IL-17/Th17 au cours de l'inflammation chronique : ciblage des interactions cellulaires

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N°d'ordre NNT : 2016LYSE1146

THESE de DOCTORAT DE L'UNIVERSITE DE LYON
opérée au sein de
l'Université Claude Bernard Lyon 1

Ecole Doctorale N° accréditation
ECOLE DOCTORALE INTERDISCIPLINAIRE SCIENCES SANTE

Spécialité de doctorat : Sciences
Discipline : Immunologie

Soutenue publiquement le 22/09/2016, par :
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RESUME FRANÇAIS

Lors de l'inflammation chronique, les cellules immunitaires, dont les lymphocytes Th17 (LTh17), migrent au niveau du site inflammatoire et interagissent avec les cellules mésenchymateuses locales. Deux contextes inflammatoires différents, la polyarthrite rhumatoïde (PR) et le psoriasis (Pso), ont servi de cadre pour étudier le rôle de ces interactions cellulaires sur la production de cytokines pro-inflammatoires, principalement l'IL-17, ainsi que l'identification des mécanismes impliqués.

L'utilisation d'un système de co-culture entre cellules mésenchymateuses (synoviocytes PR ou fibroblastes de peau Pso) et cellules mononucléées du sang périphérique mimant la situation *in vivo*, a permis d'étudier l'effet de ces interactions. Le contact cellulaire suffisait à l'induction de la sécrétion d'IL-6, d'IL-8 ou d'IL-1β. En revanche, la forte sécrétion d'IL-17 nécessitait le contact cellulaire mais également l'activation du TCR. L'inhibition de la podoplanine (pdpn), molécule d'interaction exprimée par différents types cellulaires (cellules mésenchymateuses mais également LTh17), diminuait significativement la production d'IL-17. Toutefois, cette inhibition n'était pas totale, c'est pourquoi une étude en collaboration est en cours afin d'identifier d'autres molécules impliquées.

Cette étude a donc montré que les interactions entre cellules mésenchymateuses et cellules immunitaires jouent un rôle majeur dans la sécrétion de cytokines pro-inflammatoires, notamment dans la forte production d'IL-17. La podoplanine semble largement impliquée dans ce mécanisme, ce qui en fait une cible thérapeutique potentielle pour bloquer l'activité Th17 pathogène pendant l'inflammation chronique.

TITRE ANGLAIS

IL-17/Th17 during chronic inflammation: Targeting of cellular interactions

RESUME ANGLAIS

During chronic inflammation, immune cells, including Th17 lymphocytes, migrate to the inflammatory site and interact with the local mesenchymal cells. In two inflammatory contexts, rheumatoid arthritis (RA) and psoriasis (Pso), the aim of this work was to study the effect of cellular interactions on pro-inflammatory cytokine production, with a focus on IL-17, and to identify the involved mechanisms.

Using a co-culture system between mesenchymal cells (RA synoviocytes or Pso skin fibroblasts) and peripheral blood mononuclear cells mimicking the *in vivo* situation, allowed studying the effect of these cell interactions. The cell contact alone was sufficient to induce IL-6, IL-8 and IL-1β secretion. On the contrary, the heightened IL-17 production required the cell contact and the TCR activation. The inhibition of the podoplanin (pdpn), interaction molecule expressed by different cell types (including mesenchymal cells but also Th17 lymphocytes), decreased significantly the IL-17 production. Nevertheless, this inhibition was only partial, which leads to a collaboration in order to identify other involved molecules.

In conclusion, this study showed that cell interactions between mesenchymal cells and immune cells play a major role in the pro-inflammatory cytokine production, leading to a heightened IL-17 secretion. The podoplanin molecule seems play a crucial role in this mechanism, and thus pdpn could be a potential therapeutic target to block Th17 cell pathogen activity during chronic inflammation.

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REMERCIEMENTS

Je remercie le Professeur Pierre Miossec de m'avoir donné l'opportunité de réaliser ce travail de thèse et d'avoir fait preuve de soutien et de disponibilité. Je tiens également à le remercier pour la confiance et l'autonomie qu'il m'a accordées tout au long de ce travail.

Je remercie les Professeurs Alexander So et Hubert Marotte d'avoir accepté d'être les rapporteurs de ce travail. Qu'ils trouvent ici le témoignage de ma gratitude.

Mes remerciements s'adressent également au Docteur Marie-Nathalie Sarda pour avoir accepté de faire partie de ce jury, un peu à la dernière minute et de toujours apporter la bonne humeur au laboratoire.

Un grand merci également à tous les gens du laboratoire :
A N'Diémé, qui a su me conseiller et me guider à chaque moment de cette thèse et qui a supporté mes « p'tites questions », merci pour ton aide précieuse, ta patience et ta bonne humeur. A Ana-Maria, merci de ta gentillesse et de ton implication qui a permis de récupérer pas mal d'échantillons et d'avoir pris le temps de nous faire connaître les consultations. A Fabien, toujours présent pour donner de bons conseils et assurer le bon fonctionnement du labo. A Paola, pour les bons moments passés ensemble au boulot comme à l'extérieur, on aura finalement été au bout! A Audrey, pour sa bonne humeur partagée et sa curiosité. A Samira, pour la découverte de la microscopie et des belles images et pour les pauses papotages. A Marie, dernière arrivée au labo mais une implication à toute épreuve et une compagnie de bureau bien agréable. A Julie, aide inconditionnelle dans l'administration, merci pour ton efficacité et tes bonnes astuces en couture, heureuse que tu reviennes vite !
A Bilal, Arnaud, Thomas, Giulia et tous les autres qui ont fait partie de cette bonne ambiance au laboratoire.

Je remercie également tous les gens de Biomérieux, avec qui nous partageons le quotidien, merci pour la bonne ambiance de travail.

Enfin, un énorme merci à ma famille et mes amis, qui m'ont toujours soutenue au cours de ces longues années d'études et sans qui je ne serai pas là, et en particulier ma petite moumoune.
Et à Baptiste, qui me supporte au quotidien et me redonne de la motivation dans les moments difficiles!

LISTE DES ABBREVIATIONS

CCL20: Chemokine (C-C motif) ligand 20
CCL8: Chemokine (C-C motif) ligand 8
CCR5: C-C chemokine receptor type 5
CCR6: C-C chemokine receptor type 6
CI : Complexes immuns
CSM : Cellules souches mésenchymateuses
CXCR3: C-X-C chemokine receptor type 3
CXCR4: C-X-C chemokine receptor type 4
DC: Cellules dendritiques
ERK: Extracellular signal-regulated kinases
HIF-1α: Hypoxia-inducible factor 1α
HLA : Human leukocyte antigen
IFNγ: Interferon-gamma
IL-(X) : Interleukine-(X)
IL-23R : récepteur à l'IL-23
JNK: c-Jun N-terminal kinases
LB: Lymphocytes B
LT: Lymphocytes T
LTh (-1, -2, -17): Lymphocytes T helper (-1, -2, -17)
LTreg : Lymphocytes T régulateurs
MIP-1α: Macrophage inflammatory protein-1 alpha
MMP: Matrix metalloproteinase
MP : Méthylprednisolone
MTX : Méthotrexate
ODF : Osteoclast Differentiating Factor
PBMC : Cellules mononucléées du sang périphérique
Pdpn : Podoplanine
PHA: Phytohémagglutinine
PR: Polyarthrite Rhumatoïde
Pso: Psoriasis
RANTES: Regulated on activation, normal T cell expressed and secreted
SDF-1: Stromal cell-derived factor-1
siRNA: small interfering RNA
SN: Surnageants
TNF: Tumor necrosis factor

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Partie 1. Introduction-revue

I L'inflammation

I.1 L'inflammation aigue

L'inflammation est un ensemble de mécanismes de protection par lesquels l'organisme se défend de diverses agressions (infection par un organisme pathogène, brûlure, allergie…) et répare les tissus lésés. Le processus de l'inflammation est un processus dynamique et réversible conduisant à sa résolution. L'inflammation est traditionnellement définie par quatre mots latins, *calor* (chaleur), *dolor* (douleur), *rubor* (rougeur) et *tumor* (tuméfaction). Ces symptômes sont liés aux effets des différents agents inflammatoires présents sur le site de l'agression. Lors de la phase initiale, les premières cellules à entrer en action sont les neutrophiles et les macrophages. Une fois activés par la rencontre avec l'agent pathogène, ils déclenchent le processus de l'inflammation par la sécrétion de cytokines et chimiokines pro-inflammatoires. Les cellules et molécules du système immunitaire inné sont alors recrutées à partir du sang sur le site inflammatoire. L'immunité innée va jouer un rôle dans l'élimination directe du pathogène, mais elle permet également le déclenchement de la réponse adaptative qui va aider à l'éradication du danger. En effet, les cytokines libérées par les macrophages et les neutrophiles, premières cellules recrutées sur le site inflammatoire, vont agir sur les cellules endothéliales des vaisseaux sanguins voisins. Une augmentation du diamètre vasculaire apparaît, ce qui augmente l'afflux sanguin local, responsable de la chaleur et de la rougeur. De plus, l'activation des cellules endothéliales se manifeste par la sécrétion à leur tour de cytokines et chimiokines. La modification de leurs propriétés adhésives va alors favoriser le recrutement des leucocytes circulants. Le recrutement des macrophages et neutrophiles est ainsi suivi par la migration des monocytes et finalement des lymphocytes. La

migration de ces cellules dans le tissu et leurs effets locaux sont responsables de la douleur et de la tuméfaction.

Une fois l'agression maîtrisée, la réaction inflammatoire est arrêtée. Les macrophages vont permettre le nettoyage des débris cellulaires mais également la sécrétion de cytokines permettant la réparation du tissu par les fibroblastes (collagènes) et par les cellules endothéliales (néoangiogenèse). Des cytokines anti-inflammatoires comme l'IL-10 vont progressivement remplacer les médiateurs inflammatoires et permettre l'inhibition de leur sécrétion ainsi que de leur action. L'inflammation est alors en phase de résolution [1]. Une fois que les cellules immunitaires ne sont plus requises sur le site inflammatoire, elles vont quitter le tissu ou bien mourir par perte de signaux de survie ou par apoptose (figure 1).

I.2 L'inflammation chronique

L'homéostasie tissulaire est donc régulée par une balance sensible entre recrutement, prolifération, migration et mort des cellules impliquées (figure 1). Une dysrégulation de cette balance conduit à une réponse inflammatoire soutenue entrainant une inflammation chronique irréversible [2]. L'inflammation chronique correspond donc à un échec de l'inflammation aiguë et induit de nombreuses pathologies. L'infiltrat cellulaire sur le site inflammatoire perdure et contribue ainsi à l'hyperplasie et à la destruction du tissu. Le microenvironnement joue un rôle primordial dans ce processus. En effet, la production de cytokines et chimiokines par les cellules présentes va favoriser la survie et le maintien des cellules sur le site inflammatoire. Les raisons du maintien de l'inflammation sont encore à élucider. Cependant les mécanismes et médiateurs impliqués dans le processus de l'inflammation chronique sont similaires dans différentes maladies inflammatoires chroniques telles que la polyarthrite rhumatoïde (PR), le psoriasis (Pso), la maladie de Crohn ou encore la sclérose en plaques [3, 4]. Parmi ces agents communs, une population de cellules immunitaires appartenant aux

lymphocytes T (LT) $CD4^+$ a été très largement impliquée dans ces diverses pathologies. Ces cellules sont les lymphocytes Th17 (LTh17).

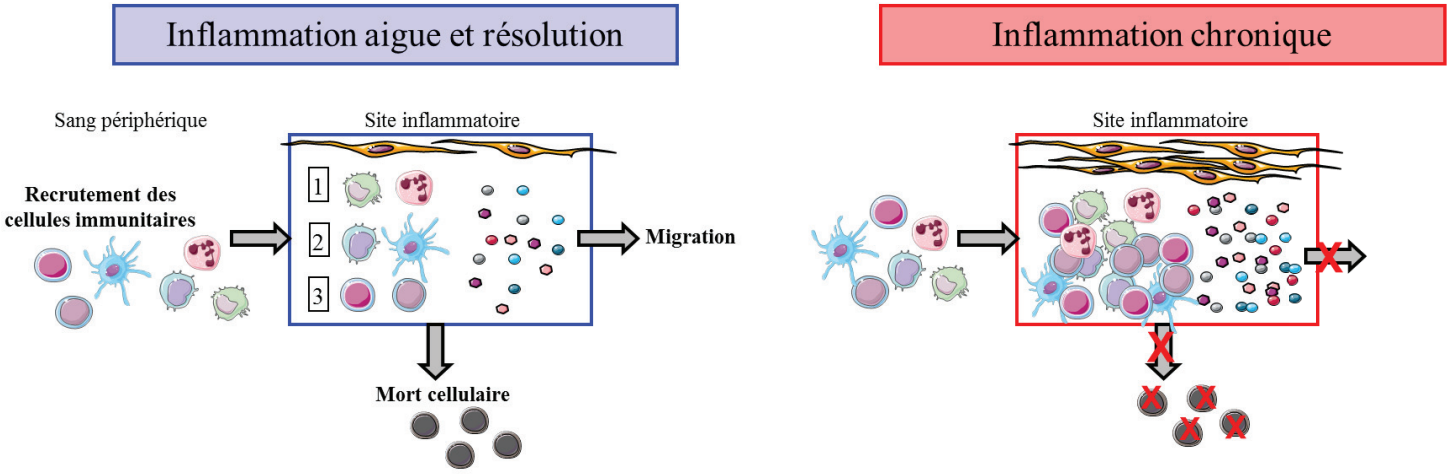

Figure 1: Lors de la phase initiale de l'inflammation, les neutrophiles et macrophages (1) sont les premières cellules recrutées. La libération de cytokines et chimiokines va alors agir sur les cellules endothéliales et permettre dans un second temps le recrutement des monocytes et cellules dendritiques (2) puis des lymphocytes (3). Une fois l'agression maîtrisée, la phase de résolution se déclenche : libération de cytokines anti-inflammatoires, migration hors du site inflammatoire et mort cellulaire. Lors de l'inflammation chronique, cette phase de résolution est absente. L'infiltrat immunitaire persiste, ce qui contribue à l'hyperplasie et à la destruction du tissu.

II Les lymphocytes Th17

Les notions fondamentales concernant les LTh17 et l'inflammation sont décrites plus en détail dans le chapitre « Th17 cells » (fin de la partie 1, p34-72), appartenant au volume « Inflammation-from molecular and cellular mechanisms to the clinic », à paraître, édition WILEY.

Le système immunitaire, composé de l'immunité innée et de l'immunité adaptative, représente la première ligne de défense de l'organisme. Ses principales fonctions sont la reconnaissance et le contrôle d'antigènes étrangers, l'induction d'une mémoire immunologique et le maintien de la tolérance des auto-antigènes. Dans le système immunitaire adaptatif, les LT CD4+ jouent un rôle critique dans l'initiation et la régulation de la réponse immunitaire, principalement par la sécrétion de cytokines spécifiques à leur sous-groupe. Les LT CD4+ ou lymphocytes T helper (LTh) sont divisés en sous-groupes selon leur profil cytokinique et leur facteur de transcription. Les deux types majeurs sont les LTh1 et les LTh2. Plus récemment un troisième lignage a été identifié, les LTh17. Ils ont en premier lieu un rôle majeur dans la protection contre les pathogènes extracellulaires et les champignons. Par la production de leur principale cytokine, l'interleukine-17 (IL-17), les LTh17 fournissent une réponse protectrice rapide et contribuent au processus inflammatoire [5]. Toutefois, les LTh17 représentent une des principales populations cellulaires pro-inflammatoires. Une sur-activation de ces cellules est largement associée au développement de plusieurs maladies inflammatoires auto-immunes [6, 7]. Malgré des marqueurs communs avec les LTh1 et les LTh2, les LTh17 requièrent des cytokines et un facteur de transcription spécifiques pour leur différenciation [8].

II.1 Différenciation chez l'homme

La différenciation des LTh17 présente trois étapes clés, une phase d'initiation, une phase d'amplification et une phase d'expansion et de stabilisation. Ces phases sont régulées par différentes cytokines. Lors de la phase d'initiation, le TFG-β en combinaison avec d'autres cytokines va jouer un rôle clé. Toutefois, si sa présence dans la différenciation des LTh17 chez la souris est indispensable, chez l'homme les résultats concernant le TGF-β sont controversés. Certaines études démontrent que le TGF-β n'est pas indispensable à la différenciation des LTh17 [9, 10] et que les combinaisons IL-1β/IL-6 [9] ou IL-1β/IL-23 [10] peuvent induire cette différenciation. Néanmoins, l'utilisation dans ces études de LT CD45RA+ et la présence de sérum et de plaquettes, sources importantes de TGF-β, rendent ces résultats difficiles à interpréter. D'autres études, utilisant des LT naïfs provenant de sang de cordon et sans sérum démontrent que le TGF-β, comme chez la souris, est un facteur indispensable pour la différenciation des LTh17 [11]. Différentes combinaisons impliquant le TGF-β, TGF-β/IL-21, TGF-β/IL-23/IL-6, sont ainsi capables d'initier la différenciation des LTh17 via l'induction du facteur de transcription RORc [11-13]. La phase d'amplification de la population LTh17 est principalement maintenue par les cytokines IL-6 et IL-1β. L'IL-6 en combinaison avec l'IL-21 est également une combinaison importante dans l'expansion des LTh17 à partir de cellules mémoires [11, 12]. Après ces différentes stimulations par TGF-β et IL-21/IL-6, les LTh17 vont exprimer le récepteur à l'IL-23 (IL-23R) et ainsi devenir sensible à cette cytokine, permettant la stabilisation et l'expansion des LTh17. Une fois différenciés, les LTh17 vont sécréter des cytokines spécifiques, dont l'IL-17, qui est leur principale cytokine.

II.2 L'IL-17

L'identification de l'IL-17, d'abord nommée CTLA-8, précède d'une dizaine d'années celle des LTh17 [14]. L'IL-17 est une cytokine pro-inflammatoire, principalement sécrétée par les LTh17. C'est un des six membres de la famille IL-17, allant de l'IL-17A, nommée IL-17, à l'IL-17F. L'IL-17 et l'IL-17F possèdent des fonctions biologiques similaires et sont génétiquement liées. Toutefois, l'IL-17 présente un effet plus important que celui de l'IL-17F. L'IL-17 se lie et agit à travers deux récepteurs, l'IL-17RA et l'IL-17RC. La distribution de ces récepteurs est ubiquitaire, permettant ainsi à l'IL-17 d'agir sur un grand nombre de cellules cibles telles que les synoviocytes, les fibroblastes ou les cellules endothéliales. L'IL-17 joue un rôle majeur dans l'immunité adaptative, principalement contre les bactéries extracellulaires et les champignons. Cependant, un excès d'IL-17 a été associé à de nombreuses maladies auto-immunes et inflammatoires chroniques, notamment la PR. L'IL-17 agit comme un médiateur de l'inflammation en induisant la production de cytokines et chimiokines pro-inflammatoires par différents types cellulaires, incluant les macrophages, les cellules dendritiques (DC), les cellules endothéliales ou encore les fibroblastes, favorisant ainsi le maintien de l'inflammation [6, 15]. L'IL-17 peut également agir directement sur le phénotype et la survie des cellules cibles [16, 17].

L'IL-17 joue donc un rôle primordial dans la chronicité de l'inflammation [18], donnant ainsi aux LTh17 une contribution majeure dans les maladies inflammatoires chroniques.

II.3 Les LTh17 et l'inflammation chronique

II.3.1 Régulation des LTh17 au cours de l'inflammation chronique

II.3.1.1 Différenciation, expansion et stabilisation des LTh17

Au cours de l'inflammation chronique, les cellules immunitaires migrent et persistent au niveau du site inflammatoire. Elles vont également sécréter des cytokines permettant la polarisation des LTh. Parmi ces cytokines, le TGF-β, l'IL-6 et l'IL-1β vont induire la différenciation des LTh17. L'activation du facteur de transcription RORc va alors permettre la sécrétion d'IL-21, agissant de manière autocrine sur les LTh17 et permettant ainsi le maintien de leur phénotype. La différenciation des LTh17 va également induire l'expression du récepteur IL-23R. Les LTh17 deviennent alors sensibles à l'IL-23, cytokine sécrétée par les cellules de l'immunité innée présentes sur le site inflammatoire, entrainant ainsi l'expansion et la stabilisation des LTh17. En plus d'une différenciation et d'une expansion grâce à l'environnement cytokinique, les LTh17 vont être recrutés directement sur le site inflammatoire via l'expression de CCR6 [19], qui lie la chimiokine CCL20, augmentée par les conditions inflammatoires et sécrétée par de nombreux types cellulaires, incluant les LTh17 eux-mêmes [20]. De plus, les LTh17 vont interagir et activer les cellules locales, induisant la continuité de la production de cytokines et chimiokines impliquées dans leur maintien. Les LTh17 contribuent ainsi à leur propre expansion.

L'exposition des LTh17 à certaines cytokines va également favoriser un phénotype pathogène. Ainsi, l'IL-23 est primordiale pour que les LTh17 soient capables d'induire une auto-immunité [21-23] et, combinée à l'IL-1β et l'IL-6, l'IL-23 induit leur phénotype pathogène [24]. De plus, l'IL-23 va augmenter l'expression du TGF-β3 endogène, indispensable à l'induction des LTh17 pathogènes [25]. Les LTh17 induits par l'IL-6 et le TGF-β3 expriment alors le GM-CSF qui fait partie de la signature des LTh17 pathogènes [25-27].

La sécrétion de cytokines et chimiokines par les cellules locales, les cellules immunitaires et les LTh17 eux-mêmes assurent ainsi la différenciation, l'expansion et la stabilisation des LTh17 tout en leur conférant un phénotype pathogène. Ces LTh17 vont alors participer très largement au maintien de l'inflammation chronique.

II.3.1.2 Phénotypes sécréteur et non sécréteur des LTh17

Les mécanismes communs à différentes pathologies inflammatoires chroniques, telles que la PR et le Pso conduisent à la différenciation et au maintien des LTh17. L'environnement inflammatoire et les interactions cellulaires vont conduire à l'activation des LTh17 et à la sécrétion de cytokines pro-inflammatoires. Les LTh17 vont ainsi sécréter de l'IL-17, leur principale cytokine effectrice. L'IL-23 va encore une fois avoir un rôle important, en favorisant la production d'IL-17. Avant leur arrivée sur le site inflammatoire, les LTh17 circulants sont caractérisés par un certain nombre de marqueurs dont l'IL-17. Toutefois, à ce stade, ces cellules ne produisent pas d'IL-17 et présentent une certaine plasticité [28-30]. Le marquage intracellulaire ne signifie pas la sécrétion de l'IL-17. En arrivant *in situ*, les LTh17 vont interagir avec les cellules stromales mésenchymateuses (CSM) dans un environnement inflammatoire, ce qui conduit à la sécrétion de l'IL-17 [31]. A ce moment-là, les cellules IL-17+ présentent une morphologie de type plasmocytaire [32]. Cette morphologie est corrélée à une augmentation de la concentration d'IL-17. Ceci indique que les LTh17 sécrétant de l'IL-17 et donc agissant sur l'inflammation ont un phénotype différent des LTh17 circulants, IL-17+ au niveau intracellulaire mais non sécréteurs.

II.3.2 Fonctions dans l'inflammation chronique

En produisant de l'IL-17, les LTh17 vont avoir un rôle pathogène majeur dans le développement de maladies auto-immunes et inflammatoires chroniques [6]. L'IL-17 possède de nombreuses fonctions biologiques en agissant sur un large panel de cellules cibles. L'IL-17 va promouvoir la sécrétion des cytokines et chimiokines pro-inflammatoires, telles que l'IL-1, l'IL-6, le TNF (Tumor Necrosis Factor) ou l'IL-8, par les cellules présentes sur le site inflammatoire, notamment les monocytes et les CSM. De plus, les interactions entre les LTh17 et les cellules locales du site inflammatoire induisent la production de MMP (Matrix Metallo Proteinase) impliquées dans la destruction matricielle [33, 34]. L'IL-17 peut également agir directement sur le phénotype et la survie cellulaire. En effet, en combinaison avec le TNF, l'IL-17 augmente la survie des synoviocytes de patients PR via l'induction de molécules anti-apoptotiques, telles que la synovioline [16] et contribue à leur phénotype agressif et invasif [17]. Les LTh17 contribuent donc largement au maintien de l'inflammation par différents mécanismes, ce qui peut expliquer leur implication dans de nombreuses maladies inflammatoires chroniques telles que le Pso ou la PR.

Des critères communs sont ainsi retrouvés dans ces maladies, comme une fréquence accrue des LTh17 et un niveau élevé de cytokines liées aux LTh17, telles que l'IL-17. L'utilisation de modèles animaux a également permis de confirmer l'implication des LTh17 via l'IL-17 dans les maladies inflammatoires chroniques et notamment dans la PR. Par exemple, dans des souris immunisées au collagène de type II, l'administration intra-articulaire d'IL-17 favorise l'arthrite au collagène avec des symptômes pathologiques similaires à la PR chez l'homme [35]. Dans le modèle d'arthrite induite par collagène (AIC), l'injection d'IL-17 exacerbe l'arthrite [36] alors que chez des souris déficientes en IL-17, l'induction de l'arthrite est supprimée [37]. De plus, l'inhibition de l'IL-17 par un anticorps diminue et ralentit la progression de l'arthrite [38]. Dans le modèle SKG, l'arthrite est inhibée dans les souris

déficientes pour l'IL-17 [39] et l'administration d'un anticorps anti-IL-17 diminue sa progression [40]. La production d'IL-17 est également nécessaire au développement spontané de l'arthrite chez la souris déficiente pour le récepteur antagoniste IL-1 [41]. Dans un autre modèle d'arthrite chez la souris, le modèle SCW (Streptococcal Cell Wall), la chronicité de la synovite destructrice nécessite la signalisation IL-17 [42]. L'IL-17 participe donc à l'induction mais également à la progression de l'arthrite, ce qui en fait un élément majeur dans sa chronicité [18].

Les LTh17, principalement par la sécrétion d'IL-17, apparaissent donc comme un sous-groupe pathogène impliqué dans l'induction et la propagation de l'inflammation chronique. Ces cellules présentent toutefois une certaine plasticité, notamment avec les lymphocytes T régulateurs (LTreg), avec qui un équilibre continuel est entretenu. Le contrôle permanent de la balance LTh17/LTreg assure l'homéostasie du système immunitaire. La dysrégulation de cette balance entraîne une sur-activation de la réponse immunitaire contribuant ainsi à l'inflammation chronique.

II.4 La balance LTh17/LTreg

Cette balance LTh17/LTreg lors des maladies auto-immunes et inflammatoires est décrite dans la revue suivante, « Th17 and regulatory T cell balance in autoimmune and inflammatory diseases », M. Noack et P. Miossec, Autoimmunity Reviews, 2014 (ci-après).

Autoimmunity Reviews 13 (2014) 668–677

Contents lists available at ScienceDirect

Autoimmunity Reviews

journal homepage: www.elsevier.com/locate/autrev

Review

Th17 and regulatory T cell balance in autoimmune and inflammatory diseases

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ARTICLE INFO

Article history:
Received 15 December 2013
Accepted 31 December 2013
Available online 11 January 2014

Keywords:
Th17
Treg
Autoimmune disease
Rheumatoid arthritis

ABSTRACT

This review focuses on the biology of T helper 17 (Th17) and regulatory T (Treg) cells and their role in inflammatory diseases, such as rheumatoid arthritis. Th17 cells represent a pro-inflammatory subset whereas Treg cells have an antagonist effect. Their developmental pathways are reciprocally interconnected and there is an important plasticity between Th17 and Treg cells. These features implicate that the Th17/Treg balance plays a major role in the development and the disease outcomes of animal model and human autoimmune/inflammatory diseases. During these diseases, this balance is disturbed and this promotes the maintenance of inflammation. Targeting the Th17/Treg imbalance can be performed at different levels such as inhibition of pro-inflammatory cytokines and their receptors, of pathogenic cells or their specific signaling pathways. Conversely, direct effects include administration or induction of protective cells, or stimulation of their specific pathways. Several clinical trials are underway and some positive results have been obtained.

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1568-9972/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.autrev.2013.12.004

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1. Introduction

The immune system with its innate and adaptive components has developed to defend the body. Its principal functions are the recognition with subsequent elimination of foreign antigens, induction of immunologic memory, and development of tolerance to self-antigens. T-lymphocytes by mediating the cellular immunity provide adaptive immunity. CD4 + T cells after being activated and differentiated into distinct effector subsets play a major role in mediating immune response through the secretion of subset-specific cytokines. Any defect in the T cell populations may result in diseases and autoimmune diseases are result from failure to sustain tolerance to self and/or cross-reactive molecules. Depending on the cytokines they produce, these T cell subsets have very different properties. Helper T cells (Th cells) include the well-defined effector Th1 and Th2 subsets as well as the more recently described Th17 cells, but also regulatory subsets like regulatory T cells (Treg cells).

The Th17 and Treg developmental pathways are reciprocally interconnected. This implicates a balance between both cell types which influences the outcome of the immune responses. Research on the immunopathogenesis of autoimmune and inflammatory diseases has made significant progress in the past few years and Th17 and Treg cells have emerged as major players in autoimmunity. Th17 cells represent a pro-inflammatory subset, which when in excess contributes to autoimmunity and tissue damage whereas Treg cells have an antagonistic effect, which when in failure also contributes to the same diseases. The Th17/Treg balance provides a basis for understanding the immunological mechanisms that induce and regulate autoimmunity and chronic inflammation.

In this review, we will give an overview of the biology of Th17 and Treg cells and their role in autoimmune diseases, with special focus on rheumatoid arthritis and multiple sclerosis, as well as how these subsets may represent new targets for treatment.

2. Description of the cell subsets

The cells of the innate immune system are the first line of defense against pathogens. CD4 + T cells have a major role in the initiation of immune responses. They provide help to other cells and take on a variety of effector functions. Upon antigenic stimulation, naive CD4 + T cells become activated, expand and differentiate into different effector subsets. Distinct types of Th cells are based on the produced cytokines and effector functions. Regarding infections, Th1 and Th2 provide effector responses to intracellular bacterial infections and parasitic pathogens, respectively. Th1 cells produce primarily interferon-γ (IFN-γ) and express the transcription factor T-bet. Their cell targets are macrophages and dendritic cells. Th2 cells produce mainly interleukin-4(IL-4), IL-13 and IL-5, and express the transcription factor GATA-3. Their cell targets are eosinophils and basophils [1].

Recently, novel cytokines, such as IL-17 [2], were identified to be produced by other Th cells. Th cells that produce IL-17 but not IFN-γ or IL-4 were named Th17 cells [3]. These cells constitute a distinct lineage of Th cells, with key role in protection against extracellular bacterial and fungal infections and autoimmunity.

The immune response needs to be controlled to avoid a permanent immune response, and chronic inflammation. For that goal, there is a type of cells known to have suppressor activity and an important role in the maintenance of self-tolerance, regulatory T cells (Treg cells) (Fig. 1). Although Treg cells can mediate any Th subset, special emphasis has been put on the Th17/Treg balance.

2.1. Th17 cells

Th17 cells were first described in mice in 2005, as the cellular source of IL-17[3–5]. However, IL-17 production by a subset of T cell clones from the RA synovium was shown already in 1999 [6]. These cells protect against fungal and extracellular bacterial infections and participate in inflammatory reactions and autoimmunity. Th17 cells secrete different cytokines (IL-17A, IL-17F, IL-21, IL-22) and require specific cytokines, such as transforming growth factor-β (TGF-β), combined with IL-6 or IL-21 and the transcription factor, RORγt, for their differentiation. CCR6, the receptor for CCL20, is highly expressed on Th17 cells and its activation induces attraction and recruitment of Th17 cells to the inflammatory site. Furthermore, coexpression of the chemokine receptors CCR4 and CCR6 or expression of CCR2 in the absence of CCR5 have been used to define human Th17 cells [1].

2.1.1. Differentiation of Th17 cells

Th17 cells exist both in mice and humans, and although there are major similarities for their development in the two species, some differences have been observed.

2.1.2. Th17 cells in mice

Naive T cells undergo differentiation into distinct lineages of Th cells, under the influence of specific cytokines. IL-18 and IL-12 drive the Th1 pathway, whereas IL-4 drives the Th2 pathway. Th17 differentiation needs a more complex combination of different cytokines. Transforming growth factor-β (TGF-β) and IL-6 are the key cytokines required to induce Th17 phenotype and these two cytokines are found sufficient for the induction of IL-17 expression in naive T cells [7–9]. TGF-β and IL-6 initiate Th17differentiation. In absence of IL-6, the differentiation of Th17 cells can also be induced by TGF-β plus IL-21 [10–12], but IL-6 is more potent. IL-21 is a member of the IL-2 cytokine family, produced by Th17 cells, and is important to amplify Th17 differentiation. Although IFN-γ and IL-4 produced by Th1 and Th2 respectively, amplify their differentiation in an autocrine loop, IL-17 cannot enhance Th17 early differentiation. IL-21 favors the autocrine loop for Th17 cells. With TGF-β, IL-21 amplifies Th17 differentiation; in its absence, the expansion of Th17 cells is defective [10–12]. Thus, TGF-β and IL-21 are major factors in the autocrine loop of Th17 cells, leading to early cell proliferation.

After differentiation initiation and expansion, IL-23, a member of the IL-12 cytokine family, is implicated in the Th17 phenotype stabilization. IL-23 deficient mice are highly resistant to the development of autoimmunity and inflammation [13,14]. Since naive T cells do not express receptors for IL-23, this cytokine cannot alone induce differentiation of naive T cells into Th17 cells [8]. TGF-β and IL-6/IL-21 induce the surface expression of IL-23 receptor on Th17 cells and these cells become then responsive to IL-23 [7,10,11]. Activation of T cells in the presence of IL-23 leads to the expansion of Th17 cells [15]. Accordingly IL-23 is not a

Th group	Function	Target cells
T-bet; IFN-γ, IL-2; Th1	Cellular immunity, Intracellular bacteria, Viruses	Macrophages, Dendritic cells
GATA3; IL-4, IL-13, IL-5; Th2	Humoral immunity, Allergic disease, Parasites	Eosinophils, Basophils
RORc; IL-17A, IL-17F, IL-21, IL-22; Th17	Autoimmunity, Extracellular bacteria, Fungi	Neutrophils
FoxP3; TGF-β, IL-10; Treg	Immune tolerance, Regulation of immune responses	Antigen presenting cells

Fig. 1. Helper T cell (Th) subgroups. The cytokine profile, the transcription factor, the function and the effector cell type that is activated are shown for each Th subgroup.

differentiation factor for Th17 cells [7–9] but acts on expansion and stabilization of the Th17 phenotype [8].

In brief, Th17 differentiation involves three steps: differentiation initiation by TGF-β and IL-6, expansion by IL-21 and stabilization by IL-23 (Fig. 2).

At the molecular level, the differentiation of Th17 cells requires a unique lineage-specific transcription factor, retinoid-related orphan receptor γt (RORγt) [16]. RORγt induces IL-17 gene in naive T cells and is required for the development of Th17 cells in the presence of IL-6 and TGF-β [16,17]. Indeed, TGF-β and IL-6, two cytokines with opposing effects, synergize to induce RORγt [7–9] via the activation of STAT3 which is required for Th17 development [18,19]. TGF-β also synergizes with IL-21 to induce RORγt and IL-17 in naive T cells [11,12].

2.1.3. Th17 cells in humans

If in mice IL-6 plus TGF-β are two cytokines necessary for Th17 development, results were for sometime conflicting for the human Th17 generation. It was argued that human Th17 differentiation was not dependent on TGF-β signaling and on the contrary, it was thought that the generation of human Th17 cells from naive precursors was inhibited by TGF-β. Furthermore, TGF-β and IL-6 failed to induce Th17 differentiation in TCR-stimulated CD45RA + human T cells. Instead, the combination of IL-1β and IL-6/IL-23 or IL-23 alone could induce Th17 differentiation [20,21]. However, these results were biased because these studies did not use genuinely naive T cells but CD45RA + Th cells purified from peripheral blood and endogenous sources of TGF-β such as serum or platelets were not carefully

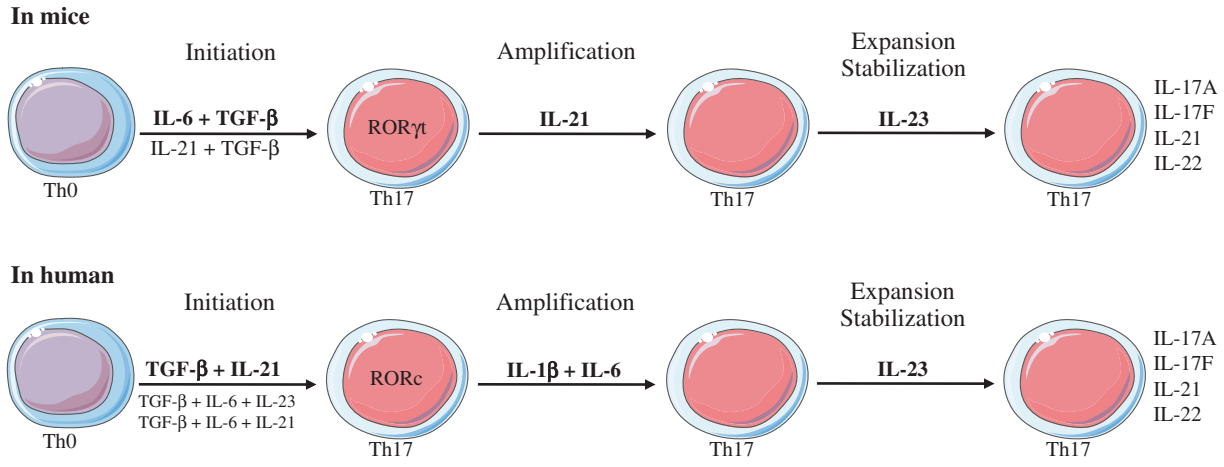

Fig. 2. Differentiation of Th17 cells in mice and in human. In mice, TGF-β and IL-6 initiate the differentiation and activate RORγt. IL-21 is induced by developing Th17 and in autocrine manner acts on Th17 cell amplification. IL-23 serves to expand and maintain Th17 cell population. In human, the differentiation is initiated by TGF-β and IL-21 which induce the transcription factor RORc. IL-1β plus IL-6 are important to enhance the amplification of Th17 cells and IL-23 to maintain the Th17 cell population.

controlled for. Subsequent studies, using naive T cells from cord blood and serum-free medium, established that TGF-β is indispensable for the differentiation of human Th17 cells from naive T cells. Furthermore, TGF-β plus IL-21 or TGF-β plus IL-6 and IL-23 or IL-6 and IL-21 can induce the expression of ROR-c, the human counterpart of murine RORγt [22,23]. The combination of TGF-β and IL-21 was sufficient to induce the differentiation of human Th17 cells from naive T cells, and IL-1β and IL-6 are important for enhancing the expansion of differentiated Th17 cells [22]. Human naive T cells do not express IL-1R and IL-23R; these receptors are induced after exposure to TGF-β and IL-6/IL-21, making the cells sensitive to IL-1β and IL-23 [22–25].

Thus, cytokine requirements for driving the differentiation of Th17 cells and their transcriptional program are likely to be very similar in both humans and mice (Fig. 2).

3. Treg cells

Regulatory T cells are a subset of CD4+ lymphocytes that were originally termed "suppressor cells" [26]. These cells play an important role in the maintenance of self-tolerance and in the modulation of overall immune responses against infections and tumor cells. Treg cells secrete TGF-β and IL-10 and require the specific cytokine TGF-β and the transcription factor FoxP3 for their differentiation. They inhibit autoimmunity; they are responsible for tolerance against self-antigens, and protect against tissue injury in infectious diseases [27]. After clearance of pathogens, they negatively regulate the immune response, thereby protecting against chronic immunopathology related to chronic infections [28,29]. Treg cells can suppress inflammation and immune responses via various mechanisms including cell-contact-dependent, for example by expressing CTLA4 which is important to inhibit immune activation by competing for costimulatory ligands on T cells; and independent ones, such as secretion of inhibitory cytokines, IL-10 or TGF-β [30].

3.1. Differentiation of Treg cells

Treg cells can be divided into two subgroups, natural Treg cells (nTreg) and inducible Treg cells (iTreg) according to the site of their maturation.

3.2. Natural Treg cells

The nTreg cells develop during normal T-cell maturation in the thymus and enter peripheral tissues where they suppress the activation of self-reactive Tcells [31]. nTreg cells are released from the thymus as a distinct lineage with FoxP3 already expressed. They are antigen specific and survive as a long-lived population in the periphery [32]. IL-2 is required for their generation and expansion, together with stimulation of the TCR/CD3 complex and co-stimulation via CD28 [33,34]. TGF-β is required for maintenance of nTreg cells after they emigrate from the thymus [32,35].

3.3. Inducible Treg cells

The iTreg cells develop directly in the peripheral lymphoid organs from naive T cells following antigen priming. iTreg cells are FoxP3 + CD4 + CD25+ cells and they mediate their inhibitory activities by producing immunosuppressive cytokines such as IL-10 and TGF-β [31]. The main factors that have been identified as crucial for the induction of FoxP3 expression in CD4+ CD25− cells are IL-2 and TGF-β [32,35]. FoxP3 is induced downstream to TGF-β signaling, after interaction with TCR [32] and it is needed for full differentiation of iTreg cells.

4. Other subsets

Accumulating evidence indicates that CD4+ T cells are more plastic than previously described, particularly Treg and Th17 cells. Thus, additional subsets have been described between these two extremes.

4.1. IL-17 + IFN-γ + cells

Some studies demonstrated that T cells can produce at the same time IL-17 and IFN-γ, the classical Th1-associated cytokine. Some FoxP3+ T cells can produce IFN-γ and IL-17 in the target organ during autoimmunity [36], and in *Candida albicans*-primed cultures, most IL-17-secreting cells also produce IFN-γ and express RORγt and T-bet [37]. IL-17/IFN-γ double positive T cells are found in significantly elevated numbers in blood from patients with chronic inflammatory disorders and could represent the most pathogenic subset. However, double positive Th17 cells are less stable and become IL-17 or IFN-γ single producing cells in inflamed tissues [38,39].

4.2. IL-17 + IL-10 + cells

Other studies showed that Th17 cells are able to produce IL-10, a cytokine secreted by Treg cells with immunosuppressive properties and potent anti-inflammatory activities. De novo generation of Th17 cells in the presence of TGF-β and IL-6 leads to the production of IL-10 concomitant with up-regulation of RORγt and IL-17 [40]. Furthermore, some pathogen-induced Th17 cells can produce IL-10 [37]. This subset with a cytokine profile of Th17 and Treg cells reflect the plasticity existing between both kinds of cells.

4.3. IL-17 + FoxP3 + cells

A first study in mice showed the presence of FoxP3+ RORγt+ T cells that have the ability to produce IL-17 [17]. This subset of CD4+ T cells was also reported in humans [41]. A subpopulation of CD4+ FoxP3+ Treg cells that express CCR6 and have the capacity to produce IL-17 is present in human peripheral blood and in lymphoid tissue. These IL-17-producing Treg cells coexpress FoxP3 and RORγt and strongly inhibit the proliferation of CD4+ responder T cells [41]. These cells may represent a subset of human CD4+ T cells that display the functional features of both Treg and Th17 cells (IL-17 production, CCR6 expression and suppressive activity). CD4 + FoxP3 + RORγt+ cells represent a transient population, able to create both Th17 and Treg cells. According to the local condition, if sensing a pro-inflammatory environment, Treg cells are able to release IL-17. Their program is turned off by IL-6 and Treg cells can be re-programmed to Th17 cells in the presence of TGF-β and IL-6 [42]. Th17 and Treg cell programs could be simultaneously induced in same T cells before they are terminally differentiated into either final lineage.

These subsets of cells implicate an important plasticity between Th17 and Treg cells and highlight the major role of Th17/Treg balance in the control and outcome of immune responses and immunopathogenesis of autoimmune and inflammatory diseases.

5. Th17/Treg balance

Th17 and Treg cells have opposite roles in the development of autoimmune and inflammatory diseases. While Th17 cells promote autoimmunity, Treg cells serve to control it and therefore play a very important role in autoimmune pathogenesis by maintaining self-tolerance and by controlling expansion and activation of autoreactive CD4+ T effector cells. The control of Th17/Treg balance appears also critical in the development of these diseases. Furthermore, developmental pathways of Th17 and Treg cells are closely related. TGF-β alone induces expression of FoxP3 [9,35] and RORγt [10] in TCR-stimulated naive CD4+ cells. Thus TGF-β is a critical factor in common

for Th17 and Treg cells, essential for inducing both RORγt and FoxP3. Despite the induction of these transcription factors, TGF-β is unable to initiate Th17 differentiation in vitro unless pro-inflammatory cytokines, as IL-6 or IL-21 are present. When these cytokines are present, the TGF-β induced-FoxP3 expression is reduced and RORγt expression is up-regulated [9,10]. In absence of significant inflammation, TGF-β promotes Treg differentiation that maintains immune tolerance. This is due to a FoxP3-mediated inhibition of the activity of RORγt, resulting in abrogation of IL-17 and IL-23 expression [17]. Furthermore, IL-6 and IL-21 have a key role in the reciprocal relationship existing between Treg and Th17 cells, by controlling the FoxP3/RORγt balance [9,10,12,18]. These cytokines inhibit the expression of FoxP3 by activating STAT3, also inhibit differentiation of Treg cells and promote TGF-β-induced RORγt expression to induce Th17 cells (Fig. 3).

5.1. Th17/Treg balance and diseases

Developmental pathways of Th17 and Treg cells are reciprocally regulated and can influence the outcome of immune responses, particularly in autoimmune and inflammatory diseases [17].

Spontaneous as well as induced diseases are associated with IL-17 in human and in mouse. Th17 cells with specificity for self-antigens are highly pathogenic and lead to the development of inflammation and severe autoimmunity. In animal models, the implication of Th17 cells was described in different diseases, including experimental autoimmune encephalomyelitis, collagen-induced arthritis. In humans, Th17 cells and their cytokines are also associated with several autoimmune and inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, psoriasis, inflammatory bowel disease and many others [43,44]. As for Th17 cells, a reduction in Treg cell numbers and/or a loss of Treg function has been observed in many animal models and human autoimmune diseases.

Nevertheless, it is important to note that the interpretation of the many studies on this subject is difficult because number and function of cells are implicated. Indeed, data on peripheral Treg cell number and function in human autoimmune diseases are contradictory and remain subject to debate [45]. Moreover, Treg cells show a certain degree of plasticity and can loss their suppressive function, especially under inflammatory conditions. Then, an increase number of Treg cells in autoimmune disease does not mean that these cells are able to control the immune response. Treg cells are crucial for restoring immune tolerance in chronic inflammation, but quality and quantity render result interpretation difficult [46].

5.2. Animal models

5.2.1. Experimental auto-immune encephalomyelitis (EAE)

EAE is an animal model of central nervous system inflammatory demyelinating disease. Accumulation of Th17 cells was the first observation to support that IL-17 contributes to the pathogenesis of EAE. Furthermore, IL-6-deficient mice have been shown to be highly resistant to the development of EAE[47] and these mice immunized with an encephalitogenic peptide have no Th17 cells infiltrating the central nervous system [9]. Conversely, an IL-17A-targeted auto-vaccine prevents EAE [13,48]and a deficiency or neutralization of IL-17 delays the onset of EAE and reduces the severity of disease [49]. Also, Treg cells are implicated in EAE. Indeed, they rapidly expand in the inflamed central nervous system, during EAE and a depletion of Treg cells exacerbates EAE, whereas supplementation through adoptive immunotherapy is protective [50]. The amelioration of EAE by β-elemene treatment is associated with the Th17/Treg balance [51] and salmon proteoglycan suppresses progression of EAE via the regulation of Th17 and Treg cells [52].

5.2.2. Collagen-induced arthritis (CIA)

CIA is a mouse model of rheumatoid arthritis. Th17 cells are implicated in this model; mice deficient in IL-17 are protected from the development of arthritis [53]. The disease is clearly mediated by IL-17 because IL-17 deficiency or treatment with IL-17RA antagonist or with IL-17 neutralizing antibody before disease onset, attenuate arthritis with decreased joint damage and reduced serum IL-6 [54]. IL-6 plays a central role in CIA model. Indeed, IL-6-deficient mice show a delayed onset and reduced severity of arthritis [55]. FoxP3-transgenic mice are resistant to CIA via reduced cellular proliferation of activated T cells

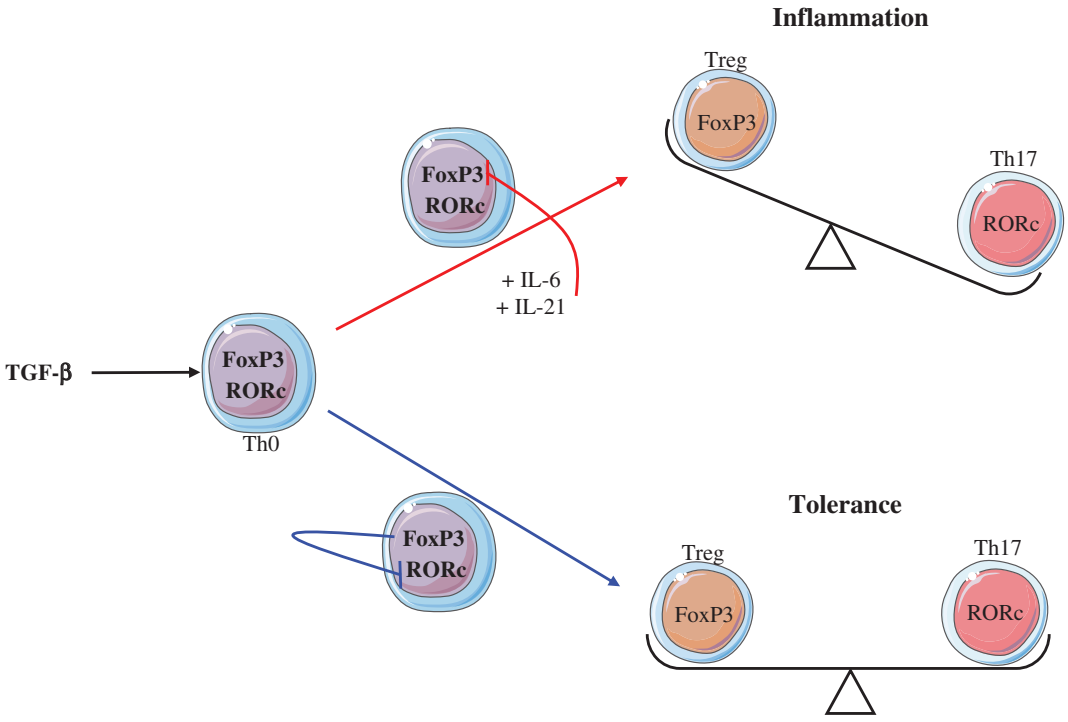

Fig. 3. Balance between Th17 and Treg cells. TGF-β is able to induce expression of FoxP3 and RORc. In presence of pro-inflammatory cytokines, such as IL-6 or IL-21, FoxP3 expression is reduced and RORc expression is up-regulated. In absence of inflammation, TGF-β promotes Treg differentiation; this is due to a FoxP3-mediated inhibition of RORc.

