



**HAL**  
open science

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

Paoline Laurent, Joelle Lapoirie, Damien Leleu, Emeline Levionnois, Cyrielle Grenier, Blanca Jurado-Mestre, Estibaliz Lazaro, Pierre Duffau, Christophe Richez, Julien Seneschal, et al.

## ► To cite this version:

Paoline Laurent, Joelle Lapoirie, Damien Leleu, Emeline Levionnois, Cyrielle Grenier, et al.. Interleukin-1 $\beta$ –Activated Microvascular Endothelial Cells Promote DC-SIGN–Positive Alternatively Activated Macrophages as a Mechanism of Skin Fibrosis in Systemic Sclerosis. *Arthritis & rheumatology*, 2022, 74 (6), pp.1013-1026. 10.1002/art.42061 . hal-03883526

**HAL Id: hal-03883526**

**<https://hal.science/hal-03883526>**

Submitted on 24 Dec 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Interleukin-1- $\beta$ -Activated Microvascular Endothelial Cells Promote DC-SIGN<sup>+</sup>**  
2 **Alternative Macrophages Associated with Skin Fibrosis in Systemic Sclerosis**

3

4 Paoline Laurent (PhD)<sup>1</sup>, Joelle Lapoirie (MD)<sup>2</sup>, Damien Leleu (PhD)<sup>1</sup>, Emeline Levionnois  
5 (MSc)<sup>1</sup>, Cyrielle Grenier (MD)<sup>1</sup>, Blanca Jurado-Mestre (MSc)<sup>1</sup>, Estibaliz Lazaro (MD,  
6 PhD)<sup>1,2</sup>, Pierre Duffau (MD, PhD)<sup>1,2</sup>, Christophe Richez (MD, PhD)<sup>1,2</sup>, Julien Seneschal (MD,  
7 PhD)<sup>2</sup>, Jean-Luc Pellegrin (MD, PhD)<sup>2</sup>, Joel Constans (MD, PhD)<sup>2</sup>, Thierry Schaefferbeke  
8 (MD, PhD)<sup>2</sup>, Isabelle Douchet (MSc)<sup>1</sup>, Dorothée Duluc (PhD)<sup>1</sup>, Thomas Pradeu (PhD)<sup>1</sup>, Carlo  
9 Chizzolini (MD, PhD)<sup>3</sup>, Patrick Blanco (MD, PhD)<sup>1,2</sup>, Marie-Elise Truchetet (MD, PhD)<sup>1,2,a</sup>,  
10 Cécile Contin-Bordes (PhD)<sup>1,2,a,\*</sup>.

11

12 <sup>1</sup>ImmunoConcEpt Laboratory, CNRS UMR 5164, Bordeaux University, F-33076 Bordeaux,  
13 France

14 <sup>2</sup>University Hospital Centre Bordeaux, F-33000 Bordeaux, France

15 <sup>3</sup>Pathology and Immunology, School of Medicine, Geneva University, Switzerland

16 <sup>a</sup>equal contribution

17

18 On behalf of the Fédération Hospitalo-Universitaire ACRONIM and Centre National de  
19 Référence des Maladies Auto-Immunes Systémiques Rares de l'Est et du Sud-Ouest (RESO),  
20 CHU Bordeaux, F-33000 Bordeaux, France

21

22

23

24

25 \*Corresponding author:

26 Dr. Cecile Contin-Bordes

27 Site de Carreire, Zone Nord, Bâtiment 1B

28 146 Rue Léo Saignat

29 33076 Bordeaux Cedex, France

30 [cecile.bordes@chu-bordeaux.fr](mailto:cecile.bordes@chu-bordeaux.fr)

31

32 The authors declare no conflict of interest with regard to the work

33

34 **Funding, grant/award info**

35 This study was supported by grants from the Foundation Arthritis and the Association des  
36 Sclérodermiques de France.

37

38 Total words count: **4491**

39

40

41

42

43

44

45

46

47 **Abstract**

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

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

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

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

68

69 **Keywords**

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

71 **Introduction**

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

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

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

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

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

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

109

110

111

112

113

114

115

116

117

118

119

120

121 **Patients and Methods**

122 Patients with SSc presenting at the University Hospital of Bordeaux, France, between March  
123 2014 and September 2016 were prospectively enrolled. All patients satisfied the classification  
124 criteria proposed by the American College of Rheumatology and the European League  
125 Against Rheumatism 2013. Patients were included in the context of the Vasculopathy and  
126 Inflammation in Systemic Sclerosis biomedical research project (CPP, 2012-A00081-42,  
127 Aquitaine). All participants provided written informed consent before inclusion. Age- and  
128 sex-matched healthy donors (HDs) were recruited at the local Blood Transfusion Centre  
129 (Etablissement Français du sang, Bordeaux). Clinical features and biological recordings are  
130 available in supplementary data section.

