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1 **TNF- α and IL-10 control CXCL13 expression in human macrophages**

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17

18 **Running title:** Macrophage CXCL13 expression and idiopathic lung fibrosis

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22 **Abbreviations:** ILD: interstitial lung disease, IPF: idiopathic pulmonary fibrosis, IF:
23 immunofluorescence, MoDM: blood monocyte-derived macrophages, AM: alveolar
24 macrophages, M-CSF: macrophage colony-stimulating factor, BAL: bronchoalveolar

25 lavages, LPS: lipopolysaccharide, IHC: immunohistochemistry, IF: immunofluorescence,
26 TNF- α : tumor necrosis factor- α (TNF- α), IL: interleukin, Ab: antibody; LOQ: limit of
27 quantification, TNFR2: TNF- α receptor 2

28

29

Final manuscript

30 **Abstract**

31 The chemokine CXCL13 controls the normal organization of secondary lymphoid tissues and
32 the neogenesis of ectopic lymphoid structures in nonlymphoid organs, particularly the lungs.
33 The progression and severity of idiopathic pulmonary fibrosis (IPF), a fatal and irreversible
34 interstitial lung disease, is predicted by the circulating blood concentrations of CXCL13.
35 While CXCL13 is produced by pulmonary tissues, it has not been determined which cells are
36 involved. This study examines CXCL13 production by lung tissue macrophages from patients
37 with IPF and the signaling pathways controlling CXCL13 gene expression in human alveolar
38 macrophages (AM) and monocyte-derived macrophages (MoDM). CXCL13 is found in
39 CD68- and CD206-positive AM from patients with IPF, and the CXCL13 gene is induced in
40 these macrophages and MoDM when they are stimulated with lipopolysaccharide. We found
41 that TNF- α and IL-10 control optimal CXCL13 gene expression in MoDM and possibly in
42 AM by activating the NF- κ B and JAK/STAT pathways, respectively. We also found that
43 blood TNF- α and CXCL13 concentrations are significantly correlated in patients with IPF,
44 suggesting that TNF- α contributes to CXCL13 production in humans. In conclusion, the
45 results of this study demonstrate that AM from patients with IPF produces CXCL13 and that
46 the NF- κ B and JAK/STAT pathways are required to induce the expression of this major
47 chemokine.

48 **Key points**

- 49 - CXCL13 is expressed in alveolar macrophages from patients with IPF
- 50 - TNF- α and IL-10 control CXCL13 expression through NF- κ B and JAK/STAT, respectively

51 **Keywords:** CXCL13, macrophage, idiopathic lung fibrosis, TNF- α , IL-10, NF- κ B

52

53 **Introduction**

54 The chemokine CXCL13, also known as B lymphocyte chemoattractant and B cell
55 attracting chemokine-1, controls the normal organization of secondary lymphoid tissues by
56 stimulating the homing of B and follicular T helper lymphocytes (1). CXCL13 is
57 constitutively expressed in these structures and is mainly produced by follicular dendritic cells
58 and stromal cells in response to lymphotoxin- β (1). CXCL13 also promotes the neogenesis of
59 ectopic lymphoid structures in nonlymphoid organs (1), especially in chronic inflammatory
60 diseases, such as ulcerative colitis and chronic obstructive pulmonary disease, and in
61 autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (2–5).
62 Follicular dendritic cells and stromal cells do not produce CXCL13 in ulcerative colitis and
63 rheumatoid arthritis lesions, but macrophages within the lymphoid structures may produce
64 CXCL13 (2).

65 Recent studies have demonstrated that CXCL13 is also a robust prognostic biomarker of
66 idiopathic pulmonary fibrosis (IPF), a progressive, fatal interstitial lung disease (ILD) (6–8).
67 Blood CXCL13 concentrations of patients with IPF are significantly elevated, and they
68 predict the severity and progression of the disease (6). The concentrations of CXCL13 mRNA
69 in pulmonary tissues from patients with IPF are significantly increased, and CXCL13 protein
70 is abundant around or within the B-cell aggregates near the fibrotic tissues (6, 7). However,
71 the cellular source of CXCL13 in lung tissues has not been determined.

72 Pulmonary macrophages play a pivotal role in IPF by secreting a variety of pro-
73 inflammatory and pro-fibrotic mediators that promote the recruitment of immune cells and the
74 activation and differentiation of lung fibroblasts (9, 10). Most alveolar macrophages (AMs) of
75 healthy subjects come from self-renewing fetal monocytes (11). In contrast, chronic exposure
76 to aggressive biological or chemical agents triggers the recruitment of circulating blood
77 monocytes that differentiate into interstitial macrophages and/or AM in the lungs (10). Blood

78 monocyte-derived macrophages (MoDMs) are intimately involved in the development of lung
79 fibrosis in murine models. The genetic deletion of blood monocyte-derived AM and the
80 selective depletion of Ly6C^{hi} circulating monocytes significantly inhibit the development of
81 bleomycin-induced lung fibrosis in mice (12, 13). Macrophage colony-stimulating factor (M-
82 CSF), which stimulates blood monocytes to differentiate into macrophages, favors the
83 development of lung fibrosis in these mice (14). Bleomycin-treated M-CSF^{-/-} mice have fewer
84 AM than normal and a lower fibrotic score than wild-type mice (14). Several indicators
85 suggest that human blood MoDM, potentially differentiated by M-CSF, also contributes to the
86 development of lung fibrosis. The concentration of M-CSF in bronchoalveolar lavages (BAL)
87 and the density of CD68-positive cells (macrophage lineage) in the pulmonary tissues of
88 patients with IPF are significantly higher than in control subjects (14, 15). Using flow
89 cytometry, Yu et al. (2016) demonstrated that most interstitial macrophages and AM from
90 patients with IPF strongly bear the CD206 mannose receptor, whose expression is potently
91 induced by M-CSF during human blood monocyte differentiation (16, 17). Finally, human
92 MoDM, differentiated *in vitro* with M-CSF, produces CXCL13 when stimulated with
93 lipopolysaccharide (LPS) (2). Taken together, these findings suggest that human pulmonary
94 macrophages can produce CXCL13. The present study was thus designed to assess this
95 hypothesis.

96 First, we used immunohistochemistry (IHC) and immunofluorescence (IF) to characterize
97 CXCL13 production by macrophages in lung tissues isolated from patients with IPF. Next,
98 we identified the cytokines and signaling pathways regulating *in vitro* CXCL13 expression
99 in human MoDM and AM. Last, we assessed the relationship between the circulating
100 concentrations of these cytokines and CXCL13 in humans suffering from IPF.

101

102 **Materials and Methods**

103 *Chemicals and reagents*

104 M-CSF was purchased from Miltenyi Biotec (Paris, France). Lipopolysaccharide (LPS)
105 (*Escherichia coli* O55:B5) and BAY-117082 (BAY) were from Sigma-Aldrich (Saint-
106 Quentin Fallavier, France). Tumor necrosis factor- α (TNF- α) and interleukin (IL)-10 were
107 obtained from PeproTech (Neuilly-sur-Seine, France); anti-TNF- α and anti-IL-10 neutralizing
108 antibodies (Abs) were provided by R&D System Europe (Biotechne, Lille, France); anti-
109 phospho-I κ B α (ser32), anti-I κ B α , anti-p100/p52, anti-RelB, anti-phospho-STAT3 (Tyr705)
110 and anti-GAPDH primary Abs were from Cell Signaling Technology (Ozyme, Montigny-le
111 Bretonneaux, France). Ruxolitinib was provided by Selleckchem (Houston, TX, USA). Si-
112 RNAs: ON-TARGETplus Control pool Nontargeting Pool (D-001810-10-05, Si-CTR), ON-
113 TARGETplus Human RelB siRNA-SMARTpool (L-004767-00-0005, Si-BelB) and ON-
114 TARGETplus Human NF κ B2 siRNA-SMARTpool (L-003918-00-0005, Si-p100/p52) were
115 purchased from Dharmacon (Horizon Discovery, UK).

116 *Immunohistochemistry*

117 Lung biopsy samples were obtained from six patients with IPF. IPF was diagnosed
118 according to the 2011 ATS/ERS/JRS/ALAT criteria, which include histopathological features
119 of usual interstitial pneumonia (18). Clinical and pulmonary functional data of the six subjects
120 are presented in Table S1 (see “Lung biopsies”). This study was approved by the Rennes
121 University Hospital Ethics Committee (Comité d'éthique, avis n°16.123). Written informed
122 consent was obtained from all subjects. Serial sections (4 μ m) were cut from paraffin-
123 embedded tissue, mounted on positively charged slides and dried at 58°C for 60 min. The
124 sections were immunostained in the Discovery Automated IHC stainer (Ventana Medical
125 Systems, Tucson, AZ) using DABMap kits or CHROMOMap DAB kits to detect CD20,

126 CD68 and CD206 or CXCL13, respectively. Briefly, paraffin was removed with EZ Prep
127 solution (8 min / 75 °C), and antigens were retrieved by incubation in CC1Tris-based buffer
128 (95-100 °C / 60 min). Endogenous peroxidase was blocked by incubation with Inhibitor-D 3%
129 H₂O₂ (Ventana) (37°C / 4 min). Sections were then rinsed and incubated (37°C / 60 min) with
130 primary Abs: mouse monoclonal anti-CD20 (Dako, M0755, clone L26, diluted 1/600), mouse
131 monoclonal anti-CD68 (M0876, clone PG-M1, DAKO, diluted 1/1000), rabbit polyclonal
132 anti-CD206 (Abcam, ab64693, diluted 1/2000), or mouse monoclonal anti-CXCL13 (R&D
133 System, AF801, diluted 1/500). Signals were enhanced with compatible biotinylated goat
134 secondary Abs for CD20, CD68 and CD206 or an Omni goat anti-mouse horseradish
135 peroxidase (HRP)-conjugated secondary Ab (Vector laboratory, Burlingame, CA, USA) for
136 CXCL13. All sections were then counterstained with hematoxylin (4 min). Finally, slides
137 were rinsed, manually dehydrated and coverslipped and scanned with the Nanozoomer 2.0 RS
138 (Hamamatsu, Tokyo, Japan). Images of whole tissue areas were captured for analysis using
139 NDPview2 software (Hamamatsu, Tokyo, Japan).