[56]. Furthermore, Treg cells are also important to the pathogenesis of CIA with exacerbation of disease following their depletion and some data showed that adoptive transfer of CD4 + CD25 + Treg cells can cure CIA [57].

5.3. Application to human inflammatory diseases

5.3.1. Multiple sclerosis (MS)

MS is a chronic inflammatory disease that leads to brain inflammation and that may involve a break in tolerance. Th17 cells are considered to be important in multiple sclerosis pathogenesis. IL-17A gene is overexpressed in biopsy samples taken from the brains of patients with MS [58] and Th17 cells are present in high proportion in active MS lesions [59]. Treg cells are also implicated in MS. It was reported that a disturbance in the development and function of Treg subpopulations and also an altered frequency of Treg cells are associated with disability status in relapsing remitting MS patients [60]. Furthermore, CD39-expressed Treg cells have the potential to suppress IL-17 production and the pathogenic Th17 cells [61]. So in MS pathogenesis, maintaining Th17/Treg balance appears to be critical.

5.3.2. Rheumatoid arthritis (RA)

RA is an autoimmune disease characterized by a chronic inflammation of the joint synovium membrane leading to bone and cartilage destruction. The chronic inflammatory process in RA indicates that immune regulation in the joint is disturbed and that may be caused by an excessive inflammatory response associated with a deficiency in the control of immune response.

T cell clones producing IL-17 were first described in RA synovial membrane and fluid [6]. Moreover, Th17 cells are more abundant in RA synovial fluid compared with RA peripheral blood [62] although increased frequencies of Th17 cells are observed in peripheral blood of RA patients [63,64]. Th17 cells exacerbate the inflammatory phase of arthritis through the activation of various kinds of cells in the inflamed joints, such as macrophages, dendritic cells or fibroblast-like synoviocytes by producing IL-17, involved in inducing and mediating pro-inflammatory responses [1]. These observations support the role of Th17 cells in the pathogenesis of RA [65], specifically at an early stage.

IL-17 was shown to be spontaneously produced by RA synovial explants and elevated production of IL-17 by T cell clones isolated from the RA synovium has been observed [6,62,66,67]. Furthermore, the production of IL-17 plays a crucial role in the development of cartilage and bone lesions as IL-17 can promote inflammation by inducing the production of pro-inflammatory mediators, including cytokines such as TNF-α, IL-6 and IL-1β, chemokines and other mediators of bone and cartilage destruction such as metalloproteinases [5,68]. Moreover, increased levels of IL-17 expression correlate with more severe joint damage. Indeed, in a clinical study of RA patients, expression of IL-17 in RA synovium biopsies was associated with an increase in both activity and severity of the disease [69].

IL-21, another cytokine produced by Th17 cells is also implicated in RA. In addition to regulating Th17 cells, IL-21 is up-regulated in the synovium, synovial fluid and serum of patients with early stage RA and promotes osteoclastogenesis in RA [70]. Therefore, different effector molecules secreted by Th17 cells can synergize to enhance disease activity and attract numerous effector T cells into inflamed tissue during RA.

Several reports described the presence of regulatory T cells in RA. However, the conclusions are conflicting. Some studies reported an increased number of Treg cells in peripheral blood and even in synovial fluid from RA patients compared with healthy controls [71,72], while other studies reported a deficit in the proportion of Treg cells in peripheral circulation of patients with early active RA [63,64,73,74]. Furthermore, some studies on the function of Treg cells in RA reveal functional abnormalities [74–77]. Treg cells isolated from patients with active RA are competent at suppressing conventional T cells proliferation but not cytokine production in culture [74]. On the contrary, some studies reported that Treg cells present a failure in their suppressive function. For example, IL-21, produced by Th17 cells renders responder T cells resistant to Treg suppression [78]. Other studies implicate that in RA environment, TNF contributes to the failure of Treg cell suppressive function [75,77]. The first study demonstrated that TNF blocks suppressive activity of Treg cells by a down-modulation of FoxP3, associated with a low capacity to suppress the proliferation and the cytokine secretion of effector cells [75]. Recently, TNF was shown as the main factor in RA synovial fluid that is responsible for Treg cell impairment [77]. In this study, they reported that TNF has no effect on FoxP3 expression but inhibits T cell function by reducing FoxP3 phosphorylation, due to an up-regulation of protein phosphatase 1 (PP1) (Fig. 4). Therefore, Treg cells in RA appear to be not completely efficient to control inflammatory response.

Considering these contradictory results on the frequency of Th17 and Treg cells, Wang and al. calculated the Th17/Treg ratio. Healthy controls have a low Th17/Treg ratio, while RA patients, especially with active RA, have a high Th17/Treg ratio [63]. The balance between Th17 and Treg cells may be abnormal in RA and this imbalance may play a pivotal role in RA pathogenesis because predominant Th17 cells can exert strong pro-inflammatory response by producing IL-17 and impaired Treg cells, partly due to the cytokine microenvironment, cannot control this immune response. Indeed, a recent study reports that Th17/Treg balance is controlled in part by TNF. TNF-induced Treg cell impairment correlates with Th17 cell development [77]. TNF, present in cytokine microenvironment, has a major role in altering the balance of Treg cells and inflammatory Th17 cells.

6. Therapeutic applications

Regarding the pathogenic mechanisms of RA, the Th17/Treg imbalance may be responsible for the development and progression of RA. The combination of the treatment that manipulates key cytokines with the treatment that target Th17/Treg imbalance may lead to novel and effective immunotherapies for RA. Targeting of Th17/Treg balance can be done at different levels: cytokines, receptors, cells or signaling pathways. Some examples of targeting are described below, taking RA as an example where many options are already in the clinic.

6.1. Inhibition of pro-inflammatory cytokines

6.1.1. TNF

The direct role of TNF in Th17 differentiation is unclear but TNF induces IL-6 production by many cells, specifically synoviocytes. Five inhibitors of TNF, including infliximab, etanercept or adalimumab, are currently used in RA [79]. The success of anti-TNF drugs in alleviating symptoms and delaying progression of RA has been attributed to their ability to antagonize the effects of TNF at the late steps of inflammation. However, anti-TNF treatments can act on Treg and Th17 cells. The failure of Treg cells to suppress the proliferation of effector cells can be reversed by treatment with infliximab [75]. Furthermore, infliximab can restore Treg suppressive function and FoxP3 phosphorylation via a reduced PP1 expression in RA cells to levels similar to those in healthy control. This is accompanied by a decrease of frequency of Th17 cells [77]. Another study showed that the functional defect of Treg cells can be restored by treatment with infliximab, possibly through the TGF-β-dependent generation of a new population of Treg cells in the periphery [80]. Similarly, the treatment with the fully humanized anti-TNF antibody adalimumab results in an increased percentage of FoxP3 + cells, endowed with the capacity to both suppress and resist conversion to Th17 cells via an IL-6-dependent mechanism. The Th17 response can be suppressed by Treg cells from patients who respond to treatment with adalimumab, via the control of monocyte-derived IL-6 production. This was not observed in RA patients who did not respond to adalimumab [81]. Other studies showed that after anti-TNF therapy, there is an

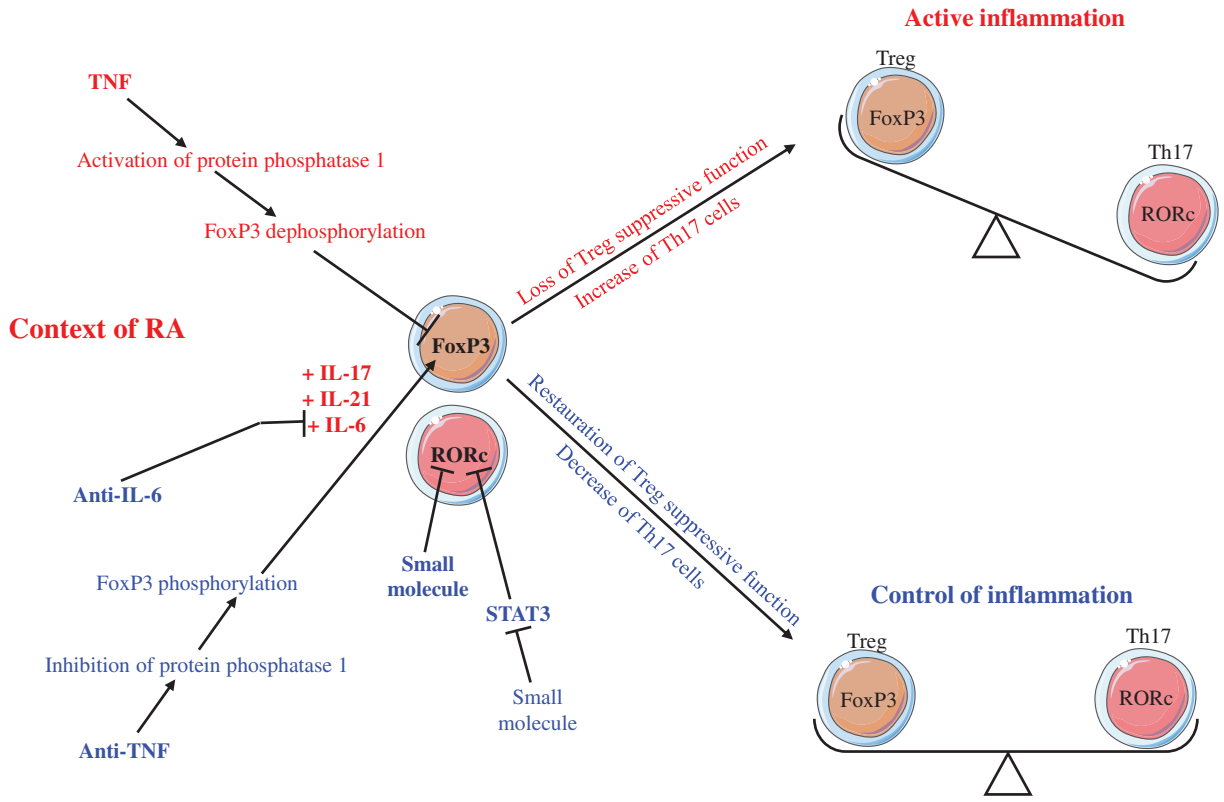

Fig. 4. Balance between Th17 and Treg cells during rheumatoid arthritis. RA cytokine microenvironment promotes Th17 cell differentiation rather than Treg cell expansion. Furthermore, pro-inflammatory cytokine as TNF is able to alter Treg suppressive function. This plays a pivotal role in persistence of active inflammation. Acting on targets implicated in Th17/Treg balance regulation is an attractive approach. By inhibiting pro-inflammatory cytokines such as IL-6 and TNF, it is possible to decrease Th17 cell population and restore Treg function, respectively. The inhibition of transcription factor RORc by small molecules also promotes the decrease of Th17 cells. These targets allow keeping an adequate balance between pathogenic Th17 cells and protective Treg cells to control inflammation.

increase in the percentage of Treg cells and a decrease of Th17 cells [74,75,81,82] (Fig. 4).

6.1.2. IL-6

Tocilizumab (TCZ) is a humanized anti-IL-6 receptor antibody, used in RA treatment [79]. It has demonstrated effectiveness in reducing disease activity and delaying joint destruction. The Th17/Treg imbalance occurring in patients with RA can be corrected by TCZ-mediated blockade of IL-6 signals, which is associated with improved clinical outcome [64]. Another study demonstrated that the Treg/Th17 ratio, which is lower in RA patients, tends to increase throughout the TCZ therapy, approaching the mean ratio exhibited by healthy controls [83].

These results indicate that blocking IL-6 function affects the balance between Th17 and Treg cells, favoring a more protective response in the context of an inflammatory disease seen in RA. This may also apply to the effect of TNF inhibition, since this also leads to reduce IL-6 production (Fig. 4).

6.1.3. IL-17

There are several ways in which members of the IL-17 protein and receptor family can be targeted. One of these options is antibodies directed against IL-17A and IL-17RA.

Two monoclonal antibodies directed against IL-17A have been tested in clinical trials: ixekizumab, a humanized IL-17A specific antibody and secukinumab, a fully human IL-17A-specific monoclonal antibody. In clinical trials with RA patients, the treatment given intravenously by secukinumab or by ixekizumab induced clinically relevant responses of variable magnitude and reduction in clinical parameters as early as one week, respectively. The rates of adverse events, including infections, were similar in the secukinumab and placebo groups [84,85].

If present studies are focused on clinical parameters, the effects of IL-17 inhibition on the Th17/Treg balance have not been evaluated. This will be interesting with IL-17 being a major cytokine in Th17 biology and in inflammation.

One monoclonal antibody directed against IL-17RA, receptor of IL-17A, brodalumab is in clinical development [86]. There was no evidence of clinical efficacy with brodalumab treatment in RA patients, who had an inadequate response to methotrexate. The reasons are unclear but may be related to the combine inhibition of IL-25 or IL-17E, which has anti-inflammatory properties. In addition to IL-17RA, which is the target of brodalumab, IL-17RC is the other chain of the IL-17A and IL-17F receptor. Inhibition of IL-17RC expression inhibits the response of synoviocytes to IL-17A [87]. However, it remains to be determined whether antagonism of IL-17RC will be pursued in the clinic. Regarding the ligand inhibition combined inhibition of IL-17A and F, and even of IL-17A, F and TNF are ready to start in the clinic.

6.2. Targeting cells

The inhibition of pro-inflammatory cytokines can impact outcomes of RA, by modulating Th17/Treg balance and so reducing inflammation. Another potential target is directly the cell. By controlling the development and the interactions of Th17 and Treg cells, it is possible to reduce inflammation and control the disease.

6.2.1. Targeting of Th17 cells

One potential target is the Th17 transcription factor RORγt, known as RORc in human, to inhibit their differentiation. This pathway has been targeted using synthetic ligands such as SR1001, a high-affinity synthetic ligand. SR1001 inhibits Th17 differentiation and function, in mice and human cells, and also reduces the severity of disease in EAE

[88]. These data demonstrate that the targeting of Th17 cells by blocking their transcription factor, without affecting other Th cell lineages, has a therapeutic potential utility in the treatment of autoimmune diseases, including RA and MS (Fig. 4).

6.2.2. Use of regulatory T cells

6.2.2.1. Adoptive Treg therapy. Transfer of polyclonal Treg cells has shown efficacy in ameliorating CIA [89]. However, an Ag-specific component is required to restore full tolerance and complete disease remission in autoimmune models. The joint specificity in Treg-mediated suppression of arthritic joint swelling is important [90]. The difficulty in transferring these results to a human setting is in generating sufficient Ag-specificity. Recently, it has been developed to an approach to rapidly generate large populations of Ag-specific Treg cells utilizing TCR gene transfer [91]. This could be translated into an applicable therapy for the treatment of RA. Another explored approach is the use of an inducible FoxP3 construct in conventional T cells. The advantages of these FoxP3 conversion approaches are mainly related to the availability of sufficient number of cells [91].

6.2.2.2. Th17/Treg balance. STAT3 and STAT5 are two transcription factors known to control the differentiation of Th17 and Treg cells, respectively. The inhibition of STAT3 by siRNA significantly decreases the proportion of Th17 cells and increases the proportion of Treg cells, among the CD4 + T cell population isolated from RA peripheral blood and RA synovial fluid (Fig. 4). In contrast, the inhibition of STAT5 has opposite effects, with an increase of Th17 cells and a decrease of Treg cells. Furthermore, no change in the production of Th1 versus Th2 signature cytokines is induced by STAT3 or STAT5 siRNA [92]. These signaling pathways could provide novel target molecules for the control of inflammation.

7. Conclusion

Th17 and Treg cells are both implicated in inflammatory and autoimmune diseases. Th17 cells are involved in the induction and propagation of pathologies whereas Treg cells inhibit autoimmunity and are responsible for tolerance against self-antigens. Th17 and Treg cells share common factors, such as TGF-β, that affect their development and generation. The balance between inflammation (Th17 cells) and tolerance (Treg cells) may influence pathology or disease outcomes in autoimmune diseases, including RA and MS. It is though that if this critical balance is deviated in favor of Th17 cells and against Treg cells, the severity of disease could be significantly enhanced. Such contribution justifies developing new therapeutic means to keep an adequate balance between pathogenic Th17 cells and protective Treg cells, for preventing the development and extension of autoimmune and inflammatory diseases.

Conflict of interest

The author declares no financial or commercial conflict of interest.

Acknowledgments

Professor Miossec is a senior member of and supported by the Institut Universitaire de France. His laboratory is supported by grants from the OPERA IHU prometteur and the EU Marie Curie Network EUTRAIN. Melissa Noack is supported by the Institut Universitaire de France.

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Frequency of Th17 CD4+ T Cells in Early Rheumatoid Arthritis: A Marker of Anti-CCP Seropositivity.

To examine the frequency and phenotype of Th17 cells in the peripheral blood of early RA (eRA) patients, **Arroyo-Villa I, et al. (PLoS One 2012;7:e42189)** isolated CD4+ T cells from the peripheral blood of 33 eRA patients, 20 established RA patients and 53 healthy controls (HC), and from the synovial fluid of 20 established RA patients (RASF), by ficoll-hypaque gradient and magnetical negative selection. After polyclonal stimulation, the frequency of Th17 and Th1 cells was determined by flow cytometry and concentrations of IL-17, IFN-γ, TNF-α and IL-10 were measured by ELISA in cell-free supernatants. When all eRA patients were analyzed together, a significantly lower percentage of circulating Th17 cells and a lower CD4-derived IL-17 secretion were observed in comparison with HC. However, after stratifying by anti-CCP antibody status, circulating Th17 cells were decreased in anti-CCP(+) but not in anti-CCP(-)-eRA. All Th17 cells were CD45RO+CD45RA- and CCR6+. Dual Th17/Th1 cells were also exclusively decreased in anti-CCP(+)-eRA. Circulating Th17 and Th17/Th1 cells were negatively correlated with anti-CCP titres. When anti-CCP(+)-eRA patients were retested one year after initiating treatment with oral methotrexate, their circulating Th17 frequency was no longer different from HC. Of note, the percentage of circulating Th1 cells and the secretion of CD4-derived IFN-γ, TNF-α and IL-10 were not different between eRA patients and HC. In established RA patients, circulating Th17 and T17/Th1 cell frequencies were comparable to HC. In RASF, both Th17 and Th1 cells were increased when compared with blood of eRA patients, established RA patients and HC. Decreased circulating Th17 levels in eRA seem to be a marker of anti-CCP seropositivity, and return to levels observed in healthy controls after treatment with methotrexate.

La balance entre inflammation (LTh17) et tolérance (LTreg) représente un mécanisme sensible, pouvant largement influencer le déroulement et l'issue de maladies inflammatoires chroniques. Lors de ces pathologies, un autre mécanisme, le contact cellulaire, permet d'influencer le déroulement de la maladie. En effet, au cours de l'inflammation chronique, les cellules immunitaires, dont les LTh17, migrent sur le site inflammatoire et interagissent alors avec les cellules mésenchymateuses locales. Ces interactions cellulaires vont avoir un rôle important dans le maintien de l'inflammation.

III Les interactions cellulaires

III.1 Au cours de l'inflammation

Lors de l'inflammation, la migration des cellules immunitaires entraîne leur interaction avec les cellules stromales mésenchymateuses (CSM) présentes sur le site inflammatoire. Ces cellules jouent un rôle primordial dans le déroulement de la résolution de l'inflammation. Elles sont capables de réguler la réponse immunitaire via différents mécanismes, influençant ainsi la phase de résolution. Les CSM peuvent inhiber la prolifération des LT [43] mais également induire leur apoptose [44], nécessaire pour le contrôle de l'inflammation. Elles sont également capables de sécréter des facteurs solubles aux propriétés immunosuppressives tels que l'IL-10. Les CSM participent donc largement au bon déroulement de la résolution de l'inflammation. Lors de l'inflammation chronique, la phase de résolution est perturbée, conduisant ainsi à la persistance de l'infiltrat immunitaire, de l'hyperplasie cellulaire et de la destruction tissulaire. Des CSM affichant un profil anormal peuvent ainsi contribuer à l'installation de l'inflammation chronique [45]. Dans diverses pathologies, les CSM affichent un phénotype différent selon que le tissu soit sain ou malade [46]. Elles peuvent donc influencer de manière importante le passage d'une réponse inflammatoire normale en inflammation chronique.

De plus, lors de différentes maladies inflammatoires chroniques, le recrutement des cellules immunitaires activées, incluant les LTh17, conduit à des interactions avec les CSM locales, ce qui favorise la prolifération cellulaire et la chronicité de l'inflammation [47-49]. L'interaction entre CSM et cellules immunitaires possède des effets réciproques, entraînant une boucle d'activation de ces cellules et permettant ainsi le maintien de l'inflammation. Par exemple, l'interaction entre LT et synoviocytes (fibroblastes du tissu synovial) est un facteur déterminant dans l'établissement de l'inflammation chronique au niveau des articulations [50, 51]. Ces interactions sont également capables d'impacter la différenciation des LT, en favorisant notamment l'expansion des LTh17 lors d'interactions entre cellules mésenchymateuses dérivées de la moelle osseuse ou du synovium et cellules mononucléées du sang périphérique (PBMC) [31]. De plus, l'IL-17 est capable d'agir directement sur ces synoviocytes afin de les activer et promouvoir ainsi la sécrétion de cytokines pro-inflammatoires telles que l'IL-6 [15].

III.2 Au cours de la PR

Les interactions cellulaires au cours de la PR vont avoir lieu au niveau de l'articulation, entre les cellules immunitaires migrantes et les synoviocytes. Ces interactions peuvent être indirectes par des facteurs sécrétés ou directes par le contact cellulaire, et aboutissent à l'activation des deux types cellulaires [52].

Les synoviocytes vont ainsi être capables d'influencer l'accumulation, l'activation et la survie des lymphocytes. La sécrétion d'IL-15, facteur de croissance des LT, va favoriser l'activation et la prolifération des LT effecteurs mais aussi des LTreg [53]. Cette cytokine va également réduire l'apoptose à la fois des synoviocytes et des LT, les deux types cellulaires exprimant son récepteur [54, 55]. La sécrétion d'IL-16 va permettre le recrutement des LTh [56]. Les synoviocytes sont capables d'inhiber l'apoptose des LT [57] mais également des

lymphocytes B (LB) [58]. De plus, l'interaction synoviocytes/LTh, et particulièrement LTh issus de patients PR, favorise la prolifération des synoviocytes [59]. Les cytokines sécrétées par les cellules immunitaires, notamment monocytes et LTh17, vont agir sur les synoviocytes. Ainsi, l'IL-17, le TNF et l'IL-1β induisent la sécrétion de CCL20 par les synoviocytes, favorisant ainsi le recrutement des monocytes et des LTh17 [60, 61]. Les LTh17 sont également capables de sécréter CCL20, alimentant ainsi leur propre recrutement [62]. Les interactions entre synoviocytes, monocytes/macrophages et LT vont également jouer un rôle important dans la destruction tissulaire [63, 64]. En effet, l'interaction synoviocytes-macrophages va favoriser l'activation des macrophages et leur différenciation en cellules multinucléées, semblables aux ostéoclastes. D'une part, les synoviocytes vont sécréter le facteur de différenciation ODF (Osteoclast Differentiating Factor) [65] et d'autre part ils vont interagir directement par la liaison CD55/CD97, exprimés par les synoviocytes et par les macrophages, respectivement [66]. Les interactions LT et monocytes favorisent également la sécrétion de cytokines pro-inflammatoires, telles que l'IL-1β et le TNF mais aussi la sécrétion de MMP [64, 67-71].

Au cours de la PR, il est donc évident que les interactions entre synoviocytes, monocytes et LT jouent un rôle primordial dans le maintien des réactions inflammatoires chroniques et destructrices.

De manière générale, nous avons pu constater que lors de l'inflammation chronique, les interactions cellulaires, directes ou indirectes, entre cellules immunitaires et CSM représentent un mécanisme primordial lors du développement de diverses pathologies inflammatoires chroniques, dont la PR et le Pso. L'implication des LTh17 dans ces mêmes maladies nous a poussés à étudier l'effet de ces interactions sur la voie LTh17/IL-17 lors de

l'inflammation chronique et également d'identifier un ou plusieurs mécanismes conduisant à la sécrétion d'IL-17, dans deux modèles principaux, la PR et le Pso (Figure 2).

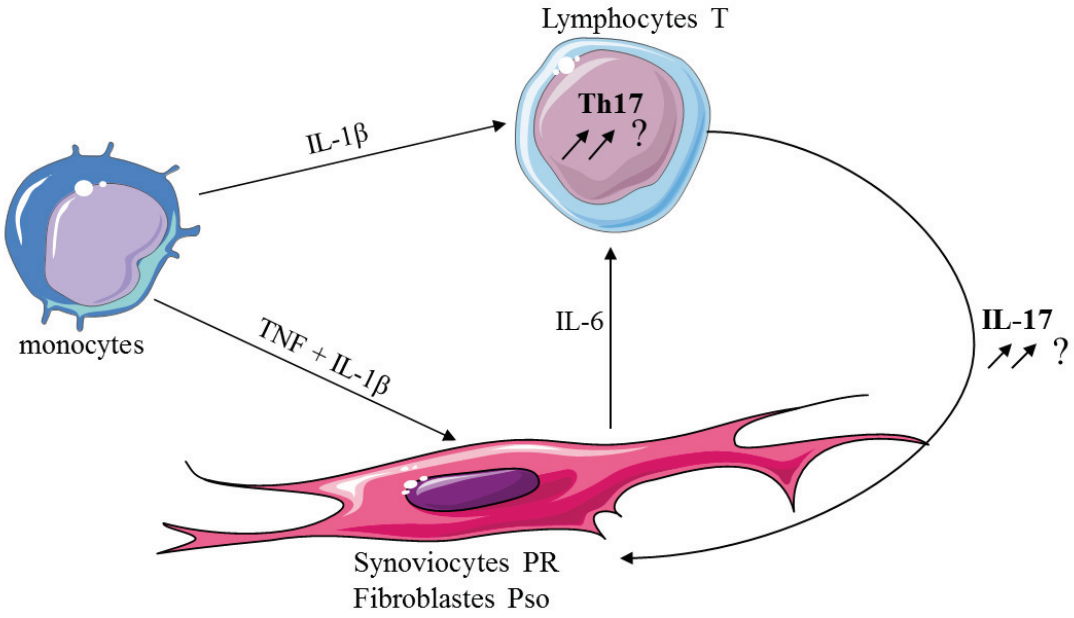

Figure 2: Lors des interactions cellulaires entre cellules mésenchymateuses (synoviocytes ou fibroblastes de peau) et cellules immunitaires, différents paramètres rentrent en jeu. D'une part, il peut y avoir des interactions directes entre les cellules pouvant induire la sécrétion de différentes cytokines telles que l'IL-1β et le TNF par les monocytes, l'IL-6 par les cellules mésenchymateuses ou encore l'IL-17 par les LTh17. Cette production entraîne d'autre part des interactions indirectes entre les cellules à travers ces cytokines. Ainsi l'IL-1β et le TNF vont stimuler les synoviocytes qui en réponse vont sécréter de l'IL-6. L'IL-6 et l'IL-1β vont influencer la différenciation des LT en LTh17 qui vont sécréter de l'IL-17, agissant en retour sur les synoviocytes. Ces interactions créent une boucle d'activation et participent au maintien de l'inflammation. Dans cette étude, les effets de ces interactions sur la voie LTh17/IL-17 sont mis en avant.

Th17 cells

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Footnotes

Mélissa Noack is supported by the Institut Universitaire de France.

Pierre Miossec is a senior member of and supported by the Institut Universitaire de France.

Introduction

The first line of defense is the immune system with its innate and adaptive components. Its main functions are to recognize and control foreign antigens, to induce an immunologic memory, and to maintain a tolerance to self-antigens. CD4+ T cells play a major role in the initiation and regulation of the immune response, mainly by secreting subset-specific cytokines after being activated and differentiated into distinct effector subsets. The CD4+ population is heterogeneous and different subsets provide effector functions during a large variety of immune reactions. Helper T cells (Th cells) are identified according to their cytokine and transcription factor profiles. The two first described major subsets were Th1 and Th2 cells. Th1 cells provide mainly a response against intracellular bacterial infections, secrete primarily interferon-γ (IFN-γ) and express the transcription factor T-bet. Th2 cells are provide mainly a response against parasitic infections and their major cytokine is interleukin-4 (IL-4) and their transcription factor GATA-3 [1]. More recently, a third distinct lineage has been identified, the Th17 subset. Th17 cells produce mainly the pro-inflammatory cytokine IL-17 and they have a key role in the protection against extracellular pathogens and fungi [1, 2]. Furthermore, over-activation of these cells has been associated with autoimmunity and inflammation [1, 3]. Despite some common markers with Th1 and Th2 subsets, Th17 cells require specific cytokines and transcription factor for their differentiation [4].

The homeostasis of the immune response is continually controlled by a balance between Th cell activation and suppression of this activation by regulatory T cells (Treg). A disruption of this homeostasis leads to an over-activation of the immune response and chronic inflammation. Th17 cells provide an immediate protective response to foreign pathogens and thus they have an important role in the induction of inflammatory process. In contrast, Th17 cells are pro-inflammatory and pathogenic underlying the development and chronicity of many autoimmune inflammatory diseases. As such there are an important target for treatment.

I. Differentiation of Th17 cells

IL-17 was identified in 1995 first in the human system as cytokine involved in inflammation and neutrophil activation [5]. The concept of IL-17 production by some but not all T cells was introduced in 1999 using T cell clones from the joint of patients with arthritis [6]. The identification of the Th17 subset was made in the mouse in 2015 [2], 10 years after the description of the cytokine [7]. The development of Th17 cells shares major similarities in mice and humans, but with some differences.

a. Th17 cell differentiation in mice

The differentiation of the distinct lineages of Th cells is driven by the influence of different cytokines and transcription factors. Transforming growth factor-β (TGF-β), an immunosuppressive cytokine [8] and IL-6, a pro-inflammatory cytokine [9] act in concert to induce the highly pro-inflammatory subset of Th17 cells. These two opposite cytokines appear to be sufficient for the induction of IL-17 expression in naïve T cells [10-12]. In the absence of IL-6, TGF-β induces FoxP3, the transcription factor of Treg cells [13, 14]. In the presence of IL-6, FoxP3 induction is inhibited and replaced by the induction of RORγt, the transcription factor of Th17 cells. Other pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) or IL-1β can enhance TGF-β and IL-6-mediated Th17 cell differentiation [12].

IL-21, a member of the IL-2 cytokine family, when combined with TGF-β can also induce Th17 differentiation [15-17]. IL-21 is less potent than IL-6 and as it is produced by Th17 cells themselves. It is thus more important in the amplification of Th17 differentiation than in its initiation. IL-21 favors the autocrine loop for Th17 cells. Thus TGF-β associated with IL-6 initiates Th17 differentiation while TGF-β associated with IL-21 is a major factor in the autocrine loop of Th17 cells.

After the initiation of differentiation and the expansion of Th17 cells, IL-23, a member of the IL-12 cytokine family, is required for the stabilization of Th17 phenotype [12]. IL-23 alone cannot induce Th17 differentiation as naïve T cells do not express the IL-23 receptor (IL-23R) [18]. IL-23R expression on Th17 cells is induced by IL-6 and IL-21 and also by IL-23 itself [15, 19, 20] and is thus found only on activated effector or memory T cells. Once activated, T cells become responsive to IL-23 and this leads to the expansion of Th17 cells [21]. IL-23 is not considered as a differentiation factor but as a major player in expansion and stabilization of Th17 phenotype. Accordingly, the differentiation of Th17 cells involves three key steps: the initiation by TGF-β and IL-6, the expansion of Th17 cells by IL-21 and the stabilization of Th17 phenotype by IL-23.

In addition to these specific cytokine patterns, the differentiation of Th17 is driven by a lineage-specific transcription factor, the retinoid-related orphan receptor γt (RORγt) [22]. Cytokines involved in the differentiation of Th17 cells (TGF-β, IL-6, IL-21 and IL-23), induce the expression of RORγt, which depends on Stat3 activation [15, 16, 23, 24]. IL-6-mediated Stat3 activation is crucial for Th17 generation [25, 26]. Another transcription factor, RORα, member of the retinoid nuclear receptor family, is also involved in the differentiation of Th17 cells. Its role is similar but not identical to that of RORγt [27]. As RORγt, RORα is strongly induced by IL-6 or IL-21 in association with TGF-β and Stat3 activation increases its expression. This suggests that Th17 cell differentiation could be driven by two lineage-specific transcriptions factors.

b. Th17 cell differentiation in humans

The two major cytokines for the differentiation of mouse Th17 cells are TGF-β and IL-6. In humans, the results concerning the role of TGF-β have been confusing. It was demonstrated that TGF-β was dispensable for Th17 differentiation [28, 29]. IL-1β in combination with IL-6 [28] or with IL-23 [29] could induce Th17 differentiation.

Nevertheless, in these studies, the use of CD45RA+ Th cells purified from peripheral blood as naïve T cells and the presence of serum and platelet contamination, which are important sources of endogenous TGF-β make the results difficult to interpret.

Other studies, using naïve T cells from cord blood and serum-free medium demonstrated that TGF-β is a key factor, indispensable for the differentiation of Th17 cells. Indeed, the presence of endogenous TGF-β from serum is sufficient to induce Th17 differentiation [30]. Different combinations of TGF-β plus cytokines, TGF-β plus IL-21, TGF-β plus IL-23 plus IL-6, can induce the transcription factor RORc, the human homolog of RORγt and therefore initiate Th17 differentiation [30-32]. Furthermore, human naïve T cells do not express the IL-1 receptor (IL-1R) and the IL-23 receptor (IL-23R), which are both induced after stimulation by TGF-β and IL-21/IL-6 [33]. Thus in humans, TGF-β has a major role in the initiation of Th17 cell differentiation and it is absolutely required for RORc induction, while IL-6 plus IL-21 is an important combination in the Th17 expansion from central memory cells [30, 31]. In conclusion, cytokine requirements and transcriptional program necessary to drive the Th17 cell differentiation in mouse and human do not appear to be so different.

c. Pathogenic Th17 cells

The contribution of these various cytokines has an effect of the final pathogenicity of the Th17 cells. TGF-β and IL-6 are required for the differentiation of Th17 cells and IL-23 for their expansion and stabilization. Nevertheless, these cytokines also play a role in the regulation of Th17 subtype functions. A continued exposure to TGF-β1 and IL-6 induces the production of IL-17 but also the production of IL-10, and these cells produce lower pro-inflammatory mediators such as IL-22 [34]. Thus IL-10 production is associated with nonpathogenic Th17 cells. A switch to a pathogenic phenotype can be induced by an IL-23 exposure [34, 35]. IL-23 is crucial to confer the Th17 cell ability to induce autoimmunity [35-37]. In the absence of TGF-β signaling, the combination of IL-1β, IL-6 and IL-23 induces

pathogenic Th17 cells [38]. Furthermore, IL-23 plays a major role in enhancing the contribution of endogenous TGF-β3 in Th17 development, which has a critical role in inducing pathogenic Th17 cells [39]. Th17 cells induced by TGF-β3 and IL-6 express GM-CSF whose expression is a part of the signature of pathogenic Th17 cells [39-41]. Others factors are associated with the Th17 pathogenic signature, including T-bet, IL-23R and IL-7R [39]. In summary, TGF-β1 and IL-6 induce nonpathogenic Th17 cells while Th17 cells induced by TGF-β3 and IL-6 are highly pathogenic. IL-23 is also required for Th17 cells to achieve their total pathogenic phenotype, notably by inhibiting the IL-10 production and by promoting TGF-β3 or GM-CSF production.

d. Circulating vs. tissue phenotypes

Th17 cells were first identified as a CD4+ T cell population producing IL-17 [42]. Their classic phenotype exhibits also the expression of RORc, IL-23R and CCR6 [43, 44]. Recently, CD161 has been identified as a marker of all circulating IL-17-producing T cells, reinforcing the specificity of the Th17 phenotype [45]. Thus, circulating Th17 cells can be characterized by the phenotype CD4+ RORc+ IL-23R+ CCR6+ CD161+ and IL-17+. Nevertheless, at this stage, Th17 cells are not producing cells and accumulating evidence indicates that CD4+ T cells are more plastic than previously described. Depending on the environment, Th17 cells can express markers of other Th subsets in addition to IL-17 and can switch their phenotype, notably into Treg cells. However, the double positive cells are less stable and become single producing cells in inflamed tissue [46-50].

In most reports, a direct association is made between Th17 cells and IL-17 secretion. The intracellular staining of IL-17, commonly used to identify Th17 cells, is thought to be an equivalent of the actual IL-17 production. Even so the intracellular presence of IL-17 inside Th17 cells does not mean by itself its secretion. *In situ*, Th17 cells interact with local mesenchymal cells in an inflammatory environment and this leads to the IL-17 secretion [51].

Furthermore, IL-17+ cells acquire a plasma cell-like morphology under *in vitro* activation and this is correlated with enhanced concentrations of secreted IL-17. In inflamed tissue, IL-17+ cells present the same plasma cell-like morphology [46]. This indicates a major discrepancy between intracellular and secreted IL-17 and it is necessary to distinguish both phenotypes, the plastic circulating Th17 cells and the tissue Th17 cells which secrete IL-17.

II. Cytokines and chemokines related to Th17 cells

a. Major cytokines and chemokines produced by Th17 cells

Th17 lymphocytes are pro-inflammatory cells that produce different cytokines and chemokines involved in the pathology of many inflammatory and autoimmune diseases [1, 4]. IL-17, IL-21, IL-22, CCL20 are the most important of these effector molecules [1, 4, 52].

IL-17A (IL-17) / IL-17F

IL-17A and IL-17F have similar biological functions and are genetically linked. IL-17A is the major cytokine produced by Th17 cells and has a stronger effect than IL-17F. IL-17 signaling goes through a heterodimeric receptor composed by two chains, IL-17RA and IL-17RC. The distribution of IL-17RA and IL-17RC diverges as IL-17RA is more distributed on immune cells while IL-17RC is preferentially expressed by non-immune cells. The binding between IL-17 and IL-17RA induces the recruitment of IL-17RC to form the complex and to transduce the signal. IL-17 plays a central role in the adaptive immune response, mainly against extra-cellular bacteria and fungi. Excess of IL-17 is involved in a growing list of autoimmune and chronic inflammatory diseases such as rheumatoid arthritis (RA), psoriasis, psoriatic arthritis, multiple sclerosis or Crohn's disease. The major role of IL-17 is to induce the production of pro-inflammatory cytokines (TNF-α, IL-6, IL-1) and chemokines (IL-8, CCL20), antimicrobial peptides (AMP) and matrix metalloproteinases by many types of cells, such as macrophages, dendritic cells, endothelial cells, or fibroblasts from different origin.

This contributes to the recruitment of neutrophils and monocytes at the inflammatory site. IL-17 also plays a role in granulopoiesis by increasing the secretion of G-CSF and GM-CSF. The many pro-inflammatory functions as well as the multiple cell targets of IL-17 make it a critical player in autoimmunity and inflammation [1, 52-55].

IL-21

IL-21 is a cytokine with potent regulatory effects on the immune system, and notably in lymphocyte regulation and inflammation [4, 52]. IL-21 is also involved in the expansion of activated B cells and in class switching of immunoglobulin isotypes [56]. IL-21 also promotes Th1 responses by enhancing IFN-γ production [57, 58] and modulates the functional development of natural killer (NK) cells [59]. Furthermore, it has a key role in the Th17 pathway, by acting in a positive feedback loop leading to the amplification of Th17 cells and to its own production [15-17]. IL-21 is essential to maintain and amplify the Th17 pool and thus it is important player in the support of the Th17 response and tissue inflammation [4, 52].

IL-22

IL-22 is a cytokine produced by terminally differentiated Th17 lymphocytes, in response to IL-23. Its receptor IL-22R is widely expressed in different tissue cells such as epithelial and endothelial cells but not on immune cells. Its important local expression is linked to the pleiotropic functions of IL-22 in the biology of epithelia, autoimmunity and infections [4, 52]. For example, by inducing AMP from various cell types such as keratinocytes [60], IL-22 plays a major role in host defense for pathogens and in the immune barrier function of epithelia [60, 61]. As a consequence, IL-22 is upregulated in skin and mucosal diseases such as psoriasis, Crohn's disease and ulcerative colitis [52]. IL-22 is a complex cytokine with both pro-inflammatory and anti-inflammatory properties, depending on the local conditions, such as during acute liver inflammation [62]. Thus, IL-22 appears as a cytokine used by Th17 cells to interact with tissues but with complex functions that depend on the environment [4, 52].

CCL20

CCL20 is a chemokine with antimicrobial and chemoattractive activities. It is produced in various tissues by mesenchymal and immune cells [63]. It attracts at the site of inflammation immature dendritic cells and Th17 cells [64]. Its receptor, CCR6 is expressed by dendritic cells, B lymphocytes and Th17 cells. Thus by secreting CCL20, Th17 cells can regulate themselves their recruitment into the inflammatory sites. [3]

b. Major cytokines acting on Th17 cells

Th17 cells contribute to severe tissue inflammation and autoimmunity. Their regulation may be driven in two ways, the persistence of Th17 pool or the inhibition of their differentiation. Two major cytokines IL-23 and IL-27 are involved in this regulation acting in opposite ways.

IL-23

IL-23 is a member of IL-12 cytokine family and it has many effects on the immune response. It is mainly produced by innate immune system such as dendritic cells and macrophages and plays a key role in the induction and chronicity of the Th17 response. IL-23 appears as a bridge between the innate and adaptive immunity [65, 66]. After the initiation of Th17 differentiation, T cells start to express the IL-23R and become responsive to IL-23. This leads to the persistence of activated Th17 lymphocytes and to the subsequent production of IL-17. IL-23 is a critical driver for the expansion and the stabilization of Th17 cells and can be the limiting factor to determine if the Th17 response is sustained and pathogenic during immune and inflammatory responses [65-67].

IL-27

To counteract the positive effect of IL-23 on Th17 cells, IL-27 acts as a negative regulatory cytokine. IL-27 was first described as an inducer of IFN-γ response [68]. IL-27 and

IL-23 belong to the IL-12 cytokine family and are both secreted by antigen-presenting cells, macrophages and dendritic cells. The anti-inflammatory properties of IL-27 down regulate the immune response during chronic inflammation. One of mechanisms is the ability to skew T cell polarization and mainly to inhibit Th17 differentiation. IL-27 can also suppress the production of pro-inflammatory cytokines, notably IL-17 and thus reducing inflammation [69, 70]. In addition to its direct activity on Th17 cells, IL-27 promotes the development of regulatory T cells [71].

III. Th17 and inflammatory diseases

Th17 cells are highly pathogenic and their deregulation could lead to the development of chronic inflammation and severe autoimmunity. Inflammation is a dynamic process with different stages. At the early stage, neutrophils are recruited first followed by monocytes and finally by lymphocytes. Th17 cells, mainly through the secretion of IL-17, increase the secretion of the pro-inflammatory cytokines IL-1, TNF and IL-6 by monocytes and other cell types. Among other secreted molecules, IL-8 is a chemokine for neutrophils and this leads to their early accumulation at the inflammatory sites. Furthermore, at these sites, Th17 cells interact with local cells and this induces the secretion of metalloproteinases (MMP) involved in matrix destruction [72, 73]. Thus Th17 cells contribute largely to inflammation by different mechanisms, which explain their involvement in several inflammatory autoimmune diseases such as Psoriasis, arthritic diseases (rheumatoid arthritis, Psoriatic arthritis or ankylosing spondylitis, multiple sclerosis or inflammatory bowel diseases [1, 3, 4, 72, 74, 75].

a. Psoriasis

Psoriasis is a chronic immune-mediated inflammatory skin disease characterized by a hyperproliferative epidermis and an immune infiltration, mainly by dendritic cells, macrophages and T cells in the dermis and neutrophils and T cells in the epidermis. Psoriasis

is considered as a Th1/Th17-based disease as both populations are found in psoriatic skin lesions [76]. The infiltrated T-cells isolated from the skin lesions present predominantly a Th17 phenotype [77]. Circulating Th17 cells are also increased in psoriatic patients [78]. These increased frequencies of Th17 cells lead to high levels of IL-17, which activates local cells such as keratinocytes to secrete pro-inflammatory chemokines and cytokines and thus to sustain chronic inflammation. Therefore, Th17 cells contribute largely to the pathogenesis of psoriasis [3, 74, 75].

b. Arthritic diseases

Arthritic diseases combine different pathologies with a common form of chronic immune-mediated inflammatory arthritis. Rheumatoid and psoriatic arthritis are characterized by progressive joint destruction with lack of repair contrasting with the ectopic new bone formation in syndesmophytes of ankylosing spondylitis [79]. Despite this major difference, Th17 cells are involved in rheumatoid and psoriatic arthritis, ankylosing spondylitis pathogenesis. Indeed, Th17 cells are increased in peripheral blood of rheumatoid arthritis [80-82], ankylosing spondylitis and psoriatic arthritis patients [83-87]. These increased frequencies of Th17 cells exacerbate the inflammatory state of arthritis through the activation of various cell types in the inflamed tissue, such as synoviocytes, dendritic cells or macrophages. Furthermore, IL-17 produced by Th17 cells in inflammatory site can synergize with other cytokines such as TNF. Interestingly, IL-17 and TNF increase osteoblastogenesis on local isolated cells such as synoviocytes or mesenchymal cells, contrasting with the destructive effect in whole bone [88, 89]. The difference between bone loss in rheumatoid arthritis and bone formation in ankylosing spondylitis could due to the cell composition of the inflammatory environment. In rheumatoid arthritis, osteoclasts are massively activated by IL-17 and TNF and they interact with osteoblasts that leads to bone destruction. Conversely, in ankylosing spondylitis, osteoclasts are not present in tendons and ligaments, and the

combination IL-17 and TNF can lead to bone formation as in syndesmophytes. The different effector molecules of Th17 cells synergize to increase the disease activity and this support the role of Th17 cells in the arthritic pathogenesis by contributing to the chronic inflammatory process [3, 74, 75].

c. Multiple sclerosis

Multiple sclerosis is a chronic inflammatory disease characterized by brain inflammation. Both innate and adaptive immune cells are involved in the inflammation of the central nervous system. As for the previous diseases, the frequencies of Th17 cells and of its related cytokines are increased in multiple sclerosis patients [90-92]. These cells have the capacity to breach the blood-brain barrier and infiltrate the central nervous system [93]. The role of Th17 cells in multiple sclerosis is reinforced by a lower ratio of Treg/Th17, which correlates with multiple sclerosis severity [91, 94]. Furthermore, different treatments used in multiple sclerosis induce a reduction of Th17 cells [74].

d. Inflammatory bowel diseases

The two major forms Crohn's disease and ulcerative colitis are characterized by a chronic relapsing inflammatory response to commensal microflora in the gut. Th17-associated cytokines are increased in the inflamed tissues and in the sera and Th17 cells are increased in peripheral blood [95, 96]. Furthermore, increased Th17/Treg ratio is associated with disease activity, notably in Crohn's disease patients [97] and activation of the IL-23/Th17 pathway is considered as a biomarker of active disease [98]. As discussed above, IL-17 is also involved in mucosal protection. Although the respective contribution of these opposite mechanisms remain to be elucidated, it is clear that this T-cell subset is important in pathogenesis [3, 74].

IV. Mechanisms in chronic inflammation

Inflammation is first a protective response and a reversible mechanism leading to the resolution at inflammatory sites. A dysregulation of the resolution and a sustained inflammatory response lead to irreversible chronic inflammation, which induces various diseases [99, 100]. T helper cells, including Th17 cells, are highly present in the inflammatory lesions. As described above, Th17 cells are a pathogenic subset largely involved in the pathogenesis of the chronic inflammatory diseases described above and the number of Th17-associated diseases keeps increasing [75]. Common mechanisms lead to their differentiation and maintenance during chronic inflammation and Th17 cells exhibit similar patterns with increased frequency and elevated Th17-cytokine levels. By activating the innate and adaptive immune cells and local cells in the inflamed site, they contribute to maintain the immune response. Th17 cells act as a bridge between adaptive and innate immunity systems to sustain chronic inflammation.

a. Differentiation, recruitment and maintenance of Th17 phenotype during chronic inflammation

During the triggering of inflammatory response, immune cells release Th-polarizing cytokines. Among them, TGF-β, IL-6 and IL-1β induce Th17 differentiation. The Th17 transcription factor RORc induces IL-21 production which acts in an autocrine pathway to maintain RORc expression and thus maintain the Th17 phenotype. Once differentiated, Th17 cells express IL-23R and become responsive to this cytokine. IL-23 produced by innate immune cells is a major driver in the maintenance of Th17 phenotype. Furthermore, CCR6 which binds only CCL20 is preferentially expressed by Th17 subset [101] that contributes to the specific recruitment of Th17 cells during inflammation. CCL20, up-regulated in inflammatory conditions, is produced by several types of epithelial and immune cells and also

by Th17 cells which can regulate themselves their migration [64]. During the inflammatory process, Th17 cells are activated and by secreting pro-inflammatory cytokines they stimulate local cells which produce in turn pro-inflammatory cytokines and chemokines. These molecules are mainly involved in Th17 differentiation and stabilization. By secreting key cytokines, by interacting with and activating local cells, Th17 cells contribute to their own maintenance. Therefore, the secretion of cytokines and chemokines by local and immune cells and by Th17 cells themselves contribute largely to the differentiation, the migration and the maintenance of Th17 cells in the inflammatory site. In addition to their own role, another subset of immune cells is crucial to the maintenance of Th17 cells, an inflammatory subset of dendritic cells (inflaDC) appearing during chronic inflammation. By secreting Th17 cell-polarizing cytokines, inflaDC induce Th17 differentiation from naïve T cells. Furthermore, they can stimulate autologous memory T cells to produce IL-17 [102]. This subset of DC is a major player in the maintenance of Th17 phenotype during chronic inflammation.

b. Secretion of IL-17 and its activity

The major effective cytokine of Th17 cells is IL-17 (Chapter 24). During chronic inflammation, the inflammatory environment and the cell interaction lead to activation of cells and release of cytokines which activate intracellular pathway. At molecular level, Stat3 by increasing RORc expression can affect IL-17 secretion. Stat3 can also bind directly the promoter *Il17*. The effective production of IL-17 depends on these two transcription factors, Stat3 and RORc. The secretion of IL-17 is also regulated by other cytokines such as IL-15, IL-18 and IL-21 which stimulate its production [103, 104]. The most efficient cytokine to induce IL-17 secretion is IL-23. Indeed, after IL-23R expression by Th17 cells, IL-23 promotes T cells to produce IL-17 and this requires Stat4 activation for a maximal production [25].

