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CXCL4 triggers monocytes and macrophages to produce PDGF-BB, culminating in fibroblast activation: Implications for systemic sclerosis

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ABSTRACT

Objective: To analyze how monocyte and macrophage exposure to CXCL4 induces inflammatory and fibrotic processes observed in Systemic sclerosis (SSc) patients.
Methods: In six independent experiments, monocytes of healthy controls (HC) and SSc patients were stimulated with CXCL4, TLR-ligands, IFNα or TGFβ and the secretion of cytokines in the supernatant was assessed by multiplex immunoassays. PDGF-BB production by monocyte-derived macrophages was quantified using immunoassays. The number of monocytes and PDGF-BB in circulation was quantified in HC and SSc patients with the Sysmex XT-1800i haematology counter and immunoassays. Intracellular PDGF-BB was quantified in monocytes by Western blot. PDGF-receptor inhibition was achieved using siRNA-mediated knockdown or treatment with Crenolanib. The production of inflammatory mediators and extracellular matrix (ECM) components by dermal fibroblasts was analyzed by qPCR, ELISA and ECM deposition assays.
Results: SSc and HC monocytes released PDGF-BB upon stimulation with CXCL4. Conversely, TLR ligands, IFNα or TGFβ did not induce PDGF-bb release. PDGF-BB plasma levels were significantly ($P = 0.009$) higher in diffuse SSc patients (n = 19), compared with HC (n = 21). In healthy dermal fibroblasts, PDGF-BB enhanced TNFα-induced expression of inflammatory cytokines and increased ECM production. Comparable results were observed in fibroblasts cultured in supernatant taken from macrophages stimulated with CXCL4. This effect was almost completely abrogated by inhibition of the PDGF-receptor using Crenolanib.
Conclusion: Our findings demonstrate that CXCL4 can drive fibroblast activation indirectly via PDGF-BB production by myeloid cells. Hence, targeting PDGF-BB or CXCL4-induced PDGF-BB release could be clinically beneficial for patients with SSc.

1. Introduction

Systemic sclerosis (SSc) is a rare systemic autoimmune disorder characterized by vascular damage and immune activation preceding the development of severe and progressive fibrosis of the skin and internal organs [1]. This deforming fibrosis can lead to serious conditions such as pulmonary fibrosis and pulmonary arterial hypertension (PAH), which further cause disease-associated morbidity and mortality [2,3]. Although the pathogenesis of SSc is still largely unknown, vascular abnormalities such as the presence of SSc-specific nailfold videocapillaroscopy (NVC) and Raynaud's phenomenon (RP) are commonly observed before the development of fibrosis [1,4]. Such vascular damage potentially leads to the infiltration of mononuclear immune cells into the fibrotic tissues, a phenomenon commonly observed in later stages of the disease [5]. A large portion of the infiltrating cells in SSc skin consists of monocytes and macrophages [6–8]. In line with this observation, in SSc monocytes we previously detected increased promoter activity and expression of the receptors for interleukin(IL)8 [9], a

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chemokine triggering migration of immune cells also found to be increased in the skin of SSc patients [10,11]. Cytokines released by such infiltrating cells activate dermal fibroblasts, thereby playing a crucial role in the development of fibrosis. Supporting this, the expression levels of *CD14*, *SERPINE1*, and more macrophage markers found in SSc skin biopsies [12], have been determined to be the strongest predictor of fibrotic exacerbation over time.

Additionally, we identified a strong increase of CXCL4, a chemokine proposed as an SSc biomarker associated with progressive fibrosis and PAH, in SSc patients [13]. CXCL4 was found to promote monocyte survival [14]. In line with this, we and others have reported an increased number of monocytes in the circulation of SSc patients [15,16]. CXCL4 can enhance the binding of monocytes to the endothelial cell wall, potentially aiding their extravasation into the tissue [17]. Further on, when used to differentiate monocytes *in-vitro*, CXCL4 was shown to induce a specific macrophage phenotype, similar to macrophages found in atherosclerotic plaques [18], while in monocyte-derived dendritic cells (moDCs) CXCL4 increased the release and mRNA stability of IL6 and tumor necrosis factor-alpha (TNFα), cytokines associated to SSc pathogenesis [19].

However, how the interaction between CXCL4 and monocytes or macrophages could affect skin fibrosis is currently unknown. Here we describe that, both in monocytes and macrophages, CXCL4 specifically drives the release of platelet derived growth factor (PDGF)-BB, a potent mitogen factor for dermal fibroblasts [20] which is majorly produced by lesional macrophages in the fibrotic skin [21]. PDGF-BB was previously found to cause increased inflammation, dermal thickening and collagen deposition in bleomycin mouse model for skin fibrosis [22]. Additionally, in fibroblast-like synoviocytes, PDGF-BB acts in concert with TGFβ to potentiate TNFα-induced release of pro-inflammatory cytokines [23]. By demonstrating that the supernatant from CXCL4-activated macrophages induces fibroblasts activation mediated via the PDGF receptor, our results provide new insights on the role of CXCL4 in SSc and suggests that PDGF-BB constitutes a key mediator of CXCL4-induced inflammation and fibrosis.

2. Materials and methods

2.1. Patient samples

Peripheral blood was drawn from patients with SSc (n = 70) and from age and gender-matched healthy control (HC) (n = 21) at the IRCCS Policlinico of Milan. Clinical characteristics of patients are shown in Table 1. Informed consent was obtained from all patients and HC enrolled in the study. All samples and clinical information were treated anonymously right after they were obtained. All patients with SSc fulfilled the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2013 classification criteria. Patients with SSc were subdivided into limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) subsets on the basis of the extent of skin fibrosis (Leroy, J Rheumatol, 1988). Patients with non-cutaneous SSc (ncSSc) met the 2013 ACR/EULAR criteria of SSc without having fibrosis at the time of recruitment. Additionally, early SSc (eaSSc) patients (n = 15) presenting with Raynaud's phenomenon in combination with either typical nailfold videocapillaroscopy abnormalities or SSc-specific autoantibodies were included in the study.

2.2. siRNA transfection

Fibroblasts were transfected using DharmaFECT1 (Thermo Scientific). Prior to transfection, cells were maintained in DMEM containing 10% FBS. 1 h before transfection the medium was replaced by Gibco OPTI-MEM (ThermoFisher) serum-reduced medium. PDGFRα and PDGFRβ specific small interfering RNA (siRNA) (20 nM) or control non-targeting siRNA (20 nM), (Thermo Scientific) were mixed with DharmaFECT1 and incubated for 20 min at room temperature prior to transfection; 24 h after transfection, medium was replaced with DMEM containing 10% FBS which was left for 24 h prior to 24 h pre-starvation followed by stimulation of the cells.

