Rôle de la réponse immunitaire de type 2 dans la réparation tissulaire : du concept au modèle pratique de la sclérodermie systémique

Paôline Laurent

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Par Paôline Laurent

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Sous la direction de : Thomas Pradeu

Membres du jury :

Madame Julie DECHANET	DR CNRS, Université de Bordeaux	Présidente
Monsieur Gérard EBERL	DR INSERM, Institut Pasteur	Rapporteur
Monsieur Jérôme AVOUAC	PU-PH, Université Paris V	Rapporteur
Madame Lucie LAPLANE	CR CNRS, Université Paris I	Examinatrice
Madame Marie-Elise TRUCHETET	MCU-PH, Université de Bordeaux	Membre invitée

Rôle de la réponse immunitaire de type 2 dans la réparation tissulaire : du concept au modèle pratique de la sclérodermie systémique

Pour beaucoup d'entre nous, y compris pour de nombreux immunologistes, le rôle du système immunitaire est restreint à un rôle de défense contre différents pathogènes, tels que les bactéries et les virus. Pourtant, il devient de plus en plus incontestable que le système immunitaire est impliqué dans de nombreux autres phénomènes que peuvent être le cancer, l'obésité et la réparation tissulaire.
Au cours de cette thèse, nous nous sommes intéressés à l'implication des cellules immunitaires, et plus particulièrement des cellules immunitaires innées, dans le mécanisme de réparation tissulaire. Par la suite, nous avons approfondi ce travail en nous focalisant sur les dérégulations de la réparation tissulaire. Ces dérégulations peuvent donner lieu notamment à des phénomènes de « sur-réparation » telle que la fibrose. La fibrose est définie comme un dépôt excessif de matrice extracellulaire par les fibroblastes en réponse à des molécules profibrotiques tels que le TGFβ ou l'IL-13. Nous nous sommes donc intéressés au rôle de la réponse immunitaire innée dans la fibrose en nous concentrant sur deux types de cellules immunitaires innées : les macrophages et les cellules lymphoïdes innées de type 2 (type 2 innate lymphoïde cells, ou « ILC2 »). Nous avons choisi comme modèle d'étude la sclérodermie systémique, maladie auto-immune caractérisée principalement par la fibrose pouvant toucher la peau et/ou les organes internes. Outre la fibrose, cette pathologie est également associée à des anomalies vasculaires et immunitaires. Les mécanismes liant ces trois caractéristiques sont encore mal définis et mal connus.
Il est donc nécessaire de comprendre la physiopathologie de cette maladie et d'établir précisément l'implication de la réponse immunitaire dans la fibrose afin d'offrir un traitement thérapeutique pour les patients sclérodermiques et plus généralement pour toutes les maladies fibrotiques.
Dans un premier temps, nous montrons, en cytométrie de flux, une diminution des ILC2 dans le sang des patients sclérodermiques par rapport aux témoins (0,007 ± 0,007% *vs.* 0,01 ± 0,01%, p=0,001). Chez les sujets sclérodermiques, cette baisse de la fréquence des ILC2 circulantes est inversement corrélée à l'atteinte de la fibrose cutanée définie par le score de Rodnan (R=-0,35, p=0,0062). Nous observons une augmentation de ces cellules dans la peau sclérodermique comparé à celle des contrôles (5,015 ± 2,8% *vs.* 2,816 ± 1,8%). Ce résultat est positivement corrélé au score de Rodnan (r=0,58, p=0,01). Nous obtenons des résultats similaires en immunofluorescence. Un phénotypage des ILC2 dermales nous a permis d'observer une diminution de l'expression de KLRG1 dans la peau des malades. En collaboration avec l'équipe du Pr. Batteux, nous avons étudié le rôle des ILC2 dans un modèle murin de sclérodermie. Nous observons une augmentation cutanée des ILC2 et cela même avant l'établissement de la fibrose au niveau de la peau des souris sclérodermiques (16677 ± 3068 *vs.* 9091 ± 474).
Puis, nous montrons, *in vitro*, que les ILC2 stimulées par le TGFβ perdent l'expression de KLRG1. Au contact des ILC2 stimulées par le TGFβ, les fibroblastes deviennent pro-fibrotique en comparaison à l'incubation avec des ILC2 non stimulées.
Ces résultats apportent de nouvelles connaissances dans la physiopathologie de la sclérodermie systémique et plus particulièrement dans la fibrose caractérisant cette maladie, ce qui offre des perspectives thérapeutiques potentielles. L'approche conceptuelle du rôle du système immunitaire dans la réparation tissulaire proposée dans cette thèse renouvelle notre vision de l'immunité et ouvre potentiellement un nouveau champ, encore sous-estimé, de thérapies ciblant le système immunitaire.

Mots clés : Réparation tissulaire, Fibrose, Réponse immunitaire de type 2, Sclérodermie systémique, ILC2, Macrophages de type 2.

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ABREVIATION

Ac	anticorps
CCL	« chemokine ligand »
CE	cellule endothéliale
CD	« cluster of differentiation »
CLP	« common lymphoid progenitor »
CPA	cellule présentatrice d'antigène
ET1	endothéline 1
FoxP3	« forkhead box 3 »
GATA3	« GATA Binding Protein 3 »
GVHD	« graft-versus-host disease »
HDMEC	« human dermal microvascular endothelial cells »
HLA	« human leukocyte antigen »
HTAP	hypertension artérielle pulmonaire
IL	« interleukine »
ILC	« Innate lymphoid cells »
ILC1	« type 1 innate lymphoid cells »
ILC2	« type 2 innate lymphoid cells »
ILC3	« type 3 innate lymphoid cells »
iILC2	« inflammatory type 2 innate lymphoid cells »
IFN	interféron
ITIM	« immunoreceptor tyrosine based inhibitory motif »
KLRG1	« Killer cell lectin-like receptor subfamily G member 1 »
KO	« knock out »
LB	lymphocyte B
LPS	lipopolysaccharide
LT	lymphocyte T
LTh	« lymphocyte T helper » ou auxiliaire
M1	macrophage de type 1
M2	macrophage de type 2

MEC	matrice extracellulaire
MMP	« matrix-metalloprotease »
nILC2	« nature type 2 innate lymphoid cells »
NK	« natural killer »
OX40L	« OX40 Ligand »
pDC	cellule dendritique plasmacytoïde
PI3K	phosphoinositide 3-kinase
RORγt	« RAR-related orphan receptor gamma »
RSC	rhinosinusite chronique
ScS	sclérodermie systémique
STAT	« signal transducer and activator of transcription »
TCR	« T cell receptor »
TGFβ	« transforming growth factor beta »
TLR	« Toll-like receptor »
TNF	« Tumor necrosis factor »
Treg	lymphocyte T régulateur
TSLP	« thymic stromal lymphopoietin »

I) Introduction

La connaissance du système immunitaire s'est bâtie au fil des années et au gré des découvertes dans le domaine de l'immunologie. Beaucoup de scientifiques ont tenté de définir son rôle. L'immunité a le plus souvent été restreinte à un simple système de défense luttant contre les micro-organismes. L'étymologie du mot « immunité » vient du latin « immunis » signifiant « exempté de, dispensé de ». L'importation de ce terme dans le domaine des sciences médicales a restreint cette définition à un simple état de défense, engendrant un biais dans le rôle du SI et le réduisant à un mécanisme de protection.

A) Contextes historiques sur l'immunologie

Le domaine de l'immunologie s'est développé en parallèle de l'accroissement des connaissances des maladies infectieuses graves et de leurs causes. Cette discipline s'est développée à partir de l'observation que des personnes guéries de certaines maladies infectieuses étaient par la suite protégées contre ces mêmes maladies. Ce fut le cas notamment avec Thucydide dès 430 avant JC lors d'une épidémie de peste ou encore avec Lady Montaigu lors d'une infection par la variole (Dinc and Ulman, 2007; Morens and Littman, 1994). Par la suite, les découvertes et les avancées dans le domaine de la vaccination, notamment avec Edward Jenner (Jenner, 1798), Louis Pasteur (Mercier and Atanasiu, 1985) et Robert Koch (Carter, 1987), ont permis l'essor d'une nouvelle discipline : l'immunologie.
Ces différentes découvertes et avancées dans le domaine de l'immunologie ont été remarquables et essentielles pour l'Homme mais elles ont participé à la restriction de la définition du système immunitaire, de son activation et de son rôle.

B) Evolution du périmètre des rôles du système immunitaire

Pour de nombreuses personnes, incluant des immunologistes, le système immunitaire est perçu comme un système de défense ayant une unique fonction de protection de l'organisme

contre des pathogènes tels que les bactéries, les virus ou les parasites. Cependant, des découvertes majeures dans le domaine de l'immunologie ont contribué à l'émergence d'une nouvelle vision du système immunitaire. En effet, le système immunitaire est impliqué directement ou indirectement, en étant la cause ou la conséquence, dans de nombreux phénomènes biologiques tels que le cancer (Chen and Mellman, 2017; de Visser et al., 2006), le vieillissement (Castelo-Branco and Soveral, 2014; Franceschi et al., 2017), les interactions avec le microbiote (Fung et al., 2017; Hooper et al., 2012), l'obésité (de Heredia et al., 2012; Saltiel and Olefsky, 2017), ou encore la réparation tissulaire (Gieseck et al., 2018; Mantovani et al., 2013).

Cette réflexion sur la pluralité fonctionnelle du système immunitaire n'est, certes pas unique, mais peu de scientifiques l'étudient expérimentalement et encore moins avec une approche conceptuelle. Beaucoup d'acteurs immunitaires impliqués dans ces différents phénomènes sont les mêmes. Les macrophages, par exemple, sont impliqués dans le cancer (Sica et al., 2006), dans le vieillissement (Plowden et al., 2004), dans les interactions avec le microbiote (Mortha et al., 2014) et dans réparation tissulaire (Wynn and Vannella, 2016). Comment une cellule immunitaire est capable de changer de rôle, de fonction selon le phénomène biologique dans lequel elle est engagée ?

Cette question nous a amené à réfléchir sur plusieurs notions :

- Le **contexte** dans lequel la cellule immunitaire est. Comme dit précédemment, une cellule immunitaire peut être impliquée dans des évènements très divers. Le contexte dans lequel la cellule agit est donc déterminant pour la fonction de la cellule immunitaire. Par exemple, deux pathologies, ayant de nombreux mécanismes en commun, que ce soient des molécules ou des cellules immunitaires, peuvent donner des symptômes extrêmement différents. Des facteurs génétiques ou environnementaux peuvent influencer le contexte dans lequel se trouve une cellule immunitaire ;
- La **localisation** de la cellulaire immunitaire. Les cellules immunitaires n'expriment pas les mêmes récepteurs selon l'organe dans lequel elles se trouvent (Picker et al., 1994). De plus, certaines cellules immunitaires sont spécifiques d'un organe donné : les kératinocytes et les cellules de Langherans sont uniquement présents dans la peau, les cellules de Kupffer ne se trouvent que dans le foie tandis que les

microglies sont localisées uniquement dans le système nerveux central. Nous pouvons légitimement penser que le microenvironnement est différent d'un organe, d'un tissu à un autre. Le microenvironnement est largement étudié dans le cadre du cancer (Fidler, 2002). Etant donné son importance dans le comportement biologique des cellules métastatiques, le microenvironnement mériterait de faire l'objet d'études plus poussées sur son implication dans d'autres phénomènes. Cette notion de localisation est intimement liée à la notion de **migration**. Outre les cellules résidentes, les cellules présentes dans les différents tissus proviennent du sang. Grâce à l'expression de récepteurs spécifiques à leur surface, les cellules migrent à un moment donné vers un tissu donné ;

- La **plasticité** des cellules immunitaires : comment une même cellule peut exprimer des récepteurs différents, produire des cytokines diverses selon le contexte et la localisation dans lesquels elle se trouve ? La notion de plasticité cellulaire est primordiale. Il est maintenant reconnu que les cellules immunitaires ont la capacité d'être plastiques (Galli et al., 2011; Huang et al., 2001; Zhou et al., 2009). Les signaux cytokiniques, les modifications épigénétiques et d'autres facteurs microenvironnementaux peuvent modifier considérablement et rapidement le phénotype de ces cellules et influencer leur fonction (Galli et al., 2011).

Au regard de l'importance de l'intégrité tissulaire dans l'homéostasie de l'organisme, nous nous sommes plus particulièrement intéressés au rôle du système immunitaire dans le mécanisme de réparation en intégrant et en insistant sur ces notions de plasticité, de localisation et de contexte. Ces éléments peuvent-ils influencer ce processus ? Les mécanismes de la réparation sont-ils similaires d'un organe à un autre ? La plasticité cellulaire est-elle impliquée dans ce processus ? Dans quels mesures le contexte est-il important ? Une dysfonction de la réponse immunitaire entraîne-t-elle une dysfonction de la réparation ?

C) Rôle du système immunitaire dans la réparation tissulaire

1. Définition de la réparation tissulaire

La réparation tissulaire est un mécanisme permettant de restaurer l'intégrité d'un tissu après que celui-ci a subi une blessure donnant lieu à une cicatrice. C'est une propriété fondamentale pour les organismes multicellulaires. C'est un mécanisme très conservé au cours de l'évolution comme en témoigne la capacité de l'éponge, un des organismes multicellulaires les plus anciens de réaliser ce processus (Nichols et al., 2006).
La réparation tissulaire se déroule en 3 étapes successives (Gurtner et al., 2008) :

- Inflammation
- Formation d'un nouveau tissu
- Remodelage

La réparation tissulaire pouvant être dérégulée, entraînant alors des situations que nous définissons comme étant pathologiques (voir ultérieurement), il paraît important de comprendre et d'analyser les voies de signalisation ainsi que tous les acteurs impliqués dans ce mécanisme.
Le porc a été dans un premier temps utilisé comme modèle dans la mesure où la peau du porc est très similaire à celle de l'homme (Schultz et al., 1987). Du fait de nombreuses difficultés rencontrées avec ce modèle, notamment en termes de coût et de manipulation de l'animal, d'autres modèles ont émergé. Ainsi, des études sur la drosophile ont permis de comprendre plus précisément le mécanisme de réparation tissulaire. Des analyses génomiques sur des drosophiles mutantes, ayant une altération de la réparation (Campos et al., 2010; Lesch et al., 2010), ont mis en évidence différentes voies de signalisation tronquées comme celles de Wnt et Grh (Mace et al., 2005). Néanmoins, la drosophile n'ayant pas d'immunité adaptative et ne faisant pas d'angiogenèse ou de fibrose, ce modèle a des limites (Razzell et al., 2011). C'est la raison pour laquelle des études sur le poisson zèbre ont été réalisées. Les acteurs et les mécanismes jouant dans les 3 phases définissant la réparation tissulaire sont très similaires entre les poissons zèbres et les mammifères (Henry et al., 2013; Richardson et al., 2013).

Ces différents modèles ont grandement aidé à comprendre l'importance du système immunitaire dans la réparation tissulaire.

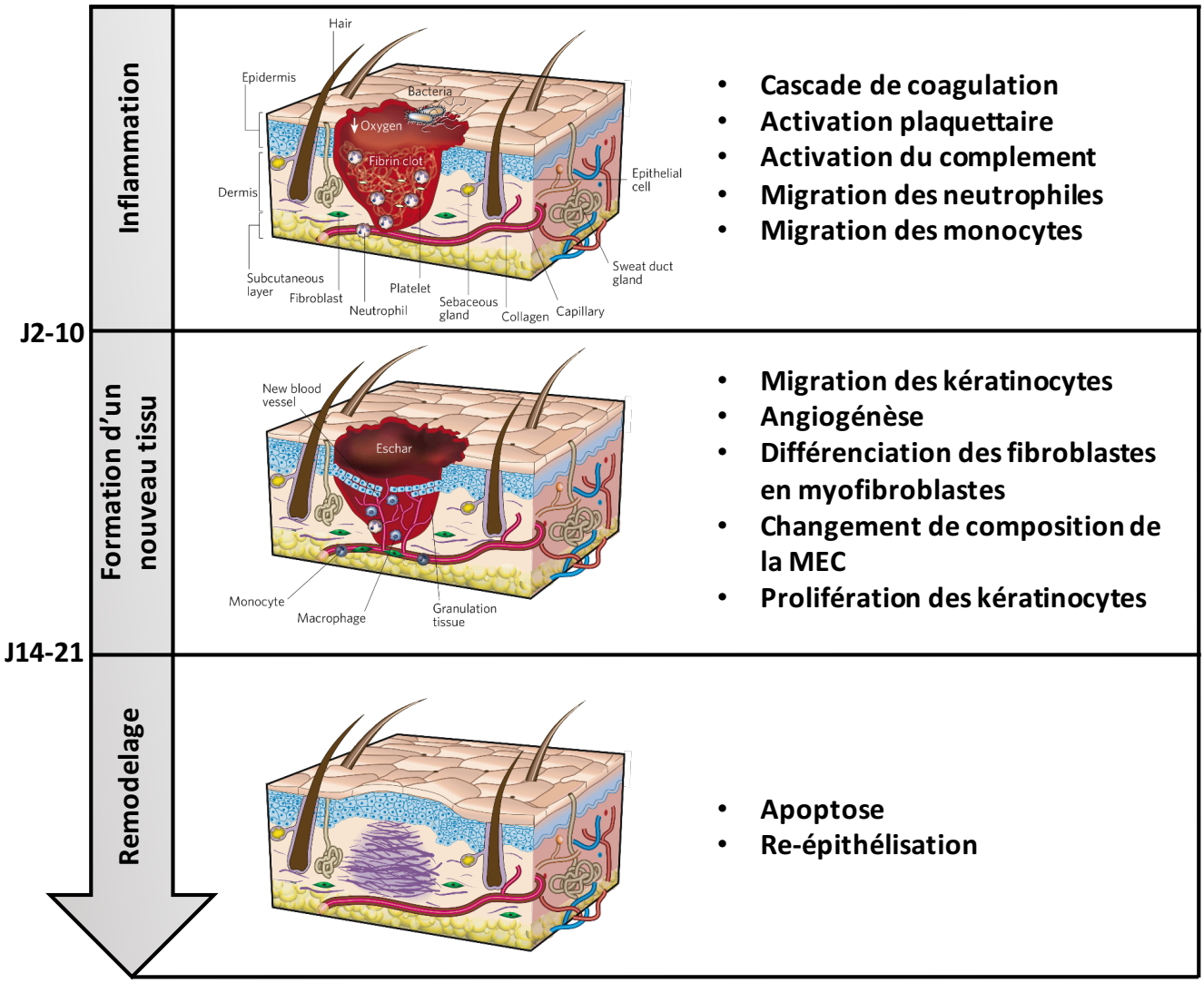

Figure 1 : Illustration de la réparation tissulaire en 3 étapes : (i) Inflammation, (ii) formation d'un nouveau tissu et (iii) remodelage. (Modifiée de Gurtner et al., 2008)

a) Importance de l'immunité innée

D'après Sabine Eming (Eming et al., 2017), Metchnikoff fut l'un des premiers à associer le système immunitaire et la réparation tissulaire dans son livre « L*eçon sur la pathologie comparée de l'inflammation »* en 1892 (Metchnikoff, 1892).

Du fait que les macrophages ont été, et sont encore, des cellules largement scrutées dans l'immunité, ils sont au centre des premières investigations du potentiel rôle du SI dans la réparation tissulaire. En effet, dès 1975 Leibovitch et Ross étudient le rôle des macrophages dans ce processus en utilisant un modèle de cochon d'Inde ayant subi une déplétion des monocytes /macrophages ; ils concluent que les principales cellules responsables de la réparation sont les macrophages (Leibovich and Ross, 1975). Dans un premier temps, le rôle des macrophages est restreint à la phase d'inflammation. Dans son article « The physiology of wound healing », Hunt souligne l'importance de ces cellules ainsi que des granulocytes dans les deux autres phases et notamment dans la formation d'une nouvelle matrice extracellulaire (MEC) (Hunt, 1988). Le rôle des macrophages a également été analysé *in vivo* dans la drosophile en utilisant une technique de « live imaging » (Stramer et al., 2007). L'utilisation du poisson zèbre a permis de mettre en évidence le rôle crucial des neutrophiles dans la réparation tissulaire et notamment dans la résolution de l'inflammation (Henry et al., 2013; Pase et al., 2012).

Puisque des organismes multicellulaires tels que la drosophile ou le poisson zèbre sont capables de réparer leur tissu lésé alors qu'ils ne possèdent pas de système immunitaire adaptatif, le rôle de l'immunité innée semble donc être primordial lors de cet événement.

b) Rôle de la plasticité cellulaire

Au cours de ma thèse, nous avons étudié le rôle des cellules immunitaires innées dans la réparation tissulaire. Cette analyse a donné lieu à la publication d'une revue de la littérature, présentée ci-dessous, sur le rôle de l'immunité au cours de la réparation tissulaire

Article de revue n°1 : Immune-Mediated Repair: A Matter of Plasticity

Laurent P, Jolivel V, Manicki P, Chiu L, Contin-Bordes C, Truchetet ME, Pradeu T

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Dans cette revue de la littérature, nous avons décrit l'implication des acteurs de l'immunité dans la réparation tissulaire. Nous avons exploré comment des types de cellules immunitaires distincts étaient impliqués dans la réparation tissulaire et comment ils interagissaient dans un processus étroitement régulé dans l'espace et dans le temps. Nous avons porté une attention particulière au concept de plasticité des cellules immunitaires qui, ces dernières années, s'est avéré fondamental pour la compréhension du processus de réparation. Dans l'ensemble, la perspective présentée ici suggère que le système immunitaire joue un rôle central dans la robustesse physiologique de l'organisme (Truchetet and Pradeu, 2018) ; en d'autres termes dans la capacité d'un organisme à maintenir ses performances malgré des perturbations, et que la plasticité cellulaire contribue à la réalisation de cette robustesse.

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MINI REVIEW
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Immune-Mediated Repair: A Matter of Plasticity

Paôline Laurent[1], Valérie Jolivel[1], Pauline Manicki[2], Lynn Chiu[1], Cécile Contin-Bordes[1,3], Marie-Elise Truchetet[1,2†] and Thomas Pradeu[1†]*

[1] ImmunoConcept, UMR5164, Immunology, CNRS, University of Bordeaux, Bordeaux, France,
[2] Rheumatology, CHU Bordeaux Hospital, Bordeaux, France, [3] Immunology, CHU Bordeaux Hospital, Bordeaux, France

Though the immune system is generally defined as a system of defense, it is increasingly recognized that the immune system also plays a crucial role in tissue repair and its potential dysregulations. In this review, we explore how distinct immune cell types are involved in tissue repair and how they interact in a process that is tightly regulated both spatially and temporally. We insist on the concept of immune cell plasticity which, in recent years, has proved fundamental for the success/understanding of the repair process. Overall, the perspective presented here suggests that the immune system plays a central role in the physiological robustness of the organism, and that cell plasticity contributes to the realization of this robustness.

Keywords: repair, plasticity, robustness, fibrosis, macrophages, neutrophils, innate lymphoid cells, transdifferentiation

> *"The medical and naturalist philosophers have been vividly struck by this tendency of the organized individual to reestablish its form, to repair its mutilations, to heal its wounds, and in this way to prove its unity, its morphological individuality (1)."*

INTRODUCTION

All organisms possess the crucial property of *robustness*, which is a system's capability to maintain its functions and performances despite perturbations (2–4). Robustness includes the capacities for each organism to build, defend, and repair itself (1, 5). The immune system, by constantly surveying the body and responding to strong anomalies, plays a key role in robustness (6, 7). In this review, we analyze how the immune system regulates tissue repair and show that one major way by which the immune system contributes to robustness is through immune cell plasticity. Notably, innate immune cells are particularly important in tissue repair, which suggests that the role of immunity in maintaining repair-oriented robustness has been conserved through evolution.

Tissue repair can be defined as the process that restores (at least partially) tissue organization after an insult (8, 9). The insult consists in damages by physical or chemical aggression, infectious agents, or "internal" stresses (10, 11). Tissue repair may be divided into three partly overlapping stages (8, 12). The first stage, *inflammation* (0–48 h after injury), immediately follows tissue damages and triggers the activation of the components of the coagulation cascade, the immune system, and inflammatory pathways. The second stage, *new tissue formation* (2–10 days after injury), is characterized by the proliferation and migration of many cell types and angiogenesis (i.e., formation of new blood vessels), and the progressive restoration of tissue function. Finally, during the third stage, *remodeling* (starts 2–3 weeks after injury and can last several months), many activated cells (e.g., endothelial cells, macrophages, and myofibroblasts) die by apoptosis or leave the site of injury, and tissues are re-epithelized.

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Reviewed by:
Kiyoshi Hirahara, Chiba University, Japan
Maryna Skok, Palladin Institute of Biochemistry (NAN Ukraine), Ukraine

***Correspondence:**
Thomas Pradeu
thomas.pradeu@u-bordeaux.fr

†These authors are co-last authors of this paper.

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The perturbation of one or several of these three stages leads to dysregulated repair and can have important pathological consequences (13). Several different disorders can be described as the result of abnormal repair. Fibrosis, for instance, is often seen as the consequence of "over-repair" or "over-healing" (8, 13), with excessive accumulation of extracellular matrix (ECM) and failure to restore tissue function and architecture (14), as observed in idiopathic pulmonary fibrosis, heart failure, and several autoimmune diseases such as systemic sclerosis (15). In contrast, ulcers can be viewed as the consequence of "under-healing" (16). Cancerous tumors can also be seen as products of an abnormal repair process, or "wounds that do not heal" (17, 18).

Though the immune system is generally defined as a system of defense, selected through evolution for its capacity to insure host protection (19–23), it is now clear that the immune system plays also an essential role in tissue repair (9, 24–26). The involvement of the immune system in repair had long been suspected (27–30), but it is only recently that a precise cellular and molecular characterization of this process has been possible. In this review, we describe the impact of the immune system on repair and dysregulated repair and emphasize the key role played by immune cell plasticity in repair (**Figure 1**) (31). The word "plasticity" is used with different and often confusing meanings. Here, we understand cell plasticity in two different and important senses. The first sense is *intra-lineage cell plasticity*, that is, changes in cell function and phenotype within a given cell lineage—for example, type 1 macrophages (M1s) turning into type 2 macrophages (M2s). This is what is sometimes called "functional plasticity" (32). The second sense is *trans-lineage cell plasticity*, that is, the switch from one lineage to another—e.g., from macrophages to fibroblasts (33). This can also be called plasticity by "transdifferentiation" (34) or by "reprogramming"—a phenomenon now known to occur in some non-immune cells (35). We show here how these two types of cell plasticity are involved in tissue repair—with a particular insistence on the first type, which is now amply demonstrated. Because the most numerous and crucial immune cells involved in tissue repair are macrophages and neutrophils, we start our review with a description of their roles and plastic capacities.

PLASTICITY OF NEUTROPHILS AND MACROPHAGES IN REPAIR

Neutrophils are part of the first line of defense against infection and are massively recruited on damage sites (36). The defense function is mainly mediated by phagocytosis, intracellular degradation, releasing of granules, and formation of neutrophil extracellular traps (NETs) after sensing dangerous stress. However, accumulating data show that neutrophils have a variety of important biological functions far beyond cytotoxicity against pathogens. Their adaptable life span, longer than previously thought (more than 5 days) (37), allows neutrophils to participate actively in the three repair stages (38).

Far from being "one-shot" weapons, long-living neutrophils are remarkably plastic. Indeed, neutrophils can differentially

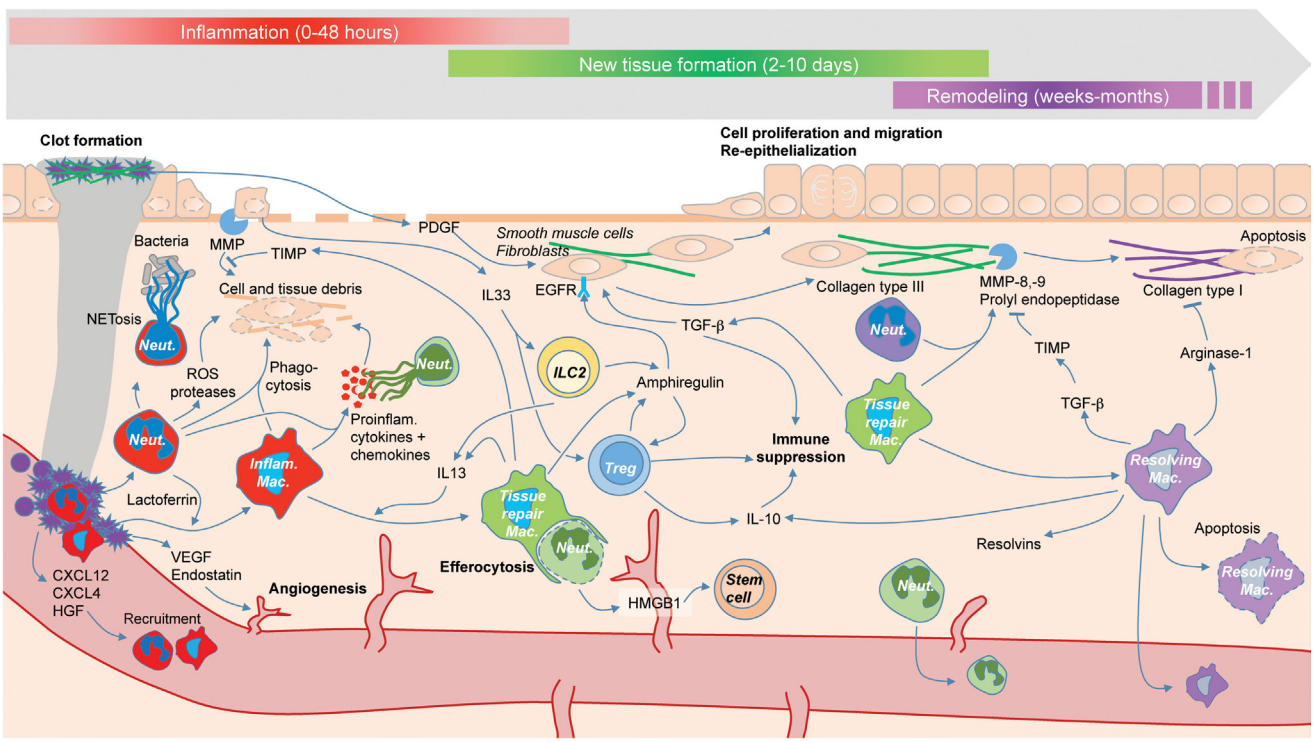

FIGURE 1 | The roles of immune cells (colored) during the three phases of tissue repair: inflammation (red), new tissue formation (green), and remodeling (purple). Tissue repair is driven by interactions between immune cells and other cell types such as fibroblasts (not colored), as well the temporary plasticity of immune cells, e.g., macrophage switch from an inflammatory phenotype (red) to a tissue repair phenotype (green) to a resolving phenotype (purple).

switch phenotypes, display distinct subpopulations under different microenvironments, and produce a large variety of cytokines and chemokines (39). Plasticity of neutrophils has been evoked recently following the controversial debate on their pro- or antitumor role, leading to the conclusion that they can be both, depending on the subsets under consideration. In repair, neutrophils can be pro- or anti-resolution of the inflammatory stage. The resolution of tissue formation depends on neutrophils for their ability to (i) produce several pro-resolving mediators (as lipoxins) (40), (ii) form NETs and aggregated NETs, according to a cell-density dependent sensing mechanism, which dismantles the pro-inflammatory gradient by degrading the inflammatory cytokines and chemokines (41), and (iii) store and release the pro-resolving protein annexin A1 (42–44).

In addition to this intra-lineage plasticity, repair-associated neutrophils are capable of trans-lineage plasticity (i.e., plasticity by transdifferentiation) (45, 46). Challenging the classic view of neutrophils as terminally differentiated leukocytes fully committed to phagocytosis, new evidence shows that neutrophils can acquire phenotypic and functional properties typically associated with professional antigen-presenting cells (APCs) (47, 48)—e.g., MHC II expression and co-stimulatory molecules (49). Such neutrophil trans-lineage plasticity is driven in part by the microenvironment composition. For example, in an inflammatory and pro-type 2 environment of a lesion, neutrophils could transdifferentiate into APCs (46). Transformation of neutrophils into APCs has also been studied in rheumatoid arthritis, where it could drive sustained inflammation, thereby preventing normal repair (50). Trans-lineage plasticity could also link innate and adaptive immunity and constitute a pivotal crossroad polarizing the response toward abnormal repair, e.g., fibrosis.

Recent research has also highlighted the plasticity of monocytes and macrophages. During the early inflammatory phase (stage 1), monocytes are attracted by neutrophils to the wound site, where they represent a predominant source of pro-inflammatory mediators and exert a phagocytic role as M1 (51). Early depletion of macrophages in mice leads to decreased inflammatory responses (52).

Type 1 macrophages drive the early inflammatory responses that lead to tissue destruction, whereas M2s (or "alternatively activated reparative macrophages") exert a central role in wound healing. Both types are needed for proper wound healing (53). The second stage of the repair process—new tissue formation—is dominated by M2s (26, 54, 55). These cells promote tissue repair by producing pro-reparative cytokines or generating a pro-type 2 microenvironment. They also present a complex heterogeneity (56). Beyond a strict M1/M2 dichotomy, a wide range of macrophage subtypes exists (57–59), and several of them are involved in repair (34). Efficient tissue repair requires inflammatory macrophages, tissue repair macrophages, and resolving macrophages (producers of resolvins, IL10, and TGF-b) (26, 34, 60).

During the remodeling phase, most macrophages undergo apoptosis or leave the wound (8). At this stage, the wound consists mostly of an acellular matrix where the collagen type III is substituted by collagen type I, thanks to the secretion of matrix metalloproteinases, notably by tissue repair macrophages. Even if current evidence is limited, macrophages might participate actively in tissue remodeling by transdifferentiation, notably into endothelial cells (61–64).

All this raises the question of whether the crucial and distinct roles played by macrophages and neutrophils in tissue repair are better explained by cell migration or by cell plasticity (65). Do subtypes of macrophages and neutrophils (M1s and M2s; pro-inflammatory and pro-resolving neutrophils) reach the tissue separately, in successive waves, or are they the product of a plastic switch from one subtype to the other? Further research is needed to answer this question.

PLASTICITY OF OTHER IMMUNE CELLS IN REPAIR

Though neutrophils and macrophages constitute the most numerous immune cells involved in tissue repair, this process also includes other immune cells—particularly γδ T cells, innate lymphoid cells (ILCs), and regulatory T cells (Tregs).

Resident T cells are present in human epidermis with both αβ and γδ subtypes. There is a lot of evidence for the involvement of γδ T cells in repair processes in mice (66). The situation is far less clear in humans. Nevertheless, some studies showed in culture models the promotion of wound healing by γδ T cells, through the production of the insulin-like growth factor 1 (67). Plasticity of γδ T cells has been only evoked in the context of cancer, where γδ T cells display either pro- or antitumor activity depending on the cytokine environment (68, 69). Further investigations are needed to demonstrate if this functional plasticity of γδ T cells is also involved in tissue repair.

Innate lymphoid cells are a recently discovered family of immune cells that includes three subsets: ILC1, ILC2, and ILC3 (70–72). These cells are found preferentially on epithelial barrier surfaces such as skin, lungs, and gut, where they protect against infection and maintain the integrity of the barriers (73, 74). ILCs are tissue-resident sentinels enriched at mucosal surfaces and have a complex crosstalk with the microenvironment. They are highly involved in tissue repair through their sentinel position and the cytokines they produce (75, 76). ILC2-secreted amphiregulin, a protein shown to orchestrate tissue repair (75), promotes wound healing by acting directly on fibroblasts, leading to ECM deposit. Key transcription factors determine the pathway of differentiation of progenitors toward an ILC1, ILC2, or ILC3 subset. ILC responses to different stimuli allow intra-lineage plasticity between the different subsets (77, 78). This plasticity between different ILC subtypes might allow for rapid innate immune responsiveness in repair (79, 80).

Regulatory T cells exert multiple functions (81) and could play a critical role in tissue repair (82). Recently, several populations of tissue-resident Tregs were identified, including special Tregs in visceral adipose tissue, muscle Tregs, and skin-resident memory Tregs (83, 84). In repair, Tregs could modulate the effector response and contribute to the redundant effect of the previously described cells by creating a dynamic microenvironment. Tissue-resident Tregs are able to rapidly respond to tissue damage, inducing tolerance and repair to prevent loss of tissue function. Tregs share these features with ILC2s and anti-inflammatory macrophages.

Thus, many elements are redundant in tissue repair. This redundancy likely illustrates the critical role played by immune-associated repair in the survival and robustness of the organism.

CELLULAR PLASTICITY IN WOUND REPAIR: A PROMISING AVENUE FOR FUTURE EXPERIMENTAL RESEARCH

Immune cells participate in the generation of a peculiar microenvironment, leading to a balance shift between the pro-inflammatory and pro-reparative sides of tissue repair. In cystic fibrosis, for example, abnormal intra-lineage neutrophil plasticity has been implicated in the unbalance of airways microenvironment, leading to chronic inflammation and inability for other cells such as macrophages to switch to resolving stages (85). Hence, manipulation of this process constitutes an innovative therapeutic approach for pathological conditions involving dysregulated repair. Here, we explore different examples of therapeutic modulations of intra-lineage plasticity, for both macrophages and neutrophils.

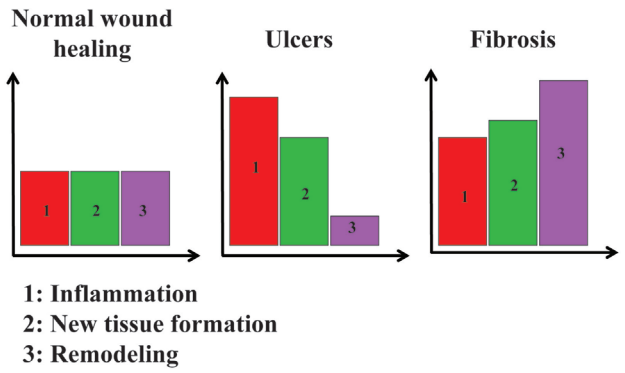

FIGURE 2 | Relative importance of inflammation, new tissue formation, and remodeling in three different situations. In normal wound healing, inflammation (1, in red), new tissue formation (2, in green), and remodeling (3, in purple) are at a basal level. In ulcers, inflammation and new tissue formation are at higher levels, whereas remodeling is lower than the basal level. In fibrosis, inflammation, new tissue formation, and remodeling are all higher than their basal levels.

Targeting Functional Plasticity during Tissue Injury

In certain conditions, accelerating tissue repair could be decisive, particularly for certain wounds (such as large or life-threatening wounds) and for certain patients (such as elderly or fragile patients). Remarkably, patients treated by immunosuppressive therapy experience a delayed wound healing, which shows that the inflammatory stage is important to induce repair. The complexity of tissue repair is due to the number of involved partners but also to the precise timing and imbrication of the phases. Therefore, isolating new targets, even of great importance, will not be sufficient if the whole balance and timing are not considered.

Numerous mediators involved in the phenotype conversion of macrophages have been described, but so far their therapeutic potential remains uncertain in humans (86). For example, *in vitro* studies showed that GM-CSF could switch inflammatory monocytes to a reparative phenotype, leading to the idea that GM-CSF could exert beneficial effects on intestinal inflammation and wound healing associated with Crohn's disease (87). Intra-lineage plasticity of macrophages could also be modulated through the VEGF-C/VEGFR3 pathway, leading to hybrid macrophages favorable to resolution (88).

Reparative neutrophils could also be modulated to accelerate the process of wound healing (39). In cardiac infarction, a temporal switch from inflammatory to resolving neutrophils has been detected (89). Programming neutrophils to the N2 subtype by a PPARγ agonist rosiglitazone could be used to obtain a beneficial role of anti-inflammatory N2 neutrophils, as shown in stroke (90).

Targeting Functional Plasticity during Chronic Injury (or in Chronic Wounds)

A chronic wound could be seen as resulting from a dysregulated repair process, with an increase of pro-inflammatory environment at the expense of the pro-resolving one (**Figure 2**). Modulating cell plasticity toward a more resolving phenotype appears an attractive strategy in that line.

Mechanical debridement of a chronic wound consists in the removal of dead tissues to improve the healing potential of the remaining healthy tissue. This removal leads to tissue re-colonization by immune cells, suggesting that they are important in the repair process. Maggot therapy could empirically modulate immune cell plasticity in addition to its mechanical role. Some data indicate that maggot secretions decrease the elastase release and the H_2O_2 production of activated neutrophils without affecting their phagocytic activity (91). Moreover, maggot secretions inhibit the production of pro-inflammatory cytokines by monocytes, skewing their phenotype from a pro-inflammatory to a pro-angiogenic type (92).

Another approach is to figure out the exact cause of the chronic pro-inflammatory stimulation and develop a therapeutic strategy specific to this cause. In some cases, the pro-inflammatory stimulation is associated with bacterial biofilms (93–95). Biofilms in acute surgical and chronic wounds appear to cause a delay in healing (95). In this composite state, bacteria are resistant against antibiotic treatment and immune responses, leading to impaired eradication of opportunistic pathogens. Biofilm-embedded bacteria release virulence factors dictated by quorum-sensing that in turn promote inflammatory mediators such as IL-1β, contributing to delayed wound re-epithelialization and healing (96, 97). Hence, dealing with biofilms has become a major challenge in chronic wound healing. Quorum-sensing blockers could be an innovative approach to treat non-healing wounds, even though clinical trials are needed to prove their relevance (98).

Targeting Plasticity in Over-Repair and Fibrotic Processes

Keloid and hypertrophic scars are pathologic fibroproliferative disorders characterized by an excess of collagen deposition in genetically predisposed patients. In hypertrophic scars, inflammatory genes are expressed at a lower level and for a longer time, with a delayed but prolonged infiltration of M2 macrophages (99).

Hypertrophic scar development is dependent on macrophages as their depletion during the subacute phase allows normal scarring in mice (100). Thus, limiting M2 activation in keloid could be a way to circumvent the hypertrophic scar.

Diffuse cutaneous systemic sclerosis (dcSSc) is a form of over-repair. SSc results from the interaction of three processes: innate and adaptive immune abnormalities, vasculopathy, and fibroblast dysfunction leading to excessive collagen and matrix components accumulation (101). Fibrotic skin is characterized by an immune cell infiltrate of various nature (15, 102–104). These cells follow a chemokine gradient, such as CCL2, partly explaining the recruitment of macrophages and the M2 polarization in SSc skin (105, 106). Limiting M2 activation and even activating M1 could be an interesting lead for dcSSc at the proper stage. The window of opportunity is critical, and studies showing the evolution of cell plasticity during SSc evolution are lacking to establish reliable therapy based on cell plasticity. Nevertheless one can assume that the number of pro-inflammatory innate cells is limited to a first phase, and then a pro-reparative profile of cells is predominant, giving a place for anti-resolving cell drugs. At the last stage, the absence of infiltrating cells could prevent the efficiency of immunological approaches.

CONCLUSION

Immunologists have tended to see the immune system as a system of defense. Yet the immune system is crucial for several "housekeeping" processes, most prominently repair (7). In some cases, it would even seem artificial to distinguish between "repair" and "defense," because a typical repair response (e.g., type 2) is used to "wall off" particular pathogens such as helminthes (107). By constantly surveying the body and responding to anomalies and through its pleiotropic roles in defense, development, and repair, the immune system is pivotal for the robustness of the organism (6). A system is "robust" when it is able to maintain its functions and performances despite perturbations (2–4). Robustness does not mean an absence of change: quite the contrary, it is through constant internal modifications that an organism can maintain its functions. We should therefore not be surprised by the main conclusion of the present review, which is that immune cell plasticity [and cell plasticity more generally (65, 108)] is essential to ensure the robustness of the organism as far as tissue repair is concerned.

Though still in its infancy, the idea of therapeutically manipulating immune cell plasticity in repair seems extremely promising. We have examined several examples where the manipulation of immune cell plasticity is already a reality, and we can only look forward to future investigations. Recently, an increasing number of tissue repair specialists have become interested in how non-mammal model organisms repair and/or regenerate (9). Crucially, the immune system plays a key role in repair and regeneration across species, and regeneration is often associated with an immunosuppressive state (109–111). Successful regeneration also presupposes cell plasticity, both intra-lineage and trans-lineage (112, 113). It is exciting to speculate that immune cell plasticity could play an important role in regeneration and that one day clinicians could manipulate this immune plasticity to skew the balance between damaging and reparative effects toward the desirable state for any given patient.

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PL, VJ, PM, LC, CC-B, M-ET, and TP wrote the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

c) *Réparation tissulaire et régénération : rôle du système immunitaire*

La capacité de régénération des organes, prédominante chez certains invertébrés et vertébrés anciens d'un point de vue phylogénétique, est peu développée chez les mammifères. Au cours de l'évolution, le système immunitaire des mammifères engendre rapidement une inflammation pour protéger l'organisme contre les infections optimisant ainsi la défense tissulaire mais entraînant en contrepartie une perte de la capacité de régénération de l'organisme (Mescher and Neff, 2005). Des études chez le têtard tendent à confirmer cette hypothèse. En effet, la perte de la capacité de régénérer du têtard coïncide avec un pic de présence de cellules immunitaires (Fukazawa et al., 2009). A l'inverse, d'autres études montrent que l'inflammation est cruciale pour la réparation tissulaire mais également pour la régénération (Godwin et al., 2017; Kyritsis et al., 2012; Willenborg et al., 2012). Durant le développement prénatal de l'Homme, le fœtus est capable de faire de la régénération, capacité qu'il perd ensuite pour l'essentiel durant la vie adulte (Colwell et al., 2003).

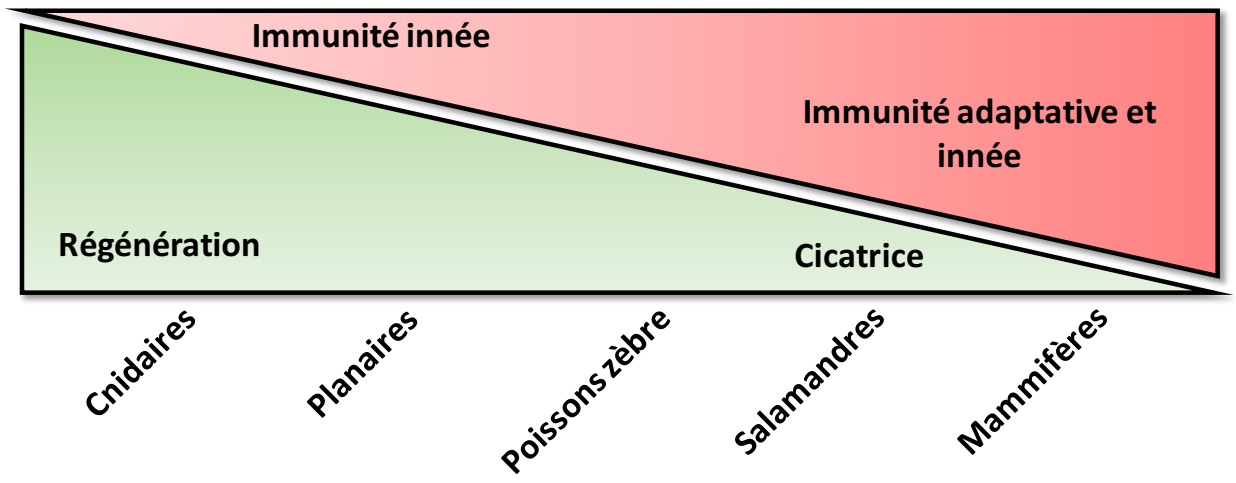


Figure 2 : Illustration des capacités régénératives et/ou cicatricielles des différentes classes d'animaux de l'évolution en fonction de la complexité de la réponse immunitaire

Il reste néanmoins encore de multiples mécanismes à comprendre afin d'envisager dans le futur la régénération comme voie thérapeutique.