IL-17 exerts various biological functions. Notably, IL-17 stimulates stromal cells to produce pro-inflammatory cytokines and chemokines, resulting in the maintenance of inflammation [5]. IL-17 can also directly act on the phenotype and survival of cells. Indeed, in combination with TNF, IL-17 increases the survival of rheumatoid arthritis synoviocytes by inducing anti-apoptotic molecules, such as synoviolin [105], and contributes to their invasive phenotype [106]. In this way, IL-17 plays a major role in the chronicity of inflammation. Mainly by secreting IL-17, Th17 cells contribute to the irreversible side of diseases induced by inflammation.

V. Plasticity of Th17 cells

Even if each Th subset requires specific cytokines and transcription factors for their differentiation, Th17 cells keep some degree of plasticity (figure 4)[3, 48-50, 107]. Thereby, under inflammatory conditions, Th17 cells display plasticity and the produced cytokines can switch from IL-17 to other pro-inflammatory cytokines, such as IFN-γ. Indeed, in blood, IL-17+ IFN-γ+ double producer cells are easily detected. Different co-expressions during inflammatory responses have been observed, IL-17/IFN-γ, RORc/T-bet, FoxP3/RORc/IL-17 or IL-17/IL-10, and these mixed phenotypes are determined by the inflammatory environment. The prime factors which affect the stability of Th subsets are also those involved in their differentiation. TGF-β plays a major role for maintenance of IL-17 expression. In the absence of TGF-β, IL-23 and IL-12 suppress IL-17 production and promote the emergence of IFN-γ expression in Th17 precursors. This plasticity toward the Th1 profile is dependent on Stat4 and T-bet transcription factors [108]. This co-expression RORc and T-bet leads to a more pathogenic phenotype of Th17 cells. On the contrary, high levels of TGF-β induce the ability to IL-17+ cells to produce also IL-10 and this is reinforced by the absence of IL-23. This phenotype is more protective due to the anti-inflammatory effect of IL-10

[109]. The presence of IL-23 is known to stabilize the Th17 phenotype but it can also mediate the switch to IFN-γ production during chronic inflammation [110]. Th17 cells express a full IL-12 receptor and thus, in response to exposure to IL-12, they switch to Th1 phenotype by expressing T-bet and secreting IFN-γ. In turn, IFN-γ increases the sensitivity of Th17 cells to IL-12 and contributes to the phenotype Th17/Th1 which co-expressed RORc and T-bet. Furthermore, the interaction with IL-4 can also lead to a switch toward Th2 subset and IL-4 production. Both phenotypes, Th1/Th17 and Th2/Th17, seem to be more aggressive and more pathogenic than unshifted Th17 cells.

Furthermore, the network between the different transcription factors also affects the stability and the plasticity of Th17 cells, and notably the inhibitory effect of Foxp3 on RORc. The development of Th17 and Treg cells are closely related by TGF-β. Indeed, TGF-β induces both transcription factors, Foxp3 and RORc but in the absence of inflammation, i.e. mainly absence of IL-6, FoxP3 represses RORc and Treg differentiation is favored. In inflammatory conditions, RORc is up-regulated and induces Th17 differentiation. A transient population made of CD4+ FoxP3+ RORc+ cells can lead to both Th17 and Treg cells according to the local conditions. The plasticity between Th17 and Treg cells is important and plays a major role in the control and outcome of immune responses and inflammation [111]. The plasticity of Th17 cells has important biological implications regarding their ability to switch to a more pathogenic phenotype such as Th17/Th1 or more protective phenotype such as Th17/Treg.

VI. Clinical targeting of Th17 cells

Considering the crucial role of Th17 cells in the pathogenesis of several chronic inflammatory diseases, they have been considered as potential targets for treatment [1, 3, 72,

107]. Various options are available to target the Th17 pathway in inflammatory diseases, cytokine targeting, signaling inhibition or transcription factor inhibitor.

To act on Th17 differentiation and amplification, inhibitors of IL-6 and IL-1β, largely involved in Th17 differentiation are currently used in the treatment of inflammatory diseases. Tocilizumab is a humanized anti-IL-6 receptor antibody; anakinra is an IL-1 receptor antagonist and canakinumab an inhibitor of IL-1. The first two are currently used in rheumatoid arthritis treatment and the last two for that of auto-inflammatory diseases [112]. In contrast, tocilizumab has no clear effect in psoriasis, psoriatic arthritis and ankylosing spondylitis [113], suggesting different mechanisms by which Th17 cells are involved in these diseases.

Another target is IL-23 as it is critical for the expansion and the stabilization of the Th17 phenotype. IL-23 can be inhibited by two antibodies directed against the p40 chain, a subunit of IL-23, ustekinumab and briakinumab. Ustekinumab is registered for the treatment of Psoriasis and Psoriatic arthritis. Other indications are in progress [114-118]. All these therapies by targeting the common p40 chain, affect both the Th17 and the Th1 pathways, and IL-17 and IFNγ, respectively. Targeting the p19 specific chain of IL-23 affects only the Th17 pathways and their cytokines IL-17, IL-21, and IL-22. The anti-IL-23 p19 inhibitors tildrakizumab and guselkumab are now tested in psoriasis and psoriatic arthritis.

Another option is to target the key transcription factor RORc [119], using small molecules. This provides a good opportunity to inhibit Th17 subset but also Th17/Th1 and Th17/Th2 shifted cells, which could be the more pathogenic ones. This will block the entire Th17 program, from Th17 development to pro-inflammatory cytokine secretion. Digoxin is an old molecule recently identified as a specific RORγt inhibitor [120]. Other molecules such as ursolic acid and SR1001 bind specifically RORα and RORγt [121]. They inhibit Th17 differentiation and function in murine and human cells and suppresses the clinical severity of

various models of autoimmune diseases in mice [122]. The use of small molecules to target the transcription factor RORc could provide a simple treatment of chronic inflammatory and autoimmune diseases.

The biologic effects of Th17 cells could also be controlled down-stream by targeting directly IL-17. Secukinumab, a fully human anti-IL-17A monoclonal antibody has been the first inhibitor of IL-17A for the treatment of psoriasis and registration for psoriatic arthritis and ankylosing spondylitis are in progress [72]. Similar indications have been selected for ixekizumab, a humanized anti-IL-17A [123]. Both antibodies have shown efficacy in rheumatoid arthritis but with a high heterogeneity in the clinical response [124, 125]. Another way to inhibit the IL-17 effect is to act downstream on IL-17 receptor complex. Brodalumab is a humanized anti-IL-17RA antibody that has shown efficacy in psoriasis [126, 127], in psoriatic arthritis and ankylosing spondylitis [128] but not in rheumatoid arthritis [129].

Conclusion

Th17 cells were identified as the third subset of T helper cells, with distinct effector functions from the Th1 and Th2 subsets. Increased knowledge of Th17 cells during the last years has been made in understanding their development and in defining their role in health and diseases. Th17 cells are important effector cells to clear many pathogens. When in excess, this subset becomes highly pathogenic in the induction and the propagation of chronic inflammation. Many diseases are influenced by the activation of the Th17 pathway, such as psoriasis, rheumatoid arthritis or multiple sclerosis.

Considering their role in inflammatory and autoimmune diseases, the inhibition or the modulation of Th17 cell differentiation and functions could be beneficial in such conditions. Impressive results have already been obtained and more strategies are developed. The final goal is a fine tuning of this balance not only to control inflammation but to induce a cure.

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Figure legends

Figure 1: Differentiation of Th17 cells in mice and in humans

In mice, TGF-β and IL-6 initiate the differentiation and activate Stat3, which induces RORγt. IL-21 is induced by developing Th17 and in autocrine manner acts on Th17 cell amplification. IL-23 serves to expand and maintain the Th17 cell population.

In humans, the differentiation is initiated by TGF-β and IL-21, which induce the transcription factor RORc. IL-1β plus IL-6 are important to enhance the amplification of Th17 cells and IL-23 to maintain the Th17 phenotype.

Figure 2: Th17 cells during chronic inflammation

During inflammation, immune cells migrate to the inflammatory site and secrete Th-polarizing cytokines. TGF-β, IL-6 and IL-1β induce Th17 differentiation via the induction of the transcription factor RORc. RORc induces IL-21 which acts in autocrine way to maintain the Th17 phenotype. Differentiated Th17 cells express IL-23R and IL-23 drives the maintenance of the Th17 phenotype.

Th17 cells express the receptor CCR6, which binds CCL20 and this interaction leads to the recruitment of Th17 cells to the inflammatory site. CCL20 is produced by mesechymal cells and also by Th17 cells themselves.

Cytokines produced by Th17 cells, and notably IL-17, stimulate local cells such as fibroblasts, synoviocytes or endothelial cells, to produce pro-inflammatory cytokines and chemokines involved in Th17 differentiation and stabilization. This way induces the chronicity of the activation of the Th17 cells.

Figure 3: Th17 phenotypes

After their expansion and stabilization, circulating Th17 cells are characterized by the phenotype CD4+ RORc+ IL-23R+ CCR6+ CD161+ and IL-17+. At this stage, Th17 cells are not producing cells and can express markers of other Th subsets and can switch their

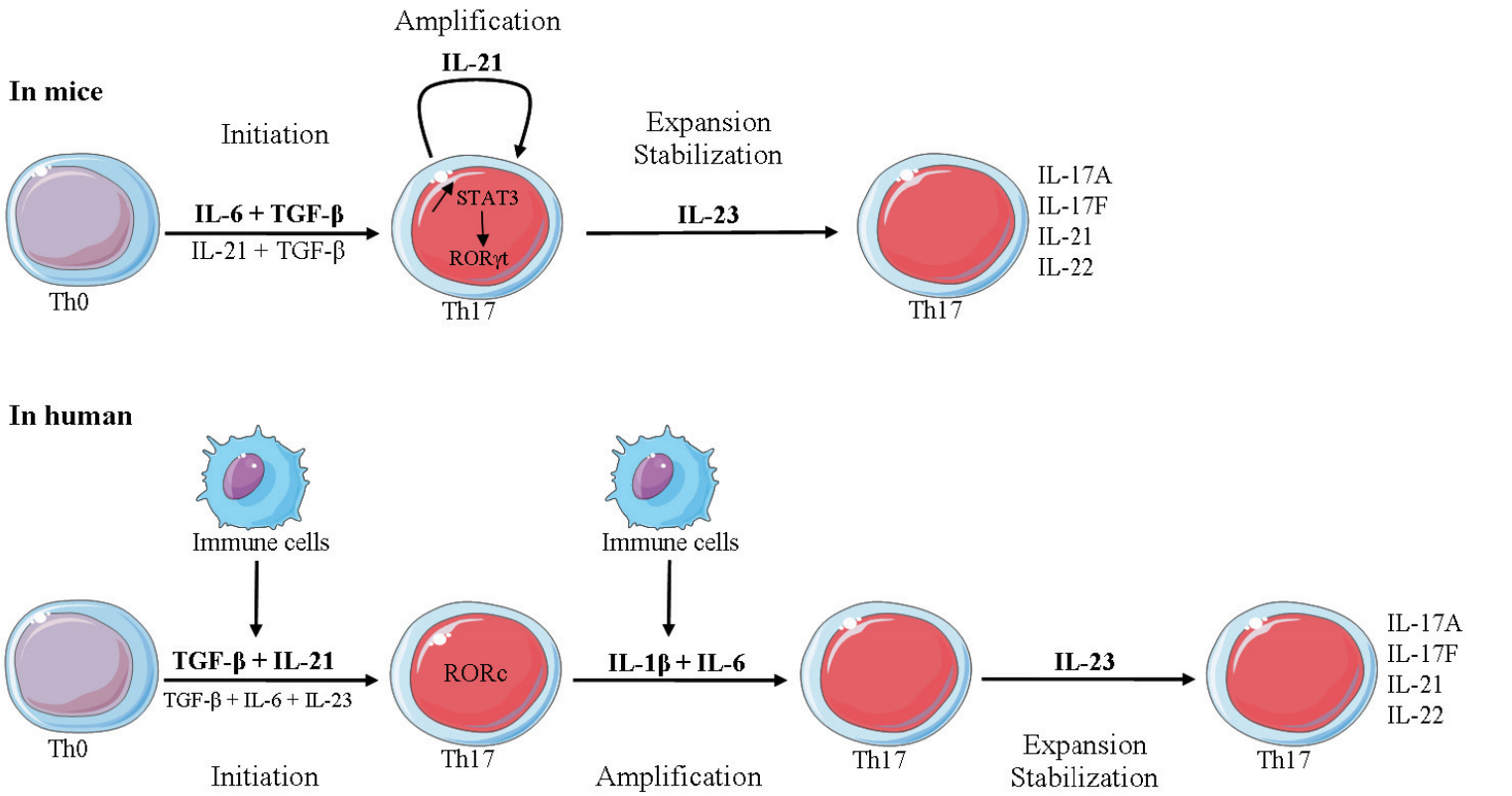
phenotype. The double positive cells are less stable and become single producing cells in the inflamed tissue, with a plasma cell phenotype.

Figure 4: Plasticity of Th17 cells

Under inflammatory conditions, the Th17 subset displays plasticity. In the absence of TGF-β, IL-23 and IL-12 suppress IL-17 production and promote T-bet expression that induces IFN-γ expression in Th17 precursors.

Exposure of Th17 cells to IL-12, which express IL-12R induces the switch to Th1 phenotype with the expression of T-bet and the secretion of IFN-γ. In turn, IFN-γ increases the sensitivity of Th17 cells to IL-12. IL-4 can lead to a switch into Th2 phenotype with GATA-3 expression and IL-4 secretion. The Th17/Th1 and Th17/Th2 phenotypes may be more aggressive and pathogenic than unshifted Th17 cells.

A high level of TGF-β can induce the secretion of IL-10 by Th17 cells, and this is increased in the absence of IL-23. This phenotype is more protective than the Th17 cells.



Modified from Noack and Miossec, Autoimmunity Reviews, 2014

Figure 1

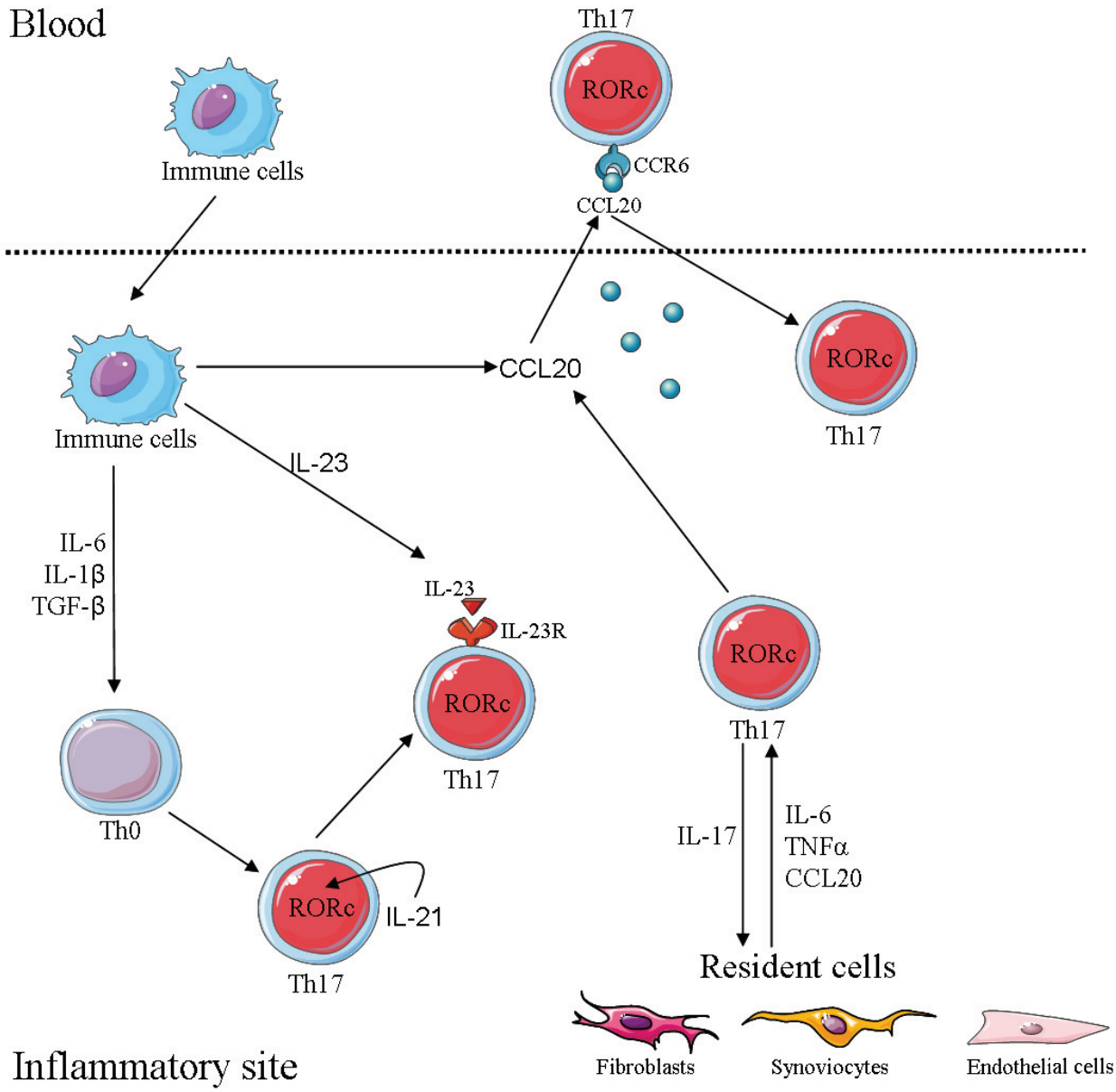

Blood
Th17
RORc
CCR6
CCL20
Immune cells
CCL20
RORc
Th17
Immune cells
IL-23
IL-6
IL-1β
TGF-β
IL-23
IL-23R
RORc
Th17
RORc
Th17
Th0
IL-17
IL-6
TNFα
CCL20
RORc
IL-21
Th17
Resident cells
Fibroblasts
Synoviocytes
Endothelial cells
Inflammatory site

Figure 2

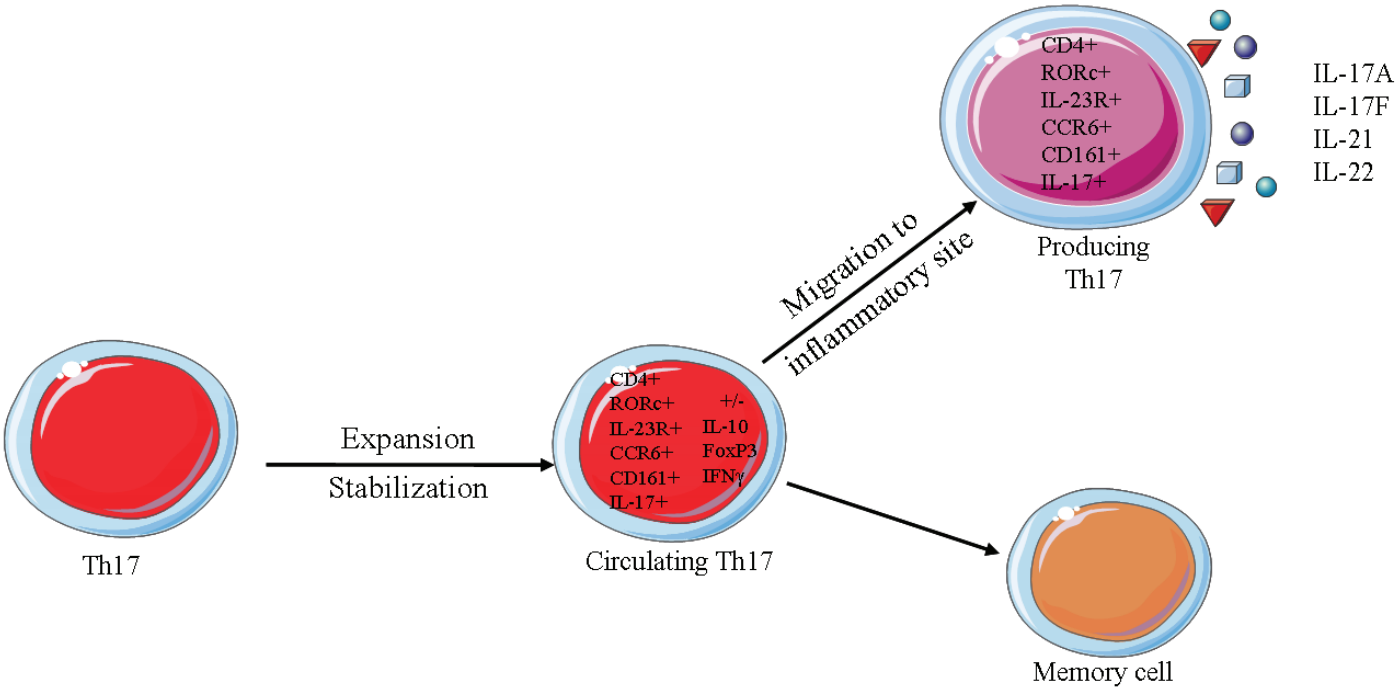
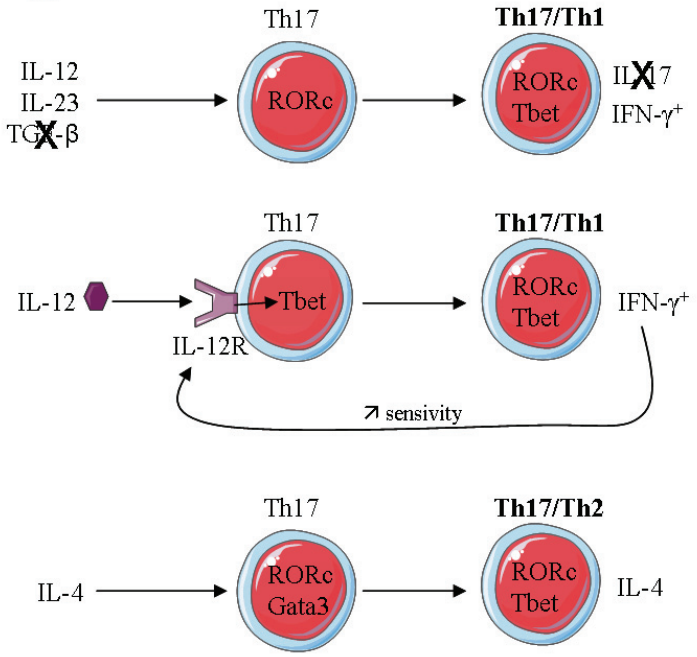

CD4+
RORc+
IL-23R+
CCR6+
CD161+
IL-17+
IL-17A
IL-17F
IL-21
IL-22
Producing
Th17
Migration to
inflammatory site
Th17
Expansion
Stabilization
CD4+
RORc+
IL-23R+
CCR6+
CD161+
IL-17+
+/-
IL-10
FoxP3
IFNγ
Circulating Th17
Memory cell

Figure 3

More pathogenic phenotypes



More protective phenotype

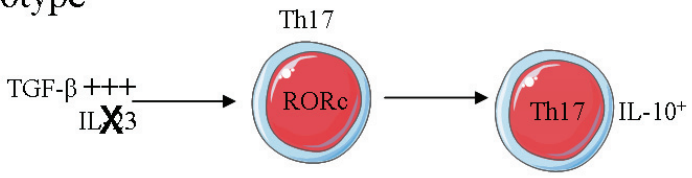


Figure 4

Partie 2. Applications aux maladies inflammatoires chroniques

Les maladies inflammatoires chroniques regroupent de nombreuses pathologies. Dans ce travail, deux pathologies seront utilisées comme modèle : la polyarthrite rhumatoïde (PR) et le psoriasis (Pso).

I La polyarthrite rhumatoïde

I.1 Etiologie

La PR est la plus fréquente des diverses formes de rhumatismes inflammatoires chroniques. Elle est considérée comme une maladie auto-immune multifactorielle et multigénique. Malgré les avancées considérables dans la compréhension de sa physiopathologie, son étiologie reste encore inconnue. Plusieurs facteurs ont toutefois été identifiés comme intervenant dans le déclenchement de la PR : des facteurs hormonaux, génétiques ou environnementaux. Parmi les facteurs environnementaux, la consommation excessive de café, une carence en vitamine D, des conditions socio-économiques défavorisées ont été classées comme facteurs de risque [72]. Dans l'environnement, certains agents infectieux, comme le virus Epstein-Barr [72], sont également des facteurs de risque. Concernant les facteurs génétiques, ils représentent un facteur de risque élevé de développer une PR. Plus d'une trentaine de polymorphismes sont associés à la PR. L'association génétique la plus forte est observée avec le gène codant pour les molécules HLA (human leukocyte antigen) de classe II. Certains allèles tels que HLA-DRB1*0401, HLA-DRB1*0404 ou HLA-DRB1*0101 sont associés au développement d'une PR en Europe [73]. Enfin, les facteurs hormonaux jouent également un rôle dans la susceptibilité à la PR. En effet, les femmes sont trois fois plus souvent atteintes que les hommes. Les changements hormonaux se

produisant pendant et après la grossesse sont également capables de modifier la susceptibilité à la PR. Ainsi, lors de la grossesse, le risque de développer une PR est faible alors que l'année suivant le post-partum, il augmente nettement, principalement à cause de l'allaitement [74].

I.2 Physiopathologie

La PR est une pathologie destructrice et handicapante ; elle provoque des érosions, des pincements et des déformations articulaires, diminuant ainsi la qualité et l'espérance de vie des patients. C'est également une maladie systémique pouvant mettre en jeu le pronostic vital, en particulier par le risque cardio-vasculaire. Le déclenchement et la pérennisation de la PR se font selon un schéma décrit en trois phases : phase d'initiation de la réponse immunitaire ; phase d'amplification de la réponse immunitaire ; phase effectrice de la réponse immunitaire et inflammation. Ces trois phases aboutissent à une destruction tissulaire, accompagnée d'un défaut de réparation ainsi que d'un infiltrat tissulaire de cellules immunitaires [49].

L'inflammation de la membrane synoviale va être responsable de la gravité de la maladie. Elle entraîne progressivement une destruction de l'os et du cartilage. L'inflammation ainsi mise en place induit une synovite chronique, caractérisée par un infiltrat de cellules immunitaires au niveau de la synoviale (macrophages, LTh, LB), ainsi que par l'interaction entre les PBMC et les CSM articulaires locales (synoviocytes). L'immunopathologie de la PR se caractérise par un dérèglement du milieu synovial, tel qu'une prolifération incontrôlée des synoviocytes, une accumulation de LTh (LTh1, LTh17), une destruction osseuse supérieure à la réparation ou encore une production importante de cytokines pro-inflammatoires et un défaut de cytokines anti-inflammatoires.

L'accumulation des synoviocytes dans l'articulation est une caractéristique majeure de la PR [75]. Ces cellules sont en effet activement impliquées dans la régulation de la réponse inflammatoire [50, 76-78]. Les synoviocytes sont des CSM qui affichent différentes

caractéristiques des fibroblastes. En situation normale, ils jouent un rôle essentiel dans l'homéostasie de l'articulation. Ils permettent de contrôler le volume du fluide synovial, de contrôler la réponse inflammatoire, de réguler la circulation leucocytaire, ou encore ils participent au maintien de la capsule articulaire [76]. Les synoviocytes apparaissent donc comme des cellules essentielles au sein de l'articulation. Chez certains patients, l'hyperplasie des synoviocytes peut précéder l'accumulation des cellules immunitaires inflammatoires, suggérant ainsi un rôle primaire de ces cellules [79]. Le nombre important de synoviocytes est dû à un déséquilibre entre prolifération, survie et apoptose de ces cellules ; la PR favorisant la survie à la mort cellulaire [80, 81]. Dans ce cas, les synoviocytes présentent des propriétés invasives et agressives. En effet, ils contribuent à la production locale de cytokines pro-inflammatoires, de médiateurs inflammatoires et d'enzymes protéolytiques telles que les MMP. Ils vont s'avérer être les principaux effecteurs de la destruction du cartilage. Les caractéristiques invasives des synoviocytes issus de patients PR ont été reportées dans différentes études [82, 83]. Au sein du laboratoire il a été montré que l'IL-17 et le TNF, cytokines retrouvées dans le milieu synovial de PR, induisent un phénotype invasif des synoviocytes. En effet, ces cytokines induisent la migration et l'invasion des synoviocytes à travers une voie dépendante de SDF-1/CXCR4 et HIF-1α [17].

De plus, grâce à l'interaction cellule-cellule, les synoviocytes vont favoriser la survie d'autres types cellulaires tels que les LT, en inhibant leur apoptose par le maintien de signaux de survie et l'absence de signaux de mort cellulaire. Ils vont également contribuer à l'initiation, à la propagation et au maintien de l'inflammation chronique. Les synoviocytes vont ainsi produire des chimiokines en réponse à la stimulation par l'IL-1 et par le TNF (MIP-1α, RANTES, SDF-1) et ainsi recruter les LT (CXCR3, CCR5, CXCR4). Ils peuvent également moduler le profil des cytokines synoviales via le contact direct avec les cellules environnantes (le contact avec les LT favorise la sécrétion de cytokines pro-inflammatoires)

[77]. Différentes études ont montré l'effet de l'interaction entre les synoviocytes et les LT [84-86]. La mise en co-culture de ces deux populations augmente la survie cellulaire, empêche l'apoptose et induit la production de cytokines (IL-17, TNF, IFNγ). Au cours de la PR, les synoviocytes sont également une source principale d'IL-6, cytokine pro-inflammatoire impliquée dans la différenciation des LTh17 [87]. Au laboratoire, il a été montré que l'interaction de cellules mésenchymateuses avec les LT favorise la différenciation des LTh17, via l'activation de la caspase-1 [31]. En effet, l'interaction des BM-MSC (Bone Marrow-Mesenchymal Stem Cells) ou des synoviocytes avec les LT présents sur le site de l'inflammation peut favoriser la différenciation des LTh17 et ainsi la production de cytokines pro-inflammatoires telles que l'IL-17, le TNF ou l'IFNγ. De plus, il a été montré que l'interaction des PBMC avec les BM-MSC inhibe la réponse Th1 et Th2, mais favorise l'expansion des LTh17.

L'IL-17, principale cytokine pro-inflammatoire produite par les LTh17, joue également un rôle central dans la chronicité de la PR. En effet, l'hyperplasie synoviale est la conséquence d'un déséquilibre entre la prolifération et l'apoptose des synoviocytes résidents. En se fixant sur son récepteur exprimé de manière ubiquitaire [88], et notamment par les synoviocytes, l'IL-17 va entrainer l'expression de la synovioline (ubiquitine ligase impliquée dans la survie cellulaire) via les voies de signalisation ERK et JNK. Ainsi, les synoviocytes échappent à l'apoptose et survivent. Dans ce cas, l'IL-17 contribue à la chronicité de la PR par sa contribution au maintien de l'inflammation et de l'hyperplasie [16].

La pathogenèse de la PR implique différents acteurs, en interaction les uns avec les autres. Ainsi, les cellules immunitaires infiltrant le tissu synovial vont interagir avec les synoviocytes locaux. Ces interactions cellulaires conduisent à une prolifération excessive de ces cellules et un maintien de l'inflammation, notamment par la sécrétion de cytokines pro-

inflammatoires. L'implication des LTh17 et de leur principale cytokine, l'IL-17, est également mise en jeu. L'étude de l'effet de l'interaction cellulaire sur cette voie est donc tout particulièrement intéressante à suivre. Ces caractéristiques se retrouvant dans d'autres pathologies inflammatoires chroniques, telles que le psoriasis, l'intérêt ne s'en trouve que renforcé, sachant que la compréhension de ces mécanismes conduit à une meilleure maitrise de ces pathologies.

I.3 Interactions cellulaires et polyarthrite rhumatoïde

Article: "Interaction among activated lymphocytes and mesenchymal cells through podoplanin is critical for a high IL-17 secretion"

L'interaction entre les lymphocytes activés et les cellules mésenchymateuses via la podoplanine est critique pour une forte sécrétion d'IL-17

Résumé:

Introduction: Au cours de la PR, maladie inflammatoire chronique, les cellules immunitaires, et notamment les LTh17, infiltrent le site inflammatoire, les articulations, ce qui permet leur interaction avec les cellules mésenchymateuses locales, les synoviocytes. Dans ce contexte, le but de ce travail était d'étudier le rôle de ces interactions cellulaires sur la sécrétion de cytokines pro-inflammatoires, principalement sur la sécrétion d'IL-17, afin de différencier l'expression intracellulaire de la sécrétion de l'IL-17 et d'identifier un mécanisme conduisant à sa forte sécrétion, avec un intérêt particulier pour la podoplanine.

Méthodes : Un système de co-culture entre PBMC et synoviocytes a été utilisé pour mimer la situation *in vivo*. Des PBMC issues de donneurs sains ou de patients atteints de PR ont été mises en co-culture pendant 48h, en présence ou non de phytohémagglutinine (PHA). Pour évaluer le rôle de la podoplanine, un anticorps dirigé contre cette molécule a été utilisé dans les co-cultures. Après 48h de culture, la production de différentes cytokines (IL-6, IL-1β et IL-17) a été mesurée par ELISA dans les surnageants et les cellules ont été marquées (CD3, CD4, IL-17 et podoplanine) et analysées par cytométrie en flux.

Résultats : Dans les conditions de contrôle (sans activation par PHA), la production d'IL-6 et d'IL-1β augmentait dans les co-cultures PBMC-synoviocytes, comparée aux PBMC seules (*p=0.02*). Aucun effet additionnel n'était observé avec l'activation des PBMC par la PHA. L'analyse par cytométrie en flux ne montrait pas de différence entre le pourcentage de LTh17 dans les PBMC activées seules ou en co-culture avec les synoviocytes (*p=0.4*), indiquant que la différenciation des LTh17 requiert d'avantage l'activation des LT que l'interaction cellulaire. A l'inverse, la production d'IL-17 était fortement augmentée dans les co-cultures PBMC activées-synoviocytes vs. PBMC activées seules (*p=0.002*). L'utilisation d'un système autologue, PBMC et synoviocytes provenant du même patient, donnait des résultats semblables, ce qui permettait d'exclure une alloréactivité et ainsi de valider notre modèle *in vitro*. De plus, l'utilisation d'un système de transwell, empêchant le contact cellule-cellule mais permettant l'échange de facteurs solubles, inhibait massivement la sécrétion d'IL-17, ce qui confirmait le rôle critique de l'interaction cellulaire dans la forte production d'IL-17. Afin de confirmer le rôle essentiel des synoviocytes et des LTh17 dans ce mécanisme, des co-cultures entre synoviocytes et clones Th17 ont été réalisées. Comme avec les PBMC, la sécrétion massive d'IL-17 était plus facilement obtenue lors des co-cultures synoviocytes-clones Th17 activés. Le rôle de la podoplanine a ensuite été étudié. La pré-incubation des

PBMC ou des synoviocytes avec un anticorps anti-podoplanine diminuait significativement la sécrétion d'IL-17, d'environ 60% en moyenne (*p=0.008*) et l'expression de la podoplanine augmentait dans les co-cultures avec PHA, ce qui corrélait avec la production massive d'IL-17. L'utilisation d'un siRNA spécifique de la podoplanine sur les synoviocytes permettait également de diminuer la sécrétion d'IL-17. Ces résultats impliquent donc largement la podoplanine dans le mécanisme menant à la forte production d'IL-17 lors de l'interaction entre PBMC et cellules mésenchymateuses.

Conclusion : L'interaction seule entre PBMC et synoviocytes est suffisante pour induire la production de cytokines pro-inflammatoires, telles que l'IL-6 et l'IL-1β. En revanche, la forte sécrétion d'IL-17 requiert deux signaux, l'activation des PBMC et l'interaction cellulaire. De plus, la molécule d'interaction podoplanine contribue largement à la production massive d'IL-17 et peut donc contribuer à la chronicité de l'inflammation. Dans ce contexte, la podoplanine peut être une cible thérapeutique potentielle pour bloquer l'activité pathogène des LTh17 pendant l'inflammation chronique.

Noack et al. Arthritis Research & Therapy (2016) 18:148
DOI 10.1186/s13075-016-1046-6

Arthritis Research & Therapy

RESEARCH ARTICLE Open Access

Interaction among activated lymphocytes and mesenchymal cells through podoplanin is critical for a high IL-17 secretion

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Abstract

Background: During chronic inflammation, immune cells, notably Th17 cells, infiltrate the inflammatory site and interact with local mesenchymal cells. Applied to rheumatoid arthritis (RA), the aim is to study the interactions between synoviocytes and peripheral blood mononuclear cells (PBMC) with a focus on the Th17 pathway and to identify a mechanism which leads to high IL-17 secretion with an interest on podoplanin.

Methods: PBMC from healthy donors and RA patients were co-cultured with RA synoviocytes during 48 h, in the presence or not of phytohemagglutinin. An antibody against podoplanin was used in co-culture. Cytokine production (IL-6, IL-1β and IL-17) was measured by ELISA and cell staining (CD3, CD4, IL-17 and podoplanin) by flow cytometry.

Results: In control conditions, IL-6 and IL-1β production was increased in PBMC-synoviocyte co-culture compared to PBMC alone ($p = 0.02$). No additional effect was observed with PBMC activation. Flow cytometry analysis showed no difference in the percentage of Th17 cells in activated PBMC alone or with synoviocytes ($p = 0.4$), indicating that Th17 differentiation requires only T cell activation. Conversely, IL-17 production was highly increased in co-cultures with activated PBMC vs. activated PBMC alone ($p = 0.002$). Transwell experiments confirm that cell-cell contact was critical for IL-17 secretion. The incubation of either PBMC or synoviocytes with an anti-podoplanin antibody decreased IL-17 secretion by 60 % ($p = 0.008$).

Conclusions: Interactions between resting PBMC and synoviocytes are sufficient to induce IL-6 and IL-1β production. Both PBMC activation and cell interactions are needed to induce a high IL-17 secretion. Podoplanin contributes at the level of both lymphocytes and synoviocytes.

Keywords: IL-17, Synoviocytes, Rheumatoid arthritis, Cell interactions, Podoplanin

Background

Interleukin-17 (IL-17) is a pro-inflammatory cytokine mainly produced by Th17 cells and involved in several chronic inflammatory diseases, such as psoriasis or rheumatoid arthritis (RA) [1, 2]. Commonly, an association is made between Th17 cells and the secretion of IL-17, using intracellular staining of IL-17 as an equivalent of actual demonstration of protein secretion. As shown in one of our previous studies, IL-17-positive cells acquire a plasma cell-like morphology under in vitro activation, which is correlated with enhanced concentrations of secreted IL-17. Moreover, in sections of inflamed tissue, IL-17+ cells have this plasma cell-like morphology [3]. The connection between IL-17 intracellular staining and IL-17 secretion thus remained to be clarified.

During chronic inflammatory diseases, the recruitment of activated immune cells, including Th17 cells, to the site of disease, leads to interactions with local mesenchymal cells and this promotes cell proliferation and the

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chronicity of inflammation [4–6]. These interactions between bone marrow-derived or synovium-derived mesenchymal cells and activated peripheral blood mononuclear cells (PBMC) promote Th17 cell expansion [7].

Regarding the crucial role of cell–cell interactions in pro-inflammatory cytokine production, a mechanism remained to be identified. Among many options, podoplanin (pdpn) was considered as a possible candidate, as a molecule involved in cell–cell interactions. Indeed, pdpn modulates IL-8 secretion during interactions between platelets and synoviocytes through its receptor CLEC-2 on platelets [8]. Moreover, the pdpn pathway is involved in different animal models of inflammatory diseases [9, 10]; notably in a mouse model of RA, pdpn is upregulated in Th17 cells compared to other Th cell subsets [9]. Pdpn expression is also upregulated in RA synovium and it might increase the migratory potential of activated synoviocytes in RA [11]. These results lead us to consider pdpn as a potential new mechanism involved in the Th17 pathway during chronic inflammation.

The aim of this study was to clarify the difference between intracellular expression from IL-17 secretion and to identify a mechanism which leads to high IL-17 secretion during interactions between PBMC and synoviocytes, with a special interest in podoplanin.

Materials and methods

Samples

RA synoviocytes were obtained from synovial tissue of RA patients undergoing joint surgery and who fulfilled the American College of Rheumatology criteria for RA [12]. Synovial tissue was minced into small pieces and then adhered in 6-well plates in Dulbecco's modified Eagle's medium (DMEM; Eurobio, Courtaboeuf, France) supplemented with 10 % fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Cells were maintained at 37 °C in a humidified 5 % carbon dioxide incubator and used between passages 4 to 9. They are fibroblast-like synoviocytes which express pdpn and secrete IL-6 after stimulation by tumor necrosis factor (TNF) [11, 13]. PBMC from healthy donors or RA patients were isolated by Ficoll-Hypaque (Eurobio, Courtaboeuf, France) density-gradient centrifugation. Adipose-derived stem cells (ASC) and human umbilical vein endothelial cells (HUVEC) were used as control cells.

T cell cloning procedure

To obtain CD4+ T cell clones, CD4+ T cells were isolated from peripheral blood mononuclear cells (PBMC) of healthy donors by immunomagnetic cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany) and further divided into the CD161 + CCR6+ and CD161- CCR6- fractions by flow cytometry cell sorting (FACSAria, BD Bioscience, Franklin Lakes, NJ, USA). Both cell fractions obtained were seeded under limiting-dilution conditions (0.5 cell/well) in round-bottom microwell plates, containing 10^5 irradiated (60 Gy [9000 rad]) allogeneic peripheral blood mononuclear cells as feeder cells, 1 % phytohemagglutinin (PHA; vol/vol), and recombinant human IL-2 (50 U/mL). Blasts were expanded in the presence of feeder cells (10^5 cells/well) plus IL-2 (50 U/mL). T cell clones were obtained and evaluated for their cytokine production activity (interferon [IFN]-γ, and IL-17) by flow cytometry after stimulation with PMA plus ionomycin, as previously described [14, 15]. T cell clones producing IL-17 alone were classified as Th17. Production of cytokines by each clone was arbitrarily considered as significant when the proportion of producing T cell blasts was >20 %. Th17 clones obtained from the CD161 + CCR6+ fraction were selected and further expanded in the presence of feeder cells plus IL-2 until they reached the number of approximately 10^6 blasts/clone, then they were transferred in 24-well plates and maintained in culture in the presence of IL-2 (50 U/mL).

Co-culture assays

Co-culture was initiated by seeding RA synoviocytes, ASC or HUVEC overnight in 96-well plates at a density of 2×10^4 cells/well in RPMI 1640 medium (Eurobio, Courtaboeuf, France) supplemented with 10 % human AB serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (complete RPMI). The next day, PBMC (1×10^5 cells/well) or Th17 clones (2×10^4 cells/well) were seeded in complete RPMI corresponding to 5:1 ratio or 1:1 ratio, respectively, in the presence or absence of phytohemagglutinin (PHA, 5 μg/ml). After 48 h, supernatants and cells were collected for analysis.

Transwell assay

RA synoviocytes were seeded in 24-well plates at a density of 1×10^5 cells/well in complete RPMI. After overnight incubation, PBMC were added directly on top of synoviocytes or in a cell culture insert (0.4 μm pore size) at a concentration of 5×10^5 cells/well, in the presence or absence of PHA (5 μg/ml). After 48 h, supernatants and PBMC were recovered for analysis.

Cell fixation

Synoviocytes or PBMC were fixed for 1 h, at 4 °C, in phosphate-buffered saline (PBS) 0.01 % paraformaldehyde before co-culture.

Enzyme-linked immunosorbent assays (ELISA)

IL-17A, IL-6, and IL-1β productions were evaluated from culture supernatants with commercially available DuoSet ELISA kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Flow cytometry

Allophycocyanin (APC), phycoerythrin (PE), PE-cyanine-7 or eFluor 450-conjugated antibodies (eBiosciences, San Diego, CA, USA) were used to stain cells. EFluor 450-CD3 (48–0038), PE-Cy7-CD4 (25–0049) and PE-pdpn (12–9381) were used for cell surface staining, according to the manufacturer's instructions. PBMC were fixed and permeabilized for APC-IL-17A (17–7179) intracellular staining. Cells were incubated in cold PBS and cold 2 % paraformaldehyde in the dark during 1 h for fixation step. For permeabilization, cells were incubated in PBS with 0.2 % Tween at 37 °C for 15 min, before intracellular staining. Flow cytometry staining buffer from eBiosciences (San Diego, CA, USA) was used for staining protocol. Analysis was done with the Kaluza software (Beckman Coulter, Brea, CA, USA).

Monocyte contribution

To remove monocytes, PBMC were pre-incubated during 2 h at 37 °C. The percentage of removal monocytes by adherence was verified with flow cytometry. This method allowed removing at least 50 % of monocytes.

Inhibition of podoplanin

A dose–response curve was performed to determine the concentration of purified anti-human podoplanin antibody (14–9381, eBiosciences, San Diego, CA, USA) required for maximal inhibition. PBMC were pre-incubated for 4 h with different concentrations of anti-podoplanin (0, 1, 5, 10 and 20 μg/ml) before co-culture assay. According to the results of dose–response curve, all experiments studying podoplanin were realized with anti-podoplanin at 5 μg/ml. A control antibody against the BetV1 allergen with no effect in the assays was used at the same concentration (anti-BetV1 Ab, Dendritics, Lyon, France).

Small interfering RNA (siRNA)

A mixture of four small interfering RNA (siRNA) provided as a single reagent (siGENOME human SMARTPool siRNA) specific for podoplanin (M-017560-01-0005) was purchased from Dharmacon, Lafayette, CO, USA. RA synoviocytes were seeded at a density of 5×10^5 cells/12-well plate before transfection and used at 80–90 % confluence. Cells were transfected with control siRNAs (siCONTROL siRNA as a negative control (mock) and siGLO peptidylpropyl isomerase B (PPIB) (cyclophilin B) as a positive control) or target siRNAs (siGENOME SMARTPool pdpn siRNA) by nucleofection (Amaxa Pharma, London, UK) according to the manufacturer's instructions (program U23; Human Dermal Fibroblast Nucleofector kit). Dose– and time–response experiments were performed to determine the best time point and the best suitable concentration of siRNA duplexes needed for efficacious RNA silencing. Cells were nucleofected with 37.6, 100 or 376 nM of pdpn siRNA (siPdpn), per 5×10^5 cells. Twenty-four hours or 48 h post-transfection, RNA was extracted and RT-PCR performed to detect the RNA silencing. The best condition was at 100 nM, at 48 h, thus this condition was used for the presented experiments.

RNA extraction and real-time PCR

Total RNA of synoviocytes was extracted using the RNeasy Mini Kit (Qiagen®, Hilden, Germany) and quantified with the Quant-it kit assay (Invitrogen™ by Thermo Fisher Scientific, Grand Island, NY, USA) following the manufacturer's instructions. cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen®) according to the manufacturer's instructions. SYBR green-based real-time qRT-PCRs were performed on the CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA, USA) using the QuantiFast SYBR green kit and QuantiTect primers (Qiagen®). Cycle threshold values were normalized with respect to the endogenous control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative expression of pdpn and PPIB genes in the different conditions was determined using the comparative threshold cycle method as described by the manufacturer.

Statistical analysis

Statistical analyses for co-culture assays were performed using two-way ANOVA. For transwell, monocytes and podoplanin experiments a paired nonparametric Wilcoxon test was used. All analyses were performed with GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). p values less than or equal to 0.05 were considered as significant.

Results

Interaction between RA synoviocytes and PBMC induces IL-6 and IL-1β production

PBMC produce pro-inflammatory cytokines, such as IL-6 and IL-1β, which are implicated in the Th17 differentiation [16–18]. Resting PBMC alone produced IL-6 at low levels and their activation by PHA had a modest effect (1.4 ± 3.4 ng/ml vs. 13.4 ± 11.8 ng/ml, Fig. 1a). IL-1β production was almost undetectable in control condition (7.2 ± 16.1 pg/ml, Fig. 1b), and PHA activation highly increased its secretion (2630.1 ± 2397.3 pg/ml, p = 0.03, Fig. 1b). Co-culture of resting PBMC and synoviocytes significantly increased IL-6 and IL-1β production compared with PBMC alone (443.0 ± 240.7 ng/ml vs. 1.4 ± 3.4 ng/ml; p = 0.0001 and 2794.4 ± 2862.2 pg/ml vs. 7.2 ± 16.1 pg/ml; p = 0.02, respectively, Fig. 1). Activation of PBMC by PHA had no additional effect in co-culture (Fig. 1). These results indicated that the cell–cell

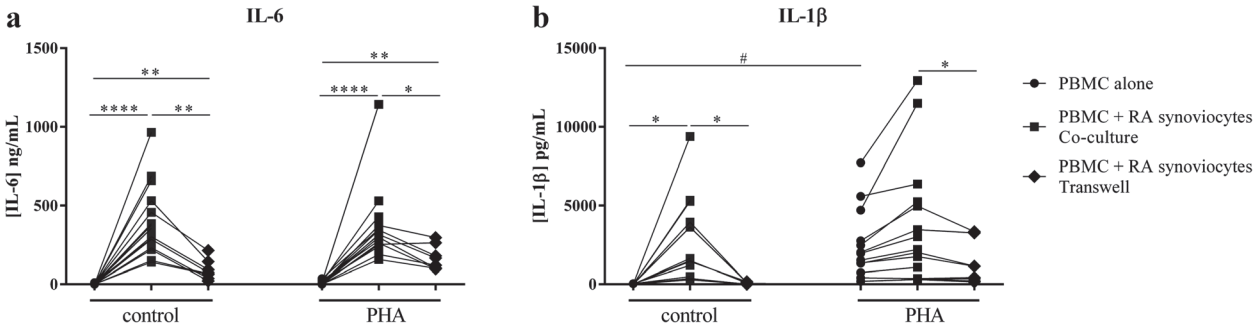

Fig. 1 Effect of interaction between synoviocytes and PBMC on IL-6 and IL-1β production. Healthy PBMC were cultured alone or in co-culture with RA synoviocytes at a 5:1 ratio for 48 h, in the presence or absence of PHA (5 μg/ml). The transwell system was used in co-cultures to inhibit cell–cell contact. Production of IL-6 (**a**) and IL-1β (**b**) in cell supernatants was measured by ELISA. Each linked dot plot represents one experiment in the different conditions. *Compares the culture conditions (PBMC alone, co-culture or transwell) with and without PHA. #Compares the activation state (control or PHA) in the culture conditions. *#$p \leq 0.05$. *IL* interleukin, *PBMC* peripheral blood mononuclear cells, *PHA* phytohemagglutinin, *RA* rheumatoid arthritis

contact was sufficient to activate the pro-inflammatory cytokine production. Furthermore, as PBMC and synoviocytes from different donors were used in the different experiments, this resulted in heterogeneity of cytokine production observed in Fig. 1.

