



HAL
open science

Neutraligands de la chimiokine CXCL12 dans l'asthme

Francois Daubeuf

► **To cite this version:**

Francois Daubeuf. Neutraligands de la chimiokine CXCL12 dans l'asthme. Pharmacologie. Université de Strasbourg, 2013. Français. NNT : 2013STRAJ112 . tel-01249541

HAL Id: tel-01249541

<https://theses.hal.science/tel-01249541>

Submitted on 4 Jan 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

*ÉCOLE DOCTORALE des Sciences de la Vie
et de la Santé*

UMR7200, Laboratoire d'innovation thérapeutique

THÈSE présentée par :

François DAUBEUF

soutenue le : 11 décembre 2013

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Pharmacologie

**NEUTRALIGANDS DE LA CHIMIOKINE
CXCL12 DANS L'ASTHME**

Jury

Pr CHANEZ Pascal

Rapporteur

Pr MAGNAN Antoine

Rapporteur

Pr CHAMBON Pierre

Examineur

Dr GALZI Jean-luc

Invité

Dr FROSSARD Nelly

Directeur de thèse

Remerciements

Ce travail a été effectué au sein de l'unité mixte de recherche UMR7200, laboratoire d'innovation thérapeutique, sous la direction de **Madame le Docteur Nelly Frossard**.

Merci de m'avoir ouvert les portes de votre laboratoire, pour votre confiance et la liberté que vous m'accordez dans mon travail.

Je tiens également à remercier vivement les membres du jury qui m'ont fait l'honneur de juger ce travail malgré leurs obligations professionnelles:

Je remercie le **Professeur Pascal Chanez**, le **Professeur Antoine Magnan** et le **Professeur Pierre Chambon** d'avoir accepté d'être rapporteurs pour ce travail.

Je remercie également le **Professeur Marcel Hibert**, directeur de l'UMR7200, pour m'avoir accueilli au sein du laboratoire d'innovation thérapeutique, mais aussi pour sa confiance et son soutien.

Je tiens tout particulièrement à remercier l'ensemble des participants et des intervenants qui ont bâti ces travaux et sans qui ces résultats n'existeraient pas.

Je remercie le **Docteur Jean-Luc Galzi**, directeur de l'UMR7242, laboratoire de Biotechnologie et signalisation cellulaire, et son équipe qui ont réalisé l'ensemble des tests fonctionnels *in vitro*. Merci à **Muriel Hachet-Haas** pour son travail, sa patience et son aide précieuse, ainsi qu'à **Dayana Abboud** pour son travail et son enthousiasme.

Je remercie également le **Docteur Dominique Bonnet** et son équipe de chimiste pour la conception et la synthèse des nouveaux neutraligands.

Merci à **Bruno Didier**, **Vincent Gasparik**, **Pierre Regenass** et **Stéphanie Riché**, pour votre bonne humeur permanente, votre disponibilité, et évidemment pour les composés.

Merci au **Professeur Jacques Haiech**, pour ses précieuses interventions et sa disponibilité lors de nos réunions de travail.

Merci à **Patrick Gizzi**, de la plateforme TechMed'ILL, pour l'ensemble des analyses physicochimiques, pour ton soutien et ton temps. Je reste disponible pour les prochaines pharmacocinétiques !

Je remercie également toute l'équipe de la plateforme IMABIO de l'IPHC: **Virgile BECKAERT**, **Ali OUADI**, **Patrice MARCHAND** et son directeur, et le **Docteur David BRASSE**. Merci à tous pour votre aide, votre disponibilité, votre bonne humeur communicative, votre temps et les "diners" improvisés lors de nos très longues journées de travail. J'espère que nous aurons l'occasion de travailler ensemble à nouveau.

Merci à **Julien Becker**, pour ton aide, ton optimisme et ta bonne humeur partagés lors de nos journées cytométrie, mais aussi pour les journées passées ensemble en compagnie du Flexivent®...

Merci aussi à **Claudine Ebel**, pour son aide technique précieuse, sans laquelle les cytomètres resteraient muets ou pire!

Merci aux personnes de la faculté, de l'ESBS, de l'GBMC, de l'ICS et nos collaborateurs que j'ai côtoyés.

Je remercie chaleureusement les membres de notre laboratoire, **Samila, Simona et Manuel**. Merci pour votre amitié, pour toutes ces idées et conseils échangés, ainsi que pour tous ces bons moments passés, au laboratoire et en dehors. Merci aussi pour votre soutien.

Merci également à **Laurent, Alaa, Jihu et Malika** pour votre confiance et pour m'avoir permis de diversifier mon travail et mes expériences.

Je ne peu m'empêcher de penser également aux animaux, rats et souris, qui m'ont accompagné et m'accompagneront encore durant mes journées et mes weekends de travail.

Merci à la **Région Alsace**, au **Labex Médalis** et au **fond de dotation " recherche en santé respiratoire"**, ainsi qu'à la **société de Pneumologie de Langue Française**, pour avoir financé mon travail.

Je terminerai en remerciant particulièrement ma famille, notamment **mes parents**. Merci pour votre soutien sans faille, merci d'avoir cru en moi et de m'avoir poussé dans la vie. Je n'en serais pas là sans votre aide et votre amour. Merci à ma mère d'avoir sans cesse aiguisé ma curiosité et merci à mon père de m'avoir fait découvrir la pêche, le premier moteur de ma vie, sans lequel je n'en serais pas là aujourd'hui. Merci à tous les deux.

Merci également à mes frères **Stéphane et Vincent**, acteurs de nos expéditions et expériences collectives. Je vous souhaite le meilleur pour vos vies respectives. Même si la distance n'aide pas, je serai toujours là pour un meuble Rubic's Cube! ou autre...

Merci à mes **grands parents** pour leur sagesse, leurs conseils avisés et leur soutien. Un grand merci à tous les autres membres de ma famille, **Nathalie, Jean-Pierre, Eléonore et Solène** pour tous les bons moments passés ensemble et vos encouragements.

Je remercie également **ma belle-famille** pour m'avoir accueilli avec tant de gentillesse.

Merci à mes **coéquipiers et amis pêcheurs du Team Garbolino Osthouse**, pour votre patience et d'avoir pallié à mon absence lors des championnats. Je me rattraperai dès la saison prochaine. Merci également à **Francis** et à **mes ex-coéquipiers du Team Sensas Multipeche Normandie**, de m'avoir fait profiter de votre expérience de la pêche et de la vie, pour votre amitié et les bons moments passés au bord de l'eau et autour d'un bon repas. Merci aussi à **Jean-Pierre** et **Patricia**, pour votre amitié, nos soirées pêche et vie au laboratoire et nos sorties.

A tous, à très bientôt au bord de l'eau !

Enfin, merci à **Christine**, ma seconde source d'inspiration (dans l'ordre chronologique) et mon moteur d'aujourd'hui. Merci de me supporter tous les jours, et pour ton soutien indéfectible, au travail comme à la maison ou au bord de l'eau. Merci pour ton aide et ton "coaching" lors des championnats passés et à venir. Je te promets de continuer à te faire voyager, de passer à nouveau du temps à tes côtés, et de terminer les meubles...

Résumé

La chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7, sont impliqués dans l'organogenèse et dans l'homéostasie ainsi que dans de nombreuses pathologies incluant l'asthme. La collaboration entre le laboratoire d'innovation thérapeutique, le laboratoire de biotechnologie et signalisation cellulaire, et la plateforme Techmed'ILL, a récemment mis en évidence un neutralisant de CXCL12, la chalcone 4, capable de bloquer la liaison de CXCL12 à ses récepteurs, et présentant une activité anti-inflammatoire dans un modèle d'asthme allergique chez la souris. Ces résultats prometteurs sont cependant ternis par la très faible solubilité du composé (9 μ M) et les possibles effets indésirables liés à la perturbation des autres fonctions de CXCL12. Ainsi, notre travail a consisté à proposer des stratégies permettant le développement de molécules actives, biodisponibles et aptes à promouvoir une action locale, et aussi à étudier le mécanisme d'action *in vivo* des neutralisants de CXCL12.

Pour cela, nous nous sommes tout d'abord appliqués à développer un modèle court d'asthme allergique chez la souris, adapté à une évaluation rapide de l'activité anti-inflammatoire des nouveaux composés, dans le but d'optimiser les échanges entre nos équipes et garantir un développement raisonné des stratégies envisagées. Nous avons mis au point un modèle rapide et reproductible, présentant les principales caractéristiques de l'asthme allergique, et apte à réduire les délais et les coûts sans nuire à la qualité des résultats.

Nous avons ensuite développé trois prodrogues solubles de la chalcone 4, inactives mais rapidement clivées en chalcone 4 active, et adaptées à une administration locale dans les voies aériennes. Leur étude *in vivo* a mis en évidence que les prodrogues inhibent le recrutement des cellules inflammatoires dans le modèle rapide d'asthme, à des doses 10000 fois plus faibles que par voie systémique, des doses aptes à limiter les effets indésirables.

Pour favoriser davantage l'action anti-inflammatoire locale du neutralisant et limiter les effets secondaires, nous avons également synthétisé un neutralisant de CXCL12 doué d'une durée de vie courte, et rapidement dégradé avant sa distribution dans l'organisme: une ante-droque. Nous avons mis en évidence que le dérivé carbonitrile-chalcone 4 est un neutralisant de CXCL12 rapidement dégradé en deux composés inactifs dans les milieux biologiques et réduit significativement l'inflammation dans le modèle rapide d'asthme uniquement lors de son administration locale. Cette approche a permis d'obtenir un neutralisant actif localement, adapté aux traitements de l'asthme, sans perturber les autres fonctions de la chimiokine CXCL12 dans l'organisme.

Enfin, l'étude de la chalcone 4 dans un modèle murin d'asthme nous a permis de mettre en évidence une activité antiasthmatique significative, agrémentée d'une diminution de l'hyperréactivité bronchique, de l'inflammation et du remodelage bronchique. Une activité liée à l'élimination rapide de la chimiokine CXCL12 du poumon (-20%). La capture de CXCL12 par le neutralisant réduit la différenciation des macrophages M1 et leur libération de cytokines pro-inflammatoires en réponse à l'allergène. La neutralisation de CXCL12 diminue aussi le recrutement des éosinophiles et des lymphocytes CXCR4+. Enfin, nos résultats mettent en évidence le rôle de CXCL12 sur l'expression du CD14 par les macrophages et son implication dans la réponse aux endotoxines, un lien qui peut être régulé par un neutralisant de CXCL12.

En conclusion, nos travaux ont permis d'apporter des éléments mécanistiques liés aux rôles de la chimiokine CXCL12 dans l'asthme, de conforter l'intérêt des neutralisants de CXCL12 pour le traitement de l'asthme allergique et présenter deux stratégies intéressantes, adaptées à une administration locale, pour limiter les effets indésirables.

Résumé en anglais

The chemokine CXCL12 and its receptors CXCR4 and CXCR7, are involved in organogenesis and homeostasis and in several diseases including asthma. The collaboration between the "laboratoire d'innovation thérapeutique", the "laboratoire de biotechnologie et signalisation cellulaire", and the Techmed'ILL platform, recently highlighted a CXCL12 neutraligand, chalcone 4, able to prevent CXCL12 binding to its receptors, and having an anti-inflammatory activity in a mouse model of allergic asthma. However, these promising results are overshadowed by the very low solubility of the compound (9 μ M) and the possible adverse effects due to the disruption of other functions of CXCL12. Thus, our work consists in proposing strategies for the development of active and bioavailable molecules able to promote local action, and also to study the *in vivo* mechanism of action of the CXCL12 neutraligands.

To do this, we initially applied to develop a short mouse model of allergic asthma, suitable for a rapid assessment of the anti-inflammatory activity of new compounds, in order to optimize exchanges between our teams and ensure rational development of the proposed strategies. We developed a short and reproducible model, presenting the main features of allergic asthma, and able to reduce time and cost without compromising the quality of results.

Following this step, we developed three soluble and nactive prodrugs of the chalcone 4, but rapidly cleaved in active chalcone 4 and adapted to local administration in the airways. Their *in vivo* study showed that the prodrugs inhibit the recruitment of inflammatory cells in the short model of asthma at doses 10,000 times lower than following systemic administration: suitable doses to minimize side effects.

To promote the benefit of local anti-inflammatory action of CXCL12 neutraligand and limit side effects, we also synthesized a CXCL12 neutraligand with a short lifespan, and rapidly degraded before its distribution in the body: an antedrug. We have demonstrated that the carbonitrile-chalcone 4 derivative is a CXCL12 neutraligand rapidly degraded in two inactive compounds in biological fluids and able to reduce significantly the inflammation in asthma, only after local administration in the short model. This approach yielded to create a neutraligand active locally, adapted to the treatment of asthma, without disturbing other functions of the CXCL12 chemokine in the body.

Finally, the study of chalcone 4 in a murine model of asthma has allowed us to highlight a significant asthma activity, characterized by a decrease in airway hyperresponsiveness, inflammation and bronchial remodeling. An activity related to the rapid elimination of the CXCL12 chemokine from the lung (-20%). The trapping of CXCL12 by the neutraligand reduced M1 macrophage activation and their release of pro-inflammatory cytokines in response to the allergen. Neutralization of CXCL12 also decreases the recruitment of eosinophils and lymphocytes CXCR4+. Finally, our results highlight the role of CXCL12 on the expression of CD14 by macrophages and its involvement in the response to endotoxin, a link that can be controlled by a neutraligand of CXCL12.

In conclusion, our work provided mechanistic elements related to the roles of chemokine CXCL12 in asthma, strengthened the interest of CXCL12 neutraligands in the treatment of allergic asthma and presents two interesting strategies tailored to local administration to limit adverse effects.

Table des matières

REMERCIEMENTS	2
RESUME	5
RÉSUMÉ EN ANGLAIS	6
TABLE DES MATIERES	7
INTRODUCTION	10
I. L'ASTHME ALLERGIQUE	11
I.1. PHYSIOPATHOLOGIE DE L'ASTHME	11
I.2. MECANISME DE L'INFLAMMATION DANS L'ASTHME	16
I.2.1. Sensibilisation et réponse à l'allergène	16
I.2.2. Les cellules inflammatoires.....	18
I.2.2.1. Les mastocytes	18
I.2.2.2. Les cellules présentatrices d'antigène.....	22
I.2.2.3. Les lymphocytes	25
I.2.2.4. Les Eosinophiles	29
I.2.2.5. Les neutrophiles	31
I.2.2.6. Les macrophages.....	34
I.2.3. L'Epithélium bronchique dans l'asthme	37
II. MODELES D'ASTHME ALLERGIQUE	42
II.1. ANIMAUX	42
II.2. SOUCHES DE SOURIS.....	45
II.3. ALLERGENES.....	46
II.4. PROTOCOLES EXPERIMENTAUX CHEZ LA SOURIS	47
II.4.1. Modèles aigus	47
II.4.2. Modèles à provocation allergénique répétée	48
II.4.3. Modèles chroniques	49
II.5. MESURE DE L'HYPERREACTIVITE BRONCHIQUE.....	51
II.5.1. Mesure non invasive	51
II.5.1. Mesure invasive de la fonction respiratoire	52
III. LES CHIMIOKINES ET LEURS RECEPTEURS	54
III.1. NOMENCLATURE ET CLASSIFICATION	54
III.2. STRUCTURES.....	56
III.3. LES RECEPTEURS DES CHIMIOKINES	58
III.4. INTERACTION CHIMIOKINE-RECEPTEUR	60
III.5. TRANSDUCTION DU SIGNAL DES RECEPTEURS DE CHIMIOKINES	62
III.6. DESENSIBILISATION ET INTERNALISATION DES RECEPTEURS	64
III.7. EXPRESSION DES CHIMIOKINES ET DE LEURS RECEPTEURS	65
III.8. ROLES PHYSIOLOGIQUES ET PHYSIOPATHOLOGIQUES DES CHIMIOKINES ET DE LEURS RECEPTEURS.....	67
III.8.1. Migration cellulaire	68
III.8.2. Inflammation.....	70
III.8.3. Prolifération cellulaire et angiogenèse.....	70

IV. LA CHIMIOKINE CXCL12 ET SES DEUX RECEPTEURS CXCR4 ET CXCR7	72
IV.1. LA CHIMIOKINE CXCL12	72
IV.2. SITES DE PRODUCTION DE CXCL12	73
IV.3. LES RECEPTEURS DE LA CHIMIOKINE CXCL12 : LE RECEPTEUR CXCR4	74
IV.3.1. Site de production du récepteur CXCR4.....	75
IV.3.2. Signalisation induite par CXCR4.....	75
IV.4. LES RECEPTEURS DE LA CHIMIOKINE CXCL12 : LE RECEPTEUR CXCR7	79
IV.4.1. Expression du récepteur CXCR7.....	80
IV.4.2. Signalisation du récepteur CXCR7.....	81
IV.5. EFFET DE L'INVALIDATION DES GENES CODANT POUR CXCL12 OU SES RECEPTEURS CXCR4 ET CXCR7.....	83
IV.6. ROLE DU COUPLE CXCL12/CXCR4 DANS L'HEMATOPOÏÈSE.....	84
IV.7. PRINCIPALES PATHOLOGIES IMPLIQUANT LES COUPLES CXCL12/CXCR4 ET CXCL12/CXCR7.....	85
IV.7.1. La chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7 dans la biologie des tumeurs.....	85
IV.7.2. La chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7 dans l'asthme	89
V. OBJECTIFS DE LA THESE	93

PUBLICATIONS.....95

PUBLICATION N°1:	96
A new rapid model of asthma in the mouse	96
PUBLICATION N°2 :	106
Prodrugs of a CXC Chemokine-12 (CXCL12) Neutraligand Prevent Inflammatory Reactions in an Asthma Model in Vivo.....	106
PUBLICATION N°3 :	112
An Antedrug of the CXCL12 Neutraligand Blocks Experimental Allergic Asthma without Systemic Effect in Mice.....	112
PUBLICATION N°4 :	125
Deciphering the mechanism of the anti-asthmatic action of the CXCL12 neutraligand chalcone 4 and links between CXCL12 and the macrophages in response to allergen	125

DISCUSSION ET PERSPECTIVES145

ANNEXES157

ANNEXE N°1:	158
Acute Ovalbumin Asthma Model In The Mouse.....	158
ANNEXE N°2:	166
Performing Bronchoalveolar Lavage in the Mouse.	166
ANNEXE N°3:	176
A Fast And Easy Flow Cytometric Method For Analysis Of The Cellular Content Of Bronchoalveolar Lavage Fluid In Mouse.....	176
ANNEXE N°4:	184
Eosinophils And The Ovalbumin Mouse Model Of Asthma	184
ANNEXE N°5 :	196
Neutralizing endogenous chemokines with small molecules Principles and potential therapeutic applications	196

BIBLIOGRAPHIE214

LISTE DES ILLUSTRATIONS

<i>Figure 1: Prévalence de l'asthme selon GINA (2006).</i>	11
<i>Tableau 1: Description des caractéristiques cliniques de l'asthme contrôlé, partiellement contrôlé, et incontrôlé selon GINA (2012).</i>	12
<i>Figure 2: Gestion du traitement de l'asthme chez l'adulte et l'enfant de plus de 5 ans selon GINA (2012).</i>	13
<i>Figure 3: Physiopathologie de l'asthme.</i>	14
<i>Figure 4: Mécanismes dans la physiopathologie de l'asthme.</i>	15
<i>Figure 5: Interactions moléculaires et mécanismes conduisant à la production d'IgE.</i>	17
<i>Tableau 2: Répartition des protéases dans les sous-populations de mastocytes chez la souris.</i>	19
<i>Figure 6: Mécanismes par lesquels le mastocyte contribue à l'établissement et au maintien de l'inflammation allergique.</i>	21
<i>Figure 7: Interactions entre les cellules présentatrices d'antigène (CPA) et l'épithélium bronchique dans la pathogénèse de l'asthme allergique.</i>	23
<i>Figure 8: Relations entre les virus, l'IFN-α, les IgE et les cellules présentatrices d'antigène (CPA) dans l'asthme.</i>	25
<i>Figure 9: Mécanismes et contrôle de la réponse immunologique par les lymphocytes Th2 dans l'inflammation allergique.</i>	26
<i>Figure 10: La différenciation des lymphocytes T helper CD4+ naïfs ou Th0.</i>	27
<i>Figure 11: Recrutement et rôles des éosinophiles dans l'inflammation allergique.</i>	30
<i>Figure 12: Rôle des neutrophiles dans l'inflammation allergique.</i>	32
<i>Figure 13: Rôles multiples des macrophages dans l'immunité et l'homéostasie.</i>	35
<i>Figure 14: Polarisation des macrophages M1 et M2.</i>	37
<i>Figure 15: Représentation schématique du rôle de l'épithélium bronchique dans l'asthme.</i>	39
<i>Figure 16: Remodelage des voies aériennes dépendant de l'épithélium bronchique dans l'asthme.</i>	40
<i>Tableau 3: Avantages et inconvénients des espèces animales utilisées dans les modèles d'asthme.</i>	44
<i>Tableau 4: Modèles d'asthme allergique aigu chez la souris.</i>	48
<i>Tableau 5: Modèles d'asthme allergique chronique chez la souris.</i>	50
<i>Tableau 6: Avantages et inconvénients des mesures invasives et non-invasives de la fonction respiratoire.</i>	52
<i>Tableau 7: Nomenclature des chimiokines humaines.</i>	55
<i>Figure 17: Les 4 sous familles de chimiokines.</i>	56
<i>Figure 18: Structure tridimensionnelle des chimiokines.</i>	57
<i>Figure 19: Représentation schématique de la forme membranaire de CX3CL1.</i>	58
<i>Figure 20: Représentation schématique d'un récepteur aux chimiokines.</i>	59
<i>Figure 21: Les chimiokines et leurs récepteurs.</i>	60
<i>Figure 22: Modèle d'interaction "chimiokine/récepteur aux chimiokines" en 2 étapes.</i>	61
<i>Figure 23: Représentation schématique résumant l'activation des protéines G hétérodimériques via les récepteurs à 7 domaines transmembranaires.</i>	62
<i>Figure 24: Principales voies de signalisation induites par la liaison des chimiokines sur leurs récepteurs.</i>	63
<i>Figure 25: Endocytose des RCPG après une activation de courte durée: mécanisme principal et devenir des récepteurs internalisés.</i>	64
<i>Figure 26: Classification des chimiokines en fonction de leur profil d'expression.</i>	66
<i>Figure 27: Expression des récepteurs aux chimiokines sur les leucocytes humains.</i>	67
<i>Figure 28: Relation entre pathologies et couples chimiokines/récepteurs.</i>	68
<i>Figure 29: Modèle en plusieurs étapes du recrutement leucocytaire.</i>	69
<i>Figure 30: Chimiokines excrétées en réponse à des agents pro-inflammatoires</i>	70
<i>Figure 31: Structure tridimensionnelle de CXCL12.</i>	73
<i>Figure 32: Principales voies de signalisation intracellulaire du couple CXCR4/CXCL12.</i>	77
<i>Figure 33: Régulation de l'activité du récepteur CXCR4</i>	79
<i>Figure 34: Alignement de séquences des récepteurs des chimiokines.</i>	80
<i>Tableau 8: Principaux sites d'expression de CXCR7 et régulation.</i>	81
<i>Figure 35: Signalisation du récepteur CXCR7.</i>	83
<i>Figure 36: Régulation et effet de CXCL12 et CXCR4/CXCR7 dans la biologie des tumeurs.</i>	88
<i>Figure 37: Rôle de la chimiokine CXCL12 et ses récepteurs CXCR4/CXCR7 dans l'asthme.</i>	92

INTRODUCTION

I. L'asthme allergique

L'asthme est une des maladies les plus fréquentes dans les pays industrialisés. Elle affecterait environ 300 millions d'individus, et sa prévalence ne cesse d'augmenter. En France, l'asthme concerne près de 3,5 millions de patients dont un tiers a moins de 15 ans. Cette maladie est responsable d'environ 2000 morts par an en France. Selon l'organisme international, *Global Initiative for Asthma* (GINA), la fréquence de l'asthme progresse à mesure que les communautés adoptent des modes de vie occidentaux et que l'urbanisation croît. Avec l'évolution de l'urbanisation, l'augmentation de l'asthme devrait se poursuivre durant les prochaines années. Le GINA estime ainsi un ajout de 100 millions d'asthmatiques supplémentaires en 2025.

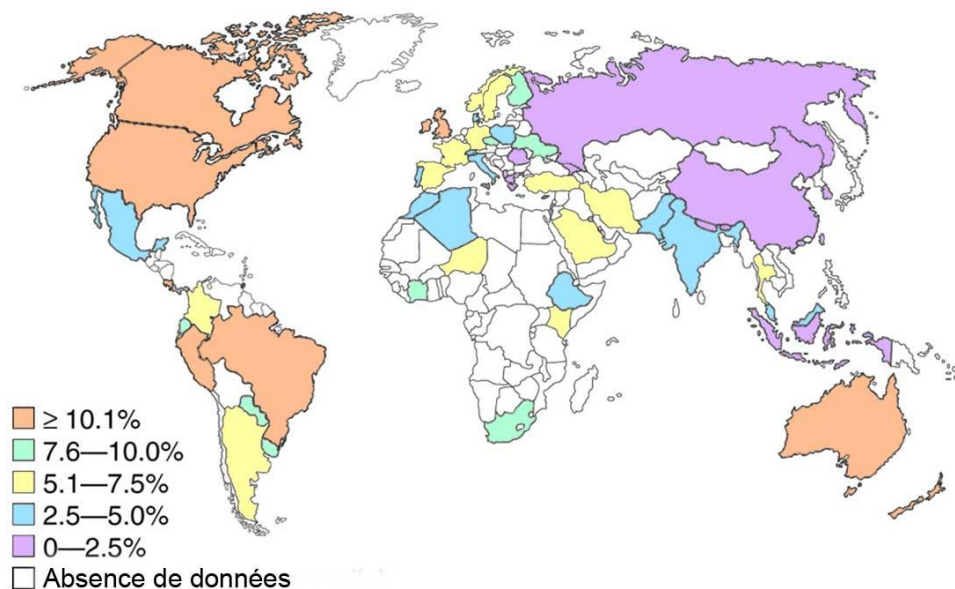


Figure 1: Prévalence de l'asthme selon GINA (2006).

I.1. Physiopathologie de l'asthme

L'asthme est une maladie inflammatoire chronique dont les symptômes majeurs sont la dyspnée, une respiration sifflante intermittente, la toux et l'essoufflement du patient. Ces symptômes sont variables et réversibles spontanément ou après traitement. L'asthme est caractérisé par la présence d'une hyperréactivité des voies aériennes : une réponse bronchique exagérée est déclenchée lors de l'exposition à divers stimuli ou facteurs déclenchant et conduisant à une bronchoconstriction, des bouchons muqueux et une augmentation de l'inflammation. L'asthme est un syndrome multifactoriel, le plus souvent associé à l'atopie, une aptitude anormale d'un individu à synthétiser des immunoglobulines E (IgE) spécifiques, dirigées contre les allergènes naturels entrant en contact

avec l'organisme par des voies naturelles. Il s'en suit une réponse immédiate caractérisée par l'activation des cellules portant les IgE spécifiques de l'allergène, en particulier les mastocytes et les macrophages. Ces cellules sécrètent alors des médiateurs, ceux-ci entraînant une obstruction bronchique. Plus tardivement, les cellules activées dans les bronches vont libérer des cytokines et des chimiokines, qui vont induire à leur tour le recrutement de cellules inflammatoires comme les éosinophiles (Barnes PJ. 1996, Tillie-Leblond et al. 2004, Murphy et al. 2010).

Le terrain génétique, le stress psychologique ou encore l'influence hormonale font partie des facteurs endogènes impliqués dans le déclenchement d'une crise d'asthme, mais aussi dans la chronicité de la maladie. Parmi les facteurs exogènes favorisant l'asthme, on compte les allergènes, les virus, les polluants atmosphériques, la fumée de tabac ou encore l'intolérance à l'aspirine. L'asthme est plus ou moins sévère et la classification actuelle comprend une distinction en différents degrés : intermittent, persistant léger, persistant modéré et persistant sévère. Afin de mieux traiter l'asthme et de réduire la morbidité, des recommandations internationales ont été initiées en 1995 par le GINA (*Global Initiative for Asthma*) et sont révisées chaque année (Tableau 1).

Caractéristiques	Contrôlé	Partiellement contrôlé	Incontrôlé
Symptômes journaliers	Aucun (2 fois ou moins par semaine)	Supérieur à 2 fois par semaine	trois ou plusieurs caractéristiques de l'asthme partiellement contrôlées
Limitation des activités	Aucun	N'importe laquelle	
Symptômes nocturnes / au réveil	Aucun	N'importe lequel	
besoin de secours/ recours aux inhalateurs	Aucun	Supérieur à 2 fois par semaine	
Fonction pulmonaire	Normale	< 80% du VEMS	

Tableau 1: Description des caractéristiques cliniques de l'asthme contrôlé, partiellement contrôlé, et incontrôlé selon GINA (2012). Un suivi médical est recommandé sur une période de 4 semaines. Plusieurs caractères sont reconnus par l'organisation pour augmenter le risque d'événements indésirables dans le futur : un mauvais suivi médical, la fréquence des exacerbations l'année précédente, la non-hospitalisation en soins intensifs lors des crises d'asthme, un faible Volume Expiratoire Maximal par Seconde (VEMS), l'exposition à la fumée de cigarette, une médication à forte dose. D'après "*Pocket Guide For Asthma Management And Prevention*", GINA, 2012.

L'asthme est une maladie particulièrement invalidante, apparaissant au cours des premières années de la vie, ou à l'âge adulte. Il n'existe, à l'heure actuelle, aucun traitement curatif. Son degré de sévérité détermine les modalités du traitement des patients (Figure 2). Ces traitements agissent sur la survenue des symptômes (bronchodilatateurs, agonistes de type β 2-adrenergiques) ou servent à limiter l'inflammation (corticoïdes) pour permettre un meilleur contrôle à long terme.

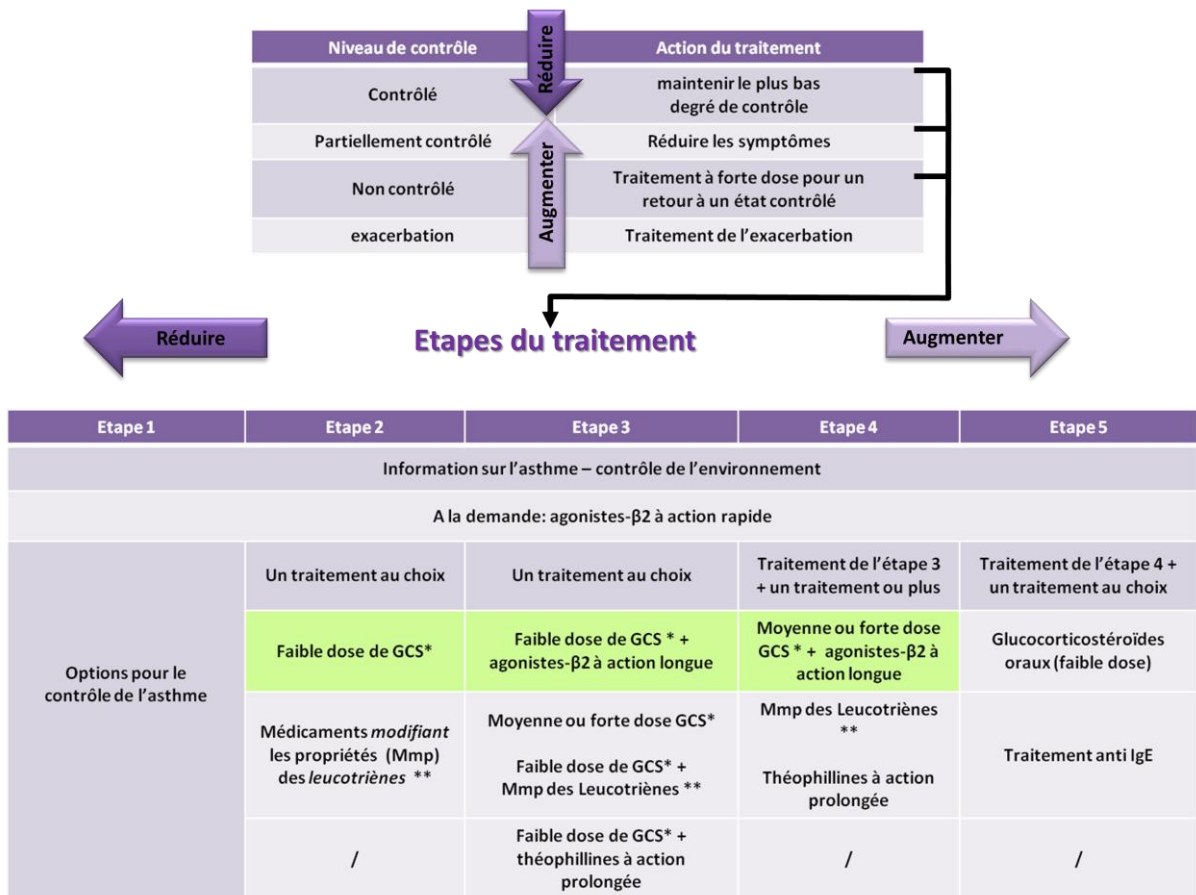


Figure 2: Gestion du traitement de l'asthme chez l'adulte et l'enfant de plus de 5 ans selon GINA (2012). Le besoin individuel de chaque patient, les préférences et la situation financière doivent être pris en compte dans le traitement du patient. Le traitement recommandé est identifié par les cases en vert. Lorsque les symptômes l'exigent, il convient d'administrer un bronchodilatateur en plus du traitement de fond quotidien. Le suivi du patient doit être régulier et le traitement doit être revu afin de rester adapté. En cas d'aggravation de l'asthme, il convient de passer à l'étape suivante. Lorsque l'asthme est sous contrôle depuis 3 mois, une réduction progressive du traitement d'entretien peut-être envisagé. *glucocorticostéroïdes inhalés (GCS), **antagoniste des récepteurs ou inhibiteur de synthèse des leucotriènes. D'après "Pocket Guide For Asthma Management And Prevention", GINA, 2012.

L'inflammation bronchique, la bronchoconstriction et l'hypersécrétion de mucus sont les mécanismes les plus souvent évoqués dans la pathophysiologie de l'asthme. Ces phénomènes conduisent à l'hyperréactivité bronchique face à des stimuli physiques, chimiques ou pharmacologiques tels que l'acétylcholine, la métacholine, l'histamine ou la prostaglandine F2α.

La gêne respiratoire est liée à la contraction du muscle lisse bronchique provoquée par des médiateurs libérés par des cellules inflammatoires, en particulier le mastocyte. Ces médiateurs sont l'histamine, la tryptase, la prostaglandine D2 (PGD2) et les leucotriènes (LT) C4 et D4. Le système nerveux autonome, contrôlant la contraction et la relaxation du muscle bronchique, est aussi impliqué. De plus, les patients asthmatiques présentent une hypertrophie du muscle lisse bronchique, une fibrose sous-épithéliale par dépôt de collagène et de myofibroblastes et un œdème qui participent aussi à l'obstruction bronchique (Figure 3) (Fahy et al. 2000). L'épaisseur de la paroi

bronchique est plus importante, le calibre des bronches est diminué et la réactivité bronchique augmentée. Une autre cause de l'obstruction bronchique est l'hypersécrétion de mucus. Les glandes à mucus et les cellules caliciformes sont plus nombreuses, hypertrophiées et participent directement à l'épaississement de la paroi bronchique.

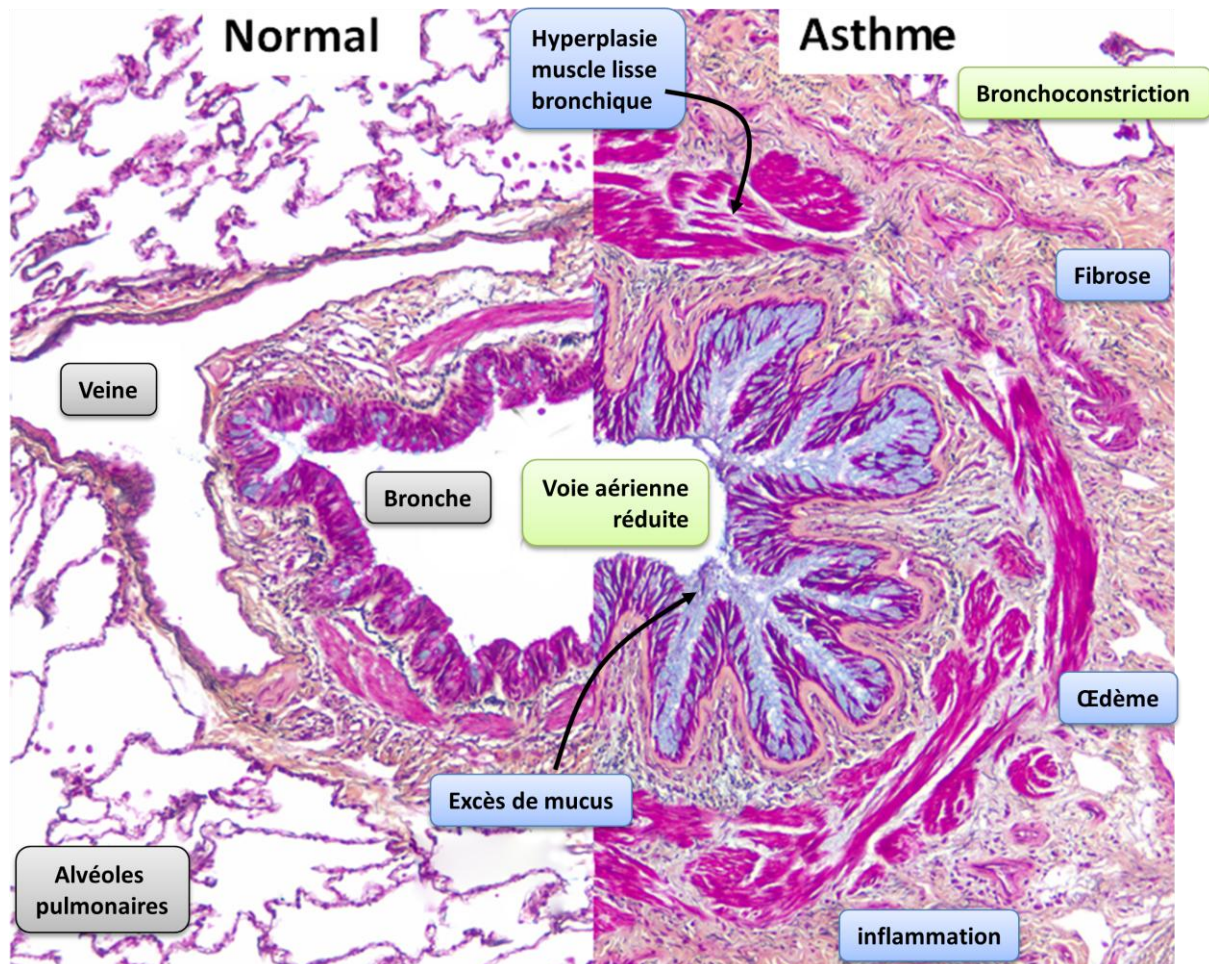


Figure 3: Physiopathologie de l'asthme. L'obstruction bronchique observée dans l'asthme est multifactorielle et met en jeu une hyperplasie du muscle lisse bronchique, une hypersécrétion de mucus, une infiltration cellulaire, un œdème, et une desquamation épithéliale. Il en résulte une diminution du calibre des bronches qui réduit le passage de l'air et induit les symptômes caractéristiques de la crise d'asthme. Adapté de Wadsworth et al. 2012.

Le processus inflammatoire dans l'asthme se caractérise par une accumulation de cellules inflammatoires dans la paroi bronchique, telles que des éosinophiles, des lymphocytes T CD4+ et CD8+, des lymphocytes B, des macrophages, des cellules dendritiques, des mastocytes et dans certains cas des neutrophiles (Barnes PJ. 2008). L'inflammation bronchique est également observée chez les asthmatiques asymptomatiques et l'importance de ces différences semble être liée à la sévérité de la maladie.

Une des conséquences de l'inflammation est la fragilité de l'épithélium. Elle se caractérise par une fragilité des cellules ciliées et caliciformes qui se détachent, dénudant la membrane basale. Ces lésions sont présentes même chez des asthmatiques peu symptomatiques. Les lésions de l'épithélium bronchique stimulent le processus de réparation qui provoque des changements structuraux de la paroi bronchique, souvent appelés "remodelage" bronchique (Barnes PJ. 1996). Il se forme un pseudo-épaississement de la membrane basale dû à un dépôt de collagène de type I, III et V et de fibronectine. Au contraire, le collagène de type IV, normalement majoritaire, est diminué dans l'asthme. Le clivage de la molécule de collagène IV libère des inhibiteurs angiogéniques, comme la tumstatin. La diminution du collagène de type IV entraîne une diminution de libération des facteurs anti-angiogéniques sous l'action de la MMP-9 (Métalloprotéinase matricielle 9 ou collagénase type IV), ce qui conduit à réduire l'apoptose de cellules endothéliales proliférantes et promeut l'angiogénèse (Burgess et al. 2010). La fibrose sous-épithéliale est aussi une cause de réduction de la lumière bronchique et de la plasticité des bronches, et participe à l'obstruction bronchique (Figure 4).

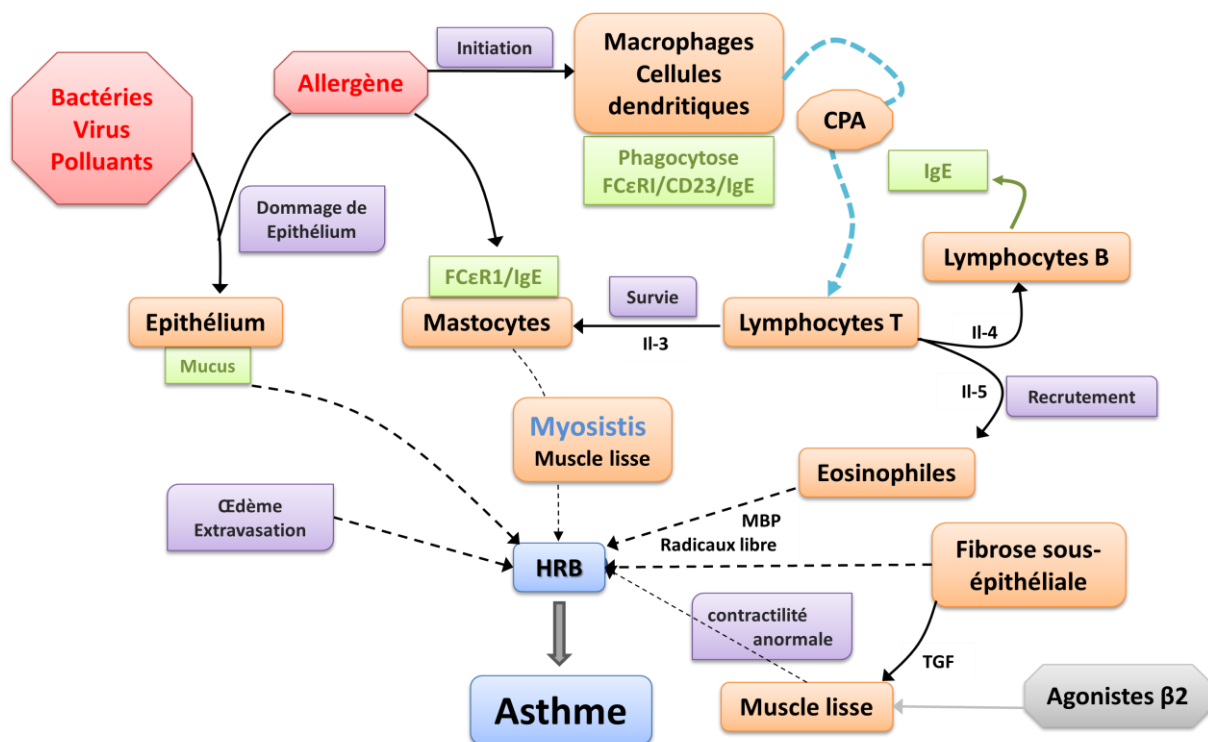


Figure 4: Mécanismes dans la physiopathologie de l'asthme. Représentation des principaux mécanismes impliqués dans le développement de l'hyperréactivité bronchique (HRB) dans l'asthme. Cellules présentatrices d'antigènes (CPA).

I.2. Mécanisme de l'inflammation dans l'asthme

I.2.1. Sensibilisation et réponse à l'allergène

L'asthme est souvent associé à l'atopie, qui se manifeste par des mécanismes dépendants des immunoglobulines E (IgE) et des lymphocytes. Les deux principaux sous-types de lymphocytes T-helper (Th) CD4⁺ sont caractérisés par leur profil de synthèse de cytokines (Fietta et al. 2009). Les Th1 produisent de l'interleukine (IL)-2 qui stimule la prolifération des lymphocytes T, de l'interféron- γ (IFN- γ) qui inhibe la synthèse d'IgE par les lymphocytes B et produisent du TNF- β (*Tumor Necrosis Factor β*). Au contraire, les lymphocytes Th2, qui sont impliqués dans l'asthme, sécrètent de l'IL-4, de l'IL-5, de l'IL-9, de l'IL-13 et de l'IL-16. Ces cytokines Th2 sont impliquées dans le développement de la réaction d'hypersensibilité. L'IL-4 permet la synthèse des IgE par différenciation des lymphocytes B en plasmocytes sécréteurs d'IgE, dirige les lymphocytes T vers une réponse Th2, empêche la mort des cellules T activées (Zamorano et al. 2003, Maes et al. 2012), contrôle les taux des récepteurs aux IgE à la surface des mastocytes (Ryan et al. 1998), des éosinophiles (Iikura et al. 2001) et des cellules musculaires lisses (Redhu et al. 2009). L'IL-4 peut également réguler l'expression des récepteurs aux cytokines et chimiokines comme l'expression des récepteurs "decoy" à l'IL-1 à la surface des macrophages (Gordon S. 2003) ou encore l'expression de CXCR4 et CCR5 à la surface des lymphocytes T (Creery et al. 2006).

Les lymphocytes T sont activés par un antigène, présenté par les cellules présentatrices d'antigènes qui expriment à leur membrane des molécules de classe II du système d'histocompatibilité (MHC II). Les cellules présentatrices d'antigènes (CPA) des voies aériennes sont constituées par des cellules dendritiques et les macrophages différenciés après avoir capturé un allergène. Dans l'asthme, Les CPA polarisent les cellules T helper naïves (Th0) en Th2 qui vont alors sécréter leurs cytokines inflammatoires (Akbari et al. 2005).

Les cytokines IL-4 et IL-13 stimulent la prolifération des lymphocytes B spécifiques de l'allergène et favorisent la production d'immunoglobuline de type E. Le processus est initié lorsque l'allergène se lie au récepteur des cellules B (*BCR*) présent sur les lymphocytes B.

L'antigène provenant d'un allergène peut être présenté par une CPA, ou directement capturé dans le milieu environnant après avoir été libéré par une cellule phagocytaire. Le *BCR* est formé d'une immunoglobuline permettant la reconnaissance de l'allergène et des CD79 responsables de la transduction du signal. L'immunoglobuline du BCR est de type IgM sur les lymphocytes matures et naïfs, mais peut être de type IgG, IgA ou IgE sur les lymphocytes post-folliculaires, générés par la prolifération des lymphocytes B activés, et destinés à devenir des plasmocytes ou des cellules B mémoires. Par rapport aux lymphocytes B naïfs, les lymphocytes B mémoire ont un BCR à forte affinité pour l'antigène, de nature IgG, IgA ou IgE.

Lorsque l'antigène se lie aux *BCR*, de type IgM lors d'une première présentation ou de type IgE chez un patient déjà sensibilisé, à la surface des lymphocytes B, il est internalisé puis présenté à la surface de la molécule MHC de classe II. Le lymphocyte B activé peut alors migrer vers les zones riches en lymphocytes T des tissus lymphoïdes secondaires où le MHC-II est reconnu par les lymphocytes T CD4+ activés (Brink et al. 2008). Le MHC-II du lymphocyte B et le récepteur CD4 du lymphocyte Th2 forment alors un complexe qui entraîne la libération rapide d'IL-4 et l'expression du ligand CD40 (CD40L) à la surface du lymphocyte Th2. Le CD40L se lie à son tour au récepteur CD40 présent à la surface de la cellule B. Cette interaction conduit à l'expression de B7 sur la cellule B qui lie à son tour le CD28 sur la cellule T et engendre une surexpression d'IL-4 (Figure 5). L'IL-4 active en retour les récepteurs de l'IL-4 de la cellule B et déclenche la transcription du locus codant pour le gène de la chaîne lourde C ϵ des immunoglobulines (Bacharier et al. 2000). L'interaction CD40-CD40L déclenche la recombinaison de l'ADN codant pour la chaîne lourde et entraîne une délétion entraînant la synthèse d'IgE. Le lymphocyte B se différencie ensuite en plasmocyte capable de synthétiser des immunoglobulines de type E, spécifiques de l'allergène (OWang et al. 2001, Vercelli. 2002).

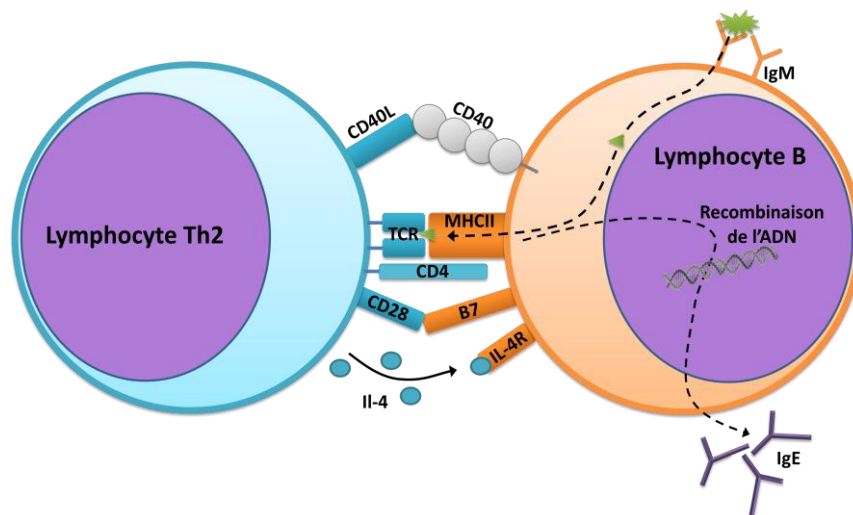


Figure 5: Interactions moléculaires et mécanismes conduisant à la production d'IgE. Représentation des principales interactions, entre les lymphocytes B et T activés, et mécanismes nécessaires au "Switch" des lymphocytes B conduisant à une production d'IgE dirigées contre un allergène. D'après Bacharier et al. 2000.

Il existe deux types de récepteurs aux IgE : les récepteurs de forte affinité (Fc ϵ R1, KD \approx 1 nM) et le récepteur de faible affinité (Fc ϵ R2 ou CD23, KD \approx 0,1-1 mM). Fc ϵ R1 est exprimé à la surface des mastocytes, des basophiles et des macrophages sous la forme de tétramère ($\alpha\beta\gamma_2$). L'expression de la chaîne β augmente l'expression à la surface de la cellule. Les récepteurs Fc ϵ R1 non occupés par des IgE ont une demi-vie de 24 heures à la surface des mastocytes alors que les récepteurs liés aux IgE semblent exprimés durant toute la vie de la cellule. La liaison aux IgE entraîne le recrutement de la kinase SyK qui active plusieurs cascades de signalisation intracellulaire conduisant à l'activation du mastocyte ou du basophile.

Le récepteur de faible affinité FcεRII (CD23) est exprimé par les lymphocytes B, les lymphocytes T, les macrophages, les monocytes, les éosinophiles et les plaquettes. Son expression est augmentée sous l'influence des IgE et de l'IL-4. L'activation du récepteur par les IgE supprime la production d'IgE par les lymphocytes B, active la phagocytose et entraîne la production de NO et la libération de cytokines pro-inflammatoires par les macrophages (Zhang et al. 2007). Le récepteur FcεRII est également associé au le MHC de classe II à la surface des cellules présentatrices d'antigènes et potentialise la présentation de l'antigène (Rosenwasser et al. 2005).

Le récepteur FcεRII peut aussi être libéré sous l'action de protéases telles que ADAM10 mais aussi par l'allergène d'acarien Der p1 provenant de l'acarien *Dermatophagoides pteronyssinus*. Le récepteur soluble FcεRII est impliqué dans la régulation de la production des IgE par les lymphocytes B. Il se lie aux IgE puis au récepteur CD21 présent sur les lymphocytes B et entraîne une inhibition de la production des IgE lorsqu'il est sous la forme monomérique ou la stimule lorsqu'il est sous la forme d'un trimère, par ailleurs plus affine que la première, avec une constante d'affinité comprise entre 10-100 nM (Acharya et al. 2010).

L'inhalation d'un allergène chez un patient allergique provoque une activation des cellules portant les IgE spécifiques de l'allergène, particulièrement les mastocytes et les basophiles présents dans les voies aériennes. Ces cellules sécrètent alors des médiateurs comme l'histamine, les enzymes protéolytiques et glycolytiques, des glycosaminoglycannes, de la PGD2 et du LTC4 qui vont stimuler la contraction du muscle lisse bronchique, stimuler les nerfs afférents, stimuler la sécrétion de mucus et induire une vasodilatation avec parfois formation d'un œdème. Dans un deuxième temps, les cellules activées libèrent des cytokines et des chimiokines dans la circulation qui vont stimuler le recrutement de cellules inflammatoires comme les éosinophiles (Broide et al. 2011).

I.2.2. Les cellules inflammatoires

I.2.2.1. Les mastocytes

C'est en 1877 que l'allemand Paul Ehrlich découvrit des cellules aux granules très denses présentes dans le tissu conjonctif. Il les nomma "Mastzellen" ou mastocytes en français en 1878, et observa que leur nombre augmentait fortement chez les patients atteints de maladies inflammatoires chroniques. Les mastocytes sont situés dans la muqueuse bronchique en plus grand nombre chez les patients asthmatiques que chez les sujets sains (Bradding et al. 1994) et sont situés dans la couche du muscle lisse bronchique et dans l'épithélium bronchique, près des vaisseaux et des terminaisons nerveuses sensibles (Bradding et al. 1994, Ammit et al. 1997). Ils sont impliqués dans l'établissement, dans le maintien de l'inflammation allergique, dans la réponse aiguë à l'allergène, ainsi que dans

hyperréactivité bronchique induite par l'allergène. Les mastocytes sont des cellules qui répondent à des "signaux de danger" et participent à l'immunité innée et acquise avec une libération immédiate ou retardée de médiateurs pré- ou néoformés impliqués dans la bronchoconstriction et l'inflammation.

Les mastocytes présentent un cytoplasme dense, envahi de granules contenant des médiateurs préformés dont des protéases comme des tryptases, des chymases et la carboxypeptidase A, ainsi que du TNF- α ou encore de l'histamine qui est connue pour la promotion de la bronchoconstriction et une vasodilatation. Les mastocytes sont souvent classés en fonction de leur emplacement et / ou du contenu des granules. Chez la souris, les mastocytes ont été classés antérieurement en deux sous-populations: les mastocytes de type muqueux et les mastocytes séreux (Tableau 2).

Sous population de Mastocytes	Tryptases	Chymases	Carboxypeptidase A
Mastocytes de type muqueux (MMC)		mMCP-1	
		mMCP-2	
Mastocytes séreux (CTMCs)	mMCP-6	mMCP-4	MC-CPA
	mMCP-7	mMCP-5	
		mMCP-9*	

Tableau 2: Répartition des protéases dans les sous-populations de mastocytes chez la souris. *Mucosal Mast Cell* (MMC), *Connective Tissue Mast Cell* (CTMC), protéase des mastocytes de souris ou mouse mast cell protease (mMCP), carboxypeptidase A (CPA). *préférentiellement exprimé dans les mastocytes de la paroi utérine. D'après Pejler et al. 2010.

Ces deux sous populations de mastocytes peuvent être identifiées à l'aide d'une coloration bleu alcian-safranine (McMenamin et al. 1996). Les granules des mastocytes de type muqueux sont riches en amines biogènes telles que l'histamine (bleu alcian +), alors que celles des mastocytes séreux sont riches en héparine (safranine +). La distribution et la population des mastocytes présents dans les voies respiratoires varient selon les espèces. Chez l'homme, les deux types de mastocytes sont présents en grand nombre dans les voies respiratoires inférieures, majoritairement autour de la trachée et le long des 6 premiers niveaux de l'arbre bronchique (Heard et al. 1990). En revanche, chez la souris, seuls des mastocytes de type muqueux sont présents, autour de la trachée et de la bronche primaire.

Les mastocytes sont issus des cellules progénitrices de la moelle osseuse exprimant CD34 et le récepteur c-Kit du SCF (stem cell factor). Ces progéniteurs entrent dans la circulation, se lient à

l'endothélium via la protéine d'adhérence cellulaire VCAM-1 (Vascular cell adhesion protein 1) et les récepteurs d'adhésion cellulaire, les intégrines $\alpha 4$ et $\beta 7$, puis migrent vers les tissus où ils deviennent matures (Hallgren et al. 2007). Les acteurs du recrutement des progéniteurs des mastocytes vers les tissus semblent être multiples. Les études *in vitro*, ont mis en évidence que de très nombreuses chimiokines (CCL1, CCL2, CCL3, CCL5, CCL7, CCL8, CCL11, CCL13, CXCL6, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13...), facteurs de croissance (SCF, NGF, TGF- β , TNF- α) et dérivés de l'acide arachidonique (PGE2, PGD2, LTB4) sont capables d'induire un chimiotactisme des progéniteurs des mastocytes (Hallgren et al. 2011, Halova et al. 2012) et sont autant d'acteurs potentiels pour le recrutement des progéniteurs des mastocytes dans les tissus. Compte tenu du nombre d'acteurs potentiels et des mécanismes compensatoires pouvant être mis en place, il s'avère difficile d'attribuer un rôle spécifique pour ses agents chimioattractants dans le trafic des mastocytes *in vivo*. Il apparaît cependant que les facteurs de croissance tels que le SCF et le NGF, ainsi que les chimiokines CXCL8, CXCL9, CXCL10 et CCL2, et le leukotriène B4 (LTB4) se démarquent et ont un rôle important dans le recrutement des progéniteurs des mastocytes (Collington et al. 2011).

D'autre part, les cytokines de type Th1 et Th2, ainsi que les IgE ne semblent pas avoir d'effet sur le recrutement des progéniteurs des mastocytes vers les tissus (Hallgren et al. 2011). En revanche, les cytokines IL-3 et IL-4 sont décrites pour induire une hyperplasie des mastocytes dans les intestins, et plus récemment les IgE comme facteur anti-apoptotique favorisant la survie des mastocytes dans les poumons (Mathias et al. 2009). La cytokine IL-9 est impliquée dans la différenciation et la maturation des mastocytes et pourrait être impliquée dans leur recrutement vers les tissus (Collington et al. 2011, Eller et al. 2011).

Lorsqu'ils sont activés, les mastocytes produisent de nombreux médiateurs lipidiques, dérivés de l'acide arachidonique, et des cytokines (Prussin et al. 2003). L'acide arachidonique est libéré de la membrane péri-nucléaire du réticulum endoplasmique et transformé en plusieurs eicosanoïdes tels que la prostaglandine D2 (PGD2), la prostaglandine E2 (PGE2) et les leukotriènes LTB4 et cystéinyl-leukotriènes LTC4, D4 et E4. Le LTC4 semble être impliqué dans la réponse inflammatoire allergique et la prolifération des mastocytes. La PGD2 est le principal prostanoloïde produit par les mastocytes et induit une vasodilatation et augmentation de la perméabilité des vaisseaux lorsqu'elle est libérée. La PGD2 peut également se lier au récepteur des prostanoloïdes D (DP) présent sur les lymphocytes T, les CPA, les basophiles, éosinophiles, les mastocytes, les cellules épithéliales et les fibroblastes, et semble alors potentialiser la réponse inflammatoire et le développement de l'asthme (Arima et al. 2011).

Dans l'asthme, les mastocytes sont une source importante de cytokines et chimiokines pro-inflammatoires (Stone et al. 2010). Ils produisent des cytokines de type Th2 incluant l'IL-4, l'IL-5 et l'IL-13, ainsi que des chimiokines comme CXCL8 (IL-8), un agent chimioattractant des neutrophiles,

impliqué dans l'angiogenèse, et CCL5 (Rantes) qui est un agent chimioattractant des éosinophiles et des lymphocytes T.

Le mastocyte est aussi une source importante de TNF- α qui promeut le recrutement de neutrophiles, la migration des cellules dendritiques et joue un rôle dans l'hyperréactivité des voies aériennes, l'inflammation bronchique et de la production de cytokines Th2 dans l'asthme (Nakae et al. 2007). Les mastocytes peuvent être activés par de nombreuses voies : la voie du complément (C3a/ C5a), le NGF (Nerve Growth Factor) via le récepteur TrkA, les ligands des TLR (Toll like Receptor), le SCF (Stem Cell Factor) via le récepteur KIT (CD117) et les IgE via le récepteur Fc ϵ R1. Dans l'asthme, la voie d'activation des mastocytes par le récepteur Fc ϵ R1 est de première importance. L'activation de Fc ϵ R1 par liaison de l'allergène aux IgE initie la libération immédiate du contenu des granules et entraîne des éternuements et une rhinorrhée dans le tractus respiratoire supérieur ; toux, bronchospasme, œdème et sécrétion de mucus dans le tractus respiratoire inférieur. Une seconde phase, plus tardive, participe à l'inflammation par la libération de médiateurs lipidiques, de cytokines et de chimiokines (Marone et al. 2005, Amin K. 2012) (Figure 6).

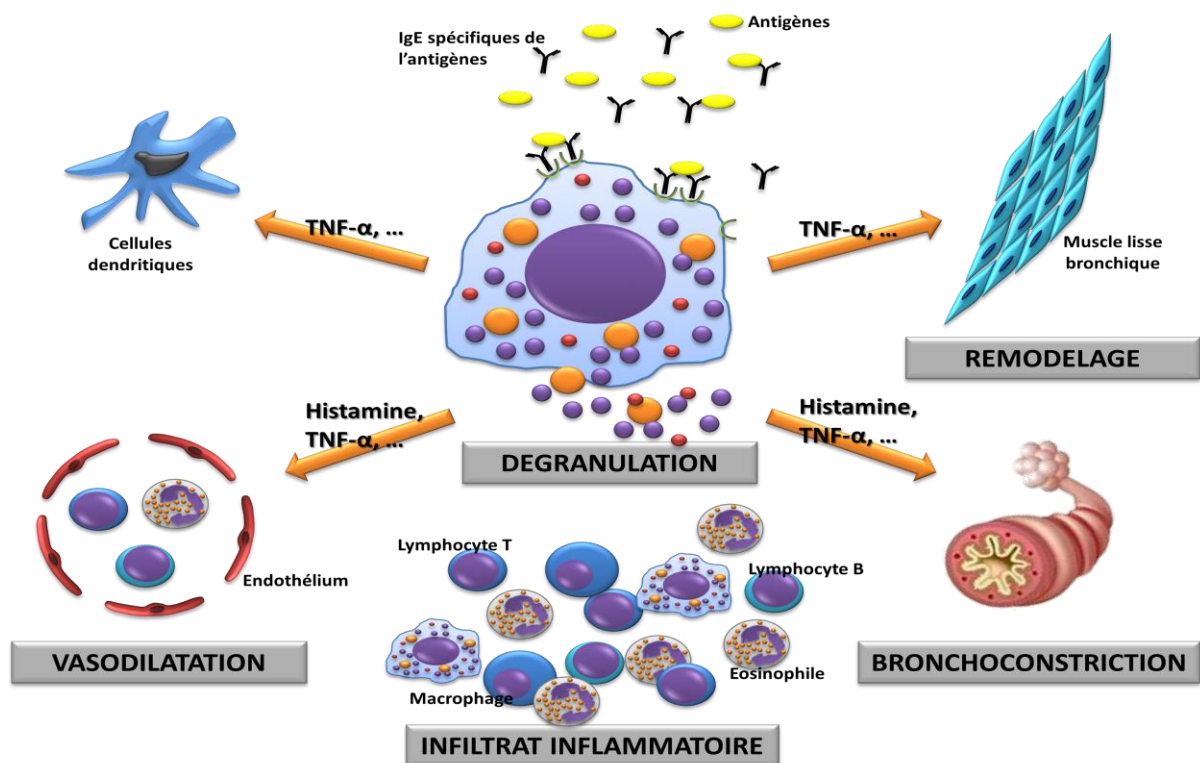


Figure 6: Mécanismes par lesquels le mastocyte contribue à l'établissement et au maintien de l'inflammation allergique.

I.2.2.2. Les cellules présentatrices d'antigène

Lorsque l'allergène pénètre dans les voies aériennes, il interagit en premier lieu avec les cellules présentatrices d'antigène. Les cellules dendritiques (*dendritic cells*, DC) sont considérées comme des cellules immunitaires spécialisées dans la présentation de l'antigène aux lymphocytes T naïfs, au contraire des macrophages, relayés au second plan. Il est cependant intéressant de noter que leur lignée, leur voie de différenciation ainsi que leur efficacité lors de la présentation de l'antigène semblent indissociables (Hume. 2008). Il apparaît aujourd'hui que les cellules dendritiques font partie du système phagocytaire mononucléaire ou MPS (*mononuclear phagocyte system*) et dérivent d'un précurseur commun avec les macrophages, présent dans la moelle osseuse, appelé progéniteur hématopoïétique spécifique des macrophages/DC ou MDP (*macrophage-DC progenitor*) (Auffray et al. 2009). Les cellules dendritiques et les macrophages répondent aux mêmes facteurs de croissance tels que le CSF-1 (*Macrophage colony-stimulating factor*), le GM-CSF (*granulocyte-macrophage colony stimulating factor*), l'IL-4, l'IFN- γ et le Flt3L. *In vivo*, aucun facteur de croissance ne semble soutenir plus spécifiquement la différenciation des DC, ni ne les sépare des MPS.

De plus, les cellules dendritiques sont définies par l'expression d'un marqueur membranaire, le CD11c, et de nombreuses études ont montré que ce marqueur est présent à la surface des macrophages et est aujourd'hui couramment utilisé pour identifier les macrophages par cytométrie en flux (Kugathasan et al. 2008, Bedoret et al. 2011, Denning et al. 2011, Poole et al. 2012, Smith et al. 2012, Daubeuf et al. 2013). Un fait d'autant plus marquant que la plupart des études portant sur les cellules dendritiques utilisent ce marqueur pour les différencier des autres leucocytes. Enfin, les cellules dendritiques ne sont pas mieux adaptées que les macrophages pour la présentation des antigènes (Toujas et al. 1997, Pozzi et al. 2005). Pour ces raisons, les cellules dendritiques ne semblent pas être un type cellulaire à part entière et différent des macrophages, mais un état d'activation des macrophages, les macrophages étant alors des cellules dendritiques immatures. Les cellules présentatrices d'antigène ou CPA sont donc des cellules capables de présenter les antigènes aux lymphocytes T et de réguler la réponse inflammatoire (Figure 7).

Les cellules du système phagocytaire mononucléaire sont soumises à un processus complexe de maturation, une "métamorphose" en CPA, débutant lors de la capture d'un antigène (Ueno et al. 2007). Ce processus comprend des modifications morphologiques, comme la perte des structures adhésives, une réorganisation du cytosquelette, l'acquisition d'une importante motilité cellulaire et la cellule perd également ses récepteurs d'endocytose / phagocytose (Trombetta et al. 2005). La cellule exprime alors des molécules de co-stimulation telles que CD40, CD80 et CD86 (Pasquier et al. 2004), ainsi que la molécule du MHC de classe II, à la surface de la cellule (Pierre et al. 1997). La CPA libère ensuite des chimiokines afin d'initier une réponse adaptée à l'antigène phagocyté, telles que CXCL1, CXCL2 et CXCL3 pour les Lymphocytes NK (*Natural Killer*) immature, CXCL8 pour les

neutrophiles ou CCL3, CCL4, CCL5, CXCL9, CXCL10 et CXCL11 pour les lymphocytes T mémoires, CXCL13 pour les lymphocytes B et T spécialisés dans les réponses humorales, CXCL19 et CXCL21 pour les lymphocytes T naïfs, et CXCL22 pour les T régulateurs ou Treg (Ueno et al. 2007, Panzer et al. 2010). Les MPS sont activées par de nombreux antigènes provenant des agents infectieux (bactéries, virus, levures, champignons et parasites), des débris cellulaires ou encore des polluants et des allergènes.

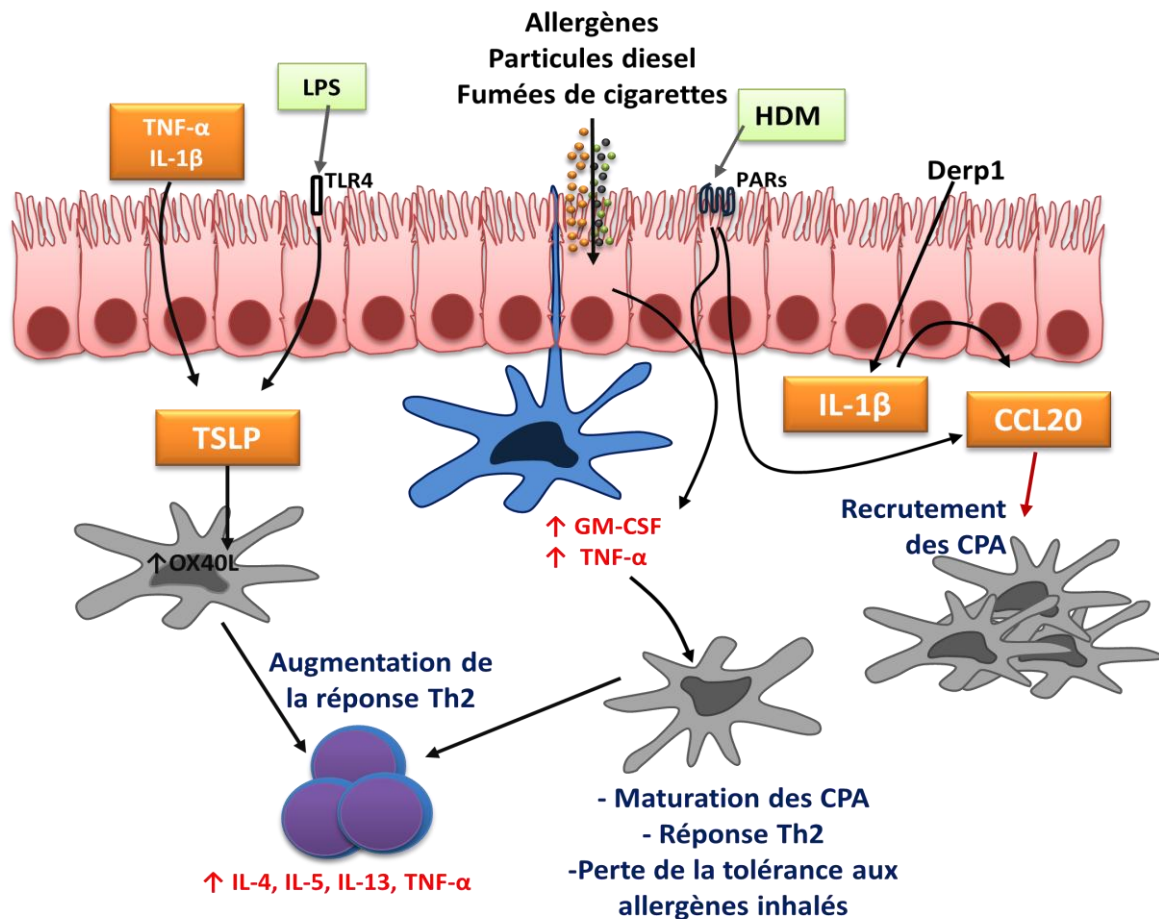


Figure 7: Interactions entre les cellules présentatrices d'antigène (CPA) et l'épithélium bronchique dans la pathogénèse de l'asthme allergique. Schéma simplifié illustrant les médiateurs impliqués dans la diaphonie entre les cellules épithéliales et les CPA et qui contribuent à l'inflammation allergique. Lipopolysaccharides des bactéries gram négatif (LPS), *Toll-like receptor 4*(TLR4), *Protease activated receptors* (PARs), *House dust mite* (HDM), protéase de *Dermatophagoides pteronyssinus*, groupe 1(Derp1). Adapté de Gill et al. 2010.

Normalement, les cellules du MPS présentes à la surface de l'épithélium bronchique, capturent les antigènes inoffensifs présents dans l'environnement, tels que les pollens et les acariens, puis maturent en CPA et interagissent avec les lymphocytes Treg et T naïfs. La production importante d'IL-10 par les lymphocytes Treg empêche alors la différenciation des T naïfs en Th2. Dans l'asthme allergique, cette tolérance est biaisée par la libération de cytokines par les mastocytes et les macrophages, notamment du TNF α , ainsi que la libération de TSLP (Thymic stromal lymphopoietin),

et d'IL-1 β par les cellules épithéliales, en réponse à l'allergène (Zhang et al. 2009, Wang. 2013). TSLP induit l'expression de la protéine OX40L (un ligand membre de la famille du TNF) par les CPA, et interagit avec le récepteur OX40 (ou CD134, membre de la famille des récepteurs du TNF) présent sur les lymphocytes T naïfs et conduit à leur différenciation en lymphocytes de type Th2, en initiant les événements de signalisation qui conduisent à la transcription de GATA-3 et la production d'IL-4, d'IL-5 et d'IL-13 (So et al. 2006). L'IL-1 β stimule la production et la libération de TSLP par l'épithélium, et le TNF α peut interagir directement avec les CPA et est connu pour son rôle perturbateur dans les mécanismes de tolérance face aux allergènes (Eisenbarth et al. 2002, Zhang et al. 2009).

Dans l'asthme, le nombre de CPA dans les voies aériennes est augmenté en réponse à un allergène. Cette augmentation est d'autant plus importante lorsque l'allergène est accompagné par des débris bactériens tels que du LPS (Lipopolysaccharides des bactéries gram négatif) qui stimulent les TLR4 (*Toll-like receptor 4*) et des protéases qui stimulent les PARs (*protease activated receptors*) présents à la surface des cellules des voies aériennes et entraînent la libération de chimiokine CCL2 et CCL20 impliquées dans le recrutement des CPA et des cytokines pro-inflammatoires comme l'IL-25, l'IL-33 et TSLP, ainsi que du GM-CSF, qui activent ensuite les CPA pour devenir pleinement compétentes dans la capture de l'antigène et induire leur migration vers les ganglions lymphatiques les plus proches (Gill. 2012). La migration des CPA est facilitée par la libération de la métalloprotéinase matricielle MMP-9 (*Matrix metalloproteinase-9*) par les leucocytes, tels que les éosinophiles, les neutrophiles et les macrophages alvéolaires, qui dégradent le collagène de type IV, le constituant majeur de la membrane basale (Vermaelen et al. 2003).

Les CPA ont également un rôle important dans la réponse antivirale car elles représentent la source la plus importante d'IFN- α et IFN- β , deux cytokines qui activent différents types de cellules du système immunitaire telles que les neutrophiles, les macrophages, les CPA, les lymphocytes NK (*Natural Killer*), B et T CD8+, afin de promouvoir la clairance virale et induire l'apoptose des cellules infectées pour empêcher la réplication virale (Wang et al. 2012). Les virus stimulent les TLRs présents à la surface des CPA, notamment les TLR7 et TLR9, et entraînent la libération d'IFN- α par les CPA. L'IFN- α est connu pour inhiber la différenciation des lymphocytes Th2 et Th17 et bloquer l'inflammation allergique. Chez les patients asthmatiques, la liaison de l'allergène aux IgE liés aux récepteurs FC ϵ RI, présents sur les CPA, inhibe la production d'IFN- α par les CPA lors de l'exposition aux virus des voies respiratoires tels que la grippe ou le rhinovirus (Gill et al. 2010). Ce mécanisme semble être impliqué dans les phénomènes d'exacerbations induites par les infections virales des voies aériennes (Figure 8).

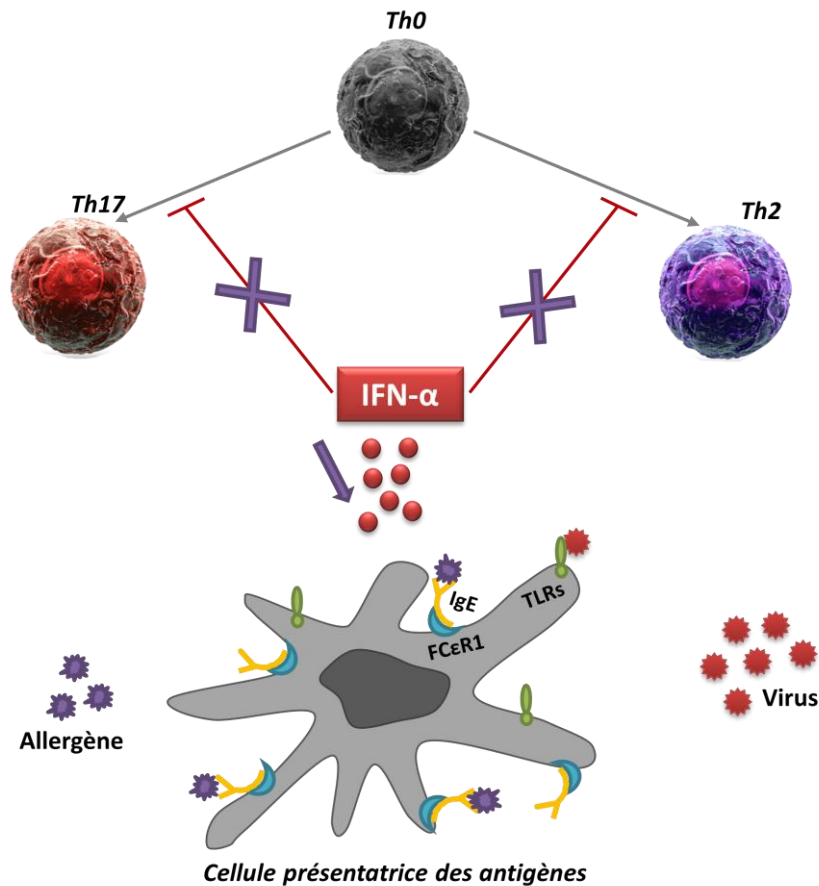


Figure 8: Relations entre les virus, l'IFN- α , les IgE et les cellules présentatrices d'antigène (CPA) dans l'asthme. Chez le patient asthmatique, l'activation des voies médiées par les IgE sur les CPA, empêche la production d'IFN- α induite par les virus. En cas d'infections virales, l'absence de production d'IFN- α pourrait promouvoir la différenciation des lymphocytes en Th2 et Th17 et contribuer aux exacerbations de l'asthme induites par les virus respiratoires. Récepteur de haute affinité pour les IgE (FC ϵ R1), *Toll-like receptor* (TLRs). Adapté de Gill et al. 2010.

I.2.2.3. Les lymphocytes

Le rôle du lymphocyte B a été établi de longue date par la mise en évidence de la synthèse d'IgE spécifiques d'allergènes dans l'asthme allergique (Ishizaka et al. 1966). En revanche, l'implication des lymphocytes T dans la réaction inflammatoire est de connaissance plus récente (Figure 9) (Romagnani et al. 1990). L'analyse de biopsies de patients asthmatiques, met en évidence la présence de nombreux lymphocytes infiltrés dans la *lamina propria* et dans l'épithélium. Ce sont majoritairement des lymphocytes T-helper de type CD4⁺ alors que les lymphocytes B sont rares. La population des lymphocytes T-helper est constituée de nombreuses sous-populations (Figure 10), classées en fonction de leurs profils de sécrétion de cytokines (Romagnani. 2001, Holgate. 2012).

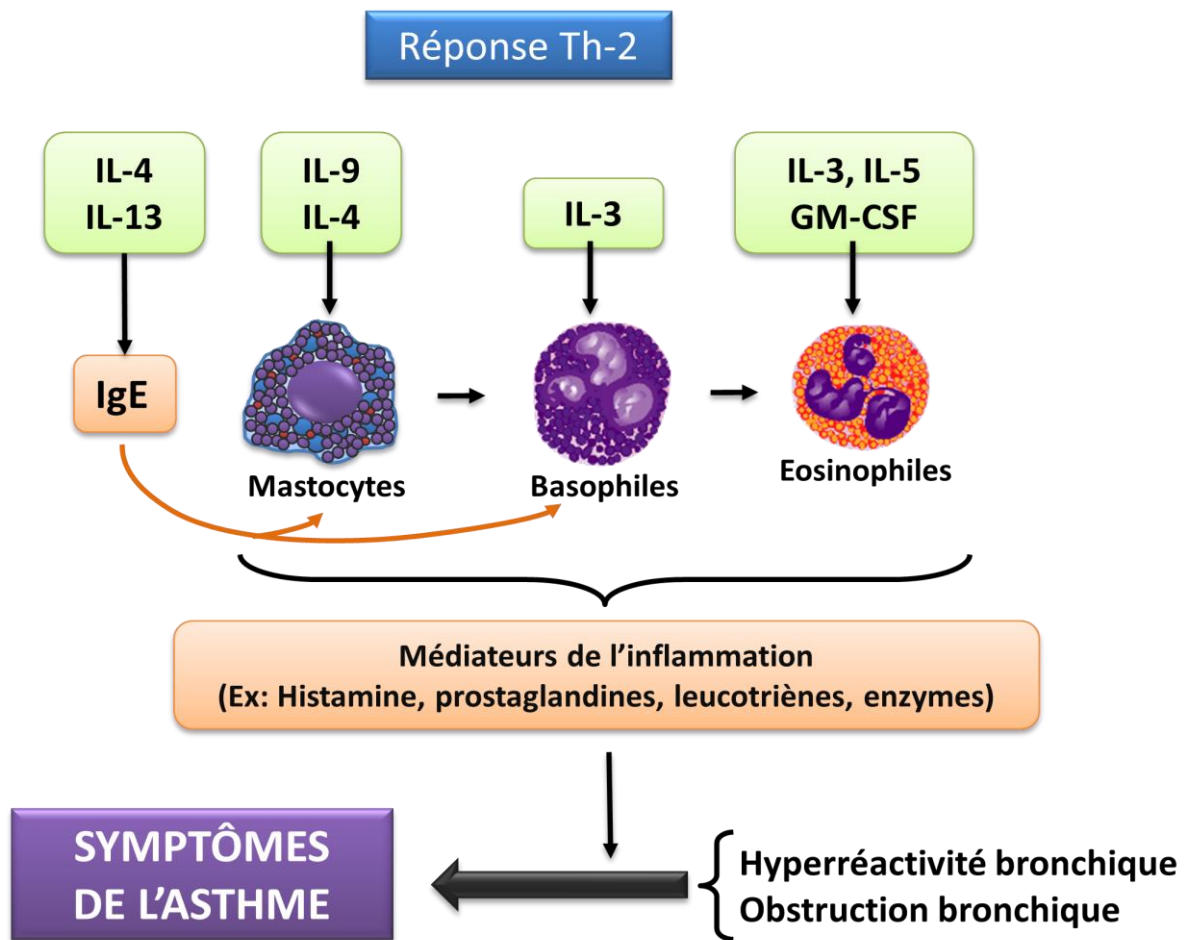


Figure 9: Mécanismes et contrôle de la réponse immunologique par les lymphocytes Th2 dans l'inflammation allergique. Les lymphocytes Th2 stimulent la synthèse des IgE par la libération d'IL-4 et d'IL-13. Les IgE sensibilisent les mastocytes et les basophiles en se fixant sur le récepteur de haute affinité des IgE (FcεRI). La libération d'IL-9 et d'IL-4, par les Th2, stimule le recrutement des mastocytes, tandis que l'IL-3 stimule la croissance et augmente le nombre de basophiles circulant. La prolifération, la différenciation et la libération des éosinophiles sont potentialisées par l'IL-3, le GM-CSF et l'IL-5. Lors de la rencontre avec un allergène, les mastocytes et les basophiles dégranulent, libérant des médiateurs préformés et nouvellement synthétisés, comme l'histamine, des leucotriènes et des cytokines qui favorisent la perméabilité vasculaire, la contraction du muscle lisse bronchique, la production de mucus, le recrutement des éosinophiles et des lymphocytes Th2. Ces mécanismes sont impliqués dans l'hyperréactivité et l'obstruction bronchique.

Les lymphocytes Th1 sont impliqués dans la sensibilisation allergique et peuvent contribuer au développement de l'asthme allergique et/ou exacerber ses manifestations (Rowe et al. 2004, van Oosterhout et al. 2005). Ils sont caractérisés par une production prédominante d'IFN- γ . Le nombre de ces cellules de profil Th1 augmente lorsque les patients sont sous corticothérapie (Krouwels et al. 1996).

Les lymphocytes Th2 renforcent la production d'IgE par la production d'IL-4 et d'IL-13 et entraînent la différenciation, le recrutement et l'activation des éosinophiles via la production d'IL-3, d'IL-5 et de GM-CSF (*Colony-Stimulating Factor*). L'IL-4 favorise également l'expression de VCAM-1 sur les cellules endothéliales, une protéine d'adhérence cellulaire indispensable au recrutement des mastocytes et

potentialise la libération de β -hexosaminidase, de LTC₄ et d'IL-8 par les mastocytes matures activés par voie IgE-dépendante (Feuser et al. 2011).

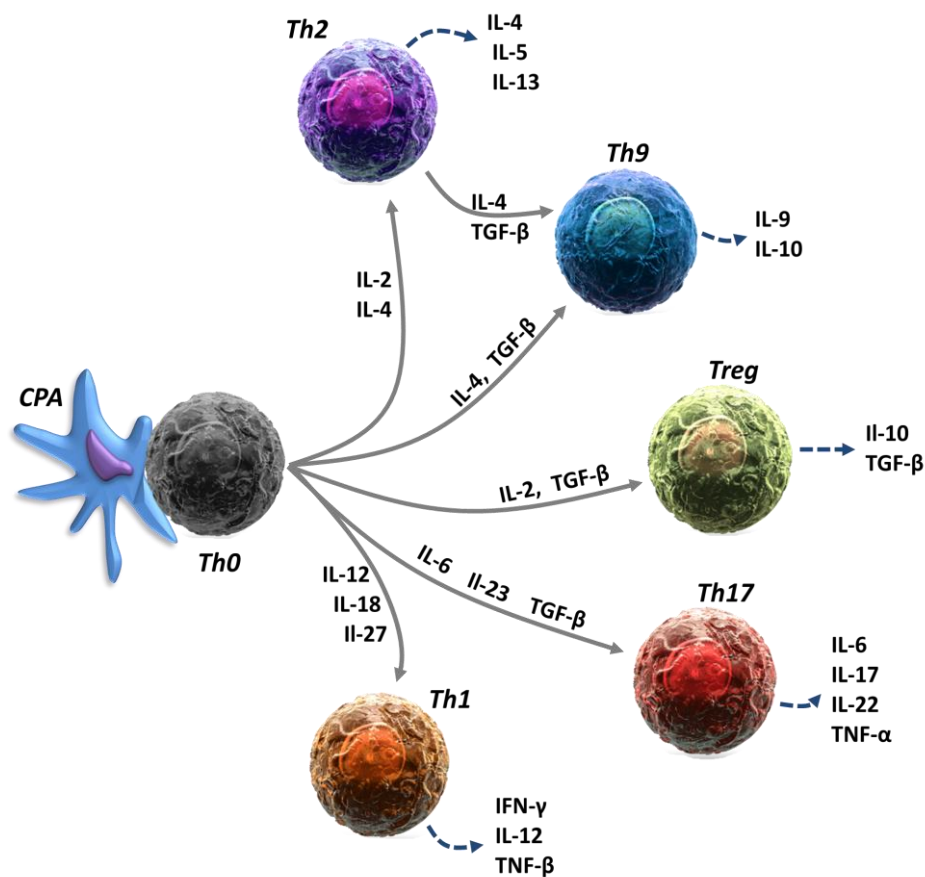


Figure 10: La différenciation des lymphocytes T helper CD4⁺ naïfs ou Th0. La différenciation des lymphocytes Th0 en cellules effectrices Th1, Th2, Th17, Treg ou Th9 dépend des cytokines présentes dans l'environnement cellulaire (ou signaux de co-stimulation) lors de la stimulation de la cellule Th0 par une cellule présentatrice de l'antigène. La différenciation des lymphocytes peut avoir lieu dans la médullaire du thymus ou dans les ganglions lymphatiques périphériques. Les cytokines libérées par les cellules Th1 peuvent inhiber la différenciation et la fonction des cellules Th2, et réciproquement. Les cytokines des lymphocytes Treg inhibent la différenciation des Th0 en Th1 et Th2 et contribuent à la génération des lymphocytes Th17 et Treg. Il a récemment été mis en évidence que l'association entre l'IL-4 et le TGF- β entraîne une différenciation des Th0 en Th9 et est aussi en mesure de "reprogrammer" les Th2 en Th9.

Les Lymphocytes Th17 sont caractérisées par la production prédominante d'IL-17A, IL-17F, IL-6, TNF- α , et IL-22. La libération d'IL-17A et d'IL-17F par les Th17 augmente à son tour la libération d'IL-6, IL-1 β , de GM-CSF (*granulocyte-macrophage colony-stimulating factor*) et chimiokines CXCL1, CXCL2, et CXCL8 par les cellules épithéliales, endothéliales et les fibroblastes, ces facteurs favorisant l'expansion des neutrophiles dans la moelle osseuse, leur survie et leur recrutement dans les voies aériennes. Dans l'asthme, l'équilibre entre éosinophilie et neutrophilie semble être déterminé par un équilibre entre l'IL-4 et IL-17, l'IL-4 ayant été décrit comme un puissant inhibiteur de l'IL-17.

L'IL-17 est aussi impliquée dans le remodelage bronchique par l'induction de l'expression de deux gènes codant pour les mucines MUC5AC et MUC5B dans les cellules épithéliales, ainsi que de plusieurs cytokines pro-fibrosantes comme l'IL-6 et l'IL-11, et favorise, avec l'IL-22, la migration des cellules musculaires lisses des voies aériennes (Halwani et al. 2013).

Une sous-population de lymphocytes T CD4⁺ et CD25⁺ également appelée lymphocyte T régulateur ou T reg est capable d'induire des phénomènes de tolérance. Ces Treg représentent environ 10 % des cellules T CD4⁺ et produisent les cytokines immuno-régulatrices TGF- β (Transforming Growth Factor) et IL-10 (Wu et al. 2007, Palomares et al. 2010). Lorsqu'ils sont activés, les Treg peuvent réguler l'activité des CPA. L'IL-10 inhibe la libération des molécules co-stimulatrices et réduit l'expression du MHC de classe II par les CPA, entraînant une diminution de sa capacité à présenter les antigènes à des lymphocytes T classiques. Les Treg, producteurs d'IL-10, régulent la prolifération et la production d'IL-5 des lymphocytes T, et sont impliqués dans les phénomènes de tolérance observés avec les immunothérapies spécifiques aux pollens de bouleau et aux acariens (Akdis et al. 2011). L'IL-10 produite par les Treg réduit la production d'IgE à la fois totaux et spécifiques de l'allergène, tout en augmentant simultanément la production des IgG4 (Meiler et al. 2008). Les Treg peuvent également avoir une action directe sur les mastocytes en empêchant leur dégranulation (Gri et al. 2008), et sur les éosinophiles en réduisant leur survie dans le poumon (Luttmann et al. 1998).

La libération de TGF- β par les Treg réduit la prolifération des lymphocytes T et B et permet donc de moduler la réponse inflammatoire et la production d'IgE, mais joue aussi un rôle dans la pathogenèse de l'asthme. Le TGF- β diminue la production d'enzymes qui dégradent la matrice extracellulaire (collagénases), augmente la production de protéines qui inhibent la production des enzymes dégradant la matrice extracellulaire (inhibiteur tissulaire de la métalloprotéase ou TIMP), et augmente la production de protéines de la matrice extracellulaire par les fibroblastes, ces activités favorisant un remodelage tissulaire (Akdis et al. 2009).

Le TGF- β contribue à la génération des lymphocytes Th17 et Treg. TGF- β dirige la conversion des cellules T effectrices en Treg et en présence d'IL-6, le TGF- β favorise la génération de lymphocytes Th17 à partir des cellules T naïves. Il a récemment été mis en évidence que l'IL-4 bloque la différenciation des lymphocytes naïfs en Treg sous l'influence du TGF- β et entraîne alors la différenciation en lymphocytes Th9 (Dardalhon et al. 2008). L'association IL-4 et TGF- β semble également pouvoir "reprogrammer" les lymphocytes Th2 en Th9 (Veldhoen et al. 2008). Ces lymphocytes Th9 perdent leurs caractéristiques précédentes, notamment leur "signature cytokinique" (production d'IL-4, d'IL-5 et d'IL-13) et produisent alors majoritairement de l'IL-9, mais aussi de l'IL-10.

La fonction physiologique de l'IL-9 semble être en quelque sorte proche de la réponse Th2. Dans l'asthme, l'IL-9 contribue à la maladie en favorisant la production des IgE induite par l'IL-4, l'expression de la chaîne α du récepteur à l'IL-5 sur les éosinophiles, ainsi qu'en induisant la production de mucus et de chimiokine, telle que CCL11, par les cellules épithéliales, et en favorisant la prolifération des mastocytes et la production de plusieurs cytokines, notamment de l'IL-5 et de l'IL-13 par les mastocytes qui en retour contribuent à l'inflammation et l'hyperréactivité des voies aériennes (Stassen et al. 2012, Tete et al. 2012).

I.2.2.4. Les Eosinophiles

Il semble exister une corrélation entre le nombre d'éosinophiles et la sévérité de l'asthme allergique, ainsi qu'avec l'hyperréactivité bronchique, mais elle est controversée (Anderson. 2008). En clinique, des études mettent en évidence que le nombre d'éosinophiles dans les voies aériennes peut être corrélé à la sévérité de l'asthme allergique (Fujitaka et al. 2011) et avec l'hyperréactivité bronchique chez les patients asymptomatiques (Schwartz et al. 2012). Des résultats controversés par d'autres études qui montrent une faible corrélation entre la sévérité de l'inflammation des voies aériennes et les marqueurs de la sévérité de l'asthme, tel que le volume d'air exhalé durant la première seconde de l'expiration forcée ou FEV1 (Leckie et al. 2000, Green et al. 2002). Il apparaît cependant que les éosinophiles permettent un suivi régulier de la maladie, d'appréhender la sensibilité à l'exacerbation et les réponses aux traitements, et représentent donc un biomarqueur utile pour guider le traitement de l'asthme allergique.

Les éosinophiles sont des leucocytes normalement destinés à l'élimination des parasites de l'organisme, en se fixant à leur surface pour déverser le contenu de leurs granules, des enzymes destinées à les détruire. Dans l'asthme, cette capacité destructrice de l'éosinophile se traduit par une altération importante de l'épithélium bronchique, laquelle influence la survenue et la pérennisation de l'hyperréactivité bronchique. Les éosinophiles activés libèrent de multiples médiateurs stockés dans les granules ou résultant d'une ré-activation membranaire. Les protéines contenues dans les granules de l'éosinophile sont majoritairement la MBP (*Major Binding Protein*) ou PRG2 (*proteoglycan 2*), une protéine fortement cationique, ainsi que l'EPO (*eosinophil peroxidase*), l'ECP (*eosinophil cationic protein*) et l'EDN (*eosinophil-derived neurotoxin*). Ces peroxydases et ribonucléases perforent les membranes et augmentent localement le stress oxydant, causant la mort cellulaire par apoptose et nécrose, et sont responsables d'atteintes du tissu nerveux. La MBP peut induire la dégranulation des mastocytes et des basophiles, et est impliquée dans le remodelage du système nerveux périphérique pulmonaire (Giembycz et al. 1999, Melo et al. 2009).

Les éosinophiles sont une source importante de médiateurs lipidiques, particulièrement le LTB₄, le LTC₄ et le PAF (Platelet Activating Factor) qui interviennent dans les phénomènes de

bronchoconstriction et dans l'augmentation de la perméabilité vasculaire (Figure 11). Ils sécrètent également des cytokines telles que IL-3, IL-4, IL-5, IL-8, IL-10, IL-13, IL-16 et IL-18, des chimiokines CCL5 et CCL11, ainsi que des facteurs de croissance : TNF- α , Gm-CSF et TGF- β (*Transforming Growth Factor*) (Rothenberg et al. 2006, Stone et al. 2010, Shamri et al. 2011). Les éosinophiles sont une source importante de TGF- β et sont impliqués dans la génération des lymphocytes Th9 et le remodelage bronchique (Akdis et al. 2009).

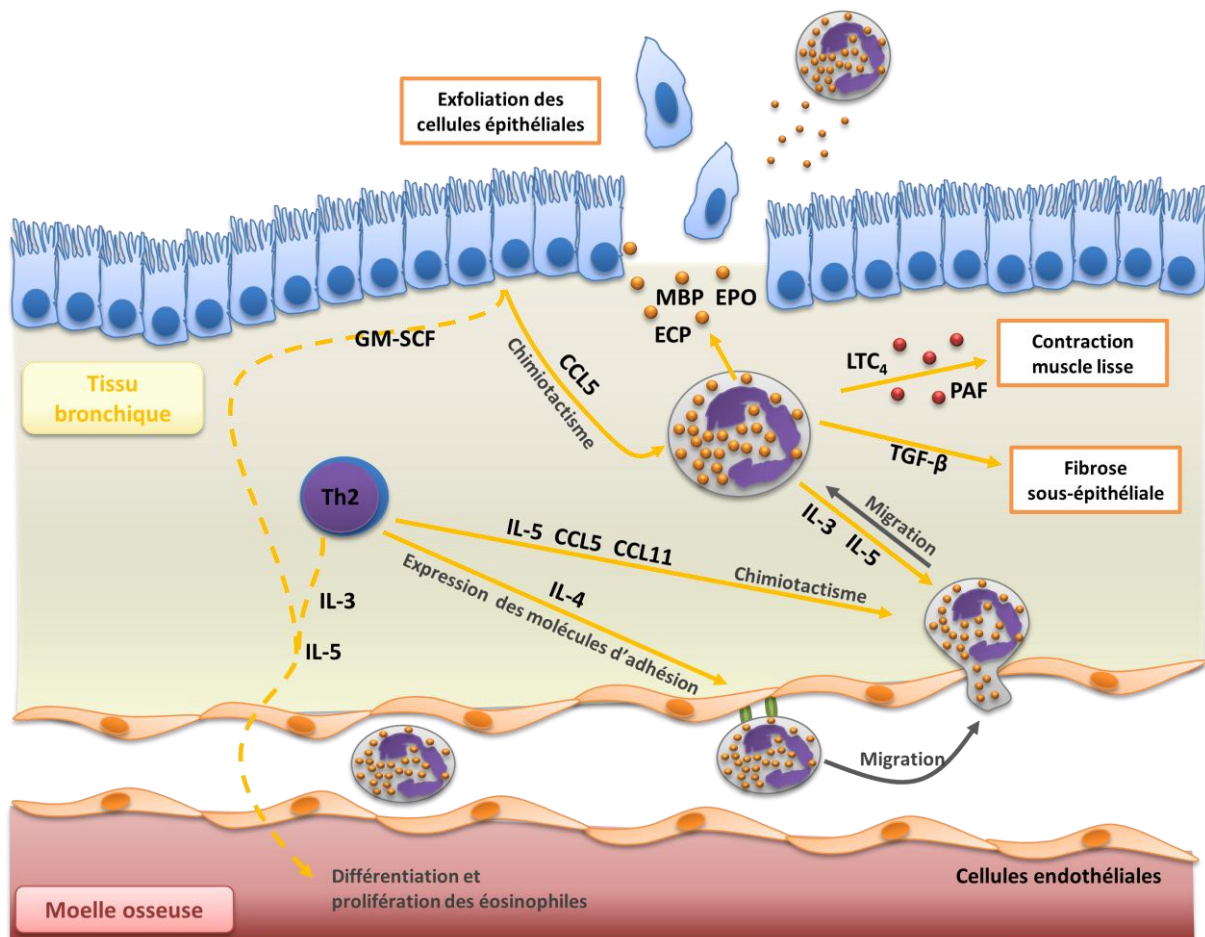


Figure 11: Recrutement et rôles des éosinophiles dans l'inflammation allergique. Représentation des principaux médiateurs impliqués dans la différenciation et la prolifération des éosinophiles, ainsi que dans la migration et le recrutement des éosinophiles vers les bronches. Les éosinophiles activés libèrent des enzymes (EPO, MBP, ECP) qui endommagent l'épithélium bronchique, mais également des médiateurs lipidiques (LTC₄ et PAF) impliqués dans la bronchoconstriction et la vasodilatation, des cytokines (IL-3, IL-5) et chimiokines (CCL5 et CCL11).

La prolifération et la différenciation des cellules précurseur des éosinophiles dans la moelle osseuse est potentialisée par l'IL-3, le GM-CSF et l'IL-5 qui sont des facteurs de croissance de l'éosinophile (Gleich 2000). La libération des éosinophiles dans la circulation sanguine est stimulée par l'IL-5. Une fois libérés de la moelle osseuse, les éosinophiles circulent dans le sang en direction des tissus. Il est à noter qu'une fois libérés dans la circulation sanguine, la demi-vie des éosinophiles dans le sang est courte (de 3 à 18h), comparativement à leur demi-vie observée dans les tissus qui est d'au moins 6

jours, ce qui suggère que les éosinophiles présents dans le sang sont rapidement recrutés vers les tissus (Wen et al. 2013). Les éosinophiles sont recrutés dans les bronches par l'IL-5 et les chimiokines CCL3, CCL5 et CCL11, libérées par l'épithélium, les macrophages, les lymphocytes et les éosinophiles eux-mêmes (Broide et al. 2011).

I.2.2.5. Les neutrophiles

De nombreux types de cellules sont impliqués dans la physiopathologie de l'asthme. La contribution des mastocytes, lymphocytes, et éosinophiles a été bien établie. Néanmoins, seulement 50% des cas d'asthme sont associés à une inflammation à éosinophiles, et dans la plupart des autres cas, l'asthme a été accompagné par une augmentation de neutrophiles dans les voies aériennes liée à une augmentation de CXCL8 (Il-8), et semble être corrélée avec la sévérité de l'asthme (Figure 12). Le nombre de neutrophiles dans les expectorations est similaire entre les patients présentant un asthme léger à modéré et les sujets sains, mais est augmenté chez les patients atteints d'asthme sévère (Fahy et al. 1995, Jatakanon et al. 1999, Porsbjerg et al. 2013) .

Dans l'asthme, les neutrophiles sont recrutés par CXCL8, le LTB₄, et le PAF (Platelet Activating Factor) libéré par les mastocytes. CXCL8 est également libérée par les cellules épithéliales et les neutrophiles eux-mêmes (Eckmann et al. 1993, Gainet et al. 1998). La libération de CXCL8 par les neutrophiles conduit à une boucle d'amplification de l'activation des polynucléaires neutrophiles et semble sous l'influence des IgE chez les patients asthmatiques. La libération de CXCL8 est spécifique de l'allergène et inhibée par un anti-IgE (Monteseirín et al. 2004) et est due à l'expression des récepteurs FCεRI et FCεRII à la surface des neutrophiles, expression qui semble être augmentée lors des périodes d'exacerbation, probablement sous l'influence des cytokines IL-4 et IL-9 (Alphonse et al. 2008). CXCL8 est également un facteur chimiotactique pour les lymphocytes T (Mukaida. 2003) et les éosinophiles activés par l'IL-5 (Schweizer et al. 1994). Ainsi, la libération de CXCL8 par les neutrophiles impliquant les IgE pourrait contribuer aux manifestations inflammatoires caractéristiques de l'asthme sévère. La libération de CXCL8 pourrait prolonger l'activation des neutrophiles, et ainsi accroître la libération d'élastase, et d'autres médiateurs qui pourraient exacerber le remodelage bronchique (Mukaida. 2003, Monteseirín. 2009).

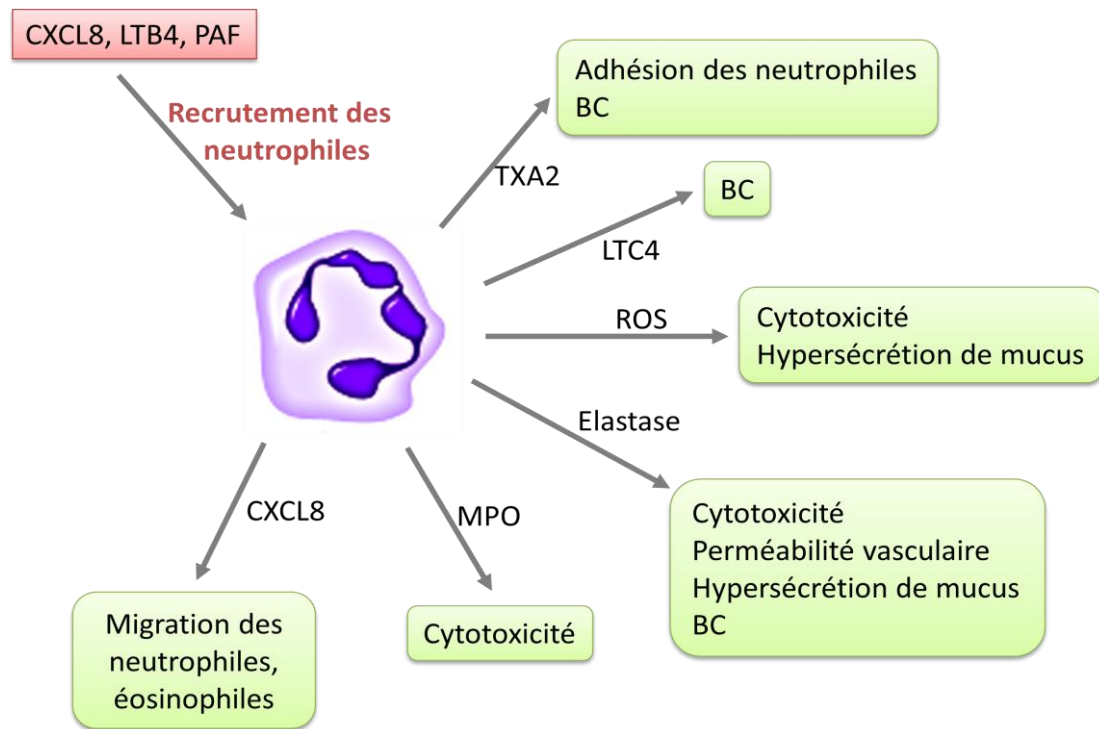


Figure 12: Rôle des neutrophiles dans l'inflammation allergique. Représentation des principaux médiateurs libérés par le neutrophile et leurs rôles dans l'asthme. Bronchoconstriction (BC).

L'élastase détruit les cellules épithéliales, augmente la perméabilité vasculaire, mais aussi stimule l'hypersécrétion de mucus et la métaplasie des cellules à mucus, et est impliquée dans la bronchoconstriction, et l'hyperréactivité bronchique (Kawabata et al. 2002). L'élastase des neutrophiles est capable de digérer la plupart des protéines de la matrice extracellulaire, dont plusieurs types de collagène, la fibronectine, les protéoglycannes, l'héparine, la fibrine, mais aussi le surfactant pulmonaire. Les rôles biologiques de l'élastase proviennent de son activité protéasique. La dégradation de la surface apicale des cellules épithéliales réduit l'inhibition des récepteurs TLR4, conférés par la matrice extracellulaire, ce qui potentialise l'activation des récepteurs TLR4 par les lipopolysacharrides (LPS) présents dans l'environnement, et engendre l'expression de chimiokine CXCL8 par les cellules épithéliales (Devaney et al. 2003, Brunn et al. 2005). La libération de CXCL8 est aussi stimulée par le clivage du récepteur CXCR1 (un récepteur de CXCL8), qui libère un fragment soluble capable d'activer le TLR2 qui augmente la transcription de CXCL8 (Hartl et al. 2007). La libération de CXCL8 sous l'action de l'élastase attire à son tour les neutrophiles.

CXCL8 entraîne également la libération de la métalloprotéinase (MMP)-9 par les neutrophiles, ce qui favoriserait la circulation des éosinophiles et des CPA vers la lumière bronchique en leur permettant de migrer plus facilement à travers la membrane basale et l'épithélium (Cataldo et al. 2002, Vermaelen et al. 2003).

L'élastase du neutrophile soutient son activité protéolytique par la dégradation de l'alpha1-antitrypsine (A1AT), un inhibiteur des sérines protéases, mais aussi celle de la MMP-9 en activant la pro-MMP-9 et en dégradant son inhibiteur naturel, l'inhibiteur tissulaire de la métalloprotéase-1 (TIMP-1) (Cantin et al. 1989, Gaggaret al. 2007).

L'élastase altère la clairance mucociliaire en inhibant la motilité ciliaire et augmente l'expression des gènes codant pour les mucines, et la sécrétion des mucines. L'élastase stimule les PARs (protease activated receptors) et entraîne la translocation de PKC δ (*Protein kinase C*) vers la membrane plasmique conduisant à la libération d'EGF et de TNF- α puis à l'activation subséquente de l'EGFR (*Epithelial growth factor receptor*) qui ralentit la motilité ciliaire et stimule la production et l'excrétion de mucus (Hamilton et al. 2001). Sous l'effet de l'élastase, les cellules épithéliales surexpriment une oxydoréductase cytoplasmique, la NADPH quinone oxydoréductase 1, qui est impliquée dans la surexpression de l'ARNm codant pour MUC5AC. En plus d'augmenter la production de mucines, l'élastase stimule la sécrétion de mucus, notamment des mucines respiratoires MUC5AC et MUC5B, de par l'activation de la PKC δ , dont la translocation du cytoplasme vers la membrane plasmique est associée à l'activation transitoire de MARCKS (*myristoylated alanine-rich C-kinase substrate*), une protéine qui chaperonne l'exocytose (Voynow et al. 2009).

L'élastase régule aussi plusieurs aspects du remodelage bronchique. Elle peut notamment engendrer un arrêt du cycle cellulaire des cellules épithéliales, un phénomène associé à une expression accrue de p27kip1, un inhibiteur des kinases dépendantes des cyclines. De plus, l'élastase diminue l'expression de ErbB2, un récepteur du facteur de croissance épidermique, EGF, à la surface des cellules de l'épithélium bronchique et entraîne une diminution de la prolifération épithéliale (Fischer et al. 2005). L'élastase contribue également aux processus destructeurs et fibrotiques des voies aériennes et du parenchyme. La dégradation de la matrice extracellulaire libère du VEGF (*Vascular endothelial growth factor*), du PDGF-AA (*Platelet-derived growth factor AA*), du PDGF-BB et du TGF- β 1 piégés dans la matrice (Hyytiäinen et al. 2004, Wada et al. 2007). Ainsi, la dégradation de la matrice extracellulaire libère des facteurs de croissance importants pour la réparation épithéliale et qui peuvent induire une fibrose sous-épithéliale (Chua et al. 2006).

L'élastase peut aussi induire une bronchoconstriction et une hyperréactivité bronchique. Le clivage de l'acide hyaluronique, un glycosaminoglycane, par l'élastase libère de la kallitréine tissulaire, une protéine clivant le kininogène en bradykinine, un puissant agent broncho-constricteur, et vasodilatateur (Forteza et al. 1999, Scuri et al. 2000).

Les neutrophiles libèrent également des ROS (Reactive Oxygen species), du TXA2 (thromboxane A2), des cytokines dont l'IL-1 β , le TNF- α , l'IL-6 et CXCL8, et du LTC4 (Monteseirín. 2009). Le LTC4 stimule la contraction du muscle lisse bronchique. En l'absence d'agents chimioattractants des éosinophiles, les neutrophiles activés par CXCL8 augmentent également la migration des éosinophiles par la libération

d'anions superoxydes, de MMP-9, de LTB4 et de PAF. Les neutrophiles activés par CXCL8 pourraient ainsi conduire à une accumulation des éosinophiles dans les voies aériennes des patients asthmatiques (Nakagome et al. 2012).

I.2.2.6. Les macrophages

Les macrophages sont des phagocytes provenant de la différenciation des monocytes sanguins et font partie du système phagocytaire mononucléaire ou MPS (*mononuclear phagocyte system*). Une fois localisés dans les tissus, les macrophages acquièrent des fonctions spécialisées, dépendantes des exigences du tissu. Dans les poumons, les macrophages alvéolaires acquièrent la capacité de libérer de grandes quantités d'oxydants cytotoxiques (ROS), pour détruire les agents pathogènes et les xénobiotiques inhalés (Brehm et al. 1996, Tasat et al. 2003). Les macrophages sont les cellules immunitaires les plus abondantes dans les tissus pulmonaires normaux et jouent un rôle critique dans les réponses immunitaires innées et spécifiques, comme dans le développement des maladies inflammatoires pulmonaires. Les macrophages pulmonaires résident à l'interface entre l'hôte et l'environnement et participent au maintien de la fonction et de la structure normale du poumon par leurs diverses fonctions : phagocytose, reconnaissance des stimuli de l'environnement, production et libération d'une grande variété de médiateurs. Les macrophages ont un rôle fondamental dans l'immunité spécifique par leurs interactions avec les lymphocytes. Les macrophages infiltrent la muqueuse bronchique et sont activés dans l'asthme (Figure 13). Les macrophages sont capables de sécréter des dérivés de l'acide arachidonique tels que la PGE2, le thromboxane (TX)-B2, le LTBA, le LTC4, le LTD4, le LTE4, les acides 5- et 15-hydroxyéicosatétraénoïques (5-HETE et 15-HETE) et les lipoxines (LXs). Ils synthétisent également un grand nombre de cytokines pro-inflammatoires comme le TNF- α , l'IL-5, l'IL-6 et l'IL-1 β , ou des cytokines anti-inflammatoires comme l'IL-10 et des chimiokines telles que CXCL8 et CCL5 (Smith et al. 2009). Les macrophages jouent un rôle important dans la réparation des tissus, par la sécrétion de facteurs de croissance comme le PDGF (Platelet Derived Growth Factor), le FGF- β (Fibroblast Growth Factor- β) et le TGF- β (Murray et al. 2011).

Dans l'asthme allergique, les macrophages ont un rôle important dans la réponse à l'allergène. La présence des récepteurs Fc ϵ RI et Fc ϵ RII (CD23) à la surface des macrophages permet la fixation des IgE et participe à la reconnaissance de l'allergène (Ying et al. 1998, Katoh et al. 1999). La reconnaissance de l'allergène entraîne la production de cytokines pro-inflammatoires telles que du TNF- α , IL-1 β , IL-6, CXCL8, CCL2 (MCP-1), CCL3 (MIP-1 α) et CCL5 (Rantes) et dans une moindre mesure, une cytokine anti-inflammatoire: l'IL-10 (Gosset et al. 1999, Qian et al. 2011).

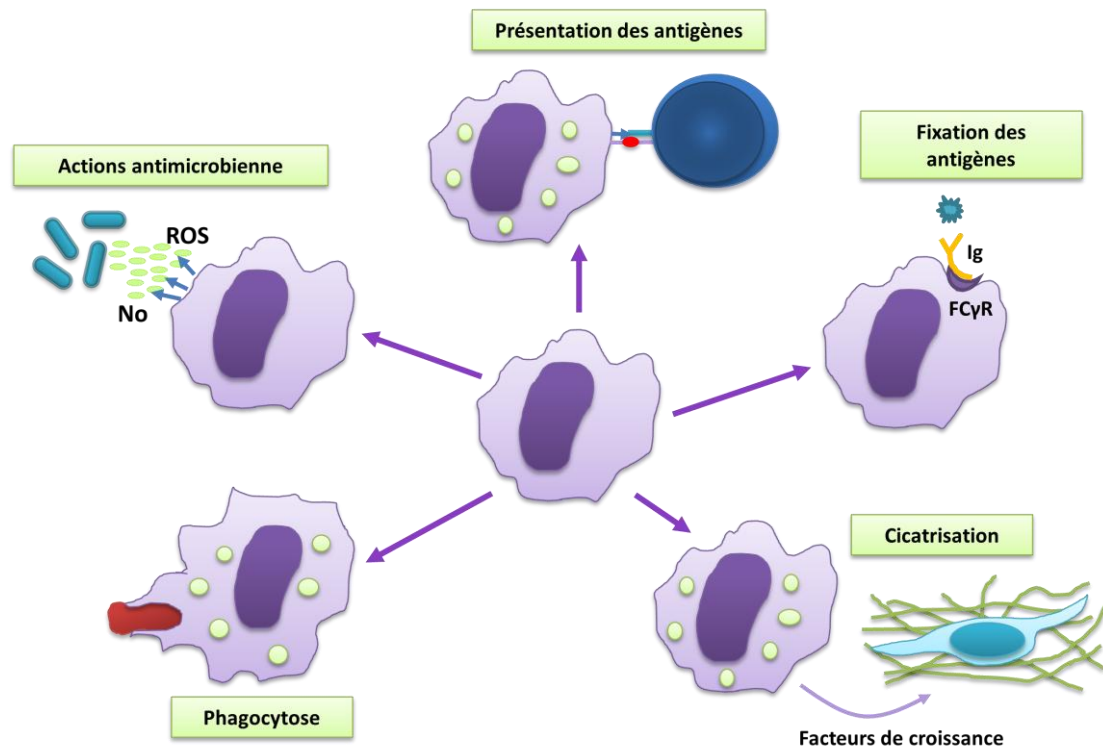


Figure 13: Rôles multiples des macrophages dans l'immunité et l'homéostasie.

La liaison de l'allergène aux IgE fixés sur les récepteurs FcεRI, serait responsable de la maturation des macrophages en CPA (Pozzi et al. 2005), une capacité soutenue par l'expression des clusters de différenciation CD80 et CD86 par les macrophages, deux récepteurs impliqués dans la présentation de l'antigène aux lymphocytes T (Balbo et al. 2001, Lim et al. 2012). La stimulation des récepteurs FcεRI peut aussi entraîner une surexpression du récepteur de l'histamine H1 et une biosynthèse accrue d'histamine (Toujas et al. 1997, Novak et al. 2013). De par la libération d'histamine, le macrophage participe à la bronchoconstriction, la vasodilatation et l'œdème observé chez les patients asthmatiques.

Les dernières avancées suggèrent que la diversité des activités biologiques des macrophages est médiée par des sous-populations phénotypiquement distinctes de cellules qui se développent en réponse aux médiateurs inflammatoires qu'ils rencontrent dans leur microenvironnement (Laskin et al. 2011, Van Loon et al. 2013). Les macrophages peuvent être différenciés en macrophages de type M1 ou M2 (Figure 14). Les macrophages sont différenciés en M1 sous l'influence des cytokines de type 1 comme l'IFN-γ et le TNF, ou après la reconnaissance de motifs moléculaires associés à des pathogènes ou PAMPS (par exemple, le lipopolysaccharide (LPS), des lipoprotéines, l'ARN double brin, ou l'acide lipoteichoïque) et de signaux de "danger" endogène tels que les protéines de choc thermique ou les protéines cytoplasmiques HMGB1 (protéines du groupe de haute mobilité 1), libérées dans l'espace extracellulaire lors de l'apoptose (Bell et al. 2006). Les macrophages M1 présentent une activité microbicide et anti-tumorale puissante, libèrent des interleukines IL-12 et IL-

23, et promeut une réponse immunitaire pro-inflammatoire de type Th1. En outre, ils exercent des activités antiprolifératives et cytotoxiques, qui résultent de la libération de ROS (les espèces réactives de l'oxygène) et de RNS (les espèces réactives de l'azote) et des cytokines pro-inflammatoires telles que de l'IL-1 β , l'IL-6 et le TNF- α (Biswas et al. 2010, Balhara et al. 2011).

Au contraire, la présence d'IL-4 et d'IL-13, deux cytokines Th2 impliquées dans le développement de l'asthme, stimule la différenciation en macrophage M2. Ces macrophages de type M2 sont souvent considérés comme assumant des fonctions anti-inflammatoires car ils inhibent la production d'un grand nombre de médiateurs pro-inflammatoires, notamment par la libération d'IL-10. Les macrophages M2 contribuent également à la résolution de l'inflammation par l'élimination des neutrophiles apoptotiques (par phagocytose) et la synthèse des médiateurs importants dans la réparation tissulaire et l'angiogenèse, comme le TGF- β , le VEGF et l'EGF (Wang et al. 2007, Barrientos et al. 2008), des facteurs qui peuvent contribuer au remodelage bronchique observé dans l'asthme (Yum et al. 2011, Song et al. 2012).

Les macrophages M2 produisent également des cytokines de type Th2 telles que l'IL-4, l'IL-10 et l'IL-13 et jouent un rôle clé dans la régulation de la fonction des lymphocytes T. Leur fonction dans l'allergie et l'asthme reste controversée, certaines études suggérant que les macrophages M2 favorisent l'inflammation allergique, notamment par la production d'IL-4 et d'IL-13 (Byers et al. 2011). Cependant, l'activation des macrophages est un processus dynamique, les mêmes cellules peuvent initialement prendre part à des réactions pro-inflammatoires ou cytotoxiques et participer ensuite à la résolution de l'inflammation (Martinez et al. 2008, Porcheray et al. 2007, Mosser et al. 2008).

In vivo, la distinction entre les macrophages de type M1 et M2 est rendue possible par des profils d'expression différents (Sica et al. 2006, Martinez et al. 2008, Clària et al. 2011, Korn et al. 2011, Krausgruber et al. 2011). Chez la souris, les macrophages M1 sont associés à des marqueurs tels que la présence du facteur de transcription IRF5 (Interferon regulatory factor 5), de iNOS (Nitric oxide synthase) dans le cytoplasme, ou encore de marqueurs membranaires tels que le récepteur CCR7 (CD197), les clusters de différenciation CD25 (chaîne alpha du récepteur de l'IL-2), CD86, CD127 (chaîne alpha du récepteur de l'IL-7), CD215 (chaîne alpha du récepteur de l'IL-15) ou encore une forte expression du MHCII. Les macrophages M2 sont eux associés à des marqueurs tels que Ym1 et Ym2 (2 petites protéines de 45kd de la famille des chitinases, des enzymes capables de dégrader la chitine, un constituant de la paroi des champignons tel que *Aspergillus fumigatus*), les clusters de différenciation CD163 (un récepteur "decoy" pour le complexe hémoglobine-haptoglobine), CD206 (récepteur du Mannose), CD209 (ou DC-SIGN pour Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) ou encore le récepteur dectin-1 (le principal récepteur au β -glycannes, un constituant des parois cellulaires des levures et de certains champignons et bactéries).

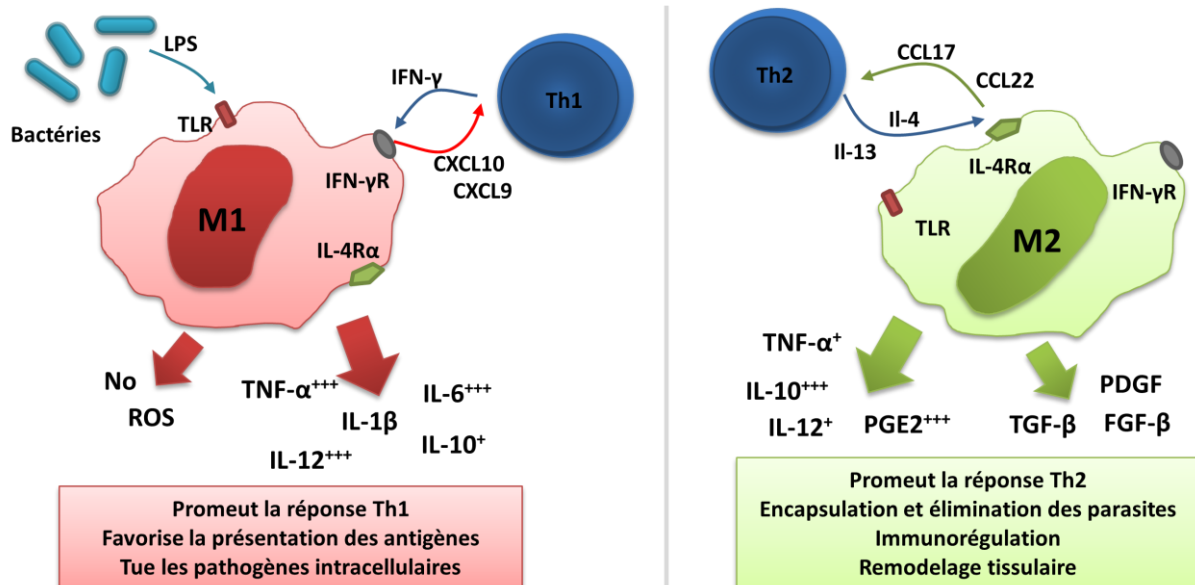


Figure 14: Polarisation des macrophages M1 et M2. Représentation des principaux médiateurs impliqués dans la polarisation et l'activation des macrophages M1 et M2. Lorsque les tissus sont endommagés, les médiateurs inflammatoires sont libérés, entraînant la mobilisation des lymphocytes et l'activation des macrophages. La présence de lipopolysaccharides (LPS de la paroi bactérienne) et la libération d'interféron- γ (IFN- γ) par les lymphocytes Th1 stimulent l'activation des macrophages qui présentent alors un phénotype M1. Cette activation conduit à la production de monoxyde d'azote (NO), d'espèces réactives de l'oxygène (ROS), d'interleukine-1 et 6 (IL-1 et IL-6) et de TNF- α , qui sont importants dans l'arsenal antimicrobien. La production d'IL-4 et IL-13 par les lymphocytes Th2 stimule l'activation de macrophages de type M2. Ces macrophages produisent des facteurs de croissance tels que le facteur de croissance dérivé des plaquettes (PDGF), le TGF- β 1 et le facteur de croissance des fibroblastes (FGF- β) qui favorisent la cicatrisation et la fibrose et participent au remodelage tissulaire. Ces macrophages produisent également de l'IL-10, une cytokine anti-inflammatoire, qui permet de réguler la production des médiateurs pro-inflammatoires.

1.2.3. L'Épithélium bronchique dans l'asthme

L'épithélium bronchique forme une barrière continue le long de l'appareil respiratoire et sa principale fonction est de séparer l'hôte de l'environnement extérieur en jouant le rôle de barrière mécanique avec une activité mucociliaire et de barrière physiologique régulant la perméabilité transépithéliale. Les fonctions structurales et de barrière de cette couche dépendent d'un ensemble de mécanismes d'adhésion intrinsèque entre les cellules épithéliales et avec la lame basale (ex: jonctions serrées, jonctions d'ancrage, desmosomes). L'épithélium bronchique est continu, de type cilié et d'épaisseur variable. Pseudostratifié au niveau de la trachée et des bronches, l'épithélium apparaît ensuite unistratifié au niveau des bronchioles. L'épithélium des bronches proximales est composé de 3 types cellulaires principaux: les cellules basales, les cellules ciliées et les cellules sécrétrices (Knight et al. 2003).

Les cellules sécrétrices composent 15 à 20% de l'épithélium bronchique, stockent et libèrent le mucus qui permet de capturer les débris inhalés, les virus et les bactéries. Le mucus est produit par les cellules caliciformes et est constitué de glycoprotéines de haut poids moléculaire, les mucines telles que MUC5AC et MUC5B, ainsi que d'acide sialique, responsable de sa viscoélasticité. Une viscoélasticité qui garantit l'épuration mucociliaire grâce au battement actif des cils présents au pôle apical des cellules ciliées. La sécrétion de mucus est accrue en cas d'agression, et les cellules sécrétrices prolifèrent en cas d'agression chronique (Rogers 2003). Un type particulier de cellules sécrétrices, les cellules "Club" (*Club cells*, anciennement dénommées cellules de Clara) produisent et libèrent des collectines (SP-A, SP-D), des lectines hydrophiles couplées à des polysaccharides ou à une partie de type collagène, qui assurent une fonction antimicrobienne et sont capables de moduler l'inflammation des voies aériennes (Lesur et al. 1996, Kasper et al. 2002). Cette dernière fonction est également assurée par la protéine CC10, produite par les cellules "Club" (Singh et al. 1997, Chen et al. 2001).

Dans le but de maintenir son rôle de barrière physique, l'épithélium bronchique est doté d'une importante capacité de régénération. Les cellules épithéliales sécrètent l'HB-EGF du côté basolatéral, où il est séquestré, tandis que les récepteurs EGFR sont situés sur le côté apical de la cellule épithéliale. L'HB-EGF est séquestré du côté basal, notamment en se liant aux héparanes sulfates présents à la surface des cellules (Higashiyama et al. 1993), mais aussi grâce aux jonctions adhérentes et aux jonctions serrées entre les cellules épithéliales intactes, qui forment une barrière imperméable (Puddicombe et al. 2000). L'altération de l'épithélium entraîne une fuite de l'EGF qui peut alors activer l'EGFR et promouvoir la réparation de l'épithélium (Davies et al. 2009). Les cellules épithéliales les plus proches de la zone altérée migrent puis prolifèrent jusqu'à leur confluence puis se différencient en cellules caliciformes et libèrent du mucus afin d'éliminer les substances étrangères via la clairance mucociliaire. Les cellules à mucus disparaissent ensuite progressivement (métaplasie) et la structure épithéliale retourne à la normale (Burgel et al. 2004). Dans l'asthme, une atteinte chronique de l'épithélium entraîne une desquamation et une perte de l'intégrité (perturbations des jonctions serrées et d'ancrages) des cellules épithéliales et conduit à une stimulation répétée des mécanismes de réparation, notamment ceux liés à l'EGF. Ces mécanismes, liés à une libération accrue, par les cellules épithéliales, de TGF- β , et d'HB-EGF (heparin-binding EGF), stimulée par l'IL-13 (Allahverdian et al. 2008), conduisent à un remodelage tissulaire caractérisé par un épaississement progressif de la paroi bronchique accompagné d'une hypertrophie et une hyperplasie du muscle lisse bronchique, ainsi qu'une hyperplasie des cellules glandulaires associée à une hypersécrétion de mucus (Lambrecht et al. 2012). Un épaississement de la membrane basale et une fibrose sous-épithéliale sont également décrits (Holgate et al. 2000, Holgate. 2008).

L'épithélium respiratoire joue aussi le rôle de barrière immunitaire par la modulation des réponses immunitaires et inflammatoires en libérant des médiateurs pro-ou anti-inflammatoires. Des anomalies morphologiques ou fonctionnelles de cet épithélium bronchique contribuent à l'initiation ou la progression du processus inflammatoire et à la pathogenèse de l'asthme (Figure 15).

Les cellules épithéliales bronchiques produisent un grand nombre de médiateurs pro- ou anti-inflammatoires. Elles peuvent initier et prolonger la réaction inflammatoire en recrutant et en modulant l'activité des cellules inflammatoires (Lambrecht et al. 2012, Nagarkar et al. 2012). Elles libèrent spontanément ou après stimulation des agents chimioattractants, incluant des métabolites de l'acide arachidonique et des chimiokines telles que CCL2 (MCP-1), CCL5 (rantes), CCL13 (BCA-1), CCL11 (éotaxine), CXCL8 (IL-8) et CXCL1 (GRO-1). Les chimiokines CCL2, CCL5 et CCL11 sont impliquées dans le recrutement des éosinophiles.

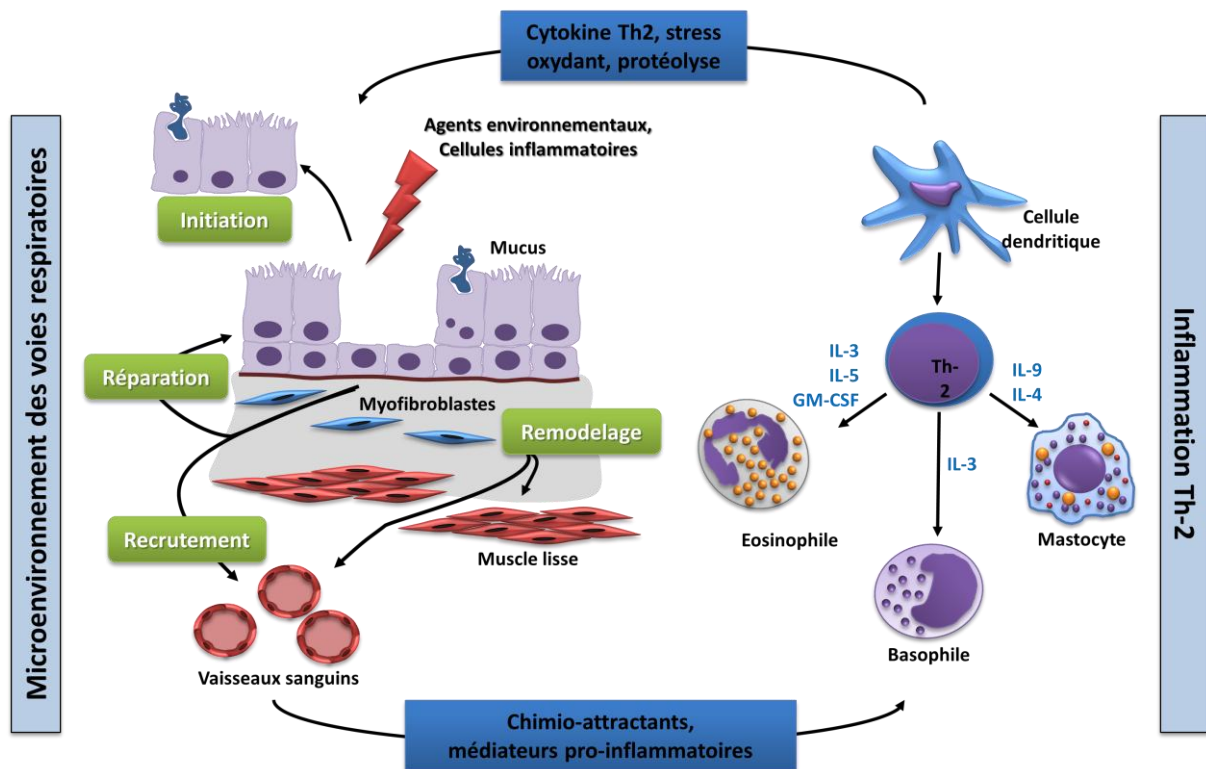


Figure 15: Représentation schématique du rôle de l'épithélium bronchique dans l'asthme. Dans l'asthme, les cellules épithéliales sont altérées et stimulées de manière chronique sous l'effet des allergènes, des polluants, et les produits oxydants et protéolytiques des cellules inflammatoires. En réponse, l'épithélium bronchique produit des facteurs de croissance pour promouvoir sa reconstruction. La production continue de ses facteurs de croissance est impliquée dans le remodelage des tissus, notamment dans la prolifération du muscle lisse et l'angiogenèse. Les cellules épithéliales produisent aussi des médiateurs pro-inflammatoires et chimio-attractants impliqués dans la réponse inflammatoire. Adapté de Lemanske et al. 2010.