2. Dérégulation de la réparation tissulaire

Cette notion de dérégulation de la réparation tissulaire est abordée précédemment dans la revue n°1. Dans la partie suivante de ma thèse, je reviendrai plus précisément sur ce concept de dérégulation en tentant d'expliquer l'origine de ce mécanisme.

a) Définition

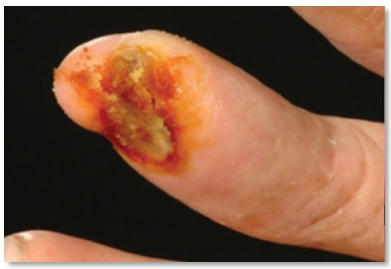
Dans certains cas, la réparation tissulaire peut donner lieu à des situations pathologiques. Bien qu'initialement bénéfique, la réparation tissulaire devient pathologique quand elle n'est pas contrôlée de manière appropriée.

i. Fibrose et chéloïde

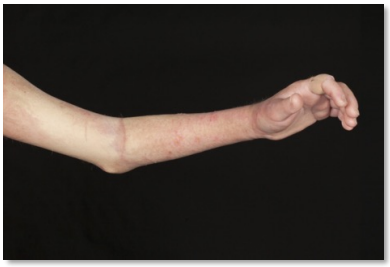
En effet, les chéloïdes ainsi que la fibrose sont caractérisés par un dépôt excessif de collagène, généralement chez des personnes prédisposées génétiquement, et peuvent être définis comme étant une sur-réparation (Laurent et al., 2018). La fibrose est le résultat pathologique de nombreuses maladies inflammatoires ainsi que de nombreuses maladies auto-immunes. Ces pathologies sont une importante cause de morbidité et de mortalité dans le monde. En effet, la fibrose peut toucher plusieurs organes tels que les poumons, les reins ou encore le foie. Les chéloïdes sont des excroissances du derme se développant habituellement sur des lésions cutanées. Ils se forment à la suite d'une cicatrisation anormale d'une plaie par exemple.

ii. Ulcère

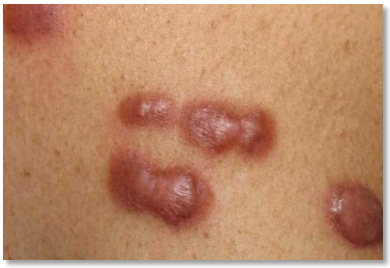
A l'inverse, les ulcères, définis comme une plaie chronique, peuvent être considérés comme une sous-réparation caractérisée par une érosion de l'épithélium. Ils sont généralement accompagnés d'une désintégration du tissu. Les causes d'un ulcère sont multiples : elles peuvent être endogènes, notamment lié au stress par exemple, mais aussi exogènes comme des actions chimiques ou mécaniques.



Ulcère



Fibrose



Cheloïde

Figure 3 : Photo représentant des ulcères (à gauche), de la fibrose (au milieu) et des chéloïdes (à droite) (Denton and Khanna, 2017, https://www.msdmanuals.com/fr/professional/troubles-dermatologiques)

b) Implication du système immunitaire

i. Rôle du système immunitaire dans la fibrose

Le rôle du SI dans l'initiation de la fibrose est indéniable mais le mécanisme précis est encore mal défini et mal compris. Il est maintenant clair que beaucoup d'éléments de l'immunité innée et adaptative participent à l'activation et la différenciation des myofibroblastes, contribuant à la progression de la fibrose (Chizzolini et al., 2011).

Deux grandes théories émergent afin de tenter d'expliquer ce phénomène de fibrose. La première suggère que le début de la fibrose est initié par une persistance de l'inflammation (Wynn and Ramalingam, 2012). En effet, dans des modèles murins de fibrose, l'apoptose des cellules épithéliales entraine une activation de la réponse inflammatoire menant à la fibrose. En d'autres termes, la boucle réparation tissulaire ne cesse jamais entraînant ainsi un dépôt de MEC en continu induisant la fibrose (Lawson et al., 2011). La deuxième théorie est fondée sur la plasticité cellulaire durant la réparation tissulaire. En effet, certains émettent l'hypothèse que la fibrose serait engendrée par une altération de la plasticité (Wynn and Vannella, 2016). Lors du passage de la deuxième à la troisième phase, les cellules présentant un phénotype « réparateur » n'adoptent pas un phénotype de « résolution ». Les cellules

« réparatrices » sécrètent du collagène et d'autres composants de la MEC de manière continue sans que les cellules de résolution n'arrêtent le phénomène.

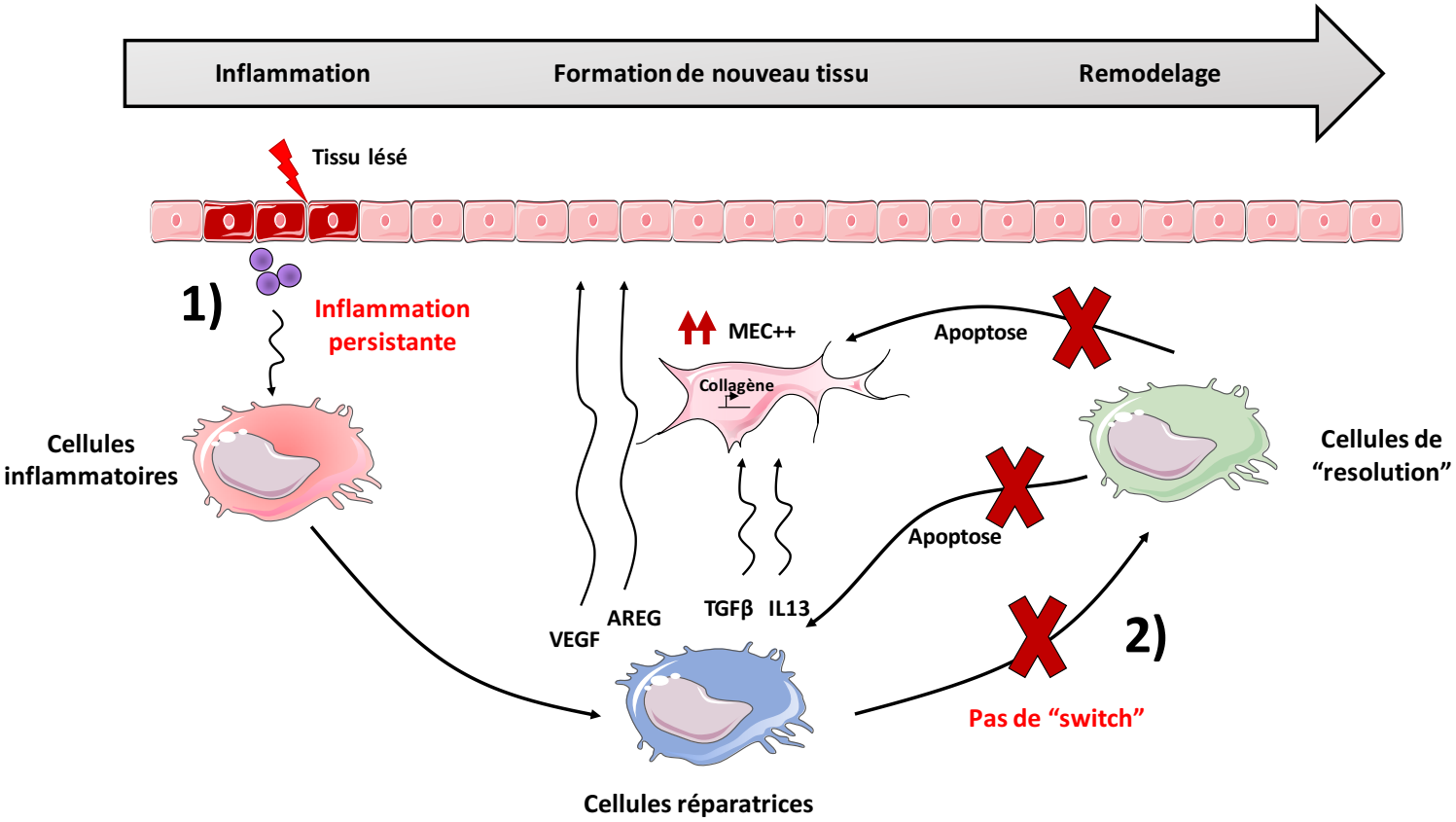


Figure 4 : Schéma représentant le rôle du système immunitaire et les potentielles raisons de l'établissement de la fibrose : 1) présence constante de la source de l'inflammation menant à une persistance de cette dernière et à une boucle continuelle de la réparation tissulaire ; 2) Altération de la plasticité : le switch entre les cellules répratrices et les cellules de résolution n'a pas lieu, entraînant une absence d'apoptose des cellules impliquées dans le renouvellement de la MEC.

ii. Rôle du système immunitaire dans les ulcères

A l'inverse de la fibrose, le rôle du système immunitaire dans le mécanisme des ulcères est très peu étudié. Quelques rares études montrent notamment une augmentation de la production de réactifs oxygénés intermédiaires par les neutrophiles (Lewkowicz et al., 2003). Des analyses en immunohistochimie mettent en évidence une infiltration de cellules immunitaires comprenant majoritairement des lymphocytes T, des lymphocytes B (LB) ainsi que des macrophages (Lee et al., 2015).

L'implication du système immunitaire dans le phénomène de fibrose et les ulcères est, certes, indéniable mais mal compris. Les causes de la fibrose sont encore méconnues. Nous nous sommes donc posé plusieurs questions : les dérégulations de la réparation tissulaire sont-elles la conséquence d'une dérégulation de la réponse immunitaire ? La localisation, la plasticité ou encore le contexte dans lequel se passe la réponse immunitaire influencent-ils cette dernière, engendrant ainsi de la fibrose ? Si tel est le cas, comment le font-ils et pouvons-nous le bloquer ?

Ces réflexions conceptuelles doivent être étayées par un modèle expérimental. La mise en place d'un schéma expérimental intégrant les concepts de localisation, de plasticité, de migration et de contexte pourrait contribuer à une meilleure compréhension de la fibrose offrant potentiellement des nouvelles perspectives thérapeutiques.

A) Modèle d'étude : la sclérodermie systémique

Parmi de nombreux modèles d'étude possibles, nous avons choisi d'étudier le rôle du système immunitaire, et plus particulièrement le rôle des cellules immunitaires innées, dans la sclérodermie systémique (ScS). La ScS regroupe les deux sous-types de dérégulation de la réparation tissulaire que nous avons définis précédemment. En effet, les patients peuvent

être atteints d'ulcères mais également d'une fibrose pouvant toucher la peau et les organes internes.

1. Généralités

La sclérodermie systémique est une maladie auto-immune rare dont la prévalence est de 50 à 300 cas/million d'habitants. Il existe une prédominance féminine avec un sex-ratio femme/homme pouvant aller jusqu'à 14/1. Le pic d'incidence de la maladie arrive vers 50 ans même s'il existe des formes précoces de la maladie (Chifflot et al., 2008).
Le pronostic de la ScS est variable et dépend de l'apparition de complications pulmonaires, rénales et/ou digestives. L'étendue de l'atteinte cutanée permet de définir deux formes cliniques principales : la forme limitée et la forme diffuse (LeRoy et al., 1988). Ces deux formes ainsi que la variabilité des atteintes de la maladie entre les individus affectés témoignent du caractère hétérogène de la maladie et rendent l'établissement d'un traitement complexe.

Le facteur déclenchant de la maladie n'est pas connu. Comme la plupart des maladies auto-immunes, elle résulte de la conjonction de plusieurs facteurs : environnementaux (silice, solvant, infection), génétiques (polymorphismes de susceptibilité à la maladie dans les gènes du complexe majeur d'histocomptabilité HLA (Human Leukocyte Antigen), du TGFβ (Transforming growth factor beta), de la molécule de co-stimulation OX40L, de l'interleukine (IL)1β ou encore du récepteur de l'IL13) et immunologiques (présence d'auto-anticorps (Ac) et cellules T auto-réactives).
Certaines études ont montré un risque accru (x3), surtout chez les sujets masculins, de développer une ScS suite à une exposition à la silice et aux solvants (Barnes and Mayes, 2012). De plus, des études génomiques ont trouvé la présence de polymorphismes chez les patients ScS, notamment dans les gènes du HLA de classe II, dans des gènes impliqués dans la régulation de la réponse immunitaire et dans le remodelage de la MEC.
Il n'y a pas de traitement spécifique et efficace pour la ScS, notamment sur la fibrose. La stratégie thérapeutique la plus répandue actuellement est de bloquer précocement le système immunitaire.

2. Physiopathologie

La ScS est une connectivite caractérisée par trois bases physiopathologiques majeures : (i) une vasculopathie, arrivant précocement au cours de la maladie ; (ii) une dérégulation de la réponse immunitaire touchant à la fois l'immunité innée et adaptative, (iii) et le phénomène de fibrose pouvant toucher simultanément la peau et les organes internes tels que le rein et les poumons (Allanore et al., 2015). Les interactions réciproques entre ces trois éléments sont indéniables mais encore mal comprises.

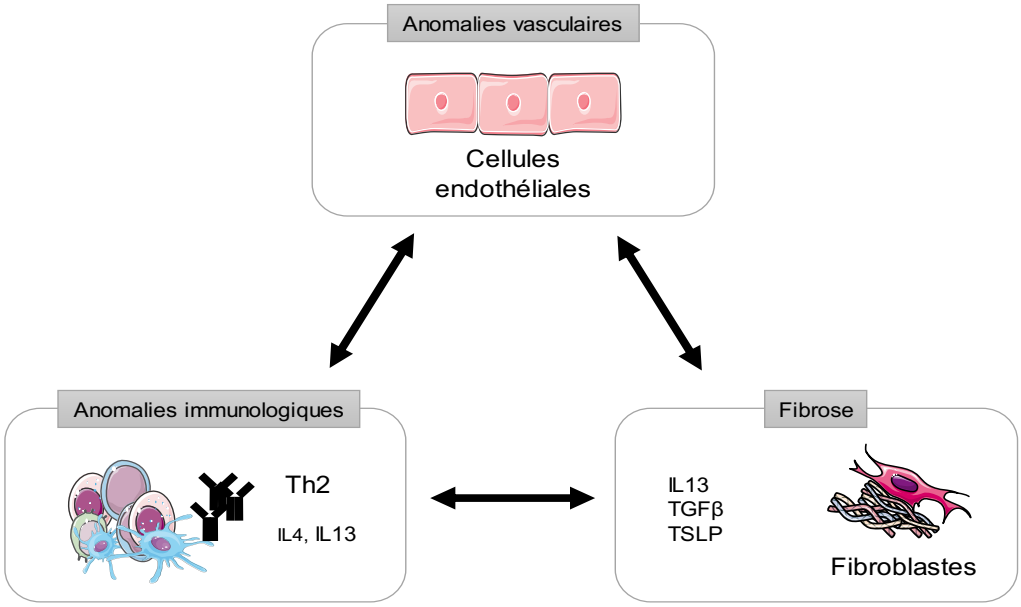


Figure 5: Physiopathologie de la ScS incluant une vasculopathie, une dérégulation de la réponse immunitaire et une fibrose

a) Anomalies vasculaires

La cellule endothéliale (CE) est la première touchée dans la ScS. Les événements vasculaires précoces se traduisent par un dysfonctionnement de l'endothélium et des lésions avec apoptose des cellules endothéliales. Les mécanismes moléculaires qui sous-tendent ces anomalies vasculaires précoces demeurent encore mal définis.

Ces anomalies vasculaires se manifestent en premier lieu par le phénomène de Raynaud et peuvent entrainer une hypertension artérielle pulmonaire (une des causes de décès chez les patients atteints de ScS (Balbir-Gurman and Braun-Moscovici, 2012)).

Les lésions vasculaires semblent débuter par une perméabilité excessive des CE entrainant une infiltration péri-vasculaire de cellules mononuclées du sang. Cette infiltration cellulaire, notamment composée de monocytes et de lymphocytes T, engendre une inflammation péri-vasculaire menant à des épisodes d'ischémie/reperfusion. Ces épisodes atténuent le stress oxydatif par la production excessive de radicaux libres oxygénés qui aggravent les dommages aux cellules endothéliales.

Une des conséquences directes de la vasculopathie au cours de la ScS est l'hypoxie tissulaire chronique. En réponse à l'hypoxie, les CE et les fibroblastes produisent des protéines de la MEC participant ainsi à la fibrose. Les CE sécrétent également du VEGF, puissant facteur angiogénique. De manière surprenante, cette surproduction de VEGF est délétère dans la ScS (Distler et al., 2007).

Le dysfonctionnement endothélial peut également résulter du déséquilibre de production de facteurs vaso-actifs se traduisant par une surproduction de vaso-constricteurs telle que l'endothéline 1 (ET1) et une sous production de vaso-dilatateurs comme l'acide nitrique ou la prostacycline (Matucci-Cerinic et al., 2013). La synthèse d'ET1 par les CE promeut à la fois l'adhésion leucocytaire, la prolifération des cellules lisses vasculaires et l'activation des fibroblastes (Varga and Abraham, 2007). Dans la ScS, elle participe à l'établissement de la fibrose en stimulant la production de collagène par les fibroblastes et en inhibant l'activité des métalloprotéinases (MMP) responsables de la dégradation de la MEC (Abraham et al., 1997; Jing et al., 2015). Un taux élevé d'ET1 est associé à une sévérité accrue des symptômes de la maladie (Abraham et al., 1997). La vasoconstriction, due notamment à l'ET1, entraîne une ischémie c'est-à-dire une diminution de l'apport sanguin. Pour atténuer ce stress oxydatif, les CE vont synthétiser des réactifs oxygénés à l'origine de la dégradation des CE (Szekanecz and Koch, 2005).

Une néo-vascularisation est alors essentielle. Ce phénomène requiert à la fois une mobilisation des progéniteurs des CE ainsi qu'une prolifération/différenciation des CE résidentes. Or, dans la ScS, le nombre de progéniteurs est réduit entraînant une altération de l'angiogenèse (Avouac et al., 2011; Distler et al., 2009).

L'activation des cellules endothéliales conduit également à la production de cytokines inflammatoires telles que le TGFβ, d'IL1α ou d'IFNγ et de chimiokines comme l'IL8 ou CCL2 (Szekanecz and Koch, 2005). Cette production de cytokines/ chimiokines, associée à la capacité des CE à exprimer des molécules d'adhésion à leur surface, facilitent la chimio-attraction et la diapédèse des LT.

Compte tenu des interactions étroites entre les plaquettes et les CE, l'activation endothéliale pourrait contribuer à l'activation des plaquettes observée chez les patients ScS via la production de réactifs oxygénés (Allanore et al., 2005; Rustin et al., 1987). En outre, les plaquettes activées participent directement à l'établissement de la fibrose via des médiateurs solubles comme la sérotonine entraînant la production excessive de MEC par les fibroblastes (Dees et al., 2011). L'activation plaquettaire peut également avoir un rôle profibrotique indirect en activant les CE via le relargage d'IL1β, entraînant ainsi la production de la TSLP (Thymic Stromal Lymphopoietin), facteur profibrotique, par les CE (Truchetet et al., 2016).

Ainsi l'amplification de l'activation plaquettaire par les CE pourrait entretenir l'activation immune et la fibrose chez les patients ScS (Ramirez et al., 2012; Scherlinger et al., 2018).

b) Anomalies immunologiques

L'immunité innée et l'immunité adaptative sont toutes les deux impliquées dans la ScS.

Les phénomènes auto-immuns et inflammatoires interviennent tôt dans le développement de la maladie avant même l'apparition de la fibrose.

Les traitements immunosuppresseurs ont une efficacité limitée dans la ScS en comparaison à d'autres pathologies auto-immunes, comme le lupus systémique érythémateux ou la polyarthrite rhumatoïde. Cela témoigne de la complexité de la maladie.

i. Immunité innée

Au cours de ma thèse, nous avons analysé le rôle potentiel de l'ensemble des acteurs de l'immunité innée impliqués dans la ScS. Cette étude a donné lieu à la publication d'une revue de la littérature sur l'implication de l'immunité innée dans la ScS .

Article de revue n°2 : Innate Immunity in Systemic Sclerosis Fibrosis: Recent Advances

Paoline Laurent†, Vanja Sisirak†, Estibaliz Lazaro, Christophe Richez, Pierre Duffau, Patrick Blanco, Marie-Elise Truchetet and Cécile Contin-Bordes

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Dans cette revue de la littérature, nous avons décrit l'implication des acteurs de l'immunité innée dans la physiopathologie de la ScS. Nous avons également décrit les éléments démontrant l'importance des processus immunitaires innés durant le développement des SSc, avec un accent particulier sur leur rôle dans l'initiation de la pathologie. Nous avons également discuté des options thérapeutiques potentielles pour moduler les cellules immunitaires innées ou la signalisation dans les SSc qui émergent de ces récentes avancées.

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Innate Immunity in Systemic Sclerosis Fibrosis: Recent Advances

Paoline Laurent[1†], Vanja Sisirak[1†], Estibaliz Lazaro[1,2], Christophe Richez[1,3], Pierre Duffau[1,2], Patrick Blanco[1,4], Marie-Elise Truchetet[1,3] and Cécile Contin-Bordes[1,4] On Behalf of the Fédération Hospitalo Universitaire ACRONIM Aquitaine's Care and Research Organization for Inflammatory and Immune-Mediated Diseases*

[1] CNRS-UMR 5164, ImmunoConcEpT, Bordeaux University, Bordeaux, France, [2] Internal Medicine Department, Bordeaux University Hospital, Bordeaux, France, [3] Rheumatology Department, Bordeaux University Hospital, Bordeaux, France, [4] Immunology and Immunogenetic Department, Bordeaux University Hospital, Bordeaux, France

Systemic sclerosis (SSc) is a heterogeneous autoimmune disease characterized by three interconnected hallmarks (i) vasculopathy, (ii) aberrant immune activation, and (iii) fibroblast dysfunction leading to extracellular matrix deposition and fibrosis. Blocking or reversing the fibrotic process associated with this devastating disease is still an unmet clinical need. Although various components of innate immunity, including macrophages and type I interferon, have long been implicated in SSc, the precise mechanisms that regulate the global innate immune contribution to SSc pathogenesis remain poorly understood. Recent studies have identified new innate immune players, such as pathogen-recognition receptors, platelet-derived danger-associated molecular patterns, innate lymphoid cells, and plasmacytoid dendritic cells in the pathophysiology of SSc, including vasculopathy and fibrosis. In this review, we describe the evidence demonstrating the importance of innate immune processes during SSc development with particular emphasis on their role in the initiation of pathology. We also discuss potential therapeutic options to modulate innate immune cells or signaling in SSc that are emerging from these recent advances.

Keywords: innate immunity, systemic sclerosis, fibrosis, sterile inflammation, future therapeutic

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****Correspondence:***
Cécile Contin-Bordes
cecile.bordes@chu-bordeaux.fr

[†]These authors have contributed equally to this work.

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INTRODUCTION

Systemic sclerosis (SSc) is a complex autoimmune disease interconnecting vasculopathy, autoimmunity, and fibrosis features. A large body of evidence has indicated that the adaptive immune system with autoreactive T cells and autoantibodies produced by B cells plays a central role in SSc pathogenesis (1). In addition, inflammatory cytokines produced by the innate immune cells have been detected in the affected tissues of both the early and late stage of SSc, suggesting a role of innate immunity both at the onset and progression of the disease (2–6). This notion was recently reinforced by genomic and genetic approaches that have been undertaken to decipher key and conserved pathophysiological pathways within organs across disease forms (7–9). Apart from genomic approaches, the study of mechanisms governing normal tissue repair has revealed physiological pathways that may be disrupted during SSc as well. The concept of unresolved tissue repair leading to sustained fibrosis has emerged based on a persistent sterile inflammation that converts a self-limited repair response to a non-resolving pathological fibrosis (10, 11). However, the initial events leading to such sterile inflammation remain unclear. Recent data showing that an imbalance in danger-associated molecular pattern (DAMP) release and/or pathogen-recognition receptor (PRR) signaling leads to sustained inflammatory cytokine production by fibroblasts or macrophages may provide the missing link in early events of SSc pathophysiology (11). In addition, plasmacytoid dendritic cell (pDC)

activation (12, 13) and type I interferon (IFNα/β, IFN-I) production has also been recently shown to contribute to SSc.

In this review, we focus on recent evidence highlighting the contribution of innate immunity during the course of SSc pathogenesis, primarily at the early stages of disease. We also discuss potential therapeutic options that may modulate innate immune cells or signaling in SSc patients.

WHAT CAN BE LEARNED FROM GENETIC STUDIES ON INNATE IMMUNE FUNCTION DURING SSc?

Major technological and analytical advances in the past 10 years have allowed the extraction of critical information from transcriptomic data such as lineage-specific gene expression, networks of interactions, and functional information (14–17). This yielded a novel field of study in the integrated comprehension of SSc pathogenesis, identifying a major contribution of innate immunity.

By analyzing three independent gene expression data sets from SSc skin biopsies, the group of Whitfield proposed interconnected functional modules involved in SSc pathogenesis, two of which involve innate immunity and are dominated by IFN, IFN-inducible genes, and type 2 macrophage (M2) signatures. The three other subnetworks were linked to adaptive immunity, fibrotic processes [response to transforming growth factor beta (TGF-β) and extracellular matrix (ECM) disassembly/wound healing], cell cycle, proliferation, and apoptosis (9). The same group recently identified a common pathogenic signature related to an "innate immune-fibrotic axis" that includes IFN-I and alternatively activated macrophages commonly referred as M2 macrophages and describes new specific pathways and hubs active in the skin and lung (8). Among shared networks, the authors found that the "innate immunity-fibrotic network" is conserved between skin and lung while the internal composition and interactions of gene expression in those tissues vary.

Such large-scale genomic studies paved the way for multiple experimental approaches to determine the molecular processes involved in patients and to establish novel therapeutic options targeting specific organs or shared pathophysiological processes.

EMERGING CONCEPT: SSc AS AN OVER-REPAIR PATHOLOGY

The ability of an organism to efficiently recover from injury whether traumatic, infectious, chemical, or internal is pivotal to maintain its integrity (18). During tissue repair, innate immune cell plasticity actively contributes to the development of an abnormal microenvironment, leading to a shift in the balance between the pro-inflammatory and pro-reparative sides of tissue repair, as recently reviewed (10).

Early SSc is characterized by a perivascular leukocyte infiltrate mainly composed of macrophages and T lymphocytes, reminiscent of the process induced during normal wound healing (19, 20). Whereas normal wound healing is accompanied by a remodeling or resolving stage, abnormal wound healing with chronic activation of immune cells such as macrophages or stromal cells like myofibroblasts fails to resolve fibrosis during SSc. Hence, SSc, specifically diffuse cutaneous forms of the disease, could be considered as a general form of over-repair. The initial trigger of the injury is still unknown, but several lines of recent evidence have brought new hypotheses on its nature.

Role of Sterile Inflammation in Unresolving Tissue Fibrosis During Scleroderma: Importance of DAMP/PRR Imbalance

Recognition of pathogen-associated molecular patterns (PAMPs) or endogenous DAMPs by innate immune cells as well as non-immune cells is the first line of response to pathogen or sterile tissue injury. DAMPs, mainly produced by epithelial cells, are heterogeneous in form encompassing early produced and highly diffusible Ca^{2+}, H_2O_2, reactive oxygen species (ROS), adenosine tri-phosphate, self-nucleic acids, but also proteins like high-mobility group protein 1, heat shock protein, S100 proteins, and fragments of the ECM. The recognition of PAMPs and DAMPs relies on cell surface, endosomal, and cytosolic PRRs that include toll-like receptors (TLRs), Nod-like receptor, Rig-I-like receptors (RLRs), cyclic GMP-AMP synthase, and receptor for advanced glycation end products. Innate immune signaling triggered by DAMPs during sterile inflammation or the persistence of pathogens such as endogenous viruses might represent an important pathway responsible for converting self-limited regenerative repair into an unresolved fibrotic process during SSc. Hence, innate immune signaling *via* TLRs was recently proposed as a key driver of persistent fibrotic response in SSc and other fibrotic-related diseases (11).

Overexpression of TLR4 and its two co-receptors CD14 and myeloid differentiation factor 2 (MD-2) has been described in SSc-affected skin and lung. TLR4 expression was mainly associated with macrophages, fibroblasts, and myofibroblasts (21). In the skin, TLR4 expression correlated to fibrosis severity measured by modified Rodnan skin score. *In vivo*, chronic TLR4 activation leads to sustained nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling, resulting in macrophage activation and a profibrotic profile (22). Work from the Varga lab recently demonstrated that endogenous DAMP activation of TLR4 can contribute to converting self-limited tissue repair responses into uncontrolled ECM deposition during SSc [for recent review, see Ref. (11)]. They proposed that fibronectin, containing alternatively spliced exons encoding type III repeat extra domain (EDA), and tenascin-C are constitutively produced by SSc fibroblasts leading to their accumulation in the skin but also in the blood. Together, fibronectin-EDA and tenascin-C act as strong profibrotic factors during SSc by binding to fibroblasts TLR4, leading to enhanced production of collagen and alpha-smooth muscle actin (α-SMA) expression (23, 24). Deletion of EDA or tenascin-C or disruption of TLR4 signaling resulted in reduced fibrotic response in a murine model of SSc. Furthermore, tensional forces generated within a rigid fibrotic microenvironment were reported to favor exposure of the EDA domain of fibronectin (25), suggesting that increased stiffness of the matrix in fibrotic tissue could favor the bioavailability and profibrotic activity of fibronectin-EDA.

Altered expression of multiple DAMPs/TLRs beyond TLR4 has been described during SSc. Indeed, increased expression of TLR9 was found in human SSc skin biopsies in both early and late stages of the disease and was mainly associated with α-SMA-positive myofibroblasts (26), and a TLR9 signature was detected in SSc skin. *In vitro* treatment of normal cutaneous fibroblasts with the TLR9 ligand unmethylated-CpG-oligodeoxynucleotides (CpG ODN) induced a profibrotic profile involving autocrine TGF-β production. Collectively, these results support the role of TLR9 signaling in SSc. Furthermore, expression of TLR2 (27) and TLR3 (28) is also increased in SSc skin fibroblasts. TLR2 was shown to respond to the endogenous ligand amyloid A, resulting in NFκB activation and increased interleukin (IL)-6 secretion causing inflammation (27). However, the role of TLR3 in SSc pathogenesis remains controversial. TLR3 activation by polyinosinic:polycytidylic acid (poly I:C) stimulates IFN-I production by fibroblasts, which in turn reduces their ability to produce ECM components (28). Conversely, such stimulation was shown to promote the expression of TGF-β by fibroblasts thus contributing to the overall fibrosis (29).

In addition to TLRs, other PRRs have been described to play a role in SSc pathogenesis. The IFN-I stimulatory property of poly I:C on SSc patient fibroblasts was shown not only to rely on TLR3 but also on intracellular RLRs (28). The inflammasome, specifically the NLRP3-inflammasome, was shown to contribute SSc pathogenesis *in vivo* (30) through the induction of the microRNA miR-155, which in turn favors excessive ECM production by fibroblasts, exacerbating SSc (31).

Studies on the contribution of TLR signaling to fibrosis in SSc as well as other fibrotic diseases have generated conflicting results (22, 26, 32–35), suggesting that whether TLR activation leads to pro- or anti-fibrotic effects depends on many factors. The nature of the stimulation (chronic vs acute), of the responding cells (immune or non-immune cells), as well as disease stage (inflammatory vs remodeling) might modulate the effects of TLRs in the fibrotic process. Profibrotic effects of TLR activation seem related to fibroblast and macrophage activation in the context of chronic stimulation, whereas epithelial and other immune cell activation in the context of acute stimulation might lead to anti-fibrotic effects. Although additional PRRs have recently been implicated in SSc, further studies are required to identify their endogenous ligands and mechanisms leading to disease. Nevertheless, PRRs and their signaling pathways may represent multiple novel therapeutic targets in SSc.

Old Players, New Pathways: Type-2 Macrophages, Platelets, and Mastocytes

Macrophages and platelets have emerged as key players not only during tissue homeostasis and repair but also fibrosis, recently reviewed in Ref. (36, 37).

We and others have defined the profibrotic role of platelets in SSc. The Distler group has shown that serotonin [5-hydroxytryptamine (5-HT)] stored in platelets strongly induces ECM synthesis in interstitial fibroblasts *via* activation of 5-HT_{2B} receptors (5-HT_{2B}) in a TGF-β-dependent manner (38). Our group discovered a pathophysiological loop active in SSc that links vasculopathy and fibrosis. Indeed, we showed that platelet activation induced the production of thymic stromal lymphopoietin (TSLP) by dermal microvascular endothelial cells in an IL-1β-dependent manner. TSLP was found to be strongly expressed in SSc skin endothelial cells and correlated to the severity of skin fibrosis. *In vitro*, TSLP was able to induce a profibrotic profile in both normal and SSc fibroblasts (39, 40).

Infiltration of macrophages in the early skin lesions of SSc patients, particularly in perivascular areas, was first detected over 20 years ago and could lead to secondary activation of adaptative system (19, 20). Since then, numerous studies have established the involvement of macrophages in SSc pathogenesis, notably their alternatively activated counterpart called M2 macrophages as reviewed in Ref. (41). Soluble CD163, a putative marker of M2 macrophages, was shown to be elevated in SSc patients' blood and associated with their poor clinical outcome (42). These observations have been reinforced by the recent genetic studies showing a prominent M2 macrophage signature in SSc-affected skin and lung (8). However, the activation of lung macrophages in SSc patients with pulmonary fibrosis is distinct from that observed in SSc skin. Activated lung-resident macrophages display a specific increase in the expression of genes related to lipid and cholesterol trafficking, suggesting a switch in their metabolism. Thus, while M2 macrophages are central to the fibrotic process both in skin and lung during SSc, distinct stimuli derived from the organ-specific microenvironment might differentially shape the plasticity of macrophages. In the recent FASSCINATE trial, molecular profiling of skin biopsies revealed that IL-6 receptor blockade by tocilizumab resulted in a reduced M2 macrophage signature observed in SSc skin (43). Accordingly, the blockade of cAMP-specific phosphodiesterase-4, which inhibits differentiation of M2 macrophages as well as IL-6 production, led to an amelioration of fibrosis in a murine model of SSc induced by bleomycin treatment (44). The same group demonstrated that nintedanib, a tyrosine kinase inhibitor targeting vascular endothelial-, fibroblast-, and platelet-derived growth factor receptors, effectively blocked myofibroblast differentiation and reduced pulmonary, dermal, and myocardial fibrosis in transgenic Fra2 mice. This effect was primarily mediated by preventing M2 macrophage accumulation in the affected tissues (45). However, the mechanisms leading to aberrant M2 macrophage polarization and the precise pathways through which M2 macrophages contribute to tissue fibrosis remain unclear. One elegant study by Eming et al. provided novel mechanistic insight to the role of M2 macrophages in fibrosis. Using a murine model of wound healing, IL-4Ra activation by IL-4 and IL-13 was demonstrated to induce the production of resting like molecule alpha by M2 macrophages, which in turn stimulates the production of enzyme lysyl-hydroxylase-2 (LH-2) ultimately contributing to persistent profibrotic collagen cross-linking in fibroblasts (46). This process was shown to be critical for transformation of the tissue into a persistent scar. In humans, Relm-β induces LH-2 in fibroblasts, and expression of both factors was reported to be increased in lipodermatosclerosis, a condition associated with excessive skin fibrosis. Whether this process contributes to SSc is still unknown. The fine mapping of specific macrophage subsets across tissues and during the course of disease, as well as elucidating of the molecular mechanisms underlying

macrophages-induced abnormal resolution, will pave the road to the development of new drugs that prevent/limit fibrosis.

Studies in patients and animal models of SSc have demonstrated that mast cells infiltrate the fibrotic skin (47, 48). This infiltration was associated with more severe disease phenotypes (48), but the function and net contribution of mast cells to fibrosis is only beginning to be understood. Mast cells have been suggested to be an important source of TGF-β and thus contribute to the overall fibrosis (49). Furthermore, a recent report using transgenic mice that develop spontaneous skin fibrosis showed a major role for mast cells specifically in inducing inflammation of the skin and the production of ECM and α-SMA by fibroblasts (50). Together with recent observations showing that mast cell deletion ameliorates experimental SSc *in vivo* (47, 51), these results indicate that mast cell targeting in SSc patients may represent an effective therapeutic approach.

Finally, other innate immune players such as natural killer (NK) cells (52, 53) and neutrophils (54) were shown to display altered properties and phenotypes in the blood of SSc patients. However, further studies are required to evaluate the role of NK cells and neutrophils in the SSc pathogenesis, especially in the settings of murine experimental models.

NEW KIDS ON THE BLOCK: pDC AND INNATE LYMPHOID CELL (ILC)

Plasmacytoid dendritic cells are innate immune cells specialized in the production of copious amounts of IFN-I (55), and thus play a key role in the initiation of antiviral immune responses (56, 57). IFN-I production by pDCs requires recognition of viral nucleic acids by TLR7 and TLR9, respectively (56, 57). pDCs were also shown to produce IFN-I in response to self-nucleic acids and consequently contribute to the development of multiple inflammatory and autoimmune disorders (58–62). An IFN-I signature, reflected by increased expression of numerous IFN-I-stimulated genes has been reported in patients with SSc (12). Furthermore, genome-wide association studies in SSc have identified polymorphisms in genes involved in the regulation of IFN-I expression in pDCs, particularly IFN-regulatory factor (IRF)-5, IRF-7, and IRF-8 (12). Approximately half of SSc patients (~50%) display an IFN-I signature within their peripheral blood mononuclear cells (63–65) and in fibrotic skin (66). The association between IFN-I signature and SSc disease activity remains controversial as no major impact of the IFN-I signature on pathological features of SSc, including extent of skin fibrosis, autoantibody specificities, and interstitial lung disease, has been reported (63, 65). However, when the profile of IFN-induced chemokines was specifically analyzed in a large cohort of SSc patients, an association was then identified with more severe SSc (67). As pDCs are an important source of IFN-I, numerous groups have investigated their role in SSc. pDCs were indeed detected in the affected skin of SSc patients (65, 68) as well as in the fibrotic skin of mice after bleomycin treatment (13). Furthermore, mice lacking fibrillin-1 (*Fbn1*), which spontaneously develop a stiff skin syndrome that recapitulates the skin fibrosis observed in SSc patients, show a high infiltration of pDCs in the affected skin (69). The frequency of pDCs is reduced in the circulation of SSc patients, likely due to their preferential recruitment into the fibrotic skin (13). Anti-topoisomerase I and anti-nuclear autoantibodies in SSc patients were shown to form immune complexes with apoptotic cell-derived constituents *in vitro* and consequently stimulate IFN-I production by pDCs (70, 71) upon uptake *via* FcγRII and the stimulation of TLR7/9 (70, 71). While such "interferongenic" properties of immune complexes may contribute to the aberrant IFN-I production, an IFN-I signature was not associated with the production of specific autoantibodies detected in the sera of SSc patients (70), suggesting that additional factors may contribute to pDC activation *in vivo*. Furthermore, pDCs in the peripheral blood or fibrotic skin of SSc patients spontaneously secrete CXC motif ligand (CXCL)-4 and IFNα (13, 68). High levels of CXCL4 in the circulation of SSc patients were associated with disease severity including skin fibrosis and pulmonary arterial hypertension (68). CXCL4 was described to potentiate pDC ability to produce IFN-I *in vitro* largely in response to TLR9 stimulation. In addition, CXCL4 was shown to induce both the expression of TLR8 and the ability to produce IFN-I in response to its specific ligands in pDCs (13). Recently, the pathogenic role of TLR8 was confirmed *in vivo* using transgenic mice that express human TLR8 and develop exacerbated skin fibrosis after bleomycin treatment compared with control animals (13). However, whether such exacerbation of disease in TLR8 transgenic animals is dependent on pDCs remains unknown, and the association between CXCL4 levels and the IFN-I signature in SSc patients has not yet been characterized. Ah Kioon et al. showed that bleomycin-induced skin fibrosis is strongly attenuated after selective pDC depletion (13). Furthermore, this model of fibrosis was associated with an IFN-I signature and increased expression of CXCL4 in the affected skin, and pDC depletion significantly reduced the occurrence of these parameters. From a therapeutic standpoint, pDC depletion ameliorated established bleomycin-induced skin fibrosis, indicating that pDCs are critical even in the maintenance of skin fibrosis. This constitutes the first study showing the deleterious impact of pDCs on SSc development *in vivo* (13). Overall, pDCs play a critical role in SSc pathogenesis; however, the molecular mechanisms through which they contribute to the disease require further investigation. This recent progress nevertheless positions SSc as another autoimmune pathology that may benefit from therapeutic targeting of pDCs using depleting or inhibitory antibodies (72).

Innate lymphoid cells were recently described as novel components of the immune system that may be considered as innate counterparts of polarized T helper cells (73). Nevertheless, knowledge on the role of ILCs in SSc remains limited. Wohlfahrt and colleagues have shown elevated numbers of ILC2 in both the peripheral blood and the affected skin of patients with SSc compared with healthy individuals, and their number correlated with the extent of cutaneous fibrosis (74). However, the increased frequency of ILC2 in SSc peripheral blood was not observed in a different study, which instead reported an elevated frequency of CD4 + ILC1 and NKp44 + ILC3 (75).

Nevertheless, in animal models of lung fibrosis induced by bleomycin, IL-33, an alarmin that has been reported to be elevated in SSc patients (76), induced the expansion of ILC2s producing the profibrotic cytokine IL-13 (77). Hence, further investigations

TABLE 1 | Potential therapeutics and therapeutics in latest clinical trials specific to innate immunity and fibrosis in SSc.

Innate immunity targeted physiopathological pathways	Target	Molecules	Drug name/ trade name	Clinical trial in SSc	Primary end-point	Result
(A) Chronic sterile inflammation						
	TLR4/MD-2 inhibition	Selective TLR4 inhibitor, lipid A mimetic	E5564/Eritoran	None for SSc, tested in sepsis (lack of efficiency)		
		Anti-TLR4	NI-0101			
		Selective TLR4 inhibitor, small molecule	T5342126	None		
	TLR4/MD-2 inhibition of DAMP	Tenascin-C A1 domain specific blocking antibody	F16	None		
		Fibronectin-EDA specific blocking antibody	F8	None		
	TLR4 downstream signaling	Small molecule binding the Cys747 of the intracellular domain of TLR4	TAK-242	None for SSc, tested in sepsis (lack of efficiency)		
	TLR7/8/9	Small molecule or oligonucleotides	CpG-52364, DV-1179, IMO 3100, IMO-8400	None		
	NFκB	PDE4 inhibitor	Crisaborole/ Eucrisa	None for SSc but Pilot Study Evaluating the Efficacy of a Topical PDE4 Inhibitor for Morphea NCT03351114	Change in dermal thickness of sentinel plaque from Baseline to 12 weeks	
	pDC	Anti-BICD2 antibody	BIIB059	None		
	Type 1 IFN	Type 1 interferon receptor sub-unit 1 blocking antibody	MEDI-546	Phase I open-label study in diffuse cutaneous SSc NCT00930683	Safety and tolerability of single or multiple intravenous doses	Decreased type I IFN gene expression in whole blood and skin for subjects with positive scores at baseline
(B) Abnormal resolution						
	Fibroblasts	Selective CB2 agonist	JBT-101/ Lenabasum	Phase II + open-labeled extension	Safety and reduction of the mRSS score	Reduction of 8.4 points in the mRSS score in the open-label extension
		Selective CB2 agonist	JBT-101/ Lenabasum	Phase III RESOLVE-1 trial NCT03398837	Change from baseline in mRSS	Expected results in 2020
	Type-2 macrophages	Anti-IL-6 receptor alpha blocking antibody	Tocilizumab/ Roactemra	Phase II FASSCINATE trial NCT01532869	Safety and difference in mean change from baseline in mRSS at week 24	Primary end-point not reached but diminished type-2 signature in the treated arm
		Tyrosine kinase inhibitor	Nintedanib	Phase III SENSCIS trial NCT02597933	Efficacy and safety in SSc patients with interstitial lung disease at week 52	
		PDE4 inhibitor	Crisaborole/ Eucrisa	No clinical trial in SSc, but pilot study evaluating the efficacy of a topical PDE4 inhibitor for morphea NCT03351114	Change in dermal thickness of sentinel plaque from Baseline to 12 weeks	
	TGF-β	TGF-β isoforms 1, 2, and 3 blocking antibody	Fresolimumab	Phase I open-label trial NCT01284322	Safety and efficacy (molecular assessment of TGF-β responsive genes and improvement in the mRSS)	Inhibition of TGF-β-regulated gene expression and improvement in the mRSS in the fresolimumab treated group

(*Continued*)

TABLE 1 | Continued

Innate immunity targeted physiopathological pathways	Target	Molecules	Drug name/ trade name	Clinical trial in SSc	Primary end-point	Result
		Soluble guanylate cyclase activator blocking TGF-β-induced release of ECM components from fibroblasts	BAY63-2521/ Riociguat	Phase II RISE-SSc trial NCT02283762	Safety and efficacy (change in mRSS at week 52) in patients with diffuse cutaneous SSc	

BDCA-2, blood dendritic cell antigen 2; DAMP, danger-associated molecular pattern; IFN, interferon; mRSS, modified Rodnan skin score; SSc, systemic sclerosis; TGF-β, transforming growth factor beta; TLR, toll-like receptor; PDE4, phosphodiesterase-4; ECM, extracellular matrix; pDC, plasmacytoid dendritic cell; MD-2, myeloid differentiation factor 2; CB2, cannabinoid receptor type 2.

are warranted to determine the role of ILC2 in the development of SSc fibrosis.

FUTURE DIRECTIONS AND THERAPEUTIC AVENUES

Significant progress has recently been made in understanding the contribution of innate immunity to SSc fibrosis. Although the precise molecular mechanisms of their action must be further defined, promising new therapeutic targets for SSc have already emerged. Such strategies include blockade of TLR4/MD-2, TLR9, or downstream signaling molecules to limit chronic sterile inflammation, modulation of macrophage polarization to promote resolution and matrix remodeling, and targeting pDCs/IFN-α. This therapeutic challenge is ongoing with many attractive new therapeutic candidates, some of which are currently being tested in Phase III clinical trials (**Tables 1A,B**). Both the evaluation of potential side effects and identification of biomarkers of patients who would benefit from such therapies are warranted in order to maximize the efficacy of treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ii. Immunité adaptative

L'immunité adaptative participe également de manière active à la physiopathologie de la ScS.

➢ Auto-anticorps et lymphocytes B

La maladie se caractérise par la présence de nombreux auto-Ac dont certains sont étroitement associés aux différentes formes cliniques de la maladie. Classiquement les auto-Ac anti-centromères sont retrouvés dans la forme limitée de la maladie alors que les auto-Ac anti Scl70 sont associés à la forme diffuse. Parallèlement, il existe d'autres auto-Ac tels que les auto-Ac anti-CE, anti-fibrilline, anti-ARN polymérase III. Le rôle de ces auto-Ac dans la physiopathologie de la maladie n'a pas encore été totalement démontré mais il existe tout de même certaines données sur le rôle des auto-Ac anti-fibrilline notamment (Kim et al., 2008). Ces auto-Ac activent les fibroblastes et stimulent la production de TGFβ. Ils sont également apparentés à une forme sévère de la maladie. De plus, il a été montré que les auto-Ac anti Scl70 induisent la production de l'interferon de type I (IFN I) par les cellules dendritiques plasmacytoïdes (pDC) en stimulant les récepteurs toll like (TLR) endosomaux 7 et 9 (Kim et al., 2008).

Il a été montré dans un modèle murin génétique de ScS (le modèle TSK) que la déplétion de LB chez une souris jeune diminue la fibrose cutanée. Cependant, la déplétion de LB chez une souris adulte ScS n'altère par le phénomène de fibrose. Cette observation suggère que les LB pourraient contribuer à l'initiation de la maladie et non au maintien de la maladie (Hasegawa et al., 2006).

➢ Lymphocytes T

Le rôle des lymphocytes T (LT) dans la physiopathologie de la ScS a été largement étudié. Les LT auto-réactifs participent dans un premier temps à l'infiltration péri-vasculaire au cours de

la phase précoce de la maladie. Puis l'infiltration lymphocytaire gagne la peau et les autres organes cibles des patients ScS.

Les Lymphocytes auxiliaires de type 2 (Th2):

Des études plus anciennes ont déterminé que l'infiltrat précoce de cellules immunitaires était majoritairement composé de lymphocytes (Fleischmajer, 1977; Roumm et al., 1984). Certaines cytokines de la réponse de type 2 participent au remodelage de la MEC telles que l'IL13 ou l'IL4. Ces cytokines, associées à une polarisation Th2, suggèrent un rôle fondamental de ces cellules dans la fibrose. Certaines études montrent pourtant une différenciation plutôt de type Th1 par les LT recrutés dans le tissu lésé (Ferrarini et al., 1990; Gruschwitz et al., 1997). Toutefois, de nombreuses autres études rapportent une accumulation préférentielle de LT produisant de l'IL4 et de l'IL13 (Mavalia et al., 1997; Salmon-Ehr et al., 1996; Scaletti et al., 2002). Dans la ScS, une différenciation des lymphocytes vers la réponse Th2 a été observée (Sakkas et al., 2006). Il existe une infiltration cutanée ainsi qu'une augmentation du taux circulant de LT auxiliaires de type 2 (Th2) (Truchetet et al., 2011) ainsi que dans les tissus affectés (Scaletti et al., 2002). Ces cellules produisent de l'IL4, IL5 et IL13. L'IL13 et l'IL4 sont considérées comme des facteurs profibrosant car elles peuvent notamment induire la production de TGFβ par les macrophages, conduisant ainsi au développement de la fibrose (Fichtner-Feigl et al., 2006). Très peu d'études portent réellement sur les Th2, elles se focalisent surtout sur les cytokines dérivées de la réponse de type 2 qui peuvent également être sécrétées par d'autres cellules immunitaires. Les macrophages produisent de l'IL4 et l'IL13 favorisant ainsi secondairement la différenciation des LT en Th2 (O'Reilly et al., 2012). De plus, un modèle murin qui a été déplété en LT ($RAG^{-}/_{-}$) développe une fibrose pulmonaire, induite par *Schistosoma mansoni*, suggérant un rôle mineur de ces cellules dans l'établissement de la fibrose (Hams et al., 2014).