2.3. Sample isolation and cell culture

Plasma samples were obtained from 2 ml undiluted whole blood centrifuged at 1700 g for 10 min. Samples were aliquoted in micronic vials, snap-frozen on dry ice and stored at −80 °C. Mononuclear cells (PBMC) were isolated from peripheral blood collected in lithium heparin tubes using Lymphoprep (Stemcell technologies) or Ficoll Paque Plus (GE Healthcare). The whole-blood monocyte count was performed with the Sysmex XT-1800i haematology counter according to manufacturer's protocol. Monocytes were isolated from PBMCs using anti-CD14 microbeads (Miltenyi Biotech, Germany) on an Automacs pro system to reach > 95% purity. For functional experiments, freshly isolated monocytes were resuspended at 2×10^6/ml in RPMI 1640 Medium, GlutaMAX™ (Gibco) supplemented with 10% FBS. For macrophage differentiation monocytes were cultured at 1×10^6/ml in RPMI medium containing 20 ng/ml M-CSF (R&D systems #216-MC-005) for 5 days followed by 2 days of polarization using 20 ng/ml M-CSF plus either a combination of 100 ng/ml IFNy (eBioscience, 14-8319-80) and 100 ng/ml LPS (R&D, 216-MC-005) for M1 macrophages or 40 ng/ml IL4 (R&D, 204-IL-010) for M2 macrophages. Macrophage conditioned supernatant was obtained by replacing the medium after polarization for RPMI medium supplemented with 1% FBS followed by 24 h culture with or without 10 μg/ml CXCL4 (Peprotech, 300–16).

2.4. Fibroblast culture

Primary dermal fibroblasts lines (N = 10) were obtained from resected material of healthy volunteers undergoing cosmetic surgery. Dermal fibroblasts isolation was performed using the Whole Skin Dissociation Kit (Miltenyi Biotech), following the manufacturer

Table 1
Characteristics of SSc patients included in the study. Data is presented as the median (interquartile range) or number (percentage). ANA: antinuclear antibodies; ACA: anticentromere antibodies; Scl-70: antitopoisomerase antibodies; mRSS: modified Rodnan Skin score; ILD: Interstitial Lung disease.

	HC (n = 21)	earlySSc (n = 15)	ncSSc (n = 27)	lcSSc (n = 24)	dcSSc (n = 19)
Age (years)	50 (44–58)	72 (46–77)	59 (50–68)	60 (51–71)	50 (45–63)
Female: n (%)	19 (90)	15 (100)	27 (100)	22 (92)	15 (79)
Disease duration (years)	–	–	9 (3–16)	19 (8–23)	13 (2–16)
ANA positive: n (%)	–	15 (100)	26 (96)	23 (96)	17 (89)
ACA positive: n (%)	–	12 (80)	20 (74)	13 (54)	0 (0)
Scl-70 positive: n (%)	–	2 (13)	1 (4)	9 (38)	11 (58)
mRSS	–	–	–	4 (3–5)	10 (5–17)
ILD: n (%)	–	0 (0)	2 (7)	7 (29)	14 (74)
Prednisone	–	2(13)	4 (15)	8 (33)	15 (79)
Other Immunosuppressant treatment[a]: n (%)	–	1 (7)	0	5 (21)	9 (47)

[a] Cyclophosphamide, methotrexate, micofenolate mofetil or biological drugs (abatacept, tocilizumab, etanercept).

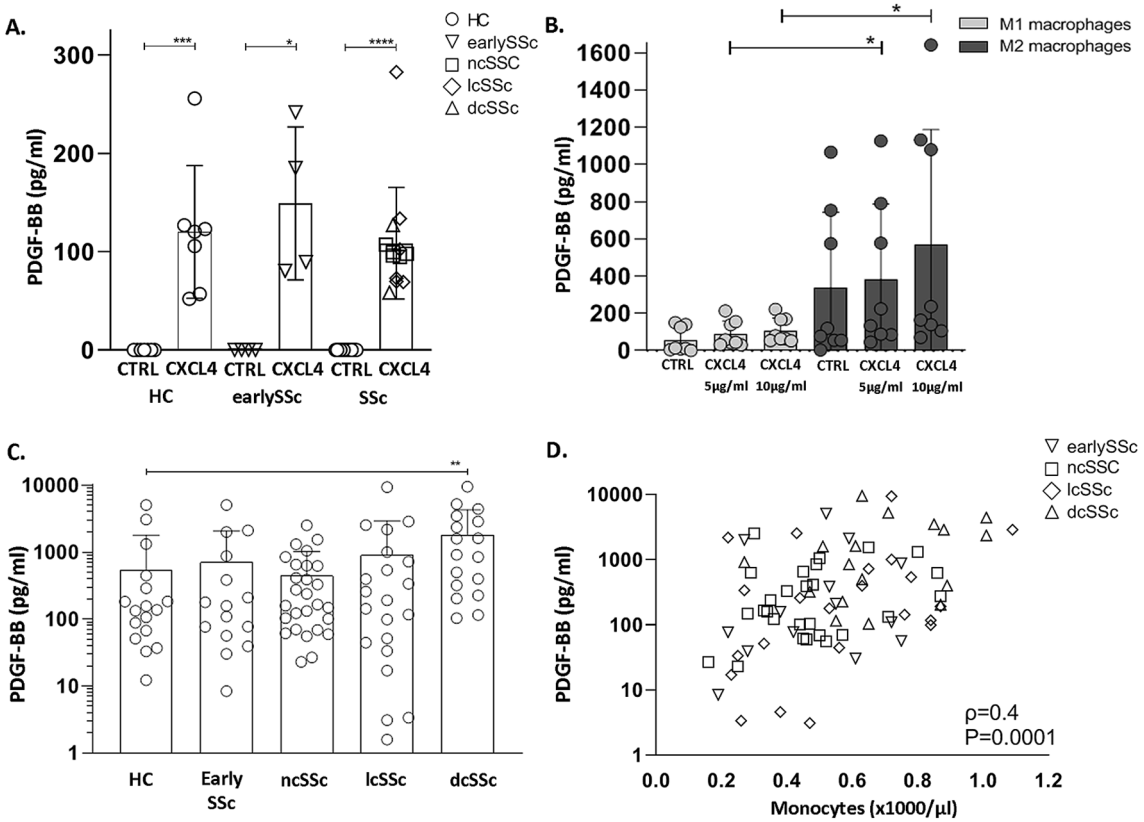

Fig. 1. PDGF-BB is released by monocytes and macrophages after CXCL4 stimulation. A) PDGF-BB concentration in supernatant of healthy (n = 7), earlySSc (n = 5) and SSc (n = 12) monocytes after 16 h in medium with 5 µg/ml CXCL4 or control medium. B) Concentration of PDGF-BB in culture supernatant of M1 (n = 8) vs M2 (n = 8) macrophages after 16 h of culture with 0, 5 or 10 µg/ml CXCL4. C) PDGF-BB concentration (mean ± sd) in plasma of HC (n = 21) or patients with earlySSc (n15) ncSSc (n = 27), lcSSc (n = 23) or dcSSc (n = 19). D) Spearman Rho's correlation between monocyte count and PDGF-BB levels in plasma of SSC patients n = 85. (A–B) Mann-Whitney *U* test. (* = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.).

protocol. Briefly, biopsies with diameter of 3 × 4 mm were digested overnight at 37 °C and processed with the gentleMACS Dissociator (Miltenyi Biotech) to obtain a single cell suspension. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Biowest) and 10,000 IU penicillin–streptomycin. Cells were used between passage 3–7. Stimulations with 10 ng/ml PDGF-BB (R&D, 220-BB) and/or TNFα (R&D, 210-TA and/or TGF-β2 (R&D, 302-B2) were performed after overnight serum starvation in medium containing 1% FBS. Where indicated, PDGFR inhibitors CP-868596/Crenolanib (Selleckchem) were used at a concentration of 1 µM and a 1 h pre-incubation was performed before stimulation. Where indicated, fibroblasts were serum-starved and cultured in conditioned supernatant of M2 macrophages.

