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Maxime Touzot

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par

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Interactions cytokiniques dans le microenvironnement inflammatoire : Analyse à large échelle de la réponse aux Interférons de Type I lors la de polarisation des Lymphocytes T auxiliaires

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INDEX

PREAMBULE 1

INTRODUCTION 2

1 LES INTERFERON DE TYPE I (IFN) 2

1.1 Historique 2

1.2 La famille des Interférons 3

2 BIOLOGIE DES INTERFERONS DE TYPE I (IFN) 4

2.1 Production cellulaire 4

2.1.1 Voie endo-cytoplasmique 4

2.1.2 Voie extra-cytoplasmique 5

2.1.3 Production in situ d'IFN 7

2.2 Un unique récepteur pour plusieurs voies de signalisation 8

2.2.1 Le récepteur IFNAR 8

2.2.2 Voies de signalisations STAT-dépendantes et indépendantes 10

2.3 Les Interferon-stimulated genes ISGs : effecteurs de la réponse IFN 14

2.4 Les IFN : médiateurs de la réponse contre les pathogènes 16

2.4.1 Réponse antivirale 16

2.4.2 Réponse antibactérienne 17

2.5 Rôle des IFN dans la prolifération et apoptose cellulaire 18

2.6 Modulation de la réponse immune par les IFN 19

2.6.1 Les IFNs indispensables à l'homéostasie des cellules immunes 19

2.6.1 IFN et immunité innée 20

2.6.3 IFN et immunité adaptative 21

3. LES LYMPHOCYTES AUXILLAIRES CD4 (Th) 22

3.1 La polarisation des Th, modèle d'intégration dynamique de signaux 23

3.1.1 Intégration de signaux extracellulaires 23

3.1.2 Intégration et dynamique des voies de signalisation 24

3.1.3 Complexité et dynamique des facteurs de transcription 25

3.1.4 Contrôle épi-génétique 26

3.2 Plasticité des Th et rôle du micro-environnement 26

4 PLASTICITE DES CELLULES DENDRITIQUES 28

4.1 Historique 28

4.2 Diversités des DC 29

4.3 DC et inflammation 30

5 LA REPONSE IFN EN PATHOLOGIE 31

5.1 L'IFN exogène : une biothérapie efficace 31

5.1.1 Traitement des néoplasies 31

5.1.2 Traitement des maladies virales 32

5.2 Une réponse IFN paradoxale en pathologie inflammatoire 33

5.2.1 Arguments expérimentaux et cliniques 30

5.2.2 Mécanisme d'action : exemple du lupus érythémateux aigu disséminé 34

5.2.3 Un effet protecteur dans le traitement des pathologies Inflammatoires 35

5.3 Facteurs modulant la réponse aux cytokines dont les IFN 36

5.3.1 Généralités 36

5.3.2 Facteurs cellulaires extrinsèques 38

5.3.3 Facteurs cellulaires intrinsèques 39

6 BASES SCIENTIFIQUES DE LA THESE **40**

6.1 Question scientifique **40**

6.2 Approche scientifique **42**

7 RESULTATS

7.1 Projet 1: Multiparametric analysis of cytokine-driven human Th17 differentiation reveals a differential regulation of IL-17 and IL-22 production. **46**

7.2 Projet 2: Environmental control of type I IFN function during Human T helper differentiation. **63**

7.3 Projet 3: Human inflammatory dendritic cells induce Th17 differentiation. **104**

DISCUSSION ET PERSPECTIVES **120**

REFERENCES **130**

ANNEXES **148**

ABBREVIATIONS

Ahr : Aryl Hydrocarbon receptor.

CBA Cytometric beads Assay

EAE Experimental Acute Encephalitis

EMA : Easy Microarray Analysis

DC : cellules dendritiques.

IFN : Type I Interferon

IRF Interferon-Responsive-Factor

ISG Interferon stimulated genes

ISRE Interferon Stimulated Response Elements

LB : Lymphocyte B

LEAD : Lupus erythemateuse aigue disséminé.

LT : Lymphocyte T

LPS Lipossacharides

MAPK : Mitogene Activated Protein Kinase

mTOR : Mechanistic Target of Rapamycin

NK : Natural killer lymphocytes

NFKb : Nuclear Factor kappa B

PAMPS : Pathogen-associated-Molecular-Pattern

PBMCS : Peripheral blood mononuclear cells

pDC : cellules dendritiques plasmacytoides

RT-PCR : Real Time Polymerase Chain reaction

SEP : Sclérose en plaques.

STAT Signal Transducers of Activation and Transcription

TCR : T-cell receptor

TF : transcription Factor

Th : lymphocytes CD4 auxilliares ou T Helper

TLR : Toll like receptor

Treg : Lymphocyte T regulateur

VIH : virus de l'immunodéficience humaine.

PREAMBULE

Le système immunitaire repose sur un ensemble de cellules qui interagissent pour garantir une défense efficace contre des pathogènes. On distingue schématiquement deux branches : l'immunité innée et l'immunité adaptative. La première correspond à une réponse immune précoce, rapide et non spécifique médiée par différents effecteurs comme les mastocytes, les granulocytes, les cellules dendritiques (DC), les lymphocytes T invariant. La deuxième consiste en une réponse plus tardive, spécifique d'un antigène et médiée par les lymphocytes T (LT) CD4 et CD8 et les lymphocytes B (LB). Ces deux branches de l'immunité coopèrent entre elles par l'intermédiaire de cellules plus spécifiques de l'immunité innée (DC, cellules plasmacytoïdes pré-dendritiques (pDC), cellules T invariantes (γδ T cells)) ; mais aussi par des médiateurs solubles (cytokines/chemokines) dont les Interférons de type I (IFN).

La découverte des interférons par ISAACS et LINDEMANN a plus de 50 ans. Les travaux de recherche sur ces cytokines ont été à l'origine des découvertes et avancées spectaculaires en thérapeutique et en recherche fondamentale. La découverte des propriétés antivirales et anti-prolifératives des IFNs a permis leurs utilisations dans le traitement des pathologies tumorales (dès 1980); des maladies virales (Hépatite C en 1990) puis dans certaines maladies neurologiques (Sclérose en plaques, 1992). Parallèlement à leur développement en thérapeutique, le clonage puis séquençage des IFNs ont permis des découvertes majeurs en biologie cellulaire notamment sur les mécanisme de transcription des ARNs, sur la coopération ente l'immunité innée et adaptative, sur les mécanisme de résistance de certains virus ….

Durant ma Thèse, je me suis intéressé à la biologie des IFN et ainsi qu'à leur fonction modulatrice dans la réponse immune adaptative. Une des questions principales a été de mieux caractériser et de prédire l'effet des IFN pendant le processus de différentiation des cellules T Helper CD4.

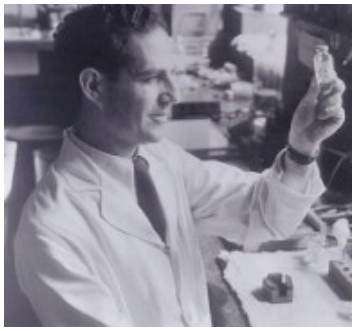
INTRODUCTION

1. LES INTERFERONS.

1.1 Historique

L'interféron a été la première cytokine individualisée par des biologistes [1]. Sa découverte revient à un virologiste anglais Alick ISAACS et un virologiste suisse Jean LINDEMANN en 1957 au National Institute for Medical Research (NIMR) à Londres. Leurs travaux reposaient sur l'interférence virale (Virus interference). Ils ont démontré à cette époque que la culture de cellule avec un premier virus « the interfering virus » bloquait la croissance d'un deuxième virus appelé « challenged virus ». Par une série d'expériences, ISAACS et LINDEMANN ont découvert que le mécanisme d'action de cette interférence provenait d'une molécule secrétée par les cellules elles-mêmes. Le nom « interféron » est à attribuer, probablement à LINDEMANN, par analogie avec les particules de Physique : « *it was time that biologists had a fundamental particle, for the physicists have so many; such as electron, neutron proton etc »*

Deux années de travaux ont permis l'identification complète de l'IFN qu'ISAACS présentait ainsi « *So far no antibiotics active against viruses have been discovered. To a large extent this is because viruses are extremely small parasites which are obliged to live inside cells, and it has not been possible to find a substance which would stop viruses from growing without at the same time harming the host cells. Interferon is the name which has been given to a new substance which prevents the growth of a number of viruses without apparently causing any gross damage to the cells. Interferon does not kill the viruses, but stops them from multiplying. This demonstration shows different aspects of the study of interferon" (Article de D. Burke en annexe)*



Alick ISAACS 1955

1.2 La famille des IFNs.

Les Interférons appartiennent à une grande famille de cytokines possédant une hélice alpha que l'on classe en 3 catégories selon le récepteur cellulaire qu'ils activent. On distingue ainsi les IFNs de type I (IFN), les interférons de Type II (IFN-γ) et les interférons de Type III (IFN-λ1 ou IL29 et IFN-λ2 ou IL-28) apparentés à la famille de l'IL-10 (IL19, IL-20, IL-22, IL24 et IL-26) [2-4]

Les Interférons de type I sont une famille de plusieurs cytokines : IFN-α, IFN-β, IFN-κ, l'IFN-ε, IFN-δ, IFN-τ et l'IFN-ω ou limitine chez la souris. Le concept des sous types d'IFN découlent des travaux de HAVELL en 1975 qui a mis en évidence les différences antigéniques entre l'interféron de fibroblaste humain (IFN-β,) et celui secrété par les leucocytes (IFN-α)[5]. Les gènes codant pour l'IFN sont apparus chez le poisson et semblent avoir évolués sous l'influence de deux pressions distinctes[6] : Une évolution verticale qui a permis la diversification des IFNs (IFN-α, IFN-β, IFN-κ, l'IFN-ε) au cours de l'évolution des espèces ; et une évolution interne conduisant à l'apparition de familles de gènes multiples notamment pour l'IFN-α (une vingtaine de gènes décrits chez la souris et l'homme). A noter que les gènes de l' IFN-α de souris présentent plus de similitudes entre eux qu'ils n'en présentent par rapport à leurs orthologues présents chez le rat ou l'homme[7]. Bien que les séquences des IFNs divergent considérablement d'une espèce à l'autre (20 à 30 % d'homologies entre les IFN-α et l'IFN-β), la structure tridimensionnelle définie par cristallographie est remarquablement conservée[8]. La raison de la maintenance et de la redondance des sous types d'IFN au cours de l'évolution est encore inconnue.

Les IFN-α et IFN-β ont été les plus étudiés et caractérisés sur le plan biologique et immunologique du fait de leur capacité à être produit par tout types cellulaires (en théorie) et par la présence d'un récepteur unique. Les 20 gènes humains de l' IFN-α codent pour 13 protéines fonctionnelles. La raison de la multiplicité des IFN-α est inconnue mais il semble que chaque sous-type possède des fonctions différentes. Leur expression qualitative et quantitative est par ailleurs dépendant du type de stimulus et du type cellulaire[9, 10]. Les autres IFNs comme l' IFN-κ, l'IFN-ε, IFN-δ, IFN-τ et l'IFN-ω ont une expression tissulaire plus spécifique[4, 11] .

2. BIOLOGIE DES INTERFERONS

2.1 PRODUCTION CELLULAIRE

La production d'IFN a été initialement décrite, en réponse à une infection virale[6], au cours de la réponse immunitaire innée. Cette dernière est caractérisée par un ensemble de modules (cellules et récepteurs) qui reconnaissent dans les minutes qui suivent l'infection d'une cellule par un pathogène[12] . L'immunité innée engendre une réponse spécifique à chaque pathogène par l'intermédiaire de divers récepteurs ou senseurs appelés PPR (Patterns Recognition Receptors) qui reconnaissent des structures conservées des pathogènes : les PAMPS (Pathogen-Associated-Moleculare Pattern)[13].

Toutes les cellules de l'organisme sont capables de produire des IFNs en quantité variable en réponse à deux types de signaux : 1/ Des signaux extracellulaires provenant de l'infection d'une cellule voisine qui activent certains récepteurs transmembranaires comme les Toll-Like recepteurs (TLR) 2/ Ou par des détecteurs cellulaires qui reconnaissent la présence intracytoplasmique d'ARN[14, 15] . L'ensemble des mécanismes de production de l'IFN est représenté par la figure 1. Deux points méritent d'être soulignés. D'une part, la localisation différente de ces récepteurs, aussi bien intra et inter cellulaire, est à l'origine de la flexibilité de la production d'IFN dans les cellules infectées. D'autre part, certains pathogènes non viraux (bactéries) ou certains stimuli inflammatoires peuvent induire une production d'IFN[14].

2.1.1 La voie endo- cytoplasmique.

La découverte d'une voie commune d'induction de l'IFN n'est que très récente (Années 2000). Elle est composée d'un ensemble de récepteurs cytoplasmiques, qui après avoir reconnu leur ligands spécifiques vont activés deux kinases clés : l'inhibiteur de NFKB (IKKε) et la TANK binding Kinase 1 (TBK1). Ces deux kinases ont pour cible nucléaire les Interferon-Responsive Factor (IRF) 3 et IRF7 nécessaire à la production d'IFN. Trois groupes de récepteurs ont été décrits : 1/ Les récepteurs reconnaissant spécifiquement des ARN composés des récepteurs Hélicase MDA5 (Melanoma differentiation-associated genes 5) et RIG-1 (Retinoic acid-inducible gene I)[16, 17] ;

2/ Des récepteurs activés par l'ADN[18, 19] 3/ et enfin, les récepteurs nucleotide-binding-oligomerization domain-containine protein (NOD) 1 et 2 [20, 21] .

MDA5 et RIG-1 sont deux récepteurs qui reconnaissent les ARNs double-brins viraux mais aussi autologues. Ils possèdent deux domaines CARD (Caspase recruiting domain) à leur extremitée N-terminale, qui leur permettent de transmettre le signal d'activation par l'intermédiaire d'une molécule adaptatrice mitochondriale appelée MAVS (Mitochondrial anti viral signaling) ou encore IPS1 (Interferon-β promotor stimulator), VISA et CARDIFF[22, 23]. L'interaction des deux hélicases avec MAVS active alors la voie classique (IKK ε et TBK1) et la voie NFκB via les deux adaptateurs FADD et RIP1.

Plusieurs récepteurs reconnaissant l'ADN viral ou bactérien (cyclic-di-GMP) comme DAI[23], STING ou DDX41 sont une alternative à l'induction des IFN par un mécanisme impliquant TBK1[18, 19, 24]. Enfin un nouveau récepteur cytoplasmique LRRFIP-1 serait capable de stimuler la transcription ISGs en phosphorylation d'une β-catenine responsable de l'acétylation de promoteurs d'ISGs [25] .

Les récepteurs NOD1 et 2 peuvent induire de l'IFN lors d'une infection bactérienne[20, 21]. Ces deux récepteurs possèdent aussi un domaine CARD dont le signal d'activation implique en aval la kinase RICK , qui interagit avec TRAF3 et MAVS pour induire l'activation d'IRF5 et IRF7 via TBK1. Deux publications ont montré ainsi que Mycobacterium Tuberculosis et Helicobacter Pylori induisent une production d'IFN par les macrophages via NOD2 et par les cellules épithéliales via NOD1 respectivement.

Chaque type cellulaire est donc capable de produire des IFNs (surtout l'IFN-β et quelques sous type d'IFN-α dont l'IFN-α4) par l'intermédiaire de ces trois voies de signalisation.

2.1.2 La voie extra cytoplasmique : restreinte mais plus spécifique.

Certaines cellules présentatrices d'antigènes (macrophages et DC) ont la capacité de produire de l'IFN après l'activation de récepteurs transmembranaires spécifiques : les Toll like recepteurs (TLR) TLR3, TRL4, TLR7 et TLR9. La famille des TLR est composée de différents récepteurs transmembranaires qui reconnaissent spécifiquement les PAMPS présent dans l'environnement. Onze TLR ont été décrits chez l'homme[26]. L'activation d'un TLR engendre par l'intermédiaire de leur domaine cytoplasmique (Toll/Interleukin-1receptor TIR) une voie de signalisation qui induit la transcription de

multiples gènes impliqués dans la réponse immune innée et adaptative[12]. Les TLR3, TLR7 TLR8 et TLR9 jouent un rôle important dans la détection de particules virales, le TLR4 reconnaissant le Lipossacharides (LPS) des bactéries. Les TLR7 et TLR8 reconnaissent l'ARN simple brin et les TLR9 l'ADN[27] .

Les cellules plasmatoïdes pré-dendritique (pDC) sont considérées comme les cellules spécialisées dans la production d'IFN[28, 29]. Elles sont capables de se transformer en DC matures après activation. Les pDC humaines expriment très fortement les molécules HLA de classe II (HLA DR), le récepteur à l'IL-3 (CD123) et spécifiquement le marqueur de type lectin BCDA2. Elles produisent tous les types d'IFN[30-32] en grande quantité (entre 3 et10 pg/cellules soit 300 fois plus que d'autres DC) après une infection virale. Apres 6 heures d'activation, 60% de l'activité transcriptionnelle des pDC correspond à la synthèse des Interferon-Stimualted Genes (ISGs)[30]. Les pDC expriment spécifiquement les TLR7 et TLR9 qui reconnaissent les ARNs simple-brin (Virus Influenza, VSV) et l'ADN double-brin respectivement [27, 33, 34]. Une fois activés, les TLR7 et 9 recrutent la molécule adaptatrice MyD88 (myeloïde differentiation molecule 88) ; qui s'associe à IRAK-4 pour activer IRF-7 et induire la production d'IFN. A noter que les pDC possèdent aussi les récepteurs MDA-5 et RIG-1 dont le rôle dans la production d'IFN reste mineure [35].

Certaines cellules dendritiques comme les Monocytes-derived Dendritic cells (MoDC) ou les macrophages peuvent aussi produire de l'IFN, en quantité plus faible que les pDC, après l'activation des TLR3 et TLR4[31]. Le signal d'activation nécessite une autre molécule adaptatrice TRIF qui s'associe à TBK1 et iKKI pour activer IRF3. Dans ce type cellulaire, il semblerait que les TLR3 et TLR4 induisent spécifiquement la production des IFN-β, l'IFN-λ et de l'IFN-α1[36] .

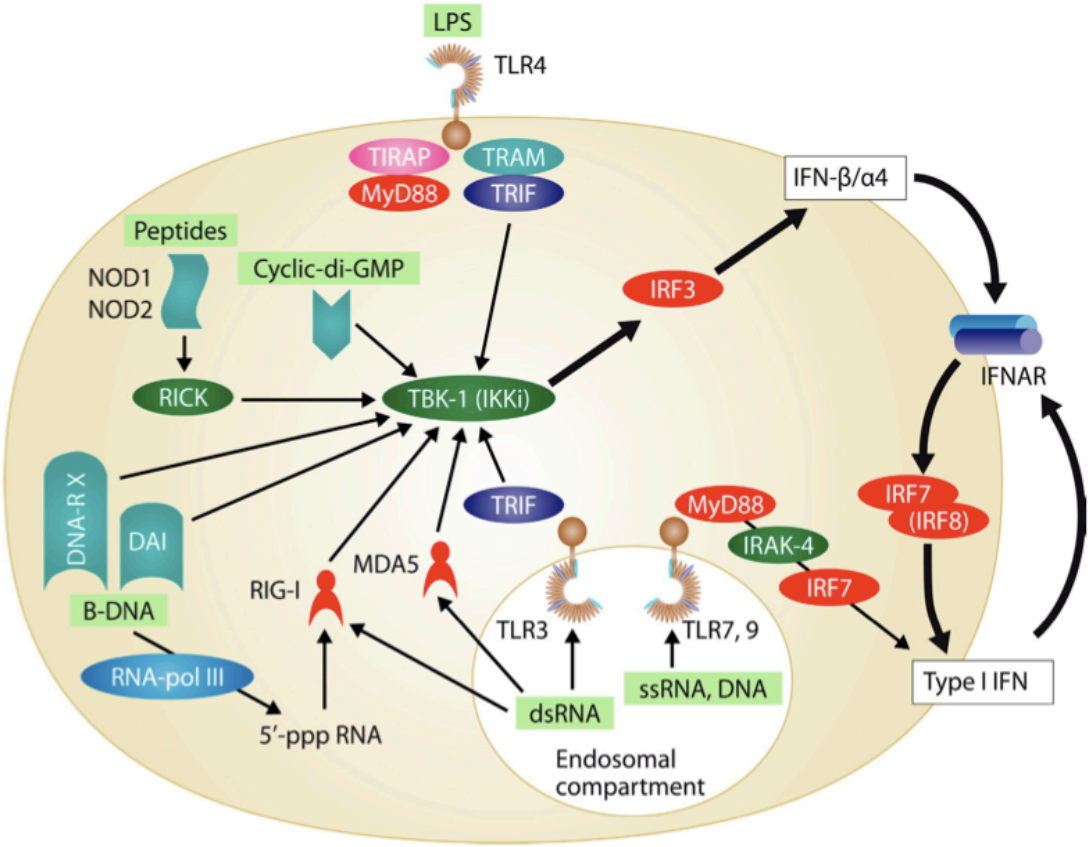


Fig. 1 : Mécanisme de production des IFN : Voies endo et extracellullaires. Adapté de Trinchieri Journal of Experimental Medecine 2010

2.1.3 Production anatomique des IFNs

Une production constitutive d'IFN-α (ARNm) est observée dans les PBMCS, et dans certains organes comme le foie, la rate, et les reins de souris[37] . Chez l'homme, le thymus serait le seul organe lymphoïde ou l'on peut détecter de l'IFN-α physiologiquement. Chaque cellule pouvant produire de l'IFN, on peut s'attendre à en trouver dans tous les tissus de manière physiologique, ou bien, dans un contexte pathologique. Dans le cadre d'infections virales, l'IFN peut être produit par les premières cellules infectées comme les cellules épithéliales bronchiques, les cellules du système nerveux central[38, 39]. L'infection peut ensuite se propager dans les organes lymphoïdes secondaires ou certains virus continuent leur réplication et induisent une production d'IFN[28] .

Chez l'homme, il a été observé de la même manière la production d'IFN par les pDC dans les ganglions de patients infectés par le virus de l'immunodéficience humaine (VIH)[40].

Des résultats similaires sont observés aussi dans des modèles d'infections par le SIV chez les primates[41] .

Dans un contexte pathologique, une preuve indirecte de la sécrétion in situ d'IFN est la présence des pDC que nous détaillerons plus loin. Ainsi, on observe ces cellules aussi bien dans des pathologies inflammatoires : psoriasis, dermatite atopique[42], maladie de Crohn[43], polyarthrite rhumatoide[44], sclérose en plaques (SEP) [45] que dans des pathologies tumorales : cancer ORL[46], lymphome de la peau .

Il faut noter par ailleurs que toutes molécules inductrices d'IFN est capable d'induire une production d'IFN in situ ou dans les ganglions drainant comme en témoignent les résultats de modèles murins[47]

Il est difficile enfin de déterminer précisément la concentration exacte d'IFN produit in situ qui dépend à la fois du type cellulaire, du nombre de cellules activées, de l'intensité du stimulus et de l'espace ou se produit la réaction. Les doses utiliseés d'IFN dans les différents systèmes expérimentaux sont très variables allant de 10 à 10000 UI/ml ou 0,1 à 100 ng/ml en fonction de l'effet recherché. Une étude chez la souris[39] a montré qu'après l'injection intra péritonéale de virus (8-24 heures), le taux d'IFN circulant est d'environ 1000 UI/ml.

2.2 RECEPTEURS ET VOIES DE SIGNALISATION

La grande spécificité et caractéristique de la réponse aux IFNs réside dans leur récepteur. En effet, les différents sous types d'IFN activent un récepteur unique qui est capable de générer des réponses biologiques complexes et différentes en fonction du stimulus initial. Ce phénomène peut être en partie expliqué par les caractéristiques, à la fois du récepteur mais aussi par la complexité des voies de signalisation cellulaires induites.

2.2.1 Un unique récepteur cellulaire IFNAR

IFNAR est un hétérodimère transmembranaire composé de deux 2 unités IFNAR1 et 2 conservées à travers l'évolution dont le gène se situe sur le chromosome 21 [4, 48, 49]. IFNAR est associé à deux tyrosines kinases intra-cytoplasmiques de la famille des Janus kinase (TYK2 et JAK1) dont la phosphorylation est à l'origine de la réponse aux IFN[50]. L'activation d'IFNAR comporte plusieurs étapes : L'IFN se fixe à l'une des sous unité puis

recrute secondairement la deuxième sous unité pour former un complexe tri-moléculaire dont la stabilité est l'élément clé de la signalisation des IFN [49, 51].

L'existence d'un unique récepteur pour les IFNs contraste avec les différences qualitatives et quantitatives des effets biologiques observés pour l'IFN-α et IFN-β. Plusieurs facteurs intrinsèques et extrinsèques au récepteur peuvent partiellement expliquer ces différences.

1/ *L'affinité des sous unités pour les IFNs* : IFNAR1 a une affinité en moyenne 1000 plus faible que IFNAR2 pour les IFNs. IFNAR1 serait le facteur limitant à la formation et la stabilisation du complexe tri-moléculaire[52]. L'affinité du récepteur dépend aussi des sous type d'IFN. Les IFN-α ont une plus faible affinité (facteur 10) que les IFN-β pour le récepteur IFNAR[53].

2/ *La concentration des récepteurs à la surface cellulaire :* La concentration d'IFNAR1 et IFNAR2 varie d'une type cellulaire à un autre [54]. Des études in vitro ont montré aussi que la fixation de l'IFN-α sur IFNAR2 entraîne le recyclage immédiat du récepteur à la surface de la cellule, contrairement à l' IFN-β dont la fixation provoque la dégradation de ce dernier [55].

3/ *Une réponse cellulaire spécifique*. La réponse IFN mesurée par le degré de phosphorylation de la voie Janus kinase dépend fortement du type cellulaire. IFN-α2 et IFN-β activent de manière équivalente STAT1/STAT2 dans des MoDC [54, 56] mais à un degré moindre pour l'IFN-α2 sur des myocardiofibroblastes humains.

4/ *La stabilité et la demi-vie* du intracellulaire du complexe tertiaire gouverne en partie les signaux intracellulaires [52].

La réponse aux IFN implique, cependant, d'autres mécanismes cellulaires qui peuvent expliquer les diversités des effets cellulaires. Un exemple typique provient des travaux de LAVOIE[57]. Dans leur modèle expérimental, L'intensité de fixation de l'IFN-α2 et l'IFN-β sur IFNAR corrèle bien avec l'effet anti-prolifératif de l'IFN et peut être modélisé. Mais cette relation n 'est pas retrouvée si on s'intéresse à la réponse antivirale.

2.2.2 Les voies de signalisation STAT-dépendantes et indépendantes.

Durant les deux dernières décennies, de nombreux travaux ont permis une meilleure compréhension des voies de signalisation en aval d'IFNAR. La réponse aux IFNs dépend de la coopération et de la régulation active de multiples voies de signalisation. On peut schématiquement distinguer : la voie d'induction des Interferon stimulated genes (ISG) régulés par les molécules STAT et une voie d'amplification des ISGs contrôlant essentiellement la transcription et la translation des ARNm. Chacune de ces voies est importante mais non suffisante pour une réponse complète.

Les voies STAT-dépendantes (Fig2). L'activation IFNAR entraine une phosphorylation et transphosphorylation des deux Janus kinase TYK2 (couplée à IFNAR1) et JAK (couplée à IFNAR2). Elles recrutent alors les médiateurs clés de la voie de signalisation : les Signaux de Transduction et d'Activation de Transcription (STAT) 2 dans un premier temps puis STAT1[58]. Il en résulte une translocation de l'hétéro-dimère STAT1-STAT2 phosphorylé dans le noyau. Ce dernier s'associe à l'Interferon Regulateur Facteur IRF-9 pour former l'Interferon-Stimulated Gene complex ISG3 [59, 60]. Ce trimère est le seul complexe capable de se fixer spécifiquement sur les éléments appelés IFN Stimulated Response Elements (ISREs) présents dans les promoteurs des ISGs. D'autres voies STAT n'impliquant pas STAT1/2 peuvent être activées par IFNAR. Par exemple, TYK2 est associée aussi à STAT5 et lors de son activation recrute la molécule adaptatrice CRKL. Le complexe CRKL activé -STAT5 migre dans le noyau et se fixe sur des séquences spécifiques GAS (Interferon Gammma Activated Site) présent dans certains promoteurs des ISGs[59]. Enfin les molécules STAT peuvent indépendamment de l'ISG3 se fixer sur des séquences de reconnaissance des promoteurs d'ISGs [61] .

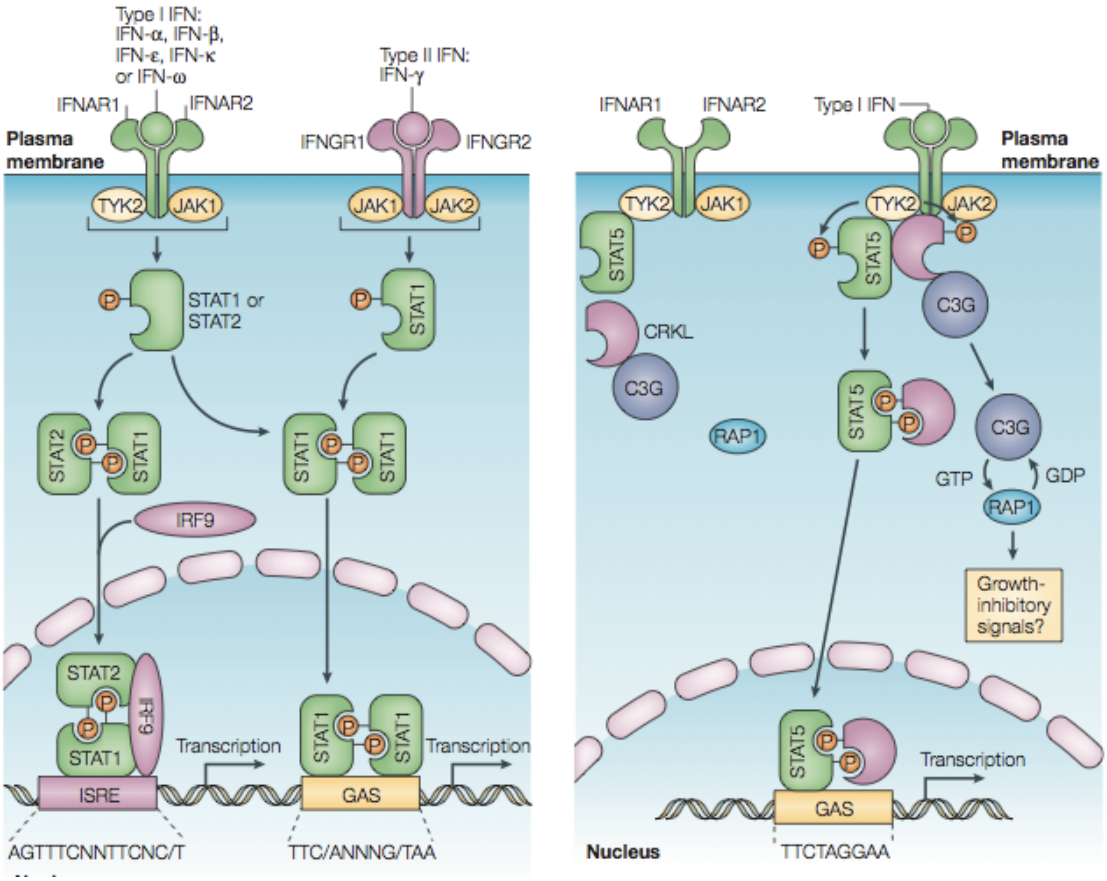


Fig 2 : Voies de signalisations STAT-dépendantes : adaptée de Platanias Nature review immunology 2005

Les voies STAT-indépendantes (Fig3). Elles participent essentiellement à l'amplification de la réponse IFN grâce à l'initiation de la translation des ARNm. Parmi elle, la voie des mTOR *(mechanistic Target of rapamycin)* et la MAPK (*Mitogene Activated Protein kinase*) [62, 63] ont été particulièrement étudiées.

La voie mTOR est une voie fondamentale dans le contrôle de différents processus cellulaires tels que la prolifération l'homéostasie cellulaire, l'autophagie[64]. Les IFNs sont capables d'activer la voie PI3K-Aky-mTOR via la phosphorylation de l'insuline-receptor-susbrate 1 (IRS1) et IRS2 par JAK1 et TYK2. mTORC1 régule deux molécules importantes en aval : la protéine ribosomal S6 (RPS6) et le répresseur (4EBP1 : eukariot

cap binding protein) qui augmente la synthèse ribosomale et l'initiation de la traduction des ARN messager [64] . Le répresseur EIF4A en augmentant la traduction de l'ARNm du FT NFKB, serait à l'origine de l'amplification de la réponse IFN- β. Par ailleurs, l'activation de mTOR exerce un rétrocontrôle positif sur la production d'IFN dans les pDC humaines et murines [65]. Le complexe mTORC2 module aussi dans la réponse IFN en contrôlant la phosphorylation de l'Akt induit par l'IFN et nécessaire à l'induction des ISGs comme l'ISG15 ou le CXCL10 [66-68]

Les MPAKS sont des serine/threonine kinase que l'on regroupe en 3 catégories : les kinases extracellulaire (ERK1, ERK2, ERK3, ERK5, ERK7), la famille des stress activated p38 et les c-jun-terminal kinases (JNK1, JNK2 and JNK3) [68]. Ces trois kinase jouent un rôle majeur dans les processus cellulaire tel que le développement, la différentiation, la prolifération et l'apoptose [69]. Les kinase p38 peuvent être induites par l'IFN [70]. Même si lien entre la voie p38 MAPK et l'initiation de la translation de l'ARN messager reste encore à démontrer, plusieurs rapports suggèrent quand même une régulation de la transcription des ISGs[70, 71]. Les IFNs activent i les kinases ERK1/2 [72], modulant la prolifération cellulaire différemment en fonction des systèmes. L'inhibition de MEK/ERK augmente l'effet anti-proliferatif de l'IFNα sur les lymphocytes CD4 ou sur des cellules myelomateuses humaines [73, 74] mais son activation augmente l'apoptose de différentes lignes cellulaires [75]. A noter que ERK1/2 contrôle aussi deux molécules MnK1 et RSK régulant la translation grâce aux deux complexes eIF4BP et l'eIF4E respectivement [66].

Rôle des micro ARNs (miRNA). Ce sont de petits ARNs capables de se fixer à l'extrémité 3'UTR des ARN messager et de les activer ou les inactiver. Les IFNs sont capables d'induire ou de réprimer certains des miRNAs nécessaires à la réplication virale [76, 77]

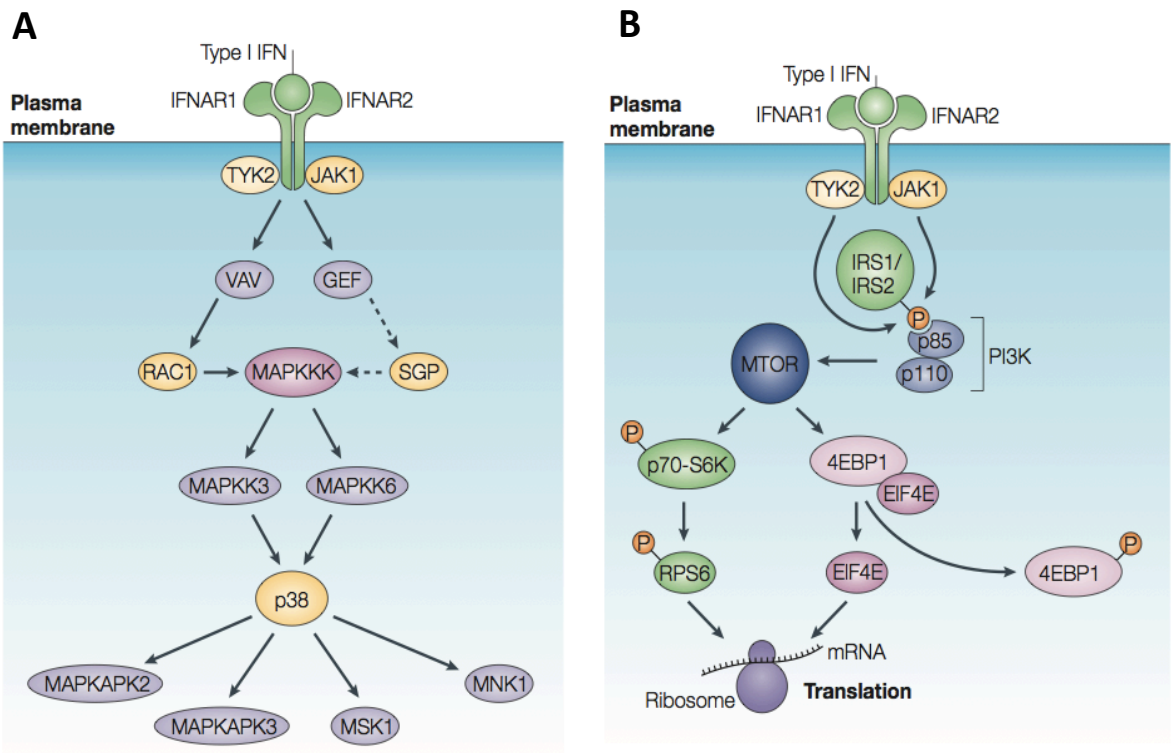


Fig3 : Voies de signalisations STAT-indépendantes **:** A/ voies des MAKP, B/ Voie mTOR qui contrôle les mécanismes de translation de l'ARN messager. Adapté de Platanias Nature review immunology 2005

Les voies de rétrocontrôle. La voie de signalisation de l'IFN est sujette à la fois à un feedback positif et négatif, ou plusieurs éléments de ces voies sont des ISGs. Les voies de signalisation cytosolique sont amplifiées par la synthèse de MDA5, RIG-1, DAI et TRIM25. La voie NFKb est inhibée par la synthèse de son inhibiteur IKBa. La voie commune de signalisation de l'IFN est amplifiée d'un coté par l'induction des molécules STAT1-2 et IRF7; et négativement réguler d'autre part par SOCS1 (inhibiteur de JAK2). Récemment une nouvelle voie constitutive de contrôle négatif de l'expression d'IRF7 par FOXO3 a été observée, et serait un facteur qui limiterait l'inflammation 2nd à la réponse antivirale[78] .

2.3 LES IFN STIMULATED GENES : Effecteur de la réponse IFN

Les voies de signalisation en aval d'IFNAR vont activer la transcription des principaux effecteurs de la réponse IFN : les Interferon Stimulated Genes ISGs. Les premiers ISGs ont été décrits il y a maintenant 30 ans grâce à l'isolation des ADNs complémentaires correspondant aux ARNm fortement induits par l'IFN [79-82]. Initialement, trois catégories d'ISGs ont été caractérisées. Les ISGs ayant une activité antivirale comme les Proteines Mx, la 2'5' oligoadenylate (2-5A) synthetase et la RNA-activated protein kinase (PKR) ; la famille des molécules (STAT) ; et enfin la famille des IFN regulator factor (IRF) [83, 84].
L'apparition des techniques d'analyse à large échelle (génomique et transcriptomique) a été une étape majeure dans l'identification et dans la caractérisation des ISGs. L'équipe de WILLIAMS à Cleveland a été la première à montrer la diversité des ISGs dans deux papiers princeps[61, 85]. En utilisant comme modèle une lignée cellulaire de fibrosarcome « HT1080 », ils ont individualisé 122 gènes différentiellement exprimés en réponse à l'IFN [85]. L'utilisation de puces à ADN plus performantes et d'autres types cellulaires (cellules dendritiques humaines et lignées cellulaires) a permis d'enrichir cette liste jusqu'à 335 gènes [61] . Cette liste n'est cependant pas définitive.

Selon les études publiées, le nombre d'ISGs varie entre 100 et 400, en fonction de trois facteurs :
Le type cellulaire étudié (LT, PBMCS, DC, cellules endothéliales, lignes cellulaires tumorales de mélanome, HepG2, HuH7, HT1080) [9, 61, 85-87] .
La capacité de détection des gènes par les puces ADN : 6800 gènes dans l'étude princeps de Der PNAS, 13000 dans les puces Affymetrix de l'étude de CERTA [88].
Les sous typse d'IFN utilisés : l'IFNα et l'IFNβ induisent respectivement 226 et 370 gènes sur des PMBCS traités par l'IFN[89] . A noter que la transcription d'ISGs semble aussi dépendre du type cellulaire par analogie à l'activation des voies de signalisation par IFNAR. SCHLAAK et coll ont montré des différences qualitatives et quantitatives dans l'induction d'un faible nombre d'ISGs sur plusieurs types cellulaires [9]. Ces résultats se rapprochent de ceux observés avec l'IFN-γ [89].

La cinétique d'induction des ISGs est variable et suit un mode biphasique. On distingue une réponse précoce d'ISGs entre 2 et 8 heures qui peut soit persister en plateau soit

diminuer rapidement ; et une réponse plus tardive entre 28 et 32 heures [86, 87]. Cette dernière peut correspondre soit à l'induction de nouveau ISGs soit à un mécanisme d'amplification de la réponse primaire.

La liste établie par le groupe de WILLIAMS en 2001 (Fig4) a été la première à proposer une classification fonctionnelle des ISGs. De nombreux ISGs ont une activité antivirale, certains interviennent dans la prolifération cellulaire, l'apoptose, la présentation et le processing des antigènes, l'activation ou la répression de transcription, les voies de signalisations intracellulaires. Depuis 2009 un consortium a mis à disposition une base de données gratuite sur les gènes induits par les Interférons de type I, II et III dans 37 espèces différentes (INTERFEROME[90]). Elle intègre les informations issues d'analyse à large échelle publiée (microarray, next generation sequencing), de cellules traitées par de l'IFN. A ce jour, elle comporte environ 2000 gènes potentiellement induits par les IFNs [91]. Cette base de données est un outil supplémentaire facilitant les analyses de pathways cellulaires, des fonctions biologiques ou des signatures transcriptomiques induites par l'IFN [91] .

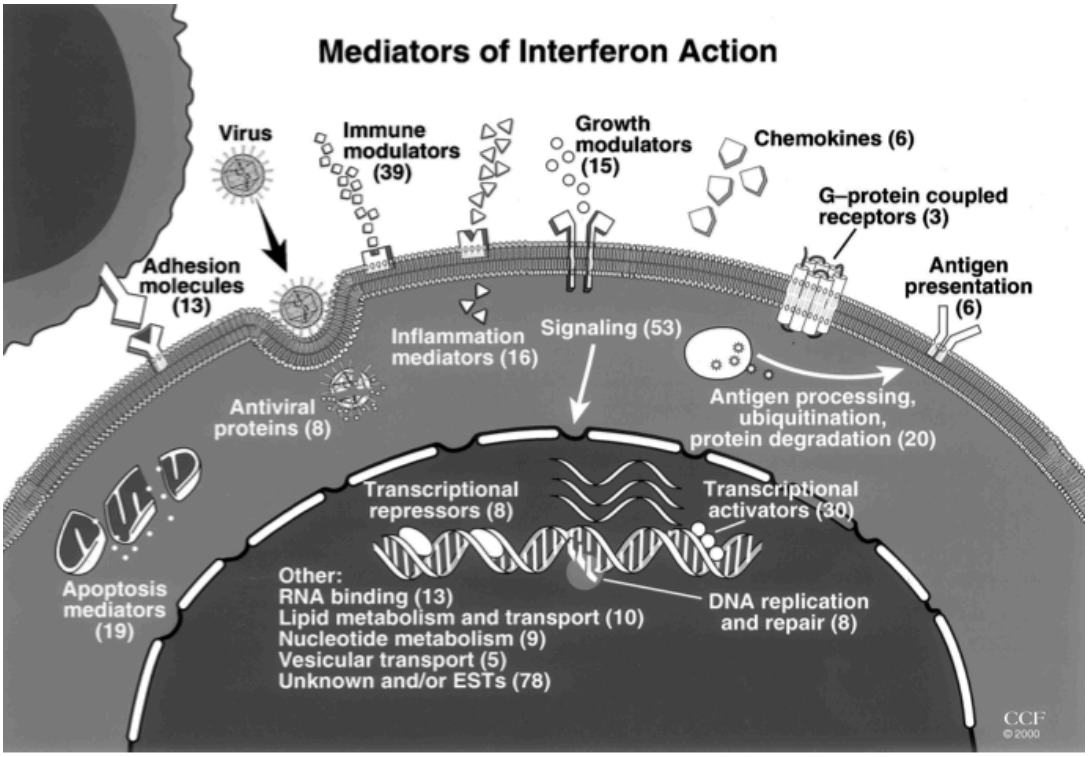


Fig 4 : Premier diagramme représentant les fonctions des ISGs Adapté de De Veer Journal of leukocyte Biology 2001.

2.4 LES IFN : MEDIATEURS DE LA REPONSE CONTRE LES PATHOGENES

2.4.1 Réponse Antivirale

Il a fallu attendre quelques décennies après les travaux de LINDEMANN pour démontrer le rôle indispensable et primordial des ISGs dans la réponse antivirale. Toute anomalie qualitative ou quantitative de la réponse IFN peut entrainer une diminution de la réponse antivirale. Les souris déficientes en IFNAR sont plus susceptibles aux infections virales [92, 93]. Chez l'homme, les mutations génétiques des voies de signalisation de l'IFN (TYK2 ou STAT) sont responsables d'infections virales létales[94-96].

Malgré le nombre d'ISGs existants, la majorité des travaux des deux dernières décennies a porté sur un nombre restreint d'entre eux. Les études ont cependant permis des découvertes fondamentales sur les mécanismes de translation et de régulation des ARNm [97]. Nous détaillons brièvement le mode d'action des principaux ISGs ayant une activité antivirale :

L'IFITM3 est une protéine transmembranaire inductible par l'IFN. C'est le seul ISG décrit capable de bloquer l'entrée des virus dans la cellule [98].

L'ISG15 est une protéine homologue de la famille des ubiquitines fortement induite par l'IFN. Son rôle est de se fixer sur une cible protéique spécifique (158 cibles potentielles recensées dont de nombreux ISGs), et d'inactiver cette dernière après le recrutement de différentes ubiquitines. Ce phénomène s'appelle l'ISGylation [99-101].

Les protéines MX GTPase sont des guanosine hydrolylases localisées dans le cytoplasme (MX2) ou le noyau (MX1). Ces protéines ciblent spécifiquement les structures de la capside virale favorisant leur dégradation [102].

La 2'5' Oligo Adenylase Synthetase (OAS1 et OAS2) et la RNAL sont deux protéines exprimées faiblement, de manière constitutive dans la cellule. Activés par l'ARN par double brin, les monomères d'OAS1 se regroupent en tetramère pour activer la RNASL. Cette dernière clive ensuite les ARN cellulaires et virales [103].

La protéine Kinase R (PKR) est exprimée de manière constitutive sous forme de monomère dans le cytoplasme et le noyau de la cellule. Comme l'OAS, la PKR est activée par l'ARN viral, et forme un dimère qui correspond à la forme enzymatique active. La PKR intervient dans différentes voies de signalisation cellulaire et notamment l'initiation de la translation médiée par EIF2α [97, 104]. D'autres ISGs antiviraux ont été

mis en évidence par leur implication dans la réplication de virus (HCV et HIV) comme la Viperin ou RSAD2, l'APOBEC3G, et le TRIM5a [105, 106].

Les progrès technologiques (expression d'ISG via des lentivirus) ont permis, récemment au groupe de RICE [107] d'approfondir les fonctions antivirales des ISGs. SCHOGGINS et coll ont testé, de manière systématique, le potentiel antiviral d'un set de 380 ISGs sur-exprimés dans des lignées cellulaires, soumises à une infection virale. Deux découvertes importantes émanent de leurs résultats.
Ils ont individualisé 3 catégories d'ISGs : un groupe minoritaire ayant un fort potentiel inhibiteur et un rôle dans le feedback positif des voies de signalisation IFN; un groupe majoritaire avec un potentiel inhibiteur plus « modeste » et de manière surprenante, un groupe capable d'activer la réplication virale. L'action antivirale de certains ISGs est plus spécifique que d'autres, et leur action synergique permet alors une inhibition quasi maximale de la réplication virale. De manière collective, les ISGs peuvent cibler toutes les étapes du cycle de réplication viral. La fonction biologique et moléculaire principale des ISGs antiviraux reste l'inhibition de la translation. Ces résultats et caractéristiques ont été confirmés depuis par un deuxième groupe sur un nombre moindre d'ISGs (288) et de virus testés (VZV et MHV8) [108]. Leur système d'analyse à large échelle est un outil intéressant pour l'identification de nouvelle cible thérapeutique [109, 110]. On ne sait cependant si les résultats de surexpression in vitro reflètent l'action des ISGs In vivo [111].

Au total, l'effet antiviral des IFN est lié principalement à un versant « immunité innée » grâce à l'effet direct des ISGs, mais aussi à un versant « immunité adaptative » par l'activation des Cellules dendritiques (DC) et des effecteurs CD8 mémoires [112]. A noter cependant qu'une sécrétion chronique d'IFN peut entrainer une dysfonction de la réponse adaptative comme dans le cas de l'infection par le VIH-1 [113].

2.4.2 Réponse Antibactérienne.

De nombreuses bactéries induisent une réponse IFN (principalement IFN-β) par la voie cytoplasmique incluant surtout les TLR2, TLR4, NOD récepteurs ou les DNA senseurs STING et MAVS [114, 115]. Les pDC peuvent aussi produire de l'IFN, lors d'une infection à staphylocoque via l'activation du TLR9. La réponse IFN est néanmoins ambivalente, à la

fois protective ou néfaste pour le système immunitaire selon le type de bactérie et la localisation de l'infection[116] .

D'une part, l'IFN-β a un effet protecteur dans des modèles murins d'infections pulmonaires à Streptocoque pneumoniae, Pseudomonas aeruginosa, Chlamidyae pneumoniae [117-120] et dans certaines infections digestives[121]. Différents mécanismes d'action ont été proposés : l'inhibition de réplication de la bactérie par augmentation de la production du tryptophane [120, 122], le recrutement des lymphocytes Natural Killer (NK), γδ T Cells et CD8 cytotoxiques [121] .

D'autre part, la réponse IFN peut aussi, inhiber la réponse immune antibactérienne. Les souris invalidées pour le récepteur IFNAR sont mieux protégées que les souris contrôle lors d'infections par des bactéries intracellulaires comme Listéria monocytogènes [123-125]. L'inhibition de l'IFN diminue les infections à Staphylocoque aureus et le risque de choc septique [126]. L'IFN participe directement à la physiopathologie des arthrites inflammatoires lors de la maladie de Lyme [127]. Récemment, une signature IFN dans les neutrophiles de patients atteints de tuberculose a été identifiée, suggérant un rôle inattendu des IFNs dans la physiopathologie de cette maladie [128].

2.5 ROLE DES IFN DANS LA PROLIFERATION ET APOPTOSE.

En parallèle de la découverte de l'effet antiviral par ISSACS, PAUCKER et coll ont observé également, un effet antiprolifératif de l'IFN sur certaines lignées cellulaires infectées [129]. De nombreux travaux ont confirmés le rôle primordial et parfois paradoxal des IFN sur la prolifération et la survie cellulaire. Les IFN sont considérés comme des cytokines ayant des effets anti-prolifératifs et pro-apoptotiques [130]. Le degré d'activation cellulaire et le micro-environnement dans lequel se trouve la cellule peuvent cependant moduler ces effets de manière inverse.

L'effet antiprolifératif des IFN est attribué à un arrêt du cycle cellulaire (prédominant sur la phase G1) par une inhibition de certaines cycline-dependant kinase Cdk [131, 132] ou l'inhibition de c-myc [133]. Ces travaux ont utilisé des lignées cellulaires cancéreuses et ont été confirmés secondairement, dans des modèles murins. Un effet similaire est retrouvé sur des LT CD4 humains pré-traités par de l'IFN mais il disparaît si l'IFN est administré de manière concomitante à l'activation des CD4 [134]. Ces résultats suggèrent que l'activation cellulaire surpasse l'effet antiprolifératif induit par l'IFN. Nous avons

confirmé ces résultat dans notre système in vitro, dans 4 contextes de polarisation différents (*Article en soumis fig suppl2*). Les IFN sont aussi capables de promouvoir la prolifération des LB et LT de manière indirect par en induisant les molécules de survie BAFF et l'IL-7 respectivement [135-137] .

L'effet de l'IFN sur la mort cellulaire programmée (apoptotique) est lui aussi à nuancer. De nombreuses études in-vitro ont démontré l'effet pro-apoptotique de l'IFN sur plusieurs lignées cellulaires et cancéreuses [138] impliquant l'action synergique d'une quinzaine d'ISGs pro-apoptique TRAIL, BAK, FAS, FASL XAF1 ou les caspases CASP4, CASP8 [85]. Le contexte dans lequel agit l'IFN peut, cependant entrainer des effets opposés. Lors d'une infection virale, l'IFN a un effet pro-apoptotique sur des pDC murins [139]. A l'inverse l'IFN augmente la survie sur des pDc fraichement isolées [140], et inhibe l'apoptose de pDC induit par les glucocorticoides grâce à l'induction des gènes anti apoptotiques Bcl –XL CFLAR/c-Flip [141]. Un effet similaire est observé sur les LT activés et des lignées de leucémie lymphoïde chronique [142, 143]. Ces effets paradoxaux sembleraient être en rapport avec l'activation de l'axe PIK3-Akt-mTOR par IFNAR. mTORC1 joue un rôle fondamentale dans la survie et prolifération cellulaire et module parfois l'apoptose [144].

2.6 MODULATION DE LA REPONSE IMMUNE PAR LES IFN.

2.6.1 IFN : molécule clé de l'homéostasie cellulaire

Les IFNs interviennent dans de nombreux processus biologiques, autres que les défenses antivirales et microbiennes. Si une forte réponse IFN est classiquement observée lors d'infection virale ou comme nous le verrons plus loin au cours de pathologies auto immunes, l'absence de production d'IFN a un impact sur le fonctionnement cellulaire.

De faibles concentrations d'IFN-β sont détectées dans différents tissus, en l'absence d'infection virale [145]. Dans les années 1980, cette sécrétion constitutive d'IFN était interprétée comme une réponse physiologique de la muqueuse aux pathogènes externes [146]. Cette sécrétion basale d'IFN-β dépend essentiellement des voies de signalisation NFKB et AT1 [147,148] ainsi que de la présence du répresseur IRF2 [149]. L'utilisation de souris génétiquement modifiées (invalidées pour le récepteur IFNAR-/- ou STAT) a

permis de mieux approfondir le rôle physiologique des IFN notamment dans l'homéostasie cellulaire et le cross-talk cellulaire.

On observe ainsi chez les souris IFNAR-/- une réponse atténuée aux cytokines comme l'IL-6 et l'IFN-γ en dehors de l'IFN. Ces résultats suggèrent un « cross-talk » entre les 3 cytokines pour lequel deux mécanismes ont été proposés. D'une part, l'activation d'IFNAR permet le recrutement des sous unités IFNγR2 et gp130 des récepteurs de l'IFN-γ et l'IL-6 respectivement [150, 151]. D'autre part, les IFNs seraient indispensable au maintien d'un niveau basal de molécules de signalisation de la famille des STAT et IRF (IRF5, IRF7) impliqués dans la signalisation d'autre cytokines [145, 152].

La sécrétion constitutive d'IFN est ainsi impliquée dans divers processus physiologiques. Alors qu'une production excessive d'IFN diminue la niche des cellules hématopoïétiques, la sécrétion basale d'IFN-β est nécessaire au maintien de cette dernière [153]. L'homéostasie des cellules du système immunitaire est perturbée chez les souris invalidées pour le récepteur IFNAR. On observe une diminution qualitative et quantitative des cellules Natural Killer (NK) (baisse de la cytotoxicité) [154] ; des lymphocytes spléniques B220 ; et des cellules myéloïdes [155, 156]. Le remodelage osseux est lui aussi altéré par un déficit qualitatif en ostéoclastes [157] . Un défaut basal de production de l'IFN pourrait aussi être impliqué dans la survenue de certains cancers hématologiques [158, 159].

Ainsi en dehors de tout contexte pathologique, une sécrétion constitutive d' IFN est indispensable pour l'homéostasie cellulaire, par un maintien dynamique et la modulation de plusieurs voies de signalisation intracellulaire [160].

2.6.2 IFN et immunité innée.

Les travaux sur des modèles murins KO pour les gènes des IRF (1,2,3,4) présentent tous des anomalies qualitatives et/ou quantitatives sur les différents effecteurs de la réponse immunitaire et notamment innée [3] . Les IFNs en présence de TNF-α et de GM-CSF permettent la différentiation et maturation complète de monocytes ou de cellules précurseurs CD34+ en DC in vivo et in vitro [161, 162]. Parallèlement, les IFN facilitent la cross présentation de peptides exogènes dans certains types de DC [163, 164], par un mécanisme impliquant l'immunoprotéasome [165]. Lors d'une infection virale, les IFNs produits par les pDC majore la cytotoxicité des NK[154, 166, 167]. Cependant, en cas de forte

concentration d'IFN, la réponse des NK peut être inhibée par un mécanisme IL-12 dépendant.

2.6.3 IFN et immunité adaptative.

Les IFNs sont des médiateurs solubles qui assurent un lien entre immunité innée et adaptative [168] . Ils modulent à la fois la réponse humorale (LB) et cellulaire (LT) de manière directe et indirecte.

L'IFN et l'IL-6 secrétés par les pDC permettent de manière synergique la différentiation des lymphocytes B en plasmocyte par un mécanisme impliquant le CD40L [169, 170]. L'IFN induit la sécrétion de BAFF, APRIL par les DC qui sont nécessaires à la survie et prolifération des Lymphocytes B [171] . Par ailleurs, le facteur IRF5 serait indispensable à la différentiation des LB [172] .

L'IFN a un rôle important dans le maintien et dans l'activation de certains sous types de lymphocytes mémoires CD8 et CD4 [173]. L'IFN, de la même manière que l'IL-12, agit comme un 3eme signal sur les LT CD8 mémoires pour maintenir l'expression permanente du programme de différentiation [174] et favoriser l'apparition de « short live effector ». Les LT CD8 sont d'ailleurs plus sensibles aux cytokines homéostatiques (IL-7 IL-15) en présence d'IFN.