Les Lymphocytes auxiliaires de type 17 (Th17):

Le rôle des Th17 dans la ScS est largement débattu. En effet, certaines études rapportent dans un premier temps une augmentation à la fois du taux d'IL17 et du nombre de Th17 dans le sang et les organes cibles chez les patients ScS (Liu et al., 2013; Lonati et al., 2014). De plus, les cytokines cruciales pour la différenciation vers la voie Th17 telles que l'IL6, le TGFβ et l'IL23, sont augmentées chez les patients ScS (Gourh et al., 2009; Komura et al., 2008). Il existe également un polymorphisme génétique du récepteur à l'IL23 dans la ScS (Agarwal et al., 2009). Yang et al. ont montré que cette augmentation des Th17, observée à la fois dans le sang et dans la peau des sujets ScS, entraîne une production accrue de collagène par les fibroblastes (Yang et al., 2014). Dans des modèle animaux, l'IL17 agit comme médiateur de la fibrose aussi bien dans la peau que dans les poumons, à la fois dans des souris TSK et dans des souris injectées avec de la bléomycine (Gasse et al., 2011; Mi et al., 2011; Okamoto et al., 2012; Wilson et al., 2010). En effet, l'une de ces études a permis de mettre en évidence qu'une déplétion d'IL17A atténue à la fois la fibrose et l'épaisseur de la peau (Okamoto et al., 2012). A l'inverse, des études chez l'homme ont montré plutôt un rôle anti-fibrotique de l'IL17 par exemple en inhibant la différenciation des fibroblastes en myofibroblastes (Brembilla et al., 2013; Truchetet et al., 2013). Dans ce sens, une étude de Nakashima et al. a également montré qu'une altération de la voie de l'IL17 contribue à une augmentation de production de collagène par les fibroblastes ScS (Nakashima et al., 2012). De plus, l'augmentation des cellules productrices d'IL17 observée dans la peau des sujets ScS est inversement corrélée à l'étendue de la fibrose cutanée (Truchetet et al., 2013).

Les lymphocytes auxiliaires de type 9 (Th9):

Le rôle des Th9 dans la ScS est très peu étudié. Une première étude montre une augmentation des Th9 dans la peau des sujets ScS corrélée à l'atteinte cutanée. Les taux sériques d'IL9 sont également augmentés chez les sujets ScS.

Les lymphocytes cytotoxiques CD8 :

Une attention limitée a été accordée au rôle des cellules CD8 + dans l'étiopathogénie et la progression de la ScS. Les cellules CD8 + peuvent avoir des propriétés autoantigènes spécifiques et pro-inflammatoires mais aussi des propriétés immunomodulatrices. Les LT CD8+ migrent vers la peau au début de la maladie et produisent de l'IL13 induisant ainsi un phénotype profibrotique à des fibroblastes normaux (Fuschiotti et al., 2013). Les CD8 mémoires résidents de la peau sont également une autre source d'IL13 entraînant la production de collagène par les fibroblastes dermaux (Li et al., 2017).

Les lymphocytes régulateurs (Treg):

La littérature concernant les Treg dans la ScS ne fournit pas de données homogènes et une vision uniforme de leur rôle et de leur fonction. En effet, certains auteurs montrent une augmentation de ces cellules chez les sujets ScS (Radstake et al., 2009a; Slobodin et al., 2010) alors que d'autres études mettent en évidence une diminution de ces cellules dans la peau et le sang des patients ScS (Antiga et al., 2010; Mathian et al., 2012). En outre, des modifications épigénétiques aberrantes telles que l'hyperméthylation du gène FOXP3 ont également été détectées dans des Tregs de patients, pouvant expliquer la diminution observée de ces cellules dans la peau de sujets ScS (Wang et al., 2014). Cependant, dans l'étude menée par Radstake *et al*., l'augmentation des Treg est associée à une diminution de leur capacité à contrôler les CD4 (Radstake et al., 2009a). De manière surprenante, les Treg présentes dans la peau des ScS participent à la production de cytokines de type 2 telles que l'IL4 et l'IL13. Ce switch Treg/Th2 participe à l'amplification conséquente d'une boucle pro-fibrotique (MacDonald et al., 2015).

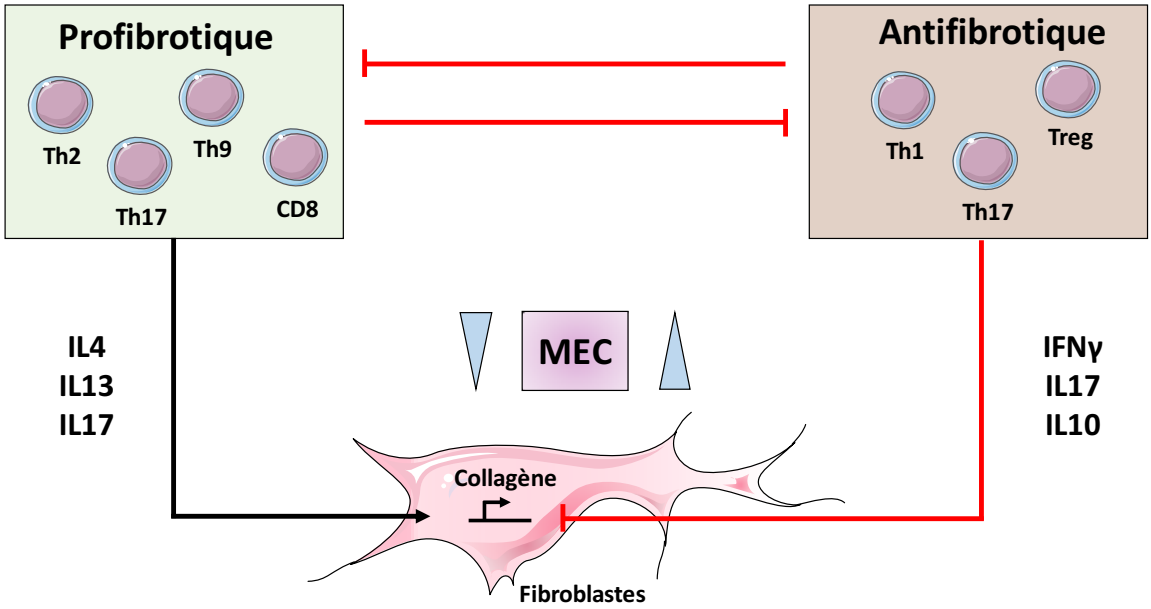


Figure 6 : Schéma résumant le rôle pro- ou anti-fibrotique des différents types de lymphocyte T helper, des LT régulateurs et des LT CD8 dans la ScS. Les Th2, les Th9, les CD8 ont un rôle profibrotique induisant la synthèse de collagène par les fibroblastes. Les Th1 et les Treg ont un rôle antifibrotique en inhibant la production de collagène par les fibroblastes. Le rôle des Th17 reste encore débattu.

Même si le rôle de certains sous-types lymphocytaires semble encore débattu, les cellules produisant de l'IL4 et/ou de l'IL13 ont un rôle profibrotique tandis que les cellules produisant de l'IFNg et/ou de l'IL10 ont un rôle antifibrotique.

c) Fibrose

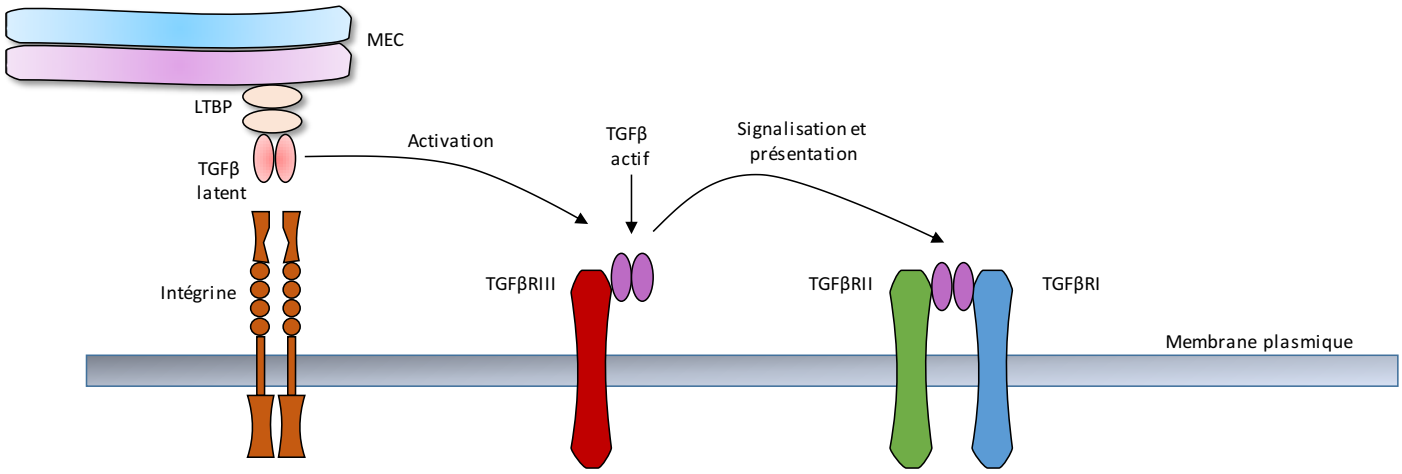
La fibrose est le trait caractéristique de la ScS. Elle affecte de nombreux organes, préférentiellement la peau mais aussi les poumons, le cœur, le tractus gastro-intestinal ainsi que les ligaments. Elle est définie par un dépôt excessif de MEC dû à une production anormalement élevée de collagène par les fibroblastes et les myofibroblastes (fibroblastes activés contractiles et sécrétoires) et une inhibition des métalloprotéinases (MPP)

responsables de la dégradation de la MEC. Des scores de fibrose, tels que le score de Rodnan, permettent d'évaluer l'étendue de la fibrose chez les patients ScS.

Dès le début des années 1970, des études ont montré une surexpression de collagène par les fibroblastes ScS en comparaison à des fibroblastes sains (Leroy, 1972; LeRoy, 1974). Bien que des études génomiques aient montré qu'environ 2000 gènes diffèrent entre une peau ScS et une peau saine, les fibroblastes ScS et sains sont génotypiquement très similaires (Gardner et al., 2006; Whitfield et al., 2003). Les fibroblastes ScS sont plus actifs et adoptent un profil pro-fibrotique en sécrétant des cytokines tel que le TGFβ. Ils sur-expriment également des intégrines, des récepteurs comme le récepteur du TGFβ ou celui de CCL2 (Rosenbloom et al., 2010; Varga and Abraham, 2007). Le phénotype activé des fibroblastes ScS peut être dû notamment à la stimulation autocrine permanente par le TGFβ (Ihn, 2008) mais également à des événements épigénétiques (Maurer et al., 2010; Pandit et al., 2011; Wang et al., 2006). En effet, dans la peau fibrotique des patients mais également dans des explants de fibroblastes ScS, l'expression du miARN antifibrotique miR-29 est diminuée dans la ScS (Maurer et al., 2010).

i. Le TGFβ

Bien que les causes des anomalies de la fibrose soient encore mal connues dans la ScS, le TGFβ semble jouer un rôle important. Il est notamment produit par des cellules mononuclées du sang ainsi que des macrophages tissulaires. La biodisponibilité du TGFβ est régulée notamment par les macrophages infiltrant les tissus ainsi que par l'activation de sa forme latente liée à la MEC. En effet, la forme latente du TGFβ se lie à des intégrines présentes à la surface du fibroblaste entrainant un changement de conformation du TGFβ qui devient actif.



`Figure 7 : Illustration de la liaison du TGFβ à son récepteur. Le TGFβ latent contenu par exemple dans la matrice extracellulaire (MEC) se fixe sur le TGFβRII. Il devient alors actif et se fixe alors sur le dimère formé par le TGFβRII et le TGFβRI.

Le TGFβ actif se lie d'abord au récepteur TGFβRIII puis au TGFβRII/ TGFβRI entraînant deux voies de signalisation possibles :

- Canonique : via le système SMAD.
- Non canonique : via un système SMAD indépendant impliquant les molécules ERK, PI3K ou encore TAK.

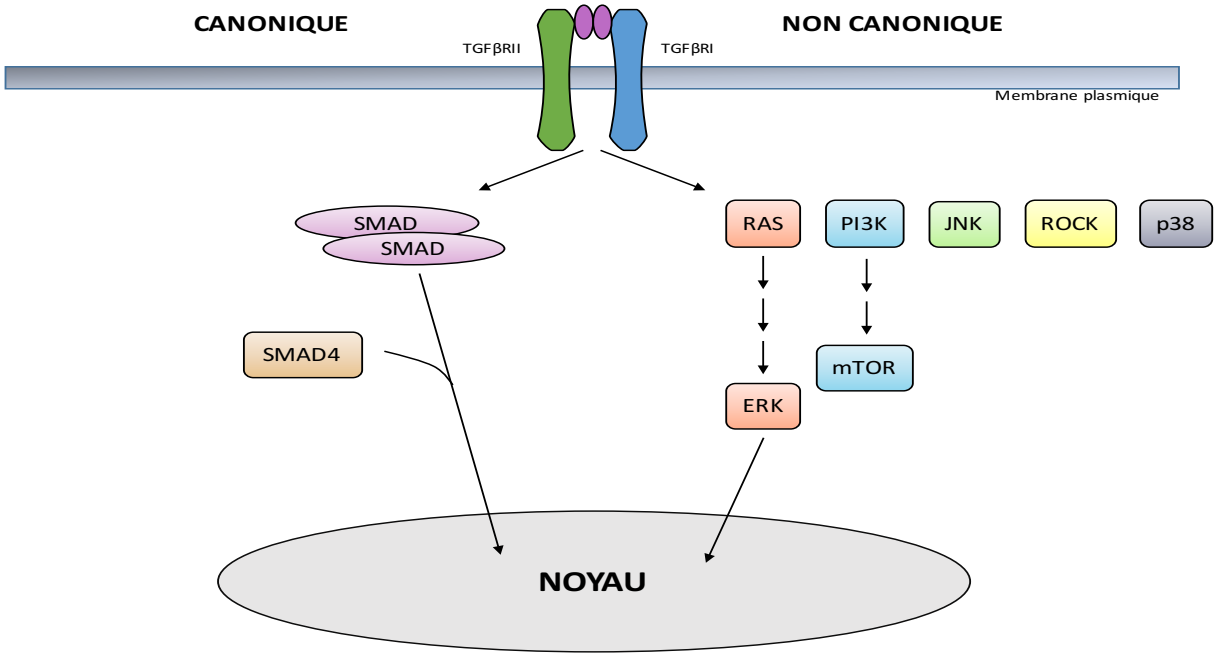


Figure 8 : Voies de signalisation du TGFβ. Une voie canonique dépendant de SMAD et une voie non canonique indépendant de SMAD impliquant des molécules suivantes telles que: RAS, PI3Kou p38.

Très peu de temps après sa découverte, le TGFβ a été considéré comme un puissant facteur profibrotique (Roberts et al., 1985, 1986), promouvant la synthèse et la sécrétion de collagène par exemple (Roberts et al., 1990) ou d'autres composants de la MEC telle que la fibronectine (Penttinen et al., 1988).

Des études d'immunohistochimies montrent que l'expression du TGFβ est augmentée dans la peau des ScS (Higley et al., 1994; Ozbilgin and Inan, 2003; Sfikakis et al., 1993). Des études génomiques montrent une augmentation de la voie du TGFβ dans la peau des ScS (Gardner et al., 2006; Sargent et al., 2010; Whitfield et al., 2003), notamment l'expression des récepteurs TGFβ RII et TGFβ RI (Kawakami et al., 1998; Pannu et al., 2006), l'expression des intégrines sur lesquelles se fixe le TGFβ (Asano et al., 2004, 2005), mais également de plusieurs témoins de l'activité du TGFβ, telles que la présence de myofibroblastes et l'expression des gènes cibles du TGFβ (Christmann et al., 2014; Farina et al., 2010; Kissin et al., 2006). L'activité du TGFβ est corrélée à l'atteinte cutanée des patients (Christmann et al., 2014; Farina et al., 2010). Dans la ScS, le TGFβ induit une augmentation de la transcription des gènes procollagène I et III

accentuant ainsi le dépôt de MEC (Roberts et al., 2003). De manière intéressante, des fibroblastes normaux exposés au TGFβ adoptent un phénotype profibrotique en produisant de grandes quantités de collagènes et de CCN2 ainsi qu'en sur-exprimant des gènes de la MEC (Varga and Pasche, 2009).

Le TGFβ peut également avoir un rôle indirect dans l'établissement de la fibrose via l'induction de la production d'ET1 (Shi-wen et al., 2007), participant ainsi à l'augmentation de l'expression de l'ET1 et de son récepteur dans la ScS (Abraham et al., 1997). L'ET1 favorise, *in vitro*, la migration de fibroblastes et leur différenciation en myofibroblastes (Xu et al., 2004)

ii. L'IL13

L'IL13 est un autre acteur majeur de l'induction de la fibrose. Elle est sécrétée par de nombreux types cellulaires comme les mastocytes, macrophages, basophiles, éosinophiles (Greenblatt and Aliprantis, 2013). Le récepteur à l'IL-13 (IL-13R) est un complexe formé de la chaîne α du récepteur de l'IL-4 (IL-4Rα) et d'une chaîne α1 (Donaldson et al., 1998; Hilton et al., 1996). L'IL-13Rα1 a une faible affinité pour l'IL-13 mais une fois associé à l'IL-4Rα, ce complexe présente une haute affinité pour l'IL-13 permettant la transmission du signal via JAK1/TYK2 et STAT6 (Roy et al., 2002; Wills-Karp and Finkelman, 2008). De plus, il existe également un récepteur spécifique pour l'IL13 : l'IL13Rα2 qui a une forte affinité et spécificité avec l'IL13 (Wynn, 2003). Son domaine intra-cytoplasmique étant court, ce récepteur est jusque-là décrit comme dépourvu action activatrice ou inhibitrice (Kelly-Welch et al., 2003). Il régulerait ainsi la fonction de l'IL13, agissant comme un leurre (Mentink-Kane et al., 2004).

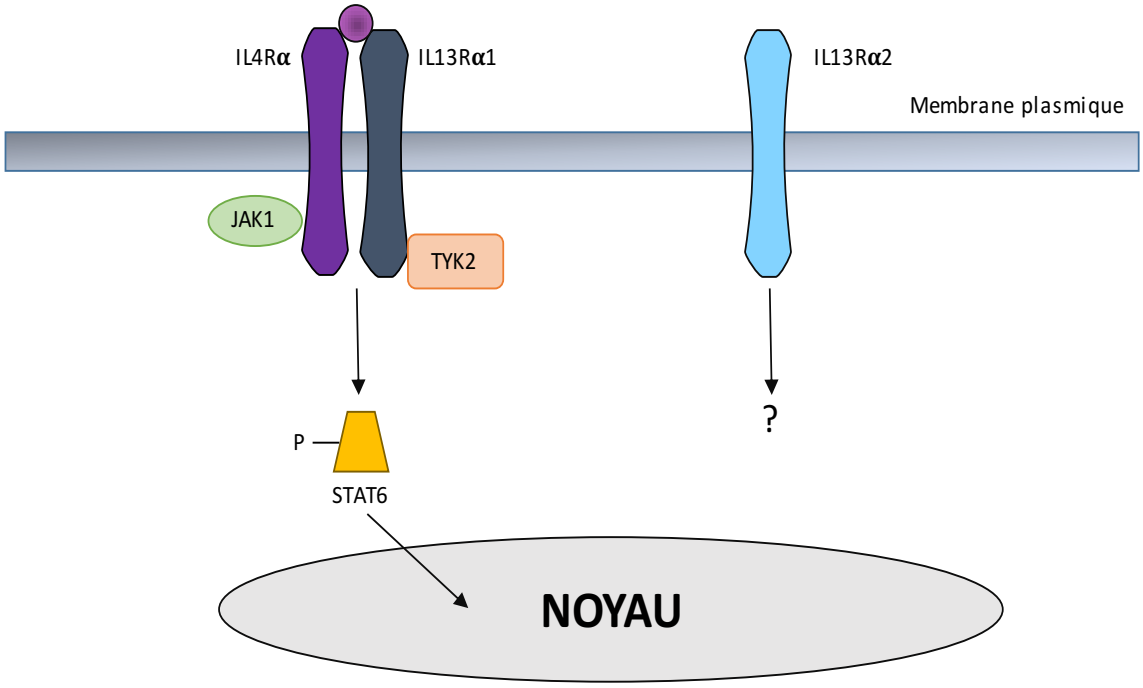


Figure 9 : Voie de signalisation de l'IL13 dépendant de JAK1, TYK2 et de pSTAT6

L'IL13 est impliquée dans de nombreuses maladies fibrotiques telles que la ScS (Wynn, 2004). En effet, des modèles *in vivo* de fibrose induite par la bléomycine ont mis en évidence une implication directe de l'IL13 dans ce processus (Aliprantis et al., 2007).

D'un point de vue génétique, des polymorphismes dans le gène de l'IL13 ou de son récepteur ont été associés à la ScS et plus particulièrement à la fibrose dermale (Granel et al., 2006a, 2006b, 2007). L'IL13 est capable à la fois d'induire la prolifération et la différenciation des fibroblastes in vitro ainsi que l'expression du collagène et d'autres gènes profibrotique tel que αSMA. Dans le cadre de la ScS, l'IL13 conduit à la sécrétion du collagène de type I par les fibroblastes dermiques par activation directe du promoteur du gène du collagène de type I (Jinnin et al., 2004). Récemment, il a été mis en évidence que des LT CD8 étaient impliqués dans la fibrose dermale chez des patients ScS via leur capacité à produire de l'IL13. Les CD8 contribuent donc en partie à l'élévation des taux sériques d'IL4 et d'IL13 observés chez les patients atteints de ScS (Fuschiotti et al., 2013). Les LB activés par l'IL13 produisent des auto-Ac dont des Ac anti-fibroblastes (McKenzie et al., 1993) qui pourraient avoir un rôle dans la fibrose notamment au niveau pulmonaire (Terrier et al., 2010). De plus, l'IL13 participe à la

diminution du processus de dégradation de la matrice favorisant ainsi la fibrose (Granel et al., 2007). De manière indirecte, l'IL13 peut également participer à la fibrose. En effet, sous l'activation de l'IL13, les macrophages produisent du TGFβ (Lanone et al., 2002; Lee et al., 2001). Ce rôle indirect est largement débattu. En effet, des études in vivo utilisant un modèle murin de fibrose IL13$^{-/-}$ montrent une complète abrogation de la fibrose malgré la présence permanente de TGFβ (Kaviratne et al., 2004).

L'utilisation de modèles animaux, notamment murin, de la ScS a permis de mieux comprendre le rôle de l'IL13 dans la fibrose associée à la maladie. En effet, l'injection sous cutanée de bléomycine chez la souris a montré que l'IL13 était sécrétée localement en quantité croissante en fonction du degré de sclérose du derme (Matsushita et al., 2004). Un autre modèle de ScS est l'induction d'une réaction chronique de greffon contre l'hôte (GVHD) chez la souris. Les souris présentent les principales caractéristiques de la ScS notamment la fibrose, les auto-Ac ainsi que des altérations vasculaires. Quand la souris receveuse du greffon est IL13$^{-/-}$, la fibrose est moins étendue ce qui témoigne du rôle de l'IL13 dans la fibrose de la ScS (Fichtner-Feigl et al., 2006).

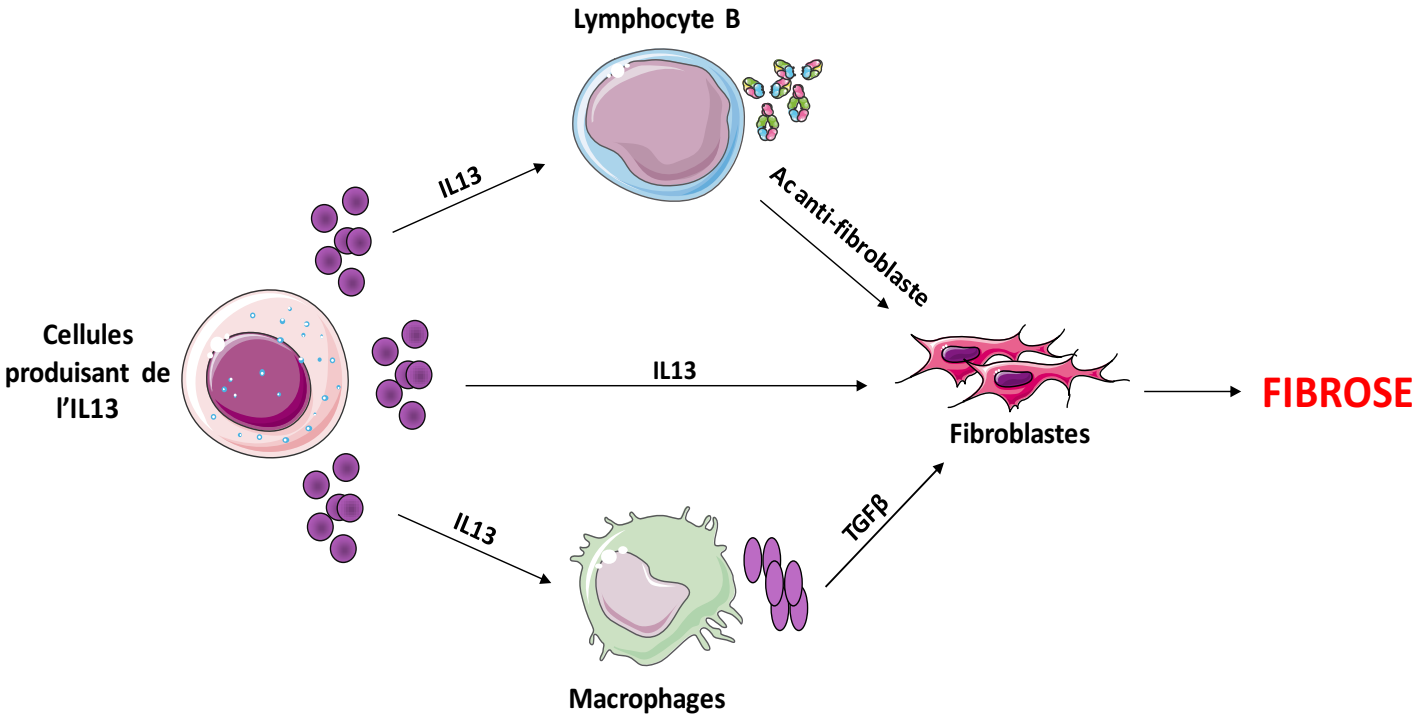


Figure 10 : Rôle de l'IL13 dans la fibrose : un rôle direct via son rôle profibrotique, un rôle indirect à la fois via l'activation des macrophages et par son action sur les lymphocytes B

iii. La TSLP

Récemment, la TSLP (Thymic Stromal Lymphopoietin) a été identifiée comme un nouvel acteur impliqué dans l'induction de la fibrose chez les patients ScS (Christmann et al., 2013; Usategui et al., 2013). Découverte en 1994 (Friend et al., 1994), elle est sécrétée par les épithéliums sous l'influence de facteurs tels que l'IL1β/α ou le LPS. Elle se lie à un récepteur hétérodimérique associant la chaine α du récepteur de l'IL7 et une chaîne commune γ (Pandey et al., 2000). Son récepteur est très répandu à la surface des cellules immunitaires notamment sur les CD, les LT et LB, les natural killer (NK), les mastocytes ainsi que les monocytes mais aussi sur des cellules non immunitaires comme les fibroblastes (Oh et al., 2011).

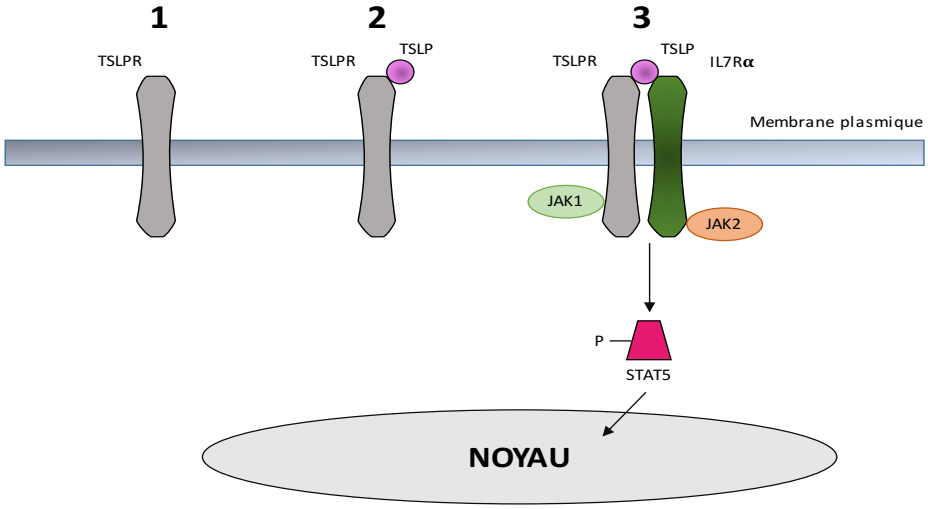


Figure 11 : Liaison et voie de signalisation de la TSLP : (i) liaison de la TSLP sur son récepteur (ii) dimérisation du complexe TSLP/TSLPR avec l'IL7Ra puis (iii) signal d'activation dépendant de JAK1/2 et pSTAT5

En terme physiopathologie, des taux élevés de la TSLP ont été détectés à la fois dans la peau de patients atteints de dermatite atypique (Oh et al., 2011) et dans celle des patients ScS (Christmann et al., 2013). Plus précisément dans la ScS, des analyses d'immunohistochimie et immunofluorescence ont permis de localiser la TSLP dans les kératinocytes de la peau et dans d'autres cellules dermales telles que les fibroblastes, les mastocytes et les cellules mononuclées (Usategui et al., 2013). La production élevée de TSLP est expliquée par la contribution de multiples cellules. Dans les fibroblastes, le taux d'ARNm de la TSLP augmente quand le TLR 3 est stimulé par un agoniste poly (I :C). Dans un modèle murin ScS, induit par

l'injection sous cutanée de bléomycine, KO pour le récepteur de TSLP, des expériences ont mis en évidence le rôle de TSLP dans la production de collagène et dans la fibrose. En effet, l'effet fibrotique, ainsi que le taux d'ARNm IL13, sont nettement diminués chez la souris KO en comparaison (Usategui et al., 2013). Notre équipe a montré récemment que sous l'activation plaquettaire, les CE produisent de la TSLP qui a une action profibrotique sur les fibroblastes (Truchetet et al., 2016).

Outre le TGFβ, l'IL13 et la TSLP, d'autres molécules peuvent intervenir dans le processus de fibrose telle que CCL2. Elle est sécrétée en très grande quantité par de nombreuses cellules, notamment les fibroblastes et les cellules endothéliales. *In vitro*, CCL2 stimule la production de collagène directement ou indirectement via le TGFβ (Distler et al., 2006).Le taux sérique de CCL2 est corrélé à l'atteinte fibrosante pulmonaire (Galindo et al., 2001; Kodera et al., 2005). L'ET1 a également un rôle profibrotique en conduisant à la migration et la différenciation des fibroblastes *in vitro* (Xu et al., 2004).

Tous ces acteurs et molécules sont des cibles thérapeutiques potentielles pour des traitements anti-fibrosants. Certaines molécules sont déjà ciblées comme l'ET1. En effet, des antagonistes du récepteur de l'ET1 sont déjà utilisés comme traitement contre l'hypertension artérielle pulmonaire et contre la récidive d'ulcères digitaux.

3. Réponse immunitaire de type 2 et sclérodermie systémique

La sclérodermie systémique est une maladie caractérisée et définie par une forte réponse de type 2. En effet, de très grandes quantités d'IL13 et d'IL4 sont retrouvées dans les tissus atteints des patients ScS.

a) Implication des macrophages de type M2 dans la ScS

Les monocytes se classent en trois sous-types selon l'expression de CD14 et de CD16 : (i) les monocytes « classiques » $CD14^{high}CD16-$, (ii) les monocytes « intermédiaires » $CD14^{high}CD16+$ et (iii) les monocytes « non classiques » $CD14^{low}CD16+$ (Ziegler-Heitbrock et al., 2010). Les monocytes restent quelques jours dans le sang puis migrent du sang vers les tissus et se différencient en macrophages. Classiquement, les macrophages sont classés en deux types : les macrophages de type 1 (M1, macrophages classiques) et les macrophages de type 2 (M2, macrophages alternatifs) (Gordon, 2003; Mosser and Edwards, 2008). En 2014, Martinez et Gordon précisent cette classification en divisant les M2 en 3 sous-types : les M2a ayant un rôle dans la défense contre les parasites, M2b impliqués dans l'immunorégulation et les M2c engagés dans la réparation (Martinez and Gordon, 2014).
Cependant, il apparaît de façon de plus en plus marquée que cette dichotomie M1/M2 est en réalité beaucoup plus complexe. Certains auteurs parlent plutôt d'un continuum d'état d'activation plutôt que de paradigme M1/M2 (Murray et al., 2014; Xue et al., 2014).

Dès la fin des années 80, Andrews *et al*. étudient le rôle des macrophages dans la ScS. Avec les outils et les techniques d'analyse disponibles à l'époque, ils montrent que les monocytes circulants des patients ScS sont très activés par rapport aux sujets contrôles (Andrews et al., 1987). Ces résultats ont été confirmés par des études plus récentes montrant notamment une augmentation des cellules inflammatoires mononuclées dans le sang et dans la peau des sujets ScS (Hussein et al., 2005). D'autres études antérieures ont également mis en évidence une infiltration macrophagique très précoce dans la peau des sujets ScS (Ishikawa and Ishikawa, 1992; Kräling et al., 1995).
L'apparition de la dichotomie M1/M2 a permis de préciser le phénotype des monocytes/macrophages étudiés lors de la ScS. L'expression de Siglec-1, marqueur spécifique des M1 est augmentée dans le sang des sujets ScS (York et al., 2007). A l'inverse, de nombreuses études montrent un taux élevé de cellules positives pour CD68, CD163 et CD204 (Higashi-Kuwata et al., 2010), tous trois marqueurs des M2 dans le sang et dans la peau ainsi qu'une augmentation du CD163 soluble dans le sang des sujets ScS (Bielecki et al., 2013; Kowal-Bielecka et al., 2013; Nakayama et al., 2012; Shimizu et al., 2012). De plus, les monocytes circulants des patients ScS ont un phénotype profibrotique par rapport aux

contrôles et semblent être impliqués dans la physiopathologie de la fibrose pulmonaire dans la ScS (Mathai et al., 2010). Une étude menée par Christmann *et al*., montre une augmentation de l'expression de CD206, un autre marqueur spécifique des M2, dans le sang des patients ScS atteints d'hypertension artérielle pulmonaire (HTAP). En effet, sous l'effet de l'IL13, les cellules CD14+ sur-expriment le CD206, suggérant que les marqueurs spécifiques des M2, trouvés augmentés chez les patients ScS, reflètent un environnement à prédominance Th2 (Christmann et al., 2011).

Précocement au cours de la maladie, les macrophages sont associés à la fois à l'inflammation et la fibrose dans le poumon et la peau des patients (Christmann et al., 2014). Les macrophages CD163+ produisent CCL19, une chimiokine impliquée dans le recrutement de cellules inflammatoires. De plus, CCL2, une molécule impliquée dans le recrutement monocytaire de la peau, est surexprimée dans la peau des patients ScS (Distler et al., 2001; Galindo et al., 2001; HASEGAWA et al., 1999; Yamamoto et al., 2001) et est corrélée à la fois avec le score de Rodnan (Bandinelli et al., 2012; Greenblatt et al., 2012; Rice et al., 2015) et la fibrose pulmonaire (Assassi et al., 2013). Sous l'action combinée de l'IL4 et de la cadhérine 11, les macrophages produisent du TGFβ et pourraient ainsi directement jouer un rôle dans la fibrose cutanée (Wu et al., 2014). Une autre étude menée par le groupe de Sabine Eming montre l'implication directe des macrophages activés par l'IL4 et l'IL13 dans la fibrose via leur capacité à produire Relmβ (Resistin-like molecule β)(Knipper et al., 2015).

La présence des deux signatures M1/M2 au cours de la ScS semble claire, mais leur rôle exact ainsi que leur mécanisme d'action restent à déterminer et préciser. Cependant, certaines molécules spécifiques des macrophages pourraient être considérées comme des biomarqueurs de la ScS tels que le CD163 (Frantz et al., 2018) et le CCL18 (Schupp et al., 2014).

b) ILC2 (Type 2 innate lymphoid cells)

i. Les ILC (innate lymphoid cells)

Les ILC2 font partie de la famille des ILC. Les ILC sont des cellules de l'immunité innée qui ont été récemment découvertes grâce à des études conjointes menées par différentes équipes

(Cella et al., 2009; Moro et al., 2010; Narni-Mancinelli et al., 2011; Price et al., 2010; Satoh-Takayama et al., 2008, 2010; Vonarbourg et al., 2010). Ces cellules sont régulées par de nombreux stimuli endogènes tels que certains neuropeptides, hormones, cytokines et autres alarmines (Artis and Spits, 2015; Vivier et al., 2018). Les ILC sont définies par une morphologie lymphoïde et plus particulièrement par une absence d'expression de molécules permettant d'identifier les autres types cellulaires tels que les récepteurs d'antigènes recombinés à médiation RAG.

➢ Classification

Les ILC se classent en deux catégories principales : les ILC cytoxiques représentant les NK et les ILC non cytotoxiques regroupant les ILC1, les ILC2 et les ILC3. Ces dernières sont définies sur la base de leur différence en termes d'expression de facteur de transcription, de molécules d'activation et de production cytokinique.

1. les ILC de type 1 (ILC1) produisant de l'IFNγ et du TNFα et qui dépendent de Tbet. Elles participent à l'immunité contre les bactéries intracellulaires et les parasites ;
2. les ILC de type 2 (ILC2) libérant principalement de l'IL4, de l'IL5, de l'IL9 et de IL13 et exprimant GATA3. Elles sont impliquées dans la défense contre les helminthes, dans la réparation tissulaire et dans l'inflammation allergique ;
3. les ILC de type 3 (ILC3) qui dépendent de RORγt et qui expriment notamment l'IL17, l'IL25 ou l'IL-22. Elles contribuent à la défense contre les bactéries extracellulaires.

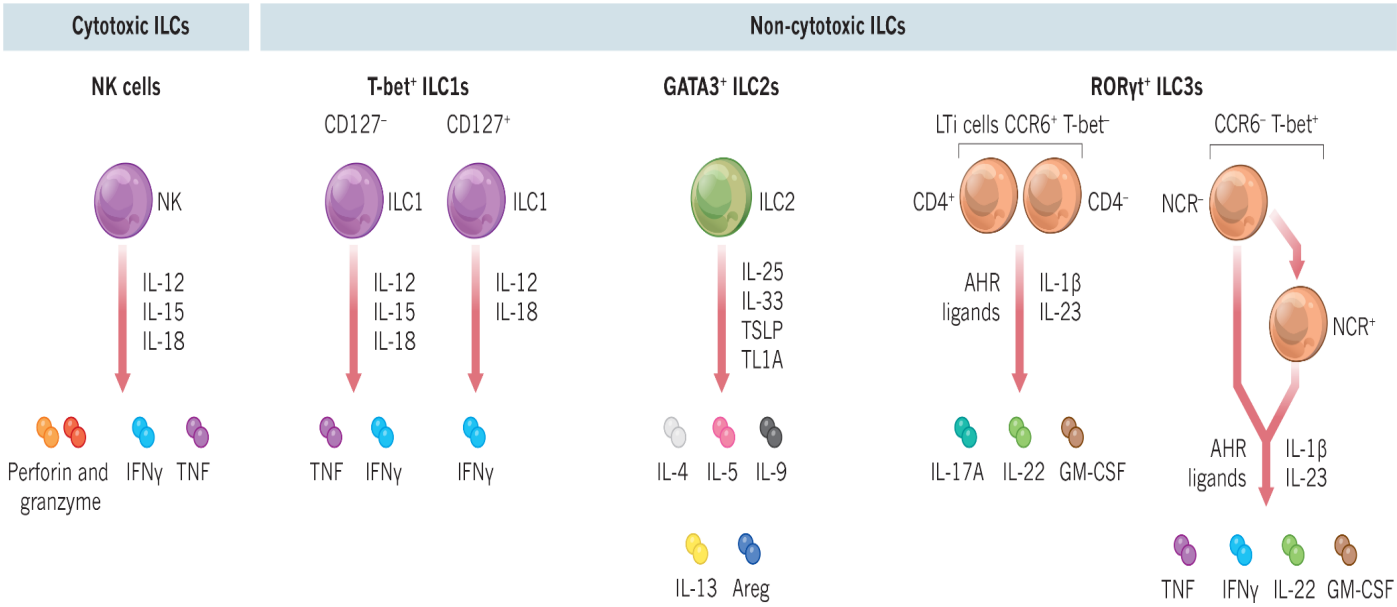


Figure 12 : Classification des ILC et de ses sous-types en fonction de leurs cytokines d'activation, de leur expression de facteur de transcription ainsi que de leur production cytokinique. Les ILC1 produisent de l'INFγ et du TNFα et expriment Tbet ; les ILC2, cellules GATA3+, sécrétent de l'IL4, IL5, IL9, IL13 et de l'amphiréguline (Areg) ; les ILC3 expriment RORγt et produisent notamment de l'IL17 et de l'IL22 (Artis, 2015)

➢ Ontogénie des ILC

Tout comme les LT adaptatifs, les ILC ont pour origine une progéniteur lymphoïde commun ou CLP (*common lymphoid progenitor*) (Gronke et al., 2016). Les facteurs de transcription exprimés au cours de la différenciation cellulaire déterminent l'engagement dans une lignée cellulaire spécifique. De la même manière, l'expression de facteur de transcription particulier dirige la différenciation cellulaire finale : l'expression de Tbet donnera lieu à des ILC1, l'expression de GATA3 ou RORα engendrera des ILC2 et l'expression de RORγt induira des ILC3 (Gronke et al., 2016).

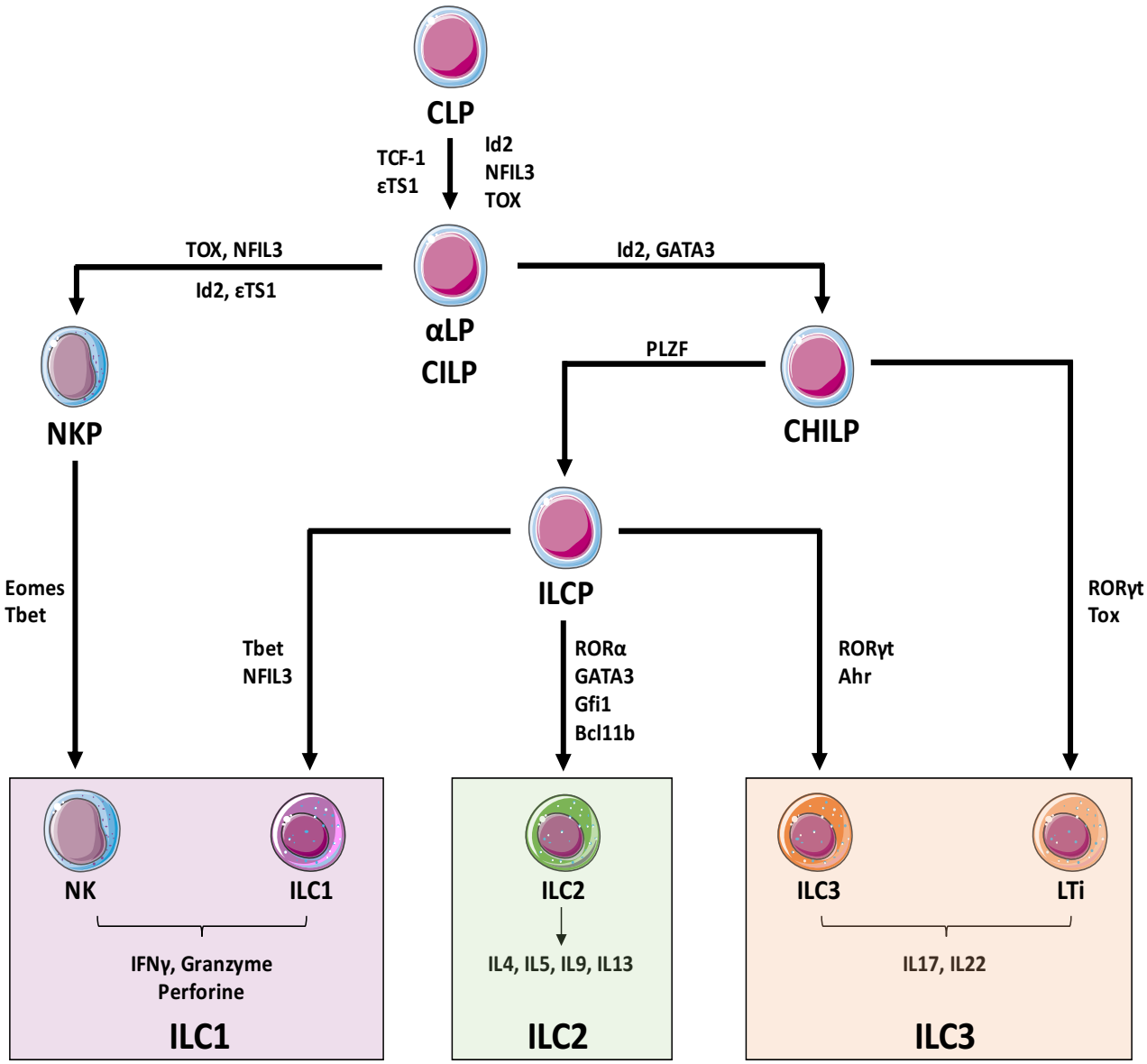


Figure 13 : Ontogénie des ILC. Les ILC dérivent d'un progéniteur lymphoïde commun (CLP). L'expression de différents facteurs de transcription au cours de la différenciation cellulaire donne lieu aux différents sous-types d'ILC (D'après Crinier et al., 2017 et Artis and Spits, 2015)

➢ Plasticité des ILC

Sous l'influence de facteurs environnementaux, les ILC sont capables d'exprimer des marqueurs et de produire des cytokines qui ne sont pas spécifiques à leur sous-groupe. Il existe une plasticité notamment fonctionnelle entre les différentes classes d'ILC à la fois chez la souris et chez l'Homme.

Plasticité des ILC1

Des études in vitro chez l'homme ont mis en évidence une plasticité des ILC1 vers les ILC3. En effet, en présence d'IL2, d'IL23 et d'IL1β, cette plasticité est contrôlée par le facteur de transcription RORγt et est favorisée en présence d'acide rétinoïque. Ces nouvelles cellules générées ont perdu la capacité de produire de l'IFNγ et sécrètent à la place de l'IL22 (Bernink et al., 2015). Ces observations obtenues *in vitro* ont été confirmées *in vivo* chez la souris. Ces résultats peuvent soulever des questions quant à l'origine des cytokines nécessaires à la différenciation (IL2, IL23 et IL1β). Cette même étude montre que des cellules dendritiques exprimant le CD104 peuvent produire ces trois cytokines et ainsi induire cette différenciation (Bernink et al., 2015).
La différenciation des ILC1 en ILC2 n'a encore jamais été observée.

Plasticité des ILC2

Des ILC2 isolées du sang humain mises en culture *in vitro* en présence d'IL2 et d'IL7 ont la capacité de produire de l'IFNγ et d'exprimer Tbet (Lim et al., 2016). Des études complémentaires ont confirmé cette observation. Des ILC2 mises en culture avec de l'IL1β expriment Tbet ainsi que le récepteur à l'IL12 et produisent de l'IFNγ en plus de l'I13. Chez la souris, des injections simultanées d'IL1β et d'IL12 entrainent le développement d'ILC2 pulmonaires positives pour Tbet et négatives pour CRTH2 (Bal et al., 2016; Ohne et al., 2016). Chez une souris infectée avec le virus de la grippe, les ILC2 sous-expriment GATA3 et surexpriment l'IL18R. Une fois mises en culture, les ILC2 produisent également de l'IFNγ.

Plasticité des ILC3

Après une stimulation conjointe, *in vitro*, de l'IL2 et du TLR2, les ILC3 humaines ont la capacité de produire de l'IL13 et de l'IL5 spécifiques des ILC2 (Crellin et al., 2010).

Sous l'influence de l'IL15 et de l'IL12 sécrétées à la fois par des cellules dendritiques conventionnelles et des monocytes, les ILC3 sont capables de générer, *in vitro*, des ILC1 (Bernink et al., 2015). Ce switch a été observé au niveau de l'intestin d'une souris atteinte de colite, participant ainsi à l'augmentation des ILC1, caractéristique de cette maladie (Bernink et al., 2013).