131

132 **Cell purification**

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

143

144

145

146 **Generation of monocyte-derived macrophages**

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

158

159 **Flow cytometry**

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

166

167 **Cytokine and chemokine assays**

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



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

175

#### 176 **Fibroblast and macrophage co-culture**

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

183

#### 184 **Quantification of mRNA expression in cutaneous tissue sections**

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

194

#### 195 **Analysis of skin biopsies**

196 3- $\mu$ m-thick FFPE sections from four healthy donors and eight patients with SSc were stained.  
197 For multiplex staining of macrophages, the sections were incubated for 1 hour at room  
198 temperature with mouse anti-human DC-SIGN and visualized using the Alexa Fluor™ 647  
199 Tyramide SuperBoost™ Kit and a goat anti-rabbit IgG following the manufacturer's  
200 instructions. Primary/secondary antibody complex was stripped by HIER and the sections  
201 were stained with mouse anti-human CD68 and rabbit anti-human cMAF primary antibodies  
202 and goat anti mouse Alexa Fluor 568 and Alexa Fluor™ 488 Tyramide SuperBoost™ Kit,  
203 goat anti-rabbit IgG secondary antibodies. Whole-tissue sections were scanned using a  
204 Nanozoomer 2.0HT (Hamamatsu). Microscopy was performed at the Bordeaux Imaging  
205 Center. Immunofluorescence reagents are detailed in supplementary data section.

206

#### 207 **Statistical analysis**

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

216

217

218

219

220

221

222

223 **Results**224 **IL-1 $\beta$ -Activated MECs promote differentiation of healthy donor monocytes into DC**225 **SIGN<sup>+</sup> AAMs *in vitro***

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

246 Neither blockade of IL-1 $\beta$  in IL-1 $\beta$ -activated MEC-CM, nor addition of recombinant IL-1 $\beta$  to  
247 non-activated MEC-CM modified DC-SIGN or CD163 expression (**Supplementary Figure**  
248 **1A**), ruling out a direct effect of IL-1 $\beta$ . Expression of CD163 and DC-SIGN were similar  
249 under GM- or M-CSF conditions (**Supplementary Figure 2**), excluding a specific effect of  
250 GM-CSF.

251 Irrespective of the stimulation conditions, IFN- $\gamma$ , TNF- $\alpha$ , IL-12p40 (**Figure 1E**), and  
252 TGF- $\beta$  (data not shown) were not detected in macrophage supernatants. IL-6 was not detected  
253 in macrophage supernatant induced by MEC-CM, and the chemokine (C-X-C motif) ligand  
254 (CXCL)8 level was comparable under all conditions tested (**Figure 1E and 1F**). In contrast,  
255 macrophages generated in the presence of IL-1 $\beta$ -activated MEC-CM produced significantly  
256 greater levels of IL-10 (p = 0.016), IL-1RA (p = 0.015), platelet-derived growth factor two B  
257 subunits (PDGF BB) (p = 0.05), CC chemokine ligand (CCL)18 (p = 0.017) and CCL2 (p =  
258 0.045) compared to non-activated MEC-CM (**Figure 1F**). As shown in **Supplementary**  
259 **Figure 1B**, IL-1 $\beta$  was not directly responsible for the increased cytokine/chemokine  
260 production. Therefore, CM from IL-1 $\beta$ -activated MECs promotes the generation of p-  
261 STAT3<sup>+</sup> DC-SIGN<sup>+</sup> AAMs, which produce IL-10 and high levels of CCL2 and CCL18.

