RMD Open
Rheumatic & Musculoskeletal Diseases

ORIGINAL RESEARCH

Circulating monocytes from systemic sclerosis patients with progressive interstitial lung disease preferentially express M2 phenotype markers: in vitro and ex vivo study

Vanessa Smith ,[1,2,3] Stefano Soldano,[4] Rosanna Campitiello,[4,5] Paola Montagna,[4] Andrea Cere,[4] Elvis Hysa,[4,5] Tamara Vojinovic,[4,6] Carmen Pizzorni,[4,6] Sabrina Paolino,[4,6] Alberto Sulli,[4,6] Emanuele Gotelli ,[4,6] Maurizio Cutolo [4,6]

To cite: Smith V, Soldano S, Campitiello R, *et al*. Circulating monocytes from systemic sclerosis patients with progressive interstitial lung disease preferentially express M2 phenotype markers: in vitro and ex vivo study. *RMD Open* 2025;**11**:e006135. doi:10.1136/rmdopen-2025-006135

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/rmdopen-2025-006135).

VS, SS and RC contributed equally.

VS, SS and RC are joint first authors.
EG and MC are joint senior authors.

Received 16 July 2025
Accepted 25 November 2025

For numbered affiliations see end of article.

Correspondence to
Professor Maurizio Cutolo;
mcutolo@unige.it

ABSTRACT

Objective To characterise phenotype and functional profibrotic M2 markers in circulating monocytes and cultured monocyte-derived macrophages (MDMs) from systemic sclerosis (SSc) patients (pts) with progressive interstitial lung disease (ILD) (prog-ILD), non-progressive ILD (no-prog-ILD) and without ILD (no-ILD).

Methods Fifty-five SSc pts and 20 age-matched healthy controls were evaluated. In 36 SSc pts, circulating monocytes expressing toll-like receptor-4 (TLR4, M1 marker) and M2 phenotype markers (CD204, CD206, CD163) were detected by flow cytometry. Moreover, MDMs of 29 SSc pts were analysed by quantitative real-time PCR and Western blotting for gene expression and protein synthesis with regard to the production of profibrotic mediators: transforming growth factor-ß1 (TGFß1), Mer tyrosine kinase receptor (MerTK) and arginase-1 (ARG1). Interleukin-6 and 4 (IL6, IL4), as well as C-C motif chemokine ligand 2 and 18 (CCL2/MCP1 and CCL18) from culture medium, were evaluated by enzyme-linked immunosorbent assay (ELISA).

Results Prog-ILD SSc pts showed a higher percentage of $TLR4^{+}CD204^{+}CD206^{+}CD163^{+}$ monocytes versus no-prog-ILD, and significantly higher compared with no-ILD SSc pts.

Interestingly, MDMs from prog-ILD SSc pts showed a significantly higher gene expression and protein synthesis of TGFβ1, and a significantly higher protein synthesis of MerTK, CD206, IL4 and CCL18 compared with no-prog-ILD SSc pts. Finally, gene expression and protein synthesis of TGFβ1, TLR4, CD206, CD163, ARG1, MerTK and IL6 were significantly increased in prog-ILD SSc versus no-ILD SSc MDMs.

Conclusions Cultured MDMs from circulating monocytes in SSc pts with prog-ILD show markedly increased profibrotic biomarkers and mediator expression, indicating an enhanced fibrotic phenotype compared to non-prog and no-ILD SSc pts.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Monocytes and macrophages, as innate immune cells, are recognised to contribute to the fibrotic process in systemic sclerosis (SSc). Hybrid M1/M2 circulating monocyte/macrophage populations are found increased in SSc patients (pts), particularly those with Interstitial Lung Disease (ILD), and have been linked to disease severity and progression.

WHAT THIS STUDY ADDS

⇒ The study provides an in-depth characterisation of circulating hybrid $TLR4^{+}$M2 monocytes and cultured monocyte-derived macrophages (MDMs) in SSc pts examining both their phenotypic and functional profibrotic characteristics. It includes comparisons across different stages of lung involvement, namely progressive ILD and non-progressive ILD, as well as absence of ILD and healthy controls, enabling comprehensive and meaningful insights. Therefore, the study highlights for the first time a prominent profibrotic attitude of cultured MDMs obtained from circulating monocytes of SSc pts with progressive ILD compared with MDMs obtained from SSc pts without ILD.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE, OR POLICY

⇒ This study may provide new insights into the immunopathogenesis of ILD in SSc by identifying specific phenotypic and functional M2 markers associated with disease progression. These insights may contribute to developing novel biomarkers for monitoring ILD severity and progression, and potentially guide targeted therapeutic strategies.

BACKGROUND

Systemic sclerosis (SSc) is an autoimmune connective tissue disorder involving a

complex interplay of genetic predisposition, epigenetic modifications and an altered immune system response.[1 2] This interplay initiates a cascade of pathological events beginning with a progressive microvascular damage, an abnormal activation of innate and adaptive immunity, as well as a fibroblast-to-myofibroblast transition, leading to tissue fibrosis.[1–4] Of note, SSc can affect multiple organs, primarily skin, heart, kidneys and lung.[5]

Interstitial lung disease (ILD) stands out as one of the most severe complications of SSc, and it is characterised by a progressive fibrosis of lung parenchyma, detectable through high-resolution CT (HRCT) and pulmonary function tests, affecting approximately 40% of patients.[6–10] Notably, the 2022 American Thoracic Society guidelines define progressive SSc-ILD based on clinical symptoms (worsening dyspnoea or cough), functional decline (forced vital capacity—(FVC) >5% or diffusing capacity of the lungs for carbon monoxide—(DLCO) >10%, in a year), and radiological progression observed within 12 months.[11 12]

Mounting evidence, focused on innate immune system components, highlighted monocytes and macrophages as potential mediators in driving the fibrotic process in SSc.[2 13 14]

Indeed, circulating inflammatory cells, primarily monocytes and T lymphocytes, are recruited and, along with tissue resident macrophages, they initiate the production of profibrotic cytokines, such as transforming growth factor-β1 (TGFβ1) and interleukin-10 (IL10).[15]

Macrophages can sense their microenvironment and dynamically adapt their behaviour through a phenotypic plasticity to perform their distinct functions in immune regulation and tissue maintenance.[16] Therefore, macrophages can polarise into five morphological and functional states, ranging from a classically (M1) phenotype with a pro-inflammatory activity to different alternatively activated (M2) macrophage subsets, according to microenvironmental stimuli and identified as M2a (or profibrotic), M2b (or regulatory), M2c (or deactivated) and M2d (or tumour associated), with different characteristics.[17–19] Although this dichotomous model of macrophage polarisation is mainly applied in in vitro models, it does not capture the full complexity of macrophage functions, which stem from their inherent plasticity. As such, M1 and M2 represent two extremes of a broader and dynamic polarisation continuum.[19] Over the years, this has led to a more refined definition of macrophage phenotypes in several autoimmune diseases (ie, rheumatoid arthritis and SSc), no longer based solely on surface markers but also incorporating transcription factors, cytokines, chemokines and gene signatures or transcriptomic profiles.[20–22] Of note, peripheral blood monocytes play a pivotal role also in the pathogenesis of inflammatory connective tissue diseases, such as rheumatoid arthritis, where $CD14^{+}$ monocytes are metabolically reprogrammed to sustain pro-inflammatory responses, with STAT3 identified as a key molecular mediator.[23]

Profibrotic M2 macrophages are phenotypically characterised by the expression of specific cell surface markers including the mannose receptor type C (CD206), macrophage and haemoglobin scavenger receptors (CD204 and CD163, respectively), and functional markers like arginase-1 (ARG1) and MER proto-oncogene receptor tyrosine kinase (MerTK). These macrophages are central in wound healing and fibrosis, primarily secreting anti-inflammatory and profibrotic molecules, including IL10 and TGFβ1.[13 24–26]

A growing body of evidence has documented the presence of profibrotic M2 macrophages in the affected tissues of SSc patients, deriving from circulating monocytes and resident macrophages activated by local mediators.[27] Interestingly, a significantly higher percentage of circulating cells belonging to the monocyte/macrophage lineage and showing hybrid M1/M2 phenotypes characterised by the co-expression of M2 markers (such as CD204, CD163, CD206) and M1 markers (such as toll-like receptor-4—TLR4, CD80 and CD86) were identified in SSc patients compared with healthy subjects.[28] In SSc patients, these hybrid phenotypes are particularly associated with the presence of ILD and elevated pulmonary artery hypertension (PAH), as well as the positivity for anti-topoisomerase antibody, a known lung involvement predictor.[28 29] Furthermore, a recent study identified high concentrations of macrophages expressing TLR4 and M2 markers in lung biopsies obtained from SSc patients undergoing lung transplantation for severe ILD, highlighting the potential dynamic role of peripheral blood monocytes in driving fibrosis after homing to the lungs.[30]

The present study aims to characterise phenotype and functional M2 markers in circulating monocytes and cultured monocyte-derived macrophages (MDMs) obtained from SSc patients with progressive ILD compared with SSc patients with non-progressive ILD and to explore the profibrotic biomarkers associated with ILD progression.