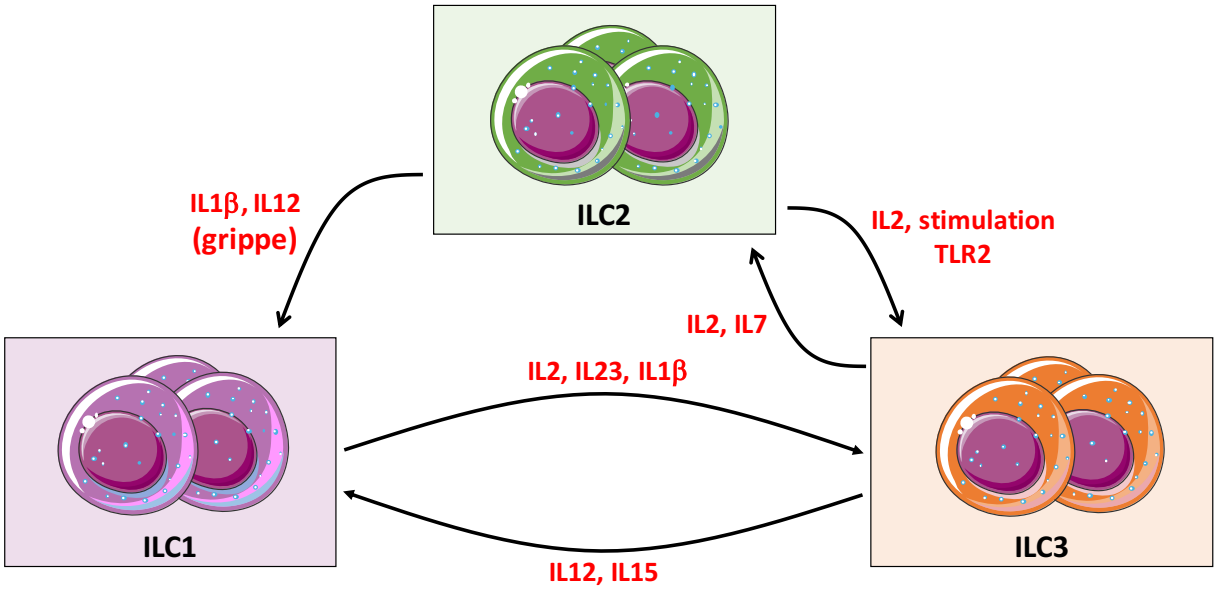


Figure 14 : Plasticité entre les différents sous-types d'ILC. La plasticité des ILC2 vers les ILC1 est favorisée par le l'IL1β et de l'IL12. L'IL2, l'IL23 et l'IL1β permettent un switch entre les ILC1 et les ILC3 tandis que l'IL12 et l'IL15 induisent le switch contraire. Les ILC3 deviennent des ILC1 grâce à l'IL2 et l'IL7 tandis qu'une stimulation du TLR2 ajoutée à de l'IL2 permet la plasticité entre les ILC2 et les ILC3

ii. Les ILC2

Les ILC2 sont des médiatrices clés de la réponse immunitaire de type 2 et sont une source cellulaire puissante d'IL5 et d'IL13 (McKenzie et al., 2014). Les ILC2 résident dans les tissus où

elles jouent un rôle important dans l'orchestration de l'inflammation de type 2 (Wojno and Artis, 2016). Il est important de noter que les ILC2 ne sont pas activées par des interactions directes avec les antigènes par l'intermédiaire du TCR. Elles expriment des PRR, notamment les TLR1, TLR4 TLR6 (Maggi et al., 2017). Elles ont également des récepteurs de cytokines rapidement libérées par des CE endommagées ou stimulées, telles que l'IL33, l'IL25 ou la TSLP. Elles sont également des récepteurs aux lipides, aux hormones, ainsi qu'à d'autres cytokines telles que l'IL45, l'IL7 et l'IL9. Ainsi, ces nombreux récepteurs leur permettent d'agir rapidement aux changements micro-environnementaux et de jouer le rôle de sentinelle au sein de l'organisme (Schuijs and Halim, 2018).

➢ Classification des ILC2

Les ILC2 sont divisées en deux sous-types : les ILC2 dites inflammatoires et les ILC2 dites natures. Il semblerait que les nILC2 soient à un stade plus tardif que les iILC2 étant donné la capacité des iILC2 à se différencier en nILC2. L'inverse n'a pour le moment pas été montré.
Outre la production d'IL17, la séparation de ces deux sous-types a été faite sur la base de l'expression de KLRG1 (killer cell lectine like receptor G1). KLRG1 est un récepteur présent sur d'autres cellules immunitaires telles que les NK, les CD8 ainsi que les CD4. KLRG1 possède un domaine intra-cytoplasmique ITIM traduisant un rôle d'inhibition de la réponse immunitaire. Toutefois, il est important de noter que l'action inhibitrice de KLRG1 est fortement associée à sa liaison avec un de ses ligands, la E-cadhérine. C'est le cas pour les ILC2 ainsi que pour les CD8 et les NK. Le rôle de KLRG1 sur les ILC2 n'a que rarement été analysé. La liaison entre la E-cadhérine et KLRG1 à la surface des ILC2 entraîne une diminution de la production d'IL4 et d'IL13 par les ILC2.
Des ILC2 murines dites inflammatoires « iILC2 » se différencient en « ILC3-like » et acquièrent la capacité de produire de l'IL17. *In vivo*, ces cellules pourraient jouer un rôle dans l'immunité antiparasitaire et antifongique (Huang et al., 2015). Dans cette même étude, une plasticité entre les iILC2 et les natures « nILC2 » est également montrée. *In vivo*, les iILC2 se développent en nILC2, dans les phases tardives d'une infection à *Nippostongylus. brasiliensis* (Huang et al., 2015).

Malgré cela, le rôle de KLRG1 sur la fonction des ILC2 et de chacune des ILC2 n'est encore pas bien déterminé et des investigations supplémentaires sont nécessaires afin de déterminer l'importance de ce récepteur sur la fonction des ILC2.

➢ Pathologies impliquant les ILC2

Il est déjà connu que les ILC2 sont impliquées dans diverses pathologies, pour la plupart des maladies inflammatoires telles que l'allergie, la dermatite atopique ou la rhinosinusite chronique.

Dermatite atopique

La dermatite atopique est une maladie inflammatoire de la peau due à des facteurs génétiques et environnementaux et caractérisée par une dysfonction de la barrière épidermale. Elle est associée à une forte réponse de type 2, comme le montrent les hauts niveaux d'IL13 et d'IL4 au niveau des lésions cutanées d'un patient atteint de dermatite atopique (Leung et al., 2004). Les ILC2 étant les plus grandes productrices d'IL13 dans la peau (Roediger et al., 2013), plusieurs équipes se sont intéressées à leur potentiel rôle dans la physiopathologie de l'AD. Les ILC2 sont enrichies dans la peau des patients atteints de dermatite atopique (Kim et al., 2013). Les ILC2 infiltrent la peau des patients dermatite atopique et produisent en grande quantité de l'IL5, de l'IL13 et de l'amphiréguline (Salimi et al., 2013). Dans cette même étude, KLRG1 est identifié comme un inhibiteur des ILC2 grâce à la fixation de la E-cadhérine. Or, le fait que l'expression de la E-cadhérine soit diminuée dans la dermatite atopique apporte un argument supplémentaire quant au rôle des ILC2 dans cette pathologie (Salimi et al., 2013). En outre, des souris transgéniques exprimant le gène de l'IL-33, piloté par le promoteur de la kératine 14, ont été générées. La production de l'IL33 restreinte à la peau entraine le développement d'une dermatite atopique chez la souris. La proportion des ILC2 produisant de l'IL-5 a augmenté significativement dans la peau lésionnelle ainsi que dans le sang périphérique, suggérant que l'activation des ILC2 par l'IL33 pourrait jouer un rôle crucial dans la pathogenèse de la dermatite atopique (Imai et al., 2013). A l'inverse, une autre étude murine démontre une activation des ILC2 dépendante de la TSLP, menant à l'inflammation de la peau (Kim et al., 2013). Dans le cadre de la dermatite atopique, des thérapies ciblant le

récepteur de l'IL13 ont été menées. Le dupilumab, un anticorps bloquant l'IL4Rα, améliore les signes et les symptômes de la maladie (Simpson et al., 2016).

Asthme et allergie

L'asthme est également caractérisé par une très forte réponse immunitaire de type 2. Le pourcentage d'ILC2 est augmenté chez les patients asthmatiques et cette hausse est d'autant plus importante que les patients présentent une forme sévère de la maladie (Smith et al., 2016). Les patients souffrant d'asthme non contrôlé ont un taux d'ILC2 produisant de l'IL13 élevé par rapport à des sujets souffrant d'asthme contrôlé. Ce taux d'ILC2 diminue lorsque les patients sont traités, suggérant que le nombre d'ILC2 pourrait être un facteur de prédiction de la sévérité de la maladie (Jia et al., 2016). Le mécanisme d'action des ILC2 et leur potentiel rôle dans la physiopathologie de l'asthme ne sont pas entièrement compris.

Cependant, les cellules épithéliales sont la principale source d'activation des ILC2 dans l'asthme de par leur capacité à produire de l'IL33, de l'IL25 et de la TSLP, entraînant le début de l'inflammation allergique. Le taux d'IL33 est augmenté à la fois dans le sérum (OSHIKAWA et al., 2012) et dans des biopsies bronchiales (Préfontaine et al., 2009) de patients asthmatiques. Les allergènes peuvent indirectement activer les ILC2 via cette sécrétion d'IL33 par les cellules épithéliales (Bartemes et al., 2012; Halim et al., 2012). Les allergènes peuvent également induire la synthèse de la TSLP, entraînant ainsi l'activation des ILC2 tout en leur conférant une résistance aux stéroïdes (Kabata et al., 2013). Une étude chez la souris a mis en évidence une autre voie d'activation des ILC2 : les leucotriènes. Les allergènes peuvent activer les ILC2 pulmonaires via le récepteur aux leucotriènes présent à leur surface, régulant ainsi la production cytokinique et la prolifération des ILC2 (Doherty et al., 2013). En complément, d'autres études *in vivo* ont montré que des souris asthmatiques sans ILC2 ont une inflammation moins sévère, une réduction de l'éosinophilie ainsi qu'une diminution de la production d'IL13 (Gold et al., 2014; Halim et al., 2012, 2014). De plus, dans un modèle murin d'asthme, les ILC2 représentent plus de la moitié des cellules produisant les cytokines de la réponse de type 2 telles que l'IL5 et l'IL13 (Klein Wolterink et al., 2012). Ces productions cytokiniques par les ILC2 entraînent notamment une hyperactivité bronchiale, une éosinophilie, une réparation tissulaire ainsi que l'activation de mastocytes dans certains cas (Lambrecht and Hammad, 2015).

Les ILC2 pourraient être une cible thérapeutique, notamment en inhibant leur activation par un Ac bloquant anti-TSLP (Gauvreau et al., 2014) ou en ciblant CRTH2 par le fevipiprant (Gonem et al., 2016).

Rhinosinusite chronique

La rhinosinusite chronique (RSC) est une pathologie courante qui est définie comme une inflammation du nez et des sinus para-nasaux. Certains patients ont également des polypes nasaux, qui sont des excroissances sur la muqueuse qui tapissent les fosses nasales et les sinus. Les patients atteints de RSC ont un taux d'ILC2 plus important dans les tissus nasaux comparé aux sujets sains (Ho et al., 2015). Plus précisément, des patients atteints de RSC avec des polypes nasaux ont un infiltrat cellulaire, majoritairement composé d'ILC2, plus important en comparaison à des patients atteints de RSC sans polypes nasaux (Nagarkar et al., 2013). Tous les sous-types d'ILC sont présents dans les polypes nasaux mais les ILC2 y sont majoritaires. Elles expriment fortement ICOS et produisent de grande quantité d'IL5 et d'IL13 (Poposki et al., 2017). Ces observations couplées au fait que l'activité de la TSLP est augmentée dans la RSC (Nagarkar et al., 2013) suggèrent un rôle des ILC2 dans la physiopathologie de cette maladie. Le nombre d'ILC2 dans les polypes nasaux d'un patient traités aux corticostéroïdes diminue, ouvrant potentiellement une nouvelle voie thérapeutique pour la RSC (Walford et al., 2014).

Implication des ILC2 dans la fibrose de la ScS

Comme dit précédemment, des modèles murins ont permis de mettre en évidence que les Th2 n'était pas un acteur primordial pour l'établissement et le développement de la fibrose (Hams et al., 2014). Nous avons également démontré une importance majeure des cellules immunitaires innées, telles que les neutrophiles et les macrophages, dans la réparation tissulaire (Laurent et al., 2017).

De plus, il a été suggéré que l'IL-33 contribue à la fibrose cutanée et pulmonaire (Rankin et al., 2010) et, plus important encore, qu'il joue un rôle clé dans le développement de la ScS (Yanaba et al., 2011). Étant donné la capacité de l'IL-33 à induire la différenciation des ILC2 (Neill et al., 2010; Spits and Di Santo, 2011a), plusieurs groupes ont étudié leur rôle potentiel dans la

fibrose. Dans des modèles animaux de fibrose pulmonaire induite par la bléomycine, l'IL-33, en plus de déclencher la polarisation des M2, induit à la fois l'expansion des ILC2 et la production d'IL-13 par les ILC2 contribuant à la fibrose (Li et al., 2014). Dans des modèles murins de fibrose hépatique induite par CCL4, l'IL-33 a également été impliquée dans l'expansion des ILC2. L'activation des ILC2, via une voie pSTAT6 dépendant, menait à des taux élevés d'IL-13 entraînant l'établissement de la fibrose hépatique (McHedlidze et al., 2013). En plus de l'IL-33, l'IL-25 a été décrite comme favorisant le développement et la différenciation des ILC2 (Neill et al., 2010; Saenz et al., 2010; Spits and Di Santo, 2011b). Hams et al, ont pour la première fois caractérisé *in vivo* le rôle des ILC2 activées par l'IL25 dans la fibrose pulmonaire via un mécanisme IL13 dépendant (Hams et al., 2014).

Dans le contexte de la ScS, des études ont rapporté des taux élevés d'IL-33 (Yanaba et al., 2011) et d'IL-25 (Lonati et al., 2014) chez des patients ScS ainsi que de la TSLP (Christmann et al., 2013; Truchetet et al., 2016), autre cytokine importante pour les ILC2 (Kim et al., 2013) suggérant un rôle des ILC2 dans la ScS.

Une étude a montré que la fréquence des ILC2 était élevée à la fois dans le sang et dans la peau des sujets ScS comparés aux sujets sains. Le nombre d'ILC2 dans le sang et dans la peau est plus grand chez des patients atteints de ScS diffuse que chez des patients atteints de ScS limitée. Il est intéressant de constater que le nombre d'ILC2 circulants et cutanés est positivement corrélé à l'étendue de la fibrose cutanée, suggérant un rôle important des ILC2 dans l'établissement de la fibrose cutanée et leur potentielle utilisation comme biomarqueur de la fibrose cutanée chez les patients ScS (Wohlfahrt et al., 2016). Les ILC1 ont également été étudiées dans le cadre de la ScS. Les auteurs ont défini des sous-types d'ILC1 en fonction de l'expression de CD4 et de CD8. Les fréquences des ILC1 CD4+ et des ILC3 NKp44+ sont augmentées dans le sang des patients ScS tandis que celles des ILC2 et des ILC1CD4- restent inchangées. De par leur forte expression du récepteur à l'IL6, les ILC1 CD4+ sont considérées comme les cellules les plus sensibles à l'IL6 (Roan et al., 2016). Ces résultats contradictoires soulèvent la question majeure de ce que l'on entend par ILC.Les taux d'IL6 ainsi que de l'IL6R soluble sont élevés chez les patients ScS (Hasegawa et al., 1998; Khan et al., 2012; Needleman et al., 1992; Radstake et al., 2009b; Sato et al., 2001). Les ILC1 CD4+ sont très probablement une source importante d'IL6R soluble et peuvent donc amplifier les réponses inflammatoires en réponse à l'IL6 (Rose-John, 2012). Le Tocilizumab, un Ac monoclonal anti-IL6R, est actuellement en essai clinique chez des patients SSc (Elhai et al., 2013) et l'évaluation de son

impact sur la pathogenèse SSc ainsi que sur les ILC permettra une meilleure compréhension de la pathophysiologie de la ScS. Ces résultats contradictoires soulèvent la question majeure de ce que l'on entend par ILC. En effet, la définition et l'identification des différents sous-types d'ILC ne sont pas les mêmes dans ces deux études. Les cellules étudiées ne sont donc pas les mêmes, soulignant la nécessité d'une classification commune des ILC et de ses sous-types.
Le rôle des ILC2 activées par ces cytokines doit être davantage étudié à l'avenir pour considérer l'axe IL-33/IL-25/ILC2 comme un axe thérapeutique potentiel.

II) Objectifs de la thèse

Nous formulons l'hypothèse que l'immunité innée de type 2 joue un rôle majeur dans la composante fibrotique de la sclérodermie systémique.
Nous avons testé cette hypothèse avec le soutien d'un travail conceptuel parallèle qui a orienté certaines de nos expériences.
Cela a conduit à la réalisation d'un travail expérimental en deux parties :
Dans une première étude, j'ai étudié l'implication des ILC, et particulièrement des ILC2, dans la fibrose au cours de la ScS.
Dans une seconde étude, je me suis intéressée aux macrophages de type 2. De nombreuses études montrent un rôle indéniable de ces cellules dans la physiopathologie de la ScS mais les mécanismes précis de l'induction de cette polarisation macrophagique de type 2 (M2) ne sont pas déterminées. Dans cette étude, nous avons tenté de lier la vasculopathie, l'altération de la réponse immunologique et la fibrose afin de trouver un traitement unique pour les patients qui soit efficace simultanément contre ces trois aspects.

Tout au long de nos expérimentations, trois concepts ont particulièrement retenu notre attention :

- La **localisation** de la cellule immunitaire. Dans notre projet, nous nous sommes focalisés sur la peau des patients sclérodermiques. Même si, selon Gurtner et ses collègues, la réparation tissulaire est similaire d'un tissu à un autre (Gurtner et al., 2008), nous pouvons légitimement penser que le microenvironnement cutané est différent des autres tissus. Les kératinocytes sont des cellules spécifiques de la peau qui sont notamment impliquées dans la fibrose au cours de la ScS (McCoy et al., 2017).
- Le **contexte** dans lequel la cellule immunitaire est. Notre modèle est un contexte pathologique mêlant à la fois de l'auto-immunité et de la fibrose, menant à un microenvironnement particulier. Cette notion de contexte est extrêmement importante. Par exemple, l'asthme et la ScS ont beaucoup de mécanismes en

commun, que ce soient des molécules ou des cellules immunitaires impliquées dans ces deux maladies. Toutefois, les symptômes de ces pathologies sont très différents soulignant l'importance du contexte dans lequel une cellule immunitaire est.

- La **plasticité** de ces cellules : il est maintenant reconnu que les cellules immunitaires ont la capacité d'être plastique (Galli et al., 2011; Huang et al., 2001; Zhou et al., 2009). Nous avons également souligné l'importance de la plasticité au cours de la réparation (Laurent et al., 2017).

Nous avons donc attaché une attention particulière à ces trois éléments qui nous paraissaient très importants pour l'élaboration de nos expériences. Est-ce que ces trois éléments sont liés ? Est-ce que le changement de fonction de la cellule immunitaire au cours de réparation tissulaire peut-être lié au contexte ou/et à sa localisation ? Est-ce que l'un ou plusieurs de ces trois éléments changent au cours de la fibrose entraînant un signal différent pour la cellule ?
Tout au long des expériences effectuées, nous avons tenté de prendre en compte ces éléments et de les intégrer dans notre schéma expérimental.
Par exemple, nous avons cultivé les ILC2 et les macrophages au contact de cytokines ou de molécules impliquées dans la ScS, notamment au niveau cutané. Nous avons, dans la mesure du possible, utilisé des cellules sclérodermiques plutôt que des cellules provenant de donneurs sains. En effet, de nombreuses études ont montré des différences notamment pour les fibroblastes dans la ScS (Bhattacharyya et al., 2012). Nous avons également effectué des expériences *in vitro* en sensibilisant les cellules avec un signal puis dans un second temps nous avons ajouté une deuxième molécule afin de mimer au maximum ce qui pouvait se passer au sein de la peau au cours de la ScS.

PROJET 1 :

Implication des ILC2 dans la fibrose au cours de la sclérodermie systémique

I) Problématique

A ce jour, aucun traitement n'est totalement efficace contre la ScS et plus particulièrement contre la fibrose. La complexité et le peu de connaissances actuelles sur les mécanismes précis de cette maladie sont des limites à la recherche d'une thérapeutique efficace.

La réponse immunitaire de type 2 joue un rôle fondamental dans la physiopathologie de la ScS. Des études récentes ont démontré le rôle des ILC2 dans la fibrose hépatique et pulmonaire.

Le but de ce projet a été d'étudier ces cellules dans le cadre de la ScS. En effet, les ILC2 et les ILC en général font l'objet de peu d'études dans cette maladie. Hormis notre laboratoire, deux autres équipes ont étudié le rôle des ILC dans la ScS. L'équipe de Steven Ziegler s'est intéressée aux ILC1 et plus précisément aux ILC1 CD4+. Ils ont ainsi montré que ces cellules pouvaient participer et amplifier l'inflammation via l'IL6. L'équipe d'Andreas Ramming s'est, quant à elle, focalisée sur les ILC2 et a montré une augmentation des ILC2 à la fois dans le sang et dans la peau des ScS, le tout corrélé à l'étendue de la fibrose cutanée. Cette étude est uniquement observationnelle et ne donne pas d'explication quant à l'implication des ILC2 dans la fibrose de la ScS.

Ce nouvel acteur immunitaire représente potentiellement une nouvelle voie d'induction de la fibrose et pourrait constituer une future cible thérapeutique.

II) Objectif du projet

Ce projet de recherche alliant à la fois une partie conceptuelle et une partie expérimentale a pour but de renouveler la vision de l'immunité afin d'ouvrir potentiellement un nouveau champ, encore sous-estimé, de thérapies ciblant le système immunitaire. Comprendre précisément l'entière complexité de celui-ci, notamment dans le mécanisme de fibrose de la ScS, permettra éventuellement de concevoir des traitements efficaces.

A travers cette réflexion et compte tenu de l'importance de la réponse de type 2 dans la fibrose, nous avons voulu étudier le rôle des ILC2 dans ce mécanisme au cours de la ScS. Ce travail a été réalisé en collaboration avec l'équipe du Pr. Batteux du laboratoire d'immunologie EA 1833 de Cochin.
Dans le cadre de ce projet, j'ai réalisé les mises au point des marquages en cytométrie de flux sur le sang et la peau des sujets sains et des sujets sclérodermiques. J'ai effectué la purification et l'amplification des ILC2 *in vitro*. J'ai également évalué l'action des ILC2 sur les fibroblastes, que j'ai préalablement extraits et purifiés de la peau, en RTqPCR.
La partie du projet concernant l'immunofluorescence de la peau saine et ScS a été mise au point par Valérie Jolivel au sein du laboratoire. Les analyses ont ensuite été faites par la société QuantaCell.
La partie *in vivo* de ce travail concernant le modèle murin de sclérodermie a été réalisée par l'équipe du Pr Batteux au sein du laboratoire EA 1833 à Paris.

III) Résumé de l'article

La sclérodermie systémique (ScS) est une maladie auto-immune complexe caractérisée par des anomalies vasculaires, une dérégulation de la réponse immunitaire ainsi qu'une fibrose.

La réponse immunitaire de type 2 a un rôle clé dans de nombreuses maladies inflammatoires et auto-immunes incluant la ScS. De plus, des études récentes ont souligné le rôle fondamental des cellules lymphoïdes de type 2 dans la fibrose.

Dans cette étude, nous mettons en évidence une diminution des ILC circulants dans le sang des patients ScS, et plus particulièrement une baisse des ILC2 et des ILC3. Nous observons une corrélation inverse entre le taux d'ILC2 circulant et l'étendue de la fibrose cutanée définie par le score de Rodnan (mRSS). A l'inverse, nous montrons une augmentation des ILC2 dans la peau des malades à la fois en cytométrie de flux et en immunofluorescence. Le nombre d'ILC2 cutanées corrèle positivement avec le score de Rodnan chez les patients ScS. Un phénotypage des ILC2 cutanées a permis de mettre en évidence une diminution, chez les sujets ScS, de l'expression de KLRG1, marqueur permettant la discrimination entre deux sous-types d'ILC2 : les ILC2 inflammatoires KLRG1+ et les ILC2 natures KLRG1-. Cependant, les ILC2 expriment plus fortement KLRG1 dans la peau des patients atteints d'une ScS diffuse progressive comparé aux ILC2 dermiques des patients atteints de ScS diffuse quiescente suggérant un switch des iILC2 vers les nILC2 au cours de la maladie. Cette observation a été confirmé en utilisant un modèle murin de ScS dans lequel la fréquence des iILC2 KLRG1^{high} diminue dans la phase chronique de la maladie qui correspond à la ScS diffuse quiescente chez l'homme. Nos études *in vitro* ont permis de montrer que sous l'influence du TGFβ, les ILC2 perdent l'expression de KLRG1. Elles deviennent alors pro-fibrotiques en induisant la production de collagène par les fibroblastes de par l'induction d'une diminution de la production d'IL10 comparé aux iILC2.

Ainsi l'ensemble de ce travail montre que par son action sur les ILC2, le TGFβ peut favoriser indirectement la fibrose au cours de la ScS via un axe KLRG1/IL10.

TGFβ-modulation of ILC2 function in SSc participates to the switch from inflammatory to fibrotic process

Paôline Laurent,[1] Mohamed Jeljeli[5], Pauline Manicki,[2] Valérie Jolivel,[1] Pauline Henrot[2]……, Christophe Richez,[1,2] Pierre Duffau,[1,3] Estibaliz Lazaro,[1,3] Thomas Pradeu[1], Patrick Blanco,[1,4] Frédéric Batteux[5], Cécile Contin-Bordes,[1,4*] Marie Elise Truchetet[1,2*]

[1]ImmunoConcEpt, UMR 5164, University of Bordeaux
[2]Rheumatology department, CHU Bordeaux Hospital, Bordeaux, France
[3]Internal Medicine department, CHU Bordeaux Hospital, Bordeaux, France
[4]Immunology department, CHU Bordeaux Hospital, Bordeaux, France
[5]Immunology department CHU Cochin Hospital, Université Paris Descartes, Paris France
* Equally contributors

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Corresponding author (ORCID, email, and full mailing address): Marie-Elise Truchetet, UMR 5164 CIRID, Université de Bordeaux, 146 rue Leo Saignat, 33076 Bordeaux, France
Phone: +33 5 57 57 92 46 Fax: +33 5 57 57 14 72 Email: marie-elise.truchetet@chu-bordeaux.fr

ABSTRACT:

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Objectives: Type 2 responses and innate immunity are thought to be involved in fibrotic processes and especially in systemic scleroderma. We aimed to study the potential role of ILC2 in this complex model of fibrosis.

Methods: Blood sampling and skin biopsies were performed in fibrotic skin for SSc patients and in pieces of brachioplasty for HD. ILCs sub-populations were measured by flow cytometry in whole blood or in CD45+ cells purified from skin biopsies. Immunofluorescence staining and semi-automatic cell count were performed on skin biopsies. Daily injection of HOCl in mice was used as systemic sclerosis mice model. Starting from HD peripheral blood, ILC2 were sorted and cultured for in vitro experiments.

Results: 73 SSc and 59 HD were prospectively enrolled at the University Hospital of Bordeaux. We showed that patients exhibited fewer ILC2 in blood. They were over-represented in the skin and positively correlated to the level of fibrosis. At the early inflammatory stage of the disease, the cutaneous ILC2 showed a stronger expression of KLRG1. In the HOCl-induced fibrosis mice model, KLRG1^{high}-ILC2 decreased at the late fibrotic stage compared to inflammatory, which indicates a switch from iILC2 to nILC2 during the fibrotic process. In vitro, KLRG1 expression in ILC2 was dramatically decreased by TGFβ. TGFβ-induced KLRG1^{low}-ILC2 had a profibrotic effect on fibroblast. This effect is mediated by a decrease of IL10 secretion by ILC2.

Conclusion: We show a new pathway by which TGFβ could indirectly modulate fibrosis via ILC2 polarization from iILC2 to nILC2.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease characterized by the occurrence of an extensive fibrosis (1). While adaptive immune system has long been considered as the main actor of SSc dysimmunity, recent observations established an important role of the innate immunity (2, 3).

Innate lymphoid cells (ILC) were recently described as important players of the innate immune system (4). The non-cytotoxic ILCs are further classified into three distinct subsets based on their cytokine production and transcription factor expression; type 2 ILCs (ILC2) are dependent on GATA3, and TSLP, IL25, and IL33 have been demonstrated as their main homeostatic factors (5). ILC2 mostly secrete IL5 and high amounts of IL13 upon stimulation (6).

In addition to mediate IgE class switching and inhibit proinflammatory cytokines, IL13 is recognized as an important mediator of fibrosis (7). Animal studies, using IL13 or IL4 transgenic mice, have suggested an even more critical role of IL13 in fibrosis over IL4 (8). Because of the unique ability of ILC2 to produce high amounts of IL13, their role in fibrosis genesis has been under scrutiny in different animal models. Hams *et al*, have characterized the role of IL25-activated ILC2 in pulmonary fibrosis *in vivo* via their adoptive transfer or specific deletion in a *Schistosoma mansoni*-induced model via an IL13 dependent signaling (9). IL33-dependent ILC2 were shown to participate to the establishment of hepatic fibrosis through the activation of pSTAT6 signaling via IL4Ra, and the production of high levels of IL13 (10).

Increased levels of circulating IL25 and IL33 have been observed in SSc conditions (11, 12), and our group found that thymic stromal lymphopoietin (TSLP) was increased in SSc and correlated to skin fibrosis (13).

ILC2 have been divided into two separate clusters responding differentially to the microenvironment. Inflammatory ILC2s (iILC2) respond to IL25 and produce IL17 in addition to IL13. Nature ILC2s (nILC2) respond to IL33 and release huge amount of IL13. The differential expression of KLRG1 (killer cell lectin like receptor G1) has been identified as a marker of this sub-division; iILC2 being KLRG1^{high} and nILC2 KLRG1^{low} (14), while its relevance in pathogenic conditions remains elusive.

In this study, we have not only demonstrated the potential role of ILC2 in the fibrotic process of SSc, but we have also shown that ILC2 may switch from iILC2 to nILC2 during the natural history of SSc, in a TGFβ dependent manner, leading to a diminished IL10 secretion and a pro-fibrotic phenotype.

METHODS

Study population

Individuals with SSc presenting at the university hospital of Bordeaux, France, were prospectively included in the study between March 2014 and September 2016. All patients satisfied the classification criteria proposed by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) 2013 (15). Patients were included in the context of the VISS (Vasculopathy and Inflammation in Systemic Sclerosis) biomedical research project founded in 2012 and approved by the institutional ethical committee (CPP, 2012-A00081-42, Aquitaine). All participants provided written informed consent before inclusion. For each patient, a disease- and organ-specific questionnaire was completed by the clinician in charge of the patient and then centralized by investigators. Clinical features (scleroderma form; sex; age at Raynaud's phenomenon (RP) onset; age at onset of the first non-RP manifestation; disease duration; and symptoms of skin, articular, heart, lung, kidney, and gastrointestinal involvement), immunologic test results (antinuclear antibodies, anti-Scl70 antibodies, and anti-centromere antibodies), imaging and functional exams (thorax CT scans, respiratory functions tests, cardiac ultrasonography and right heart catheterization) and treatments were recorded. For modified Rodnan Skin Score (mRSS) and Right Ventricle Systolic Pressure, the highest value of the medical history was registered for each patient. Interstitial lung disease was diagnosed when pulmonary function tests showed a restrictive defect with decreased diffusion capacity (DLCO) associated with several types of lesions on the thorax CT scans. Lung fibrosis was diagnosed based on specific lesions observed on the thorax CT scans, i.e., honeycomb cysts and reticular septal thickening. Punch biopsy specimens (3-4 mm) of affected mid-forearm skin were obtained for some patients. Age- and sex-matched healthy donors (HDs) were recruited at the local Blood Transfusion Centre (University Hospital, Bordeaux) for blood tests. For control skin, biopsy specimens were isolated from skin that had been discarded during plastic surgery (brachioplasty). None of the healthy individuals had dermatological disorders or were under immunosuppressive agents/glucocorticoids.

Mice

Six-week-old female BALB/c mice were purchased from Janvier Laboratory (Le Genest Saint Isle, France). Animals received human care in compliance with the guidelines implemented at

our institution (INSERM and Paris Descartes University, Ethics committee CEEA CN.023.11). All mice were housed in ventilated cages with sterile food and water ad libitum.

Animal model and experimental design

All chemicals were obtained from Sigma-Aldrich. Mice were randomly distributed into experimental (11 mice per group) and control group (10 mice/group). SSc was induced according to the protocol described by Kavian *et al.* (16). This model mimics the major human SSc features, as mice displayed the sequential development of inflammation and fibrosis of the skin and visceral organs (lung and kidney) with autoimmunity and vascular involvement (17). Therefore, at day 42, late fibrotic stage of the model is reflecting an established disease while on day 21 the model corresponds to the inflammatory stage of the disease. We induced the disease in BALB/c mice through daily injection of HOCl by local injection. A total of 300 µl of an hypochlorous acid (HOCl) solution was prepared extemporaneously by adding a NaClO solution (9.6% active chlorine) to 100 mM of KH_2PO_4 (pH 6.2) and injected intradermally into the shaved backs of mice (one injection of 150 µl in each flank), using a 27-gauge needle, every day for 6 weeks (HOCl-mice) and sacrificed at weeks 3 (apex of the inflammatory phase) and 6 (apex of the fibrotic phase). Control mice received injections of 300 µl of sterilized PBS (PBS-mice). Fibrosis induction was assessed by weekly measurement of dermal thickness, histopathological analysis, measurement of hydroxyproline content in lung and skin, determination of cutaneous and pulmonary IL-13, TGFβ, Collagen type I production by Polymerase Chain Reaction and detection of anti-DNA topoisomerase I in sera as previously described (16).

Isolation of ILC

In human experiments, blood samples (5mL) were incubated with red blood lysis (using 1X Buffer Lysis, Miltenyi) during 10 min at room temperature. After centrifugation (10 min, 1500 rpm), the staining was performed. Skin biopsy from SSc patients and healthy subjects were digested with collagenase (5mg/ml, Roche) and liberase (40µg/mL, Roche) in HBSS at 37°C for 3 hours in shaker and filtered through a 0.70 µm nylon mesh.

In mice experiments, cell suspensions from collected spleens were prepared after hypotonic lysis of erythrocytes in potassium acetate solution and filtration through a 40-µm strainer. Skin was taken from the back region of mice with a 6-mm-diamter punch (5 punches per mouse) and lung pieces from each mouse were diced using a sharp scalpel and then put 6 well plates with 1ml of Dulbecco's modified Eagle's medium containing 1% nonessential amino

acids, 1% L-glutamine, 1% sodium pyruvate, 50 units/ml penicillin, 50 mg/ml streptomycin, and 10% fetal calf serum (cDMEM, GIBCO). A mixture of collagenase (12,5 mg/mL, Roche) and liberase (100µg/mL, Roche) was added in each well. After digestion of 5 hours at 37°C, the remaining tissue was passed through a 70-µm strainer (VWR) and washed with cDMEM solution. After spinning, the pellet was re-suspended in cold-DMEM and passed through a 40-µm strainer.

Purification and amplification of ILC2

The PBMCs are isolated from healthy donor blood by Ficoll. After NK enrichment (NK cell isolation kit, Miltenyi Biotec), ILC subsets were sorted using ARIA FACs (BD). For ILC2 sorting, the following fluorochrome-conjugated were used: anti-CD1a (BL6), anti-CD3 (UCHT1), anti-CD11c (BU15), anti-CD14 (RMO52), anti-CD16 (3G8), anti-CD19 (J3-119), anti-CD34 (581), anti-CD94 (HP-3B1), anti-CD123 (AC145), anti-CD45 (J33), anti-CD117 (104D2D1), anti-CD127 (R34.34) and anti-CRTH2 (BM16) were obtained from Beckman Coulter. Anti-CD5 (UCHT2) was obtained from BD. Anti-FcεR1 (AER-37) was purchased from eBioscience and anti-CD31 (AC128) from Miltenyi Biotec. Enriched cells were incubated with APC-labeled anti-lineage (Lin) (CD1a, CD3, CD5, CD11c, CD14, CD16, CD19, CD31, CD34, CD94, CD123, FcEpsR1), KO-labeled CD45, APC/AF700-labeled CD127, PE/Cy5.5-labeled CD117 and FITC-labeled CRTH2. As previously described, ILC1 as $CD45^+Lin^-CD127^+CD117^-CRTH2^-$ cells, ILC2 as $CD45^+Lin^-CD127^+CRTH2^+$ cells, ILC3 as $CD45^+Lin^-CD127^+CD117^+CRTH2^-$ cells (18).

For RTqPCR analysis of transcription factors, 6 different donors were mixed at the same time. After sorting, the different subtypes of ILC were separated into two fractions: one part was for the study of transcription factors at day 0 (DO) while the second part was amplified during 20 days for the study of transcription factors at day 20 (D20).

For the ILC2 stimulation experiments, only the ILC2 of one donor were enriched and purified before being amplified for 20 days.

Once purified, cells are cultured in Roswell Park Memorial Institute medium containing 1% nonessential amino acids, 1% L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, and 8% fetal calf serum (cRPMI, GIBCO) in a 96-well round bottom plate in the presence of IL1β (R&D System) and IL2 (Miltenyi Biotec) both at a concentration of 10ng/ml, as previously described (19). The change of medium and the addition of cytokines is done every two days.

Stimulation of ILC2

The ILC2s were amplified for about 20 days. At the end of amplification, the cells were harvested and washed. 5.10^5 ILC2 were stimulated for 48 hours under following conditions: IL33 (Milteny Biotec), TGFβ (R&D System), IL4 (Miltenyi Biotec), TSLP (R&D System) and IL25 (Miltenyi Biotec). All these cytokines were added at a concentration of 10ng/ml. At the end of the stimulation, the supernatants were harvested and frozen at -80°C while the cells were stained.

Extraction and incubation of fibroblasts

Fibroblasts were obtained from skin lesion biopsy samples from healthy donors (20). Briefly, skin biopsy specimens were digested with 0.1% type IA collagenase at 37°Cfor 2 hours. Adherent cells were grown in cDMEM. Fibroblasts were used between the third and sixth passages.

3.10^5 fibroblasts were cultured in duplicate in a 96-well flat-bottom plate and incubated with non-stimulated ILC2 supernatants or TGFβ-stimulated ILC2 supernatants for 24 hours. TGFβ was used as a positive control of experiment and incubated with fibroblasts at a concentration of 10 ng/ml. After 24h, the supernatants were harvested and placed at -80°C and the fibroblasts were collected with a lysis buffer.

Flow Cytometry

For human sample staining, the following fluorochrome-conjugated were used for flow cytometry. Anti-CD3 (BW264/56), anti-CD5 (UCHT2), anti-CD14 (TUK4), anti-CD16 (REA423), anti-CD19 (LT19), anti-CD31 (AC128), anti-CD34 (AC136), anti-CD45 (5B11), anti-CD56 (AF12-7H3), anti-CD94 (REA113), anti-CD123 (AC145), anti-CD303 (AC144), anti-TCR αβ (BW242/412), anti-TCR γδ (11F2), anti-CD117 (A3C6E2), anti-CD127 (MB15-18C9) and anti-CLA (HECA-452) were obtained from Miltenyi. Anti-CCR10 (1B5), anti-CCR6 (11A9), anti-HLA-DR (L243) and anti-CRTH2 (BM16) were obtained from BD. Anti-FcεR1 (AER-37) and anti-KLRG1 (13F12F2) were purchased from eBioscience and anti-αSMA (1A4) from R&D systems. Anti-OX40L (ANC10G1) was obtained from Abcam.

Blood samples were incubated with APC-labeled anti-Lin (CD3, CD5, CD14, CD16, CD19, CD31, CD34, CD56, CD94, CD123, CD303, FcEpsR1,

TCR αβ and TCR γδ), APC/Cy7-labeled CD45, PE/Cy5-labeled CD127, PE/Cy7-labeled CD117, BV421-labeled CRTH2 and FITC-labeled CLA. Skin biopsy from sclerodermic patients and healthy subjects were stained with same antibodies than blood staining, adding APC-labeled αSMA, PE-labeled OX40L, PerCP/Cy5.5-labeled CCR10, BV786-labeled CCR6, PerCP/Cy5.5-labeled HLA-DR, FITC-labeled KLRG1 and PE-labeled TSLPR. As previously described, ILC were defined as $CD45^+Lin^-CD127^+$ cells, ILC1 as $CD45^+Lin^-CD127^+CD117^-CRTH2^-$ cells, ILC2 as $CD45^+Lin^-CD127^+CRTH2^+$ cells, ILC3 as $CD45^+Lin^-CD127^+CD117^+CRTH2^-$ cells (18). For *in vitro* experiments, after 48h stimulation, approximately 10^5 cells were collected and incubated with the following anti-human antibodies: FVS 510 (from BD), PE/Cy5-labeled CD127, BV421-labeled CRTH2 and FITC-labeled KLRG1 (13F12F2, purchased from eBiosciences).

For mice experiments, approximately 10^6 collected cells of spleen, skin and lung were labeled by the following anti–mice antibodies (purchased from Miltenyi unless stated otherwise): viability fixable dye, Lin negative antibody (containing CD5, CD3ε, anti-GR1 (Ly6G/C), CD45R (B220), CD11b, 7-4 and Ter-119 antibodies), CD117 (c-Kit, REA791), CD25 (IL-2Rα, REA568), CD45 (REA737), KLRG1 (2F1), CD127 (IL-7Rα, REA680) and ST2 (IL-33R, DIH9 purchased from Biolegend). Cells were first gated for small/non-granular (FSC^{low}/SSC^{low}) and live (viability fixable dye positive) leukocytes (CD45+). ILC2 are identified as Lin-, CD117 (c-Kit)-, ST2+, CD25 (IL2-Rα)+, and CD127+. ILC2 were then divided into $KLRG1^{high}$ and $KLRG1^{low}$ populations.

In general, percentages of ILC2 were calculated as percentage of lymphoid morphology CD45+ Lin- and CRTH2+ cells. Absolute values of ILC2 (for blood) were calculated as: % of ILC2 * absolute lymphocyte count (G/L)/100.

Cells were analyzed using Fortessa flow cytometer with FACSDiva software (BD) and data analysis was performed with FlowJo 10.1 software.

Tissue processing and immunofluorescence

As described previously (20), all samples were fixed in 3.8% formalin, dehydrated with graded concentrations of alcohol and finally embedded in paraffin. The tissue blocks were cut into 3 µm-thin sections with a microtome (Leica RM2255). Sections were floated in a 37°C water bath, mounted on electrostatically charged adhesion slides (SuperFrost Plus, Thermo Scientific) and dried on a heating plate for 2 hours at 56°C. Sections were dewaxed with xylene and rehydrated in decreasing concentrations of ethanol. Using the PT link instrument (Dako), a heat-induced antigen retrieval (HIER) step was performed by immersion of the

samples into a citrate buffer, pH6 (PT Module Buffer 1, Thermo Scientific) for 20 minutes at 97°C. Then, in a humidified incubation box, the sections were saturated with a blocking buffer (PBS 10mM pH7.4, 0.1% Triton X-100, 5% Normal Goat Serum (Thermo Scientific)) during 1hr at room temperature. The sections were subsequently incubated overnight at 4°C with the following antibodies diluted in the blocking buffer: rabbit anti-human CRTH2 (GPR44, Novus Biologicals), mouse anti-human CD3 (PS1, Abcam), mouse anti-human CD11b (CL1719, Novus Biologicals), mouse anti-human FcER1 (9E1, Thermo Fisher). Negative controls were systematically obtained by omitting the primary antibodies. After washing thoroughly with PBS, secondary antibodies coupled to fluorescent dyes (goat anti-rabbit AF488, Invitrogen and goat anti-mouse AF568, Invitrogen) were applied on the tissue sections for 1.5hrs at room temperature, in the dark. Nuclei were counterstained with DAPI. The slides were mounted with ProLong Gold Antifade Mountant (ThermoFisher Scientific). Whole tissue slices were scanned using the Nanozoomer 2.0HT (Hamamatsu) and 3-D acquisitions were realized with the confocal microscope SP5 (Leica).

Image extraction and cell count

Acquisition has been done with the NanoZoomer from Hamamatsu. For each acquired images, the user has visualized the ndpi files using "NDP.view2" software (Hamamatsu). Interesting parts of the virtual slides were manually selected using regions (ROI). The following steps were developed using QuantaCell homemade software solutions (Matlab scripts and C++/OpenCV programs). Virtual images were automatically cropped inside user-defined region and to export them into jpg files at a 20x resolution. A semi-automatic sample detection was performed: gray image (named im_gray) was calculated from original images (named im_orig), im_gray was filtered with morphological mathematics to make it homogeneous, automatic threshold value (named th) was calculated on im_gray using Otsu method.Manual correction of th was possible with user intervention. A binary mask (named mask_sample) was calculated using th on im_gray. In mask_sample, small objects <7500µm² were removed. Semi-automatic epiderma detection was performed. Green images (named im_green) was extracted from im_orig, im_green was filtered with morphological mathematics to make it homogeneous. Automatic threshold value (named th_green) was calculated on im_green using Otsu method. Manual correction of th_green was possible with user intervention. A binary mask (named mask_epiderma) was calculated using th_green on im_green (mask_epiderma was restricted inside mask_sample). If several objects were present in mask_epiderma then the object the closest to the sample border were kept (in order

to separated epiderma from internal green islet). A manual procedure was sometime necessary to redraw manually mask_epiderma. It was useful for very inhomogeneous epidermis.

Automatic cell quantification was performed on the processed images. Blue images (named im_blue) was extracted from im_orig. Automatic nuclei detection was done to individualize each nuclei from im_blue. It was achieved using image smoothing (to remove noise), binarization, a watershed strategy to separated contiguous nuclei, and the rejection of too small nuclei. The obtained image of nuclei is named nuclei_mask. A donut shape with a distance of 3.5µm was generated around the nuclei to define the cytoplasmic area. Cell mask (named cell_mask) is the union if nuclei_mask and the donut shape mask. Intensity statistics using average among pixel intensities were calculated: for each cell in the whole cell (nuclei + cytoplasm), for each fluorescent channel, for each cell the closest distance to the epidermis is calculated, for each cell, the position in the tissue is recorded (in the epidermis or in the "epi-dermis islet" or in the dermis). Cell classification is done according to 3 parameters (i) cell is in epidermis or in dermis, (ii) cell is positive in red or negative in red and/or green (red positivity threshold is calculated as the median value for all cells of the sample + 10 fluorescent intensity units, green positivity threshold is calculated as the median value for all cells of the sample + 50 fluorescent intensity units), (iii) cell is positive in green or negative in green. The following class counts are measured: cell count in epidermis, green positive cell count in epidermis, red negative cell count in epidermis, green positive and red negative count in epidermis, cell count in dermis, green positive cell count in dermis, red negative cell count in dermis, green positive and red negative count in dermis. Statistics are exported in Excel files for further exploitation. Montage images are automatically generated to represent: the sample boundaries, the epidermis and the cell classes.

Cells were considered as positive ILC2 when having a nucleus surrounded by green fluorescence without any red.

Reverse Transcription quantitative PCR (RTqPCR)

RNA was purified from ILC subsets and fibroblasts using RNeasy Plus Micro Kit (Qiagen). RNA concentration and purity were assessed using the Spectrophotometer DS11 (Denovix). RNA integrity number (RIN) was assessed using Agilent 2200 TapeStation (Agilent Technologies). All procedures were performed according to the manufacturer's instructions.

Total RNA was converted to cDNA using GoScript Reverse Transcription (Promega^{TM}). qPCR was performed using GoTaq Master Mix (all reagents were purchased from

Promega™). The following targets were analyzed: Tbet, GATA3, RORγt, Col1A1, MMP1 and CCL2 (see in details in Table 2). mRNA levels were normalized to 18s.

Samples were distributed in duplicate in a 384-well plate using Epmotion 5073 automated pipetting system (Eppendorf). Real-time quantitative PCR were performed using thermocycler CFX384 (Bio-Rad™).

The data were analyzed using Bio-Rad™ CFX Manager software (Bio-Rad™) and differential expressions were evaluated according to the method of ΔΔCt.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (La Jolla, CA). For populations who satisfied the Kolmogorov–Smirnov normality test, a two-tailed Student's t-test for unpaired or paired samples and one-way repeated-measures ANOVA test followed by the Bonferroni correction were used to compare the different populations according to the experimental design. When the normality test was not satisfied, the Mann-Whitney, Wilcoxon and Kruskal Wallis tests were used. Correlations were analyzed using the Spearman test. A p-value <0.05 was considered statistically significant.

RESULTS

Blood circulating ILC2 are decreased and correlates to skin fibrosis in SSc patients

To better understand the potential contribution of ILC2 in SSc pathogenesis, we first monitored total ILC population and subpopulations in the whole blood of SSc patients (SSc, n=73) and age- and sex-matched healthy donors (HD, n=59).