To investigate the importance of cell–cell contact, a transwell system was used. The insert had a pore size of 0.4 μm, which prevents direct cell–cell contact but allows the exchange of soluble factors. In this transwell system, IL-6 and IL-1β production was significantly decreased compared to control (89.1 ± 58.6 ng/ml vs. 289.5 ± 130.9 ng/ml, $p = 0.008$ and 40.1 ± 46.3 pg/ml vs. 1488.9 ± 1505.2 pg/ml, $p = 0.008$, respectively, Fig. 1). A significant decrease of IL-6 and IL-1β production in the transwell system was also observed with T cell receptor (TCR) activation by PHA, nevertheless with a tendency to a lower effect than in the control condition. This confirmed that cell–cell contact was sufficient and required to activate cells to produce pro-inflammatory cytokines. Moreover, to reinforce this result, fixed synoviocytes were used in co-cultures. In these conditions, the secretion of IL-6 and IL-1β were also induced. Nevertheless, the production was at least 50 % lower for IL-6, as synoviocytes were fixed and the level of IL-1β produced mostly by monocytes was slightly decreased (data not shown).

PBMC activation and cell interactions with synoviocytes are needed for a high IL-17 secretion

Th17 cells have been identified as a major source of IL-17 [19] and IL-6 and IL-1β, which are increased by cell interactions (Fig. 1) that are critical for Th17 differentiation [16–18]. The role of cell interactions on the IL-17 pathway was studied to distinguish the intracellular staining from the secretion of IL-17 in medium. Flow cytometry analysis showed that IL-17-positive cells were observed in culture of PBMC alone or in co-culture with synoviocytes. However, there was no difference in the percentage of IL-17-positive T cells in resting PBMC alone or cultured with synoviocytes (1.9 ± 1.7 % vs. 1.7 ± 1.6 %, respectively, Fig. 2a). The effect of PHA activation was not significant but with a tendency to higher Th17 cells with PHA (PBMC alone: 1.9 ± 1.7 % vs. 3.1 ± 1.7 %; co-culture: 1.7 ± 1.6 % vs. 3.9 ± 2.7 %, $p = 0.06$, Fig. 2a). In addition, PHA activation also increased the percentage of IL-17+ cells among the CD3 + CD4- in PBMC alone (1.1 ± 0.6 % vs. 1.8 ± 0.9 %, $p = 0.053$) and in co-culture (1.1 ± 0.7 % vs. 2.1 ± 0.7 %, $p = 0.01$); but there was no difference in the percentage between PBMC alone and co-culture (data not shown).

Actual IL-17 secretion in supernatants was measured by ELISA. Without PHA, IL-17 production was undetectable in resting PBMC (Fig. 2b); but it was present at a very low level in co-culture of PBMC and synoviocytes (1.1 ± 2.2 pg/ml). TCR activation by PHA did not increase significantly IL-17 secretion in PBMC alone (Fig. 2b). In contrast, there was a significant increased production of IL-17 in co-culture with activated PBMC (1.1 ± 2.2 pg/ml vs. 185.5 ± 220.3 pg/ml, $p = 0.002$, Fig. 2b). The activation of PBMC by anti-CD3 and anti-CD28 gave similar results as PHA activation (data not shown). These results demonstrated that the combination of TCR activation and cell–cell contact was required to obtain a high IL-17 secretion. Furthermore, TNF, which is a major cytokine involved in RA pathogenesis, is known to stimulate synoviocytes. Activation of synoviocytes by TNF was tested in co-culture without TCR stimulation, giving similar results than in the control condition (data not shown).

To confirm the crucial role of cell–cell contact in IL-17 production, the transwell system was used. This contact inhibition had no effect on Th17 differentiation, as the percentage of IL-17 positive cells was similar

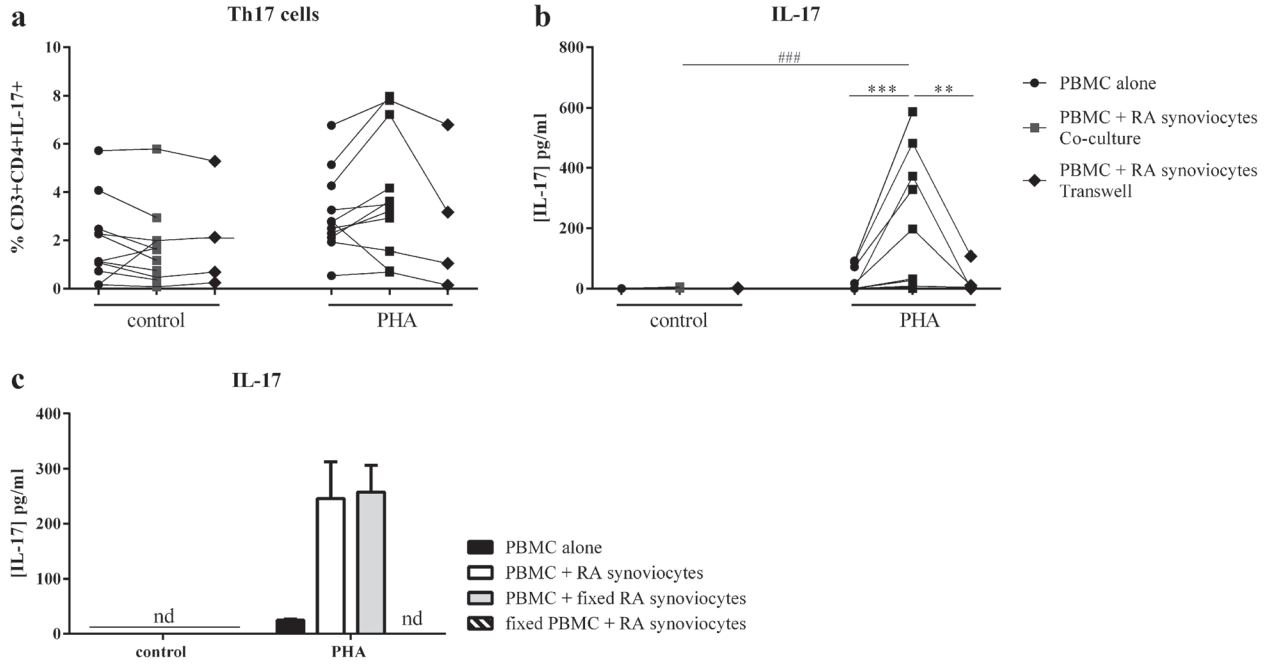

Fig. 2 Effect of interaction between synoviocytes and PBMC on Th17 differentiation and IL-17 secretion. Healthy PBMC were cultured alone or in co-culture with RA synoviocytes at a 5:1 ratio for 48 h, in the presence or absence of PHA (5 μg/ml). The transwell system was used in co-cultures to inhibit cell–cell contact and fixed synoviocytes or fixed PBMC were used. PBMC were recovered after 48 h and stained for the surface markers CD3 and CD4, and intracellular IL-17A. The percentage of CD3 + CD4 + IL-17A + is represented for each experiment (**a**). IL-17A production was measured in supernatants after 48 h, by ELISA (**b** and **c**). Each linked dot plot represents one experiment in the different conditions. *Compares the culture conditions (PBMC alone, co-culture or transwell) with and without PHA. #Compares the activation state (control or PHA) in the culture conditions. *#$p \leq 0.05$. *IL* interleukin, *PBMC* peripheral blood mononuclear cells, *PHA* phytohemagglutinin, *RA* rheumatoid arthritis

between co-culture and transwell system (2.1 ± 2.6 % vs. 2.1 ± 2.3 % without PHA; 4.3 ± 3.7 % vs. 2.8 ± 3.0 % with PHA, Fig. 2a). Conversely, without cell interactions in the PHA activation condition, IL-17 secretion was strongly reduced (265.0 ± 183.9 pg/ml vs. 30.5 ± 44.6 pg/ml, $p = 0.008$), reaching the same level as PBMC alone (30.5 ± 51.5 pg/ml vs. 26.3 ± 36.7 pg/ml, respectively, Fig. 2b). This transwell experiment clearly demonstrated that direct cell interactions between activated PBMC and RA synoviocytes were crucial for high levels of IL-17 secretion. Furthermore, using fixed synoviocytes with live activated PBMC induced IL-17 secretion at a similar level compared to co-culture with nonfixed synoviocytes. Conversely, fixed PBMC with live synoviocytes produced no IL-17 (Fig. 2c).

Co-culture between autologous cells also increases pro-inflammatory cytokine production

The cell specificity of IL-17 production induced by cell interactions between immune and mesenchymal cells was studied by comparing different mesenchymal cell types (synoviocytes and ASC) and endothelial cells (HUVEC) in co-culture. As shown in Fig. 3a, with both RA synoviocytes and ASC, interactions with PBMC induced high IL-6 production and a high IL-17 secretion in co-culture with activated PBMC (Fig. 3a). In contrast, co-culture with HUVEC did not induce IL-6 and IL-17 production (Fig. 3a). This indicated that specific interactions between fibroblast-like cells and immune cells are critical to induce high pro-inflammatory cytokine production.

To confirm that pro-inflammatory cytokine production resulting from cell interactions may occur inside the inflamed synovium, co-culture experiments with synoviocytes and PBMC from the same RA patient were tested. As observed in Fig. 3b, co-cultures with autologous cells gave similar results as co-cultures with RA synoviocytes and healthy PBMC. This indicated the absence of contribution of alloreactivity in the effect. Indeed, cell interactions were sufficient to induce IL-6 (Fig. 3b). IL-17 was markedly more produced in co-culture with autologous activated PBMC (Fig. 3b). In parallel, co-cultures between PBMC from patient 1 and synoviocytes from patient 2 and the other way around were tested. Results were similar in both systems (Fig. 3b) indicating the critical role of cell interactions in the pro-inflammatory cytokine production.

Monocytes do not contribute to the high IL-17 production

Considering the role of IL-6 and IL-1β in the Th17 pathway and the role of cell interactions in maintaining

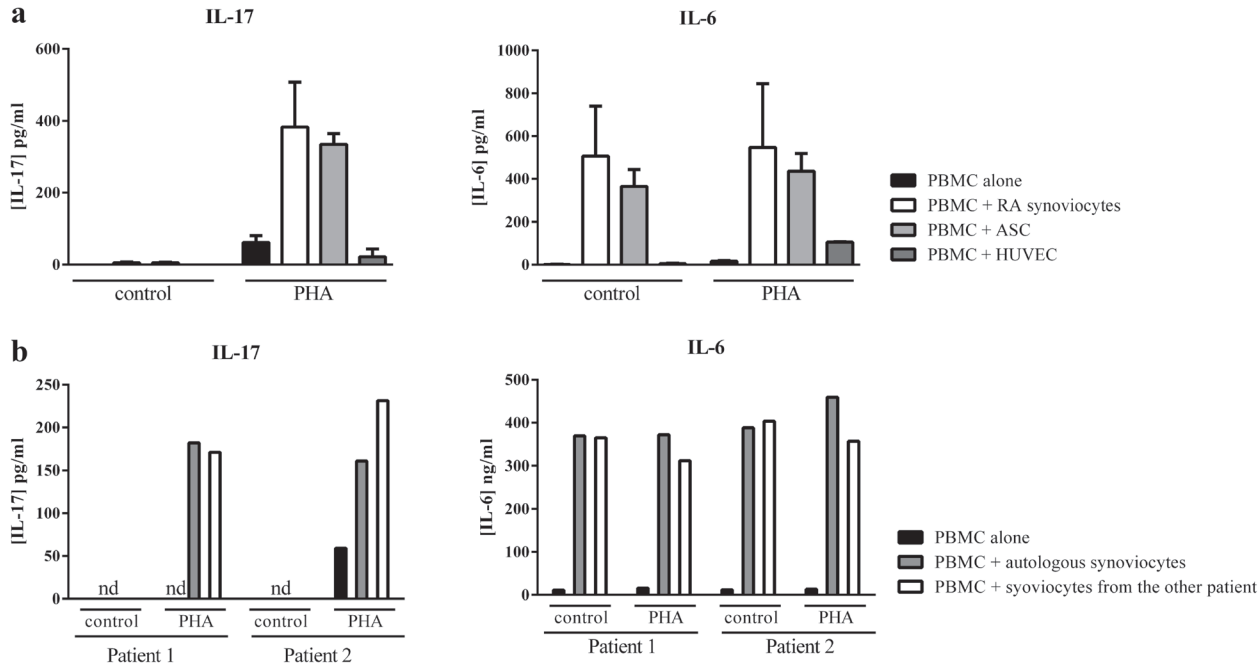

Fig. 3 Confirmation in an autologous system and with other mesenchymal cell types. Healthy PBMC were cultured alone or in co-culture with RA synoviocytes, ASC or HUVEC at a 5:1 ratio for 48 h, in the presence or absence of PHA (5 μg/ml). Levels of IL-17 and IL-6 were measured in the supernatants after 48 h by ELISA (**a**). PBMC from RA patients were cultured alone or in co-culture with autologous synoviocytes or from other patients, at the ratio 5:1 for 48 h, in the presence or absence of PHA (5 μg/ml). Levels of IL-17 and IL-6 were measured in the supernatants after 48 h by ELISA. Values are individual results (**b**). *ASC* adipose-derived stem cells, *HUVEC* human umbilical vein endothelial cells. *IL* interleukin, *PBMC* peripheral blood mononuclear cells, *PHA* phytohemagglutinin, *RA* rheumatoid arthritis

inflammation, the potential contribution of monocytes in this loop was investigated. To study their involvement in our co-culture system, monocytes were removed by adherence. As IL-1β is mainly produced by monocytes in PBMC, the reduction of IL-1β production can be considered as a good marker for the removal of monocytes. As observed in Fig. 4a, the production of IL-1β was indeed significantly inhibited in all conditions without monocytes ($p = 0.004$). In contrast, IL-6 is a pro-inflammatory cytokine produced by many cell types, including PBMC and synoviocytes. In control condition, IL-6 secretion was significantly decreased, but less than IL-1β, in culture of PBMC alone and in co-culture without monocytes (10.3 ± 8.0 ng/ml vs. 3.4 ± 2.5 pg/ml, $p = 0.003$; 532.1 ± 217.5 ng/ml vs. 431.7 ± 267.4 ng/ml, $p = 0.01$, respectively, Fig. 4b). With PHA, removal of monocytes had an effect only in PBMC alone (14.5 ± 6.1 ng/ml vs. 6.5 ± 2.3 ng/ml, $p = 0.003$, Fig. 4b). Surprisingly, IL-17 production was not affected by removing monocytes (Fig. 4c). These results showed that monocytes have no major role in the high IL-17 secretion during cell interactions, indicating the involvement of key interactions between lymphocytes and mesenchymal cells.

To confirm the crucial role of synoviocytes and Th17 cells in the high IL-17 secretion, co-cultures between synoviocytes and Th17 clones (ratio 1:1) were performed. As observed in Fig. 4d, there was no IL-1β production compared to co-cultures with PBMC. This result was expected as the major source of IL-1β was not present. In co-cultures with Th17 clones, IL-6 secretion was induced in control condition as with PBMC, even the level of production was lower than with PBMC (90.2 ± 10.0 pg/ml vs. 712.1 ± 12.5 pg/ml), and with Th17 clones, PHA activation increased IL-6 secretion (635.6 ± 12.5 pg/ml vs. 90.2 ± 10.0 pg/ml, Fig. 4e). As with PBMC, the detection of IL-17 production was possible only with PHA activation (701.7 ± 39.1 pg/ml vs. 15.2 ± 0.2 pg/ml, Fig. 4f) and the interaction with synoviocytes largely increased this secretion (7013.0 ± 458.5 pg/ml vs. 701.7 ± 39.1 pg/ml, Fig. 4f). These results confirmed the crucial role of TCR activation and of cell–cell contact in the high IL-17 production and make synoviocytes and Th17 cells the two major cell types involved in this elevated secretion.

Podoplanin plays a major role in high IL-17 secretion during co-culture between activated PBMC and RA synoviocytes

The role of direct physical cell interactions in the high IL-17 production is critical. As podoplanin (pdpn) can be

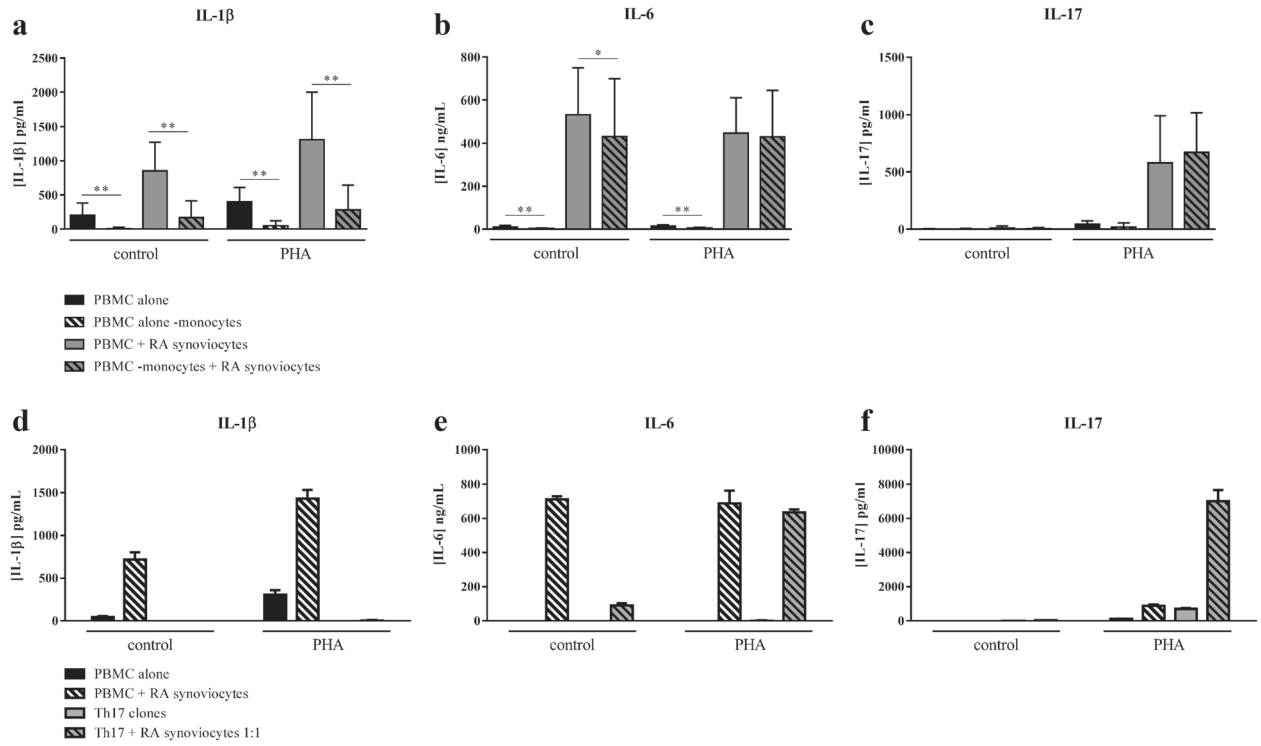

Fig. 4 Role of monocytes on pro-inflammatory cytokine production. Healthy PBMC or Th17 clones were cultured alone or in co-culture with RA synoviocytes at a 5:1 ratio or 1:1 ratio, respectively, for 48 h, in the presence or absence of PHA (5 μg/ml), and monocytes were removed or not by adherence before culture. Production of IL-1β (**a**, **d**), IL-6 (**b**, **e**) and IL-17 (**c**, **f**) were measured in supernatants after 48 h, by ELISA. *Compared conditions with and without monocytes. *$p \leq 0.05$. *IL* interleukin, *PBMC* peripheral blood mononuclear cells, *PHA* phytohemagglutinin, *RA* rheumatoid arthritis

expressed by different cell types, including synoviocytes, its potential role was studied with a blocking anti-pdpn antibody. A dose–response curve was performed with different concentrations of anti-pdpn antibody (Ab), 0, 1, 5, 10 and 20 μg/ml, to determine the optimum concentration of anti-pdpn Ab. The concentration of 5 μg/ml of antibody pre-incubated for 4 h gave the higher inhibition of cytokine production (data not shown). In the co-culture of synoviocytes and activated PBMC, the presence of anti-pdpn Ab inhibited significantly IL-17 secretion by 64.9 ± 24.0 %. ($p = 0.008$, Fig. 5a and c), IL-1β secretion (45.3 ± 25.5 % of inhibition, $p = 0.02$, Fig. 5c), but not IL-6 secretion (10.1 ± 9.0 % of inhibition, $p = 0.25$, Fig. 5c). Moreover, the effect of anti-pdpn antibody was specific as results with a control antibody were similar than without antibody (93.2 ± 31.4 pg/ml vs. 88.8 ± 29.9 pg/ml for IL-17, Fig. 5a, data not shown for IL-1β and IL-6).

The inhibition of pdpn was tested by siRNA specific for pdpn in synoviocytes. The presence of siPdpn inhibited the IL-17 production by around 30 % (74.6 ± 7.0 vs. 109.3 ± 10.5 pg/ml, Fig. 5a). This effect was specific for siPdpn as there was no inhibition of IL-17 secretion with the mock and with siPPIB (103.2 ± 5.9 pg/ml and 112.5 ± 15.6 pg/ml vs. 109.3 ± 10.5 pg/ml, respectively, data not shown). Furthermore, to verify the specificity of the siRNA, the gene expression of pdpn and PPIB was tested. In Fig. 5b, the expression of pdpn was inhibited only with the siPdpn, but not with the siPPIB (positive control) neither with the mock (negative control). The expression of PPIB was inhibited with the siPPIB but not with the siPdpn neither with the mock. This confirmed the specificity of the siPdpn.

Furthermore, with a therapeutic perspective in mind, anti-pdpn Ab was tested in an autologous system. As observed in Fig. 5d, the presence of anti-pdpn Ab decreased IL-17 (76.0 ± 26.0 % of inhibition) and IL-1β production (34.3 ± 26.2 % of inhibition), in a similar percentage as in the heterologous system. IL-6 secretion was not affected (Fig. 5d). These results in the autologous system supported the involvement of pdpn in the high IL-17 secretion during cell interactions as seen in vivo.

Pdpn has been shown to be expressed mainly by RA synoviocytes. To study the regulation of its expression during cell interactions, co-cultures were performed as described before and after 48 h, cells (synoviocytes and PBMC) were recovered and stained for pdpn. PBMC alone showed a very low percentage of pdpn + cells (1.0 ± 0.8 %

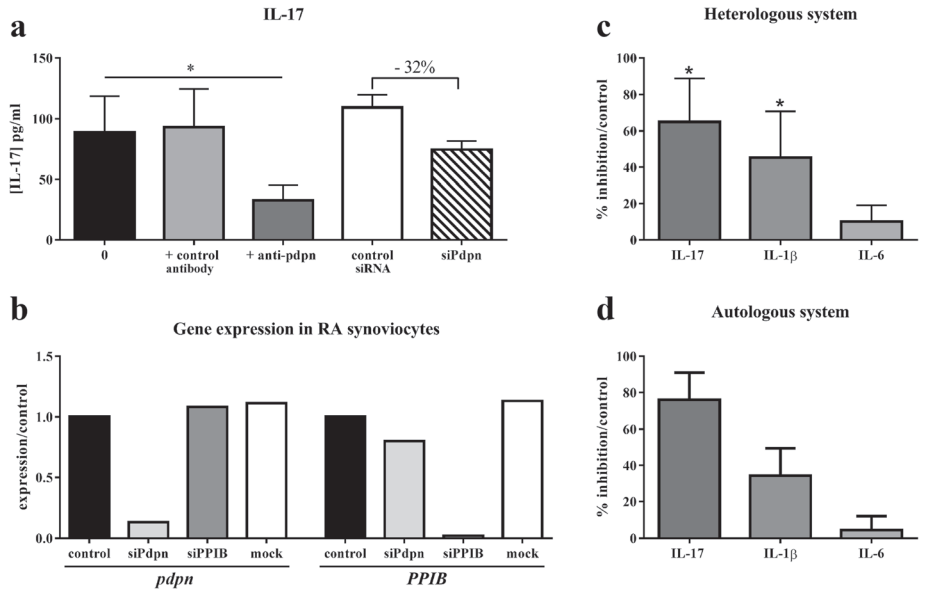

Fig. 5 The major role of podoplanin in the high IL-17 secretion. Healthy or RA PBMC were pre-incubated 4 h with or without human anti-pdpn or irrelevant antibody before co-culture with synoviocytes or autologous synoviocytes, respectively, for 48 h, in the presence of PHA (5 μg/ml). For siRNA, RA synoviocytes were transfected without (control) or with siRNA (pdpn, PPIB or mock) and then used in co-culture with healthy PBMC, in the presence of PHA, during 48 h. Levels of IL-17, IL-1β and IL-6 were measured in the supernatants after 48 h by ELISA. The concentration of IL-17 in the different conditions of culture is represented (**a**). The expression of the genes *pdpn* and *PPIB* compared to control (control = 1) (**b**) is represented as well as the percentage of cytokine inhibition compared to control in the heterologous system (**c**) and in the autologous system (**d**). *$p \leq 0.05$ compared to control. *IL* interleukin, *PBMC* peripheral blood mononuclear cells, *PHA* phytohemagglutinin, *RA* rheumatoid arthritis, *pdpn* podoplanin, *PPIB* peptidylpropyl isomerase B, *siRNA* small interfering RNA

without PHA; 0.8 ± 0.7 % with PHA, Fig. 6a and c). As synoviocytes expressed pdpn, the side scatter was focus more for PBMC. Interestingly, the percentage of pdpn + cells increased in co-culture (11.2 ± 6.2 % without PHA; 32.7 ± 8.1 % with PHA, $p = 0.04$, Fig. 6a and c). This increase was present in different populations, CD3- (9.2 ± 2.2 % without PHA; 35.9 ± 0.5 % with PHA); CD3 + CD4- (11.3 ± 3.7 % without PHA; 43.7 ± 7.2 % with PHA) and in CD3 + CD4+ (17.1 ± 5.1 % without PHA; 55.7 ± 1.5 % with PHA). Interestingly, in CD3 + CD4+, co-culture increased the pdpn expression in IL-17- (0.9 ± 0.6 % vs. 16.7 ± 5.0 % without PHA; 3.3 ± 3.4 % vs. 54.3 ± 0.9 with PHA, Fig. 6b) but this effect was major in IL-17+ cells (9.4 ± 1.1 % vs. 69.7 ± 7.6 % without PHA; 8.4 ± 8.3 % vs. 85.7 ± 2.8 % with PHA). These results indicated that pdpn expression could be regulated by cell–cell contact, with an effect mainly in Th17 cells. These results were also consistent with the increase of IL-17 secretion associated with overexpression of pdpn in both synoviocytes and activated PBMC. In addition, PBMC showed an increased size in co-culture, especially with PHA (data not shown), reflecting the high size with the plasma cell morphology observed previously in IL-17+ cells under activation and in in vivo [3].

Discussion

Cell interactions between mesenchymal and immune cells are known to induce the production of pro-inflammatory cytokines and also to affect the survival of both cell types [7, 20–22]. In the context of RA, a co-culture system between RA synoviocytes and PBMC was used to mimic the in vivo situation. Cell interactions were sufficient to provide a necessary activation state for the secretion of IL-6 and IL-1β and this is in agreement with a previous study showing that MSC-PBMC interactions increased IL-6 and IL-1β mRNA [7]. These observations reflect how high levels of pro-inflammatory cytokines, including IL-6 and IL-1β, can be produced locally by the RA synovium [23]. Furthermore, co-culture with autologous cells, PBMC and synoviocytes from the same patient, validated our co-culture in vitro model mimicking the in vivo inflammation.

IL-6 and IL-1β are known to be involved in Th17 cell differentiation [16–18]. Th17 cells in turn secrete IL-17 which acts on synoviocytes, often in synergy with other cytokines such as TNF-α, IL-1β or granulocyte-macrophage colony-stimulating factor (GM-CSF) [2, 24–26]. Considering the results on IL-6 and IL-1β production, the effect of cell interactions on the Th17 pathway was studied to differentiate the intracellular expression from the

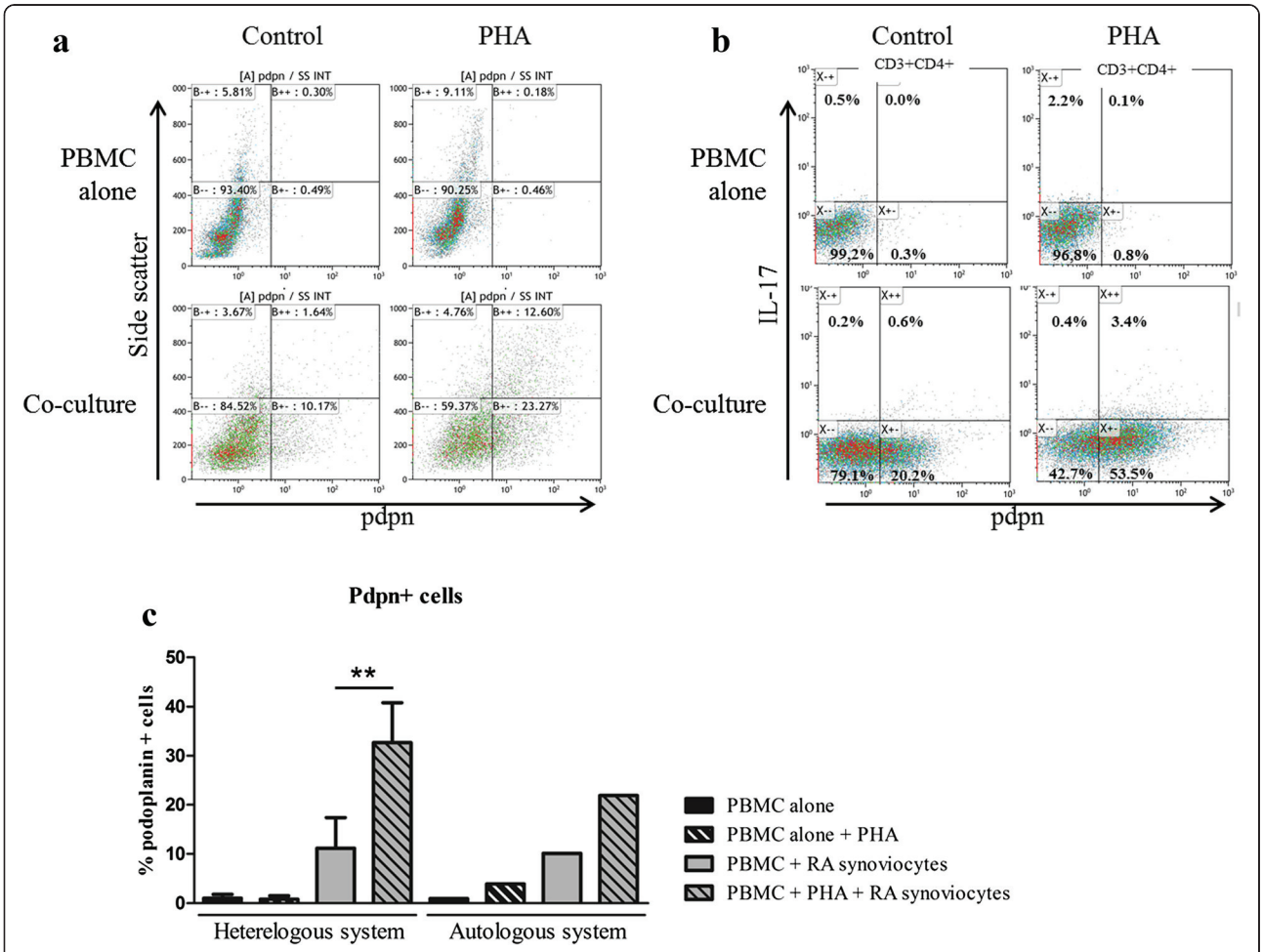

Fig. 6 Expression of podoplanin in PBMC alone or in co-culture. Healthy PBMC were cultured alone or in co-culture with RA synoviocytes at a 5:1 ratio for 48 h, in the presence or absence of PHA (5 μg/ml). Cells were recovered after 48 h and stained for pdpn (**a** and **c**) or CD3, CD4, IL-17 and pdpn (**b**). Dot plot of one experiment is represented (**a** and **b**). The percentage of pdpn + cells in the different conditions is represented (**c**). $^{*}p \leq 0.05$. *IL* interleukin, *PBMC* peripheral blood mononuclear cells, *PHA* phytohemagglutinin, *pdpn* podoplanin, *RA* rheumatoid arthritis

secretion of IL-17. The percentage of CD3 + CD4 + IL-17+ cells was evaluated in PBMC alone or in co-culture. Cell contact alone had no major effect on Th17 differentiation measured by intracellular staining. Only TCR activation had a modest effect. This indicated that Th17 differentiation requires cell activation more than cell–cell contact.

When looking at the production of IL-17 and in contrast to that of IL-6 and IL-1β, cell interactions were not sufficient to induce a high IL-17 secretion. Its production required two signals, TCR activation and cell–cell contact. Moreover, activation of synoviocytes by TNF alone in co-culture without TCR stimulation had no effect on IL-17 production. In fact, IL-17 secretion during cell interactions was dependent on T cell but not synoviocyte activation. Transwell experiments confirmed that cell interactions were crucial to have an elevated IL-17 secretion even in the presence of TCR activation.

These results reveal a major discrepancy between intracellular and secreted IL-17. The intracellular presence of IL-17 inside Th17 cells does not mean by itself a high secretion of IL-17. The presence of Th17 cells even with TCR activation was not enough to have the release of high levels of IL-17. It was necessary that activated cells could physically interact with mesenchymal cells, derived from either synovium, bone marrow, or adipose tissue. Thus, TCR activation and cell–cell contact are two needed mechanisms leading to a high IL-17 production and this differs from intracellular expression of IL-17. Both mechanisms are present during RA pathogenesis [13, 27–30], and this could explain the presence of IL-17 in the joints of RA patients [31, 32].

Furthermore, the secretion of IL-17 was variable depending on experiments which used different donors for PBMC and RA synoviocytes. This variability reflected the heterogeneity which characterizes the RA population

suggesting the variable contribution of IL-17 in the inflammatory process. The variability in IL-17 secretion observed in our experiments could explain in part the heterogeneity of the response to an anti-IL-17 treatment in RA patients [33, 34]. Such heterogeneity remains to be explained. One explanation could be the contribution of gene polymorphisms in the regulation of the Th17 pathway. *IL4R* gene polymorphisms have been associated with RA severity by increasing the Th17 cell frequency and by modulating the inhibitory effect of IL-4 on Th17 development [35] and the modulation of IL-17 production [36]. *IL-23R* polymorphisms have been implicated in IL-17A expression in RA [37].

The critical contribution of interactions between immune cells and mesenchymal cells indicated the need to identify a molecular mechanism. The limited contribution of monocytes suggested a molecule present on lymphocytes or on mesenchymal cells or on both. Pdpn, which is a type I transmembrane protein, appeared as a good candidate. Pdpn-mediated interaction of RA synoviocytes and platelets modulates IL-8 production [8]. Furthermore, in the SKG spontaneously occurring arthritis model, pdpn is upregulated in Th17 cells compared to other Th cell subsets [9] and in the synovium of RA patients [11]. In a mouse model of multiple sclerosis, mice treated with anti-pdpn present a delayed onset of symptoms [10]. Based on these observations, an antibody against pdpn was used in the co-culture system and siRNA specific for pdpn was used on synoviocytes. Both means induced an inhibition of IL-17 production and confirmed the role of pdpn in the IL-17 secretion.

These results focused on the podoplanin expressed by RA synoviocytes but it was known that Th17 cells could express pdpn, notably in an experimental arthritis model and in clinical RA [38, 39]. In accordance with this, the three different tested protocols, the pre-incubation of PBMC, the pre-incubation of synoviocytes or the pre-incubation of both cells, gave similar results (data not shown). Acting first on synoviocytes or PBMC did not affect the inhibitory effect of the anti-pdpn. This is in line with the expression of pdpn by Th17 cells and the fact that the effect of anti-pdpn could be both direct on Th17 cells and indirect by acting on synoviocytes to inhibit the IL-17 production during cell interactions. In addition, the lower percentage of inhibition obtained with siPdpn compared with the anti-pdpn Ab could also indicate the respective involvement of pdpn expressed by synoviocytes and by Th17 cells. The regulation of pdpn in PBMC and specifically in Th17 cells remains to be clarified.

The interaction between pdpn and its receptor could occur in the two directions, from synoviocytes to PBMC, or from PBMC to synoviocytes. The receptor of pdpn CLEC-2 is known to be mainly expressed by platelets [40] and also by mature dendritic cells or peripheral blood B lymphocytes [41–44]. Its expression in our co-culture system could be studied to provide a new insight on the pathway by which pdpn could influence the IL-17 secretion. Currently, there is no evidence for its expression on Th17 cells and this could also suggest a possible new receptor for pdpn. A recent study has shown that pdpn can negatively regulate CD4+ effector T cell functions through pdpn-CLEC-2 interaction [45]. A high pdpn expression was found on "nonpathogenic" Th17 lymphocytes while "pathogenic" Th17 cells expressed less pdpn. Thus, pdpn displays two divergent functions which may depend on different ligands. One ligand, such as CLEC-2 could mediate T cell inhibition while another ligand could promote inflammation by stimulating pro-inflammatory cytokine production. Furthermore, our results demonstrated that the inhibition of the interaction mediated by pdpn decreased at least by 50 % the secretion of IL-17 and of IL-1β, but not that of IL-6. Furthermore, in both PBMC and synoviocytes, pdpn expression was increased in co-culture with TCR activation which correlates with the high IL-17 production. These results suggested that cell interactions of synoviocytes with activated immune cells increased pdpn expression that contributed to the high IL-17 secretion.

If podoplanin seems to be a good potential therapeutic target, investigating the effect of cell interactions on other signaling molecules involved in Th17 differentiation and function could be interesting. Indeed, the interaction with mesenchymal stem cells (MSC) could on the one hand enhance Th17 activity [7, 46] but on the other hand it could repress Th17 molecular program through PD-1 [47]. Furthermore, IL-17A can induce soluble PD-1 (sPD-1) which level is increased in RA serum. This overexpression of sPD-1 might block the inhibitory PD-1 pathway [48]. Investigating the PD-1 pathway in co-culture system could allow identifying another mechanism. Signaling lymphocytic activation molecule (SLAM) is another candidate as it promotes the differentiation of IL-17-secreting effectors [49]. Inducible T cell costimulator (ICOS) signaling, which belongs to the CD28 costimulatory molecule superfamily, has been also shown to play a critical role in the generation and maintenance of human Th17 cells [50] and it could be another candidate.

Conclusions

This study showed that cell interactions between fibroblast-like mesenchymal cells and immune cells play a major role in pro-inflammatory cytokine production, leading to a major increase in IL-17 secretion distinct from intracellular expression. The interaction molecule podoplanin appears to have a large contribution to the high IL-17 secretion that in turn may contribute to the

chronicity of inflammation. In this context, pdpn could be a potential therapeutic target to block Th17 cell activity during chronic inflammation.

Abbreviations

ASC, adipose-derived stem cells; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; IFN, interferon; IL, interleukin; mesenchymal stem cells, MSC; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; pdpn, podoplanin; PPIB, peptidylpropyl isomerase B; RA, rheumatoid arthritis; siRNA, small interfering RNA; TCR, T cell receptor; TNF, tumor necrosis factor

Acknowledgements

The authors acknowledge Dr. Odile Damour, Laboratoire des substituts cutanés, Hôpital Edouard Herriot, for providing ASC and Pr. Francesco Annunziato, Department of Experimental and Clinical Medicine, University of Florence, for providing Th17 clones.

Funding

Mélissa Noack is supported by the Institut Universitaire de France. Ndiémé Ndongo-Thiam is supported by the IHU prometteur OPERA. Pierre Miossec is a senior member of and supported by the Institut Universitaire de France. These studies were supported in part by the IHU prometteur OPERA.

Availability of supporting data

The dataset supporting the conclusions of this manuscript is included within the manuscript.

Authors' contributions

MN carried out the experiments and drafted the manuscript. NNT participated in the experiments and helped to revise the manuscript. PM conceived the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors' information

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethical approval and consent to participate

Each individual signed an informed consent form. The protocol was approved by a committee of the hospitals of Lyon for the protection of persons participating in biomedical research.

Received: 20 April 2016 Accepted: 9 June 2016
Published online: 23 June 2016

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II Le psoriasis

II.1 Etiologie

D'autres maladies inflammatoires chroniques telles que le psoriasis (Pso) partagent des caractéristiques communes avec la PR [47]. Le Pso est une maladie inflammatoire chronique de la peau, affectant 2-3% de la population mondiale [89]. La forme la plus répandue et la plus étudiée, le psoriasis vulgaris, affecte 85 à 90% des patients psoriasiques [90]. Comme la PR, le Pso est une maladie multifactorielle dont l'étiologie reste mal définie. Des facteurs génétiques, environnementaux, une dysfonction de la barrière cutanée et immunologique sont autant de facteurs mis en jeu dans le développement du Pso [90, 91]. Au niveau génétique, au moins neuf loci chromosomiques ont été identifiés en lien avec le Pso, nommés «psoriasis susceptibility » 1 à 9 (PSOR1 à PSOR9) [92]. PSOR1 est le déterminant génétique le plus fort, représentant 30-35% de l'héritabilité de la maladie [93]. Les facteurs environnementaux sont également associés au Pso ainsi qu'à l'aggravation de cette pathologie. Dès 1872, il a été décrit qu'un traumatisme physique pouvait déclencher ou exacerber la maladie [94]. Certains médicaments comme les bétabloquants ou certaines infections telles que *Streptococcus* contribuent aussi au déclenchement du Pso. L'obésité et le stress se présentent également comme des facteurs de risque [91]. Un autre facteur important à prendre en compte dans la pathologie du Pso est le microbiote de la peau. La peau agit comme une barrière et est donc en contact direct avec l'environnement extérieur. Elle est ainsi colonisée par différents microorganismes tels que les bactéries, les champignons ou les virus [95, 96]. En condition saine, une relation symbiotique est préservée afin de protéger la peau d'une invasion de microorganismes pathogènes. Lors du Pso, une modification de ce microbiote est observée. Ainsi, au niveau des lésions psoriasiques, une augmentation des bactéries *S. pyogenes* et *S. aureus* est observable. Une modification de la composition fungique est également présente, avec notamment *Malassezia* qui représente un possible

facteur déclenchant du Pso. La modification du microbiote semble donc un facteur non négligeable dans le déclenchement et l'exacerbation de la maladie [91].

II.2 Physiopathologie

Le Pso se manifeste par l'apparition d'épaisses plaques de peau, bien définies, qui vont se détacher sous formes d'écailles blanches. Ces plaques sont le résultat d'une hyperprolifération de l'épiderme, ce qui provoque les squames. De plus, tout comme dans la PR, une infiltration de cellules immunitaires se produit dans le derme (DC, macrophages et LT) et dans l'épiderme (neutrophiles et quelques LT). Deux modèles sont actuellement proposés pour définir la physiopathologie du psoriasis. Le premier modèle propose une anomalie dans les kératinocytes, libérant des facteurs chimio-attractants pour les cellules immunitaires. Le second modèle suggère que l'infiltrat immunitaire est responsable de l'hyperprolifération des kératinocytes. Dans les deux cas, le microenvironnement et les interactions cellulaires sur le site inflammatoire jouent un rôle majeur dans le développement de la maladie. En effet, la sécrétion de chimiokines dans le microenvironnement, telles que l'IL-8 ou CCL20 va permettre le recrutement des cellules immunitaires comme les neutrophiles ou les lymphocytes, respectivement. De plus, le transport des LT du derme à l'épiderme se fait grâce à l'interaction de l'intégrine $\alpha 1\beta 1$ exprimée par les LT avec le collagène de type IV de la membrane basale de l'épiderme. Bloquer cette interaction inhibe le développement du Pso ce qui indique un rôle clé de ces interactions cellulaires [97].

Parmi les cellules de l'infiltrat immunitaire, comme dans la PR, les LTh17 vont être présents. En effet, chez les patients atteints de Pso, la fréquence des LTh17 dans le sang périphérique et dans les lésions psoriasiques et la sécrétion d'IL-17 au niveau du site inflammatoire sont augmentées [98-101]. Les LTh17 vont alors interagir aux niveaux moléculaire et cellulaire avec les cellules résidentes de la peau, comme les fibroblastes. Les

fibroblastes sont capables de soutenir l'expansion des cellules sécrétrices d'IL-17, via une augmentation de la production d'IL-23 par les DC [102]. De plus, les fibroblastes stimulés par le TNF vont sécréter un facteur de croissance de kératinocytes (KGF : Keratinocyte Growth Factor) et ainsi favoriser leur hyperprolifération. À leur tour, les kératinocytes vont sécréter de l'IL-23, favorisant l'expansion des LTh17 qui vont induire la sécrétion d'IL-8 par les kératinocytes et permettre le recrutement de neutrophiles sur le site inflammatoire. La voie IL-23/LTh17 joue donc un rôle primordial dans la physiopathologie du Pso [103]. Les LTh17 infiltrants vont également sécréter de l'IL-17 qui va activer les fibroblastes et les kératinocytes, qui en retour vont sécréter des cytokines inflammatoires (IL-6, TNF) ou des chimiokines (CCL8, CCL20).

Une autre cytokine sécrétée par les LTh17 va jouer un rôle important dans la pathologie du Pso : l'IL-22. En effet, l'IL-22 est capable d'induire la prolifération des kératinocytes [104] et d'agir en synergie avec l'IL-17 pour augmenter l'expression de peptides antimicrobiens par les kératinocytes [10, 105] et également la sécrétion d'IL-8 par les cellules épithéliales [105-107]. De plus, le niveau d'IL-22, que ce soit en ARNm ou en protéine, est augmenté dans la peau et le sang des patients atteints de Pso [108, 109].

Les interactions cellulaires et moléculaires, impliquant notamment les LTh17, jouent un rôle primordial dans la physiopathologie du Pso et présentent des mécanismes communs avec la PR. Le psoriasis peut être utilisé comme un modèle de l'inflammation chronique, la facilité d'obtention des cellules de la peau et ses caractéristiques inflammatoires communes à la PR, en font un bon outil pour étudier le rôle des cellules stromales dans les maladies inflammatoires chroniques, et notamment dans la PR.

II.3 Interactions cellulaires et psoriasis

Article: "Role of podoplanin in the high IL-17A secretion resulting from interactions between activated lymphocytes and psoriatic skin derived mesenchymal cells"

Rôle de la podoplanine dans la forte production d'IL-17 résultant des interactions entre lymphocytes activés et cellules mésenchymateuses dérivées de peau psoriasique

Résumé:

Introduction : Dans le contexte du Pso, l'infiltrat immunitaire présent sur le site inflammatoire, la peau, contient notamment des LTh17 qui vont interagir avec les CSM locales, incluant les fibroblastes de peau. L'objectif de ce travail est donc d'étudier les interactions cellulaires entre les fibroblastes de peau et PBMC, en se concentrant sur la voie LTh17 et d'identifier un mécanisme impliqué dans la forte sécrétion d'IL-17.

Méthodes : Un système de co-culture entre PBMC et fibroblastes de peau a été développé. Des PBMC de donneurs sains ou de patients atteints de Pso ont été ajoutées sur des fibroblastes issus de peau saine ou lésée de patients Pso, avec un ratio 5 :1, pendant 48h, en présence ou non d'activation par PHA. Le rôle des monocytes a été étudié en les enlevant ou non par adhérence avant la co-culture. Un anticorps anti-podoplanine a été utilisé pendant les co-cultures. Après 48h de culture, la production de différentes cytokines (IL-6, IL-8, IL-1β et IL-17) a été mesurée par ELISA dans les surnageants et les cellules ont été marquées (CD3, CD4, IL-17 et podoplanine) et analysées par cytométrie en flux.

Résultats : Sans activation du TCR, la production d'IL-8, d'IL-6 et d'IL-1β augmentait dans les co-cultures PBMC-fibroblastes comparée aux PBMC seules. Aucun effet additionnel n'était observé avec l'activation du TCR. De plus, une tendance à une plus forte production, principalement pour l'IL-6, était observée avec les fibroblastes de peau lésée. L'analyse par cytométrie en flux ne montrait aucune différence dans le pourcentage de LTh17 dans les PBMC activées seules ou en co-culture avec les fibroblastes. En revanche, la production d'IL-17 était très fortement augmentée seulement dans les co-cultures de PBMC activées et fibroblastes de peau. Le rôle des monocytes dans ces interactions cellulaires a été étudié par leur retrait des PBMC par adhérence, cette méthode permettant ainsi d'ôter au moins 50% des monocytes. Le retrait des monocytes avant les co-cultures diminuait fortement la sécrétion de toutes les cytokines mesurées, notamment de l'IL-17. Le rôle de la podoplanine a ensuite été testé par l'addition d'un anticorps anti-podoplanine dans les co-cultures. La présence de cet anticorps réduisait également la production d'IL-17 de 60% en moyenne. De plus, le pourcentage de cellules positives pour la podoplanine augmentait nettement dans les co-cultures en présence de PHA. Ceci corrélait parfaitement avec la sécrétion massive d'IL-17 et confortait l'idée de l'implication de la podoplanine dans ce mécanisme.

