



UNIVERSITÀ DEGLI STUDI DI GENOVA

**SCUOLA DI SCIENZE MEDICHE E FARMACEUTICHE
DIPARTIMENTO DI MEDICINA INTERNA E SPECIALITÀ MEDICHE**

SCUOLA DI SPECIALIZZAZIONE IN REUMATOLOGIA

Increased expression of M2 pro-fibrotic markers in circulating monocytes and cultured monocyte-derived macrophages from systemic sclerosis patients with progressive interstitial lung disease (ILD).

Relatori:

Prof. Alberto Sulli

Prof. Maurizio Cutolo

Correlatrice:

Prof.ssa Sabrina Paolino

Candidato:

Dott.ssa Rosanna Campitiello

ANNO ACCADEMICO 2023-2024

General overview on systemic sclerosis

Systemic sclerosis (SSc), also known as scleroderma, is a rare and chronic autoimmune connective tissue disease that profoundly impacts affected individuals due to its complex pathogenesis and multisystem involvement (1).

This condition is characterized by a triad of microvascular dysfunction, immune system dysregulation, and progressive fibrosis of the skin and internal organs (2).

The clinical presentation of SSc is highly variable, ranging from limited skin involvement to severe multi-organ complications, which contribute to its high morbidity and mortality (1).

The underlying mechanisms of SSc are thought to involve an intricate interplay of genetic, environmental, and immunological factors, although the exact pathogenesis remains incompletely understood (1). Endothelial cell injury, likely triggered by environmental or infectious agents in genetically predisposed individuals, initiates the pathological cascade. The resulting microvascular damage leads to chronic inflammation, immune system activation, and a dysregulated repair process, which culminates in fibroblast activation and excessive extracellular matrix (ECM) deposition (3). Over time, this process contributes to progressive fibrosis of the skin and various internal organs, profoundly affecting their structure and function (4).

In SSc patients the skin is almost universally involved and is often the first site of clinically evident disease, characterized by thickening, induration, and fibrosis. Skin changes, including pigmentation abnormalities and telangiectasias, can significantly impact the quality of life and may serve as a visible marker of underlying systemic disease. Fibrosis is not limited to the skin but extends to major organs, including the lungs, heart, gastrointestinal tract, and kidneys. Pulmonary involvement is a major driver of morbidity and mortality, with interstitial lung disease (ILD) and pulmonary arterial hypertension (PAH) representing the most severe complications (5,6). The gastrointestinal tract is another frequently involved system, where motility disorders and fibrosis of the esophagus and intestines contribute to dysphagia, malabsorption, and nutritional deficiencies (7).

Clinically, SSc is classified into two main subsets: limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc), based on the extent and distribution of skin involvement. In lcSSc, skin fibrosis is confined to areas distal to the elbows and knees, often sparing the trunk, and is associated with a more indolent disease course. Conversely, dcSSc involves widespread skin thickening, including the trunk and proximal limbs, and is frequently associated with early and severe visceral involvement (8). These subsets are further distinguished by their autoantibody profiles: anti-centromere antibodies are typically associated with lcSSc, while anti-topoisomerase I (Scl-70) and anti-RNA polymerase III antibodies are more common in dcSSc, aiding both diagnosis and prognosis (9). The earliest and most frequent clinical manifestation of scleroderma-related microangiopathy is Raynaud's phenomenon (RP), which is reported in up to 96% of patients (1,10). Indeed, RP acts as functional regulatory response to common triggers like cold exposure or emotional stressors in primary RP, while, in secondary RP, it occurs as a manifestation of underlying pathological conditions, including SSc (11,12). It is noteworthy that nailfold videocapillaroscopy (NVC) is a non-invasive, reliable diagnostic tool that enables differentiation between primary and secondary Raynaud phenomenon, with characteristic capillary features indicative of SSc (13). Of note, in SSc, the characteristic microvascular damage observed through NCV is categorized into specific "scleroderma" patterns ("early," "active," and "late"), enabling a thorough assessment and quantification of microvascular alterations. (14-17).

The microvascular abnormalities, evaluated with NCV analysis in SSc patients, highlights the role of vascular damage and dysfunction in disease initiation and progression (18).

The lung SSc involvement characterized by a progressive lung disorder is marked by the accumulation of scar tissue in the lung's air sacs (19).

This condition is present in approximately 40% of SSc patients and tends to manifest particularly within the first five years of symptom onset (20).

Commonly, SSc-ILD remains asymptomatic for a long time, however, during chest auscultation, bilateral basal crackles can occasionally be detected on chest auscultation. Nevertheless, radiographic abnormalities can be detected in 75-90% of SSc patients through high-resolution computed tomography (HRCT), which is considered the gold standard for diagnosis (21).

Indeed, ILD radiological aspects are characterized by the presence of various abnormalities, including reticulation, traction bronchiectasis, traction bronchiolectasis, honeycomb cysts, ground-glass opacities, airspace consolidation, or other interstitial changes (22).

Moreover, pulmonary function tests (PFTs) support HRCT for screening and diagnosis of SSc-ILD (23).

In 2022 the American Thoracic Society guidelines came to a consensus to evaluate three different subgroups: initial diagnosis of SSc-ILD, stable SSc-ILD, and progressive SSc-ILD (24). Although the criteria for PPF used in the 2022 guideline were extrapolated from the patient population of patients with IPF, the committee felt it appropriate to adapt the criteria to define progressive SSc-ILD after eliminating the timeline for disease progression.

Therefore, progressive SSc-ILD was defined as manifesting at least two of the following three criteria during follow-up in patients with SSc-ILD: 1) worsening dyspnea or cough; 2) declining values for forced vital capacity (FVC) >5% or diffusing capacity of the lungs for carbon monoxide (DLCO) >10% or 3) radiological evidence of disease progression (radiological interpretation of increase in the extent or severity of ILD features on computed tomography [CT] assessed visually) (24).

The heterogeneity of SSc underscores the importance of early diagnosis and subclassification to guide management and predict outcomes. Advances in understanding the molecular and cellular mechanisms of SSc have shed light on the pivotal roles of endothelial cells, immune cells, and fibroblasts in its pathogenesis (2).

Indeed, in SSc peripheral blood circulating cells, primarily monocytes and T lymphocytes, are recruited and, along with tissue resident immune macrophages, they initiate the production of pro-fibrotic cytokines, such as transforming growth factor- β 1 (TGF- β 1) and interleukine-10 (IL-10) (25). Of note, the lineage monocyte-macrophages plays a crucial role in the pathogenesis of numerous autoimmune rheumatic diseases such as rheumatoid arthritis and SSc (26,27).

Over the past few decades, mounting evidence has focused on innate immune system, primarily monocytes and macrophages, as potential mediators of the fibrotic process in SSc (2). Macrophages can sense their microenvironment and dynamically adapt their behavior through phenotypic plasticity to perform their diverse functions in immune regulation and tissue maintenance (28). Therefore, macrophages are able to polarize into five morphological and functional states, from pro-inflammatory classically (M1) to pro-fibrotic alternative (M2) macrophages, according to microenvironmental stimuli (29). Of note, dramatic imbalance of M1/M2 macrophage ratio may contribute to pathological condition such rheumatoid arthritis, systemic lupus erythematosus, and SSc (26,27,30).

The present thesis aims to characterize phenotype and functional M2 markers in circulating monocytes and cultured monocyte-derived macrophages (MDMs) from SSc patients with progressive ILD compared to SSc with non-progressive ILD and no-ILD SSc patients. Additionally a detailed NVC analysis has been performed in our cohort of patients in order to shed a light on the possible association of microvascular damage and ILD progression.

Increased expression of M2 pro-fibrotic markers in circulating monocytes and cultured monocyte-derived macrophages from systemic sclerosis patients with progressive interstitial lung disease (ILD).

Background. The pathogenesis of systemic sclerosis (SSc), includes important roles for macrophages that are involved in immune response and progressive tissue fibrosis of skin and internal organs, including the lungs. In particular, alternatively activated macrophages (M2) seem to contribute in SSc to the development of interstitial lung disease (ILD) through the synthesis of profibrotic molecules.

Objectives. To characterize phenotype and functional M2 markers in circulating monocytes and cultured monocyte-derived macrophages (MDMs) from SSc patients (pts) with progressive ILD compared to SSc with non-progressive ILD and without ILD, and to explore profibrotic signals involved in ILD development.

