PhD Thesis

The Role of Inflammation in Systemic Sclerosis

Staff-PhD Thesis Dr. Thomas Huegle (No:099044875)
Clinical Research Associate

Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom

Supervisor: Prof. J. M. van Laar

Date 8.6.2011

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Acknowledgement

First and foremost I offer my sincerest gratitude to my supervisor, Professor Jacob M. van Laar for his great support thoughout my thesis. I thank for his patience and knowledge whilst allowing me the room to work in my own way. I also thank him for his good advices not only in the laboratory but also for my carrieer planning. One simply could not wish for a more professional and friendlier supervisor and mentor.

I thank Rachel Simpson for her great technical support within the ‚TNF-project'.

I thank Christiaan Huigens for his great input, technical support and efforts and as well as his enthusiasm in the ‚TIMP-1' project.

I thank Dr. Anja Krippner for her collaboration in the ‚TNF project' and her donation of the ‚cys-TNF' mutants.

I thank Kath White for great work at the electron microscope.

I thank Dr. Venetia Bigley and Professor Matthew Collin for teaching me in dermal flow cytometry and for the kind contribution of their data of healthy skin flow cytometry and stainings of the epidermis of SSc patients.

I thank Marina Kraaij from the Department of Rheumatology for sharing her immunohistochemistry data in the ‚TNF' project.

I thank Ian Dimmick for his technical support at the flow cytometry facility.

I thank Dr. Thomas Daikeler and Profesor Ulrich Walker for their great contribution and supervision in the clinical studies.

I thank Professor John Isaacs and Professor Matthew Collin for their great work in supporting me in their role as internal PhD assessors.

I thank Line Iversen from the Department of Rheumatology, Rigshospitalet in Copenhagen, Denmark for sending me skin biospies.

I thank Philipp Schuetz from the school of public health, Harvard, USA for his statistical support.

I thank the EUSTAR database members all over the world for including patients in the database.

I thank my mentor Alan Tyndall for his mentorship.

My greatest thanks go to all Systemic Sclerosis patients that I have seen at the Freeman Hospital in Newcastle and the James Cook University Hospital in Middlesbrough. Their contribution to this work by donating blood and skin, and their willingness to help research was the most important part of this work.

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Systemic sclerosis (SSc) is a connective tissue disease of unknown etiology. It is a rare disorder with an incidence in adults of approximately 20 new cases per million per year (1). Genetic and environmental factors such as toxins and infections have been identified to be associated with SSc (2, 3). Its pathogenesis is characterized by vasculopathy, autoimmunity, and cytokine dysbalance, altogether leading to fibrosis of the skin and inner organs (Figure 1) (4). These features vary between patients, but it seems clear that SSc pathogenesis is characterized by a complex interplay between these factors. There is a high heterogeneity in clinical presentation of SSc e.g. limited versus diffuse SSc, early vs. chronic disease, or early vs. late onset SSc, that have to be taken into consideration when studying this disease (5). Along the important progress in inflammation research that has been made during the last decade in general, inflammation has also attracted increasing attention in SSc (6). Both the innate and adaptive immune system participates in SSc pathogenesis. The importance of the immune system in SSc has also been demonstrated in cases where SSc patients underwent autologous or allogeneic stem cell transplantation. Conditioning e.g. with cyclophosphamide followed by reinfusion of stem cells markedly improved the disease course (7). Cell infiltrates in SSc affected dermis mainly consist of T- and B lymphocytes, macrophages, and mast cells. These cells release profibrotic mediators which then stimulate fibroblasts to secrete extracellular matrix (ECM) proteins (8-10). The inflammatory cells are responsible for a profibrotic cytokine milieu, typically characterized by Th2 cytokines (11).

Humoral autoimmunity in the form of autoantibodies has been postulated to contribute to SSc progression, e.g. by autoantibodies against endothelial cells or fibroblasts (12). Although B-cells clearly occur in the inflamed SSc tissue and certainly are of importance, the pathogenic relevance of autoantibodies in SSc remains controversial (13).

This work contributes to the understanding of SSc by further analyses of the cellular pathogenesis and cytokines involved in SSc. Both experimental and clinical studies have been performed in this thesis, analysing different inflammatory cell types and cytokines and patient subgroups, respectively.

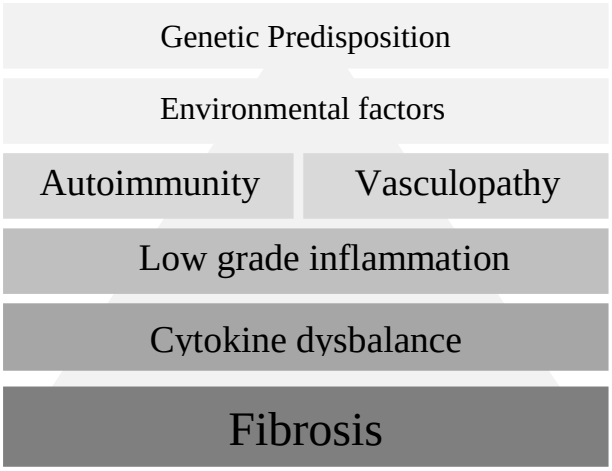

Figure 1. Sequential steps in the pathogenesis of SSc (14)

1.2 Animal models of SSc

Important lessons have been learned on the role of inflammation in SSc, by animal models (15-19). SSc animal models are classified into genetic models (e.g. TSK, Fra-2, Caveolin 1 -/- mice) or SSc-inducible models (e.g. bleomycin induced SSc, or sclerodermatous graft versus host disease) (20). The most widely used animal model of SSc is the bleomycin-induced SSc model. Bleomycin is used in humans because it has anti-tumor effects in different types of cancer. In higher doses, bleomycin can induce lung injury and pulmonary fibrosis (21) and bleomycin stimulates isolated fibroblasts to produce collagen (22). Case reports described skin fibrosis that occurred after treatment with bleomycin. Usually, mice receive bleomycin by daily subcutaneous injections over 4 weeks. When instilled in the lung, bleomycin has been used to study murine pulmonary fibrosis (23). Bleomcyin treated mice develop skin fibrosis which usually persists for several weeks after the treatment and lung fibrosis characterized by cellular infiltrates and lung

parenchymal damage. Also, anti-Scl-70 auto-antibodies develop in the course of bleomycin treatment, reflecting a state of autoimmunity or loss of tolerance to self antigens, respectively (24). The severity of skin fibrosis depends on the strain; B10.A strain e.g. shows a high susceptibility. Skin thickening in bleomycin treated mice is associated with inflammatory changes. Macrophages, eosinophils, mast cells, but also T- and B-lymphocytes are attracted and promote fibrosis presumably by activation of fibroblasts, or the differentiation into myofibroblasts. This is associated with a pro-fibrotic cytokine milieu, notably with increased levels of transforming growth factor (TGF) beta, IL-4, IL-6, IL-13 and platelet derived growth factor (PDGF) (25). Which of the above mentioned cells are mainly responsible e.g. for TGF-beta expression is elusive. In fact, inflammatory cell infiltration, cytokines and specific inflammation receptor pathway activation have been identified as 'sine qua non' for fibrosis (18, 26-30). The transfer of T-cells from bleomycin treated into healthy mice led to the development of SSc. Limitations of the bleomycin model are the lack of systemic disease features, the spontaneous improvement and possibly the overestimation of anti-inflammatory compounds (20).

As an important limitation in SSc animal models in general, not all pathogenic features that are observed in humans are found in the animals. E.g. vasculopathy, as important pathogenic feature is usually not observed in bleomycin induced skin fibrosis, scleroderma like GvHD or Tsk-1 mice. However, newer SSc animal models such as Fra-2 or UCD-200/2006 mice do present this feature and might be more useful for some studies.

Chronic GvHD occurs in a substantial part of patients who received allogeneic stem cell transplantation. Clinically, GvHD resembles SSc by skin thickening and lung fibrosis; GvHD due to microchimerism has been discussed as a possible cause for SSc (31). In the animal model, alloreactive T cells seem the most important cell type involved in the pathogenesis of chronic GVHD. As also observed in the bleomycin mouse model, naive CD4 T cells are sufficient and necessary to provoke SSc-like GvHD. In contrast, the transfer of CD8 T cells does not cause the disease phenotype.

In contrast to other models, mast cell counts decrease in mice with SSc-GVHD mice (32). Mast cell degranulation and infiltration is postulated to enhance

fibroblasts to release ECM proteins fibroblasts (32). It is however unclear what exactly stimulates mast cells and triggers the degranulation.

1.3 Clinical presentation of SSc

The disease course of SSc is chronic and highly heterogeneous with a spectrum ranging from a limited cutaneous affection to a diffuse systemic form of the disease. Raynaud's syndrome typically precedes other clinical manifestations. Skin tightening, contractures, calcification and ulcers frequently lead to functional impairment and changes in appearance.

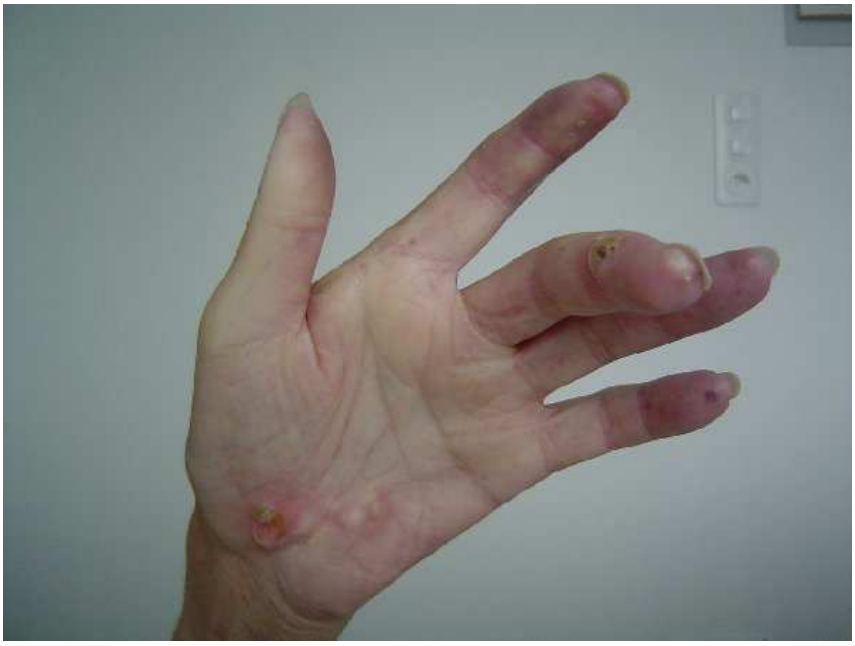

Figure 2. Hand of a patient with limited SSc showing Raynaud phenomenon, digital ulcers, calcinosis cutis and sclerodactyly. With permission of the patient.

At a later stage, fibrosis of internal organs can lead to respiratory or cardiac insufficiency or renal failure. Limited SSc is typically associated with clinical features of vasculopathy in the form of digital ulcers and pulmonary hypertension. In contrast, diffuse SSc typically manifests as skin thickening and lung fibrosis. Limited SSc is commonly associated with the CREST syndrome consisting of calcinosis cutis, Raynaud phenomenon, esophageal dysmobility, sclerodactyli and teleangiectasia. Skin involvement is the most common feature in SSc. Skin thickening and hardening are the typical findings in advanced disease. At an earlier

stage, edematous swelling, erythema, and pruritus may occur. Skin thickening is quantified by the Rodnan skin score, estimating the severity of skin thickness, pliability and fixation to underlying structures in 17 distinct areas of the body from 0 (normal) to 3 (most severe) (33).

A positive anti-centromere (ACA) autoantibody status is associated with limited disease whereas anti-topoisomerase (anti-Scl70) antibodies commonly are associated with diffuse SSc. Renal crisis occurs more frequently in patients with rapid progression of diffuse SSc. Cardiac involvement is also more prevalent in diffuse SSc patients. Patients with a rapid progression have a lower 10-year cumulative survival rate (34).

1.4 Aging and immunosenescence in SSc

Age is an important factor in the disease presentation of autoimmune diseases (5, 35). Immunosenescence is defined as changes in the immune system that develop with increasing age. This phenomenon is likely to interfere with the course of SSc. Aging can affect both the innate and adaptive immune system, both of which are involved in SSc pathogenesis. Several studies postulate an impaired function of the macrophage / monocyte cell line in older individuals (36). Impaired phagocytosis, reduced toll like receptor expression and a change in cytokine expression occur along aging processes (37). Whereas IL-6 and TNF-alpha expression of monocytes decreased in cells from aged mice, IL-10 levels increased upon stimulation (38). $CD16^+$ monocytes have attracted increasing interest in autoimmune diseases during the last years. Interestingly, $CD16^+$ expression on monocytes and polymorphonuclear neutrophils is reduced in the elderly (39).

T-lymphocyte immunity is even more affected by ageing, resulting in decreased numbers of naive T cells, impaired antigen response and proliferation, increased memory cells, and alterations in apoptosis (36). The cytokine expression pattern shifts towards a Th2 response (40). During aging, the thymus becomes atrophic and the function of the thymus reduces subsequently. This results in a decreased T-cell receptor diversity and a reduced central tolerance. Naive T-cell counts decrease with aging, and the proportion of memory T cells increases. Upon antigen stimulation, naïve $CD4^+$ T cells from aged mice produce less IL-2

compared with younger animals (41). This indicates that lymphocytes in older individuals might also react less severe on stimulation by auto-antigens.

The reduced capability of the central tolerance is compensated by peripheral tolerance, notably via T-regulatory cells. However, the function of T-regulatory cells in aged individuals seems to be impaired as well, although existing reports are controversial (42). B-cells are also decreased in the elderly, notably naïve B-cells. This is probably due to less IL-7. Specific antibodies are also decreased in the elderly and less antibodies against self-antigens have been described (43). Increased levels of antinuclear autoantibodies (ANA) in the elderly that are frequently encountered typically affect individuals with chronic disease rather than successfully aging individuals (44). Generally, elderly populations show less organ-specific autoantibodies compared to non organ-specific autoantibodies such as ANA. It is not clear which mechanisms are responsible for autoantibody production in the elderly, but failed suppressor cell function and the loss of central tolerance is likely to be involved. There are other theories why autoimmunity may increase with aging. Activation of B-cellls presentation of ‘neoantigens’ is one of them (45). Alteration of apoptosis in T-cells or the influence of long term cytomegaly infection on the immune system have also been discussed (46). A ‘risk’ phenotype of the elderly to develop autoimmune diseases consists of low B cell levels, increased $CD8^{+}CD28^{-}$ cell rates, poor T cell proliferation response, decreased CD4/CD8 ratio <1 and CMV seropositivity (47, 48).

SSc patients with a *juvenile* onset have less skin involvement and lower mortality rates, but they do suffer more frequently from overlap syndromes, typically involving skeletal muscles (49). Conversely, patients with onset of Raynaud’s phenomenon above the mean age suffer more frequently from digital ulcers, lung fibrosis, PH and diastolic heart failure (50). The mean age at onset of first non-Raynaud’s phenomenon in the EULAR Scleroderma Trials and Research (EUSTAR) database was reported 44.8 years for diffuse SSc and 47.9 years for limited SSc (50). Generally, white patients have an older age at diagnosis compared to black patients (43.8 years) (51). Among white patients, the peak incidence was between 65 and 74 years in women and >75 years in white men (51). The incidence of SSc >75 years is around 20 cases per million per year which is 2-4% of all SSc cases (51).

Late age at onset of SSc has been reported to be associated with a more aggressive disease course (52). The risk of death increases by 5% for each 1-year increase in age at diagnosis (51). The number of patients >75 years at diagnosis in previous studies however is low and naturally occurring co-morbidity in the elderly certainly influences survival analysis. Only small SSc cohorts or case series have focused on late onset SSc. Whereas some cases presented with a more severe disease course compared to patients <60 years, a more benign course, especially concerning skin involvement, has been reported by others (53-55). Despite a more severe lung involvement and a delayed diagnosis in the late onset group, the disease remained stable in patients >75 years (55). These results and the above mentioned known mechanisms of immunosenescence let to the investigation of late onset SSc. The hypothesis was that due to immunosenescence, the course of SSc in patients with a late onset of the disease is less aggressive compared to the control group.

1.5 Genetic predisposition

The discovery of genetic polymorphisms and mutations in a disease of unknown cause such as SSc is important to identify impaired signaling pathways, cytokines or receptors. Following their identification, functional analyses are usually performed to confirm a pathogenic mechanism and develop possible drug candidates. The completion of the human genome sequence and the advent of efficient and affordable genome-wide association studies (GWAS) have greatly enhanced the discovery and investigation of many new gene mutation candidates (in particular, single nucleotide polymorphisms, [SNPs]) in SSc and autoimmune diseases in general (see http://genome.gov/gwastudies). A recent study suggests that there is a familial predominance in SSc, notably affecting the prevalence of RP and interstitial lung disease. Genetic predisposition to vasculopathy is the most frequent risk among first-degree relatives in SSc pedigrees (56).

Polymorphisms of the connective-tissue growth factor (*CTGF*) gene (57), the protein tyrosine phosphatase non-receptor type 22 gene, interleukin-10 receptor gene, STAT-4 and interferon-regulating factor are associated with a genetic susceptibility to SSc (58).

Several polymorphisms have been reported within TNF-signalling or TNF-receptors: the genotype TNFA-1031T/T is associated both with diffuse SSc and limited SSc (59). Furthermore, AG/AA genotypes in position -238 and the AG genotype in position +489 of the TNF-alpha gene are significantly increased in patients with SSc. The relatively rare GG genotype in exon 6 of the TNF-R2 gene is also increased in SSc, although not significant (60).

The described polymorphism within the TNF or interferon pathway however, lack mechanistical evidence. TNF-alpha and TNF-R2 gene polymorphisms were important factors for me to carry out mechanistic research on TNF in SSc.

1.6 Cytokine environment

Cytokines are essential mediators of tissue homeostasis through their regulation of cell-growth, -interaction, -migration, and -differentiation, but also stimulation of the production and degradation of extracellular matrix (ECM). In SSc patients, imbalances of the cytokine environment have been described in tissue and serum (61). Both pro- and anti-inflammatory cytokines are increased (Table 1) (62); the exact significance of this counter-play is still elusive. In general, a shift in Th1 polarization towards Th2 cells is observed (63).

Numerous cytokines such as IL-4 and -13 are upregulated in SSc serum. They stimulate the expression of collagen in fibroblasts, and the differentiation of monocytes into fibrocytes or myofibroblasts. TGF-beta, one of the most potent profibrotic cytokines in our body, plays a key role in SSc pathogenesis as it stimulates collagen expression by fibroblasts via the SMAD transcription factor pathway and also mediates the differentiation of monocytes into myofibroblasts. In SSc skin, TGF-beta is mainly expressed perivascularly within mononuclear infiltrates (64). Once secreted, TGF-beta remains in the ECM in a latent form, until it is activated by integrins and serine proteases (65). In contrast to the findings in tissue, the TGF-beta levels in the serum of SSc patients are decreased (66).

Platelet derived growth factor (PDGF) is another important profibrotic protein. In SSc, elevated levels of PDGF have been reported in skin, serum and fluid obtained by bronchoalveolar lavage (63). It is a potent chemoattractant for monocytes, macrophages and fibroblasts. Like TGF-beta, PDGF stimulates proliferation of fibroblasts, especially in the presence of high concentrations. The exact cellular

source both of TGF-beta and PDGF is not clear. Both immune cells and fibroblasts have been shown to express the growth factors, but it remains unclear on a protein level which cells are mainly responsible for their excretion in SSc.

IL-6 is a pro-inflammatory cytokine that is elevated in SSc sera and correlates with disease severity. Fibroblasts of SSc patients produce more IL-6 compared with fibroblasts from healthy individuals. Monocyte chemoattractant protein-1 (MCP-1; also known as CCL2) is a chemotactic protein that recruits monocytes, memory T cells, and dendritic cells to the site of inflammation (6). Elevated MCP-1 levels have been described in the early stage of SSc and correlate with organ-based complications (67). Tumor necrosis factor-alpha (TNF-alpha) has been shown to be an important cytokine in inflammation although its exact role in SSc remains controversial. TNF receptors are overexpressed in SSc skin and both TNF-alpha and soluble TNF receptor levels are higher in sera of SSc patients (16).The latter correlates with inflammation and disease progression. Upregulation of TNF-alpha-converting enzyme (TACE), which sheds the receptors and soluble TNF-alpha from the cell surface, has been described in peripheral monocytes of SSc patients (68). Functional data on TNF-alpha in SSc demonstrate that it inhibits collagen expression in fibroblasts (69), presumably by a downregulation of TGF-beta receptor 2 (70).

Cytokine	*Findings in SSc*	*Translational status*
Profibrotic:		
TGF-beta	Elevated in tissue, reduced in serum, increased TGF-beta receptor expression, increased activation of latent TGF-beta by integrins	Clinical- trial with mAb. Negative outcome
IL-4, IL-13	Increased collagen production, myofibroblasts differentiation	-
PDGF	Receptor stimulation by autoantibodies	-
Inflammatory:		
IL-6	Elevated in serum	-
MCP-1	Elevated in tissue, correlation with disease activity	
Unclear:		
TNF-alpha	Increased in serum and tissue, upregulation of TACE in monocytes, inhibition of collagen production in fibroblasts	Infliximab effective in animal models and case series, negative clinical trials
Type 1 IFN	Elevated in tissue, monocyte activation	Mab under investigation in SLE, not tested in SSc

Table 1. Cytokines and chemokines involved in systemic sclerosis and current status of treatment with inhibitory antibodies. TGF-beta: transforming growth factor-beta. TGF-beta receptor; Mab: monoclonal antibody; IL: interleukin; PDGF: platelet-derived growth factor; MCP-1: monocyte chemoattractant protein-1; TNF-alpha: tumor necrosis factor-alpha; TACE: TNF-alpha-converting enzyme; IFN: interferon; SLE: Systemic lupus erythematosus

Type 1 interferons (IFNs) include IFN-alpha and -beta and have recently been shown to be involved in SSc pathogenesis. Case series described the occurrence of SSc during or after treatment with IFN-alpha or -beta in patients with hepatitis C, multiple sclerosis, and myelodysplastic syndrome, and remission or stabilization of SSc symptoms were described after cessation of treatment (71). In a previous clinical trial using IFN-alpha as a possible treatment for SSc, deleterious effects have been described (72). Increased levels of IFN are found perivascularly in the skin and serum of SSc patients (73). Compared with monocytes from healthy controls, monocytes from patients with SSc have been found to express significantly increased levels of IFN-regulated genes such as Siglec-1 (CD169) (74). Incubation of peripheral blood mononuclear cells (PBMCs) from healthy controls with sera from SSc patients (containing anti-topoisomerase I autoantibodies) led to overexpression of type 1 IFN (75) .

1.7 Vascular dysfunction

The clinical observation of Raynaud's phenomenon as a first clinical sign and the perivascular mononuclear infiltrate observed in the early phase of SSc suggest an endothelial involvement as one of the initial pathogenic steps in SSc (76). Capillaroscopy has evolved as an important diagnostic tool in SSc, especially in early disease (77). One pathogenic concept is cross-reactivity of antiviral (e.g. cytomegalovirus, CMV) antibodies with endothelial structures. A molecular mimicry in which antibodies recognize both the human CMV late protein UL94 and the integrin–novel antigen-2 (NAG-2) complex can lead to apoptosis of endothelial cells (78). Indeed, the incubation of healthy endothelial precursor cells (EPC) with serum of SSc patients leads to apoptosis, confirming that SSc sera contain apoptosis-inducing factors (79). Another possible pathogenic mechanism of vasculopathy is the insufficient supply of functional EPC. In the bone marrow of SSc patients, reduced numbers of functionally impaired EPC have been described (80). Another study showed abnormal endothelial cell differentiation from bone marrow-derived mesenchymal stem cells (MSC) (81).

Endothelin-1 (ET-1), a peptide secreted from endothelial cells is elevated in SSc. Its production leads to constriction of the underlying smooth muscle cells. ET-1 stimulates fibroblasts to produce and contract ECM. Blockade of ET-1 leads to a

reduction of type 1 collagen and alpha-smooth muscle actin (alpha-SMA) by these cells (82).

1.8 Autoimmunity

Both humoral and cellular features of autoimmunity are found in SSc. A T-cell proliferative response to type 1 collagen has been reported patients with SSc (83). Autoantibodies that are classically associated with SSc are anti-centromere antibodies (ACA) and the anti-topoisomerase I (anti-Scl-70). Although most of the infiltrating cells in affected skin in SSc are T-lympocytes, a B-cell gene expression signature has also been demonstrated (84). Increased serum levels of BAFF (B-cell activating factor), a potent B cell survival factor, have been detected and positively correlate with the severity of skin fibrosis.

The influence of B-cell in SSc has been nicely studied in the TSK mouse model which present a similar systemic autoimmunity than SSc patients (20) TSK mice show hyper-gamma-globulinemia and autoantibodies against topoisomerase I, fibrillin 1, RNA polymerase I, collagen type I, and Fc-receptors (85). CD19 expression is increased in SSc patients and CD19 signalling pathway is constantly activated in TSK mice (86). B-cell depletion in TSK mice reduces skin thickening (87).

Over the last few years, a substantial range of new autoantibodies with a possible pathogenic role in SSc have been discovered (Table 2). Anti-endothelial cell antibodies have been shown to induce apoptosis in human dermal microvascular endothelial cells (88). Anti-fibroblast antibodies directed against the protein fibrillin-1 have been detected in a significant proportion of SSc patients. They activate fibroblasts *in vitro* via the TGF-beta pathway, resulting in increased ECM production (89). Anti-matrix metalloproteinase (anti-MMP) antibodies have been shown to be directed against MMP-1 and MMP-3, which prevent ECM degradation and thus may promote fibrosis (90). Anti-platelet-derived growth factor (anti-PDGF) antibodies have been reported to recognize and activate the human PDGF receptor and to stimulate reactive oxygen species (ROS) and collagen production (12). More recently, anti-angiotensin receptor and anti-endothelin receptor autoantibodies have been demonstrated to be associated with more severe disease manifestations and to predict SSc-related mortality. (91)

Autoantibody	*Target*	*Pathogenicity*
ACA	Centromeres	Not known
Scl-70	Topoisomerase	Not known
Anti-endothelial	NAG-2	Apoptosis, ECM expression
Anti-fibroblast	Fibrillin-1	ECM expression via TGF-beta pathway
	NAG-2	fibroblast activation/ECM expression
Anti-MMP	MMP-1 and -3	Inhibition of ECM degradation
Anti-PDGF	PDGF receptor	Collagen expression, production of reactive oxygen species

Table 2. Autoantibodies in systemic sclerosis. ACA: anti-centromere antibodies; ECM: extracellular matrix; MMP: matrix metalloproteinase; PDGF: platelet-derived growth factor; NAG-2: novel antigen-2; TGF-beta: transforming growth factor-beta.

1.9 Fibrosis

Fibrosis is a biological process involving an inflammatory response and subsequent overproduction of ECM proteins. Cytokines, especially TGF-beta, have an important role in the development of fibrosis. The transcription factor T-box expressed in T cells (T-bet) has recently been identified as important regulator of skin sclerosis (92). Knockout of T-bet, the main regulator of the Th1 immune response, led to skin fibrosis via an IL-13-dependent pathway. This is associated with a predominant Th2 response, including the over-expression of TGF-beta. Interestingly, T-bet-deficient CD4 T cells were sufficient to transfer bleomycine induced pulmonary disease to unaffected SCID recipients (93). T-bet regulated transcription is of importance both for cellular members of the innate and adaptive immune system. FoxP3 positive cells upregulate their T-bet and T-bet positive T-regulatory cells accumulate at sites of TH1 cell–mediated inflammation (94). Thus, homeostasis of T-regulatory cells is dependent on T-bet, at least in a Th1 setting.

Whether or not reduced T-bet expression has a pathogenic significance in SSc, remains elusive.

A classic dogma in fibrosis is the observation that fibroblasts or myofibroblasts overproduce collagen. Another concept is the endothelial–mesenchymal transition (EMT) in which endothelial cells are transformed into matrix-producing cells such as myofibroblasts. A third, yet unproven theory is the recruitment of circulating fibrocytes (95). Fibrocytes constitute a bone marrow-derived cell type of the monocytic lineage that have a physiological function in wound healing and might be of importance in fibrosis.

