Interactions entre CCR5 et trois récepteurs couplés aux protéines G : EBI2, A2A et S1P1 : effets sur l'infection par le VIH-1, mécanismes d'action et perspectives thérapeutiques

Adeline Guigues

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Interactions entre CCR5 et trois récepteurs couplés aux protéines G : EBI2, A_{2A} et S1P1.

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Présentée par Adeline GUIGUES
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Sous la direction de Pierre CORBEAU
Et Vincent FRANCOIS

Devant le jury composé de

Martine BIARD, Directeur de Recherche, IRIM, Montpellier	Président
Elmostafa BAHRAOUI, Professeur, CHU Purpan	Rapporteur
Thomas BOURLET, Professeur, GIMAP et CHU Saint-Etienne	Rapporteur
Jérôme ESTAQUIER, Professeur, CHUL, Québec	Rapporteur
Pierre CORBEAU, Professeur, IGH et CHU Nîmes	Directeur de thèse
Vincent FRANCOIS, Chargé de Recherche, IGH, Montpellier	Co-Directeur de thèse

UNIVERSITÉ DE MONTPELLIER

Résumé

Mon laboratoire d'accueil avait montré que la densité de CCR5 à la surface des cellules cibles du VIH détermine très fortement leur infectabilité par les souches virales R5, et que les signaux d'activation cellulaire induits par la fixation virale et transitant par CCR5 favorisent une infection productive des cellules primaires. L'hypothèse de notre travail était que d'autres récepteurs couplés aux protéines G (RCPG) exprimés dans les lymphocytes T CD4+ CCR5+ pourraient également influer sur le cycle de réplication des virus R5. Pour tester cette hypothèse, les RCPGs co-exprimés avec CCR5 dans les cellules T CD4+ ont été identifiés, puis la capacité des plus exprimés d'entre eux à s'hétérodimériser avec CCR5 et à perturber l'infection par le VIH a été déterminée. Au cours de cette thèse, j'ai initié l'étude de deux de ces récepteurs, EBI2 et A_{2A}, et j'ai participé à l'étude d'un troisième, S1P1.

J'ai montré que la présence d'EBI2 ou d'A_{2A} qui chacun s'hétérodimérise avec CCR5, augmente la production virale dans des cellules HOS ou MT4, comme c'est le cas pour S1P1. Étudiant les changements au niveau des étapes du cycle viral provoqués par l'expression ou la stimulation de ces RCPGS, j'ai mis en évidence que le principal effet est une augmentation de la transcription virale. De plus, nous avons montré que la signalisation via S1P1 active le facteur de transcription NFκB et par là le promoteur du VIH. De plus, la stimulation de ce récepteur dans un modèle de cellules infectées de façon latente permet la réactivation du virus. Ce résultat ouvre la possibilité de tester la capacité d'agonistes de S1P1 à éliminer les cellules servant de réservoir chez les personnes vivant avec le VIH.

Abstract

It was shown in the laboratory that CCR5 density at the surface of primary CD4+ T cells strongly determines their R5 HIV-1 productive infectability. This is due to the fact that viral envelope - CCR5 interaction triggers signals that boost the viral life cycle. Our working hypothesis was that other G-protein coupled receptors (GPCR) co-expressed with CCR5 might also modulate HIV replicative cycle. To test this hypothesis, the GPCR coexpressed with CCR5 in CD4+ T cells have been identified, and the ability of the most highly expressed of them to dimerize with CCR5 and to interfere with the infection has been determined. During this thesis, I have initiated the study of two of these GPCR, EBI2 and A_{2A}, and participated in the study of another one, S1P1.

I have shown that the presence of EBI2 or A_{2A} that each heterodimerize with CCR5, boosts the viral production in HOS and MT4 cells, similarly to S1P1. Analyzing the stages of HIV life cycle modified by the expression and triggering of these GPCR, I unveiled that the major effect was an increase in HIV genome transcription. Furthermore, we have shown that S1P1 signaling activates the transcription factor NFκB and thereby the HIV promoter. Moreover, triggering S1P1 in an *in vitro* model of HIV reservoir cells results in the reversion of the viral latency. These findings suggest that S1P1 agonists might be used as latency reversing agents to purge the HIV reservoir.

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I. Introduction générale

A. Virus de l'immunodéficience humaine (VIH)

1. Historique

Les débuts de l'épidémie du SIDA (syndrome de l'immunodéficience humaine) ont été observés en 1981 aux États-Unis par une recrudescence de pneumocystoses et de sarcomes de Kaposi chez des personnes immunodéprimées. Ces patients présentaient un effondrement d'une sous-population lymphocytaire : les lymphocytes T CD4.

L'hypothèse d'un agent infectieux transmissible par le sang et les relations sexuelles va faire son chemin (Piot et al. 1984). Robert Gallo, qui a découvert le premier rétrovirus humain, HTLV-1, pense alors qu'il s'agit d'un agent viral proche de celui-ci. En 1983, l'équipe de Luc Montagnier isole pour la première fois le virus responsable du SIDA à partir de cellules d'un ganglion chez un patient. Il sera nommé Virus de l'immunodéficience humaine (VIH) (Barré-Sinoussi et al. 1983).

En 1986, un virus apparenté au VIH-1 est décrit il s'agit du VIH-2 (Clavel et al. 1986).

2. Epidémiologie et transmission

Fin 2016, on comptait environ 36,7 millions de personnes vivant avec le VIH, dont 1,8 million de nouvelles infections. Lors de l'année dernière 1 million de personnes sont décédées des conséquences du VIH dans le monde.

Le VIH fait partie des infections sexuellement transmissibles (IST). Ce sont alors le sperme et les sécrétions vaginales qui sont contaminants. Le VIH peut également se transmettre par le sang, ce qui fut le cas pour les transfusés ou les usagers de drogue par voie veineuse. La transmission materno-fœtale ou par l'allaitement sont des voies de contamination importantes principalement en Afrique (mondiale de la Santé 2016).

3. Physiopathologie de l'infection

L'infection par le VIH passe par plusieurs phases :

- La primo-infection qui suit la contamination pouvant s'accompagner de symptômes grippaux. Elle correspond à une forte réplication virale et à une baisse des lymphocytes T CD4.
- La phase de latence, asymptomatique où le virus se multiplie et où le nombre de lymphocytes T CD4 décroît progressivement.

- Le stade SIDA, avec une forte immunodépression. La destruction du système immunitaire entraîne une forte virémie, et l'apparition de maladies opportunistes (Levy 1993; Weber 2001).

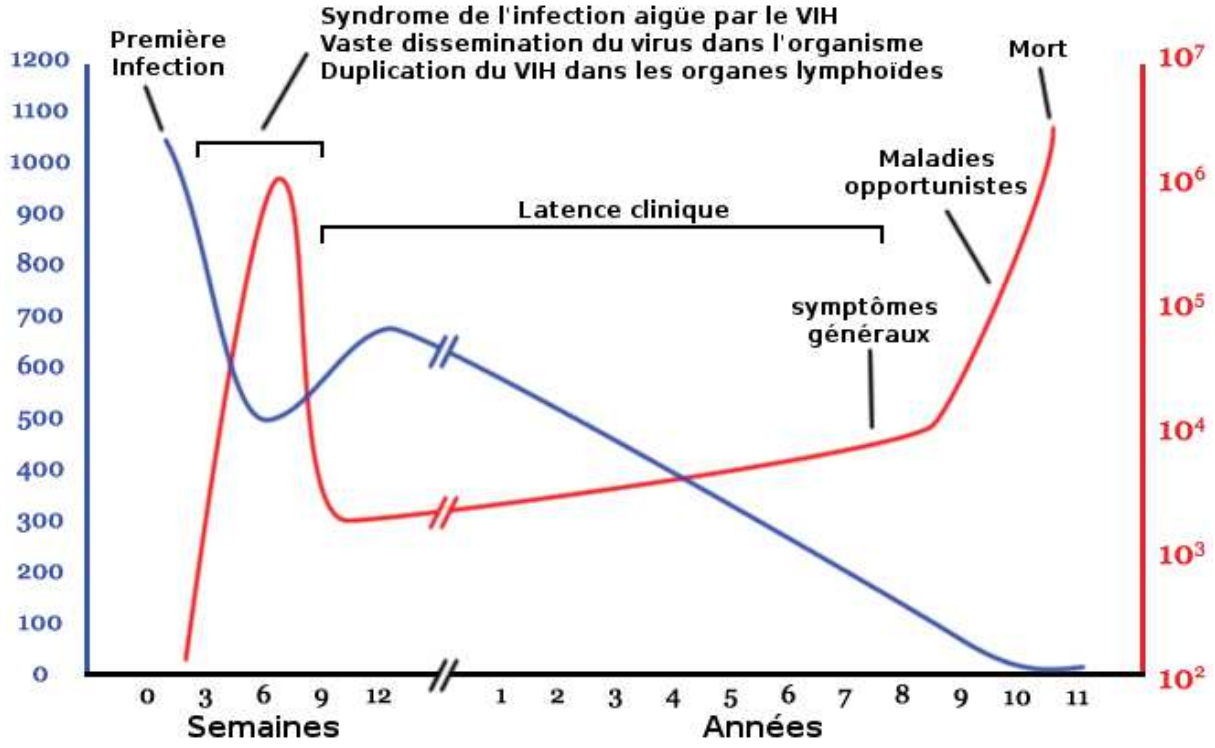


Figure 1. Évolution de l'infection par le VIH (Sanao, HIV-timecourse).

En bleu le nombre de lymphocytes T CD4 par mm3, en rouge le nombre de copies d'ARN viral par mL.

4. Structure, génome et cycle du VIH

Le VIH-1 possède deux molécules d'ARN identiques associées aux enzymes nécessaires à sa réplication : la transcriptase inverse (ou Reverse transcriptase : RT), l'intégrase et la protéase, dans un *core* cylindrique composé de la protéine p24. L'enveloppe est formée de la bicouche lipidique provenant de la membrane cytoplasmique de la cellule infectée. Elle porte les glycoprotéines : gp120 à la surface membranaire permettant la liaison du virus avec la cellule et gp41 en position transmembranaire permettant la fusion entre l'enveloppe virale et la cellule (Briggs et al. 2003).

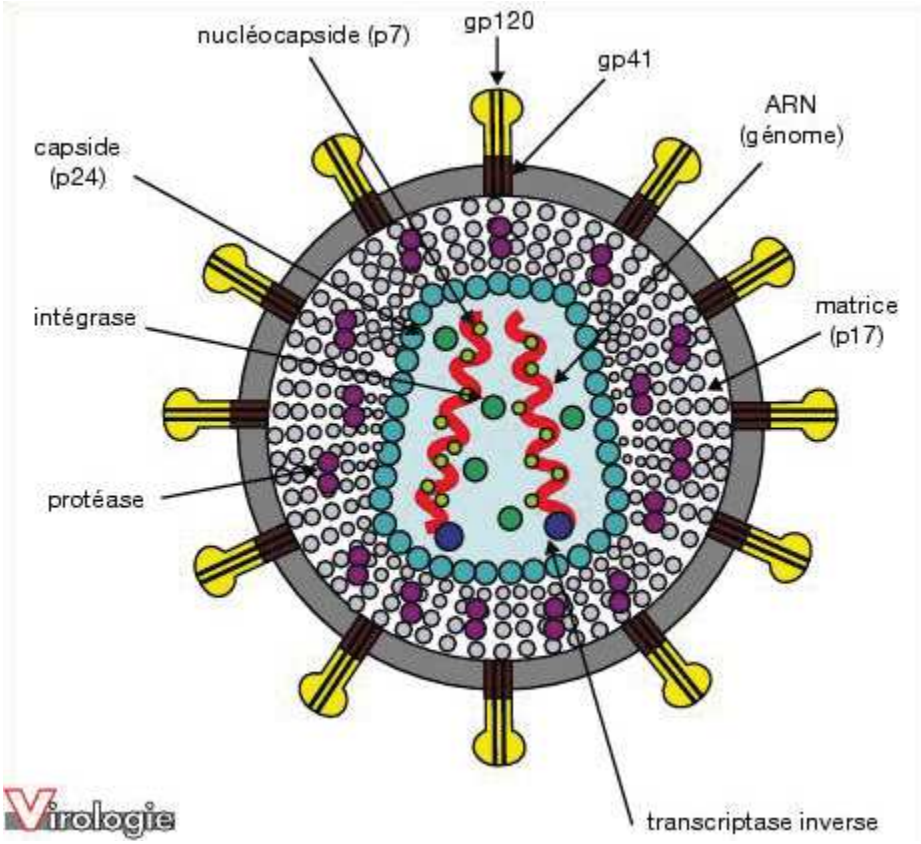


Figure 2. Structure du VIH (d'après John Libbey Eurotext).

Le génome du VIH-1 est composé de 9 gènes différents :

- *gag* code pour les protéines structurales du *core*
- *pol* code pour les enzymes virales
- *env* code pour les glycoprotéines d'enveloppe
- *tat* (transactivator of transcritption) active la transcription du génome viral
- *rev* (regulator of expression of viral proteins) permet le transport des ARNm viraux du noyau vers le cytoplasme
- *nef* (negative regulatory factor) diminue la concentration de CD4 et des molécules du complexe majeur d'histocompatibilité entraînant un mécanisme d'échappement
- *vif* (viral infectivity factor) neutralise l'enzyme APOBEC3G à activité antivirale, favorisant la propagation du virus.
- *vpr* (viral protein r) améliore la fidélité de la transcription inverse
- *vpu* (viral protein u) antagoniste du facteur de restriction Tetherin, favorise le relargage des virions (B. Chen 2016).

La première étape du cycle virale correspond à la fixation de la gp120 sur le récepteur CD4 puis sur un corécepteur, CCR5 ou CXCR4, suivie de la fusion de l'enveloppe virale et de la membrane cytoplasmique. La RT synthétise un brin d'ADN complémentaire de l'ARN viral, aboutissant ensuite à un double brin d'ADN viral qui va être intégré dans le génome cellulaire. À ce moment-là, le virus peut entrer en latence, sinon l'ADN sera transcrit en ARNm, puis traduit en protéines virales. Il s'en suit la maturation des protéines virales puis l'assemblage avant le bourgeonnement permettant la sortie de la capside entourée de la membrane cellulaire (Levy 1993).

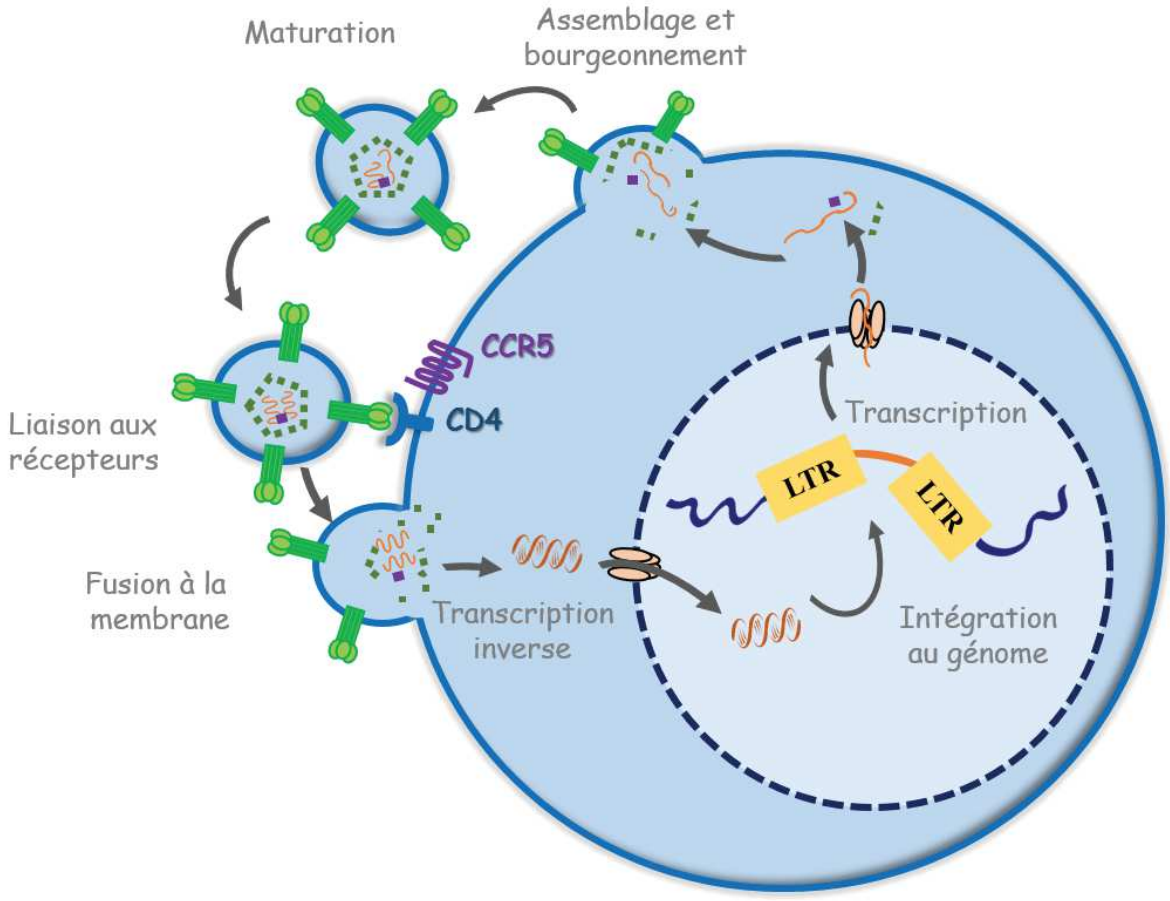


Figure 3. Schéma représentant le cycle réplicatif du VIH.

B. Présentation du projet

La plupart des souches virales VIH-1, appelées pour cette raison R5, utilisent, après avoir lié le récepteur CD4, le récepteur de chimiokines CCR5 comme corécepteur. CCR5 appartient à la classe C de la grande famille des récepteurs couplés aux protéines G (RCPGs).

Les RCPGs peuvent s'homo-dimériser et/ou former des hétéro-oligomères avec d'autres RCPGs (Pin and Prézeau 2007). Les propriétés de chacun des deux RCPGs participant à un dimère peuvent être modifiées. On sait ainsi que la densité membranaire, la liaison à des ligands extracellulaires, le couplage à des protéines G ou à d'autres molécules intracellulaires, les voies de signalisation d'un ou des deux RCPGs impliqués dans l'hétérodimère peuvent être transformés (Hillion et al. 2002; Terrillon and Bouvier 2004). Les RCPGs peuvent aussi s'hétérodésensibiliser sans contact physique. Ceci signifie que l'activation d'un RCPG à la surface d'une cellule peut induire pour un certain temps une inhibition de la capacité à s'activer d'un autre RCPG présent à la surface de cette même cellule.

Or la densité membranaire en CCR5, l'affinité de CCR5 pour l'enveloppe virale externe gp120, la signalisation via CCR5 déterminent l'efficacité de l'infection par les souches R5 (Corbeau and Reynes 2009). C'est pourquoi nous avons voulu chercher des RCPGs co-exprimés avec CCR5 à la surface des cellules T CD4+ humaines périphériques capables de moduler l'infection par des souches R5, soit par dimérisation avec CCR5, soit par hétérodésensibilisation de CCR5, soit parce qu'une de leurs voies de signalisation pourrait faciliter une étape du cycle de réplication rétrovirale.

Dans cet objectif, un tri positif des cellules exprimant CCR5 a été réalisé au laboratoire à partir de cellules T CD4+ périphériques de sujets sains, à l'aide de billes magnétiques couplées à un anticorps monoclonal anti-CCR5. Il a ensuite été déterminé par RT-PCR quantitative (TaqMan low density array, Applied Biosystems) quels étaient parmi 360 gènes humains codant pour des RCPGs, ceux exprimés dans ces lymphocytes T CD4+CCR5+. Une expression a ainsi été détectée pour 245 RCPGs, dont 39 avaient un niveau d'ARNm supérieur à la moitié de celui de CCR5.

Parmi ces 39 récepteurs, l'équipe s'est intéressée à trois récepteurs, le récepteur 1 de la sphingosine-1-phosphate (S1P1), l'Epstein-Barr virus-induced molecule 2 (EBI2) et le récepteur A_{2A} de l'adénosine. La capacité de S1P1, EBI2 et A_{2A} à se dimériser avec CCR5 a été testée en utilisant la technologie TR-FRET (Time resolved- Fluorescence resonance energy transfer), méthode dont le principe est résumé sur la figure 4. Les trois RCPGs se sont révélés capables de former des hétéromères avec CCR5.

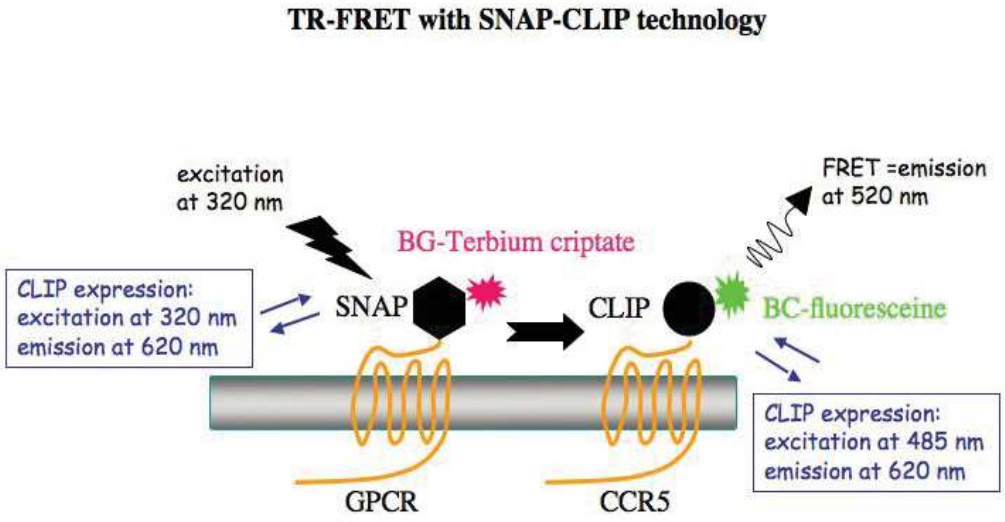


Figure 4. Figure 4. Principe du TR-FRET.

Chacun des RCPGs à tester a été cloné en fusion traductionnelle avec l'enzyme Snap qui lie spécifiquement et de manière covalente le fluorophore cryptate de terbium couplé à une benzyl-guanine (BG-cryptate). CCR5 a été cloné en fusion traductionnelle avec l'enzyme Clip, qui lie le fluorophore fluorescéine en couplage avec une benzyl-cytosine (BC-fluoresceine). Un transfert de fluorescence entre les deux fluorophores donne un signal de FRET mesurable lorsque ceux-ci sont situés à une distance inférieure à 50 Å, distance compatible avec une hétérodimérisation. Les niveaux d'expression de CCR5-CLIP et de RCPG-SNAP sont évalués par la mesure de fluorescence à 620 nM après excitation à 320 nM et 485 nM, respectivement.

C. Objectifs

Je présenterai successivement ces trois RCPGs, EBI2 en premier, A_{2A} ensuite et enfin S1P1. Pour chacun de ces récepteurs, j'exposerai d'abord ce qui est connu à ce jour les concernant. Puis j'exposerai sous forme de manuscrit, de résultats préliminaires et d'article sous presse respectivement, ce que nous avons montré comme effets de l'expression et de la stimulation de chacun de ces RCPGs sur la réplication du VIH-1.

D. Participation dans les différents projets présentés dans cette thèse

1. Projets EBI2, A_{2A}

Mon travail de thèse a porté principalement sur la caractérisation des effets des récepteurs EBI2 (Guigues et al., manuscrit en préparation, ci-après) et A_{2A} (résultats présentés ici) sur l'infection par le VIH-1 dans des lignées cellulaires HOS et MT-4 en culture.

2. Projet S1P1

Au cours de ma thèse, j'ai également participé à la caractérisation des effets de l'expression de S1P1 sur l'infection par les souches VIH-1. Ce travail avait été préalablement entrepris au laboratoire et a donné lieu à la thèse de Charline Duquenne en 2013, ainsi qu'à un manuscrit qui vient d'être accepté pour publication dans AIDS (Duquenne, Gimenez, Guigues et al., AIDS sous presse).

Ma participation dans ce projet a consisté à vérifier le caractère fonctionnel des récepteurs S1P1 et CCR5 taggués utilisés dans les expériences de FRET, en comparant la faculté de ces récepteurs à s'internaliser en présence de l'agoniste FTY720 pour SIP1 et PSC-RANTES pour CCR5. J'ai également participé à la mise au point des tests sur l'activé du LTR grâce aux cellules Hela exprimant la LTR couplée à la luciférase.

3. Projet CXCR4 (annexe 1)

J'ai participé aux expériences effectuées pour la révision de l'article concernant l'importance relative des deux isoformes de CXCR4 dans l'infection par le VIH-1 (Duquenne et al. 2014). Mon travail a consisté à déterminer les rapports des transcrits CXCR4A/CXCR4B par RT-qPCR dans des PBMC de patients infectés à différents stades de l'infection par le VIH-1.

4. Etude ACTIVIH

Lors de cette thèse j'ai pu participer au projet ACTIVIH qui a pour but de mieux caractériser les phénomènes d'activation immunitaire constante chez les patients avirémiques sous traitement. Cette étude visait à identifier et quantifier les causes de cette activation généralisée, et d'en mesurer les conséquences. Ma participation à ce sujet concerne notamment la réalisation de tests ELISA pour quantifier certains marqueurs chez ces patients.

II. EBI2

A. Structure d'EBI2

EBI2, aussi appelé GPR183, appartient à la classe A ou « rhodopsin like » des RCPGs. EBI2 peut s'hétéro-dimériser avec le récepteur de chimiokine CXCR5 (Barroso et al. 2012). Le gène EBI2 a été identifié en 1993 comme étant le gène lymphocytaire le plus activé au décours de l'infection par le virus d'Epstein-Barr (Birkenbach et al. 1993). En 2011, deux groupes ont identifié le l7α,25-hydroxycholestérol (l7α,25-OHC) et d'autres oxystérols semblables comme des ligands naturels d'EBI2 (Hannedouche et al. 2011; C. Liu et al. 2011). Ces ligands sont ubiquitaires (Kelly et al. 2011). Des cellules stromales des organes lymphoïdes secondaires (OLS) sont les principales productrices de ces ligands (Yi et al. 2012). Les résidus Arg87, Tyr112 et Tyr116, Tyr260, situés dans les deuxième (TM-2), troisième (TM-3) et sixième (TM-6) régions transmembranaires d'EBI2 respectivement, ont été impliqués dans l'interaction avec ses ligands (figure 5) (Benned-Jensen et al. 2012; L. Zhang et al. 2012).

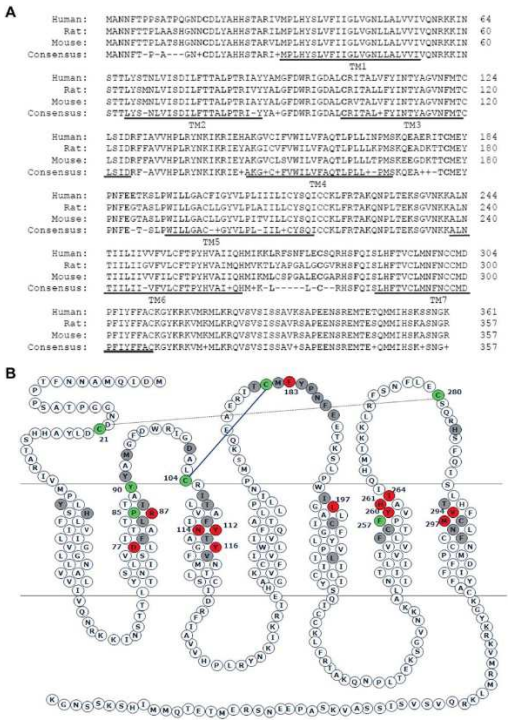


Figure 5. Séquence et structure d'EBI2 (d'après Zhang et al. 2012).

A. Comparaison des séquences d'acides aminés d'EBI2 chez l'homme, le rat et la souris.

B. Schéma des régions transmembranaires d'EBI2 humain, les résidus en rouge sont les plus importants dans la liaison récepteur-ligand.

B. Expression et rôles d'EBI2

EBI2 est exprimé par les cellules B, T CD4+ et T CD8+, les cellules dendritiques, les macrophages et les astrocytes (Rosenkilde et al. 2006). La stimulation d'EBI2 inhibe l'adénylate cyclase et induit l'activation de protéines Gαi/o, des MAP kinases ERK1/2, p38 et JNK, de Rho GTPases, la liaison à GTPγS, l'augmentation du taux intracellulaire de calcium ainsi que le recrutement de la β arrestine, provoquant prolifération et migration cellulaire (Hannedouche et al. 2011; C. Liu et al. 2011; Rosenkilde et al. 2006; Benned-Jensen et al. 2011).

Les OLS tels que la rate et les ganglions lymphatiques (GLs) sont composés de 10% de cellules stromales « architectes » au milieu desquelles se déplacent des lymphocytes. Après avoir séjourné quelques heures dans un OLS, les lymphocytes rejoignent la circulation sanguine avant de rentrer dans un nouvel OLS. Ce processus continuel permet d'organiser une immunosurveillance efficace de l'ensemble de l'organisme. Les OLS sont compartimentés afin de permettre la formation de zones permettant la survie, l'activation et la différenciation des lymphocytes B. La réponse immunitaire B dépendante des lymphocytes T ne se limite pas à la reconnaissance de l'antigène par le lymphocyte B, elle nécessite un second signal qui lui est fourni par coopération cellulaire avec un lymphocyte Tfh activé. Cette réponse est initiée par l'entrée des cellules B dans la zone T inter-folliculaire. Les lymphocytes B activés par l'antigène migrent vers l'extérieur des follicules où ils se multiplient avant de rencontrer les lymphocytes T et les cellules dendritiques folliculaires. Après cette interaction avec les cellules T, les cellules B vont se retrouver dans la région inter ou extra-folliculaire avant de se différencier en plasmocytes ou entrer dans les centres germinatifs. Ces étapes de migration et de localisation des cellules B au sein des organes lymphoïdes sont indispensables au bon déroulement de la réponse immunitaire et sont en partie médiées par EBI2 et un gradient d'oxystérols (Gatto and Brink 2013).

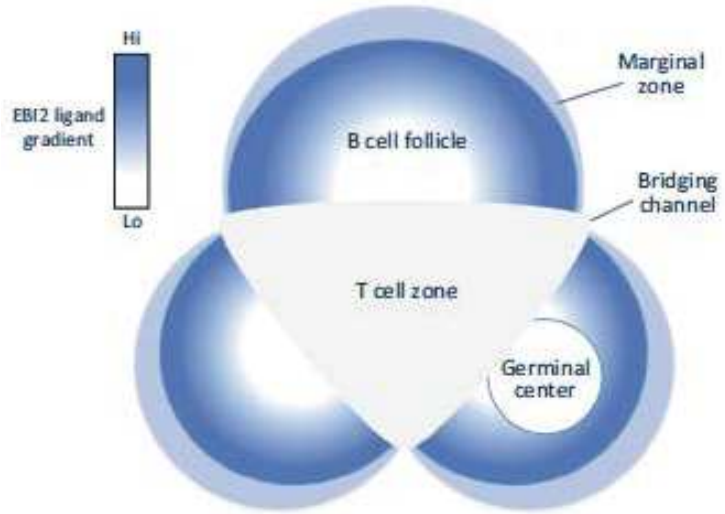


Figure 6. Gradient d'oxystérols au sein de la rate (d'après Gatto et Brink 2013).

Le positionnement des cellules B au sein des organes lymphoïdes secondaires est défini par divers signaux de chimiotactisme. Dans ce processus, EBI2 et les récepteurs aux chimiokines CCR7, CXCR5 et CXCR4 coopèrent. L'expression d'EBI2, importante à la surface des lymphocytes B naïfs et encore plus des lymphocytes B activés, pousse ces cellules à se diriger vers la périphérie des zones B et les régions extra-folliculaires où on trouve des concentrations élevées de 7α, 25-di-OHC. (Hannedouche et al. 2011; C. Liu et al. 2011) plutôt que vers le centre germinatif. Là, elles interagissent avec des lymphocytes T auxiliaires ainsi que des cellules dendritiques situées dans les zones inter-folliculaires. L'importance du rôle d'EBI2 dans la réponse humorale est soulignée par le fait que les souris EBI2$^{-/-}$ ont un déficit de production en IgM et IgG en réponse à l'immunisation contre des antigènes T-dépendants (Gatto et al. 2009; Pereira et al. 2009). Par ailleurs la présence d'EBI2 potentialise la prolifération B (Lee et al. 1998).

Les cellules T CD4+ et des populations T CD8+ expriment EBI2, répondant ainsi au l7α,25-OHC (C. Liu et al. 2011) et certaines cellules T effectrices surexpriment EBI2 (Hannedouche et al. 2011; Lund et al. 2003). EBI2 joue un rôle dans le positionnement des cellules T CD4+ au sein des organes lymphoïdes secondaires. En particulier EBI2 est responsable de la localisation des cellules T auxiliaires folliculaires (T follicular helper ou Tfh) en périphérie des zones T. Ceci leur permet d'interagir avec les cellules dendritiques activées présentes à cet endroit qui renforcent la différenciation de ces cellules Tfh (J. Li et al. 2016). Cependant, l'expression d'EBI2 n'est pas absolument nécessaire pour la différenciation en Tfh,

mais la favorise engendrant une meilleure efficacité de la réponse humorale (Gatto et al. 2009; Brink 2016).

EBI2 joue également aussi un rôle dans le positionnement des cellules dendritiques au sein des organes lymphoïdes secondaires. Au niveau de la rate, l'expression d'EBI2 à la surface de certaines cellules dendritiques conventionnelles leur permet de se situer dans les zones inter-folliculaires où passent les antigènes présents dans le sang (Yi and Cyster 2013; Gatto et al. 2013). Un autre effet de l'expression d'EBI2 dans les cellules dendritiques, a été déduit de l'étude de souris EBI2$^{-/-}$. Les cellules dendritiques plasmacytoïdes et myéloïdes de ces souris surproduisant de l'interféron (IFN) de type I après stimulation, EBI2 semble être un régulateur négatif de la production de ces cytokines (Chiang, Johnston, and Grogan 2013).

Les monocytes et les macrophages peuvent exprimer EBI2 et produire les ligands naturels de ce récepteur (Preuss et al. 2014). Cette production est stimulée par le lipopolysaccharide et les interférons de type I (IFN I) (Park and Scott 2010; Diczfalusy et al. 2009). Comme les astrocytes ont les mêmes propriétés, certains auteurs ont proposé qu'EBI2 pourrait intervenir dans les interactions entre ces deux types cellulaires (Rutkowska et al. 2016).

C. Implication d'EBI2 dans diverses pathologies

EBI2 a été impliqué dans diverses pathologies. EBI2 pourrait jouer un rôle dans diverses maladies auto-immunes et auto-inflammatoires. Un polymorphisme génétique dans la région d'*EBI2* a été mis en évidence dans des pathologies inflammatoires du tube digestif (Jostins et al. 2012) et dans le lupus érythémateux disséminé (Cantor et al. 2004; Moser et al. 1998). Un lien génétique a été retrouvé avec le locus EBI2 et le diabète de type I (Heinig et al. 2010). Récemment il a été montré qu'EBI2 pourrait intervenir dans la différenciation des ostéoclastes ainsi que dans leur mise en place au niveau des surfaces osseuses, si bien qu'il pourrait intervenir dans la polyarthrite rhumatoïde (Nevius, Gomes, and Pereira 2016). Un lien génétique a été également retrouvé avec le locus EBI2 dans la sclérose en plaques (Cantor et al. 2004; Moser et al. 1998). Chez la souris un déficit génétique en cholestérol 25-hydroxylase, enzyme responsable de la synthèse du 25-OHC à partir du cholestérol, réduit l'encéphalopathie auto-immune expérimentale, modèle de sclérose en plaques (Chalmin et al. 2015). Dans ce modèle et dans la sclérose en plaques, EBI2 a même été impliqué dans le recrutement des T encéphalitogéniques (Wanke et al. 2017).

En cancérologie, il a été montré que les monocytes/macrophages associés aux tumeurs expriment EBI2 (Eibinger et al. 2013). Une dérégulation de l'expression d'EBI2 a été retrouvée dans certaines tumeurs B (Rosenkilde et al. 2006; Benned-Jensen et al. 2012). De même une surexpression d'EBI2 a été observée dans des lymphoprolifération chez des patients infectés par EBV (Craig et al. 2007).

Les monocytes/macrophages associés aux plaques d'athérosclérose dans lesquelles des taux élevés d'oxystérols ont été retrouvés (Brown and Jessup 1999), expriment EBI2 (Rosenkilde et al. 2006; Preuss et al. 2014). Ceci a amené à proposer qu'EBI2 pourrait jouer un rôle dans le recrutement des cellules macrophagiques dans les plaques d'athérothrombose.

D. Les oxystérols chez l'Homme

Les oxystérols ou dihydroxycholestérols sont des dérivés oxydés du cholestérol, possédant comme lui 27 atomes de carbone et un noyau stérol commun. Les positions les plus communes pour l'oxydation sont au niveau du noyau stérol : 4, 5, 6 et 7, et pour la chaîne latérale : 24, 25 et 27 (Mutemberezi, Guillemot-Legris, and Muccioli 2016).

Table 1
Trivial and systematic names, as well as Lipid maps and CAS numbers of the main oxysterols.

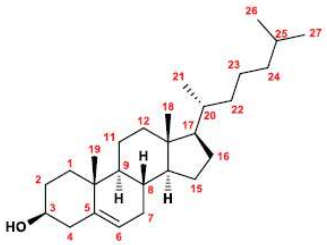
General formula	Common name	Systematic name	Abbreviation
	4β-Hydroxycholesterol	Cholest-5-en-3β,4β-diol	4β-OHC
	5α,6α-Epoxycholesterol	5α,6α-Epoxy-5α-cholestan-3β-ol	5α,6α-epoxychol
	5α,6β-Dihydroxycholesterol	Cholestane-3β,5α,6β-triol	5α,6β-di-OHC
	5β,6β-Epoxycholesterol	5β,6β-Epoxy-cholestan-3β-ol	5β,6β-epoxychol
	6α-Hydroxycholesterol	Cholestan-3β,6α-diol	6α-OHC
	7α-Hydroxycholestenone	7α-Hydroxycholest-4-en-3-one	7α-OHCnone
	7α-Hydroxycholesterol	Cholest-5-en-3β,7α-diol	7α-OHC
	7α,24(*S*)-Dihydroxycholesterol	Cholest-5-en-3β,7α,24-triol	7α,24(*S*)-di-OHC
	7α,25-Dihydroxycholesterol	Cholest-5-en-3β,7α,25-triol	7α,25-di-OHC
	7α,27-Dihydroxycholesterol	5-Cholestene-3β,7α,26-triol	7α,27-di-OHC
	7β-Hydroxycholesterol	5-Cholestene-3β,7β-diol	7β-OHC
	7β,27-Dihydroxycholesterol	(25R)-Cholest-5-en-3β,7β,27-triol	7β,27-di-OHC
	7-Dehydrocholesterol	Cholesta-5,7-dien-3β-ol	7-DHC
	7-Ketocholesterol	7-oxo-Cholest-5-en-3β-ol	7-ketochol
	7-Keto-25-hydroxycholesterol	(3β)-3,25-Dihydroxycholest-5-en-7-one	7-keto,25-OHC
	7-Keto-27-hydroxycholesterol	(3β)-3,26-Dihydroxycholest-5-en-7-one	7-keto,27-OHC
	22(*R*)-Hydroxycholesterol	Cholest-5-en-3β,22R-diol	22(*R*)-OHC
	24(*S*)-Hydroxycholesterol	Cholest-5-en-3β,24S-diol	24(*S*)-OHC
	24(*S*),25-Epoxycholesterol	24S,25-Epoxy-cholest-5-en-3β-ol	24(*S*),25-epoxychol
	24(*S*),27-Dihydroxycholesterol	Cholest-5-en-3β,24S,26-triol	24(*S*),27-di-OHC
	25-Hydroxycholesterol	Cholest-5-en-3β,25-diol	25-OHC
	27-Hydroxycholesterol	Cholest-(25R)-5-en-3β,26-diol	27-OHC
	Cholestenoic acid	3β-Hydroxycholest-5-en-25R-26-oic acid	27-CA
	Desmosterol	Cholest-5,24-dien-3β-ol	Desm

Figure 7. Tableau de nomenclature des principaux métabolites du cholestérol (tiré de Mutemberezi, Guillemot-Legris, and Muccioli 2016).

Le ligand le plus affin pour EBI2 est le 7α, 25-di-OHC, la position et l'orientation des groupes hydroxyles étant importantes pour l'efficacité d'activation d'EBI2. La forme α en position 7 du groupe hydroxyle par rapport à la forme β du 7, 25-di-OHC peut être jusqu'à 50

fois plus puissante concernant la migration induite par EBI2. L'élimination d'un groupement hydroxyle provoque des différences encore plus grandes (Hannedouche et al. 2011).

Historiquement, le métabolisme des oxystérols était vu comme une simple étape de la dégradation du cholestérol. Actuellement, en plus de leurs rôles comme métabolites intermédiaires, les oxystérols ont de nombreuses fonctions physiologiques connues. Ce sont des précurseurs des hormones stéroïdes et des acides biliaires. Ils ont un rôle clef dans la régulation de l'homéostasie cellulaire et plasmatique du cholestérol, le transport des stérols, la mort cellulaire par apoptose, l'inflammation et la signalisation. (Karuna et al. 2015). Certains oxystérols sont trouvés sous forme estérifiée à plus de 90 %. Les principaux oxystérols qui se lient à EBI2 sont le 7α-OHC, le 25-OHC, le 7α, 25-di-OHC et le 7α, 27-di-OHC qu'on trouve dans le plasma (0,3 à 0,9 ng/ml) et dans le LCR (0,02 à 0,03 ng/ml) (Mutemberezi, Guillemot-Legris, and Muccioli 2016). Les oxystérols 7α, 25-di-OHC et 7α, 25-di-OHC sont des isomères formés par hydroxylation des précurseurs 25-OHC et 27-OHC. Leurs voies de synthèse *in vivo* sont représentées dans la figure ci-dessous.

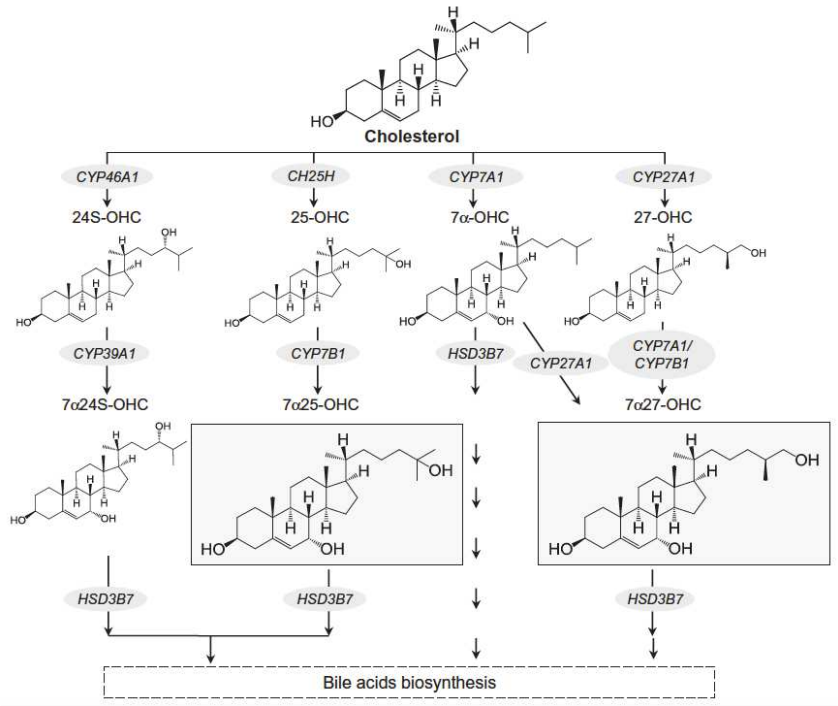


Figure 8. Les principales voies de synthèse du 7α, 25-di-OHC et 7α, 27-di-OHC (tiré de Karuna et al. 2015).

E. Antagonistes d'EBI2

La première molécule inhibitrice d'EBI2 a été trouvée par criblage à haut débit, il s'agit du GSK682753A, un antagoniste compétitif se liant dans la même région que le 7α, 25-di-OHC. Le GSK682753A bloque l'activité d'EBI2 de façon dose-dépendante avec un IC50 de 0,2 µM. Il limite seulement l'affinité de la réponse au ligand et non sa vitesse maximum (inhibiteur compétitif). Il inhibe la migration des cellules induite par les oxystérols et la prolifération cellulaire via EBI2 (Benned-Jensen et al. 2013).

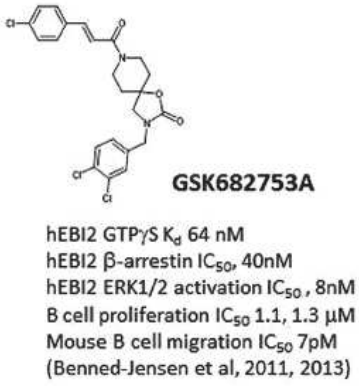


Figure 9. Structure du GSK682753A (Sun et Liu 2015)

Après identification de NIBR151 comme agoniste d'EBI2, un autre criblage de molécules pouvant bloquer l'activation d'EBI2 a permis de trouver NIBR127. Cependant cet antagoniste présentait des instabilités métaboliques et une clairance intrinsèque élevée. La molécule NIBR189 a été obtenue chimiquement par modification de NIBR127 afin d'améliorer sa stabilité métabolique et sa capacité d'inhibition. Ainsi NIBR 189 est un antagoniste puissant et sélectif, avec des propriétés pharmacologiques lui permettant une utilisation *in vitro* et *in vivo* (Gessier et al. 2014).

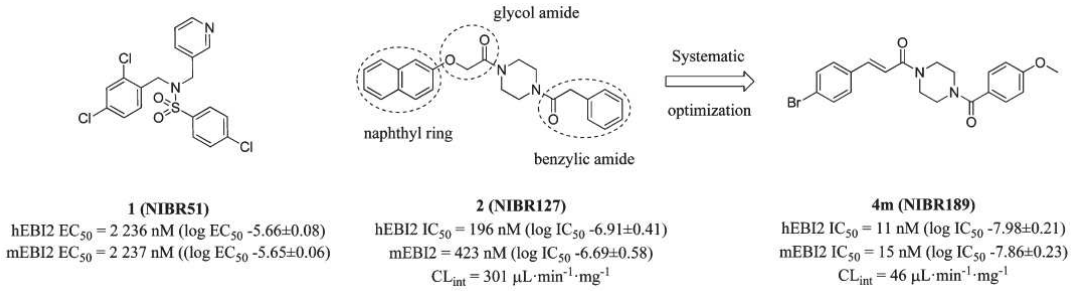


Figure 10. Structures de l'agoniste NIBR51 et des antagonistes NIBR127 et NIBR189 (Gessier et al. 2014).