2.5. Protein quantification in plasma and cell-culture supernatant

Concentrations of cytokines in plasma or secreted by cultured monocytes and macrophages was quantified using an in house Luminex immunoassay (PDGF-BB, TNFα, LAP, IL1α, IL6 MCP1, CXCL4, CXCL10, CXCL11).The quantification of IL6 and IL8 in supernatant of dermal fibroblasts was performed using PeliKine ELISA kits (M1916 and M191, Sanquin) according to manufacturer's protocol.

2.6. RNA extraction

Total RNA was isolated from monocytes, macrophages and fibroblasts using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's guideline. RNA was quantified with a Qubit (Invitrogen).

2.7. RT-qPCR analysis

RealTime-quantitative PCR (RT-qPCR) assays were performed using 2–5 ng retrotranscribed RNA obtained using the superscript IV reverse transcriptase kit (Invitrogen). Reactions were conducted using the SybrSelect mastermix (Applied Biosystems) with 350 nM specific primer pairs on a QuantStudio 12 k flex (Applied Biosystems). Cycle threshold values (Ct) of the gene of interest were normalized to the expression of the housekeeping gene GAPDH or RPL32 and analyzed using the comparative threshold cycle method. The relative expression was calculated using formula $2^{-\Delta CT}$. A list of primers used can be found in Supplementary Table 1.

2.8. Western blot analysis

Monocytes or fibroblasts were lysed in Laemmli's buffer(10% SDS, glycerol, 1 M Tris pH 6.8 and dH20) Equivalent amounts of total protein lysates were mixed with loading buffer and boiled at 95 °C for 5 min. Proteins were resolved by electrophoresis on 4–12% Bis-Tris SDS NuPAGE gels (Invitrogen) for 1 h at constant 200 V. Proteins were transferred to polyvinylidene difluoride (PVDF)membranes (Bio-Rad Laboratories), membranes were blocked in Tris-buffered saline (pH 8.0) containing 0.05% Tween-20 (Bio-Rad) and 4% milk (Bio-Rad) for 1 h at room temperature, washed and probed overnight at 4 °C with antibodies recognizing PDGF-BB (16,829, Abcam, 1:1000), p-AKT (4060 S, Cell Signaling, 1:1000) histone 3 (H3) (9715, Cell Signaling, 1:1000) or tubulin (T9026, Sigma-Aldrich, 1:10.000)). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated swine anti-rabbit or goat anti-mouse immunoglobulin secondary antibody (Dako, 1:3000), and protein visualization was performed using ECL

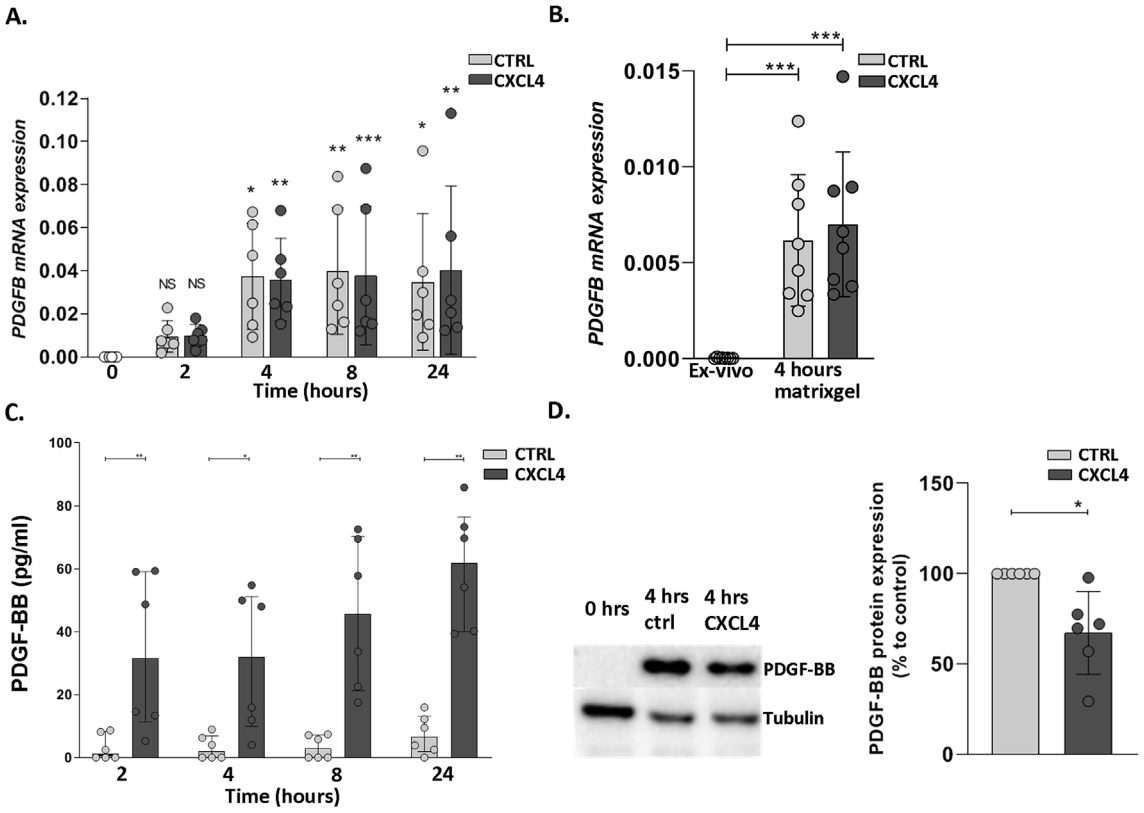

Fig. 2. CXCL4 affects PDGF-BB release through a post-transcriptional mechanism after monocyte adherence. A) mRNA expression of *PDGFB* in ex-vivo (t = 0) or cultured monocytes (n = 6) in control medium or medium supplemented with 5 μg/ml CXCL4 for up to 24 h (n = 6). (Stars indicate the significance of the difference compared with *ex-vivo* monocytes)B) *PDGFB* mRNA expression in ex-vivo monocytes (n = 8) versus monocytes (n = 8) cultured in matrigel supplemented with or without 5 μg/ml CXCL4. C) The concentration of PDGF-BB in supernatant of monocytes (n = 6) cultured in control medium or medium supplemented with 5 μg/ml CXCL4 for up to 24 h. D) Western blot quantification of intracellular PDGF-BB in ex-vivo monocytes versus monocytes cultured in control medium or medium supplemented with 5 μg/ml CXCL4 for 4 h. Representative Western blot image (left) and densitometry analysis (n = 6, right) (A B and C) Mann Withney *U* test (D) Wilcoxon matched-pairs signed rank test). (* = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.).

substrate KIT: ECL PRIME (Life Sciences #RPN2236) and a ChemiDoc MP system (Bio-Rad).