MATERIALS AND METHODS

Enrolment of SSc patients and healthy controls and blood collection

Fifty-five SSc patients were enrolled under the care of the Division of Clinical Rheumatology at the University of Genova, and Department of Rheumatology at the University of Ghent. The study was conducted after receiving approval from the Ethical Committee of the Ospedale Policlinico San Martino (237REG2015, amendment number: 002–28/05/2018) and obtaining written informed consent from all participants.

The enrolled SSc patients met 2013 ACR/EULAR classification criteria for SSc.[31] Instrumental examinations were part of their regular follow-up.[32] The presence of ILD was confirmed through routinely performed HRCT and lung function tests.[33 34]

In our cohort of patients, the definition of ILD followed the 2022 American Thoracic Society guidelines

Table 1 Demographic and clinical data of systemic sclerosis patients included in flow cytometry analysis

	SSc progressive ILD (n 8)	SSc non-progressive ILD (n 14)	SSc no-ILD (n 14)
Age (years, mean±SD)	61±13.6	66±14	66.1±21.6
Gender (female/male)	6/2	12/2	13/1
Disease duration (years, mean±SD)	7.3±4.4	7.6±6	5.6±4.4
People who smoke/people who do not smoke	1 (12)	1 (7)	0 (0)
Antibody profile n (%)			
Anti-centromere	0 (0)	4 (29)	7 (50)
Anti-Scl70	6 (75)	7 (50)	1 (7)
Anti-RNA polymerase III	0 (0)	0 (0)	0 (0)
Only ANA positive	0 (0)	0 (0)	1 (7)
Other SSc-associated antibodies*	2 (25)	6 (3)	5 (36)
ANA negative	0 (0)	0 (0)	0 (0)
Clinical manifestations n (%)			
Skin involvement (lcSSc/dcSSc)	3/5	7/7	10/4
RP	8 (100)	14 (100)	14 (100)
DUs	5 (62)	9 (64)	5 (36)
PAH	0 (0)	1 (7)	0 (0)
ILD	8 (100)	14 (100)	0 (0)
GI involvement	7 (87)	9 (64)	7 (50)
Kidney involvement	2 (25)	1 (7)	0 (0)
Pulmonary function tests (reference percentage, mean±SD)			
FVC	64±34	93±27.8	91.8±28.5
DLCO/VA	67±32	78.5±18.5	77±18.7
Radiographic pattern n (%)			
NSIP	8 (100)	14 (100)	–
UIP	0 (0)	0 (0)	–
Others	0 (0)	0 (0)	
Therapy n° (%)			
	MMF=6 (75) MTX=3 (37) RTX=0 (0) AZA=0 CYC=1 (12) HCQ=0 Ca-ant=3 (37) ACEi=5 (62) PDE5i=0 (0) ERA=2 (25) Prostanoids=8 (100) Selexipag=0 (0) Riociguat=0 (0) Nintedanib=0 (0)	MMF=5 (35) MTX=1 (8) RTX=1 (7) AZA=0 (0) CYC=0 (0) HCQ=0 (0) Ca-ant=1 (8) ACEi=6 (42) PDE5i=0 (0) ERA=3 (21) Prostanoids=14 (100) Selexipag=0 (0) Riociguat=0 (0) Nintedanib=0 (0)	MMF=3 (21) MTX=4 (29) RTX=0 (0) AZA=0 CYC=0 (0) HCQ=0 (0) Ca-ant=5 (36) ACEi=7 (50) PDE5i=0 (0) ERA=2 (14) Prostanoids=14 (100) Selexipag=0 (0) Riociguat=0 (0) Nintedanib=0 (0)

Description of age, gender, disease duration, auto-antibody profile, organ involvement, pulmonary functional tests and therapy of SSc patients included in the flow cytometry analysis.
*Other SSc-associated antibodies include anti-U3 RNP, anti-PmScl75, anti-PmScl100, anti-NOR90, anti-Th/To, anti-Ku and anti-SSA.
†
ACEi, angiotensin-converting enzyme inhibitor; ANA, anti-nuclear antibodies; AZA, azathioprine; Ca-ant, calcium channel antagonist; CYC, cyclophosphamide; dcSSc, diffuse cutaneous systemic sclerosis; DLCO, diffusing capacity for carbon monoxide; DUs, digital ulcers; ENA, extractable nuclear antigen antibodies; ERA, endothelin receptor antagonist; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; GI, gastrointestinal; HCQ, hydroxychloroquine; ILD, interstitial lung disease; lcSSc, limited cutaneous systemic sclerosis; MMF, mycophenolate mofetil; mRSS, modified Rodnan Skin Score; MSK, musculoskeletal; MTX, methotrexate; NSIP, Nonspecific Interstitial Pneumonia; PAH, pulmonary arterial hypertension; PDE5i, phosphodiesterase type 5 inhibitor; RP, Raynaud phenomenon; RTX, rituximab; Scl70, anti-topoisomerase I; UIP, Usual Interstitial Pneumonia.

Table 2 Demographic and clinical data of systemic sclerosis patients included in in vitro experiments

	SSc progressive ILD (13)	SSc non-progressive ILD (10)	SSc no ILD (6)
Age (years, mean±SD)	61.8±15.7	67±9	66.7±17
Gender (female/male)	10/3	7/3	6/0
Disease duration (years, mean±SD)	13.9±8.5	8.8±7.4	7.8±5.1
Smokers n (%)	4 (36)	2 (20)	1 (8)
Antibody profile* n (%)			
Anti-centromere	1 (8)	3 (30)	1 (16)
Anti-Scl70	6 (50)	1 (10)	2 (33)
Anti-RNA polymerase III	1 (8)	1 (10)	1 (16)
Only ANA positive	4 (33)	4 (40)	2 (33)
Other SSc-associated antibodies†	3 (25)	0 (0)	0 (0)
ANA negative	0 (0)	2 (20)	0 (0)
Clinical manifestations n (%)			
Skin involvement (lcSSc/dcSSc)	3/9	4/6	1/5
RP	12(100)	10(100)	6 (100)
DUs	7 (58)	5 (50)	3 (50)
PAH	0 (0)	1 (10)	0 (0)
ILD	13 (100)	10(100)	0 (0)
GI involvement	8 (66)	4 (40)	5 (83)
Kidney involvement	2 (16)	1 (10)	0 (0)
Pulmonary function tests (reference percentage, mean±SD)			
FVC	82±16	83±39	102.6±8.4
DLCO/VA	84.8±15	85±28	87±14
Radiographic pattern n (%)			
NSIP	13 (100)	10 (100)	–
UIP	0 (0)	0 (0)	–
Others	0 (0)	0 (0)	
Therapy n° (%)			
	MMF=6 (50) MTX=5 (41) RTX=5 (41) AZA=0 (0) CYC=0 (0) HCQ=0 (0) Ca-ant=3 (25) ACEi=5 (41) PDE5i=1 (8) ERA=6 (50) Prostanoids=8 (66) Selexipag=0 (0) Riociguat=1 (8) Nintedanib=0 (0)	MMF=1 (10) MTX=2 (20) RTX=0 (0) AZA=0 (0) CYC=1 (10) HCQ=0 (0) Ca-ant=1 (10) ACEi=4 (40) PDE5i=2 (20) ERA=2 (20) Prostanoids=6 (60) Selexipag=0 (0) Riociguat=0 (0) Nintedanib=0 (0)	MMF=6 (100) MTX=3 (50) RTX=1 (16) AZA=0 (0) CYC=1 (16) HCQ=0 (0) Ca-ant=3 (20) ACEi=1 (16) PDE5i=1 (16) ERA=1 (16) Prostanoids=6 (100) Selexipag=0 (0) Riociguat=0 (0) Nintedanib=0 (0)

Description of age, gender, disease duration, auto-antibody profile, organ involvement, pulmonary functional tests and therapy of SSc patients included in the in vitro experiments.
*Positivity for more than one SSc-specific antibodies.
†Other SSc-associated antibodies includes anti-U3 RNP, anti-PmScl75, anti-PmScl100, anti-NOR90, anti-Th/To, anti-Ku and anti-SSA.
ACEi, angiotensin-converting enzyme inhibitor; ANA, anti-nuclear antibodies; AZA, azathioprine; Ca-ant, calcium channel antagonist; CYC, cyclophosphamide; dcSSc, diffuse cutaneous systemic sclerosis; DLCO, diffusing capacity for carbon monoxide; DUs, digital ulcers; ENA, extractable nuclear antigen antibodies; ERA, endothelin receptor antagonist; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; GI, gastrointestinal; HCQ, hydroxychloroquine; ILD, interstitial lung disease; lcSSc, limited cutaneous systemic sclerosis; MMF, mycophenolate mofetil; mRSS, modified Rodnan Skin Score; MSK, musculoskeletal; MTX, methotrexate; NSIP, Nonspecific Interstitial Pneumonia; PAH, pulmonary arterial hypertension; PDE5i, phosphodiesterase type 5 inhibitor; RP, Raynaud phenomenon; RTX, rituximab; Scl70, anti-topoisomerase I; UIP, Usual Interstitial Pneumonia.

for idiopathic pulmonary fibrosis, including radiological features such as traction bronchiectasis, traction bronchiolectasis, ground-glass opacities, reticulation, honeycombing, other interstitial lung abnormalities, or a combination of these findings, as well as any recognised interstitial composite pattern.[34] To define progressive ILD in SSc, the following criteria have been adopted: FVC of at least 10% predicted, or a relative decline in FVC between 5% and less than 10% predicted, combined with a relative decline in DLCO of at least 15% predicted, within a 12-month period or worsened respiratory symptoms and increased extent of fibrosis on HRCT.[12 35]

Skin involvement was present in all subjects and was classified according to LeRoy and Medsger criteria.[36]

Demographic data and pulmonary function tests of SSc patients at time of blood sample are summarised in tables 1 and 2. Among our cohort, blood samples from 36 SSc patients were analysed with Flow Cytometry, whereas 29 underwent further in vitro analysis. Of note, 10 patients from all clinical subtypes were involved in both analyses (online supplemental file I). Finally, 25 age and sex matched healthy controls (HCs) were also enrolled into the study; 20 samples were considered for the Flow Cytometry evaluation and five for the in vitro experiments.