140 ***Immunofluorescence***

141 IF analysis was performed on the samples from the same six IPF patients. Whole-slide
142 sections (4 µm) were cut from lung tissue blocks with a microtome, transferred to charged
143 slides, and costained either for CD20 and CXCL13 or for CD68, CD206 and CXCL13 (U
144 DISCOVERY 4 plex IF; Roche Diagnostic, Meylan, France) using the Abs (at similar
145 dilutions) described in the IHC section. Proteins were visualized by incubating sections with
146 unmodified primary Ab with the corresponding HRP-conjugated secondary Ab and then
147 producing the HRP enzyme-mediated deposition of the tyramide-fluorophore that covalently
148 binds to the tissue at the site of the reaction. The primary Ab and secondary Ab-HRP
149 complexes were finally heat-inactivated. CD68 was detected with dicyclohexylcarbodiimide,
150 CD20 and CD206 with fluorescein isothiocyanate, and CXCL13 with cyanine 5. These

151 sequential reactions were repeated three times. Sections were mounted in fluoromount (Enzo
152 Life Sciences, Farmingdale, NY, USA). All manipulations were performed at the Rennes
153 H2P2 Histopathology platform (SFR UMS CNRS 3480 - INSERM 018). Stained sections
154 were examined by confocal microscopy (Zeiss LSM-880 microscope) at the Rennes
155 Microscopy Rennes Imaging Center (SFR UMS CNRS 3480 - INSERM 018). The recorded
156 images were analyzed using ImageJ software.

157 ***Bronchoalveolar lavage (BAL)***

158 BAL was obtained from seven patients with IPF. This study was approved by the local
159 Ethics Committee of Rennes University Hospital (Comité d'éthique, avis n°19.09), and all
160 patients signed written informed consent. Clinical and pulmonary functional data of the seven
161 subjects are presented in Table S1 (see "BAL"). BAL was performed under local anesthesia
162 with 2% lidocaine. A flexible fiberoptic bronchoscope was inserted into the segmental
163 bronchus of the middle lobe or into the lingula. Approximately 100 ml of sterile 0.9% saline
164 was instilled and then collected into sterile propylene tubes. The average volume obtained for
165 experimental research was approximately 10 ml. The resulting BALs were then centrifuged at
166 150 rpm for 10 min, washed once with 5 ml PBS, and suspended in GlutaMAX RPMI 1640
167 medium (Thermo Fisher Scientific, France) containing 10% heat-inactivated fetal bovine
168 serum, 2 mM L-glutamine, 20 IU/ml penicillin and 20 µg/ml streptomycin for 3 h. The
169 adherent cells (i.e., AM) were washed and cultured in complete medium in the presence or
170 absence of 20 ng/ml LPS for 24 h. At the end of the treatment, the supernatants were collected
171 and frozen at -20°C. Total RNA was extracted from AM as described below.

172 ***Human MoDM culture***

173 Buffy coats and blood samples were collected from healthy donors and from patients with
174 IPF, respectively. All subjects provided consent for their blood to be used for research

175 (Etablissement Français du Sang, Rennes, France). Clinical and pulmonary functional data of
176 the patients with IPF are presented in Table S1 (see “Blood monocytes”). The peripheral
177 mononuclear cells from buffy coats or from blood samples were collected by Ficoll gradient
178 centrifugation, and monocytes were isolated by adhesion (1 h). The monocytes were then
179 allowed to differentiate into M0 macrophages by incubation (6-7 days) in GlutaMAX RPMI
180 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 20
181 IU/ml penicillin, 20 µg/ml streptomycin and 50 ng/ml M-CSF (16). These resting MoDMs
182 (M0 macrophages) were washed and cultured in fresh complete culture medium containing 10
183 ng/ml M-CSF. RNA interference technology was used for gene silencing in some
184 experiments. MoDMs were transfected with siRNAs (30 pmol) using Lipofectamine
185 RNAiMAX (Thermo Fisher Scientific, France) according to the manufacturer’s instructions
186 and incubated for 24 h with 20 ng/ml M-CSF in complete culture medium before stimulation.

187 ***Reverse transcription quantitative polymerase chain reaction (RT-qPCR)***

188 Total RNA was extracted from cells with TRIzol (Thermo Fisher Scientific, France) and
189 reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied
190 Biosystems, Thermo Fisher Scientific, France). Quantitative PCR was performed (SYBR
191 Green methodology) on a CFX384 real-time PCR system (BioRad, Marnes-la-Coquette,
192 France) (16). The primers were provided by Sigma-Aldrich (Kiqstart primers). The specificity
193 of amplified genes was checked at the end of PCR using the comparative cycle threshold
194 method (CFX Manager™ Software). These mean Cq values were used to normalize the target
195 mRNA concentrations to those of the 18S ribosomal protein by the $2(-\Delta\Delta Cq)$ method.

196 ***Cytokine quantification***

197 The concentrations of CXCL13, TNF- α and IL-10 were measured by ELISA (Duoset
198 ELISA development system kits, R&D Systems Europe, Lille, France) according to the

199 manufacturer's instructions. The limits of quantification (LOQs) were CXCL13 - 15.2 pg/ml,
200 TNF- α - 13.4 pg/ml, and IL-10 - 31.3 pg/ml. The three cytokines were quantified in cell
201 culture media and in blood serum samples taken from 105 patients when they were diagnosed
202 with IPF. This study was approved by the Ethics Committee related to Rennes University
203 Hospital (Comité de protection des personnes Ouest V, CHU Rennes, n°2014-A00268-39),
204 and each patient signed a written informed consent form. Clinical and pulmonary functional
205 data of the 105 subjects are presented in Table S1 (see "Serum samples"). Serum samples
206 were stored at -80°C at the Biobank, Centre Hospitalier Universitaire, Rennes.

207 *Western blotting*

208 Cells were lysed with RIPA buffer containing a protease inhibitor cocktail (Roche
209 Diagnostic, Meylan, France) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich,
210 Saint-Quentin Fallavier, France) and then centrifuged. After measuring protein concentrations
211 by the Bio-Rad Protein Assay Kit, the lysates were diluted in loading buffer, heated at 95°C,
212 loaded on a 4% stacking gel and separated on a 10% gel by SDS electrophoresis as previously
213 described (16). The proteins were then transferred to nitrocellulose membranes by
214 electroblotting overnight (30 V at 4°C). After blocking, the membranes were then hybridized
215 overnight at 4°C with appropriate primary Abs and incubated with HRP-conjugated
216 secondary Abs before analysis by chemiluminescence on a ChemiDoc XRS+ System and
217 Image Lab software (Bio-Rad, Marnes-la-Coquette, France). Protein expression was
218 quantified by analyzing each visualized band by densitometry (Image Lab™ Software for
219 total protein normalization; BioRad, Marnes-la-Coquette, France).

220 *Statistical analyses*

221 All cellular endpoints were obtained using AM or MoDM from different donors. The data
222 are reported as the means \pm standard deviation (SD). Significant differences were assessed

223 using a two-way Student's *t*-test or a one-way ANOVA followed by the multirange Dunnett's
224 *t*-test for multiple comparisons. For cytokine concentrations in human blood samples,
225 significant differences were assessed using the nonparametric Mann-Whitney test.
226 Correlations between the cytokine blood concentrations were determined using Spearman's
227 rank correlation coefficient. Differences were considered to be significant when $p < 0.05$.
228

229 **Results**

230 *CXCL13 is expressed in alveolar macrophages from patients with IPF*

231 We first used IHC to detect CXCL13 in surgical lung biopsies from six patients with IPF.
232 CXCL13 staining was intense around the lymphoid aggregates containing B cells (Figure
233 S1A), which was in agreement with DePianto et al. (7). However, there was no or very weak
234 staining inside B cell aggregates. In addition, IF experiments showed that CXCL13
235 expression was mostly outside B-cell lymphoid aggregates. Thus, such pulmonary B cells
236 likely do not produce CXCL13 (Figure S1B). We studied CD68 and CD206 expression,
237 which is abundant on pulmonary macrophages from patients with IPF (15), to determine
238 whether CXCL13 was produced by macrophages that had infiltrated the lymphoid aggregates
239 (Figure S1C). There was little CD68 or CD206 staining around or within the lymphoid
240 structures, and CD68/CD206 staining was not colocalized with CXCL13 staining. We
241 therefore looked for CXCL13 in alveolar tissues where macrophages are constitutively
242 present. CD68- and CD206-positive cells (i.e., AM) were abundant in pulmonary alveoli
243 (Figure 1A). These macrophages bore intense CD206 staining. There was CXCL13 staining
244 on most AMs but not on CD68- and CD206-negative cells. The intracellular CXCL13 staining
245 was diffuse, mainly in the cytoplasm but also at the cell membrane. The biopsies from all the
246 patients provided similar images (data not shown). We also stained sections with the CXCL13
247 isotype control Ab (mouse IgG, Vector, I-2000). The isotype control staining in AM was
248 clearly less intense than the CXCL13 staining (Figure 1A), confirming the specificity of
249 CXCL13 expression in lung biopsies.

250 Multiplex IF staining showed that CXCL13 was present in CD68- and CD206-positive AM
251 (Figure 1B), while the CD68- and CD206-negative cells in the alveoli had no CXCL13
252 staining. Similar images were recorded for the five other surgical lung biopsies (Figure S2).
253 This result indicates that AM from patients with IPF likely produces CXCL13.