2.9. Extracellular matrix deposition assay

Clear flat-bottomed 96 well black imaging plates (Corning) were coated with 0.2% bovine gelatin (Sigma) solution for 1 h at 37 °C and washed with Dulbecco's PBS supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+} (DPBS+). The plates were then incubated with 1% glutaraldehyde for 30 min at room temperature followed by three washes with DPBS+ and incubation with 1 M ethanolamine for 30 min and 3 more washes with DPBS+. Fibroblasts were seeded and kept in full medium for 24 h before starvation in DMEM containing 1% FBS overnight. Cells were then stimulated with PDGF-bb (10 ng/ml) in DMEM containing 2% FBS and 50 μg/ml of L-ascorbic acid (Wako). Medium was refreshed after 48 and 96 h. After six days, cells were lysed with 0.5% vol/vol Triton X-100 containing 20 mM NH_4OH in PBS, and plates were kept at 4 °C overnight. Cellular debris was removed, and wells were fixed in ice-cold 100% methanol. Blocking with 1% normal donkey serum (Jackson ImmunoResearch) was performed for 30 min at room temperature before the samples were incubated with Collagen Pan Polyclonal antibody (anti-collagen type I, II, III, IV and V; Invitrogen - Thermo Fisher Scientific) and fibronectin (R&D Systems) for 2 h at room temperature. After washing, the secondary antibodies (IRDye 800CW and IRDye 680RD; Li-Cor) were applied for 1 h at room temperature. Quantification of the fluorescent signal reflecting the amount of protein was performed using an Odyssey Sa Infrared Imaging System (Li-Cor Biotechnology).

2.10. Statistical analysis

Correlations were calculated using Spearman's Rho test. Potential differences between experimental groups were analyzed by Wilcoxon's test, Mann-Whitney-U test, Kruskal-Wallis test, or repeated-measure ANOVA tests, as appropriate. Statistical analysis was performed using GraphPad Prism 8 software. P values less than 0.05 were considered significant.

3. Results

3.1. CXCL4 induces the release of PDGF-BB, a protein associated with SSc skin fibrosis, by monocytes and macrophages

To examine the effect of CXCL4 on the release of cytokines and chemokines by monocytes we performed six independent experiments in which we cultured $CD14^+$ monocytes of healthy controls (HC) (total n = 7) and SSc patients (total n = 17) in culture medium with and without human recombinant CXCL4. In a panel of cytokines and chemokines measured in the supernatant, we found a significant increase in the release of PDGF-bb in both healthy and SSc monocytes specifically treated with CXCL4 (Fig. 1A), but not with Toll like receptor (TLR) 3, 4 or 7/8 ligands (poly I:C, LPS, and R848 respectively), TGFβ or IFNα (Supplementary Table 2). Monocytes of earlySSc patients, as well as those of ncSSc, lcSSc and dcSSc patients, responded in a similar fashion with concern to PDGF-BB release in CXCL4-stimulated monocytes. To exclude the possibility that platelet contamination or the presence of monocyte-platelet-complexes (MPCs) could be the source of PDGF-BB

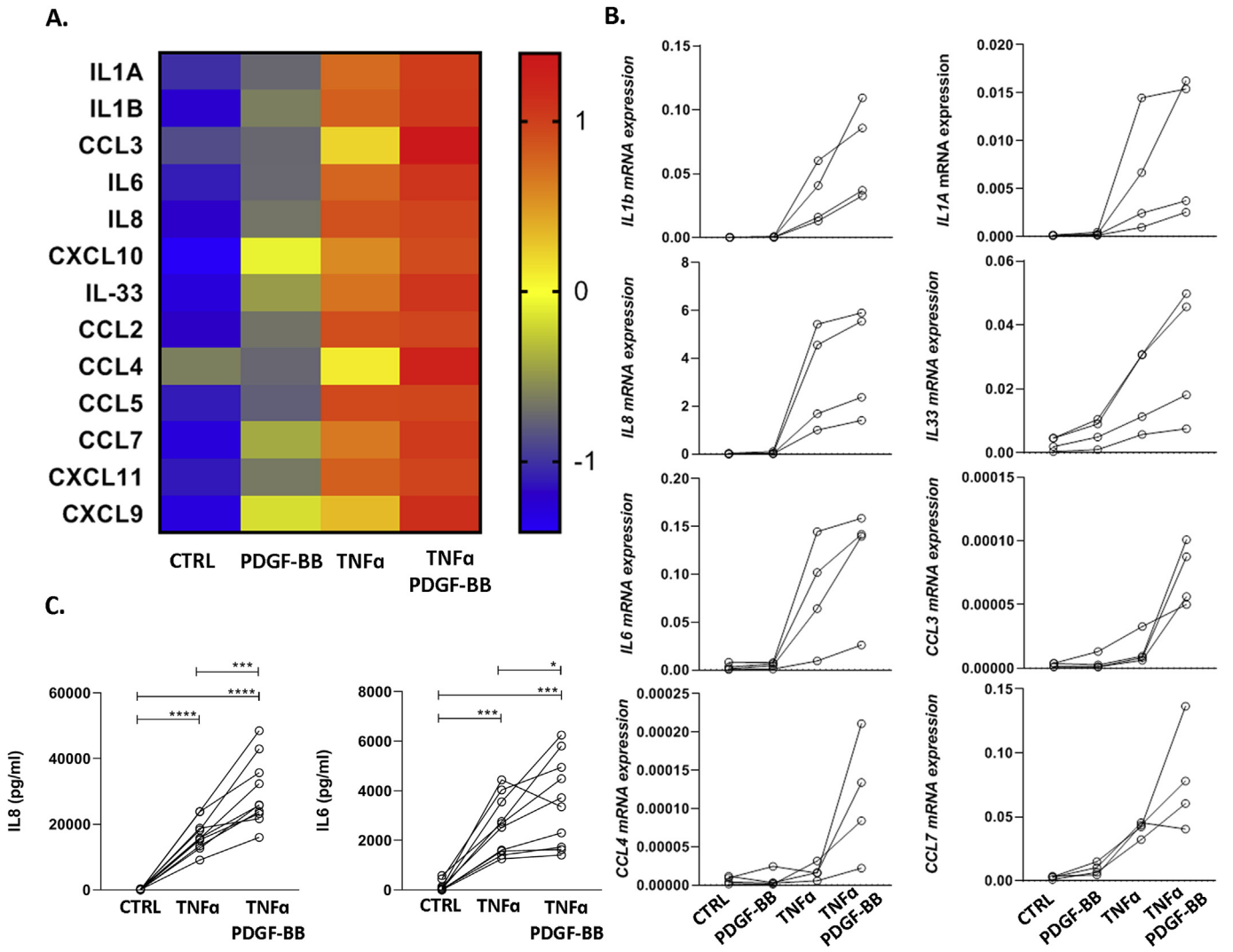

Fig. 3. PDGF-BB synergizes with TNFα to induce the expression and release of pro-inflammatory cytokines in dermal fibroblasts. A) Heatmap showing mRNA expression level (mean Z-score of ΔΔCT) of SSc associated cytokines and chemokines in dermal fibroblasts (n = 4) after 4 h culturing in control medium, or medium with 10 ng/ml PDGF-BB, 10 ng/ml TNFα or a combination thereof. B) Graphs reflecting mRNA expression of *IL1B, IL1A, IL8, IL33, IL6, CCL3, CCL4* and *CCL7* in dermal fibroblasts (n = 4) after 4 h culturing in control medium, or medium with 10 ng/ml PDGF-BB, TNFα or a combination thereof. C) ELISA quantification of the concentration of IL6 and IL8 in culture supernatant of dermal fibroblasts (n = 10) after 16 h culture in either control medium, medium with 10 ng/ml TNFα or a combination of TNFα and PDGF-BB (repeated measure one-way ANOVA with fisher LSD test). (* = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.).