L'effet de l'IFN sur la différenciation de CD4 T helper est plus complexe. Longtemps considéré comme une cytokine « pro-Th1 », les IFNs n'ont pas un effet similaire sur les différentes sous populations de Th. L'IFN majore la production d'IFN-γ des Th1 (souris et homme) mais ne peut, à lui seul, maintenir une expression stable et constante de T-bet pour une différentiation Th1 optimale [175] . Les principales cytokines Th2 (IL-4, IL-5 et IL-13) sont inhibées aussi bien par l'IFN-α et IFN-β par un mécanisme dépendant du TF GATA-3 [176] . Dans un modèle in vitro, L'IFN-α peut inhiber directement la différentiation Th17 humaine mais ces résultats ne sont pas retrouvés avec l'IFN-β, probablement du fait de condition expérimentale différente [177, 178]. Cependant l'induction d'IL-27 par les DC est un mécanisme qui explique la régulation négative des Th17 par l'IFN-β dans les modèles murins d'EAE [179, 180] . L'ensemble de ces résultats va dans le sens d'un effet pro Th1 des IFN. Etant donné la flexibilité de production de cytokines et la plasticité des Th (que nous aborderons plus loin), on ne sait pas comment l'IFN module le spectre de cytokines produites par un Th. Récemment une publication a

montré que la production d'IL-9 par les Th9 était augmentée par plusieurs cytokines dont l'IFN [181]. Dans certaines circonstances, l'IFN peut aussi induire de l'IL-10 sur des CD4 mémoires [182] ou au décours de la polarisation Th17. L'effet des IFN sur la polarisation des Th n'apparaît donc pas comme unique. Il doit être évalué sur l'ensemble des fonctions des T helper et non pas réduit à quelques cytokines dominantes.
Enfin, les IFN orchestrent la réponse immunitaire innée et adaptative en modulant la migration des cellules effectrices, par l'expression des chemokines et de leurs récepteurs. Les IFN stimulent la production CXCL10, CCL2 et CCL7 par les DC, qui permet le recrutement respectifs des cellules CXCR3+ (neutrophiles activées, LT) ou les cellules CCR2+ (Macrophages et les CD du derme) [183] .

3. LES LYMPHOCYTES T AUXILLAIRES (Th)

Les lymphocytes CD4+ T helper (Th) sont des acteurs essentiels de la réponse immune adaptative. D'origine thymique, les Th naïves circulent en périphérie jusqu'à leur activation par une DC dans les organes lymphoïdes secondaires. La rencontre entre le complexe MHC-Ag et le TCR active le programme de différenciation et l'expansion clonale de T. Ces effecteurs sont des cellules spécialisées qui sont caractérisées par des fonctions et des marqueurs spécifiques [184]. Historiquement, MOSMMAN a décrit, en 1986, chez la souris deux sous populations clonales de LT CD4 sur la base d'un panel de cytokines secrétées : Les Th1 et Th2 qui produisent respectivement soit de l'IFN-γ, IL-2, GM-CSF, IL-3 ou de l'Il-4, IL-5 [185]. Ces deux populations ont été individualisées par la suite chez l'homme. La mise en évidence de LT régulateurs induits ou naturels (iTreg, NTreg) ayant des fonctions suppressives a annoncé l'idée de diversité des T Helper [186]. Finalement, la découverte en 2005 de Th sécrétant de l'Interleukine 17 (IL-17A et IL-17F) mais aussi de l'IL-22, IL-6 et IL-21, appelé Th17, a modifié notre vision des Th et la dichotomie Th1/Th2 [187, 188, 189].

Les lymphocytes Th1, Th2 et Th17 jouent un rôle important, respectivement, dans l'éradication de pathogènes intracellulaires, helminthes, pathogènes extracellulaires et levures. Les Th1 and Th17 sont aussi impliqués dans plusieurs maladies auto-immunes et inflammatoires alors que les Th2 contribuent aux pathologies allergiques [190]. Les T régulateurs (iTreg et nTreg) sont nécessaires au maintien de la tolérance et la

modulation de la réponse immune [191]. Le spectre des Th s'est depuis élargi avec de nouvelles sous populations Th9, Th22 dont la nomenclature correspond à cytokine dominante sécrétée [192, 193]. Enfin, les « follicular Th » qui secrétent de l'IL-21, dont le rôle est d'activer les lymphocytes B au sein de structure lymphoïde secondaire ne sont pas encore considérés comme une sous population de Th.

Lors des 10 dernières années, les processus de différentiation et les fonctions des Th ont faits l'objet de nombreuses études et de découvertes majeures in vitro et in vivo. Nous aborderons de façon restreinte, dans les paragraphes suivants deux aspects importants des Th : Le processus de différenciation, le résultat de l'intégration dynamique de multiples signaux ; et la plasticité de la réponse Th dépendante du micro environnement.

3.1 Polarisation des Th résultat de l'intégration dynamique de multiples signaux

L'activation du LT requiert schématiquement 3 signaux : l'engagement du TCR avec le complexe MHC-Ag (signal 1) ; l'interaction des molécules de stimulations (inhibitrices ou activatrices) entre la DC et le LT (signal 2) ; et l'environnement cytokinique qui est un des éléments clés de la différentiation correspondant au signal 3 [184]. Le LT doit ainsi intégrer de multiples signaux qui vont chacun activer des voies de signalisation différentes pour induire les facteurs de transcription spécifiques des cellules effectrices. On conçoit ainsi que la différentiation Th est un processus complexe et dynamique. Nous détaillerons brièvement et de manière systématique les différents acteurs impliqués dans le processus de différentiation.

3.1.1 Intégration de signaux extracellulaires.

Les études in vitro chez la souris et chez l'homme ont démontré le rôle primordial des cytokines comme facteurs déterminants, de la différentiation T Helper. Une combinaison de cytokine est indispensable pour la différentiation de chaque sous populations.
L'IL-4 a été, la première cytokine décrite dans la polarisation Th2 [194] avec ou sans IL-2. Secondairement, des études ont montré le rôle adjuvant de l'IL-33 et la Thymique stromal lymphopoeitin (TSLP) [195]. La différentiation des Th1 dépend majoritairement de l'IL-12 même si les IFNs, l'IFN-γ et l'IL-18 jouent un rôle plus modéré [196,175]. Contrairement au Th1/Th2, La génération des Th17, iTreg et Th9 varie en fonction des conditions expérimentales et du modèle humain ou murin.

Chez la souris l'induction des iTreg à partir des CD4 naïves nécessite la présence d'IL-2 et de TGF-β [197] . Plusieurs combinaisons ont été essayées chez l'homme avec des résultats variables : l'IL-2 + TGF-β ; IL-10+ TGF-β ; avec ou sans Vitamine D3 ou Acide rétinoïque [198].

La même différence inter-espèce est observée pour la différentiation des Th17. La combinaison IL-6+ TGF-β est suffisante pour obtenir des Th17 murins [199, 200] mais la présence supplémentaire d'IL-1β et IL-23 est indispensable pour la différentiation optimale des Th17 humaines [201, 202]. Le rôle dominant de l'IL-1β a été récemment validée dans un modèle physiologique de différentiation Th17 [203]. Le TGF-β apparaît comme une cytokine clé dans les processus de différentiation des Th17 et des iTreg, et également dans celui des Th9 en association avec l'IL-4.

D'autres facteurs métaboliques solubles peuvent moduler la différentiation des T helper. Citons notamment les ligands de l'aryl hydrocarbon receptor (Ahr) comme la dioxin qui favorise la différentiation Th17 et Treg [204, 205], le rôle de la 1α25dhihydroxyvitaminD et les Treg [206] .

3.1.2 Intégration et dynamique des voies de signalisation.

De façon identique aux IFNs, la signalisation des cytokines polarisantes dépend de plusieurs voies intracellulaires dont celles des STAT et de la voie mTOR.

Les molécules STAT sont le point de départ des voies de signalisation des cytokines. STAT4 et à moindre mesure STAT1 sont indispensables pour la polarisation Th1 [207, 208, 209] . STAT4 est activé par l'IL-12 et permet la transcription de T-bet mais aussi de l'IL-12rB2 exerçant ainsi un rétrocontrôle positif [210, 211]. L'IL-4 peut inhiber STAT4 par un mécanisme impliquant GATA3. STAT1 aurait un rôle dans l'induction de l'IFN-γ mais non spécifique [209].

Les travaux in vitro, montrent que STAT6 est nécessaire à la polarisation Th2 grâce à l'induction de GATA3 [212] . Ces résultats ne sont pas reproduits in vivo chez la souris [213].

L'activation de STAT3 par l'IL-6, IL-21 et l'IL-23 en fait un facteur indispensable lors de la différentiation Th17 [214]. STAT3 agit par plusieurs mécanismes en induisant directement la transcription de l'IL-17 et RORc ou en inhibant FOXp3 [215, 216] .

A noter enfin que STAT5 inhibe développement des deux populations inflammatoires Th1/Th17 mais favorise celui des Th2 et iTreg.

Des publications récentes ont montré le rôle crucial de la voie mTOR dans l'homéostasie et de développement des fonctions des Th [217]. L'inhibition de mTOR permet la génération de Treg et/ou l'expansion de Treg préexistants [218] probablement par la levée de l'inhibition de FoxP3 par l'axe PIK3/AKT [219]. Les expériences sur des souris génétiquement modifiées ont mis en évidence, respectivement, l'importance de mTORC1 et mTORC2 pour la différentiation Th1/Th17 et Th2 [220, 221].

3.1.3 Complexité et dynamique des facteurs de transcription.

La différenciation Th repose sur un réseau complexe de facteurs de transcription (TF) dont le niveau d'expression (qualitatif et quantitatif) et les interactions (régulation positive et négative) sont critiques dans l'acquisition du phénotype et des fonctions des Th. La mise en évidence des TF « maitres » spécifiques permet, à la fois de mieux caractériser et définir les sous populations des Th. D'autres TF, d'expression constitutive ou induite, sont aussi indispensables à la génération optimale des T helper.

Le TF GATA-3 spécifique de la polarisation Th2 a été le premier TF identifié [222], suivi de T-bet pour les Th1 [223], FoxP3 pour les nTreg [224] et RORγT pour les Th17 [189]. Leurs découvertes ont été validées, aussi bien, dans les modèles murins et humains. L'absence de TF spécifique des Th9 ou des Th22 pose le problème très débattu de leur classification comme une sous population spécifique des Th.

Les mécanismes d'action des Master TF sont multiples pour maintenir el programme de différentiation : induction des cytokines spécifiques des Th ; régulation positive ou négative de TF, des STAT ou des cytokines. GATA3 induit la transcription de l'IL4, IL-5 et IL-13 [225,226,227] ; et inhibe plusieurs TF des Th1 dont Runx3. T-bet est induit partiellement par le remodelage du gène l'IFN-γ et l'up-régulation de l'IL-12Rβ2 [228] . T-bet n'inhibe pas en revanche l'expression de GATA3 [209].

D'autres TF moins spécifiques ont été décrits comme participant au processus de différentiation des Th. Citons RunX3, HLX, IRF1 pour les Th1 ; RORa, Baft, Runx1 et IRF4 pour les Th17 ; IRF4, c-maf GFi-1 et Dec2 pour les Th2 [229].

L'expression des TF considérée comme stable, définitive et spécifique des sous populations est aussi à nuancer. Plusieurs travaux ont montré que l'expression du TF FoxP3 est transitoire dans certaines populations de Treg. Il est décrit dans les mêmes cellules l'expression simultanées FoxP3 et T-bet ou FoxP3, runX3 et RORγT [230-232] .

Comme pour les cytokines, il existe une certaine flexibilité dans l'expression des TF. Tout Th peut exprimer plus d'un TF et/ou Master TF au cours du processus de différentiation dont le rôle est à préciser. Au total, un panel de TF dont certains Master TF contribue à la spécialisation du CD4 naïfs en Th [233].

Toutes ces données montrent que la volonté d'associer à chaque sous population de Th une cytokine, un STAT et/ou un facteur de transcription est simpliste et réductrice. La différentiation Th fait intervenir un réseau complexe, sophistiqué et dynamique de molécules, facteurs de transcription qui coopèrent ou s'opposent tout au long du processus de différentiation pour donner au Th sa spécificité et son identité cellulaire [234]

3.1.4 Contrôle épi-génétique.

Le dernier point de contrôle du processus de différentiation des Th concerne les modifications épigénétiques, pouvant intervenir au locus des gènes. On pourrait considérer ces modifications comme l'ultime prolongement des voies de signalisation intracellulaire [235]. Le contrôle épi-génétique fait intervenir différentes modifications de l'ADN et histones comme l'acétylation, la méthylation Cpg. De nombreux travaux ont soulignés le rôle primordial des modifications épi-génétiques et du remodelage de la chromatine dans les différents des Th1, Th 2 et Th17 ; ajoutant un nouveau niveau de complexité [233].

3.2 Plasticité des T helper et rôle du microenvironnement (Fig5).

Depuis 4 ans, de nombreux travaux in vitro sur le T Helper ont rapporté une certaine flexibilité dans la production de cytokines et dans l'expression des TF amorçant le concept de plasticité (ou encore « functionnal platicity) des cellules T Helper. Un exemple typique concerne l'IL-10 considérée auparavant comme une cytokine Th2, et qui aussi est secrétée par les Th1, Th2, Th17 et Treg. D'autres cytokines ont aussi la capacité d'être secrétées par plusieurs sous populations de Th comme l'IL-9 et les Th2/Th17/Th9 [236, 237]; l'IL-13 et les Th2/Th1 [238]; L'IL-22 et les Th1/Th17 et Th22 [192, 193]. La plasticité observée in vitro laisse supposer que les Th sont des cellules hautement spécialisées mais pas totalement déterminées car leur phénotype est réversible ([233]). Il

faut garder à l'esprit que la fréquence de cette flexibilité in vivo n'est pas connue, de même que les facteurs impliqués.

La plasticité des T Helper dépend des facteurs intrinsèques, mais surtout de facteurs extrinsèques qui modulent ainsi la réponse Th en pathologies. Le TGF-β est capable d'induire simultanément les deux TF RORc et FOXP3 avec un effet dominant de FOXP3 qui confère au Th son statut de Treg. Cependant, dans un milieu inflammatoire riche en IL-6, ces mêmes Treg se transforment en Th17. Ces mêmes Treg peuvent aussi en contexte inflammatoire se transformer en cellules auto-réactives destructives ou en Th17 et majorer le processus inflammatoire ([232, 239]). Koch et coll ont montré que l'IL-12 induit l'expression de T-bet dans les Treg. Ces cellules FoxP3+T-bet+ sont ainsi reprogrammées pour limiter sur le site inflammatoire l'action spécifique des Th1[231]. Les Th2 peuvent aussi être reprogrammées par les IFN au cours d'une infection virale; exprimer l'IL-12RB et devenir des cellules IL-4+/IFN-ϒ+ [240].

Il est cependant impossible de dire si ces observations in vitro reflètent le comportant in vivo des Th. Néanmoins, l'utilisation récente de système utilisant des « reporter mice » confirment la plasticité in vivo des Th17/Th1 qui varient en fonction du micro-environnement inflammatoire comme l'EAE (Experimental Acute Encephalitis) ou l'infection cutanée à Candida Albicans [241].

La plasticité des Th est une propriété fondamentale et nécessaire des Th si l'on considère que ces cellules doivent réagir devant un nombre conséquent et extrêmement variable de pathogènes. Par ailleurs, l'adaptation des cellules effectrices peut être parfois nécessaire dans des conditions particulières, pour éviter soit les effets secondaires des réactions immunes ou pour débuter des processus de réparation tissulaire après une agression par un pathogène. La production par exemple d'IL-10 par les Th1 lors d'une infection peut être justement un frein pour limiter un emballement de la réponse immune [242].

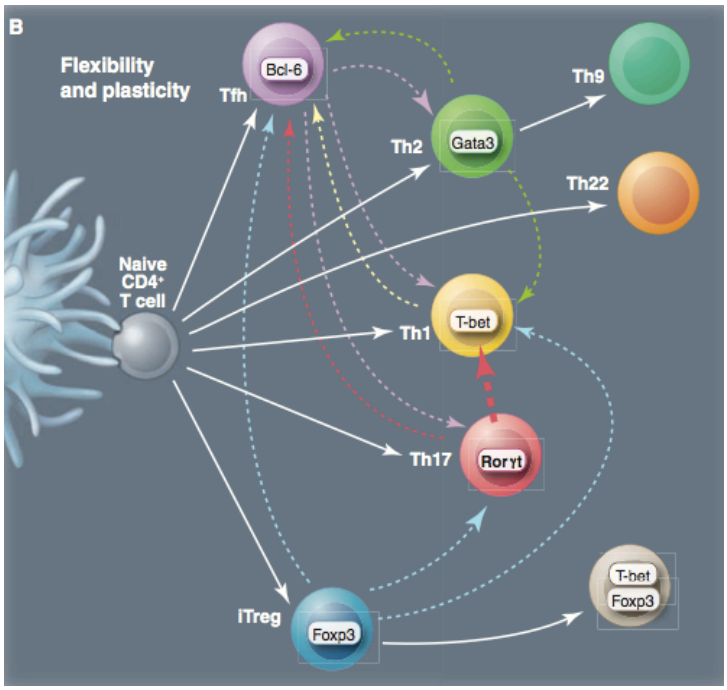


Fig 5 Plasticité et flexibilité de la réponse T Helper. Adapté de O'shea Science 2010

4 PLASTICITE DES CELLULES DENDRITIQUES.

Nous aborderons brièvement dans ce chapitre quelques caractéristiques des cellules dendritiques (DC), acteurs clés de la réponse immune qui ont fait l'objet d'une collaboration avec l'équipe de Sébastien Amigorena.

4.1 HISTOIRE DES DC.

Les DC sont une population rare de cellules hématopoïétiques appartenant à la branche de l'immunité innée. La première description des DC a été faite par PAUL LANGHERANS en 1868 sur des coupes histologiques de peau. Il faudra, cependant, attendre un siècle pour les identifier comme des cellules hématopoïétiques du système immunitaire[243]. En 1973, RALPH STEINMAN et ses collègues, ont décrit pour la première fois, une nouvelle population de cellules issue de la rate de souris[244] . Ces cellules étaient différentes des granulocytes, lymphocytes et autres cellules phagocytaires avec une morphologie bien spécifique « the cytoplasm is arranged in pseudopods of varying lentgh, width, form and number, resulting in a variety of cell shapes ranging from bipolar elongate cells to

elaborate, stellate or dendritics ones…the term « dendritic » would thus be appropriate for this particular cell type » [245]. Le lien entre ces cellules et celles observées par PAUL LANGHERANS sera fait 1985 et 1987 par SCHULER AND STEINMAN [245, 246]. Ils découvriront par la suite une de leur principale fonction à savoir leur capacité à activer les cellules T naïves dans une réaction lymphocytaire mixte (MLR); démontrant alors leur rôle essentiel dans l'initiation de la réponse immune

Les DC sont décrites comme des cellules professionnelles de présentation d'antigènes (APC) dédiées à la reconnaissance et détection de pathogène Les précurseurs des DC sont localisés dans la moelle osseuse et donnent naissance à des DC immatures circulantes qui vont se localiser dans différents tissus secondairement. Apres une agression localisée, les DC immatures capturent les antigènes (Ag) étrangers, les phagocytent et migrent vers les organes lymphoïdes secondaires (OLs). Les DC activées présentent alors l'Ag aux LT CD4 naïfs qui, après activation, régulent l'activité d'autres effecteurs comme les LT CD8 cytotoxiques et les LB. Par ailleurs, les DC expriment une variété de récepteurs comme les PAMPS ou des récepteurs d'hormones et de cytokines qui leurs permettent d'intégrer un multitude de signaux provenant du microenvironnement.

Trois critères définissent les DC selon RALPH STEINMAN [247] : 1) Morphologie (présence de dendrites), 2/ L'expression constitutive du complexe majeur d'histocompatibilité de type II (CMH II), 3/ La capacité à activer des LT dans une MLR. Les DC assurent ainsi le lien entre immunité innée et adaptative.

4.2 DIVERSITE DES DC.

Les DC représentent une catégorie hétérogène de cellules qui se reflètent à trois niveaux:

1/Les précurseurs des DC : chez l'homme, on distingue deux sous populations de précurseurs des DC circulants : les monocytes CD14+CD11c+ et les Lineages-(lin-) CD11c-CD123+ DC [248]. La première sous population exprime des marqueurs de la lignée myéloïde et donne naissance au DC « myéloïde ou conventionnelle » (mDC/cDC/DC) qui se divisent en 2 sous populations en fonction de l'expression du BDCA1 (CD1c) et BDCA3 (CD141). Les précurseurs lin-CD11c-CD123+ correspondent aux pDC.

2/Localisation anatomique. On distingue des DC circulantes (Blood DC), ou des DC résidants dans les OLs et les tissus périphériques. Les mDCs et pDCs sont des DC circulantes qui constituent une source de DC immature et précurseurs qui régénèrent continuellement le pool de DC résident en périphérie ou dans les Ols [249]. Le thymus contient des pDC, des cDC immatures et matures dont la fonction n'est pas entièrement comprise mais serait en rapport avec la régulation de la tolérance centrale [250]. Les OLs (amygdales, rate et ganglions) contiennent beaucoup de pDC et mDC [249] .

Enfin, on trouve des DC dans des régions ou l'organisme est le plus exposé à des pathogènes externes comme la peau ou d'autres épithéliums. Deux types de DC ont été décrits dans la peau : Les Cellules de Langherans (LC) et les DC interstitielles. Les LC résident dans l'épiderme et sont caractérisées par l'expression du CD1a, E-cadherin et du CD201 (C-type lectin langherin). Les DC interstitielles sont quant à elles localisées dans le derme et regroupent deux sous populations en fonction de l'expression de marqueurs spécifiques : les DC CD1ahighCD4+DC-SIGN-MMR++CD14-CD16- ou CD1alowCD4+DC-SIGN+MMR+CD14+CD16- [251] .

4/ Plasticité des réponses des DC : il existe aussi comme nous l'avons précédemment vu avec les LT une certaine plasticité dans la réponse des DC. Cette plasticité est le résultat de modifications sélectionnées aux cours de l'évolution mais aussi de facteurs environnementaux qui agissent sur les cellules[252]. Il faut noter que la plupart des observations sur la plasticité des réponses des DC sont issues d'expression in vitro a partir de précurseurs dont la maturation a été faite in Vitro ; et non à partir de DC matures « ex vivo »

Du fait d'une certaine plasticité dans leur réponse, il est alors difficile d'assigner une fonction fixe à une lignée spécifique de DC. Par exemple, la capacité des MoDC à produire de l'IL-12 et induire une réponse Th1 plutôt qu'une réponse Th2 dépend des conditions dans lesquelles ont été générées les DC [253, 254] .

4.3 DC ET REPONSE INFLAMMATOIRE.

Lors d'un processus inflammatoire dans un tissu, on assiste à un recrutement massif de plusieurs cellules du système immunitaire dont les leucocytes comme les neutrophiles et monocytes.

Il a été montre chez la souris, qu'un type spécifique de monocytes, les monocytes Ly6C^{low} équivalent des monocytes CD14+ humains, jouent le rôle de sentinelle et initie le début de la réaction inflammatoire pares la reconnaissance de PPR. Les neutrophiles arrivent ensuite sur le site inflammatoire et secrètent plusieurs molécules pro-inflammatoires (cytokines et chemokines) qui à leur tour recrutent les monocytes Ly6Clow pour amplifier la réaction. Ces derniers sont capables de se différencier soit en macrophages et phagocyter les agents pathogènes présents, soit en DCs qui alors initient la réponse immune pour contrôler l'infection [255] .

Ces DC murins, issus des monocytes Ly6C^{low}, représentent une sous population de DC appelées DC inflammatoire (ou inflammatory DC) [256, 257]. Ces dernières ont été observées dans différents modèles de maladie inflammatoire comme l'asthme ou la polyarthrite rhumatoïde [256, 258]. Les DC inflammatoires migrent aussi vers les OLs et présentent les Ag aux CD4 et CD8. Ils peuvent également transférer les Ag directement à d'autres DC [256, 258, 259]. Enfin, des travaux ont montré qu'ils activent aussi directement les cellules T mémoires présent dans les tissus périphériques[259].

L'équivalent chez l'homme des DC inflammatoires n'est pas encore bien identifié. Une sous population de DC appelée inflammatory dendritic epidermal cell (IDEC) été observé dans le derme de patient atteint de dermatite atopique [260]. Ces cellules expriment des marqueurs différents des LC et des DC du derme de patient sain [261] . On ne sait pas si ces IDEC sont une forme activée de DC de la peau ou alors une sous population de DC inflammatoires présent uniquement lors d'un inflammation. Enfin, l'importance des infDC sur les réponses Th n'est pas connue.

5. LA REPONSE IFN EN PATHOLOGIE

5.1 L'IFN EXOGENE EST UNE BIOTHERAPIE EFFICACE.

5.1.1 Traitement des néoplasies.

L'indication première de l'IFN en thérapeutique, a porté sur le traitement des cancers au milieu des années 1980 du fait de leurs effets anti-angiogéniques et pro-apoptotiques [262]. L'IFN a été initialement utilisé dans les maladies hématologiques comme la Hairy Cell Leukemia, la Leucémie Lymphoïde Chronique (LLC), et certain type

de lymphome B et T [263-265]. Leur utilisation s'est élargie ensuite à d'autres tumeurs solides comme le carcinome rénale à cellule claire, le mélanome, les ostéosarcomes et les sarcome de Kaposi [266, 267].

L'effet anti-tumoral des IFNs peut être expliqué en partie par 1/ *leur action pro-apoptotique et la régulation de la prolifération* tumorale comme précédemment décrite 2/ *la modulation des fonctions des cellules NK ou T* (migration, cytotoxicité) qui ont un rôle important dans la réponse anti-tumorale [268, 155]. 3/ *Des anomalies des voies de signalisation IFN*. Par exemple, la mutation de RNASL est associée à un risque de développement de cancer de la vessie [269]. Certains virus oncogènes comme l'HPV 16 E6 E inhibe les récepteurs cytosoliques RIG-1 et le TLR9, permettant ainsi la transformation tumorale des cellules [270] 4/ enfin *l'effet anti-angiogénique* par inhibition de la prolifération de cellules endothéliales ou de la sécrétion de VEGF [271].

5.1.2 Traitement des maladies virales.

Les hépatites B et C sont deux causes majeures de cirrhose hépatique touchant plus de 350 millions et 170 millions respectivement [272, 273] (WHO 2010). La morbidité et mortalité sont sévères en cas d'insuffisance hépatique avec un risque de développer un carcinome hépato-cellulaire dont la mortalité annuelle est de 20%. Chaque année, les complications dûes aux hépatites, sont responsables de 500.000 à 1.200.000 décès (WHO 2010). L'utilisation de l'IFN dans le traitement de l'hépatite B remonte à 1973 ou des formes impures d'IFN-α mais non d'IFN-β ont suggéré une certaine efficacité [274, 275] ; confirmée par la suite avec la disponibilité de l'IFN-α recombinant [276]. Son indication pour l'hépatite C a été par la suite approuvée en 1990. Actuellement l'IFN-α sous forme pegylé (Peg-Interferon) associée ou non à un autre immuno-modulateur la ribarivine et aux nouveaux antiviraux (DAA) constitue le traitement de référence des hépatites B et C. L'espoir de guérison varie de 50 à 90% selon les types de virus. Le mécanisme d'action des IFN au cours des hépatites n'est pas entièrement élucidé mais deux aspects ont été démontrés : 1/ les IFN sont capables d'inhiber directement le cycle de réplication virale par différents mécanismes [111] 2/ l'IFN module la réponse et le recrutement des lymphocytes sur le site inflammatoire. Certaines publications ont rapporté une corrélation entre l'expression d'ISGs et la bonne réponse au traitement antivirale [277, 278].

A noter aussi que l'IFN a été utilisé dans le traitement d'infection virale comme l'herpes ou le cytomégalovirus [279] mais remplacé depuis par des molécules plus efficaces et ayant moins d'effets secondaires.

5.2 Une réponse IFN paradoxale dans les pathologies auto-immunes.

5.2.1 Une réponse anormale à l'origine d'une auto -immunité : Arguments expérimentaux et cliniques.

Malgré le rôle fondamental des IFN dans de nombreux processus cellulaires physiologiques, plusieurs éléments expérimentaux et cliniques indiquent que l'exposition chronique à l'IFN est à l'origine d'une auto-immunité [3, 280].

Sur le plan expérimental, les modèles murins invalidants des gènes IRF ont suggéré un rôle de la réponse IFN dans les pathologies auto-immunes. Les souris déficientes en IRF1 sont protégées de phénomènes auto-immuns dans les modèles de Collagen induced Arthritis [281] ou de diabète (souris NOD) [282]. L'inactivation d'IRF2 prédispose à des lésions cutanées psoriasiformes [283], et le polymorphisme d'IRF-2 serait associé avec une prédisposition pour la dermatite atopique. Récemment, une équipe a analysé les étapes du développement des atteintes auto-immunes dans modèle murin d'une maladie génétique humaine rare (*syndrome d'Aicardi-Goutieres*), inactivant une DNAS TREX-1 [284]. Leur résultat renforce le rôle clé et probablement initiateur des IFNs dans cette maladie. Environ 20 % des patients traités par de l'IFN-α thérapeutique développent des signes biologiques d'auto immunité avec l'apparition d'auto-anticorps anti nucléaires ou DNA natifs, et ceci indépendamment de la maladie initiale [285]. Certains d'entre eux auront des manifestations cliniques notamment des thyroïdites, et parfois une maladie lupique [286-288]. L'activation des DC et l'augmentation de la production des Ac par l'IFN ont été avancés pour expliquer ces phénomènes auto-immuns [170]. Les études de transcriptome ont identifiées une « signature IFN » dans plusieurs pathologies auto-immunes : lupus érythémateux disséminé (LEAD), le psoriasis, le diabète, la maladie de Sjögren [289-291]. Ces signatures qui diffèrent d'une pathologie à l'autre, correspondent à des sous-groupes de patients et sont associées aussi bien à l'activité de la maladie qu'à la réponse à une thérapeutique [289, 291, 292]. Enfin, l'étude du polymorphisme de certains ISGs comme IRF5 ou STAT4 dans des maladies rhumatologiques (LEAD, Polyarthite Rhumatoide, Sjôgren,) renforce encore l'implication des IFNs en auto-immunité [293, 294].

5.2.2 Mécanisme d'action : exemple du lupus érythémateux disséminé

Le LEAD est une maladie auto-immune que touche particulièrement les femmes jeunes d'origine afro-américaine. La maladie systémique peut toucher de nombreux organes (peau, articulation, rein, cœur, poumon). Le pronostic fonctionnel et vital des patients est lié à la sévérité de l atteinte de certains organes comme le rein ou le poumon [295].

Le diagnostic de la maladie repose sur des critères cliniques, biologiques et immunologiques. Des nombreux travaux ont montré un rôle primordial de l'IFN et des pDC dans la pathophysiologie du LEAD. Le blocage de la production d'IFN par des Ac monoclonaux est d'ailleurs considéré comme une thérapie prometteuse, faisant l'objet d'essais cliniques de phase III [296].

L'analyse du transcriptome des PBMCS de LEAD a révélé une forte expression des nombreux ISGs par comparaison à des sujets sains [289, 297]. Cette signature est souvent associée à la présence d'IFN dans le plasma des patients [298]. La production d'IFN dépend essentiellement des pDC [299]. Ces derniers sont en nombre réduit dans le sang mais pas dans les organes lymphoïdes secondaires ou les sites inflammatoires , et présentent des marqueurs d'activation chronique [299,300]. Les pDC sont activés principalement par les complexes immuns reconnus par les TLR7 et TLR9. Cette stimulation les rend par ailleurs résistantes au mécanisme d'apoptose induits par les glucocorticoïdes, expliquant les phénomènes de résistance observés en thérapeutique [301]. Récemment, deux études ont été montré que les pDC sont aussi activés par des fragments relargués par les polynucléaires neutrophiles : les Neutrophiles Extrallular Traps (NETS) qui contiennent la LL37 ou HMBG1 [302]. Ces résultat sont à rapprocher de ceux observés dans le psoriasis : Le peptide antimicrobien LL37, présent dans les lésions, est à l'origine de l'activation des pDC et de la production d'IFN [303,304]. Dans le modèle de murin de maladie lupique rénale (souris lupique NZBR), l'inactivation de la réponse IFN entraine une disparition de lésion [305]. L'effet de IFN sur les DC est majoré par un feedback positifs sur les pDC. Les DC peuvent alors activer les effecteurs de la réponse adaptative : les LT CD8, et les LB. La réponse humorale est amplifiée par différents mécanismes (Fig. 6).

Les cellules B auto réactives, ont un phénotype d'activation chronique avec seuil d'activation diminué [306]. L'IFN favorise par ailleurs la différentiation des plasmocytes et leur production d'Ac [307] .

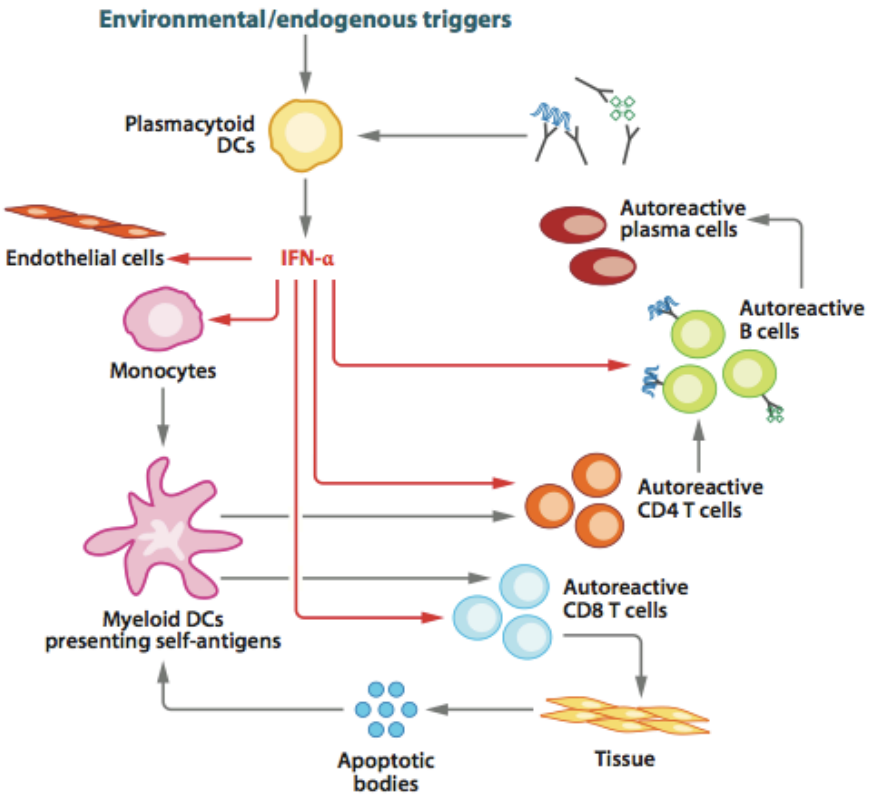


Fig 6 : Physiopathologie du lupus. **Adapté de Pascual et Banchereau.**

5.2.3 Un effet protecteur dans le traitement des pathologies inflammatoires.

De manière paradoxale, les IFNs sont utilisés en thérapeutique pour le traitement de maladies auto immunes. L'IFN-β est le traitement de référence des formes sévères de sclérose en plaques (SEP) permettant une diminution des lésions cérébrales objectivées par imagerie [308] et une diminution de 30% des rechutes chez des patients réfractaires. La réponse à L'IFN-β est cependant hétérogène avec 50 % de non répondeurs. Paradoxalement, ces patientes non répondeurs ont un taux endogène d'IFN et d'IL-17 plus élevés que chez les répondeurs [178, 309, 310].

L'IFN-α est moins efficace que l'IFN-β pour la SEP. En revanche, il est indiqué comme traitement d'attaque d'autres maladies inflammatoires touchant le système nerveux central SNC: la maladie de Behçet, et les uvéites idiopathiques [311]. Ces pathologies ont en commun l'activation anormale de lymphocytes T inflammatoires Th1 et Th17 [178, 312] . Plusieurs mécanismes d'action ont été proposés pour expliquer les effets des IFN dans des modèles murins : une diminution du recrutement des lymphocytes effecteurs sur le

site inflammatoire, de l'activation des DC (baisse de l'expression du CMH), de la prolifération des cellules T ; et une modulation de l'environnement cytokinique (IL-10, IL-27) ou de la balance entre Th1/Th17 [178, 180] et l'induction de Treg via les DC. Finalement on peut évoquer les dernières études montrant un effet bénéfique des IFN dans les maladies inflammatoires de l'intestin et certaines vascularites comme le Churg and Strauss [313, 314] .

5.3 Facteurs modulant la réponse aux cytokines dont l'IFN

5.3.1 Généralités

Les cytokines sont des médiateurs solubles (protéine ou glycoprotéine) secrétées par les cellules du système immunitaire. Leur rôle est d'assurer la communication entre les cellules de l'organisme pour en réguler l'activité et les fonctions [315,316]. Elles modulent la réponse immune soit par l'induction d'un programme transcriptionnel spécifique (effet principal) soit par des mécanismes indépendants de TF [316]. Les récepteurs des cytokines se regroupent en 6 familles selon leur conformation tridimensionnelle [317]. La majorité des récepteurs sont de type I et II qui diffèrent par la présence (I) ou absence (II) d'un motif extracellulaire WSXWS. Ils utilisent une voie de signalisation relativement simple comprenant deux médiateurs : les janus kinases et les STAT. L'induction de cette voie de signalisation entraine rapidement l'activation d'un programme de transcription spécifique à chaque membre de la famille STAT. Les mécanismes qui permettent d'expliquer comment une voie de signalisation composée de 4 JAK et 7 STAT arrive à transmettre un signal délivré par plus 30 de cytokines in vivo, sont encore inconnues [318]. La voie JAK/STAT est considérée comme la voie classique de signalisation des cytokines [319,320,321] , même si elle n'est pas exclusivement dédiée à cette signalisation[316]. D'autres voies de signalisation « alternatives » indépendamment des protéines STAT ont été décrites en réponse aux cytokines [173,322] , dont les mécanismes d'actions restent inconnues. Plusieurs modèles ont été proposés : l'utilisation de voies STAT alternatives [323], l'activation directe par JAK de voies secondaires (MAPK, PI3K, NFKB), ou l'interaction (positive ou négative) de facteurs de transcription au niveau des promoteurs des genes cibles. Une autre possibilité est la trans-activation de récepteurs non cytokines, générant ainsi plusieurs « signalosomes » qui s'intègreront par la suite pour délivrer un message unique [324,325].

Une cellule est normalement exposée à plusieurs stimuli dont l'intégration des signaux déterminera la réponse cellulaire globale. On doit donc concevoir que la réponse à une cytokine est susceptible d'être modifiée par d'autres signaux dès lors qu'ils partagent le même récepteur ou des voies de signalisation identiques ou interférentes (crosstalk).

Nous détaillerons dans les deux paragraphes suivant deux facteurs qui conditionnent la réponse à une cytokine y compris l'IFN :

1/ *des facteurs cellulaires extrinsèques* qui correspondent à un ensemble de stimuli physiques ou chimiques du microenvironnement.

2/ *des facteurs cellulaires intrinsèques* à la cellule qui sont principalement déterminés par l'identité cellulaire [234] .

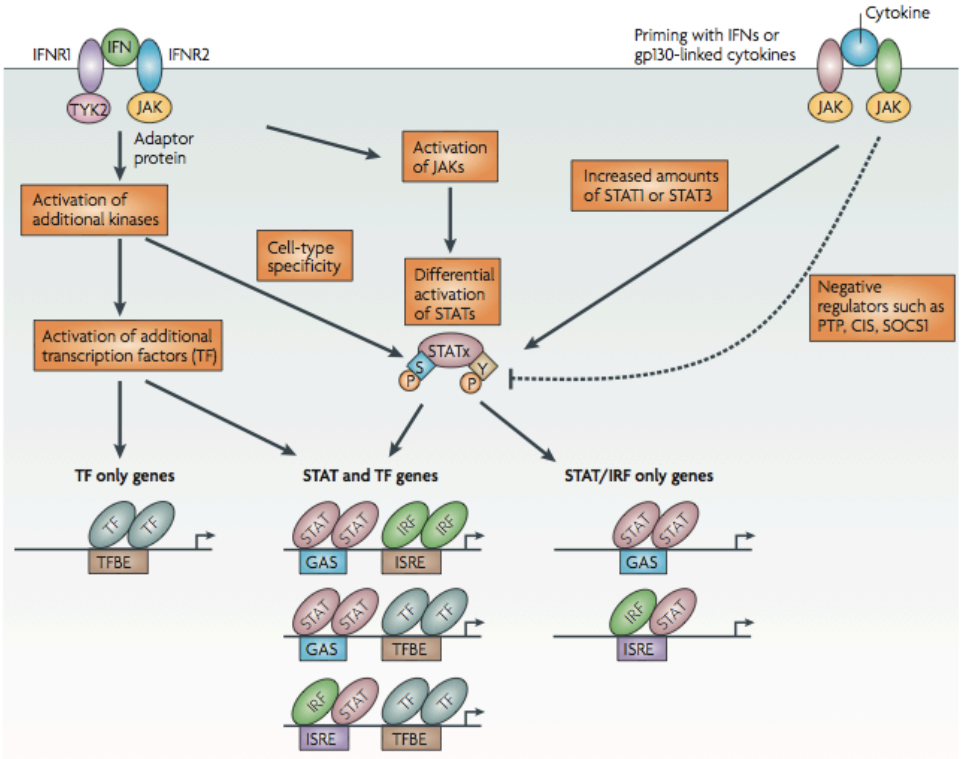


Fig.7 : Complexité de la réponse IFN. **Adapté de Borden C Nature drugs discovery 2005**

5.3.2 Facteurs cellulaires extrinsèques.

Plusieurs exemples en biologie suggèrent que l'effet des cytokines est dépendant du contexte environnemental [315]. L'environnement physique dans lequel évoluent les cellules, notamment l'expression de certaines molécules d'adhésion, conditionne leur réponse aux cytokines. Par exemple, la manière dont les cellules endothéliales prolifèrent en réponse au TGF-β est fonction de la matrice cellulaire utilisée [315].

Le TGF-β et l'IL-2 sont deux exemples de cytokine dont l'effet peut être modulé par le microenvironnement. Elles partagent d'ailleurs beaucoup de point commun avec les IFNs. Toutes deux sont des protéines très conservées au cours de l'évolution, aux effets pleïtropiques notamment sur les cellules du système immunitaire : rôle dans la prolifération et apoptose, la différenciation cellulaire, ou la modulation de la réponse immune (cellules NK, LB et LT) [326, 327].

Le TGF-β est une cytokine clé pour la génération de Treg (chez la souris). Mais en présence d'un contexte inflammatoire (IL-6) ou pro allergique (IL-4), le TGF-β reprogramme la cellule T naïve en Th17 et Th9 respectivement. Dans les cas des Th17, la modulation du signal se fait au niveau transcriptionnel. En effet, Les deux TF RORc et FOXP3 sont tous les deux induits par le TGF-β mais l'expression de RORc n'est pas suffisamment stable pour reprogrammer le Treg en Th17. En présence d'IL-6, son expression est stabilisée et renforcée par l'induction du récepteur de l'IL-23 pour maintenir le programme de différenciation Th17 [328,189]. L'IL-2 a aussi un effet « context-dependant » sur la polarisation des Th par la modulation de récepteurs de cytokines [327] . Elle augmente la prolifération des Th1 et Th2 grâce une surexpression de l'IL-12RB, et de l'IL-4R respectivement ; mais inhibe l'expression de l'IL-6R et donc la différenciation et prolifération Th17.

Plusieurs arguments sont en faveur de l'effet « context-dependant » que « context-independent » des IFNs. Le pré-conditionnement des cellules par des cytokines peut aussi moduler la réponse IFN par l'induction de nombreux inhibiteurs (SOCS, CIS, PTP) ou par la modulation d'autres voies de signalisation. On pourrait penser que l'effet des IFNs sur la polarisation des T helper dépend du milieu polarisant avec un effet « Pro Th1 » et « Pro Th9 ». Mais l'absence d'analyse systématique de l'effet des IFNs sur le profil cytokinique ou sur d'autres fonctions des Th laisse cette question en suspens. D'autres fonctions comme l'effet antiprolifératif est conservé indépendamment du

microenvironnement [134]. J'ai observé ces mêmes résultats dans mon système in vitro de différentiation des CD4 dans 4 contextes de polarisation différents (*Article en soumis fig suppl2*).

Apprécier l'effet du microenvironnement nécessiterait des analyses à large échelle pour étudier l'ensemble des ISGs modulées et une analyse systématique des principales fonctions des IFNs.

5.3.3 Facteurs cellulaires intrinsèques.

La réponse spécifique d'une cellule résulte de plusieurs facteurs : l'existence au sein d'une cellules d'un set unique de protéines et de leurs iso-formes à chaque niveau de voies de signalisation; de la structure spatiale de leurs composants, de la présence de molécules adaptatrices, de leur localisation cellulaire, de la cinétique des voies de signalisation.

L'analyse à large échelle de la réponse à l'IFN-γ dans différentes cellules immunes (LB, LT NK monocytes, macrophages) a montré une signature spécifique dépendant du type cellulaire[89]. En fonction du type de cellules tumorales, le TGF-β peut avoir un effet pro ou anti tumorale [329,330], pro ou anti apoptotique [331, 332], pro ou anti angiogénique [333].

Plusieurs hypothèses peuvent expliquer l'effet cellulaire spécifique des cytokines : 1/ des voies signalisation spécifiques à chaque sous type cellulaire : l'IL-15 active Jak2, Tyk2 and Syk dans les mastocytes, mais la signalisation passe par PI3K, Akt et NFKB dans le fibroblaste [334, 335]; 2/ l'expression spécifique de TF : les séquences GAS et ISRE n'ont pas les mêmes sites de fixation pour le TF IRF8 selon le type cellulaire modulant ainsi la réponse immune[336].

L'effet « cell-specific » sur la réponse IFN a été observé par SCHLAAK and coll [9]. Grâce à un micro-array customisé, l'expression d'un set d'ISG (150) a été analysée de manière systématique sur plusieurs lignées cellulaires et cellules primaires humaines (DC, TC, et PBMCS). Les résultats montrent que toutes les cellules sont capables d'exprimer au moins la moitié des ISGs testé. L'expression des autres ISGs dépend ensuite du type cellulaire étudié. Les effets pro et anti-apoptotiques des IFN sur les cellules T en fonction de leur état cellulaire d'activation et du niveau d'expression de STAT1/4 va dans le sens d'un effet « cell-specific ».

6 BASES SCIENTIFIQUES DE LA THESE

6.1 QUESTION SCIENTIFIQUE.

Pendant ma première année de thèse, je me suis intéressé à la caractérisation des réponses des Th et notamment à l'étude du profil cytokinique, poursuivant les travaux d'une ancienne post doctorante, Elisabetta Volpe. Ses résultats ont montré que les Th sont capables de sécréter différentes cytokines qui ensemble contribuent à la réponse globale Th. Le profil cytokinique peut être modulé differentiellement par les cytokines polarisantes. Ces données suggèrent que la production de certaines cytokines peut être co-régulé de manière identique (induction ou inhibition des 2 cytokines) ou de manière opposée (induction de l'une mais inhibition de la deuxième cytokine)

Projet 1

Dans un premier projet, j'ai cherché à analyser comment deux signaux cytokiniques (ou inputs) sont capables de moduler la fonction principale des Th (Profil cytokinique composé de 12 cytokines différentes) dans deux polarisations Th1 et Th17. Les lymphocytes Th17 secrètent principalement de l'IL-17 mais également d'autres cytokines comme l'IL-6, l'IL-10, l'IL-21, l'IFN-ϒ, le TNF-α, et l'IL-22. Cette dernière dont certaines fonctions sont similaires à l'IL-17 est aussi secrétée par le Th1

Les questions posées concernant ce projet ont été les suivantes :

- Les sécrétions de l'IL-17 et de l'IL-22 sont elles régulées de la même manière en fonction du contexte polarisant Th1 et Th17 ?
- La sécrétion d'IL-22 est elle dépendante d'une des cytokines nécessaires à la polarisation des Th17 ?

Les résultats ont fait l'objet d'une première publication en 2009 dans *Blood*.

Projet 2 (Manuscrit soumis)

Les résultats obtenus lors du projet 1 m'ont amené à élargir notre modèle d'étude de la polarisation de lymphocytes Th. J'ai décidé d'analyser de manière systématique comment une cytokine issue du microenvironnement pouvaient moduler les fonctions des Th pendant le processus de polarisation.

J'ai utilisé comme cytokine modulatrice l'IFN plusieurs raisons : 1/ L'effet de l'IFN sur les Th n'est pas entièrement élucidé 2/ l'IFN est présent dans les organes lymphoïdes secondaire lors du processus de polarisations des lymphocytes CD4 naïfs 2/ l'IFN a un effet paradoxale à la fois protecteur ou délétère dans les maladies auto-immunes et inflammatoires.

Pour évaluer au mieux l'ensemble des fonctions des Th, j'ai choisi comme approche expérimentale une analyse à large échelle du transcriptome des lymphocytes Th. L'avantage principal de cette technique est la génération de multiples output (gènes) en réponse à plusieurs inputs. L'ensemble de gènes differentiellement régulés selon les conditions peut être ensuite regroupé par modules qui nous renseignent sur les fonctions cellulaires activées ;

Au fur et mesure de la préparation du projet et des expériences de micro-array, nous nous sommes aperçus que notre schéma expérimental répondait à une question biologique plus générale sur l'effet du microenvironnement sur la réponse à une cytokine : Comme je l'ai souligné en fin d'introduction, la réponse cellulaire à une cytokine dépend à la fois de facteurs intrinsèques et extrinsèques

Nous avons reformulé notre question scientifique plus générale : **Comment le contexte polarisant des T Helper module-t-il la réponse de l'IFN ?**

Les questions posées pour ce projet ont été les suivantes :

- Comment les signaux délivrés par l'IFN et par les cytokines polarisantes sont-ils intégrés pendant le processus de différentiation des T Helper ?
- La réponse IFN est elle la même dans chaque contexte polarisant ?
- Existe-t-il une réponse IFN commune et/ou spécifique selon le contexte polarisant ?
- Ces différentes réponses ont elles un impact fonctionnelle sur le Th ?

Projet 3

Dans le cadre de l'analyse à large échelle des sous populations cellulaires, nous avons collaboré avec l'équipe de Sebastian Amigorena, sur un projet dont la question scientifique et la méthode expérimentale se rapprochaient de notre projet. L'une des post doctorantes Elodie Segura a observée chez l'homme une population des DC dites « inflammatoires » dont le phénotype est diffèrent d'autres APC comme les DC sanguines, macrophages , ou monocytes. Pour déterminer si cette population de DC inflammatoire correspond à une sous population distinctes de DC ou à une forme particulière de DC dits conventionnels (DC sanguin, macrophages…), nous avons choisi de nouveau, d'utiliser une analyse à large échelle afin de comparer les profils transcriptionnels des ces populations et de mieux les individualiser.

Les résultats ont fait l'objet d'une publication récente dans *Immunity*.

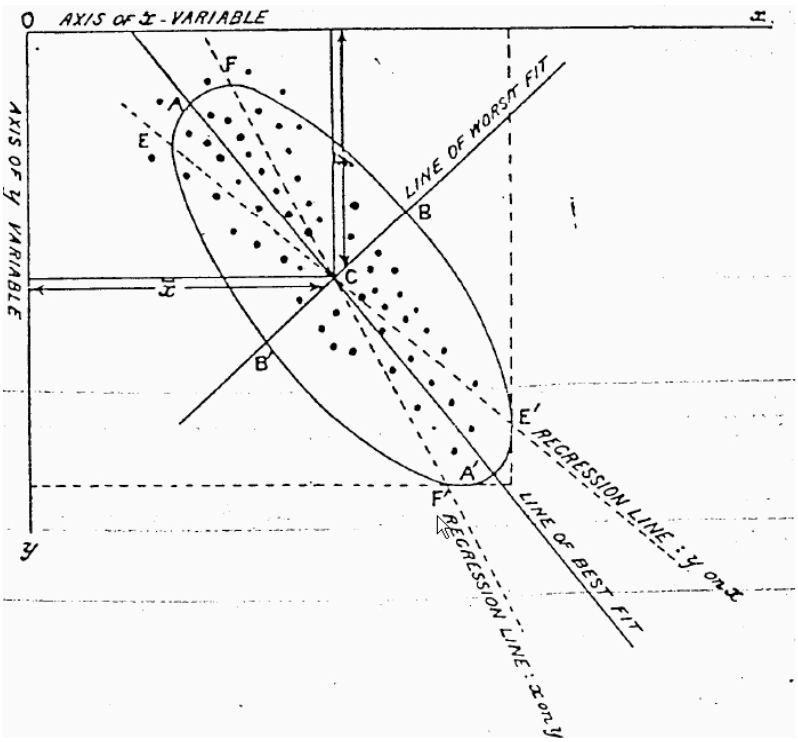
6.2 APPROCHE SCIENTIFIQUE.

Pour étudier la complexité des signaux (IFN et contexte polarisant) agissant sur le lymphocyte T, nous avons opté dans un premier temps pour une analyse à large échelle du transcriptome de cellules primaires CD4 naïves humaines après 5 jours de différentiation dans 4 contextes polarisants en présence ou non d'IFN. L'ensemble des data générées a été analysé avec plusieurs outils de bioinformatiques détaillés ci dessous. Les cibles mises en évidence par micro-array ont été validées systématiquement sur le plan de l'ARNm (quantitative real time PCR), et sur le plan protéique par des analyses multiparamétriques (ELISA, Cytométrie de Flux, Cytometrics Beads Array). La validation de « l'antiviral state » a nécessité l'infection de Th générés par des virus (HIV-1 HIV-2) en collaboration avec l'équipe de Nicolas Manel au sein du groupe U932.

L'étude de la régulation de l'IL-17 et IL-22 s'est basée sur l'analyse multiparamétrique de cytokines secrétées par des lymphocytes CD4 naïves soumises à différents contextes de polarisation.

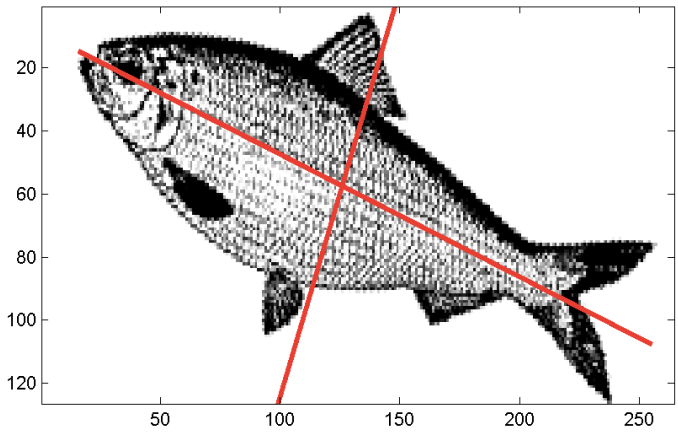
Analyse par composantes principale : généralité

L'analyse par composante principale (ACP ou PCA) est une méthode de la famille des données et plus précisément de la statistique multivariée, qui consiste à transformer/compresser des variables liées entre elles (dites « corrélées ») en nouvelles variables décorrélées les unes des autres. Ces nouvelles variables « artificielles » sont nommées « composantes principales » (PC), ou axes principaux. Il s'agit d'une approche à la fois géométrique (les variables étant représentées dans un nouvel espace, selon des directions d'inertie maximale) et statistique (la recherche portant sur des axes indépendants expliquant au mieux la variabilité – ou variance- des données). Lorsqu'on veut compresser un ensemble de **N** variables aléatoires, les ***n*** premiers axes de l'analyse en composantes principales sont les axes qui représentent le mieux la variance des **N** variables observées. Chaque axe représente un % spécifique et décroissant de la variance observée qui décroit en fonction du nombre de PC. Par exemple pour un set de données, la PC1 représente 60 % de la variance observée, la PC2 30% la PC3, 5% etc….
Voici ci dessous deux exemples pour mieux comprendre les PCA. Le premier prend sa source dans un article de Karl PEARSON publié en 1901 « the line with best fit ».



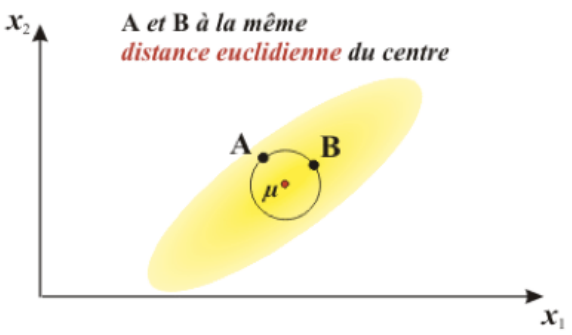
Dans le deuxième exemple, les pixels de l'image sont représentés dans un plan et considérés comme une variable aléatoire à deux dimensions. L'ACP va déterminer les

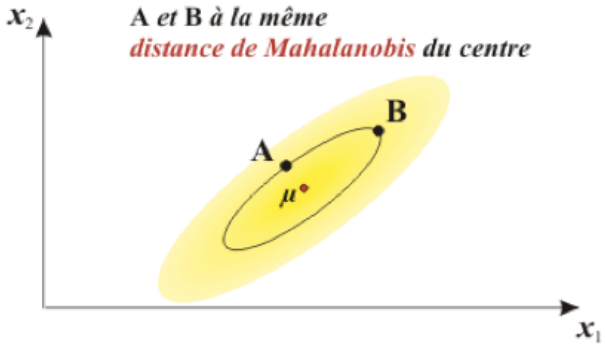
deux axes qui expliquent au mieux la dispersion de l'objet, interprété comme un nuages de points. Elle va aussi les ordonner par inertie expliquée.



Distance de Mahalanobis

Pour pouvoir comparer les profils de populations obtenus par la PCA et mesurer les « distances », entre chaque point, nous nous sommes basés sur le calcul de la distance de mahalanobis. En effet la représentation liée à la PCA n'utilise pas le système métrique euclidien ou chaque vecteur a le même poids. Chaque vecteur doit être analysé en accord avec le coefficient de pondération qui lui est attribué.