254 *LPS induces CXCL13 expression by activating NF- κ B pathways*

255 We then focused our study on the production of CXCL13 by AM and MoDM. AM were
256 isolated from BAL collected from 7 patients with IPF. AM stimulation with 20 ng/ml LPS for
257 24 h increased CXCL13 mRNA and CXCL13 protein concentrations in 7 and 4 cell cultures,
258 respectively (Figure 2A). CXCL13 expression was also significantly increased in
259 macrophages differentiated from peripheral blood monocytes of 11 patients with IPF (Figure
260 2B). It can be noted that the levels of CXCL13 secreted from activated AM were lower than
261 those secreted from “IPF” MoDM. Similarly, CXCL13 mRNA levels were strongly and stably
262 increased in the MoDM of healthy donors stimulated for 8-24 h (Figure 2C, left panel).
263 CXCL13 protein released into the culture medium was maximal after stimulation for 24 h
264 (Figure 2C, right panel). We next examined the signaling pathways mediating CXCL13 gene
265 expression in MoDM. The I κ B α kinase inhibitor BAY 11-7082 (BAY) nearly abrogated
266 CXCL13 release from LPS-stimulated MoDM (Figure 2D), which suggests that the NF- κ B
267 signaling pathways control CXCL13 gene expression. LPS is known to rapidly activate the
268 canonical NF- κ B pathway by stimulating the phosphorylation and subsequent breakdown of
269 the NF- κ B inhibitor I κ B α , which sequesters NF- κ B subunits to the cytoplasm (19). This
270 canonical pathway, activated by the I κ B kinase complex, leads to the translocation of the NF-
271 κ B dimer RelA:p50 to the nucleus. These transcription factors increase the expression of
272 several genes that regulate innate and adaptive immune responses. The factors also induce the
273 gene encoding I κ B α and stimulate the synthesis of the NF- κ B subunits NF κ B2 (p100) and
274 RelB, which mediate the noncanonical NF- κ B pathway. The phosphorylation and subsequent
275 processing of NF κ B2 (p100) catalyzed by I κ B kinase- α produce the active p52 subunit,
276 which then interacts with RelB (19).

277 Incubating MoDM with LPS for 2 h stimulated the phosphorylation and breakdown of
278 I κ B α followed by increased I κ B α synthesis after 24 h (Figure S3A). As expected,
279 pretreatment of MoDM with BAY blocked the stimulation of I κ B α synthesis. Incubation with
280 LPS for 24 h also significantly increased the levels of NF κ B2 and RelB mRNA (Figure S3B)
281 and the concentrations of the NF κ B2 (p100), p52 and RelB proteins in MoDM (Figure S3C).
282 Pretreating cells with BAY completely blocked this late production of the three noncanonical
283 NF- κ B subunits (Figure 2E). The fact that CXCL13 synthesis was also delayed in LPS-
284 stimulated human MoDM suggests that the noncanonical NF- κ B pathway activated CXCL13
285 gene transcription, as shown in murine cells (20). We tested this by transfecting MoDM with
286 siRNAs to block RelB mRNA (Si-RelB) and p100/p52 mRNA (Si-p100). Si-RelB completely
287 blocked RelB protein synthesis in LPS-stimulated MoDM but did not affect p100/p52
288 proteins (Figure 2F and S3D). Si-p100 prevented any increase in p100 and p52 proteins and
289 reduced the expression of RelB protein. This result indicates that transcription of the RelB
290 gene is activated by canonical NF- κ B pathways and regulated by noncanonical signaling (21).
291 Blocking the synthesis of RelB or p100/p52 in LPS-stimulated MoDM significantly reduced
292 their CXCL13 mRNA expression levels (Figure 2G). Thus, canonical and noncanonical NF-
293 κ B signaling pathways play major roles in the regulation of CXCL13 production by MoDM.

294 ***TNF- α and IL-10 mediate CXCL13 gene induction in LPS-stimulated MoDM***

295 We next determined the influence of early LPS-induced cytokines on CXCL13 synthesis
296 by MoDM. We investigated the roles of TNF- α and IL-10 because they were reported to
297 stimulate CXCL13 expression in activated murine stromal cells and LPS-stimulated dendritic
298 cells, respectively (22, 23). LPS rapidly increased the concentrations of both TNF- α and IL-
299 10 mRNAs in MoDM (Figure 3A). Cells stimulated for 4 to 8 h secreted the highest
300 concentration of TNF- α into the culture medium (Figure 3B). In contrast, the amount of IL-10

301 released by cells activated for 24 h gradually increased (Figure 3B). The early induction of
302 both TNF- α and IL-10 genes was fully blocked by BAY (Figure 3C), suggesting that
303 cytokines are involved in CXCL13 synthesis.

304 To explore this hypothesis, we used Abs that neutralized TNF- α (aT) and IL-10 (aI)
305 proteins. aT significantly decreased the amounts of NF κ B2 (p100) and RelB mRNAs in
306 MoDM exposed to LPS for 24 h (Figure 3D) and blocked the synthesis of p100/p52 and RelB
307 proteins (Figure 3E). In contrast, aI alone did not modify the amounts of the three
308 noncanonical NF- κ B subunits or amplify the effects of aT. However, each Ab alone
309 significantly inhibited both the induction of CXCL13 mRNA in LPS-stimulated MoDM and
310 the secretion of CXCL13 (Figures 3F-G). Interestingly, the aT/aI combination had stronger
311 inhibitory effects on CXCL13 mRNA and CXCL13 protein concentrations than did aT alone
312 (Figure 3F and 3G). These findings suggest that TNF- α mediates CXCL13 gene induction in
313 LPS-stimulated MoDM by activating NF- κ B and that IL-10 enhances its effect, leading to
314 maximal CXCL13 synthesis.

315 **IL-10 enhances TNF- α -induced CXCL13 expression by activating JAK/STAT signaling**

316 If TNF- α and IL-10 control CXCL13 gene induction in LPS-stimulated MoDM, human
317 recombinant TNF- α and IL-10 should mimic the effects of LPS in MoDM. Indeed,
318 stimulating MoDM with TNF- α for 24 h but not with IL-10 alone significantly increased both
319 NF κ B2 and RelB mRNA levels and p100, p52 and RelB protein expression (Figure 4A-B).
320 The TNF- α /IL-10 combination did not further stimulate the synthesis of the three
321 noncanonical NF- κ B subunits (Figure 4B). TNF- α increased CXCL13 mRNA and protein
322 concentrations, and its effect was significantly enhanced by IL-10 (Figure 4C-D). Finally,
323 cells stimulated with IL-10 alone contained slightly increased (up to 12-fold) CXCL13
324 mRNA concentrations and released slightly more CXCL13 (Figures 4C-D). These findings

325 confirm that TNF- α and IL-10 control CXCL13 expression in MoDM and highlight the fact
326 that IL-10 enhances CXCL13 gene induction independently of any direct NF- κ B activation.

327 The binding of IL-10 to its cognate receptor activates JAK/STAT pathways, rather than
328 NF- κ B, and especially stimulates STAT3-dependent signaling in human MoDM (24). We
329 found that LPS increased STAT3 phosphorylation in cells treated for 6 h (Figure S3E) and
330 that the level of phosphorylated STAT3 remained enhanced for 24 h (data not shown). We
331 examined the effects of ruxolitinib, a potent Janus kinase (JAK) inhibitor (25), to determine
332 whether JAK/STAT signaling pathways also mediate CXCL13 synthesis by MoDM.
333 Ruxolitinib blocked the phosphorylation of STAT3 triggered by LPS or IL-10 (Figure S3E).
334 We incubated MoDM with LPS for 4 h to stimulate TNF- α and IL-10 production and then
335 added ruxolitinib for 20 h to block the JAK/STAT pathways. Ruxolitinib did not reduce the
336 secretion of TNF- α or IL-10 (Figure 4E) and did not reduce NF κ B2 (p100) or RelB mRNA
337 expression (Figure 4F), which demonstrates that it did not interfere with the NF- κ B pathways
338 in MoDM stimulated in these experimental conditions. However, ruxolitinib blocked the
339 increase in CXCL13 mRNA induced by IL-10 (Figure S3F) and LPS (Figure 4G). These
340 results suggest that IL-10 enhances TNF- α -induced CXCL13 expression by activating the
341 JAK/STAT signaling pathway.

342 **TNF- α and IL-10 control LPS-induced CXCL13 gene expression in MoDM and AM** 343 **from patients with IPF**

344 We then examined whether TNF- α and IL-10 could also control CXCL13 expression in
345 MoDM and AM isolated from patients with IPF. As expected, the treatment of “IPF” MoDM
346 with neutralizing Ab directed against TNF- α and IL-10 significantly reduced the expression
347 of NF κ B2, RelB and CXCL13 mRNA levels induced by LPS (Figure 5A). In addition, TNF-
348 α and IL-10 significantly increased CXCL13 mRNA levels (Figure 5B), whereas ruxolitinib

349 almost completely blocked CXCL13 expression without altering NF κ B2 and RelB mRNA
350 levels (Figure 5C). Moreover, incubating AM cultures with LPS for 24 h increased NF κ B2
351 but not RelB mRNA levels, suggesting that the noncanonical NF- κ B pathway was not fully
352 activated *in vitro* (Figure 5D). Nevertheless, activated “IPF” AM treated with the aT/aI
353 combination had reduced NF κ B2 and CXCL13 mRNA levels (Figure 5D), indicating that
354 TNF- α and IL-10 may also mediate CXCL13 gene expression in activated AM from
355 patients with IPF.

356 **Serum TNF- α and IL-10 concentrations correlate with CXCL13 in patients with IPF**

357 To determine whether TNF- α and IL-10 modulate CXCL13 production in humans, we
358 examined the CXCL13, TNF- α and IL-10 concentrations in blood serum samples taken
359 from 105 patients suffering from IPF at the time of their diagnosis. The arithmetic and
360 median mean concentrations of CXCL13 were 117.94 ± 156.5 pg/ml and 58.6 pg/ml,
361 respectively. Most serum samples had no detectable TNF- α or IL-10 levels. Only 20.9%
362 (n=22) and 13.3% (n=14) of total serum samples had TNF- α or IL-10 concentrations above
363 their LOQ, respectively. Interestingly, almost all the samples with TNF- α or IL-10
364 concentrations above the LOQ had CXCL13 concentrations higher than the median
365 concentration (Figure 6A; left panel). Moreover, among those samples with TNF- α or IL-10
366 concentrations above the LOQ, 13/22 (59%) and 8/14 (57.1%) had CXCL13 concentrations
367 in the highest quartile (Figure 6A, right panel), respectively. In addition, the TNF- α
368 concentrations > LOQ (n=22), but not the IL-10 concentrations > LOQ (n=14), significantly
369 correlated with their corresponding CXCL13 concentrations (Figure 6B).