F. Oxystérols et VIH

En 1998, alors que le récepteur EBI2 est considéré comme orphelin, certains oxystérols sont testés pour leur capacité à inhiber la réplication du VIH par altération de la fluidité de son enveloppe lipidique. En effet, les oxystérols peuvent interagir avec les membranes cellulaires, modifiant leur fluidité et ainsi leurs fonctions cellulaires. Le 7-OHC, le 25-OHC et en particulier le 7, 25-di-OHC inhibent la réplication du VIH sur différents types cellulaires (CEM-SS, MT-4 et PBMC). Le cholestérol a également été testé mais ne montre pas d'activité antivirale. Cette étude suggère également une inhibition de la transcription inverse ou d'une étape antérieure à celle-ci par les oxystérols (S.-Y. Liu et al. 2013). L'expression de la Cholestérol-25-Hydroxylase ou le traitement au 25-OHC inhibe l'entrée de différents virus comme l'Herpes ou le VIH. A noter que ces effets anti-VIH des traitements aux oxystérols sont probablement dus à une action directe des molécules plutôt qu'à l'activation d'EBI2, et ceci à des concentrations non toxiques mais cependant très élevées par rapport aux concentrations physiologiques permettant l'activation du récepteur.

The EBI2 receptor is coexpressed with CCR5 in CD4+ T cells and boosts HIV-1 R5 replication

Adeline GUIGUES[1], Sandrine GIMENEZ[1], Clément METTLING[1], Damien MAUREL[2], Laurent PRÉZEAU[3], Vincent FRANÇOIS[1], Pierre CORBEAU[1,4,5]

[1]Institut de Génétique Humaine, CNRS-Université de Montpellier UMR9002, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France. [2]ARPEGE Pharmacology Screening Interactome platform facility, Institut de Génomique Fonctionnelle, 141 rue de la Cardonille, 34094 Montpellier cedex 5, France. [3]Institut de Génomique Fonctionnelle, CNRS-UMR5203, INSERM-U661, Universités Montpellier 1 & 2, UMR5203, F-34094 Montpellier Cedex 5, France. [4]Université de Montpellier, 5 Boulevard Henri IV, 34967 Montpellier cedex 2, France. [5]Centre Hospitalier Universitaire Caremeau, UF d'Immunologie, place du Pr Debré, 30029 Nîmes cedex 9, France.

The authors have declared that no conflict of interest exists

Corresponding author : Pierre Corbeau, Institute for Human Genetics, CNRS UPR1142, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France, tel : +33 434 359 932, fax: +33 434 359 901, e-mail address: pcorbeau@igh.cnrs.fr

Abstract

CCR5, a G protein-coupled receptor (GPCR), is used by most HIV strains as a coreceptor. These R5 strains bind to CCR5 and consequently trigger a signal that favors their life cycle. In this study, looking for GPCR able to interefere with these events, we unveiled that Epstein-Barr virus-induced gene 2 (EBI2) is 16-fold more expressed in primary CD4+CCR5+ T cells than CCR5. We also showed by FRET technology that CCR5 and EBI2 heterodimerize. To analyze the consequences of this coexpression we established stable cell lines transduced with *CCR5* and *EBI2*. Using HIV virions harboring β-lactamase fused to the viral protein vpr, we observed that this coexpression reduced viral entry by 50%. By contrast the amount of HIV reverse transcripts, evaluated by qPCR, was similar in cells expressing or not EBI2. Finally, the presence of EBI2 induced NFkB p65 translocation as shown by immunofluorescence. Stable and transient transfection of a reporter gene driven by HIV LTR deleted or not of its NFkB sites unraveled that the activation of this transcription factor resulted in an increase in HIV promoter activity. Globally, the result was a drastic viral overproduction in *EBI2*–transduced cells. In vivo, as the natural ligand for EBI2, 7α,25-dihydroxycholesterol (7α,25-OHC) is present in blood and lymphoid tissues, the constant EBI2 activation might boost HIV replication in CD4+ T cells. Therefore, it might be of interest to test the effect of EBI2 antagonists on the residual viral production persisting in most patients aviremic under treatment. Moreover, given that 7α,25-OHC promotes Th17 differentiation, EBI2 antagonists could also dampen the pathogenic immune activation observed in virologic responders.

Introduction

Most HIV strains, so-called R5, use the chemokine receptor CCR5 as a coreceptor in addition to the CD4 molecule [1]. HIV envelope gp120 binds to CD4 and thereafter to CCR5. This allows the next step of HIV life cycle, the fusion between virus and target cell membranes. In addition, gp120 binding to CCR5 triggers a signal that boosts HIV reverse transcription and viral promoter activation [2, 3].

CCR5 belongs to the family of G protein-coupled receptors (GPCR). Some GPCR cross-interact with each other physically and/or functionally [4]. On one hand some GPCR heterodimerize and this results in a modification of their cell surface density, of the interaction with their ligands, and/or of their signaling. On the other hand, triggering of a GPCR can reduce the capability of another GPCR to be activated, a phenomenon called heterodesensitization. As CCR5 cell surface density, CCR5-gp120 affinity and CCR5 signaling modulate HIV life cycle [2, 3, 5-7], we looked for GPCR that could interfere with these CCR5 characteristics. Here we unraveled that the GPCR Epstein-Barr virus-induced gene 2 (EBI2) is coexpressed with CCR5 in primary CD4+CCR5+ T cells and macrophages.

EBI2, also called *aka* and *GPR183*, was identified in 1993 as a gene activated by Epstein-Barr virus in a Burkitt lymphoma cell line [8]. In 2011, 7α,25-dihydroxycholesterol (7α,25-OHC) was identified as an endogenous ligand for EBI2 [9, 10]. EBI2 participates with the chemokine receptors CCR7, CXCR4 and CXCR5 in the regulation of B cell homing in secondary lymphoid tissues [11, 12]. Naïve B cells, particularly if they are activated, display high EBI2 cell surface densities that drive them to outer follicular areas. By contrast, downregulation of EBI2 cell surface expression drives B cells to central follicular areas [11, 12]. EBI2 has also been involved in B cell activation and proliferation [13]. Additionally, EBI2 is expressed in monocytes/macrophages, dendritic cells, T cells and astrocytes. The interaction between EBI2 and 7α,25-OHC has been involved in the splenic homing of dendritic cells [14, 15]. EBI2 also downregulates type I interferon responses in plasmacytoid dendritic cells [16]. In macrophages, EBI2 stimulation leads to calcium mobilization and cell migration [17, 18]. Moreover, CD4+ T differentiation into Th17 cells is driven by the agonistic effect of 7α,25-OHC on RAR-related orphan receptor gamma t (RORγt) [19] and EBI2 stimulation has been involved in multiple sclerosis [20]. Here we show that the coexpression of EBI2 with CCR5 modifies HIV replication cycle.

Results

Identification of EBI2 as a GPCR coexpressed with CCR5 in CD4+ T cells and macrophages. In a search for GPCR coexpressed with CCR5 in CD4+ T cells, we sorted CCR5+CD4+ T lymphocytes from the blood of healthy donors with magnetic beads and specific antibodies, and quantified by multi-RT-qPCR the expression of 361 *GPCR* genes. We did the same with macrophages differentiated in vitro from the circulating monocytes of the same donors. We observed that among the *GPCR* genes expressed

in peripheral CD4+CCR5+ T cells the *EBI2* gene was highly transcribed, EBI2 transcripts being 16-fold more abundant than CCR5 transcripts in these HIV target cells. EBI2 was also coexpressed with CCR5 in macrophages but EBI2 transcripts were 2-fold less abundant than CCR5 transcripts in these myeloid cells.

EBI2 forms heteromers with CCR5 in the outer cell membrane. When a GPCR heterodimerizes with another GPCR, some of its characteristics can be modified, including its cell surface density, its ligand affinity, and its signaling [21-25]. For CCR5, all these characteristics determine HIV-1 R5 replicative cycle. Therefore, we analyzed by time-resolved FRET (tr-FRET) whether EBI2 was able to form heteromers with CCR5 with a method that gives a direct assessment of receptor cross-interaction at the plasma membrane of living cells (21). To this aim, receptors were fused at their N-terminal extracellular domain with the genetically encoded tags SNAP or CLIP, that allow specific labelling with fluorescent substrates. Thereafter, SNAP-EBI2 and CLIP-CCR5 encoding plasmids were co-transfected in 293T cells, and the corresponding receptors were labeled by adding non-permeant fluorophores in the culture medium. The tr-FRET signal between the cell surface tagged-receptors was measured, as well as the fluorescence signal from each fluorophore. We observed a saturating tr-FRET signal between SNAP-EBI2 and CLIP-CCR5, typical of a specific interaction in the outer membrane (figure 1). Conversely, figure 1 shows a non-saturating tr-FRET signal between CCR5 and the beta-2 adrenergic receptor (β2AR) (24), indicating random collisions between these two receptors known not to interact with each other (23). As a positive control, CLIP-CCR5 was found to form homomers with SNAP-CCR5 (figure 1) as previously shown (25).

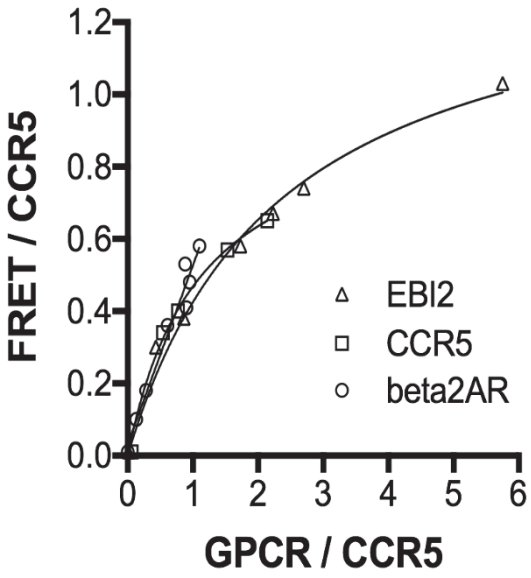


Figure 1. CCR5 forms heteromers with EBI2. Cells were cotransfected with a constant amount of CLIP-CCR5 plasmid and increasing amount of the SNAP-EBI2, SNAP-CCR5, or SNAP-β2AR plasmid, and labeled with the corresponding fluorophores. Tr-FRET signal normalized with the CLIP-CCR5 expression signal (FRET / CCR5) is represented in function of SNAP-EBI2 (Δ), SNAP-CCR5 (□) or SNAP-β2AR (O) / CLIP-CCR5 expression ratio (GPCR / CCR5). Tr-FRET signal is expressed by the Δratio = (520/620)samples – (520/620)background. The background signal corresponds to cells expressing SNAP- and CLIP-tagged proteins labeled with the same fluorophores. The curves were obtained by non-linear regressions with R2= 0.9921 and 0.9875 for SNAP-CCR5 and SNAP-EBI2, respectively, and by a linear regression for SNAP-β2AR (p value < 0.0001).

Establishment of HIV-1 R5-infectible cell lines expressing a functional EBI2 receptor. To explore the consequences of EBI2 expression on HIV-1 replicative cycle, we created CD4+CCR5+ cell lines expressing or not the *EBI2* gene. To this aim, we first cloned the *EBI2* and the *EGFP* genes separated by an IRES sequence into an HIV-1 transfer vector. Then we produced HIV-1 particles able to deliver this construct, and transduced CCR5- and CD4-positive human osteosarcoma HOS cells or lymphoid MT4 cells with these particles. As a negative control, we transduced the same cells with a *LacZ* gene. We then verified by flow cytometry that EGFP was expressed in the HOS (figure 2A) and MT4 (figure 2B) cells transduced with the EBI2-IRES-EGFP vector. We also checked that EBI2 transduction did not modify CCR5 expression (figure 2C). To ascertain that EBI2 was functional at the surface of these cells, we monitored cell culture impedance in presence or absence of the EBI2 ligand 7α,25-OHC. As shown in figure 2D and 2E, addition of 7α,25-OHC specifically modified the impedance of cells transduced with *EBI2* but not in cells transduced with *LacZ*.

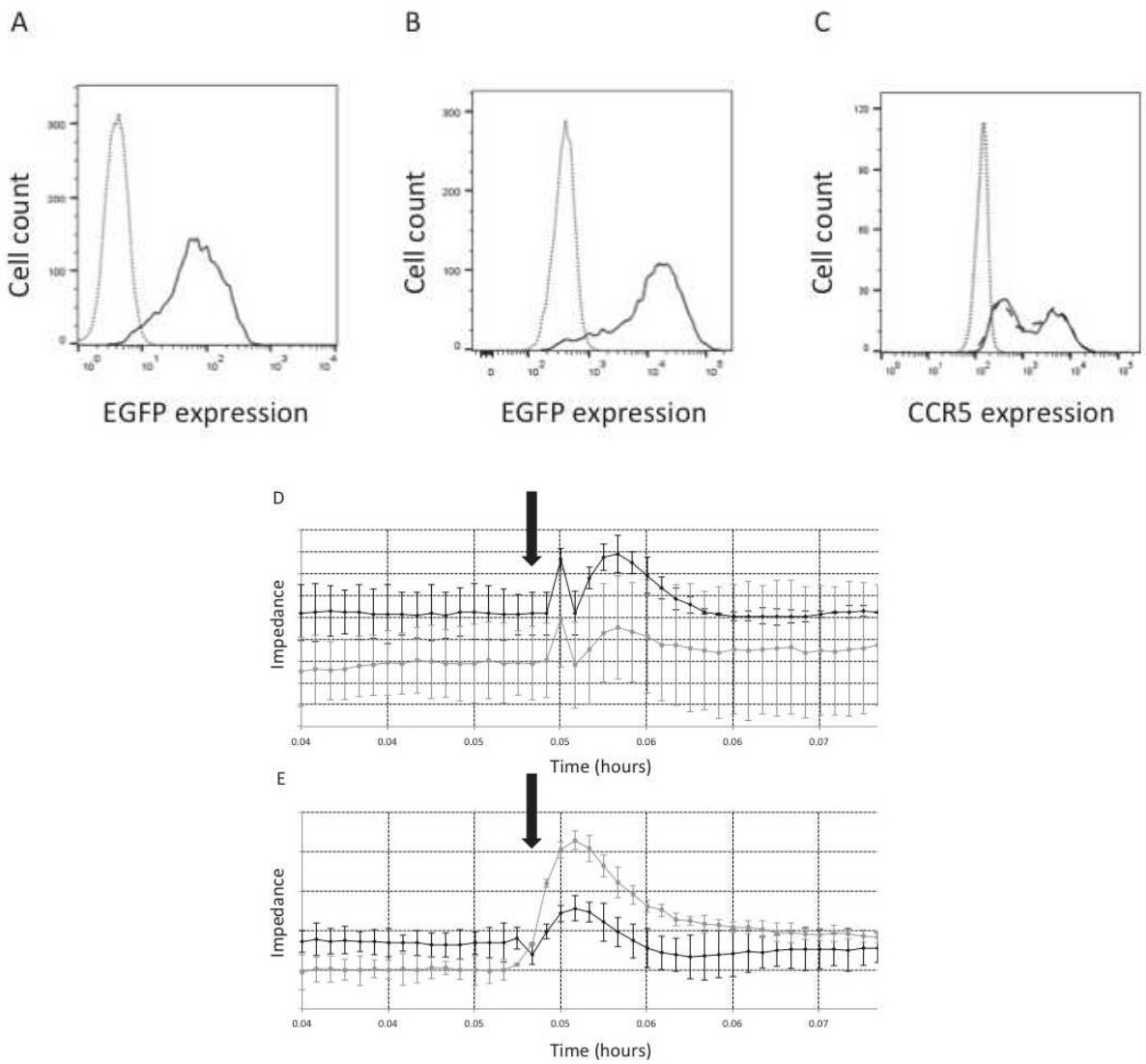


Figure 2. EBI2 expression in HOS and MT4 cells. (A) Transgene expression in HOS cells. EGFP fluorescence of HOS cells transduced with *EBI2-EGFP* (—) or *LacZ* (…). (B) Transgene expression in MT4 cells. EGFP fluorescence of MT4 cells transduced with *EBI2-EGFP* (—) or *LacZ* (…). (C) CCR5 expression in HOS cells transduced with *EBI2*. HOS cells transduced with *EBI2-EGFP* (—) or *LacZ* (- --) were indirectly labeled with a CCR5 antibody or an isotypic control (…). (D, E) EBI2 expression at the surface of *EBI2-EGFP* transduced HOS cells. HOS cells transduced with *EBI2-EGFP* (grey line) or *LacZ* (dark line) were exposed (arrow) to control culture medium (D) or to 7α,25-OHC €, and impedance was monitored over time.

EBI2 expression reduces HIV entry. As heterodimerization may modify the interactions between the GPCR involved in the heteromer and their ligands, we tested whether the presence of EBI2 modified the efficiency of HIV-1 R5 entry, as a consequence of an interference with HIV envelope-CCR5 interaction. To this aim, we used an assay measuring the delivery of an enzyme, □-lactamase, in the

course of MT4 cell infection by HIV-1 virions harboring this enzyme fused to the viral protein Vpr (Vpr-Blam). The presence of the enzyme in the target cells after infection with these virions was measured by quantifying by flow cytometry the transformation of a substrate, CCF2-MA, into a fluorescent product by □-lactamase. As shown in figure 3, the presence of EBI2 decreased HIV-1 R5 entry efficiency. In three independent experiments, this inhibition was 48.4 ± 4.9% (mean ± SEM). By comparison, preincubation of the MT4 cells with soluble CD4 before infection to inhibit viral envelope binding to CD4 resulted in a 90.7 ± 5.0% (mean ± SEM) inhibition.

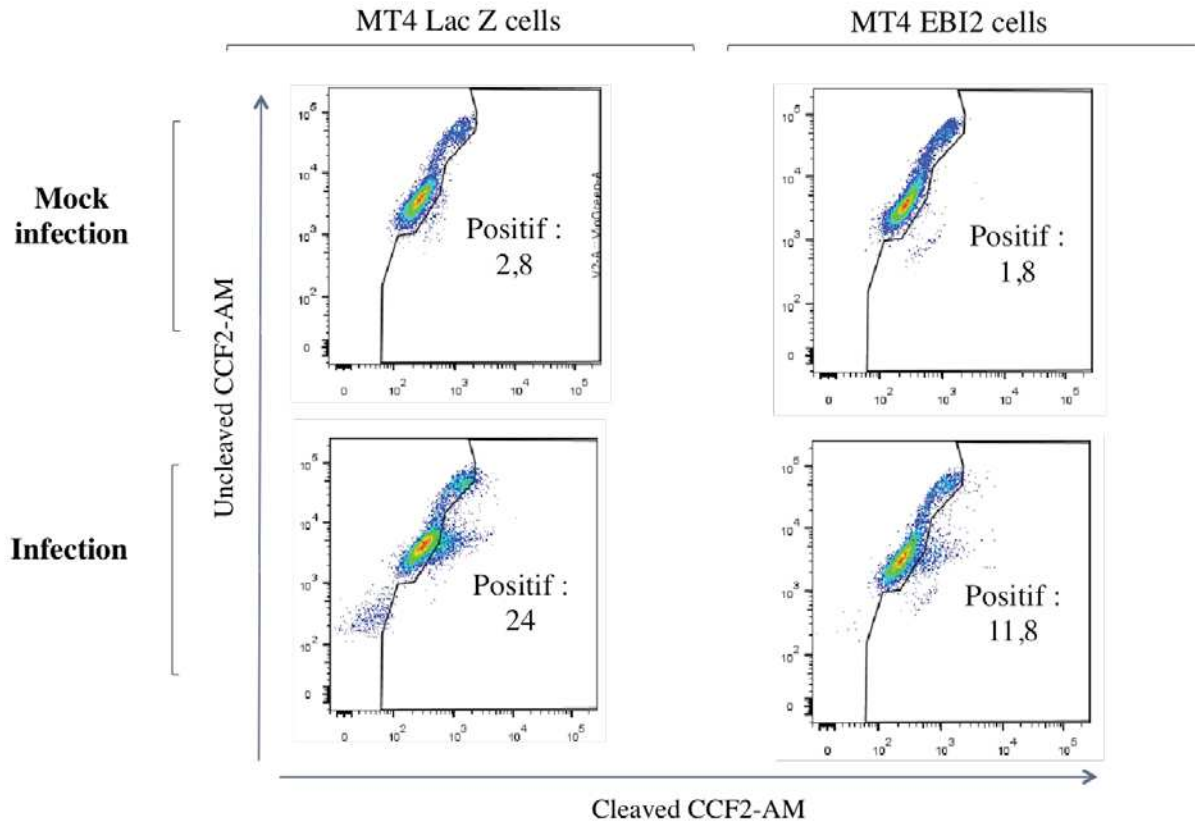


Figure 3. EBI2 expression reduces HIV entry. MT4 cells transduced with *LacZ* or *EBI2-EGFP* and cultured in duplicate were exposed or not to R5 HIV-1 particles containing Vpr-Blam, and to the CCF2-AM substrate. The fluorescence induced by CCF2-AM cleavage was then evaluated by flow cytometry, as a marker of viral entry. Representative experiment out of three experiments.

Effect of EBI2 expression on HIV RNA reverse transcription. As CCR5 signaling boosts HIV RNA reverse transcription [26], we tested the hypothesis that EBI2 signaling might have the same effect. To this aim, we infected HOS cells transduced with *EBI2* or *lacZ*, with replication defective R5 virions and monitored the appearance of early and late reverse HIV transcripts in these cells. The amount of early

(0.067 ± 0.006 and 0.070 ± 0.003 arbitrary units, p = 0.601) and late (0.012 ± 0.001 and 0.019 ± 0.003 arbitrary units, p = 0.263) transcripts were similar 3 days later in EBI2- and LacZ-expressing cells, respectively (figure 4). Considering that EBI2 has an inhibitory effect on entry, this result suggests that EBI2 expression increases early reverse transcription efficiency in target cells which compensates for entry inhibition.

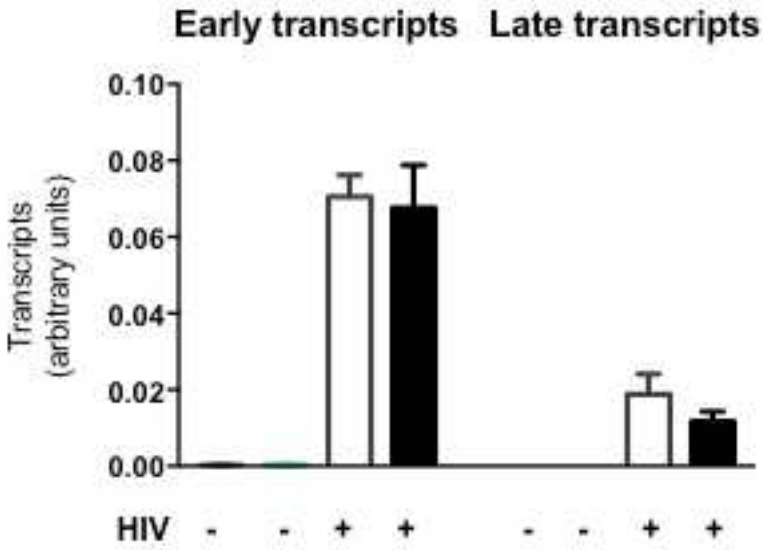


Figure 4. Effect of EBI2 expression on HIV RNA reverse transcription. HOS cells transduced with *LacZ* (open histograms) or *EBI2-EGFP* (closed histograms) were exposed or not to an R5 HIV-1 strain. The amount of early (left hand) and late (right hand) transcripts was determined by quantitative PCR.

EBI2 expression induces NFkB activation. EBI2 signaling has been reported to activate the transcription factor NFkB .(Gatto et al. 2009) Therefore, we tested by immunofluorescence whether EBI2 expression resulted in NFkB translocation. Actually, *EBI2* transduction specifically induced NFkB p65 accumulation around and in the nuclei of HeLa cells (Figure 5). This phenomenon was emphasized by the EBI2 agonist 7α,25-OHC in EBI2-transduced HeLa cells, but still not in LacZ-transduced control cells. TNF□, a cytokine known to activate NFkB, used as a positive control, induced NFkB translocation in both cell lines.

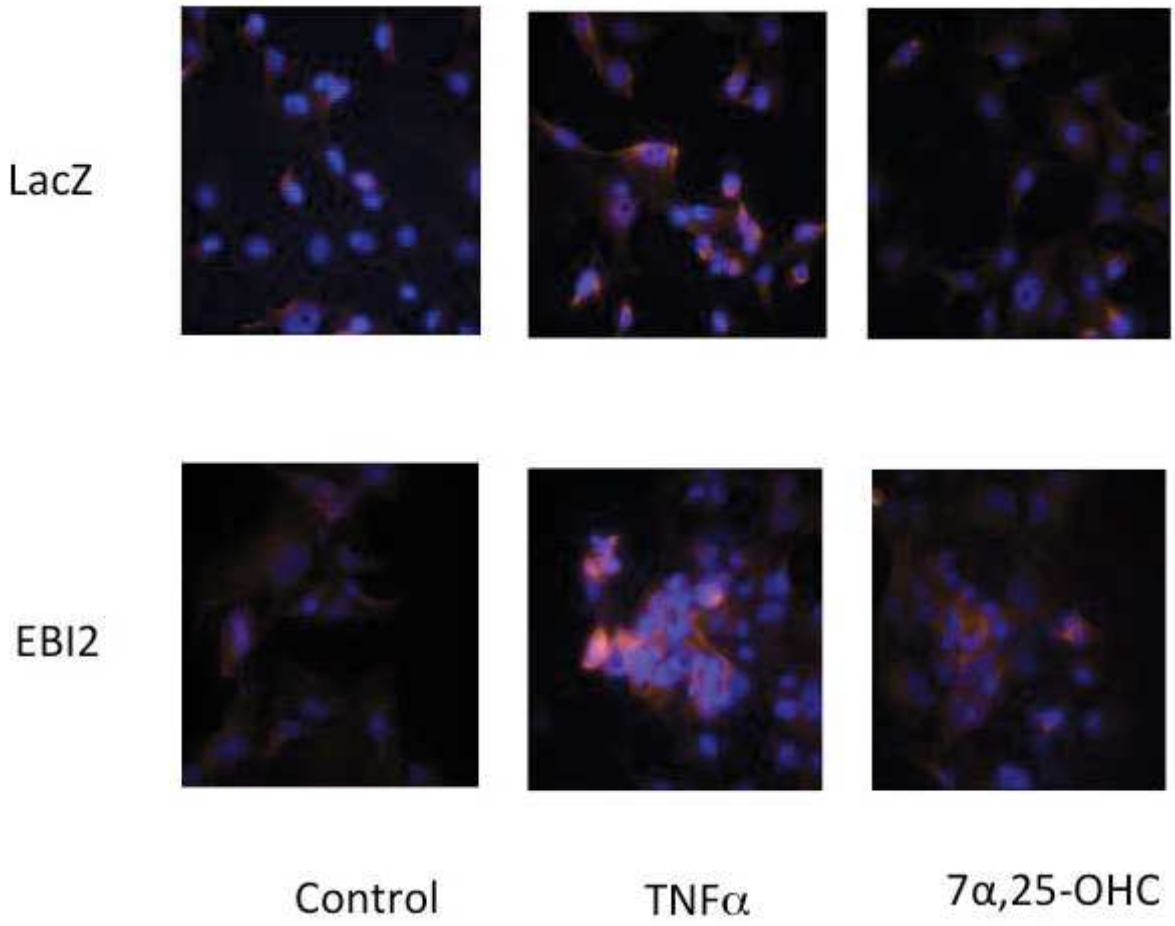


Figure 5. EBI2 signaling activates NFkB. HeLa cells transduced with *EBI2* or *LacZ* were exposed or not (control) to the EBI2 ligand 7α,25-OHC or to TNFα. Thereafter, NFκB p65 was labeled with a specific anibody and analyzed in fluorescence microscopy.

EBI2 expression activates HIV-1 promoter. As NFκB translocation is known to activate the HIV promoter LTR, we checked whether EBI2 signaling is able to induce the transcription of an LTR-driven reporter gene. To this aim, we transduced Hela-LTR.Luc cells, stably transfected with an LTR fused to a *luciferase* gene [27], with *EBI2* or *lacZ,* incubated these cells with increasing concentrations of 7α,25-OHC (0.2 to 2 μM), and monitored *luciferase* transcription. As shown in figure 6A, the EBI2 ligand induced luciferase expression in EBI2-expressing cells but not in LacZ-expressing cells (1.90 ± 0.14 versus 0.84 ± 0.02 fold increase for 0.2 μM, $p = 0.002$). TNFα, used as a positive control, induced luciferase expression in EBI2+ and EBI2- cells. To ascertain that the effect of 7α,25-OHC was mediated by EBI2 signaling, we repeated the experiment after having incubated or not the cells with the EBI2 specific antagonist NIBR189. Again 7α,25-OHC activated the LTR in EBI2-expressing cells but not in

LacZ-expressing cells even at 0.01 μM (1.41 ± 0.04 versus 1.00 ± 0.03 fold increase, p = 0.001), and this effect was inhibited by the EBI2 antagonist (Figure 6B). Similarly, when *EBI2*- and *LacZ*-transduced HOS cells were transiently transfected with a reporter *luciferase* gene driven by an HIV LTR, luciferase activity was higher in the former cells than in the latter (2823 ± 270 and 1322 ± 83 arbitrary units, respectively, p = 0.006, figure 6C). TNFα-induced luciferase expression was also higher in EBI2- than in LacZ-expressing cells (figure 6C).

Fig 6A.

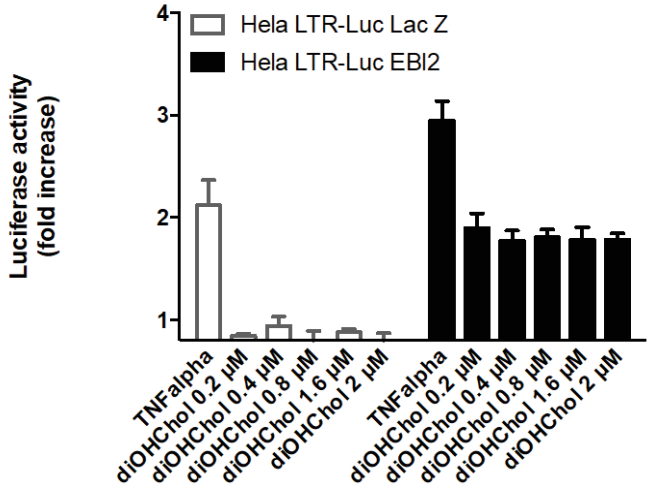


Fig 6B.

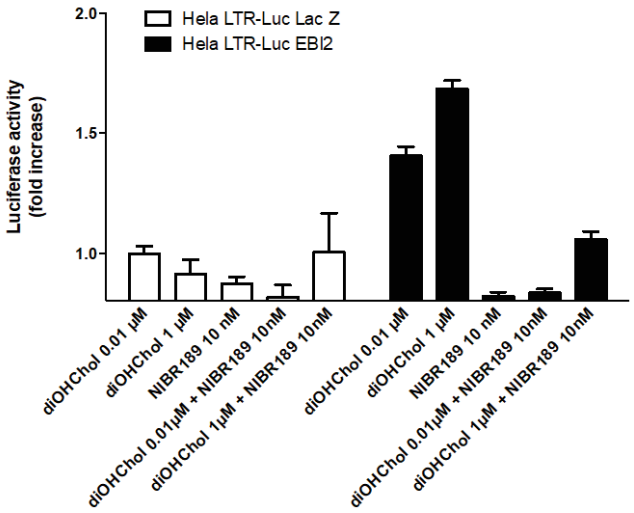


Fig 6C.

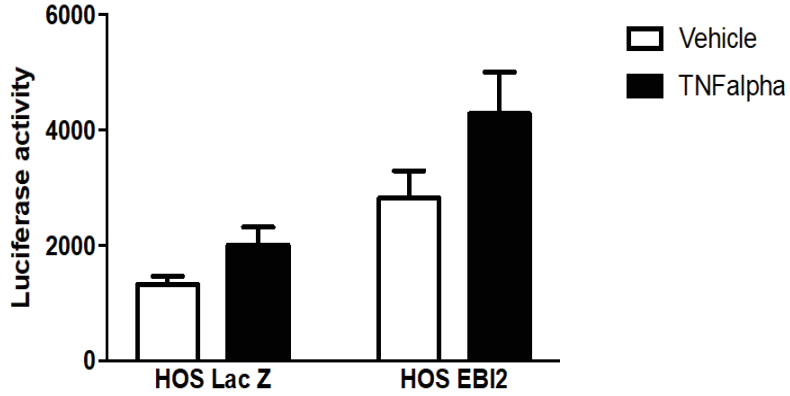


Figure 6. EBI2 signaling activates HIV-1 LTR.

(A) HeLa cells stably transfected with a *luciferase* gene driven by an HIV-1 LTR and transduced with *LacZ* (open histograms) or *EBI2-EGFP* (closed histograms) were exposed or not (negative control) to various concentrations of 7α,25-OHC or to TNFα (positive control). Thereafter, luciferase activity was measured. The increase in luciferase activity as compared with the negative controls (absence of stimulus) is represented. (B) The same experiment was performed with two different concentrations of 7α,25-OHC, and after having or not preincubated the cells with the EBI2 antagonist NIBR 189. The increase in luciferase activity as compared with the negative controls (absence of stimulus) is represented. (C) HOS cells transduced with *LacZ* or *EBI2-EGFP* and exposed (closed histograms) or not (open histograms) to TNFα (positive control) were transfected with a plasmid harboring a *luciferase* gene driven by an HIV-1 LTR. The luciferase activity measured under these different conditions is represented.

EBI2 expression increases HIV replication. As EBI2 expression activates the HIV promoter, we analyzed whether it could amplify HIV production. To this purpose we infected CD4- and CCR5-positive human osteosarcoma (HOS) cells transduced with *EBI2* or *lacZ* with a replicative HIV-1 R5 strain. EBI2-positive cells produced much more virus than the negative control lacZ-positive cells (figure 7A). As soon as day 4, p24 production was 2-fold higher in *EBI2*- than in *LacZ*-transduced cells (8.9 ± 0.6 versus 4.5 ± 0.3 ng/mL, respectively, $p = 0.003$). At day 11 the ratio was 11:1. As 25-hydroxycholesterol has been shown to block membrane fusion between viruses, including HIV, and target cell with IC_{50} of 1μM independently of EBI2 [28], we tested the effect of 7α,25-OHC on *EBI2*-transduced HOS cell infection. Interestingly, 7α,25-OHC further increased HIV replication at 0.2μM, a concentration sufficient to activate the HIV LTR (figure 7A), but not to block viral fusion [28], but inhibited it at 1μM (figure 7B). HIV replication was also increased in *EBI2*-transduced MT4 cells as

compared with *LacZ*-transduced MT4 cells (1,480 ± 49 and 719 ± 153 ng of p24/mL at day 7, respectively, p = 0.006, figure 7C).

Fig. 7A

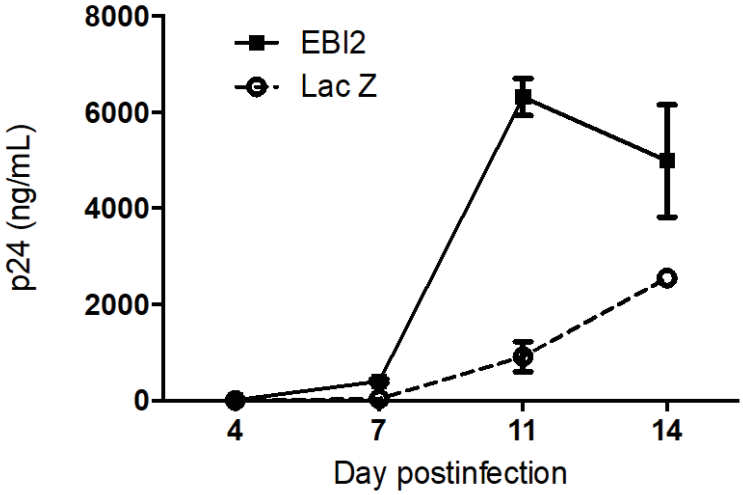


Fig. 7B

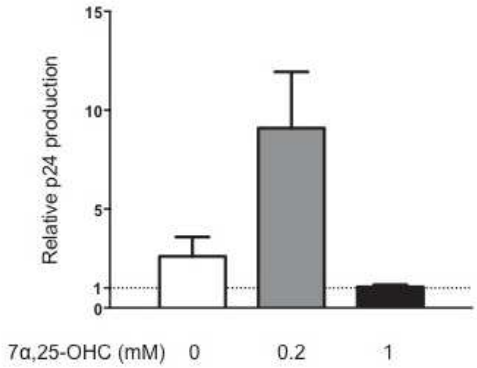


Fig. 7C

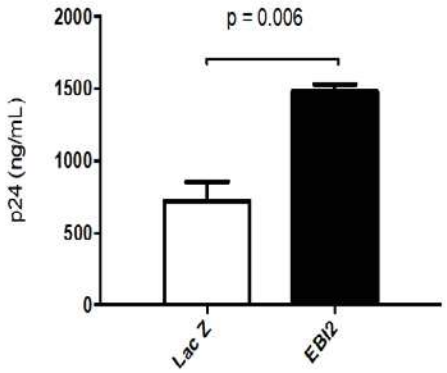


Figure 7. EBI2 expression increases HIV replication. (A) EBI2 expression increases HIV replication in HOS cells. HOS cells transduced with *LacZ* (O) or *EBI2-EGFP* (■) were exposed to an R5 HIV-1 strain and cultured. Viral production was monitored over time by measuring p24 concentration in the cell supernatant. (B) Effect of 7α,25-OHC on HIV infection of EBI2 expressing cells. HOS cells transduced with *LacZ* or *EBI2-EGFP* were exposed to an R5 HIV-1 strain and cultured in the presence

of the indicated concentrations of 7α,25-OHC. Viral production was monitored over time by measuring p24 concentration in the cell supernatant. The ratio p24 produced by EBI2-positive cells : p24 produced by LacZ-positive cells at day 7 postinfection is represented. (C) EBI2 expression increases HIV replication in MT4 cells. MT4 cells transduced with *LacZ* (open histogram) or *EBI2-EGFP* (closed hisogram) were exposed to an R5 HIV-1 strain and cultured. Seven days later, p24 concentration was measured in the cell supernatant.

Discussion

Here we show that EBI2 dimerizes with CCR5. R. Barroso et al. have reported the heterodimerization of EBI2 with another chemokine recptor, CXCR5 [29]. Of note, this dimerization resulted in a decrease in the affinity of the chemokine CXCL13 for CXCR5. Likewise, CCR5-EBI2 dimerization may reduce the affinity of CCR5 ligands, including HIV envelope, for CCR5. This could explain why we observed that EBI2 expression diminished the efficiency of HIV R5 entry into CCR5-positive cells. By contrast, EBI2 expression seemed to increase early HIV DNA reverse transcription, compensating for its inhibitory effect on viral entry. Two non exclusive mechanisms might account for this effect of EBI2 expression on reverse transcription. We have previously shown that CCR5 signaling triggered upon HIV envelope binding to CCR5 increased HIV DNA reverse transcription [3]. CCR5-EBI2 dimerization might amplify this phenomenon. On the other hand, the presence of the EBI2 natural ligand 7α,25-OHC in the culture might be responsible for an EBI2 signaling boosting the retroviral reverse transcription. The origin of 7α,25-OHC may be the fetal bovine serum added to the culture medium, since 7α,25-OHC is present in human plasma, and/or the culture cells themselves [30]. The major effect of EBI2 expression on HIV life cycle is at the level of viral genome transcription via NFkB activation. Again, 7α,25-OHC present in the culture medium and/or produced by the cultured cells might be responsible for an EBI2 signal resulting in Gαi, NFkB, and finally LTR activation.

EBI2 ligands have been detected in many lymphoid and nonlymphod tissues [31]. Moreover, 7α,25-OHC and its precursors are thought to spread from cell to cell [32]. In vivo, EBI2-positive cells might be constantly activated by these ligands. For instance the plasma concentration of 7α,25-OHC has been evaluated to be 0.9 nM [33] with an IC_{50} of 0.8 nM on EBI2 [10]. These concentrations are much lower than the concentrations at which 7α,25-OHC interferes with enveloped virus-cell fusion [28]. Thus, in vivo, the predominant effect of 7α,25-OHC might be to constantly favor HIV transcription in CD4+ T cells rather than to inhibit the fusion step of viral life cycle. This is probably particularly true in secondary lymphoid tissues where stromal cells express the two enzymes required for 7α,25-OHC synthesis, cholesterol 25-hydroxylase and 25-hydroxycholesterol 7-hydroxylase (CYP7B1) [34]. Accordingly, it would be interesting to monitor the effect of EBI2 antagonists on the residual viral production persisting in most patients aviremic under treatment [35]. Moreover, given that oxysterols,

including 7α,25-OHC, bind to RORγt and thereby promote Th17 differentiation [19], EBI2 antagonists should also dampen the pathogenic immune activation observed in virologic responders [36].

Just as CXCR5-EBI2 dimerization impairs the interaction between CXCR5 and its ligand CXCL13 [29], it is possible that CCR5-EBI2 interaction decreases the affinity of EBI2 for its natural ligands and thereby negatively modulate EBI2 physiological functions. In addition, HIV gp120 binding to CCR5 dimerized with EBI2 might exacerbate this phenomenon. Consequently, in viremic patients the function of EBI2 may be perturbed in CD4- and CCR5-positive cells. EBI2 signaling has been reported to inhibit type I interferon production in plasmacytoid and myeloid dendritic cells [16]. As HIV may infect dendritic cells via CCR5, gp120-CCR5 binding could alleviate this inhibition and participate in the type I interferon overproduction observed in the course of HIV infection [37]. EBI2 has also been shown to drive the positioning of splenic dendritic cells in marginal zone bridging channels, where they encounter blood-borne antigens [14, 15]. Impairment of this positioning consecutive to gp120-CCR5-EBI2 interaction could result in deficient CD4+ T cell activation and humoral immune response. This dysfunction might be further increased by the fact that EBI2 also guides T follicular helper T cell in secondary lymphoid organs towards the interface of the follicle and T zone, where they interact with dendritic cells [38]. Macrophages also express EBI2, in addition to CD4 and CCR5, and the enzymes necessary for 7α,25-OHC synthesis, including cholesterol 25-hydroxylase and CYP7B1 [17]. In these cells, EBI2 activation induces calcium flux and chemotaxis [17, 18, 39]. Thus, macrophage activity could similarly be impaired by circulating soluble or viral gp120.

Methods

Cells. $CD4^+$ human osteosarcoma HOS cells, human embryonic kidney 293T cells, HeLa-LTR.Luc cells [27] were cultivated in DMEM supplemented with 10% fetal bovine serum, glutamin and penicillin/streptomycin. MT4 cells were cultivated in DMEM supplemented in the same way.

Quantitative multi-RT-PCR. CD4+CCR5+ T cells (2 millions) were sorted using magnetic beads coupled to antibodies (Dynabeads Pan Mouse IgG, Invitrogen). Macrophages were obtained by *in vitro* differentiation with GM-CSF of PBMC isolated by low density gradient centrifugation. RNAs were extracted with Qiamp RNA blood (Qiagen) where an RNAse free DNAse treatment was included. cDNA was synthetized with the high capacity cDNA archive kit (Applied Biosystems). Quality of sample was controlled by Q-PCR for correct GAPDH/CCR5 ratio. cDNA from 3 donors were mixed in equimolar amounts and 750 ng of this mixture were loaded on a 384-well microfluidic cards pre-loaded with primers for 361 different GPCR and 14 housekeeping genes as positive control (TaqMan Low Density Arrays from Applied Biosystems). Q-PCR was realized on a ABI Prism 7900HT Sequence Detection System.

Time-resolved FRET. The DNA fragments for *CCR5* and *EBI2* were obtained by PCR and cloned in pRK5-HA-SNAP or pRK5-FLAG-CLIP (MS S1P1 2013[40]), resulting in a fusion of a signal peptide, an hemagglutinin (HA) or a FLAG tag, the SNAP or CLIP enzyme[40], and the receptor from the second codon to the STOP codon. The SNAP-β2AR construct has been previously described [41]. 293T cells were cotransfected with 4 ng of CLIP-CCR5 and increasing amount (1 to 25 ng) of SNAP-CCR5, SNAP-EBI2 or SNAP- β2AR, plated at 10^5 cells/well in a poly ornithine-coated 96-well plate for 30 hours, and then labeled for 2 hours at 37°C with BC-Lumi4-Tb (1 μM) and BG-Green (0.3 μM) in TagLite (TL) buffer (Cisbio Bioassays). After washing, the emission signal from the BC-Lumi4-Tb and the BG-Green fluorophores were recorded at 620 nm and 520 nm, respectively, after excitation at 337 nm and 485 nm on a time-resolved fluorimeter (PHERAstar FS, BMG Labtechnologies). In the same wells, tr-FRET signal was measured at 520 nm between 60 and 400 μs after laser excitation at 337 nm.

Flow cytometry For antibody staining, 200 000 cells were incubated in PBS with anti-human CCR5-PE-Cy5 antibody (BD Pharmingen 556889) or an isotype control (BD pharmingen 555575) for 1h at 10 μg/ml. Cells were washed and fixed in BD Cell Fix (BD Biosciences), expression was measured on a MACSquant flow cytometer. FlowJo was used to analyze data.

Transduction. HIV transfer vectors that express *CCR5, LacZ* or EBI2 were produced as described [3] transduced in 8 μg/ml polybrene (Sigma).

Infection assays with replicative virus. HOS (50 000 cells, 2 mL) or MT4 (100 000 cells, 2 mL) cells were exposed to 10 ng of Ad8, washed and cultured. Cell number was adjusted twice a week, and viral production was evaluated by measuring p24 antigen in culture supernatant (Innotest HIV Antigen mAb kit, Fujirebio, Ingen).

HIV entry assay. Virions harboring β-lactamase were produced by cotransfecting 3 millions of 293T cells per plate with 15 μg of the HIV proviral DNA pNL4.3 env-luc+, the R5 envelope plasmid pCMV-Ad8 (6 μg), a packaging plasmid (pAX2, 2 □g), and the plasmid pMM310 encoding a *Vpr* gene fused to a *β-lactamase* gene (7 μg) (S1P1-77). Virus preparations were concentrated by ultracentrifugation on 4% sucrose (1h30, 26,000 rpm, 4°C). MT4 cells were then exposed for 3.5 hours at 37°C to 500 ng of R5 Vpr-βlam virus. Cells were then washed and loaded with the CCF2-AM LiveBLAzer™ FRET-B/G Loading Kit (Life Technologies) in the presence of 15 mM Probenecid (Sigma). Cells were incubated for 1 h at room temperature in CO2-independant medium (Gibco), washed and fixed. The CCF2-AM and the cleaved CCF2-AM fluorescences (excitation at 405 nm, emission at 520 nm and 447 nM, respectively) were measured by flow cytometry on a MACSQuant analyser 10 (Mylteni).

Real-time PCR detection of HIV transcripts. To produce replication-defective HIV virions, 293T cells were co-transfected with the pNL4.3-env$^-$-luc plasmid and with the pCMV-Ad8-Env (PNAS 2002). HOS cells (100 000 cells, 1mL) were exposed to 20 ng of these virions, and lysed 24 hours later. DNA

from early and late transcripts was amplified as described [3]). Genomic CCR5 DNA was used for normalization.

LTR activation assays. Hela-LTR.Luc cells were transduced with *EBI2* or *LacZ,* exposed or not to 10 ng/mL of TNFα for 6 hours and lysed. Alternatively, HOS cells transduced with *EBI2* or *LacZ* were transiently transfected by using lipofectamine Plus (Invitrogen) with the LTR-luc reporter plasmid pL274 [42], either wild-type or mutated in the NFκB binding sites (GGGactttccgctgGGG replaced with CTCactttccgctgCTC, by Ke Zhang, a gift from Monsef Benkirane), exposed or not to 10 ng/ml of TNFα for 6 hours and lysed. Luciferase activity was then measured in cell extracts using a commercial assay (Promega).