L'épithélium bronchique peut libérer de nombreuses cytokines pro-inflammatoires comme le GM-CSF, l'IL-1 α et β , le TNF- α , l'IL-3, l'IL-6 et l'IL-13, mais aussi des facteurs de croissance comme le TGF- α , le TGF- β , l'EGF (*Epidermal Growth Factor*), l'IGF (*Insulin-like Factor*), le VEGF et le PDGF qui modulent l'activité des cellules inflammatoires. Les cellules épithéliales des sujets asthmatiques

produisent également de grande quantité de périostine, une protéine de la matrice extracellulaire, qui stimule la production du TGF- β et modifie la synthèse de collagène par les myofibroblastes des voies aériennes (Sidhu et al. 2010, Sehra et al. 2011).

L'IL-13 semble avoir un rôle important dans la pathologie de l'asthme, mis en évidence chez les souris transgénique surexprimant IL-13 dans les *Club cells* (Zhu et al. 1999). Ces souris transgéniques présentent une inflammation éosinophilique des voies aériennes accompagnée d'une forte production de CCL11 (éotaxine), d'une métaplasie des cellules à mucus, une fibrose bronchique, une obstruction des voies aériennes, et une hyperréactivité à la métacholine. Ainsi, l'IL-13 stimule les cellules épithéliales et induit la production CCL11 impliquée dans le recrutement des éosinophiles (Matsukura et al. 2001), mais peut aussi augmenter la production de mucus, induire des lésions épithéliales et une hyperréactivité bronchique, et favoriser la fibrose bronchique (Kuperman et al. 2002). L'IL-13 induit aussi une augmentation du nombre de cellules à mucus, diminue le nombre de cellules ciliées et diminue la fréquence des battements ciliaires (Figure 16) (Wadsworth et al. 2012).

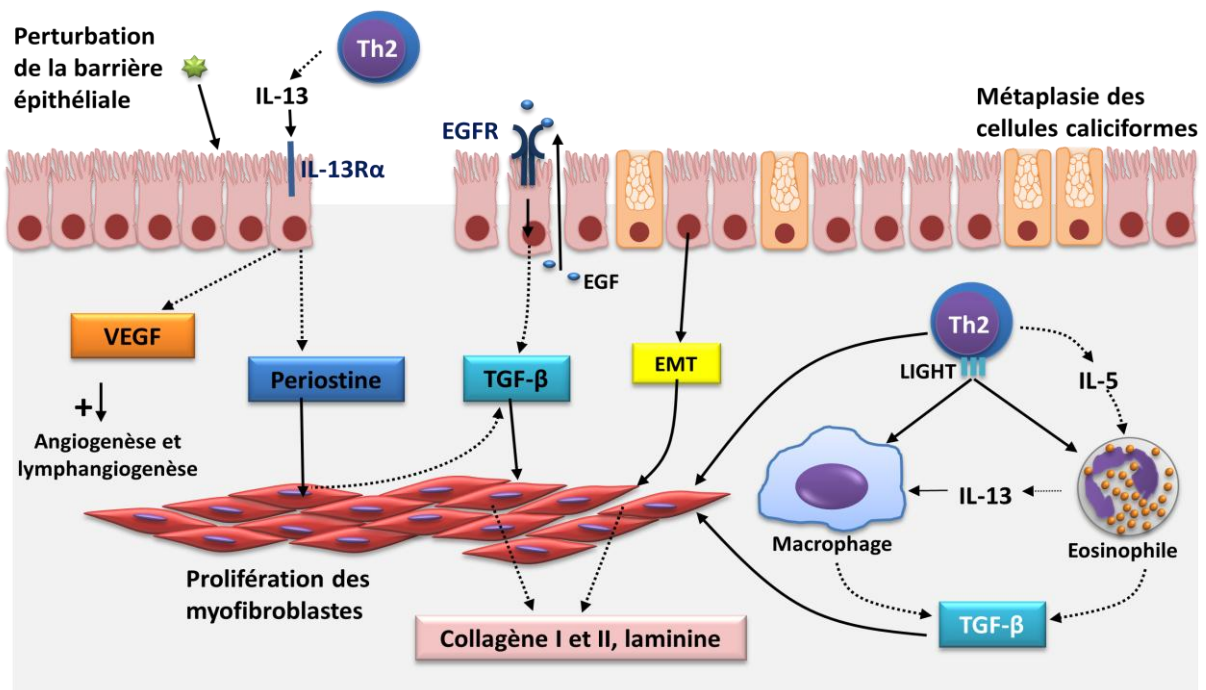


Figure 16: Remodelage des voies aériennes dépendant de l'épithélium bronchique dans l'asthme. L'altération chronique de l'épithélium entraîne la production de cytokines et de facteurs de croissance comme le TGF- β et le VEGF impliqués dans la prolifération du muscle lisse bronchique, l'angiogenèse et la lymphangiogenèse. Les jonctions épithéliales sont altérées par les enzymes protéolytiques, permettant la fuite de l'EGF vers la lumière bronchique et l'activation des récepteurs EGFR, stimulant la prolifération des cellules endothéliales et la réparation de l'épithélium, mais aussi leur différenciation en cellules caliciformes. Ces cellules libèrent également de la périostine qui augmente la production de TGF- β et conduit à la prolifération des myofibroblastes et la synthèse de collagène. Les éosinophiles sont aussi une source de TGF- β qui peut influencer la production d'IL-5 par les lymphocytes Th2. Un processus de transition épithélio-mésenchymateuse (Epithelial-mesenchymal transition, EMT) peut éventuellement se mettre en place. Adapté de Lambrecht et al. 2012.

Les cellules épithéliales libèrent aussi des médiateurs anti-inflammatoires comme la PGE2, l'IL-10, le 15(S)-HETE et le TGF- β . La PGE2 diminue la production de chimioattractants et l'IL-10 réduit la production de TNF- α et d'IL-1 β par les macrophages tandis qu'ils inhibent la prolifération des lymphocytes et sont impliqués dans les phénomènes de tolérance (Akdis et al. 2011, Sastre et al. 2012). Les cellules épithéliales libèrent d'importantes quantités de 15(S)-HETE qui inhibent la production de leucotriènes par les neutrophiles, le chimiotactisme des neutrophiles induit par le LTB4 et la production d'anion superoxyde. Le TGF- β inhibe la libération de cytokines par les macrophages et inhibe la libération d'IL-8 (CXCL8) par les cellules épithéliales bronchiques (Lambrecht et al. 2012).

Ainsi, l'asthme allergique est une maladie inflammatoire chronique des voies aériennes, impliquant de nombreux facteurs environnementaux, et dont les processus physiologiques et immunologiques sous-jacents sont complexes et nécessitent encore d'être explorés. Pour cela, de nombreux modèles animaux sont développés et utilisés pour élucider la pathophysiologie de l'asthme, ainsi que pour identifier et évaluer de nouvelles cibles thérapeutiques.

II. Modèles d'asthme allergique

De nombreux modèles animaux d'asthme allergique ont été développés afin de permettre l'étude de la pathogenèse de la maladie et de développer de nouvelles thérapies. Ces modèles sont basés sur la modélisation du comportement clinique de l'asthme allergique, et ont conduit à mieux comprendre la physiopathologie pulmonaire. Parmi les animaux utilisés, la souris est aujourd'hui l'espèce majoritaire. Les souris ne sont pas naturellement sujettes à l'asthme, ce qui implique qu'une réaction artificielle, semblable à l'asthme, doit être induite dans les voies respiratoires. Les modèles d'asthme chez la souris reproduisent rarement toutes les caractéristiques de la maladie humaine, mais elles développent des symptômes qui modélisent l'asthme allergique et sont caractérisés par une inflammation des voies aériennes avec une hyperéosinophilie, une hyperréactivité bronchique (HRB), une élévation des IgE, et un remodelage des voies aériennes avec hyperproduction de mucus et dépôt de collagène.

Il existe néanmoins des différences entre l'asthme induit chez la souris et la pathophysiologie humaine. L'HRB à la méta-choline mesurée chez la souris est transitoire et consécutive aux provocations allergéniques alors que chez l'homme, elle est présente en permanence chez les patients asthmatiques, même en l'absence de symptômes. Les modèles d'asthme allergique chez la souris ne sont pas des répliques exactes de la maladie humaine, mais les observations effectuées dans ces modèles murins soutiennent la plupart des paradigmes existants.

II.1. Animaux

Il existe de nombreuses espèces animales utilisées pour modéliser l'asthme allergique. Souris, rats, cochons d'Inde, furets, chiens, moutons, singes et chevaux ont été utilisés pour étudier les processus inflammatoires et les altérations de la fonction des voies aériennes. Chaque animal possède des avantages et des inconvénients et reproduit plus ou moins fidèlement l'asthme allergique (Tableau 3). L'animal le plus utilisé est sans conteste la souris. La souris présente l'avantage de produire préférentiellement des IgE en réponse à un allergène et offre la possibilité d'explorer des mécanismes détaillés des réactions allergiques en raison de la disponibilité de nombreux "réactifs immunologiques" tels que cytokines recombinantes et anticorps. De plus, les souches de souris consanguines permettent le transfert de cellules entre des animaux de même souche pour l'évaluation de la fonction des facteurs et cellules spécifiques. En plus de ces dernières, la souris présente une gamme importante d'outils génétiques puisqu'il est possible de générer des souris génétiquement modifiées. Cet arsenal est complété par une reproduction facile et une courte

période de gestation. L'utilisation de la souris a donc permis de nombreux progrès dans la compréhension des mécanismes impliqués dans l'asthme allergique et dans le développement des médicaments. Cependant, en raison de différences physiologiques considérables, entre la souris et l'homme, l'extrapolation des résultats à l'homme doit être judicieuse. Parmi les différences observées, la souris ne présente pas d'HRB spontanée et l'hyperplasie du muscle lisse bronchique est plus difficile à démontrer, de même que l'implication du système nerveux dans l'asthme. De plus, les modèles d'asthme allergique chez la souris pourraient présenter, pour la plupart, une résolution spontanée des différents symptômes après l'arrêt des provocations allergéniques et dans certains cas, un phénomène de tolérance ou de désensibilisation à l'allergène pouvant résulter de l'administration de dose trop élevée et/ou répétée d'allergène. Ces phénomènes pourraient dépendre de la souche de souris, des conditions d'élevages, des allergènes employés et de leur qualité (présence de contaminants), et être en partie attribués à l'absence de cofacteurs environnementaux comme la pollution et les infections pulmonaires (Eisenbarth et al. 2002, Swirski et al. 2002, Jaspers et al. 2009, Maes et al. 2010). L'analyse de ces données est d'autant plus difficile, que les conditions d'élevages sont souvent mal définies (état sanitaire, filtration, saison) et que seulement quelques modèles publiés ont fait l'objet d'une étude approfondie de l'évolution et de la persistance des symptômes mesurés tels que l'inflammation, le remodelage bronchique ou l'HRB.

Parmi les autres animaux utilisés durant ces 2 dernières décennies, le cochon d'Inde ou cobaye a permis l'étude du bronchospasme en réponse à l'allergène et de l'HRB, notamment l'hyperréactivité aux agonistes cholinergiques (aussi histamine, PGs, TxA2, autres), et la production d'anticorps de type IgG1 et IgE. Le cochon d'Inde a l'avantage de présenter une réponse immédiate à l'allergène, caractérisée par un bronchospasme consécutif à la provocation allergénique, suivie d'une phase retardée de bronchoconstriction (6 à 24h après l'administration de l'allergène) accompagnée d'un infiltrat leucocytaire, puis d'un remodelage bronchique et une HRB (Zhou et al. 1999, Che, et al. 2001, Smith et al. 2005, Jung et al. 2010, Evans et al. 2012). La réponse inflammatoire chez le cochon d'Inde est conforme à l'asthme observé chez l'homme, composée d'éosinophiles et de neutrophiles, et a permis d'identifier l'importance de l'inflammation des voies aériennes dans l'altération de la fonction respiratoire. Il existe cependant de nombreuses limites à ce modèle telles que l'absence de souches consanguines, le nombre limité de "réactifs biologiques" disponibles et de séquençage du génome ou encore la prédominance des IgG1 plutôt que des IgE (Canning et al. 2008).

Le rat a également été utilisé par de nombreux chercheurs. La réponse anaphylactique à un allergène implique majoritairement les IgE, ce qui permet l'étude du contrôle de la synthèse des IgE spécifiques de l'allergène. Autre avantage, le rat est susceptible de produire une HRB persistant durant plusieurs jours (7 jours) et l'on peut différencier la réponse immédiate et la phase tardive suite à la provocation (Xu et al. 2002). Cependant, la plupart des modèles développés et disponibles à ce jour

requièrent une étape de sensibilisation par injection de l'allergène, en combinaison avec des adjuvants comme l'hydroxyde d'aluminium (alum, Al(OH)₃), l'adjuvant de Freund ou *Bordetella pertussis* (Kucharewicz et al. 2008), plutôt qu'une sensibilisation par inhalation comme elle est utilisée chez le cochon d'Inde et est développée chez la souris durant ces dernières années. Comme pour le cochon d'Inde, les "réactifs immunologiques" spécifiques du rat ne sont pas aussi abondants que ceux disponibles pour la souris. Ce sont des limitations importantes à l'étude des réponses allergiques des voies respiratoires.

Les plus gros animaux comme le lapin, le singe, le mouton et le cheval ont été utilisés mais ils sont difficiles à manipuler et trop coûteux (Ali et al. 1994, Iwashita et al. 2008, Kier et al. 2011). Le cheval est le seul animal qui développe naturellement des troubles respiratoires caractérisés par une obstruction aiguë des voies respiratoires lorsqu'il est exposé à des poussières (Liccardi et al. 2012).

La souris est donc l'animal le plus utilisé, mais il convient de prendre en considération plusieurs éléments distincts dont les interactions sont exceptionnellement complexes, comme le fond génétique de la souris, l'allergène utilisé, le protocole expérimental et les paramètres mesurés.

Animal	Avantages	Inconvénients
Souris	Production d'IgE en réponse à l'allergène Réactifs immunologiques très nombreux Nombreuses souches consanguines Elevage facile et relativement peu coûteux Période de gestation courte	Ne présente pas d'HRB spontanée Musculature limitée des bronches Différences anatomiques du poumon Ne répond pas à l'histamine
Rat	Production d'IgE en réponse à l'allergène Produit une HRB durable (7 jours) Réponse immédiate et tardive	Les modèles disponibles requiert une sensibilisation par voie systémique et l'utilisation d'adjuvant Réactifs immunologiques moins abondants
Cochon d'inde	Le poumon est la cible de l'anaphylaxie Réponse immédiate et tardive	IgG1 majoritaire Absence d'animaux consanguins Réactifs immunologiques spécifiques peu abondants
Lapin	Réponse immédiate et phase tardive Le poumon est la cible de l'anaphylaxie Production d'IgE en réponse à l'allergène	Immunisation néonatale requise pour une phase tardive Réactifs immunologiques peu abondants
Grands Animaux	Le cheval développe naturellement de l'asthme Le singe est sensible aux <i>Ascaris</i> et à l'HDM (<i>house dust mite</i> : acariens)	Difficile à manipuler Coûteux

Tableau 3: Avantages et inconvénients des espèces animales utilisées dans les modèles d'asthme. Chaque espèce animale possède des avantages et des inconvénients et reproduit plus ou moins fidèlement l'asthme allergique humain (Ali et al. 1994, Iwashita et al. 2008, Shin et al. 2009, Kier et al. 2011).

II.2. Souches de souris

La réponse des souris de différentes souches à un protocole expérimental de sensibilisation est très variable dans les modèles d'asthme allergique. Certaines souches répondent fortement alors que d'autres paraissent résistantes (Morokata et al. 1999, Ewart et al. 2000). Les souches A/J et AKR/J présentent une HRB importante à la métacholine alors que les souris FVB/NJ, C3H/HeJ et DBA/2 sont relativement résistantes et présentent une faible réaction inflammatoire ainsi qu'une réponse à la métacholine modérée dans les conditions de sensibilisation utilisant un mélange d'OVA (400µg/kg) et d'alum (80mg/kg) au jour 0 et 14, suivi d'une provocation à l'OVA par aérosol (1%, 1 heure) au jour 21, l'inflammation et l'HRB à la métacholine ayant été mesurées 24h, 48h et 72h après la provocation allergénique (Whitehead et al. 2003).

Parmi les souches de souris, les BALB/c et les C57BL/6 sont les plus fréquemment employées en raison de leurs réponses immunologiques bien caractérisées. Les souris BALB/c développent une réponse immunitaire préférentiellement de type Th2, avec production d'IgE spécifiques de l'allergène, une HRB et une robuste inflammation éosinophilique des voies aériennes localisée principalement autour des vaisseaux (veines et artères pulmonaires) et des bronches, primaires et secondaires. Inversement, les souris C57BL/6 présentent des réactions immunitaires à dominance Th1, et des réactions allergiques limitées par rapport aux souris BALB/c, en particulier une faible production d'IgE et une plus faible HRB à la métacholine. Étonnamment, les souris C57BL/6 présentent une inflammation robuste avec une infiltration importante d'éosinophiles dans les tissus (Morokata et al. 1999, Shinagawa et al. 2003).

La disponibilité d'animaux génétiquement modifiés permet d'étudier l'influence des gènes dans la sensibilisation et le développement de l'inflammation allergique des voies respiratoires. Le séquençage complet du génome des souris C57BL/6, terminé en 2005, en a fait une souche privilégiée pour la génération de souris génétiquement modifiées (IL-4, IFN-γ, IL-6, Nlr4, Casp1, Naip5, cPLA-2, T-bet, c-maf, GATA-3, Foxp3, TGF-β3, RORγT, TSLP, Rag2,...) (Kuperman et al. 2005, Hausding et al. 2008, Lightfield et al. 2008, Martínez-Sanz et al. 2008, Nurieva et al. 2008, Takatori et al. 2008, Capitini et al. 2009, Hener et al. 2011). Quelques souris génétiquement modifiées utilisées pour étudier l'asthme allergique ont été générées sur souche Balb/c (STAT6, STAT4, TLR-4, TLR-9, IL-13, ...). L'utilisation de souris génétiquement modifiées, souris knock-out, avec délétion partielle ou totale, permanente ou conditionnelle, ou souris transgéniques par surexpression d'un gène, permet d'étudier la contribution d'une protéine et d'identifier les voies impliquées dans la physiopathologie de l'asthme. Ces études nécessitent un examen minutieux des effets observés, et de prendre en compte la possibilité d'un contournement des mécanismes de régulation ou de contrôle, perturber l'expression d'autres gènes ou encore être compensées par des voies inconnues.

II.3. Allergènes

Un certain nombre d'allergènes ont été utilisés dans des modèles animaux d'asthme allergique. Le principal allergène utilisé est l'ovalbumine de poulet (OVA). L'OVA est relativement peu coûteuse, elle peut être hautement purifiée. Les épitopes immuno-dominants ont été bien caractérisés, et des peptides recombinants ont été produits. L'OVA purifiée peut être préparée sans protéase ou endotoxine, des produits qui peuvent interférer avec le processus de sensibilisation et sont abondants dans d'autres allergènes. (Fuchs et al. 2008).

Pour la sensibilisation, l'OVA a été classiquement associée à un adjuvant et injectée pour induire la production d'anticorps et obtenir une réponse inflammatoire (Conrad et al. 2009), mais peut aussi être effective sans adjuvant, après administration intraperitonéale (Reber et al. 2013), locale (Eisenbarth et al. 2002, Yu et al. 2006) ou épicutanée (Spergel et al. 1998). Suite à la sensibilisation, une série de provocations par voie intranasale ou par inhalation permet d'obtenir une réponse inflammatoire dans les voies aériennes.

Dans la plupart des souches de souris, l'inhalation de dose trop élevée et/ou répétée d'OVA conduit au développement d'un phénomène de tolérance ou de désensibilisation à l'allergène plutôt qu'une exacerbation des symptômes allergiques. De nombreux modèles montrent une réduction de l'inflammation, du remodelage et de l'HRB après 2 à 6 semaines de provocation allergénique, une tolérance qui semble apparaître d'autant plus vite que les provocations allergéniques sont nombreuses (généralement composées d'une exposition de 30 à 60 min/jour à un aérosol d'une solution de 1 à 2,5% d'OVA). Dans ces modèles, ceux comportant plus de 3 provocations allergéniques par semaine entraînent le développement d'une tolérance dès 3 semaines (Swirski et al. 2002), alors que les modèles comportant 3 provocations allergéniques par semaine développent généralement une tolérance à partir de la 6^{ème} semaine (Sakai et al. 2001, Van Hove et al. 2007). De plus, la tolérance persiste après l'arrêt des provocations allergéniques (Yiamouyiannis et al. 1999, Schramm et al. 2004).

Bien que l'allergène soit un composant important dans l'induction de l'asthme allergique, d'autres facteurs, comme les lipopolysaccharides (LPS, endotoxines de la paroi bactérienne) peuvent y contribuer car ils sont omniprésents dans l'environnement et plus encore dans les animaleries (Lieutier-Colas et al. 2001). Les lipopolysaccharides semblent essentiels pour la sensibilisation à l'OVA chez la souris, comme cela est montré dans un modèle de sensibilisation à l'OVA par voie intranasale, où la présence de LPS est indispensable au développement d'une hyperéosinophilie, d'une HRB et pour augmenter la production d'immunoglobulines consécutive aux provocations allergéniques (Eisenbarth et al. 2002).

Les extraits ou les protéines purifiées dérivées d'allergènes puissants connus chez l'homme comme les acariens de la poussière de maison (House Dust Mite, HDM ; *Dermatophagoïdes pteronyssinus* Der p 1), le cafard, l'ambrosie ou herbe à poux (*ambrosia artemisiifolia*), ou des champignons tels que *aspergillus fumigatus* sont de plus en plus utilisés comme allergènes chez la souris (Lukacs et al 2002, Ulrich et al 2008, Gregory et al. 2011, Pakdaman et al. 2011, Wang et al. 2011). Ces allergènes induisent des crises d'asthme chez l'homme et certaines études démontrent leur capacité à induire une inflammation chronique des voies respiratoires et le remodelage bronchique chez la souris. La réponse est également différente en fonction des souches et des allergènes.

II.4. Protocoles expérimentaux chez la souris

À l'heure actuelle, il n'existe pas de protocole expérimental normalisé et chaque laboratoire a développé ses propres protocoles. On peut cependant les distinguer en modèles aigus et "chroniques".

II.4.1. Modèles aigus

Les modèles de réponse allergique aiguë aux allergènes inhalés ont été largement utilisés pour élucider les mécanismes qui sous-tendent les réponses immunologiques et inflammatoires dans l'asthme et l'identification et l'investigation de nouvelles cibles pour le contrôle de l'inflammation allergique.

Tous les modèles sont constitués d'une première phase, dite de sensibilisation, suivie d'une seconde phase, dite de provocation allergénique. La phase de sensibilisation est composée d'une ou plusieurs administrations systémiques ou locales d'allergène, avec ou sans adjuvant. Les adjuvants comme l'hydroxyde d'aluminium sont connus pour favoriser le développement des réponses de type Th2 (Brewer et al 1999, McKee et al 2009). La phase de provocation intervient généralement 2 à 4 semaines après la sensibilisation. Les allergènes sont alors administrés par aérosol ou par voie intranasale ou intratrachéale.

Ces modèles sont de courte durée, présentent une grande reproductibilité et comprennent des niveaux élevés d'IgE spécifiques de l'allergène, un influx important d'éosinophiles dans les voies respiratoires et une HRB. Cependant, le remodelage des voies aériennes est généralement faible et la plupart des autres paramètres (HRB, inflammation à éosinophiles) sont transitoires (Hogan et al. 2003). Les modèles aigus (Tableau 4) ont été beaucoup utilisés en première intention dans le développement de nouvelles thérapeutiques (Nials et al. 2008).

Souche	Sexe	Allergène	Sensibilisation	Exposition	Provocation	Réponses aux provocations	Références
Balb/c	Femelle	OVA		Aérosol	10 min/jour pendant 10 jours	Jour 11: Augmentation réponse muscle lisse <i>ex vivo</i>	Larsen et al. 1992
C57BL6	Mâle	OVA	OVA+Alum (i.p.) Jour 0	Aérosol	30 min/jour jours 7 à 14	Jour 15: éosinophiles	Brusselle et al. 1994
B6D2F1/J	Mâle	OVA	OVA+Alum (i.p.) Jour 0 et 5	Aérosol	2x60 min/ jour jour 12	Jour 13: IgE, éosinophiles	Kung et al. 1994
C57BL6	Mâle	OVA	OVA+Alum (i.p.) Jour 0 et 12	Aérosol	2x30 min/jour jour 24, puis 30 min/jour, J26,28,30 et 32	Jour 33: HRB, éosinophiles, oedeme	Hogan et al. 1997
Balb/c	Mâle	OVA	OVA (i.p.) Jour 0, 2, 4, 6, 8, 10, 12	Intratrachéale	20µg, jours 18, 21, 24	Jour 25: éosinophiles	Blyth et al. 1996
Balb/c	Mâle	OVA	OVA+Alum (i.p.) Jour 0 et 7	Intranasale	75µg, jour 18	Jour 21: HRB, éosinophiles	Hessel et al. 1997
Balb/c	Femelle	HDM		Intranasale	25µg, 5 jours/semaine, 3 semaines	HRB, éosinophiles, mucus, IgE	Fredriksson et al. 2012
Balb/c	Mâle & femelle	OVA	OVA+Alum (i.p.) Jour 0 et 14	Inhalation	20min/jour jours 28 à 30 et 32	Jour 33: HRB, éosinophiles	Kuperman et al. 1998
Balb/c	Mâle	OVA	OVA+Alum (i.p.) Jour 0 et 7	Intranasale	10µg, jours 17 à 20	Jour 21: HRB, éosinophiles, mucus	Hachet-Haas et al. 2008
C57BL6	Mâle	OVA	OVA+Alum (i.p.) Jour 0, 7 et 14	Intranasale	100µg, jours 20 à 22	Jour 23: HRB, éosinophiles	Caceres et al. 2009
Balb/c	Femelle	OVA	OVA+Alum (i.p.) Jour 0 et 12	Inhalation	jours 18 à 23	Jour 24: HRB, éosinophiles	McMillian et al. 2005
Balb/c	Femelle	OVA	OVA+Alum (i.p.) Jour 0 et 14	inhalation	jour 28 à 30	Jour 31: HRB, éosinophiles	Tomkinson et al. 2001
C57BL6	Mâle	OVA	OVA+Alum (i.p.) Jour 0, 7 et 14	Intranasale	100µg, jours 19 à 23	Jour 25: HRB, éosinophiles	Marion et al. 2011
A/J et C3H/HEJ	Mâle	HDM	100µg (i.n.), jour 0 et 14	Intranasale	jour 21	Jour 24: HRB, éosinophiles	Lewkowich et al. 2005
C57BL6	Mâle	HDM	HDM+alum (i.p.) jour 0	inhalation	jour 14 à 21	Jour 22: HRB, éosinophiles	Tournoy et al. 2000
Balb/c	Mâle & femelle	HDM	100µg (i.n.), jour 0, 7 et 14	Intranasale	100µg, Jour 21	Jour 24: éosinophiles	Draijer et al. 2013
Balb/c	Mâle & femelle	HDM	100µg (i.n.), jour 0	Intranasale	5x10µg, jours 7 à 11	Jour 14: éosinophiles	Draijer et al. 2013
Balb/c	Mâle & femelle	HDM	25µg (i.n.), jour 0,2,4, 7, 9, 11	Intranasale	25µg, jour 14, 16, 18	Jour 21: éosinophiles	Draijer et al. 2013

Tableau 4: Modèles d'asthme allergique aigu chez la souris. (Liste non-exhaustive)

II.4.2. Modèles à provocation allergénique répétée

L'HRB, l'inflammation à éosinophiles et la métaplasie des cellules à mucus peuvent être induites par les premières expositions à un allergène chez la souris sensibilisée et sont décrites dans les modèles aigus, ce qui contraste avec la pathologie humaine où l'exposition à l'allergène est répétée et chronique. Par conséquent, des modèles à provocation allergénique répétée ont été développés

pour étudier la réponse inflammatoire allergique. Dans ces modèles, les animaux sont exposés à l'allergène 2 à 6 semaines après une première provocation allergénique, quand l'éosinophilie des voies aériennes et l'HRB sont résolues et retournées aux niveaux de base. Cette seconde phase de provocation entraîne une nouvelle hyperéosinophilie des voies aériennes, souvent une neutrophilie, une accumulation de lymphocytes et une fonction respiratoire altérée. L'avantage de ce protocole est de pouvoir contrôler la cinétique des paramètres inflammatoires induite par une administration unique d'allergène (Kanehiro et al. 2001, Koga et al. 2013).

II.4.3. Modèles chroniques

L'asthme est défini comme une maladie inflammatoire chronique des voies aériennes liée à des modifications structurales comme une fibrose sous-épithéliale, une métaplasie des cellules à mucus, un épaissement du muscle lisse bronchique et une angiogenèse. Bien que les modèles aigus et répétés permettent d'étudier de nombreux aspects de la pathologie humaine, plusieurs laboratoires ont étudié l'administration chronique de l'allergène chez la souris, dans le but de simuler plus étroitement la maladie humaine. Différents modèles ont été développés en augmentant le nombre d'expositions aux allergènes sur plusieurs semaines. L'OVA ainsi que d'autres allergènes tels que l'extrait d'acariens de la poussière de maison, HDM, et les pollens de graminées ont été utilisés.

Quelques articles montrent que l'utilisation d'OVA pour l'élaboration de modèle chronique conduit à l'élaboration d'une tolérance et une diminution progressive de l'HRB et de l'éosinophilie des voies aériennes (Jungsuwadee et al. 2004, Kumar et al. 2008). Un phénomène lié à l'inhalation de dose trop élevée ou répétée d'OVA, et qui dépend également de la souche de souris utilisée. Les souris A/J ne semblent pas présenter ce phénomène de tolérance alors que chez les Balb/c et les C57BL6, la tolérance peut apparaître très rapidement (Shinagawa et al. 2003, McMillan et al. 2004).

Au contraire, il semblerait que l'utilisation d'allergènes plus pertinents, tels que l'HDM ou les pollens de graminées, entraîne moins de phénomènes de tolérance (Johnson et al. 2004, Moreira et al. 2011). Cet état de fait nécessite cependant d'être nuancé, car il semble important de noter que très peu des modèles publiés ont fait l'objet d'une étude cinétique, la plupart faisant état d'un phénotype mesuré à un point terminal. Dans ces modèles d'exposition chronique à l'allergène, il semble que quelques caractéristiques persistent après l'arrêt des provocations allergéniques. Quelques publications montrent un remodelage persistant, notamment les dépôts de collagène autour des bronches et la production de mucus, mais la persistance de l'HRB et de l'inflammation varie en fonction des protocoles (Johnson et al. 2004, McMillan et al. 2004, Kumar et al. 2004, Lloyd. 2007, Goplen et al. 2009). Ces modèles chroniques (Tableau 5) sont le plus souvent utilisés pour étudier la physiopathologie de l'asthme, mais sont peu utilisés pour l'évaluation des nouveaux agents thérapeutiques (Kumar et al. 2008, Nials et al. 2008, Wegmann. 2008).

Souche	Sexe	Allergène	Sensibilisation	Exposition	Provocation	Réponses aux provocations	Références
Balb/c	femelle	OVA	OVA+Alum (i.p.) Jour 0 et 14	Aérosol	2,5%, Jour 21, 30min/jour, 3jours/semaine, 6/8 semaines	HRB, Infiltration épithélium par éosinophiles, remodelage	Temelkowski et al. 1998
C57BL6	mâle	OVA	OVA+Alum (i.p.) Jour 0, 7 et 14	Aérosol	1% 60min/jour, 5jours/semaine, 6 semaines	HRB, éosinophiles, remodelage après 3 semaines, tous diminués après 6 semaines	Yiamouyiannis et al. 1999
Balb/c	mâle	OVA	OVA+Alum (i.p.) Jour 0, 7 et 14	Aérosol	1%, 60min/jour, 2jours/semaine, 4 semaines	HRB, éosinophiles, epaississement épithélium	Braun et al. 1999
Balb/c	femelle	OVA	OVA+Alum (i.p.) Jour 0 et 14	Intranasale	500µg, Jours 14, 27, 28, 47, 61, 73, 74, 75	HRB, éosinophiles, mucus, collagène	Henderson et al. 2002
Balb/c	femelle	HDM		Intranasale	25µg/jour, 5jours/semaine, 7 semaines	HRB, éosinophiles, mucus, IgE	Johnson et al. 2004
Balb/c	mâle	OVA	OVA+Alum (i.p.) Jour 0 et 5	Aérosol	2%, Jour 17, 30min/jour, 3jours/semaine, 6 semaines	HRB, éosinophiles, réponse aigue et tardive	Fernandez-Rodriguez et al. 2008
Balb/c	femelle	DRA mix (acariens, pollen, Aspergillus et OVA)	Imject® alum + DRA mix (s.c.) Jour 0, 4, 7 et 11	Intranasale	DRA mix, (5/50/5/60µg) 2x/semaine semaines 3 à 10	(Semaine 14) HRB, éosinophiles, mucus, collagène Mastocyte dans l'épithélium	Goplen et al. 2009
C57BL6	mâle et femelle	HDM		Intranasale	0,05AU/ml, 5jours/semaine, 10 semaines	HRB, éosinophiles, mucus	Allen et al. 2012
C57BL7	Femelle	HDM		Intranasale	10µg, 5jours/semaine, 6 semaines	HRB, éosinophiles, mucus, IgE	Moreira et al. 2011
C57BL8	mâle et femelle	HDM		Intranasale	50µg, 3jours/semaine, 6 semaines	HRB, éosinophiles, mucus, IgE	Le Cras et al. 2011
Balb/c	femelle	HDM		Intranasale	25µg/jour, 5jours/semaine, 5 semaines	HRB, éosinophiles, mucus	Botelho et al. 2011
Balb/c	mâle	HDM		Intranasale	25µg/jour, 4jours/semaine, 5 semaines	HRB, éosinophiles, mucus, collagène	Tomlinson et al. 2011
Balb/c	mâle et femelle	HDM		Intranasale	40µg/jour, 4jours/semaine, 5 semaines	HRB, éosinophiles	Simeone-Penney et al. 2007
Balb/c	mâle	HDM		Intranasale	25µg/jour, 2jours/semaine, 5 semaines	HRB, éosinophiles, mucus, E-cadherin, IgE	Post et al. 2012

Tableau 5: Modèles d'asthme allergique chronique chez la souris. (Liste non-exhaustive)

II.5. Mesure de l'hyperréactivité bronchique

La principale caractéristique clinique de l'asthme est une obstruction réversible des voies aériennes, accompagnée d'une hyperréactivité bronchique aux agents broncho-constricteurs et à des stimuli non spécifiques. Chez l'homme, l'inhalation d'un allergène peut induire une réponse immédiate et / ou une réponse tardive, mais la plupart des mesures de la réactivité bronchique le sont en réponse à l'inhalation d'agonistes cholinergiques tels que la métacholine ou l'acétylcholine, quelques heures après la provocation allergénique. L'exposition des souris à un allergène se traduit par une hausse significative de la réactivité bronchique à ces agonistes par rapport aux contrôles non exposés (Figure 22).

II.5.1. Mesure non invasive

Les approches non invasives sont utilisées pour évaluer la fonction respiratoire chez les animaux non anesthésiés, placés dans une chambre plethysmographique (EMKA technologies, Tableau 6). La réactivité des voies aériennes à la métacholine est mesurée et comparée à la réponse au solvant (solution saline ou tampon phosphate). Les différents paramètres mesurés à l'aide du pléthysmographe en réponse à l'administration d'un agent broncho-constricteur, dont les temps d'expiration et d'inspiration, permettent de calculer les valeurs de Penh (*enhanced Pause*), correspondant à l'allongement du temps de pause expiratoire observé chez les individus présentant une réduction du calibre bronchique (Hamelmann et al. 1997, Lomask 2006). Les mesures non invasives ont de nombreux avantages par rapport aux méthodes invasives : elles sont techniquement moins exigeantes et peuvent être utilisées pour suivre l'HRB dans le temps chez un même animal. Il apparaît généralement que les résultats obtenus par des manipulateurs expérimentés à l'aide d'approches non invasives sont corrélés à ceux obtenus par des méthodes invasives. Bien plus rapide et plus facile que la mesure invasive de la fonction respiratoire, cette approche nécessite néanmoins un calibrage attentif et est mieux adaptée aux souches présentant une forte HRB, telles que les souris A/J, AKR/J, BALB/c ou 129/Sv (Whitehead et al. 2003). Les résultats sont dépendants de la qualité du flux d'air dans la chambre, de la bonne maintenance du matériel, mais aussi du protocole. Pour ce dernier, la multiplication des nébulisations humidifie les filtres et les pneumotachographes, et impacte la qualité du flux d'air et la mesure. La validité de cette méthode a été remise en question et a engendré de nombreux débats car il est difficile d'exclure l'influence des voies aériennes supérieures et le Penh est un indice calculé et non une valeur physiologique (Adler et al. 2004, Hoymann et al. 2007). Aujourd'hui, la mesure de la réactivité bronchique est le plus souvent réalisée de manière non invasive puis confirmée par des méthodes invasives (Vanoirbeek et al. 2010).



Méthodes	Non-invasive	Invasive
Avantages	Permet des mesures répétées chez le même animal Mesure sur une période prolongée Mesure de la respiration normale, sans anesthésie ni trachéotomie ou instrumentation Rapide et facile	Précision Contourne les modifications des voies aériennes supérieures Basée sur des principes physiologiques
Inconvénients	Interférence des voies aériennes supérieures Sujet aux artefacts (mouvements) Pas de mesure directe de la mécanique pulmonaire Le Penh est un paramètre calculé Nécessite une maintenance intensive	Nécessite une anesthésie: modifications des réponses aux agents bronchoconstricteurs Nécessite une trachéotomie Nécessite une ventilation mécanique Long Mesure terminale Nécessite une expertise
Exemple de Matériel disponible	Pléthysmographe corps entier, animal non contraint, EMKA technologies 	Flexivent®, SCIREQ 

Tableau 6: Avantages et inconvénients des mesures invasives et non-invasives de la fonction respiratoire.
 Adapté de Glaab et al. 2007.

II.5.1. Mesure invasive de la fonction respiratoire

Les méthodes invasives validées, utilisent la méthode des oscillations forcées (Flexivent®, SCIREQ, Figure 22). Elles permettent de mesurer directement la mécanique pulmonaire chez la souris et peuvent être présentées comme le standard actuel pour la mesure de la physiologie des voies aériennes. L'HRB peut être évaluée directement par la mesure de la résistance des voies aériennes en réponse à la métacholine ou l'acétylcholine (inhalation ou injection intraveineuse) ou tout agent

broncho-contraction. Couramment, la résistance mesurée est la somme des résistances des voies aériennes et du tissu, mais ces dernières peuvent être différenciées à l'aide d'un protocole adapté. Les mesures invasives sont plus précises et permettent de mesurer des changements transitoires par évaluation au temps donné. L'insertion d'une canule trachéale permet de ne mesurer que les paramètres pulmonaires en esquivant les voies aériennes supérieures. Cependant, la mise en place de la canule oblige la réalisation d'une trachéotomie, ce qui exclut la possibilité de réaliser des mesures répétées ou un suivi prolongé, nécessite l'utilisation d'anesthésiques, d'une ventilation mécanique, et exige une certaine expertise (Irvin et al. 2003, Bates et al. 2003, Glaab et al. 2007).

La mesure invasive de la fonction respiratoire nécessite que la ventilation de l'animal soit prise en charge par le ventilateur mécanique, en l'absence de réaction de la part de l'animal. Pour cela, cette méthode implique l'utilisation de doses importantes d'anesthésique et requiert l'utilisation d'agent myorelaxant tel que la xylazine, et dans certains cas de curarisant tels que le pancuronium (Shalaby et al. 2010, McGovern et al. 2013). L'utilisation d'anesthésique peut perturber la mesure de la résistance des voies aériennes en l'absence d'agent broncho-constricteur et ne permet pas de mesurer le bronchospasme en réponse aux allergènes et aux endotoxines (LPS).

L'utilisation des modèles animaux a permis et permet de mieux comprendre les mécanismes, les interactions, et les liens entre les facteurs et les cellules. Parmi les liens mis en évidence, les chimiokines ont une place importante et sont impliquées dans un grand nombre de processus physiologiques, les réponses immunitaires et les processus physiopathologiques.

III.les chimiokines et leurs récepteurs

Les chimiokines sont des cytokines de faible poids moléculaire caractérisées par leur propriété à induire la migration des leucocytes (Baggiolini, 1998). Quarante-six chimiokines et 21 récepteurs de chimiokines de type récepteur couplé aux protéines G ou RCPG ont été décrits (Bendall et al. 2005, Zlotnik et al. 2012). Les chimiokines sont sécrétées dans les sites inflammatoires ; elles forment des gradients qui permettent de produire des signaux directionnels pour le chimiotactisme des leucocytes (Sallusto et al. 2008) et sont des médiateurs des réponses immunitaires et inflammatoires. Les chimiokines participent également à un grand nombre de processus physiologiques impliqués dans l'homéostasie, la maturation de précurseurs hématopoïétiques, le développement des organes lymphoïdes et l'angiogénèse.

III.1. Nomenclature et classification

Les premières chimiokines ont été caractérisées dans les années 70 avec la découverte du PF4 (*platelet factor 4*), qui est aujourd'hui classé dans la famille des chimiokines de type CXC et nommé CXCL4 (Levine et al. 1976, pour revue: Bendall et al. 2005). Les autres molécules apparentées ont été découvertes quelques années plus tard et regroupées sous différents noms: SIS (*Small Inducible Secreted*, Brown et al. 1989) qui regroupait des chimiokines de type CC ou bien « scy » (*small cytokines*, Beall et al. 1992) et « intercrine » (Mukaida et al. 1991), qui regroupaient des chimiokines de type CC et CXC. Le terme définitif de chimiokines, pour « cytokines chimio-attractantes », sera adopté en 1992 lors du troisième congrès international sur les cytokines. Bien que la nomenclature officielle des chimiokines fût décidée en 1995 par le comité NC-IUPHAR, les premières dénominations de chaque chimiokine restent communément utilisées dans les publications scientifiques (Tableau 7). Les chimiokines se caractérisent par un motif commun N-terminal contenant des cystéines (Cys) qui permettent de les distinguer en quatre sous-familles sur la base du nombre et de l'espacement de ses cystéines (Figure 17): les deux principales sous-familles sont les CXC-chimiokines (groupe α) et les CC-chimiokines (groupe β). Les deux premiers résidus cystéines des CXC-chimiokines sont séparés par un seul acide aminé et ceux des CC-chimiokines sont adjacents. Une autre sous-famille, composée de deux membres, a été décrite. XCL₁ ou lymphotactine- α , et XCL₂ ou lymphotactine- β sont les deux seules C-chimiokines ; elles ont la particularité de ne posséder que deux cystéines (Kleiner et al. 1994). CX3CL1 ou fractalkine est l'unique CX3C-chimiokine, dont trois acides aminés séparent les deux premières cystéines (Bazan et al. 1997).

Famille	Nom Commun	Récepteurs
Famille CXC		
CXCL1	<i>Gro-α, Growth related oncogene-α</i>	CXCR1, CXCR2
CXCL2	<i>Gro-β, Growth related oncogene-β</i>	CXCR2
CXCL3	<i>Gro-γ, Growth related oncogene-γ</i>	CXCR2
CXCL4	<i>PF-4, Platelet derived factor-4</i>	CXCR3
CXCL5	<i>ENA-78, Epithelial cell derived neutrophil activating factor-78</i>	CXCR2
CXCL6	<i>GCP-2, Granulocyte chemoattractant protein-2</i>	CXCR1
CXCL7	<i>NAP-2, Neutrophil activating protein-2</i>	CXCR2
CXCL8	<i>IL-8, Interleukin 8</i>	CXCR1, CXCR2
CXCL9	<i>MIG, Monokine</i>	CXCR3
CXCL10	<i>IP-10, Interferon inducible protein-10</i>	CXCR3
CXCL11	<i>I-TAC, Interferon inducible T cell a chemoattractant</i>	CXCR3
CXCL12	<i>SDF-1, Stromal-derived factor-1</i>	CXCR4
CXCL13	<i>BCA-1, B cell activating chemokine</i>	CXCR5
CXCL14	<i>BRAK, Breast and kidney chemokine</i>	?
CXCL16	<i>Leukotactin-1</i>	CXCR6
Famille CC		
CCL1	<i>I-309</i>	CCR8
CCL2	<i>MCP-1, Monocyte chemoattractant protein-1</i>	CCR2, CCR11
CCL3	<i>MIP-1α, Macrophage inflammatory protein-1α</i>	CCR1, CCR3, CCR5
CCL4	<i>MIP-1β, Macrophage inflammatory protein-1β</i>	CCR1, CCR5
CCL5	<i>RANTES, Regulated Activation Normal T cell Expressed and Secreted</i>	CCR1, CCR3, CCR5
CCL6	<i>Protéine C10</i>	CCR1
CCL7	<i>MCP-3, Monocyte chemoattractant protein-3</i>	CCR1, CCR2, CCR3, CCR11
CCL8	<i>MCP-2, Monocyte chemoattractant protein-2</i>	CCR1, CCR2, CCR3, CCR5, CCR11
CCL9 / CCL10	<i>MIP1-γ, Macrophage inflammatory protein-1-γ</i>	CCR1
CCL11	<i>Eotaxin-1</i>	CCR3
CCL12	<i>MCP-5, Monocyte chemoattractant protein-5</i>	CCR2, CCR11
CCL13	<i>MCP-4, Monocyte chemoattractant protein-4</i>	CCR2, CCR3, CCR5, CCR11
CCL14	<i>HCC-1, Haemofiltrate CC chemokine ou MIP-1d, Macrophage inflammatory protein-1-d</i>	CCR1, CCR5
CCL15	<i>HCC-2, Haemofiltrate CC chemokine 2 ou Lkn-1, leukotactin 1</i>	CCR1, CCR3
CCL16	<i>HCC-4, Haemofiltrate CC chemokine 4 ou LEC, liver expressed chemokine</i>	CCR1, CCR2, CCR8
CCL17	<i>TARC, Thymus and activation regulated chemokine</i>	CCR4, CCR8
CCL18	<i>PARC, Pulmonary and activation regulated chemokine</i>	CCR6
CCL19	<i>MIP-3β, Macrophage inflammatory protein-3-β ou ELC, Epstein Barr virus induced receptor ligand chemokines</i>	CCR7, CCR10, CCR11
CCL20	<i>MIP-3α, Macrophage inflammatory protein-3-α ou LARC, liver and activation regulated chemokine</i>	CCR6, CCR10,
CCL21	<i>SLC, Secondary lymphoid tissue chemokine</i>	CCR7, CCR11
CCL22	<i>MDC, Macrophage derived chemokine</i>	CCR4
CCL23	<i>MPIF-1</i>	CCR1
CCL24	<i>Eotaxin-2 ou MPIF-2, Myeloid progenitor inhibitory facto- 2</i>	CCR3
CCL25	<i>TECK, Thymus expressed chemokine</i>	CCR9, CCR11
CCL26	<i>Eotaxin-3</i>	CCR3
CCL27	<i>CTACK, cutaneous T-cell attracting chemokine ou ESKine, Embryonic stem cell chemokine</i>	CCR10
CCL28	<i>Mec, Mucosae-Associated Epithelial Chemokine</i>	CCR3, CCR10
Famille C		
XCL1	<i>Lymphotactin</i>	XCR1
XCL2	<i>SCM1b, Single C motif-1 ou Lymphotactin-β</i>	XCR2
Famille CX3C		
CX3CL1	<i>Fractakine / neurotactin</i>	CX3CR1

Tableau 7 : Nomenclature des chimiokines humaines. Classification IUPHAR et nomenclature usuelle. Adapté de Zlotnik et al. 2000, Lean et al. 2002, Ma et al. 2004, Bendall et al. 2005, Zissel et al. 2012.

Les chimiokines sont de petites protéines basiques de 70 à 130 acides aminés (soit 8 à 14 kDa) présentant 20 à 30% d'homologie entre les 4 sous-familles, jusqu'à 70% entre les CC-chimiokines et jusqu'à 90% d'homologie entre les CXC-chimiokines.

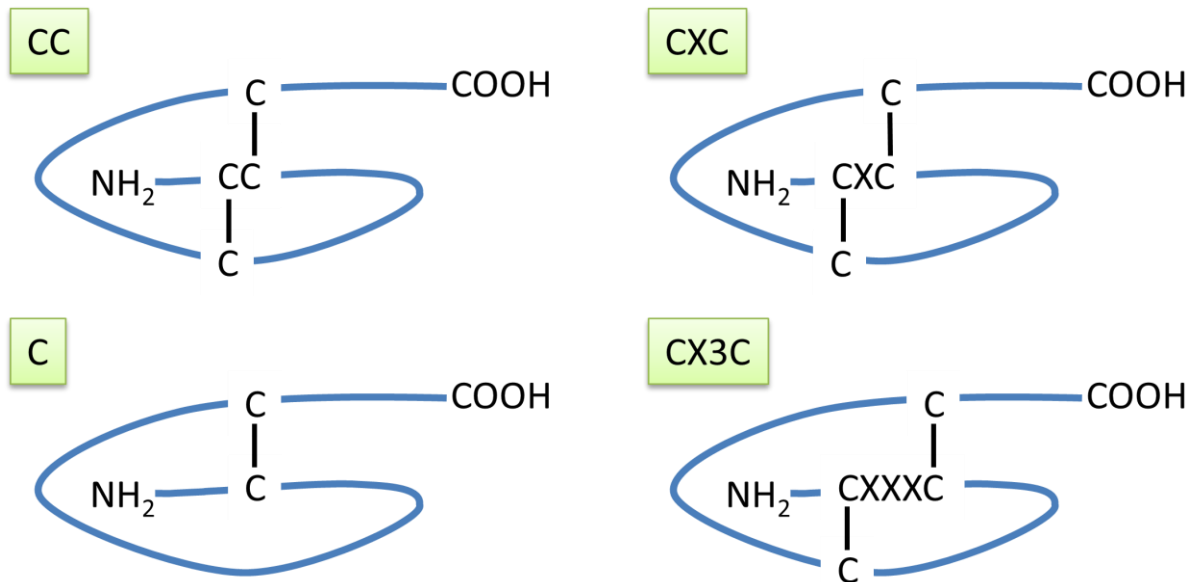


Figure 17: Les 4 sous familles de chimiokines. Les chimiokines sont divisées en 4 sous-familles sur la base du nombre et de l'espacement des cystéines conservées au niveau de leur région amino-terminale.

III.2. Structures

Les structures tridimensionnelles de plusieurs chimiokines ont été déterminées par cristallographie aux rayons X (CXCL12: Murphy et al. 2010) et par résonance magnétique nucléaire (RMN) (pour revue: Ziarek et al. 2012, CX3CR1: Park et al. 2012, CCR5: Schnur et al. 2013). La relative conservation de la composition en acides-aminés des CC et CXC chimiokines entraîne de fortes similarités au niveau de leurs structures secondaires et tertiaires.

La formation de deux ponts disulfures entre les cystéines 1 et 3 (Cys1-Cys3) et les cystéines 2 et 4 (Cys2-Cys4) confère une structure tertiaire commune et caractéristique des chimiokines. Cette structure conservée est composée d'une région amino-terminale flexible qui précède la première cystéine. Ce domaine est suivi d'une région en boucle appelée boucle N (*N loop*) suivie d'un feuillet constitué de trois brins β antiparallèles reliés entre eux par les boucles 30s, 40s et 50s. Ce motif rigide fournit une base solide sur laquelle s'ancre le domaine carboxy-terminal organisé en hélice α qui suit la dernière cystéine (Cloue et al. 1995). La structure tridimensionnelle est stabilisée à la fois par les deux ponts disulfures et par des liaisons hydrophobes entre des résidus de l'hélice α carboxy-terminale et des résidus du feuillet β (Figure 18). La portion N-terminale flexible et la boucle rigide sont les deux sites majeurs d'interaction des chimiokines avec leurs récepteurs (Onuffer et al. 2002)

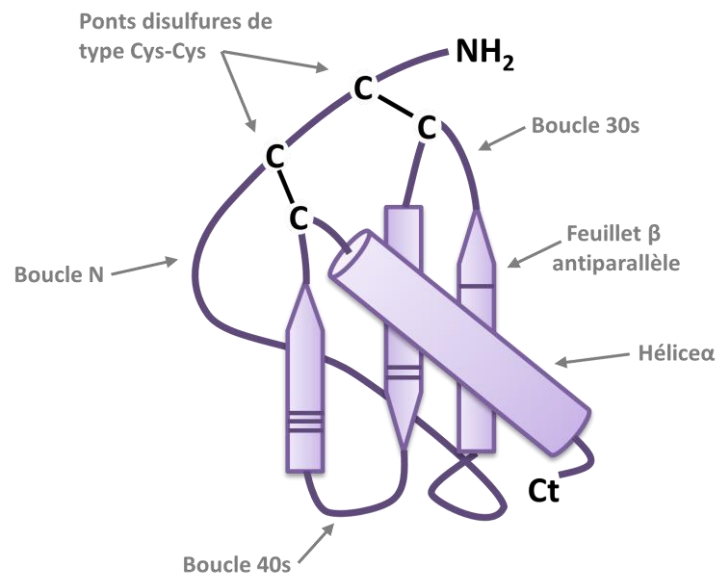


Figure 18: Structure tridimensionnelle des chimiokines. Adapté de Galzi et al. 2010.

C'est sous la forme de monomère que la plupart des chimiokines ont une activité biologique, mais elles sont capables de former des oligomères, créant de nouvelles structures pouvant avoir un rôle fonctionnel *in vivo* ; ce mécanisme serait nécessaire par exemple à l'activité chimiotactique de CCL2 et CCL5 (Proudfoot et al. 2003).

Les chimiokines telles que CCL2, CCL3, CCL5, CCL8, CXCL8 et CXCL12 ont tendance à l'oligomérisation lorsqu'elles sont fortement concentrées, notamment lorsqu'elles interagissent avec les glycosaminoglycannes (GAGs) de la matrice extracellulaire par des interactions ioniques et se trouvent concentrées à la surface des membranes cellulaires (Kuschert et al. 1999, Lau et al. 2004, Crown et al. 2006, Ziarek et al. 2013). A l'exception d'une forme de CCL27 qui est transloquée dans le noyau, les chimiokines comportent un peptide signal permettant leur sécrétion (Gortz et al. 2002).

Cependant, les chimiokines CXCL1 et CXCL16 ne sont pas libérées dans le milieu extracellulaire sous forme soluble. Elles sont produites sous forme de protéines membranaires et sont composées d'un domaine chimiokine, d'un tronc mucine, d'un segment transmembranaire et d'une partie intracellulaire (Figure 19) (Hermand et al. 2008). Sous cette forme, elles jouent le rôle de molécule d'adhésion. Ces deux chimiokines peuvent être libérées après clivage par les protéases de la famille ADAM (*A Disintegrin And Metalloproteinase*) (Ludwig et al. 2007).

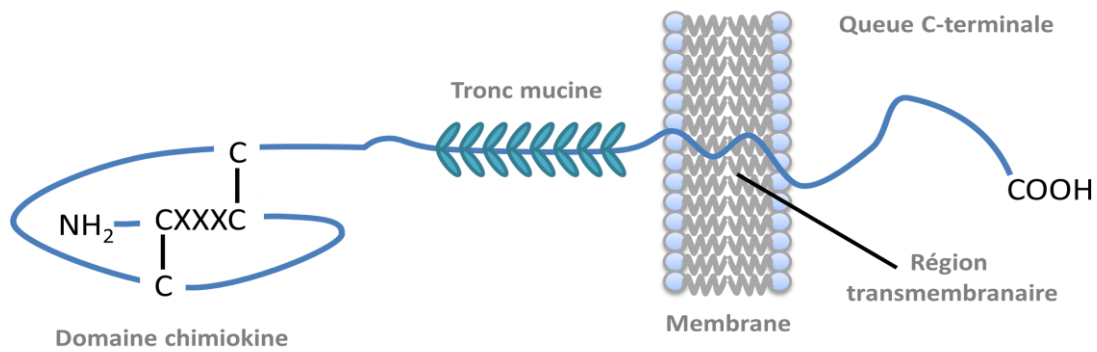


Figure 19: Représentation schématique de la forme membranaire de CX3CL1. Sous cette forme, cette chimiokine est composée de 4 domaines: un domaine chimiokine, un tronc mucine (site de glycosylation), un segment transmembranaire et une partie intracellulaire. Adapté de Hermand et al. 2010.

III.3. Les récepteurs des chimiokines

Les chimiokines interagissent avec des récepteurs couplés aux protéines G (RCPG). Une vingtaine de récepteurs a été caractérisée et regroupée en quatre sous-familles: CCR, CXCR, CX3CR et XCR en fonction de la classe des chimiokines qui les activent. De manière générale, une même chimiokine peut activer différents récepteurs et réciproquement, un même récepteur peut lier plusieurs chimiokines, toujours de la même classe (CC ou CXC) (Allen et al. 2007). A ces récepteurs classiques on peut ajouter D6, CCX-CKR (*Chemocentryx-chemokine receptor*) et DARC ou Duffy (*Duffy antigen receptor for chemokine*). Ces récepteurs lient les chimiokines mais ne semblent pas transmettre de signal intracellulaire et serviraient de leurre/piège. (Feng et al. 2009)

Les récepteurs aux chimiokines sont des protéines de 320 à 380 acides aminés. Ils sont constitués de sept domaines riches en résidus hydrophobes sous forme d'hélices α transmembranaires (TM1-TM7), reliées entre elles par 3 boucles hydrophiles extracellulaires (ECL1-ECL3) et 3 boucles intracytoplasmiques (ICL1-ICL3). Ils possèdent un domaine N-terminal extracellulaire (environ 40 acides aminés) et un domaine carboxy-terminal intracellulaire. Des ponts disulfures sont présents entre les boucles EC1 et EC2 ainsi qu'entre le domaine N-terminal et la boucle EC3 (Figure 20). Les récepteurs aux chimiokines possèdent un motif DRY (Asp-Arg-Tyr) au niveau de la boucle intracellulaire ICL2 qui est nécessaire à la liaison aux protéines G. Le domaine C-terminal contient des sites de phosphorylation sur résidus sérine et thréonine impliqués dans la signalisation et dans la désensibilisation du récepteur (Allen et al. 2007).

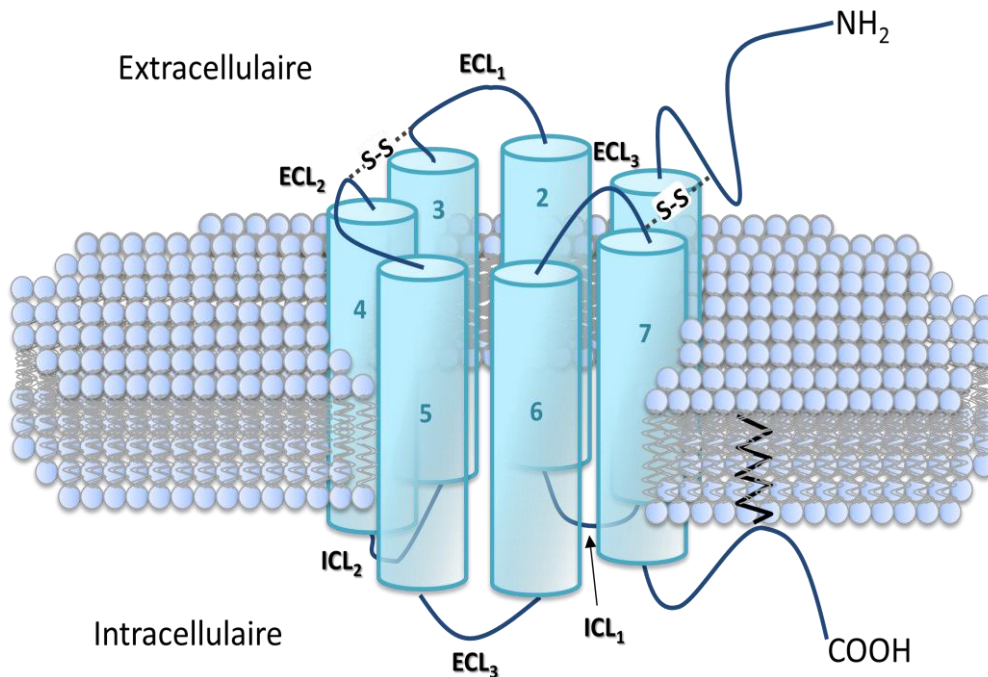


Figure 20: Représentation schématique d'un récepteur aux chimiokines. RCPG constitué de 7 segments transmembranaires (TM1-7), 3 boucles extracellulaires (ECL1-3), 3 boucles intracytoplasmiques (ICL1-3), un domaine N-terminal extracellulaire et un domaine carboxy-terminal intracellulaire. Des ponts disulfures sont présents entre les boucles ECL1 et ECL2 ainsi qu'entre le domaine N-terminal et la boucle ECL3.

Les récepteurs aux chimiokines possèdent un fort degré d'homologie au niveau des régions transmembranaires mais de grandes variations au niveau des domaines extra-membranaires.

Ils existent sous forme de monomères et pourraient dans certains cas former des oligomères, généralement des homo- ou hétéro-dimères (Thelen et al. 2010). Les récepteurs CCR2, CCR5 et CXCR4 forment des homodimères et pourraient former des hétéro-dimères. Les récepteurs CXCR7 et CXCR4 peuvent notamment former des hétérodimères lors de la fixation de CXCL12 et ainsi moduler l'activité de la chimiokine (Levoye et al. 2009). L'affinité des chimiokines pour leurs récepteurs varie en fonction du couple ligand/récepteur et du type de cellules présentant le récepteur mais reste de l'ordre du nanomolaire. Les chimiokines ont une activité agoniste, mais il a été montré que certaines chimiokines peuvent avoir également une activité « antagoniste » (Allen et al. 2007). Ces chimiokines peuvent se lier à un récepteur, sans l'activer et empêcher son activation par une chimiokine agoniste. Un phénomène rencontré par exemple entre les chimiokines CXCL8, CXCL9 et CXCL10, agonistes du récepteur CXCR3, mais qui peuvent se lier au récepteur CCR3 et empêcher la fixation des chimiokines CCL5, CCL7, CCL8, CCL11, CCL13 et CCL24 et leur activité chimiotactique (Loetscher et al. 2001). Le système des chimiokines et de leurs récepteurs est dit redondant car une chimiokine peut se lier à plusieurs récepteurs et les récepteurs ont la faculté de répondre à plusieurs chimiokines (Figure 21).

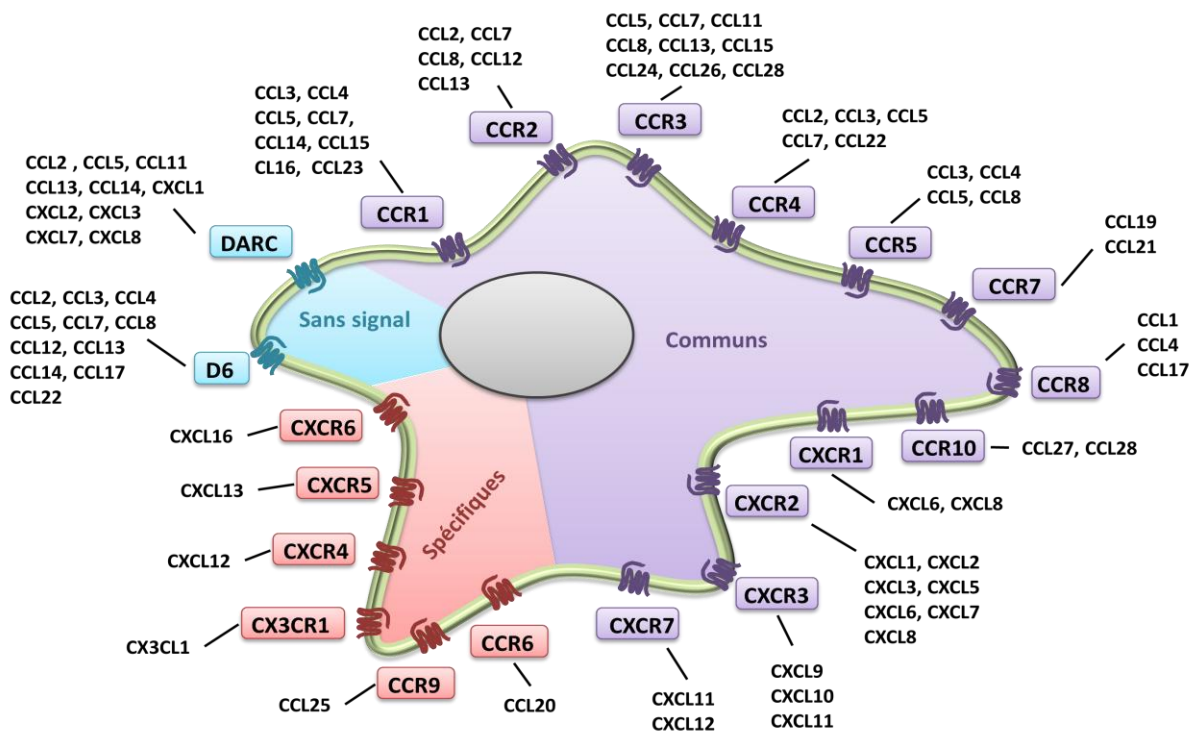


Figure 21: Les chimiokines et leurs récepteurs. Adapté de Lazennec et al. 2010.

Ce n'est cependant pas le cas de toutes les chimiokines et de tous les récepteurs. Par exemple, CX3CL1 ne se fixe qu'au récepteur CX3CR1 et est son seul ligand. De plus, les récepteurs aux chimiokines sont répertoriés en fonction de la classe de leurs ligands mais il existe des exceptions. Les récepteurs leurres DARC et D6 sont connus pour lier à la fois des CC et des CXC chimiokines avec des affinités identiques (Hansel et al. 2011). Les 3 ligands agonistes de CXCR3, qui sont CXCL9, CXCL10 et CXCL11, sont également antagonistes du récepteur CCR3 (Xanthou et al. 2003). Cette redondance permettrait d'augmenter l'efficacité du recrutement cellulaire lors des processus inflammatoires et explique pourquoi l'absence ou la non-fonctionnalité d'une chimiokine ou d'un récepteur n'entraîne pas nécessairement des anomalies majeures. A ce jour, seule l'absence de CXCR4 et de CXCR7 ou de leur ligand CXCL12 est létale (Nagasawa et al. 1996; Tachibana et al. 1998; Sierro et al. 2007).

III.4. Interaction chimiokine-récepteur

L'interaction entre la chimiokine et le récepteur est réalisée en deux étapes: le ligand reconnaît d'abord un site de liaison de faible affinité présent sur la partie N-terminale extracellulaire du récepteur qui entraîne un changement de conformation de ce dernier (Rojo et al. 1999, Szpakowska et al. 2012). Cette première étape rend possible la suivante: l'interaction entre la partie N-terminale

de la chimiokine et les boucles extracellulaires du récepteur (Figure 22). Ce second site de liaison fait intervenir la portion la plus variable chez toutes les chimiokines et est donc déterminant pour l'affinité et la spécificité de la liaison ligand-récepteur. De plus, un certain nombre d'acides aminés et de tyrosines sulfatées localisées au niveau du domaine N-terminal des récepteurs contribuent également à la haute affinité de liaison des chimiokines.

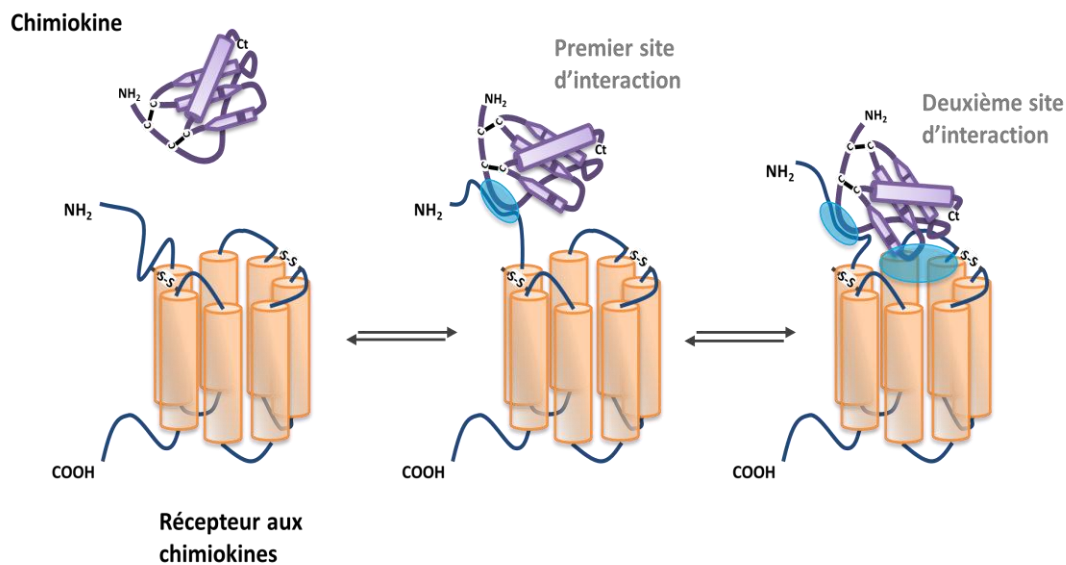


Figure 22: Modèle d'interaction "chimiokine/récepteur aux chimiokines" en 2 étapes. Le ligand reconnaît d'abord la partie amino-terminale du récepteur, puis interagit de façon plus spécifique avec les boucles extracellulaires. Adapté de Szpakowska et al. 2012.

Les résidus chargés des boucles du récepteur participent à son activation et à l'initiation de la transduction du signal (Allen al. 2007). Les 2 sites d'interaction des chimiokines sont courts (quelques acides aminés) et proches, grâce à la présence des 2 ponts disulfures hautement conservés.

A la surface des cellules, l'interaction des chimiokines avec leurs récepteurs est modulée par la présence de protéoglycannes sur la membrane plasmique (Kuschert et al. 1999). Les chimiokines peuvent se lier avec une forte affinité par l'intermédiaire d'un motif BBXB (B=résidu basique), à ces glycosaminoglycannes (GAG) chargés négativement à la surface cellulaire. Cette fixation non spécifique augmente leur concentration locale et facilite leur fixation aux récepteurs. La liaison aux GAG protège les chimiokines contre la dégradation, permet leur stockage et leur présentation au récepteur, et favorise l'activation cellulaire *in vivo* (Ali et al. 2000). Les chimiokines sont connues pour leurs fixations aux GAGs sont CCL2, CCL3, CCL4, CCL5, CCL8, CXCL8, CXCL12 (Kuschert et al. 1999, Lau et al. 2004, Crown et al. 2006, Ziarek et al. 2013).

III.5. Transduction du signal des récepteurs de chimiokines

La liaison d'une chimiokine à son récepteur induit un changement conformationnel permettant l'activation de la protéine G hétérotrimérique associée au récepteur. La cascade de signalisation induite dépend de la nature de la protéine G et des effecteurs activés. Les protéines G sont formées d'une sous-unité α liée à un complexe $\beta\gamma$. Elles sont inactives lorsque leurs sous-unités sont liées au GDP et deviennent actives quand le GDP est échangé pour du GTP. Sous forme active, la protéine G hétérotrimérique se dissocie en une sous-unité α liée au GTP et en un dimère $\beta\gamma$. Les sous unités libres sont alors capables d'interagir avec différents effecteurs tels que des adénylate cyclase, phosphodiesterase, phospholipase C, canaux Ca^{2+} voltage-dépendant, phosphatidylinositol-kinase de type 3 (PI3K), responsables de la réponse physiologique (Figure 23) (Allen et al. 2007, Offermanns 2003). Il existe plus de 20 sous-unités α regroupées en 4 familles: α_s , α_i , α_q et $\alpha_{12/13}$, ainsi que 6 sous-unités β et 12 γ (Maghazachi 2000). Les récepteurs des chimiokines sont principalement associés à des protéines $G_{\alpha i}$ sensibles à la toxine de *Bordetella pertussis* (PTX) ou à des membres de la famille $G_{\alpha q}$.

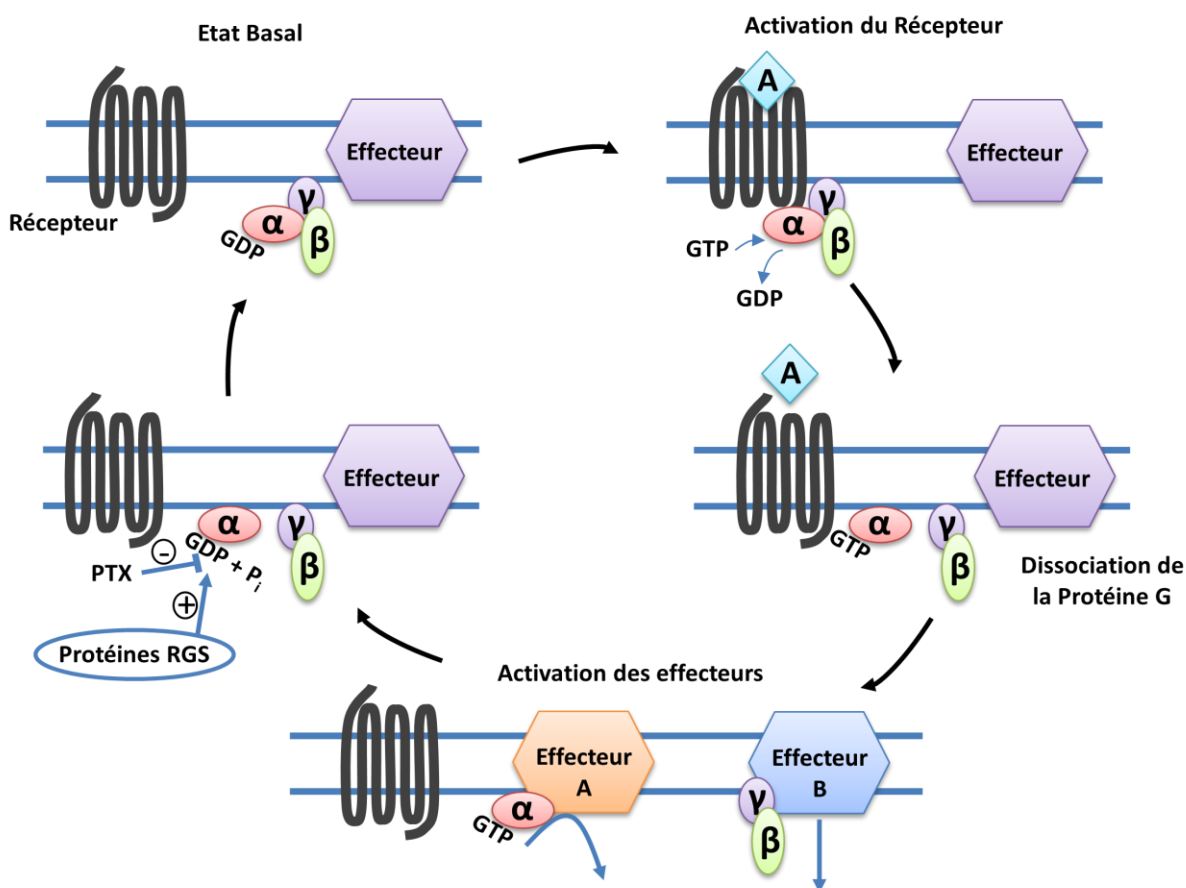


Figure 23: Représentation schématique résumant l'activation des protéines G hétérodimériques via les récepteurs à 7 domaines transmembranaires. Agoniste (A), toxine pertussique (PTX), protéines régulatrices de la signalisation des protéines G (RGS). Adapté de Offermanns 2003.

L'effecteur le plus courant est la phospholipase C β (PLC β) qui interagit avec la sous-unité $\beta\gamma$ (Kelley et al. 2006). Cette interaction permet de générer de l'inositol (1,4,5 trisphosphate (IP3) et du diacylglycérol (DAG) à partir du phosphatidylinositol (4,5)-bis phosphate (PI). L'IP3 induit la libération de Ca^{2+} des stocks intracellulaires et permet une augmentation de la concentration cytoplasmique en calcium. En présence de calcium, le DAG active différentes isoformes de protéines kinases C (PKC) qui catalysent la phosphorylation de protéines comme les MAPKs (*Mitogen-Activated Protein Kinases*) (Naor et al. 2009). La fixation des chimiokines sur leurs récepteurs peut également stimuler la phospholipase D (PLD) et des complexes d'adhésion tels que PYK2 (*proline-rich tyrosine kinase 2*) également appelé RAFTK (*adhesion focal tyrosine kinase*), appartenant à la sous-famille des FAK (*Focal Adhesion Kinase*) (Yin et al. 2003). Les sous-unités $\beta\gamma$ associées aux récepteurs de chimiokines peuvent également activer la phosphatidylinositol 3-kinase (PI3K) qui active à son tour la protéine kinase B (PKB) (Figure 24) (Macoska JA. 2011, Offermanns. 2003). Cependant, la signalisation des récepteurs des chimiokines peut être indépendante de la protéine G et dans certains cas, comme pour les récepteurs CXCR4 et CCR5, conduire au recrutement et à l'activation de la voie JAK-STAT et accroître la prolifération et la survie cellulaire (Ahr et al. 2005, Wong et al. 2003). Dans le cas du récepteur CXCR7, la liaison de la chimiokine, entraîne l'association avec la β -arrestine et permet à son tour d'induire des cascades signalétiques menant à l'activation des MAP Kinases (*mitogen activated protein kinases*), telles que les MAPK 42/44 ou ERK1/2, sans interagir avec les protéines G hétérotrimériques (Rajagopal et al. 2010). Ces cascades de signalisation des récepteurs des chimiokines sont impliquées dans la régulation de différentes fonctions biologiques comme la migration, la survie, la différenciation, la prolifération cellulaire.

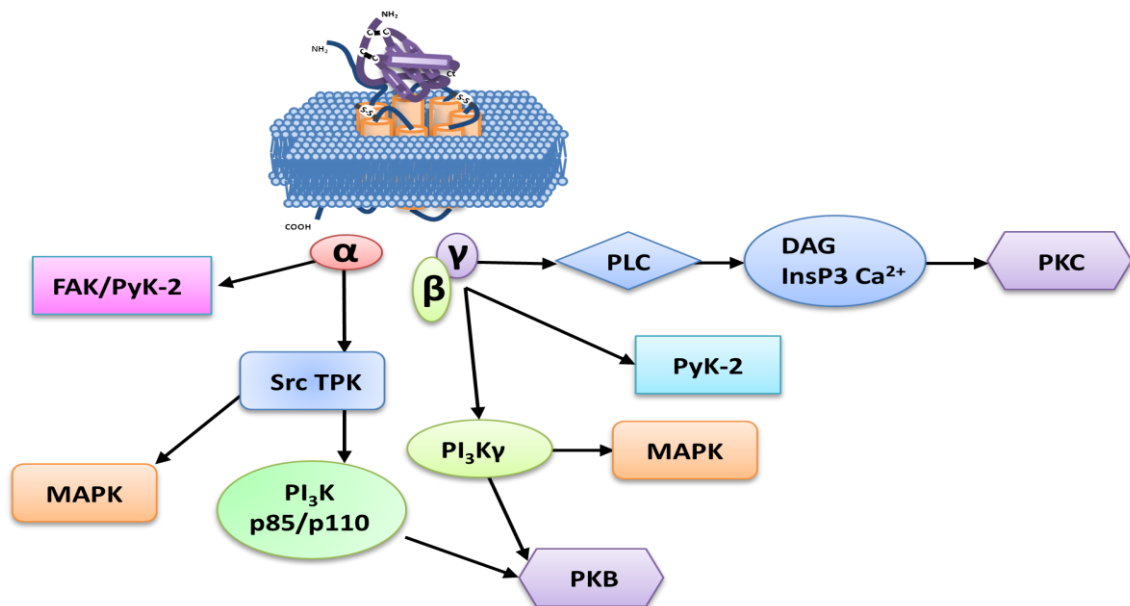


Figure 24: Principales voies de signalisation induites par la liaison des chimiokines sur leurs récepteurs. Phospholipase C (PLC), Protéine Kinase C (PKC), Protéine Kinase B (PKB), MAPK, FAKp125, PI3K, PIK, DiAcylGlycérol (DAG), Inositol 1,4,5-trisphosphate (IP3). Adapté de Thelen 2001 et Olson et al. 2002.

III.6. Désensibilisation et internalisation des récepteurs

L'activité des récepteurs des chimiokines est régulée soit par la désensibilisation du récepteur, qui concourt à limiter dans le temps le signal d'activation du récepteur, soit par l'internalisation de celui-ci (Bennett et al. 2011). Ce processus est nécessaire au maintien de la capacité des cellules à réagir à un gradient de chimiokines. Lorsqu'une chimiokine se lie à un récepteur, ce dernier interagit avec une protéine G qui se dissocie en sous-unités α -GTP et $\beta\gamma$, capables d'activer des effecteurs cellulaires. Très rapidement le domaine C-terminal du récepteur est phosphorylé par les kinases des récepteurs couplés aux protéines G (*G-protein-coupled receptor kinases* ou GRK), ce qui a pour effet d'augmenter son affinité pour la β -arrestine. Cette première étape est appelée l'étape de désensibilisation ou de phosphorylation. La β -arrestine peut ensuite interagir avec la protéine adaptatrice AP-2 et établir un pont moléculaire entre le complexe récepteur- β -arrestine et la principale protéine de la membrane de la vésicule d'endocytose: la clathrine. Ce phénomène a été décrit pour plusieurs récepteurs tels que CXCR4 et CXCR7 (Brühl et al. 2003; Berchiche et al. 2011; Luker et al. 2010). Dans les vésicules d'endocytose, le pH acide et la présence de phosphatases permet de déphosphoryler les récepteurs internalisés qui sont alors recyclés à la membrane plasmique où ils peuvent à nouveau être stimulés (Figure 25). Certains récepteurs ne recyclent pas et sont dirigés vers le compartiment lysosomal pour y être dégradés tels que CXCR2B et CXCR3 (Cottrell et al. 2012). Il existe une seconde voie d'internalisation qui ne conduit pas à la formation de vésicules de clathrine. Cette seconde voie dépend des cavéoles: des micro-domaines membranaires très organisés, riches en cholestérol et composés de cavéolines, un ensemble de protéines qui forment des oligomères et permettent l'invagination de la membrane plasmique. Les cavéosomes fusionnent ensuite avec les endosomes les plus proches (Borroni et al. 2010).

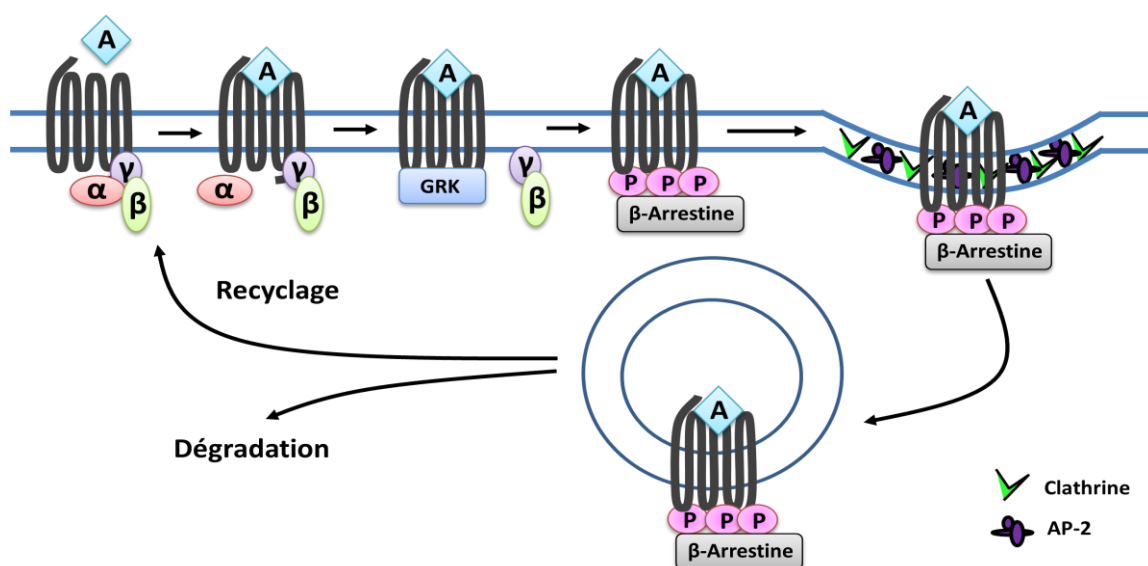


Figure 25: Endocytose des RCPG après une activation de courte durée: mécanisme principal et devenir des récepteurs internalisés. Protéine Adaptatrice (AP-2), G-protein-coupled receptor kinases (GRK). Adapté de Bennett et al. 2011.

III.7. Expression des chimiokines et de leurs récepteurs

Les chimiokines se différencient en 3 profils d'expression (Figure 26) (Rossi et al. 2000, Zlotnik et al. 2000 et 2006). Certaines chimiokines telles que CX3CL1, CCL17, CCL18, CCL19, CCL20, CCL21, CCL25 ou encore CXCL12 et CXCL13 sont produites de manière constitutive et sécrétées au niveau de tissus ou organes. Ces chimiokines sont impliquées dans l'homéostasie et peuvent participer au développement (CXCL1, CCL8, CXCL12), à l'angiogenèse (CXCL12) ou à la régulation de la production et de la distribution normales des leucocytes (CCL3, CCL19, CCL21, CCL25, CXCL13). Ces chimiokines participent notamment à la migration des lymphocytes vers les organes lymphoïdes lors de la surveillance immunitaire (Raman et al. 2011).

D'autres chimiokines sont considérées comme inflammatoires telles que CCL2, CCL5, CCL11 et CXCL8, et ne sont produites qu'en cas d'inflammation ou d'infection. Elles peuvent être produites par le tissu lui-même (endothélium vasculaire, épithélium) ou par les leucocytes (macrophages, lymphocytes T...). Leur rôle est d'induire la migration des leucocytes vers le site inflammatoire et d'activer les cellules immunitaires pour la mise en place d'une réponse immunitaire. Elles peuvent également être impliquées dans l'angiogenèse (CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL8, CXCL9, CXCL10), la lymphangiogenèse (CXCL12) et dans le développement des tumeurs (CCL2, CCL3, CCL4, CCL5, CCL22, CXCL12) (Belperio et al. 2000, Abroun. 2008, Raman et al. 2011, Zhuo et al. 2012).

Certaines chimiokines peuvent être à la fois constitutives et inflammatoires. CXCL12, par exemple, est exprimée de façon constitutive dans de nombreux tissus comme la moelle osseuse, l'intestin et le poumon mais peut également être surexprimée dans certaines pathologies (Berghuis et al. 2012, Lee et al. 2012, Sheu et al. 2012), le plus souvent en réponse à l'hypoxie (Hitchon et al. 2002, Ceradini et al. 2004, Miller et al. 2005, Yin et al. 2013). Cependant, la surexpression de la chimiokine CXCL12 ne semble pas nécessaire à son rôle majeur dans l'inflammation, notamment dans le recrutement des leucocytes et des cellules progénitrices impliquées dans les phénomènes de réparation et/ou de remodelage (Nanki et al. 2000, Wright et al. 2002, Wang et al. 2008, Karin 2010). En effet, le rôle du couple CXCL12/CXCR4 a été mis en évidence dans de nombreuses pathologies, notamment à l'aide d'antagoniste du récepteur CXCR4 ou d'agents neutralisants de CXCL12, sans qu'une surexpression de la chimiokine ne soit mesurée (Balabanian et al. 2002, Lukacs et al. 2002, Meiron et al. 2008, Hachet-Haas et al. 2008, Doyle et al. 2011).

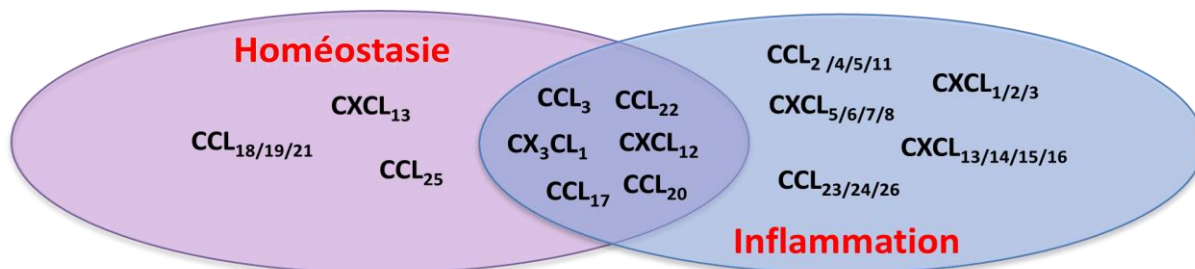


Figure 26: Classification des chimiokines en fonction de leur profil d'expression. Selon cette classification, il existe 3 types de chimiokines: certaines sont produites de façon constitutive et sécrétées au niveau de certains tissus ou organes et sont impliquées dans l'homéostasie; d'autres sont considérées comme inflammatoires et ne sont produites qu'en cas d'inflammation ou d'infection; et enfin certaines chimiokines peuvent être à la fois constitutives et inflammatoires (Liste non exhaustive).

Les récepteurs aux chimiokines sont principalement exprimés par les leucocytes. De manière générale, les CXC chimiokines exercent leur effet chimiotactique sur les neutrophiles et les lymphocytes alors que les CC chimiokines ont un spectre plus large et agissent aussi sur les monocytes, les basophiles et les éosinophiles. La C chimiokine agit principalement sur les lymphocytes T et CX3CL1 sur les monocytes/macrophages, les lymphocytes T et les cellules NK. Cependant, les récepteurs aux chimiokines sont aussi présents sur d'autres types cellulaires, notamment les cellules épithéliales et endothéliales mais aussi sur les cellules musculaires lisses, les neurones et les cellules de la microglie (Moser et al. 2004, Adler et al. 2005, Cartier et al. 2005, Ebert et al. 2005).

L'expression des récepteurs aux chimiokines sur les leucocytes semble assez bien définie (Figure 27). Ainsi, CXCR1 et CXCR2 sont exprimés très majoritairement par les neutrophiles, qui sont principalement impliqués dans la défense antimicrobienne. Les monocytes, les éosinophiles et les basophiles expriment des récepteurs communs et d'autres qui leur sont propres comme CCR5 sur les monocytes ou CCR3 sur les éosinophiles et basophiles. Les lymphocytes sont capables d'exprimer la quasi-totalité du répertoire des récepteurs aux chimiokines et certains comme CCR9, CCR10 et CXCR6 ne sont exprimés que par les lymphocytes T (Moser et al. 2001 et 2004, Abroun 2008). Le profil d'expression des récepteurs aux chimiokines d'un lymphocyte peut permettre de définir l'état de maturation et d'activation de celui-ci. Par exemple, l'expression de CCR7 par les lymphocytes T CD8+ mémoires (CD45RA-) différencie les cellules T mémoires centrales (CCR7+) recirculant vers les organes lymphoïdes secondaires, des cellules T mémoires effectrices ayant un tropisme pour les sites inflammatoires où elles peuvent interagir avec les cellules présentatrices d'antigène ou CPA (Unsoeld et al. 2002, Poholek et al. 2009). De même, les cellules présentatrices d'antigène immatures expriment plutôt CCR1, CCR2 et CCR5 alors que les matures expriment CCR4 et CCR7, ces derniers permettant la migration vers les organes lymphoïdes secondaires (Sallusto et al. 2000 et 2008).

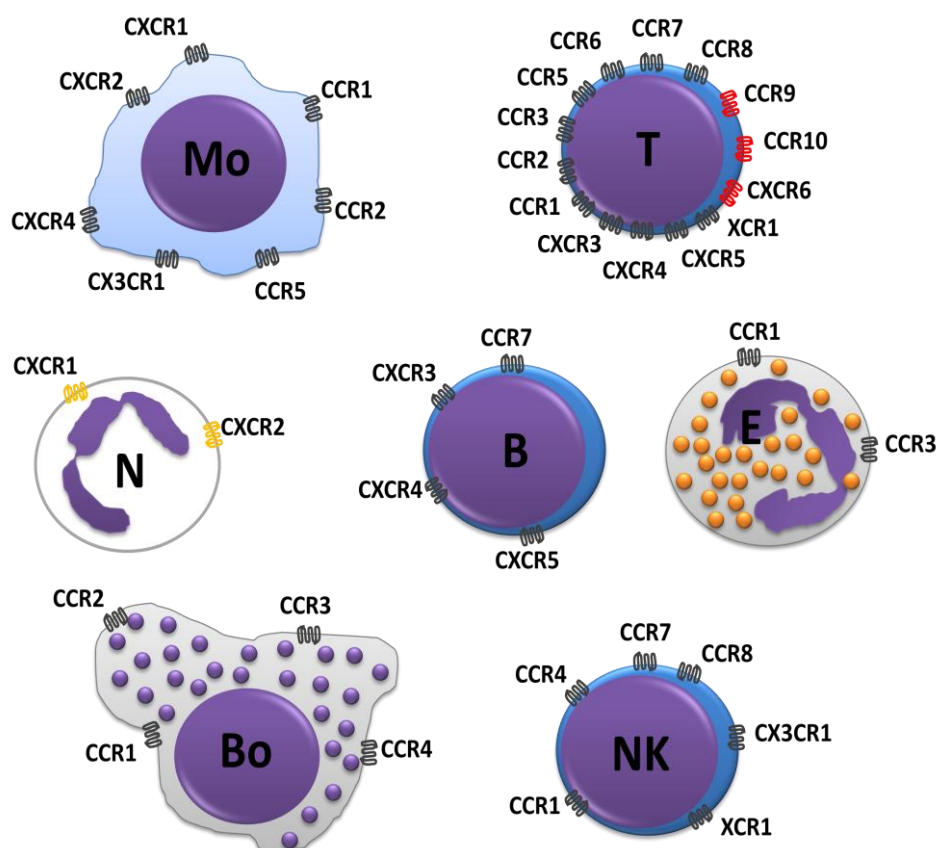


Figure 27: Expression des récepteurs aux chimiokines sur les leucocytes humains. Monocytes (Mo), lymphocytes T (T), lymphocytes B (B), lymphocytes NK (NK), neutrophile (N), basophile (Bo), eosinophile (E). Adapté de Pease 2011)

III.8. Rôles physiologiques et physiopathologiques des chimiokines et de leurs récepteurs

Les chimiokines sont capables d'orchestrer la migration et l'activation des leucocytes et des cellules souches et interviennent dans le maintien de l'homéostasie et participent aux épisodes inflammatoires en permettant le recrutement des cellules immunitaires et la défense de l'organisme contre les agents pathogènes. Cependant, la production de chimiokines inflammatoires peut devenir excessive et incontrôlée. La réponse immunitaire est alors activée de manière inappropriée et exacerbée. Les maladies inflammatoires sont le fait de ces dysfonctionnements, notamment dans les lésions athéromateuses, les rejets de greffe, les réactions allergiques ou encore les pathologies auto-immunes. Il a ainsi été montré que les chimiokines et leurs récepteurs peuvent être associés à la sclérose en plaques, la polyarthrite rhumatoïde, l'athérosclérose, l'asthme, la fibrose, le cancer (Figure 28).

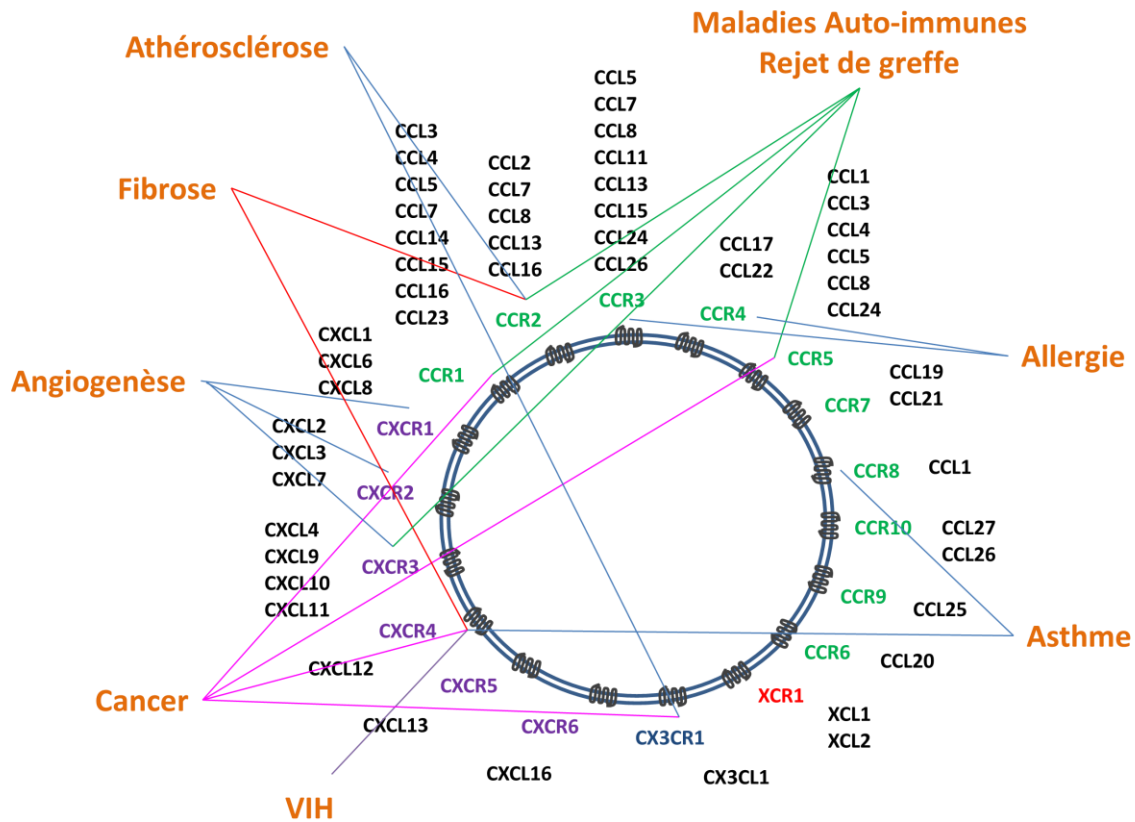


Figure 28: Relation entre pathologies et couples chimiokines/récepteurs. Les associations entre les récepteurs aux chimiokines, leurs ligands et les pathologies représentées sur la figure ne sont pas exhaustives mais montrent la complexité de ces relations. Ces rapprochements ont été obtenus par l'analyse de l'expression des chimiokines dans des biopsies, l'utilisation de modèles murins et des études de polymorphismes chez l'homme. Adapté de Sharma 2010.

III.8.1. Migration cellulaire

Principal rôle des chimiokines et de leurs récepteurs, la migration cellulaire et plus particulièrement la migration des leucocytes est impliquée dans le développement, l'homéostasie et l'inflammation. Les chimiokines et leurs récepteurs interviennent dès la maturation des leucocytes à partir de précurseurs de la moelle osseuse ou du thymus (Moser et al. 2004). Les profils d'expression des récepteurs aux chimiokines sur ces précurseurs hématopoïétiques varient et sont accompagnés par une production spécifique de chimiokines au niveau des différents microenvironnements. Ce système sophistiqué permet la relocalisation des précurseurs dans différents compartiments tissulaires pendant les phases de maturation successives. Ainsi, les chimiokines impliquées dans l'homéostasie permettent la migration des lymphocytes dans les organes lymphoïdes pour la surveillance immunitaire. D'autres chimiokines sont nécessaires pour attirer les monocytes et les cellules présentatrices d'antigène immatures au niveau du site d'inflammation, diriger les CPA matures vers les vaisseaux lymphatiques, recruter à la fois les cellules T et les CPA dans l'organe lymphoïde le plus proche, et enfin permettre la migration des cellules effectrices vers le site

inflammatoire. Les chimiokines et leurs récepteurs sont donc impliqués dans les migrations cellulaires nécessaires à l'initiation des réponses immunitaires.

Certaines chimiokines, comme CCL2, promeuvent la synthèse d'intégrines par les cellules endothéliales et favorisent ainsi la fixation des leucocytes circulants aux cellules endothéliales des capillaires sanguins. La chimiokine CXCL8 quant à elle augmente la perméabilité vasculaire et stimule la diapédèse des polynucléaires neutrophiles activés (Semple et al. 2009). Ces deux processus permettent l'infiltration des leucocytes vers le site inflammatoire (Figure 29). D'autre part, la spécificité de certaines chimiokines vis-à-vis de leurs cibles permet d'organiser la migration des leucocytes. Ainsi, les lymphocytes Th1 sont attirés de manière dose-dépendante par les CC-chimiokines CCL3 et CCL5 qui sont sans effet sur les lymphocytes Th2, au contraire des chimiokines comme CCL2, CCL17, CCL22 et CXCL12 qui exercent leur chimio-attractivité sur les lymphocytes Th1 et Th2 (Siveke JT et al. 1998, Andrew et al. 2001, Sebastiani et al. 2005). Il a été également mis en évidence que CXCL12 était important dans la migration des cellules souches myéloïdes durant le développement, permettant une migration des cellules du foie foetal vers la moelle osseuse. Enfin, la chimiokine CCL11 (éotaxine) recrute les éosinophiles de manière spécifique vers les tissus où elle est sécrétée (Romagnani et al. 2002).

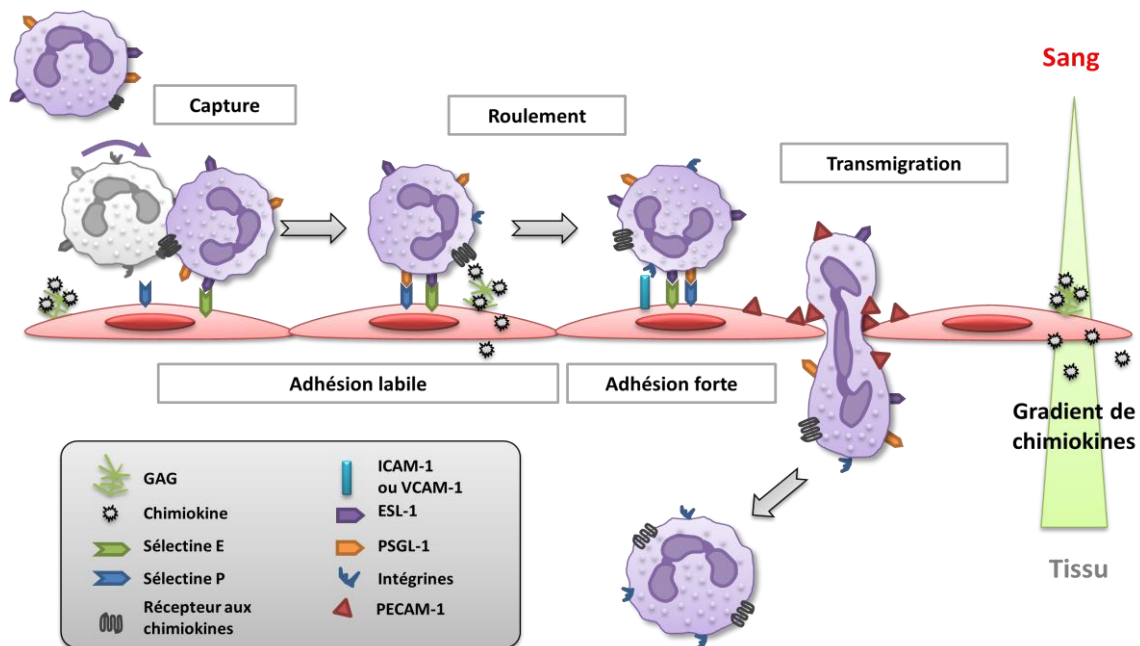


Figure 29: Modèle en plusieurs étapes du recrutement leucocytaire. Les leucocytes se fixent à la surface des cellules endothéliales à l'aide des interactions entre les sélectines et leur ligand (ESL-1 et PSGL-1). Ensuite, les chimiokines présentées par les GAGs sur les cellules endothéliales permettent l'activation des récepteurs aux chimiokines qui entraîne l'expression des intégrines à la surface des leucocytes qui peuvent alors adhérer fortement aux molécules d'adhésions (ICAM-1 ou VCAM-1) présentes sur la paroi vasculaire. Les leucocytes subissent alors un réarrangement de leur cytosquelette leur permettant de franchir la barrière endothéliale ; c'est la transmigration. Les leucocytes peuvent ensuite migrer dans les tissus en direction du gradient de chimiokine. ICAM-1 (*InterCellular Adhesion Molecule 1*), VCAM-1 (*Vascular cell adhesion protein 1*), PSGL-1 (*P-selectin Glycoprotein Ligand*), ESL-1 (*E-selectin ligand*), PECAM-1 (*platelet/endothelial cell adhesion molecule 1*). Adapté de van Buul et al. 2004, Rijcken et al. 2007 et Constantin et al. 2008.

III.8.2. Inflammation

La sécrétion des chimiokines a été détectée dans un grand nombre de maladies inflammatoires (Johnson et al. 2004). Les chimiokines sont responsables de l'activation et de l'accumulation des leucocytes dans les tissus enflammés. Chaque maladie inflammatoire semble posséder un profil leucocytaire caractéristique et spécifique des chimiokines produites. Ainsi, le recrutement des neutrophiles est généralement accompagné d'une augmentation significative de la concentration en CCL2 et CXCL8, comme dans les pneumonies aiguës d'origine bactérienne, les méningites virales ou la polyarthrite rhumatoïde (Szekanecz et al. 2010, Xia et al. 2011). L'accumulation de lymphocytes T dans les lésions granulomateuses, caractéristiques de la lèpre tuberculoïde et de la sarcoïdose, est accompagnée d'une forte concentration de CXCL10.

Dans la maladie de Crohn, une maladie inflammatoire de l'intestin, le passage de la phase aiguë à la phase chronique, caractérisé par une raréfaction des neutrophiles remplacés par des lymphocytes et des macrophages, est accompagné d'une modification du panel de chimiokines produites (MacDermott et al. 1998). Dans le psoriasis, les lésions cutanées contiennent des neutrophiles et des lymphocytes T activés et présentent une forte concentration en CXCL8, CXCL10 et CCL2 (Luster, 1998). Les chimiokines inflammatoires sont excrétées en réponse à des agents pro-inflammatoires, notamment les cytokines IL-1 β , l'IL-4, le TNF α et l'IFN γ impliquées dans la surproduction de nombreuses chimiokines (Figure 30) (Teran et al. 1999, Pechkovsky et al. 2000, Terada et al. 2001, Liu et al. 2007, Matsumiya T et al. 2010).

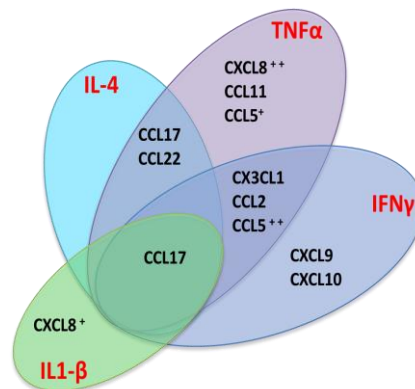


Figure 30: Chimiokines excrétées en réponse à des agents pro-inflammatoires, en particulier par les cellules épithéliales, les macrophages et les lymphocytes en réponse aux cytokines IL-1 β , IL-4, TNF α et IFN γ .

III.8.3. Prolifération cellulaire et angiogénèse

Outre les activités pro-inflammatoires, les chimiokines régulent également la prolifération de différents types cellulaires et sont impliquées dans l'angiogénèse et dans le développement de tumeurs malignes. La chimiokine CXCL1 est sécrétée par les cellules de mélanome et possède un effet prolifératif autocrine. Une étude *in vitro* a également mis en évidence que des cellules

primaires tumorales provenant de mélanomes intra-oculaires expriment les récepteurs CXCR1, CXCR2 et CXCR4, et peuvent migrer selon un gradient de chimiokine CXCL12 ou CXCL8 (Di Cesare et al. 2007). Une activité chemoattractante est également mise en évidence sur des lignées cellulaires provenant d'ostéosarcome (Perissinotto et al. 2005). Ces auteurs ont également mis en évidence l'importance de l'axe CXCL12-CXCR4 dans la rétention des cellules métastatiques dans les organes éloignés de la tumeur primaire. Ils ont en effet montré que l'administration d'un peptide anti-CXCR4 (T134) empêche la formation de métastases dans le poumon suite à l'administration intraveineuse des cellules tumorales d'ostéosarcome. D'autre part, le couple CCL21-CCR7 est lui impliqué dans la formation des métastases dans les ganglions lymphatiques dans de nombreux types de cancer tels que le cancer du sein, le cancer colorectal ou encore le cancer du poumon non à petite cellule, un phénomène lié à l'abondance de CCL21 dans les ganglions lymphatiques (Raman et al. 2007).

Les chimiokines sont également engagées dans la régulation de l'angiogenèse, en coopération avec d'autres facteurs tels le FGF β (*fibroblast growth factor β*) et le VEGF (*vascular endothelial growth factor*). Ainsi, CXCL1, CXCL5 et CXCL12, comme la plupart des chimiokines CXC, dopent le chimiotactisme et la prolifération des cellules endothéliales et ainsi stimulent l'angiogenèse. De plus, l'hypoxie augmente l'expression de CXCR4 (via HIF1- α) sur les cellules endothéliales, ce qui amplifie l'action de CXCL12. A l'inverse, des chimiokines comme CXCL4 (PF4) et CXCL10 inhibent la prolifération des cellules endothéliales (Verbeke et al. 2010). Il a été mis en évidence que la chimiokine CXCL4 peut inhiber l'activité pro-angiogénique du bFGF (*basic fibroblast growth factor*) et du VEGF, en empêchant leur liaison avec leurs récepteurs, par liaison directe avec ces facteurs de croissance ou par compétition en empêchant leur fixation aux glycosaminoglycanes (GAG), une étape importante qui stabilise les interactions entre ces facteurs de croissance et leur récepteurs. CXCL4 peut aussi agir comme un antagoniste des intégrines et réduire ainsi l'adhérence des cellules endothéliales au micro-environnement extracellulaire et leur migration (Wang et al. 2013). L'activité angiostatique de CXCL10 reste encore mal connue mais pourrait, à l'image de CXCL4, être due à sa forte affinité pour les GAGs qui empêcherait la liaison du bFGF et du VEGF aux GAGs (Campanella et al. 2010).

En résumé, les chimiokines et leurs récepteurs sont donc impliqués dans l'homéostasie et dans la défense de l'organisme contre les agents pathogènes, mais également dans de nombreuses pathologies, et notamment dans le cancer et l'inflammation.

Parmi ces chimiokines, il a été mis en évidence que la chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7 ont un rôle fondamental dans l'organogenèse et dans l'homéostasie, mais aussi dans l'asthme allergique.

IV. La chimiokine CXCL12 et ses deux récepteurs CXCR4 et CXCR7

IV.1. La chimiokine CXCL12

Les protéines SDF1 α et SDF1 β (stromal cell derived factor 1) sont les premières chimiokines identifiées par clonage à partir d'une lignée de cellules stromales de la moelle osseuse murine (Tashiro et al. 1993). Ces protéines ont été clonées à partir des cellules stromales humaines (Shirozu et al. 1995) et ont été identifiées comme des cytokines induisant la prolifération des précurseurs des cellules B dépendantes des cellules stromales. Elles avaient donc aussi été appelées « facteurs stimulant la croissance des cellules B » (PBSF : Pre-B cell growth Stimulating Factor) (Nagasawa et al. 1994 et 1996). Elles sont aujourd'hui classées dans la famille des chimiokines au motif CXC et sont appelées CXCL12 α et CXCL12 β . La chimiokine CXCL12 existe sous forme soluble mais elle peut aussi se lier aux glycosaminoglycannes présents à la surface des cellules et joue alors un rôle dans l'adhésion et dans la transmigration des leucocytes (Janowski. 2009, Bryant et al. 2012).

Les deux variants CXCL12 α (68 acides aminés ou AA) et CXCL12 β (72 acides aminés) sont codés par un seul gène qui subit un épissage alternatif. CXCL12 β possède 4 acides-aminés supplémentaires à l'extrémité carboxy-terminale. Ces deux formes alternatives ont des niveaux d'expression et des fonctions identiques. Quatre autres variants de CXCL12 ont été identifiés, qui contiennent respectivement 30 AA (CXCL12 γ), 31 AA (CXCL12 δ), 1 AA (CXCL12 ϵ) et 51 AA (CXCL12 ϕ) supplémentaires à l'extrémité C-terminale par rapport à CXCL12 α (Rueda et al 2008).

Chez l'homme, CXCL12 est la seule chimiokine de type CXC dont le gène est localisé sur le chromosome 10 alors que les gènes codant pour toutes les autres chimiokines de type CXC sont portés par les chromosomes 4 et 5. Elle est produite de façon constitutive par de nombreuses cellules comme les cellules endothéliales, les cellules épithéliales et les cellules stromales. CXCL12 est très bien conservée entre les espèces. Seul le remplacement d'une valine par une isoleucine en position 18 diffère entre l'homme et la souris. Cette conservation à travers l'évolution suggère un rôle fondamental de CXCL12 dans l'homéostasie et le développement (Shirozu et al. 1995). La chimiokine CXCL12 se lie au récepteur CXCR4 ou LESTR/fusin (Oberlin et al. 1996) et au récepteur CXCR7 ou RDC1 (*Receptor Dog ADNc*) (Balabanian et al. 2005).

Bien que présentant de nombreuses caractéristiques communes à la famille des chimiokines CXC, la structure de la chimiokine CXCL12 (Figure 31) possède une singularité la rapprochant de la famille des chimiokines CC : l'orientation des ponts disulfures est identique à celle des chimiokines de la famille CC. Cette différence résulte sans doute de l'existence d'une proline entre les cystéines 9 et 11 (Gozansky et al. 2005). De plus, une récente étude phylogénétique a établi une relation plus étroite entre CXCL12 et les chimiokines CC qu'avec les chimiokines CXC (Murphy et al. 2010).

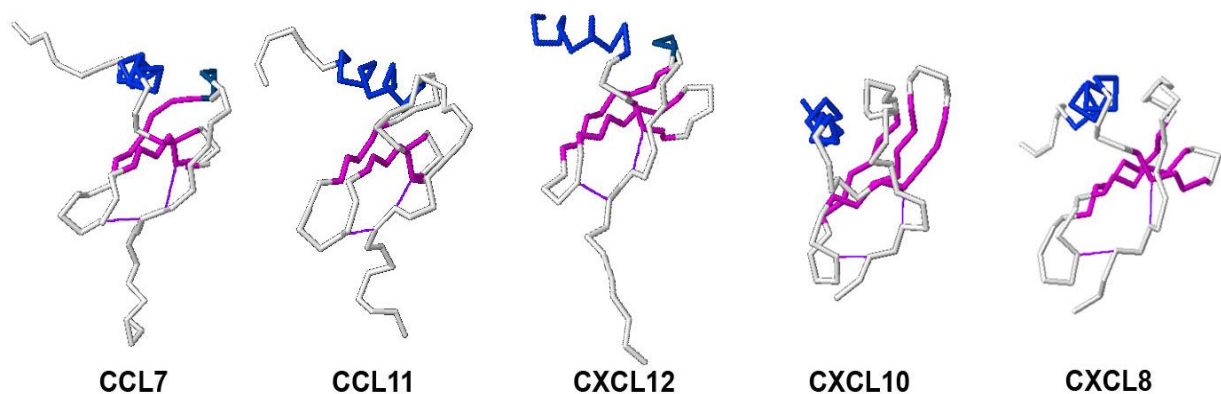


Figure 31 : Structure tridimensionnelle de CXCL12. Figure illustrant la structure 3D de la chimiokine CXCL12 et l'orientation des ponts disulfures (violet) comparativement à deux chimiokines CC (CCL7 et CCL11) et deux chimiokines CXC (CXCL8 et CXCL10). Les brins β sont représentés en rose, l'hélice α carboxy-terminale en bleu, les boucles 30s, 40s et 50s en blanc et la boucle N en vert foncé. Structures 3D obtenues et orientées à partir de la base de données PDB (*Protein Data Bank*).

IV.2. Sites de production de CXCL12

L'expression de CXCL12 est constitutive dans de nombreux tissus (Janowski. 2009). CXCL12 est produite chez le fœtus par les cellules épithéliales biliaires des plaques ductales du foie fœtal et par les cellules mésothéliales des cavités coelomiques (Lewellis SW et al. 2012), et est indispensable au développement du fœtus. Chez l'adulte, la chimiokine CXCL12 est détectée dans les cellules stromales mais aussi endothéliales, épithéliales et dendritiques dans différents tissus comme le foie, le poumon, la glande surrénale, la moelle osseuse et dans les cellules gliales, les astrocytes et certains types de neurones dans le système nerveux central (Banisadr et al. 2003, Karin 2007).

L'isoforme CXCL12- β semble être exprimé moins abondamment que l'isoforme CXCL12- α et semble être lié au système vasculaire. Il a été mis en évidence que les cellules endothéliales des microvaisseaux cérébraux chez les souris expriment spécifiquement CXCL12- β et que cette isoforme est surexprimée suite à une ischémie cérébrale focale (Stumm et al. 2002). Cette surexpression est associée à l'infiltration de cellules exprimant CXCR4, telles que les macrophages. CXCL12- β a également un effet plus important que les autres variants sur l'angiogenèse des cellules endothéliales micro-vasculaires humaines (Manetti et al. 2009). Une estimation de la répartition de CXCL12- β (ARNm) chez l'homme a révélé leur présence dans les organes hautement vascularisés tels que le foie, la rate, la moelle osseuse et les reins, et leur absence dans le cerveau (Yu et al. 2006).

CXCL12- γ est l'isoforme prédominante dans le cerveau de rat adulte, en remplacement de CXCL12- β qui est abondant durant la vie embryonnaire (Gleichmann et al. 2000). Chez l'homme et la souris, CXCL12- γ a principalement été mis en évidence dans le cœur (Pillarsetti et al. 2001, Yu et al. 2006), son expression pulmonaire basale est faible (Rueda et al 2008). Le domaine C-terminal enrichi en

acides aminés basiques de CXCL12- γ renforce de manière spectaculaire sa fixation aux GAGs (Rueda et al, PlosOne 2008). Suite à un infarctus expérimental chez la souris, la quantité de CXCL12- γ reste inchangée alors que l'expression de CXCL12- α est augmentée. Les rares données portant sur CXCL12- γ indiquent que son expression est associée aux organes les plus sensibles aux infarctus tels que le cerveau et le cœur.

Les variants d'épissage CXCL12- δ , CXCL12- ϵ et CXCL12- ϕ ont été décrits récemment dans les tissus humains et semblent abondants dans le pancréas. CXCL12- ϵ et CXCL12- ϕ ont été mis en évidence dans le cœur et le foie, ainsi que dans le rein chez le fœtus et l'adulte. CXCL12- δ a également été détecté dans la rate, le foie et le poumon chez le fœtus. Les différents variants d'épissage semblent présenter la même réponse cellulaire, mais être actifs dans différents tissus et conditions physiologiques ou pathologiques (pour revue, Janowski 2009).

IV.3. Les récepteurs de la chimiokine CXCL12 : le récepteur CXCR4

C'est en menant des recherches sur les récepteurs orphelins que le gène codant le récepteur CXCR4 est identifié pour la première fois. Il est caractérisé comme un récepteur à sept domaines transmembranaires et tout d'abord classé dans la famille des récepteurs du neuropeptide Y comme récepteur NPY Y3 (Herzog et al. 1993). Les auteurs montrent également que ce récepteur présente une homologie avec le récepteur de l'interleukine 8.

Dans le même temps, l'ADNc codant pour une protéine de 352 AA compatible avec la séquence d'un RCPG est également isolé d'une banque d'ADN de monocytes humains (Loetscher et al. 1994). Ces auteurs démontrent que ce RCPG, nommé LESTR (*Leukocyte-derived Seven Transmembrane domain Receptor*) est identique à 92,7% au récepteur NPY Y3 mais qu'il ne lie pas le NPY, ni la plupart des agents chimiotactiques connus. Ces auteurs démontrent également qu'il est présent dans les lymphocytes et les polynucléaires neutrophiles. En étudiant l'infection des lymphocytes CD4 par le VIH, Feng et ses collaborateurs mettent en évidence l'existence d'un cofacteur nécessaire à la fusion et l'infection des lymphocytes par le VIH. Ils l'identifient à un récepteur appartenant à la famille des RCPG et le nomment "Fusin" (Feng et al. 1996).

En parallèle, le récepteur murin "*pre-B-cell-derived chemokine receptor*" (PB-CKR/CXCR4) est identifié comme récepteur fonctionnel de la chimiokine CXCL12 (Nagasawa al. 1996) et le rôle protecteur de CXCL12 dans l'infection des lymphocytes par le VIH est également mis en évidence. Ce récepteur murin est cloné et sa séquence protéique présente, respectivement, 86% et 96% d'identité avec les formes bovines et humaines (Heesen et al. 1996, Nagasawa al. 1996). Récemment, il a été mis en évidence que le récepteur CXCR4 peut également lier l'ubiquitine extracellulaire, une protéine aux propriétés anti-inflammatoires (Majetschak et al. 2003), dans de nombreuses lignées cellulaires de

monocytes/macrophages murins et humains (Saini et al. 2010) et induire une réponse calcique et une baisse de l'AMPc (IC50 = 104 nM contre 49nM pour CXCL12) pouvant être inhibées par un antagoniste de CXCR4, l'AMD3100. L'ubiquitine possède aussi une activité chimiotactique sur les THP1, mais présente un index chimiotactique deux fois plus faible que CXCL12, et contrairement à CXCL12, l'ubiquitine ne se lie pas à la partie amino-terminale du récepteur, mais interagit uniquement avec les boucles extracellulaires du récepteur CXCR4 et ne bloque pas l'infection des cellules P4.R5 MAGI par le VIH (Saini et al. 2011).

Le récepteur CXCR4 présente un poids moléculaire de 40kDa et appartient à la famille des récepteurs à sept domaines transmembranaires couplés aux protéines G α i. La protéine CXCR4 murine diverge de la forme humaine, principalement au niveau de la seconde boucle extracellulaire qui présente une insertion de 7 AA (Q181-G187). De plus, elle présente 1 site de glycosylation à l'extrémité N-terminale, alors que 2 sont présents chez l'homme. Au contraire, le motif de liaison aux protéines G α i dans la troisième boucle cytoplasmique et le motif riche en sérine à l'extrémité C-terminale sont très conservés et préservent la liaison aux protéines G α i et le domaine de signalisation de ces récepteurs (Heesen et al. 1996, Bussillo et al. 2007).

IV.3.1. Site de production du récepteur CXCR4

Le récepteur CXCR4 est exprimé sur les cellules hématopoïétiques (Sharma M et al. 2011) et par les leucocytes circulants, incluant les neutrophiles, les monocytes, les lymphocytes B et T, les macrophages et les cellules dendritiques. Il est également exprimé dans le poumon, le cœur et le cerveau, notamment par les cellules endothéliales du système vasculaire, par les cellules épithéliales, et dans les neurones du système périphérique ou central, dans la microglie et les astrocytes. Une concentration importante de cellules exprimant CXCR4 a été identifiée dans les muqueuses génitales et digestives, ainsi que dans les glandes salivaires et le placenta. De plus, l'expression récepteur CXCR4 a été détectée dans 23 types de cancer (Balkwill F. 2004) où il est impliqué dans la migration et la survie des cellules cancéreuses (Furusato B et al. 2010, Hattermann K et al. 2012).

IV.3.2. Signalisation induite par CXCR4

Le récepteur CXCR4 appartient à la famille des récepteurs à sept domaines transmembranaires couplés aux protéines G α i sensibles à la toxine pertussis (PTX). Le prétraitement avec la PTX, catalyse l'ADP-ribosylation au niveau d'une cystéine située près de la partie C-terminale du récepteur, et prévient l'activation de la protéine G nécessaire à la phosphorylation des sérines en partie C-terminale du récepteur (Busillo JM et al. 2010).

La liaison de la chimiokine CXCL12 sur le récepteur CXCR4 active des voies de signalisations dépendantes de l'activation de la protéine G α i. La protéine G α i est capable d'inhiber l'adénylate cyclase et d'activer des kinases apparentées à la famille Src, tandis que G β y est libérée et active la phospholipase C- β (PLC- β) et la PI3K (*phosphatidylinositol 3 kinase*), conduisant à la régulation des processus tels que la transcription des gènes, la migration cellulaire et l'adhérence cellulaire (Figure 32) (Offermanns S. 2003, Roland J et al. 2003).

L'activation de PLC- β , entraîne le clivage du PIP2 (*phosphatidyl-inositol-diphosphate*) en IP3 (*Inositol trisphosphate*) et DAG (diacylglycerol). L'IP3 est une molécule soluble, capable de diffuser à travers le cytoplasme, en direction du réticulum endoplasmique, où il est capable de se lier à un récepteur Ins3PR (*inositol-3-phosphate receptor*) présent sur un canal calcique. La liaison de l'IP3 à Ins3PR déclenche l'ouverture du canal Ca²⁺ et la libération de Ca²⁺ dans le cytoplasme et conduit à l'activation de protéines kinases telles que PKC (*protein kinase-C*), les MAPK (*Mitogen-activated protein kinase*) (Fernandis et al. 2003). La libération de calcium intracellulaire en réponse à l'activation du récepteur CXCR4 par CXCL12 peut être utilisée pour le criblage d'agents pharmacologiques dans le but d'identifier des inhibiteurs de l'interaction CXCR4/CXCL12 (Hachet-Haas et al. 2008)

L'activation de la PI3K, entraîne ensuite la phosphorylation d'effecteurs tels que Pyk-2 (*proline-rich tyrosine kinase 2*), la FAK (*Focal adhesion kinase*), la paxilline, NCK (*non-catalytic region of tyrosine kinase adaptor protein 1*), ainsi qu'un adaptateur protéique (Crk), et promeut le chimiotactisme. La PI3K peut également activer la kinase AKT ou "protéine kinase B" qui peut conduire à l'activation de la voie NF- κ B, impliquée dans la transcription de nombreux gènes, mais peut aussi phosphoryler la protéine pro-apoptotique BAD (*Bcl-2-associated death*) et la dissocie du complexe Bcl-2/Bcl-X, perdant ainsi sa fonction pro-apoptotique (Suzuki et al. 2001).

Il a également été mis en évidence que la sous unité G α i peut inhiber l'adénylate cyclase (Dwinell et al. 2003) et entraîner une baisse de l'AMPc qui peut à son tour affecter la régulation homéostatique du transport des électrolytes par les canaux ioniques, comme cela a été mis en évidence in vitro sur une lignée cellulaire de carcinome du côlon, mais peut aussi affecter l'activation de la PKA (protéine kinase A) (Takahashi et al. 2002). En condition basale, la PKA est associée à NF- κ B et AKIP1 (A kinase interacting protein 1), dans le cytoplasme, et son domaine catalytique est masqué par I κ B (King et al. 2011). Une augmentation d'AMPc (en réponse au TNF α ...) entraîne la dissociation de la PKA, la phosphorylation de la protéine P65 du complexe NF- κ B par la PKA, la translocation de NF- κ B jusqu'au noyau et l'activation de la transcription des gènes cibles.

Ainsi, l'inhibition de l'adénylate cyclase permettrait de réguler l'activité de NF- κ B. L'activation de CXCR4 peut également influencer le cytosquelette via les petites GTPases représentées par Rho, Rac et Cdc 42 et promouvoir le chimiotactisme (Teicher et al. 2010).

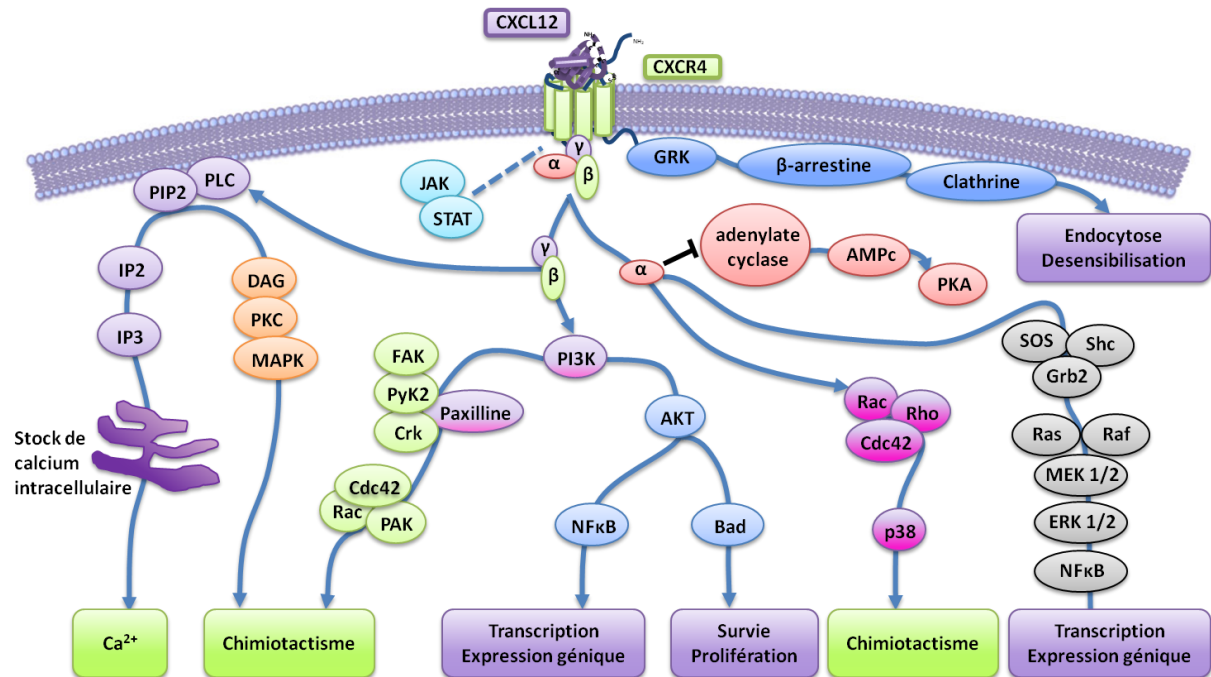


Figure 32: Principales voies de signalisation intracellulaire du couple CXCR4/CXCL12. La stimulation de CXCR4 par la chimiokine CXCL12 induit l'activation de différentes voies de signalisation qui peuvent entraîner le chimiotactisme, la survie cellulaire et / ou la prolifération, la régulation de la transcription des gènes. D'après Teicher et al. 2010.

La présence de 15 sérines et 3 thréonines sur la boucle C-terminale intracellulaire du récepteur CXCR4 (308-KFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESSESSFHSS-352) permet des interactions indépendantes de la protéine G. La liaison de CXCL12 à CXCR4 induit l'interaction de CXCR4 avec différentes GRK (*G-proteincoupled receptor kinase*) soit GRK2, GRK3, GRK5 et GRK6 (Figure 33). L'interaction de CXCR4 avec GRK2, GRK3 et GRK6 entraîne une phosphorylation du récepteur au niveau C-terminal et conduit au recrutement de la β -arrestine (Busillo et al. 2010).

Ce mécanisme résulte en un phénomène de désensibilisation et permet ainsi de réguler l'activité du récepteur CXCR4 comme cela a été mis en évidence *in vitro* sur des fibroblastes de peau provenant de patients atteints du syndrome WHIM (*Warts, Hypogammaglobulinemia, Infections, and Myelokathexis syndrome*) (Balabanian et al. 2008). Ces cellules présentent une diminution importante de la production de GRK3 qui conduit à une diminution du recrutement de la β -arrestine lors de l'activation du récepteur CXCR4 par CXCL12 entraînant une diminution de l'internalisation et de la désensibilisation du récepteur concomitante à une augmentation du chimiotactisme.

