MSc Pharmacy Master Project Report

Comparison of Monocyte Subpopulations and Circulating Fibrocytes in SSc and SSc-ILD: the search for a SSc-ILD Progression Biomarker

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Content

Abstract

Systemic sclerosis (SSc) is a rare, heterogeneous auto-immune disease that is characterised by inflammation, progressive fibrosis and vascular injury. One of the main complications of SSc is Interstitial Lung Disease (ILD); a form of lung fibrosis. Although there are methods to diagnose and monitor SSc-ILD, there is an urgent need to predict progression of SSc towards SSc-ILD. Finding a biomarker for SSc-ILD for prediction and more efficient monitoring would increase prospects for SSc-ILD patients. Monocytes and fibrocytes are associated with SSc-ILD. Furthermore, certain markers expressed on monocytes have an association with SSc-ILD or fibrotic manifestations in general, such as CD206, CD86, HLA-DR and CD169. One aim of this project was to investigate the expression of monocyte subpopulations and of CD206, CD86, HLA-DR and CD169 in these subpopulations within SSc-, SSc-ILD- and healthy control (HC) groups. Another aim was to look into the differences in the enumeration of circulating fibrocytes within SSc-, SSc-ILD and HC groups. To reach these aims, flow cytometry analysis of frozen PBMC's of 22 SSc-, 18 SSc-ILD- and 5 healthy individuals was performed, using a selected monocyte or fibrocyte antibody-panel for staining. Comparison of the expression of the monocyte subpopulations in the subject-groups exhibited no significant differences. There were significant differences observed in the expression of HLA-DR and CD169 in the intermediate and non-classical populations within the subject-groups. Especially noteworthy, was the higher expression of CD169 in the intermediate population of the SSc-ILD group compared to the SSc-group. For the second aim of the study, no results were acquired due to limitations in the set-up of the study. Overall, this project has shown that CD169 is a promising marker that has association with SSc-ILD. More research might elucidate this relation even further and may even indicate CD169 as an effective SSc-ILD progression biomarker.

1. Introduction

1.1. Systemic sclerosis

Systemic sclerosis (SSc) is a heterogenic autoimmune disease that is classified as a rheumatic disease. It is a chronic, systemic, connective tissue disease that involves inflammation, progressive fibrosis and vascular injury. SSc pathophysiology involves various organs including the skin, the kidneys, the heart, the lungs and the gastrointestinal tract. SSc is a rare and unpredictable disease (1), that occurs in countries all over the world. It has an incidence of 10-50 cases per million people per year and a prevalence of approximately 30-340 cases per million people (2). SSc amplifies in its severity when it progresses into systemic sclerosis interstitial lung disease (SSc-ILD) (1). 80% of SSc patients are reported to have lung abnormalities and of these patients, 30-40% develop ILD that is clinically significant (2). SSc-ILD is a potentially fatal disease, making it a burdensome disease for the patients that are afflicted (1). It is even the main cause of SSc-related deaths, being responsible for 35% of the SSc mortality (3). Risk factors that are associated with ILD in SSc patients are African-American race, the dcSSc subtype, male gender, presence of various SSc biomarkers such as the anti-SCL 70 antibody and antinuclear antibody (ANA), specific pulmonary function test- (PFT) and imaging results (4), cardiac involvement, hypothyroidism and a younger age (3).

There are few treatment options available for SSc-ILD. For instance mycophenolate mofetil, cyclophosphamide, nintedanib and tocilizumab have been shown to attenuate the progression of SSc-ILD, but are not curative (4) (5). Furthermore, the progression of SSc-ILD is also unpredictable and not all SSc-ILD patients will develop organ complications. This variability in SSc-ILD makes it difficult to decide which patients will progress to clinically meaningful SSc-ILD and which will not (4). For this reason, there is a need to find effective methods of monitoring SSc and the possible progression to SSc-ILD.

1.2. Symptoms and clinical manifestations

1.2.1. Systemic sclerosis

Typical clinical manifestations in SSc are sclerodactyly, telangiectasias, digital ulcer and abnormal nailfold capillaroscopy (2). However, the earliest symptoms that are manifested in SSc are often puffy fingers and Raynaud's phenomenon. This is problematic, as these two symptoms are not specific for SSc and occur also in other connective tissue diseases. This makes the diagnosis of very early SSc difficult, and a delay of 6,1 years in the diagnosis of SSc has been shown after the manifestation of Raynaud's phenomenon (6). When the disease has developed into a more advanced state, diagnosis becomes less challenging, as often more distinct features such as thickening of the skin, microvascular changes, disease specific antibodies and symptoms of internal organ involvement are present (6).

1.2.2. Systemic sclerosis interstitial lung disease

Internal organ complications, such as ILD, appear early on in SSc disease and have a subclinical presentation (6). Once ILD patients do develop symptoms, the ones that are most manifested are non-productive cough, fatigue and dyspnoea. The latter will at first only be present during exertion, but as SSc-ILD progresses the symptom will eventually also be present during resting phases. Physical examination of SSc-ILD patients will often show more clinical features, that are also frequently used in the diagnosis of SSc-ILD (2). For instance, chest auscultation may reveal "velcro-type" crackles that are a typical acoustic finding for ILD (7). Velcro-type crackles are pathological lung sounds that are irregular, short and explosive (7). Overall, patients will eventually experience a decline in lung function, the extent of which depends on the progression of pulmonary fibrosis (8).

1.3. Classification of SSc subsets

SSc may present with a wide range of clinical features, differing from patient to patient in the time course of the disease (6). In order to understand the disease course in individual patients better, SSc has been categorised into three different subsets; diffuse cutaneous systemic sclerosis (dcSSc), limited cutaneous systemic sclerosis (lcSSc) and SSc sine scleroderma (9). DcSSc is characterised by skin involvement in the regions of the limbs and trunk proximal to the knees and elbows, and in the face (1). There is an association with severe internal organ fibrosis, especially pulmonary fibrosis, cardiac fibrosis and renal fibrosis. Overall, dcSSc has a very aggressive disease course and the prospects are often low (9). LcSSc is on the other hand characterised by skin involvement in the regions distal to the knees and elbows and the nature of the skin involvement in lcSSc is milder compared to the skin involvement in dcSSc (1). LcSSc is associated with a relatively slow disease course, in which involvement of the internal organs occurs at a later stage in the disease (9). Lastly, SSc sine scleroderma is very rare and only present in 5% of the SSc patients. In this disease subtype, the patients present with SSc features such as digital ulcers and Raynaud's phenomenon, but skin involvement is absent (1). In order to make a definitive diagnosis of SSc, the classification criteria of the American College of Rheumatology (ACR) and the 2013 European League Against Rheumatism (EULAR) are used. These classification criteria panels are used to score the SSc symptoms and features that are manifested in a patient, in order to decide on the SSc subset and the related severity of the disease in the patient and to link this to the most effective available treatment option (1). ILD can appear in each of these SSc subsets (9).

1.4. Diagnosis of SSc-ILD

For the diagnosis of ILD, various imaging techniques and PFTs are used. An example of an imaging technique that is used is high-resolution computed tomography (HRCT). HRCT is used to examine the severity of lung involvement in patients. It is very effective for this purpose and it is the only non-invasive method to diagnose ILD (4). With the use of HRCT, certain lung pathological patterns could be made visible that are indicative of the nature of the lung involvement. The two most common patterns that occur in ILD are non-specific interstitial pneumonitis (NSIP) and usual interstitial pneumonitis (UIP). Establishing the presence of these patterns of lung involvement in a patient can help in the quantification of the extent of fibrosis and thus in the selection of a treatment. However, a downside to the use of HRCT is the radiation exposure. For this reason, it has been recommended that HRCT is not routinely used in the monitoring of SSc/SSc-ILD patients (4).

PFTs are safe and cost-effective ways for investigating the presence of ILD. Examples of PFTs that are commonly used for this purpose are total lung capacity (TLC), diffuse capacity of carbon monoxide (DCLO) and forced vital capacity (FVC). However, compared to HRCT, PTFs are less specific and sensitive, especially in the earlier stages of ILD. Nevertheless, PFTs are adequate for monitoring patients that have already been diagnosed with ILD and they are routinely performed in SSc-ILD patients for the first 3-5 years from the onset of disease (4). PFTs performed in ILD patients often reveal restriction and reduction of gas-exchange (2).

As mentioned, SSc-ILD often presents as asymptomatic, especially in the earliest stages of the disease. This lack of symptoms in the very early phase of the disease, makes early diagnosis of SSc-ILD very difficult. Because of this late diagnosis, the chances of development of irreversible organ damage increase significantly, limiting the therapeutics that are effective in the treatment (6). For this reason, progressive schemes for screening and monitoring have been designed. These progressive schemes can be used for diagnosis and progression of ILD in SSc patients (4).

1.5. Pathogenesis of SSc-ILD

SSc-ILD is a complex disease in which various pathological processes are involved, together forming a clinical picture of inflammation, endothelial involvement, thickening of the pulmonary interstitium and

alveolar epithelial damage (4). The exact causes of SSc and SSc-ILD are yet to be elucidated. However, it is presumed that SSc is triggered by environmental circumstances in individuals that have genetic susceptibility (8). The first trigger that leads up to the onset of SSc is possibly lung injury, or more specifically injury to vascular endothelial cells and alveolar epithelial cells (10). There are several possible causes of this injury, such as autoantibodies, viruses, toxins and ischemia-reperfusion (11). The lung injury will consequently cause a cascade of reactions, such as the generation of reactive oxygen species (ROS) and the release of substances such as serotonin, platelet-derived growth factor and thrombin. This eventually leads to effects such as platelet activation, tissue hypoxia and vascular remodelling (11). Furthermore, the alveolar and vascular injury also lead to activation of the innate and adaptive immune system, leading to the activation of cells such as T_H2 -cells (11), T_H17-cells, T_H22-cells (12), dendritic cells, monocytes and macrophages (11). These immune cells release various cytokines, such as IL-4, IL-6 and IL-13, that have direct profibrotic properties (12) (2). Stimulation of fibroblast growth, fibroblast chemotaxis induction and stimulation of the synthesis of extracellular matrix proteins fibronectin and collagen type I and II are such profibrotic properties (13) (14). One especially important cytokine with profibrotic properties is TGF-beta. TGF-beta promotes fibroblast survival and causes fibroblast activation and differentiation into myofibroblasts, which leads to extracellular matrix (ECM) deposition, remodelling of the connective tissue and stiffness. Pericytes, epithelial cells and endothelial cells can also differentiate into myofibroblasts, which processes are directly induced by lung injury (11). The accumulation of myofibroblasts eventually leads to fibrosis in the lungs. This pulmonary fibrosis is the result of two main processes: an abnormal healing process and an abnormal expansion of mesenchymal cells (10). The expansion of mesenchymal cells is believed to mediate the transition of the injury phase in the alveolar epithelium and vascular endothelium into subsequently an inflammatory phase and a fibrotic phase (2).

Thus, the progression of SSc-ILD is mainly caused by an interplay of fibrotic and inflammatory processes. The inflammatory processes are mainly regulated by macrophages originating from circulating monocytes and by T-cells. The fibrotic processes are mainly regulated by myofibroblasts. As mentioned, myofibroblasts originate from various cell types, such as fibroblasts. Fibroblasts also have various origins, one of which is that they are formed out of or activated by circulating fibrocytes. Eventually, these processes lead to tissue remodelling and organ dysfunction (2). As fibrocytes and monocytes are respectively the peripheral precursors of the local effector cells fibroblasts and macrophages (15) (16), elucidation of their role and phenotype in SSc-ILD may lead to more insights regarding the pathogenesis of SSc-ILD. When the phenotype of the ILD-related circulating monocytes and fibrocytes is explicated (17) (16), this may present promising potential biomarkers for the diagnosis and prognosis of SSc-ILD.

1.6. Monocytes in SSc-ILD

1.6.1. Monocytes: origin and function

Monocytes are circulating blood cells that originate in the bone marrow from a dividing common myeloid progenitor (15). They make up approximately 10% of the peripheral leukocytes and have a half-life of approximately 3 days (15). Monocytes are part of the mononuclear phagocyte system, which is a system that has important functions in homeostasis, stimulation and regulation of the adaptive and innate immune system and tissue remodelling. Monocytes are primarily precursor cells that differentiate into macrophages or dendritic cells. Upon tissue damage, monocytes are recruited to the site of injury where they then differentiate into the effectors cells (15).