Immunofluorescence assay. HOS cells transduced with *EBI2* or *LacZ* were exposed or not for 2 hours to 10 ng/ml TNFα and labeled for 45 min with an NFκB p65 antibody (F-6, Santa Cruz), followed by a secondary fluorescent anti-mouse antibody (A546), and finally with Dapi at 5 μg/ml for nucleus staining.

Statistical analysis. Values are expressed as the mean ± SEM. Differences were analyzed with the one-tailed unpaired Student's *t* test.

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G. Conclusion EBI2

EBI2 s'hétérodimérise avec CCR5. L'interaction entre ces deux récepteurs à la membrane cellulaire pourrait être à l'origine de la diminution de l'entrée virale en présence d'EBI2 que nous avons observée. En se dimérisant avec CCR5, EBI2 pourrait modifier la conformation de CCR5 et par là la force de l'interaction entre ce corécepteur et l'enveloppe rétrovirale.

Cependant malgré cette réduction partielle de l'entrée virale, l'effet global d'EBI2 est une amplification de la production virale. Effectivement la stimulation d'EBI2 par son ligand naturel, le 7α, 25-OHC, augmente la transcription virale à partir du LTR. Cet effet est bien spécifique d'EBI2 puisqu'il est supprimé en présence de l'antagoniste NIBR189. En revanche les étapes post traductionnelles ne semblent pas impactées par la présence d'EBI2 (figure 11). Par la suite, il sera intéressant d'identifier la voie de signalisation déclenchée par la stimulation d'EBI2 responsable de l'activation du LTR ainsi que les éventuels facteurs de transcription impliqués.

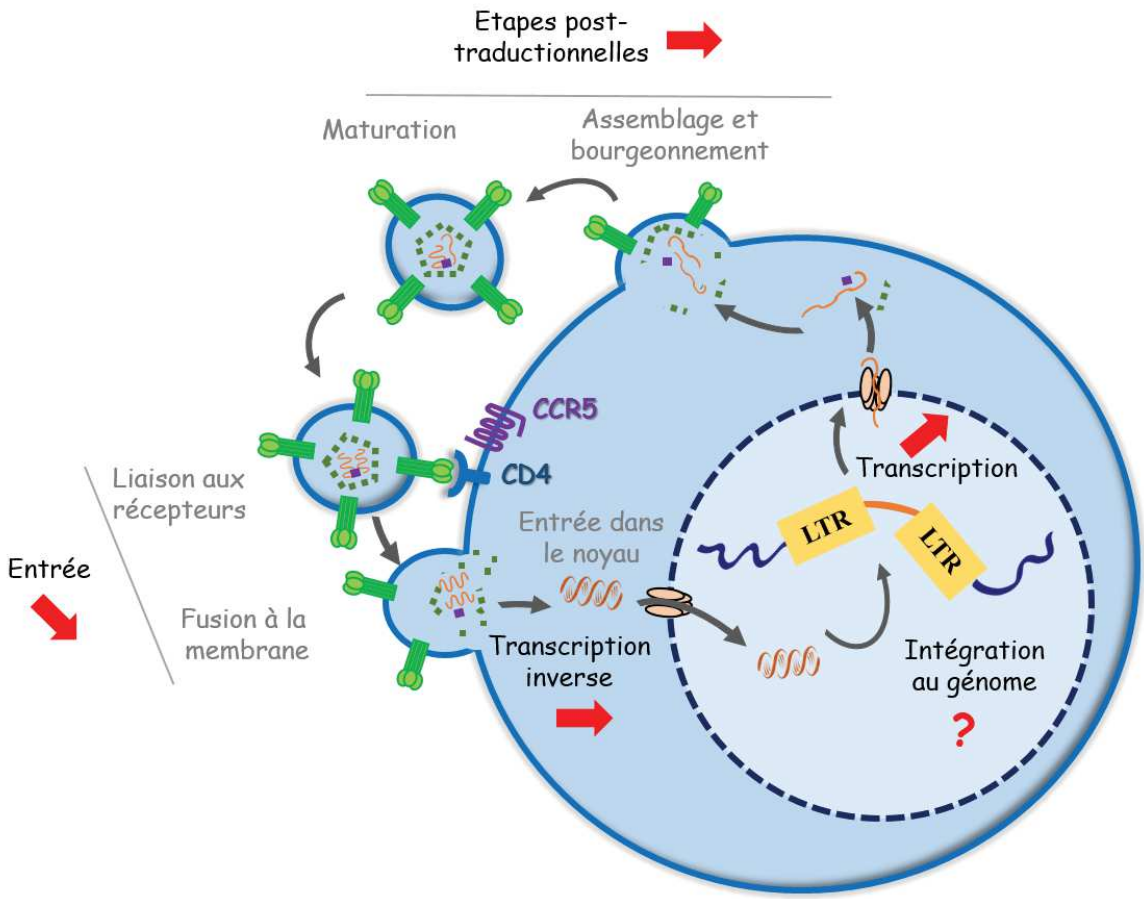


Figure 11. Schéma récapitulatif des étapes du cycle viral modifiées par EBI2.

Cette observation que des agonistes d'EBI2 induisent l'activation du LTR ouvre la voie à l'hypothèse que ces ligands pourraient avoir la capacité de lever la latence virale dans les cellules réservoirs. A l'heure où des agents ayant cette capacité (latency reversing agents ou LRA) sont activement recherchés dans une perspective de purge des réservoirs viraux, où les effets in vivo sur ces réservoirs des premiers LRA sont encore décevants et où ces agents sont potentiellement toxiques, la mise en évidence de nouveaux candidats LRA est intéressante.

Par ailleurs si EBI2 modifie la conformation et la fonctionnalité de CCR5, il se pourrait que réciproquement la fonctionnalité d'EBI2 soit changée suite à sa dimérisation avec CCR5, en particulier en présence de la gp120 rétrovirale. Cette hypothèse amène à la possibilité que chez les patients virémiques, le chimiotactisme via EBI2 lors de la réponse humorale soit perturbé. Ceci pourrait contribuer aux dysfonctionnements des cellules B observés chez les patients non traités. De plus la stimulation de CCR5 par la gp120 pourrait entraîner celle d'EBI2, favorisant l'activation polyclonale des cellules B, également observée chez les patients virémiques.

III. A_{2A}

A. Le récepteur à l'adénosine

L'adénosine est un nucléoside purinique qui joue un rôle important dans le transfert d'énergie sous forme adénosine triphosphate (ATP) et dans la signalisation via l'adénosine monophosphate cyclique (AMPc). Cependant, l'adénosine joue un rôle direct dans l'inflammation et l'activation immunitaire (Gessi et al. 2000). Elle est également produite abondamment, potentiellement par tout type cellulaire, dans les situations de stress comme l'hypoxie et l'inflammation. Etant donné qu'elle peut affecter un large spectre de fonctions biologiques, sa concentration intra- ou extracellulaire reste basse et contrôlée à partir de l'axe ATP/ADP/AMP. Sa concentration extracellulaire est de 10 à 200 nM en conditions d'homéostasie, mais peut monter localement de 10 à 100 uM en cas d'hypoxie (Fredholm, 2007). L'adénosine provient de l'action des ecto-pyrases comme CD39 qui hydrolysent l'ATP et l'ADP en AMP, puis l'ecto-4-nucléotidase CD73 convertit l'AMP en adénosine (Yegutkin 2008). En situation physiologique, l'AMP intracellulaire est plutôt transformé en inosine monophosphate, mais en situation de stress sa transformation en adénosine est favorisée. CD39 et CD73 sont ubiquitaires (Chadwick and Frischauf 1998; Kaczmarek et al. 1996; Thompson et al. 2004) et très présents à la surface des lymphocytes et des cellules endothéliales (Airas and Jalkanen 1996; Deaglio et al. 2007; Kansas, Wood, and Tedder 1991; Pulte et al. 2007; Ryzhov et al. 2011; Thomson et al. 1990). Leur expression est renforcée en cas d'hypoxie (Synnestvedt et al. 2002). L'adénosine, elle, est transformée dans les cellules en inosine par l'adénosine déaminase (ADA) et en AMP par l'adénosine kinase. Par ailleurs le flux de l'adénosine entre les compartiments intracellulaire et extracellulaire est régulé par des « equilibrative nucleoside transporters » (ENT). En situation physiologique, l'ENT favorise l'entrée de l'adénosine dans les cellules. En cas d'hypoxie tissulaire, comme c'est le cas au décours d'un sepsis, la production d'adénosine à partir de l'AMP est accrue, pendant que sa dégradation est réduite du fait de modifications des taux (S. Kobayashi, Zimmermann, and Millhorn 2000) et des activités (Decking et al. 1997) des enzymes responsables. L'inflammation peut également augmenter le taux d'ecto-4-nucléotidase et diminuer le taux et l'activité d'ADA et de l'adénosine kinase (Nakav et al. 2010). En conséquence les taux d'adénosine s'élèvent chez l'homme en situation d'endotoxémie (Ramakers et al. 2011) et de choc septique (Martin et al. 2000). L'inflammation est régulée de manière globalement opposée par L'ATP et l'adénosine ; l'ATP produite sur les sites d'inflammation par les cellules immunitaires activées et les cellules endommagées la renforce, tandis que l'adénosine produite à partir de l'ATP sur ces sites ralentit le processus inflammatoire et l'activation immunitaire, permettant ainsi un rétrocontrôle négatif de ces

processus (Fig 12). L'effet global et les effets spécifiques de l'ATP et de l'adénosine dans l'inflammation et dans la réponse immunitaire dépendent donc de la régulation fine de la concentration extracellulaire de ces deux acteurs, ainsi que de la densité de leurs récepteurs respectifs à la surface des cellules impliquées dans cette réponse.

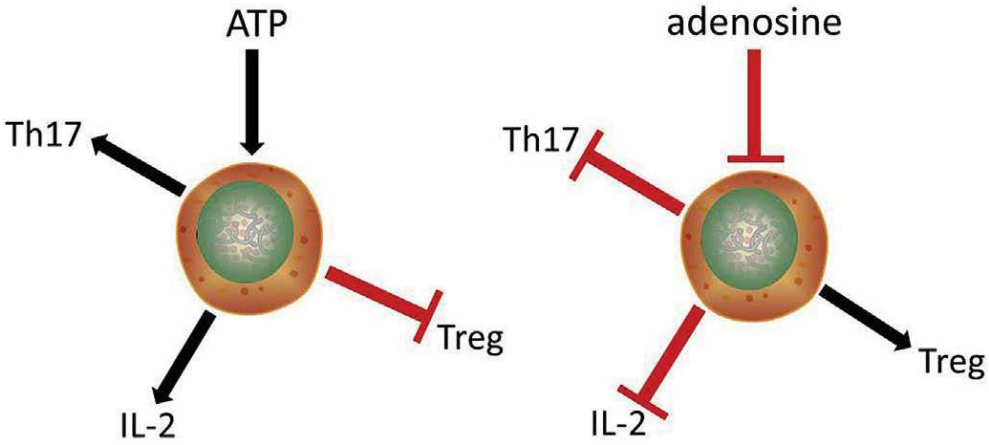


Figure 12. Les effets de l'ATP et de l'adénosine sur les cellules T (tiré de Faas, Sáez, et de Vos 2017).

Dans ces processus, les actions de l'adénosine passent par sa liaison avec quatre récepteurs à l'adénosine (Ras) qui sont des RCPGs : A_1, A_{2A}, A_{2b} et A_3, de structure primaire et propriétés pharmacologiques différentes, exprimés dans de nombreux types cellulaires en plus des cellules immunitaires. Les effets induits par la stimulation de ces récepteurs vont de la vasodilatation, l'inhibition de l'agrégation plaquettaire, la modulation du système sympathique à l'inhibition de l'activation immunitaire (Rongen et al. 1997).

Le récepteur A_1 est fortement exprimé au niveau du cœur où il régule la consommation d'oxygène et la conduction électrique jouant ainsi un rôle protecteur du muscle cardiaque, ainsi que dans le cerveau où il influence les mécanismes de la douleur. Il inhibe l'adénylate cyclase et les canaux calciques voltage dépendant, active les canaux potassiques, augmente le calcium intracellulaire et l'inisitol triphosphate (IP3) par l'activation de la phospholipase Cγ (J.-F. Chen, Eltzschig, and Fredholm 2013).

L'étude du récepteur A_3 est complexe car il est exprimé dans de nombreux tissus ; globalement, il est protecteur du système cardiovasculaire, neuroprotecteur et anti-inflammatoire dans le cerveau. C'est le seul RA surexprimé dans les cellules cancéreuses et inflammatoires, ce qui en fait une cible thérapeutique privilégiée. Des agonistes semblent déjà prometteurs dans le traitement de l'arthrite et du psoriasis (Pier Andrea Borea, Pharmacol rev

2017). Comme A_1, il inhibe l'adenylate cyclase, stimule la phospholipase C et B, et module les taux de calcium. (Sachdeva and Gupta 2013).

Au contraire d'A_1 et A_3, les récepteurs A_2 (A_{2A} et A_{2B}) stimulent l'adénylate cyclase et augmentent ainsi l'AMPc intracellulaire. A_{2B} est exprimé dans de nombreux tissus et organes, et contrairement à A_{2A} qui a une forte affinité pour l'adénosine, il est activé surtout dans des conditions de fortes concentrations d'adénosine lors d'hypoxie, d'ischémie ou d'inflammation (Ryzhov et al. 2008). Des effets opposés sont décrits dans diverses pathologies, A_{2B} pouvant être pro- ou anti-inflammatoire, pro- ou anti-tumoral (Sun and Huang 2016).

A_{2A} joue un rôle protecteur du myocarde comme A_1, et de par son implication dans le relargage du glutamate et de la dopamine au niveau du cerveau, c'est une cible thérapeutique de choix pour la dépression, la douleur, l'insomnie, l'addiction aux drogues et la maladie de Parkinson (figure 13). A noter que dans le cerveau, A_{2A} agit par interaction négative avec le récepteur à la dopamine D2, un agoniste de A_{2A} agissant comme antagoniste de D2 (Ferré et al. 1997).

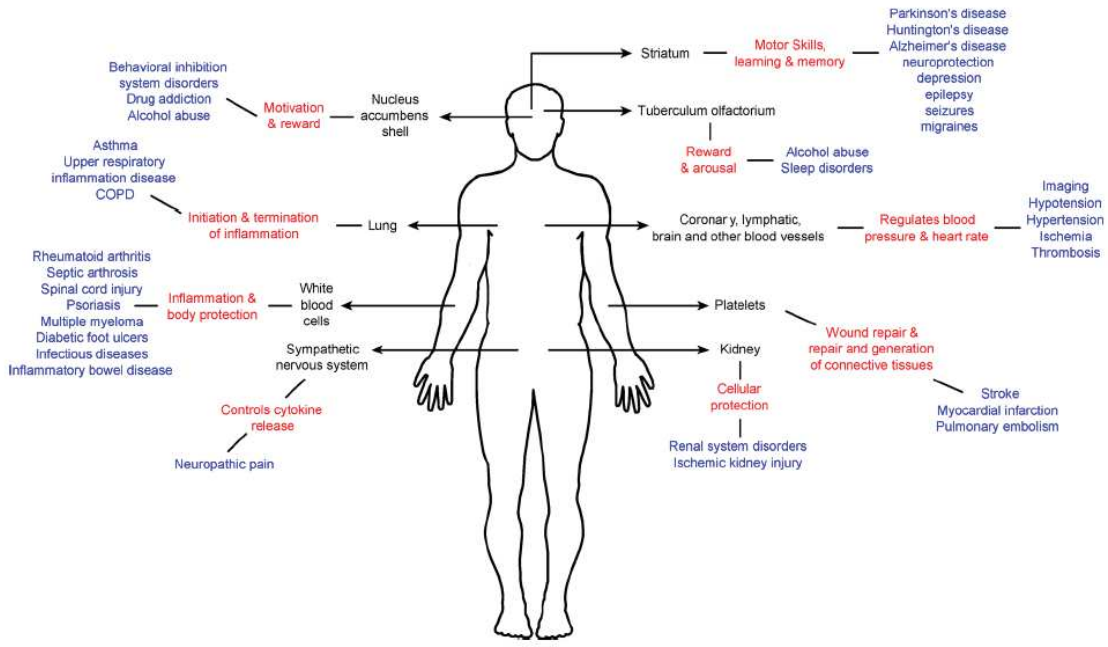


Figure 13. A2A : expression (en noir), fonctions (en rouge) et pathologies associées (en bleu), (tiré de Lera Ruiz, Lim, et Zheng 2014).

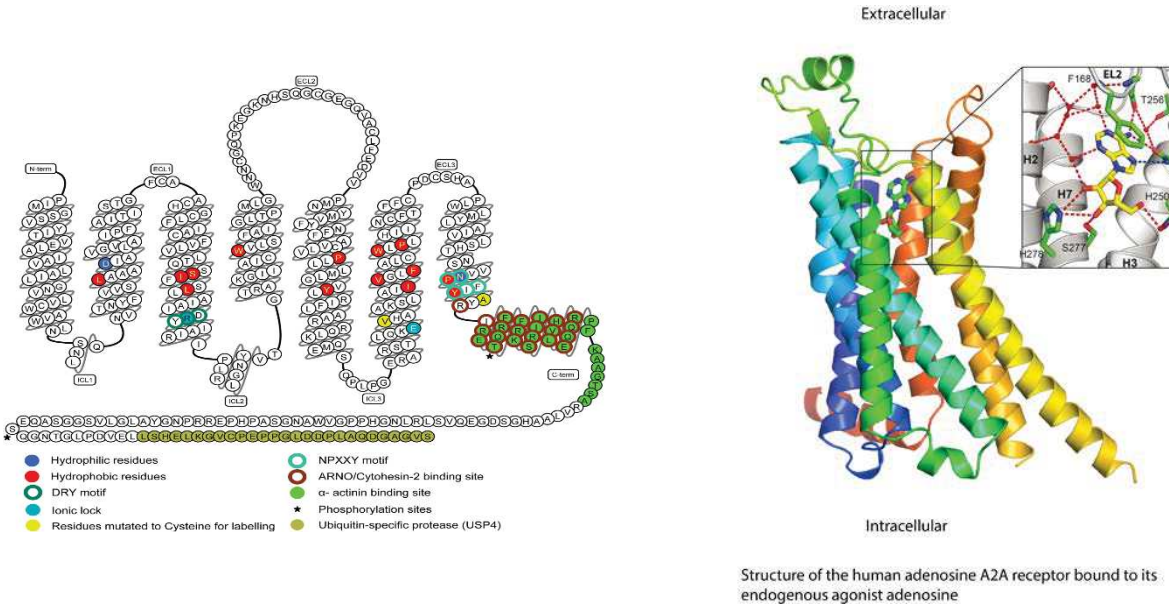
A_{2A} est couplé aux protéines Gs. L'augmentation d'AMPc active la protéine kinase A (PKA) et l'EPAC (exchange factor activated by cAMP). L'activation de la PKA aboutit à la phosphorylation de NFAT et de CREB (cAMP responsive element-binding protein) qui en liant

la sous-unité p65 inhibe l'activation de NFκB (Jimenez et al. 2001; Parry and Mackman 1997). L'activation de la PKA aboutit aussi à l'inhibition de la voie Ras/MAP kinase et de la voie NFAT (Panther et al. 2001). Par ailleurs l'activation des MAP kinases ERK1/2 et de RhoA a été observée (Mosenden and Taskén 2011; Klinger, Freissmuth, and Nanoff 2002).

1. Structure et ligands d'A_{2A}

Le récepteur A_{2A} présente une structure composée de sept domaines transmembranaires typique des RCPGs. A la suite du septième domaine transmembranaire une courte hélice parallèle à la surface cytoplasmique est considérée comme le huitième domaine transmembranaire (Fig. 14A). Les différences entres les récepteurs de l'adénosine se font au niveau de la longueur et de la fonction du domaine extracellulaire N-terminal, du domaine intracellulaire C-terminal et des boucles intra et extracellulaires (de Lera Ruiz, Lim, and Zheng 2014a).

Les analyses de la structure tridimensionnelle d'A_{2A} lié à l'agoniste naturel adénosine ou à l'agoniste inverse ZM241385 permettent de mieux comprendre comment s'effectue le passage entre l'état inactivé (stabilisé par l'agoniste inverse) et l'état activé du récepteur (stabilisé par l'agoniste). Ce dernier était le seul capable de se coupler aux protéines G et de les activer (Zhukov et al. 2011; Lebon et al. 2011) (Fig. 14B), et de manière plus générale de mieux comprendre la dynamique d'activation d'un RCPG par son ligand. Ces études ont permis de définir la forme de la poche de liaison qui est profonde, plane en cavité étroite pour l'accueil de noyaux polyaromatiques. Ces informations structurelles orientent vers des approches pour la conception de nouveaux ligands d'A_{2A}. Cependant des résultats inattendus sont parfois obtenus du fait de la différence conformationnelle entre le récepteur cristallisé stable et la nature dynamique du récepteur natif (de Lera Ruiz, Lim, and Zheng 2014a).



A. B.

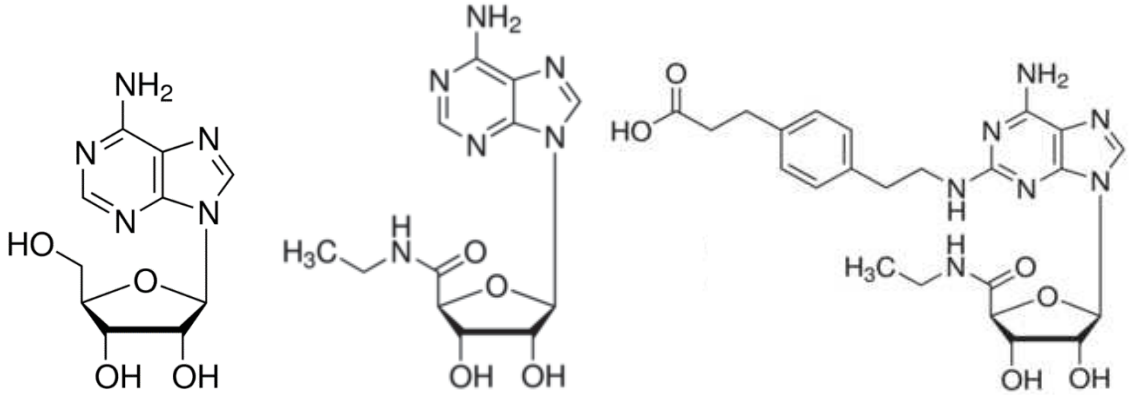
Figure 14. Structure du récepteur A2A. A. Structure primaire à 7 domaines transmembranaires (tiré de Prosser et al. 2017). B. Structure 3D (Lebon et al. 2011).

Les RAs constituent une cible pharmacologique de choix dans de nombreuses situations pathologiques. Les approches pharmacologiques prennent en compte que l'adénosine est rapidement métabolisée par diverses enzymes, que les récepteurs sont exprimés dans de nombreux tissus, et la signalisation par les RAs est parfois redondante. C'est pourquoi des ligands de haute affinité et spécificité ont été générés pour chacun des récepteurs. Néanmoins, on constate un phénomène de tolérance, c'est-à-dire une réduction de la réponse à une dose spécifique de ligand lors d'un usage chronique ou répété (Jacobson et al. 1996). Ce phénomène, dû entre autres à une diminution de l'expression des récepteurs ou à leur internalisation, peut-être réduit par l'usage d'antagonistes, ou d'agonistes partiels qui agissent de manière prédominante comme des antagonistes sur les sites où la signalisation endogène par l'adénosine est suffisante (Lambert EACCP 2004). Ces agonistes partiels ont aussi l'avantage de limiter les effets secondaires par rapport à un agoniste complet, car leur spectre d'action sur les RAs est plus réduit. Une autre approche consiste à utiliser des molécules sous forme inactive ou « prodrug », c'est-à-dire activées uniquement au niveau du tissu désiré par l'expression locale d'une enzyme, comme c'est le cas pour A_{2A} avec l'activation du CD73 dans les tissus enflammés (Flögel et al. 2012).

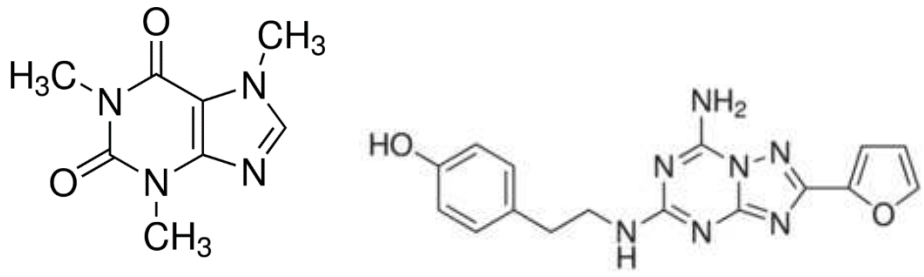
L'adénosine a une forte affinité pour A_{2A} (Ki = 700nM). Des agonistes ont été obtenus à partir de l'adénosine, les études ont conclu à la nécessité du maintien de la base et à la

modification au niveau de la position 2 de la purine ou en position 5 du ribose. Le N-ethylcarboxamidoadénosine (NECA) est un agoniste non sélectif des RAs obtenu par ajout d'un groupe alkylamide en position 5 du ribose, ce qui lui confère une plus grande affinité que l'adénosine (Ki = 20nM). Le CGS21680, obtenu à partir du NECA par ajout d'un phenylethylaminopropanoate en position 3 de l'adénine, est l'agoniste le plus sélectif d'A_{2A} (Ki = 27nM).

Il existe également de nombreux antagonistes. La caféine est un antagoniste naturel non sélectif des RAs (Ki = 23400 nM). Elle modifie le rythme et le niveau de contraction du muscle cardiaque et des muscles lisses, ainsi que la signalisation dans le système nerveux central. Sa consommation largement répandue à l'échelle mondiale doit être prise en compte dans les traitements utilisant les ligands des RAs. Le ZM241385 est un antagoniste efficace et plutôt sélectif d'A_{2A} (Ki = 0,8 nM). Sa nature bicyclique avec plusieurs possibilités de liaisons hydrogènes lui confère une solubilité dans l'eau (de Lera Ruiz, Lim, and Zheng 2014b).



A. Adénosine *B. NECA* *C. CGS21680*



D. Caféine *E. ZM241385*

Figure 15. Structure de l'agoniste naturel adénosine, des agonistes NECA et CGS21680 et des antagonistes caféine et ZM241385.

2. A_{2A} et système immunitaire

A_{2A} est exprimé dans la rate et le thymus, à la surface des cellules T, des cellules NK, des monocytes et des macrophages, des cellules dendritiques, des polynucléaires et des plaquettes.

Globalement sa stimulation aboutit à une immunosuppression (Blackburn et al. 2009; György Haskó and Pacher 2008; György Haskó et al. 2008), conséquence en particulier de l'inhibition des voies NFκB et JAK/STAT (Majumdar and Aggarwal 2003; Yoshimura, Naka, and Kubo 2007), ainsi qu'à la protection des tissus vis-à-vis des dommages inflammatoires par ses propriétés vasodilatatrices et de genèse de nouveaux vaisseaux sanguins.

A_{2a} est le principal récepteur pour l'adénosine présent à la surface des lymphocytes T. Son expression est augmentée en cas d'activation T (Deaglio et al. 2007; Lappas, Rieger, and Linden 2005). La stimulation d'A_{2A} inhibe la production d'IL-2, d'IFNγ et de cytokines pro-inflammatoires, la prolifération cellulaire, l'apoptose postactivation, l'activité des cellules T cytotoxiques, l'expression de la molécule de co-activation CD40L, mais induit celle des récepteurs inhibiteurs PD-1 et CTLA4 et la production de la cytokine anti-inflammatoire IL-10 (Raskovalova et al. 2007; Sevigny et al. 2007; Muñoz et al. 1990; Erdmann et al. 2005; Himer et al. 2010). Elle inhibe la différenciation des cellules T en Th17 (Zarek et al. 2008). En revanche l'activation d'A_{2A} favorise l'expression de Foxp3 et par là, la différenciation des cellules Treg (Parry and Mackman 1997). Par ailleurs ces cellules Treg surexpriment CD39 et CD73, ce qui leur permet de générer de l'adénosine immunosuppressive (Ralevic and Burnstock 1998). La stimulation d'A_{2a} à la surface des cellules iNKT (« invariant natural killer T cells ») réduit la production d'IFNγ et augmente celle d'IL-4, d'IL-10 et de TGFβ par ces cellules (Nowak et al. 2010). L'adénosine inhiberait également l'activité cytotoxique des cellules NK (Häusler et al. 2011).

Dans les cellules B, l'activation d'A_{2A} par l'adénosine influe sur les voies de signalisation NF-κB via l'augmentation d'AMPc et de la PKA, elle diminue leur prolifération. (Bours et al. 2006).

La liaison de l'adénosine à A_{2A} à la surface des monocytes/macrophages et des cellules dendritiques réduit la production de TNFα, de la chimiokine inflammatoire MIP-1β, d'IL-6, d'IL-8 et d'IL-12 par ces cellules présentatrices d'antigène, leur capacité à induire la différenciation des cellules T en Th1 et favorise leur production d'IL-10 (Kreckler et al. 2006; G. Haskó et al. 2000; Wall et al. 2009; Bouma et al. 1994; Hamano et al. 2008; Le Moine et al.

1996; K. Li et al. 2008). Par ailleurs la stimulation des macrophages et des cellules dendritiques par le LPS induit l'expression d'A_{2A} à leur surface (Hamano et al. 2008; Murphree et al. 2005; M. Yang et al. 2010; Schnurr et al. 2004; Fossetta et al. 2003; Panther et al. 2001). L'adénosine inhiberait également la maturation et l'activation des cellules dendritiques et favoriserait la différenciation des macrophages en cellules de phénotype M2 (Csóka et al. 2012).

Au niveau des cellules polynucléaires, l'activation d'A_{2A} inhibe la phagocytose, la production de dérivés oxygénés, l'expression membranaire de la molécule d'adhésion VLA-4 et les capacités de liaison à l'endothélium (Salmon and Cronstein 1990; Cronstein et al. 1990; Zhao et al. 1996; Cronstein et al. 1992). L'expression d'A_{2A} à la surface des polynucléaires est accrue en cas de sepsis chez l'Homme (Kreth et al. 2009).

Ces différents effets immunosuppresseurs jouent des rôles établis dans de nombreuses situations pathologiques. Ainsi la signalisation via A_{2A} atténue les lésions de divers organes suite à une ischémie suivie de re-perfusion (Day et al. 2004; Okusa et al. 1999; Sharma et al. 2010; Glover et al. 2005). Au niveau du foie, des agonistes d'A_{2A} ont montré des effets bénéfiques dans des modèles animaux d'hépatotoxicité ou de transplantation hépatique (Raskovalova et al. 2007; Sevigny et al. 2007; Tang et al. 2007). Au niveau du rein, ces agonistes se sont révélés bénéfiques dans des modèles expérimentaux de glomérulonéphrite et de néphropathie diabétique (G. E. Garcia et al. 2008; Norton et al. 1992). Ils jouent également un rôle protecteur dans des modèles de pneumopathie allergiques ou autoimmunes, de détresse respiratoire aigüe (Fozard et al. 2002; M. Thiel et al. 2005) et de lésion artérielle (McPherson et al. 2001). Les souris $A_{2A}^{-/-}$ soumises à un choc endotoxémique produisent plus de cytokines inflammatoires et survivent moins bien que des souris sauvages pour ce gène (Ohta and Sitkovsky 2001). Réciproquement des agonistes d'A_{2A} améliorent la survie dans un modèle murin de sepsis (Sullivan et al. 2004; Moore et al. 2008). Chez l'Homme, l'injection intraveineuse d'adénosine diminue la production d'IL-6 déclenchée par l'administration de LPS (Soop et al. 2003). De nombreuses cellules cancéreuses surexpriment CD39 et CD73 (Bastid et al. 2013) permettant la production d'adénosine, ce qui pourrait contribuer à l'échappement tumoral à la réponse immunitaire. De plus l'adénosine pourrait favoriser la prolifération tumorale, la néoangiogenèse et les métastases (Antonioli et al. 2013). En conséquence les récepteurs à l'adénosine deviennent des cibles thérapeutiques en cancérologie.

3. A_{2A} et VIH

L'adénosine interviendrait dans le contrôle d'infections virales chroniques comme par le VIH ou le cytomégalovirus ; en effet, les patients séropositifs pour ces virus ont une fréquence plus élevée de Treg CD39+, ou une expression et une activité augmentées de CD39 sur les lymphocytes, et donc une production accrue d'adénosine, ce qui résulte en une diminution de la réponse antivirale (Aandahl et al. 2004). L'inhibition ou le blocage de CD39 lève alors l'effet suppresseur des Treg sur les lymphocytes (Nikolova et al. 2011; Schulze et al. 2011).

Inversement, la stimulation d'A_{2A} induit une désensibilisation hétérologue de CCR5 (N. Zhang 2006). De plus, la stimulation d'A_{2A} par Adonis, un anticorps monoclonal se comportant comme un agoniste spécifique à durée d'action prolongée inhibe la prolifération des cellules T, et entraîne une diminution de l'expression des co-récepteurs du VIH, CXCR4 et CCR5, de façon dose dépendante, sans modifier le niveau du récepteur CD4 (By et al. 2010).

B. Matériel et méthodes

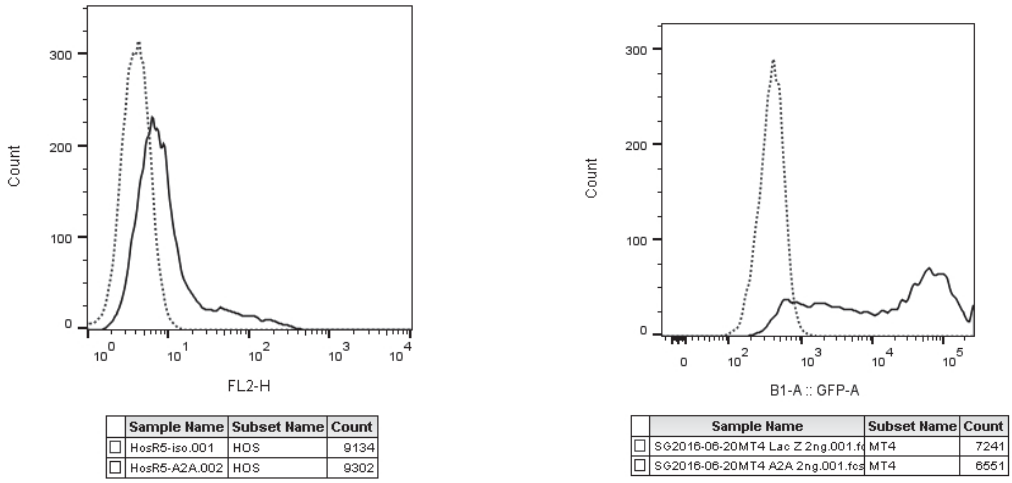
Les matériels et méthodes de cette partie sont les mêmes que ceux utilisés pour le récepteur EBI2. Les ligands d'A_{2A} ainsi que leurs concentrations sont précisés en légende des figures.

C. Résultats

1. Obtention de lignées HOS et MT-4 exprimant A_{2A}

a. Expression d'A_{2A} à la surface des lignées cellulaires

La lignée cellulaire HOS (Human OsteoSarcoma) a été transduite successivement par deux vecteur lentiviraux, l'un délivrant le corécepteur CCR5, puis l'autre délivrant la séquence du récepteur A_{2A} couplée à l'eGFP (A_{2A}-IRES-eGFP). Nous avons ainsi obtenu des cellules HOS coexprimant A_{2A} et CCR5, ou en contrôle négatif LacZ et CCR5. L'expression putative des récepteurs a été mesurée en cytométrie de flux par mesure de la fluorescence de l'eGFP dans les HOS transduites (Fig. 16A). La lignée cellulaire MT-4 (cellules T humaines transformées par HTLV-1) a été transduite comme la lignée HOS (Fig. 16B).



A. B.

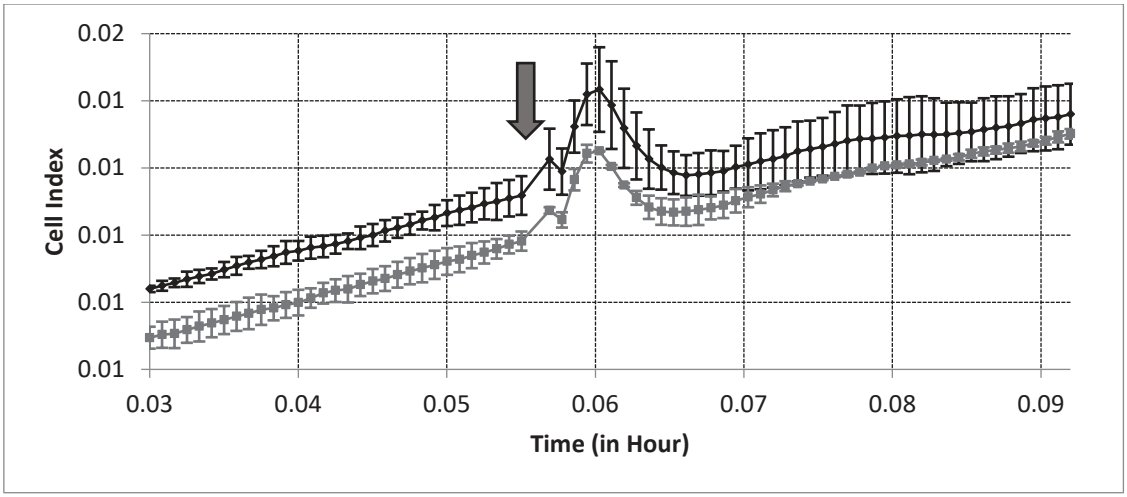
Figure 16. Expression d'A2A-eGFP après transduction de A2A-IRES-eGFP dans les cellules HOS (A) et les cellules MT-4 CCR5+ (B). *Contrôle avant transduction en pointillé, cellules transduites en ligne pleine.*

Les lignées HOS et MT4 transduites pour la construction A_{2A}-IRES-eGFP expriment donc bien l'eGFP, témoin de la transduction et de l'expression de cette construction.

b. Vérification de la présence de récepteurs A_{2A} fonctionnels à la surface des cellules HOS

Nous avons utilisé le système Xcelligence qui permet une mesure d'impédance des cellules en culture, et ceci en continu, en temps réel et sans marquage. Cette mesure donne des informations qualitatives globales sur l'état biologique cellulaire (adhésion, prolifération, morphologie) qui sont en relation avec la surface de contact de l'ensemble des cellules. Nous avons testé la réponse des cellules HOS au ligand CGS21680.

A.



B.

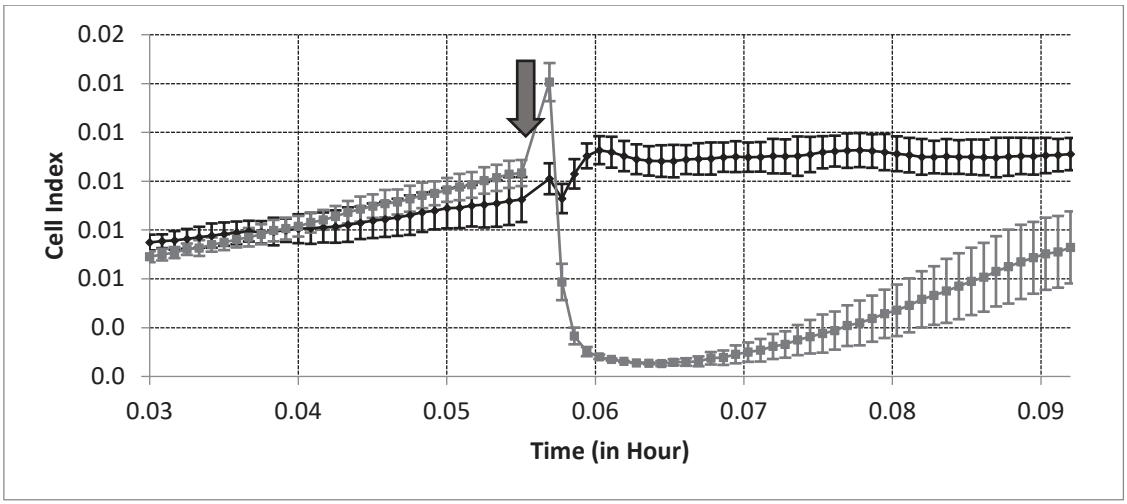


Figure 17. Vérification de la présence de récepteurs A2A fonctionnels.

En gris les cellules HOS transduites pour A_{2A}, en noir les cellules contrôles LacZ. A. Contrôle de la réaction des cellules HOS au milieu de culture (flèche). B. Effet de l'ajout d'un ligand d'A_{2A} sur les cellules HOS. Ajout de 100 µM de CGS21680 à 1100 µM (flèche). En ordonnée, l'index cellulaire traduit les variations d'impédance mesurées sur la grille sur laquelle les cellules sont déposées.

Les deux lignées HOS Lac Z et HOS A_{2A} réagissent de manière semblable à une stimulation par du milieu de culture (Fig. 17A), alors que seules les HOS A_{2A} répondent fortement à un agoniste spécifique d'A_{2A}, le CGS21680 (Fig. 17B). Le récepteur A_{2A} est donc exprimé et de manière fonctionnelle à la surface des HOS transduites avec la construction A_{2A}-IRES-GFP.

2. Effets d'A_{2A} sur l'infection par le VIH-1

Pour évaluer l'effet de l'expression d'A_{2A} lors d'une infection réplicative, des cellules HOS CCR5+ A_{2A} et HOS CCR5+ LacZ (en contrôle négatif) ont été infectées avec 10 ng/mL d'un virus réplicatif R5. L'infection a été mesurée par la concentration de p24 dans le surnageant de culture.

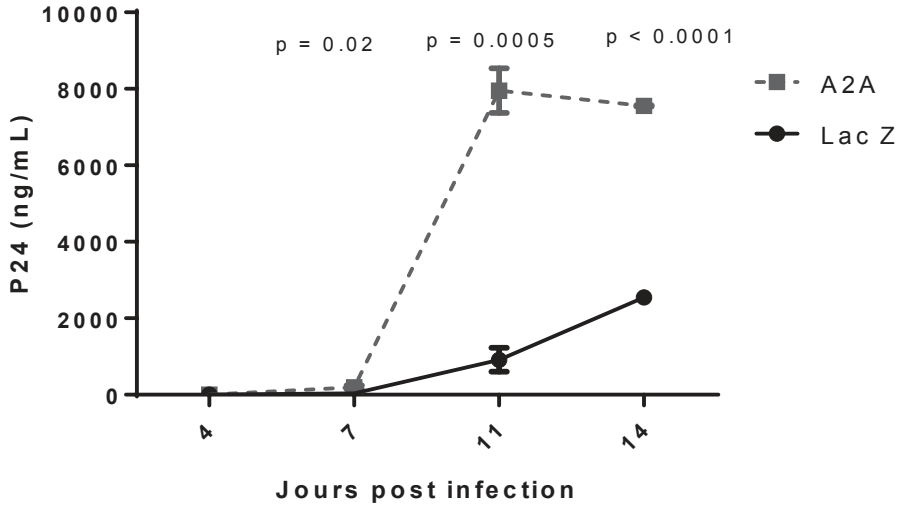


Figure 18. Infection par un virus réplicatif R5 de cellules HOS CCR5+ exprimant A2A ou LacZ

Nous avons réalisé la même expérience su les lignées MT-4 A_{2A}+ et MT-4 LacZ+. Là aussi, la présence d'A_{2A} a favorisé la réplication virale.

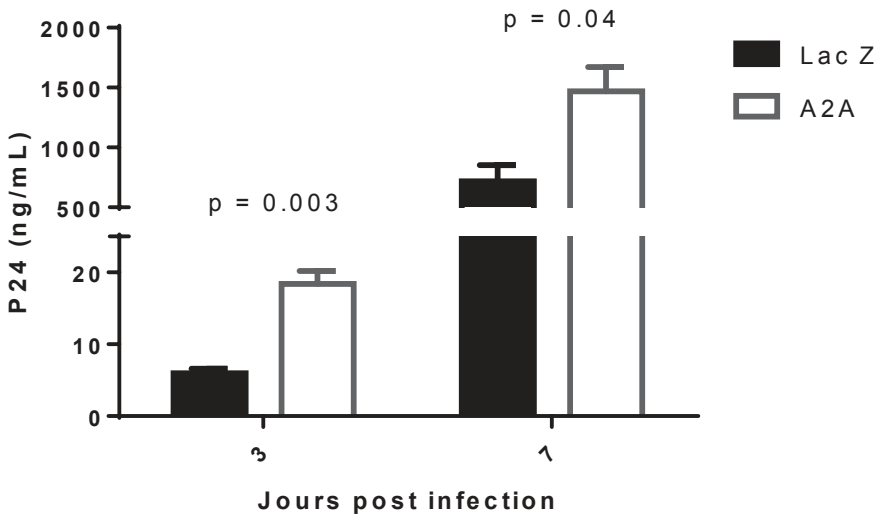


Figure 19. Infection par un virus réplicatif R5 de cellules MT-4 exprimant A2A ou LacZ.

L'expression d'A_{2A} favorise donc nettement la production virale sur plusieurs cycles de réplication, que ce soit avec des cellules HOS comme avec des cellules MT-4.

3. Influence d'A_{2A} sur les étapes du cycle viral

a. Mesure de l'entrée virale

Nous avons mesuré l'entrée virale grâce à des virions dont la protéine VPR est fusionnée avec l'enzyme β-Lactamase. Lors de la fusion de ce virus à la membrane, l'enzyme pénètre dans le cytosol et clive le substrat CCF2, modifiant ainsi la fluorescence de ce substrat de la Lactamase mesurée en cytométrie. L'entrée virale est évaluée par le pourcentage des cellules MT-4 CCR5+ devenues fluorescentes (Monel et al. 2012).

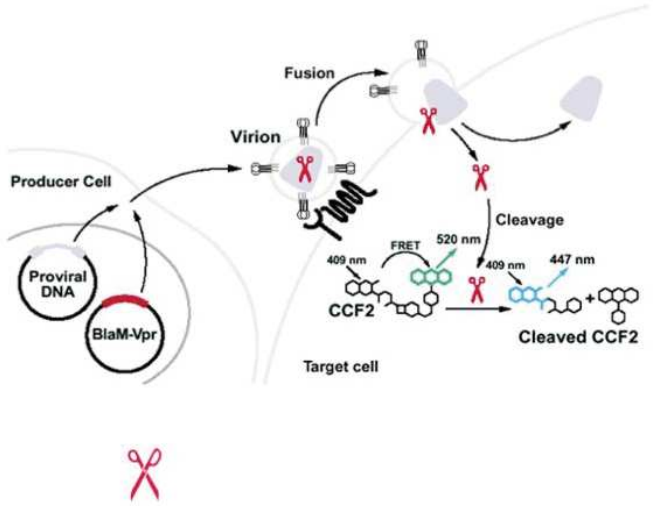


Figure 20. Schéma de la technique « VPR-βlam » permettant la mesure quantitative d'entrée des virions dans les cellules

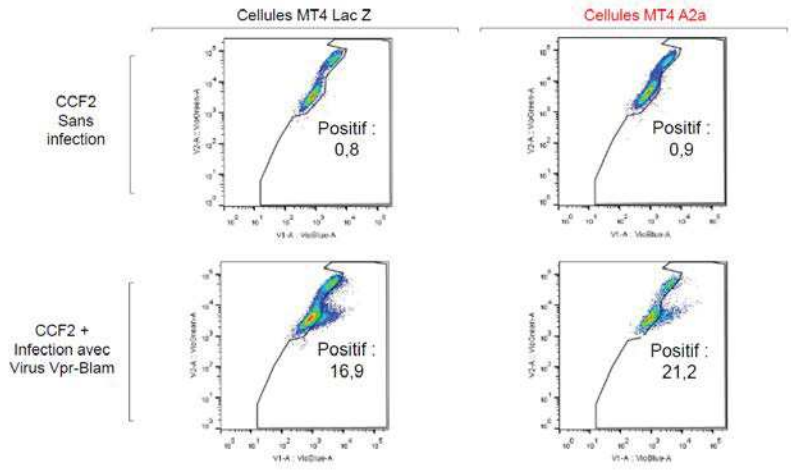


Figure 21. Images de cytométrie montrant le pourcentage de cellules positives pour le substrat CCF2 clivé après exposition à un virus VPR-βlam pseudotypé avec une enveloppe R5 (moyenne de triplicatas).