Taking into account that the ECM undergoes a constant turnover, the impaired breakdown of the matrix may also result in fibrosis. More specifically, the inhibition of metalloproteinases by tissue inhibitors of metalloproteinases (TIMPs) results in a reduced ECM breakdown. In SSc, levels of both TIMP-1 and -2 are raised in serum and correlate with disease severity (96). SSc fibroblasts express higher levels of TIMP-1 mRNA compared with healthy controls (97).

1.10 Inflammatory cell infiltration and the loss of tolerance in SSc

Several factors such as cytokines or chemokines which trigger the influx of immune cells are important in the initiation of inflammation. On the other side, the resolution of inflammation and restoration of tolerance has been increasingly recognized in inflammation research (6). Or, in other words, the problem with inflammation often is not how it starts, but how it fails to subside (98). The switch from a pro- into an anti-inflammatory cell phenotype and cytokines, apoptosis, and transport of cell debris, respectively seem to be essential events for the resolution of inflammation (98).

The ‘orchestration’ of inflammation is pivotal in understanding and probably also treating auto-inflammatory disorders. In SSc, both the innate and adaptive arms of the immune system are involved. Skin and lung biopsies from SSc patients showed increased numbers of lymphocytes, monocytes and mast cells, typically located around affected vessels (8, 10, 76, 99, 100). This is in line with SSc animal models such as bleomycine, silica or asbestos induced SSc, where inflammatory cell infiltration, cytokines and specific inflammation receptor pathway activation

have been identified as ‘sine qua non’ for fibrosis (18, 26-30) (see animal model section). The first cells entering SSc induced tissue damage are monocytes (8). Monocytes which differentiate into macrophages or dendritic cells once they have left the circulation are an important source of inflammatory cytokines such as TNF-alpha and chemokines thus attracting other cell types. In this phase, macrophages belong to the so called M1 type (101). However, monocytes can also differentiate into fibrocytes, pericytes or myofibroblasts and therefore have the capacity to produce ECM (95). In wound healing, a specific macrophage phenotype which is involved in tissue repair has been postulated (102). A M2 phenotype of macrophages orchestrate the ECM in wound by secretion of tissue proteases and thus actively remodelling the tissue (101). Older studies confirm already that mice with non functioning macrophages have a delayed wound healing (103).

1.11 Mast cells

Mast cells are granule-containing secretory cells resident in the connective tissue, notably in the skin, respiratory system and gastrointestinal tract. Activation such as in hypersensitivity or anaphylaxis leads to the release of various tissue mediators, including vasoactive amines, proteinases and also TGF-beta (104, 105). Their proximity to fibroblasts makes mast cell products available to fibroblasts and stimulates them to produce collagen (105, 106).

Mast cells promote wound healing by secretion of growth mediators and interaction with other cell types. They release histamine, prostaglandins and leukotriens, causing early vasodilatation and venule permeability, but they also release inflammatory mediators such as TNF-alpha, IL-6 and IL-1. Mast cells accumulate around injured tissue were they release mast cell tryptase. Tryptase not only stimulates collagen expression by fibroblasts, but also triggers wound contraction by stimulating alpha-SMA expression. Animal models showed that wound contraction is insufficient in the absence of mast cells (107). Mast cells interact with fibroblasts via gap-junctions (108).

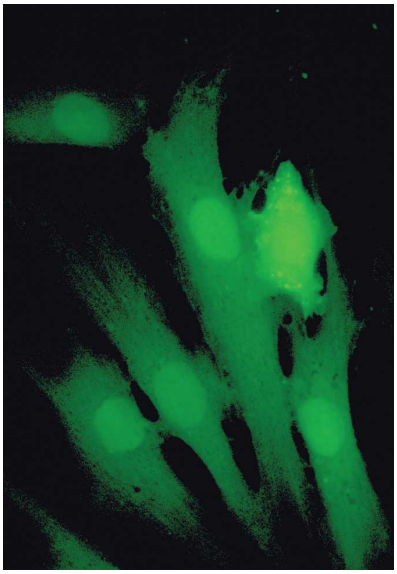

Figure 3. RMC-1 Mast cells passing Calcein Acetomethoxy dye into fibroblasts. Taken from (109).

Several mechanisms can provoke mast cell degranulation. In IgE mediated reactions, antigen binds with mast cell surface IgE. This is notably encountered in anaphylaxis or helmintic infections were metabolic products of the helmints bind IgE. But also tissue injury, mechanical stress, heat, irradiation, toxins, venoms or complement are known to induce mast cell degranulation (110).

Mast cells also express several receptors by which they can be activated and degranulated. Of those, Fc receptors such as activating IgG receptor FcγRIIa (CD32a) in the resting state, and, in the presence of interferon-γ, the high affinity activating FcγRI (CD64) (111). Other receptors expressed on mast cells are C3a and C5a receptors, IL-3R, IL-4R, IL-5R, IL-9R, IL-10R, GM-CSFR, IFN-γR and CCR3, CCR5, CXCR2, CXCR4, nerve growth factor receptor, and toll-like receptors (TLRs).

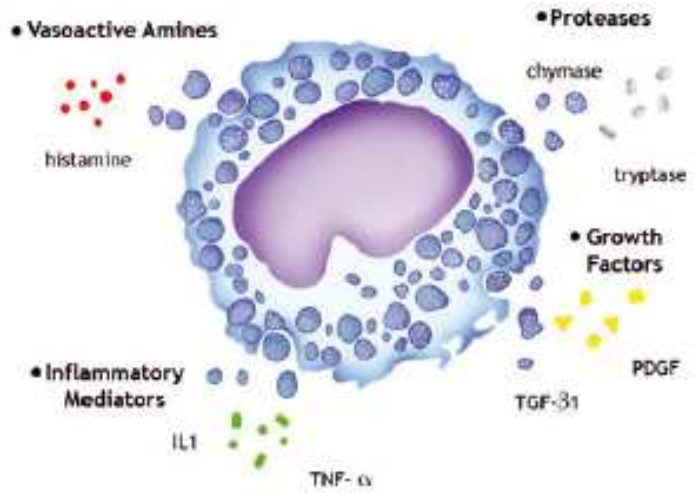

Figure 4. key inflammatory mediators produced by mast cells. IL, interleukin; TNF, tumour necrosis factor; TGF, transforming growth factor; PDGF, platelet-derived growth factor. Taken from (112).

In SSc, the number of mast cells is increased both in involved and uninvolved skin and the number of de-granulated mast cells is increased in the involved but not in the uninvolved skin (113).

The results obtained in this thesis support the hypothesis of inflammation as a key factor in the aetiology of SSc: The roles of mast cells and monocytes as members of the innate and lymphocytes as member of the adaptive immune system are further clarified. It is illustrated that mast cells in SSc are activated, appear in higher numbers, and importantly, are the major source of transforming growth factor (TGF)-beta, a pivotal growth factor in SSc (10). The factor triggering mast cell degranulation in SSc, however remain elusive.

1.12 TNF and TNF-receptors in SSc

Tumor necrosis factor (TNF) alpha is a highly pleiotropic cytokine with paramount influence on the initiation and orchestration of inflammation in general (114). It also has been postulated that TNF-alpha is a key mediator of the transition from inflammation to fibrosis (17). One of the core projects of this thesis was to investigate the role of TNF alpha receptors (TNF-R) in SSc. This was based on the fact that TNF-R are profoundly involved in autoimmunity and even profibrotic effects of TNF-R have been postulated (115). A distinct upregulation of TNF-R has been demonstrated in SSc skin samples, notably TNF-R2 expression on monocuclear cell infiltrates in SSc is high (16). The role of TNF in fibrosis is controversial (116). It is commonly accepted that TNF alpha *per se* inhibits

collagen expression of fibroblasts, at least in healthy individuals (69, 117). TNF-knock out animals fail to develop asbestos, silica or bleomycine induced lung fibrosis (29) and accordingly, TNF-blockade has been shown to prevent fibrosis in bleomycine or silica animal models (118). In this context, TNF-R2 seems to be of special importance in the development of fibrosis as TNF-R2 knock out mice are protected from fibrosis (119).

Until recently, TNF alpha signalling was mainly supposed to function via TNF-R1, which possesses a death domain and activates a pro-inflammatory cascade including NF-kappaB (120, 121) (Figure 5). In contrast to TNF-R1, TNF-R2 has no death domain and signals via the recruitment of a so called TRAF-interacting domain.

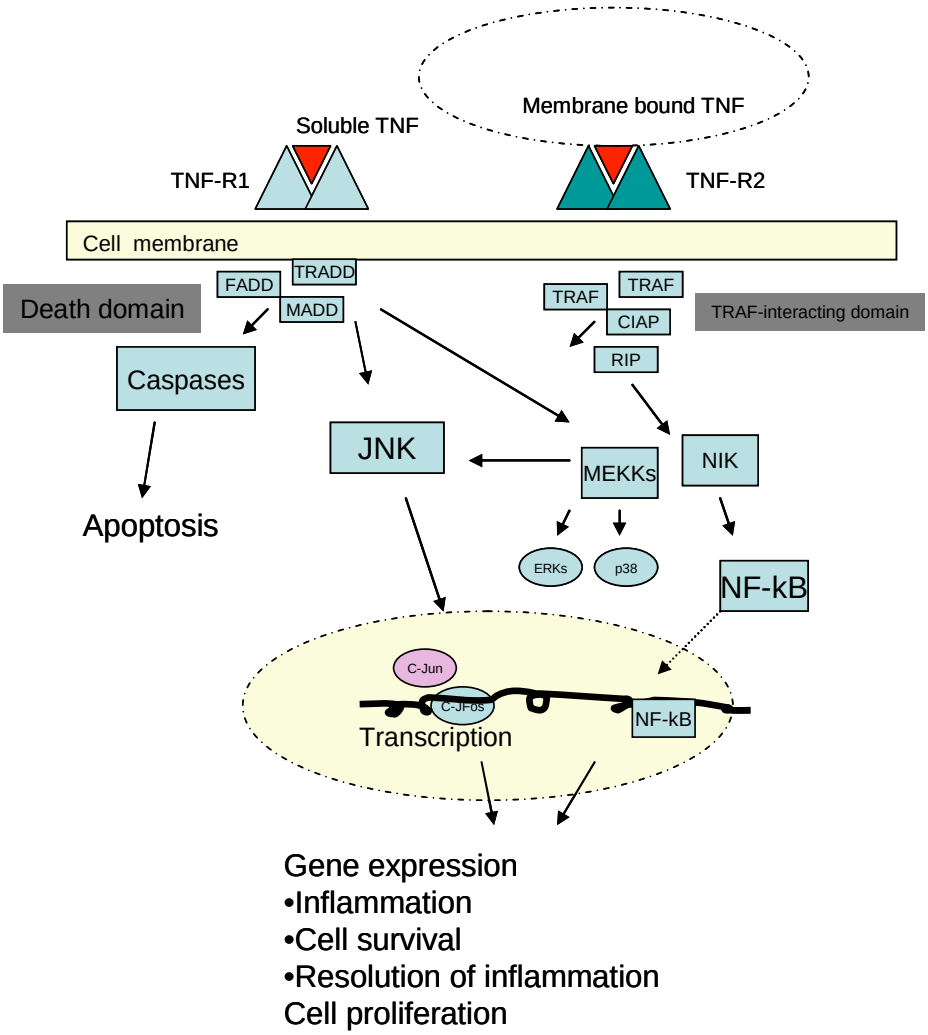

Figure 5. Downstream TNF-R signalling. TRADD: TNF Receptor Associated Death Domain, RIP: Receptor Interacting Protein, RIP: TRAF =TNF Receptor Associated Factor.

The main role of TNF-R2 was believed to be restricted to a bystander role in form of ligand passing to TNF-R1 (120). However, within the last decade, important structural and functional properties have revealed an increasing role of TNF-R in inflammation and notably the resolution of inflammation. In chronic inflammation models, TNF- and TNF-R deficient mice develop more severe inflammation (122). One hypothesis for the observation of possible TNF regulated immunosuppressive feedback effects was that TNF promotes the expansion of T-regulatory cells. Indeed it was demonstrated that active or resting T-regulatory cells express more TNF-R2 than other T-effector cells (123) Interestingly, and possibly corresponding to clinical observations in sepsis where acute inflammation is followed by a immunosuppressive state (124). TNF abrogates inhibitory T-regulatory function on T-effector cells proliferation initially. This is followed, however, by a restoration of T-regulatory function, after longer exposure to TNF. Similar to IL-2, TNF upregulates FoxP3 expression on T-regulatory cells (123). This effect was not observed on TNF-R2 knock out animals, supporting an immunosuppressive role of this receptor in inflammation. The duration of TNF exposure is also important for T-cell signalling: chronic TNF-alpha exposure impairs TCR-signalling via TNF-R2 but not TNF-R1 (125). Shedding of TNF-R2 from the cell surface by TNF alpha converting enzyme (TACE), releases TNF-R2 (126). Subsequently, TNF-R2 can inactivate TNF (as ‘natural’ TNF-blocking compound) and therefore represents an anti-inflammatory mechanism, which has been successfully translated into clinical medicine by the development of etanercept (127) Also a physiological *intrinsic* anti-inflammatory and even profibrotic property, has been attributed to TNF-R2 by triggering e.g. IL-10 expression (119, 123, 128-132). Profibrotic effects upon prolonged TNF exposure might be supported by the fact that in animal models of sustained airway inflammation, TNF drives remodeling of blood vessels and lymphatics (26).

It remains elusive why TNF blockade is an efficient treatment in humans in some autoimmune diseases and has little effect in others. In RA, spondylarthritis or inflammatory bowel diseases, anti-TNF compounds have been successfully translated into clinical application. On the other side, anti-TNF treatment does not lead to remission in all patients, or in others, only for a short period of time (133). Furthermore, autoimmune epiphenomena have been described under TNF-blockade, such as episodes of lupus, multiple sclerosis or neuropathy (134). In

SSc, a clinical trial with infliximab failed to demonstrate a significantly positive effect on skin thickening albeit the collagen expression in the dermis was significantly lower compared to the control (135). These findings reflect the necessity for a deeper understanding of TNF biology in SSc before the right SSc patients can be selected for treatment.

In this thesis, multicolour flow cytometry of the skin was for the first time applied to investigate TNF-R expression on various inflammatory dermal cell subsets. Furthermore, the availability of TNF-R selective TNF mutants for this study allowed a precise functional analysis of both receptors. Indeed, lymphocytes overexpress IL-6, an important proinflammatory and profibrotic cytokine, mainly upon TNF-R1 activation. TGF-beta, a key profibrotic cytokine, is expressed in first line upon TNF-R2 activation. It could also be demonstrated that, *in vitro*, TNF-R2 as activation marker, is overexpressed on the cell surface after CD3/28 stimulation. In healthy CD3/28 activated lymphocytes, stimulation of TNF-R2 led to an increased expression of the anti-inflammatory cytokine IL-10 when compared to activated lymphocytes from SSc patients. This might point to a dysregulation of the resolution of inflammation process.

1.13 Therapeutic strategies

1.13.1 Background therapy

Most SSc patients will use symptomatic medication, such as proton-pump inhibitors for reflux, calcium channel antagonists for vasodilatation, and an angiotensin-converting enzyme (ACE) inhibitor or angiotensin (AT) -II receptor antagonist for the prevention of renal crisis. ACE or AT-II inhibitors inhibit TGF-beta production and have shown antifibrotic effects in liver fibrosis; the significance for that in SSc however has not been investigated so far. Other background medication such as aspirin and statins are prescribed depending on co-morbidity and cardiovascular risk factors. In vitro, statins can also reduce TGF-beta production. Further treatment depends on the stage of the disease and the organs involved. In the following, we discuss already available therapeutics or upcoming strategies of the different pathogenic features of SSc.

1.13.2 Vasoactive medication

Digital ulcers and Raynaud's phenomenon lead to a reduction in quality of life and pulmonary hypertension and renal crisis are life-threatening manifestations of SSc. Much effort has therefore been put into development of an efficient treatment of SSc vasculopathy. ET-1 has been demonstrated to participate directly in the vascular damage (136). The ET-1 inhibitor bosentan is currently approved for use in pulmonary hypertension (grade 2 to 4) and the prevention of digital ulcers in SSc. A positive effect of bosentan was illustrated by a long-term improvement and disease stability in patients with grade 3 pulmonary hypertension (137). This was confirmed in another study in which bosentan improved New York Heart Association class and hemodynamics (138). Other ET-1 inhibitors that are successfully used in pulmonary hypertension are sitaxsentan and ambrisentan. A recent analysis suggested a lower incidence of adverse events and possibly a higher efficacy of sitaxsentan as compared to bosentan, but these findings need to be confirmed in an independent study (139).

The continuous intravenous application of epoprostenol is effective in the treatment of Raynaud's phenomenon secondary to SSc; it decreases the frequency and severity of attacks and induces healing of digital ulcers. In a controlled trial, a positive effect on exercise capacity and cardiopulmonary hemodynamics has been demonstrated (140). Patients with connective tissue-associated pulmonary hypertension have also benefited from treatment with sildenafil, a phosphodiesterase inhibitor (141). As shown recently, sildenafil also seems to be capable to be effective in healing digital ulcers (142).

1.13.3 Immunosuppressive agents

Up to 60% of SSc patients receive glucocorticoids, although there is no clear evidence for its efficacy (143). A significant proportion of SSc patients are treated with an immunosuppressive treatment, mostly consisting of cyclophosphamide, methotrexate, azathioprine, or hydroxychloroquine. Thus far, clear evidence of efficacy exists only for cyclophosphamide, which has been shown to be effective in SSc lung disease and skin involvement (144). Unfortunately, the positive effects, seem to disappear within one year after treatment cessation (145). Methotrexate is widely used in SSc patients with diffuse cutaneous disease. A recent case series provided some evidence for its efficacy, with a good response of skin involvement

that worsened after withdrawal of the treatment (146). Mycophenolate mofetil (MMF) has shown positive effects on SSc-related interstitial lung disease in several retrospective studies (147, 148). In addition to its immunosuppressive effect, MMF also seems to have an inhibitory effect on TGF-beta. A prospective, Phase I, open-label study of MMF is currently ongoing (www.clinicaltrials.gov identifier: NCT00433186). Another ongoing study is that of treatment with high-dose intravenous immunoglobulins (www.clinical trials.gov identifier NCT00348296).

1.13.5 TNF-blockage in SSc and other targeted therapy approaches

In several retrospective case series, TNF-alpha inhibitors showed positive effects both on arthritis and skin in SSc (149). Infliximab has been effective in the Bleomycin mouse model (118). In a prospective, open-label trial, however, 16 patients with diffuse SSc who received monthly infliximab infusions showed no improvement of the skin score after 26 weeks (135). Abatacept, a recombinant fusion protein that blocks T cell activation, has been approved for the treatment of RA. A randomized, double-blind, placebo-controlled clinical trial of abatacept versus placebo in patients with diffuse SSc is ongoing (www.clinicaltrials.gov identifier: NCT00442611) although the current state (from 16.5.2011) is ‘unknown.’

Rituximab is a monoclonal antibody directed against the CD20 transmembrane protein present on B cells (150). Given the potential pathogenetic role of autoantibodies, B-cell depletion is also being studied in SSc. In a mouse model of SSc, rituximab was found to cause reduction in skin fibrosis, autoantibody titers, and hypergammaglobulinemia. However, this positive effect was not observed in the chronic phase of the disease. A study in eight patients receiving rituximab demonstrated an improvement in the skin score, dermal hyalinised collagen content, and numbers of dermal myofibroblasts at 24 weeks (151). A more recent study in 15 patients with diffuse cutaneous SSc found no improvement in skin fibrosis or autoantibody titer with rituximab treatment, despite efficient B cell depletion being demonstrated (152).

Neutralizing anti-TGF-beta antibodies have been studied in the early diffuse SSc in 45 patients. The skin score improved both in the investigational and placebo arms, but a significant difference was not observed (153). Currently, the p144 peptide inhibitor of TGF-beta is being explored for the topical use in SSc

(www.clinicaltrial.gov identifier: NCT00574613). The current state of this study (date 16.5.2011) also is ‘unknown’.

1.13.6 Antifibrotic agents

Imatinib mesylate

Imatinib was developed to target the tyrosine kinase ABL which is translocated in chronic myelogenous leukemia. In addition to ABL, imatinib also targets other kinases such as c-Kit and the PDGF receptor. Tyrosine kinases such as the PDGF receptor participate in the activation of fibroblasts. In addition, ABL plays a role in the downstream signaling of TGF-beta (154). Data from animal models have demonstrated a reduction in fibrosis both in bleomycin-induced lung and skin disease as well as in obstructive renal fibrosis after imatinib treatment (155-157). Fibrosis also ameliorated in the later stages of SSc (158).

In five patients with SSc interstitial lung disease treated with 200 mg imatinib per day and cyclophosphamide intravenously every 3 weeks, only one patient had an improvement in lung function however (159).

In an ongoing Phase II trial, 18 patients received 400 mg imatinib per day. Acceptable tolerability and an improvement of the modified Rodnan Skin Score (mRSS) has been shown in an interim analysis (160) albeit no statistical significance could be detected. In another report, 15 patient with systemic sclerosis have been treated with up to 600 mg imatinib mesylate for one year (161). Over two thirds of the patient developed considerable side affects, typically edema, but also rash, diarrhea and notably worsening of scleroderma in two patients. In the five patients who completed the trial, a slightly improved forced vital capacity and skin thickening was observed.

Oral collagen

Collagen has been identified as a possible autoantigen in SSc. The rationale for the oral application of type 1 collagen is the induction of tolerance. In a prospective, multicenter trial, 168 patients were treated with 500 mg/day of type 1 collagen. Although no statistical significance was demonstrated in the primary endpoints, the skin status improved significantly in SSc patients with long disease duration (162).

Roseglitazone

Rosiglitazone is an agent that has been developed for the treatment of type 2 diabetes. It is an agonist of the peroxisome proliferator-activated receptor (PPAR). PPAR stimulation abrogates collagen expression and TGF-beta-dependent myofibroblast differentiation. It has now been demonstrated in the bleomycin mouse model that rosiglitazone is effective in reducing skin inflammation and dermal fibrosis (163).

Cannabinoid receptor (CB2)

CB2 is the receptor for the marijuana component delta9-tetrahydrocannabinol. It has been implicated in the regulation of atherosclerosis and is now being investigated in SSc. In the bleomycin mouse model, CB2 has clearly shown antifibrotic effects (164). Clinical trials in SSc however, have not been carried out yet.

1.13.7 Cellular therapy

Hematopoietic stem cell transplantation (HSCT) in autoimmune diseases is aimed at “resetting” the dysregulated immune system by immunoablative therapy followed by reinfusion of previously isolated hematopoietic stem cells (165). HSC may originate either from the patient him/herself (autologous) or from a human leukocyte antigen (HLA)-matched individual, typically a family member (allogeneic). Advantages of autologous HSCT are a lower treatment-related mortality (TRM) due to the absence of a graft-versus-host disease (GVHD), which occurs in 20–40% allogeneic HSCT. On the other hand, a postulated graft-versus-autoimmunity effect (analogous to a graft-versus-leukemia effect) has been suggested for allogeneic HSCT. Prospective, multicenter trials in SSc are currently ongoing for autologous HSCT whereas only case reports are available for allogeneic HSCT.

1.13.7.1 Autologous HSCT

Autologous HSCT is a multistep procedure. Firstly, HSC are mobilized by administration of intravenous cyclophosphamide and granulocyte-stimulating factor (G-CSF). Stem cells are then collected by leukapheresis. After conditioning with high-dose cyclophosphamide, stem cells are reinfused to shorten aplasia and thus the risk of bleeding or infection.

The main mechanistic effects in autologous HSCT are achieved by eradication of autoaggressive “effector T and B cells” and the induction of regulatory T cells. There is evidence that autologous HSCT may restore tolerance, despite the use of host cells.

In a French–Dutch collaborative study involving 26 patients for whom long-term follow-up data were available, event-free survival, defined as survival without mortality, relapse, or progression of SSc resulting in major organ dysfunction, was 64.3% (95% confidence interval, CI 47.9–86%) at 5 years (166). Skin thickening and performance status improved markedly, and organ dysfunction stabilized. Similar results have been reported in a North-American study (167). However, relapses occurred in one-third of the cases, typically after 2–4 years (166). The TRM rate in the autologous setting is approximately 10% (JM van Laar, personal communication).

Currently, there are three prospective, multicenter studies ongoing investigating the safety and efficacy of autologous HSCT in SSc. A total of 146 patients have thus far been randomized in the European ASTIS (Autologous Stem Cell Transplantation International Scleroderma) trial, and accrual is expected to be complete by the end of 2009. The two other trials are SCOT (Scleroderma Cyclophosphamide or Transplantation Trial) and ASSIST (American Sclerderma Stem Cell versus Immune Suppression Trial).

Allogeneic HSCT

In allogeneic HSCT, stem cells are obtained from matched family members or matched unrelated donors. The conditioning regimen includes cytotoxic agents such as fludarabine or busulphan and anti-thymocyte globulin with or without total body irradiation. The conditioning treatment is mainly performed to allow a significant engraftment of the donor stem cells. In order to reduce TRM, non-myeloablative regimens are increasingly applied. Immunosuppressive therapy with methotrexate or cyclosporine is given to prevent GVHD.

Thus far, the available data are from case series, which demonstrate that allogeneic HSCT can lead to a persistent remission or cure of the underlying autoimmune disease. Four patients with SSc have been treated with allogeneic HSCT to date. The first two, who received myeloablative conditioning, showed improvement of skin thickening and resolution of ground glass opacities on chest

CT (168). However, one died from a *Pseudomonas* sepsis 18 months after the treatment. Two other patients, who underwent non-myeloablative conditioning, remained in full remission for 3 years without signs of GVHD (169, 170). Given the lack of available matched donors, the risks associated with allogeneic HSCT, and the excellent results with autologous HSCT, it is unlikely that prospective trials of allogeneic HSCT will be performed in SSc.

Mesenchymal stem cell transplantation

MSC are bone marrow-derived stromal cells that give rise to cells such as chondrocytes and osteocytes. In addition to their differentiation capacity, MSC have immunomodulatory effects and are bystanders in hematopoeisis. A therapeutic benefit of MSC transplantation (MSCT) has been shown in GVHD, which is considered to share several pathogenic features with SSc (171). Five patients suffering from severe, diffuse SSc were elected for this treatment (172). MSC were obtained by bone marrow aspiration of cross-gender related donors by bone marrow aspiration. Adherent cells were cultured in fresh frozen human plasma and platelet lysate. All five patients showed an improvement of their skin status and acral ulcers following MSCT. Organ functions stabilized and no treatment toxicity occurred.

Pathogenic concept	*Pathological correlate*	*Possible therapeutical intervention*
Genetic Predisposition	SNP's	Downstream targets
Environmental trigger	unclear, Bleomycine, Gadolinum, CMV infection?	Unclear
Autoimmunity	Adaptive: Autoantibodies, T-cell response	Immunosuppression (Glucocorticoids, Cyc, MMF), HSCT
	Innate: unclear, TLR-activation	-
Vasculopathy	Vasoconstriction, ischemia/hypoxia, endothelial apoptosis,	Vasodilatation, ET-1 antagonists, Prostacyclin, phosphodiesterase

	ET-1 overexpression	inhibitors
Low grade inflammation	CRP, BSR, TNF-alpha, cellular infiltrate	Immunosuppression, targeted therapy, anti-TNF-alpha, anti-IL-6
Fibrosis	TGF-beta production, collagen deposition, MMP/ TIMPs dysregulation, myofibroblast differentiation	Imatinib, anti-TGF-beta, Roseglitazone, collagen desensibilitation

Table 3. Pathogenic concepts of SSc and translational interventions. SNP: single nucleotide polymorphism, CMV: cytomegalo virus, Cyc: cyclophosphamide, MMF: motil mycophenolate, HSCT: human stem cell transplantation, TLR: toll like receptor, ET-1: endothelin 1, CRP: C-reactive protein, BSR: blood sedimentation rate, TNF: tumor necrosis factor, TIMP: tissue inhibitor metalloproteinase

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CHAPTER 2

Tumor necrosis factor (TNF) alpha activated T-cells promote Fibrosis in Systemic Sclerosis

2.1 Introduction

Systemic sclerosis (SSc) is an autoimmune multisystem disease which leads to excessive fibrosis of skin and inner organs. It is characterized by vasculopathy and persistent low grade inflammation in tissues which ultimately result in an increased deposition of extra-cellular matrix by fibroblasts and myofibroblasts (1). Around 50% of patients with untreated SSc show a prominent inflammatory infiltrate in the dermis (2). The inflammatory cell infiltrate mainly consists of lymphocytes, mast cells and cells of the monocyte/macrophage lineage (3-6) and the cell infiltrate in the dermis correlates with skin thickening (2). Conversely, Langerhans cells in the epidermis are reduced in SSc (7). There is growing evidence that the interaction between T-cells and fibroblasts influence the expression of extracellular matrix in SSc (8). T-cells can also trigger the differentiation of haematolopoietic cells into fibrocytes (9) and fibroblast into myofibroblasts, respectively. Myofibroblasts are key fibrotic effector cells in SSc and originate from resident fibroblasts or pericytes that have migrated into the lesional area (10). Activated T-cells were predominant in dermal infiltration in SSc in one study (5) and, interestingly, a recent study showed that T-cell transfer from bleomycine treated mice to healthy animals could provoke SSc (11). Indeed, imunosuppressive therapy is effective in patients with SSc lung fibrosis (12) and lymphoablative treatment with anti thymocyte globulin (ATG) followed by autologous heamatopoietic stem cell transplantion (HSCT) has been shown to reverse skin thickening and vasculopathy (13). However, these treatment regimens are only effective in a subset of patients, presumably related to the high heterogeneity of SSc and timing of treatment, respectively. A better

understanding both of the cellular and cytokine orchestration of inflammation is required to develop more targeted therapies in SSc.