[24], platelet-free monocytes and MCPs were separated using FACS sorting and stimulated with CXCL4. Both cell fractions displayed a similar response to CXCL4 (Supplementary Figs. 1A and 1B). Additionally, since platelets are enucleate cells and do not display transcriptional activities [25], we could prove that PDGF-BB protein is produced in monocytes by blocking *de novo* transcription, which prevented PDGF-BB production (Supplementary Fig. 1C). Activated monocytes differentiate into both inflammatory and profibrotic macrophages (M1 and M2 macrophages, respectively) in the affected skin of SSc patients [26]. Thus, we used *in-vitro* differentiated monocyte-derived M1 and M2 macrophages to validate our findings. A dose-dependent increase of PDGF-BB release was observed in both cell types upon stimulation with CXCL4. Furthermore, in M2 macrophages, both basal and CXCL4-induced production of PDGF-BB was higher than in M1 macrophages (Fig. 1B). In order to investigate whether the CXCL4-induced PDGF-BB release by monocytes potentially leads to increased levels of PDGF-BB in the circulation of SSc patients, we isolated plasma and performed a monocyte count for HC (n = 21) early SSc (n = 15) and SSc patients with various subsets of SSc (n = 70). Compared with HC, we found a significant increase of PDGF-BB in the plasma of patients with extensive skin fibrosis (dcSSc) (Fig. 1C). We observed no significant difference in PDGF-BB plasma levels when comparing patients treated with and without prednisone or immunosuppressant drugs (IS), suggesting that treatment has no major influence on plasma PDGF-BB levels (Supplementary Fig. 2A, 2B and 2C). Furthermore, we observed that SSc patients suffering from ILD do not show significantly higher PDGF-BB plasma levels compared to patients without ILD (Supplementary Fig. 2D). Conversely, we found that SSc patients who tested positive for antinuclear antibodies (ANA), but not for anticentromere antibodies and antitopoisomerase antibodies (ACA and Scl-70, respectively), displayed higher PDGF-BB plasma levels (Supplementary Fig. 2E). Furthermore, a significant correlation was observed between the number of circulating monocytes and the concentration of PDGF-BB in the plasma of SSc patients (Fig. 1D).

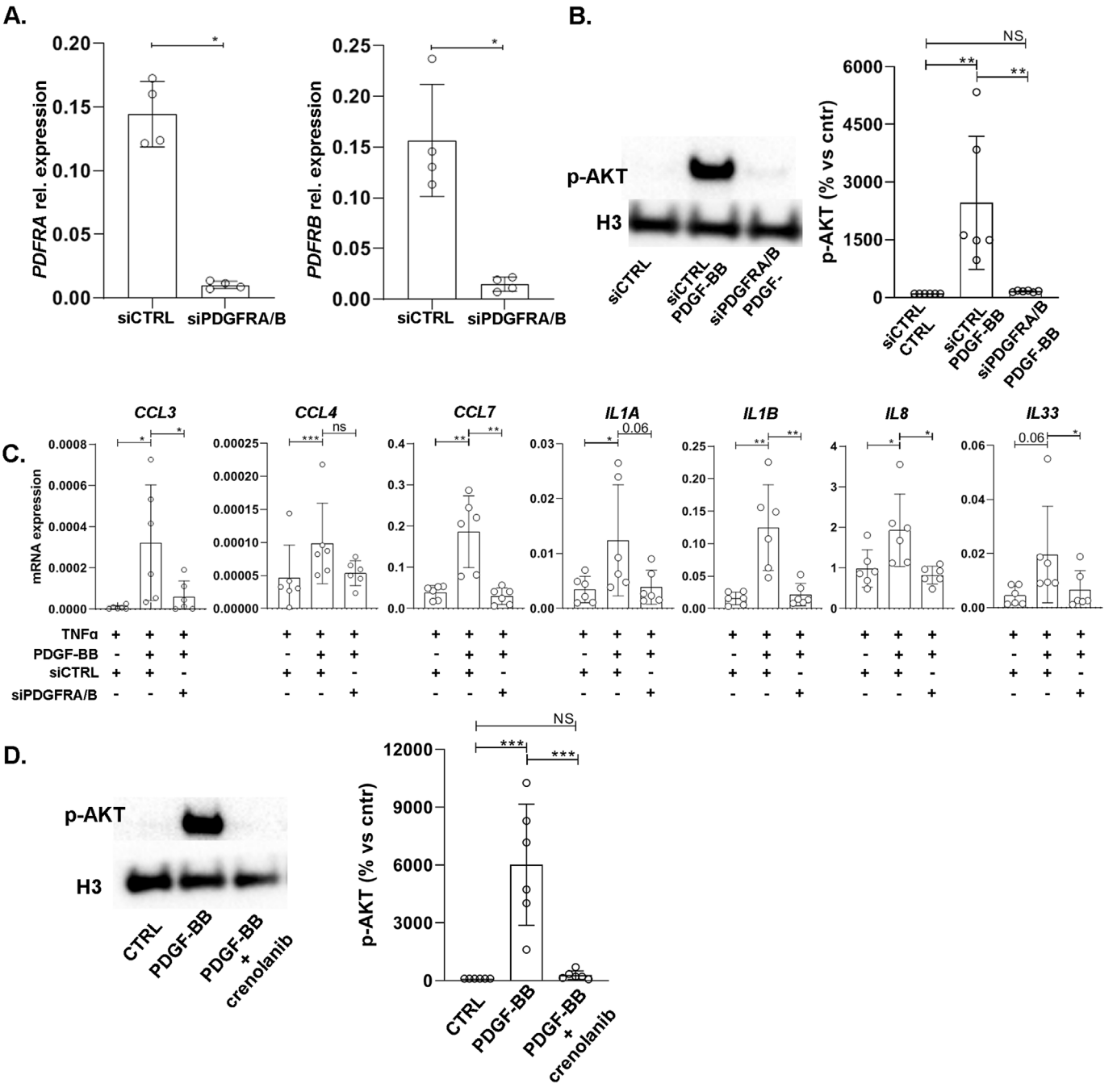

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Fig. 4. PDGF-receptor inhibition abrogates the pro-inflammatory potential of PDGF-BB. A) *PDGFRA* and *PDGFRB* mRNA expression in dermal fibroblasts (n = 4) after non-targeting control siRNA transfection (siCtrl) and combined PDGFRA and PDGFRB siRNA transfection (siPDGFRA/B). B) Western blot detection and densitometry analysis of phosphorylated AKT in siCtrl or siPDGFRA/B transfected dermal fibroblasts(n = 6) cultured for 30 min is control medium or medium supplemented with 10 ng/ml PDGF-BB. C) mRNA expression of *CCL3, CCL4, CCL7, IL1A, IL1B, IL8* and *IL33* in siCtrl or siPDGFRA/B transfected dermal fibroblasts (n = 6) cultured for 4 h in medium with 10 ng/ml TNFα or a combination of 10 ng/ml TNFα and PDGF-BB. D) Western blot detection and densitometry analysis of phosphorylated AKT in untreated or Crenolanib pre-treated dermal fibroblasts (n = 6) cultured for 30 min in control medium or in medium supplemented with 10 ng/ml PDGF-BB. E) mRNA expression of *CCL3, CCL4, CCL7, IL1A, IL1B, IL8* and *IL33* in untreated or Crenolanib pre-treated dermal fibroblasts (n = 6) cultured for 4 h in medium with 10 ng/ml TNFα or a combination of 10 ng/ml TNFα and PDGF-BB. (repeated measure one-way ANOVA with fisher LSD test). * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.).