370

371 Discussion

372 In this study, we demonstrated that CXCL13 is expressed in AM and MoDM from patients
373 with IPF. CXCL13 expression was increased *in vitro* in LPS-stimulated AM and MoDM, and
374 was regulated by both canonical and noncanonical NF- κ B signaling. LPS rapidly stimulated
375 the expression of TNF- α and IL-10, which then mediate CXCL13 gene induction by
376 activating the NF- κ B and JAK/STAT pathways, respectively. TNF- α and IL-10 are also
377 mediators of LPS-induced CXCL13 gene expression in AM. In addition, we show that TNF- α
378 concentrations significantly correlate with the corresponding CXCL13 concentrations in the
379 serum of patients with IPF, suggesting that this major pro-inflammatory cytokine may
380 regulate CXCL13 expression in humans.

381 IHC confirmed the finding of DePianto et al. (2015) that CXCL13 staining is intense
382 around the B-cell aggregates in lung tissues from patients with IPF (7). The staining was not
383 clear inside the cells, indicating that the protein could be secreted and then deposited on
384 fibrillary structures. Carlsen et al. (2) demonstrated that released CXCL13 is indeed
385 associated with extracellular fibrils such as fibronectin in ulcerative colitis lesions. Our
386 studies also revealed only faint CD68 and CD206 staining around and within B cell
387 aggregates and no colocalization of these markers with CXCL13, indicating that macrophages
388 are probably not the source of CXCL13 in these lymphoid structures. In contrast, our results
389 showed that CD68- and CD206-positive macrophages in pulmonary alveoli contain CXCL13,
390 unlike other cells in these structures. Moreover, stimulating human AM from patients with
391 IPF induced CXCL13 gene expression and CXCL13 release. Thus, pulmonary macrophages
392 are probably a source of CXCL13 in these patients. Nevertheless, we have still to determine
393 whether other pulmonary cells secrete CXCL13 to explain the notably high concentration of
394 CXCL13 around the B-cell aggregates.

395 Our RNA silencing experiments showed that blocking p100/p52 and RelB synthesis
396 significantly reduced CXCL13 mRNA expression in LPS-stimulated MoDM, confirming that
397 the noncanonical NF- κ B pathway contributes significantly to CXCL13 gene expression in
398 human macrophages, as in other murine and human cells (20, 26). However, we did find that
399 optimal CXCL13 gene expression in macrophages required the coordinated activation of the
400 NF- κ B pathway by TNF- α and the JAK/STAT pathway by IL-10.

401 TNF- α activated the noncanonical NF- κ B pathway in resting M0 MoDM, unlike IL-10,
402 and only the neutralization of TNF- α blocked NF κ B2 and RelB gene induction in LPS-
403 stimulated MoDM. TNF- α alone significantly increased CXCL13 gene expression, and its
404 neutralization prevented the LPS-dependent induction of CXCL13 mRNA. TNF- α activates
405 the canonical NF- κ B pathway by binding to the ubiquitous TNF receptor 1, and it also binds
406 to TNF receptor 2 (TNFR2), which triggers p100 processing and activates the noncanonical
407 pathway (27). Nonetheless, the distribution of TNFR2 is considerably more restricted and
408 limited to a few cell types, including human MoDM (28). The processing of p100 is catalyzed
409 by NF- κ B-inducing kinase (NIK); it activates I κ B kinase- α dimers that, in turn,
410 phosphorylate p100 and trigger its partial breakdown by the proteasome to generate the p52
411 subunit (29). Basal p100 processing is low in most cells because the TRAF2/TRAF3/cIAP
412 ubiquitin ligase complex constantly mediates ubiquitination and rapid proteasomal breakdown
413 of NIK (29). Activating cells with lymphotoxin- β receptor agonists or TNF- α -induced
414 TNFR2 stimulates TRAF2 breakdown which, in turn, stabilizes NIK protein synthesis and
415 promotes p100 processing. We did not measure NIK production by MoDM, but as the
416 differentiation of monocytes into macrophages increases NIK synthesis (30), the constitutive
417 concentration of stabilized NIK protein was probably sufficient to promote p100 processing.

418 Neutralizing IL-10 protein did not prevent activation of the noncanonical NF- κ B pathway,
419 but it significantly reduced CXCL13 gene expression in LPS-stimulated MoDM. Conversely,
420 IL-10 did not activate NF- κ B pathways, although it produced a small increase in the amounts
421 of CXCL13 mRNA in resting MoDM. We interpret these results as demonstrating that IL-10
422 activates CXCL13 gene expression by an alternative mechanism that is not dependent on NF-
423 κ B. The selective JAK inhibitor ruxolitinib significantly reduced the increases in CXCL13
424 mRNA in both LPS- and IL-10-stimulated MoDM. Ruxolitinib neither prevented the release
425 of TNF- α and IL-10 nor reduced the production of p100/p52 and RelB in activated cells,
426 suggesting that it specifically blocks CXCL13 gene expression by inhibiting the JAK/STAT
427 pathway. We did not further examine this additional pathway, but there is evidence that the
428 transcription factor STAT3 is involved in the effects of LPS and IL-10. First, STAT3
429 enhances CXCL13 gene expression in other cell types (31); second, LPS and IL-10 activate
430 STAT3 in macrophages (24, 32); and third, we show that ruxolitinib fully blocked the
431 activation of STAT3 induced by LPS and IL-10 in MoDM.

432 We also found evidence that IL-10 strongly enhanced TNF- α -induced CXCL13 gene
433 expression in MoDM. Stimulating MoDM with TNF- α in combination with IL-10 enhanced
434 the increase in CXCL13 mRNA triggered by TNF- α alone. Moreover, treating MoDM with
435 Abs against both TNF- α and IL-10 reduced CXCL13 gene expression more than incubation
436 with TNF- α alone. This finding suggests that the IL-10-activated JAK/STAT pathway
437 enhances the noncanonical NF- κ B-dependent CXCL13 gene expression induced by TNF- α .
438 STAT3 appears to increase the activity of the noncanonical NF- κ B pathway by different
439 mechanisms. STAT3 that has been acetylated by the cAMP-response element-binding protein
440 (CREB)-binding protein (CBP)/p300 can enhance p100 processing to p52 in several human
441 cell types (33). However, this phenomenon is unlikely to occur in MoDM, as IL-10 did not

442 increase the p52 protein concentration induced by TNF- α , and neutralizing IL-10 did not
443 reduce the amount of p52 protein in LPS-stimulated cells. Blocking STAT3 production
444 represses the RelB:p52-dependent induction of the cyclooxygenase 2 gene in human placental
445 cells (34). In this latter study, Yu et al. (2015) showed that STAT3 was colocalized with RelB
446 and p52 in the cytoplasm and nuclei of these cells, suggesting that STAT3 physically interacts
447 with RelB/p52 to increase either their translocation to the nucleus or their transcriptional
448 activity (34). Taken together, these results support the notion that STAT3 cooperates with
449 noncanonical NF- κ B to induce maximal CXCL13 gene expression in MoDM. Additional
450 experiments are now warranted to validate this hypothesis. IL-10 may not be involved in the
451 regulation of CXCL13 gene expression only in MoDM; it may also function in dendritic cells,
452 since blocking IL-10 significantly reduced the production of CXCL13 by LPS-stimulated
453 dendritic cells (23). Finally, our data indicate that TNF- α and possibly IL-10 contribute to
454 CXCL13 production in patients with IPF. Stimulating *in vitro* MoDM and AM with LPS
455 increased the expression of the genes encoding NF κ B2 and CXCL13, while blocking TNF- α
456 and IL-10 production significantly reduced the increase in CXCL13 mRNA. However, the
457 RelB mRNA concentration in LPS-stimulated AM was not increased. This reduced RelB
458 synthesis may limit the formation of RelB:p52 dimers and consequently the expression of
459 CXCL13 mRNA. Our results clearly show that the amounts of CXCL13 secreted by activated
460 IPF AM in response to LPS stimulation are considerably lower than those released from IPF
461 MoDM. The concentrations of CXCL13 in the blood serum of patients with IPF also provide
462 valuable information. Most blood serum samples that contained CXCL13 concentrations
463 below the median CXCL13 concentration contained no detectable TNF- α or IL-10. In
464 contrast, 48.1% of the serum samples with the highest CXCL13 concentrations (the fourth
465 quartile) had TNF- α concentrations above their LOQ, and 29.6% of these high-CXCL13

466 samples had similarly high IL-10 concentrations. Moreover, the highest TNF- α
467 concentrations (> LOQ) were strongly correlated with those of CXCL13. As TNF- α controls
468 CXCL13 gene induction *in vitro*, this correlation suggests that the cytokine may also promote
469 CXCL13 production in patients with IPF. In addition, it may be hypothesized that both TNF-
470 α and IL-10 might contribute to CXCL13 expression in lung tissues in patients with IPF.
471 Indeed, TNF- α and IL-10 are present in human lung tissues, and the concentrations of IL-10
472 mRNA in pulmonary biopsies from patients with IPF are higher than those from controls (35).
473 Bergeron et al. (2003) also reported that hyperplastic alveolar epithelial cells were a
474 prominent source of IL-10 in lung tissues from patients with IPF. TNF- α and IL-10 are also
475 secreted by B and T cells, dendritic cells and macrophages present in lung tissues. We did not
476 investigate why the blood TNF- α and IL-10 concentrations could be quantified in only a
477 small number of IPF patients. However, since TNF- α and IL-10 are typically produced in
478 response to pro-inflammatory stimuli, it seems likely that blood TNF- α , IL-10 and CXCL13
479 concentrations were especially increased in IPF patients who had inflammation prior to blood
480 sampling. Furthermore, clinical studies on large cohorts will be required to confirm that some
481 patients with IPF have substantial blood TNF and/or IL-10 concentrations and to investigate
482 the clinical consequences of these increased cytokine concentrations.

483 In conclusion, our results indicate that CXCL13 is produced by AM in patients with IPF
484 and that TNF- α and IL-10 regulate its production. More generally, we identified the crucial
485 roles of the NF- κ B and JAK/STAT pathways in CXCL13 gene expression in human
486 inflammatory MoDM.