Tumor necrosis factor (TNF) alpha is a pleiotropic cytokine which is mainly known for its proinflammatory properties. It has been successfully targeted in multiple inflammatory diseases such as rheumatoid arthritis or spondylarthropathy (14). TNF-alpha also has immunosuppressive feedback effects (15, 16) as well as profibrotic properties (17). The latter has also been demonstrated in several animal models (18). A TNF-mediated cytokine network with TNF-alpha as initiating cytokine, triggering other cytokines in chronic inflammatory lesions has been discussed (18, 19). The heterogenic effects of TNF might be attributed to two different forms of TNF, a membrane bound form and after cleavage, the soluble form TNF (sTNF) (20). TNF binds to two different receptors, the TNF-receptor (TNF-R)1 (p55) and TNF-R2 (p75). Whereas TNF-R1 is mainly stimulated by sTNF, TNF-R2 is also activated by membrane bound TNF, thus dependent on cell contact e.g. with macrophages (21-23). TNF-R2 is predominantly expressed on the plasma membrane, whereas TNF-R1 largely resides in the trans-Golgi with only a small subset of receptors appearing on the cell surface (24). TNF-R1 is ubiquitously expressed at a low level. Its activation leads either to apoptosis or NF-kB dependent gene expression. In contrast, TNF-R2 is preferentially expressed on haematoietic and endothelial cells. Both activated lymphocytes and T-regulatory cells highly express TNF-R2 (15, 25). Proliferation of peripheral T cells appears to be mediated by TNF-R2 costimulation (26, 27). The signalling of TNF-R2 which lacks the intracellular death domain, also involves NFkB stimulation and costimulates lymphocytes, but further functional effects are less clear. Several recent reports have postulated that TNF-R2 is involved in fibrogenesis (17): The lack of TNF-R2 prevented the occurrence of fibrosis in the bleomycine mouse model (18) and reduced collagen expression in intestinal fibroblasts (17). TNF-R2 also exhibits anti-inflammatory effects on tolerogenic antigen presenting cells (28).

It has been demonstrated in animal models lacking the receptorTNF-R2 over-expression and subsequent shedding by proteases has anti-inflammatory effects by binding and inactivating sTNF. Furthermore, prolonged TNF exposure of T-regs restore their suppressive influence on T-effector cells (15).

In SSc, the role of TNF is controversial (29). TNF-receptor knock out mice are protected against asbestos induced lung fibrosis (30). In these mice, reduced TGF-

beta levels have been recognized whereas TNF-alpha levels per se remained elevated (30). In antigen presenting cells, TNF-R2 mediates a tolerogenic effect (28). As shown by immunohistochemistry (IHC), both TNF-R are over-expressed in monocuclear cells in the SSc skin, especially TNF R2 which is expressed on most cells (31). The soluble forms of TNF-R1 and R2 are increased in the blood and correlate with disease activity (31). Upon direct cell-to-cell contact, lymphocytes from SSc patients inhibit collagen expression by fibroblasts via membrane bound TNF, an effect that is reduced in SSc fibroblasts (32). On the other hand, T-cells in SSc dermis overexpress IL-4, a Th-2 specific profibrotic cytokine. So far, the net effect of T-cells on fibroblasts and their differentiation into myofibroblasts is unclear. sTNF has been shown to mediate the transition from pulmonary inflammation to fibrosis and soluble TNF-alpha expression is a prerequisite for TGF-beta expression (33). Experimental models of lung fibrosis respond to TNF alpha antagonist (34). In clinical studies, the blockage of TNF with etanercept or infliximab has recently been investigated in different SSc cohorts. Etanercept, the soluble TNF-R2, was effective in ameliorating SSc-associated arthrtitis and reducing skin thickening (35). In another recent study, although not significantly, a trent towards improvement of the skin score after treatment with infliximab was obersrved; a reduced serum concentration of procollagen could be demonstrated and the secretion of collagen by fibroblasts was decreased (36). Possible explanations for the stable skin score in this study are that most patients were already in a chronic stage where the vascularity and thus drug delivery in the tissue is largely impaired. Another reason could be an inhibition of sTNF but not membrane bound TNF which has possibly a more important impact on immune effector cells in SSc.

The aim of this study was to identify which mononuclear cells express TNF-R1 and -R2 in SSc dermis and to compare their expression levels with disease characteristics. We further performed mechanistic studies of the TNF-R on SSc lymphocytes and investigated a potential pro-fibrotic effect upon their stimulation. Finally we tested our hypothesis in patients who underwent lymphoablative treatment followed by heamatopoietic stem cell transplantation.

2.2 METHODS

Patient recruitment

Patients with SSc were recruited from the Freeman hospital in Newcastle or the James Cook University Hospital in Middlesbrough, U.K. Ethical approval and written consent was obtained (REC number: 09/H0905/11). All patients were 18 years or older and fulfilled the ACR criteria for SSc according to LeRoy (37). 13 patients were included in this study, 8 had diffuse SSc and 5 limited SSc. One patient with diffuse SSc who received autologous haematopoietic stem cell transplantation (HSCT) underwent two skin biopsies, one before and one six months after HSCT. The exact treatment proceedure of HSCT is explained elsewhere. Additional 19 skin biopsies from a Dutch SSc cohort in Leiden served as a control group and were analysed by IHC. Healthy skin samples were obtained from breast of patients undergoing breast reduction surgery.

Flow cytometry of skin

The multicolour flow cytometry technique of skin cells has been adapted from the group of Prof . M. Collin, Institute of Cellular Medicine, Newcastle University (38). After informed consent was obtained, a 4 mm punch biopsy from the dorsal side of the forearm of SSc patients was performed in 13 patients under local anaesthesia (1% Lidocain). One patient underwent a second biopsy after haematopoietic stem cell transplantation. The skin was digested with 10 U/ml dispase (Gibco BRL) for 1 hour in Exvivo medium. The epidermis was removed with a scalpel and the dermis was digested in 1.6 mg/ml collagenase type 4 (Worthington's) overnight in Exvivo medium. Cells were re-suspended and blocked with 1% mouse IgG (Sigma) for 10 minutes at 4℃. Cells were stained with labelled an tibodies for CD45, HLA-DR, CD14, CD16, CD1a, CD3, CD4, CD19 (all Becton Dickinson) and TNF-1 and -2 (R&D systems), respectively (see table 1) on ice for 1h. Multicolour flow cytometry with prior adequate compensation procedures were performed using a BD LSRII cytometer and the FACS Diva software. Macrophages are identified and excluded from the analysis on the basis of autofluorescence at 488/610 nm.

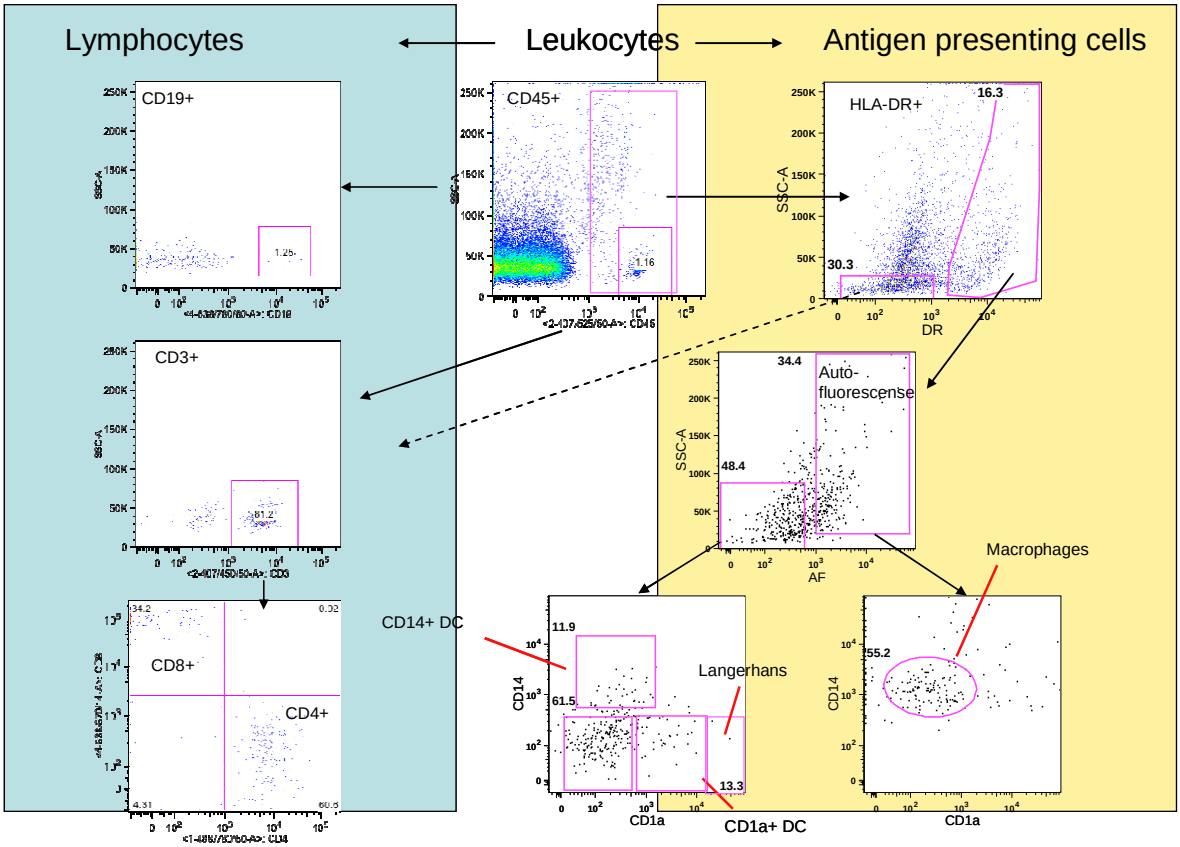


Figure 1. Schematic illustration of flow cytometry of the skin**.** 4 mm punch biopsies from the forearm were digested with dispase for 1 hour to remove the epidermis and overnight in collagenase. Then, cells were resuspended and stained for flow cytometry. Leukocytes were identified by $CD45^+$ cells (centre) and include macrophages, monocytes, dendritic cells, Langerhans cells, mast cells and lymphocytes. Autofluorescence (AF) was recorded in the FL1 channel (488-nm laser and 530/30 band-pass). In a 'dendritic cell panel' (on the right), cells were stained with CD45 APC-H7, CD14 Horizon blue, CD16 PE Cy7 and CD1a APC. $CD45^+$ cells were devided into $HLA\text{-}DR^+$ which represent antigen presenting cells and HLA-DR- SSC^{low} which are lymphocytes and $HLA\text{-}DR\text{-}SSC^{high}$ mast cells. Macrophages were identified by their AF. $CD1a^+$ dendritic cells and $CD14^+$ monocytes were identified on $HLA\text{-}DR^+AF^-$ cells. Langerhans cells were identified as $CD45^+HLA^-DR^+AF^-CD1a^{bright}$.
In a 'lymphocyte panel' (on the left) we stained with CD45 Pacific orange, HLA-DR PerCP Cy.5, CD4 PE-Cy7, CD8 APC, CD3 V450 and APC-H7. $CD45^+SSC^{low}$ cells were selected to identify T-(CD3) and B-(CD19) cells. $CD3^+$ cells were assessed for CD4 and CD8 expression. Backgating of $CD3^+$ cells corresponded to the $CD45^+HLA^-DR^-SSC^{low}$ cells from the 'dendritic cell' panel.

Flow cytometry of peripheral blood

Most antibodies used for blood flow cytometry correspond to those used for the skin. CD11c was used instead of CD1a for dendritic cell staining. Antibodies were added to 100 µl whole blood for 1 hour and incubated on ice. Red cells were lysed subsequently with BD Lysis Buffer and washed in buffer.

Antibody	Fluorochrome
HLA	PerCP Cy5 (5µl)
CD45	APC-H7 (5µl),Pacific orange (5µl)
CD14	Horizon blue (5µl) Qdot605 (1µl)
CD16	PE Cy7 (5µl)
CD1a	APC (5µl)
CD3	FITC (5µl),PE (5µl) V450 (5µl)
CD19	FITC (5µl) PE (5µl), APC-H7 (5µl)
TNF-R1	FITC(2.5µl) (Isotype 0.25 µl)
TNF-R2	PE (10µl) (Isotype 10 µl)

Table 1. Antibodies for skin flow cytometry.

Antibody	Fluorochrome
HLA	PerCP Cy5 (5µl)
CD45	APC-H7 (5µl)
CD14	Q-dot 605 (1µl)
CD16	PE Cy7 (5µl)
CD11c	APC(5µl)
Linage	FITC (CD3,19,56 each 5µl)/ PE (CD3,19,56 each 5µl)
TNF-R1	FITC (2.5µl) (Isotype 0.25 µl)
TNF-R2	PE (10 µl) (Isotype 10 µl)

Table 2. Antibodies for blood flow cytometry.

CD3 cell isolation

40 ml whole blood was collected during standard outpatient procedures in EDTA containing tubes (BD Bioscience). The samples were processed within four hours of collection. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by ficoll-hypaque density gradient centrifugation (Axis-shield PoC AS, Oslo, Norway). $CD3^+$ lymphocytes were isolated from total PBMC according to the manufacturer's protocol with the $CD3^+$ MACS beads isolation kit (Miltenyi biotec GmbH Bergisch Gladbach, Germany) described in detail elsewhere (39). In short,

$40x10^6$ PBMC were suspended in PBS supplemented with 1% foetal-calf serum (Sigma-Aldrich) and EDTA (2mM) plus 40 µl of anti-$CD3^+$ Miltenyi magnetic beads on ice for 20 minutes. Total mixture was past through a magnetic isolation column (Miltenyi Biotec). Purified $CD3^+$ monocytes were removed from the column and tested for purity by flow cytometry. Flow cytometric analyses showed a >95% purity for $CD3^+$ cells. Cells were then cultured in Dulbecco medium (DMEM) containing 10% fetal calf serum and 2mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in an incubator at 37℃ containi ng 5% CO_2.

TNF-R stimulation and T-cell activation by CD3/28 beads

CysTNF mutations are selective agonists for TNF-R1 or –R2 (table 2) and the physiological soluble TNF (sTNF) stimulate mainly TNF-R1 and probably to a minor extend also TNF-R2. The latter is controversial and currently being investigated by A. Krippner-Heidenreich (personal communication).

CysTNF mutants were added to 1 x 10^6 $CD3^+$ cells (isolated from peripheral blood, according to methods above) and cultured in DMEM containing 10% fetal calf serum and 2mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. CysTNF concentrations were titrated from 10 ng/ml to 100 ng/ml and cells were incubated from 4h to 48h. IL-6 expression was most prevalent after stimulation of the lymphocytes at a concentration of 100 ng/ml cysTNF for 24h. Cells were then harvested and processed for expression analysis as described below (Fig. 2).

In a second set of experiments, $CD3^+$ lymphocytes were identically isolated and cultured in the same medium in presence of CD3/28 T-cell expander Dynabeads (Invitrogen) at a 1:1 bead T cell ratio for 24h. Then, cysTNF mutants were added in the same concentration for further 24h before cells were harvested.

Ligand / mutation	**Effect**
CysTNF 32W/86T (stock 1 mg/ml)	Stimulation of TNF-R1
CysTNF 193N (stock 0.9 mg/ml)	Stimulation of TNF-R2
Soluble TNF (stock 0.5 mg/ml)	Stimulation of TNF-R1, possibly –R2

Table 3. Agonists for TNF-receptors.

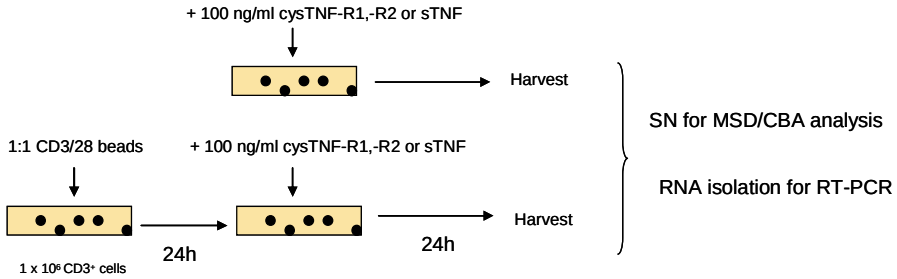


Figure 2. Schematic illustration of lymphocytes by cysTNF with or without prior treatment with CD3/28 activation beads. R1= receptor 1, R2= receptor 2. SN: supernatant

Gene expression

RNA from $CD3^+$ T-lymphocytes was isolated using the RNA mini kit from Qiagen according to the manufacturer's protocol. The RNA concentration was measured by Nanodrop 1000. 200-750ng of RNA was treated with DNAse for 30 minutes at 37℃ then reverse transcribed to cDNA using random hexamers (Invitrogen) and moloney murine leukaemia virus reverse transcriptase (MMLV-RT) enzyme (Invitrogen) according to the manufacturer's protocol. 20ng of cDNA, 1µl of 30µM forward primer and 1µl of 30µM reverse primer, 0.2µl of 100µM probe and 0.2µl of reference dye. Taqready master mix (Sigma) was used to analyse expression. Samples were analysed in triplicate and normalised to the housekeeping gene (18S) using AB7500 (applied biosystems) qPCR machine and program. Relative expression to the housekeeping gene was calculated using: $(2^{\Delta\Delta\text{cycle time}})^{-1}$. The primers used for IL-6 were: forward primer 5'- TACCCCCAGGAGAAGATT -3', reverse primer 5'- AAGGTTCAGGTTGTTTTC -3', probe 7 from universal probe library (Roche Applied Science).

Primers and probe for IL-10: forward primers 5'- GGTGATGCCCCAAGCTGA -3', reverse primer 5'- GCCTTGCTCTTGTTTTCA -3' and probe 65 from universal probe library (Roche Applied Science). Primers and probes for TGF-beta: forward primer: 5'- TGACAGCAGGGATAACACACT -3', reverse primer 5'- GCCGCACGCAGCAGTTCTTCT -3' and probe 68 from the universal probe library (Roche Applied Science). Primers and probe for 18S: forward primer 5'- CGAATGGCTCATTAAATCAGTTATGG -3', reverse primer 5'- TATTAGCTCTAGAATTACCACAGTTATCC -3' and probe 5'-FAM-TCCTTTGGTCGCTCGCTCCTC –TAMRA-3'. Primers and 18S probe were from

Sigma-aldrich. qPCR settings were applied according to Taqready mix manufacturers protocol.

ELISA

The custom MSD protocol was applied. 25µl HSC Assay Diluent were added to each well and incubated for 30 minutes with shaking (300-1000rpm) at room temperature. 25µl of calibrator or sample were added to each of the necessary wells. The plate was sealed with adhesive film and incubated for 2 hours with shaking (300-1000rpm) at room temperature. The plates were washed 3x with MSD wash (DPBS with Tween-20 at 0.05%) then 25µl of Detection Antibody Solution was added per well. The plate was sealed with adhesive film and incubated for 1-2 hours with shaking (300-1000rpm) at room temperature. Wells were washed 3x with MSD wash then 150µl of Read Buffer T 2X were added to each well.

Western blotting

Supernatants were removed before cell lysis with the lysis buffer (20mM Tris pH7.5, 1mM EDTA, 100mM NaCl, 1% Triton X-100, 0.5% DOC, 0.1% SDS
1XPIT complete protease-inhibitor cocktail (Roche Diagnostics). This was then spun to remove cell debris (4˚C for 10 minutes 15000 RPM). 4X SDS sample buffer (125 mM Tris pH6.8, 20% Glycerol, 6% SDS, 0.02% Bromphenolblue, 10% β Mercaptoethanol) was diluted to a 1X concentration and added then the sample was cooked (95˚C for 5minutes). Samples were run through a 6.5% acrylamide gel with a 4.5% acrylamide stacking gel, the gel was run at 50V until sample was in second gel then at 150V until the end of the run. Gel was in gel chamber filled with SDS buffer (25 mM Tris pH 8.3, 192mM Glycine and 0.1% SDS). Membranes were soaked in methanol prior to tank blotting. Blotting chamber was built as: fibre pad, whatman filter sheet, acrylamide gel, whatman filter sheet, fibre pad. Blotting chamber soaked in western blotting buffer (25 mM Tris pH 8.3, 192mM Glycine, 20% Methanol) and placed in running chamber filled with cold western blotting buffer and an icepack. Transfer ran for 3 hours at 100mA (for 2 gels).

Unspecific binding sites on the membrane were blocked by incubating overnight in cold room with 5% low fat milk in TBS-T (50mM Tris pH 7.6, 150mM NaCl, 0.1% Tween 20). Alpha SMA antibody was diluted 1:1000 in 5% milk with TBS-T, alpha SMA antibody used was Mouse Anti Human Actin [alpha smooth muscle actin isoform] monoclonal antibody (Millipore). Membrane was incubated with primary antibody (alpha SMA) for 1 hour at room temperature. The membrane was washed three times with TBS-T before incubation for 1 hour at room temperature with a secondary antibody (Polyclonal Goat anti mouse immunoglobulins/ HRP (Dako) diluted 1:20000 in 5% milk with TBS-T). The membrane was then washed a further three times with TBS-T. Millipore Immobilon ™ western chemiluminescent HRP substrate kit used for detection as the manufacturer's protocol. Membrane were developed on an x-ray film.

Membrane was then stripped by incubating at 50˚C for 25 minutes with a stripping buffer (Tris-HCL: 7.8grams Tris in 1000ml dH20, pH to 6.0, 2% SDS (20g SDS in the 1L) with 331µl β-mercaptoethanol added fresh for 50ml). The membrane was then ready to block again for incubation with Collagen or GAPDH. Primary antibody for collagen: Rabbit anti-human type 1 polycolnal antibody (chemicon international) diluted 1:200 in 5% milk with TBS-T. Secondary antibody for collagen: Polyclonal Goat anti Rabbit Immunoglobulins/HRP (Dako) diluted 1:20000 in 5% milk with TBS-T. Primary antibody for GAPDH: Monoclonal mouse anti rabbit glyceraldehyde-3-phosphate dehydrogenase (HY-Test) diluted 1:500 in 5% milk with TBS-T. Secondary antibody for GAPDH: Polyclonal Goat anti mouse immunoglobulins/ HRP (Dako) diluted 1:20000 in 5% milk with TBS-T.

Patient	Age	Sex	Subtype	Disease duration since first onset of non-Raynaud	Comorbidity	Autoantibody status	mRSS	Immunosuppressive treatment
1	62	M	Diffuse	2	Mantle cell lymphoma	neg.	25	none
2	58	W	Diffuse	2		ANA	30	Cyclophosphamid
3	45	W	Diffuse	3	Lymphoma, vasculitis, chronic renal failure	neg.	35	Steroids
4	75	M	Limited	35		neg.	6	None
5	60	W	Limited	18		neg.	10	Cyclophosphamid
6	65	W	Diffuse	4		Scl-70	13	Cyclophosphamid
7	65	W	Diffuse	3		ANA, anti-RNA	28	MMF
8	75	W	Limited	8		anti-centromer	4	none
9	76	M	Diffuse	5	Asbestos	Scl-70	35	Cyclophosphamid
10	49	W	Limited	12	RA overlap	ANA	9	none
11	48	W	Limited	4		ANA	4	none
12	50	W	Diffuse	3		neg.	30	MTX, Steroids
13	62	M	Diffuse	2		Scl-70	32	Cyclophosphamid

Table 4. Clinical characteristics of SSc patients in which skin biopsies were performed at their forearms.

2.3 RESULTS

Identification of leukocyte subsets in the dermis of SSc patients by flow cytometry.

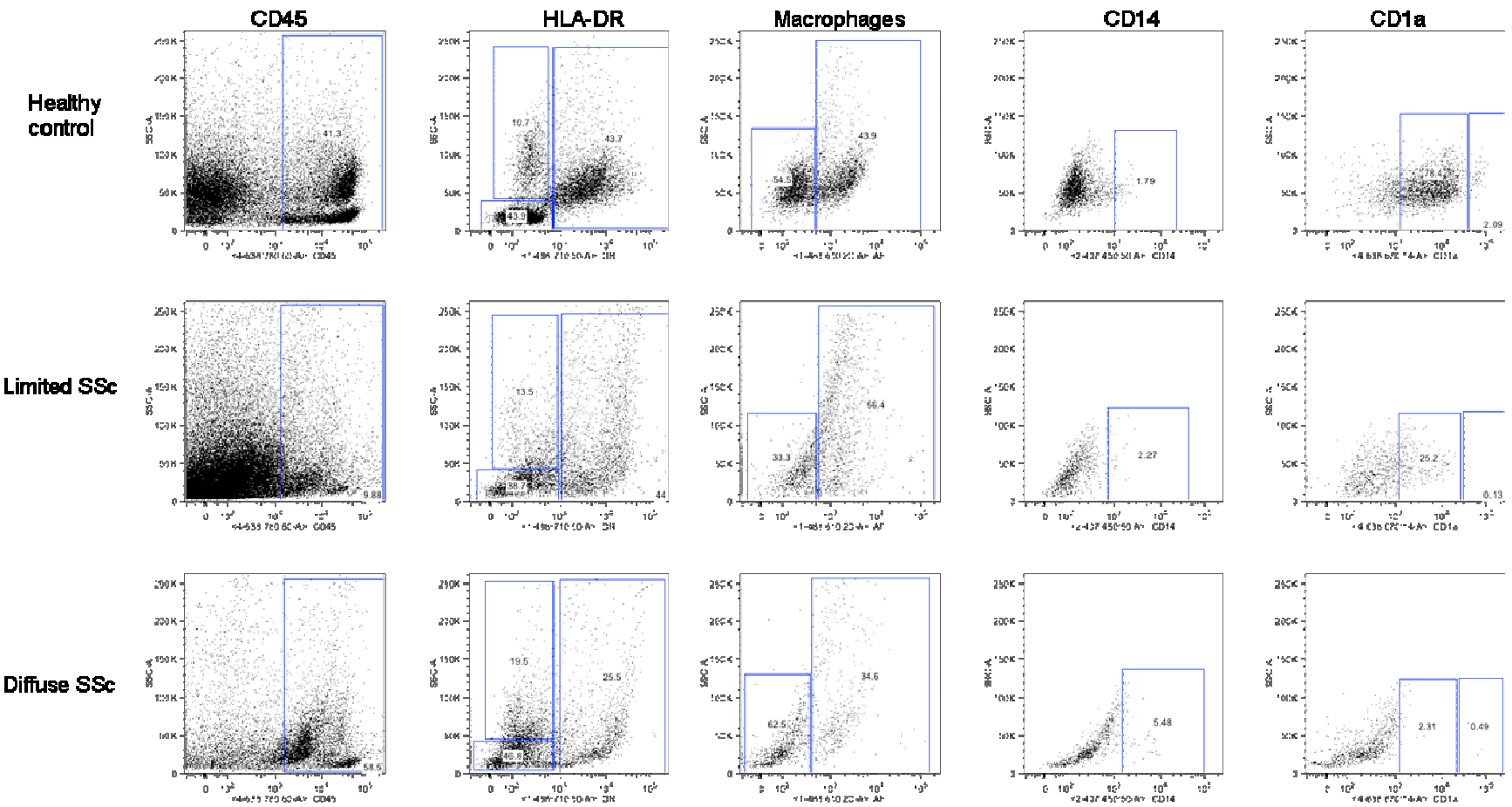
Skin samples of involved skin from the forearm of 13 SSc patients and from the breast of 1 healthy individual undergoing breast reduction surgery were obtained. 8 patients were classified as diffuse SSc, 5 patients had limited SSc. Figure 3A shows the gating strategy in two representative patients with diffuse and limited SSc and the healthy control. Dermal leukocytes were identified as $CD45^+$ cells within the live cell gate (not shown). $CD45^+$ cells were subsequently divided into $HLA\text{-}DR^+$ antigen presenting cells, $HLA\text{-}DR^-SSC^{high}$ mast cells and $HLA\text{-}DR^-SSC^{low}$ lymphocytes according to previous studies (38). Macrophages were identified by autofluoresence as $CD45^+HLA\text{-}DR^+AF^+$ cells. $CD1a^+$ and $CD14^+$ cells were gated out of the $CD45^+HLA\text{-}DR^+AF^-$ cell subset. Langerhans cells were identified as $CD45^+HLA\text{-}DR^+AF^-CD1a^{bright}$.

