UNIVERSITY OF LIVERPOOL

Defining the Neutrophil Phenotype in Systemic Sclerosis.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Theresa Corrinne Barnes

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I declare that this thesis entitled:

"Defining the neutrophil phenotype in systemic sclerosis"

is entirely my own work.

Candidate: **THERESA CORRINNE BARNES**

Supervisors: **Prof R.J.Moots**

School of Clinical Sciences

University of Liverpool.

Prof S.W.Edwards

School of Biological Sciences

University of Liverpool.

Dr M.E.Anderson

School of Clinical Sciences

University of Liverpool.

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Abstract.

Background: Systemic sclerosis (SSc) is a disease characterised by a triad of immunological abnormalities, endothelial cell dysfunction and fibrosis. Neutrophils are the most abundant circulating leukocyte. They contain several mediators which when released can lead to modulation of the inflammatory response, cause endothelial cell activation and injury and eventually lead to fibrosis. The peri-endothelial cell environment in SSc has the potential to lead to neutrophil activation and indeed this has been previously described in the literature in terms of reactive oxygen species (ROS) generation. In this thesis I aim to explore the hypothesis that: *"Neutrophils are activated in SSc and contribute to endothelial cell activation and damage"*.

Methods: The functional phenotype of SSc neutrophils was explored *in vitro*. Functions investigated included; ROS generation, chemotaxis, integrin expression, apoptosis and CD16 expression. The protein expression of *ex vivo* SSc neutrophils was compared to healthy control neutrophils. The DIGE technology was used to explore the pan-proteome and iTRAQ was used to focus in on the plasma membrane proteome. The role of neutrophil elastase was explored in SSc by examining the serum neutrophil elastase concentration and activity and correlating these to clinical manifestations of disease. The role of neutrophil: endothelial cell interactions was modelled *in vitro* using live cell imaging by confocal microscopy looking for evidence of endothelial cell activation (E-selectin expression) and apoptosis. Experiments examined the role of neutrophil derived mediators in these interactions.

Results: Functional studies revealed that SSc neutrophils are hypofunctional in terms of spontaneous ROS generation and chemotaxis *in vitro*. This may reflect *in vivo* activation. SSc neutrophils are similar in terms of integrin expression and baseline apoptosis to control neutrophils. Pan-proteomic studies reveal neutrophil activation in SSc since changes in protein expression mirror those seen in response to neutrophil activators TNFα and LPS. Proteomic studies also point to neutrophil priming *in vivo*. Serum neutrophil elastase concentrations and activity were not elevated in SSc, however, discrepancies between concentration and activity suggest a functional deficiency in elastase inhibitors in SSc serum. Serum elastase activity and concentrations were found to be lower in RNP (ribonucleoprotein) positive patients indicating that different mechanisms maybe involved in different SSc subtypes. *In vitro* models demonstrate that SSc serum causes endothelial cell activation and apoptosis and that neutrophils are essential for this effect. Serine proteases seem to play an important role in inducing apoptosis and IL-6 trans signalling is involved in endothelial cell activation and apoptosis. Neutrophils do not express IL-6 but are dominant sources of the soluble IL-6R which is essential for trans signalling.

Conclusions: There is evidence that neutrophils are activated in SSc though; no specific activating signature is identified. In co-cultures, neutrophils are essential for endothelial cell activation and apoptosis in response to SSc serum. Neutrophil mediators including serine proteases and IL6R are likely to play important roles in this effect. Therefore neutrophils may play an important part in the propagation of inflammation and endothelial cell activation which eventually leads to the fibrotic phenotype which is characteristic of this disease.

Publications and presentations.

Publications:

Barnes TC, Spiller D, Anderson ME, Edwards SW, Moots RJ. Endothelial activation and apoptosis mediated by neutrophil-dependent interleukin-6 trans signalling: a novel target for systemic sclerosis? *Submitted to Ann Rheu Dis*.

Oral presentations:

A potential role for interleukin-6 in the pathogenesis of systemic sclerosis. BSR. Birmingham. April 2010.

Interleukin-6 in systemic sclerosis. UK Scleroderma. Leeds. March 2010.

Targeting interleukin-6: potential new therapeutic options in Systemic Sclerosis. North West Rheumatology Club. Nov 2009. Winner of Mike Chalk trophy.

Systemic sclerosis serum increases endothelial cell apoptosis and activation in neutrophil-endothelial cell co-cultures: A role for Interleukin-6. PRISM conference Manchester. Oct 2009. Winner of best presentation prize.

Invited speaker. Neutrophil proteomics in systemic sclerosis. Proteomics methods forum. Liverpool. Oct 2007.

Invited speaker. Innate immune system in systemic sclerosis: low grade inflammation. Pulmonary artery hypertension, scleroderma and vasculopathy meeting. Liverpool. July 2007.

Separation anxiety. UK Scleroderma. Manchester. Jan 2007.

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Poster presentations:

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Barnes T, Spiller D, Anderson M, Edwards S, Moots R. Systemic sclerosis serum increases endothelial cell apoptosis and activation in neutrophil-endothelial cell co-cultures: A role for interleukin-6. Clinical and Experimental Rheumatology 2010: 37.

Barnes T, Cross A, Naylor E, Anderson M, Edwards S, Moots R. Neutrophil cytoskeletal abnormalities in systemic sclerosis. Rheumatology 2008; 47(suppl 2): ii304.

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Abbreviations.

AEBSF 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride

AIF apoptosis inducing factor

ANA anti-nuclear antigen

ANOVA analysis of variance

APC allophycocyanin

ATP adenosine triphosphate

BAFF B-cell activating factor

BCA bicinchoninic acid

BSA bovine serum albumin

CCP cyclic citrullinated protein

CEPs circulating endothelial precursor cells

CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CRP C-reactive protein

CTGF connective tissue growth factor

DAMP damage associated molecular pattern

dcSSc diffuse cutaneous systemic sclerosis

DEPC diethylpyrocarbonate

DHR dihydrorhodamine

DIGE differential gel electrophoresis

DLCO diffusion lung capacity for carbon monoxide

DMARDs disease modifying anti-rheumatic drugs

DNA deoxyribonucleic acid

dNTP deoxyribonucleotide triphosphate

DTT dithiothreitol

ECL electrochemiluminescence

ECM extracellular matrix

EDTA ethylenediaminetetraacetic acid

EGTA ethylene glycol tetraacetic acid

ELISA Enzyme-linked immunosorbent assay

E-selectin endothelial selectin

ET endothelin

FACs fluorescence activated cell sorting

FGF fibroblast growth factor

FITC fluorescein isothiocyanate

fMLP f-Met-Leu-Phe

FVC forced vital capacity

GAPDH glyceraldehyde-3-phosphate dehydrogenase

GCSF granulocyte stimulating factor

GMCSF granulocyte macrophage colony-stimulating factor

HBSS Hank's buffered salt solution

HDMECs human dermal microvascular endothelial cells

HEL N^{ε}-(hexanoyl)lysine

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF hypoxia inducible factor

HLA human leukocyte antigen

HRP horseradish peroxidase

HUVECs human umbilical vein endothelial cells

IAA indoleacetic acid

ICAM intercellular adhesion molecule

IEF isoelectric focussing

IL Interleukin

IPG immobilised pH gradient

IQR interquartile range

iTRAQ isobaric tag for relative and absolute quantitation

JAM junction adhesion molecule

lcSSc limited cutaneous systemic sclerosis

LCP-1 lymphocyte cytosolic protein 1

LDH lactate dehydrogenase

LPS lipopolysaccharide

LTB_4 leukotriene B4

LTBP latent TGF binding protein

LTGF latent TGF

MCP monocyte chemotactic peptide

MDA malondialdehyde

MHC major histocompatibility complex

MMP matrix metalloproteinases

MMTS methyl methanethiosulfonate

MPO myeloperoxidase

mRNA messenger ribonucleic acid

MS multiple sclerosis

MWCO molecular weight cut off

NADPH reduced nicotinamide adenine dinucleotide phosphate

NPP nitrophenol phosphate

PAF platelet activating factor

PAGE poly acrylamide gel electrophoresis

PAMP pathogen associated molecular pattern

PAR proteinase activated receptor

PBMCs peripheral blood mononuclear cells

PBS phosphate buffered saline

PCR polymerase chain reaction

PDGF platelet derived growth factor

PE phycoerythrin

PECAM platelet endothelial cell adhesion molecule

PI propidium iodide

α_1PI α_1 proteinase inhibitor

PIPES piperazine-N,N'-bis (2-ethanesulfonic acid)

PMA phorbol myristate acetate

PMN polymorphonuclear

PMSF phenylmethanesulfonylfluoride

PTFE polytetrafluoroethylene

Q-TOF quadrupole time of flight

RA rheumatoid arthritis

RAGE receptor for advanced glycation end

RCDC reducing agent compatible, detergent compatible

RhF rheumatoid factor

RNP ribonucleoprotein

ROS reactive oxygen species

RP Raynaud's phenomenon

RPMI Roswell park memorial institute

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SLE systemic lupus erythematosus

SSc systemic sclerosis

TCEP tris(2-carboxyethyl)phosphine

TCR T-cell receptor

TEAB triethylammonium bicarbonate

TGFβ transforming growth factor β

TIMP tissue inhibitor of metalloproteinases

TLR toll like receptors

TNFα tumour necrosis factor α

TSK tight skin mouse

UPLC ultra-performance liquid chromatography

VCAM vascular cell adhesion molecule

VEGF vascular endothelial cell growth factor

vWF von Willebrand factor

αSMA α smooth muscle actin

Chapter 1: Introduction.

1.1 Background

SSc is a multisystem, connective tissue disease. It has an estimated prevalence of 0.1% and is more common in women than men with a male to female ratio of 1:4.

Scleroderma, the cardinal symptom of SSc, is a fibrotic thickening of the skin and can be found alone or in association with systemic involvement (Fig.1).

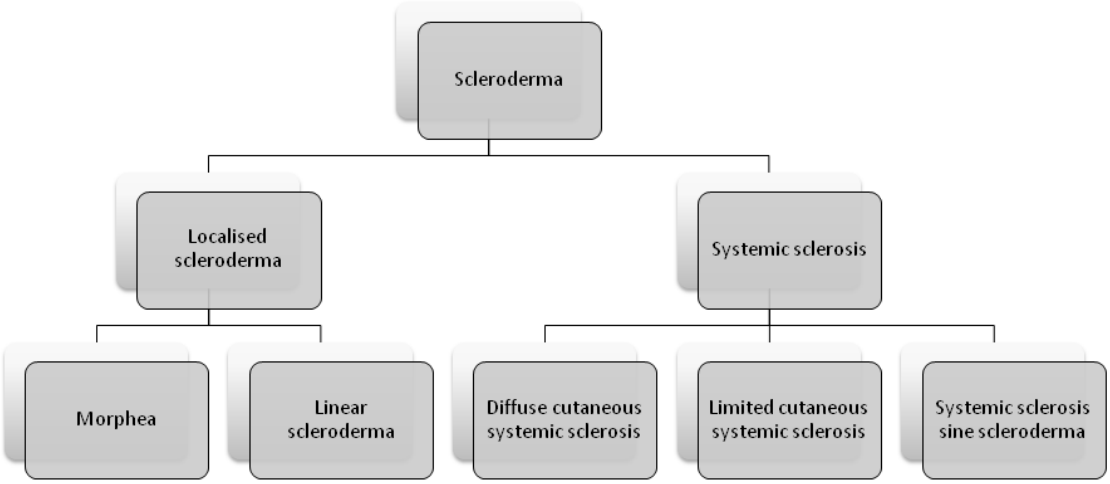


Fig. 1. Classification of scleroderma. Thickening of the skin may be localised or there may be systemic involvement. In systemic disease, skin thickening may be distal only (limited), involve proximal skin (diffuse) or may be absent (sine scleroderma).

The clinical features of SSc fall into 3 main categories: vascular dysfunction including Raynaud's phenomenon (RP), microvascular and macrovascular damage resulting in ischaemia and pulmonary hypertension, fibrosis involving the skin and internal organs, and immune abnormalities including typical autoantibodies and evidence of immune cell activation (Fig.2).

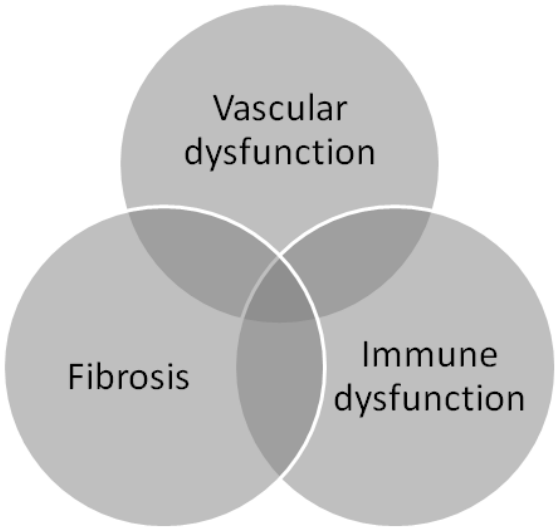


Fig.2. The central pathological components of systemic sclerosis

Diffuse cutaneous SSc (dcSSc) involves skin thickening both proximal and distal to the elbows, knees and neck. It is associated with anti-topoisomerase I antibodies, lung fibrosis and renal crisis. Limited cutaneous SSc (lcSSc) involves skin thickening distal to the elbow, knee and neck, it is associated with anti-centromere antibodies, late pulmonary artery hypertension, calcinosis, telangiectasia and prominent microvascular disease (Table 1).

