Interleukin-1β–Activated Microvascular Endothelial Cells Promote DC-SIGN–Positive Alternatively Activated Macrophages as a Mechanism of Skin Fibrosis in Systemic Sclerosis

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Interleukin-1-β-Activated Microvascular Endothelial Cells Promote DC-SIGN+ Alternative Macrophages Associated with Skin Fibrosis in Systemic Sclerosis

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Abstract

Objective: To characterize the role of interleukin (IL)-1β and skin microvascular endothelial cells (MECs) in the generation of alternatively-activated macrophages (AAMs) and explore their fibrotic role during systemic sclerosis (SSc).

Methods: Conditioned medium from MECs purified from healthy donors (HD) or SSc patient's skin were used to generate monocytes-derived macrophages. Flow cytometry, multiplex protein assessment, RTqPCR and tissue immunofluorescence were used to characterize MEC-induced AAM polarization. Co-culture experiments were conducted to assess the role of MEC-induced AAM on fibroblast activation. AAMs were characterized in skin of healthy donors and SSc patients using multiparametric immunofluorescence and multiplexed measurements of gene expression. A skin AAM-specific score was developed to perform correlations with clinical features.

Results: IL-1β-activated MECs from SSc patients induced monocytes to differentiate into DC-SIGN$^+$ AAMs, which produced high levels of CCL18, CCL2, and CXCL8 but low level of IL-10. DC-SIGN$^+$ AAMs favor pro-inflammatory fibroblasts and were enriched in perivascular regions of highly fibrotic SSc skin. A novel transcriptomic macrophage signature defined from our findings correlated with the extent of skin fibrosis (Spearman r = 0.6; p=0.0018) and was associated with early disease and lung involvement.

Conclusion: Our work sheds new light on the vicious circle implicating IL-1β unabated secretion, microvascular endothelial cells activation and generation of DC-SIGN AAM in fibrosis during scleroderma.

Keywords

Systemic Sclerosis, Innate Immunity, Fibrosis, transcriptomic signature, autoimmune disease

Introduction

Systemic sclerosis (SSc) is a rare autoimmune connective tissue disease characterized by microangiopathy and fibrosis [1]. The pathophysiology of SSc is unclear but involves a complex network of interactions between the microvascular system, activation of autoimmune processes, and chronic activation of fibroblasts [2, 3].

Monocyte-derived macrophages exhibit a wide spectrum of polarization states, which are determined by the local environment in which they differentiate. Based on *in vitro* stimulation and analysis of membrane and soluble markers, monocyte-derived macrophages are classified as inflammatory (M1 or type-1) and alternatively activated macrophages (AAM), also called M2. M1 macrophages originate in response to microbial factors, such as lipopolysaccharide (LPS) and proinflammatory cytokines (interferon-gamma [IFN-γ], tumor necrosis factor-alpha [TNF-α], and interleukin [IL]-1β) [4]. AAMs comprise numerous subsets depending on the milieu in which they differentiate: IL-4 or IL-13 (M2a); immune complexes with IL-1β or LPS (M2b); IL-10 and transforming growth factor-beta (TGF-β); or glucocorticoids (M2c), Toll-like receptor (TLR), and IL-6 (M2d) [4].

Based on analysis of circulating markers and immunohistochemistry [5, 6], the role of AAMs in fibrosis during the course of SSc has been reinforced by transcriptomics and systems biology [7, 8]. In mouse models of SSc, a cAMP-specific phosphodiesterase-4 blocker or the tyrosine kinase inhibitor nintedanib alleviated fibrosis in part by limiting polarization toward AAMs [9, 10]. Moreover, in the FASSCINATE trial, molecular profiling of skin biopsies and analysis of circulating cytokines revealed that IL-6 receptor blockade by tocilizumab downregulated the AAM signature in SSc skin [11]. However, fibrinogenic monocytes harboring both M1 and M2 markers are present in patients with SSc and interstitial lung disease [12], and transcriptomic analysis of skin revealed associated M1 and M2 signatures in early SSc [13]. This suggests that the classical M1/M2 classification rapidly finds limitations

as it does not reflect the tissue-macrophage diversity induced by dynamic and complex microenvironmental changes, which mediate macrophage long-term imprinting (trained innate immunity), polarization state, and plasticity [14].

Altogether, these observations suggest that macrophages participate in fibrotic processes in mouse models and in human SSc although the underlying mechanisms still largely unknown especially in human settings.

Human studies (for recent reviews see [15, 16]) and animal models have highlighted the role of the inflammasome and IL-1β release in lung and skin fibrosis [17-19]. Our group recently reported that activated platelets promote IL-1β-dependent production of thymic stromal lymphopoietin by endothelial cells (ECs) which in turn, activate dermal fibroblasts and enhancing collagen deposition [20]. We evaluated here whether IL-1β-activated microvascular endothelial cells (MECs) indirectly favor fibrotic processes by promoting a microenvironment that modulates polarization of cutaneous macrophages during SSc.