Monocytes can be divided into three different subpopulations, based on the expression of certain extracellular markers: the non-classical population, the intermediate population and the classical population (table 1). Non-classical monocytes are associated with anti-inflammation. During inflammation, these monocytes will locally differentiate into anti-inflammatory macrophages that have a function in the repair of damaged tissue. The intermediate monocytes are associated with pro-

inflammation. They are responsible for the production of various pro-inflammatory cytokines such as IL-6, IL-1β, TNFα and IL-12. Moreover, these monocytes have a function in T-cell activation and antigen presentation. They specifically contribute to the promotion of Th17-cells, which are involved in fibrotic processes. Lastly, the classical monocytes are also associated with pro-inflammation. These monocytes have a function in phagocytosis, they produce inflammatory cytokines, and they locally differentiate into inflammatory macrophages (18).

Table 1. Overview of the three monocyte subpopulations. Next to the surface markers that were used to identify the different monocyte subpopulations, some other markers that are predominantly expressed by the concerning subpopulation are given. Furthermore, a limited overview of the cytokine signature of the subpopulations is displayed. TNF-α: tumour necrosis factor-α, IL: interleukin, CCL2: chemokine ligand 2, G-CSF: granulocyte colony stimulating factor.

Monocyte subpopulation	Surface markers	Cytokine signature
Non-classicals	CD16+/++/CD14-/+ (17) (19) (20) CD11c (21) CD36 (21) CD45 (20) HLA-DR (20) CD33 (22) CD86 (22) CD29 (23) CD132 (23)	TNF-α (23) (19) IL-1β (23) (19) IL-6 (23) (19) IL-8 (23) (19)
Intermediates	CD16+/++/CD14++ (17) (19) (20) HLA-DR (21) CD11c (21) CD36 (21) CD86 (20) CCR5 (20) CD39 (23) CD275 (23) CD305 (23)	TNF-α (23) IL-6 (23) (18) IL-8 (23) IL-10 (23) IL-1β (18) IL-12 (18)
Classicals	CD16-/CD14++ (17) (19) (20) CD36 (21) CCR2 (21) CD64 (20) CD35 (23) CD38 (23) CD89 (23)	TNF-α (23) IL-1β (23) IL-10 (23) CCL2 (19) G-CSF (19) IL-8 (19) IL-6 (19)

1.6.2. Markers and monocyte markers

Several markers were selected in the monocyte panel: CD16, CD14, CD206, CD86, HLA-DR, CD169 and CD66b. These markers all have a specific function. Firstly, the transmembrane Fcy receptor CD16 (24) and the lipopolysaccharide receptor CD14 (25) are markers that are expressed on monocytes and that are used to gate for the three different monocyte subpopulations; the non-classicals, the intermediates and the classicals (20), see table 1. Next, mannose receptor C type 1 (MRC1), more commonly known as CD206, is a cell-surface protein. It is present on the M2 monocytes/macrophages and therefore considered an anti-inflammatory marker (26) (27). The marker CD86 is often used as an M1 monocyte marker and is therefore associated with pro-inflammation (26). Major histocompatibility complex II cell surface receptor, or HLA-DR, plays an important role during infections; it causes the initiation of the inflammatory cascade (28). Furthermore, in autoimmune diseases and HIV infections it has been shown that the expression of HLA-DR on cytotoxic T-cells (CTLs) is increased (29). The

marker CD169 is part of the Siglec family (30). It has been shown that CD169 has a high expression in certain monocyte populations of SSc patients and has an even higher expression in pulmonary arterial hypertension patients and patients with tissue disease. Moreover, an increased expression of CD169 has been found in the monocytes of patients with rheumatoid arthritis (17). Lastly, CD66b is a granulocyte activation marker and is a useful marker for excluding granulocytes in PBMC samples (31).

1.6.3. Monocytes, markers and SSc-ILD association

Because monocytes and the markers they express have an association with SSc-ILD, they may be qualified as potential progression biomarkers for this SSc subtype. As there is currently no effective method to determine the progression of SSc to SSc-ILD, SSc-ILD is often diagnosed when the disease has already progressed to a more severe stage, in which treatment options are limited. It is of interest to investigate if there is a significant difference in the expression of monocyte subpopulations and the markers that are expressed in these populations in SSc patients compared to SSc-ILD patients. In doing so, an effective way of predicting progression of SSc towards SSc-ILD may be found. For this reason, the research question of the first focus in this project is "Which circulating monocyte subpopulations are expressed in SSc and SSc-ILD patients and is the expression of CD206, CD86, HLA-DR and CD169 in these subpopulations significantly different?". Answering this question may be a step towards finding a progression biomarker for SSc-ILD and thus in improving the prospects for SSc-ILD patients.

1.7. Fibrocytes in SSc-ILD

1.7.1. Fibrocytes: origin and function

Circulating fibrocytes are related to monocytes in their origin and they have been described as peripheral blood fibroblast-like cells (32) (33). Fibrocytes are very rare cells that comprise approximately 0,1%-0,5% of the nonerythrocytic cells in the blood (33). They are mesenchymal progenitor cells that are derived from the bone marrow (32) (34). Although the exact origin of fibrocytes is unknown, they are thought to be derived from the myeloid lineage (33). It has also been suggested that in certain situations, formation of fibrocytes can occur in the bloodstream or even locally in the tissues, during wound healing responses. The formation of fibrocytes out of precursors and the differentiation of fibrocytes into other cells is regulated by various factors, such as growth factors, cytokines and immunoglobulin's (33). One of the most important regulators of fibrocyte differentiation is the cytokine TGF-β1. This cytokine is produced or activated by various cell types, such as endothelial cells, epithelial cells, eosinophils, macrophages and monocytes (33). Upon release by these cells, TGF- β1 forms a complex with latent TGF-β binding proteins, which is then integrated into the ECM. Proteolytic cleavage by proteases of the ECM or one of the components in the TGF-β1-complex, causes release of TGF-β1. TGF-β1 then causes differentiation of fibrocytes into myofibroblasts (35) (33). There are many other cells and factors involved in the activation and inhibition of fibrocyte formation and differentiation, for some of which the pathways are yet to be elucidated (33).

The main function of fibrocytes is tissue repair and wound healing. They are recruited to the sites of injury from the circulation into the tissues, where their differentiation is completed. It has been shown that 3 days after injury, there is a peak recruitment of fibrocytes from the blood. At day 5 after injury, there is a peak recruitment of fibrocytes to the injured tissue. Of the cells that infiltrate the wounded tissue, fibrocytes comprise approximately 10% (33). Fibrocytes resemble both macrophages as well as fibroblasts with regard to their function; like macrophages they have pro-inflammatory properties and like fibroblasts they have tissue remodelling properties (33). Moreover, they are capable of antigen presentation (36), phagocytic activity (37), excretion of lysosomal peptides (38), production of chemokines, cytokines and growth factors (39) (40) (41), secretion of ECM proteins, promotion of wound closure (42) and angiogenesis (40) (41). In addition, fibrocytes do not only differentiate into fibroblasts and myofibroblasts, but they are also capable of differentiating into adipocytes, which have a function in the formation of new tissue (43).

1.7.2. Markers and fibrocyte markers:

Like monocytes, fibrocytes express a variety of markers that can be used in their identification and quantification. However, the phenotype of fibrocytes is less well defined than it is for monocytes, making the gating process challenging. The markers CXCR4, CD45 and collagen-I were selected to gate for the fibrocytes. In order to gain more insight into the phenotype of fibrocytes, some other markers that are reportedly present on fibrocytes were also selected for the fibrocyte panel, namely CD16, CD14, CD34 and HLA-DR. Furthermore, in order to exclude NK cells, T-cells, neutrophils and B-cells from the analysis, respectively the markers CD56, CD3, CD15 and CD19 were selected for the fibrocyte panel (16).

The selected markers for the fibrocyte panel have each a specific function. First, C-X-C chemokine receptor 4 (CXCR4) is a chemokine receptor that binds to the ligand CXCL12 (44). Fibrocytes have a high expression of CXCR4 (16). Interaction between CXCL12 and CXCR4 leads to the migration of the fibrocytes into injured tissue (45). Next, CD45 is a transmembrane glycoprotein also called tyrosine phosphatase (46). Because CD45 is a hematopoietic marker (47), it is expressed on fibrocytes and therefore a useful marker in gating for fibrocytes (16). CD34 is a transmembrane phosphoglycoprotein that is also expressed by fibrocytes. There is relatively little known about the function of CD34, which is yet to be elucidated (16) (48). Lastly, collagen type I is a major element of the extracellular matrix (ECM) (49) and is expressed as an intracellular marker by fibrocytes. In a PBMC fraction, fibrocytes are the only cell-types that express collagen-I (50).

1.7.3. Fibrocytes and SSc-ILD association

As described above, fibrocytes are involved in tissue repair and wound healing. However, imbalance in these processes can lead to the formation of fibrosis. The prominent role of fibrocytes in the fibrotic process has led to their association with various fibrotic diseases. Hence, fibrocytes also have an association with SSc-ILD. For this reason, the second focus of the project will be led by the research question: “What is the difference in the level of circulating fibrocytes between SSc and SSc-ILD patients and are circulating fibrocytes a potential marker for SSc-ILD?”.

2. Materials and Methods

2.1. Study design and Subjects

The design of this study was a cross-sectional study. PBMC's were isolated from fresh blood obtained from patients and healthy individuals. 40 SSc patients were included in the study, of which 18 were diagnosed with ILD. 5 healthy individuals were included as healthy controls. All the individuals that participated in this study had given consent by means of a signed consent form. The clinical and demographical information of the patients and healthy controls is given in table 2. The patients were recruited based on their diagnosis of SSc or SSc-ILD and were selected from the CALC-SSc cohort study. SSc and SSc-ILD patients were diagnosed by a physician of the UMCG. The ACR/EULAR classification criteria were used in the diagnosis of the SSc patients and the TLC, FVC and DCLO were used to diagnose ILD in the SSc patients. The modified Rodnan skin score was used to assess the severity of the skin involvement in the SSc patients. Healthy controls were selected and matched to the patients based on age and gender (17).

Table 2. Presentation of the clinical and demographic information of the patients and the healthy controls that participated in the study. Data are shown as the mean with standard deviation, as a number or as a percentage. TLC: total lung capacity, FVC: forced vital capacity, DCLO: diffuse capacity of carbon monoxide (17).

	SSc patients (n=22)	SSc-ILD patients (n=18)	Healthy controls (n=5)
Female gender (%)	19 (86)	10 (56)	4 (80)
Male gender (%)	2 (9)	4 (22)	1 (20)
Unknown gender (%)	1 (5)	4 (22)	0
Age (years)	63 ± 11,7	62 ± 9,3	55 ± 6,4
Disease duration (years)	14,6 ± 10,5	10,8 ± 11,1	
Skin thickening (n)	16	12	
Modified Rodnan skin score	2,8 ± 2,3	3,2 ± 3,5	
Current digital ulcer (n)	2	3	
Past digital ulcer (n)	6	6	
Pitting scars (n)	7	7	
Telangiectasia (n)	15	10	
Abnormal nailfold capillaroscopy (n)	13	11	
Calcinosis (n)	7	8	
Puffy fingers (n)	3	1	
Sclerodactyly (n)	15	13	
Raynaud's phenomenon (n)	21	18	
Abnormality heart (n)	1	5	
Abnormality gastro-intestinal tract (n)	14	10	
Abnormality kidney (n)	5	5	
Abnormality lung (n)	7	11	
TLC	68 ± n/a	62 ± 2,8	
FVC	n/a	63,8 ± 5,3	
DCLO	58,8 ± 7,9	53,9 ± 10,5	
Pulmonary involvement (n)	0	18	

PAH (n)	0	1
ILD (n)	0	18
Anti Scl 70 (n)	1	4
ACR score	12,7 ± 5,6	14,1 ± 5,9

2.2. Blood processing

Blood samples of the patients and healthy individuals were collected in 10 ml heparin tubes. PBMC's were isolated by Ficoll density-gradient centrifugation and the cells were stored as $1x10^7$ cells per cryo-tube in RPMI with 10% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO), according to the PBMC isolation protocol (appendix I). The isolated PBMC's were placed in the -80°C freezer in isopropanol containers for at least 24 hours, after which they were transferred to a nitrogen freezer until further use.

