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Original article

Hypoxia and TLR9 activation drive CXCL4 production in systemic sclerosis plasmacytoid dendritic cells via mtROS and HIF-2α

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Abstract

Objective. SSc is a complex disease characterized by vascular abnormalities and inflammation culminating in hypoxia and excessive fibrosis. Previously, we identified chemokine (C-X-C motif) ligand 4 (CXCL4) as a novel predictive biomarker in SSc. Although CXCL4 is well-studied, the mechanisms driving its production are unclear. The aim of this study was to elucidate the mechanisms leading to CXCL4 production.

Methods. Plasmacytoid dendritic cells (pDCs) from 97 healthy controls and 70 SSc patients were cultured in the presence of hypoxia or atmospheric oxygen level and/or stimulated with several toll-like receptor (TLR) agonists. Further, pro-inflammatory cytokine production, CXCL4, hypoxia-inducible factor (HIF) -1α and HIF-2α gene and protein expression were assessed using ELISA, Luminex, qPCR, FACS and western blot assays.

Results. CXCL4 release was potentiated only when pDCs were simultaneously exposed to hypoxia and TLR9 agonist ($P < 0.0001$). Here, we demonstrated that CXCL4 production is dependent on the overproduction of mitochondrial reactive oxygen species (mtROS) ($P = 0.0079$) leading to stabilization of HIF-2α ($P = 0.029$). In addition, we show that hypoxia is fundamental for CXCL4 production by umbilical cord CD34 derived pDCs.

Conclusion. TLR-mediated activation of immune cells in the presence of hypoxia underpins the pathogenic production of CXCL4 in SSc. Blocking either mtROS or HIF-2α pathways may therapeutically attenuate the contribution of CXCL4 to SSc and other inflammatory diseases driven by CXCL4.

Key words: CXCL4, plasmacytoid dendritic cells, hypoxia, TLRs, mtROS, HIF-2α, systemic sclerosis

BASIC SCIENCE

Introduction

SSc is an autoimmune disease characterized by vascular alteration, immune dysregulation and fibrosis of the skin and internal organs [1]. Vascular alterations are manifested by reduced density and loss of capillaries, which leads to impaired oxygenation in the tissue of SSc patients. Oxygen flow is even further reduced by excessive deposition of the extracellular matrix, making a low oxygen level (hypoxia) one of the characteristic features of SSc [2]. Previously, we identified chemokine (C-X-C motif) ligand 4 (CXCL4) as a new biomarker for SSc [3], and we demonstrated that CXCL4 in these patients is mainly produced by plasmacytoid dendritic cells (pDCs) [3].

CXCL4 is largely viewed as a pro-inflammatory chemokine, which in monocytes can boost the production of

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Rheumatology key messages

- Hypoxia and TLR9 are crucial factors for CXCL4 production in healthy control plasmacytoid dendritic cells.
- CXCL4, mtROS and HIF-2α are increased in plasmacytoid dendritic cells from systemic sclerosis patients.
- Blocking mtROS and/or HIF-2α significantly reduced CXCL4 production in plasmacytoid dendritic cells from healthy controls and systemic sclerosis patients.

IL-6 and TNF-α cytokines [4, 5]. In T cells, CXCL4 induces the production of IL-17 [6] and drives the production of Th2 cytokines (IL-4, IL-5 and IL-13), while suppressing Th1 cytokines [7]. CXCL4 is involved in several inflammatory and fibrotic diseases such as intestinal IBD, psoriasis, cystic fibrosis, liver fibrosis and cancer [8–13].

Interestingly, the level of CXCL4 in SSc patients was found to be the highest in a very progressive subset of the disease [3], where impaired tissue oxygenation and inflammation are the highest, indicating that hypoxia could be a relevant factor for CXCL4 release. Furthermore, these patients have high release of endogenous toll-like receptor (TLR) agonists. These locally produced TLR agonists play an important role in SSc pathogenesis by driving inflammation and fibrosis [14, 15]. Together, accumulating evidence implicates a role for CXCL4 in inflammatory and fibrotic diseases such as SSc, which makes CXCL4 an attractive therapeutic target. One way of blocking the side effects of CXCL4 is inhibiting its production. Here we explored several potential mechanisms of CXCL4 production by systematically testing the effects of hypoxia and TLR activation on pDCs.

Methods

Patient cohort

The study was performed according to the guidelines of the Declaration of Helsinki and meets the approval of Ethical and Review committee of the Institutional Review and Ethical Board of University Medical Center Utrecht and Maastad Ziekenhuis of Rotterdam, and University of Milan and Fondazione IRCCS Ospedale Maggiore Policlinico, Italy. The Ethical Committee approval was obtained in November 2011 (Ethical approval number 12-466 and 13-697).

Blood from patients and sex- and age-matched healthy controls (HCs) was obtained from the University Medical Center Utrecht and the Maasstad Hospital, Rotterdam (the Netherlands). All patients provided informed written consent approved by the local institutional medical ethics review boards prior to inclusion in this study. Samples and clinical information were treated anonymously immediately after collection. Patients fulfilled the ACR/EULAR 2013 classification criteria for SSc [16] and the demographics and clinical characteristics of the patients are detailed in Supplementary Table 1, available at *Rheumatology* online. Briefly, we will refer to non-cutaneous SSc (ncSSc) as patients with no skin fibrosis involvement and to limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) as patients with skin fibrosis involvement (schematically represented in Supplementary Fig. 1, available at *Rheumatology* online [17–19]).

Reagents, catalogue numbers and company name are listed in Supplementary Key resource Table 2, available at *Rheumatology* online.

Methodological details

Cell isolation

Peripheral blood mononuclear cells (PBMCs) from HCs and SSc patients were isolated by Ficoll gradient. pDCs were isolated using an autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity was routinely assessed by flow cytometry and above 92% for pDCs.

pDC stimulation

Half a million pDCs per ml were cultured in RPMI-GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 10.000 I.U. penicillin–streptomycin and IL-3 (10 ng/ml), and then incubated either in atmospheric (21% O_2) or hypoxic conditions (1% O_2 in a Rasquinn (Ruskinn Technology LtdSuite, UK) invivO2 1000 hypoxic chamber). Cells were acclimated for 30 min before being exposed to different stimuli and/or inhibitors. Cells were stimulated with the TLR agonists CpG-C (TLR9, 1 μM), CpG-A (TLR9, 1 μM), loxoribine (TLR7, 1 μg/ml), R848 (TLR7/8, 2 μg/ml) or CL075 (TLR8, 5 mM) for 16 h in an incubator at 37°C and 5% CO_2.