L'entrée virale du virus VPR-βlam pseudotypé avec une enveloppe R5 est donc augmentée de 25 % en présence d'A_{2A} par rapport au contrôle LacZ.

b. Effets d'A_{2A} sur les rétrotranscrits viraux précoces et tardifs

Nous avons voulu voir si l'expression d'A_{2A} influençait la rétrotranscription. Pour cela, les cellules HOS CCR5+ exprimant ou non A_{2A} ont été infectées avec 20 ng d'un virus non réplicatif de type R5 pendant 72 heures, puis après lavage et lyse, une qPCR permettant de quantifier les rétrotranscrits précoces ou tardifs a été réalisée (Lin et al. 2002) (Fig. 22).

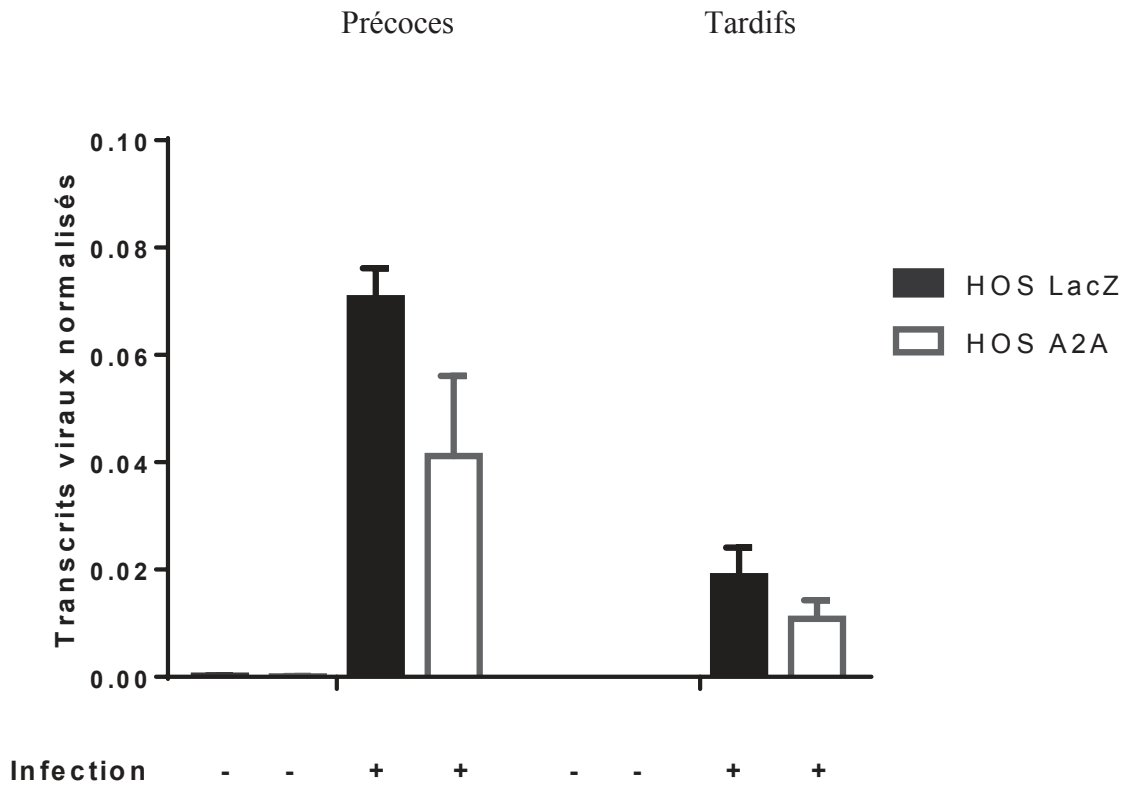


Figure 22. Effets de l'expression d'A2A sur la production de rétrotranscrits viraux précoces et tardifs. *La normalisation a été effectuée par rapport à CCR5.*

Si nous détectons une tendance de A_{2A} à diminuer la rétrotranscription virale dans les cellules HOS, la différence n'est pas significative, ni pour les rétrotranscrits précoces, ni pour les tardifs.

c. Effets d'A_{2A} sur la transcription à partir de la LTR virale

La présence d'A_{2A} pourrait activer des facteurs de transcription qui augmentent l'expression génique du VIH. Nous avons étudié l'effet de la stimulation d'A_{2A} par ses ligands sur l'activation du LTR. Pour cela, nous avons transduit avec la construction A_{2A}-eGFP ou avec LacZ, des cellules Hela préalablement transfectées de façon stable avec le LTR fusionné au gène de la luciférase (LTR-luc). Ces cellules ont été exposées 6h à une gamme de concentration de deux agonistes d'A_{2A}, le CGS21680 et le NECA, puis nous avons mesuré l'activité luciférase (Fig. 23).

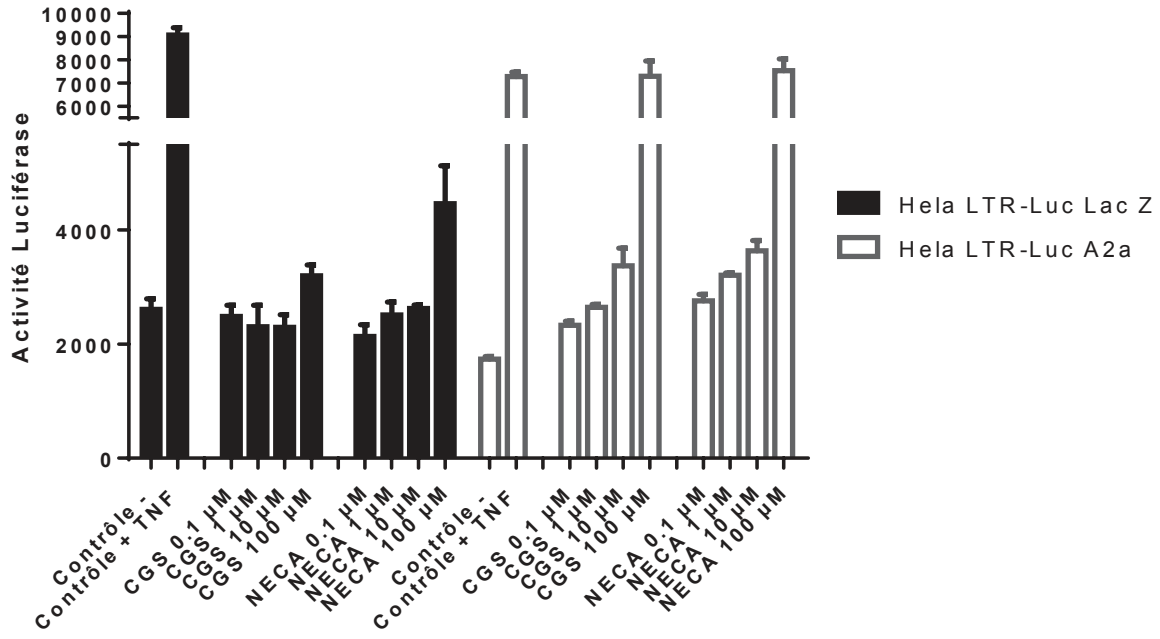
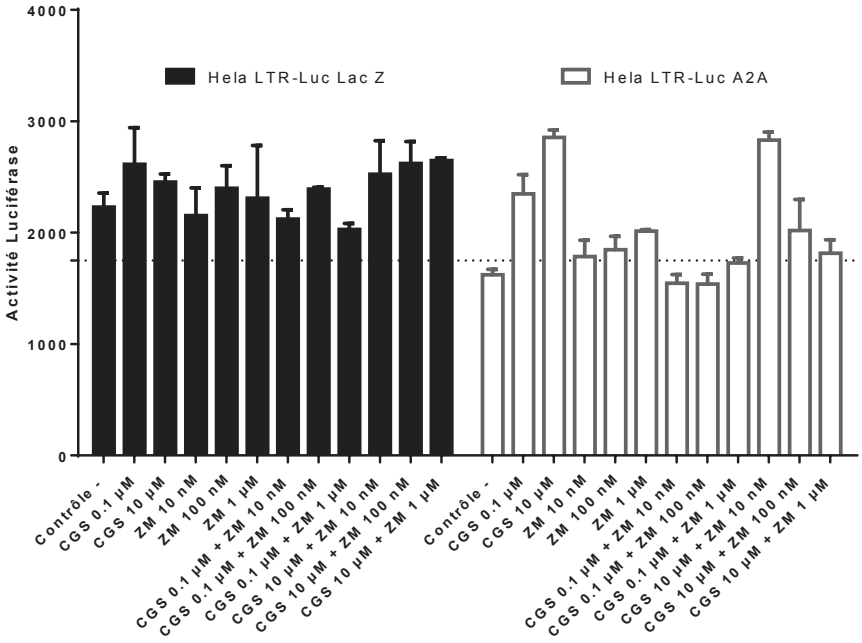


Figure 23. Effet des agonistes CGS21680 et NECA sur l'activation de la LTR.

L'expression d'A_{2A} sans ajout de ligands ne modifie pas l'activité luciférase des cellules, mais sa stimulation par les agonistes CGS21680 ou NECA ajouté dans le milieu de culture active de façon dose dépendante le promoteur assurant la transcription du VIH. Notons qu'on trouve un effet aspécifique à forte concentration (100 µM) de NECA et de CGS sur les lignées, donc un effet indépendant du récepteur A_{2A}.

Pour confirmer la spécificité d'action des agonistes d'A_{2A}, nous avons ensuite testé l'effet de l'antagoniste ZM241385, ajouté 1h avant les agonistes (Fig. 24).

A.



B.

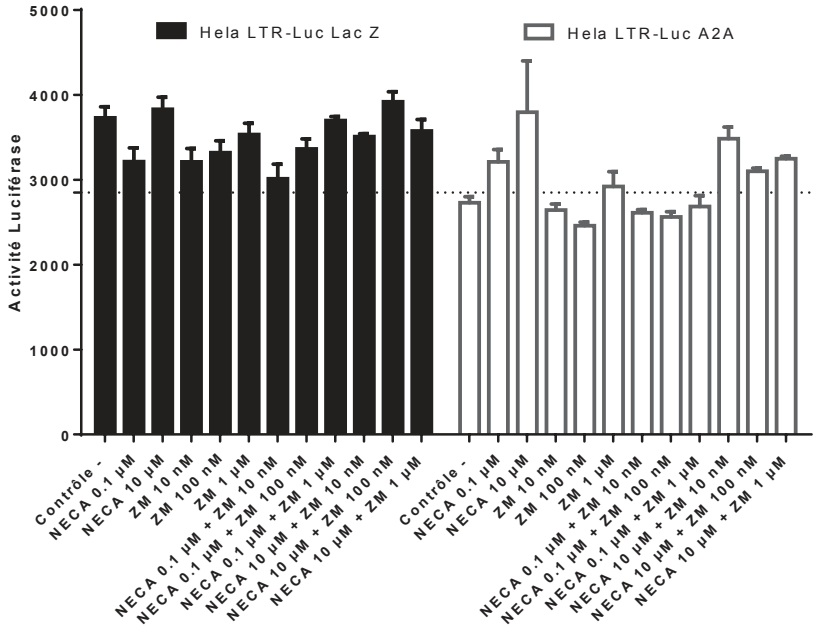


Figure 24. Effet de l'antagoniste d'A2A ZM 241385 sur l'activation de la LTR. Annulation des effets de agonistes par l'agoniste. A. Agoniste CGS21680 B. NECA

L'antagoniste ZM41385, spécifique de A_{2A}, n'a donc aucun effet *per se* sur l'expression de la LTR. Par contre, il annule ou diminue nettement l'effet des agonistes, ce qui confirme que l'action de ces derniers sur la LTR passe par la stimulation du récepteur A_{2A}. L'activation d'A_{2A}

par ses agonistes CGS21680 ou NECA entraîne donc une augmentation de l'activité des promoteurs LTR du VIH.

d. Absence d'effet d'A_{2A} sur les étapes post-traductionnelles

Afin de tester si l'expression d'A_{2A} pouvait favoriser une étape post-transcriptionnelle du cycle viral, nous avons comparé la concentration de la protéine p24 intracellulaire après lyse du culot à celle de la p24 présente dans le surnageant de culture des cellules infectées (Fig. 25). Pour cela nous avons infecté les cellules et mesuré la p24 (Fig. 18).

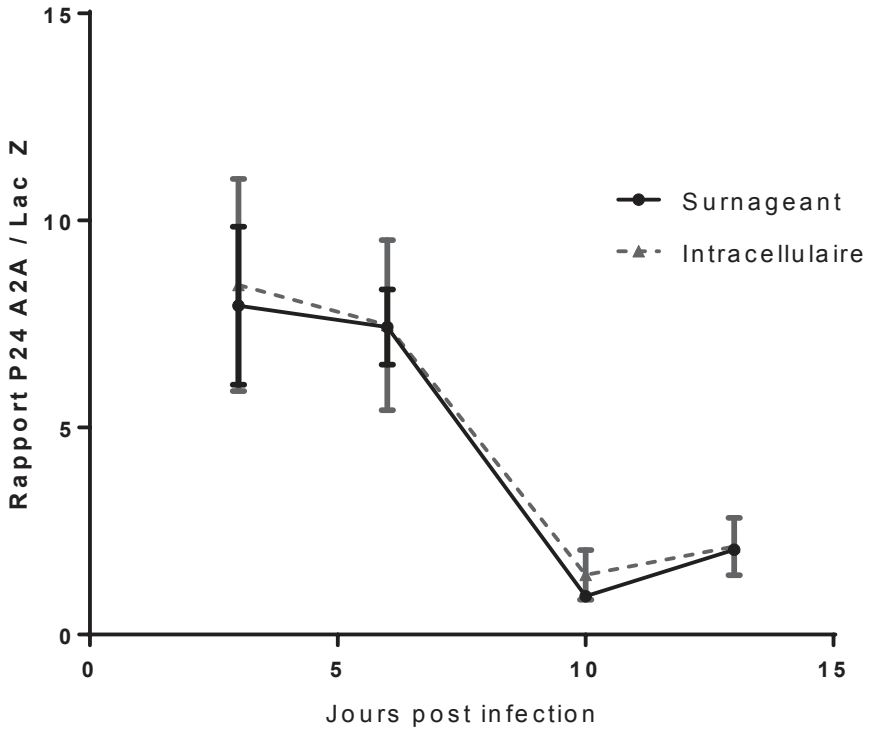


Figure 25. Absence d'effet d'A2A sur la répartition de la p24 produite par des cellules HOS infectées. *Le ratio entre p24 est calculé entre les HOS A_{2A} et les HOS LacZ, et représenté pour la p24 intracellulaire (ligne interrompue) ou la p24 présente dans le surnageant (ligne pleine).*

Les deux courbes se superposent, signifiant que l'effet d'A_{2A} sur la production de la protéine d'enveloppe est le même pour la p24 intracellulaire comme extracellulaire. L'expression d'A_{2A} ne modifie donc pas le relargage des particules virales.

D. Conclusion A_{2A}

In vitro, la présence du récepteur A_{2A} à la membrane de cellules HOS ou MT4 favorise la réplication du VIH-1, et ceci sans ajout de ligand dans le milieu de culture.

Comme EBI2, A_{2A} s'hétérodimérise avec CCR5. L'interaction entre A_{2A} et CCR5 à la surface cellulaire facilite en revanche légèrement l'entrée virale. Là aussi, en se dimérisant avec CCR5, A_{2A} pourrait modifier la conformation de CCR5 et cette fois-ci renforcer l'interaction entre ce corécepteur et l'enveloppe rétrovirale.

La stimulation d'A_{2A} par les agonistes NECA et CGS augmente la transcription virale à partir de la LTR, et ceci de manière spécifique, comme l'indique l'annulation de cet effet par l'ajout préalable d'un antagoniste. L'absence d'effet d'A_{2A} sur la transcription sans ajout de ligand pourrait être due au fait qu'A_{2A} tend à diminuer la prolifération cellulaire (Bours et al. 2006). Notons l'effet aspécifique des agonistes à haute concentration, qui pourrait s'expliquer par un effet direct des agonistes sur la membrane cellulaire (comme c'est décrit pour les oxystérols, voir partie EBI2) ou par la stimulation d'un autre récepteur qu'A_{2A}. Ici encore, il sera intéressant d'identifier la voie de signalisation déclenchée par la stimulation d'A_{2A} responsable de l'activation du LTR ainsi que les éventuels facteurs de transcription impliqués.

Pour expliquer le fait que l'ajout de ligand est nécessaire pour activer la transcription virale alors que la simple présence d'A_{2A} suffit à favoriser la production virale après infection *in vitro*, nous avons émis l'hypothèse que l'enveloppe virale (via la protéine gp120), en se liant à CCR5 qui interagit avec A_{2A}, pourrait être responsable de l'activation indirecte d'A_{2A}.

Par ailleurs, nous n'avons pas détecté d'effet d'A_{2A} sur la transcription inverse. A noter que nous avons utilisé les transcrits CCR5 pour normaliser les résultats de qPCR, ce qui pourrait fragiliser ce résultat dans la mesure où il est montré que la stimulation d'A_{2A} par le ligand adonis, un anticorps anti-A_{2A} à effet prolongé « agonist-like », diminue la production des protéines CCR5 et CXCR4 (By et al. 2010), effet qui pourrait compenser une éventuelle augmentation modérée des rétrotranscrits. Cependant nos expériences de mesure des rétrotranscrits ont été réalisées en absence de stimulation d'A_{2A} par un ligand, ce qui probablement invalide cette hypothèse.

Enfin, l'invariabilité du rapport p24 intracellulaire / p24 extracellulaires après infection indique une absence d'effet d'A_{2A} sur une étape postérieure à la traduction des protéines virales.

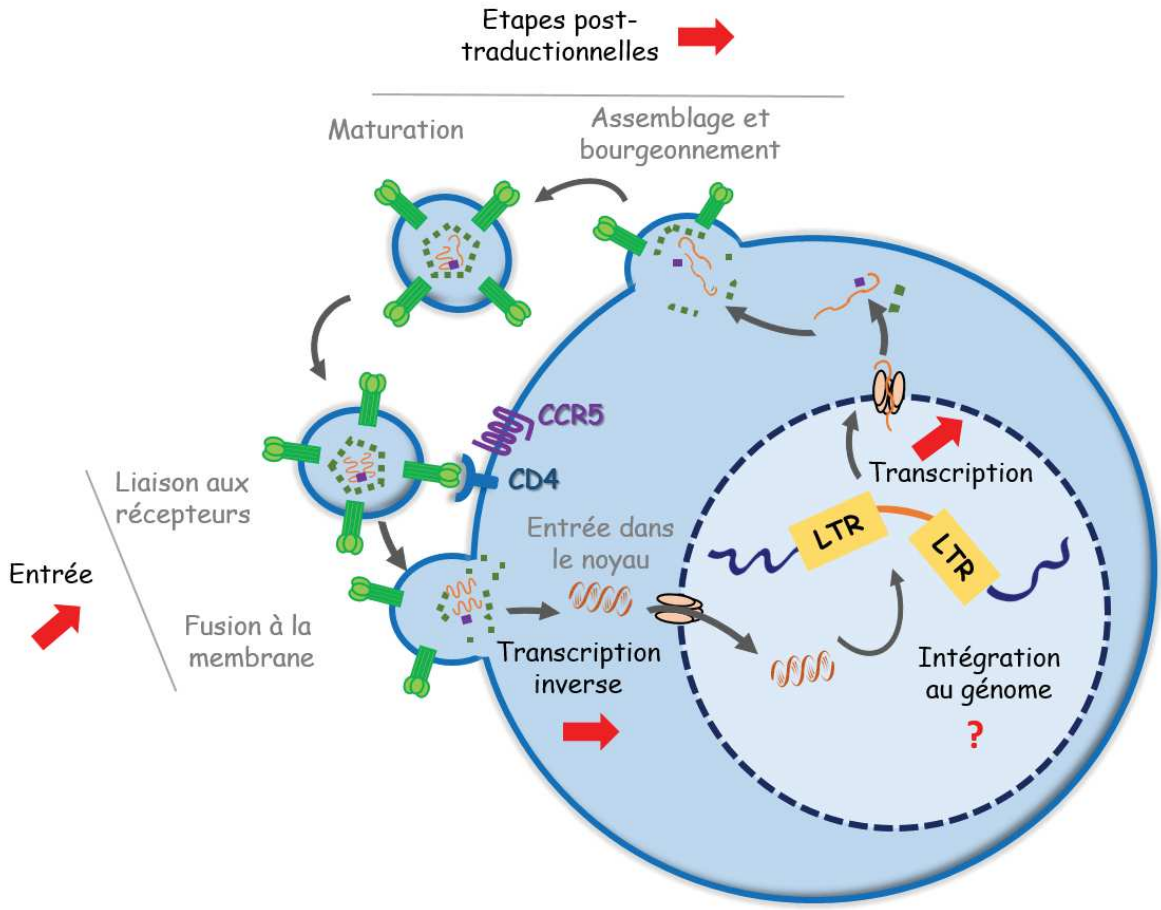


Figure 26. Schéma des étapes du cycle viral modifiées par A2A

Il va être important de déterminer par quel mécanisme A_{2A} favorise la transcription virale. Il est improbable que, contrairement à S1P1, cet effet ne passe pas par l'activation de NFκB. En effet, une expérience récente montre que l'activation du LTR n'est pas modifiée lorsque le site de liaison de NFκB du promoteur viral est inactivé par mutation. Par ailleurs, A_{2A} est connu pour diminuer l'expression de NFκB et NFAT (voir introduction).

Cette observation que des agonistes d'A_{2A} induisent l'activation du LTR ouvre aussi la voie à l'hypothèse que ces ligands pourraient avoir la capacité de lever la latence virale dans les cellules réservoirs. Il sera intéressant de tester l'effet des agonistes d'A_{2A} sur la latence virale, seuls et en combinaison avec d'autres LRA.

Par ailleurs si A_{2A} modifie la conformation et la fonctionnalité de CCR5, il se pourrait que réciproquement la fonctionnalité d'A_{2A} soit réduite suite à sa dimérisation avec CCR5, en particulier en présence de la gp120 rétrovirale. Cette hypothèse amène à la possibilité que chez les patients virémiques, les effets inhibiteurs d'A_{2A} sur le système immunitaire soient diminués ce qui pourrait contribuer à l'activation immunitaire observée chez ces patients.

IV. S1P1

A. S1P

La sphingosine-1-phosphate (S1P) est un sphingolipide, composant des membranes cellulaires eucaryotes, qui a de nombreuses fonctions biologiques en tant que molécule de signalisation. S1P est synthétisé à partir de la céramide puis de la sphingosine dans la plupart des tissus où il est présent à faible concentration, mais les érythrocytes et les cellules endothéliales qui sont considérés comme la source majeure de S1P plasmatique (L. Yang et al. 1999; Pappu et al. 2007; Venkataraman et al. 2008) assurent une concentration élevée de S1P dans le sang et la lymphe (30 nM à 1 μM) (L. Yang et al. 1999; Rosen and Goetzl 2005; Pappu et al. 2007; Venkataraman et al. 2008). Sa concentration intracellulaire est étroitement régulée par diverses enzymes et dépend d'un équilibre entre sa synthèse par des sphingosine kinases, sa reconversion en sphingosine par des S1P phosphatases et sa dégradation par la S1P lyase, cette dernière étant en partie responsable d'une faible concentration dans les tissus (S. M. Mandala 2001; Spiegel and Milstien 2011). A noter que S1P peut être produit extracellulairement à partir de la sphingosine et de l'ATP par la SphK1 relarguée constitutivement par l'endothélium vasculaire (Ancellin et al. 2002). Son transport à travers les membranes dépend de transporteurs de la famille des ATP-Binding Cassette (ABC) (N. Kobayashi et al. 2006; Mitra et al. 2006). Durant l'inflammation, les plaquettes et les mastocytes contribuent à une synthèse accrue de S1P (Sano et al. 2002; Brinkmann 2007). À l'extérieur des cellules, S1P est lié à l'albumine ou l'ApoM (Christoffersen et al. 2011).

S1P agit de manière paracrine ou autocrine, comme second messager intracellulaire ou comme ligand de cinq récepteurs couplés aux protéines G (RCPG) de classe A, les récepteurs S1P1-5 ou S1PR, pour lesquels il a une affinité de l'ordre du nanomolaire (Spiegel and Milstien 2011; Rosen and Goetzl 2005). Ces récepteurs peuvent être exprimés simultanément dans les cellules et sont couplés à différentes protéines G (Gi/o, Gq ou G12/13). En tant que second messager, S1P contrôle notamment l'homéostasie calcique (Ghosh, Bian, and Gill 1994; Spiegel and Milstien 2011) et peut agir directement dans le noyau où il influence l'équilibre dynamique de l'acétylation des histones et ainsi la transcription de certains gènes cibles (Maceyka et al. 2012; Pyne, Bittman, and Pyne 2011). S1P régule de nombreuses fonctions comme l'angiogenèse et le remodelage du cytosquelette. Contrairement à ses précurseurs, la céramide et la sphingosine, qui sont pro-apoptotiques, S1P induit la prolifération et la survie cellulaire et est un médiateur de l'adhésion et de la migration cellulaire (Hla 2003; Spiegel and Milstien 2011). Des gradients locaux de S1P influencent les migrations de plusieurs types cellulaires.

B. S1P1

Le récepteur 1 de la sphingosine-1-phosphate (S1P1), également nommé Endothelial differentiation gene 1 (EDG1), est exprimé dans les cellules endothéliales et la plupart des cellules du système immunitaire incluant les lymphocytes B et T, les natural killer (NK), les cellules dendritiques, les macrophages et les neutrophiles (G. Zhang et al. 1999; Y. Liu et al. 2000; Matloubian et al. 2004; Choi, Lee, and Chun 2008). L'ARNm de S1P1 est trouvé dans de nombreux organes comme le cerveau, les poumons, la rate, les reins, le foie et le coeur (Kluk and Hla 2002; Brinkmann 2007).

L'obtention de la structure cristallographique du récepteur S1P1 lié à un antagoniste (Hanson et al. 2012) est une aide précieuse pour le développement de nouveaux ligands spécifiques de S1P1. Ce récepteur est associé exclusivement à la protéine Gαi. Sa stimulation accroît l'activité de la phospholipase C qui augmente le calcium intracellulaire libre, d'ERK qui induit la prolifération, de la phosphatidylinositol-3-kinase (PI3K) et de la petite GTPase Rac qui permettent la migration et la vasodilatation, et de la protéine kinase Akt qui entraine la survie. Par ailleurs, la stimulation de S1P1 inhibe l'adénylate cyclase, ce qui entraine la diminution de l'AMPc (Okamoto et al. 1998; Windh et al. 1999; Kluk and Hla 2002). Les embryons mutés pour S1P1 développent un réseau vasculaire mais meurent *in utero* par défaut de cellules musculaires lisses et de péricytes.

L'inactivation ciblée de S1P1 au cours du développement chez la souris grâce au système *Cre-Lox* a permis de montrer le rôle de S1P1 dans l'angiogenèse, dans l'intégrité de la barrière endothéliale et dans la migration des cellules T et B (C. H. Liu et al. 1999; Y. Liu et al. 2000; J. G. Garcia et al. 2001; Singleton et al. 2005).

De même que les thymocytes ont besoin de sortir du thymus pour exercer leurs fonctions (Cyster 2005; Cyster and Schwab 2012; Brewer 2013), la sortie des lymphocytes des organes lymphoïdes est essentielle pour assurer l'immuno-surveillance des tissus. L'expression transitoire de S1P1 sur les cellules T et B du thymus et des ganglions lymphatiques permet leur sortie dans la circulation sanguine et lymphatique où la concentration en S1P est plus élevée. Il s'en suivra l'internalisation du récepteur en réponse à sa liaison avec son ligand. On observe d'ailleurs que l'administration de S1P, en diminuant l'expression membranaire de S1P1, entraîne le maintien des lymphocytes dans les organes lymphoïdes secondaires.

S1P1 est régulé par le facteur de transcription Krüppel-like factor 2 (KLF2) qui active sa transcription dans les cellules T (Bai et al. 2007). Lors de la stimulation des cellules T, le

marqueur d'activation CD69 (cluster of differentiation 69) forme des complexes avec S1P1 entrainant alors son internalisation, ce qui empêche la sortie des lymphocytes des ganglions lymphatiques. Aussi, lors de la maturation des thymocytes, il y a tout d'abord expression de CD69 permettant la sélection des thymocytes puis, la surexpression de KLF2 permet l'expression de S1P1 entrainant la réduction d'expression de CD69 membranaire et la sortie des T naïfs du thymus (Spiegel and Milstien 2011). S1P1 joue également un rôle sur la barrière endothéliale qui peut avoir des conséquences sur la migration des lymphocytes empêchant leur sortie des organes lymphoïdes secondaires par la formation de jonctions adhérentes (J. G. Garcia et al. 2001).

S1P1 empêche le développement des cellules Treg par la voie Akt et affecte leur migration vers et hors la périphérie du thymus en contrecarrant les signaux de rétention de CCR7, de façon similaire au mécanisme de sortie des cellules T effectrices des nœuds lymphatiques (Ishimaru et al. 2012). Chez l'homme le traitement par le FTY720 entraine une augmentation des cellules Treg mais une diminution des cellules T mémoires centrales (Serpero et al. 2013).

L'expression variable de S1P1 à la surface des cellules B leur permet de se déplacer au sein des organes lymphoïdes secondaires avec d'autres récepteurs comme CCR7, CXCR4, CXCR5, EBI2. Une forte concentration de S1P dans la zone marginale pousse les cellules vers une faible concentration dans les follicules, permettant la réexpression du récepteur à ce niveau (Rivera, Proia, and Olivera 2008).

S1P1 participe à la migration des cellules dendritiques matures vers les nœuds lymphatiques (Rathinasamy et al. 2010). S1P1 est également exprimé dans les monocytes et macrophages, régulant leur migration et leur activation. Chez la souris le traitement au FTY720 est responsable d'une diminution du nombre de monocytes circulant (Lewis et al. 2013).

On trouve une expression de S1P1 dans les cellules T CD4 isolées chez des patients souffrant d'arthrite rhumatoïde. S1P1 augmente l'expression induite par le TNFα du facteur nucléaire κB (NFκB). Dans les modèles de polyarthrite rhumatoïde, des antagonistes spécifiques de S1P1 préviennent ou améliorent la maladie en régulant à la hausse l'expression de CD69 dans les lymphocytes, bloquant leur sortie du thymus (Takeshita et al. 2012).

C. Agonistes et antagonistes de S1P1

Il existe de nombreux agonistes et antagonistes de S1P1 (Urbano et al. 2013; Guerrero, Urbano, and Roberts 2016). Dans le travail qui suit, nous avons utilisé le SEW2871 et le FTY720.

Le SEW2871 est un agoniste spécifique de S1P1 qui active les voies ERK1/2, Akt et Rac, induisant l'internalisation du récepteur avec une demi-vie courte (Jo et al. 2005). Il est à noter qu'après internalisation, les récepteurs peu ubiquitinylés, comme c'est le cas du récepteur S1P1 stimulé par S1P ou SEW2871, recyclent du compartiment endosomal tardif à la membrane plasmique, alors que les récepteurs très ubiquitinylés sont conduits aux lysosomes puis dégradés, comme dans le cas de S1P1 stimulé par FTY720-P.

Le FTY720, ou Fingolimod, est une molécule immunomodulatrice dérivée de la myriocine. Il lui est préféré pour ses effets moins toxiques et plus immunosuppresseurs, et a été approuvé par la FDA en 2010 pour son utilisation contre la sclérose en plaque (SEP) (Skoura and Hla 2009; Chun and Brinkmann 2011). Sa forme phosphorylée FTY720-P a une forte affinité pour S1P1 (Kd = 0,3 nM), mais elle se lie également aux récepteurs S1P3-5. A noter que, contrairement à S1P, FTY720-P ne stimule que la protéine Gi et antagonise l'effet de S1P sur la voie Gq d'autres S1PR (Sensken et al. 2008). De même, alors que S1P3 est internalisé par S1P, il ne l'est pas par FTY720-P (Sensken et al. 2008). A noter qu'un modulateur spécifique de S1P1, le ponesimod, est actuellement en phase III dans le traitement de la SEP.

FTY720-P agit initialement en tant qu'agoniste de S1P1 pour augmenter les fonctions de la barrière endothéliale, réduire la transmigration des lymphocytes et ainsi induire une lymphopénie (Singer et al. 2005). Cependant il agit également comme antagoniste fonctionnel en induisant l'endocytose et la dégradation du récepteur (Brinkmann et al. 2002), ceci en provoquant sa phosphorylation et sa polyubiquitinylation en C terminal, son endocytose via la cascade dépendant de la β-arrestine, puis sa dégradation par protéolyse (Gräler and Goetzl 2004; Matloubian et al. 2004; Oo et al. 2007, 2011). Il entraîne alors une lymphopénie et une séquestration des cellules T dans les organes lymphoïdes secondaires (S. Mandala et al. 2002), empêchant ainsi l'infiltration intracérébrale des lymphocytes T auto-réactifs et la démyélinisation (Chiba et al. 1998; S. Mandala et al. 2002). Par son effet immunosuppresseur, FTY720 est également utilisé contre le rejet lors des transplantations d'organes et est en essai pour le traitement d'allergies et de plusieurs autres maladies inflammatoires (Brinkmann 2007)

et des études précliniques suggèrent l'usage du FTY720 dans diverses maladies du système nerveux central (O'Sullivan, Velasco-Estevez, and Dev 2017).

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Reversing HIV latency via sphingosine-1-phosphate receptor 1 signaling

AQ1 Charline Duquenne[a,*], Sandrine Gimenez[a,*], Adeline Guigues[a,*], Benjamin Viala[a], Caroline Boulouis[a], Clément Mettling[a], Damien Maurel[b], Noëlie Campos[c], Etienne Doumazane[d], Laetitia Comps-Agrar[d], Jamal Tazi[e], Laurent Prézeau[d], Pierre Corbeau[a,f,g,*] and Vincent François[a,*]

Objective: In this study, we looked for a new family of latency reversing agents.

AQ4 **Design:** We searched for G-protein-coupled receptors (GPCR) coexpressed with CCR5 in primary CD4$^+$ T cells that activate infected cells and boost HIV production.

AQ5 **Methods:** GPCR coexpression was unveiled by RT-PCR. We used FRET to analyze the dimerization with CCR5 of the expressed GPCR. Viral entry was measured by flow cytometry, reverse transcription by quantitative PCR, NFκB translocation by immunofluorescence, LTR activation using a gene reporter assay and viral production by p24 quantification.

Results: Gαi-coupled sphingosine-1-phophate receptor 1 (S1P1) is highly coexpressed with CCR5 on primary CD4$^+$ T cells and dimerizes with it. The presence of S1P1 had major effects neither on viral entry nor on reverse transcription. Yet, S1P1 signaling induced NFκB activation, boosting the expression of the HIV LTR. Consequently, in culture medium containing sphingosine-1-phophate, the presence of S1P1 enhanced the replication of a CCR5−, but also of a CXCR4-using HIV-1 strain. The S1P1 ligand FTY720, a drug used in multiple sclerosis treatment, inhibited HIV-1 productive infection of monocyte-derived dendritic cells and of SCID mice engrafted with human peripheral blood mononuclear cells. Conversely, S1P1 agonists were able to force latently infected peripheral blood mononuclear cells and lymph node cells to produce virions *in vitro*.

Conclusion: Altogether these data indicate that the presence of S1P1 facilitates HIV-1 replicative cycle by boosting viral genome transcription, S1P1 antagonists have anti-HIV effects and S1P1 agonists are HIV latency reversing agents.

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AQ2 [a]Institut de Génétique Humaine, CNRS-Université de Montpellier UMR9002, [b]ARPEGE Pharmacology Screening Interactome platform facility, Institut de Génomique Fonctionnelle, [c]Laboratoire coopératif SPLICOS SAS, IGMM-CNRS-UMR5535, [d]Institut de Génomique Fonctionnelle, CNRS-UMR5203, INSERM-U661, Université Montpellier, UMR5203, Montpellier Cedex 5, [e]Institut de Génétique Moléculaire de Montpellier, CNRS-UMR 5535, Montpellier, [f]Université de Montpellier, Montpellier Cedex 2, and [g]Centre Hospitalier Universitaire Caremeau, UF d'Immunologie, place du Pr Debré, Nîmes Cedex 9, France.

AQ3 Correspondence to Pierre Corbeau, MD, PhD, Institute for Human Genetics, CNRS UPR1142, 141 rue de la Cardonille, 34396 Montpellier Cedex 5, France.

Tel: +33 434 359 932; fax: +33 434 359 901; e-mail: pcorbeau@igh.cnrs.fr

[*] Charline Duquenne, Sandrine Gimenez, Adeline Guigues, Pierre Corbeau and Vincent François contributed equally to the article.

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Introduction

HAART do not eradicate HIV because of the ability of this retrovirus to integrate its genome in a quiescent form into target cells with a long lifespan. As long as they are not activated, these so-called reservoir cells do not express viral components and are therefore eliminated neither by HIV cytopathogenicity, nor by the antiviral immune response. Hence, latency reversing agents (LRA), able to force reservoir cells to produce virions, and thereby to induce their destruction, have been looked for. Various LRA candidates have been tested, but the ability of these LRA to reactivate HIV genome expression is limited. Some latent proviruses seem to even be refractory to LRA. Moreover, none of these LRA is so far specific for reservoir cells, and many of them present with dose-dependent toxicity. It is therefore of importance to identify new classes of LRA.

After binding to $CD4^+$, the main HIV-1 strains (R5) utilize the chemokine receptor CCR5 as a coreceptor to enter and activate target cells. CCR5 belongs to the large family of G protein-coupled receptors (GPCR). GPCR may form homo-oligomers as well as hetero-oligomers [1]. Hetero-oligomerization can modulate receptor cell surface density, ligand specificity and/or affinity, coupling to G protein or other intracellular mediators and/or the nature and intensity of the signaling pathway [2–7]. GPCR can also influence each other by heterodesensitization. As we and others have shown that CCR5 signaling boosts HIV replication [8,9], we hypothesized that other GPCR coexpressed with CCR5 at the $CD4^+$ T-cell surface could deliver signals able to reactivate HIV gene expression in reservoir cells. This could happen via at least three potential mechanisms. If such a GPCR dimerized with CCR5, ligands for this GPCR might trigger CCR5 signaling and thereby HIV DNA transcription. Reciprocally, the fact that CCR5 dimerized with this GPCR might modify the signaling of this GPCR resulting in HIV LTR activation. Finally, even if such a GPCR did not dimerize with CCR5, ligands for this GPCR might trigger a signaling pathway able to activate HIV LTR.

To test this hyporhesis, we screened the GPCR family for receptors expressed in $CCR5^+CD4^+$ T cells and able to interfere with HIV infection. Our approach yielded sphingosine-1-phophate (S1P) receptor 1 (S1P1) as a potential candidate. S1P1 is expressed in most immune cell types including T and B lymphocytes, NK cells, dendritic cells, macrophages and neutrophils [10,11]. It plays a critical role in T-cell and B-cell egress from thymus and lymph nodes toward high S1P concentrations present in body fluid compartments [10–16]. Investigating the influence of S1P1 triggering on HIV infection, we observed that S1P1 agonists are LRA.

Methods

Cells

$CD4^+CCR5^+$ T cells were sorted from peripheral blood mononuclear cells (PBMC) with specific antibody-coupled magnetic beads (Dynabeads Pan Mouse IgG; Invitrogen) by positive selection. To obtain dendritic cells, monocytes were first isolated from PBMC with CD14 antibody-coated magnetic beads (Milteny), and cultivated in supplemented RPMI 1640 for 7 days in presence of 10 ng/ml GM-CSF and 50 ng/ml IL4 (Immunotools). Macrophages were obtained by in-vitro differentiation with GM-CSF of PBMC isolated by low-density gradient centrifugation. AQ6

Quantitative multi-RT-PCR

RNA was extracted with QIAmp RNA blood (Qiagen). cDNA was synthetized with the cDNA archive kit (Applied Biosystems). DNA from three donors were mixed and 750 ng of this mixture was loaded on a 384-well microfluidic cards preloaded with primers for 361 different GPCR and 14 housekeeping genes as positive control (TaqMan Low Density Arrays from Applied Biosystems). qPCR was realized on an ABI Prism 7900HT Sequence Detection System.

Time-resolved FRET

The 293T cells were cotransfected with plasmids expressing SNAP-tagged or CLIP-tagged receptors, plated at 10^5 cells/well in poly-ornithine-coated 96-well plates. After 30 h, SNAP and CLIP tags were labeled with the fluorophores BC-Lumi4-Tb (1 μmol/l) and BG-Green (0.3 μmol/l) in TagLite (TL) buffer (Cisbio Bioassays) for 2 h at 37 °C. The emission signal from the BC-Lumi4-Tb and the Green fluorophore were recorded at 620 and 520 nm, respectively, after excitation at 337 and 485 nm on a time-resolved fluorimeter (PHERAstar FS, BMG Labtechnologies). In the same wells, time-resolved FRET (tr-FRET) signal was measured at 520 nm between 60 and 400 μs after laser excitation at 337 nm.

Flow cytometry

Cells (2×10^5) were incubated with PE-Cy5-conjugated anti-CCR5 mAb 2D7 (Pharmingen), or antihuman S1P1 mAb 21813 (R&D Systems) for 1 h on ice at 10 μg/ml, followed by a 1 : 100 dilution of FITC-conjugated $F(ab')_2$ fragment goat antimouse IgG (H+L, Beckman Coulter) after washing, or with an isotype control. Cells were then washed, fixed in CellFIX (Becton Dickinson) and analyzed on a FACScalibur (Becton Dickinson).

Transduction

HIV transfer vectors that express *CCR5*, *CXCR4*, *LacZ* and *S1P1* (pWPXL-S1P1) were produced as previously described [17]. The pWPXL-S1P1 plasmid was obtained by cloning a BamH1-Spe1 fragment after PCR amplification of an S1P1 cDNA (clone EDG0100000

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from Missouri cDNA Resource Center) into the pWPXL lentiviral vector.

Infection assays with replicative virus

Human osteosarcoma (HOS) cells were exposed to 10 ng/ml of p24 equivalent of the R5 strain Ad8 or of the X4 strain NL4.3, washed, and cultured in supplemented DMEM medium. MT4 cells were exposed to 10 ng/ml of p24 equivalent of the R5 strain Ad8 or to 40 ng/ml of p24 equivalent of the X4 strain NL4.3, washed, and cultured in supplemented RPMI-1640 medium. Dendritic cells were preincubated with Vpx-containing virus-like particles to neutralize SAMHD1 [18]. Dendritic cells were then exposed or not to 100 nmol/l of FTY720-P 1 day prior infection, plated at 10^5 cells/ml, infected overnight with 5 ng/ml of p24 equivalent of the R5 strain Ad8, washed and cultured in supplemented RPMI-1640 medium. FTY720-P was added at each passage. Cell number was adjusted twice a week, and HIV p24 production was measured in cell supernatant using Innotest HIV Antigen mAb kit (Fujirebio, Ingen).

In-vitro model of HIV latency

PBMC from healthy patients (2×10^6 cells/ml) were exposed to 5 ng/ml of p24 equivalent of the R5 strain Ad8, washed five times and cultured in X-VIVO 15 (Lonza) medium supplemented with 100 IU/ml of IL-2. At the indicated time and further on, ligands were added to the culture medium. Cells extracted from the lymph node of an organ donor (2×10^6 cells/ml) were exposed to 14 ng/ml of p24 equivalent of the primary R5 strain MP578, washed five times and cultured in X-VIVO 15 medium supplemented with 10 IU/ml of IL-2. At the indicated time and further on, S1P was added to the culture medium.

Generation of hu-PBL-SCID mice

SCID mice are from cb17/Icr-Prkdc^{scid}/Crl genotype. Animals were engrafted by intraperitoneal injection of 30×10^6 PBMC obtained from a healthy donor. Mice with human immunoglobulin plasma concentration above 100 μg/ml were infected with 1000 TCID50 of JR-CSF HIV-1 virus in 100 μl. Daily force-feeding with 100 μl FTY720 (Cayman) at 60 μg/ml dissolved in distilled water or vehicle was begun 1 day before infection and continued for 12 days. Viral load was measured by qPCR with mpliPrep/COBAS TaqMan HIV-1 test (Roche).

HIV entry assay

We used the previously described Vpr–β-lactamase (Vpr–Blam) assay [19] to measure the efficiency of HIV core penetration into the cytosol of target cells. Virus stocks were produced by cotransfecting 3 millions of 293T cells with 15 μg of the HIV proviral DNA pNL4.3 env-luc+, an R5 or X4 envelope plasmid (pCMV-Ad8 or pVB34, respectively, 6 μg), a packaging plasmid (pAX2, 2 μg), and the plasmid encoding the *Vpr* gene fused to the *β-lactamase* gene (pMM310). Virus preparations were concentrated by ultracentrifugation on 4% sucrose (1 h 30 min, 26 000 rpm, 4 °C). MT4 cells were exposed for 3.5 h to 500 ng of p24 of R5 or to 1 μg of p24 of X4 Vpr–Blam virus. Cells were then washed and loaded with the CCF2-AM LiveBLAzer FRET-B/G Loading Kit (Life Technologies) in the presence of 15 mmol/l Probenecid (Sigma). Cells were incubated for 1 h at room temperature, washed and fixed. CCF2-AM and cleaved CCF2-AM fluorescence (excitation at 405 nm, emission at 520 nm and 447 nmol/l, respectively) was measured on a MACSQuant analyzer 10 (Mylteni). AQ7

Real-time PCR detection of HIV transcripts

To produce replication-defective HIV virions, 293T cells were cotransfected with the pNL4.3-env$^-$-luc and pCMV-Ad8-Env plasmids. HOS cells were exposed to 70 ng of p24 equivalent of defective virions and lysed 24 h postinfection. DNA from early and late transcripts was amplified as described [17].

Infection assays with replicative NFKB+/− and NFAT+/− viruses

NFKB+ and NFKB− viruses were obtained from plasmid pILIC, a derivative of pNL4.3 with only one LTR or NFKB− pILIC in which both NFKB binding sites present in the LTR promoter are mutated (GGGactttccgctgGGG is replaced by CTCactttccgctg CTC). NFAT+ and NFAT− viruses were obtained from pNL4−3 mutated at the NFAT binding site [20]. MT4 cells were infected in triplicate with 20 ng/ml of these X4 viruses.