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631

632 **Figure Legends**

633 **Figure 1: CXCL13 is expressed in CD68- and CD206-positive AM.** The lung tissue from a
634 patient with IPF was explored for CD68, CD206 and CXCL13 by IHC (A) and IF (B). (B)
635 upper panels: x 65. Red circles indicate the regions at higher magnifications. Red arrowheads
636 indicate cells negative for CD68/CD206 and CXCL13. IHC was also performed using the
637 CXCL13 isotype control Ab to confirm specific CXCL13 expression in AM. Similar images
638 of CD68, CD206 and CXCL13 staining by IHC were obtained with lung tissues from six
639 patients with IPF.

640 **Figure 2: CXCL13 expression is induced in activated AM and MoDM and is controlled**
641 **by NF- κ B.** AM and MoDM from patients with IPF (A, B) and MoDM from healthy donors
642 (C-G) were cultured without (M0) or with 20 ng/ml LPS for 24 h or other indicated times. In
643 some experiments, MoDMs were first treated with 5 μ M Bay (C-E) or transiently transfected
644 with control, RelB or p100/p52 siRNA for 24 h (F-G). Relative CXCL13 mRNA was
645 measured by quantitative RT-PCR and normalized to endogenous ribosomal 18S RNA levels.
646 Data are expressed relative to mRNA levels found in cells stimulated with LPS (A, B), in M0
647 cells (C) or in cells transfected with control siRNA (SiC) and stimulated with LPS (G),
648 arbitrarily set at 1. CXCL13 concentrations in cultured media were measured by ELISA. In
649 (E-F), cells were lysed, and protein expression was analyzed by Western blotting. Protein
650 expression was quantified by analyzing each visualized band by densitometry. The results are
651 the means \pm SD of 7 (A), 11 (B), 4 (C, left panel), 5 (C, right panel), 4 (D), 4 (E), and 7 (G)
652 independent experiments. * p <0.05, ** p <0.01, *** p <0.001 versus LPS (A, B), M0 (C) “0”
653 LPS (D, E) and “SiC” LPS (G)

654 **Figure 3: CXCL13 expression is mediated by TNF- α and IL-10 in LPS-stimulated**
655 **MoDM.** MoDMs were untreated (M0, 0) or treated with 5 μ M BAY or 2 μ g/ml neutralizing

656 Abs directed against TNF- α (aT) or IL-10 (aI) and then stimulated with 20 ng/ml LPS for 1 h
657 (C), 24 h (D-G) or the indicated times (A, B). Relative mRNA levels were determined by
658 quantitative RT-PCR and normalized to endogenous ribosomal 18S mRNA levels. Data are
659 expressed relative to mRNA levels found in M0 cells (A) or cells stimulated with “0” LPS (C,
660 D, F), arbitrarily set at 1. Concentrations of TNF- α , IL-10 and CXCL13 secreted in the culture
661 media were quantified by ELISA. In (E), cells were lysed, and protein expression was
662 analyzed by Western blotting. Protein levels were quantified by analyzing each visualized
663 band by densitometry. The results are expressed as the means \pm SD of 7 (A), 5 (B), 3 (C), 3
664 (D left panel), 7 (D, right panel), 4 (E), 7 (F) and 5 (G) independent experiments. * p <0.05,
665 ** p <0.01, *** p <0.001 versus “M0” (A, B), “0” LPS (C-G).

666 **Figure 4: IL-10 enhances CXCL13 expression induced by TNF- α through the**
667 **JAK/STAT pathway.** MoDMs were untreated (M0) or stimulated with 20 ng/ml TNF- α (T)
668 and/or IL10 (I) for 24 h or with 20 ng/ml LPS for 24 h. In (B), cells were lysed, and protein
669 expression was analyzed by Western blotting. Protein levels were quantified by analyzing
670 each visualized band by densitometry. Relative mRNA levels were determined by quantitative
671 RT-PCR and normalized to endogenous ribosomal 18S RNA levels. Data are expressed
672 relative to mRNA levels found in M0 cells (A, C) or in cells stimulated with “0” LPS (F-G),
673 arbitrarily set at 1. Concentrations of TNF- α , IL10 and CXCL13 secreted in the culture media
674 were quantified by ELISA. The results are expressed as the means \pm SD of 6 (A, left panel), 4
675 (A, right panel), 7 (B), 9 (C), 6 (D), 4 (E), 5 (F-G)) independent experiments. * p <0.05,
676 ** p <0.01, *** p <0.001 versus M0 (A-D) or “0” LPS (E-G).

677 **Figure 5: Effects of neutralizing Abs directed against TNF- α (aT) or IL-10 (aI) on AM.**
678 MoDM (A-C) and AM (D) from patients with IPF were untreated (0) or treated with 2 μ g/ml
679 aT and/or aI and then stimulated with 20 ng/ml T/I or 20 ng/ml LPS for 24 h. Relative mRNA

680 levels were determined by quantitative RT-PCR and normalized to endogenous ribosomal
681 18S RNA levels. Data are expressed relative to mRNA levels found in cells stimulated with
682 “0” LPS, arbitrarily set at 1. The results are expressed as the means \pm SD of 11 (A) and 4 (B-
683 D) independent experiments. * p <0.05, ** p <0.01, *** p <0.001 versus “0” LPS (A, C-D) or M0
684 (B).

685 **Figure 6: TNF- α , IL-10 and CXCL13 concentrations in the serum of patients with IPF.**

686 Concentrations of TNF- α , IL-10 and CXCL13 in the blood serum of 105 patients with IPF
687 were measured by ELISA. In (A), TNF- α and IL-10 concentrations were referred to CXCL13
688 median “M” (A, left panel) or quartiles “Q” (A, right panel). The numbers of values of TNF- α
689 or IL-10 concentrations above their respective LOQs are indicated in italics. (B) Correlations
690 between the values of the blood serum concentrations of CXCL13 and those of TNF- α and
691 IL10 above their respective LOQs. r_s : Spearman's rank correlation coefficient; ns: not
692 significant. *** p <0.001