RESULTATS

7.1 Publication 1

Multiparametric analysis of cytokine-driven human Th17 differentiation reveals a differential regulation of IL-17 and IL-22 production.

Volpe E, Touzot M, Servant N, Marloie-Provost MA, Hupé P, Barillot E, Soumelis V.
Blood. 17, (2009)

Dans ce premier projet, nous avons étudié les mécanismes de régulation de l'IL-22 dans deux sous populations lymphocytaires pro-inflammatoires Th1 et Th17. L'IL-22 est une cytokine appartenant à la famille de l'IL-10 ; qui joue un rôle primordial dans les défenses anti-bactériennes au niveau des muqueuses. Elle est aussi impliquée dans la physiopathologie de maladie inflammatoire comme le Psoriasis ou la Maladie de Crohn. L'IL-22 et l'IL-17 partagent plusieurs fonctions commune et mais leurs relations n'est pas clairement définie.

A partir d'analyses multiparamétriques des profils cytokiniques des Th1 et Th17, nous avons mis en évidence une régulation différentielle de l'IL17 et l'IL-22. Dans notre système in vitro de différentiation T Helper, la sécrétion de l'IL-22 est plus corrélée avec celle de l'IFN-γ que l'IL-17 (en protéine et ARN) et RORc (ARN).

Pour analyser la régulation de l'IL-22 au cours de la différenciation Th1 et Th17, nous avons testé la capacité des différentes cytokines polarisantes à induire de l'IL-22 et l'IL-17. Nous avons montré que seul l'IL-12 et l'IL-23 sont capables d'induire la production d'IL-22. Les taux observés d'IL-22 induits par l'IL-23 étaient cependant plus élevés qu'avec le cocktail de cytokine « Th17 » suggérant la présence des facteurs inhibiteurs de l'IL-22. En testant des combinaisons des différentes cytokines du cocktails Th17, nous avons observé que le TGF-β qui est indispensable à la différenciation optimale des Th17 inhibe la production de l'IL-22 .

Nos résultats montrent ainsi que l'IL-22 n'est pas une cytokine spécifique des Th17 et que sa régulation dépend de plusieurs cytokines du microenvironnement inflammatoire.

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Brief report

Multiparametric analysis of cytokine-driven human Th17 differentiation reveals a differential regulation of IL-17 and IL-22 production

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T helper 17 (Th17) cells produce IL-17 but can also make tumor necrosis factor, interleukin (IL)–6, IL-10, IL-21, and IL-22. These cytokines collectively contribute to the functional outcome of the Th response. IL-22 plays a critical role in some Th17-associated diseases, such as psoriasis, but its relationship to IL-17 remains controversial. Here, we used a systematic multiparametric analysis of Th-17-associated cytokines, which revealed the unexpected finding that the regulation pattern of IL-22 was most closely related to interferon-γ, the prototypical Th1 cytokine, and not to IL-17. To explain this observation, we systematically tested the role of Th1- and Th17-inducing cytokines. We could show that IL-12 and IL-23 induced high levels of IL-22 but no IL-17. Conversely, transforming growth factor-β inhibited IL-22 production but promoted IL-17. Thus, IL-17 and IL-22 are differentially regulated during cytokine-induced Th cell differentiation. This has important implications for the understanding and pharmacologic manipulation of Th17-associated pathologies. (Blood. 2009;114:3610-3614)

Introduction

Naive T cells can develop into different T helper (Th) subsets with different cytokine profiles and distinct effector functions.[1,2] The Th17 subset produces interleukin (IL)–17, which is particularly important in the activation of antimicrobial defense and autoimmunity.[3] However, it became clear that Th17 cells can also produce other Th cytokines, such as tumor necrosis factor (TNF), IL-6, IL-10, IL-21, and IL-22.[4,5] The interplay between IL-17 and coproduced cytokines could critically affect the global outcome of a Th17 response and modulate the balance between pathogenesis and protection.[5]

The relationship between IL-17 and IL-22 is of particular interest. IL-22 exerts similar functions to IL-17, both contributing to the control of extracellular bacterial infection by induction of a strong mucosal immunity.[2] The IL-17 and IL-22 expression is often linked to proinflammatory processes, such as psoriasis and Crohn disease.[2,6-8] These observations are consistent with a coregulation of IL-17 and IL-22. However, IL-22 can be produced by non-Th17 cell types[9-11] independently of IL-17 production. Furthermore, IL-22 also has specific functions, such as induction of tissue-repair and wound-healing responses protecting from liver disease[12,13] or myocarditis,[14] where IL-17 is not implicated. This evidence suggests a different regulation between IL-17 and IL-22 production. Thus, the relationship between IL-17 and IL-22 remains unclear.

Methods

Purification of naive CD4$^+$ T lymphocytes from adult blood

Peripheral blood naive CD4$^+$ T cells (CD4$^+$ CD45RA$^+$ CD25$^-$ CD45RO$^-$) or effector memory CD4 T cells (CD4$^+$CD45RA$^-$CD27$^-$) were isolated from peripheral blood mononuclear cell using immunomagnetic depletion (Miltenyi Biotec) and FACSAria sorting (BD Biosciences) as previously described.[4] All cells were used with the approval of the Institutional Review Board of Institut Curie, and blood donors gave their informed consent for research use of buffy coats in accordance with the Declaration of Helsinki.

T helper cell differentiation assay

Naive CD4$^+$ T cells were cultured in Yssel medium (gift of Hans Yssel, Inserm) containing fetal calf serum as previously described.[4] Stimulation was performed with cytokines (R&D Systems) and Dynabeads CD3/CD28 (Invitrogen) for 5 days. After 24 hours of restimulation, supernatants were harvested for cytokine detection and cells were collected for transcriptional analysis.

Analysis of cytokine production

IL-17 (R&D Systems), IL-21 (eBioscience), and IL-22 (PeproTech) were measured by enzyme-linked immunosorbent assay (ELISA), IL-4, IL-5, IL-6, IL-10, IL-13, interferon-γ (IFN-γ), and TNF by cytometric bead assay Flex Sets (BD Biosciences). The cells were stained with the red LIVE/DEAD (Invitrogen) to distinguish the living from the dead cells. Cytokine-producing cells were analyzed by intracellular staining after addition of brefeldin (10 μg/mL) during the last 3 hours of restimulation. Cells were fixed and permeabilized using the Staining Buffer Set (eBioscience), stained with anti–IL-22 Alexa 647 (eBioscience), anti–IFN-γ V-450 (BD Biosciences PharMingen), anti–IL-17 Alexa-488 (eBioscience), washed, and then analyzed by flow cytometry (Cyan; Dako North America).

Real-time quantitative RT-PCR

Total RNA was extracted by RNeasy Microkit (QIAGEN) and processed as previously described.[4] The following probes were used: *IL17A*

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(Hs00174383_m1), *IL22* (Hs00220924_m1), *RORC* (Hs01076112_m1), *RORA* (Hs00536545_m1), *AHR* (Hs00169233_m1), *TBx21* (Hs00203436_m1), and *IL26* (Hs00218189_m1). For each sample, mRNA abundance was normalized to the amounts of the ribosomal protein *L34* (Hs00241560_m1).

Statistical analysis

Data used to perform the clustering were corrected for the donor effect by applying a linear model. To summarize the information, the replicates were aggregated within each condition to their barycentric value for each cytokine. Hierarchical clustering analysis was performed using a distance based on Pearson correlation and Ward criteria as an agglomerative method. For pairwise comparisons, we used a nonparametric 2-tailed Wilcoxon test. *P* values less than .05 were considered statistically significant. We used the Pearson correlation coefficient to assess the significance of correlation between IL-17A, IL-22, TNF, IFN-γ protein, or *IL17* and *IL22* mRNA with *RORC*, *RORA*, *AHR*, and *TBx21*.

Results and discussion

To assess the relationship between Th17-derived cytokines, we generated a variety of Th subsets, including Th0, Th1, and Th2 as controls, together with optimal and suboptimal Th17 subsets (supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), as previously described.[4] We analyzed the production of 10 Th-derived cytokines, and we studied the relation between them by clustering analysis. This strategy enables an unbiased insight into the similarities between Th cytokines, according to their pattern of regulation in different experimental conditions.[15] The segregation of IFN-γ, IL-4, and IL-17 in 3 different clusters confirmed the distinct phenotype of Th1, Th2, and Th17 cells, respectively. IL-5 and IL-13 were most closely related to IL-4, consistent with a similar pattern of regulation of these Th2 cytokines. The cytokine showing the strongest association with IL-17 production was TNF (Figure 1A). Unexpectedly, we found that IL-22, which is often described as a Th17 cytokine,[16] correlated most closely to IFN-γ, the prototypical Th1 cytokine (Figure 1A). These results were confirmed by pairwise correlation of cytokine levels: IL-22 and IL-17 production did not significantly correlate; IL-22 correlated with IFN-γ; and IL-17 positively correlated with TNF but not with IFN-γ (Figure 1B). Interestingly, the adjacent positioning of the human *IL22* and *IFNγ* genes on chromosome 12q14[17] also supports a similar regulation of IL-22 and IFN-γ production. In addition, we could also analyze the expression of *IL26*, which was recently associated with human Th17 lineage.[18,19] We confirmed that *IL26* is mainly expressed under Th17 conditions (supplemental Figure 1A), and we observed that it significantly correlated with *IL17*, but not with *IL22* expression (supplemental Figure 1B).

To investigate whether the IL-22–producing cells preferentially coproduce IFN-γ rather than IL-17, we analyzed the IL-17 and IFN-γ production by IL-22$^+$ cells using in vitro differentiated Th cells and ex vivo purified effector memory CD4 T cells. Interestingly, the majority of IL-22$^+$ cells did not produce either IL-17 or IFN-γ under Th0 and Th17 conditions (86.8% ± 4.1% and 83.1% ± 5.0%, respectively; supplemental Figure 2A-C). In similar conditions, the IL-22$^+$ cells mainly coproduced IFN-γ rather than IL-17 (33.47% ± 21.18% compared with 1.6% ± 1.1%; supplemental Figure 2A,C). This further supports a coregulation of IL-22/IFN-γ in cytokine-induced Th cells. In contrast, IL-22–producing cells among circulating effector memory T cells similarly produced IFN-γ and IL-17, although the majority were IL-22 single producers (supplemental Figure 2B-C).

Next, we assessed the relationship between IL-17 and IL-22 at the level of transcription factors. We confirmed in our human system that *RORC*, *RORA*, and *AHR*[20,21] were specifically induced in Th17 conditions (Figure 1C). To address their relationship with IL-17 and IL-22 production, we analyzed their expression in several experimental conditions, which were inducing variable levels of IL-17 and IL-22 (supplemental Table 1). We confirmed a strong correlation between *IL17A* and *RORC* expression,[4,19] and we showed, for the first time, that human *IL17A* correlated also with *RORA* and *AHR* expression (Figure 1D). We observed a similar correlation between these transcription factors and IL-17F (supplemental Figure 3). In contrast, IL-22 did not correlate with any of the Th17-related transcription factors that we have analyzed (Figure 1E). In the mouse, IL-22 production is dependent on AHR,[21] suggesting a different transcriptional regulation of human and mouse IL-22 production. In our system, IL-22 and not IL-17 expression significantly correlated with *TBx21*, the major Th1 transcription factor, indicating a stronger relationship between IL-22 and IFN-γ compared with IL-17 (Figure 1E). Overall statistical and computational approaches revealed that IL-17 and IL-22 production was differentially regulated during cytokine-induced Th cell differentiation. This prompted us to investigate the underlying factors implicated in the differential regulation of IL-17 and IL-22.

We and others recently showed that human IL-17 induction requires IL-1β, IL-6, IL-23, and transforming growth factor-β (TGF-β).[4,22] The previous clustering analysis (Figure 1A) showed that IL-22 behaved more similarly to Th1 than to Th17 cytokines, suggesting that Th1- or Th17-inducing cytokines may explain the differential regulation of IL-17 and IL-22. To address this hypothesis, we systematically tested the role of single cytokines promoting Th1 and/or Th17 differentiation in the induction of IL-22 and IL-17 by naive CD4 T cells. We found that individual inflammatory cytokines (IL-1β, IL-6, TNF) and TGF-β did not induce IL-22 (Figure 2A) or IL-17 production (Figure 2B). On the contrary, naive T cells cultured in the presence of IL-12 (Th1 condition) or IL-23 produced high levels of IL-22 but not IL-17 (Figure 2B). This indicated that IL-23 and IL-12 are 2 underlying factors explaining the differential regulation of IL-17 and IL-22 revealed by statistical methods. These findings are consistent with previous results reporting a role for IL-23 in dermal inflammation and acanthosis in a psoriasis mouse model.[8] The importance of IL-12 in inducing IL-22 is supported by a previous study showing a reduced IL-22 secretion by T cells purified from IL-12- and IL-12 receptor-mutated patients.[23] This also suggests that IL-22/IFN-γ coproduction could play an important role in Th1-associated diseases. In summary, different environmental conditions can mediate the production of IL-17 or IL-22, potentially explaining immune responses not characterized by the coexpression of IL-17 and IL-22.[12-14]

Notably, the IL-23–mediated IL-22 production was higher than the expression found in the optimal Th17 condition (Figure 2A). This suggested that some of the other Th17-inducing components could have an inhibitory role in IL-22 production. To test this hypothesis, we measured the IL-22 production in cells stimulated with IL-23 combined to inflammatory cytokines or TGF-β. The combination of inflammatory cytokines induced low levels of IL-22 and did not affect the IL-23–mediated IL-22 production. In contrast, IL-22 was significantly inhibited by

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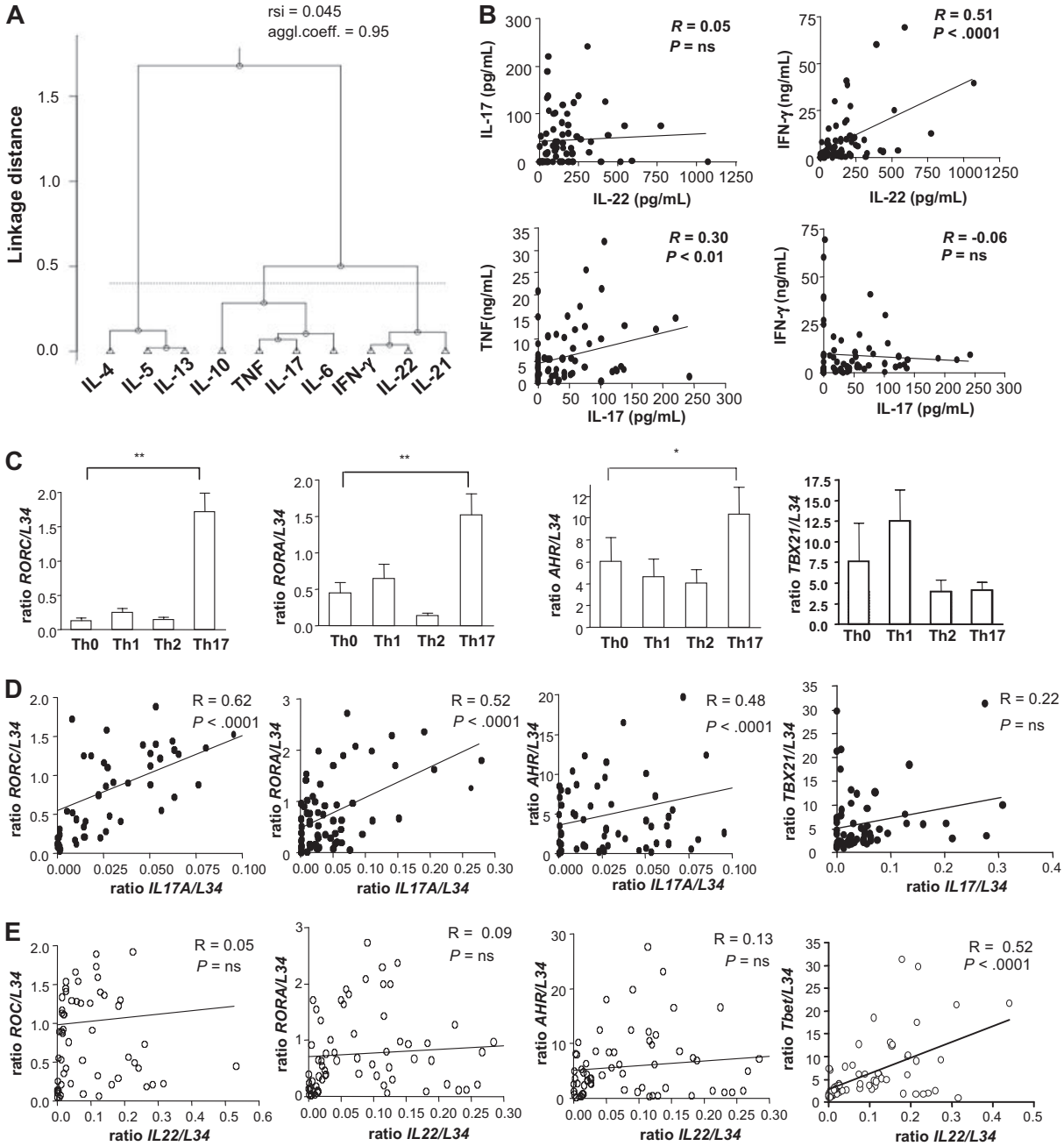

Figure 1. IL-22 regulation is most closely related to IFN-γ rather than IL-17. Naive T cells were cultured under Th0 (no polarizing cytokines), Th1 (1 ng/mL IL-12), Th2 (25 ng/mL IL-4), Th17 (10 ng/mL IL-1β, 20 ng/mL IL-6, 10 ng/mL TNF, 1 ng/mL TGF-β, 100 ng/mL IL-23), suboptimal Th17 (absence of individual Th17-promoting cytokines) in the presence of anti-CD3 + anti-CD28. Protein and transcript analyses were performed after 24 hours of restimulation with anti-CD3 + anti-CD28. Supplemental Table 1 contains more details. (A) Clustering analysis of Th cytokines produced in all experimental conditions using a Pearson correlation–based distance. Cytokines were separated in clusters by comparing their linkage distance. The agglomerative coefficient reflects the structure of the data (values close to 1 indicate well-separated clusters), and resampling similarity index (*rsi*) evaluates the robustness of the clustering. (B) Graphs of amounts of IL-17 or IL-22 protein were correlated to IFN-γ or TNF levels, using Pearson correlation. R indicates correlation coefficient. (C) RT-PCR for expression of *RORC*, *RORA*, *AHR*, *TBx21* in Th0, Th1, Th2, and Th17 conditions. Threshold cycle values were normalized to mRNA of ribosomal protein *L34* gene. Data were normalized to the maximal value obtained for each donor. Data are mean ± SEM of 9 donors. (D-E) Graphs of *IL17A* and *IL22* transcript levels, obtained from 9 independent experiments with cells cultured as previously described, were correlated to *RORC*, *RORA*, *AHR*, and *TBx21* transcript levels, using Pearson correlation. R indicates correlation coefficient.

TGF-β (Figure 2C). Inflammatory cytokines and not TGF-β could induce low levels of IL-17 in IL-23–stimulated cells (Figure 2D). IL-23– and IL-12–mediated IL-22 production was inhibited by TGF-β in a dose-dependent manner (Figure 2E-F). A comparable inhibition of IL-22 by TGF-β was also observed under Th17-promoting conditions (Figure 2G). In contrast, IL-17 production in the same culture conditions was promoted by TGF-β (Figure 2H), confirming the differential IL-17/IL-22 regulation. Notably, TGF-β did not affect IL-17 and IL-22 production by already differentiated Th17 cells (supplemental Figure 4), indicating that TGF-β acts during the early events of Th differentiation. The ability of TGF-β to inhibit IL-22 production (Figure 2G) as well as to induce apoptosis of Th1 cells[24] may collectively contribute to enhancing Th17 differentiation.

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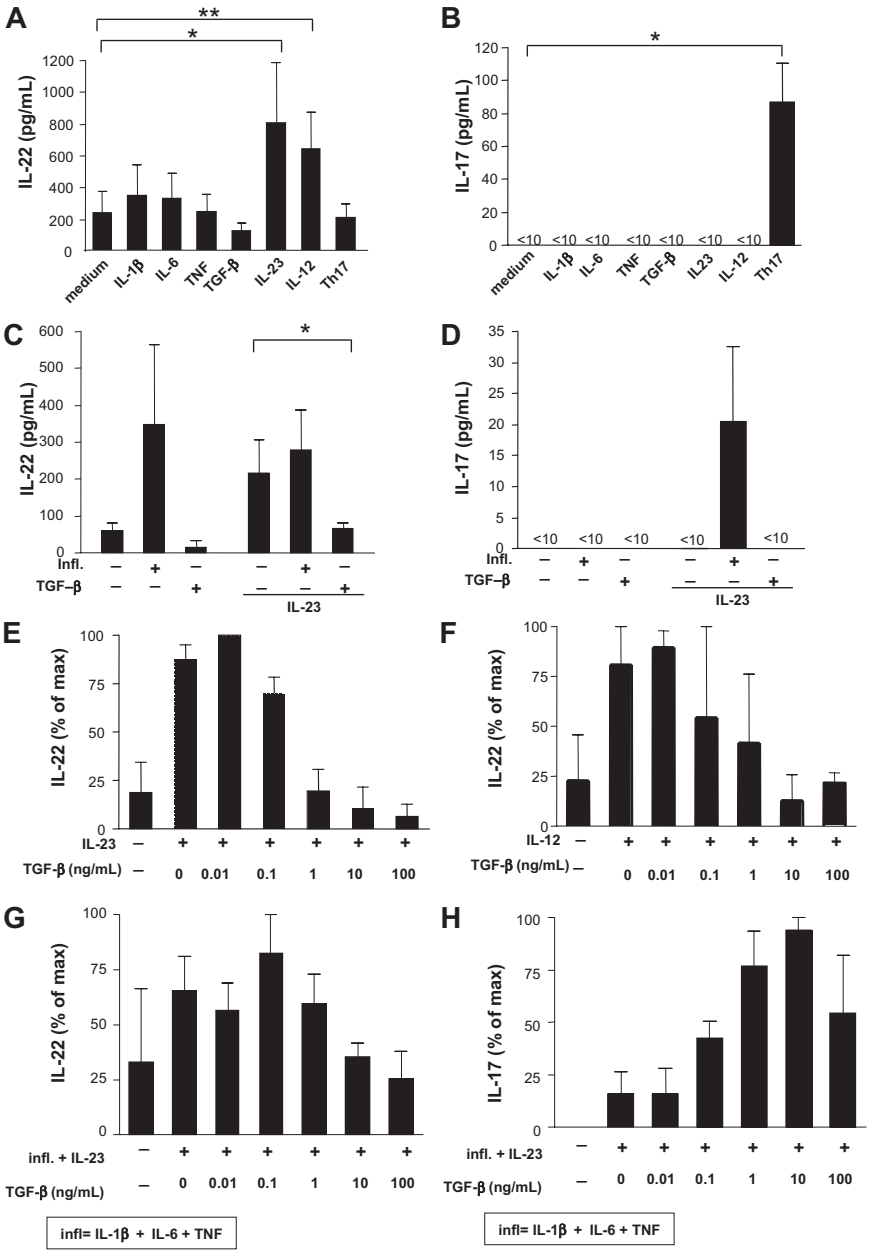

Figure 2. IL-12, IL-23, and TGF-β differentially regulate IL-17 and IL-22 production. (A-D) ELISA assay for IL-22 and IL-17 production by naive T cells differentiated for 5 days in the presence of anti-CD3 + anti-CD28 and different cytokines; IL-17 and IL-22 production in supernatant was measured after 24 hours of restimulation with anti-CD3 + anti-CD28. Data are mean ± SEM of 4 donors. ***$P < .001$, **$P < .01$, and *$P < .05$ (Wilcoxon test). (E-H) ELISA for production of IL-22 and IL-17 by naive T cells differentiated with IL-23 or IL-12 or proinflammatory cytokines + IL-23 and different concentrations of TGF-β. Cytokines were measured after 24 hours of restimulation with anti-CD3 + anti-CD28. Cytokine amounts detected were normalized to the maximum value obtained for that cytokine across the whole set of condition, for each donor. Data are mean ± SEM of 3 donors.

In conclusion, we demonstrated that the production of human IL-17 and IL-22 is differentially regulated during cytokine-induced Th cell differentiation. Although our study does not exclude that IL-17 and IL-22 may be coregulated in other systems,[16] it provides evidence that IL-22 is not a Th17-specific cytokine, and may be more broadly implicated in Th1- and IL-23–driven responses.

Acknowledgments

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Authorship

Contribution: E.V. designed and performed the experiments and contributed to writing the paper; M.T. and M.-A.M.-P. performed some experiments; N.S. and P.H. did computational and statistical analysis; E.B. supervised the computational and statistical analysis; and V.S. supervised the study and contributed to experimental design and the writing of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Figure S1.**IL26 specifically correlates with IL17 expression**

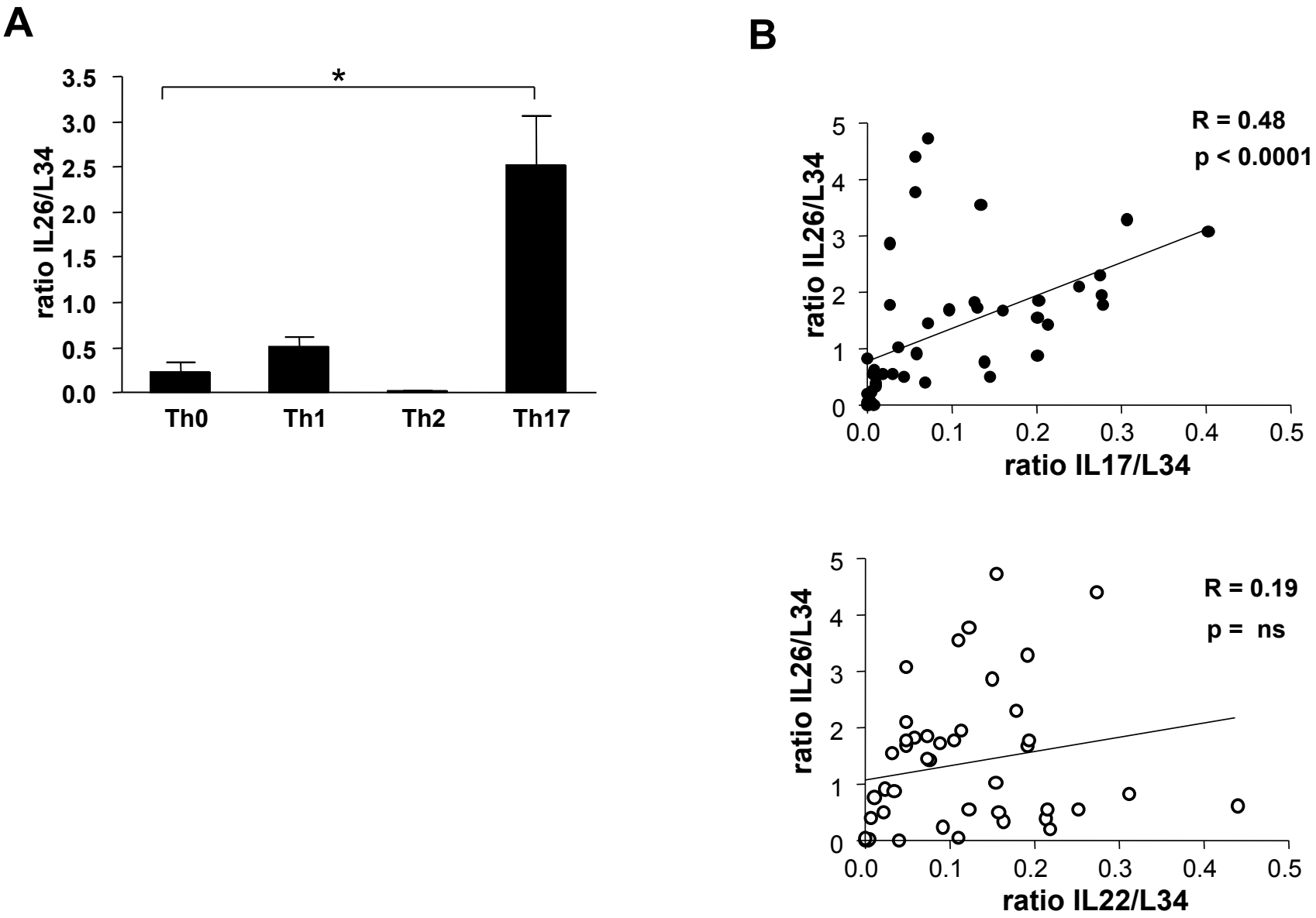
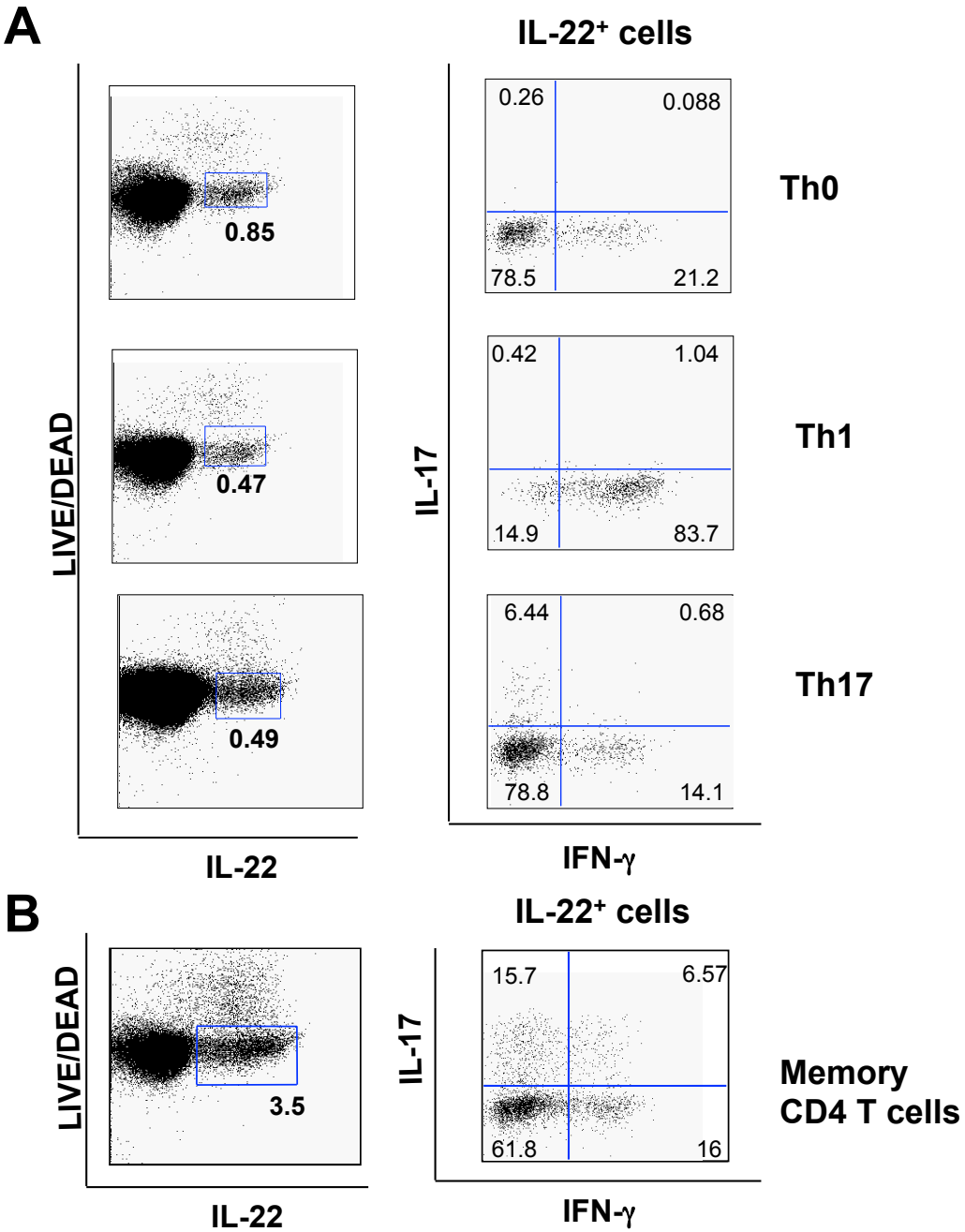


Figure S2. **IL-22-producing cells are mostly IL-17⁻**



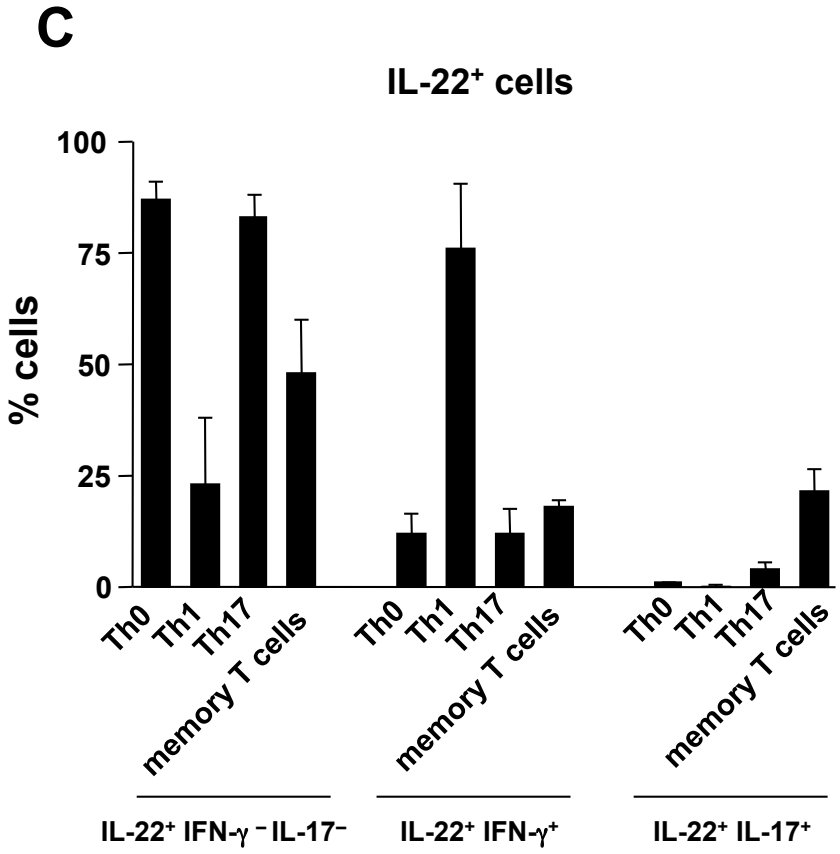


Figure S3. **IL17F correlates with RORC, RORA and AHR expression**

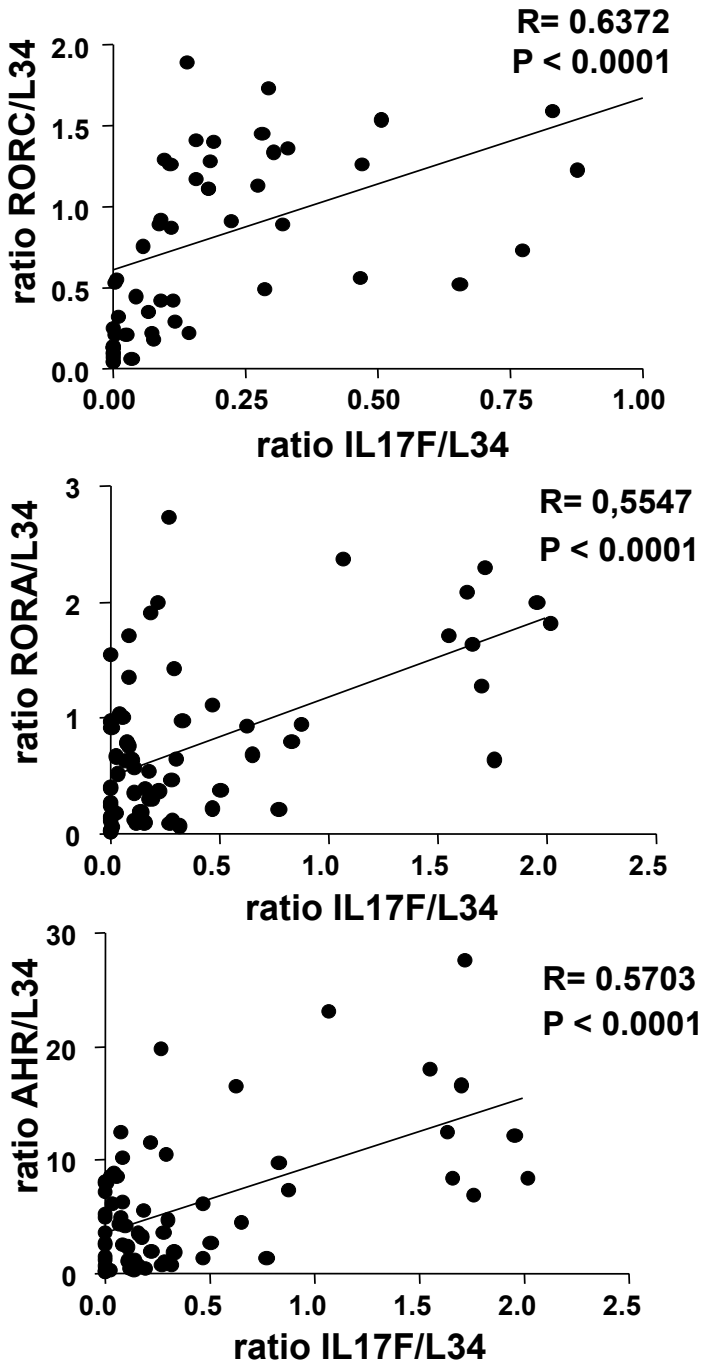
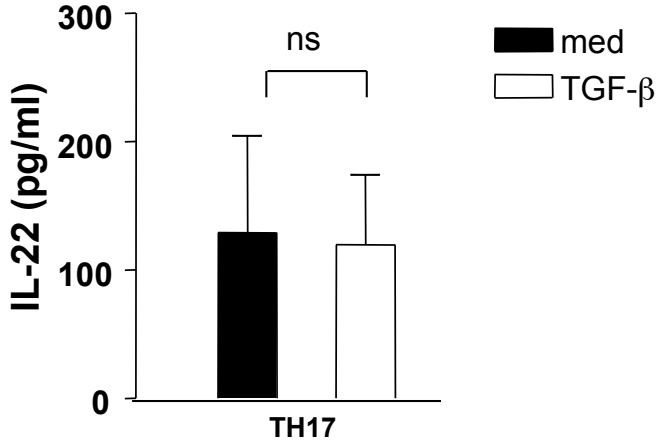


Figure S4. **TGF-β role in memory Th17 subsets**



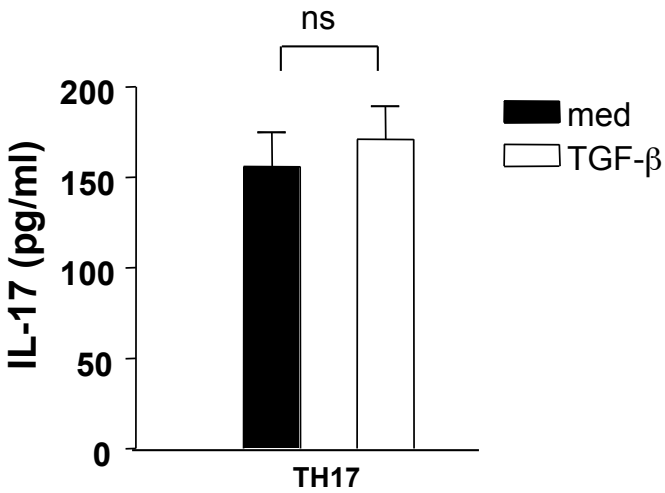


Table S1. **Experimental conditions used for the multiparametric study**

	Th0	Th2	Th1	Th17	Th17 without IL-1b	Th17 without IL-6	Th17 without TNF	Th17 without TGF-b	Th17 without IL-23	N exp.
IL-4	-	+	-	-	-	+	+	+	+	6
IL-12	-	-	+	-	-	+	+	+	+	6
IL-1b	-	-	-	+	-	+	+	+	+	6
IL-6	-	-	-	+	+	-	+	+	+	6
TNF	-	-	-	+	+	+	-	+	+	6
TGF-β	-	-	-	+	+	+	+	-	+	6
IL-23	-	-	-	+	+	+	+	+	-	6
Total samples										54

Supplementary Figures

Table S1. Experimental conditions used for the multiparametric study

We generated Th0, Th1, Th2, Th17 optimal and suboptimal conditions, with indicated cytokines. Complete data sets (9 polarizing conditions) were obtained from 6 independent donors for experiment of Figure 1 and B. Further donors were added for experiments of Figures 1C-E.

Figure S1. IL26 specifically correlates with IL17 expression

A) IL-26 transcript level was measured in cells cultured under Th0, Th1, Th2, Th17 condition. Ct values were normalized to mRNA of ribosomal protein L-34 gene. Mean ± s.e.m. of 5 donors is shown.

B) IL-26 transcript level obtained from 5 independent experiments with cells cultured as described in Table S1, was correlated to IL-17 and IL-22 transcript levels, using Pearson correlation. *R*: correlation coefficient.

Figure S2. IL-22-producing cells are mostly IL-17-

A-B) Naïve T cells under Th0 (no polarizing cytokines); Th1 (IL-12); Th17 (IL-1β, IL-6, TNF, TGF-β, IL-23) or effector memory CD4 T cells ($CD4^+$ $CD45RA^-$ $CD27^-$) were cultured in presence of anti-CD3/CD28 for 5 days. After 6h of re-stimulation we detected by FACS intracellular staining the viability (LIVE/DEAD), IL-22, IL-17 and IFN-γ production. Among viable IL-22-producing cells, we measured the percentage of IL-17- and IFN-γ-producing cells. Results are from one representative of 3 independent experiments.

C) Percentage of IL-22 single producers, IL-22/IFN-γ and IL-22/IL-17 co-producing cells were analysed on total viable IL-22-producing cells in Th0, Th1, Th17 and effector memory T cells. Data are represented as mean ± s.e.m. of 3 donors.

Figure S3. IL-17F correlates with RORC, RORA and AHR expression

IL-17F transcript level, obtained from 9 independent experiments with cells cultured as described in Table S1, was correlated to RORC, RORA, AHR transcript levels, using Pearson correlation. *R*: correlation coefficient.

Figure S4. TGF-β role in memory Th17 subset

ELISA assay for IL-22 and IL-17 production by Th cells differentiated for 5 days under Th17 (IL-1β, IL-23, TGF-β, IL-23) was measured after 24 h of re-stimulation in presence of anti-CD3 + anti-CD28 with or without TGF-β. Data are represented as mean ± s.e.m. of 3 donors. ns = $P > 0.05$ (Wilcoxon test).

7.2 Publication 2 : soumis

Environmental control of type I IFN function during Human T helper cell differentiation.

Maxime Touzot, Maximilien Grandclaudon, Takeshi Satoh, Antonio Cappuccio, Carolina Martinez, Nicolas Servant, Nicolas Manel, Vassili Soumelis.

Dans ce projet, nous nous sommes intéressés à la modulation de la réponse IFN par différents microenvironnements inflammatoires en utilisant comme modèle expérimental la différentiation des T Helpers. Nous avons caractérisé dans un premier temps la signature transcriptomique des IFN dans un contexte de polarisation neutre de CD4. Cette signature composée en grande majorité d'ISGs antiviraux est différente de celle induite par les cytokines polarisantes et semble persister après restimulation des LT. Dans un deuxième temps, nous avons comparé la signature IFN générée dans un contexte neutre des signatures IFN générées lors de la polarisation Th1 Th et Th17. Nous avons observé une grande flexibilité de la réponse IFN en fonction du contexte polarisant aussi bien sur des fonctions très conservées comme la réponse antivirale que sur d'autres fonctions biologiques des T Helpers. Ces observations à l'échelle du transcriptome ont été par la suite validées au niveau ARN et protéique. Une des nouveautés de notre travail est la mise en évidence d'une modulation de « l'antiviral state » dans les sous populations des Th qui a pour conséquence une plus grande susceptibilité des Th17 lors d'infections virales comme le HIV-1 et HIV-2. Notre travail souligne aussi la complexité et la flexibilité de la réponse IFN dans contexte physiologique mais aussi pathologique.

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Abstract

Beyond their antiviral properties, Type I interferons (IFN) are innate cytokines with pleiotropic effects on cellular functions including immune response. In chronic inflammatory conditions, IFN may have either protective or detrimental effects that remain poorly understood. We hypothesized that the microenvironment may determine the IFN response. Using a systematic large-scale approach, we analysed IFN effects during human T helper cell differentiation in the context of four distinct cytokine microenvironments (Th0, Th1, Th2 and Th17). We identified two types of IFN response.: 1) a conserved transcriptional program comprising mostly antiviral genes and observed across all tested Th contexts; 2) a flexible response dictated by the cytokine milieu. IFN induced specifically CXCL10 and IFN-γ in Th1, and CCL20 and IL-17 in Th17 cells. The cytokines produced by Th0 and Th2 cells were the most globaly affected by IFN, with an inhibition of IL-4, IL-5, IL-13 but an increase in IL-10, IL-6 and IL-3. Antiviral state was also modulated in a Th specific manner, with a lesser protection to HIV-1 and HIV-2 infection in the Th2 and Th17 contexts. Our results reveal a large scale plasticity of the IFN response that may underlie the effects of endogenous and exogenous IFN in different physiopathological contexts.

Introduction

Type I interferons (IFN) form a family of innate cytokines produced by host cells in response to viral infections (Isaacs and Lindenmann 1957). IFN-α and IFN-β are the best characterized and broadly expressed (Decker et al. 2005), with 13 IFN-α isoforms and one IFN-β isoform. Binding to a unique ubiquitous receptor, they activate a well-described signaling cascade (Trinchieri; Theofilopoulos et al. 2005) leading to the induction of more than 300 Interferon Stimulated Genes (ISGs) (de Veer et al. 2001; Indraccolo et al. 2007). ISGs have antiviral activities, and also control cell growth, apoptosis, and innate immunity (Schoggins et al.; Marrack et al. 1999; Chawla-Sarkar et al. 2003; Dondi et al. 2003; Theofilopoulos et al. 2005). However, IFN has also a complex and less well characterized role in adaptive immunity, by activating immature DC (Prchal et al. 2009), maintaining B cell survival and antibody responses, and modulating proliferation and differentiation of T Helper (Th) subsets (Theofilopoulos et al. 2005) .

IFN are effective biotherapies in chronic viral infections, malignancies and immune-mediated diseases (e.g multiple sclerosis (MS), beçhet's disease) (Borden et al. 2007). On the other side, IFN play also a central role in the pathophysiology of immune and infectious diseases, such as systemic lupus erythematosus (SLE) (Bennett et al. 2003; Guiducci et al. 2009), dermatomyositis (DM) (Guiducci et al. 2009), type 1 diabetis (Guiducci et al. 2009), and tuberculosis (Berry et al.). Inhibition of IFN is now considered to be a new challenging therapeutic strategy, with ongoing clinical trials in SLE (Merrill et al.). Hence, understanding the molecular mechanisms of action of IFN is of major importance, with potential implications in the treatment of numerous human diseases.

A common feature of all these pathophysiological conditions is that exogenous (therapeutic) or endogenously produced IFN will act in the context of different inflammatory settings, characterized by a diversity of tissues and cellular microenvironments. During an infectious event, IFN may be secreted in lymph nodes with other cytokines that drive Th polarisation. Although the IFN system is highly conserved during evolution (Krause and Pestka 2005), and the IFN response has been well characterized in model cellular systems, we hypothesized that its function may be modulated by diverse microenvironments composed by different signals, which may interact to generate a diversity of IFN signatures, rather than a unique response. Such flexibility in IFN responses is suggested by the multiplicity of IFN effects in patients bearing diverse pathologies, which remains largely unexplained.

To address our hypothesis, we decided to focus on $CD4^+$ T cells as IFN targets, and to use T helper polarizing cytokine environments as representative of diverse inflammatory contexts. We considered four prototypical polarizing contexts (Th0, Th1, Th2 and Th17) (Zhou et al. 2009) and addressed the IFN response in these four contexts. By combining in a systematic manner large scale and specific functional analyses, we could identify a core of conserved context-independent ISG, but also demonstrate the emergence of important IFN functions driven by specific cytokine environments.

Results

IFN-α induces a specific transcriptional signature during T helper differentiation.

To address the flexibility of IFN function in diverse inflammatory environments, we used $CD4^+$ T helper cell differentiation as a model. Naive $CD4^+$ T cells were cultured for 5 days in the presence of polyclonal stimulation (anti-CD3/CD28) in four distinct polarizing cytokine contexts, defined as Th0, Th1, Th2, and Th17, as previously described (Volpe et al. 2008), in the presence and absence of IFN-α. We selected IFN-α and not IFN-β because of its broad and better described implication in autoimmune disorders and its use in therapeutic.

To control the specificity of our system, we systematically measured the production of the prototypical cytokines IFN-γ, IL-4 and IL-17 by Flow Cytometry and ELISA and the expression of the master Th-specific transcription factors T-bet, GATA-3, RORc and Foxp3 in each context (**supplementary fig. 1)**. As expected, Th1 cells expressed preferentially IFN-γ and T-bet, Th2 expressed IL-4 and GATA-3, Th17 expressed IL-17 and RORc (Suppl. Fig 1). We also checked that IFN was not inhibiting T cell proliferation at day 3 using CFSE (**supplementary fig. 2A).** However, the expansion rate at day 5 was reduced by 30% to 40% in all conditions by IFN (**supplementary fig.2B**).

Transcriptional profiles were generated using Hugenes ST1.1 Affymetrix chips. Cells were harvested and lysed after 5 days of culture in distinct polarizing conditions, and after 4 hours of subsequent re-stimulation with anti-CD3/CD28 without additional cytokines (**Fig 1A, 1B, 1C**). This two time points mimic two physiological steps in the Th response : 1) the transcriptional program occuring during the Th differentiation (Day 5), 2) the transcriptional program induced after the recognition of the cognate antigen by the Th effector (Day 5+ 4h

restim.). First, we focused on the neutral IFN signature based on differential gene expression in order to characterize the effects of IFN in the unpolarized Th0 condition **(Fig. 1A)**. We identified an IFN signature comprising 76 and 71 genes, at Day 5 and Day 5 +4h restimulation, respectively, which were differentially regulated by IFN (**Fig. 1B and supplementary table 1)**. Most of the genes were upregulated (55 out of 76, and 53 out of 71, respectively). We found that the top-induced genes were conserved at both time points, including well-known ISGs such as MX1, IFI44, IFI44L, XAF-1, IFI27 that mediate the antiviral state in IFN-treated cells (**Fig. 1C and Fig. 1D).** In the IFN signature at 4 hours, we found genes related to chemotactism, such CXCL-10 (up-regulated) and the chemokine receptor CCR4 (down-regulated) (**Fig. 1D)**. To further address the functionnal modules within the IFN-α signature, we performed enrichment analysis (**Fig. 1E**). Three GO classes were found differentially regulated in a statistically significant manner at both time points: response to virus, vesicular fraction, and microsome. Signal transduction and regulation of IkB kinase were also statistically significant at Day 5.

These results suggest that IFN confers a specific signature to $CD4^+$ T cells composed of anti-viral ISGs that persists even after polyclonal restimulation.

The IFN-α signature is distinct from Th1, Th2 and Th17 signatures

Having defined the IFN signature in a neutral Th0 condition, we asked whether this was distinct from the Th1, Th2, and Th17 signatures. The Th-specific signatures was defined by differentially gene expression beetwen Th0 vs Th1, Th2 and Th17, respectively (**2A)**. We used two complementary methods. First, we looked at the global impact of IFN-α on the genes involved in Th cell differentiation. We identified 746 differentially expressed genes between the five conditions Th0, Th1, Th2, Th17, Th0+IFN-α according to stringent criteria (see materials and methods). We sought to determine if the effect of IFN-α on Th0 was more related to any of the four classical profiles generated (Th0, Th1, Th2, Th17). We used clustering analysis to evaluate similarities among all the profiles for all donors (**Fig. 2B).** Th2 and Th17 profiles were distinct from the Th0, Th1 and IFN-α. Cluster analysis revealed that Th0 and Th1 profiles were more similar to each other than to IFN-α, suggesting that IFN-α induces more variation than IL-12 (used for Th1 differentiation) at the transcriptional level.

In a second step, we compared the IFN-α signature and the specific Th signatures**.** The Th signatures were defined as the differentially expressed genes between the Th0 and the specific Th condition Th1, Th2, Th17 **(Fig. 2C).** These signatures contained known Th-specific genes, such as IFN-γ in Th1, GATA-3 in Th2, and IL-17F in Th17 (**Fig. 2D)**, validating the relevance of each signature**.** We found that the IFN-α signature at day 5 contained a specific set of genes with little overlap with Th1 (12 out of 72, 16.7 %), Th2 (9 out of 133, 6.7%) and Th17 (12 out of 236, 5%) signatures, respectively (**Fig. 2C**). For example, we found, that IL12Rbeta2, SPATSL2 and the anti-apoptotic gene CHMP5 were common to the Th1, Th2 and Th17 signature respectively. Only one gene, the integrin PECAM-1, was decreased by IFN-α as well as in Th1 and Th17 conditions at a comparable level (**Fig. 2E).** Similar results were obtained with the IFN-α and Th signatures at Day 5+ 4h restim (data not shown). Thus, our data show that IFN-α induces a unique signature on Th cells distinct from a Th1, Th2, and Th17 signatures. It indicates that IFN-α is modulating Th cell differentiation in a specific manner, and does not simply duplicate known effects of standard Th polarizing cytokines.

Flexibility of the IFN-α signature in diverse T helper polarizing cytokine environments

We showed that IFN-α induces a specific signature in Th cells. However, Th differentiation is conditioned by a complex cytokine context, corresponding to diverse pathophysiological environments. We thus asked to what extent the function of IFN may be modulated by the cytokine milieu. First, we compared the standard IFN-α signature (in Th0) with each of the IFN-α signatures obtained in a Th1, Th2, and Th17 cytokine environments (**Fig. 3A**). Surprisingly, we found that the majority of IFN-modulated genes was dependent on the cytokine context. We identified 65, 67 and 65 genes for the IFN-α signatures in Th1, Th2 and Th17 contexts, respectively (**Fig 3A**). We observed that the neutral IFN-α signature had less than 30% genes in common with the IFN-α signatures in the 3 distinct polarizing contexts (**Fig 3A**). To have a global view of the IFN-α response in distinct Th contexts, we quantified the overlap between each of the IFN-α signatures in Th1, Th2 and Th17 (**Fig. 3B**). Only 12 genes were common between the 3 IFN-α signatures, representing a conserved core of response to IFN-α. Most of these genes (9 out of 12) are known to have an anti-viral action. We confirmed by RT-PCR a stable up-regulation induced by IFN-α of four of these

genes (MX1, OAS1, IFI6 and RSAD2 aka Viperin) (**Fig. 3D**). However, the level of induction of these genes was flexible with a lower modulation in Th17 context.

Strikingly, the majority of the genes of the IFN-α signatures were specific to a given Th context, suggesting the emergence of novel IFN functions driven by diverse Th cytokine environments. To get a deeper insight into the functions underlying each of the IFN-α signatures in Th0, Th1, Th2, and Th17, enrichment analysis was performed. The results confirmed the context-dependent effect of IFN-α during Th cell differentiation at the functional level (**Fig. 3D**). Among the 15 IFN-induced functional pathways, the most conserved across Th polarizing environments was "response to virus", found in three Th contexts (**Fig 3C**), followed by purine metabolism and RIG-I receptor signalling pathway found in Th0 and Th2 contexts. However, most of the enriched pathways (11 out of 15) were induced by IFN only in a single Th context, confirming an emergence of novel properties of IFN. Among these, IFN-α induced specifically functional pathways related to nucleotide metabolic process and HIV1 elongation transcription process in a Th17 context, suggesting a different response of IFN-stimulated Th17 cells to HIV1 infection. Similarly, IFN-α modulated specifically a biological function related to lipid metabolism in a Th1 context. Similar results were obtained with the analysis of the IFN-α signature at Day 5+ 4h restimulation **(Supplementary Fig. 3**).

Overall, our results emphazised at the individual gene and global pathway levels, the flexibility of the IFN response according to diverse inflammatory environments.

A chemokine/chemokine receptor module defining emergent context-specific IFN-α functions

Most of the emergent functions of IFN were related to RNA or DNA metabolism that control transcription and replication. However we also found genes related to immune functions that characterize Th cells. In order to get a deeper insight into emergent IFN functions in Th cells, we used a second but complementary method. We focused on four main « modules » related to biological functions that characterize Th cells: cytokine, transcription factor, chemokine and receptor. We identified among all IFN signatures, the ISGs that were involved in these four modules (supplementary **Table 2**). IFN-α down regulated GATA-3 expression in a neutral context in the microarray data but this was not confirmed by RT-PCR in all context (supplementary **fig. 4**)

Analysis of the IFN-α signature in Th1, Th2 and Th17 revealed that IFN-α regulated specific components of the chemokine and chemokine receptors module (CXCL10 in Th1, CCL20 in Th17, CCR4 in Th0) in Th cells in a context-dependent manner. To validate the microarray data at the protein level in a systematic manner, we investigated the level of the known chemokine ligand/receptors CXCL10/CXCR3, CCL22/CCR4 and CCL20/CCR6 for Th17 cells (Acosta-Rodriguez et al. 2007). These chemokine receptors can also discriminate the Th subsets as Th1 are CXCR3+ cells, Th2 are CCR4+ cells and Th17 expressed both CCR4 and CCR6 (Acosta-Rodriguez et al. 2007). The results obtained were matching microarray data. The induction of CXCL10 by IFN-α was confirmed to be specific for Th0 and Th1 cells. Th1 cells showed the highest increase with mean values ranging from 514 +/- 198 pg/ml to 1532 +/- 856 pg/ml **(fig. 4A)** without any increase of the homologous receptor CXCR3. The down-regulation of CCR4 expression by IFN-α was confirmed in Th0 and Th1 conditions with statistical significance ($p<0,05$) **(fig. 4B).** CCR4 that is specific for Th2 and Th17 memory cells (Acosta-Rodriguez et al. 2007) was not modulated by IFN-α. We also measured CCL22, which binds CCR4, without any modulation by IFN-α **(fig. 4A).** Finally, CCL20 secretion was specifically increased by IFN-α in a Th17 context, with levels ranging from 1071+/-502 pg/ml to 3241+/-1005 pg/ml **(Fig. 4A).** CCR6 was detected only in the Th17 cells without any modulation by IFN-α maybe due to an already optimal surface expression. Thus, the results obtained here confirm that IFN induced a specific chemokine secretion pattern that was dictated by the Th cytokine environment.