Conclusion : Les interactions entre PBMC et fibroblastes de peau induisent la sécrétion des cytokines pro-inflammatoires telles que l'IL-8, l'IL-6 et l'IL-1β. L'activation des PBMC et l'interaction cellulaire sont primordiales dans la forte production d'IL-17, qui ne reflète pas l'expression intracellulaire. Les monocytes semblent jouer un rôle dans ce mécanisme, potentiellement par l'expression de la podoplanine.

Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12830

Role of podoplanin in the high interleukin-17A secretion resulting from interactions between activated lymphocytes and psoriatic skin-derived mesenchymal cells

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Summary

In the context of psoriasis, T helper type 17 (Th17) cells infiltrate the inflammatory site and interact with local mesenchymal cells, including skin fibroblasts. The aim of this work was to study the interactions of skin-derived fibroblasts with peripheral blood mononuclear cells (PBMC) with a focus on the Th17 pathway and to identify a mechanism which leads to a high interleukin (IL)−17 secretion. A co-culture system between PBMC and skin fibroblasts was developed. Healthy and patient PBMC were added to non-lesional or lesional skin fibroblasts at a 5:1 ratio for 48 h in the presence or not of activation with phytohaemagglutinin (PHA). Monocytes were removed or not by adherence before the co-culture. An anti-podoplanin antibody was also used during the co-culture. Cytokine production (IL-8, IL-6, IL-1β and IL-17) was measured by enzyme-linked immunosorbent assay (ELISA) and cell staining (CD3, CD4, IL-17 and podoplanin) by flow cytometry. Without T cell receptor (TCR) activation, IL-8, IL-6 and IL-1β production increased in PBMC-fibroblast co-culture compared to PBMC alone. No additional effect was observed with TCR activation, with no difference in the Th17 cell percentage in activated-PBMC alone or co-cultured. Conversely, IL-17 production was increased highly only in co-cultures between control and patient activated-PBMC and skin fibroblasts. Removal of monocytes decreased cytokine production, notably that of IL-17. Addition of an anti-podoplanin antibody decreased IL-17 secretion by 60%. Interactions between resting PBMC and fibroblasts induce the IL-8, IL-6 and IL-1β production. PBMC activation and cell interactions are critical for a high IL-17 secretion. Podoplanin contributes largely to this massive IL-17 secretion.

Keywords: cell interactions, IL-17, immune cells, podoplanin, psoriasis, skin fibroblasts

Accepted for publication 16 June 2016
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Introduction

Psoriasis is a chronic inflammatory disease characterized by a hyperproliferation of keratinocytes and an infiltrate of immune cells [1,2]. Although the pathogenesis is not yet totally understood, recent studies highlighted the involvement of the T helper type 17 (Th17) pathway in disease development and presentation [3,4]. Interleukin (IL)-17, the major proinflammatory cytokine produced by Th17 cells, plays a key role in chronic inflammation [5–7]. In psoriasis, Th17 frequency is increased in peripheral blood and in psoriatic lesions and IL-17 secretion is up-regulated at inflammatory sites [8–11]. The major role of IL-17 in psoriasis pathogenesis has been confirmed by the impressive clinical improvement seen in patients undergoing treatment with IL-17 inhibitors [12,13].

The recruitment of activated immune cells, including Th17 cells, to the disease site leads to interactions with local cells. This promotes the proliferation of mesenchymal cells such as fibroblasts in the skin or synoviocytes in the synovium, leading to the chronicity of inflammation [2,14]. In the context of psoriasis, interaction molecules are crucial for the disease development [15]. In another chronic inflammatory context, rheumatoid arthritis (RA), cell

interactions between synovium-derived mesenchymal cells and activated peripheral blood mononuclear cells (PBMC) promote IL-17 secretion [16].

Among the immune cells, monocytes could play a key role as they secrete cytokines (IL-6 and IL-1β) involved in Th17 differentiation [17–19] and are activated in psoriatic patients [20]. Furthermore, monocytes can express an interaction molecule, podoplanin (pdpn). Although its physiological functions are still poorly documented, pdpn is known to play a crucial role in the development of the lymphatic system, in cell motility and in epithelial–mesenchymal transition [21,22]. This transmembrane protein is also expressed in hyperproliferative psoriatic epidermis [23,24] and modulates IL-8 secretion during platelet–synoviocyte interactions in RA [25]. This suggests that pdpn could be involved in the high level of proinflammatory cytokine secretion, specifically of IL-17, during cell interactions.

Here, the aim was to study the role and the mechanism of the interactions between psoriatic skin fibroblasts and immune cells on the Th17 pathway, reproducing the early step of cell contact *in situ*. For this we used an *in-vitro* model with activated immune cells in contact with skin fibroblasts from psoriasis patients to study the effect of blocking of cell interactions seen in psoriasis skin.

Materials and methods

Samples

Skin fibroblasts were obtained from skin biopsies of psoriatic patients who fulfilled the Classification Criteria for Psoriasis or Psoriatic Arthritis. Punch biopsies of 4 or 5 mm were performed after local anaesthesia with xylocaine 1%. Biopsies of lesional skin defined by a clinical inflammatory aspect and non-lesional skin by a clinical normal aspect and 2 cm away from an active plaque were obtained from the same patient. The biopsies were minced and adhered in six-well plates in Dulbecco's modified Eagle's medium (DMEM; Eurobio, Courtaboeuf, France) supplemented with 10% fetal bovine serum (heat-inactivated FBS; Life Technologies, Carlsbad, CA, USA), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 incubator and used between passages 4 and 9. PBMC from healthy donors or from patients were isolated by Ficoll-Hypaque (Eurobio) density-gradient centrifugation. Each individual signed an informed consent form. The protocol was approved by the committee for the protection of people participating in biomedical research under the number AC-2010-11-64.

Co-culture assays

Co-culture was initiated by seeding fibroblasts overnight in 96-well plates at a density of $2{\cdot}10^4$ cells/well in RPMI-1640 medium (Eurobio) supplemented with 10% human AB serum (Etablissement Français du Sang, La Plaine Saint-Denis, France), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (complete RPMI). The next day, PBMC ($1{\cdot}10^5$ cells/well) were seeded in complete RPMI at a ratio of 5 : 1, in the presence or absence of phytohaemagglutinin (PHA, 5 μg/ml) or antibodies against CD3 and CD28 (anti-CD3/28, 5 μg/ml). After 48 h, supernatants and PBMC were collected for analysis.

Transwell assay

Fibroblasts were seeded in 24-well plates at a density of $1{\cdot}10^5$ cells/well in complete RPMI. After overnight incubation, PBMC were added directly on top of fibroblasts or in a cell culture insert (0·4 μm pore size) at a concentration of $5{\cdot}10^5$ cells/well in the presence or absence of PHA (5 μg/ml). After 48 h, supernatants and PBMC were recovered for analysis.

Enzyme-linked immunosorbent assays (ELISA)

IL-17A, IL-8, IL-6 and IL-1β productions were evaluated from culture supernatants with commercially available Duoset ELISA kits (DY317, DY208, DY206 and DY201 catalogue numbers, respectively), according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Flow cytometry

Allophycocyanin (APC), fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cyanin-7 or eFluor 450-conjugated antibodies (eBiosciences, San Diego, CA, USA) were used to stain cells. EFluor 450-CD3 (48-0038), PE-Cy7-CD4 (25-0049) and PE-pdpn (12-9381) were used for surface staining, according to the manufacturer's instructions. PBMC were fixed and permeabilized for APC-IL-17A (17-7179) intracellular staining. Cells were incubated in cold phosphate-buffered saline (PBS) and 2% paraformaldehyde for 1 h in the dark for fixation step. Cells were permeabilized in PBS with 0.2% Tween at 37°C for 15 min before intracellular staining. Analysis was performed with KALUZA software.

Monocyte contribution

One of the simplest and most common ways to isolate monocytes is by adherence [26,27]. To remove part of the monocytes, PBMC were preincubated 2 h at 37°C. This method allowed the removal at least 50% of the monocytes, as defined by CD14 staining and IL-1β production.

Inhibition of pdpn

A dose–response curve was performed to determine the optimum concentration of anti-human pdpn (14-9381; eBiosciences, San Diego, CA, USA). PBMC were preincubated for 4 h with different concentrations (0, 1, 5, 10 and 20 μg/ml) before co-culture assay. According to the highest inhibition of cytokine production (data not shown), all experiments studying pdpn were performed with 5 μg/ml.

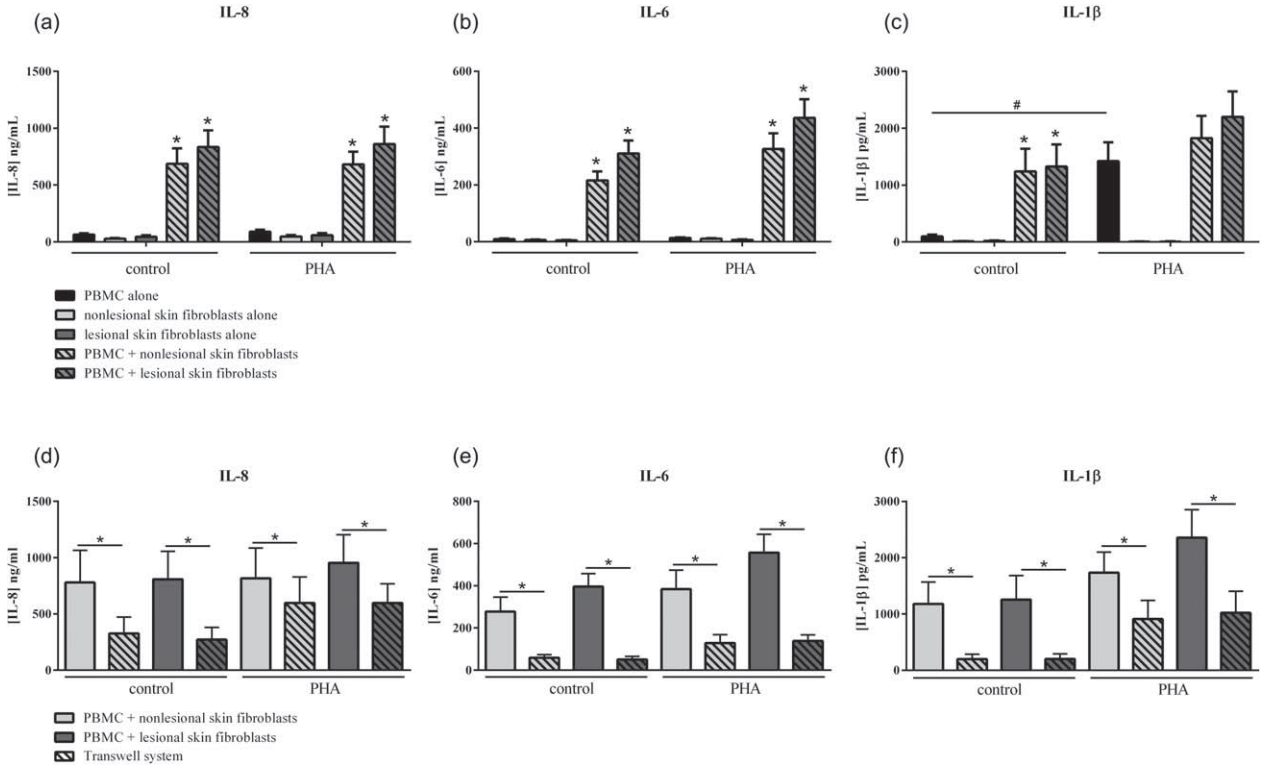

Fig. 1. Effect of interactions between skin fibroblasts and peripheral blood mononuclear cells (PBMC) on interleukin (IL)−8, IL-6 and IL-1β production. Healthy PBMC and psoriatic skin fibroblasts were cultured alone or in co-culture at a ratio of 5 : 1 for 48 h in the presence or absence of phytohaemagglutinin (PHA) (5 μg/ml). The Transwell system was used to inhibit cell–cell contact. The production of IL-8 (a,d), IL-6 (b,e) and IL-1β (c,f) in cell supernatants was measured by enzyme-linked immunosorbent assay (ELISA). *Compares PBMC alone and co-cultures (a,b,c); #compares control and PHA (a,b,c); *compares co-cultures and Transwell system (d,e,f); *#, $P \leq 0{\cdot}05$. Results are represented as mean ± standard error of the mean (s.e.m.), $n = 15$ experiments from five psoriatic patients (skin fibroblasts) and three healthy donors (PBMC) (a,b,c) and $n = 9$ experiments for Transwell experiments (d,e,f).

A monoclonal antibody directed against the BetV1 allergen and not involved in the cell interactions was used as control antibody at the same concentration (anti-BetV1 Ab; Dendritics, Lyon, France).

Statistical analysis

Statistical analyses for co-culture assays were performed using two-way analysis of variance (ANOVA). For Transwell, monocytes and pdpn experiments a paired non-parametric Wilcoxon test was used. For pdpn staining, an unpaired Mann–Whitney test was used. All analyses were performed with Graph Pad Prism version 6 software. *P*-values less than or equal to 0·05 were considered significant.

Results

Interactions between skin fibroblasts and PBMC induce IL-8, IL-6 and IL-1β production

PBMC and skin fibroblasts produce proinflammatory chemokines and cytokines such as IL-8, a mediator involved in the migration of neutrophils in psoriasis, and IL-6 and IL-1β, both involved in Th17 differentiation. In culture of psoriatic skin fibroblasts alone, either non-lesional or lesional, the production of IL-8, IL-6 and IL-β was low and PHA activation had no effect (Fig. 1a–c). In contrast, skin fibroblasts were fully responsive to IL-17, as the production of IL-8 and IL-6 was increased with IL-17 *versus* control (non-lesional skin fibroblasts: $4{\cdot}5 \pm 1{\cdot}4$ *versus* $6{\cdot}3 \pm 2{\cdot}2$ ng/ml for IL-6 and $27{\cdot}0 \pm 6{\cdot}1$ *versus* $48{\cdot}3 \pm 9{\cdot}4$ ng/ml for IL-8; lesional skin fibroblasts: $5{\cdot}2 \pm 1{\cdot}4$ *versus* $12{\cdot}9 \pm 5{\cdot}1$ ng/ml for IL-6 and $40{\cdot}4 \pm 9{\cdot}4$ *versus* $81{\cdot}9 \pm 24{\cdot}8$ ng/ml for IL-8). Similar results for IL-8 and IL-6 were found in culture of PBMC alone, with or without PHA (Fig. 1a,b). In PBMC alone, IL-1β production was detectable in the control condition (i.e. without PHA activation) ($98{\cdot}6 \pm 96{\cdot}3$ pg/ml), but PHA activation highly increased its secretion ($1422{\cdot}8 \pm 789{\cdot}8$ pg/ml, $P = 0{\cdot}03$, Fig. 1c). IL-8, IL-6 and IL-1β production was increased significantly in the control condition in PBMC-non-lesional skin fibroblast co-culture *versus* PBMC alone ($688{\cdot}1 \pm 390{\cdot}9$ ng/ml *versus* $64{\cdot}3 \pm 39{\cdot}6$ ng/ml, $P \leq 0{\cdot}001$; $216{\cdot}1 \pm 94{\cdot}9$ ng/ml *versus* $9{\cdot}3 \pm 7{\cdot}8$ ng/ml, $P \leq 0{\cdot}002$; and $1241{\cdot}2 \pm 1166{\cdot}5$ pg/ml *versus* $98{\cdot}6 \pm 96{\cdot}3$ pg/ml, $P \leq 0{\cdot}05$, respectively, Fig. 1a–c);

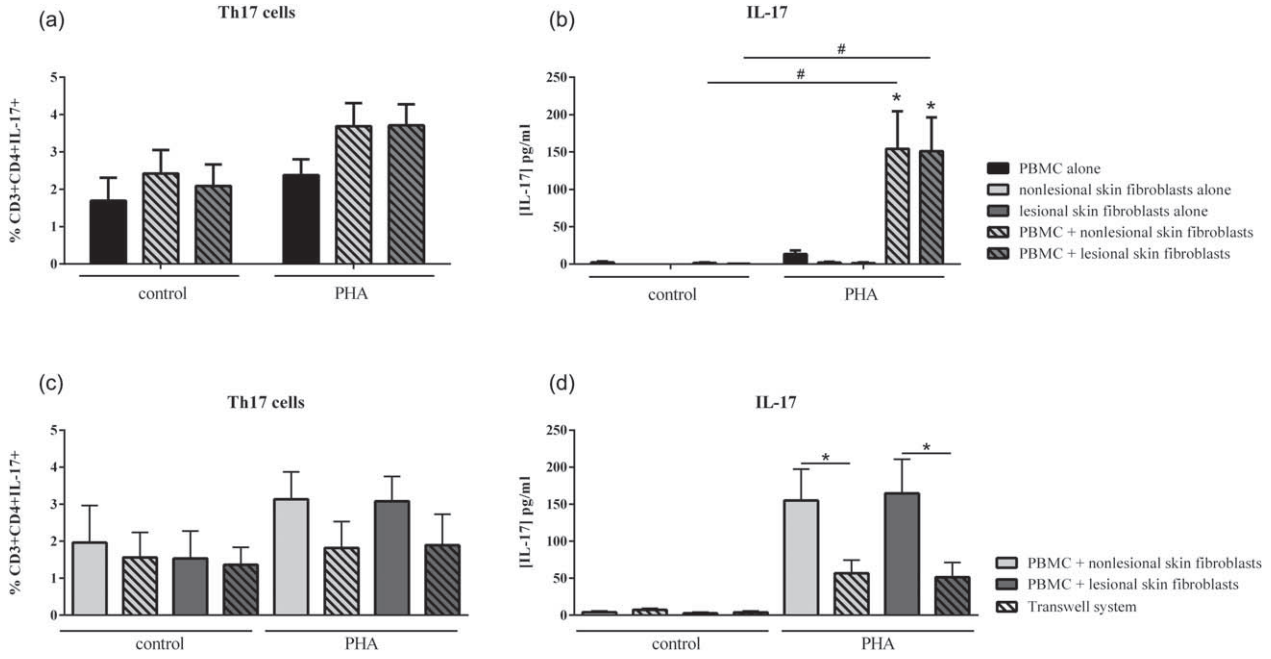

Fig. 2. Effect of interactions between skin fibroblasts and peripheral blood mononuclear cells (PBMC) on T helper type 17 (Th17) differentiation and interleukin (IL)-17 secretion. Healthy PBMC and psoriatic skin fibroblasts were cultured alone or in co-culture at a ratio of 5 : 1 for 48 h in the presence or absence of phytohaemagglutinin (PHA) (5 μg/ml). The Transwell system was used in co-cultures to inhibit cell–cell contact. PBMC were recovered after 48 h and stained with surface markers CD3 and CD4 and with intracellular IL-17A. The percentage of $CD3^+CD4^+IL\text{-}17A^+$ is represented (a,c). IL-17A production was measured in supernatants after 48 h by enzyme-linked immunosorbent assay (ELISA) (b,d). *Compares PBMC alone and co-cultures and # compares control and PHA (a,b). *Compares co-cultures and Transwell system (c,d). *$P \leq 0{\cdot}05$. Error bars represent standard error of the mean (s.e.m.), $n = 15$ for (a,b) and $n = 9$ for (c,d).

and also in PBMC-lesional skin fibroblast co-culture (835·1 ± 395·4 ng/ml *versus* 64·3 ± 39·6 ng/ml, $P \leq 0{\cdot}001$; 311·2 ± 147·0 ng/ml *versus* 9·3 ± 7·8 ng/ml, $P \leq 0{\cdot}002$; and 1325·9 ± 1258·8 pg/ml *versus* 98·6 ± 96·3 pg/ml, $P \leq 0{\cdot}05$, respectively, Fig. 1a–c). Activation of PBMC by PHA had no additional effect in co-culture (Fig. 1). These data indicated that cell–cell contact was sufficient to activate the proinflammatory cytokine production. Moreover, a tendency to higher production was observed in co-cultures with lesional compared to co-cultures with non-lesional skin fibroblasts, mainly for IL-6 (Fig. 1).

To investigate the importance of cell–cell contact, a Transwell system was used. The insert prevents direct cell interactions but allows the exchange of soluble factors. In this system, the production of IL-8, IL-6 and IL-1β was decreased significantly compared to control (326·3 ± 334·4 ng/ml *versus* 780·0 ± 706·7 ng/ml for IL-8, 58·8 ± 32·2 ng/ml *versus* 277·4 ± 136·9 ng/ml for IL-6 and 199·4 ± 150·3 pg/ml *versus* 1177·8 ± 1011·3 pg/ml for IL-1β, $P \leq 0{\cdot}008$ with non-lesional skin fibroblasts; 272·4 ± 246·2 ng/ml *versus* 807·8 ± 485·9 ng/ml for IL-8, 50·5 ± 30·0 ng/ml *versus* 396·1 ± 129·3 ng/ml for IL-6 and 200·2 ± 171·4 pg/ml *versus* 1255·7 ± 1087·3 pg/ml for IL-1β, $P \leq 0{\cdot}008$ with lesional skin fibroblasts, Fig. 1d–f). A significant decrease of IL-8, IL-6 and IL-1β production with the Transwell was also observed with PHA (Fig. 1d–f). This confirmed that cell interactions were sufficient and required to activate the proinflammatory cytokine production.

PBMC activation and cell interactions are needed to have a high IL-17 secretion

The production of IL-17 has been associated with the Th17 cells [28]. The role of cell interactions on the IL-17 pathway was studied to distinguish intracellular staining from actual secreted IL-17. The Th17 cells were gated in the $CD3^+CD4^+$ population. $IL\text{-}17^+$ cells were observed in culture of PBMC alone or in co-cultures with non-lesional or lesional skin fibroblasts. However, no difference in the percentage of $IL\text{-}17^+$ T cells was observed (Fig. 2a). The effect of PHA activation showed a tendency towards a higher Th17 cell percentage, but was not significant (1·7 ± 1·5% *versus* 2·4 ± 1·2% for PBMC alone; 2·4 ± 2·0% *versus* 3·7 ± 2·0% with non-lesional skin fibroblasts and 2·1 ± 1·8% *versus* 3·7 ± 1·8% with lesional skin fibroblast, Fig. 2a).

Actual IL-17 secretion in supernatants was measured by ELISA. In the control condition without PHA, IL-17 production was almost undetectable in PBMC and co-cultures (Fig. 2b). T cell receptor (TCR) activation by PHA did not increase IL-17 secretion significantly in PBMC alone compared to the control condition (13·3 ± 14·1 pg/ml *versus*

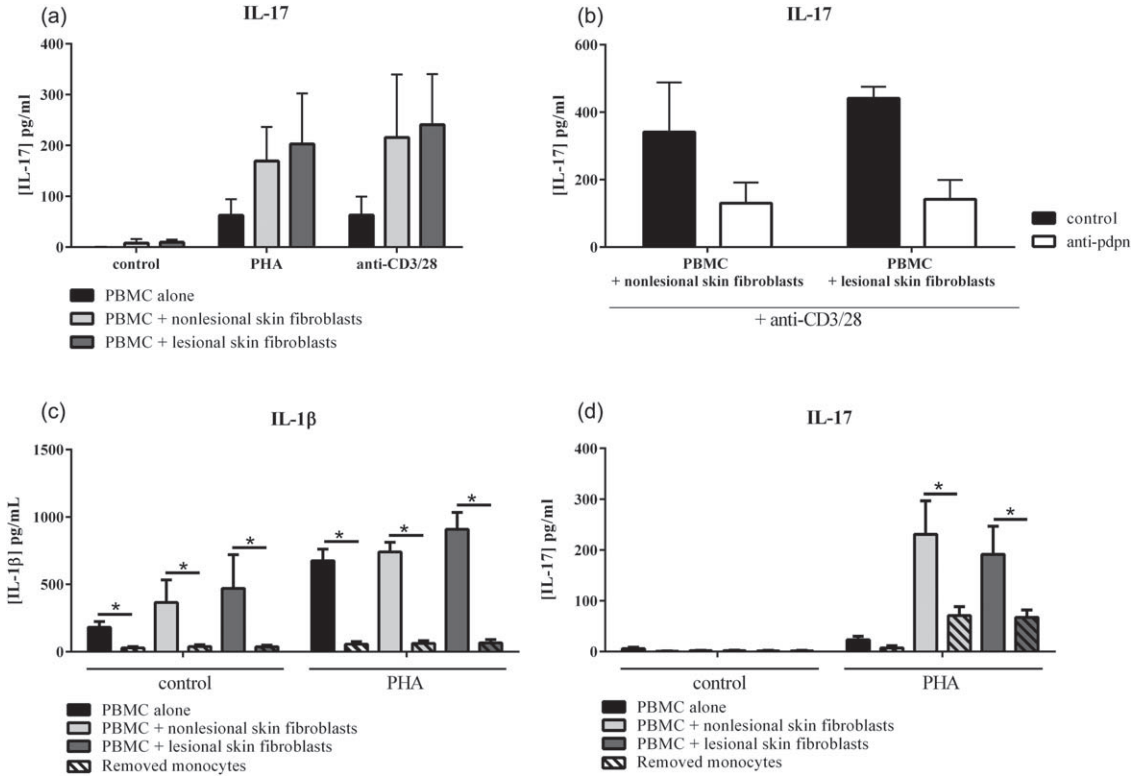

Fig. 3. Role of monocytes in proinflammatory cytokine production. Healthy peripheral blood mononuclear cells (PBMC) were cultured alone or in co-culture with psoriatic skin fibroblasts at a ratio of 5 : 1 for 48 h in the presence or absence of phytohaemagglutinin (PHA) (5 μg/ml) or anti-CD3/28 (5 μg/ml). An antibody against podoplanin was added to the culture (b), and monocytes were removed or not by adherence before culture (c,d). The production of IL-17 (a,b,d) and IL-1β (c) was measured in supernatants after 48 h by enzyme-linked immunosorbent assay (ELISA). *Compares conditions with and without monocytes. *$P \leq 0{\cdot}05$. Results are represented as mean ± standard error of the mean (s.e.m.).

2·2 ± 3·5 pg/ml, Fig. 2b). In contrast, there was a massive production of IL-17 in co-culture with activated PBMC (154·2 ± 151·9 pg/ml with non-lesional skin fibroblasts, $P = 0{\cdot}0006$ and 151·1 ± 134·0 pg/ml with lesional skin fibroblasts, $P = 0{\cdot}0007$, Fig. 2b). PBMC activation by anti-CD3/CD28 gave similar results to PHA (Fig. 3a). These results demonstrated that the combination of TCR activation and cell–cell contact was required to induce a high IL-17 secretion. An important conclusion is that the results with fluorescence activated cell sorter (FACS) staining of intracellular IL-17 do not reflect the amount of IL-17 which is secreted, and thus functional.

To confirm the crucial role of cell interactions in IL-17 production, the Transwell system was used as above. In the control condition, this contact inhibition had no effect on Th17 differentiation, as the percentage of IL-17$^+$ cells was similar between co-culture and the Transwell system (Fig. 2c). Conversely, without cell interactions in the activation condition, IL-17 secretion was reduced massively (155·0 ± 98·7 pg/ml *versus* 56·8 ± 44·6 pg/ml with non-lesional skin fibroblasts, $P = 0{\cdot}008$ and 164·7 ± 108·0 pg/ml *versus* 51·4 ± 42·0 pg/ml with lesional skin fibroblasts, $P = 0{\cdot}008$, Fig. 2d). This Transwell experiment demonstrated clearly that physical interactions between activated PBMC and fibroblasts were crucial for high levels of IL-17 secretion, as would be the case in psoriasis skin.

In addition, we confirmed that IL-17 secretion could be induced with a specific TCR activation. Co-cultures were realized in the presence of antibodies against CD3 and CD28 (anti-CD3/28) in comparison with PHA. As observed in Fig. 3a, IL-17 production increased only in co-cultures with activation, and the level was similar to PHA or anti-CD3/28 (non-lesional skin fibroblasts: 169·2 ± 67·4 pg/ml with PHA and 215·5 ± 124·1 pg/ml with anti-CD3/28; lesional skin fibroblasts: 203·0 ± 99·4 pg/ml with PHA and 240·7 ± 99·5 pg/ml with anti-CD3/28, Fig. 3a).

Monocytes contribute to induce proinflammatory cytokine production, including IL-17

With regard to the role of monocytes in the production of IL-8, IL-6 and IL-1β, their potential contribution was investigated using our co-culture system. As monocytes are a large source of IL-1β in PBMC, the reduction of IL-1β production reflected the effect of monocyte removal. However, a good response to PHA implies the presence of some

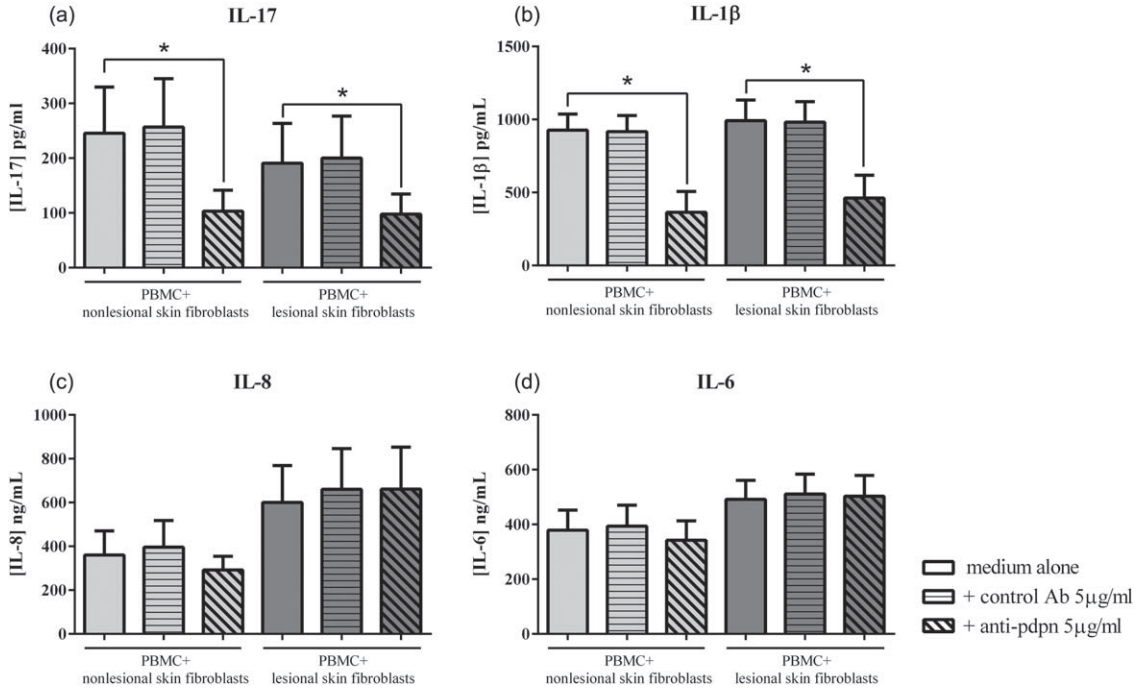

Fig. 4. Major role of podoplanin (pdpn) in high interleukin (IL)-17 secretion. Healthy peripheral blood mononuclear cells (PBMC) were preincubated for 4 h with or without human anti-pdpn or irrelevant control antibody before co-culture with psoriatic skin fibroblasts for 48 h in the presence of phytohaemagglutinin (PHA) (5 μg/ml). Levels of IL-17 (a), IL-1β (b), IL-8 (c) and IL-6 (d) were measured in the supernatants after 48 h by enzyme-linked immunosorbent assay (ELISA). $^*P \leq 0{\cdot}05$. Results are represented as mean ± standard error of the mean (s.e.m.), $n = 7$. The values for control antibody were similar to those with medium alone (245·6 ± 177·8 *versus* 256·8 ± 187·4 pg/ml for IL-17, 926·7 ± 215·7 *versus* 917·4 ± 213·5 pg/ml for IL-1β, 360·5 ± 170·4 *versus* 396·6 ± 187·4 ng/ml for IL-8 and 378·7 ± 142·8 *versus* 393·9 ± 148·5 ng/ml for IL-6, with non-lesional skin fibroblasts; 190·7 ± 136·3 *versus* 200·2 ± 143·1 pg/ml for IL-17, 991·7 ± 283·1 *versus* 981·8 ± 280·3 pg/ml for IL-1β, 600·1 ± 283·8 *versus* 660·1 ± 312·2 ng/ml for IL-8 and 491·5 ± 121·6 *versus* 511·2 ± 126·5 ng/ml for IL-6, with lesional skin fibroblasts).

monocytes, as obtained by adherence. As observed in Fig. 3c, removal of monocytes by adherence inhibited the production of IL-1β significantly in all conditions ($P = 0{\cdot}004$). Interestingly, IL-17 production was also decreased significantly in co-cultures between activated-PBMC and skin fibroblasts without monocytes ($P = 0{\cdot}008$, Fig. 3d). IL-8 and IL-6 are proinflammatory cytokines produced by many cell types, including monocytes and skin fibroblasts. In all conditions, IL-8 secretion was decreased significantly ($P \leq 0{\cdot}02$, data not shown). Except in the control condition of PBMC alone, IL-6 production was also decreased in conditions without monocytes ($P = 0{\cdot}004$, data not shown). These results showed that monocytes have a major role in proinflammatory cytokine production, and notably in the massive IL-17 secretion resulting from cell interactions between immune cells and mesenchymal cells in the persistence of inflammation.

Pdpn plays a major role in high IL-17 secretion during co-culture between activated-PBMC and psoriatic skin fibroblasts

To identify a mechanism for the interactions, we focused our attention on membrane molecules and considered the possible role of pdpn. Pdpn can be expressed by different cell types: skin fibroblasts, synoviocytes and by immune cells such as monocytes and Th17 cells. Focusing upon pdpn expressed by monocytes and T cells, PBMC were preincubated with anti-pdpn antibody. In co-cultures with activated-PBMC, the presence of anti-pdpn antibody inhibited IL-17 secretion significantly by 64·1 ± 18·0% with non-lesional skin fibroblasts (245·6 ± 177·8 *versus* 103·3 ± 69·2 pg/ml, $P = 0{\cdot}002$, Fig. 4a) and by 56·2 ± 20·8% with lesional skin fibroblasts (190·7 ± 136·3 *versus* 97·7 ± 73·4 pg/ml, $P = 0{\cdot}002$, Fig. 4a). IL-1β secretion was also inhibited (64·0 ± 22·3% of inhibition, 926·7 ± 215·7 *versus* 364·2 ± 263·6 pg/ml with non-lesional skin fibroblasts, $P = 0{\cdot}002$ and 53·8 ± 27·0% of inhibition, 991·7 ± 283·1 *versus* 462·1 ± 264·9 pg/ml with lesional skin fibroblasts, $P = 0{\cdot}002$, Fig. 4b), but neither IL-8 secretion (Fig. 4c) nor IL-6 secretion (Fig. 4d). In the presence of TCR activation with anti-CD3/28, the addition of anti-pdpn antibody also inhibited IL-17 secretion by 60% with non-lesional skin fibroblasts (341·0 ± 104·2 *versus* 130·3 ± 43·2 pg/ml, Fig. 3b) and by 70% with lesional skin fibroblasts (441·1 ± 24·2 *versus* 141·6 ± 40·8 pg/ml, Fig. 3b). IL-1β secretion was inhibited by around 60% in both co-cultures while IL-6 and IL-8 secretion was not affected (data not shown). These results

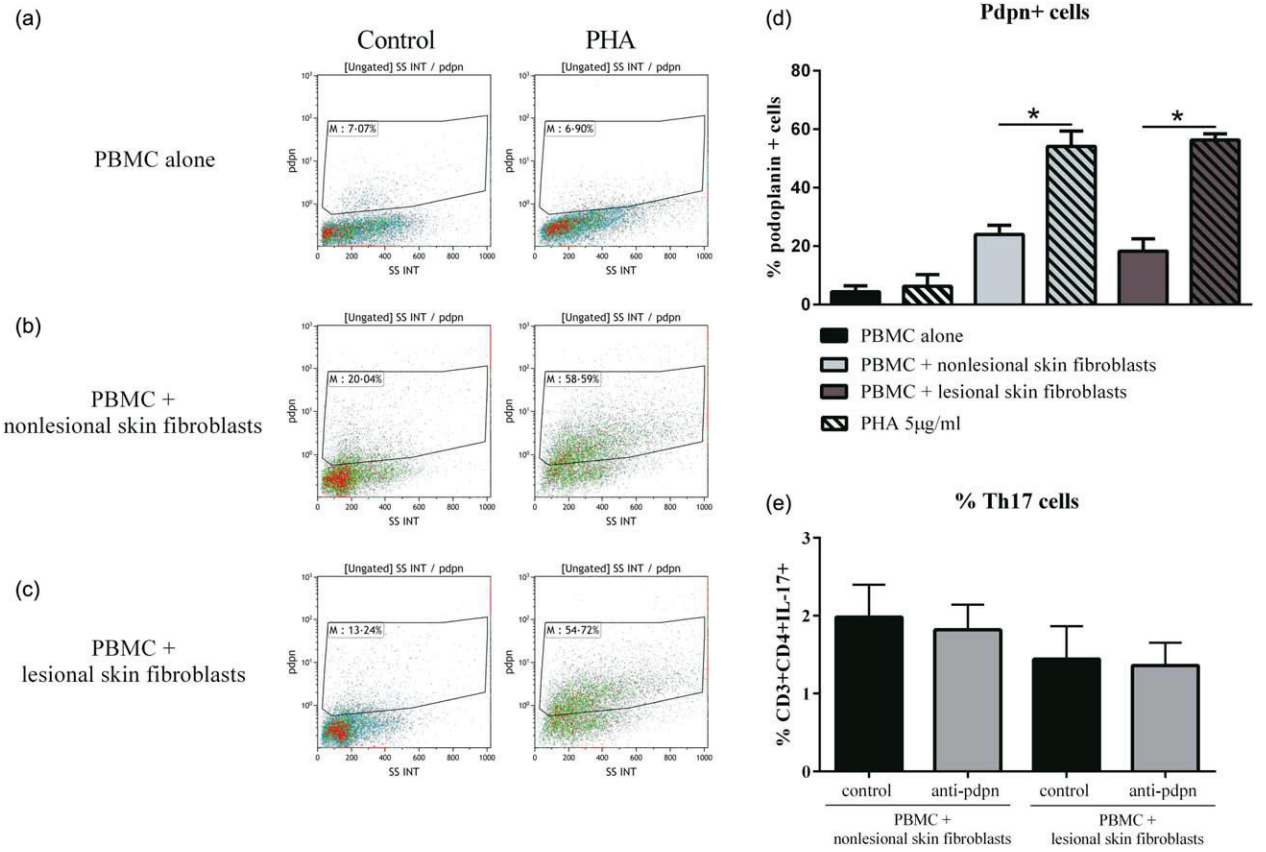

Fig. 5. Expression of podoplanin (pdpn) in peripheral blood mononuclear cells (PBMC) alone or in co-culture. Healthy PBMC were cultured alone or in co-culture with psoriatic skin fibroblasts at a ratio of 5 : 1 for 48 h in the presence or absence of phytohaemagglutinin (PHA) (5 µg/ml). Cells were recovered after 48 h and stained with anti-pdpn antibody. Dot-plot of one experiment is represented (a,b,c). The percentage of $pdpn^+$ cells in the different conditions is represented (d). Co-cultures with anti-pdpn antibody were performed and PBMC were recovered after 48 h and stained for surface markers CD3 and CD4, and with intracellular IL-17A. The percentage of $CD3^+CD4^+IL\text{-}17A^+$ is represented (e). $^*P \leq 0{\cdot}05$. Error bars represent standard error of the mean (s.e.m.) (d).

indicated that anti-pdpn antibody had an inhibitory effect on IL-17 and IL-1β, independently of PBMC activation.

To study the regulation of its expression during cell interactions after 48 h in co-culture, cells were recovered and stained for pdpn. PBMC alone showed a very low percentage of $pdpn^+$ cells (4·3 ± 2·1% in control; 3·2 ± 4·1% with PHA, Fig. 5a,d). However, the percentage of $pdpn^+$ cells increased in control co-cultures, as psoriatic skin fibroblasts express this protein (24·0 ± 3·1% with non-lesional skin fibroblasts; 18·3 ± 4·2% with lesional skin fibroblasts, Fig. 5b–d). Interestingly, PHA activation increased the number of $pdpn^+$ cells in co-cultures significantly compared to control ($P = 0{\cdot}03$, Fig. 5b–d). Furthermore, the addition of anti-pdpn did not affect the differentiation of Th17 cells (2.0 ± 0·4% *versus* 1·8 ± 0·3% with non-lesional skin fibroblasts; 1·4 ± 0·4% *versus* 1·4 ± 0·3% with lesional skin fibroblasts, Fig. 5e). These results were consistent with the increase of IL-17 secretion associated with over-expression of pdpn on both skin fibroblasts and activated PBMC.

Use of an autologous system of co-culture gives similar results and excludes the allogeneic reaction

To exclude an allogeneic reaction, the experiments were realized by using PBMC and skin fibroblasts from the same patients. As observed in Fig. 6a–d, the results on cytokine production (IL-17, IL-1β, IL-8 and IL-6, Fig. 6a–d, respectively) in co-cultures and with the removal of monocytes were similar to those with healthy PBMC. Furthermore, when the anti-pdpn antibody was added in autologous co-culture, the secretion of IL-17 (Fig. 6e) and IL-1β (Fig. 6f) was decreased while the production of IL-8 and IL-6 was unchanged (non-lesional skin fibroblasts: 994·4 ± 103·7 *versus* 1039·0 ± 29·2 ng/ml for IL-8 and 518·4 ± 30·3 *versus* 492·7 ± 41·6 ng/ml for IL-6; lesional skin fibroblasts: 1131·2 ± 53·3 *versus* 1082·1 ± 27·4 ng/ml for IL-8 and 835·3 ± 16·2 *versus* 798·7 ± 44·3 ng/ml for IL-6, data not shown). The results using an autologous system were coherent with the results obtained with healthy PBMC and thus excluded the contribution of an allogeneic reaction.

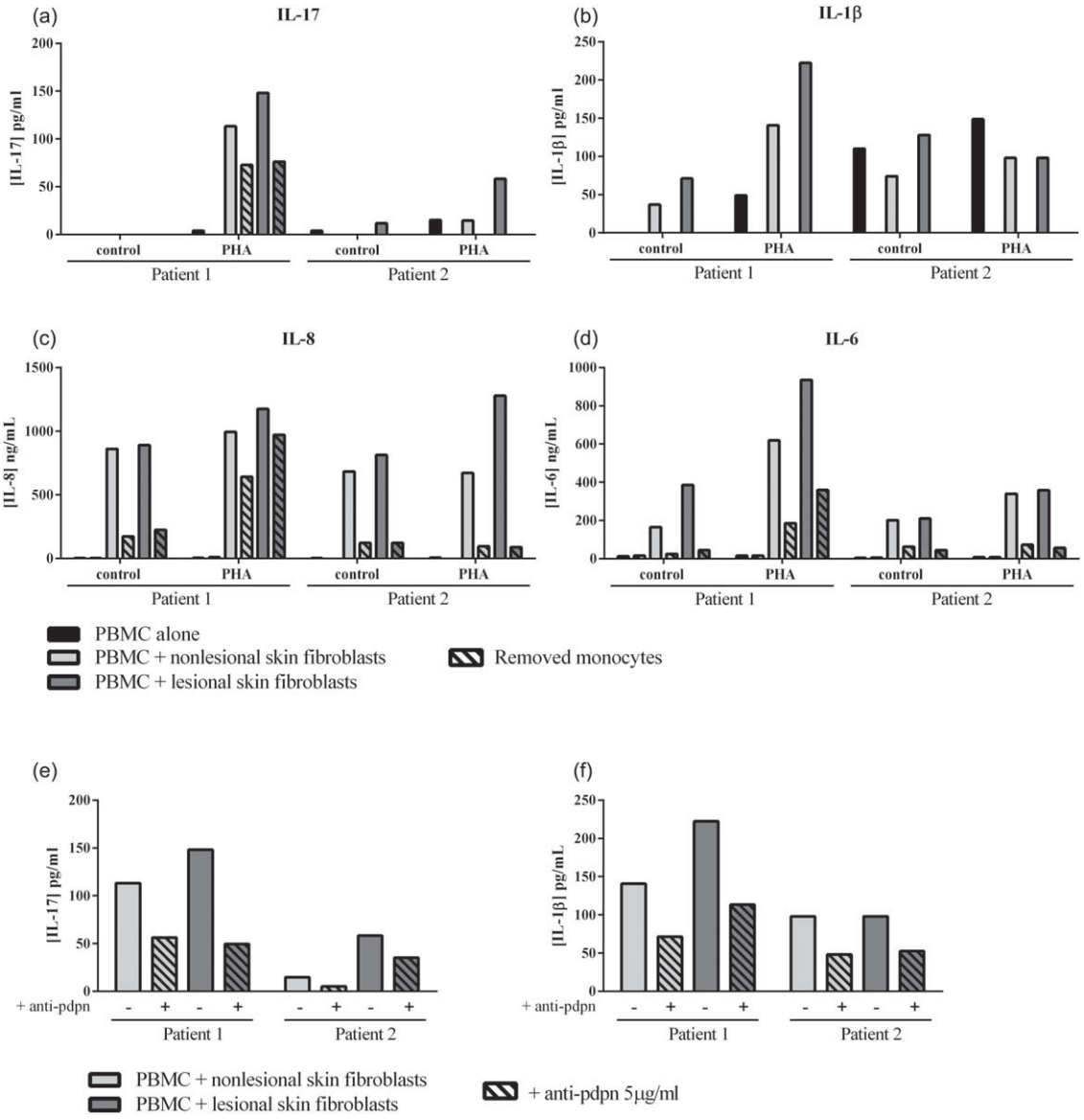

Fig. 6. Exclusion of allogeneic reaction by using an autologous system. Peripheral blood mononuclear cells (PBMC) and psoriatic skin fibroblasts from the same patient (autologous system) were cultured alone or in co-culture at a ratio of 5 : 1 for 48 h in the presence or absence of phytohaemagglutinin (PHA) (5 μg/ml) and monocytes were removed or not by adherence before culture (a,b,c,d). Autologous cells were co-cultured in the presence of PHA at a 5 : 1 ratio for 48 h with or without anti-pdpn antibody (e,f). Levels of IL-17 (a,e), IL-1β (b,f), IL-8 (c) and IL-6 (d) were measured in the supernatants after 48 h by enzyme-linked immunosorbent assay (ELISA). Results are represented as mean ± standard error of the mean (s.e.m.); $n = 2$.

Discussion

Cell interactions between mesenchymal and immune cells are critical for the local production of proinflammatory cytokines, with an effect on cell survival [16,17,29,30]. In psoriasis, the role of interactions on proinflammatory cytokine secretion, with a main focus on IL-17, was investigated to differentiate intracellular staining from secreted IL-17 and to evaluate the mechanisms involved in these cell interactions.

A co-culture system between skin fibroblasts and PBMC was used to reproduce the *in-vivo* early step of cell–cell interaction between migrating immune cells and local mesenchymal cells and the use of an autologous cell system validated this model. Our findings demonstrated that cell interactions were sufficient to provide a necessary activation state for the secretion of IL-8, IL-6 and IL-1β. These results were in agreement with a previous study showing that bone marrow mesenchymal cells in interaction with PBMC increased IL-6 and IL-1β mRNA [16]. These observations reflect how high levels of proinflammatory cytokines, including IL-8, IL-6 and IL-1β, can be found in psoriatic lesions [31]. The migrating preactivated immune

cells interact with resident cells as skin fibroblasts, and this leads to an increased production of proinflammatory cytokines. The variability of cytokine production between non-lesional and lesional skin fibroblasts could depend upon the site of biopsy and the degree of inflammation. Fibroblasts in the psoriatic lesion and nearby may have a more severe inflammatory phenotype, and thus induce a higher proinflammatory cytokine secretion.

Among these cytokines, IL-6 and IL-1β are involved in Th17 differentiation. Th17 cells, in turn, secrete IL-17, which acts on different targets, including skin fibroblasts [32], and this promotes skin inflammation. Considering the results on IL-6 and IL-1β, the effect of cell interactions on Th17 pathway was studied to differentiate intracellular from secreted IL-17. The percentage of $CD3^+CD4^+$ $IL\text{-}17^+$ cells was evaluated in PBMC alone or in co-culture. Cell contact alone had no major effect on Th17 differentiation. Only TCR activation had a modest effect. This indicated that early Th17 differentiation requires cell activation more than cell–cell contact.

When looking at IL-17 production, and in contrast to IL-8, IL-6 and IL-1β, cell interactions were not sufficient to induce high IL-17 secretion in supernatants. This production required two signals, TCR activation and cell–cell contact. Moreover, activation of skin fibroblasts by TNF alone in co-culture without TCR stimulation had no effect on IL-17 production (data not shown). Thus, IL-17 secretion during cell interactions was dependent upon both T cell activation and fibroblast contact. Transwell experiments confirmed that cell interactions, and thus the fibroblast contribution, were crucial to induce a massive IL-17 secretion even in presence of TCR activation.

These results outline a key point which has not been taken into account previously: there is a major discrepancy between intracellular staining and secreted IL-17. The intracellular presence of IL-17 inside Th17 cells does not imply its high secretion. Activated cells have to interact physically with mesenchymal cells, derived from either skin, synovium, bone marrow. TCR activation and cell–cell contact are two necessary mechanisms leading to high IL-17 production, and this differs from its intracellular expression. Both mechanisms are present during psoriasis pathogenesis [2], and this could explain not only the presence, but even more the secretion, of IL-17 in psoriatic skin lesions [8–11].