Flow cytometry and gating strategy

To investigate the presence of circulating monocytes showing a M2 phenotype and circulating TLR4$^+$M2 monocytes, Flow Cytometry analysis was performed directly on whole blood samples obtained from 37 of enrolled SSc patients and 20 of enrolled HCs, using Navios Flow Cytometer and Kaluza analysis software (Beckman Coulter, Brea, California, USA); 3 SSc patients were excluded from the analysis since they were under therapy with nintedanib (a tyrosine 2 kinase inhibitor). A volume of 0.1 mL of blood was incubated with the antibody mix for 15 min at room temperature in dark conditions. At the end of incubation, red blood cells were lysed and leucocytes post-fixed. A total of 5×10^6 cells and detecting more than 30 events in the smallest subset were investigated.[37 38] The gating strategy used in this study started from the detection of the monocyte population characterised as CD45$^+$CD14$^+$CD16$^+$cells in the leucocyte population (CD45$^+$cells) excluding both lymphocytes and granulocytes, as it has been shown in the online supplemental file 2 and recently described in our recent study.[30]

The monocyte lineage was characterised by investigating the cell membrane markers CD14, CD16 and CD45, using conjugated primary antibodies anti-human CD14-FITC (Miltenyi Biotech, Bergisch Gladbach, Germany), CD16-APC AlexaFluor 700, and CD45-Krome Orange (Beckman Coulter).

The M1 phenotype of circulating monocytes was defined through the detection of cell membrane markers CD80 and TLR4 using conjugated primary antibodies anti-human CD80-PEVio770 (Miltenyi Biotech) and TLR4-BV421 (Becton Dickinson-BD Biosciences, Franklin Lakes, New Jersey, USA), whereas to identify M2 phenotype, the specific cell membrane markers CD163, CD204 and CD206 were investigated using conjugated primary antibodies anti-human CD163-PEVio615, CD204-PE and CD206-PerCPVio700 (Miltenyi Biotech).

Moreover, to exclude the presence of dendritic cells, CD1c was investigated using the conjugated primary anti-human CD1c-APC Cy7 antibody (BioLegend, San Diego, California, USA).

The value in the box plots showing the percentage of the investigated subsets of circulating monocytes is referred to the leucocyte population.[38]

Isolation of circulating monocytes and in vitro differentiation into monocyte-derived macrophages (MDMs) from SSc patients and HC

In a subgroup of 29 enrolled SSc patients not under nintedanib therapy, venous blood samples (20 mL) were collected for the in vitro evaluation. Our cohort of patients was composed as follows: SSc patients with progressive ILD (n=13), SSc patients with non-progressive ILD (n=10) and no-ILD SSc patients (n=6). Moreover, a subgroup of 5 enrolled HCs was included in the analysis.

Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation from venous blood samples using Lympholyte-H cell separation medium (Cedarline, Burlington, Canada). Monocytes were isolated from PBMCs using the EasySep human monocyte enrichment kit without CD16 depletion (Stemcell Technologies, Vancouver, Canada), and their viability and purity were assessed by Flow Cytometry. The isolated monocytes were plated in tissue culture dishes (Eppendorf, Hamburg, Germany) at the concentration of 2.5×10^6 cells and stimulated with phorbol myristate acetate (PMA, 5 ng/mL; Sigma-Aldrich, St. Louis, Missouri, USA) in growth medium (Roswell Park Memorial Institute medium with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% L-glutamine, Euroclone, Milan, Italy) for 24 hours to induce their differentiation into MDMs, in accordance with several studies.[30 39 40] At the end of PMA stimulation, the medium was removed, and cultured MDMs were maintained for an additional 24 hours in growth medium without PMA or any other stimulation. At the end of culture time, cell supernatant was collected and stored at -80°C for the evaluation of production of TGFβ1, cytokines (IL6 and IL4) and chemokines (C-C motif chemokine ligand 2 and 18; CCL2/MCP1 and CCL18), whereas the cells were washed in Dulbecco's Phosphate Buffer Saline 1x (DPBS1x; Euroclone) and lysed using lysis buffer (Norgen Biotech, Thorold, Canada) to allow the isolation of RNA and proteins necessary to evaluate both gene expression and/or protein synthesis of TGFβ1, MerTK, ARG1, CD206, CD163, CD204 and TLR4.

Quantitative real-time PCR (qRT-PCR)

RNA was isolated using the RNA/Protein Purification Plus kit (Norgen Biotech) and quantified by nanodrop to assess its integrity and purity. For each experimental condition, first-strand complementary DNA was synthesised from 1 µg of total RNA, using QuantiTect Reverse Transcription Kit (Qiagen, Milan, Italy). The qRT-PCR was performed on an Eppendorf Realplex four mastercycler (Eppendorf) using a SYBR green mastermix detection system in a total volume of 10 µL, loaded in triplicate.

M2 phenotype markers were investigated using the specific primers for human CD204 (NM_002445), CD206 (NM_002438), CD163 (NM_004244) along with M1 phenotype marker TLR4 (NM_003266), and primers for human MerTK (NM_006343) and TGFβ1 (NM_000660), as functional M2 markers. Primers for human β-actin (NM_001101) were used as housekeeping gene. Gene expression values have been calculated using the comparative ΔΔ cycle threshold (ΔΔCT) method and corresponded to the expression level (fold-increase) of the target gene in cultured MDMs obtained from SSc patients compared with cultured MDMs obtained from HCs.[41] The melting curve was included in all qRT-PCR assays to confirm the specificity of the SYBR green assay.

Western blotting analysis

Proteins were isolated using the RNA/Protein Purification Plus kit (Norgen Biotech) and quantified by Bradford method. Subsequently, 10 µg of protein was separated via electrophoresis on pre-cast 4–20% gradient tris-glycine gels (GenScript, Piscataway, USA) and then transferred onto a nitrocellulose membrane (BioRad, Milan, Italy). After 1 hour in blocking solution, membranes were incubated overnight at 4°C with primary antibodies against human CD204, TLR4 (dilution 1:400 and 1:200, respectively; Santa Cruz Biotechnologies, Dallas, Texas, USA), CD206, CD163 and MerTK (dilution 1:500, Cell Signalling Technology, Danvers, Massachusetts, USA), ARG1 (dilution 1:500, Sigma-Aldrich, Darmstadt, Germany) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, dilution 1:1,000; Santa Cruz Biotechnologies). Membranes were subsequently incubated with specific horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:2,000; Cell Signalling Technology) for 1 hour at room temperature. Protein synthesis was detected using an enhanced chemiluminescence system (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Scientific, Rockford, USA) and the densitometric analysis was performed by the UVITEC Image Analysis System (UVITEC, Cambridge, UK).

For each sample (SSc patients and HCs), the value of the protein synthesis of the investigated molecules was normalised to that of the GAPDH (as housekeeping protein). The resulting value of each sample of SSc patients was then normalised to that of the corresponding HCs (considered as the unit value).