Disease subtype	Clinical associations
lcSSc	Telangiectasia, calcinosis, late onset pulmonary hypertension.
dcSSc	Tendon friction rubs, early and significant organ involvement, renal crisis, interstitial lung disease, myocardial disease.

Table 1. Clinical features of limited and diffuse systemic sclerosis.

More recently the definition of SSc has been broadened to try to diagnose patients early in the disease, before fibrosis has become established. These criteria for diagnosis are outlined in Table 2.

Diagnosis	Criteria
Limited SSc (lSSc)	Objective RP + either abnormal NC or SSc specific autoantibodies†.
	Subjective RP + both abnormal NC and SSc specific autoantibodies†.
Limited cutaneous SSc (lcSSc)	As for lSSc + distal skin thickening.
Diffuse cutaneous SSc (dcSSc)	As for lSSC + proximal skin thickening.

† anti-centromere, anti-topoisomerase, anti- fibrillarin, anti-PM-scl, anti-fibrillin, anti-RNA polymerase I or III in a titre of ≥1:100. NC= nailfold capillaroscopy.

Table 2. Criteria for the classification of early systemic sclerosis [2].

Gastrointestinal symptoms are common, occurring in both disease subtypes and can include oesophageal dysmotility with reflux, small bowel bacterial overgrowth due to dysmotility resulting in diarrhoea and malabsorption, constipation due to large bowel dysmotility and faecal incontinence.

The pathogenesis of SSc remains unknown, but there is now a relatively consistent hypothesis regarding at least parts of the mechanism.

1.2 The role of the endothelial cell in SSc pathogenesis.

Historically, the first important clues to the pathogenesis of SSc came from histological studies of lesional skin from affected patients. Biopsies show typical changes in the microvasculature including: a fall in total blood vessel area, perivascular oedema, a mononuclear perivascular infiltrate. There are endothelial cell abnormalities culminating in denudation of the endothelial cell layer, basement membrane thickening and reduplication. There is also an increase in dermal collagen and extracellular matrix deposition [5].

The University of California at Davis (UCD) 200 chickens develop almost all of the features of scleroderma and have been used as an animal model for the disease. Serial tissue

biopsies from these animals have shown that endothelial cell apoptosis is the earliest change seen in the pathogenesis of the disease preceding the inflammatory cell infiltrate and the development of fibrosis [6 7]. This implies that induction of endothelial cell apoptosis is likely to be the initiating lesion in SSc.

Endothelial cell apoptosis has also been reported in SSc lesional skin [8], though the mechanism is again unclear, and *in vitro* models have shown that the sera of a proportion of SSc patients contain anti-endothelial cell antibodies which have been implicated in the induction of apoptosis [9 10]. However, for the rest of the patients the situation remains unclear. Reactive oxygen species (ROS) and proteases have been implicated *in vitro* but there is no convincing evidence for their *in vivo* involvement [11 12]. Some authors contend that there is no direct evidence for endothelial cell apoptosis *in vivo* [13].

In addition, there is evidence of endothelial cell activation. Endothelial cells produce more nitric oxide *ex vivo*, and also display an increase in the expression of adhesion molecules. Serum levels of soluble adhesion molecules (sICAM, sVCAM and sE-selectin) as well as other markers of endothelial cell activation (vWF and endothelin) are elevated in patients with SSc and have been reported to correlate with disease activity [14-20].

As well as evidence of endothelial cell activation there is evidence for endothelial cell dysfunction in patients with SSc. Endothelial dependent vasodilatation, measured following iontophoresis of acetylcholine on to the digital vessels by laser Doppler, is decreased in patients with SSc [21-23].

The implications of endothelial cell activation are that the usually anti-inflammatory, anti-coagulant properties of the endothelial cell lining become pro-inflammatory and pro-coagulant [15]. Activated endothelial cells express leukocyte adhesion molecules increasing leukocyte adhesion and promoting infiltration. In addition, activated endothelial cells secrete chemokines including interleukin-8 (IL-8) and monocyte chemotactic peptide (MCP-1) that

also increase the leukocyte infiltration. They also secrete a number of pathogenically relevant cytokines and growth factors including endothelin which is pro-fibrotic and vasospastic as well as interleukin-6 which is profibrotic and promotes chronic inflammation (Fig. 3).

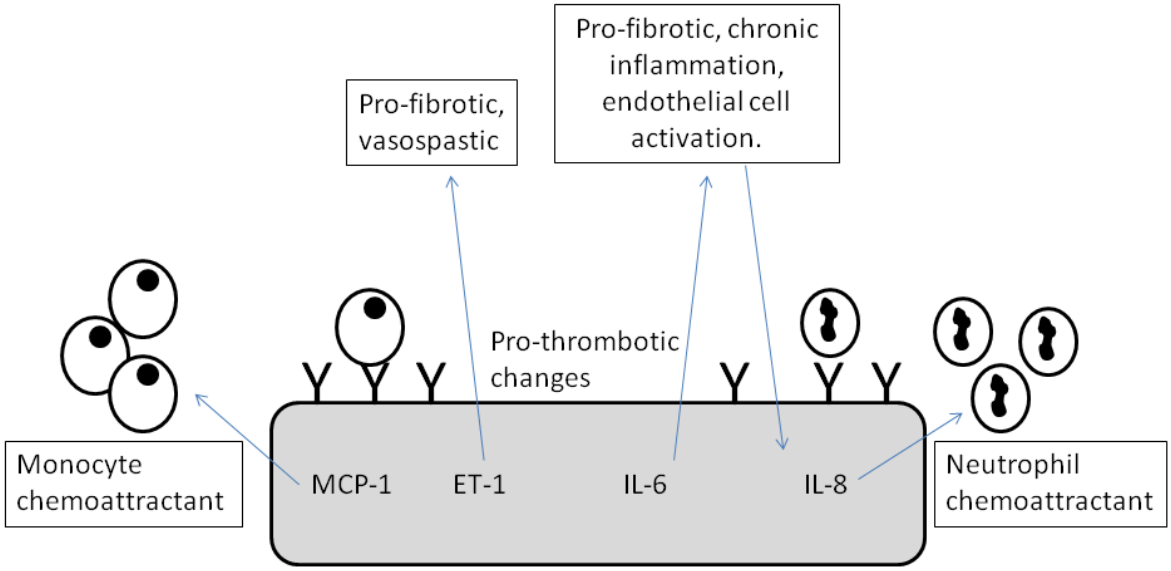


Fig. 3. Endothelial cell activation. Endothelial cell activation leads to an increase in leukocyte adhesion molecules, and release of leukocyte chemotactins and a pro-thrombotic phenotype. In addition, there is release of cytokines: ET-1=endothelin-1; IL-6=interleukin-6; IL-8=interleukin-8; MCP-1= monocyte chemoattractant peptide.

The role of endothelial apoptosis is less clear, but apoptotic cells are likely to be phagocytosed by tissue macrophages and dendritic cells leading to the potential presentation of self antigen to T cells, which could promote autoimmunity. In addition, apoptotic endothelial cells are adhesive to platelets and leukocytes and can therefore activate the alternative complement and coagulation cascades. This may result in microthrombosis and consequent tissue ischaemia that may perpetuate the process. Laplante *et al* have shown that apoptotic endothelial cell conditioned media can inhibit fibroblast apoptosis, induce myofibroblast differentiation and increase production of type 1 collagen. This mimics the pro-fibrotic fibroblast phenotype seen in SSc [24].

1.3 The role of the fibroblast in SSc pathogenesis

Fibroblasts have a central role in the pathogenesis of SSc. Fibroblasts isolated from SSc lesional skin have a unique phenotype that mimics fibroblast activation by TGFβ [25-28].

Of particular interest is the fact that fibroblasts appear to be constitutively activated in *ex vivo* culture for several passages. They express increased collagen mRNA and protein [26 29-31], and constitutively express profibrotic and proinflammatory cytokines (e.g. IL-6, IL-1α), growth factors (e.g. CTGF) and chemokines (e.g. IL-8 and MCP-1) [30 32-36].

Fibroblasts are heterogeneous depending on their tissue of origin and the surrounding environment [37]. Activated fibroblasts differentiate to a myofibroblast phenotype which is identified by the expression of αSMA. Although there is no increase in the absolute number of skin fibroblasts in SSc there is an increase in the number of myofibroblasts [38]. It is not clear whether the abundance of activated fibroblasts found in SSc lesional skin is a response to activating pro-inflammatory cytokines, or whether it represents the clonal expansion of a subpopulation of highly activated cells, although there is supporting evidence for both [27].

Aberrant apoptotic responses can potentially be responsible for clonal expansion. During the normal response to injury or inflammation, there is fibroblast activation and an increase in extracellular matrix deposition. This is usually followed by a period of remodeling which involves matrix metalloproteinases (MMPs) and their regulators, tissue inhibitors of matrix metalloproteinases (TIMPs). Finally, this is followed by a period of resolution that is accompanied by apoptosis of activated fibroblasts. In SSc there is increased expression of TIMPs, therefore inhibiting the breakdown of the extracellular matrix [39-42]. In addition, there is a paucity of fibroblast apoptosis and SSc fibroblasts *in vitro* are resistant to Fas-mediated apoptosis [43 44]. SSc fibroblasts constitutively express increased concentrations of TIMP-1 and are also more sensitive to the mitogenic effects of TIMP-1, supporting the clonal expansion theory [45].

Several candidate cytokines for paracrine fibroblast activation exist. IL-1β, IL-6 and MCP-1 are elevated in the serum of patients with SSc and can give rise to fibroblast activation. Endothelial cell conditioned media increases DNA and protein synthesis in cultured SSc fibroblasts more than in normal fibroblasts [46]. Antibodies against fibroblast growth factor (FGF) and IL-1β abrogate the effect of the conditioned media on DNA and protein expression, respectively [46].

Several autoregulatory loops have been identified that might account for the autonomous phenotype seen *in vitro*. These include the MCP-1 signalling axis, IL-6 and TIMP-1 [33 39 45 47]. Constitutive expression of IL-1α by SSc fibroblasts increases the expression of IL-6 and PDGF that are capable of further fibroblast activation [35].

Activated T-cells interact with fibroblasts via CD40 expressed on fibroblast cell membranes. SSc fibroblasts have an increased expression of CD40 and ligation of this increases the production of IL-6, IL-8 and MCP-1 [48]. Ligation also increases the expression of a co-stimulatory molecule (CD80) on SSc fibroblasts. None of these changes were induced by ligation of CD40 on control fibroblasts [48]. These data imply that direct cell contact may also have a role in fibroblast activation in SSc.

Autoantibodies directed against fibroblasts have been detected in a proportion of patients with SSc and have been implicated in fibroblast stimulation [49].

1.4 Cytokines and growth factors in SSc

1.4.1 Transforming growth factor-β

TGFβ is believed to play a major role in SSc as the typical fibroblast phenotype outlined above closely mimics the effects of TGFβ stimulation. Genome wide studies of lesional skin have demonstrated significant differences in TGFβ-related genes in the skin of diffuse SSc patients compared to controls [50].

There are contradictory studies of the tissue expression of TGFβ, some show an increase in TGFβ staining by histocytochemistry of lesional skin whereas others do not [36]. This may be explained by a study that demonstrated that TGFβ expression was increased at the "leading edge" of the lesional skin and therefore may be implicated in the initiation and propagation of the fibrosis [51]. Therefore, the discrepancy in biopsy based histocytochemistry may depend on the site of the biopsy. If it is taken from an established lesion, in may be devoid of TGFβ, whereas a biopsy from skin adjacent to established fibrosis may be within the "leading edge".

SSc fibroblasts do not produce excessive amounts of TGFβ and they are not more sensitive to TGFβ than normal fibroblasts. In fact, SSc fibroblasts appear to be less sensitive *ex vivo* to TGFβ than normal fibroblasts [27]. It has been suggested that this may reflect insensitivity following excessive *in vivo* exposure. Consistent with this hypothesis, there is an increase in TGF receptor expression on the surface of SSc fibroblasts [27]. *In vitro* studies reveal that over-expression of TGFβ receptor 1 is sufficient to reproduce the activated phenotype of SSc fibroblasts and is dependent on autocrine TGFβ secretion, but not on receptor kinase activity. Reversing the over expression using siRNA normalized the fibroblast phenotype [52].

The canonical TGFβ signalling pathway involves Smad proteins, including Smad 2 and 3 which lead to characterised responses to TGF, and Smad 7 which is an inhibitor of TGFβ signalling. Immunohistochemical analysis demonstrated increased Smad 2 and 3 expression in SSc non-lesional skin with increased translocation of the protein to the nucleus [53]. Skin involvement estimated by the Rodnan skin score correlates with levels of phosphorylated Smad 3 in protein extracts from SSc fibroblasts [54]. Some investigators have reported a decrease in the expression of Smad 7 in SSc whilst others report no difference [54].