Patients and Methods

Patients with SSc presenting at the University Hospital of Bordeaux, France, between March 2014 and September 2016 were prospectively enrolled. All patients satisfied the classification criteria proposed by the American College of Rheumatology and the European League Against Rheumatism 2013. Patients were included in the context of the Vasculopathy and Inflammation in Systemic Sclerosis biomedical research project (CPP, 2012-A00081-42, Aquitaine). All participants provided written informed consent before inclusion. Age- and sex-matched healthy donors (HDs) were recruited at the local Blood Transfusion Centre (Etablissement Français du sang, Bordeaux). Clinical features and biological recordings are available in supplementary data section.

Cell purification

Human dermal microvascular endothelial cells (MECs) were obtained from healthy and SSc skins as described in [21] (Supplementary Table 2). MEC-conditioned medium (MEC-CM) was produced by culturing MECs with or without IL-1β (50 ng/mL, R&D Systems) for 24 hours in MV2 medium supplemented with 1% penicillin/streptomycin and 8% FCS (PromoCell). For fibroblast, dermis was incubated in Hank's buffered salts solution containing 0.1% type IA collagenase (Sigma) for 3 hours at 37°C. Pelleted cells were suspended in Dulbecco's modified Eagle's medium with 1% penicillin/streptomycin and 10% FCS (Gibco). Monocytes were obtained from the blood of HDs recruited at Bordeaux Blood Transfusion Centre. After Ficoll (Eurobio), monocytes were purified by immunomagnetic sorting using CD14 microbeads (Miltenyi). Purity (> 90%) was assessed by flow cytometry.

Generation of monocyte-derived macrophages

Monocytes (5×10^4) were incubated for 6 days in 24-flat-bottom-well plates in a 1:1 volume of complete RPMI medium (supplemented with 1% penicillin/streptomycin, 8% FCS) with MV2 medium (medium condition), or MEC-CM. Cultures were supplemented with 5 ng/mL GM-CSF or 50 ng/mL M-CSF (Miltenyi) and fresh cytokines were added at day 3. When indicated, ET-1R antagonist (macitentan, 100 ng/mL, gift from Actelion Pharmaceuticals Ltd.), anti-IL-6R (Tocilizumab, 100 ng/mL, Roche), anti-IL-1β (Canakinumab, 150 µg/mL, Novartis) antibodies, recombinant IL-6 (100 ng/mL) or recombinant ET-1 (10 ng/mL) were added. To potentiate blockade of the receptors, ET-1R antagonist and anti-IL-6R were pre-incubated for 1 hour with monocytes before the addition of MEC-CM. To prevent quick engagement of IL-1β receptor by IL-1β contained in MEC-CM, blocking anti-IL-1β was pre-incubated with MEC-CM for 1 hour before the culture with monocytes.

Flow cytometry

STAT1 and 3 phosphorylation was assessed in monocytes after incubation for 20 minutes with MECCM using dedicated flow cytometry kit from Miltenyi. Macrophage membrane marker expression was evaluated by flow cytometry using PerCP-labeled CD14, APC-labeled CD206, VioBlue-labeled CD209, APCCy7-labeled CCR2, PECy7-labeled CCR7 (Miltenyi), and PE-labeled CD163 (BD). Cells were analyzed using a FACS Canto II flow cytometer with FACSDiva (BD), and data analysis was performed using FlowJo software version 10.1.

Cytokine and chemokine assays

IL-6, CXCL8, CXCL10, PDGFBB, IL-1Ra, GM-CSF, GCSF, CCL2, CCL5, IL-12p40, TNF-α, and IFN-γ in MECs, macrophage and fibroblast supernatants were quantified using a multiplex assay (Biolegend). IL-10 and ET-1 (Abcam) were assessed by enzyme-linked

immunosorbent assay (ELISA). To ensure the specificity of cytokine/chemokine measurements in macrophage supernatants, cytokine/chemokine levels were also quantified in MEC-CM cultured without macrophages for 6 days and were deduced from the total levels in macrophage culture supernatant.

Fibroblast and macrophage co-culture

Macrophages were intensive washed (three times with 10 mL PBS) to eliminate any remaining cytokines/chemokines from the supernatant, before they were seeded (3×10^4) in the lower chamber of Transwell microplates. Fibroblasts were seeded (3×10^4) in the upper chamber and cultured for 24 hours. *Col1A1*, *Col1A2*, *MMP1*, *MMP2*, *TIMP1*, *TIMP2*, *CCL2, IL-6, CXCL10, CCL5, IL-1R* and *CFS3* mRNA expression was quantified by qRT-PCR as previously described [20]. Probe for RTqPCR are detailed in supplementary data section.

Quantification of mRNA expression in cutaneous tissue sections

NanoString nCounter gene expression assay was performed on RNA extracted from formalin-fixed paraffin-embedded (FFPE) skin sections from HDs (n = 10) and patients with SSc (n = 24) using the High-Pure FFPET RNA Isolation Kit (Roche). The enriched gene signature comprised mRNAs of *CD68*, *CD163*, *CD209* (DC-SIGN), *MRC1* (CD206), *cMAF*, *STAT3*, *IL-10*, *CCL18*, *CCL2*, *CXCL8*, *CX3CL1*, *CX3CR1*, *CSF1R*, *CCR2*, *CFS1*, *IL-1R1*, and *IL-1RA*. Natural logarithm-transformed expression values of genes were scaled and visualized as a clustergram using the heatmap R package. The macrophage score was determined for each sample as previously described [22], and the fold-change between each patient and the HD group was computed.