2.3. FACS staining

For the staining of the PBMC's, two different panels were used; a monocyte panel and a fibrocyte panel. The protocol for the staining of each of these panels is given in appendix III. Before staining, the frozen PBMC's were first resuscitated in RPMI medium with 10% FCS and gentamicin (500 ml RPMI with 3 ml gentamicin), according to the protocol given in appendix II. $0,5x10^6$ cells were used for each staining panel. In the monocyte panel the following antibodies with conjugated fluorochromes were used; CD16 – PE Cy7 (Biolegend), CD14 – BV605 (Biolegend), CD206 – PE (BD Biosciences), CD86 – APC (BD Biosciences), CD66b – FITC (Biolegend), HLA-DR – BV786 (BD Biosciences) and CD169 – BV421 (Biolegend). For the fibrocyte panel the following antibodies with conjugated fluorochromes were used CXCR4 – BV605 (Biolegend), CD45 – PerCP-Cy5.5 (BD Biosiences), CD34 – APC-Cy7 (Biolegend), HLA-DR – BV786 (BD Biosciences), CD14 – PE (IQ products), CD16 – PE-Cy7 (Biolegend), Collageen-I – FITC (Rockland), CD56 – APC (BD Biosciences), CD15 – APC (Biolegend), CD3 – APC (BD Biosciences), CD19 – AF700 (eBioscience).

In the monocyte panel, the fluorochrome-conjugated antibodies were added to 50 ul of Brilliant Stain Buffer (BD Biosciences), after which $0,5x10^6$ cells in 100 µl medium were added to this buffer. After an incubation time of 30 minutes in the dark, the cells were incubated in the dark with 2 ml FACs Lysing Buffer, 10x concentrate (BD Biosciences) for no longer than 10 minutes. Following centrifugation and aspiration of the supernatant, the cells were washed two times with 2 ml of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). The cells were resuspended in 300 µl 1% BSA in PBS and stored in the dark at 4˚C, for no longer than 24 hours. Analysis of the stained PBMC's was performed using the NovoCyte Quanteon FACs machine from Agilent, using the NovoSampler Q from Agilent.

In the fibrocyte panel, the fluorochrome-conjugated antibodies were added to 50 ul of Brilliant Stain Buffer, after which $0,5x10^6$ cells in 100 µl medium were added. After an incubation time of 30 minutes in the dark, the cells were washed with 2,5 ml of 5% FCS in PBS. Hereafter, the cells were fixated with Fixation medium A (Reagent A) from the Cell Fixation and Permeabilization Kit (Nordic MUbio) and incubated for 15 minutes in the dark. Before permeabilization with Permeabilization Medium B (Reagent B) from the Cell Fixation and Permeabilization Kit (Nordic MUbio) and addition of the 5x diluted Collagen-I – FITC antibody, the cells were washed with 2,5 ml of 5% FCS in PBS. After permeabilization of the cells and addition of the intracellular antibody, the cells were incubated for 30 minutes in the dark. Thereafter, the cells were washed with 2,5 ml of 5% FCS in PBS and resuspended in 300 µl 5% FCS in PBS. The stained cells were stored at 4˚C in the dark for no longer than 24 hours. Analysis of the stained PBMC's was performed using the NovoCyte Quanteon FACs machine from Agilent, using the NovoSampler Q from Agilent.

2.4. Flow cytometry and gating

The samples were measured on the Quanteon 1 and 2 at the FACs centre at the UMCG, using the program Novoexpress. For both the monocyte and the fibrocyte panel, single stains of the applied markers were used for the compensation of the measurements. For each measurement with a stained sample, an unstained sample was used as a negative control; the samples stained with the selected marker panel were indicated as the “mixed tubes” and the unstained samples were indicated as the “unstained tubes”.

The gating strategy of the monocyte panel was partially based on the paper by Schneider et al, 2021 (17). Autofluorescence was differentiated from true positive signals by means of fluorescence minus one (FMO) for the markers in the monocyte panel. The gating strategy for the monocyte panel is shown in figure 1. First, the monocytes were gated in the forward- and side-scatter plot. Hereafter, doublets and granulocytes were excluded. Following this, CD16 and CD14 were used to gate for the three different monocyte sub-populations, in which the expression of the markers CD206, CD86, HLA-DR and CD169 was analyzed, by plotting these markers against the forward scatter. The expression of CD206, CD86, HLA-DR and CD169 was also investigated by plotting these markers in a histogram in which the count of these markers within the three monocyte subpopulations was visualized.

The gating strategy for the fibrocyte panel was based on the paper by Heukels et al, 2018 (16). Also for this panel, true positive signals were established by FMO. The gating strategy for the fibrocyte panel is shown in figure 9. CD45 positive cells were selected by plotting CD45 against the forward scatter and by gating for the positive population. In the CD45 positive population, the CD56/CD15/CD3 markers were plotted against the forward scatter and the negative population was selected. CD19 signals were excluded by plotting CD19 against forward scatter and gating for the negative population. Hereafter, CXCR4 was plotted against collagen-I and the double positive population was selected, which was considered to be the fibrocyte population. Expression of the markers CD16, CD14, HLA-DR and CD34 in the fibrocyte population was investigated by plotting these markers against the forward scatter as well as by plotting a histogram of these markers in which the count of CD16, CD14, HLA-DR and CD34 was visualized within the fibrocyte population.

2.5. Analysis

The data was analysed using the Novoexpress software from Agilent. The dot plots and histograms created with this software were exported to PDF’s and numerical data was extracted by exportation to subsequently an Excel CSV file and an Excel worksheet.

In the analysis of the flow cytometry data, two different parameters were used to define the expression of the cell-populations and their markers. The percentage population (%population) parameter was used, in which the positive and the negative populations were distinguished from one another. The value of this parameter indicated the number of events (being an equivalent for the number of cells) that was positive or negative for a specific marker within a certain population of cells. The %population gives the percentage that is gated in a dot plot with respect to the parent population. The other parameter that was used was the Median Fluorescence Intensity (MFI). This parameter gives the median-value for the level of expression of a marker on the cells within a selected population. Thus, the higher the MFI, the higher the expression of a marker on the cells in a population (51). The median values of the histograms that were plotted for the various markers were used to determine the MFI of these markers in the different monocyte subpopulations. The MFI for CD86, HLA-DR and CD169 in the monocyte subpopulations was calculated by subtracting the median from the unstained sample population from the stained sample population.

The mean and the standard deviation (SD) of the %population and the MFI of the total monocyte population, the different subpopulations populations, the HLA-DR populations, the CD169 populations and the CD86 populations were plotted using GraphPad Prism version 5.0.

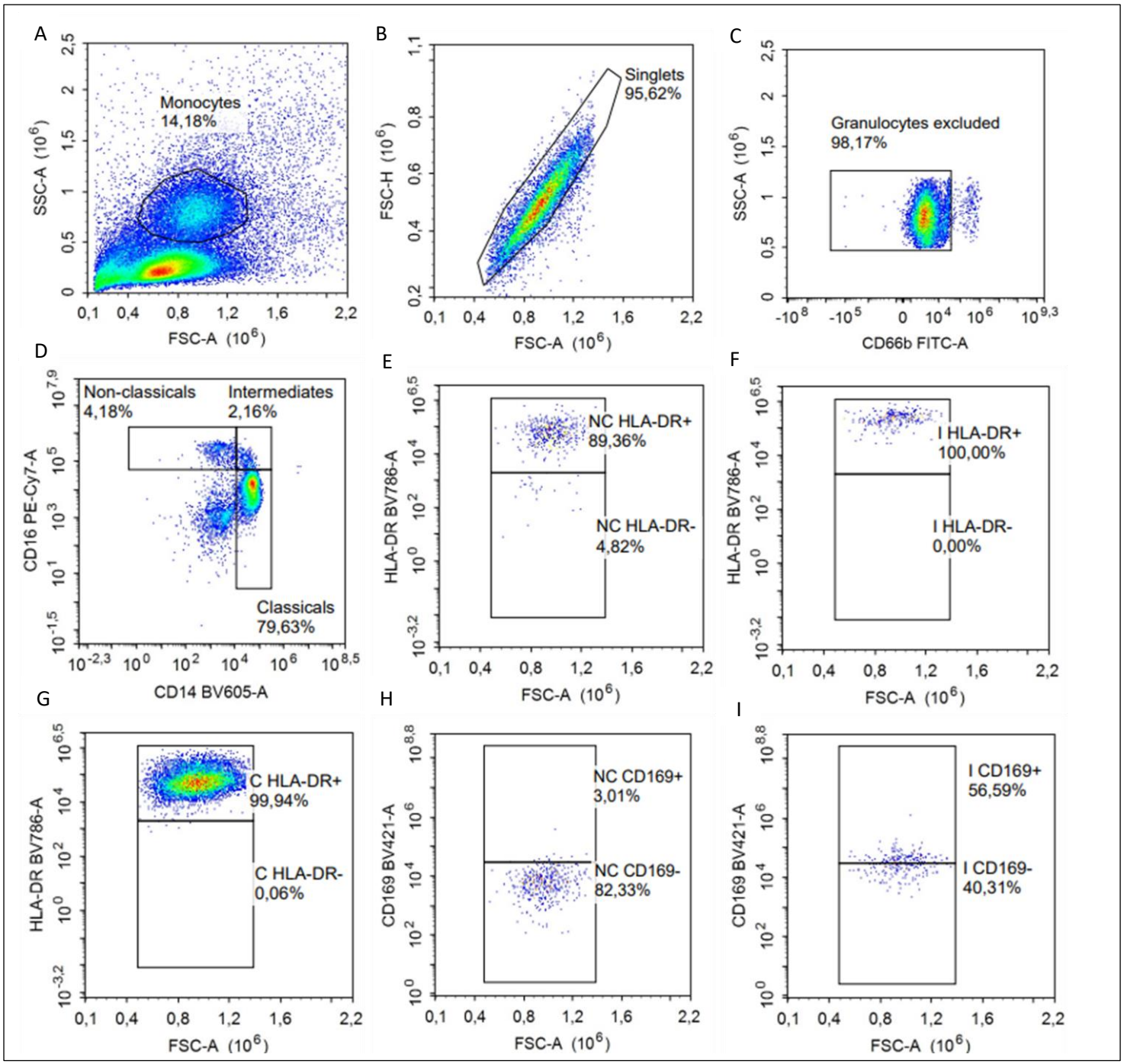
2.6. Statistics

Statistical analysis of the data was performed using GraphPad Prism version 5.0. The results of the SSc patient group, the SSc-ILD patient group and the healthy control group were compared with each other using one-way ANOVA Kruskall-Wallis test. A 95% confidence interval was applied and thus $p < 0,05$ was considered statistically significant. When the one-way ANOVA Kruskall-Wallis test pointed out a significant difference between the groups, a Students T-test and Mann-Whitney U test were performed in which each subject group was compared with the other two subject groups. For these comparisons there was also a 95% confidence interval applied and thus $p < 0,05$ was considered statistically significant.

3. Results

3.1. Circulating monocytes

In total, frozen PBMC samples from 40 patients were stained with the monocyte panel and analysed on the Quanteon. Of those patients, 22 were diagnosed with SSc and 18 were diagnosed with SSc-ILD. In figure 1, the original gating strategy is shown for gating of the monocyte subpopulations.



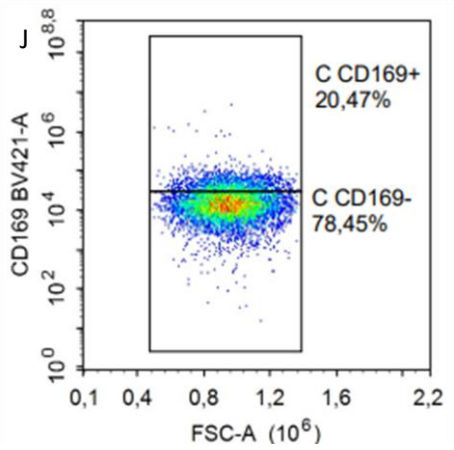


Figure 1. Gating strategy for the circulating monocytes. The %populations are given in each plot and + and – signs indicate positive and negative populations. This analysis was performed on stained PBMC's from patient S21 A: monocytes are selected in the forward- and sidescatter plot. B: doublets are excluded by gating for the singlets. C: granulocytes are excluded by gating for the CD66b negative population. D: CD16 and CD14 are used to gate for the non-classical, intermediate and classical monocyte subpopulations. E: In the non-classical subpopulation, the HLA-DR positive population is selected. F: In the intermediate population, the HLA-DR positive population is selected. G: In the classical population, the HLA-DR positive population is selected. H: In the non-classical population, the CD169 positive population is selected. I: In the intermediate population, the CD169 positive population is selected. J: In the classical-population the CD169 positive population is selected.