Inhibition of mitochondrial reactive oxygen species and hypoxia-inducible factors

To inhibit mitochondrial reactive oxygen species (mtROS) we used mitoQ (10 nM) (provided by Mike Murphy, MRC Mitochondrial Biology Unit, University of Cambridge, UK), and hypoxia-inducible factor (HIF)-1α and HIF-2α were inhibited using bisphenol A dimethyl ether (10 nM) and HIF-2 antagonist 2 (10 nM), respectively.

Transfection of pDCs using siRNA

Transfection of pDCs was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) transfection reagent. HIF-1α, HIF-2α or control non-targeting silencing RNA (siRNA) (5 nM) were mixed

with Optimem and Plus (Thermo Fisher Scientific, Waltham, MA, USA) reagent and incubated for 20 min at room temperature prior to transfection for 6 h. After transfection cells were incubated either under atmospheric or hypoxic conditions as described above.

RNA sequencing

RNA sequencing was performed by Beijing Genomics Institute (Hong Kong) using 100 ng RNA per sample to prepare RNAseq libraries (TruSeq Stranded kit, Illumina San Diego, CA, USA) after poly(A) capture according to the manufacturer's instructions. Pooled libraries of equimolar concentration were sequenced according to manufacturer's protocols using the Illumina HiSeq 2000 sequencer. Each sample generated $\sim 2 \times 10^7$ paired-end (100 bp) reads. FastQC was used to perform a quality check and, using STAR aligner [20], samples were aligned to a reference human genome (GRCh38 build 79). HTSeq-count was used to calculate the read counts per gene [21]. DESeq2 was used to perform the differential gene expression analysis of HCs and SSc patients as described before. To obtain normalized read counts, variance stabilizing transformation was used [22].

mtROS measurement

mtROS production was quantified using MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol, measured in FACS CANTO II Flow cytometer and analysed with BD FACSDiva software (BD Biosciences, San Jose, CA, USA).

Quantification of cytokine production level

Cell-free supernatants were analysed by ELISA for CXCL4 and IFN- α. TNF-α, IL-6 and IL-8 were measured using human single-plex assays (Bio-Rad Laboratories, Hercules, CA, USA) and read on a Bio-Plex 200 system (Bio-Rad).

RT-PCR and quantitative (q)PCR

RNA from pDCs was isolated using the Allprep RNA/DNA (Qiagen, Hilden, Germany) kit. Total RNA was reverse-transcribed using SuperScript II RT (Thermo Fisher Scientific, Waltham, MA, USA). Duplicate PCR reactions were performed using SYBR green or TaqMan with a Quantstudio Real-Time PCR detection system (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was amplified using specific primers listed in Supplementary Key resource Table 3, available at *Rheumatology* online. Relative levels of gene expression were calculated by normalizing to *GUSB* or *B2M* housekeeping genes. Fold changes of mRNA were calculated by using the formulas $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$.

Intracellular flow cytometry in pDCs

pDCs were stained extracellularly first with CD123 and CD303 at 4°C using optimized concentrations of antibody for 15 min. After being fixed and permeabilized using Fix-Perm (eBioscience/Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol, pDCs were stained with either anti-HIF-1α or anti-HIF-2α antibody. Data were acquired using a Fortessa flow cytometer and analysed using FlowJo software (BD Biosciences, San Jose, CA, USA). Protein levels were quantified and represented as mean fluorescence intensity. Antibodies used are listed in Supplementary Key resource Table 4, available at *Rheumatology* online).

Immunohistochemical analysis

Cryosections of skin from SSc patients or HCs were cut at 5 μm and stained for HIF-1α (1:20), HIF-2α /EPAS1 (1:50), BDCA4/CD304-phycoerythrin (PE) (1:50) and 4′,6-diamidino-2-phenylindole (DAPI) (1:500). Secondary antibodies used were goat anti-mouse AF594 (Thermo Fisher Scientific, cat. no. A11005, 1:300) and goat anti-rabbit AF594 (Thermo Fisher Scientific, cat. no. A11012, 1:300). Isotype controls were used to test for non-specific binding: mouse IgG2b-PE (R&D Systems, Minneapolis, MN, USA, cat. no. IC0041P) and rabbit IgG polyclonal (Abcam, Cambridge, UK, cat. no. ab27478).

In short, 5 μm cryosections were air-dried for 1 h, after which antigen retrieval was performed with acetone; sections were subsequently blocked with 2.5% goat serum in 1% BSA/PBS. Primary antibody in 1% BSA/PBS + 1% goat serum was applied for 1 h and sections were washed three times in Tris-buffered saline–Tween 20 (TBST) before secondary antibody in 1% BSA/PBS + 1% goat serum was applied for 30 min. After three washes of TBST, a second primary antibody in 1% BSA/PBS + 1% goat serum was applied for 1 h and sections were again subsequently washed three times in TBST. Staining of nuclei was performed with DAPI in PBS for 7 min after which sections were washed three times in PBS before being mounted and closed with FluorSave reagent (Merck, cat. no. 345789-20ML).

Microscopic analysis

Fluorescent pictures were taken on a confocal microscope (Carl Zeiss Microscopy, Oberkochen, Germany, cat. no. LSM710) with Zen 2009 software and subsequently analysed using ImageJ software (imagej.net, FIJI build).

OP9 cells harvest and culture

On day 0 OP9 cells, a cell line derived from mouse bone marrow stromal cell [23], were seeded on a 24-well plate at a final concentration of 20 000 cells per well in 750 μl of MEM α medium enriched with 20% FBS and 1% penicillin and streptomycin. Cells were kept at 37°C and 5% CO_2 overnight.

$CD34^+$ cell culture

Umbilical cord derived CD34 cells, isolated from cord blood donated to the UMC Utrecht hospital, were thawed in a 37°C water bath, diluted in PBS and loaded on FBS. After one wash in PBS, cells were resuspended in MEM α enriched with 20% FBS, 1% penicillin and streptomycin. Cells were plated on top of OP9 cells, at a final concentration of 25 000 cells per well. Medium was supplemented with IL-7 (15 ng/ml) (PeproTech

Cranbury, NJ, USA) and FMS-like tyrosine kinase 3 ligand (FLT-3L) (15 ng/ml) (Cellgenix, Freiburg Germany). Cells were then incubated for 14 days either in hypoxia (5% O_2) or at atmospheric oxygen level (AOL ; 21% O_2) at 5% CO_2. Every 2 days complete MEM α medium enriched with IL-7 and Flt-3L was added. On day 14 the cells were stimulated with CpG-C either in hypoxia (5% O_2) or in AOL (21% O_2) with 5% CO_2.