3.2. CXCL4 affects the release but not the production of PDGF-BB by monocytes

Given that the signaling pathways activated by CXCL4 in monocytes are not fully known, we sought to identify whether the release of PDGF-BB by monocytes is regulated at the transcriptional level. qPCR quantification of *PDGFB* mRNA expression revealed that in both CXCL4-stimulated and untreated monocytes, *PDGFB* gene expression was equally induced upon culturing the cells in tissue culture plates. The induction of *PDGFB* mRNA expression was observed after plating cells for 2 h and reached a plateau after 4 h (Fig. 2A), after which expression levels stayed relatively constant up to at least 96 h (data not shown). To exclude the possibility that PDGF-BB production was just an effect of the tissue plate culture conditions, monocytes were cultured in matrigel to resemble the extracellular environment of the skin. We could validate that a strong induction of *PDGFB* mRNA expression was also observed upon culturing the cells in matrigel, regardless of the addition of CXCL4 (Fig. 2B). Furthermore, even though the mRNA expression level of *PDGFB* was similar in CXCL4-stimulated and unstimulated monocytes, PDGF-BB protein was only detected in the supernatant of CXCL4-stimulated conditions, as early as 2 h after culture initiation (Fig. 2C). Using Western blot analysis to quantify the level of intracellular PDGF-BB, we could observe that monocytes do not express PDGF-BB *ex-vivo* but only upon adherence (Fig. 2D). Finally, we found that CXCL4 reduced the level of intracellular PDGF-BB protein, possibly as a consequence of the increased release of the protein in this condition (Fig. 2C and D).

3.3. PDGF-BB synergizes with TNFα to induce the expression and release of pro-inflammatory cytokines in dermal fibroblasts

Autoimmunity and inflammation are both early hallmarks of SSc that precede the onset of fibrosis [4]. Hence, we sought to determine whether PDGF-BB could contribute to the development of an inflammatory phenotype in dermal fibroblasts. qPCR analysis indicated that PDGF-BB has a marginally potentiating effect on the gene expression of inflammatory cytokines and chemokines in dermal fibroblasts (Fig. 3A and B). However, in combination with TNFα, a cytokine associated with SSc pathogenesis [27] and induced by CXCL4 in monocytes (Supplementary Table 2), PDGF-BB strongly enhanced the expression of pro-inflammatory mediators (Fig. 3A and B) and the release of IL6 and IL8 (Fig. 3C).

3.4. Inhibiting the expression or the functionality of the PDGF receptor using siRNA knockdown or crenolanib abrogates the pro-inflammatory potential of PDGF-BB

To verify whether the pro-inflammatory effect of PDGF-BB is mediated by its traditional signaling pathway and to see if this effect could be abrogated by the inhibition of its receptor, we made use of siRNA techniques to silence the expression of PDGFRα (*PDGFRA*) and -Rβ (*PDGFRB*), the two tyrosine kinase receptors of PDGF-BB, in dermal fibroblasts. Silencing of PDGFR in dermal fibroblasts resulted in an average reduction of *PDGFRA* and *PDGFRB* mRNA expression of 93 and 89%, respectively (Fig. 4A). The phosphorylation of Protein Kinase B (PKB or AKT), a known signaling event downstream of PDGF-BB/PDGF receptor interaction [28], was suppressed after combined knockdown of *PDGFRA* and *PDGFRB* (Fig. 4B). Furthermore, PDGF receptor knockdown prevented the induction of various chemokines (*CCL3, CCL4, CCL7* and *IL8*) and cytokines (*IL1A, IL1B* and *IL33)* by TNFα and PDGF-BB (Fig. 4C). A comparable inhibition of these genes was observed after knockdown of the PDGF receptor in dermal fibroblasts stimulated with the supernatant of CXCL4-conditioned M2 macrophages (Supplementary Fig. 3). These results indicate that the CXCL4-induced release of PDGF-BB by macrophages has a functional effect on dermal fibroblasts and that blocking PDGF signaling dampens the inflammatory response in these cells. Additionally, similar results with regards to the phosphorylation of AKT (Fig. 4D) and the expression of chemokines and cytokines in dermal fibroblasts upon PDGF-BB and TNFα stimulation (Fig. 4E) were obtained using Crenolanib, a small molecule inhibitor of the PDGF receptor.

3.5. CXCL4-induced PDGF-BB derived from M2 macrophages results in increased deposition of collagen and fibronectin by dermal fibroblasts

The aberrant production of ECM molecules, such as collagen and fibronectin, by dermal fibroblasts is a known hallmark of SSc-related skin fibrosis [29]. To assess the potential effect of PDGF-BB on the production of these proteins, dermal fibroblasts were stimulated with PDGF-BB for 6 days. PDGF-BB induced a significant increase in the deposition of both collagen and fibronectin (Fig. 5A). Besides enhancing TNFα-induced inflammation, the combination of TNFα and PDGF-BB led to more ECM deposition compared to either stimulus alone or medium control (Supplementary Fig. 4). Given the increased presence of CXCL4 and M2 macrophages in the skin of SSc patients [6,7,13] we investigated how CXCL4-exposed M2 macrophages can indirectly affect ECM deposition in dermal fibroblasts. Using ECM deposition assays we found that the supernatant of CXCL4-activated M2 macrophages was able to induce the enhanced deposition of both fibronectin and collagen by dermal fibroblasts (Fig. 5B and Supplementary Figs. 4B and 4C). The concentration of PDGF-BB detected in the supernatant of both control and CXCL4-treated M2 macrophages correlated significantly with the amount of collagen and fibronectin deposited by the dermal fibroblasts (Fig. 5C). Remarkably, Crenolanib strongly inhibited ECM deposition in fibroblasts exposed to CXCL4-conditioned M2 macrophage supernatant. These data indicate the potential of Crenolanib in inhibiting not only inflammatory, but also pro-fibrotic, responses which are associated with SSc pathogenesis (Fig. 5D).

