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

3

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

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12 ¹ImmunoConcEpt Laboratory, CNRS UMR 5164, Bordeaux University, F-33076 Bordeaux,
13 France

14 ²University Hospital Centre Bordeaux, F-33000 Bordeaux, France

15 ³Pathology and Immunology, School of Medicine, Geneva University, Switzerland

16 ^aequal 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

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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

31

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47 **Abstract**

48 **Objective:** To characterize the role of interleukin (IL)-1 β 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 β -activated MECs from SSc patients induced monocytes to differentiate into
60 DC-SIGN⁺ AAMs, which produced high levels of CCL18, CCL2, and CXCL8 but low level
61 of IL-10. DC-SIGN⁺ 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 β 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- γ], tumor necrosis factor-
82 alpha [TNF- α], and interleukin [IL]-1 β) [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 β or
84 LPS (M2b); IL-10 and transforming growth factor-beta (TGF- β); 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 β release in lung and skin fibrosis [17-19]. Our group
104 recently reported that activated platelets promote IL-1 β -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 β -activated
107 microvascular endothelial cells (MECs) indirectly favor fibrotic processes by promoting a
108 microenvironment that modulates polarization of cutaneous macrophages during SSc.

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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 β (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.

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146 **Generation of monocyte-derived macrophages**

147 Monocytes (5×10^4) 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 β (Canakinumab, 150 μ 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 β receptor by IL-1 β contained in MEC-CM, blocking anti-IL-1 β 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 α , and IFN- γ 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 (3×10^4) in
179 the lower chamber of Transwell microplates. Fibroblasts were seeded (3×10^4) 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- μ 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 #### $p < 0.0001$.

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223 **Results**224 **IL-1 β -Activated MECs promote differentiation of healthy donor monocytes into DC**225 **SIGN⁺ 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 β -
228 activated MECs from healthy donors (HDs) modulate the polarization of monocyte-derived
229 macrophages. As shown in **Figure 1A**, IL-1 β -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 β -
236 activated MEC-CM compared to non-activated CM ($p = 0.028$) (**Figure 1B**). CM from IL-1 β -
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 β -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 β in IL-1 β -activated MEC-CM, nor addition of recombinant IL-1 β to
247 non-activated MEC-CM modified DC-SIGN or CD163 expression (**Supplementary Figure**
248 **1A**), ruling out a direct effect of IL-1 β . 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- γ , TNF- α , IL-12p40 (**Figure 1E**), and
252 TGF- β (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 β -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 β was not directly responsible for the increased cytokine/chemokine
260 production. Therefore, CM from IL-1 β -activated MECs promotes the generation of p-
261 STAT3⁺ DC-SIGN⁺ AAMs, which produce IL-10 and high levels of CCL2 and CCL18.

262

263 **IL-1 β -activated SSc MECs favor generation of DC-SIGN⁺ 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 β -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 β -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 β -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 β -activated SSc MECs compared to HD ($p = 0.009$). Of note,
279 although it was barely detectable, irrespective of stimulation in HDs, IL-1 β -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 β -stimulated MECs from patients with
283 SSc promote generation of DC-SIGN⁺ 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⁺ 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- α , and IFN- γ 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 β 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 β -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 β -activated MECs (**Figure 3B**). We tested whether IL-6 receptor blockade by
295 tocilizumab in IL-1 β -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 β -activated MECs decreased slightly but significantly both the percentage and
300 MFI (data not shown) of DC-SIGN⁺ cells ($p = 0.0003$; and $p = 0.002$). The proportion of
301 pSTAT3⁺ 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 β promotes the production of IL-6 and ET-1 by MECs, favoring monocyte
307 polarization toward DC-SIGN⁺ AAMs.

308

309 **AAM induced by IL-1 β -activated MEC from SSc patients promote proinflammatory** 310 **fibroblasts**

311 To analyze their role in fibrosis and inflammation, we co-cultured DC-SIGN⁺ 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 (*CollA1* and *CollA2*) genes were assessed. Also,
315 the cytokine and chemokine global production was quantified in the co-culture supernatants.