Viability assessment on pDCs

Cell death was assessed by Annexin V-7AAD staining. Cells were stained with Annexin V (1:100 dilution, BD Biosciences) and 7AAD (1:100 dilution, BD Pharmingen) and measured using a FACS Canto Flow Cytometer. The data were further analysed with BD FACS DIVA software.

Quantification and statistical analysis

Where appropriate, the Mann–Whitney test, unpaired Kruskal–Wallis test or paired one-way analysis of variance was performed using Graph Pad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA). *P*-values less than 0.05 were considered to be statistically significant.

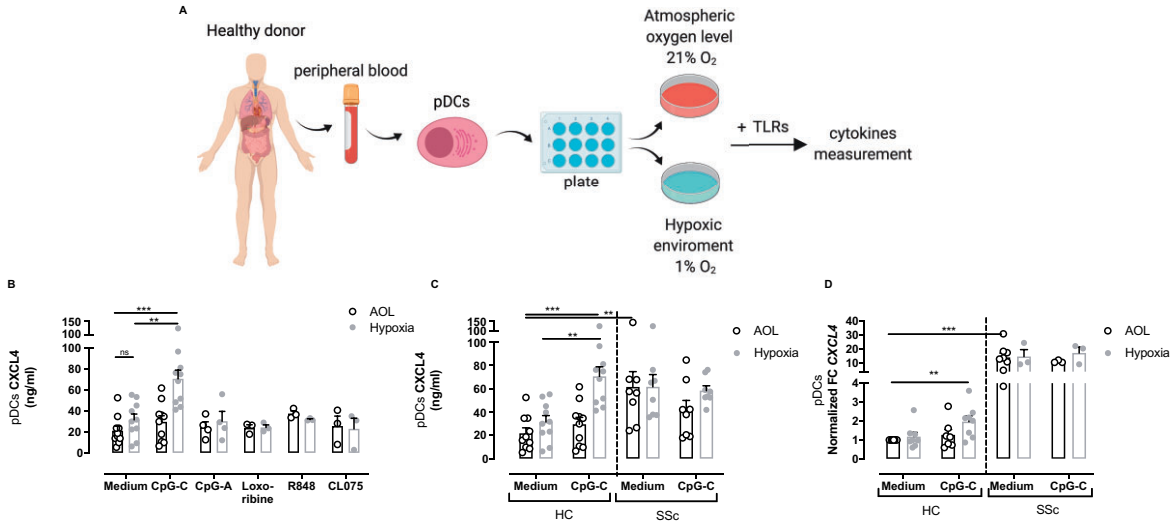
Results

pDC CXCL4 production is dependent upon hypoxia and TLR9

pDCs were exposed to hypoxia and/or TLR agonists after which CXCL4 production was quantified. The exposure to hypoxia alone or to individual TLR agonists alone did not induce CXCL4 production at protein or RNA level (Fig. 1B and D). However, the production of CXCL4 was increased upon co-exposure of hypoxia and TLR9 agonist, CpG-C ($P < 0.0001$ at the protein level, $P = 0.0057$ at the mRNA level) (Fig. 1B and D). CXCL4 production was not increased when the cells were co-exposed to hypoxia and other TLR agonists (Fig. 1B). Therefore, in the following experiments, pDCs were stimulated only with CpG-C.

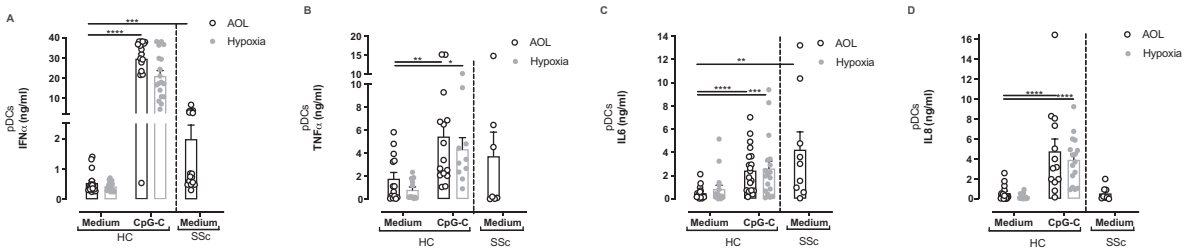
Our data confirmed a high level of CXCL4 production by unstimulated pDCs from SSc patients both at the protein (Fig. 1C) and the mRNA level (Fig. 1D) compared with HC pDCs in atmospheric conditions ($P = 0.0021$). In addition, CXCL4 production of SSc pDCs was not further increased after hypoxia, TLR9 stimulation or the combination of them, neither at the mRNA nor at the protein level (Fig. 1C and 1D).

Fig. 1 Hypoxia and TLR9 are the factors leading to increased CXCL4 production in plasmacytoid dendritic cells



(**A**) Schematic layout of the experimental design created with Biorender. (**B**) CXCL4 levels quantified in the supernatant of pDCs isolated from healthy participants upon hypoxia or atmospheric oxygen level and challenged with either TLR9 agonist (CpG-C, $n = 12$, or CpG-A, $n = 4$), TLR7 agonist (loxoribine, $n = 3$), TLR7/8 agonist (R848, $n = 3$) or TLR8 agonist (CL075, $n = 3$) for 16 h. (**C**) Supernatant CXCL4 levels secreted by pDCs of healthy participants ($n = 17$) and patients with SSc ($n = 8$) triggered with TLR9 agonist (CpG-C) incubated under hypoxic or normoxic conditions for 16 h. (**D**) CXCL4 mRNA expression level represented as normalized fold change (FC) in healthy participants ($n = 8$) and SSc ($n = 8$) pDCs, incubated in hypoxic or atmospheric condition, that were challenged with TLR9 (CpG-C) for 16 h. Bars show mean (S.E.M.). Grey and white edges respectively represent hypoxic and atmospheric conditions. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, using Mann–Whitney test. AOL: atmospheric oxygen level; CXCL4: chemokine (C-X-C motif) ligand 4; pDC: plasmacytoid dendritic cell; TLR, toll-like receptor.