3.1.1. Comparison of %population of monocyte subpopulations in the SSc-, SSc-ILD and HC group

In the monocyte populations of the various samples, the non-classical, the intermediate and the classical populations were analysed, as can be seen in figure 2a, 2b and 2c. There were no significant differences found between the mean-values of the %populations of the SSc group, the SSc-ILD group and the HC group for the non-classicals, the intermediates or the classicals. The groups were compared using the one-way ANOVA Kruskall Wallis test.

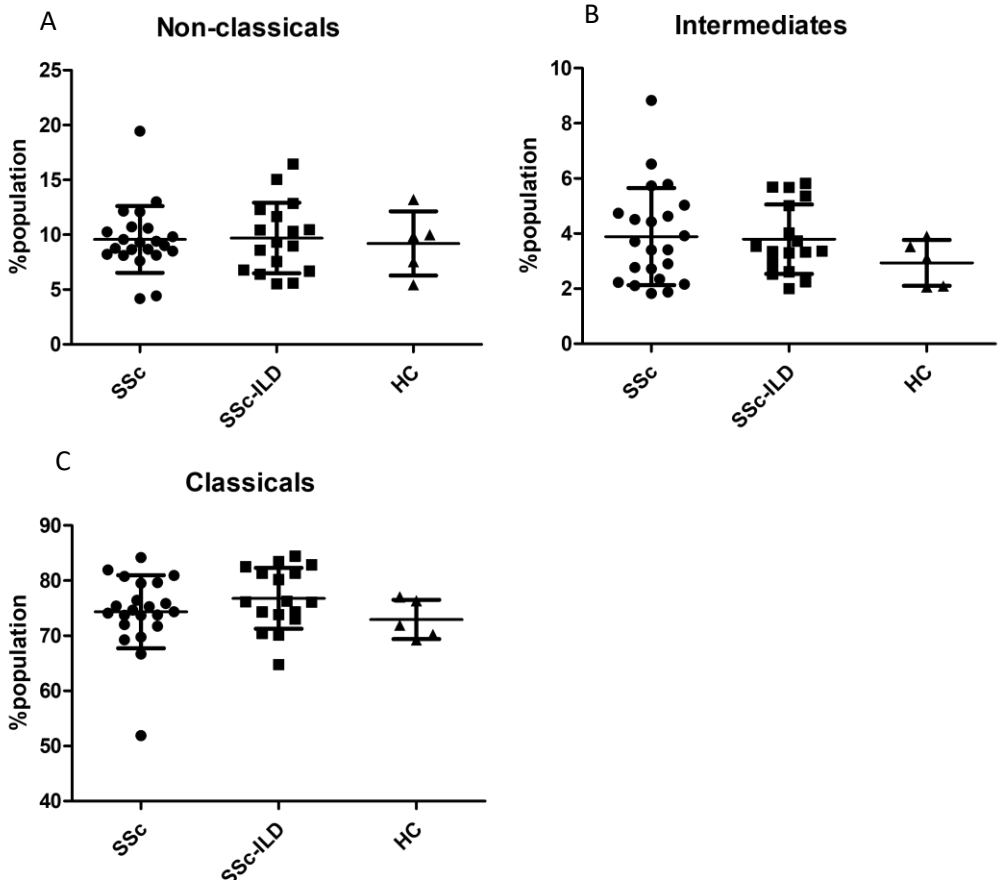


Figure 2. In the three figures, the mean and the standard deviation (SD) of the percentage populations of the three separate monocyte subpopulations are shown for the SSc group, the SSc-ILD group and the HC-group. A: The mean and the SD are shown for the non-classical %populations in the SSc-, SSc-ILD- and HC group. B: The mean and the SD are shown for the intermediate %populations in the SSc-, SSc-ILD- and HC group. C: The mean and the SD are shown for the classical %populations in the SSc-, SSc-ILD- and HC group.

3.1.2. Comparison of %population of HLA-DR in monocyte subpopulations in the SSc-, SSc-ILD and HC-group

In the various monocyte subpopulations, the expression of HLA-DR was investigated. This was done by looking at the %population of the HLA-DR expressing monocyte subpopulations. As can be seen in figure 3a, the mean of the %population of the HLA-DR expressing non-classical population was for the SSc group 91,06% (SD=4,998), 90,35% (SD=6,295) for the SSc-ILD group and 84,04% (SD=3,431) for the HC group. These mean-values were compared using the one-way ANOVA Kruskall Wallis test, which resulted in a p-value of 0,0446. This indicated that there was a significant difference between the mean-values of these groups. Comparison of the SSc- and the SSc-ILD group, the SSc- and the HC group and the SSc-ILD- and the HC group using the students T-test resulted in the respective p-values of 0,9892, 0,0096 and 0,0483. Thus, there was no significant difference between the SSc- and the SSc-ILD group. However, there was indeed a significant difference between the SSc- and the HC group and the SSc-ILD- and the HC-group. For the intermediate population, the mean of the %population of HLA-DR in the SSc group was 99,74% (SD=0,5823), the mean of the %population of HLA-DR in the SSc-ILD group was 99,20% (SD=1,027) and the mean of the %population of HLA-DR in the HC group was 98,95% (SD=0,7660). Comparison of these mean-values using the one-way ANOVA Kruskall Wallis test resulted

in a p-value of 0,0051, indicating a significant difference. Comparison of the mean value of the SSc group with the SSc-ILD group, the SSc group with the HC group and the SSc-ILD group with the HC group using the students T-test resulted in the respective p-values of 0,0045, 0,0259 and 0,2165. There was no significant difference between the SSc-ILD- and the HC group, but there was a significant difference between SSc- and the SSc-ILD group and the SSc- and the HC group. There were no significant differences for the mean-values for the %population of HLA-DR in the classical and total monocyte populations in the SSc-, the SSc-ILD- and the HC group.

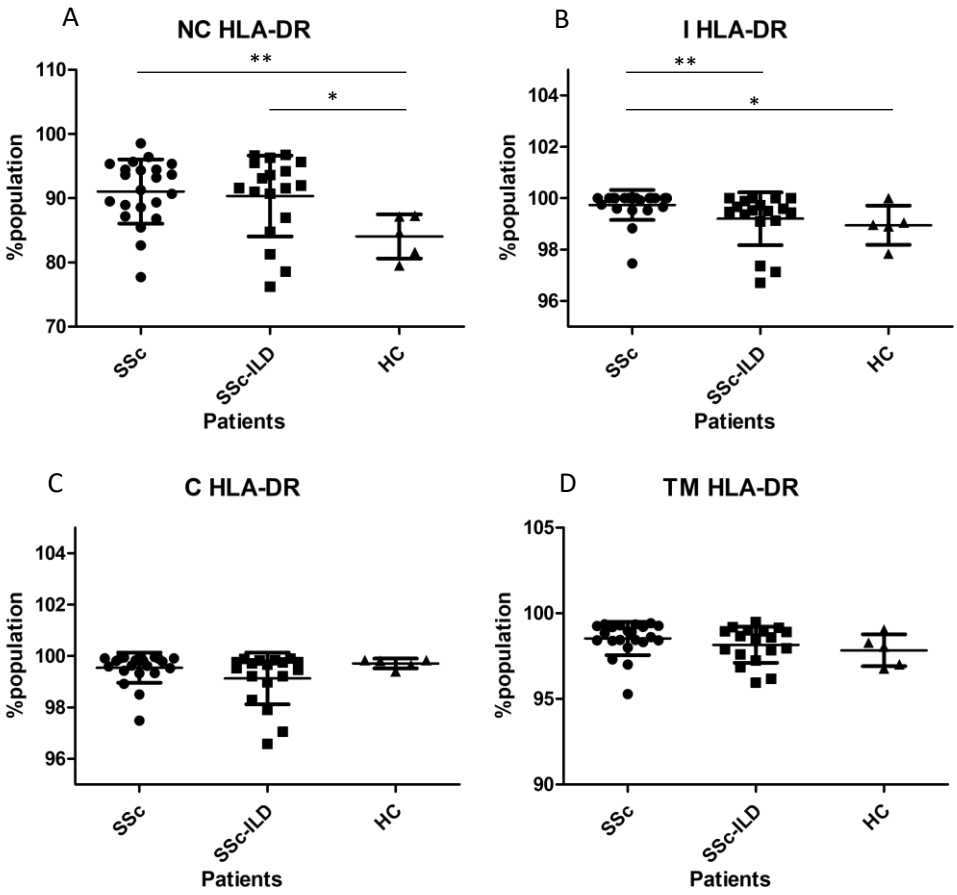


*Figure 3. The mean %population and the corresponding SD-values are plotted for the HLA-DR expressing monocyte subpopulations in the SSc and SSc-ILD groups. In A: the mean and SD for the HLA-DR positive populations in the non-classical monocyte population are shown, in B: the mean and SD for the HLA-DR positive populations in the intermediate populations are shown, in C: the mean and SD for the HLA-DR positive populations in the non-classical populations are shown and in D: the mean and SD for the HLA-DR positive populations in the total monocyte population are shown. *p≤0,05, **p≤0,01.*

3.1.3. Comparison of MFI of HLA-DR in monocyte subpopulations in the SSc-, SSc-ILD and the HC group

The mean-values for the MFI of HLA-DR in the different monocyte subpopulations for the SSc-, the SSc-ILD- and the HC group were examined, see figure 4. There were no significant differences for the MFI mean-values for HLA-DR in the non-classical, intermediate or classical populations between the SSc-, SSc-ILD and HC groups. The groups were compared using the one-way ANOVA Kruskall Wallis test.

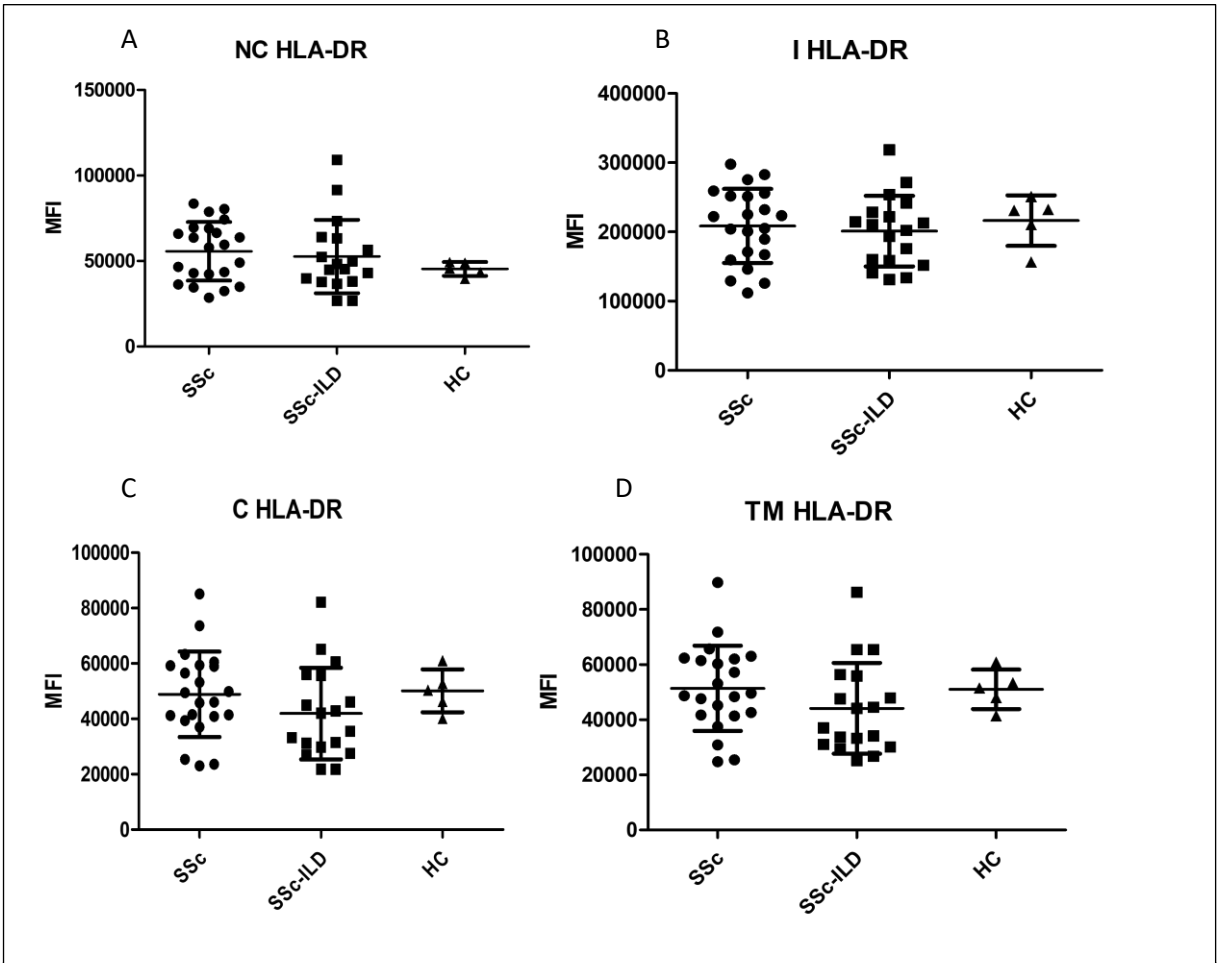


Figure 4. The mean MFI values for the HLA-DR expression in the monocyte subpopulations are plotted with the corresponding SD value, for the SSc-, the SSc-ILD and the HC groups. A: the mean of the MFI for HLA-DR in the non-classical population is shown for the SSc-, the SSc-ILD- and the HC group. B: the mean of the MFI for HLA-DR in the intermediate population is shown for the SSc-, the SSc-ILD- and the HC group. C: the mean of the MFI for HLA-DR in the classical population is shown the SSc-, the SSc-ILD- and the HC group. D group. D: the mean of the MFI for HLA-DR in the total monocyte population is shown for the SSc-, the SSc-ILD- and the HC group.