Environmental control of IFN-induced Th cell polarization

Analysis of the chemokine module confirmed the flexible response to IFN-α of Th cells in distinct cytokine environments. We aimed to assess whether a similar effect was observed in the cytokine module that represents the most characterized functional outcome of Th cells. IFN-α has been shown to modulate the production of the prototypical cytokines of Th cells (Huber et al.). Only IFN-γ was upregulated in the IFN-α signature in a Th1 and Th17 environment (supplementary **Table 2**). This can be explained taking in consideration the difference in kinetics of transcription, post-transcriptional modification and/or secretion of each cytokines.

To better clarify the role of IFN-α on Th cytokine secretion, we measured the protein levels of 14 different Th-derived cytokines in the supernatant 24 hours after restimulation. We first looked at the prototypical cytokines IFN-γ, IL-4, IL-17 and IL-10 by ELISA and cytometric bead array (**Fig. 5A**). All of these cytokines were modulated by IFN-α in a context-dependent manner. IFN-γ was significantly increased in Th1 and Th17 conditions but not in a Th0 or Th2 context, matching the mRNA data. IL-4 secretion was inhibited by IFN only in Th0 and Th2 environments. This decrease was not associated to an inhibition of GATA-3 whose expression remained stable after 5 days of differentiation (supplementary **Fig. 4).** Moreover, Th2 cells increased specifically IL-10 secretion, without modification in FoxP3 nor c-MAF expression at the mRNA level. We found surprisingly that IFN-α also increased RORc expression and IL-17 production in a Th17 context (**Fig. 5A and** supplementary **Fig. 5**).

The effect of IFN-α on Th polarization has often been reduced to the modulation of their prototypical cytokines IL-4, IL-17 and IFN-γ. However, Th cells are able to produce a broader array of cytokines that participate to the global Th responses (Volpe et al. 2008; Volpe et al. 2009). To which extent IFN-α is able to modulate the global cytokine profile of each Th cells has remained elusive. To address this issue, we used the Principal Component Analysis (PCA) as a computational approach to analyze the full dataset of fourteen cytokines in eight culture conditions (**Fig. 5B**). Each point of the PCA plane represents the reduction in two dimensions of the fourteen-dimensional cytokine profiles for six independent donors. Principal component 1 and 2 represent respectively 41% and 19 % of all the variance in the dataset. IFN determined a shift of the cytokine profiles (as defined by the direction and the length of the vectors) that differs in Th0, Th1, Th2 or Th17. PCA confirmed the context-dependent effect of IFN-α on the cytokine profile in each Th cytokine environment. Only the modulation of the Th0 and Th2 profiles by IFN-α was statistically significant at the global according to a Multivariate Anova Test (supplementary **Fig. 5**). However, this could not exclude significant changes at the individual cytokine level. Next, we aimed to know whether the change in the cytokine profile was due to modulation of one specific cytokine or a combination of a group of them. By looking at individual cytokines, we found that IFN-α modulated a specific set of cytokines in each polarizing context **(Fig. 5D).** In a Th1 environment, IFN increased IFN-γ and to a lesser extent IL-10. In a Th2 environment, IFN inhibited IL-4, IL-9 and IL-13, while increasing IL-10, IL-6, IL-3 and

TGF-β. Finally, Th17 modulation was explained by an increase in IL-17, IL-21, IFN-γ, and an inhibition of IL-22.

Overall, the context-dependent effects of IFN-α that we observed at the global transcriptional signature were confirmed at the protein level within the chemokine and cytokine modules. These results also validated the concept that the immune modulating functions of IFN are highly flexible, including the mostly conserved antiviral functions, as evidenced by the analysis of multiple IFN signatures.

Flexibility of the antiviral state induced by IFN-α leads to a susceptibility to viral infection in a Th subset-dependent manner.

Deeper analysis of the core response suggests also that the polarizing context influences the antiviral state induced by IFN (**Fig. 3C**). Validation of antiviral ISGs at the RNA level showed quantitative differences among each subset (**Fig. 3D**). This was confirmed also by the analysis of expression of other antiviral ISGs from the microarray data (data not shown). Antiviral state was mostly affected in Th2 and Th17 environments. In the latter, we observed a 2 to 4 fold less induction of RSDA2 and MX1 by IFN. This lower Mx1 induction was further confirmed at the protein level (**Fig 6A and Fig. 6B**). Thus, we wondered whether these changes of the antiviral state have a functional impact on Th cells during viral infection. We differentiated distinct Th subsets in the presence and absence of IFN, and infected them with 2 different lymphotropic GFP-reporter viruses derived from HIV-1 and HIV-2 for 48H (supplementary **fig.6).** HIV receptors (CD4, CXCR4, CCR5) are themselves regulated by the T-Helper differentiation program, this may be a confounding factor (Baumann et al. 2004). Thus, we used VSV-G-pseudotyped viruses that are not dependent on the expression of these receptors.

We observed that Th0, Th1 and Th2 cells polarized in the presence of IFN were less infected by HIV-2, as compared to the same cells polarized in the presence of IFN. This IFN-induced protection was dose-dependent (16% vs 32%; 14,8% vs 28,2%; and 21,1% vs 37,2% respectively for a multiplicity of infection of 1000) (**Fig. 6C**). However, IFN had less impact on the protection of Th17 cells, with a non-significant decrease in infection from 27 to 22%. When Th cells were challenged with our mutant HIV-1, only Th0 and Th1 cells were significantly protected when prior cultured with IFN (17,4% vs 25,6% and 27,2% vs 41,7% respectively) (**Fig. 6D**).

Collectively, these data show that the cytokine environment also modulated the antiviral response induced by IFN in a context-specific manner. This provides a functional level of validation of the environmental plasticity of IFN effects during Th cell polarization.

DISCUSSION

Previous study have demonstrated that some IFN response (e.g apoptosis, anti-proliferative effect) were cell-specific. This suggested that the effects of IFN may be regulated by the nature of the cellular target, implying intrinsic mechanisms selected by the ontogeny of these different cell types. In our study, we demonstrate that extrinsic factors may be determinant in modulating IFN signatures in a given cell type. We analyzed IFN responses at the large-scale transcriptome level. The results revealed a previously unsuspected flexibility in IFN-induced transcriptional programs depending on Th polarizing cytokine context, with functional impact.

Anti-viral functions of IFN are the most conserved across evolution, and correspond to an essential role in innate immunity, including in lower vertebrates, such as fish *(Krause and Pestka 2005)*. This enables to protect the host cells from viruses in a cell-intrinsic manner, independently of adjuvant effects on immune cells (Garcia-Sastre and Biron 2006). In our study, it is notable that the most conserved genes in IFN signatures across different Th contexts is predominantly composed of anti-viral ISGs, indicating that this response is also conserved in the mammalian immune system where several levels of fine regulation are implicated. Anti-viral ISGs are also conserved in transcriptional signatures observed in patients treated with IFN for a variety of diseases *(Berry et al.; Baechler et al. 2003; Bennett et al. 2003; Weinstock-Guttman et al. 2003; Baechler et al. 2007; Asselah et al. 2009)*. For example, the following antiviral ISGs (MX1, MX2, PKKR, OAS1, OAS2, RSAD2, IFI6, IFI44 or ISG15) are induced by IFN in patients with chronic Hepatitis C (HCV) (Asselah et al. 2009), MS *(Serrano-Fernandez et al.; Weinstock-Guttman et al. 2003; Malucchi et al. 2008)* . They can be also found as a major part of IFN signature in inflammatory or infectious disease (Berry et al.; Baechler et al. 2003; Bennett et al. 2003; Banchereau and Pascual 2006). However, our study identified a surprisingly large number of genes, which are induced and/or modulated by IFN only in specific Th contexts, and may enable a more appropriate response to specific physiopathological conditions. We can hypothesize that

these emergent functions have appeared later in evolution, together with the increase of complexity in the adaptive branch of the immune system.

Among emergent Th-specific functions, we identified a chemokine module, which was confirmed at the protein level (**Fig. 4**). The most striking result was observed for CCL20, which was greatly enhanced by IFN in a Th17 context, but not in the others Th environments. To our knowledge, this is the first report of secretion of CCL20 in human Th17 cells. CCL20 is known to attract CCR6+ cells (Schutyser et al. 2003), including Th17 cells (Esplugues et al.). Thus, CCL20 and IL-17 upregulation by IFN in a Th17 context may serve as a mechanism to amplify pre-existing Th17 responses. Modulation of CCL20 may also be important since recent reports have emphasized its role in the migration of Th17 cells in the small intestine where their generation and elimination are controlled (Esplugues et al.). Other cells types are known to express CCR6, such as Mucosal Associated Invariant T (MAIT) cells. Recent studies have suggested that MAIT cells may reduce pathogenic Th1 responses in MS (Miyazaki et al.). This could explain a another regulatory function of IFN in this context.

Analysis of the cytokine module also shed new light into the effect of IFN on T helper differentiation cells that remains controversial. IFN is still described as a "Th1 dominant" cytokine even if IFN is not sufficient to induce a stable Th1 phenotype (Rogge et al. 1998). Further studies emphasized this property as IFN inhibited both human and mice Th2-driven polarisation (Huber et al.) or Th17 driven polarizaton (Moschen et al. 2008; Prinz et al. 2008). However, recent studies raise the question of a "unique" effect of IFN on Th cells. IFN was able to induce human Th17 (Axtell et al.), increase human Th9 differentiation (Wong et al.), and induce in the presence of IL-10 a regulatory phenotype during CD4 differentiation in a mouse model (Dikopoulos et al. 2005). Our results shed new light on the role of IFN during Th cell differentiation, and provide definite evidence that the cytokine context is able to condition IFN responses. Part of the ongoing controversy may be due to the comparison of type IFN effects in different contexts. In our study, we performed a systematic analysis in all major Th cytokine differentiation conditions (Th1, Th2, Th17), and analyzed a diversity of effector Th output cytokines, instead of restricting our study to a given Th subset. In this manner, we could dissect the variability in IFN responses, and associate it to specific sets of both input and output Th cytokines. In our system, IFN potentialize both pro-inflammatory Th1 and Th17, with a specific increase of their master transcription factor T-bet and RORc. This finding contrasts with other reports (Moschen et

al. 2008) showing the inhibitory effect of IFN-α. The difference of experimental setting (medium, combination of cytokine to generate Th17) may explain this discrepancy.

We also observed that the polarizing context influences the antiviral response. The expression of antiviral ISGs microarray or by RT-PCR was similar in Th0 and Th1 context but decreased in Th2 and mainly Th17 context. This flexibility in the antiviral response leads to reduced proctection of Th cells during viral infection. We used HIV-1 infection as it infects CD4+ T cells. IFN has clear antiviral effects against HIV-1 replication in vitro and in vivo (Azzoni et al.; Goujon and Malim). However, immune control of lentiviral infection in macaque models and humans is associated with resorbption of IFN gene expression signature, while chronic activation is associated with a sustained IFN response signature (Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque). Such discrepancy suggest that the functions of IFN are likely dependent on the context. We found the antiviral state induced I Th17 were less protective to HIV-1 and HIV-2 infection as compared to Th0 or Th1 context. Previous reports have shown that Th17 cells were reduced either in blood or in the lamina propria of HIV-1-infected patients (Gosselin et al.; Brenchley et al. 2008). Thus, The "lower" antiviral state induced by IFN during HIV infection may contribute to this phenomenon. These results contrast also with our findings that IFN were able potentialized both Th1 and Th17 proinflammatory response according to the cytokine and chemokine module. A combination of ISGs is required to obtain a strong and complete control of virus replication *(Schoggins et al.).* In our system, the quantitative difference of expression in many ISGs may explain at least the flexibility of the antiviral response in different Th context. Overall our data strongly support that antiviral state is context-dependent and virus-specific.

Our data raise a new level of complexity in IFN responses but may also explain such a diversity of IFN response in therapeutics or in inflammatory disease as revealed by microarray analysis. The inflammatory environment (extrinsic factors) and the cellular type of cells involved in the physiopathological will drive a specific IFN response with a core response and a « specific disease response ».

In summary, our study provides definite evidence that the effects of IFN on a given target cell may be of two types: 1) a conserved response predominantly made of anti-viral ISGs, 2) a flexible response determined by extrinsic environmental factors, causing the emergence of

novel functionalities. Therefore, we may anticipate that the effects of IFN in different types of inflammatory diseases might follow the same rule. It will be important to define and characterize these two types of response in order to explain the variable effects of IFN observed in patients with diverse inflammatory diseases. In pathophysiological setting of infection, we could also hypothesize that endogenous IFN will have different functional consequences in a bacterial versus a viral inflammatory environment. Our results and analysis framework can serve as a basis to address these questions, and to further progress in our understanding of the complexity of type IFN-induced regulation of cellular functions.

MATERIALS AND METHODS

Purification of naive CD4+ T lymphocytes from adult blood. Peripheral blood mononuclear cells were separated by Ficoll-Hypaque centrifugation (Amersham Biosciences) from buffy coats obtained from healthy donors (Saint Antoine-Crozatier Blood bank, Paris). $CD4^+$ T lymphocytes were then purified by immunomagnetic depletion with the human $CD4^+$T cell Isolation Kit II (Miltenyi Biotec), followed by staining with allophyco-cyanin-anti CD4 (VIT4 ; Miltenyi Biotec), phycoerythrin-anti-CD45RA (BD), fluorescein-isothiocyanate-anti-CD45RO (BD Bioscience) and phycoerythrin 7-anti-CD25 (BD bioscience). Naive $CD4^+$ T cells sorting of $CD4^+CD45RA^+CD45RO^-CD25^-$ had a purity of over 99% with a FACSAria (BD Bioscience).

T helper cell differentiation assay. Naive $CD4^+$ T cells were cultured in 48-well plates (Falcon) at a density of 8 x 10^4 cells per well in X-VIVO 15 serum free medium (Lonza) in the presence of Dynabeads CD3/CD28 T cell expander (at the ratio of one bead per cell; Invitrogen) and polarized into Th0, Th1, Th2, Th17 with the following cytokines: none for Th0 (non polarized condition); IL-12 (10 ng/ml; R&D Systems) for Th1; IL-4 (25 ng/ml; R&D Systems) for Th2; IL-1β (10 ng/ml; Peprotech), IL-6 (20 ng/ml; Peprotech), IL-23 (100 ng/ml; R&D Systems) and TGF-β (1 ng/ml; Peprotech) for Th17. IFN-α (Miltenyi) was added at 10 ng/ml. After 5-6 days, cells were collected and washed extensively and their viability was determined by triptan blue exclusion. Cells were re-stimulated at a density of

1 x 10^6 cells/ml for 5 hours (for Flow cytometry intracellular staining) or for 24 h (For ELISA, Cytometry Beads Array CBA and RT-PCR)

T cells proliferation assay : Sort-purified Naive $CD4^+$ T cells were carboxyfluorescein succinimidylester (CFSE)-labeled (Invitrogen) at a concentration of 1μM. Cells were then cultured in 48-well plates (Falcon) at a density of 8 x 10^4 cells per well in X-VIVO 15 serum free medium (Lonza) in the presence of Dynabeads CD3/CD28 T cell expander (at the ratio of one bead per cell; Invitrogen) and polarized into Th0, Th1, Th2, Th17 with or without IFN-α. At Day three, cells were harvested, washed twice and stained with allophyco-cyanin-anti CD4 (VIT4 ; Miltenyi Biotec), for 30 minutes at 4°C before analyzed by flow cytometry (LSRII, Becton Dickinson).

Analysis of cytokine and chemokine production. The following cytokines and chemokines were measured in culture supernatants by ELISA : IL-17 (R&D System), IL-22 (R&D System), IL-21(eBioscience), CCL20 (R&D System). IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IFN-γ, TNF-α, LT-α, TFG-β and CXCL10 were measured by CBA (BD Bioscience) according to the manufacturer's instructions.

Intracellular and surface staining: For intracellular cytokine staining, cells were incubated for 5 h with PMA (100 ng/ml; Sigma), and ionomycin (500ng/ml; Sigma). Brefeldin (10 μg/ml eBioscience) was added during the final 3 h 30 of re-stimulation. After 5 hours of stimulation, cells were harvested, washed and stained with the live/dead kit (Invitrogen) for 30min at 4°C. Cells were then fixed with the IC fixation buffer (eBioscience) and permeabilized for 45' at 21°C with the permeabilization buffer (eBioscience). Cells were then stained for 30' at 21°C with the corresponding fluorescence-labeled antibodies: fluorescein-isothiocyanate-conjugated anti-IL-17 (BL168, Biolegend), phycoerythrin-indotricarbocyanine-conjugated anti-IFN-γ (4S.B3, eBioscience), allophyco-cyanin-conjugted anti-IL-4 (8D4-8; eBioscience) and phyco-erythrin-conjugated anti-IL-10 (JES3-9D7, eBioscience)**.** Cells producing IFN-γ, IL-17, IL-4 were analyzed by flow cytometry (LSRII, Becton Dickinson). For the analysis of surface markers, cells were stained by incubation for 15' on ice with the corresponding fluorescence-labeled antibodies: phyco-erythrin anti-CCR6 (11A9, BD Bioscience), AlexaFluor647 anti-CCR4 (TG6/CCR4, Biolegend) and AlexaFluor647 anti-CXCR3 (G025H7, Biolegend). For the detection of Mx1 protein, cells were incubated as for cytokine staining. Cells were then stained for 30' at

21°C with a primary Rabbit antiboby against Mx1 (Abcam ab95926) , wahsed and stained with a secondary antibody (Donkey anti rabbit cy5, Jackson Immunoresearch).

Virus production and infection of CD4+T cells : Three different plasmid were used **:**HIV-GFP (NL4-3 Δ*vif*Δ*vpr*Δ*vpu*Δ*env*Δ*nef* with the GFP open reading frame in place of *nef*), HIV-2 ROD9 Δenv GFP (ROD9 Δ*env*Δ*nef* with the GFP open reading frame in place of *nef*), and CMV-VSVG have been described previously (Manel et al.) **.** Viral particles were produced by transfection of 293FT cells with 3µg DNA and 8µl TransIT-293 (Mirus Bio); for HIV1-GFP, 0.4 µg CMV-VSVG and 2.6 µg HIV-GFP; for HIV2-GFP, 0.4 µg CMV-VSVG and 2.6 µg HIV-2 ROD9 Δenv GFP. One day after transfection, media was removed, cells were wash out once and fresh media was added. Viral supernatants were harvested one day later and debris was removed by using 0.45µm-syringe filter. Virus titers were measured on GHOST X4R5 cells titration as previously described (Manel et al.) .

At day 5 of Naïve T cell differentiation, cells were harvested, counted and resuspended in fresh media at the concentration of – million per ml with 8 µg/ml protamine and 100 µl was aliquoted in round-bottomed 96-well plates. For infection, 100µl of media or dilution of virus supernatants. Forty-eight hours after infection, cells were fixed using 1%paraformaldehyde and GFP-positive cells were evaluated by FACSVerse (BD).

Real-time quantitative RT-PCR. Total RNA was extracted with an RNeasy Micro kit (Qiagen). A mixture containing random hexamers, oligo$(dT)_{15}$ (Promega) and SuperScript II Reverse Transcriptase (Invitrogen) was used for cDNA synthesis. Transcripts were quantified by real-time quantitative PCR on a Lightcycler400 sequence detector (Roche) with Applied Biosystems predesigned TaqMan Gene Expression Assays and Absolute QPCR master mix (Roche). The following probes were used (Applied Biosystems assay identification numbers in parentheses): FoxP3 (Hs00203958_m1), GATA-3 (Hs00231122_m1), T-bet (Hs00203436_m1), RORc (Hs01076112_m1), AHR (Hs00169233_m1) and c-Maf (Hs00193519_m1)**.** For each sample, mRNA abundance was normalized to the amount of ribosomal protein L34 (Hs00241560_m1).

Affymetrix microarray hybridization. Naive $CD4^+$ T cells were differentiated with anti-CD3 plus anti-CD28 in Th0, Th1, Th2, and Th17 for 5 days and re-stimulated with anti-CD3 plus anti-CD28. Microarray analyses were performed at two time points: either before re-stimulation (Day 5) or four hours after re-stimulation (Day 5+ 4H restim.). For each condition, 500pg of RNA were used to synthesize targets using the WT-Ovation™ Pico

RNA amplification system (Nugen, Bemmel,The Netherlands). Labelled DNA was hybridized on the Affymetrix human Gene ST1.1, an oligonucleotide 28,000-gene microarray processed on an Affymetrix GeneTitan device. The data derive from three independent donors.

Statistical analysis and data mining. 1/ A nonparametric two-tailed Wilcoxon test or a T-student test was used for pair-wise comparisons of cytokines. 2/ Multivariate Anova (MANOVA) test was used for comparison of the Th cytokine profile implemented in Matlab software. A data matrix was created containing 48 rows (8 Th profiles with 6 donors) and 14 columns (cytokine concentrations). The matrix was used as a input to the function manova1. Intergroup distances were computed by using the output gmdist. Clustering wath done with the function manovacluster. *P* values of 0,05 or less were considered statistically significant. 3/ *Analysis of microarray*: the data derived from three independent experiments were normalized using the RMA algorithm and bioinformatics analysis was performed using GeneSpring GX 7.3 (Agilent, Palo Alto, CA) or EMA (Bioinformatique, Institut Curie) on R software. Probes with a signal < 20 were excluded. For IFN-α signature, differential gene expression was defined according to the two following criteria: Fold Change > 2; p-value < 0.05 (T-test). Data for the clustering and principal component analysis were processed and analyzed as previously described (Volpe et al. 2008) . To analyze the genes differentially expressed among Th0, Th0+IFN-α, Th1, Th2 and Th17, we selected the genes according to the following criteria: Anova Test with a Tukey HSD test p<0,05 and a false discovery rate (Benjamini Hochberg) p< 0,05. The Euclidean correlation distance and the Ward's criteria as an agglomerative method were used for hierarchical clustering analysis. 4/ GO analysis were performed using the Molecular Signatures Database (MSigDB).

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Touzot *et al.,* 2012) and are accessible through GEO Series accession number GSE

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Authors Contributions

M.T performed experiments, drafted and co-wrote the manuscript; M.T, N.S, A.C did computational and statistical analysis. C.M, M.G, T.S performed part of the experiments. V.S designed and supervised the study andwrote the manuscript.

Figure legends

Figure 1 IFN-α induces a conserved signature during the differentiation of naïve CD4 T cell that is still conserved after restimulation of cells.

(A) Sorted naives CD4 T cells were cultured for five days with anti CD28/CD3 beads in the presence or not of IFN-α. Half of the cells were collected after five days for RNA extraction. The other half were washed and re-stimulated with anti CD28/CD3 beads for 4 hours. Microarrays chips were hybridized both at day five (Day 5) and after 4 hours of restimulation (= Day 5+ 4h restim.). **(B)** Hierarchical clustering of the 76 and the 72 genes that represent the *IFN-α signature* at Day 5 and at Day 5+ restim. respectively. *IFN-α signature* was defined as the IFN modulated genes in Th0 according to two criteria: Fold change (FC) > 2 and p values< 0.05 (**see Methods**). **(C)** Relative gene expression (log value) of the fifteen top induced of *IFN-α signature* at Day 5. Data represent the mean (+/- SD) of 3 independent donors. **(D)** Relative gene expression (log value) of the fifteen top induced of *IFN-α signature* at Day 5+ 4h restim. Data represent the mean (+/- SD) of 3 independent donors. **(E)** Enrichment analaysis of the *IFN-α signature* at Day 5 according to the Moleculare Signature database.

Figure 2 The IFN-α signature is distinct from Th1, Th2 and Th17 signatures

(A) Sorted naives CD4 T cells were cultured for five days in 4 different polarizing contexts (Th0, Th1, Th2 and Th17) with anti CD28/CD3 beads or in neutral condition (Th0) in the presence or not of IFN-α. Half of the cells were collected after five days for RNA extraction. The other half were washed and re-stimulated with anti CD28/CD3 beads for 4 hours. Micro arrays chips were performed both at day five (Day 5) and after 4 hours of restimulation (= Day 5+ 4h restim.). **(B)** Clustering of the 746 differentially regulated genes (see materials and methods) among the five conditions (Th0, Th0+IFN-α, Th1, Th2 and Th17) by a euclidean correlation distance. Culture conditions are separated into clusters by comparison of their linkage distance. Each point represents one individual donor. **(C)** Venn diagram representing the overlap between the *IFN-α signature* and the specific Th signature (Th1, Th2 or Th17). E.g : Th1 signature was defined by the genes differentially regulated between Th1 and Th0 at Day 5 according to the following criteria: Fold change (FC) > 2 and p values< 0.05 (**see Methods**). **(D)** Example of specific genes of Th signature. **(E)** Example

of common genes of Th signature and *IFN-α signature*. For **(D)** and **(E)** Values of the three individual donors are expressed in arbitrary units.

Figure 3 IFN-α signature is modulated by the polarizing context leading to conserved and emergent biological functions.

(A) Sorted naive CD4 T cells were cultured for five days in 4 different polarizing contexts (Th0, Th1, Th2 and Th17) with anti CD28/CD3 beads in the presence or not of IFN-α. Half of the cells was collected after five days for RNA extraction. The other half was washed and re-stimulated with anti CD28/CD3 beads for 4 hours. Microarrays chips were hybridyzed both at day five (Day 5) and after 4 hours of restimulation (= Day 5+ 4h restim.). **(B)** Venn diagram representing the overlap between the *IFN-α signature* in Th0 and the IFN-α signature in the 3 different polarizing contexts (Th1, Th2 or Th17). E.g : *IFN-α signature* in Th1 was defined by the genes differentially regulated between the Two conditions Th1+ IFN-α and Th1 at Day 5 according to the following criteria: Fold change (FC) > 2 and p values< 0.05. **(C)** Venn diagram representing the overlap between the *IFN-α signature* in the 3 different polarizing contexts (Th1, Th2 or Th17). **(D)** Validation of some antiviral genes that belong to the core response of IFN-α (MX1, OAS1, RSAD2 and IFI6) by RT-PCR. Data represent mean +/- SD of five independent experiments $p < 0.05$; **, $p < 0.01$; ns: non significant (paired T-test).**(E)** Enrichment analysis for the *IFN-α signature* in Th0, Th1, Th2 and Th17**.**

Figure 4 IFN-α modulates specifically the chemokine/chemokine receptor of each T Helper subsets.

ELISA, cytometric bead assay and Flow cytometry analysis of **(A)** CXCR3, CCR4 and CCR6 **(B)** IP-10, CCL22 and CCL20. CD4 T cells were polarized in Th0, Th1, Th2 and Th17 cells with anti CD28/CD3 beads in the presence or absence of IFN-α for five days. Cells were re-stimulated with anti CD28/CD3 beads. Flow cytometry was performed after 12 hours. Supernatants were collected after 24hours for chemokine analysis. ND, non detectable; *, $p < 0.05$; **, $p < 0.01$ (Wilcoxon test (N<6) or T-student test (N<6)). Data represent five to six independent experiments

Figure 5 IFN-α has a context-dependent effect on the cytokine profile of each Th subset, by modulating set of cytokines.

Naïve T cells were differentiated with anti-CD3/CD28 in Th0, Th1, Th2, and Th17 +/- IFN-α for 5 days and re-stimulated for 24h with anti-CD3 plus anti-CD28 to collect supernatant. **(A)** ELISA and/or cytometric bead assay of IL-4, IL-17, IL-10 and IFN-γ in supernatants. *, $p < 0.05$; ** , $p < 0.01$ (Wilcoxon test). Data are the mean of eight independent experiments. **(B)** PCA of the cytokine profile (IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL17, IL-21, IL-22, IFN-γ, LT-α, TNF-α and TGF-β) in 8 experimental conditions: Th0, Th1, Th2, Th17 +/- IFN-α. Points represent the mean of all values for 6 donors. Ellipses represent the standard deviation for each condition along the principal components analysis. The red line represents the "shift" of the Th profile. A MANOVA test was applied to discriminate statistical difference between the global cytokine profiles *, $p < 0.05$; **, $p < 0.01$). **(C)** Heatmap represents the modulation of individual cytokine secretion by IFN-α in the Th0, Th1, Th2 and Th17 contexts. Red represents increased and black decreased values. Fourteen cytokines were measured by ELISA and/or CBA (**methods**). For each cytokine, the mean value obtained from 6 individuals donors was normalized (center and reduced) in all conditions (Th0, Th1, Th2, Th17 +/- IFN-α). Modulation was then defined by difference (Δ) of the normalized data according to the presence or not of IFN-α.

Figure 6 Antiviral state induced by IFN-α confers a specific susceptibility to viral infection in a Th subset-dependent manner.

Naïve T cells were differentiated with anti-CD3 plus anti-CD28 in Th0, Th1, Th2, and Th17 +/- IFN-α for 5 days. Mx1 protein was evaluated by Intracellular cytokine staining in the 8 contexts. (**A**) FACS plot from one experiment representative of five independent experiments and **(B)** Quantification of Mx1 protein in all the five donors. The same cells were then infected with HIV-1 or HIV-2 tagged with GFP (with increasing dose of virus), in the presence of IL-2 for 48H. Infected cells, defined by GFP+ cells were evaluted by Flow cytometry. **(C)** Infection of Th cells with HIV-2. MOI : multiple of Infection. Data are the mean +/- SD of five independent experiments. **(D)** Infection of Th cells with HIV-1. Data are the mean +/- SD of five independent experiments. Ns, non significant, *, $p < 0.05$; ** , $p < 0.01$ (T-test)

Supplementary figure 1 Cytokine production and Transcription Factor expression in the four polarizing contexts.

Naïve T cells were differentiated with anti-CD3 plus anti-CD28 in Th0, Th1, Th2, and Th17 for 5 days and re-stimulated for 24h with anti-CD3 plus anti-CD28 to collect supernatant. **(A)** Intracellular cytokine staining of IL-4, IL-17 and IFN-γ in the four polarizing contexts after 5 days of differentiation. Data are from one experiment representative of four independent experiments. **(B)** ELISA and/or cytometric bead assay of IL-4, IL-17 and IFN-γ in supernatants. *, $p < 0.05$; **, $p < 0.01$ (Wilcoxon test). Data are the mean of eight independent experiments. **(C)** RT-PCR analysis of the expression of T-bet, GATA-3 and RORc mRNA in naïve T cells differentiated with anti-CD3 plus anti-CD28 in Th0, Th1, Th2, and Th17 for 5 days. Cycling threshold values are normalized to those of L34. *, $p < 0.05$; ** , $p < 0.01$ (T-test). Data are the mean +/- SD of five independent experiments.

Supplementary figure 2 IFN-α modulates the proliferation of T helper cells in a similar manner.

(A) CFSE proliferation of naive T cells stimulated by with anti-CD3 plus anti-CD28 in Th0, Th1, Th2, and Th17 in the presence or not of IFN-α for 3 days. Data are from one experiment representative of three independent experiments. **(B)** Expansion rate (ER) of T Helper cells (Th0, Th1, Th2 and Th17) +/- IFN-α after 5 days of stimulation with anti-CD3 plus anti-CD28. ER was measured by triptan blue count. *, $p < 0.05$; ** , $p < 0.01$ (Wilcoxon test). Data are the mean of eight independent experiments.

Supplementary figure 3 IFN-α signature after restimulation is still modulated by the polarizing context.

(A) Venn diagram representing the overloap between the *IFN-α signature* at Day 5+ 4h restim. in the 3 different polarizing contexts (Th1, Th2 or Th17). **(B)** Enrichment in the biological functions for the *IFN-α signature* at Day 5+ 4h restim. in Th0, Th1,Th2 and Th17**.**

Supplementary figure 4 Modulation of the Master transcription factors of T Helper cells by IFN-α.

RT-PCR analysis of the expression of T-bet, GATA-3, RORc, FOXP3, AHR, c-MAF mRNA in naïve T cells differentiated with anti-CD3 plus anti-CD28 in the four polarizing contexts +/- IFN-α for 5 days. Cycling threshold values are normalized to those of ribosomal protein L34. *, $p < 0.05$; ** , $p < 0.01$ (T-test). Data are the mean of three to four independent experiments.

Supplementary figure 5 IFN-α modulates specifically the Th0 and Th0 cytokine profile.

Manova-cluster of the 8 experimental conditions: Th0, Th1, Th2, Th17 +/- IFN-α. The 8 cytokine profiles (IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL17, IL-21, IL-22, IFN-γ, LT-α, TNF-α and TGF-β) were analysed and separated into cluster according to the mahalanobis distance that estimates the interdistance beetween each pair of groups. A MANOVA test was applied to discriminate statistical difference between two profiles *, $p < 0.05$; **, $p < 0.01$).

Supplementary figure 6 Antiviral state induced by IFN-α confers a specific susceptibility to viral infection in a Th subset-dependent manner.

Naïve T cells were differentiated with anti-CD3 plus anti-CD28 in Th0, Th1, Th2, and Th17 +/- IFN-α for 5 days. The same cells were then infected with HIV-2 tagged with GFP (with increasing dose of virus), in a presence of IL-2 for 48H. Infected cells, defined by GFP+ cells were evaluted by Flow cytometry. Data represents the strategy of gating**.**

Supplementary table 1 : IFN signatures.

List of the 76 and the 71 genes that represent the *IFN-α signature* at Day 5 and at Day 5+ restim. respectively. *IFN-α signature* was defined as the IFN modulated genes in Th0 according to two criteria: Fold change (FC) > 2 and p values< 0.05

Supplementary table 2 : Genes differentially expressed among IFN signatures in the four modules.

We focused on four main « modules » related to biological functions that characterize Th cells: cytokine, transcription factor, chemokine and receptor. We identified among all IFN signatures, the ISGs that were involved in these four modules

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IFN-α signature at D5 : List of the 76 genes.

Probe Set ID	Gene Symbol	p-value (t-test)	mean ratio (log)
7902541	IFI44L	0,0000	4,80
7902553	IFI44	0,0102	3,58
8068713	MX1	0,0003	3,40
8103563	DDX60	0,0002	2,92
8148572	LY6E	0,0149	2,76
7976443	IFI27	0,0306	2,59
8004184	XAF1	0,0062	2,56
8085579	---	0,0058	2,55
8124183	---	0,0496	2,36
8040080	RSAD2	0,0029	2,35
8047272	SPATS2L	0,0113	2,23
8074606	USP18	0,0142	2,23
7902205	IL12RB2	0,0370	2,15
7929065	IFIT1	0,0220	2,13
8167163	CXorf24	0,0174	2,11
8092348	LAMP3	0,0297	2,00
7958884	OAS1	0,0016	1,95
7929052	IFIT3	0,0347	1,95
8096361	HERC5	0,0053	1,87
8068697	MX2	0,0289	1,82
8051501	EIF2AK2	0,0108	1,82
8094259	LAP3	0,0160	1,79
7958895	OAS3	0,0003	1,74
8007446	IFI35	0,0069	1,71
8060503	SNORD57	0,0129	1,63
7958913	OAS2	0,0020	1,61
8052331	PNPT1	0,0386	1,57
7914127	IFI6	0,0002	1,57
8090018	PARP9	0,0057	1,56
8005809	LGALS9	0,0133	1,55
7947027	UEVLD	0,0095	1,54
8140967	SAMD9	0,0287	1,52
7971661	MIR15A	0,0396	1,47
7896817	ISG15	0,0013	1,46
8103755	FBXO8	0,0122	1,45
7929072	IFIT5	0,0261	1,37
8082100	PARP14	0,0040	1,36

8103601	DDX60L	0,0013	1,35
8154785	CHMP5	0,0075	1,33
8073242	ADSL	0,0298	1,32
8152626	---	0,0228	1,30
8048940	SP100	0,0146	1,30
7972888	PCID2	0,0078	1,29
8131335	---	0,0140	1,27
7991777	C4orf46	0,0134	1,25
8119198	FTSJD2	0,0180	1,19
8035304	BST2	0,0002	1,16
8140971	SAMD9L	0,0411	1,16
8078688	---	0,0438	1,15
7957467	C12orf29	0,0479	1,14
8123714	---	0,0407	1,11
7951467	ALKBH8	0,0130	1,10
8056285	IFIH1	0,0498	1,09
8016018	SLC25A39	0,0092	1,09
7955425	ATF1	0,0243	1,09
7910591	C1orf57	0,0443	1,08
8099668	---	0,0338	1,07
8096335	HERC6	0,0362	1,05
7979802	---	0,0232	1,05
8150565	RNF170	0,0175	1,03
7919589	HIST2H3D	0,0129	1,03
8104760	TARS	0,0496	1,01
8083605	RSRC1	0,0339	1,00
8016847	TRIM25	0,0359	1,00
8072687	MCM5	0,0124	1,00
8091656	METT5D1	0,0442	-1,02
8122222	PDE7B	0,0259	-1,05
7917530	---	0,0267	-1,07
8017599	PECAM1	0,0060	-1,09
7915504	ELOVL1	0,0146	-1,10
8004219	---	0,0392	-1,14
7954559	PPFIBP1	0,0051	-1,17
8058969	---	0,0020	-1,35
8150550	CHRNA6	0,0231	-1,38
8098193	---	0,0476	-1,41
8112033	ARL15	0,0375	-1,43

IFN-α signature at H4 : List of the 71 genes.

Probe Set ID	Gene Symbol	p-val (t-test)	mean ratio (log)
7902541	IFI44L	0,0001	4,13
7902474	---	0,0113	3,05
7902553	IFI44	0,0002	2,88
7976443	IFI27	0,0010	2,63
8012852	---	0,0055	2,38
7958884	OAS1	0,0010	2,35
8009253	---	0,0000	2,34
8068713	MX1	0,0030	2,27
8164694	---	0,0024	2,18
8149248	---	0,0009	2,08
8040080	RSAD2	0,0153	2,03
7950370	---	0,0214	2,00
8004184	XAF1	0,0010	1,99
8005809	LGALS9	0,0052	1,87
8118979	---	0,0355	1,82
8101126	CXCL10	0,0185	1,81
8049540	LRRFIP1	0,0120	1,80
7964787	IFNG	0,0213	1,64
8103563	DDX60	0,0012	1,56
7914127	IFI6	0,0001	1,53
7929052	IFIT3	0,0330	1,49
8112274	ELOVL7	0,0065	1,49
8144699	---	0,0127	1,47
8051501	EIF2AK2	0,0017	1,45
8148572	LY6E	0,0042	1,39
7969048	---	0,0294	1,37
8047272	SPATS2L	0,0213	1,35
7938035	TRIM22	0,0091	1,34
7946426	---	0,0177	1,31
7896817	ISG15	0,0129	1,27
7908312	PRG4	0,0241	1,26
8013450	LGALS9B	0,0058	1,25
8145977	PLEKHA2	0,0085	1,23
8133623	---	0,0021	1,19
8139975	---	0,0018	1,19
8115865	BOD1	0,0469	1,18
8140971	SAMD9L	0,0132	1,17

8034512	SNORD41	0,0315	1,17
7971296	EPSTI1	0,0057	1,17
8068697	MX2	0,0011	1,15
7929047	IFIT2	0,0339	1,14
7958913	OAS2	0,0132	1,09
8118207	SNORA38	0,0304	1,08
8140967	SAMD9	0,0125	1,07
8125766	BAK1	0,0012	1,07
8121043	ORC3L	0,0100	1,07
7929072	IFIT5	0,0344	1,06
8096335	HERC6	0,0143	1,06
8135080	AP1S1	0,0427	1,05
8066528	PIGT	0,0259	1,05
8092348	LAMP3	0,0362	1,04
8139458	LOC100128364	0,0136	1,02
8103601	DDX60L	0,0396	1,00
8122198	---	0,0475	-1,01
8017344	LOC100129112	0,0212	-1,03
8129214	MCM9	0,0198	-1,05
8141490	PMS2L1	0,0312	-1,06
8164215	SNORA65	0,0479	-1,07
8156263	SPIN1	0,0102	-1,07
7926105	GATA3	0,0127	-1,08
8112746	WDR41	0,0032	-1,11
7997904	ZNF778	0,0225	-1,14
8136889	---	0,0316	-1,15
8154866	---	0,0232	-1,17
8155327	ALDH1B1	0,0199	-1,24
8066254	LOC388796	0,0148	-1,27
8078442	CCR4	0,0002	-1,28
8059648	---	0,0196	-1,37
7903022	SNORD21	0,0235	-1,43
8039945	---	0,0101	-1,99
7911566	---	0,0099	-1,99

Table 1

		Fold change induced by IFN-α			
		Th0	Th1	Th2	Th17
Cytokines	IFN-Y	1,09	2,73	1,05	2,82
Receptors	CCR4	0,41	0,36	0,52	0,94
	IL-12Rβ2	4,42	1,29	2,89	1,70
Chemokines	CCL-1	0,88	0,32	1,03	0,74
	CCL-20	0,91	1,00	1,09	2,75
	IP-10	3,39	1,11	1,60	1,25
Transcription factors	GATA3	0,46	0,72	0,98	0,82

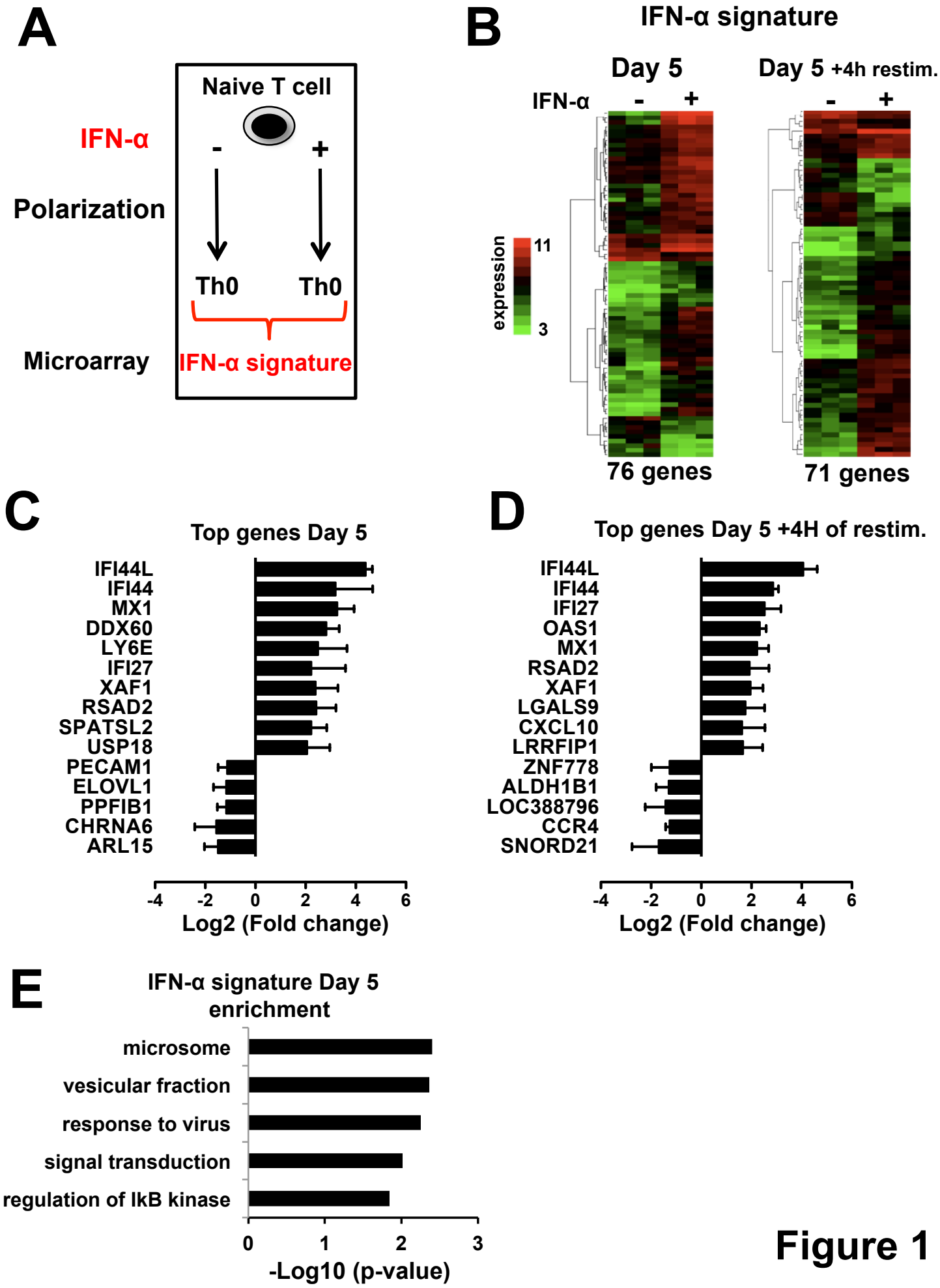

A
Naive T cell
IFN-α
-
+
Polarization
Th0
Th0
Microarray
IFN-α signature
B
IFN-α signature
Day 5
Day 5 +4h restim.
IFN-α
-
+
-
+
expression
11
3
76 genes
71 genes
C
Top genes Day 5
IFI44L
IFI44
MX1
DDX60
LY6E
IFI27
XAF1
RSAD2
SPATSL2
USP18
PECAM1
ELOVL1
PPFIB1
CHRNA6
ARL15
-4
-2
0
2
4
6
Log2 (Fold change)
D
Top genes Day 5 +4H of restim.
IFI44L
IFI44
IFI27
OAS1
MX1
RSAD2
XAF1
LGALS9
CXCL10
LRRFIP1
ZNF778
ALDH1B1
LOC388796
CCR4
SNORD21
-4
-2
0
2
4
6
Log2 (Fold change)
E
IFN-α signature Day 5 enrichment
microsome
vesicular fraction
response to virus
signal transduction
regulation of IkB kinase
0
1
2
3
-Log10 (p-value)

Figure 1

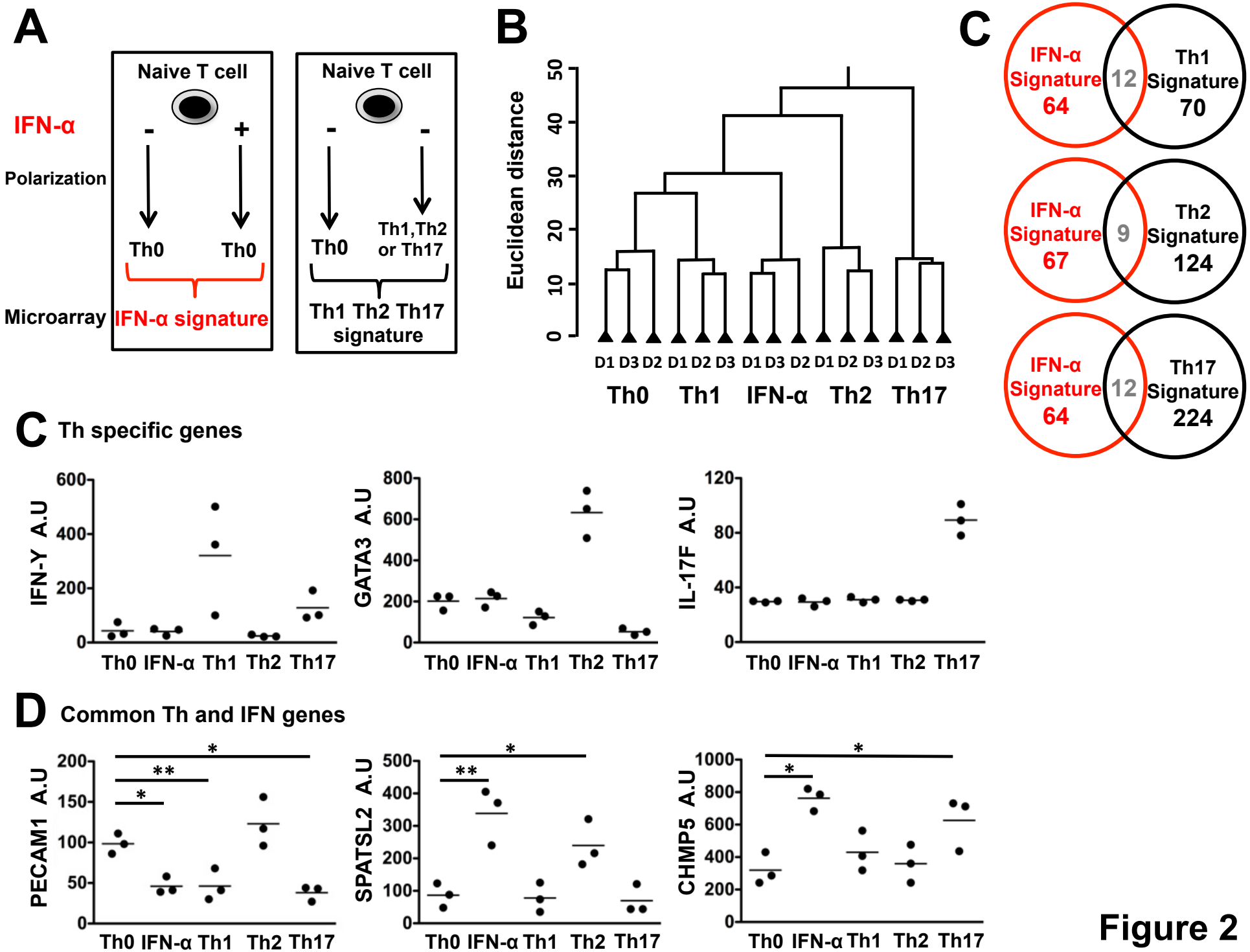

A
Naive T cell
IFN-α
-
+
Polarization
Th0
Th0
Microarray
IFN-α signature
Naive T cell
-
-
Th0
Th1,Th2 or Th17
Th1 Th2 Th17 signature
B
Euclidean distance
0
10
20
30
40
50
D1 D3 D2 D1 D2 D3 D1 D3 D2 D1 D2 D3 D1 D2 D3
Th0 Th1 IFN-α Th2 Th17
C
IFN-α Signature 64
12
Th1 Signature 70
IFN-α Signature 67
9
Th2 Signature 124
IFN-α Signature 64
12
Th17 Signature 224
C Th specific genes
IFN-Y A.U
GATA3 A.U
IL-17F A.U
Th0 IFN-α Th1 Th2 Th17
D Common Th and IFN genes
PECAM1 A.U
SPATSL2 A.U
CHMP5 A.U
Th0 IFN-α Th1 Th2 Th17

Figure 2

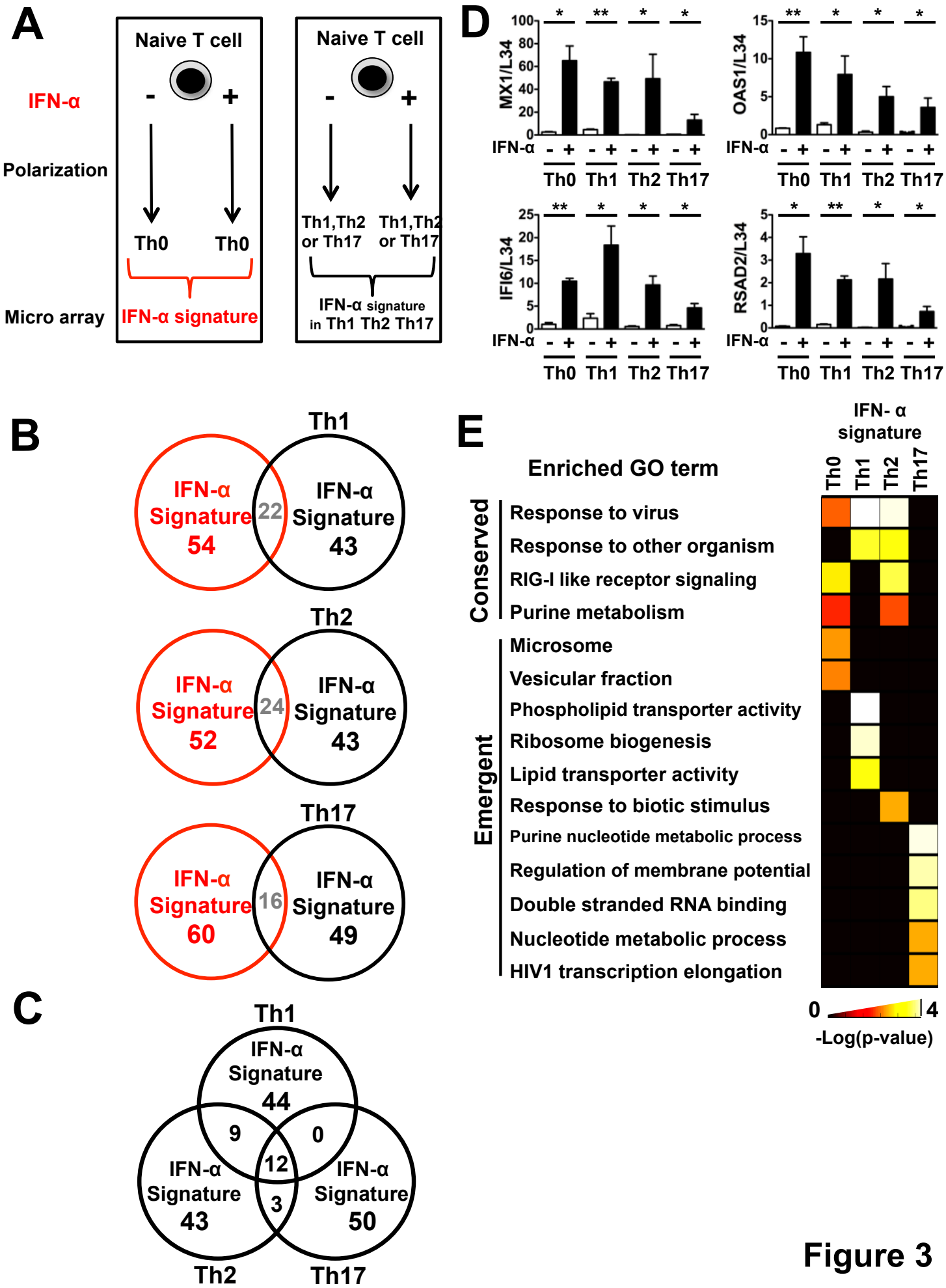

A
Naive T cell
IFN-α
-
+
Polarization
Th0
Th0
Micro array
IFN-α signature
Naive T cell
-
+
Th1,Th2 or Th17
Th1,Th2 or Th17
IFN-α signature in Th1 Th2 Th17
D
MX1/L34
OAS1/L34
IFI6/L34
RSAD2/L34
IFN-α
Th0 Th1 Th2 Th17
B
Th1
IFN-α Signature 54
22
IFN-α Signature 43
Th2
IFN-α Signature 52
24
IFN-α Signature 43
Th17
IFN-α Signature 60
16
IFN-α Signature 49
C
Th1
IFN-α Signature 44
9
0
12
3
IFN-α Signature 43
IFN-α Signature 50
Th2
Th17
E
IFN- α signature
Enriched GO term
Th0 Th1 Th2 Th17
Conserved
Response to virus
Response to other organism
RIG-I like receptor signaling
Purine metabolism
Emergent
Microsome
Vesicular fraction
Phospholipid transporter activity
Ribosome biogenesis
Lipid transporter activity
Response to biotic stimulus
Purine nucleotide metabolic process
Regulation of membrane potential
Double stranded RNA binding
Nucleotide metabolic process
HIV1 transcription elongation
0
4
-Log(p-value)