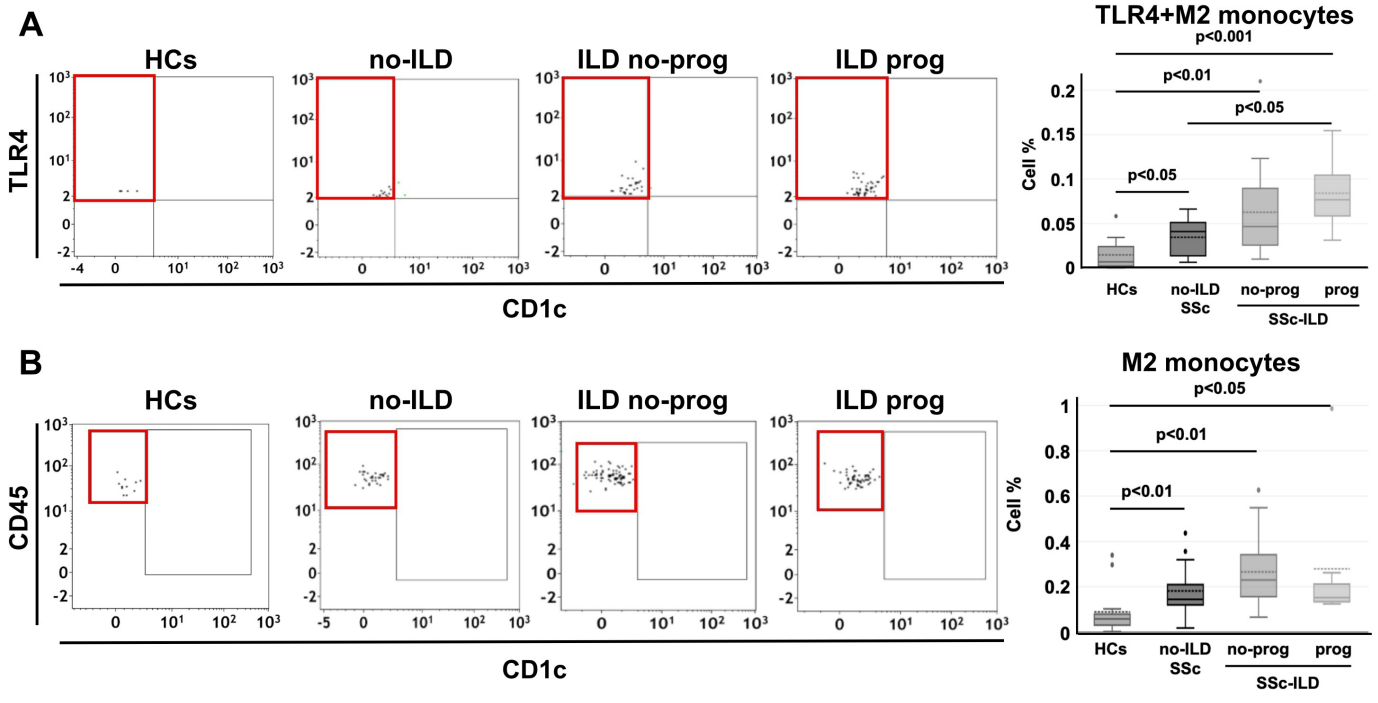

Figure 1 Flow Cytometry analysis of circulating monocytes from SSc patients with progressive ILD, non-progressive ILD, no-ILD and from HCs. Representative Flow Cytometry analysis of (A) TLR4$^+$M2 monocytes (CD1c$^-$CD80$^-$TLR4$^+$CD163$^+$CD204$^+$CD206$^+$CD14$^+$CD16$^+$CD45$^+$cells) and (B) M2 monocytes (CD1c$^-$CD80$^-$TLR4$^-$CD163$^+$CD204$^+$CD206$^+$CD14$^+$CD16$^+$CD45$^+$cells) in the peripheral blood of 8 SSc patients with progressive ILD (SSc-ILD prog), 14 with non-progressive ILD (SSc-ILD no-prog), 14 without ILD (SSc no-ILD) and 20 healthy controls (HCs) and related box plot representation of the cell percentage. ILD, interstitial lung disease; SSc, systemic sclerosis.

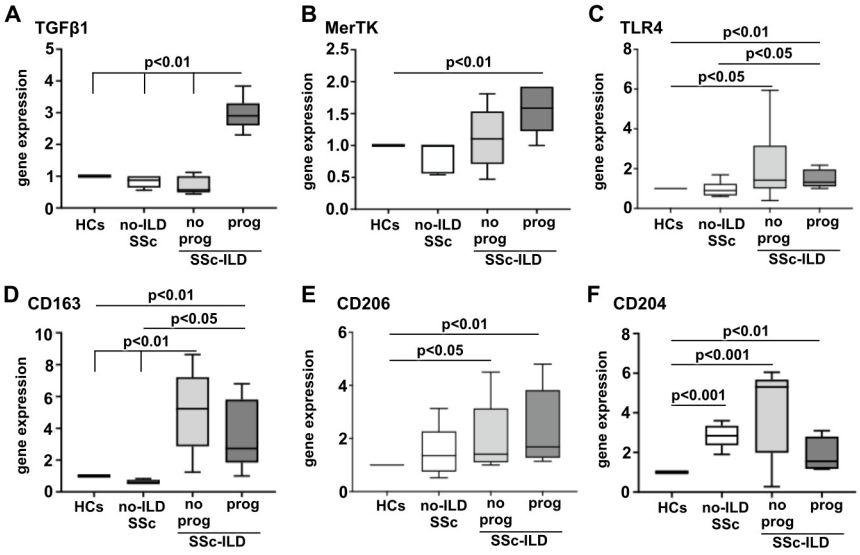

Figure 2 Gene expression of TLR4, functional and cell surface markers of profibrotic M2 phenotype in cultured MDMs obtained from SSc with progressive ILD, SSc with non-progressive ILD, no-ILD SSc and HCs. (A–F) Evaluation by quantitative real-time PCR (qRT-PCR) of the gene expression of TGFβ1, MerTK, TLR4, CD163, CD206 and CD204 in cultures of monocyte-derived macrophages (MDMs) obtained from 13 SSc patients with progressive ILD (SSc-ILD prog), 10 with non-progressive ILD (SSc-ILD no-prog), 6 without ILD (SSc no-ILD) and 5 healthy controls (HCs). The resulting value of the gene expression of each molecule in cultured MDMs from SSc-ILD prog, SSc-ILD no-prog and SSc no-ILD patients was compared with that obtained in cultured HC-MDMs (taken as unit value). Data are reported as median with a range of fold increase compared with HCs. ILD, interstitial lung disease; MerTK, Mer tyrosine kinase receptor; SSc, systemic sclerosis; TLR4, toll-like receptor-4; TGF, transforming growth factor.

Enzyme-linked immunosorbent assay (ELISA)

The endogenous level of the active form of TGFβ1 was determined in cell supernatants by ELISA assay using Ella automated immunoassay system, in accordance with the manufacturer's protocol (Bio-techne, Minneapolis, Minnesota, USA). In the first phase of the procedure (sample activation step), 80 µL of sample was mixed with 20 µL of HCl 1 n and incubated for 10 min at room temperature to acidify the sample. At the end of incubation, the acidification of samples was neutralised by adding 20 µL of NaOH 1.2 N / HEPES 0.5 M solution to obtain an activated cell culture supernatant, which was subsequently diluted five-fold with a specific sample diluent (20 µL of activated sample was added to 80 µL of sample diluent). At the end of this phase, 50 µL of each diluted sample was transferred into the TGFβ1 Ella cartridge.

In the same cell supernatants, the levels of IL6, IL4, CCL2/MCP1 and CCL18 were also investigated by ELISA using the Sandwich-ELISA principle, in accordance with the manufacturers' protocol (Elabscience, Houston, Texas, USA). A volume of 0.1 mL each of diluted standard, blank and sample was dispensed into the appropriate wells, incubated for 90 min, and then 0.1 mL of biotinylated detection antibody was added and incubated for 60 min always at 37°C. At the end of this step, each well was washed three times and subsequently 0.1 mL of HRP conjugate working solution was added and incubated for 30 min; after a second washing step, the wells were incubated with 0.1 mL of Substrate reagent solution and incubated for an additional 30 min. At the end of the incubation time, 50 µL of stop solution was added to each well and the optical density (OD value) of each well was determined at once with a micro-plate reader set to 450 nm.

Levels of TGFβ1, IL6, IL4, CCL2/MCP1 and CCL18 were expressed as pg/mL.

Statistical analysis

Statistical analysis was carried out by non-parametric Mann–Whitney U test using GraphPad Prism (V.8.4.0, GraphPad Software, San Diego, California, USA) to compare gene and protein expression of investigated M2 markers between cultured MDMs obtained from HCs, SSc patients no-ILD and SSc-prog ILD patients, SSc-non-prog ILD patients. Any p value lower than 0.05 has been considered as statistically significant. Results of qRT- PCR and Western blotting were analysed and graphically reported as median with range. Statistical analyses included the χ^2 test with Yates correction and Kruskal-Wallis test (H) with post hoc analysis (Dunn-Bonferroni) for group comparisons, and Spearman correlation analysis to examine relationships between monocyte subsets and capillary density. These analyses were performed using DATAtab Team (2025).