Fig. 2 Hypoxia is an important factor for CXCL4 production but not for other cytokines



Measurement of IFN-α (**A**), TNF-α (**B**), IL-6 (**C**) and IL-8 (**D**) in the supernatant of healthy pDCs ($n = 17$ IFN-α, $n = 15$ TNF-α, $n = 20$ IL-6, $n = 15$ IL-8) cultured in AOL or hypoxic conditions and co-stimulated with TLR9 agonist CpG-C and SSc pDCs ($n = 17$ IFN-α, $n = 7$ TNF-α, $n = 9$ IL-6, $n = 8$ IL-8) for 16 h. Bars show mean (S.E.M.). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ using Mann–Whitney test. AOL: atmospheric oxygen level; CXCL4: chemokine (C-X-C motif) ligand 4; pDC: plasmacytoid dendritic cell.

Hypoxia and TLR9 specifically regulate the production of CXCL4 by pDCs but not other cytokines

To examine the specificity of production of CXCL4 in response to hypoxia and TLR9 stimulation, we also assessed the production of other cytokines relevant to SSc such as IFN-α, TNF-α, IL-6 and IL-8 for pDCs (Fig. 2A–D). TLR stimulation alone was sufficient to significantly increase the production of these cytokines (IFN-α: $P < 0.0001$; TNF-α: $P = 0.0051$; IL-6: $P < 0.0001$; IL-8: $P < 0.0001$) by HC pDCs, while hypoxia alone and co-exposure to hypoxia with TLR9 had no further effect on the production of these cytokines (Fig. 2A–D). Hence the concerted action of hypoxia and endosomal TLR9 signalling was specific in inducing CXCL4 production.

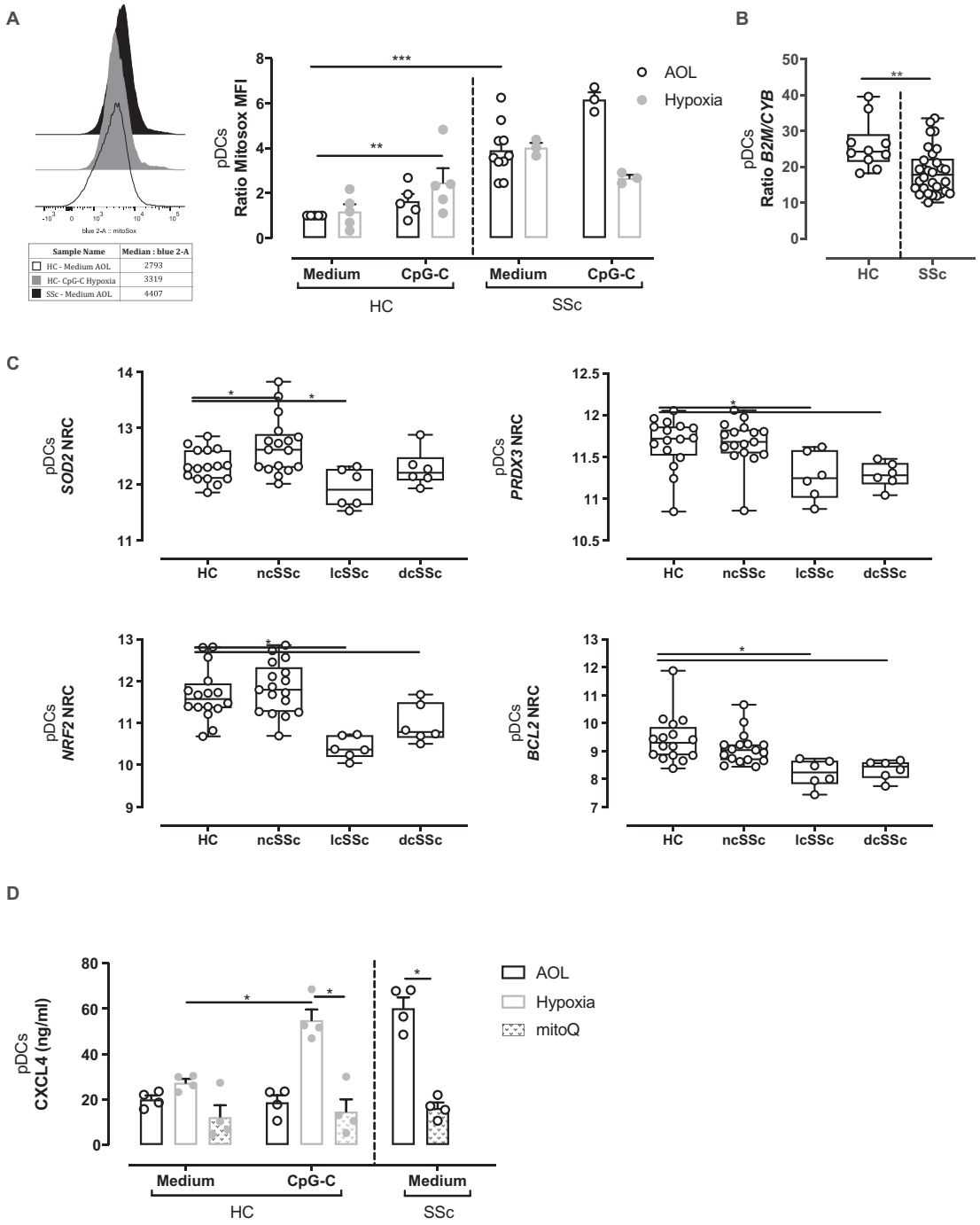
Increased mtROS production in SSc patient pDCs is associated with instability of mtDNA and is essential for CXCL4 production