A higher percentage of dermal leukocytes per live cells were observed in skin from diffuse SSc patients (23.9% range 14.4-65.6) compared to limited SSc patients (9.8% range 5.4-17.3; p=0.03) (Figure 3B). Leukocytes of SSc patients were lower compared to skin from the healthy control, suggsting a higher number of stromal cells in SSc dermis. $HLA\text{-}DR^-SSC^{low}$ lymphocytes also were significantly higher in patients with diffuse SSc (46.6%, range 32.2-53.0; vs. 28.7%, range 17.8-37.9; p=0.03). Mast cells infiltration did not differ between groups (21.7% range 16.1-42.4 vs. 24.5% range 10.7-44.0; p=0.75) but was higher compared to the control. No significant difference was found in $CD1a^+$ cell percentages between limited (4.1%, range 0.9-5.9) vs. diffuse SSc (1.0%, range 0.5-1.6; p=0.09) and $CD14^+$ cells (0.6%; range 0.1-1.3 vs. 0.7%, range 0.3-1.4; p=0.66). $CD1a^+$ cell numbers were higher and $CD14^+$ cells were lower than in the healthy control. Macrophages were significantly more prevalent in limited SSc patients (21.0%, range 16.2-31.4 vs. 11.3%, range 8.5-14.3; p=0.007) and higher than in healthy control. Lymphocyte subsets were analysed by flow cytometry in two patients with limited and diffuse SSc, respectively (Figure 3C). $CD45+SSC^{low}$ cells were selected to identify T- ($CD3^+$) and B-($CD19^+$) cells. $CD3^+$ cells were assessed for CD4 and CD8 expression. Back-gating of $CD3^+$ cells corresponded to the CD45+HLA-DR-

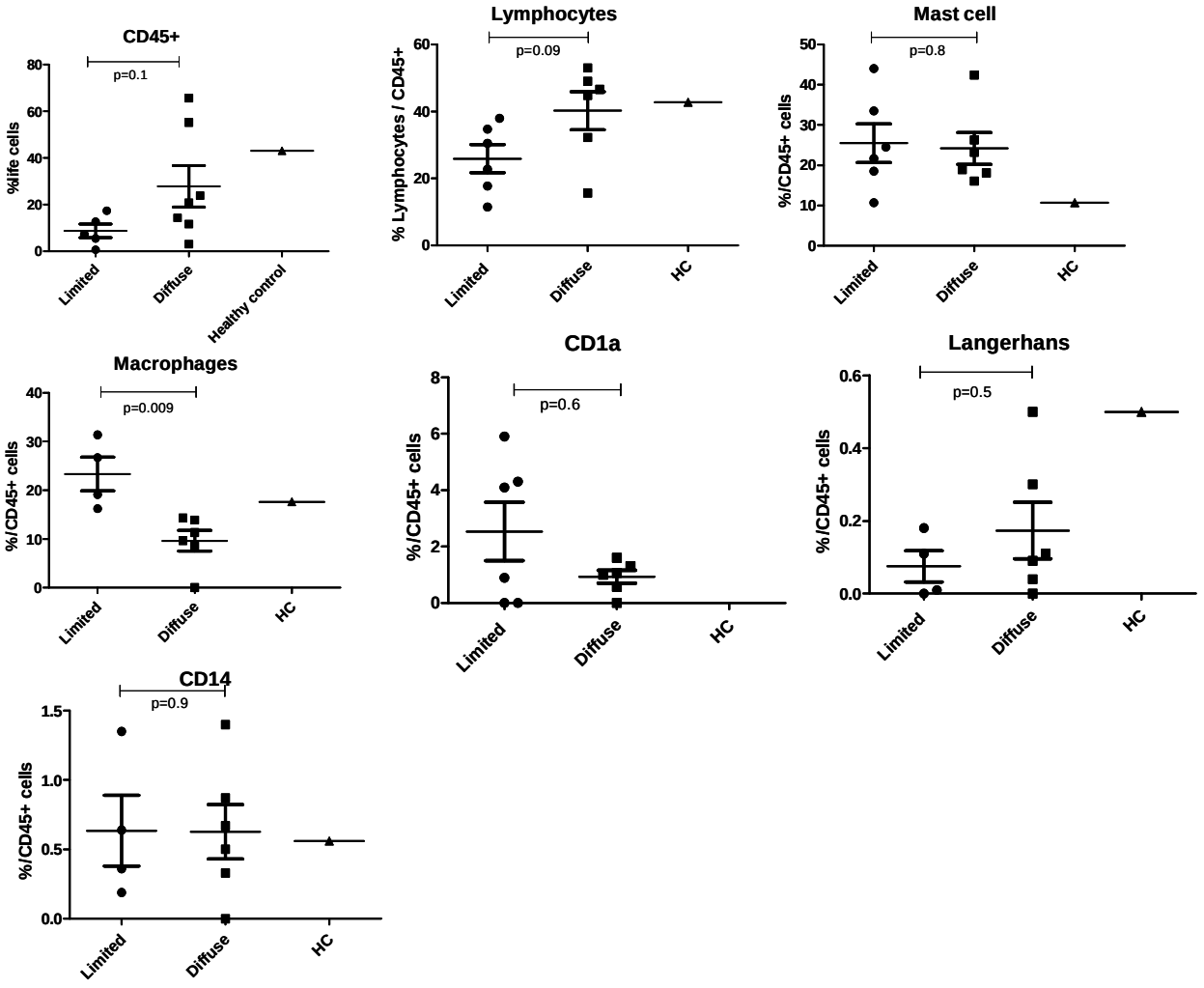
SSC^{low} cells from the previous experiments. Of $CD45^+CD3^+$ cells $CD4^+$ positivity ranged from 56.8% to 65.0% and CD8 positivity from 35.0-40.2%. Only 1-5% of CD3 cells were CD4CD8 double positive. The patient with diffuse SSc had a higher percentage of $CD3^+$ cells. In this patient $CD4^+$ cells were predominant (67.7%) compared to $CD8^+$ cells (26%). B-cell percentage per $CD45^+$ cells in this patient was relatively low with 2.1%.

Figure 3. Flow cytometry of skin in SSc patients. 4 mm punch biopsies from the forearm of 13 SSc patients were digested with dispase for 1 hour to remove the epidermis and subsequently over night in collagenase. Cells were resuspended and stained for flow cytometry. Leukocytes were identified by $CD45^+$ cells and include macrophages, DC, mast cells and lymphocytes. Autofluorescence (AF) was recorded in the FL1 channel (488-nm laser and 530/30 band-pass).
A: In a 'dendritic cell panel', cells were stained with CD45 APC-H7, CD14 Horizon blue, CD16 PE Cy7 and CD1a APC. $CD45^+$ cells were devided into HLA-DR+ which represent antigen presenting cells and HLA-DR- SSC^{low} which are lymphocytes and HLA-DR-SSC^{high} which are mast cells. For the further gating strategy, $CD1a^+$ and $CD14^+$ cells were identified on HLA-DR^+ and AF negative cells. Langerhans cells were identified as $CD45^+HLA^-DR^+AF^-CD1a^{bright}$. **B:** Leukocyte subset percentages per $CD45^+$ cells were compared between patients with limited versus diffuse SSc and one healthy control. Significant differences were found in lymphocytes which were higher in diffuse SSc patients. Conversely, macrophages were significantly more prevalent in patients with limited SSc. **C:** Skin of three patients was stained in a 'lymphocyte panel' which included CD45 Pacific orange, HLA-DR PerCP Cy.5, CD4 PE-Cy7, CD8 APC, CD3 V450 and APC-H7. $CD45^+SSC^{low}$ cells were selected to identify T- (CD3) and B-(CD19) cells. $CD3^+$ cells were assessed for CD4 and CD8 expression. Backgating of $CD3^+$ cells corresponded to the $CD45^+HLA^-DR^-SSC^{low}$ cells from the previous experiments. Of $CD45^+CD3^+$cells $CD4^+$ positivity ranged from 56.8% to 65.0% and CD8 positivity from 35.0-40.2%. Only 1-5% of $CD3^+$ cells were double positive for CD4 and CD8.
D: CD45+ cells, $CD45^+HLA^-DR^-SSC^{low}$ lymphocytes, $CD45^+HLA^-DR^+AF^-CD1a^+$ dendritic cells, CD45+HLA-DR+AF-$CD1a^{bright}$ Langerhans cells, $CD45^+HLA^-DR^-SSC^{high}$ mast cells and $CD45^+HLA^-DR^+AF^-CD14^+$ monocytes were quantified as percentage of $CD45^+$ cells. Percentages of cell subsets were correlated with the modified Rodnan Skin Score (mRSS) which assesses the skin thickening in SSc. A positive correlation between skin thickening and lymphocytes and a negative correlation with $CD1a^+$ and macrophages was seen. In contrast, no correlation with skin thickening could be detected for mast cells or $CD14^+$ monocytes.

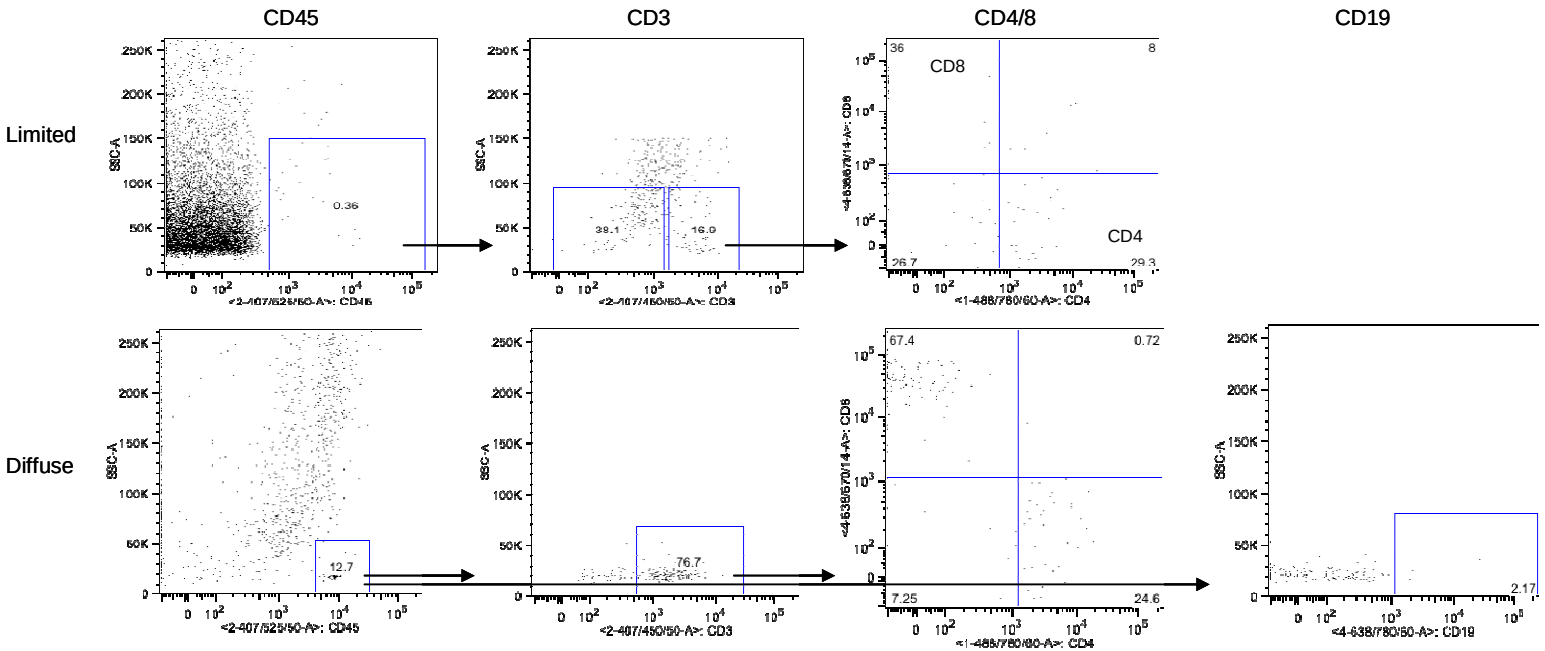
A


CD45
HLA-DR
Macrophages
CD14
CD1a
Healthy control
Limited SSc
Diffuse SSc

B


CD45+
p=0.1
%life cells
Limited
Diffuse
Healthy control
Lymphocytes
p=0.09
% Lymphocytes / CD45+
Limited
Diffuse
HC
Mast cell
p=0.8
%/CD45+ cells
Limited
Diffuse
HC
Macrophages
p=0.009
%/CD45+ cells
Limited
Diffuse
HC
CD1a
p=0.6
%/CD45+ cells
Limited
Diffuse
HC
Langerhans
p=0.5
%/CD45+ cells
Limited
Diffuse
HC
CD14
p=0.9
%/CD45+ cells
Limited
Diffuse
HC

C


CD45
CD3
CD4/8
CD19
Limited
Diffuse
CD8
CD4
0.36
38.1
16.9
36
8
28.7
29.3
12.7
76.7
67.4
0.72
7.25
24.6
2.17
SSC-A
<2-407/525/50-A>: CD45
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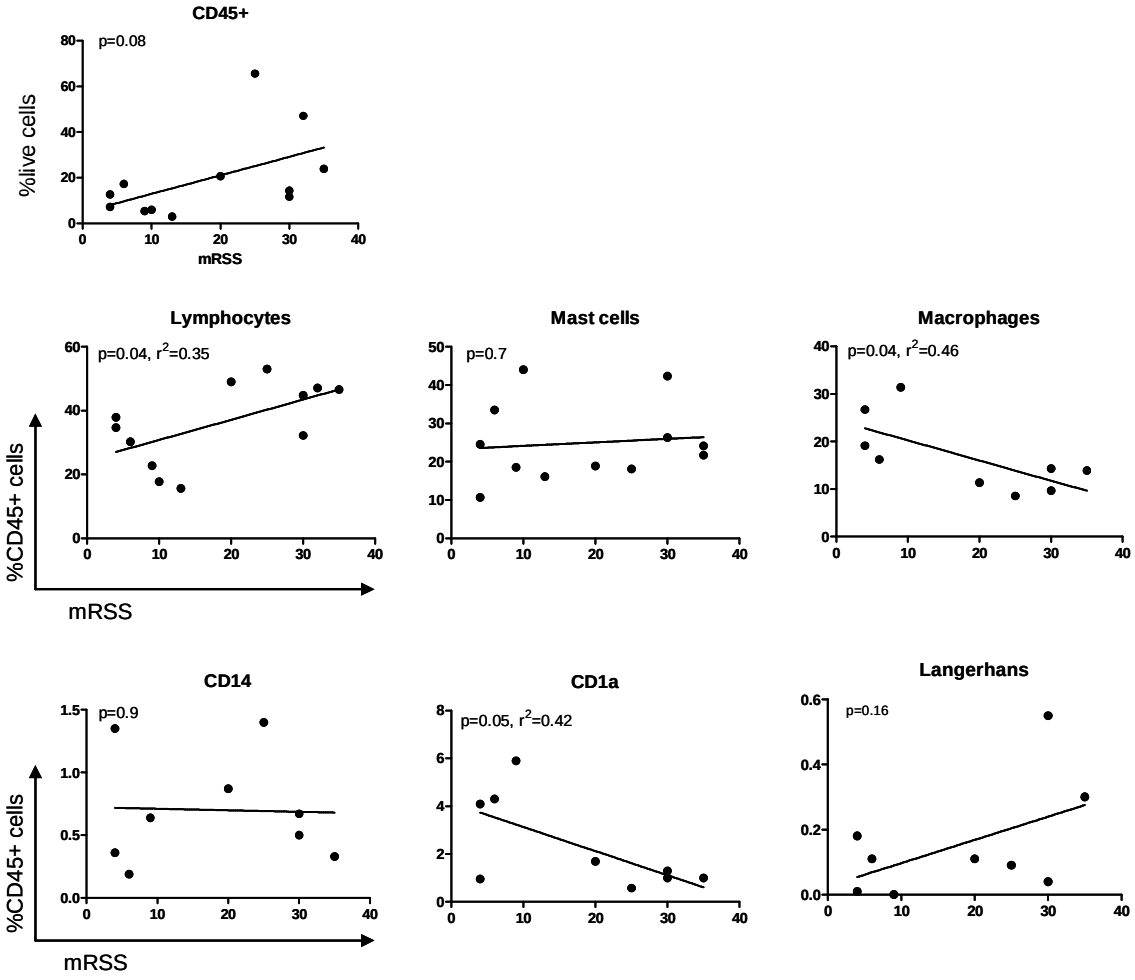
<1-488/780/60-A>: CD4
<4-638/780/50-A>: CD19

D



Lymphocyte infiltration positively correlates, whilst macrophage infiltration negatively correlates with skin thickening.

The number of $CD45^+$ cells per live cells increased with skin thickening (measured as mRSS) (p=0.08) (Figure 3D). $CD45^+HLA\text{-}DR^-SSC^{low}$ lymphocyte infiltration significantly correlated with mRSS (p=0.04). There was no correlation of mast cells and $CD14^+$ percentages with skin thickening but both macrophages (p=0.04) and $CD1a^+$ (p=0.05) cells negatively correlated with skin thickening and were more prevalent in patients with limited SSc. The number of Langerhans cells in the dermis did not correlate with skin thickening. $CD1a^+$ Langerhans cell number in the *epidermis* was assessed by IHC. Numbers per visual field were lower in patients with diffuse SSc, compared to patients with limited SS or healthy control skin, respectively (data not shown).

These results were confirmed by IHC where we found significantly more $CD3^+$ T-cells in patients with diffuse SSc but lower numbers of $CD1a^+$ and $CD14^+$ cells as compared to patients with limited SSc (Figure 4A). Mast cell infiltration identified by toluidine blue staining did not depend on the subtype but was higher in patients with recent onset of non-Raynaud phenomenon symptoms (see mast cell chapter). High numbers of lymphocytes in the dermis of patients with diffuse SSc was confirmed by electron microscopy (Figure 4B).

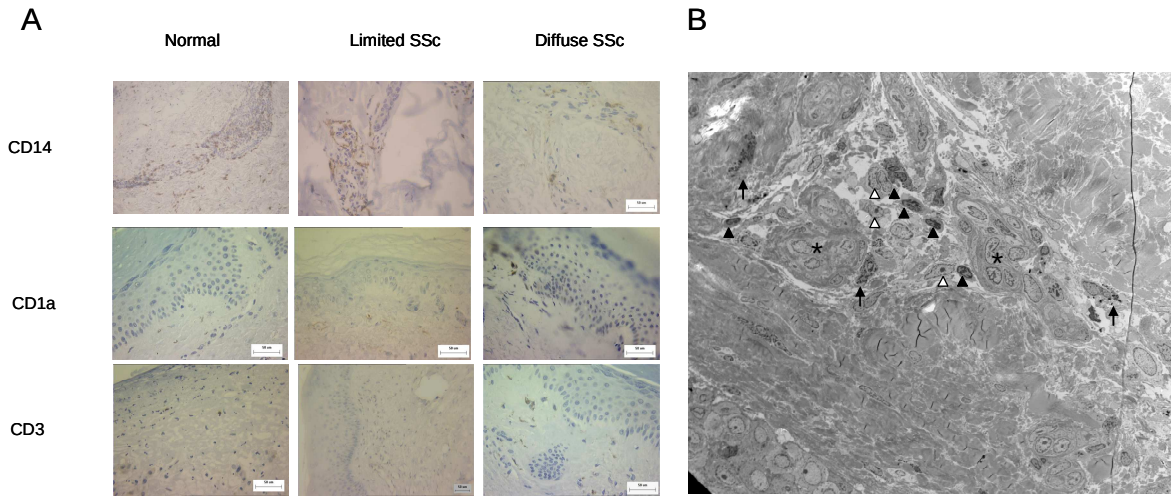


Figure 4. Inflammatory cell subsets in SSc dermis assessed by immunohistochemistry and electron microscopy. Higher numbers of $CD14^+$ and $CD1a^+$ cells were found in the dermis of patients with limited SSc whereas lymphocytes were more prevalent in diffuse SSc (Figure 4A). Electron microscopy (Figure 4B) of the dermis of a patient with diffuse SSc confirms the high number of lymphocytes (black arrowheads), mast cells (black arrows) and antigen presenting cells (white arrowheads). Blood vessels are indicated by asterisks.

TNF-receptor expression of dermal leukocyte subsets and in peripheral blood.

TNF-R 1 and 2 expression was analyzed by flow cytometry on $CD45^+HLA\text{-}DR^-SSC^{low}$ lymphocytes, $CD45^+HLA\text{-}DR^+AF^+$ macrophages, $CD45^+HLA\text{-}DR^+AF^-CD14^+$ cells and $CD45^+HLA\text{-}DR^+AF^-CD1a^+$ dendritic cells (Fig. 5A). Both TNF-R1 and- R2 expression was predominant on lymphocytes. To a minor extent, TNF-R1 was also expressed on $CD14^+$ and $CD1a^+$ cells. On lymphocytes, TNF-R2 expression

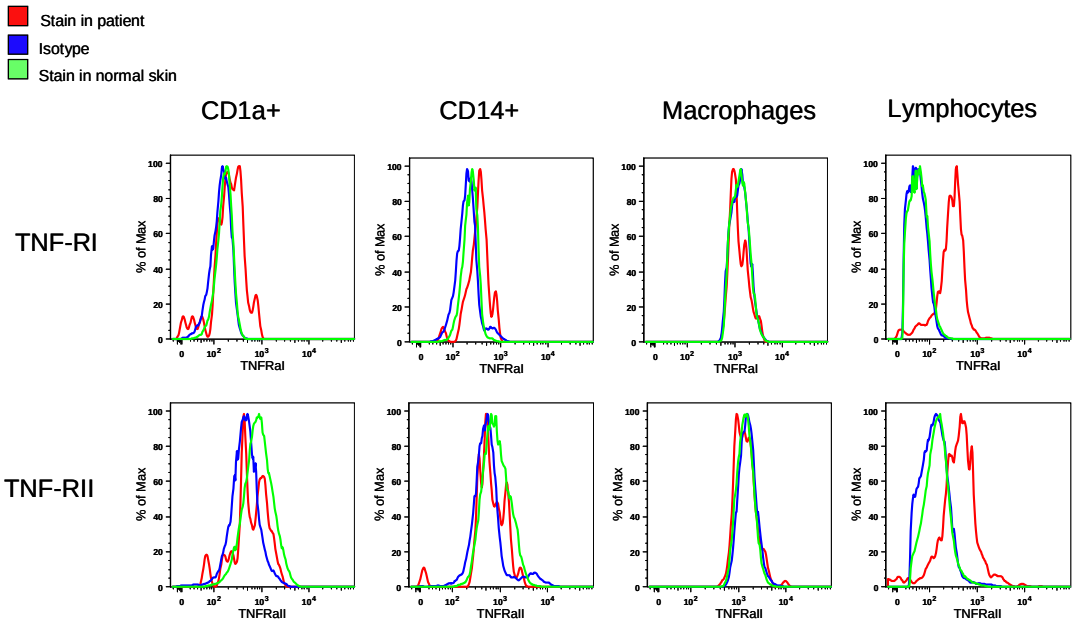
measured by mean intensity fluorescence (MIF) compared to isotype controls on lymphocytes was 1.8 fold, whereas MIF of TNF-R1 expression only was 1.1 fold compared to the isotype control (Fig. 5B). TNF-R2 but not TNF-R1 expression correlated significantly with skin thickening (p=0.02) (Fig. 5C). The three patients with the highest skin scores and progressive disease course showed the highest TNF-R2 expression on dermal lymphocytes.

TNF-R expression on lymphocyte subsets was confirmed by concomitant staining with CD3, CD4 and CD8 in two SSc patients. In the patient with diffuse SSc, TNF-R2 was expressed on $CD4^+$ and $CD8^+$ subsets. In contrast, TNF-R1 was not or slightly expressed (Fig. 5D).

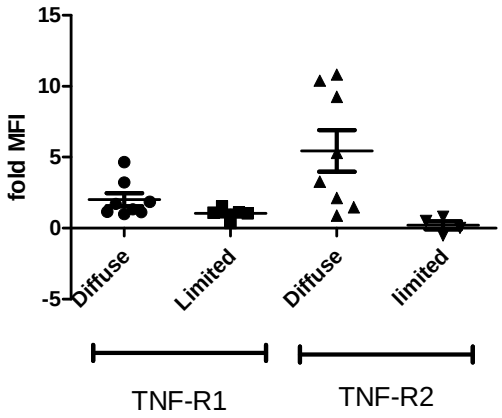
We also analyzed TNF-R expression on lymphocytes in peripheral blood in 11 patients, 4 of those also had received skin biopsies. TNF-R2 was significantly upregulated in SSc patients compared to healthy controls, whereas no expression of TNF-R1 could be detected (Figure 5C). In the 4 patients with concomitant skin and blood analysis, we found an upregulation of TNF-R2 both in skin and in blood. The patient with the highest TNF-R2 expression pattern had the highest mRRS, a progressive disease course and did not receive prior cyclophosphamid treatment.

Figure 5. TNF-R1 and R2 expression in SSc skin and peripheral blood. Dermis of 13 SSc patients was processed and stainded as described in material and methods. FITC-labeled TNF-R1 and PE-labeled TNF-R2 antiserum was added to the antibody panel. TNF-R1 and –R2 expression was evaluated in SSc patients and a healthy control on macrophages, $CD1a^+$ dendritic cells, $CD14^+$ monocytes and lymphocytes. Both TNF-R1 and -R2 expression was most distinctively expressed on lymphocytes; to a minor extent, TNF-R1 was also expressed on $CD1a^+$ dendritic cells and $CD14^+$ monocytes (Fig. 5A). TNF-R2 was significantly higher expressed on SSc patients with a diffuse disease subset, whereas TNF-R1 expression (which is mainly intracellular) was lower and had been detected only in a few patients (Figure 5B). In contrast to TNF-R1, TNF-R2 expression did correlate with skin thickening (Fig. 5C). Analysis of T-cell subsets revealed that TNF-R1 and R2 were expressed on CD3, CD4 and CD8 positive cells (5D).