Analysis of skin biopsies

3-µm-thick FFPE sections from four healthy donors and eight patients with SSc were stained. For multiplex staining of macrophages, the sections were incubated for 1 hour at room temperature with mouse anti-human DC-SIGN and visualized using the Alexa Fluor™ 647 Tyramide SuperBoost™ Kit and a goat anti-rabbit IgG following the manufacturer's instructions. Primary/secondary antibody complex was stripped by HIER and the sections were stained with mouse anti-human CD68 and rabbit anti-human cMAF primary antibodies and goat anti mouse Alexa Fluor 568 and Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, goat anti-rabbit IgG secondary antibodies. Whole-tissue sections were scanned using a Nanozoomer 2.0HT (Hamamatsu). Microscopy was performed at the Bordeaux Imaging Center. Immunofluorescence reagents are detailed in supplementary data section.

Statistical analysis

Statistical analysis was performed using Prism (GraphPad, La Jolla, CA). For distributions that satisfied the Shapiro–Wilk normality test, a two-tailed Student's t-test for unpaired or paired samples and one-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni correction were used to compare populations. When the normality test was not satisfied, the Mann-Whitney or Wilcoxon tests were used. Correlations were analyzed by Spearman test. Values of $p < 0.05$ were considered indicative of statistical significance. Data are means ± SEM, (#) was used for t-test and (*) for analysis of variance. * or #, $p < 0.05$; ** or ##, $p < 0.01$ *** or ###, $p < 0.001$; **** or #### $p < 0.0001$.

Results

IL-1β-Activated MECs promote differentiation of healthy donor monocytes into DC SIGN$^+$ AAMs *in vitro*

AAMs play a pivotal role in fibrosis during scleroderma. However, the mechanisms underlying AAM polarization in affected tissues are unknown. We evaluated whether IL-1β-activated MECs from healthy donors (HDs) modulate the polarization of monocyte-derived macrophages. As shown in **Figure 1A**, IL-1β-activated MEC conditioned medium (MEC-CM) induced a significant increase in the expression of phospho-STAT3 (pSTAT3), in terms of both the percentage and mean fluorescence intensity (MFI) compared to medium alone and non-activated MEC-CM. The expression of pSTAT1 was not detected under any condition (**Figure 1A**). The transcription factor cMAF, which regulates the expression of numerous alternative- and tumor-associated macrophage-related genes in mouse and human [23] was significantly upregulated at day 6 in macrophages generated in the presence of IL-1β-activated MEC-CM compared to non-activated CM ($p = 0.028$) (**Figure 1B**). CM from IL-1β-activated MECs induced significant upregulation of surface CD163 expression compared to medium alone, and there was a non-significant trend toward increased expression compared to non-activated MEC-CM ($p = 0.06$) (**Figure 1C and 1D**). The same observation held true for MFI. The AAM-associated marker CD206 was induced under all conditions, as expected, considering the presence of GM-CSF in the culture. DC-SIGN expression (% and MFI) was significantly increased in macrophages derived from monocytes cultured in the presence of IL-1β-activated MEC-CM compared to medium alone and non-activated MEC-CM (%, $p = 0.008$ and MFI, $p = 0.03$). In contrast, the M1 macrophage marker CCR7 was not detected and CCR2 expression was unchanged under all conditions (**Figure 1C** and data not shown).

Neither blockade of IL-1β in IL-1β-activated MEC-CM, nor addition of recombinant IL-1β to non-activated MEC-CM modified DC-SIGN or CD163 expression (**Supplementary Figure 1A**), ruling out a direct effect of IL-1β. Expression of CD163 and DC-SIGN were similar under GM- or M-CSF conditions (**Supplementary Figure 2**), excluding a specific effect of GM-CSF.

Irrespective of the stimulation conditions, IFN-γ, TNF-α, IL-12p40 (**Figure 1E**), and TGF-β (data not shown) were not detected in macrophage supernatants. IL-6 was not detected in macrophage supernatant induced by MEC-CM, and the chemokine (C-X-C motif) ligand (CXCL)8 level was comparable under all conditions tested (**Figure 1E and 1F**). In contrast, macrophages generated in the presence of IL-1β-activated MEC-CM produced significantly greater levels of IL-10 ($p = 0.016$), IL-1RA ($p = 0.015$), platelet-derived growth factor two B subunits (PDGF BB) ($p = 0.05$), CC chemokine ligand (CCL)18 ($p = 0.017$) and CCL2 ($p = 0.045$) compared to non-activated MEC-CM (**Figure 1F**). As shown in **Supplementary Figure 1B**, IL-1β was not directly responsible for the increased cytokine/chemokine production. Therefore, CM from IL-1β-activated MECs promotes the generation of p-STAT3$^+$ DC-SIGN$^+$ AAMs, which produce IL-10 and high levels of CCL2 and CCL18.

IL-1β-activated SSc MECs favor generation of DC-SIGN$^+$ macrophages with distinct functional capabilities.