Une autre équipe a montré que la perte de GRK6 conduit également à une suractivité du récepteur CXCR4 (Vroon et al. 2004). Les auteurs montrent que le chimiotactisme des neutrophiles GRK6^{-/-} est augmenté, que les récepteurs CXCR4 ne se désensibilisent pas et que l'administration sous-cutanée de G-CSF à des souris déficientes en GRK6 mobilise trois fois moins de neutrophiles dans le sang, une diminution de la mobilisation des neutrophiles pouvant être expliquée par une rétention plus importante des cellules dans la moelle osseuse due à la suractivité du récepteur CXCR4. Étonnamment, contrairement aux neutrophiles, le chimiotactisme des lymphocytes B et T pour CXCL12 est inhibé en l'absence de GRK6 (Fong et al. 2002). Cette différence d'activité chimiotactique entre les lymphocytes et les neutrophiles pourrait être expliquée par l'activation de voies de signalisation distinctes entre ces cellules en réponse à CXCR4.

In vitro, sur des cellules HEK-293T, la phosphorylation des sérines 324/5, 330 et 339 par GRK6 permet le recrutement de la β -arrestine et inhibe le flux calcique, tandis que la phosphorylation des résidus entre les sérines 346 et 352 par GRK3 peut agir de concert avec GRK6 et participer à l'activation de ERK1/2 en altérant la conformation de la β -arrestine (Busillo et al. 2010). Les auteurs montrent également que les β -arrestines 2 et 3 n'ont pas les mêmes effets sur la voie de signalisation de CXCR4 et montrent que la β -arrestine 3 est impliquée dans la désensibilisation du récepteur tandis que la β -arrestine 2 peut activer ERK1/2 et nécessite la phosphorylation du récepteur CXCR4 par GRK6 et GRK3. Des résultats en accord avec une étude précédente qui montre aussi une activation de ERK1/2 via la β -arrestine 2 pour le récepteur de l'angiotensine II (Ahn et al. 2004). Il est intéressant de noter que ces résultats ont été obtenus sur la même lignée cellulaire, les HEK-293T, et qu'ils n'ont pour le moment pas été confirmés sur une autre lignée cellulaire ou sur des cultures primaires, et pourraient être spécifiques de la lignée utilisée.

D'autre part, la phosphorylation des résidus entre les sérines 346 et 352 par GRK2 inhibe l'activation des flux calciques et l'activation de ERK1/2 en réponse à l'activation de CXCR4 par CXCL12 (Busillo et al. 2010). La surexpression de GRK2 augmente l'internalisation du récepteur CXCR4 suite à l'activation par CXCL12 et accroît le phénomène de désensibilisation (Orsini et al. 1999). Des résultats qui laissent à penser que la phosphorylation des résidus par GRK2 promeut la liaison à la β -arrestine 3. GRK2 pourrait aussi interagir directement avec MEK et contrôler l'activation de ERK1/2 induite par la liaison de la chimiokine à son récepteur (Jiménez-Sainz et al. 2006).

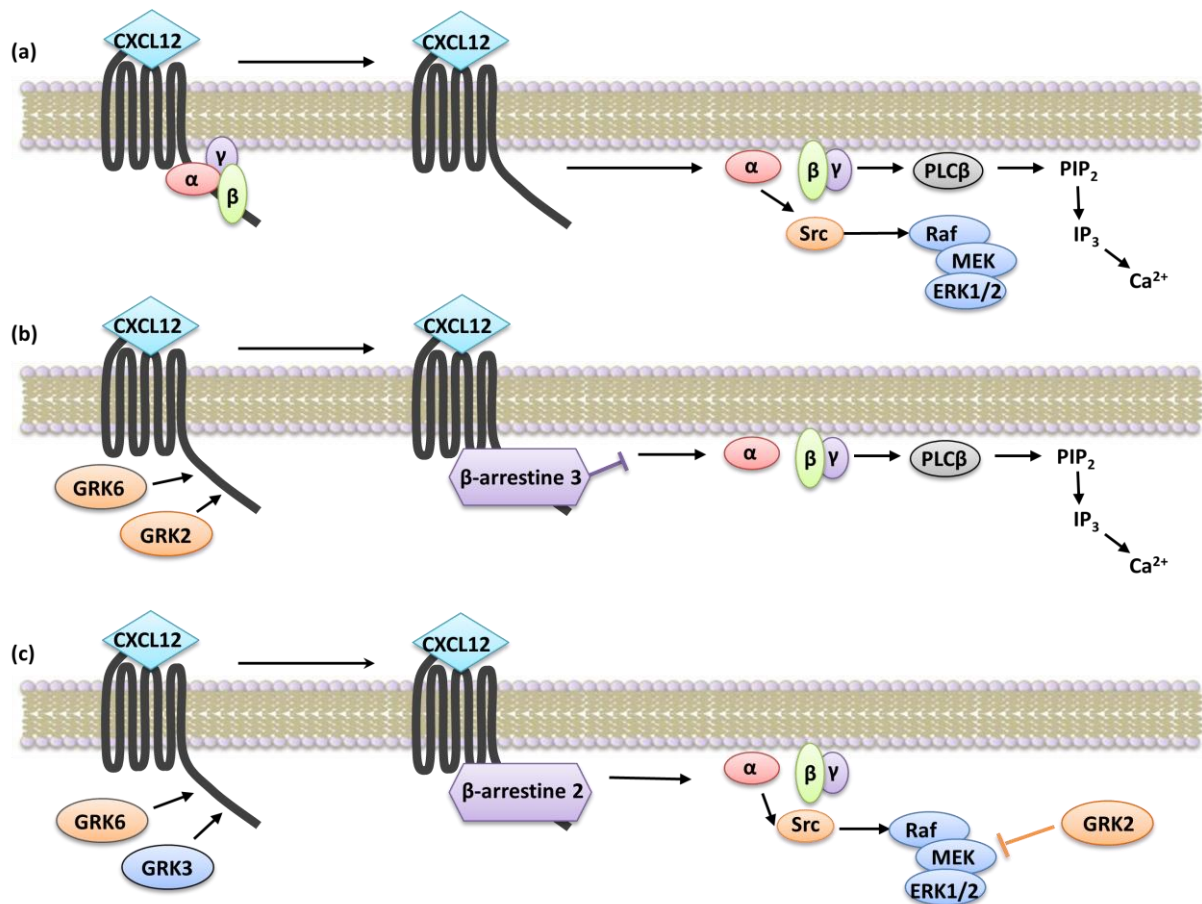


Figure 33: Régulation de l'activité du récepteur CXCR4. (a) Suite à la liaison de CXCL12, le récepteur CXCR4 active plusieurs cascades de signalisation induisant une libération des stocks de calcium intracellulaire et la phosphorylation de ERK1/2. (b) La phosphorylation de CXCR4 par GRK6 (Sérines 324/5, 330 et 339) et GRK2 (résidus entre les sérines 346 et 352) induit le recrutement de l'arrestine3, ce qui atténue l'activation de la protéine Gαi et la libération de calcium. Phospholipase C-β (PLCβ), Phosphatidylinositol diphosphate (PIP₂), inositol triphosphate (IP₃). (c) La phosphorylation de CXCR4 par GRK6 et GRK3 (résidus entre les sérines 346 et 352) conduit au recrutement de l'arrestine 2 et permet une activation complète de ERK1/2. GRK2 pourrait interagir avec MEK et réguler l'activation de ERK1/2. (D'après Busillo et al. 2010)

IV.4. Les récepteurs de la chimiokine CXCL12 : le récepteur CXCR7

Le récepteur CXCR7 est connu depuis 1989 sous le nom de RDC1 « Receptor Dog ADNc » lors de sa mise en évidence dans une banque d'ADNc de chien (Libert et al. 1989). Il est aussi connu sous d'autres noms tels que les GPR159, GPRN1, CMKOR1 (*chemiokine orphan receptor 1*), AW541270 et *chemocentrin-chemokine receptor 2* (CCX-CKR2). Orphelin durant de nombreuses années, le premier ligand de CXCR7 a été identifié en 2005: CXCL12 (Balabanian et al. 2005). Il a été montré que CXCL12 possède une affinité environ 10 fois supérieure pour CXCR7 ($K_d \approx 0.2-0.4$ nM) que pour CXCR4 ($K_d \approx 2-4$ nM) (Naumann et al. 2010). Depuis, il a également été mis en évidence que CXCR7 lie la chimiokine CXCL11/ ITAC (*interferon-inducible T cell chemoattractant*) avec une forte affinité. Ce

second ligand peut perturber l'interaction CXCL12/CXCR7 (Burns et al. 2006). En outre, CXCL11 peut également se lier à un autre récepteur de chimiokine : CXCR3 (Cole et al. 1998). Bien que CXCR7 soit hautement conservé chez les mammifères, la position du gène varie : ainsi chez la souris, le gène CXCR7 se situe sur le chromosome 1 alors que le gène CXCR7 humain se situe sur le chromosome 2 (où les gènes codant pour CXCR1, CXCR2, et CXCR4 sont également situés). Comme tous les récepteurs des chimiokines, le récepteur CXCR7 est un récepteur à 7 domaines transmembranaires (Zlotnik et al. 2000), mais il n'est cependant pas couplé aux protéines G. Cette caractéristique peut être liée à des modifications dans la séquence DRYLAIV nécessaires à la liaison aux protéines G hétérotrimériques et situées au niveau de la boucle ICL2 (Figure 34) (Thelen et al. 2008).

CX3C1	T	V	I	S	I	D	R	Y	L	A	I	V	L	A	A	N	S	M	N	N	R	T
XCR1	T	I	M	T	I	H	R	Y	L	S	V	V	S	P	L	S	T	L	R	V	P	T
CCR1	I	L	L	T	I	D	R	Y	L	A	I	V	H	A	V	F	A	L	R	A	R	T
CCR2	I	L	L	T	I	D	R	Y	L	A	I	V	H	A	V	F	A	L	K	A	R	T
CCR3	I	L	L	S	I	D	R	Y	L	A	I	V	H	A	V	F	A	L	R	A	R	T
CCR4	M	L	M	S	I	D	R	Y	L	A	I	V	H	A	V	F	S	L	R	A	R	T
CCR5	I	L	L	T	I	D	R	Y	L	A	V	V	H	A	V	F	A	L	K	A	R	T
CCR6	T	C	I	S	M	D	R	Y	I	A	I	V	Q	A	T	K	S	F	R	L	R	S
CCR7	L	C	I	S	I	D	R	Y	V	A	I	V	Q	A	V	S	A	H	R	H	R	A
CCR8	T	L	M	S	V	D	R	Y	L	A	V	V	H	A	V	Y	A	L	K	V	R	T
CCR9	M	C	I	S	V	D	R	Y	I	A	I	A	Q	A	M	R	A	H	T	W	R	E
CCR10	A	C	I	S	A	D	R	Y	V	A	I	A	R	A	L	P	A	G	P	R	P	S
CCRL1	A	C	I	S	I	D	R	Y	V	A	V	T	K	V	P	S	Q	S	G	V	G	P
CXCR1	A	C	I	S	V	D	R	Y	L	A	I	V	H	A	T	R	T	L	T	Q	K	R
CXCR2	A	C	I	S	V	D	R	Y	L	A	I	V	H	A	T	R	T	L	T	Q	K	R
CXCR3	A	C	I	S	F	D	R	Y	L	N	I	V	H	A	T	Q	L	Y	R	R	G	P
CXCR4	A	F	I	S	L	D	R	Y	L	A	I	V	H	A	T	N	S	Q	P	R	K	L
CXCR5	A	C	I	A	V	D	R	Y	L	A	I	V	H	A	V	H	A	Y	R	H	R	R
CXCR6	T	C	I	T	V	D	R	F	I	V	V	V	K	A	T	K	A	Y	N	Q	Q	A
CXCR7	T	C	M	S	V	D	R	Y	L	S	I	T	Y	F	T	N	T	P	S	S	R	K
CCBP2	S	C	M	S	L	D	K	Y	L	E	I	V	H	A	Q	P	Y	H	R	L	R	T
DUFY	G	C	H	A	S	L	G	H	R	L	G	A	G	Q	V	P	G	L	T	L	G	L

Figure 34 : Alignement de séquences des récepteurs des chimiokines. La région encadrée représente la séquence N-terminale de la seconde boucle intracellulaire ICL2 comprenant le motif DRYLAIV. D'après Thelen et al. 2008.

IV.4.1. Expression du récepteur CXCR7

Chez l'homme et la souris, l'expression de CXCR7 est plus élevée dans les tissus embryonnaires que chez l'adulte (Thelen et al. 2008). CXCR7 est exprimé par les lymphocytes B et T, les monocytes et les neutrophiles (Balabanian et al. 2005, Sánchez-Martín et al. 2013). L'expression du récepteur CXCR7 est corrélée à la capacité des lymphocytes B à se différencier en plasmocytes après activation, ce qui suggère que CXCR7 est un marqueur des cellules B mémoires compétentes pour devenir des cellules sécrétrices d'anticorps (Sierro et al. 2007). Il a également été mis en évidence que l'expression de CXCR7 par les cellules B mémoires augmente la survie de ces cellules (Infantino et al. 2006).

De plus, CXCR7 est exprimé dans les cellules neuronales, dans les cellules vasculaires et gliales et par de nombreuses lignées cellulaires tumorales (comme dans certains types de cancer du sein, du poumon et du cerveau), par les cellules du foie fœtal et par le placenta (Tableau 8) (Sánchez-Martín et al. 2013).

Type cellulaire	Expression ARNm	Expression à la membrane	Expression intracellulaire
Lymphocytes T	Population CD4+ Faible expression dans les lymphocytes T purifiés	Moelle osseuse CD4+ Lymphocytes sanguins	Oui
Lymphocytes B	Faible expression dans les lymphocytes B purifiés	Oui	n.d.
Monocytes	Faible expression dans les monocytes purifiés	Monocytes sanguins	Oui
Macrophages	n.d.	n.d.	Associés aux tumeurs
Cellules dendritiques	n.d.	Augmentation de l'expression en présence de LPS, TNF- α	n.d.
Cellules NK	n.d.	Oui	n.d.
Granulocytes	n.d.	Moelle osseuse	n.d.
Plaquettes	n.d.	Oui	n.d.
Neurones	Migration corticale		
Cellules gliales	n.d.	Présence en hypoxie	n.d.
CSM	Oui	Oui	Oui
Cellules progénitrices rénales	Présence en hypoxie		n.d.
Endothéliales	Augmentation de l'expression en présence de TNF- α ou en hypoxie		
Cellules cancéreuses	Augmentation de l'expression en présence d'IL-8 et VEGF Augmentation ou diminution en cas d'hypoxie (dépendante de la lignée cellulaire)		

Tableau 8: Principaux sites d'expression de CXCR7 et régulation. Cellules souches mésenchymateuses (CSM). D'après Sánchez-Martín et al. 2013.

IV.4.2. Signalisation du récepteur CXCR7

Contrairement au récepteur CXCR4, plusieurs études ont montré que l'activation du récepteur CXCR7 n'induit pas l'association de protéines G hétérotrimériques, ni de libération de calcium intracellulaire (Burns et al. 2006; Sánchez-Martín et al. 2013). Cependant, d'autres études mettent en évidence que CXCR7 peut se lier à la sous-unité $G\alpha_i$. Une première équipe a mis en évidence sur une lignée cellulaire provenant d'un rhabdomyosarcome qui exprime CXCR7 et CXCR4 (RH30) que le

chémotactisme de ces cellules pour CXCL12 et CXCL11 (Transwell®) peut être inhibé par la toxine pertussique (PTX) connue pour prévenir l'interaction de la sous-unité protéique G α i avec les RCPG (Grymula et al. 2010). Les auteurs montrent que l'inhibition du chimiotactisme des cellules RH30 pour CXCL11 est corrélée avec l'inhibition de la phosphorylation de la MAPK et de AKT induite par CXCL11. Cette étude ne montre cependant pas de lien direct entre CXCR7 et G α i, et ne tient pas compte de l'existence possible d'hétérodimères CXCR7/CXCR4 qui pourraient expliquer l'activation de la sous-unité protéique G α i en réponse à CXCL11.

Dans les cellules Jurkat (une lignée de lymphocytes T immortalisée) exprimant CXCR7 et CXCR4, CXCL12 active les voies ERK1/2 et AKT, même en présence d'un anticorps bloquant dirigé contre CXCR4 (12G5, R&D system), indiquant que l'activation de CXCR7 est impliquée dans le chimiotactisme, et promeut la survie des cellules. Cependant, la liaison de CXCL12 à CXCR7 n'entraîne pas de flux calcique, et l'activation de voies ERK1/2 et AKT est insensible à la PTX, indiquant que leur activation ne nécessite pas la liaison du récepteur CXCR7 avec la sous unité G α i (Kumar et al. 2012). Par ailleurs, il a été démontré par BRET sur des cellules HEK293 surexprimant G α i-Rluc (renilla luciférase) et CXCR7-YFP, que CXCR7 était associé à la protéine G α i inactive de manière constitutive et que G α i n'est pas activée lors de l'ajout de CXCL12 (Levoye et al. 2009).

D'autre part, la stimulation de CXCR7 par la chimiokine CXCL12 induit le recrutement de la β -arrestine indépendamment de l'interaction du récepteur avec la protéine G hétérotrimérique dans les cellules HEK293 (Rajagopal et al. 2010). De plus, l'interaction du récepteur CXCR7 avec la β -arrestine dépend de l'activité de la GRK2 (*G protein-coupled receptor kinases 2*), qui permet la phosphorylation de CXCR7 et la fixation de la β -arrestine (Lipfert et al. 2013). Contrairement au récepteur CXCR4, la liaison de CXCL12 (ou CXCL11) au récepteur CXCR7 stabilise l'association avec la β -arrestine et permet l'activation des cascades de signalisation menant à l'activation des MAP Kinases, en particulier ERK1/2, sans interagir avec les protéines G hétérotrimériques (Lipfert et al. 2013).

Aussi, CXCR7 peut former des hétérodimères avec le récepteur CXCR4 et, ainsi, affaiblir la signalisation des protéines G médiées par CXCR4 (Levoye et al. 2009). CXCR7 peut ainsi moduler les diverses fonctions du récepteur CXCR4. Cette action est couplée à son rôle de récepteur leurre («decoy») où CXCR7 permet de piéger la chimiokine CXCL12 et ainsi de réduire la stimulation du récepteur CXCR4 (Figure 35) (Memi et al. 2013, Sánchez-Martín et al. 2013). Certaines études montrent que CXCR7 se retrouve dans des vésicules d'endosomes précoces situées sous la membrane plasmique et qu'il existe un recyclage permanent de CXCR7 entre cette membrane et les vésicules endosomales. Ce mécanisme assurerait une réserve cytoplasmique de CXCR7 dans le but d'accroître rapidement le piégeage de CXCL12 (Mahabaleshwar et al. 2012).

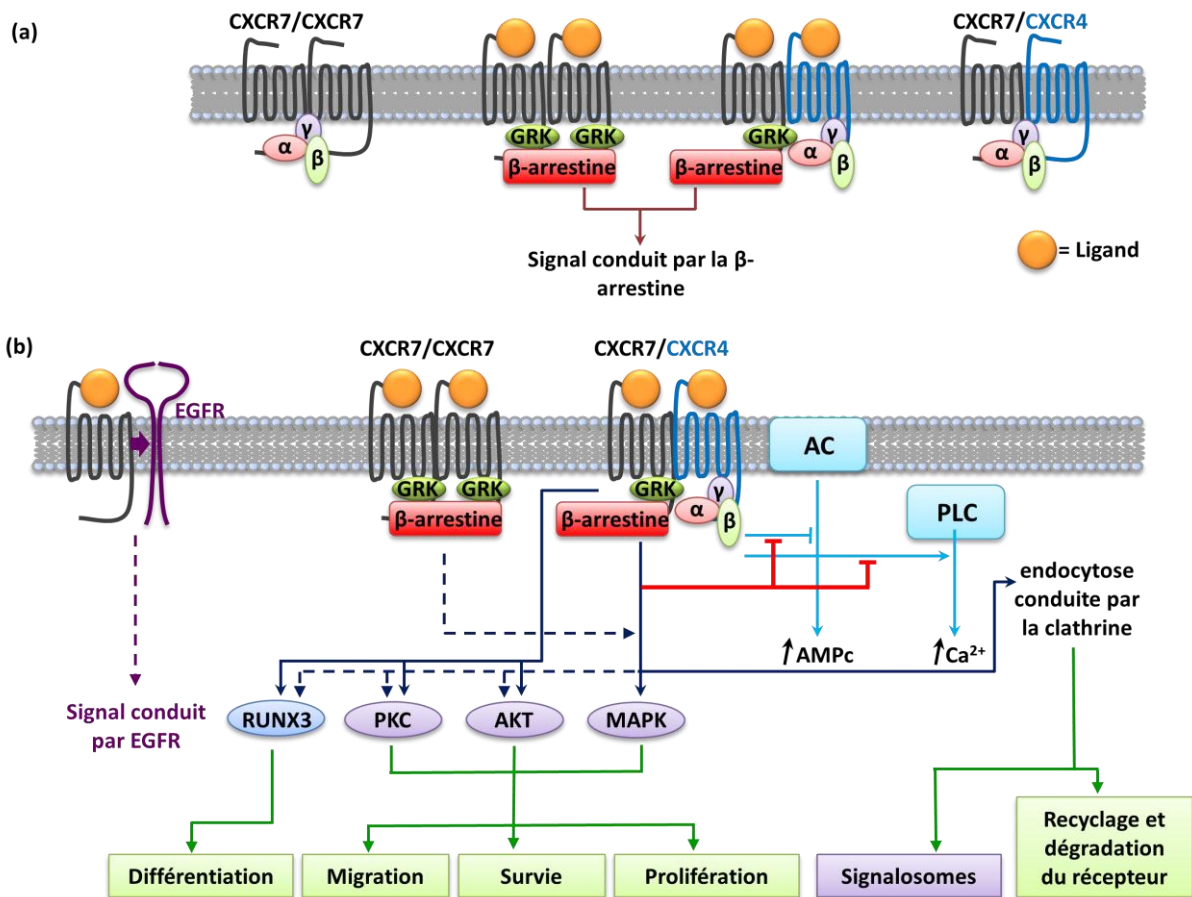


Figure 35: Signalisation du récepteur CXCR7. (a) Modèle représentant les différentes interactions entre les homodimères de CXCR7 ou les hétérodimères CXCR4/CXCR7 et la β-arrestine ou la protéine G hétérotrimérique suite à la liaison de CXCL12. En absence de la chimiokine, CXCR7 et CXCR4 sont constitutivement associés aux protéines Gαi. Pour les hétérodimères, la chimiokine CXCL12 (et CXCL11 pour CXCR7) se lie au récepteur approprié, active la protéine Gαi liée à CXCR4 et entraîne le recrutement de la β-arrestine par CXCR7 sous l'action de GRK. Il peut en résulter un signal faussé et conduit par la β-arrestine. (b) Voies de signalisation décrites (ligne pleine) ou suggérées (lignes pointillées) pour CXCR7. Ce récepteur transmet le signal via la β-arrestine et inhibe le signal induit par Gαi. Récepteur du facteur de croissance épithéliale (EGFR), adénylate cyclase (AC), Phospholipase C (PLC), Kinases couplées aux protéines G (GRK), protéine kinase C (PKC), protéine kinase activant la mitogénèse (MAPK), Facteur de transcription Runt (*Runt-related transcription factor 3*, RUNX3), Adénosine monophosphate cyclique (AMPC). (D'après Sánchez-Martín et al. 2013)

IV.5. Effet de l'inactivation des gènes codant pour CXCL12 ou ses récepteurs CXCR4 et CXCR7

Les rôles importants de CXCL12, CXCR4 et CXCR7 dans l'embryogenèse et dans le développement ont été mis en évidence chez l'animal après inactivation de ces gènes. Les souris invalidées pour le gène codant CXCL12 (CXCL12^{-/-}) ou CXCR4 (CXCR4^{-/-}) ne sont pas viables. Elles présentent d'importantes anomalies de l'hématopoïèse caractérisées par une diminution sévère de la lymphopoïèse B dans le foie fœtal et la moelle osseuse, une diminution de la myélopoïèse dans le foie fœtal et une absence de myélopoïèse dans la moelle. De plus, ces souris présentent des anomalies du système vasculaire et gastro-intestinal et des malformations du tissu cérébelleux. L'examen histologique révèle une

architecture déformée dans les tissus du cervelet, avec une couche de cellules granulaires externes atténuée et une ectopie des cellules de Purkinje (Nagasawa et al. 1996; Ma et al. 1998; Tachibana et al. 1998; Zou et al. 1998). En revanche, deux études ont montré que les souris invalidées pour le gène CXCR7 (CXCR7^{-/-}) ont un développement normal du système hématopoïétique et nerveux et développent uniquement des anomalies du système cardio-vasculaire ou des malformations des valves cardiaques (Sierro et al. 2007, Biben et al. 2007; Gerrits, van Ingen Schenau et al. 2008), ce qui se traduit par la mort de 70% des souris CXCR7^{-/-} dans la première semaine après la naissance.

IV.6. Rôle du couple CXCL12/CXCR4 dans l'hématopoïèse

La chimiokine CXCL12 est impliquée dans la mobilisation et la prolifération des cellules souches de la moelle osseuse. Il a été mis en évidence qu'elle permet une entrée rapide et efficace des cellules progénitrices CD34+ dans la moelle osseuse lors d'une transplantation de moelle osseuse. En effet, l'administration préventive ou subséquente de G-CSF lors de la perfusion des cellules de moelle osseuse, diminue le niveau de CXCL12 dans la moelle osseuse. CXCL12 est libérée suite à la dégradation des glycosaminoglycanes (GAGs) par les enzymes protéolytiques, notamment MMP-9, elles-mêmes libérées par les neutrophiles stimulés par le G-CSF. La libération de CXCL12 par la moelle osseuse augmente ainsi le chimiotactisme des cellules progénitrices CD34+/CXCR4+ du sang vers la moelle osseuse (Roberts et al. 2005, Bogunia-Kubik et al. 2009). La chimiokine CXCL2 augmente également l'adhésion des cellules à VCAM-1 à la surface des cellules endothéliales et facilite ainsi la transmigration des cellules progénitrices du sang vers la moelle osseuse (Broxmeyer HE. 2008). Il a été mis en évidence par co-immunoprécipitation, sur une lignée cellulaire tumorale provenant d'un patient atteint d'une macroglobulinémie de Waldenström (un cancer hématologique), que le récepteur CXCR4 stimulé par CXCL12 peut interagir directement avec l'intégrine VLA-4 (*Very Late Antigen-4*) (Ngo et al. 2008). La stimulation du récepteur CXCR4 par CXCL12 active VLA-4 et augmente l'interaction entre VLA-4 et VCAM-1 promouvant ainsi l'adhésion des cellules immatures CD34+/CXCR4+ du sang ombilical *in vitro* (Peled et al. 2000). Un phénomène également mis en évidence et impliqué dans la transmigration des lymphocytes T activés CXCR4+ *in vitro* (Bryant et al. 2012) et *in vivo*, en régulant la transmigration des neutrophiles, préalablement isolés de la moelle osseuse et injectés par voie intraveineuse à des souris, du sang vers la moelle osseuse et leur rétention dans la moelle osseuse (Petty et al. 2009).

D'autre part, la chimiokine CXCL12 et son récepteur CXCR4 sont impliqués dans la rétention des cellules hématopoïétiques dans la moelle osseuse. La chimiokine CXCL12 est produite de manière constitutive par les cellules stromales, se lie aux GAGs à la surface de ces cellules lorsqu'elle est libérée et permet de retenir les cellules souches hématopoïétiques et les cellules progénitrices

hématopoïétiques exprimant CXCR4. Cette particularité est utilisée pour le recrutement des cellules hématopoïétiques dans le sang en utilisant l'AMD3100, un antagoniste du récepteur CXCR4, qui entre en compétition CXCL12 et entraîne une fuite des cellules hématopoïétiques dans la circulation sanguine. L'administration de l'AMD3100 permet ainsi de collecter les cellules hématopoïétiques pour les transplantations. Le couple CXCL12/CXCR4 promeut également la survie des cellules souches hématopoïétiques, l'adhésion de ces cellules à la surface des cellules de la moelle osseuse, stromales et endothéliales, leur migration transendothéliale et leur chimiotactisme selon le gradient de CXCL12 de la moelle osseuse. Le couple CXCL12/CXCR4 joue donc un rôle important dans la rétention et la greffe des cellules hématopoïétiques. (Laurence 2006, Broxmeyer 2008, Sharma et al. 2011).

IV.7. Principales pathologies impliquant les couples CXCL12/CXCR4 et CXCL12/CXCR7

Il a été suggéré que le couple CXCL12/CXCR4 joue un rôle dans diverses pathologies inflammatoires comme l'allergie (Gonzalo et al. 2000, Lukacs et al. 2002, Hachet-Haas et al. 2008), la polyarthrite rhumatoïde (Chung et al. 2010, Bryant et al. 2012), les maladies inflammatoires de l'intestin (Werner et al. 2013), le lupus (Wang et al. 2010), le syndrome WHIM (*warts, hypogammaglobulinemia, infections, and myelokathexis*) (Balabanian et al. 2008 et 2012, Beaussant et al. 2012, Liu et al. 2012), ainsi que dans l'hypertension artérielle pulmonaire (Young et al. 2009, Montani et al. 2011, Gambaryan et al. 2011, Yu et al. 2011, Costello et al. 2012). Les récepteurs CXCR4 et CXCR7 sont des corécepteurs d'entrée de certaines souches du VIH (Kuritzkes. 2009). De plus, plusieurs études ont montré le rôle pro-tumoral des couples CXCL12/CXCR4 et CXCL12/CXCR7 dans différents types de cancers (Furusato et al. 2010, Lazennec et al. 2010, Hattermann et al. 2013, Wald et al. 2013).

IV.7.1. La chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7 dans la biologie des tumeurs

Plusieurs études ont montré des rôles importants du couple CXCL12/CXCR4 ainsi que du couple CXCL12/CXCR7 dans la métastase, la prolifération et l'angiogenèse des différentes variétés de cancers comme le cancer de la prostate, le cancer du poumon, le cancer pancréatique (pour revue, Hattermann et al. 2012) (Figure 36).

L'expression du récepteur CXCR4 par les cellules tumorales est également un marqueur du potentiel invasif/métastatique dans certains cancer du poumon, de l'œsophage, du colon ou encore des os (Perissinotto et al. 2005, Parker et al. 2012, Lu et al. 2013, Zhang et al. 2013) et peut être augmentée en réponse à CXCL12 (Sánchez-Martín et al. 2013). *In vitro*, la stimulation des cellules A10 et PC-3 par CXCL12 durant 24h accroît l'expression des ARNm codant pour CXCR4 et accentue l'expression du

récepteur CXCR4 à la surface des cellules (Kukreja et al. 2005, Pan et al. 2012, Chen et al. 2012). Ces études montrent que la stimulation de CXCR4 par CXCL12 entraîne la phosphorylation des effecteurs MEK1/2 et ERK 1/2, ainsi que l'activation de NF- κ B, mesurée par chimiluminescence (*NF- κ B luciferase reporter assay*) (Pan et al. 2012). Dans les cellules PC-3, la surexpression de NF- κ B amplifie l'expression de CXCR4 en réponse à CXCL12, tandis que l'inhibition de la voie NF- κ B (vecteur adénoviral codant pour un mutant de NF- κ B trans-dominant négatif) réduit l'expression du récepteur CXCR4 (Kukreja et al. 2005). La surexpression du récepteur CXCR4 en réponse à CXCL12 augmente la viabilité cellulaire (MTT), la transmigration (Transwell®) et l'adhésion de ces cellules aux cellules endothéliales (*in vitro*, HUVECs). La chimiokine CXCL12 peut être produite au sein de la tumeur par les myofibroblastes, et favorise la croissance des tumeurs par l'activation de la prolifération, la survie et l'angiogenèse des cellules cancéreuses (Orimo et al. 2005, Peng et al. 2013).

L'activation du récepteur CXCR4 par CXCL12 entraîne une augmentation d'expression des facteurs proangiogéniques tels que le VEGF et CXCL8 (Wang et al. 2011). Chez la souris, la surexpression de CXCL12 diminue l'expression de l'enzyme glycolytique PGK1 (*phosphoglycérate kinase 1*), un régulateur négatif de l'expression du VEGF et de CXCL8, ce qui intensifie l'expression du VEGF et de CXCL8 et promeut l'angiogenèse (Dai et al. 2007).

Ce phénomène pourrait également être amplifié ou être suppléé, dans le cas d'une faible expression de CXCR4, par la surexpression de CXCR7 par certaines cellules tumorales (Shiozawa et al. 2008). Les auteurs ont mis en évidence que la surexpression de CXCR7 dans la lignée cellulaire PC-3 augmente la production du VEGF et CXCL8 et stimule l'angiogenèse et la croissance tumorale *in vitro* et *in vivo* chez les souris SCID. L'inhibition de l'expression de CXCR7 (siRNA) dans ces mêmes cellules réduit la libération du VEGF et de CXCL8 et diminue l'angiogenèse et la croissance tumorale. L'implication du récepteur CXCR7 dans l'angiogenèse a été confirmée *in vivo* dans un modèle de métastases du cancer du sein, chez des souris SCID, dans lequel la surexpression de CXCR7 (transfection du gène CXCR7 dans les cellules MTLn3) intensifie l'angiogenèse et la croissance tumorale (Hernandez et al. 2011)

Les récepteurs CXCR7 et CXCR4 seraient aussi impliqués dans les phénomènes de résistance aux thérapies anti-cancéreuses telles que le témozomolide, la camptothécine ou la gemcitabine (Hattermann et al. 2010, Arora et al. 2013). La co-stimulation *in vitro* de lignées cellulaires tumorales (A764 et U334) par CXCL12 inhibe l'induction de l'apoptose induite par le témozomolide ou la camptothécine, une inhibition levée par l'ajout d'un antagoniste de CXCR7, le composé CCX733 (Hattermann et al. 2010). La résistance à la gemcitabine est le résultat d'une surexpression du récepteur CXCR4, mise en évidence sur deux lignées de cellules du cancer du pancréas (MiaPaCa et Colo357) et est dépendante de la génération de ROS (*reactive oxygen species*) induite par le

traitement des cellules par la gemcitabine comme le démontre l'inhibition de la surexpression du récepteur CXCR4 en présence de N-acetyl-Lcysteine, un puissant anti-oxydant (Arora et al. 2013).

La génération de ROS entraîne l'activation de HIF- α (*hypoxia inducible factor-1*) qui conduit à la surexpression de CXCR4, un effet probablement médié par l'enzyme PHD2 (protein prolyl hydroxylase 2) (Niecknig et al. 2012) et pouvant être lié à une oxydation et une perte de fonction de la PHD2 induite par les ROS, ou à la baisse du taux d'oxygène intracellulaire consécutif à la formation excessive de ROS.

Indépendamment des dérivés réactifs de l'oxygène, l'hypoxie tumorale conduit à une inhibition des enzymes prolyl-hydroxylases telles que PHD2 (*protein prolyl hydroxylase 2*), qui utilisent l'oxygène comme co-substrat, ce qui inhibe l'hydroxylation de la sous-unité alpha de HIF- α (*hypoxia inducible factor-1*) puis sa liaison aux protéines pVHL et son ubiquitination, et stoppe sa dégradation (Semenza et al. 2004). La protéine HIF- α non-hydroxylée est stable et est alors transportée dans le noyau, se lie à des cofacteurs comme le p300/CBP et induit la transcription de nombreux gènes codant pour des facteurs de croissance angiogéniques et de survie, des enzymes glycolytiques, des transporteurs de glucose (Sharp et al. 2001). Une étude menée sur des HEK-293 montre que le gène codant pour CXCR4 est une cible de HIF-1 α et que son promoteur présente 4 potentiels HREs (hypoxia-response elements) localisés 2.6Kb en amont du site de départ de la transcription du gène CXCR4 (Staller et al. 2003).

Il existe une corrélation entre la surexpression de CXCR4 et de CXCR7 et l'activation de HIF-1 α dans les adénocarcinomes pancréatiques, et la forte expression de ses récepteurs dans les tumeurs diminue fortement la survie des patients (Maréchal et al. 2009). *In vitro*, la mise en culture de cellules souches mésenchymateuses en condition hypoxique durant 24h (3% O₂, 5% CO₂ et 92% N₂) entraîne aussi une forte surexpression des récepteurs CXCR4 et CXCR7 qui peut être inhibée par l'ajout de ARNi spécifique de HIF-1 α (Liu et al. 2010).

Comme cela est montré avec la gencitabine, la surexpression de CXCR4 en réponse à l'activation de HIF-1 α augmente le chimiotactisme des cellules pour CXCL12 (Transwell®) et la capacité de ces cellules à envahir une matrice (Matrigel®), un pouvoir invasif médié par l'expression de métalloprotéinases et de sérine-protéases et mis en évidence par l'utilisation d'inhibiteurs des métalloprotéinases et de sérine-protéases (Arora et al. 2013).

La stimulation du récepteur CXCR4 par CXCL12 entraîne notamment la libération de MMP-9 et MMP-12, sous la dépendance de PI3K et AKT (Chinni et al. 2009). La libération de MMP-9 par les cellules tumorales permet de dégrader la matrice extracellulaire et favorise la migration des cellules tumorales, l'invasion des tissus et les métastases (Wang et al. 2011). Une invasion amplifiée par l'activation des intégrines VLA-4 par le couple CXCL12/CXCR4 qui accroît l'adhésion des cellules tumorales à VCAM-1 et favorise la transmigration des cellules (Ngo et al. 2008).

Enfin, inhiber les récepteurs CXCR4 ou CXCR7 permet de diminuer la prolifération tumorale et les métastases : la neutralisation de CXCR4 par des anticorps spécifiques (Muller, Homey et al. 2001) ou l'inhibition de son expression par des siRNA (Liang, Yoon et al. 2005) diminue la métastase des cellules du cancer du sein. De plus, l'inhibition du récepteur CXCR4 par l'antagoniste T140 diminue l'adhésion cellulaire dans le cancer pulmonaire à petites cellules (Hartmann, Burger et al. 2005). L'utilisation de l'antagoniste de CXCR4 (AMD3100) réduit la métastase des cellules du cancer de l'ovaire (Kajiyama, Shibata et al. 2008). Enfin, l'inhibition de CXCR7 par des siRNA ou par des antagonistes (CCX754) dans les cellules LLC (*Lewis lung carcinoma cells*) aboutit à une diminution de la croissance tumorale *in vitro* et de la taille des tumeurs et augmente la survie des souris *in vivo* (Burns et al. 2006).

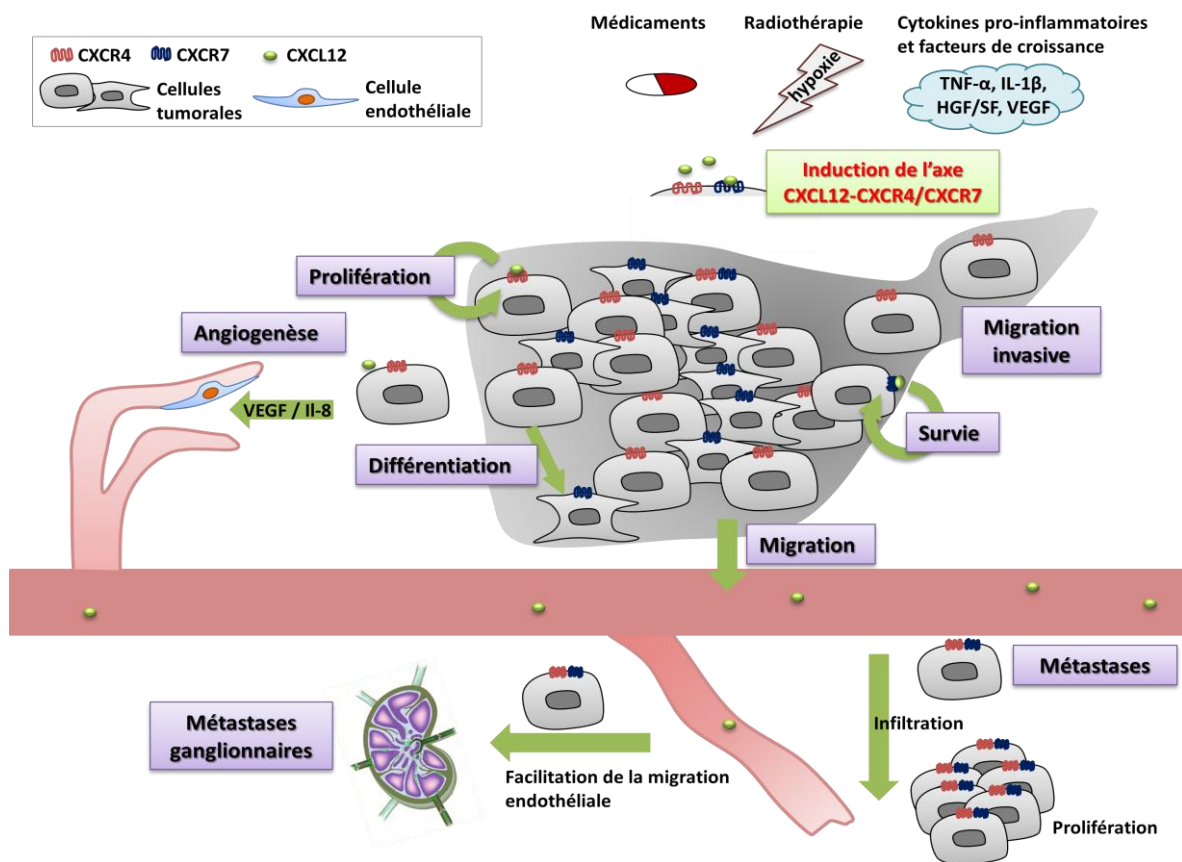


Figure 36: Régulation et effet de CXCL12 et CXCR4/CXCR7 dans la biologie des tumeurs. CXCL12 est produit par les tissus environnant la tumeur. CXCR4 est exprimé de manière prédominante par les cellules à la périphérie de la tumeur et promeut l'angiogenèse et l'infiltration des tissus environnants. Dans le cas des gliomes, les cellules différenciées expriment préférentiellement CXCR7. La co-expression des 2 récepteurs est le plus souvent mise en évidence sur les cellules en cours de différenciation. L'expression de CXCL12 et ses récepteurs peut être induite par des facteurs endogènes (cytokines, facteurs de croissance) associés à l'inflammation, le temozolomide et les agents alkylants utilisés dans les thérapies, et l'hypoxie provoquée par une expansion tumorale rapide ou la radiothérapie. Facteur de nécrose tumorale (TNF), facteur de croissance des hépatocytes (HGF/SF), facteur de croissance de l'endothélium vasculaire (VEGF). (D'après Hattermann et al. 2012).

IV.7.2. La chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7 dans l'asthme

Les rôles de la chimiokine CXCL12 et de ses récepteurs CXCR4 et CXCR7 dans l'asthme allergique sont encore inconnus, mais leur implication dans la maladie est avérée depuis plusieurs années (Figure 37).

Dans un modèle d'asthme allergique aigu à l'ovalbumine chez la souris, l'utilisation d'anticorps neutralisant CXCR4 ou CXCL12 a permis de réduire l'éosinophilie et l'hyperréactivité des voies aériennes, alors que la surexpression des récepteurs CXCR4 par les leucocytes (grâce à l'utilisation de rétrovirus) augmente l'inflammation pulmonaire (Gonzalo et al. 2000). L'administration systémique d'un antagoniste du récepteur CXCR4, l'AMD3100, diminue l'inflammation et la résistance des voies aériennes et réduit les taux d'IL-4 et d'IL-5 bronchiques, deux cytokines produites par les lymphocytes Th2 dans un modèle murin d'asthme aigu aux allergènes de cafard (Lukacs et al. 2002). L'utilisation de l'AMD3100 a également permis de mettre en évidence l'implication de CXCR4 dans un modèle murin d'asthme fongique à *Aspergillus fumigatus* (Hogaboam et al. 2005).

D'autre part, une augmentation du nombre de cellules CXCL12⁺ dans les tissus pulmonaires a été mise en évidence chez les patients asthmatiques par rapport aux sujets sains (Hoshino et al. 2003). La concentration de la chimiokine CXCL12 dans le lavage bronchoalvéolaire est augmentée chez les patients asthmatiques et le nombre de leucocytes dans le lavage bronchoalvéolaire est augmenté proportionnellement à la concentration de CXCL12 (Negrete-García et al. 2010). Une augmentation de la concentration plasmatique de CXCL12 a aussi été mesurée chez un groupe d'enfants asthmatiques (Hung et al. 2007). En outre, cette étude clinique a montré que la prise de Montelukast, un antagoniste des récepteurs des leucotriènes, réduit la concentration plasmatique de CXCL12 et permet de réduire la chemoattractivité de CXCL12 sur les monocytes THP-1 *in vitro*. Cette action pourrait provenir de l'activité inhibitrice du Montelukast sur la voie NF-κB (Maeba et al. 2005), ce qui conduirait à une diminution de l'expression de CXCR4 à la surface des cellules. En effet, l'étude par retard sur gel ou EMSA (*Electrophoretic Mobility Shift Assay*) de NF-κB, a révélé que le facteur de transcription NF-κB peut se lier au promoteur de CXCR4 et induire sa transcription (Helbig et al. 2003). La surexpression de NF-κB est connue pour accroître l'expression de CXCR4 et l'inhibition de la voie NF-κB pour réduire l'expression du récepteur CXCR4 (Kukreja et al. 2005). L'inhibition de la voie NF-κB serait également susceptible de réduire la production de la chimiokine CXCL12, comme cela a été montré, sur des cellules MTEC1 (*mouse thymic medullary-type epithelial cell 1*) transfectées surexprimant mEET (mouse estrogen enhanced transcript), en réponse à l'œstrogène (Jin et al. 2003). L'œstrogène réduit la translocation NF-κB dans ces cellules et inhibe l'expression de CXCL12. De plus, l'absence de p52NF-κB, dans des fibroblastes embryonnaires de souris déficients pour la sous-unité p52 de NF-κB (knock out), inhibe la production de CXCL12 et confirme que la voie NF-κB est impliquée dans la production de CXCL12 (Kew et al. 2012).

Chez l'homme, l'exposition à un allergène diminue l'expression de CXCR4 par les cellules de la moelle osseuse CD34+, ainsi que le taux de CXCL12 dans la moelle osseuse, ce qui pourrait réduire la rétention des cellules progénitrices dans la moelle osseuse et ainsi promouvoir la fuite des cellules (Dorman et al. 2005). Ce phénomène pourrait favoriser le recrutement des cellules progénitrices épithéliales nécessaire à la ré-épithélialisation de l'épithélium et dépendant de CXCL12 (Gomperts et al. 2006), mais aussi le recrutement des fibrocytes CXCR4+ et promouvoir le remodelage bronchique (Gomperts et al. 2007, Garibaldi et al. 2013). De plus, les récepteur CXCR4 et CXCR7 sont exprimés par les lymphocytes T et sont impliqués dans le recrutement des lymphocytes T par CXCL12 (Balabanian et al. 2005, Chung et al. 2010). L'utilisation d'un anticorps dirigé contre CXCR7 inhibe le chimiotactisme des splénocytes et des neutrophiles en réponse à CXCL12 (Zhang et al. 2009). Les deux récepteurs, CXCR4 et CXCR7, sont aussi impliqués dans la migration trans-endothéliale des cellules circulantes. La stimulation du récepteur CXCR4 par CXCL12 active l'intégrine VLA-4 et augmente l'interaction entre VLA-4 et VCAM-1 promouvant ainsi l'adhésion des cellules circulantes à la surface des cellules endothéliales et facilite ainsi la transmigration des cellules progénitrices du sang vers la moelle osseuse (Broxmeyer HE. 2008). L'expression de VCAM-1 est, quant à elle, intensifiée suite à l'exposition à un allergène, notamment sous l'influence de TNF- α /TNFR1 (Hirata et al. 1998, Zhou et al. 2007).

Par ailleurs, il est aujourd'hui démontré que l'asthme peut entraîner une hypoxémie, et plus encore une hypoxie locale des tissus pulmonaires résultant de l'œdème pulmonaire, du remodelage bronchique et de l'infiltrat inflammatoire (Voelkel et al. 2009, Duong et al. 2011, Rangasamy et al. 2011). Ces phénomènes sont accompagnés d'une néo-vascularisation dans laquelle la chimiokine CXCL12 et ses récepteurs semblent impliqués (Hoshino et al. 2003). L'hypoxie augmente l'expression des ARNm codant pour les récepteurs CXCR4 et CXCR7 sous la dépendance de HIF-1 α (Liu et al. 2010), et peut aussi accroître l'expression de CXCL12, comme cela a été mis en évidence *in vivo* chez la souris (Costello et al. 2008). La présence du récepteur CXCR7 sur les cellules endothéliales humaines a été confirmée et semble augmentée chez les patients présentant une hypertension pulmonaire ou HTAP associée à l'hypoxie (Costello et al. 2012), et également chez un modèle murin d'HTAP en réponse à une hypoxie. Le taux plasmatique de CXCL12 est également plus élevé chez les patients souffrant d'HTAP et le récepteur CXCR7 serait impliqué dans la régénération, la réparation et la prolifération des cellules endothéliales du poumon (Costello et al. 2012). L'utilisation d'un antagoniste de CXCR7, CCX771, peut réduire la prolifération des cellules vasculaires pulmonaires et le remodelage dans un modèle d'HTAP chez des souris (hypoxie 10% d'O₂, de P1 à P12) (Sartina et al. 2012), une inhibition cependant contestée par une autre équipe (Gambaryan et al. 2011) qui montre que chez des souris plus âgées (8 à 10 semaines) et exposées durant 2 jours à des conditions hypoxiques (10% d'O₂), l'administration du composé CCX771 seul ne permet pas de réduire l'HTAP.

Dans ce modèle, l'expression du récepteur CXCR4 est augmentée dans les poumons et l'utilisation d'un antagoniste de CXCR4, l'AMD3100, réduit le remodelage vasculaire et l'accumulation péri-vasculaire des cellules progénitrices c-kit+/sca-1+ (Gambaryan et al. 2011, Yu et al. 2011).

Ainsi, CXCL12 et ses récepteurs participeraient à la régénération, la réparation et la prolifération des cellules endothéliales, mais également au recrutement et à la migration trans-épithéliale des cellules progénitrices comme les fibrocytes et les cellules progénitrices et épithéliales, et à l'angiogenèse par la production de VEGF et CXCL8.

Les éosinophiles occupant une place importante dans la physiopathologie de l'asthme, la présence du récepteur CXCR4 et l'activité chimioattractrice de CXCL12 sur les éosinophiles ont été étudiées. Une première démonstration a mis en évidence l'absence d'expression membranaire de CXCR4 sur les éosinophiles circulants humains provenant de volontaires sains et a mis en évidence qu'elle peut être induite après mise en culture durant 24h (RPMI 1640, 10% SVF) en l'absence et en présence de TNF- α , l'INF- γ et le TGF- β *in vitro* (Nagase et al. 2000). Les mêmes auteurs ont mis en évidence une très faible expression de CXCR4 (anticorps anti-CXCR4, 12G5, BD pharmingen) sur les éosinophiles du lavage bronchoalvéolaire, chez des patients présentant une éosinophilie des voies aériennes (Nagase et al. 2001). Par la suite, une étude a mis en évidence l'expression du récepteur CXCR4 sur les éosinophiles (Liu et al. 2003). D'après cette étude menée sur 9 patients asthmatiques, une partie des éosinophiles circulants (30%) expriment CXCR4 à leur surface (anticorps anti-CXCR4, Clone ?, R&D). L'expression du récepteur CXCR4 n'est pas modifiée 48h après provocation allergénique et les éosinophiles présents dans le lavage bronchoalvéolaire présentent une expression de CXCR4 équivalente aux éosinophiles circulants. Cependant, la référence de l'anticorps n'est pas mentionnée, l'utilisation d'un contrôle positif n'est pas décrit, et les mesures effectuées dans ces deux articles n'ont pas été réalisées sur des volontaires sains, ce qui ne permet pas de comparer et contrôler les résultats publiés.

D'autre part, le chimiotactisme des éosinophiles sous l'influence de CXCL12 a été mesuré *in vitro* (Nitta et al. 2007) et montre que les éosinophiles circulants de volontaires sains (CD3-, CD14-, CD16- et CD19-, Macs[®]) peuvent se déplacer selon un gradient croissant de CXCL12 (Taxiscan[®], 1h). Cependant, ces résultats ont été obtenus après mise en culture des cellules durant 8h (RPMI-HEPES, 10% SVF), dans le but d'induire l'expression du récepteur CXCR4. Une étude plus récente met en évidence une faible, très variable et non significative migration (Transwell[®], 4h) des cellules progénitrices des éosinophiles (CD34+, CD45+, IL-5R α +) et des éosinophiles circulants matures (Ficol-, CD16-, Macs[®]) provenant de patients asthmatiques en réponse à la chimiokine CXCL12 (Punia et al. 2011). Il est intéressant de noter que l'expression de CXCR4 à la surface des éosinophiles n'a pas été mesurée dans ces deux études, mais que les résultats concordent avec ceux publiés précédemment (Nagase et al. 2000): les éosinophiles présentent naturellement une très faible expression de CXCR4

et un chimiotactisme limité pour CXCL12, mais l'expression de CXCR4 peut être amplifiée et les éosinophiles sont alors sensibles et migrent selon un gradient de CXCL12.

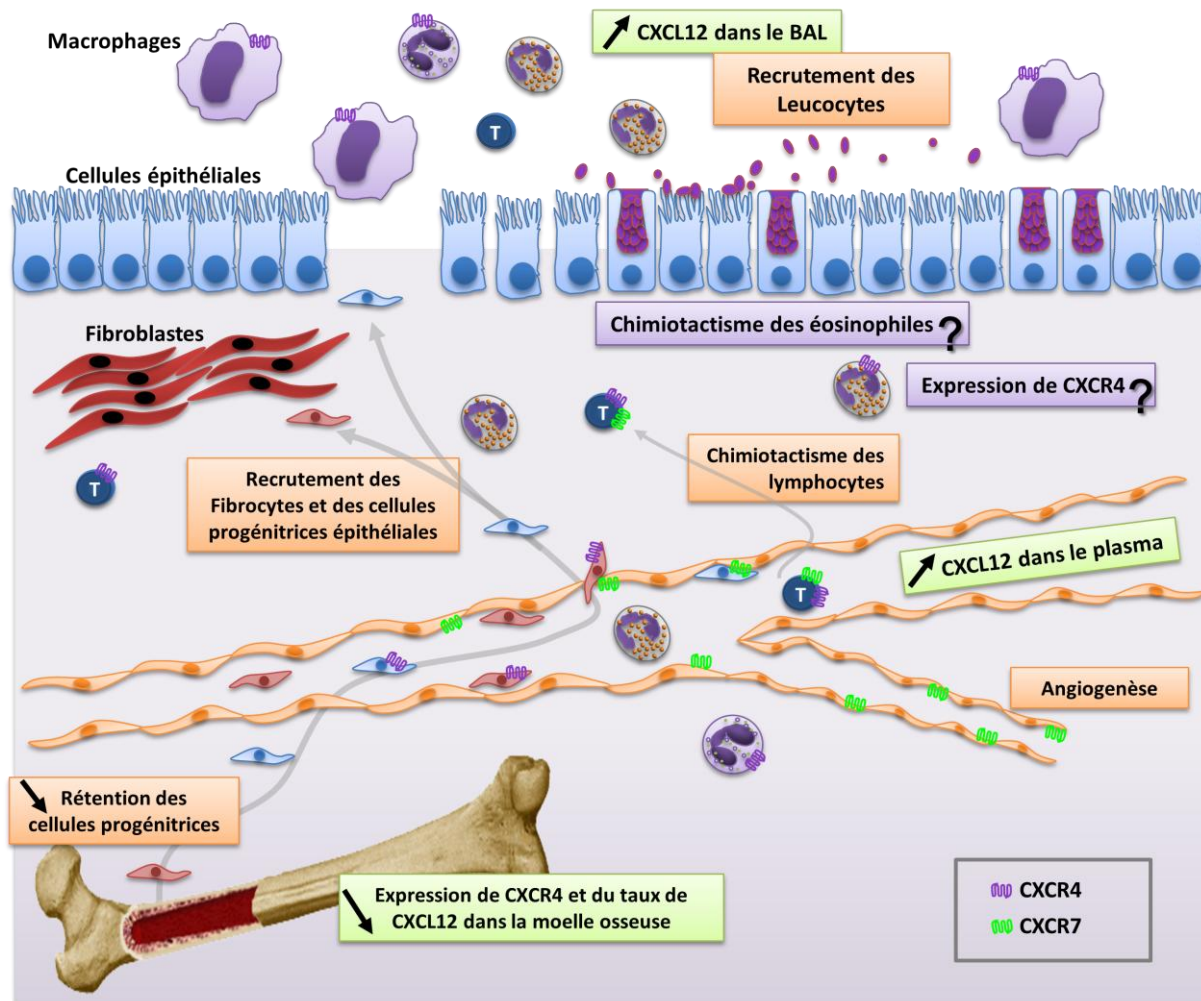


Figure 37: Rôle de la chimiokine CXCL12 et ses récepteurs CXCR4/CXCR7 dans l'asthme. Chez l'homme, la concentration de la chimiokine CXCL12 est augmentée dans le lavage bronchoalvéolaire (LBA) et dans le plasma des patients asthmatiques tandis qu'elle est diminuée dans la moelle osseuse, de même que l'expression du récepteur CXCR4 à la surface des cellules CD34⁺ (Dorman et al. 2005). La diminution de l'expression de CXCL12 et CXCR4 dans la moelle osseuse pourrait promouvoir la fuite des cellules progénitrices. Les fibrocytes et les cellules progénitrices épithéliales CXCR4⁺ sont recrutées dans le poumon sous l'attraction de CXCL12. Chez l'homme, le nombre de leucocytes présent dans le LBA est proportionnel à la concentration en CXCL12 mesurée dans le LBA, tandis que le chimiotactisme des éosinophiles en réponse à CXCL12 reste controversé. Le récepteur CXCR7 est impliqué dans l'angiogenèse.

Ainsi, il a été mis en évidence que la chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7 sont impliqués dans l'asthme allergique. Cependant, les outils thérapeutiques disponibles (Plerixafor, AMD3100) ou en cours de développement clinique (NOX-12, un anti-CXCL12) permettant d'inhiber l'action de CXCL12, sont indiqués pour le recrutement des cellules de la moelle osseuse, un effet systémique indésirable dans le cas du traitement de l'asthme. Le développement de molécules actives, bio-disponibles et favorisant une action locale, pourrait présenter une alternative intéressante dans le cas du traitement de l'asthme.

V. Objectifs de la thèse

L'asthme est une maladie inflammatoire complexe dépendant de facteurs environnementaux et génétiques. Elle résulte d'une activation inappropriée du système immunitaire. Cette maladie inflammatoire bronchique est caractérisée par une hyperréactivité bronchique réversible, une hyperéosinophilie sanguine et bronchique, un œdème de la muqueuse ainsi qu'un remodelage des tissus. L'inflammation persistante provoque des modifications morphologiques des bronches qui se caractérisent par un dépôt de collagène dans et sous la membrane basale associé à une prolifération de myofibroblastes (fibrose sous-épithéliale), l'hypertrophie des cellules productrices de mucus, la perte des cellules épithéliales ciliées ainsi que l'hypertrophie et l'hyperplasie des cellules musculaires lisses bronchiques. C'est une maladie chronique, pour laquelle de nombreuses études montrent un rôle des chimiokines et de leurs récepteurs dans la réponse à l'allergène, dans l'inflammation et le remodelage bronchique (Pease 2006, Pease et al. 2011, Velazquez et al. 2011). Les chimiokines peuvent donc être considérées comme des candidats au sein des facteurs moléculaires jouant un rôle dans l'asthme.

Parmi les chimiokines impliquées, plusieurs études ont montré que la chimiokine CXCL12 peut être une cible intéressante pour le traitement de l'asthme. L'utilisation d'antagonistes spécifiques du récepteur CXCR4, l'AMD3100, d'anticorps bloquant de CXCR4 ou d'anticorps neutralisant la chimiokine CXCL12 permet de réduire l'infiltrat de cellules inflammatoires dans des modèles d'asthme chez la souris (Gonzalo et al. 2000, Lukacs et al. 2002). Notre laboratoire a récemment identifié une petite molécule chimique capable de neutraliser l'interaction entre CXCL12 et CXCR4, et mis en évidence son activité anti-inflammatoire dans un modèle d'asthme aigu chez la souris (Hachet-Haas et al. 2008). Le criblage de la chimiothèque patrimoine de notre laboratoire (alors laboratoire de pharmacochimie de Strasbourg) sur un test fonctionnel in vitro de transfert d'énergie de fluorescence lors de la liaison entre le ligand CXCL12 couplé au Texas Red (CXCL12-TR) et le récepteur CXCR4 couplé à l'eGFP, a permis d'identifier une molécule : la chalcone 4. Elle inhibe la liaison de CXCL12 à ses récepteurs CXCR4 et CXCR7 avec une forte affinité et bloque les réponses cellulaires (élévation de Ca⁺⁺, baisse d'AMPc) et le chimiotactisme des cellules exprimant CXCR4. Nous avons montré que la chalcone 4 neutralise la chimiokine par interaction directe avec celle-ci et non avec le récepteur CXCR4 et l'empêche d'interagir avec ses récepteurs cibles. Cette analogie avec le mécanisme d'action des anticorps neutralisants nous a conduit à nommer de cette molécule, un neutraligand, qui constitue un concept nouveau de blocage d'un peptide ligand avec une petite molécule. En raison de son mode d'action qui est la neutralisation de la chimiokine et non de ses récepteurs CXCR4 et CXCR7, ce neutraligand présente un fort potentiel car il neutralise la liaison de

CXCL12 à CXCR4 et CXCR7, et n'affecte pas la capacité d'activation des récepteurs, ni leurs signalisations intracellulaires en réponse à leurs autres ligands (ubiquitine et CXCL11 respectivement). Ainsi, l'utilisation d'un neutraligand de CXCL12 pourrait présenter une alternative intéressante aux antagonistes et présenter moins d'effets secondaires dans le cas du traitement de l'asthme. Une collaboration entre l'institut de recherche de l'ESBS (JL. Galzi) et le laboratoire d'innovation thérapeutique de la faculté de pharmacie (LIT, N. Frossard), a permis de mettre en évidence que l'administration de la chalcone 4 réduit l'invasion des voies aériennes par les éosinophiles dans un modèle d'asthme chez la souris (Hachet-Haas et al. 2008). Cependant, ces résultats prometteurs sont ternis par une très faible solubilité dans les tampons aqueux (9 μ M). Les chimistes du LIT (M. Hibert et D. Bonnet) ont initié l'optimisation de la structure de la chalcone 4, chef de file "neutraligand de CXCL12", afin d'augmenter son affinité et améliorer sa solubilité. Dans cette collaboration, nous avons pu proposer une stratégie permettant le développement de molécules actives et bio-disponibles et promouvoir une action locale, tout en évitant les effets systémiques indésirables sur les autres fonctions liées à CXCL12.

L'objectif de cette thèse est de valider et renforcer le concept de "neutraligand de CXCL12" comme outil en physiologie et physiopathologie à l'aide de dérivés de chalcones et de modèles d'asthme chez la souris, de participer au développement de molécules plus solubles et plus actives, et/ou permettant de se prémunir des effets systémiques indésirables, ainsi que d'étudier l'activité antiasthmatique de la chalcone 4 et son mécanisme d'action.

Ainsi, notre but a tout d'abord été de mettre au point un modèle rapide d'asthme afin d'optimiser notre collaboration avec les chimistes du LIT (M. Hibert et D. Bonnet) et le laboratoire de l'ESBS (JL. Galzi). Pour cela, nous avons développé et validé un modèle rapide et reproductible d'asthme allergique à l'ovalbumine (en 8 jours), offrant une réponse semblable au modèle conventionnel en 21 jours et permettant d'évaluer rapidement l'activité anti-inflammatoire *in vivo* des composés pharmacologiques (**publication n°1**).

Afin d'améliorer la solubilité aqueuse de la chalcone 4, des prodrogues solubles ont été synthétisées par les chimistes du LIT dans le but de permettre un traitement local, par voie intranasale chez la souris et mimant l'administration par inhalation chez l'homme, pour augmenter l'activité anti-inflammatoire des neutraligands de CXCL12 *in vivo* dans l'asthme (**publication n°2**).

Ensuite, dans le but de favoriser une action anti-inflammatoire locale du neutraligand et limiter les effets secondaires, nous avons exploré la possibilité de produire un neutraligand de courte durée d'action, une anté-droque de la chalcone 4, rapidement dégradé avant sa distribution dans l'organisme et les tissus non visés (**publication n°3**).

Enfin, nous avons étudié l'activité antiasthmatique de la Chalcone 4 dans un modèle d'asthme conventionnel (21-jours) et mis en évidence son mécanisme d'action original (**publication n°4**).

PUBLICATIONS

Publication n°1:

A new rapid model of asthma in the mouse

François Daubeuf, Jean-Luc Galzi, and Nelly Frossard

(En préparation pour publication)

L'hyperéosinophilie des voies aériennes est une des cibles majeures des candidats médicaments anti-inflammatoires développés pour le traitement de l'asthme. Evaluer l'activité anti-inflammatoire *in vivo* d'une nouvelle stratégie ou d'un nouveau composé, dans l'asthme, requiert l'utilisation de modèles animaux, pour la plupart, longs, coûteux et peu adaptés au criblage de ces nouvelles thérapeutiques.

Notre travail s'inscrit dans le développement conjoint de nouveaux neutraligands, en partenariat avec les équipes de chimistes de notre laboratoire, le laboratoire de Biotechnologie et signalisation cellulaire (UMR7242, ESBS), et la plateforme Techmed'ILL, respectivement en charge de la synthèse, de l'étude de l'activité *in vitro* et enfin de la mesure des caractéristiques physico-chimiques des composés. Notre rôle est l'évaluation de l'activité *in vivo* des molécules d'intérêt. Dans ce contexte, notre but a été de développer un nouveau modèle d'asthme allergique, reproductible et de courte durée, permettant d'évaluer rapidement l'activité anti-inflammatoire des nouveaux composés afin de garantir un développement raisonné de ces nouvelles stratégies, ainsi qu'une utilisation optimisée des ressources.

Pour le développement de ce modèle, nous avons comparé les paramètres obtenus dans ce modèle rapide d'asthme allergique (8 jours) en réponse à l'ovalbumine à ceux obtenus dans le modèle conventionnel nécessitant 21 jours. Les résultats mettent en évidence la robustesse de l'inflammation des voies aériennes dans le modèle mis au point, une reproductibilité comparable au modèle conventionnel, une étonnante similarité des caractéristiques mesurées, et ont révélé la possibilité d'engendrer un remodelage et une hyperréactivité des voies aériennes dans notre modèle rapide.

A new rapid model of asthma in the mouse

François Daubeuf^{1\$#}, Jean-Luc Galzi[‡] and Nelly Frossard^{\$#*}

^{\$}Laboratoire d'Innovation Thérapeutique, UMR7200, CNRS-Université de Strasbourg, Faculté de Pharmacie, 67400 Illkirch, France, [‡]Laboratoire de Biotechnologie et Signalisation Cellulaire, UMR7242, CNRS / Strasbourg University, Ecole de Biotechnologie, 67400 Illkirch, France, and [#]Laboratory of Excellence MEDALIS, Université de Strasbourg, 67400 Illkirch, France

Airway hypereosinophilia is a main characteristic feature of the asthmatic airways, and one of the major cellular targets for anti-asthma drug candidates. The commonly used 21-day ovalbumin-induced asthma mouse model is expensive and time consuming. Here we have developed a new short model of asthma that will allow to evaluate rapidly and efficiently the anti-inflammatory potential of new drug candidates or strategies.

Toward this aim, mice were sensitized intraperitoneally with ovalbumin in the presence of alum on 3 consecutive days, and challenged intranasally three days after for another 3 consecutive days with ovalbumin. Mice were killed on day 8. Airway hyperresponsiveness (AHR, Flexivent® and whole body plethysmography), airway eosinophilia and Th2 cytokines in the bronchoalveolar lavage fluid, serum ovalbumin-specific immunoglobulins as well as lung mucus production, collagen deposition in the lung, and mast cell infiltration were quantified and compared to those observed in the commonly used 21-day model. We found that the short model (8-day model) mimics all features observed in the 21-day model. In addition, a similar reproducibility was observed in the 8-day model and the 21-day models. The evaluation of a treatment with anti-inflammatory drug was exemplified with dexamethasone (100 µg/kg), showing a significant decrease in airway eosinophilia (60±4%). Thus, we have developed and exemplified a rapid, easy, reproducible, and predictive asthma model to ovalbumin that is suitable to efficiently study new therapeutic strategies for the anti-inflammatory treatment of asthma.

This work was supported by funds from CNRS, the Institut National pour la Santé et la Recherche Médicale, and the Agence Nationale pour la Recherche.

I FD was supported by a joint fellowship from the Fonds de Recherche en Santé Respiratoire from Société de Pneumologie de langue française (SPLF) and the Region Alsace.

* To whom correspondence should be addressed. E-mail: nelly.frossard@unistra.fr.

The prevalence of asthma is increasing worldwide (Jones *et al.*, 2011) and animal models play an important role in the development of new therapeutics or strategies for the treatment of asthma.

Allergic asthma is a chronic inflammatory disease of the airways characterized by infiltration of T cells and eosinophils in the airways associated with Airway HyperResponsiveness (AHR) and remodelling, as well as elevated allergen-specific immunoglobulins E (Barnes, 1996; Barnes, 2008). Th2 cells play a central role in the pathogenesis of asthma, as they secrete IL-4 to switch B-cells towards IgE synthesis, and IL-5, which promoting eosinophil recruitment (Barnes PJ, 1998; Larche *et al.*, 2003). Eosinophils are found in the airway wall, and may be recovered in the bronchoalveolar lavage fluid (BALF). They produce and release numerous toxic and pro-inflammatory mediators, cytokines and chemokines contributing to airway inflammation and causing damage of the airway tissue and nerves (Humbles *et al.*, 2004).

The main treatment of inflammation in asthma are the glucocorticoids (GINA, 2012). However, in the long run, glucocorticoids may impair anabolic processes, and be responsible for side effects. One objective for asthma treatment is to find new active drugs with fewer side effects to help reduce glucocorticoid dosing and secondary effects.

The commonly used asthma mouse models consist of sensitization and challenge with a foreign protein, as for instance ovalbumin (OVA), in 21 to 28 days duration models (Nials *et al.*, 2008). Maintaining mice in the animal house is expensive, time- and space-consuming, and necessitates an adequate planning of the experiments. Therefore, analysis of the effect of new anti-inflammatory compounds in the pharmaceutical industry or research laboratories requires several weeks to collect *in vivo* data. A rapid mouse model where eosinophils invade the airways specifically would thus be very attractive for teams specialized in chemical biology and pharmacology of new potential therapeutics.

We therefore developed and validated a reproducible and reliable model of airway hypereosinophilia in Balb/c mice occurring within 8 days of OVA sensitization and challenge, and compared it with the commonly used asthma 21-day model in terms of inflammation (number of infiltrated cells and cytokine levels in BALF), remodelling (lung histology, mucus and collagen measurement), serum OVA-specific Igs, as well as airway responsiveness measured by plethysmography and the Flexivent® technique. We clearly show equivalence of results between the two models, except for airway hyperresponsiveness that is two fold lower than in the 21-day model.

METHODS

Mice – Female and male age-matched Balb/cJ mice were used (Janvier Labs, Le Genest Saint Isle, France). Animals were maintained under controlled environmental conditions in conventional husbandry at constant temperature (20 ± 2 °C) with a relative humidity (50 ± 10 %) and 12 hr/12 hr light-dark cycle (lighting 07:00-19:00). Mice were bred in polycarbonate cages with 4 mice per cage (PCT2L12SHT, Allentown) with bedding made from spruce wood chips (Safe). The room air was ventilated at 10 air changes per hour according to the European recommendations. Food (standard diet, Mucedola) and tap water were available *ad libitum*. Cages with bedding were changed weekly. Animal experimentation was conducted with the approval of the government body that regulates animal research in France.

Allergen sensitization and challenge – For each model, mice were sensitized by intraperitoneal (i.p.) injections of a mixture contain 50 µg OVA (Sigma-Aldrich, A5503) and 2 mg aluminium hydroxide (Sigma-Aldrich, 23918-6) in 0.1 ml saline. Mice were challenged intranasally (i.n.) with 10 µg OVA in 25 µl saline (12.5 µl/nostril). Control mice received i.p. injections of a mixture containing 50 µg OVA and 2 mg aluminium hydroxide in 0.1 ml saline, and i.n. administrations of saline alone. Intranasal administrations were performed under anaesthesia induced by an i.p. injection of 50 mg/kg ketamine (Imalgene 1000, Merial, Villeurbanne, France) and 3.3 mg/kg xylazine (Rompun 2%, Bayer, Loos, France). For the 8-day protocol, mice were sensitized on days 0, 1 and 2 and challenged on days 5, 6 and 7. For the 21-day protocol, mice were sensitized on days 0 and 7 and challenged on days 18, 19, 20 and 21 (figure 1). At the indicated time points, mice were killed by i.p. injection of 150 mg/kg ketamine and 10 mg/kg xylazine and the bronchoalveolar lavage (BAL) were collected. Lungs were perfused and collected.

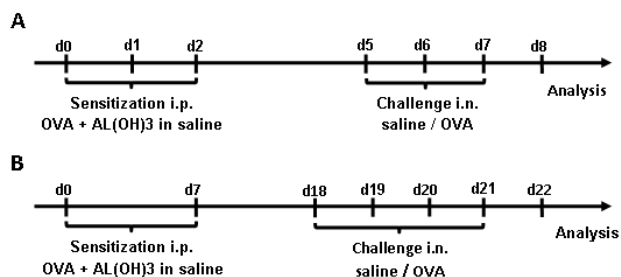


FIGURE 1. Comparative description of the 8-day and 21-day models of hyper eosinophilia in Balb/c mice immunised and challenged with OVA or saline. A. Rapid 8-day hyper eosinophilia model. B. Commonly use 21-day asthma model.

Treatment – To validate the model for use in the research of anti-inflammatory strategies, we explored its reactivity to the best known comparator treatment, *i.e.* glucocorticoids, as requested by the drug agencies. Mice were treated with the glucocorticoids budesonide (1mg/kg) (AstraZeneca) or dexamethasone (1mg/kg or 100µg/kg) (Sigma-Aldrich, D2915) in 0.2 ml saline by i.p. injection one hour before each challenge.

Collection of bronchoalveolar lavage fluid – BAL and differential cell counts were performed as described previously (Daubeuf *et al.*, 2012). Briefly, BAL was performed 24 h after the last OVA challenge. Mice were sacrificed by i.p. injection of 150 mg/kg ketamine and 10 mg/kg xylazine. A plastic canula was inserted into the

trachea, and airways were lavaged by 10 instillations of 0.5 ml ice-cold saline supplemented with 2.6 mM EDTA (saline-EDTA). BALF recovered from the two first lavages were centrifuged (10,000g for 10 min at 4°C), and the resulting supernatant was stored at -20°C for cytokine measurements.

Total and differential cell counts in BAL – BALF recovered from the eight last lavages were pooled and centrifuged (300g for 5 min at 4°C) to pellet cells. Erythrocytes were lysed by hypotonic shock by addition of 1.5 ml distilled water, followed by addition of 0.5 ml KCl (0.6 M). Cells were centrifuged and resuspended in 500 µl ice-cold saline-EDTA, and total cell counts were determined using an haemocytometer (Neubauer's chamber). Differential cell counts were assessed on cytologic preparation obtained by cytocentrifugation (Cytospin 4, Shandon Ltd) of 200 µl of diluted BALF (250,000 cells/ml in ice-cold saline-EDTA). Slides were stained with Hemacolor (Merck), and determinations were performed by counting at least 400 cells on each slide. The number of cells was expressed as absolute numbers from total cell counts.

Histological examination – Lung tissues were fixed in 4% formaldehyde and paraffin-embedded, 6 µm sections were cut, mounted on Superfrost glass slides (Fischer Scientific) and stained with Periodic Acid-Schiff (PAS), Masson's trichrome or toluidine blue (Sigma-Aldrich). Mucus production was quantified using a 5 points-grading system described by Tanaka *et al.*, (2001). The hyperplasia of the goblet cells within the epithelial lining was expressed as a percentage of goblet cells within the epithelial layer. To minimize sampling errors, the 5-point scoring system (grade 0–4) was adopted: grade 0 (no goblet cells); grade 1, <25%; grade 2, 25–50%; grade 3, 50–75%; grade 4, ≥75%. The mean scores of hyperplasia of goblet cells were calculated in 9 sections from 6 animals.

Counting of toluidine blue-positive mast cells was performed in at least three different fields for each lung section, and reported as a ratio to the length of the bronchus perimeter measured with the Cell dimension software coupled to a DP72 camera (Olympus). Mean scores were obtained from counts on 9 sections from 6 animals.

Colorimetric assay of mucus and collagen – Mucus in BALF and lung homogenate was measured by a mucus colorimetric assay: 100 µl BALF or lung homogeante were added to 20 µl acid mucus reagent (0,2% periodic acid, 7% acetic acid), incubated for 1 hour at 37°C and 20 µl mucus dye reagent (sodium metabisulfite 20%, pararosaniline 1%, 10N hydrochloric acid 1%) were added. Samples were incubated for 30 min at 37°C and optical density was read at 555 nm. Collagen in lung homogenate was measured using a collagen colorimetric assay. Right lobes of the lung were kept frozen at -20°C until use. They were grounded with an UltraTurax® and centrifuged at 10,000 g for 10 min at 4°C. The supernatants were used for collagen measurement: 10 µl samples were added to 200 µl collagen dye reagent (Sirius red 0.017%, and picric acid 0.8% in absolute ethanol), vortexed for 30 min at 200 rpm at room temperature, centrifuged at 10000 g for 10 min at 4°C and supernatants were removed. Pellets were solubilized with 200 µl Alkali collagen reagent (absolute ethanol 20%, sodium dodecyl sulfate 1.3%, 2N sodium hydroxide 12%) and optical density measured at 540 nm.

Measurement of cytokines and OVA-specific antibodies – IL-4 and IL-5 measurements in BALF were performed by ELISA

(BD Pharmingen) according to the manufacturer's instructions. Ovalbumin-specific IgE, IgG1 and IgG2a plasma levels were determined by enzyme-linked immunosorbent assay. Microtiter plates were coated overnight at 4°C with the capture antibody: anti-mouse IgE, IgG1 or IgG2a at 2 µg/well in carbonate buffer, pH 9.5 (BD Pharmingen, clone R35-118, R19-15 and A85-1). After three washings with phosphate-buffered saline (PBS) containing 0.05% Tween-20, microtiter plates were blocked for 1h at 37°C with 10% bovine serum albumin in PBS. After three washings with PBS-Tween 0.05%, plasma samples were incubated for 2 h at room temperature. After three washings with PBS-Tween 0.05%, ovalbumin-HRP (BUF048, Abdsertec) was added to the wells and incubated for 1 h at room temperature. The plates were washed and tetramethylbenzidine (Pharmingen), a horseradish peroxidase substrate, was added and coloration allowed to develop for 15–20 min. The reaction was stopped by addition of 0.5M H₂SO₄ and absorbance intensity was read at 450nm. Serum levels of ovalbumin-specific Igs were expressed as optical densities (O.D).

Measurement of airway responsiveness by whole body plethysmography – Twenty-four hours after the last OVA or saline challenge, airway responsiveness to increasing concentrations of methacholine (MCh) (0 to 0.3 M; Sigma-Aldrich, A2251) was measured in unrestrained conscious and spontaneously breathing animals by whole-body barometric plethysmography (Emka Technologies, Paris, France), using the enhanced pause (PenH) as an index of airway obstruction as described (Daubeuf *et al.*, 2013). Briefly, mice were stabilized in the plethysmograph chamber for 30 min until stable baseline, and then exposed to aerosolized saline (30 sec) as a control. Then, mice were challenged every 20 min with increasing doses of aerosolized MCh (0.05, 0.1, 0.2 and 0.3M) for 30 sec each, and airway responsiveness was recorded for 5 min.

Measurement of airway resistance by forced oscillation technique – Other groups of mice were used to measure airway resistance twenty-four hours after challenge, airway hyperresponsiveness to methacholine was assessed using a

forced oscillation technique (Flexivent®, SCIREQ, Montreal, Canada) and as described (Daubeuf *et al.*, 2013). Mice were anesthetized with an i.p. injection of 6 ml/kg of xylazine (Rompun®;0.25%), followed 50 minutes later by an i.p. injection of 6ml/kg of pentobarbital sodium (0.9%). The trachea was exposed and an 18-gauge metal needle was inserted into the trachea. Airways were connected to a computer-controlled small animal ventilator, and quasi-sinusoidally ventilated with a tidal volume of 10 ml/Kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. After measurement of a baseline, each mouse was challenged for 10 sec with an aerosol of solvent generated by an in-line nebulizer and administered directly through the ventilator before PBS and MCh challenge at 50 mg/ml. For calculation of methacholine effect, the peak response was used as the mean of the three maximal values, and integrated for calculation of airway resistance (R). Airway resistance was expressed as cm H₂O.s.ml⁻¹.

Statistical analysis □ Differences between groups were tested for statistical significance using a two-way ANOVA and Bonferroni post-hoc test or a one-way ANOVA followed by Tukey post-test. Data were considered significantly different when p value <0.05.

RESULTS

Development of an 8-day model of allergen-induced airway hyper eosinophilia

– The sensitization scheme used OVA and alum. Balb/c mice were immunised by i.p. administration on D0, D1 and D2. They were then exposed to i.n. OVA in order to induce eosinophil recruitment. We evaluated the number of eosinophils recovered in BAL fluid after i.n. OVA challenges on two (D5 and D6; 7-day protocol) *versus* three consecutive days (D5, D6 and D7; 8-day protocol). Mice were anesthetized on D7 or D8, respectively, and BAL were performed. Comparative results obtained in the 7-day *vs* 8-day protocols are presented on figure 2A.

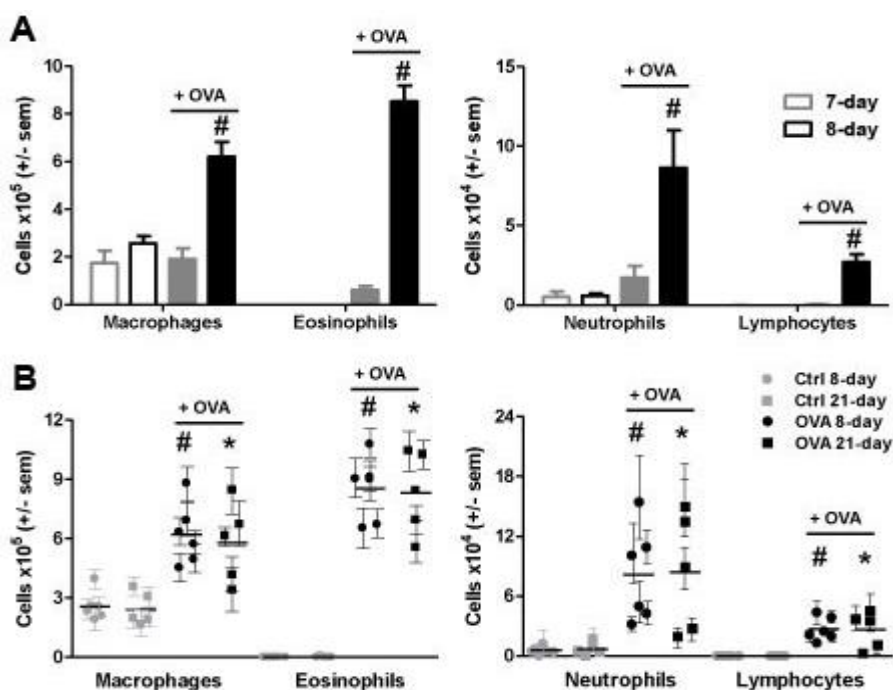


FIGURE 2. Rapid allergen-induced airway hyper eosinophilic models in Balb/c mice.

A. Development of the 8-day model: Balb/c mice were immunised and challenged with OVA or saline and analysis was performed on day 7 (gray, 2 challenges on D5 and 6) or 8 (white, 3 challenges on D5, 6 and 7) after the first immunisation with OVA. Numbers of macrophages, eosinophils, neutrophils and lymphocytes in BALF are shown. Blocks are means and bars are SEM values (n = 6 per group). **B.** Reproducibility of the 8-day model: Balb/c mice were immunised and challenged with OVA or saline, and analysis performed 8-days (dots) or 21-days (square) after the first sensitization. Numbers of macrophages, eosinophils, neutrophils and lymphocytes in BALF are shown. Each dot represent the mean value of results from n= 6 independent studies including 6 mice/study group. Blocks are means and bars are SEM values (n = 6 per group). # indicates p<0.05 between control and OVA groups in the 8-day model and * indicates p<0.05 between control and OVA groups in the 21-day model.

Mice developed slight inflammation in the 7-day protocol with few eosinophils in the BALF. By contrast, marked airway inflammation comprised of significant influx of macrophages and eosinophils ($8.7 \pm 0.4 \times 10^5$, more than 50% of total cells), and few neutrophils ($8.3 \pm 2.1 \times 10^4$) and lymphocytes ($2.6 \pm 0.2 \times 10^4$) was recovered in BAL fluid in the 8-day protocol. Control mice immunized by i.p. injection of OVA and alum on D0, D1, D2 and challenge with saline did not allow any inflammatory response. The 8-day model is the model of choice for studied rapidly the anti-inflammatory activity for the anti-inflammatory treatment of asthma.

Reproducibility of the 8-day model - In order to evaluate the activity of new therapeutic strategies to reduce influx of airway eosinophils in allergic diseases, it is essential to have a model with a high reproducibility. We therefore compared results of 6 independent experiments using this new rapid hypereosinophilia model vs 6 independent experiments using the commonly used 21-day asthma model (figure 2B). Figure 2B shows similar cell infiltration between the 8-day model and the 21-day model, indicating that the 8-day model is as reproducible and as robust as the 21-day model.

Response to glucocorticoids - Use of this 8-day model for drug discovery requires validation by the effect of glucocorticoids. The activity of 2 glucocorticoids, dexamethasone and budesonide, administered i.p. 1 hour before each OVA challenge, is presented on figure 3 in this 8-day model. At low dose, dexamethasone (0.1 mg/kg) significantly inhibited the eosinophil influx in BALF by 60%, and at higher dose (1 mg/kg) totally blocked eosinophil influx. Budesonide at 1 mg/kg also totally decreased eosinophil infiltration. We thus clearly demonstrated that our new model is highly reproducible and sensitive to the major treatment of airway inflammation in allergic diseases, the glucocorticoids dexamethasone and budesonide.

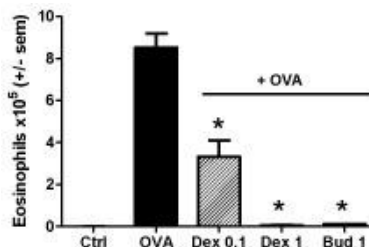


FIGURE 3. **Comparative effect of glucocorticoids:** Balb/c mice were immunised and challenged with OVA or saline and treated i.p. with dexamethasone or budesonide one hour before each OVA challenge. Eosinophils are shown. Blocks are means and bars are SEM values (n = 6 per group). # indicates $p \leq 0.05$ to OVA groups.

OVA-specific immunoglobulins in the 8-day and 21-day models - To further characterize our model, OVA-specific IgE, IgG1 and IgG2A levels were measured in plasma of mice sensitized and challenged with OVA as compared with control mice sensitized with OVA and challenged with saline (figure 4A). OVA-specific IgE ($185 \pm 19\%$ vs. control mice) and IgG1 ($168 \pm 44\%$) were significantly increased in the 8-day model. By contrast, OVA-specific IgG2A levels were not modified after OVA challenge in any model. Levels of OVA-specific IgE, IgG1 and IgG2A were similar in the 8-day and 21-day models, indicating that the immune response was similar in both models, and characteristic of an allergen-specific Th2 response.

Th2 cytokine levels in the 8-day and 21-day models - To characterise the rapid model in more detail, we explored the levels of the eosinophil chemoattractant IL-5 and of the IgE-switching cytokine IL-4 in BALF. Levels of IL-5 and IL-4 are represented on figure 4B. Data show as expected that IL-5 and IL-4 levels are increased ($82 \pm 14\%$ and $94 \pm 17\%$ respectively) in BALF from mice sensitized and challenged with OVA as compared with controls. The increase was similar in both 8-day and 21-day models.

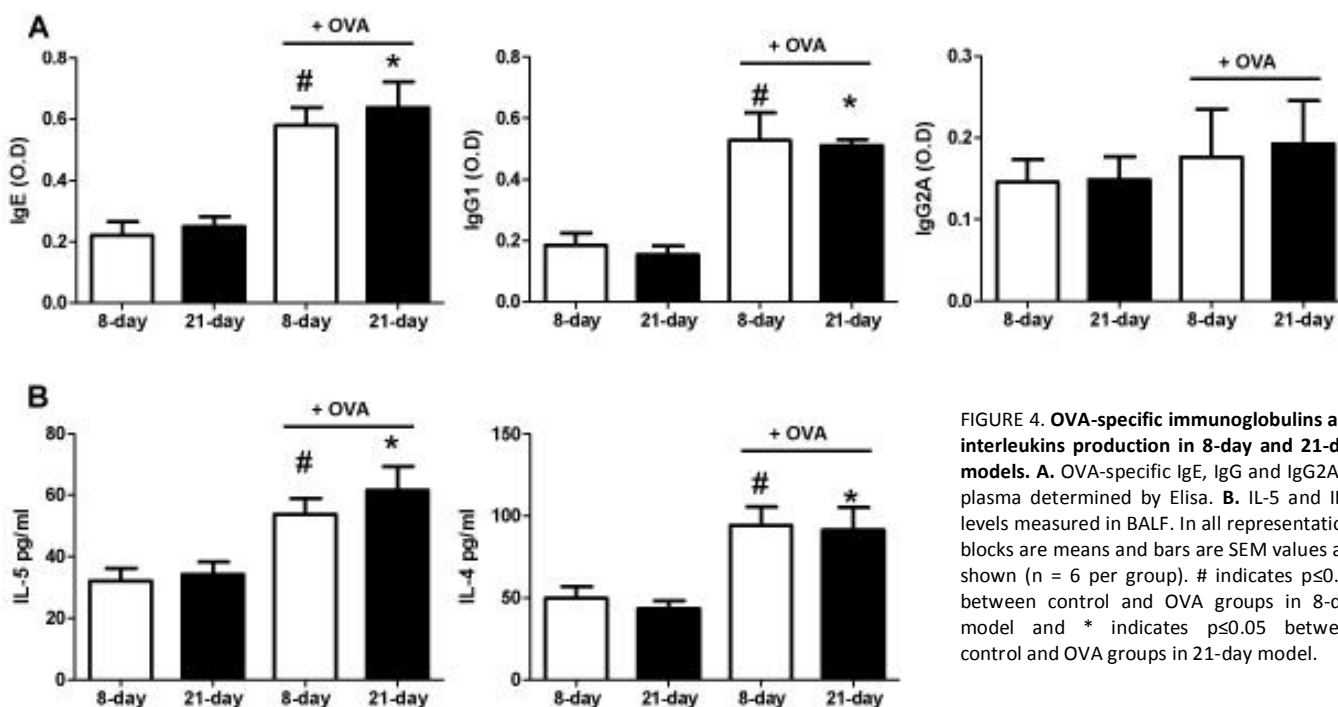


FIGURE 4. **OVA-specific immunoglobulins and interleukins production in 8-day and 21-day models.** A. OVA-specific IgE, IgG and IgG2A in plasma determined by Elisa. B. IL-5 and IL-4 levels measured in BALF. In all representation, blocks are means and bars are SEM values are shown (n = 6 per group). # indicates $p \leq 0.05$ between control and OVA groups in 8-day model and * indicates $p \leq 0.05$ between control and OVA groups in 21-day model.

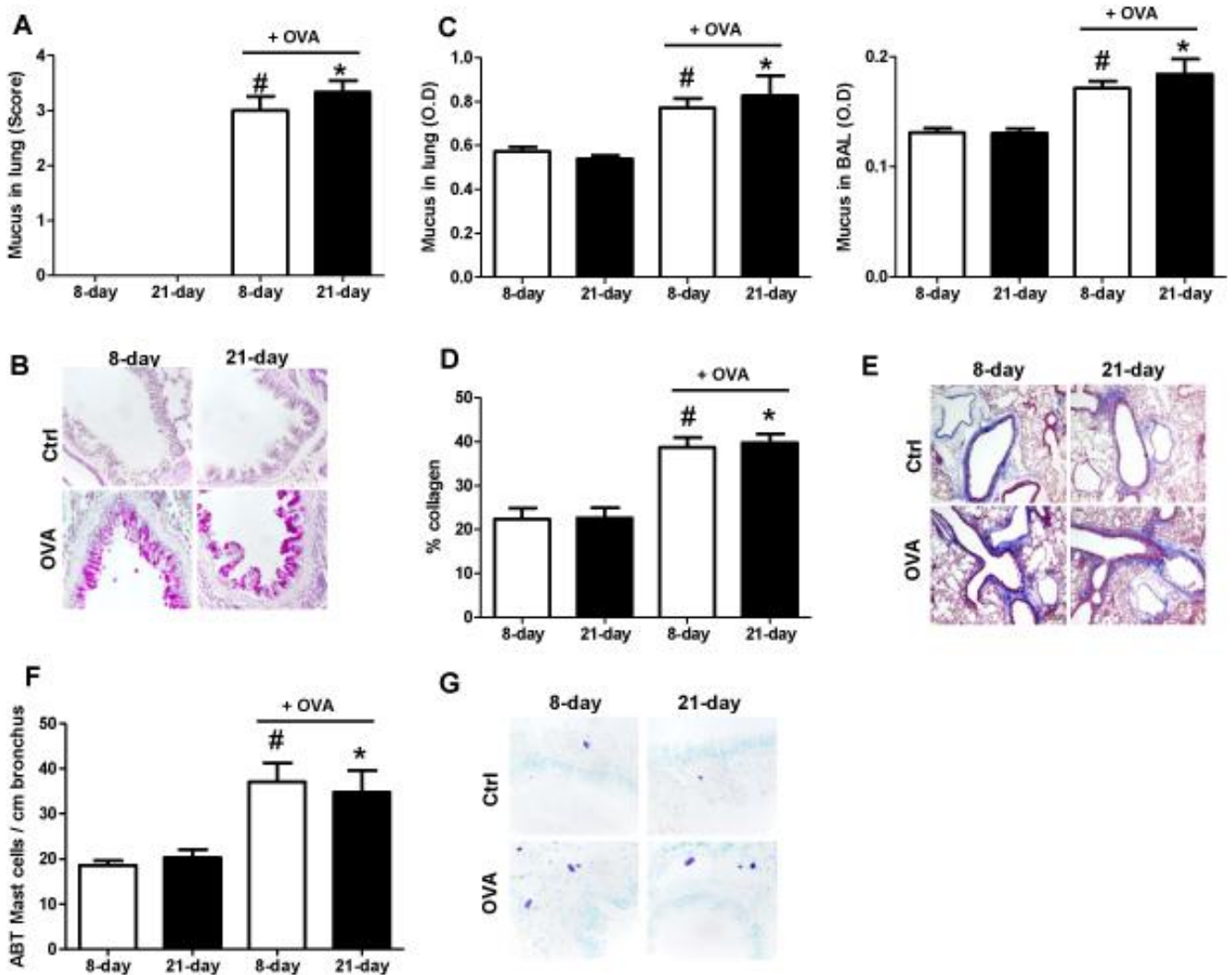


FIGURE 5. Excessive mucus production, collagen synthesis and mast cells infiltrate in 8-day and 21-day models. A. Excessive mucus production: Periodic acid Schiff (PAS) stained sections were scored as described in the materials and methods. B. Representative sections stain with PAS. C. Mucus in lung and BALF measured by mucus colorimetric assay. D. Collagen in lung measured by collagen colorimetric assay. E. Representative sections stain with Masson's trichrome showed collagen deposition (in blue) around the bronchus and vessels. F. Density of toluidine blue-positive mast cells. G. Toluidine blue staining of lung, showing infiltration of mast cells in the peribronchial tissues (magnification, x200). In all representation, blocks are means and bars are SEM values (n = 6 per group) are shown. # indicates $p \leq 0.05$ between control and OVA groups in 8-day model and * indicates $p \leq 0.05$ between control and OVA groups in 21-day model.

Mucus hypersecretion, collagen production and mast cell infiltrate—We compared airway remodelling occurring in the 8-day vs 21-day model.

Mucus production was quantified both histologically on lung sections using a 5 points-grading score of goblet cells stained in light red in the epithelium (Tanaka *et al.*, 2001) on paraffin-embedded lung sections (Figure 5A and 5B) and by use of a mucus colorimetric assay in BALF and in the lung homogenate. The score of mucus-producing goblet cells in the epithelium shows that mucus production was markedly enhanced in mice sensitized and challenged with OVA as compared with controls mice challenged with saline. The increase was comparable between 8-day (3 ± 0.2) and 21-day model (3.3 ± 0.2). Quantification performed with the mucus colorimetric assay confirms the increase in mucus production in lung as well as in BALF after OVA challenge in sensitized animals (Figure 5C), and that the increase is similar in the 8-day ($29 \pm 4\%$) and the 21-day models ($34 \pm 9\%$).

Collagen was studied in lung homogenate using a

colorimetric assay (Figure 5D). The percentage of collagen was increased in mice sensitized and challenged with OVA as compared with control. A similar increase was observed between the 8-day ($79 \pm 16\%$) and 21-day models ($82 \pm 11\%$). In parallel, representative sections labelled for collagen with the Masson's trichrome staining show the increase of collagen deposition around the bronchi and vessels in the OVA groups (Figure 5E).

Mast cells are known to play an important role in the inflammatory response in asthma. We compared the mast cells density in the lung of mice sensitized and challenged with OVA between the 8-day and 21-day models. Toluidine blue positive mast cells count are represented on figure 5F. The data show that mast cell numbers were increased in response to allergen in both models, with a similar increase between the 8-day ($74 \pm 17\%$) and 21-day models ($69 \pm 20\%$). Representative sections show mast cells stained with the toluidine blue staining technique, and coloured in purple on figure 5G.

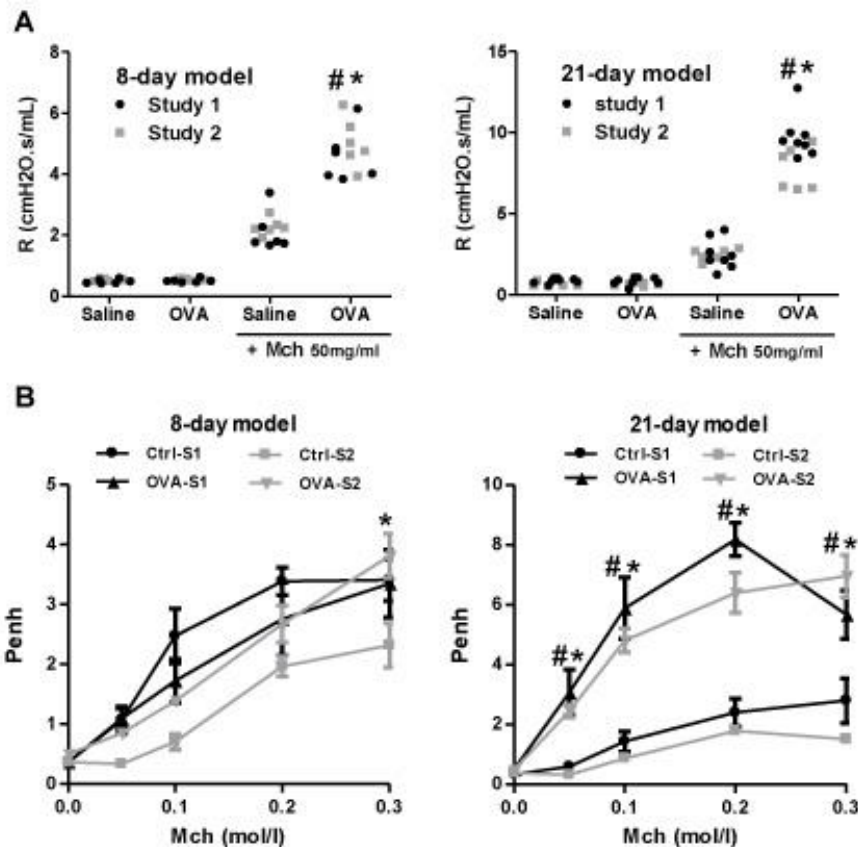


FIGURE 6. Airway reactivity to methacholine. **A.** Airway resistance (R) expressed as cm H₂O.s.mL⁻¹ in response to methacholine (50mg/mL) was assessed by the Flexivent® technique 24 hours after the last OVA challenge. Individual values obtained in a first study are in Black and in a second study are in grey (n = 6 mice/group). **B.** Airway reactivity to methacholine was measured using a whole body plethysmograph. Enhanced pause (PenH) responses to increasing doses aerosolized methacholine 24 hours after the last OVA challenge. Dots are means and bars are SEM values (n = 6 mice/group). Values obtained in a first study are in Black and in a second study are in grey. # indicates p ≤ 0.05 between control and OVA groups in the first study and * indicates p ≤ 0.05 between control and OVA groups in the second study.

Thus, our data show that this new rapid hyper eosinophilia model also exhibits mucus production, collagen synthesis and mast cell infiltrate, three characteristics of the asthma disease.

Airway responsiveness in the 8-day and 21-day models – We compared airway responses to methacholine in the 8-day vs 21-day models in two independent experiments as represented in black (study 1) and grey (study 2) on figure 6. The forced oscillation technique (Flexivent®) was used to measure the airway resistance in response to nebulized MCh (50 mg/ml). Figure 6A show that airway resistance was significantly increased in mice sensitized and challenged with OVA as compared to control mice challenged with saline both in the 8-day and the 21-day models. This was reproduced in two independent experiments. Airway resistance was increased by 2-fold in the 8-day model and by 4.5-fold in the 21-day model. Evaluation of airway obstruction (PenH) with unrestrained whole body plethysmography, in response to increasing doses of MCh (0.05, 0.1, 0.2 and 0.3M)(figure 6B), show that the measurement is unreliable in the 8-day model, with important variability and a low reactivity to MCh, in contrast to the significant and reproducible increase of the AHR in the 21-day model (4- to 5-fold increase). Therefore, these data show

the presence of airway hyperresponsiveness in the 8-day model, although to a lesser extent than in the 21-day model.

DISCUSSION

The aim of this study was to develop a rapid model of airway eosinophil recruitment in the mouse, with a Th2 profile characteristic of an “asthma-type” response. For this purpose, we used OVA as the allergen and alum as the adjuvant. Acute sensitization protocols require multiple systemic administration of the allergen in the presence of an adjuvant. Adjuvants such as aluminium hydroxide (Al(OH)₃) are known to promote the development of the Th2 phenotype by the immune system when it is exposed to an antigen (Brewer et al., 1999; McKee et al., 2009). Ovalbumin is seldom implicated in human asthma, and alternative allergens with greater clinical relevance, such as house dust mite and cockroach extracts have been successfully used to develop asthma models in mice (Ulrich et al., 2008; Lukacs et al., 2002). However, OVA derived from chicken egg is a frequently used allergen that induces, in a reproducible manner, a rapid and robust allergic airway inflammation with airway hyperresponsiveness and remodelling. The acute ovalbumin models are used in the development of drugs and therapeutic strategies (Nials et al., 2008), as corticosteroids (Trifilieff et al., 2000), leukotriene antagonists (Eum et al.,

2003) and anti-IL-5 (Hamelmann et al., 1999) strategies, which are now on the market.

We found that mice immunized with OVA/alum i.p. on 3 consecutive days (day 0, 1 and 2) and challenged with intranasal OVA on 3 consecutive days (day 5, 6 and 7) developed a strong airway eosinophilia, in contrast to mice challenged with OVA on 2 consecutive days only. This correlated with an increase in IL-5 levels in BALF on day 8. This clearly shows that three repeated exposures to OVA are necessary to develop significant and important recruitment of inflammatory cells, in particular eosinophils, in the airways. This is in accordance with the requirement illustrated in the review by Kumar and collaborators (Kumar et al., 2008), reporting that a 3 day-delay after allergen challenge is necessary for the development of asthma features (Kung et al., 1994, Kuperman et al., 1998, Trifilieff et al., 2000).

Bronchoalveolar lavage is a rapid and effective procedure for studying inflammation in the airways after euthanasia (Andreasen, 2003; Daubeuf et al., 2012) and results in the assessment of eosinophils, neutrophils, macrophages and lymphocytes counts. In this study, lavages were performed with 500 μ L solution, i.e. a volume corresponding to the mouse tidal volume, therefore preventing destruction of the lung structure. With this low volume, lavages were repeated 10 times in order to optimize cells recovery as previously reported (Daubeuf et al., 2012).

With this optimized technique, we found that the 8-day rapid model led to development of a strong airway eosinophil influx in response to OVA challenge as compared to saline challenge in sensitized mice. We also note that sensitization itself with OVA/Alum did not allow development of any airway inflammation in the absence of allergen challenge. Our new 8-day model allows recruitment of a high number of eosinophils in BALF (8×10^5), a number that is similar to or enhanced as compared to the recruitment reported by other investigators using longer immunization protocols (McMillan et al., 2005; Tomkinson et al., 2001; Henderson et al., 1996; Tanaka et al., 2000 et 2001; Sarpong et al., 2003; Wue et al. 2003, Fernandez-Rodriguez et al., 2008; Ble et al., 2008; 2009; Delayre-Orthez et al., 2004; 2005a; 2005b; 2008; Deschamps et al., 2011; Hachet-Haas et al., 2008; Zhang et al., 2009). In order to surmount any eventual difference coming from the lavage technology, we compared these results in our rapid 8-day model to those in the commonly used 21-day model. We clearly show that the number of eosinophils measured in the BALF in this rapid model was identical to that found in the 21-day model. We show from 6 independent studies, that the mean values of cell recruitment are equally distributed between the 8-day and the 21-day models for eosinophils, macrophages, neutrophils and lymphocytes. In addition, this comparison allows to determine the reproducibility of the technique, with a similarity for the 8-day and the 21-day models.

We showed that, in 6 independent studies, the variance is lower in the 8-day than in the 21-day model for macrophages (2.3 vs. 4.1×10^5 cells²), eosinophils (2.6 vs. 4.2×10^5 cells²), neutrophils (32.9 vs. 35.6×10^4 cells²) and lymphocytes (1.4 vs. 3.4×10^4 cells²).

We further characterized our model in greater details, and found this rapid immunization protocol was able to induce increased levels of OVA-specific IgE and IgG1 in the plasma, similar to those induced by the 21-day protocol, as

well as similar enhanced levels of IL-5 and IL-4 in the BALF in both models. These findings show that, although the immunization protocol is very short, it is sufficient to induce allergen-specific Th2-type responses in the airways. The most likely explanation for the development of airway eosinophilia is that IL-5 produced by Th2 cells was responsible for recruitment to and survival of eosinophils in the airways (Rothenberg et al., 2006). In addition, in our rapid 8-day model, we also measured an excess of mucus production, collagen deposition and mast cell infiltration in sensitized and challenged mice as compared to controls. Surprisingly, for all parameters mentioned above, no differences were observed between the 8-day and 21-day models. The inflammatory and the remodelling responses were comparable, and suggest a similar mechanism of action. This is an important point, that enhances the interest of this rapid 8-day model.

At that step, the question was to know whether this rapid 8-day model was compatible with the study of anti-inflammatory drugs or strategies. To do so, we evaluated the response of our model to two compounds recommended for the treatment of asthma in humans (see "Global Initiative for Asthma" guidelines and used as positive reference controls in various studies in mice (Kumar et al., 2003; Ben et al., 2008; Li et al., 2012; Reber et al. 2012; Firinci et al., 2013; Mushaben et al., 2013). Dexamethasone and budesonide are two corticosteroids very active in their anti-asthmatic effect in conventional models. We show that our rapid 8-day model was sensitive to low doses of glucocorticoids: dexamethasone (100 μ g/kg, i.p.) significantly decreased airway hyper-eosinophilia by 80%. This result ascertains that the rapid 8-day model may be used to study the activity of anti-inflammatory drugs in asthma.

Additionally, the measurement of airway responsiveness to metacholine was assessed by two exploratory techniques: by whole body plethysmography and the forced oscillation (Flexivent®) technique. Balb/c mice are commonly used for the study of this asthma feature, characterized by a high bronchial hyperresponsiveness in sensitized and challenged animals (Brewer et al., 1999; Taube et al., 2004). Accordingly, our results show that the airway parameters measured, i.e. airway resistance and Penh, were significantly increased by 4.5-fold in OVA sensitized and challenged mice as compared to control mice in the 21-day model. In the 8-day model by contrast, these parameters were not affected in a similar proportion. Whole body plethysmography shows a non reproducible increase in airway reactivity (only in study 2) that was very modest (a 2-fold increase in Penh) as compared to control mice. In contrast, we measured a significant and reproducible increase of the airway resistance with the Flexivent® technique. It is now well established that a correlation exists between the invasiveness of a measurement technique and its precision (Glaab et al. 2007; Vanoirbeek et al., 2010). This is in accordance with the reproducibility of the assessment of AHR with the Flexivent technique in the 8-day model. However, AHR developed in the 8-day model was lower than in the 21-day model. According to our results of a similarity in each other parameter developed between the 8-day and the 21-day models, we can only speculate that the time of airway infiltration by eosinophils may not be long enough to induce AHR: 24h or 48h respectively, according to the kinetic of the eosinophil influx, measured in the BALF.

Supporting this view is that AHR usually correlates with the presence of eosinophils and a therapeutic intervention reducing eosinophils also reduces AHR (Foster et al., 2005; Lee et al., 2004; Cho et al., 2004; Humbles et al., 2004). A further possibility is that other unmeasured parameters may affect airway hyperresponsiveness between the 8-day and the 21-day models, as for instance airway smooth muscle proliferation, abnormal contractility, epithelial damage, subepithelial fibrosis, angiogenesis or changes in the myositis interaction (Barnes, 1996; Ozier et al., 2011).

In conclusion, we here present a rapid asthma model with asthma-like features, that will allow to reduce time and cost of research of anti-inflammatory and anti-asthma strategies without loss in the quality of the results. Our data presented confirm that the 8-day model is a rapid and reproducible model, which may be used as a predictive testing for drug-induced reduction of airway hyper-eosinophilia. Our model may be suitable to efficiently study new therapeutic strategies for asthma-like hyper-eosinophilia and associated features.

REFERENCES

- Andreasen CB. (2003). Bronchoalveolar lavage. *Vet Clin Small Anim* 33 : 69–88.
- Barnes PJ. (1996) Pathophysiology of asthma. *Br J Clin Pharmacol.* 4, 3–10.
- Barnes PJ, Chung KF, Page CP. (1998) Inflammatory mediators of asthma: an update. *Pharmacol Rev.* 50, 515–96.
- Barnes PJ. (2008) The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest.* 118(11):3546-56.
- Ben S, Li X, Xu F, Xu W, Li W, Wu Z, Huang H, Shi H, Shen H. (2008) Treatment with anti-CC chemokine receptor 3 monoclonal antibody or dexamethasone inhibits the migration and differentiation of bone marrow CD34 progenitor cells in an allergic mouse model. *Allergy.* 63(9):1164-76
- Blé F-X, Cannet C, Zurbrugg S, Karmouty Quintana H, Frossard N, Trifilieff A, Beckmann N. (2008) Allergen-induced lung inflammation in actively sensitized mice assessed by MRI. *Radiology,* 248: 834-43, 2008.
- Blé F-X, Cannet C, Zurbrugg S, Gérard C, Frossard N, Beckmann N, Trifilieff A. (2009) Activation of the lung S1P1 receptor reduces allergen-induced plasma leakage in mouse. *Brit J Pharmacol,* 158: 1295-301, 2009.
- Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J. (1999) Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J Immunol.* 15;163(12):6448-54.
- Brewer JP, Kisselgof AB, Martin TR. (1999) Genetic variability in pulmonary physiological, cellular, and antibody responses to antigen in mice. *Am J Respir Crit Care Med.* 160(4):1150-6.
- Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, *et al.* (2004) Inhibition of airway remodeling in IL-5-deficient mice. *J Clin Invest.* 113, 551–60.
- Daubeuf F., Frossard N. (2012) Performing Bronchoalveolar Lavage in the Mouse. *Current Protocols in Mouse Biology.* 2: 167-175
- Daubeuf F., Reber L., Frossard N. (2013) Measurement of Airway Responsiveness on Vigil and Unrestrained Mouse. <http://www.bio-protocol.org/wenzhang.aspx?id=328>
- Daubeuf F., Reber L., Frossard N. (2013) Measurement of Airway Responsiveness in the Anesthetized Mouse. <http://www.bio-protocol.org/wenzhang.aspx?id=645>
- Delayre-Orthez C, Becker J, De Blay F, Frossard N, Pons F. (2004) Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin Exp Allergy,* 34: 1789-1795.
- Delayre-Orthez C, Becker J, Guenon I, Lagente V, Auwerx J, Frossard N, Pons F. (2005a) PPARalpha downregulates airway inflammation induced by lipopolysaccharide in the mouse. *Respir Res,* 6: 91.
- Delayre-Orthez C, Becker J, de Blay F, Frossard N, Pons F. (2005b) Exposure to endotoxins during sensitization prevents further endotoxin-induced exacerbation of airway inflammation in a mouse model of allergic asthma. *Int Arch Allergy Clin Immunol,* 138: 298-304.
- Delayre-Orthez C, Becker J, Auwerx J, Frossard N, Pons F. (2008) Suppression of allergen-induced airway inflammation and immune response by the peroxisome proliferator-activated receptor-alpha agonist fenofibrate. *Eur J Pharmacol,* 581, 177-184.
- Deschamps K, Cromlish W, Weicker S, Lamontagne S, Huszar SL, Gauthier JY, Mudgett JS, Guimond A, Romand R, Frossard N, Percival MD, Slipetz D, Tan CM. (2011) Genetic and pharmacological evaluation of Cathepsin S in a mouse model of asthma. *Am J Respir Cell Mol Biol,* 45: 81-7.
- Eum, S. Y., Maghni, K., Hamid, Q., Campbell, H., Eidelman, D. H. and Martin, J. G. (2003). Involvement of the cysteinyl-leukotrienes in allergen-induced airway eosinophilia and hyperresponsiveness in the mouse. *Am. J. Respir. Cell Mol. Biol.* 28, 25-32
- Fernandez-Rodriguez S, Ford WR, Broadley KJ, Kidd EJ. (2008) Establishing the phenotype in novel acute and chronic murine models of allergic asthma. *Int Immunopharmacol.* 8(5):756-63. Epub 2008 Feb 22.
- Fırıncı F, Karaman M, Cilaker-Mıcılı S, Bağrıyanık A, Uzuner N, Karaman O. (2013) The effect of atorvastatin on lung histopathology in a murine model of chronic asthma. *Allergol Immunopathol (Madr).* S0301-0546(13)00238-3.
- Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. (2005) Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J exp Med.* 183, 195–201.
- GINA, Global Initiative for Asthma (2012) Pocket Guide For Asthma Management And Prevention. Updated December 2012.
- Glaab T, Taube C, Braun A, Mitzner W.(2007) Invasive and noninvasive methods for studying pulmonary function in mice. *Respir Res.* 14;8:63.
- Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW. (1997) Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med.* 156, 766-75
- Hamelmann, E., Cieslewicz, G., Schwarze, J., Ishizuka, T., Joetham, A., Heusser, C. and Gelfand, E. W. (1999). Anti-IL-5 but not anti-IgE prevents airway inflammation and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 160, 934-941
- Henderson WR Jr, Lewis DB, Albert RK, Zhang Y, Lamm WJ, Chiang GK, Jones F, Eriksen P, Tien YT, Jonas M, Chi EY.(1996) The importance of leukotrienes in airway

- inflammation in a mouse model of asthma. *J Exp Med.* 184(4):1483-94.
- Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, *et al.* (2004) A critical role for eosinophils in allergic airways remodeling. *Science* 305,1776–9.
- Kung TT, Jones H, Adams GK 3rd, Umland SP, Kreutner W, Egan RW, Chapman RW, Watnick AS (1994) Characterization of a murine model of allergic pulmonary inflammation. *Int Arch Allergy Immunol.* 105(1):83-90.
- Kumar RK, Herbert C, Thomas PS, Wollin L, Beume R, Yang M, Webb DC, Foster PS. (2003) Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. *J Pharmacol Exp Ther.* 307(1):349-55.
- Kumar RK, Herbert C, Foster PS. (2008) The "classical" ovalbumin challenge model of asthma in mice. *Curr Drug Targets.* 9(6):485-94. Review.
- Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. (1998) Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp Med.* Mar 16;187(6):939-48.
- Jones SC, Iverson D, Burns P, Evers U, Caputi P, Morgan S. (2011) Asthma and ageing: an end user's perspective - the perception and problems with the management of asthma in the elderly. *Clin Exp Allergy.* 41(4):471-81
- Larche M, Robinson DS, Kay AB. (2003) The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 111, 450–63.
- Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, *et al.* (2004) Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 305, 1773–6.
- Li H, Zhu S, He S, Hao L. (2012) Anti-inflammatory effects of moxifloxacin on rat airway smooth muscle cells exposed to allergen: Inhibition of extracellular-signal-regulated kinase and nuclear factor- κ B activation and of interleukin-8 and eotaxin synthesis. *Respirology.* 17(6):997-1005.
- Lukacs NW, John A, Berlin A, Bullard DC, Knibbs R, Stoolman LM. (2002) E- and P-selectins are essential for the development of cockroach allergen-induced airway responses. *J Immunol.* 15;169(4):2120-5.
- McKee AS, Munks MW, MacLeod MK, Fleenor CJ, Van Rooijen N, Kappler JW, Marrack P. (2009) Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J Immunol.* 1;183(7):4403-14.
- McMillan SJ, Xanthou G, Lloyd CM. (2005) Therapeutic administration of Budesonide ameliorates allergen-induced airway remodelling. *Clin Exp Allergy.* 35(3):388-96.
- Mushaben EM, Brandt EB, Hershey GK, Le Cras TD (2013) Differential effects of rapamycin and dexamethasone in mouse models of established allergic asthma. *PLoS One.* 8(1):e54426.
- Nials AT, Uddin S. (2008) Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech.* 1(4-5):213-20.
- Ozier A, Allard B, Bara I, Girodet PO, Trian T, Marthan R, Berger P. (2011) The pivotal role of airway smooth muscle in asthma pathophysiology. *J Allergy (Cairo).* 2011:742710.
- Rothenberg ME, Hogan SP. (2006) The eosinophil. *Annu Rev Immunol.* 24, 147–74.
- Sarpong SB, Zhang LY, Kleeberger SR. (2003) A novel mouse model of experimental asthma. *Int Arch Allergy Immunol.* 132(4):346-54.
- Tanaka H, Kawada N, Yamada T, Kawada K, Takatsu K, Nagai H. (2000) Allergen-induced airway inflammation and bronchial responsiveness in interleukin-5 receptor alpha chain-deficient mice. *Clin Exp Allergy.* 30(6):874-81.
- Reber LL, Daubeuf F, Nemska S, Frossard N. (2012) The AGC kinase inhibitor H89 attenuates airway inflammation in mouse models of asthma. *PLoS One.* 7(11):e49512.
- Tanaka H, Masuda T, Tokuoka S, Komai M, Nagao K, Takahashi Y, Nagai H. (2001) The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm Res.* 50, 616-24.
- Taube C, Dakhama A, Gelfand EW. (2004) Insights into the pathogenesis of asthma utilizing murine models. *Int Arch Allergy Immunol.* 135(2):173-86.
- Tomkinson A, Duez C, Cieslewicz G, Pratt JC, Joetham A, Shanafelt MC, Gundel R, Gelfand EW. (2001) A murine IL-4 receptor antagonist that inhibits IL-4- and IL-13-induced responses prevents antigen-induced airway eosinophilia and airway hyperresponsiveness. *J Immunol.* 166(9):5792-800.
- Trifilieff A, El-Hashim A, Bertrand C. (2000) Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *Am J Physiol Lung Cell Mol Physiol.* 279(6):L1120-8.
- Ulrich K, Hincks JS, Walsh R, Wetterstrand EM, Fidock MD, Sreckovic S, Lamb DJ, Douglas GJ, Yeadon M, Perros-Huguet C, Evans SM. (2008) Anti-inflammatory modulation of chronic airway inflammation in the murine house dust mite model. *Pulm Pharmacol Ther.* 21(4):637-47.
- Vanoirbeek JA, Rinaldi M, De Vooght V, Haenen S, Bobic S, Gayan-Ramirez G, Hoet PH, Verbeke E, Decramer M, Nemery B, Janssens W. (2010) Noninvasive and invasive pulmonary function in mouse models of obstructive and restrictive respiratory diseases. *Am J Respir Cell Mol Biol.* 42(1):96-104.
- Wu AY, Chik SC, Chan AW, Li Z, Tsang KW, Li W. (2003) Anti-inflammatory effects of high-dose montelukast in an animal model of acute asthma. *Clin Exp Allergy.* 33(3):359-66
- Zhang Z, Hener P, Frossard N, Kato S, Metzger D, Li M, Chambon P. (2009) Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma. *Proc Natl Acad Sci USA,* 106(5):1536-41.

Publication n°2 :

Prodrugs of a CXC Chemokine-12 (CXCL12) Neutraligand Prevent Inflammatory Reactions in an Asthma Model in Vivo

Vincent Gasparik, François Daubeuf, Muriel Hachet-Haas, François Rohmer, Patrick Gizzi, Jacques Haiech, Jean-Luc Galzi, Marcel Hibert, Dominique Bonnet, and Nelly Frossard

ACS Medicinal Chemistry Letters., 2012, 3 (1), pp 10–14.

Il a précédemment été mis en évidence que la chalcone 4, neutralise la chimiokine CXCL12 et inhibe la liaison de la chimiokine aux récepteurs CXCR4 et CXCR7 et l'activation résultante (Hachet-Haas et al. 2008). De plus, l'administration systémique de la chalcone 4 inhibe efficacement l'afflux d'éosinophiles dans un modèle aigu d'asthme chez la souris. Cette activité in vivo, est cependant limitée par la faible solubilité du composé chalcone 4 dans les tampons aqueux (9 μM dans un tampon HEPES).

Dans le but de contrecarrer ce manque de solubilité, des analogues solubles de la chalcone 4 ont été conçus et synthétisés, pour tenter d'augmenter l'activité anti-inflammatoire du neutraligand dans l'asthme allergique et permettre une administration locale.

Nos résultats montrent que l'addition de groupements : phosphate (chalc4-P), L-serine, ou sulfate permet d'augmenter fortement (5 à 10000 fois) la solubilité dans les tampons aqueux selon les analyses physicochimiques réalisées. Cependant, ces composés ne présentent plus d'activité neutraligand de la chimiokine CXCL12 par eux-mêmes, mais peuvent être hydrolysés dans les milieux physiologiques comme montré dans le sérum ($T_{1/2} = 120\text{min}$ pour la chalc4-P) et l'homogénat de poumon de souris ($T_{1/2} = 15\text{min}$ pour la chalc4-P). Ces composés se comportent comme des prodrogues : des composés inactifs, dégradés après administration in vivo, en un composé actif : la chalcone 4.

L'étude in vivo de ces prodrogues montre que l'administration locale de la prodrogue chalc4-P, par voie intranasale, permet d'inhiber le recrutement des éosinophiles dans les voies aériennes, dans notre modèle rapide d'asthme allergique, avec une concentration inhibitrice médiane de 10nmol/kg. L'administration locale des prodrogues phosphate, L-serine, ou sulfate à la dose de 30nmol/kg, met en évidence une activité similaire (67, 69 and 64% respectivement).

Ainsi la conception de prodrogues du composé chalcone 4 permet une administration locale, une activité anti-inflammatoire du neutraligand de CXCL12 à de très faible doses, et offre une stratégie intéressante pour le développement de médicament candidat pour le traitement de l'asthme

Prodrugs of a CXC Chemokine-12 (CXCL12) Neutraligand Prevent Inflammatory Reactions in an Asthma Model in Vivo

Vincent Gasparik,[†] François Daubeuf,[†] Muriel Hachet-Haas,[‡] François Rohmer,[†] Patrick Gizzi,[‡] Jacques Haiech,[†] Jean-Luc Galzi,[‡] Marcel Hibert,^{*,†} Dominique Bonnet,[†] and Nelly Frossard[†]

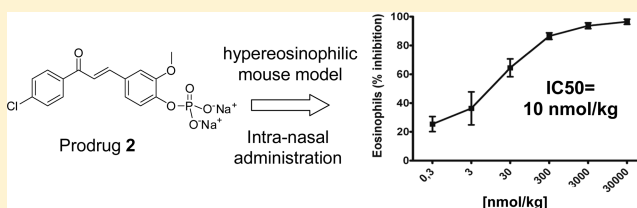
[†]Laboratoire d'Innovation Thérapeutique, UMR 7200 CNRS/Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

[‡]Biotechnologie et signalisation cellulaire, UMR 7242 CNRS/Université de Strasbourg, ESBS, Bld Sébastien Brant, 67412 Illkirch, France

S Supporting Information

ABSTRACT: Chalcone 4 (compound 1) is a small molecule that neutralizes the CXC chemokine CXCL12 and prevents it from acting on the CXCR4 and CXCR7 receptors. To overcome its poor solubility in aqueous buffers, we designed highly soluble analogues of compound 1, phosphate, L-seryl, and sulfate, all inactive by themselves on CXCL12 but when cleaved in vivo into 1, highly active locally at a low dose in a mouse airway hyper eosinophilia model.

KEYWORDS: prodrug, solubility, CXCL12 chemokine, CXCR4 receptor, GPCR, asthma



The chemokine CXCL12 plays a pivotal role in normal and pathological situations, including brain development, hematopoiesis, and chronic inflammation.¹ In screening a proprietary chemical library,² we recently identified compounds belonging to the chalcone family and preventing CXCL12 from binding to its CXCR4 or CXCR7 receptors.³ These compounds have an original mechanism of action: they bind to the chemokine rather than to the receptors. The highest affinity molecule, chalcone 4³ (compound 1 in Scheme 1, now commercialized by Sigma-Aldrich, C7870), inhibits both chemotaxis of human peripheral blood lymphocytes and eosinophil infiltration in a mouse model of airway hyper eosinophilia.³ The observation of these effects highlights the enormous potential of such a ligand neutralization strategy.^{3–5} Because of its mechanism of action, compound 1 does not affect the resting/basal level of the receptors for either calcium or chemotactic responses.³ By analogy to the activity of neutralizing antibodies, we named compound 1 a “neutraligand”.⁴ It is a tool that complements traditional antagonists of the CXCR4 receptor, such as AMD3100, AMD3465, ALX40-4C, and T22/T140,^{6–8} but has the disadvantage of poor solubility in aqueous buffer (9 μ M). The aim of the work reported here was to increase its solubility to enable in vivo access to its pharmacological targets. We report the design and synthesis of highly soluble derivatives of compound 1 that behave as prodrugs^{9,10} converted into the active compound 1 and have in vivo anti-inflammatory activity at low doses upon intranasal administration in a mouse model of asthma.

The requirements for suitable prodrugs of compound 1 were (1) inclusion of a functional group enzymatically cleavable in vivo,¹¹ (2) improved aqueous solubility to facilitate in vivo intranasal administration, and (3) rapid and efficient synthesis to allow large-scale preparation. To fulfill these requirements, we decided to introduce three types of functional groups on the

hydroxyl moiety of compound 1: a phosphate, an L-seryl, and a sulfate group. Prodrugs 2, 4, and 5 were prepared according to Scheme 1 starting from compound 1, itself obtained following a Claisen–Schmidt reaction with a 67% overall yield.¹²

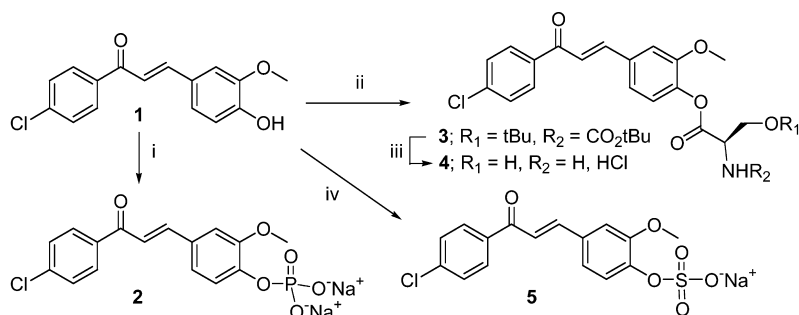
The phosphate derivative 2 was obtained by phosphorylation of the phenol group with dibenzylphosphonate in CCl_4 , followed by benzyl removal in the presence of trimethylsilylbromide (37% overall yield).¹³ An alternative one-pot procedure involved phosphorylation of the 4-hydroxyphenyl in the presence of phosphorus oxichloride, followed by basic hydrolysis to obtain the disodium phosphate analogue with a 45% yield.¹⁴ The L-serine conjugate of compound 1 was synthesized in two steps. First, an ester was prepared with Boc-L-Ser(tBu)-OH in the presence of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDCI) and a catalytic amount of *N,N*-dimethyl-4-aminopyridine (4-DMAP). Subsequent deprotection in acidic media provided the expected prodrug 4 as the hydrochloride salt, with a 70% overall yield. The sulfate analogue 5 was obtained by direct sulfation of the 4'-hydroxyphenyl with a chlorosulfonic acid/pyridine mixture and then treatment with aqueous NaOH to obtain the sodium salt (34%).¹⁵ The structure and purity of all compounds were assessed by ¹H, ¹³C NMR, high-resolution mass spectrometry (HRMS), and RP-HPLC analyses.

Thermodynamic solubility was measured in an aqueous 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer (pH 7.4, 22 °C). As expected, the solubility of the phosphate 2 was very high, so high that saturation was not achieved at a 30 mM

Received: April 21, 2011

Accepted: December 9, 2011

Published: December 9, 2011

Scheme 1. Prodrugs **2**, **4**, and **5** Preparation^a from Compound **1**

^aReagents: (i) (a) POCl₃ (3 equiv), Et₃N (10 equiv), CH₂Cl₂, room temperature, overnight; (b) aqueous NaOH. (ii) Boc-Ser(*t*Bu)-OH (1.5 equiv), DMAP (0.1 equiv), EDCI (1.5 equiv), CH₂Cl₂, room temperature, 1 h. (iii) 4 N HCl/dioxane, 60 °C, 4 h. (iv) (a) Chlorosulfonic acid (10 equiv), pyridine, room temperature, overnight; (b) aqueous NaOH.

Table 1. Characterization of Compounds **1**, **2**, **4**, and **5**

compd	isolated yield (%)	CHI ^a	Log <i>D</i> calcd ^b	solubility (μM) ^c	stability ^d half-life (<i>T</i> _{1/2})			binding inhibition (%) ^e
					PBS	murine serum	lung homogenate	
1		90	3.4	9 ± 1	>10 h	>10 h	>6 h	90 ± 10 (<i>K</i> _i = 53 ± 31 nM)
2	40	43	1.0	>30000	>10 h	2 h	<15 min	5 ± 2
4	70	76	2.7	42 ± 3	12 min	<5 min	<5 min	15 ± 5
5	34	64	2.1	1434 ± 3	>10 h	>10 h	30 min	4 ± 3

^aDetermined by RP-HPLC¹⁴ (C18 Luna column, 2 mL/min, 365 nm, solvent A = pH 7.4 ammonium acetate buffer, solvent B = acetonitrile). ^bCalculated from CHI. ^cMeasured at pH 7.4, HEPES buffer, 24 h of incubation, 22 °C. ^d37 °C. ^eInhibition of CXCL12-TR binding to EGFP-CXCR4 determined by FRET at 5 μM.

concentration. Its solubility is thus at least 3000 times higher than the 9 μM solubility of compound **1**. The solubility measured for the sulfate **5** under the same experimental conditions was 1430 μM, that is, greater than that required for satisfactory drug development. The yield of the serine **4** was better than that of the other two compounds (70%), but its solubility was only moderately better than that of compound **1** (4.6-fold; 42 μM as compared to 9 μM).

The partition coefficients of compounds **1**, **2**, **4**, and **5** at pH 7.4, expressed as Log *D*, are derived directly from measurements of their chromatographic hydrophobicity index (CHI), as compared with reference compounds.¹⁶ Log *D* decreased from 3.4 for the original compound **1** to 2.7 for the serine **4**, 2.1 for the sulfate **5**, and 1.0 for the phosphate **2**.