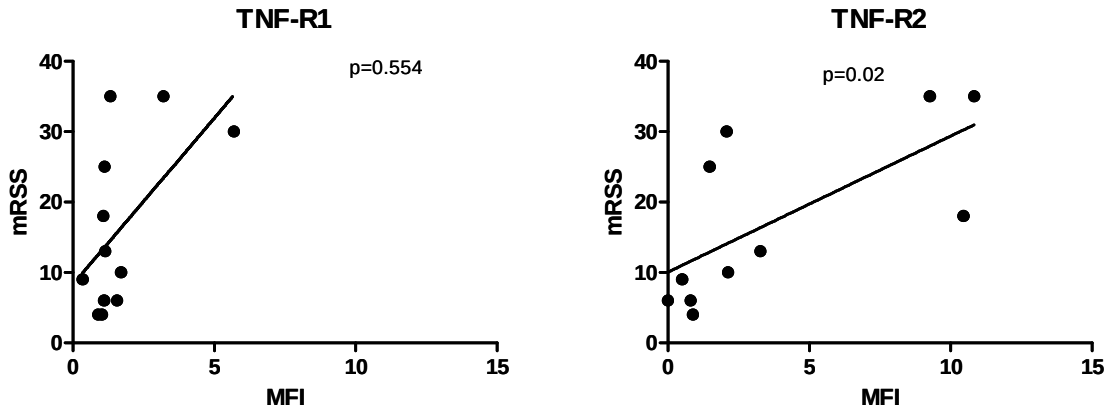
A


Stain in patient
Isotype
Stain in normal skin
CD1a+
CD14+
Macrophages
Lymphocytes
TNF-RI
TNF-RII
% of Max
TNFRaI
TNFRaII

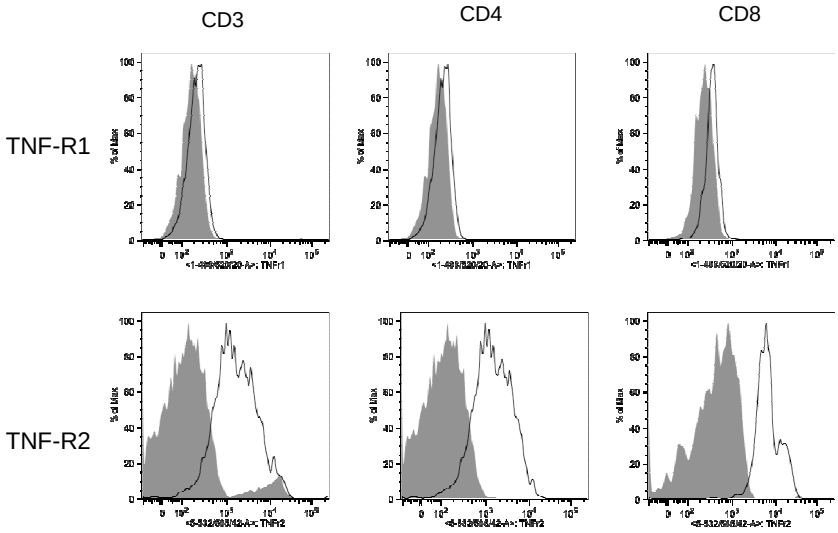
B


fold MFI
15
10
5
0
-5
Diffuse
Limited
Diffuse
limited
TNF-R1
TNF-R2

C


TNF-R1
p=0.554
mRSS
MFI
TNF-R2
p=0.02
mRSS
MFI

D


CD3
CD4
CD8
TNF-R1
TNF-R2

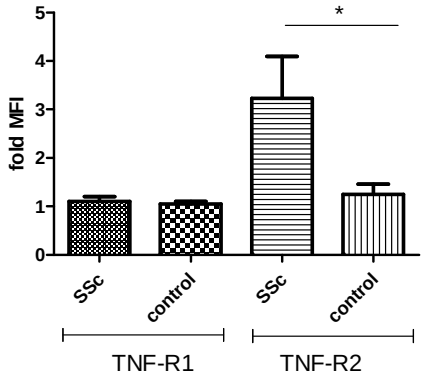


Figure 6. TNF-R2 was significantly upregulated on lymphocytes TNF-R expression on lymphocytes in peripheral blood from 11 SSc patients and healthy controls. from SSc patients compared to healthy controls. TNF-R1 was not upregulated.

SSc lymphocytes overexpress IL-6 and TGF-beta upon selective TNF-receptor stimulation, but their IL-10 expression is impaired.

$CD3^+$ cells were isolated from peripheral blood of 8 diffuse SSc patients from the same patient pool receiving skin biopsies and 8 healthy controls by Ficoll and magnetic beads as described in the methods section. $1x10^6$ cells were subsequently stimulated for 24h with 100 ng/ml selective TNF-alpha mutants: cysTNF-R1 selectively stimulates TNF-R1 whereas cysTNF-R2 selectively stimulates TNF-R2. Soluble TNF (sTNF) stimulates predominantly TNF-R1 and to a minor extent TNF-R2. Both resting T-cells and priorly CD3/28 activated cells were analyzed. In resting T-cells, IL-6 expression of cultured lymphocytes from SSc patients was higher than in control lymphocytes (Figure 7A). Notably stimulation with CysTNF-R1 led to a higher IL-6 expression in SSc lymphocytes. IL-10 expression was detected after CD3/28 activation (7B). IL-10 mainly increased after costimulation of TNF-R2. Conversely, costimulation of SSc lymphocytes with TNF agonists did not lead to IL-10 upregulation as seen in normal lymphocytes. This might indicate that lymphocytes of SSc patients lack a regulatory potential upon TNF-stimulation and therefore the resolution of inflammation might be impaired. Stimulation of TNF-R2 led to a higher TGF-beta expression SSc lymphocytes (7C).

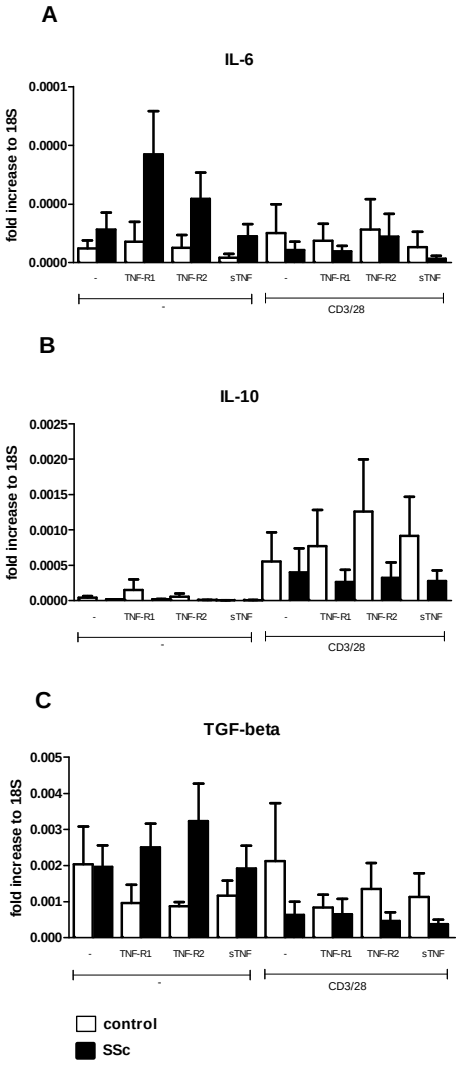


Figure 7. Cytokine expression of SSc lymphocytes after co-stimulation with TNF agonists**.** CD3+ cells were isolated from peripheral blood of 8 diffuse SSc patients by magnetic beads and cultured in DMEM + 10% fetal calf serum. 100 ng/ml of selective TNF mutants CysTNF-RI and CysTNF-R2 or soluble TNF were added to the cells for 24 hours. In resting T-cells, IL-6 expression of SSc lymphocytes was higher than in control lymphocytes (7A), notably upon stimulation of TNF-RI. Stimulation of TNF-R2 led to a higher TGF-beta expression SSc lymphocytes (7C). After stimulation with CD3/28 beads (activated), IL-10 expression as observed in healthy lymphocytes after stimulation of TNF-R2 was impaired in SSc lymphocytes (7B).

CD-3$^+$ lymphocyte correlate with alpha-SMA$^+$ myofibroblast infiltration in SSc dermis

A possible correlation between CD3$^+$ T-cells or CD20$^+$ B-cells and myofibroblasts was investigated in an independent patient cohort of 18 patients by IHC. Skin biopsies were obtained from affected and unaffected skin. In accordance to our flow cytometry results CD3$^+$ cell infiltration of affected skin was higher in patients with diffuse SSc compared to limited SSc (p=0.03)(Figure 8 and 9). CD3$^+$ infiltrates were mainly found in affected SSc skin (Figure 10). However, low grade CD3$^+$ cell infiltration was also prevalent in unaffected skin. Higher infiltrates of alpha-SMA positive cells are observed only in affected skin. Conversely, no alpha-SMA positive cells were seen in the most samples of unaffected skin. For quantification, cell infiltrates were blindly scored from 0-3. 3 was regarded as high, 2 as intermediate, 1 as low and 0 as no cell infiltration. We found a weak but significant correlation between CD3 cells (p=0.038, r^2=0.24) and CD20+ cells (p=0.0041, r^2=0.24) and alpha-SMA positive cells in affected SSc skin (Figure 11).

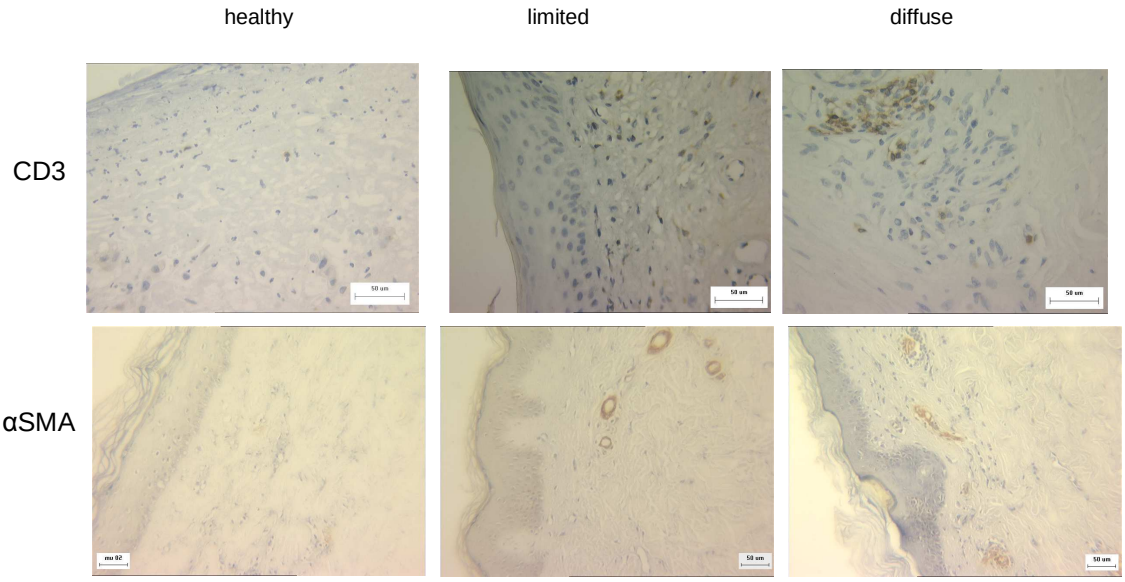


Figure 8. Immunohistochemistry of CD3$^+$ and alpha-smooth muscle actin (αSMA) cells in skin of a healthy individual and patients with limited or diffuse SSc. Single CD3$^+$ cells are seen in normal skin. In limited small accumulation of T-cells are seen, whereas abundant cell infiltrates are observed in diffuse SSc. A similar pattern is seen for αSMA expressing pericytes and myofibroblasts.

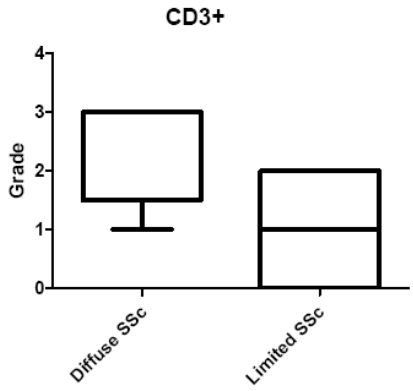
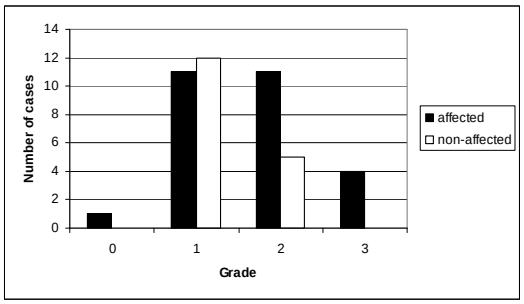


Figure 9. Dermal $CD3^+$ cells quantified in immunohistochemistry are higher in patients with diffuse vs. limited SSc**.** Postive cell infiltrates were scored from 0-3 in an independent dutch cohort of 18 SSc patients. 3 was regarded as high infiltration, 2 as intermediate, 1 as low and 0 as no cell infiltration. Similar to the flow cytometry data, $CD3^+$ infiltrates were higher in diffuse SSc patients.

A



B

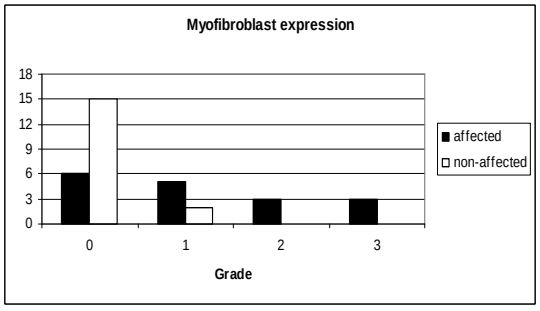


Figure 10. CD-3 lymphocyte and alpha-SMA+ cell infiltration assessed by immunohistochemistry from affected or unaffected sites in SSc patients.
Postive cell infiltrates were scored from 0-3 in an independent dutch cohort of 19 SSc patients. 3 was regarded as high infiltrate, 2 intermediate, 1 were low and 0 no positive CD3 cells. CD3 infiltrates were mainly found in affected SSc skin. Low grade infiltration is also prevalent in unaffected skin. In contrast, no alpha-SMA positive cells are seen in the most samples of unaffected skin. Higher infiltrates of alpha-SMA positive cells are observed only in affected skin.

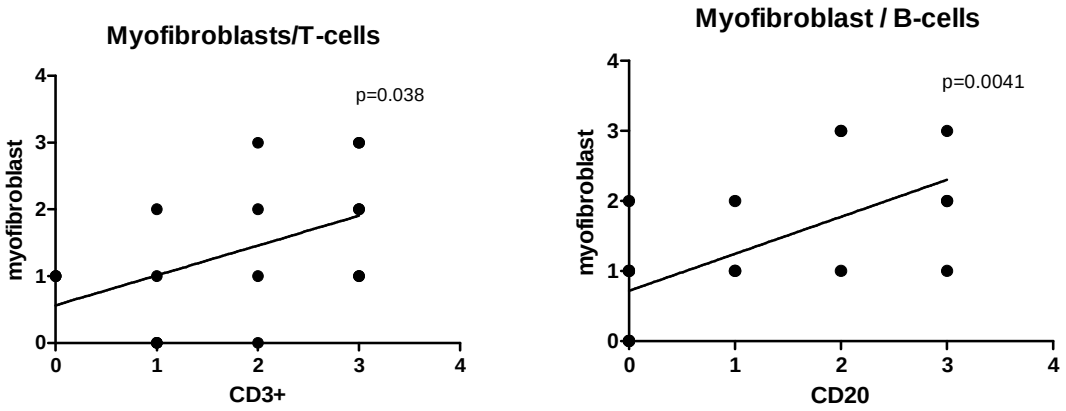


Figure 11. CD3 T-cell and B-cell infiltration correlate with myofibroblast infiltration in dermis of SSc patients. Skin biopsies were obtained in 18 patients with SSc. $CD3^+$ T-cells and alpha-SMA+ myofibroblasts were identified by IHC. Cell infiltration was scored blinded from 0= no infiltration, 1 = low infiltration, 2= intermediate and 3=high infiltration. There was a significant correlation both between $CD3^+$ ($p=0.038$, $r^2=0.24$) and $CD20^+$ ($p=0.0041$, $r^2=0.24$) cells and myofibroblast infiltration.

Conditioned media of TNF-activated lymphocytes from four SSc patients promote alpha-SMA expression by healthy or SSc fibroblasts.

$CD3^+$ lymphocytes from four SSc patients and four healthy controls were isolated by positive selection using magnetic beads as described above and stimulated in independent experiments for 24h with CysTNF-R1, R2, sTNF or no TNF. Healthy or SSc fibroblasts were then incubated with the conditioned media for further 24h and alpha-SMA expression detected by Western blotting (Fig. 12). Compared to healthy lymphocytes, conditioned medium from TNF naive SSc lymphocytes had an inhibitory effect on alpha-SMA expression. In contrast to healthy lymphocytes, supernatant of CysTNF-R1, R2 or sTNF stimulated lymphocytes restored the induction of alpha-SMA expression. This shows that resting SSc T-cells might be profibrotic towards healthy fibroblasts after TNF-R1, R2 or sTNF costimulation. The inhibitory effect of unstimulated lymphocytes is abolished in fibroblasts from SSc patients.

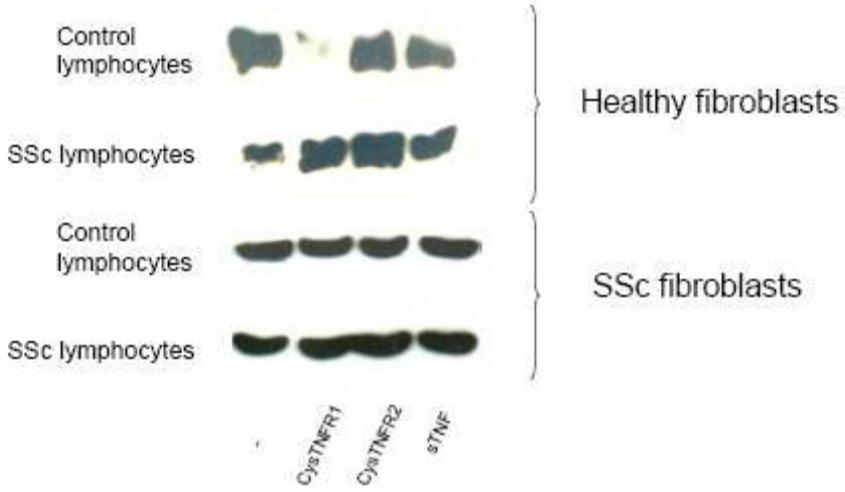


Figure 12. Effect of conditioned media of TNF-stimulated lymphocytes on alpha-SMA expression by healthy or SSc fibroblasts. Supernatant (SN) of unstimulated SSc lymphocytes inhibit alpha-SMA expression in healthy fibroblasts but not in SSc fibroblasts. In contrast to healthy lymphocytes, TNF stimulation of SSc lymphocytes restores the induction of alpha-SMA expression.

Dermal $CD3^+$ T-cell and myofibroblast infiltration is reversible after HSCT. $CD3^+$ cells and myfibroblast infiltration was assessed in a patient with diffuse SSc pre- and 6 months post HSCT. Skin thickening has improved partly in this patient; skin biopsies were taken from the same localisation at the forearm. $CD3^+$ T-cell infiltration and alpha-SMA positive myofibroblast infiltration was assessed by IHC, showing a clear reduction both of $CD3^+$ lymphocytes and myofibroblasts (Fig. 13).

TNF-receptor overexpression is reversible after HSCT. We measured dermal TNF-R1 and R2 expression by flow cytometry in correspondence to previous experiments. In this patient, we found a complete downregulation of TNF-R1 and R2 six months post HSCT (Fig. 14).

Protracted lymphopenia in peripheral blood after HSCT. Similar to the observations in SSc dermis of the patient 6 months after HSCT, we also found a protracted lymphopenia, notably of CD4+ helper cells. In contrast, T-supressor cells and NK-cells were increased after HSCT (Fig. 15).

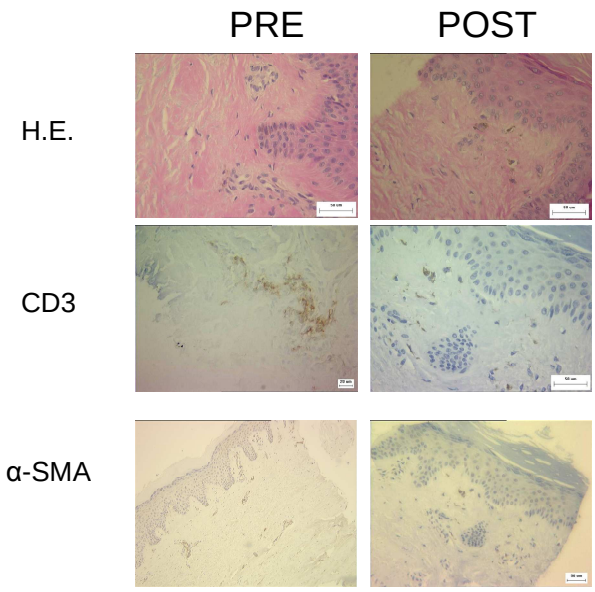


Figure 13. CD3 and alpha-SMA expression in skin from a patient with diffuse SSc before and 6 months after autologous haematopoietic stem cell transplantation (HSCT). Skin biopsies from the forearm were obtained and stained with hematoxilin/eosin or with CD3 or alpha-SMA. The cellular infiltrate before transplant has widely reduced after the procedure. Abundant $CD3^{+}$ cells are seen before HSCT, whereas only single CD3 positive cells are found after. The same is the case for alpha-SMA positive cells.

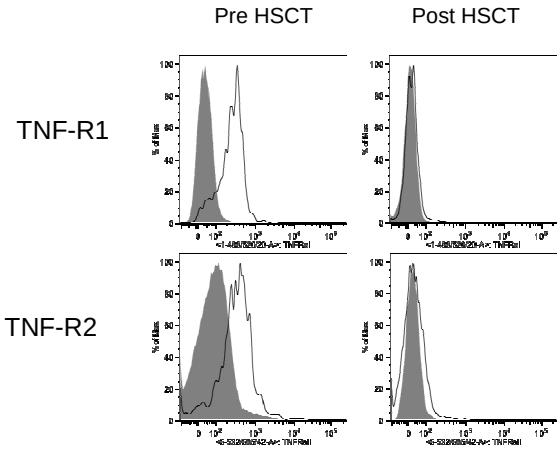


Figure 14. TNF-R up-regulation on lymphocytes reverses after autologous HSCT. Skin from the forearm of a patient with diffuse SSc who underwent autologous HSCT was digested with dispase and collagenase and prepared for flow cytometry. Lymphocytes were identified as CD45+HLA-DR+AF- SSC^{low} cells. TNF-R were stained with FITC (TNF-R1) or PE (TNF-R2). After the 'reset' of HSCT, lymphocytes loose their activation.

	Pre HSCT absolute count (%)	Post HSCT absolute count (%)
Peripheral blood:		
T Lymphocytes (CD3+)	768 (74)	189 (26)
T Suppressor Lymphocytes (CD3+CD8+)	114 (12)	125 (18)
T-helper Lymphocytes (CD3+CD4+)	607 (62)	61 (9)
CD3+CD4+CD8+	3 (0)	10 (1)
Lymphocytes CD45+	1035	716
NK cells (CD16+CD56+)	107 (10)	133 (18)
B-cells (CD19+)	164 (15)	381 (52)

Table 5 shows a table of peripheral blood cell subsets before and six months after HSCT. T-cells in peripheral blood remain suppressed 6 months after autologous HSCT. In contrast, T-suppressor lymphocytes, NK-cells and B-cells increase after the treatment. HSCT: haematopoietic stem cell transplantation.

2.4 DISCUSSION

This study underlines the importance of lymphocytes in SSc pathology and provides functional evidence for the association between lymphocyte infiltration and myofibroblast differentiation. For the first time, flow cytometry was applied for the analysis of *dermal* leukocytes in patients with SSc. The advantage of dermal flow cytometry is the possibility of multicolour flow analysis whereas in IHC co-staining is technically difficult and usually limited to the detection of a few concomitant antigens. Furthermore, the amount of counted events is considerably higher in flow cytometry (38). Here we analysed between 767 and 12243 $CD45^+$ leukocytes per staining, around 100.000 cells per skin biopsy. This is considerably

higher compared to IHC where analyses typically comprise counted cells of a few high power fields.

One limitation of flow cytometry is the need to treat the tissue with collagenase. Surface antigens have to be collagenase resistant in order to be analyzed by this method. This has been tested beforehand in this study. Also, skin biopsies have to be processed immediately, which can be demanding logistically.

It has been previously demonstrated that leukocyte infiltration occurs in SSc dermis in SSc (3-6). Lymphocytes, macrophages and mast cells have been identified as key infiltrating cells. Here we give evidence that in patients with diffuse SSc, lymphocytes are more prevalent whereas in skin of patients with limited SSc, macrophages are more prevalent. Lymphocyte infiltration correlates with skin thickening, suggesting an active role of lymphocytes in SSc fibrogenesis. This is in line with previous findings where T-cell transfer from bleomycine-treated mice led to fibrosis and autoantibody production in healthy mice (11). Blocking T-cell influx by CD3-antiserum abrogated lung fibrosis in bleomycine-treated mice and diseased T-cells stimulated fibroblast proliferation (40). It has therefore been postulated that (auto)activated T-cells in SSc stimulate fibroblasts and that T-cell control or regulation might be impaired, respectively. One explanation for the stimulation of fibroblasts could be the fact that lymphocytes (e.g. T-regulatory) cells in diffuse SSc secrete growth factors such as TGF-beta. Limited SSc has a more vasculopathic aspect compared to diffuse SSc were skin and lung fibrosis are more prevalent. The observation that macrophage infiltration is higher in the limited form of SSc is in line with a more ‘atherosclerotic’ pathogenesis, were macrophages infiltration in plaques and vessel wall, respectively are key pathogenic elements.

In contrast, Langerhans cells in the epidermis are reduced in diffuse SSc patients (7). We had similar findings here in the dermis, albeit this did not reach significance, probably to the low sample number.

We analysed TNF-alpha receptors as the role of these receptors in SSc is controversial and putative regulatory functions especially of TNF-R2 have been postulated in other settings. T-cells activated by CD3 ligation seem to inhibit collagen expression of fibroblasts in cell to cell contact by membrane associated TNF-alpha (32). On the other hand, TNF-receptors are necessary for bleomycine

induced fibrosis and TNF-alpha blockade can inhibit scleroderma in bleomycine-treated mice (41, 42).

In line with previous findings, we found TNF-R2 upregulation mainly on lymphocytes (31). Conversely, TNF-R1 expression is ubiquitous, with most of the receptors being stored intracellularly, therefore not detectable by flow cytometry. In this study, TNF-R1 was expressed on a low level on lymphocytes, $CD1a^+$ and $CD14^+$ cells. TNF-R2 expression measured by mean intensity fluorescence was considerably higher and correlated with skin thickening, suggesting a profibrotic role of this receptor in SSc. The predominant upregulation of TNF-R2 has also been demonstrated in bleomycine treated mice (18) but also in renal allograft rejection (43).

Functional studies of TNF-R were performed in CD3+ T-cells obtained from peripheral blood of SSc patients. Interestingly, the baseline expression of IL-6 which is an inflammatory but also highly profibrotic cytokine was already higher in lymphocytes from SSc patients. This data was shown both in rtPCR and ELISA analyses.

Upon selective TNF-R stimulation IL-6 expression was significantly higher in SSc patients, notably after TNF-R1 stimulation. The baseline of TGF-beta expression did not differ between SSc patients and healthy controls, but after selective TNF-R2 stimulation, TGF-beta expression was higher SSc patients. IL-10, a potent anti-inflammatory cytokine, was expressed after CD3/28 antigen stimulation. Interestingly CD3/28 activated SSc lymphocytes did not upregulate IL-10, neither IL-6 or TGF-beta. This might be due to an intrinsic dysfunction of SSc lymphocytes, a chronic TNF-alpha stimulation which renders lymphocytes tolerant towards antigen stimulation (44), or the influence of treatment such as cyclophosphamide. The lack of IL-10 upregulation might have implications e.g. in form of a disturbed resolution of inflammation.

As a limitation of this study we performed functional studies in lymphocytes from peripheral blood and not isolated from skin. In the next experiments we demonstrated in a control cohort, that there is a correlation between lymphocytes and myofibroblasts in SSc dermis. This finding suggested that T-cell can trigger the differentiation of fibroblasts into myofibroblasts. To find this out we incubated healthy fibroblasts in conditioned medium from SSc lymphocytes which were previously treated with TNF-agonists. The results here showed different results.

Preliminary data indicate that conditioned medium from SSc T-cells inhibit the alpha SMA production of healthy fibroblasts. This is in line with previous findings where T-cell membranes extracted from dermal lymphocytes inhibited fibroblasts in producing collagen (32). This effect was attributed to TNF-alpha itself. Conditioned medium of TNF-alpha activated T-cells however triggered alpha SMA expression. One possible reason for the inhibition of alpha-SMA production might be the content of Interferon gamma which we have not measured in this study. We postulate that the higher amount of IL-6 after costimulation of lymphocytes by TNF-alpha increases the alpha-SMA expression by fibroblasts. Previous studies already demonstrated that $CD4^+$ cells control the differentiation of monocytes into collagen producing fibrocytes (45). This observation has been obtained from four consecutive patients in two independent experiments. Clearly, the effect of SSc lymphocytes with or without prior TNF-R activation on fibroblasts needs confirmation in a larger number of patients. The exact mechanism by which TNF-activated lymphocytes stimulate fibroblasts remains elusive at this point. This is currently investigated by the group of Professor van Laar.