We next determined whether MECs purified from SSc skin would behave as normal MECs. CM from IL-1β-activated SSc MECs increased pSTAT3, compared to HD conditions (**Figure 2A**). The magnitude of pSTAT3 induction was significantly higher with SSc CM both in terms of the percentage of positive cells and MFI while no significant difference was observed between HD and SSc non-activated condition (**Figure 2B**). IL-1β-activated SSc MEC-CM significantly increased cMAF (**Figure 2C**) and DC-SIGN (**Figure 2D**) expression

in macrophages compared to non-activated MEC CM. No difference in cMAF and DC-SIGN expression was observed between IL-1β-activated HD and SSc MEC-CM induced macrophages. CD163 and CD206 expression was comparable between the SSc and HD conditions (**Figure 2C**), as was that of CCR7 and CCR2 (data not shown).

While CCL18, IL-1RA, and PDGF BB production by macrophages were increased under the HD and SSc conditions, consistent with AAMs, CCL2 was markedly upregulated ($p = 0.038$). Surprisingly, despite cMAF induction, IL-10 was not produced by macrophages generated in the presence of IL-1β-activated SSc MECs compared to HD ($p = 0.009$). Of note, although it was barely detectable, irrespective of stimulation in HDs, IL-1β-activated SSc MECs strongly upregulated production of the M1-associated marker CXCL8 ($p = 0.009$; **Figure 2D**).

Altogether, these data demonstrate that IL-1β-stimulated MECs from patients with SSc promote generation of DC-SIGN$^+$ AAMs producing no IL-10 but high CCL2 and CXCL8 compared to MECs from HDs, thus harboring mixed AAM and M1 features.

IL-6 and endothelin-1 are involved in the generation of DC-SIGN$^+$ macrophages

To identify soluble factors responsible for the skewing of normal monocytes toward AMMs, we measured in MEC-CM the levels of several soluble promoters of macrophage polarization. While IL-4, IL-13, eotaxin, GM-CSF, M-CSF, TNF-α, and IFN-γ were not or barely detectable in all CM tested (data not shown), IL-6 was strongly induced in the presence of IL-1β but was not detected in CM of non-activated MECs (**Figure 3A**). Interestingly, the IL-6 level was higher in the CM of IL-1β-activated MECs from SSc patients. Even non-statistically significant, we observed a trend toward increased production of ET-1 in the CM of IL-1β-activated MECs (**Figure 3B**). We tested whether IL-6 receptor blockade by tocilizumab in IL-1β-activated MEC-CM would affect macrophage differentiation. IL-6

blockade significantly reduced the percentage (**Figure 3C and 3D**) and the MFI (data not shown) of pSTAT3 and DC-SIGN. ET-1, a key mediator in SSc, has been recently involved in AAM polarization [24]. Addition of macitentan (inhibitor of ET-1 receptors A and B) to CM from IL-1β-activated MECs decreased slightly but significantly both the percentage and MFI (data not shown) of DC-SIGN$^+$ cells ($p = 0.0003$; and $p = 0.002$). The proportion of pSTAT3$^+$ cells decreased significantly (**Figure 3C and 3D**) but the MFI was unchanged (data not shown).

As shown in **Figure 3C and 3D**, addition of recombinant IL-6 or ET1 to non-activated MEC-CM significantly increased DC-SIGN and p-STAT3 expression. IL-6 and ET-1 redundantly but not synergistically enhanced STAT3 phosphorylation (**Figure 3D**). Therefore, IL-1β promotes the production of IL-6 and ET-1 by MECs, favoring monocyte polarization toward DC-SIGN$^+$ AAMs.

AAM induced by IL-1β-activated MEC from SSc patients promote proinflammatory fibroblasts

To analyze their role in fibrosis and inflammation, we co-cultured DC-SIGN$^+$ AAMs with HD fibroblasts in Transwells. After 24 hours, fibroblasts were harvested and the mRNA levels of inflammatory (*CCL2, IL-1RA, IL-6, CXCL10, CCL5, CFS3*), matrix remodeling (*MMP-1*, *MMP-2*, *TIMP-1*), and extracellular matrix (*Col1A1* and *Col1A2*) genes were assessed. Also, the cytokine and chemokine global production was quantified in the co-culture supernatants.

Expression of *Col1A1*, *Col1A2*, *MMP-2*, and *TIMP1* by fibroblasts was unchanged irrespective of the stimulation conditions and the origin of the ECs (**Supplementary Figure 3**). In contrast, strong and reproducible increases in *MMP-1* and *CCL2* mRNA expression were observed in HDs and patients with SSc (**Supplementary Figure 3** and **Figure 4A**). Although macrophages induced in the presence of IL-1β-activated MEC-CM from patients