The stability of the four compounds was evaluated by LC-MS in three media: phosphate buffer saline (PBS), mouse serum, and mouse lung homogenates (Table 1). All experiments were conducted with extemporaneously prepared solution. Compound **1** at 10 μM is stable after incubation in all three media (*T*_{1/2} > 6–10 h). The phosphate **2** showed good stability in PBS, with 50% of it converted into **1** after 2 h of incubation in mouse serum (Table 1 and Figure 1) and within less than 15 min in lung homogenates (Table 1). The serine ester **4** was rapidly hydrolyzed into **1** with a *T*_{1/2} = 12 min in PBS; it disappeared in less than 5 min in mouse serum and lung homogenate. This instability is the combined effect of the strong electron withdrawing effect of the protonated aminogroup activating the ester linkage toward hydrolysis and intramolecular assistance of this aminogroup, as already reported for α-aminoester prodrugs.¹⁷ The sulfate **5** was stable in PBS and murine serum (*T*_{1/2} > 10 h) but rapidly converted into **1** in lung homogenates (*T*_{1/2} = 30 min). We therefore confirmed the high rate of conversion of each prodrug candidate into **1** in lung homogenates (Table 1).

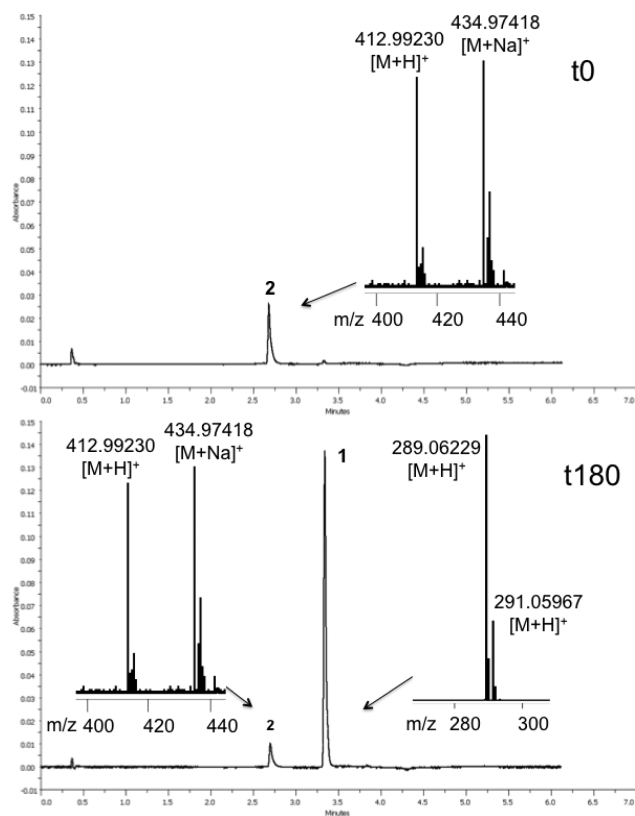


Figure 1. RP-HPLC chromatogram (C18 Luna column, 2 mL/min, 365 nm, solvent A = pH 7.4 ammonium acetate buffer, solvent B = acetonitrile). Gradient: 0–0.2 min, 0% B; 0.2–2.7 min, 0–100% B; 2.7–3.2 min, 100% B; 3.2–3.4 min, 100–0% B; and 3.4–6.2 min, 0% B. Positive ESI-MS spectrum of compound **2** at *t*₀ and *t*₁₈₀ at 37 °C in mouse serum.

In vitro inhibition of binding of Texas Red (TR)-labeled CXCL12 (CXCL12-TR, 100 nM) to enhanced green fluorescent protein (EGFP)-tagged CXCR4 by compounds **1**, **2**, **4**, and **5** at 5 μ M was determined by fluorescence resonance energy transfer (FRET) (Table 1 and Figure 2) as previously described.³

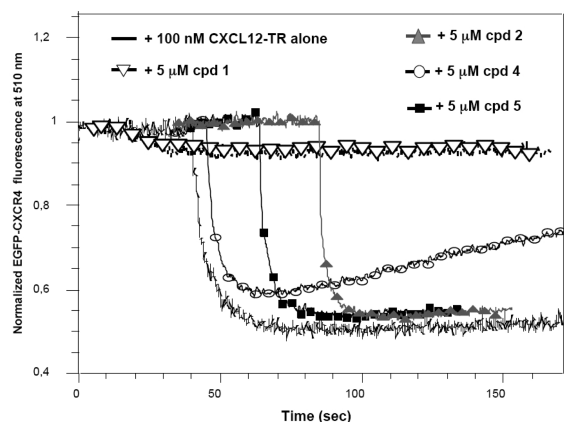


Figure 2. Real-time monitoring of fluorescent CXCL12-TR to EGFP-CXCR4 at 510 nm as a function of time. Upon addition of CXCL12-TR (100 nM), the fluorescence intensity at 510 nm declines as a result of interactions between CXCL12 and CXCR4. Compound **1** inhibits binding of CXCL12-TR to EGFP-CXCR4, whereas prodrugs **2**, **4**, and **5** (5 μ M) do not.

Compound **1**, the reference compound, at 5 μ M reduces this binding by 90% with a K_i of 53 nM. Compounds **2**, **4**, and **5** are, as expected, almost inactive in this in vitro binding assay (Figure 2). When serine was substituted in **4**, prolonged incubation times tended to inhibit CXCL12 binding to CXCR4. The inadequate stability of **4** in buffer or serum (Table 1), due to the progressive conversion of the inactive prodrug **4** into the active **1**, may explain this result.

The in vivo activity of the prodrugs was assessed in an 8 day mouse model of airway hyper eosinophilia in ovalbumin (OVA)-sensitized mice challenged with OVA or control saline. Eosinophil number in the bronchoalveolar lavage fluid increased significantly after OVA challenge, as compared with saline, in OVA-sensitized animals, as well as macrophage, neutrophil,

and lymphocyte recruitment, although those were more moderate (Figure 3). Because topical administration is a well-accepted and preferred route for treatment of inflamed airways, especially in asthma, we developed our model with intranasal administration of the compounds in mice to mimic the inhalation route used in humans.

We first investigated the activity of compound **1** and compared it with that of phosphate **2**, the most soluble compound, after intranasal administration to the airways (2 h before each challenge with OVA or saline on 3 consecutive days) (Figure 3A). We evaluated the activity of **1** and **2** administered at doses of 22 nmol/kg, that is, the dose corresponding to the maximal solubility of **1** in PBS (9 μ M, 25 μ L/mouse). The compounds per se had no effect in the control unsensitized groups. In the OVA-challenged mice, **1** had no effect on inflammatory cell recruitment (Figure 3A). By contrast, the phosphate **2** significantly inhibited OVA-induced eosinophil (56 \pm 9%) and neutrophil recruitment, with a trend toward significance for lymphocytes (Figure 3A). The dose–response activity of phosphate **2** on eosinophil recruitment showed an IC_{50} of 10 nmol/kg (Figure 3B), thereby suggesting that the prodrug provides more efficient distribution of compound **1** in the target tissue. Contrastingly, no effect of **2** was noticed on macrophage recruitment, corroborating results on the activity of anti-CXCR4 or -CXCL12 neutralizing antibodies¹⁸ or CXCR4 antagonist.¹⁹ LC-MS analysis shows that **1** appears in lung tissue as early as 15 min after intranasal administration of **2**, thus revealing its hydrolysis into the active **1**, thereby delivered at high concentrations to the lung.

The activity of the other two prodrugs, the serine **4** and sulfate **5** derivatives, was compared to that of the phosphate **2** in the same airway hyper eosinophilia mouse model. The intranasally administered dose was 30 nmol/kg. The activity levels of **4** and **5** shows similarity to that of **2** with significant reduction of the recruitment of eosinophils (67, 69, and 64% inhibition, respectively) and neutrophils by the three prodrugs and at a lower level for lymphocytes, with no effect on macrophage recruitment (Figure 4). This clearly indicates that all three prodrugs release the same amount of the active species **1** when administered to the airways. This finding is consistent with the rapid release of **1** from the three different prodrugs in lung homogenates in vitro (Table 1), its limited distribution in

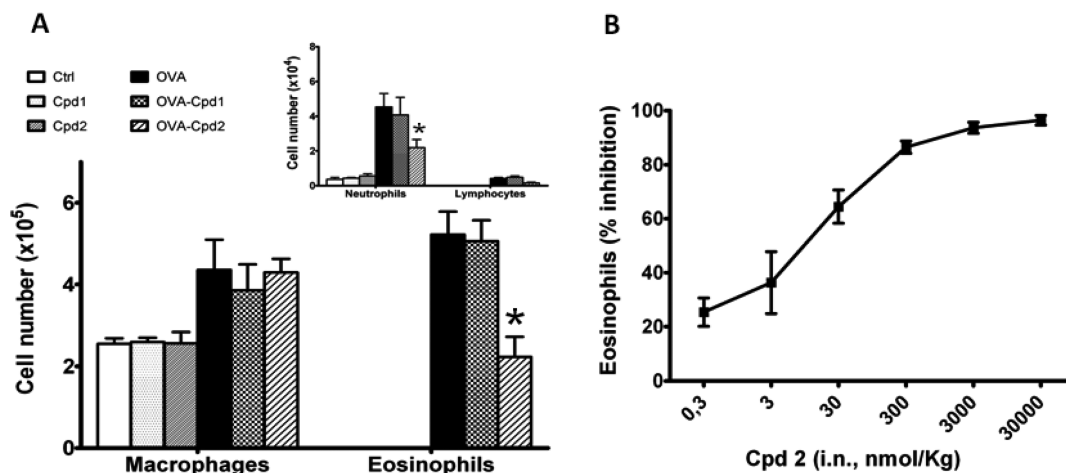


Figure 3. (A) Intranasal treatment with compounds **1** and **2** (22 nmol/kg in PBS) 2 h before each challenge in the 8 day mouse model of hyper eosinophilia. Blocks (means) and bars (SEM) of $n = 6$ mice/group. * $p \leq 0.05$ as compared to saline-treated OVA group. (B) Dose–response inhibition of eosinophil number of compound **2** (percent). Means (filled squares) and bars (SEM) of $n = 6$ mice/group.

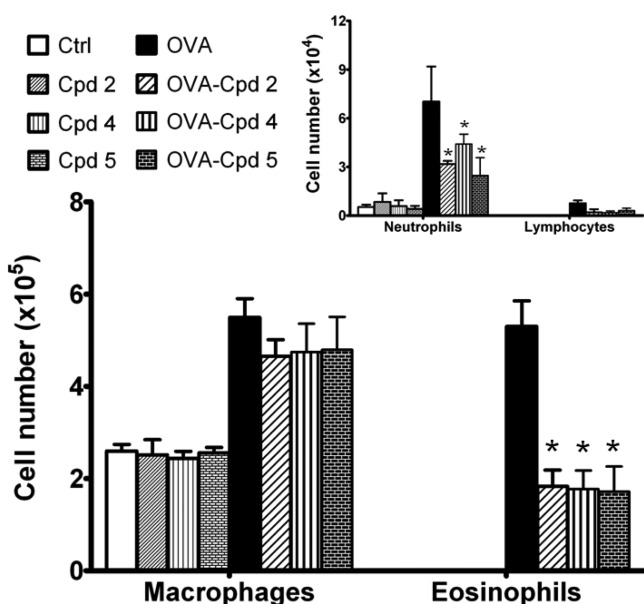


Figure 4. Intranasal administration of compounds 2, 4, and 5 (30 nmol/kg) 2 h before each challenge in a hypereosinophilic mouse model. Absolute numbers of cells in BAL are shown. Means (blocks) and SEM (bars) of $n = 6$ mice/group. * $p \leq 0.05$ as compared to OVA.

vivo, and efficient trapping of the pro-inflammatory chemokine, CXCL12, locally in the inflamed lung tissue.

Three analogues of compound 1, a neutral ligand of the CXC chemokine CXCL12, bearing a phosphate, an L-seryl, and a sulfate moiety, are highly soluble and bioavailable when administered to the airways. They behave as prodrugs, remaining inactive until compound 1 is released. Intranasal application of each of these three analogues inhibits eosinophil recruitment in the airways by $\geq 50\%$ at a dose as low as 30 nmol/kg and without any signs of toxicity. This prodrug strategy, which results in a low-dose application for topical effect, therefore, appears to be a powerful strategy for obtaining highly soluble compounds that act directly in the airways and promote the desired local action while avoiding systemic adverse effects on other CXCL12-related functions.

EXPERIMENTAL PROCEDURES

For (*E*)-4-(3-(4-chlorophenyl)-3-oxoprop-1-enyl)-2-methoxyphenylphosphate, sodium salt 2, under argon atmosphere, POCl₃ (186 μ L, 2 mmol) was dissolved in anhydrous CH₂Cl₂ (8 mL); the mixture was then cooled to 0 °C, and anhydrous triethylamine (700 μ L, 5 mmol) was added. After 5 min of stirring, compound 1 (114 mg, 0.39 mmol dissolved in 1 mL of anhydrous CH₂Cl₂) was added dropwise to the reaction mixture. The resulting mixture was stirred for 1 h at 0 °C and then allowed to warm to room temperature overnight. The crude mixture was then evaporated to dryness under reduced pressure to remove the excess NEt₃ and POCl₃. The residue was dissolved in CH₂Cl₂ and evaporated to dryness again. The procedure was repeated twice. Finally, the residue was dissolved in a tetrahydrofuran–water solution (2/1 v/v, 1.5 mL), and the mixture was vigorously stirred for 1 h. The solution was diluted with water (500 μ L) and cooled down to 0 °C. A 0.1 N, NaOH aqueous solution was added dropwise to reach a pH of 12. After concentration under reduced pressure, compound 5 was isolated by flash chromatography on an RP18 column, with a linear gradient of acetonitrile in water. Following a freeze/drying step, compound 2 was recovered as a yellow solid (74 mg, 45% yield).

For (*R,E*)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)-2-methoxyphenyl-3-(*tert*-butoxy)-2-(*tert*-butyloxycarbonylamino)-propanoate 3, under argon atmosphere, 1 (100 mg, 0.35 mmol) was dissolved in

anhydrous CH₂Cl₂ (1.5 mL), and *N,N*-dimethyl-4-aminopyridine (4-DMAP) (4 mg, 0.034 mmol) was added. To this solution was added a solution of Boc-L-Ser(*Ot*Bu)-OH (136 mg, 0.52 mmol) and EDCI hydrochloride (100 mg, 0.52 mmol) in anhydrous CH₂Cl₂ (1.5 mL), after it was prepared, and stirred for 15 min at room temperature in the dark. The combined mixture was stirred at room temperature in the dark for 1 h, the solvent was removed, and the crude product was purified by chromatography on Si40 silica (3/7 ethyl acetate/heptane). Compound 3 was recovered as a pale yellow oil (170 mg, 92% yield).

For (*R,E*)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)-2-methoxyphenyl 2-amino-3-hydroxypropanoate, hydrochloride salt 4, compound 3 (51 mg, 0.096 mmol) was dissolved in a mixture of dioxane and 4 N HCl aqueous solution (1/1 v/v, 510 μ L). The mixture was heated at 60 °C for 3 h. After completion, the precipitate was recovered by filtration and triturated twice with ether. After it was dried, 4 was obtained as a pale yellow solid (30 mg, 76% yield).

For (*E*)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)-2-methoxyphenyl sulfate, sodium salt 5, under argon atmosphere, 1 (100 mg, 0.35 mmol) was dissolved in anhydrous pyridine (10 mL), and the mixture was cooled to –10 °C. Chlorosulfonic acid (233 μ L, 3.5 mmol) was added dropwise (warning: violent reaction!). The mixture was stirred at room temperature overnight. The solvent was then evaporated under reduced pressure, and the residue was dissolved in water and acidified to pH 2 by addition of 1 N HCl aqueous solution. The mixture was extracted three times with CH₂Cl₂. The combined organic layers were concentrated under reduced pressure. The residue was dissolved in EtOH (1 mL) and alkalinized with 50% NaOH aqueous solution (1 equiv). The solvent was evaporated under reduced pressure at room temperature, and the crude oily residue was purified by flash chromatography on Reverse-Phase 18 column (eluent water-acetonitrile). Compound 5 was recovered as a yellow solid (46 mg, 34% yield).

After mice were exposed for 15 min to intranasally administered compounds 2, 4, and 5, they were killed, and their lungs were collected, homogenized, and solid–liquid extracted with acetonitrile. After 3000g centrifugation, the supernatant was collected, evaporated, and resuspended in 50% water–50% acetonitrile, 0.1% TFA before injection. Analyses were conducted with RP-HPLC, as shown in Figure 1, or LC-MS.

The activity of each compound was assessed in vivo in an 8 day model of hypereosinophilia. Briefly, 9 weeks old male Balb/c mice were sensitized by intraperitoneal injection of 50 μ g of OVA (grade V, Sigma-Aldrich) adsorbed on 2 mg of aluminum hydroxide (Sigma-Aldrich) in 0.1 mL of saline on days 0, 1, and 2. Mice were challenged intranasally [10 μ g of OVA in 25 μ L of saline (12.5 μ L/nostril)] on days 5, 6, and 7. Control mice received intraperitoneal and intranasal administrations of saline alone. Intranasal administrations were performed under anesthesia with intraperitoneal 50 mg/kg ketamine and xylazine at 3.33 mg/kg. Food and water were supplied ad libitum. Animal experimentation was conducted with the approval of the government body that regulates animal research in France. Two hours before each OVA or saline challenge, compound 1 or 2, 4, or 5 in PBS (25 μ L) was administered intranasally.

Bronchoalveolar lavage (BAL) was performed 24 h after the last OVA challenge. Mice were deeply anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. A plastic canula was inserted into the trachea, and airways were lavaged by 10 instillations of 0.5 mL of ice-cold saline supplemented with 2.6 mM ethylenediaminetetraacetic acid (EDTA) (saline-EDTA). BAL fluids were centrifuged (300g, 5 min, 4 °C) to pellet cells, and erythrocytes were lysed by hypotonic shock. Cells were resuspended in 500 μ L of ice-cold saline-EDTA, and total cell counts were determined with a hemocytometer (Neubauer's chamber). Differential cell counts were assessed on cytologic preparations (Cytospin, Shandon Ltd.) (250 000 cells/mL in ice-cold saline-EDTA) stained with Hemacolor (Merck) on counts of at least 400 cells and expressed as absolute numbers or percentage of total cell number.

■ ASSOCIATED CONTENT

■ Supporting Information

Detailed synthesis and full characterization of compounds **1**, **2**, **4**, and **5**, the solubility and stability determinations, and the in vitro binding experiments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +33368854232. Fax: +33368854310. E-mail: mhibert@unistra.fr.

Funding

This work was supported by the French "Ministère de la Recherche", ANR, CNRS, INSERM, Région Alsace, and Strasbourg University.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We warmly thank Stéphanie Riché for her continuous involvement in this work. We thank Pascale Buisine and Patrick Wehrung for MS analyses and Cyril Antheaume for NMR analyses. We thank Pascal Villa for screening.

■ ABBREVIATIONS

BAL, bronchoalveolar lavage; CHI, chromatography hydrophobicity index; 4-DMAP, *N,N*-dimethyl-4-aminopyridine; EDCl, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide; EDTA, ethylenediaminetetraacetic acid; EGFP, enhanced green fluorescent protein; FRET, fluorescence resonance energy transfer; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HRMS, high-resolution mass spectrometry; OVA, ovalbumin; SEM, standard error of the mean; TR, Texas Red; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol

■ REFERENCES

- (1) Fernandez, E. J.; Lolis, E. Structure, function, and inhibition of chemokines. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 469–499.
- (2) Hibert, M. F. French/European academic compound library initiative. *Drug Discovery Today* **2009**, *14*, 723–725.
- (3) Hachet-Haas, M.; Balabanian, K.; Rohmer, F.; Pons, F.; Franchet, C.; Lecat, S.; Chow, K. Y.; Dagher, R.; Gizzi, P.; Didier, B.; Lagane, B.; Kellenberger, E.; Bonnet, D.; Baleux, F.; Haiech, J.; Parmentier, M.; Frossard, N.; Arenzana-Seisdedos, F.; Hibert, M.; Galzi, J. L. Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J. Biol. Chem.* **2008**, *283*, 23189–23199.
- (4) Galzi, J. L.; Hachet-Haas, M.; Bonnet, D.; Daubeuf, F.; Lecat, S.; Hibert, M.; Haiech, J.; Frossard, N. Neutralizing endogenous chemokines with small molecules. Principles and potential therapeutic applications. *Pharmacol. Ther.* **2010**, *126*, 39–55.
- (5) Veldkamp, C. T.; Ziarek, J. J.; Peterson, F. C.; Chen, Y.; Volkman, B. F. Targeting SDF-1/CXCL12 with a ligand that prevents activation of CXCR4 through structure-based drug design. *J. Am. Chem. Soc.* **2010**, *132*, 7242–7243.
- (6) Zhang, W. B.; Navenot, J. M.; Haribabu, B.; Tamamura, H.; Hiramatu, K.; Omagari, A.; Pei, G.; Manfredi, J. P.; Fujii, N.; Broach, J. R.; Peiper, S. C. A point mutation that confers constitutive activity to CXCR4 reveals that T140 is an inverse agonist and that AMD3100 and ALX40–4C are weak partial agonists. *J. Biol. Chem.* **2002**, *277*, 24515–24521.
- (7) Hu, J. S.; Freeman, C. M.; Stolberg, V. R.; Chiu, B. C.; Bridger, G. J.; Fricker, S. P.; Lukacs, N. W.; Chensue, S. W. AMD3465, a novel CXCR4 receptor antagonist, abrogates schistosomal antigen-elicited

(type-2) pulmonary granuloma formation. *Am. J. Pathol.* **2006**, *169*, 424–432.

(8) Hatse, S.; Princen, K.; De Clercq, E.; Rosenkilde, M. M.; Schwartz, T. W.; Hernandez-Abad, P. E.; Skerlj, R. T.; Bridger, G. J.; Schols, D. AMD3465, a monomacrocyclic CXCR4 antagonist and potent HIV entry inhibitor. *Biochem. Pharmacol.* **2005**, *70*, 752–761.

(9) Kumpulainen, H.; Heimbach, T.; Oliyai, R.; Oh, D.; Jarvinen, T.; Savolainen, J. Prodrugs: Design and clinical applications. *Nat. Rev. Drug Discovery* **2008**, *7*, 255–270.

(10) Huttunen, K. M.; Raunio, H.; Rautio, J. Prodrugs—from Serendipity to Rational Design. *Pharmacol. Rev.* **2011**, *63*, 750–771.

(11) Testa, B.; Mayer, J. M. *Hydrolysis in Drug and Prodrug Metabolism Chemistry, Biochemistry and Enzymology*; Verlag Helvetica Chimica Acta and Wiley-VCH: Zurich, 2003; pp 470–474 and 549–590.

(12) Manna, F.; Chimenti, F.; Fioravanti, R.; Bolasco, A.; Secci, D.; Chimenti, P.; Ferlini, C.; Scambia, G. Synthesis of some pyrazole derivatives and preliminary investigation of their affinity binding to P-glycoprotein. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4632–4635.

(13) Silverberg, L. J.; Dillon, J. L.; Vemishetti, P. A simple, rapid and efficient protocol for the selective phosphorylation of phenols with dibenzyl phosphite. *Tetrahedron Lett.* **1996**, *37*, 771–774.

(14) Palanki, M. S. S.; Akiyama, H.; Campochiaro, P.; Cao, J.; Chow, C. P.; Dellamary, L.; Doukas, J.; Fine, R.; Gritzen, C.; Hood, J. D.; Hu, S.; Kachi, S.; Kang, X.; Klebansky, B.; Kousba, A.; Lohse, D.; Mak, C. C.; Martin, M.; McPherson, A.; Pathak, V. P.; Renick, J.; Soll, R.; Umeda, N.; Yee, S.; Yokoi, K.; Zeng, B.; Zhu, H.; Noronha, G. Development of prodrug 4-chloro-3-(5-methyl-3-[[4-(2-pyrrolidin-1-ylethoxy)phenyl]amino]1,2,4-benzotriazin-7-yl)phenyl benzoate (TG100801): A topically administered therapeutic candidate in clinical trials for the treatment of age-related macular degeneration. *J. Med. Chem.* **2008**, *51*, 1546–1559.

(15) Fairley, B.; Botting, N. P.; Cassidy, A. The synthesis of daidzein sulfates. *Tetrahedron* **2003**, *59*, 5407–5410.

(16) Valko, K.; Bevan, C.; Reynolds, D. Chromatographic hydrophobicity index by fast-gradient RP HPLC: A high-throughput alternative to log P log D. *Anal. Chem.* **1997**, *69*, 2022–2029.

(17) Bundgaard, H.; Falch, E.; Jensen, E. A Novel Solution-Stable, Water-Soluble Prodrug Type for Drugs Containing a Hydroxyl or an NH-Acidic Group. *J. Med. Chem.* **1989**, *32*, 2503–2507.

(18) Gonzalo, J.-A.; Lloyd, C. M.; Peled, A.; Delaney, T.; Coyle, A. J.; Gutierrez-Ramos, J.-C. Critical Involvement of the Chemotactic Axis CXCR4/Stromal Cell-Derived Factor-1 α in the Inflammatory Component of Allergic Airway Disease. *J. Immunol.* **2000**, *165*, 499–508.

(19) Lukacs, N. W.; Berlin, A.; Schols, D.; Skerlj, R. T.; Bridger, G. J. AMD3100, a CXCR4 Antagonist, Attenuates Allergic Lung Inflammation and Airway Hyperreactivity. *Am. J. Pathol.* **2002**, *160*, 1353–1360.

An Antedrug of the CXCL12 Neutraligand Blocks Experimental Allergic Asthma without Systemic Effect in Mice

François Daubeuf, Muriel Hachet-Haas, Patrick Gizzi, Vincent Gasparik, Dominique Bonnet, Valérie Utard, Marcel Hibert, Nelly Frossard and Jean-Luc Galzi,

Journal of Biological Chemistry. 2013; 288(17):11865-76

La chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7 sont impliqués dans la croissance cellulaire tumorale, la survie et le recrutement de cellules immunitaires et inflammatoires ainsi que dans l'asthme, mais sont aussi indispensables à l'organogenèse au cours du développement, et ont des rôles importants dans l'homéostasie chez l'adulte. Le traitement de l'asthme à l'aide d'un composé neutralisant le couple CXCR4/CXCL12 doit donc être délivré aussi sélectivement que possible dans les voies aériennes dans le but de réduire les effets indésirables. Afin de promouvoir l'activité locale du neutraligand de CXCL12, et limiter les effets systémiques, nous avons généré un neutraligand de courte durée de vie, dérivé de la chalcone 4 (chalc4), la carbonitrile-chalcone 4 (CN-chalc4).

Nous montrons que la CN-Chalc4 est un neutraligand de CXCL12, par la mesure *in vitro* de l'inhibition du signal FRET entre CXCL12-TR et CXCR4-eGFP, et que la CN-chalc4 est rapidement dégradé dans un homogénat de poumon ($T_{1/2} = 35\text{min}$ vs. $T_{1/2} > 16\text{h}$) en deux produits inactifs: le par-chlorobenzoylacetone nitrile (pBCA) et la vanilline protégée (VanP).

In vivo, nous montrons que le composé CN-chalc4 inhibe, de manière dose-dépendante, le recrutement des éosinophiles dans les voies aériennes, dans notre modèle rapide d'asthme chez la souris, lorsqu'il est administré par voie locale (intranasale). Son activité est maximale à une dose de 300nmol/kg (48% d'inhibition) et similaire à la chalc4. Dans les mêmes conditions, les composés pBCA et VanP restent inactifs

Administrées par voie systémique (intrapéritonéale), le composé chalc4 inhibe le recrutement des éosinophiles (45%), alors que le composé CN-chalc4 est inactif.

L'absence d'effet par voie systémique est due à la dégradation rapide du neutraligand CN-chalc4 en deux fragments inactifs, et peut être défini comme une ante-drogue: un principe actif localement et dégradé en produits inactifs avant leur distribution dans l'organisme. Cette approche permet une activité locale du neutraligand de CXCL12, dans les maladies des voies respiratoires, sans perturber les autres rôles de la protéine cible dans l'organisme.

An Antedrug of the CXCL12 Neutraligand Blocks Experimental Allergic Asthma without Systemic Effect in Mice*

Received for publication, January 3, 2013, and in revised form, February 27, 2013. Published, JBC Papers in Press, February 28, 2013, DOI 10.1074/jbc.M112.449348

François Daubeuf^{†§1,2}, Muriel Hachet-Haas^{§¶1,3}, Patrick Gizzi^{§¶4}, Vincent Gasparik^{†§4}, Dominique Bonnet^{†§}, Valérie Utard^{§¶1}, Marcel Hibert^{†§}, Nelly Frossard^{†§}, and Jean-Luc Galzi^{§¶1,5}

From the [†]Laboratoire d'Innovation Thérapeutique, UMR 7200 CNRS/Université de Strasbourg, Faculté de Pharmacie, 74 Route du Rhin, 67401 Illkirch, France, the [¶]Laboratoire de Biotechnologie et Signalisation Cellulaire, Ecole de Biotechnologie de Strasbourg, UMR 7242 CNRS/Université de Strasbourg, Ecole Supérieure de Biotechnologie, Bd. Sébastien Brant, 67412 Illkirch, France, and the [§]Laboratory of Excellence MEDALIS, Université de Strasbourg, 67400 Illkirch, France

Background: The chemokine CXCL12 and its receptor CXCR4 are widely distributed and contribute to the physiopathology of inflammation.

Results: Recruitment of eosinophils in the inflamed airway is selectively attenuated by short lived antagonists that block CXCL12-mediated activation of CXCR4.

Conclusion: CXCL12/CXCR4 signaling regulates local leukocyte-mediated inflammation.

Significance: Antedrugs of neutraligands allow dissecting the physiological role of chemokines, especially when expression occurs in multiple tissues.

The chemokine receptor CXCR4 and its chemokine CXCL12 are involved in normal tissue patterning but also in tumor cell growth and survival as well as in the recruitment of immune and inflammatory cells, as successfully demonstrated using agents that block either CXCL12 or CXCR4. In order to achieve selectivity in drug action on the CXCR4/CXCL12 pair, in particular in the airways, drugs should be delivered as selectively as possible in the treated tissue and should not diffuse in the systemic circulation, where it may reach undesired organs. To this end, we used a previously unexploited Knoevenagel reaction to create a short lived drug, or soft drug, based on the CXCL12-neutralizing small molecule, chalcone 4, which blocks binding of CXCL12 to CXCR4. We show that the compound, carbonitrile-chalcone 4, blocks the recruitment of eosinophils to the airways in ovalbumin-sensitized and challenged mice *in vivo* when administered directly to the airways by the intranasal route, but not when administered systemically by the intraperitoneal route. We show that the lack of effect at a distant site is due to the rapid degradation of the molecule to inactive fragments. This approach allows selective action of the CXCL12 neutraligands although the target protein is widely distributed in the organism.

Chemokines are small proteins that play critical roles in the development and function of various tissues in vertebrates. In the adult, they regulate the directional migration of leukocytes under normal and pathological conditions. As a rather general rule, chemokines and their G protein-coupled receptors display redundancy and binding promiscuity (*i.e.* several chemokines may bind to the same receptor set) (1), whereas a few chemokines play a pivotal and non-redundant homeostatic role. A singular case is that of the CXCL12/SDF1 chemokine and its receptor CXCR4, which are both conserved during evolution from jawless fish to humans and appear essential during normal embryogenesis and organogenesis (2–4). CXCL12 is constitutively expressed by stromal, epithelial, and endothelial cells in primary lymphoid organs (including bone marrow and thymus) and secondary lymphoid organs, such as spleen and ganglia (5). Disruption of either the *CXCL12* (5) or the *CXCR4* (4) gene is lethal during mouse embryogenesis, illustrating the prominent role of CXCL12 and CXCR4 in the patterning of embryonic tissue formation through progenitor cell migrations. Suppression of CXCL12/X4 interaction upon treatment with granulocyte(-macrophage) colony-stimulating factor (GM-CSF or G-CSF) (6, 7) or with the selective CXCR4 antagonist AMD 3100 promotes neutrophilia (8). In the adult, CXCR4 and CXCL12 maintain stem cell niches in the bone marrow and contribute to the proliferation of hematopoietic progenitors (9, 10).

CXCL12 and CXCR4 are also important players in pathophysiological situations (11–14), including AIDS (15–17), the unusual form of neutropenia reported as WHIM syndrome (18–20), or carcinogenesis (11, 14, 21). In addition, CXCR4 and CXCL12 are also implicated in inflammation. They contribute to promoting transendothelial migration of lymphocytes (22) and invasion of inflamed tissues, as illustrated in the airways of animal models of asthma (23–27), in the pulmonary vasculature in pulmonary arterial hypertension (28), and in fibropro-

* This work was supported by funds from CNRS, the Institut National pour la Santé et la Recherche Médicale, and the Agence Nationale pour la Recherche (ANR).

✂ Author's Choice—Final version full access.

¹ Both authors contributed equally to this work.

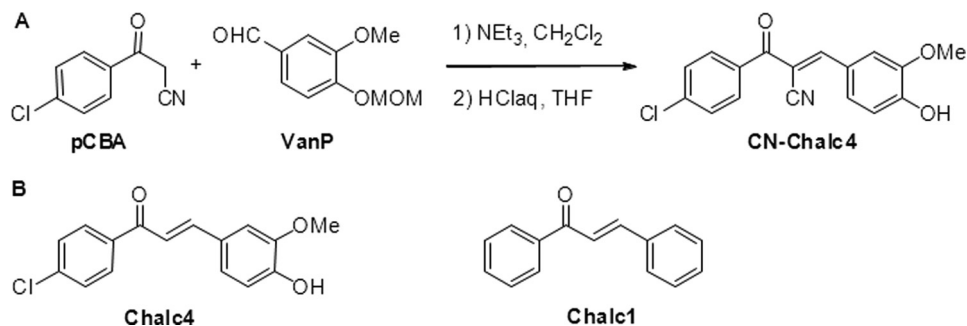
² Supported by a fellowship from the Fonds de Recherche en Santé Respiratoire from Société de Pneumologie de langue française (SPLF) and the Region Alsace.

³ Supported by a fellowship from ANR.

⁴ Supported by a fellowship from CNRS.

⁵ To whom correspondence should be addressed. Tel.: 33-3-6-68-02-84-21; E-mail: galzi@unistra.fr.

Antedrug of the CXCL12 Neutraligand and Asthma



SCHEME 1. A, synthesis of CN-chalcone 4 (CN-Chalc4) following a Knoevenagel condensation of pCBA with 3-methoxy-4-(methoxymethoxy)benzaldehyde (VanP). B, structure of reference compounds chalcone 4 (Chalc 4) and unsubstituted chalcone (Chalc 1).

liferative tissue in a murine model of obliterative bronchiolitis after heterotopic tracheal transplantation (29).

CXCL12 and CXCR4 were long thought to be the exclusive interactors of each other until the recent discovery that the orphan G protein-coupled receptor, CXCR7, also binds CXCL12 as well as CXCL11 (30, 31). CXCR7 is expressed by endothelial cells and cardiomyocytes and is essential in heart development (32, 33). CXCR7 does not elicit clear responses to CXCL12 but clearly associates with the CXCR4 protein to modulate its sensitivity for CXCL12 (33, 34).

The physiological and pathophysiological importance of CXCL12, CXCR4, and CXCR7 has prompted the launching of drug discovery programs aiming at blocking HIV entry, inhibiting cancer cell proliferation, or reducing inflammatory responses. The most advanced compound is the CXCR4 antagonist AMD 3100, which has been approved for treatment of lymphoproliferative disorders (Plerixafor®). It displays efficacy in humans in mobilizing CXCR4⁺ progenitor cells (10, 35–38) upon acute administration. Use of AMD 3100 is currently being evaluated for other therapeutic indications, such as glioblastoma and the WHIM syndrome (39, 40). It is, however, endowed with side effects, mainly cardiotoxicity (41), which is an expected problem if one considers the multiplicity of tissues expressing CXCR4 as well as the variety of diseases in which CXCR4 is implicated. According to a recent report (42), AMD 3100 is presumed to act as an agonist of the CXCR7 receptor, a property that might account for potential secondary effects of AMD 3100.

An alternative strategy consists in preventing the agonist-receptor interaction by neutralizing the endogenous ligands. In this context, we have identified a compound that belongs to this category of pharmacological agents (*i.e.* a small neutralizing compound binding to CXCL12), chalcone 4 (Scheme 1), which prevents CXCL12 binding either to CXCR4 or CXCR7 (21, 23, 24, 26, 43, 44). Chalcone 4 blocks responses of CXCR4 to CXCL12 *in vitro* without affecting the basal level receptor activity and displays anti-inflammatory effects in a murine model of asthma *in vivo*.

In order to favor desired local anti-inflammatory action of the neutraligand at the expense of undesired distant effects, we explored the possibility of generating a short lived neutraligand prone to efficient biodegradation before it distributes in the body and reaches unwanted tissues. To this end, we describe here the synthesis of a molecule using previously unexploited Knoevenagel/retro-Knoevenagel reactions to generate so called

soft drugs or antedugs (45). Characterization of the functional properties of the new short lived carbonitrile-chalcone, *in vitro* and *in vivo*, shows that it is active when administered locally and inactive after systemic administration.

EXPERIMENTAL PROCEDURES

Chemistry—Reagents were obtained from commercial sources and used without any further purification. Thin-layer chromatography was performed on silica gel 60F₂₅₄ plates. Flash chromatography was performed on silica gel (puriFlash® 30 μm, Interchim) or C18 (puriFlash® 30 μm, Interchim) pre-packed columns on a SpotII Ultima from Armen. NMR spectra were recorded on a Bruker AV400 spectrometer. Chemical shifts (δ) are reported in ppm, and coupling constants (*J*) are expressed in Hz. Analytical HPLC analyses were performed on an Eclipse XBD-C18 column (5 μm, 46 × 150 mm; Agilent) using the following conditions: flow rate, 1 ml/min; Solvent A, 0.1% aqueous TFA; Solvent B, 0.1% TFA in CH₃CN; gradient, 5–100% B developed over 15 min; detection at 220/254/365 nm. Retention times (*t_R*) from analytical reverse phase HPLC are reported in min. LC/MS spectra were obtained on an Agilent HPLC single quadrupole spectrometer (1200RRLC/1956b-SL) equipped with a THERMO Hypersyl column (1.9 μm, 1 × 30 mm) using an Agilent Multimode ion source. High resolution mass spectrometry spectra were obtained on an Accurate-Mass Q-ToF spectrometer from Agilent using electrospray ionization. For (*E*)-2-(4-chlorobenzoyl)-3-(4-hydroxy-3-methoxyphenyl)acrylonitrile (CN-chalcone 4)⁶, *p*-chloro-benzoyl-acetonitrile (1 g, 5.606 mmol) and 3-methoxy-4-(methoxymethoxy) benzaldehyde (1.1 g, 5.606 mmol) were dissolved in dry CH₂Cl₂ (15 ml). Dry NEt₃ (78 μl, 0.56 mmol) and pulverized activated 4-Å molecular sieves (1 g) were added. The mixture was stirred at room temperature, monitoring the progress of the reaction by TLC. After 20 h, the molecular sieve was filtered off, and the organic layer was concentrated *in vacuo* to dryness. The residual crude orange solid was recrystallized from aqueous EtOH to afford (*E*)-2-(4-chlorobenzoyl)-3-(3-methoxy-4-(methoxymethoxy)phenyl) acrylonitrile as a yellow solid (1.01 g, 50% yield). *R_f* = 0.38 (heptane-ethyl acetate: 7–3); mp = 140–1 °C; ¹H NMR (CDCl₃): *d* 3.51 (s, 3H), 3.96 (s, 3H), 5.32 (s, 2H), 7.23 (d,

⁶ The abbreviations used are: CN-chalcone 4, carbonitrile-chalcone 4; BALF, bronchoalveolar lavage fluid; BRET, bioluminescence resonance energy transfer; HP-βCD, hydroxypropyl-β-cyclodextrin; OVA, ovalbumin; TR, Texas Red; pCBA, *para*-chlorobenzoylacetonitrile.

$J = 8.6$ Hz, 1H), 7.43 (dd, $J = 8.6, 2.1$ Hz, 1H), 7.48 (d, $J = 8.5$ Hz, 2H), 7.83 (d, $J = 8.5$ Hz, 2H), 7.88 (d, $J = 2.1$ Hz, 1H), 8.00 (s, 1H); ^{13}C NMR (CDCl_3): δ 55.9, 56.3, 95.2, 106.5, 112.8, 116.2, 117.9, 125.7, 128.3, 129.1, 130.8, 134.8, 139.8, 150.3, 151.8, 156.1, 188.1.

(*E*)-2-(4-Chlorobenzoyl)-3-(3-methoxy-4-(methoxymethoxy)phenyl)acrylonitrile was dissolved in THF (10 ml) in the presence of a 1 N HCl aqueous solution (5 eq, 14 ml). The resulting mixture was stirred at room temperature, monitoring the progress of the conversion by HPLC. After 12 h, the solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 and washed with water until neutralization. The solvent was removed, and deprotected CN-chalcone 4 was recovered as a yellow solid in quantitative yield (886 mg). $R_f = 0.27$ (heptane-ethyl acetate: 8–2); mp = 162–3 °C (recrystallized from aqueous EtOH); ^1H NMR (CDCl_3): δ 3.98 (s, 3H), 6.26 (br s, 1H), 7.01 (d, $J = 8.3$ Hz, 1H), 7.41 (dd, $J = 8.3, 2.1$ Hz, 1H), 7.48 (d, $J = 8.6$ Hz, 2H), 7.82 (d, $J = 8.6$ Hz, 2H), 7.91 (d, $J = 2.1$ Hz, 1H), 8.00 (s, 1H); ^{13}C NMR (CDCl_3): δ 56.4, 105.9, 111.5, 115.3, 118.1, 124.7, 129.1, 129.5, 130.7, 134.6, 139.6, 147.0, 151.4, 156.0, 188.0. reverse phase HPLC purity >97%; high resolution mass spectrometry calcd. for $\text{C}_{17}\text{H}_{13}\text{ClNO}_3$ 314.0506; found: 314.0518 ($\text{M} + \text{H}$)⁺. Chalcone 1 and chalcone 4 were provided by the French national chemical library.

In Vitro and in Vivo Experiments—Chalcone stock solutions were prepared in sterile DMSO and then stored at –20 °C until use. The human chemokines CXCL12 and CXCL12-TR were purchased from Almac. The cAMP biosensor CAMYEL was kindly provided by Dr. Lily Jiang (Dallas, TX).

Determination of Solubility and Stability—Solubility and stability studies were performed on a Gilson HPLC system with a photodiode array detector, an autosampler, and a Valco injector. Data acquisition and processing were performed with Trilution LC version 2.0 software. Measurements were carried out at 21 ± 1 °C. A 2.6- μm Kinetex column (50 \times 4.6 mm) was used for stability studies in biological media, and a 5- μm Luna C18(2) column (50 \times 4.6 mm) was used for solubility and chemical stability analysis. Both columns were purchased from Phenomenex. The injection volume was 20 μl , the mobile phase flow rate was 2 ml/min, and the following program was applied for the elution: 0–0.2 min, 0% B; 0.2–2.7 min, 0–100% B; 2.7–3.2 min, 100% B; 3.2–3.4 min, 100–0% B; 3.4–6.1 min, 0% B. Solvent B was HPLC grade acetonitrile (Sigma-Aldrich CHROMASOLV). The aqueous solvent contained 0.1% trifluoroacetic acid, and the detection wavelength was 365 nm.

Solubility—Thermodynamic solubility was measured by dissolving the compounds up to saturation in a pH 7.4 phosphate-buffered saline (PBS) with the following composition: 137.5 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , and 1.4 mM KH_2PO_4 supplemented or not with 10% hydroxypropyl- β -cyclodextrin (HP- β CD). Samples were shaken for 24 h at 21 ± 1 °C. Saturation was confirmed by the presence of undissolved powder. After ultracentrifugation, the concentration in the supernatant was measured by an HPLC procedure using a calibration curve established for each compound by diluting a 10 mM DMSO stock solution to adapted concentrations. Due to rapid degradation of CN-chalcone 4, solubility was determined after 2 h of

shaking. The indicated value given for CN-chalcone 4 is thus an estimate of the solubility.

Chemical Stability—Stability of compounds was assessed in PBS, pH 7.4, with or without 10% HP- β CD at 20 °C up to 24 h. For each compound, the 10 mM DMSO stock solution was diluted to a final incubation concentration of 10 μM with 0.1% DMSO. 20 μl of sample were removed at t_0 , 1, 2, 4, 6, 8, 10, and 24 h and directly injected onto the HPLC. The percentage of remaining test compound relative to t_0 was measured by monitoring the peak area on the chromatogram.

Stability in Mouse Serum—Stability of chalcones was determined in mouse serum with or without 10% HP- β CD at 37 °C up to 16 h. For each compound, the 10 mM DMSO stock solution was diluted in serum to a final concentration of 20 μM with 1% DMSO. For the measurements with HP- β -cyclodextrin, a solution of PBS (pH 7.4) containing 10% (v/w) HP- β CD was saturated with compound powder. The saturated solution was then diluted in murine serum to a final compound concentration of 20 μM . The mixture was divided into five aliquots. The incubation of each aliquot was stopped at t_0 and 30 min, 1, 2, and 16 h for chalcone 4 and at t_0 and 15, 30, 45, and 60 min for CN-chalcone 4 by adding one volume of ice cold acetonitrile. Samples were stirred for 3 min, sonicated for 3 min, and then centrifuged at 4 °C before HPLC injection. The percentage of remaining test compound relative to t_0 was measured by monitoring the peak area on the chromatogram.

Stability in Lung Homogenate—Stability of chalcones was determined in mouse lung homogenate with or without HP- β CD 10% at 37 °C. Lung homogenate was prepared separately with a Fastprep® (Q-BIOgene, Illkirch, France) in PBS (one lung homogenized in 1 ml of buffer). For each compound, the incubation solutions, the sampling, and the extraction conditions were prepared as described under “Stability in Mouse Serum.”

Cell Culture—Human embryonic kidney 293 cells expressing the fusion receptor EGFP-hCXCR4 (stable cell lines (26)) were cultured to ~80% confluence in 75-cm² flasks in minimum Eagle's medium with Earle's salt supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% antibiotics (penicillin/streptomycin) and replated twice a week. HepG2 cells were grown to ~80% confluence in 75-cm² flasks in MEM with Earle's salt supplemented with 10% fetal calf serum, 2 mM glutamine, 1% antibiotics (penicillin/streptomycin), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen) and replated twice a week.

Binding Experiments—FRET-based binding experiments were carried out as described (26, 46). Human embryonic kidney 293 cells expressing the fusion receptor EGFP-hCXCR4 were harvested in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4 , pH 7.4) supplemented with 5 mM EDTA, pH 7.4, centrifuged, and resuspended in HEPES-bovine serum albumin (BSA) buffer (10 mM HEPES, 137.5 mM NaCl, 1.25 mM MgCl_2 , 1.25 mM CaCl_2 , 6 mM KCl, 10 mM glucose, 0.4 mM NaH_2PO_4 , 0.1% bovine serum albumin (w/v), pH 7.4). Cells were used at a concentration of 10^6 cells/ml. Apparent affinities of the different ligands were determined by real-time fluorescence monitoring ligand-receptor interactions. Time-based recording of binding was initiated by adding 100

Antedrug of the CXCL12 Neutraligand and Asthma

nM CXCL12-TR (Texas Red-labeled CXCL12) to the 0.5-ml cell suspension. Fluorescence emitted at 510 nm (excitation at 470 nm) was recorded at 21 °C using a Fluorolog 2 spectrofluorimeter (SPEX) and sampled every 0.3 s. Binding of CXCL12-TR to EGFP-labeled CXCR4 was detected as a reversible decline of emission at 510 nm, due to energy transfer from excited EGFP to TR. For competition experiments, the fluorescent chemokine was preincubated for 1 h at room temperature with or without various concentrations of each chalcone. Then the pre-mix was added, and fluorescence was recorded until equilibrium was reached (300 s). Data were analyzed using Kaleidagraph 3.08 software (Synergy Software, Reading, PA).

cAMP Determination—CXCR4 receptor coupling to adenylyl cyclase was assessed by measuring the dose-dependent inhibitory effects of chemokine with or without chalcone on forskolin-stimulated cAMP accumulation. To facilitate studies of cAMP regulation, we used a developed bioluminescence resonance energy transfer (BRET) sensor for cAMP, CAMYEL (cAMP sensor using YFP-Epac-RLuc), which can quantitatively and rapidly monitor intracellular concentrations of cAMP in cells (47). HEK EGFP-hCXCR4 cells were cultured to ~80% confluence in 75-cm² flasks in MEM with Earle's salt supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% antibiotics (penicillin/streptomycin). Cells were plated on 10-cm plates and transfected with 10 µg of CAMYEL plasmid using the calcium phosphate precipitation method. Two days later, cells were plated in 96-well solid white tissue culture plates (Greiner) at a density of 60,000 cells/well the day before cAMP assay. Cells were serum-starved in Hanks' balanced salt solution, pH 7.4 (Sigma-Aldrich) for 30 min at 37 °C before treatments (80 µl/well). The BRET assay was carried out with a Victor Light plate reader (PerkinElmer Life Sciences). Emission signals from *Renilla* luciferase and YFP were measured sequentially using a BRET1 filter set (475-30/535-30). Stimulations were initiated by injection of 20 µl of 5× concentrated ligands prepared in Hanks' balanced salt solution supplemented with 500 mM isobutylmethylxanthine. After 5 min, 10 µl of a Hanks' balanced salt solution of coelenterazine-H (2 µM) were added, and plates were incubated for 10 min at 37 °C before reading.

Cytotoxicity—The capacity of the cells to carry out reduction reactions after drug treatment was estimated with the fluorescent Alamar Blue reagent (AbD Serotec, Oxford, UK). Proliferating cells cause the change from the oxidized blue and non-fluorescent Alamar Blue form (resazurin) to a reduced, pink, highly fluorescent form (resorufin) that can be detected using fluorescence monitoring (excitation 560 nm; emission 590 nm). HepG2 or HEK 293 cells were seeded at a density of 30,000 cells/well on a 96-well plate and allowed to settle overnight before treatment. The dye was added to the cells together with the test substances (5-fold concentrated stock solution prepared in culture medium or in PBS with 10% HP-βCD), and the fluorescence was measured after 24-h incubation, using a Flex Station (Molecular Devices).

Murine Model of Hypereosinophilia—The *in vivo* activity of the compounds was assessed in an 8-day model of hypereosinophilia created by immunization and challenge to ovalbumin in mice. Briefly, 9-week-old male BALB/c mice were sensitized by intraperitoneal injection of a mixture of 50 µg of ovalbumin

(OVA, grade V; Sigma-Aldrich) adsorbed on aluminum hydroxide (2 mg; Sigma-Aldrich) in 0.1 ml of saline on days 0, 1, and 2. Mice were challenged intranasally with 10 µg of OVA in 25 µl of saline (12.5 µl/nostril) on days 5, 6, and 7. Control mice received intraperitoneal and intranasal administrations of saline alone. Intranasal administrations were performed under anesthesia with 50 mg/kg ketamine and 3.33 mg/kg xylazine given intraperitoneally. Food and water were supplied *ad libitum*. Animal experimentation was conducted with the approval of the government body that regulates animal research in France. The chalcone compounds were administered either by the intraperitoneal or intranasal routes. In a first set of experiments, mice received either chalcone 4 or carbonitrile-chalcone 4 solubilized in PBS with 10% HP-βCD (C0926, Sigma) by intranasal injection 2 h before each nasal OVA or saline challenge. In a second set of experiments, mice received CN-chalcone 4, vanillin, or parachlorobenzoylacetonitrile intranasally, in PBS with HP-βCD 10%, 2 h before each OVA or saline challenge. In the last set of experiments, mice received chalcone 4 or CN-chalcone 4 in suspension in carboxymethylcellulose 1% or solubilized in PBS with HP-βCD 10% and administered by intraperitoneal injection 2 h before each OVA or saline challenge. Collection of bronchoalveolar lavage fluid (BALF) was performed 24 h after the last OVA challenge. Mice were deeply anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. A plastic canula was inserted into the trachea, and airways were lavaged by 10 instillations of 0.5 ml of ice-cold saline supplemented with 2.6 mM EDTA (saline-EDTA). Total and differential cell counts in the BALF were performed after centrifugation (300 × *g* for 5 min at 4 °C) to pellet cells. Erythrocytes were lysed by hypotonic shock by the addition of 1.5 ml of distilled H₂O, followed by the addition of 0.5 ml of 0.6 M KCl. Cells were centrifuged and resuspended in 500 µl of ice-cold saline-EDTA, and total cell counts were determined using a hemocytometer (Neubauer's chamber). Differential cell counts were assessed on cytologic preparation obtained by cytocentrifugation (Cytospin 3, Shandon Ltd.) of 200 µl of diluted BALF (250,000 cells/ml in ice-cold saline-EDTA). Slides were stained with Hemacolor (Merck), and counts were performed on at least 400 cells for each preparation. Differential counts were expressed as absolute numbers or as a percentage of the total number of cells.

Cytochrome *c* Oxidase Activity—Lung and heart were homogenized separately with an UltraTurax® (IMLAB, Lille, France) in PBS, pH 7.4 (one organ in 1 ml of buffer). Lung homogenate was diluted at 1:2 and heart homogenate at 1:5 in PBS. 100 µl of each diluted homogenate were placed in a microplate, and 100 µl of assay reagent (10 mM tetramethyl-*p*-phenylenediamine, 2 mM sodium ascorbate, 20 mM KH₂PO₄) were added. The microplate was placed immediately in a microplate spectrophotometer reader, and optical density kinetic ($\lambda = 610$ nm) was measured every 20 s for 30 min. Results are expressed as $V_{\max}/100 \mu\text{g}$ of protein (OD/min/100 µg of protein).

RESULTS

Carbonitrile-chalcone 4 Neutralizes CXCL12 *In Vitro* and *In Vivo*—CN-chalcone 4 was prepared by condensing *para*-chlorobenzoyl-acetonitrile (pCBA) with protected vanillin (*VanP*)

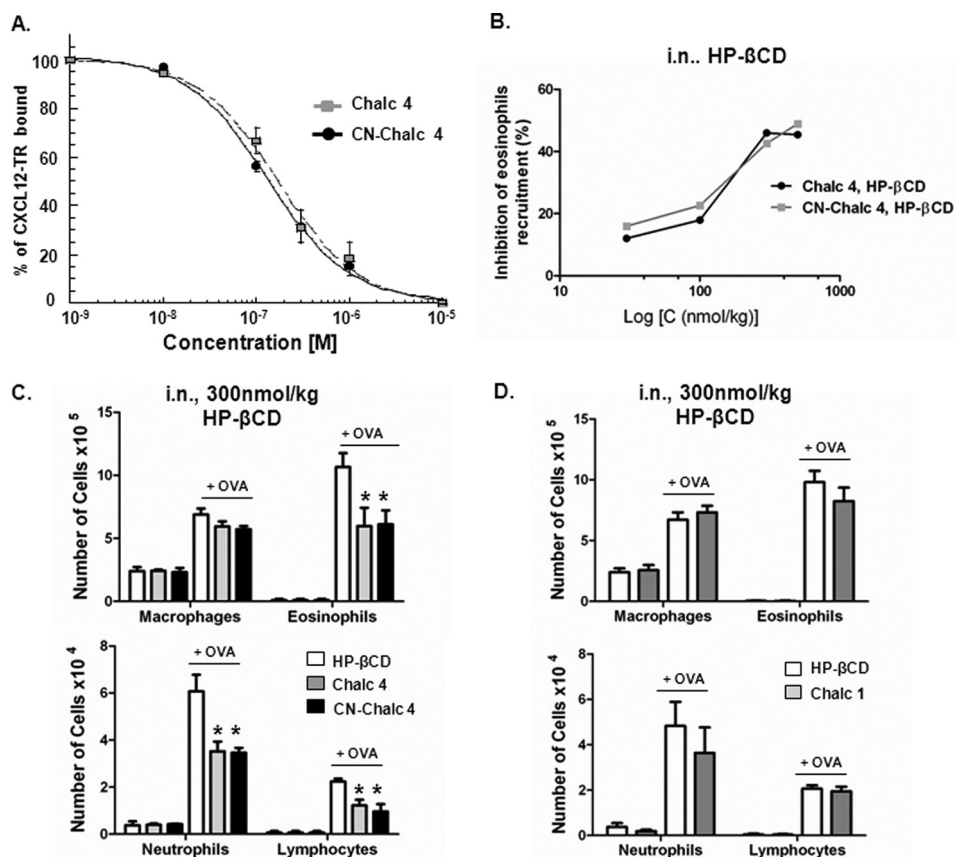


FIGURE 1. Carbonitrile-chalcone reduces inflammation in a mouse model of allergic eosinophilic airway inflammation. *A*, *in vitro* inhibition of CXCL12 binding to CXCR4 receptor by chalcone 4 (*Chalc 4*) and CN-chalcone 4 (*CN-Chalc 4*). Inhibition of CXCL12 binding to CXCR4 as a function of increasing concentration of chalcone 4 (gray squares) and CN-chalcone 4 (black circles) is monitored using FRET intensity variation. The fluorescence of cells expressing EGFP-labeled CXCR4 is followed at 510 nm as a function of time. Upon the addition of Texas Red-labeled CXCL12 (CXCL12-TR, 100 nM), fluorescence intensity at 510 nm declines as a result of interaction between CXCL12-TR and EGFP-CXCR4, which causes FRET. The ordinate axis reports the intensity of FRET as a percentage of the control value (100 nM CXCL12-TR alone). *K_i* values were derived from the IC₅₀ values determined from competition curves using the Cheng and Prusoff relationship (65). *K_i* values are 53 ± 31 nM (chalcone 4) and 45 ± 57 nM (CN-chalcone 4). Each data point represents the mean ± S.D. (error bars) of three experiments. *B*, *in vivo* dose-response effect of a topical treatment with chalcone 4 and CN-chalcone 4 in an 8-day mouse model of hyper-eosinophilia. BALB/c mice were sensitized and challenged with OVA or saline. Chalcone 4 (gray line) or CN-chalcone 4 (black line) solubilized in 10% HP-βCD were administered intranasally 2 h before each challenge. The percentage of inhibition of eosinophil is shown. Data points (squares) are means of *n* = 6 determinations. *C* and *D*, effect of topical (intranasal; *i.n.*) treatment with chalcone 4, CN-chalcone 4 (*C*), and chalcone 1 (*Chalc 1*) (*D*) in the 8-day mouse model of hyper-eosinophilia. BALB/c mice were sensitized and challenged with OVA or saline. Drugs (300 nmol/kg) were administered intranasally 2 h before each challenge in HP-βCD 10% (vehicle). Absolute numbers of macrophages, eosinophils, neutrophils, and lymphocytes in BALF are shown. Bars represent means, and error bars show S.E. values (*n* = 6/group). *, *p* ≤ 0.05 in comparison with the saline-treated OVA group.

as described in Scheme 1. CN-chalcone 4 is the homolog of chalcone 4, formerly described as a ligand of CXCL12, that inhibits chemokine binding to both CXCR4 and CXCR7 receptors (21, 23, 26). CN-chalcone 4 prevents binding of Texas Red-labeled CXCL12 (CXCL12-TR, 100 nM) to EGFP-tagged CXCR4, determined by fluorescence resonance energy transfer (26) (Fig. 1A) with similar affinity as chalcone 4 (*K_i* = 45 ± 57 nM for chalcone 4 *versus* *K_i* = 53 ± 31 nM for CN-chalcone 4). This inhibition of CXCL12 binding to CXCR4 consequently blocks CXCL12-evoked CXCR4 cellular signaling (inhibition of cAMP production, shown in Fig. 4B) and trafficking (data not shown).

The *in vivo* activity of CN-chalcone 4 was assayed in a recently developed 8-day mouse model of airway hyper-eosinophilia (24). In this model, mice are sensitized by intraperitoneal injection of OVA (50 μg) adsorbed on 2 mg of aluminum hydroxide in 0.1 ml of saline on days 0, 1, and 2. Mice are then challenged intranasally with 10 μg of OVA in 25 μl of saline (12.5 μl/nostril) on days 5, 6, and 7. Drugs to be tested can be

administered either systemically by the intraperitoneal route or locally by the intranasal route. The intranasal route mimics the inhalation exposure used in humans, which is the preferred and well accepted administration for inflamed airway treatment, in particular asthma. Thus, unless otherwise stated, drugs were administered intranasally in this study. Due to limited solubility of chalcone 4 (9 ± 1 μM) and CN-chalcone 4 (16 ± 2 μM) in saline buffer, drugs were dissolved in physiological solutions complemented with HP-βCD (10%, w/w). Under such conditions, chalcone 4 and CN-chalcone 4 could be dissolved at maximal concentrations reaching 690 ± 44 and 493 ± 36 μM, respectively.

Administering these solutions at 25 μl/mouse intranasally allows a maximal dose approximating 300–500 nmol/kg. Eosinophil counts in BALF are dose-dependently inhibited by both chalcone 4 and CN-chalcone 4 up to 50% at doses of 300–500 nmol/kg, which can be reached as limits of drug solubility (Fig. 1B).

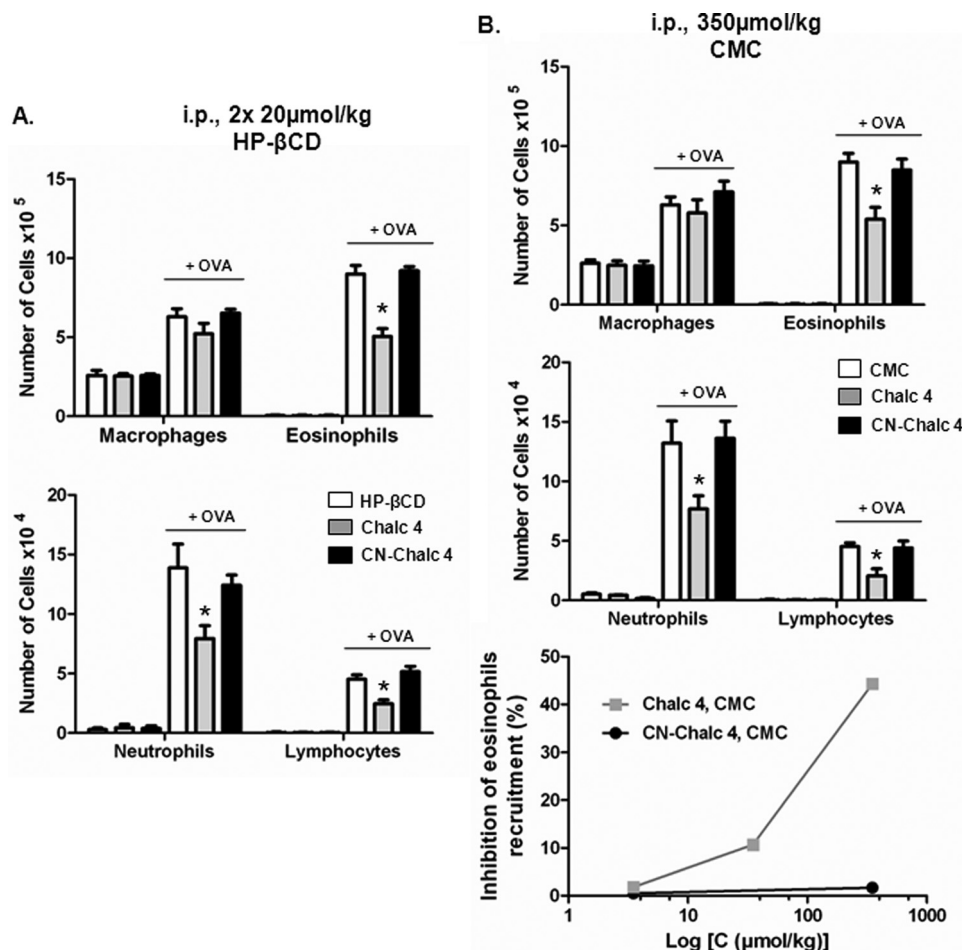


FIGURE 2. **CN-chalcone 4 is an antedrug.** Systemic effect of chalcone 4 and CN-chalcone 4 in two vehicles. Systemic (intraperitoneal (*i.p.*)) treatment with chalcone 4 (*Chalc 4*) and CN-chalcone 4 (*CN-Chalc 4*) in the 8-day mouse model of hypereosinophilia is shown. BALB/c mice were sensitized and challenged with OVA or saline. Drugs (two administrations of 20 μmol/kg/day for 3 days in 10% HP-βCD (vehicle) (A) or 350 μmol/kg once a day for 3 days in 1% carboxymethylcellulose (CMC) as a vehicle (B)) were administered intraperitoneally 2 h before each OVA challenge. Absolute numbers of macrophages, eosinophils (top), neutrophils, and lymphocytes (middle) in BALF are shown. Bars show means, and error bars show S.E. values ($n = 6/\text{group}$). *, $p \leq 0.05$ in comparison with the saline-treated OVA group. Dose intensity relationship of eosinophil recruitment in the intraperitoneal route is shown for chalcone 4 and CN-chalcone 4 up to the maximal dose (bottom).

BALF infiltrate with macrophages, eosinophils, neutrophils, and lymphocytes was determined under treatment with intranasal chalcone 4 and CN-chalcone 4 at 300 nmol/kg. Fig. 1C shows that neither vehicle (10% HP-βCD) nor any of the chalcones by themselves elicit any cell recruitment in the airways. After ovalbumin challenge, a significant increase in the number of eosinophils and macrophages occurs (Fig. 1C, top). Polymorphonuclear neutrophils and lymphocytes were also significantly present in BALFs after OVA challenge, albeit at ~10–20-fold lower levels than eosinophils (Fig. 1C, bottom). Significant reduction of eosinophil recruitment is noted with chalcone 4 administered intranasally (300 nmol/kg). In addition, we note that the new molecule CN-chalcone 4 is as potent as chalcone 4.

In order to further document the specificity of chalcone 4 and CN-chalcone 4 action, we tested the activity of the unsubstituted chalcone backbone (*chalc 1* in Scheme 1) already reported as inactive upon CXCL12 binding to CXCR4 (26). Fig. 1D shows that chalcone 1 does not promote any inflammatory response *per se*; nor does it significantly attenuate eosinophil or any other inflammatory cell recruitment in the airways.

These results therefore indicate that the inactive chalcone chemotype can be substituted by functional groups to inhibit CXCL12 binding to CXCR4 to become active as an attenuator of allergen-induced inflammatory responses. CN-chalcone 4 is as active as chalcone 4 to neutralize CXCL12 *in vitro* and to inhibit eosinophilic airway inflammation *in vivo*. This shows that the introduction of the carbonitrile group in chalcone 4 does not affect the activity and potency of the compound.

CN-chalcone 4 Is Active Locally but Not Systemically—We then compared the *in vivo* activity of CN-chalcone 4 administered systemically by the intraperitoneal route with that of chalcone 4 (Fig. 2). Chalcone 4 and CN-chalcone 4 were solubilized in HP-βCD (10%), or in carboxymethylcellulose (CMC), allowing administrations either of $2 \times 20 \mu\text{mol/kg}$ (Fig. 2A) or of a 350 μmol/kg (Fig. 2B) dose of compound, respectively. Chalcone 4 and CN-chalcone 4 could thus be administered intraperitoneally at doses 40- or 700-fold higher than those used for intranasal administration. These doses correspond to the maximal possible dose because the solubility of chalcones is limited by the concentration of vehicle (carboxymethylcellulose or HP-βCD) and because higher doses of vehicle lead to toxic

effects on animals. Fig. 2 displays results of inflammatory cell recruitment in BALF of OVA-sensitized mice receiving the compounds or vehicles.

Fig. 2A shows the effect of twice daily treatment with 20 $\mu\text{mol/kg}$ chalcone 4 or CN-chalcone 4 in 10% HP- βCD administered intraperitoneally. Neither HP- βCD alone nor any of the two drugs had any effect on inflammatory cell recruitment (macrophages, eosinophils, neutrophils, or lymphocytes) in the naive airways. When administered 1 h before the challenging doses of OVA, chalcone 4 shows anti-inflammatory properties by significantly reducing eosinophils, neutrophils, and T cell counts in BALF (Fig. 2A). By contrast, CN-chalcone 4 did not affect any inflammatory cell counts. Repeating the experiment with carboxymethylcellulose as the excipient allowed administration at doses as high as 350 $\mu\text{mol/kg}$ (Fig. 2B). Again, only chalcone 4 exhibits dose-dependent anti-inflammatory activity in the airways with significant inhibition of macrophage recruitment in addition to inhibition of eosinophil, neutrophil, and lymphocyte influx. These experiments reveal that, in contrast to chalcone 4, CN-chalcone 4 is inactive at inhibiting airway inflammation *in vivo* when administered at a distance from the airways. Therefore, introducing a carbonitrile group in chalcone 4 affects its distribution or metabolism.

CN-chalcone 4 Is Rapidly Degraded in Biological Media—One major effect of the introduction of the carbonitrile group on chalcone 4 is detected on the stability of CN-chalcone 4 in biological media. Compound stability was assessed using HPLC detection (Fig. 3) after incubation in various media. In contrast to chalcone 4, which is stable for hours in buffer and tissue homogenates (Table 1), CN-chalcone 4 is rapidly degraded in phosphate-buffered saline ($t_{1/2} = 6$ h) and even more rapidly in murine serum ($t_{1/2} = 20$ min) or lung homogenate ($t_{1/2} = 25$ min). 10% HP- βCD not only solubilizes the molecule but also significantly improves its stability. CN-chalcone 4 half-life reaches 40 min in lung homogenate in the presence of HP- βCD . Whatever the experimental condition, the half-life of CN-chalcone 4 is significantly shorter than that of chalcone 4, possibly accounting for the lack of effect when using the intraperitoneal route rather than the intranasal administration.

Because the carbonitrile group is an electron-attracting group, its presence on CN-chalcone 4 facilitates nucleophilic attack by water molecules, a reaction that ultimately leads to the hydrolysis of the molecule and production of pCBA and vanillin (*Van*) as shown in Scheme 2. This is indeed observed on reverse-phase HPLC chromatograms (Fig. 3B), showing that hydrolysis of CN-chalcone 4 is accompanied by the concomitant appearance of its two constituents, which represent the major degradation products in biological media (Fig. 3C).

Because CN-chalcone 4 degradation occurs rapidly, we investigated the biological activity of its degradation products with regard to CXCL12 neutralization, cell signaling, and *in vivo* inhibition of eosinophil recruitment in the airways. Fig. 4A shows that CN-chalcone 4 dose-dependently prevents CXCL12 binding to CXCR4 with maximal inhibition beyond 1 μM . Neither vanillin nor pCBA exhibits any binding-neutralizing activity at concentrations up to 10 μM , indicating that binding inhibition is indeed due to CN-chalcone 4 itself.

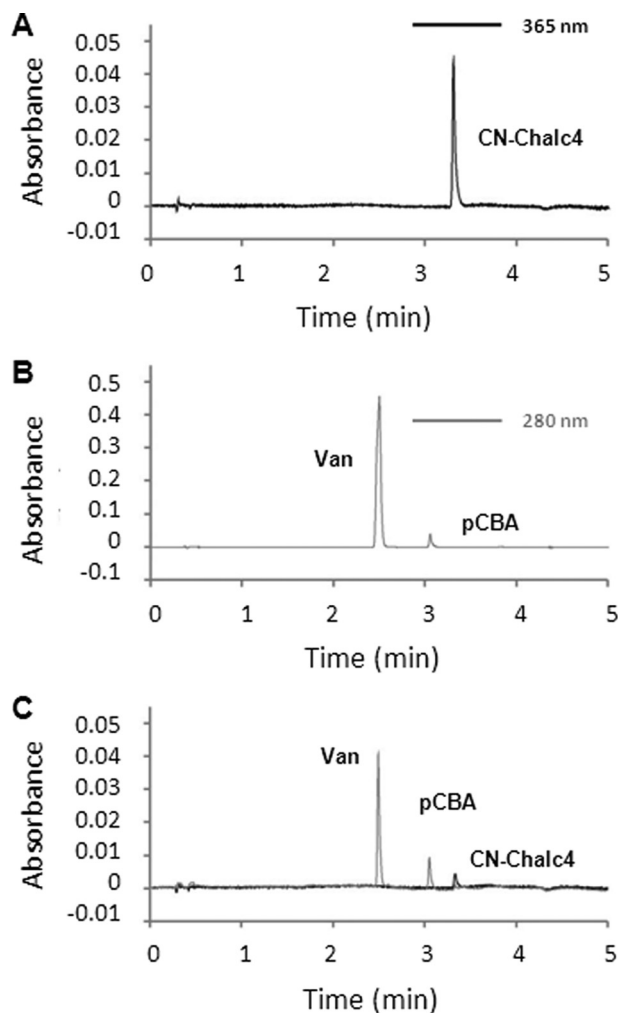


FIGURE 3. CN-chalcone 4 is rapidly hydrolyzed in murine serum. Chromatograms were obtained with a Luna C18(2) 5- μm , 4.6 \times 50-mm column. Gradient elution was as follows: 0–0.2 min, 0% B; 0.2–2.7 min, 0–100% B; 2.7–3.2 min, 100% B; 3.2–3.4 min, 100–0% B; 3.4–6.1 min, 0% B (A, water, 0.1% trifluoroacetic acid; B, acetonitrile). A, chromatogram (at $\lambda = 365$ nm) of 20 μl of the reference compound CN-chalcone 4, dissolved at 10 μM in water/acetonitrile (1:1, v/v) ($t_R = 3.31$ min). B, chromatogram (at $\lambda = 280$ nm) of 20 μl of the reference compounds vanillin (*Van*) and pCBA, dissolved at 100 μM in water/acetonitrile (1:1, v/v) ($t_R(\text{Van}) = 2.47$ min and $t_R(\text{pCBA}) = 3.02$ min). C, chromatogram (at $\lambda = 280$ nm and 365 nm) of 20 μl of CN-chalcone 4 dissolved at 20 μM and incubated for 1 h in murine serum. The solution was diluted with one volume of acetonitrile before injection in the HPLC. Peaks detected at $t_R = 2.47$ min, $t_R = 3.02$ min, and $t_R = 3.31$ min correspond to vanillin, pCBA, and CN-chalcone 4 (*CN-Chalc4*), respectively.

Inhibition of CXCL12 effect on cAMP formation in transfected HEK293 cells overexpressing the human CXCR4 receptor has also been characterized. As shown in Fig. 4B, forskolin (1 μM) evokes an increase in intracellular cAMP that is potently blocked by CXCL12 (3 nM). This blocking effect of CXCL12 is dose-dependently counteracted by either chalcone 4 or CN-chalcone 4 (1–10 μM) but not by the CN-chalcone 4 degradation products vanillin (10 μM) and pCBA (10 μM). The derived IC_{50} values are equal to 4.1 ± 0.3 and 6.9 ± 0.4 μM for chalcone 4 and CN-chalcone 4, respectively.

In vivo effects of the degradation products have also been tested on OVA-sensitized and -challenged mice. Intranasal administration of vanillin and pCBA at the same dose as CN-chalcone 4 in Fig. 1 (300 nmol/kg) has no effect on eosinophil

Antedrug of the CXCL12 Neutraligand and Asthma

recruitment in the airways (Fig. 4C), showing their lack of activity *in vivo* that matches their lack of *in vitro* activity on CXCL12.

Thus, CN-chalcone 4, which is as active as chalcone 4, is subject to spontaneous hydrolysis in buffered aqueous solu-

TABLE 1

Stability of chalcone 4 versus CN-chalcone 4 in different media

Compound stability was measured either in HEPES buffer, pH 7.4, or murine serum, or lung homogenates supplemented or not with 10% HP- β CD, as indicated. Incubation of compound was stopped at 15 and 30 min and 1, 2, 16, and 24 h, depending on the compound. Values in parentheses represent the percentage of starting molecule recovered at the indicated time.

	Stability half-life ($t_{1/2}$)	
	Chalcone 4	CN-chalcone 4
PBS	>10 (98% at 10 h) ^a	6
PBS, 10% β -cyclodextrine	>10 (95% at 10 h) ^a	16.3
Murine serum	>16 (96% at 16 h)	0.3
Murine serum, 10% β -cyclodextrine	>16 (91% at 16 h)	1.3
Lung homogenate	>6 (60%) ^a	0.4
Lung homogenate, 10% β -cyclodextrine	>16 (66% at 16 h)	0.6

^a Data are taken from Ref. 24.

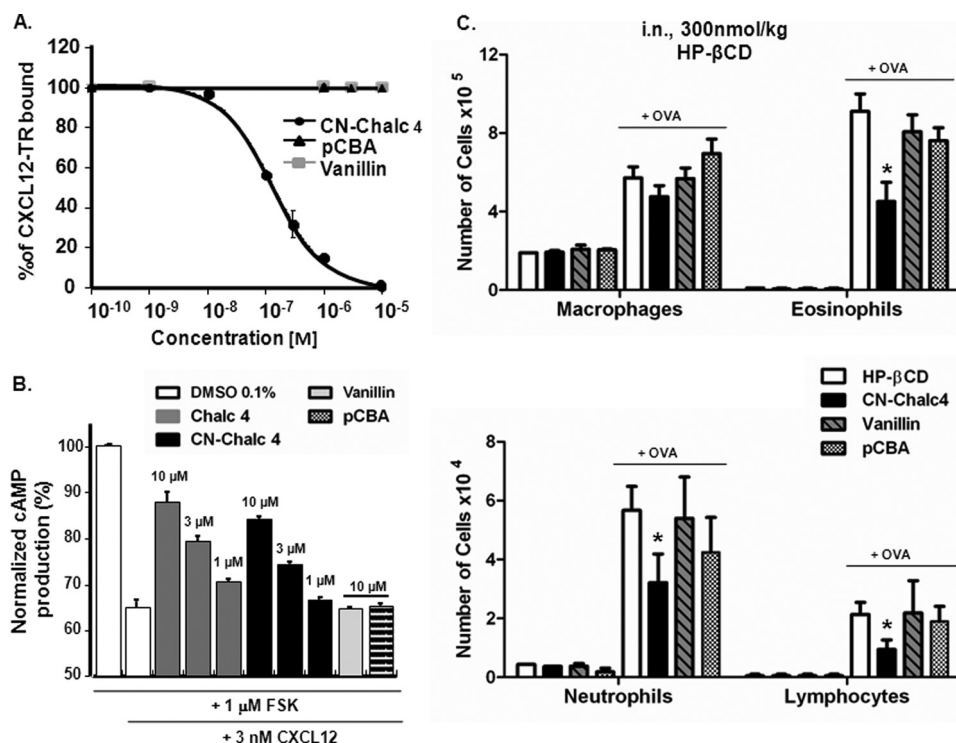
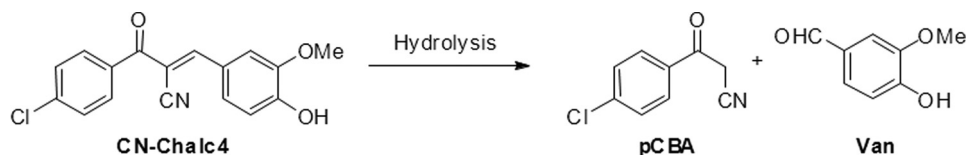


FIGURE 4. CN-chalcone 4 degradation products are inactive. A, CN-chalcone 4 (CN-Chalc 4) degradation products (up to 10 μ M) do not inhibit CXCL12-TR binding to EGFP-CXCR4 detected by FRET as in Fig. 1A. Each data point represents the mean \pm S.D. of three independent experiments performed in triplicates. B, CN-chalcone 4, but not its degradation products, dose-dependently inhibits CXCL12 action on forskolin-evoked cAMP responses in HEK EGFP-CXCR4 cells. The first two bars report the maximal production of cAMP (%) triggered by 1 μ M forskolin and its inhibition by 3 nM CXCL12. The following bars show that chalcone 4 (Chalc 4) and CN-chalcone 4 (1, 3, and 10 μ M), but not vanillin or pCBA (10 μ M), inhibit the CXCL12 effect on cAMP production. Each bar represents the mean \pm S.D. (error bars) of three independent experiments performed in triplicates. C, topical treatment with chalcone 4, CN-chalcone 4, vanillin, and pCBA in the 8-day mouse model of hyper-eosinophilia. BALB/c mice were immunized and challenged with OVA or saline. Treatments (300 nmol/kg) or HP- β CD 10% (vehicle), were administered intranasally 2 h before each challenge. Absolute numbers of macrophages, eosinophils, neutrophils, and lymphocytes in BAL are shown. Bars show means, and error bars show S.E. values ($n = 6$ /group). *, $p \leq 0.05$ in comparison with the saline-treated OVA group.

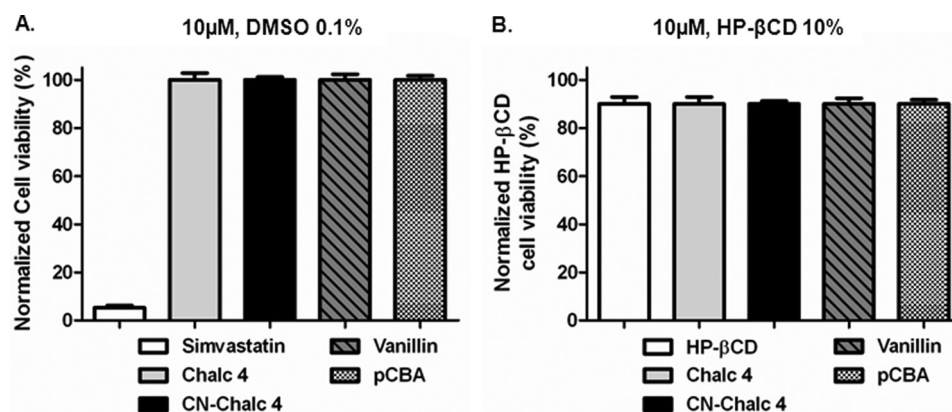


FIGURE 5. **Cytotoxicity of chalcone derivatives on HepG2 cells using a resazurin reduction assay.** Cell viability is expressed as the percentage of untreated control cells (A) and as 10% HP- β CD-treated cells (B). Cells were exposed to 10 μ M compounds for 24 h. Each exposure was preceded by equilibrium setting for 16 h in growth medium at 37 $^{\circ}$ C, 5% CO₂. The dye was added to the cells together with the test substances at a final concentration of 10%. Simvastatin (100 μ M) was used as a positive control. All samples contained 0.1% DMSO. Data are expressed as means \pm S.D. (error bars) ($n = 3$). *Chalc 4*, chalcone 4; *CN-Chalc 4*, CN-chalcone 4.

The positive cytotoxicity control molecule was simvastatin (100 μ M) (48).

In order to increase the low chalcone 4 solubility ($9 \pm 1 \mu$ M in physiological medium), we also used HP- β CD in this study. Although HP- β CD displays some toxicity on its own at 10% in culture medium, as was described previously (66, 67), there was no further cytotoxicity of any of the compounds (Fig. 5B) as compared with cells treated with HP- β CD alone.

General toxicity was evaluated *in vivo* in mice that received CN-chalcone 4 intraperitoneally at a dose of 350 μ mol/kg (100 mg/kg) per day during three consecutive days. As reported in Table 2, there was no body or spleen weight loss nor any modification of cytochrome *c* oxidase activity in lung and heart, indicating no toxicity of CN-chalcone 4 administered at the highest active dose.

DISCUSSION

Our results show the anti-inflammatory effect of a rapidly hydrolyzable CXCL12 neutraligand in an airway hypereosinophilia model. Carbonitrile-chalcone 4 is an efficient blocker of CXCL12 binding to CXCR4 and of the associated inhibition of cAMP production. However, in biological fluids, CN-chalcone 4 is rapidly degraded into two inactive metabolites, vanillin and pCBA, the two compounds that served as synthetic building blocks for its production. When administered locally in the airways by the intranasal route, CN-chalcone 4 efficiently inhibits eosinophil, neutrophil, and T cell recruitment at a low dose. By contrast, it remains without any anti-inflammatory effect in the airways when administered systemically by the intraperitoneal route even at doses 100–1000-fold higher. This is opposed to the systemic effect of chalcone 4 and demonstrates that CN-chalcone 4 behaves as an antedrug or soft drug acting at the administration site that is degraded prior to wider distribution.

Three groups, including ours (24–27), described that when CXCR4 signaling is inhibited, either with antibodies (25), with CXCR4 antagonists (27), or with CXCL12-neutralizing small molecules (26), invasion of lungs by eosinophils is reduced by \sim 50%. This piece of evidence highlights a functional role of CXCR4 and of its ligand either in the allergic response onset or in its maintenance. The question as to whether airway inflam-

TABLE 2
General toxicity

Absence of general toxicity of CN-chalcone 4. After OVA challenge leading to hypereosinophilia and CN-chalcone 4 treatment, whole animals and spleens were weighed. Whole animal weights were compared with those of untreated animals (body weight as a percentage of that for control animals). Spleen weight is given in mg. Cytochrome *c* oxidase activity was determined in lung and heart, and maximal activity (V_{\max}) is reported as the variation of tetramethyl-*p*-phenylenediamine 610-nm optical density/min/100 μ g of protein.

	Weight		Cytochrome <i>c</i> oxidase (V_{\max})	
	Body	Spleen	Lung ($\times 10^2$)	Heart ($\times 10^3$)
	%	mg		
Carboxymethylcellulose	99.9 \pm 4	124 \pm 7	15.1 \pm 1.6	25.6 \pm 2.6
CN-chalcone 4	99.6 \pm 6	125 \pm 11	13.8 \pm 2.8	28.5 \pm 3.2
OVA	99.8 \pm 6	128 \pm 6	12.8 \pm 2.0	23.1 \pm 3.1
OVA + CN-chalcone 4	99.6 \pm 6	123 \pm 4	12.7 \pm 1.6	29.9 \pm 3.5

mation stimulates CXCL12 production continues to be debated because immunohistochemical detection in lung tissue shows no change (25), whereas immunochemical determination in BALF (49) and gene expression in lung (50) indicate that CXCL12 is up-regulated. The expression of CXCR4, on the other hand, is higher in BAL CD4⁺ T cells of human asthmatics as compared with their peripheral blood CD4⁺ lymphocytes (51) and is up-regulated by the proinflammatory cytokine IL-4 in CD4⁺ T cells, including Th2 cells (25, 52–54). This renders significant response to CXCL12 likely to occur in the airway, whatever the regulation of CXCL12 expression. In addition, CXCR4 is also expressed in eosinophils (55, 56). Eosinophils have a migratory response to CXCL12 comparable with that evoked by eotaxin.

The mode of action of neutraligands opens the way to new therapeutic strategies especially for airway diseases, because (i) chalcone 4 and its analogs are active through the intranasal route, and (ii) they act on a new target, namely CXCL12, the ligand of CXCR4 and CXCR7 chemokine receptors. Thus, the mode of action of chalcone 4 (26) and its analogs chalcone 4-phosphate (24) and CN-chalcone 4 (this work) appears as complementary to that of classical receptor antagonists because the blockade of the chemokine is without any effect on the receptor. In particular, it is neither a partial agonist of CXCR4 nor an activator of CXCR7 (42, 57, 58), as was described

Antedrug of the CXCL12 Neutraligand and Asthma

for AMD 3100 and in other instances with RANTES (regulated on activation normal T cell expressed and secreted) analogs acting on the CCR5 receptor (59). Therefore, the mechanism of action of chalcone 4 and its analogs deserves to be exploited in drug development programs.

Another concern was raised regarding the large tissue distribution of CXCR4, which can be the cause of possible side effects of CXCR4-targeting drugs. The use of systemically administered AMD 3100 confirmed the risk of side effects resulting from general CXCR4 inhibition. This was illustrated on leukocyte maturation in the bone marrow (27) and on cardiac function (36, 37, 60). We therefore generated a short lived readily hydrolyzable analog of the initial compound, chalcone 4, and show here that CN-chalcone 4 is as active as chalcone 4 on airway inflammation when administered by the intranasal route, whereas it is inactive when delivered systemically using the intraperitoneal route. It therefore typically behaves as an antedrug or soft drug.

The general principles and reactions that are used for antedrug structures include various cleavable chemical functions, such as carboxylic esters and amides, oximes, thioester, spi-roenones, or lactones (45, 61). In designing carbonitrile-chalcone 4, we here make use of the Knoevenagel and retro-Knoevenagel reactions (62) yielding the desired compound due to a reversible aldolization reaction (63) that has never been exploited in the antedrug field before. The biologically active compound, carbonitrile-chalcone 4, is readily hydrolyzed in aqueous media with a half-life of a few tens of min and yields vanillin and pCBA, which both serve as synthetic building blocks for the preparation of carbonitrile-chalcone 4. The probable hydrolysis mechanism involves the addition of one water molecule according to a Michael addition on the α - β unsaturated conjugated system. Hydration of the double bond is presumably facilitated by the presence of the electron-attracting nitrile group. The resulting enolic structure then evolves toward production of the initial reactants vanillin and pCBA according to a retroaldolization reaction (64). We show here that neither the reactants nor carbonitrile-chalcone 4 display any toxic effect *in vivo* or in HepG2 cells *in vitro*.

In conclusion, our results show a strong activity of a chalcone 4 derivative, carbonitrile-chalcone 4, displaying only local and no systemic effect due to a short lifetime in biological fluids, therefore playing the role of an antedrug, which is particularly interesting when the airways are considered. The various chalcone 4 derivatives that we have generated in this and previous works will serve as tools to understand CXCR4, CXCR7, and CXCL12 functions in the airway inflammation process. In particular, the sequence of events and their dependence on CXCL12 activity will be important elements in the characterization of CXCL12 as a drug target in airway inflammation. The mechanism of action of chalcone 4 and its analogs deserves to be exploited in drug development programs because blockade of the chemokine is without any effect on the receptor spontaneous activity as opposed to the most widely encountered pharmacological action of G protein-coupled receptor antagonists.

REFERENCES

1. Johnson, Z., Schwarz, M., Power, C. A., Wells, T. N., and Proudfoot, A. E. (2005) Multi-faceted strategies to combat disease by interference with the chemokine system. *Trends Immunol.* **26**, 268–274
2. Dambly-Chaudière, C., Cubedo, N., and Ghysen, A. (2007) Control of cell migration in the development of the posterior lateral line. Antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. *BMC Dev. Biol.* **7**, 23
3. Takabatake, Y., Sugiyama, T., Kohara, H., Matsusaka, T., Kurihara, H., Koni, P. A., Nagasawa, Y., Hamano, T., Matsui, I., Kawada, N., Imai, E., Nagasawa, T., Rakugi, H., and Isaka, Y. (2009) The CXCL12 (SDF-1)/CXCR4 axis is essential for the development of renal vasculature. *J. Am. Soc. Nephrol.* **20**, 1714–1723
4. Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., and Littman, D. R. (1998) Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**, 595–599
5. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382**, 635–638
6. Lévesque, J. P., Hendy, J., Takamatsu, Y., Simmons, P. J., and Bendall, L. J. (2003) Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by G-CSF or cyclophosphamide. *J. Clin. Invest.* **111**, 187–196
7. Petit, I., Szyper-Kravitz, M., Nagler, A., Lahav, M., Peled, A., Habler, L., Ponomaryov, T., Taichman, R. S., Arenzana-Seisdedos, F., Fujii, N., Sandbank, J., Zipori, D., and Lapidot, T. (2002) G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat. Immunol.* **3**, 687–694
8. Liles, W. C., Broxmeyer, H. E., Rodger, E., Wood, B., Hübel, K., Cooper, S., Hangoc, G., Bridger, G. J., Henson, G. W., Calandra, G., and Dale, D. C. (2003) Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. *Blood* **102**, 2728–2730
9. Broxmeyer, H. E., Cooper, S., Kohli, L., Hangoc, G., Lee, Y., Mantel, C., Clapp, D. W., and Kim, C. H. (2003) Transgenic expression of stromal cell-derived factor-1/CXC chemokine ligand 12 enhances myeloid progenitor cell survival/antiapoptosis *in vitro* in response to growth factor withdrawal and enhances myelopoiesis *in vivo*. *J. Immunol.* **170**, 421–429
10. Broxmeyer, H. E., Orschell, C. M., Clapp, D. W., Hangoc, G., Cooper, S., Plett, P. A., Liles, W. C., Li, X., Graham-Evans, B., Campbell, T. B., Calandra, G., Bridger, G., Dale, D. C., and Srour, E. F. (2005) Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J. Exp. Med.* **201**, 1307–1318
11. Balkwill, F. (2004a) The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin. Cancer Biol.* **14**, 171–179
12. Balkwill, F. (2004b) Cancer and the chemokine network. *Nat. Rev. Cancer* **4**, 540–550
13. Balkwill, F., and Mantovani, A. (2010) Cancer and inflammation. Implications for pharmacology and therapeutics. *Clin. Pharmacol. Ther.* **87**, 401–406
14. Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verástegui, E., and Zlotnik, A. (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**, 50–56
15. Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A., and Mackay, C. R. (1997) The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 1925–1930
16. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) HIV-1 entry cofactor. Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877
17. Oberlin, E., Amara, A., Bachelier, F., Bessia, C., Virelizier, J. L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J. M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., and Moser, B. (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**, 833–835
18. Bachelier, F. (2010) CXCL12/CXCR4-axis dysfunctions. Markers of the

- rare immunodeficiency disorder WHIM syndrome. *Dis. Markers* **29**, 189–198
19. Balabanian, K., Lagane, B., Pablos, J. L., Laurent, L., Planchenault, T., Verola, O., Lebbe, C., Kerob, D., Dupuy, A., Hermine, O., Nicolas, J. F., Latger-Cannard, V., Bensoussan, D., Bordignon, P., Baleux, F., Le Deist, F., Virelizier, J. L., Arenzana-Seisdedos, F., and Bachelier, F. (2005) WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood* **105**, 2449–2457
 20. Wetzler, M., Talpaz, M., Kleinerman, E. S., King, A., Huh, Y. O., Gutterman, J. U., and Kurzrock, R. (1990) A new familial immunodeficiency disorder characterized by severe neutropenia, a defective marrow release mechanism, and hypogammaglobulinemia. *Am. J. Med.* **89**, 663–672
 21. Chow, K. Y., Brotin, É., Ben Khalifa, Y., Carthagena, L., Teissier, S., Dancckaert, A., Galzi, J. L., Arenzana-Seisdedos, F., Thierry, F., and Bachelier, F. (2010) A pivotal role for CXCL12 signaling in HPV-mediated transformation of keratinocytes. Clues to understanding HPV-pathogenesis in WHIM syndrome. *Cell Host Microbe* **8**, 523–533
 22. Cinamon, G., Shinder, V., and Alon, R. (2001) Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. *Nat. Immunol.* **2**, 515–522
 23. Galzi, J. L., Hachet-Haas, M., Bonnet, D., Daubeuf, F., Lecat, S., Hibert, M., Haiech, J., and Frossard, N. (2010) Neutralizing endogenous chemokines with small molecules. Principles and potential therapeutic applications. *Pharmacol. Ther.* **126**, 39–55
 24. Gasparik, V., Daubeuf, F., Hachet-Haas, M., Rohmer, F., Gizzi, P., Haiech, J., Galzi, J. L., Hibert, M., Bonnet, D., and Frossard, N. (2012) Prodrugs of a CXC chemokine-12 (CXCL12) neutraligand prevent inflammatory reactions in an asthma model in vivo. *ACS Med. Chem. Lett.* **3**, 10–14
 25. Gonzalo, J. A., Lloyd, C. M., Peled, A., Delaney, T., Coyle, A. J., and Gutierrez-Ramos, J. C. (2000) Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 α in the inflammatory component of allergic airway disease. *J. Immunol.* **165**, 499–508
 26. Hachet-Haas, M., Balabanian, K., Rohmer, F., Pons, F., Franchet, C., Lecat, S., Chow, K. Y., Dagher, R., Gizzi, P., Didier, B., Lagane, B., Kellenberger, E., Bonnet, D., Baleux, F., Haiech, J., Parmentier, M., Frossard, N., Arenzana-Seisdedos, F., Hibert, M., and Galzi, J. L. (2008) Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J. Biol. Chem.* **283**, 23189–23199
 27. Lukacs, N. W., Berlin, A., Schols, D., Skerlj, R. T., and Bridger, G. J. (2002) AMD3100, a CXCR4 antagonist, attenuates allergic lung inflammation and airway hyperreactivity. *Am. J. Pathol.* **160**, 1353–1360
 28. Montani, D., Perros, F., Gambaryan, N., Girerd, B., Dorfmueller, P., Price, L. C., Huertas, A., Hammad, H., Lambrecht, B., Simonneau, G., Launay, J. M., Cohen-Kaminsky, S., and Humbert, M. (2011) C-kit-positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial hypertension. *Am. J. Respir. Crit. Care Med.* **184**, 116–123
 29. Xu, J., Torres, E., Mora, A. L., Shim, H., Ramirez, A., Neujahr, D., Brigham, K. L., and Rojas, M. (2008) Attenuation of obliterative bronchiolitis by a CXCR4 antagonist in the murine heterotopic tracheal transplant model. *J. Heart Lung Transplant.* **27**, 1302–1310
 30. Balabanian, K., Lagane, B., Infantino, S., Chow, K. Y., Harriague, J., Moepps, B., Arenzana-Seisdedos, F., Thelen, M., and Bachelier, F. (2005) The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J. Biol. Chem.* **280**, 35760–35766
 31. Burns, J. M., Summers, B. C., Wang, Y., Melikian, A., Berahovich, R., Miao, Z., Penfold, M. E., Sunshine, M. J., Littman, D. R., Kuo, C. J., Wei, K., McMaster, B. E., Wright, K., Howard, M. C., and Schall, T. J. (2006) A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J. Exp. Med.* **203**, 2201–2213
 32. Gerrits, H., van Ingen Schenau, D. S., Bakker, N. E., van Disseldorp, A. J., Strik, A., Hermens, L. S., Koenen, T. B., Krajinac-Franken, M. A., and Gossen, J. A. (2008) Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. *Genesis* **46**, 235–245
 33. Sierro, F., Biben, C., Martínez-Muñoz, L., Mellado, M., Ransohoff, R. M., Li, M., Woehl, B., Leung, H., Groom, J., Batten, M., Harvey, R. P., Martínez-A, C., Mackay, C. R., and Mackay, F. (2007) Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 14759–14764
 34. Levoe, A., Balabanian, K., Baleux, F., Bachelier, F., and Lagane, B. (2009) CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* **113**, 6085–6093
 35. Devine, S. M., Flomenberg, N., Vesole, D. H., Liesveld, J., Weisdorf, D., Badel, K., Calandra, G., and DiPersio, J. F. (2004) Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J. Clin. Oncol.* **22**, 1095–1102
 36. Flomenberg, N., Devine, S. M., DiPersio, J. F., Liesveld, J. L., McCarty, J. M., Rowley, S. D., Vesole, D. H., Badel, K., and Calandra, G. (2005) The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood* **106**, 1867–1874
 37. Flomenberg, N., DiPersio, J., and Calandra, G. (2005) Role of CXCR4 chemokine receptor blockade using AMD3100 for mobilization of autologous hematopoietic progenitor cells. *Acta Haematol.* **114**, 198–205
 38. Liles, W. C., Rodger, E., Broxmeyer, H. E., Dehner, C., Badel, K., Calandra, G., Christensen, J., Wood, B., Price, T. H., and Dale, D. C. (2005) Augmented mobilization and collection of CD34+ hematopoietic cells from normal human volunteers stimulated with granulocyte-colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. *Transfusion* **45**, 295–300
 39. McDermott, D. H., Liu, Q., Ulrick, J., Kwatema, N., Anaya-O'Brien, S., Penzak, S. R., Filho, J. O., Long Priel, D. A., Kelly, C., Garofalo, M., Littel, P., Marquesen, M. M., Hilligoss, D., Decastro, R., Fleisher, T. A., Kuhns, D. B., Malech, H. L., and Murphy, P. M. (2011) The CXCR4 antagonist plerixafor corrects panleukopenia in patients with WHIM syndrome. *Blood* **118**, 4957–4962
 40. McDermott, D. H., Lopez, J., Deng, F., Liu, Q., Ojode, T., Chen, H., Ulrick, J., Kwatema, N., Kelly, C., Anaya-O'Brien, S., Garofalo, M., Marquesen, M., Hilligoss, D., DeCastro, R., Malech, H. L., and Murphy, P. M. (2011) AMD3100 is a potent antagonist at CXCR4(R334X), a hyperfunctional mutant chemokine receptor and cause of WHIM syndrome. *J. Cell Mol. Med.* **15**, 2071–2081
 41. Hendrix, C. W., Collier, A. C., Lederman, M. M., Schols, D., Pollard, R. B., Brown, S., Jackson, J. B., Coombs, R. W., Glesby, M. J., Flexner, C. W., Bridger, G. J., Badel, K., MacFarland, R. T., Henson, G. W., and Calandra, G. (2004) Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *J. Acquir. Immune Defic. Syndr.* **37**, 1253–1262
 42. Kalatskaya, I., Berchiche, Y. A., Gravel, S., Limberg, B. J., Rosenbaum, J. S., and Heveker, N. (2009) AMD3100 is a CXCR7 ligand with allosteric agonist properties. *Mol. Pharmacol.* **75**, 1240–1247
 43. Balabanian, K., Brotin, E., Biajoux, V., Bouchet-Delbos, L., Lainey, E., Fenneteau, O., Bonnet, D., Fiette, L., Emilie, D., and Bachelier, F. (2012) Proper desensitization of CXCR4 is required for lymphocyte development and peripheral compartmentalization in mice. *Blood* **119**, 5722–5730
 44. Bouchery, T., Dénécé, G., Attout, T., Ehrhardt, K., Lhermitte-Vallarino, N., Hachet-Haas, M., Galzi, J. L., Brotin, E., Bachelier, F., Gavotte, L., Moulia, C., Bain, O., and Martin, C. (2012) The chemokine CXCL12 is essential for the clearance of the filaria *Litomosoides sigmodontis* in resistant mice. *PLoS One* **7**, e34971
 45. Khan, M. O., Park, K. K., and Lee, H. J. (2005) Antedrug. An approach to safer drugs. *Curr. Med. Chem.* **12**, 2227–2239
 46. Maillet, E. L., Pellegrini, N., Valant, C., Bucher, B., Hibert, M., Bourguignon, J. J., and Galzi, J. L. (2007) A novel, conformation-specific allosteric inhibitor of the tachykinin NK2 receptor (NK2R) with functionally selective properties. *FASEB J.* **21**, 2124–2134
 47. Jiang, L. I., Collins, J., Davis, R., Lin, K. M., DeCamp, D., Roach, T., Hsueh, R., Rebres, R. A., Ross, E. M., Taussig, R., Fraser, I., and Sternweis, P. C. (2007) Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *J. Biol. Chem.* **282**, 10576–10584
 48. Waldhauser, K. M., Brecht, K., Hebeisen, S., Ha, H. R., Konrad, D., Bur, D., and Krähenbühl, S. (2008) Interaction with the hERG channel and cytotoxicity of amiodarone and amiodarone analogues. *Br. J. Pharmacol.* **155**, 585–595
 49. Negrete-García, M. C., Velazquez, J. R., Popoca-Coyotl, A., Montes-Vi-

Antedrug of the CXCL12 Neutraligand and Asthma

- zuet, A. R., Juárez-Carvajal, E., and Teran, L. M. (2010) Chemokine (C-X-C motif) ligand 12/stromal cell-derived factor-1 is associated with leukocyte recruitment in asthma. *Chest* **138**, 100–106
50. Fulkerson, P. C., Zimmermann, N., Hassman, L. M., Finkelman, F. D., and Rothenberg, M. E. (2004) Pulmonary chemokine expression is coordinately regulated by STAT1, STAT6, and IFN- γ . *J. Immunol.* **173**, 7565–7574
51. Thomas, S. Y., Banerji, A., Medoff, B. D., Lilly, C. M., and Luster, A. D. (2007) Multiple chemokine receptors, including CCR6 and CXCR3, regulate antigen-induced T cell homing to the human asthmatic airway. *J. Immunol.* **179**, 1901–1912
52. Eddleston, J., Christiansen, S. C., and Zuraw, B. L. (2002) Functional expression of the C-X-C chemokine receptor CXCR4 by human bronchial epithelial cells. Regulation by proinflammatory mediators. *J. Immunol.* **169**, 6445–6451
53. Jourdan, P., Abbal, C., Noraz, N., Hori, T., Uchiyama, T., Vendrell, J. P., Bousquet, J., Taylor, N., Pène, J., and Yssel, H. (1998) IL-4 induces functional cell-surface expression of CXCR4 on human T cells. *J. Immunol.* **160**, 4153–4157
54. Wang, J., Harada, A., Matsushita, S., Matsumi, S., Zhang, Y., Shioda, T., Nagai, Y., and Matsushima, K. (1998) IL-4 and a glucocorticoid up-regulate CXCR4 expression on human CD4⁺ T lymphocytes and enhance HIV-1 replication. *J. Leukoc. Biol.* **64**, 642–649
55. Nagase, H., Miyamasu, M., Yamaguchi, M., Fujisawa, T., Ohta, K., Yamamoto, K., Morita, Y., and Hirai, K. (2000) Expression of CXCR4 in eosinophils. Functional analyses and cytokine-mediated regulation. *J. Immunol.* **164**, 5935–5943
56. Nagase, H., Miyamasu, M., Yamaguchi, M., Kawasaki, H., Ohta, K., Yamamoto, K., Morita, Y., and Hirai, K. (2000) Glucocorticoids preferentially upregulate functional CXCR4 expression in eosinophils. *J. Allergy Clin. Immunol.* **106**, 1132–1139
57. Kim, H. Y., Hwang, J. Y., Kim, S. W., Lee, H. J., Yun, H. J., Kim, S., and Jo, D. Y. (2010) The CXCR4 antagonist AMD3100 has dual effects on survival and proliferation of myeloma cells *in vitro*. *Cancer Res. Treat.* **42**, 225–234
58. Trent, J. O., Wang, Z. X., Murray, J. L., Shao, W., Tamamura, H., Fujii, N., and Peiper, S. C. (2003) Lipid bilayer simulations of CXCR4 with inverse agonists and weak partial agonists. *J. Biol. Chem.* **278**, 47136–47144
59. Wells, T. N., and Proudfoot, A. E. (1999) Chemokine receptors and their antagonists in allergic lung disease. *Inflamm. Res.* **48**, 353–362
60. Rusconi, S., Lo Cicero, M., Viganò, O., Sirianni, F., Bulgheroni, E., Ferramosca, S., Bencini, A., Bianchi, A., Ruiz, L., Cabrera, C., Martinez-Picado, J., Supuran, C. T., and Galli, M. (2009) New macrocyclic amines showing activity as HIV entry inhibitors against wild type and multi-drug resistant viruses. *Molecules* **14**, 1927–1937
61. Khan, M. O., and Lee, H. J. (2008) Synthesis and pharmacology of anti-inflammatory steroidal antedrugs. *Chem. Rev.* **108**, 5131–5145
62. Rajasekaran, K., Sarathi, A., and Ramalakshmi, S. (2008) Micellar catalysis in the retro-Knoevenagel reaction of ethyl- α -cyanocinnamates. *J. Chem. Sci.* **120**, 475–480
63. Guthrie, J. P., Cossar, J., Cullimore, P. A., Kamkar, N. M., and Taylor, K. F. (1983) The retroaldol reaction of chalcone. *Can. J. Chem.* **61**, 2621–2626
64. Roberts, D. L., and Rowland, R. L. (1962) Macrocyclic diterpenes α - and β -4,8,13-duvatriene-1,3-diols from tobacco. *J. Org. Chem.* **27**, 3989–3995
65. Cheng, Y., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–3108
66. Davis, M. E., and Brewster, M. E. (2004) Cyclodextrin-based pharmaceuticals. Past, present and future. *Nat. Rev. Drug Discov.* **3**, 1023–1035
67. Gould, S., and Scott, R. C. (2005) 2-Hydroxypropyl- β -cyclodextrin (HP- β -CD). A toxicology review. *Food Chem. Toxicol.* **43**, 1451–1459

Publication n°4 :

Deciphering the mechanism of the anti-asthmatic action of the CXCL12 neutraligand chalcone 4 and links between CXCL12 and the macrophages in response to allergen

François Daubeuf, Dominique Bonnet, Virgile BECKAERT, Ali OUADI, Patrice MARCHAND, David BRASSE, Patrick Gizzi, Dayana ABOUD, Marcel Hibert, Jean-Luc Galzi and Nelly Frossard

En préparation

La chimiokine CXCL12 et ses récepteurs sont impliqués dans l'asthme allergique, comme l'a démontré avec succès l'utilisation d'agents bloquant CXCL12 ou CXCR4 (Gonzalo et al. 2000, Lukacs et al. 2002). Ces résultats sont confortés par l'utilisation de neutraligand de CXCL12 (Hachet-Haas et al. 2008, Gasparik et al. 2011, Daubeuf et al. 2012), qui bloquent la liaison de CXCL12 à CXCR4 et CXCR7, et permet d'inhiber le recrutement des éosinophiles dans les voies aériennes dans des modèles aigu d'asthme allergique chez la souris. Nous avons recherché si l'action anti-inflammatoire était associée à une activité antiasthmatic plus complète. Nous avons étudié l'activité anti-asthmatic et le mécanisme d'action d'un neutraligand de CXCL12, la chalcone 4 (chalc-4) administrée in vivo par voie locale, dans un modèle murin d'asthme aigu à l'ovalbumine.

Nous montrons que l'administration locale de la chalcone 4 réduit les symptômes de l'asthme tels que l'hyperréactivité bronchique à la métacholine observée par plethysmographie chez l'animal vigile non contraint (45%) et chez l'animal anesthésié (37%, Flexivent®), l'hyperéosinophilie des voies aériennes (54%), ainsi que le remodelage bronchique comme la production de mucus (84%) et la déposition de collagène (78%).

Nous mettons également en évidence la relation singulière entre la pharmacocinétique et l'activité antiasthmatic de ce neutraligand. Pour cela, un dérivé de la chalcone 4 a été conçu par substitution du chlore par de l'iode (chalc4-I), qui présente la même activité de neutralisation de CXCL12 et la même activité anti-inflammatoire que la chalc4. Le suivi par imagerie SPECT de ce neutraligand iodé¹²³ (chalc4-I¹²³) et l'étude pharmacocinétique de la chalc4 (UHPLC-LC-MS/MS) montrent que le neutraligand quitte très rapidement le poumon ($T_{1/2} < 5\text{min}$) et la circulation sanguine pour être éliminée dans l'urine et la bile. L'élimination rapide de la chalc4 est liée à l'élimination de la chimiokine (concentration plasmatique de CXCL12 $\times 1000$, 5 min après administration i.n. de chalc4 et $t_{1/2} < 10\text{min}$), et conduit à une réduction significative de la quantité de CXCL12 dans le poumon (20% après 4 administrations 4 jours consécutifs). Le traitement par la chalc4 entraîne une réduction du nombre de macrophages de type M1 (CD206⁻, CCR7⁺) (62%), et diminue la réponse des macrophages à CXCL12 (-98% de TNF α) et à l'ovalbumine (-99% de TNF α et -91% d'IL-5).

Ainsi, nous montrons que l'administration locale d'un neutralisant de CXCL12, neutralise et entraîne la chimiochine CXCL12 présente dans le poumon, modifie la réponse des macrophages alvéolaires à l'allergène et conduit à réduire la réponse inflammatoire, l'hyperactivité bronchique à la métacholine et le remodelage bronchique dans un modèle murin d'asthme aigu.

Deciphering the mechanism of the anti-asthmatic action of the CXCL12 neutraligand chalcone 4 and links between CXCL12 and the macrophages in response to allergen

François Daubeuf[§], Dominique Bonnet[§], Virgile Beckaert[§], Ali Ouadi[§], Patrice Marchand[§], David Brasse[§], Patrick Gizzi[#], Dayana Abboud[#], Marcel Hibert[§], Jean-Luc Galzi[#], Nelly Frossard[§]

§ Laboratoire d'Innovation Thérapeutique, UMR7200 CNRS/Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France ; # Laboratoire de Biotechnologie et Signalisation Cellulaire, UMR 7242 CNRS/Université de Strasbourg, Ecole Supérieure de Biotechnologie, Bd Sébastien Brant, 67412 Illkirch, France ; § Imabio, Institut Pluridisciplinaire Hubert CURIE, 23 rue du loess, 67037 Strasbourg, France.

The chemokine receptor CXCR4 and its chemokine CXCL12 are not only involved in normal tissue patterning, but also in tumor cell growth and survival, and in the recruitment of immune and inflammatory cells as well as asthma. These functions were successfully demonstrated using agents that block either CXCL12 or CXCR4. We recently described the interest of CXCL12 neutraligand, which blocks binding of CXCL12 to CXCR4 and/or CXCR7, in asthma like model in mouse, although the *in vivo* neutraligand mechanism of action has not been studied. Here we show that local administration of the Chalcone 4 compound reduces asthma symptoms characterized by an inhibition of airway hyperresponsiveness (45%), inflammatory recruitment (54%) and bronchial remodeling in an acute mice model of asthma (mucus: 84%, collagen : 78%). The pharmacokinetics of the chalcone 4 highlights that CXCL12 is very quickly trapped by the neutraligand and eliminated from the lung (-20% after 4 administrations). This phenomenon is correlated with the decrease of M1 macrophages in the bronchoalveolar lavage (65%), and the inhibition of the cytokines release by alveolar macrophages in response to CXCL12 (TNF- α : -98%) and ovalbumin (TNF- α : -99%, IL-5: -91%) able to modulate the recruitment of inflammatory cells. Interestingly, we show that macrophages activation and differentiation in response to allergen, may be regulated by a CXCL12 neutraligand.

Chemokines and their receptors play critical roles in development, homeostasis and pathological conditions. In adult, they regulate the recruitment and activation of leukocytes under normal condition but also in inflammatory responses or infectious disease [9, 21]. The chemokine CXCL12 and its CXCR4 and CXCR7 receptors play pivotal roles in normal and pathological situations including AIDS [7,20,46], the unusual form of neutropenia reported as the WHIM syndrome [1,2] or carcinogenesis [3,12,40]. In inflammation, CXCR4 and CXCL12 contribute to promoting transendothelial migration of lymphocytes [13], and invasion of inflamed tissues, as illustrated airways asthma model animals [15,25,27,28,36], the pulmonary vasculature in pulmonary arterial hypertension [39], and fibroproliferative tissue in a murine model of obliterative bronchiolitis after heterotopic tracheal transplantation [54]. CXCR7 does not elicit clear responses to CXCL12 but clearly associates with the CXCR4 protein, to modulate its sensitivity for CXCL12 [34,52], and CXCR7 is also known to be a scavenger receptor for CXCL12 [30,43].

Contrary to CXCR4 and CXCR7 receptors antagonists, we promote an alternative strategy which consisted in preventing the agonist-receptor interaction by neutralizing the endogenous ligands with pharmacological agents [24]. These neutraligands prevent CXCL12 binding either to CXCR4 or CXCR7 [15,25,28]. Chalcone 4 blocks responses of CXCR4 to CXCL12 *in vitro* without affecting the basal level receptor activity, and displays anti-inflammatory effects in a murine model of asthma *in vivo*. We recently described the interest of CXCL12 neutraligand in two asthma like model in mouse,

although the *in vivo* neutraligand mechanism of action was poorly described.

In the lung, CXCL12 chemokine has been described to be expressed at the surface of epithelial cells [65] and in macrophages [49], and it has been demonstrated that CXCL12 regulates monocyte-macrophage differentiation and CD14 expression, a part of the lipopolysaccharide "sensing apparatus" [49]. Macrophages represent the sentinel leukocyte in lung and M1/M2 alternative activation of macrophages in pulmonary diseases, as in allergic asthma, appears to be implicated in the pathogenesis [67,68,69]. Several studies reveal an inappropriate activation during asthma responses [66]. These findings suggested that macrophages may be a target of the anti-inflammatory activity of the CXCL12 neutraligand.

In addition to macrophages, neutralizing the CXCL12 chemokine may interfere with the chemotaxis of numerous cells in response to allergen and implicated in asthma. The CXCR4 receptor is expressed on fibroblasts, lymphocytes, mast cells progenitors, and has been described to be expressed on eosinophils and all of these cells presents a chemotaxis activity to CXCL12 [70,71,73,74]. These observations have been confirmed *in vivo*, under administration of the CXCR4 antagonist AMD3100, that reveals the inhibition of the trafficking response and accumulation of CXCR4+ cells in disease, as fibroblast [72], eosinophils and lymphocytes [36]. However, the role of the CXCL12 chemokine in the recruitment of mast cells in the tissue has not been elucidated in diseases but a recent study showed that CXCL12 may mediate mast cells recruitment in lymph node in response to

jet fuel application on mouse skin [75]. In consequence, the anti-inflammatory activity of CXCL12 neutraligands in allergic asthma may be one of numerous anti-asthmatics activities.

In this study, we found that the local administration, by intranasale route, of the chalcone 4 neutraligand, in a mouse model of allergic asthma, decrease airway hyperresponsiveness, bronchial remodeling as mucus hypersecretion, goblet cells hyperplasia, and collagen accumulation around bronchus, and inflammatory cells recruitment associated with allergen challenges. Unexpectedly, the study of the pharmacokinetics of the chalcone 4 highlights that neutraligand anti-asthmatics activities were correlate not only with neutralization of the CXCL12 chemokine but reveals that CXCL12 is very quickly trapping and eliminated from the lung. More surprisingly, this trapping is correlated with the decrease of M1 macrophages in the airways and lead to the inhibition of the cytokines release by alveolar macrophages. Our results suggest that CXCL12 release from macrophages may modulate activation and differentiation of macrophages and production of TNF- α and Th-2 cytokines implicated in the recruitment of inflammatory cells in response to allergen

METHODS

Mice. Nine-week-old male Balb/cJ mice were purchased from Janvier Laboratories (France). Animals were maintained under controlled environmental conditions in conventional husbandry. Mice were kept in polycarbonate cages with 4 mice per cage (PCT2L12SHT, Allentown) with bedding made from spruce wood chips (Safe) and enriched with play tunnels. The room air was ventilated at 10 air changes per hour according. The animal room was maintained at constant temperature (20 ± 2 °C) with a relative humidity (50 ± 10 %) and 12 hr/12 hr light-dark cycle (lighting 07:00-19:00) was imposed. Food (standard diet, 4RF21, Mucedola) and tap water were available ad libitum. Cages with bedding and play tunnels were changed weekly. Animal experimentation was conducted with the approval of the government body that regulates animal research in France.

Allergen sensitization and challenge. Nine week-old Balb/c mice were sensitized (i.p.) on days 0 and 7 with 50 μ g chicken egg albumin (OVA, Grade V, Sigma-Aldrich, A5503) adsorbed on 2 mg aluminium hydroxide (alum) in saline (23918-6, Sigma-Aldrich). Mice were challenged on days 17, 18, 19 and 20 by intranasal (i.n.) instillations of 10 μ g OVA in saline or with saline alone for controls (12.5 μ l/nostril). These challenges were performed under anesthesia (i.p.) with 50mg/kg ketamine and 3.33mg/kg xylazine as reported [16].

Treatment with Chalcone 4. The CXCL12 neutraligands, chalcone 4 compound (Chalc4, [28]) and I-chalcone 4 (I-Chalc4) was administered by i.n. route. Mice received chalc4 solubilized in PBS with 2-Hydroxypropyl- β -cyclodextrin 10% (HP β CD, C0926, Sigma) by intranasal (i.n.) administration, two hours before each intranasal OVA or saline challenge. Intranasal administrations were performed under anaesthesia (i.p.) with 50 mg/kg ketamine (Imalgene®, Merial) and 3.33 mg/kg xylazine (Rompun®, Bayer).

Chalcone 1 (Pharmacokinetic) and chalcone 4 were provided by La Chimiothèque Nationale, France. (French National

Chemical Library.

(<http://chimiotheque-nationale.enscm.fr/?lang=en>)

Total and differential cell counts in bronchoalveolar lavage (BAL) fluids. BAL and differential cell counts were performed as described previously [17]. Briefly, collection of bronchoalveolar lavage fluid (BALF) was performed 24 h after the last OVA challenge. Mice were deeply anaesthetized by i.p. injection of 150 mg/kg ketamine and 10 mg/kg xylazine. A plastic canula was inserted into the trachea and airways were lavaged by 10 instillations of 0.5 mL ice-cold saline supplemented with 2.6 mM EDTA (saline-EDTA).

Total and differential cell counts in the BALF were performed after centrifugation (300 g for 5 min at 4 °C) to pellet cells. Erythrocytes were lysed by hypotonic shock by addition of 1.5ml distilled H₂O, followed by addition of 0.5ml KCL 0.6M. Cells were centrifuged and resuspended in 500 μ L ice-cold saline-EDTA and total cell counts were determined using a haemocytometer (Neubauer's chamber). Differential cell counts were assessed on cytologic preparation obtained by cytocentrifugation (Cytospin 4, Shandon Ltd) of 200 μ L of diluted BALF (250 000 cells/mL in ice-cold saline-EDTA). Slides were stained with Hemacolor (Merck), and counts were performed on at least 400 cells for each preparation. Differential counts were expressed as absolute numbers or as a percentage of the total number of cells. BAL and differential cell counts were performed as described previously

Measurement of airway responsiveness by whole body plethysmography. Airway responsiveness to aerosolized methacholine (MCh) at increasing concentrations (0 - 0.3 M; A2251, Sigma-Aldrich) was measured in unrestrained conscious and spontaneously breathing animals by whole-body barometric plethysmography (Emka Technologies), using the enhanced pause (penh) as an index of airway obstruction as reported [18]. Briefly, mice were stabilized in the plethysmograph chamber for 30 min until stable baseline, and then exposed to aerosolized saline (30 sec) as a control. Then, mice were challenged every 20 min with increasing doses of aerosolized MCh for 30 sec each, and airway responses recorded for 5 min.

Measurement of airway resistance by the forced oscillation technique. Airway responsiveness to methacholine was assessed using the forced oscillation technique (Flexivent®, SCIREQ, Montreal, Canada) on another study groups of mice as described previously [19]. Mice were anesthetized with an intraperitoneal injection of 6ml/kg of xylazine (Rompun®;0.25%), followed 50 minutes later by an intraperitoneal injection of 6ml/kg of pentobarbital sodium (0.9%). The trachea was exposed and an 18-gauge metal needle was inserted into the trachea. Airways were connected to a computer-controlled small animal ventilator, and quasi-sinusoidally ventilated with a tidal volume of 10 ml/Kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. Each mouse was challenged for 10 sec with an aerosol, generated with an in-line nebulizer and administered directly through the ventilator: PBS for the baseline and followed by MCh challenge at 50 mg/ml. For calculation of the effect of methacholine, the peak response was used as the mean of the three maximal

values, and integrated for calculation of airway resistance (R), expressed as cm H₂O.s.ml⁻¹.

Histological analysis. Lung tissues were fixed (4% paraformaldehyde) and paraffin-embedded. 3- μ m sections were cut, mounted on Superfrost glass slides (Fischer Scientific), and stained with HES or periodic acid-Schiff (PAS) or toluidine blue or Picrosirius (all powder from Sigma-Aldrich). To determine the severity of the mucus production, the perimeters of bronchus with epithelial cells containing the mucus were measured and reported to the total bronchus perimeter. Area of picrosirius stained collagen was measured and toluidine blue positive mast cells were counted and reported to the slide area. Measure were realized with the *Cell dimension software* coupled to a DP72 camera (Olympus) on 6 levels for each animal.

ELISAs. IL-4 and IL-5 (BD Pharmingen), CCL11 and CXCL12 (R&D systems) ELISA kits were used according to the manufacturer's instructions. IL-4, IL-5 and CCL11 were quantified in BAL fluids recovered 24h after the last OVA challenge and CXCL12 were measured in plasma, BAL, urine, and in the supernatants obtained from the lung or gall-bladder tissues after they were grounded with an UltraTurax® and centrifuged at 10,000 g for 10 min at 4°C.

Ovalbumin-specific IgE plasma level were determined by ELISA. Microtiter plates were coated overnight at 4°C with the capture antibody: anti-mouse IgE at 2 μ g/well in carbonate buffer, pH 9.5 (BD pharmingen, clone R35-118). After three washings with phosphate-buffered saline containing 0.05% Tween-20, microtiter plates were blocked for 1 h at 37 °C with 10% bovine serum albumin in phosphate buffered saline. After three washings with phosphate-buffered saline containing 0.05% Tween-20, plasma samples were incubated for 2 h at room temperature. After three washings with phosphate-buffered saline containing 0.05% Tween-20, an ovalbumin-HRP (BUF048, AbDserotec) was added to the wells and incubated for 1 h at room temperature. The plates were washed and the horseradish peroxidase substrate, tetramethylbenzidine (BD Pharmingen) was added and coloration allowed to develop for 15–20 min. The reaction was stopped by addition of 0.5 M H₂SO₄ and absorbance intensity was read at 450 nm. Plasma levels of ovalbumin-specific Igs were expressed as optical densities (O.D).

Colorimetric assay of mucus, collagen and tryptase. Mucus in BALF was measured by a mucus colorimetric assay: 100 μ l samples were added to 20 μ l acid mucus reagent (0,2% periodic acid, 7% acetic acid), incubated 1 hour at 37°C and 20 μ l mucus dye reagent (sodium metabisulfite 20%, pararosaniline 1%, 10N hydrochloric acid 1%) were added. Samples were incubated for 30 min at 37°C and optical density was measured at 555 nm. Collagen in the lung was measured using a collagen colorimetric assay. Right lobes of the lung were kept frozen at -20°C until use. Lung were grounded with an UltraTurax® and centrifuged at 10,000 g for 10 min at 4°C. The supernatants were used for collagen measurement: 10 μ l samples are added to 200 μ l collagen dye reagent (Sirius red 0.017%, and picric acid 0.8% in absolute ethanol), vortexed for 30 min at 200 rpm at room temperature, centrifuged at 10000 g for 10 min at 4°C and supernatants were removed. Pellets were solubilized with 200 μ l Alkali

collagen reagent (absolute ethanol 20%, sodium dodecyl sulfate 1.3%, 2N sodium hydroxide 12%) and optical density measured at 540 nm.

Tryptase in BMMC culture supernatant was measured by a tryptase colorimetric assay: 50 μ l samples were added to 50 μ l Tryptase reagent (0.6mM Z-Lys-SBZL, 1mM DTNB) and optical density was measured at 410 nm every 20sec to 30min. Tryptase activity in culture supernatant was expressed as optical densities (O.D).

Flow Cytometry. Macrophages, eosinophils, neutrophils and lymphocytes in BALF were identified by flow cytometry analysis using LSRII® cytometer (BD bioscience) as described previously (Daubeuf et al. 2013). Briefly, BALF were centrifuged (300 g for 5 min at 4 °C) to pellet cells and cells were resuspend in 1ml of cold PBS-EDTA 3mM. In a black microplate (0030601807, Eppendorf), 150 μ l of BAL cells were add to 5 μ l of FCblock (553142, BD bioscience) and incubated 20min at room temperature and antibodies were added: CD11C-FITC (557400, BD bioscience), I-A/I-E-PerCPCy5.5 (107626, BioLegend), Gr-1-Pacific Orange (RM3030, Invitrogen), CD11b-APCAlex750 (557657, BD bioscience), CD45-AlexaFluor700 (103128, BioLegend). Antibodies were incubated 30min at room temperature and 5 μ l of 7AAD (BD bioscience) was added and flow cytometry analysis was performed immediately. To detect CXCR4+ and CXCL12+ cells, CXCR4-PE (12-9991, eBioscience) and CXCL12-biotinylated (MAB350, R&D systems) antibodies and Steptavidin-eFluor 650NC (94-4317, eBioscience) was added with the primary antibody cocktail. In others samples, to identified M1 and M2 macrophages, CD197-PE (CCR7, MCA2367PET, AbDserotec) and CD206-biotinylated (MCA2253BT, AbDserotec) antibodies and Steptavidin-eFluor 650NC (94-4317, eBioscience) were added with the primary antibody cocktail.

Alveolar macrophages, BMMC and HEK culture. Mast cells exocytosis: Bone marrow cells derived from 6-week-old mice were cultured for 6 weeks in DMEM medium, supplemented with 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 μ g/ml), 10 ng/ml recombinant murine IL-3 (R&D Systems) and 12.5 ng/ml recombinant murine SCF (R&D Systems). Bone marrow-derived cultured MCs (BMCMCs) phenotype was confirmed by flow cytometry, using anti-Fc ϵ R1 α (MAR-I) and anti-c-KIT (2B8) antibodies (eBioscience). For exocytose analysis, BMCMCs (>98% Fc ϵ R1 α +c-KIT⁺ and >80% toluidine blue positive) were cultured in 24 wells culture plate in complete media: 2.10⁶ cells/400 μ l/well. For stimulation with antigen crosslinking, the BMCMCs were incubated for 16 h at 37°C with anti-OVA antibodies (serum of asthma like mice). Cells were washed once with complete media, pre-treated with solvent (DMSO) or chalc4 (10 μ M) in presence or absence of CXCL12 (50nM) for 20min and were stimulated with PBS or OVA (100 ng/ml) for 30min at 37°C. Supernatant was collected in a Protein LoBind Deepwell plate (0030504100, Eppendorf) and store at -20°C.

Alveolar macrophages release: on day 21, BALF cells from each group of treated mice (HP β CD, chalc4, OVA and OVA-chac4) were centrifuged (300 g for 5 min at 4 °C) to pellet cells and cells were re-suspended in 0.5ml of RPMI 1640

medium, supplemented with 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 µg/ml) and were dispatched in a culture 96 well microplate and incubated 3 hours at 37°C. The microplate was then vortex (1200rpm, 1min) to resuspend all unfixed cells in supernatant and supernatants were immediately removed. The microplate was immediately wash with complete media. Alveolar macrophages were stimulated with LPS (100ng/ml) OVA (100ng/ml) and CXCL12 (50nM) in complete media for 16h at 37°C. Supernatant was collected in a Protein LoBind® Deepwell plate (0030504100, Eppendorf) and store at -20°C and cells were fixed by dehydration with a Concentrator-plus® (Eppendorf) at room temperature and store at room temperature and protect from the light.

Chemokine snatching by Chalcone 4: HEK cells were cultured in Lab-tek® chamber, in DMEM medium supplemented with 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 µg/ml). HEK cells were coated with exogenous CXCL12-TR (10 nM) for 10 min, rinsed with complete DMEM medium, incubated 20 min with 10 µM Chalc4 or chalc1 in complete DMEM medium, rinsed with PBS and fixed with 4% formalin. HEK cells were observed with a confocal microscope (Leica TSC-SPE).

Pharmacokinetic of chalcone 4 and its glucuronid in mouse plasma, BALF, lung and urine. Pharmacokinetic was realized at the TechMed^{ILL} platforme recognized by international ISO9001 certification. Chalcone 4 and its glucuronid were quantified using a triple quadrupole coupled to an ultra-high performance liquid chromatography system (LC-MS 8030, Shimadzu). Data acquisition and analysis were performed using LabSolutions version 5 software. Measurements were carried out at 40°C. A 1.7 µm Kinetex column (50 X 2.1 mm) purchased from Phenomenex was used. The mobile phase flow rate was fixed at 0.5 mL/min and the following program was applied for the elution: 0-1.2 min, 5-95% B; 1.2-1.4 min, 95% B; 1.4-1.42 min, 95-5% B and 1.42-2.8 min, 5% B. Solvent A consisted of 0.05% formic acid in water and solvent B was HPLC grade acetonitrile. 1 µL was injected. The mass spectrometer was interfaced with the UHPLC system using an electrospray ion source. The instrumental parameters were as follows: nebulizer gas, 2 L/min; DL temperature, 250°C; block heater temperature, 400°C; interface voltage, 4.5 kV; collision energy, -40 and -20V. The collision gas used was argon at 230 kPa. The MRM transitions were m/z 287.0 → 272.1, and 463.0 → 272.1 respectively for chalcone 4 and glucuronid. The dwell time was set to 40 msec and the pause time to 3 msec. Blood, BALF, lung, and urine were collected from mice at the following times: 2, 5, 10, 15, 30 and 60 min (3 mice per time point). Absolute quantification was done for chalcone 4 from linear calibration curves. Only relative quantification was performed for the glucuronid.

Quantification in plasma. Blood samples were collected from mice into microtubes containing EDTA as an anticoagulant (Terumo, Capiject®, NM243), and centrifuged immediately (2 000 g, 10 min, 4°C). An aliquot (200 µL) of each plasma sample was accurately transferred to a microcentrifuge tube and frozen in liquid nitrogen. Samples were then stored at -80°C. Blank plasma samples were spiked with chalcone 4 stock solution to prepare calibration samples. Predetermined

volumes of chalcone 4 stock solution were added separately to 200 µL of blank plasma to obtain concentrations of 14, 72 and 144 ng/mL. Chalcone 4 and its metabolite were extracted from the plasma samples by acetonitrile precipitation. 500 µL of acetonitrile were added to each sample, tubes were vortex-stirred and centrifuged (15 000 g, 5 min, 4°C). Supernatants were analyzed by the LC-MS/MS method.

Quantification in lung. Lungs were perfused with saline-EDTA (2.6mM) then rapidly taken and homogenized in 400 µL normal saline with an UltraTurax®. Blank homogenates samples were used to prepare calibration samples (6, 29, 58 ng). Chalcone 4 was extracted from the lung samples by acetonitrile. 1000 µL of solvent were added to each lung homogenate sample. After vortexing for 3 min, the samples were centrifuged (15 000 g, 5 min, 4°C). Supernatants were analyzed by the LC-MS/MS method.