In our last set of experiments we showed that this effect might be reversible. We analyzed the effect of HSCT on dermal infiltration of $CD3^+$ lymphocytes and myofibroblasts. Both $CD3^+$ T-cells and myofibroblasts infiltration decreased strikingly 6 months after the intervention. On detected lymphocytes, previously overexpressed TNF-R1 and R2 were not longer detected, suggesting a reset of lymphocytes from an activated into a normal state. The lower infiltration by leukocytes was also shown in peripheral blood were we observed a protracted decrease of CD4 T-helper cells but an increase of T-suppressor lymphocytes.

In conclusion, we show that lymphocyte infiltration is of importance in SSc skin pathogenesis, presumably by interfering with the myofibroblast differentiation process. TNF-R2 is upregulated on dermal lymphcytes. TNF-R stimulation on lymphcytes has possibly profibrotic effects by IL-6 and TGF-beta production. TNF-R overexpression in the skin is reversible after HSCT.

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CHAPTER 3

Peripheral blood monocytes from systemic sclerosis patients express and secrete functionally active TIMP-1

Christiaan A Huigens[1], Thomas Hügle[1] (equally contributed),

Tess Stanly[1], Bridget Griffiths[2], Jasper Broen[3], Tim Radstake[3], Andreas Gessner[1], Jacob M. van Laar MD[1].

[1] Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK; [2] Dept of Rheumatology, The Freeman Hospital, Newcastle upon Tyne, UK; [3] Dept of Rheumatology, University Medical Centre Nijmegen, The Netherlands

Submitted for publication

ABSTRACT

OBJECTIVE: To investigate whether monocytes can act as a source of TIMP-1 in systemic sclerosis (SSc).

METHODS: $CD14^+$ Monocytes were isolated from blood of SSc patients and healthy controls using immunomagnetic beads. MMP-1 and TIMP-1 expression and secretion was measured by qRT-PCR and ELISA respectively. The functional effect of monocyte-derived TIMP-1 was studied in a MMP-1 activity assay.

RESULTS: The expression of TIMP-1 in freshly isolated SSc monocytes was higher compared to healthy control monocytes (mean increase 3.1, SD 2.8, p=0.03). The addition of SSc serum to cultures of healthy monocytes increased expression of TIMP-1, but not of MMP-1, when compared to culutures with serum of healthy controls (mean increase 1.9, SD 1.2, p=0.01) and RA patients 1 (mean increase 1.7, SD 1.1, p=0.04). The secretion of TIMP-1 by healthy monocytes cultured in SSc sera also was significantly higher when compared to cultures with sera from healthy controls or RA patients (mean 16.8 (SD 9) vs. 9.1 (SD 2.4) and 6.9 (SD 2.4) ng), p=0.01, p=0.04 respectively). Culture supernatants from monocytes cultured in SSc serum inhibited *in vitro* matrix degradation by 59% (SD 2%) when compared to culture supernatants of monocytes cultured in healthy control serum (p=0.02).

CONCLUSION: Circulating monocytes from SSc patients overexpress TIMP-1. TIMP-1 can be induced in healthy monocytes by soluble serum factors from SSc patients. Our findings suggest that circulating monocytes from SSc patients may contribute to matrix deposition via perturbation of the TIMP/MMP balance in favor of TIMP-1.

3.1 INTRODUCTION

Systemic sclerosis (SSc) is a rare autoimmune connective tissue disease characterised by vasculopathy and fibrosis of the skin and inner organs. Low grade inflammation with tissue infiltration by mononuclear cells plays an important role in fibrogenesis [1]. Fibrosis in SSc is characterised by extensive accumulation of extracellular matrix, notably collagen, glycosaminoglycans and proteoglycans [2]. In addition to increased secretion of ECM components, impaired breakdown of ECM can also contribute to fibrosis [3]. ECM breakdown is mainly mediated by matrix metalloproteinases (MMP), a family of zinc dependent endopeptidases which are capable of degrading all matrix components [4]. Tissue inhibitors of metalloproteinase (TIMP) selectively inhibit these peptidases and can therefore inhibit ECM digestion [4, 5]. The TIMP family consists of four members; of those TIMP-1 is a key enzyme as it can inhibit most MMPs. [4]. Increased concentrations of TIMP-1 have been measured in serum of SSc patients [6, 7]. Fibroblasts isolated from the skin of SSc patients have been shown to overexpress TIMP-1 [8, 9]. Several studies report an increase in the ratio of TIMP/MMP in SSc both in tissue and blood [7, 10-12]. In wound healing studies TIMP expressed by inflammatory cells such as monocytes or macrophages is important in tissue remodelling [13]. Recent studies have identified circulating monocytes as key inflammatory cells in SSc, showing profound dysregulation of gene expression [14, 15].

We postulated that monocytes can act as a source of TIMP-1 in SSc and thereby contribute to the imbalance of TIMP-1/MMP which underlies fibrosis.

3.2 MATERIALS AND METHODS

Study participants.

23 patients with SSc, 16 healthy controls and 11 patients with active rheumatoid arthritis (DAS28 score >2.6) were included in the study. All SSc patients fulfilled the ACR criteria according to LeRoy . Informed consent was obtained from all patients and controls. The study was approved by the ethics committees from the participating centres. Table 1 shows disease characteristics and treatment status of the patients with SSc.

Disease characteristics	SSc patients (n=23)
Female	80%
Disease duration. Years (Range)	8 (2-37)
Diffuse disease (%)	15 (65%)
Auto-antibodies (ACA / Scl-70 / ANA)	7 / 6 / 9
Rodnan skin score (Range)	11.6 (0-35)
Lung Fibrosis (%)	7 (39%)
Cyclophosphamide treated.	14 (61%)

Table 1. Twenty-three patients with SSc were included in the study. Data on gender, disease duration, limited or diffuse disease, auto-antibodies, rodnan skin score, lung involvement and cyclophosphamide treatment is provided. ACA: anti-centromere autoantibodies, Scl-70: anti-Scl70 autoantibodies, ANA: anti-nuclear autoantibodies.

Sample collection and cell purification.

Whole blood was collected during standard outpatient procedures in either serum and/or EDTA containing tubes (BD Bioscience). The samples were processed

within 4 hours of collection. Serum tubes were centrifuged at 1800g for 10 minutes according to the manufacturer's protocol. Total serum was harvested, aliquoted and frozen at -20℃. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by Ficoll-hypaque density gradient centrifugation (Axis-shield). $CD14^+$ monocytes were isolated from total PBMC according to the manufacturer's protocol with the $CD14^+$ MACS beads isolation kit (Miltenyi- Biotec). In short, PBMC's were suspended in PBS supplemented with 1% fetal-calf serum (Sigma-Aldrich) and EDTA (2mM) plus $CD14^+$ Miltenyi magnetic beads on ice for 20 minutes. The total mixture was passed through a magnetic isolation column (Miltenyi Biotec). Purified $CD14^+$ monocytes were removed from the column and tested for purity by flow cytometry. Flow cytometric analyses showed a >95% purity for $CD14^+CD3^-$ cells.

Cell culture.

Cell culture experiments were carried out with $CD14^+$ monocytes from healthy donors. Up to $2*10^6$ cells per well of a standard 24 well plate (Costar) were cultured for 4-24 hours in 250µl of Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with penicillin (100 units/ml), streptomycin (100 ug/ml), L-glutamin (2 mM) (all Sigma) and 10% serum from patients with SSc, patients with RA or healthy controls. Serum concentration was optimised through titration (Supplement 1). Cells were incubated at 37℃ in atmosphere containing 5% CO_2.

The real time fluorescence based TIMP activity assay requires the use of serum-free culture supernatant to exclude TIMP's present in the serum. In order to obtain serum free culture supernatant, monocytes were cultured for 2 hours as described

above. After 2 hours, cells were washed twice with PBS and incubated in DMEM supplemented with penicillin, streptomycin and L-glutamin for additional 4 hours.

TIMP-1 and MMP-1 expression analyses by quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR).

RNA from adherent or freshly isolated monocytes was isolated using the RNA mini kit (Qiagen) according to the manufacturer's protocol. 200-750 ng of RNA was treated with DNAse for 30 minutes at 37℃ and rever se transcribed (RT) to cDNA with the use of random hexamers (Invitrogen) and the moloney murine leukemia virus-reverse transcriptase (MMLV-RT) enzyme (Invitrogen) according to the manufacturer's protocol. 20 ng of cDNA, 0.6 μM forward and reverse primer, 0.3μM probe and 0.2μl reference dye in 20μl total volume per well was used for TIMP-1 expression analyses by qPCR. MMP-1 expression was analysed using SYBERGREEN taqready mix (Sigma). Samples were analysed in triplicate and normalised to the 18S housekeeping gene using the AB7500 (Applied Biosystems) qPCR machine and program. Relative expression levels to the average healthy control were calculated using the following equation: $(2^{\wedge}\text{Delta Delta CT})^{-1}$. The primers and probe sequences for TIMP-1: forward primer 5'-GACGGCCTTCTGCAATTCC -3' reverse primer 5'-GTATAAGGTGGTCTGGTTGACTTCTG -3' probe 5'-FAM-ACCTCGTCATCAGGGCCAAGTTCGT -TAMRA-3'. Primers and probe sequences for 18S: forward primer 5'-GAATGGCTCATTAAATCAGTTATGG-3' reverse primer 5'-TATTAGCTCTAGAATTACCACAGTTATCC-3' probe 5'-FAM-TCCTTTGGTCGCTCGCTCCTC- TAMRA-3'. Primer sequences for MMP-1: forward primer 5'-AGTGACTGGGAAACCAGATGCTGA-3' reverse primer 5'-

GCTCTTGGCAAATCTGGCCTGTAA-3'. Primers, probes and 2x Taqready mastermix (with and without sybergreen) were obtained from Sigma-Aldrich. qPCR settings according to the Taqready mix manufacturer's protocol.

TIMP-1 Enzyme-Linked Immunosorbent Assay (ELISA).

The TIMP-1 protein concentration in conditioned and unconditioned medium and sera was measured by ELISA as described before [16]. In short, plates (Costar) were coated with the primary antibody [16] overnight at 4℃ in phosphate buffered saline (PBS, Sigma-Aldrich) and blocked with bovine serum albumin (BSA) (Sigma-Aldrich) in PBS (50 mg/ml) overnight at 4℃ or for 2 hours at room temperature. The secondary biotinylated antibody was incubated for 2 hours at room temperature. Signal development was performed with HRP/Streptavidin for 30 minutes at room temperature. OPD substrate and hydrogen peroxide in phosphate citrate buffer (all Sigma-Aldrich) were added. When colour development was sufficient, 50 µl of sulphate was added to stop the reaction. Fluorescence was measured with a plate reader (Tecan, Sunrise) at 490nm wave length. All samples were run in duplicate and diluted one in eight in order to fall within the detection limits of the assay (0-90 ng/ml). A titration of recombinant human TIMP-1 was used as calibration curve (Range 5-90 ng/ml) [16].

Real time fluorescence based TIMP activity assay.

The functional effect of increased levels of TIMP-1 in monocytes was studied by a real time fluorescence based TIMP activity assay [17]. In this assay serum free culture supernatants derived from monocytes pre-incubated with 10% SSc patient

or healthy control serum is added to FS-6 (=fluorogenic substrate) and activated recombinant human MMP-1. The effect of supernatant on the breakdown rate of FS-6 by the activated MMP-1 is visualised using a Perkin Elmer LS50b plate reader.

ProMMP-1 (generously donated by Dr. J. Milner) was activated by APMA (0.675 mM) (Sigma) in 0.1M Tris, pH 7.5, 0.1 M NaCl, 10mM CaCl2, 0.05% Brij-35, 0.1% PEG 6000 for 4 hours at 37℃. Subsequently, MMP-1 was incubated with serum free culture supernatants from monocytes pre incubated with either SSc patient or healthy control serum or plain DMEM for 15 minutes at 37℃. The mixture of culture supernatant and activated recombinant human MMP-1 was then added to separate wells containing FS-6 substrate (500 µM, Calbiochem). Fluorescence was measured by the Perkin Elmer LS50b exciting at 325nM and measured at 400nM wave length. For each sample, the rate of breakdown was compared to the standard containing MMP-1 (6µg/ml), FS-6 (50µM) and unconditioned medium (plain DMEM, Sigma). Titration experiments using recombinant human TIMP-1 were performed to study the MMP-1 concentration optimal for detecting differences in TIMP concentrations (Supplement 3).

Statistical analysis.

Differences in expression levels, protein concentration or substrate breakdown between SSc and control groups in all experiments were defined using a two tailed T-test. P-values <0.05 were considered significant.

3.3 RESULTS

Serum TIMP-1 concentrations are higher in SSc patients versus healthy controls.

TIMP-1 concentrations in sera from 18 SSc patients and 13 healthy controls were measured by ELISA (Figure 1A). The mean serum TIMP-1 concentration in SSc patients was 941 ng/ml (SD 325 ng/ml) compared to 637 ng/ml (SD 270 ng/ml) in healthy individuals (p=0.01), confirming previous work. Serum TIMP-1 concentrations did not correlate with Rodnan skin score, disease onset, lung involvement, treatment status or autoantibody status.

TIMP-1 but not MMP-1 is up-regulated in circulating monocytes from SSc patient.

TIMP-1 and MMP-1 expression in CD14+ monocytes from 15 patients with SSc and 11 healthy controls was analysed (Figure 1B). Seven patients had diffuse SSc, 6 of these patients were positive for Scl-70 auto-antibodies. The other patients had limited disease and were positive for either ACA or ANA. TIMP-1 expression in monocytes from patients with SSc, after normalisation to the 18S housekeeping gene, was significantly higher compared to monocytes from healthy controls (p=0.03). Mean expression of TIMP-1 in SSc monocytes was 3.1 fold (SD 2.8) higher compared to healthy control monocytes. The level of expression of TIMP-1 did not correlate with autoantibody status of the SSc patients. MMP-1 levels were

below the detection limit of the assay in CD14+ monocytes from both healthy controls and SSc patients.

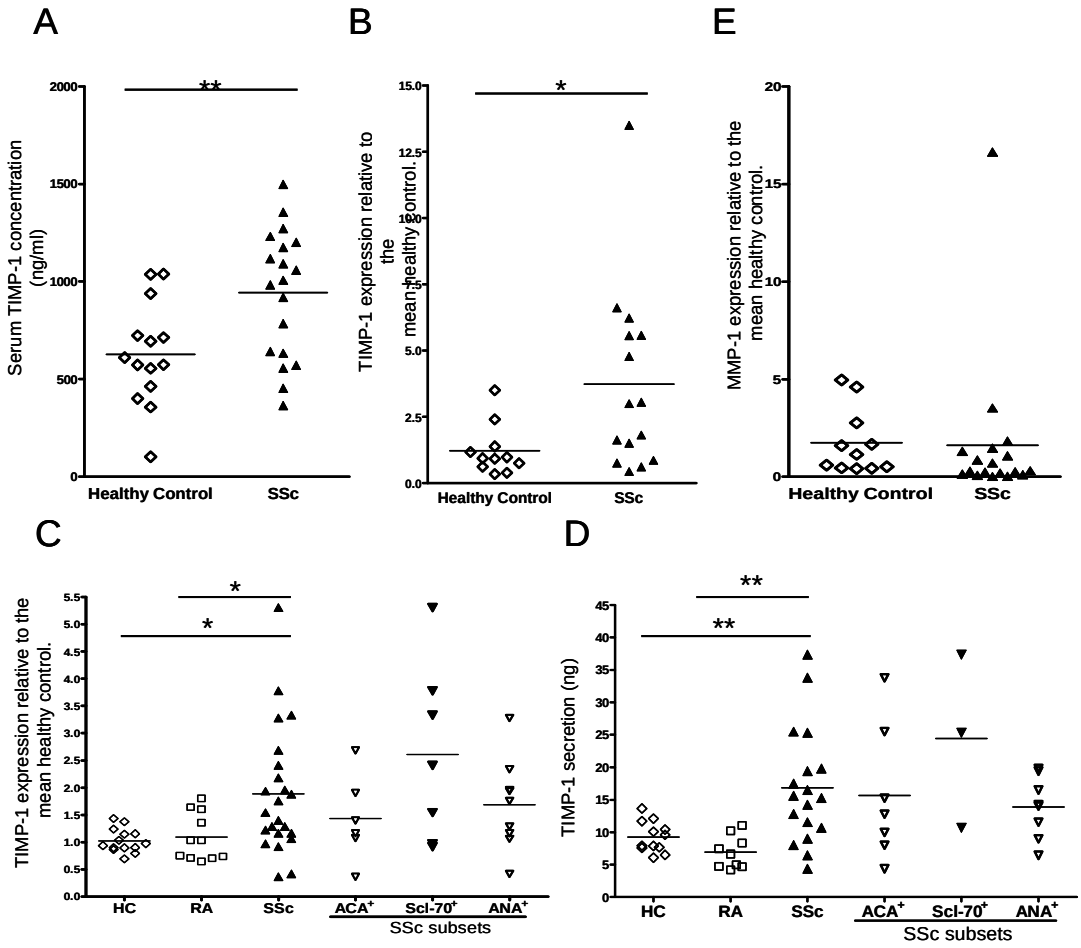

Figure 1. Overexpression of TIMP-1 in SSc CD14+ monocytes and the effect of SSc serum factors on TIMP-1 levels in CD14+ monocytes from healthy donors. A. TIMP-1 serum protein concentration by ELISA in SSc and healthy control serum (ng/ml). B. TIMP-1 expression levels in freshly isolated circulating $CD14^+$ monocytes from patients with SSc and healthy controls by qPCR. C and D. 4 hour expression (C) and secretion (D) of TIMP-1 by cultured CD14+ monocytes from healthy donors incubated in 10% SSc, RA or healthy control serum. E. MMP-1 expression by qPCR in $CD14^+$monocytes cultured in 10% SSc or healthy control serum. Data show mean concentration, expression or secretion for the individual groups and SSc patient subgroups as defined by autoantibody positivity. B, C, D. Expression levels, normalised to the 18S housekeeping gene, are relative to the mean healthy control. Each dot represents the mean effect of one patient/control sample in multiple experiments (N=2-4). ACA: anti-centromere antibody, Scl-70: anti-Scl70 antibodies, ANA: anti-nuclear antibodies. *p<0.05, **p<0.01

Serum from patients with SSc up-regulates TIMP-1 in healthy monocytes. Monocytes from healthy donors were incubated in 10% serum from patients with SSc, healthy controls or serum from patients with rheumatoid arthritis (RA). Mean TIMP-1 expression levels of SSc serum cultured monocytes were 1.9 (SD 1.2) and 1.7 (SD 1.1) fold higher compared to the mean of healthy control or RA patient serum cultured monocytes respectively (p=0.01, p=0.04). TIMP-1 expression was higher in monocytes cultured in sera containing Scl-70 auto-antibodies (mean expression levels were 2.6 vs 1.9 fold increased compared to mean healthy control sera (SD 1 – 4.25, p=ns)) (Figure 1C). Likewise, TIMP-1 protein secretion over 4 hours as measured by ELISA was significantly higher after culture of monocytes from healthy donors in SSc sera as compared to healthy control or RA sera (16.8 (SD 9.0) vs. 9.3 (SD 2.4) and 7.0 (SD 2.4) ng respectively) (p=0.0083, p=0.042 respectively). The highest TIMP-1 levels were measured in cultures with anti-Scl-70 positive sera (mean secretion of 24.4 ng (SD 13.4 ng) in 4 hours opposed to a mean of 16.8 ng (SD 9.0 ng) for all SSc sera) (Figure 1D).

While MMP-1 levels of freshly isolated monocytes were undetectable, cultured monocytes did express low levels of MMP-1. MMP-1 expression levels were not significantly different (p=0.92) for monocytes cultured in SSc or healthy control serum (Figure 1E).

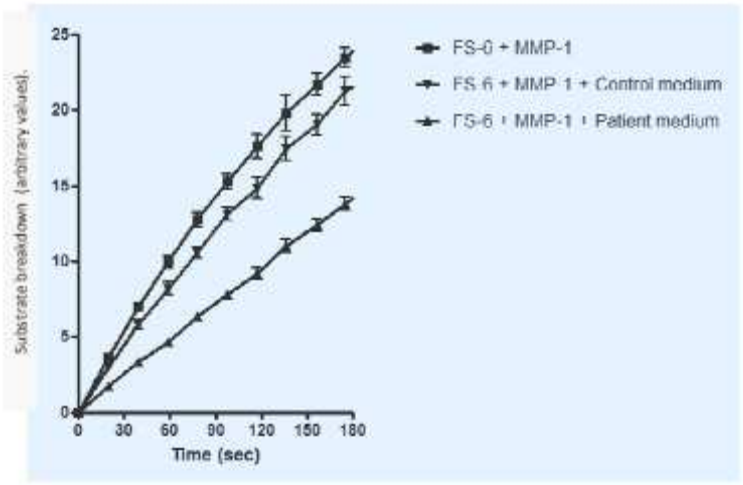

Figure 2. Functional TIMP-1 in supernatants from SSc serum-activated monocytes in MMP-1 enzyme activity. Conditioned medium from SSc-serum activated monocytes significantly inhibits MMP-1 mediated substrate breakdown. Data of a representative experiment (n=3) are shown. (*= P<0.05 compared to the values of FS-6 +MMP-1)

Supernatants from monocyte-cultures impairs substrate breakdown by MMP-1.

To investigate whether elevated levels of monocyte-expressed and secreted TIMP-1 impairs substrate breakdown by MMP-1 we applied a functional matrix breakdown assay. FS-6 breakdown by MMP-1 was significantly (P=0.02) lower in the presence of supernatant from monocytes pre-incubated with SSc serum compared to healthy control serum or plain DMEM (Figure 2). A 59% (SD 2%) decrease in MMP-1 activity was demonstrated when culture supernatant from monocytes activated by SSc compared to those activated by healthy control serum was added to equal doses of active MMP-1. Data shown are from one representative experiment (n=3). In all 3 experiments sera of 3 different SSc patients and controls was tested. TIMP-1 concentrations in the supernatants of monocytes cultured in SSc serum was higher in all experiments as compared to

supernatants of monocytes cultured in serum from healthy controls. (supplementary data)

3.4 DISCUSSION

The importance of the innate immune system including circulating monocytes in the pathogenesis of systemic sclerosis (SSc) is increasingly recognized. Traditionally, monocytes are sees as prototypic pro-inflammatory cells, but recent evidence has shown that their functional capabilities extends beyond those of cytokine production alone. In the present study we wanted to explore the possibility that SSc monocytes can contribute to tissue fibrosis through preferential up-regulation of TIMP-1 as opposed to MMPs. This was based on the observation of increased serum TIMP-1 concentrations in SSc patients [6, 7] and the fact that some macrophage subsets have a repair phenotype.

Our findings indeed strongly suggest that monocytes can play an important role in fibrogenesis. We found that TIMP-1 but not MMP-1 gene expression is higher in freshly isolated SSc monocytes compared to healthy control monocytes. Interestingly, serum from SSc patients induced expression and secretion of TIMP-1 but not MMP-1 in healthy monocytes, thus generating a profibrotic state [7, 10-12]. This shift in balance towards TIMP-1was functionally relevant as shown by the results from the matrix degradation assay. These results could translate into increased matrix deposition in skin, blood vessels and internal organs. Our findings suggest that factor(s) in serum from SSc patients are responsible for the imbalance between TIMP-1/MMP as observed in SSc monocytes.

The observation of profibrotic properties of monocytes are in line with recent findings on the identification of two different subsets of monocytes/macrophages: a pro-inflammatory (M1) and a profibrotic (M2) subset. The M2 subset, characterized by expression of CD163 and CD202 and an important source of the pro-fibrotic cytokines such as TGF-β, has been identified in SSc skin and serum [18]. Another study found that in SSc patients with interstitial lung disease, $CD14^+$ monocytes, in response to LPS, are skewed towards the pro-fibrotic phenotype and overexpress the M2 marker, CD163 and IL-10 and CCL-18, both known to stimulate collagen secretion by fibroblasts [19].

Taken together, our data indicate that SSc monocytes are skewed towards a pro-fibrotic state. Monocytes could thus play a role in the accumulation of collagen by inhibiting breakdown as a result of perturbation of the balance of TIMP/MMP in favor of TIMP-1. The exact mechanism by which monocytes become profibrotic remains to be investigated. The cytokine and or auto-antibody milieu is likely to play a major role in this regard.

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CHAPTER 4

Mast Cells are a Source of Transforming Growth Factor (TGF) beta in Systemic Sclerosis

Thomas Hügle[1,2], Vanessa Hogan[1], Kathryn White[3], Jacob M. van Laar[1]

[1] Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, United Kingdom
[2] Department of Rheumatology, Felix-Platter-Spital, University Hospital of Basel, Basel, Switzerland
[3] EM research services, Newcastle University, United Kingdom

Published in Arthritis and Rheumatism 2011;63(3):795-9.

ABSTRACT

Objective. To describe the cellular source of transforming growth factor (TGF) beta in the dermis of patients with systemic sclerosis (SSc).

Methods. We performed gold labelled immuno electron microscopy (EM) on skin biopsies of seven SSc patients and three healthy controls. For TGF-beta quantification, gold particles per square micron were calculated. Mast cell origin was confirmed and quantified by toluidine blue staining and light microscopy. De-granulation was assessed on toluidine blue sections and EM images.

Results. In all patients, active TGF-beta was uniquely observed in mast cell vesicles, some of which were released into the extracellular space. Patients with progressive SSc and a more recent onset of non-Raynaud's phenomenon symptoms had higher numbers of mast cells and gold particles per mast cell. Mast cells in healthy controls also contained active TGF-beta but in contrast to SSc samples showed a resting character with no or low-level de-granulation and uniformly dense osmiophilic vesicles.

Conclusion. De-granulation of skin mast cells can be an important mechanism of TGF-beta secretion in SSc.

4.1 INTRODUCTION

Systemic sclerosis (SSc) is a heterogeneous connective tissue disease presenting with low grade inflammation and vasculopathy, which together lead to fibrosis of skin and inner organs. Transforming growth factor (TGF) beta is a key player in fibrosis and has shown to be of paramount importance in SSc (1). It has been convincingly demonstrated that TGF-beta contributes to fibrosis via the activation of increased levels of TGF-beta receptors on fibroblasts (2), leading to activation of the Smad pathway and stimulation of fibroblasts to express collagen or differentiate into myofibroblasts (3). There are reports of several cell lines expressing or storing TGF-beta in SSc. Notably fibroblasts, endothelial cells, thrombocytes, monocytes and macrophages are considered to synthesise TGF-beta (4). In SSc dermis, the expression of TGF-beta is most prominent around vessels. It is associated with infiltrating mononuclear cells and it co-localizes with type-1 collagen expression (5-7). However so far it has not been demonstrated which of these cells are mainly responsible for TGF-beta expression in SSc.

Mast cells are vesicle-containing secretory cells resident in the connective tissue, notably in the skin, respiratory system and gastrointestinal tract. Activation such as in hypersensitivity or anaphylaxis leads to the release of various tissue mediators, including vasoactive amines, proteinases and also TGF-beta (8, 9). Their proximity to fibroblasts makes mast cell products available to fibroblasts and stimulates them to produce collagen (4, 9).

In SSc, the number of mast cells is increased both in involved and uninvolved skin, but the number of de-granulated mast cells is increased only in involved skin (10).

In this report we demonstrate by electron microscopy (EM) immunogold-labelling that mast cells are a major source of TGF-beta in SSc.

4.2 PATIENTS AND METHODS

4 mm dermal punch skin biopsies of involved skin from the forearm were obtained from 7 SSc patients (4 with diffuse cutaneous SSc and 3 patients with limited cutaneous SSc) (Table 1), all of which fulfilled the ACR criteria for SSc (11). The disease course was categorized as improving, stable or progressive depending on

the change of skin thickening and/or organ function in the year preceding skin sampling. Control skin samples were obtained from the forearm in one, and from breast-reduction surgery in two healthy individuals. Ethical approval and written consent was obtained before the intervention. Skin biopsies were fixed in 1% paraformaldehyde and embedded in LR white resin. For immuno-staining we used a monoclonal mouse antiserum reactive against active human TGF-beta1,2 isoforms (R&D systems) and a gold labelled secondary antibody. Staining with gold-labelled secondary antibody without primary antibody served as a negative control in each experiment (Fig. 1). Sections were examined on a Philips CM100 transmission EM.