3.1.4. Comparison of %population of CD169 in monocyte populations in the SSc-, the SSc-ILD- and the HC group

The %population of the CD169 expressing monocyte subpopulations was investigated in the SSc-, SSc-ILD- and HC group, see figure 5. The mean-values of the %population of the CD169 expressing intermediate populations were 35,80% (SD=19,40), 51,40% (SD=23,39) and 53,83% (SD=9,298) for respectively the SSc-, the SSc-ILD- and the HC group. Analysis of these groups using the one-way ANOVA Kruskall Wallis test resulted in a p-value of 0,0347, indicating that there was a significant difference between the mean-values. Analysis of the SSc- and the SSc-ILD group, the SSc- and the HC group and the SSc-ILD and the HC group using the students T-test resulted in the respective p-values of 0,0457, 0,0267 and 0,6818. Thus, there was a significant difference between the SSc- and the SSc-ILD group and the SSc- and the HC group. However, there was no significant difference between the SSc-ILD- and the HC group. There were no significant differences found for the mean-values of the %population for the CD169 expression non-classical-, classical- and total monocyte populations between the SSc-, the SSc-ILD- and the HC groups.

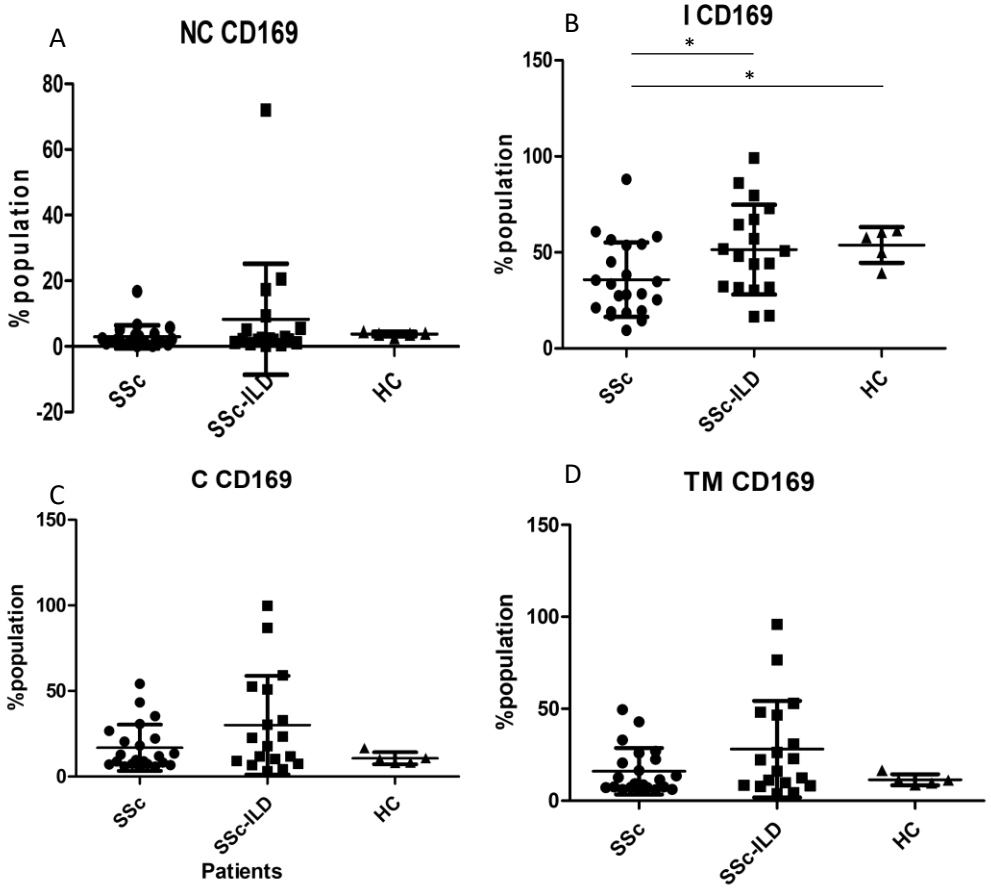


*Figure 5. The mean and the corresponding SD of the %population of the CD169 expressing monocyte populations are plotted for the SSc-, the SSc-ILD- and the HC group. A: the mean and SD of the CD169 population in the non-classical monocyte population are shown. B: the mean and SD of the CD169 population in the intermediate monocyte population are shown. C: the mean and SD of the CD169 population in the classical monocyte population are shown. D: the mean and SD of the CD169 population in the total monocyte population are shown. *p≤0,05.*

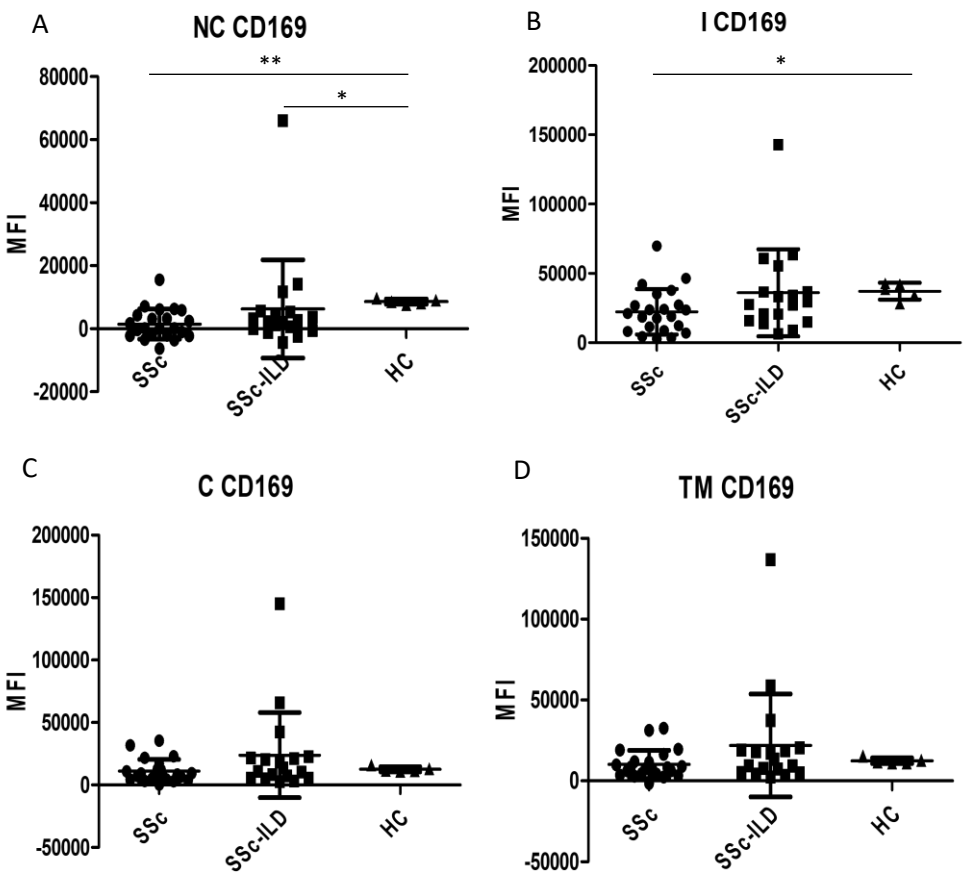


*Figure 6. The mean-values and the SD-values are plotted for the MFI of CD169 expressed in the various monocyte subpopulations in the SSc-, the SSc-ILD and the HC groups. A: the mean and SD are plotted for the MFI of CD169 in the non-classical population. B: the mean and SD are plotted for the MFI of CD169 in the intermediate population. C: the mean and SD are plotted for the MFI of CD169 in the classical population. D: the mean and SD are plotted for the MFI of CD169 in the total monocyte population. *p≤0,05, **p≤0,01.*

3.1.5. Comparison of MFI of CD169 in monocyte subpopulations in the SSc-, SSc-ILD and the HC group

The MFI of CD169 in the different monocyte subpopulations was investigated and the corresponding mean-values for the SSc-, the SSc-ILD- and the HC groups were compared, see figure 6. The mean-values of the MFI of CD169 in the non-classical population for the SSc-, the SSc-ILD- and the HC group were respectively 1497 (SD=4806), 6310 (SD=15580) and 8633 (SD=804,4). Comparison of these groups using the one-way ANOVA Kruskall Wallis test resulted in a p-value of 0,0085, indicating a significant difference between these mean-values. Comparison of the SSc- and the SSc-ILD group, the SSc- and the HC group and the SSc-ILD- and the HC group using the students T-test resulted in the respective p-values of 0,2953, 0,0020 and 0,0279. There was no significant difference between the SSc- and the SSc-ILD group, but there was significant difference between both the SSc- and the HC group and the SSc-ILD- and the HC group. The mean-values of the MFI of CD169 in the intermediate population for the SSc-, the SSc-ILD- and the HC group were respectively 22300 (SD=16360), 36040 (SD=31420) and 37130 (SD=6095). Comparison of these groups using the one-way ANOVA Kruskall Wallis test resulted in a p-value of 0,0306, indicating a significant difference between these mean-values. Comparison of the SSc-

and the SSc-ILD group, the SSc- and the HC group and the SSc-ILD- and the HC group using the students T-test resulted in the respective p-values of 0,0893, 0,0193 and 0,1921. Thus, there was no significant difference between the SSc- and the SSc-ILD group or the SSc-ILD- and the HC group, but there was a significant difference between the SSc- and the HC group. No significant differences were found for the mean-values of the MFI of CD169 in the classical or total monocyte population between the SSc-, the SSc-ILD- or the HC groups.

3.1.6. Analysis of CD206 and CD86 monocyte populations

3.1.6.1. Difficulties with the CD206 marker

In the assembly of the monocyte panel, the two markers CD206 and CD86 had been selected, amongst other markers. However, during the analysis of the PBMC samples using this monocyte panel, it was found that the CD206 antibody was not giving an adequate signal. In the dot plots for the FMO's of CD206 and CD86 (figure 7), the populations were positioned at the same height. Compared to the population in the dot plot of the FMO of CD206, the population in the dot plot of the FMO of CD86 had shifted to the left.

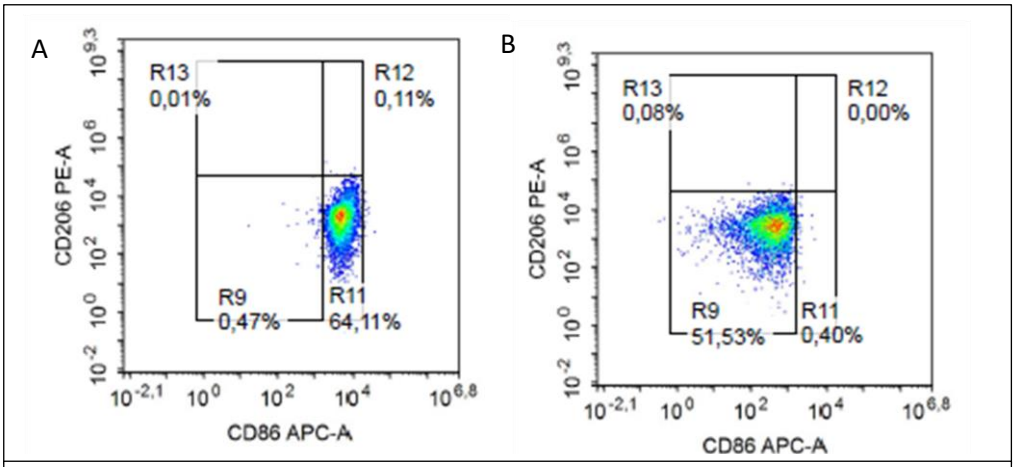


Figure 7. A: Dot plot of the FMO of the marker CD206, in which can be seen that in the absence of CD206, a signal is caused due to CD86. This signal is considered as CD86 positive and CD206 negative. B: Dot plot of the FMO of the marker CD86, in which can be seen that in the absence of CD86 a signal is caused. However, this signal appears to be the CD206/CD86 double negative population and not the CD206 positive/CD86 negative population.