262

263 **IL-1 $\beta$ -activated SSc MECs favor generation of DC-SIGN<sup>+</sup> macrophages with distinct**  
264 **functional capabilities.**

265 We next determined whether MECs purified from SSc skin would behave as normal MECs.  
266 CM from IL-1 $\beta$ -activated SSc MECs increased pSTAT3, compared to HD conditions (**Figure**  
267 **2A**). The magnitude of pSTAT3 induction was significantly higher with SSc CM both in  
268 terms of the percentage of positive cells and MFI while no significant difference was  
269 observed between HD and SSc non-activated condition (**Figure 2B**). IL-1 $\beta$ -activated SSc  
270 MEC-CM significantly increased cMAF (**Figure 2C**) and DC-SIGN (**Figure 2D**) expression

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

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

282 Altogether, these data demonstrate that IL-1 $\beta$ -stimulated MECs from patients with  
283 SSc promote generation of DC-SIGN<sup>+</sup> AAMs producing no IL-10 but high CCL2 and  
284 CXCL8 compared to MECs from HDs, thus harboring mixed AAM and M1 features.

285

### 286 **IL-6 and endothelin-1 are involved in the generation of DC-SIGN<sup>+</sup> macrophages**

287 To identify soluble factors responsible for the skewing of normal monocytes toward AMMs,  
288 we measured in MEC-CM the levels of several soluble promoters of macrophage polarization.

289 While IL-4, IL-13, eotaxin, GM-CSF, M-CSF, TNF- $\alpha$ , and IFN- $\gamma$  were not or barely  
290 detectable in all CM tested (data not shown), IL-6 was strongly induced in the presence of IL-  
291 1 $\beta$  but was not detected in CM of non-activated MECs (**Figure 3A**). Interestingly, the IL-6  
292 level was higher in the CM of IL-1 $\beta$ -activated MECs from SSc patients. Even non-  
293 statistically significant, we observed a trend toward increased production of ET-1 in the CM  
294 of IL-1 $\beta$ -activated MECs (**Figure 3B**). We tested whether IL-6 receptor blockade by  
295 tocilizumab in IL-1 $\beta$ -activated MEC-CM would affect macrophage differentiation. IL-6

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

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

308

### 309 **AAM induced by IL-1 $\beta$ -activated MEC from SSc patients promote proinflammatory** 310 **fibroblasts**

311 To analyze their role in fibrosis and inflammation, we co-cultured DC-SIGN<sup>+</sup> AAMs with HD  
312 fibroblasts in Transwells. After 24 hours, fibroblasts were harvested and the mRNA levels of  
313 inflammatory (*CCL2*, *IL-1RA*, *IL-6*, *CXCL10*, *CCL5*, *CFS3*), matrix remodeling (*MMP-1*,  
314 *MMP-2*, *TIMP-1*), and extracellular matrix (*Coll1A1* and *Coll1A2*) genes were assessed. Also,  
315 the cytokine and chemokine global production was quantified in the co-culture supernatants.

316 Expression of *Coll1A1*, *Coll1A2*, *MMP-2*, and *TIMP1* by fibroblasts was unchanged  
317 irrespective of the stimulation conditions and the origin of the ECs (**Supplementary Figure**  
318 **3**). In contrast, strong and reproducible increases in *MMP-1* and *CCL2* mRNA expression  
319 were observed in HDs and patients with SSc (**Supplementary Figure 3** and **Figure 4A**).  
320 Although macrophages induced in the presence of IL-1 $\beta$ -activated MEC-CM from patients

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

339 Taken together, these results show that IL-1 $\beta$ -activated MECs from patients with SSc  
340 promote DC-SIGN<sup>+</sup> AAMs, which prompt fibroblasts to adopt a proinflammatory phenotype.

341

342 **The DC-SIGN<sup>+</sup> AAM transcriptomic skin signature is a marker of early disease and a**  
343 **high fibrotic score**