RESULTS

Demographic data of SSc patients with progressive ILD compared with patients with non-progressive ILD and no-ILD enrolled for the studies

The cohort of patients enrolled for the Flow Cytometry analysis had the following demographic characteristics:

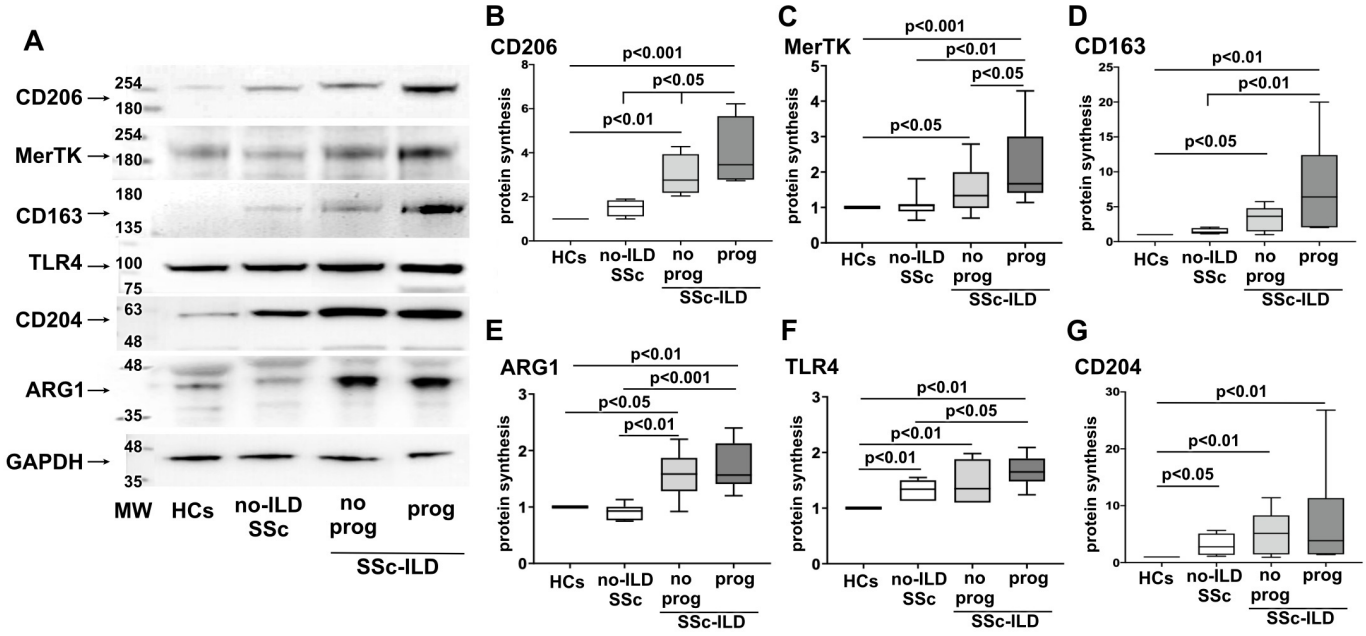

Figure 3 Protein synthesis of TLR4, functional and cell surface markers of profibrotic M2 phenotype in cultured MDMs obtained from SSc with progressive ILD, SSc with non-progressive ILD, no-ILD SSc and HCs. (A) Evaluation by Western blotting and (B–G) related densitometric analysis of the protein synthesis of CD206, MerTK, CD163, ARG1, TLR4 and CD204 in cultures of monocyte-derived macrophages (MDMs) obtained from 13 SSc patients with progressive ILD (SSc-ILD prog), 10 with non-progressive ILD (SSc-ILD no-prog), 6 without ILD (SSc no-ILD) and 5 healthy controls (HCs). The value of protein expression of the investigated molecules was normalised to that of the corresponding GAPDH in cultured MDMs from SSc-ILD prog, SSc-ILD no-prog, no-ILD SSc and HCs. The resulting value of the protein synthesis of each molecule in cultured MDMs from progressive SSc-ILD, non-prog SSc-ILD and no-ILD SSc patients was compared with that obtained in cultured HC-MDMs (taken as unit value). Data are reported as median with a range of fold increase compared with HCs. ILD, interstitial lung disease; MerTK, Mer tyrosine kinase receptor; SSc, systemic sclerosis; TLR4, toll-like receptor-4;

the mean age was 61±13.6 years in SSc progressive ILD patients, 66±14 years in SSc non-progressive ILD patients, and 66.1±21.6 years in no-ILD SSc patients; the mean disease duration was 7.3±4.4 years in SSc progressive ILD patients, 7.6±6 years in SSc non-progressive ILD patients, and 5.6±4.4 years in no-ILD SSc patients . There were 6 female and 2 male SSc progressive ILD patients, 12 female and 2 male SSc non-progressive ILD and 13 female and 1 male no-ILD SSc patients.

Demographics, SSc organ involvement and therapies are summarised in table 1.

The cohort of patients enrolled for the in vitro analysis had the following demographic characteristics: the mean age was 61.8±15.7 years in SSc progressive ILD patients, 67±9 years in SSc non-progressive ILD patients and 66.7±17 years in no-ILD SSc patients; the mean disease duration was 13.9±8.5 years in SSc progressive ILD patients, 8.8±7.4 years in SSc non-progressive ILD patients and 7.8±5.1 years in no-ILD SSc patients . There were 9 female and 3 male SSc progressive ILD patients, 7 female and 1 male SSc non-progressive ILD and 6 female and 0 male no-ILD SSc patients.

Demographics, SSc organ involvement and therapies are summarised in table 2.

Increased percentage of circulating TLR4$^+$M2 monocytes in SSc patients with progressive ILD compared with patients with non-progressive ILD and no-ILD

Flow Cytometry was conducted to examine the phenotype of circulating monocytes, specifically those showing a hybrid TLR4$^+$M2 and M2 phenotypes, based on the gating strategy described in the online supplemental file 2.

Results showed that SSc patients with progressive ILD were characterised by a significantly higher percentage of circulating hybrid TLR4$^+$M2 monocytes (TLR4$^+$CD80$^-$CD204$^+$CD206$^+$CD163$^+$CD14$^+$CD16$^+$CD45$^+$cells) compared with no-ILD SSc patients ($p<0.05$) (figure 1A). Moreover, the percentage of these cells was higher in SSc patients with progressive ILD compared with those patients with non-progressive ILD (not statistically significant) (figure 1A).

No differences in the percentage of circulating monocytes showing an M2 phenotype (TLR4$^-$CD80$^-$CD204$^+$CD206$^+$CD163$^+$CD14$^+$CD16$^+$CD45$^+$cells) in SSc patients with progressive ILD compared with SSc patients with non-progressive ILD and no-ILD SSc patients were observed (figure 1B).

The results also showed that the percentage of circulating TLR4$^+$M2 monocytes and M2 monocytes was significantly higher in SSc patients compared with HCs (no-ILD

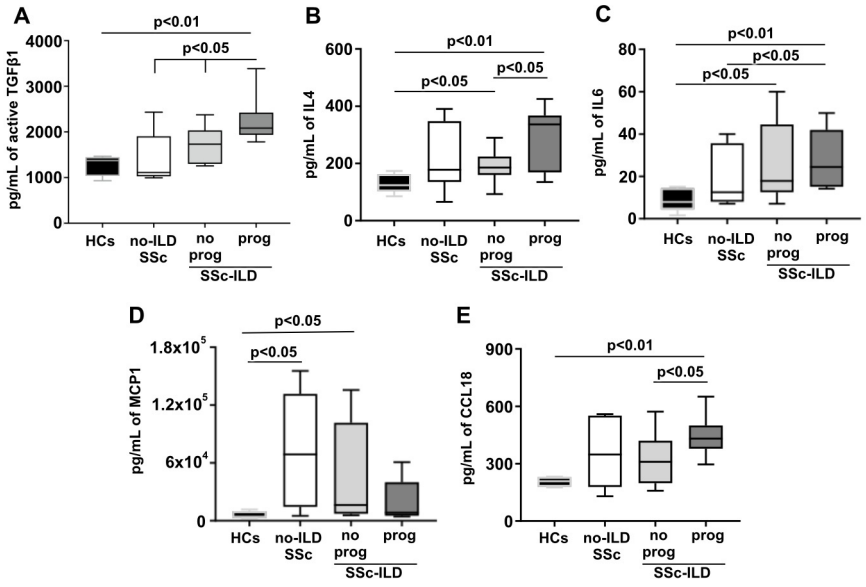

Figure 4 Levels of TGFβ1, IL6, IL4, CCL2/MCP1 and CCL18 in cell supernatant of cultured MDMs obtained from from SSc with progressive ILD, SSc with non-progressive ILD, no-ILD SSc and HCs.(A–E) Evaluation by ELISA of the release of active TGFβ1, IL6, IL4, MCP1 and CCL18 in the conditioned medium of cultured monocyte-derived macrophages (MDMs) obtained from 13 SSc patients with progressive ILD (SSc-ILD prog), 10 with non-progressive ILD (SSc-ILD no-prog), 6 without ILD (SSc no-ILD) and 5 healthy controls (HCs). The levels of TGFβ1, cytokines and chemokines in cultured MDMs from SSc-ILD prog, SSc-ILD non-prog and no-ILD SSc, as well as in cultured HC-MDMs, were reported as pg/mL. ILD, interstitial lung disease; SSc, systemic sclerosis; TGF, transforming growth factor, IL, Interleukin; MCP1, monocyte chemoattractant protein-1; CCL-18, C-C Motif Chemokine Ligand 18

SSc: p<0.01 and p<0.05; SSc with non-progressive ILD: p<0.01 for both cell subsets; SSc with progressive ILD: p<0.001 and p<0.05) (figure 1). No statistically significant correlations were found between the percentage of TLR4^{+}M2 monocytes and FVC, DLCO or modified Rodnan Skin Score.