TGFβ also signals through non-canonical pathways, in particular the c-Abl non-receptor tyrosine kinase that can be inhibited by Imatinib, and the early growth response-1 protein that can mediate increased transcription of collagen in response to TGFβ [55].

The production of TGFβ and its activation involves highly complex regulatory mechanisms. It is secreted in a latent form (latent TGFβ), containing a latency associated peptide which is cleaved to form the active TGFβ. Latent TGFβ is also associated with a binding protein (LTGF binding protein (LTBP)) which serves to bind LTGF to the extracellular matrix. This provides a large pool of latent TGFβ, stored in the ECM ready for rapid release on stimulation. Proteolytic cleavage of the active form can be performed by matrix metalloproteinases, serine proteases and integrins [56]. TGFβ can also be dissociated from the latency-associated peptide by thrombospondin [57]. This complicated system affords multiple levels of control over the activity of TGFβ.

Animal models support a prominent role for TGFβ in SSc and anti-TGFβ strategies can prevent fibrosis in these animal models. However, many of the animal models incompletely represent the SSc phenotype and therefore the applicability of these strategies to humans is debatable [54].

To date, strategies to block TGFβ in humans have been less successful than in the animal models. The human monoclonal antibody metelimumab was given in a randomized controlled trial to 45 patients with SSc, but no improvement was seen in skin scores or other disease manifestations. There was an increase in adverse events in the treatment arm but concerns about increased autoimmune events were not realised [58]. Potential reasons for the failure include the restricted isotype and low binding affinity of the antibody used, the low number of patients studied and the short duration of the trial.

Other strategies to block TGFβ are in the early stages of development and the results in clinical trials are eagerly awaited. The protein tyrosine kinase inhibitor Imatinib has been shown to block non-canonical TGFβ signalling, in addition to its other actions and could contribute to its beneficial effects in animal models of SSc. Imatinib will be discussed in greater detail later.

1.4.2 Vascular endothelial growth factor

Vascular endothelial cell growth factor (VEGF) is another important mediator in SSc. Endothelial cell expression of VEGF is transcriptionally induced by hypoxia via the transcription factor HIF1α. It is a potent angiogenic growth factor, increasing the recruitment of endothelial cell progenitor cells from the bone marrow and acting as an endothelial cell mitogen. It also has additional effects on inflammation including the induction of cytokines (IL-6) and chemokines (IL-8, GROα) [59].

Despite the fact that there is ischaemia and a measurable hypoxia in the lesional skin of patients with SSc, there is no concomitant increase in angiogenesis. Instead, there is a disorganized architecture of the microvessels and an overall decrease in the number of vessels.

Despite the lack of angiogenesis however, there is an increase in the tissue expression of VEGF in lesional and non-lesional skin. There is also an increase in the tissue expression of the VEGFRs 1 and 2. The increased expression does not correlate with the levels of HIF1α and is likely, therefore, to be as a result of HIF1-independent mechanisms [60]. Cytokines such as TGFβ, IL-6, IL-1β and PDGF are all raised in patients with SSc and are capable of increasing the expression of VEGF. They may have an, as yet, unconfirmed role in VEGF over-expression in SSc.

It appears that the kinetics of VEGF expression may be important in determining the response to VEGF. In a mouse model where VEGF expression is inducible by the administration of tetracyclines, experiments were conducted to explore the effects of VEGF kinetics. It seems that prolonged expression of VEGF, as seen in patients with SSc, can cause a disorganized and ineffective angiogenesis that very much resembles the picture seen in SSc [61]. An alternative explanation for the lack of angiogenesis seen in response to elevated VEGF in SSc is there that is also an increase in the expression of opposing,

angiostatic factors. The data regarding this is contradictory, and so far there is no convincing positive evidence for an increase in such a factor [62].

There is some *in vitro* evidence to suggest that endothelial cells isolated from patients with SSc have a defective response to VEGF in a Matrigel tube formation assay [63]. Investigators using circulating endothelial precursor cells (CEPs) isolated from SSc patients as a model for SSc endothelial cells have shown that SSc CEPs have a decreased up-regulation of VEGFR1 in response to hypoxia [64]. This may help explain the apparent resistance to the pro-angiogenic effects of VEGF seen in SSc, however there is some evidence to suggest that VEGFR1 has an inhibitory role in VEGF signalling. In addition, it has not been completely established that CEPs are a good model for mature endothelial cell function. CEPs from patients with SSc are less able to differentiate *in vitro* than CEPs from healthy controls [65]. Therefore, data derived from CEPs should only be taken as circumstantial evidence at the present time.

1.4.3 Platelet derived growth factor

Platelet derived growth factor (PDGF) is a potent mesenchymal mitogen. It has effects on fibroblasts including replication, survival, myofibroblast differentiation and migration and is produced by a number of cell types including fibroblasts, endothelial cells and mononuclear leukocytes. Release can be stimulated by injury, hypoxia and inflammatory cytokines, such as IL1-β.

Four isoforms of PDGF exist, A, B, C and D. PDGF C and D are secreted in a latent form requiring proteolytic cleavage for activation. These form homodimers, while the A and B isoforms can form heterodimers. These dimers signal through two receptors PDGFRα and PDGFRβ. PDGF-AA signals exclusively through PDGFRα, PDGF-BB signals through PDGFRα, αβ, and β, PDGF-AB and C signal through PDGFRα and αβ, and PDGF-D signals preferentially through PDGFRβ (Fig.4).

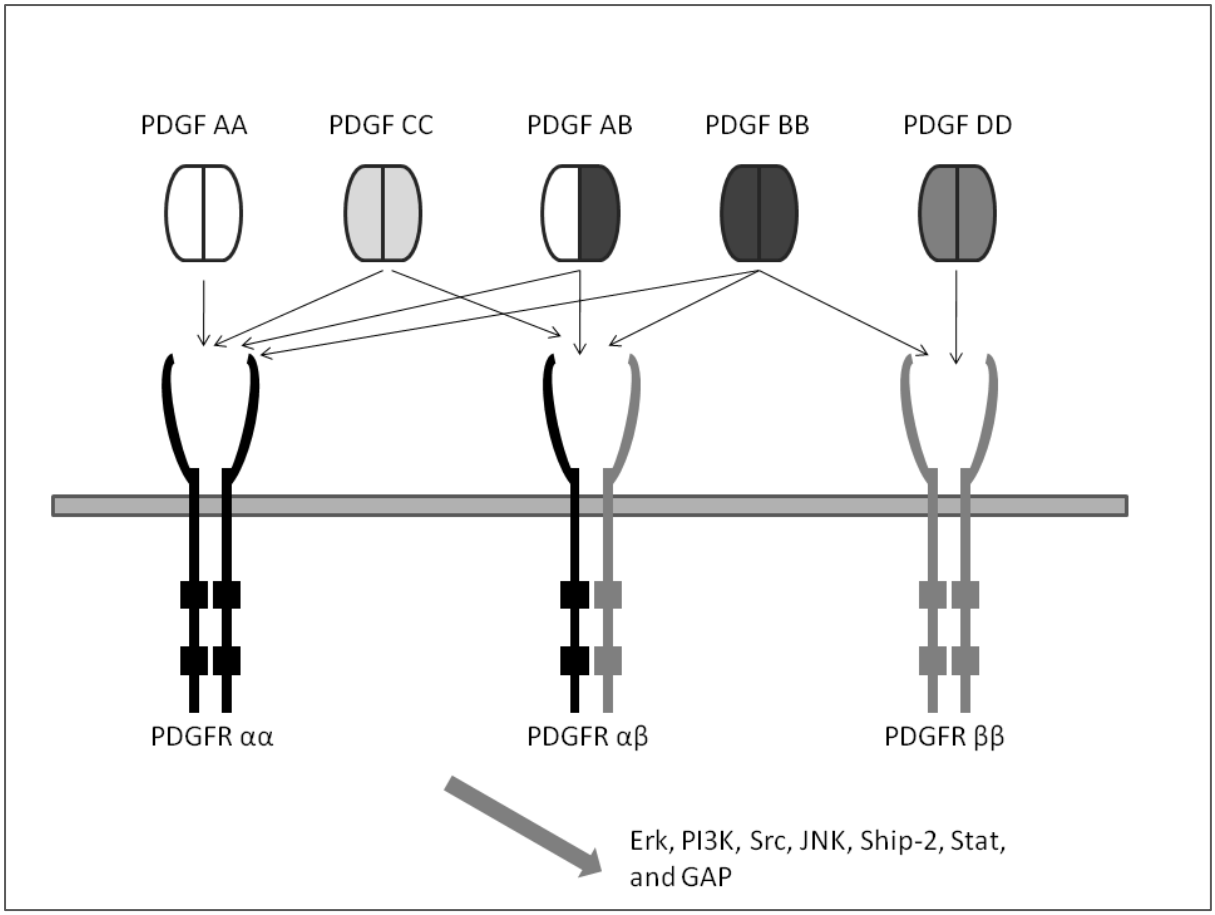


Fig.4. Platelet derived growth factor (PDGF) signalling. PDGFs stimulate dimerisation of α and β receptors and consequent autophosphorylation leads to activation of intracellular signalling cascades.

Expression of PDGF and its receptors are increased in SSc. Immunohistochemistry demonstrates that there is increased PDGF and PDGFR expression in the lesional skin of patients with SSc [66 67], localised to the endothelial cells and mononuclear leukocyte infiltrate, hair follicles and stromal cells. PDGF is almost undetectable in healthy control skin. PDGF A and B are also elevated in the bronchoalveolar lavage fluid of patients with SSc [68].

Fibroblasts isolated from patients with SSc, when treated with TGFβ demonstrate increased expression of PDGFRα which is a unique feature of SSc fibroblasts and is not found in healthy control fibroblasts [69]. This translated into an increase in the mitogenic response to PDGF-AA. Other investigators have shown that low dose TGFβ stimulates a mitogenic

fibroblast response by inducing the autocrine production of PDGF-AA. However, at higher doses PDGFRα expression was down-regulated in a negative feedback mechanism [70]. SSc fibroblasts mount a mitogenic response to TGFβ over a wide range of concentrations. In turn, PDGF-AA and AB up-regulate the expression of TGFβR2 on dermal fibroblasts [71]. In summary, PDGF and TGFβ appear to work in concert to increase the mitogenic and profibrotic fibroblast phenotype.

IL-1α is constitutively expressed by SSc fibroblasts in culture and induces PDGF and IL-6 expression [35]. PDGF and IL-6 can act together to increase cell proliferation and induce the production of collagen, respectively [35]. The serine protease thrombin can also induce the expression of PDGF-AA and PDGFRα and is increased in bronchoalveolar lavage fluid of patients with SSc [72].

There is evidence that the sera of patients with SSc may contain antibodies directed against PDGFR. These appear to be functional as they induce tyrosine phosphorylation of the downstream signalling protein ERK1/2, increase fibroblast ROS generation and induce fibroblast collagen production. Therefore, these antibodies are of potential pathogenic relevance in SSc, though independent reproduction of this data is still required to confirm this finding [73].

Many partially successful anti-fibrotic agents may exert their effects through blocking PDGF signalling. No treatments are yet available to treat fibrosis in SSc, because, at least in theory, once fibrosis is established, targeting profibrotic cytokines is only likely to prevent progression at best, and is unlikely to mediate improvement.

Imatinib is a protein tyrosine kinase inhibitor that can block the activity of PDGFR. It has been used for the treatment of chronic myeloid leukaemia and can regress bone marrow fibrosis in this condition. It has been trialed in animal models of SSc including the bleomycin-induced fibrosis model and the tight skin mouse model (TSK-1) [74]. These studies have given

different information regarding the possible utility of this agent in SSc. Imatinib was able to prevent the development of fibrosis in the bleomycin model but this gave no information about its ability to treat established fibrosis. Therefore, the investigators administered Imatinib to the TSK-1 mice and were able to demonstrate not only a decrease in myofibroblast differentiation but also a regression of established fibrosis. Imatinib drastically decreased the production of extracellular matrix, but no effect has been demonstrated on the production of enzymes that break this down e.g. MMPs. This may indicate that regression occurs purely by decrease in matrix production allowing the natural pool of enzymes to break down the fibrotic areas [74].

The fact remains however, that although these are good models of fibrosis, they are incomplete models of SSc. In particular, the potentially antiangiogenic actions of Imatinib remain a concern in a cohort of patients who already lack angiogenesis and may have ischaemic symptoms [75].

One case study in a patient with SSc treated with Imatinib exists. Fibroblasts from the patient were explanted and treated with TGFβ in the presence and absence of Imatinib. Imatinib decreased the proliferation and collagen mRNA production by fibroblasts in response to TGFβ. In addition, the patient demonstrated a clinical improvement [76]. Interim results from 2 trials using Imatinib in SSc were reported at the American College of Rheumatology meeting, 2009.