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

346 ten HDs and twenty-four SSc skin biopsies by nanostring technology. *CD68* mRNA  
347 expression was significantly upregulated in patients with SSc compared to HDs (0.75 log<sub>2</sub>-  
348 fold change, adjusted  $p = 0.03$ ). The fold change in *CD68* mRNA expression significantly  
349 correlated to the extent of cutaneous fibrosis assessed by the modified Rodnan Skin Score  
350 (mRSS) ( $r = 0.5$ ,  $p = 0.008$ , **Supplementary Figure 4A**) and to *CD163* ( $r = 0.92$ ,  $p < 0.0001$ ,  
351 **Supplementary Figure 4B**) and *DC-SIGN* ( $r = 0.57$ ,  $p < 0.0033$ , **Supplementary Figure 4C**)  
352 but not IL-10 (**Supplementary Figure 4D**) expression. Based on our *in vitro* data, we then  
353 defined a supervised macrophage gene signature comprising mRNAs of *CD68*, *CD163*,  
354 *CD209* (DC-SIGN), *MRC1* (CD206), *cMAF*, *STAT3*, *IL-10*, *CCL18*, *CCL2*, *CXCL8*, *CX3CL1*,  
355 *CX3CR1*, *CSF1R*, *CCR2*, *CFS1*, *IL-1R1*, and *IL-1RA*. Principal component analysis (PCA)  
356 and unsupervised clustering of enriched macrophage marker genes enabled differentiation of  
357 the patients and HDs. Interestingly, some patients clustered with HDs, but others did not  
358 (**Figure 5A and 5B**). To evaluate whether the signature was associated with clinical features,  
359 we calculated a score based on the mean log<sub>2</sub> fold change in gene expression as described in  
360 [22]. As shown in **Figure 5C**, the score was increased in patients with diffuse cutaneous SSc  
361 compared to those with limited cutaneous SSc (**Figure 5C**) and in patients with pulmonary  
362 fibrosis (**Figure 5D**). Patients with a shorter disease duration had a significantly higher score  
363 than those with a longer disease duration (**Figure 5E**), but this needs to be confirmed because  
364 of the small number of patients with a shorter disease duration. Moreover, the score was  
365 strongly correlated with the mRSS (**Figure 5F**) ( $r = 0.6$ ,  $p = 0.001$ ).

366 Taken together, the DC-SIGN<sup>+</sup> AAM signature we defined is associated with the  
367 clinical characteristics of SSc; a high score being associated with severe skin and lung fibrosis  
368 and a shorter disease duration.

369



370 **Perivascular localization and enrichment in SSc skin of DC-SIGN<sup>+</sup>CD68<sup>+</sup> macrophages**  
371 **correlate with the extent of fibrosis**

372 To confirm the presence and gain insight into the localization of DC-SIGN<sup>+</sup>CD68<sup>+</sup> AAMs, we  
373 performed immunofluorescence analysis of skin biopsies from eight patients with SSc and  
374 four HDs. We first analyzed global cutaneous CD68<sup>+</sup> macrophage infiltration and their  
375 topographical location (within the dermis and around the vessels [morphologically  
376 determined]). As shown in **Figure 6A**, CD68<sup>+</sup> macrophages were detected in HD and SSc  
377 skin. However, their topographical distribution differed between SSc and HD and within SSc.  
378 In four SSc skin samples, CD68<sup>+</sup> macrophages were present in the dermis, as in HDs (**Figure**  
379 **6A**, patients SSc1 and SSc3). In the four other SSc skin samples, CD68<sup>+</sup> macrophages were  
380 enriched in perivascular areas (**Figure 6A**, representative staining, patients SSc5 and SSc6).  
381 The total number of CD68<sup>+</sup> macrophages quantified using automated counting was  
382 significantly increased in patients with an mRSS > 10 compared to those with an mRSS < 10  
383 and the HDs (**Figure 6B**). This result was consistent with the nanostring data as the four  
384 patients with a high mRSS had greater fold increases in *CD68* mRNA expression compared to  
385 the four with a low mRSS, which were similar to the HDs (p = 0.029; **Supplementary Figure**  
386 **4E**). This was the result of an increased number and proportion of perivascular CD68<sup>+</sup>  
387 macrophages in skin biopsies from individuals with a high mRSS (**Figure 6D**).

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

395 CD68<sup>+</sup>DC-SIGN<sup>+</sup> macrophages were increased in the dermis but they were significantly  
396 enriched in perivascular areas compared to HDs and patients with SSc with a low fibrotic  
397 score (**Figure 6F**). The patients with barely detectable CD68<sup>+</sup>DC-SIGN<sup>+</sup> macrophages  
398 (**Figure 6E**) clustered with the HDs heat map analysis (**Figures 5B**). In contrast, patients with  
399 a high mRSS did not cluster with the HDs (**Figure 5B**), exhibited greater CD68<sup>+</sup> macrophage  
400 infiltration and enhanced perivascular CD68<sup>+</sup>DC-SIGN<sup>+</sup> expression (**Figure 6C-F**).  
401 Therefore, CD68<sup>+</sup>DC-SIGN<sup>+</sup> skin macrophages are enriched in the perivascular areas of  
402 patients with SSc with a high mRSS, supporting a role for SSc endothelial cells to favor DC-  
403 SIGN<sup>+</sup> AAMs generation that correlates to skin fibrosis. Moreover, the macrophage gene  
404 signature enables discrimination of patients with skin-infiltrating DC-SIGN<sup>+</sup> AAMs.  
405

406 **Discussion**

407 Our findings provide insight into the role of MECs in macrophage polarization in SSc. By  
408 means of *in vitro*, *in situ*, and transcriptomic approaches we showed that IL-1 $\beta$  prompts SSc  
409 MECs to induce differentiation of monocytes into DC-  
410 SIGN<sup>+</sup>CCL18<sup>high</sup>CCL2<sup>high</sup>CXCL8<sup>high</sup>IL-10<sup>low</sup> AAMs, which are associated with fibrosis.