The frequency as well as the absolute number of total ILC (defined as Lin-CD45+CD127+, Fig. 1A), were decreased in SSc patients compared to HD (0.04 ± 0.02% *vs.* 0.09 ± 0.07%, p<0.0001 and 0.0009 ± 0.0003 *vs.* 0.002 ± 0.001, p=0.0004; Fig. 1B and supplementary Fig. 1A respectively). Considering the subpopulation we observed that 17.3% of circulating ILCs were ILC2 in HD *vs*. 15% in SSc whereas 52% of circulating ILCs were ILC1 in HD *vs.* 74% in SSc and 25% were ILC3 *vs.* 11% in SSc (Fig. 1C).

While circulating ILC1 (defined as Lin-CD45+CD127+CRTH2-CD117-, Fig. 1A) frequency and absolute number were similar in SSc patients and HD (0.033 ± 0.019% *vs.* 0.039 ± 0.025% and 0.0006 ± 0.0003 *vs.* 0.0008 ± 0.0004, Fig. 1D and supplementary Fig. 1B respectively), ILC2 (defined as Lin-CD45+CD127+CRTH2+, Fig. 1A) frequency and absolute number were significantly lower in SSc than in HD (0.007 ± 0.007% *vs.* 0.01 ± 0.01%, p=0.001 and 0.0001 ± 0.000009 *vs.* 0.0003 ± 0.0001, p=0.0006, Fig 1E and supplementary Fig. 1C respectively). The ILC3 frequency and absolute number were also significantly decreased in peripheral blood of SSc compared with HD (0.005 ± 0.006% *vs.* 0.03 ± 0.03%, p<0.0001 and 0.000009 ± 0.0001 *vs.* 0.0004 ± 0.0003, p<0.0001, Fig. 1F and supplementary Fig. 1D respectively).

In order to evaluate the clinical relevance of these observations, we correlated ILC parameters to different clinical data of specific interest in SSc. We observed an inverse correlation between the fibrotic skin score (as defined by the modified Rodnan skin score, mRSS) and the frequency or absolute number of ILC2 (Spearman r=-0.35, p=0.0062 and supplementary Fig. 1E, spearman r=-0.39, p=0.01, Fig. 1G). No correlation was observed between neither ILC1 nor ILC3 frequencies and mRSS (supplementary Fig. 1F and data not shown). Pulmonary fibrosis or vasculopathy were not associated with the ILC frequencies or absolute values (data not shown). The percentage of CLA (cutaneous lymphocyte-associated antigen)-expressing ILC2 was specifically reduced in SSc patients compared to HD (6.043 ± 1.707 % *vs.* 16.06 ± 3.409 %, p=0.0003; Fig. 1H). Collectively, these data demonstrate the SSc phenotype is associated with significant decrease in the frequency of circulating ILC2, which is correlated with skin fibrotic.

Clinical parameters are associated to preferential dermis *vs.* epidermis localization of ILC2 in SSc patients.

The decreased number in the blood of patients and the correlation between circulating ILC2 and skin fibrotic score prompt us to analyze ILC content in SSc skin biopsies. In order to gain more insight into the *in situ* quantification and the distribution of ILC2, we performed immunofluorescence analysis of the SSc and HD skin biopsies. As a lineage marker, we used a cocktail of anti-FcεRI, anti-CD3 and anti-CD11b antibodies, while anti-CRTH2 was chosen as a detection marker for ILC2; Lin-DAPI+CRTH2+ being considered as ILC2 (Fig. 2A). In order to ensure an objective and reproducible measurement, the entire skin sections were scanned and analyzed through an automated process allowing a separate count of the cells in the epidermis and dermis. Seventeen healthy subjects and 30 SSc patient skin sections (representative staining for HD, Fig. 2A and SSc patient, Fig. 2B) were included. Analysis of ILC2 distribution and quantification revealed that the number of ILC2 per surface area (mm^2) was increased in SSc patients compared to HD in both epidermis and dermis (respectively 448 ± 376 *vs*. 200 ± 280 and 5.6 ± 7.2 *vs*. 1.1 ± 1.8 in, p=0.02 and 0.01, Fig 2C and 2D). We also found a significant increased proportion of ILC2 in SSc compared to HD in epidermis (0.09 ± 0.07 *vs*. 0.05 ± 0.05, $p<0.05$, Fig. 2E) that was even more pronounced in the dermis (0.7 ± 0.8 *vs*. 0.15 ± 0.19, $p<0.005$, Fig. 2F).

ILC2 are differentially distributed through epidermis and dermis both in HD and SSc. Comparing epidermis *vs.* dermis, in HD and SSc patients, the number of ILC2/mm^2 was significantly higher in the epidermis than in the dermis (200 ± 68 *vs*. 1.1 ± 0.4 and 448 ± 68 *vs*. 5.6 ± 1.3 in HD and SSc respectively, p=0.004 and <0.0001, Fig. 2C and D). When looking at ILC2/cell on the contrary, they were significantly lower in epidermis than in dermis especially in SSc patients (0.04 ± 0.01 *vs*. 0.1 ± 0.04 and 0.08 ± 0.01 *vs*. 0.7 ± 0.1 in HD and SSc respectively, p=0.04 and 0.0001, Fig. 2E, 2F and 2G).

Only the number of ILC2/mm^2 in the dermis was positively correlated with skin fibrosis assessed by mRSS at the time of biopsy (Spearman r=0.5, p=0.006 and spearman r=0.16, p=0.4 respectively, Fig. 2H and 2I). In addition, dermal ILC2s in the skin appeared to be geographically close to vascular structures but their number was not increased in patients. Interestingly, only patients presenting with digital ulcers shows an increased proportion of t epidermal ILC2s over dermal ones (322 ± 80 *vs*. 575 ± 103 and, p=0.04, Fig. 2J).

Overall, these results show that ILC2 are increased in SSc skin, with a different repartition (dermis vs epidermis) related to clinical parameters.

KLRG1^{high} ILC2 are recruited in the skin of scleroderma patients at inflammatory stage

Total ILCs and subpopulations were quantified in skin samples of SSc patients or HD, after enzymatic digestion, by flow cytometry. A representative staining depicting the gating strategy of ILCs and subpopulation categorization is shown in Fig 3A. We found that the percentage of total ILCs was increased in SSc skin extract compared to HD (6.639 ± 3.5 % *vs*. 4 ± 2.1 % of CD45+ cells, p=0.01, Fig. 3B), 69% of ILCs being ILC2 in HD *vs*. 77% in SSc patients. In the meantime, 27% of ILCs were ILC1 in HD *vs.* 19% in SSc (Fig. 3C). ILC3 were undetectable in the skin.

Subtype analysis revealed a significant increase in the ILC2 in SSc skin biopsy compared to HD (5.015 ± 2.8 % *vs*. 2.816 ± 1.8 % of CD45+ cells, p=0.008, Fig. 3D). ILC1 frequency among CD45+ cells was similar in SSc and HD skin (1.29 ± 0.8% *vs*. 1.07 ± 0.7% of CD45+ cells, Fig. 3E) even if their proportion among ILCs was decreased in SSc compared to HD (Fig. 3C). This demonstrated that the increase of ILC2 among skin CD45+ cells reflects not only the overall increase in ILC but also an imbalance in the proportion of the different ILC subtypes within SSc skin. The increased percentage of ILC2 among CD45+ skin cells correlated to cutaneous fibrosis measured by the mRSS (Spearman r=0.58, p=0.01, Fig. 3F). No correlation was observed between ILC1 frequency and mRSS (Fig. 3G).

We next extensively phenotype the ILC2 skin infiltrate and did not detect any difference in the percentage of ILC2 cells expressing HLA-DR, OX40L, CCR6, CCR10, CLA and TSLPR in SSc skin biopsies compared to healthy skin (Fig. 3H). Notably, we found a significant decrease in KLRG1+ ILC2 in SSc skin compared to healthy skin (13.76 ± 6.55 % *vs*. 3.07 ± 1.8 % of ILC2, p=0.05, Fig. 3H). Interestingly, we observed a more intense expression of KLRG1 (KLRG1^{high}) in rapidly progressive diffuse cutaneous SSc (inflammatory phase of the disease) than in late diffuse cutaneous SSc (sclerotic phase of the disease) or in HD (representative result in Fig. 3I and 826 ± 235 *vs.* 122 ± 62 expressed in MFI, p=0.003, Fig. 3J).

Altogether, these results strongly suggest that ILC2 are recruited in fibrotic skin of SSc patients with a preferential location of iILC2 (KLRG1^{high}) in inflammatory SSc skin.

ILC2 are increased and switch from iILC2 towards nILC2 in the hypochlorous acid-induced murine model of systemic sclerosis

In order to decipher the kinetic of imbalance ILC2 homeostasis during the different phases of SSc (from the inflammatory to the established fibrotic stages), we used the HOCL-induced mouse model. As expected, we observed an increase in dermal thickness, OH proline dosages and expression of TGF-β (data not shown). We analyzed ILC2 in the spleen and the skin to reflect circulating cells and targeted organ respectively.

The absolute number and frequency of ILC2 in the spleen of affected mice (chronic stage) were similar compared to that of unaffected mice (Fig. 4A and data not shown), whereas they were significantly decreased in affected mice between inflammatory and chronic stages (5385 ± 916 *vs*. 2583 ± 281 respectively, p=0.01, Fig 4B). In contrast, skin ILC2s were increased both in terms of absolute count and frequency in HOCl mice at chronic stage (3.8 ± 0.2 *vs*. 2.6 ± 0.2 %, p=0.02 and 25702 ± 3376 *vs*. 10235 ± 1652, p=0.008 respectively, Fig. 4C and 4D). This increase was positively correlated with the collagen content of the skin at chronic stage (measured by the hydroxyproline content in μg/mg) (r=0.74, p<0.001, Fig. 4E) and with the expression of IL13 in the skin (r=0.70, p=0.0005, Fig. 4F). Globally, KLRG1^{high}ILC2 are increased in affected skin compared to unaffected in absolute number (1783 ± 472 *vs*. 603 ± 101, p=0.01, Fig 4G). When the dynamic process of fibrosis is considered, ILC2 global absolute number increased from inflammatory to late chronic phase in skin (27887 ± 3152 *vs*. 13870 ± 1513, p<0.01) but KLRG1^{high}ILC2 subset dramatically decreased in the same time (2016 ± 503 *vs*. 1068 ± 136 in inflammatory and chronic stages respectively, p=0.01, Fig. 4I). Taken together, these data showed the increased presence of ILC2 in fibrotic organs of an inducible scleroderma mouse model, persisting in the established fibrotic phase, with however a switch to a particular subtype with a low expression of KLRG1, nILC2.

TGFβ is involved in the switch of iILC2 to nILC2 leading to a pro-fibrotic subset through a decreased secretion of IL10

To learn more about the direct role of ILC2 in fibrosis process, a functional *in vitro* study was conducted. We sorted ILC2 from peripheral blood and obtained a pure cell population as shown in Fig. 5A and 5B. As expected, GATA3 was highly expressed in every subset of ILC (21). The cells most expressing GATA3 were ILC3 but ILC2 phenotype was validated as they expressed less tbet than ILC1 and less RORγt than ILC3. Extracted ILC2 represent a small number of cells making their functional study difficult. Because of this small number, we expanded them with IL-1β and IL-2. At the end of the culture period we tested their phenotype, and observed that 90% of amplified cells were CD127+CRTH2+ (Fig. 5C). After

20 days of culture, IL1β–generated ILC2 expressed higher level of transcription factors, including GATA3 than at D0 (Fig. 5D).

We tested different cytokines of interest in SSc on IL1β–generated ILC2s to identify which one could be involved in the modulation of KLRG1 (Fig. 5E). IL4, TSLP and IL25 did not modify the expression of KLRG1 (Fig. 5F). IL33 slightly reduced KLRG1 expression on ILC2 (20.01 ± 3.065 *vs.* 26.21 ± 5.07 for IL33-stimulated and no stimulated ILC2 respectively, p=0.03, Fig. 5F). In the meantime, TGFβ dramatically decreased KLRG1 expression on ILC2 (5.071 ± 1.34 *vs.* 26.21 ± 5.07 for TGFβ-stimulated and not stimulated ILC2 respectively, p=0.004, Fig. 5F). This decrease was also present when evaluating the number of ILC2 KLRG1^{high}, (0.68 ± 0.35 *vs.* 12.12 ± 1.87 for TGFβ-stimulated and no stimulated ILC2 respectively, p=0.04, Fig. 5G and 5H) reflecting the virtual disappearance of a sub-population of ILC2 strongly expressing KLRG1.

To evaluate the function of TGFβ-treated ILC2, supernatant of ILC2 activated under different conditions was added to skin fibroblast. As expected, TGFβ induced an increase of Col1A1 expression (2.47 ± 0.47-fold *vs.* medium Fig. 5G) and a decrease of MMP1 and CCL2 mRNA when directly incubated with skin fibroblasts (0.72 ± 0.12-fold and 0.49 ± 0.22-fold *vs.* medium respectively, Fig. 5H). The SN of non-stimulated ILC2 did not affect COL1A1 mRNA (1.05 ± 0.1-fold, Fig. 5I), while the incubation of fibroblasts with SN from TGF-stimulated ILC2 increased COL1A1 mRNA (2.049 ± 0.35-fold, p=0.05, Fig. 5J). The co-incubation of fibroblasts with SN from TGFβ-stimulated ILC2 with anti-TGFβ blocking antibody does not affect this result. A direct effect of TGFβ present in the SN is so eliminated, especially since the effect of the TGFβ-stimulated ILC2 SN on MMP1 and CCL2 is very different from that TGFβ alone. Interestingly, ILC2 SN (with or without TGFβ stimulation) dramatically increase MMP1 and CCL2 mRNA expression (9.5 ± 2.5-fold and 23.7 ± 6.6-fold Fig. 5J and 5K). We assessed cytokine production by ILC2 after TGFβ stimulation to determine the mechanism of this profibrotic effect. IL4 and IFNγ were barely undetectable in both conditions. Secretion levels of IL5 and IL9 were similar whatever the TGFβ-stimulated status of the ILC2 (Fig. 5L). More surprisingly we did not find any differences between the level of IL13 secretion in both SN (Fig. 5L). In the meantime, IL10 secretion by ILC2 was significantly reduced by the TGFβ stimulation (1510 ± 587 *vs.* 610 ± 212 pg/ml, p=0.05, Fig. 5L). To test whether the decrease of IL10 could be involved in the fibrotic effect of nILC2, we tested both the effect of adding or blocking IL10 in the co-incubation of fibroblasts with TGFβ-stimulated ILC2 SN. Inhibiting IL10 in the SN of ILC2

led to an increase of the collagen expression of fibroblasts cultured with the unstimulated ILC2 SN (0.96 ± 0.1-fold *vs.* 2.5 ± 0.5-fold, p=0.01, Fig. 5M). In the meantime, adding IL10 in the SN of TGFβ-stimulated ILC2 led to a dramatic decrease of its pro-collagen effect (1.8 ± 0.1-fold *vs.* 0.3 ± 0.1-fold, p=0.005, Fig. 5M).

Collectively, we showed from cultured ILC2 that the switch from iILC2 to the nILC2 form was favored by TGFβ, and that the pro-fibrotic effect of this subset of ILC2 depended largely on the level of IL10 secretion.

DISCUSSION

With the present study, we demonstrate the presence and the potential role of ILC2 in an autoimmune dependent fibrotic process using the model of SSc. We showed that ILC2 are early involved in the inflammatory process and switched to from iILC2 in the inflammatory phase to nILC2 during the sclerotic stage. Same kind of switch has been previously described in a very different condition, i.e. helminthic infection (14). The originality of our demonstration is based on the key role of TGFβ, cytokine of high relevance in SSc microenvironment, and the identification of a new pathway through IL10 secretion.

ILCs patrol environmental interfaces to defend against infection and protect barrier integrity. Given the growing attention paid to the role of the innate immune system in SSc, it was not surprising that these cells, novel and important actors of innate immunity, should be considered in this pathology. In 2016, two different publications have been issued on the subject. Surprisingly, the first one showed an increased number of CD4+ group 1 innate lymphoid cells (22). This result raises the major question of what is defined as an ILC. In our work, we relied on the extensive study of ILC and help of Professor Eric Vivier's team (18). The CD4+ cells were excluded from our analysis and Roan's results are therefore not comparable to ours. As for ourselves, we did not find any increase in ILC1 in the blood of SSc patients. A previous report was in the line of our hypothesis, but Wohlfarht *et al.* have shown elevated levels of ILC2 in both blood and the affected skin of patient with SSc compared to healthy subject (23). We were very interested in determining the causes of this discrepancy and we compared our results extensively. This difference can be explained technically by the fact that (i) in our case we gated on PBMCs by excluding granulocytes, (ii) we used different antibodies to characterize ILCs using CRTH2 and not ST2 to determine positive cells, (iii) we left CD5 in the lineage, thereby eliminating some of the T cells that would have lost CD4 expression. CD5+ ILCs are thought to be functionally immature very close from the progenitor (24). CD5 could also be expressed in a slight proportion of ILC1, ILC2 and ILC3, but these cells become functionally active secreting ILCs when turning CD5- cells. As a result we did not look at exactly the same cells as the Wohlfahrt's group, which may explain our discrepancies in results (25). In the skin the gating is different and the importance of the CD5 is dimmer, so we found concordant results. We robustly confirmed the result obtained on the skin by two different techniques including a semi-automatic quantification with mapping.

The increase in ILC2 in the skin of SSc patients raises the question of whether these cells are the result of local proliferation, switch/local polarization or migration from the periphery. The question is not fully solved, however the decrease in peripheral frequency and absolute number, and kinetics of ILC2 in mice would be in favor of the migration hypothesis. Our mouse model, allows a comparison between inflammatory phase (early) and fibrotic phase (late) of the disease (17). We found a significant decrease of ILC2 in the spleen in HOCL- mice at chronic versus inflammatory phase suggesting that these cells could have migrated in target organs such as skin. Same theory could apply to ILC3, which are dramatically decreased in human peripheral blood but cells from target organs such as intestines have to be isolated to find out the answer (26). Complementary mechanisms are conceivable since ILCs have already demonstrated their great plasticity (25). One can imagine that an environmental modification in SSc would be in favor of an ILC2 phenotype as we and other already demonstrated that TSLP was clearly increased in the skin of patients (13, 27, 28). Polarization from an undifferentiated ILC would be possible. A common helper innate lymphoid cell progenitor (CHILP) characterized by its expression of Id2 (which encodes transcriptional repressor inhibitor of DNA binding 2, Id2), and an ILC precursor (ILCP) expressing high level of Zbtb16 (that encodes promyelocytic leukemia zinc finger protein, PLZF) were identified (6). However this type of cell has been found in bone marrow of mice, even if their existence in other tissue such as skin has not been demonstrated so far (29). A study identified a RORγt-expressing ILC progenitor population in human, which is capable of developing into all ILC subsets and NK cells (30). Several distinct ILC precursors with pan-ILC (giving rise to all ILCs) or subset-restricted potentials have been described in both mouse and man.

It should be noted that amplification in the presence of IL2 and IL1β slightly modifies the cultured ILC2. As previously demonstrated, IL1β promoted an increase in Tbet without decreasing GATA3 expression or modifying phenotypic cell expression (19). It is therefore not surprising to see the increase in these two factors at the end of the 20 days of amplification. Both the increase in GATA3 mRNA expression and the cell surface phenotype after 20 days (CRTH2+CD127+) suggest that the cells at the end of amplification are still ILC2. With these cultured cells, we showed that the contribution to fibrosis of ILC2 is not straight, as the supernatant of these cells does not directly induce collagen production by fibroblasts. Nonetheless the activation of fibroblasts as evidenced by MMP1 and MCP1 production could lead to a pro-fibrotic profile in a facilitating environment. ILC2 could be either rather pro-resolving or rather pro-inflammatory. The environment plays a major role on cellular plasticity (31, 32). There are arguments in the literature that infusion of IL33 and thus

activation of the IL33-ILC2 axis would have a beneficial effect on renal ischemic lesions in mice (33).

Here we demonstrated the importance of another axis. KLRG1 expression is used to separate iILC2 ($KLRG1^{high}$) and nILC2 ($KLRG1^{low}$). iILC2 are transient progenitors of ILC and develop into nILC2 or ILC3-lile cells (34). In our work, iILC2 highly contributed to the increase of total ILC2 in the inflammatory phase notably in mice. Interestingly, we observed less dermal iILC2 in the chronic phase. These observations corroborate Huang's results showing, an increase in iILC2 after *Nippostrongylus brasiliensis* infection followed by a switch of these cells to nILC2 in order to participate in worm elimination (14). Here, we may provide the first evidence of a switch from iILC2 to nILC2 in the skin in a human pathological condition. During the chronic phase, iILC2 switch into nILC2 and participate to fibrosis. As previously showed for CD8, TGFβ decreased the expression of KLRG1 in ILC2 (35). Given the high relevance of TGFβ in human SSc (36) and mouse skin during the chronic phase, TGF could be at the origin of the switch between iILC2 and nILC2 during the chronic phase in SSc. Contrary to what was previously thought the pro-repair profile of the nILC2 obtained in TGFβ condition does not involve IL13 but rather a decrease in IL10 secretion by these cells. Our results corroborate and broadly substantiate the previous demonstration of the anti-fibrotic property of IL10 (37, 38).

To conclude, we confirmed the role of ILC2 in SSc and we showed the first evidence of new TGFβ/IL10/ILC2 axis in the pathophysiology of fibrosis and in SSc specifically. This indirect role of TGFβ, through a functional switch of ILC2 in human pathological condition highlights the crucial importance of adapting to a precise timing to treat the disease.

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TABLES

Table 1. Demographic, clinical and biologic characteristics of SSc population.

	Patients with lcSSc (n=50)	Patients with dcSSc (n=23)	All SSc patients (n=73)	p[†]
Female (%)	39 (78)	9 (40)	48 (65.8)	
Age at onset, mean ± SD years[‡]	48.7 ± 13.2	49.9 ± 14.5	49 ± 13.5	ns
Disease duration, mean ± SD years[‡]	10.7 ± 6.0	11.9 ± 9.47	11.5 ± 8.6	ns
RP (%)	50 (100)	23 (100)	73 (100)	ns
Digitals ulcers (%)	20 (40)	7 (30.8)	27 (37)	0.02
MRSS, mean ± SD	5.9 ± 5.5	24.6 ± 12.7	11.1 ± 11.6[§]	<0.0001
PAH (%)	7 (14)	1 (4.3)	8 (11)[¶]	ns
Interstitial lung disease (%)	11 (22)	13 (56.5)	24 (32.9)[#]	ns
Lung fibrosis (%)	7 (14)	1 (4.3)	8 (11)[#]	ns
Renal crisis (%)	1 (2)	0 (0)	1 (1.4)[¶]	ns
Antinuclear autoantibody positive	50 (100)	23 (100)	73 (100)	ns
Anti-centromere antibody positive	25 (50)	1 (4.34)	26 (35.6)	ns
Anti-topoisomerase antibody positive	4 (8)	11 (47.8)	15 (20.5)	ns
Anti-ARNIII polymerase antibody positive	1 (2)	1 (4.34)	2 (2.7)	ns

∗ Except where indicated otherwise, values are the number (%). MRSS = modified Rodnan skin thickness score; PAH = pulmonary hypertension. [†]Limited cutaneous systemic sclerosis (lcSSc) versus diffuse cutaneous SSc (dSSc).[‡]Age at onset of symptoms other than Raynaud's phenomenon (RP) and disease duration duration since symptoms other than RP. § Data were available for 34 patients. ¶ Data were available for 36 patients. # Data were available for 35 patients.

FIGURE LEGENDS

Figure 1: Characterization of ILC in a blood of patients with systemic sclerosis (SSc) and healthy donors (HD). (A) Representative dot plot of circulating ILC (CD45+, Lin-, CD127+) and ILC subsets (ILC1 (CD45+, Lin-, CD127+, CD117-, CRTH2-), ILC2 (CD45+, Lin-, CD127+, CRTH2+) and ILC3 (CD45+, Lin-, CD127+, CD117+, CRTH2-)) in HD and SSc blood. (B) Flow cytometry analysis of ILC frequency in blood of HD and SSc. (C) Proportion of ILC1 (white), ILC2 (black) and ILC3 (grey) in blood of HD and SSc (D-F) Percentage of circulating ILC1, ILC2 and ILC3 in HD and SSc blood. (G) Negative correlation between percentage of circulating ILC2 and the extent of cutaneous fibrosis characterized by rodnan score (mRSS). (H) Percentage of CLA+ cells gated on ILC2 in HD and SSc blood. Bar graphs show data as mean ± SEM (n=59 and 73 for ILC and ILC subsets frequencies in HD and SSc respectively). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$. HD= healthy donor, SSc= sclerodermic.

Figure 2: Characterization of cutaneous ILC2 in patients with systemic sclerosis (SSc) and healthy donors (HD) in immunofluorescence. (A and B) Immunofluorescence assay using anti-CRTH2-based immunofluorescence (green) and anti-lineage (CD3, CD11b and FcεR1)-based immunofluorescence (red) was performed to detect CRTH2+Lin- ILC2 in HD and SSc skin. (C and D) Number of ILC2 per mm2, respectively, in epidermis and dermis of HD and SSc skin. (E and F) Percentage of ILC2 per total cell count respectively in epidermis and dermis of HD and SSc skin. (G) Percentage of ILC2 (per total cell count) in both epidermis and dermis of HD and SSc skin. (H and I) Correlation between the extent of cutaneous fibrosis and number of ILC2 in dermis and epidermis respectively. (J) Number of ILC2 per mm2 in epidermis in skin of patients without digital ulcers and patients affected by digital ulcers. Bar graphs show data as mean ± SEM (n=17 and 30 for HD and SSc respectively). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

Figure 3: Characterization of ILC in a skin of patients with systemic sclerosis (SSc) and healthy donors (HD). (A) Representative dot plot of cutaneous ILC (CD45+, Lin-, CD127+) and ILC subsets (ILC1 (CD45+, Lin-, CD127+, CD117-, CRTH2-), ILC2 (CD45+, Lin-, CD127+, CRTH2+) and ILC3 (CD45+, Lin-, CD127+, CD117+, CRTH2-)) in HD and SSc skin. (B) Flow cytometry analysis of ILC frequency in skin of HD and SSc. (C) Proportion of ILC1 (white), ILC2 (black) and ILC3 (grey) in skin of HD and SSc. (D and E) Percentage of ILC2 and ILC1 among CD45+ lymphoid cells in HD and SSc skin. (F) Positive correlation between percentage of ILC2 among CD45+ cells and the extent of cutaneous fibrosis characterized by rodnan score (mRSS). (G) Correlation between percentage of ILC1 among CD45+ cells and the extent of cutaneous fibrosis characterized by rodnan score (mRSS). (H) Percentage of HLA-DR, OX40L, CCR10, CCR6, CLA, TSLPR and KLRG1 positive cells among ILC2 in HD and SSc skin. (I-J) Fluorescence intensity (MFI) of KLRG1 cells in HD, quiescent diffuse SSc and progressive diffuse SSc skin. Bar graphs show data as mean ± SEM (n=19 and 18 for ILC and ILC subsets frequencies in HD and SSc respectively). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$

Figure 4: Characterization of ILC2 in spleen, lung and skin of PBS and HOCl-injected mice at the end of chronic skin. (A) Absolute number of ILC2 in spleen of PBS or HOCl mice during inflammatory phase; (B) Absolute number of ILC2 in spleen HOCl mice during inflammatory and chronic phases; (C and D) Percentage and absolute number of ILC2 in skin of PBS or HOCl mice. (E) Positive correlation between absolute number of ILC2 in skin and the appearance of fibrosis measured by the hydroxyproline content (OH proline) in HOCl-injected mice; (F) Positive correlation between absolute number of ILC2 and IL13 mRNA expression in skin of HOCl mice model; (G) Absolute number of ILC2 KLRG1^{high} in skin at the end of inflammatory phase (D20) in PBS and HOCl-injected mice; (H) Absolute number of ILC2 in skin at the end of inflammatory and chronic phases in HOCl-injected mice; (I) Absolute number of ILC2 KLRG1^{high} in skin at the end of inflammatory and chronic phases in HOCl-injected mice.

Bar graphs show data as mean ± SEM (n=4 for PBS-injected mice and n=6 for HOCl-injected mice). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

Figure 5: TGFβ exert an indirect fibrotic role by favoring the switch from inflammatory (KLRG1^{high} ILC2) to nature. (A) Representative dot blot of ILC2 (CD45+Lin-CD127+CRTH2+) sorting; (B) Verification of the purity of the cell sorting; (C) ILC2 phenotype after 20 days of amplification with IL2 and IL1β; (D) RTqPCR analysis of ILC2 after 20 days of amplification for Tbet, GATA3 and RORγt. Relative expression of Tbet, GATA3 and RORγt mRNA in ILC2 at D20 were normalized with the expression at D0 using ΔΔCt method; (E) Representative dot blot of KLRG1 expression, in flow cytometry, in unstimulated ILC2 (medium) and in ILC2 stimulated with rIL33, rTGFβ, rIL4, rTSLP and rIL25 after 20 days of amplification. (F) Percentage of KLRG1+ cells in unstimulated ILC2 and IL33, TGFβ, IL4, TSLP and IL25-stimulated ILC2. (G) Representative dot plot of the KLRG1 expression level characterized by KLRG1^{high} and KLRG1^{low} cells in unstimulated ILC2 and TGFβ-stimulated ILC2. (H) Percentage of KLRG1^{high} cells in unstimulated ILC2 and TGFβ-stimulated ILC2. (I-K) RTqPCR analysis of Col1A1, MMP1 and CCL2 mRNA expression in fibroblasts incubated with TGFβ, supernatant of unstimulated ILC2 and supernatant of TGFβ-stimulated ILC2 with or without blocking antibodies anti-IL1β; (L) Secretion of IL5, IL9, IL13 and IL10 assessed by cytokine bead assay (CBA); (M) RTqPCR analysis of Col1A1 expression in fibroblasts incubated with supernatant of unstimulated ILC2 and supernatant of TGFβ-stimulated ILC2 Data were analyzed using ΔΔCt method.

Bar graphs show data as mean ± SEM. (n=3 and n=4 for RTqPCR analysis in ILC and fibroblasts respectively). Comparison between groups was calculated using the paired Wilcoxon test and Kruskal Wallis tests. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

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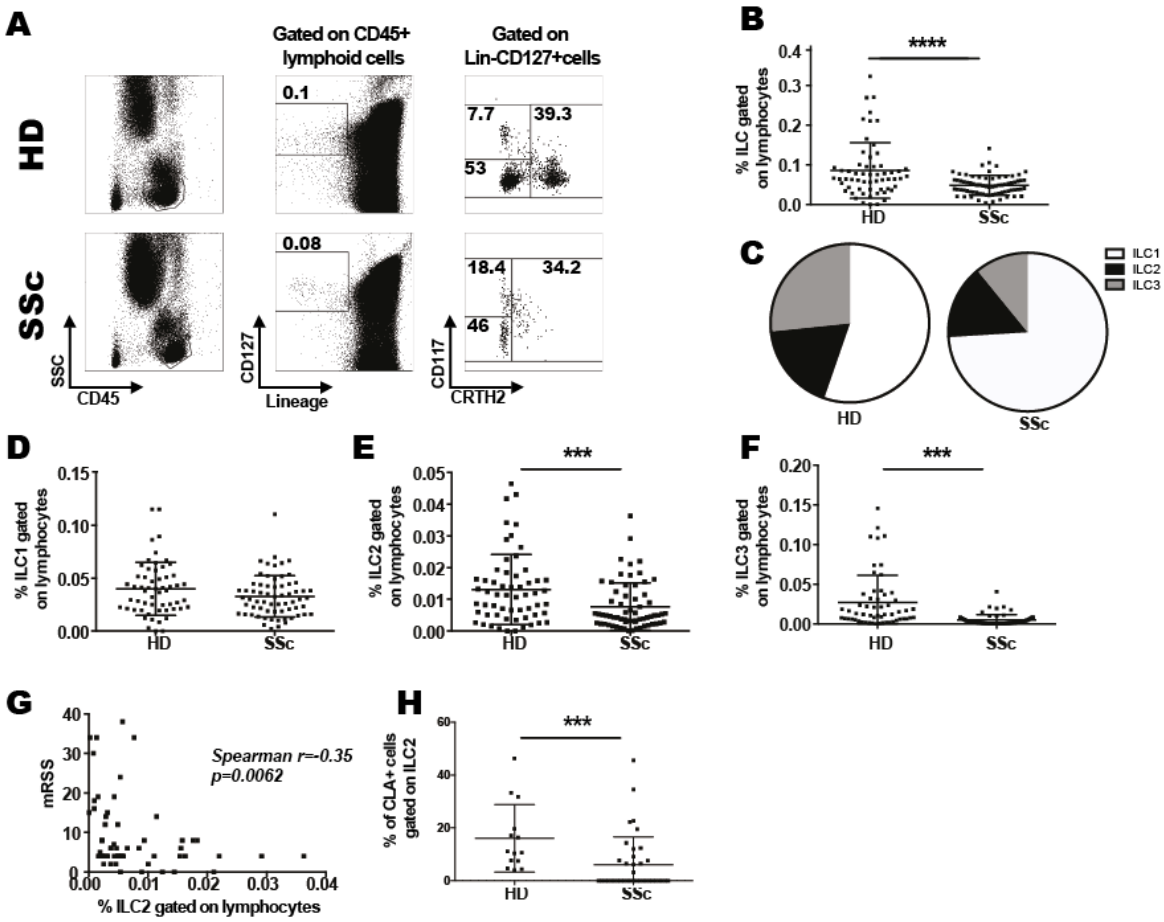

Figure 1: Characterization of ILC in a blood of patients with systemic sclerosis (SSc) and healthy donors (HD). (A) Representative dot plot of circulating ILC (CD45+, Lin-, CD127+) and ILC subsets (ILC1 (CD45+, Lin-, CD127+, CD117-, CRTH2-), ILC2 (CD45+, Lin-, CD127+, CRTH2+) and ILC3 (CD45+, Lin-, CD127+, CD117+, CRTH2-)) in HD and SSc blood. (B) Flow cytometry analysis of ILC frequency in blood of HD and SSc. (C) Proportion of ILC1 (white), ILC2 (black) and ILC3 (grey) in blood of HD and SSc (D-F) Percentage of circulating ILC1, ILC2 and ILC3 in HD and SSc blood. (G) Negative correlation between percentage of circulating ILC2 and the extent of cutaneous fibrosis characterized by rodnan score (mRSS). (H) Percentage of CLA+ cells gated on ILC2 in HD and SSc blood. Bar graphs show data as mean ± SEM (n=59 and 73 for ILC and ILC subsets frequencies in HD and SSc respectively). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$. HD= healthy donor, SSc= sclerodermic.

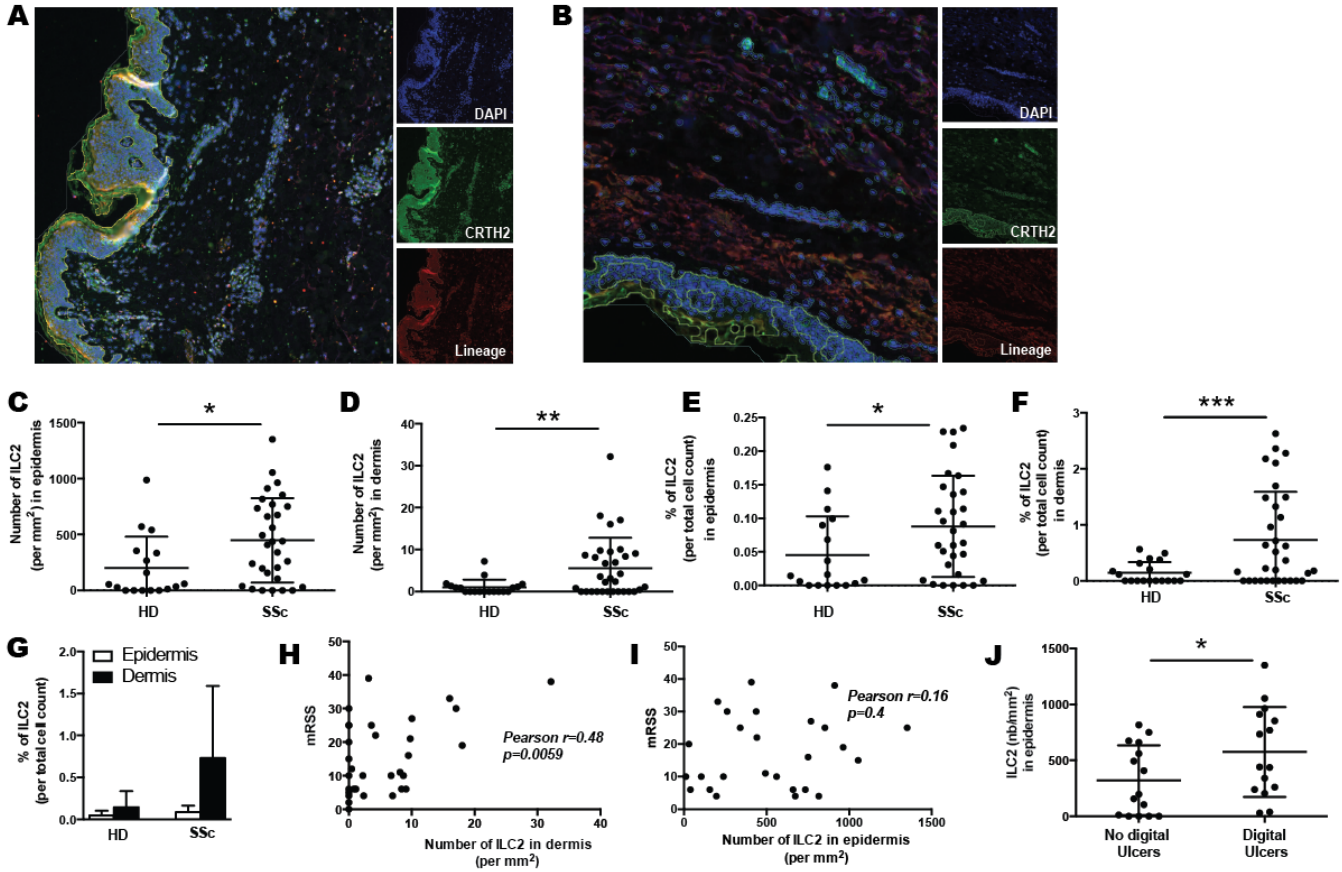

Figure 2: Characterization of cutaneous ILC2 in patients with systemic sclerosis (SSc) and healthy donors (HD) in immunofluorescence. (A and B) Immunofluorescence assay using anti-CRTH2-based immunofluorescence (green) and anti-lineage (CD3, CD11b and FcεR1)-based immunofluorescence (red) was performed to detect CRTH2+Lin- ILC2 in HD and SSc skin. (C and D) Number of ILC2 per mm2, respectively, in epidermis and dermis of HD and SSc skin. (E and F) Percentage of ILC2 per total cell count respectively in epidermis and dermis of HD and SSc skin. (G) Percentage of ILC2 (per total cell count) in both epidermis and dermis of HD and SSc skin. (H and I) Correlation between the extent of cutaneous fibrosis and number of ILC2 in dermis and epidermis respectively. (J) Number of ILC2 per mm2 in epidermis in skin of patients without digital ulcers and patients affected by digital ulcers. Bar graphs show data as mean ± SEM (n=17 and 30 for HD and SSc respectively). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

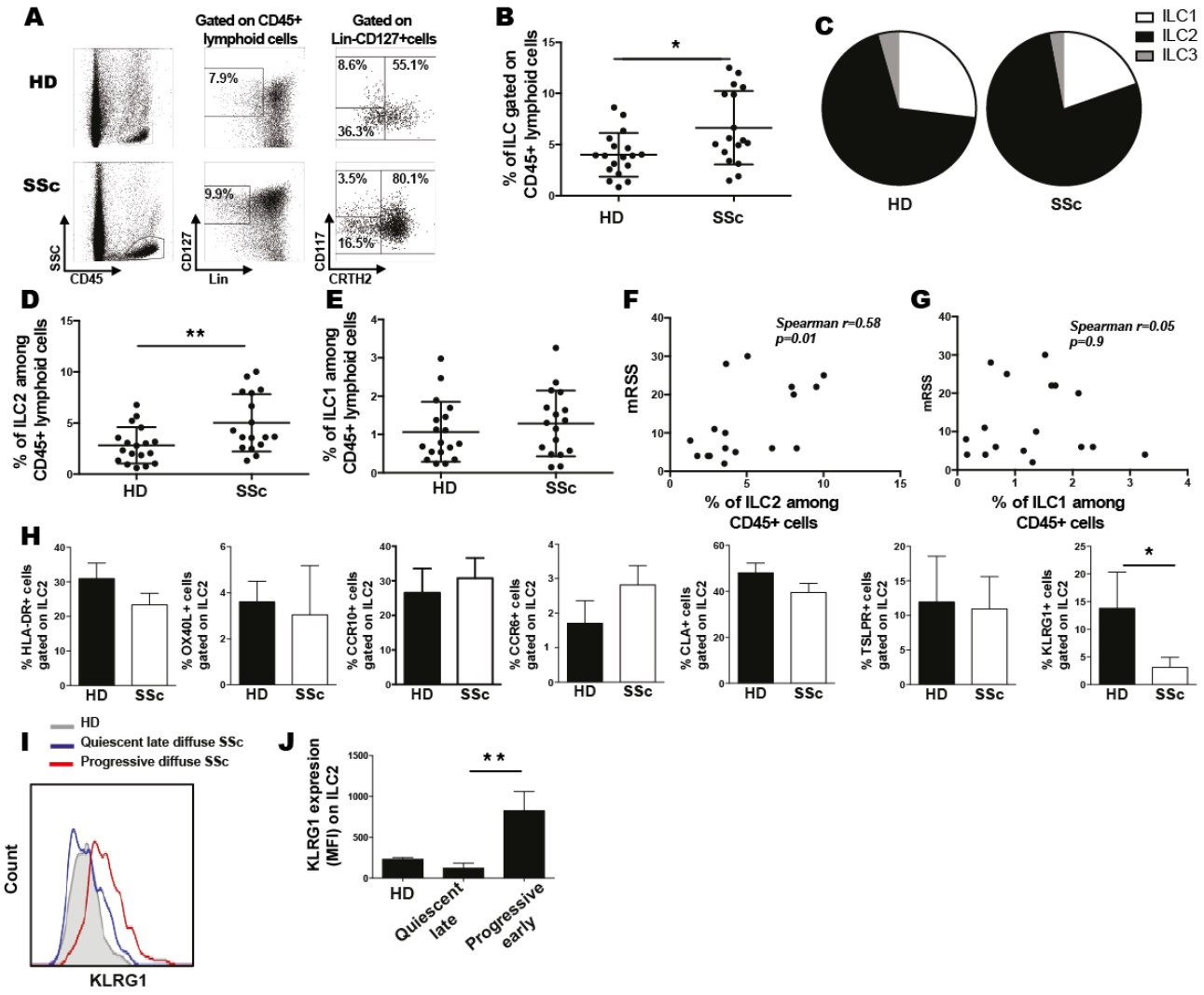

Figure 3: Characterization of ILC in a skin of patients with systemic sclerosis (SSc) and healthy donors (HD). (A) Representative dot plot of cutaneous ILC (CD45+, Lin-, CD127+) and ILC subsets (ILC1 (CD45+, Lin-, CD127+, CD117-, CRTH2-), ILC2 (CD45+, Lin-, CD127+, CRTH2+) and ILC3 (CD45+, Lin-, CD127+, CD117+, CRTH2-)) in HD and SSc skin. (B) Flow cytometry analysis of ILC frequency in skin of HD and SSc. (C) Proportion of ILC1 (white), ILC2 (black) and ILC3 (grey) in skin of HD and SSc. (D and E) Percentage of ILC2 and ILC1 among CD45+ lymphoid cells in HD and SSc skin. (F) Positive correlation between percentage of ILC2 among CD45+ cells and the extent of cutaneous fibrosis characterized by rodnan score (mRSS). (G) Correlation between percentage of ILC1 among CD45+ cells and the extent of cutaneous fibrosis characterized by rodnan score (mRSS). (H) Percentage of HLA-DR, OX40L, CCR10, CCR6, CLA, TSLPR and KLRG1 positive cells among ILC2 in HD and SSc skin. (I-J) Fluorescence intensity (MFI) of KLRG1 cells in HD, quiescent diffuse SSc and progressive diffuse SSc skin. Bar graphs show data as mean ± SEM (n=19 and 18 for ILC and ILC subsets frequencies in HD and SSc respectively). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$

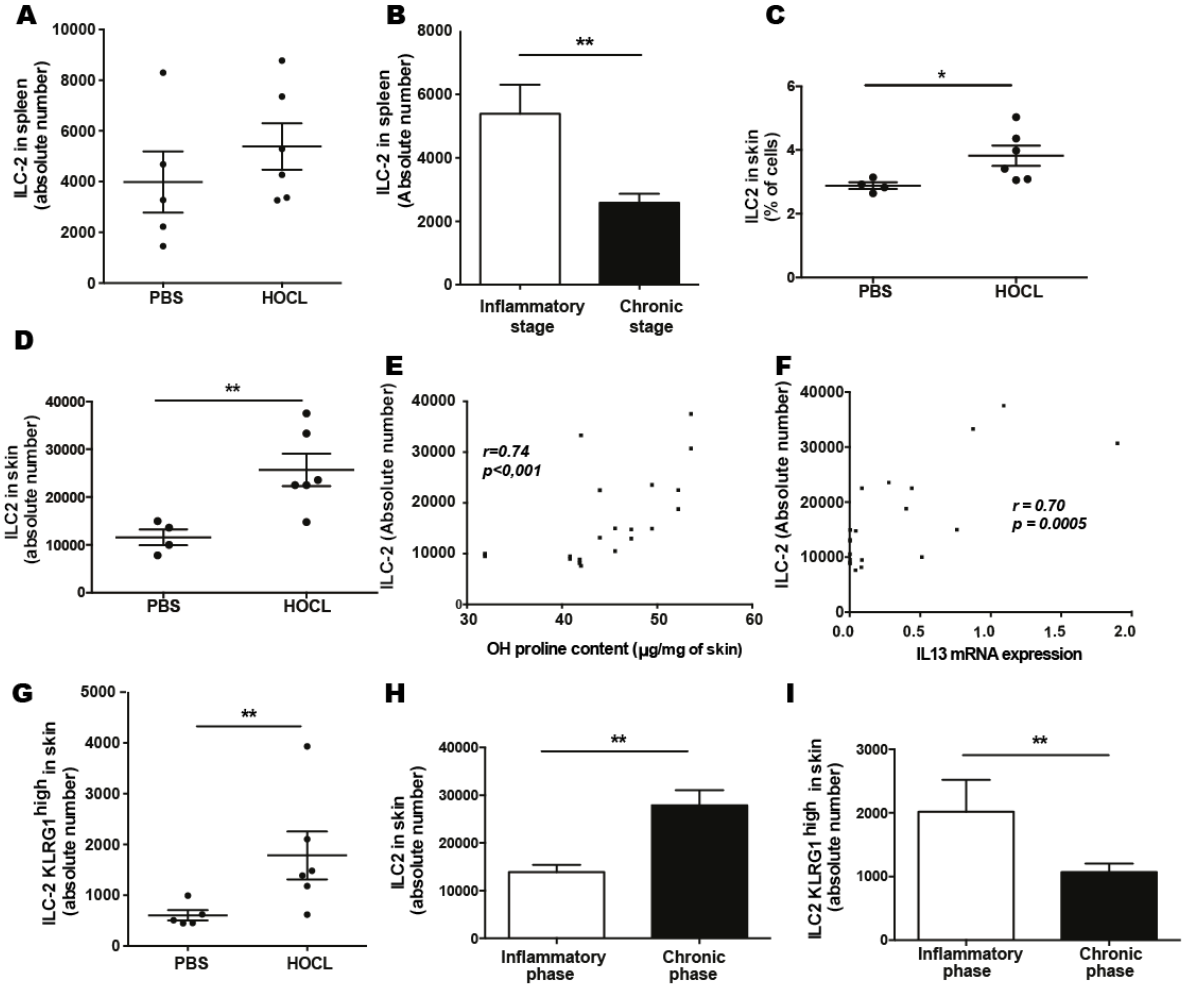

Figure 4: Characterization of ILC2 in spleen, lung and skin of PBS and HOCl-injected mice at the end of chronic skin. (A) Absolute number of ILC2 in spleen of PBS or HOCl mice during inflammatory phase; (B) Absolute number of ILC2 in spleen HOCl mice during inflammatory and chronic phases; (C and D) Percentage and absolute number of ILC2 in skin of PBS or HOCl mice. (E) Positive correlation between absolute number of ILC2 in skin and the appearance of fibrosis measured by the hydroxyproline content (OH proline) in HOCl-injected mice; (F) Positive correlation between absolute number of ILC2 and IL13 mRNA expression in skin of HOCl mice model; (G) Absolute number of ILC2 KLRG1^{high} in skin at the end of inflammatory phase (D20) in PBS and HOCl-injected mice; (H) Absolute number of ILC2 in skin at the end of inflammatory and chronic phases in HOCl-injected mice; (I) Absolute number of ILC2 KLRG1^{high} in skin at the end of inflammatory and chronic phases in HOCl-injected mice.