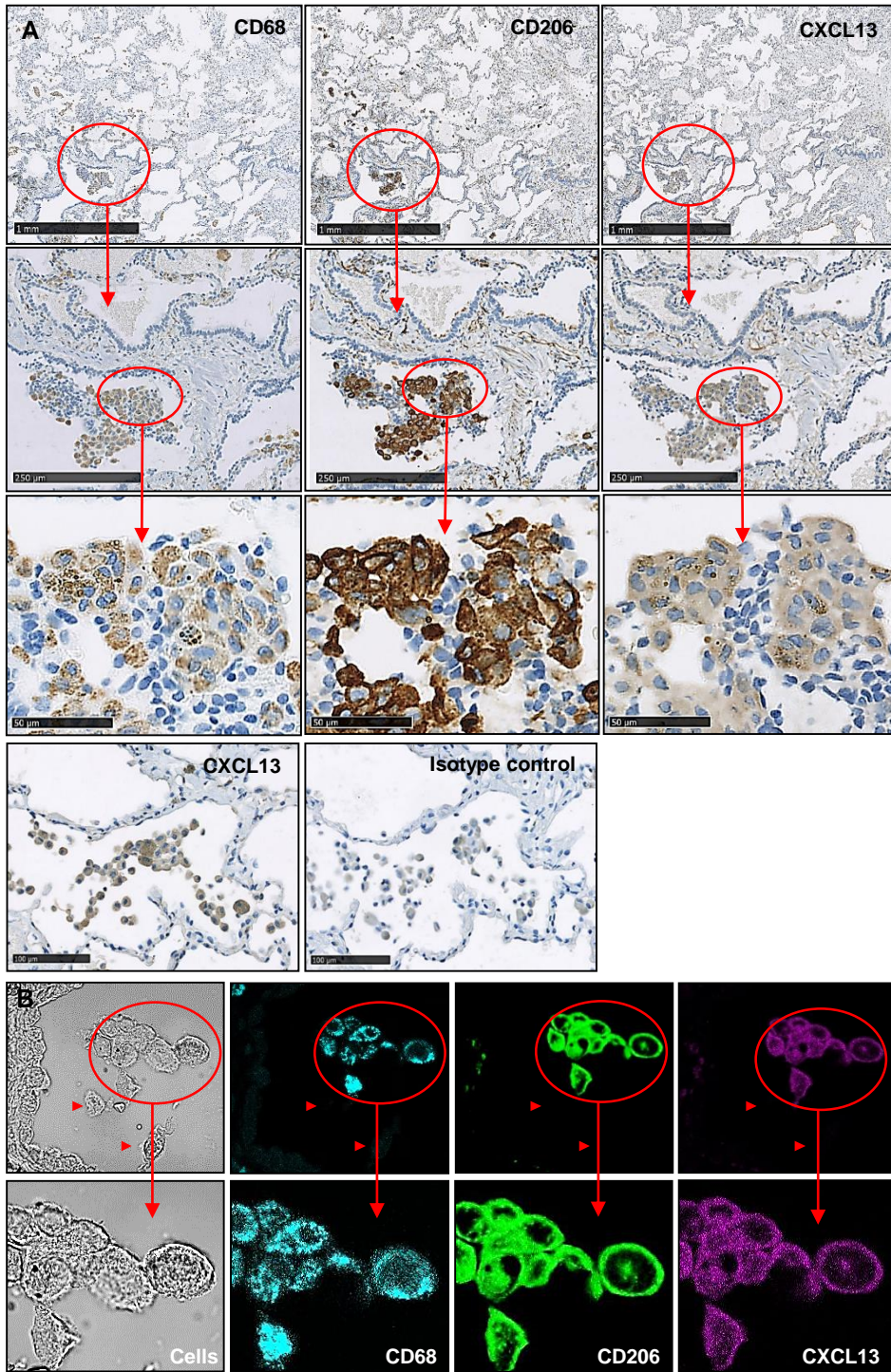


Figure 1

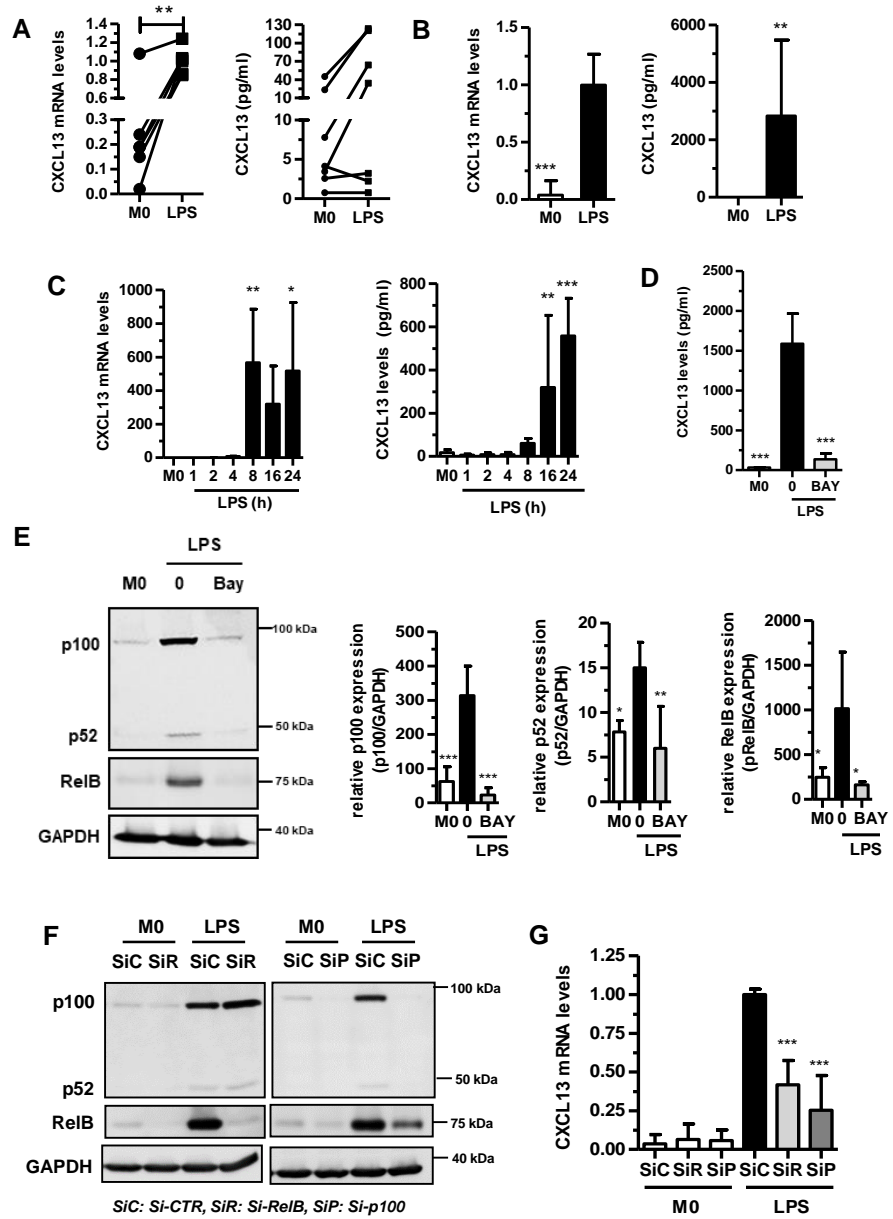


Figure 2

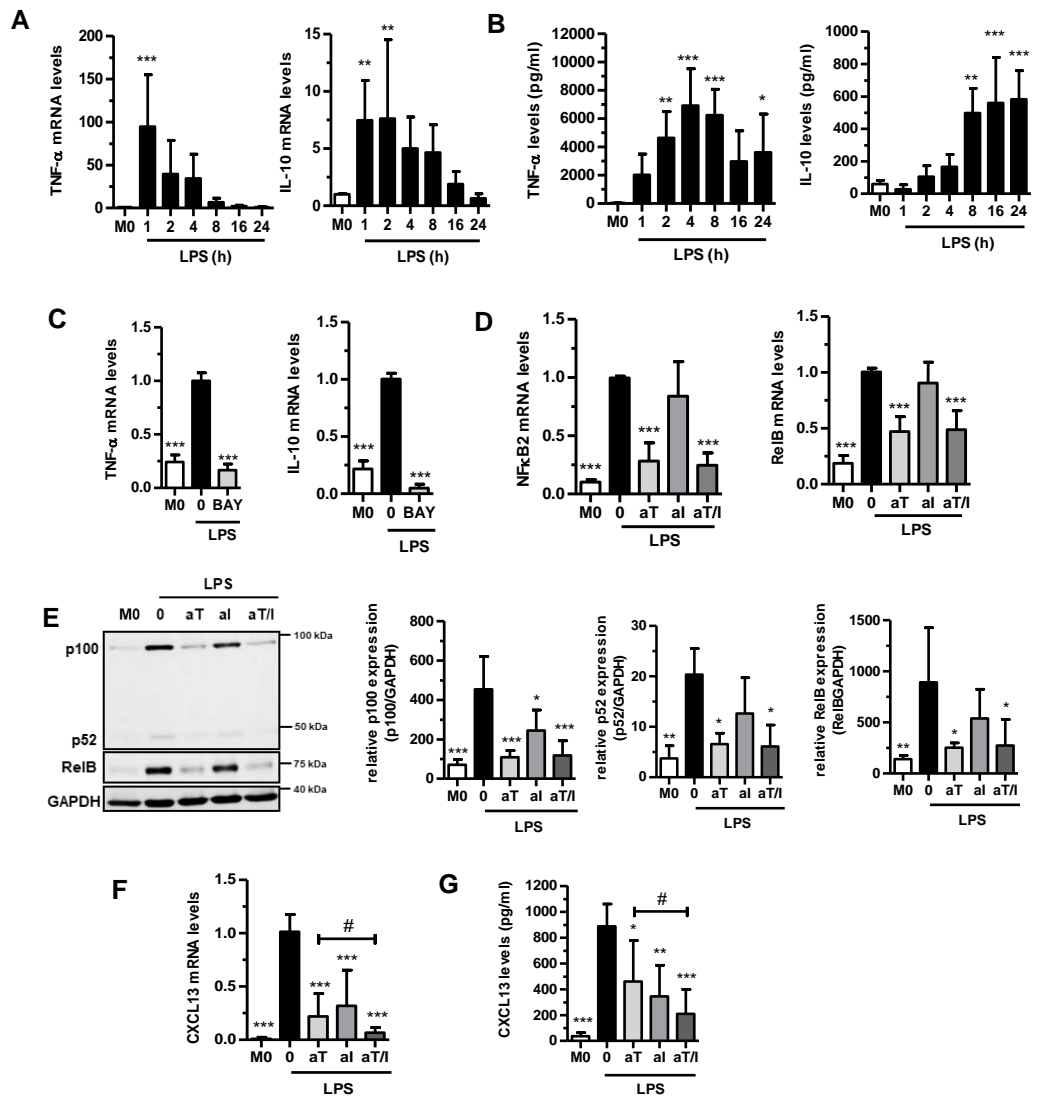


Figure 3

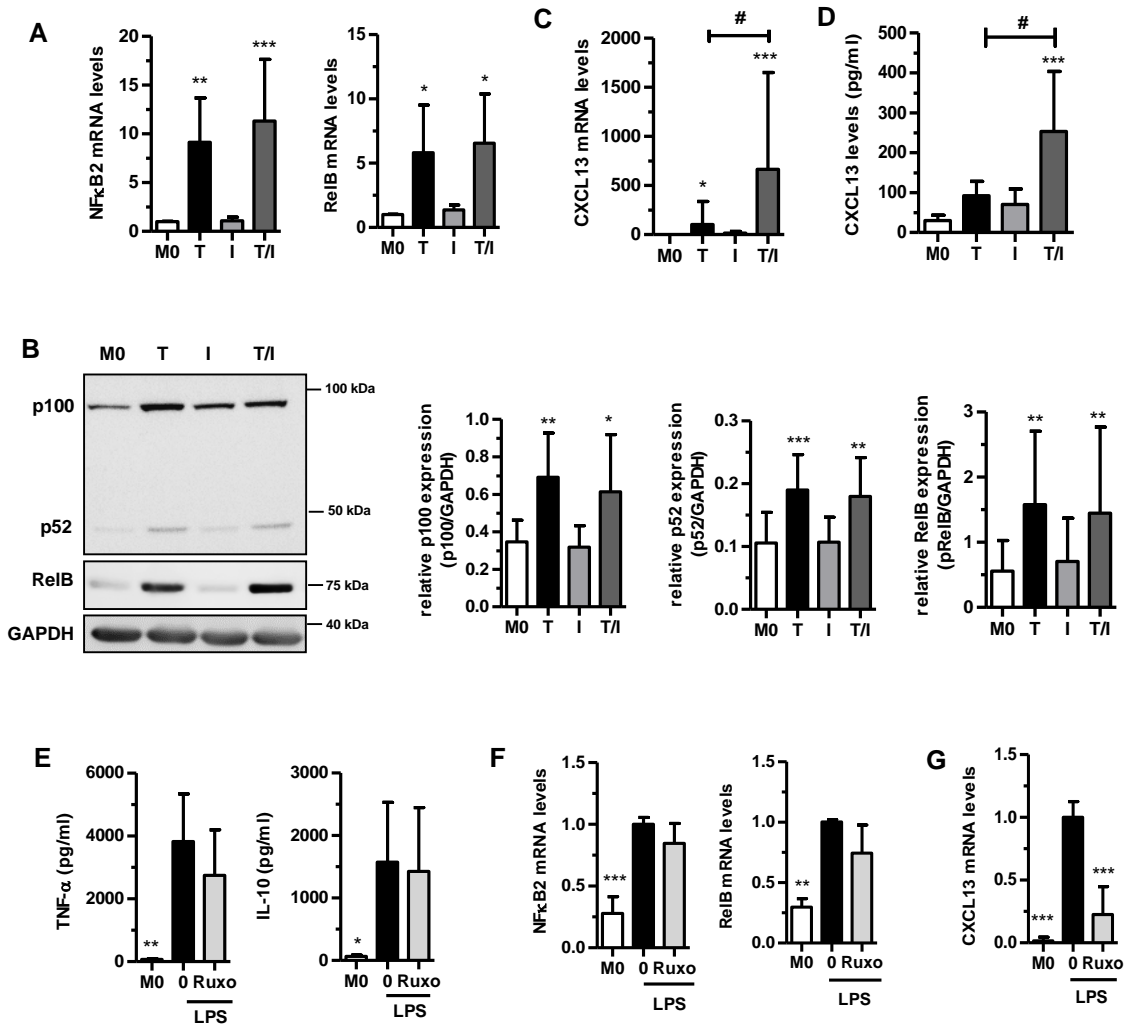


Figure 4

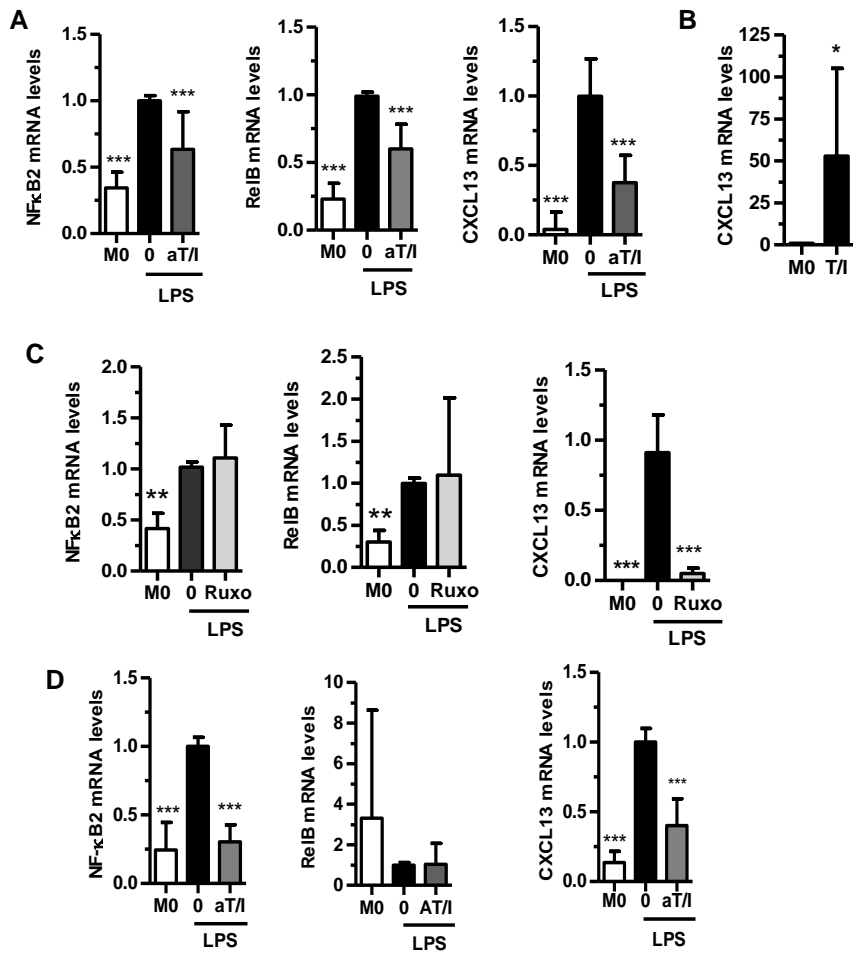


Figure 5

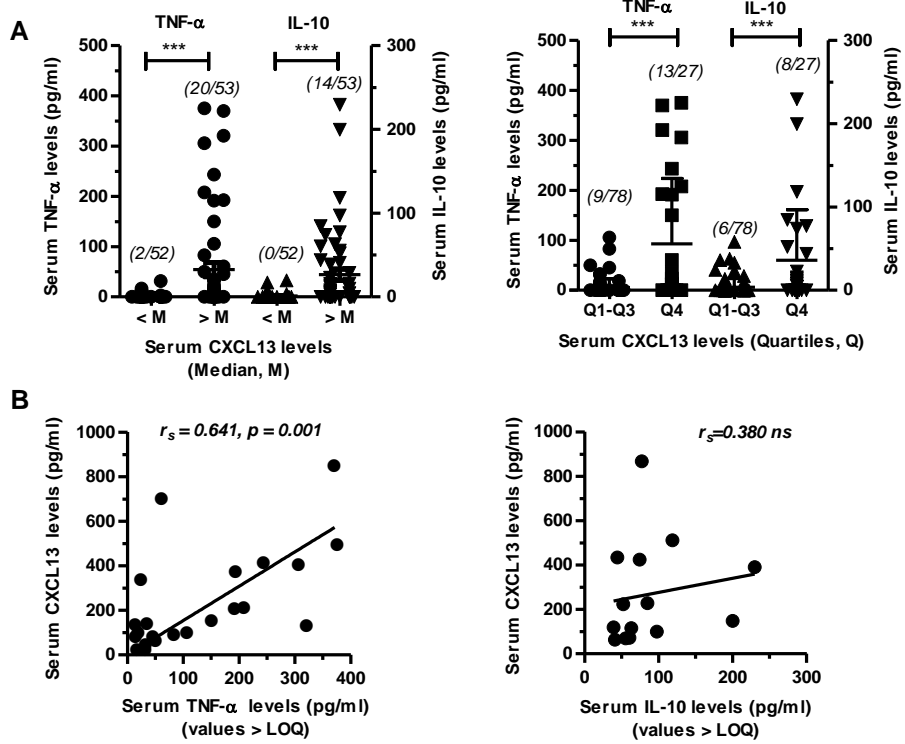


Figure 6

Table S1. Clinical and functional characteristics of patients with IPF

Variable	Mean (SD) unless otherwise specified			
	Lung biopsies	BAL	Blood monocytes	Serum samples
Number of patients	6	7	11	105
Sex (M:F)	6:0	6:1	10:1	80:25
Age at diagnosis (years)	70.0 (5.1)	75.6 (10.1)	73.8 (8.3)	75.0 (7.9)
Smoker (Y:N)	3:3	4:3	8:3	67:38
BMI (kg/m ²)	28.3 (2.0)	28.2 (2.9)	25.5 (2.5)	26.5 (3.8)
FVC % predicted	74.5 (15.6)	85.2 (19.1)	78.7 (13.3)	82.0 (16.7)
DLco % predicted	46.5 (10.9)	50.6 (11.9)	52.5 (14.1)	43.8 (13.6)
Treatment (no:pirf/nint)	6:0	1:6	3:8	105:0