Increased gene expression of MerTK and TGFβ1 in cultured MDMs obtained from SSc patients with progressive ILD compared with patients with non-progressive ILD and no-ILD

Cultured MDMs obtained from SSc patients with progressive ILD showed a significant upregulation of the gene expression of TGFβ1 compared with MDMs obtained from both SSc patients with non-progressive ILD and no-ILD SSc patients (p<0.01) (figure 2A).

Moreover, these MDMs obtained from SSc patients with progressive ILD were characterised by a higher gene expression of MerTK compared with MDMs obtained from SSc patients with non-progressive ILD and no-ILD SSc patients (mean fold increase: 1.6±0.4 vs. 1.1±0.5, and vs 0.82±0.3, respectively), whereas the gene expression of TLR4 was significantly higher compared with no-ILD SSc patients (p<0.05) (figure 2B–C).

In any case, MDMs obtained from SSc patients with non-progressive ILD showed an increased TLR4 gene expression compared with MDMs obtained from no-ILD SSc patients (non-statistically significant) (figure 2C).

Of note, cultured MDMs obtained from SSc patients with ILD (both progressive and non-progressive) were characterised by a significant upregulation of the gene expression of CD163 compared with MDMs obtained from no-ILD SSc patients (p<0.05; p<0.01) (figure 2D).

Moreover, CD206 gene expression was found significantly upregulated in cultured MDMs obtained from SSc patients both with progressive and non-progressive ILD compared with HCs (p<0.01; p<0.05), whereas, CD204 gene expression was significantly upregulated in MDMs obtained from all SSc patients' groups (p<0.001 for MDMs of non-progressive and no-ILD SSc patients; p<0.01 for MDMs of SSc patients with progressive ILD vs HC-MDMs) (figure 2E,F).

Increased protein synthesis of TLR4, MerTK and M2 markers in cultured MDMs obtained from SSc patients with progressive ILD compared with patients with non-progressive ILD and no ILD.

Cultured MDMs obtained from SSc patients with progressive ILD were characterised by a significant increase in protein synthesis of CD206, MerTK, CD163, ARG1 and TLR4 compared with MDMs obtained from no-ILD SSc patients (CD206: p<0.05; MerTK and CD163: p<0.01; ARG1: p<0.001; TLR4: p<0.05) (figure 3A–E). Moreover, the protein synthesis of CD206 and MerTK was significantly higher in cultured MDMs obtained from SSc patients with progressive ILD compared with non-progressive ILD SSc patients (p<0.05 for both), whereas CD163 protein synthesis showed a trend to higher value that was non-statistically significant (mean±SD protein synthesis CD163: 7.79±6.8 vs 3,23±1.8) (figure 3A–D).

Of note, cultured MDMs obtained from SSc-ILD patients (both progressive and non-progressive) showed

a significant increase in protein synthesis of ARG1 compared with MDMs obtained from no-ILD SSc patients (p<0.01 for SSc patients with non-progressive ILD; p<0.001 for SSc patients with progressive ILD) and they also showed a significantly higher protein synthesis of CD206, MerTK, CD163 and ARG1 compared with MDMs obtained from HCs (non-progressive SSc-ILD patients: p<0.01 for CD206; p<0.05 for MerTK, CD163 and AGR1; progressive SSc-ILD patients: p<0.001 for CD206 and MerTK, p<0.01 for CD163 and AGR1) (figure 3A–E). A supplementary file shows this in more detail (see online supplemental file 3).

Increased production and release of profibrotic cytokines, chemokines and IL6 in cultured MDMs obtained from SSc patients with progressive ILD compared with patients with non-progressive ILD, no-ILD and HC.

Based on the results obtained at gene and protein level, the production and release of profibrotic growth factor TGFβ1, IL4, CCL2/MCP1, CCL18 as well as pro-inflammatory IL6 were investigated by ELISA assay in the cell supernatants of cultured MDMs obtained from all SSc patient groups and HCs.

Cultured MDMs obtained from SSc patients with progressive ILD were characterised by a significantly higher production and release of the active form of TGFβ1 compared with MDMs obtained from SSc patients with non-progressive ILD, no-ILD SSc patients (p<0.05 for both), and compared with MDMs obtained from HCs (p<0.01) (figure 4A). Cultured MDMs obtained from SSc patients with progressive ILD were also characterised by a significantly higher production and release of IL4 and CCL18 compared with MDMs obtained from SSc patients with non-progressive ILD (p<0.05 for both profibrotic molecules) (figure 4B and E). Of note, the level of CCL18 was significantly higher only in cultured MDMs obtained from SSc patients with progressive ILD compared with HC-MDMs (p<0.01), whereas CCL2/MCP1 levels were significantly higher in cultured MDMs obtained from no-ILD SSc patients and SSc patient with non-progressive ILD (p<0.05) (figure 4D and E). Cultured MDMs obtained from SSc-ILD patients (both progressive and non-progressive) were characterise by a significantly higher production and release of IL6 compared with HC-MDMs (p<0.01 and p<0.05) (figure 4C). Moreover, the culture medium level of IL6 was significantly higher in MDMs from progressive ILD SSc patients compared with MDMs obtained from no-ILD SSc patients (p<0.05) (figure 4C).

Finally, these results confirmed that the production of all the investigated profibrotic cytokines and chemokines, together with that of IL6, was significantly higher in cultured MDMs obtained from all SSc patients compared with MDMs obtained from HCs (p<0.05 for all molecules) (online supplemental file 4).

DISCUSSION

The investigation shows for the first time that cultured MDMs originating from circulating monocytes of SSc patients with progressive ILD are characterised by a significantly higher combination of biomarkers and protein production for a strong profibrotic phenotype including significant increase of protein synthesis and/or gene expression for functional mediators of fibrosis compared with non-progressive ILD, as well as no-ILD SSc patients.

Therefore, the finding of this study seems to align with previous ones showing an increased prevalence of hybrid M1/M2 phenotypes in SSc patients with lung involvement and linking these cells to ILD development and severity.[28]

As a matter of fact, the in vitro results revealed that cultured MDMs obtained from SSc patients with progressive ILD seem to be characterised by a higher profibrotic activity due to their significant increased gene expression and protein release of TGFβ1 and secretion of IL4 and CCL18 compared with MDMs from non-progressive SSc-ILD patients.

Moreover, the significant upregulation of MerTK (a marker associated with efferocytosis and fibrogenesis), ARG1, CD206 and CD163 at both gene and protein level compared with MDMs obtained from no-ILD SSc patients further supports the high profibrotic activity of these cells and their involvement at least in the lung fibrotic process.[42]

Interestingly, MerTK is a member of the Tyro-3, Axl and TAM receptor tyrosine kinase family, implicated in various fibrotic conditions, including pulmonary and hepatic fibrosis, and it plays a role in recognising apoptotic cells during efferocytosis.[43]

Notably, MerTK induces TGFβ1 expression, which activates fibrotic cells, such as fibroblasts, into myofibroblasts enhancing collagen production during wound healing.[44] Similarly, in lung fibrosis, macrophages highly expressing MerTK have been shown to drive TGFβ1 production, which promotes fibroblast-to-myofibroblast transition, leading to tissue fibrosis.[45] Of note, the TGFβ/Smad and Wnt/β-catenin signalling pathways are activated in M2 macrophages stimulating the fibrotic process.[44] The significantly higher secretion of TGFβ1 observed in cultured MDMs obtained from progressive ILD SSc patients might be triggered by a metabolic reprogramming toward an increased glycolytic pathway involving GLUT1 and NADPH-mediated mechanisms, and a mitochondrial dysfunction allowing for an increased oxygen species (ROS) production, as already observed in alveolar macrophages and fibroblasts in lung fibrosis.[46–48]

Indeed, the enhanced gene expression and protein synthesis of MerTK in cultured MDMs obtained from SSc patients with progressive ILD observed in this study further support its critical role in driving fibrosis and highlighting the profibrotic function of MerTK positive macrophage.[39 49] In addition, the higher synthesis and release of the active form of TGFβ1, together with IL4 and CCL18 observed in cultured MDMs from SSc patients with progressive ILD, highlight these cells as pivotal mediators in the interplay between immune

signalling and fibrotic tissue remodelling, as already observed in pulmonary fibrosis.[50] Moreover, CCL18 is highly increased in pulmonary fibrotic disease processes and is considered a profibrotic mediator together with TGFβ1 responsible for fibroblast-to-myofibroblast differentiation and collagen overproduction.[51]

The identification of macrophages co-expressing TLR4 and the M2 cell surface markers (CD206, CD163, CD204) in fibrotic parenchyma of SSc-ILD seems to reinforce their potential role as pathogenic elements in the SSc-related lung involvement.[30] Reflecting this observation, the higher percentage of circulating hybrid TLR4$^+$M2 monocytes in patients with progressive ILD compared with non-progressive ILD patients and significantly higher compared with no-ILD SSc patients reported in the study suggests a possible involvement of these cells in the progression of lung fibrosis. The absence of CD1c (marker for dendritic cells) further validated the monocyte-specific nature of these circulating cells.[52]

In addition, the higher production and release of IL6 by cultured MDMs obtained from SSc-ILD patients (both progressive and non-progressive), associated with a higher gene and protein expression of TLR4, M2 markers (ie, ARG1, CD163 and CD206) and CCL18, seem to confirm not only the plasticity of these cells but also their possible phenotypic traits characteristic of tumour-associated macrophages.[53 54]

A recent study highlighted the association of serum interferon α–2a and chemokines such as CCL8, CCL19, CXCL10 and CXCL11 with disease severity and prognosis in SSc, with CCL8 emerging as a potent monocyte chemoattractant—underscoring the central role of the monocyte–macrophage lineage in both the production and response to these inflammatory mediators.[55]

Notably, a recent in vitro study has reported for the first time that nintedanib, a tyrosine kinase inhibitor and antifibrotic drug currently approved for the SSc-ILD treatment, significantly downregulates the expression of MerTK in cultured ILD-SSc MDMs.[39] This finding offers new insights into how nintedanib may mitigate fibrosis by interfering with pathways involving the tyrosine kinase receptor MerTK.[56] Nowadays, tailoring pharmacological treatment requires consideration not only of the patient's clinical phenotype but also of their immune cell profile.