3.1.6.2. *Comparison of MFI of CD86 in monocyte subpopulations of the SSc-, SSc-ILD- and HC group*

The MFI of CD86 in the three monocyte subpopulations and in the total monocyte population was examined, see figure 8. No significant differences were found for the mean-values of the MFI of CD86 in the non-classical, intermediate, classical or total monocyte populations between the SSc-, the SSc-ILD- and the HC groups. The groups were compared using the one-way ANOVA Kruskall Wallis test.

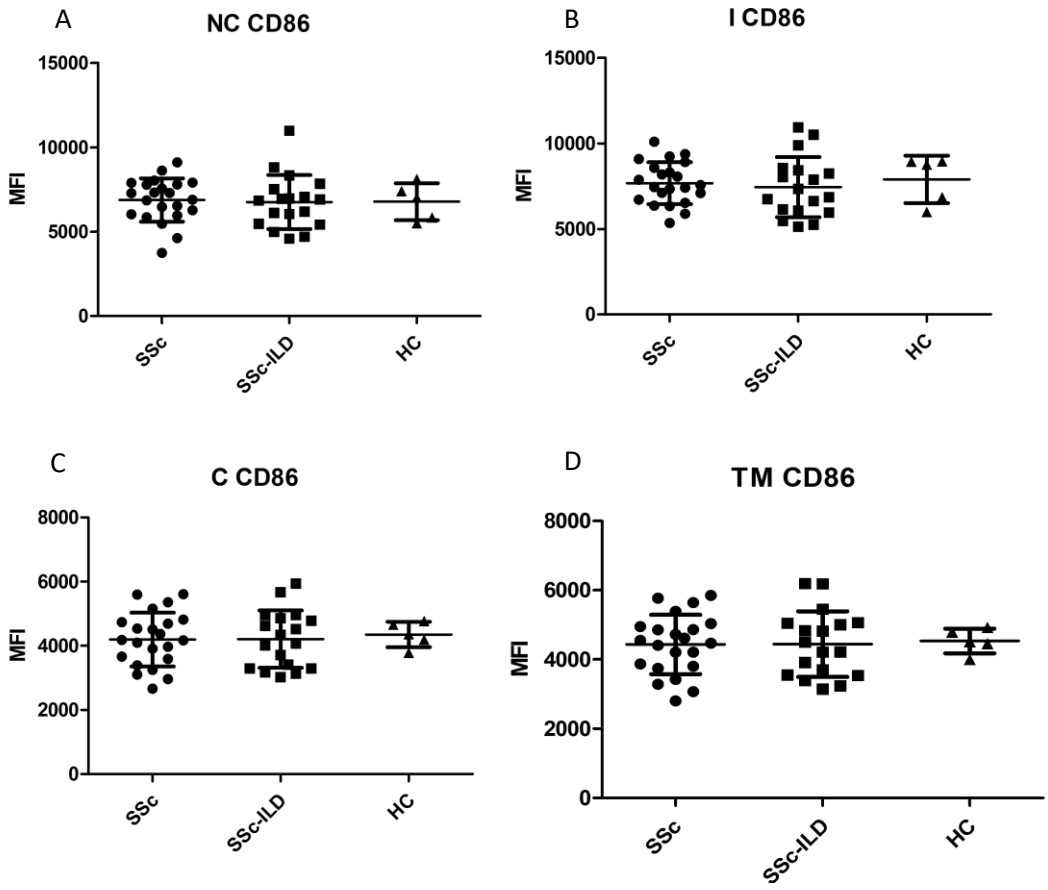


Figure 8. The mean-values and the SD-values of the MFI of CD86 in the various monocyte subpopulations plotted for the SSc-, the SSc-ILD- and the HC group. A: the mean and SD are shown for the MFI of CD86 in the non-classical population. B: the mean and SD are shown for the MFI of CD86 in the intermediate population. C: the mean and SD are shown for the MFI of CD86 in the classical population. D: the mean and SD are shown for the MFI of CD86 in the total monocyte population.

3.2. Circulating fibrocytes

In this second focus of the project, the aim was to evaluate the presence of circulating fibrocytes. In figure 9, the original gating strategy is shown for gating of the circulating fibrocytes. In total, 13 patients had been investigated using the fibrocyte panel. During the analysis of the PBMC samples with the fibrocyte panel, several problems occurred. In figure 9e, there is no clear CXCR4/collagen-I double positive population present, there is only one relatively large population visible. Based on the FMO gates, this population is collagen-I positive, but not CXCR4 positive.

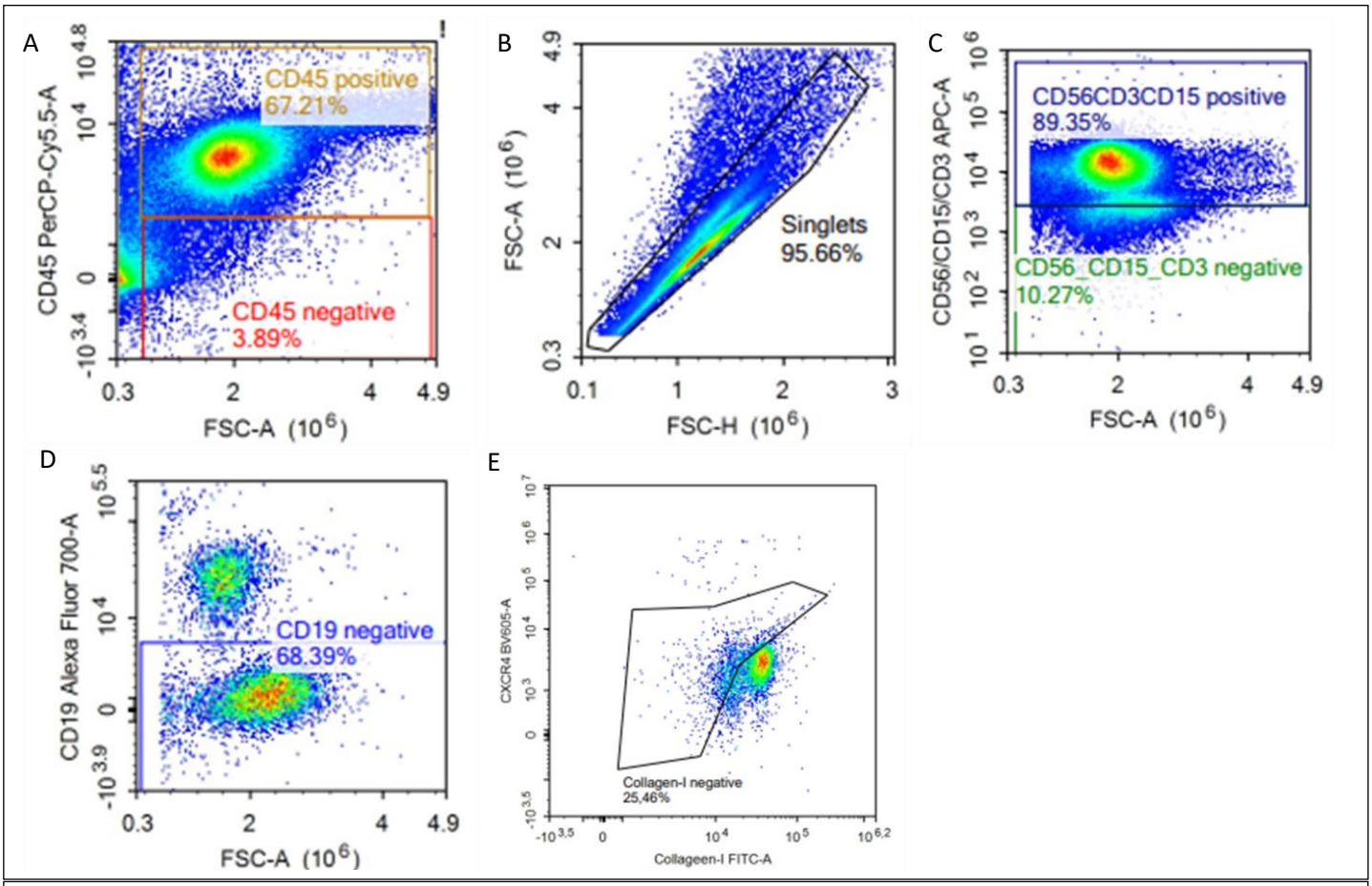


Figure 9. Gating strategy for circulating fibrocytes. The results are from staining of the PBMC's of SSc patient S01. The %populations are given in each plot and "+" and "-" signs indicate positive and negative populations. A: the CD45 positive population is selected. B: the doublets are excluded by selecting the singlets. C: the CD56/CD3/CD15 negative population is selected. D: the CD19 negative population is selected. E: the CXCR4 high/Col-I double positive population are assumed to be the circulating fibrocytes.

4. Discussion and conclusions

4.1. Circulating monocytes

One of the objectives of this project was to investigate the expression of the three monocyte subpopulations in SSc-, SSc-ILD- and HC individuals. The focus was to see if there was a difference in the expression of the markers CD206, CD86, HLA-DR and CD169 within these monocyte subsets for the three different subject-groups. With this objective, the intention was to answer the research question: "Which circulating monocyte subpopulations are expressed in SSc and SSc-ILD patients and is the expression of CD206, CD86, HLA-DR and CD169 in these subpopulations significantly different?". Finding a biomarker for SSc-ILD for prediction and more efficient monitoring of the disease would increase prospects for SSc-ILD patients, as this would make the selection of a treatment more fitting for the disease stage of a patient. Also, treatments could be started earlier, before progression of SSc-ILD to a more severe stage, making the treatment more effective.

Monocytes have been reported to be associated with the SSc and SSc-ILD pathogenesis (17) (52) (53) (54) (55). As mentioned, monocytes are the precursor cells of macrophages, which have a role in the inflammatory processes in SSc-ILD. Therefore, it is reasonable that monocytes also have an association with this disease. It has been shown that the expression of monocytes is increased in the SSc patients compared to healthy controls (17) (52) and that the expression of monocytes is also elevated in SSc-ILD patients (54) (55). Furthermore, a higher count of monocytes has been associated with fibrotic manifestations in SSc (52), also supporting a possible association of SSc-ILD with an increased number of monocytes.

Another association between SSc-ILD and monocytes, is based on the markers that are expressed by the monocytes. The expression of these markers can vary during certain physiological and pathological situations. Because some markers have a higher expression in certain diseases, this makes them potentially very suitable as progression biomarkers for the concerning disease. Some of the markers expressed by monocytes have been associated with the SSc and/or SSc-ILD disease manifestations. For instance, several markers have been shown to have a higher expression on monocytes in SSc patients. This is specifically the case for the markers HLA-DR, CD169 (17) and CD86 (56). Because the pathogenesis of SSc and SSc-ILD differ from each other, the expression of the markers HLA-DR, CD169 and CD86 may also be different in SSc-ILD patients compared to SSc patients. Moreover, the markers CD169 and CD206 have been shown to have a higher expression in SSc and SSc-ILD compared to various other disease, such as sarcoidosis and lung cancer (54) (57). Looking at the difference in the expression of the markers CD206, CD86, HLA-DR and CD169 between SSc and SSc-ILD is therefore also promising. Finally, some markers have been associated specifically with fibrosis, this is the case for CD206 and CD169, even increasing a potential association of these markers with SSc-ILD (17).

No significant differences were found in the expression of the monocyte subpopulations between the SSc-, the SSc-ILD group and the HC group. For expression of the various markers in the monocyte subpopulations, some significant differences were found between the three subject-groups, namely for the markers HLA-DR and CD169. For the marker CD86, no significant differences were found in the MFI of CD86 for any of the subpopulations between any of the subject groups.