411 Animal models have highlighted the role of the inflammasome and IL-1 $\beta$  in lung and  
412 skin fibrosis. IL-1 receptor type I (IL-1R)-knockout mice showed reduced fibrosis in both  
413 cutaneous and deep tissue wounds [25], and IL-1 $\beta$  transient overexpression induced by an  
414 adenoviral gene promoted severe progressive tissue fibrosis in the rat lung [19]. In a murine  
415 model of fibrosis induced by silica exposure, blocking production of IL-1 $\beta$  in *nlrp3*-null mice  
416 alleviated fibrosis [17]. In bleomycin-induced lung injury, a model of idiopathic pulmonary  
417 fibrosis, specific blockade of IL-1R1 reduced inflammation and fibrosis, and exogenous IL-1 $\beta$   
418 administration alone was sufficient to mimic bleomycin-induced lung pathology [18]. In  
419 human SSc, studies have shown association between elevated levels of IL-1 $\beta$  and skin or lung  
420 fibrosis [26-28]. These data suggest that IL-1 $\beta$  is an important mediator of lung and skin  
421 inflammation and fibrosis but the precise mechanisms are still unclear, notably in human  
422 settings. Induction of myofibroblasts differentiation and collagen production by IL-1 $\beta$ -  
423 induced miR-55 expression [26], as well as endothelial to mesenchymal transition together  
424 with IL-6 and TGF- $\beta$ 2 [29] have been proposed. Finally, the use of blockers of IL-1 $\beta$  are  
425 scarce in both mouse [18, 30] and human SSc [31], and yielded conflicting results suggesting  
426 that the effect of IL-1 $\beta$  inhibition is time and context-dependent. In this line, recent  
427 transcriptomic analysis of skin biopsies from the PRESS cohort of patients with early diffuse  
428 SSc showed that IL-1 $\beta$  was one of the three most-expressed cytokines, suggesting a role in  
429 the early phase of SSc [13].

430

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

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

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

454 In a mouse model of allograft kidney rejection, DC-SIGN<sup>+</sup>Ly6C<sup>low</sup> macrophages  
455 induced expansion of Foxp3-expressing regulatory T cells and suppressed CD8<sup>+</sup> T-cell

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

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

476 Finally, we developed a new DC-SIGN<sup>+</sup> AAM gene signature based on *in vitro* data to  
477 distinguish patients according to their skin fibrosis score. Of note, the signature only shares  
478 CD163, CX3CR1, and IL-10RA with the M2 macrophage subnetwork defined by Whitfield  
479 [7]. High score patients have more severe fibrosis, a shorter disease duration and pulmonary  
480 complications.

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

486  
487 **Limitations of the study:** our work gives insight onto the role of microvascular endothelial  
488 cells in fibrosis through the induction of type 2 macrophages endowed with peculiar cytokines  
489 production and pro-inflammatory fibroblasts induction. However, our study find several  
490 limitations among them the limited number of different SSc endothelial cells for macrophages  
491 induction and testing did not allow defining potential sub-groups. We did not assessed the  
492 functional consequences of altered cytokine/chemokines production by IL-1 $\beta$  activated SSc  
493 MEC induced DC-SIGN AAM, and further studies are warranted to determine how it  
494 mechanistically relates to SSc notably within tissue. Moreover, a more detailed phenotypic  
495 and functional characterization of perivascular cutaneous DC-SIGN<sup>+</sup> macrophages by  
496 immunofluorescence and/or single cell RNA-seq analysis is needed to better understand their  
497 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

506 **Acknowledgments**

507 We are grateful to the Groupe de Recherche en Immunologie Clinique at Bordeaux University  
508 Hospital for assistance with the nanostring analysis. We thank Sébastien Marais from the BIC  
509 for help with image analysis and the Macha Nikolski's group for bioinformatics analysis.