It has previously been demonstrated that hypoxia increases the production of reactive oxygen species (ROS) from mitochondria [24]. We identified an increased basal level of mtROS in SSc pDCs ($P = 0.0007$) (Fig. 3A). Increased production of mtROS may result in the progressive destruction of mitochondrial DNA (mtDNA) and consequent loss of mitochondrial function [25]. We found a significant overall reduction in the mtDNA copy number, expressed as the ratio *B2M/CYB*, in SSc patient pDCs ($P = 0.0011$) (Fig. 3B). In addition, we looked at the expression of genes that act as antioxidants to clear mtROS, such as *SOD2* (superoxide dismutase 2), *PRDX3* (thoredoxin-dependent peroxide reductase), *NRF2* (nuclear factor erythroid-derived 2, which regulates antioxidant gene transcription) and *BCL2* (B cell lymphoma 2) in different subsets of SSc. Accordingly to the literature [17–19], patients were classified as non-cutaneous SSc (ncSSc), limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) and schematically represented in Supplementary Fig. 1, available at *Rheumatology* online. We found a significant reduction in the expression of *NRF2* in lcSSc ($P = 0.0001$) and dcSSc ($P = 0.043$), *PRDX3* in lcSSc ($P = 0.0325$) and dcSSc ($P = 0.0356$), *BCL2* in lcSSc ($P = 0.0087$) and dcSSc ($P = 0.0197$), and *SOD2* in lcSSc ($P = 0.0402$) (Fig. 3C). In HC pDCs we observed an increased production of mtROS after exposure to hypoxia and TLR9 ($P = 0.0079$), which mimicked the high levels of mtROS observed in SSc pDCs ($P = 0.0007$) (Fig. 3A). In order to assess the role of mtROS in CXCL4 production, HC pDCs were incubated in the presence of mitoQ, a specific mtROS inhibitor. We observed a reduction of the CXCL4 production in cells co-exposed to hypoxia and TLR agonist in the presence of mitoQ, demonstrating an important role of mtROS in CXCL4 production in HC pDCs ($P = 0.029$) (Fig. 3D). Also, in pDCs from SSc patients, mitoQ reduced the production of CXCL4 ($P = 0.028$). Furthermore, mitoQ did not affect cell viability (Supplementary Fig. 2, available at *Rheumatology* online).

HIF-2α expression is increased in SSc pDCs and is essential for CXCL4 production

The HIFs are stabilized upon exposure to hypoxia, TLR triggering and/or increased production of mtROS [26]. Therefore, we first measured the expression of HIFs in pDCs from SSc patients, where we found an increased expression of *HIF-2α* mRNA ($P < 0.0001$) (Fig. 4A). We observed no differences in *HIF-1α* RNA level in any of the disease subsets (Fig. 4B). To confirm this observation, we quantified HIF-2α and HIF-1α protein in SSc pDCs, where we confirmed the higher presence of HIF-2α in patients ($P = 0.014$), suggesting that hypoxia responses in SSc patient pDCs are due to HIF-2α rather than HIF-1α (Fig. 4C). We observed no expression of HIF-3α in pDCs (data not shown). Next, we looked at the co-expression of HIFs and pDCs ($BDCA4^{+}$) in the skin of SSc patients and HCs. We found significant increase of HIF-2α $BDCA4^{+}$ cells ($P = 0.037$) and decrease of HIF-1α/$BDCA4^{+}$ ($P = 0.041$) cells in the skin of SSc patients when compared with HCs (Fig. 4D, Supplementary Fig. 3, available at *Rheumatology* online). In addition, we found an increase of HIF-2α and

Fig. 3 mtROS are essential for CXCL4 production in plasmacytoid dendritic cells



(**A**) On the left, representative plot of the MFI of mitoSOX dye in pDCs from HCs and SSc patients exposed to either AOL or hypoxia and TLR9 agonist CpG-C for 16 h; on the right, FACS analysis of the MFI of mitoSOX dye for mtROS in pDCs from five HCs and 10 SSc patients exposed to either AOL or hypoxia and TLR9 agonist CpG-C for 16 h. (**B**) Quantification of mitochondrial DNA copies expressed as ratio between B2M and CYB in freshly isolated pDCs of HCs and SSc patients. (**C**) RNAseq analysis of SOD2, PRDX3, NRF2 and BCL2 in pDCs from HCs and SSc patients. (**D**) ELISA measurement of CXCL4 in pDCs (four HCs and four SSc patients) exposed to either AOL or hypoxia and

decrease of HIF-1α in the epidermis ($P = 0.0079$ and $P = 0.04$ respectively). Similarly, we observed an increment of HIF-2α in the dermis ($P = 0.013$). We observed no statistical difference in HIF-1α of SSc skin compared with HC skin (Supplementary Fig. 3, available at *Rheumatology* online). Next, we quantified the protein level of HIF-2α and HIF-1α by western blot in pDCs from HCs and we observed that both HIF-2α and HIF-1α were increased after exposure to hypoxia (Fig. 4E).

In order to confirm the link between mtROS and HIFs, we measured the RNA expression of *HIF-2α* and *HIF-1α* in pDCs from HCs and SSc patients co-exposed to hypoxia and TLR9, both in the presence of mtROS inhibitor mitoQ. We observed reduction of *HIF-2α* RNA expression in pDCs from HCs exposed to hypoxia and TLR9 as in pDCs from SSc patients when mitoQ was added to the mediums ($P = 0.029$). Further, we observed no differences in *HIF-1α* RNA level (Fig. 4F).

To further assess the importance of HIF-2α in CXCL4 production, specific inhibitors for HIF-2α or HIF-1α were added to the culture. Inhibition of HIF-2α, but not HIF-1α, in both HC ($P = 0.007$) and SSc ($P = 0.038$) pDCs resulted in a reduction of CXCL4 production (Fig. 4G), with no effect on cell viability (Supplementary Fig. 2, available at *Rheumatology* online).

In order to confirm the obligatory role of HIF-2α in CXCL4 production, HIF-2α or HIF-1α was silenced in HC pDCs with subsequent exposure to hypoxia and TLR9 agonist. We observed that HIF-2α silencing in pDCs led to a significant reduction in CXCL4 production ($P = 0.0022$), while HIF-1α silencing did not (Fig. 4H). The efficiency of the silencing was 54.7% for HIF-2α and 58.8% for HIF-1α (Supplementary Fig. 4, available at *Rheumatology* online).

Hypoxia is fundamental for the production of CXCL4 in $CD34^+$ derived pDCs

Umbilical cord (uc) $CD34^+$ derived pDCs were differentiated for 14 days either in AOL or in hypoxia, and after exposed to TLR9 ligand CpG-C for 16 h (Fig. 5A). We screened the cytokine production of the ucCD34 derived pDCs that were differentiated in AOL and observed that they were able to produce IFN-α ($P = 0.0093$), TNF-α ($P = 0.081$) and IL-6 ($P = 0.04$) but no CXCL4 after TLR9 exposure (Fig. 5B). Interestingly, ucCD34 derived pDCs differentiated under hypoxia (5%) were able to produce CXCL4 (Fig. 5C). These results indicate that hypoxia plays a fundamental role in not only the production but also the ability to produce CXCL4 by pDCs.