Figure 3

Figure 4

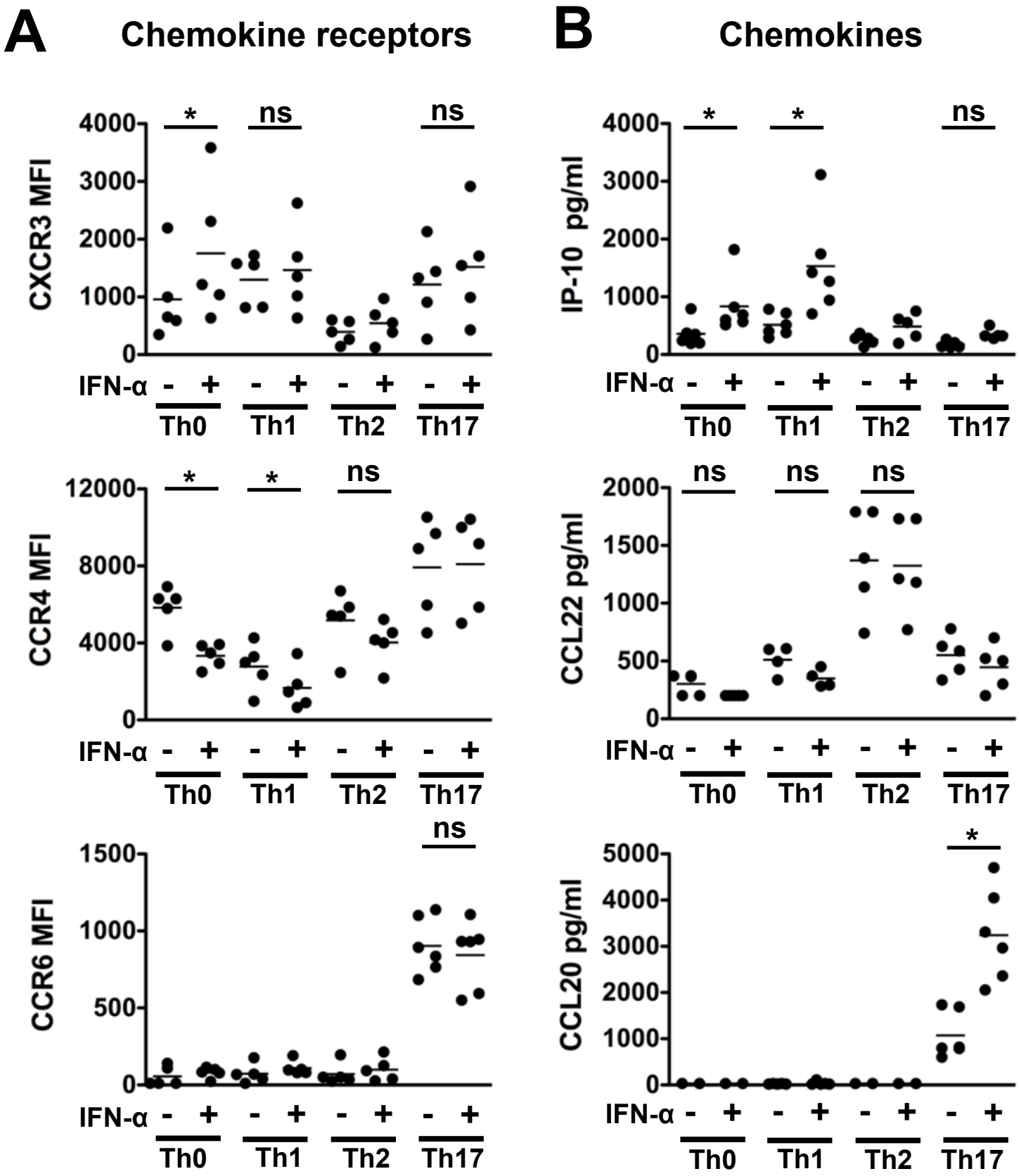
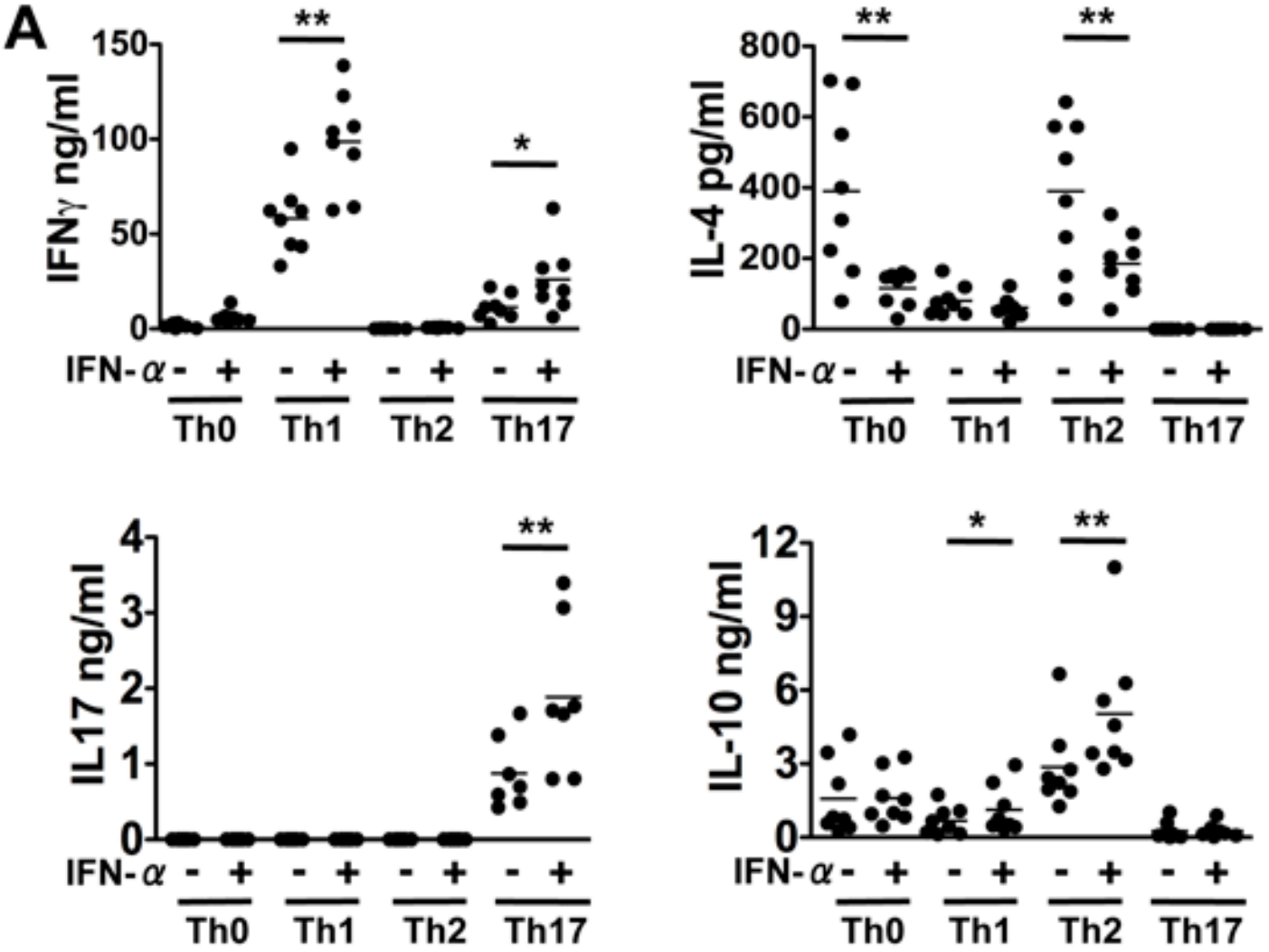
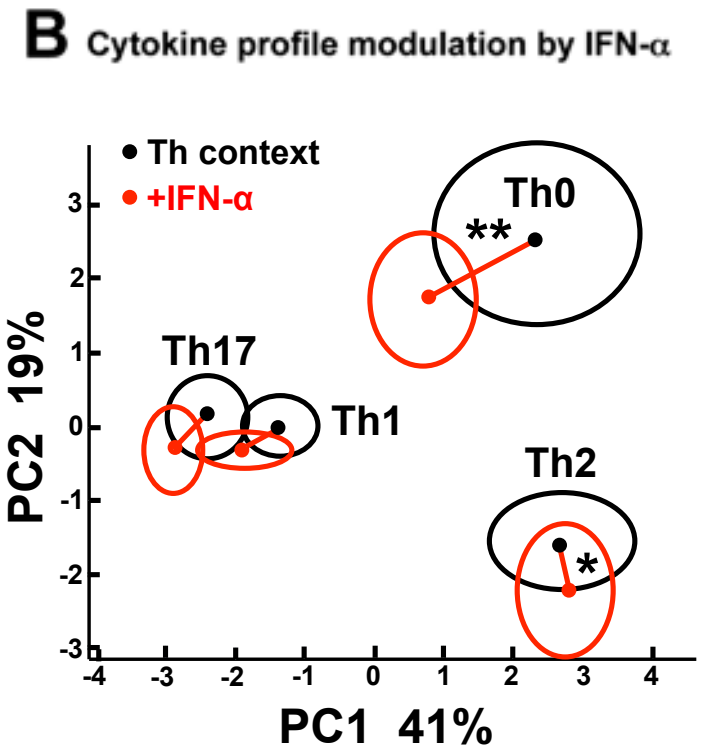

A
Chemokine receptors
B
Chemokines
CXCR3 MFI
IP-10 pg/ml
CCR4 MFI
CCL22 pg/ml
CCR6 MFI
CCL20 pg/ml
IFN-α
Th0
Th1
Th2
Th17
*
ns

Figure 5


A
IFNγ ng/ml
150
100
50
0
**
*
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17
IL-4 pg/ml
800
600
400
200
0
**
**
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17
IL17 ng/ml
4
3
2
1
0
**
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17
IL-10 ng/ml
12
9
6
3
0
*
**
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17


B Cytokine profile modulation by IFN-α
Th context
+IFN-α
Th0
**
Th17
Th1
Th2
*
PC2 19%
PC1 41%
3
2
1
0
-1
-2
-3
-4
-3
-2
-1
0
1
2
3
4

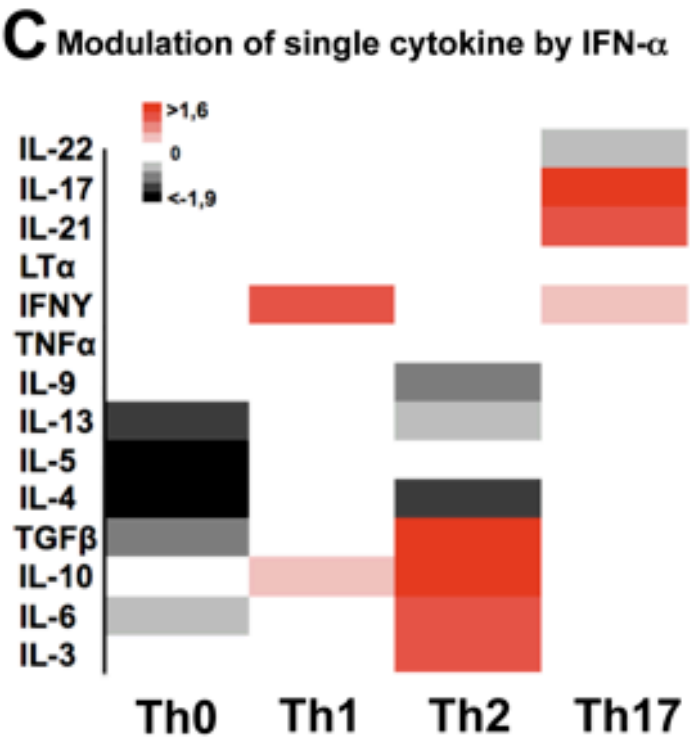
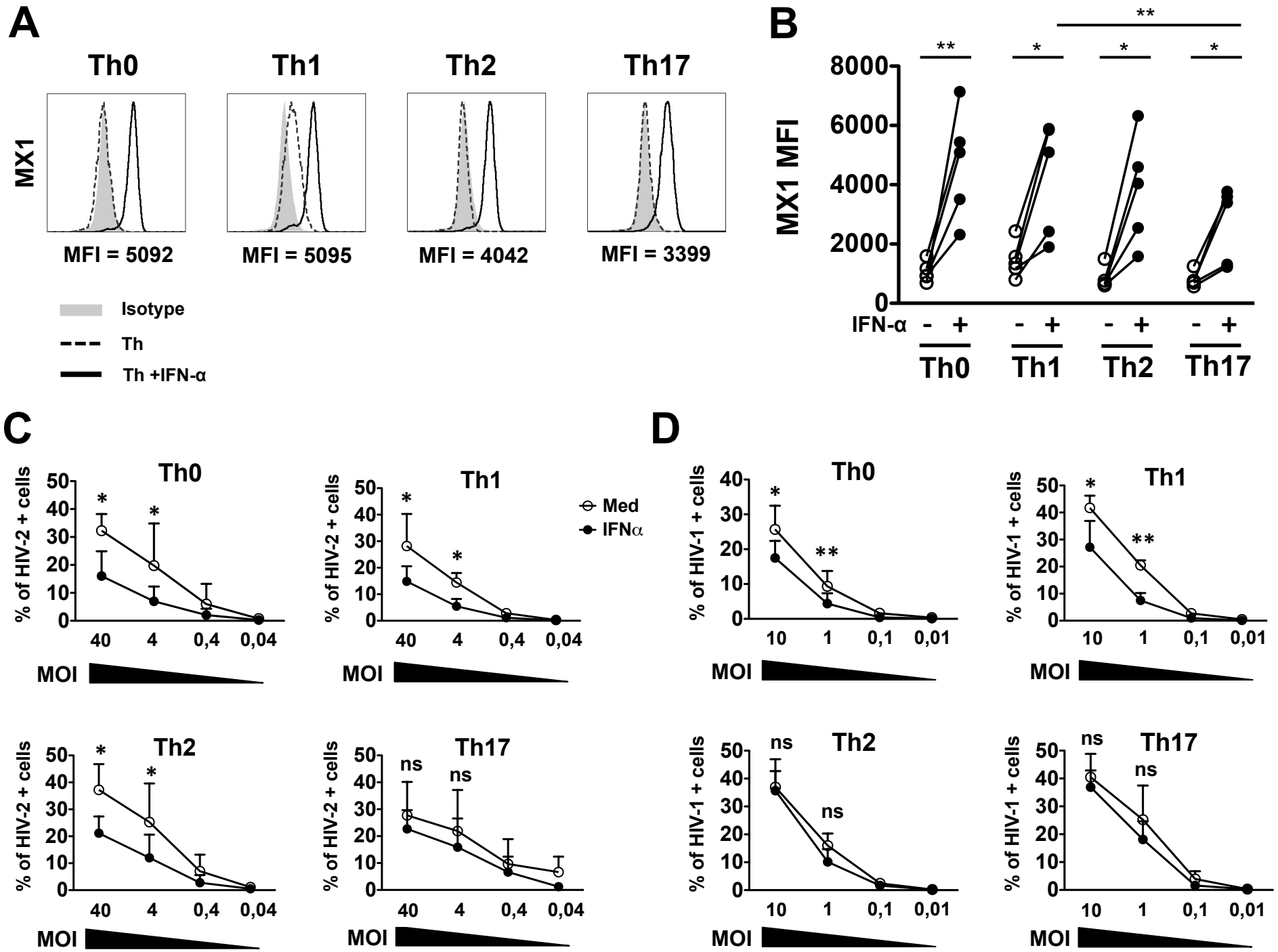
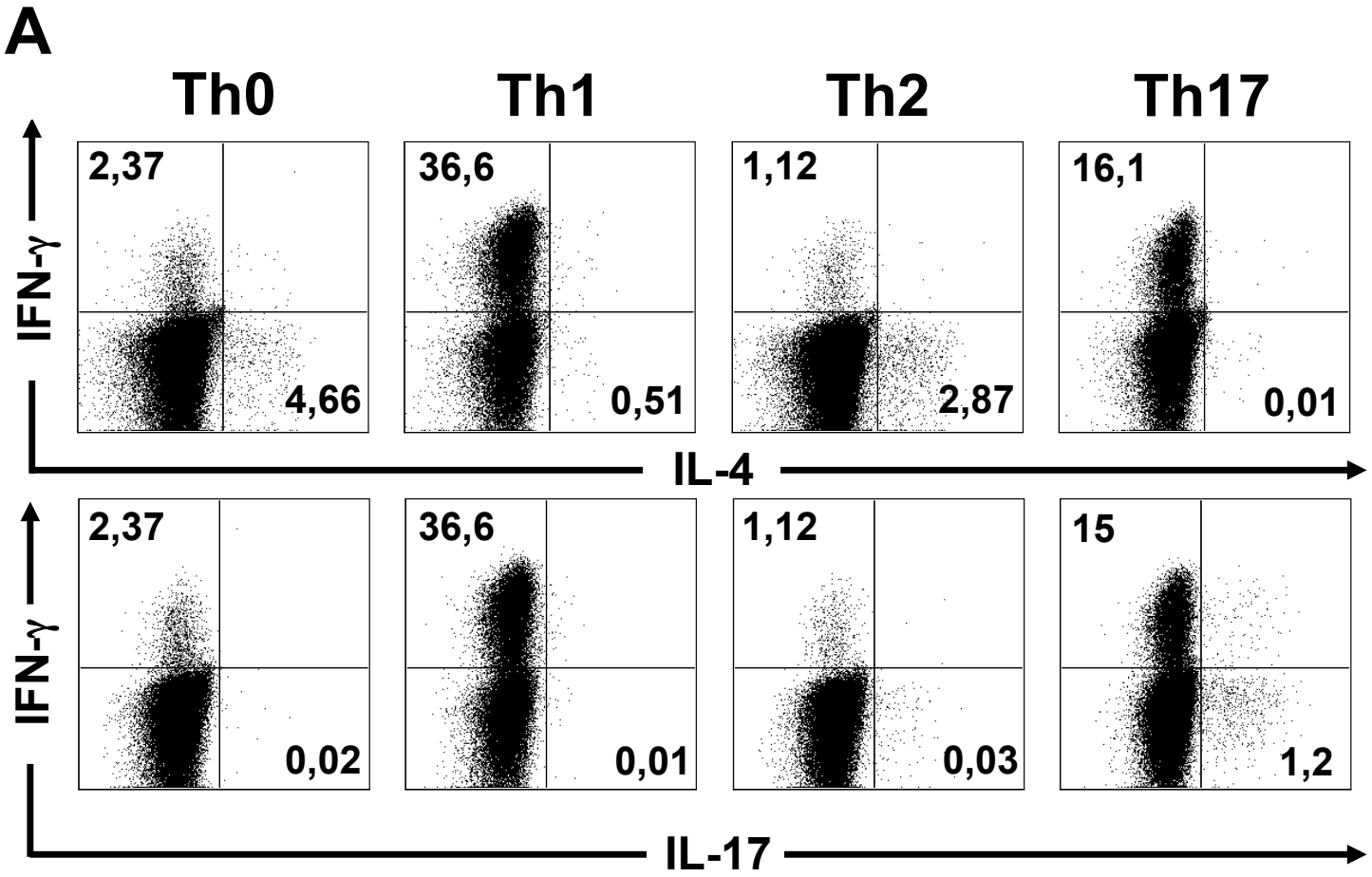
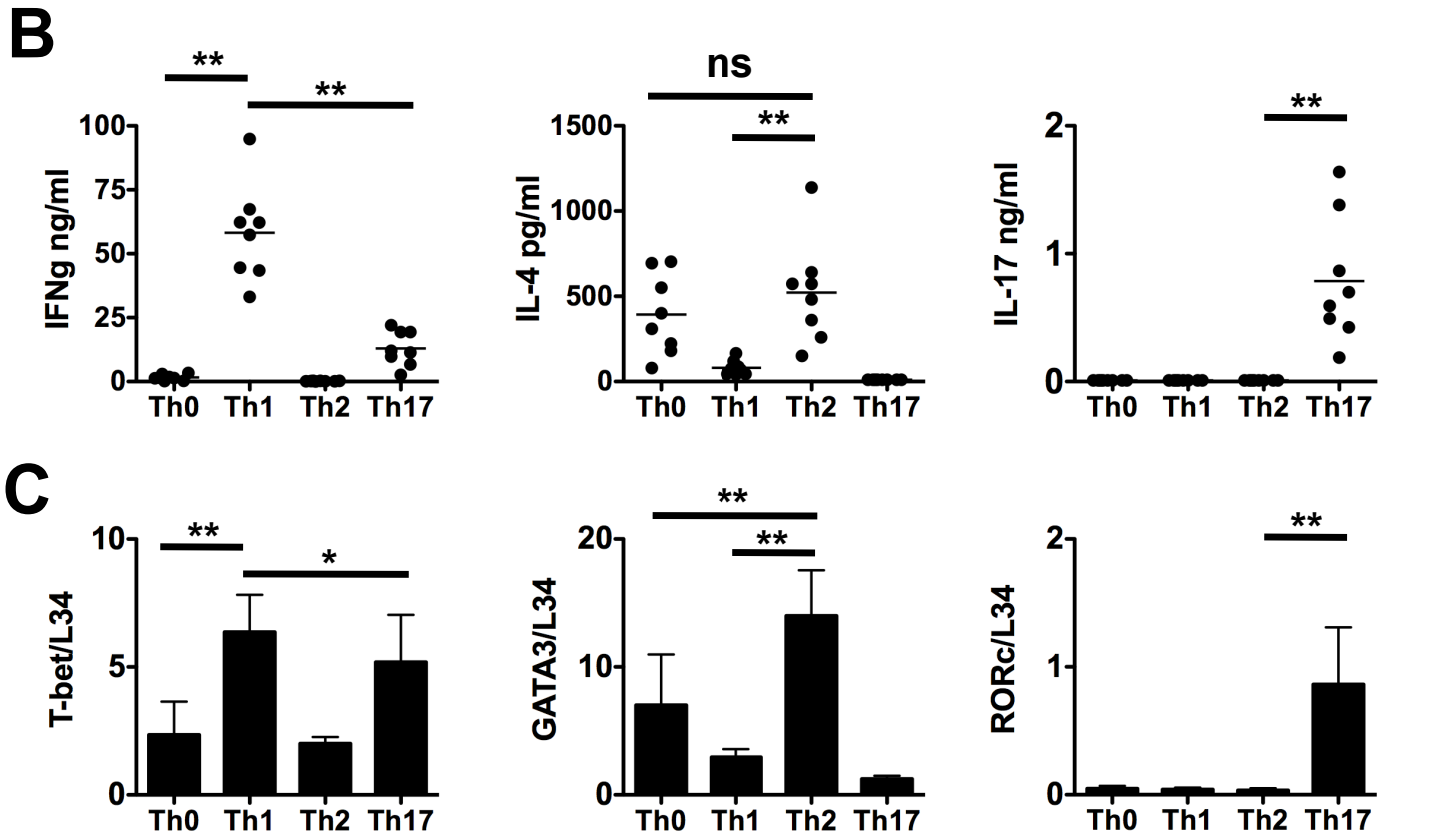

C Modulation of single cytokine by IFN-α
>1,6
0
<-1,9
IL-22
IL-17
IL-21
LTα
IFNY
TNFα
IL-9
IL-13
IL-5
IL-4
TGFβ
IL-10
IL-6
IL-3
Th0 Th1 Th2 Th17

Figure 6

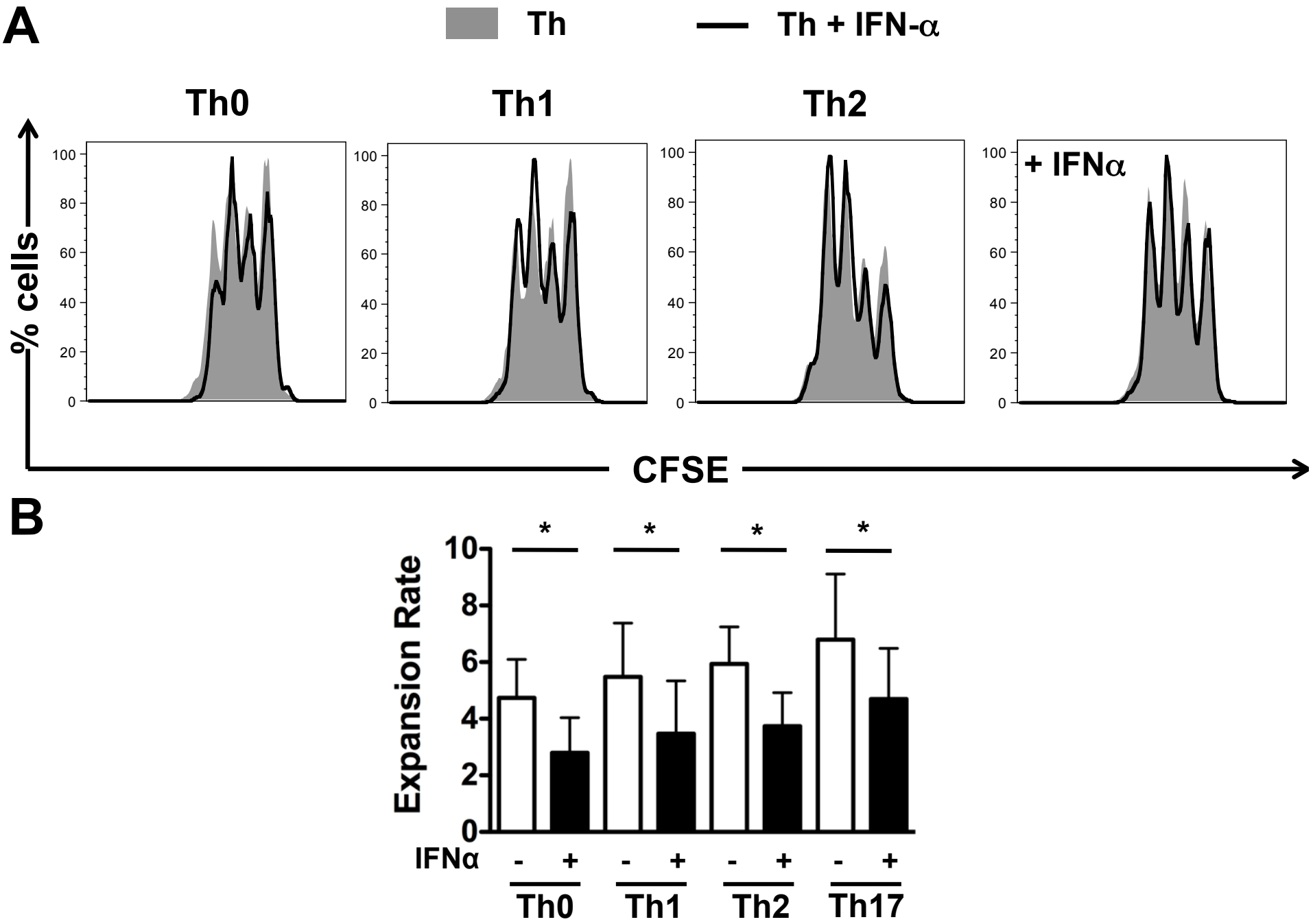

A
Th0
Th1
Th2
Th17
MX1
MFI = 5092
MFI = 5095
MFI = 4042
MFI = 3399
Isotype
Th
Th +IFN-α
B
MX1 MFI
8000
6000
4000
2000
0
IFN-α - + - + - + - +
Th0
Th1
Th2
Th17
C
D
% of HIV-2 + cells
% of HIV-1 + cells
MOI
40
4
0,4
0,04
10
1
0,1
0,01
Med
IFNα
ns

Supplementary fig 1



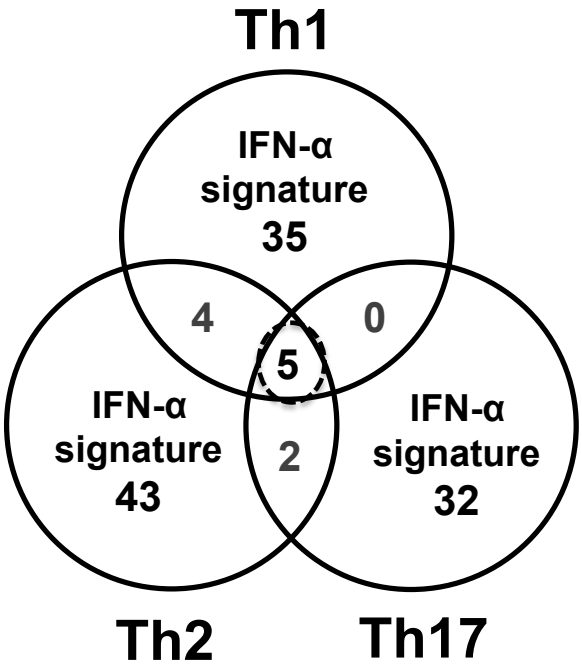


Supplementary fig 2

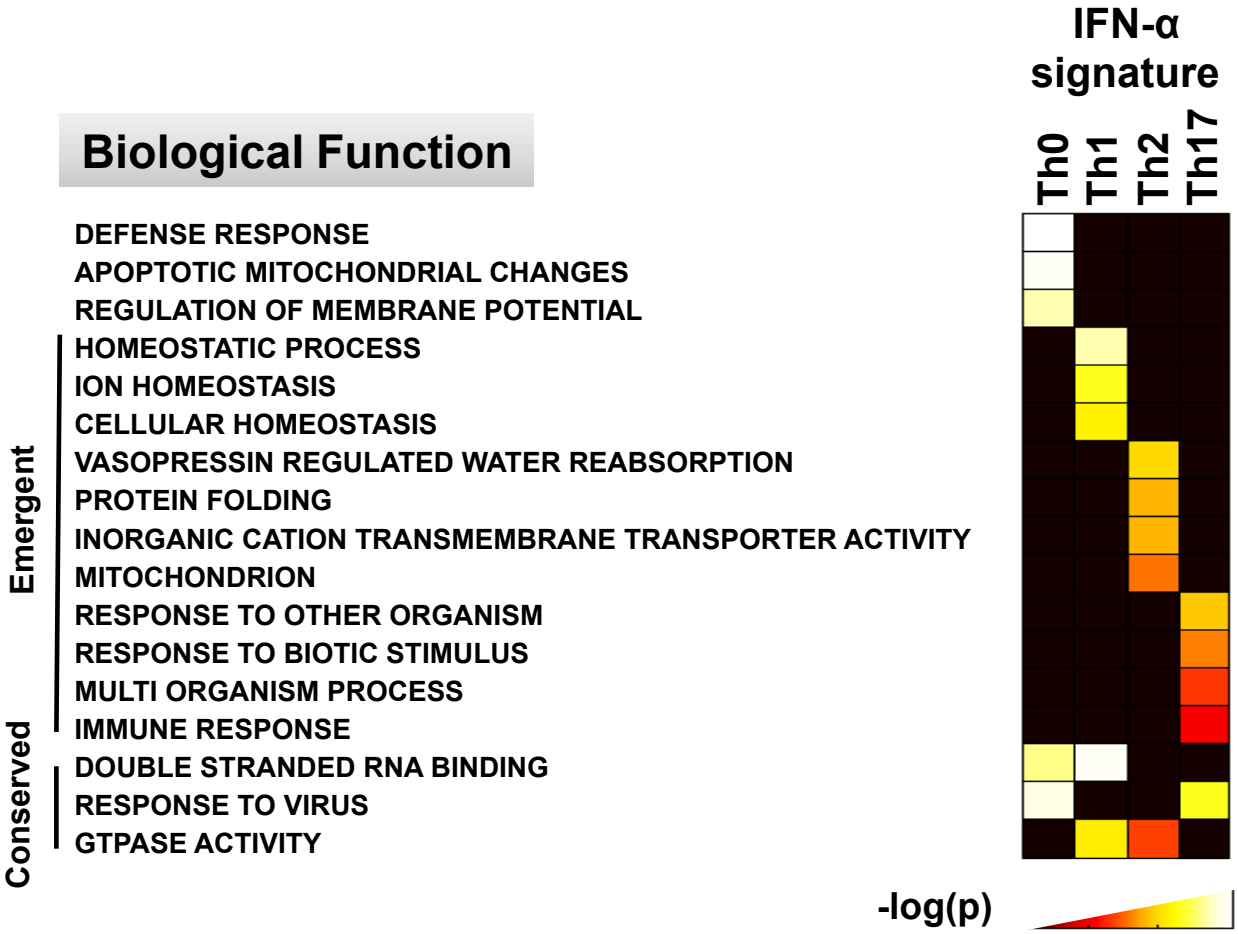


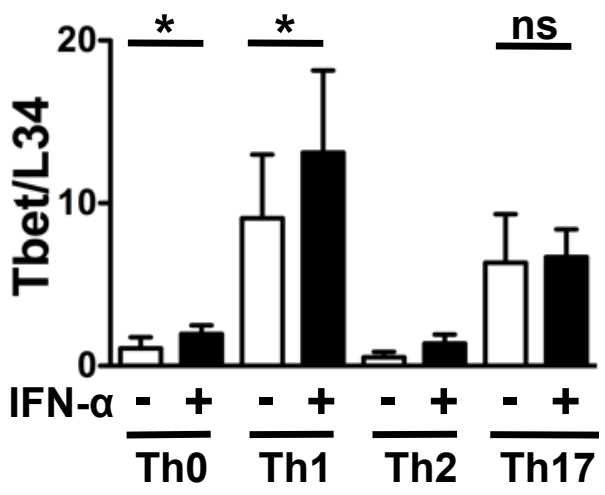
Supplementary fig 3

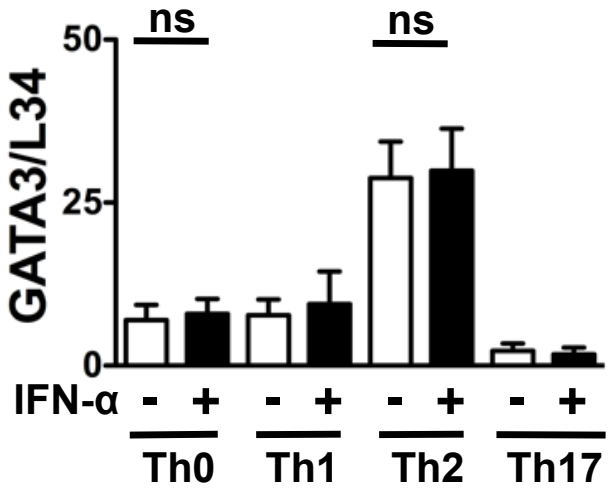
A

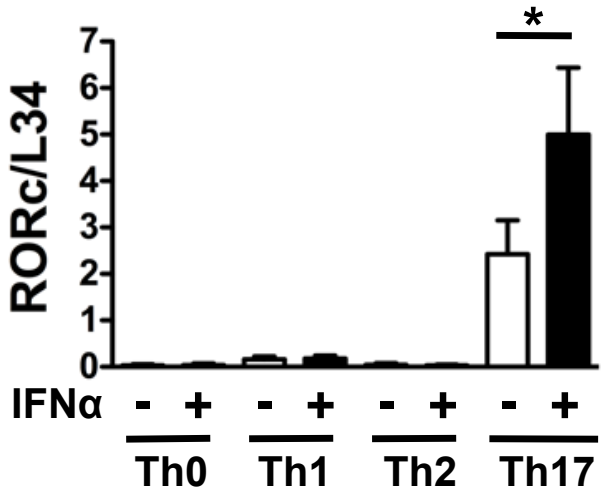


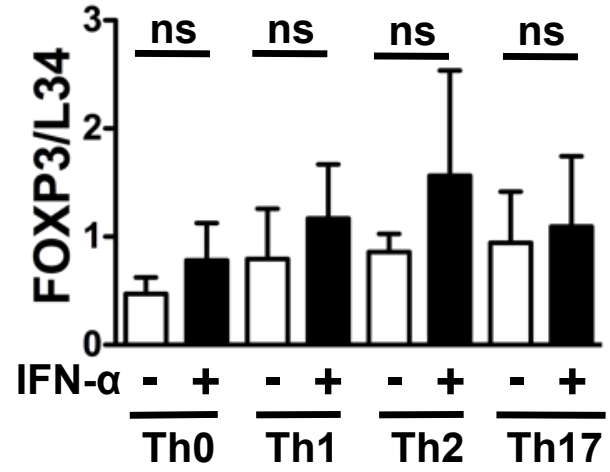
B

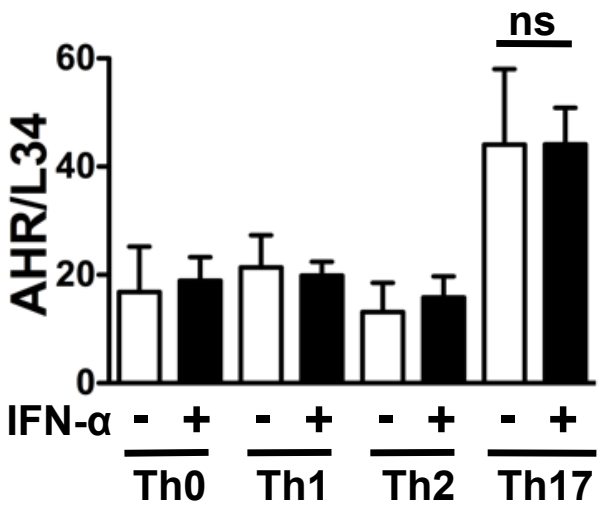


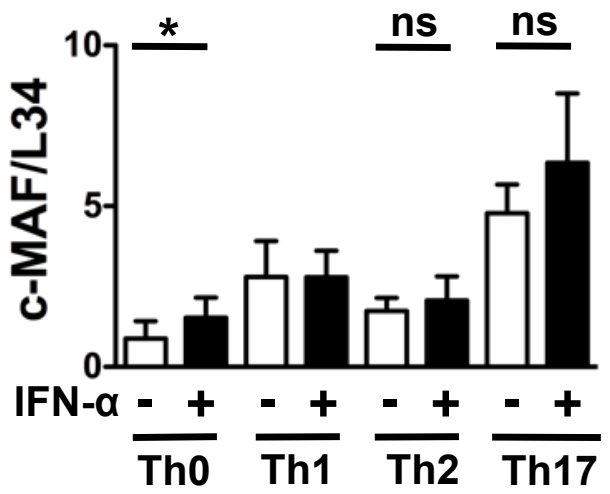

Tbet/L34
20
10
0
*
*
ns
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17


GATA3/L34
50
25
0
ns
ns
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17

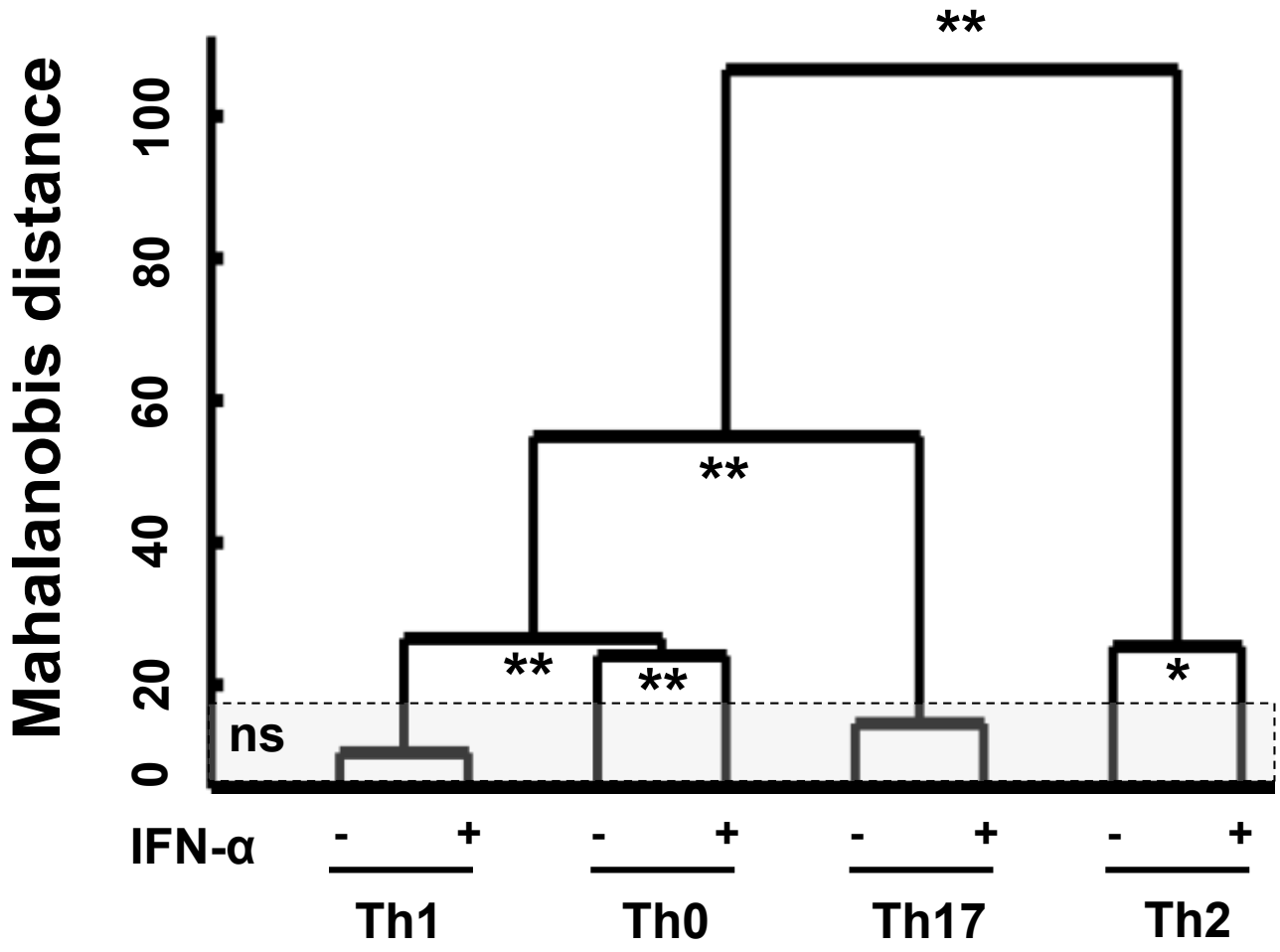

RORc/L34
7
6
5
4
3
2
1
0
*
IFNα - + - + - + - +
Th0 Th1 Th2 Th17


FOXP3/L34
3
2
1
0
ns
ns
ns
ns
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17

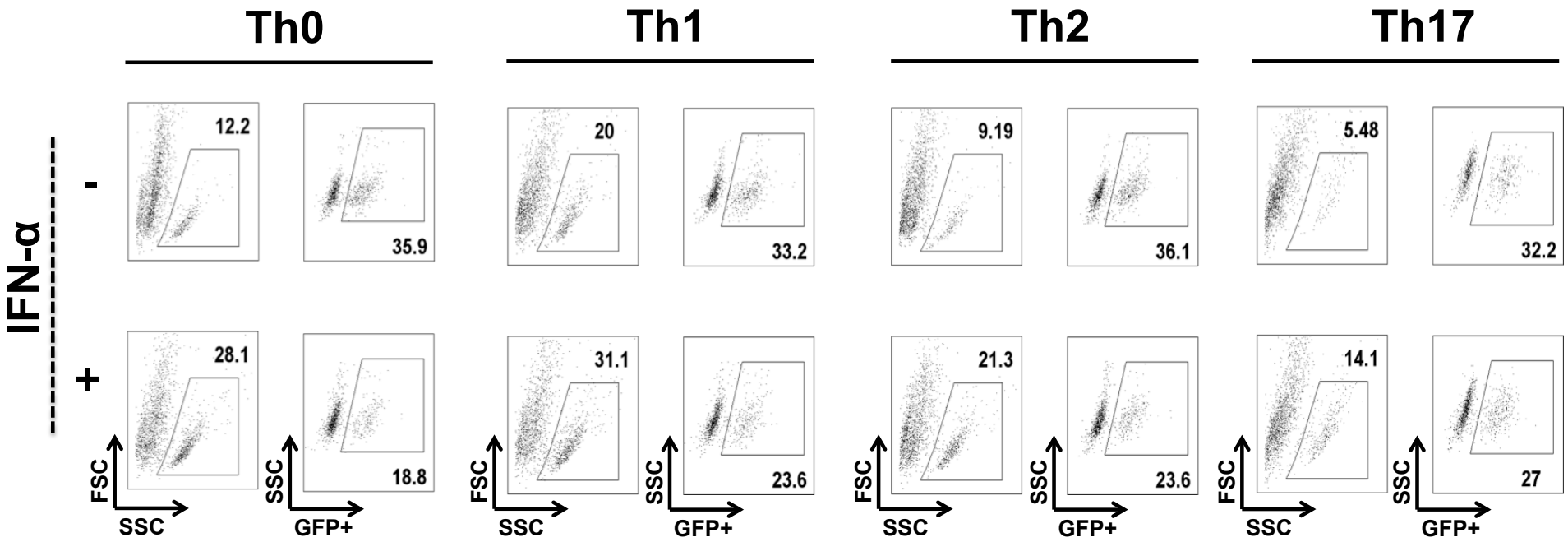

AHR/L34
60
40
20
0
ns
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17


c-MAF/L34
10
5
0
*
ns
ns
IFN-α - + - + - + - +
Th0 Th1 Th2 Th17

Supplementary fig 5



Supplementary fig 6



7.3 Publication 3

Human inflammatory dendritic cells induce Th17 differentiation.

Elodie Segura, Maxime Touzot, Armelle Bohineust, Antonio cappuccino, Anne Hosmalin, Marc Dalod, Vassili Soumelis, Sebastian Amigorena.

Immunity 2013

Pour cette collaboration avec l'équipe de Sebastian Amigorana, nous avons de nouveau utilisé des analyses à large échelle afin de mieux caractériser l'origine d'une sous population de cellule dendritique DC humaine, les DC inflammatoires (infDC).

Il existe chez l'homme et la souris, plusieurs types de DC qui diffèrent par leur ontogénie, leurs fonctions et leurs localisations anatomiques. Au cours de phénomènes inflammatoires, il a été individualisé, chez la souris, une population distincte de DC inflammatoires (InfDC). Peu de travaux existent sur cette population de DC chez l'homme.

Elodie Segura a identifié dans deux contextes inflammatoires distincts une population spécifique de infDC. Ces derniers présentent des critères histologiques et un phénotypique différent (CD11c+, BDCA1+, HLADR+, MMR+, CC11b+ Sirpa+) de macrophages inflammatoires ou des DC sanguins.

Pour déterminer si cette population de infDC correspond à une sous-population distincte de DC ou à une forme particulière de DC activée, nous avons comparé les profils transcriptionnels des infDC avec 4 autres populations.

Nous avons observés par deux méthodes complémentaires (Analyse par Composante Principale et Clustering), que le profil transcriptionnel des InfDC était différent de celui des Macrophages inflammatoires, des DC sanguins, des monocytes CD14+CD16- et des monocytes CD14-CD16+. L'analyse spécifique des modules fonctionnels des DC (Antigen Proceessing, endocytosis, et chemotactisme) a révèlé que les infDC partageaient certaines fonctions des DC sanguins (Antigen Proceessing) mais aussi des macrophages inflammatoires (endocytosis, et chemotactisme). Enfin, nous avons observé, également, que les InfDC exprimaient certains facteurs de transcription spécifique de la lignée myéloïde murins, suggérant que le développement des InfDC a les caractéristiques proches de ceux des DC et mais aussi des macrophages.

Finalement, nous avons montré que notre signature transcriptomique des infDC avait une grande similitude avec celles de MoDC déjà publiées.

En analysant ainsi de manière globale et plus spécifique le programme transcriptionnel des InfDC, l'ensemble de ces data suggère que les InfDC humains sont probablement issus des monocytes mais présentent des fonctions communes avec les DC sanguines et les macrophages inflammatoires.

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Human Inflammatory Dendritic Cells Induce Th17 Cell Differentiation

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SUMMARY

Dendritic cells (DCs) are critical regulators of immune responses. Under noninflammatory conditions, several human DC subsets have been identified. Little is known, however, about the human DC compartment under inflammatory conditions. Here, we characterize a DC population found in human inflammatory fluids that displayed a phenotype distinct from macrophages from the same fluids and from steady-state lymphoid organ and blood DCs. Transcriptome analysis showed that they correspond to a distinct DC subset and share gene signatures with in vitro monocyte-derived DCs. Moreover, human inflammatory DCs, but not inflammatory macrophages, stimulated autologous memory CD4$^+$ T cells to produce interleukin-17 and induce T helper 17 (Th17) cell differentiation from naive CD4$^+$ T cells through the selective secretion of Th17 cell-polarizing cytokines. We conclude that inflammatory DCs represent a distinct human DC subset and propose that they are derived from monocytes and are involved in the induction and maintenance of Th17 cell responses.

INTRODUCTION

Numerous studies in mice have shown that dendritic cells (DCs) are heterogenous and comprise several subtypes with different phenotypes and functional properties (Heath and Carbone, 2009). In the steady state, committed DC progenitors originating from the bone marrow give rise to lymphoid organ-resident DCs and to migratory tissue DCs (Merad and Manz, 2009). During inflammation appears an additional DC subset, termed "inflammatory DCs" (infDCs), that differentiates from Ly6C^{hi} monocytes recruited to the site of inflammation (León et al., 2007). These in vivo differentiated monocyte-derived DCs (Mo-DCs) appear during pathogenic inflammation (Domínguez and Ardavín, 2010; Greter et al., 2012) and in models of inflammatory diseases, such as asthma (Hammad et al., 2010) and rheumatoid arthritis (Campbell et al., 2011). InfDCs can be modeled in vitro by bone-marrow derived DCs (BM-DC) cultured with GM-CSF (Xu et al., 2007), although GM-CSF seems dispensable for their development in vivo (Greter et al., 2012). InfDCs migrate to lymphoid organs where they can present antigens to both CD4$^+$ and CD8$^+$ T cells (Ballesteros-Tato et al., 2010; Hammad et al., 2010; León et al., 2007) or transfer antigens to resident DCs (Ersland et al., 2010). In addition, infDCs can activate memory T cells directly in peripheral tissues (Wakim et al., 2008). Depending on the inflammatory environment, infDCs induce T helper 1 (Th1) cell (León et al., 2007; Nakano et al., 2009) or Th2 cell-mediated responses (Hammad et al., 2010; Kool et al., 2008). Many inflammatory environments also trigger an additional Th cell subtype, Th17 cells, which secrete interleukin-17 (IL-17) and mediate host defense against extracellular bacteria and fungi. Th17 cell responses during inflammation are most likely also induced and maintained by DCs, but the precise type of DCs involved has not been characterized.

In humans, distinct subsets of DCs have also been evidenced. Blood conventional DCs can be separated into BDCA1$^+$ and BDCA3$^+$ DCs (Dzionek et al., 2000). Several DC subsets have been identified in human skin: dermal CD1a$^+$ DCs and CD14$^+$ DCs and epidermal Langerhans cells (Klechevsky et al., 2008). Although the ontogeny of human DCs remains poorly understood, it is now clear that these DC subsets differ considerably from the widely used in vitro model of Mo-DCs differentiated in the presence of GM-CSF and IL-4 (Robbins et al., 2008). An additional subset of epidermal DCs, termed inflammatory dendritic epidermal cells (IDECs), has been described in the skin of atopic dermatitis patients (Wollenberg et al., 1996). These cells express surface markers different from Langerhans cells and dermal

DCs from healthy skin. It is unclear, however, whether IDECs are an activated form of skin DCs or represent a distinct subset that appears only during inflammation. Similarly, a population of CD11c$^+$ cells observed in the skin of psoriasis patients, but absent from healthy skin, was proposed to represent a type of infDCs (Zaba et al., 2009) and to be similar to a population of blood myeloid cells expressing 6-sulfo LacNAc (slanDC) (Hansel et al., 2011). These blood cells, however, were recently shown to be a subset of CD16$^+$ monocytes rather than bona fide DCs (Cros et al., 2010). Therefore, although it has been known for many years that monocytes have the potential to differentiate into DCs in vitro (Sallusto and Lanzavecchia, 1994), the in vivo counterpart of human Mo-DCs awaits identification.

In the present study, we have characterized the DCs present in inflammatory environments in humans. We showed that these DCs represent a distinct DC subset, most likely derived from monocytes, that induces Th17 cell differentiation through the release of Th17 cell-polarizing cytokines.

RESULTS

DCs with a Distinct Phenotype Are Present in Human Inflammatory Fluids

In order to identify potential infDCs in humans, we analyzed myeloid cell populations in two different inflammatory environments: synovial fluid from rheumatoid arthritic patients and inflammatory tumor ascites from untreated cancer patients. In both series of samples, we observed the presence of CD11c$^+$ HLA-DR$^+$ cells that could be divided into two main populations: CD16$^+$BDCA1$^-$ cells and CD16$^-$BDCA1$^+$ cells (Figures 1A and 1B). BDCA1$^+$ cells from synovial fluid (Figure 1C) and tumor ascites (Figure 1D) displayed typical DC morphology with numerous dendrites, distinct from the macrophage-like morphology of CD16$^+$BDCA1$^-$ cells (Figures 1C and 1D). BDCA1$^+$ cells represented around 15% of CD11c$^+$ HLA-DR$^+$ cells in synovial fluid (Figure 1E) and 25% in tumor ascites (Figure 1F). Another defining feature of DCs is their ability to activate T cells. In an allogeneic mixed leukocyte reaction, BDCA1$^+$ cells were potent inducers of CD4 T cell proliferation, whereas CD16$^+$ BDCA1$^-$ cells were poor stimulators (Figures 1G and 1H). Constitutive expression of major histocompatibility class II molecules, dendritic morphology, and T cell activation ability therefore identify BDCA1$^+$ cells as bona fide DCs. By contrast, CD16$^+$ BDCA1$^-$ cells can be defined as macrophages.

A detailed phenotypic analysis showed that these DCs express CD206, CD11b, and CD172a (Sirpα), similar to CD16$^+$ BDCA1$^-$ macrophages (Figure 2A). DCs also express CD14, although at lower levels than macrophages. DCs, but not macrophages, express CD1a and FcεRI, whereas neither cell type expressed CD209 (DC-SIGN). This phenotype was distinct from that of blood BDCA1$^+$ DCs (Figure 2B). In addition, no population of CD11c$^+$ HLA-DR$^+$ CD14$^+$ BDCA1$^+$ CD206$^+$ cells could be found in noninvaded human lymph nodes from untreated breast cancer patients (Figure 2C), in human tonsils from healthy patients (data not shown), or in spleens from pancreatic cancer patients (Figure 2D). Of note, in the spleens of patients with gastric cancer, which is associated with chronic inflammation (Grivennikov et al., 2010), we observed CD11c$^+$ HLA-DR$^+$ CD14$^+$ BDCA1$^+$ FcεRI$^+$ CD206$^+$ cells (Figure 2E), indicating that this population of DCs can also be found in secondary lymphoid organs in some inflammatory situations. These results therefore identify a novel population of DCs present in inflammatory environments. These cells will be referred to as infDCs.

Inflammatory DCs Represent a Distinct DC Subset

To address whether human infDCs correspond to a distinct subset or to an activated form of conventional DCs, we used Affymetrix microarrays to compare the transcriptomes of DCs and macrophages purified from five inflammatory ascites to that of purified CD14$^+$CD16$^-$ and CD14^{dim}CD16$^+$ monocytes and BDCA1$^+$ DCs from the blood of four healthy donors.

To assess the reliability of the microarray analysis, we compared the expression of messenger RNA (mRNA) encoding CD14, CD16 and CD1c (BDCA1) across the different populations analyzed (Figure 3A). We found that mRNA expression of these markers correlated with the known surface phenotype of the different populations. Probe sets with a value higher than 25 fluorescence units were selected for analysis. Using a one-way ANOVA test (with a False Discovery Rate of 0.01), we identified 5,459 genes (corresponding to 5,760 probe sets) that were differentially expressed among the five populations. We next selected genes whose expression varied with a minimum fold change of 2 when compared to the gene expression of infDCs (n = 2,012) and used them to explore the relationship between the five cell types examined based on hierarchical clustering (Figure 3B). This analysis showed that infDCs were more closely related to inflammatory macrophages than to other populations (Figure 3C). This finding was further supported by principal component analysis, which showed in addition that the gene expression pattern of infDCs was closer to both inflammatory macrophages and blood BDCA1$^+$ DCs than to monocytes (Figure 3D). We conclude that infDCs represent a distinct DC subtype.

Inflammatory DCs Share Molecular Features with Both Conventional DCs and Inflammatory Macrophages

Focusing on the major functions of DCs, we selected differentially expressed genes corresponding to the biological functions of antigen processing (GO 0019882), chemotaxis (GO 0006935), and endocytosis (GO 0006897) (the full list of genes is presented in Table S1 available online). Hierarchical clustering using these sets of genes showed that the antigen processing signature of infDCs was closely related to that of blood BDCA1$^+$ DCs (Figure 4A), suggesting that antigen processing and presentation is a cardinal feature of infDCs. By contrast, the chemotaxis and endocytosis transcriptomic pathways of infDCs were closer to that of inflammatory macrophages than to those of other populations, most likely reflecting the effect of the inflammatory environment on the properties of infDCs and macrophages. We also analyzed the expression of pathogen-recognition receptors (PRR) by the different populations (Figure 4B). The PRR expression pattern of infDCs was similar to that of inflammatory macrophages and close to that of CD14$^+$ monocytes, suggesting a specialization in the type of pathogens detected by infDCs as compared to conventional BDCA1$^+$ DCs.

To gain some insight into the development of infDCs, we analyzed the expression of transcription factors (Figure 4C) and growth factor receptors (Figure 4D) involved in myeloid

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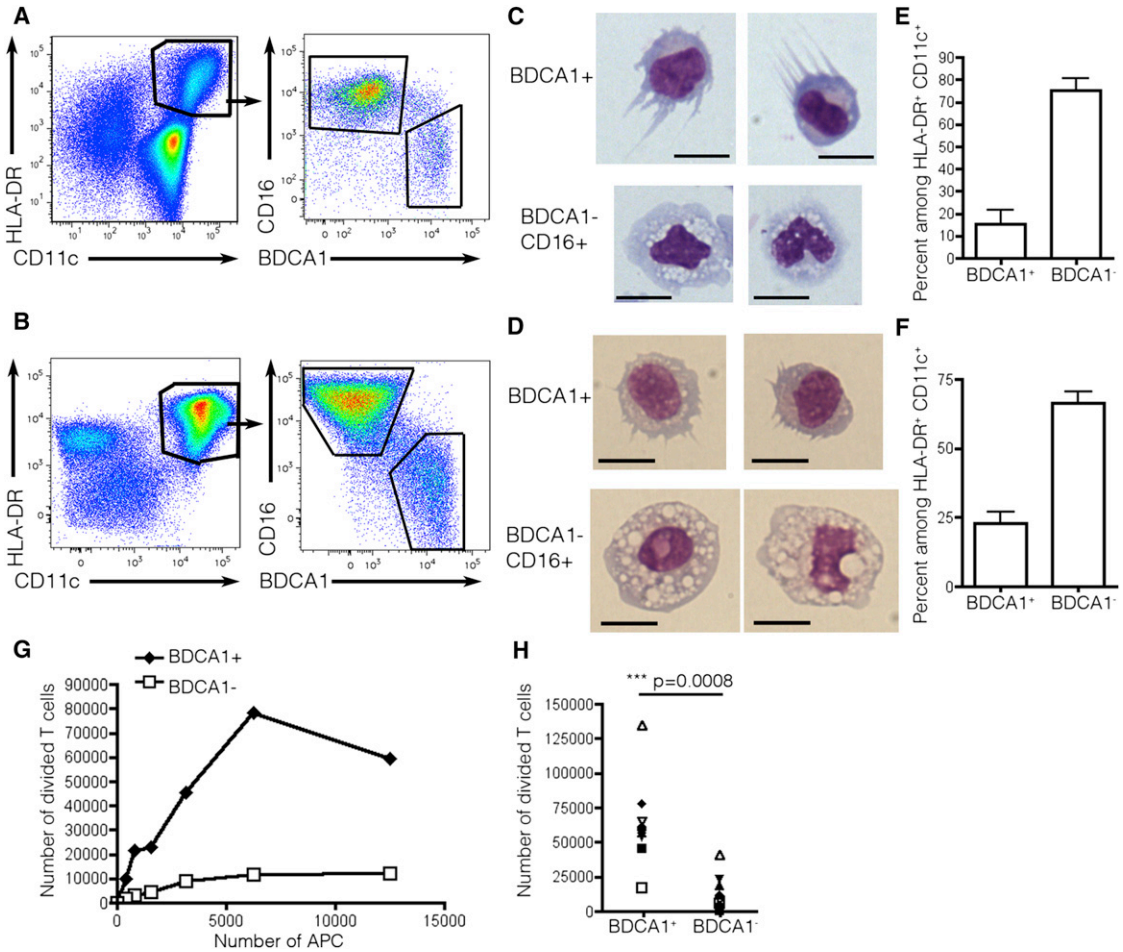

Figure 1. Identification of Dendritic Cells in Human Inflammatory Fluids

Light density cells from arthritic synovial fluid (A) or tumor ascites (B) were stained with anti-HLA-DR, CD11c, BDCA1, and CD16 antibodies and analyzed by flow cytometry. One representative experiment out of 4 (A) or 12 (B) is shown. Sorted HLA-DR$^+$ CD11c$^+$ CD16$^-$ BDCA1$^+$ and HLA-DR$^+$ CD11c$^+$ CD16$^+$ BDCA1$^-$ cells from arthritic synovial fluid (C) or ascites (D) were analyzed by microscopy after Giemsa/May-Grünwald staining. Scale bar represents 10 μm. One representative experiment out of 3 (C) or 8 (D) is shown. Percentage of CD16$^-$ BDCA1$^+$ and CD16$^+$ BDCA1$^-$ cells among HLA-DR$^+$ CD11c$^+$ cells from arthritic synovial fluid (E) or ascites (F). Mean ± SD is shown, n = 4 (E) or n = 20 (F).