with SSc and HDs resulted in similar levels of *MMP1* mRNA, CCL2 was reproducibly and significantly higher in IL-1β-activated SSc MEC-CM, both at the mRNA and the protein levels ($p = 0.04$ and $p= 0.017$ respectively, **Figure 4A and 4B**). The IL-6 protein level was also significantly higher in the presence of macrophages induced by IL-1β-activated SSc-MECs compared to HD-MEC-CM (**Figure 4B**, $p= 0.028$). IL-6 mRNA was up regulated in fibroblasts cultured with IL-1β-activated MECs derived macrophages, but irrespective of the MEC origin, suggesting that both macrophages and fibroblasts contributed to the increased protein production observed in culture supernatant from IL-1β-activated MECs condition (**Figure 4C**) . Finally, the CCL5, CXCL10, G-CSF, and IL-1RA protein levels were also increased in IL-1β-activated MEC-CM conditions, irrespective of the source of the MECs (**Figure 4B**). However, as depicted in **Figure 4C**, IL1-RA and CCL5 transcripts were undetectable in any condition tested suggesting that both proteins were produced by macrophages. In sharp contrast, G-CSF expression was strongly up-regulated in fibroblasts cultured in the presence of macrophages generated with both HD and SSc IL-1β-activated MEC, confirming the contribution of fibroblasts in its production (**Figure 4C**). Interestingly, CXCL10 expression was reproducibly increased in fibroblasts cultured with macrophages generated with IL-1β-activated SSc MEC, while results from HD were more inconsistent, as seen at the protein level (**Figure 4B**).

Taken together, these results show that IL-1β-activated MECs from patients with SSc promote DC-SIGN$^+$ AAMs, which prompt fibroblasts to adopt a proinflammatory phenotype.

The DC-SIGN$^+$ AAM transcriptomic skin signature is a marker of early disease and a high fibrotic score

We next evaluated whether a specific macrophage transcriptomic signature could be used to classify the severity and systemic involvement of SSc. We quantified mRNA expression in

ten HDs and twenty-four SSc skin biopsies by nanostring technology. *CD68* mRNA expression was significantly upregulated in patients with SSc compared to HDs (0.75 log_2-fold change, adjusted p = 0.03). The fold change in *CD68* mRNA expression significantly correlated to the extent of cutaneous fibrosis assessed by the modified Rodnan Skin Score (mRSS) (r = 0.5, p = 0.008, **Supplementary Figure 4A**) and to *CD163* (r = 0.92, p < 0.0001, **Supplementary Figure 4B**) and *DC-SIGN* (r = 0.57, p < 0.0033, **Supplementary Figure 4C**) but not IL-10 (**Supplementary Figure 4D**) expression. Based on our *in vitro* data, we then defined a supervised macrophage gene signature comprising mRNAs of *CD68*, *CD163*, *CD209* (DC-SIGN), *MRC1* (CD206), *cMAF*, *STAT3*, *IL-10*, *CCL18*, *CCL2*, *CXCL8*, *CX3CL1*, *CX3CR1*, *CSF1R*, *CCR2*, *CFS1*, *IL-1R1*, and *IL-1RA*. Principal component analysis (PCA) and unsupervised clustering of enriched macrophage marker genes enabled differentiation of the patients and HDs. Interestingly, some patients clustered with HDs, but others did not (**Figure 5A and 5B**). To evaluate whether the signature was associated with clinical features, we calculated a score based on the mean log2 fold change in gene expression as described in [22]. As shown in **Figure 5C**, the score was increased in patients with diffuse cutaneous SSc compared to those with limited cutaneous SSc (**Figure 5C**) and in patients with pulmonary fibrosis (**Figure 5D**). Patients with a shorter disease duration had a significantly higher score than those with a longer disease duration (**Figure 5E**), but this needs to be confirmed because of the small number of patients with a shorter disease duration. Moreover, the score was strongly correlated with the mRSS (**Figure 5F**) (r = 0.6, p = 0.001).

Taken together, the DC-SIGN$^+$ AAM signature we defined is associated with the clinical characteristics of SSc; a high score being associated with severe skin and lung fibrosis and a shorter disease duration.

Perivascular localization and enrichment in SSc skin of DC-SIGN$^+$CD68$^+$ macrophages correlate with the extent of fibrosis

To confirm the presence and gain insight into the localization of DC-SIGN$^+$CD68$^+$ AAMs, we performed immunofluorescence analysis of skin biopsies from eight patients with SSc and four HDs. We first analyzed global cutaneous CD68$^+$ macrophage infiltration and their topographical location (within the dermis and around the vessels [morphologically determined]). As shown in **Figure 6A**, CD68$^+$ macrophages were detected in HD and SSc skin. However, their topographical distribution differed between SSc and HD and within SSc. In four SSc skin samples, CD68$^+$ macrophages were present in the dermis, as in HDs (**Figure 6A**, patients SSc1 and SSc3). In the four other SSc skin samples, CD68$^+$ macrophages were enriched in perivascular areas (**Figure 6A**, representative staining, patients SSc5 and SSc6). The total number of CD68$^+$ macrophages quantified using automated counting was significantly increased in patients with an mRSS > 10 compared to those with an mRSS < 10 and the HDs (**Figure 6B**). This result was consistent with the nanostring data as the four patients with a high mRSS had greater fold increases in *CD68* mRNA expression compared to the four with a low mRSS, which were similar to the HDs ($p = 0.029$; **Supplementary Figure 4E**). This was the result of an increased number and proportion of perivascular CD68$^+$ macrophages in skin biopsies from individuals with a high mRSS (**Figure 6D**).