LTR activation assays

HeLa–LTR–Luc cells [21] were exposed for 10 h to 100 nmol/l of FTY720-P, to SEW2871 at the indicated concentrations, or to 30 μg/ml of the anti-S1P1 mAb 21813 (R&D Systems) and lysed. Luciferase activity was measured in cell extracts using a commercial assay (Promega). Alternatively, HeLa cells were transiently transfected by using lipofectamine Plus (Invitrogen) with the LTR-luc reporter plasmid pL274 [22], either wild-type or mutated in the NFκB binding sites (pL274 with the same mutations as in the NFKB− pILIC). FTY720-P (100 nmol/l) or 10 ng/ml TNFα were added 24 h later for 6 h and luciferase activity was measured. AQ8

Immunofluorescence assay

HeLa cells were exposed for 2 h to 100 nmol/l FTY720-P or to 10 ng/ml TNFα, labeled for 45 min with an NFκB p65 antibody (F-6, Santa Cruz) followed by a secondary fluorescent antimouse antibody, and with Dapi (5 μg/ml).

Statistics

Values are expressed as the mean ± SEM. Differences were analyzed with unpaired Student's *t* test.

Study approval

Animal studies were approved by the local Animal Care Committee. Lymph node extractions from organ donors were approved by the Agence de la Biomédecine.

Results

Identification of the *GPCR* genes coexpressed with CCR5 in $CD4^+$ T cells and macrophages

We looked for GPCR expression in $CD4^+CCR5^+$ T cells sorted from the PBMC of healthy donors. Out of 361 *GPCR* genes, we detected the expression of 250 of them in $CD4^+CCR5^+$ T lymphocytes by multi-RT-PCR. In particular, S1P1 mRNA was 33-fold more abundant in these cells than CCR5 mRNA.

Sphingosine-1-phosphate receptor 1 forms heteromers with CCR5 in the outer cell membrane

We then tested the ability of S1P1 to oligomerize with CCR5 by tr-FRET. Both receptors were fused at their N-terminal extracellular domain with the genetically encoded tags SNAP and CLIP, that allow specific labeling with nonpermeant fluorophores. SNAP-S1P1 and CLIP-CCR5 encoding plasmids were cotransfected in 293T cells, and the tr-FRET signal between cell surface tagged-receptors was measured [23]. We observed a superposition of the saturation curves with the two lowest CCR5 amounts (Fig. 1a), indicating that under these conditions the interactions between CCR5 and S1P1 are specific [24]. The beta-2 adrenergic receptor, known not to interact with CCR5 [25], gave a nonsaturating tr-FRET signal [26] with CLIP-CCR5 (Fig. 1b). As a positive control, CLIP-CCR5 was found to form homomers with SNAP-CCR5 as previously shown [27]. Conversely, no interaction was found between S1P1 and the other HIV coreceptor, CXCR4, whereas CXCR4 formed homomers (Fig. 1c).

Sphingosine-1-phosphate receptor 1 expression increases HIV replication

To analyze the consequences of S1P1 coexpression on HIV infection, we transduced HOS cells expressing $CD4^+$ and CCR5 with either an *S1P1* or a negative control *lacZ* transgene. The two cell lines thus obtained displayed the same cell surface CCR5 densities (Fig. 2a). We also checked that the former, but not the latter cell line expressed S1P1 (Fig. 2b). We then infected both

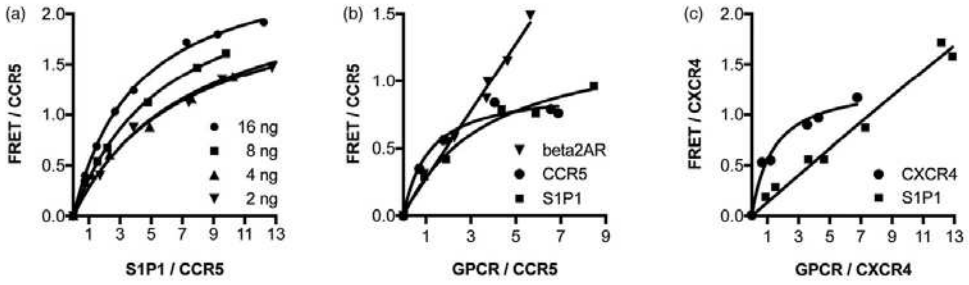

Fig. 1. CCR5 forms heteromers with sphingosine-1-phophate receptor 1. (a) Time-resolved FRET saturation curve of CLIP-CCR5 with SNAP-sphingosine-1-phophate receptor 1. Cells were cotransfected with a constant amount of CLIP-CCR5 plasmid (2 ng, ▲; 4 ng, ▼; 8 ng, ■ or 16 ng, •) and increasing amounts of SNAP-sphingosine-1-phophate receptor 1 plasmid (1–50 ng), and labeled with a mixture of BC-Lumi4-Tb (1 μmol/l) and BG-green (0.3 μmol/l). Time-resolved FRET signal normalized with the CLIP-CCR5 expression signal is represented in function of SNAP-sphingosine-1-phophate receptor 1/CLIP-CCR5 expression ratio. Time-resolved FRET signal is expressed by the $\Delta ratio = (520/620)_{samples} - (520/620)_{background}$. The background signal corresponds to cells expressing SNAP-tagged and CLIP-tagged proteins labeled with a mixture of BC-Lumi4-Tb (1 μmol/l) and O6-BG (0.3 μmol/l), used to prevent low unspecific SNAP tag labeling with BC-Lumi4-Tb. Note that the two curves with the lowest CLIP-CCR5 amount have similar $FRET_{max}$ and $FRET_{50}$ values. The curves were obtained by nonlinear regressions with $R^2 = 0.9940$, 0.9901, 0.9984 and 0.9986 for CLIP-CCR5 = 2, 4, 8 and 16 ng, respectively. (b) Time-resolved FRET saturation curve of CLIP-CCR5 with SNAP-sphingosine-1-phophate receptor 1, SNAP-CCR5 or SNAP-beta-2 adrenergic receptor. For each curve, cells were cotransfected with a constant amount of CLIP-CCR5 plasmid (2 ng) and increasing amounts of the SNAP plasmid (1–25 ng) and labeled as in (a). Time-resolved FRET signal was normalized as in (a), and represented in function of SNAP-sphingosine-1-phophate receptor 1 (■), SNAP-CCR5 (•) or SNAP-beta-2 adrenergic receptor (▼)/CLIP-CCR5 expression ratio. The curves were obtained by nonlinear regressions with $R^2 = 0.9741$ and 0.9844 for SNAP-CCR5 and SNAP-sphingosine-1-phophate receptor 1, respectively, and by a linear regression for SNAP-beta-2 adrenergic receptor ($P < 0.001$). (c) Time-resolved FRET saturation curve of CLIP-CXCR4 with SNAP-sphingosine-1-phophate receptor 1 or SNAP-CXCR4. For each curve, cells were cotransfected with a constant amount of CLIP-CXCR4 plasmid (2 ng) and increasing amounts of the SNAP plasmid (1–25 ng) and labeled as in (a). Time-resolved FRET signal was normalized as in (a), and represented in function of SNAP-sphingosine-1-phophate receptor 1 (■) or SNAP-CXCR4 (•).

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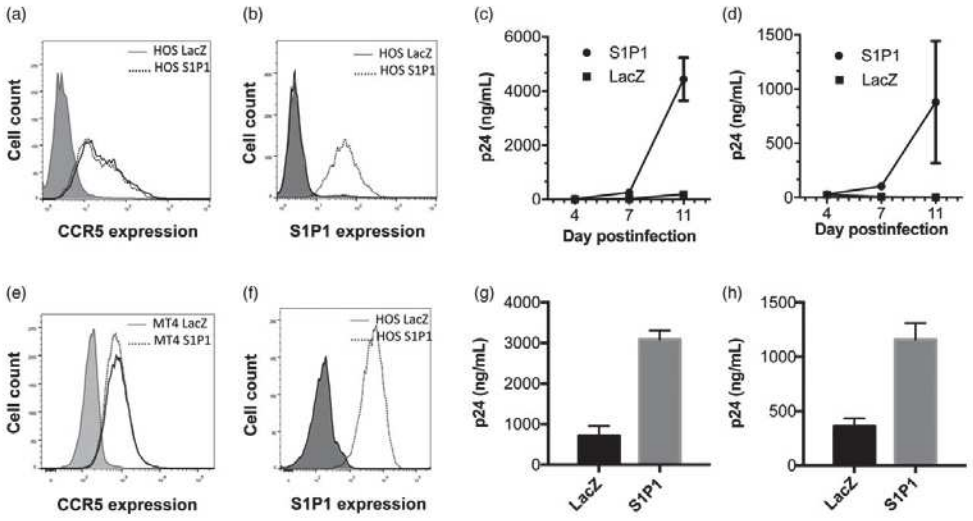

Fig. 2. Sphingosine-1-phophate receptor 1 expression increases HIV replication. (a) CCR5 expression on human osteosarcoma cells transduced with *sphingosine-1-phophate receptor 1* or *LacZ*. Fluorescence of human osteosarcoma cells transduced with *sphingosine-1-phophate receptor 1* (dotted line) or *LacZ* (full line), and labeled with a CCR5 antibody (open histogram) or an isotype control (closed histogram). (b) Sphingosine-1-phophate receptor 1 expression on human osteosarcoma cells transduced with *sphingosine-1-phophate receptor 1* or *LacZ*. Fluorescence of human osteosarcoma cells transduced with *sphingosine-1-phophate receptor 1* (dotted line) or *LacZ* (full line), and labeled with an sphingosine-1-phophate receptor 1 antibody (open histogram) or an isotype control (closed histogram). (c and d) Sphingosine-1-phophate receptor 1 expression increases HIV replication in human osteosarcoma cells. Human osteosarcoma cells transduced with *sphingosine-1-phophate receptor 1* (•) or *LacZ* (■) were exposed to an R5 (c) or an X4 (d) HIV-1 strain and cultured. Viral production was monitored over time by measuring p24 concentration in the cell supernatant of biological triplicates. The experiment was repeated thrice with the same results. (e) CCR5 expression on MT4 cells transduced with *sphingosine-1-phophate receptor 1* or *LacZ*. Fluorescence of MT4 cells transduced with *sphingosine-1-phophate receptor 1* (dotted line) or *LacZ* (full line), and labeled with a CCR5 antibody (open histogram) or an isotype control (closed histogram). (f) Sphingosine-1-phophate receptor 1 expression on MT4 cells transduced with *sphingosine-1-phophate receptor 1* or *LacZ*. Fluorescence of MT4 cells transduced with *sphingosine-1-phophate receptor 1* (dotted line) or *LacZ* (full line), and labeled with an sphingosine-1-phophate receptor 1 antibody (open histogram) or an isotype control (closed histogram). (g and h) Sphingosine-1-phophate receptor 1 expression increases HIV replication in MT4 cells. MT4 cells transduced with *sphingosine-1-phophate receptor 1* (gray hisogram) or *LacZ* (black histogram) were exposed to an R5 (g) or an X4 (h) HIV-1 strain and cultured. Seven days later, p24 concentration was measured in the cell supernatant of biological triplicates. The experiment was repeated thrice with the same results.

cell lines with an R5 or an X4 strain, and observed that S1P1-positive cells produced much more virus than the lacZ-expressing cells (Fig. 2c and d). At day 11, the mean p24 concentrations in the supernatant of S1P1-expressing cells were 4441 and 879 ng/ml, whereas they were 189 and 2 ng/ml in the supernatant of lacZ-expressing cells after R5 and X4 infection, respectively, We obtained similar results with the lymphoid cell line MT4 (Fig. 2e–h).

Sphingosine-1-phosphate receptor 1 expression boosts HIV genome transcription via NFκB

To examined whether the presence of S1P1 influenced HIV entry, we compared viral entry into MT4 cells transduced or not with *S1P1*. Cells were infected with R5 or X4 envelope-pseudotyped virions harboring the enzyme β-lactamase fused to the Vpr protein [28]. β-lactamase activity of the exposed cells was then measured by flow cytometry as a marker of viral entry. S1P1 expression increased by 30.2 ± 7.4% R5 (Fig. 3a), but not X4 (Fig. 3b) entry. Preincubation of target cells with a soluble form of $CD4^+$ prevented viral entry (Fig. 3a and b).

To study whether the presence of S1P1 modified reverse transcription efficiency, we exposed *S1P1*-transduced or *lacZ*-transduced HOS cells to R5 virions, and quantified the presence of early and late reverse transcripts in the infected cells. As for the entry, we observed a slight increase in HIV DNA content in S1P1-expressing cells,

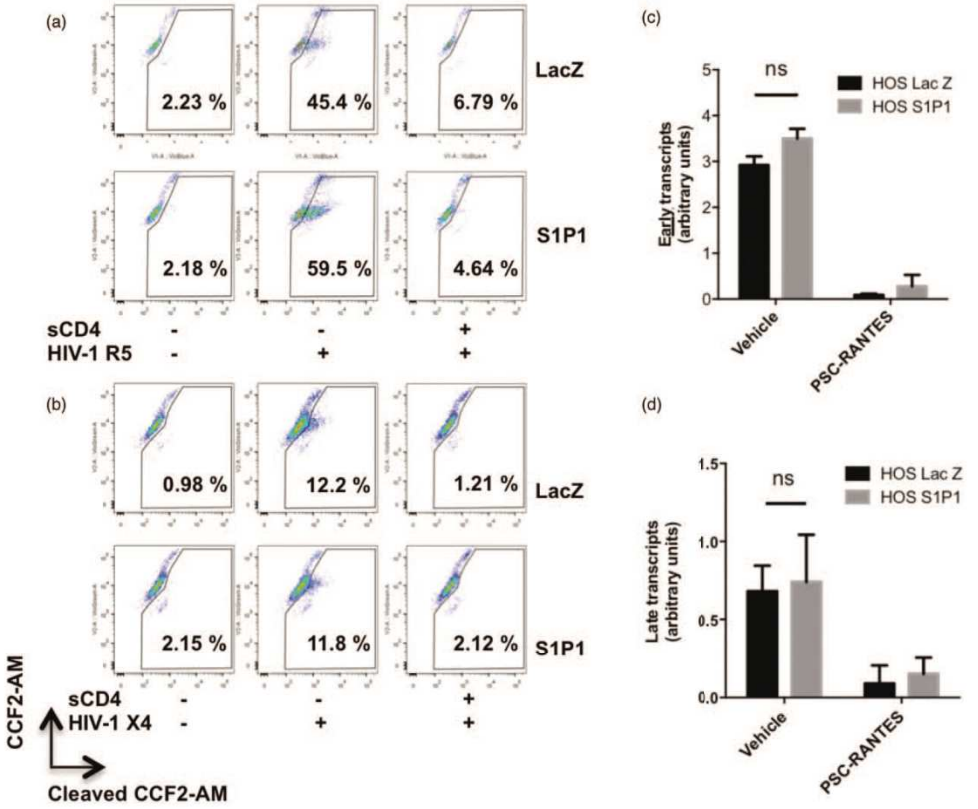

Fig. 3. Effect of sphingosine-1-phophate receptor 1 expression on HIV entry and reverse transcription. (a and b) Effect of sphingosine-1-phophate receptor 1 expression on HIV entry. MT4 cells were preincubated or not with soluble $CD4^+$, exposed or not to HIV-1 particles containing Vpr–β-lactamase and pseudotyped with either an R5-tropic (a) or an X4-tropic (b) envelope, and thereafter to the CCF2-AM substrate. Cells containing the fluorescent cleaved substrate were then quantified by flow cytometry to assess viral entry. Representative experiment out of five for R5, and three for X4. (c and d) Effect of sphingosine-1-phophate receptor 1 expression on HIV DNA reverse transcription. Human osteosarcoma cells were transduced with *sphingosine-1-phophate receptor 1* (gray histograms) or *LacZ* (black histograms), preincubated or not (vehicle) with a CCR5 ligand (PSC-RANTES), exposed to a replicative defective R5 strain, and the amount of early (c) and late (d) transcripts was determined by quantitative PCR on biological triplicates. ns, non significant.

but this difference was NS (Fig. 3c and d). Thus, the presence of S1P1 in target cells did not modify reverse transcription efficiency.

As S1P1 triggering activates NFκB via $G_{\alpha i}$ proteins [29–31], we questioned whether S1P1 expression could increase HIV transcription. HeLa cells stably transfected with an LTR fused to a *luciferase* reporter gene [21] were transduced with *S1P1* or *lacZ* gene. We observed that S1P1-expressing HeLa cells displayed a two-fold increase in luciferase expression compared with lacZ-expressing HeLa cells (Fig. 4a). As shown in Fig. 4a, preincubation of the *S1P1*-transduced cells with an anti-S1P1 antibody abolished LTR activation, suggesting that this activation was triggered via S1P1 by S1P present in the bovine serum added to the culture medium [32]. Conversely, addition to the cell culture of FTY720-P, a compound with an initial S1P receptor agonist effect [33], further increased luciferase expression (Fig. 4a). The same phenomenon was observed with the specific S1P1 agonist SEW2871 [34], in a dose-dependent manner (Fig. 4b).

We next explored whether S1P1 expression resulted in NFκB activation. Actually, we observed the accumulation of NFκB p65 protein at perinuclear and nuclear sites in

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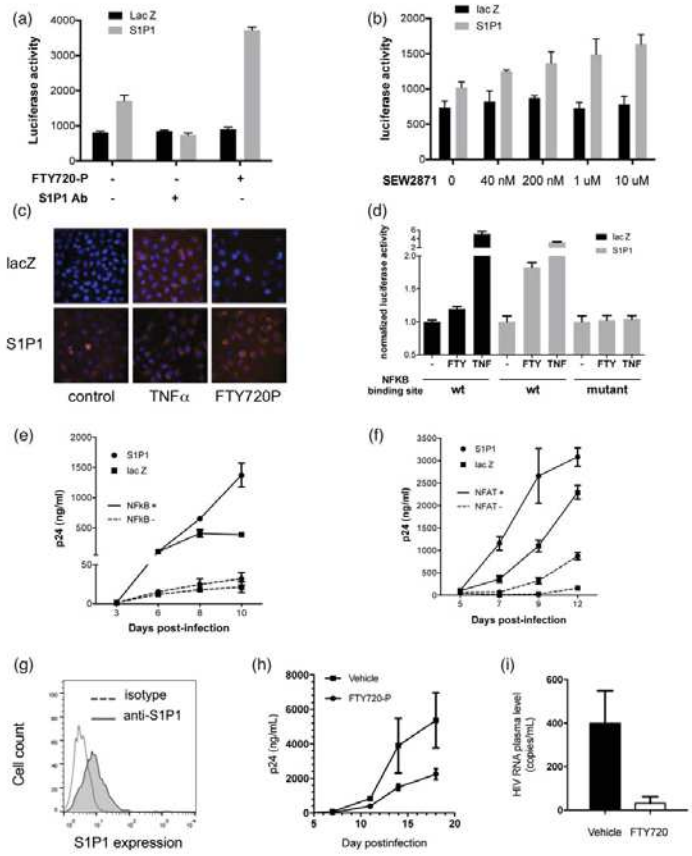

Fig. 4. Sphingosine-1-phophate receptor 1 expression induces an NFκB-dependent HIV genome activation and FTY720-P inhibits HIV-1 R5 infection *in vitro* and *in vivo*. (a) Sphingosine-1-phophate receptor 1 expression activates HIV LTR. HeLa cells harboring an HIV-1 LTR-driven *luciferase* gene were transduced with *sphingosine-1-phophate receptor 1* (gray histograms) or *LacZ* (black histograms), and cultured in triplicate in presence or absence of an antisphingosine-1-phophate receptor 1 antibody or the sphingosine-1-phophate receptor 1 ligand FTY720-P for 10 h. Thereafter, luciferase activity was measured in cell lystates. (b) Sphingosine-1-phophate receptor 1 signaling results in a dose-dependent activation of HIV LTR. The same experiment as in (a) was performed in presence of increasing concentrations (0–10 μmol/l) of the sphingosine-1-phophate receptor 1 agonist SEW2871 for 20 h. (c) Sphingosine-1-phophate receptor 1 signaling activates NFκB. HeLa cells transduced with *sphingosine-1-phophate receptor 1* or *LacZ* were exposed or not (control) to the sphingosine-1-phophate receptor 1 ligand FTY720-P or to TNFα. Thereafter, NFκB p65 was labeled with a specific anibody and analyzed in fluorescence microscopy. (d) Sphingosine-1-phophate receptor 1 signaling activates HIV LTR via NFκB. HeLa cells transduced with *sphingosine-1-phophate receptor 1* (gray histograms) or *LacZ* (black histograms) were transiently transfected with a *luciferase* gene driven by an LTR harboring (wt) or not (mutant) NFκB binding sites, and cultured in triplicate in presence or absence of the sphingosine-1-phophate receptor 1 ligand FTY720-P (FTY) or of TNFα (tumor necrosis factor). Thereafter, luciferase activity was measured in cell lystates. The increase in luciferase activity as compared with the negative controls is represented. (e) Sphingosine-1-phophate receptor 1 expression boosts HIV replication via NFκB. MT4 cells transduced with *sphingosine-1-phophate receptor 1* (•) or *LacZ* (■) were exposed in triplicate to an HIV strain harboring (—, NFκB+) or not (—, NFκB−) NFκB binding sites in its LTR promoter. Viral production was monitored overtime by measuring p24 concentration in the cell supernatant. (f) Sphingosine-1-phophate receptor 1 expression does not boost HIV replication via NFAT. The same experiment as in (e) was performed but with an HIV strain harboring (—, NFAT+) or not (—, NFAT−) NFAT binding sites in its LTR promoter. (g) Sphingosine-1-phophate receptor 1 expression on dendritic cells. Monocytes were differentiated into dendritic cells *in vitro*, and labeled with an antisphingosine-1-phophate receptor 1 antibody (closed histogram) or an isotype control (open histogram). Cell fluorescence was then assessed by flow cytometry. (h) FTY720-P reduces

S1P1-expressing cells (Fig. 4c). This translocation was increased in these cells by FTY720-P. TNFα did the same in S1P1-positive and S1P1-negative cells. To further assess the role of NFκB in S1P1-driven LTR activation, HeLa cells transduced or not with *S1P1* were transiently transfected with plasmids carrying the *luciferase* gene under the control of an LTR deleted or not of the NFκB binding sites, and exposed to FTY720-P. As expected, FTY720-P induced the expression of the *luciferase* gene driven by the wild-type LTR in S1P1-positive cells. By contrast, the reporter gene driven by the NFκB-deleted LTR was transcribed in S1P1-expressing cells neither under FTY720-P nor under TNFα stimulus (Fig. 4d). We confirmed that NFκB is involved in S1P1-induced HIV-1 genome activation by infecting *S1P1*-transduced or *lacZ*-transduced MT4 cells with X4 HIV strains harboring an LTR deleted (NFκB−) or not (NFκB+) of its NFκB binding sites. As expected NFκB+ virus production was higher in S1P1-positive than in S1P1-negative cell cultures. And this difference was abrogated in cell cultures infected with the NFκB− virus (Fig. 4e). As a control we performed the same experiment with viruses harboring an LTR deleted (NFAT−) or not (NFAT+) of the NFAT binding site. This time, we observed no consequence of this deletion on the effect of the presence of S1P1 on viral production (Fig. 4f). Altogether, our data show that the major effect of S1P1 expression is an increase in viral genome transcription mediated by NFκB.

An sphingosine-1-phosphate receptor 1 antagonist reduced HIV production in primary immune cells *in vitro* and *in vivo*

FTY720-P has an initial S1P1 agonist effect, as observed in Fig. 4a, but it can also display an antagonist effect on the long term due to S1P1 internalization and degradation. We reasoned that FTY720-P could inhibit HIV production in culture. Therefore, we decided to test the effect of FTY720-P on the infection of primary immune cells. We chose to study this effect on in-vitro infection of monocyte-derived dendritic as these cells display S1P1 receptors at their surface (Fig. 4g). We neutralized the HIV restriction factor SAMHD1 by exposing these dendritic cells to pseudo viral particles delivering vpx, and infected them with an R5 strain in presence or absence of FTY720-P. FTY720-P reduced viral production (Fig. 4h). We also tested the effect of FTY720 on HIV-1 R5 infection *in vivo*. SCID mice were reconstituted by intraperitoneal injection of human PBMC. The animals were then force-fed with FTY720, that is converted into its phosphorylated active form FTY720-P *in vivo*, or with vehicle, and infected with an HIV-1 R5 strain. We observed a 13-fold decrease in plasma viral load in FTY720-treated mice as compared with the negative controls (31 ± 31 HIV versus 398 ± 106 HIV RNA copies/ml, $P = 0.014$, Fig. 4i).

Sphingosine-1-phosphate receptor 1 agonists induce reactivation of HIV-1 in peripheral blood mononuclear cells and in lymph node cells

We surmised that S1P1 agonists might reverse HIV latency in reservoir cells. To generate an in-vitro model of reservoir cells, we infected PBMC or lymph node cells with a primary R5 strain in absence of stimulation. Under these conditions, cells transiently produce viral particles and return to a nonproductive quiescent state. At this stage, CD3 and CD28 stimulation induced a second round of viral production (Fig. 5a). As compared with nontreated cultures (26.6 ± 8.7 pg/ml of p24), exposition of quiescent infected PBMC to S1P (118.1 ± 41.9 pg/ml, $P = 0.029$, Fig. 5b and c) or to the S1P1 agonist SEW2871 (112.1 ± 38.5 pg/ml of p24, $P = 0.028$, Fig. 5b and c) provoked a rebound in HIV p24 production, 2 and 6 days after the S1P1 ligand addition. FTY720-P also induced p24 production, but this de-novo production was NS (Fig. 5b). S1P addition to a culture of lymph node cells infected in nonactivating conditions after the peak of viral production also provoked a viral rebound (Fig. 5d). The amount of virus produced 72 h after S1P stimulation was significantly higher than in absence of stimulation (27.3 ± 9.1 and 0.9 ± 0.2 pg/ml of p24, respectively, $P = 0.050$, Fig. 5e).

Discussion

In an effort to identify new GPCR that interfere with HIV replication in immune target cells, we analyzed *GPCR* genes expression in $CCR5^+CD4^+$ T cells by RT-PCR. We found that the *S1P1* gene is highly transcribed in $CCR5^+CD4^+$ T cells. S1P1 is required for the exit of B and T lymphocytes from secondary lymphoid organs [11]. At the nervous system level, S1P1 is involved in myelination, astrogliosis and microglial production of proinflammatory cytokines [35]. S1P1 also regulates angiogenesis, cardiac development and vascular barrier integrity [36,37]. We show that S1P1 is able to form heteromers with CCR5 on the outer cellular membrane. It has been previously reported that CCR5 forms homo-

replicative R5-HIV infection in dendritic cells. Differentiated DCs were preincubated with Vpx-containing virus-like particles, exposed or not to FTY720-P for 24 h, and infected in triplicate with the R5 strain Ad8. Viral production was monitored over time by measuring p24 concentration in the cell supernatant. (i) FTY720 inhibits replicative HIV-1 R5 infection in humanized SCID mice. SCID mice reconstituted with human BPMC, were fed with FTY720 (open box) or vehicle (closed box), and infected with a replicative R5 HIV-1 virus. Representation of the viral loads measured at days 6, 9 and 12 postinfection are pooled.

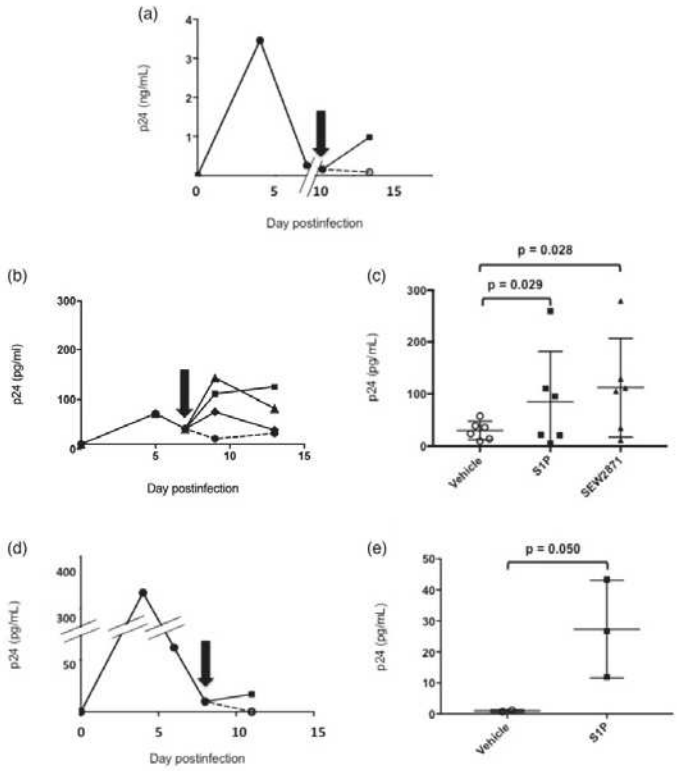

Fig. 5. Sphingosine-1-phophate receptor 1 agonists induce viral production in an in vitro model of HIV reservoir cells. (a) Establishment of an in-vitro model of HIV reservoir cells. Resting PBMC were exposed to an HIV-1 R5 strain, and stimulated (■) or not (o) after the peak of viral production (arrow) with anti-CD3 and anti-CD28 antibodies. Mean viral production was monitored over time by measuring p24 concentration in the cell supernatant. (b and c) Sphingosine-1-phophate receptor 1 agonists induce viral production in an in-vitro model of HIV reservoir PBMC. Resting PBMC were exposed to an HIV-1 R5 strain, and stimulated or not (o) after the peak of viral production (arrow) with sphingosine-1-phophate (■), SEW2871 (▲) or FTY720-P (◆). Mean viral production was monitored over time by measuring p24 concentration in the cell supernatant (b). p24 concentrations at days 9 and 13 in the supernatant of PBMC stimulated or not as described in (b) with sphingosine-1-phophate or SEW2871 (c). (d and e) sphingosine-1-phophate induces viral production in an in-vitro model of HIV reservoir lymph node cells. Resting lymph node cells were exposed to an HIV-1 R5 strain, and stimulated (■) or not (o) after the peak of viral production (arrow) with sphingosine-1-phophate. Mean viral production was monitored over time by measuring p24 concentration in the cell supernatant (d). p24 concentrations in the supernatant of lymph node cells stimulated or not with sphingosine-1-phophate as described in (d) at day 11 (e).

oligomers and coimmunoprecipitates with the opioid receptors [38] independently of ligand binding [27]. Furthermore, mu opioid receptor and CCR5 heterodimerize and cross desensitize each other [39]. CCR5 has also been found to heterodimerize with CCR2 [40], CXCR3 [41], and CXCR4 [42]. It is also known that interaction of CCR5 with the mu and delta opioid receptors [43–46] and with other nonchemokine [47–54] or chemokine [40–42] GPCR modulate HIV infectability via heterodimerization or heterodesensitization. Here we show that the major consequence of S1P1 expression in HIV infectible cells is an increase in viral gene expression via the transcription factor NFκB. The fact that the early steps of HIV life cycle are not strongly modified by the presence of S1P1, and that its presence boosts X4 as well as R5 strain replication whereas S1P1 and CXCR4 do not dimerize, argues for an absence of role for S1P1-CCR5 heteromerization in S1P1 effect on HIV life cycle. In particular, S1P1 ability to boost HIV genome transcription appears no to be linked to its dimerization with CCR5. For in-vitro infection experiments, cells are grown into culture medium AQ9

containing fetal bovine serum, a source of S1P. Moreover, S1P is produced by several cell types and may act in an autocrine or paracrine manner [55]. It is therefore possible that the effects we observed result from S1P1 stimulation by S1P present in the medium and/or produced by the cells.

We observed an HIV RNA plasma level decrease in FTY720-treated humanized mice, consistently with the results obtained by Murooka *et al.* [56] in a similar experiment. These authors attributed this phenomenon to the blocking of migratory T cells egress from the lymph nodes into efferent lymph vessels, and to the interruption of T-cell recirculation, hence limiting HIV dissemination [56]. Given our data, it may now be added that there is an additive inhibitory effect of this functional S1P1 antagonist on HIV infection. Two studies reported the effect of FTY720 administration on simian HIV (SHIV) infection. Kersh *et al.* [57] injected the drug into infected rhesus macaques, whereas Morris *et al.* [58] challenged FTY720-treated pigtail macaques by repeat vaginal exposures to the virus. In these experiments, FTY720 was administrated weekly rather than continuously. Given the bimodal action of the drug on S1P1 activation, this difference may be important. The authors reported no inhibitory effect of the S1P1 ligand, neither on SHIV susceptibility, nor on viremia. Nonetheless, it is interesting to note that in the former study, SHIV plasma levels raised after most of the injections of FTY720 at a functional dose of 0.1 mg/kg. This in-vivo effect in a primate model of HIV infection is consistent with our data.

It is possible that CCR5-S1P1 dimerization alters S1P1 functions. CCR5 ligands might further modify the response of S1P1 to S1P, and thereby hinder the T-cell egress from thymus and lymph nodes. This raises the interesting possibility that gp120, by binding to CCR5, might be responsible for the effects of HIV-1 on T-cell production via S1P1 [59], and for impaired T-cell responses to S1P in HIV-1-infected lymph nodes [60]. Such a mechanism might also be involved in the adenopathies observed in viremic patients [61].

It could be of interest to study the effect of FTY720, or of more recent S1P1 antagonists [35], in persons living with HIV, in as much as FTY720 is already used to treat multiple sclerosis [62]. In addition to the inhibitory effect on HIV DNA transcription we describe here, S1P1 antagonists could have additional beneficial effects. Actually, S1P1 signaling has been reported to enhance Th17 polarization [63] and to decrease the differentiation of thymic Treg precursors as well as the function of mature Treg cells [64,65]. Thus, blocking this signaling could downregulate the immune activation that is deleterious even in patients aviremic under treatment [66].

HIV latency is the consequence of combined mechanisms. Therefore, the reversion of this phenomenon may need drugs acting on different levels. Moreover, antilatency agent association should allow to reduce the posology of each agent and thereby their toxicity. It will be of interest to look for a synergistic effect between S1P1 agonists and other LRA.

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Author contributions: C.D. and S.G. transduced the cells, acquired, analyzed and interpreted flow cytometry data, LTR activation and infection assays. A.G. performed HIV entry assays and quantified HIV transcripts. B.V. and C.B. carried out HIV infection assays. D.M., E.D., L.C.-A., L.P. and V.F. were in charge of the FRET analyses. N.C. and J.T. managed the infection of SCID mice. V.F. and P.C. contributed to the conception and design of the study, analyzed and interpreted data, and wrote the article.

Conflicts of interest

There are no conflicts of interest.

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D. Conclusion S1P1

Comme EBI2 et A_{2A}, S1P1 s'hétérodimérise avec CCR5. Comme pour A_{2A}, l'interaction entre S1P1 et CCR5 à la surface cellulaire facilite légèrement l'entrée virale. Là aussi, comme avec A_{2A}, l'interaction entre S1P1 et CCR5 pourrait modifier la conformation de CCR5 et renforcer l'interaction entre ce corécepteur et l'enveloppe rétrovirale.

Cependant, de même que pour EBI2 et A_{2A}, l'effet global de S1P1 est une amplification de la production virale. Ceci est dû à une activation du LTR via le facteur de transcription NFkB (figure 27).

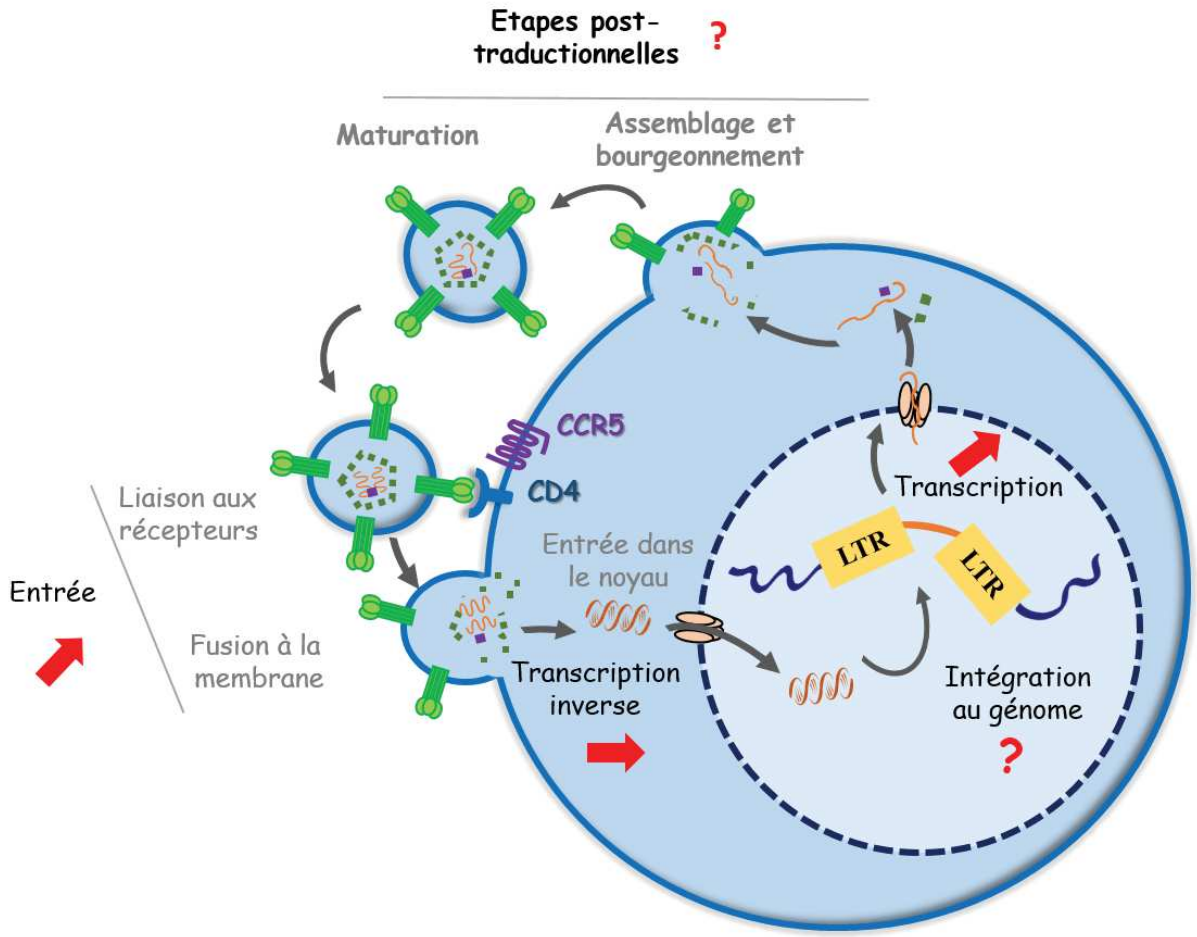

Figure 27. Schéma récapitulatif des étapes du cycle viral modifiées par S1P1

En conséquence des agonistes de S1P1 ont la capacité de lever la latence virale in vitro. Il sera intéressant de tester l'effet de ces agonistes ex vivo sur des cellules réservoirs de patients avirémiques et de chercher un effet synergique avec d'autres LRA.

Il sera aussi intéressant de tester si la fonctionnalité de S1P1 est réduite suite à sa dimérisation avec CCR5, en particulier en présence de la gp120 rétrovirale. Cette hypothèse pourrait expliquer en partie la séquestration des cellules T dans les organes lymphoïdes secondaires observée chez ces patients et la levée de cette séquestration consécutive à la mise en place d'un traitement antirétroviral.

V. Conclusion générale

Depuis la découverte du VIH, le traitement de la maladie et la qualité de vie des patients ont considérablement évolué, notamment grâce aux multithérapies qui ciblent différentes étapes du cycle viral. Bien que les nouveaux traitements permettent de lutter efficacement contre le VIH en réduisant la virémie à un niveau très bas, aucun ne permet encore d'éradiquer le virus de l'organisme, condition nécessaire à la guérison. Un point clé réside dans l'incapacité actuelle des traitements à éliminer les cellules réservoirs qui continuent à abriter le virus sous une forme silencieuse ou en latence, en particulier des cellules T CD4+ mémoires quiescentes infectées. Les stratégies mises en œuvre pour répondre à cet écueil visent à lever la latence du réservoir viral en stimulant la transcription du génome viral. L'approche « shock and kill » utilise des LRA pour réactiver la production virale et ainsi éliminer les cellules nouvellement productrices de virus.

Les virus R5 utilisent comme corécepteur CCR5 qui est un RCPG. Je présente ici une étude de trois RCPGs qui s'hétérodimérisent avec le corécepteur CCR5 et qui modifient l'infection par le VIH. Cette thèse examine l'influence de la modulation de l'expression et/ou de l'activation d'EBI2, A_{2A} et S1P1 par leurs ligands sur le cycle viral et vise à en comprendre les mécanismes. Les résultats obtenus ouvrent des perspectives thérapeutiques pour l'élimination des cellules réservoirs.

Les trois récepteurs EBI2, A2A et S1P1 influent sur la réplication du VIH lorsqu'ils sont exprimés dans des lignées cellulaires. L'effet global de l'expression de chacun de ces RCPGs est une augmentation de la production virale qui résulte d'une stimulation de la transcription du virus intégré au génome cellulaire. L'analyse des étapes du cycle viral montre quelques différences entre ces récepteurs. Leur présence ou leur stimulation peut favoriser une étape, la diminuer ou bien ne pas avoir de conséquences remarquables à ce niveau, dépendant du récepteur étudié (figure 28).

Les ligands des trois RCPGs étudiés ici pourraient donc être des candidats LRA pour leurs effets stimulant sur la production virale. Il sera intéressant de tester des agonistes d'EBI2 et d'A_{2A} pour lever la latence, mais également de tester des agonistes de S1P1 en synergie avec d'autres LRA décrits par ailleurs.

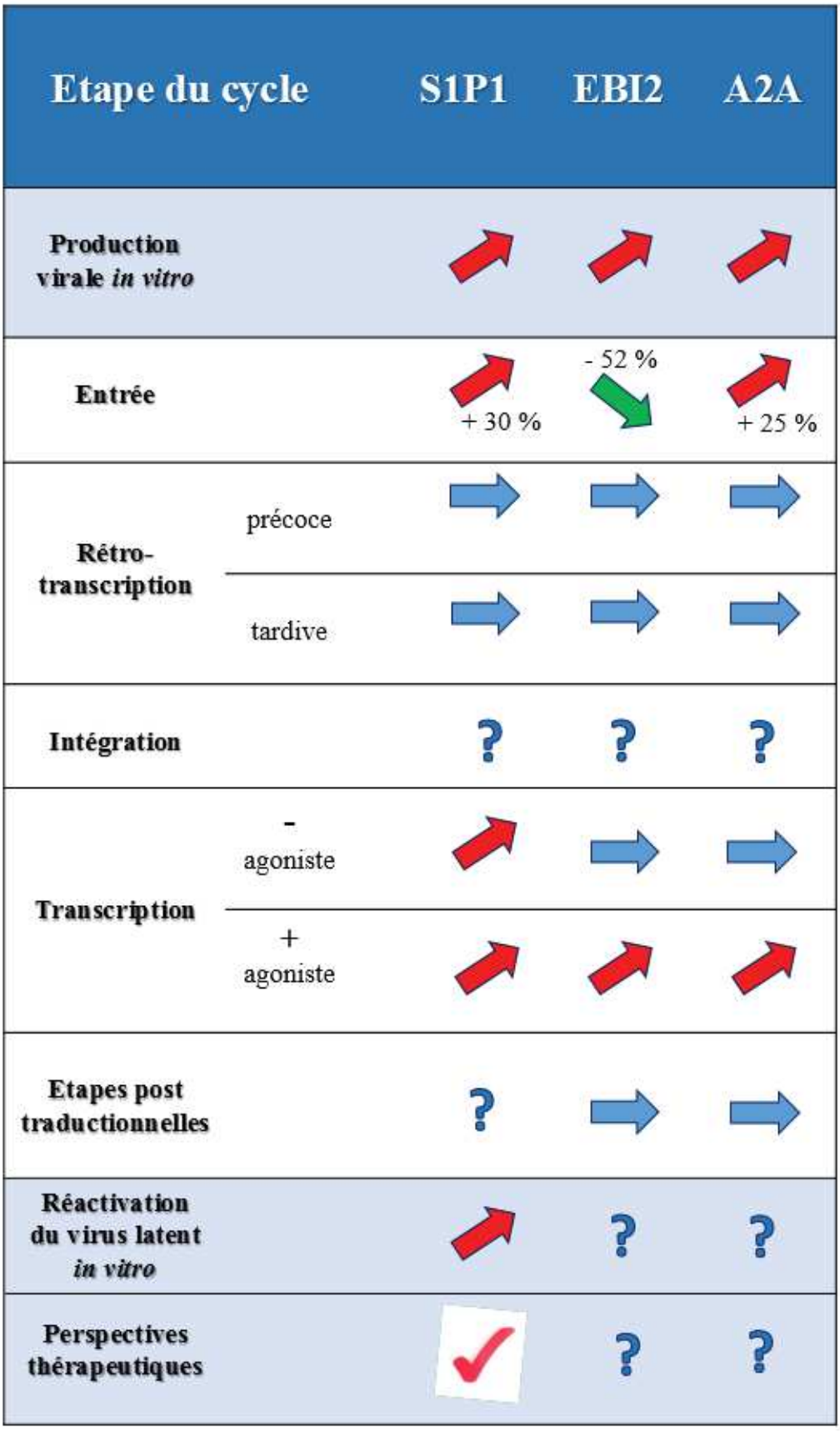

Figure 28. Tableau récapitulatif des effets des récepteurs sur les étapes du cycle viral.

VI. Annexe 1 : CXCR4

En début d'infection par le VIH-1 les virions utilisent principalement CCR5 comme corécepteur et sont appelés R5. Ceci même si des virions R5X4 ou X4, capables d'utiliser CXCR4 comme corécepteur, étaient présents dans l'inoculum. Ce n'est généralement qu'à un stade avancé chez environ 40 % des patients que des souches virales X4 utilisant CXCR4 comme corécepteur émergent (Schuitemaker et al. 1991), alors qu'elles étaient parfois pré-existantes dans l'organisme infecté. De même la quasi-totalité des individus homozygotes pour l 'allèle *D32CCR5*, qui n'expriment pas de molécule CCR5 à la surface de leurs cellules, sont résistants à l'infection par le VIH. Ces données suggèrent que chez un individu dont le système immunitaire est intact, les souches utilisant CXCR4 pourraient être contre-sélectionnées. Ce ne serait qu'une fois que des souches R5 ont affaibli ce système immunitaire que des souches R5X4 et X4 pourraient se développer.

L'émergence de souches X4 s'accompagne d'une accélération de la progression de la maladie (Tersmette et al. 1988). Ceci justifie l'importance d'étudier le phénomène de commutation R5→X4.

Le tropisme R5 semble en grande partie conditionné par la boucle V3 de la gp120. Ainsi les charges électriques des positions 11, 24 et 25 jouent un rôle dans ce tropisme, mais aussi les glycosylations. Cependant d'autres acides aminés semblent jouer un rôle comme l'isoleucine en position 326, et des acides aminés situés dans le bridging sheet, dans les domaines V1/V2 et C4, mais aussi dans la gp40.