316 Expression of *CollA1*, *CollA2*, *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 β -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 β -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 β -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 β -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 β -activated MECs condition
329 (**Figure 4C**). Finally, the CCL5, CXCL10, G-CSF, and IL-1RA protein levels were also
330 increased in IL-1 β -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 β -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 β -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 β -activated MECs from patients with SSc
340 promote DC-SIGN⁺ AAMs, which prompt fibroblasts to adopt a proinflammatory phenotype.

341

342 **The DC-SIGN⁺ 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 log₂-
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 log₂ 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⁺ 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⁺CD68⁺ macrophages**
371 **correlate with the extent of fibrosis**

372 To confirm the presence and gain insight into the localization of DC-SIGN⁺CD68⁺ AAMs, we
373 performed immunofluorescence analysis of skin biopsies from eight patients with SSc and
374 four HDs. We first analyzed global cutaneous CD68⁺ macrophage infiltration and their
375 topographical location (within the dermis and around the vessels [morphologically
376 determined]). As shown in **Figure 6A**, CD68⁺ 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⁺ macrophages were present in the dermis, as in HDs (**Figure**
379 **6A**, patients SSc1 and SSc3). In the four other SSc skin samples, CD68⁺ macrophages were
380 enriched in perivascular areas (**Figure 6A**, representative staining, patients SSc5 and SSc6).
381 The total number of CD68⁺ 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⁺
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⁺ macrophages in
389 skin sections. Perivascular CD68⁺ 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⁺ CD68⁺ macrophages, similar to the HDs (**Figure 6E**
392 and **Supplementary Figure 5**). cMAF was not detected in CD68⁺ macrophages in HD skin
393 sections and was inconsistently expressed in CD68⁺ macrophages from patients with SSc
394 (**Figure 6E** and **Supplementary Figure 5**). In patients with SSc with a high mRSS, not only

395 CD68⁺DC-SIGN⁺ 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⁺DC-SIGN⁺ 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⁺ macrophage
400 infiltration and enhanced perivascular CD68⁺DC-SIGN⁺ expression (**Figure 6C-F**).
401 Therefore, CD68⁺DC-SIGN⁺ 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⁺ AAMs generation that correlates to skin fibrosis. Moreover, the macrophage gene
404 signature enables discrimination of patients with skin-infiltrating DC-SIGN⁺ 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 β prompts SSc
409 MECs to induce differentiation of monocytes into DC-
410 SIGN⁺CCL18^{high}CCL2^{high}CXCL8^{high}IL-10^{low} AAMs, which are associated with fibrosis.

411 Animal models have highlighted the role of the inflammasome and IL-1 β 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 β 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 β 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 β
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 β and skin or lung
420 fibrosis [26-28]. These data suggest that IL-1 β 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 β -
423 induced miR-55 expression [26], as well as endothelial to mesenchymal transition together
424 with IL-6 and TGF- β 2 [29] have been proposed. Finally, the use of blockers of IL-1 β are
425 scarce in both mouse [18, 30] and human SSc [31], and yielded conflicting results suggesting
426 that the effect of IL-1 β 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 β 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 β is an important mediator of lung and skin inflammation~~
432 ~~and fibrosis. However, evidence for IL-1 β 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 β 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 β 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 β 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⁺ 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⁺Ly6C^{low} macrophages
455 induced expansion of Foxp3-expressing regulatory T cells and suppressed CD8⁺ 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⁺ CD68⁺ 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⁺ 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⁺ 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⁺ 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⁺ 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 β sustained
482 secretion, microvascular endothelial cells activation and generation of DC-SIGN⁺ 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 β 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⁺ 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

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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

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, Zammiti 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.

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663 **Figure Legends**

664

665 **Figure 1. IL-1 β -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-1 β -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 β -
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 β -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 β -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-1 β -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 β -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 β -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 1 β -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 β -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 β -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 β -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 β -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 β -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 β -activated MEC-CM from HDs
713 and patients with SSc. UND : undetectable.

714

715 **Figure 5. Enriched DC-SIGN⁺ 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⁺CD68⁺ macrophages are enriched in perivascular area in SSc**
724 **fibrotic skin. (A)** Staining of CD68⁺ 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⁺ macrophages (cells/mm²) 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⁺ 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/mm² **(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⁺DC-SIGN⁺ 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