Quantification in urine and bronchoalveolar lavage. Urine was collected with a syringe through the bladder and BALF was obtained as describe previously. Airways were lavaged by 2 instillations of 0.5 mL ice-cold saline supplemented with 2.6 mM EDTA. Urine and BALF samples volumes were variable. Samples were extracted using solid-phase extraction. For each sample, the whole volume was applied on a cartridge (Strata C18-E (55µm, 70A) 50 mg/1mL) after conditioning with 1 mL methanol followed by 1 mL water. The cartridges were then washed with 1 mL water and finally eluted with 1 mL methanol. The solvent was evaporated under vacuum. The dry samples were then dissolved in 100 µL water/acetonitrile 1/1 v/v and analyzed by the LC-MS/MS method. Calibration was performed from blank samples spiked with stock solution of chalcone 4 before the SPE procedure (BAL: 9, 43, 86 ng and urine: 1, 3, 14 ng).

SPECT analysis. In order to recognize different organs, prior to SPECT analysis, a CT image was recorded on the AMISSA platform, a homemade multimodality imaging system for small animals combining X-ray, SPECT and PET devices. The mCT delivered a 3D reconstructed volume of the animal in real time [76]. Targeting of I123-chalcone 4 was monitored via the mSPECT imaging technique. Compound I123-chalc4 was administered by intranasale route in anesthetized mice. mSPECT imaging was performed 4 to 7 minutes post-intranasale administration of 10 MBq of I123-Chalcone 4. The mSPECT system consists of a four head detection gamma camera. Each head comprises five-separated detection modules arranged along a circle of 58 mm with the pinhole as the center. A detection module consists of a YAP:Ce matrix of 8 x 8 scintillating crystals 2.3 x 2.3 x 28 mm³ each coupled to a multianode photomultiplier 8 x 8 (Hamamatsu H 8804). The distance from the pinhole to the axis of rotation is 28 mm and the distance between the pinhole and the crystal is 58 mm which results in a magnification factor of 2.07 [77]. Images were reconstructed using the OSEM (Ordered Subset Expectation Maximisation) iterative algorithm adapted for pinhole imaging. Images were viewed and quantified using the Anatomist freeware (http://brainvisa.info/index_f.html).

Statistical analysis.

Data are presented as means ± SEM. Differences between groups were tested for statistical significance using a two-way ANOVA and Bonferroni post-hoc test or a one-way ANOVA

followed by Tukey post-test. Data were considered significantly different when $p < 0.05$. In figure representing results obtained in repeated studies: statistic analysis was conducted in each study and minimal significance was represented. Data were not pooled for statistic analysis.

RESULTS

Chalcone 4 reduces airway hyperactivity to metacholine in a mouse model of asthma

The *in vivo* activity of chalcone 4 (chalc4) was assayed in a 21-day classic mouse model of asthma. In this model, Balb/c mice are sensitized by i.p. injection of ovalbumin (50 μ g; OVA) adsorbed on 2mg aluminium hydroxide in 0.1 ml saline, on days 0 and 7. Mice are then challenged intranasally with 10 μ g OVA in 25 μ l saline (12.5 μ l per nostril) on days 17, 18, 19 and 20 or saline alone for controls groups. Chalc4 was administered locally 2 hours before OVA or saline challenges by intranasal route at the dose of 300 nmol/kg in 10% HP β CD. The intranasal route mimics the inhalation exposure used in humans, which is the preferred and well accepted administration for inflamed airway treatment, in particular asthma. We previously describes that the chalc4 solubility was limited in physiological solutions and required to be complemented with HP β CD (10%, W/W). Under such condition, the local administration (i.n.) of 300 nmol/kg chalc4 presented the maximal inhibition of the eosinophil influx in a 8-day mouse model of airway hypereosinophilia [15].

Airway reactivity was determined under treatment with i.n. chalc4 at 300 nmol/kg, 24 hours after the last challenge. **Figure 1A** shows airway reactivity to increasing doses of aerosolized methacholine (MCh) was assayed by non-invasive whole body plethysmography measuring the enhance pause [Penh]. Results shows that vehicle (HP β CD) and the Chalc4 presented the same normal airway reactivity to methacholine. OVA-sensitization and subsequent challenge led the development of airway hyperresponsiveness (AHR) as compare to HP β CD and Chalc4 groups challenged with saline. Treatment with Chalc4 significantly inhibited AHR in OVA sensitized/challenged mice by 45 \pm 7%. In addition, the protocol was repeated to assayed airway reactivity by Flexivent[®] technique in anaesthetized mice (**Figure 1B**). Nebulization of PBS showed a similar baseline airway resistance 24h after the last saline or OVA challenge in HP β CD or Chalc4 treated mice whereas nebulization of MCh (50mg/ml) increased airway reactivity in HP β CD and Chalc4 groups challenged with saline and showed AHR in OVA challenged mice. Treatment with Chalc4 inhibited AHR by 37 \pm 8% and confirmed the reduction of the AHR in response to the local treatment with Chalc4. Similar results are shown in elastance (**supplementary Figures S1A**). Analysis of compliance obtain with Flexivent[®] shows a significant decrease in OVA-treated mice. This decrease is no inhibit with chalcone 4 and baseline are not modify in control mice that

received HP β CD and chalcone 4 and challenged with saline (**supplementary Figure S1B**).

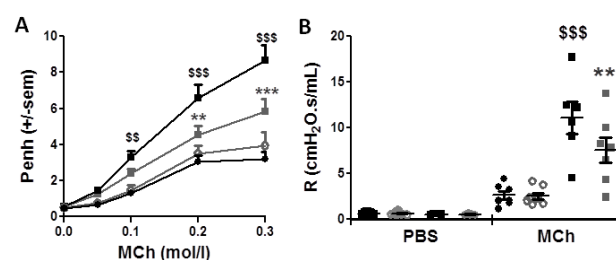


Figure 1. The CXCL12 neutraligand, chalcone 4 reduces airway hyperactivity to metacholine in a mouse model of asthma. Balb/c mice were sensitized to OVA and challenged with OVA or saline. Chalc4 (grey line) or HP β CD (black line) were administered i.n. (300nmol/kg) 2h before each OVA (square) or saline (dots) challenge. **A.** Airway reactivity to increasing dose of metacholine measured by whole body plethysmography in vigil and unrestrained mice. The ordinate axis reports Penh, dots and squares represent means and error bars show S.E. values (n=12/group). **B.** Airway resistance (R, cmH₂O.s.ml⁻¹) in response to aerosolized PBS and MCh (50mg/ml, i.e. 0.26 M, in PBS) assessed by Flexivent[®] technique in anaesthetized mice. Dots and squares are individual values, lines are means and error bars show S.E. values (n=8/group). Statistical analysis consisted of two-way ANOVA and Bonferroni post-test and was conducted with Prism software (GraphPad). \$\$ P \leq 0.01; \$\$\$ P \leq 0.001 vs HP β CD group and **P \leq 0.01, ***P \leq 0.001 vs OVA group.

Topical administration of chalc4 reduces inflammatory cell infiltration and remodeling

Lung tissues collected 24h after the last challenge showed marked peribronchial inflammation in response to OVA challenge (**Figure 2A**). Chalc4 inhibited this infiltration of inflammatory cells. Periodic acid-schiff (PAS) staining revealed a strong goblet cell hyperplasia and mucus overexpression in OVA sensitized/challenged mice (**Figure 2B**). This feature was also significantly inhibited by Chalc4, by 68 \pm 3% (**Figure 2C**) and by 84 \pm 5% in BALF (**Figure 2D**). In complement, Picro-Sirius stained sections showed an increase of collagen deposition around bronchus and vessels in OVA challenged mice as compare to saline challenged mice (**Figure 2E**) Measures on sections revealed that treatment with chalc4 reduced significantly collagen deposition (-84 \pm 18%, **Figure 2F**) and was confirmed in lung homogenate with collagen colorimetric assay (-78 \pm 6%, **Figure 2G**). BALF infiltrate with macrophages, eosinophils, neutrophils and lymphocytes under treatment with i.n. Chalc4 was investigate and repeated 3 times. **Figure 3A** shows that neither vehicle (HP β CD) nor Chalc4 by themselves do elicit any cell recruitment in the airways. After ovalbumin challenge, significant increase in the number of eosinophils, macrophages, neutrophils and lymphocytes occurs (Black squares). Significant reduction in recruitment of eosinophil (-49 \pm 7%, -54 \pm 9% and -59 \pm 6%), neutrophil (-55 \pm 5%, -64 \pm 8% and -82 \pm 8%) and lymphocyte (-45 \pm 3%, -62 \pm 6% and -63 \pm 4%) are noted with Chalc4 treatment (grey squares). However, macrophages recruitment was not significantly reduce in neither protocol.

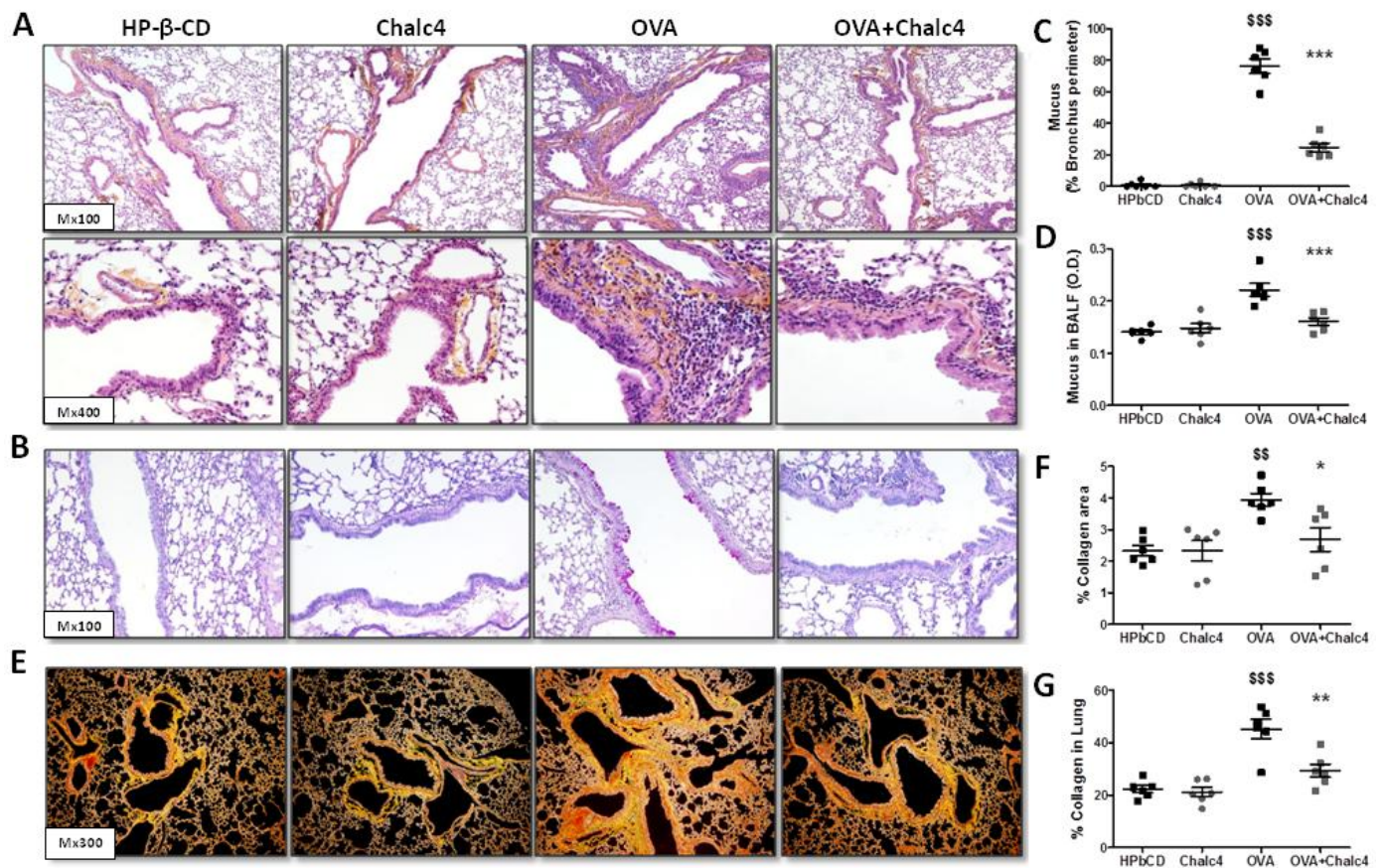


Figure 2. Topical administration of Chalc4 reduces lung tissue inflammatory cell infiltration and remodeling. **A.** HES stained sections demonstrating peribronchial inflammatory infiltrates 24 hours after the last OVA challenge (magnification x100 and x400). **B-D.** Effect of topical treatment with Chalc4 on mucus-producing goblet cells and mucus excretion. **B.** Periodic acid Schiff (PAS) stained lung sections demonstrating hyperplasia of mucus-producing goblet cells (magnification x100). **C.** Mucus-producing goblet cells hyperplasia was measured in lung section from OVA sensitized mice challenged with OVA (squares) or saline (dots) and treated with Chalc4 (grey) or HPβCD (black). The percentage of bronchus perimeter presenting mucus-producing goblet cells is shown. Dots and squares are individual values, lines are means and error bars show S.E. values (n=6/group). **D.** Mucus excretion in BALF measured by mucus colorimetric assay. Dots and squares are individual values, lines are means and error bars show S.E. values (n=6/group). **E-G.** Effect of topical treatment with Chalc4 on collagen deposition in the lung. **E.** Picro-Sirius stained lung sections demonstrating collagen deposition around bronchus and vessels (magnification x300). **F.** Collagen deposition was measured in lung section from OVA sensitized mice challenged with OVA (squares) or saline (dots) and treated with Chalc4 (grey) or HPβCD (black). The percentage of collagen deposition area in lung section area is shown. Dots and squares are individual values, lines are means and error bars show S.E. values (n=6/group). **G.** Collagen in lung homogenate was measured by collagen colorimetric assay and represented as % of collagen in total proteins. Dots and squares are individual values, lines are means and error bars show S.E. values (n=6/group). Statistical analysis consisted of one-way ANOVA and Tukey post-test was conducted with Prism software (GraphPad). ^{\$\$\$} P<0.01; ^{\$\$\$\$} P<0.0001 vs HPβCD group and *P<0.05; **P<0.01; ***P<0.001 vs OVA group.

In addition, **Figure S2** shows the inhibition of the inflammatory cell recruitment in the lung by chalcone 4 is not accompanied with a modification of the white blood cell formula 24h after the last challenge. This result shows that treatment with chalcone 4 reduce infiltration of leukocytes in the lung without lead to an increase of leukocytes in the blood. In order to further document the activity of the chalcone 4 and because mast cells are known to be implicated in myositis and in inflammation in response to the allergen, we studied the activity of the compound on mast cells. Contrary as expected, **Figure 3B** shows that treatment with chalcone 4 does not inhibited the increase number of mast cells in the lung tissue observed in response to OVA challenges. Furthermore, we investigated the mast cell degranulation *in vitro*, in an antigen cross-linking assay for OVA targeting the endogenous FcεRI receptor in sensitized BMDCs incubated with specific anti-OVA antibody. **Figure 3C** shows that OVA increase significantly mast cell tryptase release in culture supernatant

of BMDC stimulated by IgE receptor cross-linking (+145±17%) and does not potentiate with CXCL12. Treatment with chalcone 4 does not impact the release of mast cell tryptase. These results therefore indicate that the chalcone 4 inhibits the inflammatory cell recruitment and the remodeling in the airway occurs in response to the allergen but seems not active on mast cells allergen specific response.

Chalcone 4 reduce cytokines promoting eosinophils recruitment but not the allergen immune response

We next assessed the levels of Th2 cytokine IL-5 (**Figure 4A**) and of the chemokine CCL11 (**Figure 4B**) in BAL fluid, collected 24 h after the last challenge, and known to promote eosinophils recruitment. OVA-treated mice showed significantly increased levels of IL-5 and CCL11 as compare to saline challenged groups. These increases are inhibited by chalcone 4 up to -75±9 % and -52±4 % respectively, without modifying baseline levels of control mice (HPβCD).

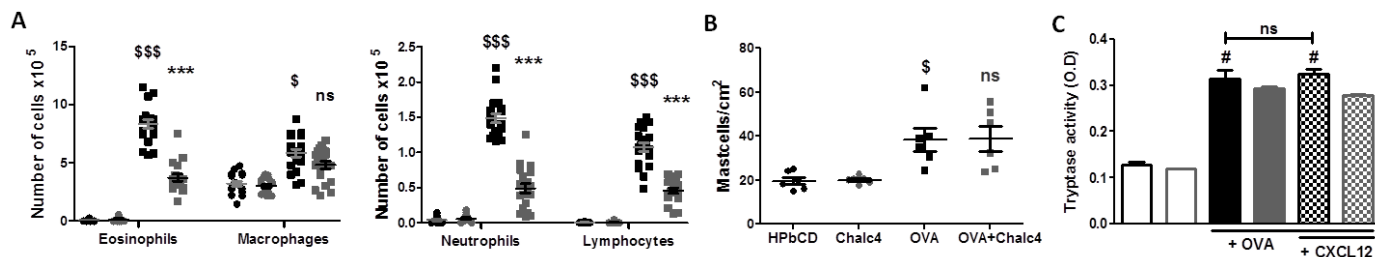


Figure 3. Chalc4 inhibits number of leukocytes in BALF but no interfere with mast cells recruitment in lung and mast cells release. **A.** Effect of local administration of 300 nmol/kg Chalc4 (grey) or HPβCD (black) on absolute number of eosinophil, macrophages, neutrophils and lymphocytes in BAL fluid 24h after the last challenge in Balb/c mice sensitized to OVA and challenged with OVA (square) or saline (dots). Dots and squares are individual values obtained in 3 independent studies, lines are means and error bars show S.E. values (n=6+6+8/group). **B.** Toluidine blue positive mast cells were counted on toluidine blue stained lung sections and report to section area as mast cells / cm². Dots and squares are individual values, lines are means and error bars show S.E. values (n=6/group). **C.** Mast cells release was investigated in vitro. Six week old BMMCs were incubated 16h with specific anti-OVA antibodies and stimulated 30min with PBS (empty blocks) or OVA (100ng/ml, full blocks) for targeting the endogenous FcεRI receptor. BMMCs were pretreated 20 min before OVA stimulation with solvent (DMSO, in black) or chalc4 (10μM, in grey), in presence or absence of CXCL12 (50nM). Tryptase activity was measured in culture supernatant with a colorimetric assay. Blocks are means and error bars show S.E. values (n=3/group: BMMC culture derived from 3 mice). Statistical analysis consisted of two-way ANOVA with Bonferroni post-test and one-way ANOVA with Tukey post-test was conducted with Prism software (GraphPad).[§] P<0.05; ^{§§} P<0.01; ^{§§§} P<0.001 vs HPβCD group and ^{***}P<0.001 vs OVA group. [#] P<0.05 vs PBS-DMSO group.

In contrast, the Th2 cytokine IL-4 in BALF and the OVA-induced immunoglobulin (Ig) in plasma are not decrease. **Figure 4C** and **4D** shows OVA challenges significantly increase levels of IL-4 (+473±41%) and OVA-specific IgE (+87±5%). Treatment with chalcone 4 not reduce IL-4 and both OVA-specific IgE, IgG1 and IgG2a levels (**supplementary Figures S3**).

Again, chalcone inhibits pro-eosinophilic signals according to previous significant inhibition of eosinophil influx. Yet, treatment with chalcone 4 not impact the allergen specific immune response.

Local administration of chalcone 4 inhibits M1 macrophages activation in the lung and prevent cytokine release by macrophages.

As the CXCL12 seems regulated monocyte-macrophages differentiation and activation [49], we investigate the implication of the chalcone 4 in the response of macrophages in the lung in the model of asthma.

First, we investigate the effect of the *in vivo* treatment with chalcone 4 on the macrophages response. BALF were collected 24h after the last challenge and cells were distributed on 96 wells plate, incubated 3 h at 37°C in RPMI 1640 medium, vortexed and non-adherent cells were immediately

eliminated in supernatant. Adherent cells were identified as macrophages (F4/80+) over 97% and homogeneity of proportion and distribution in each well was controlled (**supplementary Figure S4**).

Figure 5A shows the response *ex-vivo* of alveolar macrophages to 50mM CXCL12. Addition of CXCL12 in culture medium lead to secretion of TNFα in supernatant. Although all alveolar macrophage population respond to CXCL12, we find that macrophages from OVA-sensitized and challenged mice exhibit a greatest release of TNFα as compare to macrophages from OVA-sensitized mice challenged with saline. Treatment of mice with chalcone 4 abolishes the great output of TNFα (-98±9 %), without modifying the baseline levels of control mice. According to this results and because macrophages as know to be the major source of TNFα in the lung, we assessed to measure TNFα in the lung homogenate (**supplementary Figure S5A**). TNFα level is significantly increases in the lung homogenate in OVA-treated mice and significantly inhibits by chalcone 4 administrations (-62±11%).

As macrophages seem to be affected by CXCL12 and the treatment with chalcone 4, we measured the release of TNFα (**supplementary Figure S5B**) and IL-5 (**Figure 5B**) by alveolar macrophages in response to OVA.

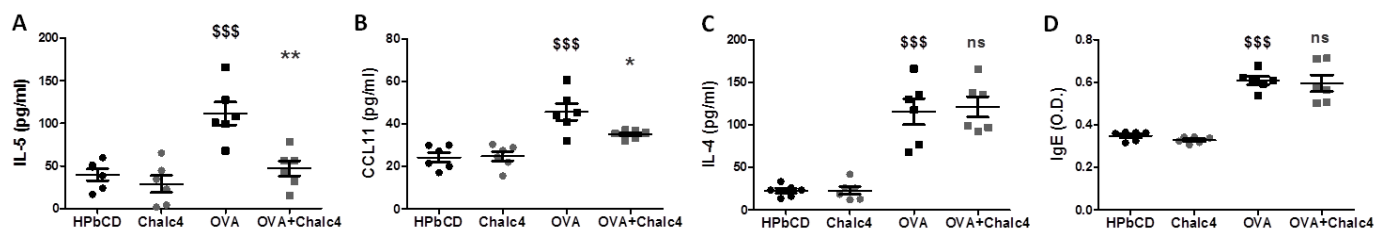


Figure 4. Chalc4 reduces eosinophil chemoattractant agents but remain without effect on immunization against OVA. **A-C.** BAL fluid was collected 24h after the last OVA challenge. The levels of IL-5 (**A**), CCL11 (**B**) and IL-4 (**C**) were determined using ELISA in OVA sensitized mice challenged with OVA (squares) or saline (dots) and treated with Chalc4 (grey) or HPβCD (black). Dots and squares are individual values, lines are means and error bars show S.E. values (n=6/group). **D.** Level of OVA-specific IgE in plasma. Dots and squares are individual values, lines are means and error bars show S.E. values (n=6/group). Statistical analysis consisted of one-way ANOVA and Tukey post-test was conducted with Prism software (GraphPad). ^{§§§} P<0.001 vs HPβCD group and ^{*}P<0.05; ^{**}P<0.01 vs OVA group.

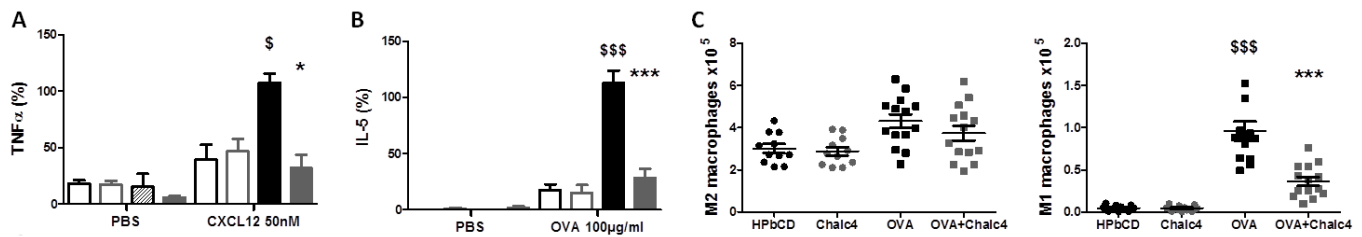


Figure 5. Treatment with Chalc4 inhibits cytokines release by alveolar macrophages in response to CXCL12 or OVA and reduces the recruitment of M1 macrophages in BALF. **A and B.** Alveolar macrophages of OVA sensitized mice challenged with OVA or saline and treated with Chalc4 (grey) or HPβCD (black) were collected in BALF 24h after the last OVA challenge, isolated in 96-wells microplates and stimulated overnight (16h) with CXCL12 (50nM) or OVA (100ng/ml). The levels of TNFα (**A**) and IL-5 (**B**) were determined using ELISA in supernatant. Blocks are means obtained in 2 independent studies and error bars show S.E. values (n=3+6/group). **C.** Direct flow cytometry analysis was used to measure M1 (CD197+, CD206-) and M2 (CD206+) macrophages in BALF, in OVA sensitized mice challenged with OVA (squares) or saline (dots) and treated with Chalc4 (grey) or HPβCD (black). Dots and squares are individual values obtained in 2 independent studies, lines are means and error bars show S.E. values (n=6+8/group). Statistical analysis consisted of one-way ANOVA with Tukey post-test and two-way ANOVA with Bonferroni post-test was conducted with Prism software (GraphPad). [§] P≤0.05; ^{\$\$\$} P≤0.001 vs HPβCD group and ^{*}P≤0.05; ^{***}P≤0.001 vs OVA group.

Ovalbumin increase release of TNFα and IL-5 by macrophages with a significantly greatest release in macrophages from OVA-sensitized and challenged mice. Treatment of mice with chalcone 4 inhibit the release of TNFα (-99±8 %) and IL-5 (-91± 5%) by macrophages and not modify baseline in control mice.

Second, we investigate the activation of macrophages in BAL by direct flow cytometry analysis of BALF cells in twice experiments. **Figure 5C**, reveals that the M2 macrophage population (CD206+) is stable in mice regardless of mice treatment and is not affect by OVA challenges. On the contrary, ovalbumin challenges increase significantly the M1 macrophage population (CD206-, CD197+) as compare to saline challenge and is significantly reduce by chalcone 4 (-61±9 % and -62±11 %). HPβCD and chalc4 administration in saline challenged mice not modify the baseline population of M1 macrophages.

Local treatment with Chalc4 reduces the level of the chemokine CXCL12 in the lung

Regarding to chalcone 4 is a neutraligand of the CXCL12 chemokine, we assay the level of CXCL12 in lung. Lung tissues were homogenate, centrifuge and supernatant were used to measure CXCL12. **Figure 6** shows the percent of CXCL12 chemokine in lung. Surprisingly, our results show that CXCL12 is not increase in OVA-treated mice as compare to saline challenged mice. Treatment with chalcone 4 significantly reduce the level of CXCL12 in the lung by -16±3 % in saline challenged mice and by -20±2% in OVA challenged mice as compare to HPβCD treated mice. Study was reproduced twice with similar results: chalcone 4 significantly reduce the level of CXCL12 in the lung by -25±3 % in saline challenged mice and by -20±2 % in OVA challenged mice as compare to HPβCD treated mice.

The dose-response curve of CXCL12 detect by ELISA (**supplemental Figure S6**) in absence or presence of HPβCD and Chalc4 is not impact and comfort the ability of the ELISA to detect CXCL12 and the results show above.

Chalcone 4 disappears rapidly from the lungs after intranasal administration

Iodo-chalcone 4 is the homolog of the chalcone 4 and prevents binding of Texas Red-labeled CXCL12 (CXCL12-TR, 100

mM) to EGFP-tagged CXCR4, determined by fluorescence resonance energy transfer (FRET) with similar affinity as chalcone 4 (Ki = 45 ± 57 nM for chalcone 4 vs Ki = 25 ± 35 nM for I-chalcone 4). The *in vivo* activity of the I-chalcone 4 was assayed in a recently developed 8-day mouse model of airway hypereosinophilia [12] and compare to chalcone 4. Compound were administered intranasally in HP-βCD (10% w/w) at the dose of 300 nmol/kg, 2 hours before OVA challenges. **Supplementary Figure S7** shows that eosinophil counts in BALF are inhibited in like manner by both chalcone 4 and I-chalcone 4 respectively by 48±4% and 45±5%. We notice that the molecule I-chalcone 4 is as potent as chalcone 4. **Figure 7A** and **7B** shows the monitoring of I₁₂₃-chalcone 4 with single photon emission computed tomography (SPECT) after intranasal administration. Following the administration, I₁₂₃-chalcone 4 is distribute in the airway between nose, trachea and lung and is equally present in the gall-bladder. At time = 25min, SPECT-CT image reconstitution shows the chalcone 4 disappearing rapidly of the lung and is newfound in bowels and bladder. SPECT-CT image reconstitution shows that only one hour after intranasal administration, I₁₂₃-chalcone 4 is no longer present in lung, the compound is exclusively found in gall-bladder, bowels and bladder. These results is confirm by the measure of the I₁₂₃-chalcone 4 isotope activity in tissues 5 min and 30 min after intranasal.

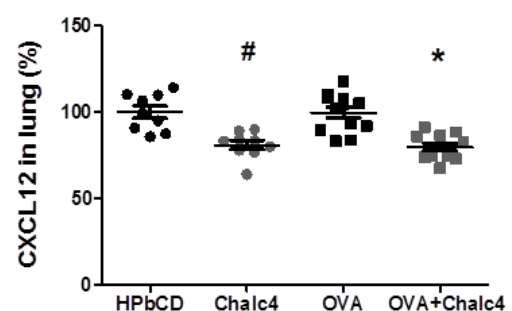


Figure 6. Treatment with Chalc4 reduces the level of the chemokine CXCL12. Lung of OVA sensitized mice challenged with OVA (squares) or saline (dots) and treated with Chalc4 (grey) or HPβCD (black) were perfused, collected and homogenate then the chemokine CXCL12 level was determined using ELISA. Dots and squares are individual values obtained in 2 independent studies, lines are means and error bars show S.E. values (n=6+6/group). Statistical analysis consisted of one-way ANOVA and Tukey post-test was conducted with Prism software (GraphPad). [#] P≤0.05 vs HPβCD group and ^{*}P≤0.05 vs OVA group

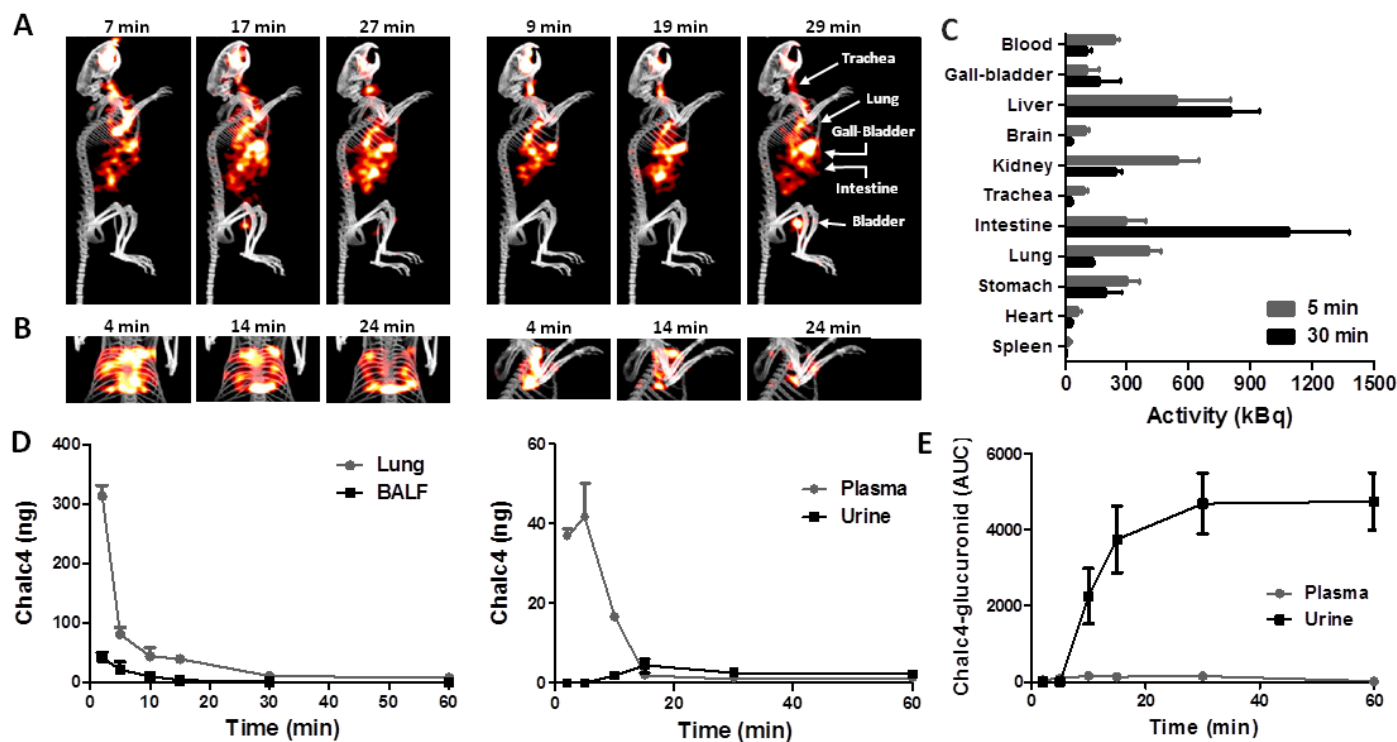


Figure 7. Chalc4 is rapidly eliminated from the lung after intranasal administration. A-C. Chalc4-¹²³I was administered i.n. with Chalc4 to a final dose of 300nmol/kg and followed by tomography (SPECT) in Blab/c mice. Representative SPECT-CT image reconstitution of Chalc4-¹²³I distribution in whole body (A) and lung (B). C. Chalc4-¹²³I specific activity in several organs 5min and 30 min after intranasal administration. D. Lung, BALF, plasma and urine concentration-time curve of Chalc4 after i.n. administration (300 nmol/kg). Dots are means and error bars show S.E. values (n=3). E. Plasma and urine concentration-time curve of Chalc4-glucuronid metabolite following Chalc4 i.n. administration (300 nmol/kg). Dots are means and error bars show S.E. values (n=3).

Figure 7C shows the fast distribution of the radioactive compound and elimination of the chalcone 4 in bile and urine. I123-chalcone 4 through the lung tissues, drive to the liver and kidneys in blood, and eliminate in bile and urine. Because SPECT image reconstitution is limited by image acquisition time and to precise pharmacokinetic of the chalcone, chalcone 4 was administered intranasally and BALF, plasma, urine and lung were harvest and frozen in liquid nitrogen to measure chalcone 4 (**Figure 7D**) and metabolite (**Figure 7E**) in UHPLC-MS/MS. Concentration-time curve of local administration of 300 nmol/kg chalcone 4 show a quick decrease in BALF and lung (6 fold less, of 313ng to 44 ng between 2 and 10 min) and is accompanied with an increase of chalcone 4 in plasma, with a peak at 5 min (42 ng) then the compound decrease quickly to 2 ng at 15 min (20 fold less). Chalcone 4 can be measure in urine with a peak at 20 min (4.5 ng). In the same time, concentration-time curves of Chalcone 4 glucuronid, the major metabolite determined in microsomal stability assay, show a fast increase of chalcone 4 glucuronid in plasma and in a large amount in urine with a maximum at 30 min (**Figure 7E**)

Therefore, the intranasal administration deliver the chalcone 4 into the airway and especially in lung and is follow by a quick absorption, distribution and elimination of the chalcone and is characterized by half-life times usually considered as "fatal" in the development of a drug: $T_{1/2} < 5$ min in lung (the target), $T_{1/2} < 10$ min in plasma and an elimination $>95\%$ of the compound in less than 30 min.

Chalc4 intranasal administration take away the chemokine CXCL12

Our result highlight that chalcone 4 is active in a mouse model of asthma after local administration and eliminate from the lung tissues in few minutes and more broadly from the mouse tissues in less than 30 min. This fact reveal a gap between the activity and the pharmacokinetic.

As chalcone 4 elimination occurs rapidly, we investigated its neutraligand activity. We measure the CXCL12 concentration in plasma and BALF after intranasal administration of chalcone 4 or chalcone 1, the unsubstituted chalcone backbone already report as inactive on CXCL12 binding to CXCR4 [15, 28]. **Figure 8A** shows that i.n. administration of chalcone 4 increase by 1000 fold the plasma concentration of CXCL12. The kinetic is the same as chalcone 4, with a peak at 5min follow by a rapid back to standard concentration, in 1h. The concentration-time curve measured in BAL shows the same profile. In contrast, i.n. administration of chalcone 1 does not promote any increase in CXCL12 concentration in BAL and plasma. In addition, each i.n. administration of chalcone 4 induce an increase of CXCL12 in plasma 5 min after instillation (**Figure 8B**) and reveal that the increase observed is lower after 3 administrations. **Figure 8C** shows the same change in BALF. In parallel, **Figure 8D** shows the decrease of CXCL12 in lung homogenate after each intranasal administration of chalcone 4 up to $-20 \pm 4\%$. These results highlight that local treatment with the chalcone 4, neutralizes the chemokine CXCL12 of the lung tissues and take it away into the bloodstream where they are removed rapidly. This phenomenon appears to be very fast and linked to the pharmacokinetic of the chalcone 4.

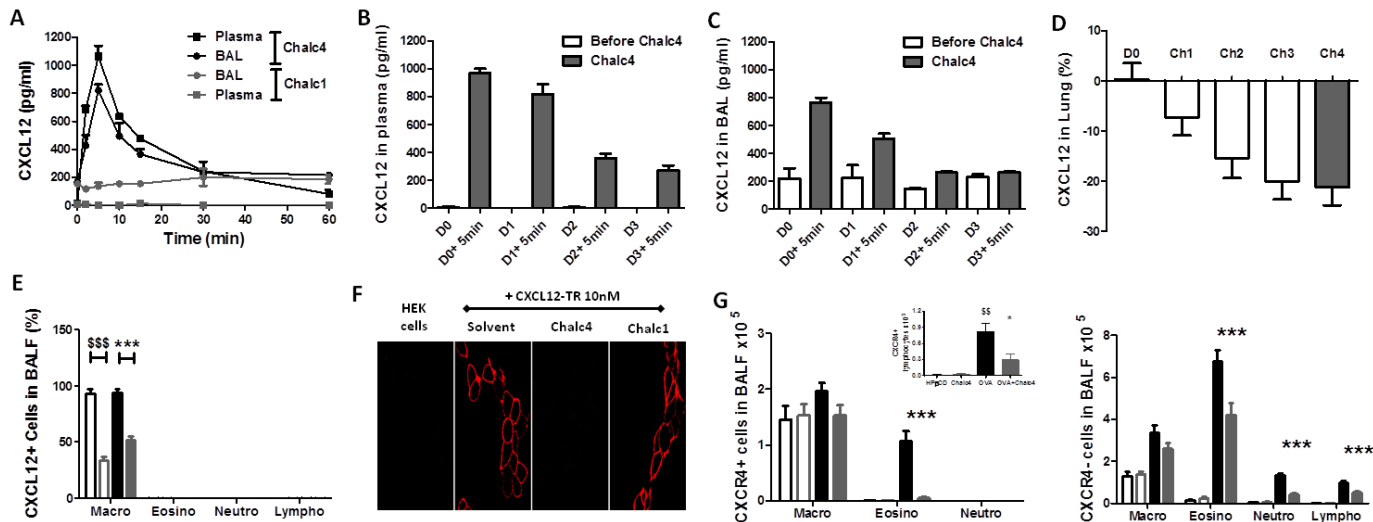


Figure 8. Chalc4 intranasal administration take away the chemokine CXCL12. **A.** Plasma and BALF concentration-time curve of CXCL12 after i.n. administration of 300 nmol/kg Chalc4 or Chalc1. CXCL12 level was determined using ELISA. Dots are means and error bars show S.E. values (n=3). **B and C.** CXCL12 concentration in plasma and BALF before and 5 min after 1 to 4 daily administrations of Chalc4 (300 nmol/kg). Blocks are means and error bars show S.E. values (n=3). **D.** CXCL12 concentration in lung 24 hours after 1 to 4 daily administrations of Chalc4 (300nmol/kg). Blocks are means and error bars show S.E. values (n=3). **E.** Flow cytometry analysis of CXCL12 on the surface of BALF inflammatory cells from control (empty blocks) or OVA sensitized and challenged mice (full blocks) following incubation 30min with 5 μ M Chalc4 (grey) or HP β CD (black). Data are represented as % of CXCL12+ cells in respective cell populations. Blocks are means and error bars show S.E. values (n=3). **F.** Confocal image of HEK cells coated with CXCL12-TR 10 nM before and after incubation with 10 μ M Chalc4 or chalc1. **G.** Flow cytometry analysis of CXCR4+ macrophages, eosinophils, neutrophils and lymphocytes from OVA sensitized Balb/c mice challenged with OVA (full blocks) or saline (empty blocks) and treated with Chalc4 (grey) or HP β CD (black). Blocks are means obtained in 2 independent studies and error bars show S.E. values (n=6+8/group). Statistical analysis consisted of two-way ANOVA with Bonferroni post-test was conducted with Prism software (GraphPad). ^{SSS} P \leq 0.001 vs HP β CD group and ^{***}P \leq 0.001 vs OVA group.

Moreover, the ability of the chalcone 4 to neutralize and capture the chemokine CXCL12 was confirmed on BALF cells by flow cytometry. In **Figure 8E**, we show that unlike other BALF cells, alveolar macrophages are naturally coated with CXCL12 in OVA sensitized mice challenged with saline or OVA. *Ex vivo* incubation with chalcone 4 leads a significant decrease in CXCL12 (50%) on the membrane of macrophages (**Figure S8**).

In **Figure 8F**, the use of HEK cells and exogenous CXCL12-TR confirmed the ability of chalcone 4 to capture the chemokine bind at the cell surface. CXCL12-TR is bind at the cell surface then chalcone 4 is added in the culture medium and incubated. After rinse, pictures show CXCL12-TR is suppress from the cell membrane.

Furthermore, flow cytometry analysis of the expression of the receptor CXCR4 on BALF inflammatory cells (**Figure 8G and S9**) shows this expression is limit to a fraction of macrophages ($\pm 40\%$), eosinophils ($\pm 15\%$) and lymphocytes ($\pm 1.4\%$). In BALF, no neutrophils and only few lymphocytes express the receptor CXCR4. CXCR4+ macrophages population seems to be stable between OVA sensitized mice challenged with saline or OVA and treated with HP β CD or chalcone 4. OVA sensitization and challenges increase significantly CXCR4+ eosinophils in BALF. Treatment with chalcone 4 significantly abolishes the influx of CXCR4+ eosinophils ($-98\pm 1\%$). Regarding to CXCR4+ lymphocytes, OVA challenges increase significantly the recruitment of CXCR4+ lymphocytes in BALF and this is significantly inhibited by treatment with chalcone 4 ($-69\pm 6\%$).

Macrophages release CXCL12 in response to allergen

While flow cytometry analysis reveals that macrophages expressed CXCL12 at the membrane, immunocytofluorescence against CXCL12 clearly demonstrated that alveolar macrophages from BAL expressed CXCL12 in the cytoplasm and suggests that macrophage could release CXCL12 (**Figure 9A**). The *in vitro* stimulation of alveolar macrophages by LPS or ovalbumin followed 24h after by the measure of CXCL12 chemokine in supernatants confirmed that macrophages is able to release the CXCL12 chemokine (**Figure 9B**). Surprisingly, macrophages from OVA sensitized and challenged mice release more CXCL12 than macrophages from control mice and support that CXCL12 release should be increase after ovalbumin challenges.

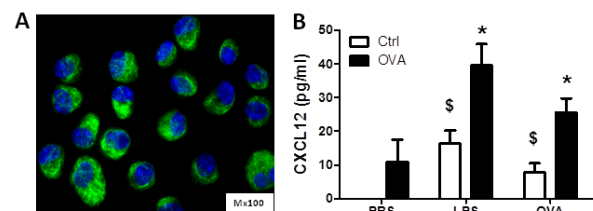


Figure 9. Macrophages release CXCL12 in response to LPS and allergen. **A.** Immunocytofluorescence of CXCL12 chemokine (green) on permeabilised cytopun macrophages. Nuclei are counterstain with DAPI (blue). **B.** Alveolar macrophages of OVA sensitized mice challenged with OVA (black) or saline (white) were collected in BALF 24h after the last OVA challenge, isolated in 96-wells microplates and stimulated overnight (16h) with LPS (100ng/ml) or OVA (100ng/ml). The levels of CXCL12 was determined using ELISA in supernatant. Blocks are means and error bars show S.E. values (n=6/group). Statistical analysis consisted of two-way ANOVA with Bonferroni post-test was conducted with Prism software (GraphPad). $\$$ P \leq 0.05 vs Ctrl/PBS group, *P \leq 0.05 vs Ctrl group, $\$ \$ \$$ P \leq 0.001 vs HP β CD group, and ^{***}P \leq 0.001 vs OVA group.

DISCUSSION

Our results show the anti-asthmatic effect of a CXCL12 neutraligand in a mouse model of acute allergic asthma. We previously described that the chalcone 4 is an efficient blocker of CXCL12 binding to CXCR4, and of the associated inhibition of cAMP production [28]. This compound is stable in biological fluids but also poorly soluble in physiological solutions and require to be complemented with HP β CD (10%, W/W) in order to be administrate locally at an efficient dose [15,25].

Several models of allergic asthma have been developed in mice and have revealed that disparities exist between mouse strains, protocols and the response against allergens. The ability to induce asthma features varies greatly among the different strains. Among the most widely used strain, BALB/c mice typically mount Th2-dominated immune responses, and the induction of parameters of allergic responses such as allergen-specific IgE, AHR, and eosinophilic airway inflammation are robust. Although all models promote a T helper type 2 (Th2) cell-based pulmonary inflammation, mouse models of allergic asthma are classically divided in 2 groups as acute and chronic models [51]. Acute models are defined as an artificial asthma-like reaction induced in the airways in few weeks and are generally constituted by a sensitization phase with adjuvant and a challenge phase composed with multiple local ovalbumin administration [32,45]. In contrast, chronic models have been developed to more closely mimic the human disease defined as a chronic inflammatory and required to multiply allergen exposures over many weeks without adjuvant and with extracts or purified proteins derived from potent human allergens such as HDM extract and/or grass pollen. Nevertheless, it appears that the acute ovalbumin models was used in development of many drugs and therapeutic strategies as corticosteroids, calcineurin inhibitor, thromboxane antagonist, leukotriene antagonist, anti-IgE, anti-IL-5, anti-IL-4, anti TNF- α and are preferred models to develop anti-asthmatic strategies [45].

For these reasons, we decided to assess the anti-asthmatic properties of chalcone 4 using an acute model of allergic asthma consisted of sensitization of Balb/c mice by intraperitoneal (i.p.) injection of chicken egg ovalbumin (OVA) adsorbed on the adjuvant aluminum hydroxide (alum), followed by repetitive intranasal (i.n.) challenges with OVA.

In this acute model to OVA, local treatment with 300nmol/kg chalcone 4, by the intranasal route, decrease significantly asthma symptoms as AHR, inflammatory cells recruitment and remodeling in the lung. Airway hyperresponsiveness (AHR) to methacholine was assessed with two exploratory techniques, by barometric whole body plethysmography and the forced oscillation technique (Flexivent®). AHR in response to MCh induce by ovalbumin is significantly inhibited by the local administration of chalcone 4 in non-invasive and was confirmed in invasive methodology. Inhibition of mucus production and collagen deposition in the lung by the chalcone 4 is accompanied with an inhibition of inflammatory cell recruitment in the lung and a significant decrease of eosinophils, neutrophils and lymphocytes in BALF. This concurs with the chalcone 4-

induced inhibition of Th2 cytokine production as demonstrated in this study for IL-5, CCL11 in BALF and TNF α in lung homogenate.

Surprisingly, chalcone 4 does not reduce IL-4 production measured in BALF and does not affect OVA-specific immunoglobulins levels in plasma. IL-4 is predominantly produce by Th2 lymphocytes and IL-4 is known to promote activated B-cell proliferation, the differentiation of B cells into plasma cells and to induce B-cell class switching to IgE [37]. Moreover, chalcone 4 does not affect mast cell recruitment and also their ability to release protease and inflammatory mediators in response to ovalbumin. This results suggests that CXCL12 is not implicated in the recruitment of mast cells although mast cells are known to expressed CXCR4 and precursor of mast cell population can be recruited by CXCL12 [26,70]. The absence of activity of CXCL12 neutraligand on mast cell is support by the down regulation of CXCR4 observed upon IgE-mediated antigen stimulation [38]. However, our result about mast cells requires to be moderate because it was demonstrated that the presence of adjuvant (alum) leads to inflammation, IgE production and airway hyperresponsiveness independent of mast cells [78], suggesting that an asthma model without alum may be more relevant to study mast cells recruitment to CXCL12. On the other hand, elevated level of IL-4 was report of induce mastocytosis in allergic response [10] and promote mast cells to produce IL-4 in response to an allergen linked to the complex formed by high-affinity Fc receptor (Fc γ RI) and IgE [47,50] which suggests that elevated IL-4 level may be implicated in the maintain of mast cell recruitment.

Before the first challenge with allergen, one of the prominent immune system cells found in the airways is the alveolar macrophages. Traditionally characterized as the first line of defense against foreign invaders, the role of alveolar macrophages extends to developmental processes, maintenance of tissue homeostasis and have been implicated in the development and progression of asthma [8]. Macrophages are also known to express CXCR4 [33] and can be able to release CXCL12 [49]. In addition, activation of the receptor CXCR4 by the chemokine CXCL12 may lead to a rapid extracellular release of TNF α [5] and macrophages are one of major origin of TNF α in the lung [48]. TNF α is an important pro-inflammatory cytokine in asthma notably involved as a chemoattractant for neutrophils and eosinophils and in the activation of, and cytokine release by, T cells [4]. According to these facts, alveolar macrophages may represent an important target in the treatment with CXCL12 neutraligand. Our results show that 24 hours after the last challenge, alveolar macrophages in BALF expressed CXCR4 and are coated with CXCL12. We shows that alveolar macrophages from OVA sensitized mice are able to release TNF α in response to CXCL12, and chalcone 4 suppress the increase observed in macrophages from OVA challenged mice. Alveolar macrophages release also TNF α and IL-5 in response to OVA and they are reduce by treatment with chalcone 4.

Classically (M1) and alternatively (M2) activated alveolar macrophages has been differentiate by flow

cytometry with two classic membrane markers: the CCR7 receptor (CD197) for M1-like macrophages [57,58,59,60,61] and the mannose receptor (CD206) for M2-like macrophage [56,61,62,63,64]. Analysis of alveolar macrophages activation state reveals that BALF macrophage population is constituted by 100% of M2 activated macrophages in control Balb/c mice sensitized with OVA/alum. Ovalbumin challenges induce M1 activated macrophages influx in BALF and is reduce by chalcone 4. M1 activated macrophages are described to be pro-inflammatory and release profusely TNF α [6,41], and also to be associated with antigen presentation function [82]. In addition, the marker used to study the polarization of M1 macrophages, the CCR7 receptor, is also know to be a marker for maturation of antigen presenting cells or APC [83,84] and is involved in the migration of APCs to lymph nodes in response to the chemokines CCL19 and CCL21 [85,86]. Maturation of APCs is also accompanied by overexpression of the CXCR4 receptor [87] and the CXCL12 chemokine contributes to the migration of CCR7+ cells to the lymph nodes [88,89,90,91]. These findings suggests that the cell population identified as M1 macrophage (CD45+, CD11b+, GR1-, MHCII+, CD11c+, CCR7+) may also be considered as APCs. Chalcone 4 reduces the activation of APC-like cells and suggests that CXCL12 is implicated in the activation of macrophages in response to allergen. chalcone 4 may be also susceptible to disrupt the migration of APCs to the lymph nodes, and contributes to reduce the allergic response.

In addition, our results show alveolar macrophages represent an important source of IL-5, therefore regulation of macrophage activation state and their response to OVA by chalcone 4, decreases eosinophil recruitment in the airways following the inhibition of the recruitment and activation of the lymphocytes, but also directly with a decrease of IL-5 release. The inhibition of the eosinophil influx is complete by the direct neutralization of CXCL12 by chalcone 4. Flow cytometry analysis of CXCR4+ cells in BALF show that OVA sensitization and challenges induce the recruitment of CXCR4+ eosinophils in BALF. In OVA sensitized/challenged mice, CXCR4+ eosinophils represented 14% of the eosinophil population and is totally abolished by chalcone 4 treatment. The percent of CXCR4+ eosinophils measured in BALF, is consistent with previous studies that have demonstrated a low expression of CXCR4 on eosinophils in blood or in BALF of asthmatic patients [35, 42].

The dissection of the pharmacokinetic of the chalcone has led us to study the interaction between CXCL12 and the neutraligand and leads to describe an original mechanism. Local administration of chalcone 4 take away the chemokine CXCL12 through the lung, in the blood stream where they are removed rapidly. Chalcone 4 is eliminated by the liver, in the bile or in urine in the form of glucuronid-chalcone 4. For CXCL12, the chemokine is not found in bile or urine (data not shown) and its rapid elimination of the blood stream suggests the presence of active mechanisms. CXCR7 is known to be a scavenger receptor for CXCL12 [30,43], is highly expressed on endothelial cells and a recent article suggests that CXCR7 on the surface of the vascular endothelium can regulate the plasma concentration of the chemokine CXCL12 [92]. According to these findings, we

suggest that CXCL12 in the blood stream binds to CXCR7 receptors to be internalized and degraded.

Furthermore, the neutralization of CXCL12 by chalcone 4 in the lung leads to record a pharmacokinetic usually considered as "fatal" and which usually results in the elimination of the drug candidate [31]. The pharmacokinetic is a key component in the development of new drug and often considered before *in vivo* activity in the choice of drug candidate. Our results show the necessity to be careful in the setting up of an early drug discovery plan and put question about the place of the pharmacokinetic in the rational governing the early drug discovery and the choice of new candidate.

In our study, the chalcone 4 is able to bind CXCL12 chemokine and loosed the chemokine from glycosaminoglycans (GAG) at the cells surface and its pharmacokinetic is correlate with the trapping of CXCL12. Repeated intranasal administration of chalcone 4 results in a decrease of the chemokine CXCL12 in the lung. After 4 intranasales, the amount of CXCL12 measured by ELISA is reduce by 20% in control and OVA challenged balb/c mice. Moreover, in our hands, CXCL12 production is not affect in OVA sensitized and challenged mice. This observation is also report with immunohistochemical detection in lung tissue shows no change [27], while immunochemical determination in BALF [44] and gene expression in lung [23] conclude that CXCL12 is up-regulated. The most likely explanation is that CXCL12 production appears to be dependant of the mouse strain [80].

In addition, our results confirmed that macrophages are able to release CXCL12 according to the literature [49] and suggest that M1 macrophages are more sensitive to LPS or OVA. Moreover, Sánchez-Martín describe an autocrin release of CXCL12 in macrophages able to increase CD14 expression in response to LPS. In accord with this description, our results suggest that allergen may induce a similar autocrin release of CXCL12, able to module macrophage activation and APC like differentiation.

In conclusion, our results show a strong antiasthmatic activity of chalcone 4 administered intranasally in a mouse model of acute asthma. Local administration of chalcone 4 neutralizes then takes away the chemokine CXCL12 and reduce CXCL12 in lung by 20% after four intranasales administrations. This trapping of CXCL12 by the neutraligand inhibits the macrophages differentiation in M1 macrophages in response to the allergen and reduces the release of pro-inflammatory cytokines by macrophages. These findings may lead in the decrease of the recruitment / activation of T cells. The action of chalcone 4 treatment on macrophages seems also directly implicated in the inhibition of the recruitment of neutrophils and eosinophils. In addition, neutralizing CXCL12 decrease the chemo-attractant activity of CXCL12 to CXCR4+ eosinophils and CXCR4+ lymphocytes. Finally, our results highlight the role of CXCL12 on macrophages activation and differentiation and allow us to propose an original link between the CXCL12 chemokine and macrophages in the response to allergen.

ACKNOWLEDGEMENT

This work was supported by the "Fonds de Dotation Recherche en Santé Respiratoire, Appel d'Offres Blanc 2010". FD is a recipient of a fellowship from the Laboratory of excellence Medalis Initiative of Excellence (IdEx), Strasbourg University, France.

REFERENCES

1. Bachelierie, F., CXCL12/CXCR4-axis dysfunctions: Markers of the rare immunodeficiency disorder WHIM syndrome. *Dis Markers*, 2010. 29(3-4): p. 189-98.
2. Balabanian, K., B. Lagane, J.L. Pablos, L. Laurent, T. Planchenault, O. Verola, C. Lebbe, D. Kerob, A. Dupuy, O. Hermine, J.F. Nicolas, V. Latger-Cannard, D. Bensoussan, P. Bordigoni, F. Baleux, F. Le Deist, J.L. Virelizier, F. Arenzana-Seisdedos, and F. Bachelierie, WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood*, 2005b. 105(6): p. 2449-57.
3. Balkwill, F., The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin Cancer Biol*, 2004a. 14(3): p. 171-9.
4. Berry M, Brightling C, Pavord I, Wardlaw A. TNF-alpha in asthma. *Curr Opin Pharmacol*. 2007 Jun;7(3):279-82.
5. Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, Vescevi A, Bagezza G, Kollias G, Meldolesi J, Volterra A. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. *Nat Neurosci*. 2001 Jul;4(7):702-10.
6. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol*. 2010 Oct;11(10):889-96. doi: 10.1038/ni.1937. Epub 2010 Sep 20.
7. Bleul, C.C., L. Wu, J.A. Hoxie, T.A. Springer, and C.R. Mackay, The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc Natl Acad Sci U S A*, 1997. 94(5): p. 1925-30.
8. Boorsma CE, Draijer C, Melgert BN. Macrophage heterogeneity in respiratory diseases. *Mediators Inflamm*. 2013;2013:769214.
9. Bouchery, T., G. Denece, T. Attout, K. Ehrhardt, N. Lhermitte-Vallarino, M. Hachet-Haas, J.L. Galzi, E. Brotin, F. Bachelierie, L. Gavotte, C. Moulia, O. Bain, and C. Martin, The chemokine CXCL12 is essential for the clearance of the filaria *Litomosoides sigmodontis* in resistant mice. *PLoS One*, 2012. 7(4): p. e34971
10. Burton OT, Darling AR, Zhou JS, Noval-Rivas M, Jones TG, Gurish MF, Chatila TA, Oettgen HC. Direct effects of IL-4 on mast cells drive their intestinal expansion and increase susceptibility to anaphylaxis in a murine model of food allergy. *Mucosal Immunol*. 2013 Jul;6(4):740-50.
11. Byers DE, Holtzman MJ. Alternatively activated macrophages and airway disease. *Chest*. 2011 Sep;140(3):768-74.
12. Chow, K.Y., E. Brotin, Y. Ben Khalifa, L. Carthage, S. Teissier, A. Danckaert, J.L. Galzi, F. Arenzana-Seisdedos, F. Thierry, and F. Bachelierie, A pivotal role for CXCL12 signaling in HPV-mediated transformation of keratinocytes: clues to understanding HPV-pathogenesis in WHIM syndrome. *Cell Host Microbe*, 2010. 8(6): p. 523-33.
13. Cinamon, G., V. Shinder, and R. Alon, Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. *Nat Immunol*, 2001. 2(6): p. 515-22.
14. Costello CM, McCullagh B, Howell K, Sands M, Belperio JA, Keane MP, Gaine S, McLoughlin P. A role for the CXCL12 receptor, CXCR7, in the pathogenesis of human pulmonary vascular disease. *Eur Respir J*. 2012. 39(6):1415-24.
15. Daubeuf F, Hachet-Haas M, Gizzi P, Gasparik V, Bonnet D, Utard V, Hibert M, Frossard N, Galzi JL. An Antedrug of the CXCL12 Neutraligand Blocks Experimental Allergic Asthma without Systemic Effect in Mice. *J Biol Chem*. 2013. 288(17):11865-76
16. Daubeuf F., Frossard N. Acute Asthma Models to Ovalbumin in the Mouse. *Current Protocols in Mouse Biology*. March 2013
17. Daubeuf F., Frossard N. Performing bronchoalveolar lavage in the mouse. *Current Protocols in Mouse Biology*. June 2012.
18. Daubeuf F., Reber L., Frossard N. Measurement of Airway Responsiveness on Vigil and Unrestrained Mouse. <http://www.bio-protocol.org/wenzhang.aspx?id=328>
19. Daubeuf F., Reber L., Frossard N. Measurement of Airway Responsiveness in the Anesthetized Mouse. <http://www.bio-protocol.org/wenzhang.aspx?id=645>
20. Feng, Y., C.C. Broder, P.E. Kennedy, and E.A. Berger, HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*, 1996. 272(5263): p. 872-7
21. Fernandez, E. J.; Lolis, E. Structure, function, and inhibition of chemokines. *Annual Review of Pharmacology and Toxicology* 2002, 42, 469-499.
22. Flomenberg, N., S.M. Devine, J.F. Dipersio, J.L. Liesveld, J.M. McCarty, S.D. Rowley, D.H. Vesole, K. Badel, and G. Calandra, The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood*, 2005. 106(5): p. 1867-74.
23. Fulkerson, P.C., N. Zimmermann, L.M. Hassman, F.D. Finkelman, and M.E. Rothenberg, Pulmonary chemokine expression is coordinately regulated by STAT1, STAT6, and IFN-gamma. *J Immunol*, 2004. 173(12): p. 7565-74.
24. Galzi, J.L., M. Hachet-Haas, D. Bonnet, F. Daubeuf, S. Lecat, M. Hibert, J. Haiech, and N. Frossard, Neutralizing endogenous chemokines with small molecules. Principles and potential therapeutic applications. *Pharmacol Ther*, 2010. 126(1): p. 39-55.
25. Gasparik, V., F. Daubeuf, M. Hachet-Haas, F. Rohmer, P. Gizzi, J. Haiech, J.L. Galzi, M. Hibert, D. Bonnet, and N. Frossard, Prodrugs of a CXCR4 chemokine ligand 12, CXCL12, neutraligand active in vivo in a new asthma model. *ACS Med Chem Letters*, 2011. 3: p. 10-14.
26. Godot V, Arock M, Garcia G, Capel F, Flys C, Dy M, Emilie D, Humbert M. H4 histamine receptor mediates optimal migration of mast cell precursors to CXCL12. *J Allergy Clin Immunol*. 2007 Oct;120(4):827-34.
27. Gonzalo, J.A., C.M. Lloyd, A. Peled, T. Delaney, A.J. Coyle, and J.C. Gutierrez-Ramos, Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 alpha in the inflammatory component of allergic airway disease. *J Immunol*, 2000. 165(1): p. 499-508.
28. Hachet-Haas, M., K. Balabanian, F. Rohmer, F. Pons, C. Franchet, S. Lecat, K.Y. Chow, R. Dagher, P. Gizzi, B. Didier, B. Lagane, E. Kellenberger, D. Bonnet, F. Baleux, J. Haiech, M. Parmentier, N. Frossard, F. Arenzana-Seisdedos, M. Hibert, and J.L. Galzi, Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J Biol Chem*, 2008. 283(34): p. 23189-99.
29. Hendrix, C.W., A.C. Collier, M.M. Lederman, D. Schols, R.B. Pollard, S. Brown, J.B. Jackson, R.W. Coombs, M.J. Glesby, C.W. Flexner, G.J. Bridger, K. Badel, R.T. MacFarland, G.W. Henson, and G. Calandra, Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *J Acquir Immune Defic Syndr*, 2004. 37(2): p. 1253-62.
30. Hoffmann F, Müller W, Schütz D, Penfold ME, Wong YH, Schulz S, Stumm R. Rapid uptake and degradation of CXCL12 depend on CXCR7 carboxyl-terminal serine/threonine residues. *J Biol Chem*. 2012 Aug 17;287(34):28362-77.
31. Hughes JP, S Rees, SB Kalindjian and KL Philpott. Principles of early drug discovery. *Br J Pharmacol*. 2011 March; 162(6): 1239-1249.
32. Kumar RK, Herbert C, Foster PS. The "classical" ovalbumin challenge model of asthma in mice. *Curr Drug Targets*. 2008 Jun;9(6):485-94.
33. Lee C, Liu QH, Tomkowicz B, Yi Y, Freedman BD, Collman RG. Macrophage activation through CCR5- and CXCR4-mediated gp120-elicited signaling pathways. *J Leukoc Biol*. 2003 Nov;74(5):676-82.
34. Levoye, A., K. Balabanian, F. Baleux, F. Bachelierie, and B. Lagane, CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood*, 2009. 113(24): p. 6085-93.
35. Liu LY, Jarjour NN, Busse WW, Kelly EA. Chemokine receptor expression on human eosinophils from peripheral blood and bronchoalveolar lavage fluid after segmental antigen challenge. *J Allergy Clin Immunol*. 2003 Sep;112(3):556-62.
36. Lukacs, N.W., A. Berlin, D. Schols, R.T. Skerlj, and G.J. Bridger, AMD3100, a CXCR4 antagonist, attenuates allergic lung inflammation and airway hyperreactivity. *Am J Pathol*, 2002. 160(4): p. 1353
37. Maes T, Joos GF, Brusselle GG. Targeting interleukin-4 in asthma: lost in translation? *Am J Respir Cell Mol Biol*. 2012 Sep;47(3):261-70.
38. Matsuura J, Sakanaka M, Sato N, Ichikawa A, Tanaka S. Suppression of CXCR4 expression in mast cells upon IgE-mediated antigen stimulation. *Inflamm Res*. 2010 Feb;59(2):123-7.

39. Montani, D., F. Perros, N. Gambaryan, B. Girerd, P. Dorfmüller, L.C. Price, A. Huertas, H. Hammad, B. Lambrecht, G. Simonneau, J.M. Launay, S. Cohen-Kaminsky, and M. Humbert, C-kit-positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2011. 184(1): p. 116-23.
40. Müller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verastegui, and A. Zlotnik, Involvement of chemokine receptors in breast cancer metastasis. *Nature*, 2001. 410(6824): p. 50-6.
41. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. *J Leukoc Biol*. 2011 Apr;89(4):557-63.
42. Nagase H, Miyamasu M, Yamaguchi M, Fujisawa T, Kawasaki H, Ohta K, Yamamoto K, Morita Y, Hirai K. Regulation of chemokine receptor expression in eosinophils. *Int Arch Allergy Immunol*. 2001;125 Suppl 1:29-32.
43. Naumann U, Cameroni E, Pruenster M, Mahabaleshwar H, Raz E, Zerwes HG, Rot A, Thelen M. CXCR7 functions as a scavenger for CXCL12 and CXCL11. *PLoS One*. 2010 Feb 11;5(2):e9175.
44. Negrete-Garcia, M.C., J.R. Velazquez, A. Popoca-Coyotl, A.R. Montes-Vizuet, E. Juarez-Carvajal, and L.M. Teran, Chemokine (C-X-C motif) ligand 12/stromal cell-derived factor-1 is associated with leukocyte recruitment in asthma. *Chest*, 2010. 138(1): p. 100-6.
45. Nials AT, Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech*. 2008 Nov-Dec;1(4-5):213-20.
46. Oberlin, E., A. Amara, F. Bachelier, C. Bessia, J.L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J.M. Heard, I. Clark-Lewis, D.F. Legler, M. Loetscher, M. Baggiolini, and B. Moser, The CXCL chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature*, 1996. 382(6594): p. 833-5.
47. Ochi H, De Jesus NH, Hsieh FH, Austen KF, Boyce JA. IL-4 and -5 prime human mast cells for different profiles of IgE-dependent cytokine production. *Proc Natl Acad Sci U S A*. 2000 Sep 12;97(19):10509-13.
48. Parameswaran N, Patali S. Tumor necrosis factor- α signaling in macrophages. *Crit Rev Eukaryot Gene Expr*. 2010;20(2):87-103.
49. Sánchez-Martín L, Estechea A, Samaniego R, Sánchez-Ramón S, Vega MÁ, Sánchez-Mateos P. The chemokine CXCL12 regulates monocyte-macrophage differentiation and RUNX3 expression. *Blood*. 2011 Jan 6;117(1):88-97.
50. Sewell WA, Scurr LL, Orphanides H, Kinder S, Ludowyke RI. Induction of interleukin-4 and interleukin-5 expression in mast cells is inhibited by glucocorticoids. *Clin Diagn Lab Immunol*. 1998 Jan;5(1):18-23.
51. Shin YS, Takeda K, Gelfand EW. Understanding asthma using animal models. *Allergy Asthma Immunol Res*. 2009 Oct;1(1):10-8.
52. Sierro, F., C. Biben, L. Martinez-Munoz, M. Mellado, R.M. Ransohoff, M. Li, B. Woehl, H. Leung, J. Groom, M. Batten, R.P. Harvey, A.C. Martinez, C.R. Mackay, and F. Mackay, Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci U S A*. 2007. 104(37): p. 14759-64.
53. Wetzler, M., M. Talpaz, E.S. Kleinerman, A. King, Y.O. Huh, J.U. Gutterman, and R. Kurzrock, A new familial immunodeficiency disorder characterized by severe neutropenia, a defective marrow release mechanism, and hypogammaglobulinemia. *Am J Med*, 1990. 89(5): p. 663-72.
54. Xu, J., E. Torres, A.L. Mora, H. Shim, A. Ramirez, D. Neujahr, K.L. Brigham, and M. Rojas, Attenuation of obliterative bronchiolitis by a CXCR4 antagonist in the murine heterotopic tracheal transplant model. *J Heart Lung Transplant*, 2008. 27(12): p. 1302-10.
55. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990;249(4975):1431-1433
56. Bhatia S, Fei M, Yarlagadda M, Qi Z, Akira S, Saijo S, Iwakura Y, van Rooijen N, Gibson GA, St Croix CM, Ray A, Ray P. Rapid host defense against *Aspergillus fumigatus* involves alveolar macrophages with a predominance of alternatively activated phenotype. *PLoS One*. 2011 Jan 5;6(1):e15943.
57. Murphy BS, Sundareshan V, Cory TJ, Hayes D Jr, Anstead MI, Feola DJ. (2008) Azithromycin alters macrophage phenotype. *J Antimicrob Chemother*. 61(3):554-60.
58. Badylak SF, Valentin JE, Ravindra AK, McCabe GP, Stewart-Akers AM. (2008) Macrophage phenotype as a determinant of biologic scaffold remodeling. *Tissue Eng Part A*. 14(11):1835-42.
59. Zeyda M, Gollinger K, Kriehuber E, Kiefer FW, Neuhofner A, Stulnig TM. (2010) Newly identified adipose tissue macrophage populations in obesity with distinct chemokine and chemokine receptor expression. *Int J Obes (Lond)*. 34(12):1684-94.
60. Kodumudi KN, Woan K, Gilvary DL, Sahakian E, Wei S, Djeu JY. (2010) A novel chemoimmunomodulating property of docetaxel: suppression of myeloid-derived suppressor cells in tumor bearers. *Clin Cancer Res*. 16(18):4583-94.
61. J, Riek AE, Weng S, Petty M, Kim D, Colonna M, Cella M, Bernal-Mizrachi C. (2012) Endoplasmic reticulum stress controls M2 macrophage differentiation and foam cell formation. *J Biol Chem*. 287(15):11629-41.
62. Pechkovsky DV, Prasse A, Kollert F, Engel KM, Dentler J, Luttmann W, Friedrich K, Müller-Quernheim J, Zissel G. Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. *Clin Immunol*. 2010 Oct;137(1):89-101
63. Müller U, Stenzel W, Köhler G, Werner C, Polte T, Hansen G, Schütze N, Straubinger RK, Blessing M, McKenzie AN, Brombacher F, Alber G. IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with *Cryptococcus neoformans*. *J Immunol*. 2007 Oct 15;179(8):5367-77.
64. Wentworth JM, Naselli G, Brown WA, Doyle L, Phipson B, Smyth GK, Wabitsch M, O'Brien PE, Harrison LC. Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. *Diabetes*. 2010 Jul;59(7):1648-56.
65. Gomperts BN, Belperio JA, Rao PN, Randell SH, Fishbein MC, Burdick MD, Strieter RM. Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury. *J Immunol*. 2006 Feb 1;176(3):1916-27.
66. Moreira AP, Hogaboam CM. Macrophages in allergic asthma: fine-tuning their pro- and anti-inflammatory actions for disease resolution. *J Interferon Cytokine Res*. 2011 Jun;31(6):485-91.
67. Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: agents of defense or destruction? *Annu Rev Pharmacol Toxicol*. 2011;51:267-88.
68. Siddiqui S, Secor ER Jr, Silbart LK. Broncho-alveolar macrophages express chemokines associated with leukocyte migration in a mouse model of asthma. *Cell Immunol*. 2013 Feb;281(2):159-69.
69. Pappas K, Papaioannou AI, Kostikas K, Tzanakis N. The role of macrophages in obstructive airways disease: Chronic obstructive pulmonary disease and asthma. *Cytokine*. 2013 Sep 28. pii: S1043-4666(13)00688-1.
70. Halova I, Draberova L, Draber P. Mast cell chemotaxis - chemoattractants and signaling pathways. *Front Immunol*. 2012 May 25;3:119.
71. Debes GF, Dahl ME, Mahiny AJ, Bonhagen K, Campbell DJ, Siegmund K, Erb KJ, Lewis DB, Kamradt T, Hamann A. Chemotactic responses of IL-4-, IL-10-, and IFN-gamma-producing CD4+ T cells depend on tissue origin and microbial stimulus. *J Immunol*. 2006 Jan 1;176(1):557-66.
72. Mehrad B, Burdick MD, Strieter RM. Fibrocyte CXCR4 regulation as a therapeutic target in pulmonary fibrosis. *Int J Biochem Cell Biol*. 2009 Aug-Sep;41(8-9):1708-18.
73. Nitta N, Tsuchiya T, Yamauchi A, Tamatani T, Kanegasaki S. Quantitative analysis of eosinophil chemotaxis tracked using a novel optical device -- TAXIScan. *J Immunol Methods*. 2007 Mar 30;320(1-2):155-63.
74. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest*. 2004 Aug;114(3):438-46.
75. Limón-Flores AY, Chacón-Salinas R, Ramos G, Ullrich SE. Mast cells mediate the immune suppression induced by dermal exposure to JP-8 jet fuel. *Toxicol Sci*. 2009 Nov;112(1):144-52.
76. D. Brasse, B. Humbert, C. Mathelin, M. C. Rio and J. L. Guyonnet, Towards an inline reconstruction architecture for micro-CT systems. *Phys. Med. Biol.*, 2005, 50, 5799–5811.
77. Z. El Bitar, B. Leh, V. Bekaert, D. Huss, D. Brasse and IEEE, in IEEE Nuclear Science Symposium/Medical Imaging Conference, IEEE, Dresden, Germany, 2008, pp. 3076–3079.

78. Nakae, S., L. H. Ho, et al. (2007) Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J Allergy Clin Immunol* 120(1): 48-55.
79. Lichtman SN, Wang J, Lemasters JJ. (1998) LPS receptor CD14 participates in release of TNF-alpha in RAW 264.7 and peritoneal cells but not in kupffer cells. *Am J Physiol*. 275(1 Pt 1):G39-46.
80. Bouchery T, Dénécé G, Attout T, Ehrhardt K, Lhermitte-Vallarino N, Hachet-Haas M, Galzi JL, Brotin E, Bachelier F, Gavotte L, Moulia C, Bain O, Martin C. (2012) The chemokine CXCL12 is essential for the clearance of the filaria *Litomosoides sigmodontis* in resistant mice. *PLoS One*. 2012;7(4):e34971. doi: 10.1371/journal.pone.0034971. Epub 2012 Apr 12.
81. Kumar A, Kremer KN, Dominguez D, Tadi M, Hedin KE. Ga13 and Rho mediate endosomal trafficking of CXCR4 into Rab11+ vesicles upon stromal cell-derived factor-1 stimulation. *J Immunol*. 2011 Jan 15;186(2):951-8.
82. Lawrence T, Natoli G. (2011) Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat Rev Immunol*. 11(11):750-61
83. Jin P, Han TH, Ren J, Saunders S, Wang E, Marincola FM, Stronck DF. Molecular signatures of maturing dendritic cells: implications for testing the quality of dendritic cell therapies. *J Transl Med*. 2010 Jan 15;8:4. doi: 10.1186/1479-5876-8-4.
84. Poole JA, Thiele GM, Alexis NE, Burrell AM, Parks C, Romberger DJ. Organic dust exposure alters monocyte-derived dendritic cell differentiation and maturation. *Am J Physiol Lung Cell Mol Physiol*. 2009 Oct;297(4):L767-76. doi: 10.1152/ajplung.00107.2009. Epub 2009 Jul 31.
85. Rivas-Caicedo A, Soldevila G, Fortoul TI, Castell-Rodríguez A, Flores-Romo L, García-Zepeda EA. (2009) Jak3 is involved in dendritic cell maturation and CCR7-dependent migration. *PLoS One*. 4(9):e7066.
86. Johnson LA, Jackson DG. (2013) Control of dendritic cell trafficking in lymphatics by chemokines. *Angiogenesis*. Nov 15
87. Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR, Qin S, Lanzavecchia A (1998) Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* 28(9):2760-2769
88. Stutte S, Quast T, Gerbitzki N, Savinko T, Novak N, Reifenberger J, Homey B, Kolanus W, Alenius H, Förster I. (2010) Requirement of CCL17 for CCR7- and CXCR4-dependent migration of cutaneous dendritic cells. *Proc Natl Acad Sci U S A*. 107(19):8736-41.
89. Umemoto E, Otani K, Ikeno T, Verjan Garcia N, Hayasaka H, Bai Z, Jang MH, Tanaka T, Nagasawa T, Ueda K, Miyasaka M. (2012) Constitutive plasmacytoid dendritic cell migration to the splenic white pulp is cooperatively regulated by CCR7- and CXCR4-mediated signaling. *J Immunol*. 189(1):191-9.
90. Bai Z, Hayasaka H, Kobayashi M, Li W, Guo Z, Jang MH, Kondo A, Choi BI, Iwakura Y, Miyasaka M. (2009) CXC chemokine ligand 12 promotes CCR7-dependent naive T cell trafficking to lymph nodes and Peyer's patches. *J Immunol*. 182(3):1287-95.
91. Humrich JY, Humrich JH, Averbek M, Thumann P, Termeer C, Kämpgen E, Schuler G, Jenne L. (2006) Mature monocyte-derived dendritic cells respond more strongly to CCL19 than to CXCL12: consequences for directional migration. *Immunology*. 117(2):238-47
92. Berahovich RD, Zabel BA, Lewén S, Walters MJ, Ebsworth K, Wang Y, Jaen JC, Schall TJ. (2013) Endothelial Expression of CXCR7 and the Regulation of Systemic CXCL12 Levels. *Immunology*

SUPPLEMENTAL INFORMATION

METHODS

Measurement of airway resistance by the forced oscillation technique. Airway responsiveness to methacholine was assessed using the forced oscillation technique (Flexivent®, SCIREQ, Montreal, Canada) and as described previously [19]. Mice were anesthetized with an intraperitoneal injection of 6ml/kg of xylazine (Rompun®;0.25%), followed 50 minutes later by an intraperitoneal injection of 6ml/kg of pentobarbital sodium (0.9%). The trachea was exposed and an 18-gauge metal needle was inserted into the trachea. Airways were connected to a computer-controlled small animal ventilator, and quasi-sinusoidally ventilated with a tidal volume of 10 ml/Kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. Each mouse was challenged for 10 sec with an aerosol, generated with an in-line nebulizer and administered directly through the ventilator: PBS for the baseline and followed by MCh challenge at 50 mg/ml. For calculation of the effect of methacholine, the peak response was used as the mean of the three maximal values, and integrated for calculation of airway elastance (E) and compliance (C). Airway elastance is expressed as cm H₂O.ml⁻¹ and airway compliance as ml.cm H₂O⁻¹

ELISAs. TNF α , (R&D systems) ELISA kits were used according to the manufacturer's instructions. TNF α were quantified in the culture supernatants and TNF α was also quantified in the supernatants obtained from the lung tissues after they were grounded with an UltraTurax® and centrifuged at 10,000 g for 10 min at 4°C.

Ovalbumin-specific IgG1 and IgG2a plasma levels were determined by ELISA. Microtiter plates were coated overnight at 4°C with the capture antibody: anti-mouse IgG1 or IgG2a at 2 μ g/well in carbonate buffer, pH 9.5 (BD pharmingen, R19-15 and A85-1). After three washings with phosphate-buffered saline containing 0.05% Tween-20, microtiter plates were blocked for 1 h at 37 °C with 10% bovine serum albumin in phosphate buffered saline. After three washings with phosphate-buffered saline containing 0.05% Tween-20, plasma samples were incubated for 2 h at

room temperature. After three washings with phosphate-buffered saline containing 0.05% Tween-20, an ovalbumin-HRP (BUF048, Abdsrotec) was added to the wells and incubated for 1 h at room temperature. The plates were washed and the horseradish peroxidase substrate, tetramethylbenzidine (BD Pharmingen) was added and coloration allowed to develop for 15–20 min. The reaction was stopped by addition of 0.5 M H₂SO₄ and absorbance intensity was read at 450 nm. Plasma levels of ovalbumin-specific Igs were expressed as optical densities (O.D).

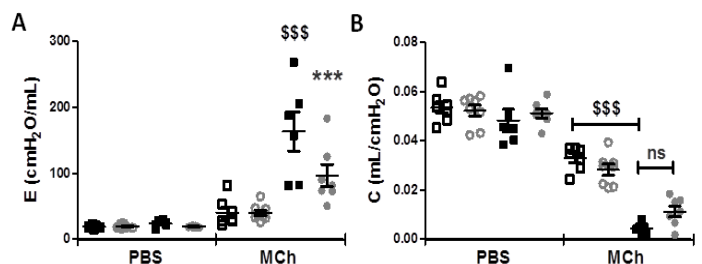
Alveolar macrophages culture. On day 21, BALF cells from each group of treated mice (HP β CD, chalc4, OVA and OVA-chac4) were centrifuged (300 g for 5 min at 4 °C) to pellet cells and cells were re-suspended in 0.5ml of RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 μ g/ml) and were dispatched in a culture 96 well microplate and incubated 3 hours at 37°C. The microplate was then vortex (1200rpm, 1min) to resuspend all unfixed cells in supernatant and supernatant were immediately removed. The microplate was immediately wash with complete media. Alveolar macrophages were stimulated with OVA (100ng/ml) in complete media for 16h at 37°C. Supernatant was collected in a Protein LoBind Deepwell plate (0030504100, Eppendorf) and store at -20°C and cells were fixed by dehydration with a Concentrator plus (Eppendorf) at room temperature and store at room temperature and protect from the light.

Cells were incubated with F4/80-AlexaFluor®488 and propidium iodide in 100 μ l of cold PBS-BSA 1% for 1h at 4°C and cells were wash with cold PBS. Purity and quantity of macrophages per well was analysis using Celigo® S Imaging Cell Cytometer (Brooks).

White blood cells formula. White blood cells were analysis using ADVIA 120® System (Siemens Healthcare). Samples of Blood were collected day 21 in inferior vena cava and placed in EDTA-CAPJECT® Micro Collection Tubes (T-MQK, Terumo®) and stored in ice. White blood cells were analysis 5 hours after collection.

FIGURES

Figure S1. Effect of chalcone 4 on the development of airway hyperreactivity assessed by Flexivent® technique in a mouse model of asthma. Balb/c mice were sensitized to OVA and challenged with OVA or saline. Chalc4 (grey line) or HP β CD (black line) were administered i.n. (300nmol/kg) 2h before each OVA (square) or saline (dots) challenge. **A.** Airway elastance (cm H₂O.ml⁻¹) in response to aerosolized PBS and MCh (50mg/ml, i.e. 0.26 M, in PBS). **B.** Airway compliance (ml.cm H₂O⁻¹) in response to aerosolized PBS and MCh (50mg/ml, i.e. 0.26 M, in PBS). Dots and squares are individual values, lines are means and error bars show S.E. values (n=8/group). Statistical analysis consisted of two-way ANOVA and Bonferroni post-test and was conducted with Prism software (GraphPad).^{SSS} P \leq 0.001 vs HP β CD group and *******P \leq 0.001 vs OVA group.



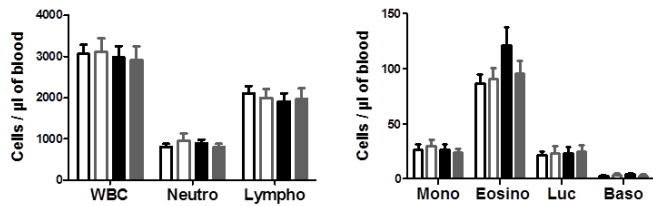


Figure S2. Effect of Chalc4 on white blood cell formula. WBC was determined with ADVIA 120[®] System in blood samples collected on day 21 in OVA sensitized mice challenged with OVA (full blocks) or saline (empty blocks) and treated with Chalc4 (grey) or HPβCD (black). Number of white blood cells (WBC), neutrophils (Neutro), lymphocytes (Lympho), monocytes (Mono), eosinophil (Eosino), large unstained cells (LUC) and basophils (Baso) are represented. Blocks are means and error bars show S.E. values (n=8/group).

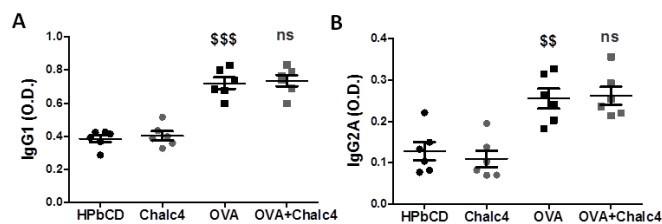


Figure S3. Effect of Chalc4 on plasma OVA-specific Ig levels. A and B. Levels of OVA-specific IgG1 and IgG2a in plasma. Dots and squares are individual values, lines are means and error bars show S.E. values (n=6/group). Statistical analysis consisted of one-way ANOVA and Tukey post-test was conducted with Prism software (GraphPad). ⁵⁵ P≤0.001 and ⁵⁵⁵ P≤0.001 vs HPβCD group.

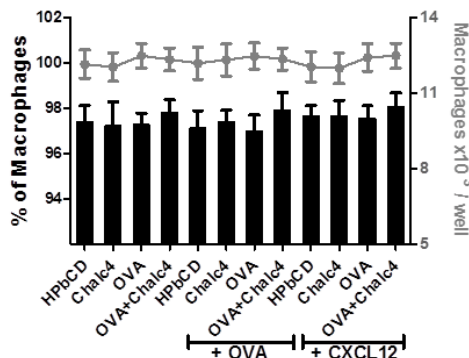


Figure S4. Homogeneity in number and proportion of alveolar macrophages in 96 wells plate. Alveolar macrophages of OVA sensitized mice challenged with OVA or saline and treated with Chalc4 (grey) or HPβCD (black) were collected in BALF 24h after the last OVA challenge, isolated in 96-wells microplates and stimulated overnight (16h) with CXCL12 (50nM) or OVA (100ng/ml). Celigo[®] S Imaging Cell Cytometer (Brooks) was used to determine the number of CF4/80+ cells and the number of PI+ (propidium iodide) cells in each well. Results were expressed in percent of macrophage (F4/80+/PI+) in wells (Black blocks with left Y axis). Grey dots are numbers of macrophages in wells (right Y axis). Blocks and Dots are means and error bars show S.E. values.

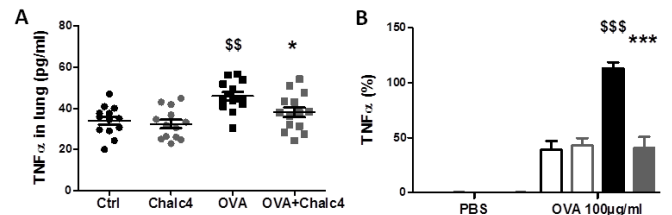


Figure S5. Effect of Chalc4 on TNFα level in the lung and TNFα release by alveolar macrophages in response to OVA. A. Lungs were collected 24h after the last OVA challenge and homogenate with UltraTurax[®]. The levels of TNFα was determined using ELISA in OVA sensitized mice challenged with OVA (squares) or saline (dots) and treated with Chalc4 (grey) or HPβCD (black). Dots and squares are individual values obtained in 2 independent studies, lines are means and error bars show S.E. values (n=6+8/group). B. Alveolar macrophages of OVA sensitized mice challenged with OVA or saline and treated with Chalc4 (grey) or HPβCD (black) were collected in BALF 24h after the last OVA challenge, isolated in 96-wells microplates and stimulated overnight (16h) with OVA (100ng/ml). The levels of TNFα was determined using ELISA in supernatant. Blocks are means obtained in 2 independent studies and error bars show S.E. values (n=3+3/group). Statistical analysis consisted of one-way ANOVA with Tukey post-test was conducted with Prism software (GraphPad). ⁵⁵ P≤0.01, ⁵⁵⁵ P≤0.001 vs HPβCD group and *P≤0.05, ***P≤0.001 vs OVA group.

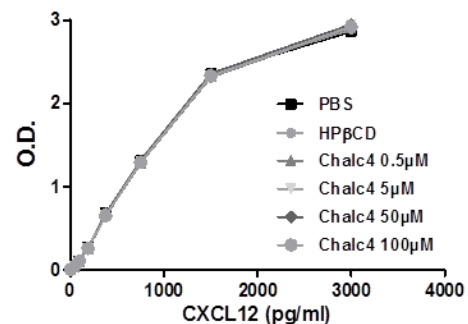


Figure S6. Effect of Chalc4 on CXCL12 detection by ELISA. Dose response curve to CXCL12 incubated in PBS, HPβCD 10% and with increasing dose of Chalc4 (0.5µM to 100µM). Dots are means.

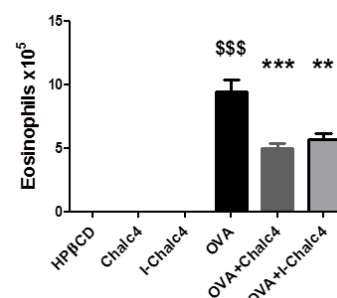


Figure S7. Effect of Chalc4 and I-Chalc4 on eosinophil recruitment in a 8-day mouse model of airway hyper-eosinophilia. Effect of local administration of 300 nmol/kg Chalc4 (grey), I-chalc4 (light grey) or HPβCD (black) on absolute number of eosinophil in BAL fluid 24h after the last challenge in Balb/c mice sensitized to OVA and challenged with OVA or saline. Blocks are means and error bars show S.E. values (n=6/group). Statistical analysis consisted of one-way ANOVA with Tukey post-test was conducted with Prism software (GraphPad). ⁵⁵⁵ P≤0.001 vs HPβCD group and **P≤0.01, ***P≤0.001 vs OVA group.

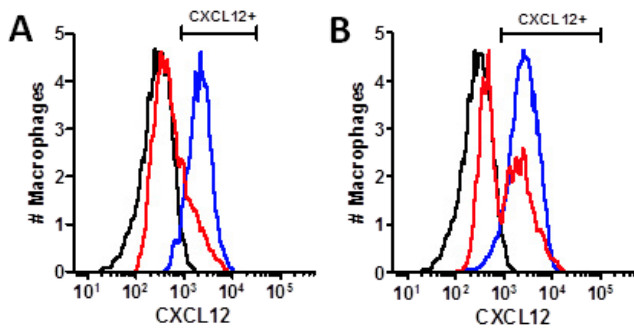


Figure S8. Effect of Chalc4 on expression of CXCL12 on the surface of macrophages. Flow cytometry analysis of CXCL12 on the surface of BALF macrophages from OVA sensitized mice challenged with saline (A) or OVA (B) following incubation 30min with 5 μ M Chalc4 (red histogram) or HP β CD (blue histogram). Black histogram: unstained macrophages.

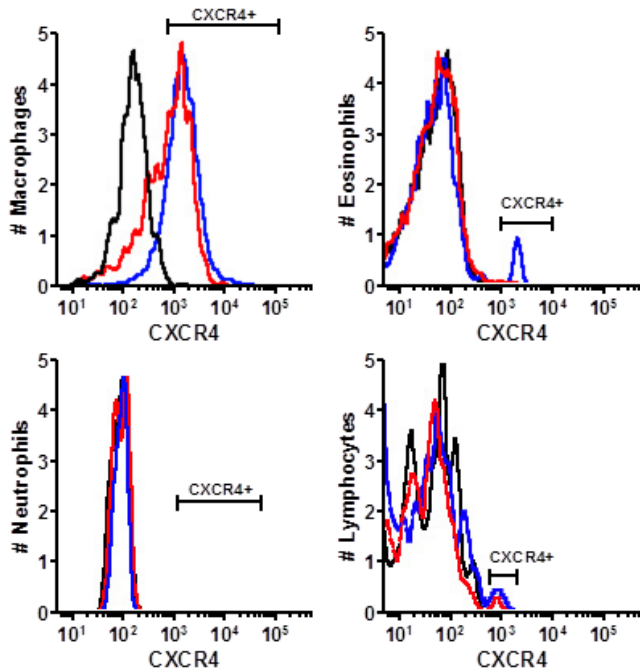


Figure S9. Effect of Chalc4 on CXCR4+ cells in BALF. Flow cytometry analysis of CXCR4 on the surface of BALF cells from OVA sensitized/challenged mice treated with Chalc4 (red histogram) or HP β CD (blue histogram). Black histogram: unstained cells.

DISCUSSION ET PERSPECTIVES

Ce travail est le résultat d'une étroite collaboration entre le laboratoire d'Innovation Thérapeutique (UMR7200, faculté de pharmacie), le laboratoire de biotechnologie et signalisation cellulaire (UMR7242, ESBS), et la plateforme Techmed'ILL. La synergie née de cette collaboration a permis le développement de neutraligands de la chimiokine CXCL12, la chalcone 4, la mise en évidence de son activité anti-asthmatique dans un modèle aigu d'asthme allergique et de son mode d'action original.

L'implication de CXCL12 et de ses récepteurs CXCR4 et CXCR7 dans l'asthme a notamment été mis en évidence chez la souris par l'utilisation d'anticorps neutralisant soit CXCR4 ou CXCL12, ou encore l'utilisation d'un antagoniste du récepteur CXCR4: le composé AMD3100. Ces stratégies permettent de réduire l'éosinophilie et l'hyperréactivité des voies aériennes, tandis que la surexpression du récepteur CXCR4 par les leucocytes, suite au transfert du gène médié par un vecteur rétroviral, augmente l'inflammation pulmonaire (Gonzalo et al. 2000, Lukacs et al. 2002, Hogaboam et al. 2005). Il apparaît ainsi que les modèles d'asthme allergique chez la souris sont adaptés à l'étude de l'activité *in vivo* de nos neutraligands de la chimiokine CXCL12. Nous avons ainsi évalué l'activité anti-inflammatoire *in vivo* de la chalcone 4, le premier neutraligand de CXCL12 mis en évidence par nos équipes (Hachet-Haas et al. 2008).

Cependant, l'utilisation d'un modèle aigu d'asthme allergique en 21 jours est coûteux et consommateur d'espace dans l'animalerie car il requiert le maintien des animaux sur une période de 4-5 semaines. Ce modèle nécessite également une organisation et une planification rigoureuse et s'avère peu adapté à l'évaluation de l'activité anti-inflammatoire *in vivo* de nouveaux neutraligands de CXCL12.

Développement d'un modèle rapide d'asthme allergique chez la souris.

La première partie de ma thèse a consisté à mettre au point et valider un modèle de courte durée d'asthme allergique chez la souris, caractérisé par une hyperéosinophilie robuste dans les voies aériennes, afin de garantir une évaluation rapide de l'activité anti-inflammatoire des nouveaux composés. Nous pourrions ainsi optimiser nos échanges avec les autres équipes, chimistes, biochimistes et pharmacologues moléculaires, dans le but de garantir un développement raisonné de nos stratégies.

Forts de notre expérience sur les modèles d'asthme allergique à l'ovalbumine qui permettent d'induire une inflammation rapide, robuste et reproductible des voies aériennes, nous avons envisagé d'adapter le modèle conventionnel cité précédemment (Hachet-Haas et al. 2008). Notre travail a permis de mettre au point un modèle rapide d'hyperéosinophilie bronchique chez la souris Balb/c, en 8 jours. Ce modèle est constitué d'une étape de sensibilisation à l'ovalbumine composée de trois administrations intrapéritonéales consécutives les trois premiers jours, suivie de trois

provocations allergéniques par voie intranasale au jours 5, 6 et 7 (**publication n°1**). Nous avons montré que la sensibilisation à l'ovalbumine nécessite l'utilisation d'hydroxyde d'aluminium de qualité (**publication n°A1**), un adjuvant connu pour favoriser le développement du phénotype Th2 par le système immunitaire exposé à un antigène (McKee et al. 2009). De plus, nous montrons qu'une durée de trois jours est indispensable au recrutement d'un grand nombre d'éosinophiles dans les voies aériennes (**publication n°1 et n°A4**), et que la répétition journalière des provocations allergéniques sur 3 jours permet d'accroître la réponse inflammatoire dans les voies aériennes et de garantir un afflux important d'éosinophiles ($\geq 50\%$ des cellules présentes dans le lavage bronchoalvéolaire ou LBA), ainsi qu'une reproductibilité suffisante permettant de limiter le nombre d'animaux à 6 animaux par groupe (**publication n°1 et n°A4**). La reproductibilité de ce modèle court est équivalente au modèle conventionnel en 21 jours (**publication n°1**).

Nous avons démontré que l'infiltrat inflammatoire est équivalent à celui mesuré dans le modèle 21 jours et que l'afflux d'éosinophiles est également en accord avec l'augmentation significative d'IL-5, principale cytokine impliquée dans le recrutement des éosinophiles (Rothenberg et al. 2006), d'IL-4 responsable de la différenciation lymphocytaire B en plasmocyte sécréteur d'immunoglobulines E (IgE) spécifiques de l'ovalbumine (**publication n°1**). La répétitions des provocations allergéniques permet de maintenir une libération élevée des cytokines IL-4 et IL-5 et promeut ainsi la réponse inflammatoire (**publication n°A4**). La répétition des provocations allergéniques permet de maintenir une libération élevée des cytokines IL-4 et IL-5 et promeut ainsi la réponse inflammatoire (**publication n°A4**). Nos résultats confirment ainsi que le protocole d'immunisation mis en place, bien que très court, est suffisant pour induire une réponse allergique de type Th2, spécifique de l'allergène, conduisant au recrutement des cellules inflammatoires dans les voies aériennes.

L'inflammation induite dans le modèle court est également accompagnée, comme dans le modèle conventionnel, d'une augmentation du nombre de mastocytes autour de la bronche primaire et d'un remodelage bronchique caractérisé par une hyperplasie des cellules caliciformes et une augmentation des dépôts de collagène autour des bronches (**publication n°1**). Nos résultats mettent en évidence que la réponse à l'allergène est très similaire entre ces deux modèles et que seule la réactivité bronchique à la métacholine diffère. Le modèle 8 jours présente une hyperréactivité dont l'amplitude est plus faible que dans le modèle 21 jours (réactivité bronchique 2 fois plus élevée par rapport aux animaux témoins, pour le modèle 8 jours, et 4 fois plus élevée dans le modèle 21 jours). Cette hyperréactivité est difficile à mesurer par pléthysmographie sur animaux vigiles, mais bien caractérisée sur animaux anesthésiés (Flexivent®). L'hyperréactivité bronchique mise en évidence dans le modèle court reste cependant trop faible pour permettre d'évaluer l'activité d'un composé sur ce paramètre avec un faible nombre d'animaux (*National Centre for the Replacement, Refinement and Reduction of Animals in Research*).

Nous avons ainsi développé un modèle rapide, présentant les principales caractéristiques de l'asthme allergique et capable de réduire les délais et les coûts. Toutes les données confirment que le modèle 8 jours est un modèle rapide et reproductible, adapté à l'étude de nouvelles stratégies thérapeutiques pour le traitement de l'hyperéosinophilie des voies aériennes associée à l'asthme (mesuré dans le LBA, **publication n°A2**).

Développement de dérivés de la chalcone 4, promotion d'une activité local du neutraligand adaptée au traitement de l'asthme

Dans un deuxième temps, nous avons parfait le concept de neutraligand de CXCL12 dans l'asthme. Il a été montré précédemment que l'administration systémique de la chalcone 4 inhibe efficacement l'afflux d'éosinophiles dans le modèle aigu d'asthme en 21 jours chez la souris (Hachet-Haas et al. 2008), une activité *in vivo* qui est cependant limitée par la faible solubilité du composé dans les tampons aqueux (9 μ M dans un tampon HEPES). Dans le but d'accroître la distribution du neutraligand dans les voies aériennes et d'augmenter ainsi l'accès à sa cible pharmacologique et son efficacité, nous avons envisagé une administration locale du neutraligand, par voie intranasale. Trois dérivés solubles ont été conçus et synthétisés par l'ajout d'une fonction solubilisante constituée d'un groupement phosphate, sérine ou sulfate (**publication n°2**).

Nous avons montré que ces dérivés étaient plus solubles que la chalcone 4 (jusqu'à 3000 fois plus pour la chalcone 4 phosphate), mais qu'ils n'inhibent pas la liaison de la chimiokine CXCL12 à CXCR4, mesurée *in vitro* par FRET. Ils ne sont donc pas neutraligands de CXCL12. L'étude physicochimique de ces composés à la plateforme Techmed^{ILL} a cependant révélé que les trois composés sont rapidement clivés en chalcone 4, le principe actif, en présence d'un homogénat pulmonaire. Les dérivés synthétisés se comporte donc comme des prodrogues de la chalcone 4. Les mesures physicochimiques ont mis en évidence que les 3 dérivés présentent une demi-vie inférieure à 30 min, la chalcone 4 sulfate étant le composé clivé le plus lentement, et que le dérivé chalcone 4 sérine est le moins stable en solution. La chalcone 4-phosphate est le composé le plus soluble et le plus rapidement clivé en chalcone 4 dans les milieux biologiques, deux critères plébiscités pour en faire une prodrogue efficace (Stella et al. 1985, Rautio et al. 2007), et représente donc le composé le mieux adapté pour l'étude de l'activité anti-inflammatoire *in vivo*.

L'administration locale, par voie intranasale, de la chalcone 4 et de la chalcone 4 phosphate (chalc4-P) à la dose maximale de 22nmol/kg (compte-tenu de la faible solubilité de la chalc4 dans le PBS: 22 μ M), a montré que seule la chalc4-P réduit l'inflammation des voies aériennes, permettant une diminution du recrutement des éosinophiles, des neutrophiles et des lymphocytes. Cette activité est corroborée par la courbe dose-réponse de la chalc4-P qui révèle une concentration inhibitrice médiane par la chalc4-P de 10nmol/kg (**publication n°2**). Ces résultats mettent en évidence l'intérêt

d'un neutraligand soluble et suggèrent que la prodrogue permet une distribution plus efficace de la chalcone 4 dans le tissu cible. L'étude *in vivo* des prodrogues sérine et sulfate montre une activité anti-inflammatoire similaire à la prodrogue chalc4-P à la dose de 30 nmol/kg ce qui indique que les trois prodrogues libèrent la même quantité de chalcone 4 active lorsqu'elles sont administrées localement dans les voies aériennes, un résultat cohérent avec la libération rapide de la chalcone 4 mesurée *in vitro* dans les homogénats de poumon. Ainsi, nous avons développé dans cette étude trois composés prodrogues solubles et adaptés à une application topique à très faibles doses dans les voies aériennes, des résultats corroborés par l'activité systémique des prodrogues. Nous avons montré que l'administration systémique (i.p.) de la prodrogue par le biais de la chalc4-P permet de réduire le recrutement des éosinophiles dans le modèle court d'asthme allergique, et présente une activité équivalente à la chalcone 4, avec une concentration inhibitrice médiane proche de 350µmol/kg (**effet dose-réponse non publié**). Ce résultat met en évidence que la dose administrée dans le cas du traitement local est très faible, soit 10 000 fois plus faible que par voie systémique pour une activité équivalente (**publication n°2**).

Les prodrogues du neutraligand chalcone 4 représentent une stratégie intéressante, dans le cas du traitement de l'asthme, car leur activité à des doses très faibles permettraient de réduire les effets indésirables des traitements sur toutes les autres fonctions associées à la chimiokine CXCL12 en dehors du poumon. Une telle stratégie a aussi été décrite dans le cas des glucocorticoïdes (Derendorf et al. 2006). Le bénéfice de cette stratégie semble d'autant plus importante que la chimiokine CXCL12 et ses récepteurs CXCR4 et CXCR7 soient impliqués dans l'inflammation, le cancer ou encore le syndrome d'immunodéficience acquise lié au VIH, la chimiokine CXCL12 est aussi impliquée dans l'homéostasie et permet notamment la rétention des précurseurs hématopoïétiques dans la moelle osseuse (Suarez-Alvarez et al. 2012). Aujourd'hui, l'antagoniste de CXCR4, l'AMD 3100 (Plerixafor, MOZOBILTM, solution injectable), est commercialisé pour le traitement de troubles lymphoprolifératifs chez l'homme (Liles et al. 2005). Il y est utilisé pour sa capacité à mobiliser les cellules progénitrices CXCR4+, une activité également démontrée chez la souris (Broxmeyer et al. 2005). Un autre composé, dirigé contre la chimiokine CXCL12, le NOX-A12 (solution injectable), est en cours de développement clinique (phase II) et pressenti pour le traitement de la leucémie lymphoïde chronique (Vater et al. 2013), une fois encore pour sa capacité à recruter les cellules de la moelle osseuse dans le sang.

Ainsi la conception de prodrogues solubles du composé chalcone 4 permet une administration locale, une activité anti-inflammatoire du neutraligand de CXCL12 à de très faibles doses, et offre une stratégie intéressante pour le développement de médicament candidat pour le traitement de l'asthme en limitant les effets adverses.

Dans un second travail, nous avons cherché à développer un neutraligand actif localement et ne présentant pas d'activité systémique, de manière à éviter toute dissémination de l'activité dans l'organisme. Parmi les composés synthétisés, nous avons montré que le composé carbonitrile-chalcone 4 (CN-chalc4) inhibe efficacement la liaison de la chimiokine CXCL12 à CXCR4 *in vitro* (FRET), et présente une activité identique à la chalcone 4 (**publication n°3**). Néanmoins, de même que la chalcone 4, le dérivé CN-chalc4 présente une très faible solubilité dans les tampons aqueux ($9\pm 1 \mu\text{M}$ et $16\pm 2 \mu\text{M}$, respectivement). Afin de permettre une administration locale *in vivo*, nous avons solubilisé ces composés dans une solution contenant 10% de 2-hydroxypropyl β -cyclodextrine, une formulation compatible avec une administration intranasale (Loftsson et al. 1996, Tewes et al. 2008) et qui permet d'augmenter la solubilité des composés ($690\pm 44 \mu\text{M}$ pour la chalc4 et $493\pm 36 \mu\text{M}$ pour la CN-chalc4).

Nous avons démontré que l'administration locale *in vivo* du dérivé CN-chalc4 permet d'inhiber le recrutement des éosinophiles dans les voies aériennes selon une courbe dose-réponse similaire à la chalcone 4. La comparaison des courbes doses-réponses des CN-chalc4 et chalc4 avec celle obtenue avec la chalc4-P met en évidence que la prodrogue chalc4-P a une concentration inhibitrice médiane 50 fois plus faible (500 nmol/kg versus 10 nmol/kg , respectivement) (**publications n°2 et 3**). De plus, la formulation des neutraligands CN-chalc4 et chalc4 permet de mesurer une activité anti-inflammatoire après administration locale, mais elle reste inférieure à celle atteinte par les prodrogues à la même dose. Cette différence pourrait être expliquée par une distribution augmentée dans les poumons pour les prodrogues par rapport à la distribution obtenue avec des dérivés moins solubles (**publication n°2 et 4**).

L'administration systémique (i.p.) des composés CN-chalc4 et chalc4 a révélé que le dérivé CN-chalc4 est inactif, contrairement à la chalcone 4 qui permet de réduire significativement l'inflammation des voies aériennes. La formulation des composés, en solution dans la 2-hydroxypropyl β -cyclodextrine ou en suspension dans une solution contenant 1% de carboxyméthyl cellulose, est sans effet sur l'activité systémique (**publication n°3**). L'étude des propriétés physicochimiques de la CN-chalc4 a mis en évidence que l'absence d'effet à un site distant est liée à une dégradation rapide du composé en deux fragments inactifs, comme démontré *in vitro* dans du sérum de souris et dans un homogénat de poumon. Nous avons ainsi démontré que la CN-chalc4 est un neutraligand avec une durée de vie courte (demi-vie de 35 min) qui peut être défini comme une ante-droge: un composé actif localement et dégradé en produits inactifs avant leur distribution dans l'organisme. Cette approche a permis d'obtenir un neutraligand de CXCL12 actif localement, adapté au traitement des maladies des voies aériennes, sans perturber les autres rôles de la chimiokine CXCL12 dans l'organisme.

Mode d'action du neutraligand chalcone 4 dans l'asthme

Parallèlement au développement des prodrogues et de l'ante-droge de la chalcone 4, nous avons étudié le mécanisme d'action de la chalcone 4 et son activité anti-asthmatique (**publication n°4**). Pour cela, nous avons utilisé le modèle conventionnel d'asthme allergique à l'ovalbumine en 21 jours chez la souris Balb/c. Nous avons montré que ce modèle est adapté à l'étude de la réactivité bronchique chez la souris Balb/c et présente une inflammation robuste et reproductible de type Th2, avec augmentation du nombre de mastocytes et qu'il permet d'étudier l'hypersécrétion de mucus et de collagène (**publication n°1**). Ce modèle aigu d'asthme allergique, plus classiquement utilisé que le modèle court (modèle 8 jours développé pour la sélection des composés) comprend une phase de provocation allergénique de 4 jours consécutifs, suffisant pour induire la plupart des caractéristiques de l'asthme allergique et adaptée à l'étude de l'activité de composés pharmacologiques : une chronologie parfaitement établie, traitement de courte durée et quantité de composé raisonnable. Ces caractéristiques en font les modèles les plus utilisés dans le développement des stratégies anti-asthmatiques (Nials et al. 2008). En particulier, une hyperréactivité bronchique (HRB) à la métacholine bien reproductible est observée dans ce modèle 21 jours.

Activité antiasthmatique: Nous avons montré que l'administration locale (i.n.) de chalcone 4 réduit l'hyperréactivité bronchique mesurée par plethysmographie chez l'animal vigile et non contraint. Cette réduction est confirmée par la mesure de la réactivité bronchique chez l'animal anesthésié (Flexivent®) par la mesure de la résistance des voies aériennes (**publication n°4**). Cette diminution de l'HRB est accompagnée d'une réduction significative et reproductible de l'hyperéosinophilie des voies aériennes, d'une diminution d'IL-5 et CCL11 (éotaxine), les deux principaux agents chimioattractants des éosinophiles. Le recrutement des neutrophiles et des lymphocytes dans le lavage bronchoalvéolaire est également diminué, ainsi que la production de mucus et la déposition de collagène. Il apparaît cependant que ce traitement par un neutraligand de la chimiokine CXCL12 ne permet pas de réduire les taux d'IL-4 libérée en réponse à l'ovalbumine, ainsi que des IgE plasmatiques, indiquant que l'activité de la chalcone 4 n'a pas pour cible les lymphocytes B producteurs d'IgE. Ces derniers résultats, réduction de la production d'IL-5 et pas de modification de production d'IL-4, ainsi que les effets connexes (hyperéosinophilie et production d'IgE) ont également été confirmés dans le modèle court d'asthme allergique (**résultats non présentés**), permettant d'asseoir ces résultats dans le contexte de l'asthme allergique. La chalcone 4 ne réduit pas non plus le recrutement de mastocytes dans le poumon, laissant à penser que la neutralisation de CXCL12 n'est pas active pour influencer ce recrutement de cellules mastocytaires, dont les progéniteurs expriment pourtant CXCR4 (Matsuura et al. 2010). Il a été mis en évidence que la présence d'adjuvant (alum) aboutit à une inflammation, une production d'IgE et une hyperréactivité

bronchique indépendante des mastocytes (Nakae et al. 2007). Ces résultats ne nous permettent pas d'avancer sur cette hypothèse puisqu'une diminution de l'inflammation et de l'HRB sont observées sous l'influence de la chalcone 4, et que l'infiltrat mastocytaire n'est pas modifié. Il nous paraît maintenant utile d'évaluer le rôle de la chalcone dans un modèle sans adjuvant qui permettra d'étudier la réponse des mastocytes à CXCL12.

Pharmacocinétique: Nous avons mis en évidence une relation singulière existant entre la pharmacocinétique et la pharmacodynamie de la chalcone 4 lors de son administration locale. Dans l'objectif d'utiliser une analyse par imagerie SPECT, une collaboration entre les chimistes de notre équipe (Dr Dominique Bonnet) et de l'institut pluridisciplinaire Hubert Curien à Cronenbourg (Dr Ali Ouadi) a permis de synthétiser une chalcone iodée, dont l'activité est identique à celle de la chalcone 4 (neutraligand de CXCL12 inhibant le recrutement d'éosinophiles *in vivo* dans le modèle court d'asthme). Cette iodo-chalcone a été marquée par l'Iode¹²³ radioactif (chalc4-I¹²³) (**publication en cours de rédaction**). L'étude pharmacocinétique de la chalc4-I¹²³ réalisée par SPECT et par activimétrie révèle que le neutraligand est rapidement éliminé du poumon (30 min) vers la circulation sanguine (pic à 5 min) pour être éliminé dans les urines, principalement sous la forme glucuroconjuguée, et dans la bile qui relargue alors le composé dans les intestins pour être éliminé dans les fèces. L'élimination de la chalcone 4 dans la bile et sous forme glucuroconjuguée dans les urines indique que la chalcone 4 est filtrée et métabolisée par le foie (Hagenbuch 2010). De plus, nous avons montré que cette absorption rapide de la chalcone 4, des poumons vers le sang, est concomitante à la présence de la chimiokine CXCL12 mesurée dans le sang, selon une cinétique parallèle à celle du composé (**publication n°4**). Nous en déduisons que la liaison de la chimiokine CXCL12 au neutraligand dans le tissu pulmonaire permet de la libérer des glycosaminoglycannes auxquels elle est liée à la surface cellulaire pour former un gradient de concentration et que ceci conduit à son élimination des poumons. Un tel phénomène de "capture" mis en jeu par la chalcone 4 a été validé *in vitro* sur des cellules HEK293 par microscopie confocale où la chimiokine CXCL12-TR exogène préalablement liée à la surface des cellules peut être capturée par la chalcone 4 et éliminée. Nous montrons également qu'une incubation rapide *ex vivo* des macrophages du LBA en présence de chalcone 4 (5µM, 30min), suivie d'un rinçage, entraîne une forte diminution du nombre de macrophages CXCL12+ (cytométrie en flux).

De plus, nous avons montré que l'élimination de la chimiokine CXCL12 engendrée par l'administration de chalcone 4 diminue la concentration de CXCL12 dans le poumon après chaque administration de chalcone 4, et conduit à une réduction significative de 20% de la quantité de CXCL12 présente dans le poumon après 4 administrations intranasales (**publication n°4**).

Macrophages: L'étude histologique et cytologique de la distribution de CXCL12 dans le poumon et les cellules inflammatoires met en évidence que la chimiokine est présente à la surface des cellules de l'épithélium bronchique, ainsi qu'à la membrane et dans le cytoplasme des macrophages (**publication n°4**, Gomperts et al. 2006). Nous avons donc étudié le rôle de la chimiokine CXCL12 et l'effet de la chalcone 4 sur les macrophages, représentant des cellules sentinelles du poumon. Une étude par cytométrie en flux des cellules du LBA permettant d'identifier efficacement les populations leucocytaires: éosinophiles, neutrophiles, lymphocytes T et B et macrophages (**publication n°A3**), à laquelle nous avons adjoint un anticorps anti-CXCL12, a confirmé la présence de la chimiokine CXCL12, à la surface des macrophages, contrairement aux autres leucocytes présents dans le LBA et qu'une proportion importante de ces macrophages exprime aussi le récepteur CXCR4 (**publication n°4**). Nous avons observé que le nombre de macrophages est augmenté en réponse à l'ovalbumine dans nos modèles d'asthme et n'est pas significativement diminué par le traitement par les neutralisants de CXCL12, chalcone 4, prodrogues et ante-droge (**publication n°2, 3 et 4**). Ces constats nous ont amenés à étudier l'état d'activation des macrophages en macrophages de type M1 ou M2. Pour cela, nous avons utilisé des marqueurs extracellulaires connus pour être spécifique de l'état d'activation des macrophages: l'expression du récepteur CCR7 (CD197) pour les macrophages M1 (Murphy et al. 2008, Badylak et al. 2008, Zeyda et al. 2010, Kodumudi et al. 2010, Riek et al. 2012, Mercurio et al. 2013, Vasconcelos et al. 2013) et un récepteur au mannose (CD206) pour les macrophages M2 (Muller et al. 2007, Pechkovsky et al. 2010, Wentworth et al. 2010, Bhatia et al. 2011, Riek et al. 2012).

L'étude par cytométrie en flux de l'activation des macrophages en macrophages de type M1 (CCR7+, CD206-) ou M2 (CD206+, CCR7-) a mis en évidence que le traitement local par la chalcone 4 diminue significativement le recrutement des macrophages de type M1 alors que la population des macrophages de type M2 reste stable (**publication n°4**).

Le phénotype d'activation M1 des macrophages est associé à une activité pro-inflammatoire accrue (TNF- α), et à la fonction de présentation d'antigène (Lawrence et al. 2011). Nous montrons *ex-vivo*, que les macrophages du LBA des souris sensibilisées et provoquées à l'ovalbumine et traitées par la chalc4, libèrent moins de TNF- α et d'IL-5 en réponse à l'ovalbumine, mais également ne libèrent plus de TNF- α en réponse au CXCL12 exogène.

D'autre part, le marqueur utilisé pour étudier le phénotype d'activation M1 des macrophages, le récepteur CCR7, est aussi connu pour être un marqueur de la maturation des cellules présentatrices d'antigène ou CPA (Poole et al. 2009, Jin et al. 2010) et est impliqué dans la migration de ces cellules vers les ganglions lymphatiques en réponse aux chimiokines CCL19 et CCL21 (Rivas-Cañedo et al. 2009, Johnson et al. 2013). La maturation des CPA est aussi accompagnée d'une surexpression du récepteur CXCR4 (Sallusto et al. 1998) et plusieurs publications mettent en évidence la contribution

de la chimiokine CXCL12 dans la migration des cellules CCR7+ vers les ganglions lymphatiques (Humrich et al. 2006, Bai et al. 2009, Stutte et al. 2010), notamment par l'utilisation d'AMD3100, qui inhibe *in vitro* le chimiotactisme des CPA en réponse à CXCL12 et CCL21 et *in vivo* le recrutement des CPA CCR7-/- vers les follicules lymphoïdes de la rate (Umemoto et al. 2012). Ces résultats suggèrent que la population identifiée comme macrophage M1 (CD45+, CD11b+, GR1-, MHCII+, CD11c+, CCR7+) est constituée de macrophages activés qui pourraient être en cours de différenciation en CPA. Le neutralisant de la chimiokine CXCL12 permet de réduire la quantité de CXCL12 dans le poumon et permettrait ainsi de prévenir ou limiter l'activation des macrophages en réponse à l'allergène.

Perspectives

Nos résultats montrent que l'administration locale de la chalcone 4 entraîne une très forte augmentation de la concentration plasmatique de CXCL12, qui diminue ensuite rapidement, pour retrouver une concentration basale une heure après l'administration de la chalcone 4 (**publication n°4**). L'élimination rapide de CXCL12 dans le sang évoque la présence de mécanismes actifs. Cependant, le ou les mécanismes impliqués dans l'élimination de CXCL12 présente dans le sang suite à l'administration de la chalcone 4, restent inconnus. Un récent article suggère que l'expression de CXCR7 à la surface de l'endothélium vasculaire permet de réguler la concentration plasmatique de la chimiokine CXCL12 (Berahovich et al. 2013), un mécanisme conforté par le rôle de récepteur "piège" de CXCR7 (Naumann et al. 2011). L'administration systémique d'un antagoniste du récepteur CXCR7, le composé CCX771, augmente la concentration plasmatique de la chimiokine CXCL12, suggérant que l'inhibition de CXCR7 perturbe la régulation de la concentration plasmatique de CXCL12 (Berahovich et al. 2013). Il semble donc indispensable de confirmer le rôle du récepteur CXCR7 dans l'élimination de la chimiokine CXCL12 plasmatique suite l'administration de la chalcone 4. La démonstration pourrait être réalisée en prétraitant des souris par le composé CCX771 avant l'administration locale de la chalcone 4 et la mesure de CXCL12 dans le plasma.

D'autre part, nous montrons que la chalcone 4 réduit le remodelage bronchique, notamment les dépôts de collagènes péri-bronchiques, mais nous ignorons si le neutralisant inhibe le recrutement des fibrocytes liés au développement de l'asthme (Nihlberg et al. 2006) . La chimiokine CXCL12 et ses récepteurs sont connus pour être impliqués dans le recrutement des fibrocytes CXCR4+/CD34+ (Gomperts et al. 2007, Garibald et al. 2013) qui contribueraient à la genèse d'une fibrose sous-épithéliale (Schmidt et al. 2003, Nihlberg et al. 2006), et qui peuvent aussi produire des médiateurs pro-angiogéniques et favoriser l'angiogenèse (Hartlapp et al. 2001). De plus, la chimiokine CXCL12 semble aussi impliquée dans la migration et la prolifération des cellules endothéliales (Costello et al. 2012), notamment en induisant l'expression des facteurs pro-angiogéniques tels que le VEGF et

CXCL8 par son action sur CXCR7, et pourrait être impliquée dans la néo-vascularisation observée dans l'asthme. L'augmentation de la microcirculation des voies aériennes dans l'asthme est accompagnée d'une augmentation du flux sanguin et de la perméabilité microvasculaire entraînant la formation d'œdème dans la paroi bronchique. Ce phénomène contribue à l'épaississement de la paroi des bronches, et conduit à un rétrécissement de la lumière bronchique lors de la contraction du muscle lisse et amplifie l'hyperréactivité bronchique (Ribatti et al. 2009). Ainsi, la neutralisation de la chimiokine CXCL12 par la chalcone 4 pourrait diminuer le recrutement des fibrocytes et réduire la néo-angiogenèse. Nous montrons que l'administration de la chalcone 4 est associée à une diminution du recrutement des éosinophiles et des lymphocytes CXCR4+ dans les voies aériennes (**publication n°4**) et il semble donc intéressant de mesurer l'activité de la chalcone 4 sur le recrutement des fibrocytes et la néo-angiogenèse. Pour cela, nous utiliserons un modèle d'asthme chronique, connu pour présenter un recrutement important de fibrocytes et une néo-angiogenèse, et nous mesurerons l'activité du neutralisant sur le recrutement des fibrocytes (CD34+, CXCR4+) et l'angiogenèse (CD45-, CD31+, CXCR7+ et ou CXCR4+) par cytométrie en flux après dissociation des cellules du poumon.

Le choix d'un modèle sans adjuvant nous permettra aussi d'étudier le rôle de la chimiokine CXCL12 et l'action du neutralisant sur le recrutement des mastocytes qui pourra être mesuré par des techniques histologiques (Bleu de toluidine) ou après mise en culture des cellules dissociées du poumon en présence de SCF et d'IL-3, pour mesurer le recrutement des progéniteurs des cellules mastocytaires (Dahlin et al. 2011).

Nos travaux mettent en évidence que la chimiokine CXCL12 est impliquée dans l'activation des macrophages alvéolaires et la libération des cytokines pro-inflammatoires, TNF- α et IL-5, et de la chimiokine CXCL12, en réponse à l'ovalbumine (**publication n°4**). D'autre part, une récente étude met en évidence que la chimiokine CXCL12 est impliquée dans la régulation de l'expression du marqueur CD14 à la surface des macrophages en réponse au LPS (Sánchez-Martín et al. 2013). Les auteurs ont mis en évidence que le LPS stimule la libération de la chimiokine CXCL12 par les macrophages et active les récepteurs CXCR4. Cette activation autocrine de CXCR4 accroît l'expression du récepteur CD14 qui fait partie du complexe de détection des lipopolysaccharides (LPS) et est connu pour potentialiser la libération du TNF- α en réponse au LPS (Lichtman et al. 1998).

L'ensemble de ces données suggèrent que l'activation des macrophages en réponse à l'allergène pourrait aussi impliquer l'activation autocrine de CXCR4 par CXCL12. Nous envisageons d'étudier le rôle des récepteurs FC ϵ RI et FC ϵ RII et des immunoglobulines IgE dans la libération de la chimiokine CXCL12 et des cytokines pro-inflammatoires par les macrophages, en réponse à l'ovalbumine. Pour cela, nous utiliserons des macrophages alvéolaires de souris sensibilisées et non sensibilisées, traités

ou non par des anticorps bloquants les récepteurs FC et par la chalcone 4. La libération des cytokines et de CXCL12 sera mesurée en réponse à l'ovalbumine, de même que l'expression des récepteurs CXCR4 et CCR7.

Enfin, l'implication de la chimiokine CXCL12 dans de nombreuses pathologies inflammatoires ainsi que dans la fibrose pulmonaire (Balkwill et al. 2004), l'hypertension artérielle pulmonaire (Young et al. 2009, Montani et al. 2011, Gambaryan et al. 2011, Yu et al. 2011, Costello et al. 2012) et certain cancer (Furusato et al. 2010, Lazennec et al. 2010, Hattermann et al. 2013, Wald et al. 2013), permet d'envisager d'autres applications à un neutralisant de CXCL12. En ce sens, des travaux sont en cours au sein du laboratoire sur la bronchiolite oblitérante (modèle murin de greffe hétérotopique de trachée, Simona Nemska) et la dermatite atopique (modèle murin en réponse au Calcipotriol ou MC903, Dayana Abboud) qui doivent être confirmés, mais les premiers résultats ont mis en évidence des effets bénéfiques du traitement par la chalcone 4.

Conclusion

En conclusion, nos travaux nous ont permis de mettre en évidence l'activité antiasthmatique des neutralisants de CXCL12 dans l'asthme allergique. La synthèse de dérivés de chalcone 4 a validé deux stratégies intéressantes, adaptées au traitement local de l'asthme allergique permettant d'en limiter les effets systémiques: les prodrogues solubles permettent une administration locale à de très faibles doses, tandis que la conception d'une ante-droge permet d'obtenir un neutralisant de courte durée de vie, dégradé avant sa distribution dans l'organisme. Ces travaux sont l'exemplification du potentiel de molécules neutralisant l'activité de la chimiokine CXCL12 sur ses récepteurs comme agents anti-inflammatoires et antiasthmatiques, selon les modèles d'asthme utilisés.

Nos travaux décrivent le mécanisme d'action de la chalcone 4 administrée in vivo par voie intranasale dans un modèle murin d'asthme allergique. La chalcone 4 neutralise CXCL12 au niveau pulmonaire et l'entraîne hors du tissu bronchique, perturbant ainsi le chimiotactisme des cellules inflammatoires, en particulier les éosinophiles et les lymphocytes, et modifiant la réponse des macrophages alvéolaires à l'allergène. Ces effets conduisent à une réduction de la réponse inflammatoire, de l'hyperréactivité bronchique et du remodelage bronchique. L'ensemble de ces résultats soutient l'intérêt de l'utilisation de neutralisants de CXCL12 pour le traitement de l'asthme allergique.

ANNEXES

Annexe n°1:

Acute Ovalbumin Asthma Model In The Mouse.

François Daubeuf and Nelly Frossard

Current Protocols in Mouse Biology. March, 2013

<http://www.currentprotocols.com/WileyCDA/CPUnit/refId-mo120202.html>

Cet article décrit les procédures à suivre pour obtenir des modèles aigus d'asthme chez la souris. Les protocoles décrits engendrent le développement des principales caractéristiques de l'asthme en réponse à un allergène, l'ovalbumine. Elles présentent les modèles en présence d'un adjuvant, l'hydroxyde d'aluminium ou alum afin d'accroître la réponse Th2 à l'allergène. Le suivi de ces procédures permet le développement de modèles aigus, de manière efficace et reproductible, chez les souris de souche BALB/c ou C57BL6, et permet l'évaluation de l'activité de candidats-médicaments et/ou de stratégies anti-asthmatiques.

Acute Asthma Models to Ovalbumin in the Mouse

François Daubeuf¹ and Nelly Frossard¹

¹Laboratoire d'Innovation Thérapeutique, Faculté de Pharmacie, UMR7200 CNRS/Université de Strasbourg, Illkirch Cedex, France

ABSTRACT

Human asthma is defined as a chronic inflammatory disease of the airways. Animal models are required to study asthma pathophysiology and identify and/or evaluate new therapeutic strategies. Several models of asthma have been developed in mice to mimic asthma symptoms, and can be divided in two groups as acute and chronic models. They are characterized by airway hyperresponsiveness (AHR), inflammation, and remodeling. Several experimental procedures have been implemented, the one mostly used being acute asthma models to ovalbumin. It comprises a sensitization step in the presence of aluminum hydroxide as an adjuvant, and a second step where mice are challenged with the allergen introduced directly into the airways to induce the modeled asthma features. This article describes procedures to efficiently and reproducibly obtain acute asthma features in mice, with ovalbumin as the allergen, which allow group comparisons and/or assessment of the activity of drug candidates. *Curr. Protoc. Mouse Biol.* 3:31-37 © 2013 by John Wiley & Sons, Inc.

Keywords: asthma • allergy • inflammation • bronchial hyperresponsiveness

INTRODUCTION

Human allergic asthma is defined as a chronic inflammatory disorder of the airways and is characterized by airway inflammation and hyperresponsiveness (Meurs et al., 2008; Berend et al., 2008) with airway structural modifications corresponding to “airway remodeling;” this includes excessive mucus secretion, fibroblast proliferation with collagen deposition as “subepithelial fibrosis,” smooth muscle thickening, and increased vascularity (Bai and Knight, 2005; Ozier et al., 2011). To study the airway responses to an allergen and develop anti-asthma strategies, several animal asthma models have been developed. The mouse is nowadays the most widely used species. Mice do not spontaneously develop asthma, and it is therefore necessary to generate an artificial asthma-like reaction in the airways. The most commonly used strain for antigen challenge models is the BALB/c, but the C57BL/6 or A/J strains have also been used successfully for allergen challenge studies and development of asthma features (Kumar et al., 2008). Allergens that may have clinical relevance, such as house dust mite and cockroach extracts (Lukacs et al., 2002; Ulrich et al., 2008), have been successfully developed for asthma models in the mouse. Ovalbumin (OVA) derived from chicken egg is a more frequently used allergen since it induces a robust allergic bronchial inflammation, particularly where acute allergic response is concerned. Asthma models to OVA reproduce many features of clinical asthma, and are very useful to identify novel therapeutic targets and strategies. Many different sensitization and challenge protocols have been developed.

As a general statement, acute sensitization protocols require multiple systemic administration of the allergen in the presence of an adjuvant. Adjuvants such as aluminum hydroxide [Al(OH)₃] are known to promote the development of a Th2 phenotype of the immune system exposed to an antigen (Brewer et al., 1999; McKee et al., 2009), which is

required for a validated acute asthma model. In addition, these protocols require multiple airway ovalbumin challenges administered either by aerosol or by the intranasal route.

In this short protocol, we describe the optimized procedures to sensitize and challenge mice in order to develop acute asthma with OVA as the allergen, and which warrant the best reproducibility. We present three models, detailing the different steps; each model represents a suitable alternative dependent on the mouse strain and on parameters to be measured.

ACUTE OVALBUMIN ASTHMA MODELS

This protocol describes the procedure for sensitizing and challenging mice with OVA, thereby inducing the development of reproducible and robust asthma models. Acute models of asthma to ovalbumin require several sensitization steps to ovalbumin in the presence of aluminum hydroxide, leading to a high inflammatory cell recruitment (Fig. 1), which can be measured in the bronchoalveolar lavage collected as described (Daubeuf and Frossard, 2012). The following steps may be used for several asthma models; they are dependent on the number of sensitizations and challenges, the adjuvant, the dose of ovalbumin used, and the time between sensitizations and/or challenges. The controls for experiments will be mice sensitized with ovalbumin in presence of $\text{Al}(\text{OH})_3$ and challenged with solvent (saline) alone.