Methods. Thirty-seven SSc patients (mean age 65 ± 13 years; 10 SSc with progressive ILD, 14 SSc with non-progressive ILD, 13 not affected by ILD (no-ILD) SSc patients), fulfilling the 2013 ACR/EULAR criteria for SSc, and 19 age-matched healthy controls (HC), were recruited at the Division of Clinical Rheumatology of Genova and Ghent Universities, after obtaining Ethical Committee approval and patients' informed consent. Circulating monocytes expressing macrophage scavenger receptors (CD204, CD163), mannose receptor-1 (CD206), identifying M2 macrophages, and toll-like receptor-4 (TLR4, M1 marker) were detected by Flow Cytometry (Navios Flow Cytometer) For the *in vitro* study were enrolled 28 SSc patients (mean age 64 ± 14 years; 6 no-ILD SSc patients, 10 SSc with non-progressive ILD, 12 SSc with progressive ILD and 5 age-matched HC. All cultured monocytes were differentiated into MDMs (phorbol myristate acetate) and maintained in Roswell Park Memorial Institute growth medium) for 24 hours. Gene expression and protein synthesis of the above-mentioned markers as well as Mer tyrosine kinase (MerTK), which is a functional marker expressed in M2 macrophages with intensive profibrotic activity (4), were evaluated by quantitative real-time polymerase chain reaction (PCR) and Western blotting (WB). Nailfold video capillaroscopy (NVC) analysis has been performed (Horus, Adamo srl, Italy). Statistical analysis was carried out using non-parametric tests.

Results. At Flow Cytometry analysis SSc pts with progressive ILD showed a significantly higher percentage of circulating TLR4⁺CD204⁺CD206⁺CD163⁺monocytes compared to SSc no-ILD pts ($p<0.05$). In addition, the percentage of these cells was found higher (not statically significant (ns)) in SSc pts with progressive ILD compared to those with non-progressive ILD.

Of note, cultured MDMs obtained from SSc patients with progressive ILD showed a significant higher gene expression and protein synthesis of TGF β 1 compaerd to MDMs obtained from non-progressive ILD and no-ILD SSc pts ($p<0.05$). Moreover, an higher upregulation of MerTK gene expression level was found more evident (fold increase) in progressive ILD compared to non-progressive ILD and no ILD SSc pts (ns).

Furthermore, gene expression of TLR4 was found significantly upregulated ($p<0.05$) and CD163 was found of higher in MDMs obtained from SSc pts with progressive ILD compared to MDMs compared to no-ILD SSc pts.

Interestingly, cultured MDMs obtained from SSc pts with progressive ILD showed a significantly increased protein synthesis of CD206, CD163, and MerTK compared to no-ILD SSc MDMs (CD206 and CD163: $p<0.05$; MerTK: $p<0.01$). Once again, the protein synthesis

of CD206, CD163 and MerTK was found numerically increased (ns) in MDMs obtained from SSc with progressive ILD compared to non-progressive ILD SSc pts.

In all the experiments the presence of TGF β 1, MerTK, TLR4, and M2 phenotype makers in MDMs from ILD SSc pts were found significantly higher compared to HC ($p < 0.01$ and $p < 0.05$). Capillary loss and significant prevalence of "Late" NVC scleroderma pattern ($p < 0.01$) has been observed in SSc patients with progressive ILD vs no-ILD.

Conclusions. The study shows for the first time that the functional M2 markers, TGF β 1 and MerTK ($p < 0.05$ for TGF β 1), are significantly more expressed at gene and protein level on cultured MDMs from progressive vs, non progressive SSc pts. Generally, SSc pts with ILD showed a significant higher cell percentage of hybrid TLR4 (M1 marker) and M2 markers on circulating monocytes compared to no-ILD SSc pts ($p < 0.05$). Interestingly, a higher trend of expression for the last markers was found in progressive vs non-progressive ILD pts (ns). In addition, progressive ILD patients showed at NVC, a significant for both reduction of the capillary number and higher prevalence of "Late" scleroderma pattern vs no-ILD ones. In conclusion, the significant increase of circulating hybrid TLR4⁺M2 monocytes, as well as increased MerTK and TGF β 1 (M2 markers) expression on cultured MDMs in ILD-SSc patients seems to represent potential biomarkers of progressive lung fibrosis at least in SSc.

Keywords Systemic sclerosis, Fibrosis, Interstitial lung disease, M2 macrophages

1. Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease, characterized by early vascular damage and immune system activation, and by progressive skin and organ fibrosis (1). Of note, SSc can affect multiple organs, such as skin, heart, kidneys, gastrointestinal, musculoskeletal system and lungs (7).

SSc-related interstitial lung disease (ILD) stands out as one of the most severe complications, affecting approximately 40% of SSc patients, particularly within the first five years of symptom onset (5,6,20).

Of note, SSc-ILD is characterized by progressive fibrosis of lung parenchyma, detectable through high-resolution computed tomography (HRCT) and pulmonary function tests. (21,23).

Notably, the 2022 American Thoracic Society guidelines define progressive SSc-ILD based on clinical symptoms (worsening dyspnea or cough), functional decline (FVC $> 5\%$ or DLCO $> 10\%$ in a year), and radiological progression observed within 12 months (24, 31).

To date, the pathogenesis of SSc remains incompletely understood but it may involve a complex interplay of genetic predisposition, epigenetic modifications and environmental exposures, and altered immune system response (4).

Of note, this interplay initiates a cascade of pathological events beginning with progressive microvascular damage and abnormal activation of both innate and adaptive immunity, leading to tissue fibrosis(2). As matter of facts, together with the progressive fibrotic damage, the activation of innate and adaptive immune responses represents a pivotal driver of SSc pathophysiology (32). Indeed, pro-fibrotic mediators such as transforming growth factor- β 1 (TGF β 1) activate fibroblasts, promoting to excessive deposition of extracellular matrix (ECM) components including collagen, resulting in fibrosis of the skin and internal organs (3, 33).

Over the past few decades, mounting evidence has focused on innate immune system components, primarily monocytes and macrophages, also as potential mediators of the fibrotic process in SSc (2,27).

Indeed, circulating inflammatory cells, primarily monocytes and T lymphocytes, are recruited and, along with tissue resident macrophages, they initiate the production of pro-fibrotic cytokines, such as TGF- β 1 and interleukine-10 (IL-10) (25).

Macrophages can sense their microenvironment and dynamically adapt their behavior through phenotypic plasticity to perform their diverse functions in immune regulation and tissue maintenance (28). Therefore, macrophages can polarize into five morphological and functional states, from classically (M1) to alternative (M2) macrophages, according to microenvironmental stimuli (29).

Pro-fibrotic M2 macrophages are phenotypically characterized by the expression of specific cell surface markers including the mannose receptor type C (CD206), macrophage scavenger receptor 1 (CD204), and hemoglobin scavenger receptor (CD163), and functional markers like MER proto-oncogene receptor tyrosine kinase (MerTK). These macrophages are central to wound healing and fibrosis, primarily secreting anti-inflammatory and pro-fibrotic molecules, including IL-10 and TGF β 1 (34).

Noteworthy, dramatic imbalance of M1/M2 macrophage ratio is implicated in various pathological conditions, including rheumatoid arthritis, systemic lupus erythematosus, and SSc (26,27,30).

A growing body of evidence has documented the presence of pro-fibrotic M2 macrophages in the affected tissues of SSc patients, deriving from circulating monocytes and resident macrophages activated by local mediators (35). Interestingly, circulating cells belonging to the monocyte/macrophage lineage and showing an hybrid M1/M2 phenotypes (characterized by the co-expression of M2 markers such as CD204, CD163, CD206, and M1 markers such as TLR4, CD80, and CD86) were identified in SSc patients (36). These hybrid phenotypes, which are significantly more prevalent in SSc patients compared to HC, are particularly associated with the presence of interstitial lung disease (ILD) and elevated pulmonary artery pressure (PAH), as well as SSc patients positive for anti-topoisomerase antibody, a known lung involvement predictor (36,37).

Furthermore, a recent study identified high concentrations of TLR4⁺M2 macrophages in lung biopsies 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 (38).

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

2. Materials and methods

2.1. SSc patients and HC

Blood samples were obtained from 51 SSc patients under the care of the Division of Clinical Rheumatology at the University of Genova, Italy and Ghent University. 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. All SSc patients met 2013 ACR/EULAR classification criteria for SSc (9). Instrumental examinations were part of their regular follow-up (39). The presence of ILD was confirmed through routinely performed HRCT and lung function tests (40,41). In our cohort of patients, the definition of ILD followed the 2022 American Thoracic

Society guidelines for IPF 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 recognized interstitial composite pattern (41). To define progressive ILD in SSc, the following criteria have been adopted: a decline in forced vital capacity (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 diffusing capacity for carbon monoxide (DLCO) of at least 15% predicted, within a 12-month period or worsened respiratory symptoms and increased extent of fibrosis on HRCT (42). Skin involvement was present in all subjects and was classified according to LeRoy and Medsger criteria (43). Demographic data and pulmonary function tests of SSc patients at time of blood sample are summarized in table 1. and table 2. Finally, 25 age and sex matched were enrolled into the study.