The critical involvement of cell interactions indicated the need to identify a molecular mechanism. The contribution of monocytes was first investigated, as they secrete the key cytokines involved in Th17 differentiation and are activated in patients with psoriasis [20]. To study this involvement, monocytes were removed by adherence before co-culture. The reduction of IL-1β production confirmed that the removal was efficient. Interestingly, the production of IL-8 and IL-6 was also decreased significantly. Furthermore, this removal inhibited largely the IL-17 production induced by the cell interactions between activated PBMC and fibroblasts. This suggested that monocytes are a major player in the process inducing proinflammatory cytokine production and specifically the massive IL-17 secretion. In return, IL-17 will increase the production of IL-1β and other cytokines by monocytes.

The mechanism by which monocytes are involved in the interaction-induced IL-17 production remained to be identified. We selected pdpn as a candidate. Indeed, this interaction molecule is expressed by human monocytes [33] and RA synoviocytes [25]. Furthermore, pdpn is expressed in hyperproliferative psoriatic epidermis [23,24], but its contribution to disease remains to be clarified further. Based on these observations, PBMC were preincubated before co-culture with the anti-pdpn antibody to block the pdpn expressed by monocytes and T cells. Our results demonstrated that the inhibition of the pdpn-mediated interaction decreased the secretion of IL-17 and IL-1β by at least 50%, but not of IL-8 and IL-6. Furthermore, pdpn expression was increased in co-culture with TCR activation which correlates with the massive IL-17 production. These results suggested that cell interactions of fibroblasts with activated-immune cells increased pdpn expression that contributed to high IL-17 secretion. Pdpn could be one mechanism by which monocytes and their activation contribute to IL-17 secretion during cell interactions.

The mechanistic knowledge on cellular pdpn functions comes in part from cancer studies [21]. Pdpn is involved in cell motility, and notably in dendritic cell (DC) migration. DCs express the pdpn receptor C-type lectin-like receptor 2 (CLEC-2), and the interaction with pdpn expressed by stromal cells induces DC migration to lymph nodes [34]. Furthermore, pdpn can bind CCL21, a chemotactic factor for mature DC and T cell migration [21]. CCR7 ligands, including CCL21, can then stimulate the production of IL-23 by DCs leading to the IL-23-dependent generation of pathogenic Th17 cells [35]. Moreover, different animal models have indicated the contribution of the pdpn pathway to various inflammatory diseases [36,37]. In the human situation, pdpn expression is up-regulated in RA synovium [38], and pdpn-mediated interaction between RA synoviocytes and platelets modulates IL-8 production [25]. Furthermore, pdpn expressed by fibroblasts may contribute to tissue organization in germinal centres as well as during tumour formation [39]. Clearly, more studies are needed, but the present results indicate the link between pdpn, IL-17 and chronic inflammation.

In conclusion, this study provides an important concept by showing that cell interactions between psoriatic skin fibroblasts and blood-derived immune cells play a major role in proinflammatory cytokine production, leading to a massive IL-17 secretion distinct from, and not reflected by, intracellular expression. Monocytes and the interaction molecule pdpn contribute widely to this high IL-17 secretion. In this context, pdpn could be a potential therapeutic

target to block Th17 cell activity during chronic inflammation.

Acknowledgements

The authors acknowledge Dr Ana-Maria Moldovan, Hospital Edouard Herriot, for providing skin biopsies. These studies were supported in part by the IHU prometteur OPERA.

Author contributions

M. N. carried out the experiments and drafted the manuscript; she is supported by the Institut Universitaire de France. N'D. N.-T. participated in the experiments and helped to revise the manuscript; she is supported by the IHU prometteur OPERA. P. M. conceived the study and helped to draft the manuscript. He is a senior member of and supported by the Institut Universitaire de France. All authors read and approved the final manuscript.

Disclosure

The authors have no conflicts of interest to declare.

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Partie 3. Applications aux traitements

I Introduction aux biothérapies

Dans des contextes inflammatoires chroniques tels que la PR et le Pso, les chapitres précédents démontrent que les interactions cellulaires entre cellules mésenchymateuses et cellules immunitaires jouent un rôle primordial dans la sécrétion massive d'IL-17. De plus, l'IL-17 est largement associée à l'inflammation chronique, notamment dans le cadre de la PR et du Pso, ce qui fait de cette cytokine une cible thérapeutique attractive. La revue suivante fait état de l'IL-17 et de son ciblage dans l'inflammation chronique et notamment dans la PR (« IL-17 in chronic inflammation : from discovery to targeting », A. Beringer, M. Noack et P. Miossec, Trends in Molecular Medicine, 2016).

Trends in Molecular Medicine

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Review

IL-17 in Chronic Inflammation: From Discovery to Targeting

Audrey Beringer,[1] Melissa Noack,[1] and Pierre Miossec[1,*]

Interleukin-17 (IL-17) is a cytokine which elicits protection against extracellular bacterial and fungal infections and which plays important roles in inflammation. However, when produced in excess, it contributes to chronic inflammation associated with many inflammatory and autoimmune disorders. This has made IL-17 an attractive therapeutic target. The present review describes the structure of the IL-17 family, the IL-17 receptor complex, and the cells producing IL-17. The contributions of IL-17 to disease as well as new IL-17-based treatment options are discussed. Finally, the results of IL-17 or IL-17 receptor inhibitors in clinical trials are detailed. With a fruitful outlook, drug registration has now been granted for psoriasis psoriatic arthritis and ankylosing spondylitis, and also bears great potential in a growing number of conditions.

Introduction

The proinflammatory cytokine IL-17 was described fairly recently and is becoming an important therapeutic target for a growing number of chronic inflammatory diseases [1]. IL-17 plays a key role in host defense against extracellular bacterial and fungal infections [2]. Excess contribution of IL-17 has been associated with several inflammatory disorders including psoriasis, psoriatic arthritis (PsA), rheumatoid arthritis (RA), and ankylosing spondylitis (AS). A first antibody against IL-17 (anti-IL-17) was approved in 2015 for the treatment of psoriasis. Other IL-17 pathway inhibitors are currently being tested for an increasing number of clinical indications relevant to various conditions [3].

This review first describes the structure and signaling pathways of IL-17 and IL-17-producing cells. The key functions of IL-17 are analyzed in the context of disease to introduce the treatment strategies. Finally, we highlight the benefits and limitations of inhibitors targeting IL-17 and the IL-17 receptor (IL-17R).

This discussion is timely, as it represents a good example of 'translational research', highlighting recent advances. It shows how quickly information from the discovery of IL-17 and T helper 17 (Th17) cells has been translated into the development of a successful therapy.

IL-17 and IL-17R Family Members and IL-17 Signaling: The Basics

IL-17 Family

Human cytotoxic T lymphocyte-associated antigen 8 (CTLA8) was identified in 1993 and named IL-17 in 1995 [4]. The first bioactivity of human IL-17 was described in 1996 by showing the production of IL-6 and IL-8 from RA synoviocytes in response to IL-17. This immediately linked IL-17 to inflammation through IL-6 and to neutrophil recruitment through IL-8 [5].

Sequence screening identified an IL-17 family comprising six members from IL-17A (the first described IL-17) to IL-17F (Figure 1). IL-17A and F are the closest members, with 50% homology. They are secreted as IL-17A and IL-17F homodimers and as IL-17A/F heterodimers [6,7].

Trends

Interleukin-17 (IL-17) is a proinflammatory cytokine that plays a key role in host defense against extracellular bacterial and fungal infections.

T helper 17 (Th17) cells play a key role in the production of IL-17.

Increased production and contribution of IL-17 have been associated with several inflammatory disorders, including psoriasis, psoriatic arthritis, rheumatoid arthritis, and ankylosing spondylitis.

The first antibody against IL-17 was approved by the FDA and EMA in 2015 for the treatment of psoriasis.

Other IL-17 inhibitors are under development for a growing number of clinical indications. These include bispecific molecules targeting IL-17A and IL-17F as well as tumor necrosis factor (TNF) and IL-17A.

Inhibitors of Th17 differentiation include those that target IL-23 and the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt).

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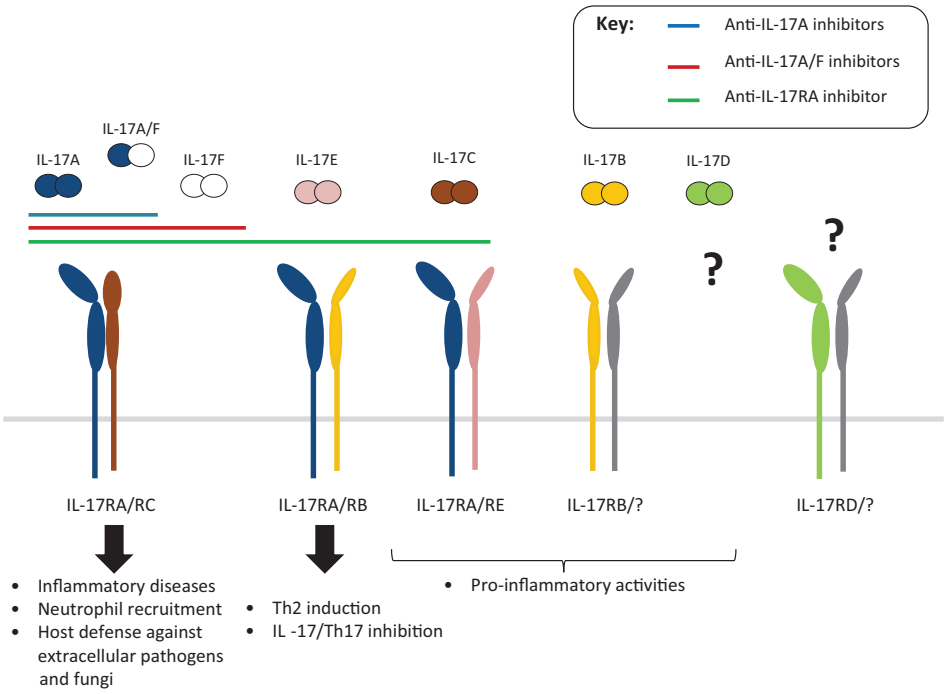


Figure 1. Interleukin (IL)-17 Cytokine and Receptor Family. IL-17 homodimer or heterodimer ligands bind various receptor complexes. IL-17A and IL-17F bind the IL-17 receptor (IL-17R)A and IL-17RC complex. IL-17E or IL-25 binds the IL-17RA and IL-17RB complex. Bars represent the various antibodies in clinical development that target IL-17A (blue), IL-17A and IL-17F (red), or IL-17RA (green).

They share most of their activities, with IL-17A being more potent than IL-17F and IL-17A/F having an intermediate activity [8]. IL-17B, IL-17C, and IL-17D are classified as proinflammatory cytokines but their role is not fully known. By contrast, IL-17E, also known as IL-25, has the lowest homology and is involved in Th2 cell responses against parasites and allergy [7]. IL-25 regulates IL-17 function and this could possibly occur via competition at the receptor level [9].

IL-17R Family

IL-17R was identified in 1995 as a new type of cytokine receptor [10]. The IL-17R family was later described with five subunits, from IL-17RA to IL-17RE (Figure 1). IL-17A, IL-17F, and IL-17A/F bind the same receptor complex comprising IL-17RA and IL-17RC subunits [11,12]. IL-17RA is also a receptor subunit of the receptor for IL-25, comprising IL-17RA and IL-17RB. This is important when targeting IL-17RA, which blocks the proinflammatory pathways mediated by IL-17A, -17F, and -17A/F but also the anti-inflammatory response mediated by IL-25 (Figure 1).

IL-17R Signaling

All receptor subunits have a single transmembrane domain and the binding of IL-17A to the IL-17RA/RC complex recruits the ubiquitin ligase Act1 via the SEF/IL-17R (SEFIR) domain [11]. Act1 recruits tumor necrosis factor (TNF) receptor-associated factor 6 (Traf6) leading to the activation of nuclear factor kappa B (NF-κB) and the mitogen-activated protein (MAP) kinase pathways. Such activation upregulates many inflammatory genes, particularly the neutrophil-specific CXC chemokines [13,14].

IL-17-Producing Cells

Th17 Cells

The production of IL-17 by a subset of T cells was discovered in 1999 using T cell clones from the joints of RA patients [15]. The results were then confirmed in mice and the term Th17 subset was introduced in 2005 in the mouse as a T helper subset distinct from Th1 and Th2 cells [16–18]. IL-12 had been identified as the key cytokine for the production of interferon gamma (IFNγ), the signature cytokine of the Th1 pathway. The new cytokine IL-23 was found to be associated with the Th17 pathway [19]. Th17 cell differentiation involves an initiation step in the presence of transforming growth factor beta (TGF-β) and IL-21 or IL-6, which induce the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt) (human counterpart RORC). Then amplification occurs with IL-1β and IL-6 or IL-21, which induces the expression of IL-23R. This leads to the final step of stabilization with IL-23 [20]. The key cytokines produced by human activated Th17 cells are IL-17A, IL-17F, IL-21, and IL-22.

Th17 cell differentiation is also linked to the differentiation of $CD4^+$ regulatory T cells (Tregs). Because of their opposite effects on the immune response, the Th17/Treg balance is critical in maintaining immune homeostasis. In inflammatory conditions, Tregs are defective and can be converted into Th17 cells [21,22].

Other Sources of IL-17

IL-17 is also produced in both humans and mice by innate immune cells in peripheral tissues such as lungs, intestinal mucosa, and skin [23]. They control the immediate IL-17 response to stress or tissue injury. Their list keeps growing and includes $CD8^+$ T cells, γδ T cells, invariant natural killer T cells (iNKT), natural killer (NK) cells, natural Th17 cells, lymphoid tissue inducer (LTi) cells, and group 3 innate lymphoid (ILC3) cells [13,23,24].

Macrophages, neutrophils, and mast cells in both humans and mice have been reported as another source of IL-17 [7,23]. However, this remains controversial. Although mast cells may stain positive for IL-17 in sections of inflamed tissue, demonstration of active IL-17 mRNA expression has been difficult, suggesting that mast cells might engulf IL-17, serving as a local reservoir [25].

The Biology of IL-17A

IL-17 has pleiotropic effects on multiple cell types. It plays a key role in host defense against infections but also in the development and chronicity of inflammatory disorders.

Role of IL-17 in Host Defense

Th17 cells and other IL-17-producing cells protect the host against extracellular bacterial and fungal infections at epithelial and mucosal surfaces. To control infection, IL-17 promotes granulopoiesis leading to neutrophilia by increasing granulocyte colony-stimulating factor (G-CSF) and migration in response to neutrophil chemoattractants such as IL-8/CXCL8 [5]. The production of chemoattractants for lymphocytes, dendritic cells, and monocytes is also induced by IL-17. CCL20 is an important chemokine that attracts Th17 cells by binding to CCR6, the receptor for CCL20, which is also a marker of Th17 cells [26].

Overexpression of IL-17 in the lung enhances *Klebsiella pneumoniae* clearance and mouse survival [27]. Mice deficient in IL-17 and/or IL-17RA have a higher susceptibility to several extracellular bacteria, specifically *Staphylococcus aureus* [28,29]. IL-17 is also essential in controlling fungal infections [30]. After skin infection with *Candida albicans*, IL-23- and IL-17-deficient mice show delayed skin healing and a higher fungal burden, which are improved by exogenous administration of IL-17A [31].

In humans, this phenotype is reproduced in patients with hyper-IgE syndrome caused by a genetic mutation in the STAT3 gene leading to a reduced number of Th17 cells, defective production of IL-17, and Th2 activation leading to increased production of IgE. They suffer severe *S. aureus* and *C. albicans* infections [32]. Moreover, chronic mucocutaneous candidiasis has been associated with genetic deficiencies of IL-17RA, IL-17F, Act1, IL-17RC, and RORC and occurs in patients with autoantibodies against IL-17A, IL-17F, and IL-22 [32–34].

IL-17 and Inflammation

IL-17A and IL-17F act on various isolated cells in both humans and mice, such as endothelial cells, macrophages, fibroblasts, osteoblasts, and chondrocytes. This increases the production of proinflammatory cytokines from monocytes [TNFα, IL-1β, IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), G-CSF] [1]. IL-17 acts on mesenchymal cells from synovium and skin to induce chemokines leading to neutrophil (IL-8/CXCL8), lymphocyte (CCL20), and macrophage recruitment [35–37]. CCL20 drives the recruitment of Th17 and dendritic cells to the inflammatory site [26]. In turn, Th17 cells are activated and produce inflammatory mediators leading to chronic inflammation [38]. IL-17 contributes to cartilage destruction by stimulating the expression of cartilage-degrading enzymes [39] and to bone destruction by enhancing the expression of receptor activator of NF-κB ligand (RANKL) on osteoblasts that activate RANK-positive osteoclasts [40].

IL-17 alone is often poorly active but it can synergize with other inflammatory cytokines such as TNFα, IL-1β, IL-22, IFNγ, and GM-CSF, leading to increased production of inflammatory mediators such as IL-6 and IL-8 [6,37,41]. This results from a combination of mechanisms. IL-17 stabilizes the mRNA expression activated by TNFα, leading to increased and prolonged protein production [42]. In addition, IL-17 induces TNFα receptor II expression, which increases the response to TNFα [41]. As a consequence, a combination of IL-17 and TNFα inhibitors has greater efficacy in arthritis progression than the monotherapies in mouse models [43]. Similar results have been obtained with *ex vivo* cultures of explants taken from the synovium and bone of arthritis patients [40].

The interactions between IL-17 and TNFα that lead to increased inflammation are the main basis for targeting both IL-17 and TNFα, either with a single bispecific molecule or with two inhibitors. This approach could be interest in patients with an inadequate response to TNFα inhibitors [44].

The Role of IL-17 in Inflammatory Diseases

IL-17 thus has two opposite contributions. Its deficiency leads to reduced control of infections, but its overproduction can lead to several chronic inflammatory diseases.

Psoriasis

Psoriasis is an immunological skin disease characterized by chronic inflammation with proliferation of keratinocytes and accumulation of immune cells, specifically Th17 cells. Expression and production of IL-17 by skin-infiltrating cells is increased in psoriatic skin lesions [45]. It is well known that IL-17 acts on keratinocytes to induce the expression of several chemokines leading to the recruitment and accumulation of neutrophils, T cells, and dendritic cells.

Rheumatic Diseases

PsA is a chronic inflammatory arthritis leading to distal joint destruction. It affects 20–30% of psoriasis patients but can also be observed in the absence of skin manifestations. High levels of Th17 cells and $CD8^+$ $IL\text{-}17^+$ T cells are found in human peripheral blood and synovial fluid, correlating with disease activity [46]. *In vitro*, IL-17 treatment of PsA synoviocytes induces higher levels of IL-6, IL-8, and matrix metalloproteinase 3 (MMP-3) than that of osteoarthritis synoviocytes, suggesting a link between IL-17 and local tissue changes in the joint [47].

RA is another chronic inflammatory disease characterized by synovitis and destruction of bone and cartilage. The first demonstration of the production of IL-17 at the site of a human disease was shown in 1999 using RA synovium explants [35]. The addition of an anti-IL-17 antibody to supernatants of these RA synovium cultures reduced the production of IL-6 by synoviocytes exposed to these supernatants. Moreover, elevated levels of IL-17A have been found in serum and synovial fluid of RA patients, correlating with disease activity [48]. Antibodies against IL-17A or IL-17RA have been shown to reduce synovial inflammation and prevent joint destruction in the collagen-induced arthritis mouse model as well as in human synovium and bone explants [40,49].

AS affects the sacroiliac joints and the spine. In contrast to the destructive aspects of RA and PsA, AS leads to ectopic bone formation in the spine, referred to as syndesmophytes. Elevated levels of IL-17 and IL-23 are found in the serum of AS patients [50]. High levels of Th17 cells are also found in their peripheral blood and synovial fluid [51]. In the sacroiliac joint, IL-17 may be produced by cells other than Th17 cells [25,52]. ILC3 cells, which express high levels of IL-17 and IL-22, are expanded in the peripheral blood, synovial fluid, gut, and bone marrow of AS patients, indicating that overall systemic changes occur in these patients [53].

Multiple Sclerosis (MS)

MS is a chronic inflammatory autoimmune disease of the central nervous system (CNS) characterized by the destruction of myelin by autoreactive pathogenic T cells. Early results showed increased expression of IL-17 in the brain at autopsy. IL-17-expressing cells are abundant in active CNS lesions in MS models and patients [54,55].

Crohn's Disease (CD)

CD is an inflammatory bowel disease characterized by local mucosal inflammation in the intestine. The contribution of IL-17 in CD remains unclear. IL-17 production results in intestinal inflammation in some mouse studies but is protective in others [56,57]. In some but not other studies of CD patients, the number of Th17 cells and the expression of their related cytokines have been reported to be increased in intestinal biopsies and to correlate with disease activity [58,59]. Another study has indicated that local inflammation leads to greater conversion of Tregs into Th17 cells [60]. Together these results suggest that IL-17 could play a dual role in disease activity and protection from mucosal damage.

Other Diseases

An increasing number of diseases has been associated with the IL-17 pathway, including asthma, chronic obstructive pulmonary disease (COPD), lupus, hidradenitis suppurativa, polymyalgia rheumatica, giant cell arteritis, Behçet disease, dry-eye syndrome, and Sjögren's syndrome. However, for most it remains unclear whether the association is pathogenic and whether it could justify attempting IL-17 targeting.

Tools to Target the IL-17 Pathway

Studies using cell systems as well as samples from patients and animal models have provided a strong justification for targeting IL-17 in human diseases, with the goal of controlling the harmful (and/or painful) manifestations associated with chronic inflammation. Two major options are currently being developed to target the IL-17 pathway, one acting directly on IL-17A and IL-17F or IL-17RA and the other acting upstream on the differentiation of Th17 cells (Table 1 and Figure 2, Key Figure).

Direct Targeting of IL-17A, IL-17F, and IL-17RA

Targeting the cytokine or its receptor with monoclonal antibodies (mAbs) is the most direct and specific strategy. The first two anti-IL-17A antibodies tested in the clinic were secukinumab

Table 1. Drug Candidates Targeting IL-17 or its Receptor IL-17RA and Their Current Clinical Status

Drug	Manufacturer					
		Psoriasis	PsA	AS	RA	Other Indications
IL-17A Inhibitors						
Secukinumab (AIN457), Consentyx™	Novartis	Approved	Approved	Approved	Phase III	
Ixekizumab (LY2439821)	Lilly	Submitted	Phase III		Phase II	
CNTO 6785	Janssen				Phase II	COPD (Phase II)
CJM112	Novartis	Phase I/II				Hidradenitis suppurativa (Phase II)
BCD 085	Biocad					Healthy subjects (Phase I)
IL-17A and IL-17F Inhibitors						
Bimekizumab (UCB-4940)	UCB	Phase I	Phase I		Phase II	
ALX-0761 (MSB 0010841)	Merck Serono Ablynx	Phase I				
IL-17A and TNFα Inhibitors						
ABT-122	AbbVie		Phase II		Phase II	
COVA322	Janssen/ Covagen	Phase I/II	Preclinical	Preclinical	Preclinical	
IL-17RA Inhibitors						
Brodalumab (AMG 827)	Valeant Pharmaceuticals	Phase III		Phase III	Phase II	

Clinical status is based on data from Clinicaltrials.gov.

(AIN457, Consentyx™), a fully human IgG1κ anti-IL-17A mAb, and ixekizumab (LY2439821), a humanized IgG4 antibody (Table 1). Several other antibodies, such as CNTO 6785, CJM112, and BCD085, are now in clinical trials.

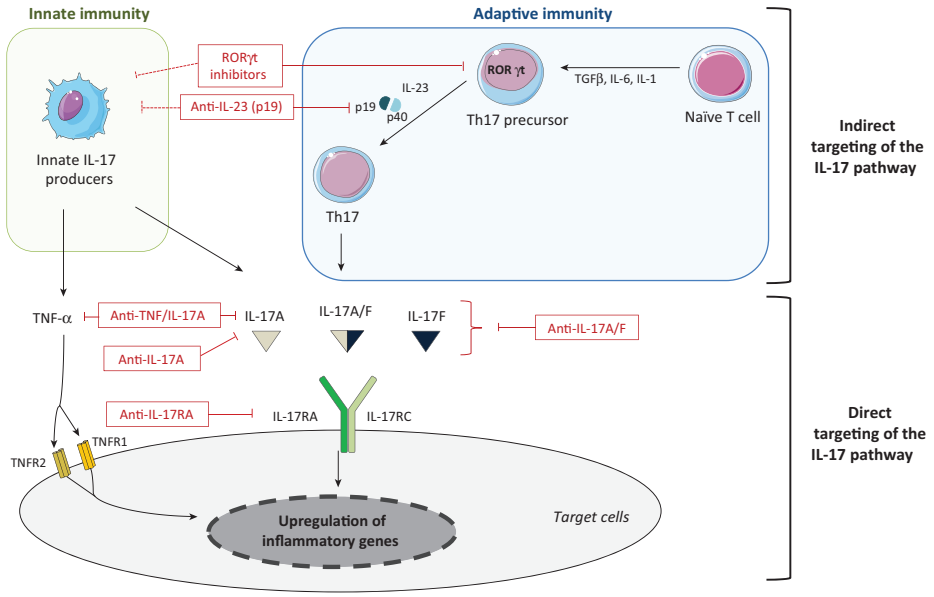
Based on the contribution of IL-17F to inflammation in addition to IL-17A, biomolecules targeting common motifs shared by IL-17A and IL-17F are now in clinical development, including the nanobody ALX-0761 and the mAb bimekizumab [61].

To target the IL-17 receptor, brodalumab (AMG 827) is a fully human IgG2 that selectively blocks signaling through the IL-17RA chain of the IL-17 receptor. This antibody inhibits human IL-17A, IL-17F, and IL-17C but also IL-25 (Figure 1).

Based on the synergistic interactions between TNFα and IL-17 described above, bispecific molecules are now in clinical development. ABT-122 is a dual variable domain immunoglobulin (DVD-Ig™) molecule with one site binding TNFα and the other IL-17A [62,63]. COVA322 is a fusion molecule comprising the fully human anti-TNFα antibody adalimumab with an anti-IL-17A fynomer [64].

Key Figure

Therapeutic Strategies for Targeting the Interleukin (IL)-17 and T helper 17 (Th17) Pathways



Antibody against IL-17A (Anti-IL-17A)	Antibody against IL-17A and IL-17F (Anti-IL-17A/F)	Antibody against TNFα and IL-17A (Anti-TNFα/IL-17A)	Antibody against IL-17 receptor type A (Anti-IL-17RA)
Secukinumab (Novartis)	Bimekizumab (UCB)	ABT-122 (AbbVie)	Brodalumab (Valeant)
Ixekizumab (Lilly)	ALX-122 (Merck Serono/Ablynx)	COVA322 (Janssen/Covagen)	
CNTO 6785 (Janssen)			
CJM112 (Novartis)			
BCD 085 (BIOCAD)			

Trends in Molecular Medicine

Figure 2. The IL-17 pathway can be targeted directly using monospecific antibodies against IL-17A (anti-IL-17A) and IL-17 receptor (IL-17R)A. Bispecific antibodies can target IL-17A and tumor necrosis factor alpha (TNFα) or IL-17A and IL-17F. The IL-17 pathway can be targeted indirectly by acting on the differentiation of Th17 cells with inhibitors of IL-23 using a specific antibody against the IL-23 p19 subunit and with inhibitors of the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt).

Indirect Targeting of the IL-17 Pathway

IL-23 acts upstream of IL-17. It is a heterodimeric ligand comprising the IL-23-specific p19 subunit and the common p40 subunit shared with IL-12. Specific inhibitors of IL-23 described as IL-23p19 antibodies include tildrakizumab (MK-3222, SCH-900222), guselkumab (CNTO 1959), AMG 139, LY3074828, and BI 655066 [61]. These inhibitors act on IL-17A, IL-17F, IL-21, and IL-22 production.

RORγt controls the differentiation of Th17 cells and its targeting will reduce the production of IL-17A, IL-17F, IL-21, and IL-22. Several small molecules such as the synthetic ligand SR1001 can bind RORγt and suppress its transcriptional activities *in vitro* and in mouse models such as experimental autoimmune encephalomyelitis [65–67]. They are now at an early stage of development.

Clinical Results with Inhibitors of IL-17 or IL-17R

A summary of the clinical results with antibodies directly targeting the IL-17 pathway is shown in Table 1.

Psoriasis

The key clinical marker for psoriasis response is the Psoriasis Area and Severity Index (PASI), which measures changes in skin lesion area from baseline. Secukinumab has been evaluated in psoriasis, with the largest number of clinical trials. The first report, published in 2010, showed that the percentage of patients achieving PASI 75 at week 12 was higher with secukinumab than with placebo [68]. Importantly, and for the first time, a drug could achieve a PASI 100 response. Using the same read-out, secukinumab was found to be superior to two registered drugs for psoriasis: etanercept, a TNFα inhibitor [69], and ustekinumab, an inhibitor of the p40 common chain shared by IL-12 and IL-23 [70]. The FDA and EMA approved this anti-IL-17 in 2015 for the treatment of adult patients with moderate to severe plaque psoriasis, with an initial dose of 300 mg subcutaneously at weeks 0, 1, 2, 3, and 4 followed from week 8 by 300 mg once monthly.

The first results with ixekizumab (anti-IL-17A mAb), published in 2012, showed that 40% of patients on the drug achieved PASI 100 versus 0% with placebo [71]. Two recent Phase III clinical studies confirmed these results compared with placebo and etanercept [72]. The first results with brodalumab (anti-IL-17RA antibody), published in 2012, showed that 75–80% of patients on the drug achieved PASI 90 versus 0% with placebo [73]. The results were independent of the presence of arthritis [74]. In a recent Phase III trial, a PASI 100 response rate was possibly more common with brodalumab than with ustekinumab [75]. Based on these results, a dossier for registration has been submitted for ixekizumab and brodalumab.

Regarding the inhibition of IL-23, rather similar results have been seen with guselkumab, which was later found to be more active than adalimumab, a TNFα inhibitor [76,77]. Tildrakizumab showed efficacy against placebo in Phase I and II trials [78,79].

PsA

In addition to the PASI score for skin, PsA response is evaluated in part with the American College of Rheumatology 20 response rate (ACR20), which measures percentage change in disease activity from baseline.

In the first Phase II trial, the response to secukinumab did not meet the ACR20 primary end point [80]. However, this was not the case in two larger Phase III trials, which also showed an effect on radiographic joint damage [81,82]. Now, secukinumab is EMA and FDA-approved for PsA results with ixekizumab and with brodalumab also demonstrated a higher ARC 20 response rate with the drug [83].

AS

In AS, response to treatment is based on the level of improvement of the Assessment of Spondyloarthritis International Society criteria (ASAS20, 50, 70), which measures percentage change in disease activity from baseline.

In a Phase II study, the response to secukinumab was rapid, since at week 6 the ASAS20 response rate was 59% with secukinumab versus 24% with placebo [84]. In a longer follow-up of up to 2 years, sustained clinical improvement was accompanied by regression of spinal inflammation [85]. Secukinumab is now EMA and FDA-approved for AS.

RA

RA response rates to treatment are mostly evaluated using ACR and Disease Activity Score 28 (DAS28) response rates.

The first results on secukinumab in RA, published in 2010, showed positive results based on the ACR20 clinical response [68]. In another Phase II trial, the same primary efficacy end point was not achieved at week 16 [86]. In a 52-week trial, RA patients who failed to respond to methotrexate and other biologics showed improvement after long-term treatment with secukinumab, with better patient-reported outcomes [87,88]. Analysis of individual response rates showed a high degree of heterogeneity. In a subanalysis, genetic markers associated with the MHC type I polymorphism *HLA-DRB1** shared epitope and with high rheumatoid factor levels (but not with anti-CCP antibody positivity) were linked to a better clinical response [89].

Ixekizumab was administered in a first in-human Phase I trial in patients with RA. The first results, published in 2010, showed better ACR and DAS28 indices with the drug than with placebo [90]. Similar conclusions were reached in a Phase II study in biologic-naïve patients and in patients with an inadequate response to TNFα inhibitors [91]. By contrast, no response was seen with brodalumab [92,93]. No explanation has been proposed for the lack of clinical effect of anti-IL-17RA brodalumab, but it is possible that, unlike anti-IL-17 antibodies, it might result in inhibition of the anti-inflammatory cytokine IL-25.

MS

The use of cytokine inhibitors in MS has been limited as the initial proof-of-concept trials with TNFα inhibitors in MS showed increased inflammatory lesions [94]. Although there are many studies employing the experimental autoimmune encephalomyelitis MS mouse model to support the inhibition of IL-17 [95], only secukinumab has been tested in MS. A Phase II trial showed a 60% decrease of new MRI lesions compared with placebo, with a trend of reduction of the annual relapse rate [96,97].

CD

Secukinumab was tested for CD in two Phase II studies. No positive effect was found; rather, treatment resulted in increased disease activity and a higher rate of serious adverse events in some patients [98–100]. Two Phase II trials with brodalumab reached the same conclusion [84]. These negative results may be explained by the protective function of IL-17 in the intestine and the differential contributions of IL-23 and IL-17, as suggested by mouse models of colitis [101].

Other Diseases

The extent of information on other conditions is limited. The effects of secukinumab and brodalumab on various symptoms, including lung function in asthma, do not support an important contribution of IL-17 in clinical improvement [102]. Secukinumab did not appear to influence the severity of dry-eye syndrome [103].

Adverse Events

As predicted from the role of IL-17 in host defense and neutrophil biology, the rate of mild or moderate infections in patients treated with IL-17 inhibitors has been generally higher [99]. Also as predicted, *Candida* infections have been more common in patients treated with IL-17A and IL-17RA inhibitors [69,72]. However, the severity has been documented as being lower than in patients with genetic defects affecting the IL-17 pathway [33]. Cases of reduced neutrophil count have also been reported [73]. Importantly, cases of tuberculosis reactivation, a problem noted with TNF inhibition, have not been reported with IL-17 inhibition [99].

Cases of stroke and myocardial infarction have been observed in a recent study of secukinumab in PsA but not in other diseases, or on treatment with other drugs [82]. In addition, suicidal ideation and behaviors were observed in patients taking brodalumab for psoriasis [104]. No mechanism has been proposed for this unpredicted adverse event, which has not been seen with the other IL-17A inhibitors. Only post-authorization safety studies and real-life use of the

drugs in large numbers of patients will allow a full assessment of the safety profile and the risk: benefit ratio.

Concluding Remarks

Inhibition of IL-17A and IL-17RA has already provided a major improvement in the care of psoriasis, achieving a level of response not seen before. Drug registration has been obtained for PsA and AS. These are already impressive achievements for a molecule discovered in 1995 and identified as a clinical target in 1999.

Other options based on IL-17 biology are now being tested, including bispecific antibodies against IL-17A and IL-17F and against TNFα and IL-17A, which may be of interest in cases of observed lack or loss of response to anti-TNFα therapy [43]. In parallel, molecules targeting Th17 cells and related pathways are under active development. Differences in efficacy and tolerance are already emerging among these options.

It is clear, however, that a better understanding of patient heterogeneity will be needed to achieve accurate and improved personalized medicine (see Outstanding Questions). Moreover, additional research is needed to identify patients with IL-17-driven diseases as well as the various components that contribute to those diseases [105]. Nevertheless, the process has begun.

Outstanding Questions

What are the respective contributions of Th17 cells versus other IL-17-producing cells in an inflammatory response?

What are the positive and negative long-term consequences of IL-17 pathway inhibition?

As the IL-17R antibody brodalumab blocks several IL-17 family cytokines, including the anti-inflammatory IL-25, will this inhibitor show a gain of effectiveness and/or more side effects compared with anti-IL-17A antibodies?

What are the positive and negative consequences of IL-17A versus IL-17A and IL-17F inhibition?

The bispecific anti-TNFα/IL-17 antibodies are promising biotherapies because of the common synergistic interactions between these two cytokines. Will blocking both TNFα and IL-17 exacerbate the risk of infection? As the bispecific anti-TNFα/IL-17 antibodies are already in clinical trials, is there a benefit to combining an anti-TNFα drug with an anti-IL-17 drug to modify the dose or sequence of administration?

The therapeutic response of IL-17 pathway inhibitors appears to be heterogeneous in diseases such as RA. Regarding safety and efficacy, which criteria or biomarkers would be useful in selecting patients for IL-17 pathway inhibitor trials?

What is the contribution of IL-17 in other diseases such as lupus, scleroderma, and vasculitis? Could IL-17 pathway inhibitors be useful in these disorders?

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Si l'inhibition de l'IL-17 fournit une nette amélioration chez les patients psoriasiques, il existe une large hétérogénéité dans la réponse chez les patients atteints de PR. Les thérapies actuelles sont donc toujours largement étudiées et perfectionnées afin d'améliorer la qualité de vie des patients ainsi que de limiter les effets secondaires. Les stéroïdes sont ainsi la thérapie anti-inflammatoire la plus ancienne et la plus classique utilisée dans le cadre de la PR. Le méthotrexate (MTX) est également un traitement clé dans la PR, souvent en association avec les stéroïdes. Toutefois, ces deux thérapies engendrent divers effets secondaires, et peuvent conduire à une non-réponse ou à une résistance face au traitement. De plus récentes thérapies, les biothérapies, sont alors mises en jeu, en combinaison ou non avec les stéroïdes et le MTX. Les études actuelles se focalisent sur l'aspect clinique de ces traitements et peu de choses sont documentées concernant l'aspect cellulaire. Le chapitre suivant traite donc des effets des différentes thérapies utilisées couramment au cours du traitement de la PR sur la production de cytokines résultant des interactions cellulaires, en se concentrant sur les stéroïdes et les biothérapies.

II Effet des biothérapies sur l'inflammation, vision cellulaire

Article: "Differential effects of arthritis-related biotherapies in an *in vitro* co-culture model with immune cells and synoviocytes"

Effets différentiels des biothérapies liées à l'arthrite dans un modèle *in vitro*, utilisant des cellules immunitaires et des synoviocytes

Résumé :

Introduction : Au cours des maladies inflammatoires chroniques, telles que la PR, les stéroïdes, comme la méthylprednisolone (MP), et les biothérapies sont couramment utilisés. Les études actuelles se concentrent surtout sur l'aspect clinique délaissant les effets au niveau cellulaire. Dans le contexte de la PR, le but de ce travail était d'étudier les effets de différents traitements au niveau cellulaire, en utilisant un modèle *in vitro* d'interactions entre CSM et PBMC.

Méthodes : Des PBMC de donneurs sains sont mises en co-culture avec des synoviocytes de patients atteints de PR pendant 48h, en présence d'une activation par PHA. Une dose-réponse de MP (0.001, 0.01, 0.1, 1 et 10 µg/ml) ainsi que différentes biothérapies (Infliximab, Tocilizumab, Abatacept et Rituximab) à deux concentrations (10 et 100 µg/ml) ont été testées dans ce système. La combinaison MP/biothérapie a également été ajoutée aux co-cultures (0.01 µg/ml et 10 µg/ml, respectivement). La production de plusieurs cytokines (IL-17, IL-6, IL-1β, IFN-γ et IL-10) a été mesurée par ELISA après 48h de culture.

Résultats : L'addition de MP dans les co-cultures inhibait la production de cytokines. La sécrétion d'IL-17 et d'IL-6 était inhibée de manière dose-dépendante alors que l'IL-1β, l'IFN-γ et l'IL-10 étaient réduites dès la dose la plus faible de 0.001 µg/ml. La réponse aux différentes biothérapies était très dépendante de chaque traitement. La production d'IL-17 était inhibée seulement par le Tocilizumab ($p=0.004$) et de manière dose-dépendante ($p=0.04$), alors que la sécrétion d'IL-6 était diminuée seulement par l'Infliximab ($p\leq0.002$) sans dose-réponse. La concentration d'IL-1β était réduite dans toutes les conditions ($p\leq0.03$). Le Tocilizumab et le Rituximab inhibaient la production d'IFN-γ seulement à 10µg/ml

($p \leq 0.03$) alors que l'Abatacept avait un effet seulement à 100 µg/ml ($p=0.01$). L'Infliximab réduisait clairement la production d'IFN-γ aux deux concentrations ($p=0.004$). La sécrétion d'IL-10 était principalement inhibée par l'Infliximab et le Tocilizumab ($p \leq 0.004$). La combinaison MP/biothérapie ne montrait pas d'effet additionnel sur l'inhibition de la production de cytokines pro-inflammatoires résultant des interactions cellulaires. L'effet inhibiteur de la MP pouvait même être annulé par l'addition de biothérapie, surtout pour l'IFN-γ. Concernant la cytokine anti-inflammatoire IL-10, en présence de la combinaison MP/biothérapie, sa concentration était plus faible que le contrôle mais plus élevée qu'en présence de MP seule, principalement pour le Rituximab.

Conclusion : Les stéroïdes inhibent clairement la sécrétion de cytokines pro-inflammatoires, à partir de très faibles doses. L'effet anti-inflammatoire au niveau cellulaire des biothérapies est dépendant de leur mécanisme d'action. La combinaison MP/biothérapie n'améliore pas l'effet inhibiteur sur les cytokines pro-inflammatoires mais pourrait plutôt avoir un effet bénéfique en agissant sur la sécrétion d'IL-10, cytokine anti-inflammatoire. Toutefois, la réponse IL-10 présente une certaine hétérogénéité liée aux différents donneurs. Il serait intéressant d'analyser l'association possible de cette hétérogénéité au polymorphisme de l'IL-10, déjà associé à la PR.

Differential Effects of Steroids and Arthritis-Related Biotherapies in an *in vitro* Co-culture Model with Immune Cells and Synoviocytes

Running title: Steroids and biotherapies on cell interactions

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Abstract

Background: During rheumatoid arthritis (RA), steroids and biotherapies are used alone and combined. Efficacy has been established in clinical trials but their differential effects at the cellular level are less documented. Thus, the aim was to study these cellular effects by using an *in vitro* model with synoviocytes interacting with peripheral blood mononuclear cells (PBMC) to reproduce the interactions in the RA synovium.

Methods: Activated-PBMC were co-cultured with RA synoviocytes during 48h. A dose-response of methylprednisolone (MP) was tested and different biotherapies (Infliximab, Etanercept, Adalimumab, Tocilizumab, Abatacept and Rituximab) were added alone or in combination with MP. Cytokine production (IL-17, IL-6, IL-1β, IFN-γ and IL-10) was measured by ELISA.

Results: Addition of MP to co-cultures inhibited the production of all cytokines. The response to the biotherapies alone was treatment-dependent. IL-17 production was inhibited only by Tocilizumab ($p=0.004$) while IL-6 was decreased only by Infliximab ($p\leq0.002$). IL-1β level was affected in all conditions ($p\leq0.03$). IFN-γ production was mainly decreased by Infliximab ($p=0.004$) and IL-10 by Infliximab and Tocilizumab ($p\leq0.004$). The combination MP and biotherapy did not induce an additional effect; even it could cancel the inhibitory effect of MP, mainly for IFN-γ. The combination MP and biotherapy induced a higher IL-10 secretion than MP alone, mainly with Rituximab.

Conclusion: Steroids inhibited clearly the secretion of all cytokines, and low doses were as potent. The anti-inflammatory effect of biotherapies was dependent on their mechanism of action. MP and biotherapy combination did not enhance the inhibitory effect on pro-inflammatory cytokines but could have a beneficial effect by increasing IL-10 production.

Keywords: methylprednisolone, biotherapies, rheumatoid arthritis, cell interaction, pro-inflammatory cytokines

Introduction

Rheumatoid arthritis is a chronic inflammatory disease leading to joint destruction (1). If its aetiology is not well defined, currently, different treatments are available to treat the clinical symptoms. Steroids (also named glucocorticoids or corticosteroids) are the oldest and the most classical anti-inflammatory therapy used for many chronic inflammatory diseases other than RA. They act by inhibiting multiple inflammatory genes (encoding cytokines, chemokines…) that are activated during chronic inflammation (2-4). However their use is associated with adverse events mainly infections specifically at high dosage. Moreover, non-responsiveness or resistance can be observed (5). Another key drug is methotrexate (MTX), the most common treatment of RA. MTX improves clinical parameters in RA patients but severe adverse events could lead to discontinuation (6-8).

The heterogeneity of the response to steroids and MTX has led to a combination therapy with biotherapies. In RA, anti-TNF is the most used biotherapy. Many studies described the improvement of symptoms and physical functions (9). Nevertheless, adverse events, mainly elevated risk of infections (10) are known and prevention measures are in place. Furthermore, biotherapies need to be adjusted to each patient because of the response. Current studies focus on the clinical level of the treatment efficacy but the mode of action at the cellular level is less documented.

In the RA joint synovium, the inflammation leads to the recruitment of immune cells that interact with local mesenchymal cells or synoviocytes. These interactions contribute to the chronicity of inflammation, notably by increasing the pro-inflammatory cytokine production and the cell survival of synoviocytes (11-13). In our previous studies, the effects of cell interactions on pro-inflammatory cytokine production were studied by using an *in vitro* model of co-culture between mesenchymal cells and peripheral blood mononuclear cells. These studies showed that cell interactions were critical for massive pro-inflammatory cytokine secretion. The use of an autologous system validated this model as mimicking the *in vivo* situation (14, 15).

Herein, in the RA inflammatory context, the aim was to compare the effects of all the current biotherapies against TNF, IL-6, CD20 and CTLA4, with or without steroids by using this *in vitro* cell-cell interaction model looking at cytokine production.

Materials and methods

Samples

RA synoviocytes were obtained from synovial tissue of RA patients undergoing joint surgery and who fulfilled the American College of Rheumatology criteria for RA (16). Synovial tissue was minced into small pieces and then adhered in 6-well plates in Dulbecco's modified Eagle's medium (DMEM; Eurobio, Courtaboeuf, France) supplemented with 10% foetal bovine serum (FBS; Life Technologies, Carlsbad, USA), 2mM L-glutamine and 100U/ml penicillin/streptomycin. Cells were maintained at 37°C in a humidified 5% carbon dioxide incubator and used between passages 4 to 9. PBMC from healthy donors or RA patients were isolated by Ficoll-Hypaque (Eurobio, Courtaboeuf, France) density-gradient centrifugation. Each individual signed an informed consent form. The protocol was approved by a committee for the protection of persons participating in biomedical research.

Co-culture assays

Co-culture was initiated by seeding RA synoviocytes overnight in 96-well plates at a density of $2x10^4$ cells/well in RPMI 1640 medium (Eurobio, Courtaboeuf, France) supplemented with 10% human AB serum, 2mM L-glutamine and 100U/ml penicillin/streptomycin (complete RPMI). The next day, PBMC ($1x10^5$ cells/well) were pre-incubated in complete RPMI with or without different treatments and then seeded corresponding to 5:1 ratio, in the presence of phytohemagglutinin (PHA, 5µg/ml). After 48h, supernatants and PBMC were collected for analysis (17).

Treatments

A dose-response of Methylprednisolone (MP) was done with 0, 0.001, 0.01, 0.1, 1 and 10 µg/ml. The concentration of 0.01 µg/ml was used in combination with biotherapies. Two concentrations of biotherapies were used in co-culture, 10 and 100 µg/ml. Infliximab (Remicade, anti-TNF), Tocilizumab (Roactemra, anti-IL-6 receptor), Abatacept (Orencia, CTLA4 Ig), Rituximab (Mabthera, anti-CD20), Etanercept (Enbrel, anti-TNF) and Adalimumab (Humira, anti-TNF) were tested.

Enzyme-linked immunosorbent assays (ELISAs)

IL-17A, IL-6, IL-1β, IFN-γ and IL-10 production was evaluated from culture supernatants with commercially available Duoset ELISA kits, according to the manufacturer's instructions (R&D system, Minneapolis, USA).

Statistical analysis

Statistical analyses were performed using paired Wilcoxon test. All analyses were performed with Graph Pad Prism 6 software. *p* values less than or equal to 0.05 were considered as significant.

Results

Principle of the *in vitro* model

During chronic inflammatory arthritis, immune cells infiltrate the inflammatory synovium, and interact with the local mesenchymal cells, as synoviocytes. This promotes the secretion of various cytokines and chemokines. To mimic this event, an *in vitro* model of co-culture was used. In the RA context, synoviocytes from patients were cultured in the presence of activated-PBMC. These cell-cell interactions lead to massive cytokine production. In control condition, the cell contact between synoviocytes and PBMC is sufficient to induce IL-6 and IL-1β production. A high IL-17 secretion is obtained in co-culture with activated-PBMC (14). Using this model, the effect of treatments on cytokine production resulting from cell interactions could be studied (figure 1).

Effects of Methylprednisolone

The effect of MP was evaluated in the co-culture system, using a dose-response curve. Different concentrations of MP were tested: 0, 0.001, 0.01, 0.1, 1 and 10 µg/ml. The impact on pro- and anti-inflammatory cytokine production was measured in the supernatants of cultures after 48h. As observed in figure 2, IL-17 secretion was significantly decreased from 0.1 µg/ml (62.3 ± 24.7 vs. 112.8 ± 33.8 pg/ml, *p=0.03*) but there was already a decreased production starting from 0.01 µg/ml (76.6 ± 27.8 vs. 112.8 ± 33.8 pg/ml, *p=0.06*). The inhibition followed a dose-response. IL-6, IL-1β and IFN-γ production significantly decreased starting with the lowest concentration of 0.001 µg/ml (234.7 ± 30.2 vs. 291.8 ± 20.2 ng/ml, for IL-6; 90.4 ± 31.7 vs. 230.5 ± 77.8 pg/ml, for IL-1β, *p=0.008*; 234.4 ± 136.5 vs. 552.3 ± 217.6 pg/ml, for IFN-γ, *p=0.03*). The dose effect was clear for IL-6 secretion and almost for IL-1β; nevertheless for IFN-γ the maximum effect was reached from the lowest concentration. If MP inhibited well the production of pro-inflammatory cytokines, the secretion of the anti-inflammatory cytokine IL-10 was also inhibited by the addition of MP. This decreased production was significant from the lowest concentration of 0.001 µg/ml (32.0 ± 12.8 vs. 70.8 ± 23.6 pg/ml, *p=0.02*) and was not clearly dose-dependent.
These results confirmed the anti-inflammatory potential of MP in *in vitro* system, but showed that MP also decreased the production of anti-inflammatory cytokine IL-10.

Effects of biotherapies alone

The effects of the current biotherapies for RA treatment were first tested alone. After preliminary experiments, two doses, 10 and 100 µg/ml, were selected and tested for their effect on cytokine production. As shown in figure 3, the effects of the addition of biotherapies in co-culture were measured on the production of the pro-inflammatory cytokines IL-17, IL-6, IL-1β and IFN-γ and the anti-inflammatory cytokine IL-10.