4.1.1. No significant differences in expression monocyte subpopulations between SSc-, SSc-ILD and HC groups

From the results can be observed that there were no significant difference between the SSc-, the SSc-ILD- the HC group in the expression of the monocyte subpopulations. It is peculiar that no differences were found between these subject groups, as the expectation was that the expression of the monocyte subpopulations would be higher in SSc patients compared to healthy controls and in SSc-ILD patients compared to SSc patients. This expectation was based on reports in which was indicated that the

expression of monocytes was increased in SSc patients compared to healthy controls (17) (52) (54) (55) and in SSc-ILD patients compared to healthy controls (54) (55). Furthermore, monocytes also have been shown to have an association specifically with fibrotic manifestations (55). This further substantiated the expectation that the expression of monocytes in SSc-ILD patients would be higher compared to the expression of monocytes in SSc patients, as SSc-ILD is also a fibrotic disease. The lack of a significant difference between the monocyte subpopulations of SSc- and SSc-ILD patients in this project does not necessarily diminish the possibility of there being a difference between the monocyte subpopulations of SSc- and SSc-ILD patients in general. It is possible that the sample size in this project was relatively small and that more samples need to be analysed to draw more reliable conclusions. In previous research, the subject groups were larger than in this project, indicating that investigation of a larger subject group might exhibit significant differences, not only for comparison between SSc patients and healthy controls, but also for comparison between SSc- and SSc-ILD patients.

4.1.2. No CD206 positive populations were observed

In none of the samples a CD206 positive population was found. In the FMO for the CD86 marker, it was observed that CD206 had not caused a signal and that the signal that was present was in fact what appeared to be the double negative population for CD206/CD86. This was concluded based on the position of the signal present in the FMO dot plot of CD206. Here, the signal was caused by CD86 and the population was thus considered to be CD86 positive. The signal was clearly caused by CD86, because when the FMO of CD86 was compared with the dot plot of the unstained sample, the population that was considered to be the CD86 positive population clearly shifted to the right compared to the negative population in the unstained plot. The signal that was seen in the FMO of CD86 was at the same height as the signal in the FMO of CD206, and also at the same height as the double negative population of CD206/CD86. Therefore, the signal in the FMO of CD86 could not have been caused by the CD206 marker, as the signal of this marker should have been positioned above the CD86 positive population and above the CD206/CD86 double negative population.

It thus appeared that addition of the CD206 marker did not cause a CD206 positive signal. In order to verify this, the FMO for the monocyte panel was performed a second time. However, the results were the same as for the first FMO measurement. Because the first assumption was that the problem was being caused by the antibody, a new CD206 antibody was ordered. This new CD206 marker was a different clone than the previously used CD206 marker. However, when the FMO was performed with this new CD206 marker, the results were still the same as the results that were obtained with the old antibody. Two different possible causes for this problem were suggested. The first possibility had to do with the state of the PBMC's. In this project the PBMC's were frozen after isolation and resuscitated before staining. In other studies, the staining often occurred using fresh PBMC's. It is possible that the process of freezing and resuscitation of the PBMC's influenced the expression of CD206 on the monocytes, which resulted in the absence of a CD206 positive signal. Another possible reason that no positive signal for CD206 was found in any of the samples, is that CD206 may be a maturation marker. In the article by Wright et al, 2021, it was reported that CD206 was expressed by macrophages with a more mature phenotype. Macrophages that were CD206 negative had a less mature phenotype, resembling the monocytes (58). Thus, it may be possible that during the maturation of monocytes into macrophages, the expression of CD206 is acquired, explaining the absence of CD206 on monocytes. However, in previous articles, CD206 expressing monocytes were found, thus contradicting the findings in the article of Wright et al, 2021 (17). In order to gain more insight into the cause of the absence of a CD206 positive signal in the analysed samples, in the future fresh PBMC's should be stained with this monocyte panel.

4.1.3. HLA-DR expression in monocyte subpopulations

In this project, whilst analysing the expression of HLA-DR in the non-classical, intermediate, classical and total monocyte populations in the three different subject-groups, it was observed that the expression of HLA-DR was significantly different in the non-classical and the intermediate populations.

There was no significant difference in the expression of HLA-DR in the classical and total monocyte subpopulations between the three subject groups. In the non-classical population, a significant difference was found between the SSc- and HC group and the SSc-ILD and HC group. Both the SSc- and the SSc-ILD group had a higher expression of HLA-DR in the non-classical population compared to the HC group. In the intermediate subpopulation, a significant difference was found between the SSc- and SSc-ILD group and the SSc- and HC group. The SSc group had a relatively higher HLA-DR expression compared to the SSc-ILD group and compared to the HC group. These results are conflicting with the expectation, which was partially based on the findings by Schneider et al, 2021 (17). In the research by Schneider et al, 2021, it was found that there was a higher expression of HLA-DR in all monocyte subpopulations in SSc patients compared to healthy controls (17). However, in this project the expression of HLA-DR was only higher in SSc patients compared to HC individuals in the non-classical and intermediate populations. Furthermore, it was expected that the expression of HLA-DR would be higher in SSc-ILD patients compared to SSc patients, which was not the case in any of the monocyte subpopulations. HLA-DR is an activation marker (17) of monocytes (59) which has been found to have a higher expression in auto-immune diseases (17). As monocyte activation has been shown to be associated with the disease progression of autoimmune diseases (60), it is likely that the monocytes have become activated to a higher extent in the disease course of SSc-ILD patients compared to the disease course of SSc patients. Therefore, it was expected that the HLA-DR marker would have a higher expression in SSc-ILD patients, as these patients have presumably an increased activation of monocytes and therefore also an increased expression of HLA-DR.

In the analysis of the MFI data, no significant differences were observed in the expression of HLA-DR in the monocyte subpopulations or in the total monocyte population between the three subject groups. Thus, the expression of HLA-DR on the monocytes in the different monocyte subpopulations did not differ between the SSc-, SSc-ILD or HC group. This is also contradicting earlier reports, in which the MFI expression of HLA-DR was higher in all monocyte subpopulations of SSc patients compared to HC patients (17).

Thus, the results on the expression of HLA-DR in the monocyte subpopulations did not match the expectations. Compared to other research, in this project relatively few participants were included in the analysis, which is a possible cause for the deviating results, as compared to the expectation. Continuation of this research may present with results that are more in line with the expectation (17).

4.1.4. CD169 expression in monocyte subpopulations

The %population of CD169 was only significantly different between the subject-groups in the intermediate population. It was shown that the CD169 expression was higher in the SSc-ILD patient group compared to the SSc patient group. Also, the CD169 expression was higher in the HC group compared to the SSc group. The HC group had a higher expression of CD169 compared to the SSc group was not according to expectation. CD169 has been associated with SSc, as in earlier reports the expression of this marker was shown to be higher in SSc patients compared to healthy controls (17). Therefore, the expectation was that the SSc group would have a higher expression of CD169 compared to the HC group. The higher expression of CD169 in the SSc-ILD group compared to the SSc group was expected. CD169 has been associated with inflammation (61) and fibrosis (17) (53) (57) as it is a representative marker for the dcSSc subtype (17) and it has been shown to have a higher expression in SSc-ILD compared to other diseases such as sarcoidosis and lung cancer (54) (57). These findings suggest that CD169 may also have a higher expression in SSc-ILD compared to SSc. Although this hypothesis is confirmed by the higher expression of CD169 in the intermediate population in the SSc-ILD group compared to the SSc group, it is contradicted by the expression of CD169 in the other monocyte populations.

The MFI expression of CD169 was significantly different in the non-classical and the intermediate populations. In the non-classical population, the SSc- and SSc-ILD groups had a relatively lower MFI

expression of CD169 compared to the HC group. In the intermediate population the SSc group had a relatively lower MFI expression of CD169 compared to the HC group. These results are not in line with the expectation for the MFI of CD169 in the different subpopulations and subject groups. The expectation was that the SSc-ILD group would have a higher MFI of CD169 in the classical and intermediate populations compared to the MFI of CD169 in the classical and intermediate populations of the SSc- and HC group. This expectation was based on the findings by Schneider et al, 2021, where they found a higher MFI of CD169 in the classical and intermediate populations in dSSc patients compared to lSSc patients and HC individuals. As mentioned before, dSSc compared to lSSc has a more aggressive course and it is associated with internal organ fibrosis (9). Because the expression of CD169 in dSSc was increased compared to the expression of CD169 in lSSc, there is a possible association between CD169 and fibrotic manifestations. As ILD is a form of internal organ fibrosis in SSc, it was thus expected that the MFI of CD169 in SSc-ILD patients would be higher compared to the MFI of CD169 in SSc patients.

As was previously mentioned, the number of samples that were analysed in this project was relatively low. Therefore, a better insight in the CD169 expression in the monocyte subpopulations may be acquired when the subject group is extended. Nevertheless, the results of the %population of CD169 in the intermediate population showed a promising association of CD169 with SSc-ILD.

4.1.5. Monocytes conclusion

For the expression of CD86 within the monocyte subpopulations, no significant differences were found between any of the subject-groups. For the expression of CD206 in the monocyte subpopulations, no reliable conclusions could be drawn from the results that were obtained in this project, as the cause of the absence of a positive CD206 signal was not identified. It was also difficult to draw conclusions from the results on the expression of the monocyte subpopulations and the expression of HLA-DR and CD169 in the monocyte subpopulations, due to the relatively small subject-groups. Analysis of more SSc-, SSc-ILD and HC individuals might shed more light on the relation between the monocyte subpopulations and their markers with SSc-ILD. However, in this project it has been shown that CD169 is a promising potential progression marker for SSc-ILD. This because of the previous reports on CD169 in other articles (17) (53) (57) combined with the higher expression of CD169 in the intermediate population of the SSc-ILD group found in this project. Further research on this marker, with a larger subject group, may present with more promising prospects for CD169 as a SSc-ILD progression biomarker.

4.2. Circulating fibrocytes

Another objective of this project was to investigate the expression of circulating fibrocytes in SSc patients compared to SSc-ILD patients. By doing so, the intent was to answer the research question: “What is the difference in the level of circulating fibrocytes between SSc and SSc-ILD patients and are circulating fibrocytes a potential marker for SSc-ILD?”. However, as was mentioned in the results, there were problems with the analysis of the samples stained with the fibrocyte panel.

It has been shown that fibrocytes have a higher expression in patients with SSc-ILD compared to healthy controls (55). In one specific research, fibrocytes were found in the lungs of SSc-ILD patients whilst no fibrocytes were found in the lungs of healthy control patients (47). Moreover, reports have been made on the association between fibrocytes and pulmonary fibrosis (62) and on their elevated expression in idiopathic pulmonary fibrosis (IPF) (63) (16). These findings even strengthen implications of the possible role of fibrocytes within the SSc-ILD pathogenesis. Therefore, circulating fibrocytes may be a potential progression biomarker for SSc-ILD and thus a potential tool for predicting the progression of SSc into SSc-ILD.

According to previously published articles (16) (50), circulating fibrocytes express collagen-I and “high” CXCR4. CXCR4 is also expressed by monocytes, but monocytes express “dim” CXCR4, enabling the

distinction between monocytes and fibrocytes based on the level of CXCR4 expression. Furthermore, in PBMC samples, fibrocytes are the only cells that express collagen-I (50). This fact makes this gating strategy in theory very effective for finding fibrocytes. However, as can be seen in figure 9e, there is no clear double positive population for CXCR4/collagen-I, making it very difficult to determine if there are circulating fibrocytes present in the sample or not. The absence of a double positive CXCR4/collagen-I population could be the effect of either two causes: either there were no fibrocytes present in that sample or there were fibrocytes present, but due to one or more variables in the experiment it was not possible to detect them. The first possibility was linked to the question that was to be answered with the experiment, "are there fibrocytes present in these samples or not?". Therefore, it was not possible to confirm or reject this possibility. For this reason, the focus was placed on the second possibility: "Were there variables in the experiment that prevented the detection of fibrocytes?". In order to confirm or reject the second possibility, the entire experiment set-up had to be evaluated. Several variables in the experiment were considered and eventually the most likely bottlenecks were appointed. First the antibodies and their efficacy were considered. Looking at the results of the analysis of a PBMC sample with the fibrocyte panel in figure 9, it appeared that the CXCR4 and/or collagen-I antibodies were not working properly: there was no clear positive signal for CXCR4 and the collagen-I positive population seemed relatively too large. Several experiments were executed to investigate the efficacy of the CXCR4 and collagen-I antibodies. From these experiments it was concluded that the CXCR4 antibody was working but the protocol for staining with the CXCR4 antibody had to be changed; addition of CXCR4 should have taken place after the permeabilization step in the fibrocyte staining protocol. Furthermore, from these experiments it was concluded that the collagen-I antibody was binding unspecific.