A comprehensive immune cell characterisation is therefore crucial to achieve an in-depth patient stratification and pro-active personalised therapeutic approaches.[57]

Some limitations of the study are present. The small sample size and cross-sectional design limit the ability to establish causal relationships between macrophage phenotypes and ILD progression. This limitation will be solved by ongoing larger studies.

In some analyses, the lack of statistical significance differences between progressive and non-progressive ILD SSc groups may have been influenced by the dynamic and evolving nature of the disease, as well as by the intrinsic heterogeneity of ILD and the limited sample size, reflecting a typical variation within the cohort.

A further limitation of this study is that the ongoing immunosuppressive treatments were allowed, reflecting a real-life clinical context. Nevertheless, patients treated with antifibrotic agents, such as nintedanib, were excluded from the analysis (online supplemental file 1).

To further elucidate the profibrotic role of TLR4$^+$M2 monocytes and their direct interaction with mesenchymal cells, especially fibroblasts, additional in vitro co-culture studies are needed to confirm the connection between these two cell types, increasing the powerful and novel central function of these hybrid monocytes/macrophages in SSc lung fibrosis.[57] Indeed, the characterisation of TLR4$^+$M2 monocytes and MDMs from SSc patients, particularly those with progressive ILD, is ongoing in order to investigate their functional status, gene expression profile and their possible capability to induce in vitro the activation of fibroblasts and extracellular matrix production.

Future research should also investigate the interaction between M2 macrophages and other immune cells, as well as their involvement in microvascular damage in SSc and other CTDs.

Moreover, based on the results of the study, it might be interesting to elucidate if the higher profibrotic activity of circulating monocytes and MDMs obtained from SSc-ILD patients, primarily in those with a progressive ILD, may be correlated/linked with an alteration of the immunometabolic shifting from oxidative phosphorylation to glycolysis to fuel their phenotype and protein production, as well as with an alteration of the mitochondrial oxidative stress and turnover, both involved in the activation of TGFβ1 signalling pathway and in the promotion of a profibrotic phenotype in alveolar macrophages.[46 47]

Of note, other pathophysiological mechanisms may further intervene in the dynamics of SSc microvascular damage and related symptoms, such as gender, the increase in nocturnal inflammatory reactions (ie, increased cytokine and growth factor synthesis) that follow the circadian rhythm, as seen in rheumatoid arthritis synovitis.[58 59]

Finally, longitudinal studies, already planned, are required to validate these findings and explore whether therapeutic modulation of these pathways can significantly influence clinical outcomes.

CONCLUSION

The investigation shows for the first time that cultured MDMs originating from circulating monocytes of SSc patients with progressive ILD are characterised by a significantly higher combination of biomarkers and protein production for a strong profibrotic phenotype (CD206, CD163, IL4, IL6 and CCL18), including significant increase of protein synthesis and/or gene expression for functional mediators of

fibrosis (TGFβ1, MerTK, ARG1) compared with non-progressive ILD, as well as no-ILD SSc patients.

Present results represent the basis of our ongoing investigations to further characterise the potential role of such circulating monocyte phenotype, to be considered as possible predictive biomarker of evolution versus progressive ILD at least among ILD-SSc patients.

Author affiliations
[1]Department of Rheumatology and Internal Medicine, University Hospital Ghent, Ghent, Flanders, Belgium
[2]Unit for Molecular Immunology and Infammation, Flemish Institute for Technological Research, Mol, Flanders, Belgium
[3]Department of Internal Medicine, Ghent University Hospital, University of Ghent, Ghent, Belgium
[4]Laboratory of Experimental Rheumatology and Academic Division of Clinical Rheumatology, Department of Internal Medicine and Medical Specialities (Di.M.I.), University of Genoa, Genoa, Liguria, Italy
[5]Department of Experimental Medicine (DIMES), University of Genoa, Genova, Italy
[6]IRCCS Ospedale Policlinico San Martino, Genoa, Liguria, Italy

Acknowledgements Laboratory for Experimental Rheumatology, Academic Division of Clinical Rheumatology of the University of Genova (IRCCS Ospedale Policlinico San Martino) and Ghent University Hospital (Department of Rheumatology) are members of the European Reference Network on Rare Connective Tissue and Musculoskeletal Diseases (ERN- ReCONNET), as well as Centres of Excellence for Imaging of EULAR (European Alliance of Associations for Rheumatology). MC is chair of the EULAR study group on neuroendocrine immunology of RMDs (NEIRD). VS is a senior clinical investigator of the Research Foundation - Flanders (Belgium) (FWO) (1.8.029.20N). The FWO was not involved in study design, collection, analysis and interpretation of data, writing of the report, or in the decision to submit the article for publication. Dr Paola Contini was involved in the partial acquisition of flow cytometry samples.

Contributors MC is the guarantor. MC and VS: conceptualisation, data curation, methodology, writing – original draft, writing – review and final editing. SS: conceptualisation, flow cytometry and proteomic analysis, in vitro experiments, writing – original draft, writing – review and editing. RC: Patient enrolment, sample collection, formal analysis, in vitro experiments, data curation, writing – review and editing. EG: Conceptualisation, methodology, writing, review and editing. PM: molecular biology assays and data analysis of gene expression. AC: Patient enrolment and in vitro experiments. CP, EH, SP, TV and AS: Review and approve.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by the Ethical Committee of the Ospedale Policlinico San Martino (237REG2015, amendment number: 002–28/05/2018) and obtained written informed consent from all participants. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

ORCID iDs
Vanessa Smith https://orcid.org/0000-0001-6271-7945
Emanuele Gotelli https://orcid.org/0000-0002-4732-0306
Maurizio Cutolo https://orcid.org/0000-0002-5396-0932

REFERENCES

1 Volkmann ER, Andréasson K, Smith V. Systemic sclerosis. *Lancet* 2023;401:304–18.
2 Cutolo M, Soldano S, Smith V. Pathophysiology of systemic sclerosis: current understanding and new insights. *Expert Rev Clin Immunol* 2019;15:753–64.
3 Tsou PS, Varga J, O'Reilly S. Advances in epigenetics in systemic sclerosis: molecular mechanisms and therapeutic potential. *Nat Rev Rheumatol* 2021;17:596–607.
4 Denton CP, Khanna D. Systemic sclerosis. *Lancet* 2017;390:1685–99.
5 Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. *N Engl J Med* 2009;360:1989–2003.
6 Tyndall AJ, Bannert B, Vonk M, *et al*. Causes and risk factors for death in systemic sclerosis: a study from the EULAR Scleroderma Trials and Research (EUSTAR) database. *Ann Rheum Dis* 2010;69:1809–15.
7 Elhai M, Meune C, Boubaya M, *et al*. Mapping and predicting mortality from systemic sclerosis. *Ann Rheum Dis* 2017;76:1897–905.
8 Bergamasco A, Hartmann N, Wallace L, *et al*. Epidemiology of systemic sclerosis and systemic sclerosis-associated interstitial lung disease. *Clin Epidemiol* 2019;11:257–73.
9 Perelas A, Silver RM, Arrossi AV, *et al*. Systemic sclerosis-associated interstitial lung disease. *Lancet Respir Med* 2020;8:304–20.
10 Hoffmann-Vold A-M, Maher TM, Philpot EE, *et al*. The identification and management of interstitial lung disease in systemic sclerosis: evidence-based European consensus statements. *Lancet Rheumatol* 2020;2:e71–83.
11 Wells AU, Walsh SLF, Adegunsoye A, *et al*. Identification of progressive pulmonary fibrosis: consensus findings from a modified Delphi study. *Respir Res* 2024;25:448.
12 Raghu G, Remy-Jardin M, Richeldi L, *et al*. Idiopathic Pulmonary Fibrosis (an Update) and Progressive Pulmonary Fibrosis in Adults: An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline. *Am J Respir Crit Care Med* 2022;205:e18–47.
13 Campitiello R, Soldano S, Gotelli E, *et al*. The intervention of macrophages in progressive fibrosis characterizing systemic sclerosis: A systematic review. *Autoimmun Rev* 2024;23:103637.
14 Brown M, O'Reilly S. The immunopathogenesis of fibrosis in systemic sclerosis. *Clin Exp Immunol* 2019;195:310–21.
15 Liakouli V, Ciancio A, Del Galdo F, *et al*. Systemic sclerosis interstitial lung disease: unmet needs and potential solutions. *Nat Rev Rheumatol* 2024;20:21–32.
16 Murray PJ, Allen JE, Biswas SK, *et al*. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* 2014;41:14–20.
17 Degboé Y, Poupot R, Poupot M. Repolarization of Unbalanced Macrophages: Unmet Medical Need in Chronic Inflammation and Cancer. *Int J Mol Sci* 2022;23:1496.
18 Peng Y, Zhou M, Yang H, *et al*. Regulatory Mechanism of M1/M2 Macrophage Polarization in the Development of Autoimmune Diseases. *Mediators Inflamm* 2023;2023:8821610.
19 Cutolo M, Soldano S, Smith V, *et al*. Dynamic macrophage phenotypes in autoimmune and inflammatory rheumatic diseases. *Nat Rev Rheumatol* 2025;21:546–65.
20 Hanlon MM, McGarry T, Marzaioli V, *et al*. Rheumatoid arthritis macrophages are primed for inflammation and display bioenergetic and functional alterations. *Rheumatology (Sunnyvale)* 2023;62:2611–20.
21 Al-Adwi Y, Westra J, van Goor H, *et al*. Macrophages as determinants and regulators of fibrosis in systemic sclerosis. *Rheumatology (Sunnyvale)* 2023;62:535–45.
22 Kurowska-Stolarska M, Alivernini S. Synovial tissue macrophages in joint homeostasis, rheumatoid arthritis and disease remission. *Nat Rev Rheumatol* 2022;18:384–97.
23 McGarry T, Hanlon MM, Marzaioli V, *et al*. Rheumatoid arthritis $CD14^+$ monocytes display metabolic and inflammatory dysfunction, a phenotype that precedes clinical manifestation of disease. *Clin Transl Immunology* 2021;10:e1237.
24 Luo M, Zhao F, Cheng H, *et al*. Macrophage polarization: an important role in inflammatory diseases. *Front Immunol* 2024;15:1352946.