Gordon *et al* reported on an open label study of Imatinib in 30 SSc patients with a primary endpoint of safety. They reported 156 adverse events, 24 serious adverse events and 1 death. Only 16 patients were able to complete the study. They did however, report improvements in skin score, FVC, DLCO and histologic features on skin biopsy.

Pope *et al* reported on a placebo-controlled, randomized, double blind, mechanistic study. They only enrolled 10 patients and the trial was stopped due to the high number of adverse

events. Only 4 patients managed to complete the study. No benefits were seen in this small number of patients.

1.4.4 Interleukin-6.

Interleukin-6 (IL-6) is increased in the serum of patients with SSc and this correlates with disease activity [77-83]. Immunocytochemical studies reveal an increase in IL-6 staining in the lesional skin of patients with SSc. Unlike other pro-inflammatory cytokines, IL-6 is found in late as well as early biopsy specimens implying that it has a continuing role in disease pathogenesis [79]. IL-6 is a pro-fibrotic cytokine that increases collagen and extracellular matrix production by fibroblasts [84]. It also increases the expression of mononuclear cell chemokines such as MCP-1 and VEGF in fibroblasts and endothelial cells [85-88]. IL-6 may have a role in the proliferation of synovial fibroblasts in rheumatoid arthritis [89]. However, these authors only found a pro-proliferative effect in one synovial sample out of 4 and further examination of this and other synovial samples demonstrated an anti-proliferative effect [90]; experiments with dermal fibroblasts show that IL-6 inhibits proliferation [91].

IL-6 can induce myofibroblast differentiation [92], and also has an activating role for endothelial cells, increasing the expression of cell surface leukocyte receptors, and increasing cytokine (IL-6) and chemokine expression (MCP-1 and IL-8) [93 94].

IL-6 also has an important role in the transition from acute to chronic inflammation and the maintenance of chronic inflammation [95-97]. This occurs through the activation of T-cells, delaying T-cell apoptosis and altering the chemokine profile from neutrophil attractants (IL-8) to monocyte attractants (MCP-1) [98 99]. In addition, IL-6 may increase neutrophil apoptosis, though this is controversial [95 100-102].

IL-6 has a role in the differentiation of B-cells towards plasma cells and may account for the hypergammaglobulinaemia which is a feature of SSc. Finally, IL-6 may have a role in promoting autoimmune phenomena which are one of the clinical hallmarks of this disease [99].

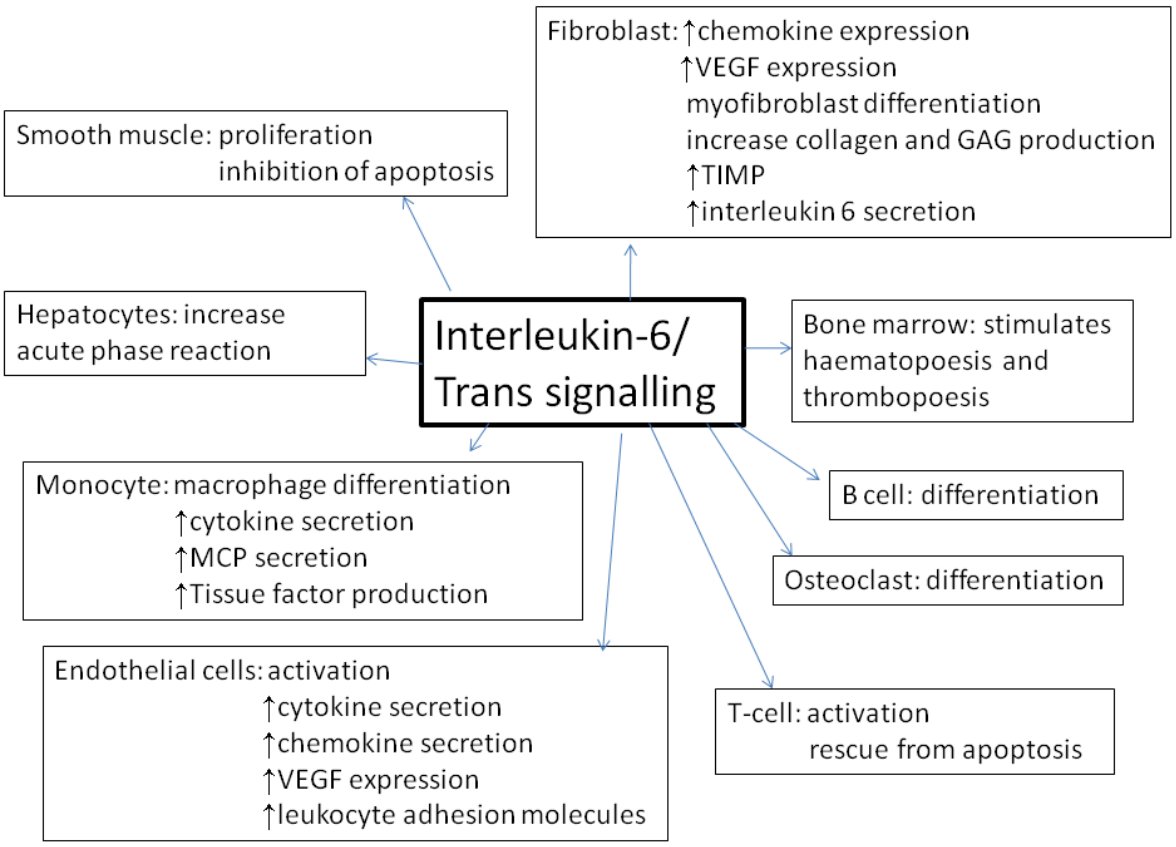


Fig. 5. Biological actions of interleukin-6 and interleukin-6 trans signalling *in vivo* and *in vitro*.

1.4.5 Monocyte chemoattractant protein-1

MCP-1 is a chemokine of the C-C type that has been reclassified as CCL2. It is predominantly a monocyte/macrophage chemoattractant and mainly signals through the chemokine receptor CCR2. However, there is significant overlap: many chemokines signal through multiple chemokine receptors and chemokine receptors may respond to ligation by multiple chemokines. In addition to its effect on chemotaxis, there is additional evidence that MCP-1 may have a profibrotic role.

In rat lung fibroblasts, it increases the production of collagen by increasing TGFβ [103]. CCL2 seems to have an additional direct effect on collagen mRNA levels *in vitro,* increasing transcripts for type 1 collagen in human dermal fibroblasts with kinetics that suggest that it is a direct phenomenon [104]. The difference noted between rat lung fibroblast and human

dermal fibroblast response to CCL2 may reflect the well-described phenotypic heterogeneity of fibroblasts. The authors, however, did not directly explore collagen protein expression in response to CCL2. Distler *et al,* in contrast, cultured both normal and SSc fibroblasts *in vitro* with CCL2 and found no increase in collagen protein expression [105]. This may reflect the fact that the increased transcripts are not translated, or more likely may be related to differences in the CCL2 concentrations used. Yamamoto *et al* used 10ng/ml whereas Distler *et al* used 100 and 400ng/ml. Serum concentrations of CCL2 are around 200pg/ml but local tissue concentrations are unknown.

CCR2 was co-expressed with α-smooth muscle actin (αSMA) a myofibroblast marker, in a small proportion of dermal fibroblasts, and the proportion of total fibroblasts with this phenotype was higher in SSc than in controls. In this population of fibroblasts, specific inhibition of CCL2 or CCR2 attenuated the expression of αSMA implying a role for CCL2 in myofibroblast differentiation [106].

CCL2 is increased in the serum and bronchoalveolar lavage fluids of patients with SSc and is associated with renal disease and pulmonary fibrosis [82 107 108]. T-cell lines isolated from SSc patients express more CCL2 than controls when stimulated with PMA and ionomycin *in vitro* [82]. PBMCs isolated from patients with SSc have an enhanced spontaneous production of CCL2 in culture [109].

CCL2 expression is increased in the skin of patients with SSc and is almost undetectable in controls [105 108 110]. It is localized to infiltrating monocytes, keratinocytes, fibroblasts and endothelial cells in patients with both limited and diffuse disease. The CCR2 is also increased in the skin of patients with SSc but is associated with early disease in the diffuse subset. It was located within myofibroblasts, pericytes, lymphocytes, macrophages and endothelial cells.

Fibroblasts in culture also spontaneously produce increased amounts of CCL2 compared to controls, with a dampened increase in CCL2 production in response to hypoxia [33]. An autocrine positive feedback loop also exists, as CCL2 is shown to increase CCL2 mRNA expression in SSc fibroblasts but not control fibroblasts [111]. Distler *et al* reported no increase in constitutive CCL2 expression by SSc fibroblasts but a greater induction of the chemokine in response to PDGF in SSc fibroblasts than controls [105].

A functional single nucleotide polymorphism in the promoter region of CCL2 has been reported in association with SSc by 2 groups, in Germany and Slovenia [112 113]. However, two larger European cohorts failed to demonstrate such an association [114 115]. The populations are of similar ethnic origins, and the differences are likely to be due to sample size.

In vitro models of SSc have shown an important role for CCL2 in monocyte chemotaxis. Dermal fibroblasts explanted from the skin of SSc patients or controls were co-cultured below an endothelial cell line monolayer (1E-7). The transmigration of mononuclear cells (U937) through the endothelial cells was measured. The proportion of transmigrating cells was much greater towards SSc fibroblasts than controls. In addition, fibroblast-conditioned media from SSc and control fibroblast cultures were used to stimulate transmigration through the endothelial cell layer, and again the conditioned media from the SSc fibroblasts had a more profound response. This was unrelated to any effect on leukocyte adhesion molecule expression on the surface of endothelial cells, but was blocked by an anti-CCL2 antibody [116]. This data implies a role for fibroblast derived CCL2 in increasing the mononuclear cell infiltrate that is characteristic of SSc histology.

1.4.6 Endothelin-1.

Endothelin-1 is one of three closely-related vasoactive peptides, endothelin-1,2 and 3. As well as its prominent role in vasoconstriction, it has pleiotropic effects on fibroblasts and endothelial cells. Its expression is induced by TGFβ, a potent profibrotic cytokine whose role

in SSc is outlined above [117]. In smooth muscle, endothelin-1 is associated with proliferation, contraction and production of profibrotic cytokines such as TGFβ, CTGF and PDGF [118-121]. In fibroblasts, it causes myofibroblast differentiation as well as increased production of cell surface leukocyte adhesion molecules and the production of extracellular matrix proteins [122-125]. The effects of endothelin-1 on endothelial cells include an increase in the expression of leukocyte adhesion molecules and production of nitric oxide [121]. Endothelin-1 also has actions on macrophages with an increase in the release of free radicals and the production of chemokines (IL-8 and CCL2) and cytokines (TGFβ) [121 126]. Endothelin-1 thus has interesting roles which may be relevant, not only to the vasospastic Raynaud's phenomenon, but also the tissue remodeling which is a feature of the vasculopathy and fibrosis in this disease.

Endothelin-1 expression is increased in the serum and bronchoalveolar lavage fluid of patients with SSc [127-129]. Elevated levels of endothelin-1 have been found in the skin, lungs, blood vessels and kidney of patients with SSc [130-133]. Increased endothelin receptor expression has been found in presclerotic and early diffuse skin lesions.

Preliminary results with the endothelin antagonist Bosentan were initially very exciting as it normalized the gene expression of explanted SSc fibroblasts [134]. In a large study of patients with primary and secondary pulmonary artery hypertension, Bosentan significantly improved the symptoms of patients with pulmonary hypertension and this was also true in a sub-group analysis of SSc patients [135]. A follow up study went on to demonstrate an additional increase in patient survival [136]. However, later studies were not so positive. Bosentan failed to make a difference in SSc associated pulmonary fibrosis [137 138]. Trials where the primary outcome measure was digital ulcer healing, showed that Bosentan had no effect on digital ulcer healing but it did decrease the rate of new ulcer formation [139].

Mechanistic studies have attempted to examine the effects of Bosentan on endothelial cell function. One study reported no change in endothelial cell dependent- and independent-

vasodilatory responses, no changes in capillary permeability, capillary density, capillary structure or serum markers of endothelial dysfunction in 15 patients treated for 12 weeks with Bosentan [140]. However, this study suffered from small sample size, especially given the wide variability in the techniques used and the lack of control patients.

Another study, with a control arm showed an improvement in endothelial dependent vasodilatory responses in just 10 min with an endothelin receptor type A specific inhibitor (BQ-123) [141]. These results are exciting but somewhat surprising in terms of the kinetics, and need to be independently reproduced.

1.4.7 Other cytokines.

The expression of many other cytokines have been inconsistently associated with SSc including IL-1β, IL2, IL4, IL8, IL10, TNFα and others. However, the roles of these cytokines have been less rigorously explored and therefore are not reviewed here.

1.5 Leukocytes in SSc.

There is significant evidence to imply a role for the immune system in SSc. Histological specimens consistently demonstrate an immune cell infiltrate in lesional biopsies from patients with SSc. This is usually reported as a mononuclear infiltrate, but one study by Hussein *et al* also implicated eosinophils and neutrophils [142]. Bronchoalveolar lavage fluids are also associated with a leukocyte infiltrate.