4. Discussion

SSc is a severe autoimmune disease with a high risk of life-threatening complications. Although mortality has decreased over the last decades, current treatment options in SSc are mainly disease-modifying or symptomatic [30]. The development of deforming skin fibrosis typifies SSc and causes decreased mobility and quality of life. The cause of the overproduction of ECM underlying this skin fibrosis are still unclear. Mononuclear cell infiltrates, mainly consisting of lymphocytes and monocyte-derived macrophages, have been observed at the early stage of the development of skin fibrosis in SSc [31] and are associated with the aggravation thereof [12]. Additionally, we previously found that the number of monocytes in circulation is increased in SSc patients

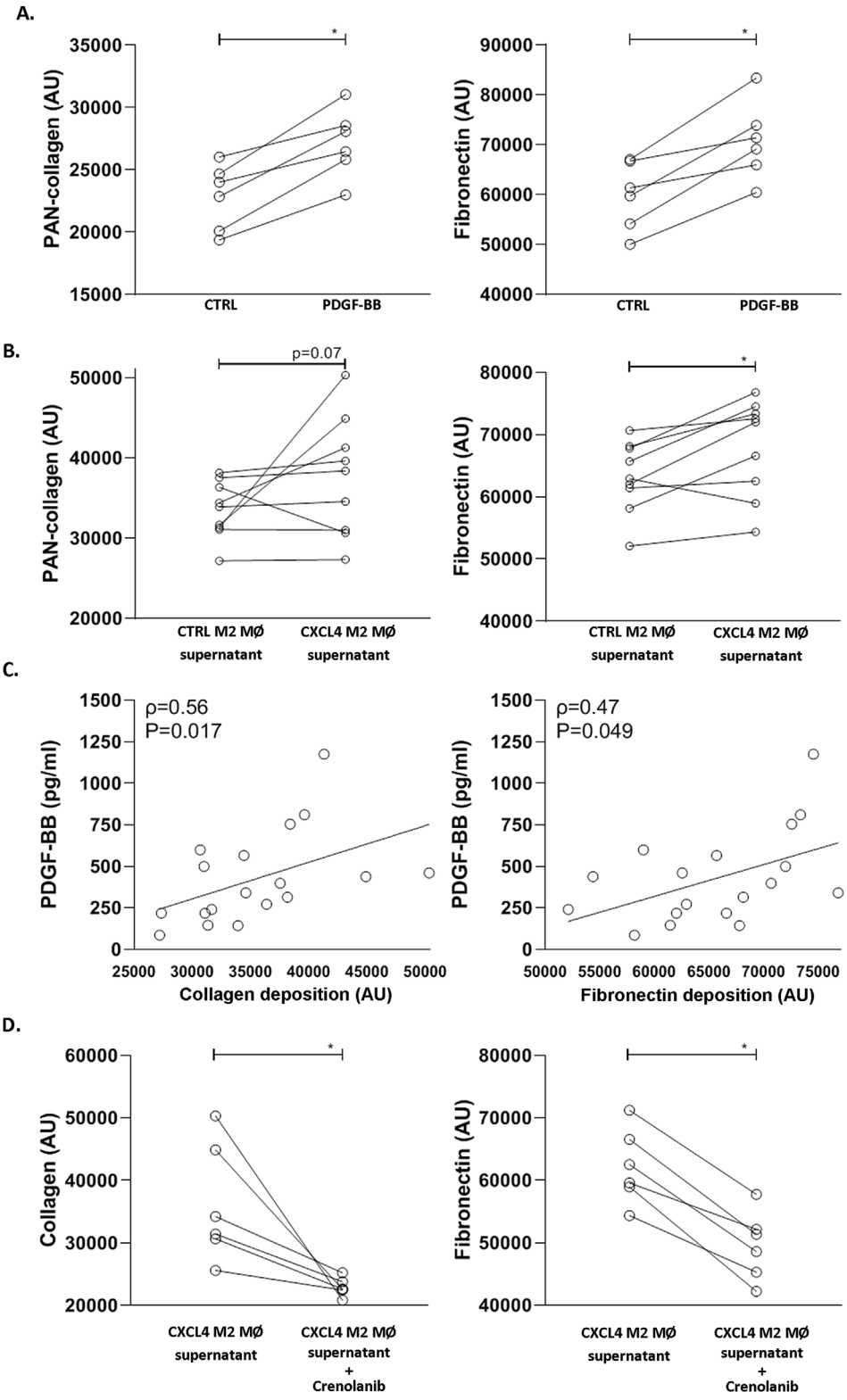

Fig. 5. CXCL4-induced PDGF-BB release causes increased deposition of collagen and fibronectin by dermal fibroblasts. A) Quantification of collagen (left) and fibronectin (right) deposition by dermal fibroblasts (n = 6) after 6 days of culture in control medium or medium supplemented with 10 ng/ml PDGF-BB. B) Quantification of collagen (left) and fibronectin (right) deposition by dermal fibroblasts after 6 days of culture in supernatant of either control M2 macrophages (n = 9) or M2 macrophages stimulated with 10 μg/ml CXCL4 (n = 9) for 24 h. C) Spearman Rho correlation of the concentration of PDGF-BB in the M2 macrophage supernatant samples and the amount of collagen (left) and fibronectin (right) deposition by dermal fibroblasts after 6 days of supernatant culture. D) quantification of collagen (left) and fibronectin (right) deposition by dermal fibroblasts after 6 days of culture in supernatant of CXCL4-conditioned M2 macrophages(n = 6) in the presence or absence of 1 μM Crenolanib. (Mann-Whitney U tests). (* = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.) AU = arbitrary units; MØ = macrophages.

and correlates with the mRSS [15].

CXCL4 is a chemokine released by platelets and various immune cells in pathological conditions [32,33]. Increased presence of CXCL4 was previously detected in the skin and circulation of SSc patients [13,34]. Here we describe how the CXCL4/PDGF-BB signaling axis in monocytes and macrophages can induce both a pro-inflammatory and a pro-fibrotic phenotype in dermal fibroblasts.

Increased levels of PDGF-BB and the PDGF receptor-β were found in the lesional skin of SSc patients [35,36]. Here, in line with previous findings [37], we observed increased concentrations of PDGF-BB in plasma of diffuse SSc patients. Of interest, SSc patients who tested positive for ANA, but not for the SSc specific ACA or Scl-70 [38] displayed higher PDGF-BB plasma levels. We found that monocytes rapidly induce the expression of *PDGFB* mRNA and PDGF-BB protein upon adhesion and maintain this expression not only over time, but also over the course of differentiation to macrophages. Although PDGF-BB is