2.2. Flow cytometry and gating strategy

In the peripheral blood of 37 SSc patients, the monocyte lineage was characterized by investigating the surface markers CD14, CD16 and CD45, using conjugated primary antibodies anti-human CD14-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany), CD16-APC AlexaFluor 700, and CD45-Krome Orange (Beckman Coulter, Brea, California, USA). The M1 phenotype of circulating monocytes was defined through the detection of surface markers CD80 and TLR4 using conjugated primary antibodies anti-human CD80-PEVio770 (Miltenyi Biotec) and TLR4-BV421 (Becton Dickinson-BD Biosciences, Franklin Lakes, New Jersey, USA), whereas to identify M2 phenotype, the surface markers CD163, CD204 and CD206 were investigated using conjugated primary antibodies anti-human CD163-PEVio615, CD204-PE and CD206-PerCPVio700 (Miltenyi Biotec).

Moreover, to exclude the presence of dendritic cells in the monocyte population, CD1c was investigated using the conjugated primary anti-human CD1c-APC Cy7 antibody.

Flow cytometry analysis was performed using Navios Flow Cytometer and Kaluza analysis software, evaluating a total of 5×10^6 cells and detecting more than 30 events in the smallest subset investigated (44,45).

The gating strategy used in this study started from the detection of the monocyte population characterized as CD45⁺CD14⁺CD16⁺ cells in the leukocyte population (CD45⁺ cells) excluding both lymphocytes and granulocytes, as recently been described by our group (38). The value in the box plots of the percentage of the investigated subsets of circulating monocytes is referred to the leukocyte population (45).

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

In a subgroup of 20 enrolled SSc patients not under therapy with nintedanib, (Tyrosin 2 kinase inhibitor), 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=12), SSc patients with non-progressive ILD (n=10) and no-ILD SSc patients (n=6). Moreover, 5 HC were included in the analysis.

Monocytes were isolated using the EasySep human monocyte enrichment kit without CD16 depletion (Stemcell Technologies, Vancouver, Canada). 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 (RPMI) 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 (46).

At the end of PMA stimulation, the medium was removed, and cultured MDMs were maintained for additional 24 hours in growth medium without any stimulation. Cell supernatant was collected and stored at -80°C, 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 protein synthesis of the above-mentioned M2 markers and TLR4.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated using the RNA/Protein Purification Plus kit (Norgen Biotech) and quantified by nanodrop to assess its integrity. For each experimental condition, first-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA, using QuantiTect Reverse Transcription Kit (Qiagen, Milan, Italy). The qRT-PCR was performed on an Eppendorf Realplex 4 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,) as cell membrane markers, and primers for human MerTK (NM_006343), TGFβ1 (NM_000660), as functional M2 markers. Primers for human β-actin (NM_001101) was used as housekeeping gene. Gene expression values have been calculated using the comparative $\Delta\Delta$ cycle threshold ($\Delta\Delta$ CT) method and corresponded to the expression level (fold-increase) of the target gene (47). The melting curve was included in all qRT-PCR assays to confirm the specificity of the SYBR green assay.

2.5. Western blotting

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 Signaling Technology, Danvers, Massachusetts, USA) 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 Signaling 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 HC), the value of the protein synthesis of the investigated molecules was normalized to that of the GAPDH as housekeeping protein. The resulting value of each sample of SSc patients was then normalized to that of the corresponding HC (considered as the unit value).

2.6. 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-technie, Minneapolis, Minnesota, USA). In the first phase of the procedure (sample activation step), 80μL of sample was mixed with 20μL of 1 N HCl and incubated for 10 min at room temperature to acidify the sample. At the end of incubation, the acidification of samples was neutralized by adding 20μL of 1.2 N NaOH/0.5 M HEPES solution to obtain an activated cell culture supernatant, which was subsequently diluted 5 folds with a specific sample diluent (20μL of activated sample was added to 80μL of sample diluent). At the end of this phase, 50μL of each diluted sample was transferred into the TGFβ1 Ella cartridge. TGFβ1 level was expressed as pg/mL.

2.7. Nailfold capillaroscopy examination

In order to analyze the NVC parameters, the images, were recorded using a 200 × magnification optical probe (Horus, Adamo srl Italy), connected to an image analysis software at the videocapillaroscopy Service at the Division of Rheumatology of the University of Genova. As for standard protocol each patient waited for a minimum of 15 min in a room at a temperature range of 20–22 °C before NVC (13). The NVC parameters included the NVC SSc patterns, according to Cutolo et al. and was followed the EULAR Study Group on Microcirculation in Rheumatic Diseases standardized capillaroscopy evaluation (13,14). The absolute number of capillaries refers to the average count of capillaries per linear millimeter (mm) across all NVC images analyzed during the examination. It is worth noting that semiquantitative scoring systems assess NVC characteristics, including capillary dilations, microhemorrhages, giant capillaries, reduced capillary density, altered microvascular architecture, and angiogenesis (17). The scores were determined by assigning a rank from 0 to 3 for each domain based on the severity of NVC changes: 0 indicating no changes, 1 for changes affecting ≤33%, 2 for 33–66% changes, and 3 for changes involving ≥66%. The overall score was obtained by summing the grades assigned to each domain, with higher scores indicating greater microvascular damage.

2.8. Statistical analysis

Statistical analysis was carried out by non-parametric Mann–Whitney U test using GraphPad Prism (version 8.4.0, GraphPad Software, San Diego, CA, USA) to compare gene and protein expression of investigated M2 markers between cultured MDMs obtained from HC, SSc patients no-ILD, 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 analyzed and graphically reported as median with range. Statistical analyses included the Chi-Square test (χ^2) with Yate 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).

2.9. Ethics statement

Blood sample analyses were carried out in accordance with the recommendations of IRCCS Ospedale Policlinico San Martino (Genova, Italy) with written informed consent from all

patients as standard procedures. The blood collection was approved by the Ethics Committee of IRCCS Ospedale Policlinico San Martino (273-reg-2015).

3. Results

3.1. Demographic data of SSc patients with progressive ILD compared to 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: the mean age was 61 ± 13.6 years in SSc progressive ILD patients, 66 ± 14 years SSc in non progressive ILD and 66.1 ± 21.6 years in no-ILD SSc patients; the mean disease duration was 7.1 ± 4.4 years in SSc progressive ILD patients, 7.6 ± 6.6 years SSc non progressive ILD and 5.6 ± 4.4 years SSc patients no-ILD; there were 8 female and 2 male SSc progressive ILD patients, 12 female and 2 male SSc non progressive ILD and 6 female and 0 male no-ILD SSc patients.

Demographics, SSc organ involvement, and therapies are summarized in Table I.

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 SSc non progressive ILD and 66.7 ± 17 years no-ILD SSc patients; the mean disease duration was 13.9 ± 8.5 years in SSc progressive ILD patients, 8.8 ± 7.4 years SSc non progressive ILD and 7.8 ± 5.1 years SSc patients no-ILD; 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 summarized in Table II.

3.2. Increased percentage of circulating TLR4⁺M2 monocytes in SSc patients with progressive ILD compared to 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.

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

No differences in the percentage of circulating monocytes showing a M2 phenotype (TLR4⁻CD80⁻CD204⁺CD206⁺CD163⁺CD14⁺CD16⁺CD45⁺ cells) in SSc patients with progressive ILD compared to SSc patients with non-progressive ILD and no-ILD SSc patients was observed (Figure 1).

The results also showed that the percentage of circulating TLR4⁺M2 monocytes and M2 monocytes was significantly higher in SSc patients compared to HC (no-ILD 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).

3.3. Increased gene expression of MerTK and TGF β 1 in cultured MDMs obtained from SSc patients with progressive ILD compared to 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 TGF β 1 compared to MDMs obtained from both SSc patients with non-progressive ILD and no-ILD SSc patients ($p < 0.05$) (Figure 2).

Moreover, the MDMs obtained from SSc patients with progressive ILD were characterized by a higher gene expression of MerTK compared to MDMs obtained from SSc patients with non-progressive ILD and no-ILD SSc patients (mean fold increase: 1.7 ± 0.2 vs. 1.4 ± 0.87 , and 1.2 ± 0.66 , respectively), whereas the gene expression of TLR4 was significantly higher compared to no-ILD SSc patients ($p < 0.05$) (Figure 2).