4.2.1. Fibrocytes conclusion

Finding proof of the presence of fibrocytes by means of flow cytometry is challenging. In the future, this experiment set-up can be used for answering the research question: "What is the difference in the level of circulating fibrocytes between SSc and SSc-ILD patients and are circulating fibrocytes a potential marker for SSc-ILD?". Now that the problems with the fibrocyte panel and the staining protocol have been established, alterations can be made to eliminate the causes of the problems. Firstly, the CXCR4 antibody should be added after the permeabilization step in the fibrocyte staining protocol. Secondly, another collagen-I antibody should be selected for the fibrocyte staining panel, so that unspecific binding will be prevented. Once these changes are integrated in the experiment set-up, differences in expression of circulating fibrocytes in SSc patients and SSc-ILD patients can be investigated and the search for a SSc-ILD progression biomarker can be continued and prospects for SSc-ILD patients might potentially be increased.

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Appendix I – Protocol PBMC isolation

Materials:

- Lymfoprep™ (Serumwerk Bernburg AG for Alere Technologies AS, Oslo, Norway) (sterile, at room temperature)
- RPMI medium 1640 (1x) (Gibco) + gentamycin (500 ml + 3 ml, at room temperature)
- RPMI + 20% DMSO (16 ml + 4 ml, on ice)
- RPMI + 20% FCS (16 ml + 4 ml, on ice)
- ZAP-OGLOBIN II lytic reagent (Beckman Coulter, Canada LP)

Methods:

1. Keep the peripheral blood in the 10 ml lithium heparin tubes on the roller bank if not using immediately.
2. Add 4 ml lymphoprep to 6 x 15 ml glass tubes.
3. Pour the blood from the lithium heparin tubes in a 50 ml tube.
4. Add RPMI + gentamycin to the blood approximately 1:1 and homogenize.
5. Carefully add 8 ml of diluted blood to the lymphoprep, divide residual blood over the tubes.
6. Centrifuge the tubes with sufficient support at the bottom, with program 6 (2400 rpm, break 0, 20 minutes).
7. Remove the supernatant down to 0,5 cm above the white ring by aspiration.
8. In a circular movement, collect the white part to a new 15 ml tube, pooling together the white part of 2 tubes into one new 15 ml tube.
9. Add RPMI + gentamycin and homogenize.
10. Centrifuge to wash with program 7 (1800 rpm, brake 5, 10 minutes).
11. Remove the supernatant by aspiration.
12. Resuspend the cell pellets with 10 ml RPMI + gentamycin, dividing the 10 ml over the three tubes. Pool the pellets together in one tube.
13. Count the cells: add 40 µl of the cell suspension to 20 ml of counting suspension + 3-4 drops of ZAP-OGLOBIN II lytic reagent, count the cells with the Z1 Coulter Particle Counter, Beckman Coulter.
14. Centrifuge the rest of the cell suspension with program 4 (1200 rpm, brake 5, 10 minutes).
15. Remove the supernatant by aspiration.
16. Resuspend the cells in RPMI + 20% FCS, so that the end concentration is $1x10^7$ cells in 10% FCS + 10% DMSO (50 million cells: resuspend with 2,5 ml RPMI + 20% FCS, divide over tubes, add 0,5 ml RPMI + 20% DMSO to each tube).
17. Divide the cell suspension over the cryotubes on ice, add RPMI + 20% DMSO.
18. Place the tubes in the isopropanol freezing container (room temperature, use up to 5 times).
19. Place the isopropanol freezing container in the -80˚C freezer.
20. After at least 24 hours of freezing, put the tubes in the nitrogen container.

Appendix II – Protocol PBMC resuscitation

Preparation:

- Check if there is enough medium for resuscitation of the PBMC's:
    - Prepare under sterile conditions, in the down-flow cabinet:
        - 500 ml RPMI + 3 ml gentamicin
        - In a 50 ml tube: RPMI + gentamicin + 10% FCS → 5 ml FCS + 45 ml RPMI + gentamicin.
- Put the water bath on at 37°C.
- Check if the required antibodies are present.
- Prepare an ice-container with tweezers.
- Prepare the necessary amount of 10 ml tubes (glass tubes with white cap) and label them with the corresponding patient and antibody/mix/unstained.
- Check if there is enough FACS lysing solution.
    - 5 ml of stock FACS lysing buffer filled up to 50 ml with demi water in a 50 ml tube.
    - Coat the 50 ml tube with aluminum foil.
- Check if there is enough Wash Buffer:
    - 1% BSA in PBS (2,5 ml of 20% BSA→ fill up to 50 ml with PBS)
    - 5% FCS in PBS (2,5 ml → fill up to 50 ml with PBS)
- Calculate the necessary amount of cell solution (100 µl/tube) and prepare the necessary amount of RNA-free Eppendorf cups for Trizoling of the residual PBMC's.
- Label the RNA-free Eppendorf cups for Trizoling: Trizoled PBMC's, Patient number, date.
- Prepare a cup for counting the resuscitated PBMC's:
    - 20 ml counting suspension + 3-4 drops of ZAP-OGLOBIN II lytic reagent.
- Prepare a 15 ml tube (glass tube with white cap) with 9 ml of RPMI + 10% FCS.

PBMC resuscitation:

- Collect the isolated PBMC's from the specific patient from the nitrogen freezer at the basement, bring an ice container and tweezers.
- Thaw the PBMC's in the cryo-tubes in the water bath (37˚C): stir the cryo-tube in the water bath while regularly checking if the cells are thawing. Do this until a small clump of ice is left in the tube.
- Transfer the thawed PBMC's into the 15 ml tube (glass tubes with white cap) with 9 ml of RPMI + 10% FCS.
- Use 1 ml of RPMI + 10% FCS to give the cryo-tube a wash, and transfer this 1 ml also to the glass tube.
- Homogenize the cell solution.
- Pipet 40 µl of resuscitated PBMC's to the counting cup.
- Count the amount of cells: desired amount = 10.000.000 cells (1.000.000 cells/ml in 10 ml → 10.000.000 cells).
- Centrifuge the tube for 6 minutes at 1400 rpm with brake 0.
- Remove the supernatant by aspiration.
- Resuspend the cell pellet in the necessary amount of medium. In case of 10.000.000 cells: in 2 ml of RPMI + 10% FCS medium.
    - Cells are now at a concentration of 10.000.000 cells in 2 ml = 5.000.000 cells/ml.
- Pipet 100 µl of this cell suspension to all the prepared 10 ml tubes: pipet at the bottom of the tube!
    - Amount of cells: 100 µl of 5.000.000 cells/ml = 0,5 x 10^6 cells in 100 µl.

Appendix III – Protocol staining of isolated PBMC's

For tubes with addition of Brilliant Stain Buffer:

- Add 50 µl of BD Horizon Brilliant Stain Buffer to all tubes.
- Add each fluorescent reagent at the recommended volume per test.
- Add 100 µl of the human cells to each tube (0,5 x 10^6 cells per cup are needed (5 x 10^6 cells/ml)).
- Vortex tube contents.
- Incubate 30 minutes the suspended cells protected from light at room temperature.
- For the intracellular markers tubes: follow instructions under "*Intracellular marker tubes".
- To the rest of the tubes: Add 2 ml of FACS Lysing solution to lyse the red blood cells (start a timer when commencing with the pipetting).
- Vortex the tubes gently.
- Incubate for 10 minutes at room temperature in the dark (no longer!).
- Centrifuge at 2500 rpm for 2 minutes (break 9), and remove the supernatant until about 100-200 µl of fluid is left, without disturbing the cell pellet.
- Resuspend the cells in the 100-200 µl fluid by capping the tubes and shaking them whilst they are in the tube-rack.
- Wash twice with 2 ml Wash Buffer (1% BSA in PBS):
    - Add 2 ml of Wash Buffer to each tube.
    - Centrifuge at 2500 rpm for 2 minutes (break 9).
    - Remove the supernatant by aspiration until about 100-200 µl of fluid is left.
    - Resuspend the cells in the 100-200 µl of fluid by capping the tubes and shaking them whilst they are in the tube-rack.
    - Add 2 ml of Wash Buffer.
    - Centrifuge at 2500 rpm for 2 minutes, break 9.
    - Remove the supernatant without disturbing the cell pellet.
- Resuspend cells in 300 µl wash buffer, and analyze on Quantion. Samples can be stored at 4˚C in the dark, for maximum 24 hours.

*Staining with intracellular markers:

- Wash the cells with 2,5 ml of Wash Buffer (5% FCS in PBS). Centrifuge for 5 minutes, at 1800 rpm, break 9.
- Remove the supernatant until about 100-200 µl of fluid is left, without disturbing the cell pellet.
- Resuspend the cells in the 100-200 µl fluid by capping the tubes and shaking them whilst they are in the tube-rack and resuspend with the pulse vortex.
- Add 100 µl of Reagent A (Fixation medium; Caltag) to each of the intracellular marker tubes, and incubate for 15 minutes in the dark at room temperature.
- Wash the cells with 2,5 ml of Wash Buffer (5% FCS in PBS). Centrifuge for 5 minutes, at 1800 rpm, break 9.
- Remove the supernatant until about 100-200 µl of fluid is left, without disturbing the cell pellet.
- Resuspend the cells in the 100-200 µl fluid by capping the tubes and shaking them whilst they are in the tube-rack and resuspend with the pulse vortex.
- Add 100 µl of Reagent B (Permeabilization medium; Caltag) to each tube, and add the fluorochrome-conjugates Ab's against the intracellular proteins.
- Incubate for 30 minutes at RT in the dark.
- Wash the cells with 2,5 ml of Wash Buffer (5% FCS in PBS) and centrifuge for 5 minutes at 1800 rpm, break 9.
- Resuspend the cells 300 µl of Wash Buffer (5% FCS in PBS) and analyze on the Quantion.

For the TANG tubes:

- For the FACs procedure 100 µl with 0,5 x 10^6 cells per cup are needed (5 x 10^6 cells/ml).
- Add fluorescent-conjugated Moab's, the surface markers (see the tables), to the corresponding tubes, there has to be 1 unstained tube.
- Vortex the tubes gently.
- Incubate for 15 minutes at room temperature in the dark.
- For the intracellular markers tubes: follow instructions under "*Intracellular marker tubes".
- To the rest of the tubes: Add 2 ml of 1x FACS Lysing solution to lyse the red blood cells (start a timer when commencing with the pipetting).
- Vortex the tubes gently.
- Incubate for 10 minutes at room temperature in the dark (no longer!).
- Centrifuge at 2500 rpm for 2 minutes (break 9), and remove the supernatant until about 100-200 µl of fluid is left, without disturbing the cell pellet.
- Resuspend the cells in the 100-200 µl fluid by capping the tubes and shaking them whilst they are in the tube-rack.
- Wash twice with 2 ml Wash Buffer (1% BSA in PBS):
    - Add 2 ml of Wash Buffer to each tube.
    - Centrifuge at 2500 rpm for 2 minutes (break 9).
    - Remove the supernatant by aspiration until about 100-200 µl of fluid is left.
    - Resuspend the cells in the 100-200 µl of fluid by capping the tubes and shaking them whilst they are in the tube-rack.
    - Add 2 ml of Wash Buffer.
    - Centrifuge at 2500 rpm for 2 minutes, break 9.
    - Remove the supernatant without disturbing the cell pellet.
- Resuspend cells in 300 µl wash buffer, and analyze on Quantion. Samples can be stored at 4˚C in the dark, for maximum 24 hours.

Table 3: FACS Monocyte mix tube:

MoAb		**Cat. No.**	**Volume (µl):**
CD16	PE cy7		5
CD14	BV 605		5
CD206	PE		5
CD86	APC		5
CD66b	FITC		5
HLA-DR	BV786		5
CD169	BV421		5

Table 4: FACS Fibrocyte mix tube:

MoAb		**Cat. No.**	**Volume (µl):**
CXCR4	BV605		2,5
CD45	PerCP-Cy5.5		10
CD34	APC-Cy7		5
HLA-DR	BV786		5
CD14	PE		5
CD16	PE-Cy7		5
Collagen-1	FITC	Intercellular marker	1 (from 5x dilution)
CD56	APC	Dump channel	5
CD15	APC	Dump channel	5
CD3	AF700	Dump channel	5
CD19	Af700	Dump channel	5