Discussion

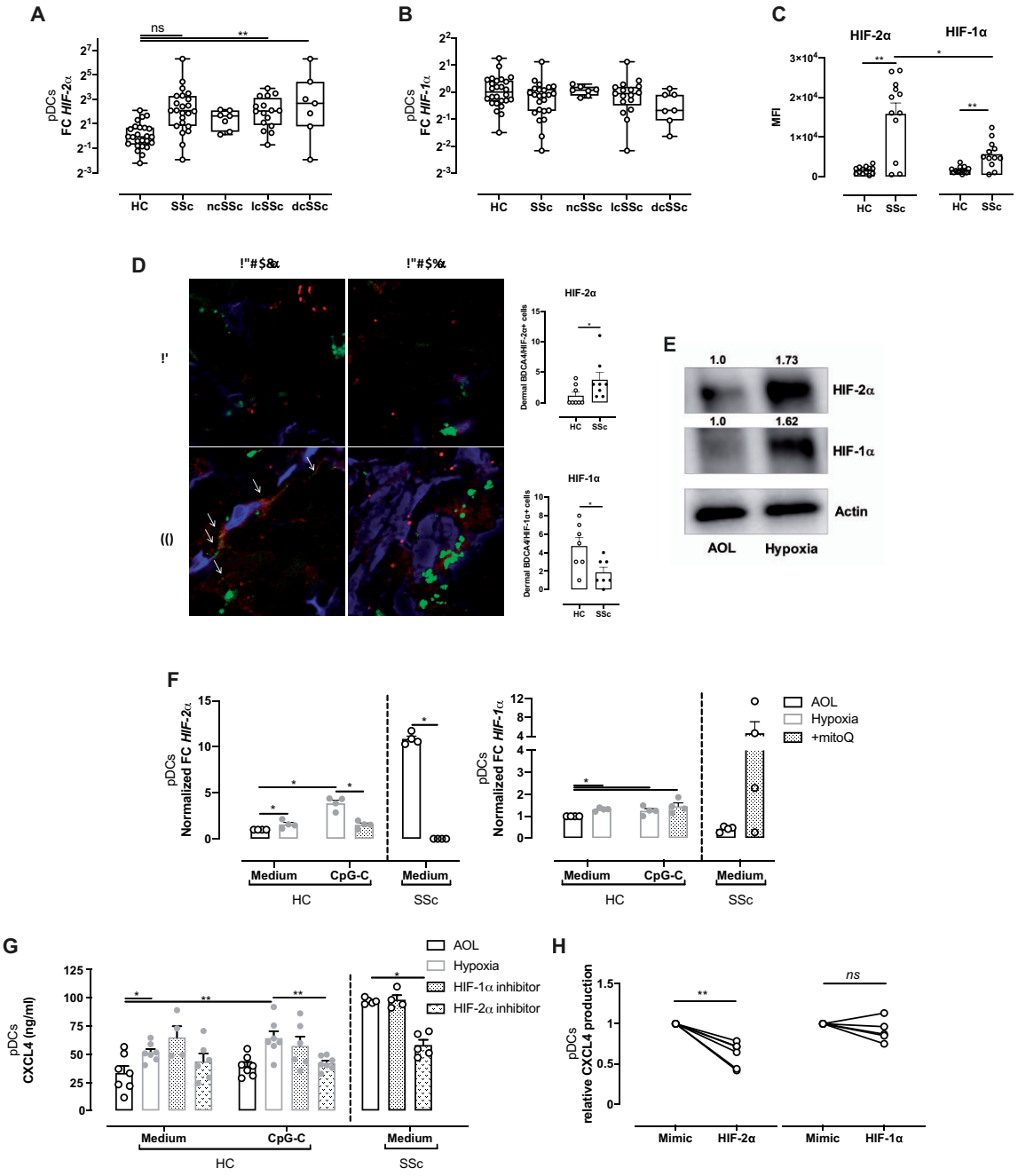
We identified hypoxia as a key factor responsible for the production of CXCL4 by pDCs. Hypoxia is often considered a driving force behind many pathological hallmarks of SSc [27]. Our results suggest that the hypoxic environment makes pDCs more prone to respond to viral or bacterial infection or endogenous ligands via TLR signalling, culminating in high CXCL4 production and acceleration of disease progression. Recently our group observed that CXCL4 promotes genetic imprinting of DCs making them more prone to TLR stimulation and changes DCs in pro-fibrotic cells via direct matrix production as well as indirectly by inducing myofibroblast transition [28, 29]. Together with the observations presented here, this suggests that CXCL4 plays an essential role in the vicious circle of inflammation, hypoxia and fibrosis as observed in SSc. Hence, for the first time we provide a link between hypoxia and disease progression which is orchestrated by CXCL4, an observation with great therapeutic relevance.

Hypoxia could be seen as a generalized phenomenon in SSc patients, due to Raynaud's phenomenon and the vasculopathy responsible for the typical vascular alteration of SSc [30]. Therefore, we speculate that this vasculopathy is responsible for a condition of chronic hypoxia in SSc. Also, the events responsible for Raynaud's phenomenon could take place in organs different from the skin, affecting the circulatory cells. Furthermore, it has been shown that hypoxia leads to oxidative stress via excessive production of ROS in dendritic cells [31]. Recently it has been shown that mtROS, rather than other sources of ROS, are responsible for the production of inflammatory cytokines [32, 33]. In this study, we found an increased basal level of mtROS being produced by pDCs isolated from SSc patients. Increased production of mtROS in the cell can lead to alteration in mitochondria such as a decrease in mtDNA copy numbers [34]. In fact, we observed a reduction in mtDNA copy numbers in pDCs from SSc patients consistent with their exposure to excessive mtROS production. To further explore the involvement of mtROS in SSc pDCs, we investigated the expression of genes that play a crucial role in maintaining cellular redox homeostasis in the mitochondria, such as *SOD2* or *NRF2*. The expression of these genes was significantly reduced in SSc pDCs, especially in patients with fibrotic involvement (lcSSc, dcSSc). Targeting one of these genes could restore mtROS homeostasis in SSc patients. For