Infliximab is a monoclonal antibody which binds TNF. Infliximab decreased the production of IL-17 at 10 µg/ml, without reaching significance (78.5 ± 22.1 vs. 94.8 ± 26.1 pg/ml, *p=0.07*, figure 3A) and also at 100 µg/ml (70.3 ± 24.3 vs. 94.8 ± 26.1 pg/ml, *p=0.055*, figure 3A). On the other hand, the IL-6 production was decreased around 30-35%, without a clear dose-response (223.7 ± 23.1 vs. 321.1 ± 30.1 ng/ml, for 10 µg/ml, *p=0.001*; 209.2 ± 27.5 vs. 321.1 ± 30.1 ng/ml, for 100 µg/ml, *p=0.002*, figure 3A). The secretion of IL-1β and IFN-γ was the most inhibited by the addition of Infliximab, with a decrease around 60-70% (100.8 ± 28.1 vs. 250.7 ± 66.7 pg/ml, for 10 µg/ml and 74.5 ± 26.8 vs. 250.7 ± 66.7 pg/ml, for 100 µg/ml, for IL-1β, *p=0.002*; 166.0 ± 67.9 vs. 532.0 ± 163.9 pg/ml, for 10 µg/ml and 158.0 ± 55.9 vs. 532.0 ± 163.9 pg/ml, for 100 µg/ml, for IFN-γ, *p=0.004*, figure 3A). Furthermore, Infliximab also decreased by about 30% the production of IL-10 (46.3 ± 18.7 vs. 62.8 ± 17.5

pg/ml, for 10 µg/ml, *p=0.05*; 38.0 ± 16.2 vs. 62.8 ± 17.5 pg/ml, for 100 µg/ml, *p=0.002*, figure 3A).

Tocilizumab is a monoclonal antibody which binds the IL-6 receptor. Tocilizumab inhibited significantly the production of IL-17 either at 10 µg/ml (62.0 ± 19.4 vs. 94.8 ±26.1 pg/ml, *p=0.004*, figure 3B) either at 100 µg/ml (53.7 ± 16.7 vs. 94.8 ± 26.1 pg/ml, *p=0.004*, figure 3A), with a dose effect (*p=0.04*, figure 3B). While IL-1β production was also significantly decreased by the addition of Tocilizumab (106.5 ± 30.8 vs. 250.7 ± 66.7 pg/ml, for 10 µg/ml; 105.1 ± 30.4 vs. 250.7 ± 66.7 pg/ml, for 100 µg/ml, *p=0.002*, figure 3B), without a clear dose effect, the secretion of IL-6 was similar to the control (293.4 ± 33.1 vs. 321.1 ± 30.1 ng/ml for 10 µg/ml; 293.3 ± 34.9 vs. 321.1 ± 30.1 ng/ml, for 100 µg/ml, figure 3B). The IFN-γ secretion was significantly reduced with 10 µg/ml (340.1 ± 105.1 vs. 532.0 ± 163.9 pg/ml, *p=0.03*, figure 3B) but without reaching significance with 100 µg/ml (370.2 ± 137.0 vs. 532.0 ± 163.9 pg/ml, *p=0.09*, figure 3B), suggesting that high dose is not necessarily more efficient. The production of IL-10 was also significantly decreased by the addition of Tocilizumab (37.9 ± 12.6 vs. 62.8 ± 17.5 pg/ml, for 10 µg/ml; 27.1 ± 9.2 vs. 62.8 ± 17.5 pg/ml, for 100 µg/ml, *p=0.002*, figure 3B).

Two biotherapies targeting specifically cells were also tested, Abatacept and Rituximab. Abatacept is a fusion protein (CTLA-4-Ig) which interacts with B7 (ligand of CD28 which is a T cell activation molecule). The addition of Abatacept did not affect IL-17 and IL-6 secretion at 10 µg/ml (96.1 ± 25.8 vs. 94.8 ± 26.1 pg/ml for IL-17; 309.5 ± 42.1 vs. 321.1 ± 30.1 ng/ml for IL-6, figure 3C) and at 100 µg/ml (83.2 ± 24.5 vs. 94.8 ± 26.1 pg/ml for IL-17; 291.9 ± 40.6 vs. 321.1 ± 30.1 ng/ml for IL-6, figure 3C). The production of IL-1β was significantly decreased by about 45%, without a dose-effect (143.1 ± 40.4 vs. 250.7 ± 66.7 pg/ml, for 10 µg/ml; 132.0 ± 40.5 vs. 250.7 ± 66.7 pg/ml, for 100 µg/ml, *p=0.002*, figure 3C). IFN-γ secretion was altered in a dose-dependent pattern, as there was a significant decrease between 10 and 100 µg/ml (356.6 ± 123.4 vs. 274.7 ± 95.2 pg/ml, respectively, *p=0.04*, vs. 532.0 ± 163.9 pg/ml for the control, figure 3C). IL-10 was slightly decreased only with the higher dose of 100 µg/ml (48.9 ± 13.1 vs. 62.8 ± 17.5 pg/ml, *p=0.03*, figure 3C).

Rituximab is a monoclonal antibody against CD20. As observed in figure 3D, Rituximab had no effect on the secretion of IL-17, IL-6 and IL-10. The concentration of 10 µg/ml decreased significantly IL-1β production (188.8 ± 60.6 vs. 250.7 ± 66.7 pg/ml, *p=0.04*) and also IFN-γ secretion (370.2 ± 137.0 vs. 532.0 ± 163.9 pg/ml, *p=0.02*). At 100 µg/ml, their production was not significantly decreased and as for Tocilizumab, this suggests that increasing the dose may not be necessary and even could have an opposite effect.

In summary, a global inhibitory effect of all biotherapies was observed but with differences between treatments. Infliximab and Tocilizumab had an effect higher and extended than Abatacept and Rituximab. This could be explained by a broader effect of Infliximab and Tocilizumab acting on cytokines and their receptors contrasting with the cell specificity of Abatacept and Rituximab.

Effects of various anti-TNF comparison

Various inhibitors of TNF are currently used with different modes of action. Infliximab was compared to two other TNF inhibitors, Etanercept, a soluble receptor, and Adalimumab, a monoclonal antibody like Infliximab. As observed in figure 4, at both concentrations, only Adalimumab decreased significantly the secretion of IL-17 by about 30% (70.1 ± 24.0 vs. 99.2 ± 28.4 pg/ml for 10 µg/ml; and 67.1 ± 22.0 vs. 99.2 ± 28.4 pg/ml for 100 µg/ml, *p=0.008*). The three biotherapies had a similar effect on IL-6 and IFN-γ, with an inhibition by about 30% and 70%, respectively, with no clear dose effect (Figure 4). For IL-1β, Infliximab seemed the most effective at both concentrations, with a significant higher efficiency at 100

µg/ml (51.8 ± 15.8 vs. 230.3 ± 70.3 pg/ml for Infliximab, *p=0.004*; 133.0 ± 49.1 vs. 230.3 ± 70.3 pg/ml for Etanercept, *p=0.004*; and 138.4 ± 52.0 vs. 230.3 ± 70.3 pg/ml for Adalimumab, *p=0.004*). The three anti-TNF inhibited significantly the IL-10 production, without dose-effect. A difference between the three biotherapies was observed, significantly only at 10 µg/ml. Infliximab decreased less IL-10 production (47.0 ± 20.7 vs. 63.1 ± 19.3 pg/ml for 10 µg/ml, *p=0.07*; and 39.4 ± 17.9 vs. 63.1 ± 19.3 pg/ml for 100 µg/ml, *p=0.004*), followed by Etanercept (31.2 ± 14.5 vs. 63.1 ± 19.3 pg/ml for 10 µg/ml, *p=0.03*; and 25.8 ± 15.5 vs. 63.1 ± 19.3 pg/ml for 100 µg/ml, *p=0.008*); and the most inhibitory effect was obtained with Adalimumab (18.7 ± 9.0 vs. 63.1 ± 19.3 pg/ml for 10 µg/ml, *p=0.004*; and 18.9 ± 9.9 vs. 63.1 ± 19.3 pg/ml for 100 µg/ml, *p=0.004*). Overall, the three anti-TNF displayed a similar effect. Nevertheless, Infliximab was the more potent on IL-1β inhibition and less than the others on IL-10 secretion.

Combination of MP and biotherapies

The effect of treatment combination is not well understood in patients and thus the basis for combining drugs remains unclear. The effect of MP and biotherapies, alone or in combination was compared. The lowest effective doses of MP, 0.01 µg/ml and of biotherapies, 10 µg/ml, were used alone or in combination to evaluate their impact on cytokine production. MP alone inhibited significantly the IL-17 production (58.9 ± 23.4 vs. 94.8 ± 26.1 pg/ml, *p=0.02*, figure 5A) as Tocilizumab alone (62.0 ± 19.4 vs. 94.8 ± 26.1 pg/ml, *p=0.004*, figure 5A). Infliximab, Abatacept and Rituximab alone did not inhibit the IL-17 production (Figure 5A). In combination, the inhibitory effect of MP and Tocilizumab was not increased and the inhibition was still around 40% (56.5 ± 20.6 vs. 94.8 ± 26.1 pg/ml, *p=0.004*, figure 5A). The combination of MP and Infliximab or Abatacept tended to increase the IL-17 production compared to MP alone (73.3 ± 20.8 pg/ml and 85.7 ± 27.8 pg/ml, respectively, vs. 58.9 ± 23.4 pg/ml, *p=0.07*, figure 5A). The combination of MP and Rituximab significantly inhibited the MP effect and the level of IL-17 was similar to the control (102.3 ± 30.6 vs. 94.8 ± 26.1 pg/ml, *p=0.03*, figure 5A).

For IL-6 secretion, MP and Infliximab alone decreased significantly IL-6 secretion compared to control (167.7 ± 21.0 pg/ml and 78.5 ± 22.1 pg/ml, respectively, vs. 321.1 ± 30.1 pg/ml, *p=0.001*, figure 5B); and the combination of both increased significantly the inhibitory effect compared to MP alone (131.3 ± 13.6 vs. 167.7 ± 21.0 pg/ml, *p=0.04*, figure 5B). The combination of MP with other biotherapies (Tocilizumab, Abatacept and Rituximab) did not change the decreased IL-6 production induced by MP alone (Figure 5B).

IL-1β production was decreased in a similar way between MP alone, Infliximab, Tocilizumab or Abatacept alone and the combination of both (Figure 5C). Nevertheless, Rituximab alone inhibited IL-1β secretion but to a less extent that MP alone, compared to control (188.8 ± 60.6 pg/ml, *p=0.04*, and 104.5 ± 59.2 pg/ml, *p=0.002*, respectively, vs. 250.7 ± 77.8 pg/ml); and the combination of both cancelled the large inhibitory effect of MP alone (218.4 ± 70.4 vs. 104.5 ± 59.2 pg/ml, *p=0.03*, figure 5C).

Except Abatacept, biotherapies (Infliximab, Tocilizumab and Rituximab) or MP alone decreased significantly the production of IFN-γ compared to control (Figure 5D). The combination of MP and Infliximab led to a similar inhibition than with MP alone (252.2 ± 110.6 vs. 254.2 ± 169.2 pg/ml compared to 532.0 ± 163.9 pg/ml for control, figure 5D). The combination of MP with Tocilizumab and Rituximab significantly reduced the inhibition of IFN-γ secretion compared to MP alone (389.3 ± 159.4 pg/ml, *p=0.03*, and 415.1 ± 178.1 pg/ml, *p=0.01*, respectively, vs. 254.2 ± 169.2 pg/ml, figure 5D) and it was the same tendency for combination of MP and Abatacept (*p=0.08*, figure 5D).

Herein, the results showed that the combination even at low doses did not necessarily result in an additional effect on the inhibition of cytokine production.

Effect on IL-10 secretion

Given some unexpected results on pro-inflammatory cytokines with the combination of MP and biotherapy, the next step was to detect a potential effect on the anti-inflammatory cytokine IL-10. The dose of 0.01 µg/ml of MP alone decreased IL-10 production compared to control (28.0 ± 9.1 vs. 62.8 ± 17.5 pg/ml, *p=0.01*, figure 6). Infliximab and Tocilizumab alone inhibited significantly the IL-10 secretion (46.3 ± 18.7 pg/ml, *p=0.05*, and 37.9 ± 12.6 pg/ml, *p=0.002*, respectively, vs. 62.8 ± 17.5 pg/ml, figure 6) while Abatacept and Rituximab did not affect the IL-10 level compared to control (63.8 ± 23.7 pg/ml and 61.6 ± 21.2 pg/ml, respectively, vs. 62.8 ± 17.5 pg/ml, figure 6). In the presence of the combination of MP and biotherapy, the IL-10 level was lower than control but higher than MP alone, mainly with Rituximab, without reaching significance (48.1 ± 16.3 vs. 28.0 ± 9.1 pg/ml, *p=0.3*, figure 6). These results indicated that the beneficial effect of combination of MP and biotherapy could come from a reduction of the inhibitory effect of MP on IL-10 production.

Discussion

In this study, the interest is focused on the effects of steroids and biotherapies alone and combined on cytokine production resulting from cell interactions between mesenchymal cells and immune cells.

Steroids are very effective anti-inflammatory drugs but the adverse effects limit their use. There is no doubt that steroids present a strong potential for frequent and serious side effects, increasing with longer use and higher dosage (18). Side effects of chronic steroid therapy are dose-related. At a low-dose (<7.5mg/day), many studies suggest that steroid treatment is relatively safe in RA (19, 20) and even a very low dose (<5mg/ml), can be sufficient to maintain remission without severe adverse events (21) but a better clinical response (22-25). To identify their effects at the cellular level, an *in vitro* model based on interactions between mesenchymal cells and PBMC was used to mimic the *in vivo* inflammatory state. A dose-response with MP was done to evaluate its effects on cytokine production. The production of pro-inflammatory cytokines was clearly inhibited by the addition of MP in co-culture with differences between cytokines. If IL-17 and IL-6 production decreased according to a dose-response, IL-1β and IFN-γ secretion was already inhibited with the lowest concentration of 0.001µg/ml. A low dose of MP appears sufficient to induce an inhibitory effect on pro-inflammatory cytokine production. Furthermore, from 10 µg/ml the cytokine production, notably IL-6 and IL-1β, appeared to increase compared to 1 µg/ml. In the clinic, this would suggest high dosage of MP might not be needed to obtain its anti-inflammatory effect through cytokine inhibition and that high doses could have the opposite effect. In addition, the presence of MP even at the lowest dose also inhibited the secretion of IL-10. This may explain the increased risk of infection for the treated patients.

The use of biotherapies during chronic inflammatory diseases, such as RA, has been a major step in improved care. The benefits on clinical symptoms are well established while the effect at cellular levels is less documented. Our *in vitro* system demonstrated in previous studies that cell interactions between mesenchymal including synoviocytes from RA patients or skin fibroblasts from psoriatic patients and immune cells resulting in massive pro-inflammatory cytokine production as observed in the *in vivo* situation (14, 15). Our system was used to evaluate the effect of current biotherapies at cytokine production level.

TNF is a pro-inflammatory cytokine largely involved in the pathogenesis of RA (1, 26), notably by stimulating synoviocytes to produce IL-6. As expected, TNF inhibition led to a decreased production of IL-6 which is also a pro-inflammatory cytokine involved in the activation of T cell differentiation. The decrease of IL-6 level could result in the decreased IL-1β and IFN-γ levels. The most used anti-TNF treatments are Infliximab, Etanercept and Adalimumab and some studies showed differences in efficacy and adherence between them (27, 28). When compared at the cellular level, the three therapies displayed a similar inhibitory effect on pro-inflammatory cytokine production. The major difference appeared on IL-10 production, with Infliximab inhibiting less IL-10 production compared to the two others.

Two thirds of the RA patients respond to anti-TNF leaving space for other options (27). Tocilizumab is a recombinant humanized monoclonal antibody against the IL-6 receptor (IL-6R), preventing IL-6 binding to both membranous and soluble IL-6R (29, 30). In our *in vitro* system, Tocilizumab decreased significantly IL-1β and IL-17 secretion (26, 31, 32). Both IL-6 and IL-1β are involved in the Th17 differentiation. Thus the inhibition of IL-6R followed by the decrease of IL-1β could explain the observed inhibition of IL-17 secretion. The secretion of IL-6 was not decreased by the addition of Tocilizumab. Indeed, in RA patients, the serum level of IL-6 increases after Tocilizumab administration, as IL-6 does not bind its receptor (33). Nevertheless, its signalling pathway is well inhibited as confirmed by the decreased production of CRP in patients.

In addition to cytokine inhibition, other biotherapies target cells. In this way, two other biotherapies, Abatacept and Rituximab were tested on cytokine production. Abatacept is a fusion protein of the extracellular domain of CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and the Fc region of IgG1. It prevents from T cell activation by CD28 by increasing the inhibitory effect of CTLA-4 on T cell activation (34). In our *in vitro* system, the major inhibited cytokine was IL-1β. The decreased of IFN-γ production could be explained by the direct effect of Abatacept on T cell activation. In addition, IL-1β is involved in IFN-γ secretion by T cells (35, 36) and NK cells (37). Thus, Abatacept may have a direct effect on T cell activation and an indirect effect by inhibiting IL-1β production.

Rituximab is directed against CD20, and induces the death of B cells (38). Herein, Rituximab decreased only IL-1β and IFN-γ production, at low dose. As Rituximab acts directly on B cells, it could be interesting to study the *in vitro* effect on immunoglobulin production. Indeed, patients positive for anti-citrullinated protein antibodies (ACPA) appear to respond better to Rituximab (39-41).

Steroids and biotherapies are two different ways to diminish inflammation, by reducing cytokine production. The first conclusion is that high doses were not more efficient than low doses, for both steroids and biotherapies. The effect of their combination each at low dose is more complex. Addition of a biotherapy seemed to reduce the effect of MP alone. At the same time, the combination reduced the inhibitory effect on IL-10 production of the two treatments used alone. At the end, the combination may increase the net anti-inflammatory effects of IL-10 with a suppressor effect on numerous pro-inflammatory cytokines such as TNF, IL-1β or IL-6, all involved in RA pathogenesis. The next step will be to evaluate this time the effects of methotrexate alone and combined with biotherapies, as done routinely in patients with RA and other inflammatory diseases.

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Funding
Mélissa Noack is supported by the Institut Universitaire de France.
Ndiémé Ndongo-Thiam is supported by the IHU prometteur OPERA.
Pierre Miossec is a senior member of and supported by the Institut Universitaire de France.

Conflict of interests
The authors declare that they have no competing interests.

Author contributions
MN carried out the experiments and drafted the manuscript. NNT participated in the experiments and helped to revise the manuscript. PM conceived the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Figure legends

Figure 1. ***Principle of the*** **in vitro** ***co-culture model.***

Co-culture between synoviocytes and activated-PBMC leads to cell interactions promoting the secretion of cytokines. Cell-cell contact induces IL-1β, TNF or IL-10 production by monocytes. In turn, TNF and IL-1β stimulate synoviocytes to produce IL-6. Secreted cytokines influence T lymphocyte differentiation, as IL-6 and IL-1β for Th17 cells, and then interactions with synoviocytes promotes the release of their cytokine, as IL-17 which in turn stimulates synoviocytes. The co-culture reproduces the cell interactions existing in the inflammatory site and the cytokine environment. Using this model, the effect of treatments on cytokine production resulting from cell interactions can be studied.

Figure 2. ***Effect of the dose-response of Methylprednisolone on cytokine production*****.**

Healthy PBMC activated by PHA (5µg/ml) were pre-incubated or not with different doses of Methylprednisolone. Then, PBMC were co-cultured with RA synoviocytes at a ratio 5:1 for 48h. The production of IL-17, IL-6, IL-1β, IFN-γ and IL-10 in cell supernatants was measured by enzyme-linked immunosorbent assay (ELISA). *, $p \leq 0.05$. Results are represented as mean ± SEM, n=8.

Figure 3. ***Effect of biotherapies on cytokine production*****.**

Healthy activated-PBMC were pre-incubated or not with two doses of biotherapies, 10µg/ml or 100µg/ml and co-cultured with RA synoviocytes at a ratio 5:1 for 48h. Infliximab (A), Tocilizumab (B), Abatacept (C) and Rituximab (D) were tested. After 48h, the production of IL-17, IL-6, IL-1β, IFN-γ and IL-10 in cell supernatants was measured by ELISA. *, $p \leq 0.05$. Results are represented as mean ± SEM, n=11.

Figure 4. ***Comparison of different anti-TNF biotherapies***.

Healthy activated-PBMC were pre-incubated or not with two doses of anti-TNF biotherapies, 10µg/ml or 100µg/ml and co-cultured with RA synoviocytes at a ratio 5:1 for 48h. Infliximab, Etanercept and Adalimumab were compared. After 48h, the production of IL-17, IL-6, IL-1β, IFN-γ and IL-10 in cell supernatants was measured by ELISA. *,#, $p \leq 0.05$. * compares anti-TNF vs. control and # compares the different anti-TNF. Results are represented as mean ± SEM, n=10.

Figure 5. ***Effect of the combination of Methylprednisolone and biotherapy on pro-inflammatory cytokine production***.

Healthy activated-PBMC were pre-incubated or not with Methylprednisolone alone (0.01µg/ml), biotherapy alone (10µg/ml), or with the combination of both treatments. Then, PBMC were co-cultured with RA synoviocytes at a ratio 5:1 for 48h. Infliximab (A), Tocilizumab (B), Abatacept (C) and Rituximab (D) were tested. After 48h, the production of IL-17, IL-6, IL-1β and IFN-γ in cell supernatants was measured by ELISA. *, #, $p \leq 0.05$. * compares with control and # compares combination MP/biotherapy vs. MP alone. Results are represented as mean ± SEM, n=11.

Figure 6. ***Effect of the combination of Methylprednisolone and biotherapy on anti-inflammatory cytokine, IL-10.***

Healthy activated-PBMC were pre-incubated or not with Methylprednisolone alone (0.01µg/ml), biotherapy alone (10µg/ml), or with the combination of both treatments. Then, PBMC were co-cultured with RA synoviocytes at a ratio 5:1 for 48h. Infliximab, Tocilizumab, Abatacept and Rituximab were tested. After 48h, the production of IL-10 in cell supernatants was measured by ELISA. *, $p \leq 0.05$. * compares with control. Results are represented as mean ± SEM, n=10.

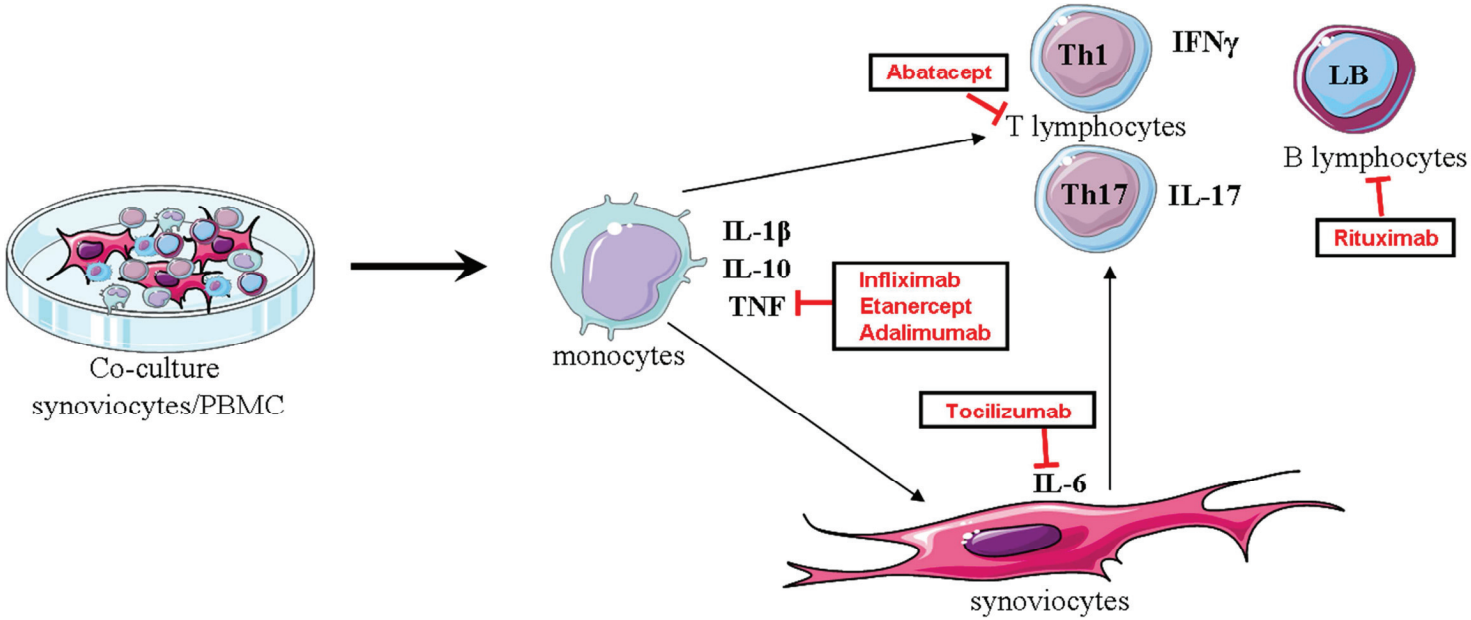

Co-culture
synoviocytes/PBMC
monocytes
IL-1β
IL-10
TNF
Infliximab
Etanercept
Adalimumab
Abatacept
Th1
IFNγ
T lymphocytes
Th17
IL-17
LB
B lymphocytes
Rituximab
Tocilizumab
IL-6
synoviocytes

Figure 1

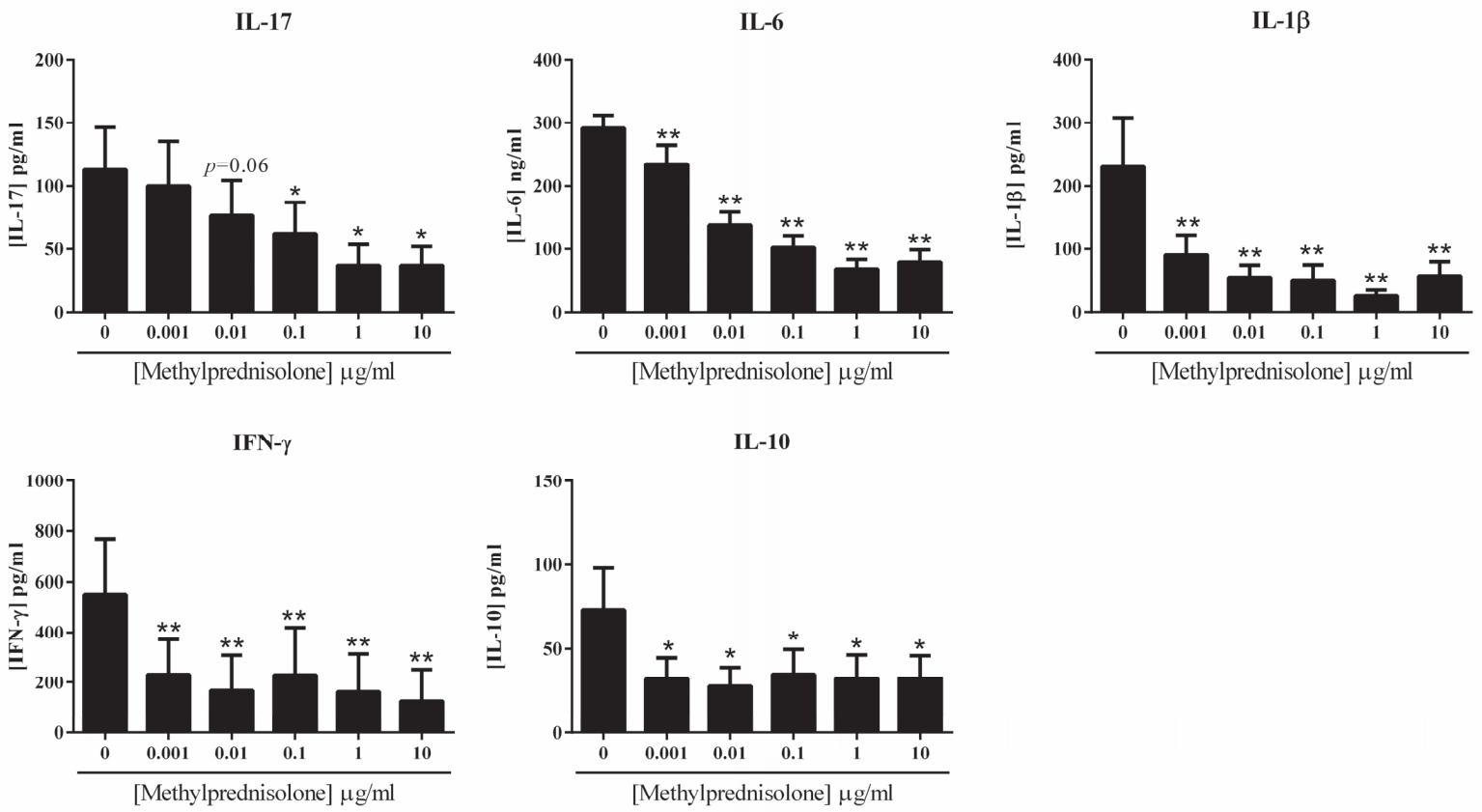

IL-17
[IL-17] pg/ml
200
150
100
50
0
p=0.06
*
*
*
0
0.001
0.01
0.1
1
10
[Methylprednisolone] µg/ml
IL-6
[IL-6] ng/ml
400
300
200
100
0
**
**
**
**
**
0
0.001
0.01
0.1
1
10
[Methylprednisolone] µg/ml
IL-1β
[IL-1β] pg/ml
400
300
200
100
0
**
**
**
**
**
0
0.001
0.01
0.1
1
10
[Methylprednisolone] µg/ml
IFN-γ
[IFN-γ] pg/ml
1000
800
600
400
200
0
**
**
**
**
**
0
0.001
0.01
0.1
1
10
[Methylprednisolone] µg/ml
IL-10
[IL-10] pg/ml
150
100
50
0
*
*
*
*
*
0
0.001
0.01
0.1
1
10
[Methylprednisolone] µg/ml

Figure 2

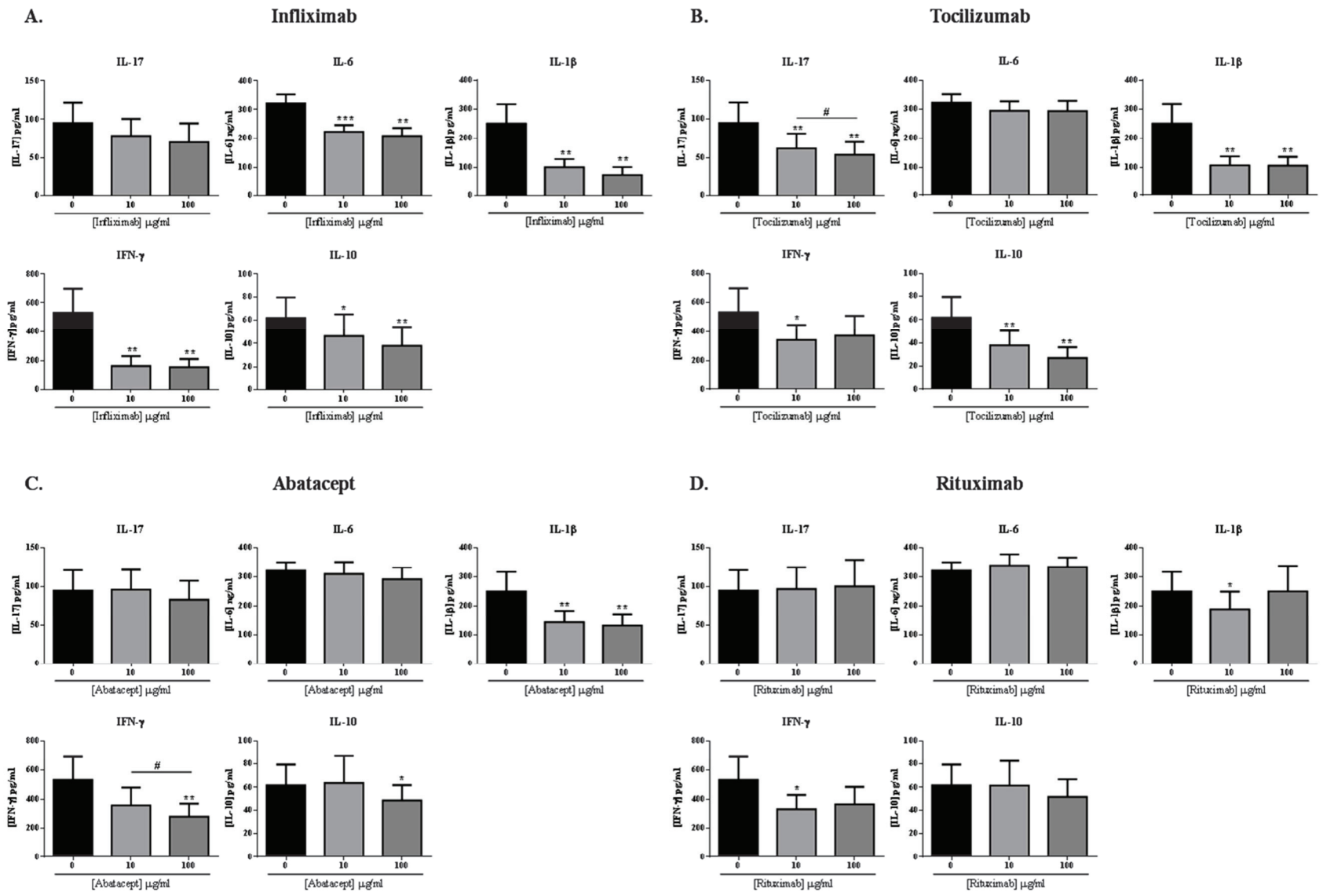

A.
Infliximab
IL-17
IL-6
IL-1β
IFN-γ
IL-10
[Infliximab] µg/ml
B.
Tocilizumab
[Tocilizumab] µg/ml
C.
Abatacept
[Abatacept] µg/ml
D.
Rituximab
[Rituximab] µg/ml

Figure 3

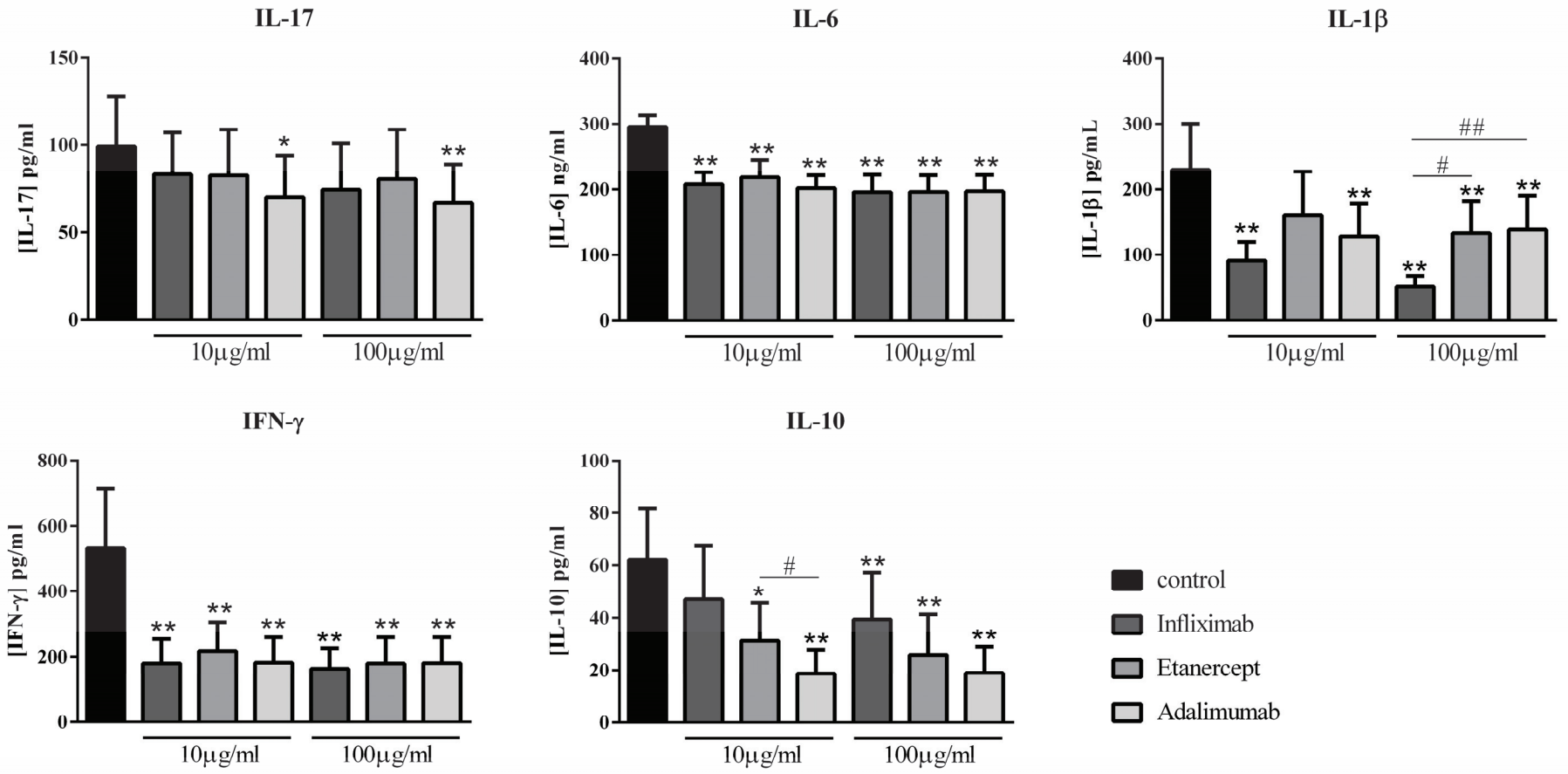

IL-17
[IL-17] pg/ml
150
100
50
0
10μg/ml
100μg/ml
IL-6
[IL-6] ng/ml
400
300
200
100
0
IL-1β
[IL-1β] pg/mL
##
#
IFN-γ
[IFN-γ] pg/ml
800
600
400
200
IL-10
[IL-10] pg/ml
80
60
40
20
control
Infliximab
Etanercept
Adalimumab

Figure 4

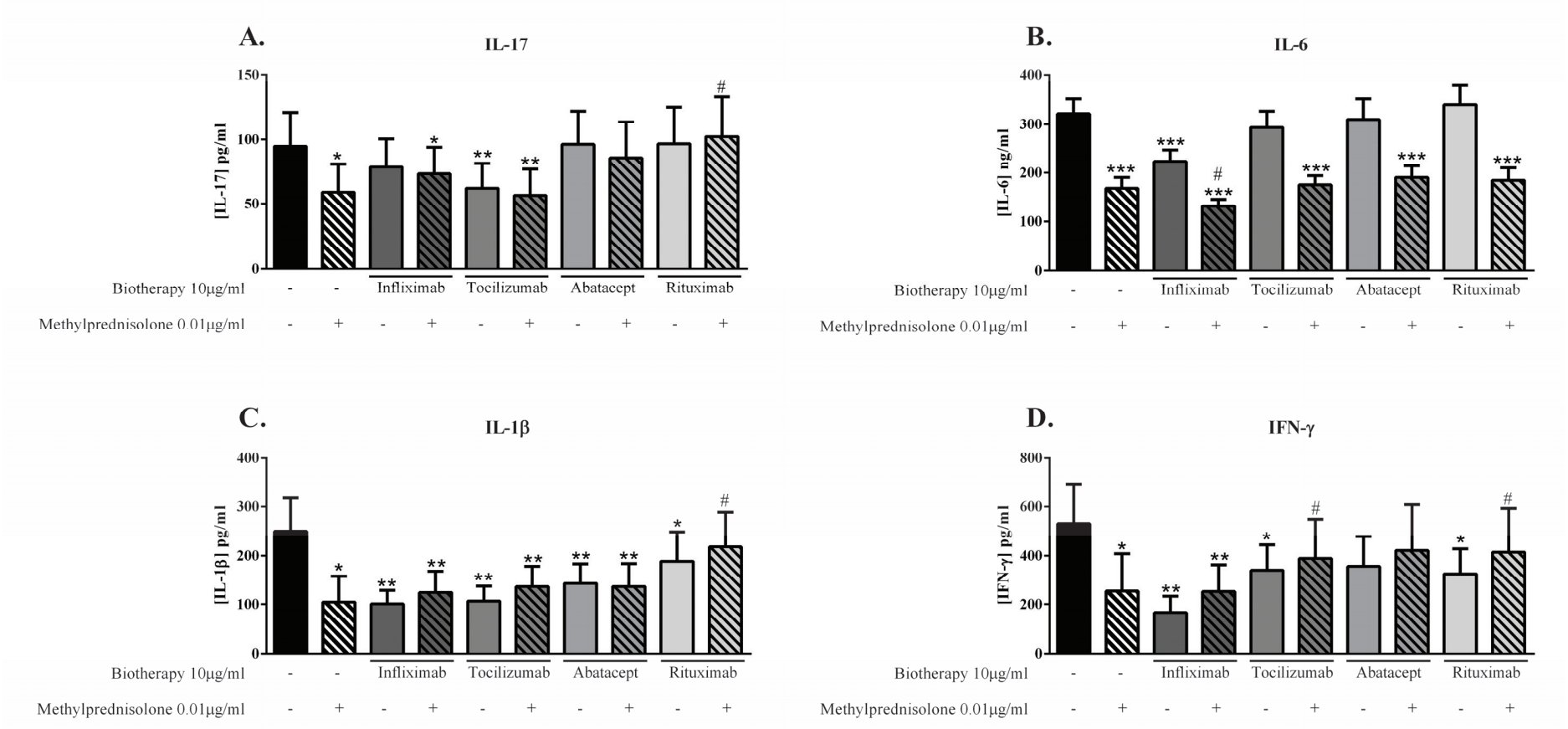

A.
IL-17
[IL-17] pg/ml
150
100
50
0
B.
IL-6
[IL-6] ng/ml
400
300
200
100
0
C.
IL-1β
[IL-1β] pg/ml
400
300
200
100
0
D.
IFN-γ
[IFN-γ] pg/ml
800
600
400
200
0
Biotherapy 10µg/ml - - Infliximab Tocilizumab Abatacept Rituximab
Methylprednisolone 0.01µg/ml - + - + - + - + - +

Figure 5

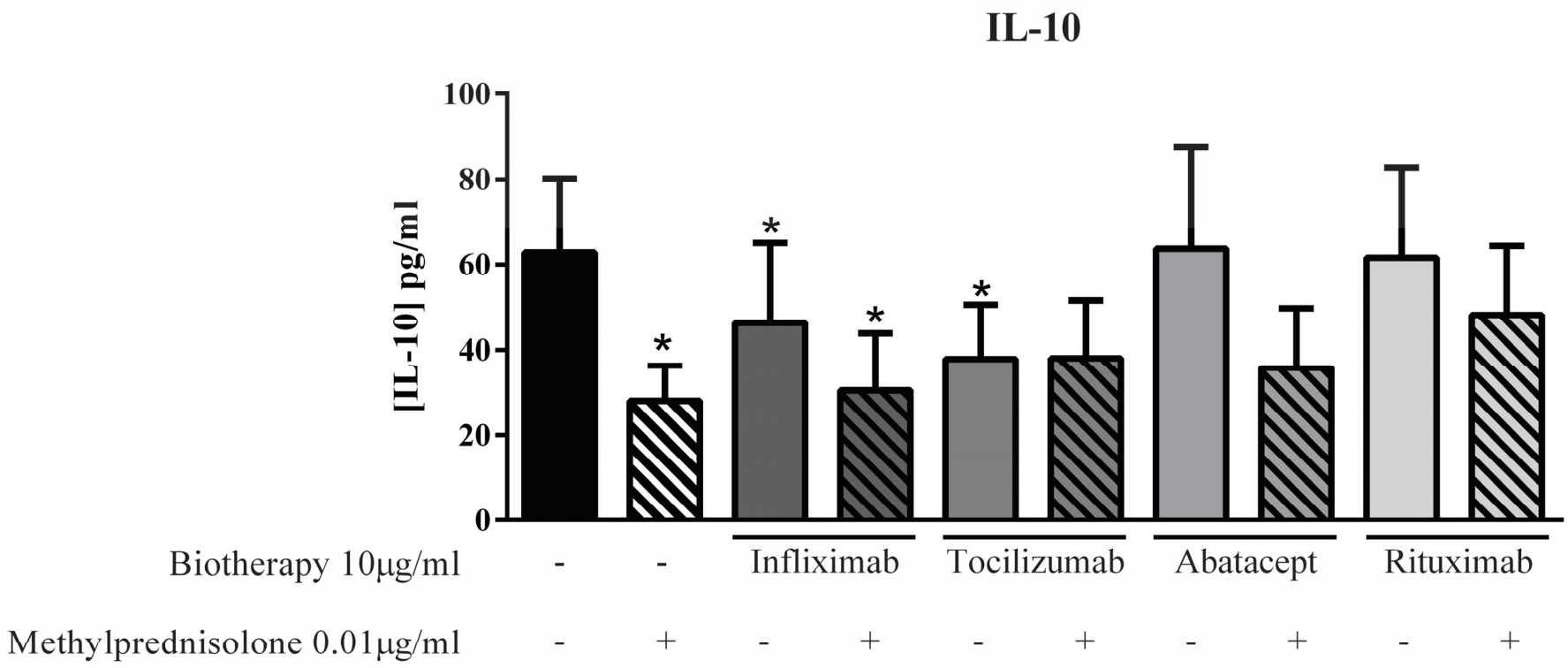

IL-10
[IL-10] pg/ml
100
80
60
40
20
0
*
*
*
*
Biotherapy 10µg/ml
-
-
Infliximab
Tocilizumab
Abatacept
Rituximab
Methylprednisolone 0.01µg/ml
-
+
-
+
-
+
-
+
-
+

Figure 6

III Identification d'une ou plusieurs molécules

Une meilleure compréhension de l'effet des traitements actuels serait un pas vers une médication plus personnalisée des patients. Toutefois, il reste important de chercher d'autres mécanismes pouvant fournir de nouvelles cibles thérapeutiques. Dans ce cadre, nous nous sommes intéressés à la podoplanine, molécule d'interaction.

III.1 La podoplanine

III.1.1 Définition

La podoplanine (pdpn) est une molécule d'interaction transmembranaire de type I, exprimée dans de nombreux tissus tels que le cerveau, le cœur, les poumons ou les organes lymphoïdes. Son principal récepteur est CLEC-2, principalement exprimé par les plaquettes [110] mais également par les DC matures ou les LB du sang périphérique [111, 112]. La pdpn présente un large panel de fonctions allant du rôle dans le développement du système lymphatique, en passant par la motilité cellulaire et allant jusqu'à un rôle dans la transition épithéliale-mésenchymateuse. Toutefois, malgré une expression sur plusieurs types cellulaires, son rôle physiologique reste peu connu [113].

III.1.1.1 La podoplanine dans le développement

Si le rôle biologique de la pdpn reste mal défini, l'observation des souris déficientes pour la pdpn a permis d'établir son rôle crucial dans le développement et le fonctionnement normal des poumons, du cœur ou encore du système lymphatique. Les souris déficientes pour la pdpn présentent une défaillance respiratoire, entraînant leur mort à la naissance. En effet, l'absence de pdpn inhibe la différenciation des pneumocytes entraînant le sous-développement des alvéoles pulmonaires [114]. La mortalité accrue chez les souris déficientes pour la pdpn

est également liée à des perturbations dans le développement normal du cœur, résultant probablement d'une diminution de la capacité de la transition épithéliale-mésenchymateuse des cellules [115-117]. De plus, les souris déficientes pour la pdpn présentent des vaisseaux lymphatiques anormaux, incapables de réguler correctement la circulation lymphatique [118]. Chez la souris adulte, l'expression de la pdpn est également requise pour un maintien d'une bonne architecture vasculaire [119]. L'interaction entre la pdpn exprimée par les cellules endothéliales lymphatiques et son récepteur CLEC-2 exprimé par les plaquettes induit l'agrégation et l'activation de ces dernières, ce qui est primordial dans l'homéostasie des vaisseaux lymphatiques [120-122]. L'interaction monocytes/plaquettes via pdpn/CLEC-2 favorise également la formation des vaisseaux lymphatiques [123]. En plus de son rôle dans le développement du système lymphatique, la pdpn est impliquée dans celui du système lymphoïde, notamment dans le maintien de l'architecture des organes lymphoïdes. En effet, chez les souris déficientes pour la pdpn, les ganglions lymphatiques sont majoritairement absents, et ceux qui se développent sont extrêmement désorganisés [124]. De plus, les cellules endothéliales lymphatiques et les cellules fibroblastiques réticulaires, les deux principaux types de cellules stromales lymphoïdes expriment fortement la pdpn [125], qui va alors interagir avec CLEC-2 exprimé par les DC, permettant une migration efficace de ces cellules dans les ganglions lymphatiques, nécessaire pour l'initiation de la réponse immunitaire [111].

III.1.1.2 Interactions de la podoplanine

Si CLEC-2 est le seul récepteur connu de la pdpn, celle-ci peut toutefois se lier à d'autres protéines. La pdpn peut ainsi se lier avec une forte affinité à CCL21. Cette interaction va notamment avoir des implications intéressantes pour le trafic des lymphocytes dans la zone T des ganglions lymphatiques [126, 127]. L'interaction de la pdpn avec CD44, impliqué dans la migration et l'adhésion cellulaire mais également dans l'activation des lymphocytes, va

jouer un rôle important dans les cancers. Les deux molécules, CD44 et pdpn, se retrouvent simultanément surexprimées dans les lignées cellulaires de cancer agressif et CD44 est requis pour la migration induite par la pdpn [128]. La pdpn favorise ainsi la progression des cellules cancéreuses et donc les métastases. Toutefois, la pdpn peut également se lier au CD9, qui agit comme un suppresseur de tumeur dans de nombreux cancers. Cette interaction aboutit à une diminution des métastases et inhibe également l'agrégation des plaquettes médiée par la pdpn, en interférant dans la liaison avec CLEC-2 [129]. Ces résultats peuvent expliquer pourquoi dans certaines études la pdpn est associée à une diminution de la survie des patients cancéreux et à une augmentation des métastases alors que dans d'autres études, l'effet inverse est observé [130]. La pdpn est donc une molécule d'interaction complexe, nécessitant des études complémentaires afin d'identifier ses multiples fonctions dépendantes de la nature de ses interactions.