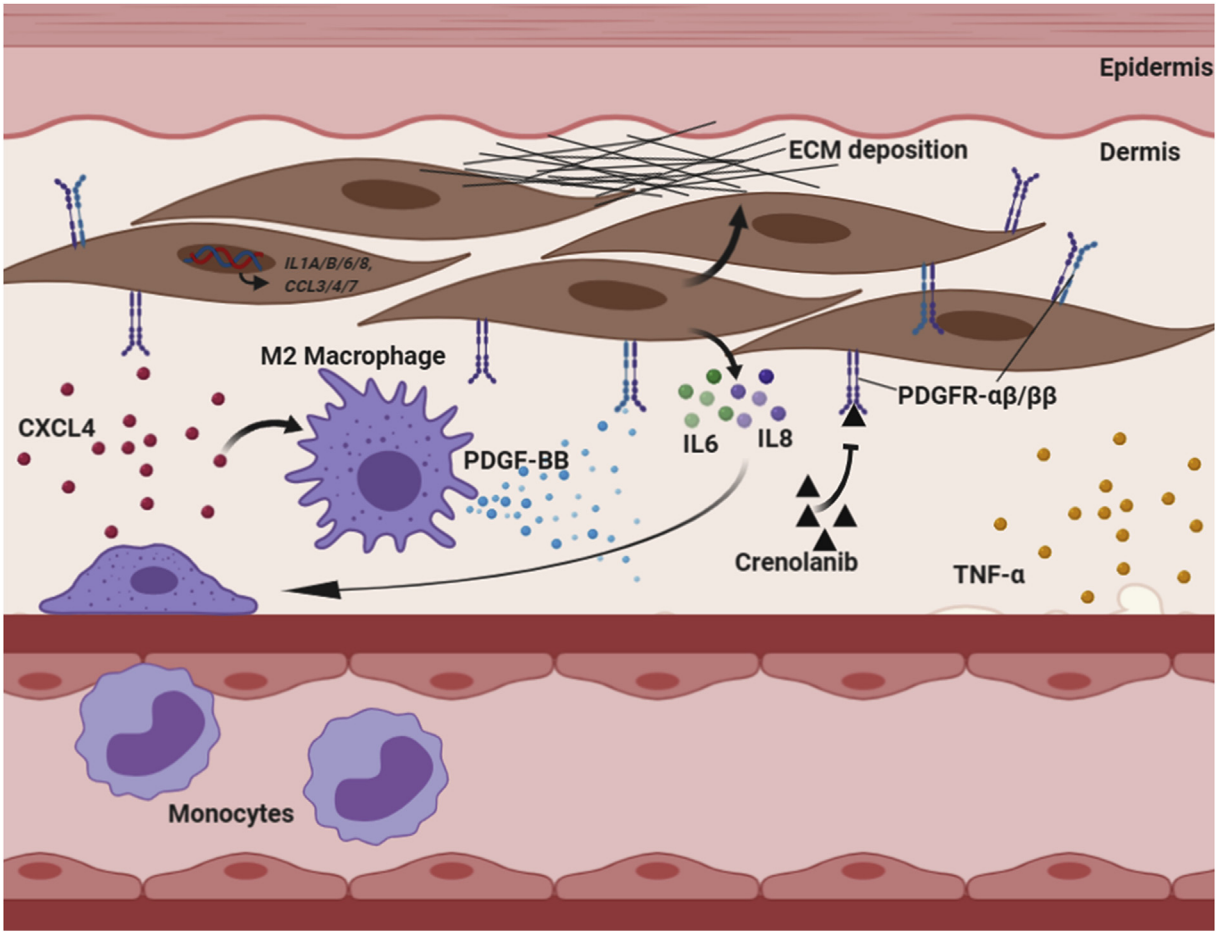

Fig. 6. Suggested mechanism of action of CXCL4 induced PDGF-BB release inducing an inflammatory and pro-fibrotic phenotype in dermal fibroblasts. CXCL4 present in the skin and circulation of SSc patients activates monocytes and M2 macrophages inducing the release of PDGF-BB. PDGF-BB contributes to the instigation of inflammation and ECM deposition, reflected by the increased release of IL6 and IL8 and the enhanced deposition of fibronectin and collagen. Blocking the PDGF-receptor using Crenolanib abrogates both the inflammation and ECM deposition in dermal fibroblasts.

present intracellularly in cultured monocytes, we found that the release of PDGF-BB could only occur after stimulation with CXCL4. Interestingly, we did not detect PDGF-BB release upon stimulation with IFNα, TGFβ, and various TLR-ligands, suggesting that PDGF-BB secretion could be specifically triggered by CXCL4. Additionally, we observed that the number of monocytes in circulation correlated with the plasma level of PDGF-BB in SSc patients. These data, combined with the observation that CXCL4 levels are increased in SSc patients, suggest that monocytes may be a significant source of this growth factor *in-vivo*.

The development of fibrosis in SSc patients is commonly preceded by autoimmunity and inflammation [4]. TNFα is a main pro-inflammatory factor implicated in SSc pathogenesis which was found to be increased in the skin and circulation of SSc patients [39,40]. By screening a panel of cytokines and chemokines implicated in the pathogenesis of SSc, we found that PDGF-BB displayed a synergistic effect on the expression of various TNFα-induced inflammatory mediators in dermal fibroblasts. The expression levels of IL6 and members of the IL1 family, IL1α, IL1β and IL33, were associated with the perpetuation of a fibrogenic phenotype in dermal fibroblasts [41–43]. The infiltration of mononuclear cells into the skin is considered a major driving force instigating the inflammation, followed by fibrosis, in SSc patients. Hence, the increased production of these chemokines by fibroblasts is likely to aggravate fibrotic processes. Here, we found that PDGF-BB contributes to the increased expression of *CCL2,3,4,5,* and *7*, as well as *CXCL8,9,10,* and *11*, chemokines previously shown to be involved in leukocyte recruitment and fibrosis in SSc [44].

Aside from promoting inflammation, PDGF-BB has been shown to exacerbate fibrosis in the bleomycin mouse model of SSc [22] and reported to play a crucial role in a variety of other fibrotic conditions, such as pulmonary, liver and cardiac fibrosis [45]. PDGF-BB was initially considered to mainly function as a chemotactic and mitogenic factor for fibroblasts in sites of tissue damage. However, studies have shown that PDGF-BB also leads to increased differentiation of fibroblasts into myofibroblasts [46], contractile and ECM-producing cells which are commonly observed in fibrotic SSc lesions [47]. Using genome-scale gene expression profiling of SSc skin, Mahoney et al. recently demonstrated the altered expression of a cluster of genes implicated in aberrant PDGF signaling in the skin of SSc patients [48]. In line with these findings, we observed that dermal fibroblasts deposited more collagen and fibronectin when treated with PDGF-BB. Additionally, stimulation of dermal fibroblasts with CXCL4-conditioned M2 macrophage supernatant also induced a pro-inflammatory and pro-fibrotic phenotype in dermal fibroblasts, similarly to what was observed using recombinant proteins. Lastly, we showed that inhibiting the PDGF receptors using siRNA knockdown or the PDGF receptor inhibitor Crenolanib could prevent the inflammatory and fibrogenic phenotype induced by human recombinant PDGF-BB and CXCL4-conditioned macrophage supernatant.

The use of PDGF receptor inhibitors in SSc has been hampered by the lack of compounds with high specificity. Clinical trials on imatinib (Gleevec), an unspecific kinase inhibitor targeting both TGFβ signaling, stem cell factor (SCF) and PDGF receptor signaling, reported moderate but inconsistent improvement of skin fibrosis [49,50]. Crenolanib is a more potent and specific PDGF receptor inhibitor than Imatinib [51].

Thus, it is plausible that its use in the clinics could prove beneficial, as recently demonstrated by therapeutic efficacy in a pre-clinical model of SSc [52].

Overall, our data show that CXCL4 promotes the release of PDGF-BB, thereby enhancing the expression of pro-inflammatory cytokines and the deposition of ECM molecules in dermal fibroblasts (Fig. 6). These results indicate that targeting the CXCL4-PDGF-BB axis can be clinically relevant for SSc.

Author statement

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Declarations of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2020.102444.

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