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

Of note, cultured MDMs obtained from SSc patients with ILD (both progressive and non-progressive) were characterized by a significant upregulation of the gene expression of CD163 compared to MDMs obtained from no-ILD SSc patients ($p < 0.05$; $p < 0.01$) (Figure 2). As expected, CD206 gene expression was significantly upregulated in cultured MDMs obtained from SSc patients both with progressive and non-progressive ILD compared to HC ($p < 0.05$), whereas, CD204 gene expression was significantly upregulated in MDMs obtained from all SSc patients' groups ($p < 0.05$ for all vs. HC-MDMs) (Figure 2).

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

Cultured MDMs obtained from SSc patients with progressive ILD were characterized by a significant increase in protein synthesis of CD206, CD163 and MerTK compared to MDMs obtained from no-ILD SSc patients (CD206 and CD163: $p < 0.05$; MerTK: $p < 0.01$) (Figure 3). Moreover, the protein synthesis of these M2 markers was higher in cultured MDMs obtained from SSc patients with progressive ILD compared from those obtained from SSc patients with non-progressive ILD, although the trend to higher values was non statistically significant (mean \pm SD protein synthesis CD206: 3.37 ± 1.9 vs. 2.96 ± 0.95 ; CD163: 7.79 ± 6.8 vs $3,23 \pm 1.8$; MerTK: 2.33 ± 0.7 vs. 1.4 ± 0.4) (Figure 3).

Of note, only cultured MDMs obtained from SSc-ILD patients (both with progressive and non-progressive ILD) showed a significant increase in protein synthesis of CD163 and MerTK compared to MDMs obtained from HC ($p < 0.01$ for SSc patients with progressive ILD; $p < 0.05$ for SSc patients with non-progressive ILD, for both molecules) (Figure 3).

Again as expected, Western blotting analysis confirmed that cultured MDMs obtained from all SSc patients' groups were characterized by a significant increase of the protein synthesis of M2/M1 markers, namely CD206 and CD204, TLR4, compared to MDMs obtained from HC (CD206 and CD204: $p < 0.05$ for MDMs no-ILD SSc; $p < 0.01$ for MDMs of progressive and non-progressive ILD; TLR4: $p < 0.05$ for all SSc patients' groups) (Figure 3).

3.5. Increased production and release of TGF β 1 in cultured MDMs obtained from SSc patients with progressive ILD compared to patients with non progressive ILD, no-ILD and HC

Based on the results at gene expression level, the production and release of the active form of the pro-fibrotic growth factor TGF β 1 was investigated by ELISA assay in the cell supernatants of cultured MDMs obtained from SSc patients and HC.

Cultured MDMs obtained from SSc patients with progressive ILD were characterized by a significantly higher production and release of the active form of TGF β 1 compared to MDMs obtained from SSc patients with non-progressive ILD and from no-ILD SSc patients ($p < 0.05$

vs. both), as well as compared to MDMs obtained from HC ($p < 0.01$) (Figure 4). These results on protein synthesis and release confirmed those obtained at gene expression level.

3.6. NCV analysis in ILD SSc patients

These findings at NVC analysis indicate that capillary density is significantly reduced in patients with progressive ILD compared to those with no-ILD SSc patients (Table III).

Data shows a higher statistically significant prevalence of “Late” scleroderma pattern SSc patients with progressive ILD vs no-ILD SSc patients ($\chi^2_{(1,46)} = 12.3$, $p < 0.01$).

Spearman correlation analysis demonstrated a negative relationship between TLR4+CD80-M2 monocytes and capillary density (measured as capillaries per linear mm at NVC analysis), suggesting that higher levels of TLR4+CD80-M2 monocytes are associated with reduced capillary density ($r(34) = -0.3581$, $p < 0.05$).

4. Discussion

This study highlights for the first time that SSc patients with progressive ILD show a significantly higher percentage of circulating hybrid TLR4⁺M2 monocytes compared to patients with non-progressive ILD and no-ILD, suggesting an involvement of these cells to contribute to the lung fibrosis progression. The absence of CD1c (marker for dendritic cells), further validated the monocyte-specific nature of these circulating cells (48).

Notably, the presence of these hybrid monocytes was previously found non significant in HC, supporting the actual results that suggest their potential relevance as a biomarker of ILD progression in SSc. The finding of this study aligns with previous one showing an increased prevalence of M1/M2 hybrid phenotypes in SSc patients with lung involvement, linking these cells to ILD development and severity (49).

The *in vitro* evaluation of MDMs in SSc patients revealed that cultured macrophages obtained from SSc patients with progressive ILD showed a significant upregulation of MerTK, TGF β 1, and CD163 at both the gene and protein level compared to MDMs obtained from no-ILD SSc patients. These findings suggest that macrophages in progressive ILD might adopt a profibrotic phenotype capable of driving fibroblast/myofibroblasts activation and excessive extracellular matrix protein production (i.e. collagen). The overexpression of MerTK, a marker associated with efferocytosis and fibrogenesis, further supports the involvement of this M phenotype in the fibrotic process (50).

Interestingly, MerTK, is a member of the Tyro-3, Axl, and TAM receptor tyrosine kinase family, and is implicated in various fibrotic conditions, including pulmonary and hepatic fibrosis, and plays a role in recognizing apoptotic cells during efferocytosis (51).

Notably, MerTK induces TGF β 1 expression, which activates fibrotic cells, such as fibroblasts, into myofibroblasts, enhancing collagen production during wound healing (52). Similarly, in lung fibrosis, macrophages with a high MerTK expression have been shown to drive TGF β 1 production, which further activates fibrotic cells, including fibroblasts, leading to tissue fibrosis (53).

Indeed, the TGF β /Smad and Wnt/ β -catenin signaling pathways are activated in M2 macrophages stimulating the fibrotic process (54).

The identification of macrophages co-expressing TLR4 and the M2 cell surface markers (CD206, CD163, CD204) in fibrotic parenchyma of SSc-ILD reinforce their potential role as pathogenic elements and/or biomarkers for SSc-related lung involvement (38).

Therefore, 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 macrophage MerTK positive (55).

In addition, the higher synthesis and release of the active form of TGF β 1 observed in MDMs from SSc patients with progressive ILD highlight these macrophages as pivotal mediators in the interplay between immune signaling and fibrotic tissue remodeling. This underscores their potential as a promising therapeutic target for managing ILD.

Notably, a recent *in vitro* study has reported for the first time that nintedanib, a tyrosine kinase inhibitor and antifibrotic drug currently used in SSc-ILD treatment, significantly downregulates the expression of MerTK. This finding offers new insights into how nintedanib may mitigate fibrosis by interfering with pathways involving the tyrosine kinase receptor MerTK (46).

Concerning NCV analysis, the higher prevalence of "Late" scleroderma pattern in SSc patients with progressive ILD observed in the present manuscript might serve as a potential morphological and quantifiable marker for identifying patients at risk of more severe lung fibrosis, offering a critical insight for patient management and prognosis.

On the other hands, HRCT is widely recognized as the gold standard for diagnosing and monitoring ILD-SSc, whereas NVC, a non-invasive, cost-effective imaging technique, has gained significant attention as a further potential biomarker for microvascular damage in ILD SSc patients (56).

Recent studies further substantiate the role of NVC in ILD, highlighting its value in detecting microvascular changes associated with disease progression. For instance, Smith et al. discuss the potential of NVC as a proxy for lung involvement in connective tissue diseases, emphasizing its utility in ILD management (57).

Furthermore, a detailed analysis of the EUSTAR database showed a significant correlation between the progression of NCV abnormalities in SSc patients and a higher annual risk of developing ILD (58).

Interestingly, a recent manuscript revealed that both CTD-ILD and non-CTD-ILD exhibit NVC abnormalities that worsen with disease duration (59).

Indeed, the association of advanced NVC findings of altered microcirculation with ILD progression, reinforces its value as a non-invasive tool for early detection and monitoring (57).

Additionally, NVC detects characteristic microvascular abnormalities, such as capillary loss, which are classified into distinct scleroderma patterns ("Early," "Active," and "Late") and this latter pattern, characterized by severe capillary loss has been associated with anti-topoisomerase autoantibodies (SCL70), frequently observed in ILD-SSc (13,60). As mentioned, hybrid monocytes co-expressing of M1 and M2 surface markers was found associated to ILD presence in SSc patients and correlated with ILD severity (lower DLCO% and higher ratio FVC/DLCO), as well as SCL70 positivity (37).