SSc is characterised by specific autoimmune phenomena, the majority of patients express auto-antibodies and the auto-antibody profile correlates with disease expression. Recently, potentially pathogenic autoantibodies have been reported e.g. anti-fibroblast antibodies, anti-endothelial cell antibodies and antibodies directed against the PDGF receptor [10 49 73 143].

Recent pan-genome studies of gene expression in SSc have shown that the disease is strongly associated with HLA phenotype, which again implies a significant role for the immune system [144] [145].

Autologous stem cell transplants have been used for patients with autoimmune diseases including SSc, the aim of which is to ablate the cells of the adaptive immune system and allow them to reconstitute and develop a new concept of self, thus eliminating autoreactive T and B lymphocytes. Autologous stem cell transplants have, in small studies, shown clinical benefit in SSc, including an improvement of fibrosis on biopsy and improvement of vascular abnormalities on nailbed capillaroscopy. In addition, stabilisation of symptoms has been noted. Initial protocols have been improved to give a more favourable risk: benefit ratio and larger studies with control (cyclophosphamide) arms are now under way in Europe and North America. These studies should clarify the role for stem cell transplantation, but the fact that re-setting the adaptive immune system can lead to signs of clinical improvement implies a role for the immune system in the perpetuation if not the initiation of the disease process [146].

1.5.1 T-lymphocytes.

T lymphocytes are seen in the skin of patients with SSc, although there is not an increased number compared to healthy controls [142]. However, T-cells in the skin of patients appear to be activated as they express higher levels of activation markers CD69 and HLA-DR [147] [148]. T-cells in the peripheral blood of patients show an increase in the ratio of $CD4^+$ T helper cells to $CD8^+$ cytotoxic T-cells [142]. In addition, there is a decrease in the number of regulatory T-cells (T-reg) in patients with SSc [149]. T-regs have an important role in negatively regulating T-cell functions and in particular in regulating autoimmune activation. Although patients with SSc have a smaller proportion of T-reg cells, their T-regs have an increased expression of CD25, implying chronic activation. Functional studies have not been performed on the Treg cells of SSc patients, and this would be important in the future to clarify their role in disease pathogenesis.

One of the most interesting findings is that T-cells from patients with SSc seem to show monoclonal or oligoclonal expansion [150]. This implies antigen-driven T-cell activation, though the specific antigen has not yet been identified. Microchimerism, leading to a type of graft versus host disease, and topoisomerase 1 have been suggested as possible antigenic stimuli. However, inducing autoreactivity against topoisomerase 1 in mice does not result in a SSc like phenotype. This may reflect human/murine differences, or may reflect the fact that anti-topoisomerase antibodies alone are not sufficient to cause the disease phenotype.

Some investigators propose that T-cells from the skin and circulation of patients with SSc show a predominantly type 2 T-helper cell phenotype and as such secrete profibrotic cytokines such as IL-4 and IL-13 [151]. However, a synthesis of the available published literature is rather contradictory and no clear pattern of cytokine expression emerges when serum or bronchoalveolar lavage fluid is analysed. This is likely to be due to the changes in the immune response depending on the stage and the activity of the disease, and may also reflect the overall heterogeneity of the disease. One group of investigators identified a novel subset of T-cells which were $CD4^+/CD8^+$ double positive cells in SSc [152]. These cells were identified in both skin derived T-cell lines and *in vivo* in the skin of SSc patients. In culture, this distinct subset of cells produced very high levels of IL-4.

T-cells from patients with SSc have an increased propensity to migrate spontaneously across endothelial cell layers [148]. It is intriguing therefore, that investigators have found a decrease in the $CD8^+$ T-cell expression of L-selectin, a T-cell adhesion molecule that is thought to promote adhesion of T-cells to endothelial cells and subsequent migration [153]. However, it is not the primary molecule mediating migration and these processes are somewhat separate; indeed expression of adhesion molecules CD11a, CD49d, CD29 and CD44 were elevated in lymphocytes recovered following endothelial transmigration [148].

Cytotoxic γδ T-cells are T-cells that have cytolytic capabilities and are not MHC restricted: they are capable, therefore, of interacting with intact, unprocessed self antigen. There are

reports that they accumulate in the lesional skin and lungs of patients with SSc and a polymorphism in the T-cell receptor γ gene is associated with SSc [154-156].

Cytotoxic T-cells collected from the PBMC fraction of SSc patient blood following stimulation and immuno-magnetic separation were compared to similarly-isolated cells from matched controls. Cytotoxic T-cells isolated from SSc patients demonstrated increased adhesion to unstimulated endothelial cells in culture, increased proliferation when incubated with irradiated endothelial cells, and increased cytotoxicity towards endothelial cells but not fibroblasts. The cytotoxicity towards endothelial cells was reversed by anti-TCRγδ and anti-granzyme antibodies implicating activation of the granzyme/perforin cytotoxicity mechanism and interactions involving the TCRγδ [157]. Therefore, T-cell endothelial cell interactions may be important in causing the endothelial cell damage that is implicated in the pathogenesis of SSc.

1.5.2 B-lymphocytes.

B cells and plasma cells form a minor part of the mononuclear cell infiltrate seen in patients with SSc. However, gene expression experiments show that genes associated with B-cells are associated with SSc skin [145].

Naïve B-cells are increased in the circulation of patients with SSc but memory B-cells are decreased [158]. B-cells in the circulation of patients with SSc express higher levels of activation markers (CD80, CD86 and CD95) and CD19 [158]. CD19 modulates B-cell function, and in mice, over-expression promotes autoantibody production.

There is a generalized hypergammaglobulinaemia associated with SSc and 95% of patients with SSc express autoantibodies. These autoantibodies segregate with disease phenotype and some are thought to have potentially pathogenic roles including anti endothelial cell antibodies that can cause endothelial cell activation and apoptosis. They may also have a role in antibody dependent cellular cytotoxicity [10 143].

Bleomycin administration in wild type mice induces fibrosis, but development of the phenotype is inhibited in CD19 deficient mice [159]. B-cell depletion in neonatal but not adult Tsk mice resulted in decreased fibrosis as did antagonism of B-cell activating factor (BAFF) [160] [161]. Finally, blockade of CD40/CD40 ligand interactions in Tsk neonatal mice inhibited fibrosis and autoantibody generation [162].

Serum levels of BAFF are increased in patients with SSc and B-cells from patients express increased levels of BAFF receptor [163]. BAFF signalling promotes B-cell survival and may be implicated in the rescue of autoreactive B-cells.

In the Tsk mouse model, infusion of bone marrow derived from Tsk mice into normal littermates results in the development, over time, of a Tsk/SSc phenotype [164]. This was not a graft versus host response as control mice were infused with normal littermate bone marrow, and sibling tissue grafts did not elicit any graft versus host response. Interestingly, transfusing differentiated Tsk B-cells into healthy littermates transferred antibody production but did not result in a fibrotic phenotype. Infusion of T-cells alone did not alter the phenotype at all but infusion of both T and B-cells resulted in a very mild fibrotic phenotype.

Tsk mice crossed with $CD4^{-/-}$ mice had much less fibrosis but still developed autoantibodies. Tsk mice crossed with $CD8^{-/-}$ mice developed similar levels of fibrosis but fewer autoantibodies. Tsk mice crossed with $Rag^{-/-}$ mice, which are deficient in both T and B-cells, still develop the same fibrotic phenotype [165]. These data show that the adaptive immune system alone is not sufficient to explain the full phenotype in animal models and that different subsets of immune cells may play unique roles in the pathogenesis. These findings may be specific to the mouse models alone but are persuasive evidence that other cell types, the precise environment and perhaps the genetic background may play additional and important roles in the disease phenotype.

T-bet$^{-/-}$ mice when exposed to bleomycin produce a florid fibrogenic response. However, when crossed with B and T-cell deficient mice these mice still have a fibrogenic response to bleomycin, arguing against a significant role for the adaptive immune system in this phenotype. When the same T-bet $^{-/-}$ mice are crossed with IL-13$^{-/-}$ mice no fibrotic response is seen in response to bleomycin, therefore arguing for an important role for IL-13. The source of this IL-13 remains unclear [166].

1.5.3 Monocytes.

Monocytes or histiocytes (monocyte derived tissue dwelling cells) are the predominant cell type in the mononuclear cell infiltrate of early SSc [142]. There is, however, no significant increase in monocytes in the bronchoalveolar lavage fluid of patients with SSc compared to controls [142]. Circulating monocytes are not increased in number, but monocytes isolated from the circulation have several important features when cultured *ex vivo*.

Monocytes from SSc patients spontaneously, and in response to phorbol esters, produce increased amounts of superoxide anion in culture compared to cells isolated from healthy controls or patients with primary Raynaud's phenomenon [167]. Higher levels of superoxide generation were associated particularly with early diffuse disease. This could contribute to the increased oxidative stress that may be important in this disease (see later section).

Monocytes from SSc patients spontaneously express higher concentrations of TGF1β in culture than healthy control cells [168]. They also produce higher levels of spontaneous- and LPS-stimulated IL-6 in culture than control monocytes [169].

Monocytes from patients with SSc have an increased expression of interferon-responsive genes [170-172], which include Siglec-1/sialoadhesin that is induced by type 1 interferons, and AIF1 that is induced by γ-interferon. SSc serum can induce interferon-α release from PBMCs especially when the sera contains anti-topoisomerase antibodies [173]. This increase in "interferon signature" may reflect innate immune system activation via Toll like receptors

(TLRs). Other consequences of TLR activation in monocyte/macrophages include the production of IL-1, TNFα and IL-6 which may drive inflammation and fibrosis, as outlined above [174].

One group has identified constitutive production of IL-1 by cultured SSc monocytes [175]. However, others were unable to reproduce these findings, though they used unfractionated PBMCs in their study [176]. The latter used a functional assay to measure IL-1 concentration and their data showed evidence for an inhibitor of IL-1 in SSc PBMC culture supernatants.

1.6 Hypoxia in SSc.

SSc is characterized by microvascular abnormalities and an overall capillary loss in lesional skin. In addition, the deposition of extracellular matrix in the lesional tissue increases the distance between cells and their blood supply and interrupts the diffusion of oxygen to the tissues. Thus, one would expect to find that the lesional tissues in SSc suffer from a degree of hypoxia. Two separate groups have confirmed that this is the case [62 177]. Involved skin was found to be hypoxic and the degree of hypoxia correlated with the skin thickness, adding weight to the hypothesis that the laying down of extracellular matrix contributes to hypoxia [177]. Oxygen levels in lesional (but not non-lesional) skin were found to be around 3% [62], sufficient to induce hypoxia induced gene expression.

Hypoxia induced gene expression frequently occurs via an increase in HIF1α stability and a resultant HIF1α-dependent gene transcription. Therefore, it is somewhat surprising that HIF1α expression is not increased in the lesional skin of patients with SSc; in fact the expression seems to be lower than in controls [60 62]. The reason is not clear, but possible reasons will be explored in later chapters. What is apparent, however, is that proteins that are typically increased in response to hypoxia are elevated in the skin of patients with SSc, including VEGF, PDGF and endothelin-1, as mentioned above. It is possible that hypoxia-independent mechanisms increase the levels of these growth factors or perhaps the

expression is controlled by alternative hypoxia-inducible pathways e.g. HIF2α, HIF3α or activating protein-1 (AP-1).

Hypoxia may have an active role in perpetuating the disease process in SSc. Hypoxia is present in the skin of patients of SSc as mentioned, but is not a persistent feature of Raynaud's phenomenon. Although short-term hypoxia causes adaptive responses to increase angiogenesis and vasculoneogenesis and changes in vascular tone to increase blood flow, long term hypoxia results in plastic changes. These changes include proliferation of smooth muscle cells that may result in intimal hyperplasia, as seen in pulmonary artery hypertension and SSc renal crisis and fibrosis. In addition, persistently increased levels of VEGF, which can be induced by hypoxia, can result in disorganised angiogenesis and vasculoneogenesis that can closely resemble the SSc phenotype [61].

Hypoxia leads to an induction of collagen genes and collagen-modifying enzymes in many tissue types, including endothelial cells. Dermal fibroblasts express increased concentrations of extracellular matrix proteins in response to hypoxia in culture [178-180]. This change was time-dependent and proportional to the degree of hypoxia. In a mouse model of normobaric hypoxia, increased secretion of extracellular matrix proteins occurred as soon as 24h [181]. Antibodies against TGF1β abrogated the effects of hypoxia on fibroblast extracellular matrix expression in healthy control and SSc fibroblasts in culture [180]. These data imply a central role for TGF1β in hypoxia-induced fibrosis.

1.7 Oxidative stress in SSc.

There is accumulating evidence that oxidative stress may be important in the pathogenesis and perpetuation of SSc. Ischaemia-reperfusion injury, which is presumed to occur after repeated episodes of Raynaud's phenomenon, causes an increase in free radicals in the form of ROS and reactive nitrogen species. In addition, inflammation leads to increased production of reactive species.