(G and H) Different numbers of sorted BDCA1$^+$ or BDCA1$^-$ from arthritic synovial fluid or ascites were cultured with CFSE-labeled allogeneic CD4 T cells for 6 days. T cell proliferation was assessed by flow cytometry.

(G) One representative experiment out of 8 (3 for synovial fluid and 5 for ascites).

(H) Number of divided T cells after culture with 6,250 antigen-presenting cells. Symbols represent cells from the same donor (open symbol = synovial fluid, filled symbol = ascites).

development in mice (Geissmann et al., 2010). The expression of mRNA encoding IRF8 and BATF3 was not selective among the five cell populations analyzed. The expression of mRNA encoding EGR1, EGR2, and CSFR1 (all involved in macrophage differentiation in mice) was equally high in infDCs and inflammatory macrophages, as compared to blood BDCA1$^+$ DCs. By contrast, the expression of mRNA encoding MAFB (which is also involved in macrophage differentiation in mice) was high in macrophages and absent in infDCs. Importantly, the expression of mRNA encoding IRF4 and FLT3, which are both involved in DC differentiation in mice, was high in blood BDCA1$^+$ DCs and infDCs and very low in macrophages and monocytes. Finally, we analyzed the expression of mRNA encoding ZBTB46, a transcription factor specific of the conventional DC lineage in both mice and humans that is also expressed in murine Mo-DCs (Meredith et al., 2012; Satpathy et al., 2012). We found that infDCs and blood BDCA1$^+$ DCs, but not inflammatory macrophages or monocytes, expressed mRNA encoding ZBTB46. These results suggest that the developmental pathway of infDCs has characteristics of both DC and macrophage development.

Inflammatory DCs Are Enriched for Gene Signatures of Monocyte-Derived DCs

To address whether infDCs are the in vivo equivalents of Mo-DCs, we designed a two-step strategy. First, we identified gene signatures for macrophages, BDCA1$^+$ DCs, blood CD16$^+$ or CD14$^+$ monocytes, and in vitro-generated Mo-DCs using different sets of publicly available human expression data

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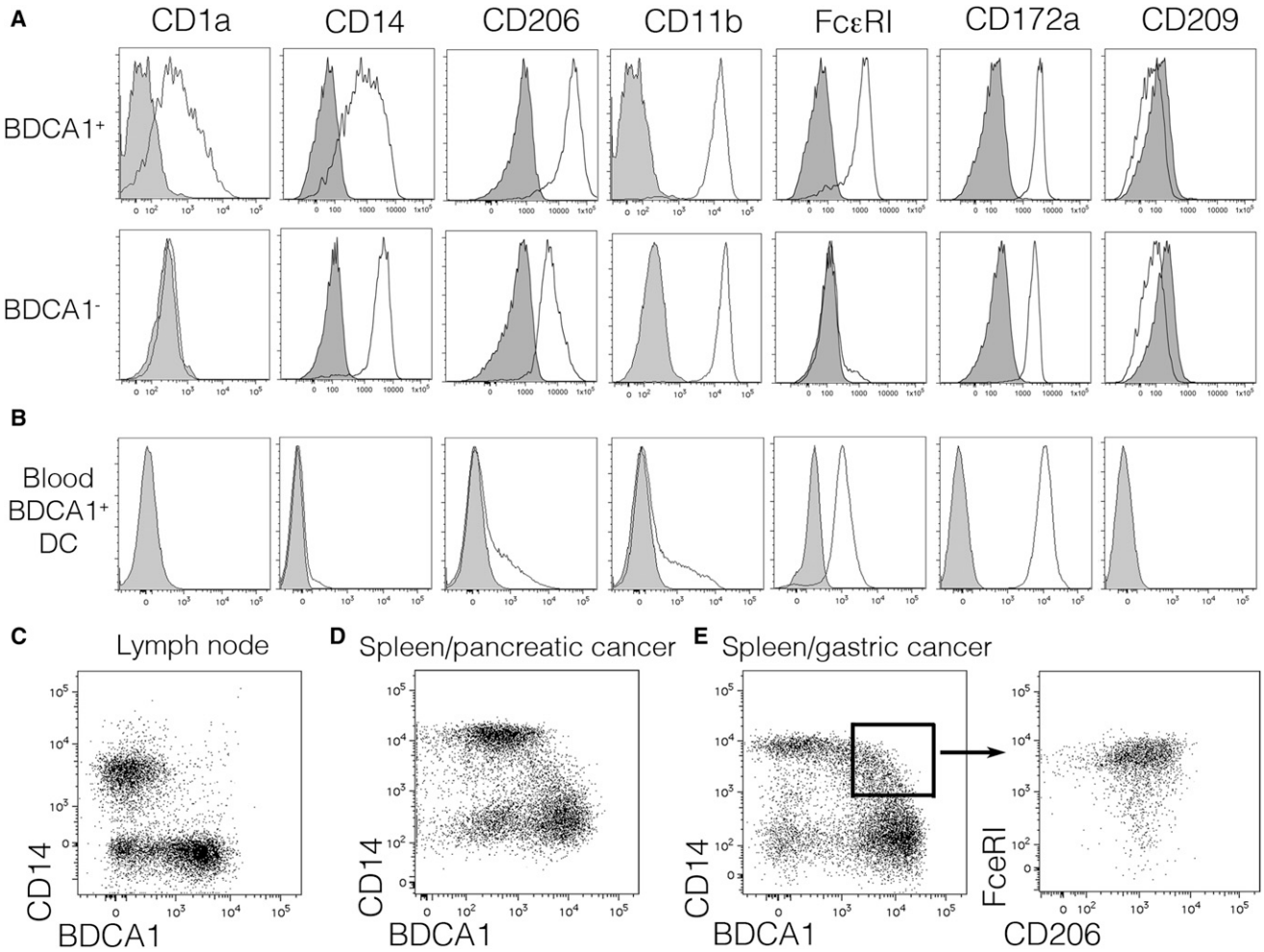

Figure 2. Dendritic Cells from Inflammatory Fluids Display a Distinct Phenotype

(A) Light density cells from arthritic synovial fluid or ascites were stained with anti-HLA-DR, CD11c, BDCA1, CD16 and CD14, CD206, CD1a, CD11b, FcεRI, CD172a, CD209, or control isotype antibodies and analyzed by flow cytometry. BDCA1$^+$ cells were gated as HLA-DR$^+$ CD11c$^+$ CD16$^-$ BDCA1$^+$ and BDCA1$^-$ cells gated as HLA-DR$^+$ CD11c$^+$ CD16$^+$ BDCA1$^-$. One representative experiment out of 8 (2 for synovial fluid and 6 for ascites) is shown.

(B) Blood PBMC were stained for HLA-DR, CD11c, CD16, BDCA1, and CD14, CD206, CD1a, CD11b, FcεRI, CD172a, CD209, or control isotype antibodies and analyzed by flow cytometry. Blood DCs were gated as HLA-DR$^+$ CD11c$^+$ CD16$^-$ BDCA1$^+$. Representative results of six independent experiments.

(C) HLA-DR$^+$ CD11c$^+$ lymph node cells were stained for CD14 and BDCA1. Representative results of four independent experiments.

(D and E) HLA-DR$^+$ CD11c$^+$ spleen cells from pancreatic cancer patients (D) or gastric cancer patients (E) were stained for CD14, BDCA1, FcεRI, and CD206. Representative results of three (D) or two (E) independent experiments.

(Crozat et al., 2010a; Robbins et al., 2008). Then, we compared the transcriptomes of the five populations that we have isolated for the expression of these signature genes.

To identify gene signatures (or GeneSets), we analyzed gene expression data from primary immune cell subsets isolated from tonsil (tonsil BDCA1$^+$ DCs), lung (alveolar macrophages), blood analyzed directly ex vivo (CD14$^+$ monocytes, CD16$^+$ monocytes, BDCA1$^+$ DCs, B lymphocytes, CD4 T cells, CD8 T cells, NK cells, neutrophils), or after 30 hr of in vitro culture in the absence of specific stimuli (resting monocytes, resting B lymphocytes, resting T cells). We also included data sets from in vitro-generated cells (peripheral blood mononuclear cell-derived macrophages, monocyte-derived macrophages, and Mo-DCs). To establish GeneSets specific for Mo-DCs, macrophages, BDCA1$^+$ DCs, blood CD14+ monocytes, and CD16+ monocytes, we selected for each of these five cell populations genes expressed at least 2-fold higher than in all the other cell types examined (Crozat et al., 2010b) (the list of genes for each GeneSet can be found in Table S2).

We then tested whether these GeneSets were enriched in infDCs, inflammatory macrophages, blood CD16$^+$ or CD14$^+$ monocytes, or blood BDCA1$^+$ DCs by performing pairwise comparisons using gene set enrichment analysis (GSEA). For each analysis, the output could be represented as a bar code characterized by two parameters: the normalized enrichment score (NES) and the false discovery rate statistical q value (for more details, see Experimental Procedures). As internal controls, GSEA showed that blood BDCA1$^+$ DCs were more enriched than infDCs for the BDCA1 DC gene signature, whereas inflammatory macrophages were more enriched for the macrophage

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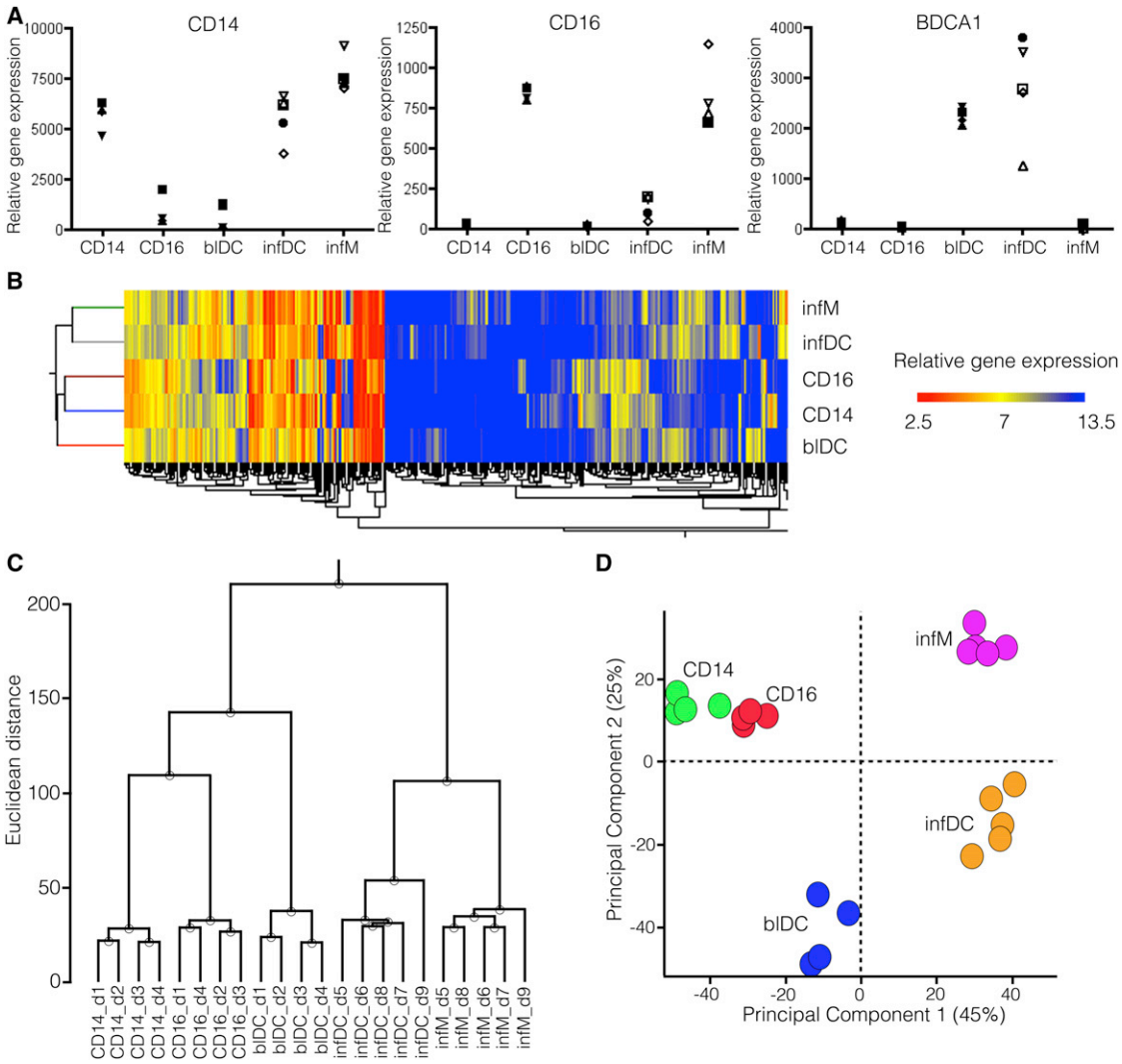

Figure 3. Human Inflammatory Dendritic Cells Represent a Distinct Subset

Gene expression profiles of purified blood CD14$^+$CD16$^-$ monocytes (CD14), CD14^{dim}CD16$^+$ monocytes (CD16), and BDCA1$^+$ DCs (bIDC) from four healthy donors, and purified inflammatory DCs (infDC) and inflammatory macrophages (infM) from five ascites were analyzed by Affymetrix microarrays.

(A) Expression of mRNA encoding CD14, CD16, and CD1c (BDCA1).

(B) We selected 2,012 genes for analysis based on differential expression and fold change > 2 compared to infDC and clustered on a heat map.

(C) Hierarchical clustering of the different samples using the 2,012 selected genes.

(D) Principal component analysis of differentially expressed genes. Principal component 1 and 2 (PC1 and PC2, respectively) were selected as the axes explaining most of the data variance.

gene signature than infDCs (Figure 5A, right panels). GSEA indicated that infDCs were more enriched for the Mo-DC gene signature than blood BDCA1$^+$ DCs or inflammatory macrophages (Figure 5A, left panels).

In order to obtain a wider view of GeneSet enrichment in the purified populations, we represented the bar code information for each pairwise GSEA as a dot whose color corresponded to the cell in which the gene signature was more represented. The dot area was proportional to the NES and the color intensity indicated the q value (a dark, large dot indicated a strong enrichment; Figures 5A and 5B). When compared to the four other cell types studied, inflammatory macrophages were always enriched for the macrophage signature (four red dots highlighted by a rectangle in the second upper panel; Figure 5B), which was not the case for other GeneSets (there was always at least one green dot per column). For instance, inflammatory macrophages were enriched for the Mo-DC gene signature when compared to blood CD14$^+$ or CD16$^+$ monocytes, or to BDCA1$^+$ DC, but not when compared to infDCs. Therefore, the macrophage signature was the dominant gene signature for inflammatory macrophages. Similarly, the BDCA1$^+$ DC signature was dominant for blood BDCA1$^+$ DCs, the CD16 monocyte signature was the dominant gene signature for blood CD16$^+$ monocytes, and the monocyte signature was the dominant gene signature for blood CD14$^+$ monocytes (however, this GeneSet only comprised six genes, so results should be taken with caution)

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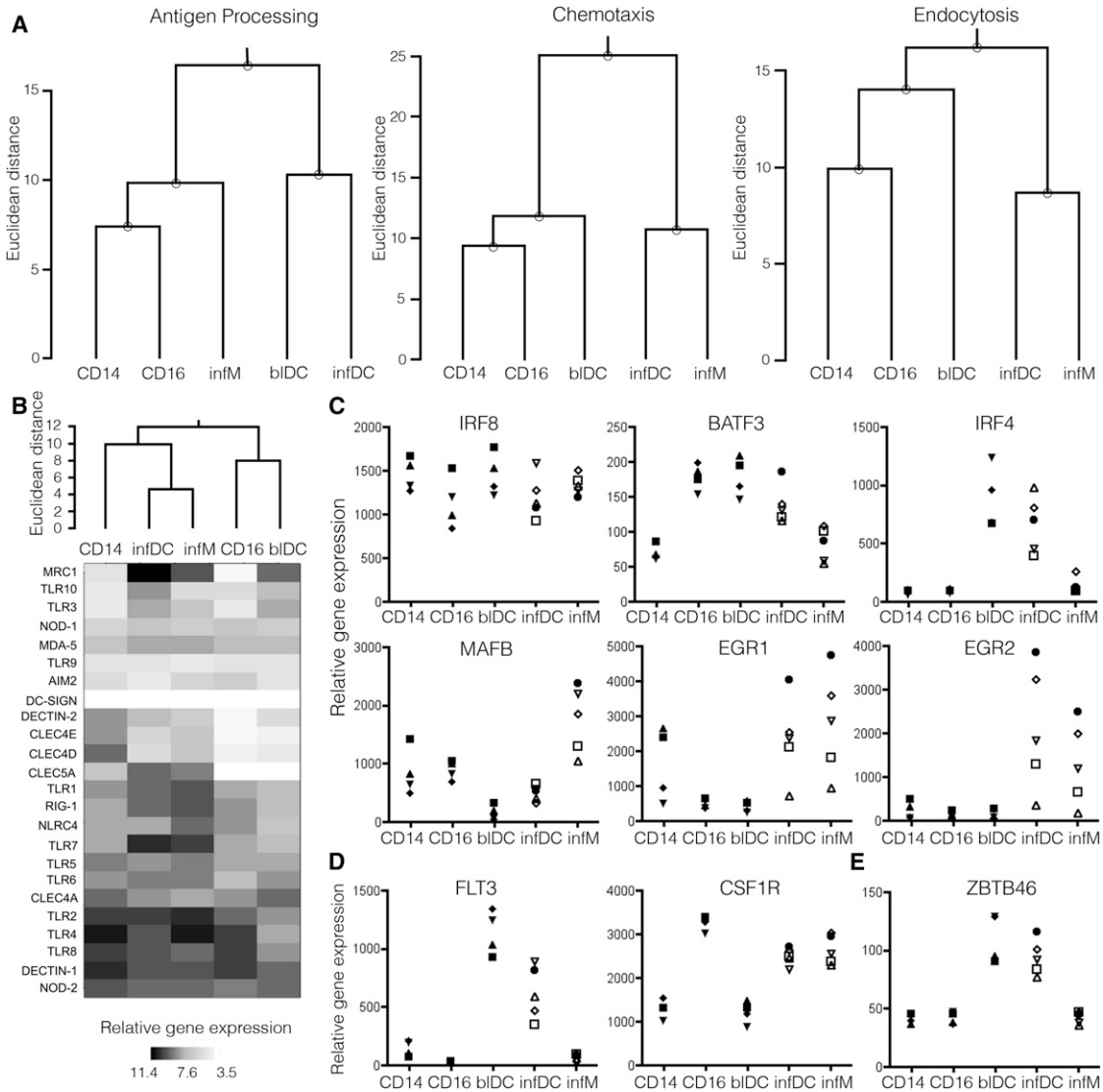

Figure 4. Human Inflammatory Dendritic Cells Share Transcriptomic Signatures with Both Blood Dendritic Cells and Inflammatory Macrophages

(A) Hierarchical clustering using sets of differentially expressed genes corresponding to the Gene Ontology biological functions of antigen processing (53 genes), chemotaxis (73 genes), or endocytosis (100 genes). Populations analyzed were blood $CD14^{+}CD16^{-}$ monocytes (CD14), $CD14^{dim}CD16^{+}$ monocytes (CD16) and $BDCA1^{+}$ DCs (blDC), and inflammatory DCs (infDC) and inflammatory macrophages (infM). See also Table S1.

(B) Heat map representing the relative gene expression of pathogen recognition receptors.

(C) Relative gene expression of selected transcription factors.

(D) Relative gene expression of selected growth factor receptors.

(Figure 5B). These results show that the gene signatures defined here are relevant to distinguish the different myeloid populations analyzed.

In order to clarify the relationship of infDCs to the other purified populations, a similar analysis was applied (Figure 5B, upper panel). The Mo-DC signature was the dominant gene signature for infDCs (Figure 5B, four red dots highlighted by a rectangle in the upper panel) suggesting that, similar to murine infDCs and BM-DCs (Xu et al., 2007), human infDCs are the in vivo counterparts of Mo-DCs. infDCs were also enriched for the macrophage gene signature (second column of the upper panel, three red dots), which in this case cannot be attributed to the inflammatory environment because in vitro-generated macrophages were included to establish the gene signature and possibly reflects molecular programs resulting from the differentiation from monocytes. Finally, infDCs were also enriched for the $BDCA1^{+}$ DC gene signature (third column of the upper panel, three red dots), consistent with our previous conclusions that infDCs expressed molecular signatures involved in DC functions. We conclude that infDCs were more enriched for the Mo-DC gene signature than all other populations analyzed and therefore most likely represent the in vivo equivalents of Mo-DCs. These

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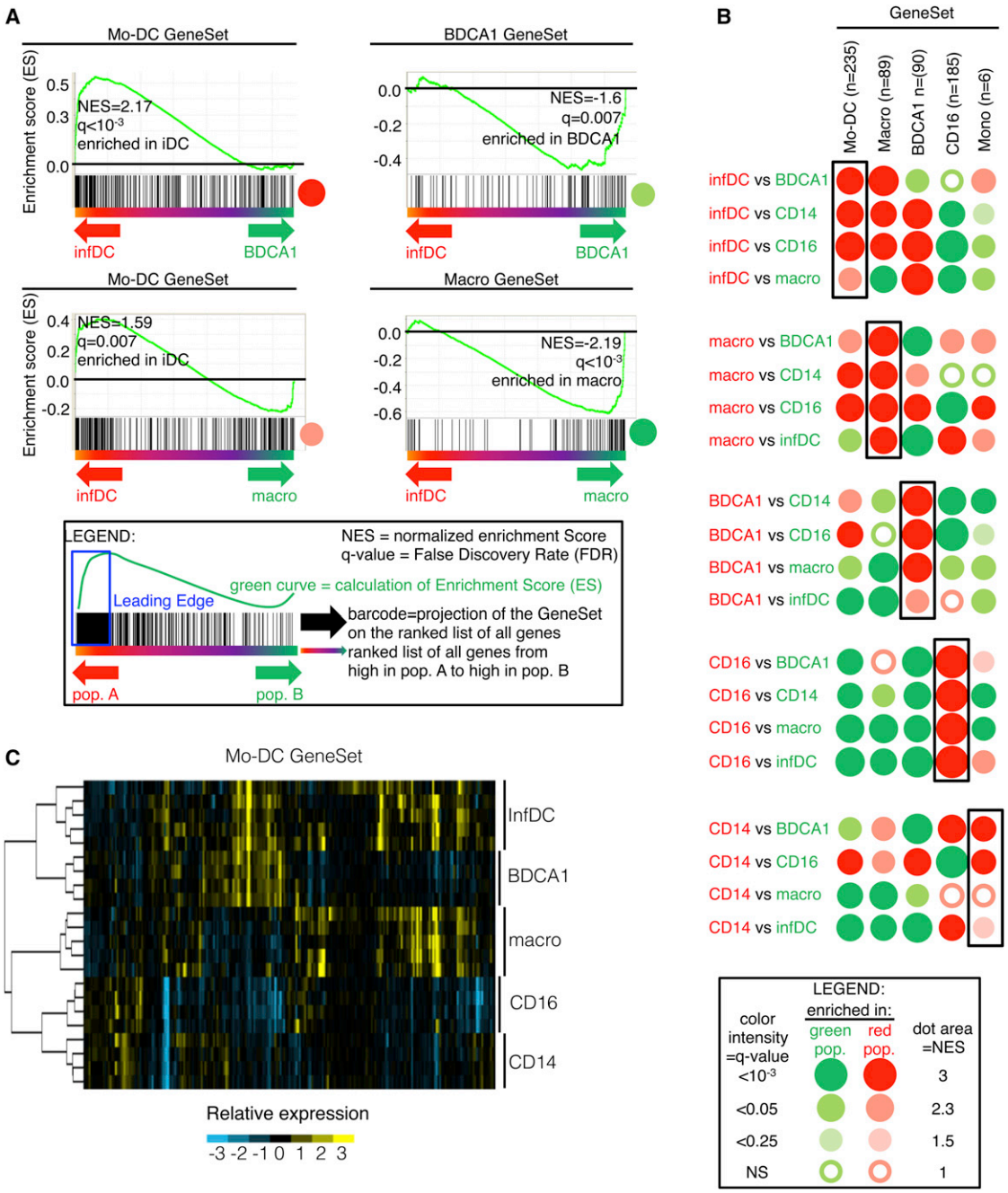

Figure 5. Human Inflammatory Dendritic Cells Are Enriched for Monocyte-Derived Dendritic Cell Gene Signature

GSEA of the gene signature (GeneSet) of Mo-DCs, macrophages (macro), blood and tonsil BDCA1$^+$ DCs (BDCA1), blood CD16$^+$ monocytes (CD16), and blood CD14$^+$ monocytes (mono) was performed.

(A) GSEA results for pairwise comparisons involving infDCs. The GSEA output is represented as a bar code characterized by two parameters: the normalized enrichment score (NES) and the false discovery rate statistical value (q).

(B) Dot blot representation of all pairwise GSEA comparisons between infDCs, inflammatory macrophages (macro), blood CD16$^+$ (CD16) or CD14$^+$ (CD14) monocytes, and blood BDCA1$^+$ DCs (BDCA1). GeneSets comprise different number of genes (n). Dot color corresponds to the font color of the population in which the GeneSet is enriched. The dot area is proportional to the NES, which varies from 1 (no enrichment) to a maximum of 5 (all genes of the GeneSet are expressed to higher levels in the same population). The color intensity is indicative of the false-discovery rate statistical q value, which estimates the likelihood that the enrichment of the GeneSet represents a false-positive finding. See also Table S2.

(C) Hierarchical clustering of gene expression for the Mo-DC GeneSet. Data is shown in Log2 centered to median expression across all samples (i.e., values around the median are shown in black, values above in yellow, and values below in blue).

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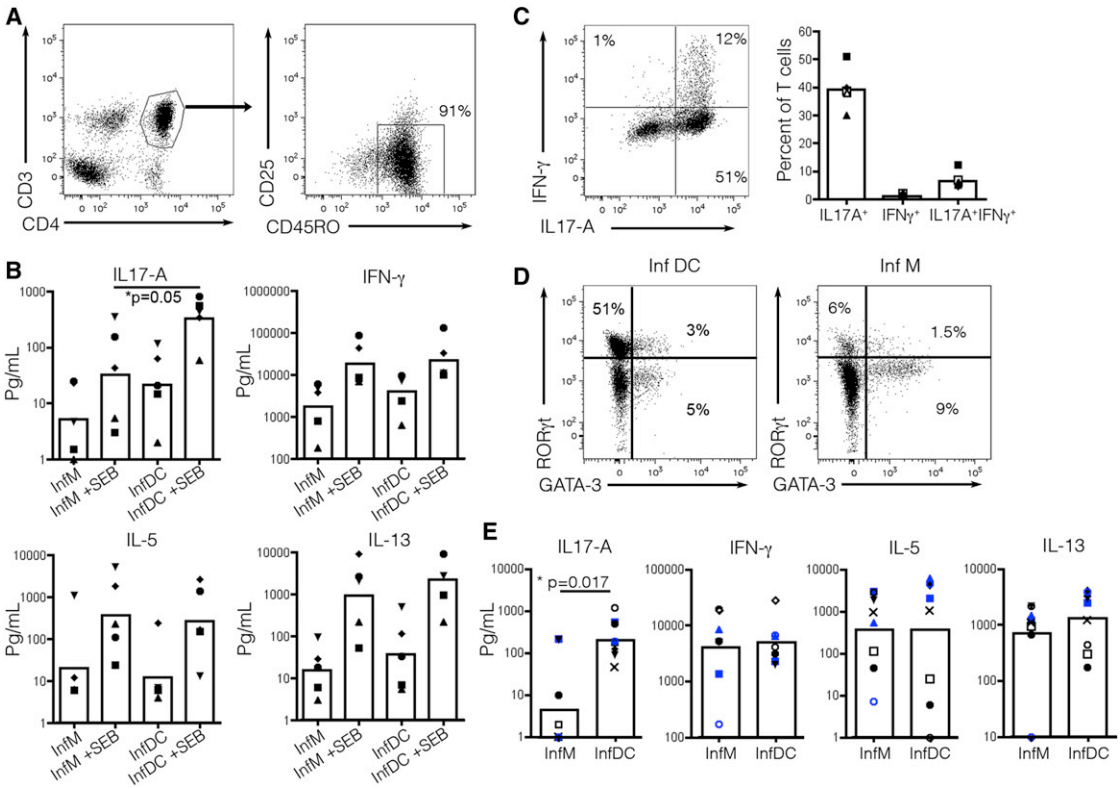

Figure 6. Human Inflammatory Dendritic Cells Induce Th17 Cells

(A) Cells from ascites were analyzed for the expression of CD3, CD4, CD25, and CD45RO. Representative result of five independent experiments.

(B) Purified macrophages (inf M) or DCs (inf DC) from ascites were cultured for 6 days with autologous ascites memory CD4 T cells in the presence or absence of SEB. After washing and T cell restimulation, cytokine secretion was measured in the supernatant. Symbols represent results from the same donor (n = 5). Mean is shown.

(C) Purified DCs from ascites were cultured for 18 hr with autologous ascites' memory CD4 T cells in the presence of SEB, then fixed, permeabilized, and stained for IFN-γ and IL-17A. Representative result of five independent experiments and percentage of each population among T cells are shown. Symbols represent results from the same donor (n = 5). Mean is shown.

(D) Purified macrophages or DCs from ascites were cultured for 6 days with allogeneic naive CD4 T cells. Cells were fixed, permeabilized, and stained for GATA-3 and RORγt. Representative result of four independent experiments. (E) Purified macrophages or DCs from arthritic synovial fluid or ascites were cultured for 6 days with allogeneic naive CD4 T cells before washing and T cell restimulation. Cytokine secretion was measured in the supernatant. Symbols represent results from the same donor (n = 3 for synovial fluid, blue symbols, n = 8 for ascites and n = 6 for blood). Mean is shown.

results also suggest that infDCs, like Mo-DCs and macrophages, are derived from monocytes.

Because the Mo-DC signature was highly enriched in infDCs, inflammatory macrophages and blood BDCA1$^+$ DCs, we next evaluated whether the shared sets of genes between these populations were similar or distinct. Hierarchical clustering showed that infDCs express higher number of genes from the Mo-DC signature than BDCA1$^+$ DCs or macrophages (compare the amount of yellow in the infDC versus the BDCA1 or the macrophage samples; Figure 5C). This analysis also showed that the genes from the Mo-DC signature shared between infDCs and blood BDCA1$^+$ DCs were different from the genes shared by infDCs and inflammatory macrophages, confirming that infDCs display molecular features of both conventional DCs and inflammatory macrophages.

Inflammatory DCs Induce Th17 Cell Differentiation Ex Vivo

We then analyzed the functional properties of infDCs. Inflammatory Mo-DCs in the mouse have been shown to activate memory T cells directly in the tissues (Wakim et al., 2008). We therefore addressed whether infDCs from ascites could activate CD4$^+$ T cells isolated from the same ascites. CD4$^+$ T cells in tumor ascites were mainly memory T cells, as shown by the expression of CD45RO and absence of CD25 (Figure 6A). InfDCs, but not inflammatory macrophages, could stimulate ascites' memory CD4$^+$ T cells to produce IL-17A (Figure 6B). When incubated with a superantigen, *Staphylococcus aureus* Enterotoxin B (SEB), both cell types could induce autologous memory CD4$^+$ T cells to produce IFN-γ, as well as low amounts of IL-13 and IL-5 (Figure 6B). Virtually no IL-10 was detected with either cell population (data not shown). In the presence of SEB, both infDCs and macrophages activated ascites' memory CD4$^+$ T cells to produce IL-17A, but infDCs were significantly more efficient (Figure 6B). Most IL-17A-secreting T cells did not produce IFN-γ, whereas all IFN-γ-secreting T cells also secreted IL-17A (Figure 6C). These results show that infDCs are potent stimulators of Th17 cells as compared to inflammatory macrophages.

Because infDCs can also be found in secondary lymphoid organs (Figure 2E), we then addressed the ability of these cells

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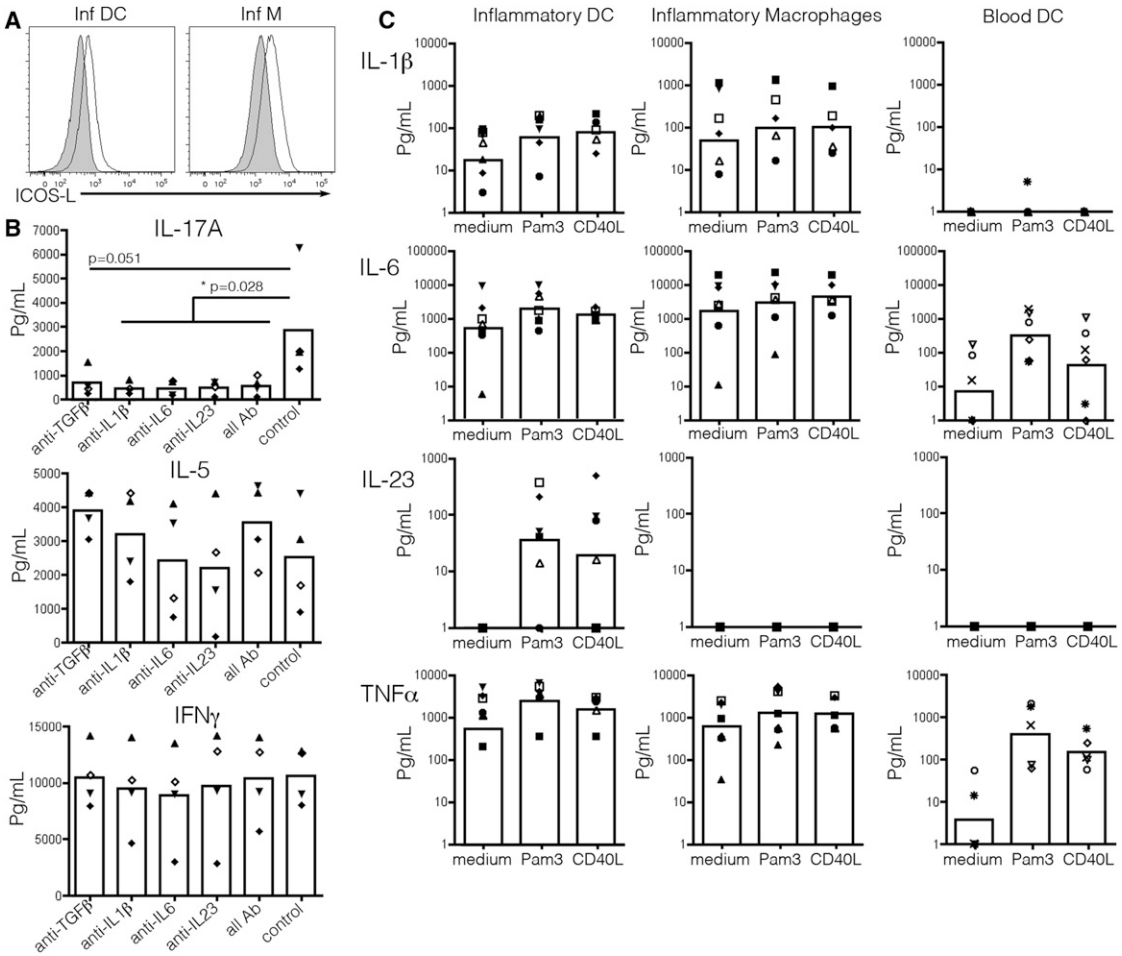

Figure 7. Only Inflammatory Dendritic Cells Secrete Th17 Cell-Polarizing Cytokines

(A) Light density cells from ascites were stained with anti-HLA-DR, CD11c, BDCA1, CD16. and ICOS-L or control isotype antibodies and analyzed by flow cytometry. infDCs were gated as HLA-DR$^+$ CD11c$^+$ CD16$^-$ BDCA1$^+$ and inflammatory macrophages (infM) were gated as HLA-DR$^+$ CD11c$^+$ CD16$^+$ BDCA1$^-$. Representative results of four independent experiments.

(B) Purified DCs from ascites were cultured for 6 days with allogeneic naive CD4 T cells in the presence or absence of blocking antibodies, or isotype control, before washing and T cell restimulation. Cytokine secretion was measured in the supernatant. Symbols represent results from the same donor (n = 4). Mean is shown.

(C) Purified macrophages or DCs from ascites or DCs from blood of healthy donors were cultured for 24 hr with or without Pam3Csk4 (Pam3) or dimerized CD40-Ligand (CD40L). Cytokine secretion was measured in the supernatant. Symbols represent results from the same donor (n = 7 for ascites and n = 6 for blood). Mean is shown. See also Figure S1.

to polarize naive CD4$^+$ T cells by analyzing the Th profiles induced directly ex vivo by infDCs and inflammatory macrophages (Figures 6D and 6E). InfDCs and inflammatory macrophages induced naive allogeneic CD4$^+$ T cells to produce very low amounts of IL-13 and IL-5, and similar amounts of IFN-γ (Figure 6E). Virtually no IL-10 was detected with either cell population (data not shown). By contrast, only infDCs induced significant levels of the Th17 cell cytokine IL-17A. Consistent with this, while very few CD4$^+$ T cells cultured with inflammatory macrophages expressed RORγt (Figure 6D), a high proportion of the Th cells induced by infDCs expressed exclusively RORγt and not GATA-3, the master regulators of Th17 and Th2 lineages respectively (Ivanov et al., 2006; Zheng and Flavell, 1997) (Figure 6E). We conclude that infDCs, but not inflammatory macrophages, are potent inducers of Th17 cell-mediated responses ex vivo.

Only Inflammatory DCs Secrete Th17 Cell-Polarizing Cytokines

Finally, we investigated the molecular mechanisms of this specialization. Costimulation through the ICOS/ICOS-L axis has recently been shown to be important for Th17 cell differentiation (Paulos et al., 2010). We found that ICOS-Ligand was expressed at low amounts by both infDCs and inflammatory macrophages (Figure 7A) and therefore could not explain their differential Th17 cell polarization ability.

In humans, IL-6, IL1-β, TΓΦβ, and IL-23 have been proposed to induce the development of Th17 cells (Acosta-Rodriguez et al., 2007; Manel et al., 2008; Volpe et al., 2008; Wilson et al., 2007). In order to address the requirement for these cytokines in infDC-induced Th17 cell polarization, CD4$^+$ naive T cells were incubated with allogeneic infDCs in the presence of

blocking antibodies to TGFβ, IL-1β, IL-6, IL-23, a cocktail of all the antibodies or an isotype control (Figure 7B). The blocking antibodies did not affect the differentiation of naive T cells into Th1 (as measured by the secretion of IFN-γ) or Th2 (as measured by the secretion of IL-5). By contrast, the secretion of IL-17A was severely impaired by the blocking antibodies, as compared to isotype control.

We then analyzed the cytokines secreted by the different antigen-presenting cells (Figure 7C). We analyzed the secretion of IL-12p70 after activation with a cocktail of CD40-ligand and IFN-γ in the presence or absence of Pam_3Csk_4 (Figure S1). Only infDCs, but not macrophages, secreted IL-12p70. After activation by Pam_3Csk_4, a TLR2/TLR1 ligand, or by CD40-ligand to mimic the interaction with T cells, infDCs secreted levels of IL1-β, IL-6, and TNF-α similar to that of inflammatory macrophages and higher than those secreted by blood BDCA1$^+$ DCs, even after stimulation. By contrast, stimulated infDCs, but no other cell type tested, secreted IL-23 after activation. We conclude that infDCs induce Th17 cell polarization through the selective secretion of Th17 cell polarizing cytokines, notably IL-23.

DISCUSSION

We have identified a population of human DCs present in two different inflammatory environments, ascites from untreated ovary and breast cancer patients, and synovial fluid from rheumatoid arthritis patients. InfDCs displayed a unique phenotype, including several distinctive markers absent from conventional DCs. InfDCs were potent stimulators of Th17 cells as compared to macrophages.

We propose that infDCs are the human equivalents of murine monocyte-derived inflammatory DCs. Human and mouse inflammatory DCs share phenotypic similarities: both DC types express CD11b (Greter et al., 2012; León et al., 2007), CD206 (Segura et al., 2009), CD172a (Greter et al., 2012), and FcεRI (Hammad et al., 2010). A cardinal feature of mouse infDCs is that they are derived from Ly6C^{hi} monocytes and not from committed DC progenitors (Guilliams et al., 2009; León et al., 2007; Naik et al., 2006). Transcriptome analysis showed that human infDCs express transcription factors involved in both DC and macrophage development, suggesting that infDCs have a specific developmental pathway. In addition, infDCs were specifically enriched for the Mo-DC gene signature and are therefore most likely derived from monocytes rather than from DC precursors. Nevertheless, whether infDCs originate from CD14$^+$ monocytes, which have been shown to be the equivalents of murine Ly6C^{high} monocytes (Cros et al., 2010), or from CD16$^+$ monocytes, could not be determined by our analysis and remains open for future investigation (GSEA suggests that infDCs were more enriched for the CD14$^+$ monocyte gene signature than for the CD16$^+$ monocyte one, but the former only included 6 genes limiting the reliability of the results, Figure 5B). Finally, mouse Mo-DCs express the DC lineage-specific transcription factor *Zbtb46* (Satpathy et al., 2012), and CSF1R/CD115 has recently been shown to be important for mouse infDC development in vivo (Greter et al., 2012). Both *ZBTB46* and *CSFR1* were highly expressed in human infDCs, suggesting a similar developmental pathway.

Two series of studies claimed previously to describe inflammatory DCs in the skin of atopic dermatitis (Wollenberg et al., 1996) and psoriasis (Zaba et al., 2009) patients. Similar to infDCs, IDECs express HLA-DR, CD11c, BDCA1, CD1a, FcεRI, CD206, and CD11b (Guttman-Yassky et al., 2007; Wollenberg et al., 2002). These cells are distinct from Langerhans cells, because they do not express Langerin and do not display Birbeck granules (Wollenberg et al., 1996). Even if the transcriptome, morphology, or ontogeny of IDECs has not been analyzed, because they appear only in lesional skin, it seems reasonable to hypothesize that IDECs are actually "inflammatory" DCs. IDECs, however, were not related to Th17 cell environments, because the acute phase of atopic dermatitis is initiated by Th2-type inflammation, whereas the chronic phase of the disease is dominated by a Th1-type response (Bieber, 2010). Using in vitro differentiated Mo-DC as a model of IDECs, it has been proposed that IDECs play a role in initiating Th1 cells differentiation (Novak et al., 2004), although the type of Th responses induced by naturally-occurring IDECs has not been investigated.

The population of HLA-DR$^+$ CD11c$^+$ cells that appears in inflammatory skin lesions from psoriasis patients have been termed "inflammatory dermal" DCs (Zaba et al., 2009). Because the surface markers studied were different from the defining phenotypic markers of IDECs, it is difficult to determine whether these cells are similar to IDECs and infDCs. These "inflammatory dermal" antigen-presenting cells induce Th1 and some Th17 cell polarization when cultured with allogeneic total T cells (Zaba et al., 2009). They do not express BDCA1 and were recently proposed to be similar to slanDC (Hansel et al., 2011), a population of CD16$^+$ antigen-presenting cells found in the blood (Schäkel et al., 2002). It is not clear, however, whether these "inflammatory dermal" antigen-presenting cells are actual DCs or activated monocytes or macrophages. Indeed, morphological evidence is lacking and these cells share a number of features with dermal macrophages, including expression of phenotypic markers and inflammatory cytokines (Fuentes-Duculan et al., 2010; Zaba et al., 2010). In addition, slanDC have been recently shown to be a subset of CD16$^+$ monocytes by transcriptomic and functional analysis (Cros et al., 2010).

Monocytes are generally considered to be very plastic cells. Mouse monocyte-derived infDCs have been shown to induce Th1- (León et al., 2007; Nakano et al., 2009) or Th2-mediated responses (Hammad et al., 2010; Kool et al., 2008) depending on the pathology. Moreover, human monocytes can be differentiated in vitro into phenotypically and functionally diverse Mo-DCs with selective Th cell polarization abilities depending on the growth factors and cytokines used, suggesting that Mo-DCs may be polarized differently depending on the environment (Geissmann et al., 2010). Our results show that in the case of tumor ascites and rheumatoid arthritis, infDCs induce preferentially Th17 cells. Whether other inflammatory environments induce other types of Th cell polarization in vivo remains open for future investigation.

The cytokines directing Th17 cell differentiation have been extensively studied using in vitro stimulation of CD4$^+$ T cells with recombinant cytokines. IL-6, IL1-β, TΓΦβ, and IL-23 have been proposed to be involved in human Th17 cell polarization, but whether all these cytokines are essential has been controversial (Acosta-Rodriguez et al., 2007; Manel et al., 2008; Volpe

et al., 2008; Wilson et al., 2007). Here, we took advantage of the discovery of naturally-occurring Th17 cell-polarizing DCs to revisit this issue and we found that blocking IL-6, IL1-β, TΓΦβ, or IL-23 impaired Th17 cell polarization ex vivo. Our results therefore confirm that all these cytokines are required for Th17 cell differentiation. Recently, two types of human Th17 cells have been described that are induced by different types of pathogens, *Candida albicans* and *Staphyloccocus aureus* (Zielinski et al., 2012). Similar to *Candida albicans*-induced Th17 cells, infDC-induced Th17 cells secreted IL-17 and IFN-γ and their differentiation required IL-1β, showing that this Th17 cell profile is also induced during sterile inflammation.

Th17 cells have been implicated in the pathogenesis of several autoimmune or inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, asthma, inflammatory bowel disease, and psoriasis (Hu et al., 2011), as well as in tumors (Zou and Restifo, 2010). A large number of studies, both in humans and in mouse models, have evidenced a pathogenic role for IL-17 in these diseases, and IL-17 has become a major therapeutic target. Preclinical studies showed that blocking IL-17 with neutralizing antibodies suppresses collagen-induced arthritis (Lubberts et al., 2004) or ameliorates symptoms in experimental autoimmune encephalomyelitis (Hofstetter et al., 2005). Moreover, treating patients with blocking antibodies against IL-23 has shown promising results in the treatment of psoriasis (Kimball et al., 2008; Krueger et al., 2007) and Crohn's disease (Sandborn et al., 2008). Our study shows that infDCs are the main inducers of Th17 cells in arthritic joints, and possibly in other inflammatory settings, through the secretion of IL-23. The set of selective markers for infDCs that we define here should allow the analysis of frequency and the function of these cells in human inflammatory diseases.

EXPERIMENTAL PROCEDURES

Cell Isolation

Samples of ovarian or breast tumor ascites from untreated patients were obtained from Hôpital de l'Institut Curie (Paris) and samples of synovial fluid were obtained from Hôpital Cochin (Paris) in accordance with Institut Curie and INSERM ethical guidelines. Cells were isolated after centrifugation on a Ficoll gradient (Lymphoprep, Greiner Bio-One) followed by cell sorting on a FACSAria instrument (BD Biosciences). Samples of lymph nodes from untreated cancer patients undergoing diagnostic surgery were obtained from Hôpital de l'Institut Curie (Paris) in accordance with the Institut Curie and INSERM ethical guidelines. Only lymph nodes considered healthy (noninvaded) after anatomopathological examination were included in the study. Lymph node samples were cut into small fragments, digested with 0.1 mg/mL Liberase TL (Roche) in the presence of 0.1 mg/mL DNase (Roche) for 20 min before addition of 10 mM EDTA. Cells were filtered on a 40 μm cell strainer (BD Falcon) and washed. DCs were enriched by depletion of cells expressing CD3, CD15, CD19, CD56, and CD235a using antibody-coated magnetic beads and magnetic columns according to manufacturer's instructions (Miltenyi). Spleen samples were obtained from pancreatic or gastric cancer patients undergoing surgery at Hôpital de l'Institut Curie or Hôpital Saint-Antoine in accordance with institutional guidelines. Light density cells were obtained by centrifugation on a Ficoll gradient. For two of the pancreatic cancer patients, splenic DCs were previously shown to be nonactivated (Nascimbeni et al., 2009). Buffy coats from healthy donors were obtained from Etablissement Français du Sang. PBMC were prepared by centrifugation on a Ficoll gradient.

Flow Cytometry

See Supplemental Experimental Procedures for the list of antibodies used. Cells were analyzed on a LSR II (BD Biosciences), FACSVerse (BD Biosciences), or MACSQuant (Miltenyi Biotec) instrument. Data was analyzed with FlowJo (Tree Star).

Morphological Analysis

Cells were subjected to cytospin and colored with May-Grunwald/Giemsa staining. Pictures were taken with a CFW-1308C color digital camera (Scion Corporation) on a Leica DM 4000 B microscope.

MLR

Allogeneic $CD4^+$ T cells were isolated from healthy donors' PBMC by negative selection using a $CD4^+$ T cell isolation kit (Miltenyi Biotec). $CD4^+$ T cells were labeled with 5 μM CFSE and cultured (5×10^4 cells/well) with different numbers of antigen-presenting cells. After 6 days, T cell proliferation was assessed by flow cytometry.

Affymetrix Microarray Hybridization

The Affymetrix data has been deposited in GEO (Accession number GSE40484). See Supplemental Experimental Procedures for cell purification strategies. RNA was extracted using the Rneasy Micro Kit (QIAGEN) according to the manufacturer's protocol. For each condition, 100 ng of polysomal-bound RNA were employed to synthesize double-stranded cDNA using two successive reverse-transcription reactions according to the standard Affymetrix protocol. Labeled DNA was hybridized on the Affymetrix human Gene ST1.1, an oligonucleotide 28,000-gene microarray processed on an Affymetrix GeneTitan device.

Statistical Analysis and Data Mining

Statistical analysis was performed using GeneSpring GX 7.3 (Agilent), R package EMA, and Matlab softwares. Principal component analysis was performed as previously described (Volpe et al., 2008). See Supplemental Experimental Procedures for more details.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed as previously described (Crozat et al., 2010a; Robbins et al., 2008). See Supplemental Experimental Procedures for more details.

Cytokine Secretion

Cells (2.5×10^4 cells/well) were incubated during 24 hr in Yssel medium in the presence or absence of 2 μg/mL Pam3Csk4 (Invivogen), 1 μg/mL dimerized CD40-ligand (Alexis), 1 μg/mL dimerized CD40-ligand and 1000 IU/mL IFNγ (Miltenyi), or 1 μg/mL dimerized CD40-ligand and 1000 IU/mL IFN-γ and 2 μg/mL Pam3Csk4. Supernatants were collected and kept at −20°C. Cytokine secretion was assessed by CBA (BD Biosciences) or ELISA (for IL-23, eBioscience).

T Helper Cell Polarization

Naive $CD4^+$ T cells were isolated from healthy donors' PBMC by negative selection followed by cell sorting on a FACSAria instrument (BD Biosciences). Naive $CD4^+$ T cells were gated as $CD4^+CD25^-CD45RA^+CD45RO^-$. Ascites memory $CD4^+$ T cells were isolated from by cell sorting on a FACSAria instrument. Ascites $CD4^+$ T cells were gated as $CD11c^-CD4^+CD25^-CD45RO^+$. Antigen-presenting cells (2×10^4 cells/well) were cultured with blood naive or ascites memory $CD4^+$ T cells (5×10^4 cells/well) for 6 days in Yssel medium. For memory T cells, cells were cultured in the presence or absence of 0.5 μg/mL *Staphylococcus aureus* Enterotoxin B (SEB) (Sigma). In some experiments with naive $CD4^+$ T cells, cells were cultured in the presence of 2.5 μg/mL anti-TGFβ, 2.5 μg/mL anti-IL-1β, 2.5 μg/mL anti-IL-6, 0.5 μg/mL anti-IL-23, or 2.5 μg/mL control isotype (all from R&D systems). After washing, cells were incubated with anti-CD3/CD28 beads (Invitrogen) for 24 hr in X-VIVO 15 serum free medium (Lonza). Supernatants were collected and kept at −20°C. Cytokine secretion was assessed by CBA (BD Biosciences). For 6 day cultures with naive $CD4^+$ T cells, cells were fixed and permeabilized with intracellular staining reagents according to manufacturer's instructions (eBioscience) and stained with eFluor660 anti-GATA3 and PE anti-RORγt (eBioscience). For intracellular staining on ascites' memory $CD4^+$ T cells, cells were analyzed after 18 hr of culture in the presence of SEB and an additional 3 hr in the presence of brefeldin A (Sigma). Cells were stained with APC-Cy7

anti-CD3 (BD Biosciences), then fixed with Cytofix/Cytoperm (BD Biosciences) according to manufacturer's instructions. Cells were stained with FITC anti-IFNγ (IOTest) and PE anti-IL17A (eBioscience).

Statistical Analysis

Wilcoxon matched-paired test or Mann-Whitney test were performed using Prism (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.10.018.

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DISCUSSION

Dans les prochains paragraphes, nous aborderons dans un premier temps les avantages et les inconvénients des modèles expérimentaux utilisés dans nos trois projets.
Nous verrons par la suite si les résultats obtenus répondent aux questions scientifiques posées. Nous diviserons la discussion en 2 parties : l'une centrée sur la biologie des cytokines notamment au cours de la polarisation des Th ; l'autre s'intéressant à l'apport des analyses à large échelle dans l'analyse de population cellulaire.

1. Optimisation et analyse du système expérimental.

1.1 Optimisation d'un système de culture in vitro.

* Tout au long de nos expériences, nous avons essayé d'utiliser un système qui soit le plus fidèle à un contexte physiologique in vivo. Cette démarche est indispensable afin de transposer les conclusions obtenues à partir d'un modèle expérimental à une situation physiologique. La manipulation du modèle humain est plus difficile et moins physiologique que celle du modèle murin dans lequel la grande majorité des découvertes en immunologie a été faite. Cependant, toutes les découvertes faites chez la souris ne sont pas transposables systématiquement chez l'homme. Par exemple, la phosphorylation de STAT4 par les IFN est observée chez l'homme mais pas chez la souris, entraînant une réponse différente lors de la différentiation Th1 et Th2 [175] . Cette disparité inter-espèce a été aussi constatée dans le projet I. En effet, la corrélation entre la production d'IL-22 et l'expression du FT AHR décrite chez la souris, n'a pas été confirmée dans notre modèle.
* Pour les trois projets présentés précédemment, nous avons utilisé à chaque fois des cellules primaires (LT CD4 naïfs ou DC) obtenues grâce à des méthodes de purification et des marqueurs spécifiques. Apres un tri cellulaire par cytométrie de flux par un marquage CD4+CD25-CD45RO-CD45RA+, la pureté de LT naïf obtenue est de l'ordre de 99%. Ces méthodes nous permettent ainsi de diminuer la variabilité interindividuelle.
* La polarisation Th représente un parfait modèle d'intégration de signaux extracellulaire qui conduit à l'induction d'un programme de transcription spécifique. La majorité des études et découvertes sur la polarisation des Th humains ou murins

proviennent d'études in vitro [185, 229, 233]. Nous avons utilisé des conditions expérimentales de polarisation « classiques » publiées par différents groupes dont le nôtre, notamment pour la différentiation Th17 [201]. La seule différence de notre protocole par rapport à d'autres groupes est l'absence d'anticorps bloquant anti IL-12 et anti IL-4. Le principal défaut de ce système in vitro est qu'il est impossible de savoir s'il reflète exactement le processus de différentiation des Th in vivo.

* Nous avons initialement étudié l'IFN-α et l'IFN-β, pour ne retenir que l'IFN-α pour deux raisons : d'une part du fait de sa plus grande implication en physiologie et en thérapeutique ; et d'autre part car l'induction des ISGs et des cytokines était qualitativement et quantitativement moins importante avec l'IFN-β rendant l'analyse statistique plus difficile. La dose utilisée d'IFN-α (10ng/ml) a été déterminée après titration et se rapproche des taux sériques des IFN en thérapeutique (2-3 ng/ml).

1.2 Caractéristique de l'analyse Bio-informatique.

Les projets II et III ont eu recours à une analyse à large échelle du transcriptome de différentes populations cellulaires soumises ou non à des stimuli extracellulaires. Ce type d'analyse permet à la fois une vision globale de l'état cellulaire, et donne à chaque gène la même chance d'émerger comme un facteur clé d'une voie de signalisation de manière non biaisée.

Dans le projet II, nous avons analysé le transcriptome de CD4 différenciés en Th0, Th1, Th2, Th17 en présence ou non d'IFN, pour comprendre comment l'intégration de deux signaux extracellulaires module les fonctions cellulaires des Th. Des puces Affymetrix ont été réalisées dans 12 conditions (Th0, Th1, Th2, Th17 +/- IFNα/β) chez trois donneurs à deux temps précis de la polarisation (J5 et J5 + 4 heures de restimulation antigénique). Il nous a paru nécessaire d'étudier le transcriptome à 2 temps différents compte-tenu de la dynamique d'expression des TF. Ces 2 temps correspondent à 2 situations physiologiques distinctes : la fin du processus de différentiation où les Th expriment de manière « constante » un programme de transcription spécifique (J5), et le programme de transcription déclenché après l'activation du Th par la reconnaissance d'un antigène spécifique (J5 + 4 heures de restimulation antigénique).