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. Longitudinal studies are required to validate these findings and explore whether therapeutic modulation of these pathways can influence clinical outcomes. Future research should also investigate the interaction between M2 macrophages and other immune cells, as well as their involvement in microvascular damage.

In conclusion, the detection of significant increased of circulating hybrid TLR4⁺M2 monocytes, as well as the increased expression of MerTK and TGF β 1 (M2 functional markers) on cultured MDMs in ILD-SSc patients, might represent potential biomarkers of progressive lung fibrosis at least in SSc. Further studies are ongoing to investigate the functional mechanisms linking hybrid TLR4⁺M2 monocytes to progressive fibrosis.

5. Conclusion

This manuscript highlights the presence of circulating hybrid TLR4+M2 monocytes with elevated MerTK and TGF β 1 expression in SSc patients with progressive ILD. These patients also exhibited reduced capillary density and a higher prevalence of the "Late" scleroderma pattern. Together, these findings suggest that TGF β 1, MerTK, hybrid TLR4+M2 monocytes, and the "Late" scleroderma pattern at NVC could serve as potential biomarkers for progressive lung fibrosis in SSc.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MC and VS: conceptualization, data curation, methodology, writing – original draft, writing – review & editing.

SS: conceptualization, data curation, formal analysis, methodology, writing – original draft, writing – review & editing.

RC: Patient enrollment, formal analysis, data curation, writing – review & editing.

EG: Formal analysis, Writing – review & editing.

AC: Patient enrollment, formal analysis.

EH: Review & approving.

SP: Review & approving.

CP: Review & approving.

AS: Review & approving.

Funding

The author(s) declare that no financial support for the research, authorship, and/or publication of this article.

Acknowledgments

IRCCS Ospedale Policlinico San Martino and Laboratory for Experimental Rheumatology, Academic Division of Clinical Rheumatology 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 Centers of Excellence for Imaging of EULAR (European Alliance of Associations for Rheumatology). VS is 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, nor in the decision to submit the article for publication.

Conflict of interest

The authors state that the research was carried out without any commercial or financial relationships that could be perceived as a potential conflict of interest.

Figure legends.

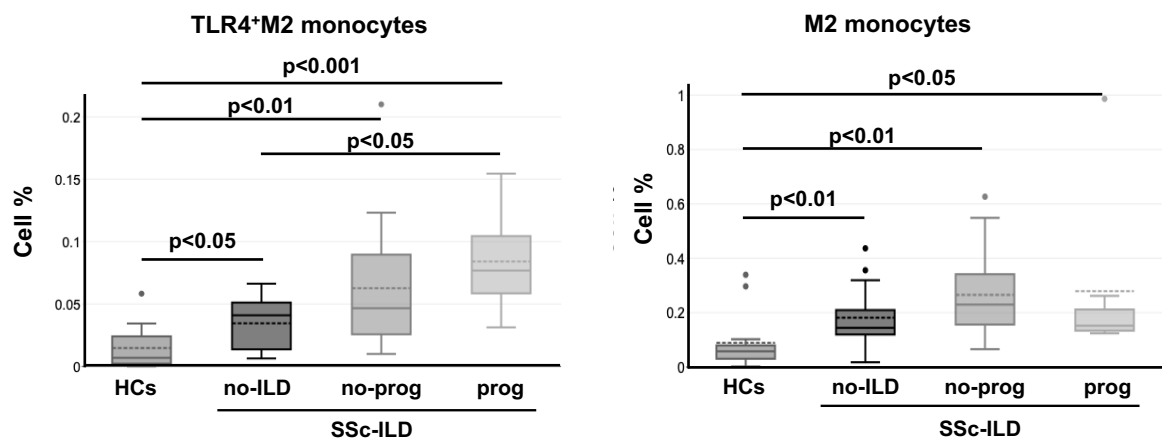


Figure 1. Flow cytometry analysis of circulating monocytes from progressive ILD SSc, non-progressive ILD, no-ILD SSc patients and HC-MDMs Representative flow cytometry analysis to identify TLR4+M2 monocytes (CD1c-CD80-TLR4+CD163+CD204+CD206+cells) and M2 monocytes (CD1c-CD80-TLR4-CD163+CD204+CD206+cells) from 10 progressive ILD SSc , 14 non-progressive ILD, 13 no-ILD SSc and 20 HC in the peripheral blood of related box plot representation of the percentage of these cells was included.

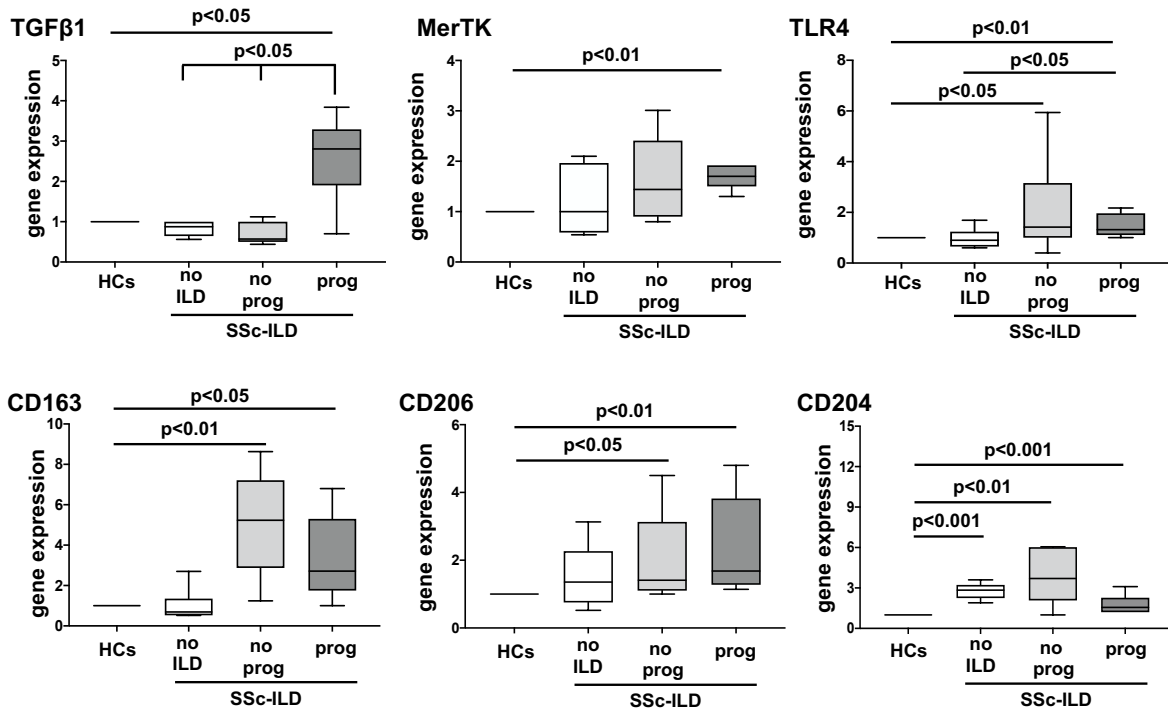


Figure 2. Gene expression of TLR4, functional and cell surface markers of profibrotic M2 phenotype in cultured MDMs obtained from progressive ILD SSc, non-progressive ILD, no-ILD SSc and HC (taken as unit value).

Evaluation by quantitative real time polymerase chain reaction (qRT-PCR) of the gene expression of TGFβ1, MerTK, TLR4, CD163, CD206 and CD204 in cultures of monocyte-derived macrophages (MDMs) obtained from 12 progressive ILD SSc, 10 non-progressive ILD, 6 no-ILD SSc and 5 HC. The resulting value of the gene expression of each molecule in cultured MDMs from progressive SSc, non-prog SSc and no-ILD SSc patients was compared with that obtained in cultured HC-MDMs (taken as unit value). Data are reported as median with range of fold increase compared to HC.