These free radicals are highly reactive and contribute to what is termed “oxidative stress”. Many cell types can produce ROS; phagocytes (neutrophils and macrophages) have a NADPH dependent oxidoreductase and the ROS are used as an antimicrobial system. Other cell types including endothelial cells, chondrocytes, lymphocytes and fibroblasts express NADPH-oxidase which can produce $OH^{\cdot}$, $O_2^{\cdot -}$, and H_2O_2.

Free radicals can cause damage to proteins, lipids and DNA and can lead to irreversible cell membrane damage and cell death via necrosis or apoptosis. Endothelial cells are particularly vulnerable to free radical damage as they lack the protective enzyme, catalase [182]. Products of lipid peroxidation can also be cytotoxic and alter T-cell/macrophage interactions [183]. Oxidation of lipoproteins can result in products which are chemotactic for monocytes, and cytotoxic to endothelial cells and smooth muscle cells [184]. In addition, they can stimulate the formation of connective tissue and promote interactions between monocytes and endothelial cells [185 186].

Fig. 6. Oxidative stress in the pathogenesis of SSc. Oxidative stress leads to endothelial damage, perivascular immune cell infiltrate and fibrosis. The process is self perpetuating. This material has been removed from the abridged version but can be found in Simonini et al [3] Figure 1.

ROS also act as second messengers in many receptor ligand pathways, and can lead to the differentiation of many cell types including immune cells, fibroblasts, pericytes and vascular smooth muscle. VEGF, PDGF and TGFβ can all act through NADPH oxidase dependent pathways. Angiotensin II and thrombin can also activate NADPH oxidase [11].

By-products of oxidative damage can be measured in the plasma and urine. Malondialdehyde (MDA) is the end product of lipid peroxidation and serum levels have been validated as marker of oxidant stress. Levels were found to be 10-fold higher in SSc serum

than in control serum especially in early disease [187] [188]. Erythrocyte MDA levels were also elevated in patients with SSc [188]. Isoprostanes, which are products of free radical catalysed arachidonic acid peroxidation, can be measured in various biological fluids. They are stable in urine where non-invasive monitoring can be performed. Levels change in models of oxidant injury and in response to antioxidant treatments, but are not affected by the lipid content of the diet. Their levels are increased in SSc and are associated with early stages of the disease, but not with primary Raynaud's phenomenon [189]. They correlate with pulmonary fibrosis and with nailfold capillary abnormalities, but not with the degree of skin involvement [190] [191].

Cracowski *et al* demonstrated that not only were the F_2-isoprostanes elevated in the urine of patients with SSc, but that levels correlated with post-occlusive hyperaemia, a measure of endothelial cell function in these patients [192].

Serum 8-isoprostane levels are increased in diffuse and limited SSc, and correlate negatively with lung function and positively with renal blood flow. Levels also correlated with serum IgG concentrations and anti-agalactosyl levels [190]. This last point is of interest as agalactosyl IgG is thought to self-aggregate to form immune complexes [193] [194]. Immune complexes can bind to the Fc receptors of neutrophils and monocytes, activating them and increasing their production of ROS.

The same authors also found elevated levels of another marker of oxidative stress, namely N^{ε}-(hexanoyl)lysine (HEL), to be elevated in SSc. They did not identify any difference between limited and diffuse disease but again, found levels to be correlated with the anti-agalactosyl IgG antibody concentration and rheumatoid factor titre [195]. In this study however, they also found that patients with systemic lupus erythematosus (SLE) and dermatomyositis had elevated levels HEL, and in fact levels were even higher in these patients. Although these patients can suffer with Raynaud's phenomenon, they do not tend to have major episodes of ischaemia-reperfusion as seen in SSc. This might argue against ischaemia-

reperfusion being the main instigator of oxidative stress and may point towards inflammation as the cause of increased oxidative stress, as inflammation is more prominent in these autoimmune conditions. Equally, primary Raynaud's phenomenon is not associated with an increase in markers of oxidative stress and therefore if ischaemia-reperfusion is involved in the generation of ROS in SSc, it is more likely to be due to the chronic ischaemia that can occur in this condition [11].

One group of investigators chose to measure markers not only of lipid peroxidation (ROOH), but also markers of protein oxidative damage (sulphydryl and carbonyl groups) in the plasma. They found that the markers of lipid peroxidation were raised in SSc. While sulphydryl groups were decreased in the plasma, consistent with oxidative protein damage in SSc; carbonyl groups, however, were unchanged. Markers of lipid peroxidation correlated with changes measured by nailfold capillaroscopy, whereas markers of oxidative protein damage correlated with Rodnan's skin score and pulmonary fibrosis (carbonyl) and gastrointestinal involvement (sulphydryl) [196]. The reasons for these discrepancies are unclear, but *in vitro* oxidative changes to proteins occur rapidly *ex vivo* and therefore there may be non-specific changes occurring in the plasma samples, especially the samples for carbonyl assessment that were frozen after 1h and analysed after 24h. The study is also limited by the relatively small sample size, and so the associations with disease manifestations might be less specific were the sample size to be increased.

Other markers of lipid peroxidation include diene-conjugates which were also raised in the serum of patients with SSc and were higher both in the limited form of the disease, and early in the disease course [3].

Genetic polymorphisms in the glutathione-S-transferase enzyme (which acts as a free-radical scavenger) are associated with SSc [197]. The anti-oxidant capacity in SSc also appears to be decreased [198]. Elements such as selenium and vitamins such as vitamin-C, vitamin-E and carotene are decreased in patients with SSc, and this does not appear to be

due to a dietary anomaly [19 199]. Malabsorption does not seem to contribute to the decreased plasma levels except when there is evidence of bacterial overgrowth [200]. It may simply be, however, that the normal anti-oxidant capacity is overwhelmed by persistent elevations in ROS, resulting in a consumptive decrease in detectable anti-oxidants.

Attempts have been made to assess the total anti-oxidant capacity in venous blood from patients with SSc compared to controls, using a ferric reducing assay [201]. It was found that the total anti-oxidant capacity was unchanged in SSc. The reason for this is unclear and appears to counter the previously published evidence that consistently shows a decrease in individual antioxidants. This may suggest that unmeasured antioxidants may be compensating for the relative lack of anti-oxidants measured in other studies. The venous levels of antioxidants may not be as important as the local tissue levels where ROS are being generated and oxidative damage is occurring.

Exposure of endothelial cells to ROS results in damage to the cell membrane and degeneration and reduplication of the basement membrane, changes that can be typically seen on histological samples from patients with SSc. ROS also increase the expression of adhesion molecules and increase vascular permeability. Damage to the endothelial cell layer by ROS results in a loss of vascular tone control and a pro-thrombogenic state [11].

There is evidence that ROS can cause damage to and fragmentation of antigens in a metal-dependent manner, resulting in the exposure of neo-antigens and the promotion of SSc associated auto-antigen responses [202].

Oxidative stress is reported to promote fibrosis. Fibroblasts explanted from the skin of SSc patients spontaneously produce more O_2^- and H_2O_2 through the NADPH oxidase complex than control fibroblasts [203]. Control fibroblasts can be stimulated to produce ROS in response to IL1-β, PDGF BB, TGFβ and H_2O_2 but SSc fibroblasts cannot be stimulated by these factors to produce any further increase in ROS, implying that they are maximally stimulated

already [203]. Constitutive ROS generation in SSc fibroblasts was not decreased in response to superoxide dismutase, catalase, IL-1Ra, anti-TGFβ or anti-PDGF BB. However, it was decreased by the intracellular anti-oxidant *N*-acetyl cysteine. In addition, this anti-oxidant decreased the proliferative capacity and collagen mRNA expression of SSc fibroblasts [203]. This data implies that constitutive ROS generation by SSc fibroblasts may occur via a positive autocrine feedback mechanism and that it may be involved in the fibrogenic phenotype of these cells.

ROS can increase the release of the profibrotic cytokine TGFβ from pulmonary epithelial cells and can activate TGFβ by displacing it from latency-associated peptide [204 205]. H_2O_2 can stimulate the Ets dependent transcription of TGFβ and collagen I synthesis [206 207].

The hypothesis that oxidative stress has a pivotal role in SSc pathogenesis would be stronger if treatment with anti-oxidants resulted in evidence of improvement in clinical trials. However, results so far have been disappointing [208-210]. This may result from the high turnover of ROS, which are highly reactive, the inability to control local levels of oxidant stress and limitation of trials to date. Clinical trials in SSc are hampered by the lack of dynamic, sensitive, reproducible outcome measures, the relative rarity of the condition and the inherent heterogeneity in the disease phenotype. Another group of therapies that may impact this process are vasodilators, which may decrease ROS by suppressing vasospastic events and therefore decreasing ischaemia reperfusion. To this end, prostacyclins have been associated with a normalization in oxidative stress [211]. Prostacyclins also have direct effects on phagocyte function and therefore may have an additional affect on immune generated ROS.

1.8 Neutrophils in SSc.

Neutrophils have been largely overlooked in SSc. This may be due to the fact that neutrophils do not form a major part of the infiltrate in SSc lesional skin and also because

SSc sclerosis is not traditionally thought of as an inflammatory disease. Although SSc is not usually associated with a rise in inflammatory markers, inflammation, depending on the definition used, is very much a part of this disease, in this respect it shares many features with atherosclerosis. Inflammation is more prominent in the early stages of the SSc, which is associated with an immune cell infiltrate in lesional tissues and higher expression of pro-inflammatory cytokines. However, inflammatory features persist throughout the disease, including markers of endothelial cell activation and continued tissue expression of IL-6. Oxidative stress continues throughout the disease and may be the result of ischaemia-reperfusion injury and immune cell activation. Ischaemia–reperfusion is associated with a pronounced neutrophil activation and binding of neutrophils to endothelial cells, which then initiates a local inflammatory cascade.

Neutrophils are not prominent in the immune cell infiltrate of lesional skin in SSc; however, Hussein *et al* showed that, unlike lymphocytes, neutrophils are numerically increased in the skin of patients with SSc [142]. Endothelial cells are activated in SSc and there is an increase in the expression of cell adhesion molecules on the surface of endothelial cells. Therefore, it is likely that neutrophil adhesion to SSc endothelial surfaces will be increased. If, however there is no chemotactic pressure for neutrophils to infiltrate into the skin they may not form a significant part of the infiltrate. Endothelial cells produce increased levels of neutrophil chemoattractants e.g. IL-8 in SSc early in the disease [79]. The role of neutrophils in inflammatory processes is usually an acute and relatively transient one, and therefore examining lesions at a single static time point may not reveal the true role for neutrophils in this disease.

Neutrophils are the most abundant circulating leukocyte. They are capable of producing ROS in significant quantities and are a potent source of proteases. ROS from neutrophils could make a significant contribution to the oxidative stress seen in SSc, if ROS were released locally at the endothelial surface. Proteases also play complex roles in disease.

Proteases can be directly cytotoxic but in addition, it is becoming increasingly clear that proteases can play important regulatory roles in cell signalling.

1.8.1 Neutrophil ROS and reactive nitrogen species generation in SSc

The published literature regarding neutrophil ROS generation in SSc is highly contradictory. This may reflect the heterogeneity of the disease and the recruitment of different disease populations by different groups. However, it more likely reflects the different methodologies used. There are numerous methods for measuring neutrophil ROS generation but each has their limitations and specificity for different species of oxidant. Some methods, for instance only measure either intracellular (DHR-123) or extracellular ROS (horseradish peroxidase dependent oxidation of phenol red). Others, for instance luminol-enhanced chemiluminescence, are capable of measuring both intra- and extra-cellular ROS. Another important difference between assays is the precise nature of the ROS measured. Some specifically measure superoxide (cytochrome C) whilst others measure H_2O_2 (DHR-123, phenol red), and for others the precise species measured is unclear (chemiluminescence). It is important to note that DHR-123 can be affected by reactive nitrogen species as well as ROS, both of which have been implicated in SSc. The last important point to consider is whether and how neutrophils were separated from whole blood samples. Many isolation methods which rely on sedimentation over substances such as dextran, have been shown to activate neutrophils, and techniques using density gradients are preferable [212-214]. It is possible to measure ROS generation in whole blood, which has many advantages; cells are not activated by separation procedures and the assays can reproduce the physiological environment preserving many of the cytokine and leukocyte interactions that occur *in vivo*. However, whole blood techniques do not discriminate which cell type is producing ROS, and while neutrophils have been shown to be the main producers of ROS in whole blood, this may depend on the exact circumstances, and other cell types in peripheral blood are capable of producing ROS (e.g. monocytes).

Some papers report an increase in ROS production by SSc neutrophils compared to controls. Maslen *et al*. in 1987 initially reported that following Percoll gradient isolation and using horseradish peroxidase dependent oxidation of phenol red dye to measure extracellular H_2O_2 secretion, unstimulated SSc neutrophils produced more ROS than controls [215]. In addition, they reported that fMLP stimulated ROS production was no different in SSc neutrophils compared to controls when corrected for endogenous production. They did, however, find that the increase in ROS generation in response to immune complexes was greater in SSc neutrophils than in controls. They hypothesized that this may reflect an increase in Fc receptor expression on the surface of SSc neutrophils as immune complex induced ROS production is mediated by these receptors. In support of this hypothesis, they found that SSc neutrophils increased the proportion of EA-rosette formation for given concentrations of sensitizing IgG. However, they did not directly measure the Fc receptors (CD16 or CD32) on the neutrophil surface. The same authors went on to perform further studies with similar methodologies and showed that PMA stimulated neutrophils from SSc patients produced similar ROS levels compared to controls.