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

520

521 **References**

- 522 1. Denton CP and Khanna D. Systemic sclerosis. *Lancet* 2017;390:1685-1699.
- 523 2. Asano Y. Systemic sclerosis. *J Dermatol* 2018;45:128-138.
- 524 3. Cutolo M, Soldano S, and Smith V. Pathophysiology of systemic sclerosis: current  
525 understanding and new insights. *Expert Rev Clin Immunol* 2019;15:753-764.
- 526 4. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdts S, et al. Macrophage  
527 activation and polarization: nomenclature and experimental guidelines. *Immunity*  
528 2014;41:14-20.
- 529 5. Manetti M. Deciphering the alternatively activated (M2) phenotype of macrophages in  
530 scleroderma. *Exp Dermatol* 2015;24:576-8.
- 531 6. Nakayama W, Jinnin M, Makino K, Kajihara I, Makino T, Fukushima S, et al. Serum  
532 levels of soluble CD163 in patients with systemic sclerosis. *Rheumatol Int*  
533 2012;32:403-7.
- 534 7. Mahoney JM, Taroni J, Martyanov V, Wood TA, Greene CS, Pioli PA, et al. Systems  
535 level analysis of systemic sclerosis shows a network of immune and profibrotic  
536 pathways connected with genetic polymorphisms. *PLoS Comput Biol*  
537 2015;11:e1004005.
- 538 8. Taroni JN, Greene CS, Martyanov V, Wood TA, Christmann RB, Farber HW, et al. A  
539 novel multi-network approach reveals tissue-specific cellular modulators of fibrosis in  
540 systemic sclerosis. *Genome Med* 2017;9:27.
- 541 9. Huang J, Maier C, Zhang Y, Soare A, Dees C, Beyer C, et al. Nintedanib inhibits  
542 macrophage activation and ameliorates vascular and fibrotic manifestations in the Fra2  
543 mouse model of systemic sclerosis. *Ann Rheum Dis* 2017;76:1941-1948.
- 544 10. Maier C, Ramming A, Bergmann C, Weinkam R, Kittan N, Schett G, et al. Inhibition  
545 of phosphodiesterase 4 (PDE4) reduces dermal fibrosis by interfering with the release  
546 of interleukin-6 from M2 macrophages. *Ann Rheum Dis* 2017;76:1133-1141.
- 547 11. Khanna D, Denton CP, Jahreis A, van Laar JM, Frech TM, Anderson ME, et al. Safety  
548 and efficacy of subcutaneous tocilizumab in adults with systemic sclerosis  
549 (faSScinate): a phase 2, randomised, controlled trial. *Lancet* 2016;387:2630-2640.
- 550 12. Trombetta AC, Soldano S, Contini P, Tomatis V, Ruaro B, Paolino S, et al. A  
551 circulating cell population showing both M1 and M2 monocyte/macrophage surface  
552 markers characterizes systemic sclerosis patients with lung involvement. *Respir Res*  
553 2018;19:186.
- 554 13. Skaug B, Khanna D, Swindell WR, Hinchcliff ME, Frech TM, Steen VD, et al. Global  
555 skin gene expression analysis of early diffuse cutaneous systemic sclerosis shows a  
556 prominent innate and adaptive inflammatory profile. *Ann Rheum Dis* 2020;79:379-  
557 386.
- 558 14. Locati M, Curtale G, and Mantovani A. Diversity, Mechanisms, and Significance of  
559 Macrophage Plasticity. *Annu Rev Pathol* 2020;15:123-147.
- 560 15. De Luca G, Cavalli G, Campochiaro C, Bruni C, Tomelleri A, Dagna L, et al.  
561 Interleukin-1 and Systemic Sclerosis: Getting to the Heart of Cardiac Involvement.  
562 *Front Immunol* 2021;12:653950.
- 563 16. Xu D, Mu R, and Wei X. The Roles of IL-1 Family Cytokines in the Pathogenesis of  
564 Systemic Sclerosis. *Front Immunol* 2019;10:2025.
- 565 17. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, et al. The  
566 Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci*  
567 U S A 2008;105:9035-40.