For quantification we calculated the mean number of gold particles per square micron in all detected mast cells per sample. Toluidine blue (Sigma) stained sections and light microscopy were used to confirm mast cells. To determine the prevalence of mast cells we counted toluidine blue positive cells per x100 magnification field. De-granulation was assessed both by toluidine blue staining and EM and scored blinded as absent (-), mild (+), moderate (++) or extensive (+++).

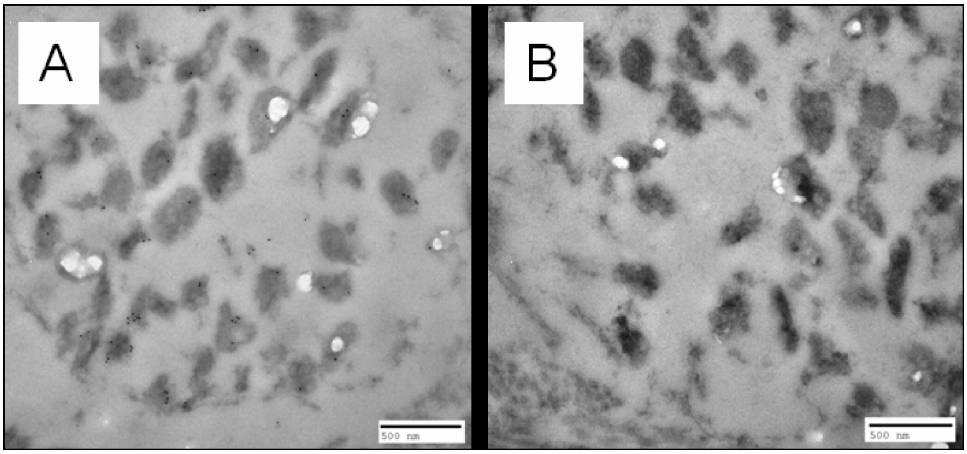

Figure 1. Negative control. A. Immuno electron microscopy with anti-TGF-beta antiserum and secondary, gold-labelled antibody. B. The same experiment without primary antibody.

4.3 RESULTS

Active TGF-beta was uniquely detected in mast cells. Figure 2A shows a mast cell in proximity of fibroblasts and a proliferating vessel. On higher magnification (Figure 2B), TGF-beta is seen abundantly in the vesicles of this mast cell. Some TGF-beta containing vesicles are released into the extracellular space (Figure 2C). To confirm that these were mast cells serial sections were stained with toluidine blue and examined with light microscopy or processed for immuno-EM, respectively (Fig 2 D,E). No other cells, notably lymphocytes, fibroblasts or macrophages were positive for TGF-beta in the patients.

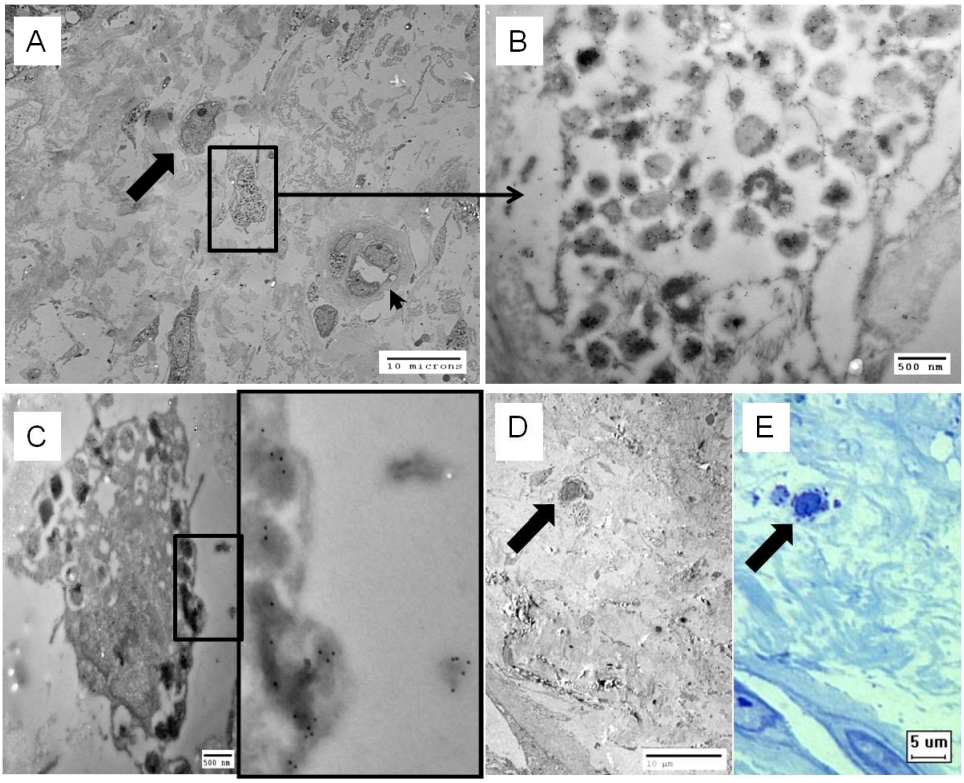

Figure 2. Immuno electron microscopy and –histology of the dermis of SSc patients. A. A mast cell with typical morphology is seen in close proximity to a fibroblast (arrow) and a proliferated vessel (arrowhead). **B**. On a higher magnification, abundant gold particles are seen in the mast cell vesicles. **C**. TGF-beta is released by mast cell de-granulation in the extracellular space. **D** and **E**. The nature of mast cells are confirmed in serial sections stained with immunohistochemistry and toluidine blue.

Mast cells were more prevalent (≥ 4 per visual field) in patients with progressive disease and a more recent SSc onset (≤ 3 years) (Table 1). The two patients with

the highest modified Rodnan Skin Score also had the highest numbers of mast cells with up to 6 toluidine blue stained cells in a single field (Figure 4). Patients with stable or improving disease had very few mast cells (≤ 1 per visual field).

The number of gold-labelled TGF-beta molecules per square micron in mast cells was also higher in patients with a progressive disease course compared to those with a stable course. One patient with a relatively high skin score who had successfully been treated with hematopoietic stem cell transplantation prior to skin sampling only had low mast cell numbers and gold particles per mast cell. We found mast cell de-granulation in five out of seven patients. One patient had diffuse scattering of TGF-beta containing vesicles throughout the dermis, three patients had moderate de-granulation whereas mild or no de-granulation was found in other three patients.

Mast cells in all three healthy samples also contained TGF-beta confined to vesicles with a mean number of 19 gold-labelled TGF-beta molecules per cell. However, two healthy samples had a dermal mast cell number per field of 2 with mild de-granulation and one healthy individual had a maximum of 5 mast cells per field without de-granulation. With standard EM we observed heterogeneity in mast cell vesicle density in the SSc samples compared to a more homogeneous pattern of osmiophilic structures in healthy controls (Figure 3). Some SSc mast cell vesicles were fused in the form of a ‘tunnel’. These findings reflect an active state of mast cells in SSc skin.

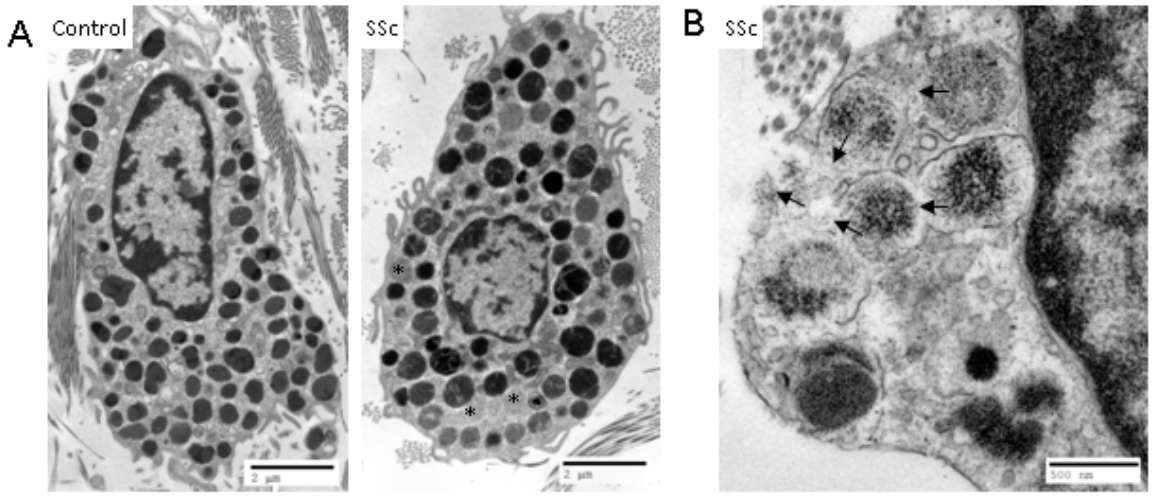

Figure 3. Standard electron microscopy illustrating mast cell vesicle morphology. A**.** Mast cells of healthy individuals show uniformly dense osmiophilic vesicles whereas vesicle density in SSc samples was more heterogeneic (asterisks) indicating a higher mast cell activity. **B.** High

magnification of mast cell vesicles in SSc patients showed a fusion of vesicle membranes with each other and the cell membrane, respectively (arrows).

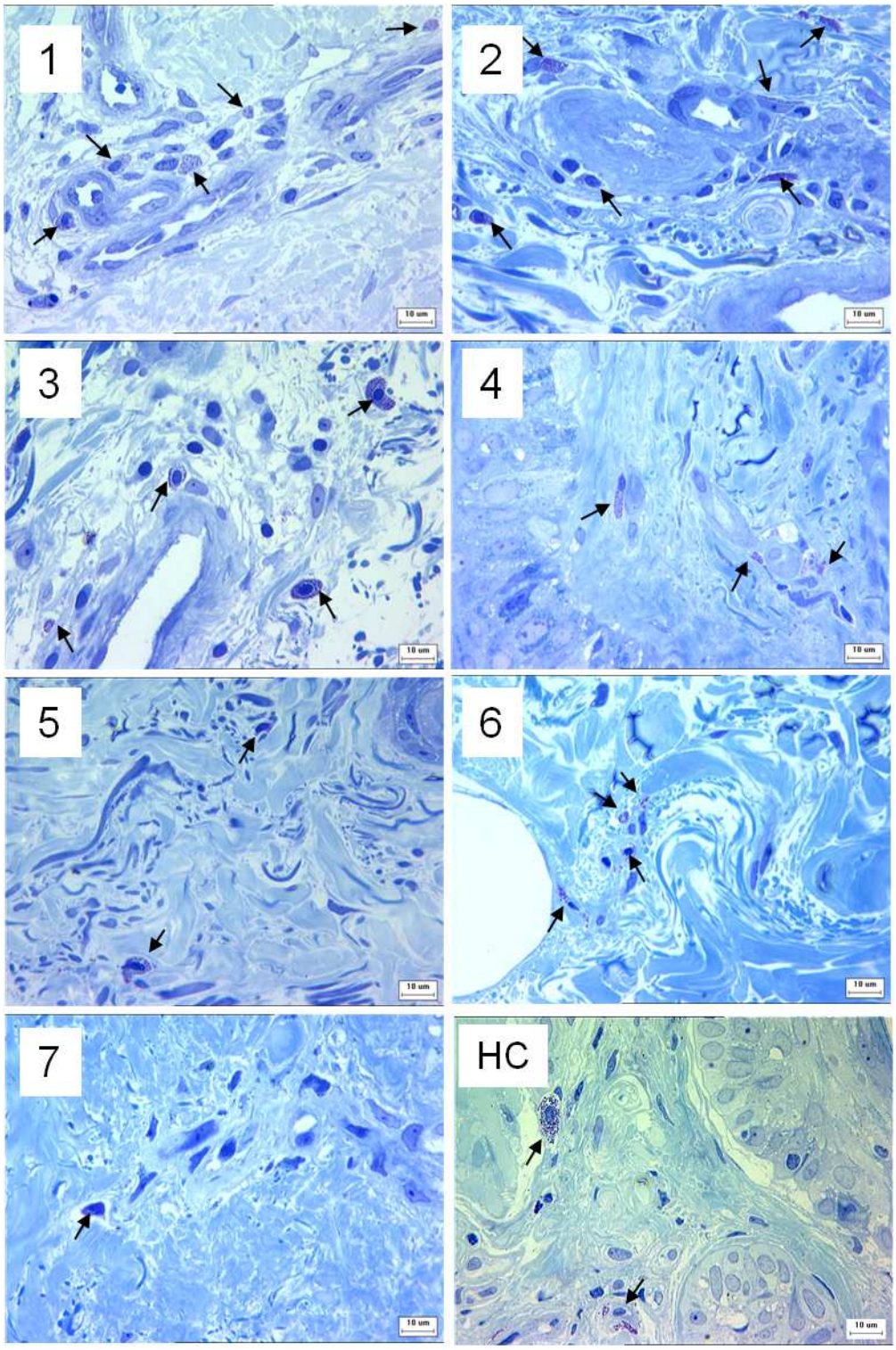

Figure 4. Dermal mast cell infiltration in SSc patients 1-7 and a healthy control shown by positive Toluidine blue staining (arrows). Patients numbers refer to table 1. Patient 1-3 who suffered from progressive disease course had higher numbers of mast cells in the dermis (magnification x100). HC: healthy control.

4.4 DISCUSSION

In this study we identified de-granulating mast cells as a major source of active TGF-beta in skin of SSc patients. Both the number of mast cells and detectable TGF-beta in mast cells are higher in patients with a more recent onset and progressive disease, respectively. This suggests that the release of TGF-beta by mast cells and trafficking in vesicles could be of importance in the pathogenesis of SSc, e.g. through inflammatory cell attraction or fibroblast stimulation. Functional analyses, however, will be necessary to confirm the pro-fibrotic role of mast cells in SSc. Furthermore we cannot exclude that immuno-EM is not sensitive enough to detect lower levels of TGF-beta when expressed in other cells. It is possible that TGF-beta anchored in the latent TGF-beta complex could not be detected in these experiments because of a masked epitope.

Nonetheless, our analysis demonstrates that de-granulating mast cells are a source of TGF-beta in SSc and thus might actively contribute to fibrosis by activation of fibroblasts. This is in line with findings in other fibrotic diseases where mast cells promote collagen deposition and represent a potential therapeutic target (12, 13). As expected, physiological TGF-beta storage in vesicles of resting mast cells from healthy individuals was found. In contrast, ongoing de-granulation, a higher number of mast cells and mast cell activity reflected by the heterogenic vesicle coloration and fusion of vesicles indicate a higher TGF-beta turnover by mast cells in SSc patients.

Patient	Gender	Age (years)	Disease duration (years since first non-Raynaud)	Subtype	mRRS	Immunosuppressive treatment	Disease course	Max. number of mast cells/field	Gold particles/sq micron	Mast cell de-granulation
1	Male	76	3	Diffuse	35	Cyc	Progressive	6	11.7	++
2	Female	58	2	Diffuse	30	MTX	Progressive	6	9.1	+++
3	Female	55	3	Limited	9	-	Progressive	4	9.8	++
4	Female	46	3	Limited	8	-	Stable	3	7.8	++
5	Male	57	10	Diffuse	20	Cyc	Stable	2	6.0	-
6	Female	63	12	Limited, RA overlap	9	-	Stable	4	6.9	+
7	Female	57	4	Diffuse	28	Autologous HSCT	Improving	1	1.5	-

Table 1. Patient characteristics, mast cell numbers and TGF-beta labelling per mast cell. Mast cells were quantified as toluidine blue positive cells. The maximal number of infiltrating cells per x100 magnification field is indicated. Gold particles per square micron were counted in all identified mast cells per biopsy. De-granulation was assessed by toluidine blue staining and electron microscopy and scored as absent (-), mild (+), moderate (++) or extensive (+++). MRSS: modified Rodnan Skin Score, RA: rheumatoid arthritis, HSCT: hematopoietic stem cell transplantation. Cyc: Cyclophosphamide, MTX: Methotrexate.

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CHAPTER 5

Sclerosing skin disorders in association with multiple sclerosis. Coincidence, underlying autoimmune pathology or interferon induced?

T Hügle, S Gratzl, T Daikeler, D Frey, A Tyndall, U A Walker

Published in Annals of Rheumatic Diseases. 2009;68(1):47-50.

ABSTRACT

Objectives: To describe and analyse the manifestation of sclerosing skin disorders in patients with multiple sclerosis (MS). Case reports: We describe three patients with relapsing remitting MS who developed skin sclerosis while receiving interferon (IFN)-beta treatment and review nine further cases of systemic sclerosis (SSc) in MS from the

literature. Of all 12 patients reported, eight had limited cutaneous SSc, three had diffuse cutaneous SSc and one patient had an antisynthetase syndrome. Localised scleroderma such as morphoea was not described. The mean age at diagnosis was 25.2 years for MS (range 12 to 51) and 38.3 years for SSc (range 16 to 66). Eleven patients developed SSc after the onset of MS and manifested with skin sclerosis after a mean of 14.9 years (range 1 to 45). In five patients IFN-beta was commenced before the development of skin sclerosis (mean 4.6 years, range 1 to 8 years). There was no relationship between the onset of skin sclerosis and MS activity. With the exception of one individual, all patients had antinuclear antibodies. Conclusions: Sclerosing skin disorders may develop in the course of MS. The relatively early age of SSc onset in patients with MS suggests a genetic predisposition and/ or an IFN-associated trigger.

5.1 INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease mediated by autoreactive T cells. Although a report suggested an increased prevalence of Sjögren's syndrome, an association between MS and connective tissue diseases (CTD) has not been confirmed in a large cohort.[1] Interferon (IFN)-beta is frequently used in the treatment of relapsing-remitting MS. IFN-beta may stimulate autoantibody production, induce autoimmune disorders in the thyroid and other organs, and precipitate Raynaud's phenomenon.[2] A parallel occurrence of MS and systemic sclerosis (SSc) has only been described in a few individuals. Here, we describe three patients who were referred to our tertiary care hospital suffering from MS and having developed a sclerosing skin disorder while receiving IFN-beta treatment. We review the evidence for a link between both autoimmune conditions, and the triggering of skin sclerosis by IFN.

5.2 CASE REPORTS

Patient 1

A 54-year-old female patient had developed relapsing-remitting MS at the age of 12 years. Since 47 years of age, she had received corticosteroids and IFN-b, under which her MS remained stable. At the age of 53 years under continued IFN-treatment she noticed Raynaud's phenomenon with oedema, sclerodactyli and hyperpigmentation of the nail beds. Digital ulcers, telangiectasias or synovitis were not present. She had dilated and distorted nail fold capillaries. There were no symptoms of gastro-oesophageal reflux. Echocardiography, lung function tests and a high resolution computed tomography of the chest were normal, but anticentromere autoantibodies were positive (titre 1:5210). Treatment with IFN-beta was not discontinued. The skin status remained stable and follow-up echocardiography and lung function test were normal after 2 years.

Patient 2

A 66-year-old woman had developed relapsing remitting MS at 21 years of age. IFN-beta treatment was commenced at 58 years of age and had led to a complete stabilisation of the disease. Eight years after the initiation of IFN-b, she noted Raynaud's phenomenon, arthralgia, myalgia and sclerodactyli. She had not

developed digital ulcers, telangiectasias, dysphagia or sicca symptoms. A nail-fold capillaroscopy and a computerised tomography of the chest were normal. Her antinuclear antibody (ANA) titre was 1:80, anti-Jo-1 and anti-SSA autoantibodies were positive. After 5 months without cessation of IFN, her skin status remained stable. At this time, a gastric carcinoma was diagnosed.

Patient 3

A 59-year-old man was known for relapsingremitting MS since the age of 51 years. Since then he had received IFN-beta although this treatment could not fully prevent recurrent episodes of MS progression. Since the age of 58, Raynaud's phenomenon, sclerodactily and digital ulcers were observed. He had no dysphagia. Echocardiography, high resolution computed tomography and lung function testing did not reveal evidence of cardiac or pulmonary involvement. His nail-fold capillaroscopy was normal but anticentromere autoantibodies were positive (titre 1:1280).

We have described three patients with relapsing remitting MS who developed a sclerosing skin disorder 7–8 years after the initiation of IFN-beta treatment. Two patients were diagnosed with limited cutaneous scleroderma (lcSSc) and the third patient had skin sclerosis in association with antisynthetase autoantibodies

In the literature, we found nine further cases of MS in association with SSc (table 1). Six cases had lcSSc, and three had diffuse cutaneous SSc (dcSSc). Localised scleroderma such as morphoea or ''en coup de sabre'' has not been described. Eight individuals had MS prior to the onset of SSc; in only one individual MS developed after the onset of SSc. One patient, who had had Raynaud's phenomenon for 11 years, developed SSc 1 year after receiving IFN for MS. All but one patient had elevated ANA titres. The onset of skin sclerosis was dissociated from MS activity in most of the patients. In all 12 patients, the mean age of MS diagnosis was 25.2 years (range 12–51). The mean age at the onset of skin sclerosis was 38.3 years (range 16–66). Skin sclerosis was therefore preceded by the MS by a mean of 14.9 years (range

1–45). Five of the 11 patients who developed skin sclerosis after the onset of MS had received IFN-b. After the initiation of IFN treatment, skin sclerosis developed after a mean of 4.6 years (range 1–8). Interestingly, lung fibrosis did not progress and

Raynaud’s phenomenon improved after cessation of IFN treatment in one MS patient.3 The literature also reports three patients who developed skin sclerosis while receiving IFN-a, another type 1 IFN (table 1). Patients had either simultaneous chronic hepatitis C virus infection or myeloproliferative disease and had developed skin sclerosis after a mean of 1.2 years (range 0.5–1.7) of IFN-alpha treatment. All patients were female and all had high-titre ANAs. Two patients had SSc, one patient had a PM-Scl positive collagenosis.

	Age at onset of first disease (years)	Gender	Time of IFN treatment prior to second disease (years)	Age at onset of second disease (years)	Clinical features of SSc	Nail-fold capillaroscopy	ANA titre	ANA specificity
SSc in the course of MS								
Patient 1	12	Female	6 (IFN-beta)	53	sclerodactily, RP	Positive	1:5210	Centromere
Patient 2	21	Female	8 (IFN-beta)	66	RP, limited scleroderma, puffy hands	Negative	1:80	SSA, Jo1
Patient 3	51	Male	7 (IFN-beta)	58	sclerodactily, digital ulcers		1:1280	Centromere
Pelidou et al 2007	26	Female	1 (IFN-beta)	34	RP, limited scleroderma, digital ulcers, lung fibrosis, oesophageal dysmotility, stabilisation after IFN cessation	ND	1:5120	
Spadaro et al 1999	20	Female	1 (IFN-beta)	27	diffuse scleroderma, digital ulcers	Positive	Positive	Scl70, U1RNP
Igarashi et al 1989	27	Female	No IFN	39	RP, diffuse scleroderma, lung fibrosis, oesophageal dysmotility	ND	Positive	Scl70
Gorodkin et al 2004	37	Female	No IFN	47	RP, limited scleroderma, digital ulcers, calcinosis, oesophageal dysmotility		Positive	Negative
Gorodkin et al 2004	28	Female	No IFN	ND	RP, sclerodactily, oesophageal dysmotility		ND	Centromere
Jawad et al 1997	22	Female	No IFN	43	RP, limited scleroderma, oesophageal dysmotility	ND	1:320	
Trostle et al 1986	15	Female	No IFN	16	RP, limited scleroderma digital pitting scars, telangiectasias, calcinosis, lung fibrosis	ND	1:320	
Trostle et al 1986	16	Female	No IFN	19	RP, diffuse scleroderma ND 1:40 –			
MS in the course of SSc								
Chroni et al 2002	20	Female	No IFN	22	Limited scleroderma (face and hands)	ND	1:640	Scl70
SSc in the course of IFN treatment for chronic HCV infection								
Tahara et al 2007	50	Female	1.5 (IFN-alpha)	68	limited scleroderma (fingers, forearms)	Negative	1:1280	
Solans et al 2004	47	Female	0.5 (IFN-alpha)	47	sclerodactily, telangiectasias, calcinosis, lung fibrosis, improvement after IFN discontinuation	Positive	1:1280	PM-Scl
SSC in the course of IFN treatment for chronic myelogenous leukaemia								
Beretta et al 2004	52	Female	1.7 (IFN-alpha)	54	Limited scleroderma (fingers, wrists), lung fibrosis, alveolitis, PAH, oesophageal dysmotility	Positive	Positive	Scl70

Table 1. The three patients in our case series and all nine found in literature are listed. Patients who developed skin sclerosis after receiving IFN-alpha treatment for HCV infection and myeloproliferative disease are also listed. ANA, antinuclear antibody; HCV, hepatitis C virus; IFN, interferon; MS, multiple sclerosis; ND, not done/not described; PAH, pulmonary arterial hypertension; RP, Raynaud's phenomenon; SSc, systemic sclerosis.

5.3 DISCUSSION

The cluster of the three cases described here and a further nine patients found in the literature prompted us to investigate a possible relation between MS and SSc. The presence of these two autoimmune diseases in the same individual raises the question whether or not this association reflects pure coincidence, an underlying predisposition to develop both autoimmune diseases, or the stimulation of skin sclerosis by IFN. A gadolinium triggered dermopathy could be ruled out based on a

normal renal function and the presence of Raynaud's phenomenon in these patients.

Although there is no clear proof of other autoimmune complications in patients with MS themselves, an increased risk for autoimmune disease was demonstrated in first-degree relatives of patients with MS.[4] Conversely, approximately one-third of SSc

patients were found to have at least one additional autoimmune disease.[5] The observation that the onset of skin sclerosis in patients with MS was 9 years before the mean age reported in the large European Scleroderma Trial and Research cohort (EUSTAR)[6] may also be interpreted as an underlying genetic predisposition. To this end, it is noteworthy that an otherwise rare allele in the CD45 gene has been found in relatively high frequency in both, patients with MS and SSc.[7] This CD45 allele may enhance T cell receptor signalling and may thus represent a genetic risk factor for both autoimmune diseases.[7] Elevated ANA titres are found in up to 45% of patients with MS. Although ANA titres usually remain low in patients with MS, they are known to be increased by IFN-beta exposure.[8] Large observational studies have described the induction of new autoantibodies in up to 61%, and autoimmune complications in 4–19% of IFN-treated individuals.[2] The dynamics of de novo autoantibody induction in patients with MS by IFN appears to be delayed, however, as ANA were usually not observed during the first 6 months of IFN treatment. In most of the patients

with MS summarised here, it is unknown if ANA were present before IFN was commenced. It is, however, well known that autoimmune thyroiditis, a frequent complication of IFN treatment, develops preferentially in subjects harbouring preexisting antithyroid autoantibodies.[2] Among the CTDs, de novo

manifestations of systemic lupus erythematosus have been observed under IFN-a and IFN-beta treatment,[9] the duration of IFN exposure ranging from 1 month to 7 years. Conversely, systemic lupus erythematosus was observed to resolve in some cases in which IFN-beta was discontinued.[10] A placebo-controlled randomised study has examined IFN-a as a possible treatment for SSc.[11] In this trial, IFN actually worsened some SSc manifestations and even increased SSc mortality,[11] supporting the possibility that IFN contributes to skin sclerosis. In MS, autoreactive T cells are thought to play an important pathogenic role. In SSc, activated fibroblasts and endothelial cells have mainly been implicated in the vascular damage and fibrosis,
but an antigen-driven T helper 2 (Th2) cell activation with consequent enhancement of interleukin 4 expression was also identified in the aetiology of active SSc.[12] A similar Th2 cytokine pattern also characterises patients with MS after the initiation of
IFN-beta treatment.[13] Because Th2 cytokines were shown to activate fibroblasts, it is conceivable that the IFN-mediated Th2 shift contributes to the development of skin sclerosis.[14] The possible role of IFN as a trigger of skin sclerosis is further supported by cases in which fibrotic complications resolved or stabilised after IFN cessation.[15] On the other hand, only five patients with MS had received IFN before the appearance of SSc. We thus conclude that IFN may be a possible, albeit not necessary cofactor for the development of SSc in patients with MS. The decision as to whether IFN treatment should be discontinued in such cases can only be interdisciplinary and should be individualised on basis of the activity of both diseases,
the available therapeutic options, and the patient’s personal priorities.