Deux isoformes du récepteur CXCR4 ont été identifiées chez l'Homme : CXCR4-A et CXCR4-B. Le récepteur CXCR4-B est plus long de 5 acides aminés en N terminal et les 4 acides aminés suivants sont différents. Ces deux isoformes présentent également des différences fonctionnelles au niveau du chimiotactisme (Gupta and Pillarisetti 1999).

Nous nous sommes intéressés aux différences entre CXCR4-A et CXCR4-B en tant que corécepteur du VIH-1.

The Journal of Immunology

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The Two Human CXCR4 Isoforms Display Different HIV Receptor Activities: Consequences for the Emergence of X4 Strains

Charline Duquenne,* Christina Psomas,[†,‡] Sandrine Gimenez,* Adeline Guigues,* Marie-Josée Carles,[§] Claudine Barbuat,[¶] Jean-Philippe Lavigne,[§,‖,#] Albert Sotto,[¶,‖,#] Jacques Reynes,[†,‡,‖] Paul Guglielmi, Clément Mettling,* Vincent François,*[,1] and Pierre Corbeau*[,‖,††,1]**

CXCR4 is a chemokine receptor that plays key roles with its specific ligand, CXCL12, in stem cell homing and immune trafficking. It is also used as a coreceptor by some HIV-1 strains (X4 strains), whereas other strains (R5 strains) use an alternative coreceptor, CCR5. X4 strains mainly emerge at late stages of the infection and are linked to disease progression. Two isoforms of this coreceptor have been described in humans: CXCR4-A and CXCR4-B, corresponding to an unspliced and a spliced mRNA, respectively. In this study, we show that CXCR4-B, but not CXCR4-A, mediates an efficient HIV-1 X4 entry and productive infection. Yet, the chemotactic activity of CXCL12 on both isoforms was similar. Furthermore, HIV-R5 infection favored CXCR4-B expression over that of CXCR4-A. In vitro infection with an R5 strain increased CXCR4-B/CXCR4-A mRNA ratio in PBMCs, and this ratio correlated with HIV RNA plasma level in R5-infected individuals. In addition, the presence of the CXCR4-B isoform favored R5 to X4 switch more efficiently than did CXCR4-A in vitro. Hence, the predominance of CXCR4-B over CXCR4-A expression in PBMCs was linked to the ability of circulating HIV-1 strains to use CXCR4, as determined by genotyping. These data suggest that R5 to X4 switch could be favored by R5 infection–induced overexpression of CXCR4-B. Finally, we achieved a specific small interfering RNA–mediated knockdown of CXCR4-B. This represents a proof of concept for a possible gene-therapeutic approach aimed at blocking the HIV coreceptor activity of CXCR4 without knocking down its chemotactic activity. *The Journal of Immunology*, 2014, 193: 4188–4194.

The chemokine receptor CXCR4 is vital for embryonic development and plays key roles in myelopoiesis, homeostasis, maintenance of the immune system, and cancer progression (1). Mutations in CXCR4 that increase its responsiveness to the chemokine ligand CXCL12 result in an immunodeficiency called WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) syndrome (2). One consequence of increased agonist-induced CXCR4 signaling in this syndrome is an impaired egress of leukocytes, particularly neutrophils, from the bone marrow, causing neutropenia and, possibly, leukopenia.

*Institut de Génétique Humaine, Unité Propre de Recherche 1142 du Centre National de la Recherche Scientifique, 34396 Montpellier Cedex 5, France; [†]Infectious Diseases Department, Montpellier University Hospital, 34295 Montpellier Cedex 5, France; [‡]Unité Mixte Internationale 233, Institut de Recherche pour le Développement–Université Montpellier 1, 34294 Montpellier Cedex 5, France; [§]Microbiology Department, Nîmes University Hospital, 30029 Nîmes Cedex 9, France; [¶]Infectious Diseases Department, Nîmes University Hospital, 30029 Nîmes Cedex 9, France; [‖]Faculté de Médecine, Université Montpellier 1, 34967 Montpellier Cedex 2, France; [#]INSERM U1047, 30908 Nîmes Cedex 9, France; **Unité Mixte de Recherche 5235, Centre National de la Recherche Scientifique–Université Montpellier 2, 34095 Montpellier, France; and [††]Immunology Department, Nîmes University Hospital, 30029 Nîmes Cedex 9, France

[1]V.F. and P.C. contributed equally to this work.

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Address correspondence and reprint requests to Prof. Pierre Corbeau, Institut de Génétique Humaine, Unité Propre de Recherche 1142 du Centre National de la Recherche Scientifique, 141 Rue de la Cardonille, 34396 Montpellier Cedex 5, France. E-mail address: pcorbeau@igh.cnrs.fr

Abbreviations used in this article: FPR, false-positive rate; LTR, long terminal repeat; N-term, NH2-terminal extremity; siRNA, small interfering RNA.

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Together with the chemokine receptor CCR5, used by HIV-1 R5 strains, CXCR4 is the coreceptor for dual tropic HIV-1 R5X4 (CCR5- and CXCR4-using) and X4 (CXCR4-using) strains, in addition to the main receptor CD4 (3). HIV-1 R5 strains predominate in the early stages of disease, and the proportion of CXCR4-using strains increases with the duration of infection (4). Of note, the emergence of CXCR4-using strains correlated with an acceleration of disease progression (3). One factor that could favor this R5 to X4 switch is the increase in $CD4^+$ T cell CXCR4 surface density observed in about one third of HIV-1–infected individuals with a CD4 count < 400 cells/μl (5). This is demonstrated when CXCR4 is overexpressed at the surface of HIV-1 R5–infected cells, facilitating the emergence of CXCR4-using HIV-1 strains (6, 7). Moreover, patients with a high $CD4^+$ T cell surface CXCR4 density harbor (R5)X4 strains more frequently than do patients with physiological levels of CXCR4 expression (5). CXCR4 overexpression observed in advanced disease could be mediated, in part, by IL-7, which is overproduced as a consequence of $CD4^+$ T cell lymphopenia (6, 8).

Although CCR5 antagonists have been successfully developed as anti-HIV drugs (9), the long-term use of CXCR4 antagonists is limited by the physiological roles played by CXCR4 (10). CCR5 and CXCR4 antagonists usually inhibit the binding of their natural ligands to CCR5 and CXCR4; however, the role of CCR5 is dispensable, whereas the role of CXCR4 is not, over the long term.

Two human isoforms of CXCR4 have been described. They are coexpressed in PBMCs (11). The main isoform, CXCR4-B, is coded by a spliced mRNA (Fig. 1). The long variant (CXCR4-A) is coded by an unspliced mRNA containing a distinct 5′ untranslated

region and a coding region initiated from an alternative start codon (Fig. 1). The CXCR4-A receptor has a longer NH2-terminal extremity (N-term) that is different from the CXCR4-B isoform by the first 9 aa residues (Fig. 1).

Because CXCR4 N-term was shown to be involved in HIV-1 X4 binding (12–14), we compared the capability of the both the A and B isoforms to support HIV-1 infection and chemotaxis.

Materials and Methods

Cell culture

$CD4^+CCR5^+$ HOS, HEK-293T, $CD4^-CCR5^-$ HeLa, and $CD4^+CXCR4^+$ HeLa-P4 cells were cultivated in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Lonza) at 37°C and 5% CO_2. $CD4^+CCR5^-CXCR4^+$ CEM cells, $CD4^+CCR5^-CXCR4^+$ MT2 cells, and human PBMCs were cultivated in RPMI 1640 supplemented the same as DMEM and under the same conditions. Cell viability was quantified after staining with 0.4% trypan blue (Sigma) with an automated cell counter (MACSQuant; Miltenyi Biotec) using cell-counting chamber slides (Invitrogen).

Transduction

The CXCR4 cDNA was obtained via the AIDS Research Reagent Program (Rockville, MD) from Dr. Nathaniel Landau (New York University School of Medicine, New York, NY). It was amplified to give CXCR4-A (corresponding to PubMed sequence NM_001008540 from nucleotide 297 to 1375) or CXCR4-B (corresponding to PubMed sequence NM_003467 from nucleotide 36 to 1154). Each cDNA was inserted downstream of the CMV promoter in the lentiviral transfer vector pHR-BX (15). These CXCR4 transfer vectors were cotransfected with the HIV packaging plasmid pAX2 and the plasmid pMD2G, which codes for the vesicular stomatitis virus envelope glycoprotein G, in HEK-293T cells by the calcium phosphate method. Supernatants were collected at day 2 posttransfection and concentrated on sucrose (Sigma) by ultracentrifugation at 17,000 × *g* for 1.5 h at 4°C. HOS or HeLa cells were plated in 24-well culture plates and exposed to the same amount of the p24 equivalent of HIV vectors harboring the CXCR4-B or CXCR4-A cDNA in 8 μg/ml Polybrene (Sigma) and centrifuged at 200 × *g* for 90 min at 30°C. Cells were washed 24 h posttransduction, amplified, and controlled for CXCR4 membrane expression by flow cytometry.

Single-round infection assay

To produce replication-defective HIV virions, pNL4.3-Luc.R^-.E^- transfer plasmid harboring a defective viral *env* gene with a *firefly luciferase* gene inserted in the viral *nef* gene was cotransfected with the R5 envelope plasmid pCMV-Ad8-Env, the X4 envelope plasmid VB34, or the vesicular stomatitis virus envelope glycoprotein G envelope plasmid pDM2G (AIDS Research Program) into HEK-293T cells at a molecular ratio of 2:1. The virions produced were collected in the culture supernatant 48 h posttransfection, filtrated at 0.45 μm, and concentrated on sucrose by centrifugation. For the single-round infection assay, HOS cells were cultivated in triplicates in 96-well culture plates at a density of 0.25×10^6 cells/ml and infected with the various virions. In some experiments, cells were incubated for 1 h with CXCL12 (PeproTech) at various concentrations before infection. Cells were washed twice in PBS 24 h postinfection and kept in culture for 72 h. Cells were washed once in PBS and lysed with 50 μl lysis buffer, and the firefly luciferase activity was measured on a luminometer using a commercial kit (Luciferase Assay System; Promega).

Multiple-round infection assay

The HIV-1 R5 Ad8 strain (AIDS Reagent Program) was expanded in $CD4^+$ $CCR5^+$ HOS cells. The HIV-1 X4 NL4.3 DNA (AIDS Reagent Program) was transfected into HEK-293T cells by the calcium phosphate method, and the collected virions were amplified in the human $CD4^+CXCR4^+$ CEM cell line. HOS cells were plated in 24-well culture plates at a density of 0.04×10^6 cells/ml. Cells were infected in triplicates with 10 ng/ml the p24 equivalent of Ad8 or NL4.3 overnight, washed with PBS, and kept in culture; cell number was adjusted twice a week. Virus production was monitored by measuring p24 gag concentration in the cell supernatant using a commercial ELISA (INNOTEST HIV Ag mAb; Ingen).

Detection of early and late reverse transcripts

Reverse transcripts were quantified as previously described (16).

Quantification of CXCR4-A and CXCR4-B mRNA

mRNA from 5×10^6 PBMCs was extracted using a commercial kit (High Pure RNA Isolation Kit; Roche). Reverse transcription was carried out using random primers (Promega) and Superscript III reverse transcriptase (Invitrogen) at 50°C for 30 min, 55°C for 30 min, and 70°C for 15 min. cDNA was amplified with the appropriate primers—5′-CTTGCTGA-ATTGGAAGTGAATG-3′ and 5′-GGTGGGCAGGAAGATTTTATTG-3′ for CXCR4-A and 5′-CAGCAGGTAGCAAAGTGACG-3′ and 5′-ATG-GAGTCATAGTCCCCTGAGC-3′ for CXCR4-B—in a LightCycler 480 (Roche) with SYBR Green, following the manufacturer's recommendations. Cellular genomic S14 (5′-ACCAGTCACACGGCAGATG-3′ and 5′-GGGGAAGGAAAAGAAGGAAGAA-3′) was used for normalization. A standard curve was established by analyzing serial dilutions of a positive-control plasmid. The PCR cycle at which the amplification signal entered the exponential range was used to quantify the cellular DNA. CXCR4-A and S14 annealing were realized at 64°C, and CXCR4-B annealing occurred at 69°C. To quantify mRNA in infected cells, 5×10^6 PBMCs, at a density of 2×10^6 cells/ml, were exposed for 18 h to 350 ng p24 equivalent of the R5 strain Ad8 or the R5X4 strain 89.6, washed twice in PBS, and cultivated for 4 d.

RNA interference

HeLa cells were cultured in a 24-well plate at a density of 0.025×10^6 cells/ml and transfected with 2 nM the anti–CXCR4-B small interfering RNA (siRNA) 5′-GCAGCAGGUAGCAAAGUGA-3′ or a nontargeting pool of siRNA (Thermo-Scientific) with INTERFERin (Polyplus Transfection). Three days posttransfection, cells were infected with 10 ng/ml p24 equivalent of the X4 strain NL4.3 for 24 h, washed in PBS, and cultured; cell number was adjusted twice a week. siRNA transfection was repeated once a week.

Flow cytometry

For CXCR4 staining, 2×10^5 cells were incubated in PBS supplemented with 0.2% BSA (Sigma) and labeled with the anti-human CXCR4 mAb 12G5 or an isotype control (BD Biosciences) for 1 h on ice at a final concentration of 10 μg/ml. After washing, cells were incubated with a 1:100 dilution of Goat $F(ab')_2$ fragment anti-mouse IgG (H+L)-FITC (Beckman Coulter) for another hour on ice. Cells were then washed, fixed in BD Cell Fix, and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Chemotaxis assay

Chemotaxis of HOS cells in response to CXCL12 was measured across 8-μm-pore polycarbonate filters in 24-well Transwell chambers, as previously described (17). HOS–CXCR4-A or HOS–CXCR4-B cells were added to the upper chamber of each Transwell (0.1×10^6 cells in 100 μl 1% FCS RPMI 1640). CXCL12, at various concentrations, was added to the lower chamber under a volume of 600 μl. The plates were incubated for 4 h at 37°C in 5% CO_2. Cells that had migrated into the lower chamber were collected and enumerated with a cell counter (MACSQuant).

R5 to X4 switch

PBMCs from healthy donors (2×10^6 cells/ml) were activated with 1 μg/ml PHA-M (Sigma) and 100 IU/ml IL-2 (PeproTech) for 3 d in culture medium, exposed to the plasma of an HIV-1 R5–infected subject, and cultured in 100 IU/ml IL-2. Then, we performed a coculture between these infected PBMCs and HOS–CXCR4-A or HOS–CXCR4-B cells. HOS cell number was adjusted twice a week by the addition of uninfected HOS cells. Viral production was monitored by measurement of gag p24 in cell supernatant twice a week. R5 to X4 switch was evaluated using MT2 cells cultured at a density of 0.1×10^6 cells/ml in infected HOS–CXCR4-A and HOS–CXCR4-B cell supernatants, as previously described (7).

Statistical analysis

All data are representative of at least three different experiments. Values are expressed as the mean ± SD. Differences in infection, chemotaxis, LTR expression, and CXCR4 isoforms mRNA ratio were analyzed with the unpaired Student *t* test. Spearman rank correlations were used to evaluate the link between CXCR4-B/CXCR4-A mRNA ratio and viremia or false-positive rate (FPR).

Ethics statement

The observational study involving patients was approved by the institutional review board Sud Méditerrannée IV. Written informed consent was obtained from all participants.

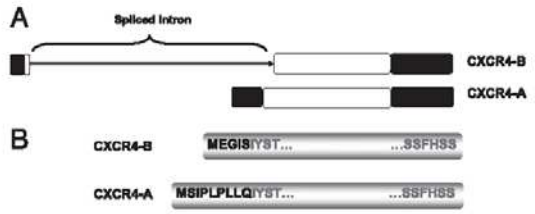

FIGURE 1. CXCR4-A and CXCR4-B isoforms. (**A**) Schematic representation of the mRNA coding for the CXCR4-B and CXCR4-A isoforms. Open boxes correspond to the coding sequences, and closed boxes to the noncoding sequences. (**B**) Aminoacid sequence of the CXCR4-A and –B isoforms.

Results

CXCR4-A is less efficient than CXCR4-B as an HIV-1 coreceptor

To study the characteristics of the two isoforms of CXCR4 (Fig. 1), we cloned the cDNA corresponding to each isoform in an HIV-1 vector and transduced $CD4^+CCR5^+$ HOS cells. Thus, we obtained two cell lines, HOS-CXCR4-A and HOS-CXCR4-B, which expressed the same cell surface density in each isoform (mean fluorescence intensity of 20 and 24, respectively, Fig. 2A–C). This remained stable over time. Thereafter, we evaluated the ability of both isoforms to support HIV-1 infection. With the aim of comparing one-round X4 infection in the two cell lines, we first exposed them to HIV-1 virions harboring a genome with a deletion in the *env* gene, a *luciferase* gene fused to the *nef* gene, and pseudotyped with an X4 envelope. Fig. 2D shows that, after a single cycle, HIV-1 expression, as quantified by luciferase activity, was higher in HOS–CXCR4-B cells than in HOS–CXCR4-A cells (luciferase activity of 93,559 ± 20,314 and 21,418 ± 7,568 arbitrary units, respectively [$p = 0.029$], for a viral input of 20 ng; luciferase activity of 177,473 ± 10,453 and 88,405 ± 15,607 arbitrary units, respectively [$p = 0.009$], for a viral input of 50 ng; and luciferase activity of 481,896 ± 35,250 and 152,031 ± 11,503 arbitrary units, respectively [$p < 0.001$], for a viral input of 100 ng). When we performed the same experiment with virions pseudotyped with an HIV-1 R5 envelope (Fig. 2E) or the G protein

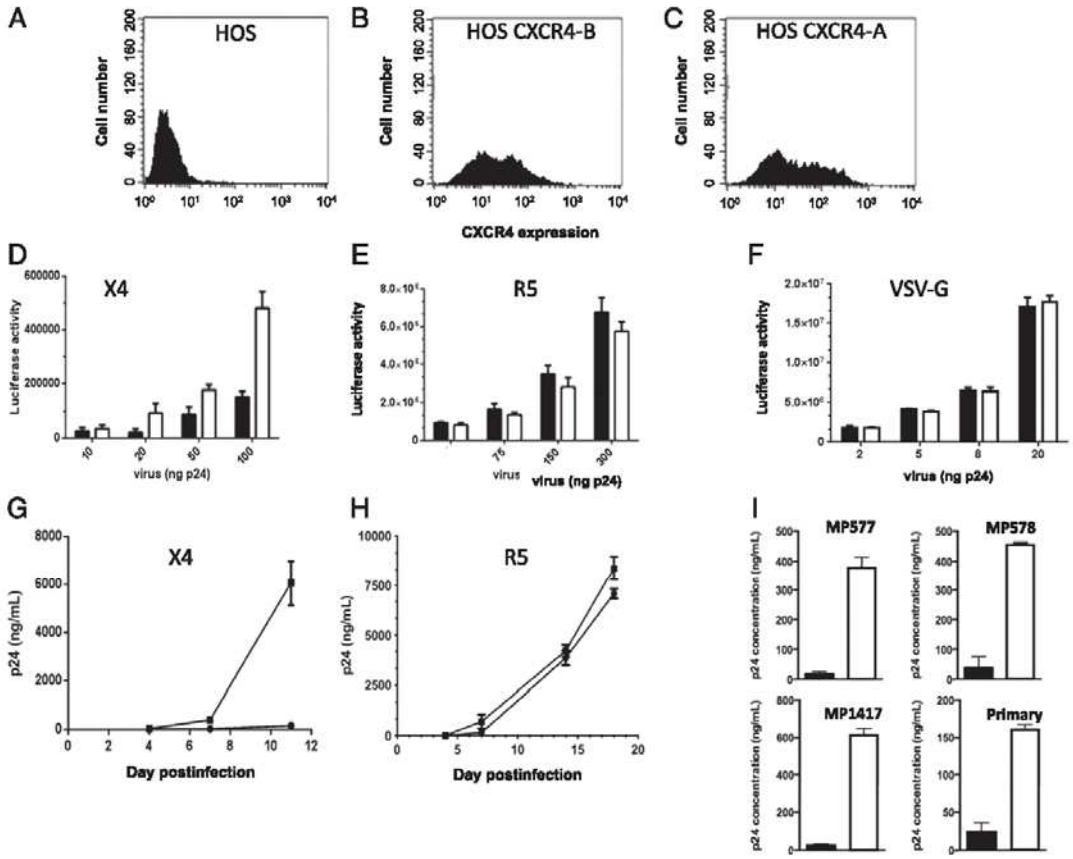

FIGURE 2. HIV-1 X4 infectability of HOS cells transduced with CXCR4-B or CXCR4-A cDNA. (**A–C**) CXCR4 expression on HOS cells transduced with CXCR4-B or CXCR4-A cDNA. CXCR4 expression at the surface of nontransduced cells (A) or cells transduced with CXCR4-B (B) or CXCR4-A (C) cDNA was analyzed by immunolabeling and flow cytometry. (**D–F**) Efficiency of one round of HIV-1 infection. HOS–CXCR4-A (filled bars) and HOS–CXCR4-B (open bars) cells were exposed to *env*-defective HIV-1 harboring the *luciferase* marker gene and pseudotyped with an HIV-1 X4 (D), an HIV-1 R5 (E), or a vesicular stomatitis virus (F) envelope at various quantities. Luciferase activity was measured in cell lysates 72 h later. (**G–I**) Efficiency of replicative HIV-1 infection. HOS–CXCR4-A (●) and HOS–CXCR4-B (■) cells were exposed to the wild-type HIV-1 X4 strain NL4.3 (G) or the wild-type HIV-1 R5 strain AD8 (H), and viral production was monitored over time by measuring gag p24 concentration in the culture supernatant. (I) The amount of virions produced by HOS–CXCR4-A (filled bars) and HOS–CXCR4-B (open bars) cells after 2 wk of infection with the subtype F2 MP577, the subtype CRF02 MP578, or the subtype G MP1417 X4 strains, as well as a primary X4 virus, was quantified by measuring gag p24 concentration in the culture supernatant.

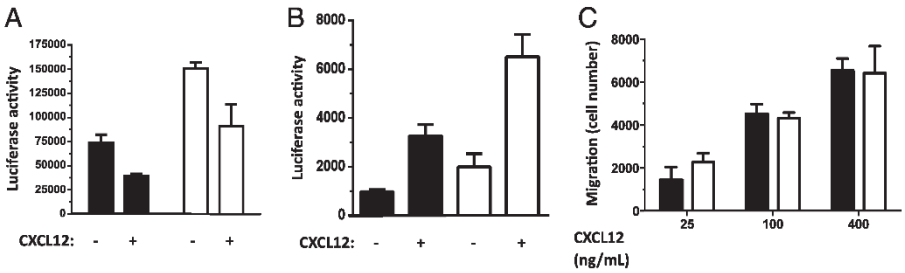

FIGURE 3. Effects of CXCL12 on HOS cells transduced with CXCR4-A or CXCR4-B cDNA. (**A**) Inhibitory effect of CXCL12 on X4 infection. HOS–CXCR4-A (filled bars) and HOS-CXCR4-B (open bars) cells were preincubated or not with 5 μg/ml of CXCL12 and subsequently exposed to an *env*-defective HIV-1 X4 virus harboring the *luciferase* marker gene and pseudotyped with an X4 envelope. Luciferase activity was measured in cell lysates 72 h later. (**B**) Activating effect of CXCL12 on HIV-1 LTR. HeLa cells harboring the *luciferase* marker gene driven by an HIV-1 LTR were transduced with CXCR4-A (filled bars) or CXCR4-B (open bars) cDNA and exposed to 1 μg/ml of CXCL12. The effect of CXCR4 signaling on LTR activity was estimated 72 h later by measuring luciferase activity in cell lysates. (**C**) Chemotactic effect of CXCL12. Migration of CXCR4-A–transduced (filled bars) or CXCR4-B–transduced (open bars) cells toward various concentrations of CXCL12.

of the vesicular stomatitis virus (Fig. 2F) instead of an HIV-1 X4 envelope, no difference in viral production was observed between the two cell lines. This difference was accentuated when we exposed the two cell lines to the replicative CXCR4-using strain NL4.3 (Fig. 1G), to three other laboratory X4 strains from different subtypes, or to a primary X4 strain (Fig. 2I). In contrast, a wild-type R5 strain replicated as efficiently in HOS–CXCR4-B cells as in HOS–CXCR4-A cells (Fig. 2H).

CXCR4-A is as efficient as CXCR4-B as a chemokine receptor

We wanted to know whether the response of the two isoforms of CXCR4 to their natural ligand, the chemokine CXCL12, was similar. First, we compared the ability of CXCL12 to inhibit X4 infection in both cell lines. Fig. 3A shows that preincubation of HOS–CXCR4-A and HOS–CXCR4-B cells with a suboptimal concentration of CXCL12 inhibited viral production with the same efficiency (inhibition of 53 ± 10% and 56 ± 6%, respectively, $p = 0{,}810$). Second, because CXCR4 activation was shown to induce HIV gene expression (18), we analyzed the effect of CXCL12 binding to each CXCR4 isoform on LTR activation. To this end, we transduced $CD4^-CCR5^-$ HeLa cells stably transfected with a reporter *luciferase* gene driven by the HIV-1 LTR with CXCR4-A or with CXCR4-B cDNA, exposed them to 1 μg/ml of CXCL12, and measured luciferase activity 24 h later. As shown in Fig. 3B, LTR activity was enhanced to a similar level upon CXCL12 exposure in both cell lines (3.33 ± 0.51-fold and 3.27 ± 0.8-fold, respectively, $p = 0.953$). Third, we measured the chemotactic effect of CXCL12 on both cell lines. HOS–CXCR4-A and HOS–CXCR4-B cells migrated toward increasing concentrations of the chemokine in a similar fashion (1448 ± 418 and 2266 ± 291 cells, respectively [$p = 0.249$], at a CXCL12 concentration of 25 ng/ml; 4527 ± 314 and 4323 ± 177 cells, respectively [$p = 0.629$], at a CXCL12 concentration of 100 ng/ml; and 6545 ± 389 cells and 6417 ± 891 cells, respectively [$p = 0.907$], at a CXCL12 concentration of 400 ng/ml, Fig. 3C). These findings indicate that, although CXCR4-A is used less efficiently than CXCR4-B by X4 virions to infect $CD4^+$ cells, the responses of both isoforms to CXC12 are undistinguishable in terms of inhibition of HIV infection, LTR activation, and chemotaxis.

X4 entry is more efficient in CXCR4-B–expressing cells than in CXCR4-A–expressing cells

The CXCR4 N-term region is involved in X4 envelope binding to CXCR4 (12–14). Because the two CXCR4 isoforms do not have the same sequence at this extremity, we tested whether this resulted in a difference in X4 infectivity due to entry kinetics. To test this hypothesis, we exposed both cell lines to a wild-type X4 strain in the presence or absence of 1 μM of the CXCR4 antagonist AMD3100, which is known to inhibit the binding of X4 envelopes to CXCR4. As expected, 72 h postinfection the HOS–CXCR4-B cells produced more virus (72.7 ± 0.6 ng/ml) than the HOS–CXCR4-A cells (32.9 ± 0.5 ng/ml, $p < 0.001$), and cells preincubated with the CXCR4 antagonist produced no virions (Fig. 4A). At 17 h postinfection, we used quantitative PCR to measure the amount of early reverse transcripts harbored by the cells. As shown in Fig. 4B, nearly twice as many early reverse transcripts were detected in HOS–CXCR4-B cells (2.39 ± 0.17 arbitrary units) than in HOS–CXCR4-A cells (1.52 ± 0.05 arbitrary units, $p = 0.007$). We

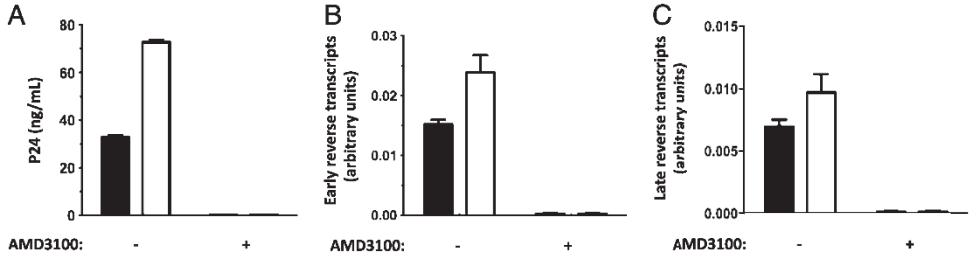

FIGURE 4. Efficiency of HIV-1 X4 entry and reverse transcription in HOS cells transduced with CXCR4-A or CXCR4-B cDNA. HOS–CXCR4-B and HOS–CXCR4-A cells were preincubated or not with the CXCR4 antagonist AMD3100 and exposed to the wild-type X4 strain NL4.3. Seventeen hours postinfection, the quantity of early (**B**) and late (**C**) reverse transcripts was determined in cell lysates by quantitative PCR. (**A**) To control infection, the amount of gag p24 Ag was measured 3 d later in the culture supernatant.

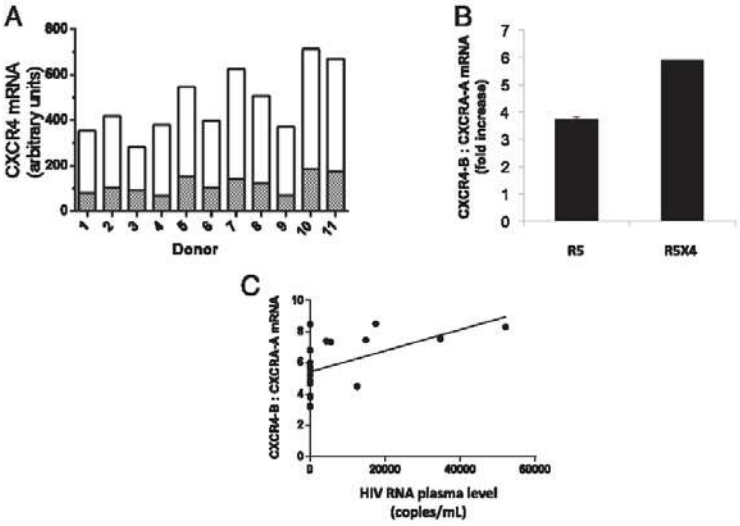

FIGURE 5. CXCR4-A and CXCR4-B mRNA expression during the course of HIV-1 R5 infection. (**A**) Expression of the mRNA encoding CXCR4-A (stippled pattern) and CXCR4-B (open bar) in PBMCs of 11 healthy donors was quantified by RT-PCR. (**B**) Increase in CXCR4-B/CXCR4-A mRNA ratio in PBMCs of a healthy donor infected in vitro by the R5 strain AD8 or the R5X4 strain 89.6 compared with noninfected PBMCs. (**C**) Correlation between CXCR4-B/CXCR4-A mRNA ratio in PBMCs of 19 HIV-1 R5–infected persons and their viral load. The lower viral RNA plasma concentrations were <20 copies/ml for nine patients and 39, 54, and 101 copies/ml for the others.

also quantified late reverse transcripts in both cell lines and noted that the difference persisted but did not increase ($p = 0.039$, Fig. 4C). Neither early nor late reverse transcripts were amplified in AMD3100-treated cells (Fig. 4B, 4C). We concluded that the CXCR4-B isoform allowed more efficient X4 virion entry into target cells than did the CXCR4-A isoform.

R5 infection induces CXCR4-B expression in vitro and in vivo

The level of physiological expression of the two CXCR4 isoforms was measured by quantitative RT-PCR in the PBMCs of healthy donors. Fig. 5A shows that the PBMCs of all of the volunteers expressed both isoforms, the proportions of CXCR4-A and CXCR4-B forms being 10–30% and 70–90%, respectively. Next, we studied the effect of R5 infection on this expression. For this purpose, we exposed the nonactivated PBMCs of a healthy donor to an R5 strain. At day 6, we detected viral production in the culture supernatant. At that time, R5 infection induced an increase in the CXCR4-B/CXCR4-A mRNA ratio in the PBMCs (Fig. 5B). Concurrently, we infected the same PBMCs with the R5X4 strain 89.6. R5X4 infection also induced an increase in the CXCR4-B/CXCR4-A mRNA ratio (Fig. 5B). We then quantified the CXCR4-B/CXCR4-A mRNA ratio in the PBMCs of 19 individuals infected with R5 strains, either under antiretroviral therapy or not. We observed a correlation between this ratio and viral load of these patients ($r = +0.601$, $p = 0.054$, Fig. 5C). Thus, R5 infection favors CXCR4-B expression over CXCR4-A expression in vitro, as well as in vivo.

CXCR4-B induces R5 to X4 switch more rapidly than CXCR4-A

CXCR4 overexpression facilitates X4 replication (7). Moreover, we showed that CXCR4 overexpression at the surface of target cells infected with an R5 strain favors the emergence of X4 strains (6, 7). In line with these observations, because X4 strains replicate more easily in CXCR4-B^+ cells than in CXCR4-A^+ cells, we questioned whether the presence of CXCR4-B at the surface of R5-infected cells could facilitate R5 to X4 switch compared with CXCR4-A. To answer this question, we exposed HOS–CXCR4-A and HOS–CXCR4-B cells to an R5 strain and monitored the appearance of CXCR4-using strains in the cell supernatant. As shown in Fig. 6A, X4 strains, identified by their ability to induce syncytia in $CD4^+CCR5^-CXCR4^+$ MT2 cells, emerged more rapidly in the HOS–CXCR4-B culture than in the HOS–CXCR4-A culture. The overexpression of CXCR4-B in the course

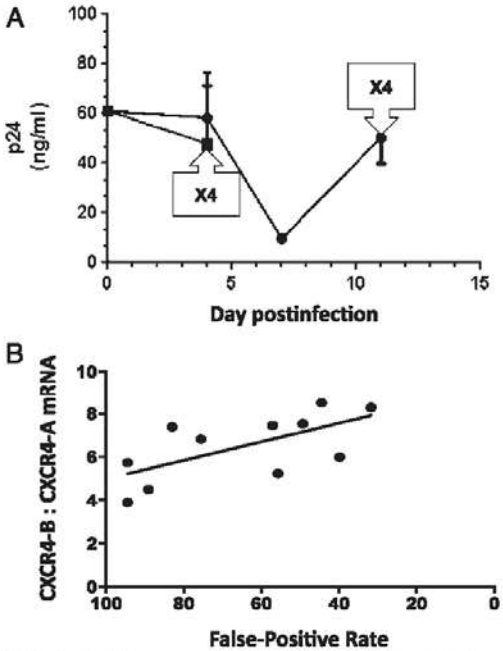

FIGURE 6. CXCR4-B expression and R5 to X4 switch. (**A**) Delay of R5 to X4 switch in HOS–CXCR4-A and HOS–CXCR4-B cells. HOS–CXCR4-A (●) and HOS–CXCR4-B (■) cells were infected with a primary R5 strain, and viral production was monitored over time by measuring gag p24 concentration in the culture supernatant. The emergence of X4 virions, monitored by infecting MT2 cells with the culture supernatant, is indicated. (**B**) Correlation between CXCR4-B/CXCR4-A mRNA ratio in PBMCs of 11 HIV-1 R5–infected persons and the R5/X4 phenotype of their circulating virions, as determined by the FPR calculated by the Geno2pheno algorithm.

of R5 infection might increase the risk for emergence of CXCR4-using strains in vivo. A surrogate marker of the risk for R5 to X4 switch is the FPR score, calculated by the Geno2pheno algorithm using the sequence of the V3 region of the envelope. The FPR corresponds to the risk for falsely predicting as R5 a virus that is, in fact, X4. Recently, the FPR score was shown to be correlated with the percentage of X4 strains detected by ultradeep sequencing (19). Therefore, we measured the CXCR4-B/CXCR4-A ratio of mRNA in the PBMCs of 11 R5-infected individuals and compared it with their FPR score. As shown in Fig. 6B, we observed a strong inverse correlation between these two parameters ($r = -0.633$, $p = 0.039$).

The specific knockdown of CXCR4-B as an anti-HIV therapy

Because both CXCR4 isoforms are expressed in $CD4^+$ T cells and mediate chemotaxis toward CXCL12, whereas only the B isoform supports a highly productive HIV-1 X4 infection, the specific downregulation of CXCR4-B could be envisaged as an anti–HIV-1 X4 strategy. To test this possibility, we designed an siRNA complementary with a sequence in CXCR4-B that is absent in CXCR4-A and transfected it into $CD4^+$ HeLa-P4 cells that coexpress both CXCR4 isoforms. The siRNA resulted in downregulation of the CXCR4-B mRNA (from 13.8 ± 0.1 to 5.2 ± 0.1 arbitrary units, $p < 0.001$, Fig. 7B), whereas CXCR4-A remained unaffected (Fig. 7A). Consequently, we observed a decrease in cell surface CXCR4 density (Fig. 7C–E). Specific inhibition of the CXCR4-B isoform resulted in a drastic decrease in X4 infectability of HeLa-P4 cells, as shown by the difference in viral production at day 7 postinfection (764 ± 109 versus 266 ± 44 ng/ml, $p = 0.013$) and day 11 postinfection (1448 ± 181 versus 283 ± 100 ng/ml, $p = 0.005$, Fig. 7F).

Discussion

In the current study, we provide evidence that the B isoform of CXCR4 is used by HIV-1 strains to enter and productively infect target cells much more efficiently than the A isoform, whereas both isoforms are used with the same efficiency by the natural ligand CXCL12; HIV-1 R5 infection favors CXCR4-B expression over that of CXCR4-A, a phenomenon that facilitates the emergence of CXCR4-using HIV-1 strains; and it is possible to downregulate CXCR4-B mRNA without modifying CXCR4-A mRNA expression.

Our observation that the chemotactic response of $CXCR4\text{-}A^+$ and $CXCR4\text{-}B^+$ cells to CXCL12 is similar is in contradiction to the data reported by Gupta and Pillarisetti (11). One possible explanation for this discrepancy is a technical difference; they worked with stably transfected rat basophil leukemia cell lines, whereas we worked with human cells transduced with the open reading frames of each CXCR4 isoform. Stable transfectants need to be selected, and this selection may randomly isolate clones deficient for characteristics other than the one for which they are being selected. In Gupta and Pillarisetti's study (11), no positive control verified that the chemotactic defect observed in the cells stably transfected with the CXCR4-A open reading frame was specific. In contrast, as shown in Fig. 2, all of the cells that we transduced expressed CXCR4, and we did not need to perform any selection to obtain cells expressing one or the other CXCR4 isoform. Moreover, we confirmed this result with two other assays in which we measured the ability of each CXCR4 isoform to mediate the effect of CXCL12 on HIV-1 infection and HIV-1 LTR activation.

The gp120 and CXCL12 binding sites overlap but remain distinct, and the involvement of the N-term in CXCR4 coreceptor activity is the present paradigm. Substitution of the CXCR4 N-term with the one derived from CCR5 impairs the fusion capability of X4 strains (12). Moreover, Tyr-7, Tyr-12, and Tyr-21 (13), as well as Glu-15 and Glu-32 (14), play a role in CXCR4-mediated viral entry. In contrast, Doranz et al. (20) reported that the first 27 residues of CXCR4 are not necessary for activation by CXCL12.

Interestingly, the induction of CXCR4-B expression over that of CXCR4-A as a consequence of HIV-1 R5 infection could be

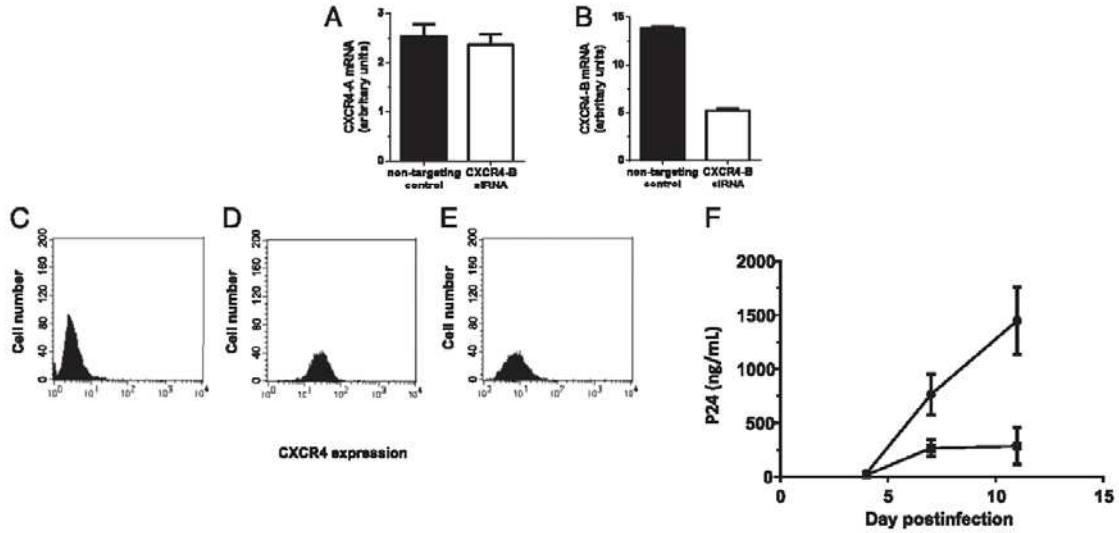

FIGURE 7. Specific downregulation of CXCR4-B by RNA interference. HeLa-P4 cells were transfected with an siRNA specific for CXCR4-B mRNA or a negative-control siRNA. CXCR4-A (**A**) and CXCR4-B (**B**) mRNA were quantified in transfected cells. CXCR4 expression at the surface of cells transfected with the nonspecific (**D**) or the specific (**E**) siRNA were analyzed by immunolabeling and flow cytometry. (**C**) HeLa cells labeled with an irrelevant isotype-matched Ab were used as a negative control. (**F**) HeLa-P4 cells transfected with the nonspecific (●) or the specific (■) siRNA were exposed to the wild-type HIV-1 X4 strain NL4.3, and viral production was monitored over time by measuring gag p24 concentration in the culture supernatant.

a factor that facilitates the emergence of CXCR4-using strains. Combined with the increase in $CD4^+$ T cell surface CXCR4 density observed in one third of the patients during the course of the infection, this preferential expression of the CXCR4 isoform, which is the most efficient as an HIV-1 coreceptor, could favor the expansion of X4 strains. The increase in $CD4^+$ T cell surface CXCR4 density and in CXCR4-B expression over that of CXCR4-A could promote X4 emergence via a common mechanism. This mechanism is probably a higher amplification of the rare HIV-1 strains able to use CXCR4 as a coreceptor, that randomly appear as a consequence of mutations in the *envelope* gene of R5 strains rather than via the induction of these mutations. Of note, the increase in CXCR4-B/CXCR4-A ratio under R5 infection is clearly not limited to infected cells. If this were the case, and because <1% of the circulating $CD4^+$ T cells are infected in vivo, we could not have detected any correlation between this ratio and HIV RNA plasma level. The molecular mechanisms responsible for the variability in CXCR4-B/CXCR4-A ratio among the tissues, as well as for its increase under HIV-1 R5 infection, remain to be elucidated.

The discrepancy between the dual nature of CXCR4 as an HIV-1 coreceptor and its chemokine receptor functions opens a potential new therapeutic strategy. The use of CXCR4 antagonists in HIV-1 infection has been limited by the fact that they blocked CXCL12 function. Using a complementary siRNA, we were able to specifically downregulate CXCR4-B expression. By transducing hematopoietic stem cells with a transgene encoding such a short hairpin RNA, together with another transgene coding for CXCR4-A, it will be possible to generate cells that specifically express the CXCR4-A isoform, are partly resistant to HIV-1 X4 infection, and are able to respond to CXCL12 signals. Such a strategy could be combined with the gene-therapeutic approaches that are currently being tested to make target cells resistant to HIV-1 R5 infection.

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Disclosures

The authors have no financial conflicts of interest.

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VI. Annexe 2 : étude ACTIVIH

A. Etat des connaissances

L'infection par le VIH-1 s'accompagne d'une activation immunitaire généralisée. Cette activation est réduite sous traitement antirétroviral efficace mais souvent de façon incomplète. Or cette activation immunitaire résiduelle a deux effets délétères. D'une part elle entretient le déficit immunitaire. D'autre part elle fait le jeu de morbidités dites non liées au SIDA comme l'athérothrombose, les troubles neuro-cognitifs, la stéatose hépatique, le syndrome métabolique, l'ostéoporose, l'atteinte rénale, l'insuffisance de production hématopoïétique, une fragilité générale, voire certains cancers.

Précédemment les études avaient analysé, chez les patients avirémiques sous traitement antirétroviral, appelés « répondeurs virologiques », certaines causes, certains types, et certaines conséquences de cette activation immunitaire. Nous avons réalisé maintenant une étude globale des causes, types et conséquences de l'activation immunitaire. Notre objectif général a été d'établir des corrélations entre les causes de l'activation immunitaire chez les patients avirémiques sous traitement, le type d'activation (T CD4+, T CD8+, B, NK, monocytaire, polynucléaire, endothéliale, plaquettaire, inflammatoire) et les conséquences de cette activation (déficit immunitaire persistant et morbidités non liées au déficit immunitaire). Ces corrélations devraient permettre de proposer des mécanismes physiopathologiques (par exemple les lipopolysaccharides issus de la translocation bactérienne activent les monocytes via TLR4, lesquels monocytes produisent du facteur tissulaire favorisant l'athérothrombose) qui pourront être testés expérimentalement. La mise en évidence de ces mécanismes pourrait déboucher sur de nouvelles stratégies thérapeutiques les ciblant (dans le cas ci-dessus par exemple l'emploi d'antagonistes de TLR4). Ce travail pourrait permettre aussi d'identifier des marqueurs biologiques prédictifs de complications favorisées par l'activation immunitaire chronique et ainsi d'améliorer le dépistage de ces complications pour une prise en charge plus précoce.

1. Les causes d'activation immunitaire résiduelle chez les répondeurs virologiques

Les principales causes d'activation immunitaire chez les répondeurs virologiques sont :

- la production de produits viraux (Palmer et al. 2008) pouvant stimuler le système immunitaire soit par la réponse immunitaire spécifique (stimulation xénogénique), soit par effet activateur direct (activation des cellules dendritiques plasmacytoïdes par l'ARN rétroviral par exemple)

- l'infection *de novo* voire la réplication rétrovirale persistante, ont également un effet activateur direct sur les cellules infectées ; en effet, l'accumulation de rétrotranscrits même lors d'un cycle d'infection avorté, induit l'apoptose via la voie des caspases et une réaction inflammatoire (Doitsh et al. 2010)

- la présence d'agents pathogènes responsables de coinfections actives, en particulier du cytomégalovirus (CMV) et du virus de l'hépatite C (HCV) induiraient une costimulation immunitaire permanente durant l'infection par le VIH-1, qui pourrait expliquer la progression plus rapide de cette dernière chez les patients coinfectés ; on peut alors parler de « charge des copathogènes »

- la translocation microbienne, qui est le passage des produits microbiens de la lumière intestinale vers l'organisme, secondaire à la destruction de la barrière immunitaire que constitue le tissu lymphoïde associé au tube digestif (GALT) dès les stades précoces de l'infection par le VIH-1 (Brenchley et al. 2004)

- la lymphopénie T CD4+ probablement via la production d'interleukine (IL)-7, qui a une activité proinflammatoire (Sereti et al. 2009) ; en effet, l'administration d'IL-7 chez les non répondeurs immunologiques sous HAART induit non seulement une meilleure reconstitution immunitaire, mais également une augmentation de l'activation cellulaire T CD8+

- l'immunosénescence; le profil immunologique des personnes âgées présente de nombreuses similitudes avec celui des sujets infectés par le VIH-1; la sénescence, et en particulier l'immunosénescence, est un facteur additionnel à l'origine de l'activation immunitaire (Effros, Cai, and Linton 2003), essentiellement via un phénotype proinflammatoire

- un déficit en cellules T CD4+ de type régulatrices (Treg) ; les cellules Treg exercent un effet inhibiteur sur les autres sous-populations T CD4+ (Th1, Th2, Th17) et une carence en activité T CD4+ régulatrice pourrait favoriser l'activation immunitaire (Kanwar, Favre, and McCune 2010).