In agreement with this study, Czirjak *et al*. also reported an increase in basal ROS production in SSc whole blood using luminol enhanced chemiluminescence, which measures both intracellular and extracellular ROS [216]. They also reported that zymosan stimulation of neutrophils resulted in a similar percentage increase in ROS generation in both SSc and control neutrophils. They made the assumption that the main source of ROS was neutrophils, but it is interesting to note that others have demonstrated an increase in unstimulated ROS production in SSc monocytes and the magnitude of this increase was much greater than that seen in SSc neutrophils [167]. Therefore, it is not quite clear whether the assumptions regarding whole blood assays are valid. Czirjak *et al*. also reported that there were, on average, slightly higher levels of immune complexes in SSc serum compared to controls, but no relationship was found with the functional tests. They did not find any increase in EA cell binding.

Finally, a recent paper using whole blood to examine intracellular H_2O_2 production using DHR-123 found that basal ROS generation was actually decreased in SSc neutrophils compared to controls [217]. This study had strengths because it benefited from the advantages of whole blood assay systems i.e. maintaining *in vivo* cellular and cytokine interactions but in addition it allowed the authors to explore which cell type was producing the ROS. So, ROS generation in this system can be measured by cellular fluorescence on a flow cytometer, while the different cell types can be gated separately on the flow cytometer, according to typical forward and side scatter properties. Then, fluorescence generation in response to ROS can be assessed in neutrophil and monocyte populations separately. Also, this method can take into account cellular heterogeneity as the fluorescence of each cell will be recorded and the variance in ROS generation can be assessed. The disadvantage of this system however, is that DHR-123 is an intracellular probe which is converted to a fluorescent species following interaction with cellular esterases and H_2O_2. Experiments have shown that intracellular ROS generation does not reflect production by plasma membrane NADPH oxidase and passive diffusion into the cell [218]. In fact, scavenging of extracellular ROS makes no difference to ROS measurements by DHR-123. Therefore, this method does not account for the potentially important secretion of ROS by neutrophils.

The decrease in ROS production seen by this group therefore may reflect the maintenance of important cellular and cytokine reactions or it may reflect an inability to capture ROS secretion that is measured by other methods. All of these studies, therefore, may reflect an increase in ROS secretion by NADPH oxidase and a decrease in intracellular ROS generation. It is also important to note that DHR-123, used in this study can also react with reactive nitrogen species and increased nitric oxide production by neutrophils has been inferred in one study [219].

One group has reported that the adenosine receptor A_{2B} expression is down regulated in SSc neutrophils compared to controls and they postulated that as adenosine is known to

decrease neutrophil ROS generation, that down regulation of this receptor may contribute to the observed increase in SSc neutrophil ROS generation [220].

1.8.2 Other neutrophil functions.

Other neutrophil functions have been occasionally investigated in SSc. Neutrophil chemotaxis and random migration have been studied by two groups: the first reported no differences in chemotaxis or random migration of SSc neutrophils compared to controls, the other reported decreased SSc neutrophil chemotaxis [216 221]. The differences may reflect methodological differences, since they used different stimulants for chemotaxis, and the former group did not stipulate how long they allowed migration to take place. Both groups used the modified Boyden chamber and the leading-front method. In our experience, this method is difficult to reproduce consistently, and is operator dependent. Both groups used dextran sedimentation to isolate neutrophils, which can cause significant neutrophil activation, and therefore small but relevant changes in function may have been negated.

The same reports also explored neutrophil phagocytosis. Neither group found any difference in the ability of neutrophils to phagocytose opsonised yeast, although the above comments about neutrophil preparation should also be taken into account [216 221].

No group has directly compared degranulation or protease release from SSc neutrophils compared to controls. However, one group explored the serum levels of neutrophil elastase and found them to be elevated in SSc serum and to correlate with markers of SSc lung disease [222].

1.8.3 Neutrophil function following treatment with Iloprost.

Iloprost is a prostacyclin agonist that is known to be useful in the treatment of vascular dysfunction in SSc, not only due to short term vasodilatory effects but also because of longer term, immuno-modulatory effects lasting up to 3 months. It is

therefore extremely interesting to note that Iloprost infusions (*in vivo*) have a profound effect on neutrophil function (*ex vivo*). Mazzone *et al*. examined neutrophil ROS generation and integrin expression before and 6h after a continuous infusion of Iloprost, and found a significant decrease in ROS generation and integrin expression in patients with SSc [223].

However, this effect was not disease specific and was also seen in patients with ischaemia due to peripheral vascular disease, with no clinical evidence of SSc. Therefore, this seems to be a consequence of ischaemia rather than SSc specifically. Most patients with ischaemia do not generate auto-antibodies, or experience the widespread fibrosis that is a feature of SSc. This study implies an important role in endothelial dysfunction and damage, but does not suggest a wider role for neutrophils in the disease phenotype. The dynamics of the repeated ischaemia seen in SSc, however, may provoke the fibrotic and autoimmune phenotypes, and neutrophils may still play an important role in this.

1.9 Summary.

There is evidence that neutrophils could be important in the pathogenesis of SSc. Although, they do not form the predominant immune cell infiltrating lesional skin in SSc patients, they are found at increased levels in the skin compared to controls [142]. In addition, these features are only seen in skin with established fibrosis. Neutrophils are often an early and transient feature of immune cell infiltrates giving way later to chronic inflammatory cells, such as monocytes and cells of the adaptive immune system.

Neutrophil chemotactins are elevated in the serum, the lesional tissue and bronchoalveolar lavage fluids in patients with SSc and endothelial cells express increased levels of leukocyte adhesion molecules [79 224-226]. Therefore, it seems likely

that neutrophils would be attracted to endothelial cell lesions that they may be initiating inflammation and the resultant fibrosis in this disease. Binding of neutrophils to leukocyte adhesion molecules on endothelial cells, would lead to neutrophil activation.

Finally, there is some evidence to suggest that neutrophils are activated in this disease and in particular that they produce ROS that could contribute to the increased oxidative stress in SSc, which may itself contribute to perpetuation of endothelial injury.

ROS generation however, is not the only feature of neutrophil biology that could potentially lead to endothelial injury. Neutrophils can release proteases, express and release cytokines, and release cytokine receptors, all of which could contribute to endothelial cell activation and dysfunction. Neutrophils, once thought to be rather limited in their immune repertoire, have recently been shown to have multiple and complex functions in inflammation, including their ability to interact with cells of the adaptive immune system via expression of MHC class II, antigen presentation and cytokine production [227].

Previous studies have shown that the adaptive immune system alone is not sufficient for the fibrotic phenotype in animal models[165], pointing to the importance of investigating the innate immune system in this disease, including the role of the neutrophil.

1.10 Aims of this thesis.

Conditions exist in SSc that are likely to promote neutrophil activation. Neutrophil derived mediators, including ROS, proteases, cytokines and cytokine receptors could lead to pathogenically relevant effects on endothelial cell activation and damage, promotion of chronic inflammation and fibrosis.

In this thesis, I aim to explore the hypothesis that: *"Neutrophils are activated in SSc and contribute to endothelial cell activation and injury"*.

The specific aims are:

1. To define the functional phenotype of neutrophils isolated from patients with SSc. This will involve measurements of ROS generation, chemotaxis, apoptosis, integrin expression and CD16 expression (reported in Chapter 3).
2. To explore the *ex vivo* protein expression of SSc neutrophils compared to control neutrophils looking specifically for evidence of neutrophil priming or activation. The pan-proteome will be explored using the DIGE technology (Chapter 4) and the membrane proteome will be specifically explored using the iTRAQ technology (Chapter 5).
3. To investigate a possible role for neutrophil elastase in SSc (Chapter 6).
4. To model interactions between neutrophils and endothelial cells *in vitro* and to explore the role of neutrophil-derived mediators in these interactions (Chapter 7).

Chapter 2: Materials and methods.

2.1 Materials.

Annexin V-FITC (Biosource), Anti-CD11b antibody APC conjugated (Miltenyi), Anti-CD16 antibody APC conjugated, mitotracker red, trizol, GAPDH primers, sIL6R ELISA (Invitrogen), 4 Nitrophenylphosphate disodium, mouse polyclonal anti-H3 histone antibody, mouse monoclonal anti-α tubulin antibody, ATP, catalase, elastase, interleukin-6, iodoacetamide, luminol disodium salt, lysophosphatidylcholine, MMTS, phalloidin-FITC, N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide propidium iodide, TCEP, TEAB, TritonX-100, CHAPS, colloidal coomassie blue, PMSF, AEBSF (Sigma), BCA protein assay kit, direct IP kit, Albumin/IgG dye based depletion kit (Pierce), calcein-AM, rabbit monoclonal anti-neutrophil elastase antibody, TNF (Calbiochem), sheep polyclonal anti-S100A8 antibody (R&D systems), CD14 positive selection kit (Stem cell technologies), anti-CD16 antibody FITC conjugated (BD biosciences), CD3 positive selection beads (Dynal), anti-CD62E antibody APC conjugated, rabbit monoclonal anti-MHC class I antibody, anti-ICAM1 antibody PE conjugated, rabbit polyclonal anti SERPIN B1 antibody, rabbit polyclonal anti-interleukin-6 antibody (Abcam), complete mini protease inhibitor tablets, GMCSF (Roche), DHR-123 (molecular probes), human dermal microvascular endothelial cells, endothelial cell growth media MV (Promocell), mouse monoclonal anti eosinophil peroxidase antibody (Chemicon), hanging cell culture insert, Micron MWCO devices, Immobilon ECL, luminex 13plex assay kit (Millipore), ITRAQ multiplex kit, trypsin (Applied biosystems), PMN elastase ELISA, sICAM1 ELISA (Bender), Polymorphprep (Axis Shields), random primers, trypsin TLCK (Promega), Rapid Romanowsky stain (HD supplies), RPMI+ 2mM glutamine+ 25mM HEPES (Gibco), Sorvall tubes 5ml, non-TC coated white 96 well plates (Fisher), Urea, Thiourea, Cy2, Cy3, Cy5 (GE Healthcare), MgAc, acetonitrile, ambic (BDH), 30% polyacrylamide, Bio-Lytes 3-10, IPG strips 17cm pH 3-10 non linear, RCDC protein assay (Bio-Rad), DTT (Melford), RNAeasy RNA extraction kit (Qiagen), sheep monoclonal Anti-MPO antibody (Biodesign).

2.2 Methods.

2.2.1 Neutrophil isolation.

The study was approved by the Sefton Local Research Ethics Committee, in accordance with the Helsinki declaration. Informed written consent was taken from patients with SSc [2] and from healthy volunteers. 30ml of heparinised venous blood was taken from the subjects.

Peripheral blood was separated into neutrophil and mononuclear cell fractions using Polymorphprep (as described in the manufacturer's instructions). Contaminating erythrocytes were removed using ammonium chloride lysis buffer ($KHCO_3$ 3.4mM, NH_4Cl 155mM, EDTA 96.7µM). Neutrophils were routinely examined for purity using morphological analysis of cytospins after staining with Rapid Romanowsky; purity was >95% immediately after isolation. Neutrophils were resuspended in RPMI 1640 +25mM HEPES +2mM glutamine at a concentration of $5x10^6$ cells/ml.

2.2.2 Extraction of mRNA.

Neutrophils were isolated from control and SSc blood as previously described and resuspended in RPMI 1640 +25mM HEPES +2mM glutamine at $5x10^6$/ml. $1x10^7$ neutrophils were pelleted for each sample. The supernatant was removed and 1ml of TRIzol reagent was added to the cell pellet. The TRIzol was mixed by pipetting until the cell pellet was completely lysed. The lysate was incubated for 5 min at room temperature. 200µl of chloroform was added in the fume hood. The mixture was agitated for 15s, and incubated for 5 min at room temperature. The sample was centrifuged at 12000*xg* for 15 min at 4˚C to separate the sample into RNA, DNA and protein layers. The upper phase (RNA) was removed to a clean, RNase free microfuge tube and an equal volume of isopropanol was added and incubated overnight at -20˚C to precipitate the RNA. RNA precipitate was stored at -80˚C until required or converted immediately to cDNA for polymerase chain reaction (PCR).

2.2.3 Preparation of cDNA.

RNA precipitate was collected by centrifugation at 12000*xg* for 30 min at 4˚C. The supernatant was discarded and the pellet washed with 70% (v/v) ethanol/ DEPC treated ddH_2O. The pellet was recovered by centrifugation at 12000*xg* for 5 min, the supernatant removed and the pellet air dried. The sample was resuspended in RNase free H_2O and quantified using the Nanodrop (Thermo) (OD 260/280nm). The sample was cleaned using a RNA extraction kit (Qiagen RNeasy) according to the manufacturer's instructions for clean up.