25 Cutolo M, Campitiello R, Gotelli E, *et al*. The Role of M1/M2 Macrophage Polarization in Rheumatoid Arthritis Synovitis. *Front Immunol* 2022;13:867260.
26 Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008;8:958–69.
27 Lescoat A, Lecureur V, Varga J. Contribution of monocytes and macrophages to the pathogenesis of systemic sclerosis: recent insights and therapeutic implications. *Curr Opin Rheumatol* 2021;33:463–70.
28 Soldano S, Trombetta AC, Contini P, *et al*. Increase in circulating cells coexpressing M1 and M2 macrophage surface markers in patients with systemic sclerosis. *Ann Rheum Dis* 2018;77:1842–5.
29 Trombetta AC, Soldano S, Contini P, *et al*. A circulating cell population showing both M1 and M2 monocyte/macrophage surface markers characterizes systemic sclerosis patients with lung involvement. *Respir Res* 2018;19:186.
30 Gotelli E, Soldano S, Feghali-Bostwick C, *et al*. Prevalence of hybrid TLR4^{+}M2 monocytes/macrophages in peripheral blood and lung of systemic sclerosis patients with interstitial lung disease. *Front Immunol* 2024;15:1488867.
31 van den Hoogen F, Khanna D, Fransen J, *et al*. 2013 classification criteria for systemic sclerosis: an American college of rheumatology/European league against rheumatism collaborative initiative. *Ann Rheum Dis* 2013;72:1747–55.
32 Smith V, Scirè CA, Talarico R, *et al*. Systemic sclerosis: state of the art on clinical practice guidelines. *RMD Open* 2018;4:e000782.
33 DeMizio DJ, Bernstein EJ. Detection and classification of systemic sclerosis-related interstitial lung disease: a review. *Curr Opin Rheumatol* 2019;31:553–60.
34 Vandecasteele E, Melsens K, Vanhaecke A, *et al*. Incidence, prevalence and long-term progression of Goh algorithm rated interstitial lung disease in systemic sclerosis in two independent cohorts in flanders: A retrospective cohort study. *Semin Arthritis Rheum* 2021;51:969–76.
35 Distler O, Highland KB, Gahlemann M, *et al*. Nintedanib for Systemic Sclerosis-Associated Interstitial Lung Disease. *N Engl J Med* 2019;380:2518–28.
36 LeRoy EC, Medsger TA. Criteria for the classification of early systemic sclerosis. *J Rheumatol* 2001;28:1573–6.
37 Arroz M, Came N, Lin P, *et al*. Consensus guidelines on plasma cell myeloma minimal residual disease analysis and reporting. *Cytometry B Clin Cytom* 2016;90:31–9.
38 Cossarizza A, Chang H-D, Radbruch A, *et al*. Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *Eur J Immunol* 2021;51:2708–3145.
39 Soldano S, Smith V, Montagna P, *et al*. Nintedanib downregulates the profibrotic M2 phenotype in cultured monocyte-derived macrophages obtained from systemic sclerosis patients affected by interstitial lung disease. *Arthritis Res Ther* 2024;26:74.
40 Cutolo M, Soldano S, Gotelli E, *et al*. CTLA4-Ig treatment induces M1–M2 shift in cultured monocyte-derived macrophages from healthy subjects and rheumatoid arthritis patients. *Arthritis Res Ther* 2021;23:306.
41 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–8.
42 Gheibi Hayat SM, Bianconi V, Pirro M, *et al*. Efferocytosis: molecular mechanisms and pathophysiological perspectives. *Immunol Cell Biol* 2019;97:124–33.
43 Mohning MP, Thomas SM, Barthel L, *et al*. Phagocytosis of microparticles by alveolar macrophages during acute lung injury requires MerTK. *Am J Physiol Lung Cell Mol Physiol* 2018;314:L69–82.
44 Hu M, Yao Z, Xu L, *et al*. M2 macrophage polarization in systemic sclerosis fibrosis: pathogenic mechanisms and therapeutic effects. *Heliyon* 2023;9:e16206.
45 Darby IA, Laverdet B, Bonté F, *et al*. Fibroblasts and myofibroblasts in wound healing. *Clin Cosmet Investig Dermatol* 2014;7:301–11.
46 Perrot CY, Karampitsakos T, Herazo-Maya JD. Monocytes and macrophages: emerging mechanisms and novel therapeutic targets in pulmonary fibrosis. *Am J Physiol Cell Physiol* 2023;325:C1046–57.
47 Feng L, Chen X, Huang Y, *et al*. Immunometabolism changes in fibrosis: from mechanisms to therapeutic strategies. *Front Pharmacol* 2023;14:1243675.
48 Yin X, Choudhury M, Kang J-H, *et al*. Hexokinase 2 couples glycolysis with the profibrotic actions of TGF-β. *Sci Signal* 2019;12:eaax4067.
49 Morse C, Tabib T, Sembrat J, *et al*. Proliferating SPP1/MERTK-expressing macrophages in idiopathic pulmonary fibrosis. *Eur Respir J* 2019;54:1802441.
50 Jiang Y, Cai R, Huang Y, *et al*. Macrophages in organ fibrosis: from pathogenesis to therapeutic targets. *Cell Death Discov* 2024;10:487.
51 Prasse A, Pechkovsky DV, Toews GB, *et al*. CCL18 as an indicator of pulmonary fibrotic activity in idiopathic interstitial pneumonias and systemic sclerosis. *Arthritis Rheum* 2007;56:1685–93.
52 Heger L, Hofer TP, Bigley V, *et al*. Subsets of CD1c+ DCs: Dendritic Cell Versus Monocyte Lineage. *Front Immunol* 2020;11:559166.
53 Wang J, Niu H, Kang J, *et al*. Macrophage Polarization in Lung Diseases: From Mechanisms to Therapeutic Strategies. *Immunol Invest* 2025;54:743–69.
54 Nguyen TN, Nguyen-Tran HH, Chen CY, *et al*. IL6 and CCL18 Mediate Cross-talk between VHL-Deficient Kidney Cells and Macrophages during Development of Renal Cell Carcinoma. *Cancer Res* 2022;82:2716–33.
55 Gerges E, Cauvet A, Schwarz M, *et al*. Correction to: Association of serum interferon alpha-2a levels with disease severity and prognosis in systemic sclerosis. *Rheumatology (Sunnyvale)* 2025;64:3172.
56 Tian Y, Chen S, Yu B, *et al*. Identification of biomarkers and immune infiltration associated with sexes in SSc: a bioinformatics and machine learning. *Rheumatology (Oxford)* 2025;64:3806–15.
57 Komura K, Yanaba K, Bouaziz J-D, *et al*. Perspective to precision medicine in scleroderma. *Front Immunol* 2023;14:1298665.
58 Cutolo M, Villaggio B, Otsa K, *et al*. Altered circadian rhythms in rheumatoid arthritis patients play a role in the disease's symptoms. *Autoimmun Rev* 2005;4:497–502.
59 Cutolo M, Sulli A, Barone A, *et al*. Macrophages, synovial tissue and rheumatoid arthritis. *Clin Exp* 1993;11:331–9.