III.1.1.3 Régulation de la podoplanine

La régulation de la pdpn implique différents facteurs selon le type cellulaire, ce qui peut aussi être une des raisons à ses nombreuses fonctions physiologiques. Prox-1, régulateur majeur de la différenciation des cellules endothéliales lymphatiques, contrôle l'induction de la pdpn dans ces cellules [131]. L'IL-3, qui stimule la différenciation des cellules hématopoïétiques, est également capable d'augmenter l'expression de la pdpn mais aussi de Prox-1 [132]. Dans de nombreux cancers, la pdpn est principalement régulée par le facteur de transcription AP-1, composé des protéines Jun et Fos, Fos pouvant se lier directement au promoteur de la pdpn [133]. En plus de ces facteurs, il a été montré que la présence de cytokines pro-inflammatoires pouvait augmenter l'expression de la pdpn au cours de pathologies telles que la PR ou le Pso. En effet, la présence d'IL-1β, de TNF ou de TGF-β augmente l'expression de la pdpn par les synoviocytes [134]. De manière similaire, les

kératinocytes traités avec du TGF-β, de l'IL-6, de l'IFN-γ ou de l'IL-22 surexpriment la pdpn [135]. Il apparaît ainsi que l'inflammation favorise l'expression de la pdpn.

III.1.1.4 La podoplanine dans l'inflammation

En plus de son expression par les cellules stromales, la pdpn peut être exprimée par certaines cellules immunitaires, dont les LT, les monocytes et les macrophages [123, 124, 136, 137]. Dans ce cas, l'expression de la pdpn est associée à des maladies auto-immunes et inflammatoires. Ainsi, les LTh17 générés *in vitro* ou provenant du système nerveux central de souris EAE (Encéphalomyélite Allergique Expérimentale) expriment la pdpn [124]. Dans un modèle de PR, les souris SKG, il a été montré que l'expression de la pdpn est augmentée seulement dans la sous-classe des LTh17 [138]. Toujours dans le contexte PR, la pdpn est capable de réguler la sécrétion d'IL-8 lors des interactions cellulaires entre plaquettes et synoviocytes via CLEC-2 [139]. L'expression de la pdpn est également augmentée sur les synoviocytes de patients PR et peut promouvoir le potentiel migratoire de ces cellules activées [134]. De plus, sa signalisation a été impliquée dans différents modèles animaux de maladies inflammatoires [124, 138], et notamment de PR. Sa surexpression est également observée dans l'épiderme hyperprolifératif de patients atteints de Pso [135, 140]. La pdpn semble donc être impliquée à différents niveaux de l'inflammation, avec notamment un rôle dans la pathogénicité des LTh17 et du phénotype invasif des synoviocytes mais également dans la modulation de la production de cytokines résultant du contact cellulaire. Ces arguments font de la pdpn un nouveau mécanisme potentiellement impliqué dans la voie LTh17 lors de l'inflammation chronique, notamment lors de la PR et du Pso.

Dans ce cadre, son rôle a été étudié dans la production de cytokines pro-inflammatoires lors de l'interaction entre CSM (synoviocytes ou fibroblastes de peau) et cellules immunitaires (cf. articles de la partie 2, chapitre I.3 p80-91 et chapitre II.3 p97-107).

III.1.2 Résumé des résultats

L'ajout d'un anticorps anti-pdpn dans le système de co-cultures PBMC-synoviocytes ou PBMC-fibroblastes de peau inhibe significativement la sécrétion d'IL-17, d'au moins 60% (Figure 1A), mais également la sécrétion d'IL-1β (Figure 1B). En revanche, la production d'IL-6 et d'IL-8 n'est que peu ou pas affectée par l'inhibition de la pdpn (Figure 1, C et D). Afin de confirmer le rôle de la pdpn dans la forte sécrétion d'IL-17 résultant du contact cellulaire, les synoviocytes ont été transfectés avec un siRNA spécifique de la pdpn. L'inhibition de la pdpn par siRNA entraine une inhibition de la sécrétion d'IL-17 (Figure 2). Toutefois, cette inhibition est inférieure à celle obtenue avec l'ajout de l'anticorps anti-pdpn. Cela peut s'expliquer d'une part par le fait que le siRNA va inhiber la pdpn nouvellement synthétisée et non celle déjà présente à la surface des cellules. D'autre part, cela renforce l'idée que l'interaction via la pdpn entre les cellules mésenchymateuses et les cellules immunitaires se fait à double sens, et dans ce cas le siRNA n'agit que partiellement du fait d'une inhibition de la pdpn des cellules mésenchymateuses mais pas de celle des cellules immunitaires.

Afin de vérifier l'expression de la pdpn lors des co-cultures, un marquage pdpn a été réalisé après les 48h de co-cultures. Le pourcentage de cellules positives augmente dans les co-cultures comparé aux PBMC seules et surtout en présence d'activation des PBMC (11.2 ± 6.2 % vs. 1.0 ± 0.8 % sans PHA; 32.7 ± 8.1 % vs. 0.8 ± 0.7 % avec PHA, figure 3). De manière intéressante, parmi les cellules CD3+ CD4+, le pourcentage de cellules IL-17-pdpn+ augmente en co-culture (0.9 ± 0.6 % vs. 16.7 ± 5.0 % sans PHA; 3.3 ± 3.4 % vs. 54.3 ±

0.9 % avec PHA, figure 3B) mais cette augmentation est nettement supérieure dans les cellules IL-17+ (9.4 ± 1.1 % vs. 69.7 ± 7.6 % sans PHA; 8.4 ± 8.3 % vs. 85.7 ± 2.8 % avec PHA). Cette augmentation corrèle avec la sécrétion massive d'IL-17 observée lors de l'interaction entre PBMC activées et cellules mésenchymateuses. De plus, afin de confirmer l'expression de la pdpn par les LTh17, des clones Th17 ont été utilisés. En culture seuls, environ 20% des clones Th17 expriment la pdpn (Figure 4A). Lors de la co-culture, les cellules IL-17+ sont quasiment toutes positives pour la pdpn (Figure 4A). Ces résultats sont en accord avec l'augmentation du pourcentage de cellules pdpn+ lors des co-cultures en présence de PHA (Figure 3) et sont également en accord avec la forte sécrétion d'IL-17 obtenue en co-cultures de synoviocytes et LTh17 activés (Figure 4B). De plus, l'inhibition de la sécrétion d'IL-17 en présence d'anti-pdpn dans les co-cultures synoviocytes-clones Th17 activés (Figure 4B) confirme le rôle de la pdpn dans la production massive d'IL-17 résultant des interactions entre cellules mésenchymateuses et lymphocytes activés. D'autres études sont toutefois nécessaires afin d'identifier la contribution de la pdpn exprimée par les LTh17 et les synoviocytes mais aussi pour déterminer le récepteur impliqué.

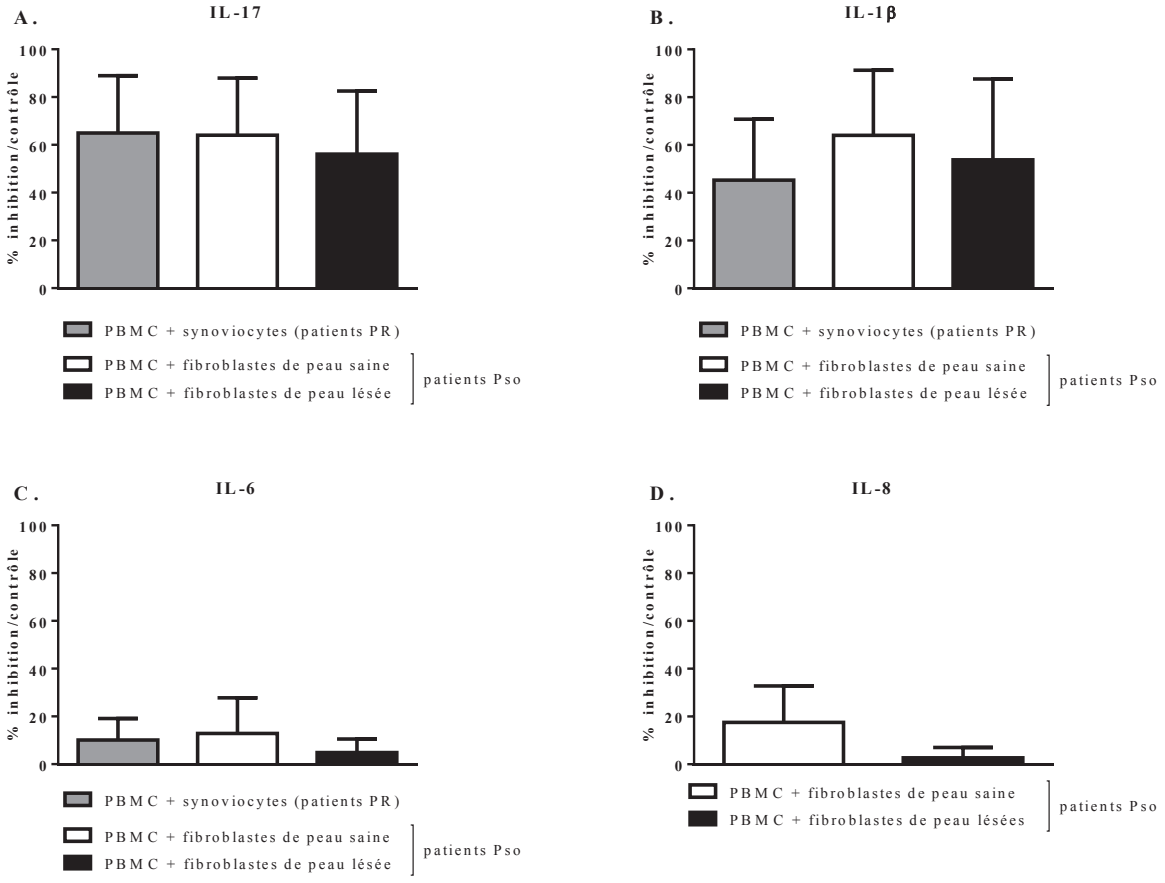


Figure 1: Des co-cultures entre PBMC de donneurs sains, activées et pré-incubées ou non pendant 4h avec un anticorps anti-podoplanine, et CSM de patients (synoviocytes PR ou fibroblastes de peau Pso) ont été réalisées. Après 48h de co-culture, les surnageants ont été récupérés et les cytokines IL-17 (A), IL-1β (B), IL-6 (C) ou IL-8 (D) dosées par ELISA. Le pourcentage d'inhibition de sécrétion avec l'anti-pdpn comparé au contrôle est représenté.

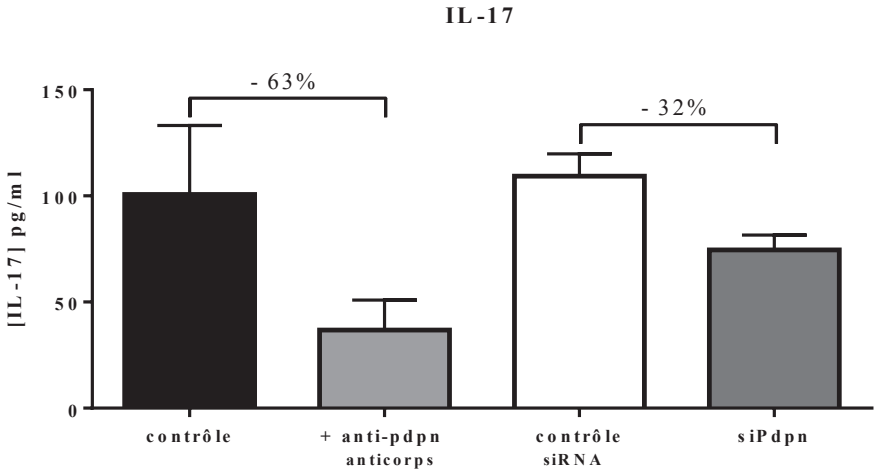


Figure 2: Des PBMC de donneurs sains ont été pré-incubées pendant 4h avec ou sans anticorps anti-podoplanine avant la co-culture de 48h avec des synoviocytes de patients PR, en présence de PHA. Pour les siRNA, les synoviocytes PR ont été transfectés sans (contrôle) ou avec un siRNA spécifique pour la podoplanine (siPdpn) et ensuite utilisés en co-culture avec des PBMC de donneurs sains, en présence de PHA, pendant 48h. La production d'IL-17 a été mesurée dans les surnageants après 48h. Les concentrations d'IL-17 pour les différentes conditions de cultures sont représentées.

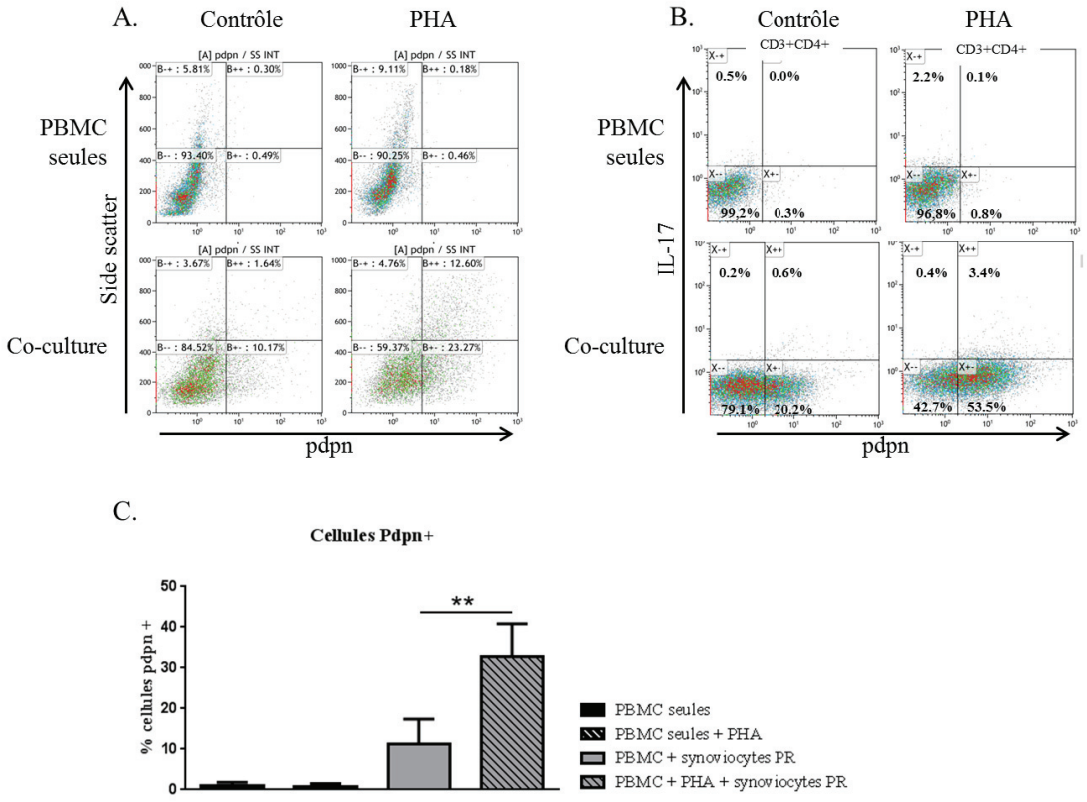


Figure 3: Des PBMC de donneurs sains ont été mises en culture seules ou en co-culture avec des synoviocytes de patients PR à un ratio 5:1, en présence ou non de PHA. Après 48h de culture, les cellules ont été récupérées et marquées pour la pdpn (A et C) ou pour CD3, CD4, IL-17 et pdpn (B). Le « dot plot » d'une expérience (A et B) et le pourcentage de cellules pdpn+ dans les différentes conditions de culture (C) sont représentés.

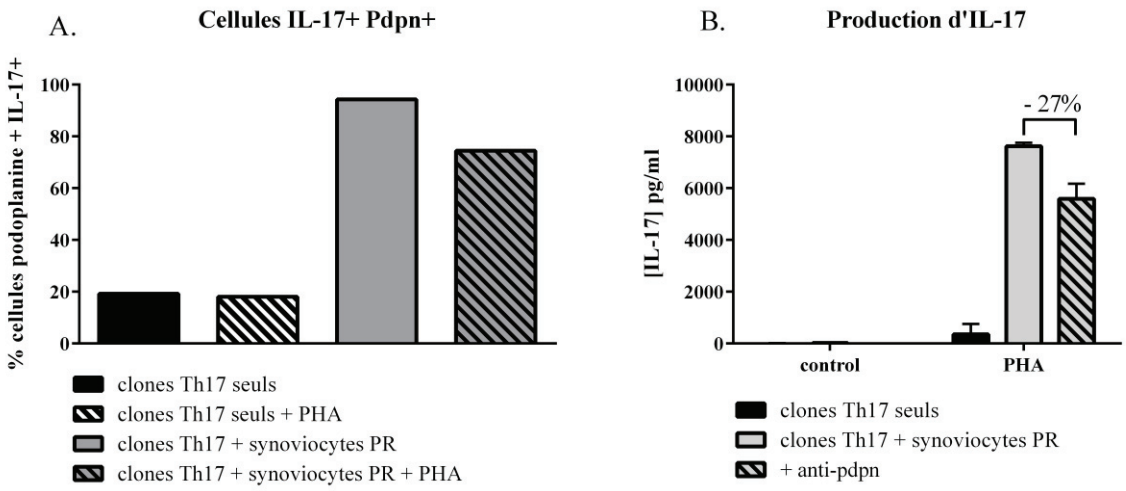


Figure 4: Des clones Th17 ont été mis en culture seuls ou avec des synoviocytes de patients PR, à un ratio 1:1, en présence ou non de PHA (A). Les clones Th17 ont également été pré-incubés pendant 4h avec ou sans anticorps anti-podoplanine avant la co-culture de 48h avec des synoviocytes de patients PR (B). Après 48h, les cellules ont été récupérées et marquées pour pdpn et IL-17 et la production d'IL-17 a été mesurée dans les surnageants. Le pourcentage de cellules IL-17+ pdpn+ (A) et la concentration d'IL-17 (B) dans les différentes conditions de cultures sont représentés.

III.2 A la recherche d'autres molécules

La molécule d'interaction podoplanine semble clairement jouer un rôle dans la sécrétion d'IL-17 lors du contact cellulaire, cellules mésenchymateuses-cellules immunitaires. Toutefois, l'inhibition de la production d'IL-17 en présence d'un anticorps anti-pdpn présente un certain degré d'hétérogénéité et n'est que rarement de 100%. L'identification d'autres molécules pourrait donc compléter la compréhension de ce mécanisme complexe. Dans cet objectif, une collaboration a été développée avec une société lyonnaise, spécialisée dans la production d'anticorps monoclonaux, Dendritics. Possédant une large collection de surnageants (SN) d'anticorps spécifiques des synoviocytes, le but est de se servir de leur collection pour effectuer un screening et sélectionner puis identifier la cible des anticorps d'intérêt. Pour cela, nous avons testé ces SN dans notre système de co-culture et étudier l'effet sur la production des cytokines (IL-17 et IL-6), l'inhibition de la sécrétion d'IL-17 étant le critère principal de sélection.

Le travail a donc commencé par l'analyse de 75 surnageants d'anticorps spécifiques des synoviocytes. Des co-cultures synoviocytes-PBMC activées ont été réalisées en présence de ces SN. Après 48h, la production d'IL-17 et d'IL-6 a été mesurée par ELISA. Comme observé dans la figure 1A, si certains surnageants n'avaient aucun effet sur la production d'IL-17, d'autres en revanche inhibaient sa sécrétion mais pouvaient également l'augmenter. Les effets sur la production d'IL-6 semblaient similaires à ceux observés sur l'IL-17, avec toutefois une efficacité moindre (Figure 1B). Basé principalement sur l'effet sur la production d'IL-17, nous avons fait une première sélection de 10 SN d'anticorps, encadrés en rouge sur la figure 1, dont voici la liste : 8288 inhibiteur, 8290 activateur, 8296 activateur, 8301 inhibiteur, 8314 inhibiteur, 8335 inhibiteur, 8340 inhibiteur, 8349 inhibiteur, 8350 inhibiteur et 8353 inhibiteur.

Dans un second temps, à partir de ces surnageants sélectionnés, les anticorps ont été purifiés par l'équipe de Dendritics. Nous avons ensuite testé ces anticorps purifiés afin de confirmer leur rôle. Certains présentant plusieurs clones de purification, nous avons testé tous les clones afin de savoir lequel représentait notre anticorps d'intérêt. Trois concentrations ont été utilisées, 0.1, 1 et 10 µg/ml. Les co-cultures ont été faites en présence ou non des différents anticorps purifiés. Après 48h de culture, les surnageants ont été récupérés et la production d'IL-17 et d'IL-6 a été mesurée par ELISA. La figure 2 présente le pourcentage d'inhibition de la production par rapport au contrôle. L'inhibition de l'IL-17 atteignait au maximum 45% (Figure 2A) et celle de l'IL-6 30% (Figure 2B). Cela nous a permis de confirmer ou non le rôle des anticorps présélectionnés. Ainsi, les deux SN qui semblaient plutôt activateur de la sécrétion d'IL-17, le 8290 et le 8296, ne présentaient plus cette activité (Figure 2A). Ceci indique que le premier effet observé était certainement dû à autre chose présent dans le SN. Les SN affichant une activité inhibitrice lors de la première étape semblaient conserver cet effet après purification des anticorps. Toutefois, certains présentaient un effet inhibiteur moindre comparé à l'effet du SN correspondant, tel que le 8350.

Nous avons également voulu vérifié que ces anticorps étaient spécifiques des synoviocytes. Pour cela, les synoviocytes ont été incubés avec les différents anticorps sélectionnés et un anticorps secondaire couplé FITC a été ajouté afin d'analyser le marquage par cytométrie en flux. Les résultats obtenus montraient que les synoviocytes étaient positifs pour la plupart des anticorps mais pas tous. Afin de vérifier si l'activation des cellules pouvait induire l'expression des molécules ciblées par les anticorps négatifs, les synoviocytes ont été activés par IL-17 et TNF, 24h avant le marquage. Cette activation ne changeait pas les résultats (Figure 2C).

A partir de ces résultats, nous avons effectué une deuxième sélection d'anticorps purifiés afin de poursuivre les expériences. Nous avons ainsi gardé 6 anticorps, listés ci-après avec leurs caractéristiques.

- 8314 : inhibiteur, négatif sur synoviocytes
- 8335 : inhibiteur, négatif sur synoviocytes
- 8288 (deux clones) : inhibiteur, positif sur synoviocytes
- 8349 (clone 2) : inhibiteur, positif sur synoviocytes
- 8340 : inhibiteur, positif sur synoviocytes
- 8301 : inhibiteur, positif sur synoviocytes

A ce stade, l'étape suivante consiste à refaire des co-cultures avec ces anticorps, en testant une dose-réponse plus large, de 0.001 à 100 µg/ml, afin d'établir une concentration d'utilisation optimale. La positivité à ces anticorps sera également testée sur d'autres types cellulaires, afin de savoir s'ils sont spécifiques exclusivement aux synoviocytes ou si leur cible peut être exprimée par d'autres cellules telles que les fibroblastes, les cellules endothéliales ou les cellules immunitaires (DC, lymphocytes). Cela permettra également de déterminer le type cellulaire exprimant la cible des anticorps négatifs pour les synoviocytes.

Une fois cela établi, l'étape clé de cette collaboration sera l'identification de la cible de ces anticorps. Dans ce cadre, le clonage par expression sera utilisé, ce qui permettra de récupérer ensuite facilement et en grande quantité l'ADNc (ADN complémentaire) de la protéine d'intérêt. Ces ADNc seront ensuite comparés à des librairies d'ADNc afin d'établir des correspondances et ainsi d'identifier nos protéines d'intérêt.

Grâce à cette collaboration, différentes molécules impliquées dans la sécrétion d'IL-17 résultant des interactions cellulaires pourraient être identifiées. Ceci permettrait de mieux

comprendre ce mécanisme complexe et de fournir de nouvelles cibles thérapeutiques potentielles.

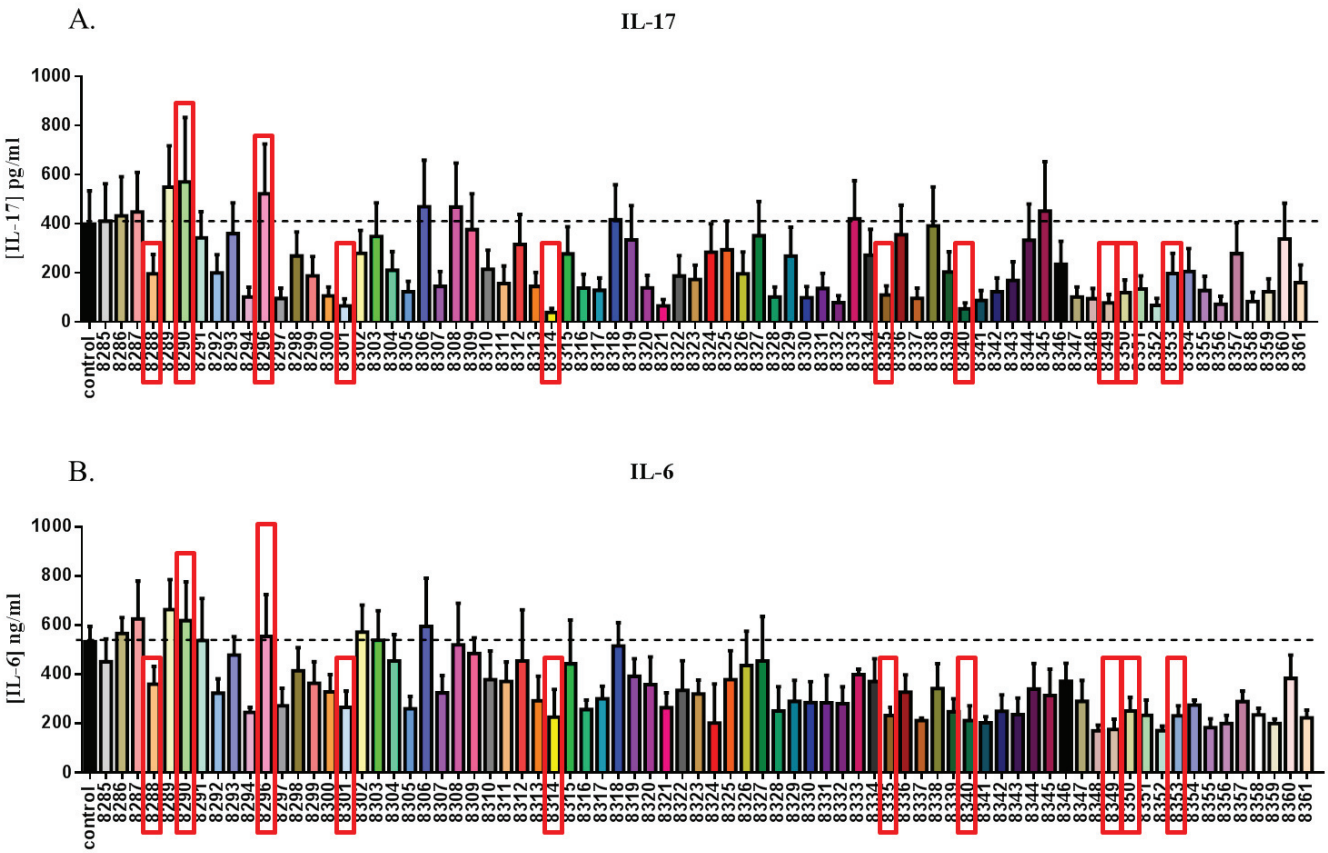
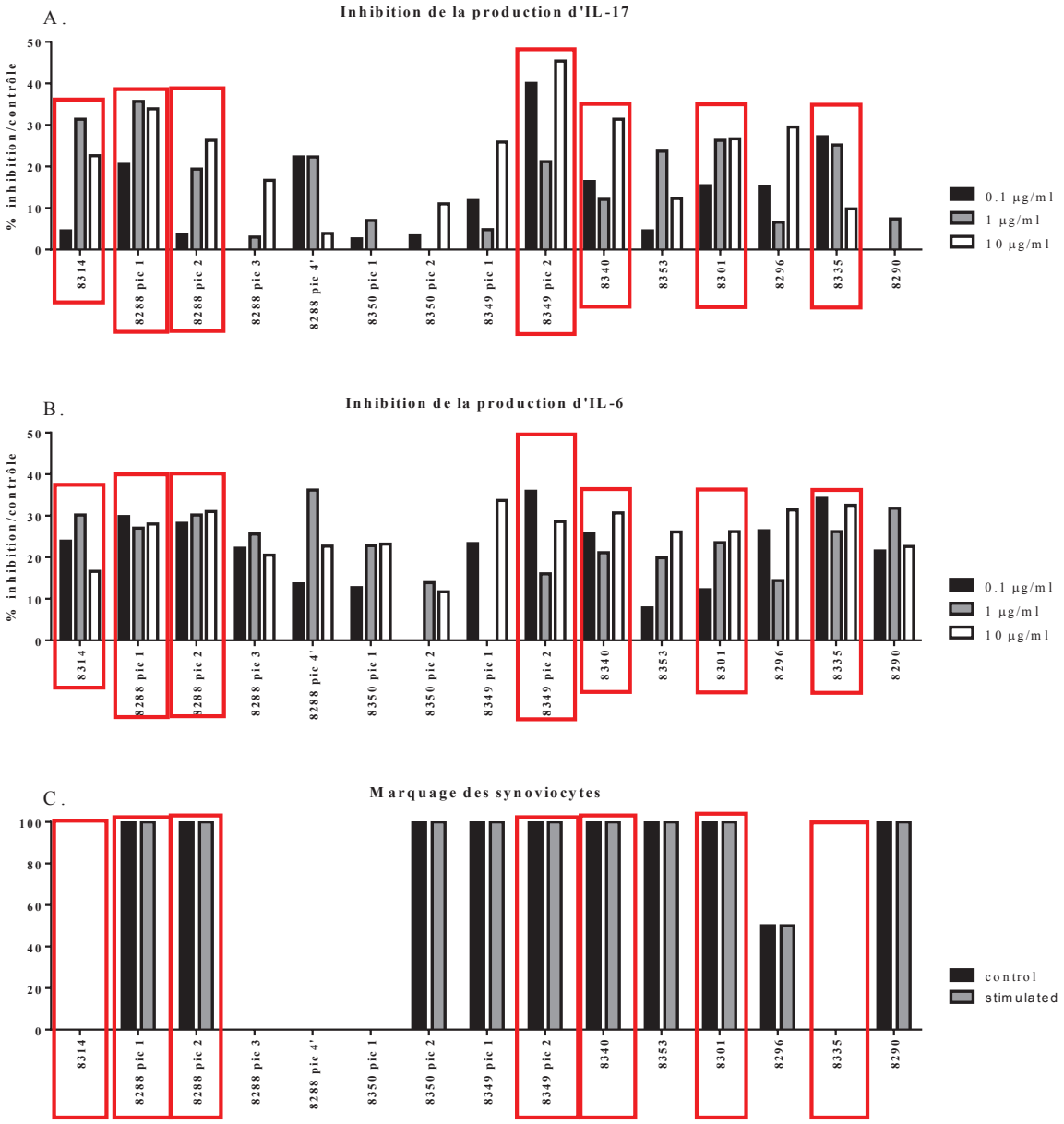


Figure 1: Des co-cultures entre PBMC activées de donneurs sains et synoviocytes de patients PR ont été réalisées en présence ou non d'une série de surnageants d'anticorps fournis par la société Dendritics. Après 48h de culture, la production d'IL-17 et d'IL-6 a été mesurée dans les surnageants par ELISA. Les concentrations d'IL-17 (A) et d'IL-6 (B) sont représentées.



<u>Figure 2</u>: Des co-cultures entre PBMC activées de donneurs sains et synoviocytes de patients PR ont été réalisées en présence ou non d'anticorps purifiés. Après 48h de culture, la production d'IL-17 et d'IL-6 a été mesurée dans les surnageants par ELISA. Les concentrations d'IL-17 (A) et d'IL-6 (B) sont représentées. Des synoviocytes activés ou non par IL-17 + TNF ont été incubés en présence de ces anticorps et d'un anticorps secondaire FITC permettant l'analyse par cytométrie en flux. Les résultats du marquage sont représentés (C) avec 100=positif, 0=négatif.

Partie 4. Conclusion générale et perspectives

L'objectif principal de ce travail était d'étudier l'effet des interactions cellulaires entre cellules stromales mésenchymateuses, issues de différentes origines, et cellules immunitaires, dans un contexte inflammatoire chronique. Pour cela, nous avons utilisé un système de co-culture permettant de mimer les interactions présentes sur le site inflammatoire. Des PBMC étaient mises en contact avec des CSM, soit des synoviocytes issus de patients PR, soit des fibroblastes de peau issus de patients Pso. Ce système reproduit l'étape précoce de ce qui se passe *in vivo* sur le site inflammatoire, à savoir l'interaction entre les cellules migrantes du sang périphérique et les CSM locales. L'utilisation d'un système autologue (PBMC et CSM du même patient) nous a permis de valider ce modèle et d'exclure une alloréactivité. Dans notre étude, nous nous sommes concentrés sur l'effet de ces interactions sur la voie LTh17 et principalement sur la sécrétion d'IL-17.

Dans un premier temps, nous avons démontré que l'interaction seule entre CSM (synoviocytes ou fibroblastes de peau) et PBMC était suffisante pour induire la sécrétion de différentes cytokines pro-inflammatoires telles que l'IL-6, l'IL-1β ou l'IL-8. Ces résultats sont en accord avec une étude précédente du laboratoire montrant que l'interaction entre cellules mésenchymateuses de moelle osseuse et PBMC permettait une augmentation de l'ARNm de l'IL-6 et de l'IL-1β [31]. Ces observations reflètent comment de fortes concentrations de cytokines pro-inflammatoires peuvent être détectées sur le site inflammatoire, que ce soit l'articulation pour la PR ou la peau psoriasique pour le Pso [141, 142].

L'intérêt de notre étude s'est ensuite porté sur la voie LTh17/IL-17. L'IL-6 et l'IL-1β étant deux cytokines impliquées dans la différenciation des LTh17 [9, 10, 12], nous avons tout d'abord regardé l'effet des interactions cellulaires sur la différenciation des LTh17. Les

résultats indiquaient que l'interaction n'avait qu'un faible effet sur cette différenciation et que l'activation semblait plus impliquée dans ce phénomène. Nous avons ensuite déterminé l'impact sur la sécrétion d'IL-17. Dans ce cas, l'interaction cellulaire seule n'était pas suffisante pour induire une sécrétion massive de cette cytokine, l'activation du TCR était également requise. Ceci indiquait que contrairement à l'IL-6 et à l'IL-1β, l'IL-17, cytokine pro-inflammatoire spécifique des LTh17 requiert deux signaux, l'activation du TCR et l'interaction cellulaire. De plus, cette augmentation de l'IL-17 n'était pas corrélée à une augmentation de la différenciation des LTh17, ce qui démontre une discordance entre le marquage intracellulaire et la présence de la molécule active. Ceci renvoie à un problème de définition dans les LTh17. En effet, le marquage intracellulaire de l'IL-17 est utilisé comme un marqueur des LTh17 mais il ne reflète pas nécessairement la sécrétion de l'IL-17 comme nous avons pu le constater. Ainsi, il est important de différencier les LTh17 non sécréteurs, qui se retrouvent plutôt dans la circulation et les LTh17 sécréteurs, qui nécessitent l'environnement inflammatoire (interactions cellulaires et facteurs solubles) pour libérer la molécule effectrice et qui se retrouvent donc *in situ*.

Dans le cas de la PR, la présence de cet environnement inflammatoire dans l'articulation induit la sécrétion d'IL-17 avec toutefois une grande hétérogénéité chez les patients. Le niveau d'IL-17 bioactive est ainsi associé à la sévérité de la maladie [143]. Toutefois, l'efficacité des traitements anti-IL-17 dans la PR reste inférieure à celles des anti-TNF ou anti-IL-6, alors que l'implication de l'IL-17 dans la pathogenèse de la PR est largement démontrée. Cette variabilité de production d'IL-17 pourrait donc en partie expliquer les réponses très différentes observées chez les patients atteints de PR [144, 145]. De plus, la présence d'auto-anticorps dirigés contre des cytokines pro-inflammatoires telles que l'IL-1α est un marqueur de bon pronostic dans la PR [146, 147]. La liaison de l'anticorps avec son antigène forme un complexe immun (CI). Dans le cas de l'IL-17, la détection des

auto-anticorps anti-IL-17 et des CI combinés à l'IL-17 bioactive pourrait représenter des biomarqueurs intéressants dans la prédiction de la réponse au traitement anti-IL-17 [148]. Cette anticipation permettrait un traitement personnalisé et adapté à chaque patient. Une autre possibilité est de prévoir un moyen d'action différent de l'inhibition de l'IL-17 sécrétée. Agir en amont, pour diminuer directement la sécrétion d'IL-17 peut être un moyen de réduire les effets néfastes de l'IL-17 plus généralement. Cela nécessite de comprendre les mécanismes impliqués dans la production d'IL-17.

Dans ce cadre, nos expériences ont montré un rôle critique de l'interaction cellulaire entre CSM de différentes origines et cellules immunitaires dans la production de l'IL-17. Toutefois les mécanismes impliqués restaient inconnus. Dans le but d'identifier des acteurs de ce processus, nous nous sommes déjà intéressés au rôle que pouvaient avoir les monocytes, capables d'interagir à la fois avec les CSM mais également avec les lymphocytes (Figure 2). Après leur retrait par adhérence, les mêmes co-cultures que précédemment ont été réalisées. De manière surprenante, les résultats obtenus divergeaient entre synoviocytes et fibroblastes de peau. Dans le contexte PR, le retrait des monocytes lors des co-cultures n'avait que peu ou pas d'impact sur la sécrétion de l'IL-17. A l'inverse, dans le contexte Pso, la sécrétion de toutes les cytokines pro-inflammatoires testées était significativement diminuée, y compris celle de l'IL-17. Ces résultats suggèrent que malgré des caractéristiques communes aux cellules mésenchymateuses, selon leur origine, des différences de mécanismes conduisant à la production de cytokines inflammatoires lors du contact cellulaire peuvent apparaître.

Après avoir établi le rôle majeur des monocytes dans la sécrétion de l'IL-17 lors des interactions cellulaires entre fibroblastes et cellules immunitaires, nous avons voulu savoir par quel mécanisme cela se produisait et qui pourrait également être impliqué dans la PR. Nous nous sommes donc intéressés à la molécule d'interaction la podoplanine (pdpn). En effet, la

pdpn peut être exprimée par les monocytes [123] mais elle est également exprimée dans l'épiderme psoriasique hyperprolifératif de patients Pso et dans la synoviale de patients PR [134, 135, 140]. Nous avons donc utilisé un anticorps anti-pdpn dans nos co-cultures afin d'étudier son rôle lors des interactions cellulaires. Dans le contexte Pso, la sécrétion d'IL-17 et d'IL-1β était diminuée d'au moins 50% en présence de l'anticorps. De plus, l'expression de la pdpn était augmentée dans les co-cultures, surtout en présence d'activation des PBMC, ce qui corrélait avec la sécrétion massive d'IL-17. La pdpn pourrait donc être un des mécanismes par lequel les monocytes contribueraient à la forte sécrétion d'IL-17 lors des interactions cellulaires. Toutefois, afin de confirmer ce mécanisme, l'étude de la pdpn sur les monocytes devrait être approfondie. Un double marquage CD14/pdpn pourrait confirmer l'expression de la pdpn par les monocytes et vérifier la régulation de son expression par ces cellules au cours des co-cultures. De plus, un ciblage de la pdpn spécifiquement sur les monocytes pourrait être envisagé afin de confirmer le rôle des monocytes via la pdpn.

La pdpn étant exprimée également par les fibroblastes ou les synoviocytes, elle peut donc influencer la sécrétion de cytokines via d'autres cellules que les monocytes. En outre, il a été montré que l'interaction entre synoviocytes et plaquettes, médiée par la pdpn, pouvait moduler la sécrétion d'IL-8 [139]. Dans notre contexte de PR, l'ajout de l'anticorps anti-pdpn dans les co-cultures inhibait en moyenne de 60% la sécrétion d'IL-17. Afin de confirmer le rôle de la pdpn exprimée par les synoviocytes, nous avons utilisé un siRNA spécifique de la pdpn sur nos synoviocytes. Dans ce cas, la production d'IL-17 était également diminuée mais de manière moins importante que lors de l'ajout de l'anticorps. Ce résultat peut s'expliquer d'une part par le fait que le siRNA bloque l'expression de la podoplanine néoformée et non celle déjà exprimée à la surface des cellules. D'autre part, cela peut impliquer la pdpn exprimée par d'autres cellules, notamment les LTh17. Dans un modèle d'arthrite, la pdpn est surexprimée dans les LTh17 en comparaison des autres sous-groupes de LT [138].

L'utilisation de trois protocoles différents, pré-incubation des PBMC ou des synoviocytes ou des deux types cellulaires avec l'anti-pdpn avant les co-cultures donnait les mêmes résultats d'inhibition de la sécrétion d'IL-17. Ajouté au plus faible pourcentage d'inhibition obtenu avec le siRNA comparé à l'anticorps, ceci pourrait indiquer l'implication de la pdpn exprimée par les synoviocytes et par les LTh17. Dans cette optique, nous avons utilisé des clones Th17 dans nos expériences afin de mettre en avant l'interaction synoviocytes/LTh17. Les mêmes co-cultures ont été réalisées. Les résultats avec les clones Th17 étaient similaires à ceux obtenus avec les PBMC, la concentration d'IL-17 produite étant toutefois plus élevée, comme attendu. Ceci confirmait le rôle des synoviocytes et des LTh17 dans la sécrétion massive d'IL-17 lors de leur interaction cellulaire. De plus, l'ajout d'un anticorps anti-pdpn diminuait d'environ 30% la sécrétion d'IL-17. Cette inhibition est inférieure à celle obtenue avec les PBMC. Toutefois, il faut noter que l'expérience a été faite avec un seul type de clones Th17. Il serait nécessaire de réitérer l'expérience avec d'autres clones afin de déterminer si le pourcentage d'inhibition présente une certaine hétérogénéité, comme cela est le cas avec les PBMC ou si l'inhibition est moins importante avec les clones Th17. Cette différence peut également être due au fait que la concentration en IL-17 est bien plus élevée avec les clones présents en plus grand nombre. Nous avons également réalisé un marquage IL-17/pdpn afin d'évaluer l'expression de la pdpn par les LTh17 dans notre système. En culture seuls, environ 20% des clones Th17 exprimaient la pdpn, avec ou sans activation par PHA. En revanche, en co-culture, la quasi-totalité des cellules IL-17+ exprimaient la pdpn. Cette augmentation était similaire avec ou sans activation, ce qui indique que le contact cellulaire des LTh17 avec les synoviocytes permet l'induction de l'expression par les LTh17. Nous avons également réalisé ce double marquage IL-17/pdpn dans nos co-cultures avec PBMC afin de confirmer ces résultats. De la même manière que pour les clones, les cellules IL-17+ devenaient quasiment toutes positives pour la pdpn lors de l'interaction avec les synoviocytes.

Le rôle de la pdpn exprimée par les synoviocytes a été mis en évidence grâce aux siRNA, il semble maintenant intéressant de continuer l'étude en confirmant le rôle de la pdpn exprimée par les LTh17.

Ces résultats montrent l'implication de la pdpn dans la sécrétion massive d'IL-17 lors des interactions cellulaires entre cellules mésenchymateuses et cellules immunitaires, avec une interaction à double sens. Pour poursuivre, il serait intéressant d'identifier par quel récepteur ces interactions se font. En effet, le récepteur principal de la pdpn, CLEC-2, a été récemment impliqué dans la régulation négative des fonctions des LT effecteurs [149]. Ces fonctions antagonistes de la pdpn, dans notre cas plutôt inflammatoire et dans l'autre cas plutôt anti-inflammatoire, pourraient impliquer différents récepteurs. Dans un premier temps, nous pourrions regarder l'expression de CLEC-2 dans notre système, puis dans un second temps chercher à identifier un autre récepteur potentiellement engagé dans l'interaction cellulaire avec la pdpn.

La pdpn semble être une molécule importante dans la sécrétion d'IL-17 induite par les interactions cellulaires. Toutefois, son inhibition n'entraine qu'une diminution partielle de la production d'IL-17, ce qui laisse penser que le mécanisme est bien plus complexe et implique d'autres molécules. Dans ce contexte, nous avons établi une collaboration avec une société lyonnaise, Dendritics afin d'identifier d'autres molécules. A partir d'une collection des SN cellulaires contenant des anticorps spécifiques pour les synoviocytes, nous avons réalisé un screening d'un échantillon de cette collection. Pour cela, les co-cultures PBMC activées-synoviocytes ont été réalisées en présence de ces SN. En se basant sur l'effet inhibiteur observé sur la sécrétion d'IL-17, nous avons sélectionné certains SN dont les anticorps ont ensuite été purifiés par Dendritics. Après une deuxième sélection des anticorps purifiés, nous voulons maintenant identifier les cibles de ces anticorps. Pour cela nous utiliserons le clonage par expression, ce qui nous permettra ensuite de comparer les ADNc

obtenus avec des librairies disponibles. Nous espérons ainsi pouvoir identifier d'autres acteurs clés dans ce mécanisme et ainsi des cibles thérapeutiques potentielles pour l'inflammation chronique.

De nouvelles cibles thérapeutiques seraient un progrès réel pour le traitement des maladies inflammatoires chroniques telles que la PR ou le Pso. Toutefois, le temps de développer ces nouvelles thérapies, il est important de bien connaître celles couramment utilisées. Dans la PR, les stéroïdes et le MTX sont deux thérapies fréquemment utilisées, seules ou en association avec différentes biothérapies. Les études actuelles concernant les traitements des patients sont le plus souvent focalisées sur l'aspect clinique au dépend de l'aspect cellulaire. Dans ce contexte, nous nous sommes donc intéressés à l'effet des stéroïdes et des biothérapies au niveau cellulaire, et notamment sur la production de cytokines résultant de l'interaction entre cellules mésenchymateuses et cellules immunitaires, grâce à notre système de co-culture. Dans un premier temps, l'utilisation d'une dose-réponse de méthylprednisolone (MP) a démontré qu'une faible dose était suffisante pour induire un effet inhibiteur sur la production de cytokines pro-inflammatoires (IL-6, IL-1β, IFN-γ et IL-17). Toutefois, cette inhibition s'accompagnait de celle de l'IL-10, cytokine anti-inflammatoire. Dans le même temps, deux concentrations de biothérapies ont été testées afin de déterminer un potentiel effet dose. De manière générale, la concentration la plus faible était suffisante pour induire les effets anti-inflammatoires spécifiques à chaque biothérapie testée (Infliximab, Tocilizumab, Abatacept et Rituximab). Ces premiers résultats montraient ainsi que de fortes doses n'étaient pas forcément nécessaires pour obtenir l'effet escompté.

Dans un second temps, nous avons voulu testé l'effet additionnel MP/biothérapie, comme ces traitements sont souvent utilisés en combinaison chez les patients. Nous sommes partis des concentrations efficaces les plus faibles pour la MP et pour les biothérapies, ajoutées en

combinaison dans nos co-cultures. De manière surprenante, les résultats obtenus n'étaient pas ceux attendus, à savoir un effet additionnel de l'inhibition de la production des cytokines pro-inflammatoires. Cet effet inhibiteur n'était pas augmenté et avait même tendance à diminuer selon les combinaisons. Une étude plus approfondie pour expliquer le mécanisme de cet effet est nécessaire, mais ces résultats suscitent tout de même une certaine réflexion quant à l'utilisation de ces combinaisons. Néanmoins, si les effets sur les cytokines pro-inflammatoires étaient quelque peu déconcertants, il s'est dégagé un résultat intéressant concernant la cytokine anti-inflammatoire IL-10. L'effet de la combinaison MP/biothérapie sur l'IL-10 peut être divisé en trois groupes : le premier présente un effet inhibiteur additionnel sur la production d'IL-10, le second ne montre aucun changement par rapport à l'effet de la MP seule et le troisième révèle une augmentation de la sécrétion d'IL-10. L'IL-10 peut avoir un effet suppresseur sur différentes cytokines pro-inflammatoires telles que le TNF, l'IL-1β, l'IL-6 ou l'IL-17, toutes impliquées dans la pathogenèse de la PR. Le gène de l'IL-10 présente également un certain polymorphisme, dans la région promotrice, avec trois principaux polymorphismes mononucléotidiques [150]. De plus, différentes études ont démontré une association entre le polymorphisme de l'IL-10 et la PR [151-156] et le génotype de l'IL-10 peut également être associé à la réponse aux stéroïdes [157] ou aux biothérapies telles que l'anti-TNF [156]. Ces données montrent que le polymorphisme de l'IL-10 pourrait influencer la réponse aux traitements chez les patients, et dans notre étude pourrait expliquer l'hétérogénéité des réponses. Une analyse approfondie sur l'association potentielle entre le polymorphisme de l'IL-10 et la réponse à la combinaison MP/biothérapie pourrait ainsi fournir une orientation pour des traitements adaptés à chaque patient. De plus, le MTX étant un traitement clé dans la PR, il semble primordial d'étendre cette étude avec le MTX.

Ces derniers résultats concernant l'IL-10 nous poussent également à nous intéresser aux LTreg, source majeur d'IL-10. Notre étude se focalisant sur la voie LTh17, les LTreg sont d'autant plus une piste intéressante. En effet, les voies de développement de ces deux types cellulaires sont réciproquement connectées et une importante plasticité existe entre les LTh17 et les LTreg [158]. Pendant la PR, cette balance LTh17/LTreg est perturbée, ce qui renforce la chronicité de l'inflammation. Dans la suite de ce travail, il serait donc intéressant d'étudier le rôle des interactions cellulaires et de leur inhibition sur les LTreg, en faisant toujours le parallèle avec la voie LTh17. Ceci permettrait de mieux comprendre le mécanisme et peut-être réguler cette balance LTh17/LTreg, afin de prévenir le développement de maladies auto-immunes et inflammatoires chroniques.

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