Bar graphs show data as mean ± SEM (n=4 for PBS-injected mice and n=6 for HOCl-injected mice). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

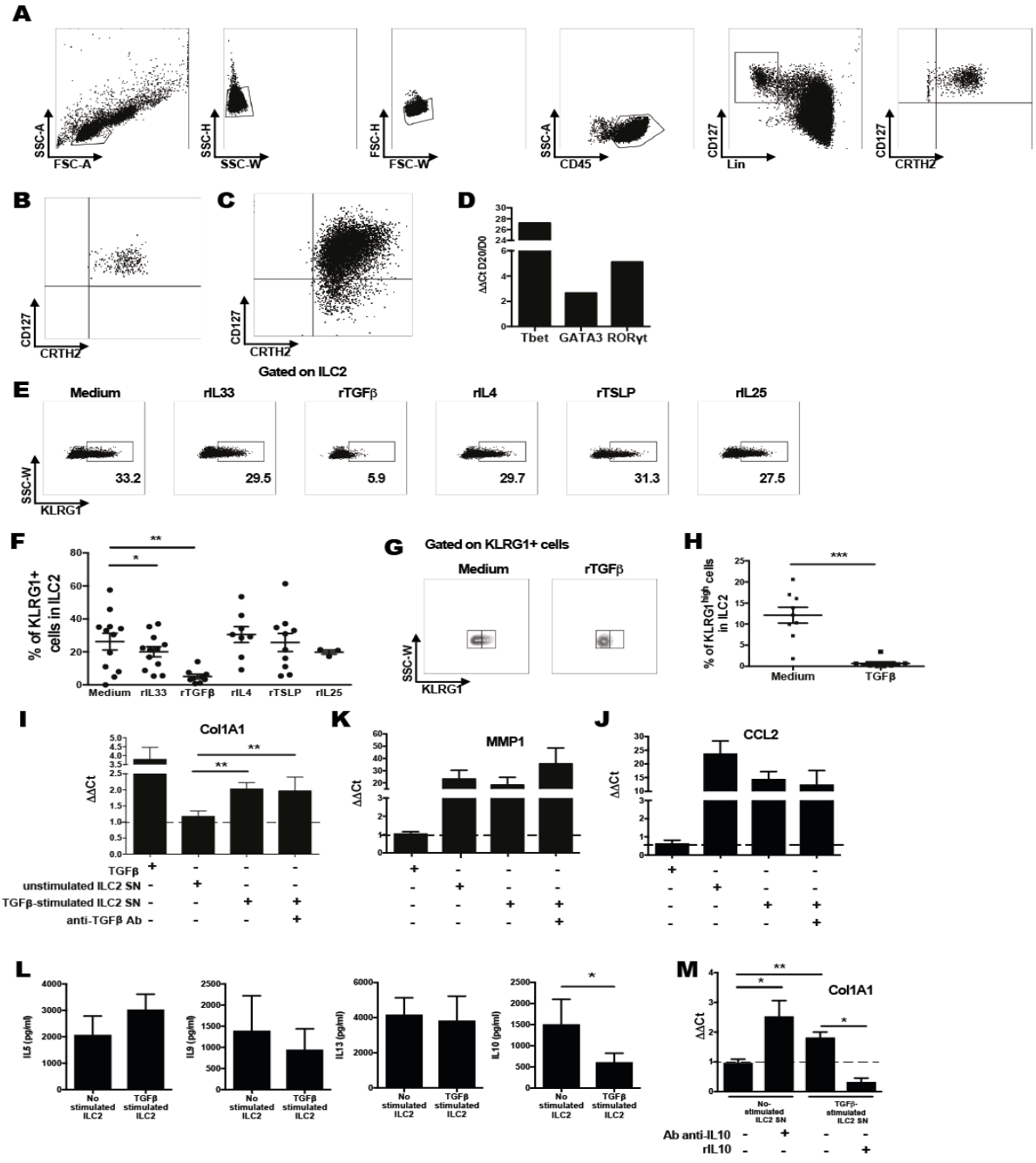

Figure 5: TGFβ exert an indirect fibrotic role by favoring the switch from inflammatory (KLRG1^{high} ILC2) to nature. (A) Representative dot blot of ILC2 (CD45+Lin-CD127+CRTH2+) sorting; (B) Verification of the purity of the cell sorting; (C) ILC2 phenotype after 20 days of amplification with IL2 and IL1β; (D) RTqPCR analysis of ILC2 after 20 days of amplification for Tbet, GATA3 and RORγt. Relative expression of Tbet, GATA3 and RORγt mRNA in ILC2 at D20 were normalized with the expression at D0 using ΔΔCt method; (E) Representative dot blot of KLRG1 expression, in flow cytometry, in unstimulated ILC2 (medium) and in ILC2 stimulated with rIL33, rTGFβ, rIL4, rTSLP and rIL25 after 20 days of amplification. (F) Percentage of KLRG1+ cells in unstimulated ILC2 and IL33, TGFβ, IL4, TSLP and IL25-stimulated ILC2. (G) Representative dot plot of the KLRG1 expression level characterized by KLRG1^{high} and KLRG1^{low} cells in unstimulated ILC2 and TGFβ-stimulated ILC2. (H) Percentage of KLRG1^{high} cells in unstimulated ILC2 and TGFβ-stimulated ILC2. (I-K) RTqPCR analysis of Col1A1, MMP1 and CCL2 mRNA expression in fibroblasts incubated with TGFβ, supernatant of unstimulated ILC2 and supernatant of TGFβ-stimulated ILC2 with or without blocking antibodies anti-IL1β; (L) Secretion of IL5, IL9, IL13 and IL10 assessed by cytokine bead assay (CBA); (M) RTqPCR analysis of Col1A1 expression in fibroblasts incubated with supernatant of unstimulated ILC2 with or without blocking antibody anti-IL10 and supernatant of TGFβ-stimulated ILC2 with or without rIL10. Data were analyzed using ΔΔCt method.
Bar graphs show data as mean ± SEM. (n=3 and n=4 for RTqPCR analysis in ILC and fibroblasts respectively). Comparison between groups was calculated using the paired Wilcoxon test and Kruskal Wallis tests. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

PROJET 2 :

Implication des macrophages de type 2 dans la fibrose au cours de la sclérodermie systémique

I) Problématique

Les macrophages de type 2 jouent très probablement un rôle dans la ScS qui reste encore mal connu malgré de nombreuses études. La plasticité macrophagique représente une difficulté supplémentaire. En effet, quels sont les mécanismes moléculaires entrainant un changement de polarisation ? Comme passe-t-on d'une polarisation macrophagique de type 1, majoritaire au début de maladie, à une polarisation macrophagique de type M2 ?

II) Objectif du projet

Ce projet de recherche vise à établir un lien entre les trois caractéristiques majeures de la ScS : la vasculopathie, les anomalies immunologiques et la fibrose. Comprendre les différentes interactions entre ces trois éléments pourrait permettre une meilleure perspective thérapeutique en ciblant spécifiquement des molécules liées à la vasculopathie et entraînant une dérégulation de la réponse immunitaire et la fibrose.

A travers ce projet, nous souhaitons démontrer le rôle indirect de la vasculopathie sur la fibrose via la dérégulation de la réponse immunitaire.
Ce projet a été commencé en 2014 lors de mon stage de master 2 au sein du laboratoire. Par la suite, Joëlle Lapoirie et Blanca Jurado (Master 2) et moi-même avons contribué au projet

III) Résumé de l'article

La sclérodermie systémique (ScS) est principalement caractérisée par une fibrose qui est irréversible. La réponse immunitaire de type 2 semble impliquer dans ce processus fibrotique. Au-delà des cellules Th2 (lymphocytes auxiliaires de type 2), les macrophages de type 2 ont un rôle important durant la fibrose mais les mécanismes précis impliqués restent confus. Dans cette étude, nous avons dressé une cartographie histologique des macrophages cutanés montrant leur localisation préférentielle près des cellules endothéliales micro-vasculaires de la peau (HDMEC). Nous avons ensuite démontré que les HDMEC, via leur production d'IL6 et d'ET1, induisaient un profil M2 aux macrophages générés de par leur expression de CD163, DC-SIGN et pSTAT3 et de leur capacité à produire de l'IL10 et de l'IL6. Ces macrophages générés vont ensuite induire des niveaux élevés de MMP1 et de CCL2 par les fibroblastes suggérant un rôle plutôt pro-inflammatoire de ces derniers. Ce travail démontre une nouvelle voie dans la physiopathologie de la ScS liant la vasculopathie, les réponses immunitaires et la fibrose ouvrant de nouvelles perspectives thérapeutiques dans cette maladie.

Article in preparation

IL-1-β activated microvascular endothelial cells promote M2-like cells: consequences on fibrosis during scleroderma

P.Laurent[1], J.Lapoirie[1], E.Levionnois[1], C.Grenier[1], B. Jurado-Mestre[1], E.Lazaro[1,2], P.Duffau[1,2], C.Richez[1,2], J Seneschal[2], Jean-Luc Pellegrin[2], Joël Constans[2], Thierry Schaeverbeke[2], Marie-Sylvie Doutre[2,] I.Douchet[1], C.Chizzolini[3], P.Blanco[1,2], ME.Truchetet[1,2], C.Contin-Bordes[1,2].

[1]ImmunoConcEpt, UMR 5164, University of Bordeaux

[2]CHU Bordeaux Hospital, Bordeaux, France

[3]Immunology department, University Hospital and School of Medicine, Geneva, Switzerland

Corresponding author
Cecile Contin-Bordes
Cecile.bordes@chu-bordeaux.fr

Abstract

Systemic sclerosis (SSc) is mainly characterized by an irreversible fibrosis in which type 2 response plays a crucial role. On the top of Th2 cells, it is undeniable that alternative-activated macrophages (M2) have an important role during fibrosis but the precise mechanisms involved in their recruitment and activation are still unclear. In this study, we show deep alterations in the circulating monocytes population and skin macrophages, showing their preferential localization in dermis close to endothelial cells (EC). We demonstrate that IL-1β-activated EC induced a M2-like profile in macrophages reminiscent of pro-resolving macrophages, as they express pSTAT3, CD163 and DC-SIGN and produce IL-10. IL-6 and endothelin-1 (ET-1) secreted by IL-1β-activated EC are key players in this process. In turn, M2-like induced macrophages promote synthesis of MMP-1 and CCL-2 transcripts in fibroblasts suggesting a pro-inflammatory and pro-remodelling role. This work revealed a new pathway in SSc linking vasculopathy, immune responses and fibrosis and can open up new therapeutic perspectives in this disease.

Introduction

SSc is a rare autoimmune connective tissue disease characterized by a microangiopathy and fibrosis of the skin and internal organs. The disease has a heterogeneous presentation and

severity related to the extent of skin fibrosis and internal organ involvement. The pathophysiology of scleroderma remains incompletely understood but involves a complex network of interactions between the microvascular system, activation of autoimmune processes, and chronic activation of fibroblasts leading to the deregulated production of extracellular matrix, and finally fibrosis (Denton and Khanna, 2017; Varga and Abraham, 2007). The nature and the sequence of these reciprocal interactions remain debated, but constitute an important area of investigation in order to better assess SSc pathogenesis and to propose new therapeutic strategies.

Macrophages have emerged as key players in tissue maintenance and repair as well as in fibrosis (for recent review see (Gieseck et al., 2018)), and they have been implicated in the pathophysiology of scleroderma. In human SSc, macrophages infiltrate skin lesions adopting a perivascular localization in early forms of scleroderma. Moreover, skin-infiltrating macrophages express CD163 and/or CD204, two markers of M2-type macrophages (Manetti, 2015). Serum levels of soluble CD163 are increased in scleroderma patients and are associated with pulmonary fibrosis (Nakayama et al., 2012) . Patients presenting a limited form of scleroderma and pulmonary hypertension, are characterized by an increased expression of mRNA coding for IL13 and MRC1 (c-type mannose receptor 1) at the monocyte level (Christmann et al., 2011). Finally, transcriptome analysis of pulmonary biopsies of SSc patients presenting a diffuse interstitial pulmonary disease has shown a macrophagic-type signature including CD163 and CCL18 overexpression (Christmann et al., 2014). Very recently, the implication of M2 polarized macrophages in two mouse models of SSc have been described (Huang et al., 2017; Maier et al., 2017).

Altogether, these observations strongly suggest that macrophages from SSc patients are M2-polarized, and participate to the fibrotic process of the disease. However, the precise mechanisms involved in their recruitment and/or polarization are not yet elucidated.

Our group recently described a new pathophysiological loop linking platelet activation, endothelial activation and fibrosis in human scleroderma (Truchetet et al., 2016). In this work, we showed that the close contact between activated platelets and the microvascular endothelium promotes an IL1-β-dependent production of TSLP (thymic stromal lymphopoietin) by endothelial cells, which is in turn involved in the activation of cutaneous fibroblasts, and the deposition of collagen.

We now provide evidences that endothelial cells could act indirectly on the fibrotic process through the modulation of the cutaneous immune responses. We show that IL1-β activated

microvascular endothelial cells promote M2-like cells reminiscent of pro-resolving macrophages, which are involved in fibroblast activation.

Altogether those data provide new clue on the role of EC in the inflammatory process during scleroderma.

Material and methods

Patients

Individuals with SSc presenting at the university hospital of Bordeaux, France, were prospectively included in the study between March 2014 and September 2016. All patients satisfied the classification criteria proposed by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) 2013 (van den Hoogen et al., 2013). Patients were included in the context of the VISS (Vasculopathy and Inflammation in Systemic Sclerosis) biomedical research project founded in 2012 and approved by the institutional ethical committee (CPP, 2012-A00081-42, Aquitaine). All participants provided written informed consent before inclusion. For each patient, a disease- and organ-specific questionnaire was completed by the clinician in charge of the patient and then centralized by investigators. Clinical features (scleroderma form; sex; age at Raynaud's phenomenon (RP) onset; age at onset of the first non-RP manifestation; disease duration; and symptoms of skin, articular, heart, lung, kidney, and gastrointestinal involvement), immunologic test results (antinuclear antibodies, anti-Scl70 antibodies, and anti-centromere antibodies), imaging and functional exams (thorax CT scans, respiratory functions tests, cardiac ultrasonography and right heart catheterization) and treatments were recorded. For modified Rodnan Skin Score (mRSS) and Right Ventricle Systolic Pressure, the highest value of the medical history was registered for each patient. Interstitial lung disease was diagnosed when pulmonary function tests showed a restrictive defect with decreased diffusion capacity (DLCO) associated with several types of lesions on the thorax CT scans. Lung fibrosis was diagnosed based on specific lesions observed on the thorax CT scans, i.e., honeycomb cysts and reticular septal thickening. Punch biopsy specimens (3-4 mm) of affected mid-forearm skin were obtained for some patients. Age- and sex-matched healthy donors (HD) were recruited at the local Blood Transfusion Centre (Etablissement Français du sang, Bordeaux) for blood tests.

Symptoms of vasculopathy included digital ulcer, pulmonary arterial hypertension, and/or renal crisis.

Cell purification

HDMEC (Human dermal microvascular endothelial cells) were obtained from healthy and SSc skins. As previously described (Truchetet et al., 2016), skin was incubated overnight with dispase (Roche) at 4°C. After separating the dermis from the epidermis, the dermis is placed in a petri dish with 3 mL of HBSS. Using forceps, pressure is applied to the dermis to extract the endothelial cells. HBSS is harvested and centrifuged. The cell pellet was resuspended with MV2 medium. The dermis is then incubated with HBSS containing 0.1% type IA collagenase for 3 hours at 37°C to purify fibroblasts. The solution was filtered through 0,7 um nylon and centrifuged. Pellet cells were resuspended with cDMEM (Dulbecco's Modified Eagle Medium+1% penincillin/streptomycin+10% FCS (Fetal calf serum), GIBCO). Fibroblasts were used between the third and sixth passages.
Monocytes were obtained from blood of HD recruited at Blood Transfusion Centre. Briefly, peripheral blood mononuclear cells were isolated by Ficoll (Eurobio) and then monocytes were purified by immunomagnetic sorting using CD14 microbeads (Miltenyi). Purity (>90%) was measured by flow cytometry using APC-labeled CD14 (Milteny).

Preparation of endothelial cell supernatants

HDMEC were cultivated in MV2 (cMV2+1%penicillin/streptomycin+8%FCS, PromoCell) in normoxia (21% O2) or hypoxia (1% O2). IL-1β (50ng/mL R&D System) was added in culture in normoxic condition. Surpernatants were harvested and frozen at -80°C. IL1β-activated EC supernatant was called SN* and no-activated EC supernatant was called SN.

Cytokines and Chemokines assay

IL-6, IL-8, IL-1Ra, GM-CSF, CCL2, CCL3 CCL4 and CCL5 in EC supernatants were assay by multiplex (Biolegend) according to the manufacturer's protocol. IL-10 and ET-1 were measured by ELISA (Abcam).

IL-12p40, TNFα and IFNγ were measured using multiplex (Biolegend). Active TGFβ was measured by ELISA (R&D system) after acidification (HCl 1N) of monocyte supernatants.

Activation of monocytes

2.10^5 monocytes were incubated in 100µl of cRPMI (Roswell Park Memorial Institute, 1%penicillin/streptomycin+8%FCS+5ng/ml GM-CSF, GIBCO) with or without 100µl of MV2 or EC-conditioned supernatants in a 96 round-bottom well-plate. For some conditions, ET-1 (10 ng/mL, Sigma-Aldrich), IL-6 (100 ng/mL, Preprotech), IL-1β (50 ng/mL, R&D), anti-ET-1R (100 ng/mL, kindly gift from Actelion Pharmaceuticals Ldt), anti-IL-6R (100 ng/mL) and anti-IL-1β (150 µg/mL) were added. The negative control ("medium") consisted of 100µl MV2 medium and 100µl cRPMI.

STAT phosphorylation was assessed after 20 min using Perm Buffer Set A (Miltenyi). Monocytes were stained PE-labeled phosphoSTAT1 (Miltenyi) and APC-labeled phosphoSTAT3 (Miltenyi).

Phenotyping of monocytes was performed after 48h using DAPI (Sigma-Aldrich), APC-labeled CD14 (Miltenyi), PE-labeled CD163 (BD) and FITC-labeled CD206 (Miltenyi).

Macrophage differentiation was determined at 6 days using PerCP-labeled CD14, APC-labeled CD206, VioBlue-labeled CD209, APCCy7-labeled CCR2 (all purchased from Miltenyi) and PE-labeled CD163 (BD).

Viability (>80%) was controlled by flow cytometry using a viability marker (DAPI or FVS 510 from BD).

Whole blood phenotyping

PE-labeled CD163 (BD), FITC-labeled CD206 (Miltenyi), PE-Cy7-labeled CD16 (Beckman coulter) and Vio-Blue-labeled CD14 (Miltenyi) expression was assessed on whole blood of HD and SSc patients by flow cytometry (FACS Canto II) and analyzed using Flowjo software.

Immunofluorescence

Coculture fibroblasts/macrophages

Macrophages and fibroblasts were cultivated using transwell. 3.10^4 fibroblasts were in upper chamber of transwell and 3.10^4 were in lower chamber during 24hours. Fibroblasts were harvested and lysed with Lysis Buffer.

RTqPCR

RNA was purified from ILC subsets and fibroblasts using RNeasy Plus Micro Kit (Qiagen). RNA concentration and purity were assessed using the Spectrophotometer DS11 (Denovix). RNA integrity number (RIN) was assessed using Agilent 2200 TapeStation (Agilent Technologies). All procedures were performed according to the manufacturer's instructions.
Total RNA was converted to cDNA using GoScript Reverse Transcription (Promega™). qPCR was performed using GoTaq Master Mix (all reagents were purchased from Promega™). The following targets were analyzed: Col1A1, MMP1 and CCL2 (see in details in Table 1). mRNA levels were normalized to 18s.
Samples were distributed in duplicate in a 384-well plate using Epmotion 5073 automated pipetting system (Eppendorf). Real-time quantitative PCR were performed using thermocycler CFX384 (Bio-Rad™).
The data were analyzed using Bio-Rad™ CFX Manager software (Bio-Rad™) and differential expressions were evaluated according to the method of ΔΔCt.

Results

Monocytes subsets and phenotype are altered in SSc patients

Monocytes are classically separated into three subsets depending on the expression of CD14 and CD16 REF (**Figure 1A**). We showed that the frequency of $CD14^{high}$/CD16- monocytes is significantly increased in SSc blood compared to HD (**Figure 1B**, 62.69% ± 3.128 *vs.* 82.15% ± 2.2 for HD and SSc respectively, *p<0.0002*). In contrast, the number of $CD14^{low}$/CD16+ is decreased in patients (**Figure 1C**, 10.49% ± 1.65 *vs.* 3.56% ± 0.52 for HD and SSc respectively, *p=0.0011*) whereas the rate of $CD14^{high}$/CD16+ is similar between SSc and HD (**Figure 1D**,

9.64% ± 2.478 *vs.* 6.38% ± 1.076 for HD and SSc respectively, *p=0.4159*). Overall, the proportion of monocyte subsets is altered in SSc patients compared to HD (**Figure 1E**).

We observed a decrease expression of two type-2 markers CD163 and CD206 in SSc $CD14^{low}$/CD16+ monocytes (CD163, 23.77% ± 7.005 *vs.* 11.74% ± 2.85 for HD and SSc respectively, *p=0.031*; CD206, 41.77% ± 12.02 *vs.* 13.08% ± 3.514 for HD and SSc respectively, *p=0.0313*) while SSc $CD14^{high}$/CD16- cells express lower levels of CD206 (16.84% ± 2.678 *vs.* 7.10% ± 1.854 for HD and SSc respectively, *p=0.002*). Similar levels of both markers were detected in $CD14^{high}$/CD16- cells from SSc patients or HD (**Figure 1F and 1G**). Interestingly, percentages of CD163+$CD14^{low}$CD16+ are positively correlated with the extent of cutaneous fibrosis representing by the modified Rodnan score (mRSS) (**Figure 1H,** Spearman test, r=0.89, *p=0.006*). The frequency of $CD14^{low}$/CD16+ is increased in blood of patients with vasculopathy compared to patients without vasculopathy (**Figure 1I,** 8.983± 1.254 *vs.* 5.143 % ± 1.6 respectively, *p=0.035*).

Taken together these results suggest an alteration in proportion and phenotype of monocyte subsets in SSc patients.

Type-2 Macrophages are increased in SSc skin

In order to gain more insight in the alteration of macrophages within affected tissues, we performed immunofluorescence in SSc and HD skin according to Barros and colleagues (Barros et al., 2013)

We provide evidence that CD68+cMAF+ cells, reminiscent of pro-resolving type-2 Macrophages, were increased in SSc skin as shown in Figure 2.

This part of the work still ongoing.

IL-1β but not hypoxia induces chemokines release by Human Dermal Microvascular Endothelial Cells (HDEMC)

As vasculopathy is a prominent and early feature during scleroderma, we next assess the ability of HDMEC to modulate monocyte migration.

Our previous work showed that activated platelets, through the release of IL-1β, favors the production of the profibrotic cytokines TSLP by HDMEC during SSc (Truchetet et al., 2016).

To test the capacity of IL-1β-activated HDMEC to attract monocytes to damaged tissues, we measured CCL2, CCL3, CCL4 and CCL5 in non-activated HDMEC (SN) and in IL-1β (SN*) HDMEC supernatants. CCL2 was secreted in both SN and SN* compared to medium condition but IL-1β induced a strong increase of the chemokine production (**Figure 3A**, 158.96 pg/ml (SN) *vs* 828.10 pg/ml (SN*)). CCL4 and CCL5 were only produced in the presence of IL-1β (**Figures 3B and 3C**) while CCL3 were undetectable in any conditions tested (data not shown). Fractalkine measurements were ongoing.

As the direct consequence of vasculopathy during SSc is chronic tissue hypoxia, we then assess whether hypoxia might modulate the production of CCL2, CCL3, CCL4 and CCL5 by HDMEC. To that end, HDMEC were cultured in normoxic (21% O_2) or hypoxic (1% O_2) conditions for 24 hours, in the presence or nor of IL-1β. To validate the hypoxic state of HDMEC, we measured lactic acid production in cell supernatant (James et al., 1999). As expected, we found that lactic acid production was induced in HDMEC cultured in hypoxic conditions (166 and 182 ng/µL for SN and SN* respectively, positive cut-off 100 ng/µL, colorimetric reaction), confirming a hypoxic state of the cells (**Supplementary Figure 1**). Our results indicate that hypoxia does not potentiate the production of CCL2, CCL3, CCL4 and CCL5 by non-activated HDEMC. Interestingly, while hypoxia do not affect IL-1β-induced CCL2 production by HDMEC, it seems to limit IL-1β-induced CCL4 and CCL5 production (**Figure 3A-C**).

Altogether, these results show that IL-1β induces the production of monocytes chemoattractive factors by HDMEC suggesting that IL-1β derived from activated platelets might favor the monocyte's migration into tissue during scleroderma.

IL-1β but not hypoxia induces a M2-like phenotype by HDMEC

In human SSc, macrophages infiltrate skin lesions adopting a perivascular localization in early forms of the disease (Higashi-Kuwata et al., 2010). To test whether IL-1β activated HDMEC could modulate monocyte differentiation and whether hypoxia might potentiate this process, we incubated healthy donors monocytes with medium, SN or SN* for six days. We then assessed the expression of CCR7, CD163 and CD206, DC-SIGN, and CCR2 (**Figure 4A**). As shown in **Figure 4B**, the expression of CD163 is higher in SN*-induced macrophages compared to medium and SN-induced macrophages (21.09%±7.64%, 14.26%±5.197, 57.33%±6.89% in medium, SN and SN* respectively, $p=0.013$ for medium and SN*, $p=0.0025$ for SN and SN*)

while CD206 expression was comparable in each condition (data not shown). Of interest, monocytes incubated in the presence of SN* strongly expressed the type-2 associated macrophage DC-SIGN marker (**Figure 4C,** 10.47%±4.602%, 11.84%±3.054, 41.88%±9.575% in medium, SN and SN* respectively, *p=0.025* for medium and SN*, *p=0.024* for SN and SN*). In sharp contrast, CCR7 (M1 marker) and CCR2 (M2c marker) expression remained unchanged in each condition (data not shown). In parallel, we measured STAT1 and STAT3 phosphorylation in monocytes after 6 hours activation in the presence of medium, SN or SN*. STAT3 phosphorylation was induced in monocytes incubated with IL-1β-activated HDMEC conditioned supernatant (**Figure 4D,** 2.6%±1.42% (medium) vs 3.2%±1.01% (SN) vs 18.8%±2.7% (SN*), *p=0.029*), while expression of pSTAT1 was undetectable in each condition (data not shown). Addition neither anti-IL-1β blocking antibody in SN* inhibited pSTAT3, CD163 or DC-SIGN increased expression, nor adding recombinant IL-1β in the SN induced the expression of the three markers showing that the observed effect was not IL-1β-mediated (**Supplementary Figure 1**).

We finally measured cytokine production in macrophages supernatant after 6 days of culture in the presence of HDMEC conditioned medium. The production of IL-10 and IL-8 were higher in SN*-stimulated macrophage supernatant (SN*Mφ) compared to SN-stimulated macrophage supernatant (SNMφ) while they were not or barely detectable in medium-stimulated macrophage supernatant (MedMφ) (**Figure 4E and 4F**). GM-CSF and IL-6 were only detectable in SN*-stimulated macrophage supernatant (SN*Mφ). The level of IL-1Ra was similar in both SNMφ and SN*Mφ (**Figure 4F**). Regardless of the stimulation conditions, macrophages did not secrete IFN-γ, TNF-α, IL-12p40 and amphiregulin (data not shown). We verified that all of these cytokines were not or barely detectable in SN or SN* (data not shown). As shown in **Figures 5A-F**, similar results were obtained in normoxic and hypoxic conditions suggesting that hypoxia is not involved in M2-like cells generation by HDMEC.

Altogether, these results suggest that IL-1-β-activated HDMEC induce monocyte differentiation into cells adopting a "M2-like" phenotype and producing IL-10, reminiscent of pro-resolving macrophages.

IL6 and Endothelin-1 (ET-1) derived from IL-1β-activated HDMEC induce M2-like phenotype

To identify the soluble factors responsible for this differentiation, we quantified cytokines in HDMEC conditioned medium. Among the cytokines tested, IL-6 and ET-1 production were strongly induced in SN* supernatants (56.68±30.81 pg/mL (SN) *vs* 126.3±49.11 pg/mL (SN*) for ET-1; 200±99.98 pg/mL (SN*) for IL-6), while they were not, or barely, detectable in control conditions (**Figure 5A**).

In order to assess the role of Il-6 and ET-1 produced by HDMEC in the monocyte differentiation toward an M2-like phenotype, we used anti-IL-6 receptor blocking antibody and inhibitor of ET-1 receptors A and B.

As shown in (**Figures 5B-E**), blocking either IL-6R or ET-1R inhibits CD163, DC-SIGN and pSTAT3 expression.

Altogether, those data indicate that IL-1β-activated HDMEC produce IL-6 and ET-1 that promote M2-like macrophages differentiation.

HDMEC induced M2-like cells promote pro-inflammatory and pro-remodelling profile on fibroblast

In order to analyse the effect of HDMEC-induced M2-like macrophages on fibroblasts, co-cultures were carried out and the expression of inflammatory and fibrotic genes were studied.

None of SN or SN*-differentiated macrophages (SNMφ and SN*Mφ respectively) modify Col1A1, Col1A2, MMP-2 and TIMP1 expression in fibroblasts (**Figure 6A, B, D and F**). In sharp contrast, SN*-differentiated macrophages induced strong increased-expression of MMP-1 (**Figure 6C**, 1.508 ± 0.28-fold (SN), 6.36 ± 2.322-fold (SN*) *vs* medium, p=0.049) and CCL2 (**Figure 6E**, 4.478 ± 2.998-fold (SN), 21.61 ± 6.484-fold (SN*) *vs* medium, p=0.05).

Altogether, those data indicate that IL-1β-activated HDMEC promote “pro-resolving-like” M2 macrophages able to induce a potent inflammatory and pro-remodelling profile by fibroblasts.

HDMEC from SSc patient induce pro-resolving M2 like cells with enhanced ability to promote CCL2 production by fibroblasts

In order to test whether HDMEC from SSc patients have altered ability to induce the observed "M2-like"phenotype compared to healthy donors, we first measured IL-6 and ET-1 in HDMEC supernatant from one patient diffuse SSc and HD. We observed a strong increase in IL-6 and

in a lesser extent ET-1 production by SN*-activated HDMEC from SSc compared to SN*-activated HDMEC from HD (301.5169 pg/ml (SN* HD) *vs* 83415,150 pg/ml (SN* SSc).

We next assessed the phenotype induced by the HDMEC conditioned supernatants from SSc or HD on macrophages after 6 days culture as previously described. In a similar way to healthy subjects, HDMEC conditioned supernatant, we observed an increased expression of DC-SIGN and pSTAT3 macrophages differentiated in the presence of IL-1-β activated SSc HDMEC supernatant suggestive of an M2-like polarization. Interestingly, we showed that the increase in pSTAT3 expression is higher in the presence of SSc SN* than with HD SN* (38.3%±11.17% (HD) vs 55.85%±11.28% (SSc), *p=0.03*). We also observed trend toward increase in DC-SIGN expression under the same conditions although non-statistically significant (41.88%±9.575% (HD) vs 56.37%±2.745% (SSc)).

Finally, macrophages induced in the presence of IL-1β activated SSc HDMEC (SSc SN*Mφ). promote similar level of MMP1 mRNA in healthy fibroblasts compared with macrophages induced in the presence of IL-1β activated HD HDMEC (HD SN*Mφ), while the mRNA level of CCL2 appeared to be reproducibly higher in fibroblasts incubated with SSc SN*Mφ.

Taken together those results show that IL-1β activated HDMEC from SSc patient keep the ability to induce "M2 pro-resolving-like" cells that in turn promote pro-remodelling phenotype by fibroblasts and increased CCL2 production compared to healthy HDMEC.

Discussion

Macrophages have emerged as key players in tissue maintenance and repair as well as in fibrosis (Wynn and Vannella, 2016), and they have been implicated in the pathophysiology of scleroderma (Manetti, 2015; Stifano and Christmann, 2016). In human SSc, macrophages infiltrate skin lesions adopting a perivascular localization in early forms of scleroderma (Higashi-Kuwata et al., 2010). Moreover, skin-infiltrating macrophages express CD163 and/or CD204, two markers of M2-type macrophages (Higashi-Kuwata et al., 2010). Serum levels of soluble CD163 are increased in scleroderma patients and are associated with pulmonary fibrosis (Shimizu et al., 2012). Patients presenting a limited form of scleroderma and pulmonary hypertension, are characterized by an increased expression of mRNA coding for IL-13 and MRC1 (c-type mannose receptor 1) at the monocyte level (Christmann et al., 2011). Finally, transcriptome analysis of pulmonary biopsies of SSc patients presenting a diffuse interstitial pulmonary disease has shown a macrophagic-type signature including CD163 and CCL18

overexpression (Christmann et al., 2014). Very recently, the implication of M2 polarized macrophages in two mouse models of SSc have been described (Huang et al., 2017; Maier et al., 2017).

These findings indicate that M2 macrophages may contribute to the persistence of fibrotic disease in patients with SSc. Targeting M2 macrophages might therefore re-establish physiological tissue homeostasis and allow reversal of fibrosis. Yet, the mechanisms involved in macrophages differentiation and activation towards the M2 pathway in scleroderma are not fully understood, and the potential role of microvascular endothelial cell has been not described so far.

In this study, we confirmed alterations in monocytes/macrophages populations during scleroderma and brought new insight on the role of microvascular endothelial cells in this process. We showed that IL-1β is able to profoundly modify cytokines and chemokines production by normal as well SSc microvascular endothelial cells associated to alteration of monocyte circulation and their differentiation in tissue. We demonstrated that IL-1β promote the production of CCL2, CCL4 and CCL5 (fractalkine dosage is ongoing) by HDMEC that might account in observed alteration of monocytes subtypes. Even if the decreased in $CD14^{low}CD16+$ monocyte sub-population in SSc we observed is contradictory to the work of Lescoat et al (Lescoat et al., 2017), we confirm the correlation between this population and the severity of skin fibrosis suggesting that CD16+ monocytes might play a role in the pathogenesis of scleroderma fibrosis. This subtype can differentiate into macrophage after extravasation into inflammatory tissues through the interaction between endothelial cell expressing fractalkine (CX3CL1) and its cognate receptor CX3CR1 (Geissmann et al., 2003). In human SSc, fractalkine is strongly expressed on endothelial cells in the affected skin and lung tissues while the numbers of monocytes/macrophages expressing CX3CR1 are increased in the lesional skin and lung tissues (Hasegawa et al., 2005). The decrease in CD163 expression we observed in $CD14^{low}CD16+$ might be due to the migration of $CD163+CD14^{low}CD16+$ monocytes from blood to affected tissues such as skin or lungs, or to an oxidative-stress mediated proteolytic cleavage of membrane-bound CD163 in line with increased soluble CD163 levels in SSc patients (Frantz et al., 2018).

Our results indicate that not only IL-1-β activated HDMEC are able to favour monocyte recruitment to affected tissue, but also induce the differentiation of recruited monocytes into M2-like macrophages in a mechanism dependent on IL-6 and ET-1 production. Hence, our results suggests that IL-1-β released by activated platelets in the vicinity of microvascular

endothelial cells that might contribute to the high levels of IL-6 and ET-1 observed in SSc that correlate positively with cutaneous fibrosis (Sato et al., 2001; Vancheeswaran et al., 1994). Platelet-derived IL-1-β activated HDMECs could also directly contribute to the increase in the number of M2 macrophages seen in the skin of SSc subjects (Manetti, 2015). We provide evidence that macrophages differentiated in the presence of IL-1β-activated HDMEC, express CD163, DC-SIGN and pSTAT3, and secrete IL-10, IL-6, IL-8 and GM-CSF but no TGFβ, TNFα or IL-1Ra suggesting that the generated macrophages adopt a pro-resolving phenotype. Clinical trials using a monoclonal antibody against the IL-6 receptor, tocilizumab, are currently in phases 2 and 3. If only slight improvements are observed in skin fibrosis and lung function, the analysis of gene expression profiles in skin lesions of SSc patients treated with tocilizumab revealed a critical role of monocyte-macrophage lineage cells in the development of skin fibrosis and the involvement of IL-6 in the activation of those cells (Khanna et al., 2016a). Clinical trials using Macitentan, an ET-1 receptor antagonist, have also been initiated but the results are inconclusive (Khanna et al., 2016b). Our results questioned the interest of combination of blocking IL6 and ET-1 receptors as well as inhibiting platelet activation in future therapeutic perspective. In this line, we are conducting a clinical trial using clopidogrel in early phase of scleroderma to assess whether blocking platelet activation could prevent or limit fibrosis.

These "pro-resolving-like" cells induce a higher production of MMP-1 and CCL2 by fibroblasts suggesting a pro-remodelling and pro-inflammatory effect on fibroblasts. These observations hold true using HDMEC purified from SSc patient suggesting that this process might operate in patients. Indeed, CCL2, produced by both HDMEC and fibroblasts, might perpetuate monocyte migration into the damaged skin, amplifying the inflammatory loop at work in patients affected tissue. The role of MMP-1 is more paradoxical and questioning. Surprisingly, it has been shown in animal models that inhibition of MMP prevents the progression of fibrosis (Corbel et al., 2001; Creemers et al., 2001; Ro et al., 2007). In bleomycin-induced fibrosis, a mice model of SSc, the administration of MMP inhibitor is useful in preventing experimental fibrosis by increasing the activity of MMP-2 and MMP-9 (Corbel et al., 2001). Interestingly, MMP-1 is increased in fibroblasts of early-phase SSc patients and decreased in fibroblasts of late phase patients (Kuroda and Shinkai, 1997) suggesting that, similarly to mice model of SSc for MMP-2 and MMP-9, the increased activity of MMP-1 may contribute to fibrosis in early phase of human SSc. MMP-1, and MMPs in general, digest existing ECM to allow de novo

ECM synthesis and further fibrosis. The activation of MMP-1 can also allow the migration of ECM-producing cells as well as release cytokines present in the tissue that will activate the ECM-producing cells (Jinnin, 2010). Hence, during SSc, platelet-activated microvascular endothelial cell might contribute to increased MMP-1 expression by fibroblasts both directly through IL-6 secretion, and indirectly through their ability to promote M2 like macrophages.
To our knowledge, this is the first study shedding light on a new pathophysiological loop linking vasculopathy, macrophage activation and fibrotic process during scleroderma.
This paves the road toward the early blockade of platelet activation to limit both direct fibroblast activation but also through limitation of immune activation such as M2-like cell differentiation. It also emphasized the role of EC-derived IL-6 and ET-1 in M2-polarization and questioned the potential effect of IL-6 and/or ET-1 receptor blockers in limiting M2-like cell accumulation during the early phase of the disease.

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Figure legends

Figure 1 : Monocytes subsets repartition and phenotype are altered in SSc patient

(A) Representative staining of circulating monocyte subsets and expression of CD163 and CD206 in $CD14^{high}$CD16-, $CD14^{high}$CD16+ and $CD14^{low}$CD16+ monocytes. (B-D) Percentage of $CD14^{high}$CD16-, $CD14^{high}$CD16+ and $CD14^{low}$CD16+ monocytes in HD and SSc blood. (E) Proportion of monocyte subsets in HD and SSc blood. (F-G) Expression of CD163 and CD206 in $CD14^{high}$CD16-, $CD14^{high}$CD16+ and $CD14^{low}$CD16+ monocytes in HD and SSc blood. (H) Correlation between the percentage of CD163+ $CD14^{low}$CD16+ and the extent of fibrosis assessed by the modified Rodnan skin score. (I) Frequency of $CD14^{high}$CD16+ in SSc patient with or without vasculopathy.

Bar graphs show data as mean ± SEM. Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

Figure 2 : Number of "M2-like" macrophages are higher in SSc skin compared to HD skin

Figure 3 : IL-1b-activated HDMEC produce CCL2, CCL4 and CCL5.

(A-C) Assay of CCL2, CCL4 and CCL5 in culture medium, in the supernatant of non-activated HMEC (SN) and in the supernatant of IL-1β-activated HDMEC (SN*).

Figure 4 : IL-1b-activated HDMEC induce "M2-like" polarization

(A) Representative staining of CD163, DC-SIGN and pSTAT3 expression in medium, SN or SN*-incubated macrophages after 6 days' culture. (B-D) Cumulative data obtained from x independent experiments. (E-F) Production of IL-10, IL-8, IL-6, GM-CSF and IL-1Ra in medium, SN or SN*-stimulated macrophages supernatants.

Bar graphs show data as mean ± SEM. Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$

Figure 5 : Hypoxia does not induce nor potentiate M2 polarization induced by HDMEC

(A-B) Representative staining of CD163, DC-SIGN and pSTAT3 in hypoxic or normoxic macrophages incubated with medium, SN and SN*. (C-F) CD163, DC-SIGN and pSTAT3 expressions and IL-10 production in medium, SN and SN*-stimulated macrophages in hypoxia or normoxia conditions.

Bar graphs show data as mean ± SEM (n=4). Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$

Figure 6 : IL-1b-activated HDMEC produce IL-6 and ET-1 triggering "M2-like" polarization

(A) Production of IL-6 and ET-1 by medium, SN or SN*. (B) Representative staining of CD163, DC-SIGN and pSTAT3 in medium, SN or SN*-incubated macrophages. (C-E) Cumulative data from x independent experiments.

Bar graphs show data as mean ± SEM. Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$

Figure 7 : Macrophages differentiated in the presence of IL-1b-activated HDMEC supernatant have a pro-inflammatory and pro-remodelling activities on fibroblasts

(A-F) RTqPCR analysis of Col1A1, Col1A2, MMP1, MMP2, CCL2 and TIMP-1 mRNA in fibroblasts incubated with TGFb, SN-stimulated macrophages (SNMΦ) and SN*-stimulate (SN*MΦ) macrophages.

Bar graphs show data as mean ± SEM from 3 independent experiments. Comparison between groups was calculated using the paired Wilcoxon test and Kruskal Wallis tests. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

Figure 8 : HDMEC from SSc patient induced pro-resolving M2 like cells with enhanced ability to promote CCL2 production by fibroblasts compared to healthy donor HDMEC

(A) Production of IL-6 and ET-1 by non activated-HDMEC and SN or SN*-activated HDMEC from HD and SSc. (B) Representative staining of DC-SIGN and pSTAT3 expression in macrophages generated in the presence of healthy donor or SSc HDMEC supernatants after 6 days culture. (C-D) Cumulative data obtained from 3 independent experiments. (E-F) RTqPCR analysis of MMP1 and CCL2 mRNA expression in fibroblasts incubated with TGFb, or macrophages generated in the presence of supernatant form IL-1-b activated or nonactivated HDMEC from HD and SSc. The data show two independent experiments.

Bar graphs show data as mean ± SEM. Comparison between groups was calculated using the paired Wilcoxon test and Kruskal Wallis tests. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

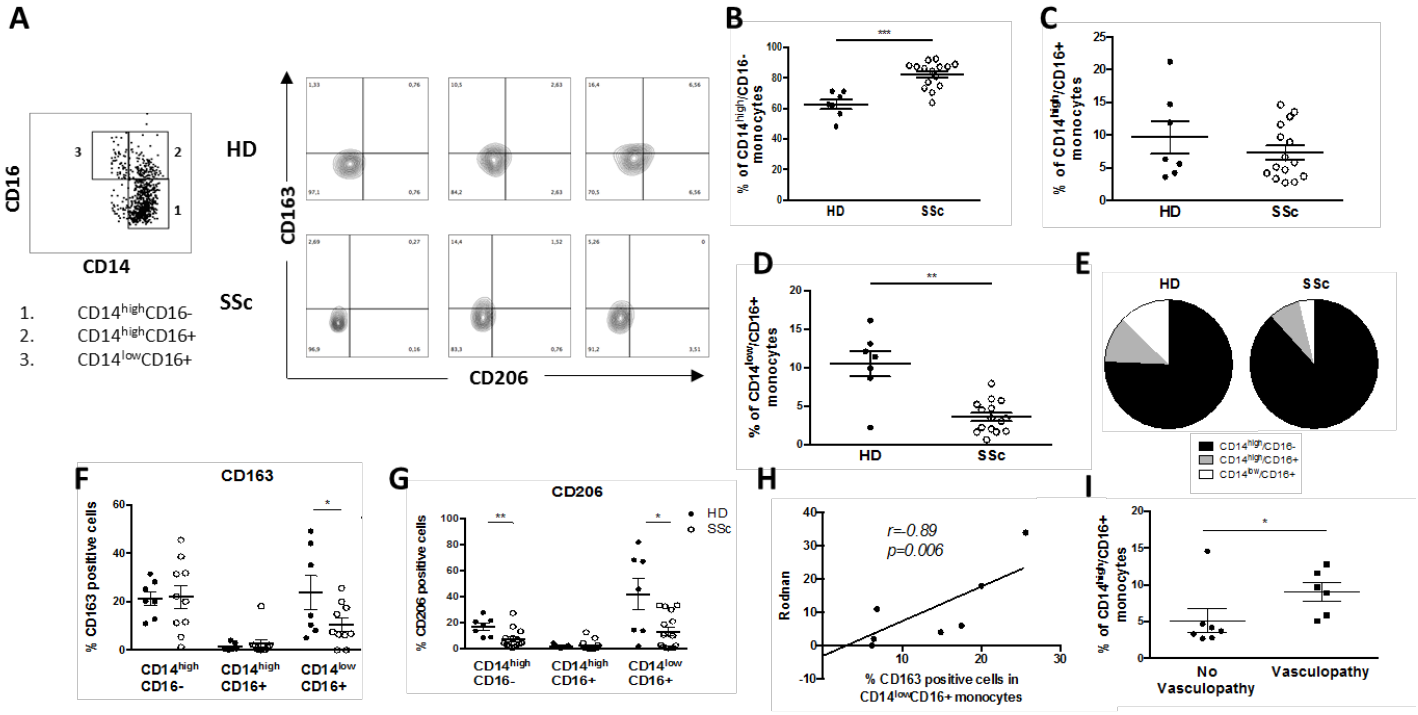


Figure 1 : Monocytes subsets repartition and phenotype are altered in SSc patient

(A) Representative staining of circulating monocyte subsets and expression of CD163 and CD206 in $CD14^{high}CD16-$, $CD14^{high}CD16+$ and $CD14^{low}CD16+$ monocytes. (B-D) Percentage of $CD14^{high}CD16-$, $CD14^{high}CD16+$ and $CD14^{low}CD16+$ monocytes in HD and SSc blood. (E) Proportion of monocyte subsets in HD and SSc blood. (F-G) Expression of CD163 and CD206 in $CD14^{high}CD16-$, $CD14^{high}CD16+$ and $CD14^{low}CD16+$ monocytes in HD and SSc blood. (H) Correlation between the percentage of CD163+ $CD14^{low}CD16+$ and the extent of fibrosis assessed by the modified Rodnan skin score. (I) Frequency of $CD14^{high}CD16+$ in SSc patient with or without vasculopathy.