- 568 18. Gasse P, Mary C, Guenon I, Noulin N, Charron S, Schnyder-Candrian S, et al. IL-  
569 1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation  
570 and fibrosis in mice. *J Clin Invest* 2007;117:3786-99.
- 571 19. Kolb M, Margetts PJ, Anthony DC, Pitossi F, and Gauldie J. Transient expression of  
572 IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J*  
573 *Clin Invest* 2001;107:1529-36.
- 574 20. Truchetet ME, Demoures B, Eduardo Guimaraes J, Bertrand A, Laurent P, Jolivel V,  
575 et al. Platelets Induce Thymic Stromal Lymphopoietin Production by Endothelial  
576 Cells: Contribution to Fibrosis in Human Systemic Sclerosis. *Arthritis Rheumatol*  
577 2016;68:2784-2794.
- 578 21. Henrot P, Laurent P, Levionnois E, Leleu D, Pain C, Truchetet ME, et al. A Method  
579 for Isolating and Culturing Skin Cells: Application to Endothelial Cells, Fibroblasts,  
580 Keratinocytes, and Melanocytes From Punch Biopsies in Systemic Sclerosis Skin.  
581 *Front Immunol* 2020;11:566607.
- 582 22. Danaher P, Warren S, Dennis L, D'Amico L, White A, Disis ML, et al. Gene  
583 expression markers of Tumor Infiltrating Leukocytes. *J Immunother Cancer*  
584 2017;5:18.
- 585 23. Liu M, Tong Z, Ding C, Luo F, Wu S, Wu C, et al. Transcription factor c-Maf is a  
586 checkpoint that programs macrophages in lung cancer. *J Clin Invest* 2020;130:2081-  
587 2096.
- 588 24. Soldano S, Pizzorni C, Paolino S, Trombetta AC, Montagna P, Brizzolara R, et al.  
589 Alternatively Activated (M2) Macrophage Phenotype Is Inducible by Endothelin-1 in  
590 Cultured Human Macrophages. *PLoS One* 2016;11:e0166433.
- 591 25. Thomay AA, Daley JM, Sabo E, Worth PJ, Shelton LJ, Harty MW, et al. Disruption of  
592 interleukin-1 signaling improves the quality of wound healing. *Am J Pathol*  
593 2009;174:2129-36.
- 594 26. Artlett CM, Sassi-Gaha S, Hope JL, Feghali-Bostwick CA, and Katsikis PD. Mir-155  
595 is overexpressed in systemic sclerosis fibroblasts and is required for NLRP3  
596 inflammasome-mediated collagen synthesis during fibrosis. *Arthritis Res Ther*  
597 2017;19:144.
- 598 27. Artlett CM, Sassi-Gaha S, Rieger JL, Boesteanu AC, Feghali-Bostwick CA, and  
599 Katsikis PD. The inflammasome activating caspase 1 mediates fibrosis and  
600 myofibroblast differentiation in systemic sclerosis. *Arthritis Rheum* 2011;63:3563-74.
- 601 28. Martinez-Godinez MA, Cruz-Dominguez MP, Jara LJ, Dominguez-Lopez A, Jarillo-  
602 Luna RA, Vera-Lastra O, et al. Expression of NLRP3 inflammasome, cytokines and  
603 vascular mediators in the skin of systemic sclerosis patients. *Isr Med Assoc J*  
604 2015;17:5-10.
- 605 29. Maleszewska M, Moonen JR, Huijkman N, van de Sluis B, Krenning G, and Harmsen  
606 MC. IL-1beta and TGFbeta2 synergistically induce endothelial to mesenchymal  
607 transition in an NFkappaB-dependent manner. *Immunobiology* 2013;218:443-54.
- 608 30. Birnhuber A, Crnkovic S, Biasin V, Marsh LM, Odler B, Sahu-Osen A, et al. IL-1  
609 receptor blockade skews inflammation towards Th2 in a mouse model of systemic  
610 sclerosis. *Eur Respir J* 2019;54.
- 611 31. Mantero JC, Kishore N, Ziemek J, Stifano G, Zammitti C, Khanna D, et al.  
612 Randomised, double-blind, placebo-controlled trial of IL1-trap, rilonacept, in systemic  
613 sclerosis. A phase I/II biomarker trial. *Clin Exp Rheumatol* 2018;36 Suppl 113:146-  
614 149.
- 615 32. Conde P, Rodriguez M, van der Touw W, Jimenez A, Burns M, Miller J, et al. DC-  
616 SIGN(+) Macrophages Control the Induction of Transplantation Tolerance. *Immunity*  
617 2015;42:1143-58.