Fig. 3 Continued

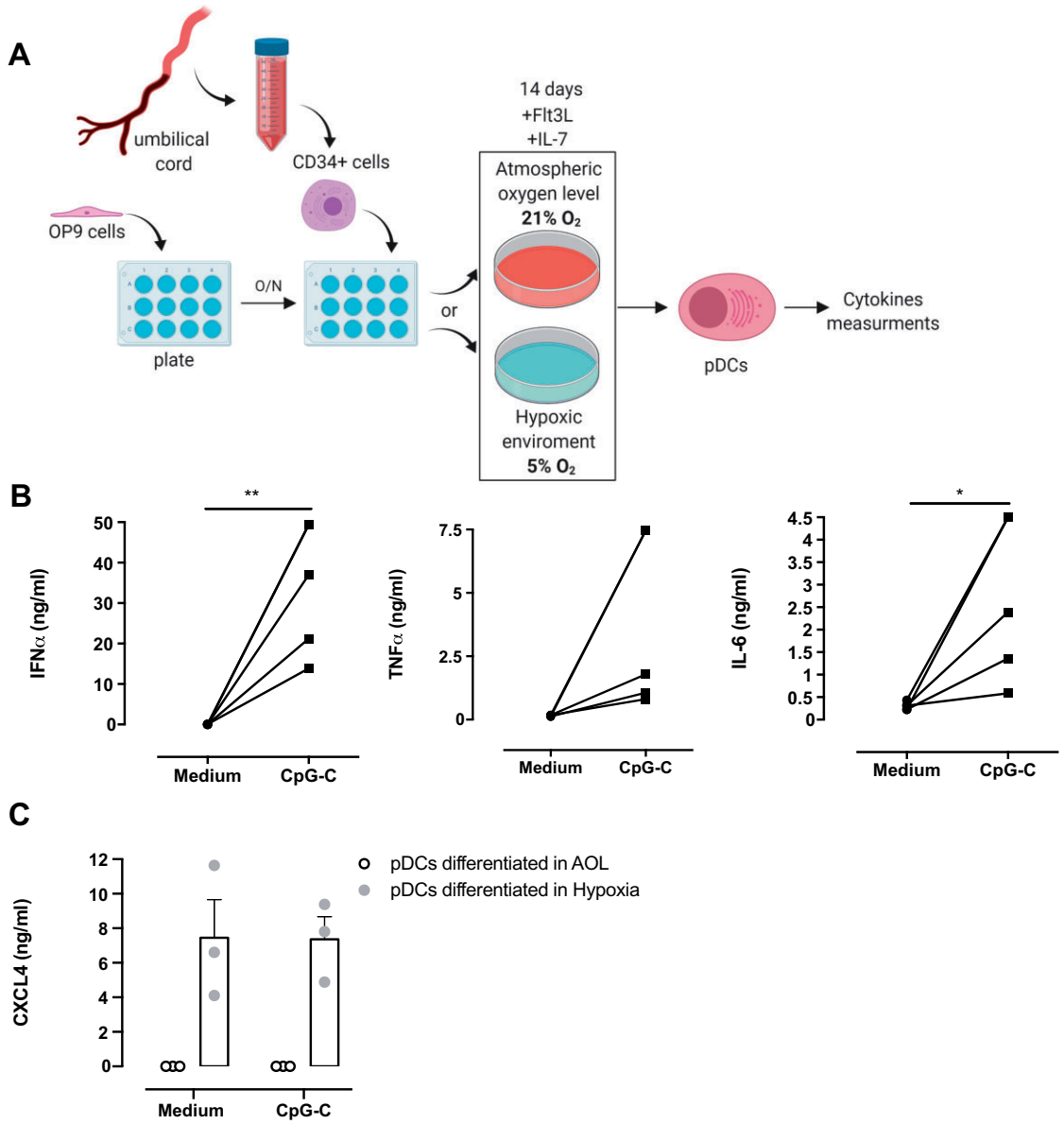
TLR9 agonist CpG-C ± MitoQ for 16 h. Bars show mean (S.E.M.). Grey and white edges respectively represent hypoxic and atmospheric conditions. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, using ordinary one-way analysis of variance (C) and Mann–Whitney test (A, B, D, E). AOL: atmospheric oxygen level; BCL2: B cell lymphoma 2; CXCL4: chemokine (C-X-C motif) ligand 4; dcSSc: diffuse cutaneous SSc; HC: healthy control; lcSSc: limited cutaneous SSc; MFI: mean fluorescence intensity; mtROS: mitochondrial reactive oxygen species; ncSSc: non-cutaneous SSc; NRC: normalized read count; NRF2: nuclear factor erythroid-derived 2; pDC: plasmacytoid dendritic cell; PRDX3: thoredoxin-dependent peroxide reductase; SOD2: superoxide dismutase 2; TLR, toll-like receptor.

Fig. 4 HIF-2α is essential for the production of CXCL4 by plasmacytoid dendritic cells



(**A**, **B**) qPCR quantification of HIF-2α (**A**) and HIF-1α (**B**) genes in freshly isolated pDCs from HCs and SSc patients. (**C**) Quantification of HIF-2α and HIF-1α protein expression as MFI in freshly isolated pDCs from 13 HCs and 12 SSc patients. (**D**) Co-localization of HIF-2α or HIF-1α and BDCA4 in skin from SSc and HCs. Representative images are shown on the left. On the right quantification of double staining from four SSc patients and four HCs. (**E**) Western blot quantification of HIF-2α and HIF-1α in pDCs from three poured donors, cultured either in AOL or hypoxia for 16 h. Numbers above bands represent relative protein quantification normalized to actin. (**F**) qPCR quantification of *HIF-2α* and *HIF-1α* genes in pDCs (four HCs and four SSc patients) exposed to either AOL or hypoxia and TLR9 agonist CpG-C ± MitoQ for 16 h. (**G**) ELISA measurement of CXCL4 in pDCs from seven HCs and five SSc patients cultured in atmospheric or hypoxic condition ± CpG-C with the addition of either HIF-1α inhibitor or HIF-2α inhibitor to the culture medium for 16 h. (**H**) Measurement of CXCL4 production in pDCs from five HCs exposed to hypoxia and CpG-C and transfected with a non-targeting (mimic) siRNA or either HIF-2α or HIF-1α siRNA, expressed as relative

Fig. 5 Hypoxia is the fundamental factor to enable CXCL4 production by plasmacytoid dendritic cells