We also analyzed the expression of DC-SIGN and cMAF in CD68$^+$ macrophages in skin sections. Perivascular CD68$^+$ macrophages from patients with SSc with a high mRSS (SSc 5 to 8) expressed DC-SIGN, whereas patients with SSc with a low mRSS (SSc 1 to 4) had no or barely detectable DC-SIGN$^+$ CD68$^+$ macrophages, similar to the HDs (**Figure 6E** and **Supplementary Figure 5)**. cMAF was not detected in CD68$^+$ macrophages in HD skin sections and was inconsistently expressed in CD68$^+$ macrophages from patients with SSc (**Figure 6E** and **Supplementary Figure 5)**. In patients with SSc with a high mRSS, not only

CD68$^+$DC-SIGN$^+$ macrophages were increased in the dermis but they were significantly enriched in perivascular areas compared to HDs and patients with SSc with a low fibrotic score (**Figure 6F**). The patients with barely detectable CD68$^+$DC-SIGN$^+$ macrophages (**Figure 6E**) clustered with the HDs heat map analysis (**Figures 5B**). In contrast, patients with a high mRSS did not cluster with the HDs (**Figure 5B**), exhibited greater CD68$^+$ macrophage infiltration and enhanced perivascular CD68$^+$DC-SIGN$^+$ expression (**Figure 6C-F**).

Therefore, CD68$^+$DC-SIGN$^+$ skin macrophages are enriched in the perivascular areas of patients with SSc with a high mRSS, supporting a role for SSc endothelial cells to favor DC-SIGN$^+$ AAMs generation that correlates to skin fibrosis. Moreover, the macrophage gene signature enables discrimination of patients with skin-infiltrating DC-SIGN$^+$ AAMs.

Discussion

Our findings provide insight into the role of MECs in macrophage polarization in SSc. By means of *in vitro*, *in situ*, and transcriptomic approaches we showed that IL-1β prompts SSc MECs to induce differentiation of monocytes into DC-SIGN^{+}CCL18^{high}CCL2^{high}CXCL8^{high}IL-10^{low}AAMs, which are associated with fibrosis.

Animal models have highlighted the role of the inflammasome and IL-1β in lung and skin fibrosis. IL-1 receptor type I (IL-1R)-knockout mice showed reduced fibrosis in both cutaneous and deep tissue wounds [25], and IL-1β transient overexpression induced by an adenoviral gene promoted severe progressive tissue fibrosis in the rat lung [19]. In a murine model of fibrosis induced by silica exposure, blocking production of IL-1β in *nlrp3*-null mice alleviated fibrosis [17]. In bleomycin-induced lung injury, a model of idiopathic pulmonary fibrosis, specific blockade of IL-1R1 reduced inflammation and fibrosis, and exogenous IL-1β administration alone was sufficient to mimic bleomycin-induced lung pathology [18]. In human SSc, studies have shown association between elevated levels of IL-1β and skin or lung fibrosis [26-28]. These data suggest that IL-1β is an important mediator of lung and skin inflammation and fibrosis but the precise mechanisms are still unclear, notably in human settings. Induction of myofibroblasts differentiation and collagen production by IL-1β-induced miR-55 expression [26], as well as endothelial to mesenchymal transition together with IL-6 and TGF-β2 [29] have been proposed. Finally, the use of blockers of IL-1β are scarce in both mouse [18, 30] and human SSc [31]**,** and yielded conflicting results suggesting that the effect of IL1-β inhibition is time and context-dependent. In this line, recent transcriptomic analysis of skin biopsies from the PRESS cohort of patients with early diffuse SSc showed that IL-1β was one of the three most-expressed cytokines, suggesting a role in the early phase of SSc [13].

~~These data suggest that IL-1β is an important mediator of lung and skin inflammation and fibrosis. However, evidence for IL-1β involvement in human SSc is scarce and the mechanisms are unclear. Nonetheless, transcriptomic analysis of skin biopsies from the PRESS cohort of patients with early diffuse SSc showed that IL-1β was one of the three most-expressed cytokines, suggesting a role in the early phase of SSc [13].~~

M-CSF alone or in combination with IL-4, CSF-1, and IL-1β are inducers of DC-SIGN in human and mouse macrophages [32-34]; by contrast, GM-CSF suppresses DC-SIGN expression [33]. In this study, upregulation of DC-SIGN was independent of IL-4 and M-CSF, because IL-4 was not detected in CM of MECs or differentiated macrophages and was induced in the absence of M-CSF but in the presence of GM-CSF. Rather, EC-derived IL-6 and endothelin-1 contributed to upregulation of DC-SIGN in macrophages, even in the presence of GM-CSF. Importantly and contrary to a prior report, IL-1β had no direct effect on DC-SIGN induction [34].

Enhanced pro-inflammatory and pro-fibrotic CCL2, IL-6, CXL10, and CXCL8 levels has been described in SSc, but the precise mechanisms contributing to their production is still unclear [35-37]. Here we show that not only DC-SIGN$^+$ AAMs induced by SSc ECs produced increased levels of CCL2 and CXCL8, but they also favor CCL2, IL-6 and CXCL10 production by fibroblasts highlighting a new dynamic interplay in SSc contributing to sustained inflammation. The role of increased MMP-1 expression is paradoxical. In bleomycin-induced fibrosis, a mouse model of SSc, MMP inhibitor prevents experimental fibrosis by increasing MMP-2 and -9 activity [38]. Interestingly, MMP-1 expression is increased and decreased in fibroblasts from patients with early and late SSc, respectively [39], suggesting that increased MMP-1 activity contributes to fibrosis in early SSc.