- des perturbations métaboliques activatrices pour le système immunitaire comme l'augmentation des taux de lipoprotéines oxydées.

2. Les types d'activation immunitaire

Tous les acteurs, centraux ou périphériques, de l'immunité peuvent être activés au cours de l'infection chronique par le VIH-1: cellules T et NKT, cellules B, cellules NK, monocytes/macrophages, cellules dendritiques, polynucléaires, plaquettes, cellules endothéliales, cellules épithéliales, inflammation, coagulation, etc… Cet état d'activation immunitaire contraste souvent avec une capacité réduite de développer une réponse immunitaire efficace contre un stimulus spécifique. Même si le traitement antirétroviral améliore cette situation, habituellement il ne la normalise pas.

Lors de l'infection par le VIH-1 les cellules T sont soumises à une stimulation immunitaire répétée étant à l'origine de la sénescence cellulaire T, ainsi que de la limitation du répertoire cellulaire T (croissance continue des cellules mémoires spécifiques d'un nombre limité d'antigènes et épuisement des réserves des sous-populations cellulaires naïves) (Gorochov et al. 1998). La destination finale de la majorité des cellules T CD4+ activées est l'apoptose consécutive à l'activation. Même si les cellules T CD8+ expriment également des marqueurs d'activation, d'apoptose et de sénescence, elles possèdent une plus grande capacité de prolifération et de survie à l'activation (Kovacs et al. 2001) comparativement aux cellules T CD4+.

Les cellules B sanguines surexpriment également des marqueurs d'activation (Lane et al. 1983), de prolifération (Ho et al. 2006), de différenciation (Moir et al. 2008) et d'apoptose (Moir et al. 2004). En conséquence, le taux des immunoglobulines plasmatiques et des autoanticorps circulants est-il augmenté (Ng 1996), ainsi que le risque de pathologies hématologiques malignes de type B (Biggar 2001).

L'activation de la lignée monocytaire-macrophagique chez les sujets infectés paraît être responsable du défaut de chimiotactisme (Smith et al. 1984) et de phagocytose (Musher et al. 1990), de la diminution du métabolisme oxydatif (T. P. Chen et al. 1993) et de la capacité microbicide (Pittis et al. 1997), ainsi que de la prépondérance des phénotypes pro-inflammatoires des ces deux populations cellulaires (Canque et al. 1996; Weiss et al. 1989; Thieblemont et al. 1995).

Les polynucléaires neutrophiles présentent des capacités microbicides altérées (Mastroianni et al. 2000), contrastant avec une réponse inflammatoire exagérée à certains stimuli (Cassone et al. 1997).

Les cellules endothéliales peuvent être activées par des cytokines inflammatoires (Krishnaswamy et al. 1999) et certaines protéines virales (tat, nef et gp120) (Bussolino et al. 2001). Elles secrètent alors des cytokines et des chimiokines (Krishnaswamy et al. 1999).

Les plaquettes présentent des signes d'activation, essentiellement chez les personnes virémiques (Karpatkin, Nardi, and Green 2002), et peuvent être à l'origine d'un état d'hypercoagulabilité caractérisé par l'augmentation des D-dimères (Kuller et al. 2008) persistante sous HAART (Neuhaus et al. 2010).

D'une manière plus globale, les niveaux des cytokines pro-inflammatoires sont élevés dans le plasma (Kuller et al. 2008; Emilie et al. 1990), le liquide cérébro-spinal (Corasaniti et al. 2003) et les ganglions lymphatiques (Emilie et al. 1990).

Nous avons retenu pour cette étude les marqueurs d'activation immunitaire qui ont été identifiés comme pouvant persister sous traitement antirétroviral efficace.

3. Les conséquences de l'activation immunitaire

L'activation immunitaire résiduelle sous traitement antirétroviral efficace peut avoir deux types de conséquence :

- la persistance de la destruction de cellules T CD4+ (Février, Dorgham, and Rebollo 2011) pouvant aboutir à une moindre réponse immunologique au traitement et de façon plus générale à un déficit immunitaire résiduel. Ainsi, le niveau d'activation immunitaire globale ou cellulaire T CD8+ est un meilleur facteur prédictif de la progression de la maladie que la charge virale (Giorgi et al. 1999).
- le développement de pathologies non liées au SIDA. De nombreuses études ont établi que l'activation immunitaire chez les patients infectés par le VIH-1 traités ou non était associée à certaines morbidités non liées au SIDA (Deeks and Phillips 2009; Deeks 2011; Effros et al. 2008). Parmi celles-ci, nous avons choisi de nous concentrer sur l'atteinte hépatique, le syndrome métabolique, l'ostéoporose et les troubles rénaux. Il faut ajouter que certains traitements antirétroviraux peuvent accroître le risque de développement de certaines de ces comorbidités.

B. Hypothèses de l'étude

Les hypothèses de base de cette étude ont été les suivantes :

1 - différents facteurs étiologiques peuvent se combiner chez chaque répondeur virologique pour induire de l'activation immunitaire

2 – on pourrait alors distinguer chez ces patients différents profils d'activation immunitaire, conséquences de combinaisons particulières de facteurs étiologiques

3 – certaines étiologies pourraient donc causer préférentiellement certains profils d'activation immunitaire et certains profils d'activation immunitaire pourraient alors causer préférentiellement certaines morbidités.

C. Objectifs de l'étude

Notre principal objectif a été d'évaluer, chez des patients infectés par le VIH-1 et avirémiques sous traitement les formes (T CD4+, T CD8+, B, NK, monocytaire, polynucléaire, endothéliale, plaquettaire, inflammatoire) de l'activation immunitaire généralement présente chez ces patients et de voir si l'on pouvait définir des profils d'activation immunitaire.

Notre objectif secondaire a été d'explorer si certains de ces profils d'activation immunitaire pouvaient être reliés à certaines morbidités.

D. Critères de jugement

1. Diagnostic des causes d'activation immunitaire

- *Production virale résiduelle persistante* : présence d'ARN VIH plasmatique détectable
- *Translocation microbienne* : quantification de l'ADN bactérien plasmatique et diagnostic d'espèce bactérienne par PCR 16sRNA. La quantification de l'ADN bactérien se fait à l'aide d'une PCR utilisant des amorces amplifiant les parties communes du génome des différentes espèces bactériennes ; le diagnostic d'espèce est réalisé à l'aide d'une amplification par PCR des parties spécifiques de chaque génome bactérien, suivie d'un séquençage des amplicons.
- *Immunosénescence:* détermination du nombre et du pourcentage de cellules T CD4+ et T CD8+ circulantes naïves (CD45RA+) et mémoires (CD45RA-), sénescentes (CD57+)
- Déficit en Treg: détermination du nombre et du pourcentage de cellules T CD4+CD25hiCD127lo
- *Coinfections* HBV, HCV, HEV, CMV, EBV

2. Diagnostic des types d'activation immunitaire

- *Activation T CD4+ et T CD8+*: détermination du nombre et du pourcentage de cellules T CD4+ et T CD8+ circulantes activées (HLA-DR+ et/ou CD38+)
- *Activation B* : mesure de la concentration plasmatique d'immunoglobulines
- *Activation NK* : détermination du nombre et du pourcentage de cellules NK circulantes activées (HLA-DR+ et/ou CD69+), sénescentes (CD57+CD56-)
- *Activation monocytaire* : mesure de la concentration plasmatique de sCD14, sCD163
- *Activation polynucléaire* : détermination du nombre et du pourcentage de polynucléaires circulants CD64+ et de la densité membranaire en CD64 des polynucléaires CD64+
- *Activation endothéliale* : mesure de la concentration sanguine des complexes [t-PA/PAI-1], de la thrombomoduline soluble et de l'EPCR soluble
- *Activation plaquettaire* : détermination du nombre de microparticules d'origine plaquettaire
- *Inflammation* : mesure de la concentration plasmatique d'IL-6, de CRP (ultrasensible), de sTNR1et d'IFNα

3. Diagnostic des marqueurs biologiques des comorbodités

- Bilan clinique de syndrome métabolique : tension artérielle, tour de taille, tour de hanche
- Bilan biologique de syndrome métabolique : glycémie à jeun, HbA1c, triglycérides, LDL-cholestérol, HDL-cholestérol, insulinémie

E. Perspectives

Cette étude a permis d'avoir une vue d'ensemble des causes et types de l'activation immunitaire chez les répondeurs virologiques. La thématique de l'activation immunitaire a un

intérêt particulier sur ce terrain, avec des multiples implications cliniques. Si on objective des causes persistantes d'activation immunitaire, on pourra suggérer un traitement spécifique pour chaque cause afin de les contrôler et limiter ainsi les conséquences en termes de déficit immunitaire persistant et morbidités non liées au déficit immunitaire.

Notre étude transversale va être complétée par une étude longitudinale. Le suivi des morbidités apparaissant au cours du temps au sein de la même cohorte de patients permettra de mettre en évidence de biomarqueurs prédictifs de ces morbidités. De plus des sous-études de la morbidité cardio-vasculaire, hépatique et neuro-cognitive feront l'objet de projets ultérieurs au sein de la même population de patients, afin d'établir des associations possibles entre le profil immunitaire et les différents types de comorbidités.

Par ailleurs, il est très probable que les voies pathogènes que nous allons étudier, reliant activation immunitaire chronique et les morbidités citées, soient une des clefs du vieillissement "physiologique", ce qui pourrait être confirmé par d'autres études sur d'autres cohortes de patients.

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Research Paper

One of the immune activation profiles observed in HIV-1-infected adults with suppressed viremia is linked to metabolic syndrome: The ACTIVIH study☆

Christina Psomas [a,b,1], Mehwish Younas [a,1], Christelle Reynes [c], Renaud Cezar [d], Pierre Portalès [e], Edouard Tuaillon [f], Adeline Guigues [a], Corinne Merle [b], Nadine Atoui [b], Céline Fernandez [b], Vincent Le Moing [b,g,h], Claudine Barbuat [i], Grégory Marin [j], Nicolas Nagot [j], Albert Sotto [h,i], Jean-François Eliaou [e,h], Robert Sabatier [c], Jacques Reynes [b,g,h], Pierre Corbeau [a,d,h,*]

[a] Institute for Human Genetics, CNRS UPR1142, 141 rue de la Cardonille, 34396, Montpellier cedex 5, France
[b] Infectious Diseases Department, University Hospital, 80 avenue A. Fliche, 34295 Montpellier cedex 5, France
[c] Institute for Functional Genomics, Montpellier University, UMR5203, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France
[d] Immunology Department, University Hospital, Place du Pr Debré, 30029 Nîmes cedex, France
[e] Immunology Department, University Hospital, 80 avenue A. Fliche, 34295 Montpellier cedex 5, France
[f] Microbiology Department, University Hospital, 371 Av. du Doyen Gaston Giraud, 34295 Montpellier cedex 5, France
[g] IRD UMI 233, INSERM U1175, Montpellier University, 911 avenue Agropolis, 34294 Montpellier cedex 5, France
[h] Montpellier University, 5 Boulevard Henri IV, 34967 Montpellier cedex 2, France
[i] Infectious Diseases Department, University Hospital, Place du Pr Debré, 30029 Nîmes cedex, France
[j] Medical Informatics Department, University Hospital, 39 Avenue Charles Flahault, 34090 Montpellier cedex 5, France

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ABSTRACT

Immune activation in HIV-1-infected individuals is reduced under antiretroviral therapies, but persists, resulting in various morbidities. To better characterize this phenomenon, using a panel of 68 soluble and cell surface markers, we measured the level of activation in circulating CD4+ and CD8+ T cells, B cells, monocytes, NK cells, polynuclear and endothelial cells as well as of inflammation and fibrinolysis in 120 virologic responders over 45 years of age. As compared with age- and sex-matched uninfected individuals, we observed a persistence of activation in all the cell subpopulations analyzed, together with marks of inflammation and fibrinolysis. Two independent hierarchical clustering analyses allowed us to identify five clusters of markers that varied concurrently, and five patient groups, each with the same activation profile. The five groups of patients could be characterized by a marker of CD4+ T cell, CD8+ T cell, NK cell, monocyte activation or of inflammation, respectively. One of these profiles was strongly associated with marks of metabolic syndrome, particularly with hyperinsulinemia (OR 12.17 [95% CI 1.79–82.86], $p = 0.011$). In conclusion, our study unveils biomarkers linked to metabolic syndrome that could be tested as predictive markers, and opens the way to new therapeutic approaches tailored to each patient group.

1. Introduction

Today, about 90% of treated persons living with HIV are aviremic (Delaugerre et al., 2015). Yet, 6 to 24% of these virologic responders do not restore their CD4 count as they should, exposing them to the risk of immune deficiency-linked morbidities (Corbeau and Reynes, 2011). The main reason for that is the persistence of a state of hyperactivity of the immune system in these patients (Corbeau and Reynes, 2011). This residual immune activation also fuels the so-called non-AIDS-linked morbidities such as atherothrombosis, osteoporosis, metabolic syndrome, neurocognitive disorders, liver steatosis, kidney failure, frailty, and some types of cancer (Hunt, 2012). These comorbidities are now the major cause of morbi-mortality in treated patients (Lau et al., 2007).

HIV infection is characterized by the high level of immune activation induced in the human organism. Various causes may combine to provoke this immune hyperactivity (Younas et al., 2015). A first cause is the presence of the virus itself, detected by the immune system as a xenoantigen and a danger. For example, gp120 has recently been shown to activate monocytes and macrophages via TLR4 (Del Corno et al., 2016). A second cause is the infection process that triggers a DNA detection system resulting in inflammatory cytokine production (Doitsh

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* Corresponding author at: Institute for Human Genetics, CNRS UPR1142, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France.
E-mail address: pcorbeau@igh.cnrs.fr (P. Corbeau).

[1] CP and MY contributed equally to this work.

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et al., 2014). A third cause is the virus-induced deterioration of the gut barrier that opens the way for intestinal microbes to enter the human organism (Zevin et al., 2016). A fourth cause is the microbes that co-infect the organism (Boulougoura and Sereti, 2016), as for instance hepatitis (Kovacs et al., 2008) or herpes viruses (Sheth et al., 2008). A fifth set of causes is linked to some effects of HIV on the immune system itself: CD4+ T lymphopenia (Zonios et al., 2008; Sereti et al., 2009), immunosenescence (Effros et al., 2003) and an imbalance in the CD4+ T cell subpopulations (Kanwar et al., 2010). Finally, metabolic dysregulation may also be a source of immune activation (Aounallah et al., 2016; Dagenais-Lussier et al., 2015; Palmer et al., 2016). For instance, lipid peroxidation may fuel monocyte activation (Zidar et al., 2015). Moreover, the oxidative stress in the course of HIV infection might increase inflammatory cytokines production (Chaudhri and Clark, 1989). Combined antiretroviral therapies (cART) reduce all the sources of immune activation without abolishing them (Palmer et al., 2008; Guadalupe et al., 2003; Gonzalez et al., 2009; Stone et al., 2005). Consequently, immune activation is only partly downregulated in aviremic patients under treatment.

Previous works have shed a partial light on this phenomenon. The adaptative and the innate immune systems, including inflammation, are both involved. T cell activation has been particularly studied. Many authors have shown the persistence of marks of CD4+ and CD8+ T cell activation (Hunt et al., 2003), exhaustion (Cockerham et al., 2014), and senescence (Stone et al., 2005) under cART. The same is true for NK cells (Lichtfuss et al., 2012; Mavilio et al., 2005). Likewise, B cells remain unquiet in virologic responders, as testified by the presence of hyperglobulinemia (De Milito et al., 2004). In the same type of patients, high peripheral blood levels of soluble CD14 (sCD14) and soluble CD163 (sCD163) have also been reported (Lichtfuss et al., 2012; Burdo et al., 2011; Rajasuriar et al., 2010; Wallet et al., 2010; Kamat et al., 2012), considered as signs of monocyte/macrophage hyperactivity. Even neutrophils present with marks of activation in HIV-infected subjects, including high cell surface expression of the program death ligand 1 PD-L1 (Bowers et al., 2014) and of the adhesion molecules CD11b and CD18, as well as low cell surface expression of CD62L (Elbim et al., 1994), and PD-L1 expression has been shown to remain elevated on ART (Bowers et al., 2014). Inflammation is also evident in HIV patients, and for instance the routine marker CRP and the soluble form of TNFα receptor II has been reported to be incompletely reduced under treatment (Brazille et al., 2003; Aukrust et al., 1999; French et al., 2009; Calmy et al., 2009; Guimaraes et al., 2008; Regidor et al., 2011; Baker et al., 2011; van Vonderen et al., 2009; Funderburg et al., 2010; Shikuma et al., 2011). Various soluble markers have been used to monitor the activation of endothelial cells in the course of HIV infection, and some of them have been reported to be still increased after 12 years of ART (Rhönsholt et al., 2013). Finally, high levels of the main marker of fibrinolysis D-dimer have been observed (Kuller et al., 2008; Rodger et al., 2009; Neuhaus et al., 2010) that are not normalized under cART (Neuhaus et al., 2010).

Many studies have explored different aspects of this chronic immune, endothelial and coagulation activation, but we still lack a global picture of this phenomenon. Also, whether all patients have the same profile of activation or if various profiles may be distinguished remains unknown. This question is of major importance if we want to elucidate the specific role played by the different causes of immune activation, and for instance if some causes will favour some types of immune activation and other causes other types. It is also of importance if we want to have a better insight into the links between immune activation and comorbidities. A global analysis of immune activation might help us to assess if some profiles of immune activation favour specifically some morbidities. It is with this aim in mind, that we carried out an extensive characterization of the immune, endothelial and coagulation activation in HIV patients aviremic under treatment. This study enabled us to define five different profiles of immune activation in virologic responders.

2. Materials and methods

2.1. Study design

We carried out a cross-sectional observational study at two University Hospitals in France (Montpellier and Nîmes). Eligible patients were HIV-1-infected adults (age >45 years) with pretherapeutic CD4 cell counts of 350 cells per μL or less, CD4 cell counts of 200 cells per μL or more in the last 6 months, and plasma viral load below 50 copies per mL for four time points each separated by 6 months while on a potent, stable combination antiretroviral regimen. Exclusion criteria were: pregnancy, breastfeeding, immunomodulatory therapy, anticancer chemotherapy, active hepatitis B or C virus infection, decompensated cirrhosis, and any non-infectious disease susceptible to impact on the immune system. The Ethics Committee of the University Hospital of Montpellier approved this study. All patients provided written informed consent.

2.2. Flow cytometry

Monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), energy-coupled dye (ECD), PE-Cyanine5.5 (PC5.5), PE-Cyanine7 (PC7), allophycocyanine (APC), APC/Alexa700, or APC/Alexa750 were purchased from Beckman Coulter. The antibodies were used in the following combinations; CD57-FITC/CD279-PE/CD45RA-ECD/C2-PC5.5/CD27-PC7/CD8-APC/CD4-APC700/CD3-APC750, CD8-APC/CD4-APC700/CD3-APC750/CD38-PE/HLADR-PC7/CD20-FITC, CD3-APC750/CD16 APC/HLADR-PC7/CD56-PC5.5/CD69-PE/CD57-FITC, CD4-FITC/CD45RA-ECD/CD25 PC7/FOXP3-APC/CD127-APC750. Whole blood collected into EDTA tubes was stained within 1 hour for 10 min at room temperature in the dark with cocktail of antibodies and fixed using IntraPrep Permeabilization Reagent Kit (Beckman Coulter) according to manufacturer's protocol. Cells were run on a Navios flow cytometer and results were analyzed by using Kaluza software (Beckman Coulter). A minimum 20,000 lymphocytes were gated to analyze the subpopulations. Representative gating strategies and images of flow plots are shown in Supplementary Fig. 1. We controlled the inter-run variability with the same batch of FlowSet Pro Beads (Beckman Coulter). The targets were defined on the samples settings (voltages). During the study, no voltage adjustment has been necessary to keep the beads into their respective defined targets.

2.3. Metabolic and soluble immunologic markers in peripheral blood

ELISA was used to quantify soluble TNF receptor I (sTNFRI), sCD14 and sCD163 (Quantikine, R&D systems), as well as tissue Plasminogen Activator, soluble Thrombomodulin, and soluble Endothelial Protein C Receptor (Asserachrom, Stago) in plasma collected into EDTA Vacutainer tubes (Becton Dickinson) and frozen. CRP and immunoglobulins were measured by turbidimetry in serum collected into SST II Vacutainer tubes, and D-dimers in blood collected into CTAD Vacutainer tubes (Becton Dickinson). Blood triglyceride, high-density lipoprotein (HDL) cholesterol, and insulin levels were quantified in serum collected into SST II Vacutainer tubes (Becton Dickinson) after 8 hours of fasting.

2.4. Statistical analysis

We used Wilcoxon tests to compare patients and donors, and Spearman rank correlations to evaluate the link between biomarkers with *p*-values multiplicity-adjusted by the False Discovery Rate method. We sorted the dataset with two independent hierarchical clustering analyses using respectively correlation and Euclidean distance to measure proximities/distances between markers and patients. The heatmap was generated using the *heatmap* function of the software R. The classifications used Ward (on the squared distance) as a metric. The distance was the euclidean for the patients and 1-abs (correlation) for markers. We used ANOVA results corrected by False Discovery Rate for multiple

testing, to determine the frequency of activation markers significantly different for at least one group of patients with regards to the other ones. Logistic regressions were carried out in order to study the relationship between profiles of immune activation and metabolic syndrome. Univariate analyses were first performed, and an adjustment by the age was thereafter executed. To study the relationship between activation markers of various components of the immune, endothelial and coagulation systems, we performed spearman correlations, and a Principal Component Analysis (PCA) after standardization of our variables. PCA allowed us to analyze multiple correlated values simultaneously, and to represent it as a set of new orthogonal variables called principal components, linear combinations of the variables. Then, the analysis of the components was carried out in order to highlight the degree of correlation between the variables. Statistical analyses were carried out using SAS Enterprise Guide V4.3 (SAS Institute Inc.), and R V3.1.1 (The R Foundation for Statistical Computing). A p-value lower than 0.05 was considered statistically significant.

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4. Results

4.1. Study subjects

Between April 29 and September 17, 2014, we recruited 22 female and 98 male HIV virologic responders (Table 1). Ninety-five percent of them were Caucasians. For all patients included, mean CD4 cell count was 688 (SD 326) cells per μL with a mean CD4:CD8 ratio of 0.99 (SD 0.52). Mean duration of HIV infection was 17.2 (SD 7.4) years with a mean pretherapeutic CD4 cell count of 192 (SD 108) cells per μL. 29% were current smokers, and 19% former smokers. The percentages of patients presenting with hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), or Epstein-Barr virus (EBV) infection were 72%, 42%, 5%, 91%, and 98% respectively. Sex- and age-matched HIV-uninfected and HIV-infected, viremic, untreated caucasians were enrolled as negative and positive controls, respectively.

4.2. Activation markers

We selected markers known to be modified in untreated HIV patients, and potentially in treated HIV patients (Younas et al., 2015). At the T lymphocyte surface, we chose the activation markers HLA-DR and CD38, as well as the inhibitory receptor Programmed cell Death 1 (PD-1 or CD279). CD45RA and CD27 were used to distinguish naïve

Table 1
Bioclinical and therapeutic characteristics of the study populations. ND, not determined; NA, not applicable; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.

Characteristic		HIV−	HIV+ treated	HIV+ untreated
Number of individuals		20	120	10
Age	Mean (±SD)	54.6 (±6.4)	56.5 (±8.0)	52.8 (±10.2)
	Median (min–max)	53.0 (46.0–69.0)	55.0 (45.0–83.0)	50.5 (36.0–68.0)
Male sex	N (%)	16 (80)	98 (82)	8 (80)
Caucasians	N (%)	20 (100)	114 (95)	10 (100)
% CD4+ T cell	Mean (±SD)	ND	34.7 (±9.8)	29.6 (±10.7)
	Median (min–max)	ND	34.0 (15.0–62.0)	28.0 (16.0–47.0)
CD4 count (/mm^3)	Mean (±SD)	ND	688 (±326)	574 (±283)
	Median (min–max)	ND	593 (243–2044)	509 (166–1153)
CD4:CD8 ratio	Mean (±SD)	ND	0.99 (±0.52)	0.65 (±0.33)
	Median (min–max)	ND	0.90 (0.20–3.30)	0.60 (0.20–1.20)
Pretherapeutic nadir CD4 count	Mean (±SD)	NA	192 (±108)	515 (±304)
(cells/μL)	Median (min–max)	NA	187 (1–458)	442 (130–1153)
Pretherapeutic viremia (RNA copies/ml)	Mean (±SD)	NA	393,985 (±1,314,356)	58,833 (±55,700)
	Median (min–max)	NA	104,156 (60–12,000,000)	35,234 (1220–170,318)
Years of HIV infection	Mean (±SD)	NA	17.2 (±7.4)	1.1 (±1.6)
	Median (min–max)	NA	18.0 (3.6–30.4)	0.3 (0.1–4.2)
Duration of viral suppression (months)	Mean (±SD)	NA	102 (±47)	NA
	Median (min–max)	NA	95 (25–217)	NA
NRTI	N (%)	NA	108 (90)	NA
NNRTI	N (%)	NA	46 (38)	NA
Protease inhibitor	N (%)	NA	61 (51)	NA
Integrase inhibitor	N (%)	NA	34 (28)	NA
HBs Ag+	N (%)	ND	3 (2)	0 (0)
Anti-HBs Ab+	N (%)	ND	53 (44)	6 (60)
Anti-HBc Ab+	N (%)	ND	50 (42)	2 (20)
HCV coinfection	N (%)	ND	6 (5)	0 (0)
CMV coinfection	N (%)	ND	109 (91)	9 (90)
EBV coinfection	N (%)	ND	118 (98)	10 (100)
HAV coinfection	N (%)	ND	87 (72)	7 (70)
Systolic blood pressure (mmHg)	Mean (±SD)	ND	128 (±16)	124 (±14)
	Median (min–max)	ND	127 (100–181)	128 (100–136)
Diastolic blood pressure (mmHg)	Mean (±SD)	ND	80 (±11)	82 (±10)
	Median (min–max)	ND	80 (60–120)	82 (60–94)
Waist circumference (cm)	Mean (±SD)	ND	91 (±11)	89 (±10)
	Median (min–max)	ND	91 (65–128)	85 (82–108)
Lipodystrophy	N (%)	ND	40 (33)	0 (0)
Insulinemia (μUI/ml)	Mean (±SD)	ND	11 (±7)	7 (±5)
	Median (min–max)	ND	9 (1–45)	7 (2–17)
Triglyceridemia (mM/L)	Mean (±SD)	ND	1.86 (±1.75)	1.55 (±1.59)
	Median (min–max)	ND	1.57 (0.58–17.60)	1.10 (0.64–5.70)
HDL serum level (mM/L)	Mean (±SD)	ND	1.45 (±0.61)	1.25 (±0.55)
	Median (min–max)	ND	1.33 (0.16–4.20)	1.17 (0.50–2.42)

and central memory from effector memory T cells. CD57 upregulation along with CD27 and/or CD28 loss were used to characterize T lymphocyte senescence. In addition to these markers we also measured CD38 overexpression to evaluate CD8+ T cell activation (Tuaillon et al., 2009). NK cell activation was monitored analyzing HLA-DR and CD69 expression, NK cell dysfunction CD56 loss, and NK cell senescence CD57 gain. B cell hyperactivity was assessed by quantifying IgG, IgA, and IgM in the serum. The plasma level of sCD14 and sCD163 were used as indicators of monocyte/macrophage activation, and that of sTNFRI and C-reactive protein (CRP) as indicators of inflammation. Endothelium activation was explored by looking for an increase in soluble Endothelial Protein C Receptor (sEPCR) and in tissue Plasminogen Activator (tPA), and for a decrease in soluble Thrombomodulin (sThrombomodulin) plasma levels. In a subgroup of patients, D-dimer plasma concentration was measured as well as CD64, CD62L, and PD-L1 polynuclear surface density.

4.3. Immune activation in virologic responders is global

As compared with HIV-negative controls, we observed in patients aviremic under cART an increase in the percentages of activated, HLA-DR+, CD4+ T cells (16 ± 8 and 22 ± 13, $p = 0.015$), of activated, HLA-DR+ (45 ± 15, 54 ± 18, $p = 0.042$), and senescent, CD57+, CD8+ T cells (18 ± 18, 32 ± 14, $p = 0.001$), and of activated, HLA-DR+ (33 ± 22, 52 ± 22, $p = 0.002$), dysfunctional, CD56- (8 ± 4, 11 ± 7, $p = 0.030$), and senescent, CD57+ (33 ± 16, 44 ± 18, $p = 0.012$) NK cells (Fig. 1). Yet, these percentages were lower than those observed in non-treated HIV-infected individuals. We also observed an increase in the plasma level of sCD163 (279 ± 158 ng/mL, 467 ± 282 ng/mL, $p = 0.033$), and sTNFRI (0.9 ± 0.3 ng/mL, 1.2 ± 0.5 ng/mL, $p = 0.012$), even though these levels were below those measured in treatment-naïve patients. CD64 polynuclear surface density was higher in treated patients than in healthy controls (1.0 ± 0.3 versus 0.8 ± 0.1 arbitrary units, $p = 0.030$). As compared with virologic responders (95 ± 56 ng/mL) sThrombomodulin concentration was higher in control subjects (142 ± 29 ng/mL, $p = 0.008$), but lower in viremic patients. Finally, IgG, IgA, IgM, and D-dimer plasma concentrations in aviremic patients were lower than in viremic patients, and higher than in noninfected controls (data not shown). Thus, all the components of the innate and adaptative immune system we tested, as well as the endothelium and the coagulation, appeared globally activated in the HIV-1-infected individuals we analyzed, even though they had been aviremic for years.

4.4. Links between markers within each component of the immune and endothelial systems

We looked for correlations between the various markers within each cell subpopulation. In CD4+ T cells (Table 2), expression of the

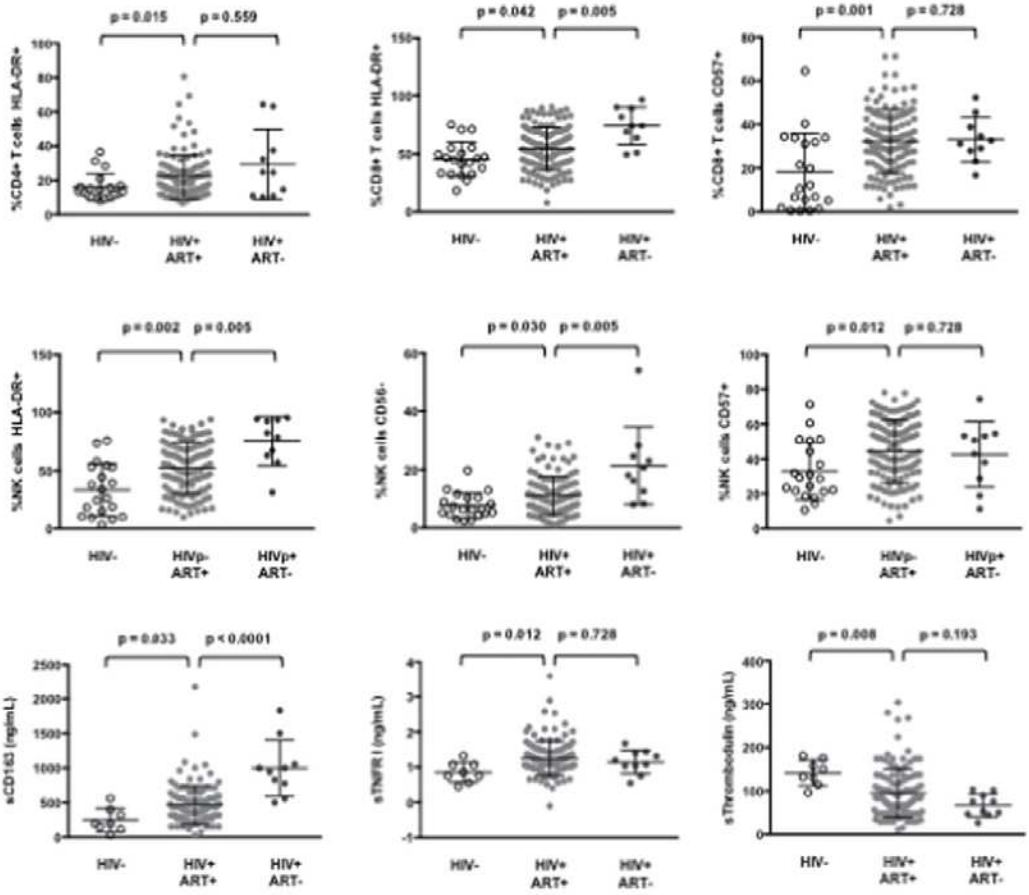

Fig. 1. Immune activation in virologic responders. Percentages of various cell populations and plasma levels of soluble markers in healthy donors (HIV-), treated (HIV+ ART+), and untreated (HIV+ ART–) HIV patients. Data are presented as mean values and 95% confidence intervals; *p*-values are shown.

activation marker HLA-DR was linked to that of the inhibitory receptor PD-1, and of markers of senescence (CD57 expression with and without loss of CD28 and/or CD27). In CD8+ T cells (Table 3), we found no correlation between HLA-DR and CD38 expression. Moreover, in these lymphocytes, HLA-DR expression, but not that of CD38, was linked to markers of senescence. Of note, in CD8+ T cells, PD-1 expression was linked neither to these two markers of activation, nor to markers of senescence. In NK cells (Table 4), the two activation markers HLA-DR and CD69 were linked together but not to the marker of senescence CD57, but this latter was linked to the marker of dysfunction CD56-. Of note, the levels of the monocyte/macrophage activation markers sCD14 and sCD163 were not linked together ($r = 0.087$, $p = 0.344$). By contrast, the plasma concentrations of IgG and IgA ($r = 0.424$, $p < 0.001$), and IgG and IgM ($r = 0.352$, $p < 0.001$) were linked together.

4.5. Correlations between the various markers of the immune, endothelial, and coagulation activations

As we observed a hyperactivity in all the components we analyzed, we wondered whether the level of activity of some of these components were linked, either because their activation results from common causes and/or because they induce each other. To answer this question, we systematically looked for correlations between markers characterizing the activation of the various components of the immune, endothelial and coagulation systems (Table 5). As characteristic markers, we chose HLA-DR expression on CD4+ T cells, CD38 overexpression on CD8+ T cells, CD69 expression on NK cells, CD62L expression on polynuclear cells, sCD14, IgG, tPA, D-dimer and sTNFRI plasma levels for monocyte, B cell, endothelial, coagulation activation and inflammation, respectively. The only links we uncovered was between HLA-DR expression on CD4+ T cells and sCD14 level ($r = 0.200$, $p = 0.028$) on one hand, and sTNFRI and D-dimer levels ($r = 0.215$, $p = 0.011$) on the other hand.

In order to comprehensively analyze more globally correlations between the various activation markers and to obtain a visual representation, a PCA was computed, after standardization of the variables (Fig. 2). We decided to keep two principal components after performing an elbow test. These two components represented about 35% of the model's variance (22.70 + 11.96). The results corroborated the conclusions above. For instance, in CD4+ T cells, we found a strong correlation between markers of senescence (CD57 expression with and without loss of CD28 and/or CD27), and of activation (HLA-DR) and PD-1. By contrast, D-dimer levels appeared not to be linked to CD4+ T cell and CD8+ T cell markers.

4.6. Identification of different profiles of immune activation in patients aviremic under cART

To test the hypothesis of the existence of different profiles of immune activation in patients aviremic under treatment, we sorted the patients and the 68 markers of lymphocyte, NK cell, monocyte, endothelium activation and inflammation by two independent hierarchical clustering analyses (Fig. 3). This clustering outlined the correlations, or the absence thereof, existing between some markers. Some of these correlations were expected. For instance the correlation between the percentages of CD8+ T cells positive for HLA-DR and for CD57 shown in Table 3 is illustrated by their proximity in the heatmap (8th and 10th markers from the right end). This clustering also unveiled the unexpected absence of links between other markers, such as between sCD14 and sCD163, as previously mentioned, situated far apart from each other in the heatmap. It also showed unexpected links between other markers. For instance, the marker of NK activation HLA-DR was linked to the marker of endothelium activation tPA ($r = 0.261$, $p = 0.004$). On the other hand, tPA appeared also linked to monocyte/macrophage activation as estimated by the level of sCD163 ($r = 0.244$, $p = 0.007$).

The hierarchical clustering analysis also identified five groups of patients, presenting with very different profiles of immune activation, and 2 outliers (Fig. 3). The levels of more than 80% of the markers were on average significantly different for at least one group of patients with regards to the other ones. The diversity of the activation profiles was evident when selected activation markers were analyzed (Fig. 4). As compared with the other groups, group 1 (T8 profile) was characterized by a high percentage of central memory (CD45RA-CD27+) CD8+ T cells (31 ± 13 versus $23 \pm 10\%$, $p = 0.0007$), group 2 (inflammation profile) by a high level of the inflammation marker sTNFRI (1.5 ± 0.3 versus 1.2 ± 0.5 mg/L, $p = 0.040$), and group 3 (NK profile) by a high number of activated, CD69+, NK cells (41 ± 43 versus 31 ± 60 cells/μL, $p = 0.033$). Group 4 (T4 profile) had the highest proportion of CD4+ T cells expressing CD38 ($65 \pm 9\%$ versus $55 \pm 12\%$, $p < 0.001$), and group 5 (monocyte profile) the highest level of the monocyte activation marker sCD163 (671 ± 520 ng/mL versus 446 ± 279 ng/mL, $p = 0.009$). To simplify the identification of the five profiles of immune activation, we looked for a small number of markers able to characterize each patient group. Using only 8 biomarkers, we were able to classify the patients in the five groups with an error rate of 8.5%.

4.7. Relationship between a profile of immune activation and metabolic syndrome

According to various studies, the frequency of metabolic syndrome has been reported as 14%–26% (Tebas, 2008) in HIV patients. This syndrome is characterized by the presence of at least 3 of the following components: hypertension, elevated waist circumference or waist-hip ratio, impaired glucose tolerance, and high triglyceride and/or low HDL cholesterol (Alberti et al., 2009). As the metabolic syndrome has been linked to immune activation (Esser et al., 2014), we looked for a relationship between this non-AIDS-linked morbidity and the immunological profiles we defined, by carrying out logistic regressions. Univariate analyses showed that, as compared with the other groups, patient group 2 presented with higher mean systolic and diastolic blood pressures, higher waist circumference, and lower HDL cholesterol

Table 2
Correlations between CD4+ T cell markers of activation.

	% CD4+ T cells HLA-DR+	% CD4+ T cells PD-1+	% CD4+ T cells CD57+	% CD4+ T cells CD57+CD28−	% CD4+ T cells CD57+CD28−CD27−
% CD4+ T cells HLA-DR+		$r = 0.393$ $p < 10^{-4}$	$r = 0.585$ $p < 10^{-4}$	$r = 0.584$ $p < 10^{-4}$	$r = 0.542$ $p < 10^{-4}$
% CD4+ T cells PD-1+			$r = 0.375$ $p < 10^{-4}$	$r = 0.374$ $p < 10^{-4}$	$r = 0.326$ $p < 10^{-4}$
% CD4+ T cells CD57+				$r = 0.998$ $p < 10^{-4}$	$r = 0.976$ $p < 10^{-4}$
% CD4+ T cells CD57+CD28−					$r = 0.976$ $p < 10^{-4}$
% CD4+ T cells CD57+CD28−CD27−					

Table 3
Correlations between CD8+ T cell markers of activation.

	% CD8+ T cells HLA-DR+	% CD8+ T cells CD38+	% CD8+ T cells CD38hi	% CD8+ T cells HLA-DR+CD38+	% CD8+ T cells PD1+	% CD8+ T cells CD57+	% CD8+ T cells CD57+CD28−	% CD8+ T cells CD57+CD28−CD27−
% CD8+ T cells HLA-DR+				$r = 0.615$ $p < 10^{-4}$		$r = 0.503$ $p < 10^{-4}$	$r = 0.494$ $p < 10^{-4}$	$r = 0.462$ $p < 10^{-4}$
% CD8+ Tcells CD38+			$r = 0.598$ $p < 10^{-4}$	$r = 0.443$ $p < 10^{-4}$				
% CD8+ T cells CD38hi				$r = 0.354$ $p < 10^{-4}$				
% CD8+ T cells HLA-DR+CD38+						$r = 0.506$ $p < 10^{-4}$	$r = 0.493$ $p < 10^{-4}$	$r = 0.436$ $p < 10^{-4}$
% CD8+ T cells PD-1+								
% CD8+ T cells CD57+							$r = 0.996$ $p < 10^{-4}$	$r = 0.905$ $p < 10^{-4}$
% CD8+ T cells CD57+CD28−								$r = 0.907$ $p < 10^{-4}$
% CD8+ T cells CD57+CD28−CD27−								

levels, but these differences did not reach significance. The frequency of hyperinsulinemia (insulinemia >24.9 mIU/L; OR 12.17 [95% CI 1.79–82.86], $p = 0.011$, Fig. 5A), and of high (>2.85 mM) triglyceridemia (OR 4.18 [95% CI 1.08–16.19], $p = 0.038$, Fig. 5B) was significantly elevated in group 2 as compared with the other groups. Moreover, the proportion of patients presenting with lipodystrophia was also higher in group 2 than in the other groups (OR 4.87 [95% CI 1.36–17.39], $p = 0.015$, Fig. 5C). Thus, the immune activation profile 2 was linked to major marks of one of non-AIDS comorbidities, the metabolic syndrome. Univariate analysis showed that, as compared with the other groups, patients in group 2 were slightly older (OR 1.08 [95% CI 1.01–1.16], $p = 0.023$). A multivariate analysis, adjusted with age as a covariate, still highlighted the risk of lipodystrophia (OR 4.37 [95% CI 1.17–16.33], $p = 0.028$) and a risk of hyperinsulinemia that was even higher (OR 17.06 [95% CI 2.14–135.60], $p = 0.007$) in the group 2.

5. Discussion

In this study, we report a global evaluation of the state of activation of the immune, endothelial and coagulation systems in 120 treated patients aviremic for at least 2 years. A first conclusion is that these patients overall present with a generalized hyperactivity of these systems.

A second conclusion is that many markers of the activation of a cell subpopulation are not equivalent. Analyzing intra-subpopulation correlations, we noticed that HLA-DR expression and CD38 overexpression on CD8+ T cells were not correlated (Table 3). This is in line with the fact that these markers may not be coexpressed in vitro (Chadburn et al., 1992) and in vivo (Espinosa et al., 2013). It is also in agreement with the initial observation that CD38, but not HLA-DR, is a predictive marker of the disease progression (Giorgi et al., 1993). Clearly, the expression of these two markers is largely independent, probably driven by different mechanisms of activation, and leading to different consequences. Accordingly, CD8+ T cell senescence, as indicated by CD57 appearance and CD27 and/or CD28 loss, correlated with HLA-DR, but not with CD38 expression (Table 3). Strikingly, PD-1 expression was linked to none of these two activation markers (Table 3). This emphasizes the notion that the percentage of CD8+ T cells positive for PD-1 is dependent on the stage of differentiation, PD-1 being mostly expressed at an early and intermediate stage of differentiation, rather than on the level of activation (Sauce et al., 2007). Indeed, in our study, the percentage of CD8+ T cells expressing PD-1 was very strongly (data not shown) correlated with the percentage of CD8+ T cells CD45RA-CD27+. Consequently, alternative markers need to be used to measure exhaustion, as for instance CD160 (Vigano et al., 2014). A striking observation is also that sCD14 and sCD163 are situated far apart in the biomarker clustering. The absence of correlation between these two monocyte/macrophage activation markers has been reported elsewhere (Patro et al., 2016). This is in line with the fact that sCD163, but not sCD14, has been correlated with HIV load, and may normalize under antiretroviral therapy (Castley et al., 2014). Likewise, sCD163, but not sCD14, has been linked to the level of anti-CMV IgG (Hodowanec et al., 2015) and to liver fibrosis (Mueller et al., 2015). Altogether these data emphasize the idea that these two markers likely do not reflect the same activation pathway.

Another important finding of our study is that the various pathways we analyzed are not often activated concurrently. In the individuals we examined, most of the components of the immune, endothelial and coagulation systems, as evaluated by selected markers, appear to be independently activated. This raised the interesting hypothesis that virologic responders might present with different profiles of activation.

The ascendant hierarchical classification enabled us to cluster activation markers that fluctuate in the same way. Apart from some expected correlations, this classification uncovered some unexpected ones. Indeed, to our knowledge, correlations between NK and endothelium activation had never been previously reported in HIV infection. The fact that activated endothelial cells may release fractalkine, a chemokine known to activate NK cells (Robinson et al., 2003) might be a mechanism warranting further study. Likewise, no links between monocyte/

Table 4
Correlations between NK cell markers of activation.

	% NK cells HLA-DR+	% NK cells CD69+	% NK cells HLA-DR+CD69+	% NK cells CD56-	% NK CD57+
% NK cells HLA-DR+		$r = -0.501$ $p < 10^{-4}$	$r = 0.395$ $p < 10^{-4}$		
% NK cells CD69+			$r = 0.270$ $p = 0.0029$		
% NK cells HLA-DR+CD69+					$r = -0.335$ $p = 0.0479$
% NK cells CD56-					$r = 0.270$ $p = 0.0002$
% NK cells CD57+					

Table 5
Correlations between markers of immune, endothelial and thrombolysis activation.

	% CD4+ T cells HLA-DR+	% CD8+ T cells CD38hi	% NK cells CD69+	CD62L polynuclear cell surface density	sCD14	IgG	sTNFR I	tPA	D-dimer
% CD4+ T cells HLA-DR+		–			$r = 0.200$ $p = 0.0284$				
% CD8+ T cells CD38hi									
% NK cells CD69+									
CD62L polynuclear cell surface density									
sCD14									
IgG									
sTNFR I					–				$r = 0.215$ $p = 0.0107$
tPA									
D-dimer									

macrophage and endothelium activation had been described so far in HIV patients. The fact that tPA has been reported to induce microglial activation via surface annexin II (Siao and Tsirka, 2002) might account for the correlation between tPA and sCD163 we uncovered. These examples highlight the interest of our global approach that may unveil interactions between the components of the immune, endothelial and coagulation systems.