Materials

- Ovalbumin grade V (Sigma-Aldrich, cat. no. A5503)
- Sterile saline
- Aluminum hydroxide (Sigma-Aldrich, cat. no. 23918-6)
- 9-week-old male BALB/c or C57BL/6 mice
- Anesthetics (50 mg/kg Ketamine/3 mg/kg Xylazine), stored at 4°C
- Precision balance (0.1 mg)
- 50-ml Falcon tubes
- 1.5-ml microtubes, sterile
- 5 ml sterile culture tubes
- 15-ml sterile centrifuge tubes
- Rotator mixer
- 1-ml sterile syringes
- 25-G needles
- Vortex mixer

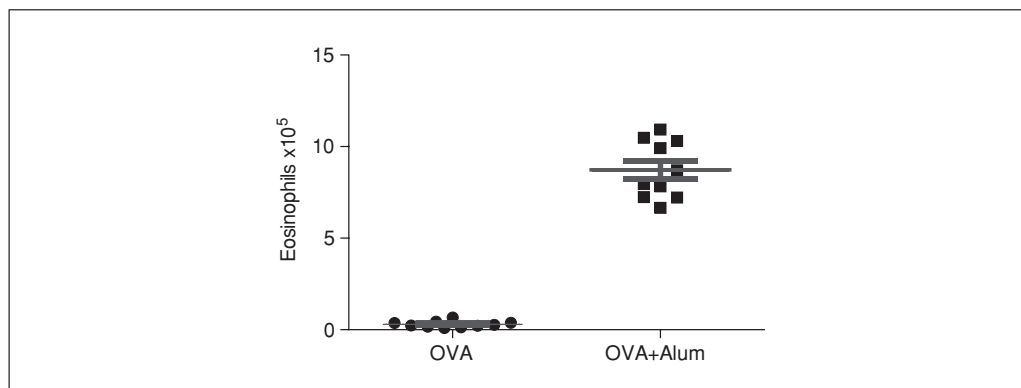


Figure 1 Number of eosinophils in bronchoalveolar lavage (BAL) fluid in the 8-day model with and without aluminum hydroxide. Number of eosinophils in BAL of mice sensitized to OVA in the presence or absence of alum after OVA challenges in the 8-day hyper eosinophilia model. Dots are individuals values and bars are means and SEM values ($n = 10$).

Sterile tips
Precision pipets (20 μ l and 1000 μ l)
Heating blanket or heating lamp

Prepare the ovalbumin and aluminum hydroxide

1. Prepare a sterile 4 mg/ml solution of ovalbumin as follows:
 - a. Weigh 200 mg ovalbumin.
 - b. Dissolve it in 50 ml cold sterile saline in a 50-ml Falcon tube.
 - c. Mix well.
2. Distribute 20 ml of the solution as 1-ml aliquots into 1.5-ml microtubes. Immediately store the aliquots up to 1 year at -80°C .

To be used for the 23-day model.
3. Take 20 ml of the solution, dilute it 1/2 (2 mg/ml) and distribute as 1-ml aliquots into microtubes. Immediately store aliquots up to 1 year at -80°C .
4. Take 4 ml of the remaining 4 mg/ml solution and dilute it 1:10 in cold sterile saline. Mix and distribute as 1-ml aliquots in microtubes. Immediately store aliquots up to 1 year at -80°C .

To be used for the 8-day and 21-day models.
5. In addition, transfer 1 ml sterile saline into microtubes and 4 ml saline into sterile culture tubes. Immediately store the aliquots up to 1 year at -80°C .

You should have a total of sixty tubes containing 1ml sterile saline and forty tubes containing 4 ml saline.
6. As soon as the aluminum hydroxide powder [$\text{Al}(\text{OH})_3$, alum] arrives at the laboratory, weigh aliquots of 80 mg in 5-ml sterile culture tubes. Store the alum aliquots protected from light at room temperature.

Be very careful while choosing the aluminum hydroxide reagent (see Fig. 2).

Perform ovalbumin sensitization with aluminum hydroxide

7. Before use, bring an aliquot of 2 mg/ml ovalbumin solution and a 4-ml aliquot of sterile saline to room temperature.
8. Freshly prepare a suspension containing ovalbumin and aluminum hydroxide diluted in sterile saline. Take an aliquot of aluminum hydroxide (80 mg), add 1 ml ovalbumin solution (2 mg/ml) and 3 ml sterile saline.
9. Gently homogenize the ovalbumin+ $\text{Al}(\text{OH})_3$ suspension for 4 hr at 4°C on a rotator mixer to adsorb ovalbumin on aluminum hydroxide.

Aluminum hydroxide is not entirely soluble and will be presented as a suspension.
10. Before use in animals, bring the solutions to room temperature (18° to 23°C).
11. Hold the mouse in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail with fingers.
12. Use 1-ml syringes and 25-G needles to inject the suspension and administer 4 ml/kg per mouse by intraperitoneal injection.

Gently homogenate the suspension between each injection.

Ovalbumin challenge

This point concerns three different models: the 8-day model and the 21-day-model are performed in BALB/c mice; the 23-day-model is performed in C57BL/6 mice.

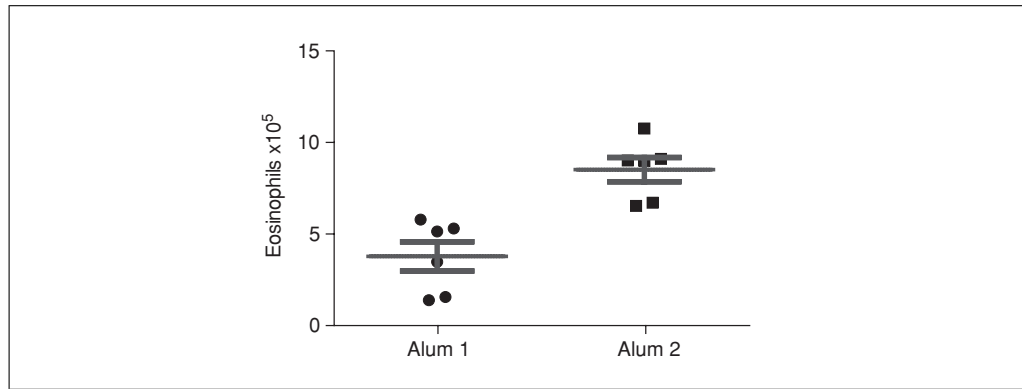


Figure 2 Influence of the quality of aluminum hydroxide on the number of eosinophils in BAL. Number of eosinophils in BAL in ovalbumin-sensitized and challenged mice in the 8-day asthma model. Alum 1: mice were sensitized with aluminum hydroxide from Merck (cat. no. 101091); Alum 2: mice were sensitized with aluminum hydroxide from Sigma-Aldrich (cat. no. 239186). Dots are independent studies ($n = 6$ mice/study) and bars are means with SEM values ($n = 6$ independent studies).

13. Before use, bring an aliquot of 0.4 mg/ml OVA solution (for the 8-day and the 21-day models) or an aliquot of 4 mg/ml OVA solution (for the 23-day model) to room temperature (18° to 25°C).
14. Vortex for 15 sec to mix.
15. Anesthetize the mice as follows:
 - a. Hold the mouse in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail with fingers.
 - b. Use 1-ml syringes and 25-G needles to inject the anesthetic solution; inject 4 ml/kg of a 12.5 mg/ml ketamine and 0.83 mg/ml xylazine solution per mouse intraperitoneally.
 - c. Place the mouse in the cage and wait until it is anesthetized. Check that vibrissae do not move any more.
16. Hold the mouse in your hand in a vertical position with its head up and its rear legs down. Administer 12.5 μ l OVA solution or saline alone for controls in each nostril by using sterile tips and a 20- μ l precision pipet.

Solution has to be administered drop by drop, slowly and very carefully.
17. Keep the mouse in your hand in a vertical position for at least 1 min so that the solution can be distributed low in the airways, and check that the mouse breathes normally.

If the mouse does not breathe normally, perform a thorax massage by pressing the rib cage several times, quickly but carefully.
18. Next, place the mouse in a horizontal decubitus on a heating blanket until the mouse is completely awake.

**SUPPORT
PROTOCOL**

**Acute Asthma
Models to
Ovalbumin in
the Mouse**

MODELS OF ASTHMA INDUCED BY OVALBUMIN

This protocol describes three models of asthma induced by ovalbumin, as the allergen, in presence of an adjuvant, aluminum hydroxide, in mice: the rapid 8-day model (Gasparik et al., 2012), and the commonly used 21-day model (Hachet-Haas et al., 2008) in BALB/c mice, and a 23-day model for C57BL/6 mice (Caceres et al., 2009). In these models (Fig. 3), BALB/c or C57BL/6 mice develop an ovalbumin-specific IgE production, airway inflammatory cell recruitment, and airway remodeling. Airway

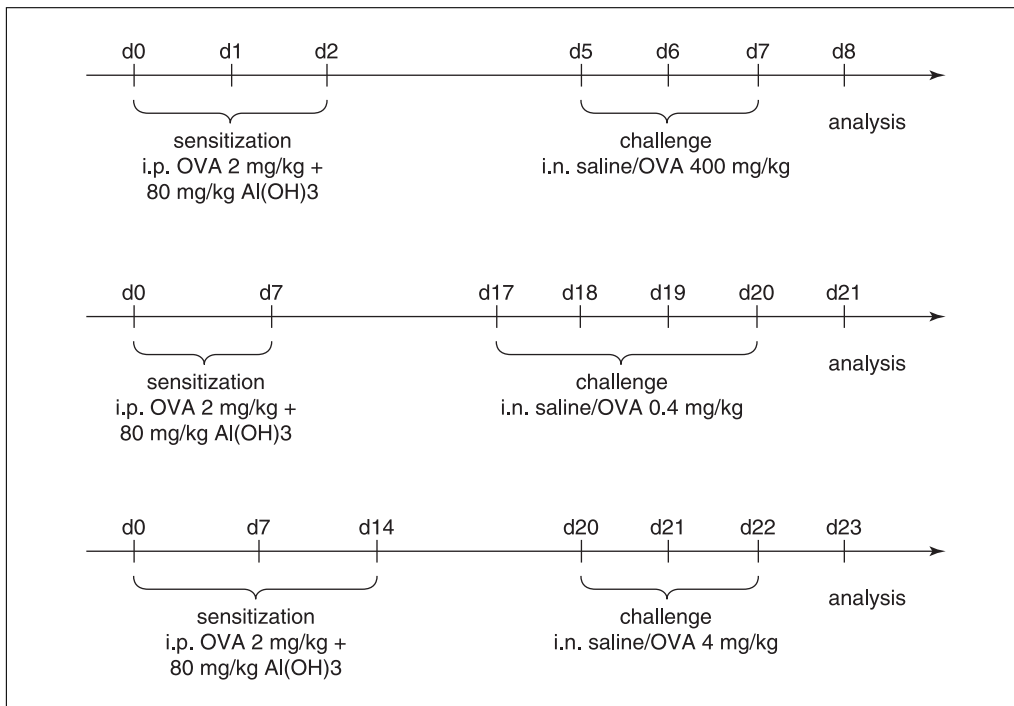


Figure 3 Acute asthma models to ovalbumin in mice. Three models of acute asthma composed of sensitization steps with intraperitoneal (i.p.) administration of ovalbumin and aluminum hydroxide, and intranasal (i.n.) challenges with ovalbumin or saline for control mice.

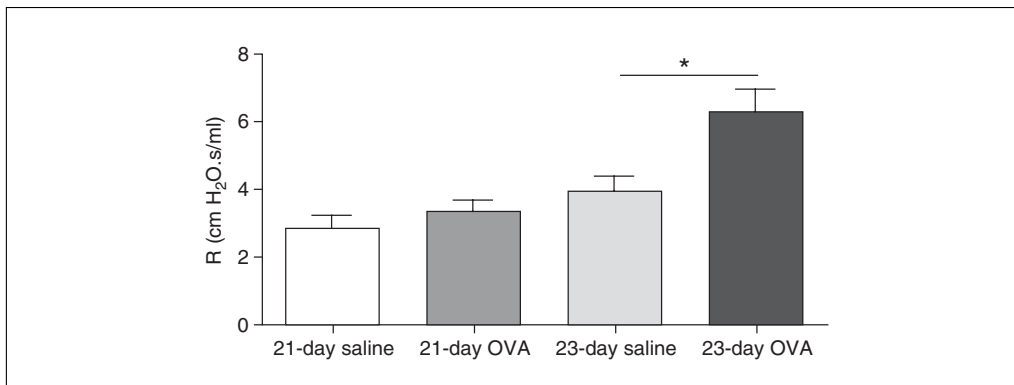


Figure 4 Measurement of airway resistance in the C57Bl/6 strain of mice in the 21-day and the 23-day asthma models. Airway resistance (R) in response to a single dose of aerosolized methacholine (50 mg/kg) was assessed by the Flexivent technique. Blocks are means, and bars are SEM values ($n = 12$). * $p \leq 0.05$.

hyperresponsiveness (AHR) to methacholine can be measured in BALB/c mice in the 21-day model, either by unrestrained whole-body barometric plethysmography (PenH measurement) or by the Flexivent techniques (resistance, compliance, and elastance measurements). AHR can be measured in BALB/c in the 8-day (Gasparik et al., 2012), and in C57bl/6 in the 23-day (Caceres et al., 2009) asthma models by the Flexivent technique (Fig. 4). For materials, see the Basic Protocol.

8-day model

- 1a. Sensitize mice i.p. with 4 ml/kg of the OVA-Alum suspension (2 mg/kg ovalbumin and 80 mg/kg aluminum hydroxide) on days 0, 1, and 2 as described in the Basic Protocol, steps 7 to 12.
- 2a. Anesthetize and challenge mice intranasally (i.n.) with the 0.4 mg/ml ovalbumin solution by administering 12.5 μ l per nostril on days 5, 6, and 7.

- 3a. Perform measurements on day 8, i.e., 18 to 24 hr after the last challenge.

21-day model

- 1b. Sensitize mice i.p. with 4 ml/kg of the OVA-Alum suspension (2 mg/kg ovalbumin and 80 mg/kg aluminum hydroxide) on days 0 and 7 as described in the Basic Protocol, steps 7 to 12.
- 2b. Anesthetize and challenge mice i.n. with the 0.4 mg/ml ovalbumin solution by administering 12.5 μ l per nostril on days 18, 19, 20, and 21.
- 3b. Perform measurements on day 22, i.e., 18 to 24 hr after the last challenge.

23-day model

- 1c. Sensitize mice i.p. with 4 ml/kg of the OVA-Alum suspension (2 mg/kg ovalbumin and 80 mg/kg aluminum hydroxide) on days 0, 7, and 14 as described in the Basic Protocol, steps 7 to 12.
- 2c. Anesthetize and challenge mice i.n. with the 4 mg/ml ovalbumin solution by administering 12.5 μ l per nostril on days 20, 21, and 22.
- 3c. Perform measurements on day 23 i.e., 18 to 24 hr after the last challenge.

COMMENTARY

Background Information

The acute asthma models are very useful for studying the effects of anti-asthma drugs and new therapeutic strategies, since they allow appraisal of airway inflammatory cell infiltrate and cytokine expression, airway hyper-responsiveness, and bronchial wall remodeling. The frequently used allergen in acute asthma models is ovalbumin from chicken egg. This allergen leads to a rapid, robust, and reproducible allergic response in regard to inflammatory cell recruitment, AHR, and remodeling (Delayre-Orthez et al., 2004, 2005). However, ovalbumin is seldom implicated in human asthma, and in recent years, several models have been developed with more realistic allergens that may have greater clinical relevance, such as house dust mite (HDM) or cockroach extracts. HDM and cockroach extracts are now used in chronic asthma models to study the mechanisms underlying the asthmatic disease. These models take long to develop and are very “drug-consuming” when therapeutic strategy is concerned as administered in a chronic manner.

We have developed the ovalbumin-induced 8-day asthma model to rapidly screen the activity of drug candidates in vivo, which requires less compounds, or antibodies, etc. This acute model is very reliable and reproducible, and allows rapid results as a Go-NoGo in the development of drug-candidates. Nowadays, the acute ovalbumin models described in this article have been used in the development of many drugs and therapeutic strategies such as

corticosteroids, calcineurin inhibitors, thromboxane antagonists, leukotriene antagonists, anti-IGE, anti-IL-5, anti-IL-4, and anti-TNF- α monoclonal antibodies, and remain important as first models to develop anti-asthma strategies (Nials et al., 2008).

Critical Parameters

The validity of results obtained from pulmonary phenotyping where genetically modified mice are concerned is likewise largely dependent on the methods used. Asthma models require intranasal administration for airway challenge. Intranasal challenge is a simple technique, easy to perform, but it requires expertise to obtain reproducible responses. This step should be performed by the same investigator within the same protocol to optimize the quality and reproducibility of results. In addition, we advise to keep aliquots of both ovalbumin and aluminum hydroxide powders, which will avoid opening stock bottles and help prevent bacterial and fungal contamination to guarantee the best reproducibility on inflammation. Concerning ovalbumin powder, which must be stored at 4°C, each handling of the bottle at room temperature (to weigh an aliquot for instance) will cause water condensation in the bottle and hydration of ovalbumin powder that will promote protein degradation and fungal proliferation.

Troubleshooting

Intranasal administration is the key for success of good reproducibility. If the mouse is not

Table 1 Time Considerations

Step	Time required
Preparation	4 hr
Sensitization	10 min
Challenges	15 min

sufficiently anesthetized, the challenge will not be effective since the mouse will eliminate the solution through the nostrils. Checking the vibrissae reaction is essential before intranasal administration. Moreover, when anesthesia has to be repeated on the same day, e.g., for successive intranasal administration of a drug-candidate, followed 1 to 2 hr later by ovalbumin challenge, the dose of anesthetics will need to be increased in order to optimize the second administration.

Time Considerations

Table 1 summarizes the timelines associated with completing these procedures for one mouse. Note that the preparation of ovalbumin-aluminum hydroxide solution requires mixing 4 hr before the intraperitoneal administration. For sensitization and challenge, the time does not increase linearly with the number of mice.

Literature Cited

- Bai, T.R. and Knight, D.A. 2005. Structural changes in the airways in asthma: Observations and consequences. *Clin Sci (Lond)*. 108:463-477.
- Berend, N., Salome, C.M., and King, G.G. 2008. Mechanisms of airway hyperresponsiveness in asthma. *Respirology* 13:624-631.
- Brewer, J.M., Conacher, M., Hunter, C.A., Mohrs, M., Brombacher, F., and Alexander, J. 1999. Aluminum hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J Immunol*. 163:6448-6454.
- Caceres, A.I., Brackmann, M., Elia, M.D., Bessac, B.F., del Camino, D., D'Amours, M., Witek, J.S., Fanger, C.M., Chong, J.A., Hayward, N.J., Homer, R.J., Cohn, L., Huang, X., Moran, M.M., and Jordt, S.E. 2009. A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proc. Natl. Acad. Sci. U.S.A.* 106:9099-9104.
- Daubeuf, F. and Frossard, N. 2012. Performing bronchoalveolar lavage in the mouse. *Curr. Protoc. Mouse Biol.* 2:167-175.
- Delayre-Orthez, C., Becker, J., De Blay, F., Frossard, N., and Pons, F. 2004. Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin. Exp. Allergy* 34:1789-1795.
- Delayre-Orthez, C., Becker, J., de Blay, F., Frossard, N., and Pons, F. 2005. Exposure to endotoxins during sensitization prevents further endotoxin-induced exacerbation of airway inflammation in a mouse model of allergic asthma. *Int. Arch. Allergy Clin. Immunol.* 138:298-304.
- Gasparik, V., Daubeuf, F., Hachet-Haas, M., Rohmer, F., Gizzi, P., Haiech, J., Galzi, J.-L., Hibert, M., Bonnet, D., and Frossard, N. 2012. Prodrugs of a CXCL12 neutraligand active in vivo in a new asthma model. *ACS Med. Chem. Lett.* 3:10-14.
- Hachet-Haas, M., Balabanian, K., Rohmer, F., Pons, F., Franchet, C., Lecat, S., Chow, K.Y., Dagher, R., Gizzi, P., Didier, B., Lagane, B., Kellenberger, E., Bonnet, D., Baleux, F., Haiech, J., Parmentier, M., Frossard, N., Arenzana-Seisdedos, F., Hibert, M., and Galzi, J.-L. 2008. Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J. Biol. Chem.* 283:23189-23199.
- Kumar, R.K., Herbert, C., and Foster, P.S. 2008. The "classical" ovalbumin challenge model of asthma in mice. *Curr. Drug Targets* 9:485-494.
- Lukacs, N.W., John, A., Berlin, A., Bullard, D.C., Knibbs, R., and Stoolman, L.M. 2002. E- and P-selectins are essential for the development of cockroach allergen-induced airway responses. *J. Immunol.* 169:2120-2125.
- McKee, A.S., Munks, M.W., MacLeod, M.K., Fleenor, C.J., Van Rooijen, N., Kappler, J.W., and Marrack, P. 2009. Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J. Immunol.* 183:4403-4414.
- Meurs, H., Gosens, R., and Zaagsma, J. 2008. Airway hyperresponsiveness in asthma: Lessons from in vitro model systems and animal models. *Eur. Respir. J.* 32:487-502.
- Nials, A.T. and Uddin, S. 2008. Mouse models of allergic asthma: Acute and chronic allergen challenge. *Dis. Model Mech.* 1:213-220.
- Ozier, A., Allard, B., Bara, I., Girodet, P.O., Trian, T., Marthan, R., and Berger, P. 2011. The pivotal role of airway smooth muscle in asthma pathophysiology. *J. Allergy (Cairo)* 2011:742710.
- Ulrich, K., Hincks, J.S., Walsh, R., Wetterstrand, E.M., Fidock, M.D., Sreckovic, S., Lamb, D.J., Douglas, G.J., Yeadon, M., Perros-Huguet, C., and Evans, S.M. 2008. Anti-inflammatory modulation of chronic airway inflammation in the murine house dust mite model. *Pulm. Pharmacol. Ther.* 21:637-647.

Annexe n°2:

Performing Bronchoalveolar Lavage in the Mouse.

François Daubeuf and Nelly Frossard

Current Protocols in Mouse Biology. June, 2012

<http://www.currentprotocols.com/WileyCDA/CPUnit/refId-mo110201.html>

Cet article décrit une procédure, rapide, peu onéreuse et hautement reproductible pour effectuer un lavage bronchoalvéolaire (LBA) chez la souris et analyser les populations leucocytaires dans ce LBA. Le LBA permet d'étudier l'afflux cellulaire dans les maladies des voies aériennes telles que l'asthme, la BPCO et l'inflammation aigüe. Le comptage et l'identification des cellules peuvent être combinés avec des méthodes comme l'ELISA, l'immunohistochimie, la RT-qPCR ou encore la spectrométrie de masse (détection et mesure des eicosanoïdes, des composés pharmacologiques), dans le but d'évaluer les composantes inflammatoires comme les cytokines, les facteurs de croissance, des analytes, les récepteurs exprimés à la membrane cellulaire ou la distribution de composés thérapeutiques. Cette procédure décrit la méthodologie et l'ensemble des résultats validant la technique.

Performing Bronchoalveolar Lavage in the Mouse

François Daubeuf¹ and Nelly Frossard¹

¹UMR 7200 CNRS—Université de Strasbourg, Laboratoire d'Innovation Thérapeutique, Faculté de Pharmacie, Illkirch, France

ABSTRACT

Bronchoalveolar lavage (BAL) is a simple technique commonly used in humans to sample the contents of the epithelial lining fluid and determine the cellular and molecular composition of the pulmonary airways. In murine models, BAL makes it possible to sample immunological and inflammatory cell populations; it is indispensable for studying cell influx in disease models of the airways such as asthma and COPD. Cell counts can be combined with methods such as ELISA, immunoblot, immunohistochemistry, quantitative polymerase chain reaction, and HPLC to assess such inflammatory components as cytokines, growth factors, analytes, and receptors expressed at the cell membrane. Performing BAL in a reproducible manner is a hallmark of airway research in the mouse. Several procedures may be implemented. This unit describes a basic, rapid, inexpensive, and highly reproducible procedure to collect BAL fluid and cells that can be counted efficiently and reproducibly. *Curr. Protoc. Mouse Biol.* 2:167-175 © 2012 by John Wiley & Sons, Inc.

Keywords: bronchoalveolar lavage • inflammation • airways • lung • asthma • COPD

INTRODUCTION

Bronchoalveolar lavage (BAL) is an invaluable technique to evaluate the inflammatory response and explore immune mechanisms in the lung (Hunninghake et al., 1979). In humans, it is commonly used in the clinic to diagnose lung diseases, infections, inflammation, and cancer (Henderson, 1994). This procedure makes it possible to study the components of the epithelial lining fluid. Levels and phenotypes of inflammatory cells infiltrating the airways can be assessed, for they are recovered in the BAL fluid (BALF) after transmigration through the epithelial layer. BAL is also used to quantify the protein composition of the airways. In experimental studies in small animals, BAL is a rapid and effective procedure for studying inflammation in the airways after euthanasia (Andreasen, 2003). Hence, BAL is generally a terminal experiment in animals.

In animal models of inflammatory or infectious diseases, BAL is most commonly used to study the influx of inflammatory cells in the airways, such as the eosinophils in allergic asthma for instance. To study this influx, BAL is performed and differential cells counted by optical microscopy: eosinophils, neutrophils, macrophages, and lymphocytes (Delayre-Orthez et al., 2004, 2005a,b; 2008; Hachet-Haas et al., 2008; Blé et al., 2008, 2009; Zhang et al., 2009; Deschamps et al., 2011; Reber et al., 2012). For more specific assessments, flow cytometric methods can be applied, e.g., to identify T cell phenotypes in the cellular composition of BALF cells in asthma models (Van Rijt et al., 2004; Reber et al., 2012).

Surprisingly, however, there is no one standardized BAL procedure used in the large number of publications and teams in the field. There are as many ways to perform BAL as there are research laboratories using BAL. This variability causes difficulty in the interpretation and comparison of the results.

In this short unit, we describe a basic, easy-to-use, inexpensive, and reproducible BAL procedure that makes it possible to collect most of the cells infiltrating the airway lumen of the mouse.

BRONCHOALVEOLAR LAVAGE

This protocol describes a basic, rapid, inexpensive, and highly reproducible procedure for collecting BAL cells and fluid. BAL may be used to assess the number and phenotype of cells, compound levels for pharmacokinetics, the composition of proteins such as cytokines, chemokines, growth factors, and mucus, and enzyme activity including but not limited to myeloperoxidase, alkaline phosphatase, and lactate dehydrogenase. To optimize cell recovery, lavage needs to be performed several times; to prevent destruction of the lung structure, the total volume of lavage should not exceed airway volume. In the mouse, this volume is 500 μ l, and the optimal number of lavages is ten. However, to assess protein composition accurately, the initial concentrated supernatant, from the first two lavages, must be collected, centrifuged, and frozen. The cell pellet will be added to the one collected from the other eight lavages also centrifuged to pellet cells; the fluid is generally discarded.

Materials

Anesthetic (Ketamine 50 mg/kg-Xylazine 3 mg/kg)
Sterile saline with EDTA added (2.6 mM) on ice

1-ml sterile syringes
21- and 25-G sterile needles
23-G sterile needles, optional (if sampling blood)
Scissors
Cotton thread no. 40
21-G lavage tubing, carefully placed over a 21-G needle
5-ml polypropylene tubes
Centrifuge

Perform a tracheostomy

1. Anesthetize the mouse by intraperitoneal injection of the anesthetic using a 1-ml syringe equipped with a 25-G needle.
2. Place the animal in the dorsal decubitus position.
At this step, blood can be collected within the inferior vena cava after opening the abdominal cavity, with a 1-ml syringe equipped with a 23-G needle.
3. Using scissors, make a small incision in the neck skin on the trachea and open the skin upwards to expose the salivary glands. Separate these to expose the sternohyoid muscle.
4. Incise the muscle longitudinally and expose the trachea.
5. Place a cotton thread under the trachea.
6. Make a small semi-excision of the trachea to allow a 21-G lavage tube to pass into the trachea. Take care not to cut through the trachea.
7. Stabilize the tube and needle by attaching them with a cotton thread.

Collect lavage fluid

8. Load a 1-ml syringe with 0.5 ml sterile saline-EDTA.
9. Place the 1-ml syringe in the 21-G lavage tube and inject 500 μ l saline-EDTA into the lung.

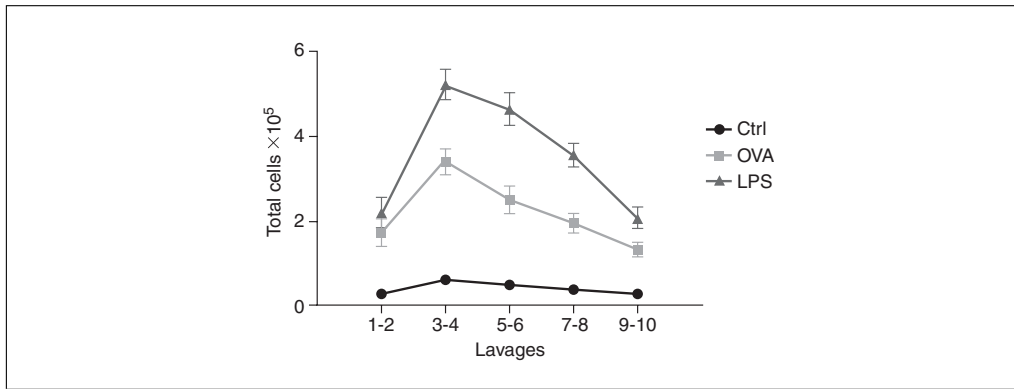


Figure 1 Evolution of total cell count per lavage in bronchoalveolar lavage (BAL). Total cells in BALF from control naive (Ctrl), ovalbumin-sensitized and -challenged (OVA), and *Escherichia coli* lipopolysaccharide-treated (LPS) mice. Cells from the first and second lavages 1-2, and from lavages 3-4, 5-6, 7-8, and 9-10 were stored in saline-EDTA at 4°C, and total cells were counted in a hemacytometer (Neubauer). Data show that lavage must be done 10 times for the most efficient cell recovery. Dots are means and bars are SEM values ($n = 6$)

10. Massage the chest for 10 sec.
11. Aspirate saline for the first lavage.
12. Remove the syringe from needle and inject the recovered lavage fluid into a 5-ml tube placed on ice.
13. Repeat the procedure 10 times per animal (Fig. 1). Centrifuge the initial concentrated supernatant from the first two pooled lavages for 5 min at $300 \times g$, 4°C. Freeze the supernatant for further analysis, and keep the cell pellet.
14. Pool the other eight lavages recovered and centrifuge them for 5 min at $300 \times g$, 4°C, to pellet the cells. Add the pellet to the cell pellet from the first two lavages.
15. Store the pellets on ice (max 6 hr).

The lungs can be perfused at this stage and collected for histology studies, PCR, ELISA, immunoblot analysis, etc.

CELL COUNTS

After BAL collection, erythrocytes must be removed from the cell pellet so that only the white blood cells are kept (this step must be performed as soon as possible and no more than 6 hr after collection). Total leukocytes should be counted manually with a hemacytometer (e.g., a Neubauer chamber). Finally, the cells must be cytospun and stained for differential counting, either with Diff-Quick staining within 1 hr, since Diff-Quick requires freshly cytospun cells, or with the home-made staining described below.

Materials

- BAL (see Basic Protocol 1)
- Sterile deionized H₂O (H₂O_d)
- Sterile potassium chloride solution (0.6 M)
- Sterile saline (0.9% NaCl) with EDTA (2.6 mM) on ice
- Diff-Quick staining kit (Hemacolor; Merck, cat. no. 1.11661.0001) containing:
 - Diff-Quick fixative
 - Solution I
 - Solution II

BASIC PROTOCOL 2

BAL in the Mouse

Reagent HMS-A (Home-Made stain A, methanol 100%)
Reagent HMS-B (Eosin 0.075%, H₂O)
Reagent HMS-C (methylene blue 0.06%, toluidine blue 0.04%, H₂O)

Centrifuge

Hemocytometer (Neubauer or other)

Cytofunnel (Shandon; Thermo Scientific, cat. no. 5991040)

Shandon filter cards (Shandon; Thermo Scientific, cat. no. 5991022)

Superfrost slides (MENZEL-GLÄSER; Thermo Scientific, cat. no. LCSF)

Cytospin (Shandon; Thermo Scientific, cat. no. A78300002)

Conventional brightfield microscope

Automated stainer Shandon Varistain XY (with low agitation)

Perform hemolysis

1. Centrifuge the BAL for 5 min at 300 × g, 4°C. Discard the supernatant.
2. Add 1500 µl distilled water to the cell pellets.
3. Wait for 10 sec, add 500 µl 0.6 M KCl, and homogenize by inverting.
4. Centrifuge the BAL for 5 min at 300 × g, 4°C. Discard the supernatant.
5. Add 500 µl saline-EDTA to the cell pellet and homogenize by inverting.

Obtain the total cell count

6. Place 5 µl of the cell suspension on a hemacytometer.

The total cell count should be performed within 24 hr after BAL (Fig. 2).

7. Count the cells and calculate the total cell concentration. Calculate the total number of cells as a function of the volume of lavage fluid recovered.

Cytospin

8. Homogenize by inverting and dilute with saline-EDTA to obtain a final concentration of 250,000 cells/ml.

BALF can be preserved at 4°C. If this is to be done, cytopinning must be performed within 3 days of BAL (Fig. 3).

9. Place the slide covered with a Shandon filter card clipped to the cytofunnel in the cytospin.
10. Place 200 µl of the cell suspension in the cytofunnel.

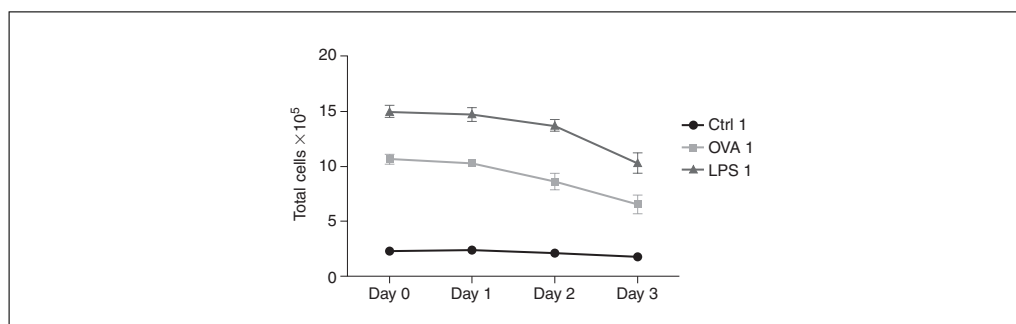


Figure 2 Evolution over time in the total number of cells counted in BALF from control (Ctrl 1), OVA-sensitized and -challenged (OVA 1), or LPS-treated (LPS 1) mice. Cells were stored in saline-EDTA at 4°C; total cells were counted in a Neubauer hemacytometer on days 0, 1, 2, and 3. Data show that total cell count should be performed within 24 hr after BAL. Dots are means and bars are SEM values ($n = 3$).

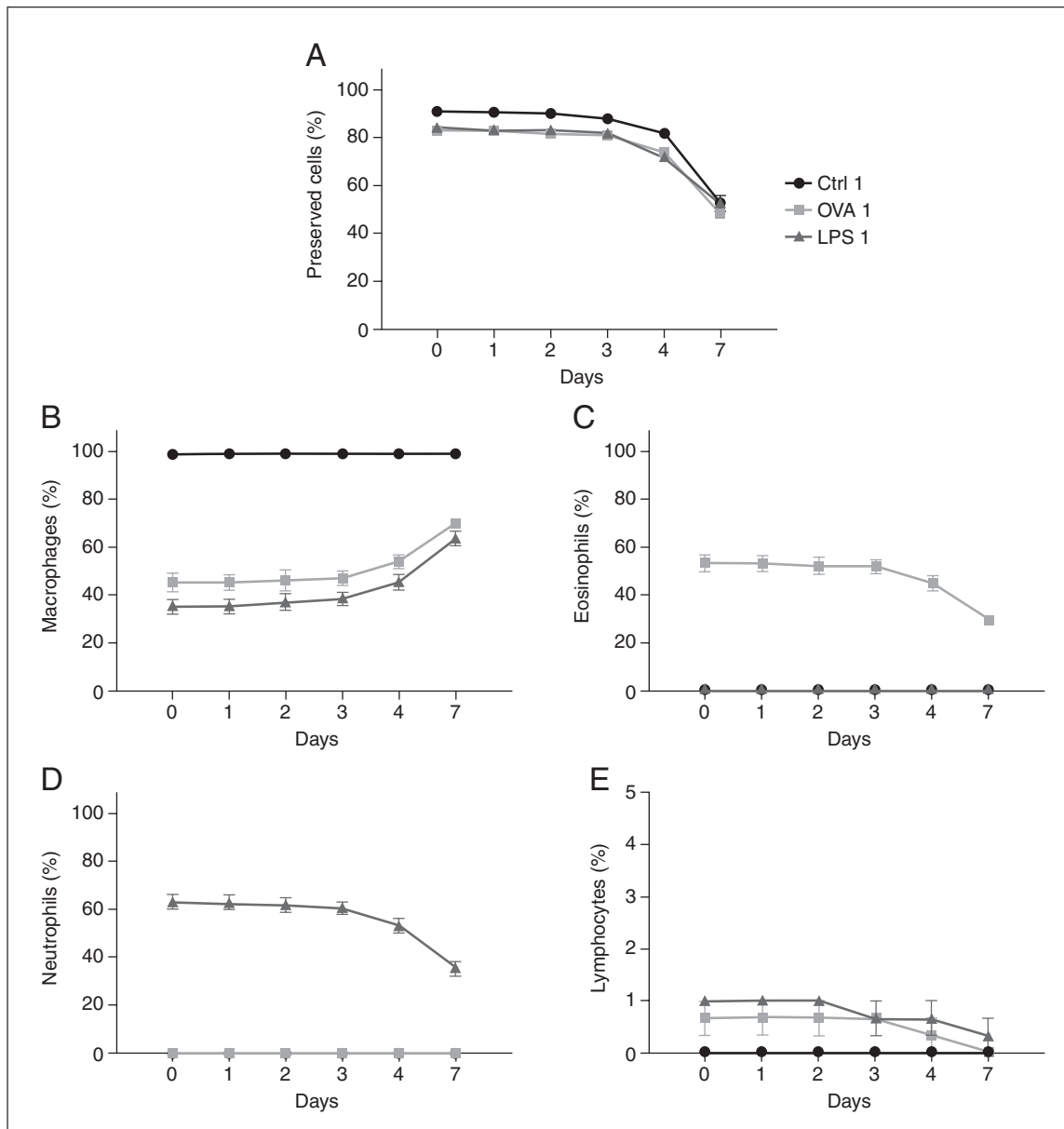


Figure 3 Evolution over time of differential cell counts on cytospinning after BAL storage at 4°C. Cells were stored in saline-EDTA at 4°C and cytospun and stained on days 0, 1, 2, 3, 4, or 7. The data show that cells (A) can be stored a few days to avoid immediate cytospinning but that they must be cytospun within 3 days after the BAL for correct differential cell count. After more than 3 days, the cells become fragile, and fewer eosinophils (C), neutrophils (D) and lymphocytes (E) are preserved on cytospinning, resulting in an increased percentage of the more “stable” macrophages (B) after 3 days. Dots are means and bars are SEM values ($n = 3$).

11. Centrifuge for 10 min at 700 rpm in a Cytospin.

12. Let the slide air-dry and proceed to cell staining.

Cell staining

Two different stainings are detailed below, the commercial Diff-Quick staining being possible with less than an hour of cytospinning; the home-made staining can be used at any time.

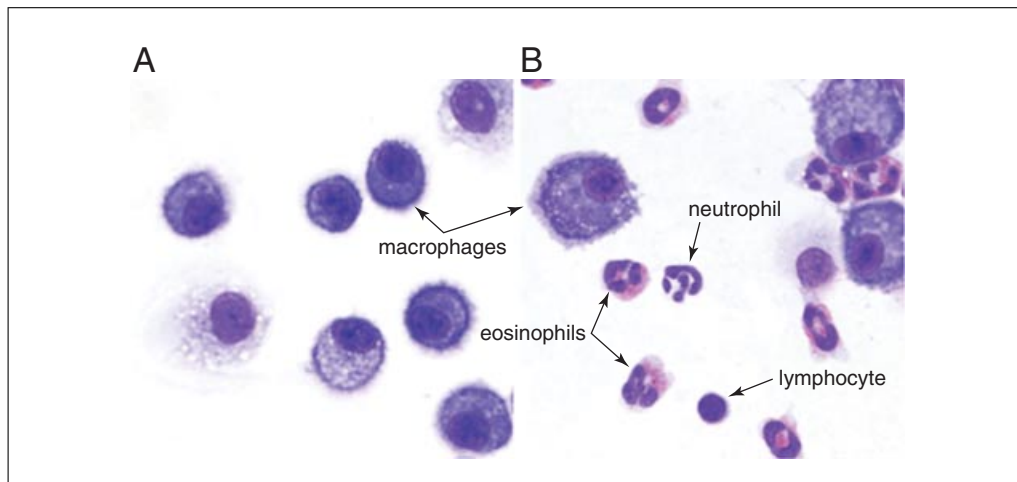


Figure 4 Photograph of cytopun BAL cells stained with Diff-Quick. **(A)** Control BAL; **(B)** BAL from ovalbumin-sensitized and challenged mice. BAL cells show macrophages, eosinophils, neutrophils and lymphocytes in OVA-challenged animals.

Diff-Quick staining

13a. Immerse the slide for 15 sec in Diff-Quick fixative reagent.

14a. Immerse the slide for 30 sec in Diff-Quick solution I.

15a. Immerse the slide for 15 sec in Diff-Quick solution II.

16a. Rinse the slide for 5 sec in tap water.

17a. Let the slide air-dry.

18a. Count and identify 400 cells under light microscopy.

Magnification $\times 1000$; oil immersion (Fig. 4).

Home-made staining (HMS)

This HMS staining is better if performed in an automated stainer.

13b. Immerse the slide for 15 sec in reagent HMS-A.

14b. Immerse the slide for 30 sec in reagent HMS-B.

15b. Immerse the slide for 25 sec in reagent HMS-C.

16b. Rinse the slide for 15 sec in tap water.

17b. Let the slide air-dry.

18b. Count and identify 400 cells under light microscopy.

Magnification $\times 1000$; oil immersion (Fig. 4).

COMMENTARY

Background Information

BAL appears to be the leading technical approach for studying airway inflammation in animal models of airway diseases, and thus for examining the effect of new anti-inflammatory drugs and therapeutic strategies in airway diseases.

Two approaches are commonly used to identify and count cells from BAL: the standard manual morphologic counting (described in this procedure) and the flow cytometric method (Van Rijt et al, 2004; Mathers et al, 2006). These methods are typically complementary. Manual counting may appear very

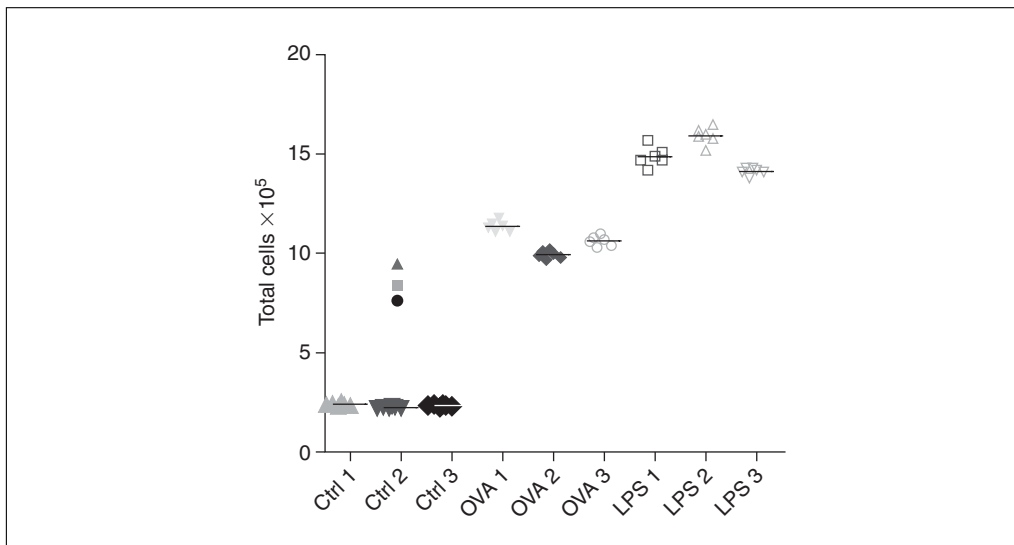


Figure 5 Interindividual reproducibility of total cell counts. Cells in saline-EDTA were cytospun and counted on a hemacytometer after BAL. Dots are individual values and horizontal bars are means ($n = 6$).

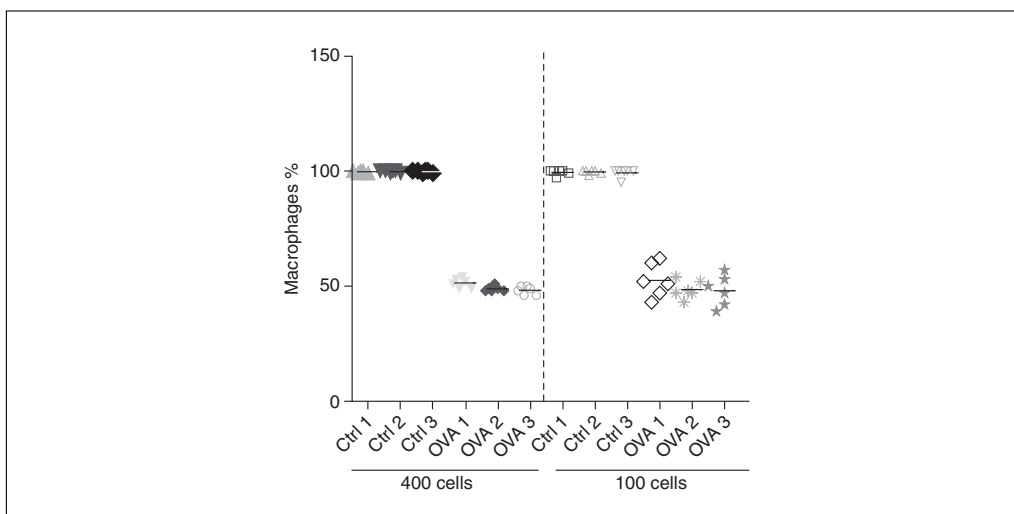


Figure 6 Interindividual reproducibility of differential cell counts. Cells in saline-EDTA were cytospun and stained before the differential cell count; 100 (right panel) or 400 (left panel) cells were counted and identified on six replicated cytopspins. Data show that counting and identifying 400 cells is necessary and sufficient for good reproducibility. Dots are individual values and horizontal bars are means ($n = 6$).

tedious but is an extremely reproducible (Figs. 5 and 6) and inexpensive technique and does not need to be performed very soon after the lavage, unlike flow cytometry. In addition, cytospun cells can be stored for years for later colorimetric staining—except Diff-Quick staining that should be performed within less than an hour of cytopinning—or immunohistochemical or immunofluorescence labeling.

Flow cytometry takes more time to perform and is more expensive, but necessary in order to be able to use a one-pot step to study spe-

cific cell types, such as B, Th1, Th2, TReg, Th17 lymphocytes and dendritic cells within BAL.

Critical Parameters

The BAL procedure is a simple technique and is easy to perform, but it requires expertise if it is to be reproducible. This step should be performed by the same investigator within the same protocol to optimize the quality and reproducibility of results. All other steps are investigator-independent.

Table 1 Timeline for Procedures

Step	Time required
BAL	15 min
Hemolysis	15 min
Total cell count	5 min
Cytospin	15 min
Diff-Quick stain	3 min
Differential counting	15 min

Troubleshooting

Diff-Quick staining requires fresh cells, within an hour of cytospinning, in order to obtain good staining that allows accurate identification of the structures and cells, in particular for eosinophil granules (see Fig. 3).

Diff-Quick can be replaced by other stains for use with cytospun cells, performed later on, after several days or months, with the same results, like our home-made stain described here above, which we recommend to all researchers. However, using our home-made stain without an automated stainer will require a precise time adjustment.

Unstained cytospun cells must be stored at room temperature. It is important to avoid the humidity occurring in refrigerated conditions, because water will destroy cells and cause mold growth.

Time Considerations

Table 1 summarizes the timelines associated with these procedures. Note that several steps are very time-consuming. However, the time does not increase linearly with the number of mice. In addition, staining can be automated, so that all slides are stained in 3 min.

Acknowledgments

The authors warmly thank T. Le Meur for his excellent technical assistance. This work was supported by the Eumodic European Grant.

Literature Cited

- Andreasen, C.B. 2003. Bronchoalveolar lavage. *Vet. Clin. Small Anim.* 33:69-88.
- Blé, F.-X., Cannet, C., Zurbrugg, S., Karmouty Quintana, H., Frossard, N., Trifilieff, A., and Beckmann, N. 2008. Allergen-induced lung inflammation in actively sensitized mice assessed by MRI. *Radiology* 248:834-843.
- Blé, F.-X., Cannet, C., Zurbrugg, S., Gérard, C., Frossard, N., Beckmann, N., and Trifilieff, A. 2009. Activation of the lung S1P1 receptor

reduces allergen-induced plasma leakage in mouse. *Brit. J. Pharmacol.* 158:1295-1301.

- Delayre-Orthez, C., Becker, J., De Blay, F., Frossard, N., and Pons, F. 2004. Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin. Exp. Allergy* 34:1789-1795.
- Delayre-Orthez, C., Becker, J., Guenon, I., Lagente, V., Auwerx, J., Frossard, N., and Pons, F. 2005a. PPARalpha downregulates airway inflammation induced by lipopolysaccharide in the mouse. *Respir. Res.* 6:91.
- Delayre-Orthez, C., Becker, J., de Blay, F., Frossard, N., and Pons, F. 2005b. Exposure to endotoxins during sensitization prevents further endotoxin-induced exacerbation of airway inflammation in a mouse model of allergic asthma. *Int. Arch. Allergy Clin. Immunol.* 138:298-304.
- Delayre-Orthez, C., Becker, J., Auwerx, J., Frossard, N., and Pons, F. 2008. Suppression of allergen-induced airway inflammation and immune response by the peroxisome proliferator-activated receptor-alpha agonist fenofibrate. *Eur. J. Pharmacol.* 581:177-184.
- Deschamps, K., Cromlish, W., Weicker, S., Lamontagne, S., Huszar, S.L., Gauthier, J.Y., Mudgett, J.S., Guimond, A., Romand, R., Frossard, N., Percival, M.D., Slipetz, D., and Tan, C.M. 2011. Genetic and pharmacological evaluation of Cathepsin S in a mouse model of asthma. *Am. J. Respir. Cell Mol. Biol.* 45:81-87.
- Hachet-Haas, M., Balabanian, K., Rohmer, F., Pons, F., Franchet, C., Lecat, S., Chow, K.Y., Dagher, R., Gizzi, P., Didier, B., Lagane, B., Kellenberger, E., Bonnet, D., Baleux, F., Haiech, J., Parmentier, M., Frossard, N., Arenzana-Seisdedos, F., Hibert, M., and Galzi, J.-L. 2008. Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J. Biol. Chem.* 283:23189-23199.
- Henderson, A.J.W. 1994. Bronchoalveolar lavage. *Arch. Dis. Childhood* 70:167-169.
- Hunninghake, G.W., Gadek, E., Kawanami, O., Ferrans, V.J., and Crystal, R.G. 1979. Inflammatory and immune processes in the human lung in health and disease: Evaluation by bronchoalveolar lavage. *Am. J. Pathol.* 97:149-206.
- Mathers, R.A., Evans, G.O., Bleby, J., and Tornow, T. 2006. Total and differential leucocyte counts

- in rat and mouse bronchoalveolar lavage fluids using the Sysmex XT-2000iV. *Comp. Clin. Path.* 16:29-39.
- Reber, L.L., Daubeuf, F., Plantinga, M., De Cauwer, L., Gerlo, S., Waelput, W., Van Calenbergh, S., Tavernier, J., Haegeman, G., Lambrecht, B.N., Frossard, N., and de Bosscher, K. 2012. A fully dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a murine model of asthma. *J. Immunol.* 188:3478-3487.
- Van Rijt, L.S., Kuipers, H., Vos, N., Hijdra, D., Hoogsteden, H.C., and Lambrecht, B.N. 2004. A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *J. Immunol. Methods* 288:111-121.
- Zhang, Z., Hener, P., Frossard, N., Kato, S., Metzger, D., Li, M., and Chambon, P. 2009. Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma. *Proc. Natl. Acad. Sci. U.S.A.* 106:1536-1541.

Annexe n°3:

A Fast And Easy Flow Cytometric Method For Analysis Of The Cellular Content Of Bronchoalveolar Lavage Fluid In Mouse.

François Daubeuf, Julien. Becker, Claudine. Ebel, Juan Antonio Aguilar-Pimentel,
Yann. Hérault and Nelly Frossard

En préparation

Dans le but de déterminer avec précision la composition leucocytaire du lavage bronchoalvéolaire chez la souris, nous avons développé et validé une procédure rapide, en une étape, d'analyse par cytométrie en flux.

La validation de ce protocole de cytométrie en flux a été contrôlée par l'analyse morphologique des différentes populations de leucocytes, après tri des cellules à l'aide d'un analyseur-trieur à haut débit. La reproductibilité du protocole a également été contrôlée, ainsi que son utilisation dans différents modèles de maladies inflammatoires des voies aériennes chez la souris.

A fast and easy flow cytometric method for analysis of the cellular content of bronchoalveolar lavage fluid in mouse

François DAUBEUF§, Julien BECKER#, Claudine EBEL*, Juan Antonio AGUILAR-PIMENTEL\$, Yann HERAULT #, Nelly FROSSARD§ 1

§ Laboratoire d'Innovation Thérapeutique, UMR7200 CNRS/Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France; # Institut Clinique de la Souris (ICS), 1 rue Laurent Fries, BP 10142, Parc d'Innovation, 67404 Illkirch-Graffenstaden Cedex, France; * Institut de Génétique et de Biologie Moléculaire et Cellulaire, IGBMC, 1 Rue Laurent Fries, 67400 Illkirch-Graffenstaden; \$ German Mouse Clinic, Helmholtz Zentrum München - Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany.

ABSTRACT

Mouse models are increasingly used to study airway diseases. To characterize airway inflammation in asthma models, cell composition of the bronchoalveolar lavage fluid (BALF) is commonly used. Leukocytes are generally fixed on cytopsin, identified after May–Grünwald Giemsa or Diff-Quick® staining as eosinophils, neutrophils, macrophages and lymphocytes by morphological criteria, and counted under optical microscopy. Identification of macrophages from activated T cells is not always easy. We here describe a rapid, easy-to-use, one-step and evolutive flow cytometric method to perform differential cell counts, and compared it to the counts based on morphological criteria on Diff-Quick®-stained cytopsin. Identification of cells from BAL was based on forward and side scatter characteristics (FSC and SSC) and a simultaneous one-step staining with antibodies for T cells (CD3), B cells (CD19), neutrophils (GR-1), eosinophils and macrophages (CD11c). Validation of this flow cytometric protocol was performed by morphological analysis of flow-sorted cellular subsets.

Keywords: Flow cytometry • bronchoalveolar lavage • inflammation • airways • lung

INTRODUCTION

Numerous mouse models of airway disease were developed and are widely used to study the pathophysiology of airway disorder as allergic asthma, acute inflammation and fibrosis (Shin et al. 2009, Nials et al. 2008, Daubeuf et al. 2013, Kips et al. 2003, Mouratis et al. 2011, Vodovotz et al. 2006). In all of these models, the recruitment of leukocytes into the lung is a critical parameters used to measure the effect of anti-inflammatory drugs or strategies (Nials et al. 2006).

Bronchoalveolar lavage (BAL) is an essential technique to measure inflammatory response and explore immune mechanisms in the lung (Hunninghake et al. 1979). It is commonly used in the clinic to diagnose lung diseases, infections, inflammation, and cancer (Henderson et al. 1994). This procedure makes it possible to study the components of the epithelial lining fluid. Levels and phenotypes of inflammatory cells infiltrating the airways can be assessed, for they are recovered in the BAL fluid (BALF) after transmigration through the epithelial layer. BAL is also used to quantify the protein composition of the airways.

In experimental studies in small animals, BAL is a rapid and effective procedure for studying inflammation in the airways after euthanasia (Andreasen 2003). The cellular composition of the BALF is the reflect of the lung inflammatory cells with eosinophils, neutrophils, macrophages and lymphocytes (Ble et al 2008; 2009; Delayre-Orthez et al 2004; 2005a; 2005b; 2008; Deschamps et al 2011; Hachet-Haas et al 2008; Zhang et al 2009). The cellular composition of BAL fluid (BALF) is commonly determined on cytopsin stained with either May-

Grünwald Giemsa or Diff-Quick® and using morphological criteria by optical microscopy (Daubeuf et al. 2012). Some authors have therefore used the endogenous cyanide-resistant peroxidase activity of eosinophils or the intracellular presence of major basic protein to enumerate these cells in BAL fluid (Lee et al. 1997) and equally with Haematology analyser as Sysmex XT-2000i or ADVIA 120 (Mathers et al. 2007). However, morphological criteria are insufficient to discriminate between activated T and B cells that required to identified CD (cluster of differentiation) molecules as cell surface markers (Van Rijt et al. 2004).

Today, democratization of multi-color flow cytometers with 6 or more color and the development of a wide range of fluorescently labeled antibodies allow to consider possible to analyse rapidly and efficiently several cell types in large numbers of samples. With flow cytometry, a substantial number of cells can be analyzed as compared with the number of cells counted by an investigator (10,000 events or more vs. the traditional 200–400 counted cells in morphological analysis). In addition, flow cytometry analysis of many samples simultaneously is less time consuming compared with preparing, staining and counting cytopins.

In comparison, flow cytometry analysis promises to be more rapid and reliable and requires expertise in handling, have a multi-color cytometer and many antibodies whereas manual counting is basic, easy to use, inexpensive, reproducible, boring and time consuming. Nonetheless, our experience shows that the majors limits are the false ideas about flow cytometry as need complex protocol, the weakness of fluorescent antibodies and there time limited conservation.

Here, we describe a rapid, easy to use, reliable and evolutive flow cytometric method for differential cell counts of BALF cells by staining with a combination of commercially available antibodies to identifies T and B lymphocytes, eosinophils, neutrophils and macrophages in BALF and exceed prejudiced ideas.

TOTAL CELL COUNTS

After BAL collection, total leukocytes are counted manually with a hemocytometer (e.g. Neubauer or Malassez chamber). Total leukocytes may be counted indifferently before or after flow cytometry analysis but it is necessary to concentrate BAL cells before flow cytometry analysis.

Materials

BALF from mouse with inflamed airways comparatively to controls (see Daubeuf et al. 2012)

Phosphate buffered saline containing EDTA (3 mM) kept on ice

Centrifuge (300 g)

Conventional bright field microscope

Hemocytometer (Neubauer or other)

Concentrate the BAL cells

1. Centrifuge BAL (5 min, 300 g).
2. Discard supernatant.
3. Add 500 μ L PBS-EDTA on pellet cells and homogenize by inverting.

Obtain the total cell count

4. Place 5 μ L of the cell suspension on a hemocytometer.
The total cell count should be performed within 24 h after BAL (see Daubeuf et al. 2012).
5. Count cells and calculate the total cell concentration.
6. Calculate the total number of cells as a function of the volume of lavage fluid recovered.

FLOW CYTOMETRY PROCEDURE AND ANALYSIS

After BAL cells were concentrated, leukocytes were labeled with antibodies to identified macrophages, eosinophils, neutrophils and lymphocytes T and B. This protocol describe a fast, one-step and easy antibodies staining.

Materials

Concentrated BAL cells

Phosphate buffer saline with EDTA (3 mM) on ice

FC Block™ (553142, BD bioscience)

7-AAD (559925, BD bioscience)

Antibodies anti-mouse: CD11C-FITC (557400, BD bioscience), I-A/I-E-PercpCy5,5 (107626, BioLegend), CD19-PE-Cy7 (552854, BD bioscience), CD3-EFlour 450 (48-0032-82, eBioscience), CD11b-APC Alx750 (557657, BD bioscience), CD45-AlxFluor700 (103128, BioLegend), Ly-6G (Gr-1)-PE (12-5931-85, eBioscience)

5-ml polystyrene round bottom test tube (352058, BD Falcon™)

Sterile tips

Precision pipets (2.5 μ l, 20, 200 and 1000 μ l)

LSR II Flow cytometer or FACS Aria II (BD bioscience)

FlowJo® software

Block the Fc-mediated adherence of antibodies

1. Homogenize BAL cells by vortexing slowly (1000rpm, 5sec) and distribute 150 μ l of each BAL cells in a microplate 96/U or in microtubes
2. Add 5 μ l of FC Block™ in each well
3. Incubate 10min to room temperature

Antibody solution

4. Add 1ml of PBS-EDTA in a 1.5-ml microtube and store on ice

This volume is required to 36 samples

5. Add antibodies: 3µl of CD11C-FITC, 1µl of I-A/I-E-PercpCy5,5, 1.5µl of CD19-PE-Cy7, 3µl of CD3-EFlour 450, 2µl of CD11b-APC Alx750, 3.5µl of CD45-AlxFluor700 and 1.5µl of Ly-6G (Gr-1)-PE antibody
6. Homogenize by vortexing

Antibody labeling and flow cytometry analysis

7. Distribute 25µl of the antibody solution in each well containing BAL cells in the microplate or in the microtubes.
8. Incubate 30min to room temperature
9. Add 5µl of 7-AAD (pre-dilute, 1:3 in PBS-EDTA) in each well or microtube
10. data to Flow Jo software to differentiate and count cells (*Fig. 1*)
11. Export results to Excel to calculate the percent of each leukocytes population to live cells population. Use these percent and the total number of BAL cells (Basic protocol one) to obtain the number each leukocytes in BAL.

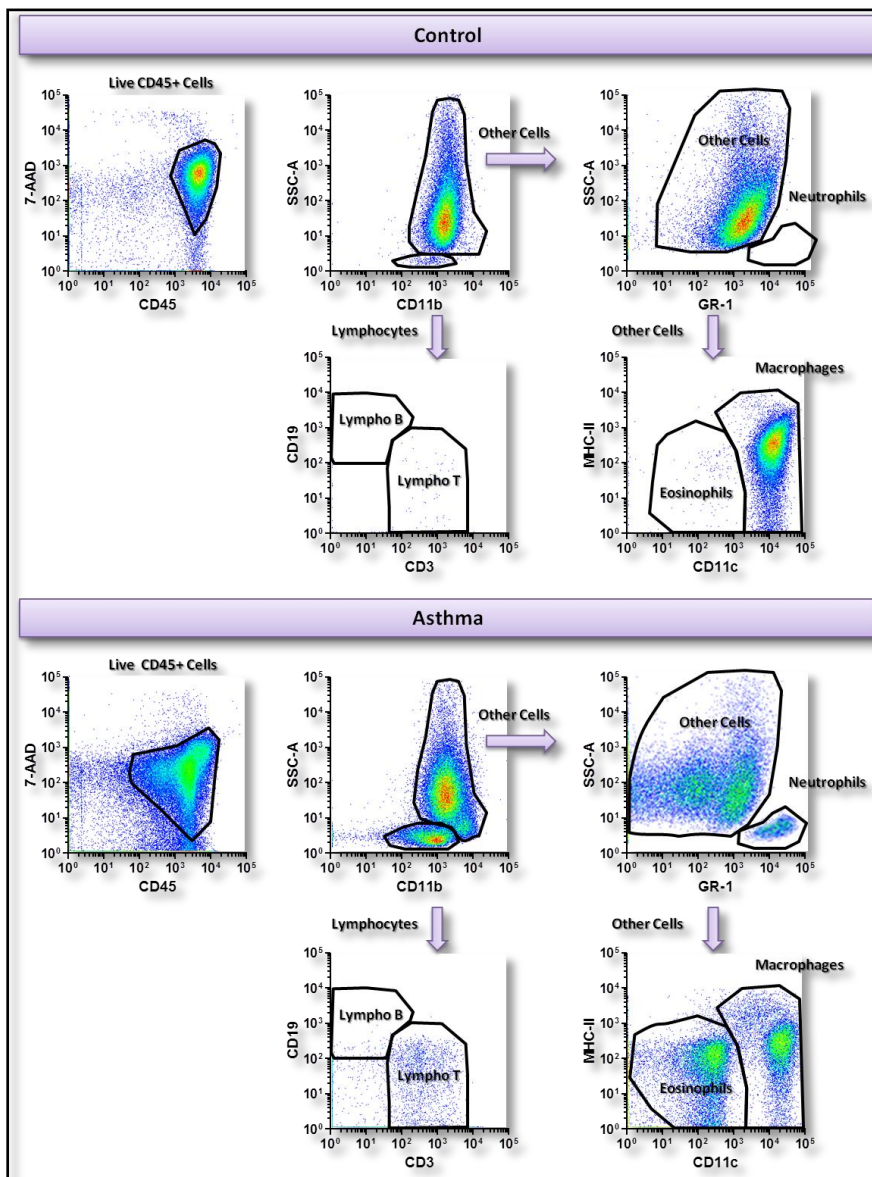


Figure 1: Analysis of multi-color Flow cytometry labeling of BALF cells. FlowJo software is used to select BALF cells, on 2-D plot, in naive mice (Control) and in a model of asthma induced by ovalbumin (Asthma). In first, live CD45+ leukocytes are selected to eliminate debris, erythrocytes and dead cells, then lymphocytes are gated on SSC-A/CD11b plot to discriminate CD19+ B cells and CD3+ T cells. Others cells are analyzed with SSC-A/GR1 plot to identify neutrophils and MHC-II/CD11c plot are used to separate eosinophils and macrophages in the remaining cells. This procedure identify 98.7±1.4% of live leukocytes.

COMMENTARY

Background Information

BAL appears to be an essential approach for studying airway inflammation in animal models of airway diseases and needs to identify and count leukocytes. Today, several possibilities are offered by the development of cytometry: the standard manual morphologic counting (described in procedure daubeuf et al. 2012) and the flow cytometric method (Van Rijt et al. 2004, and described in this procedure). Manual counting appears wearisome, inexpensive, reproducible and controlled method and may be delayed as compared to flow cytometry that required to be achieved during the day and disposal of a multi-color cytometer. From this point of view, manual counting seems more attractive but flow cytometry is useful to separate macrophages from activated T cells and necessary to study specific cell types, such as B, Th1, Th2, TReg, Th17 lymphocytes or dendritic cells. In our hands, flow cytometry reveals an important fact: our results show that lymphocytes are frequently confounded with macrophages in manual counting whereas they are perfectly discriminated in flow cytometry. Moreover, lymphocytes are a leading population in airway disease (Fig. 2).

Our protocol is designed to identify macrophages, eosinophils, neutrophils and lymphocytes T and B with efficient and to be fast, easy to use, reproducible and upgradable. It is possible to add antibodies to identify another subset or receptors found on previously described cells.

However, it subsists another false idea: the limited conservation of antibodies. Our experience confirms the great stability of the antibodies described in this procedure. They can be used during several years and were stable to repeated temperature fluctuation from 4°C to room temperature.

Critical parameters

Hemolysis, centrifugation and rinse steps were avoided to warranty the BAL cell viability, stability and quality of labeling. Moreover, we recommend to beware about tubes and microplate quality. Microtubes with low adsorption prevent surface coating of diluted antibodies (in PBS-EDTA without BSA or serum) and quality of microplate must be controlled to prevent macrophage adhesion. In complement, phosphate saline buffer with EDTA is essential to limit macrophage activation and required endotoxin-free PBS to prevent cell activations and gage quality, purity and reproducibility of the flow cytometry procedure and analysis (Fig. 3)

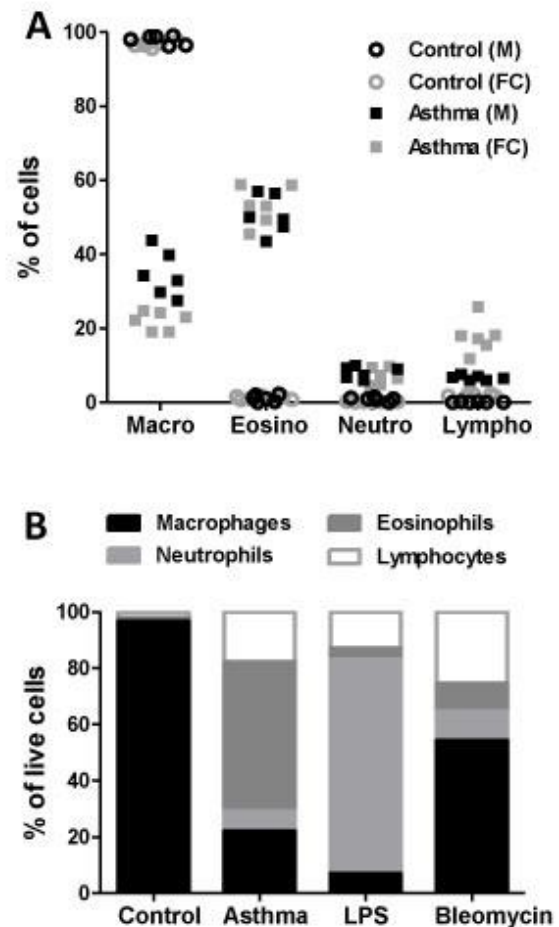


Figure 2: Cell population in BALF in airway disease. A. Comparison between manual morphologic count (M) and flow cytometry analysis (FC) in naive mice (Control) and in a model of asthma induced by ovalbumin (Asthma). Data shows that flow cytometry is useful to separate macrophage from lymphocytes whereas manual and flow cytometry are perfectly comparable for eosinophils and neutrophils. **B.** Example of flow cytometry analysis in three models of airway disease: a model of asthma induced by ovalbumin, an acute inflammatory model induced by intranasal administration of Escherichia coli Lipopolysaccharide (LPS, 200µg/kg, BALF were performed 24 h after) and a model of pulmonary fibrosis with bleomycin (one intranasal administration, 1mg/kg, BALF were performed 14 days after).

Troubleshooting

Because of the absence of hemolysis step, manual total cell counting with a hemocytometer could be difficult when the number of erythrocytes is too high. In this case, our experience shows that only few apparatus are adapted to count precisely leukocytes in BAL in samples containing debris and erythrocytes.

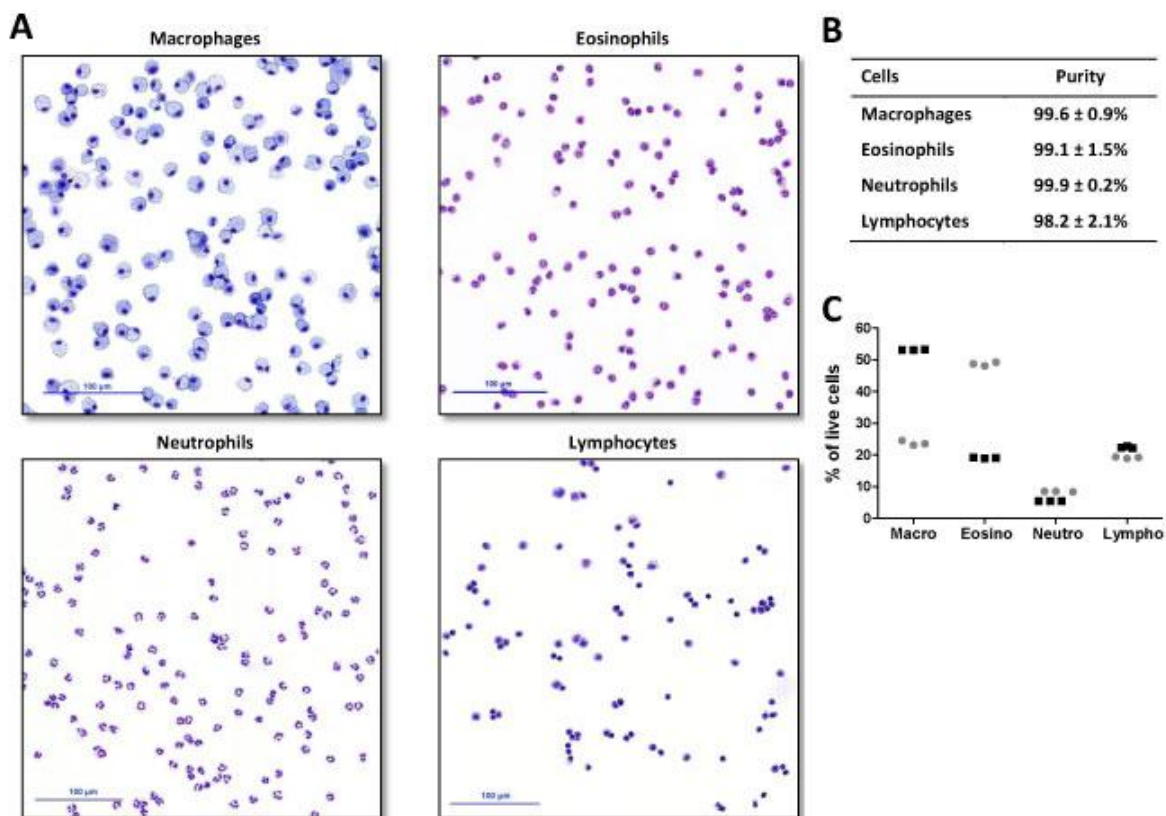


Figure 3: Purity and control quality of the one-step flow cytometry procedure. **A.** Flow cytometry procedure are used on a cell sorter ARIA II (BD bioscience) and sorted cells were cytopun and stained with Diff-Quick®. **B.** Sorted macrophages, eosinophils, neutrophils and lymphocytes were counted on cytopsin to determine the efficacy and the purity of the flow cytometry procedure. **C.** Intra-individual reproducibility of the flow cytometry procedure on BALF cells is represented in two asthmatic mice (gray dots and black squares).

Three apparatus are useful and validate: the ADVIA 120 hemocytometer or the Cellometer Auto X4 and the Celigo® S Imaging Cell Cytometer with fluorescent dye nuclear staining, respectively acridin orange or propidium iodine (Fig 4A). Conventional cell counters using Bright field images or impedance-based particle detection appear few discriminate in heterogenic samples and can't be used with BALF (Fig 4B).

Time considerations

The following table summarizes the timelines associated with these procedures

Note that several steps are very time-consuming. The time does not increase linearly with the number of mice so we present the time necessary for 20 samples.

Step	Time required
BAL cells concentration	15 min
Manual total cell count	60 min
Antibodies labelling	60 min
Flow cytometry	60 min
Analysis	90 min

LITERATURE CITED

- Andreasen CB. 2003. Bronchoalveolar lavage. *Vet Clin Small Animf* 33 : 69–88, 2003.
- Blé F-X, Cannet C, Zurbrugg S, Gérard C, Frossard N, Beckmann N, Trifilieff A. (2009) Activation of the lung SIP1 receptor reduces allergen-induced plasma leakage in mouse. *Brit J Pharmacol*, 158: 1295-301.
- Daubeuf F., Frossard N. (2012) Performing bronchoalveolar lavage in the mouse. *Current Protocols in Mouse Biology*. June 2012.
- Daubeuf F., Frossard N. (2013) Acute Asthma Models to Ovalbumin in the Mouse. *Current Protocols in Mouse Biology*. March 2013
- Delayre-Orthez C, Becker J, Auwerx J, Frossard N, Pons F. (2008) Suppression of allergen-induced airway inflammation and immune response by the peroxisome proliferator-activated receptor-alpha agonist fenofibrate. *Eur J Pharmacol*, 581, 177-184.
- Delayre-Orthez C, Becker J, De Blay F, Frossard N, Pons F. (2004) Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin Exp Allergy*, 34: 1789-1795.

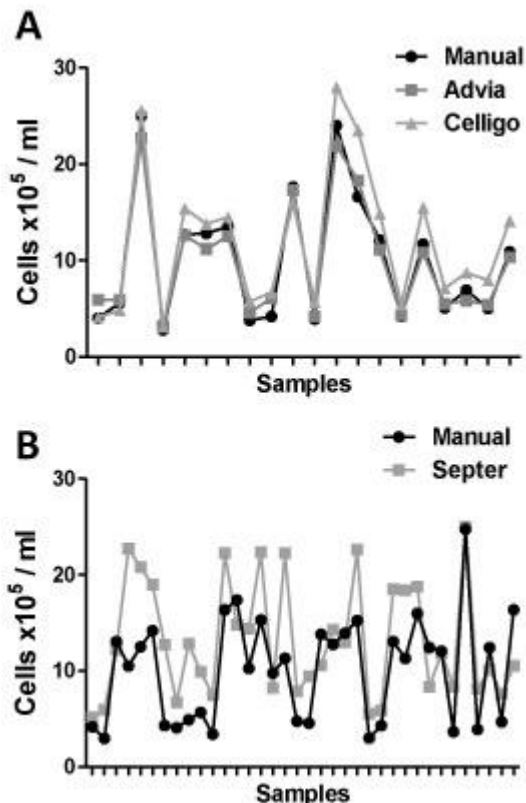


Figure 4: Total cell counting can be replaced only with few cell counter. A. Total cell count realized with ADVIA 120 and Celligo® and compared to manual count with a hemocytometer. **B.** Comparison between total cell counting with manual method or with an impedance-based particle detection counter

Delayre-Orthez C, Becker J, de Blay F, Frossard N, Pons F. (2005) Exposure to endotoxins during sensitization prevents further endotoxin-induced exacerbation of airway inflammation in a mouse model of allergic asthma. *Int Arch Allergy Clin Immunol.* 138: 298-304.

Delayre-Orthez C, Becker J, Guenon I, Lagente V, Auwerx J, Frossard N, Pons F. (2005) PPARalpha downregulates airway inflammation induced by lipopolysaccharide in the mouse. *Respir Res.* 6: 91.

Deschamps K, Cromlish W, Weicker S, Lamontagne S, Huszar SL, Gauthier JY, Mudgett JS, Guimond A, Romand R, Frossard N, Percival MD, Slipetz D, Tan CM. (2011) Genetic and pharmacological evaluation of Cathepsin S in a mouse model of asthma. *Am J Respir Cell Mol Biol,* 45: 81-7.

Gregory LG, Lloyd CM. (2011) Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* (9):402-11.

Hachet-Haas M, Balabanian K, Rohmer F, Pons F, Franchet C, Lecat S, Chow KY, Dagher R, Gizzi P, Didier B, Lagane B, Kellenberger E, Bonnet D, Baleux F, Haiech J, Parmentier M, Frossard N, Arenzana-Seisdedos F, Hibert M, Galzi J-L. (2008) Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J Biol Chem,* 283(34): 23189-99.

Henderson AJW. (1994) Bronchoalveolar lavage. *Archives of Disease in Childhood.* 70: 167-169.

Hunninghake GW, Gadek JE, Kawanami O, Ferrans VJ, Crystal RG. (1979) Inflammatory and immune processes in the human lung in health and disease: evaluation by bronchoalveolar lavage. *Am J Pathol.* 97(1):149-206.

Kips JC, Anderson GP, Fredberg JJ, Herz U, Inman MD, Jordana M, Kemeny DM, Lötval J, Pauwels RA, Plopper CG, Schmidt D, Sterk PJ, Van Oosterhout AJ, Vargaftig BB, Chung KF. (2003) *Murine models of asthma.* *Eur Respir J.* 22(2):374-82.

Kumar RK, Herbert C, Foster PS. (2008) The "classical" ovalbumin challenge model of asthma in mice. *Curr Drug Targets.* 9(6):485-94.

Lee, J.J., McGarry, M.P., Farmer, S.C., Denzler, K.L., Larson, K.A., Carrigan, P.E., Brenneise, I.E., Horton, M.A., Haczku, A., Gelfand, E.W., Leikauf, G.D., Lee, N.A. (1997) Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J. Exp. Med.* 185, 2143

Mathers R. A., G. O. Evans, J. Bleby, T. Tornow (2007) Total and differential leucocyte counts in rat and mouse bronchoalveolar lavage fluids using the Sysmex XT-2000iV. *Comparative Clinical Pathology.* Volume 16, Issue 1, pp 29-39

Mouratis MA, Aidinis V. (2011) Modeling pulmonary fibrosis with bleomycin. *Curr Opin Pulm Med.* 17(5):355-61.

Nials AT, Uddin S. (2008) Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech.* 1(4-5):213-20.

Shin YS, Takeda K, Gelfand EW. (2009) Understanding asthma using animal models. *Allergy Asthma Immunol Res.* 1(1):10-8.

van Rijt LS, Kuipers H, Vos N, Hijdra D, Hoogsteden HC, Lambrecht BN. (2004) A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *J Immunol Methods.* 288(1-2):111-21.

Vodovotz Y, Chow CC, Bartels J, Lagoa C, Prince JM, Levy RM, Kumar R, Day J, Rubin J, Constantine G, Billiar TR, Fink MP, Clermont G. (2006) In silico models of acute inflammation in animals. *Shock.* 26(3):235-44.

Annexe n°4:

Eosinophils And The Ovalbumin Mouse Model Of Asthma

François Daubeuf and Nelly Frossard

Article à paraître dans "Methods in Molecular Biology – Eosinophils",

Dans cet article, Nous décrivons des méthodes pour obtenir un afflux d'éosinophiles dans les voies aériennes dans un modèle d'asthme allergique . Les modèles d'asthme allergique présentés offrent une grande reproductibilité et entraînent le recrutement de nombreux éosinophiles dans le poumon. Ce recrutement est mesuré dans le lavage bronchoalveolaire et est accompagné d'une hyperréactivité des voies aériennes et d'un remodelage bronchique. Nous présentons ici le modèle conventionnel d'asthme allergique à l'ovalbumine, en 21 jours, les ajustements nécessaires pour un modèle rapide d'hyperéosinophilie allergique des voies aériennes en 8 jours, et un modèle en 57 jours, adapté à l'utilisation des souris C57BL/6, dans le but de développer une hyperéosinophilie associée à une hyperréactivité bronchique.

Eosinophils And The Ovalbumin Mouse Model Of Asthma

F. Daubeuf and N. Frossard

UMR7200 CNRS-Strasbourg University, Laboratoire d'Innovation Thérapeutique, Illkirch, and Laboratory of Excellence Medalis, Strasbourg, France

Summary

Mouse models of asthma are essential to understand asthma pathogenesis and eosinophil recruitment in the airways, and to develop new therapeutic strategies. Animal models try mimicking all features of the human disease including airway hyperresponsiveness (AHR), eosinophilic inflammation, and remodeling, which are the typical asthma-related characteristics. The mouse is now the species of choice for asthma research due to the availability of transgenic animals and of wide array of specific reagents and techniques available. Cellular responses may be studied with innovative imaging and flow cytometry methods while lung mechanics may be precisely measured by the forced oscillation technique, and airway responsiveness approached by barometric plethysmography in awoken and unconstrained animals. Here, we describe procedures to generate acute models of hypereosinophilic asthma in mice, with ovalbumin as the allergen. The presented allergic asthma models offer a large and reproducible eosinophil recruitment, measured in the bronchoalveolar lavage (BAL), accompanied with airway hyperresponsiveness, inflammation and remodeling, and are particularly suited to assess the activity of drug candidates. We here present the classical 21-day allergic asthma model to ovalbumin, and adjustments for a rapid 8-day model of airway allergic hypereosinophilia, and a more chronic 57-day model suitable for C57BL/6 mice to develop airway hyperresponsiveness together with airway eosinophilic inflammation and remodeling.

Key words: Allergic asthma, Mouse model, Ovalbumin

1. Introduction

Human allergic asthma is defined as a chronic eosinophilic inflammatory disorder of the airways, and is characterized clinically by airway hyperresponsiveness (AHR) and intermittent, reversible airway obstruction (1). Histopathological features of asthma show the presence of a large eosinophilic infiltrate in the bronchi, and structural changes grouped as "airway remodelling" including subepithelial fibrosis, goblet cell hyperplasia, airway smooth muscle thickening, and increased vascularity. It is accepted that AHR and remodeling result from repeated exposure to allergen, that lead to chronic inflammation of the airways (2). Studies in laboratory animals have produced a large amount of knowledge on the mechanisms responsible for allergic asthma.

Among animal models, the mouse is now the most widely used species. Mice are not naturally prone to asthma but asthma-like artificial reactions may be induced in the respiratory tract to develop inflammation and symptoms that are similar to those of the human disease. Some differences persist however in the pathophysiology of asthma between mouse and human. AHR in mice follows allergen challenge, and is transient while it is present at any time in human even in the absence of symptoms (3). Chronic models appear difficult to develop in mice, probably due to the absence of environmental cofactors such as pollution, lung infection, and also probably to the employed allergen. These observations suggest that allergic asthma models in mice do not exactly replicate the human disease, but the observations made in these mouse models support most existing paradigms and are useful to develop new anti-asthma strategies (4).

The most commonly used strain for allergen challenge models and eosinophil recruitment is the Th2-type BALB/c mice, but the C57BL/6 or A/J strains have also been used successfully for allergen challenge studies and development of asthma features (5). A/J mice seem more sensitive to allergen and able to develop chronic models (6). Allergens that may have clinical relevance, such as house dust mite or cockroach extracts (7, 8), have been recently successfully developed for asthma models in the mouse. However, ovalbumin (OVA) derived from chicken egg is until now a more frequently used allergen since it induces a robust and reproducible allergic bronchial inflammation, particularly in acute allergic response (9). Various sensitization and challenge procedures have been developed to induce acute asthma symptoms to OVA. For priming, allergen sensitization requires multiple systemic administrations of OVA in the presence of an adjuvant via intraperitoneal route. Aluminium hydroxide ($\text{Al}(\text{OH})_3$) is a commonly used adjuvant to promote the development of a Th2 immune response to OVA (10). After the sensitization period allowing IgE production (usually from 5 to 21 days), all protocols require local challenges with the allergen. OVA may be applied to the airways by nebulization, or by intratracheal (i.t) or intranasal (i.n.) instillation (11,12). Following these procedures, mice develop key features of clinical asthma, including increased levels of allergen specific IgE in serum, eosinophil and Th2 cell infiltration into the airways referred to as airway inflammation, mucus hyperproduction and collagen deposition as airway remodeling, and AHR to methacholine.

The influx of inflammatory cells in the airways, in particular the eosinophil infiltration, is studied in the bronchoalveolar lavage (BAL) (20). BAL is performed after euthanasia and differential cells are counted by optical microscopy as eosinophils, neutrophils, macrophages, and lymphocytes (15). For more specific assessments, flow cytometric methods can be applied, e.g. to identify T cell phenotypes in the cell composition of BAL fluid in asthma models (21).

We here describe the optimized procedures to sensitize and challenge mice to ovalbumin, in order to develop reproducible features of airway inflammation, e.g. eosinophil infiltration assessed in BAL fluid, remodeling and hyperresponsiveness. To do so, we present the classical acute 21-day asthma model in Balb/c mice and special adjustments for a rapid 8-day model of airway allergic hyper eosinophilia and a more chronic 57-day model suitable for C57BL/6 mice to develop airway hyperresponsiveness associated with inflammation and remodeling. In a following step, we describe an easy-to-perform, inexpensive, and reproducible manner to obtain bronchoalveolar lavage to measure inflammatory cell recruitment in mouse airways.

2. Materials

Prepare all solutions using sterile and pyrogen-free commercial saline (NaCl 0.9%, B. Braun, Boulogne, France), and all reagents on ice. Ovalbumin (OVA) solutions are prepared, aliquoted and frozen ready-to-use to increase reproducibility of the experiment.

2.1. Ovalbumin Solutions And Aluminium Hydroxide Powder

1. Prepare a sterile 2 mg/ml solution of OVA as follows: weigh 80 mg ovalbumin (Sigma-Aldrich, A5503) and dissolve it in 40 ml cold sterile saline in a 50 ml Falcon tube with a vortex (at maximal speed, *see Note 1*)
2. For sensitization procedures in the 8-day and 21-day models, take 25 ml of this solution, and distribute as 1.1-ml aliquots into 1.5-ml microtubes. Immediately freeze and store aliquots at -80°C .
3. For challenge procedures in the 8-day and 21-day models, take 8 ml of the remaining 2 mg/ml solution and dilute it $1/5^{\text{th}}$ by adding 32 ml cold sterile saline in a 50-ml Falcon tube with a vortex (at maximal speed, *see Note 1*). Distribute as 1-ml aliquots in 1.5-ml microtubes. Immediately store aliquots at -80°C .
4. For sensitization and challenge procedures in the 57-day model, take 8 ml of the 2 mg/ml solution and dilute it $1/2.5^{\text{th}}$ by adding 12 ml cold sterile saline in a 50-ml Falcon tube with a vortex (at maximal speed, *see Note 1*). Distribute as 1-ml aliquots in 1.5-ml microtubes. Immediately store aliquots at -80°C .
5. In addition, prepare aliquots of ready-to-use sterile saline for control experiments. Transfer 1 ml sterile saline into 1.5-ml microtubes and 4 ml saline into sterile culture tubes (BD Falcon, 352003). Immediately store the aliquots at -80°C .
6. Directly when the aluminium hydroxide powder [$\text{Al}(\text{OH})_3$, alum, Sigma-Aldrich, cat. no. 239186] (*see Note 2*) arrives at the lab, weigh aliquots of 80 mg in 5-ml sterile culture tubes. Be careful to store alum aliquots protected from light at room temperature.

2.2. Ovalbumin And

Conjugation of OVA to alum is a critical step to guarantee the

Alum Conjugates

reproducibility of the sensitization.

1. Before use, bring an aliquot of 2 mg/ml OVA solution and an aliquot of 4-ml sterile saline to room temperature.
2. Take an aliquot of alum (80 mg), add 1 ml OVA solution (2 mg/ml) and 3 ml sterile saline. This suspension should be prepared fresh every day.
3. Gently homogenize the OVA-alum suspension for 4 hr at 4°C on a rotator mixer to allow adsorption of OVA on alum. (Alum is not entirely soluble and this will be presented as a suspension).
4. Before administration to animals, bring the suspension to room temperature on a rotator mixer for 10 min (18° to 23°C).

2.3 Anaesthetic Solution

1. In a 15 ml Falcon tube, add 1.5 ml of a 100 g/L commercial solution of ketamine (Merial, Imalgene[®]), 0.5 ml of a 20 g/L commercial solution of xylazine (Bayer, Rompun[®] 2%) and 10 ml of sterile saline. The prepared solution contains 12.5 mg/ml ketamine base and 0.83 mg/ml xylazine base from hydrochloride. This preparation may be stored at 4°C for 10 days.
2. Before use in animals, bring the solution to room temperature. Inject 100 µl of the anaesthetic solution per mouse (25 g), i.e. 4 ml/kg. The administered dose is 50 mg/kg ketamine and 3.3 mg/kg xylazine.

2.4 Anaesthetic Solution for bronchoalveolar lavage procedure

1. In a 15 ml Falcon tube, add 4.5 ml of a 100 g/L commercial solution of ketamine (Merial, Imalgene[®]), 1.5 ml of a 20 g/L commercial solution of xylazine (Bayer, Rompun[®] 2%) and 6 ml of sterile saline. The prepared solution contains 37.5 mg/ml ketamine base and 24.9 mg/ml xylazine base from hydrochloride. This preparation may be stored at 4°C for 10 days.
2. Before use in animals, bring the solution to room temperature. Inject 100 µl of the anaesthetic solution per mouse (25 g), i.e. 4 ml/kg. The administered dose is 150 mg/kg ketamine and 10 mg/kg xylazine.

2.5 Solutions for bronchoalveolar lavage procedure

1. Saline-EDTA 2.6 mM: in a sterile 1-l DURAN[®] glass bottle (Schott, 21801545), add 970 mg of EDTA (ethylene diamine-tetra acetic acid disodium salt dihydrate, Sigma-Aldrich, E1644), 9 g of Sodium chloride (Sigma-Aldrich, S7653) and 1-l of ultrapure water. Add a PTFE coated stir bars in the bottle and place it on a magnetic stirrer (Stuart, CB161) for 30 min until dissolved. Adjust pH to 7.3 with a 2 N sodium hydroxide solution (Fluka, 71474). This preparation may be stored at 4°C for 1 week.
2. Potassium chloride 0.6 M: in a sterile 500-ml DURAN[®] glass bottle (Schott, 21801545), add 22,36 g of potassium chloride (Sigma-Aldrich, P9541) and 500 ml of ultrapure water.

3. Methods

This procedure describes how mice sensitized and challenged to OVA will develop reproducible and robust allergic asthma features, in particular eosinophil infiltration to be recovered in the bronchoalveolar lavage. Mice should be sensitized intraperitoneally, then challenged by intranasal administration at the indicated times (**Fig.1**). Repeated exposure to OVA is required to induce cytokine production and increase eosinophil recruitment (**Fig.3**). Eosinophils recovered in the bronchoalveolar lavage fluid are presented on **Fig.2**. Control mice are sensitized with the OVA-alum suspension for the 8-day and 21-day models or with OVA in the absence of adjuvant for the 57-day model. They are challenged with solvent (saline) alone.

Asthma features will be assessed twenty-four hours after the last OVA challenge: AHR may be measured by whole body plethysmography as described (13) or the forced oscillation technique (14) and inflammatory cell recruitment in particular eosinophils assessed in the collected bronchoalveolar lavage (15) described below and presented on **Fig.2**.

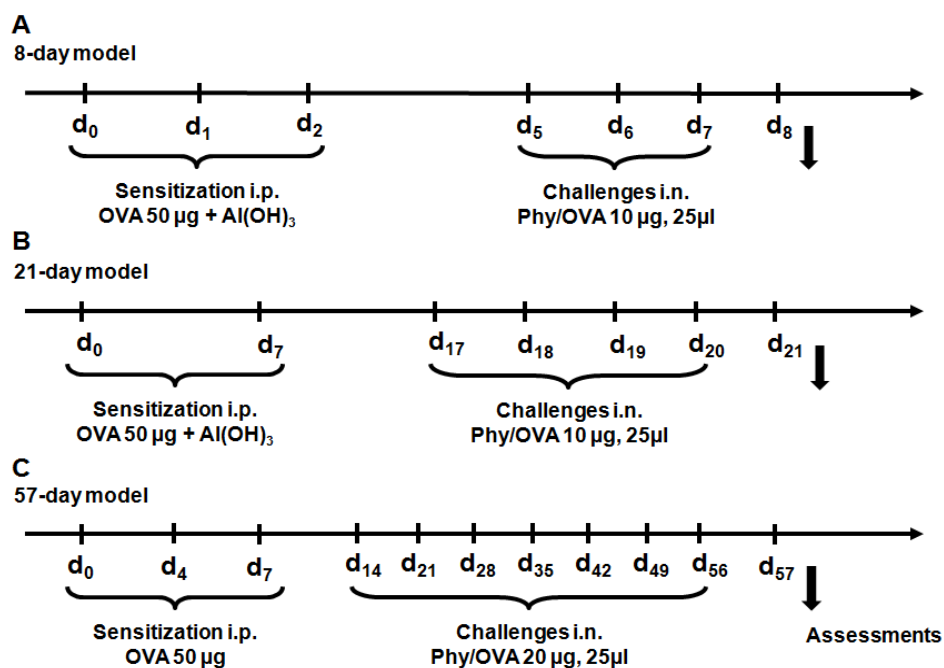


Figure 1: Allergic asthma models to ovalbumin in mice. (A) The 8-day rapid model of hyper eosinophilia is composed of a sensitization step with intraperitoneal (i.p.) administrations of OVA-alum suspension on days 0, 1 and 2, followed by intranasal (i.n.) challenges with ovalbumin or saline for control mice on days 5, 6 and 7. (B) The 21-day acute model of asthma is composed of a sensitization step with i.p. administrations of OVA-alum suspension on days 0 and 7, followed by i.n. challenges with ovalbumin or saline for control mice on days 17, 18, 19 and 20. (C) The 57-day chronic model of asthma is composed of a sensitization step with i.p. administrations of ovalbumin alone (no adjuvant) on days 0, 4 and 7, followed by i.n. challenges with ovalbumin or saline for control mice weekly for 7 weeks. Assessments of AHR, OVA-specific IgE production, airway inflammatory cell recruitment, and airway remodeling are performed twenty-four hours after the last OVA challenge.

3.1. Perform ovalbumin sensitization with adjuvant in the 8-day and 21-day models

1. Take the OVA-alum suspension (see §2.2).
2. Hold the mouse (*see Note 3*) in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail with fingers.
3. Use 1-ml syringes and 25-G needles to inject the OVA-alum suspension and administer 4 ml/kg, i.e. 100 μ l per mouse (of 25 g) by intraperitoneal injection. Gently homogenize the suspension between each injection (*see Note 4*). Each mouse receives 50 μ g OVA adsorbed on 2 mg alum in saline.

3.2. Perform ovalbumin sensitization in the 57-day model

1. Bring an aliquot of 0.8 mg/ml OVA solution to room temperature, and vortex for 5 sec at maximal speed (*see Note 1*).
2. Hold the mouse (*see Note 3*) in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail with fingers.
3. Use 1-ml syringes and 25-G needles to inject OVA and administer 5 ml/kg, i.e. 125 μ l per mouse (of 25 g) by intraperitoneal injection. Each mouse receives 50 μ g OVA in saline.

3.3. Ovalbumin challenge

1. Bring an aliquot of 0.4 mg/ml (8-day and 21-day models) or 0.8 mg/ml (57-day model) OVA solution to room temperature and vortex for 5 sec at maximal speed (*see Note 1*).
2. Mice should be anaesthetized for intranasal administration. Anesthetize mice as follows: Hold the mouse in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail with fingers and use 1-ml syringes and 25-G needles to inject 4 ml/kg of the anaesthetic solution (*see Note 5*) by intraperitoneal injection.
3. Place the mouse in the cage and wait until vibrissae do not move any more.
4. Hold the mouse in your hand in a vertical position with its head up and its rear legs down. Administer, drop by drop, 12.5 μ l OVA solution in each nostril or of saline alone for controls, by using sterile tips and a 20- μ l precision pipette (*see Note 6*). Each mouse receives 10 μ g (8-day and 21-day models) or 20 μ g (57-day model) of OVA.
5. Keep the mouse in your hand in a vertical position for at least 1 min so that the solution can be distributed in the airways, and check that the mouse breathes normally (*see Note 7*).
6. Next, place the mouse in a horizontal decubitus on a heating blanket until the mouse is completely awoken.

3.4. Bronchoalveolar lavage procedure

1. Mice should be anaesthetized for BAL procedure as follows: Hold the mouse in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail with fingers and use 1-ml syringes and 25-G needles to inject 4 ml/kg of the anaesthetic solution (*see 2.4*) by intraperitoneal injection.
2. Place the animal in a dorsal decubitus position until the mouse is deeply anesthetized.
3. Using scissors, make a small incision in the neck skin. Separate

salivary glands, incise the sternohyoid muscle to expose the trachea, and place a cotton thread (black haberdashery cotton thread no. 40) under the trachea.

4. Make a small semi-incision of the trachea to allow a 21-G lavage tube to pass into the trachea (*see Note 8*). Stabilize the tube and needle by attaching them with a cotton thread

6. Load a 1-ml syringe (Terumo, BS-01T) with 0.5 ml sterile saline-EDTA, place it in the 21-G lavage tube and inject 0.5 ml saline-EDTA into the lung. Massage the chest for 10 sec, re-aspirate saline of the first lavage, and keep the recovered lavage fluid into a 5-ml tube placed on ice.

7. Repeat the procedure 10 times per animal. (*see Note 9*)

8. Pool lavages recovered and centrifuge them for 5 min at $300 \times g$ at 4°C to pellet the cells. Discard the supernatant and add 1500 μl ultrapure water to the cell pellet for erythrocyte hemolysis. Wait for 10 sec, add 500 μl KCl (0.6 M), and homogenize by inverting.

9. Centrifuge the BAL fluid for 5 min at $300 \times g$ at 4°C . Discard the supernatant, add 500 μl saline-EDTA to the cell pellet, and homogenize by inverting.

3.4. Inflammatory cell counting

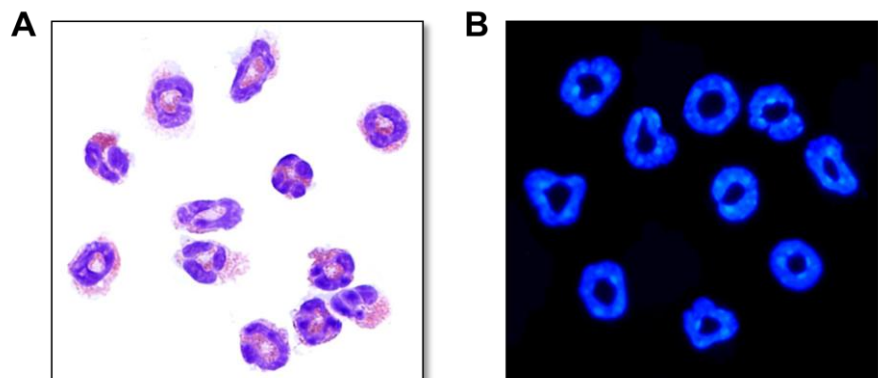
1. For total cell count: place 5 μl of the cell suspension on a hemocytometer (Neubauer, Malassez, or other), count the cells and calculate the total cell number in BALF.

2. For differential cell count: homogenize by inverting and dilute with saline-EDTA to obtain a final concentration of 250,000 cells/ml. In a cytofunnel (CytoSpin™ 4 Cytocentrifuge), place 200 μl of the cell suspension. Centrifuge for 10 min at 700 rpm then let the slide air-dry and proceed to cell staining.

3. Cell staining with Diff-Quick staining kit (Hemacolor; Merck, cat. no. 1.11661.0001): immerse the slide for 15 sec in Diff-Quick fixative reagent, for 30 sec in Diff-Quick solution I then for 15 sec in Diff-Quick solution II and rinse the slide for 5 sec in tap water. Let the slide air-dry (*see Note 10*).

4. Count and identify 400 cells under light microscopy on each slide. Magnification $\times 1000$; oil immersion. Manual counting allows to identify eosinophils, neutrophils, macrophages, and lymphocytes. The percentage of each cell population is reported to the total number of cells, and the absolute number of each cell population is then calculated in BALF.

Figure 2: Photograph of eosinophils recovered in BALF in the 21-day acute model of asthma to ovalbumin in mice. (A) Bright field photograph of cytopun eosinophils stained with Diff-Quick. (B) Fluorescent photograph of eosinophils stained with DAPI to highlight the typical circular nucleus of murine eosinophils.



4. Notes

1. OVA is a protein that may be difficult to dissolve in saline. In our experience, it is better to use a high speed vortex, with maximal speed $\geq 3000\text{rpm}$ (Eppendorf MixMate[®]) to dissolve OVA rapidly and prevent the formation of a turbid suspension composed of hydrated OVA particles.

2. Be very careful while choosing the aluminium hydroxide reagent. The quality of aluminium hydroxide influences the number of eosinophils recovered in BAL in the 8-day and 21-day model. We recommend to use aluminium hydroxide from Sigma-Aldrich (cat. no. 239186).

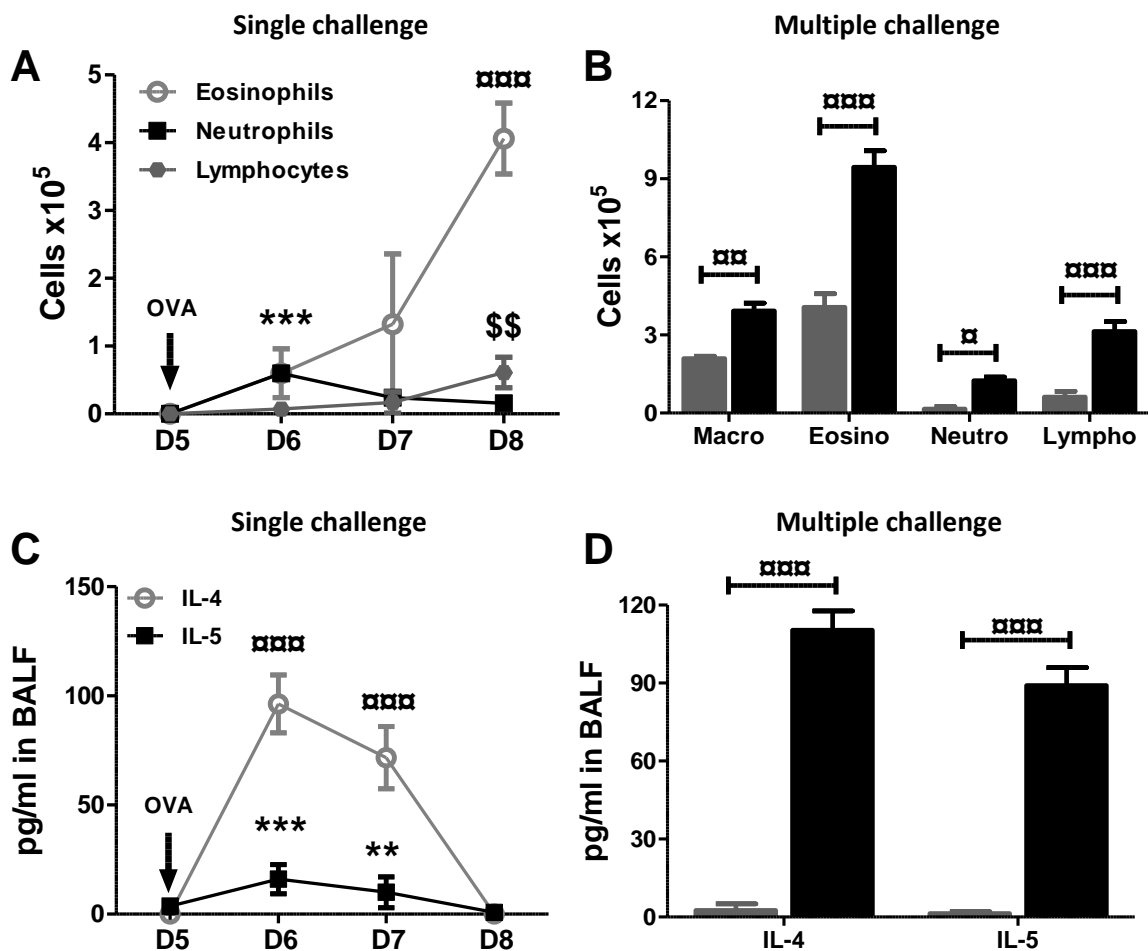


Figure 3: Repeated exposure to OVA is required to induce both eosinophil recruitment and cytokine production. Example of the 8-day model. (A) Evolution of absolute numbers of eosinophils, neutrophils and lymphocytes in the bronchoalveolar lavage fluid (BALF) 24h (D6), 48h (D7) and 72h (D8) after a single challenge to OVA (performed on D5 and represented by the arrow) in mice sensitized to OVA. Dots are means and bars are SEM values (n=3 to 6 mice per group). ^{****}P \leq 0.001 vs D5 for eosinophils, ^{***}P \leq 0.001 vs D5 for neutrophils and ^{\$\$}P \leq 0.01 vs D5 for lymphocytes. (B) Absolute numbers of macrophages (Macro), eosinophils (Eosino), neutrophils (Neutro) and lymphocytes (Lympho) in BALF on D8 after a single challenge to OVA on D5 (grey blocks) or 3 challenges to OVA on 3 consecutive days (D5, D6 and D7) (black blocks). Blocks are means and bars are SEM values (n=6 mice per group). [†]P \leq 0.05; ^{††}P \leq 0.01; ^{†††}P \leq 0.001 (C) Evolution of IL-4 and IL-5 cytokine levels recovered in BALF 24h, 48h and 72h after a single challenge to OVA on D5 (represented by the arrow). Dots are means and bars are SEM values (n=3 to 6 mice per group). ^{†††}P \leq 0.001 vs D5 for IL-5 and ^{**}P \leq 0.01, ^{***}P \leq 0.001 vs D5 for IL-4. (D) Levels of IL-4 and IL-5 recovered in BALF on D8 after a single challenge to OVA on D5 (grey blocks) or 3 challenges to OVA on 3 consecutive days (D5, D6 and D7) (black blocks). Blocks are means and bars are SEM values (n=6 mice per group). ^{†††}P \leq 0.001.

3. These protocols are developed to be used with nine week-old male or female Balb/c mice to study AHR, OVA-specific IgE production, airway inflammatory cell recruitment, and airway remodeling (16-19). C57BL6 mice may be used to study eosinophilic airway inflammation in the 8-day and 21-day models, but AHR cannot be measured (Fig.4). To measure AHR together with eosinophil assessment in C57BL6 mice, we recommend the 57-day allergic asthma model. In addition, the described steps may be used for various other asthma models. Most of asthma models are dependent on the number of sensitizations and challenges, the dose of OVA and alum used, and the time between sensitizations and/or challenges.
4. Gently homogenize the suspension by 3-4 repeated reversals of the tube/syringe between each use so that the suspension does not drop at the bottom of the tube/syringe.
5. Anaesthesia is key to successful intranasal administration. Too light anaesthesia reduces reproducibility of inflammatory cells recruitment, and too higher anaesthesia enhances respiratory failure during and after i.n. administration. It may be necessary to adapt the dose of anaesthetic to warrant the best quality of anaesthesia. It often requires to increase the dose over time.
6. Solution has to be administered drop by drop, slowly and very carefully to maximize the distribution of OVA into the airways. Intranasal challenge is a simple technique, easy to perform, but it requires expertise to obtain reproducible responses.
7. If the mouse does not breathe normally, perform a thorax massage by pressing the rib cage several times, quickly but carefully. Please take care to identify each mouse who stops breathing, because this may affect AHR and eosinophilic inflammation of the airways.

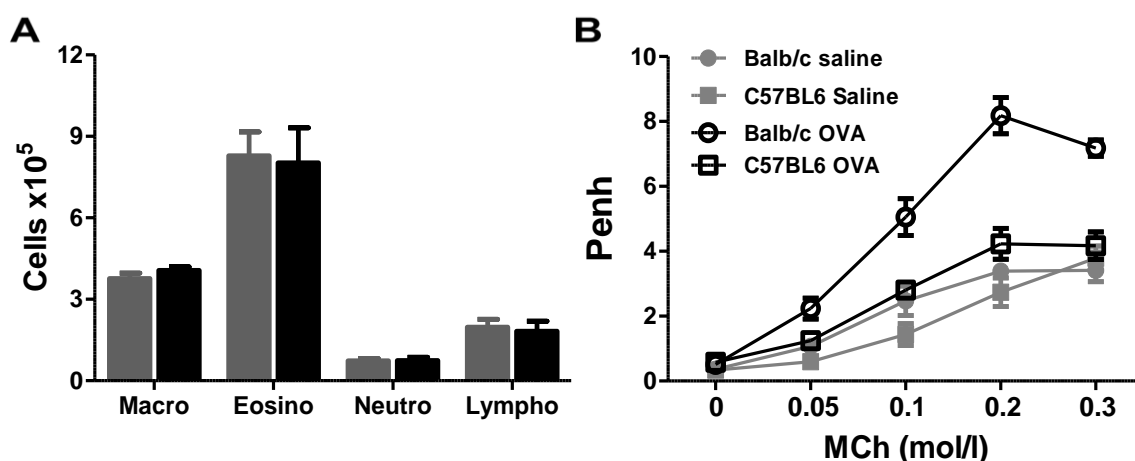


Figure 4: Comparison of inflammatory cell recruitment and airway hyperresponsiveness between Balb/c and C57BL6 in the 21-day allergic asthma model. (A) Inflammatory cells in BALF on day 21 in Balb/c mice (grey blocks) or in C57BL6 mice (black blocks). Blocks are means and bars are SEM values. **(B)** Airway hyperresponsiveness to metacholine measured by plethysmography on vigil and unrestrained mice (EMKA Technologies, Paris, France) on day 21. Dots are means and bars are SEM values.

8. While semi-incising the trachea, take care not to cut through the trachea. For tracheotomy, use a 21-G lavage tubing, carefully placed over a 21-G needle or a single-use Terumo surshield® IV catheter (Terumo, 1SR*SFA2025A).

9. Supernatant from the first two lavages may be pooled and separated from the eight following lavages for cytokine or mucus analysis, since a higher concentration is present in the 2 first lavages. In this case, centrifuge the initial two first lavages for 5 min at $300 \times g$ at 4°C , then freeze the supernatant for further analysis, and add the cell pellet to the eight following lavages.

10. We describe the staining of cytopinned cells with the commercial Diff-Quick staining : this staining requires to be performed in less than an hour of cytopinning. Other commercial staining may be useful as RAL 555 (RAL-diagnostics) or could be advantageously replaced by a home-made staining described in (15).

Acknowledgements

This work was supported by the "Fonds de Dotation Recherche en Santé Respiratoire, Appel d'Offres Blanc 2010". FD is a recipient of a fellowship from the Laboratory of Excellence Medalis Initiative of Excellence (IdEx), Strasbourg University, France.

References

1. Global initiative for asthma (GINA). 2012. Pocket Guide For Asthma Management And Prevention. <http://www.ginasthma.org/>
2. Berend, N., Salome, C.M., and King, G.G. (2008) Mechanisms of airway hyperresponsiveness in asthma. *Respirology* 13:624-631.
3. Bates JH, Rincon M, Irvin CG. (2009) Animal models of asthma. *Am J Physiol Lung Cell Mol Physiol*. 297(3):L401-10
4. Epstein MM. (2006) Are mouse models of allergic asthma useful for testing novel therapeutics? *Exp Toxicol Pathol*. 57 Suppl 2:41-4.
5. Kumar RK, Herbert C, Foster PS. (2008) The "classical" ovalbumin challenge model of asthma in mice. *Curr Drug Targets*. 9(6):485-94.
6. Shinagawa K, Kojima M. (2003) Mouse model of airway remodeling: strain differences. *Am J Respir Crit Care Med*. 168(8):959-67.
7. Lukacs NW, John A, Berlin A, Bullard DC, Knibbs R, Stoolman LM. (2002). E- and P-selectins are essential for the development of cockroach allergen-induced airway responses. *J Immunol*. 169(4):2120-5
8. Ulrich, K., Hincks, J.S., Walsh, R., et al. (2008) Anti-inflammatory modulation of chronic airway inflammation in the murine house dust mite model. *Pulm. Pharmacol. Ther*. 21:637-647.
9. Nials AT, Uddin S. (2008) Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech*. 1(4-5):213-20.
10. McKee, A.S., Munks, M.W., MacLeod, M.K., et al. (2009) Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J. Immunol*. 183:4403-4414.
11. Zosky GR, Sly PD (2007) Animal models of asthma. *Clin Exp Allergy*. 37(7):973-88.
12. Daubeuf F., Frossard N. Acute Asthma Models to Ovalbumin in the Mouse. *Current Protocols in Mouse Biology*. March 2013
13. Daubeuf, F., Reber L. and Frossard, N. (2013) Measurement of Airway Responsiveness on

- Vigil and Unrestrained Mouse, <http://www.bio-protocol.org/wenzhang.aspx?id=328>
14. Daubeuf, F., Reber L. and Frossard, N. (2013) Measurement of Airway Responsiveness in the Anesthetized Mouse, <http://www.bio-protocol.org/wenzhang.aspx?id=645>
 15. Daubeuf, F. and Frossard, N. (2012) Performing bronchoalveolar lavage in the mouse. *Curr. Protoc. Mouse Biol.* 2:167-175.
 16. Delayre-Orthez, C., Becker, J., De Blay, F., Frossard, N., and Pons, F. (2004) Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin. Exp. Allergy* 34:1789-1795. 12.
 17. Daubeuf F, Hachet-Haas M, Gizzi P, Gasparik V, Bonnet D, Utard V, Hibert M, Frossard N, Galzi JL. (2013) An Antedrug of the CXCL12 Neutraligand Blocks Experimental Allergic Asthma without Systemic Effect in Mice. *J Biol Chem.* 288(17):11865-76 23.
 18. Gasparik, V., F. Daubeuf, M. Hachet-Haas, F. Rohmer, P. Gizzi, J. Haiech, J.L. Galzi, M. Hibert, D. Bonnet, and N. Frossard (2011) Prodrugs of a CXC chemokine ligand 12, CXCL12, neutraligand active in vivo in a new asthma model. *ACS Med Chem Letters.* 3: p. 10-14
 19. Hachet-Haas, M., Balabanian, K., Rohmer, F., et al. (2008) Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J. Biol. Chem.* 283:23189-23199.
 20. Andreasen, C.B. (2003) Bronchoalveolar lavage. *Vet.Clin. Small Anim.* 33:69-88.
 21. Reber, L.L., Daubeuf, F., Plantinga, M., De Cauwer, L., Gerlo, S., Waelput, W., Van Calenbergh, S., Tavernier, J., Haegeman, G., Lambrecht, B.N., Frossard, N., and de Bosscher, K. (2012) A fully dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a murine model of asthma. *J. Immunol.* 188:3478-3487.

Annexe n°5 :

**Neutralizing endogenous chemokines with small molecules
Principles and potential therapeutic applications**

Jean-Luc Galzi,, Muriel Hachet-Haas, Dominique Bonnet, Francois Daubeuf, Sandra Lecat,
Marcel Hibert, Jacques Haiech, Nelly Frossard

Pharmacol Ther. 2010 Apr;126(1):39-55.

La régulation des réponses cellulaires à des cytokines et des chimiokines implique de nombreuses étapes de régulations et de contrôles. Une des possibilités est le piégeage ou la neutralisation des cytokines et des chimiokines, et peut permettre la validation des voies de signalisation dans les conditions physiologiques et pathophysiologiques, mais aussi concourir au développement de molécules thérapeutiques. Cet revue explore le domaine de la neutralisation des ligands et tente de déterminer dans quelle mesure les petites molécules chimiques pourraient se substituer aux anticorps neutralisants pour des approches thérapeutiques .



Neutralizing endogenous chemokines with small molecules Principles and potential therapeutic applications

Jean-Luc Galzi^{a,*}, Muriel Hachet-Haas^a, Dominique Bonnet^b, Francois Daubeuf^b, Sandra Lecat^a,
Marcel Hibert^b, Jacques Haiech^b, Nelly Frossard^b

^a IREBS, FRE3211, Ecole Supérieure de Biotechnologie de Strasbourg, Boulevard Sébastien Brant, 67412 Illkirch, France

^b Laboratoire d'Innovation Thérapeutique, UMR7200, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

ARTICLE INFO

Keywords:

Chemokines
Cytokines
GPCRs
Decoy receptors
Neutralizing ligands
Small molecule

ABSTRACT

Regulation of cellular responses to external stimuli such as hormones, neurotransmitters, or cytokines is achieved through the control of all steps of the complex cascade starting with synthesis, going through maturation steps, release, distribution, degradation and/or uptake of the signalling molecule interacting with the target protein. One possible way of regulation, referred to as scavenging or neutralization of the ligand, has been increasingly studied, especially for small protein ligands. It shows innovative potential in chemical biology approaches as well as in disease treatment. Neutralization of protein ligands, as for example cytokines or chemokines can lead to the validation of signalling pathways under physiological or pathophysiological conditions, and in certain cases, to the development of therapeutic molecules now used in autoimmune diseases, chronic inflammation and cancer treatment. This review explores the field of ligand neutralization and tries to determine to what extent small chemical molecules could substitute for neutralizing antibodies in therapeutic approaches.

© 2010 Published by Elsevier Inc.

Contents

1. Introduction	39
2. Natural cytokine and chemokine neutralization	40
3. Potential therapeutic interest of soluble decoy proteins	43
4. Validation of chemokines in signalling pathways and pathology: importance of anti-chemokine antibodies	45
5. Neutralizing cytokines and chemokines with small chemical compounds	46
6. Concluding remarks and perspectives.	49
Acknowledgments	50
References	50

1. Introduction

Deciphering biological signalling pathways makes use of convergent approaches including direct gene manipulation or downstream information processing intermediates such as messenger RNAs, proteins or signalling small molecules/hormones or their metabolites. Gene manipulation, in particular gene deletion/invalidation, is one of the most widely used approaches to determine the function of a gene

and of its products. It presents the major advantage of selectively altering one gene structure or expression so that a given phenotype, when observed, is generally closely associated with the gene of interest and to its products. On the other hand, gene deletion or overexpression can be induced, but not yet in a reversible manner, so that control experiments must be carried out on wild type animals in which developmental or compensatory effects may not have taken place in a comparable manner (Chensue et al., 2001; Auwerx et al., 2004; Brown et al., 2005; Yang et al., 2006). Chemical biology approaches, i.e. methods that use chemical tools to elucidate the function of a protein in a given signalling pathway, and are at the frontier between pharmacology, chemistry and biophysics, are useful too and show complementarity with genetic approaches. They also offer the possibility to transpose small molecule tools into drugs when

Abbreviations: BSA, Bovine serum albumin; DARC, Duffy antigen receptor for chemokines; GAG, Glycosaminoglycans; GPCR, G-protein-coupled receptor; IL, Interleukin; LPS, Lipopolysaccharide; MS, Multiple sclerosis.

* Corresponding author. Tel.: +33 368 85 47 59.

E-mail address: galzi@unistra.fr (J.-L. Galzi).

pathological issues are coming into play during the assessment of the protein function. The advantages and drawbacks of the chemical biology approach are mirror images of the genomic approach. Reversibility of the effect of a molecule can be studied on the same living individual upon cessation of molecule administration. On the other hand, molecules are rarely specific for a given target protein, and the claimed selectivity of a compound generally follows an inverse relationship with the extent of side effects (Wermuth, 2006). Antibodies, and in particular monoclonal antibodies, have arisen as potential substitutes to both the genetic and the chemical biology approaches in the sense that they exhibit quasi-exclusive selectivity for a protein target and that the interruption of treatment leads to reversal of their effects. Antibodies offer in addition the possibility of target interactions, especially large protein–protein interactions, that are difficult to perturb with small molecules. This has led to the exponential development of antibodies or antibody fragments (Chames et al., 2009; Nelson & Reichert, 2009; Wesolowski et al., 2009) for therapeutic purposes. Antibodies are powerful tools in laboratory research because they can be developed much faster than small chemical molecules (see below). They have thus been largely used to validate the involvement of proteins in signal transduction pathways, and as potential target for drug development. On the other hand, antibodies have intrinsic limitations that constrain their use for biological systems exploration. With some exceptions, antibodies and antibody fragments do not cross biological barriers, such as the intestinal or blood brain barriers. The consequence is that antibodies must be injected and most central nervous system proteins will not be reached. Also, antibodies cannot reach intracellular target proteins unless they cycle to the plasma membrane.

For all these reasons, the chemical biology approach using small molecules as tools or drugs remains a useful and valid strategy. In this article, we review examples of small chemical molecules that can be used to neutralize small signalling proteins such as chemokines or cytokines. The reader should appreciate that only a few examples are known to date. The reason for this is that all neutralizing molecule discoveries that are presented here were serendipitous, and specifically designed experimental approaches are only just entering starting blocks. Small molecules are being searched to inhibit protein–protein interactions, with a focus on intracellular compartments and cancer related interactions, or brain function exploration (Berg, 2003; Arkin & Wells, 2004; Arkin, 2005; Wells & McClendon, 2007; Blazer & Neubig, 2009). These will not be reviewed here. We will focus mainly on the family of small signalling proteins, the chemokines, which constitute a well adapted biological system to develop neutralizing small molecules. Examples from other cytokines will be discussed as well.

1.1. Chemokines and chemokine receptors

Chemokines are small secreted chemotactic cytokines endowed with multiple activities. Their main function is chemical attraction of leukocytes, but they also contribute to the regulation of organ development during ontogeny. In inflammation, the chemotactic signal given by chemokines leads to egress of leukocytes from the blood circulation across the walls of small blood vessels. To do this, chemokines that are produced on the site of inflammation cross the endothelial cell wall and remain immobilized on the luminal surface of the endothelium. Circulating leukocytes, depending on their chemokine receptor expression will then be attracted and directed towards the inflamed site along the chemotactic gradient. Chemokines, in addition to attracting cells, contribute to the regulation of gene expression on target cells and help to control cell proliferation and apoptosis, for instance in angiogenesis.

Chemokines are also subdivided into several functional groups depending on whether their expression is constitutive or inducible by inflammatory signals, and also on their capacity to stimulate or inhibit angiogenesis, especially in tumors (Vandercappellen et al., 2008). The

CXC chemokines in particular exert angiogenic or angiostatic activities depending on the presence of an ELR (Glu-Leu-Arg) motif in their N-terminal portion (Addison et al., 2000). As important regulators of cell migration, therapeutic intervention of the chemokine system(s) includes infectious diseases, intra-organism alert systems possibly leading to autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis or lupus erythematosus, as well as allergic disorders such as asthma, inflammatory bowel disease, transplant rejection, neuropathies or dermatitis.

More than 50 chemokines are known (Wells et al., 2006). The chemokine structure (Figs. 1 and 3) comprises an N-terminal loop region, three-strand anti-parallel beta-sheets forming the typical core fold of the chemokines and a C-terminal alpha helix which overlays the beta-sheet. CC, CXC and CX3C chemokines comprise in addition two disulfide bridges linking the N-terminal domain with the loop separating sheet 1 and sheet 2 and the N-terminal domain with the end of sheet 3. In order to allow gradients to be formed at the vicinity of the site of release, chemokines bind to extracellular matrix components, i.e. the negatively charged glycosaminoglycans (GAGs), by means of their positively charged amino acids. These positive amino acids form distinct clusters at the surface of the chemokine depending on whether the chemokine belongs to the CC, CXC or CX3C group (Laguri et al., 2008). In the CXC chemokine group, the GAG-binding area is on the side of the protein that does not interact with the receptor (Amara et al., 1999; Santiago et al., 2006; Murphy et al., 2007) and, mutation of the positive amino acids that bind to GAGs does not alter chemokine binding to the receptor (Amara et al., 1999; Proudfoot et al., 2001), and interaction with heparan sulfates does not change the equilibrium binding affinity of the chemokine for its receptor (Valenzuela-Fernandez et al., 2001). In the CC group of chemokines, in contrast, there is significant overlap between receptor binding and GAG-binding areas which, in the case of CCL5 for instance, has influence on receptor subtype-specific interactions (Proudfoot et al., 2001).

Chemokines signal through G proteins coupled to seven transmembrane receptors which are classified according to the chemokines they bind (CXCR, CCR, CX3CR and XCR) (Murphy, 2002). The chemokine receptor family groups twenty G-protein-coupled receptors (GPCRs) and covers extremely diverse physiological responses. As a general rule, structural promiscuity between GPCRs accounts for frequently observed problems of ligand selectivity among subtypes. Reciprocally, GPCR ligands, in particular chemokines, are grouped in small chemical families, so that neutralizing the ligand rather than the receptor may allow good focus on a subset of targeted signalling pathways.

Along with several other signalling proteins (Alcami & Smith, 1992; Colotta et al., 1993; Pitti et al., 1998; Rahaman et al., 2002; Bezerra et al., 2005; Bamiás et al., 2008; de Moura et al., 2009; Fili et al., 2009; Funke et al., 2009; Mueller et al., 2009; Scola et al., 2009), chemokines are subject to natural modulation of their concentrations by proteins to which they bind (Fig. 2) without leading to typical signalling (Murphy, 2000; Alcami, 2003; Graham & McKimmie, 2006; Mantovani et al., 2006; Murphy et al., 2007; Graham, 2009; Pruenster et al., 2009). These proteins may be endogenously encoded to modulate chemokine functions or expressed by exogenous sources like pathogens or parasites with the aim of escaping the host immune system (see below). These naturally occurring “scavenger” or “decoy” proteins act as “interceptors” – i.e. intercepting receptors – that neutralize the action of the chemokine. We shall briefly review these systems because they validate the concepts of ligand neutralization, before considering approaches to unnatural neutralization.

2. Natural cytokine and chemokine neutralization

Besides metabolic regulation of hormone or peptide production such as enzymatic degradation, transport (Mortier et al., 2008), a captivating aspect of response regulation is scavenging of ligands by molecules that bind to it and modulate its biological function. This

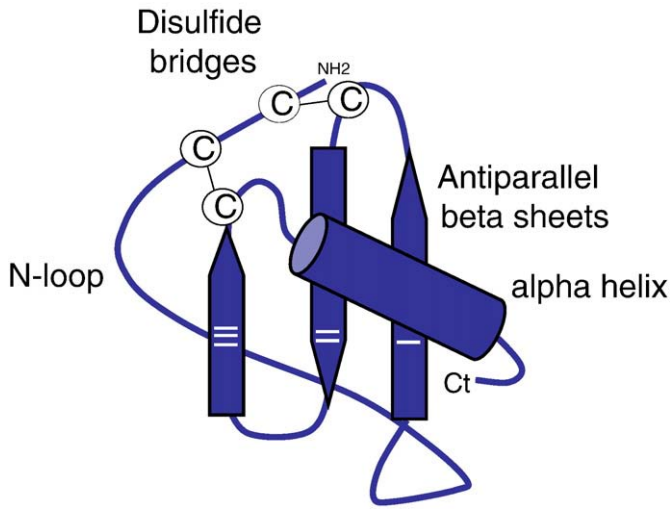


Fig. 1. Folding of chemokines: chemokine adopts a typical structure with 3 anti-parallel β -strands and one carboxy terminal helix. C–C denotes disulfide bridges.

has been illustrated in the past 25 years with the identification of endogenous receptor-like structures that do not lead to conventional signalling in response to small protein ligands but rather seem to contribute to their blockade or elimination (Colotta et al., 1993; Colotta et al., 1995; Bezerra et al., 2005; Mantovani et al., 2006; Mantovani et al., 2007; Thelen & Thelen, 2008; Mantovani et al., 2008; Bamias et al., 2008; Bonecchi et al., 2008b; de Moura et al., 2009; Mueller et al., 2009; Scola et al., 2009). These receptor-like molecules, which can be soluble (Colotta et al., 1993; de Moura et al., 2009; Funke et al., 2009) or membrane-bound (Mantovani et al., 2006; Scola et al., 2009), have been termed “decoy” or “scavenger” proteins. They however serve physiological as well as pathophysiological functions.

Decoy proteins for interleukins IL-1 (Colotta et al., 1993), IL-22 (de Moura et al., 2009), IL-13 (Caput et al., 1996; Rahaman et al., 2002), death ligands TRAIL (Bellail et al., 2009) and CD95L (Pitti et al., 1998), activators of NF- κ B-RANK (Simonet et al., 1997; Khosla, 2001) or complement (Cain & Monk, 2002; Scola et al., 2009) generally exhibit ligand selectivity and/or specificity. Those for chemokines (Mantovani et al., 2006; Graham, 2009) display poor ligand selectivity.

2.1. Endogenous chemokine interceptors

There are three, possibly four, endogenous proteins that belong to the structural family of G-protein-coupled receptors, bind chemokines with limited to low selectivity, do not signal toward G-protein-dependent pathways but keep the capacity to internalize and transport the bound chemokine across the plasma membrane. These proteins, DARC, D6, CCX-CKR and possibly CXCR7, act as uptake or re-uptake proteins that trap the ligand, internalize it and direct it towards degradation, possibly also towards transcytosis. These proteins play important roles in inflammation, development, and chemokine-associated diseases such as cancer (Graham & McKimmie, 2006; Mantovani et al., 2006).

2.1.1. Duffy antigen receptor for chemokines

Duffy antigen receptor for chemokines, DARC, binds both CC (CCL-2, -5, -7, -11, and -13) and CXC (CXCL-1, -3, -5, -6, -8, and -11) inflammatory chemokines as well as the homeostatic chemokine CCL14. It is a G-protein-coupled receptor-like protein that lacks the capacity to stimulate G proteins.

DARC is expressed at high levels in the cell membrane of erythrocytes where it was shown to contribute to clearing circulating chemokines (Darbonne et al., 1991). Supporting this role in chemokine clearance, lack of DARC protein is associated with an exaggerated inflammatory response to lipopolysaccharide LPS (Dawson et al., 2000), while overexpression of the protein leads to diminished angiogenesis (Bonecchi et al., 2008a). Further supporting the importance of DARC in

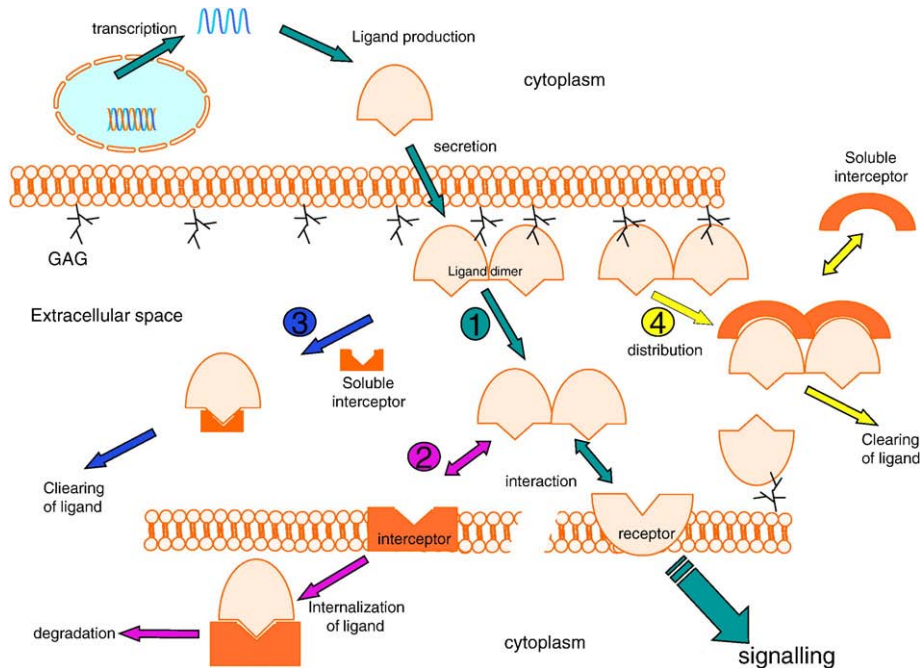


Fig. 2. Examples of different possible routes that can be followed by chemokines/cytokines in the presence of neutralizing macromolecules. Route 1 leads to signalling in the target cell expressing the chemokine/cytokine receptor. Route 2 is used either endogenously or by pathogens. Binding of chemokines takes place without signalling. This event may either lead to degradation of the chemokine or to its transcytosis. Route 3 is used by pathogens that express soluble proteins capable of binding chemokines, generally with moderate affinity and selectivity, and prevents them from normal signalling to the immune system. In Route 4, the neutralizing molecule prevents chemokine binding to glycosaminoglycans. The resulting effect is a collapse of the chemotactic gradient that abolishes leukocyte attraction in the inflamed tissue.

chemokine clearance, circulating CXCL1 is mostly associated with red blood cells in wild type mice, while it is found in the plasma in DARC^{-/-} mice. Accordingly, in a mouse model of acute lung injury, LPS-induced polymorphonuclear leukocyte migration in the alveolar space is elevated two-fold in knock-out animals (Reutershan et al., 2009). The chemokine sequestering function of DARC is also clinically validated as preventing/reducing tumor cell growth, as was demonstrated in breast cancer (Wang et al., 2006b).