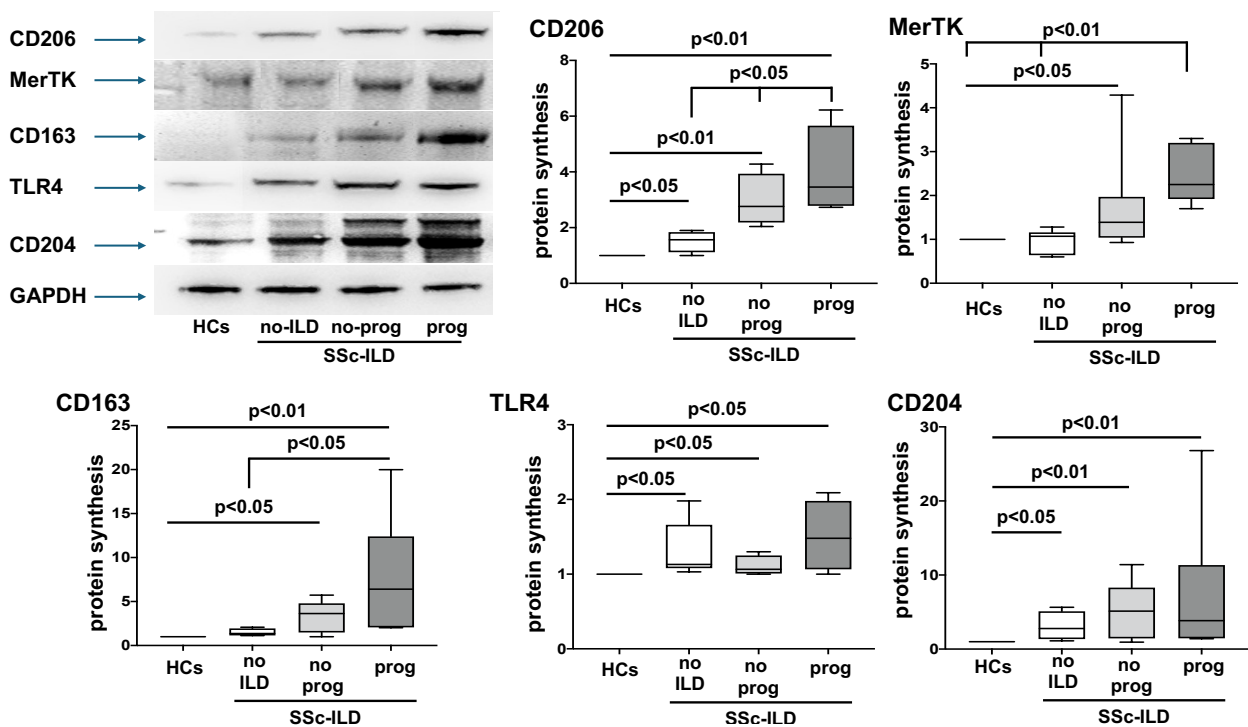


Figure 3. Protein synthesis of TLR4, functional and cell surface markers of profibrotic M2 phenotype in cultured MDMs obtained from progressive ILD SSc , non-progressive ILD, no-ILD SSc and HC.

Evaluation by Western blotting and related densitometric analysis of the protein synthesis of CD206, MerTK, CD163, TLR4 and CD204 in cultures of monocyte-derived macrophages (MDMs) obtained from 12 progressive ILD SSc , 10 non-progressive ILD, 6 no-ILD SSc and 5 HC. The value of protein expression of the investigated molecules was normalized to that of the corresponding GAPDH in cultured MDMs from progressive ILD SSc , non-progressive ILD, no-ILD SSc and HC.

The resulting value of the protein synthesis of each molecule in cultured MDMs from progressive SSc, non-prog SSc and no-ILD SSc patients was compared with that obtained in cultured HC-MDMs (taken as unit value). Data are reported as median with range of fold increase compared to HC.

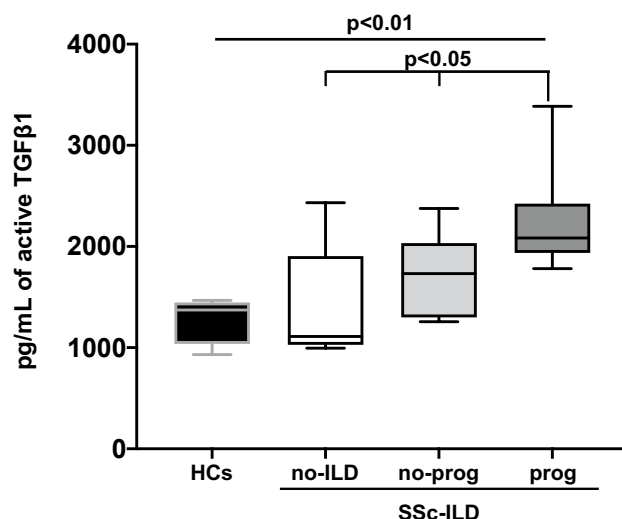


Figure 4. TGFβ1 protein synthesis of cultured MDMs obtained from healthy subjects, SSc patients without ILD and SSc patients with non-progressive and progressive ILD.

Evaluation by ELISA of the release of active TGFβ1 in conditioned medium of cultured monocytes-derived macrophages (MDMs) obtained from 12 progressive ILD SSc, 10 non-progressive ILD, 6 no-ILD SSc and 5 HC. The resulting value of TGFβ1 protein synthesis in cultured MDMs from progressive SSc, non-prog SSc and no-ILD SSc patients was compared with that obtained in cultured HC-MDMs (taken as unit value). Data are reported as median with range of fold increase compared to HC.

Table I

Demographic and clinical data of SSc patients included in Flow cytometry evaluation.

	SSc progressive ILD (10)	SSc non progressive ILD (14)	SSc no-ILD (13)
Age (years, M± SD)	61±13.6	66±14	66.1±21.6
Gender (Female/Male)	8/2	12/2	6/0
Disease duration (years, M±SD)	7.1±4.4	7.6±6	5.6±4.4
Smoker/non smoker	1(10)	1(7)	0(0)
ANTIBODY PROFILE^a n (%)			
Anti-centromere	0 (0)	4 (29)	7 (64)
Anti-Scl70	8 (80)	7 (50)	1 (9)
Anti-RNA polymerase III	0 (0)	0 (0)	0 (0)
Only ANA positive	0 (0)	0 (0)	1 (9)
Other SSc-associated antibodies ^b	2 (20)	6 (3)	3 (27)
ANA negative	0(0)	0 (0)	0 (0)
CLINICAL MANIFESTATIONS n (%)			
Skin involvement (lcSSc / dcSSc)	3/7	7/7	9/4
RP	10 (100)	14 (100)	13 (100)
DUs	8 (80)	9 (64)	5 (38)
PAH	0 (0)	1 (7)	0 (0)
ILD	10 (100)	14 (100)	0 (0)
Gastrointestinal involvement	7 (70)	9 (64)	7 (54)
Kidney involvement	2 (20)	1(7)	0 (0)
PULMONARY FUNCTION TESTS (reference percentage, M±SD)			
FVC	74±33.6	93±27.8	105±23
DLCO/VA	75.3±29.5	78.5±18.5	84.2±17.3
THERAPY n° (%)			
	MMF=6(60)	MMF=5(35)	MMF=3(25)
	MTX=3(30)	MTX=1(8)	MTX=4(33)
	RTX=1(10)	RTX=1(7)	RTX=0(0)
	AZA=0	AZA=0	AZA=0
	CYC=1(10)	CYC=0(0)	CYC=0(0)
	HCQ=0	HCQ=0	HCQ=0(0)

	Ca-ant =3(30) ACEi=6(60) PDE5i=0(0) ERA=3(30) Prostanoids=10(100) Selexipag =0(0) Riociguat =0(0) Nintedanib=3(30)	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)	Ca-ant =4(30) ACEi=7(53) PDE5i=0(0) ERA=2(15) Prostanoids=11(85) Selexipag = 0 Riociguat = 0 Nintedanib= 0(0)
NVC scleroderma patterns (mean ± SD)			
“Early”	1(10)	3 (22)	1 (7)
“Active”	2 (20)	6 (42)	10 (77)
“Late”	7 (70)	5 (35)	2 (15)
NVC FINDINGS (mean ± SD)			
Mean capillary absolute number per linear millimetre	4.1±1.1	6±1.8	6.4±1.4
Dilated capillaries (score 0-3)	1.8±0.4	1.9±0.3	1.7±0.5
Giant capillaries (score 0-3)	0.8±0.8	0.8±0.8	1.3±0.75
Haemorrhages (score 0-3)	0.5±0.5	0.7±0.5	0.8±0.6
Neoangiogenesis (score 0-3)	1.3±0.7	1.6±0.7	1.4±0.5
Capillary loss (score 0-3)	1.7±0.8	1.1±0.8	0.9±0.5

^aPositivity for more than one antibody was observed.

^bIncludes the following autoantibodies: anti-RNA polymerase III, anti-U3 RNP, anti-PmScl75, anti-PmScl100, anti-NOR90, anti-Th/To, anti-Ku and anti-SSA.