Bar graphs show data as mean ± SEM. Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

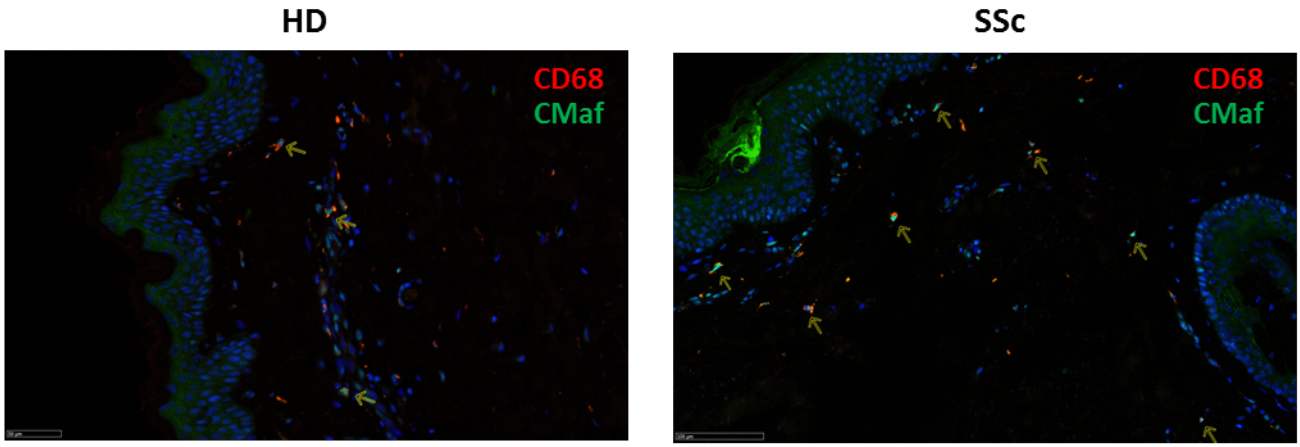


Figure 2 : Number of "M2-like" macrophages are higher in SSc skin compared to HD skin

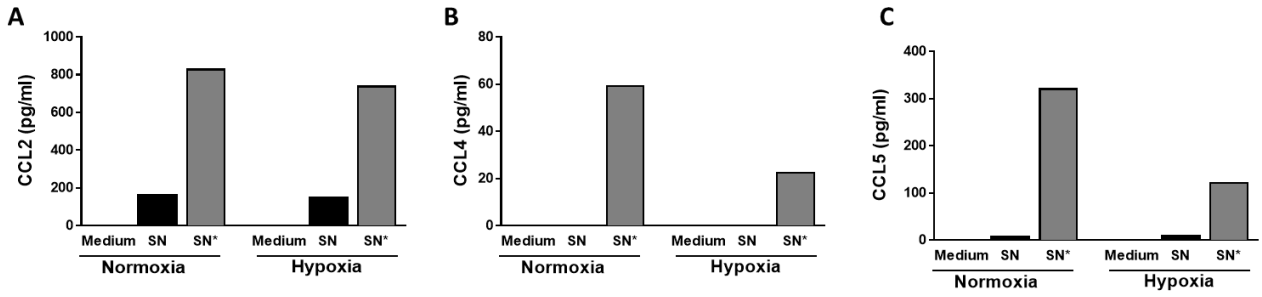


Figure 3 : IL-1β-activated HDMEC produce CCL2, CCL4 and CCL5.
(A-C) Assay of CCL2, CCL4 and CCL5 in culture medium, in the supernatant of non-activated HMEC (SN) and in the supernatant of IL-1β-activated HDMEC (SN*).

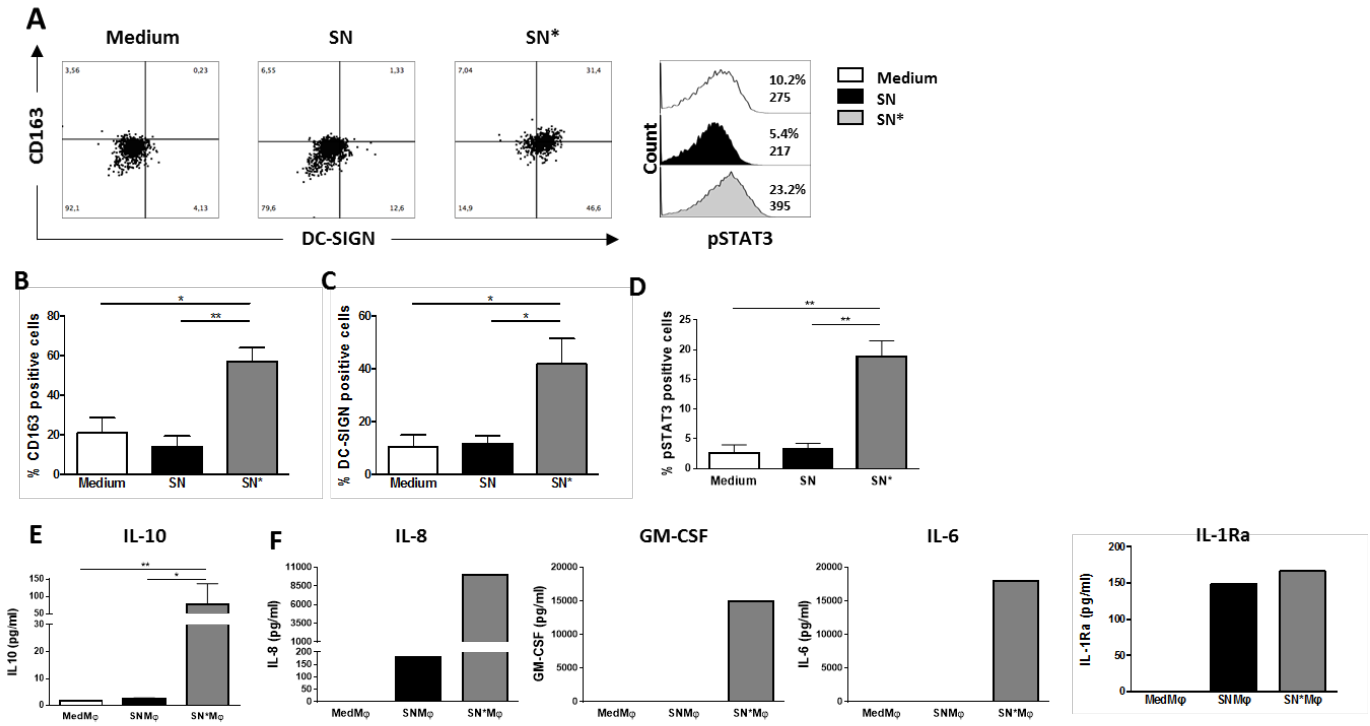


Figure 4 : IL-1β-activated HDMEC induce "M2-like" polarization

(A) Representative staining of CD163, DC-SIGN and pSTAT3 expression in medium, SN or SN*-incubated macrophages after 6 days culture. (B-D) Cumulative data obtained from 4 independent experiments. (E-F) Production of IL-10, IL-8, IL-6, GM-CSF and IL-1Ra in medium, SN or SN*-stimulated macrophages supernatants.

Bar graphs show data as mean ± SEM. Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$

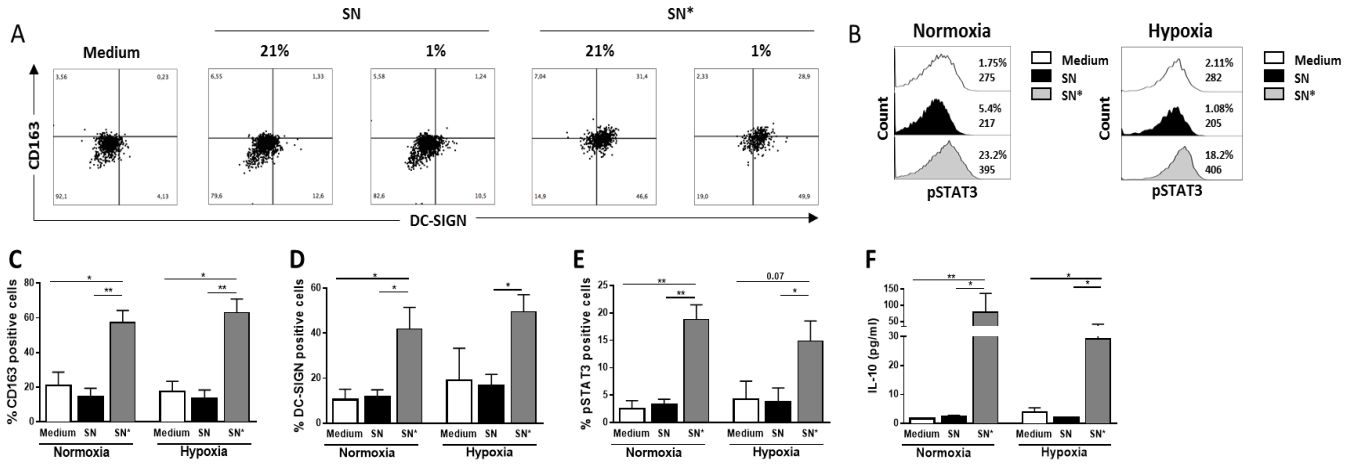


Figure 5 : Hypoxia does not induce nor potentiate M2 polarization induced by HDMEC

(A-B) Representative staining of CD163, DC-SIGN and pSTAT3 in hypoxic or normoxic macrophages incubated with medium, SN and SN*. (C-F) CD163, DC-SIGN and pSTAT3 expressions and IL-10 production in medium, SN and SN*-stimulated macrophages in hypoxia or normoxia conditions.

Bar graphs show data as mean ± SEM (n=4). Comparison between groups was calculated using the unpaired Mann-Whitney test.
*, P < 0.05; **, P < 0.01 ***, P < 0.001

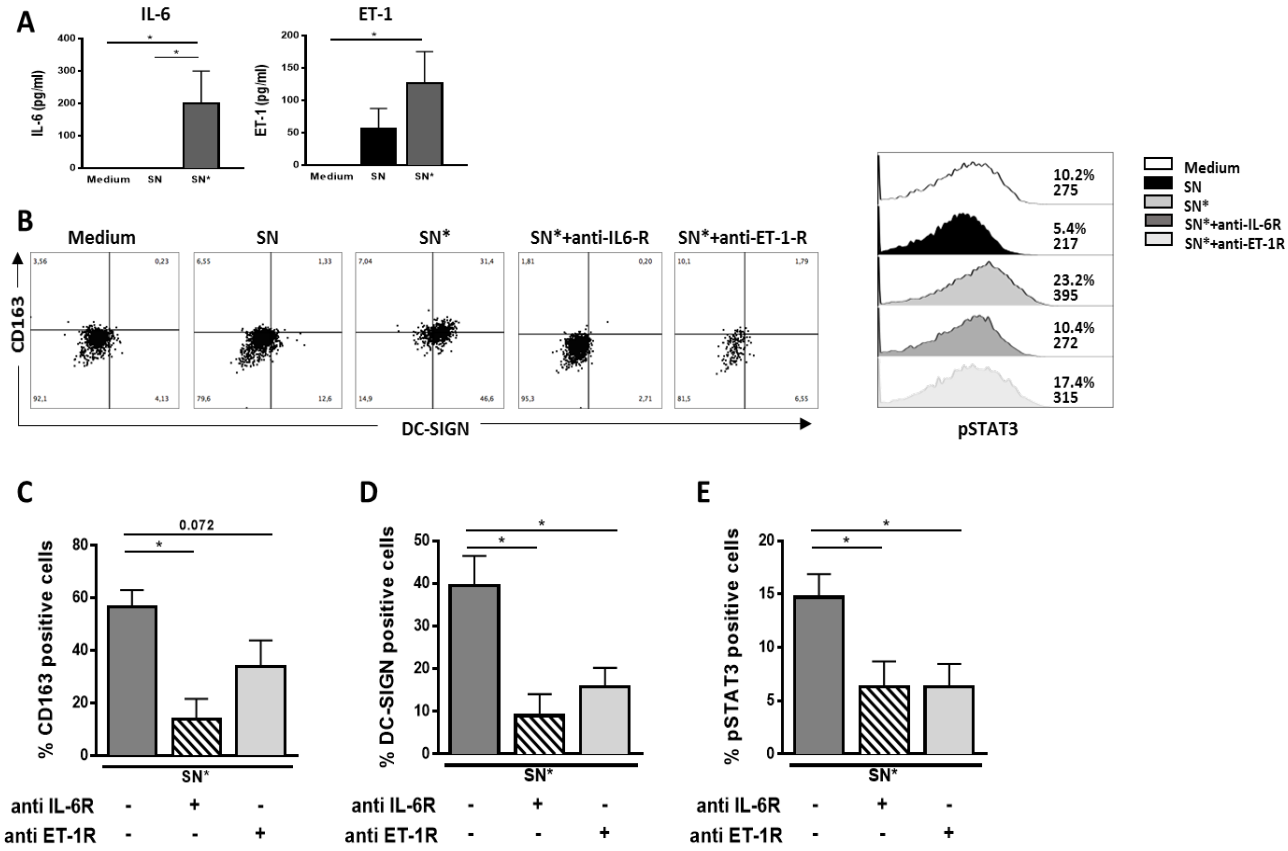


Figure 6 : IL-1β-activated HDMEC produce IL-6 and ET-1 triggering "M2-like" polarization

(A) Production of IL-6 and ET-1 by medium, SN or SN*. (B) Representative staining of CD163, DC-SIGN and pSTAT3 in medium, SN or SN*-incubated macrophages. (C-E) Cumulative data from x independent experiments.

Bar graphs show data as mean ± SEM. Comparison between groups was calculated using the unpaired Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$

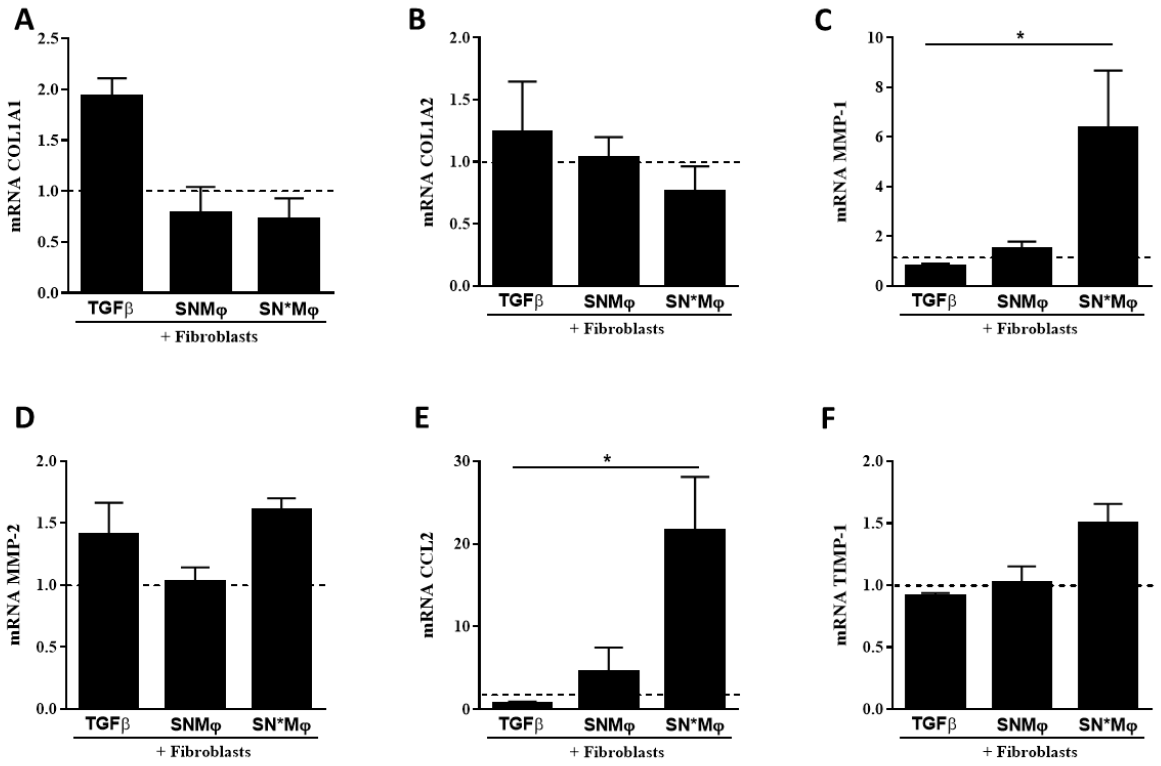


Figure 7 : Macrophages differentiated in the presence of IL-1β-activated HDMEC supernatant have a pro-inflammatory and pro-remodelling activities on fibroblasts

(A-F) RTqPCR analysis of Col1A1, Col1A2, MMP1, MMP2, CCL2 and TIMP-1 mRNA in fibroblasts incubated with TGFβ, SN-stimulated macrophages (SNMφ) and SN*-stimulate (SN*Mφ) macrophages.

Bar graphs show data as mean ± SEM from 3 independent experiments. Comparison between groups was calculated using the paired Wilcoxon test and Kruskal Wallis tests. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.

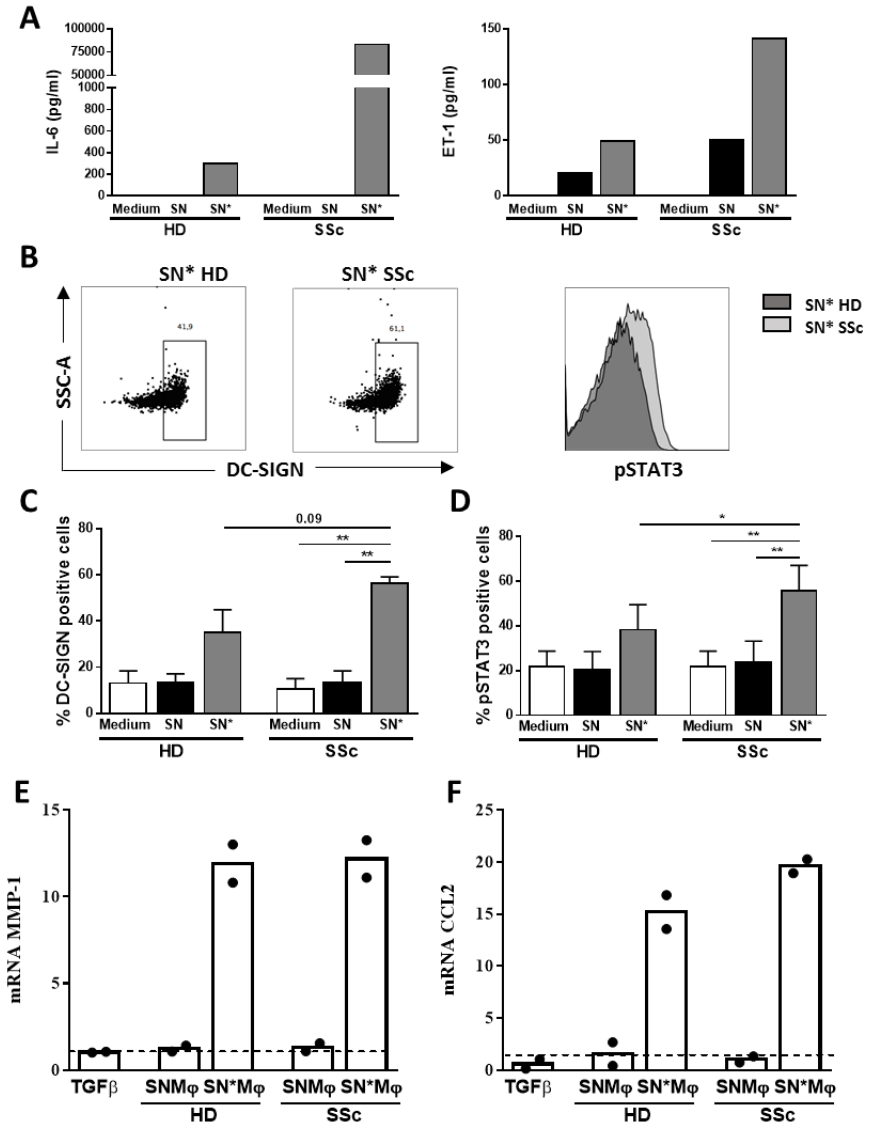
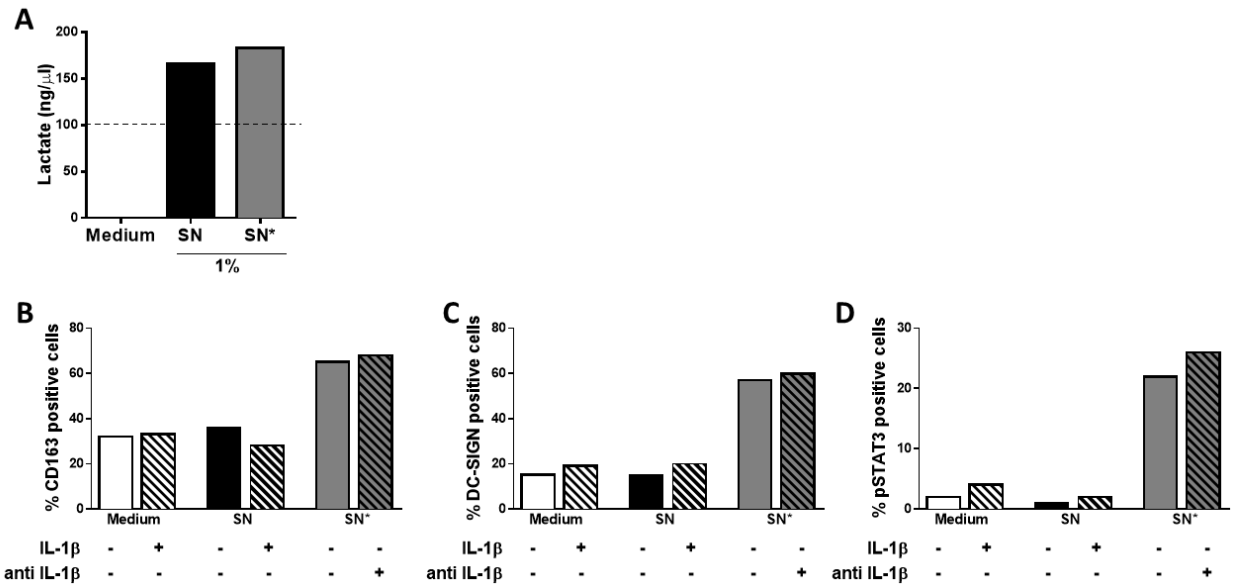


Figure 8 : HDMEC from SSc patient induced pro-resolving M2 like cells with enhanced ability to promote CCL2 production by fibroblasts compared to healthy donor HDMEC

(A) Production of IL-6 and ET-1 by non activated-HDMEC and SN or SN*-activated HDMEC from HD and SSc. (B) Representative staining of DC-SIGN and pSTAT3 expression in macrophages generated in the presence of healthy donor or SSc HDMEC supernatants after 6 days culture. (C-D) Cumulative data obtained from 3 independent experiments. (E-F) RTqPCR analysis of MMP1 and CCL2 mRNA expression in fibroblasts incubated with TGFβ, or macrophages generated in the presence of supernatant form IL-1-β activated or nonactivated HDMEC from HD and SSc. The data show two independent experiments.

Bar graphs show data as mean ± SEM. Comparison between groups was calculated using the paired Wilcoxon test and Kruskal Wallis tests. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$.



Supplementary Figure 1 :
(A) Production of lactate by SN and SN* activated-HDMEC under hypoxic conditions (B-D) Expression of CD163, DC-SIGN and pSTAT3 after 6 days of incubation with: medium +/- IL-1β, SN +/- IL-1β and SN* +/- anti-IL-1β.

III) Discussion

La sclérodermie systémique est une maladie auto-immune dont la physiopathologie est encore mal comprise. Elle est principalement définie par trois caractéristiques : (i) une vasculopathie, (ii) une dérégulation de la réponse immunitaire (iii) et une fibrose pouvant affecter la peau et les organes internes. Malgré de nombreux essais cliniques menés, la sclérodermie systémique reste une maladie sans traitement spécifique notamment pour la part fibrotique.

Les ILC2, cellules récemment décrites, sont des cellules résidentes dans les tissus jouant un rôle de sentinelle. Tandis que de plus en plus d'études montrent une implication de ces cellules dans la fibrose hépatique et pulmonaire, leur potentielle implication dans la ScS a jusqu'ici été très peu étudiée.

Les ILC2, un rôle fibrotique dans la ScS

Par un marquage sur sang total, j'ai caractérisé les ILC dans leur ensemble et les ILC1, les ILC2 et les ILC3. J'ai montré que la fréquence des ILC était diminuée dans le sang des patients ScS comparé à des HD. Cette diminution observée était due à la fois à la baisse des ILC2 et des ILC3 circulantes. Contrairement à la fréquence des ILC3 circulantes, le pourcentage d'ILC2 sanguines était corrélé à l'étendue de l'atteinte cutanée chez les patients ScS. Suivant ce résultat très intéressant, j'ai donc mesuré et phénotypé les différents sous-types d'ILC cutanés. J'ai observé une augmentation du nombre d'ILC2 tandis que les ILC3 étaient indétectables dans les deux types de peaux (saines et HD), suggérant un site de migration préférentiel différent pour les ILC3. Il est intéressant de noter que les patients ScS peuvent également atteints de troubles gastro-intestinaux. Etant donné le lien fort entre l'intestin et les ILC3 (Mortha et al., 2014), de futures investigations pourraient être menées afin d'étudier le rôle potentiel des ILC3 dans la ScS au niveau intestinal. Nos réflexions conceptuelles ont également été présentes lors de l'analyse des résultats. En effet, la diminution des ILC2 sanguines chez les patients SCS, nous a dans un premier temps surpris. Nous nous sommes rattachés alors à un de nos trois concepts majeurs : la **localisation**. Même si la ScS comporte des anomalies vasculaires, la fibrose a lieu au niveau des tissus et notamment au niveau de la

peau. Après l'analyse cutanée montrant une augmentation des ILC2 chez les patients, nous nous sommes également attachés à prendre en compte toutes les interprétations possibles de ce résultat. En effet, l'hypothèse première était que les ILC2 migraient du sang vers la peau. Toutefois, deux autres conjectures étaient également envisagées : la prolifération plus importante des ILC2 résidentes dans la peau ScS comparée à celle de la peau saine et la **plasticité** entre les ILC1, également présente au niveau cutané, vers les ILC2. Des expériences supplémentaires devront être faites afin d'étayer ces hypothèses.

J'ai ensuite phénotypé les ILC2 dermiques et j'ai mis en évidence une diminution de l'expression de KLRG1 chez les sujets ScS. Cependant, les ILC2 de patients atteints d'une ScS diffuse progressive exprimaient plus fortement KLRG1 comparé aux ILC2 de patients atteints de SCS diffuse quiescente suggérant une plasticité des iILC2 vers les nILC2 dépendant du microenvironnement cutané spécifique à la ScS.

L'implication des ILC2 dans la ScS a également été étudié dans un modèle murin induit de ScS en collaboration avec le Pr. Batteux. Le modèle d'induction de la ScS par HOCl présente un avantage majeur : il sépare la progression de la maladie en 2 phases (i) une phase inflammatoire où la fibrose n'est pas encore établie et (ii) une phase chronique où la fibrose apparait. Ces deux phases correspondent respectivement à la phase progressive et à la phase quiescente de la ScS diffuse chez l'Homme. Ce modèle nous a permis de confirmer le switch entre les iILC2 et les nILC2 au cours de la maladie avec une diminution d'ILC2 KLRG1^{high} dans la phase chronique de la maladie.

In vitro, après avoir amplifié les ILC2 purifiées du sang selon un protocole déjà établi (Bal et al., 2016; Ohne et al., 2016), j'ai mis en évidence sous l'action du TGFβ, l'expression de KLRG1 diminuait à la surface des ILC2. Des études fonctionnelles m'ont permis de montrer que les ILC2 stimulées avec du TGFβ avait un rôle pro-fibrotique en induisant une transcription plus importante de collagène par les fibroblastes du fait de leur production plus faible d'IL10, cytokine ayant un rôle anti-fibrotique (Nelson et al., 2000). La perte d'expression de KLRG1 peut-elle induire la diminution d'IL10 par les ILC2 ? Le rôle inhibiteur de KLRG1 est dépendant de SHIP1 (Tessmer et al., 2007). SHIP dégrade PIP3 (Phosphatidylinositol (3,4,5)-triphosphate) régulant ainsi la fonction de PI3K (phosphoinositide 3-kinase) et Akt (Tessmer et al., 2007). Or, une inhibition d'Akt entraîne une augmentation de la production d'IL10 (Martin et al., 2003). Nous pouvons donc supposer que suite à une exposition au TGFβ, les ILC2 perdent l'expression de KLRG1 entraînant ainsi une perte d'inhibition de PI3K/Akt et menant à une diminution de

la production d'IL10 par les nILC2 (ILC2 KLRG1^{low}) qui deviennent alors pro-fibrotique. Des thérapeutiques futures pourraient éventuellement cibler les iILC2 au cours de la ScS afin d'éviter une plasticité des iILC2 vers les nILC2 responsables de l'établissement de la fibrose.

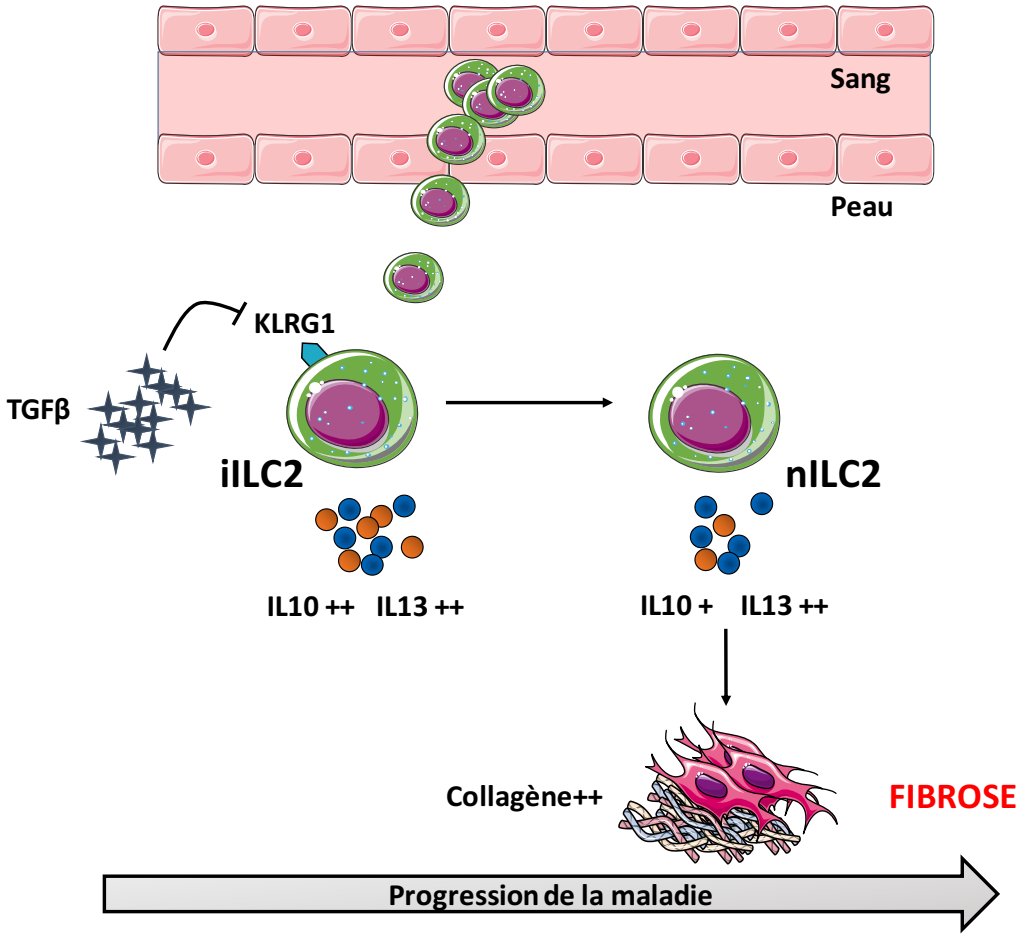


Figure 15 : Schéma résumant les travaux obtenus au cours de ma thèse sur l'étude de l'implication des ILC2 dans la fibrose de la ScS

Les M2, un rôle inflammatoire dans la ScS

Ce projet, encore en cours, a pour l'instant permis de mettre en évidence une altération de la proportion des trois sous-types de monocytes dans le sang de patients ScS comparé aux sujets sains. Cette altération est notamment due à une diminution de la fréquence des monocytes CD14^{low}CD16+ chez les patients ScS. J'ai également montré une diminution de l'expression de

CD163 à la surface des $CD14^{low}CD16+$. De manière surprenante, l'intensité de l'expression de CD163 à la surface de ces cellules est corrélée positivement à l'étendue de la fibrose cutanée. Une étude menée en 2010 montre que ces $CD14^{low}CD16+$ circulent au plus près de l'endothélium afin de surveiller des signes d'inflammation et de dommages tissulaires. Ces cellules sont rapidement capables de migrer à travers l'endothélium vers le tissu lésé (Cros et al., 2010).Cette diapédèse se fait grâce à l'interaction entre la fractalkine (CX3CL1), exprimée par les CE, et son ligand CX3CR1 présent à la surface des monocytes. Dans la ScS, la fractalkine est fortement exprimée par les CE dans la peau lésée et les poumons tandis que le nombre de monocytes/macrophages exprimant CX3CR1 est augmenté dans la peau et les poumons (Hasegawa et al., 2005). J'ai montré une diminution de CD163 à la surface des $CD14^{low}CD16+$ et une corrélation positive entre l'intensité d'expression de ce marqueur et l'étendue de la fibrose cutanée. Les monocytes $CD14^{low}CD16+CD163+$ semblent donc rapidement migrer du sang vers la peau afin de participer à la fibrose. La CE, pouvant produire également CCL2 (Deshmane et al., 2009), peut induire la migration des monocytes $CD14^{high}CD16-$ exprimant très fortement CCR2 (Weber et al., 2000). Des dosages supplémentaires des surnageants des CE devront être effectuées afin de mesurer la production de chimiokines par les CE. Une fois dans la peau, les macrophages sont localisés près des vaisseaux.

L'induction de la polarisation macrophagique tournée vers le type 2 n'est pas connue dans le contexte de la ScS. Etant donné la localisation préférentielle des macrophages dans la peau ScS, nous avons émis l'hypothèse que les CE pourraient être responsables de la polarisation macrophagique de type 2 dans la ScS. La conséquence directe de la vasculopathie durant la maladie est l'hypoxie chronique (Beyer et al., 2009). Nous avons donc testé la capacité des HDMEC hypoxiques à induire la polarisation macrophagique. Cependant, nos expériences *in vitro* n'ont pas permis de mettre en évidence une induction de la différenciation M2 par l'hypoxie. Toutefois, le **contexte** de la ScS reste très important. En effet, l'expérience devra être réitérée avec des CE ScS, qui sont différentes des CE saines et pouvant ainsi réagir autrement à l'hypoxie. De plus, l'hypoxie n'a pas lieu continuellement chez le patient ScS, mais est plutôt caractérisée par une succession d'état hypoxique et normoxique. Une expérience mimant cet enchaînement de conditions d'hypoxie et de normoxie pourrait être envisagée.

Nous avions déjà montré précédemment un rôle indirect des plaquettes dans la fibrose via l'activation des CE (Truchetet et al., 2016). Nous avons supposé que les plaquettes pouvaient également être impliquées dans la polarisation macrophagique de type 2 via leur action sur

les CE. Pour cela, nous avons modélisé cette activation des CE par les plaquettes par la stimulation des CE par de l'IL1β. Sous l'effet des HDMEC activées par l'IL1β, les macrophages générés expriment plus fortement le CD163, DC-SIGN et pSTAT3, marqueurs spécifiques des M2. Ils produisent également de l'IL10, de l'IL6, de l'IL8 et du GM-CSF. A l'inverse de l'IL6, le rôle des trois autres cytokines est mal connu dans la ScS. Cette différenciation est dépendante de la production d'IL6 et d'ET1 par les HDMEC. L'IL8 est un chimiokine qui permet notamment la migration de neutrophiles (Kunkel et al., 1991). Les neutrophiles sont une source importante de ROS dans la ScS amplifiant alors la boucle de la vasculopathie et la fibrose (Abdulle et al., 2018; Barnes et al., 2012). Le taux sérique d'IL10 est augmenté chez les patients ScS (Hasegawa et al., 1997) tandis que la fréquence les LB produisant de l'IL10 est diminuée (Forestier et al., 2018).

Afin d'analyser le rôle des macrophages générés, j'ai effectué des co-cultures entre ces macrophages et des fibroblastes en utilisant un transwell. J'ai observé que les macrophages stimulés avec du surnageants de HDMEC activées avec de l'IL1b induisaient une transcription plus importante de CCL2 et de MMP1. Tout comme les CE, les fibroblastes participent donc à la chimioattraction des monocytes $CD14^{high}CD16$- ayant une activité de phagocytose importante (Wong et al., 2012), dans la peau des ScS où leur rôle reste encore à déterminer. La production de MMP1 par les fibroblastes est augmentée dans les phases précoces de la maladie puis est diminuée dans les phases tardives (Kuroda and Shinkai, 1997) suggérant que la production de MMP1 est dépendante du **contexte** et de la **localisation**. De manière paradoxale, l'injection d'inhibiteurs de MMP dans des souris provoquait une inhibition de la progression de la fibrose (Corbel et al., 2001; Creemers et al., 2001; Ro et al., 2007). La digestion de la MEC par les MMP pourrait permettre la libération de cytokines pro-fibrotique piégées dans la MEC, comme le TGFβ résultant à la fibrose tissulaire. De plus, le microenvironnement ScS est probablement nécessaire aux macrophages que nous avons générés afin qu'ils exercent leur réelle fonction au sein du tissu lésé.

Des expériences sont actuellement en cours afin de finir ce projet et de répondre aux nombreuses interrogations encore non résolues.

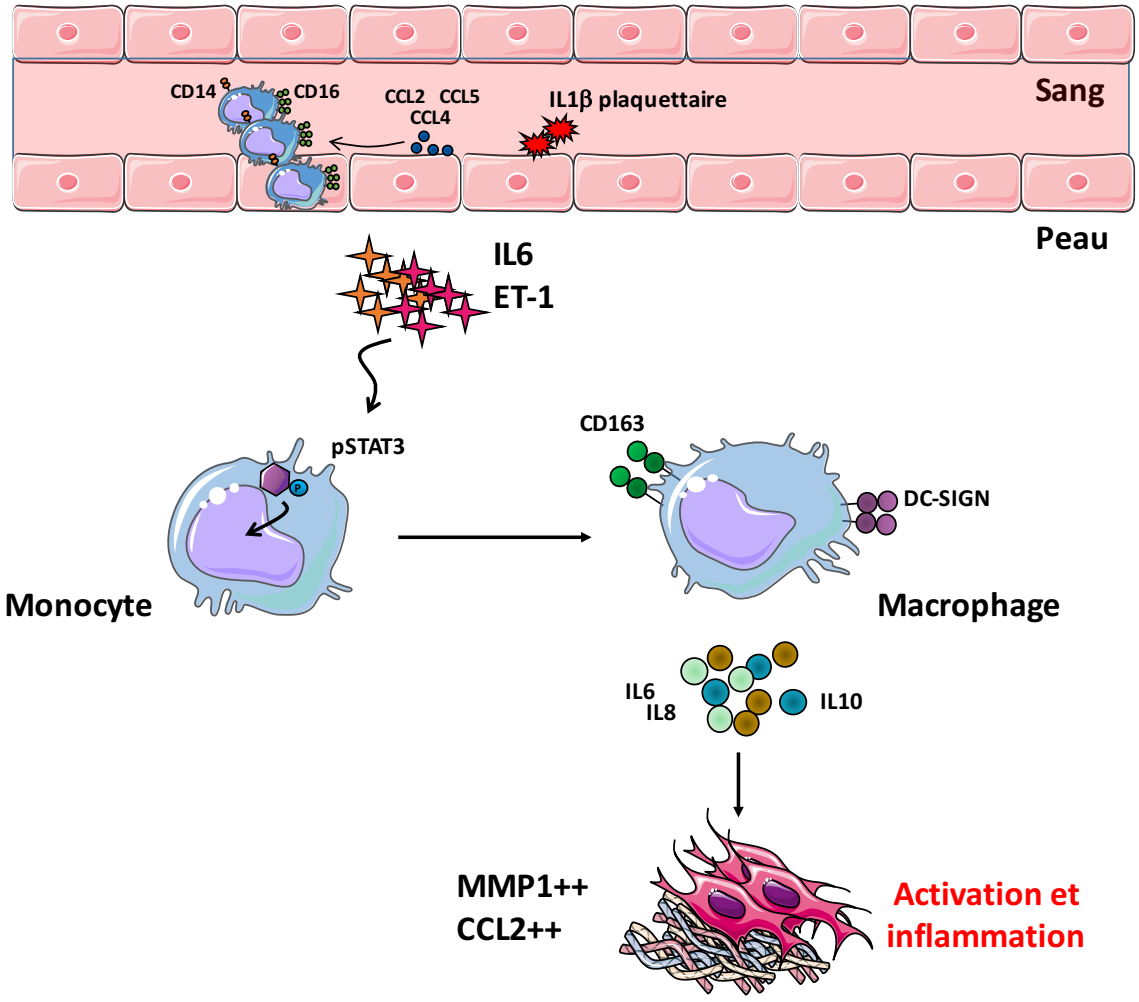


Figure 16 : Schéma résumant les résultats obtenus au cours de ma thèse sur l'implication des macrophages de type 2 dans la ScS

Le **contexte** et la **localisation** des cellules étudiées étant fondamentaux, nous avons essayé ou envisagé de travailler, dans la mesure du possible, avec des cellules de la peau ScS. Au cours de nos expériences, cela a quelquefois été impossible à faire, menant à plusieurs interrogations telles que : les ILC2 sclérodermiques sont-elles différentes des ILC2 provenant de la peau saine ?

Pour répondre à cette question, la technique du Nanostring permettant de faire une étude transcriptomique de la peau sur près de 770 gènes sera réalisée.

Nous avons aussi commencé une collaboration avec le Pr. Eric Vivier à Marseille, qui est actuellement en cours afin de faire du « RNA single cell analysis » sur des ILC dermiques de ScS comparé aux ILC dermiques de sujets sains. Cette étude pourrait permettre de souligner la **plasticité** des ILC dans la peau des malades en les classant par des clusters autres que les clusters classiques des ILC : ILC1, ILC2 et ILC3.

Conclusion générale

En conclusion, mon travail de thèse a permis de mettre en évidence le rôle crucial des ILC2 dans la fibrose ainsi que le rôle pro-inflammatoire des macrophages de type 2 au cours de la ScS. La plasticité des ILC2 ainsi que celle des macrophages apparaît comme une cible thérapeutique de choix dans le traitement contre la ScS. Tandis que le blocage de cette plasticité cellulaire semble être une solution de premier plan, il pourrait être également intéressant d'aider le système immunitaire à stopper lui-même ce switch cellulaire. Peut-on envisager d'étendre ces observations à la fibrose d'autres organes touchés dans la ScS et même plus généralement à d'autres maladies fibrosantes ?

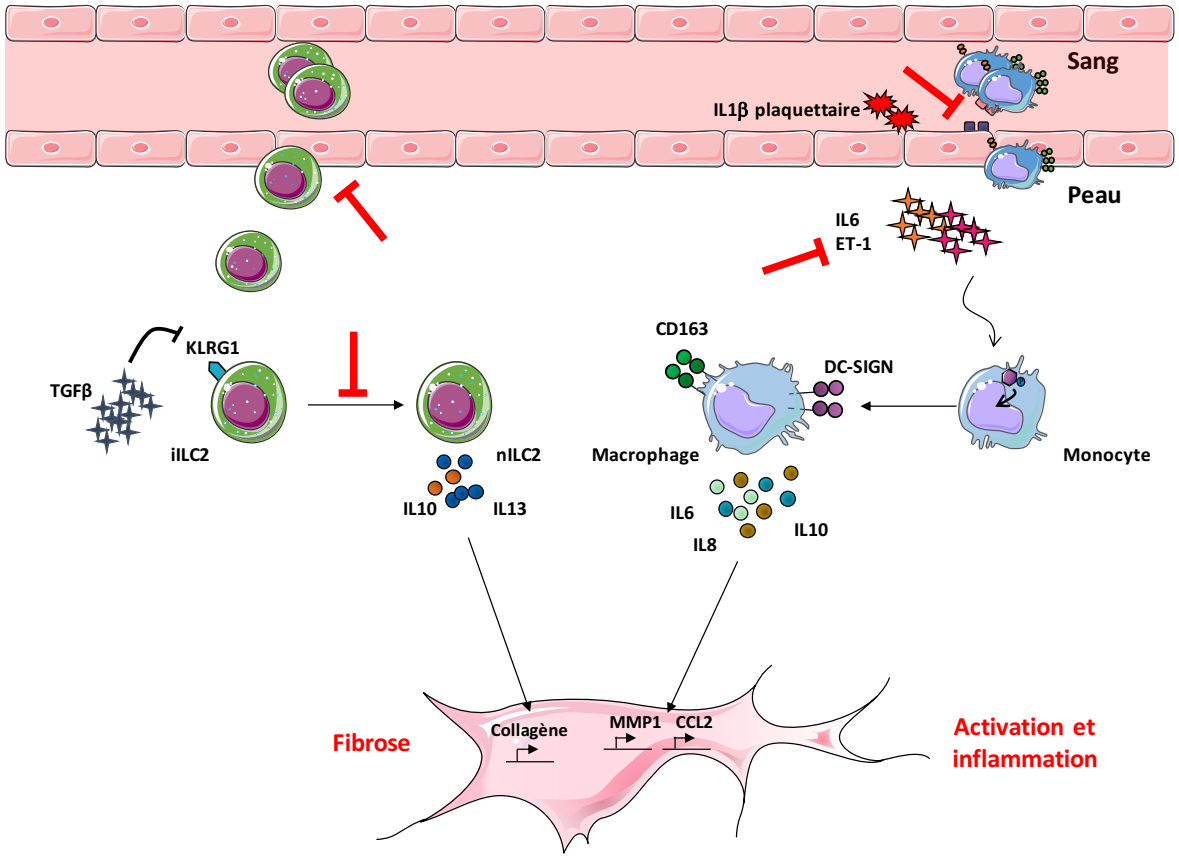


Figure 17 : Schéma intégrant l'ensemble des résultats obtenus durant ma thèse et les potentielles perspectives thérapeutiques qu'ils offrent

Ces travaux apportent toutefois des éléments nouveaux et intéressants dans la compréhension de la ScS.

Cette approche conceptuelle du rôle du système immunitaire dans la fibrose a été fondée sur trois concepts clés : la localisation de la fibrose, le contexte dans lequel la fibrose intervient et la plasticité des cellules impliquées dans la fibrose. Cette nouvelle vision de l'implication du SI dans la fibrose offre potentiellement un nouveau champ, encore sous-estimé, de voies thérapeutiques potentielles.

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ANNEXE

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Platelets Induce Thymic Stromal Lymphopoietin Production by Endothelial Cells

Contribution to Fibrosis in Human Systemic Sclerosis

Marie-Elise Truchetet,[1] Béatrice Demoures,[2] Jorge Eduardo Guimaraes,[3] Anne Bertrand,[4] Paôline Laurent,[2] Valérie Jolivel,[2] Isabelle Douchet,[2] Clément Jacquemin,[2] Liliane Khoryati,[2] Pierre Duffau,[1] Estibaliz Lazaro,[1] Christophe Richez,[1] Julien Seneschal,[4] Marie-Sylvie Doutre,[4] Jean-Luc Pellegrin,[4] Joël Constans,[4] Thierry Schaeverbeke,[4] Patrick Blanco,[1] and Cécile Contin-Bordes,[1] on behalf of the Fédération Hospitalo-Universitaire ACRONIM

Objective. **To investigate the relationship between vascular damage and fibrosis in systemic sclerosis (SSc) by testing the hypothesis that platelets contribute to skin fibrosis via the activation of human dermal microvascular endothelial cells (HDMECs) and subsequent production of profibrotic mediators.**

Methods. **A total of 203 SSc patients and 30 healthy donors were prospectively enrolled between 2012 and 2015 at the University Hospital of Bordeaux. Immunohistochemistry and immunofluorescence analyses were performed on skin biopsy sections from 18 SSc patients and 5 healthy donors. Serum thymic stromal lymphopoietin (TSLP) levels were measured by enzyme-linked immunosorbent assay in the entire cohort. HDMECs and fibroblasts were purified from biopsy sections. Extracellular matrix production by cultured fibroblasts was assessed by real-time quantitative polymerase chain reaction.**

Results. **Serum TSLP levels were significantly increased in SSc patients compared to healthy donors ($P < 0.0001$) and were associated with a higher frequency of vasculopathy ($P = 0.02$). The proportion of TSLP-positive dermal cells was increased in the skin of SSc patients compared with healthy donors ($P < 0.0001$) and was correlated with fibrosis (modified Rodnan skin thickness score) ($r = 0.6146$, $P = 0.0001$). In SSc dermis, TSLP was mainly expressed by CD31-positive endothelial cells. In vitro, activated platelets induced TSLP production by HDMECs in an interleukin-1β–dependent manner. SSc fibroblasts responded differently according to their original TSLP environment.**

Conclusion. **Taken together, these results identify HDMECs as contributors to TSLP production in SSc and suggest a potential mechanism by which platelets may profoundly affect the fibrotic process in SSc.**

Systemic sclerosis (SSc) is a complex systemic autoimmune disease that is characterized by microvascular dysfunction, immune activation, and interstitial and perivascular fibrosis affecting the skin and internal organs (1). The interrelationships between these processes are unknown, and the elucidation of the interplay between these 3 pathologic hallmarks would enhance knowledge of the pathogenesis of this disease and,

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[1]Marie-Elise Truchetet, MD, PhD, Pierre Duffau, MD, PhD, Estibaliz Lazaro, MD, PhD, Christophe Richez, MD, PhD, Patrick Blanco, MD, PhD, Cécile Contin-Bordes, PhD: CNRS-UMR 5164 Immuno ConcEpT, Bordeaux University and Bordeaux Hospital, Bordeaux, France; [2]Béatrice Demoures, Paôline Laurent, Valérie Jolivel, PhD, Isabelle Douchet, Clément Jacquemin, PhD, Liliane Khoryati, PhD: CNRS-UMR 5164 Immuno ConcEpT, Bordeaux University, Bordeaux, France; [3]Jorge Eduardo Guimaraes: CNRS-UMR 5164 Immuno ConcEpT, Bordeaux University, Bordeaux, France, and Federal University of Alagoas, Maceió, Alagoas, Brazil; [4]Anne Bertrand, MD, Julien Seneschal, MD, PhD, Marie-Sylvie Doutre, MD, Jean-Luc Pellegrin, MD, Joël Constans, MD, Thierry Schaeverbeke, MD, PhD: Bordeaux Hospital, Bordeaux, France.