- 618 33. Dominguez-Soto A, Sierra-Filardi E, Puig-Kroger A, Perez-Maceda B, Gomez-  
619 Aguado F, Corcuera MT, et al. Dendritic cell-specific ICAM-3-grabbing nonintegrin  
620 expression on M2-polarized and tumor-associated macrophages is macrophage-CSF  
621 dependent and enhanced by tumor-derived IL-6 and IL-10. *J Immunol* 2011;186:2192-  
622 200.
- 623 34. Schenk M, Fabri M, Krutzik SR, Lee DJ, Vu DM, Sieling PA, et al. Interleukin-1beta  
624 triggers the differentiation of macrophages with enhanced capacity to present  
625 mycobacterial antigen to T cells. *Immunology* 2014;141:174-80.
- 626 35. Carvalheiro T, Horta S, van Roon JAG, Santiago M, Salvador MJ, Trindade H, et al.  
627 Increased frequencies of circulating CXCL10-, CXCL8- and CCL4-producing  
628 monocytes and Siglec-3-expressing myeloid dendritic cells in systemic sclerosis  
629 patients. *Inflamm Res* 2018;67:169-177.
- 630 36. Codullo V, Baldwin HM, Singh MD, Fraser AR, Wilson C, Gilmour A, et al. An  
631 investigation of the inflammatory cytokine and chemokine network in systemic  
632 sclerosis. *Ann Rheum Dis* 2011;70:1115-21.
- 633 37. Schmidt K, Martinez-Gamboa L, Meier S, Witt C, Meisel C, Hanitsch LG, et al.  
634 Bronchoalveolar lavage fluid cytokines and chemokines as markers and predictors  
635 for the outcome of interstitial lung disease in systemic sclerosis patients. *Arthritis Res*  
636 *Ther* 2009;11:R111.
- 637 38. Corbel M, Lanchou J, Germain N, Malledant Y, Boichot E, and Lagente V.  
638 Modulation of airway remodeling-associated mediators by the antifibrotic compound,  
639 pirfenidone, and the matrix metalloproteinase inhibitor, batimastat, during acute lung  
640 injury in mice. *Eur J Pharmacol* 2001;426:113-21.
- 641 39. Kuroda K and Shinkai H. Gene expression of types I and III collagen, decorin, matrix  
642 metalloproteinases and tissue inhibitors of metalloproteinases in skin fibroblasts from  
643 patients with systemic sclerosis. *Arch Dermatol Res* 1997;289:567-72.
- 644 40. Jeljeli M, Riccio LGC, Doridot L, Chene C, Nicco C, Chouzenoux S, et al. Trained  
645 immunity modulates inflammation-induced fibrosis. *Nat Commun* 2019;10:5670.
- 646 41. Sharma A, Kumar M, Aich J, Hariharan M, Brahmachari SK, Agrawal A, et al.  
647 Posttranscriptional regulation of interleukin-10 expression by hsa-miR-106a. *Proc*  
648 *Natl Acad Sci U S A* 2009;106:5761-6.
- 649 42. Stoecklin G, Tenenbaum SA, Mayo T, Chittur SV, George AD, Baroni TE, et al.  
650 Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. *J*  
651 *Biol Chem* 2008;283:11689-99.
- 652 43. van Liempt E, Bank CM, Mehta P, Garcia-Vallejo JJ, Kawar ZS, Geyer R, et al.  
653 Specificity of DC-SIGN for mannose- and fucose-containing glycans. *FEBS Lett*  
654 2006;580:6123-31.

656

657

658

659

660

661

662

663 **Figure Legends**

664

665 **Figure 1. IL-1 $\beta$ -activated HD MECs induce DC-SIGN<sup>+</sup> cMAF<sup>+</sup> 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-1 $\beta$ -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-1 $\beta$ -  
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-1 $\beta$ -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-1 $\beta$ -activated SSc MECs induce DC-SIGN<sup>+</sup> 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-1 $\beta$ -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-1 $\beta$ -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-1 $\beta$ -activated MECs produce IL-6 and ET-1, triggering DC-SIGN<sup>+</sup>**  
693 **alternative macrophage polarization. (A-B)** Production of IL-6 and ET-1 by non- and IL-  
694 1 $\beta$ -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-1 $\beta$ -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-1 $\beta$ -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-1 $\beta$ -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-1 $\beta$ -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 1 $\beta$ -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

712 incubated with macrophages stimulated with non- and IL-1 $\beta$ -activated MEC-CM from HDs  
713 and patients with SSc. UND : undetectable.

714

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

722

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

735

736