In a mouse model of allograft kidney rejection, DC-SIGN$^+$Ly6C^{low} macrophages induced expansion of Foxp3-expressing regulatory T cells and suppressed CD8$^+$ T-cell

proliferation in part, by releasing IL-10 in a manner dependent on DC-SIGN and TLR4 triggering [32]. Batteux and colleagues reported recently that repeated injection of a low dose of LPS reduced disease severity associated with the induction of ICOS ligand, DC-SIGN, and IL-10 production by dermal macrophages [40]. In contrast, we showed that perivascular DC-SIGN$^+$ CD68$^+$ macrophages are associated with fibrosis in patients with SSc. Interestingly, we provide *in vitro* evidence of a lack of IL-10 production by DC-SIGN$^+$ AAMs induced by SSc ECs despite cMAF induction, that might contribute to enhance inflammation and fibrosis in tissues. Hsa-miR-106a microRNA and tristetraprolin RNA-binding molecule have been involved in post-transcriptional degradation of IL-10 mRNA in human myeloid cells and mouse macrophages respectively [41, 42]. Whether post-transcriptional alterations account for the absence of IL-10 production by DC-SIGN$^+$ AAMs induced by SSc ECs still to be determined. Although some patients with SSc exhibited high *IL-10* expression in the skin according to nanostring analysis, there was no correlation between *CD68* and *IL-10* mRNA, suggesting that IL-10 was not being expressed by macrophages contrary to DC-SIGN.

Following inflammation and tissue damage, large amounts of carbohydrates are released, including those containing mannose or fucose residues or LewisX, which are potent DC-SIGN agonists [43]. Hence, liberation of carbohydrates by dying ECS or neutrophils in the vicinity of DC-SIGN$^+$ macrophages modulates their immune function during SSc. Whether triggering DC-SIGN on macrophages affects their immune functions and restores their IL-10 secretion is unknown.

Finally, we developed a new DC-SIGN$^+$ AAM gene signature based on *in vitro* data to distinguish patients according to their skin fibrosis score. Of note, the signature only shares CD163, CX3CR1, and IL-10RA with the M2 macrophage subnetwork defined by Whitfield [7]. High score patients have more severe fibrosis, a shorter disease duration and pulmonary complications.

In conclusion, our work sheds new light on the vicious circle implicating IL-1β sustained secretion, microvascular endothelial cells activation and generation of DC-SIGN$^+$ AAMs which trigger proinflammatory fibroblasts, contributing to the fibrotic process in scleroderma. Targeting this pathophysiological loop may open new therapeutic perspectives. The findings may also be relevant to other autoimmune and inflammatory fibrotic diseases.

Limitations of the study: our work gives insight onto the role of microvascular endothelial cells in fibrosis through the induction of type 2 macrophages endowed with peculiar cytokines production and pro-inflammatory fibroblasts induction. However, our study find several limitations among them the limited number of different SSc endothelial cells for macrophages induction and testing did not allow defining potential sub-groups. We did not assessed the functional consequences of altered cytokine/chemokines production by IL-1β activated SSc MEC induced DC-SIGN AAM, and further studies are warranted to determine how it mechanistically relates to SSc notably within tissue. Moreover, a more detailed phenotypic and functional characterization of perivascular cutaneous DC-SIGN+ macrophages by immunofluorescence and/or single cell RNA-seq analysis is needed to better understand their contribution to the fibrotic process.

498 **Authors' Contributions**

499 PL, JL, EL, CG, ID, DL and BJM performed and analyzed the experiments. PL, BA and CCB
500 performed statistical analyses. EL, PD, CR, JS, JLP, JC and TS recruited the participants and
501 provided samples and clinical data. BA, DD, TP, CC, and PB provided intellectual input and
502 edited the manuscript. CCB designed and supervised the study and the analysis of
503 experiments. CCB wrote the manuscript with MET. CCB and MET jointly supervised the
504 study.

505

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510

511 **Ethical approval information**

512 Patients were included in the context of the Vasculopathy and Inflammation in Systemic
513 Sclerosis biomedical research project (CPP, 2012-A00081-42, Aquitaine). All participants
514 provided written informed consent before inclusion.

515

516 **Data sharing statement**

517 The authors confirm that the data supporting the findings of this study are available within the
518 article [and/or] its supplementary materials.

519 **Patient and Public Involvement** not applicable

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662

663 **Figure Legends**

664

665 **Figure 1. IL-1β-activated HD MECs induce DC-SIGN$^+$ cMAF$^+$ alternative**
666 **macrophages. (A)** Representative flow cytometry histograms (numbers representing the
667 MFI) and cumulative data (5 independent experiments) of pSTAT3 and pSTAT1 expression
668 (percentages and MFIs) in monocytes incubated with medium, or non- or IL-1β-activated
669 MEC-CM from HDs. **(B)** *cMAF* mRNA expression in macrophages at day 6. Data are from
670 three independent experiments. **(C)** Representative histograms of CD163, CD206, DC-SIGN,
671 CCR7, and CCR2 expression in macrophages incubated with medium or non- or IL-1β-
672 activated MEC-CM from HDs. **(D)** Representative dot plots and cumulative data (5
673 independent experiments) of CD163, CD206 and DC-SIGN expression (percentages and
674 MFIs) in macrophages incubated with medium or non- or IL-1β-activated MEC-CM from
675 HDs. **(E)** Representative heat map of cytokine and chemokines levels (ng/mL) at day 6. **(F)**
676 Data are from five independent experiments. To ensure specificity, cytokine/chemokine levels
677 were measured in MEC_CM without macrophages for 6 days and deduced from those in
678 macrophage supernatant.