M:F: male:female; BAL: bronchoalveolar lavages; BMI: body mass index; FVC: forced vital capacity, DLco: diffusion capacity for carbon monoxide; pirf/nint: pirfenidone or nintedanib

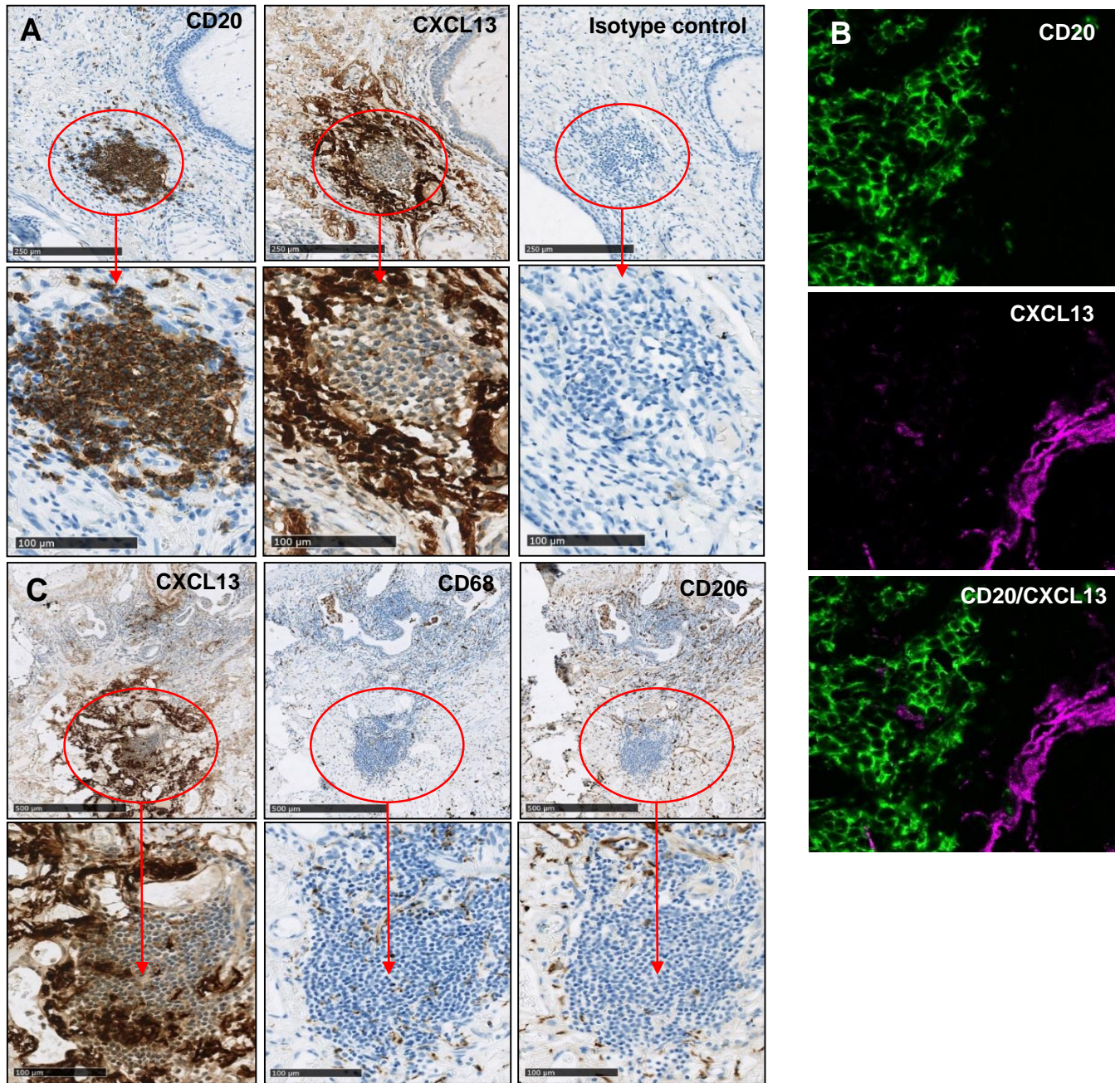


Figure S1: Expression of CXCL13 (A, C), CD68 and CD206 (C) around or within CD20-positive lymphoid aggregates on surgical lung biopsy isolated from a patient with IPF were analyzed by IHC as described in the “Materials and methods” section. Immunostaining was also performed using the CXCL13 isotype control Ab (A). Red circles indicate the region shown at a higher magnification in panels (A, C). (B) IF staining was performed to confirm CXCL13 expression inside and around CD20-positive lymphoid aggregates, as described in the “Materials and method” section. Similar images of CXCL13, CD68 and CD206 staining by IHC were obtained with lung tissues isolated from six patients with IPF.