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CHAPTER 6

Late Onset Systemic Sclerosis - A systematic survey of the EULAR Scleroderma Trials and Research (EUSTAR) group database

Thomas Hügle[1], Philipp Schuetz[2], Thomas Daikeler[3], Alan Tyndall[3], Marco Matucci-Cerinic[4], Ulrich A. Walker[3], Jacob M. van Laar[1], and EUSTAR coauthors

[1] Musculoskeletal Research Group, Newcastle University, United Kingdom
[2] Harvard School of Public Health, Boston, U.S.A.
[3] Department of Rheumatology,Basel University, Switzerland
[4] Department of Biomedicine, Div Rheumatology AOUC, Denothe Centre, University of Florence, Florence, Italy

Published in Rheumatology 2011;50(1):161-5

ABSTRACT

Objective. The clinical course of systemic sclerosis (SSc) depends on subtype, organ involvement and age. Few data are reported on patients suffering from late onset SSc.

Methods. We analyzed data from 8,554 patients prospectively followed in the EULAR Scleroderma Trials and Research (EUSTAR) group database. Late onset SSc was defined as onset of non-Raynaud's disease features at or beyond 75 years of age. Disease characteristics, clinical features, disease course and mortality were evaluated.

Results. A total of 123 patients with SSc onset at or beyond 75 years of age were identified. Compared to patients <75 years they had more frequently limited than diffuse SSc and a higher prevalence of anti-centromere autoantibodies. Fewer old patients had digital ulcers. The modified Rodnan´s skin score, the prevalence of lung fibrosis and renal crisis did not differ significantly between groups. Pulmonary hypertension (PH) measured by echocardiography was more prevalent in the late onset group, as well as arterial hypertension and diastolic dysfunction. Late onset SSc remained a positive predictor for PH in multivariate analyses. No significant difference of the two groups in skin score or diffusion capacity was observed during follow up. Mortality due to SSc was higher in the late onset group, but the survival time from diagnosis was longer compared to the younger patients.

Conclusion. Late onset SSc shows a distinct clinical presentation and outcome. Patients with late onset SSc suffer more frequently from the limited subtype and PH but fewer patients have digital ulcers. PH may in part be determined by underlying cardiovascular disease.

6.1 INTRODUCTION

Systemic Sclerosis (SSc) is a heterogeneous connective tissue disease. Whereas vasculopathy in form of digital ulcers or pulmonary hypertension (PH) is the leading clinical feature in the limited form, diffuse SSc is associated with progressive fibrosis of skin and inner organs. The clinical presentation also depends on age. Patients with juvenile onset SSc have less skin involvement and lower mortality but suffer more frequently from overlap syndromes, notably with skeletal muscle involvement (116). In contrast, patients with onset of Raynaud's phenomenon above the mean age suffer more frequently from digital ulcers, lung fibrosis, PH and diastolic heart failure (117). The mean age at onset of first non-Raynaud's phenomenon in the EULAR Scleroderma Trials and Research (EUSTAR) database was reported 44.8 years for diffuse SSc and 47.9 years for limited SSc (117). In a North American SSc cohort study, white patients had an older age at diagnosis (55.5 years) compared to black patients (43.8 years) (118). Among white patients, the peak incidence was between 65 and 74 years in women and >75 years in white men (118). The incidence of SSc >75 years is around 20 cases per million per year which is 2-4% of all SSc cases (118).

Data indicate that a late age at onset of SSc is associated with more aggressive disease (119). In fact, the risk of death increases by 5% for each 1-year increase in age at diagnosis (118). The number of patients >75 years at diagnosis in previous studies however is low and naturally occurring co-morbidity in the elderly certainly influences survival analysis. Only small SSc cohorts or case series have focused on late onset SSc. Whereas some cases presented with a more severe disease course compared to patients <60 years, a more benign course, especially concerning skin involvement, has been reported by others (120-122). Despite a more severe lung involvement and a delayed diagnosis in the late onset group, the disease remained stable in patients >75 years (122).

In this study, we describe disease characteristics, progression and mortality of late onset SSc in a larger patient cohort by analyzing the large collection of the EUSTAR database.

6.2 PATIENTS AND METHODS

Data sample

We reviewed the minimal essential dataset of the EUSTAR database. The recruitment, structure and content of the database have been described previously (117). For SSc classification, ACR criteria and clinical subsets were defined according to LeRoy et al. (97). All participating centres have obtained ethics committee approval and enter data for all consecutively consenting patients.

Definitions

Late onset SSc was defined as age ≥75 years at onset of first non-Raynaud's phenomenon. This age cut off has been chosen as representative for geriatric patients and in accordance with previous studies (122). First onset of non-Raynaud's phenomena was chosen to assess specifically the first occurrence of manifest organ involvement.

Data analysis

Patients were dichotomised according to age (< or ≥75 years) and data analysed cross-sectionally at study entry. We analysed follow up and survival data in all patients with ≥1 follow up visits. Both ACR criteria positive and negative patients were included in the main dataset in order to keep atypical or early SSc cases in the analysis. As a sensitivity analysis, we repeated our analysis limited to the subset patients with positive ACR criteria only.

Dyspnoea was defined as NYHA grade 3 or 4, arterial hypertension as blood pressure measured >140 mmHg systolic or >90 mmHg diastolic. Pulmonary fibrosis was diagnosed by computer tomography. Lung restriction was defined as vital capacity <80%. PH (defined as systolic pulmonary artery pressure >40 mmHg), diastolic dysfunction and reduced left ventricular ejection fraction (LVEF) were diagnosed according to echocardiographic results reported by the cardiologist. Patients without tricuspid regurgitation were considered as not having PH. For disease course analysis endpoints were defined as relative DLCO decline of ≥25% from baseline or mRSS decline of ≥3 points, which corresponded to 25% of the median baseline mRSS in the cohort.

Statistical analysis

Discrete variables are expressed as counts and continuous variables as medians and interquartile ranges (IQR). Two-group comparison was performed with Wilcoxon-Mann-Whitney tests and a Kruskal-Wallis one-way analysis of variance was used for multiple-group comparisons. Associations of late onset SSc and other factors with PH were calculated in logistic regression models. We used unadjusted models and multivariate models adjusted for confounders and report odds ratios (OR). Effect modification of late onset SSc for the association of diastolic dysfunction and PH was tested by including an interaction term into the multivariate regression model. Because the frequency of missing data was low (<2-5% per variable and <10% for the multivariate model), we only present a complete case analysis and did not perform imputation of missings. All testing was two-tailed and p values less than 0.05 were considered significant. All calculations were performed using STATA 11.0 (Stata Corp, College Station, Texas).

6.3 RESULTS

Basic description

At the time of data censoring (1st of June 2009), 8,554 patients were included in the EUSTAR database. 123 patients with a late onset of non-Raynaud's phenomenon at an age of ≥75 were identified. Of these, 74.3% fulfilled the ACR criteria for SSc whereas ACR criteria were fulfilled in 84.4% of the patients <75 years (p=0.002) (table 1). The median disease duration both of Raynaud's (4.4 (IQR 2-14) vs. 10.1 (IQR 4-20) months; p=<*0.01*) and non-Raynaud's phenomena (6.8 (IQR 2.8-14.3) vs. 2.1 (IQR 0.8-3.0) months; p=<*0.01)* at diagnosis was lower in the late onset group. The median disease duration of non-Raynaud's phenomena symptoms at study inclusion was also lower in the late onset group (30 (IQR 15-48) vs. 75 (IQR 36-144) months; p=0.01).

Significantly more patients in the ≥75 years group had limited SSc (74.1% vs. 58.2%: p=0.001) whereas 17.8% vs. 32.5% suffered from diffuse disease

(p=0.001). The late onset group had a higher proportion of positive anti-centromere-autoantibodies (54.2% vs. 33.4%; p<0.001). Anti-Scl70 auto-antibodies were more prevalent in the younger group 32.3% vs. 23.0% (p=0.034). ANA positivity was similar in both groups (94.2 vs. 92.1%; p=0.4). Significant differences for the same covariates were also found in the subsets of ACR criteria positive patients.

	Age <75	**Age ≥75**	***p value***
Total number of patients	8431	123	
Demographic characteristics			
Average age at inclusion, median (IQR)	55 (45.6-64.8)	79.6 (78.2-81.7)	*<0.0001***
Female Sex (%)	86.4	89.3	*0.36*
Clinical characteristics			
Clinical subtype			
- limited (%)	58.2	74.1	*0.001***
- diffuse (%)	32.5	17.8	*0.001***
- other (%)	9.1	8	*0.67*
Disease History			
Age at onset of Raynaud's phenomenon (years)	41 (28-52)	75 (64-78)	*<0.01*
Age at onset of non-Raynaud's phenomenon (years)	45 (33-56)	78 (76-80)	*<0.01**
Disease duration of non-Raynaud's phenomenon at diagnosis (months)	6.8 (2.8-14.3)	2.1 (0.8-3.0)	*<0.01*
Duration of Raynaud's phenomenon at diagnosis (months)	10.1 (4.4-20.4)	4.4 (2.2-14.5)	*<0.01*
ARC criteria fulfilled	84.4	74.3	*0.002**
Autoantibody status			
- Anti-centromere (%)	34.4	54.2	*<0.001***
- Anti-Scl-70 (%)	32.3	23	*0.034**
- ANA (%)	92.1	94.2	*0.4*

Table 1. Demographic features at inclusion according to SSc onset by age at first non-Raynauds disease feature.

Clinical features

The median mRSS was similar in both age strata (7 vs. 7; p=0.57) (table 2). The late onset group had significantly less digital ulcers (22.1% vs. 30.1%; p=0.03). Patients with late onset SSc had higher rates of arterial hypertension (40.6% vs. 20.2%; p<0.001), abnormal diastolic function (29% vs. 16.1%; p<0.001) and conduction blocks (9.7% vs. 21.8%; p<0.001). PH was more prevalent in the late onset group (35% vs. 20%; p<0.001) whereas there was no difference in median DLCO in both groups (53% vs. 45%; p=0.3); the late onset group did not suffer more frequently from dyspnoea (40.9% vs. 34.3%; p=0.13). Similar results and statistical significant differences were also found in the subset of ACR criteria positive patients, except for CK elevation, which was similar in both groups.

To investigate whether late onset SSc was an independent risk factor for PH we calculated logistic regression analysis (supplementary data). Late onset SSc was a significant predictor for PH with an unadjusted Odds ratio of 2.1 (95%CI 1.5-3.1). In addition, we found reduced LVEF (OR 4.78 (95%CI 3.90-5.86), abnormal diastolic function (OR 3.4 (95%CI 3.0-3.8), lung fibrosis (OR 2.7 (95%CI 2.5-3.1), conduction block (OR 2.64 (95%CI 2.2-3.0) and arterial hypertension (OR 1.82 (95%CI 1.55-2.14) to be significant univariate predictors for PH. All those factors remained independent predictors for PH in multivariate logistic regression analysis. We found no evidence for effect modification of late onset SSc (p of interaction term = 0.2), indicating that diastolic dysfunction is a risk factor for PH in all age groups.

Parameter	**Age <75**	**Age ≥75**	***P value***
MRSS	7 (3-14)	7 (3-12)	*0.57*
Synovitis (%)	15.8	15.5	*0.94*
Joint contracture (%)	30.1	26.4	*0.38*
Digital ulcer (%)	31.3	22.1	*0.03**
Tendon friction rubs (%)	10.5	10	*0.84*
Muscle weakness (%)	25.7	21.4	*0.28*
Muscle atrophy (%)	12.8	10.6	*0.46*
Proteinuria (%)	5.8	6.7	*0.68*
Raynaud’s phenomenon (%)	95.2	92.6	*0.19*

Conduction block (%)	9.7	21.8	*<0.001***
Diastolic function abnormal (%)	16.1	29.6	*<0.001***
Diastolic failure (%)	5.8	6.7	*0.68*
Pulmonary hypertension (echocardiographic) (%)	20	35	*<0.001***
Lung restrictive defect (%)	30.7	29.8	*0.83*
Lung fibrosis (%)	36.4	30.1	*0.16*
Esophageal symptoms (%)	67.0	59.8	*0.09*
Intestinal symptoms (%)	23.2	26.2	*0.77*
Renal crisis (%)	2.2	0.82	*0.29*
Dyspnoea (%)	34.3	40.9	*0.127*
Palpitation (%)	23.8	21.3	*0.50*
CK elevation (%)	8.2	3.3	*0.05**
Arterial hypertension (%)	20.2	40.6	*<0.001***
Elevated acute phase reaction (%)	29.9	44.5	*0.001***
DLCO (% of normal)	53(-76)	45 (-73)	*0.30*
Reduced left ventricular function (%)	5.2	6.3	*0.6*

Table 2. Prevalence of clinical features in patients aged ≥75 vs. <75 years. Data are presented as median (interquartile range).

Disease course

All patients with ≥1 follow ups were analyzed with respect to the course of DLCO and mRSS. Severe and moderate impairment of DLCO (defined as a DLCO < 50% and <65%) was found in 20% and 22% of late onset patients, compared to 20% and 27% of control patients. During follow up, 17% of late onset and 11% of control patients had a decline of at least 25% from baseline DLCO (p=0.38). Late onset patients had lower median mRSS at study inclusion (4 (IQR 3-7) vs 7 (IQR 4-13), p=0.002), which was also confirmed in multivariate analysis adjusted for disease duration (p=0.02). The decline of 3 or more points of mRSS was also similar in both groups (28% in late onset patients vs. 34% of control patients (p= 0.4).

Overall and disease-specific Mortality

The overall mortality in the 4,081 patients with available follow up information was 6.9% (22% in the late onset group and 6.7% in the early onset group,

p<0.001). Overall, 178 deaths (63%) were attributed to SSc. The SSc-specific mortality rate was 12.2% in late onset patients with a median survival time of 49 months (IQR 22-92) and significantly lower in early onset patients (4.3%, p=0.01), but with a shorter median survival of 41 months (IQR 22-73).

6.4 DISCUSSION

This study aimed to characterize clinical features, disease progression and mortality in late onset SSc. Unlike reported in previous studies, SSc in the late onset group was diagnosed earlier then in the control group which might be due to a higher frequency of medical consultation or less extensive and invasive diagnostic investigation in this subgroup (122). Patients with a late onset suffered more frequently from limited SSc and had significantly higher rates of anti-centromere autoantibodies. Despite the higher prevalence of limited disease, they had less digital ulcers. This suggests a milder disease course in late onset SSc patients but might be biased by a shift of patients with mild disease into the late onset group and the shorter disease duration at study inclusion, respectively. Conversely, PH was more prevalent in late onset SSc. However, diastolic dysfunction, arterial hypertension and conduction blocks which were also more prevalent in the late onset group, can lead to left ventricular hypertrophy and thus result non-specifically in PH (123). Unfortunately, right heart catheterization was performed in none of the late onset patients. The dataset in the present form did not allow us to determine whether the higher frequency of diastolic dysfunction and conduction blocks in the late onset group was caused by left ventricle hypertrophy or cardiac fibrosis due to SSc. Also, false negative results from echocardiography due to the absence of detectable tricuspid regurgitation jet cannot be excluded, although there are no data to suggest that this would affect elderly SSc patients more than younger patients. The course of both interstitial lung disease and the skin involvement remained stable in the majority of both groups. This is in line with previous reports of a stable course of signs in elderly SSc, but low mRSS values especially found in limited SSc are

relatively unsensitive and therefore only of limited value as a marker for disease progression (122).

ACR criteria were fulfilled in fewer patients in the late onset group. This might indicate that, apart from an earlier study inclusion, older SSc patients suffer from a more atypical disease course, possibly influenced by co-medication or co-morbidities such as atherosclerosis. Studies in patients with late onset systemic lupus erythematosus (SLE) have shown similar results where they had more frequently Raynaud's phenomona, arterial hypertension, organ damage and a higher mortality (124). Conversely, and in accordance with the present findings, the clinical course of late onset SLE was described as less aggressive (124). It is also noteworthy that Raynaud's phenomenon in the elderly has different characteristics and pathogenic mechanisms compared to younger individuals (125, 126).

As expected, more patients died in the older group. Taken into account the international nature of the EUSTAR database, mortality could not be compared with an available age-matched control group. Unexpectedly, in patients whose death was attributed to SSc, the median survival time was longer in the older patients, furthermore suggesting a more protracted course of SSc in the elderly.

Limitations of this study include missing information concerning treatment modalities and co-medication, and a possible bias related to differences in diagnostic work-up and treatment of younger compared to older patients. Inter-individual and centre bias might also influence the results.

Taken together, limited disease and PH measured by echocardiography are more prevalent in the elderly, but these patients suffer less from digital ulcers. SSc unrelated left heart hypertrophy has to be considered especially in late onset SSc before the diagnosis of PH can effectively be made and treatment started, respectively.

6.5 KEY MESSAGES

In late onset SSc, the limited subtype and anti-centromere autoantibodies are more prevalent but patients suffer less from digital ulcers.

PH is partly influenced by underlying cardiovascular co-morbidity such as arterial hypertension or diastolic dysfunction.

Although the mortality is higher in late onset SSc, the time from diagnosis to death is longer compared to the control group.

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CHAPTER 7

CONCLUSION

SSc is a complex multisystem disease. It is characterized by the interplay between inflammatory and autoimmune processes and influenced by intrinsic factors such as genetic predisposition, gender, age as well as extrinsic factors like environmental triggers, drugs or infection.

This piece of work contributes to the understanding of the orchestration of inflammation in SSc and the transition from inflammation to fibrosis in several aspects.

Chapter 1 describes multicolour flow cytometry of fresh skin biopsies of SSc patients. This technique has been applied in SSc for the first time. Flow cytometry of the skin permitted the analysis of significantly higher number of dermal cells and a thorough description of their phenotype, compared to immunohistochemistry.

Lymphocyte infiltration correlated with skin thickening in a small cohort of 13 SSc patients, the highest lymphocyte numbers occurring in patients with severe diffuse SSc. In contrast, higher numbers of macrophages were found in patients with limited disease. This is in line with previous findings of immunohistochemistry studies and confirms the hypothesis that diffuse and limited SSc have a distinct pathogenesis. Patients with limited SSc typically have clinical signs of vasculopathy such as digital ulcers, Raynaud's phenomenon and pulmonary hypertension and show signs of enhanced atherosclerosis. The observation of macrophage infiltration here is similar to macrophage invasion in atherosclerosis, notably in atherosclerotic plaques.

How do lymphocytes then trigger fibrosis? The hypothesis of this study was that lymphocytes in SSc dermis 1. secrete profibrotic cytokines and 2. trigger differentiation from fibroblasts to myofibroblasts. We expected a Th2 weighted immune response, possibly also increased amounts of T-regulatory cells, as the latter are, despite impaired function, increased in peripheral blood.

We found that TNF-R are overexpressed in the dermis and blood of SSc patients. Whereas TNF-R1 was upregulated on the cell surface, the induction of TNF-R2 expression was observed in most diffuse SSc patients. Other studies showed TNF-R2 expression on T-regulatory cells. We therefore postulate that dermal lymphocytes at least try to exhibit a regulatory function, probably as an attempt to resolute ongoing inflammation.

Functional analysis using TNF-R1 and –R2 selective TNF mutants showed a distinct cytokine expression pattern in lymphocytes compared to healthy controls: upon TNF-R stimulation. Both important profibrotic cytokines and growth factor IL-6 and TNF-beta were higher expressed on SSc lymphcytes. TNF-R1 seems to trigger IL-6 expression and TNF-R2 primarily stimulates TGF-beta expression. The exact subcellular signaling events are elusive but it is likely that NFkB is involved as transcription factor. Interestingly, naturally occurring IL-10 production after antigen-specific stimulation (here by CD3/28 beads) after TNF-R2 stimulaton was *impaired* in SSc lymphocytes. This implicates that on the one side we observe a profibrotic state of SSc lymphocytes (by increased expression of IL-6 and TGF-beta) and on the other side a reduced anti-inflammatory capacity of SSc lymphocytes.

As a proof of concept, we show in a SSc patient that after autologous HSCT, CD3 cell infiltration decreased clearly and so did their TNF-R expression and as well IL-6 expression. Myofibroblast infiltration was also decreased after the intervention, supporting our hypothesis.

This is in line with 1. animal models of SSc where TNF-blockade inhibits the development of SSc, and 2. clinical findings in humans where a trend towards a decreasing skin thickening and reduced dermal collagen production was observed after treatment with infliximab. In the latter trial, however, no significance of anti-TNF treatment could be observed. Why did this happen? We postulate that anti-TNFalpha treatment was performed too late as the patients suffered from SSc for several years in average. Possibly, an earlier treatment would have been more efficient. Secondly, TNF-R2 is mainly stimulated via membrane bound TNFalpha e.g. on infiltrating macrophages or resident mast cells. Anti-TNFalpha compounds might not have been able to diffuse into the fibrotic skin and might not have sufficiently inhibited membrane bound TNFalpha. Third, we think that due to different pathogenic aspects, not

all SSc patients necessarily benefit from TNFalpha blockers. Our work here displays the fact that not all 13 patients had an upregulation of TNF-R and the grade of inflammation is variable in each individual. Maybe only patients with a high TNF-R status benefit from this treatment. As currently discussed in other diseases, a more personalized therapeutic approach will be required also for SSc patients.
Taken together, both animal models and clinical oberservations in humans support the profibrotic role of TNFalpha-costimulated lymphocytes.
The conclusion of these data is the following: drug delivering of TNFalpha-inhibitors into fibrotic tissue should be improved, e.g. by a small molecule specifically inhibiting TNF-R2. Also, compounds in direction of supporting the resolution of inflammation (such as IL-10) should be developed and the exact impairment in the TNF-R2 downstream signaling should be further studied.

In chapter two, it is demonstrated that monocytes as members of the innate immune system also contribute to fibrosis by their increased expression of functionally active TIMP-1 and thus have an active role in the ECM turn over. We found out that factors in the serum trigger TIMP-1 expression, yet it is unclear which factors exactly are responsible for this observation.
These findings are in line with the previously described activation of monocytes in blood and tissue where monocytes were shown to bear a type-1 interferon fingerprint (e.g. shown by a Siclec-1 expression). So we postulate that a type-1 interferon stimulus (e.g. in form of a viral infection) could trigger TIMP-1 expression.
Further experiments would include the stimulation and subsequent inhibition of monocytes with interferon alpha or beta, toll like receptors and cytokines such as TNF-alpha, IL-6, IL-10 or IL-13. It would also be important to demonstrate TIMP-1 expression by monocytes or macrophages in the tissue e.g. by immunohistochemistry.

Chapter three shows that mast cell degranulation is more prevalent in dermis of SSc patients and mast cells in fact are the most important source of active TGF-beta in the dermis in SSc. One crucial question remains what factor causes mast cell degranulation in SSc? Again, a possible answer could be the

clinical observation of patients who underwent type-1 interferon treatment and subsequently developed SSc. Type-1 interferons can indeed provoke mast cell degranulation. As a next experiment, healthy mast cells of a cell line could be incubated with sera from SSc patients and degranulation could be measured e.g. by measurement of tryptase. Then, factors in the sera can further be identified. The dynamic of mast cell degranulation is also of interest. Apparently the degranulation has a continuous low-mid level dynamic, also called (piece meal degranulation) rather than a full blown degranulation as seen in anaphylaxy. The reason for this is not clear, apart from an ongoing stimulus, there could be an impairment of the resolution of degranulation or a structural defect of mast cell vesicles, respectively. Mast cells can also degranulate via antigen stimulation, toll like receptors or Fc receptors. Therefore, their expression on the cell surface as well as downstream signaling of these receptors should be studied in detail in order to demonstrate a ‘mast cell autoimmunity’ in SSc patients.

Chapter four is a clinical study following the observation of SSc cases after type-1 IFN treatment for multiple sclerosis or other diseases. Despite the low patient number this analysis is supported by several investigations where a type-1 fingerprint of monocytes has been found. It is demonstrated in several cases that SSc occurred with a delay typically of 6-12 months after IFN treatment. This implicates that SSc 1. could be a consequence of a viral infection (which is associated with high levels of type-1 IFN) and 2. this IFN trigger might not occur close to the SSc onset. This hypothesis is in line with autoantibodies which are sometimes found years before the onset of SSc.

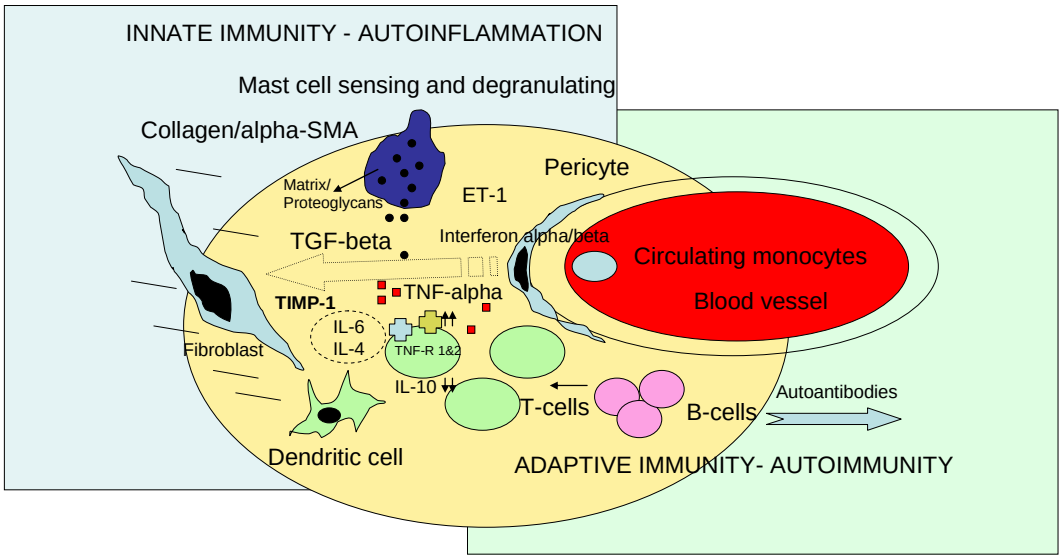
Chapter five: Age is an important factor for SSc and autoimmune diseases in general. The reason is ‘immunosenesence’, the fact that our immune system alters during our life span. Despite controversial data, the dogma has been that in older patients SSc has a worse course. In this project I used data from the world largest SSc database EUSTAR to analyze clinical features and outcome of SSc patients ≥75 years. In contrast to the above mentioned dogma, patients with a late onset of SSc in fact had relatively stable course of SSc. Older individuals at diagnosis of SSc live longer compared to younger

individuals. As a conclusion, older individuals that are often more fragile with more comorbidity and polymedication might need a less intense or distinct treatment strategy.

This was a purely clinical study project, but opens the door for further laboratory based research questions: e.g. in which functional status is the innate or adaptive immune system in older SSc patients versus younger or age matched healthy individuals?

Taken together, I conclude that new treatment strategies should build on knowledge of in the orchestration of inflammation in SSc as well as SSc subtypes, time course and age of the patients.

Disease Model



Schematic illustration of the context between the data gained in this thesis. Mast cells as 'connective tissue sensors' are activated in SSc and degranulate e.g. as response to an exogenous or endogenous type-1 interferon stimulation. Mast cell granula contain TGF-beta and various other cytokines, growth factors and tryptase. This leads to the attraction of inflammatory cells, vasodilatation and probably also fibrosis via the ongoing secretion of TGF-beta. Monocytes are early infiltrating cells typically secreting proinflammatory cytokines. We show however that monocytes also secrete functionally active TIMP-1, therefore inhibiting MMP-mediated matrix degradation. Monocytes might also differentiate into mesenchymal cells such as myofibroblasts. Lymphocytes are activated in SSc skin, which demonstrated by their expression of TNF-R2. In first line, activated T-lymphocytes raise an adaptive immune response by stimulating B-cells to produce antibodies. Activated lymphocytes also secrete IL-10 to resolve the inflammation, an effect which is triggered via TNF-R2 stimulation. The failure of resolving inflammation in SSc leads to persisting expression of TGF-beta by mast cells, TIMP-1 by monocytes and IL-10 by lymphocytes. The 'net effect' of this cytokine imbalance results in fibrosis.

SMA: smooth muscle actin, TGF-beta: transforming growth factor, ET-1: endothelin-1, TIMP-1: tissue inhibitor of metalloproteinases, TNF: tumor necrosis factor. TNF-R: tumor necrosis factor receptor, MMP: metalloproteinase.