Dans le projet III, nous avons choisi spécifiquement 5 populations cellulaires de DC facilement individualisables ex vivo CD14, CD16, cDC, InfDC et les macrophages.

L'extraction d'ARN a été faite selon un procédé rigoureux avec un contrôle qualitatif et quantitatif. Nous avons choisi les puces Affymetrix (Hugenes ST1.1) comportant environ 33000 probeset. Les données générées ont été analysées avec le logiciel Genespring GX 7.3 (Agilent, Palo Alto, CA). Différentes méthodes statistiques ont été utilisées pour l'analyse des puces du projet IFN-T helper et celles du projet des cellules dendritiques inflammatoires d'Elodie Segura. Plusieurs logiciels on été utilisés pour l'analyse bio informatique : Ingenuity Pathway, Molecular Signature Database (MSigDB), et l'application EMA (Easy Microarray Analysis) sur le logiciel R (R software) développée par l'équipe de bioinformatique de l'Institut Curie.

2. Limite de notre système expérimental.

Le faible nombre de donneur (n=3) a limité la puissance d'analyse des micro arrays et donc le nombre potentiel d'ISGs induits. La faible stringence des analyses statistiques nous expose aussi à un grand nombre de faux négatifs et positifs. Cependant, la majorité des gènes identifiés a été validée par RT-PCR ou en protéines, confirmant le caractère robuste de nos données. Les analyses de pathways type Gene Ontology peuvent cependant être biaisées car elles se basent sur un ensemble de données publiées dans différents systèmes.

Toutes les découvertes (molécules ou fonctions) issues d'analyses à large échelle doivent être validées sur le plan protéique et sur le plan fonctionnel. Tous les modules mis en évidence n'ont pas pu être validés sur le plan fonctionnel comme le module chemokine par exemple (voir annexe 2). Nous avons observé que l'IFN majore la production de CXCL10 et CCL20 dans les Th1 et Th17 évoquant un mécanisme d'auto amplification des réponses pro-inflammatoires. Partant de cette hypothèse, nous avons testé dans un modèle de migration cellulaire (transwell), le pouvoir « chemoattractant » des surnageants des Th1, Th17 +/- IFN sur leurs cellules cibles (lymphocytes mémoires CD4+CD45RO+CXCR3+ pour Th1 et CD4+CD45RO+CCR6+ pour les Th17). Malheureusement, aucune différence de migration n'a été mise en évidence avec les différents surnageants. La faible concentration des chemokines (2-5 ng/ml) dans nos surnageants comparées à celles de nos contrôles positifs (de 50 à 100ng/ml) constitue peut-être une limite du système, pouvant expliquer ces résultats négatifs. On ne peut exclure que cette production soit suffisante in vivo pour attirer d'autres cellules CCR6+

dans un espace plus réduit. Le CCL20 est une chemokine qui possède aussi les propriétés des Beta-defensines [337,338] et peut inhiber la prolifération de bactéries comme l'Escherichia Coli [338]. Afin d'évaluer l'effet « antimicrobien » de nos surnageants Th17 ou Th17+IFN, nous avons mis en culture des doses croissantes d'E.COLI dans ces différents milieux (sans antibiotiques). Aucun effet sur la prolifération bactérienne n'a été mis en évidence.

3. Nos résultats répondent-ils entièrement aux questions scientifiques posées ?

3.1 Comment le microenvironnement module-t-il l'effet d'une cytokine ?

Le Projet I portant sur la régulation IL-17 et IL-22, a montré à une échelle réduite comment deux cytokines produites par une même cellule peuvent être régulées différemment selon le contexte polarisant. Nous avons observés que les cytokines de la famille de l'IL-12 (IL-12 et IL-23, impliquée dans la polarisation Th1, et Th17) peuvent induire d'IL-22, notamment l'IL-23. En revanche, le TGF-β qui est nécessaire à l'induction optimale de Th17 va de manière opposée, inhiber la production d'IL-22. Cet effet du TGF-β n'est pas observé sur des CD4 mémoires. Ce résultat suggère que l'intégration du signal délivré par le TGF-β a modifié la capacité de production des cytokines des Th17. Cet effet du microenvironnement sur la réponse cytokinique va être confirmé à plus large échelle dans le projet II.

Dans ce dernier, nous avons caractérisé dans un contexte neutre de polarisation des CD4, une signature IFN différente de celle du programme de différenciation Th1, Th2 et Th17. Une majorité de ces gènes persiste après re-stimulation polyclonale des LT suggérant une certaine stabilité du programme de transcription IFN. La nouveauté de ce travail réside dans la caractérisation de 2 réponses IFN : une réponse commune aux 4 contextes Th et une réponse flexible déterminée par le contexte de polarisation.

Les fonctions antivirales de l'IFN sont très conservées au cours de l'évolution[6], ceci indépendamment de l'espèce, et du type cellulaire étudié. Nos résultats montrent que cette réponse persiste pour un groupe d'ISGs antiviraux donnés dans différents environnements cytokinique comme le suggéraient certaines études. La réponse antivirale reste néanmoins flexible pour d'autres ISGs. Cette propriété intrinsèque à l'IFN peut expliquer le fait que la réponse antivirale soit retrouvée majoritairement dans

de nombreuses signatures de transcriptome de maladies virales (Hepatite C) ou maladies inflammatoires [277,339, 340, 341,289].

L'analyse des voies de signalisation a mis en évidence 15 fonctions biologiques émergentes de l'IFN très diverses, aussi bien métaboliques, antivirale ou en rapport avec la réponse immunitaire. Nous avons identifié par exemple des gènes liée au chemotactisme (CXCL-10, CCL-20) qui ont été validés au niveau du transcrit ARN et au niveau protéique.

La flexibilité de l'effet d'une cytokine peut résulter de la modulation de plusieurs facteurs clés de la voie de signalisation de cette dernière (exemple : kinase, FT, expression des récepteurs). Nous avons observé dans notre système la modulation par l'IFN de plusieurs FT, récepteur de cytokines clés dans la polarisation T Helper. Par exemple, l'effet pro Th17 de l'IFN dans notre système s 'explique par : l'induction du FT RORc, et du récepteur de l'IL-1β. Ce dernier a été validé à la fois en ARN et en protéine et participe probablement à une amplification des voies de signalisation de l'IL-1β (voir annexe 1), cytokine majeure dans la différentiation Th17.

Nos deux projets mettent ainsi évidence la flexibilité de la réponse IFN et de la réponse au TGF-β dépendant de facteurs extrinsèques. Nos données confirment le concept d'un effet « dépendant du contexte» des cytokines décrit dans les années 70 même si ce dernier considérait plus l'effet de l'environnement physique que chimique. On peut se demander dès lors si ce concept peut être généralisé à l'ensemble des cytokines. Enfin, la flexibilité de la réponse à l'IFN pourrait ainsi expliquer les différents effets observés des IFN en thérapeutique et en physiopathologie. Nos résultats et notre système actuel ne permettent pas de savoir lequel des facteurs extrinsèques ou intrinsèques déterminent le plus la réponse IFN.

3.2 Impact fonctionnel de la flexibilité de la réponse antivirale.

La deuxième nouveauté de notre travail a été de valider, par des tests fonctionnels, le caractère flexible de la réponse antivirale. L'étude des micro-array a montré que la réponse antivirale est qualitativement et quantitativement différente dans les 4 populations de T Helper notamment en Th17 (diminution du nombre et de l'intensité des ISGs). Ces résultats ont été validés par la suite sur 6 gènes en RT-PCR et en protéine pour un gène (Mx1). Nous avons alors émis l'hypothèse que « l'antiviral state » induit

dans les sous populations Th par l'IFN, ne leur conférait pas la même capacité de défense lors d'une infection virale. L'infection des Th (générés en présence ou non d'IFN) par deux virus (HIV1 et HIV2) a révélé 1/ une susceptibilité plus importante aux infections virales de la populations Th17 ; 2/ que l'antivirale state induit dans certaines populations de TH (Th2 et Th17) ne les protégeait pas des infections virales. Ces résultats sont en accord des publications récentes montrant que l'HIV infecte préférentiellement les CD4 Th17[342], avec les observations de déplétion des lymphocytes CD4 de la muqueuse gastrique lors d'infection par le HIV ou le SIV [343,344, 345].

3.3 Confirmation de la flexibilité des T helper

Nos deux travaux (projet 1 et 2) insistent sur la nécessité de ne pas réduire les fonctions des Th à une cytokine et à un facteur de transcription clé (Master TF). Un Th se définit par un ensemble de cytokines inductrices, de FT dont l'expression est dynamique, de récepteurs cellulaires, et d'un ensemble de cytokines et chemokines. L'intégration de ces données par module permet ainsi de mieux définir les profils des Th dans notre système.

* Dans le projet 1, l'analyse de la régulation IL-22 au cours de la polarisation Th1 et Th17 a révélée des aspects différents de sécrétion des cytokines appartenant à la famille de l'IL-10 (IL-22 et IL-26). Dans notre système, la production d'IL-22 est corrélée avec celle de l'IFN-γ et un profil Th1. IL est à noter que le locus de l'IL-22 se situe juste à coté de celui de l'IFN-γ. La production L'IL-26 est quant à elle corrélé avec l'expression de RORc, RORa et AHR spécifique du profil Th17, et non pas celle d'IL-17.
* Dans le projet 2, nous avons observé que l'IFN majore les propriétés inflammatoires des populations Th1 et Th17. Ainsi, l'IFN augmente l'expression de T-bet, la sécrétion d'IFN-γ et de CXCL-10 pendant polarisation Th1; et de façon similaire, l'expression de ROR-c, de l'IL-17A (ARN/protéine), l'IL-17F (ARN) et du CCL-20 lors de la polarisation Th17. Le CCL20 est une chemokine secrétée habituellement par les DC, qui attire les cellules CCR6+ dont les Th17. L'induction des cytokines et chemokines clés des Th1 et Th17 (IFN-γ/CXCL10 et IL-17/CCL-20 respectivement) pourrait servir à un mécanisme d'amplification des réponses pro-inflammatoires.
* Notre système a mis en évidence un autre aspect de la régulation des Th2 par les IFNs. Le profil Th2 est celui qui est le plus modulé par les IFNs. Ces derniers ont toujours été

décrits comme inhibant les Th2 et surtout les cytokines majeures IL-4 IL-5 et IL-13 que nous avons aussi confirmé. L'analyse du profil cytokine révèle que ces cellules sécrètent en contre partie majoritairement de l'IL-10, IL-6, IL-3 et du TFG-β. Contrairement à une étude récente [176], l'expression du FT GATA-3 n'est pas inhibée. On observe une légère augmentation de FoxP3 de manière non significative. L'expression d'IL-10 et de FoxP3 leur confère un profil « régulateur » qui n'a pas été encore validé sur le plan fonctionnel. L'absence de contrôle positif pour la sécrétion du TFG-β (Surnageant de T régulatrice) rend difficile son interprétation. Contrairement aux Th1 et Th17, l'IFN « reprogramme » les Th2 en modulant surtout le profil cytokinique mais pas le profil des chemokines et récepteurs. Ces résultats vont dans le sens d'une flexibilité des T helpers.

3.4 Intérêt du transcriptome dans la caractérisation de population cellulaire.

L'étude du transcriptome fournit l'analyse la plus complète de l'état « statique ». L'intérêt est la génération de multiples data qui peuvent être ensuite analysées par groupes mais aussi individuellement. Les différents outils de bioinformatique disponibles offrent de nombreuses possibilités d'analyse : analyse de groupe de gènes par modules fonctionnels, caractérisation de voies de signalisation ou des FT en amont ou en aval du signal étudié, analyse individuelle de gènes, comparaison des échantillons utilisés selon les modules individualisés…

Les techniques dites « omics » se sont largement répandues depuis 15 ans. Appliquée à un organe ou à une cellule, l'analyse du transcriptome permet la comparaison de différentes populations sur un ensemble de marqueurs ou d'étudier la réponse cellulaire à différents stimuli dont des pathogènes[346]. En recherche clinique, l'analyse de transcriptome est couramment utilisée et permet de définir des « signatures spécifiques ». Ces dernières peuvent avoir un intérêt diagnostique, définir l'activité de la maladie, identifier des cibles thérapeutiques ou des mécanismes physiopathologiques[289].

Dans les deux projets (IFN et cellules dendritiques), nous avons choisi de caractériser des populations cellulaires (projet II/III) ainsi que l'effet de cytokine sur l'état cellulaire (Projet II) à partir de l'étude du transcriptome cellulaire.

Nous avons individualisé des signatures spécifiques pour chaque population cellulaire avec une méthodologie statistique adaptée. Pour réduire la complexité des data générées (2500 gènes pour la signature DC et environ 75 à 250 gènes pour la signature

IFN), nous avons utilisé des outils de bioinformatique afin de mieux apprécier les profils d'expression de gènes entre nos différentes populations et quantifier ces différences par des tests statistiques adaptés.
Nous avons ainsi observé des différences entre des sous types de populations cellulaires (Projet DC), ou des états d'activation cellulaire variés (Cellules Th en présence ou non d'IFN) ; à la fois au niveau globale (ensemble des gènes différentiellement exprimés) mais aussi à un niveau plus réduit (modules fonctionnels définis dans un second temps). Les mêmes résultats ont été observés par des analyses indépendantes d'un groupe de 14 cytokines définissant une des fonctions des Th. Ces validations à deux niveaux différents (global et réduit), aussi bien en ARN et protéines renforcent notre concept.

L'analyse du transcriptome est cependant limitée par son caractère statique. Le module cytokine en est un parfait exemple. Toutes les cytokines produites pour les Th ne sont pas sécrétées selon les mêmes modalités et la même cinétique, après l'activation de la cellule. C'est une des raisons possibles pour laquelle seul l'IFN-γ a été retrouvé dans nos signatures après la restimulation des cellules.
Dans le projet DC inflammatoire, nous avons utilisé les analyses à large échelle pour déterminer l'origine des infDC : sont-elles issues des DC sanguins ou de monocytes ? Les résultats ne permettent pas de répondre définitivement à cette question. L'analyse globale et l'analyse spécifique de module (présentation d'Ag, chemotactism, endocytose) révèlent que cette population présente des caractéristiques différentes des autres APC. Deux arguments suggèrent cependant que les infDC sont issues de monocytes : 1/ la signature des infDC est enrichie en gènes de MoDC, 2/ ils expriment le FT zbtzb46, spécifique de la lignée des DC « conventionelles » murine qui est aussi exprimé par les Mo-DC[348] 3/ ils expriment aussi le FT CSF1R qui est important pour le développement des infDC dits murins in vivo[347, 348].
Au final, les analyses à larges échelles sont indispensables pour apprécier au mieux le complexe réseau cellulaire mais nécessitent une puissance suffisante, et des outils complémentaires pour approfondir et valider définitivement les données observées.

PERSPECTIVES

Les données générées au cours de la thèse posent de nouvelles questions biologiques, d'une manière générale sur les mécanismes d'interactions de signaux cellulaires mais aussi plus spécifiques sur le rôle antiviral des IFN ou sur la polarisation Th, par exemple.

1. Approfondir la modulation de « l'antiviral state » :

Une analyse approfondie de la modulation de « l'antiviral state » dans les Th est nécessaire pour explorer les mécanismes de la réponse antivirale. La validation par ARN et protéine d'autre d'ISGs est indispensable pour confirmer les données la modulation qualitative et quantitative des ISGs par les contextes Th. La validation par RT PCR d'une dizaine d'ISGs est prévue. Certains comme la viperin (RSASD2), ou XAF-1 qui ne sont pas induits en Th17 sont des candidats à étudier en priorité. Nous avons essayé de quantifier la protéine Viperin dans nos différentes conditions par cytométrie de flux. Malheureusement, les Ac disponibles actuellement et notre système ne permettent de détecter de manière significative cette protéine**.** Il sera ensuite intéressant de **c**omprendre quels ISGs ou groupe d'ISGs sont responsables de la relative protection dans les Th face à une infection Pour tester cette hypothèse, nous envisageons de réprimer de manière systématique et spécifique des ISGs antiviraux par ShRNA afin d'évaluer leur rôle dans la réponse antivirale spécifique des Th.
Toutes nos data sont issues d'un modèle de différentiation in vitro. Il est important et nécessaire de confirmer les différences de l'antivirale state dans les sous populations de Th mémoires (Th1, Th2, Th17 et aussi T-reg), pour généraliser nos conclusions. Ce projet est actuellement en cours.

2. Exploitations des données de micro-array.

L'ensemble des résultats présentés concerne essentiellement l'effet de l' IFNα sur les Cellules Th. Nous disposons des données similaires (transcriptomiques) avec l'IFN-β qui ouvre de nombreuses perspectives: existe-il également deux réponses (commune et flexible) à l'IFN-β ? Quelles sont les différences entre la signature IFNβ et IFNα ? L'IFNβ et IFNα induisent-ils le même antiviral state ?

Indépendamment de l'effet des IFN sur la polarisation des Th, les données des micro-array sur les 4 Types de Th peuvent servir de base afin de mieux caractériser les programmes transcriptionnels des Th et d'étudier plus spécifiquement les fonctions des sous populations. Une partie des données de micro-array est étudiée par l'équipe de Gorgio Trincheri (NIH) dans une collaboration portant sur l'individualisation de nouveaux FT des Th.

3. Mécanisme d'Intégration de signaux extracellulaire.

Notre modèle donne un aperçu de la complexité d'intégration de signaux composés de cytokines. Il offre une vision limitée car il manque une multitude de cytokines intervenantes, de molécules de co-stimulations et autres facteurs environnementaux comme les nutriments et/ou hormones qui vont moduler le programme de différentiation des Th. Plusieurs études ont montré que les interactions multiples entre les éléments du microenvironnement peuvent changer leur effet sur la fonction cellulaire[141]. Nous avons développé dans notre laboratoire des méthodes d'analyses pour étudier comment deux signaux sont intégrés au niveau cellulaire et comment les différents mécanismes d'intégration (synergie, additivé, répression) modulent les multiples fonctions cellulaires. (Raphael Zollinger, manuscript en préparation).

Comprendre, dans un milieu inflammatoire, quel signal extracellulaire dominera l'autre et que sera leur impact sur les fonctions cellulaires est un enjeu majeur.

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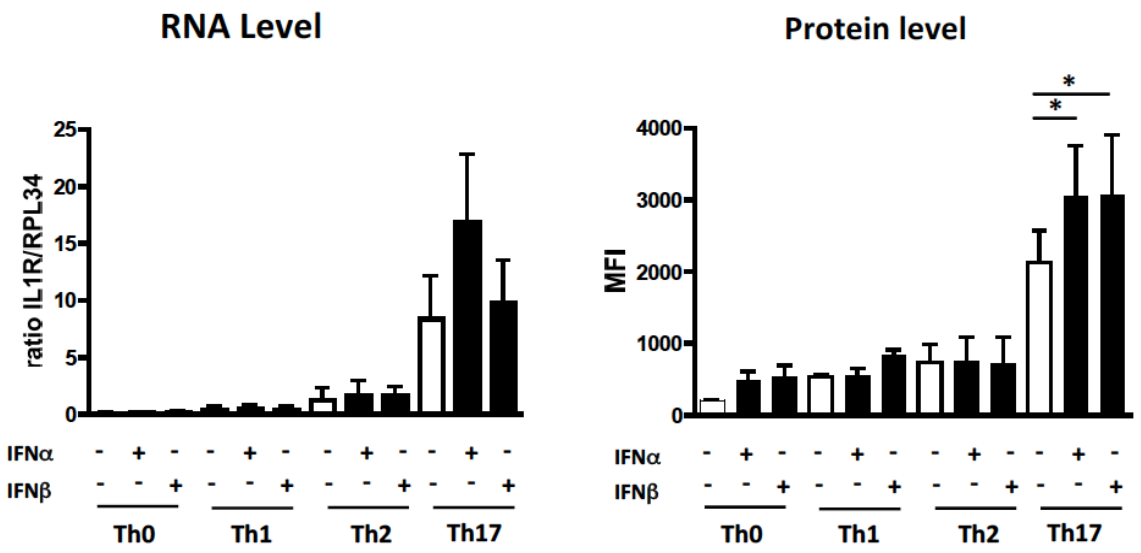
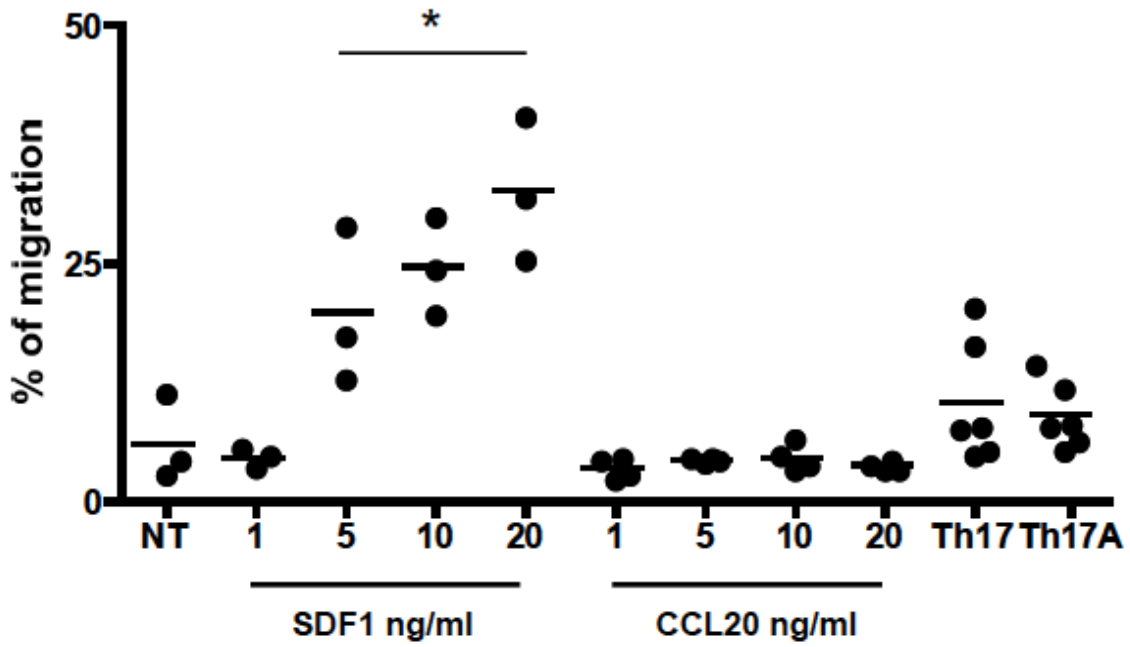
ANNEXE



Annexe 1 Both IFN-α and IFN-β increase IL-1R during Th17 differentiation.

Naïve T cells were differentiated with anti-CD3 plus anti-CD28 in Th0, Th1, Th2, and Th17 +/- IFN-α or IFN-β for 5 days. IL1R was evaluated at RNA and protein in the 12 contexts. (A) RNA level of IL1-RB at day five. (B) IL-1RB level by intracellular cytokine staining after 12 Hours of restimulation by beads anti CD3/CD28.. Data are the mean +/- SD of 6 independent experiments. Ns, non significant, *, $p < 0.05$; ** , $p < 0.01$ (Wilcoxon Test)



Annexe 2 Supernantants from Th17 or Th17 +IFN induce similar level of memory T cells migration.

CCR6+CD45RO+CD4 memory T cells were seeded in equal numbers in the upper chamber of an uncoated transwell system. Lower chambers were filled either with supernatants from Th17 or Th17+IFNa culture, and two controls SDF-1 and CCL20 in a dose dependent manner. Data are the mean of 3 independents donors, and 3 differents supernatants. Ns, non significant, *, $p < 0.05$; **, $p < 0.01$ (T-Test)

Critical role of IL-21 in modulating T_H17 and regulatory T cells in Behçet disease

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Background: Behçet disease (BD) is a chronic systemic inflammatory disorder of unknown etiology.
Objective: To determine the nature of T cells driving inflammatory lesions in BD.
Methods: T cell homeostasis and cytokines production were analyzed in peripheral blood and brain inflammatory lesions from 45 adult patients with BD (active and untreated BD [n = 25] and patients in remission [n = 20]) and 20 healthy donors, using Luminex, flow cytometry, immunohistochemistry, and immunofluorescence analysis.
Results: We found a marked increase in T_H17 cells and a decrease in the frequency of $CD4^+$ forkhead box $P3^+$ regulatory T cells (Tregs) in peripheral blood that were induced by IL-21 production and that correlate with BD activity. The addition of serum from patients with active BD in a sorted $CD4^+$ T cells culture of healthy donors induced a significant and dose-dependent production of IL-17A and a decrease in forkhead box P3 expression. We demonstrated the presence of IL-21– and IL-17A–producing T cells within the cerebrospinal fluid, brain parenchyma inflammatory infiltrates, and intracerebral blood vessels from patients with active BD and central nervous system involvement. The stimulation of $CD4^+$ T cells with IL-21 increased T_H17 and T_H1 differentiation and decreased the frequency of Treg cells. Conversely, IL-21 blockade with an IL-21R-Fc restored the T_H17 and Treg homeostasis in patients with BD.
Conclusion: We provided here the first evidence of the critical role of IL-21 in driving inflammatory lesions in BD by promoting T_H17 effectors and suppressing Treg cells. IL-21 represents a promising target for novel therapy in BD. (J Allergy Clin Immunol 2011;128:655-64.)

Key words: *Behçet disease, T_H17, T_H1, regulatory T cells, IL-21, vasculitis, autoimmunity*

Behçet disease (BD) is a chronic systemic inflammatory disorder at the crossroad between autoimmune and autoinflammatory syndromes.[1] It is characterized by recurrent episodes of oral and genital ulcers, uveitis, and central nervous system (CNS) involvement.[2,3] Although the pathogenesis of BD remains poorly characterized, it is currently thought, as with many autoimmune or autoinflammatory syndromes, that certain infectious (in particular, *Streptococcus sanguis*) and/or environmental factors are able to trigger symptomatology in individuals with particular genetic variants.[2] In common with ankylosing spondylitis and psoriatic arthropathy, BD shares MHC class I association. HLA-B51 is the most strongly associated known genetic factor to BD.[4] However, it accounts for less than 20% of the genetic risk, which indicates that other genetic factors remain to be discovered. Recently, genome-wide association studies from Japan and Turkey identified an association at IL23R and IL12RB2 locus.[5,6] The implication of T cells and polymorphonuclear leukocytes is supported by pathological studies showing perivascular infiltration of memory T cells and polymorphonuclear leucocytes within vasculitic lesions in patients with BD who have arterial and CNS involvement.[7] However, the nature of T cells driving inflammatory lesions remains elusive.

Here, we first demonstrated the promotion of T_H17 responses and the suppression of regulatory T cells (Tregs) that were induced by IL-21 production and that correlate with BD activity. We demonstrated the presence of IL-21– and IL-17A–producing T cells within the cerebrospinal fluid (CSF), brain parenchyma inflammatory infiltrates, and intracerebral blood vessels from patients with active BD (aBD) and CNS involvement. The stimulation of $CD4^+$ T cells with IL-21 increased T_H17 and T_H1 differentiation and decreased the frequency of Treg cells. Conversely, IL-21 blockade with an IL-21R-Fc restored the T_H17 and Treg cells' homeostasis in patients with BD. Our findings suggest that IL-21 exerts a critical role in the pathogenesis of BD, thus providing a promising target for novel therapy.

METHODS

Patients

The study population consisted of 45 consecutive adult patients (25 men and 20 women; mean age, 40 years; range, 23-72 years) fulfilling the international criteria for BD.[8] Patients were divided into two groups: patients with untreated aBD (n = 25) and patients in remission of BD (rBD; n = 20).

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Abbreviations used
aBD: Active Behçet disease
BD: Behçet disease
CNS: Central nervous system
CSF: Cerebrospinal fluid
Fc: Fragment constant
FoxP3: Forkhead box P3
HD: Healthy donor
rBD: Behçet disease in remission
TCM: Central memory T cells
Treg: Regulatory T cell

Patients with aBD were defined as patients with severe posterior or pan-uveitis and/or CNS involvement, in the absence of corticosteroids or immunosuppressant agents. Patients with rBD were defined by the absence of severe clinical manifestation and increased inflammatory parameters. Patients in remission were on corticosteroids (mean dosage, 5.2 mg/d; n = 12), immunosuppressant agents (n = 7), or were left untreated (n = 6). Blood samples from healthy donors (HDs) were obtained from Etablissement Français du Sang (Hôpital Pitié-Salpêtrière). The study was performed according to the Helsinki declaration. All donors gave informed consent.

Analysis of cell surface markers and forkhead box P3 expression

PBMCs were stained with the following conjugated mAbs at predetermined optimal dilutions for 30 minutes at 4°C: CD3-ECD, CD4-PCy7, CD4-ECD, CD8-PCy7, CD8-APC, CD10-APC, CD16-FITC, CD19-ECD, CD27-PE, CD28-FITC, CD45RO-FITC, CD45RA-APC, CD56-PE, HLA-DR-PCy7 (Beckman Coulter, Villepinte, France), CD25-PE, CD38-PCy7, CD56-FITC, CD62L-FITC, IgD-FITC (BD Pharmingen, Le Pont-De-Claix, France), CCR7-PE (R&D Systems, Lille, France), and CD127-FITC (eBioscience, Paris, France). Intracellular detection of forkhead box P3 (FoxP3) was performed on fixed and permeabilized cells using an appropriate buffer (eBioscience). Data were acquired using a Navios flow cytometer and analyzed with the CXP analysis software (Beckman Coulter).

Analysis of cytokine production

PBMCs from the 45 patients with BD and the 20 HDs and CSF mononuclear cells from 3 patients with active CNS involvement were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich) in the presence of brefeldin A (BD Pharmingen). Cells were then permeabilized with a Cytofix/Cytoperm buffer (BD Pharmingen); stained with IFN-γ-FITC (BD Pharmingen), IL17A-Alexa Fluor 647 (eBioscience), and IL-21-Alexa Fluor 647 (Biolegend, Saint Quentin en Yvelines, France); and acquired. Fresh PBMCs from patients with aBD and HDs were also cultured in a serum-free medium (X-vivo 20; Lonza, Levallois-Perret, France) supplemented with 2% penicillin-streptomycin with various conditions: medium alone and anti-CD3/CD28 mAbs. After 5 days of culture, cells were collected and restimulated for 4 hours with PMA/ionomycin, and an analysis of intracellular IL-17A and IFN-γ production and FoxP3 expression was performed. Cytokine levels in serum and culture supernatants were measured by using ELISA and Luminex (Invitrogen, Cergy Pontoise, France). IL-21 was also measured in the CSF from 8 patients with BD with active CNS involvement, 8 patients with BD without CNS involvement, and 3 patients with inactive connective tissue disorders. The IL-21 ELISA had a threshold sensitivity of 16 pg/mL.

Purification of CD4$^+$ T lymphocytes from patients with BD

Peripheral total CD4$^+$, CD4$^+$CD25$^-$, CD4$^+$CD25$^+$, and CD4$^+$CD25^{++} T cells were isolated from PBMCs by using immunomagnetic depletion (Miltenyi Biotec, Paris, France) and FACSAria sorting (BD Biosciences) with a purity of each population being more than 97%. Purified CD4$^+$ T cell populations were cultured in X-vivo 20 supplemented with 2% penicillin-streptomycin (1×10^6 cells/mL) and stimulated in 48-well plates coated with anti-CD3/CD28 mAbs with various conditions: anti-CD3/CD28 alone, recombinant human IL-21 (50 ng/mL; BioVision, Lyon, France), and recombinant human IL-21R/fragment constant (Fc) chimera (100 μg/mL, R&D Systems). FoxP3 expression was analyzed by using flow cytometry as previously described, and intracellular cytokine production was analyzed after restimulation with PMA/ionomycin and flow cytometry.

TABLE I. Phenotypical analysis of T, B, and NK cell subsets in patients with Behçet disease and healthy donors

Measurement	HD	aBD	rBD
CD4$^+$ T cells (n, %)			
Total	65.8 (11.2)	54.1 (15.0)*	60.8 (15.6)
Naive	28.4 (13.2)	29.4 (16.0)	29.6 (18.9)
CM	23.3 (10.7)	27.2 (17.1)	17.3 (11.5)
TEM	36.4 (13.3)	31.8 (16.8)	35.5 (20.2)
tTEM	12.0 (7.0)	11.9 (15.1)	17.6 (14.4)
HLA DR$^+$	24.8 (13.3)	20.9 (17.6)	25.4 (15.4)
CD25$^-$	66.8 (15.0)	69.7 (9.8)	67.9 (9.6)
CD25$^+$	28.8 (14.3)	25.4 (10.4)	26.9 (9.1)
CD25^{++}	1.9 (0.8)	3.6 (1.8)†	3.6 (1.5)‡
CD8$^+$ T cells (n, %)			
Total	29.2 (9.5)	34.9 (12.4)	29.7 (8.8)
Naive	25.5 (14.5)	40.3 (17.4)†	28.8 (16.3)
CM	5.8 (4.0)	9.1 (8.0)	4.4 (3.5)
TEM	29.3 (11.0)	17.4 (8.6)†	25.4 (18.1)
tTEM	39.4 (19.9)	33.2 (18.6)	41.4 (19.8)
HLA DR$^+$	52.7 (19.5)	45.1 (19.7)	50.6 (20.1)
CD25$^-$	90 (11.4)	85.1 (12.9)	85.8 (10.6)
CD25$^+$	10.0 (11.4)	13.3 (9.9)	13.9 (10.3)
Ratio CD4/CD8	2.6 (1.3)	1.9 (1.0)*	2.4 (1.0)
CD19$^+$ B cells (n, %)			
Total	9.6 (4.6)	8.6 (5.4)	8.1 (4.3)
Immature	4.4 (3.2)	3.1 (3.3)	3.5 (4.4)
Naive	45.1 (21.3)	45.5 (20.0)	41.3 (20.5)
MZ	26.3 (18.1)	26.4 (14.7)	30.2 (18.2)
CS	19.9 (12.1)	23.1 (12.7)	23.2 (12.0)
Plasmablast	0.7 (0.4)	1.3 (1.1)	0.8 (0.6)
CD3$^-$CD56$^+$ NK cells	10.8 (7.7)	7.6 (5.1)	6.7 (4.3)

CM, Central memory subset (CD45RA$^-$CD62L$^+$); *CS,* class switched memory subset (IgD$^-$CD27$^+$); *MZ,* marginal zone subset (IgD$^+$CD27$^+$); Naive B cells (IgD$^+$CD27$^-$); Naive subset (CD45RA$^+$CD62L$^+$); *NK,* natural killer (CD3$^-$CD56$^+$); *TEM,* effector memory subset of T cells (CD45RA$^-$CD62L$^-$); *tTEM,* terminally differentiated EM T cells (CD45RA$^+$CD62L$^-$CD27$^-$CD28$^-$). Comparisons were performed between patients with aBD and HDs and patients with rBD and HDs.
*$P < .05$.
†$P < .01$.
‡$P < .001$.

Immunohistochemical analysis

IL-21$^+$, IL-17A$^+$, chemokine (C-C motif) ligand (CCL) 20$^+$, and chemokine (CXC motif) ligand (CXCL) 8$^+$ cells were detected on fixed, paraffin-embedded samples from 2 patients with BD with active CNS involvement (necropsic samples) and 2 normal controls (from the Banque d'échantillons biologiques de recherche en neurologie). Dewaxed slides were submitted to antigen retrieval by heating in a citrate buffer with pH 6.0. Before incubation with primary antibodies, the Fc receptor was blocked with 2% BSA. Slides were incubated overnight with polyclonal goat antihuman IL-17A (dilution 1:20; R&D Systems), polyclonal rabbit IL-21 (dilution 1:20; 500-P191; PeproTech, Neuilly sur Seine, France), polyclonal goat antihuman CXCL8 (dilution 1:20, R&D Systems), and polyclonal rabbit

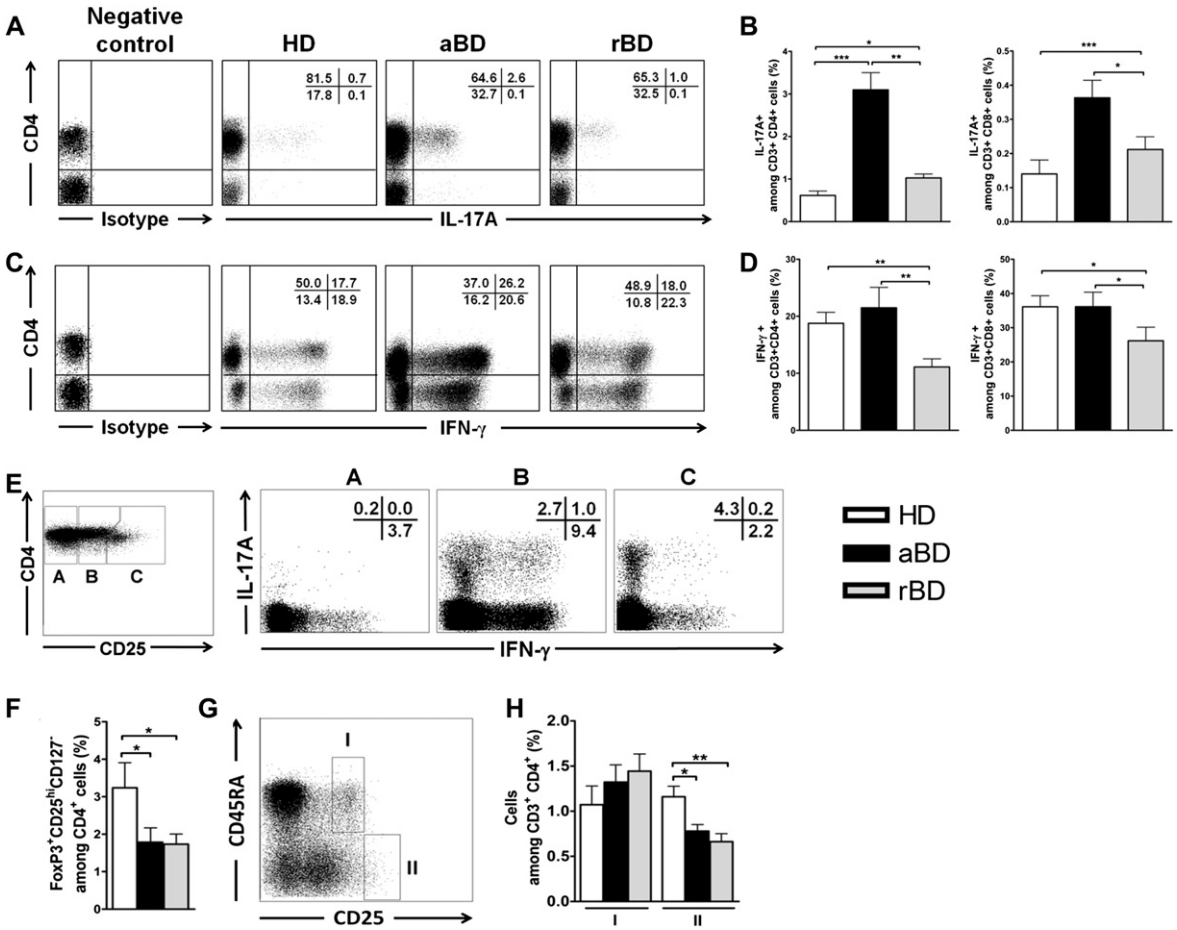

FIG 1. Increased T_H17 effectors and decreased FoxP3$^+$CD25^{hi}CD127$^-$ Treg cells in BD. Representative dot plots of IL-17A– **(A)** and IFN-γ **(C)**–producing CD4$^+$ after gating on CD3$^+$. Frequencies of IL-17A– **(B)** and IFN-γ **(D)**–producing T cells from 20 HDs, 25 patients with aBD, and 20 resting patients with BD. Analysis of IL-17A and IFN-γ production by CD4$^+$ T cells according to CD25 expression **(E)**. Frequencies of Treg cells **(F)**. Dot plots **(G)** and frequencies **(H)** of resting *(box I)* and activated Treg cells *(box II)*. $*P < .05$; $**P < .01$; $***P < .001$.

antihuman CCL20 (working dilution 1:30; Abcam, Paris, France) antibodies. Antibody binding was visualized with diaminobenzidine tetrahydrochloride (Dako, Trappes, France). For the negative control, proper isotype controls were used. CD3$^+$IL-17A$^+$ and CD3$^+$IL-21$^+$cells were detected on the same samples. Slides were incubated overnight at 4°C with polyclonal goat antihuman IL-17A (dilution 1:10) or polyclonal rabbit antihuman IL-21 (dilution 1:10) and monoclonal mouse antihuman CD3 antibody (Clone F7.2.38, Dako). Slides were then incubated for 1 hour at room temperature with Cy3-conjugated goat antirabbit antibody or biotinylated polyclonal antigoat antibody, then with APC-conjugated streptavidin, and finally with Cy2-conjugated goat antirabbit antibody (working dilution 1:200, Invitrogen), mounted in Fluoromount (Amersham, Orsay, France), and evaluated under fluorescence microscopy.

Data analysis

Data are presented as a mean (SEM) for continuous variables and as a percentage for qualitative variables. The Fisher exact test was used to compare qualitative variables, and nonparametric Mann-Whitney and Wilcoxon tests were used to compare continuous variables, as appropriate. A P value of <.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 4.0 and InStat version 3.0 for Windows (GraphPad Software, San Diego, Calif).

RESULTS

Phenotypical analysis of T cell subsets in patients with BD

To identify perturbations in T cell homeostasis, frequencies of naive, central memory T cells (TCMs), effector memory T cells (TEMs), and terminally differentiated TEM (tTEM) cells were compared among CD4$^+$ or CD8$^+$ T cells subsets from patients with aBD, patients with rBD, and HDs (Table I). No difference was found among CD4$^+$ T cell, B cell, and NK cell subsets. Among CD8$^+$ T cells, naive T cells were increased in patients with aBD compared with HDs and patients with rBD (40.3% vs 25.5% and 28.8%; $P = .007$ and $P = .049$, respectively). In contrast, TEMs were decreased in patients with aBD compared with HDs (17.4% vs 29.3%; $P = .002$).

T_H17 cells are increased in the peripheral blood in BD and correlate with disease activity

We examined the frequency of IL-17A– and IFN-γ–producing T cells after 4 hours of stimulation with PMA and ionomycin (Fig 1). We found a marked enrichment in IL-17A–producing

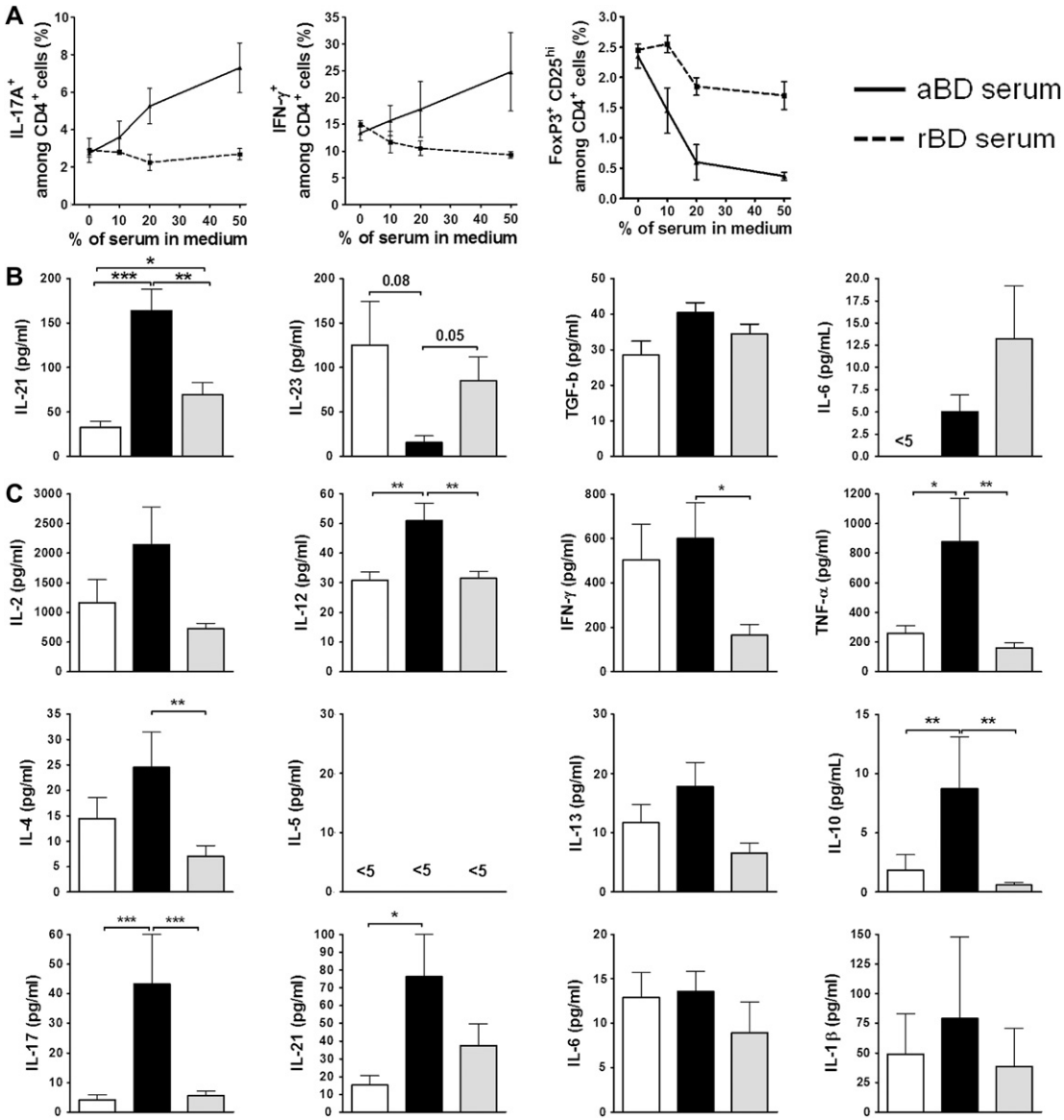

FIG 2. Sera from patients with BD contains increased IL-21 level and modulates predominant T_H17 cytokines modulating T_H1 and T_H17 differentiation and FoxP3 expression in HDs. Frequencies of IL-17A$^+$, IFN-γ$^+$, and FoxP3$^+$ cells among sorted CD4$^+$ T cells after 5 days culture with anti-CD3/anti-CD28 and with increasing concentrations of sera **(A)** in patients with aBD and 20 patients with rBD (3 independent experiments). T_H17 cytokine levels in the sera of HDs, patients with aBD, and those with rBD **(B)**. T_H1, T_H2, and T_H17 cytokine levels in culture supernatants in BD **(C)**. Cytokines were assessed in 20 HDs, 25 patients with aBD, and 20 patients with rBD, except for IL-23 and TGF-β, which were assessed in 10 HDs, 18 patients with aBD, and 18 patients with rBD. *$P < .05$; **$P < .01$; ***$P < .001$.

CD45RO$^+$CCR6$^+$CD4$^+$ T cells (T_H17) in patients with aBD compared with those who had rBD and in HDs (3.1% vs 1.0% and 0.6%; $P < .0001$ for both) (Fig 1, *A* and *B*), whereas IFN-γ–producing CD4$^+$ and CD8$^+$T cells (T_H1) did not differ significantly between patients with aBD and HDs. In contrast, T_H1 cells were increased in patients with aBD compared with patients with rBD (Fig 1, *C* and *D*). Immunosuppressant therapy in patients with rBD may explain the decrease in IFN-γ production by T cells. We next examined the IL-17A and IFN-γ production in purified CD4$^+$ T cells from patients with BD, according to the CD25 expression, after 5 days of stimulation with anti-CD3/CD28 (Fig 1, *E*). CD4$^+$CD25$^+$ T cells (B) were enriched in both IL-17A– and IFN-γ–producing cells, whereas CD4$^+$CD25^{++} T cells (C) produced mainly IL-17A (Fig 1, *E*).

Decreased Tregs in peripheral blood from patients with BD

We next explored a possible defect in T cell regulation associated with the T_H17 and T_H1 imbalance. We found that CD4$^+$FoxP3$^+$CD25^{hi}CD127$^-$ Treg cells were decreased in peripheral blood from patients with aBD and rBD compared with HDs (1.78% and 1.73% vs 3.2%; $P = .02$) (Fig 1, *F*). We analyzed the expression of CD45RA and CD25 among the CD4$^+$ T cells in

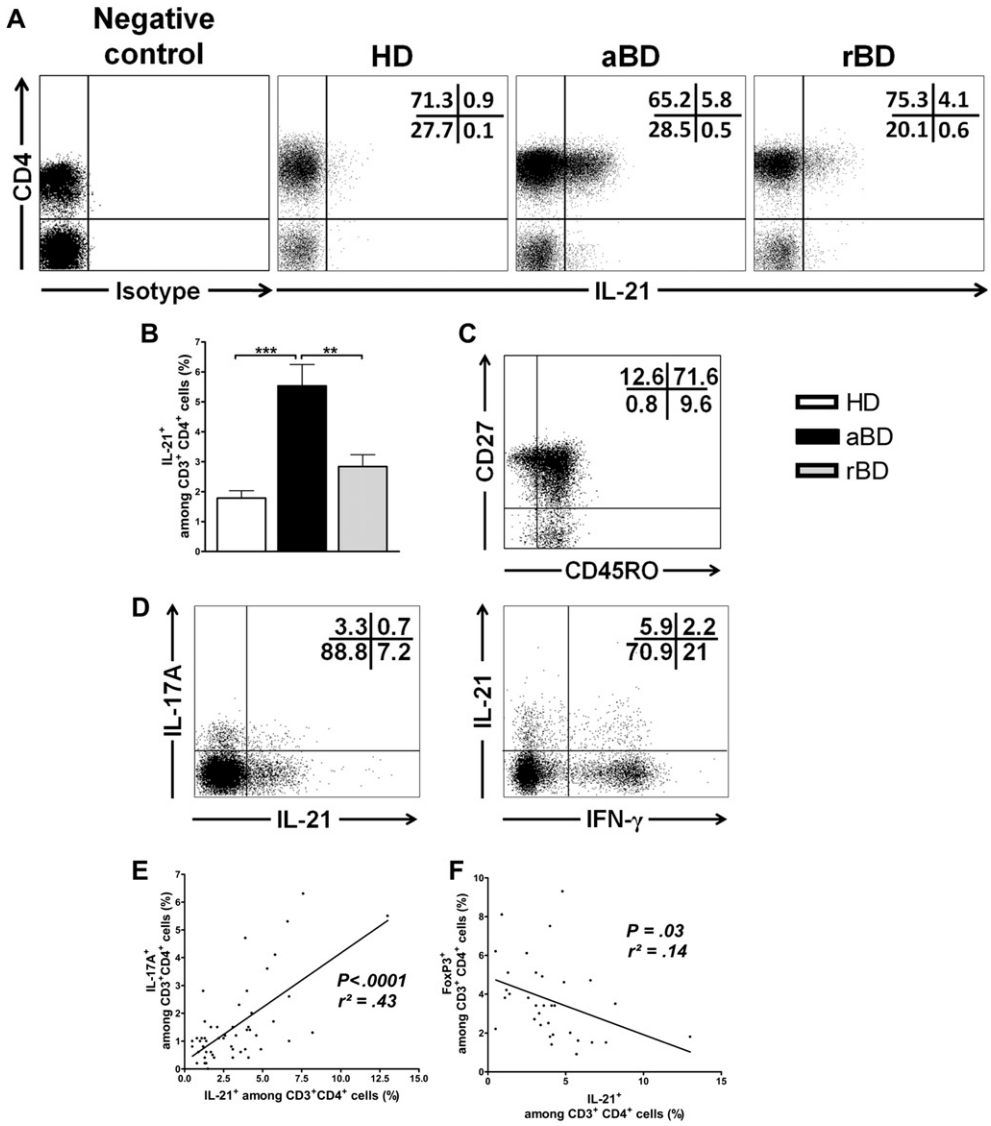

FIG 3. IL-21 is produced by central memory CD4$^+$ T cells and correlates with T_H17 response and FoxP3 expression. Dot plots **(A)** and frequencies of IL-21–producing CD4$^+$ T cells **(B)** after PMA/ionomycin stimulation. IL-21$^+$ is produced by central memory CD4$^+$ T cells **(C)**. Dot plot of IL-21–, IL-17A–, and IFN-γ–producing CD4$^+$ T cells after PMA/ionomycin stimulation **(D)**. Correlations between IL-21–producing CD4$^+$ T cells and T_H17 cells **(E)** and FoxP3$^+$ Tregs **(F)**. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

order to better delineate CD45RA$^+$CD25^{++} resting Tregs (rTregs) and CD45RA$^-$CD25^{+++} activated Tregs (aTregs) (Fig 1, *G*). aTreg cells were decreased in patients with aBD and rBD compared with HDs (0.8% and 0.7% vs 1.2%; $P = .02$ and $P = .014$, respectively) (Fig 1, *H*).

Sera from patients with BD modulate T_H1 and T_H17 differentiation and FoxP3 expression in healthy controls

We determined whether serum from patients with BD was able to modulate T cell differentiation and FoxP3 expression in healthy controls. The frequency of IL-17A– and IFN-γ–producing T cells and FoxP3 expression were evaluated after 5 days of stimulation of purified CD4$^+$ T cells from healthy controls with anti-CD3/CD28, in the presence of various proportions of serum from patients with aBD and rBD in culture medium. The frequency of IL-17A– and IFN-γ–producing T cells increased in a dose-dependent manner with sera from patients with aBD compared with that from patients with rBD (2.8%, 3.6%, 5.3%, and 7.3% of T_H17 and 13.4%, 15.7%, 17.8%, and 24.8% of T_H1 with 0%, 10%, 20%, and 50% of serum concentrations from patients with aBD compared with 2.9%, 2.8%, 2.3%, and 2.7% of T_H17 and 15.1%, 11.7%, 10.5%, and 9.3% of T_H1 with 0%, 10%, 20%, and 50% of serum concentrations from patients with rBD, respectively). FoxP3 expression decreased similarly in a dose-dependent manner with sera from patients with aBD compared with that from patients with rBD (2.4%, 1.5%, 0.6%, and 0.4% serum concentrations from patients with aBD compared with 2.5%, 2.6%, 1.9%, and 1.7% serum concentrations from patients with rBD) (Fig 2, *A*).

Increased IL-21 in the serum of patients with BD

Levels of cytokines promoting IL-17A production (IL-21, IL-23, TGF-β, and IL-6) were measured in serum from patients with aBD and rBD and from HDs (Fig 2B). IL-21 level was markedly increased in patients with aBD compared with those who had rBD and in HDs (164 pg/mL vs 69.5 and 32.9 pg/mL; $P = .004$ and $P = .0002$, respectively). In contrast, levels of IL-23 (15.5, 85.5, and 125.4 pg/mL), TGF-β (40.4, 34.4, and 28.5 pg/mL), and IL-6 (13.3, 5.0, and <5 pg/mL) did not differ significantly among patients with aBD, patients with rBD, and HDs. Levels of T_H1 (IL-2, IL-12, IFN-γ, and TNF-α), T_H2 (IL-4, IL-5, and IL-13), and T_H17 cytokines (IL-1β, IL-6, IL-17A, and IL-21) were also measured in culture supernatants after 4 hours of stimulation with PMA/ionomycin (Fig 2, *C*). IL-17A and IL-21 levels were markedly increased in patients with aBD compared with patients with rBD and HDs (43.3 pg/mL vs 5.5 and 4.2 pg/mL; $P = .0001$ for both and 76.3 pg/mL vs 37.6 and 15.3 pg/mL; $P = .19$ and $P = .03$, respectively). Similarly, TNF-α (877.2 pg/mL vs 159.2 and 258.6 pg/mL; $P = .002$ and $P = .03$, respectively), IL-12 (50.9 pg/mL vs 31.5 and 30.8 pg/mL; $P = .005$ and $P = .009$, respectively), and IL-10 levels (8.7 pg/mL vs 0.6 and 1.9 pg/mL; $P = .001$ and $P = .006$, respectively) were significantly increased in patients with aBD compared with patients with rBD and HDs. IFN-γ level was higher in patients with aBD compared with patients with rBD (599 pg/mL vs 165 pg/mL; $P = .012$) but not when compared with HDs (599 pg/mL vs 504 pg/mL; $P = .94$). No difference was found between groups for the levels of remaining cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, and IL-13) (Fig 2, *C*).

IL-21 is produced by central memory $CD4^+$ T cells and correlates with T_H17 response and FoxP3 expression

Given the increased levels of IL-21 in serum and culture supernatants, we analyzed intracellular production by T cells by using flow cytometry. Levels of IL-21–producing $CD4^+$ T cells were markedly increased in patients with aBD compared with patients with rBD and HDs (5.5% vs 2.8% and 1.8%; $P = .002$ and $P < .0001$, respectively) (Fig 3, *A* and *B*). IL-21–producing $CD4^+$ T cells displayed the phenotype of a TCM, as indicated by the expression of CD45RO and CD27 (Fig 3, *C*). Most of the IL-21–producing $CD4^+$ T cells did not produce IL-17A or IFN-γ (Fig 3, *D*). Double-positive IL-21^+ and IL-17^+CD4^+ cells were a minor population corresponding to less than 1% of the whole $CD4^+$ T cell population. Increase in IL-21–producing $CD4^+$ T cells was positively correlated with T_H17 ($r^2 = 0.43$; $P < .0001$) and negatively correlated with FoxP3 Treg cells ($r^2 = 0.14$; $P = .03$) in patients with BD (Fig 3, *E* and *F*). In addition, in patients with aBD, IL-21–producing $CD4^+$ T cells were inversely correlated with aTreg cells ($r^2 = 0.45$; $P = .009$) but not with rTreg cells ($r^2 = .03$; $P = .38$).