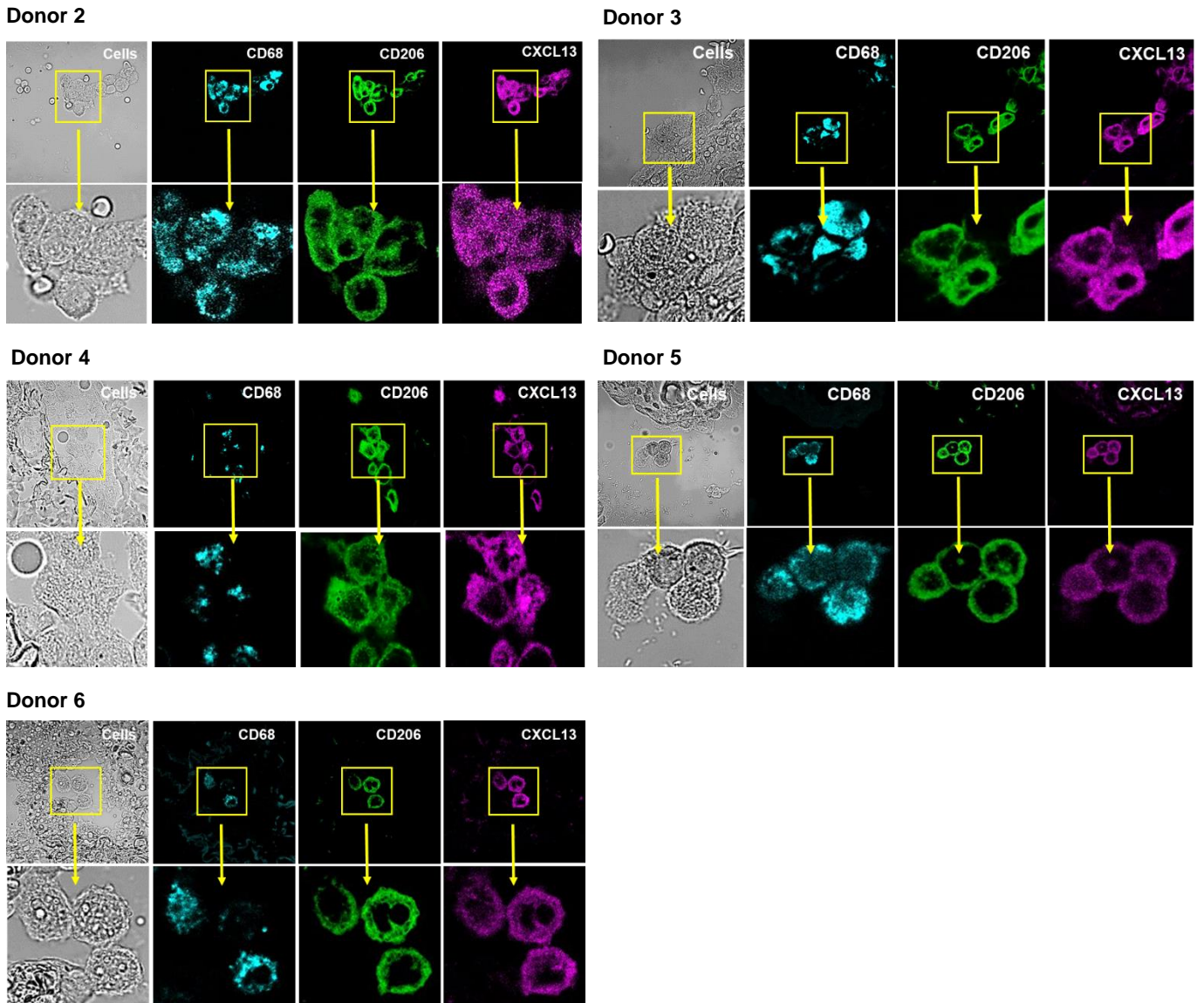


Figure S2: Expression of CD68, CD206 and CXCL13 in alveolar tissues isolated from five different patients with IPF. Multiplex IF staining was performed as described in the “Materials and method” section. Yellow squares indicate the region shown at a higher magnification in the panels.

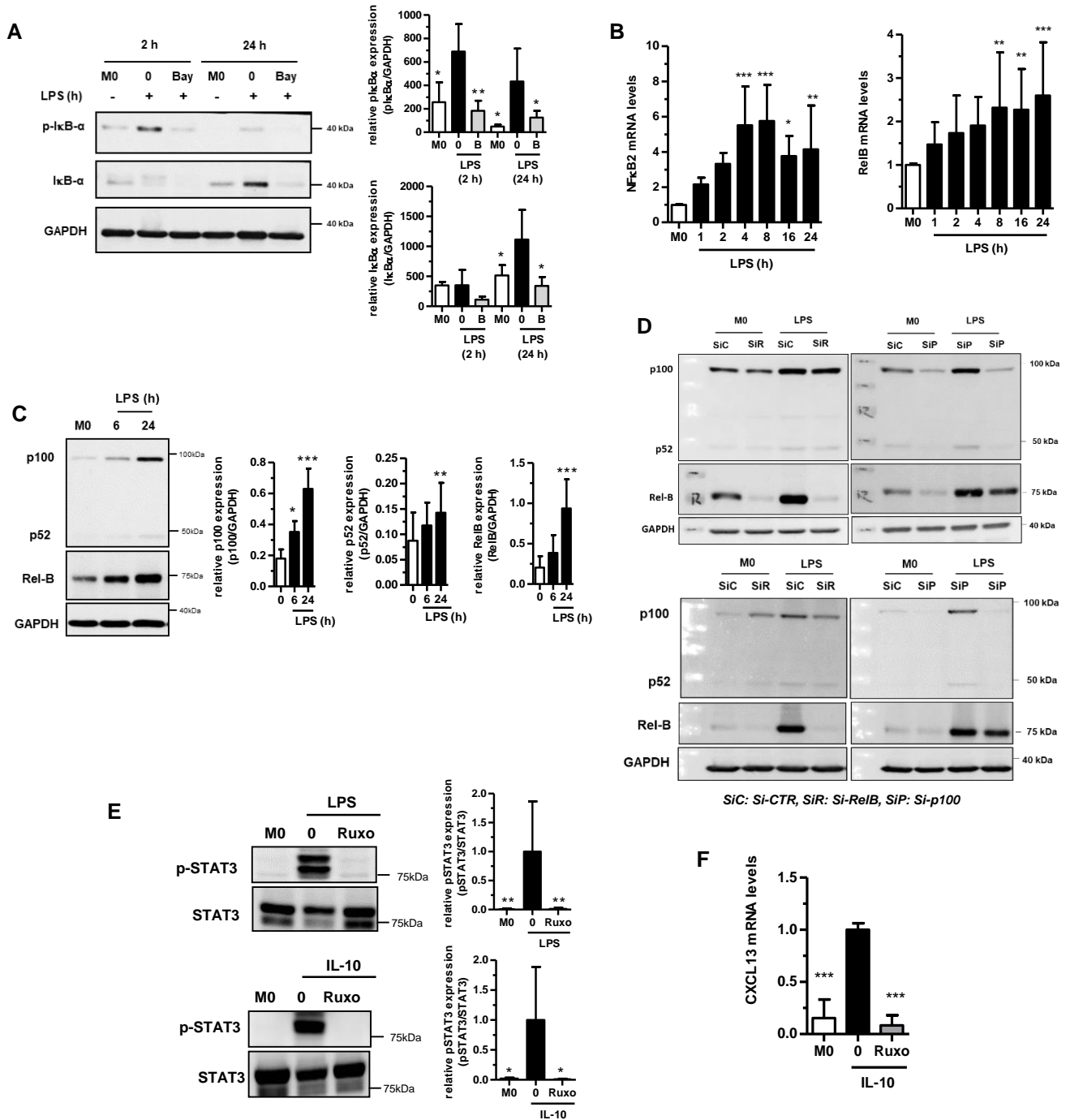


Figure S3: Human MoDM were left untreated (M0) or treated with 5 μ M BAY 11-7082 (BAY) (A) or 5 μ M ruxolitinib (Ruxo) (E, F) and then stimulated either with 20 ng/ml LPS for 6 h (E) or for the indicated times (A-C), or with 10 ng/ml IL-10 for 10 min (E) or 24 h (F). In (D), two independent cell cultures were transiently transfected with control, RelB or p100/p52 siRNA for 24 h and then stimulated with 20 ng/ml LPS for 24 h. In (A, C-E), cells were lysed and total proteins were extracted as described in Materials and Methods. Protein expressions were determined by Western blotting and the visualized bands were analyzed by densitometry using Image Lab™ Software for total protein normalization (BioRad, Marnes-la-Coquette, France). In (B, F), relative mRNA levels were determined by quantitative RT-PCR and normalized to endogenous ribosomal 18S RNA levels. Data are expressed relative to mRNA levels found in M0 cells (B) or “0” IL-10 (F), arbitrarily set at 1. Results are expressed as means \pm SD of 4 (A, C, E lower panel), 6 (B, E upper panel) and 3 (F) independent experiments. * p <0.05, ** p <0.01, *** p <0.001 versus 0 “LPS” (A, E upper panel), M0 (B, C) or “0” IL-10 (E lower panel, F).