Abbreviations: M: mean; SD: standard deviation; ANA: anti-nuclear antibodies; ENA: extractable nuclear antigen antibodies; RP: Raynaud phenomenon; DUs: digital ulcers; PAH: pulmonary arterial hypertension; ILD: interstitial lung disease; lcSSc: limited cutaneous systemic sclerosis; dcSSc: diffuse cutaneous systemic sclerosis; MSK: musculoskeletal; mRSS: modified Rodnan Skin Score; NVC: nailfold videocapillaroscopy; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; DLCO: diffusing capacity for carbon monoxide; MMF: mycophenolate mofetil; MTX: methotrexate; RTX: rituximab; AZA: azathioprine; CYC: cyclophosphamide; HCQ: hydroxychloroquine; Ca-ant: calcium channel antagonist; ACEi: angiotensin-converting enzyme inhibitor; PDE5i: phosphodiesterase type 5 inhibitor; ERA: endothelin receptor antagonist.

Table II
Demographic and clinical data of SSc patients included in *in vitro* experiments (cultured MDMs) .

SSc (n)	SSc progressive ILD (12)	SSc non progressive ILD (10)	SSc no ILD (6)
Age (years, M± SD)	61.8±15.7	67±9	66.7±17
Gender (Female/Male)	9/3	7/1	6/0
Disease duration (years, M±SD)	13.9±8.5	8.8±7.4	7.8±5.1
Smokers n (%)	4(36)	2(20)	1(8)
ANTIBODY PROFILE^a n (%)			
Anti-centromere	1(8)	3(30)	1(16)
Anti-Scl70	6(50)	1(10)	2(33)
Anti-RNA polymerase III	1(8) 4(33)	1(10) 4(40)	1(16) 2(33)
Only ANA positive	3(25)	0(0)	0(0)
Other SSc-associated antibodies ^b	0(0)	2(20)	0(0)
ANA negative			
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)
Gastrointestinal involvement	8(66)	4(40)	5(83)
Kidney involvement	2(16)	1(10)	0(0)
PULMONARY FUNCTION TESTS (reference percentage, M±SD)			

FVC	82±16	83±39	102.6±8.4
DLCO/VA	84.8±15	85±28	87±14
THERAPY n° (%)			
	MMF=6(50)	MMF=1(10)	MMF=6(100)
	MTX=5(41)	MTX=2(20)	MTX=3(50)
	RTX=5(41)	RTX=0(0)	RTX=1(16)
	AZA=0	AZA=0	AZA=0
	CYC=0	CYC=1(10)	CYC=1(16)
	HCQ=0	HCQ=0	HCQ=0(0)
	Ca-ant =3(25)	Ca-ant=1(10)	Ca-ant =3(20)
	ACEi=5(41)	ACEi=4(40)	ACEi=1(16)
	PDE5i=1(8)	PDE5i=2(20)	PDE5i=1(16)
	ERA=6(50)	ERA=2(20)	ERA=1(16)
	Prostanoids=8(66)	Prostanoids=6(60)	Prostanoids=6(100)
	Selexipag=0(0)	Selexipag=0(0)	Selexipag = 0
	Riociguat=1(8)	Riociguat=0(0)	Riociguat = 0
	Nintedanib=0(0)	Nintedanib=0(0)	Nintedanib=0(0)
NVC patterns			
“Early”	1(8)	0(0)	0(0)
“Active”	1(8)	3(30)	3(50)
“Late”	6(50)	3(30)	1(16)
Normal	0(0)	0(0)	0(0)
Non-specific alterations	1(8)	1(10)	2(33)
Not performed	3(25)	2(20)	0(0)
NVC findings			
(Mean±SD)			

Mean capillary number per linear millimetre	4.5±1.7	6.1±1.5	6.5±1.6
Capillary loss (score 0-3)	2.2±1	1.1±0.8	0.5±0.5
Dilated capillaries (score 0-3)	1.3±0.5	1.3±0.5	1.8±0.4
Giant capillaries (score 0-3)	0.4±0.5	0.5±0.8	0.8±0.75
Haemorrhages (score 0-3)	0.7±0.5	0.4±0.5	0.7±0.8
Neoangiogenesis (score 0-3)	1.2±0.7	0.6±0.9	1.3±0.8

^aPositivity for more than one antibody was observed.

^bIncludes the following autoantibodies: anti-RNA polymerase III, anti-U3 RNP, anti-PmScl175, anti-PmScl100, anti-NOR90, anti-Th/To, anti-Ku and anti-SSA.

Abbreviations: N: number; M: mean; SD: standard deviation; ANA: anti-nuclear antibodies; ENA: extractable nuclear antigen antibodies; RP: Raynaud phenomenon; DUs: digital ulcers; PAH: pulmonary arterial hypertension; ILD: interstitial lung disease; lcSSc: limited cutaneous systemic sclerosis; dcSSc: diffuse cutaneous systemic sclerosis; MSK: musculoskeletal; nailfold videocapillaroscopy; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; DLCO: diffusing capacity for carbon monoxide; MMF: mycophenolate mofetil; MTX: methotrexate; RTX: rituximab; AZA: azathioprine; CYC: cyclophosphamide; HCQ: hydroxychloroquine; Ca-ant: calcium channel antagonist; ACEi: angiotensin-converting enzyme inhibitor; PDE5i: phosphodiesterase type 5 inhibitor; ERA: endothelin receptor antagonist.

Table III
Statistical analysis of nailfold videocapillaroscopy abnormalities in SSc patients

SSc (n) (Mean±SD)	SSc progressive ILD (16)	SSc non progressive ILD (20)	SSc no ILD (20)	p value ^a	P value ^b
Number capillary per linear mm	4.8±1.3	5.6±1.3	6.4±1.1	ns	<0.05
Capillary loss score (0-3)	1.8±0.9	1.2±0.7	0.7±0.5	ns	<0.01

P-values equal or lower than 0.05 were considered statistically significant.

^a P-values comparisons between ILD progressive and ILD non progressive SSc patients

^b P-values comparisons between ILD progressive and no-ILD SSc patients

Bibliography

1. Volkman ER, Andréasson K, Smith V. Systemic sclerosis. *Lancet*. 2023;401(10373):304-318.
2. Cutolo M, Soldano S, Smith V. Pathophysiology of systemic sclerosis: current understanding and new insights. *Expert Rev Clin Immunol*. 2019;15(7):753-764.
3. van Caam A, Vonk M, van Den Hoogen F, et al. Unraveling SSc pathophysiology; the myofibroblast. *Front Immunol*. 2018;9:2452–2463.
4. Denton CP, Khanna D. Systemic sclerosis. *Lancet*. 2017;390(10103):1685-99.
5. Tyndall AJ, Bannert B, Vonk M, Airò P, Cozzi F, Carreira PE, 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(10):1809-15.
6. Elhai M, Meune C, Boubaya M, Avouac J, Hachulla E, Balbir-Gurman, et al. Mapping and predicting mortality from systemic sclerosis. *Ann Rheum Dis*. 2017;76(11):1897-1905.
7. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. *N Engl J Med*. 2009;360:1989–2003.
8. Wollheim FA. Classification of systemic sclerosis. Visions and reality. *Rheumatology (Oxford)*. 2005;44(10):1212-6.
9. Van den Hoogen F et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis*. 2013;72(11):1747-55.
10. Cutolo M, Sulli A, Smith V. Assessing microvascular changes in systemic sclerosis diagnosis and management. *Nat Rev Rheumatol*. 2010;6(10):578-87.
11. Hughes M, Herrick AL. Raynaud's Phenomenon. *Best Pract Res Clin Rheumatol*. 2016;30:112–132.
12. Pauling JD, Hughes M, Pope JE. Raynaud's phenomenon - an update on diagnosis, classification and management. *Clin Rheumatol*. 2019;38:3317–30.
13. Smith V, Herrick AL, Ingegnoli F, Damjanov N, De Angelis R, Denton CP, et al. Standardisation of nailfold capillaroscopy for the assessment of patients with Raynaud's phenomenon and systemic sclerosis. *Autoimmun Rev*. 2020;19(3):102458.
14. Cutolo M, Sulli A, Pizzorni C, Accardo S. Nailfold videocapillaroscopy assessment of microvascular damage in systemic sclerosis. *J Rheumatol*. 2000;27(1):155-160.
15. Cutolo M, Smith V. Detection of microvascular changes in systemic sclerosis and other rheumatic diseases. *Nat Rev Rheumatol*. 2021;17(11):665–677.
16. Cutolo M, Pizzorni C, Sulli A. Identification of transition from primary Raynaud's phenomenon to secondary Raynaud's phenomenon by nailfold videocapillaroscopy: comment on the article by Hirschl et al. *Arthritis Rheum*. 2007;56(6):2102-2104.
17. Sulli A, Secchi ME, Pizzorni C, Cutolo M. Scoring the nailfold microvascular changes during the capillaroscopic analysis in systemic sclerosis patients. *Ann Rheum Dis*. 2008;67(6):885-887.
18. Gilbane AJ, Denton CP, Holmes AM. Scleroderma pathogenesis: a pivotal role for fibroblasts as effector cells. *Arthritis Res Ther*. 2013;15:215.