Increased production of IL-21, IL-17A, and IFN-γ in cerebrospinal fluid from patients with active BD with central nervous system flare

Pathological studies support the implication of T cells and polymorphonuclear leukocytes in the inflammatory lesions of patients with BD with CNS involvement. We analyzed the CSF level of IL-6 and IL-21 in patients with BD with (BD CNS^+) or without (BD CNS^-) CNS flare (Fig 4, *A*) and in inactive controls. Significant levels of IL-21 were detectable only in the CSF from patients with aBD with CNS involvement (47.5 pg/mL in BD CNS^+ vs undetectable levels in BD CNS^- and healthy controls). The IL-6 level was also detectable in the CSF from patients with aBD with CNS involvement (9.6 pg/mL vs 5.9 pg/mL in BD CNS^- and 1.3 pg/mL in healthy controls). We compared the frequencies of IL-17A– and IFN-γ–producing T-cells in the peripheral blood and CSF from 3 patients with aBD with CNS involvement (Fig 4, *B* and *C*). The levels of IL-17A–producing $CD4^+$ and $CD8^+$ T cells were dramatically increased in the CSF than in the peripheral blood from patients with aBD (8.6% vs 1.9% of $CD4^+$ T cells and 1.6% vs 0.4% of $CD8^+$ T cells, respectively). Levels of

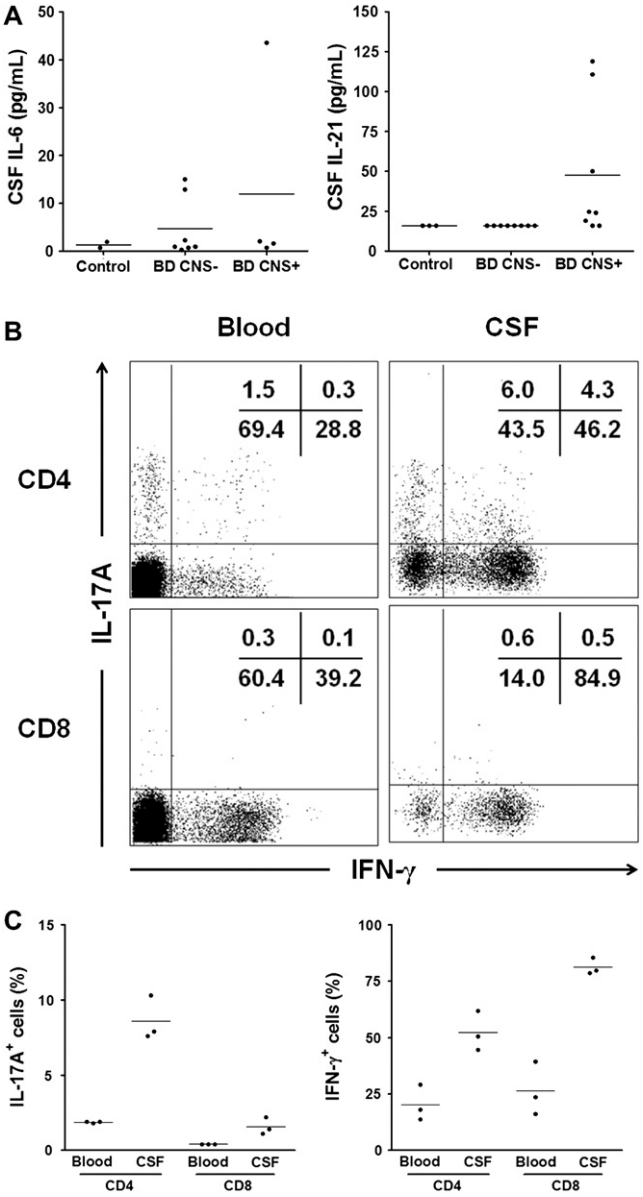

FIG 4. Increased production of IL-21, IL-17A, and IFN-γ in the CSF from patients with BD with CNS flare. IL-6 and IL-21 levels in the CSF from control and patients with BD with (BD CNS^+) or without (BD CNS^-) CNS involvement **(A)**. Dot plots **(B)** and frequencies **(C)** of IL-17A– and IFN-γ–producing T cells after PMA/ionomycin stimulation in the peripheral blood and CSF from patients with BD with CNS involvement.

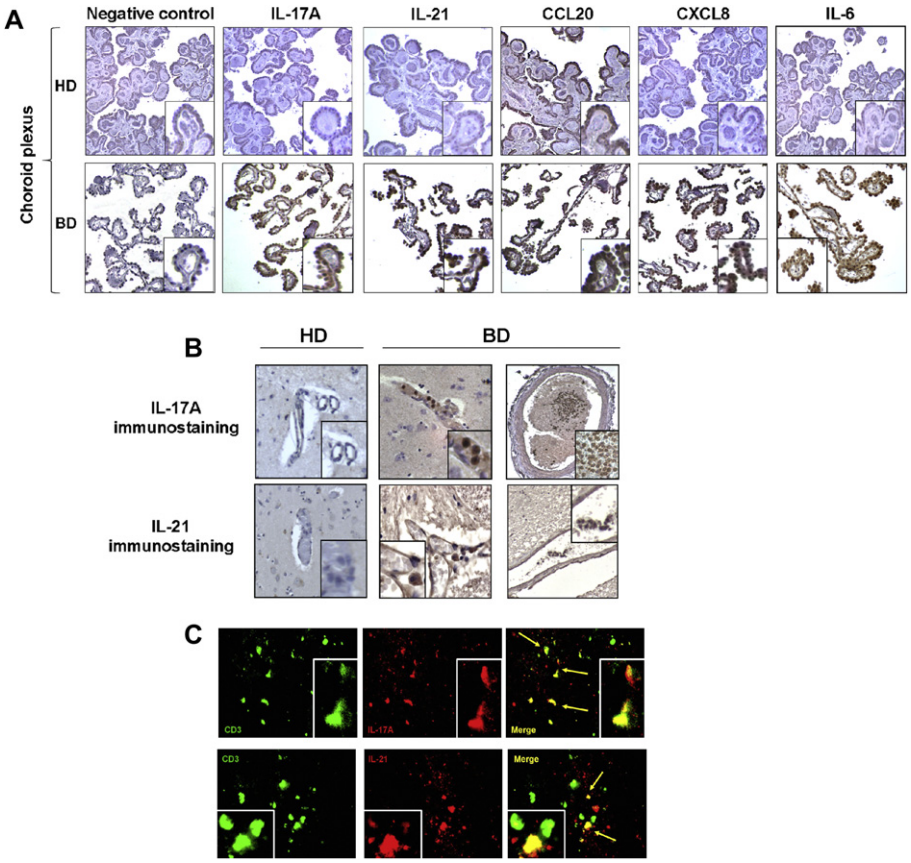

FIG 5. Strong IL-21 and IL-17A expression in CNS inflammatory lesions from patients with BD. Immunohistochemical analysis of IL-21, IL-17A, CCL20, CXCL8, and IL-6 expression in the choroid plexus from patients with aBD and from HDs **(A)**. IL-17A and IL-21 are expressed in intracerebral blood vessels from patients with BD **(B)**. IL-17A and IL-21 are expressed in inflammatory brain lesions and colocalized with CD3 in patients with aBD with CNS involvement **(C)**.

IFN-γ–producing $CD4^+$ and $CD8^+$ T cells were also dramatically increased in the CSF than in the peripheral blood from patients with aBD (52.3% vs 20.3% of $CD4^+$ T cells and 85.4% vs 39.3% of $CD8^+$ T cells, respectively).

Strong IL-21 and IL-17A expression within central nervous system inflammatory lesions from patients with active BD

Immunohistochemical analysis of paraffin-embedded necropsic brain tissue specimens from 2 patients with aBD with CNS involvement and 3 normal brain controls was used to investigate the pattern of expression of IL-21, IL-17A, IL-6, CCL20, and CXCL8, which is a potent chemoattractant of T_H17 cells and polymorphonuclear leukocytes. The brain's choroid plexus in patients with BD showed a strong expression of IL-21, IL-17A, IL-6, and CXCL8, as compared with normal brain controls (Fig 5, *A*). Although a uniform staining of CCL20 by choroid plexus epithelial was noted in normal controls, a higher expression of CCL20 was observed in patients with BD (Fig 5, *A*). IL-17A and IL-21 were expressed within intracerebral blood vessels and in brain parenchyma infiltrates from patients with BD compared with normal controls (Fig 5, *B*). Immunofluorescence microscopy analysis of brain parenchyma from patients with BD showed the expression of IL-17A and IL-21, and we observed the colocalization of IL-17A and IL-21 with CD3, indicating the presence of T_H17 cells and IL-21–producing T cells (Fig 5, *C*).

IL-21 is critical in modulating T_H17 differentiation and FoxP3 expression in patients with BD

We then stimulated purified $CD4^+$ T cells for 5 days with anti-CD3/CD28 with or without human recombinant IL-21 (rHuIL-21). The adjunction of rHuIL-21 increased T_H17 cells (2.1% -2.6% of T_H17 cells in HDs with and without rHuIL-21 and 3.8%-5.7% in patients with aBD; $P = .04$ and .03, respectively) and, to a lesser extent, T_H1 cell frequencies (10.9%-12.0% of T_H1 cells in HDs with and without rHuIL-21 and 12.6%-15.8% in patients with aBD; $P = .30$ and .31, respectively) (Fig 6, *A-C*), and it decreased FoxP3 expression (3.3%-1.8% of $FoxP3^+CD25^{hi}CD4^+$ T cells in HD with and without rHuIL-21 and 0.5%-0.3% in patients with aBD; $P = .004$ and .03, respectively) (Fig 6, *D* and *E*). The effect of rHuIL-21 in promoting T_H17 differentiation was mainly observed on purified $CD25^+$ and $CD25^{++}$ $CD4^+$ T cells (Fig 6, *F*).

Lastly, we analyzed the effect of IL-21 blockade by using IL-21R/Fc chimera on T_H17, T_H1, and Treg cells. The adjunction of IL-21R/Fc decreased the proportion of T_H17 (5.2% to 6.8% and 2.6% of IL-17A–producing $CD4^+$ T cells with anti-CD3/CD28 alone, anti-CD3/CD28 plus rHuIL-21, and anti-CD3/CD28 plus IL-21R/Fc, respectively) and T_H1 cells (13.2% to 20.8% and

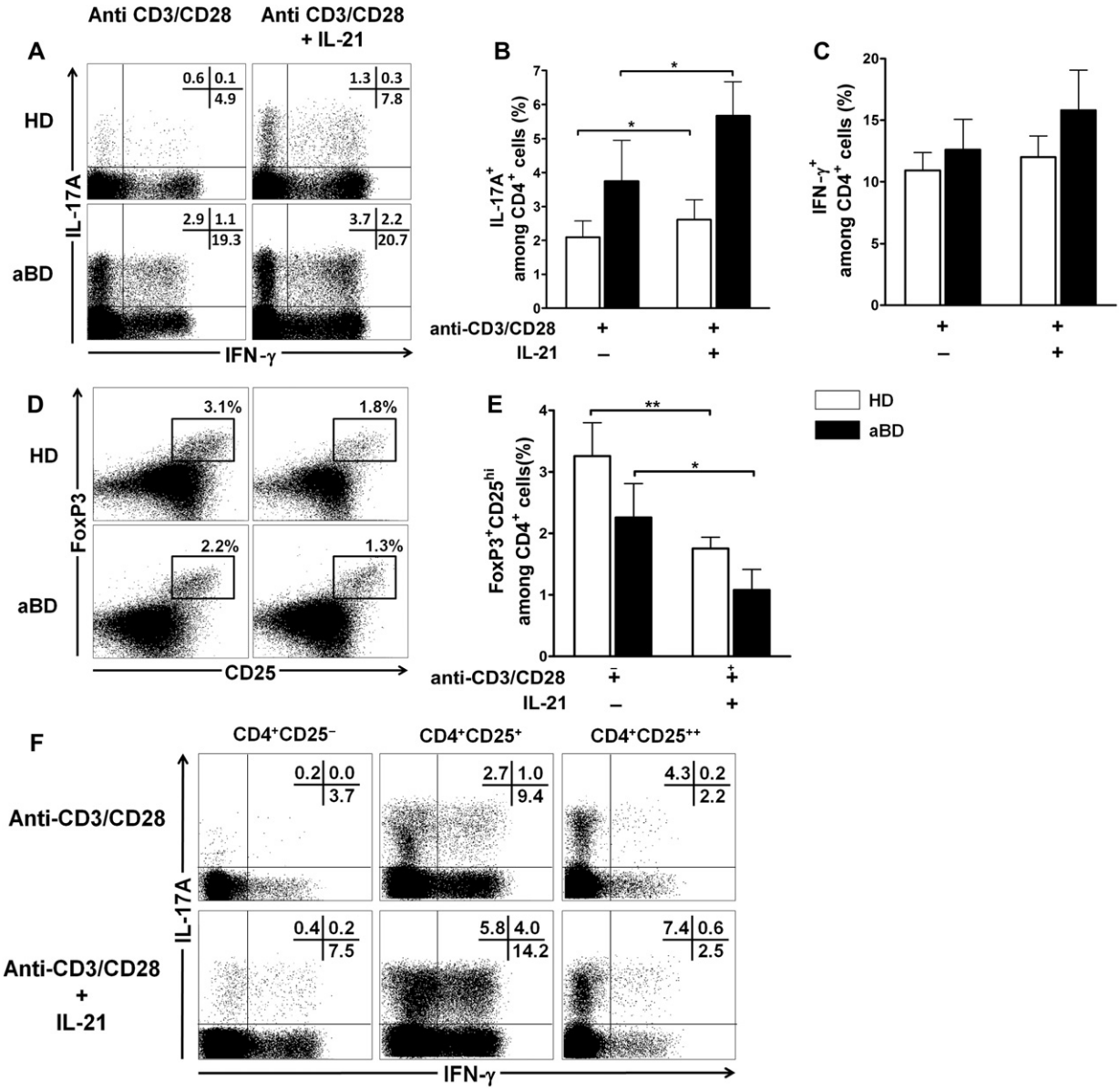

FIG 6. IL-21 is critical in modulating T_H17 response and FoxP3 expression in BD. Dot plots **(A)** and frequencies of IL-17A **(B)** and IFN-γ **(C)**–producing cells and $FoxP3^+$ expression **(D, E)** in purified $CD4^+$ T cells in patients with aBD and in HDs after anti-CD3/CD28 stimulation with and without IL-21. IL-21 promotes T_H17 and T_H1 differentiation in $CD4^+CD25^+$ T cells and exclusively promotes T_H17 differentiation in $CD4^+CD25^{++}$ T cells **(F)**. Histograms are representative of 5 patients in at least 3 independent experiments. $*P < .05$; $**P < .01$; $***P < .001$.

11.3% of IFN-γ–producing $CD4^+$ T cells, respectively) (Fig 7, *A* and *C*) and increased $FoxP3^+$ Treg cells (2.5% to 1.5% and 3.4%, respectively) (Fig 7, *B* and *C*).

DISCUSSION

Herein, we first demonstrate a marked increase in T_H17 cells and a decreased frequency of Treg cells in peripheral blood that correlate with BD activity. In contrast, the balance between T_H1 and T_H2 differentiation was not tilted. Along this line, mice that were deficient in IRF-4–binding protein, a protein that inhibits IL-17A production by controlling the activity of IRF-4 transcription factor,[9] rapidly developed a large-vessel vasculitis, sharing similarities with human BD because of an inappropriate synthesis of IL-17A.[10] The role of T_H17 cells has recently been demonstrated in giant-cell arteritis, another model of human large-vessel vasculitis.[11] Strikingly, the addition of serum from patients with aBD in sorted $CD4^+$ T cells culture from HDs induced a significant and dose-dependent production of IL-17A and a decrease in FoxP3 expression. We then intended to characterize the mechanism that promotes T_H17 differentiation and suppresses Treg cells. We did not find increased serum levels of T_H17-promoting cytokines such as IL-1β, IL-6, IL-23, and TGF-β in patients with BD compared with HDs. In contrast, serum levels of IL-21 and IL-21–producing $CD4^+$ T cells were dramatically increased in peripheral blood from patients with BD.

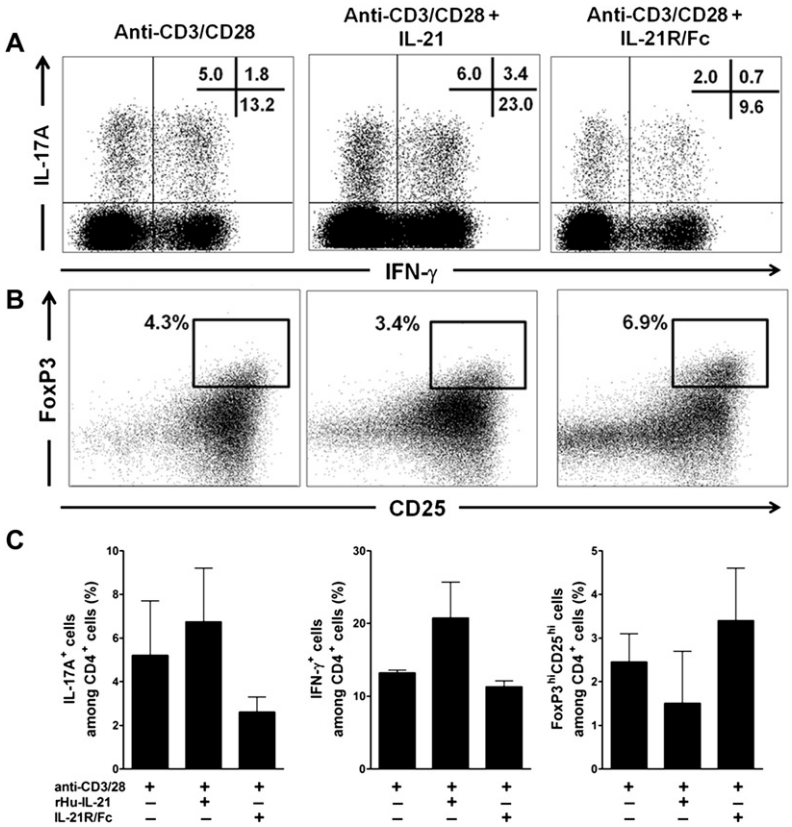

FIG 7. Blockade of IL-21 with IL-21R/Fc restores effector and regulatory T cells homeostasis. Dot plots **(A, B)** and histograms **(C)** of IL-17A and IFN-γ production and FoxP3 expression **(B)** in purified $CD4^+$ T cells after anti-CD3/anti-CD28 stimulation with and without IL-21 or IL-21R/Fc in patients with aBD. Data are representative of 3 independent experiments in 3 patients with aBD.

IL-21 is the most recently identified of the type 1 cytokine-family members.[12] IL-21 is produced by activated $CD4^+$ T cells but targets a much broader range of cells.[13] In patients with BD, IL-21–producing $CD4^+$ T cells displayed the phenotype of TCMs. Increased expression of IL-21 has been detected in two mouse models of autoimmunity: the BXSB.B6-Yaa^+/J mouse, which is a model of SLE, and the nonobese diabetic mouse.[14,15] IL-21 was shown to tilt the balance between Treg cells and T_H17 cells.[16] We observed a decreased frequency of activated/memory Treg cells in patients with BD, whereas the proportion of resting/naive Treg cells was within the normal range. The adjunction of IL-21 on stimulated purified $CD4^+$ T cells decreased FoxP3 expression. Consistent with our findings, IL-21 was recently shown to suppress the conversion of rTreg cells into aTreg cells,[16,17] supporting the deleterious effect of IL-21 on Treg cells' homeostasis. In addition, mice deficient in IRF-4–binding protein, which rapidly developed a large-vessel vasculitis similar to BD, also showed an inappropriate IL-21 synthesis.[10]

We next demonstrated the presence of IL-21– and IL-17A–producing T cells within the CSF, choroid plexus, brain parenchyma inflammatory infiltrates, and intracerebral blood vessels from patients with aBD with CNS involvement. Levels of IFN-γ–producing $CD4^+$ and $CD8^+$ T cells were also dramatically increased in the CSF from these patients. IL-17A– and IFN-γ–producing T cells are observed in the CSF and inflammatory CNS lesions of patients with multiple sclerosis.[18,19] Interestingly, we found a strong expression of CCL20, a potent chemoattractant for T_H17, and CXCL8, known to be a potent chemoattractant for inflammatory polymorphonuclear leukocytes, in the choroid plexus in patients with BD. In addition, the choroid plexus in patients with BD showed strong and uniform expression of IL-21 and IL-17A. IL-21 has proinflammatory effects that result from the induction of the expression of CXCL8 by macrophages. Taken together, our data suggest that IL-21 drives T_H17 effectors' differentiation and chemoattractants for T_H17 and polymorphonuclear leukocytes in CNS inflammatory lesions of patients with BD. Leukocyte entry into the CNS is restricted, in part, because of the blood-brain barrier. However, it is now well established that lymphocytes can also enter the CNS through the choroid plexus.[20,21] Recently, Reboldi et al provided a molecular and anatomical basis for distinguishing between constitutive and inflammatory pathways of T-cell entry into the CNS, with a critical role for the choroid plexus in the control of immune surveillance of the CNS.[22] Their findings in experimental autoimmune encephalitis (EAE) support a 2-step model of EAE pathogenesis in which a first wave of $CCR6^+$ T_H17 cells leads to the CCR6-independent recruitment in the CNS of a second wave of T cells, including T_H1 cells and inflammatory leukocytes. Our data in patients with BD with active CNS involvement are consistent with this hypothesis. The presence of T_H1 cells and polymorphonuclear leukocytes, in addition to T_H17 cells, in the brain and CSF of patients with BD supports this hypothesis.[23] IL-17A synergized with TNF-α, a cytokine efficiently targeted in BD,[24] for the induction of CXCL8 and CCL20, allows the recruitment of polymorphonuclear leucocytes and T_H17 cells within target tissues.[25] A similar

effect of IL-17A, in synergy with TNF-α, on epithelial cells of the choroid plexus in patients with BD could be observed.

Lastly, we showed that IL-21 was critical in modulating T_H17 differentiation and FoxP3 expression in patients with BD. The adjunction of IL-21 on stimulated purified $CD4^+$ T cells increased T_H17 and T_H1 cells' frequencies and decreased FoxP3 expression. The blockade of the IL-21 pathway with IL-21R-Fc restored the balance between Treg cells and T_H17 cells by suppressing IL-17A production and increasing FoxP3 expression by $CD4^+$ T cells.

In conclusion, we demonstrate the implication of IL-21 in the peripheral blood and CNS inflammatory lesions of patients with BD. IL-21 modulates T_H17 differentiation and FoxP3 expression and correlates with BD activity. Our findings suggest that IL-21 exerts a critical role in the pathogenesis of BD and represents a promising target for novel therapy.

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Key messages

- **IL-21 promotes T_H17 responses and suppresses regulatory T cells in Behçet disease (BD) and correlates with BD activity.**
- **IL-21– and IL-17A–producing T cells are enriched within inflammatory brain lesions from patients with BD.**

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A dive into the complexity of type I interferon antiviral functions

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COMMENTARY ON:
A diverse range of gene products are effectors of the type I interferon antiviral response. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM. Nature. 2011 Apr 28;472(7344):481–485. Copyright (2011). Abstract reprinted by permission from Macmillan Publishers Ltd.

http://www.ncbi.nlm.nih.gov/pubmed/21478870

Abstract: *The type I interferon response protects cells against invading viral pathogens. The cellular factors that mediate this defense are the products of interferon-stimulated genes (ISGs). Although hundreds of ISGs have been identified since their discovery more than 25 years ago, only a few have been characterized with respect to antiviral activity. For most ISG products, little is known about their antiviral potential, their target specificity, and their mechanisms of action. Using an overexpression screening approach, here we show that different viruses are targeted by unique sets of ISGs. We find that each viral species is susceptible to multiple antiviral genes, which together encompass a range of inhibitory activities. To conduct the screen, more than 380 human ISGs were tested for their ability to inhibit the replication of several important human and animal viruses, including hepatitis C virus, yellow fever virus, West Nile virus, chikungunya virus, Venezuelan equine encephalitis virus, and human immunodeficiency virus type-1. Broadly acting effectors included IRF1, C6orf150 (also known as MB21D1), HPSE, RIG-I (also known as DDX58), MDA5 (also known as IFIH1), and IFITM3, whereas more targeted antiviral specificity was observed with DDX60, IFI44L, IFI6, IFITM2, MAP3K14, MOV10, NAMPT (also known as PBEF1), OASL, RTP4, TREX1, and UNC84B (also known as SUN2). Combined expression of pairs of ISGs showed additive antiviral effects similar to those of moderate type I interferon doses. Mechanistic studies uncovered a common theme of translational inhibition for numerous effectors. Several ISGs, including ADAR, FAM46C, LY6E, and MCOLN2, enhanced the replication of certain viruses, highlighting another layer of complexity in the highly pleiotropic type I interferon system.*

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Introduction

Type I interferons are a family of major innate immune cytokines produced by host cells in response to viral infection [1]. Since their discovery 50 years ago, fundamental and biomedical research has greatly improved our understanding of their molecular mechanisms of action, and led to the development of the first "cytokine-based" therapy in the 70s, now licensed worldwide for viral disease, malignant and even immune disorders [1,2].

Interferon remains the therapeutic backbone of chronic hepatitis C. The standard of care, in HCV genotype 1 infected patients, is the addition of direct-acting antivirals (DAAs) with a protease inhibitor (telaprevir or boceprevir) to pegylated interferon plus ribavirin [3].

The type I interferon family is composed of 5 members in humans: the well described IFNα and IFNβ, along with IFNκ, IFNε, IFNω that are less characterized, and more tissue targeted [4,5].

There are 13 IFNα and one IFNβ isoforms, all acting through a unique ubiquitous heterodimeric receptor IFNAR1/IFNAR2. Downstream signaling pathways have been extensively described: phosphorylation of tyrosine kinases JAK1 and TYK2 results in the recruitment of STAT1 and STAT2 which migrate into the nucleus and associate with IFN regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 (ISGF3). This complex then activates the transcription of all the IFN Stimulated Genes (ISGs), which mediate diverse cellular effects in the infected cell. The study of highly induced ISGs (MX1, OAS, dsRNA-activated protein kinase PKR) led to fundamental discoveries concerning the translational control and regulation of RNA stability [6].

Unresolved questions

The function of many ISGs, however, remains unknown, limiting our ability to manipulate IFN in a rational manner and predict its therapeutic and side effects. In particular, it is not known

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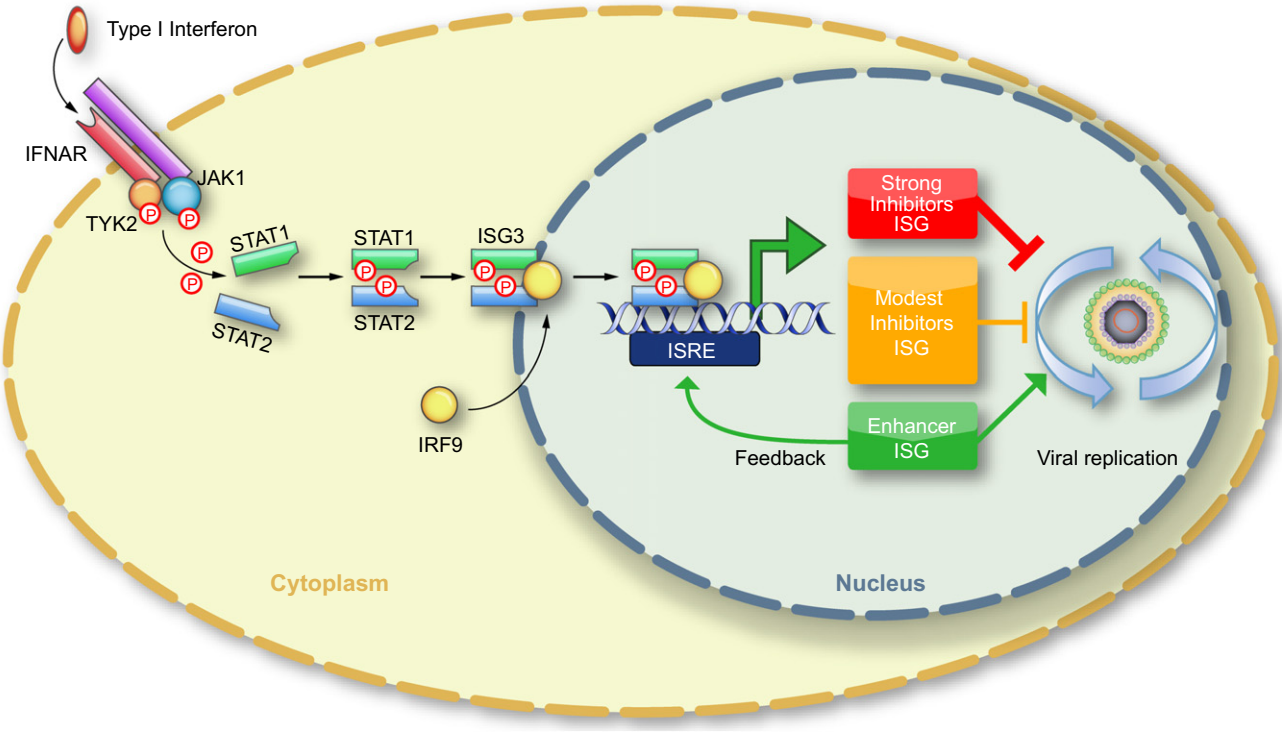

Fig. 1. New view of ISG's function in viral replication. Interferon stimulated genes (ISG) can be divided in 3 groups: strong inhibitors, modest inhibitors or enhancers. ISGs use multiple strategies to inhibit viral replication: either by targeting specific phase of viral replication (e.g. primary translational inhibition) or/and by potentiating IFN response by a positive feedback loop. IFNAR, Interferon receptor; ISRE, Interferon response stimulating elements, IRF9, Interferon response factor 9.

whether all ISGs share the same antiviral potential and/or mechanism of action.

In the issue of April 2011 of *Nature*, Schoggins and colleagues succeeded in answering these questions [7]. They proposed a new model to analyze the antiviral function of ISG in a systematic and large-scale manner. They developed a cell-based assay using a lentiviral vector co-expressing an ISG and a red fluorescent protein, TagRFP, in order to overexpress the ISG in different cell types. They subsequently challenged these cells with different green fluorescent proteins (GFP)-expressing viruses (including HCV) to assess the inhibitory capacity of all the ISG on viral replication by flow cytometry.

Interestingly, they identified 3 main categories of ISGs for each virus: a small group with strong inhibitory effect that probably has a feedback into the IFN-mediated signaling pathway; a major group with moderate inhibitory functions, and a small group that surprisingly enhances viral replication. Moreover, the use of combinations of two inhibitory ISGs increased the inhibition to 90% for HCV, HIV, and yellow fever virus replication.

Nucleic acid binding, hydrolase, and helicase activities were the main molecular functions of the ISG. The authors then investigated the potential mechanism of action of selected inhibitory ISGs. Translational inhibition appears to be a common mechanism of ISG-mediated antiviral effect which correlates with percent of inhibition. In the case of HCV, IRF1, IRF2, IRF7 MDA5, RIG-I, MAP3K14, and OASL were the most efficient ISG and inhibited primary translation by 25–70% after 4 h of infection. None of them was able to significantly impair viral entry into the cell.

These results support the concept that the downstream effectors of Type I interferon exploit multiple strategies to block viral replication at an early stage, in an additive manner. Some of the ISGs, however, have the paradoxical effect of enhancing viral replication at least in this experimental model.

Novelty of this article

This is the first study on IFN downstream effectors to screen such large numbers of ISGs (380) in a systematic manner. Moreover, the reported findings point out new differences between ISGs in terms of viral replication and mechanism of action, which change our current view of ISG function (Fig. 1).

Some ISGs have broad effects on different viruses (IRF1, C6orf150, RIG-1, MDA5) whereas others are more target-specific (IFI44l, IFI6, OASL, IFIT3M). Even if they don't share the same mechanism of action, they can have additive effects to maximize viral inhibition. Capacity of viral inhibition varies among ISGs, and the authors showed for the first time that few of them could indeed enhance viral replication. It would be interesting now to test the ISGs on other viruses in order to have a complete view of ISGs functions.

Perspectives, unanswered questions

An important question remains whether the *in vitro* over-expression of the ISGs reflects *in vivo* expression. It is crucial to validate the targeted set of ISGs on *in vivo* or *ex vivo* samples. To date, several studies on liver gene expression in chronic hepatitis C have already identified a type I interferon signature (MX1, OAS1, IFI27, viperin) [8–11]. None of these molecules appear to have a

strong "inhibitory potential" for HCV replication according to the Shoggins study. Interestingly, in chronic hepatitis C, prior to the initiation of treatment, gene expression profiles differ between non-responders and responders. The most notable changes in gene expression are mainly observed in the IFN stimulated genes [12]. A two-gene signature (*IFI27* and *CXCL9*) was able to predict treatment response. Interestingly, the baseline liver levels of expression of IFN stimulated genes were higher in non-responders than in sustained virological responders. The failure to respond to exogenous PEG-IFN in non-responders could indicate a blunted response to IFN. This suggests that IFN stimulated genes are already maximally induced in non-responders.

Furthermore, it seems also that some ISGs can enhance HCV replication but these were not described in details. Another paradoxical finding is that HCV through NS3-4A expression may inhibit the RIG-1 and MDA pathway that was found to be the most efficient inhibitor of HCV replication [13]. Follow up studies are necessary to extend and validate the Schoggins' findings in complementary model systems, as well as on patient material.

Conclusions

In their study, Schoggins and colleagues bring new insight into the effector mechanisms of type I IFN responses. The understanding of antiviral mechanisms of IFN is crucial for the discovery of new treatment biomarkers for efficacy and toxicity. Moreover, there is a need for improvement of IFN therapy with regard to the clinical side effect and viral resistance. Focus on specific sets of ISGs could lead to the development of a more targeted therapy, by specifically inhibiting viral replication, while diminishing the side effects observed with type I IFNs. Future investigation and therapeutic clinical trials will be crucial to validate the potential of using ISGs *in vivo*.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding of conflict of interest with respect to this manuscript.

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I first met Alick Isaacs in November, 1955.

He immediately struck me as an extremely intelligent and very lively person; though little did I know that those next few years would lead to a major discovery – that of interferon – and also that working with him was going to set the course of my own scientific career.

Alick Isaacs was a very bright medical scientist who had started his career in virology after completing his medical training, working first with Professor Stuart Harris at Sheffield University, then in Australia at the Walter and Eliza Hall Institute in Melbourne and after coming back to Britain, at the National Institute for Medical Research (NIMR) in north London, one of the premier research laboratories in Britain and funded by the Medical Research Council. While in Australia he had been studying virus ‘interference’. It had been known for some years that treatment of cells in culture with one virus (the interfering virus) blocked the growth of a second virus, called the challenge virus. It was not an immunological phenomenon, nor did the first virus, the interfering virus have to multiply, since heat inactivated influenza virus was effective against challenge with either the infectious virus or other unrelated viruses, such as vaccinia. However, the mechanism of virus interference was completely unknown. Jean Lindenmann was a Swiss virologist, also medically trained, who had come to work at the National Institute for Medical Research for one year, and brought with him some interesting observations that he had made in Switzerland on virus interference, and it was discussion of those experiments with Alick Isaacs that led to their initial experiments.', 'I was 25 and had just come back from the United States, after spending two years as a post-doctoral research fellow at Yale University, where I had been working on the isolation and structure of some novel nucleosides, which had been isolated from a Caribbean sponge, and which contained arabinose rather than ribose as the sugar. One of these nucleosides was later to enter cancer chemotherapy as AraC. My first degree, from the University of Birmingham in England, was in chemistry and I had stayed on to work for a Ph.D. on steroids, so I had a background in natural product chemistry. I had gone to the US in September, 1953 by boat – everyone traveled by boat in those days – on the Cunard liner ‘Georgic’, and there amongst the large number of Americans returning from a summer in Europe was a young man named Jim Watson, who had just published with Francis Crick, that famous letter in Nature, which with it’s memorable conclusion: “It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying

mechanism for the genetic material", was to set the course of the biosciences for the next 50 years. In addition, I was newly married to a Yale graduate, liable for military service in the British army, and I had no job. I was grateful to be offered two very different jobs in Britain – one working on rocket fuel development and the other on the biochemistry of viruses at NIMR. I jumped at the NIMR job and was lucky enough to be given exemption from military service. I had a 3 year appointment as a member of the Chemistry Division, not the Virology Division, since Sir Christopher Andrewes, the head of Virology, was resolutely opposed to any scientist being a member of his division. For him, virology was a medical subject, accessible only to medical graduates, and was not taught for example, in any undergraduate science course in Britain at that time.

My first project was to determine the nucleic acid content of influenza virus, which was known to be an RNA virus, but how much RNA was uncertain, and there was some evidence that the amount of RNA depended on the way the virus was grown. Neither was it certain whether the virus also contained DNA — such was our ignorance. I spent the next eighteen months determining the RNA content of two forms of the purified virus (spheres and filaments) by the extremely laborious method of hydrolyzing the viral RNA to mononucleotides, separating them by ion-exchange chromatography, and using UV absorption to determine the relative amounts of each nucleotide. The work was published with Alick and the head of my Chemistry Division as co-authors; my first paper with Alick of many [1], though not on interferon.

Towards the end of this period, I started discussing with Alick what I should do next. I had in mind a very ambitious project that involved labelling purified influenza virus with radioactive phosphorus and following it through the infectious process. In retrospect, it would have been a disaster — the radioisotope would have gone everywhere and nothing interpretable would have resulted. He suggested, as an alternative, that I might like to help him "with something interesting that we are doing on interference." "We" was Jean Lindenmann and himself, the time was a March, 1957, and interferon was only a few weeks old. Jean had, I believe, suggested the name interferon – Alick once complained to me that he thought that it was "time that biologists had a fundamental particle, for the physicists have so many; such as electron, neutron proton etc." However that did not stop Lord Hailsham, a senior lawyer, trained in the classics and the Chairman of the MRC at that time, objecting that it was a nasty hybrid word with both Latin and Greek roots! By then, though, the name had stuck. Alick and Jean worked well together; they had adjacent rooms on the second floor of the Institute, and since Alick was also Director of the World Influenza Centre, he had a large laboratory that tended to be our communal workspace. Jean supplied an admirable foil for Alick's mercurial, effervescent temperament, while I had a chemical training which came in useful.

Interferon had been discovered by a series of experiments planned to test quite another hypothesis. It was the early days of virology (the steam age, as Sir Christopher Andrewes would

say, referring rather disparagingly to the dream age that would follow – molecular biology and all that which he did not believe in!), and no one really knew how animal viruses worked – indeed it was suggested that the viral coat was left outside the cell, as did bacteriophage. Alick and Jean were testing this by seeing whether any viral property – and they chose interference – was still associated with the outer coat membrane of the cell, and could be washed off. What they found was not the viral coat from outside the cell, but the interferon newly made inside the cell. It was their perception that there was something unusual going on, when the small effects that were observed could easily have been dismissed as experimental error, as well as the formulation of a testable hypothesis that was the real insight.

The system was very crude. The virus that was used to stimulate interferon production was heat-inactivated influenza virus, which could interfere but not multiply, and the cells used were pieces of chorioallantoic membrane, cut from a 10-day-old fertile hen's egg. The virus preparations were not very potent and one of the improvements we made soon after I joined the collaboration was the use of ultraviolet-inactivated virus instead of heat-inactivated virus. Interferon was estimated by challenging the treated cells with infectious influenza virus and then measuring virus growth by hemaglutination titration. Influenza has the capacity to bind to chick red cells, so called agglutination, and finding the dilution of virus that gave partial agglutination provided a simple, though insensitive, measure of the amount of virus present. It was necessary to test, in sextuplicate, at least three two-fold dilutions of the interferon sample to get a response onto the dose-response curve. The amount of virus produced in interferon-treated cells was measured by diluting the virus produced in serial two-fold steps in plastic plates, and then adding chicken red blood cells. The endpoint of the titration was the well with partial agglutination, and a reciprocal of the interferon dilution, the interferon titer.

The experiments took hours to titrate, involving little more than purely mechanical operations, and this left time to talk. Alick was the leader in conversation, and ideas for new experiments, political discussion, or identification of snatches of opera that he would sing made the time pass quickly. Alick, too, was adept at determining where the endpoint of the titration was, and with the aid of a hand lens, could do it long before the rest of us, so he had often planned the next experiment before the red cells were really settled. Occasionally, in his impatience to start the next experiment, he mistook the endpoint and then the experiment was abandoned before it had even been started! It was immensely stimulating, and very different from the chemistry I had being doing, for that was a mature discipline, and this was so new!

By March, 1957 Alick and Jean had established the basic phenomenon and, together with the electron microscopist Robin Valentine, had looked hard for virus particles in the interferon preparations, since it was quite possible the interference detected in the fluids was due to residual virus particles. Two papers were written, and were sent to the most prestigious journal of the time, the *Proceedings of the Royal Society*, but there was still much to do. The first of

these two papers [2] described the production of interferon by treatment of chick chorioallantoic membranes *in vitro* with heat-inactivated influenza virus, and went on to show that interferon could be distinguished from heat-activated influenza virus by several properties; interferon was non-hemagglutininating, its activity was not neutralized by viral antiserum, and it was not sedimented by high speed centrifugation. However it was not possible to decide whether interferon was a cellular product formed in response to virus infection, or a part of the heated virus itself, or whether possibly it represented an abortive attempt at virus multiplication. The second paper [3], which was quite short, described attempts at visualisation of interferon.

It was quite easy to plan and carry out the experiments that characterized the system further, and these were published in a series of papers in the *British Journal of Experimental Pathology*. I still have my laboratory notebooks from those early years, and my first experiment, dated March 4th, 1957 was headed “Dialysis of interferon” – we did not even know whether interferon would pass through a dialysis membrane or not! A second experiment, started on the same day, was to test whether interferon activity was destroyed by shaking a crude preparation with ether. It was, and it was another hint that interferon was a macromolecule. A series of experiments to characterize the stability of interferon at different pH’s followed and then several experiments to see whether interferon did really behave like a macromolecule, either a polysaccharide or more likely, a protein. I found that it was precipitated with ammonium sulphate, (experiments carried out in early May, 1957), and that it was degraded by treatment with the proteolytic enzyme trypsin, and also that it was inactivated by shaking with butanol but not inactivated with periodate – suggesting it was not a polysaccharide. The stability of interferon at pH2 gave us an opportunity to test whether it was destroyed by the proteolytic enzyme pepsin, and it was, confirming that interferon was a protein, and if it was a protein, then presumably it could be purified, possibly relatively easily. The first of these conclusions was true, but the second took a long time and was much more difficult.

The first paper of the series in the *British Journal of Experimental Pathology* with the title: “Studies on the production, mode of action and properties of interferon” [4], was the only paper for which Jean and I were coauthors with Alick, before Jean went back to Switzerland in September 1957. Alick wrote papers very quickly; he would take the laboratory notebooks home and produce a first draft by the next morning, and so we were able to submit this paper as early as July 23rd, 1957. In brief, it described a system that we used to make interferon for the next few years, some experiments showing a need for cell metabolism before interferon could be effective and others showing the lack of specificity of interferon’s action. It also described the well-known pH2 stability, precipitation by ammonium sulphate, and inactivation by trypsin. Thus this early paper established a substantial number of the basic parameters of the interferon system. We had, of course, no idea as to how complex the system was going to be – Alick and Jean’s first paper had been called “The interferon”, as if it was a single substance, and it was not until David Tyrrell later showed that interferon was often species specific that we realized that there was more than one interferon.

The next paper, rather frugally titled “Further studies on interferon” [5], was submitted on November 7th, 1957, and described the use of ultraviolet inactivated virus as a much more efficient producer of interferon and showed that the time of a irradiation was very important in determining the yield, small amounts of irradiation producing high yields, whereas longer periods of irradiation led to a complete loss of effectiveness. These experiments are now most readily interpreted as a measure of the capacity of the virus to form double-stranded RNA which was, in turn, the actual inducer.

The final paper in that early series was modestly called “Mode of action of interferon” [6]; it seems incredible, looking back, that we could have thought that the problem was that simply solved. This short, rather complicated paper, showed that pre-treatment of cells with interferon, followed by the induction by inactivated virus led to an increased yield of interferon, a phenomenon called ‘priming’. This effect has now been explained by the induction of otherwise rate-limiting transcription factors required to produce interferon messenger RNA. However we knew nothing at that time about transcription factors of course, and at the time we advanced a rather complicated, though ingenious, interpretation of what we had observed. Rereading the paper after all these years, it strikes me that the conclusion of that paper is remarkably dense and strikingly void of any molecular interpretation. It is of course more a comment on how descriptive our understanding of cellular processes was at that time. In the event, these results were pushed to one side, when a simple and elegant experiment by Joyce Taylor in 1964 showed that interferon production was inhibited by treatment of virus-infected cells with actinomycin, and since it was known that actinomycin blocked DNA-directed-RNA synthesis, and since the interferon was induced by infection with an actinomycin-resistant virus, it was clear that cellular DNA must be involved. Though that explained the cell specificity of interferon very neatly and provided no insight as to the actual process, it did provide the essential molecular framework for much of the work that followed in the early sixties.

One of the most striking characteristics of the interference phenomenon, which we now believed was mediated by interferon, was that one virus can interfere with the growth of a number of unrelated viruses. It was therefore important to see how broad the antiviral effect was and the next paper in the series [7], published in October 1958, showed that the chick chorion of the 12-day old fertile hen’s eggs could be used to measure the protective effect of interferon against vaccinia virus. A similar protective effect was found against two other poxviruses, cow-pox and ectromelia, although herpes simplex appeared to be more resistant. This was the first demonstration that interferon was active in vivo, although only in the fertile hen’s egg, not in an animal. More importantly, it really did look as if interferon had a wide specificity and this raised the important practical question of whether interferon could be developed as an antiviral antibiotic. The last paper in the series reported on “Some factors affecting the production of interferon” [8], showing a general correspondence between the capacity of influenza virus to produce interference and interferon.

By then interest in interferon was growing and already the focus was shifting to the possible utilisation of interferon. Virus infections were very important medically, there were no antiviral drugs and vaccine development was in its infancy. Alick Isaacs and I wrote a general article titled “Interferon: A possible check to Virus Infections” [9] which was published in the British weekly science journal The New Scientist in June, 1958. Interferon even made the Flash Gordon cartoon! We were also honored by an invitation to present our results at a Conversazione (a reception with food, wine and scientific exhibits) for the Fellows of the Royal Society (Alick was elected a Fellow in 1966) in May 1958, and I have a copy of our abstract which started:

“So far no antibiotics active against viruses have been discovered. To a large extent this is because viruses are extremely small parasites which are obliged to live inside cells, and it has not been possible to find a substance which would stop viruses from growing without at the same time harming the host cells. Interferon is the name which has been given to a new substance which prevents the growth of a number of viruses without apparently causing any gross damage to the cells. Interferon does not kill the viruses, but stops them from multiplying. This demonstration shows different aspects of the study of interferon….”

We had a rather simple series of posters and demonstrations which showed this distinguished body of senior scientists, all Fellows of the Royal Society or their guests, some of the early results and its promise. We were all dressed up, quite appropriately, in dinner jackets, and I remember that we were asked to present our demonstration a second time to an event to which only the really ‘great and good’ were invited. For this event we had to wear white tie and tails, which I didn"t possess and had to hire. I vividly remember dressing up in our very modest little North London flat, and sitting down with my wife to eat in my splendor, and she complimented me by putting on an evening dress, as we sat at the kitchen table, before going off to the great event. It was a heady time; I was only 28.

However, some problems were surfacing. The first indication was a puzzling positive result – we could get protection against the growth of vaccinia virus in the rabbit skin using chick interferon. That did not cause us any concern at the time because it had still not been shown, as it was so clearly later by David Tyrrell, that interferon was species specific and that chick interferon was not active in rabbit cells. But as soon as it was, we had to ask ourselves why was our preparation of chick interferon preventing the growth of vaccinia in the rabbit skin? It struck us that this might be due to traces of ultraviolet inactivated virus coming through from the cells in which the interferon had been prepared, contaminating the interferon and adding to the interference affect. If that was so, how many of the other results were due to traces of UV inactivated virus as a contaminant? This troubled us greatly, and it coincided with criticism of

the interpretation of our results in the US, where interferon was being called “misinterpreton” and several eminent US virologists were dismissing the effects as due to traces of virus. Alick was very depressed by this reaction, and it was the first sign of a series of depressive setbacks which dogged him over the next few years. He was off work for a month or two and I spent that time repeating all the initial experiments with interferon which had been treated at pH2 in order to destroy any UV inactivated virus, so as to be quite sure that the effects we had been observing, and publishing, were due to interferon and not to traces of contaminating virus. To our relief, all the early experiments held up, and it was not necessary to publish any retraction or corrections.

Two lines of inquiry dominated our time for the next few years. The first was to see whether interferon could really be developed as an effective antiviral agent in the UK. In the late fifties the outcome of the penicillin story still grated in Britain; the perception was that a British discovery had been “handed over” to the Americans during the war, they had then developed an industrial production process which had been patented, and we were now paying royalties on that process in order to obtain the drug we had discovered. So the MRC was under considerable political pressure to determine whether and how interferon could be developed as an effective antiviral agent in the UK. The actual discovery of interferon was patented, although the legal process was so cumbersome, and was so delayed as result of challenge from the United States, that the patent did not come into effect for some years afterwards. However that patent did provide some royalty income at a time when interferon was being developed on a large scale, so it was not wasted.

More to the point, a novel groundbreaking collaboration was built between the MRC and three major pharmaceutical companies working in the UK: Glaxo Laboratories, ICI Pharmaceuticals and Burroughs Wellcome, later to become the Wellcome Foundation. This was set up about 1958 and worked until the mid-sixties with the specific aim of making enough interferon to do an effective clinical trial. The collaboration brought new skills and new people into the field: Karl Fantes from Glaxo, and Norman Finter from Burroughs Wellcome were outstandingly valuable additions, and many other resources became available. For example, the standardization of the interferon unit, the development of better methods for large scale production, and experienced development management skills from the pharmaceutical industry all brought benefits. I was a member of that Committee throughout its life, and Alick was chairman. He was not a good chairman; my experience is that research academics rarely have the necessary skills to steer a mixed academic/industrial project forward, and in retrospect a professional, experienced manager from one of the pharmaceutical companies should have chaired the whole process, but we were feeling our way at that time towards effective research/industry collaborations and what is obvious now was not obvious then. Partly because of Alick’s style and partly because of his series of illnesses, the collaboration had its up’s and down’s, and on a number of occasions went off down blind alleys, but it did achieve its initial objective of a trial against a vaccinia virus challenge in the upper arm of unvaccinated volunteers at the Common Cold Research Unit at Salisbury in the spring and summer of 1962. So the outcome was two edged: on the one hand,

the collaboration had shown that interferon could be used in humans against a virus challenge, but on the other hand, it was not practical to prepare either enough interferon, or to deliver it early enough to be a either a useful prophylactic or a therapeutic.

So other systems to determine the parameters had to be explored, and herpes infection of the rabbit eye was one system which did give a useful clinical outcome. But the whole clinical development of interferon was put on hold then for some years, partly because of our inability to make enough interferon – a problem not solved until the development of large scale production in human cells by Kari Cantell in Helsinki, using human leukocytes, and by Norman Finter in the UK, using human lymphoblastoid cells, and finally by the production of interferon by gene cloning in the early eighties. The other new driver which emerged in the seventies was the claim that interferon could be used effectively against human cancer, but that is quite another story.

The other line, which was my responsibility, and filled my time until the early sixties when I began to work on other aspects of interferon production, was its purification. Early experiments had shown to our satisfaction that it was a protein: suitable purification procedures were then being rapidly developed, and there was expertise available in NIMR, especially in the group working around Rodney Porter, who was awarded the Nobel Prize for elucidation of the structure of antibodies from work he did at this time. So all looked good. The object was twofold: to prepare material that was could be used in clinical trials, and to establish exactly what sort of physico-chemical entity interferon was. In the event, this took years and the story became increasingly complex as it emerged that there was not just one human interferon but many: α, β and γ, and also multiple varieties of interferon α and all this was unknown when I started serious work on interferon purification in the summer of 1958. Nor had we any idea how high the specific activity of interferon would turn out to be: our best preparations had about 1000 units per ml and with a specific activity of about 109 units per mg; we had only about ten micrograms of the interferon in our 10 litres of starting material. So although we scaled the process up, ultimately working with ten litre batches, the amount of material we were trying to purify was very small, and because interferon was readily absorbed on to surfaces or became attached to other proteins present in the crude preparation, and also because it emerged that column purification procedures would only work effectively with high loads of interferon protein, it is clear that in retrospect that the desired outcome, that of making and characterizing pure interferon in a year or two was a hopeless task.

But we pressed on and scaled up in using larger and larger bottles and more and more eggs but still using the initial process of treating chorioallantoic membranes with ultraviolet inactivated virus. This was before the use of tissue culture systems which were then being developed on an industrial scale for polio vaccine production, and it was long before the production on the multi-thousand litre scale, developed by the Wellcome Foundation for the production of human interferon. We did all our own assays, and Friday, Saturday morning and all day Monday was

taken up with the assays from experiments that had been run on Tuesday and Wednesday. It was a difficult task and looking back, it was impossible, but of course that is hindsight with its 20:20 vision. So I filled twelve laboratory note books with experiments aimed at developing a multi-stage purification process for chick interferon. I established a partial process by the spring of 1960, just before I left NIMR at the end of March, 1960 to take up a university lectureship. It was, as the Duke of Wellington said of the battle of Waterloo “the nearest run thing you ever saw in your life” since my contract expired in the summer of 1960, and with a wife and two children, I had to get a job. My original three year contract had been extended for another two, mainly as the Director rather tactlessly explained, to keep the lab going while Alick was unable to work. So I had no long-term future there and I looked for a job where I could continue working on interferon and the biochemistry of viruses, and I needed another virologist in the same university to help get me going. In 1960 that restricted my choice to just one or two universities, and there were only a few jobs going every year in biochemistry anyway. So I was fortunate to get a lectureship in biochemistry at the University of Aberdeen to start in April 1960, and that gave me a very firm deadline for the completion of the purification work.

I vividly remember taking the last ten litre batch through the purification process desperately hoping that nothing would go wrong. And in those days it often did. The fraction collectors, which were essential for collecting the eluate from the ion exchange columns we were using for the multistage process, were made in the Institute workshop and were unreliable because they worked on a siphon system, the filling of the siphon triggering the move to the next test tube. But as soon as protein started to be eluted from the column, the surface tension changed, the siphon started siphoning continuously and the crucial eluate went all over the cold room floor. So I used to work late at night watching over the fraction cutter, and get into the laboratory as early as I could, often about six am when I was doing a big run. We did manage to get enough material through the multistage procedure from the final ten litre batch to give us enough biologically active product to characterize by starch gel electrophoresis – polyacrylamide gels had not been invented – and to do an analysis in an analytical ultracentrifuge. The material was homogeneous on both counts, had a molecular weight of about 63,000 and we really thought we had a homogeneous product [10]. At this point the removal van had taken away our furniture to Scotland, and we were living in our small flat with two children on a day to day basis desperately trying to finish before I had to drive to Aberdeen with the family. So we thought we had made it, but the product turned out not to be pure interferon but chicken albumin, to which some interferon was hydrophobically bound. There was indeed one protein, and it was associated with biological activity, but it was not pure interferon, and it took some years before other workers in United States and Britain completed the task. After one modest attempt to continue purification work in Aberdeen, I decided this was a project that was impossible to continue in an academic setting, and switched to a study of the mechanism of interferon production. However, my paper had unequivocally shown that the biological activity of interferon was associated with a purifiable protein, and that interferon was not just a figment of the imagination which, like the Cheshire cat, faded way as soon as it was inspected.

But back to 1957. It was a very special summer; for it is not often that at 27, one can publish effectively every experiment, and that of course helped me when I did come to leave NIMR. Over the three years 1957 to 1960, interferon had been firmly established as the mediator of virus interference, as a protein which was purifiable, and as an important new lead in dealing with virus infections. It was also a marvelous learning time for me personally, trained as a chemist, working with outstanding biologists for the first time. Interferon was new, exciting and had clear medical applications. However, best of all was the company, and I shall always remember Alick, Jean, and I doing hemagglutination titrations in room 215. It was also the summer our first child was born – I remember that we called her the “interfering particle” because of lost sleep. The years slip by, and now that she is over 50, I’m reminded of the discovery that Jean and Alick made 50 years ago.

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Modulation of cytokine response by microenvironment: Large-scale analysis of Type IFN response during Human T Helper cells differentiation.

Abstract : Type I IFN (IFN) are innate cytokines produced by host cells during viral infection. It has pleiotropic and sometimes opposing, protective or detrimental effects, on both innate and adaptive immunity that remain poorly understood. Parts of IFN response may be explain by intrinsic effect (cell-specificity). My thesis was focused on the effect of the microenvironment, as present during T Helper cell differentiation, on IFN response. Using a systems level approach, we studied IFN responses during Four Human T Helper cell differentiation. We identified 1/ a conserved IFN-induced transcriptional program comprising mostly antiviral genes 2/ a flexible IFN response, leading to a different pattern of chemokine and cytokine induction by IFN in distinct Th environments. Antiviral response was also flexible with a lesser protection to HIV-1 and HIV-2 infection in Th2 and Th17 contexts. Our in vitro results suggested that environmental control might shape the effects of IFN in different physiopathological contexts
Key words: IFN, cytokine, Th, large-scale analysis, HIV

Interactions cytokiniques dans le microenvironnement inflammatoire : Analyse à large échelle de la réponse aux Interférons de Type I lors la de polarisation des Lymphocytes T auxiliaires

Résumé : Les interférons de Type I (IFN) sont des cytokines produites par les cellules en réponse à une infection virale. Les IFNs ont des effets pleïotropiques et parfois paradoxaux, protecteur ou néfaste pour l'immunité innée ou adaptative. Certains facteurs intrinsèques (type cellulaire) peuvent expliquer une partie ces discordances. Mon travail de thèse s'est intéressé à l 'effet du microenvironnement cytokinique sur la réponse IFN. En utilisant des analyses à large échelle, nous avons étudié la réponse IFN dans 4 contextes de polarisation des lymphocytes T auxiliaires (Th). Nous avons identifié 1/ un programme de transcription conservé et 2/ une réponse IFN flexible, modulant spécifiquement les principales fonctions des Th (cytokines, chemokines) en fonction du contexte polarisant. La réponse antivirale apparait aussi flexible avec une moins bonne protection des Th2 et Th17 contre l'infection par HIV-1et HIV-2. Nous résultats suggèrent que l'environnement cytokinique contrôle en partie la réponse IFN et peut ainsi moduler cette dernière dans différents contextes physiopathologiques.
Mots clés: IFN, Cytokine, Lymphocytes T auxiliaires, Analyse à large échelle, HIV

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