DARC is also expressed on endothelial cells, but under normal conditions, this expression is restricted to postcapillary venules (Fra et al., 2003). It may extend to other types of blood vessels (arteries, capillaries) during infection, inflammation or graft rejection (Segeer et al., 2000; Gardner et al., 2006). In endothelial cells, DARC protein serves as a transporter that allows chemokine transcytosis, thus leading to efficient exposure of tissue-derived inflammatory chemokines to the lumen of vessels and subsequent leukocyte recruitment and extravasation (Prunster et al., 2009).

2.1.2. D6 binds and suppresses inflammatory chemokines

Like DARC, D6 exhibits poor selectivity towards chemokines. It binds 12 different chemokines out of which none belong to the CXC group and 8 are CC pro-inflammatory molecules, implicating D6 as a probable regulator of inflammation. Noteworthy also is the fact that DARC and D6 bind 7 identical chemokines (Bonecchi et al., 2008a). D6 is expressed in lymphatic endothelial cells from non-inflamed skin, gut and lung. Upon inflammation, D6 is expressed in leukocytes, especially in those that invade inflamed tissues (Graham & McKimmie, 2006). In contrast to DARC, D6 does not promote chemokine transcytosis, but rather contributes to their degradation by directing them towards endosomes. D6 is predominantly localized in recycling endosomes capable of trafficking to and from the cell surface in the absence of ligand. In the presence of ligand, D6 can rapidly internalize chemokines; however, D6-internalized chemokines are more effectively retained intracellularly because they more readily dissociate from the receptor during vesicle acidification. These chemokines are then degraded while the receptor recycles to the cell surface (Fra et al., 2003; Galliera et al., 2004; Weber et al., 2004). The most likely physiological role of D6 thus is to clear tissues from remaining chemokines in order to prevent an excessive response, and eventually terminate the inflammatory response (Graham & McKimmie, 2006). In support of this, the lack of D6 expression, *in vivo*, results in an amplified chemokine-mediated inflammatory response (Jamieson et al., 2005; Martinez de la Torre et al., 2005). D6^{-/-} mice show high levels of inflammatory chemokines in the lymph nodes. By contrast, over-expression of D6 reduces leukocyte responses in inflammation models (Nibbs et al., 2007).

As a result of deregulated expression of chemokine receptors and chemokines in cancer, a role of D6 in carcinogenesis has been proposed (Nibbs et al., 2007; Wu et al., 2008). Due to its capacity to sequester chemokines, D6 protects from tumorigenesis in chemical treatment-evoked skin tumors (Nibbs et al., 2007). In another study, D6 has been reported to reduce intratumor levels of CCL2 and CCL5 chemokines, and consequently to inhibit proliferation and invasion of breast cancer cells *in vitro* as well as tumorigenesis and metastasis *in vivo* (Wu et al., 2008).

2.1.3. CCX-CKR binds and suppresses homeostatic chemokines

CCX-CKR is, like DARC and D6, derived from a G-protein-coupled structure, and is devoid of signalling capacity towards G proteins. It is expressed in various organs such as spleen, lymph nodes, heart, kidney, placenta, trachea and brain (Gosling et al., 2000), and in various cell types like T cells, immature dendritic cells, stromal cells, astrocytes (Dorf et al., 2000), ciliated bronchial epithelial cells in pulmonary sarcoidosis (Kriegova et al., 2006), and endothelial cells surrounding cancer cells in tumors (Feng et al., 2009).

CCX-CKR binds the homeostatic chemokines CCL19 and CCL21 which control trafficking of naive T cells, CCL25, and CXCL13 which mediate B cells and helper T cell migration (Gosling et al., 2000; Townson & Nibbs, 2002; Comerford et al., 2006), and directs them towards degradation. *In vitro*, cells expressing CCX-CKR deplete large quantities of these chemokines (Gosling et al., 2000; Townson & Nibbs, 2002; Comerford et al., 2006). Inflammation promoting signals, such as interleukin 1beta, tumor necrosis factor TNF α or interferon IFN gamma attenuate CCX-CKR mRNA levels, supporting a potential link of this interceptor with inflammation. *In vivo*, in particular in mice harbouring CCX-CKR transfected xenografts, reduced tumor growth, neovascularization and metastasis are detected (Feng et al., 2009). Also, a clinical study in breast cancer shows the natural level of CCX-CKR expression to correlate with longer survival of the patients (Feng et al., 2009).

2.1.4. Chemokine receptors as temporary interceptors: the debated case of CXCR7

The capacity of G-protein-coupled receptors to endocytose together with their ligands makes it likely that at least some of them behave as interceptors. The expression of CCR5, for example, is up regulated in T cells responding to anti-inflammatory lipids. Such modification represents a mechanism by which chemokines can be trapped and inflammation terminated (Ariel et al., 2006).

The second receptor for CXCL12 and for CXCL11, namely CXCR7, is another example of an atypical receptor. Indeed, although the receptor sequence contains the canonical DRY sequence required for coupling receptors to G proteins and, attempts to detect signalling through G proteins, activation of MAP-kinases or stimulation of PI3-kinase was unsuccessful to date (Balabanian et al., 2005; Burns et al., 2006; Dambly-Chaudiere et al., 2007) with the exception of one report on signalling through Akt (Wang et al., 2008) which may itself result from beta-arrestin recruitment (Kalatskaya et al., 2009; Luker et al., 2009a; Zabel et al., 2009). Whichever the way CXCR7 signals, it appears important during development, and in particular in heart valve formation (Sierro et al., 2007) and for stabilization of cell adhesion after migration towards CXCL12 gradients (Dambly-Chaudiere et al., 2007; Boldajipour et al., 2008). During development of the zebrafish sensory system, formation of the sensory organ, the lateral line, requires long distance migration of primordial germ cells. Dambly-Chaudiere et al. (2007) and Boldajipour et al. (2008) showed that this migration of germ cells involves a chemokinetic response to CXCL12, mediated by CXCR4, that leads to the migration of germ cells. Directionality of the migration is provided by trailing cells that express the second CXCL12 receptor, CXCR7, which prevents backward migration by depleting CXCL12, in the rear of the migrating group of cells. Consistent with this, Mazinghi et al. reported that human renal progenitor cells use both CXCR4 and CXCR7 receptors for transendothelial migration, but that CXCR7 is a major contributor for cell adhesion to endothelial cells and progenitor cell survival (Mazinghi et al., 2008). CXCR7-mediated or -enhanced adhesiveness is also clearly established in prostate cancer cells, together with improved cell survival and invasiveness (Wang et al., 2008). In the cases reported above, the contribution of CXCR7 to the physiological responses could be chemokine interception and termination of the subsequent migratory response or at least its modulation. CXCR7 indeed shows significantly higher capacity than CXCR4 to increase cell-association of CXCL12 (Luker et al., 2009b).

Another probable physiological function of CXCR7 is related to its capacity to heterodimerize (Sierro et al., 2007; Levoye et al., 2009), as can be detected by bioluminescence- or fluorescence-energy transfer in heterologous expression systems. In the more recent work by Levoye et al. (2009), CXCR7 is shown to exhibit an apparent paradoxical effect interfering with CXCR4 responses to CXCL12. CXCR7 indeed reduces responses to low CXCL12 concentrations while leaving responses to high concentrations unchanged, as compared to CXCR4 alone. This effect

is also detected in isolated human T lymphocytes. The presence of CXCR7 in cells expressing CXCR4 is to render dose–response relationships steeper than expected from the law of mass action. The net result of that interference is a conversion of CXCR4 responses to CXCL12 into an almost all or nothing type of response (Sierro et al., 2007; Levoye et al., 2009) with triggering versus non-triggering CXCL12 doses differing by only three- to five-fold. This physiological effect supports the concept open by the structural demonstrations of CXCR7 heterodimerization. Modelling of the behavior of CXCR7 as a chemokine scavenger could be of interest to discriminate among direct receptor–receptor interactions and the indirect effects of CXCR7 regulating the level of chemokine that would be available to CXCR4.

2.2. Avoidance strategies: examples of neutralizing molecules produced by pathogens

Pathogenic viruses, bacteria or parasites, have set up several strategies to escape host detection and defence systems. They use cytokine or chemokine signalling molecules (receptors and ligands) to infect host cells (Chitnis & Sharma, 2008; Hughes & Nelson, 2009). They also block cytokine signalling by producing antagonists (Damon et al., 1998) which allow to escape alert systems or to redirect them to their own benefit (McFadden et al., 1998; Sozzani et al., 1998; Alcami, 2003; Mantovani et al., 2006; Rosenkilde, 2005; Andreasen & Carbonetti, 2008). The parasite *Leishmania* infecting macrophages, for example, express functional chemokine receptors. These are used as chemoreceptors to promote chemokinesis toward chemokine-producing macrophages. This leads to an efficient *Leishmania* internalization (Roychoudhury et al., 2006) that takes place before an efficacious immune response is set up to clear the pathogen. A second example is taken from the bacterium *Bordetella pertussis*. The pathogen is reported to delay neutrophil recruitment, by slowing down chemokine production by the host (Andreasen & Carbonetti, 2008), through production of *pertussis* toxin the well known inhibitor of Gi protein mediated signalling. Also, as a third illustration, human herpesvirus 6 (HHV-6) produces a chemokine, U83A, that binds to CCR5 to modify its internalization–recycling fate (Catusse et al., 2007; Catusse et al., 2009). Indeed, at variance to other CCR5 chemokines, U83A is a CCR5 agonist that does not drive the receptor towards a clathrin-mediated endocytosis but to a delayed and long lasting caveolin-linked pathway. Combined to the fact that U83A is not recognized by DARC and D6, the viral chemokine thus facilitates clearance of all other CCR5 chemokines which can no longer activate the receptor but remain capable of being trapped by interceptors.

Relevant to the present article are the neutralizing molecules produced by viruses and multicellular pathogens that are used to neutralize the immune response of the host. Several articles and reviews describe the production by viruses, of soluble proteins able to bind chemokines sometimes simultaneously with cytokines such as interferon gamma, interleukine-1 β or tumor necrosis factor α (McFadden et al., 1998; Murphy, 2000; Alcami, 2003; Rosenkilde, 2005; Mantovani et al., 2006), all of which are implicated in the host immune response to pathogens. Two major mechanisms of action are depicted: the inhibition of cytokine–cytokine receptor interaction and the inhibition of cytokine–extracellular matrix interaction (McFadden et al., 1998), both of which being associated with improvement of cytokine clearance by elimination and/or degradation. The biologically active scavenging molecule can be a soluble protein, often mimicking the extracellular binding domain of the host cytokine receptor. It may also be a membrane-bound protein, like the decoy receptors mimicking the chemokine receptors which do not contain any soluble portions.

Representatives of soluble proteins that inhibit the interaction between chemokines and glycosaminoglycans (GAGs) from the extracellular matrix are M-T1 and M-T7 produced by the rabbit-infecting *myxoma* virus. M-T7 binds interferon gamma together with

chemokines from the CC-, CXC- and C-groups (Lalani et al., 1997). The herpesvirus homodimeric protein M3 and the glycoprotein G also belong to the group of soluble proteins inhibiting chemokine binding to GAGs (van Berkel et al., 2000; Martin et al., 2006). All exhibit original tridimensional structures that do not resemble chemokine receptors. The mechanisms by which they neutralize the immune system may be two-fold. On the one hand, GAGs are well known to contribute to the setting up and maintenance of chemokine gradients close to their sites of production. The inhibition of chemokines binding to GAGs might thus result in chemokine gradient collapses. Altered immune response that could derive from that could be attenuation of signalling intensity or unsuited, or even absence of, leukocyte targeting (Wells et al., 2006). On the other hand, the large size of soluble chemokine binding proteins could hinder the interaction with the chemokine receptor. Thus, although the targeted domain of the chemokine is the GAG-binding domain, the remainder of the large soluble protein might simultaneously prevent interactions with the cognate chemokine receptors. The use of small molecules mimicking the effects of GAG-binding proteins would help to determine the mechanism of action likely to take place.

Another example of soluble proteins produced by parasites is highlighted by recent research developments. Ticks are bloodsucking parasites that transmit the spirochete *Borrelia burgdorferi* responsible for Lyme disease (Hirschfeld et al., 1999; Hajnicka et al., 2001; Guerau-de-Arellano & Huber, 2005; Behera et al., 2006; Vancova et al., 2007; Deruaz et al., 2008). In order to survive, ticks attach and remain feeding on the host for several days–weeks. A particularity of the host–parasite interaction is the absence of an inflammatory response to ticks. This was investigated by several groups who realized that the parasite produces anti-haemostatic, anti-inflammatory and immunomodulatory substances, and secretes them in the host (Waxman et al., 1990; Valenzuela et al., 2000). Anti-chemokine molecules acting against CXCL8 (Hajnicka et al., 2001), CCL2, CCL3, CCL5 and CXCL11 (Vancova et al., 2007) were detected although their identity was not elucidated. Using an expression cloning strategy, the group of Proudfoot identified a family of small proteins, the evasins, that similar to soluble viral chemokine binding proteins, recognize and bind chemokines with various degrees of selectivity, and intercept their signalling to the host immune and anti-inflammatory systems (Frauensschuh et al., 2007; Deruaz et al., 2008). Three identified evasins bind CC (evasins-1 and -4) and CXC (evasin-3) chemokines. The fourth one, evasin-2, is still without a known ligand. The interest in these small proteins resides in their extreme efficacy to delude the immune system, and to their very small size (60–70 amino acids) that inspires searches for chemokine neutralizing motifs with the potential to become drugs.

The second general mode of action of pathogens is reminiscent of intercepting receptors described above. Human and mouse cytomegalovirus, Kaposi-associated herpesvirus and capripoxvirus produce seven transmembrane segment proteins (ORF74, US28, M33, and Q2/3L) which are analogous to G-protein-coupled receptors (Alcami, 2003; Rosenkilde, 2005). These proteins are expressed at the surface of infected cells and act, similarly to DARC or CCX-CKR, as decoy proteins that internalize chemokines and drive them towards degradation.

3. Potential therapeutic interest of soluble decoy proteins

Soon after the discovery of 50 different chemokines, the number of receptors grew to 20 members, all belonging to the G-protein-coupled receptor family for which it should be noted that one given chemokine may activate several receptor subtypes. The chemokine CXCL8 for instance activates two receptors (CXCR1 and CXCR2) and the chemokine CCL5 activates three receptors (CCR1, CCR3, and CCR5). On the other hand, a large number of chemokines may activate a single receptor subtype. This is the case for CXCR2, which is activated by CXCL-1, -2, -3, -5, -6, -7 and -8, for CCR5 that is activated by CCL-3, -4,

Table 1

Chemokine/chemokine receptor	Biological tool	Effect	Reference
CCL1 (I-309) CCR8	Anti-CCL1	Post-operational peritoneal adhesions	Hoshino et al., 2007
CCL2 (MCP-1) CCR2	Anti-CCL2	– Prostate cancer growth inhibition	Loberg et al., 2007; Li et al., 2009a
		– Infectious keratitis	Xue et al., 2007
		– Atherosclerosis	Lutgens et al., 2005
	CCR2 knock out	– Atherosclerosis/multiple sclerosis	Boring et al., 1998; Izikson et al., 2000
	CCL2 knock out	– Age-related macular degeneration/neuroinflammation	Belmadani et al., 2006; Ross et al., 2008
		– Sepsis	Lu et al., 1998
		– Atherosclerosis	Gu et al., 1998
CCL3 (MIP-1a) CCR1/CCR3/CCR5	Anti-CCL3	– Infectious keratitis	Xue et al., 2007
		– Fever	Soares et al., 2009
		– Sepsis	Takahashi et al., 2002
		– Inflammation in MS	Man et al., 2007
CCL4	CCL3 ^{-/-}	– Sepsis	Cook et al., 1995
	Anti-CCL4	– Lung inflammatory response	Bless et al., 2000
CCL5 (RANTES) CCR5/CCR1/CCR3	Anti-CCL5	– Autocrine proliferation of Hodgkin lymphoma cell lines	Boring et al., 1998; Izikson et al., 2000; Aldinucci et al., 2008; Levina et al., 2008
	CCL5 ^{-/-}	– Demyelination in MS	Glass et al., 2004
		– Glial activation	El-Hage et al., 2008
CCL6 (C10) CCR1	Anti-CCL6	– Lung inflammation and remodeling	Ma et al., 2004
		– Airway allergy and hyperresponsiveness	Hogaboam et al., 1999
		– Phagocytic activity of macrophages	Steinhauser et al., 2000
CCL7 (MCP-3) CCR2	Anti-CCL7	– Airway allergy and hypereosinophilia	Stafford et al., 1997
CCL8 (MCP-2) CCR2/CCR5			
CCL9 (MIP-1g) CCR1	Anti-CCL9	– Osteoclast differentiation	Yang et al., 2006
CCL11 (Eotaxin) CCR3	Anti-CCL11	– Airway allergy/asthma	Ding et al., 2004; Niimi et al., 2007
		– Bronchiolitis	Matthews et al., 2005
	Eotaxin ^{-/-}	– Acute inflammatory response	Rothenberg et al., 1997
CCL12 (MCP-5) CCR2/CCR5			
CCL13 (MCP-4) CCR2			
CCL14 (HCC-1) CCR1			
CCL15 (HCC-2) CCR1/CCR3			
CCL16 (HCC-4) CCR1/CCR3			
CCL17 (TARC) CCR4	Anti-TARC	– Hypereosinophilia/allergic asthma	de Lavareille et al., 2001; Schnyder-Candrian et al., 2006
		– Pulmonary infections/fibrosis	Belperio et al., 2004; Carpenter and Hogaboam, 2005
		– Lung cancer (?)	Qin et al., 2009
		– Hepatic failure	Yoneyama et al., 1998
		– Skin inflammation	Campbell et al., 1999
		– Rheumatoid arthritis	van der Voort et al., 2005
CCL18 (PARC) CCR3 (?)	Anti-CCL18		
CCL19 (ELC) CCR7			
CCL20 (MIP-3 alpha) CCR6	Anti-CCL20/anti-CCR6	– Multiple myeloma	Giuliani et al., 2008
	Anti-CCL20	– HPV infection/Langerhans cells migration	Caberg et al., 2009
		– Brain inflammation (MS/EAE)	Ambrosini et al., 2003
CCL21 (SLC) CCR7	Anti-CCL21	– Kidney fibrosis	Sakai et al., 2006; Wada et al., 2007
		– Corneal immunity	Jin et al., 2007b
		– Thymus development	Liu et al., 2005
CCL22 (MDC) CCR4	CCL21 ^{-/-} mice	– Leukemia cell survival and proliferation	Ghia et al., 2002
	Anti-CCL22	– Eosinophil activation in lung inflammation	Pinho et al., 2003
		– Lung cancer	Qin et al., 2009
		– Vascular endothelial cell migration	Son et al., 2006
CCL23 (MIPF-1) CCR3		– HIV pathogenicity	Fiorucci et al., 2007
CCL24 (Eotaxin-2) CCR3		– Intestinal immunity	Feng et al., 2006; Hieshima et al., 2008
CCL25 (TECK) CCR9	Anti-CCL25		Cuvelier and Patel, 2001
CCL26 (Eotaxin-3) CCR3			Morales et al., 1999; Reiss et al., 2001; Chen et al., 2006
CCL27 (CTACK) CCR10	Anti-CCL27	Dermatitis/skin disease	Feng et al., 2006; Hieshima et al., 2008
CCL28 (MEC) CCR10	Anti-CCL28	– Intestine and colon immunity	Grespan et al., 2008; Lemos et al., 2009
CXCL1 (Gro alpha) CXCR2	Anti-CXCL1	– Arthritis	Brown et al., 2007
		– Kidney sepsis	Issa et al., 2006
		– Airway inflammation	Brown et al., 2007
CXCL2 (Gro-beta) CXCR2	Anti-CXCL2	– Kidney sepsis	
CXCL3 (Gro gamma) CXCR2			
CXCL4 (PF4) CXCR3b			
CXCL5 (ENA-78) CXCR2	Anti-CXCL5	– Arthritis	Grespan et al., 2008; Smith et al., 2008; Lemos et al., 2009
		– Diabetes	Chavey et al., 2009
		– NSCLC growth/angiogenesis	Pold et al., 2004
CXCL6 (GCP-2)	Anti-GCP-2	– Growth SCLC	Zhu et al., 2006
		– Arthritis	Kelchtermans et al., 2007
CXCL7 (NAP-2)	Anti-NAP-2	– Thrombosis	Amiral et al., 1996; Piccardoni et al., 1996
CXCL8 (IL8) CXCR1/CXCR2	Anti-CXCL8	– Inhibition of NSCLC growth/angiogenesis	Pold et al., 2004
	Anti-CXCR1	– Inhibition of NSCLC proliferation	Zhu et al., 2004
	Anti-CXCL8	– Clearance of apoptotic cells	Iyoda et al., 2005
CXCL9 (Mig) CXCR3	Anti-CXCL9	– Brain immunity and MS	Liu et al., 2001a; Salmaggi et al., 2002
		– Transplant rejection	Belperio et al., 2003; Whiting et al., 2004; Colvin et al., 2005

Table 1 (continued)

Chemokine/chemokine receptor	Biological tool	Effect	Reference
CXCL10 (IP-10) CXCR3	Anti-CXCL10	– Axon sprouting and vasculature remodelling following injury – Inflammatory demyelination in MS – Coronavirus-induced neurological and liver damage – Transplant rejection	Glaser et al., 2004; Glaser et al., 2006 Liu et al., 2001; Narumi et al., 2002 Walsh et al., 2007 Belperio et al., 2002
CXCL11 (I-TAC) CXCR3	Anti-CXCL11	– Brain immunity	Rupprecht et al., 2005
CXCL12 (SDF-1alpha) CXCR4	Anti-CXCL12	– Autoimmune disease/lupus erythematosus – Metastases/tumor proliferation – Pulmonary hypertension/airway inflammation	Matin et al., 2002; Balabanian et al., 2003; Wang et al., 2009 Muller et al., 2001; Cardones et al., 2003; Orimo et al., 2005; Phillips et al., 2003; Pan et al., 2006; Otsuka and Bebb, 2008 Gonzalo et al., 2000; Hachet-Haas et al., 2008; Lukacs et al., 2002; Young et al., 2009 Bertolini et al., 2002; Hinton et al., 2008; Li et al., 2009b
	Anti-CXCR4	– Tumor invasion – NSCLC proliferation – Airway inflammation	
	CXCL12 ^{-/-}	– Development	Otsuka and Bebb, 2008 Nagasawa et al., 1996
CXCL13 (BCA-1) CXCR5	Anti-CXCL13	– Autoimmunity/myasthenia gravis – Arthritis – Graft rejection	Meraouna et al., 2006 Zheng et al., 2005 Lee et al., 2006
CXCL14 (BRAK, BMAC) CXCL15 (Lungkine) CXCL16 CXCR6	CXCL15 knock out Anti-CXCL16	– Sepsis – Kidney inflammation – Sepsis – Arthritis – Graft tolerance	Chen et al., 2001 Yang et al., 2008 Shimaoka et al., 2003; Xu et al., 2005
	CXCL16 ^{-/-}	– Atherosclerosis	Nanki et al., 2005 Jiang et al., 2005
CX3CL1 (fractalkine) CX3CR1	Anti-CX3CL1	– Graft tolerance – Autoimmune disease – Atherosclerosis	Aslanian and Charo, 2006 Ueha et al., 2007 Suzuki et al., 2005
	CX3CL1 knock out	– No phenotype	Schulz et al., 2007 Cook et al., 2001
XCL1 (lymphotactin) XCR1	Overexpression	– Cancer immunotherapy – Anti-infection immunotherapy	Wang et al., 2002 Yue et al., 2009

NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer; MS: multiple sclerosis; EAE: experimental autoimmune encephalomyelitis.

-5, -6, -8, -12, as well as for many other receptors (CXCR3, CCR1, CCR2, CCR3 ...) (reviewed in Wells et al., 2006). The question then arose as to which receptor and which chemokine should be targeted to decipher physiological signalling pathways and predict therapeutic approaches for disease treatment. Many research groups could help solve this problem by showing that despite chemokines and cytokines' cooperation to increase inflammatory responses, knock out or neutralization of one chemokine or chemokine receptor will induce significant attenuation of inflammation (see Table 1). Several chemokine gene disruptions result in a clear effect, as for instance knock out of CCL3 that reduces the inflammatory response to viruses such as influenza A and cytomegalovirus (Salazar-Mather et al., 1998). Similarly, the knock-out approach indicates the importance of CCL2 and its receptor in chemoattraction of neural progenitors to inflamed neural sites (Belmadani et al., 2006), that of CCL5 in glial cell activation (El-Hage et al., 2008), of CCL21 in thymus development (Liu et al., 2005) or that of CXCL12 in haematopoiesis (Nagasawa et al., 1996). Very convincing results are also obtained by using proteins or antibodies neutralizing the chemokine ligand. The Lucas and Mc Fadden groups have exploited the neutralizing effect of the *myxoma* virus M-T7 soluble protein to reduce post-operative responses in murine models of tissue engrafting (Liu et al., 2000, 2004; Bedard et al., 2003). They show that intravenous injection of M-T7 protein, that binds all types of chemokines (see Section 2.2) in rats after angioplasty-induced injury diminishes atherosclerosis and restenosis (Liu et al., 2000). This is in good agreement with the phenotype of CCL2^{-/-} mice (Gosling et al., 1999), and with the reported prevention of renal allograft rejection (Bedard et al., 2003) or reduction of aortic allograft vasculopathy through inhibition of chemokine-mediated responses (Liu et al., 2004).

Finally, the newly identified evasin proteins from ticks also display potent anti-inflammatory properties in vivo in animal models (Deruaz et al., 2008). Evasin-1, which binds CCL3 and CCL4, significantly attenuates recruitment of pro-inflammatory cells in phorbol ester-inflamed skin of D6^{-/-} mice and fibrosis in bleomycin-induced lung

injury. Evasin-3 recognizes CXCL8 and its mouse homolog KC, as well as CXCL11. It inhibits neutrophil chemotaxis in vitro, as well as neutrophil recruitment to the peritoneal cavity in mice in a model of BSA-induced arthritis.

4. Validation of chemokines in signalling pathways and pathology: importance of anti-chemokine antibodies

Chemokines are directly implicated in many physiological processes including surveillance of organism integrity, elimination of damaged cells and tissues or host defence against pathogens. To this end, they recruit the most adapted cell types on the site where intervention is needed, and promote a controlled reaction generally associated with limited inflammation. Under abnormal conditions, the inflammatory response escapes control, thus leading to pathological states such as inflammatory bowel disease, multiple sclerosis, and probably Alzheimer's disease, among others. In this case, abnormally elevated levels of chemokines, or overexpression of their receptors, lead to permanent recruitment of immune cells and to tissue damage. In a comparable manner, abnormal recognition of antigen initiates autoimmune diseases (myasthenia gravis, lupus erythematosus, Type I diabetes, rheumatoid arthritis...), where abnormally elevated levels of chemokines are detected (Matin et al., 2002; Kong et al., 2009; Wang et al., 2009). The cytokine and chemokine systems are also used by cancer cells to promote cell proliferation, tumor survival and neovascularization, or to establish metastases at distant but non-random places (Vandercappellen et al., 2008). Chemokines also contribute to tissue development (Nagasawa et al., 1996; Mahabaleshwar et al., 2008; Raz & Mahabaleshwar, 2009) by forming gradients of morphogens for migrating cells.

Table 1 summarizes all efforts made to investigate the role of the various chemokines, using approaches targeting the chemokine as directly as possible. The two major approaches, namely gene disruption and anti-chemokine antibodies, do generally lead to

convergent observations, although the same phenomenon has only rarely been studied using the two approaches.

Deletion of one of the CCL3 and CCL5 receptors, the CCR5 receptor (CCR5 Δ 32 allele found in humans) is associated with protection from HIV infection in humans (Samson et al., 1996; Kindberg et al., 2008; Lim et al., 2008) while deletion of a second receptor, the CCR1 receptor, results in protection from an excessive response to systemic inflammation in mouse models (Gerard et al., 1997). Deletion of the CCR1 and CCR5 ligand, CCL3 (MIP-1 α), results in weaker inflammatory responses to viral pathogens (Cook et al., 1995), and deletion of CCL5 (RANTES) to a reduced glial cell inflammatory response (El-Hage et al., 2008). Therefore, the absence of perfect matching between chemokines and their receptors is a cause of difficulties encountered when signalling pathways are to be traced, and molecules targeting the function of one receptor do not systematically match the effects of molecules targeting the ligand (Horuk, 2009).

Rather than knocking out chemokine or chemokine receptor genes, neutralizing antibodies, which are rapidly obtained, have been very useful in particular for chemokines. Chemokines are small proteins with a highly stable structure (Fig. 3), which renders them amenable to the development of neutralizing antibodies (Table 1).

In the case of CCL5 for instance, neutralizing antibodies allow the functional role of this chemokine in autocrine proliferation of leukemia cells (Boring et al., 1998; Izikson et al., 2000; Aldinucci et al., 2008) to be demonstrated as well as neuroinflammation in models of multiple sclerosis (Glass et al., 2004). It is interesting to note that although CCL5 binds to the same subset of chemokine receptors as CCL3, the anti-CCL5 neutralizing effect is specific because CCL3 is expressed in other cell types.

Anti-CCL1 antibodies have been used to demonstrate the contribution of CCL1/CCR8 autocrine activation of peritoneal macrophages in the formation of peritoneal adhesion, which constitutes complications in visceral surgery and inflammation (Hoshino et al., 2007).

Also antibodies to CXCL8, CCL2 or CCL5 block the antiapoptotic and proliferative effects of the corresponding tumor-derived cell lines obtained from lung, melanoma, breast ovarian or leukemia cancers (Levina et al., 2008).

Besides their functions in the immune system, the role of chemokines in cancer initiation and progression as well as in tumor

survival has been confirmed with neutralizing antibody strategies. Hence, antibodies against chemokines or chemokine receptors block tumor growth and/or migration as well as invasiveness. Various examples may be given, like i) anti-CXCL12/CXCR4 antibodies in ovarian and breast cancer (Muller et al., 2001; Scotton et al., 2002; Kwong et al., 2009), ii) anti-CXCL1 and anti-CXCL2/CXCR2 in lung cancer (Wang et al., 2006a), iii) anti-CXCL13/CXCR5 in cell lines from pancreatic or colon cancers (Meijer et al., 2006), iv) anti-CCL2/CCR2 and -CCL5/CCR5 (Vaday et al., 2006), and -CCL11/CCR2 in ovarian cancer (Levina et al., 2009), and v) anti-CCL21/CCR7 in thyroid tumor cells (Sancho et al., 2006).

5. Neutralizing cytokines and chemokines with small chemical compounds

The study of protein–protein interactions is important to understand major regulatory pathways, especially in the intracellular compartment, which is not reached by neutralizing antibodies. The difficulty associated with the study of protein–protein interactions is not only that most of the time, interacting partners are both intracellular, and thus not easily accessible for biophysical or pharmacological manipulations, but also that protein–protein interactions generally involve contact areas that are much larger than small molecules. These contact areas, in addition, are quite featureless in terms of the number of attachment points that can be exploited by medicinal chemists to develop small molecules with high affinity. It follows that small molecules at most bind with modest affinities and frequently hardly compete efficiently to inhibit the interaction between two proteins. Still, favourable cases exist in which neutralization of a protein function can be obtained with a small molecule (Arkin & Wells, 2004; Arkin, 2005; Arkin & Moasser, 2008; Blazer & Neubig, 2009). When the approach works, questions related to the mode of action of the small molecule must be addressed, in order to generalize the principles and extend the approach to other specific cases. The different mechanisms of ligand neutralization are numerous and diverse. In terms of chemical biology, the aim thus being to develop small chemical molecules blocking the function of the protein ligand, we shall not discuss molecules that inhibit synthesis, maturation or release of the protein ligand, nor molecules that modulate its catabolism, already reviewed elsewhere (Foxwell et al., 2003; Vergote et al., 2006; Mortier et al., 2008). Rather we will focus on small organic molecules that bind to the protein ligand and prevent its signalling.

Four main modes of action are encountered (Fig. 4):

- i) The small molecule competitively binds to the same site as the receptor;
- ii) The small molecule alters the quaternary structure of the protein ligand;
- iii) The protein ligand undergoes structural changes that regulate its activity: the small molecule alters tertiary structure of the protein ligand;
- iv) The small molecule interferes with ligand bioavailability.

These different modes of chemokine/cytokine neutralization will now be illustrated, and the methods to identify them discussed. These mechanisms of action have been validated, (Berg, 2003; Arkin & Wells, 2004; Arkin, 2005; Blazer & Neubig, 2009) and may be extended to chemokine neutralization.

5.1. The small molecule competitively binds to the same site as the receptor

Based upon structure–function relationship studies, many examples of peptides mimicking receptor domains and acting as inhibitors are available. In the family of chemokines and their receptors, the importance of the extracellular parts of the chemokine receptor (Zoffmann et al., 2002; Duma et al., 2007), and in particular of its amino-terminal domain, for ligand–receptor interactions has been extensively documented (Blanpain et al., 1999; Gayle et al., 1993;

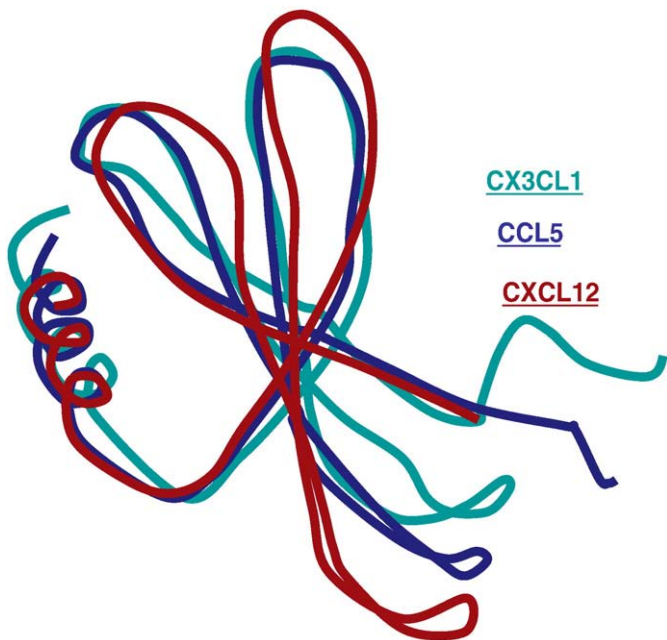


Fig. 3. Superimposition of peptide backbones from CC, CXC and CX3C chemokine groups shows that they have a canonical three dimensional structure.

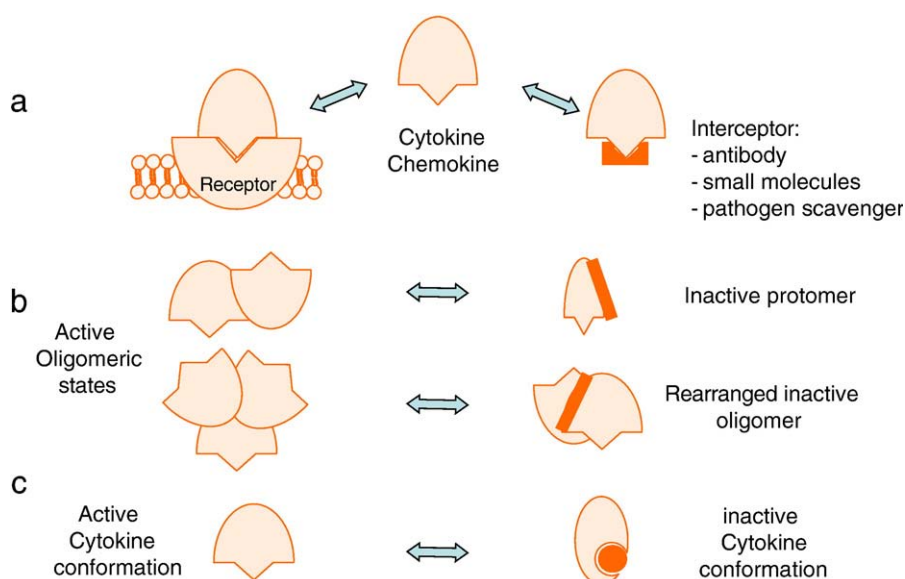


Fig. 4. Illustration of the different modes of action of neutralizing molecules. **a)** The small molecule competitively binds to the same site as the receptor. **b)** The small chemical molecule alters the quaternary structure of the protein ligand. **c)** The protein ligand undergoes structural changes that regulate its activity: the small molecule alters tertiary structure of the protein ligand.

Monteclaro & Charo, 1996, 1997; Pease et al., 1998; Ye et al., 2000; Bannert et al., 2001; Fong et al., 2002; Rajagopalan & Rajarathnam, 2004; Prado et al., 2007; Veldkamp et al., 2008). This has led to the identification of peptide fragments capable of binding to the chemokine in a manner thought to mimic the mode of interaction of the chemokine with the receptor. Peptide fragments corresponding to the 1–35 N-terminal residues from CCR3 (Mayer & Stone, 2000; Ye et al., 2000), 1–40 from CXCR1 (Clubb et al., 1994) or 2–19 from CX3CR1 (Mizoue et al., 1999; Kokkoli et al., 2005) interact with CCL24, CXCL8 and CX3CL1, respectively, with millimolar to micromolar affinities. In all three examples, the interaction area of the chemokine is located in the helical turn of the N-loop and the $\beta 1$ – $\beta 2$ and $\beta 2$ – $\beta 3$ hairpin domains, in regions of greatest flexibility and structural variability of the chemokine (Mizoue et al., 1999). These peptides have not been further used to investigate the *in vitro* or *in vivo* functions of chemokines. Their discovery however suggested that neutralizing antibodies-based approaches (Table 1) are not the only possible tools with which to inhibit chemokine functions, and paved the way to find high affinity peptides capable of neutralizing chemokines. One approach starts from natural peptides targeting chemokines as illustrated by the work on evasins (Deruaz et al., 2008) described above. Although peptidic in nature, evasins are not immunogenic, at least when secreted by feeding ticks. They are thus expected to represent valuable scaffolds to study, to make analogs and to use as non-peptidic drugs. Another approach consists in identifying new chemokine binding molecules from collections of peptides and peptidomimetics (Burger & Peled, 2009). Such peptides or peptidomimetics may either exhibit selectivity towards a single chemokine or, in contrast, poorly discriminate among chemokines such as CXCL9, CCL2, CXCL8, CXCL12 or CCL11 (Peled, A., Eizenberg, O. Vaizel-Ohayon, D. US patent 7488717). The anti-CXCL12 peptide, BKT 140, identified by surface plasmon resonance and ELISA, is currently in clinical phase I for neutropenia and anemia.

Chemokine neutralizing ligands may also not be peptidic at all. A screening campaign of an academic library of small molecules (Boeglin et al., 2007; Hibert, 2009) was designed, using a fluorescence resonance energy transfer assay (Vollmer et al., 1999; Valenzuela-Fernandez et al., 2001) in order to identify inhibitors of CXCL12–CXCR4 interactions. A chalcone molecule (4'-phenyl, 3-methoxy, 4-hydroxy chalcone) was found to be very effective ($K_i = 50$ nM) at inhibiting CXCL12 binding to CXCR4 and CXCR7, and signalling through CXCR4, including chemotaxis

in vitro and *in vivo* (Hachet-Haas et al., 2008). The molecule however was unable to block cell fusion in an *in vitro* model (Chanel et al., 2002) of HIV entry. The proposed model of chalcone binding to the chemokine rather than to its receptor could be demonstrated using tryptophan fluorescence and microcalorimetry. This was reminiscent of earlier studies describing a natural derivative of chalcones, the flavone baicalin, isolated from *Scutellaria baicalensis*, which binds to the chemokines CXCL8, CXCL12, CCL4 and CCL8, although affinities were about four orders of magnitude lower (Li et al., 2000). The compound 4'-phenyl, 3-methoxy, 4-hydroxy chalcone, modestly inhibits signalling through CXCL8, a chemokine from the same structural subgroup as CXCL12, but is not active on CCL5, at least not in the micromolar concentration range. Finally, the chalcone compound shows efficacy, *in vivo*, in a mouse model of allergic hypereosinophilic airway inflammation where it is as powerful as neutralizing antibodies to either CXCL12 or CXCR4 (Hachet-Haas et al., 2008). Although the structure of the chemokine–chalcone has not been solved, molecular modeling and preliminary NMR data (C. Veldkamp, Milwaukee University, personal communication) support the idea that the chalcone binds to the same chemokine area as do chemokine receptor-derived peptides, i.e. in the groove delineated by the N-loop hairpin and β -strands 2 and 3 of the chemokine (Fig. 5). Yet, whether chalcone perturbs the state of chemokine oligomerization remains an open question. Other chalcone molecules are reported to act as inhibitors of allergic inflammatory diseases (Meng et al., 2007). The question as to whether the chalcone backbone acts as a chemical platform for biologically active molecules is open, and the mechanism of action of the molecules *in vivo* might rely on multiple interactions with different target proteins.

The example of the IL-2 neutralizing small molecules, SP4206 and SP4160, illustrates that flexible regions of small protein ligands can be targeted by high affinity molecules binding at protein–protein interfaces that are poorly druggable, but can adjust their structure to accommodate the ligand (Arkin & Wells, 2004; Thanos et al., 2006). The IL-2 receptor mediates T-helper cell maturation and is a drug target for transplant rejection (Waldmann & O'Shea, 1998; Berard et al., 1999) and autoimmune diseases (Schippeling & Martin, 2008). Its ligand, interleukin-2 is a 15 kDa four helix bundle protein that promotes T cell growth. In a drug design program aiming at mimicking the IL-2 part that binds to the IL-2R, a peptidomimetic molecule, Ro26-4550, was discovered (Tilley et al., 1997). The molecule could inhibit IL-2 binding to its receptor with micromolar affinity, and careful

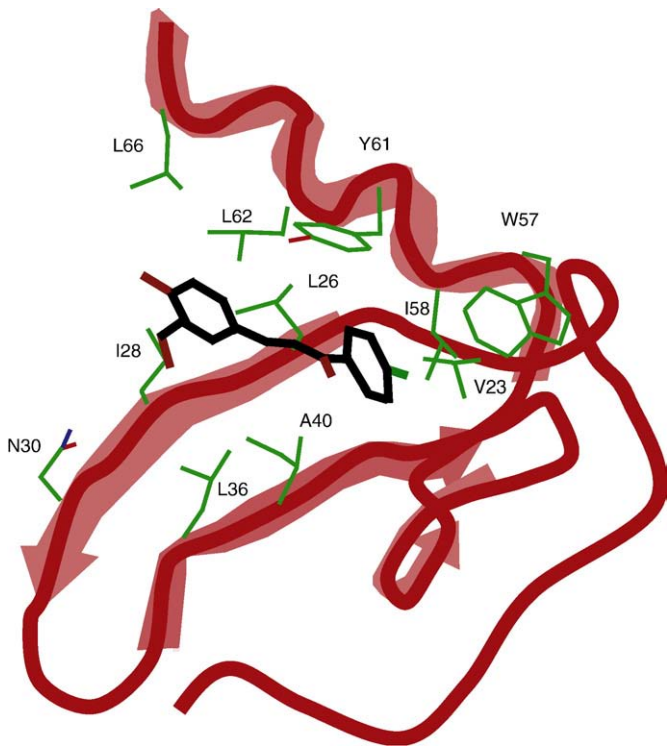


Fig. 5. Proposed model for the interaction between CXCL12 and neutralizing chalcone molecule 4. Redrawn from Hachet-Haas et al. (2008).

characterization of the mode of action led to the identification of the interleukin-2 itself being the receptor for Ro26-4550, not the IL-2R receptor protein (Emerson et al., 2003). Detailed structural analysis of Ro26-4550 interaction with IL-2, in particular using X-ray crystallography and NMR (Arkin et al., 2003) described in detail the binding area. The small chemical compound binds to a pocket that does not pre-exist as such in its absence. In other words, the binding area is the complex result of the adaptation of the local protein folds around the chemical. This particular case illustrates the difficulty to use the structure of the “empty” binding site, a priori, to predict Ro26-4550 structure or binding. However, optimization of the compounds, using X-ray crystallography of complexes, fragment-based approaches and tethering techniques, allowed the affinity of the IL-2 binding molecules to be lower to a few tens of nanomolar (Raimundo et al., 2004; Thanos et al., 2006).

Chemokines are present in the brain in glial cells as well as in neurons (Rostene et al., 2007). Concordant with their known role in the periphery, chemokines are up regulated during inflammation, and contribute to brain immunity and neuroprotection (Cartier et al., 2005; Glass et al., 2005; Madrigal et al., 2009; Omari et al., 2009). They are also involved in progenitor cell migration towards brain tumors (Magge et al., 2009), and participate in brain development and neuronal cell differentiation (Zou et al., 1998; Park et al., 2009). They are even distributed in neurons as neuromodulators are, and modify release of neurotransmitters or neuropeptides (Rostene et al., 2007). These findings, together with the development of pharmacological tools for chemokine receptors offers the possibility to better study chemokine signalling in the brain. Not only agonists or antagonists of the receptors, but also small neutralizing molecules directed against chemokines could be extremely valuable, in particular if they cross the blood brain barrier (BBB) to reach their target cytokines. One example is the conversion of the chemokine CXCL12, an agonist of CXCR4 and ligand of CXCR7, into an antagonist of CXCR3 (Vergote et al., 2006). The chalcone molecule mentioned above is probably a good pharmacological agent to study neuronal toxicity associated with CXCL12 catabolism.

Another application for potential neutralizing ligands is exemplified by the effect of anti-CCL5 antibodies. They are administered to fight the leukocyte infiltration that takes place in the brain as a result of virus-driven CCL5 production in mice. This infiltration is followed by destruction of myelin and appearance of neurological impairment (Glass et al., 2004). Antibodies to CCL5 significantly decrease macrophage accumulation in the brain and demyelination. CCL5 neutraligands should thus prove useful to investigate the incidence of CCL5 in multiple sclerosis.

5.2. The small molecule alters quaternary structure of the protein ligand

Chemokines show a natural propensity to form dimers or oligomers depending on medium composition (Veldkamp et al., 2005), biochemical environment in particular glycosaminoglycans (Crown et al., 2006), or interaction with chemokine receptors (Veldkamp et al., 2008). Homodimerization/oligomerization is well established for CC chemokines such as CCL2, CCL4, CCL5 and CCL14 (Blain et al., 2007; Jin et al., 2007a; Proudfoot et al., 2003), and heterodimerization is also experimentally supported for instance in CCL2–CCL13, CCL2–CCL11 or CCL8–CCL13 heterodimers (Crown et al., 2006). Up to now, the functional consequences of these homologous or heterologous interactions have not been well understood, but the oligomerization process may be interesting to interfere with when attempting to neutralize chemokine actions (Fig. 4). Indeed, CC chemokine dimers do not correspond to the quaternary structure that interacts with the receptor, because residues critical for receptor binding are buried in the dimer (Jin et al., 2007a), and mutations can be made to abolish dimerization (Proudfoot et al., 2003; Jin et al., 2007a). In the case of CCL2, it could be demonstrated that certain responses, in particular leukocyte attraction *in vivo*, were abolished. This selective suppression of certain responses which leaves other responses unaffected is reminiscent of previously described multiple active states of G-protein-coupled receptors (Palanche et al., 2001) that can be differentially activated by distinct agonists, or modulated by allosteric effectors (Maillet et al., 2007). If prevention of dimer formation is per se sufficient to block chemoattraction *in vivo*, then the mechanism of action of the interceptor M3 (from herpesvirus) is probably exquisitely optimized. Decoy receptor M3 (vCKBP3) binds two monomers of CCL2 per M3 homodimer (Alexander et al., 2002). Thus, even though M3 recognizes and binds the GAG-binding domain of CCL2, its aptitude to “dissolve” dimers is thus likely to represent a mechanism of inhibition of CCL2 signalling *in vivo* (Handel et al., 2008).

In the case of CXC chemokines, dimerization also takes place spontaneously, but the dimer structure differs from that of CC chemokine dimers in that residues important for receptor interaction are not buried in the dimer. CXC chemokines can thus interact with their receptors either as monomers or as dimers/oligomers. At variance with what happens when CC chemokines interact with their receptors, it was demonstrated that a CXC chemokine receptor may itself be responsible for chemokine dimerization (Veldkamp et al., 2008). Tyrosine sulfation of the N-terminal domain of CXCR4 on tyrosines 7 and 12 is indeed a key determinant of receptor-mediated dimer formation since sulfated tyrosine 7 interacts with a CXCL12 monomer, while sulfated tyrosine 12 interacts with the second monomer. Two lines of evidence support the importance of CXCL12 dimerization in signalling: i) amino acids from CXCL12 are involved both in the interaction with heparan sulfates and with CXCR4; heparan sulfates can thus negatively affect CXCL12-evoked chemotaxis *in vitro* (Murphy et al., 2007), and ii) tethering CXCL12 monomers to obtain permanent CXCL12 dimers results in a partial loss of function of CXCL12. The permanent dimer indeed shows unaltered capacity to promote intracellular calcium elevation in CXCR4 expressing cells, but is no longer capable of triggering *in vitro* chemotaxis (Veldkamp et al., 2008). Although these results are not yet totally interpreted in terms of structure–function relationships, modulation of chemokine

oligomerization represents a promising way to change cellular responses with possible important consequences *in vivo*. The unsaturated heparin disaccharide used to perturb CXCL12 dimer structure (Murphy et al., 2007) is a plausible starting chemical platform to exploit. Virtual and experimental screening of collections of molecules on chemokine dimers could lead to the discovery of neutralizing molecules, the mechanism of action of which would be prevention of dimer formation. The critical step in this kind of project would be the definition of primary and secondary assays allowing qualitative and quantitative description of the new compound effects.

TNF α is produced in response to pathogens through toll-like receptor activation, and promotes expression of many immune system effectors, including cytokines and chemokines (Balkwill, 2009) that will recruit leukocytes to the site of inflammation. If production of TNF α is excessive, chronic inflammation can develop as in rheumatoid arthritis, Crohn's disease, severe asthma or psoriasis, all diseases in which a prominent role of TNF α has been demonstrated. Anti-inflammatory therapies have been developed, based on inhibition of either the production of TNF α or the neutralization of TNF α itself (Foxwell et al., 2003). The neutralizing monoclonal antibodies, etanercept, infliximab and adalimumab have led to successful treatment in rheumatoid arthritis, and show that neutralizing antibodies can prove valuable not only in acute, but also in chronic human diseases. Yet, two problems remain after several runs of antibody optimization for human use: the mode of administration associated with dosing difficulties, and the elevated cost of treatment have motivated the search for alternative therapeutic tools. In the search for small molecules from collections of combinatorial fragments capable of inhibiting TNF α binding to its TNF-R1 receptor, He et al. (2005) discovered a small molecule inhibitor exhibiting a micromolar affinity constant. When trying to identify by X-ray crystallography the binding site of the small molecule on the large trimeric structure of TNF α , the authors realized that soaking TNF α crystals led to their destruction. Crystallization of the complex was then obtained. It revealed that the mode of action of the small molecule antagonist of TNF α is to dissociate its trimeric quaternary structure into inactive inhibitor-bound dimers with a stoichiometry of one molecule per dimer (He et al., 2005; Berg, 2006). This illustrates one of the modes of action of small molecule inhibitors that, following description of CCL2 monomer failure to signal (Handel et al., 2008), could be applied to the family of chemokine proteins, using detection of quaternary structure as a primary screen.

5.3. The protein ligand undergoes tertiary structural changes that regulate its activity and the small molecule prevents the active conformer

Soluble proteins may undergo three dimensional structural changes, either spontaneously, in a regulated manner (Monod et al., 1965) or as a mechanism of pathogenesis (Dobson, 1999). Lymphotactin is a chemokine with unusual properties. First, in contrast to most chemokines its structure is stabilized by a single disulfide bridge. Second, it has the singular property of existing as two unrelated protein folds (Kuloglu et al., 2002; Tuinstra et al., 2008). One of the two protein folds resembles the canonical chemokine structure with 3 anti-parallel β -strands and one carboxy terminal helix. This conformation binds to and activates its receptor XCR1 but does not interact with glycosaminoglycans. The second protein fold exhibits 4 anti-parallel β -strands but has no helix (Tuinstra et al., 2008). This second structure binds glycosaminoglycans with high affinity but is unable to activate the XCR1 receptor. As this example is unique to date in the field of proteins and protein ligands, its generality is questionable. It is an extreme case of the general field of change in protein conformational equilibrium, which has been tackled by many different laboratories on many different regulatory proteins to select new pharmacological tools and active drugs. Ligands that would stabilize the glycosaminoglycan binding state could reveal neutralizing molecules capable

of modulating acute allograft rejection response (Wang et al., 1998) or attenuate inflammatory bowel disease (Boismenu et al., 1996; Middel et al., 2001), as two examples of mucosal immunity in which lymphotactin is involved.

5.4. The small molecule interferes with ligand bioavailability

Chemokines activate G-protein-coupled receptors to recruit leukocytes during organogenesis, immunosurveillance, and inflammation. An important component of this process is the formation of a chemotactic gradient by immobilization of chemokines on the extracellular matrix of cells, in particular on glycosaminoglycans. Analysis of the role played by glycosaminoglycans has been carried out using mutants of CC (Proudfoot et al., 2003) or CXC/XCL (Peterson et al., 2004; Sadir et al., 2004; Johnson et al., 2004; Ali et al., 2005; Jin et al., 2007a) chemokines devoid of key residues known to bind to negatively charged sugar moieties. Suppression of GAG interactions for CCL2, CCL4, CCL5, CCL7 or XCL1 chemokines was found to abolish leukocyte recruitment *in vivo*, when injected intraperitoneally, although *in vitro* chemotaxis was not altered.

The importance of these interactions has been further highlighted by showing that GAG-binding mutants of chemokines can block the action of wild type chemokines in normal animals as well as in murine models of diseases (Johnson et al., 2004; Ali et al., 2005; Braunersreuther et al., 2008). Pathogens also target GAG-binding domains of chemokines to prevent their effects. As mentioned above (Section 2.1, avoidance strategies), the poorly selective M-T1 and M-T7 proteins from *myxoma* virus, the M3 protein from herpesvirus or glycoprotein E163 from *ectromelia* virus neutralizes chemokines upon binding to their GAG-binding domain. The likely consequence of this is that high local concentrations of chemokines giving rise to a "Velcro effect" are reduced, and gradients are disrupted. Therefore, leukocyte attraction no longer takes place. Although no small chemical molecule targeting the GAG-binding domain of chemokines has been described up to now, this portion of the protein is validated for chemical biology approaches. Noteworthy are the disaccharides that were used to solve the structure of CCL5 forming complexes with heparin-derived sugars (Shaw et al., 2004) that could be used as starting blocks for drug design. Interestingly too is the small molecule surfen, that was first used as an excipient in drug formulas before being also identified as a heparin neutralizing molecule (Hunter & Hill, 1961) with antibacterial and trypanocidal activity, which could be used to lower excessive heparan sulfate-involving interactions (Schuksz et al., 2008).

6. Concluding remarks and perspectives

Many articles now report that either genetic manipulations of receptors and chemokines, or development of pharmacological agents leads to selective alterations of a subset of responses out of a series of possible responses. One consequence is that, depending on the desired properties of the molecule to be developed for research or disease treatment, preference for a ligand of receptor or a ligand of ligand will need to be validated experimentally. Indeed, chemokines activate members of the family of G-protein-coupled receptors (GPCRs) known to exhibit a significant level (around 10%) of spontaneous isomerization towards active conformations (Lefkowitz et al., 1993a,b; Leurs et al., 2000; Palanche et al., 2001; Alewijns et al., 2000; Claeysen et al., 2000; Lecat et al., 2002). Spontaneous activity of GPCRs has well established physiological roles (Adan, 2006; Arrang et al., 2007) like control of neurotransmitter (Threlfell et al., 2008) and hormone (Ben-Shlomo et al., 2009) release in relation with higher order behaviors (Fioravanti et al., 2008), or regulation of apoptosis (Lau et al., 2009). Spontaneous activity of chemokine receptors also exists. For herpesvirus-encoded receptors this activity is linked to transforming effects (Burger et al., 1999; Holst et al., 2001). Ligands of GPCRs almost never behave as neutral molecules, and most antagonists are either weak partial agonists

(that increase the level of receptor activity) or are so called “inverse agonists” capable of diminishing the level of spontaneous activity of the receptor. Small molecules neutralizing ligands may complement the tool palette of pharmacologists since they are expected to leave the spontaneous activity of the receptors unchanged.

There are four preferred ways to identify small molecules modulating protein–protein interactions, ELISA assays, fluorescence resonance energy transfer (FRET) assays, fluorescence anisotropy (Berg, 2003) and surface plasmon resonance. We have designed a general strategy in order to find fluorescent probes that bind to a soluble protein. This fluorescence anisotropy strategy involves four steps:

- 1) Synthesis of a library of fluorescent compounds using known chemical scaffolds that exhibit low specificity. The molecules are organized around generic GPCR-preferring chemical scaffolds, are derivatized with charged, hydrophilic and hydrophobic moieties and bear a lissamine fluorophore at the end of a spacing arm,
- 2) Screening of the library of fluorescent molecules by fluorescence anisotropy measurements in order to successfully fish one fluorescent probe at least. This technique allows to set up a “mix and read” assay that readily pinpoints the probe that interacts with the protein,
- 3) Characterization of the binding properties of the probe in order to fulfil the desired requirements, i.e. neutralize binding of the ligand to its receptor, or inhibit oligomerization, or inhibit binding to GAGs, and select the desired and optimized screening assay,
- 4) Screening of libraries of unlabelled and drug-like molecules to identify high affinity molecules to use for chemical biology purposes.

The generalization of such kinds of drug discovery approaches in particular in university laboratories, is highly desirable. The increasing amount of screening data will then lead to large scale chemoinformatics and bioinformatics including data from transcriptional analyses and proteomics (Schadt et al., 2009; Weill & Rognan, 2009), development of complex network modelling as is the case in systems biology in order to establish training sets for interaction network prediction, and to use these models to predict molecule toxicity and metabolism, bioavailability and patterns of biological activity.

Acknowledgments

The authors wish to thank the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Université de Strasbourg, the Agence Nationale de la Recherche sur le SIDA (ANRS), Sidaction, and the Agence Nationale de la Recherche (ANR).

References

Adan, R. A. (2006). Constitutive receptor activity series: endogenous inverse agonists and constitutive receptor activity in the melanocortin system. *Trends Pharmacol Sci* 27(4), 183–186.

Addison, C. L., Daniel, T. O., Burdick, M. D., Liu, H., Ehler, J. E., Xue, Y. Y., et al. (2000). The CXCR2 chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXCR2 chemokine-induced angiogenic activity. *J Immunol* 165(9), 5269–5277.

Alcami, A. (2003). Viral mimicry of cytokines, chemokines and their receptors. *Nat Rev Immunol* 3(1), 36–50.

Alcami, A., & Smith, G. L. (1992). A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell* 71(1), 153–167.

Aldinucci, D., Lorenzon, D., Cattaruzza, L., Pinto, A., Gloghini, A., Carbone, A., et al. (2008). Expression of CCR5 receptors on Reed–Sternberg cells and Hodgkin lymphoma cell lines: involvement of CCL5/Rantes in tumor cell growth and microenvironmental interactions. *Int J Cancer* 122(4), 769–776.

Alewijnse, A. E., Timmerman, H., Jacobs, E. H., Smit, M. J., Roovers, E., Cotecchia, S., et al. (2000). The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H(2) receptor. *Mol Pharmacol* 57(5), 890–898.

Alexander, J. M., Nelson, C. A., van Berkel, V., Lau, E. K., Studts, J. M., Brett, T. J., et al. (2002). Structural basis of chemokine sequestration by a herpesvirus decoy receptor. *Cell* 111(3), 343–356.

Ali, S., Robertson, H., Wain, J. H., Isaacs, J. D., Malik, G., & Kirby, J. A. (2005). A non-glycosaminoglycan-binding variant of CC chemokine ligand 7 (monocyte chemoattractant protein-3) antagonizes chemokine-mediated inflammation. *J Immunol* 175(2), 1257–1266.

Amara, A., Lorthioir, O., Valenzuela, A., Magerus, A., Thelen, M., Montes, M., et al. (1999). Stromal cell-derived factor-1alpha associates with heparan sulfates through the first beta-strand of the chemokine. *J Biol Chem* 274(34), 23916–23925.

Ambrosini, E., Columba-Cabezas, S., Serafini, B., Muscella, A., & Aloisi, F. (2003). Astrocytes are the major intracerebral source of macrophage inflammatory protein-3alpha/CCL20 in relapsing experimental autoimmune encephalomyelitis and in vitro. *Glia* 41(3), 290–300.

Amiral, J., Marfaing-Koka, A., Wolf, M., Alessi, M. C., Tardy, B., Boyer-Neumann, C., et al. (1996). Presence of autoantibodies to interleukin-8 or neutrophil-activating peptide-2 in patients with heparin-associated thrombocytopenia. *Blood* 88(2), 410–416.

Andreasen, C., & Carbonetti, N. H. (2008). Pertussis toxin inhibits early chemokine production to delay neutrophil recruitment in response to *Bordetella pertussis* respiratory tract infection in mice. *Infect Immun* 76(11), 5139–5148.

Ariel, A., Fredman, G., Sun, Y. P., Kantarci, A., Van Dyke, T. E., Luster, A. D., et al. (2006). Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nat Immunol* 7(11), 1209–1216.

Arkin, M. (2005). Protein–protein interactions and cancer: small molecules going in for the kill. *Curr Opin Chem Biol* 9(3), 317–324.

Arkin, M., & Moasser, M. M. (2008). HER-2-directed, small-molecule antagonists. *Curr Opin Investig Drugs* 9(12), 1264–1276.

Arkin, M. R., & Wells, J. A. (2004). Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. *Nat Rev Drug Discov* 3(4), 301–317.

Arkin, M. R., Randal, M., DeLano, W. L., Hyde, J., Luong, T. N., Oslob, J. D., et al. (2003). Binding of small molecules to an adaptive protein–protein interface. *Proc Natl Acad Sci U S A* 100(4), 1603–1608.

Arrang, J. M., Morisset, S., & Gbahou, F. (2007). Constitutive activity of the histamine H3 receptor. *Trends Pharmacol Sci* 28(7), 350–357.

Aslanian, A. M., & Charo, I. F. (2006). Targeted disruption of the scavenger receptor and chemokine CXCL16 accelerates atherosclerosis. *Circulation* 114(6), 583–590.

Auwerx, J., Avner, P., Baldock, R., Ballabio, A., Balling, R., Barbacid, M., et al. (2004). The European dimension for the mouse genome mutagenesis program. *Nat Genet* 36(9), 925–927.

Balabanian, K., Couderc, J., Bouchet-Delbos, L., Amara, A., Berrebi, D., Foussat, A., et al. (2003). Role of the chemokine stromal cell-derived factor 1 in autoantibody production and nephritis in murine lupus. *J Immunol* 170(6), 3392–3400.

Balabanian, K., Lagane, B., Infantino, S., Chow, K. Y., Harriague, J., Moepps, B., et al. (2005). The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem* 280(42), 35760–35766.

Balkwill, F. (2009). Tumour necrosis factor and cancer. *Nat Rev Cancer* 9(5), 361–371.

Bamias, G., Siakavellas, S. I., Stamateopoulos, K. S., Chrysoschoou, E., Papamichael, C., & Sfikakis, P. P. (2008). Circulating levels of TNF-like cytokine 1A (TL1A) and its decoy receptor 3 (DcR3) in rheumatoid arthritis. *Clin Immunol* 129(2), 249–255.

Bannert, N., Craig, S., Farzan, M., Sogah, D., Santo, N. V., Choe, H., et al. (2001). Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. *J Exp Med* 194(11), 1661–1673.

Bedard, E. L., Kim, P., Jiang, J., Parry, N., Liu, L., Wang, H., et al. (2003). Chemokine-binding viral protein M-T7 prevents chronic rejection in rat renal allografts. *Transplantation* 76(1), 249–252.

Behera, A. K., Hildebrand, E., Bronson, R. T., Perides, G., Uematsu, S., Akira, S., et al. (2006). MyD88 deficiency results in tissue-specific changes in cytokine induction and inflammation in interleukin-18-independent mice infected with *Borrelia burgdorferi*. *Infect Immun* 74(3), 1462–1470.

Bellail, A. C., Tse, M. C., Song, J. H., Phuphanich, S., Olson, J. J., Sun, S. Y., et al. (2009). DR5-mediated DISC controls caspase-8 cleavage and initiation of apoptosis in human glioblastomas. *J Cell Mol Med*.

Belmadani, A., Tran, P. B., Ren, D., & Miller, R. J. (2006). Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. *J Neurosci* 26(12), 3182–3191.

Belperio, J. A., Dy, M., Murray, L., Burdick, M. D., Xue, Y. Y., Strieter, R. M., et al. (2004). The role of the Th2 CC chemokine ligand CCL17 in pulmonary fibrosis. *J Immunol* 173(7), 4692–4698.

Belperio, J. A., Keane, M. P., Burdick, M. D., Lynch, J. P., III, Xue, Y. Y., Li, K., et al. (2002). Critical role for CXCR3 chemokine biology in the pathogenesis of bronchiolitis obliterans syndrome. *J Immunol* 169(2), 1037–1049.

Belperio, J. A., Keane, M. P., Burdick, M. D., Lynch, J. P., III, Zisman, D. A., Xue, Y. Y., et al. (2003). Role of CXCL9/CXCR3 chemokine biology during pathogenesis of acute lung allograft rejection. *J Immunol* 171(9), 4844–4852.

Ben-Shlomo, A., Zhou, C., Pichurin, O., Chesnokova, V., Liu, N. A., Culler, M. D., et al. (2009). Constitutive somatostatin receptor activity determines tonic pituitary cell response. *Mol Endocrinol* 23(3), 337–348.

Berard, J. L., Velez, R. L., Freeman, R. B., & Tsunoda, S. M. (1999). A review of interleukin-2 receptor antagonists in solid organ transplantation. *Pharmacotherapy* 19(10), 1127–1137.

Berg, T. (2003). Modulation of protein–protein interactions with small organic molecules. *Angew Chem Int Ed Engl* 42(22), 2462–2481.

Berg, T. (2006). Inhibition of TNF-alpha signaling: divide and conquer. *Chem Med Chem* 1(7), 687–688.

Bertolini, F., Dell’Agnola, C., Mancuso, P., Rabascio, C., Burlini, A., Monestiroli, S., et al. (2002). CXCR4 neutralization, a novel therapeutic approach for non-Hodgkin’s lymphoma. *Cancer Res* 62(11), 3106–3112.

- Bezerra, M. C., Carvalho, J. F., Prokopowitsch, A. S., & Pereira, R. M. (2005). RANK, RANKL and osteoprotegerin in arthritic bone loss. *Braz J Med Biol Res* 38(2), 161–170.
- Blain, K. Y., Kwiatkowski, W., Zhao, Q., La Fleur, D., Naik, C., Chun, T. W., et al. (2007). Structural and functional characterization of CC chemokine CCL14. *Biochemistry* 46(35), 10008–10015.
- Blanpain, C., Doranz, B. J., Vakili, J., Rucker, J., Govaerts, C., Baik, S. S., et al. (1999). Multiple charged and aromatic residues in CCR5 amino-terminal domain are involved in high affinity binding of both chemokines and HIV-1 Env protein. *J Biol Chem* 274(49), 34719–34727.
- Blazer, L. L., & Neubig, R. R. (2009). Small molecule protein–protein interaction inhibitors as CNS therapeutic agents: current progress and future hurdles. *Neuropharmacology* 34(1), 126–141.
- Bless, N. M., Huber-Lang, M., Guo, R. F., Warner, R. L., Schmal, H., Czermak, B. J., et al. (2000). Role of CC chemokines (macrophage inflammatory protein-1 beta, monocyte chemoattractant protein-1, RANTES) in acute lung injury in rats. *J Immunol* 164(5), 2650–2659.
- Boeglin, D., Bonnet, D., & Hibert, M. (2007). Solid-phase preparation of a pilot library derived from the 2, 3, 4, 5-tetrahydro-1H-benzo[b]azepin-5-amine scaffold. *J Comb Chem* 9(3), 487–500.
- Boismenu, R., Feng, L., Xia, Y. Y., Chang, J. C., & Havran, W. L. (1996). Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. *J Immunol* 157(3), 985–992.
- Boldajipour, B., Mahabaleswar, H., Kardash, E., Reichman-Fried, M., Blaser, H., Minina, S., et al. (2008). Control of chemokine-guided cell migration by ligand sequestration. *Cell* 132(3), 463–473.
- Bonecchi, R., Borroni, E. M., Anselmo, A., Doni, A., Savino, B., Mirole, M., et al. (2008). Regulation of D6 chemokine scavenging activity by ligand- and Rab11-dependent surface up-regulation. *Blood* 112(3), 493–503.
- Bonecchi, R., Borroni, E. M., Savino, B., Buracchi, C., Mantovani, A., & Locati, M. (2008). Non-signaling chemokine receptors: mechanism of action and role in vivo. *J Neuroimmunol* 198(1–2), 14–19.
- Boring, L., Gosling, J., Cleary, M., & Charo, I. F. (1998). Decreased lesion formation in CCR2^{-/-} mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 394(6696), 894–897.
- Braunersreuther, V., Steffens, S., Arnaud, C., Pelli, G., Burger, F., Proudfoot, A., et al. (2008). A novel RANTES antagonist prevents progression of established atherosclerotic lesions in mice. *Arterioscler Thromb Vasc Biol* 28(6), 1090–1096.
- Brown, H. J., Lock, H. R., Wolfs, T. G., Buurman, W. A., Sacks, S. H., & Robson, M. G. (2007). Toll-like receptor 4 ligation on intrinsic renal cells contributes to the induction of antibody-mediated glomerulonephritis via CXCL1 and CXCL2. *J Am Soc Nephrol* 18(6), 1732–1739.
- Brown, S. D., Chambon, P., & de Angelis, M. H. (2005). EMPRESS: standardized phenotype screens for functional annotation of the mouse genome. *Nat Genet* 37(11), 1155.
- Burger, J. A., & Peled, A. (2009). CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. *Leukemia* 23(1), 43–52.
- Burger, M., Burger, J. A., Hoch, R. A., Oades, Z., Takamori, H., & Schraufstatter, I. U. (1999). Point mutation causing constitutive signaling of CXCR2 leads to transforming activity similar to Kaposi's sarcoma herpesvirus-G protein-coupled receptor. *J Immunol* 163(4), 2017–2022.
- Burns, J. M., Summers, B. C., Wang, Y., Melikian, A., Berahovich, R., Miao, Z., et al. (2006). A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med* 203(9), 2201–2213.
- Caberg, J. H., Hubert, P., Herman, L., Herfs, M., Roncarati, P., Boniver, J., et al. (2009). Increased migration of Langerhans cells in response to HPV16 E6 and E7 oncogene silencing: role of CCL20. *Cancer Immunol Immunother* 58(1), 39–47.
- Cain, S. A., & Monk, P. N. (2002). The orphan receptor C5L2 has high affinity binding sites for complement fragments C5a and C5a des-Arg(74). *J Biol Chem* 277(9), 7165–7169.
- Campbell, J. J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., et al. (1999). The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 400(6746), 776–780.
- Caput, D., Laurent, P., Kaghad, M., Lelias, J. M., Lefort, S., Vita, N., et al. (1996). Cloning and characterization of a specific interleukin (IL)-13 binding protein structurally related to the IL-5 receptor alpha chain. *J Biol Chem* 271(28), 16921–16926.
- Cardones, A. R., Murakami, T., & Hwang, S. T. (2003). CXCR4 enhances adhesion of B16 tumor cells to endothelial cells in vitro and in vivo via beta(1) integrin. *Cancer Res* 63(20), 6751–6757.
- Carpenter, K. J., & Hogaboam, C. M. (2005). Immunosuppressive effects of CCL17 on pulmonary antifungal responses during pulmonary invasive aspergillosis. *Infect Immun* 73(11), 7198–7207.
- Cartier, L., Hartley, O., Dubois-Dauphin, M., & Krause, K. H. (2005). Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. *Brain Res Brain Res Rev* 48(1), 16–42.
- Catusse, J., Clark, D. J., & Gompels, U. A. (2009). CCR5 signalling, but not DARC or D6 regulatory, chemokine receptors are targeted by herpesvirus U83A chemokine which delays receptor internalisation via diversion to a caveolin-linked pathway. *J Inflamm (Lond)* 6, 22.
- Catusse, J., Parry, C. M., Dewin, D. R., & Gompels, U. A. (2007). Inhibition of HIV-1 infection by viral chemokine U83A via high-affinity CCR5 interactions that block human chemokine-induced leukocyte chemotaxis and receptor internalization. *Blood* 109(9), 3633–3639.
- Chames, P., Van Regenmortel, M., Weiss, E., & Baty, D. (2009). Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol* 157(2), 220–233.
- Chanel, C., Staropoli, I., Baleux, F., Amara, A., Valenzuela-Fernandez, A., Virelizier, J. L., et al. (2002). Low levels of co-receptor CCR5 are sufficient to permit HIV envelope-mediated fusion with resting CD4 T cells. *Aids* 16(17), 2337–2340.
- Chavey, C., Lazennec, G., Lagarrigue, S., Clape, C., Iankova, I., Teyssier, J., et al. (2009). CXC ligand 5 is an adipose-tissue derived factor that links obesity to insulin resistance. *Cell Metab* 9(4), 339–349.
- Chen, L., Lin, S. X., Agha-Majzoub, R., Overbergh, L., Mathieu, C., & Chan, L. S. (2006). CCL27 is a critical factor for the development of atopic dermatitis in the keratin-14 IL-4 transgenic mouse model. *Int Immunol* 18(8), 1233–1242.
- Chen, S. C., Mehrad, B., Deng, J. C., Vassileva, G., Manfra, D. J., Cook, D. N., et al. (2001). Impaired pulmonary host defense in mice lacking expression of the CXC chemokine lungkine. *J Immunol* 166(5), 3362–3368.
- Chensue, S. W., Lukacs, N. W., Yang, T. Y., Shang, X., Frai, K. A., Kunkel, S. L., et al. (2001). Aberrant in vivo T helper type 2 cell response and impaired eosinophil recruitment in CC chemokine receptor 8 knockout mice. *J Exp Med* 193(5), 573–584.
- Chitnis, C. E., & Sharma, A. (2008). Targeting the *Plasmodium vivax* Duffy-binding protein. *Trends Parasitol* 24(1), 29–34.
- Claeysen, S., Sebben, M., Becamel, C., Eglen, R. M., Clark, R. D., Bockaert, J., et al. (2000). Pharmacological properties of 5-hydroxytryptamine(4) receptor antagonists on constitutively active wild-type and mutated receptors. *Mol Pharmacol* 58(1), 136–144.
- Clubb, R. T., Omichinski, J. G., Clore, G. M., & Gronenborn, A. M. (1994). Mapping the binding surface of interleukin-8 complexed with an N-terminal fragment of the type 1 human interleukin-8 receptor. *FEBS Lett* 338(1), 93–97.
- Colotta, F., Orlando, S., Fadlon, E. J., Sozzani, S., Matteucci, C., & Mantovani, A. (1995). Chemoattractants induce rapid release of the interleukin 1 type II decoy receptor in human polymorphonuclear cells. *J Exp Med* 181(6), 2181–2186.
- Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., et al. (1993). Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 261(5120), 472–475.
- Colvin, B. L., Wang, Z., Nakano, H., Wu, W., Kakiuchi, T., Fairchild, R. L., et al. (2005). CXCL9 antagonism further extends prolonged cardiac allograft survival in CCL19/CCL21-deficient mice. *Am J Transplant* 5(9), 2104–2113.
- Comerford, I., Milasta, S., Morrow, V., Milligan, G., & Nibbs, R. (2006). The chemokine receptor CXCR2 mediates effective scavenging of CCL19 in vitro. *Eur J Immunol* 36(7), 1904–1916.
- Cook, D. N., Beck, M. A., Coffman, T. M., Kirby, S. L., Sheridan, J. F., Pragnell, I. B., et al. (1995). Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science* 269(5230), 1583–1585.
- Cook, D. N., Chen, S. C., Sullivan, L. M., Manfra, D. J., Wiekowski, M. T., Prosser, D. M., et al. (2001). Generation and analysis of mice lacking the chemokine fractalkine. *Mol Cell Biol* 21(9), 3159–3165.
- Crown, S. E., Yu, Y., Sweeney, M. D., Leary, J. A., & Handel, T. M. (2006). Heterodimerization of CCR2 chemokines and regulation by glycosaminoglycan binding. *J Biol Chem* 281(35), 25438–25446.
- Cuvelier, S. L., & Patel, K. D. (2001). Shear-dependent eosinophil transmigration on interleukin 4-stimulated endothelial cells: a role for endothelium-associated eotaxin-3. *J Exp Med* 194(12), 1699–1709.
- Dambly-Chaudiere, C., Cubedo, N., & Ghysen, A. (2007). Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. *BMC Dev Biol* 7, 23.
- Damon, I., Murphy, P. M., & Moss, B. (1998). Broad spectrum chemokine antagonistic activity of a human poxvirus chemokine homolog. *Proc Natl Acad Sci U S A* 95(11), 6403–6407.
- Darbonne, W. C., Rice, G. C., Mohler, M. A., Apple, T., Hebert, C. A., Valente, A. J., et al. (1991). Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *J Clin Invest* 88(4), 1362–1369.
- Dawson, T. C., Lentsch, A. B., Wang, Z., Cowhig, J. E., Rot, A., Maeda, N., et al. (2000). Exaggerated response to endotoxin in mice lacking the Duffy antigen/receptor for chemokines (DARC). *Blood* 96(5), 1681–1684.
- de Lavarelle, A., Roufousse, F., Schandene, L., Stordeur, P., Cogan, E., & Goldman, M. (2001). Clonal Th2 cells associated with chronic hypereosinophilia: TARC-induced CCR4 down-regulation in vivo. *Eur J Immunol* 31(4), 1037–1046.
- de Moura, P. R., Watanabe, L., Bleicher, L., Colau, D., Dumoutier, L., Lemaire, M. M., et al. (2009). Crystal structure of a soluble decoy receptor IL-22BP bound to interleukin-22. *FEBS Lett* 583(7), 1072–1077.
- Deruz, M., Frauenschuh, A., Alessandri, A. L., Dias, J. M., Coelho, F. M., Russo, R. C., et al. (2008). Ticks produce highly selective chemokine binding proteins with anti-inflammatory activity. *J Exp Med* 205(9), 2019–2031.
- Ding, C., Li, J., & Zhang, X. (2004). Bertilimumab Cambridge Antibody Technology Group. *Curr Opin Investig Drugs* 5(11), 1213–1218.
- Dobson, C. M. (1999). Protein misfolding, evolution and disease. *Trends Biochem Sci* 24(9), 329–332.
- Dorf, M. E., Berman, M. A., Tanabe, S., Heesen, M., & Luo, Y. (2000). Astrocytes express functional chemokine receptors. *J Neuroimmunol* 111(1–2), 109–121.
- Duma, L., Haussinger, D., Rogowski, M., Lusso, P., & Grzesiek, S. (2007). Recognition of RANTES by extracellular parts of the CCR5 receptor. *J Mol Biol* 365(4), 1063–1075.
- El-Hage, N., Bruce-Keller, A. J., Knapp, P. E., & Hauser, K. F. (2008). CCL5/RANTES gene deletion attenuates opioid-induced increases in glial CCL2/MCP-1 immunoreactivity and activation in HIV-1 Tat-exposed mice. *J Neuroimmune Pharmacol* 3(4), 275–285.
- Emerson, S. D., Palermo, R., Liu, C. M., Tilley, J. W., Chen, L., Danho, W., et al. (2003). NMR characterization of interleukin-2 in complexes with the IL-2Ralpha receptor component, and with low molecular weight compounds that inhibit the IL-2/IL-Ralpha interaction. *Protein Sci* 12(4), 811–822.
- Feng, L. Y., Ou, Z. L., Wu, F. Y., Shen, Z. Z., & Shao, Z. M. (2009). Involvement of a novel chemokine decoy receptor CXCR2 in breast cancer growth, metastasis and patient survival. *Clin Cancer Res* 15(9), 2962–2970.
- Feng, N., Jaimes, M. C., Lazarus, N. H., Monak, D., Zhang, C., Butcher, E. C., et al. (2006). Redundant role of chemokines CCL25/TECK and CCL28/MEC in IgA+ plasmablast

- recruitment to the intestinal lamina propria after rotavirus infection. *J Immunol* 176 (10), 5749–5759.
- Fili, S., Karalaki, M., & Schaller, B. (2009). Mechanism of bone metastasis: the role of osteoprotegerin and of the host-tissue microenvironment-related survival factors. *Cancer Lett* 283(1), 711–726.
- Fioravanti, B., De Felice, M., Stucky, C. L., Medler, K. A., Luo, M. C., Gardell, L. R., et al. (2008). Constitutive activity at the cannabinoid CB1 receptor is required for behavioral response to noxious chemical stimulation of TRPV1: antinociceptive actions of CB1 inverse agonists. *J Neurosci* 28(45), 11593–11602.
- Fiorucci, G., Olivetta, E., Chiantore, M. V., & Federico, M. (2007). Microarray analysis reveals CCL24/eotaxin-2 as an effector of the pathogenetic effects induced by HIV-1 Nef. *Curr Drug Discov Technol* 4(1), 12–23.
- Fong, A. M., Alam, S. M., Imai, T., Haribabu, B., & Patel, D. D. (2002). CX3CR1 tyrosine sulfation enhances fractalkine-induced cell adhesion. *J Biol Chem* 277(22), 19418–19423.
- Foxwell, B., Andreacos, E., Brennan, F., Feldmann, M., Smith, C., & Conron, M. (2003). Prospects for the development of small molecular weight compounds to replace anti-tumour necrosis factor biological agents. *Ann Rheum Dis* 62(Suppl 2), ii90–93.
- Fra, A. M., Locati, M., Otero, K., Sironi, M., Signorelli, P., Massardi, M. L., et al. (2003). Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J Immunol* 170(5), 2279–2282.
- Frauenschu, A., Power, C. A., Deruaz, M., Ferreira, B. R., Silva, J. S., Teixeira, M. M., et al. (2007). Molecular cloning and characterization of a highly selective chemokine-binding protein from the tick *Rhipicephalus sanguineus*. *J Biol Chem* 282(37), 27250–27258.
- Funke, B., Autschbach, F., Kim, S., Lasitschka, F., Strauch, U., Rogler, G., et al. (2009). Functional characterisation of decoy receptor 3 in Crohn's disease. *Gut* 58(4), 483–491.
- Galliera, E., Jala, V. R., Trent, J. O., Bonocchi, R., Signorelli, P., Lefkowitz, R. J., et al. (2004). Beta-arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. *J Biol Chem* 279(24), 25590–25597.
- Gardner, L., Wilson, C., Patterson, A. M., Bresnihan, B., FitzGerald, O., Stone, M. A., et al. (2006). Temporal expression pattern of Duffy antigen in rheumatoid arthritis: up-regulation in early disease. *Arthritis Rheum* 54(6), 2022–2026.
- Gayle, R. B., III, Sleath, P. R., Srinivasan, S., Birks, C. W., Weerawarna, K. S., Cerretti, D. P., et al. (1993). Importance of the amino terminus of the interleukin-8 receptor in ligand interactions. *J Biol Chem* 268(10), 7283–7289.
- Gerard, C., Frossard, J. L., Bhatia, M., Saluja, A., Gerard, N. P., Lu, B., et al. (1997). Targeted disruption of the beta-chemokine receptor CCR1 protects against pancreatitis-associated lung injury. *J Clin Invest* 100(8), 2022–2027.
- Chia, P., Stroala, G., Granziero, L., Geuna, M., Guida, G., Sallusto, F., et al. (2002). Chronic lymphocytic leukemia B cells are endowed with the capacity to attract CD4+, CD40L+ T cells by producing CCL22. *Eur J Immunol* 32(5), 1403–1413.
- Giuliani, N., Lisignoli, G., Colla, S., Lazzaretti, M., Storti, P., Mancini, C., et al. (2008). CC-chemokine ligand 20/macrophage inflammatory protein-3alpha and CC-chemokine receptor 6 are overexpressed in myeloma microenvironment related to osteolytic bone lesions. *Cancer Res* 68(16), 6840–6850.
- Glaser, J., Gonzalez, R., Perreau, V. M., Cotman, C. W., & Keirstead, H. S. (2004). Neutralization of the chemokine CXCL10 enhances tissue sparing and angiogenesis following spinal cord injury. *J Neurosci Res* 77(5), 701–708.
- Glaser, J., Gonzalez, R., Sadr, E., & Keirstead, H. S. (2006). Neutralization of the chemokine CXCL10 reduces apoptosis and increases axon sprouting after spinal cord injury. *J Neurosci Res* 84(4), 724–734.
- Glass, W. G., Hickey, M. J., Hardison, J. L., Liu, M. T., Manning, J. E., & Lane, T. E. (2004). Antibody targeting of the CC chemokine ligand 5 results in diminished leukocyte infiltration into the central nervous system and reduced neurologic disease in a viral model of multiple sclerosis. *J Immunol* 172(7), 4018–4025.
- Glass, W. G., Lim, J. K., Cholera, R., Pletnev, A. G., Gao, J. L., & Murphy, P. M. (2005). Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *J Exp Med* 202(8), 1087–1098.
- Gonzalo, J. A., Lloyd, C. M., Peled, A., Delaney, T., Coyle, A. J., & Gutierrez-Ramos, J. C. (2000). Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 alpha in the inflammatory component of allergic airway disease. *J Immunol* 165(1), 499–508.
- Gosling, J., Dairaghi, D. J., Wang, Y., Hanley, M., Talbot, D., Miao, Z., et al. (2000). Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol* 164(6), 2851–2856.
- Gosling, J., Slaymaker, S., Gu, L., Tseng, S., Zlot, C. H., Young, S. G., et al. (1999). MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J Clin Invest* 103(6), 773–778.
- Graham, G. J. (2009). D6 and the atypical chemokine receptor family: novel regulators of immune and inflammatory processes. *Eur J Immunol* 39(2), 342–351.
- Graham, G. J., & McKimmie, C. S. (2006). Chemokine scavenging by D6: a movable feast? *Trends Immunol* 27(8), 381–386.
- Grespan, R., Fukada, S. Y., Lemos, H. P., Vieira, S. M., Napimoga, M. H., Teixeira, M. M., et al. (2008). CXCR2-specific chemokines mediate leukotriene B4-dependent recruitment of neutrophils to inflamed joints in mice with antigen-induced arthritis. *Arthritis Rheum* 58(7), 2030–2040.
- Gu, L., Okada, Y., Clinton, S. K., Gerard, C., Sukhova, G. K., Libby, P., et al. (1998). Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 2(2), 275–281.
- Guerau-de-Arellano, M., & Huber, B. T. (2005). Chemokines and Toll-like receptors in Lyme disease pathogenesis. *Trends Mol Med* 11(3), 114–120.
- Hachet-Haas, M., Balabanian, K., Rohmer, F., Pons, F., Franchet, C., Lecat, S., et al. (2008). Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J Biol Chem* 283(34), 23189–23199.
- Hajnicka, V., Kocakova, P., Slavikova, M., Slovak, M., Gasperik, J., Fuchsberger, N., et al. (2001). Anti-interleukin-8 activity of tick salivary gland extracts. *Parasite Immunol* 23(9), 483–489.
- Handel, T. M., Johnson, Z., Rodrigues, D. H., Dos Santos, A. C., Cirillo, R., Muzio, V., et al. (2008). An engineered monomer of CCL2 has anti-inflammatory properties emphasizing the importance of oligomerization for chemokine activity in vivo. *J Leukoc Biol* 84(4), 1101–1108.
- He, M. M., Smith, A. S., Oslob, J. D., Flanagan, W. M., Braisted, A. C., Whitty, A., et al. (2005). Small-molecule inhibition of TNF-alpha. *Science* 310(5750), 1022–1025.
- Hibert, M. F. (2009). French/European academic compound library initiative. *Drug Discov Today*.
- Hieshima, K., Nagakubo, D., Nakayama, T., Shirakawa, A. K., Jin, Z., & Yoshie, O. (2008). Tax-inducible production of CC chemokine ligand 22 by human T cell leukemia virus type 1 (HTLV-1)-infected T cells promotes preferential transmission of HTLV-1 to CCR4-expressing CD4+ T cells. *J Immunol* 180(2), 931–939.
- Hinton, C. V., Avraham, S., & Avraham, H. K. (2008). Role of the CXCR4/CXCL12 signaling axis in breast cancer metastasis to the brain. *Clin Exp Metastasis*.
- Hirschfeld, M., Kirschning, C. J., Schwandner, R., Wesche, H., Weiss, J. H., Wooten, R. M., et al. (1999). Cutting edge: inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by toll-like receptor 2. *J Immunol* 163(5), 2382–2386.
- Hogaboam, C. M., Gallinat, C. S., Taub, D. D., Strieter, R. M., Kunkel, S. L., & Lukacs, N. W. (1999). Immunomodulatory role of C10 chemokine in a murine model of allergic bronchopulmonary aspergillosis. *J Immunol* 162(10), 6071–6079.
- Holst, P. J., Rosenkilde, M. M., Manfra, D., Chen, S. C., Wiekowski, M. T., Holst, B., et al. (2001). Tumorigenesis induced by the HHV8-encoded chemokine receptor requires ligand modulation of high constitutive activity. *J Clin Invest* 108(12), 1789–1796.
- Horuk, R. (2009). Promiscuous drugs as therapeutics for chemokine receptors. *Expert Rev Mol Med* 11, e1.
- Hoshino, A., Kawamura, Y. I., Yasuhara, M., Toyama-Sorimachi, N., Yamamoto, K., Matsukawa, A., et al. (2007). Inhibition of CCL1-CCR8 interaction prevents aggregation of macrophages and development of peritoneal adhesions. *J Immunol* 178(8), 5296–5304.
- Hughes, A., & Nelson, M. (2009). HIV entry: new insights and implications for patient management. *Curr Opin Infect Dis* 22(1), 35–42.
- Hunter, D. T., Jr., & Hill, J. M. (1961). Surfen: a quinoline with oncogenic and heparin-neutralizing properties. *Nature* 191, 1378–1379.
- Issa, R., Xie, S., Lee, K. Y., Stanbridge, R. D., Bhavsar, P., Sukkar, M. B., et al. (2006). GRO-alpha regulation in airway smooth muscle by IL-1beta and TNF-alpha: role of NF-kappaB and MAP kinases. *Am J Physiol Lung Cell Mol Physiol* 291(1), L66–L74.
- Iyoda, T., Nagata, K., Akashi, M., & Kobayashi, Y. (2005). Neutrophils accelerate macrophage-mediated digestion of apoptotic cells in vivo as well as in vitro. *J Immunol* 175(6), 3475–3483.
- Izikson, L., Klein, R. S., Charo, I. F., Weiner, H. L., & Luster, A. D. (2000). Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR2). *J Exp Med* 192(7), 1075–1080.
- Jamieson, T., Cook, D. N., Nibbs, R. J., Rot, A., Nixon, C., McLean, P., et al. (2005). The chemokine receptor D6 limits the inflammatory response in vivo. *Nat Immunol* 6(4), 403–411.
- Jiang, X., Shimaoka, T., Kojo, S., Harada, M., Watarai, H., Wakao, H., et al. (2005). Cutting edge: critical role of CXCL16/CXCR6 in NKT cell trafficking in allograft tolerance. *J Immunol* 175(4), 2051–2055.
- Jin, H., Shen, X., Baggett, B. R., Kong, X., & LiWang, P. J. (2007). The human CC chemokine MIP-1beta dimer is not competent to bind to the CCR5 receptor. *J Biol Chem* 282(38), 27976–27983.
- Jin, Y., Shen, L., Chong, E. M., Hamrah, P., Zhang, Q., Chen, L., et al. (2007). The chemokine receptor CCR7 mediates corneal antigen-presenting cell trafficking. *Mol Vis* 13, 626–634.
- Johnson, Z., Kosco-Vilbois, M. H., Herren, S., Cirillo, R., Muzio, V., Zaratin, P., et al. (2004). Interference with heparin binding and oligomerization creates a novel anti-inflammatory strategy targeting the chemokine system. *J Immunol* 173(9), 5776–5785.
- Kalatskaya, I., Berchiche, Y. A., Gravel, S., Limberg, B. J., Rosenbaum, J. S., & Heveker, N. (2009). AMD3100 is a CXCR7 ligand with allosteric agonist properties. *Mol Pharmacol* 75(5), 1240–1247.
- Kelchtermans, H., Struyf, S., De Klerck, B., Mitera, T., Alen, M., Geboes, L., et al. (2007). Protective role of IFN-gamma in collagen-induced arthritis conferred by inhibition of mycobacteria-induced granulocyte chemotactic protein-2 production. *J Leukoc Biol* 81(4), 1044–1053.
- Khosla, S. (2001). Minireview: the OPG/RANKL/RANK system. *Endocrinology* 142(12), 5050–5055.
- Kindberg, E., Mickiene, A., Ax, C., Akerlind, B., Vene, S., Lindquist, L., et al. (2008). A deletion in the chemokine receptor 5 (CCR5) gene is associated with tickborne encephalitis. *J Infect Dis* 197(2), 266–269.
- Kokkoi, E., Kasinskas, R. W., Mardilovich, A., & Garg, A. (2005). Fractalkine targeting with a receptor-mimicking peptide-amphiphile. *Biomacromolecules* 6(3), 1272–1279.
- Kong, K. O., Tan, A. W., Thong, B. Y., Lian, T. Y., Cheng, Y. K., Teh, C. L., et al. (2009). Enhanced expression of interferon-inducible protein-10 correlates with disease activity and clinical manifestations in systemic lupus erythematosus. *Clin Exp Immunol* 156(1), 134–140.
- Kriegova, E., Tsyruynyk, A., Arakelyan, A., Mrzsek, F., Ordeltova, M., Petzmann, S., et al. (2006). Expression of CXCR in pulmonary sarcoidosis. *Inflamm Res* 55(10), 441–445.
- Kuloglu, E. S., McCaslin, D. R., Markley, J. L., & Volkman, B. F. (2002). Structural rearrangement of human lymphotactin, a C chemokine, under physiological solution conditions. *J Biol Chem* 277(20), 17863–17870.
- Kwong, J., Kulbe, H., Wong, D., Chakravarty, P., & Balkwill, F. (2009). An antagonist of the chemokine receptor CXCR4 induces mitotic catastrophe in ovarian cancer cells. *Mol Cancer Ther* 8(7), 1893–1905.
- Laguri, C., Arenzana-Seisdedos, F., & Lortat-Jacob, H. (2008). Relationships between glycosaminoglycan and receptor binding sites in chemokines—the CXCL12 example. *Carbohydr Res* 343(12), 2018–2023.

- Lalani, A. S., Graham, K., Mossman, K., Rajarathnam, K., Clark-Lewis, I., Kelvin, D., et al. (1997). The purified myxoma virus gamma interferon receptor homolog M-T7 interacts with the heparin-binding domains of chemokines. *J Virol* 71(6), 4356–4363.
- Lau, P. N., Chow, K. B., Chan, C. B., Cheng, C. H., & Wise, H. (2009). The constitutive activity of the ghrelin receptor attenuates apoptosis via a protein kinase C-dependent pathway. *Mol Cell Endocrinol* 299(2), 232–239.
- Lecat, S., Bucher, B., Mely, Y., & Galzi, J. L. (2002). Mutations in the extracellular amino-terminal domain of the NK2 neurokinin receptor abolish cAMP signaling but preserve intracellular calcium responses. *J Biol Chem* 277(44), 42034–42048.
- Lee, B. P., Chen, W., Shi, H., Der, S. D., Forster, R., & Zhang, L. (2006). CXCR5/CXCL13 interaction is important for double-negative regulatory T cell homing to cardiac allografts. *J Immunol* 176(9), 5276–5283.
- Lefkowitz, R. J., Cotecchia, S., Kjelsberg, M. A., Pitcher, J., Koch, W. J., Inglese, J., et al. (1993). Adrenergic receptors: recent insights into their mechanism of activation and desensitization. *Adv Second Messenger Phosphoprotein Res* 28, 1–9.
- Lefkowitz, R. J., Cotecchia, S., Samama, P., & Costa, T. (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* 14(8), 303–307.
- Lemos, H. P., Grespan, R., Vieira, S. M., Cunha, T. M., Verri, W. A., Jr., Fernandes, K. S., et al. (2009). Prostaglandin mediates IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFN γ production. *Proc Natl Acad Sci U S A* 106(14), 5954–5959.
- Leurs, R., Rodriguez Pena, M. S., Bakker, R. A., Alewijnse, A. E., & Timmerman, H. (2000). Constitutive activity of G protein coupled receptors and drug action. *Pharm Acta Helv* 74(2–3), 327–331.
- Levina, V., Nolen, B. M., Marrangoni, A. M., Cheng, P., Marks, J. R., Szczepanski, M. J., et al. (2009). Role of eotaxin-1 signaling in ovarian cancer. *Clin Cancer Res* 15(8), 2647–2656.
- Levina, V., Su, Y., Nolen, B., Liu, X., Gordin, Y., Lee, M., et al. (2008). Chemotherapeutic drugs and human tumor cells cytokine network. *Int J Cancer* 123(9), 2031–2040.
- Leyove, A., Balabanian, K., Baleux, F., Bachelier, F., & Lagane, B. (2009). CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* 113(24), 6085–6093.
- Li, B. Q., Fu, T., Gong, W. H., Dunlop, N., Kung, H., Yan, Y., et al. (2000). The flavonoid baicalin exhibits anti-inflammatory activity by binding to chemokines. *Immunopharmacology* 49(3), 295–306.
- Li, X., Loberg, R., Liao, J., Ying, C., Snyder, L. A., Pienta, K. J., et al. (2009). A destructive cascade mediated by CCL2 facilitates prostate cancer growth in bone. *Cancer Res* 69(4), 1685–1692.
- Li, H., Yang, W., Chen, P. W., Alizadeh, H., & Niederkorn, J. Y. (2009). Inhibition of chemokine receptor expression on uveal melanomas by CXCR4 siRNA blocks tumor cell invasion and liver metastasis of uveal melanoma cells. *Invest Ophthalmol Vis Sci*.
- Lim, J. K., Louie, C. Y., Glaser, C., Jean, C., Johnson, B., Johnson, H., et al. (2008). Genetic deficiency of chemokine receptor CCR5 is a strong risk factor for symptomatic West Nile virus infection: a meta-analysis of 4 cohorts in the US epidemic. *J Infect Dis* 197(2), 262–265.
- Liu, C., Ueno, T., Kuse, S., Saito, F., Nitta, T., Piali, L., et al. (2005). The role of CCL21 in recruitment of T-precursor cells to fetal thymus. *Blood* 105(1), 31–39.
- Liu, L., Dai, E., Miller, L., Seet, B., Lalani, A., Macauley, C., et al. (2004). Viral chemokine-binding proteins inhibit inflammatory responses and aortic allograft transplant vasculopathy in rat models. *Transplantation* 77(11), 1652–1660.
- Liu, L., Lalani, A., Dai, E., Seet, B., Macauley, C., Singh, R., et al. (2000). The viral anti-inflammatory chemokine-binding protein M-T7 reduces intimal hyperplasia after vascular injury. *J Clin Invest* 105(11), 1613–1621.
- Liu, M. T., Armstrong, D., Hamilton, T. A., & Lane, T. E. (2001). Expression of Mig (monokine induced by interferon-gamma) is important in T lymphocyte recruitment and host defense following viral infection of the central nervous system. *J Immunol* 166(3), 1790–1795.
- Liu, M. T., Keirstead, H. S., & Lane, T. E. (2001). Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. *J Immunol* 167(7), 4091–4097.
- Loberg, R. D., Ying, C., Craig, M., Day, L. L., Sargent, E., Neeley, C., et al. (2007). Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. *Cancer Res* 67(19), 9417–9424.
- Lu, B., Rutledge, B. J., Gu, L., Fiorillo, J., Lukacs, N. W., Kunkel, S. L., et al. (1998). Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* 187(4), 601–608.
- Lukacs, N. W., Berlin, A., Schols, D., Skerlj, R. T., & Bridger, G. J. (2002). AMD3100, a CXCR4 antagonist, attenuates allergic lung inflammation and airway hyperreactivity. *Am J Pathol* 160(4), 1353–1360.
- Luker, K., Gupta, M., & Luker, G. (2009). Bioluminescent CXCL12 fusion protein for cellular studies of CXCR4 and CXCR7. *Biotechniques* 47(1), 625–632.
- Luker, K. E., Gupta, M., Steele, J. M., Foerster, B. R., & Luker, G. D. (2009). Imaging ligand-dependent activation of CXCR7. *Neoplasia* 11(10), 1022–1035.
- Lutgens, E., Faber, B., Schapira, K., Evelo, C. T., van Haften, R., Heeneman, S., et al. (2005). Gene profiling in atherosclerosis reveals a key role for small inducible cytokines: validation using a novel monocyte chemoattractant protein monoclonal antibody. *Circulation* 111(25), 3443–3452.
- Ma, B., Zhu, Z., Homer, R. J., Gerard, C., Strieter, R., & Elias, J. A. (2004). The C10/CCL6 chemokine and CCR1 play critical roles in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* 172(3), 1872–1881.
- Madrigal, J. L., Leza, J. C., Polak, P., Kalinin, S., & Feinstein, D. L. (2009). Astrocyte-derived MCP-1 mediates neuroprotective effects of noradrenaline. *J Neurosci* 29(1), 263–267.
- Magge, S. N., Malik, S. Z., Royo, N. C., Chen, H. I., Yu, L., Snyder, E. Y., et al. (2009). Role of monocyte chemoattractant protein-1 (MCP-1/CCL2) in migration of neural progenitor cells toward glial tumors. *J Neurosci Res* 87(7), 1547–1555.
- Mahabaleshwar, H., Boldajipour, B., & Raz, E. (2008). Killing the messenger: the role of CXCR7 in regulating primordial germ cell migration. *Cell Adh Migr* 2(2), 69–70.
- Maillet, E. L., Pellegrini, N., Valant, C., Bucher, B., Hibert, M., Bourguignon, J. J., et al. (2007). A novel, conformation-specific allosteric inhibitor of the tachykinin NK2 receptor (NK2R) with functionally selective properties. *Faseb J* 21(9), 2124–2134.
- Man, S. M., Ma, Y. R., Shang, D. S., Zhao, W. D., Li, B., Guo, D. W., et al. (2007). Peripheral T cells overexpress MIP-1 α to enhance its transendothelial migration in Alzheimer's disease. *Neurobiol Aging* 28(4), 485–496.
- Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature* 454(7203), 436–444.
- Mantovani, A., Bonocchi, R., Locati, M. (2006). Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol* 6(12), 907–918.
- Mantovani, A., Garlanda, C., Locati, M., Rodriguez, T. V., Feo, S. G., Savino, B., et al. (2007). Regulatory pathways in inflammation. *Autoimmun Rev* 7(1), 8–11.
- Martin, A. P., Canasto-Chibuque, C., Shang, L., Rollins, B. J., & Lira, S. A. (2006). The chemokine decoy receptor M3 blocks CC chemokine ligand 2 and CXC chemokine ligand 13 function in vivo. *J Immunol* 177(10), 7296–7302.
- Martinez de la Torre, Y., Locati, M., Buracchi, C., Duporcq, J., Cook, D. N., Bonocchi, R., et al. (2005). Increased inflammation in mice deficient for the chemokine decoy receptor D6. *Eur J Immunol* 35(5), 1342–1346.
- Matin, K., Salam, M. A., Akhter, J., Hanada, N., & Senpuku, H. (2002). Role of stromal-cell derived factor-1 in the development of autoimmune diseases in non-obese diabetic mice. *Immunology* 107(2), 222–232.
- Matthews, S. P., Tregoning, J. S., Coyle, A. J., Hussels, T., & Openshaw, P. J. (2005). Role of CCL11 in eosinophilic lung disease during respiratory syncytial virus infection. *J Virol* 79(4), 2050–2057.
- Mayer, K. L., & Stone, M. J. (2000). NMR solution structure and receptor peptide binding of the CC chemokine eotaxin-2. *Biochemistry* 39(29), 8382–8395.
- Mazzinghi, B., Ronconi, E., Lazzeri, E., Sagrinati, C., Ballerini, L., Angelotti, M. L., et al. (2008). Essential but differential role for CXCR4 and CXCR7 in the therapeutic homing of human renal progenitor cells. *J Exp Med* 205(2), 479–490.
- McFadden, G., Lalani, A., Everett, H., Nash, P., & Xu, X. (1998). Virus-encoded receptors for cytokines and chemokines. *Semin Cell Dev Biol* 9(3), 359–368.
- Meijer, J., Zeelenberg, I. S., Sips, B., & Roos, E. (2006). The CXCR5 chemokine receptor is expressed by carcinoma cells and promotes growth of colon carcinoma in the liver. *Cancer Res* 66(19), 9576–9582.
- Meng, C. Q., Ni, L., Worsencroft, K. J., Ye, Z., Weingarten, M. D., Simpson, J. E., et al. (2007). Carboxylated, heteroaryl-substituted chalcones as inhibitors of vascular cell adhesion molecule-1 expression for use in chronic inflammatory diseases. *J Med Chem* 50(6), 1304–1315.
- Meraoua, A., Cizeron-Clairac, G., Panse, R. L., Bismuth, J., Truffault, F., Tallaksen, C., et al. (2006). The chemokine CXCL13 is a key molecule in autoimmune myasthenia gravis. *Blood* 108(2), 432–440.
- Middel, P., Thelen, P., Blaschke, S., Polzien, F., Reich, K., Blaschke, V., et al. (2001). Expression of the T-cell chemoattractant chemokine lymphotactin in Crohn's disease. *Am J Pathol* 159(5), 1751–1761.
- Mizoue, L. S., Bazan, J. F., Johnson, E. C., & Handel, T. M. (1999). Solution structure and dynamics of the CX3C chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX3CR1. *Biochemistry* 38(5), 1402–1414.
- Monod, J., Wyman, J., & Changeux, J. P. (1965). On the nature of allosteric transitions: a plausible model. *J Mol Biol* 12, 88–118.
- Montecarlo, F. S., & Charo, I. F. (1996). The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1 α receptor, confers chemokine selectivity. Evidence for a two-step mechanism for MCP-1 receptor activation. *J Biol Chem* 271(32), 19084–19092.
- Montecarlo, F. S., & Charo, I. F. (1997). The amino-terminal domain of CCR2 is both necessary and sufficient for high affinity binding of monocyte chemoattractant protein 1. Receptor activation by a pseudo-tethered ligand. *J Biol Chem* 272(37), 23186–23190.
- Morales, J., Homey, B., Vicari, A. P., Hudak, S., Oldham, E., Hedrick, J., et al. (1999). CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc Natl Acad Sci U S A* 96(25), 14470–14475.
- Mortier, A., Van Damme, J., & Proost, P. (2008). Regulation of chemokine activity by posttranslational modification. *Pharmacol Ther* 120(2), 197–217.
- Mueller, A. M., Pedre, X., Killian, S., David, M., & Steinbrecher, A. (2009). The Decoy Receptor 3 (DcR3, TNFRSF6B) suppresses Th17 immune responses and is abundant in human cerebrospinal fluid. *J Neuroimmunol* 209(1–2), 57–64.
- Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410(6824), 50–56.
- Murphy, P. M. (2000). Viral antichemokines: from pathogenesis to drug discovery. *J Clin Invest* 105(11), 1515–1517.
- Murphy, P. M. (2002). International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacol Rev* 54(2), 227–229.
- Murphy, J. W., Cho, Y., Sachpatzidis, A., Fan, C., Hodson, M. E., & Lolis, E. (2007). Structural and functional basis of CXCL12 (stromal cell-derived factor-1 α) binding to heparin. *J Biol Chem* 282(13), 10018–10027.
- Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., et al. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CX3C chemokine PBSF/SDF-1. *Nature* 382(6592), 635–638.
- Nanki, T., Shimaoka, T., Hayashida, K., Taniguchi, K., Yonehara, S., & Miyasaka, N. (2005). Pathogenic role of the CXCL16–CXCR6 pathway in rheumatoid arthritis. *Arthritis Rheum* 52(10), 3004–3014.
- Narumi, S., Kaburaki, T., Yoneyama, H., Iwamura, H., Kobayashi, Y., & Matsushima, K. (2002). Neutralization of IFN-inducible protein 10/CXCL10 exacerbates experimental autoimmune encephalomyelitis. *Eur J Immunol* 32(6), 1784–1791.

- Nelson, A. L., & Reichert, J. M. (2009). Development trends for therapeutic antibody fragments. *Nat Biotechnol* 27(4), 331–337.
- Nibbs, R. J., Gilchrist, D. S., King, V., Ferrar, A., Forrow, S., Hunter, K. D., et al. (2007). The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J Clin Invest* 117(7), 1884–1892.
- Niimi, K., Asano, K., Shiraiishi, Y., Nakajima, T., Wakaki, M., Kagyo, J., et al. (2007). TLR3-mediated synthesis and release of eotaxin-1/CCL11 from human bronchial smooth muscle cells stimulated with double-stranded RNA. *J Immunol* 178(1), 489–495.
- Omari, K. M., Lutz, S. E., Santambrogio, L., Lira, S. A., & Raine, C. S. (2009). Neuroprotection and remyelination after autoimmune demyelination in mice that inducibly overexpress CXCL1. *Am J Pathol* 174(1), 164–176.
- Orimo, A., Gupta, P. B., Sgroi, D. C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., et al. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121(3), 335–348.
- Otsuka, S., & Bebb, G. (2008). The CXCR4/SDF-1 chemokine receptor axis: a new target therapeutic for non-small cell lung cancer. *J Thorac Oncol* 3(12), 1379–1383.
- Palanche, T., Ilien, B., Zoffmann, S., Reck, M. P., Bucher, B., Edelstein, S. J., et al. (2001). The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. *J Biol Chem* 276(37), 34853–34861.
- Pan, J., Mestas, J., Burdick, M. D., Phillips, R. J., Thomas, G. V., Reckamp, K., et al. (2006). Stromal derived factor-1 (SDF-1/CXCL12) and CXCR4 in renal cell carcinoma metastasis. *Mol Cancer* 5, 56.
- Park, M. H., Lee, Y. K., Lee, Y. H., Kim, Y. B., Yun, Y. W., Nam, S. Y., et al. (2009). Chemokines released from astrocytes promote chemokine receptor 5-mediated neuronal cell differentiation. *Exp Cell Res*.
- Pease, J. E., Wang, J., Ponath, P. D., & Murphy, P. M. (1998). The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1alpha and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation. *J Biol Chem* 273(32), 19972–19976.
- Peterson, F. C., Elgin, E. S., Nelson, T. J., Zhang, F., Hoeger, T. J., Linhardt, R. J., et al. (2004). Identification and characterization of a glycosaminoglycan recognition element of the C chemokine lymphotactin. *J Biol Chem* 279(13), 12598–12604.
- Phillips, R. J., Burdick, M. D., Lutz, M., Belperio, J. A., Keane, M. P., & Strieter, R. M. (2003). The stromal derived factor-1/CXCL12-CXC chemokine receptor 4 biological axis in non-small cell lung cancer metastases. *Am J Respir Crit Care Med* 167(12), 1676–1686.
- Piccardoni, P., Evangelista, V., Piccoli, A., de Gaetano, G., Walz, A., & Cerletti, C. (1996). Thrombin-activated human platelets release two NAP-2 variants that stimulate polymorphonuclear leukocytes. *Thromb Haemost* 76(5), 780–785.
- Pinho, V., Oliveira, S. H., Souza, D. G., Vasconcelos, D., Alessandri, A. L., Lukacs, N. W., et al. (2003). The role of CCL22 (MDC) for the recruitment of eosinophils during allergic pleurisy in mice. *J Leukoc Biol* 73(3), 356–362.
- Pitti, R. M., Marsters, S. A., Lawrence, D. A., Roy, M., Kischkel, F. C., Dowd, P., et al. (1998). Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 396(6712), 699–703.
- Pold, M., Zhu, L. X., Sharma, S., Burdick, M. D., Lin, Y., Lee, P. P., et al. (2004). Cyclooxygenase-2-dependent expression of angiogenic CXC chemokines ENA-78/CXC Ligand (CXCL) 5 and interleukin-8/CXCL8 in human non-small cell lung cancer. *Cancer Res* 64(5), 1853–1860.
- Prado, G. N., Suetomi, K., Shumate, D., Maxwell, C., Ravindran, A., Rajarathnam, K., et al. (2007). Chemokine signaling specificity: essential role for the N-terminal domain of chemokine receptors. *Biochemistry* 46(31), 8961–8968.
- Proudfoot, A. E., Fritchley, S., Borlat, F., Shaw, J. P., Vilbois, F., Zwahlen, C., et al. (2001). The BBXB motif of RANTES is the principal site for heparin binding and controls receptor selectivity. *J Biol Chem* 276(14), 10620–10626.
- Proudfoot, A. E., Handel, T. M., Johnson, Z., Lau, E. K., LiWang, P., Clark-Lewis, I., et al. (2003). Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A* 100(4), 1885–1890.
- Pruenster, M., Muddle, L., Bombosi, P., Dimitrova, S., Zsak, M., Middleton, J., et al. (2009). The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nat Immunol* 10(1), 101–108.
- Qin, X. J., Shi, H. Z., Deng, J. M., Liang, Q. L., Jiang, J., & Ye, Z. J. (2009). CCL22 recruits CD4-positive CD25-positive regulatory T cells into malignant pleural effusion. *Clin Cancer Res* 15(7), 2231–2237.
- Rahaman, S. O., Sharma, P., Harbor, P. C., Aman, M. J., Vogelbaum, M. A., & Haque, S. J. (2002). IL-13R(alpha)2, a decoy receptor for IL-13 acts as an inhibitor of IL-4-dependent signal transduction in glioblastoma cells. *Cancer Res* 62(4), 1103–1109.
- Raimundo, B. C., Oslob, J. D., Braisted, A. C., Hyde, J., McDowell, R. S., Randal, M., et al. (2004). Integrating fragment assembly and biophysical methods in the chemical advancement of small-molecule antagonists of IL-2: an approach for inhibiting protein-protein interactions. *J Med Chem* 47(12), 3111–3130.
- Rajagopalan, L., & Rajarathnam, K. (2004). Ligand selectivity and affinity of chemokine receptor CXCR1, role of N-terminal domain. *J Biol Chem* 279(29), 30000–30008.
- Raz, E., & Mahabaleswar, H. (2009). Chemokine signaling in embryonic cell migration: a fish-eye view. *Development* 136(8), 1223–1229.
- Reiss, Y., Proudfoot, A. E., Power, C. A., Campbell, J. J., & Butcher, E. C. (2001). CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J Exp Med* 194(10), 1541–1547.
- Reutershan, J., Harry, B., Chang, D., Bagby, G. J., & Ley, K. (2009). DARC on RBC limits lung injury by balancing compartmental distribution of CXC chemokines. *Eur J Immunol* 39(6), 1597–1607.
- Rosenkilde, M. M. (2005). Virus-encoded chemokine receptors—putative novel antiviral drug targets. *Neuropharmacology* 48(1), 1–13.
- Ross, R. J., Zhou, M., Shen, D., Fariss, R. N., Ding, X., Bojanowski, C. M., et al. (2008). Immunological protein expression profile in Ccl2/Cx3cr1 deficient mice with lesions similar to age-related macular degeneration. *Exp Eye Res* 86(4), 675–683.
- Rostene, W., Kitabgi, P., & Parsadaniantz, S. M. (2007). Chemokines: a new class of neuromodulator? *Nat Rev Neurosci* 8(11), 895–903.
- Rothenberg, M. E., MacLean, J. A., Pearlman, E., Luster, A. D., & Leder, P. (1997). Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J Exp Med* 185(4), 785–790.
- Roychoudhury, K., Dasgupta, B., Sen, P., Laskay, T., Solbach, W., De, T., et al. (2006). Evidence of direct interactions between the CC-chemokines CCL3, CCL4 and CCL5 and *Leishmania* promastigotes. *Mol Biochem Parasitol* 150(2), 374–377.
- Rupprecht, T. A., Koedel, U., Muhlberger, B., Wilske, B., Fontana, A., & Pfister, H. W. (2005). CXCL11 is involved in leucocyte recruitment to the central nervous system in neuroborreliosis. *J Neurol* 252(7), 820–823.
- Sadir, R., Imberty, A., Baleux, F., & Lortat-Jacob, H. (2004). Heparan sulfate/heparin oligosaccharides protect stromal cell-derived factor-1 (SDF-1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. *J Biol Chem* 279(42), 43854–43860.
- Sakai, N., Wada, T., Yokoyama, H., Lipp, M., Ueha, S., Matsushima, K., et al. (2006). Secondary lymphoid tissue chemokine (SLC/CCL21)/CCR7 signaling regulates fibrocytes in renal fibrosis. *Proc Natl Acad Sci U S A* 103(38), 14098–14103.
- Salazar-Mather, T. P., Orange, J. S., & Biron, C. A. (1998). Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1alpha (MIP-1alpha)-dependent pathways. *J Exp Med* 187(1), 1–14.
- Salmaggi, A., Gelati, M., Dufour, A., Corsini, E., Pagano, S., Baccalini, R., et al. (2002). Expression and modulation of IFN-gamma-inducible chemokines (IP-10, Mig, and I-TAC) in human brain endothelium and astrocytes: possible relevance for the immune invasion of the central nervous system and the pathogenesis of multiple sclerosis. *J Interferon Cytokine Res* 22(6), 631–640.
- Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., et al. (1996). Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 382(6593), 722–725.
- Sancho, M., Vieira, J. M., Casalou, C., Mesquita, M., Pereira, T., Cavaco, B. M., et al. (2006). Expression and function of the chemokine receptor CCR7 in thyroid carcinomas. *J Endocrinol* 191(1), 229–238.
- Santiago, B., Baleux, F., Palao, G., Gutierrez-Canas, I., Ramirez, J. C., Arenzana-Seisdedos, F., et al. (2006). CXCL12 is displayed by rheumatoid endothelial cells through its basic amino-terminal motif on heparan sulfate proteoglycans. *Arthritis Res Ther* 8(2), R43.
- Schadt, E. E., Friend, S. H., & Shaywitz, D. A. (2009). A network view of disease and compound screening. *Nat Rev Drug Discov* 8(4), 286–295.
- Schippeling, D. S., & Martin, R. (2008). Spotlight on anti-CD25: daclizumab in MS. *Int MS J* 15(3), 94–98.
- Schnyder-Candrian, S., Togbe, D., Couillin, I., Mercier, I., Brombacher, F., Quesniaux, V., et al. (2006). Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* 203(12), 2715–2725.
- Schulz, M., Fuster, M. M., Brown, J. R., Crawford, B. E., Ditto, D. P., Lawrence, R., et al. (2008). Surfen, a small molecule antagonist of heparan sulfate. *Proc Natl Acad Sci U S A* 105(35), 13075–13080.
- Schulz, C., Schafer, A., Stolla, M., Kerstan, S., Lorenz, M., von Bruhl, M. L., et al. (2007). Chemokine fractalkine mediates leukocyte recruitment to inflammatory endothelial cells in flowing whole blood: a critical role for P-selectin expressed on activated platelets. *Circulation* 116(7), 764–773.
- Scola, A. M., Johswich, K. O., Morgan, B. P., Klos, A., & Monk, P. N. (2009). The human complement fragment receptor, C5L2, is a recycling decoy receptor. *Mol Immunol* 46(6), 1149–1162.
- Scotton, C. J., Wilson, J. L., Scott, K., Stamp, G., Wilbanks, G. D., Fricker, S., et al. (2002). Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. *Cancer Res* 62(20), 5930–5938.
- Seegerer, S., Regele, H., Mac, K. M., Kain, R., Cartron, J. P., Colin, Y., et al. (2000). The Duffy antigen receptor for chemokines is up-regulated during acute renal transplant rejection and crescentic glomerulonephritis. *Kidney Int* 58(4), 1546–1556.
- Shaw, J. P., Johnson, Z., Borlat, F., Zwahlen, C., Kungl, A., Roulin, K., et al. (2004). The X-ray structure of RANTES: heparin-derived disaccharides allows the rational design of chemokine inhibitors. *Structure* 12(11), 2081–2093.
- Shimaoka, T., Nakayama, T., Kume, N., Takahashi, S., Yamaguchi, J., Minami, M., et al. (2003). Cutting edge: SR-PSOX/CXC chemokine ligand 16 mediates bacterial phagocytosis by APCs through its chemokine domain. *J Immunol* 171(4), 1647–1651.
- Sierro, F., Biben, C., Martinez-Munoz, L., Mellado, M., Ransohoff, R. M., Li, M., et al. (2007). Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci U S A* 104(37), 14759–14764.
- Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., et al. (1997). Osteopontin: a novel secreted protein involved in the regulation of bone density. *Cell* 89(2), 309–319.
- Smith, E., McGettrick, H. M., Stone, M. A., Shaw, J. S., Middleton, J., Nash, G. B., et al. (2008). Duffy antigen receptor for chemokines and CXCL5 are essential for the recruitment of neutrophils in a multicellular model of rheumatoid arthritis synovium. *Arthritis Rheum* 58(7), 1968–1973.
- Soares, D. M., Figueiredo, M. J., Martins, J. M., Machado, R. R., Kanashiro, A., Malvar Ddo, C., et al. (2009). CCL3/MIP-1 alpha is not involved in the LPS-induced fever and its pyrogenic activity depends on CRF. *Brain Res* 1269, 54–60.
- Son, K. N., Hwang, J., Kwon, B. S., & Kim, J. (2006). Human CC chemokine CCL23 enhances expression of matrix metalloproteinase-2 and invasion of vascular endothelial cells. *Biochem Biophys Res Commun* 340(2), 498–504.

- Sozzani, S., Luini, W., Bianchi, G., Allavena, P., Wells, T. N., Napolitano, M., et al. (1998). The viral chemokine macrophage inflammatory protein-II is a selective Th2 chemoattractant. *Blood* 92(11), 4036–4039.
- Stafford, S., Li, H., Forsythe, P. A., Ryan, M., Bravo, R., & Alam, R. (1997). Monocyte chemoattractant protein-3 (MCP-3)/fibroblast-induced cytokine (FIC) in eosinophilic inflammation of the airways and the inhibitory effects of an anti-MCP-3/FIC antibody. *J Immunol* 158(10), 4953–4960.
- Steinhauser, M. L., Hogaboam, C. M., Matsukawa, A., Lukacs, N. W., Strieter, R. M., & Kunkel, S. L. (2000). Chemokine C10 promotes disease resolution and survival in an experimental model of bacterial sepsis. *Infect Immun* 68(11), 6108–6114.
- Suzuki, F., Nanki, T., Imai, T., Kikuchi, H., Hirohata, S., Kohsaka, H., et al. (2005). Inhibition of CX3CL1 (fractalkine) improves experimental autoimmune myositis in SJL/J mice. *J Immunol* 175(10), 6987–6996.
- Takahashi, H., Tashiro, T., Miyazaki, M., Kobayashi, M., Pollard, R. B., & Suzuki, F. (2002). An essential role of macrophage inflammatory protein 1alpha/CCL3 on the expression of host's innate immunities against infectious complications. *J Leukoc Biol* 72(6), 1190–1197.
- Thanos, C. D., DeLano, W. L., & Wells, J. A. (2006). Hot-spot mimicry of a cytokine receptor by a small molecule. *Proc Natl Acad Sci U S A* 103(42), 15422–15427.
- Thelen, M., & Thelen, S. (2008). CXCR7, CXCR4 and CXCL12: an eccentric trio? *J Neuroimmunol* 198(1–2), 9–13.
- Threlfell, S., Exley, R., Cragg, S. J., & Greenfield, S. A. (2008). Constitutive histamine H2 receptor activity regulates serotonin release in the substantia nigra. *J Neurochem* 107(3), 745–755.
- Tilley, J. W., Chen, L., Fry, D. C., Emerson, S. D., Power, G. D., Biondi, D., et al. (1997). Identification of a small molecule inhibitor of the IL-2/IL-2R α receptor interaction which binds to IL-2. *J Amer Chem Soc* 119, 7589–7590.
- Townson, J. R., & Nibbs, R. J. (2002). Characterization of mouse CXCR-CKR, a receptor for the lymphocyte-attracting chemokines TECK/mCCL25, SLC/mCCL21 and MIP-3beta/mCCL19: comparison to human CXCR-CKR. *Eur J Immunol* 32(5), 1230–1241.
- Tuinstra, R. L., Peterson, F. C., Kutlesa, S., Elgin, E. S., Kron, M. A., & Volkman, B. F. (2008). Interconversion between two unrelated protein folds in the lymphotactin native state. *Proc Natl Acad Sci U S A* 105(13), 5057–5062.
- Ueha, S., Murai, M., Yoneyama, H., Kitabatake, M., Imai, T., Shimaoka, T., et al. (2007). Intervention of MAdCAM-1 or fractalkine alleviates graft-versus-host reaction associated intestinal injury while preserving graft-versus-tumor effects. *J Leukoc Biol* 81(1), 176–185.
- Vaday, G. G., Peehl, D. M., Kadam, P. A., & Lawrence, D. M. (2006). Expression of CCL5 (RANTES) and CCR5 in prostate cancer. *Prostate* 66(2), 124–134.
- Valenzuela, J. G., Charlab, R., Mather, T. N., & Ribeiro, J. M. (2000). Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis*. *J Biol Chem* 275(25), 18717–18723.
- Valenzuela-Fernandez, A., Palanche, T., Amara, A., Magerus, A., Altmeyer, R., Delaunay, T., et al. (2001). Optimal inhibition of X4 HIV isolates by the CXCR chemokine stromal cell-derived factor 1 alpha requires interaction with cell surface heparan sulfate proteoglycans. *J Biol Chem* 276(28), 26550–26558.
- van Berkel, V., Barrett, J., Tiffany, H. L., Fremont, D. H., Murphy, P. M., McFadden, G., et al. (2000). Identification of a gammaherpesvirus selective chemokine binding protein that inhibits chemokine action. *J Virol* 74(15), 6741–6747.
- van der Voort, R., Kramer, M., Lindhout, E., Torensma, R., Eleveld, D., van Lieshout, A. W., et al. (2005). Novel monoclonal antibodies detect elevated levels of the chemokine CCL18/DC-CK1 in serum and body fluids in pathological conditions. *J Leukoc Biol* 77(5), 739–747.
- Vancova, I., Slovák, M., Hajnická, V., Labuda, M., Simo, L., Peterkova, K., et al. (2007). Differential anti-chemokine activity of *Amblyomma variegatum* adult ticks during blood-feeding. *Parasite Immunol* 29(4), 169–177.
- Vandercappellen, J., Van Damme, J., & Struyf, S. (2008). The role of CXC chemokines and their receptors in cancer. *Cancer Lett* 267(2), 226–244.
- Veldkamp, C. T., Peterson, F. C., Pelzek, A. J., & Volkman, B. F. (2005). The monomer-dimer equilibrium of stromal cell-derived factor-1 (CXCL 12) is altered by pH, phosphate, sulfate, and heparin. *Protein Sci* 14(4), 1071–1081.
- Veldkamp, C. T., Seibert, C., Peterson, F. C., De la Cruz, N. B., Haugner, J. C., III, Basnet, H., et al. (2008). Structural basis of CXCR4 sulfotyrosine recognition by the chemokine SDF-1/CXCL12. *Sci Signal* 1(37), ra4.
- Vergote, D., Butler, G. S., Ooms, M., Cox, J. H., Silva, C., Hollenberg, M. D., et al. (2006). Proteolytic processing of SDF-1alpha reveals a change in receptor specificity mediating HIV-associated neurodegeneration. *Proc Natl Acad Sci U S A* 103(50), 19182–19187.
- Vollmer, J. Y., Alix, P., Chollet, A., Takeda, K., & Galzi, J. L. (1999). Subcellular compartmentalization of activation and desensitization of responses mediated by NK2 neurokinin receptors. *J Biol Chem* 274(53), 37915–37922.
- Wada, T., Sakai, N., Matsushima, K., & Kaneko, S. (2007). Fibrocytes: a new insight into kidney fibrosis. *Kidney Int* 72(3), 269–273.
- Waldmann, T. A., & O'Shea, J. (1998). The use of antibodies against the IL-2 receptor in transplantation. *Curr Opin Immunol* 10(5), 507–512.
- Walsh, K. B., Edwards, R. A., Romero, K. M., Kotlajich, M. V., Stohlman, S. A., & Lane, T. E. (2007). Expression of CXC chemokine ligand 10 from the mouse hepatitis virus genome results in protection from viral-induced neurological and liver disease. *J Immunol* 179(2), 1155–1165.
- Wang, A., Fairhurst, A. M., Tus, K., Subramanian, S., Liu, Y., Lin, F., et al. (2009). CXCR4/CXCL12 hyperexpression plays a pivotal role in the pathogenesis of lupus. *J Immunol* 182(7), 4448–4458.
- Wang, B., Hendricks, D. T., Wamunyokoli, F., & Parker, M. I. (2006). A growth-related oncogene/CXC chemokine receptor 2 autocrine loop contributes to cellular proliferation in esophageal cancer. *Cancer Res* 66(6), 3071–3077.
- Wang, J. D., Nonomura, N., Takahara, S., Li, B. S., Azuma, H., Ichimaru, N., et al. (1998). Lymphotactin: a key regulator of lymphocyte trafficking during acute graft rejection. *Immunology* 95(1), 56–61.
- Wang, J., Ou, Z. L., Hou, Y. F., Luo, J. M., Shen, Z. Z., Ding, J., et al. (2006). Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential. *Oncogene* 25(54), 7201–7211.
- Wang, J., Shiozawa, Y., Wang, J., Wang, Y., Jung, Y., Pienta, K. J., et al. (2008). The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. *J Biol Chem* 283(7), 4283–4294.
- Wang, Q., Yu, H., Zhang, L., Ju, D., Pan, J., Xia, D., et al. (2002). Adenovirus-mediated intratumoral lymphotactin gene transfer potentiates the antibody-targeted superantigen therapy of cancer. *J Mol Med* 80(9), 585–594.
- Waxman, L., Smith, D. E., Arcuri, K. E., & Vlasuk, G. P. (1990). Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. *Science* 248(4955), 593–596.
- Weber, M., Blair, E., Simpson, C. V., O'Hara, M., Blackburn, P. E., Rot, A., et al. (2004). The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines. *Mol Biol Cell* 15(5), 2492–2508.
- Weill, N., & Rognan, D. (2009). Development and validation of a novel protein-ligand fingerprint to mine chemogenomic space: application to G protein-coupled receptors and their ligands. *J Chem Inf Model* 49(4), 1049–1062.
- Wells, J. A., & McClendon, C. L. (2007). Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature* 450(7172), 1001–1009.
- Wells, T. N., Power, C. A., Shaw, J. P., & Proudfoot, A. E. (2006). Chemokine blockers—therapeutics in the making? *Trends Pharmacol Sci* 27(1), 41–47.
- Wermuth, C. G. (2006). Selective optimization of side activities: the SOSA approach. *Drug Discov Today* 11(3–4), 160–164.
- Wesolowski, J., Alzogaray, V., Reyelt, J., Unger, M., Juarez, K., Urrutia, M., et al. (2009). Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol* 198(3), 157–174.
- Whiting, D., Hsieh, G., Yun, J. J., Banerji, A., Yao, W., Fishbein, M. C., et al. (2004). Chemokine monokine induced by IFN-gamma/CXC chemokine ligand 9 stimulates T lymphocyte proliferation and effector cytokine production. *J Immunol* 172(12), 7417–7424.
- Wu, F. Y., Ou, Z. L., Feng, L. Y., Luo, J. M., Wang, L. P., Shen, Z. Z., et al. (2008). Chemokine decoy receptor d6 plays a negative role in human breast cancer. *Mol Cancer Res* 6(8), 1276–1288.
- Xu, H., Xu, W., Chu, Y., Gong, Y., Jiang, Z., & Xiong, S. (2005). Involvement of up-regulated CXC chemokine ligand 16/scavenger receptor that binds phosphatidylserine and oxidized lipoprotein in endotoxin-induced lethal liver injury via regulation of T-cell recruitment and adhesion. *Infect Immun* 73(7), 4007–4016.
- Xue, M. L., Thakur, A., Cole, N., Lloyd, A., Stapleton, F., Wakefield, D., et al. (2007). A critical role for CCL2 and CCL3 chemokines in the regulation of polymorphonuclear neutrophils recruitment during corneal infection in mice. *Immunol Cell Biol* 85(7), 525–531.
- Yang, S. H., Kim, S. J., Kim, N., Oh, J. E., Lee, J. G., Chung, N. H., et al. (2008). NKT cells inhibit the development of experimental crescentic glomerulonephritis. *J Am Soc Nephrol* 19(9), 1663–1671.
- Yang, M., Mailhot, G., MacKay, C. A., Mason-Savas, A., Aubin, J., & Odgren, P. R. (2006). Chemokine and chemokine receptor expression during colony stimulating factor-1-induced osteoclast differentiation in the toothless osteopetrotic rat: a key role for CCL9 (MIP-1gamma) in osteoclastogenesis in vivo and in vitro. *Blood* 107(6), 2262–2270.
- Ye, J., Kohli, L. L., & Stone, M. J. (2000). Characterization of binding between the chemokine eotaxin and peptides derived from the chemokine receptor CCR3. *J Biol Chem* 275(35), 27250–27257.
- Yoneyama, H., Harada, A., Imai, T., Baba, M., Yoshie, O., Zhang, Y., et al. (1998). Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice. *J Clin Invest* 102(11), 1933–1941.
- Young, K. C., Torres, E., Hatzistergos, K. E., Hehre, D., Suguihara, C., & Hare, J. M. (2009). Inhibition of the SDF-1/CXCR4 axis attenuates neonatal hypoxia-induced pulmonary hypertension. *Circ Res* 104(11), 1293–1301.
- Yue, Y., Xu, W., Hu, L., Jiang, Z., & Xiong, S. (2009). Enhanced resistance to coxsackievirus B3-induced myocarditis by intranasal co-immunization of lymphotactin gene encapsulated in chitosan particle. *Virology* 386(2), 438–447.
- Zabel, B. A., Wang, Y., Lewen, S., Berahovich, R. D., Penfold, M. E., Zhang, P., et al. (2009). Elucidation of CXCR7-mediated signaling events and inhibition of CXCR4-mediated tumor cell transendothelial migration by CXCR7 ligands. *J Immunol* 183(5), 3204–3211.
- Zheng, B., Ozen, Z., Zhang, X., De Silva, S., Marinova, E., Guo, L., et al. (2005). CXCL13 neutralization reduces the severity of collagen-induced arthritis. *Arthritis Rheum* 52(2), 620–626.
- Zhu, Y. M., Bagstaff, S. M., & Woll, P. J. (2006). Production and upregulation of granulocyte chemoattractant protein-2/CXCL6 by IL-1beta and hypoxia in small cell lung cancer. *Br J Cancer* 94(12), 1936–1941.
- Zhu, Y. M., Webster, S. J., Flower, D., & Woll, P. J. (2004). Interleukin-8/CXCL8 is a growth factor for human lung cancer cells. *Br J Cancer* 91(11), 1970–1976.
- Zoffmann, S., Chollet, A., & Galzi, J. L. (2002). Identification of the extracellular loop 2 as the point of interaction between the N terminus of the chemokine MIP-1alpha and its CCR1 receptor. *Mol Pharmacol* 62(3), 729–736.
- Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., & Littman, D. R. (1998). Function of the chemokine receptor CXCR4 in hematopoiesis and in cerebellar development. *Nature* 393(6685), 595–599.