679

680 **Figure 2. IL-1β-activated SSc MECs induce DC-SIGN$^+$ alternative macrophages with**
681 **altered chemokine and cytokine production. (A)** Representative flow cytometry histograms
682 (numbers representing the MFI) and **(B)** data from five independent experiments of pSTAT3
683 and pSTAT1 expression (percentages of positive cells and MFIs) in monocytes incubated
684 with medium or non- or IL-1β-activated MEC-CM from patients with SSc and HDs. **(C)**
685 *cMAF* mRNA expression in macrophages at day 6 (HD vs SSc conditions). Data are from
686 three independent experiments. **(D)** Representative flow cytometry dot plots of CD163 and

687 DC-SIGN expression in macrophages incubated with medium or non- or IL-1β-activated
688 MEC-CM from patients with SSc and HDs. **(E)** Cytokine and chemokine levels in
689 macrophage culture supernatant at day 6. Data are from five independent experiments using
690 different MECs and monocytes.
691
692 **Figure 3. IL-1β-activated MECs produce IL-6 and ET-1, triggering DC-SIGN$^+$**
693 **alternative macrophage polarization. (A-B)** Production of IL-6 and ET-1 by non- and IL-
694 1β-activated MEC from HDs (n = 4) and patients with SSc (n = 3). **(C)** Upper panels,
695 representative experiment of pSTAT3 and DC-SIGN expression in macrophages induced by
696 IL-1β-activated MEC-CM, with or without IL-6 receptor blocking antibody (tocilizumab),
697 ET-1 receptors blocker (macitentan), or both. Tocilizumab and/or macitentan were pre-
698 incubated with monocytes before the addition of IL-1β-activated MEC-CM to potentiate their
699 effect. Lower panels, pSTAT3 and DC-SIGN expression in macrophages induced by non-
700 activated MEC-CM supplemented or not with recombinant IL-6 (Rec IL-6), ET-1 (Rec ET-1),
701 or both. **(D)** pSTAT3 and DC-SIGN expression from at least four independent experiments of
702 each above-described experimental setting.
703
704 **Figure 4. Macrophages differentiated in the presence of IL-1β-activated MEC**
705 **supernatant have proinflammatory and pro-remodeling effects on fibroblasts. (A)** qRT-
706 PCR analysis of *MMP1* and *CCL2* in fibroblasts incubated with macrophages stimulated with
707 non- and IL-1β-activated MEC-CM from HDs and patients with SSc. **(B)** Global
708 cytokine/chemokine production (CCL2, IL-6, IL-1RA, G-CSF, CCL5, and CXCL10) in
709 culture supernatant of fibroblasts incubated with macrophages stimulated with non- and IL-
710 1β-activated MEC-CM from HDs and patients with SSc. Data are from three independent
711 experiments. **(C)** qRT-PCR analysis of *IL-1RA, IL-6, CXCL10, CCL5, CFS3* in fibroblasts

incubated with macrophages stimulated with non- and IL-1β-activated MEC-CM from HDs and patients with SSc. UND : undetectable.

Figure 5. Enriched DC-SIGN$^+$ alternative macrophage gene signature in skin is associated with early SSc and correlates to fibrosis. (A-B) PCA and heatmap of macrophage marker genes in the skin of twent-four patients with SSc and ten HDs. Skin macrophage score in HDs, patients with limited SSc (lSSc) and diffuse SSc (dSSc) **(C)**, in patients with or without pulmonary disorders (**D**) and in the skin of patients with SSc with a disease duration < 3 or > 3 years **(E),** and **. (F)** Correlation between macrophage score in skin and the extent of fibrosis in patients with SSc.

Figure 6. DC-SIGN$^+$CD68$^+$ macrophages are enriched in perivascular area in SSc fibrotic skin. (A) Staining of CD68$^+$ macrophages in skin sections of HDs (left panels), patients with SSc with a low mRSS (middle panels), and patients with SSc with a high mRSS (right panels). **(B)** Density of dermal CD68$^+$ macrophages (cells/mm^2) in skin sections from four HDs, four patients with SSc with a low (< 10) mRSS, and four patients with SSc with a high (> 10) mRSS. **(C and D)** Automated quantification of perivascular CD68$^+$ macrophages in skin sections from HDs, patients with SSc with a low (< 10) mRSS, and patients with SSc with a high (> 10) mRSS. Data are cells/mm^2 **(C)** or percentages of perivascular positive cells **(D)**. **(E)** Immunostaining of CD68, cMAF, and DC-SIGN in skin sections from four HDs and eight patients with SSc by tissue multiplex immunofluorescence. **(F)** CD68$^+$DC-SIGN$^+$ cells in HDs, patients with SSc with a low (< 10) mRSS and patients with SSc with a high (> 10) mRSS expressed as percentages of perivascular positive cells.