Via this global approach we were able to cluster the patients into five different immune activation profiles. The diversity of these profiles is illustrated by the fact that over 80% of the markers are variable among the

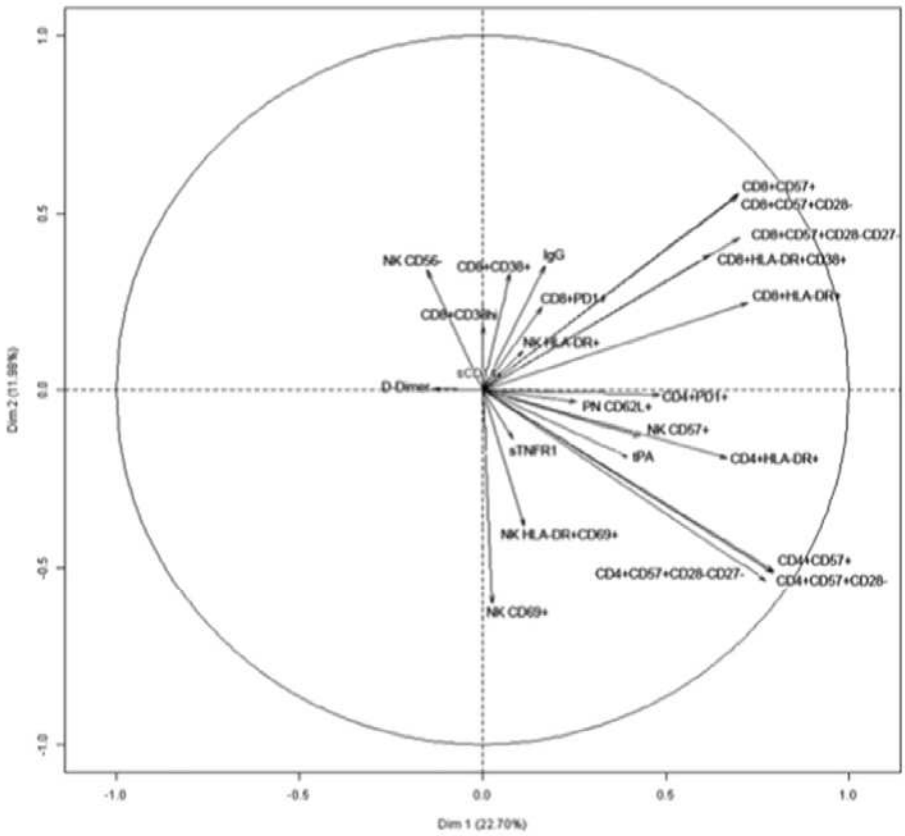

Fig. 2. Variables factor map resulting from Principal Component Analysis. The variables are represented by arrows. The elbow test was carried out in order to select the number of components to consider. This was done by plotting the components' eigenvalues according to their size and analyzing the point in the graph where the slope goes from "steep" to "flat" in order to keep only the components that are placed before the elbow, which were 2 in our case. The length of each of these arrows depends on the correlation of the variable with the component. Highly positively correlated variables are represented by arrows close to each other. Strongly negatively correlated variables are represented by arrows diametrically opposed.

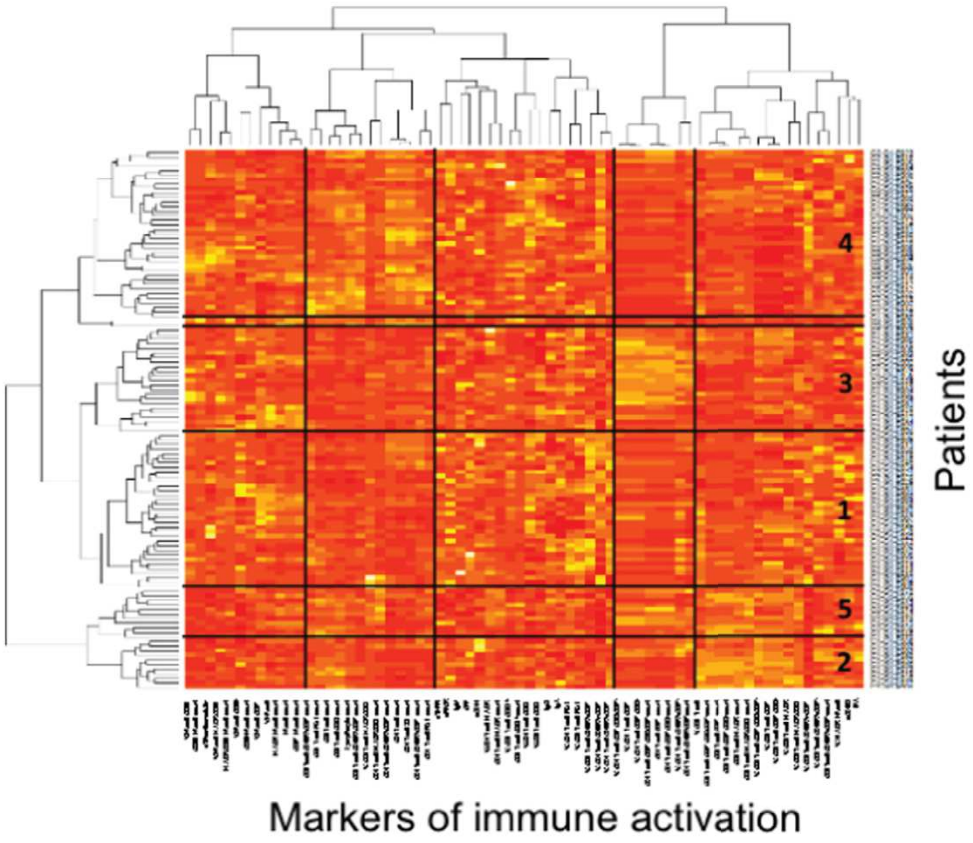

Fig. 3. Virologic responders present with different immune activation profiles. Heatmap showing the hierarchical clustering of the activation markers (vertical) as well as of the virologic responders according to their profile of activation (horizontal). Each group number is indicated.

patient groups. Moreover, each of these groups can be distinguished by a different immune activation marker. As various factors, including residual HIV production, microbial translocation, coinfections, metabolic disorders, CD4+ T cell lymphopenia, immunosenescence, and Treg deregulation, may cause immune and endothelial activation, it is tempting to speculate that these five profiles are the result of different combinations of these etiologic factors. To test this possibility, we are currently measuring markers specific for these causes of activation in the same cohort of patients in order to look for correlations between these different causes and the five profiles of activation. To evaluate whether some coinfections might be coresponsible for the immune activation profile 2, we looked for a link between markers of these co-infections and this biomarker profile. As compared with the other patient groups, we did not find any increase in the frequency of either HAV, HBV, HCV or CMV seropositivity, or EBV load in patient group 2.

It is also possible that each activation profile fuels preferentially some comorbidities as compared with the other profiles. In line with this hypothesis, we observed a significant correlation between one of the patient group (group 2) and three hallmarks of the metabolic syndrome, hypertriglyceridemia, lipodystrophia and hyperinsulinemia. Of note, a characteristic of patients in group 2 is the high level of sTNFRI, a marker of TNFα production. Interestingly, TNFα has been reported to inhibit insulin signalling via the phosphorylation of the Insulin Receptor Substrate 1 (Hotamisligil et al., 1996; Yin et al., 1998; Aguirre et al., 2000), an effect that favours the development of metabolic syndrome (Alberti et al., 2009). Moreover, the plasma level of TNFα has been reported to be correlated with the metabolic syndrome (Shoelson et al., 2006).

Our study has some limitations. First, the prevalence of sexually transmitted diseases in patients and the CMV status as well as the proportion of substance abusers in the negative controls were unknown. These factors might confound the comparisons between healthy donors and HIV adults. It must also be noted that it is a cross-sectional study. This type of study design enables to show differences in immune activation profiles and correlations with a comorbidity. Yet, a longitudinal follow-up will be needed to test the predicting value of the immune activation profile of group 2.

From a more general point of view, it will be of interest to look for correlations between other comorbidities and the immune activation profiles uncovered by the present study. If we establish correlations between other comorbidities and some immune activation profiles, the simple signature of eight markers we have shown to be able to characterize each profile could be tested for its ability to predict some morbidities driven by immune/endothelial activation. Of practical interest, the identification of this signature requires a single flow cytometry tube. In the future, such a signature might enable to tailor prevention and early diagnosis of non-AIDS-linked morbidities to each patient. Unveiling links between some causes of immune activation and some immune activation profiles, and between some immune activation profiles and some comorbidities could also have therapeutic implications. Uncovering molecular mechanisms responsible for a causative link between a profile of immune activation and atherothrombosis, liver

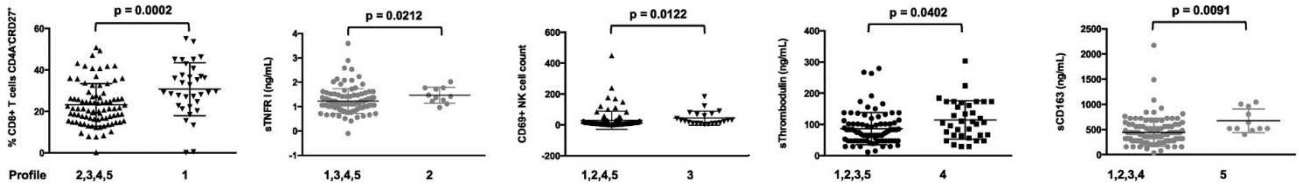

Fig. 4. Characterization of the five different immune activation profiles. Differences in the levels of key activation markers between each group of patients and the other groups are represented. Data are presented as mean values and 95% confidence intervals; *p*-values are shown.

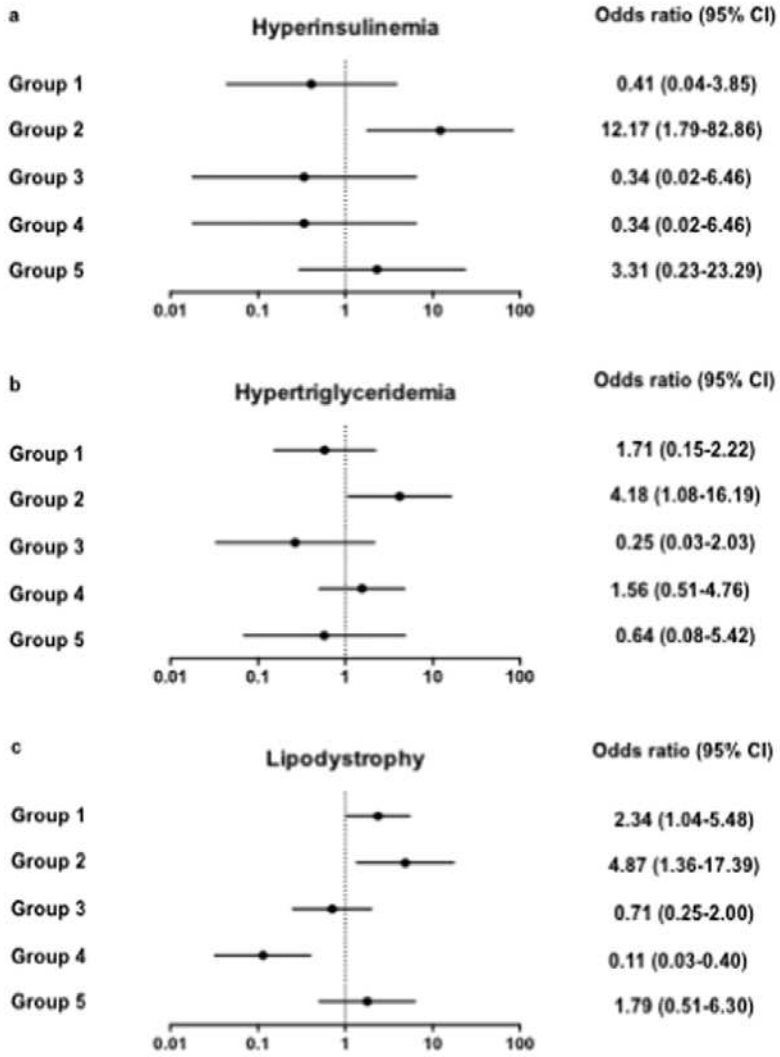

Fig. 5. Link between immune activation profile 2 and marks of metabolic syndrome. Odd ratios relating each profile of immune activation to risk of hyperinsulinemia (a), hypertriglyceridemia (b), and lipodystrophy (c). Data are presented as OR and 95% confidence intervals.

steatosis, osteoporosis or neurocognitive impairment could provide us with new therapeutic targets to prevent and attenuate these chronic diseases. Moreover, uncovering the causes (HIV and non-HIV infection, microbial translocation, metabolic dysregulation, CD4+ T cell loss, immunosenescence, and Treg dysfunction) fueling the immune activation profile of each patient could open the way to an etiologic treatment.

As these pathogenic pathways are probably not specific for HIV infection but relevant to any situation of chronic immune activation, including aging, and given the frequency of these comorbidities, these observations are of general interest. It is for instance striking that HIV infection and the autoimmune disease rheumatoid arthritis share many common comorbidities, including bone loss, atherosclerosis, metabolic syndrome, and frailty (Weyand et al., 2014). Thus, our approach could improve our knowledge on the pathogenic consequences of chronic immune activation, on the pathophysiology of the major chronic diseases it fuels, as well as on the prevention of these diseases, their early diagnosis and treatment.

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Conflicts of Interest

None.

Authors Contributions

Christina PSOMAS contributed to the conception and design of the study, the patients' enrollment, acquired, analyzed and interpreted data. Mehwish YOUNAS contributed to the conception and design of the flow cytometry study, acquired, analyzed and interpreted cell surface and soluble markers data. Renaud CEZAR, Pierre PORTALES, Edouard TUAILLON, Jean-François ELIAOU contributed to the conception and design of the flow cytometry study, acquired, analyzed and interpreted cell surface markers. Christelle REYNES, Robert SABATIER,

Grégory MARIN, Nicolas NAGOT contributed to the conception and design of the statistical study, acquired, analyzed and interpreted statistical data. Adeline GUIGUES acquired and analyzed soluble markers data. Corinne MERLE, Nadine ATOUI, Céline FERNANDEZ, Vincent LE MOING, Claudine BARBUAT, Albert SOTTO acquired, analyzed and interpreted clinical data. Jacques REYNES contributed to the conception and design of the study, analyzed and interpreted data. Pierre CORBEAU contributed to the conception and design of the study, analyzed and interpreted data, and wrote the first draft of the manuscript. All authors revised and approved the final version.

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Corrigendum

Corrigendum to "One of the Immune Activation Profiles Observed in HIV-1-Infected Adults with Suppressed Viremia is Linked to Metabolic Syndrome: The ACTIVIH Study" [EBioMedicine 8 (2016) 265–276]

Christina Psomas [a,b], Mehwish Younas [a], Christelle Reynes [c], Renaud Cezar [d], Pierre Portalès [e], Edouard Tuaillon [f], Adeline Guigues [a], Corinne Merle [b], Nadine Atoui [b], Céline Fernandez [b], Vincent Le Moing [b,g,h], Claudine Barbuat [i], Grégory Marin [j], Nicolas Nagot [j], Albert Sotto [h,i], Jean-François Eliaou [e,h], Robert Sabatier [c], Jacques Reynes [b,g,h], Pierre Corbeau [a,d,h,*]

[a] Institute for Human Genetics, CNRS UPR1142, 141 rue de la Cardonille, 34396, Montpellier, cedex 5, France
[b] Infectious Diseases Department, University Hospital, 80 avenue A. Fliche, 34295 Montpellier, cedex 5, France
[c] Institute for Functional Genomics, Montpellier University, UMR5203, 141 rue de la Cardonille, 34396 Montpellier, cedex 5, France
[d] Immunology Department, University Hospital, Place du Pr Debré, 30029 Nîmes, cedex, France
[e] Immunology Department, University Hospital, 80 avenue A. Fliche, 34295 Montpellier, cedex 5, France
[f] Microbiology Department, University Hospital, 371 Av. du Doyen Gaston Giraud, 34295 Montpellier, cedex 5, France
[g] IRD UMI 233, INSERM U1175, Montpellier University, 911 avenue Agropolis, 34294 Montpellier, cedex 5, France
[h] Montpellier University, 5 Boulevard Henri IV, 34967 Montpellier, cedex 2, France
[i] Infectious Diseases Department, University Hospital, Place du Pr Debré, 30029 Nîmes, cedex, France
[j] Medical Informatics Department, University Hospital, 39 Avenue Charles Flahault, 34090 Montpellier, cedex 5, France

The authors wish to republish high-resolution Figures in this article here. (See Figs. 1–5.)

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* Corresponding author.
E-mail address: pierre.corbeau@igh.cnrs.fr (P. Corbeau).

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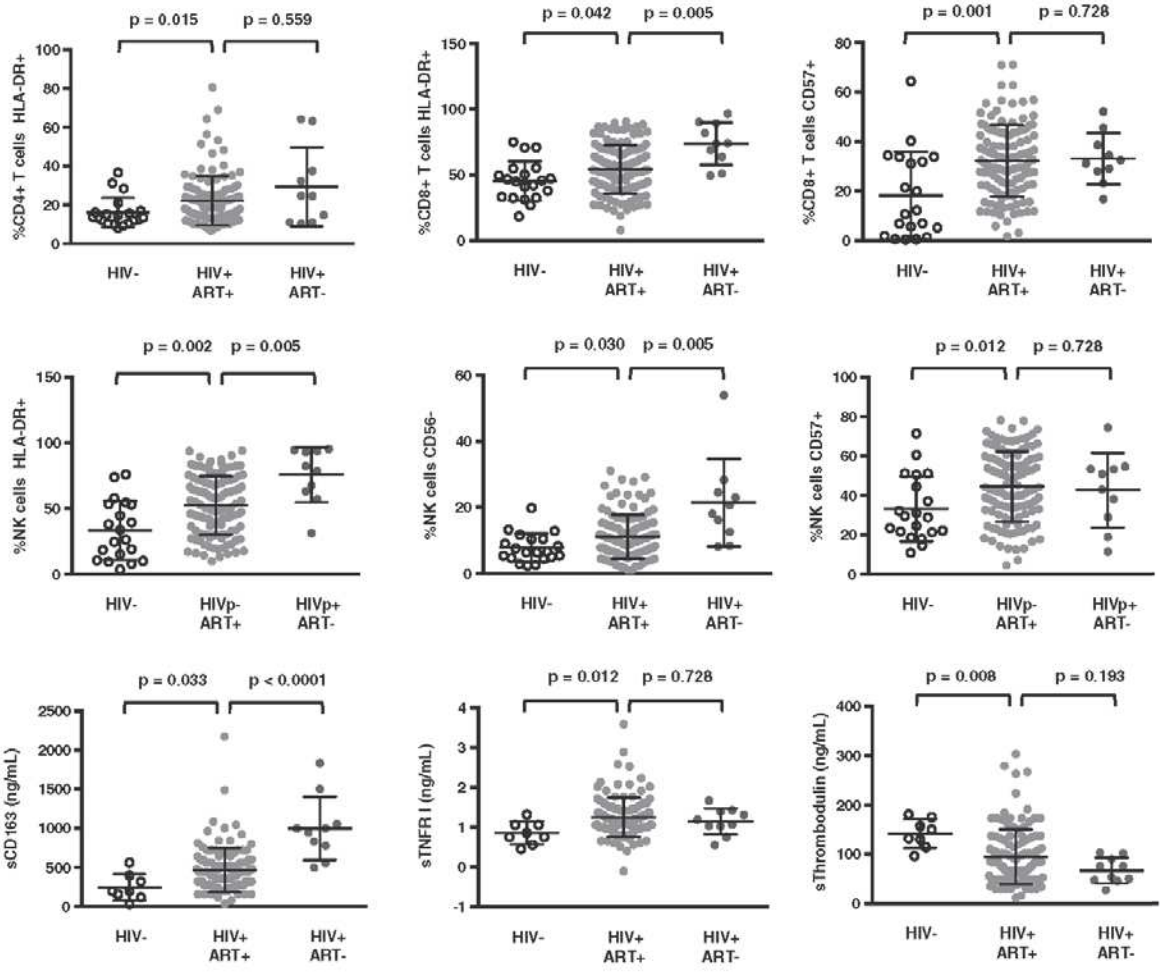

Fig. 1. Immune activation in virologic responders. Percentages of various cell populations and plasma levels of soluble markers in healthy donors (HIV-), treated (HIV+ ART+), and untreated (HIV+ ART–) HIV patients. Data are presented as mean values and 95% confidence intervals; *p*-values are shown.

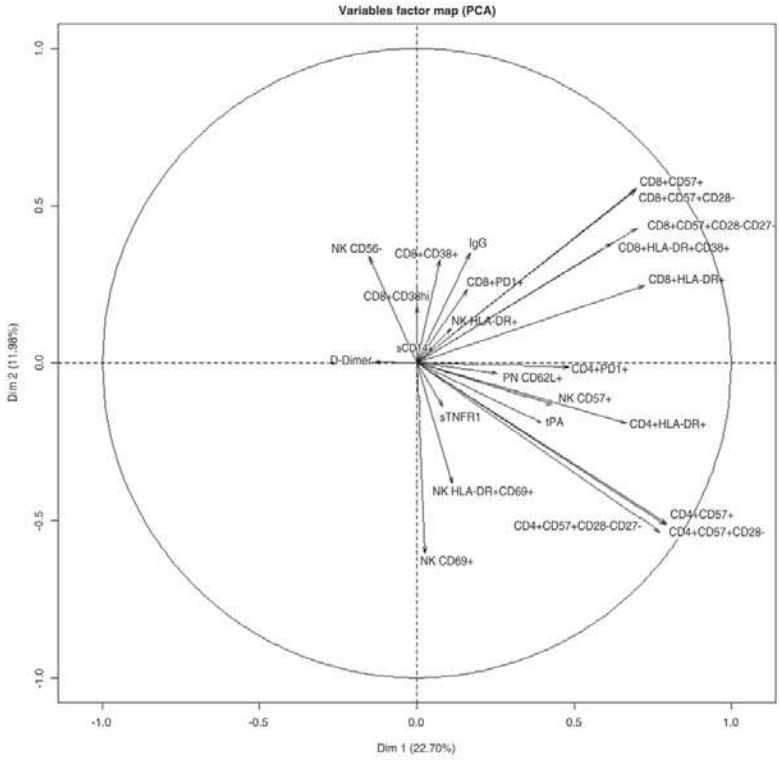

Fig. 2. Variables factor map resulting from Principal Component Analysis. The variables are represented by arrows. The elbow test was carried out in order to select the number of components to consider. This was done by plotting the components' eigenvalues according to their size and analyzing the point in the graph where the slope goes from "steep" to "flat" in order to keep only the components that are placed before the elbow, which were 2 in our case. The length of each of these arrows depends on the correlation of the variable with the component. Highly positively correlated variables are represented by arrows close to each other. Strongly negatively correlated variables are represented by arrows diametrically opposed.

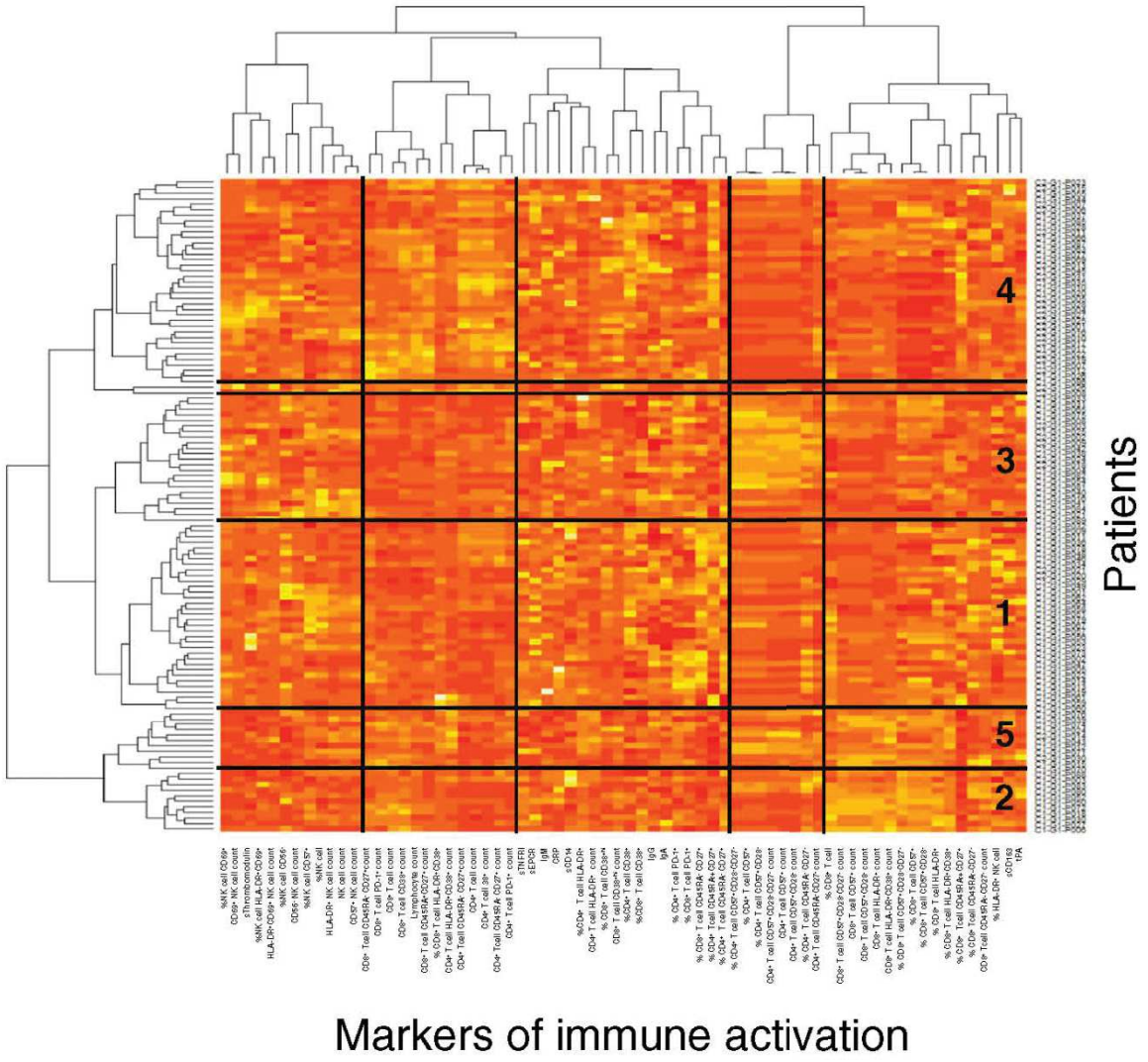

Fig. 3. Virologic responders present with different immune activation profiles. Heatmap showing the hierarchical clustering of the activation markers (vertical) as well as of the virologic responders according to their profile of activation (horizontal). Each group number is indicated.

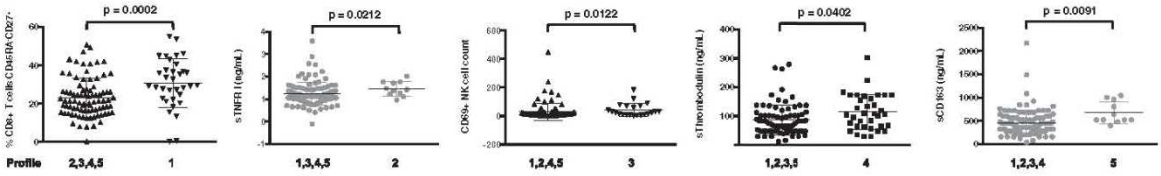

Fig. 4. Characterization of the five different immune activation profiles. Differences in the levels of key activation markers between each group of patients and the other groups are represented. Data are presented as mean values and 95% confidence intervals; *p*-values are shown.

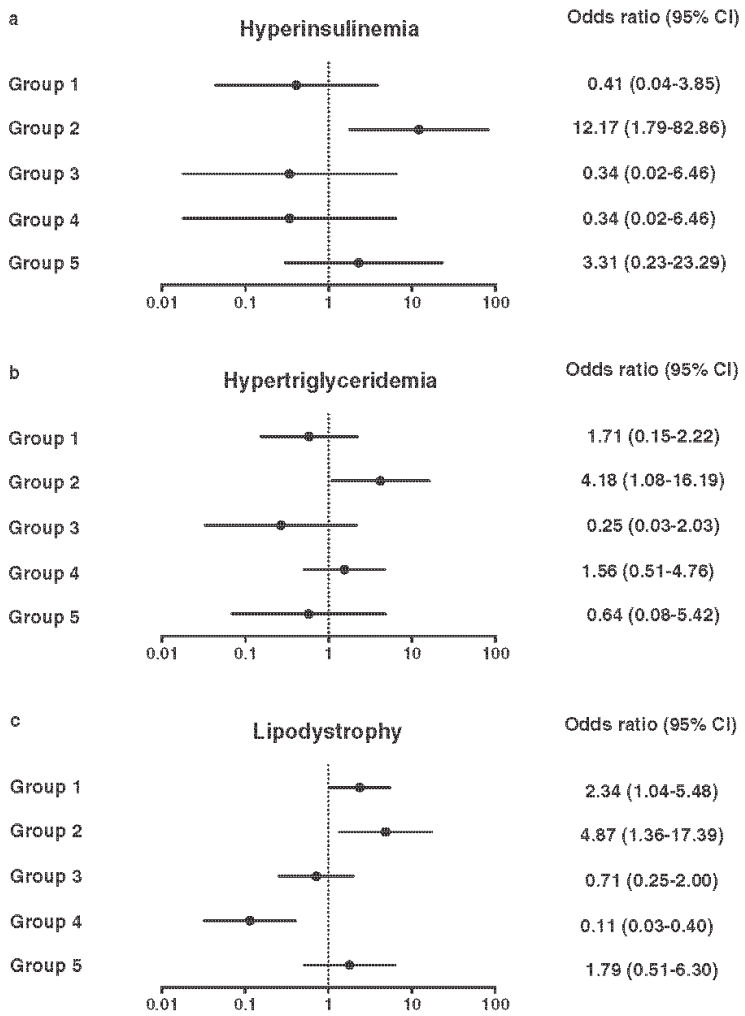

Fig. 5. Link between immune activation profile 2 and marks of metabolic syndrome. Odd ratios relating each profile of immune activation to risk of hyperinsulinemia (a), hypertriglyceridemia (b), and lipodystrophy (c). Data are presented as OR and 95% confidence intervals.

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RESEARCH ARTICLE

Plasma Level of Soluble ST2 in Chronically Infected HIV-1 Patients with Suppressed Viremia

Mehwish Younas[1], Christina Psomas[1,2], Vikram Mehraj[3], Renaud Cezar[4], Pierre Portales[5], Edouard Tuaillon[6], Adeline Guigues[1], Jacques Reynes[2,7,8], Pierre Corbeau[1,4,8,*,†] and Jean-Pierre Routy[3,9,†]

[1]*Institute for Human Genetics, CNRS UPR1142, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France,*
[2]*Infectious Diseases Department, University Hospital, 80 avenue A. Fliche, 34295 Montpellier cedex 5, France,*
[3]*Research Institute and Chronic Viral Illness Service of the McGill University Health Center, Montréal, Québec, Canada*
[4]*Immunology Department, University Hospital, Place du Pr Debré, 30029 Nimes cedex, France,*
[5]*Immunology Department, University Hospital, 80 avenue A. Fliche, 34295 Montpellier cedex 5, France,*
[6]*Microbiology Department, University Hospital, 371 Av. du Doyen Gaston Giraud, 34295 Montpellier cedex 5, France,*
[7]*UMI 233, IRD-Montpellier I University, 911 avenue Agropolis, 34294 Montpellier cedex 5, France,*
[8]*Montpellier I University, 5 Boulevard Henri IV, 34967 Montpellier cedex 2, France,*
[9]*Division of Hematology, McGill University Health Center, Montréal, Québec, Canada*

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Abstract:

Introduction:

Interleukin-33 (IL-33) is a cell damage-induced alarmin. The plasma concentration of suppression of tumorogenicity (sST2), a surrogate marker of IL-33 production, is a prognostic marker of cardiovascular disease.

Observation:

Recently, we reported that sST2 plasma levels were elevated in early HIV-1 infection and linked to markers of microbial translocation and of T cell activation.

Results:

Here we show that it is not the case in patients with suppressed viremia. Thus, IL-33 plays its alarmin role only during the early phase of the infection.

Keywords: Interleukin-33, Plasma, HIV, Viremia, Cardiovascular, Translocation.

INTRODUCTION

Interleukin-33 (IL-33), which is a member of the interleukin-1 (IL-1) cytokine family, is produced in response to the cell damage caused by infections or by breaches in tissue barrier integrity [1]. The soluble form of the IL-33 cognate receptor, suppression of tumorogenicity (sST2), acts as a decoy receptor; the plasma concentration of sST2 is a surrogate marker of IL-33 production and a prognostic marker of sepsis, acute respiratory distress, and of heart failure [1]. Recently, we reported that sST2 plasma levels were elevated in early HIV-1 infection [2]. Furthermore, sST2 levels

* Address correspondence to this author at the Professor Pierre Corbeau, Institut de Génétique Humaine CNRS UPR1142, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France, Tel: +33 4 34 35 99 32, Fax: +33 4 34 35 99 01, E-mail: pierre.corbeau@igh.cnrs.fr

† P.C. and J.-P.R. contributed equally to this work.

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correlated with the levels of intestinal-type fatty acid-binding protein (I-FABP), a marker of epithelial gut damage. We also found a correlation between plasma sST2 concentrations and the following markers of immune activation: CD8+ T-cell count, percentage of HLA-DR+CD38+CD4+ T-cells, percentage of HLA-DR+CD38+CD8+ T-cells, percentage of PD-1 CD4+ T-cells, soluble CD14 (sCD14), soluble CD40L, interferon-γ, and plasma indoleamine-2,3-dioxygenase activity. In the recent ACTIVIH study, we analyzed 68 markers of residual immune activation in 120 aviremic, HIV-1-infected adults treated with antiretroviral therapy [3]. We decided to measure this cohort's plasma sST2 concentrations.

METHODOLOGY

sST2 concentrations were determined by ELISA (Quantikine ELISA kit, R&D Systems) in stored, frozen plasma. sST2 concentrations were compared using unpaired two-tailed t test.

Findings

The 120 virologic responders we analyzed were mostly male (82%) and Caucasians (95%). Their duration of viral suppression (mean ± SD) was 102 ± 47 months, for a duration of infection of 17.2 ± 7.4 years. Their current and pretherapeutic CD4 counts were 688 ± 326 and 192 ± 108 cells/μL, respectively. Almost all of them were infected by cytomegalovirus (91%) and Epstein-Barr virus (98%), and only 5% by Hepatitis C virus. Consistent with our previous findings [2] and with those of Secemsky et al. [4], we found no difference in plasma sST2 concentrations between effectively-treated HIV-1-infected adults and age- and sex-matched healthy controls (Fig. **1a**). Although the concentration of sST2 was higher in untreated patients, this difference was not statistically significant (10.7 ± 1.0 and 9.7 ± 0.3 ng/mL, respectively, $p = 0.42$). sST2 plasma levels were not linked to the duration of viral suppression (Pearson $r < 0.01$, $p = 0.93$).

We subsequently looked for a link between plasma sST2 concentration and markers of causes of immune activation. Residual viremia, coinfections, microbial translocation, immune senescence and Treg deficiency have been identified as potential drivers of persistent immune activation in virologic responders [5]. We failed to find a correlation between plasma sST2 levels and i) residual viremia below 50 copies/mL or with frequency; ii) markers of senescence on CD4+ or CD8+ T-cells (CD27, CD28 and CD57) or on NK cells (CD57); iii) cytomegalovirus, Epstein-Barr virus, hepatitis A virus, hepatitis B virus, and/or hepatitis C virus coinfections; iv) the plasma concentrations of bacterial DNA, I-FABP or lipopolysaccharide-binding protein or v) the percentage of Treg cells. Furthermore, there was no correlation between plasma sST2 concentration and smoking tobacco.

We also looked for correlations between plasma sST2 levels and the phenotype of the immune activation observed in these patients. In the ACTIVIH study, a double hierarchical clustering of the 68 markers of immune activation and of the 120 patients resulted in the identification of 5 patient groups presenting with very different immune activation profiles [3]. This means that patients belonging to the same profile have common marks of immune activation as determined by the 68 markers we used. Looking for differences in sST2 levels among these immune activation profiles, we observed that patients with profiles 2, 3, and 4 tended to have higher sST2 concentrations than the group consisting of participants with profiles 1 or 5 (9.5 ± 0.4 and 8.5 ± 0.5 ng/mL, respectively, $p = 0.11$; Fig. **1b**). Of note, immune activation profile 2, which we previously showed to be strongly linked to an atherothrombosis-associated metabolic syndrome [3], correlated with a high sST2 concentration, which is prognostic of cardiovascular disease [6]. In line with our previous observation of a link between sST2 concentration and CD8+ T-cell count in early HIV-1 infection [2], we found here that chronically infected patients with immune activation profiles 2, 3 and 4 presented with higher CD8+ T-cell counts than patients with immune activation profiles 1 and 5 ($p = 0.02$; Fig. **1c**).

We also looked for correlations between plasma sST2 levels and each of the 68 activation markers. No correlation was observed between plasma sST2 levels and i) CD4+ or CD8+ T-cell differentiation (naïve/central memory/effector memory), CD4+ T-cell activation (HLA-DR and/or CD38), CD4+ or CD8+ T-cell exhaustion (PD-1); ii) NK cell activation (HLA-DR and/or CD69) or dysfunction (loss of CD56 expression); iii) B cell activation (IgG, IgA, and IgM plasma levels); iv) monocyte activation (sCD14, sCD163); v) neutrophil activation (CD64, PD-L1, loss of CD62L expression); vi) inflammation (CRP, soluble TNF receptor I); vii) endothelial cell activation (tissue plasminogen activator, soluble endothelial protein C receptor and thrombomodulin); and viii) fibrinolysis (D-dimer). However, we found a link between sST2 levels and the number of CD8+ T-cells expressing the activation marker HLA-DR (Pearson $r = 0.19$, $p = 0.03$; Fig. **1d**). This is illustrated in Fig. (**1e**) by the difference in HLA-DR+CD8+ T cell count in two patients presenting with sST2 plasma levels of 15.7 and 1.0 ng/mL, respectively.

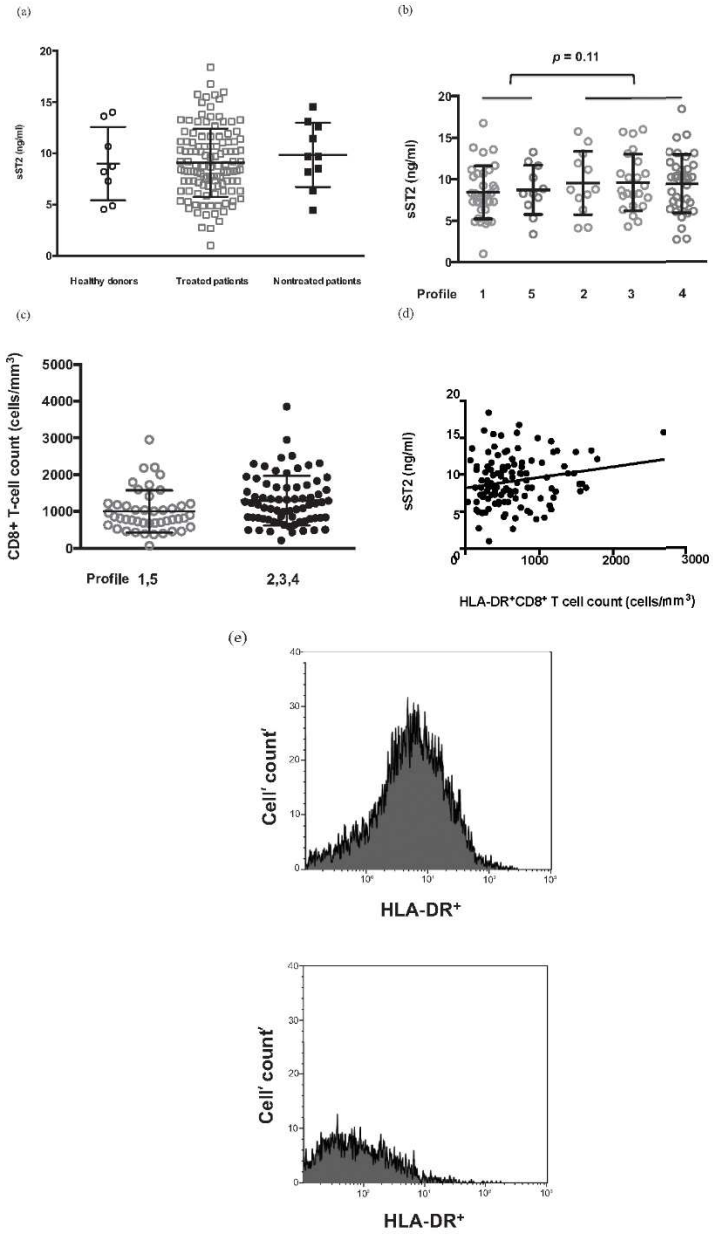

Fig. (1). Plasma sST2 concentrations during chronic HIV-1 infection. (a) Plasma sST2 concentrations in uninfected controls, and in treated or untreated, chronically infected adults. (b) Plasma sST2 concentrations in chronically infected adults with various immune activation profiles. (c) CD8+ T-cell count of patients presenting with various immune activation profiles. (d) Correlation between plasma sST2 concentration and the number of circulating CD8+ T-cells expressing CD38. (e) HLA-DR expression on CD8+ T cells from patients with sST2 levels of 15.7 (upper plot) and 2.7 (lower plot) ng/mL.

CONCLUSION

Collectively, our data showed that sST2 is mostly increased during early infection. HIV-induced gut barrier damage is major at this stage, being partly reduced under antiretroviral therapy [7]. IL-33 is produced by damaged endothelial and epithelial cells at this barrier site [1]. Accordingly, we observed a high sST2 peripheral blood concentration, linked to the levels of the microbial translocation markers I-FABP and sCD14 in early HIV infection [2]. It is thus logical that circulating sST2 levels are higher during the initial phase than during the treated chronic phase of the infection. IL-33 signaling via ST2 inhibits the development of atherosclerosis, and therefore, sST2 is thought to be proatherogenic [8]. Consequently, a slight sST2 overproduction in treated HIV patients, over years, might favour atherothrombosis. This could explain why sST2 level is a predictor of cardiovascular insufficiency in HIV patients aviremic under treatment [4] as it is in non-infected adults [6].

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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VI. Annexe 3 : productions et formations

A. Productions réalisées dans le cadre de la thèse

1. Publications

- « Reversing HIV latency via sphingosine-1-phosphate receptor 1 signaling » Charline Duquenne, Sandrine Gimenez, Adeline Guigues, Benjamin Viala, Caroline Boulouis, Clément Mettling, Damien Maurel, Noëlie Campos, Etienne Doumazane, Laetitia Comps-Agrar, Jamal Tazi, Laurent Prézeau, Pierre Corbeau et Vincent François. *AIDS-D-17-00622. (*2017)

- « Plasma Level of Soluble ST2 in Chronically Infected HIV-1 Patients with Suppressed Viremia » Mehwish Younas, Christina Psomas, Vikram Mehraj, Renaud Cezar, Pierre Portales, Edouard Tuaillon, Adeline Guigues, Jacques Reynes, Pierre Corbeau, et Jean-Pierre Routy. *The Open AIDS Journal* 11: 32‑35. doi:10.2174/1874613601711010032. (2017)

- « One of the Immune Activation Profiles Observed in HIV-1-Infected Adults with Suppressed Viremia Is Linked to Metabolic Syndrome: The ACTIVIH Study » Christina Psomas, Mehwish Younas, Christelle Reynes, Renaud Cezar, Pierre Portalès, Edouard Tuaillon, Adeline Guigues, Corinne Merle, Nadine Atoui, Céline Fernandez, Vincent Le Moing, Claudine Barbuat, Grégory Marin, Nicolas Nagot, Albert Sotto, Jean-François Eliaou, Robert Sabatier, Jacques Reynes et Pierre Corbeau. *EBioMedicine* 8 (juin): 265‑76. doi:10.1016/j.ebiom.2016.05.008. (2016)

- « Corrigendum to “One of the Immune Activation Profiles Observed in HIV-1-Infected Adults with Suppressed Viremia Is Linked to Metabolic Syndrome: The ACTIVIH Study” [EBioMedicine 8 (2016) 265-276] » *EBioMedicine* 10 (août): 318‑22. doi:10.1016/j.ebiom.2016.08.003. (2016)

- « The Two Human CXCR4 Isoforms Display Different HIV Receptor Activities: Consequences for the Emergence of X4 Strains » Charline Duquenne, Christina Psomas, Sandrine Gimenez, Adeline Guigues, Marie-Josée Carles, Claudine Barbuat, Jean-Philippe Lavigne, Albert Sotto, Jacques Reynes, Paul Guglielmi, Clément

Mettling, Vincent François et Pierre Corbeau. *Journal of Immunology (Baltimore, Md.: 1950)* 193 (8): 4188-94. doi:10.4049/jimmunol.1303298. (2014)

2. Article en préparation

- « The EBI2 receptor is coexpressed with CCR5 in CD4+ T cells and boosts HIV-1 R5 replication » Adeline Guigues, Sandrine Gimenez, Clément Mettling, Damien Maurel, Laurent Prézeau, Vincent François et Pierre Corbeau.

3. Communication affichées

- EBI2 interacts with the HIV Co-receptor CCR5 and interfers with HIV-1 infection » Adeline Guigues, Sandrine Gimenez, Clément Mettling, Pierre Corbeau et Vincent François. CBS2 days. Montpellier, 2016.

- « The A_{2A} receptor interacts with the HIV co-receptor and enhances HIV infection » Adeline Guigues, Sandrine Gimenez, Clément Mettling, Laurent Prezeau, Pierre Corbeau et Vincent François. Montpellier infectious diseases. Mèze, 2015

- « The A_{2A} receptor interacts with the HIV co-receptor and enhances HIV infection » Adeline Guigues, Sandrine Gimenez, Clément Mettling, Laurent Prezeau, Pierre Corbeau et Vincent François. IGH days. Montpellier, 2015.

- « Étude de l'effet d 'un récepteur couplé aux protéines G et de sa stimulation sur la fonction de CCR5, co-récepteur du VIH-1. » Adeline Guigues, Charline Duquenne, Sandrine Gimenez, Vincent François et Pierre Corbeau. Université des jeunes chercheurs, Sidaction. Carry le Rouet, 2013.

4. Communication orale

- « Interactions de CCR5 et d'EBI2 : conséquences sur l'infection par le VIH-1 R5 » *https://www.youtube.com/watch?v=BGqxWq-Cdpc.* Montpellier, 2016.

B. Formations suivies

Catégorie : Méthodologie et outils de la thèse

Accueil et Rencontre des Doctorants (11 décembre 2014)

Catégorie : Méthodologie de la recherche

Documentation / Dépôt électronique des thèses - Session 1 (19 janvier 2016) Université Montpellier 3 - Site Saint-Charles, salle des Colloques

Catégorie : Hygiène et sécurité

Préparation à l'habilitation conduite d'autoclaves CNRS délégation LR à Montpellier

Catégorie : Atelier technologique

Découverte du BRET pour l'étude de la transduction du signal et du criblage de molécules BioCampus Montpellier

Catégorie : Enseignement

Prise de parole en public, pédagogie interactive niveau 1 (09 décembre 2015)

Catégorie : Culture et médiation scientifique

La médiation scientifique : comment préparer et gérer un atelier de découverte scientifique destiné aux élèves du primaire CBS2 Montpellier et CEMEA Languedoc Roussillon

Catégorie : Formations scientifiques interdisciplinaires

Université des jeunes chercheurs 2013 Sidaction à Carry le Rouet

Catégorie : Outil pour la poursuite de carrière

« GESTION DU STRESS » Adopter un comportement gagnant (23 octobre 2015)

Catégorie : Insertion professionnelle

Concours Ma thèse en 180 secondes Languedoc Roussillon 2016 (11 février 2016)

Catégorie : Langues

Anglais préparation TOEIC doctorants en 3ème année +700 au test (29 septembre 2015) Université Montpellier 2, département des langues Bâtiment 5

IX. Références bibliographiques

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