BIBLIOGRAPHIE

1. Abroun S. (2008) **Chemokines in Homeostasis and Cancers**. *Yakhteh Medical Journal*, Vol 10, No 3, Pages: 155-166
2. Acharya M, Borland G, Edkins AL, Maclellan LM, Matheson J, Ozanne BW, Cushley W. (2010) **CD23/FcεRII: molecular multi-tasking**. *Clin Exp Immunol*. 162(1):12-23.
3. Adler A, Cieslewicz G, Irvin CG. (2004) **Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice**. *J Appl Physiol*. 97(1):286-92.
4. Adler MW, Rogers TJ. (2005) **Are chemokines the third major system in the brain?** *J Leukoc Biol*. 78(6):1204-9.
5. Ahn S, Shenoy SK, Wei H, Lefkowitz RJ. (2004) **Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor**. *J Biol Chem*. 279(34):35518-25.
6. Ahr B, Denizot M, Robert-Hebmann V, Brelot A, Biard-Piechaczyk M. (2005) **Identification of the cytoplasmic domains of CXCR4 involved in Jak2 and STAT3 phosphorylation**. *J Biol Chem*. 280(8):6692-700.
7. Akbari O, Umetsu DT. (2005) **Role of regulatory dendritic cells in allergy and asthma**. *Curr Allergy Asthma Rep*. 5(1):56-61.
8. Akdis CA, Akdis M. (2009) **Mechanisms and treatment of allergic disease in the big picture of regulatory T cells**. *J Allergy Clin Immunol*. 123(4):735-46
9. Akdis CA, Akdis M. (2011) **Mechanisms of allergen-specific immunotherapy**. *J Allergy Clin Immunol*. 127(1):18-27
10. Ali S, Mustafa SJ, Metzger WJ. (1994) **Adenosine-induced bronchoconstriction and contraction of airway smooth muscle from allergic rabbits with late-phase airway obstruction: evidence for an inducible adenosine A1 receptor**. *J Pharmacol Exp Ther*. 268(3):1328-34.
11. Ali S, Palmer AC, Banerjee B, Fritchley SJ, Kirby JA. (2000) **Examination of the function of RANTES, MIP-1alpha, and MIP-1beta following interaction with heparin-like glycosaminoglycans**. *J Biol Chem*. 275(16):11721-7.
12. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA. (1996) **CCR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1**. *Science*. 272(5270):1955-8.
13. Allahverdian S, Harada N, Singhera GK, Knight DA, Dorscheid DR. (2008) **Secretion of IL-13 by airway epithelial cells enhances epithelial repair via HB-EGF**. *Am J Respir Cell Mol Biol*. 38(2):153-60.
14. Allen IC, Jania CM, Wilson JE, Tekeppe EM, Hua X, Brickey WJ, Kwan M, Koller BH, Tilley SL, Ting JP. (2012) **Analysis of NLRP3 in the development of allergic airway disease in mice**. *J Immunol*. 188(6):2884-93.
15. Allen SJ, Crown SE, Handel TM. (2007) **Chemokine: receptor structure, interactions, and antagonism**. *Annu Rev Immunol*. 25:787-820.
16. Alphonse MP, Saffar AS, Shan L, HayGlass KT, Simons FE, Gounni AS. (2008) **Regulation of the high affinity IgE receptor (Fc epsilonRI) in human neutrophils: role of seasonal allergen exposure and Th-2 cytokines**. *PLoS One*. 3(4):e1921.
17. Amin K (2012) **The role of mast cells in allergic inflammation**. *Respir Med*. 106(1):9-14.
18. Ammit AJ, Bekir SS, Johnson PR, Hughes JM, Armour CL, Black JL. (1997) **Mast cell numbers are increased in the smooth muscle of human sensitized isolated bronchi**. *Am J Respir Crit Care Med*. 155(3):1123-9.
19. Anderson GP. (2008) **Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease**. *Lancet*. 372(9643):1107-19.
20. Andrew DP, Ruffing N, Kim CH, Miao W, Heath H, Li Y, Murphy K, Campbell JJ, Butcher EC, Wu L. (2001) **C-C chemokine receptor 4 expression defines a major subset of circulating nonintestinal memory T cells of both Th1 and Th2 potential**. *J Immunol*. 166(1):103-11.
21. Arima M, Fukuda T, Hallgren J, Gurish MF, Redhu NS, Saleh A, Shan L, Gerthoffer WT, Kung SK (2011) **Prostaglandin D₂ and T(H)₂ inflammation in the pathogenesis of bronchial asthma**. *Korean J Intern Med*. 26(1):8-18.
22. Arora S, Bhardwaj A, Singh S, Srivastava SK, McClellan S, Nirodi CS, Piazza GA, Grizzle WE, Owen LB, Singh AP. (2013) **An undesired effect of chemotherapy: gemcitabine promotes pancreatic cancer cell invasiveness through reactive oxygen species-dependent, nuclear factor κB- and hypoxia-inducible factor 1α-mediated up-regulation of CXCR4**. *J Biol Chem*. 288(29):21197-207.
23. Auffray C, Fogg DK, Narni-Mancinelli E, Senechal B, Trouillet C, Saederup N, Leemput J, Bigot K, Campisi L, Abitbol M, Molina T, Charo I, Hume DA, Cumano A, Lauvau G, Geissmann F. (2009) **CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation**. *J Exp Med*. 206(3):595-606.

24. Bacharier LB, Geha RS. (2000) **Molecular mechanisms of IgE regulation.** *J Allergy Clin Immunol.* 105(2 Pt 2):S547-58.
25. Bai Z, Hayasaka H, Kobayashi M, Li W, Guo Z, Jang MH, Kondo A, Choi BI, Iwakura Y, Miyasaka M. (2009) **CXC chemokine ligand 12 promotes CCR7-dependent naive T cell trafficking to lymph nodes and Peyer's patches.** *J Immunol.* 182(3):1287-95.
26. Baggiolini M. (1998) **Chemokines and leukocyte traffic.** *Nature,* 392, 565-568.
27. Balabanian K, Brotin E, Biajoux V, Bouchet-Delbos L, Lainey E, Fenneteau O, Bonnet D, Fiette L, Emilie D, Bachelierie F. (2012) **Proper desensitization of CXCR4 is required for lymphocyte development and peripheral compartmentalization in mice.** *Blood.* 119(24):5722-30.
28. Balabanian K, Lagane B, Infantino S, Chow KY, Harriague J, Moepps B, Arenzana-Seisdedos F, Thelen M, Bachelierie F. (2005) **The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes.** *J Biol Chem.* 280(42):35760-6.
29. Balabanian, K., Couderc, J., Bouchet-Delbos, L., Amara, A., Berrebi, D., Foussat, A., Baleux, F., Portier, A., Durand-Gasselín, I., Coffman, R. L., Galanaud, P., Peuchmaur, M., Emilie, D. (2003) **Role of the chemokine stromal cell-derived factor 1 in autoantibody production and nephritis in murine lupus.** *J. Immunol.* 170,3392-3400
30. Balabanian K, Levoye A, Klemm L, Lagane B, Hermine O, Harriague J, Baleux F, Arenzana-Seisdedos F, Bachelierie F. (2008) **Leukocyte analysis from WHIM syndrome patients reveals a pivotal role for GRK3 in CXCR4 signaling.** *J Clin Invest.* 118(3):1074-84.
31. Balbo P, Silvestri M, Rossi GA, Crimi E, Burastero SE. (2001) **Differential role of CD80 and CD86 on alveolar macrophages in the presentation of allergen to T lymphocytes in asthma.** *Clin Exp Allergy.* 31(4):625-36.
32. Balhara J, Gounni AS. (2012) **The alveolar macrophages in asthma: a double-edged sword.** *Mucosal Immunol.* 5(6):605-9
33. Balkwill F (2004) **The significance of cancer cell expression of the chemokine receptor CXCR4.** *Semin Cancer Biol.* 14(3):171-9.
34. Baniadr G, Skrzydelski D, Kitabgi P, Rostène W, Parsadaniantz SM. (2003) **Highly regionalized distribution of stromal cell-derived factor-1/CXCL12 in adult rat brain: constitutive expression in cholinergic, dopaminergic and vasopressinergic neurons.** *Eur J Neurosci.* 18(6):1593-606.
35. Barnes PJ. (1996) **Pathophysiology of asthma.** *Br J Clin Pharmacol.* 42(1):3-10.
36. Barnes PJ. **Immunology of asthma and chronic obstructive pulmonary disease.** *Nat Rev Immunol.* 2008 Mar;8(3):183-92.
37. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. (2008) **Growth factors and cytokines in wound healing.** *Wound Repair Regen.* 2008 Sep-Oct;16(5):585-601. doi: 10.1111/j.1524-475X.2008.00410.x.
38. Bates JH, Irvin CG. (2003) **Measuring lung function in mice: the phenotyping uncertainty principle.** *J Appl Physiol.* 94(4):1297-306.
39. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A, Schall TJ. (1997) **A new class of membrane-bound chemokine with a CX3C motif.** *Nature.* 385(6617):640-4.
40. Bedoret D, Wallemacq H, Marichal T, Desmet C, Quesada Calvo F, Henry E, Closset R, Dewals B, Thielen C, Gustin P, de Leval L, Van Rooijen N, Le Moine A, Vanderplasschen A, Cataldo D, Drion PV, Moser M, Lekeux P, Bureau F. (2011) **Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice.** *J Clin Invest.* 119(12):3723-38.
41. Beall CJ, Mahajan S, Kolattukudy PE. (1992) **Conversion of monocyte chemoattractant protein-1 into a neutrophil attractant by substitution of two amino acids.** *J Biol Chem.* 267(5):3455-9.
42. Beaussant Cohen S, Fenneteau O, Plouvier E, Rohrlisch PS, Daltroff G, Plantier I, Dupuy A, Kerob D, Beaupain B, Bordigoni P, Fouyssac F, Delezoide AL, Devouassoux G, Nicolas JF, Bensaid P, Bertrand Y, Balabanian K, Chantelot CB, Bachelierie F, Donadieu J. (2012) **Description and outcome of a cohort of 8 patients with WHIM syndrome from the French Severe Chronic Neutropenia Registry.** *Orphanet J Rare Dis.* 7:71.
43. Bell CW, Jiang W, Reich CF 3rd, Pisetsky DS. (2006) **The extracellular release of HMGB1 during apoptotic cell death.** *Am J Physiol Cell Physiol.* 291(6):C1318-25.
44. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM (2000) **CXC chemokines in angiogenesis.** *J Leukoc Biol.* 68(1):1-8.
45. Bendall L. (2005) **Chemokines and their receptors in disease.** *Histol Histopathol.* 20(3):907-26
46. Bennett LD, Fox JM, Signoret N (2011) **Mechanisms regulating chemokine receptor activity.** *Immunology.* 134(3):246-56.
47. Berahovich RD, Zabel BA, Lewén S, Walters MJ, Ebsworth K, Wang Y, Jaen JC, Schall TJ. (2013) **Endothelial Expression of CXCR7 and the Regulation of Systemic CXCL12 Levels.** *Immunology.*

48. Berchiche YA, Gravel S, Pelletier ME, St-Onge G, Heveker N. (2011) **Different effects of the different natural CC chemokine receptor 2b ligands on beta-arrestin recruitment, Gai signaling, and receptor internalization.** *Mol Pharmacol.* 79(3):488-98.
49. Berghuis D, Schilham MW, Santos SJ, Savola S, Knowles HJ, Dirksen U, Schaefer KL, Vakkila J, Hogendoorn PC, Lankester AC. (2012) **The CXCR4-CXCL12 axis in Ewing sarcoma: promotion of tumor growth rather than metastatic disease.** *Clin Sarcoma Res.* 2012 Dec 18;2(1):24. doi: 10.1186/2045-3329-2-24.
50. Bieniasz PD, Cullen BR. (1998) **Chemokine receptors and human immunodeficiency virus infection.** *Front Biosci.* 1;3:d44-58.
51. Biswas SK, Mantovani A. (2010) **Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm.** *Nat Immunol.* 11(10):889-96.
52. Blyth DI, Pedrick MS, Savage TJ, Hessel EM, Fattah D. (1996) **Lung inflammation and epithelial changes in a murine model of atopic asthma.** *Am J Respir Cell Mol Biol.* 14(5):425-38.
53. Bogunia-Kubik K, Gieryng A, Dlubek D, Lange A. (2009) **The CXCL12-3'A allele is associated with a higher mobilization yield of CD34 progenitors to the peripheral blood of healthy donors for allogeneic transplantation.** *Bone Marrow Transplant.* 44(5):273-8.
54. Bouchery T, Dénécé G, Attout T, Ehrhardt K, Lhermitte-Vallarino N, Hachet-Haas M, Galzi JL, Brotin E, Bachelerie F, Gavotte L, Moulia C, Bain O, Martin C. (2012) **The chemokine CXCL12 is essential for the clearance of the filaria *Litomosoides sigmodontis* in resistant mice.** *PLoS One.* 2012;7(4):e34971. doi: 10.1371/journal.pone.0034971. Epub 2012 Apr 12.
55. Borroni EM, Mantovani A, Locati M, Bonecchi R. (2010) **Chemokine receptors intracellular trafficking.** *Pharmacol Ther.* 127(1):1-8.
56. Botelho FM, Llop-Guevara A, Trimble NJ, Nikota JK, Bauer CM, Lambert KN, Kianpour S, Jordana M, Stämpfli MR. (2011) **Cigarette smoke differentially affects eosinophilia and remodeling in a model of house dust mite asthma.** *Am J Respir Cell Mol Biol.* 45(4):753-60.
57. Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R, Heusser CH, Howarth PH, Holgate ST. (1994) **Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines.** *Am J Respir Cell Mol Biol.* 10(5):471-80.
58. Braun A, Lommatzsch M, Mannsfeldt A, Neuhaus-Steinmetz U, Fischer A, Schnoy N, Lewin GR, Renz H. (1999) **Cellular sources of enhanced brain-derived neurotrophic factor production in a mouse model of allergic inflammation.** *Am J Respir Cell Mol Biol.* 21(4):537-46.
59. Brehm M, Schiller E, Zeller WJ. (1996) **Quantification of reactive oxygen species generated by alveolar macrophages using lucigenin-enhanced chemiluminescence--methodical aspects.** *Toxicol Lett.* 87(2-3):131-8.
60. Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J. (1999) **Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling.** *J Immunol.* 163(12):6448-54
61. Brink R. , Tri Giang Phan, Didrik Paus and Chan T. (2008) **Visualizing the effects of antigen affinity on T-dependent B-cell differentiation** *Immunology and Cell Biology* (2008) 86, 31–39;
62. Broide DH, Finkelman F, Bochner BS, Rothenberg ME. (2011) **Advances in mechanisms of asthma, allergy, and immunology in 2010.** *J Allergy Clin Immunol.* 127(3):689-95.
63. Brown KD, Zurawski SM, Mosmann TR, Zurawski G. (1989) **A family of small inducible proteins secreted by leukocytes are members of a new superfamily that includes leukocyte and fibroblast-derived inflammatory agents, growth factors, and indicators of various activation processes.** *J Immunol.* 142(2):679-87.
64. Broxmeyer HE. (2008) **Chemokines in hematopoiesis.** *Curr Opin Hematol.* 15(1):49-58
65. Broxmeyer, H. E., Orschell, C. M., Clapp, D. W., Hangoc, G., Cooper, S., Plett, P. A., Liles, W. C., Li, X., Graham-Evans, B., Campbell, T. B., Calandra, G., Bridger, G., Dale, D. C., and Srouf, E. F. (2005) **Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist.** *J. Exp. Med.* 201, 1307–1318
66. Brühl H, Cohen CD, Linder S, Kretzler M, Schlöndorff D, Mack M. (2003) **Post-translational and cell type-specific regulation of CXCR4 expression by cytokines.** *Eur J Immunol.* 33(11):3028-37.
67. Brunn GJ, Bungum MK, Johnson GB, Platt JL (2005) **Conditional signaling by Toll-like receptor 4.** *FASEB J.* 19(7):872-4.
68. Bryant J, Ahern DJ, Brennan FM. (2012) **CXCR4 and vascular cell adhesion molecule 1 are key chemokine/adhesion receptors in the migration of cytokine-activated T cells.** *Arthritis Rheum.* 64(7):2137-46.

69. Burgel PR, Nadel JA. (2004) **Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium.** *Thorax.* 59(11):992-6.
70. Burgess JK, Boustany S, Moir LM, Weckmann M, Lau JY, Grafton K, Baraket M, Hansbro PM, Hansbro NG, Foster PS, Black JL, Oliver BG. (2010) **Reduction of tumstatin in asthmatic airways contributes to angiogenesis, inflammation, and hyperresponsiveness.** *Am J Respir Crit Care Med.* 15;181(2):106-15.
71. Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold ME, Sunshine MJ, Littman DR, Kuo CJ, Wei K, McMaster BE, Wright K, Howard MC, Schall TJ. (2006) **A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development.** *J Exp Med.* 203(9):2201-13.
72. Busillo JM, Armando S, Sengupta R, Meucci O, Bouvier M, Benovic JL. (2010) **Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling.** *J Biol Chem.* 285(10):7805-17.
73. Busillo JM, Benovic JL. (2007) **Regulation of CXCR4 signaling.** *Biochim Biophys Acta.* 1768(4):952-63.
74. Byers DE, Holtzman MJ. (2011) **Alternatively activated macrophages and airway disease.** *Chest.* 140(3):768-74
75. Caceres, A.I., Brackmann, M., Elia, M.D., Bessac, B.F., del Camino, D., D'Amours, M., Witek, J.S., Fanger, C.M., Chong, J.A., Hayward, N.J., Homer, R.J., Cohn, L., Huang, X., Moran, M.M., and Jordt, S.E. (2009) **A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma.** *Proc. Natl. Acad. Sci. U.S.A.* 106:9099-9104.
76. Campana L, Bosurgi L, Bianchi ME, Manfredi AA, Rovere-Querini P. (2009) **Requirement of HMGB1 for stromal cell-derived factor-1/CXCL12-dependent migration of macrophages and dendritic cells.** *J Leukoc Biol.* 86(3):609-15.
77. Campanella GS, Colvin RA, Luster AD (2010) **CXCL10 can inhibit endothelial cell proliferation independently of CXCR3.** *PLoS One.* 5(9):e12700.
78. Canning BJ, Chou Y. (2008) **Using guinea pigs in studies relevant to asthma and COPD.** *Pulm Pharmacol Ther.* 21(5):702-20
79. Cantin A, Bilodeau G, Begin R. (1989) **Granulocyte elastase-mediated proteolysis of alpha 1-antitrypsin in cystic fibrosis bronchopulmonary secretions.** *Pediatr Pulmonol.* 7:12-17.
80. Capitini C, Herby S, Milliron M, Anver M, Mackall C, Fry T. (2009) **Bone marrow deficient in IFN- γ signaling selectively reverses GVHD-associated immunosuppression and enhances a tumor-specific GVT effect.** *Blood.* 113:5002-9
81. Cartier L, Hartley O, Dubois-Dauphin M, Krause KH. (2005) **Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases.** *Brain Res Brain Res Rev.* 48(1):16-42.
82. Cataldo DD, Tournoy KG, Vermaelen K, Munaut C, Foidart JM, Louis R, Noël A, Pauwels RA. (2002) **Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation.** *Am J Pathol.* 161(2):491-8.
83. Catusse J, Parry CM, Dewin DR, Gompels UA. (2007) **Inhibition of HIV-1 infection by viral chemokine U83A via high-affinity CCR5 interactions that block human chemokine-induced leukocyte chemotaxis and receptor internalization.** *Blood.* 1;109(9):3633-9.
84. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. (2004) **Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1.** *Nat Med.* 10(8):858-64.
85. Chen G, Zhou D, Kang BC (2001) **A comparative study on cockroach and ovalbumin sensitizations and challenge responses in Hartley guinea-pigs.** *Respir Physiol.* 125(3):239-47.
86. Chen LC, Zhang Z, Myers AC, Huang SK. (2001) **Cutting edge: altered pulmonary eosinophilic inflammation in mice deficient for Clara cell secretory 10-kDa protein.** *J Immunol.* 167(6):3025-8.
87. Chen F, Yang D, Wang S, Che X, Wang J, Li X, Zhang Z, Chen X, Song X. (2012) **Livin regulates prostate cancer cell invasion by impacting the NF- κ B signaling pathway and the expression of FN and CXCR4.** *IUBMB Life.* 64(3):274-83.
88. Chua F, Laurent GJ. (2006) **Neutrophil elastase: mediator of extracellular matrix destruction and accumulation.** *Proc Am Thorac Soc.* 3(5):424-7.
89. Chung SH, Seki K, Choi BI, Kimura KB, Ito A, Fujikado N, Saijo S, Iwakura Y. (2010) **CXC chemokine receptor 4 expressed in T cells plays an important role in the development of collagen-induced arthritis.** *Arthritis Res Ther.* 12(5):R188.
90. Chinni SR, Sivalogan S, Dong Z, Filho JC, Deng X, Bonfil RD, Cher ML. (2006) **CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells: the role of bone microenvironment-associated CXCL12.** *Prostate.* 66(1):32-48.

91. Clària J, González-Pérez A, López-Vicario C, Rius B, Titos E. (2011) **New insights into the role of macrophages in adipose tissue inflammation and Fatty liver disease: modulation by endogenous omega-3 Fatty Acid-derived lipid mediators.** *Front Immunol.* 2:49.
92. Clore GM, Gronenborn AM. (1995) **Three-dimensional structures of alpha and beta chemokines.** *FASEB J.* 9(1):57-62.
93. Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, Lin W, Boyd JG, Moser B, Wood DE, Sahagan BG, Neote K. (1998) **Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3.** *J Exp Med.* 187(12):2009-21.
94. Collington SJ, Williams TJ, Weller CL. (2011) **Mechanisms underlying the localisation of mast cells in tissues.** *Trends Immunol.* 2011 Oct;32(10):478-85.
95. Conrad ML, Yildirim AO, Sonar SS, Kiliç A, Sudowe S, Lunow M, Teich R, Renz H, Garn H. (2009) **Comparison of adjuvant and adjuvant-free murine experimental asthma models.** *Clin Exp Allergy.* 39(8):1246-54.
96. Constantin G. (2008) **Chemokine signaling and integrin activation in lymphocyte migration into the inflamed brain.** *J Neuroimmunol.* 198(1-2):20-6.
97. Cook DN. (1996) **The role of MIP-1 alpha in inflammation and hematopoiesis.** *J Leukoc Biol.* 59(1):61-6.
98. Costello CM, Howell K, Cahill E, McBryan J, Konigshoff M, Eickelberg O, Gaine S, Martin F, McLoughlin P. (2008) **Lung-selective gene responses to alveolar hypoxia: potential role for the bone morphogenetic antagonist gremlin in pulmonary hypertension.** *Am J Physiol Lung Cell Mol Physiol.* 295(2):L272-84.
99. Costello CM, McCullagh B, Howell K, Sands M, Belperio JA, Keane MP, Gaine S, McLoughlin P (2012) **A role for the CXCL12 receptor, CXCR7, in the pathogenesis of human pulmonary vascular disease.** *Eur Respir J.* 39(6):1415-24.
100. Cottrell GS. (2012) **Roles of Proteolysis in Regulation of G Protein-Coupled Receptor Function.** *Br J Pharmacol.* doi: 10.1111/j.1476-5381.2012.02234.x.
101. Creery D, Weiss W, Graziani-Bowering G, Kumar R, Aziz Z, Angel JB, Kumar A. (2006) **Differential regulation of CXCR4 and CCR5 expression by interleukin (IL)-4 and IL-13 is associated with inhibition of chemotaxis and human immunodeficiency Virus (HIV) type 1 replication but not HIV entry into human monocytes.** *Viral Immunol.* 19(3):409-23.
102. Crown SE, Yu Y, Sweeney MD, Leary JA, Handel TM (2006) **Heterodimerization of CCR2 chemokines and regulation by glycosaminoglycan binding.** *J Biol Chem.* 281(35):25438-46.
103. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, Mitsdoerffer M, Strom TB, Elyaman W, Ho IC, Khoury S, Oukka M, Kuchroo VK. (2008) **IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells.** *Nat Immunol.* 9(12):1347-55.
104. Davies DE. (2009) **The role of the epithelium in airway remodeling in asthma.** *Proc Am Thorac Soc.* 6(8):678-82.
105. Denning TL, Norris BA, Medina-Contreras O, Manicassamy S, Geem D, Madan R, Karp CL, Pulendran B. (2011) **Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization.** *J Immunol.* 187(2):733-47.
106. Derendorf H, Nave R, Drollmann A, Cerasoli F, Wurst W. (2006) **Relevance of pharmacokinetics and pharmacodynamics of inhaled corticosteroids to asthma.** *Eur Respir J.* 28(5):1042-50.
107. Devaney JM, Greene CM, Taggart CC, Carroll TP, O'Neill SJ, McElvaney NG. (2003) **Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4.** *FEBS Lett.* 544(1-3):129-32.
108. Di Cesare S, Marshall JC, Logan P, Anteck E, Faingold D, Maloney SC, Burnier MN Jr. (2007) **Expression and migratory analysis of 5 human uveal melanoma cell lines for CXCL12, CXCL8, CXCL1, and HGF.** *J Carcinog.* 29;6:2.
109. Dorman SC, Babirad I, Post J, Watson RM, Foley R, Jones GL, O'Byrne PM, Sehmi R. (2005) **Progenitor egress from the bone marrow after allergen challenge: role of stromal cell-derived factor 1alpha and eotaxin.** *J Allergy Clin Immunol.* 115(3):501-7.
110. Doyle TM, Ellis R, Park HJ, Inman MD, Sehmi R. (2011) **Modulating progenitor accumulation attenuates lung angiogenesis in a mouse model of asthma.** *Eur Respir J.* 38(3):679-87.
111. Draijer C, Robbe P, Boorsma CE, Hylkema MN, Melgert BN. (2013) **Characterization of macrophage phenotypes in three murine models of house-dust-mite-induced asthma.** *Mediators Inflamm.* 2013:632049.
112. Duong HT, Erzurum SC, Asosingh K. (2011) **Pro-angiogenic hematopoietic progenitor cells and endothelial colony-forming cells in pathological angiogenesis of bronchial and pulmonary circulation.** *Angiogenesis.* 14(4):411-22.

113. Dwinell MB, Ogawa H, Barrett KE, Kagnoff MF. (2004) **SDF-1/CXCL12 regulates cAMP production and ion transport in intestinal epithelial cells via CXCR4.** *Am J Physiol Gastrointest Liver Physiol.* 286(5):G844-50.
114. Ebert LM, Schaerli P, Moser B. (2005) **Chemokine-mediated control of T cell traffic in lymphoid and peripheral tissues.** *Mol Immunol.* 42(7):799-809.
115. Eckmann L, Kagnoff MF, Fierer J. (1993) **Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry.** *Infect Immun.* 61(11):4569-74.
116. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. (2002) **Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen.** *J Exp Med.* 196(12):1645-51.
117. Eller K, Wolf D, Huber JM, Metz M, Mayer G, McKenzie AN, Maurer M, Rosenkranz AR, Wolf AM. (2011) **IL-9 production by regulatory T cells recruits mast cells that are essential for regulatory T cell-induced immune suppression.** *J Immunol.* 186(1):83-91.
118. Ewart SL, Kuperman D, Schadt E, Tankersley C, Grupe A, Shubitowski DM, Peltz G, Wills-Karp M. (2000) **Quantitative trait loci controlling allergen-induced airway hyperresponsiveness in inbred mice.** *Am J Respir Cell Mol Biol.* 23(4):537-45.
119. F, Tian Y, Nothnagel HJ, Chu M, Kiefer H, Maier K, De Angelis AA, Marassi FM, Opella SJ. (2012) **Structure of the chemokine receptor CXCR1 in phospholipid bilayers.** *Nature.* 491(7426):779-83.
120. Fahy JV, Corry DB, Boushey HA (2000) **Airway inflammation and remodeling in asthma.** *Curr Opin Pulm Med.* 6(1):15-20.
121. Fahy JV, Woo K, Liu J, Boushey HA. (1995) **Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation.** *J Allergy Clin Immunol.* 95:843-52.
122. Feng LY, Ou ZL, Wu FY, Shen ZZ, Shao ZM. (2009) **Involvement of a novel chemokine decoy receptor CCX-CR in breast cancer growth, metastasis and patient survival.** *Clin Cancer Res.* 15(9):2962-70.
123. Feng Y, Broder CC, Kennedy PE, Berger EA. (1996) **HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.** *Science.* 272(5263):872-7.
124. Feng Y, Broder CC, Kennedy PE, Berger EA. (1996) **Pillars article: HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.** *J Immunol.* 186(11):6076-81.
125. Fernandez-Rodriguez S, Ford WR, Broadley KJ, Kidd EJ (2008) **Establishing the phenotype in novel acute and chronic murine models of allergic asthma.** *Int Immunopharmacol.* 8(5):756-63
126. Fernandis AZ, Cherla RP, Ganju RK. (2003) **Differential regulation of CXCR4-mediated T-cell chemotaxis and mitogen-activated protein kinase activation by the membrane tyrosine phosphatase, CD45.** *J Biol Chem.* 2003 Mar 14;278(11):9536-43. Epub 2003 Jan 8.
127. Feuser K, Feilhauer K, Staib L, Bischoff SC, Lorentz A. (2011) **Akt cross-links IL-4 priming, stem cell factor signaling, and IgE-dependent activation in mature human mast cells.** *Mol Immunol.* 48(4):546-52.
128. Fietta P, Delsante G. (2009) **The effector T helper cell triade.** *Riv Biol.* 102(1):61-74.
129. Fischer BM, Zheng S, Fan R, Voynow JA. (2007) **Neutrophil elastase inhibition of cell cycle progression in airway epithelial cells in vitro is mediated by p27kip1.** *Am J Physiol Lung Cell Mol Physiol.* 293:L762-768
130. Fong AM, Premont RT, Richardson RM, Yu YR, Lefkowitz RJ, Patel DD. (2002) **Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice.** *Proc Natl Acad Sci U S A.* 99(11):7478-83.
131. Forteza R, Laurodo I, Abraham WM, Conner GE. (1999) **Bronchial tissue kallikrein activity is regulated by hyaluronic acid binding.** *Am J Respir Cell Mol Biol.* 21(6):666-74.
132. Fredriksson K, Fielhaber JA, Lam JK, Yao X, Meyer KS, Keeran KJ, Zywicke GJ, Qu X, Yu ZX, Moss J, Kristof AS, Levine SJ. (2012) **Paradoxical effects of rapamycin on experimental house dust mite-induced asthma.** *PLoS One.* 7(5):e33984.
133. Fuchs B, Braun A. (2008) **Improved mouse models of allergy and allergic asthma--chances beyond ovalbumin.** *Curr Drug Targets.* 9(6):495-502.
134. Fujitaka M, Kawaguchi H, Kato Y, Sakura N, Ueda K, Abe Y. (2001) **Significance of the eosinophil cationic protein/eosinophil count ratio in asthmatic patients: its relationship to disease severity.** *Ann Allergy Asthma Immunol.* 86(3):323-9.
135. Furusato B, Mohamed A, Uhlén M, Rhim JS. (2010) **CXCR4 and cancer.** *Pathol Int.* 60(7):497-505
136. G. Brusselle, J. Kips, G. Joos, H. Bluethmann, and R. Pauwels. (1994) **Allergen-induced Airway Inflammation and Bronchial Responsiveness in Wild-type and Interleukin-4-deficient Mice.** *Am. J. Respir. Cell Mol. Biol.* Vol. 12. pp. 254-259, 1995
137. Gaggar A, Li Y, Weathington N, Winkler M, Kong M, Jackson P, Blalock JE, Clancy JP. (2007) **Matrix metalloprotease-9 dysregulation in lower airway secretions of cystic fibrosis patients.** *Am J Physiol Lung Cell Mol Physiol.* 293(1):L96-L104.

138. Gaiet J, Chollet-Martin S, Brion M, Hakim J, Gougerot-Pocidal MA, Elbim C. (1998) **Interleukin-8 production by polymorphonuclear neutrophils in patients with rapidly progressive periodontitis: an amplifying loop of polymorphonuclear neutrophil activation.** *Lab Invest.* 78(6):755-62.
139. Galzi JL, Hachet-Haas M, Bonnet D, Daubeuf F, Lecat S, Hibert M, Haiech J, Frossard N. (2010) **Neutralizing endogenous chemokines with small molecules. Principles and potential therapeutic applications.** *Pharmacol Ther.* 126(1):39-55.
140. Gambaryan N, Perros F, Montani D, Cohen-Kaminsky S, Mazmanian M, Renaud JF, Simonneau G, Lombet A, Humbert M. (2011) **Targeting of c-kit+ haematopoietic progenitor cells prevents hypoxic pulmonary hypertension.** *Eur Respir J.* 37(6):1392-9.
141. Garibaldi BT, D'Alessio FR, Mock JR, Files DC, Chau E, Eto Y, Drummond MB, Aggarwal NR, Sidhaye V, King LS. (2013) **Regulatory T cells reduce acute lung injury fibroproliferation by decreasing fibrocyte recruitment.** *Am J Respir Cell Mol Biol.* 48(1):35-43.
142. Gerrits H, van Ingen Schenau DS, Bakker NE, van Disseldorp AJ, Strik A, Hermens LS, Koenen TB, Krajnc-Franken MA, Gossen (2008) **Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice.** *Genesis.* 46(5):235-45.
143. Gienbycz MA, Lindsay MA. (1999) **Pharmacology of the eosinophil.** *Pharmacol Rev.* 51(2):213-340.
144. Gill MA (2012) **The role of dendritic cells in asthma.** *J Allergy Clin Immunol.* 129(4):889-901.
145. Gill MA, Bajwa G, George TA, Dong CC, Dougherty II, Jiang N, Gan VN, Gruchalla RS (2010) **Counterregulation between the FcεRI pathway and antiviral responses in human plasmacytoid dendritic cells.** *J Immunol.* 184(11):5999-6006.
146. **GINA**, Global Initiative for Asthma (2012) Pocket Guide For Asthma Management And Prevention. Updated December 2012.
147. Glaab T, Taube C, Braun A, Mitzner W. (2007) **Invasive and noninvasive methods for studying pulmonary function in mice.** *Respir Res.* 8:63.
148. Gleich GJ. (2000) **Mechanisms of eosinophil-associated inflammation.** *J Allergy Clin Immunol.* 105(4):651-63.
149. Gleichmann M, Gillen C, Czardybon M, Bosse F, Greiner-Petter R, Auer J. (2000) **Cloning and characterization of SDF-1γ, a novel SDF-1 chemokine transcript with developmentally regulated expression in the nervous system.** *Eur J Neurosci.* 12:1857–1866
150. Gomperts BN, Belperio JA, Rao PN, Randell SH, Fishbein MC, Burdick MD, Strieter RM. (2006) **Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury.** *J Immunol.* 176(3):1916-27.
151. Gomperts BN, Strieter RM. (2007) **Fibrocytes in lung disease.** *J Leukoc Biol.* 82(3):449-56.
152. Gonzalo JA, Lloyd CM, Peled A, Delaney T, Coyle AJ, Gutierrez-Ramos JC. (2000) **Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 alpha in the inflammatory component of allergic airway disease.** *J Immunol.* 165(1):499-508.
153. Goplen N, Karim MZ, Liang Q, Gorska MM, Rozario S, Guo L, Alam R. (2009) **Combined sensitization of mice to extracts of dust mite, ragweed, and Aspergillus species breaks through tolerance and establishes chronic features of asthma.** *J Allergy Clin Immunol.* 123(4):925-32.e11.
154. Gordon S. (2003) **Alternative activation of macrophages.** *Nat Rev Immunol.* 2003 Jan;3(1):23-35.
155. Gortz A, Nibbs RJ, McLean P, Jarmin D, Lambie W, Baird JW, Graham GJ. (2002) **The chemokine ESKine/CCL27 displays novel modes of intracrine and paracrine function.** *J Immunol.* 169(3):1387-94.
156. Gosset P, Tillie-Leblond I, Oudin S, Parmentier O, Wallaert B, Joseph M, Tonnel AB. (1999) **Production of chemokines and proinflammatory and antiinflammatory cytokines by human alveolar macrophages activated by IgE receptors.** *J Allergy Clin Immunol.* 103(2 Pt 1):289-97.
157. Gounni AS. (2009) **Proinflammatory and Th2 cytokines regulate the high affinity IgE receptor (FcεRI) and IgE-dependant activation of human airway smooth muscle cells.** *PLoS One.* 4(7):e6153.
158. Gozansky EK, Louis JM, Caffrey M, Clore GM. (2005) **Mapping the binding of the N-terminal extracellular tail of the CXCR4 receptor to stromal cell-derived factor-1α.** *J Mol Biol.* 28;345(4):651-8.
159. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, Wardlaw AJ, Pavord ID (2002) **Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial.** *Lancet.* 60(9347):1715-21.
160. Gregory LG, Lloyd CM. (2011) **Orchestrating house dust mite-associated allergy in the lung.** *Trends Immunol.* (9):402-11.
161. Gri, G., Piconese, S., Frossi, B., Manfroi, V., Merluzzi, S., Tripodo, C., Viola, A. et al. (2008) **CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction.** *Immunity.* 29: 771–781.

162. Grymula K, Tarnowski M, Wysoczynski M, Drukala J, Barr FG, Ratajczak J, Kucia M, Ratajczak MZ. (2010) **Overlapping and distinct role of CXCR7-SDF-1/ITAC and CXCR4-SDF-1 axes in regulating metastatic behavior of human rhabdomyosarcomas.** *Int J Cancer.* 127(11):2554-68.
163. Hachet-Haas M, Balabanian K, Rohmer F, Pons F, Franchet C, Lecat S, Chow KY, Dagher R, Gizzi P, Didier B, Lagane B, Kellenberger E, Bonnet D, Baleux F, Haiech J, Parmentier M, Frossard N, Arenzana-Seisdedos F, Hibert M, Galzi JL. (2008) **Small neutralizing molecules to inhibit actions of the chemokine CXCL12.** *J Biol Chem.* 283(34):23189-99.
164. Hagenbuch Bruno (2010) **Drug uptake systems in liver and kidney: historic perspective.** *Clin Pharmacol Ther.* 87(1): 39.
165. Hallgren J, Gurish MF. (2007) **Pathways of murine mast cell development and trafficking: tracking the roots and routes of the mast cell.** *Immunol Rev.* 217:8-18.
166. Hallgren J, Gurish MF. (2011) **Mast cell progenitor trafficking and maturation.** *Adv Exp Med Biol.* 716:14-28.
167. Halova I, Draberova L, Draber P. (2012) **Mast cell chemotaxis - chemoattractants and signaling pathways.** *Front Immunol.* 3:119.
168. Halwani R, Al-Muhsen S, Hamid Q. (2013) **T helper 17 cells in airway diseases: from laboratory bench to bedside.** *Chest.* 143(2):494-501.
169. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW. (1997) **Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography.** *Am J Respir Crit Care Med.* 156(3 Pt 1):766-75.
170. Hamilton, J.R., Moffatt, J.D., Frauman, A.G., Cocks, T.M. (2001) **Protease-activated receptor (PAR) 1 but not PAR2 or PAR4 mediates endothelium-dependent relaxation to thrombin and trypsin in human pulmonary arteries.** *J. Cardiovasc. Pharmacol.* 38(1), 108-19
171. Hansell C, Hurson C, Nibbs R. (2011) **DARC and D6: silent partners in chemokine regulation?** *Immunol Cell Biol.* PMC
172. Hartl D, Latzin P, Hordijk P, Marcos V, Rudolph C, Woischnik M, Krauss-Etschmann S, Koller B, Reinhardt D, Roscher AA, Roos D, Griese M. (2007) **Cleavage of CXCR1 on neutrophils disables bacterial killing in cystic fibrosis lung disease.** *Nat Med.* 13(12):1423-30.
173. Hartlapp I, Abe R, Saeed RW, (2001) **Fibrocytes induce an angiogenic phenotype in cultured endothelial cells and promote angiogenesis in vivo.** *Faseb J.* 15:2215–24
174. Hartmann TN, Burger JA, Glodek A, Fujii N, Burger M. (2005) **CXCR4 chemokine receptor and integrin signaling co-operate in mediating adhesion and chemoresistance in small cell lung cancer (SCLC) cells.** *Oncogene.* 24(27):4462-71.
175. Hattermann K, Held-Feindt J, Lucius R, Mürköster SS, Penfold ME, Schall TJ, Mentlein R. (2010) **The chemokine receptor CXCR7 is highly expressed in human glioma cells and mediates antiapoptotic effects.** *Cancer Res.* 70(8):3299-308.
176. Hattermann K, Mentlein R (2012) **An Infernal Trio: The chemokine CXCL12 and its receptors CXCR4 and CXCR7 in tumor biology.** *Ann Anat.* S0940-9602(12)00170-7.
177. Hattermann K, Mentlein R. (2013) **An infernal trio: the chemokine CXCL12 and its receptors CXCR4 and CXCR7 in tumor biology.** *Ann Anat.* 195(2):103-10.
178. Hausding M, Sauer K, Maxeiner JH, Finotto S. (2008) **Transgenic models in allergic responses.** *Curr Drug Targets.* 9(6):503-10.
179. Heard BE, Dewar A, Nunn AJ, Kay AB. (1990) **Heterogeneous ultrastructure of human bronchial mast cells: morphometric subdivision of cell types and evidence for a degranulation gradient.** *Am J Respir Cell Mol Biol.* 3(1):71-8.
180. Heesen M, Berman MA, Benson JD, Gerard C, Dorf ME. (1996) **Cloning of the mouse fusin gene, homologue to a human HIV-1 co-factor.** *J Immunol.* 157(12):5455-60.
181. Helbig G, Christopherson KW 2nd, Bhat-Nakshatri P, Kumar S, Kishimoto H, Miller KD, Broxmeyer HE, Nakshatri H. (2003) **NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4.** *J Biol Chem.* 2003 Jun 13;278(24):21631-8. Epub 2003 Apr 9.
182. Henderson WR Jr, Tang LO, Chu SJ, Tsao SM, Chiang GK, Jones F, Jonas M, Pae C, Wang H, Chi EY. (2002) **A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model.** *Am J Respir Crit Care Med.* 165(1):108-16.
183. Hener P, Friedmann L, Metzger D, Chambon P, Li M (2011) **Aggravated TSLP-induced atopic dermatitis in mice lacking Dicer in adult skin keratinocytes.** *J Invest Dermatol.* 131(11):2324-7.

184. Hermand P, Pincet F, Carvalho S, Ansanay H, Trinquet E, Daoudi M, Combadière C, Deterre P. (2008) **Functional adhesiveness of the CX3CL1 chemokine requires its aggregation. Role of the transmembrane domain.** *J Biol Chem.* 283(44):30225-34.
185. Hernandez L, Magalhaes MA, Coniglio SJ, Condeelis JS, Segall JE. (2011) **Opposing roles of CXCR4 and CXCR7 in breast cancer metastasis.** *Breast Cancer Res.* 13(6):R128.
186. Herzog H, Hort YJ, Shine J, Selbie LA. (1993) **Molecular cloning, characterization, and localization of the human homolog to the reported bovine NPY Y3 receptor: lack of NPY binding and activation.** *DNA Cell Biol.* 12(6):465-71.
187. Hessel E M, A J Van Oosterhout, I Van Ark, B Van Esch, G Hofman, H Van Loveren, H F Savelkoul, and F P Nijkamp (1997) **Development of airway hyperresponsiveness is dependent on interferon-gamma and independent of eosinophil infiltration.** *American Journal of Respiratory Cell and Molecular Biology*, Vol. 16, No. 3, pp. 325-34.
188. Higashiyama S, Abraham JA, Klagsbrun M. (1993) **Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate.** *J Cell Biol.* 122(4):933-40.
189. Hirata N, Kohrogi H, Iwagoe H, Goto E, Hamamoto J, Fujii K, Yamaguchi T, Kawano O, Ando M. (1998) **Allergen exposure induces the expression of endothelial adhesion molecules in passively sensitized human bronchus: time course and the role of cytokines.** *Am J Respir Cell Mol Biol.* 18(1):12-20.
190. Hitchon C, Wong K, Ma G, Reed J, Lyttle D, El-Gabalawy H. (2002) **Hypoxia-induced production of stromal cell-derived factor 1 (CXCL12) and vascular endothelial growth factor by synovial fibroblasts.** *Arthritis Rheum.* 46(10):2587-97.
191. Hogaboam CM, Carpenter KJ, Schuh JM, Proudfoot AA, Bridger G, Buckland KF. (2005) **The therapeutic potential in targeting CCR5 and CXCR4 receptors in infectious and allergic pulmonary disease.** *Pharmacol Ther.* 2005 Sep;107(3):314-28.
192. Hogan MB, Weissman DN, Hubbs AF, Gibson LF, Piktel D, Landreth KS. (2003) **Regulation of eosinophilopoiesis in a murine model of asthma.** *J Immunol.* 171(5):2644-51.
193. Hogan S P, A Mould, H Kikutani, A J Ramsay, P S Foster (1997) **Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyperreactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins.** *J Clin Invest.* 99(6): 1329-1339.
194. Holgate ST (2012) **Innate and adaptive immune responses in asthma.** *Nat Med.* 18(5):673-83.
195. Holgate ST. (2008) **Pathogenesis of asthma.** *Clin Exp Allergy.* 38(6):872-97.
196. Hoshino M, Aoike N, Takahashi M, Nakamura Y, Nakagawa T. (2003) **Increased immunoreactivity of stromal cell-derived factor-1 and angiogenesis in asthma.** *Eur Respir J.* 21(5):804-9.
197. Hoymann HG (2007) **Invasive and noninvasive lung function measurements in rodents.** *J Pharmacol Toxicol Methods.* 55(1):16-26.
198. Hume DA. (2008) **Macrophages as APC and the dendritic cell myth.** *J Immunol.* 181(9):5829-35.
199. Humrich JY, Humrich JH, Averbek M, Thumann P, Termeer C, Kämpgen E, Schuler G, Jenne L. (2006) **Mature monocyte-derived dendritic cells respond more strongly to CCL19 than to CXCL12: consequences for directional migration.** *Immunology.* 117(2):238-47
200. Hung CH, Jong YJ, Hua YM, Li CY, Lai YS, Yang KD, Chang HC. (2007) **Regulation of stromal cell-derived factor-1 and exhaled nitric oxide in asthmatic children following montelukast and ketotifen treatment.** *Pulm Pharmacol Ther.* 20(3):233-9.
201. Hyytiäinen M, Penttinen C, Keski-Oja J. (2004) **Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation.** *Crit Rev Clin Lab Sci.* 2004;41(3):233-64.
202. Iikura M, Yamaguchi M, Hirai K, Miyamasu M, Yamada H, Nakajima T, Fujisawa T, Ra C, Morita Y, Yamamoto K. (2001) **Regulation of surface FcepsilonRI expression on human eosinophils by IL-4 and IgE.** *Int Arch Allergy Immunol.* 124(4):470-7.
203. Infantino S, Moepps B, Thelen M. (2006) **Expression and regulation of the orphan receptor RDC1 and its putative ligand in human dendritic and B cells.** *J Immunol.* 176(4):2197-207.
204. Irvin CG, Bates JH. (2003) **Measuring the lung function in the mouse: the challenge of size.** *Respir Res.* 4:4.
205. Ishizaka K, Ishizaka T, Hornbrook MM. (1966) **Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity with gamma-E-globulin antibody.** *J Immunol.* 97(6):840-53.
206. Iwashita K, Kawasaki H, Sawada M, In M, Mataka Y, Kuwabara T. (2008) **Shortening of the induction period of allergic asthma in cynomolgus monkeys by *Ascaris suum* and house dust mite.** *J Pharmacol Sci.* 106(1):92-9.
207. Janowski M (2009) **Functional diversity of SDF-1 splicing variants.** *Cell Adh Migr.* 3(3):243-9.

208. Jaspers I, Sheridan PA, Zhang W, Brighton LE, Chason KD, Hua X, Tilley SL. (2009) **Exacerbation of allergic inflammation in mice exposed to diesel exhaust particles prior to viral infection.** Part Fibre Toxicol. 6:22.
209. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. (1999) **Neutrophilic inflammation in severe persistent asthma.** Am J Respir Crit Care Med. 160:1532-9.
210. Jiménez-Sainz MC, Murga C, Kavelaars A, Jurado-Pueyo M, Krakstad BF, Heijnen CJ, Mayor F Jr, Aragay AM. (2006) **G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits.** Mol Biol Cell. 17(1):25-31.
211. Jin C, Fu WX, Xie LP, Qian XP, Chen WF. (2003) **SDF-1alpha production is negatively regulated by mouse estrogen enhanced transcript in a mouse thymus epithelial cell line.** Cell Immunol. 223(1):26-34.
212. Jin P, Han TH, Ren J, Saunders S, Wang E, Marincola FM, Stroncek DF. **Molecular signatures of maturing dendritic cells: implications for testing the quality of dendritic cell therapies.** J Transl Med. 2010 Jan 15;8:4. doi: 10.1186/1479-5876-8-4.
213. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M. (2004) **Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling.** Am J Respir Crit Care Med. 169(3):378-85.
214. Johnson LA, Jackson DG. (2013) **Control of dendritic cell trafficking in lymphatics by chemokines. Angiogenesis.** Nov 15.
215. Johnson Z, Power CA, Weiss C, Rintelen F, Ji H, Ruckle T, Camps M, Wells TN, Schwarz MK, Proudfoot AE, Rommel C. (2004) **Chemokine inhibition--why, when, where, which and how?** Biochem Soc Trans. 32(Pt 2):366-77.
216. Jung CH, Lee JY, Park JH, Cho BJ, Sim SS, Kim CJ. (2010) **Flavonols attenuate the immediate and late-phase asthmatic responses to aerosolized-ovalbumin exposure in the conscious guinea pig.** Fitoterapia. 81(7):803-12.
217. Jungsuwadee P., Benkovsky M., Dekan G., Stingl G., Epstein M. M. (2004) **Repeated aerosol allergen exposure suppresses inflammation in B-cell deficient mice with established allergic asthma.** Int. Arch. Allergy Immunol. 133, 126–134.
218. Kajiyama H, Shibata K, Terauchi M, Ino K, Nawa A, Kikkawa F. (2008) **Involvement of SDF-1alpha/CXCR4 axis in the enhanced peritoneal metastasis of epithelial ovarian carcinoma.** Int J Cancer. 122(1):91-9.
219. Kanehiro A, Ikemura T, Mäkelä MJ, Lahn M, Joetham A, Dakhama A, Gelfand EW. (2001) **Inhibition of phosphodiesterase 4 attenuates airway hyperresponsiveness and airway inflammation in a model of secondary allergen challenge.** Am J Respir Crit Care Med. 163(1):173-84
220. Karin N (2010) **The multiple faces of CXCL12 (SDF-1alpha) in the regulation of immunity during health and disease.** J Leukoc Biol. 88(3):463-73.
221. Kasper M, Sims G, Koslowski R, Kuss H, Thuemmler M, Fehrenbach H, Auten RL. (2002) **Increased surfactant protein D in rat airway goblet and Clara cells during ovalbumin-induced allergic airway inflammation.** Clin Exp Allergy. 32(8):1251-8.
222. Katoh N, Kraft S, Weßendorf HM, Bieber T. (1999) **The high-affinity IgE receptor (FcεRI) blocks apoptosis in normal human monocytes** J Clin Invest. 2: 2
223. Kawabata K, Hagio T, Matsuoka S (2002) **The role of neutrophil elastase in acute lung injury.** Eur J Pharmacol. 451(1):1-10.
224. Kelley GG, Kaproth-Joslin KA, Reks SE, Smrcka AV, Wojcikiewicz RJ. (2006) **G-protein-coupled receptor agonists activate endogenous phospholipase Cepsilon and phospholipase Cbeta3 in a temporally distinct manner.** J Biol Chem. 281(5):2639-48.
225. Kelner GS, Kennedy J, Bacon KB, Kleyensteuber S, Largaespada DA, Jenkins NA, Copeland NG, Bazan JF, Moore KW, Schall TJ, et al. (1994) **Lymphotactin: a cytokine that represents a new class of chemokine.** Science. 266(5189):1395-9.
226. Kew RR, Penzo M, Habel DM, Marcu KB. (2012) **The IKKα-dependent NF-κB p52/RelB noncanonical pathway is essential to sustain a CXCL12 autocrine loop in cells migrating in response to HMGB1.** J Immunol. 188(5):2380-6.
227. Kier SD, Spina D, Douglas G, Herd C, Page CP. (2011) **Airway responsiveness in an allergic rabbit model.** J Pharmacol Toxicol Methods. 64(2):187-95.
228. Kim SJ, Kim CH, Ahn JH, Kim MS, Kim SC, Lee SY, Kwon SS, Kim YK, Kim KH, Moon HS, Song JS, Park SH. (2007) **Time sequence of airway remodeling in a mouse model of chronic asthma: the relation with airway hyperresponsiveness.** J Korean Med Sci. 22(2):183-91.
229. King CC, Sastri M, Chang P, Pennypacker J, Taylor SS (2011) **The rate of NF-κB nuclear translocation is regulated by PKA and A kinase interacting protein 1.** PLoS One. 6(4):e18713.

230. Knight DA, Holgate ST. (2003) **The airway epithelium: structural and functional properties in health and disease.** *Respirology.* 8(4):432-46.
231. Koga H, Miyahara N, Fuchimoto Y, Ikeda G, Waseda K, Ono K, Tanimoto Y, Kataoka M, Gelfand EW, Tanimoto M, Kanehiro A. (2013) **Inhibition of neutrophil elastase attenuates airway hyperresponsiveness and inflammation in a mouse model of secondary allergen challenge: neutrophil elastase inhibition attenuates allergic airway responses.** *Respir Res.* 14:8.
232. Korn D, Frasnich SC, Fernandez-Boyanapalli R, Henson PM, Bratton DL. (2011) **Modulation of macrophage efferocytosis in inflammation.** *Front Immunol.* 2:57.
233. Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, Hussell T, Feldmann M, Udalova IA. (2011) **IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses.** *Nat Immunol.* 12(3):231-8.
234. Krouwels FH, van der Heijden JF, Lutter R, van Neerven RJ, Jansen HM, Out TA. (1996) **Glucocorticosteroids affect functions of airway- and blood-derived human T-cell clones, favoring the Th1 profile through two mechanisms.** *Am J Respir Cell Mol Biol.* 14(4):388-97.
235. Kucharewicz I, Bodzenta-Łukaszyk A, Buczek W. (2008) **Experimental asthma in rats.** *Pharmacol Rep.* 60(6):783-8.
236. Kugathasan K, Roediger EK, Small CL, McCormick S, Yang P, Xing Z. (2008) **CD11c+ antigen presenting cells from the alveolar space, lung parenchyma and spleen differ in their phenotype and capabilities to activate naïve and antigen-primed T cells.** *BMC Immunol.* 9:48.
237. Kukreja P, Abdel-Mageed AB, Mondal D, Liu K, Agrawal KC. (2005) **Up-regulation of CXCR4 expression in PC-3 cells by stromal-derived factor-1alpha (CXCL12) increases endothelial adhesion and transendothelial migration: role of MEK/ERK signaling pathway-dependent NF-kappaB activation.** *Cancer Res.* 65(21):9891-8.
238. Kumar R, Tripathi V, Ahmad M, Nath N, Mir RA, Chauhan SS, Luthra K. (2012) **CXCR7 mediated G α independent activation of ERK and Akt promotes cell survival and chemotaxis in T cells.** *Cell Immunol.* 272(2):230-41.
239. Kumar RK, Herbert C, Foster PS. (2008) **The "classical" ovalbumin challenge model of asthma in mice.** *Curr Drug Targets.* 9(6):485-94.
240. Kumar RK, Herbert C, Kasper M (2004) **Reversibility of airway inflammation and remodelling following cessation of antigenic challenge in a model of chronic asthma.** *Clin Exp Allergy.* 34(11):1796-802.
241. Kung TT, Jones H, Adams GK 3rd, Umland SP, Kreutner W, Egan RW, Chapman RW, Watnick AS. (1994) **Characterization of a murine model of allergic pulmonary inflammation.** *Int Arch Allergy Immunol.* 105(1):83-90.
242. Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. (1998) **Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production.** *J Exp Med.* 187(6):939-48.
243. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. (2002) **Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma.** *Nat Med.* 8(8):885-9.
244. Kuperman DA, Lewis CC, Woodruff PG, Rodriguez MW, Yang YH, Dolganov GM, Fahy JV, Erle DJ. (2005) **Dissecting asthma using focused transgenic modeling and functional genomics.** *J Allergy Clin Immunol.* 116(2):305-11.
245. Kuritzkes DR. (2009) **HIV-1 entry inhibitors: an overview.** *Curr Opin HIV AIDS.* 4(2):82-7.
246. Kuschert GS, Coulin F, Power CA, Proudfoot AE, Hubbard RE, Hoogewerf AJ, Wells TN. (1999) **Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses.** *Biochemistry.* 38(39):12959-68.
247. Lambrecht BN, Hammad H. (2010) **The role of dendritic and epithelial cells as master regulators of allergic airway inflammation.** *Lancet.* 376(9743):835-43.
248. Lambrecht BN, Hammad H. (2012) **The airway epithelium in asthma.** *Nat Med.* 18(5):684-92. doi: 10.1038/nm.2737.
249. Larsen, H, Renz, J E, Loader, K L, Bradley, E W, Gelfand (1992) **Airway response to electrical field stimulation in sensitized inbred mice. Passive transfer of increased responsiveness with peribronchial lymph nodes.** *J Clin Invest.* 89(3): 747-752
250. Laskin DL, Sunil VR, Gardner CR, Laskin JD. (2011) **Macrophages and tissue injury: agents of defense or destruction?** *Annu Rev Pharmacol Toxicol.* 51:267-88.

251. Lau EK, Paavola CD, Johnson Z, Gaudry JP, Geretti E, Borlat F, Kungl AJ, Proudfoot AE, Handel TM. (2004) **Identification of the glycosaminoglycan binding site of the CC chemokine, MCP-1: implications for structure and function in vivo.** *J Biol Chem.* 279(21):22294-305.
252. Laurence AD. (2006) **Location, movement and survival: the role of chemokines in haematopoiesis and malignancy.** *Br J Haematol.* 132(3):255-67
253. Lawrence T, Natoli G. (2011) **Transcriptional regulation of macrophage polarization: enabling diversity with identity.** *Nat Rev Immunol.* 11(11):750-61.
254. Lazennec G, Richmond A. (2010) **Chemokines and chemokine receptors: new insights into cancer-related inflammation.** *Trends Mol Med.* 16(3):133-44.
255. Le Cras TD, Acciani TH, Mushaben EM, Kramer EL, Pastura PA, Hardie WD, Korfhagen TR, Sivaprasad U, Ericksen M, Gibson AM, Holtzman MJ, Whitsett JA, Hershey GK. (2011) **Epithelial EGF receptor signaling mediates airway hyperreactivity and remodeling in a mouse model of chronic asthma.** *Am J Physiol Lung Cell Mol Physiol.* 300(3):L414-21.
256. Lean JM, Murphy C, Fuller K, Chambers TJ. (2002) **CCL9/MIP-1gamma and its receptor CCR1 are the major chemokine ligand/receptor species expressed by osteoclasts.** *J Cell Biochem.* 87(4):386-93.
257. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ, Barnes PJ. (2000) **Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response.** *Lancet*, 356, pp. 2144–2148
258. Lee HJ, Lee K, Lee DG, Bae KH, Kim JS, Liang ZL, Huang SM, Suk Oh Y, Kim HY, Jo DY, Min JK, Kim JM. (2012) **Chemokine (C-X-C motif) ligand 12 is associated with gallbladder carcinoma progression and is a novel independent poor prognostic factor.** *Clin Cancer Res.* 18(12):3270-80.
259. Lemanske RF Jr, Busse WW. (2010) **Asthma: clinical expression and molecular mechanisms.** *J Allergy Clin Immunol.* 125(2 Suppl 2):S95-102.
260. Lesur O, Bernard AM, Bégin RO (1996) **Clara cell protein (CC-16) and surfactant-associated protein A (SP-A) in asbestos-exposed workers.** *Chest.* 109(2):467-74.
261. Levine SP, Wohl H. (1976) **Human platelet factor 4: Purification and characterization by affinity chromatography.** *J Biol Chem.* 251(2):324-8.
262. Levoye A, Balabanian K, Baleux F, Bachelier F, Lagane B. (2009) **CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling.** *Blood.* 113(24):6085-93.
263. Lewellis SW, Knaut H. (2012) **Attractive guidance: how the chemokine SDF1/CXCL12 guides different cells to different locations.** *Semin Cell Dev Biol.* 23(3):333-40.
264. Liang Z, Yoon Y, Votaw J, Goodman MM, Williams L, Shim H (2005) **Silencing of CXCR4 blocks breast cancer metastasis.** *Cancer Res.* 65(3):967-71.
265. Libert F, Parmentier M, Lefort A, Dumont JE, Vassart G. (1990) **Complete nucleotide sequence of a putative G protein coupled receptor: RDC1.** *Nucleic Acids Res.* 18(7):1917.
266. Liccardi G, Emenius G, Merritt AS, Salzillo A, D'Amato M, D'Amato G. (2012) **Direct and indirect exposure to horse: risk for sensitization and asthma.** *Curr Allergy Asthma Rep.* 12(5):429-37.
267. Lieutier-Colas F, Meyer P, Larsson P, Malmberg P, Frossard N, Pauli G, de Blay F. (2001) **Difference in exposure to airborne major rat allergen (Rat n 1) and to endotoxin in rat quarters according to tasks.** *Clin Exp Allergy.* 31(9):1449-56.
268. Lightfield KL, Persson J, Brubaker SW, Witte CE, von Moltke J, Dunipace EA, Henry T, Sun YH, Cado D, Dietrich WF, Monack DM, Tsolis RM, Vance RE. (2008) **Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin.** *Nat Immunol.* 9(10):1171-8.
269. Liles, W. C., Rodger, E., Broxmeyer, H. E., Dehner, C., Badel, K., Calandra, G., Christensen, J., Wood, B., Price, T. H., and Dale, D. C. (2005) **Augmented mobilization and collection of CD34 hematopoietic cells from normal human volunteers stimulated with granulocyte-colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist.** *Transfusion* 45, 295–300
270. Lim TS, Goh JK, Mortellaro A, Lim CT, Hämmerling GJ, Ricciardi-Castagnoli P. (2012) **CD80 and CD86 differentially regulate mechanical interactions of T-cells with antigen-presenting dendritic cells and B-cells.** *PLoS One.* 7(9):e45185.
271. Lipfert J, Ödemis V, Engele J. (2013) **Grk2 is an essential regulator of CXCR7 signalling in astrocytes.** *Am J Respir Crit Care Med.* 184(1):116-23.
272. Liu LY, Bates ME, Jarjour NN, Busse WW, Bertics PJ, Kelly EA (2007) **Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-alpha.** *J Immunol.* 1;179(7):4840-8.

273. Liu LY, Jarjour NN, Busse WW, Kelly EA. (2003) **Chemokine receptor expression on human eosinophils from peripheral blood and bronchoalveolar lavage fluid after segmental antigen challenge.** *J Allergy Clin Immunol.* 112(3):556-62.
274. Liu Q, Chen H, Ojode T, Gao X, Anaya-O'Brien S, Turner NA, Ulrick J, DeCastro R, Kelly C, Cardones AR, Gold SH, Hwang EI, Wechsler DS, Malech HL, Murphy PM, McDermott DH. (2012) **WHIM syndrome caused by a single amino acid substitution in the carboxy-tail of chemokine receptor CXCR4.** *Blood.* 120(1):181-9.
275. Lloyd CM (2007) **Review Building better mouse models of asthma.** *Curr Allergy Asthma Rep.* 7(3):231-6.
276. Loetscher M, Geiser T, O'Reilly T, Zwahlen R, Baggiolini M, Moser B. (1994) **Cloning of a human seven-transmembrane domain receptor, LESTR, that is highly expressed in leukocytes.** *J Biol Chem.* 269(1):232-7.
277. Loetscher P, Clark-Lewis I. (2001) **Agonistic and antagonistic activities of chemokines.** *J Leukoc Biol.* 69(6):881-4.
278. Loftsson T., Brewster M.E. (1996) **Pharmaceutical applications of cyclodextrins. Drug solubilization and stabilization.** *Journal of Pharmaceutical Sciences,* 85, pp. 1017–1025
279. Lomask M. (2006) **Further exploration of the Penh parameter.** *Exp Toxicol Pathol.* 57 Suppl 2:13-20.
280. Lu CL, Guo J, Gu J, Ge D, Hou YY, Lin ZW, Ding JY. (2013) **CXCR4 heterogeneous expression in esophageal squamous cell cancer and stronger metastatic potential with CXCR4-positive cancer cells.** *Dis Esophagus*
281. Ludwig A, Weber C. (2007) **Transmembrane chemokines: versatile 'special agents' in vascular inflammation.** *Thromb Haemost.* 97(5):694-703.
282. Lukacs NW, Berlin A, Schols D, Skerlj RT, Bridger GJ. (2002) **AMD3100, a CxCR4 antagonist, attenuates allergic lung inflammation and airway hyperreactivity.** *Am J Pathol.* 2002 Apr;160(4):1353-60.
283. Lukacs NW, John A, Berlin A, Bullard DC, Knibbs R, Stoolman LM. (2002) **E- and P-selectins are essential for the development of cockroach allergen-induced airway responses.** *J Immunol.* 169(4):2120-5.
284. Luker KE, Steele JM, Mihalko LA, Ray P, Luker GD. (2010) **Constitutive and chemokine-dependent internalization and recycling of CXCR7 in breast cancer cells to degrade chemokine ligands.** *Oncogene.* 12;29(32):4599-610.
285. Luster AD. (1998) **Chemokines--chemotactic cytokines that mediate inflammation.** *N Engl J Med.* 338(7):436-45.
286. Luttmann W, Franz P, Matthys H, Virchow JC Jr. (1998) **Effects of TGF-beta on eosinophil chemotaxis.** *Scand J Immunol.* 47(2):127-30.
287. Ma B, Zhu Z, Homer RJ, Gerard C, Strieter R, Elias JA. (2004) **The C10/CCL6 chemokine and CCR1 play critical roles in the pathogenesis of IL-13-induced inflammation and remodeling.** *J Immunol.* 172(3):1872-81.
288. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA (1998) **Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice.** *Proc Natl Acad Sci U S A.* 95(16):9448-53.
289. MacDermott RP, Sanderson IR, Reinecker HC. (1998) **The central role of chemokines (chemotactic cytokines) in the immunopathogenesis of ulcerative colitis and Crohn's disease.** *Inflamm Bowel Dis.* 4(1):54-67.
290. Macoska JA. (2011) **Chemokines and BPH/LUTS.** *Differentiation.* 82(4-5):253-60
291. Maeba S, Ichiyama T, Ueno Y, Makata H, Matsubara T, Furukawa S. (2005) **Effect of montelukast on nuclear factor kappaB activation and proinflammatory molecules.** *Ann Allergy Asthma Immunol.* 94(6):670-4.
292. Maes T, Joos GF, Brusselle GG (2012) **Targeting interleukin-4 in asthma: lost in translation?** *Am J Respir Cell Mol Biol.* 47(3):261-70.
293. Maes T, Provoost S, Lanckacker EA, Cataldo DD, Vanoirbeek JA, Nemery B, Tournoy KG, Joos GF. (2010) **Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation.** *Respir Res.* 11:7.
294. Maghazachi AA. (2000) **Intracellular signaling events at the leading edge of migrating cells.** *Int J Biochem Cell Biol.* 32(9):931-43.
295. Mahabaleswar H, Tarbashevich K, Nowak M, Brand M, Raz E. (2012) **β-arrestin control of late endosomal sorting facilitates decoy receptor function and chemokine gradient formation.** *Development.* 139(16):2897-902.
296. Majetschak M, Krehmeier U, Bardenheuer M, Denz C, Quintel M, Voggenreiter G, Obertacke U. (2003) **Extracellular ubiquitin inhibits the TNF-alpha response to endotoxin in peripheral blood mononuclear cells and regulates endotoxin hyporesponsiveness in critical illness.** *Blood.* 101(5):1882-90.
297. Makino H, Aono Y, Azuma M, Kishi M, Yokota Y, Kinoshita K, Takezaki A, Kishi J, Kawano H, Ogawa H, Uehara H, Izumi K, Sone S, Nishioka Y. (2013) **Antifibrotic effects of CXCR4 antagonist in bleomycin-induced pulmonary fibrosis in mice.** *J Med Invest.* 60(1-2):127-37.

298. Maréchal R, Demetter P, Nagy N, Berton A, Decaestecker C, Polus M, Closset J, Devière J, Salmon I, Van Laethem JL. (2009) **High expression of CXCR4 may predict poor survival in resected pancreatic adenocarcinoma.** *Br J Cancer.* 100(9):1444-51.
299. Manetti M, Liakouli V, Fatini C, Cipriani P, Bonino C, Vettori S, Guiducci S, Montecucco C, Abbate R, Valentini G, Matucci-Cerinic M, Giacomelli R, Ibba-Manneschi L. (2009) **Association between a stromal cell-derived factor 1 (SDF-1/CXCL12) gene polymorphism and microvascular disease in systemic sclerosis.** *Ann Rheum Dis.* 68(3):408-11.
300. Marion A, Hofmann Bowman, Ahlke Heydemann, Joe Gawdzik, Rebecca A. Shilling, and Blanca Camoretti-Mercado (2011) **Transgenic expression of human S100A12 induces structural airway abnormalities and limited lung inflammation in a mouse model of allergic inflammation.** *Clin Exp Allergy.* 41(6): 878–889.
301. Marone G, Triggiani M, Genovese A, De Paulis A. **Role of human mast cells and basophils in bronchial asthma.** *Adv Immunol.* 2005;88:97-160.
302. Martinez FO, Sica A, Mantovani A, Locati M. (2008) **Macrophage activation and polarization.** *Front Biosci.* 13:453-61.
303. Martínez-Sanz E, Del Río A, Barrio C, Murillo J, Maldonado E, Garcillán B (2008) **Alteration of medial-edge epithelium cell adhesion in two Tgf-beta3 null mouse strains. Differentiation.** 76:417-30
304. Mathias CB, Freyschmidt EJ, Caplan B, Jones T, Poddighe D, Xing W, Harrison KL, Gurish MF, Oettgen HC (2009) **IgE influences the number and function of mature mast cells, but not progenitor recruitment in allergic pulmonary inflammation.** *J Immunol.* 182(4):2416-24.
305. Matsukura S, Stellato C, Georas SN, Casolaro V, Plitt JR, Miura K, Kurosawa S, Schindler U, Schleimer RP (2001) **Interleukin-13 upregulates eotaxin expression in airway epithelial cells by a STAT6-dependent mechanism.** *Am J Respir Cell Mol Biol.* 24(6):755-61.
306. Matsumiya T, Ota K, Imaizumi T, Yoshida H, Kimura H, Satoh K. (2010) **Characterization of synergistic induction of CX3CL1/fractalkine by TNF-alpha and IFN-gamma in vascular endothelial cells: an essential role for TNF-alpha in post-transcriptional regulation of CX3CL1.** *J Immunol.* 15;184(8):4205-14.
307. McGovern TK, Robichaud A, Fereydoonzad L, Schuessler TF, Martin JG. (2013) **Evaluation of Respiratory System Mechanics in Mice using the Forced Oscillation Technique.** *J Vis Exp.*(75).
308. McKee AS, Munks MW, MacLeod MK, Fleenor CJ, Van Rooijen N, Kappler JW, Marrack P. (2009) **Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity.** *J Immunol.* 183(7):4403-14
309. McMenamin PG, Morrison SM, McMenamin C. (1996) **Immunomorphologic studies of mast cell heterogeneity, location, and distribution in the rat conjunctiva.** *J Allergy Clin Immunol.* 97(6):1375-86.
310. McMillan SJ, Lloyd CM (2004) **Prolonged allergen challenge in mice leads to persistent airway remodelling.** *Clin Exp Allergy.* 34(3):497-507.
311. Meiler F, Klunker S, Zimmermann M, Akdis CA, Akdis M. (2008) **Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors.** *Allergy.* 63(11):1455-63.
312. Meiron, M., Zohar, Y., Anunu, R., Wildbaum, G., Karin, N. (2008) **CXCL12 (SDF-1{α}) suppresses ongoing experimental autoimmune encephalomyelitis by selecting antigen-specific regulatory T cells.** *J. Exp. Med.* 205,2643-2655
313. Melo RC, Spencer LA, Perez SA, Neves JS, Bafford SP, Morgan ES, Dvorak AM, Weller PF. (2009) **Vesicle-mediated secretion of human eosinophil granule-derived major basic protein.** *Lab Invest.* 89(7):769-81.
314. Memi F, Abe P, Cariboni A, Mackay F, Parnavelas JG, Stumm R. (2013) **CXC Chemokine Receptor 7 (CXCR7) Affects the Migration of GnRH Neurons by Regulating CXCL12 Availability.** *J Neurosci.* 33(44):17527-37.
315. Mercalli A, Calavita I, Dugnani E, Citro A, Cantarelli E, Nano R, Melzi R, Maffi P, Secchi A, Sordi V, Piemonti L. (2013) **Rapamycin unbalances the polarization of human macrophages to M1.** *Immunology.* 140(2):179-90.
316. Miller JT, Bartley JH, Wimborne HJ, Walker AL, Hess DC, Hill WD, Carroll JE. (2005) **The neuroblast and angioblast chemotactic factor SDF-1 (CXCL12) expression is briefly up regulated by reactive astrocytes in brain following neonatal hypoxic-ischemic injury.** *BMC Neurosci.* 6:63.
317. Montani D, Perros F, Gambaryan N, Girerd B, Dorfmueller P, Price LC, Huertas A, Hammad H, Lambrecht B, Simonneau G, Launay JM, Cohen-Kaminsky S, Humbert M. (2011) **C-kit-positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial hypertension.** *Cell Mol Neurobiol.* 33(1):111-8.
318. Monteseirín J, Chacón P, Vega A, El Bekay R, Alvarez M, Alba G, Conde M, Jiménez J, Asturias JA, Martínez A, Conde J, Pintado E, Bedoya FJ, Sobrino F. (2004) **Human neutrophils synthesize IL-8 in an IgE-mediated activation.** *J Leukoc Biol.* 76(3):692-700.
319. Monteseirín J. (2009) **Neutrophils and asthma.** *J Investig Allergol Clin Immunol.* 19(5):340-54.

320. Moreira AP, Cavassani KA, Ismailoglu UB, Hullinger R, Dunleavy MP, Knight DA, Kunkel SL, Uematsu S, Akira S, Hogaboam CM. (2011) **The protective role of TLR6 in a mouse model of asthma is mediated by IL-23 and IL-17A.** *J Clin Invest.* 121(11):4420-32.
321. Morokata T, Ishikawa J, Ida K, Yamada T. (1999) **C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses.** *Immunology.* 98(3):345-51.
322. Morokata T, Ishikawa J, Yamada T. (1999) **Differential susceptibility of C57BL/6 and DBA/2 mice to ovalbumin-induced pulmonary eosinophilia regulated by Th1/Th2-type cytokines.** *Immunol Lett.* 1;70(2):127-34.
323. Mortier A, Van Damme J, Proost P. (2012) **Overview of the mechanisms regulating chemokine activity and availability.** *Immunol Lett.* 145(1-2):2-9.
324. Moser B, Loetscher P. (2001) **Lymphocyte traffic control by chemokines.** *Nat Immunol.* 2(2):123-8.
325. Moser B, Willmann K. (2004) **Chemokines: role in inflammation and immune surveillance.** *Ann Rheum Dis.* 63 Suppl 2:II84-II89.
326. Moser B, Wolf M, Walz A, Loetscher P. (2004) **Chemokines: multiple levels of leukocyte migration control.** *Trends Immunol.* 25(2):75-84.
327. Mosser DM, Edwards JP. (2008) **Exploring the full spectrum of macrophage activation.** *Nat Rev Immunol.* 8:958-69.
328. Mukaida N, Hishinuma A, Zachariae CO, Oppenheim JJ, Matsushima K. (1991) **Regulation of human interleukin 8 gene expression and binding of several other members of the intercrine family to receptors for interleukin-8.** *Adv Exp Med Biol.* 305:31-8.
329. Mukaida N. (2003) **Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases.** *Am J Physiol Lung Cell Mol Physiol.* 284(4):L566-77.
330. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verástegui E, Zlotnik A (2001) **Involvement of chemokine receptors in breast cancer metastasis.** *Nature.* 410(6824):50-6.
331. Murphy DM, O'Byrne PM. (2010) **Recent advances in the pathophysiology of asthma.** *Chest.* 137(6):1417-26.
332. Murphy JW, Yuan H, Kong Y, Xiong Y, Lolis EJ (2010) **Heterologous quaternary structure of CXCL12 and its relationship to the CC chemokine family.** *Proteins.* 78(5):1331-7.
333. Murray PJ, Wynn TA. (2011) **Protective and pathogenic functions of macrophage subsets.** *Nat Rev Immunol.* 11(11):723-37.
334. Nagarkar DR, Poposki JA, Comeau MR, Biyasheva A, Avila PC, Schleimer RP, Kato A. (2012) **Airway epithelial cells activate TH2 cytokine production in mast cells through IL-1 and thymic stromal lymphopoietin.** *J Allergy Clin Immunol.* 130(1):225-32.e4.
335. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. (1996) **Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1.** *Nature.* 382(6592):635-8.
336. Nagasawa T, Kikutani H, and Kishimoto T (1994) **Molecular cloning and structure of a pre-B-cell growth-stimulating factor.** *Proc Natl Acad Sci U S A.* 91(6): 2305-2309.
337. Nagasawa T, Nakajima T, Tachibana K, Iizasa H, Bleul CC, Yoshie O, Matsushima K, Yoshida N, Springer TA, Kishimoto T. (1996) **Molecular cloning and characterization of a murine pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 receptor, a murine homolog of the human immunodeficiency virus 1 entry coreceptor fusin.** *Proc Natl Acad Sci U S A.* 93(25):14726-9.
338. Nagase H, Kudo K, Izumi S, Ohta K, Kobayashi N, Yamaguchi M, Matsushima K, Morita Y, Yamamoto K, Hirai K. (2001) **Chemokine receptor expression profile of eosinophils at inflamed tissue sites: Decreased CCR3 and increased CXCR4 expression by lung eosinophils.** *J Allergy Clin Immunol.* 108(4):563-9.
339. Nagase H, Miyamasu M, Yamaguchi M, Fujisawa T, Ohta K, Yamamoto K, Morita Y, Hirai K. (2000) **Expression of CXCR4 in eosinophils: functional analyses and cytokine-mediated regulation.** *J Immunol.* 164(11):5935-43.
340. Nakae, S., L. H. Ho, et al. (2007) **Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice.** *J Allergy Clin Immunol* 120(1): 48-55.
341. Nakagome K, Matsushita S, Nagata M. (2012) **Neutrophilic inflammation in severe asthma.** *Int Arch Allergy Immunol.* 158 Suppl 1:96-102.
342. Nanki, T., Lipsky, P. E. (2000) **Cutting edge: stromal cell-derived factor-1 is a costimulator for CD4+ T cell activation.** *J. Immunol.* 164,5010-5014

343. Naor Z. (2009) **Signaling by G-protein-coupled receptor (GPCR): studies on the GnRH receptor.** *Front Neuroendocrinol.* 30(1):10-29.
344. **National Centre for Replacement, Reduction and Refinement of animals in research (NC3Rs).** <http://www.nc3rs.org.uk/>
345. Negrete-García MC, Velazquez JR, Popoca-Coyotl A, Montes-Vizuet AR, Juárez-Carvajal E, Teran LM. (2010) **Chemokine (C-X-C motif) ligand 12/stromal cell-derived factor-1 is associated with leukocyte recruitment in asthma.** *Chest.* 138(1):100-6.
346. Nesselova IV, Sham Y, Gao J, Mayo KH. (2008) **CXC and CC chemokines form mixed heterodimers: association free energies from molecular dynamics simulations and experimental correlations.** *J Biol Chem.* 283(35):24155-66.
347. Nihlberg K, Larsen K, Hultgårdh-Nilsson A, Malmström A, Bjermer L, Westergren-Thorsson G. (2006) **Tissue fibrocytes in patients with mild asthma: a possible link to thickness of reticular basement membrane?** *Respir Res.* 7:50.
348. Ngo HT, Leleu X, Lee J, Jia X, Melhem M, Runnels J, Moreau AS, Burwick N, Azab AK, Roccaro A, Azab F, Sacco A, Farag M, Sackstein R, Ghobrial IM. (2008) **SDF-1/CXCR4 and VLA-4 interaction regulates homing in Waldenstrom macroglobulinemia.** *Blood.* 112(1):150-8.
349. Nials AT, Uddin S. (2008) **Mouse models of allergic asthma: acute and chronic allergen challenge.** *Dis Model Mech.* 1(4-5):213-20.
350. Niecknig H, Tug S, Reyes BD, Kirsch M, Fandrey J, Berchner-Pfannschmidt U. (2012) **Role of reactive oxygen species in the regulation of HIF-1 by prolyl hydroxylase 2 under mild hypoxia.** *Free Radic Res.* 46(6):705-17.
351. Nitta N, Tsuchiya T, Yamauchi A, Tamatani T, Kanegasaki S. (2007) **Quantitative analysis of eosinophil chemotaxis tracked using a novel optical device -- TAXIScan.** *J Immunol Methods.* 320(1-2):155-63.
352. Novak N, Peng WM, Bieber T, Akdis C (2013) **FcεRI stimulation promotes the differentiation of histamine receptor 1-expressing inflammatory macrophages.** *Allergy.* 68(4):454-61.
353. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, Wang YH, Watowich SS, Jetten AM, Tian Q, Dong C. (2008) **Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages.** *Immunity.* 29(1):138-49.
354. Oberlin E, Amara A, Bachelier F, Bessia C, Virelizier JL, Arenzana-Seisdedos F, Schwartz O, Heard JM, Clark-Lewis I, Legler DF, Loetscher M, Baggiolini M, Moser B. (1996) **The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1.** *Nature.* 382(6594):833-5.
355. Offermanns S. (2003) **G-proteins as transducers in transmembrane signalling.** *Prog Biophys Mol Biol.* 83(2):101-30.
356. Olson TS, Ley K. (2002) **Chemokines and chemokine receptors in leukocyte trafficking.** *Am J Physiol Regul Integr Comp Physiol.* 283(1):R7-28.
357. Onuffer JJ, Horuk R. (2002) **Chemokines, chemokine receptors and small-molecule antagonists: recent developments.** *Trends Pharmacol Sci.* 23(10):459-67.
358. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. (2005) **Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion.** *Cell.* 121(3):335-48
359. Orsini MJ, Parent JL, Mundell SJ, Marchese A, Benovic JL. (1999) **Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization.** *J Biol Chem.* 274(43):31076-86.
360. OWang J., Watanabe. T (2001) **Antigen Presentation to Lymphocytes** *Medical ENCYCLOPEDIA OF LIFE SCIENCES*
361. Pakdaman MN, Corry DB, Luong A (2011) **Fungi linking the pathophysiology of chronic rhinosinusitis with nasal polyps and allergic asthma.** *Immunol Invest.* 40(7-8):767-85.
362. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. (2010) **Role of Treg in immune regulation of allergic diseases.** *Eur J Immunol.* 2010 May;40(5):1232-40.
363. Pan CH, Chen CW, Sheu MJ, Wu CH. (2012) **Salvianolic acid B inhibits SDF-1 α -stimulated cell proliferation and migration of vascular smooth muscle cells by suppressing CXCR4 receptor.** *Vascul Pharmacol.* 56(1-2):98-105.
364. Panzer U, Kurts C. (2010) **T cell cross-talk with kidney dendritic cells in glomerulonephritis.** *J Mol Med (Berl).* 88(1):19-26.
365. Parker CC, Kim RH, Li BD, Chu QD (2012) **The chemokine receptor CXCR4 as a novel independent prognostic marker for node-positive breast cancer patients.** *J Surg Oncol.* 106(4):393-8.

366. Pasquier B, Lepelletier Y, Baude C, Hermine O, Monteiro RC (2004) **Differential expression and function of IgA receptors (CD89 and CD71) during maturation of dendritic cells.** *J Leukoc Biol.* 76(6):1134-41. Epub 2004 Sep 15.
367. Pease JE, Williams TJ. (2006) **Chemokines and their receptors in allergic disease.** *J Allergy Clin Immunol.* 118(2):305-18
368. Pease JE. (2011) **Targeting chemokine receptors in allergic disease.** *Biochem J.* 434(1):11-24
369. Pechkovsky DV, Zissel G, Ziegenhagen MW, Einhaus M, Taube C, Rabe KF, Magnussen H, Papadopoulos T, Schlaak M, Müller-Quernheim J. (2000) **Effect of proinflammatory cytokines on interleukin-8 mRNA expression and protein production by isolated human alveolar epithelial cells type II in primary culture.** *Eur Cytokine Netw.* 11(4):618-25.
370. Pejler G, Rönnerberg E, Waern I, Wernersson S. (2010) **Mast cell proteases: multifaceted regulators of inflammatory disease.** *Blood.* 115(24):4981-90.
371. Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V, Slav MM, Nagler A, Lider O, Alon R, Zipori D, Lapidot T. (2000) **The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice.** *Blood.* 95(11):3289-96.
372. Pelus LM, Fukuda S. (2006) **Peripheral blood stem cell mobilization: the CXCR2 ligand GRObeta rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties.** *Exp Hematol.* 34(8):1010-20.
373. Peng Q, Zhao L, Hou Y, Sun Y, Wang L, Luo H, Peng H, Liu M. (2013) **Biological characteristics and genetic heterogeneity between carcinoma-associated fibroblasts and their paired normal fibroblasts in human breast cancer.** *PLoS One.* 8(4):e60321.
374. Perissinotto E, Cavalloni G, Leone F, Fonsato V, Mitola S, Grignani G, Surrenti N, Sangiolo D, Bussolino F, Piacibello W, Aglietta M. (2005) **Involvement of chemokine receptor 4/stromal cell-derived factor 1 system during osteosarcoma tumor progression.** *Clin Cancer Res.* 11(2 Pt 1):490-7.
375. Petty JM, Lenox CC, Weiss DJ, Poynter ME, Suratt BT (2009) **Crosstalk between CXCR4/stromal derived factor-1 and VLA-4/VCAM-1 pathways regulates neutrophil retention in the bone marrow.** *J Immunol.* 182(1):604-12.
376. Pierre P, Turley SJ, Gatti E, Hull M, Meltzer J, Mirza A, Inaba K, Steinman RM, Mellman I. (1997) **Developmental regulation of MHC class II transport in mouse dendritic cells.** *Nature.* 388(6644):787-92.
377. Pillarisetti K, Gupta SK. (2001) **Cloning and relative expression analysis of rat stromal cell derived factor-1 (SDF-1)1: SDF-1 alpha mRNA is selectively induced in rat model of myocardial infarction.** *Inflammation.* 25:293-300.
378. Poggi A, Maggi E, Biagiotti R, Giudizi MG, Almerigogna F, Pella N, Caligaris-Cappio F, Romagnani S, Moretta L. (1990) **A novel pathway of human B cell activation initiated by CK226 surface antigen.** *Eur J Immunol.* 20(5):1161-5.
379. Poholek AC, Craft J (2009) **Competing for help: new insights into the function of follicular helper T cells.** *Immunol Cell Biol.* 87(6):438-9.
380. Porcheray F, Viaud S, Rimaniol AC, Leone C, Samah B. (2005) **Macrophage activation switching: an asset for the resolution of inflammation.** *Clin Exp Immunol.* 142:481-89
381. Poole JA, Gleason AM, Bauer C, West WW, Alexis N, van Rooijen N, Reynolds SJ, Romberger DJ, Kielian TL. (2012) **CD11c(+)/CD11b(+) cells are critical for organic dust-elicited murine lung inflammation.** *Am J Respir Cell Mol Biol.* 47(5):652-9.
382. Poole JA, Thiele GM, Alexis NE, Burrell AM, Parks C, Romberger DJ (2009). **Organic dust exposure alters monocyte-derived dendritic cell differentiation and maturation.** *Am J Physiol Lung Cell Mol Physiol.* 297(4):L767-76.
383. Porsbjerg CM, Gibson PG, Pretto JJ, Salome CM, Brown NJ, Berend N, King GG. (2013) **Relationship between airway pathophysiology and airway inflammation in older asthmatics.** *Respirology.*
384. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, van Oosterhout AJ, Heijink IH. (2012) **The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation.** *Thorax.* 67(6):488-95.
385. Pozzi LA, Maciaszek JW, Rock KL. (2005) **Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop effector function, and differentiate into memory cells.** *J Immunol.* 175(4):2071-81.
386. Proudfoot AE, Handel TM, Johnson Z, Lau EK, LiWang P, Clark-Lewis I, Borlat F, Wells TN, Kosco-Vilbois MH. (2003) **Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines.** *Proc Natl Acad Sci U S A.* 100(4):1885-90.

387. Proudfoot AE, Power CA, Rommel C, Wells TN. (2003) **Strategies for chemokine antagonists as therapeutics.** *Semin Immunol.* 15(1):57-65.
388. Proudfoot AE, Power CA, Schwarz MK. (2010) **Anti-chemokine small molecule drugs: a promising future?** *Expert Opin Investig Drugs.* 19(3):345-55.
389. Prussin C, Metcalfe DD. (2003) **IgE, mast cells, basophils, and eosinophils.** *J Allergy Clin Immunol.* 111(2 Suppl):S486-94.
390. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. (2000) **Involvement of the epidermal growth factor receptor in epithelial repair in asthma.** *FASEB J.* 14(10):1362-74.
391. Punia N, Smith S, Thomson JV, Irshad A, Nair P, Sehmi R. (2012) **Interleukin-4 and interleukin-13 prime migrational responses of haemopoietic progenitor cells to stromal cell-derived factor-1 α .** *Clin Exp Allergy.* 42(2):255-64.
392. Qian X, Zhang J, Liu J. (2011) **Tumor-secreted PGE2 inhibits CCL5 production in activated macrophages through cAMP/PKA signaling pathway.** *J Biol Chem.* 286(3):2111-20.
393. Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, Gerard C, Lefkowitz RJ. (2010) **Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7.** *Proc Natl Acad Sci U S A.* 107(2):628-32.
394. Raman D, Baugher PJ, Thu YM, Richmond A. (2007) **Role of chemokines in tumor growth.** *Cancer Lett.* 256(2):137-65.
395. Raman D, Sobolik-Delmaire T, Richmond A. (2011) **Chemokines in health and disease.** *Exp Cell Res.* 317(5):575-89.
396. Rangasamy T, Semenza GL, Georas SN. (2011) **What is hypoxia-inducible factor-1 doing in the allergic lung?** *Allergy.* 66(7):815-7.
397. Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Järvinen T, Savolainen J. (2008) **Prodrugs: design and clinical applications.** *Nat Rev Drug Discov.* 7(3):255-70
398. Reber LL, Daubeuf F, Nemska S, Frossard N. (2012) **The AGC kinase inhibitor H89 attenuates airway inflammation in mouse models of asthma.** *PLoS One.* 7(11):e49512.
399. Ribatti D, Puxeddu I, Crivellato E, Nico B, Vacca A, Levi-Schaffer F. (2009) **Angiogenesis in asthma.** *Clin Exp Allergy.* 39(12):1815-21.
400. Rijcken E, Mennigen RB, Schaefer SD, Laukoetter MG, Anthoni C, Spiegel HU, Bruewer M, Senninger N, Kriegelstein CF. (2007) **PECAM-1 (CD 31) mediates transendothelial leukocyte migration in experimental colitis.** *Am J Physiol Gastrointest Liver Physiol.* 293(2):G446-52.
401. Rivas-Caicedo A, Soldevila G, Fortoul TI, Castell-Rodríguez A, Flores-Romo L, García-Zepeda EA. (2009) **Jak3 is involved in dendritic cell maturation and CCR7-dependent migration.** *PLoS One.* 4(9):e7066.
402. Roberts AW. (2005) **G-CSF: a key regulator of neutrophil production, but that's not all!** *Growth Factors.* 23(1):33-41.
403. Rogers DF. (2003) **The airway goblet cell.** *Int J Biochem Cell Biol.* 35(1):1-6.
404. Rojo D, Suetomi K, Navarro J. (1999) **Structural biology of chemokine receptors.** *Biol Res.* 32(4):263-72.
405. Roland J, Murphy BJ, Ahr B, Robert-Hebmann V, Delauzun V, Nye KE, Devaux C, Biard-Piechaczyk M. (2003) **Role of the intracellular domains of CXCR4 in SDF-1-mediated signaling.** *Blood.* 101(2):399-406. Epub 2002 Sep 5.
406. Romagnani S. (2001) **T-cell responses in allergy and asthma.** *Curr Opin Allergy Clin Immunol.* 1(1):73-8.
407. Romagnani S. (2002) **Cytokines and chemoattractants in allergic inflammation.** *Mol Immunol.* 38(12-13):881-5.
408. Rosenwasser LJ, Meng J. (2005) **Anti-CD23.** *Clin Rev Allergy Immunol.* 29(1):61-72.
409. Rossi D, Zlotnik A. (2000) **The biology of chemokines and their receptors.** *Annu Rev Immunol.* 18:217-42.
410. Rothenberg ME, Hogan SP. (2006) **The eosinophil.** *Annu Rev Immunol.* 24:147-74.
411. Rowe J, Heaton T, Kusel M, Suriyaarachchi D, Serralha M, Holt BJ, de Klerk N, Sly PD, Holt PG. (2004) **High IFN-gamma production by CD8+ T cells and early sensitization among infants at high risk of atopy.** *J Allergy Clin Immunol.* 113(4):710-6.
412. Ryan JJ, DeSimone S, Klisch G, Shelburne C, McReynolds LJ, Han K, Kovacs R, Mirmonsef P, Huff TF. (1998) **IL-4 inhibits mouse mast cell Fc epsilonRI expression through a STAT6-dependent mechanism.** *J Immunol.* 161(12):6915-23.
413. Saini V, Marchese A, Majetschak M. (2010) **CXC chemokine receptor 4 is a cell surface receptor for extracellular ubiquitin.** *J Biol Chem.* 285(20):15566-76
414. Saini V, Staren DM, Ziarek JJ, Nashaat ZN, Campbell EM, Volkman BF, Marchese A, Majetschak M. (2011) **The CXC chemokine receptor 4 ligands ubiquitin and stromal cell-derived factor-1 α function through distinct receptor interactions.** *J Biol Chem.* 286(38):33466-77.

415. Sakai K, Yokoyama A, Kohno N, Hamada H, Hiwada K. (2001) **Prolonged antigen exposure ameliorates airway inflammation but not remodeling in a mouse model of bronchial asthma.** *Int Arch Allergy Immunol.* 126:126–134.
416. Sallusto F, Baggiolini M. (2008) **Chemokines and leukocyte traffic.** *Nat Immunol.* 9(9):949-52.
417. Sallusto F, Lanzavecchia A. (2000) **Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression.** *Immunol Rev.* 177:134-40.
418. Sallusto F, Schaerli P, Loetscher P, Scharniel C, Lenig D, Mackay CR, Qin S, Lanzavecchia A (1998) **Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation.** *Eur J Immunol* 28(9):2760–2769
419. Sánchez-Martín L, Sánchez-Mateos P, Cabañas C (2013) **CXCR7 impact on CXCL12 biology and disease.** *Trends Mol Med.* 19(1):12-22.
420. Sastre B. and del Pozo V. (2012) **Role of PGE2 in Asthma and Nonasthmatic Eosinophilic Bronchitis.** *Mediators Inflamm.* 2012: 645383.
421. Sartina E, Suguihara C, Ramchandran S, Nwajei P, Rodriguez M, Torres E, Hehre D, Devia C, Walters MJ, Penfold ME, Young KC. (2012) **Antagonism of CXCR7 attenuates chronic hypoxia-induced pulmonary hypertension.** *Pediatr Res.* 71(6):682-8.
422. Sayana S, Khanlou H. (2009) **Maraviroc: a new CCR5 antagonist.** *Expert Rev Anti Infect Ther.* 7(1):9-19.
423. Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. (2003) **Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma.** *J Immunol.* 171(1):380-9.
424. Schnur E, Kessler N, Zherdev Y, Noah E, Scherf T, Ding FX, Rabinovich S, Arshava B, Kurbatska V, Leonciks A, Tsimanis A, Rosen O, Naider F, Anglister J. (2013) **NMR mapping of RANTES surfaces interacting with CCR5 using linked extracellular domains.** *FEBS J.* 280(9):2068-84.
425. Schramm CM, Puddington L, Wu C, Guernsey L, Gharaee-Kermani M, Phan SH, Thrall RS. (2004) **Chronic inhaled ovalbumin exposure induces antigen-dependent but not antigen-specific inhalational tolerance in a murine model of allergic airway disease.** *Am J Pathol.* 164:295– 304.
426. Schwartz N, Grossman A, Levy Y, Schwarz Y (2012) **Correlation between eosinophil count and methacholine challenge test in asymptomatic subjects.** *J Asthma.* 49(4):336-41.
427. Schweizer RC, Welmers BA, Raaijmakers JA, Zanen P, Lammers JW, Koenderman L. (1994) **RANTES- and interleukin-8-induced responses in normal human eosinophils: effects of priming with interleukin-5.** *Blood.* 33(12):3697-704.
428. Scuri M, Forteza R, Lauredo I, Sabater JR, Botvinnikova Y, Allegra L, Abraham WM. (2000) **Inhaled porcine pancreatic elastase causes bronchoconstriction via a bradykinin-mediated mechanism.** *J Appl Physiol.* 89(4):1397-402.
429. Sebastiani S, Danelon G, Gerber B, Uguccioni M. (2005) **CCL22-induced responses are powerfully enhanced by synergy inducing chemokines via CCR4: evidence for the involvement of first beta-strand of chemokine.** *Eur J Immunol.* 35(3):746-56.
430. Sehra S, Yao W, Nguyen ET, Ahyi AN, Tuana FM, Ahlfeld SK, Snider P, Tepper RS, Petrache I, Conway SJ, Kaplan MH. (2011) **Periostin regulates goblet cell metaplasia in a model of allergic airway inflammation.** *J Immunol.* 186(8):4959-66.
431. Semenza GL. (2004) **Hydroxylation of HIF-1: oxygen sensing at the molecular level.** *Physiology (Bethesda).* 19:176-82.
432. Semple BD, Kossman T, Morganti-Kossmann MC. (2009) **Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks.** *J Cereb Blood Flow Metab.* 2010 March; 30(3): 459–473.
433. Serhan CN, Chiang N. (2007) **Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus.** *Br J Pharmacol.* 153 Suppl 1:S200-15.
434. Shalaby KH, Gold LG, Schuessler TF, Martin JG, Robichaud A (2010) **Combined forced oscillation and forced expiration measurements in mice for the assessment of airway hyperresponsiveness.** *Respir Res.* 11:82.
435. Shamri R, Xenakis JJ, Spencer LA. (2011) **Eosinophils in innate immunity: an evolving story.** *Cell Tissue Res.* 343(1):57-83.
436. Sharma M, Afrin F, Satija N, Tripathi RP, Gangenahalli GU. (2011) **Stromal-derived factor-1/CXCR4 signaling: indispensable role in homing and engraftment of hematopoietic stem cells in bone marrow.** *Stem Cells Dev.* 20(6):933-46.
437. Sharma M. (2010) **Chemokines and their receptors: orchestrating a fine balance between health and disease.** *Crit Rev Biotechnol.* 30(1):1-22.
438. Sharp FR, Bergeron M, Bernaudin M (2001) **Hypoxia-inducible factor in brain.** *Adv Exp Med Biol.* 502:273-91.

439. Sheu ML, Cheng FC, Su HL, Chen YJ, Chen CJ, Chiang CM, Chiu WT, Sheehan J, Pan HC. (2012) **Recruitment by SDF-1 α of CD34-positive cells involved in sciatic nerve regeneration.** *J Neurosurg.* 116(2):432-44.
440. Shin YS, Takeda K, Gelfand EW. (2009) **Understanding asthma using animal models.** *Allergy Asthma Immunol Res.* 1(1):10-8.
441. Shinagawa K, Kojima M (2003) **Mouse model of airway remodeling: strain differences.** *Am J Respir Crit Care Med.* 168(8):959-67.
442. Shirozu M, Nakano T, Inazawa J, Tashiro K, Tada H, Shinohara T, Honjo T. (1995) **Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene.** *Genomics.* 10;28(3):495-500.
443. Sica A, Schioppa T, Mantovani A, Allavena P. (2006) **Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy.** *Eur J Cancer.* 42(6):717-27. Epub 2006 Mar 7.
444. Sidhu SS, Yuan S, Innes AL, Kerr S, Woodruff PG, Hou L, Muller SJ, Fahy JV. (2010) **Roles of epithelial cell-derived periostin in TGF-beta activation, collagen production, and collagen gel elasticity in asthma.** *Proc Natl Acad Sci U S A.* 107(32):14170-5.
445. Sierro F, Biben C, Martínez-Muñoz L, Mellado M, Ransohoff RM, Li M, Woehl B, Leung H, Groom J, Batten M, Harvey RP, Martínez-A C, Mackay CR, Mackay F. (2007) **Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7.** *Proc Natl Acad Sci U S A.* 104(37):14759-64.
446. Simeone-Penney MC, Severgnini M, Tu P, Homer RJ, Mariani TJ, Cohn L, Simon AR. (2007) **Airway epithelial STAT3 is required for allergic inflammation in a murine model of asthma.** *J Immunol.* 178(10):6191-9.
447. Singh G, Katyal SL. (1997) **Clara cells and Clara cell 10 kD protein (CC10).** *Am J Respir Cell Mol Biol.* 17(2):141-3.
448. Siveke JT, Hamann A. (1998) **T helper 1 and T helper 2 cells respond differentially to chemokines.** *J Immunol.* 15;160(2):550-4.
449. Smith AM, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW, Palmer CD, Wilde J, Foxwell BM, Gloger IS, Sweeting T, Marsh M, Walker AP, Bloom SL, Segal AW. (2009) **Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease.** *J Exp Med.* 206(9):1883-97.
450. Smith KA, Harcus Y, Garbi N, Hämmerling GJ, MacDonald AS, Maizels RM. (2012) **Type 2 innate immunity in helminth infection is induced redundantly and acts autonomously following CD11c(+) cell depletion.** *Infect Immun.* 80(10):3481-9.
451. Smith N, Johnson FJ (2005) **Early- and late-phase bronchoconstriction, airway hyper-reactivity and cell influx into the lungs, after 5'-adenosine monophosphate inhalation: comparison with ovalbumin.** *Clin Exp Allergy.* 35(4):522-30.
452. So T, Song J, Sugie K, Altman A, Croft M (2006) **Signals from OX40 regulate nuclear factor of activated T cells c1 and T cell helper 2 lineage commitment.** *Proc Natl Acad Sci U S A.* 2006 Mar 7;103(10):3740-5. Epub 2006 Feb 24.
453. Song C, Ma H, Yao C, Tao X, Gan H. (2012) **Alveolar macrophage-derived vascular endothelial growth factor contributes to allergic airway inflammation in a mouse asthma model.** *Scand J Immunol.* 75(6):599-605.
454. Spergel JM, Mizoguchi E, Brewer JP, Martin TR, Bhan AK, Geha RS. (1998) **Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice.** *J Clin Invest.* 101(8):1614-22.
455. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W (2003) **Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL.** *Nature.* 425(6955):307-11.
456. Stassen M, Schmitt E, Bopp T. (2012) **From interleukin-9 to T helper 9 cells.** *Ann N Y Acad Sci.* 1247:56-68.
457. Steen A, Schwartz TW, Rosenkilde MM. (2009) **Targeting CXCR4 in HIV cell-entry inhibition.** *Mini Rev Med Chem.* 9(14):1605-21.
458. Stella VJ, Charman WN, Naringrekar VH. (1985) **Prodrugs. Do they have advantages in clinical practice?** *Drugs.* 29(5):455-73
459. Stone KD, Prussin C, Metcalfe DD. (2010) **IgE, mast cells, basophils, and eosinophils.** *J Allergy Clin Immunol.* 125(2 Suppl 2):S73-80.
460. Stumm RK, Rummel J, Junker V, Culmsee C, Pfeiffer M, Kriegelstein J, Höllt V, Schulz S. (2002) **A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia.** *J Neurosci.* 22(14):5865-78.

461. Stutte S, Quast T, Gerbitzki N, Savinko T, Novak N, Reifenberger J, Homey B, Kolanus W, Alenius H, Förster I. (2010) **Requirement of CCL17 for CCR7- and CXCR4-dependent migration of cutaneous dendritic cells.** *Proc Natl Acad Sci U S A.* 107(19):8736-41.
462. Suárez-Álvarez B, López-Vázquez A, López-Larrea C. (2012) **Mobilization and homing of hematopoietic stem cells.** *Adv Exp Med Biol.* 741:152-70.
463. Suzuki Y, Rahman M, Mitsuya H. (2001) **Diverse transcriptional response of CD4(+) T cells to stromal cell-derived factor (SDF)-1: cell survival promotion and priming effects of SDF-1 on CD4(+) T cells.** *J Immunol.* 167(6):3064-73.
464. Swirski FK, Sajic D, Robbins CS, Gajewska BU, Jordana M, Stampfli MR. (2002) **Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-stimulating factor.** *J Immunol.* 169:3499–3506.
465. Szekanecz Z, Vegvari A, Szabo Z, Koch AE. (2010) **Chemokines and chemokine receptors in arthritis.** *Front Biosci.* 2:153-67.
466. Szpakowska M, Fievez V, Arumugan K, van Nuland N, Schmit JC, Chevigné A. (2012) **Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors.** *Biochem Pharmacol.* 84(10):1366-80.
467. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T. (1998) **The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract.** *Nature.* 393(6685):591-4.
468. Takahashi N, Tetsuka T, Uranishi H, Okamoto T. (2002) **Inhibition of the NF-kappaB transcriptional activity by protein kinase A.** *Eur J Biochem.* 2002 Sep;269(18):4559-65.
469. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, Littman DR, O'Shea JJ. (2008) **Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22.** *J Exp Med.* 206(1):35-41.
470. Tasat DR, Mancuso R, O'Connor S, Molinari B. (2003) **Age-dependent change in reactive oxygen species and nitric oxide generation by rat alveolar macrophages.** *Aging Cell.* 2(3):159-64.
471. Tashiro K, Tada H, Heilker R, Shirozu M, Nakano T, Honjo T. (1993) **Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins.** *Science.* 30;261(5121):600-3.
472. Teicher BA, Fricker SP. (2010) **CXCL12 (SDF-1)/CXCR4 pathway in cancer.** *Clin Cancer Res.* 16(11):2927-31.
473. Temelkovski J, Hogan SP, Shepherd DP, Foster PS, Kumar RK. (1998) **An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen.** *Thorax.* 53(10):849-56.
474. Terada N, Nomura T, Kim WJ, Otsuka Y, Takahashi R, Kishi H, Yamashita T, Sugawara N, Fukuda S, Ikeda-Ito T, Konno A. (2001) **Expression of C-C chemokine TARC in human nasal mucosa and its regulation by cytokines.** *Clin Exp Allergy.* 31(12):1923-31.
475. Teran LM, Mochizuki M, Bartels J, Valencia EL, Nakajima T, Hirai K, Schröder JM. (1999) **Th1- and Th2-type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts.** *Am J Respir Cell Mol Biol.* 20(4):777-86.
476. Tete S, Saggini A, Maccauro G, Rosati M, Conti F, Cianchetti E, Tripodi D, Toniato E, Fulcheri M, Salini V, Caraffa A, Antinolfi P, Frydas S, Pandolfi F, Conti P, Potalivo G, Nicoletti M, Theoharides TC. (2012) **Interleukin-9 and mast cells.** *J Biol Regul Homeost Agents.* 26(3):319-26.
477. Tewes F, Brillault J, Couet W, Olivier JC. (2008) **Formulation of rifampicin-cyclodextrin complexes for lung nebulization.** *J Control Release.* 129(2):93-9.
478. Thelen M, Muñoz LM, Rodríguez-Frade JM, Mellado M. (2010) **Chemokine receptor oligomerization: functional considerations.** *Curr Opin Pharmacol.* 10(1):38-43.
479. Thelen M, Thelen S (2008) **CXCR7, CXCR4 and CXCL12: an eccentric trio?** *J Neuroimmunol.* 198(1-2):9-13.
480. Tillie-Leblond I, Iliescu C, Deschildre A. (2004) **Physiopathology of inflammatory events in asthma.** *Arch Pediatr.* 11 Suppl 2:58s-64s.
481. Tomlinson KL, Davies GC, Sutton DJ, Palframan RT. (2010) **Neutralisation of interleukin-13 in mice prevents airway pathology caused by chronic exposure to house dust mite.** *PLoS One.* 5(10). pii: e13136.
482. Toujas L, Delcros JC, E Diez, N Gervois, G Semana, G Corradin, and Jotereau F. (1997) **Human monocyte-derived macrophages and dendritic cells are comparably effective in vitro in presenting HLA class I-restricted exogenous peptides.** *Immunology.* 91(4): 635–642.
483. Trombetta ES, Mellman I. (2005) **Cell biology of antigen processing in vitro and in vivo.** *Annu Rev Immunol.* 23:975–1028.
484. Uddin M, Levy BD. (2011) **Resolvins: natural agonists for resolution of pulmonary inflammation.** *Prog Lipid Res.* 2011 Jan;50(1):75-88.

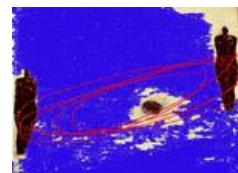
485. Ueno H, Klechevsky E, Morita R, Asford C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V, Palucka AK, Banchereau J. (2007) **Dendritic cell subsets in health and disease.** *Immunol Rev.* 219:118-42.
486. Ulrich K, Hincks JS, Walsh R, Wetterstrand EM, Fidock MD, Sreckovic S, Lamb DJ, Douglas GJ, Yeadon M, Perros-Huguet C, Evans SM. (2008) **Anti-inflammatory modulation of chronic airway inflammation in the murine house dust mite model.** *Pulm Pharmacol Ther.* 21(4):637-47.
487. Umemoto E, Otani K, Ikeno T, Verjan Garcia N, Hayasaka H, Bai Z, Jang MH, Tanaka T, Nagasawa T, Ueda K, Miyasaka M. (2012) **Constitutive plasmacytoid dendritic cell migration to the splenic white pulp is cooperatively regulated by CCR7- and CXCR4-mediated signaling.** *J Immunol.* 189(1):191-9.
488. Unsoeld H, Krautwald S, Voehringer D, Kunzendorf U, Pircher H. (2002) **Cutting edge: CCR7+ and CCR7-memory T cells do not differ in immediate effector cell function.** *J Immunol.* 169(2):638-41.
489. van Buul JD, Hordijk PL. (2004) **Signaling in leukocyte transendothelial migration.** *Arterioscler Thromb Vasc Biol.* 24(5):824-33.
490. Van Hove CL, Maes T, Joos GF, Tournoy KG. (2007) **Prolonged inhaled allergen exposure can induce persistent tolerance.** *Am J Respir Cell Mol Biol.* 36:573-84.
491. Van Loon S. L. M, Smits A. I. P. M, Driessen-Mol A, Baaijens F. P. T, Bouten C. V. C (2013) **The Immune Response in In Situ Tissue Engineering of Aortic Heart Valves.** book "Calcific Aortic Valve Disease", edited by Elena Aikawa (ISBN 978-953-51-1150-4)
492. Vanoirbeek JA, Rinaldi M, De Vooght V, Haenen S, Bobic S, Gayan-Ramirez G, Hoet PH, Verbeken E, Decramer M, Nemery B, Janssens W. (2010) **Noninvasive and invasive pulmonary function in mouse models of obstructive and restrictive respiratory diseases.** *Am J Respir Cell Mol Biol.* 42(1):96-104.
493. van Oosterhout AJ, Bloksma N. (2005) **Regulatory T-lymphocytes in asthma.** *Eur Respir J.* 26(5):918-32. Review
494. Vasconcelos DP, Fonseca AC, Costa M, Amaral IF, Barbosa MA, Águas AP, Barbosa JN. (2013) **Macrophage polarization following chitosan implantation.** *Biomaterials.* 34(38):9952-9.
495. Vater A, Sahlmann J, Kröger N, Zöllner S, Lioznov M, Maasch C, Buchner K, Vossmeier D, Schwoebel F, Purschke WG, Vonhoff S, Kruschinski A, Hübel K, Humphrey M, Klussmann S, Fliegert F. (2013) **Hematopoietic stem and progenitor cell mobilization in mice and humans by a first-in-class mirror-image oligonucleotide inhibitor of CXCL12.** *Clin Pharmacol Ther.* 94(1):150-7.
496. Velazquez JR, Teran LM (2011) **Chemokines and their receptors in the allergic airway inflammatory process.** *Clin Rev Allergy Immunol.* 41(1):76-88.
497. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, Martin B, Wilhelm C, Stockinger B. (2008) **Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset.** *Nat Immunol.* 9(12):1341-6.
498. Verbeke H, De Hertogh G, Li S, Vandercappellen J, Noppen S, Schutyser E, El-Asrar AA, Opendakker G, Van Damme J, Geboes K, Struyf S. (2010) **Expression of angiostatic platelet factor-4var/CXCL4L1 counterbalances angiogenic impulses of vascular endothelial growth factor, interleukin-8/CXCL8, and stromal cell-derived factor 1/CXCL12 in esophageal and colorectal cancer.** *Hum Pathol.* 41(7):990-1001.
499. Vercelli D. (2002) **The regulation of IgE synthesis.** *Clin Allergy Immunol.* 16:179-96.
500. Vermaelen KY, Cataldo D, Tournoy K, Maes T, Dhulst A, Louis R, Foidart JM, Noël A, Pauwels R. (2003) **Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in a mouse model of asthma** *J Immunol.* 171(2):1016-22.
501. Voelkel NF, Spiegel S. (2009) **Why is effective treatment of asthma so difficult? An integrated systems biology hypothesis of asthma.** *Immunol Cell Biol.* 87(8):601-5.
502. Voynow JA, Fischer BM, Zheng S. (2008) **Proteases and cystic fibrosis.** *Int J Biochem Cell Biol.* 40(6-7):1238-45.
503. Vroon A, Heijnen CJ, Raatgever R, Touw IP, Ploemacher RE, Premont RT, Kavelaars A. (2004) **GRK6 deficiency is associated with enhanced CXCR4-mediated neutrophil chemotaxis in vitro and impaired responsiveness to G-CSF in vivo.** *J Leukoc Biol.* 75(4):698-704.
504. Wada Y, Yoshida K, Tsutani Y, Shigematsu H, Oeda M, Sanada Y, Suzuki T, Mizuiri H, Hamai Y, Tanabe K, Ukon K, Hihara J. **Neutrophil elastase induces cell proliferation and migration by the release of TGF-alpha, PDGF and VEGF in esophageal cell lines.** *Oncol Rep.* 17:161-167.
505. Wadsworth SJ, Yang J, Dorscheid D (2012) **IL-13, Asthma and Glycosylation in Airway Epithelial Repair.** *Biochemistry, Genetics and Molecular Biology "Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology"*, book, chapter 9, ISBN 978-953-51-0864-1.
506. Wald O, Shapira OM, Izhar U. (2013) **CXCR4/CXCL12 axis in non small cell lung cancer (NSCLC) pathologic roles and therapeutic potential.** *Theranostics.* 3(1):26-33.

507. Wang A, Guilpain P, Chong BF, Chouzenoux S, Guillevin L, Du Y, Zhou XJ, Lin F, Fairhurst AM, Boudreaux C, Roux C, Wakeland EK, Davis LS, Batteux F, Mohan C. (2010) **Dysregulated expression of CXCR4/CXCL12 in subsets of patients with systemic lupus erythematosus.** *Arthritis Rheum.* 62(11):3436-46.
508. Wang BX, Fish EN. (2012) **The yin and yang of viruses and interferons.** *Trends Immunol.* 33(4):190-7.
509. Wang J, Jiao H, Stewart TL, Lyons MV, Shankowsky HA, Scott PG, Tredget EE (2007) **Accelerated wound healing in leukocyte-specific, protein 1-deficient mouse is associated with increased infiltration of leukocytes and fibrocytes.** *J Leukoc Biol.* 82(6):1554-63.
510. Wang J, Shiozawa Y, Wang J, Wang Y, Jung Y, Pienta KJ, Mehra R, Loberg R, Taichman RS. (2008) **The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer.** *J Biol Chem.* 283(7):4283-94.
511. Wang J, Wang J, Dai J, Jung Y, Wei CL, Wang Y, Havens AM, Hogg PJ, Keller ET, Pienta KJ, Nor JE, Wang CY, Taichman RS. (2007) **A glycolytic mechanism regulating an angiogenic switch in prostate cancer.** *Cancer Res.* 67(1):149-59.
512. Wang, J., Shiozawa, Y., Wang, Y., Jung, Y., Pienta, K. J., Mehra, R., Loberg, R., Taichman, R. S. (2008) **The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer.** *J. Biol. Chem.* 283,4283-4294
513. Wang JY. (2013) **The innate immune response in house dust mite-induced allergic inflammation.** *Allergy Asthma Immunol Res.* 5(2):68-74.
514. Wang Q, Diao X, Sun J, Chen Z. (2011) **Regulation of VEGF, MMP-9 and metastasis by CXCR4 in a prostate cancer cell line.** *Cell Biol Int.* 35(9):897-904
515. Wang Y, Li Y, Shan J, Fixman E, McCusker C. (2011) **Effective treatment of experimental ragweed-induced asthma with STAT-6-IP, a topically delivered cell-penetrating peptide.** *Clin Exp Allergy.* 41(11):1622-30.
516. Wang Z, Huang H. (2013) **Platelet factor-4 (CXCL4/PF-4): an angiostatic chemokine for cancer therapy.** *Cancer Lett.* 331(2):147-53.
517. Wegmann M. (2008) **Animal models of chronic experimental asthma - strategies for the identification of new therapeutic targets.** *J Occup Med Toxicol.* 3 Suppl 1:S4.
518. Wen T, Besse JA, Mingler MK, Fulkerson PC, Rothenberg ME. (2013) **Eosinophil adoptive transfer system to directly evaluate pulmonary eosinophil trafficking in vivo.** *Proc Natl Acad Sci U S A.* 110(15):6067-72.
519. Werner, Hanan Guzner-Gur, Iris Dotan (2013) **Involvement of CXCR4/CXCR7/CXCL12 Interactions in Inflammatory Bowel Disease Theranostics.** 3(1):40-46
520. Whitehead GS, Walker JK, Berman KG, Foster WM, Schwartz DA. (2003) **Allergen-induced airway disease is mouse strain dependent.** *Am J Physiol Lung Cell Mol Physiol.* 2003 Jul;285(1):L32-42. Epub 2003 Mar 7.
521. Wong MM, Fish EN. (2003) **Chemokines: attractive mediators of the immune response.** *Semin Immunol.* 15(1):5-14.
522. Wright, N., Hidalgo, A., Rodriguez-Frade, J. M., Soriano, S. F., Mellado, M., Pardo-Cabanas, M., Briskin, M. J., Teixido, J. (2002) **The chemokine stromal cell-derived factor-1 α modulates $\alpha 4 \beta 7$ integrin-mediated lymphocyte adhesion to mucosal addressin cell adhesion molecule-1 and fibronectin.** *J. Immunol.* 168,5268-5277
523. Wu K, Bi Y, Sun K, Wang C. (2007) **IL-10-producing type 1 regulatory T cells and allergy.** *Cell Mol Immunol.* 4(4):269-75.
524. Xanthou G, Duchesnes CE, Williams TJ, Pease JE. (2003) **CCR3 functional responses are regulated by both CXCR3 and its ligands CXCL9, CXCL10 and CXCL11.** *Eur J Immunol.* 33(8):2241-50.
525. Xia L, Lu J, Xiao W. (2011) **Blockage of TNF- α by infliximab reduces CCL2 and CCR2 levels in patients with rheumatoid arthritis.** *J Investig Med.* 59(6):961-3.
526. Xu KF, Vlahos R, Messina A, Bamford TL, Bertram JF, Stewart AG. (2002) **Antigen-induced airway inflammation in the Brown Norway rat results in airway smooth muscle hyperplasia.** *J Appl Physiol.* 93(5):1833-40.
527. Yiamouyiannis CA, Schramm CM, Puddington L, Stengel P, Baradaran-Hosseini E, Wolynec WW, Whiteley HE, Thrall RS. (1999) **Shifts in lung lymphocyte profiles correlate with the sequential development of acute allergic and chronic tolerant stages in a murine asthma model.** *Am J Pathol.* 154(6):1911-21.
528. Yin G, Yan C, Berk BC. (2003) **Angiotensin II signaling pathways mediated by tyrosine kinases.** *Int J Biochem Cell Biol.* 35(6):780-3.
529. Yin W, Ma L, Zhang J, Huang K, Yang Q, Guo YY, Liu SB, Liu YH, Wu YM. (2013) **The migration of neural progenitor cell mediated by SDF-1 is NF- κ B/HIF-1 α dependent upon hypoxia.** *CNS Neurosci Ther.* 19(3):145-53.
530. Ying S, Barata LT, Meng Q, Grant JA, Barkans J, Durham SR, Kay AB. (1998) **High-affinity immunoglobulin E receptor (Fc epsilon R1)-bearing eosinophils, mast cells, macrophages and Langerhans' cells in allergen-induced late-phase cutaneous reactions in atopic subjects.** *Immunology.* 93(2):281-8.

531. Young KC, Torres E, Hatzistergos KE, Hehre D, Suguihara C, Hare JM. (2009) **Inhibition of the SDF-1/CXCR4 axis attenuates neonatal hypoxia-induced pulmonary hypertension.** *Circ Res.* 104(11):1293-301.
532. Yu L, Cecil J, Peng SB, Schrementi J, Kovacevic S, Paul D, Su EW, Wang J. (2006) **Identification and expression of novel isoforms of human stromal cell-derived factor 1.** *Gene.* 374:174-9.
533. Yu L, Hales CA. (2011) **Effect of chemokine receptor CXCR4 on hypoxia-induced pulmonary hypertension and vascular remodeling in rats.** *Respir Res.* 12:21.
534. Yu M, Tsai M, Tam SY, Jones C, Zehnder J, Galli SJ. (2006) **Mast cells can promote the development of multiple features of chronic asthma in mice.** *J Clin Invest.* 2006 Jun;116(6):1633-41.
535. Yum HY, Cho JY, Miller M, Broide DH. (2011) **Allergen-induced coexpression of bFGF and TGF- β 1 by macrophages in a mouse model of airway remodeling: bFGF induces macrophage TGF- β 1 expression in vitro.** *Int Arch Allergy Immunol.* 155(1):12-22.
536. Zamorano J, M.D. Rivas, M. Pérez-G **Interleukin-4: A multifunctional cytokine** *Inmunología*, Vol. 22 / Núm 2/ Abril-Junio 2003: 215-224
537. Zboralski D, Hoellenriegel J, Maasch C, Kruschinski A, Burger JA (2012) **Mode of Action of the SDF-1/CXCL12 Inhibiting Spiegelmer NOX-A12 and Its Impact On Chronic Lymphocytic Leukemia (CLL) Cell Motility and Chemosensitization.** *Blood (ASH Annual Meeting Abstracts) 2012* 120: Abstract 318 (<http://abstracts.hematologylibrary.org/cgi/content/abstract/120/21/318>)
538. Zhang M, Murphy RF, Agrawal DK. (2007) **Decoding IgE Fc receptors.** *Immunol Res.* 37(1):1-16.
539. Zhang SS, Han ZP, Jing YY, Tao SF, Li TJ, Wang H, Wang Y, Li R, Yang Y, Zhao X, Xu XD, Yu ED, Rui YC, Liu HJ, Zhang L, Wei LX. (2012) **CD133(+)/CXCR4(+) colon cancer cells exhibit metastatic potential and predict poor prognosis of patients.** *BMC Med.* 10:85.
540. Zhang Z, Hener P, Frossard N, Kato S, Metzger D, Li M, Chambon P. (2009) **Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma.** *Proc Natl Acad Sci U S A.* 106(5):1536-41.
541. Zhang Z, Zhong W, Hall MJ, Kurre P, Spencer D, Skinner A, O'Neill S, Xia Z, Rosenbaum JT. (2009) **CXCR4 but not CXCR7 is mainly implicated in ocular leukocyte trafficking during ovalbumin-induced acute uveitis.** *Exp Eye Res.* 89(4):522-31.
542. Zhou D, Chen G, Kim JT, Lee LY, Kang BC. (1998) **A dose-response relationship between exposure to cockroach allergens and induction of sensitization in an experimental asthma in Hartley guinea pigs.** *J Allergy Clin Immunol.* 101(5):653-9.
543. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. (1999) **Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production.** *J Clin Invest.* 103(6):779-88.
544. Zhuo W, Jia L, Song N, Lu XA, Ding Y, Wang X, Song X, Fu Y, Luo Y (2012) **The CXCL12-CXCR4 chemokine pathway: a novel axis regulates lymphangiogenesis.** *Clin Cancer Res.* 18(19):5387-98.
545. Ziarek JJ, Veldkamp CT, Zhang F, Murray NJ, Kartz GA, Liang X, Su J, Baker JE, Linhardt RJ, Volkman BF. (2013) **Heparin oligosaccharides inhibit chemokine (CXC motif) ligand 12 (CXCL12) cardioprotection by binding orthogonal to the dimerization interface, promoting oligomerization, and competing with the chemokine (CXC motif) receptor 4 (CXCR4) N terminus.** *J Biol Chem.* 288(1):737-46.
546. Ziarek JJ, Volkman BF. (2012) **NMR in the Analysis of Functional Chemokine Interactions and Drug Discovery.** *Drug Discov Today Technol.* 9(4):e293-e299.
547. Zissel G, K Höhne, A Kilic, C Maier, T Goldmann, A Prasse, T Ploenes, M Trepel, H Eibel, J Müller-Quernheim (2012) **Identification of the CCL18 Receptor - Effects of CCL18 on Human Lung Fibroblasts in Pulmonary Fibrosis are Mediated via CCR6.** *Pneumologie* 2012; 66 - P3_012
548. Zlotnik A, Yoshie O, Nomiyama H. (2006) **The chemokine and chemokine receptor superfamilies and their molecular evolution.** *Genome Biol.* 7(12):243.
549. Zlotnik A, Yoshie O. (2000) **Chemokines: a new classification system and their role in immunity.** *Immunity.* 12(2):121-7.
550. Zlotnik A, Yoshie O. (2012) **The chemokine superfamily revisited.** *Immunity.* 36(5):705-16.
551. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. (1998) **Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development.** *Nature.* 393(6685):595-9.
552. Zhou Z, Connell MC, MacEwan DJ. (2007) **TNFR1-induced NF- κ B, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells.** *Cell Signal.* 19(6):1238-48.

François DAUBEUF

NEUTRALIGANDS DE LA CHIMIOKINE CXCL12 DANS L'ASTHME



Résumé

La liaison de CXCL12 à ses récepteurs CXCR4 et CXCR7 peut-être bloquée par un neutraligand de CXCL12, la chalcone 4, qui présente une activité anti-inflammatoire dans un modèle d'asthme chez la souris. Notre travail a consisté à proposer des stratégies permettant le développement de molécules biodisponibles et actives localement, et d'étudier le mécanisme d'action in vivo des neutraligands de CXCL12.

Nous avons développé un modèle court et reproductible d'asthme allergique chez la souris, adapté à une évaluation rapide de l'activité anti-inflammatoire des nouveaux composés et un développement raisonné des stratégies envisagées.

Nous avons synthétisé trois prodrogues solubles, adaptées à une administration locale, inactives et rapidement clivées en chalcone 4 active. In vivo, les prodrogues sont anti-inflammatoires à des doses susceptibles de limiter les effets indésirables.

Pour favoriser davantage l'action anti-inflammatoire locale du neutraligand de CXCL12, nous avons synthétisé une ante-droque, la carbonitrile-chalcone 4, active in vivo par administration locale et rapidement dégradée en deux composés inactifs avant sa distribution dans l'organisme.

Enfin, l'étude de la chalcone 4 a mis en évidence une activité antiasthmatisque significative, liée à l'élimination rapide de la chimiokine CXCL12 du poumon. La capture de CXCL12 par le neutraligand réduit la différenciation des macrophages M1 et leur libération de cytokines pro-inflammatoires, ainsi que le recrutement des éosinophiles et des lymphocytes CXCR4+.

En conclusion, nos travaux ont permis de conforter les rôles de la chimiokine CXCL12 dans l'asthme et présenter deux stratégies aptes à limiter les effets indésirables.

Résumé en anglais

The binding of the chemokine CXCL12 to its receptors CXCR4 and CXCR7 may be prevented by a CXCL12 neutraligand, chalcone 4, having an anti-inflammatory activity in a mouse model of allergic asthma. Our work consisted in proposing strategies for the development of active and bioavailable molecules, able to promote local action, and to study the in vivo mechanism of action of the CXCL12 neutraligands.

We initially developed a short and reproducible mouse model of allergic asthma, suitable for a rapid assessment of the anti-inflammatory activity of new compounds, in order to ensure rational development of the proposed strategies.

We developed three soluble prodrugs, adapted to local administration, inactive but rapidly cleaved in active chalcone 4. In vivo, the prodrugs have an anti-inflammatory activity at suitable doses to minimize side effects.

To promote the benefit of local anti-inflammatory action of CXCL12 neutraligand, we synthesized an antedrug, carbonitrile-chalcone 4, active locally in vivo after local administration and rapidly degraded before its distribution in the body.

Finally, the study of chalcone 4 allowed us to highlight a significant asthma activity. An activity related to the rapid elimination of the CXCL12 chemokine from the lung. The trapping of CXCL12 by the neutraligand reduced M1 macrophage activation and their release of pro-inflammatory cytokines, as decreases the recruitment of CXCR4+ eosinophils and lymphocytes.

In conclusion, our work provided mechanistic elements related to the roles of the chemokine CXCL12 in asthma, and present two interesting strategies adapted to local administration to limit adverse effects.