Ms. Demoures, Mr. Guimaraes, and Dr. Bertrand contributed equally to this work.

Address correspondence to Cécile Contin-Bordes, PhD, UMR 5164 CIRID, Université de Bordeaux, 146 Rue Leo Saignat, 33076 Bordeaux, France. E-mail: cecile.bordes@chu-bordeaux.fr.

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consequently, improve treatment options. The activation of endothelial cells (ECs) occurs very early in the course of the disease, which suggests that chronic fibroblast activation may be at least partly the result of endothelial dysfunction (1,2).

Microvascular endothelium activation precedes the intimal hyperplasia that is characteristic of vascular remodeling in SSc (3). The progressive loss of the vascular architecture is assumed to be involved in the accumulation of extracellular matrix (ECM) and chronic activation of platelets observed in SSc patients (4–7). Conversely, platelet-derived factors modulate EC activation and homeostasis in SSc (8,9). Recently, the role of platelet-derived serotonin in fibroblast activation and ECM component deposition has been described in SSc (10). However, the ability of the platelet–EC interaction to induce the secretion of factors directly involved in fibroblast activation and subsequent fibrosis in SSc remains uncertain.

Thymic stromal lymphopoietin (TSLP) is an interleukin-7 (IL-7) cytokine family member that has been implicated in various inflammatory disorders (11,12). TSLP promotes the differentiation of naive T cells into a Th2 phenotype and the secretion of various profibrotic factors, including IL-13 (13,14). TSLP expression is increased and contributes to fibroblast activation in idiopathic fibrosis (15). These effects suggest that TSLP may affect the course of fibrotic disorders, including SSc. Two recent studies have demonstrated that TSLP expression is up-regulated in the lung and skin of SSc patients (16,17), but the underlying mechanisms in humans have not been precisely determined. Conversely, in a mouse model of SSc, TSLP receptor knockout was shown to ameliorate fibrosis (17), whereas the administration of TSLP induced a partial profibrotic gene signature (16). Therefore, TSLP may induce skin fibrosis in SSc by acting both directly on fibroblasts and indirectly by modulating IL-13 expression and/or transforming growth factor β (TGFβ) signaling.

We hypothesized that in addition to their direct involvement in fibroblast activation, platelets could contribute to skin fibrosis in SSc via the activation of human dermal microvascular ECs (HDMECs) and the production of profibrotic mediators such as TSLP. We therefore investigated the potential interaction between platelet activation, microvasculopathy, and fibrosis.

PATIENTS AND METHODS

Study population. Individuals with SSc presenting to the University Hospital of Bordeaux were prospectively included in the study between April 2012 and January 2015. All patients satisfied the American College of Rheumatology/European League Against Rheumatism 2013 classification criteria for SSc (18). Patients were included in the context of the Vasculopathy and Inflammation in Systemic Sclerosis biomedical research project, which was initiated in 2012 and approved by the institutional ethics committee (CPP, 2012-A00081-42, Aquitaine). All participants provided written informed consent before inclusion. For each patient, a disease- and organ-specific questionnaire was completed by the clinician and then centralized by the investigators. Clinical features (SSc type, sex, age at onset of Raynaud's phenomenon [RP], age at onset of the first non-RP manifestation, disease duration, and symptoms of skin, articular, heart, lung, kidney, and gastrointestinal involvement), immunologic test results (antinuclear antibodies, anti–Scl-70 antibodies, and anticentromere antibodies), imaging and functional examinations (thorax computed tomography [CT] scans, respiratory function tests, cardiac ultrasonography, and right-sided heart catheterization), and treatments were recorded. For the modified Rodnan skin thickness score (MRSS) (19) and right ventricular systolic pressure (RVSP), the highest value from the medical history was registered for each patient. Patient characteristics are shown in Table 1.

Interstitial lung disease was diagnosed if pulmonary function tests showed a restrictive defect with decreased diffusing capacity for carbon monoxide (DLCO) associated with several types of lesions on the thorax CT scans. Lung fibrosis was diagnosed based on specific lesions observed on the thorax CT scans, i.e., honeycomb cysts and reticular septal thickening. Punch biopsy specimens (3–4 mm) of affected midforearm skin were obtained from 18 patients. Two control groups were used. The first group consisted of 20 patients with systemic lupus erythematosus (SLE) who had a systemic form with cutaneous and/or articular flares; of these, 12 also had renal involvement. None of the patients had a history of pulmonary arterial hypertension (PAH) or RP. The other group consisted of 30 age- and sex-matched healthy donors who presented to the local Blood Transfusion Center (University Hospital of Bordeaux) for blood tests. For control skin samples, 5 biopsy specimens were isolated from skin that had been discarded during plastic surgery (brachioplasty). None of the healthy individuals had dermatologic disorders or were receiving immunosuppressive agents or glucocorticoids.

Fibroblast and EC primary cultures. Fibroblasts were obtained from skin lesion biopsy samples from 3 healthy donors and 3 patients with SSc (2 with diffuse cutaneous SSc [dcSSc] and 1 with limited cutaneous SSc [lcSSc]) as previously described (20). Briefly, skin biopsy specimens were digested with 0.1% type IA collagenase at 37°C for 2 hours. Adherent cells were grown in Dulbecco's modified Eagle's medium containing 1% nonessential amino acids, 1% L-glutamine, 1% sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum. Fibroblasts were used between the third and sixth passages. HDMECs were obtained from biopsy samples of normal skin and were isolated after double digestion (0.1% Dispase II overnight at 4°C and 0.1% type IA collagenase at 37°C for 2 hours) of the biopsy sections. Pellets of cell suspension were then filtered through a 0.70-μm nylon mesh and cultured for at least 24 hours. Nonadherent cells were removed, and the remainder were treated with trypsin before purification using a CD31 microbeads kit (Miltenyi Biotec). Cells were cultured in MV2 EC medium (PromoCell) and used between passages 3 and 6.

Table 1. Characteristics of the SSc patient cohort*

	Patients with lcSSc (n = 133)	Patients with dcSSc (n = 70)	All SSc patients (n = 203)	P†
Female	111 (83.4)	40 (57.1)	151 (74.3)	<0.0001
Age at onset, mean ± SD years‡	50.2 ± 11.5	50.8 ± 12	50.4 ± 11.7	0.73
Disease duration, mean ± SD years‡	9.5 ± 5.9	8.5 ± 5.5	9.2 ± 5.8	0.24
Antinuclear autoantibody positive	119 (89.4)	68 (97.1)	187 (92.1)	0.052
Anticentromere antibody positive	83 (62.4)	4 (5.7)	87 (42.8)	<0.0001
Antitopoisomerase antibody positive	2 (1.5)	48 (68.5)	50 (24.6)	<0.0001
RP	128 (96.2)	69 (98.6)	197 (97)	0.66
Digital ulcers	52 (39)	48 (68.5)	100 (49.2)	<0.0001
MRSS, mean ± SD years	4.7 ± 2.6	17.8 ± 9.5	9.2 ± 7.3	<0.0001
RVSP >35 mm Hg	34 (25.9)§	25 (35.7)	59 (29.3)¶	0.87
PAH	8 (6.1)	8 (11.4)	16 (7.9)	0.28
Interstitial lung disease	23 (18.2)#	50 (71.4)	73 (37.2)**	<0.0001
Lung fibrosis	10 (7.9)	29 (41.4)	39 (19.8)	<0.0001
Renal crisis	4 (3)	5 (7.1)	9 (4.4)	0.28
Antiplatelet treatment	23 (17)	12 (17)	35 (17)	1
ERA treatment	32 (24)	33 (47)	65 (32)	0.0014
PDE5 inhibitor treatment	4 (3)	7 (10)	11 (5.4)	0.05

* Except where indicated otherwise, values are the number (%). MRSS = modified Rodnan skin thickness score; RVSP = right ventricular systolic pressure; PAH = pulmonary arterial hypertension; ERA = endothelin receptor antagonist; PDE5 = phosphodiesterase 5.
† Limited cutaneous systemic sclerosis (lcSSc) versus diffuse cutaneous SSc (dcSSc).
‡ Age at onset of Raynaud's phenomenon (RP) and disease duration since symptoms other than RP.
§ Data were available for 131 patients.
¶ Data were available for 201 patients.
# Data were available for 126 patients.
** Data were available for 196 patients.

Immunohistochemistry and cell quantification. Immunohistochemistry was performed as previously described (20), and for each patient and control, analyses were performed in duplicate. Immunohistochemical images were acquired by scanning whole-tissue sections using a NanoZoomer apparatus at the Bordeaux Imaging Centre. At least 2 sections of each skin biopsy were analyzed per individual. Each section was manually subdivided into epidermis and dermis using ImageJ software (National Institutes of Health; online at http://rsbweb.nih.gov/ij/). Annexes were excluded from the analysis. TSLP-positive cells in the dermis were semiautomatically quantified using ImageJ software. Positive cells (brown) were filtered for size and normalized to the total number of cells (blue) in each field. Representative images were manually reviewed by 2 operators (MET and BD).

Immunofluorescence. Immunofluorescence analysis was performed as previously described (20). Images were acquired using an LSM 510 META confocal laser scanning microscope (Carl Zeiss).

Real-time quantitative polymerase chain reaction (qPCR). Total RNA was isolated from trypsinized fibroblasts with a NucleoSpin RNA II extraction system (Macherey-Nagel), and complementary DNA was synthesized from 0.25 μg of total RNA using random hexamers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Gene expression was quantified by SYBR Green on an SDS 7900HT instrument (Applied Biosystems). The specific primer pairs for each gene are shown in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39817/abstract (15,21–23). Each reaction was performed in triplicate. Stable housekeeping genes GAPDH and EEF1A1 were selected for normalization. The oligonucleotides were obtained from Sigma-Aldrich. The differences were calculated using the threshold cycle (C_t) and the comparative C_t method for relative quantification.

Cytokine determinations. TSLP levels in cell culture supernatants or serum were quantified using an enzyme-linked immunosorbent assay (ELISA) kit from eBioscience according to the manufacturer's instructions. The cutoff for TSLP positivity was defined as values of ≥8 pg/ml, which corresponds to the detection limit of the ELISA.

EC activation. HDMECs were cultured in MV2 complete growth medium in 96-well flat-bottomed plates (20,000 cells per well in 200 μl) and allowed to stand overnight before the addition of different reagents (IL-1β, IL-4, IL-13, tumor necrosis factor [TNF], interferon-γ [IFNγ], lipopolysaccharide [LPS], serotonin, or tryptase) or ADP-activated or nonactivated platelets for 24 hours at 37°C. Anti–IL-1β blocker (canakinumab) or anti–serotonin receptor blocker (5HT2A and 5HT2B) (all from Abcam) were added at a final concentration of 10 μg/ml. Platelet-enriched plasma was prepared from whole blood obtained by venipuncture in an EDTA tube after a 20-minute continuous centrifugation at 1,000 rpm without acceleration. The supernatant was harvested, and 1 μM prostaglandin E_1 was added. After centrifugation (continuously for 10 minutes at 2,000 rpm), the supernatant was discarded, and Tyrode's buffer was added to obtain 8×10^8 platelets/ml.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software. For populations that satisfied the Kolmogorov-Smirnov normality test, Student's 2-tailed *t*-test for unpaired or paired samples and one-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni correction were used to compare the different populations according to the

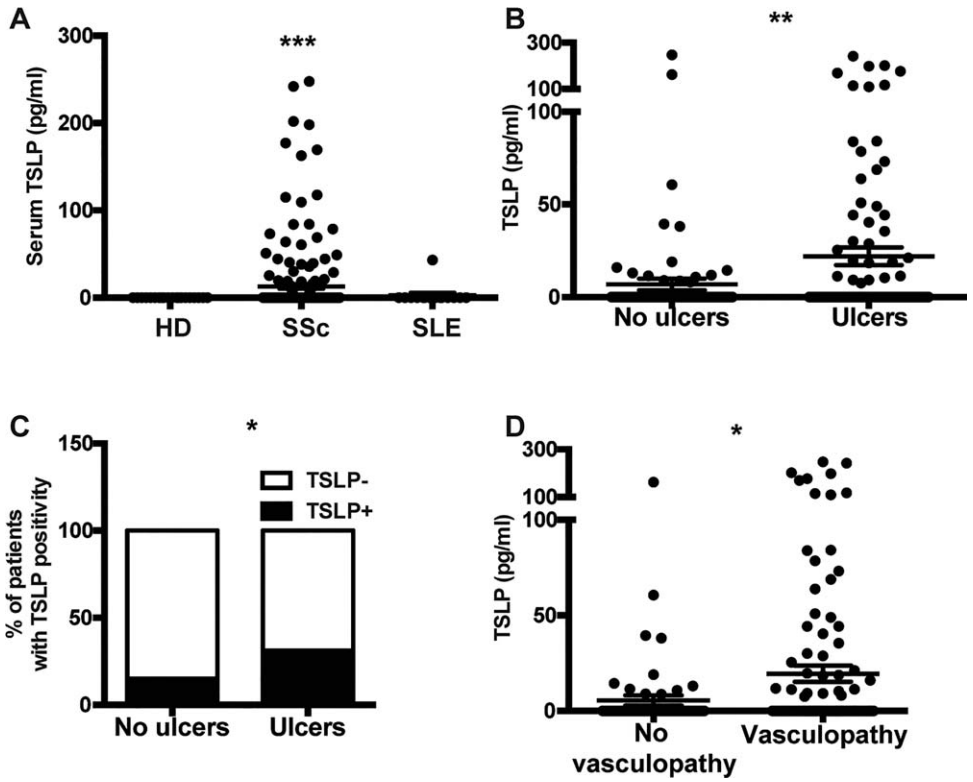

Figure 1. Overexpression of serum thymic stromal lymphopoietin (TSLP) in patients with systemic sclerosis (SSc), particularly those with digital ulcers. **A**, TSLP levels in serum from 20 healthy donors (HD), 203 patients with SSc, and 20 patients with systemic lupus erythematosus (SLE). *** = $P < 0.0001$, by Kruskal-Wallis nonparametric multiple comparison test. **B**, Serum TSLP levels in SSc patients without digital ulcers and those with digital ulcers. ** = $P = 0.0089$ by unpaired *t*-test. **C**, Percentages of patients positive for TSLP according to the presence or absence of digital ulcers. * = $P < 0.05$ by Fisher's exact test. **D**, Serum TSLP levels in SSc patients according to vascular profile. Symptoms of vasculopathy included digital ulcer, pulmonary arterial hypertension, and/or renal crisis. * = $P < 0.05$ by unpaired *t*-test. In **A**, **B**, and **D**, symbols represent individual subjects; horizontal lines and error bars show the mean ± SEM.

experimental design. When the normality test was not satisfied, the Mann-Whitney, Wilcoxon, and Kruskal-Wallis tests were used. Correlations were analyzed using Spearman's test. *P* values less than 0.05 were considered significant.

RESULTS

Increased circulating TSLP levels in sera from SSc patients and association with digital ulcers. From April 2012 to January 2015, 203 consecutive SSc patients were included in the study. Approximately two-thirds of the cohort (65.5%, n = 133) had lcSSc. The demographic characteristics and main clinical features are presented in Table 1. Serum levels of TSLP were assessed by ELISA. Serum TSLP levels were significantly higher in SSc patients than in healthy donors or SLE controls (systemic autoimmune disease control group) (mean ± SEM 12.95 ± 2.6 pg/ml versus 0 pg/ml and versus 2.9 ± 2.9 pg/ml, respectively; $P < 0.0001$ by Kruskal-Wallis test followed by Dunn's post hoc test) (Figure 1A). We observed a trend toward higher levels of TSLP in patients with dcSSc compared with patients with lcSSc, but this difference was not significant (mean ± SEM 19.02 ± 5.8 pg/ml versus 12.4 ± 3.2 pg/ml; $P = 0.07$) (Supplementary Figure 1A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39817/abstract). Patients with dcSSc were also more likely than those with lcSSc to be positive for TSLP (29% versus 21%) (data not shown).

To evaluate TSLP as a biomarker in SSc, we assessed the correlation between circulating TSLP levels and several clinical parameters. No correlation was observed between serum TSLP levels and fibrotic parameters such as MRSS, DLCO, or total lung capacity. There were also no associations between TSLP levels and disease duration or PAH diagnosed using ultrasonography (data not shown). In contrast, we observed a significant association between TSLP and vascular damage, and TSLP levels were significantly higher in patients with digital ulcers than in patients without any history of digital ulcers (mean ± SEM 22.03 ± 3.1 pg/ml

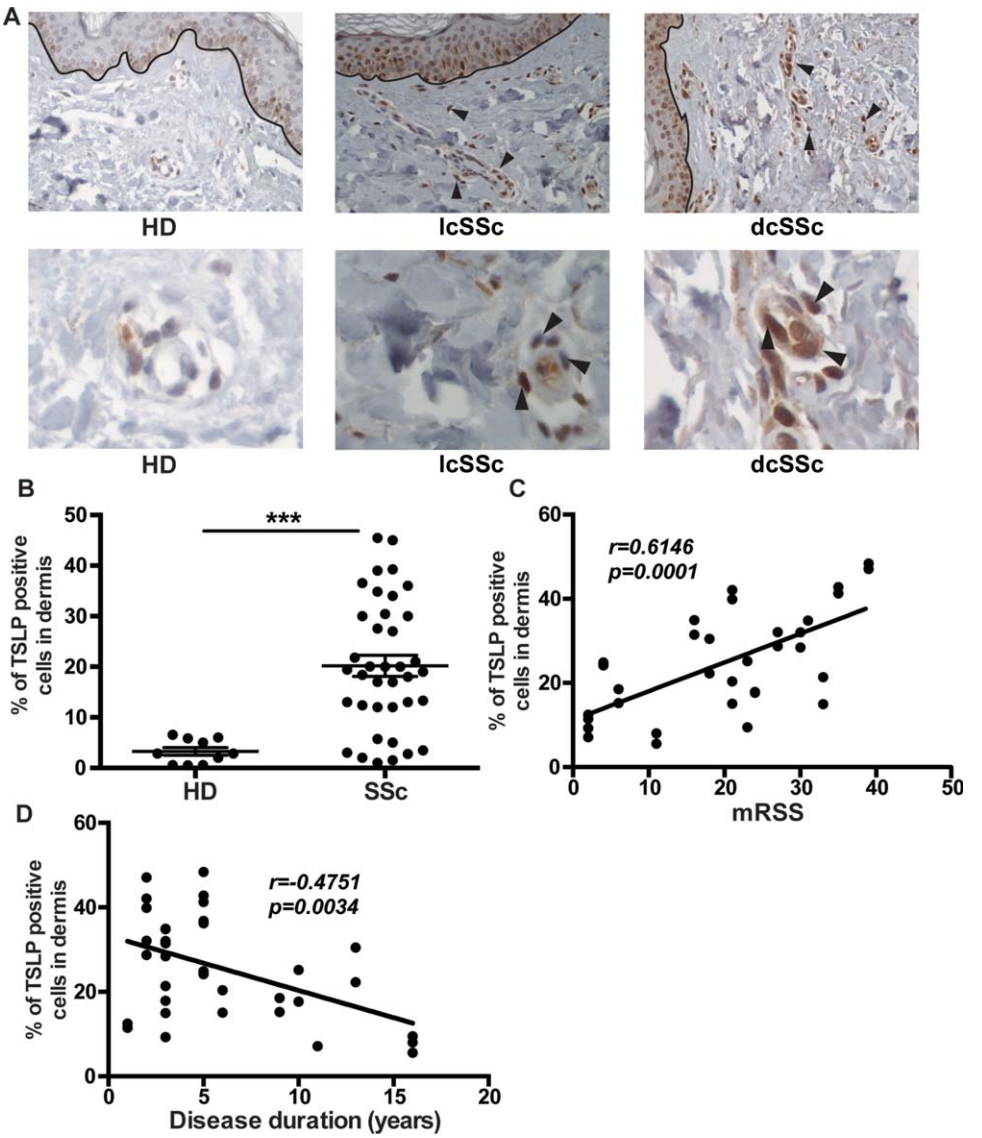

Figure 2. Overexpression of TSLP in the skin of SSc patients compared to healthy donors, and correlation of TSLP with fibrosis. **A**, Immunohistochemical staining to identify ex vivo expression of TSLP in human skin. Top, Representative immunohistochemical staining for TSLP in skin samples from a healthy donor, a patient with limited cutaneous SSc (lcSSc), and a patient with diffuse cutaneous SSc (dcSSc). Results are representative of 5 healthy donors, 8 patients with lcSSc, and 10 patients with dcSSc. Original magnification × 40. Bottom, Higher-magnification views of immunohistochemical staining for TSLP in the same subjects as in the top panel, showing the perivascular distribution of the TSLP-positive cells. **Arrowheads** indicate positive TSLP staining. Original magnification × 100. **B**, Percentage of TSLP-positive cells among the total number of cells in the analyzed section of the dermis in healthy donors and patients with SSc. Two separate slides for each of 5 healthy donors and 18 SSc patients were analyzed. Symbols represent individual samples; horizontal lines and error bars show the mean ± SEM. *** = $P < 0.0001$ by unpaired *t*-test. **C**, Correlation between percentage of TSLP-expressing dermal cells and the modified Rodnan skin thickness score (MRSS). **D**, Correlation between the percentage of TSLP-expressing dermal cells and disease duration. Correlation was determined using Spearman's test. The cell count was analyzed using ImageJ software. See Figure 1 for other definitions.

versus 6.85 ± 4.6 pg/ml; $P = 0.0089$ by unpaired *t*-test) (Figure 1B). Similarly, patients with digital ulcers were more likely to be positive for TSLP (31% versus 15%; $P = 0.01$ by Fisher's exact test) (Figure 1C). In addition, serum TSLP levels were increased in patients with at least one symptom of vasculopathy: the presence of digital ulcers, scleroderma renal crisis, and/or PAH on right-sided heart catheterization (mean ± SEM 19.48 ± 4.2 pg/ml versus 5.53 ± 2.6 pg/ml; $P = 0.022$ by unpaired *t*-test) (Figure 1D).

Endothelin receptor antagonist and phosphodiesterase 5 inhibitors are prescribed significantly more

often to patients with the diffuse form of the disease than to those with the limited form, since patients with dcSSc present with digital ulcers more frequently than do patients with lcSSc; however, no significant effect of such treatment was observed on TSLP serum levels or positivity (Supplementary Figure 2A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39817/abstract). Antiplatelet drugs are prescribed with equal frequency to patients with the limited form and those with the diffuse form of the disease, and their administration had no direct effect on serum TSLP levels (Supplementary Figure 2B). Taken together, these results suggest that serum TSLP is increased in SSc patients and is associated with vascular damage.

Overexpression of TSLP in SSc skin and correlation with fibrosis. We conducted immunohistochemical analysis of TSLP expression in skin from SSc patients and controls. Direct staining with secondary antibodies did not result in any significant fluorescence (results not shown), confirming the specificity of the staining. Further confirming the specificity of the staining, preincubation of anti-TSLP antibodies with a 10× excess of recombinant TSLP protein resulted in virtually complete abrogation of staining, with the exception of a slight border in the basal membrane (results not shown). Whereas TSLP expression was nearly undetectable in the skin of healthy individuals, we observed significant TSLP expression in skin from both patients with dcSSc and those with lcSSc, as previously described (17) (Figure 2A). TSLP expression was very low in SLE skin sections, confirming the findings obtained in sera (Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39817/abstract). Interestingly, TSLP expression was detected in the epidermis and the dermis, including in the perivascular area in dcSSc patients (Figure 2A), consistent with the findings of a previous study (16). An identical pattern of expression was observed in lcSSc patients (Figure 2A).

TSLP staining of the epidermis was fairly homogenous across the SSc populations; therefore, we focused on cells expressing TSLP in the dermis. The total number of cells and the number of TSLP-positive cells in the dermis sections of the biopsy specimens were determined. Counts were performed in duplicate, and intrinsic variation in the number of positive cells within the same biopsy specimen was very low (Supplementary Figure 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39817/abstract). Whereas the number of total cells per mm^2 did not differ significantly between the groups (mean ± SEM 1,190 ± 138.3 cells in SSc patient slides versus 1,046 ± 98.2 cells in healthy donor slides; $P = 0.4909$ by unpaired *t*-test), the proportion of TSLP-positive dermal cells was significantly increased in SSc compared to healthy donor skin (mean ± SEM 20.35 ± 2.28 cells versus 3.669 ± 0.88 cells; $P < 0.0001$ by unpaired *t*-test) (Figure 2B). A trend toward a higher percentage of TSLP-positive cells in dcSSc than in lcSSc was observed (mean ± SEM 29.92 ± 3.2% versus 20.28 ± 5.1%) (data not shown).

Dermal TSLP expression correlated with skin fibrosis as assessed by the MRSS ($r = 0.6146$, $P = 0.0001$ by Spearman's test) (Figure 2C). The dermal expression of TSLP was also inversely correlated with disease duration (since the appearance of non-RP symptoms) ($r = -0.4751$, $P = 0.0034$ by Spearman's test) (Figure 2D). Finally, dermal TSLP levels were higher in patients with pulmonary hypertension diagnosed using ultrasonography (RVSP >35 mm Hg) (mean ± SEM 33.12 ± 2.8% versus 23.58 ± 2.4%; $P = 0.0238$ by unpaired *t*-test) (data not shown). Right-sided heart catheterization had been performed in 38 patients with pulmonary hypertension and in 15 patients who underwent skin biopsy; among these patients, only 2 had PAH, preventing further analysis. We conclude that in SSc skin, TSLP expression is increased in perivascular areas, is associated with an early form of the disease, and is correlated with the extent of skin fibrosis.

HDMECs overexpress TSLP in SSc skin ex vivo and produce TSLP in vitro via an IL-1β–dependent mechanism. To further analyze the cell types with positive staining for TSLP, we performed immunofluorescence experiments. Both α-smooth muscle actin (α-SMA)–positive myofibroblasts and CD31-positive ECs stained positively for TSLP in SSc patients and negatively in healthy donors (Figure 3A). Notably, perivascular α-SMA–positive pericytes were negative for TSLP in both SSc patients and healthy donors (Figure 3A).

To confirm that ECs are a potent TSLP producer in skin, we cultured HDMECs in vitro and in the presence or absence of profibrotic cytokines (IL-4 and IL-13), proinflammatory cytokines (IL-1β, TNF, and IFNγ), and Toll-like receptor agonists. Only IL-1β increased the expression of TSLP at the protein level (mean ± SEM 651.7 ± 95.4 pg/ml versus 7.4 ± 3.5 pg/ml for HDMECs alone; $P = 0.0002$ by paired *t*-test) (Figures 3B and C) and transcript level (Figure 3D). None of the other stimuli had any effect, except for LPS, which slightly but reproducibly induced TSLP production (Figure 3B). Taken together, these results demonstrate that HDMECs are potent TSLP producers in SSc and that IL-1β is a main inducer of its secretion.

Activated platelets promote the secretion of TSLP by ECs via an IL-1β– and serotonin-dependent mechanism. Among the IL-1β–secreting cells activated in SSc patients, platelets were of significant interest (10,24).

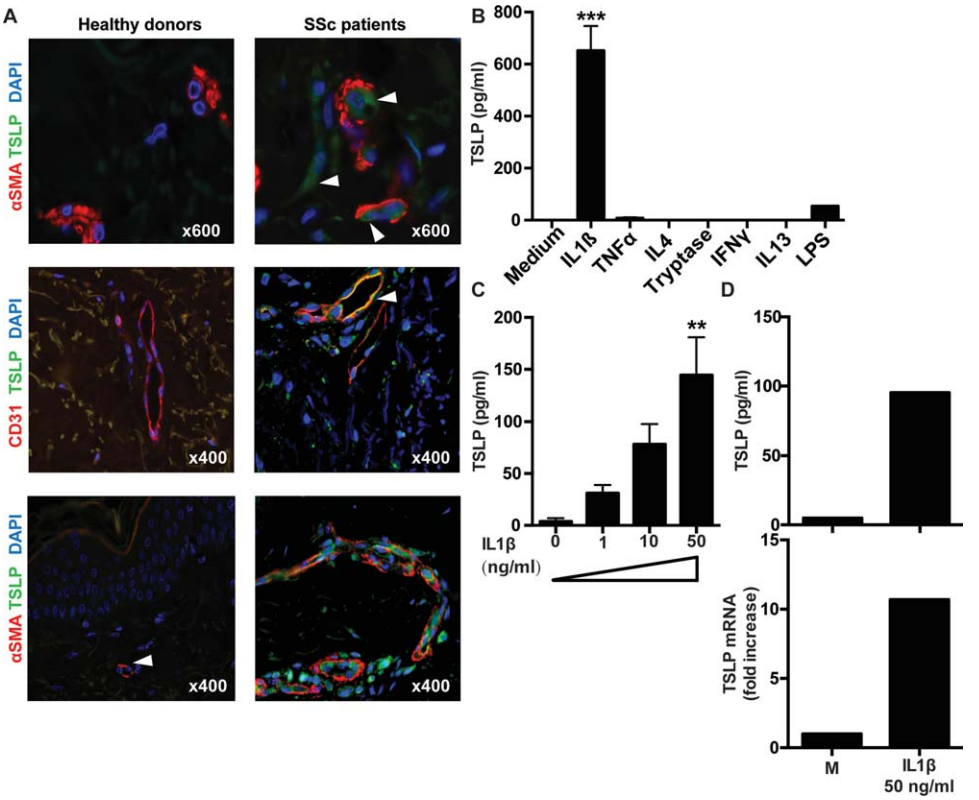

Figure 3. Endothelial cell (EC) production of TSLP ex vivo in SSc and in vitro. **A,** Indirect immunofluorescence analysis of TSLP in the skin of healthy donors and SSc patients, showing co-staining for TSLP (green) and α-smooth muscle actin (α-SMA; red) in fibroblasts (top) and pericytes (bottom) and co-staining of TSLP (green) and CD31 (red) in ECs (middle). The nuclei were stained with DAPI (blue). Yellow indicates co-stained cells. **Arrowheads** show TSLP-positive fibroblasts (top), TSLP-positive endothelial cells (middle), and TSLP-negative pericytes (bottom). **B** and **C,** Production of TSLP by interleukin-1β (IL-1β)–stimulated human dermal microvascular ECs (HDMECs). HDMECs were purified from normal skin (obtained from patients undergoing plastic surgery) and stimulated for 24 hours with either cytokines potentially involved in SSc or the Toll-like receptor 4 agonist lipopolysaccharide (LPS) (**B**) or increasing amounts of IL-1β (0–50 ng/ml) in MV2 complete medium (M) (**C**). The supernatants were harvested, and TSLP content was measured by dedicated enzyme-linked immunosorbent assay (eBioscience). Bars show the mean ± SD (n = 3 independent experiments in **B** and n = 6 independent experiments in **C**). TNF = tumor necrosis factor; IFNγ = interferon-γ. **D,** Quantification of TSLP mRNA in IL-1β–activated HDMECs by real-time quantitative polymerase chain reaction. HDMECs were stimulated with 50 ng/ml of IL-1β. Results are representative of 3 experiments. ** = $P < 0.001$; *** = $P = 0.0004$ by Kruskal-Wallis test. See Figure 1 for other definitions.

Platelets from healthy donors induced significant TSLP production by HDMECs in a dose-dependent manner (Figure 4A). TSLP production by HDMECs was further increased in the presence of ADP-activated platelets compared to unstimulated platelets (Figure 4A). Notably, TSLP production was dependent on HDMECs because activated platelets were unable to secrete a significant amount of TSLP (data not shown). To elucidate the mechanism responsible for platelet-induced TSLP production by HDMECs, we first blocked IL-1β using the monoclonal antibody canakinumab. At a 50:1 ratio of platelets to ECs, the addition of anti–IL-1β–blocking antibody returned TSLP production to basal levels (mean ± SEM 20.9 ± 3.7 pg/ml in stimulated cells versus 64.8 ± 3.9 pg/ml in unstimulated cells). At a 100:1 ratio of platelets to ECs and a 200:1 ratio of platelets to ECs, the addition of anti−IL-1β–blocking antibody induced an incomplete inhibition of TSLP production (for a 100:1 ratio of platelets to ECs, mean ± SEM 173.1 ± 0.4 pg/ml in stimulated cells versus 369.6 ± 12.7 pg/ml in unstimulated cells; for a 200:1 ratio of platelets to ECs, mean ± SEM 357.7 ± 28.4 pg/ml in stimulated cells versus 648.5 ± 13.8 pg/ml in unstimulated cells). Significance was determined by the Kruskal-Wallis test followed by a multiple comparison test. (Figure 4B). This result suggests a saturation of the blocking antibody or a role of other platelet-derived factors.

Because platelet-derived serotonin is directly involved in SSc pathogenesis (10), we added recombinant serotonin at different concentrations and observed that serotonin induced TSLP production by HDMECs

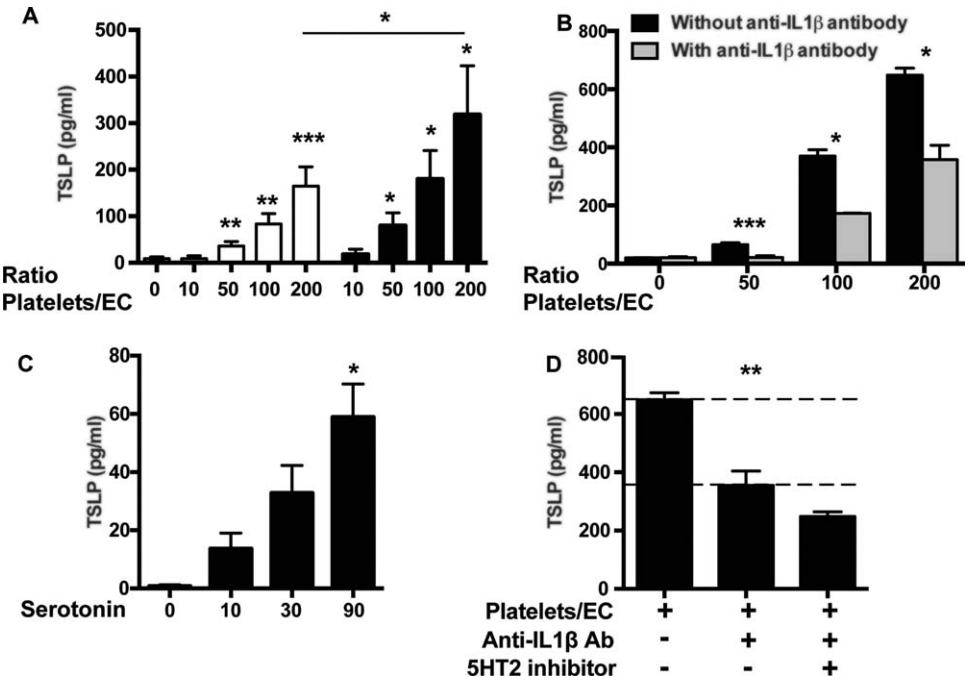

Figure 4. Platelets induce thymic stromal lymphopoietin (TSLP) production by human dermal microvascular endothelial cells (HDMECs) in an interleukin-1β (IL-1β)–dependent manner. **A**, TSLP levels in HDMECs cultured in MV2 complete medium with increasing amounts of purified nonactivated platelets (10–200 platelets per HDMEC) (open bars) and in HDMECs stimulated with purified ADP-activated platelets (solid bars). A total of 70,000 HDMECs were cultured for 24 hours in a 24-well culture plate. $* = P < 0.05$; $** = P < 0.001$; $*** = P < 0.0001$ versus unstimulated cells (except where indicated otherwise), by Kruskal-Wallis nonparametric test followed by multiple comparison post hoc test. **B**, TSLP levels in HDMECs stimulated with increasing ratios of ADP-activated platelets alone or with increasing ratios of ADP-activated platelets and an anti–IL-1β–blocking antibody. $* = P < 0.05$; $*** = P < 0.0001$, without anti–IL-1β antibody versus with anti–IL-1β antibody, by Kruskal-Wallis nonparametric test followed by multiple comparison post hoc test. **C**, TSLP levels in HDMECs stimulated with increasing doses of serotonin (0–90 ng/ml). $* = P < 0.05$ versus unstimulated cells, by paired *t*-test. **D**, TSLP levels in HDMECs stimulated with ADP-activated platelets (at a 1:200 ratio of HDMECs to platelets) with or without anti–IL-1β–blocking antibody and with or without 5HT receptor–blocking antibodies (antibodies blocking 5HTR2a and 5HTR2b). The supernatants were harvested, and TSLP content was measured using a dedicated enzyme-linked immunosorbent assay. Broken lines indicate the maximal production of TSLP (top) and the production of TSLP in the presence of anti–IL-1β–blocking antibody (bottom). Bars show the mean ± SEM of 3 independent experiments.

(mean ± SEM 58.9 ± 11.4 pg/ml for HDMECs stimulated with 90 ng/ml serotonin versus 0.75 ± 0.5 pg/ml for HDMECs alone; $P = 0.013$ by paired *t*-test) (Figure 4C). We did not observe any synergistic effect of IL-1β and serotonin on TSLP production (data not shown). The addition of serotonin receptor–blocking antibodies (antibodies blocking 5HT2A and 5HT2B) to the platelet–EC coculture significantly reduced TSLP production (mean ± SEM 649 ± 13.8 pg/ml in platelet ECs versus 335.1 ± 28.5 pg/ml in cells stimulated with canakinumab versus 242.2 ± 9.1 pg/ml in cells stimulated with canakinumab plus serotonin receptor inhibitors; $P = 0.0072$ by ANOVA) (Figure 4D). These results suggest that platelet-induced TSLP production by HDMECs is mainly due to IL-1β and, to a lesser extent, serotonin.

TSLP induction of fibrosis in humans via ECM production and collagenase inhibition. TSLP has been implicated in the fibrotic process in mouse models, but its role in humans remains unclear (16,17). Therefore, we assessed whether recombinant TSLP directly affects the activation and ECM production of dermal fibroblasts from healthy donors.

Healthy donor dermal fibroblasts were stimulated with IL-1β, TSLP, or TGFβ as a control. As expected, TGFβ induced collagen 1A1 (5.35 ± 0.9-fold increase versus medium; $P = 0.0486$ by paired *t*-test) (Figure 5A) and collagen 1A2 (data not shown) messenger RNA (mRNA) overexpression in healthy donor dermal fibroblasts. Interestingly, recombinant TSLP induced a reproducible and significant increase in collagen 1A1 (1.5 ± 0.06-fold increase versus medium; $P = 0.0188$ by paired *t*-test) (Figure 5A) and collagen 1A2 (data not shown) mRNA expression by fibroblasts. Concomitantly, recombinant TSLP significantly reduced the expression of the collagenase matrix metalloproteinase 1 (MMP-1) to the same extent as TGFβ1

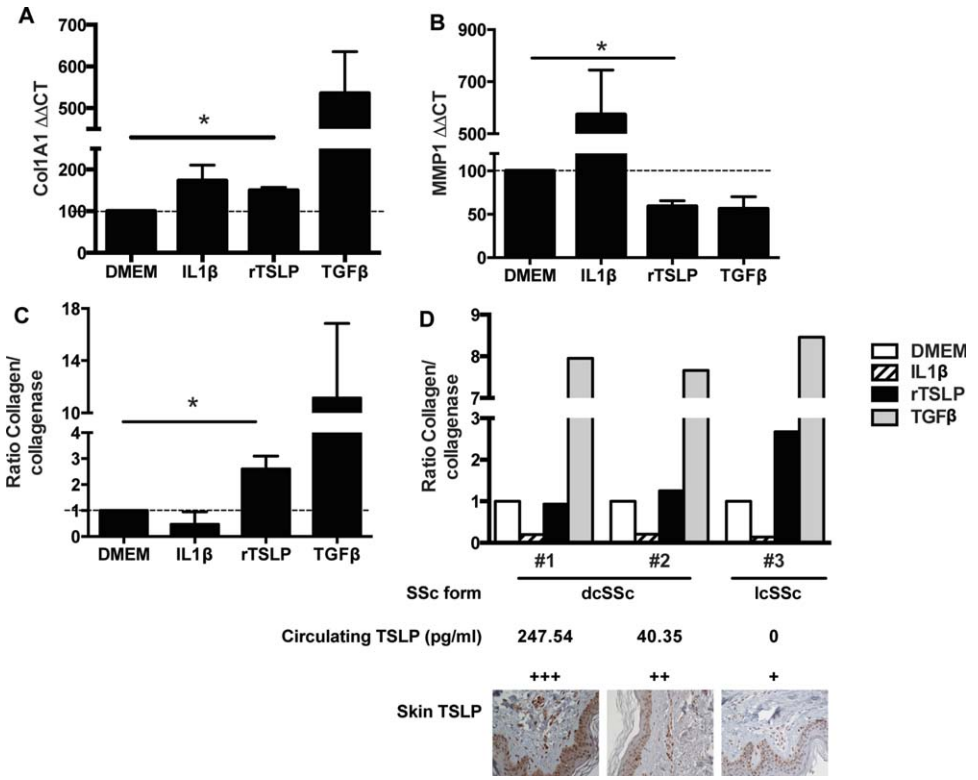

Figure 5. Thymic stromal lymphopoietin (TSLP) induces a profibrotic profile in fibroblasts from healthy donors and systemic sclerosis (SSc) patients. Human dermal fibroblasts were isolated from healthy or SSc skin and incubated for 48 hours with Dulbecco's modified Eagle's medium (DMEM), interleukin-1β (IL-1β), recombinant TSLP (rTSLP), or transforming growth factor β1 (TGFβ1). **A,** Levels of Col1A1 mRNA in activated fibroblasts from 3 different healthy donors, quantified by real-time quantitative polymerase chain reaction (qPCR) and expressed in relation to $\Delta\Delta C_t$ for DMEM as the baseline condition. **B,** Levels of matrix metalloproteinase 1 (MMP-1) mRNA in activated fibroblasts from 3 different healthy donors, quantified by real-time qPCR. **C,** Ratio of COL1A1 to MMP-1 mRNA from activated normal fibroblasts from 3 different healthy donors, determined by real-time qPCR. **D,** Ratio of COL1A1 to MMP-1 mRNA in activated scleroderma fibroblasts from 3 different patients (2 with diffuse cutaneous SSc [dcSSc] and 1 with limited cutaneous SSc [lcSSc]), determined by real-time qPCR. Circulating TSLP levels and images of TSLP expression in the skin for each patient are shown. Original magnification $\times$ 40. Bars show the mean $\pm$ SEM of 3 independent experiments. $* = P < 0.05$ by paired *t*-test.

(0.59 ± 0.06-fold increase and 0.56 ± 0.14-fold increase, respectively, versus medium; $P = 0.024$ by paired *t*-test) (Figure 5B). Conversely, the ratio of collagen to collagenase was significantly increased in the presence of recombinant TSLP, demonstrating the direct profibrotic effect of the cytokine (2.6 ± 0.3-fold increase versus medium; $P = 0.0309$ by paired *t*-test) (Figure 5C).

We also tested the effect of IL-1β, which could be present in the SSc microenvironment along with TSLP. In contrast to recombinant TSLP, the results for IL-1β were less homogeneous and did not reach statistical significance. IL-1β induced a slight increase in collagen expression (1.7 ± 0.4-fold increase versus medium; $P = 0.193$) (Figure 5A), but in sharp contrast to recombinant TSLP and TGFβ1, IL-1β increased MMP-1 expression (5.7 ± 1.7-fold increase versus medium; $P = 1.1$) (Figure 5B). Consequently, the ratio of collagen to collagenase tended to diminish, but this tendency was not significant (0.47 ± 0.3-fold increase versus medium; $P = 0.2$ by paired *t*-test) (Figure 5C).

Interestingly, recombinant TSLP and TGFβ induced strong down-regulation of monocyte chemotactic protein 1 (MCP-1) mRNA expression (0.48 ± 0.02-fold and 0.31 ± 0.09-fold increase, respectively, versus medium; $P = 0.0012$) (Supplementary Figure 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39817/abstract), whereas IL-1β highly increased the expression of MCP-1 in human dermal fibroblasts (52.7 ± 11.2-fold increase versus medium; $P = 0.0471$) (Supplementary Figure 5). All of the patient fibroblasts responded to TGFβ and IL-1β in the same manner as normal fibroblasts with respect to collagen and collagenase secretion. Intriguingly, recombinant TSLP–activated fibroblasts purified from fibrotic skin with high levels of TSLP from both of the patients with dcSSc had lower collagen:collagenase ratios

than those purified from nonfibrotic skin with low levels of TSLP from the lcSSc patient. Analysis of TSLP receptor expression by real-time qPCR (CRLF2 mRNA) did not show any differences between normal and SSc fibroblasts (Supplementary Figure 6, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39817/abstract). Our findings demonstrate that TSLP promotes a profibrotic response in human dermal fibroblasts. Moreover, the results suggest that fibroblasts from skin with high levels of TSLP have lost their ability to respond to TSLP signals in vitro.

DISCUSSION

Improved characterization of the fibrotic mechanisms involved in SSc is a keystone to developing biomarkers and effective disease-modifying therapies, a continuing unmet medical need despite recent breakthroughs (9,10,25–27). In this study, we provide evidence of a new pathogenic loop implicating activated platelets, microvascular ECs, and ECM production in SSc.

Our findings shed new light on the numerous contributors to TSLP production in SSc and extend previous research (16,17). Usategui et al (17) demonstrated that TSLP up-regulation was mainly attributable to keratinocytes and, to a lesser extent, dermal cells, such as mast cells and fibroblasts, whereas Christmann et al (16) did not observe any differences in keratinocyte staining between SSc patients and controls. By contrast, Christmann et al observed specific prominent perivascular staining that was attributed to perivascular infiltrating CD163+ macrophages in SSc patients. These discrepancies may be linked to the differences in the populations studied, in particular the stage of the disease, since perivascular infiltration, including T cells and macrophages, are classically observed in the early forms of the disease (28). A potential limitation of histologic analysis involves the locations of the biopsy. In our study, the control biopsy specimens were obtained at the time of brachioplasty and were located very close to the forearm; this should have introduced very little bias into the results compared with control samples obtained directly from the forearm.

Initially associated with allergies, deregulated expression of TSLP has now been observed in other disorders, including psoriasis, idiopathic pulmonary fibrosis, rheumatoid arthritis, and cancer (15,29–31). Notably, TSLP-expressing ECs are not classically observed in these settings. Furthermore, the association of TSLP with digital ulcers and PAH strongly suggests a direct link between SSc-associated vasculopathy and TSLP production. Consequently, EC-deregulated expression of TSLP appears more specific for SSc and might constitute a new biomarker.

Platelets are not only the sentinels of vasculature integrity but also play a role in the modulation of the innate and adaptive immune cell responses (32–34). Platelet activation has been described in SSc (4–7), but the contribution of platelets to fibrosis has only recently been suggested (10,35). Whereas platelet-derived β-thromboglobulin and CXCL4 levels are increased in bronchoalveolar lavage from SSc patients with pulmonary interstitial fibrosis (35), platelet-derived serotonin directly promotes fibroblast activation and ECM production in both SSc patients and a mouse model of SSc (10). In this study, we identified a previously unknown mechanism and demonstrated that platelet-derived IL-1β and, to a lesser extent, serotonin induce TSLP production by microvascular ECs in a dose-dependent manner. In turn, recombinant TSLP reproducibly promotes a profibrotic profile in normal fibroblasts, consistent with the profibrotic gene signature observed by Christmann et al (16) after continuous subcutaneous injection of TSLP in mice. Interestingly, platelets are also activated in SLE (34), but we did not find significant TSLP levels in either the circulation or the skin of SLE patients. SSc pathogenesis is a prototypical vasculopathy in which platelets and ECs are in tight interaction in the microcirculation, which is probably not the case in other autoimmune diseases with chronic activation of platelets, including SLE. This might account for the absence of TSLP in SLE patients. However, we cannot exclude the possibility of negative feedback mechanisms of unknown origin that prevent TSLP production by ECs in SLE patients.

In conclusion, we have defined a new pathogenic loop involving platelets, ECs, and fibroblasts in SSc patients. Our findings and the previous observation of diminished fibrosis in two mouse models of scleroderma treated with antiplatelet agents represent progress toward new clinical trials in human SSc (10).

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Contin-Bordes had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Truchetet, Seneschal, Doutre, Pellegrin, Constans, Schaeverbeke, Blanco, Contin-Bordes.

Acquisition of data. Truchetet, Demoures, Guimaraes, Bertrand, Laurent, Jolivel, Douchet.

Analysis and interpretation of data. Truchetet, Jacquemin, Khoryati, Duffau, Lazaro, Richez, Blanco, Contin-Bordes.

ROLE OF THE STUDY SPONSOR

UCB had no role in the study design or in the collection, analysis, or interpretation of the data, the writing of the manuscript, or

the decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by UCB.

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