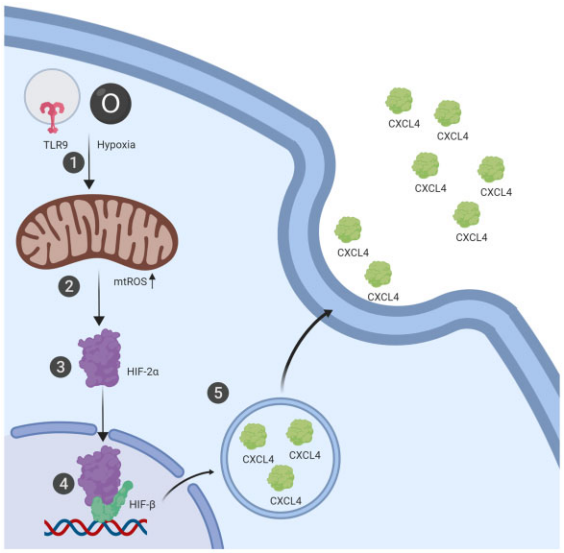


(**A**) Schematic representation of the experimental layout of the ucCD34 derived pDCs, created with Biorender. (**B**) Luminex measurement of IL-6, TNF-α and IFN-α production in ucCD34 derived pDCs differentiated in AOL and stimulated with TLR9 ligand CpG-C. (**C**) CXCL4 ELISA measurement in ucCD34 derived pDCs, differentiated either in AOL or hypoxia and subsequently exposed to TLR9 ligand CpG-C either in AOL or hypoxia. Bars show mean (S.E.M.). Grey and white edges respectively represent hypoxic and atmospheric conditions. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ using unpaired Mann–Whitney test. AOL: atmospheric oxygen level; CXCL4: chemokine (C-X-C motif) ligand 4; MFI: mean fluorescent intensity; pDC: plasmacytoid dendritic cell; TLR: toll-like receptor; uc: umbilical cord.

Fig. 4 Continued

production compared with mimic. All data represent mean (S.E.M.). Grey edges represent hypoxic condition and white bars AOL. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ using unpaired Kruskal–Wallis test (A, B) and Mann–Whitney test (C, D, E, F, G). AOL: atmospheric oxygen level; CXCL4: chemokine (C-X-C motif) ligand 4; dcSSc: diffuse cutaneous SSc; HC: healthy control; HIF: hypoxia-inducible factor; lcSSc: limited cutaneous SSc; ncSSc: non-cutaneous SSc; MFI: mean fluorescence intensity; pDC: plasmacytoid dendritic cell.

FIG. 6 Model of CXCL4 production upon exposure to hypoxia and TLR9 in plasmacytoid dendritic cells



The co-exposure to hypoxia and TLR9 agonist (**1**) leads to increase of mtROS production (**2**). mtROS stabilizes HIF-2α (**3**), which can dimerize with HIF-β and induce CXCL4 expression (**4**). CXCL4 is then transported to the plasma membrane and released (**5**). Created with Biorender. CXCL4: chemokine (C-X-C motif) ligand 4; HIF: hypoxia-inducible factor; mtROS: mitochondrial reactive oxygen species; TLR: toll-like receptor.

instance, pharmacological activation of NRF2 with the naturally occurring NRF2 activator sulforaphone could be a valid approach [35]. Interestingly, blocking mtROS with a specific mtROS inhibitor (mitoQ) significantly reduced the production of CXCL4 in pDCs.

HIFs are transcriptional activators that function as master regulators of oxygen homeostasis in the cell and are capable of influencing cell metabolism, vascular neogenesis, metabolic changes, cell proliferation and survival [36]. HIFs are stabilized upon exposure to hypoxia, TLR triggering, and production of mtROS [37, 38].

Our data show an increased expression of HIF-2α in circulatory SSc pDCs, while no significant changes were observed in the expression of HIF-1α. Moreover, we found an increased number of cells expressing HIF-2α in pDCs (BDCA4^{+}) in the skin of SSc patients when compared with HCs, while cells expressing HIF-1α in pDCs were decreased in SSc skin. Interestingly, we found that blocking mtROS with mitoQ specifically blocks the expression of *HIF-2α* but not *HIF-1α* in pDCs. Furthermore, we found that in stimulated HC pDCs and pDCs of SSc patients, inhibiting as well as silencing *HIF-2α* but not HIF-1α diminished the production of CXCL4 significantly. This observation is in line with the findings of Ryu *et al.* where, although not showing the mechanism, fibroblast like synoviocytes from rheumatoid arthritis patients overexpressing HIF-2α showed that the production of different pro-inflammatory mediators, including CXCL4, was significantly increased at the RNA level [39]. Furthermore, we observed that ucCD34 derived pDCs differentiated under AOL are able to produce cytokines such as IL-6, TNF-α and IFN-α, but not CXCL4. Interestingly, ucCD34 derived pDCs differentiated under hypoxia (5% O_2) were able to produce CXCL4, further highlighting the fundamental role of hypoxia in CXCL4 production.

Taken together, as CXCL4 plays an important role in inflammation and fibrosis, prevention of its excessive production and/or inhibition of its function provides an attractive target for a plethora of medical conditions. As clinical grade monoclonal antibodies against CXCL4 are not yet available, an alternative way to block the effects of CXCL4 is the prevention of its release.

Our study identifies hypoxia and TLR9 stimulation as a crucial factor driving CXCL4 release via increased production of mtROS leading to HIF-2α stabilization, all thus potentially interesting molecular processes for therapeutic targeting (Fig. 6). Interestingly, a few successful clinical trials have already been performed by using MitoQ to block mtROS production in ageing [40]. Also a HIF-2α antagonist (PT2385) was recently successfully used for a phase 1 clinical trial for the treatment of clear cell renal carcinoma [41]. Further studies are needed to assess the potential clinical relevance of blocking the detrimental effects of hypoxia by interference with the responsible downstream molecular processes.

In conclusion, our study reveals new insight into the pathogenesis of SSc in identifying metabolic changes in SSc pDCs, including increase of mtROS and alterations in mitochondria. This study is the first to show significant changes in the expression of HIFs in SSc patients, where HIF-2α plays an essential role.

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All the authors listed have made substantial, direct, and intellectual contribution to the work.

A.O. and W.M. had full access to the data and take responsibility for the accuracy of the performed analysis and the integrity of the data. A.O., T.R.D.J.R. and W.M. were involved in the design of the study. Execution and analysis of the results was performed by A.O. A.O., M.Z., T.C., N.V., S.S.-C., A.J.A., E.C., M.v.dK., E.M.-G., M.R., S.G.P., R.G.T., C.P.J.B. and R.G.K.W. were involved in performing experiments. A.P. was involved in analysing the gene array seq. A.O. and M.C. were involved in selection of the patients. J.T., E.T., J.vL., L.B., F.B.-M. and T.R.D.J.R. were involved in inclusion of SSc patients. All authors approved the final version after being involved in drafting and revising the article for important intellectual content.

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Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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