19. Denton CP, Wells AU, Coghlan JG. Major lung complications of systemic sclerosis. *Nat Rev Rheumatol.* 2018;14(11):682.
20. Bergamasco A, Hartmann N, Wallace L, Verpillat P. Epidemiology of systemic sclerosis and systemic sclerosis-associated interstitial lung disease. *Clin Epidemiol.* 2019;11:257-273.
21. Perelas A, Silver RM, Arrossi AV, Highland KB. Systemic sclerosis-associated interstitial lung disease. *Lancet Respir Med.* 2020;8:304–20.
22. Desai SR, Veeraraghavan S, Hansell DM, et al. CT features of lung disease in patients with systemic sclerosis. *Radiology.* 2004;232:560–7.
23. 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 Rheumatology.* 2020;2:e71–83.
24. 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(9):e18-e47.
25. 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.
26. Cutolo M, Campitiello R, Gotelli E, Soldano S. The Role of M1/M2 Macrophage Polarization in Rheumatoid Arthritis Synovitis. *Front Immunol.* 2022;13:867260.
27. Campitiello R, Soldano S, Gotelli E, Hysa E, Montagna P, Casabella A. The intervention of macrophages in progressive fibrosis characterizing systemic sclerosis: A systematic review. *Autoimmun Rev.* 2024;23(10):103637.
28. P.J. Murray, R. Langhans, D.A. Rutschman, C. Jenkins, S. Gordon. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity.* 2014;41(1):14–20.
29. Wang N, He H, Wang X, Yan Y, Wang J. Molecular mechanisms that influence the macrophage M1-M2 polarization balance. *Front Immunol.* 2014;5:614.
30. Ahamada MM, Jia Y, Wu X. Macrophage polarization and plasticity in systemic lupus erythematosus. *Front Immunol.* 2021;12:734008.
31. Wells AU, Walsh SLF, Adegunsoye A, Cottin V, Danoff SK, Devaraj A, et al. Identification of progressive pulmonary fibrosis: consensus findings from a modified Delphi study. *Respir Res.* 2024;25(1):448.
32. Fang D, Chen B, Lescoat A, Khanna D, Mu R. Immune cell dysregulation as a mediator of fibrosis in systemic sclerosis. *Nat Rev Rheumatol.* 2022;18:683–93.
33. Ayub S, Shafi T, Rasool R, Dangroo MA, Bindroo MA, Gull A. Evaluating the role of active TGF- β 1 as inflammatory biomarker in Kashmiri (North-Indian) patients with systemic sclerosis: a case-control study. *Adv Rheumatol.* 2024;64(1):91.
34. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008;8:958–69.
35. 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.

36. Soldano S, Montagna P, Campitiello R, Gotelli E, Paolino S, Pizzorni C. Increase in circulating cells coexpressing M1 and M2 macrophage surface markers in patients with systemic sclerosis. *Ann Rheum Dis*. 2018;77(12):1842–45.
37. Trombetta AC, Soldano S, Paolino S, Montagna P, Secchi ME, Cutolo M. A circulating cell population showing both M1 and M2 monocyte/macrophage surface markers characterizes systemic sclerosis patients with lung involvement. *Respir Res*. 2018;19(1):186.
38. Gotelli E, Soldano S, Feghali-Bostwick C, Montagna P, Campitiello R, Contini P. 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.
39. Smith V, Sciré CA, Talarico R, Airo P, Alexander T, Allanore Y, et al. Systemic sclerosis: state of art on clinical practice guidelines. *RMD Open*. 2018;4(Suppl1):e000782.
40. DeMizio DJ, Bernstein EJ. Detection and classification of systemic sclerosis-related interstitial lung disease: a review. *Curr Opin Rheumatol*. 2019;31:553–60.
41. Vandecasteele E, Melsens K, Vanhaecke A, Blockmans D, Bonroy C, Carton C, 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.
42. Distler O, Highland KB, Gahlemann M, et al. Nintedanib for systemic sclerosis-associated interstitial lung disease. *N Engl J Med*. 2019;380(26):2518–2528.
43. LeRoy EC, Medsger TA. Criteria for the classification of early systemic sclerosis. *J Rheumatol*. 2001;28:1573–6.
44. Arroz M, Came N, Lin P, Chen W, Yuan C, Lagoo A, et al. Consensus guidelines on plasma cell myeloma minimal residual disease analysis and reporting. *Cytometry B Clin Cytom*. 2016;90:31–39.
45. Cossarizza A, Chang HD, Radbruch A, Abrignani S, Addo R, Akdis M, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *Eur J Immunol*. 2021;51:2708–3145.
46. Soldano S, Smith V, Montagna P, Gotelli E, Campitiello R, Pizzorni C, Paolino S, Sulli A, Cere A, Cutolo M. 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.
47. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods*. 2001;25:402–8.
48. Heger L, Hofer TP, Bigley V, de Vries IJM, Dalod M, Dudziak D, et al. Subsets of CD1c+ DCs: dendritic cell versus monocyte lineage. *Front Immunol*. 2020;11:559166.
49. Soldano S, Trombetta AC, Contini P, Tomatis V, Ruaro B, Brizzolara R, Montagna P, Sulli A, Paolino S, Pizzorni C, Smith V, Cutolo M. Increase in circulating cells coexpressing M1 and M2 macrophage surface markers in patients with systemic sclerosis. *Ann Rheum Dis*. 2018;77(12):1842–1845.
50. Gheibi Hayat SM, Bianconi V, Pirro M, Sahebkar A. Efferocytosis: molecular mechanisms and pathophysiological perspectives. *Immunol Cell Biol*. 2019;97:124–33.

51. Mohning MP, Thomas SM, Barthel L, Mould KJ, McCubbrey AL, Frasch SC, 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.
52. Darby IA, Laverdet B, Bonte F, Desmouliere A. Fibroblasts and myofibroblasts in wound healing. *Clin Cosmet Investig Dermatol*. 2014;7:301–311.
53. She Y, Xu X, Yu Q, Yang X, He J, Tang XX. Elevated expression of macrophage MERTK exhibits profibrotic effects and results in defective regulation of efferocytosis function in pulmonary fibrosis. *Respir Res*. 2023;24:118.
54. Hu M, Yao Z, Xu L, Peng M, Deng G, Liu L, et al. M2 macrophage polarization in systemic sclerosis fibrosis: pathogenic mechanisms and therapeutic effects. *Heliyon*. 2023;9(5):e16206.
55. Morse C, Tabib T, Sembrat J, Buschur KL, Bittar HT, Valenzi E, Jiang Y, Kass DJ, Gibson K, Chen W, Mora A, Benos PV, Rojas M, Lafyatis R. Proliferating SPP1/MERTK-expressing macrophages in idiopathic pulmonary fibrosis. *Eur Respir J*. 2019;54(2):1802441.
56. Smith V, Thevissen K, Trombetta AC, Pizzorni C, Ruaro B, Piette Y, Paolino S, De Keyser F, Sulli A, Melsens K, Cutolo M. Nailfold capillaroscopy and clinical applications in systemic sclerosis. *Microcirculation*. 2016;23(5):364–72.
57. Smith V, Distler O, Du Four T, Cutolo M. *Rheumatology (Oxford)*. 2022;61(6):2217–2220.
58. Velauthapillai A, de Vries-Bouwstra JK, Henes J, Vonk MC, Melsens K, Smith V. Longitudinal association between nailfold capillaroscopy and incident interstitial lung disease: A EUSTAR database analysis. *J Scleroderma Relat Disord*. 2025;10:23971983241307692.
59. Acemoğlu ŞŞZ, Türk İ, Deniz PP, Aşık MA, Arslan D, Hanta İ. Relationship between nailfold capillaroscopy findings and the etiology and prognosis of interstitial lung disease. *Clin Rheumatol*. 2024;43(8):2679–2687.
60. Hysa E, Campitiello R, Sammorì S, Gotelli E, Cere A, Pesce G, Pizzorni C, Paolino S, Sulli A, Smith V, Cutolo M. Specific autoantibodies and microvascular damage progression assessed by nailfold videocapillaroscopy in systemic sclerosis: Are there peculiar associations? *Antibodies (Basel)*. 2023;12(1):3.