1µl of random primers were added to 1µl of RNA (10pg-5µg) and 1µl of 10mM dNTP mix, the total volume was made up to 13µl with nuclease free H_2O. The reaction was incubated at 65˚C for 5 min and then 1 min on ice. 4µl of 5x First Strand Buffer, 1µl of 0.1M DTT, 1µl of RNaseOUT and 1µl of SuperScript III reverse transcriptase (200u/µl) were added. The reaction was incubated at 25˚C for 5 min and 50˚C for 1 h, and the reaction stopped by heating to 70˚C for 15 min.

2.2.4 Real Time polymerase chain reaction for quantification of mRNA.

Primers were designed using Primer3Plus, and are listed in Table 3. cDNA was mixed with Quantitect at a ratio of 1:5: forward and reverse primers specific to the gene of interest were combined and diluted to 4µM with nuclease free H_2O. 6µl of cDNA mix and 4µl of primer mix were combined in a nuclease free tube and the PCR reaction monitored on the RotorGene 3000 (Corbett). Initial reactions were performed on serial dilutions of control cDNA to define the efficiency of the primers. Following this, relative quantification of mRNA expression was calculated using the modified Pfaffl equation using observed primer efficiencies and normalized to cyclophilin A expression.

Gene	Forward primer	Reverse primer	Efficiency
HIF1α	TGCTCATCAGTTGCCACTTC	TCCTCACACGCAAATAGCTG	1.2
Interleukin 6	AGGAGACTTGCCTGGTGAAA	CAGGGGTGGTTATTGCATCT	0.99
Cyclophilin A	GCTTTGGGTCCAGGAATGG	GTTGTCCACAGTCAGCAATGGT	1.4

Table 3. Primers

2.2.5 Western blotting.

2.2.5.1 Preparing samples for western blotting.

Neutrophils were isolated from control and SSc blood, as previously described; $5x10^6$ neutrophils were pelleted, the supernatant was completely aspirated and 10µl/$5x10^5$neutrophils of hot SDS-PAGE reducing buffer (see Appendix A) was added. The samples were incubated at 100˚C for 3min or until the pellet had completely lysed and then stored at -20˚C prior to use.

2.2.5.2 SDS PAGE.

Whole cell extracts were loaded onto polyacrylamide gels, the percentage acrylamide content varied according to the size of the protein of interest (see Table 4).

% acrylamide content of gels	Relative Molecular mass range (kDa)
7	50-500
10	20-300
12	10-200
15	3-100

Table 4. Acrylamide content

In general, 10µl of sample was loaded per lane but this varied according to the abundance of the protein of interest. Biotinylated ladders were loaded for reference. Following electrophoretic separation using standard running buffer (see Appendix A), proteins were transferred to nitrocellulose membranes using BioRad Mini Protean II Transfer apparatus (for transfer buffer recipe see Appendix A). Membranes were stained with Ponceau S to confirm successful protein transfer and were then destained with wash buffer (see Appendix

A). Membranes were blocked with blocking buffer (5% w/v Marvel in wash buffer) for 1h at room temperature. Membranes were briefly washed with wash buffer and then incubated with gentle agitation in primary antibody buffer (blocking buffer containing optimized concentration of antibody to the protein of interest) overnight at 4˚C. For details of antibodies used, see Table 5. Membranes were washed for 2x30s, 2x5min, 1x15min in wash buffer before incubation with secondary antibody buffer (blocking buffer containing HRP-conjugated, species specific secondary antibody and an HRP conjugated anti-biotin antibody) for 1h at room temperature. Following thorough washing (as above) the membranes were treated with excess ECL reagent (Millipore) for 5 min and then imaged on a G-BOX (Syngene). Quantification was performed using Image-J (NIH, USA) and was normalized to tubulin expression.

Protein of interest	**Clonality**	**Source**	**Concentration used**	**Host species**
Calgranulin A/B	Polyclonal	R&D	1:200	Sheep
Elastase	Monoclonal	Calbiochem	1:5000	Rabbit
SERPIN B1	Polyclonal	Abcam	1:5000	Rabbit
HLA	Monoclonal	Abcam	1:10000	Rabbit
Histone	Monoclonal	Sigma	1:10000	Rabbit
MPO	Monoclonal	Biodesign	1:10000	Sheep
Interleukin-6	Polyclonal	Abcam	1:10000	Rabbit
Tubulin A	Monoclonal	Sigma	1:10000	Mouse

Table 5. Antibodies.

2.2.5.3 Tris-Tricene SDS-PAGE for quantification of low molecular mass proteins.

Tris-Tricene SDS-PAGE was used for the improved quantification of Calgranulin-A by western blotting, as compared to traditional gels they improve the resolution of low molecular mass proteins at the same percentage of acrylamide. In addition, the gels improve the resolution of hydrophobic proteins. The acrylamide concentration of traditional gels cannot be increased beyond 15% or the gels become too brittle to handle. Tris-Tricine gels contain 13% glycerol in order to make them less brittle.

Solution component	Resolving gel (mL)	Stacking gel(mL)
30% (w/v) Acrylamide: 0.8% (w/v) bis-acrylamide	7.5	0.83
H_2O	0.44	3.72
3M Tris, 0.3% (w/v) SDS pH 8.45	5	1.55
Glycerol	2	
10% (w/v) ammonium persulphate	0.15	0.05
TEMED	0.005	0.005

Table 6. Recipe for 2x 1.5mm 15% Tris-Tricene mini gels.

Instead of standard running buffer, two separate buffers are used in the electrophoretic separation (see Fig. 7). The inner chamber between the two gels is filled with Inner chamber buffer (see Appendix A) and the outer chamber is filled with Outer chamber buffer (see Appendix A). Protein is separated at a constant current of 70-80 mA until the dye front runs off (~4h). Thereafter, the process was as per traditional western blotting.

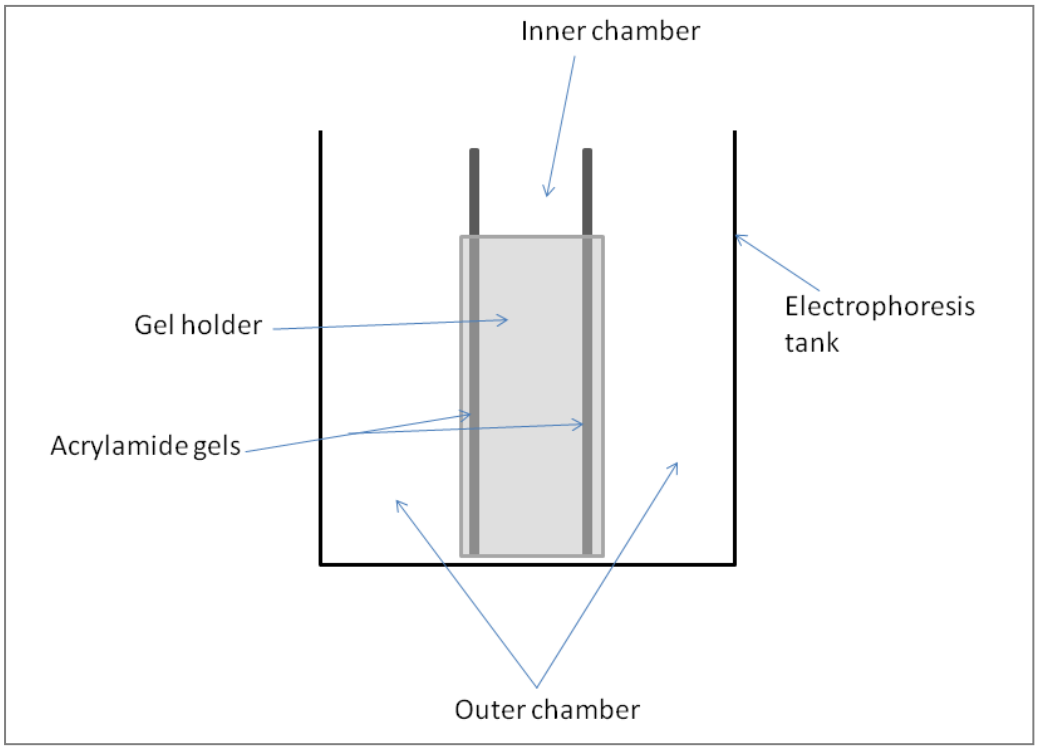


Fig. 7. Set up for Tris-tricene gels.

Chapter 3: Neutrophil function in SSc.

3.1 Introduction

Products of neutrophil activation including cytokines, ROS and granule enzymes could potentially mediate endothelial cell activation and dysfunction, which are central to the pathogenesis of SSc.

Concentrations of neutrophil chemoattractants IL-8 and LTB_4 are elevated in the lesional tissue, serum and bronchoalveolar lavage fluid of patients with SSc and endothelial cells from patients with SSc express higher levels of leukocyte adhesion molecules [79] [224-226]. Neutrophils are activated following binding to these molecules, and their longevity is increased due to a decrease in apoptosis. In addition, circulating levels of proinflammatory cytokines (TNFα, IL-1β) that are capable of activating neutrophils are elevated in the serum of some patients with SSc especially in the diffuse disease and early in the disease course [79] [82] [142]. SSc fibroblasts and mononuclear cells also constitutively express IL-1 *in vitro*: thus local levels of IL-1 may be significantly elevated in lesional tissue in the absence of elevated serum levels [35] [175].

Several studies have previously examined neutrophil function in SSc, but the results are somewhat contradictory [215] [217] [221] [228]. This is most likely due to methodological differences in the measurements of neutrophil function but also in the methods used for neutrophil separation.

Neutrophils are traditionally thought to be terminally-differentiated cells with a narrow spectrum of biological responses and limited capacity for *de novo* protein expression. However, recent evidence demonstrates that neutrophils are capable of complex roles in the immune system including: cytokine production, antigen presentation and interaction with cells of the adaptive immune system [227] [229].

In this Chapter, I explore a range of functions in SSc neutrophils compared to control neutrophils, using neutrophil separation methods which result in minimal cell activation.

3.2 Methods.

3.2.1 Luminol enhanced chemiluminescence.

Two assays were used to measure chemiluminescence. Neutrophils from healthy controls at $5x10^6$/ml were either primed with TNF for 20min or unprimed. For the tube assay, $1x10^6$ neutrophils were diluted to 750µl with HBSS warmed to 37˚C. 1µl of 10mM luminol (sodium salt) dissolved in ddH_2O was added and 250µl of either control or SSc serum was added as a stimulant. 1µl of fMLP (1µM, final conc.) was added to further samples as a positive control. Tubes were agitated and chemiluminescence was measured using a luminometer every 30 s over 45 cycles.

For the microplate assay, neutrophils from patients or controls at $1x10^6$/ml were either primed with TNF for 20min or unprimed. $5x10^4$ neutrophils were added to wells of a 96-well, white plastic, non-coated plate. HBSS was warmed at 37˚C luminol (disodium salt) was added to a final concentration of 67µM, and to separate aliquots of this mixture, PMA and fMLP were added to a final concentration of 100ng/ml and 1µM respectively. 150µl of these stimulant mixtures were added to the wells and chemiluminescence was measured immediately on a Perkin Elmer microplate reader, every 30s for 80 cycles.

3.2.2 Phagocytosis and phagocytosis-stimulated ROS generation.

Neutrophils were isolated from control and SSc blood as previously described. Cells were resuspended in RPMI 1640 +25mM HEPES +2mM glutamine at $2x10^6$/ml. $2x10^6$ cells were transferred to a FACs tube and incubated with heat inactivated, propidium iodide (PI) labeled, opsonised *S. aureus* in the following ratios for 30min in the dark at room temperature; 2.5:1, 5:1, 10:1, 15:1, 25:1 bacteria: neutrophil. Cells were spun down and

resuspended in 250µl RPMI 1640 +25mM HEPES +2mM glutamine, ROS generation was assessed by incubation of the cells with 10µM DHR-123 for 15min. PI was measured on the PECy5 channel following excitation with the 488nm laser and rhodamine-123 was measured on the FITC channel following excitation with the 488nm laser on a Dako CyAn ADP flow cytometer.

3.2.3 Neutrophil chemotaxis.

Neutrophils were isolated from control and SSc blood, as previously described, and were resuspended at $5x10^6$/ml in RPMI 1640 +25mM HEPES +2mM glutamine. Disposable chemotactic chambers (Millipore) with a 3µm PTFE membrane were used (Fig. 8). The bottom chamber was filled with 800µl of RPMI 1640 +25mM HEPES +2mM glutamine ±10nM fMLP. The top chamber was filled with 200µl ($1x10^6$ cells) of freshly-isolated neutrophils. The chambers were covered and incubated at 37˚C without agitation for 90min. The top chambers were removed from the bottom chamber and placed into an empty 24well plate. The medium in both chambers was agitated by pipetting up and down 3 times to dislodge loosely adherent cells. The number of cells in each chamber was then counted on a coulter counter. Cells moving from the top to the bottom chamber in the absence of a chemotactic gradient represent the random motility of the cells. Cells moving from the top to the bottom chamber along a fMLP chemotactic gradient have undergone chemotaxis. Often, the total number of cells in the bottom and top chambers did not account for all the cells entering the system. This was interpreted as cells adherent to the membrane or plastic-ware.

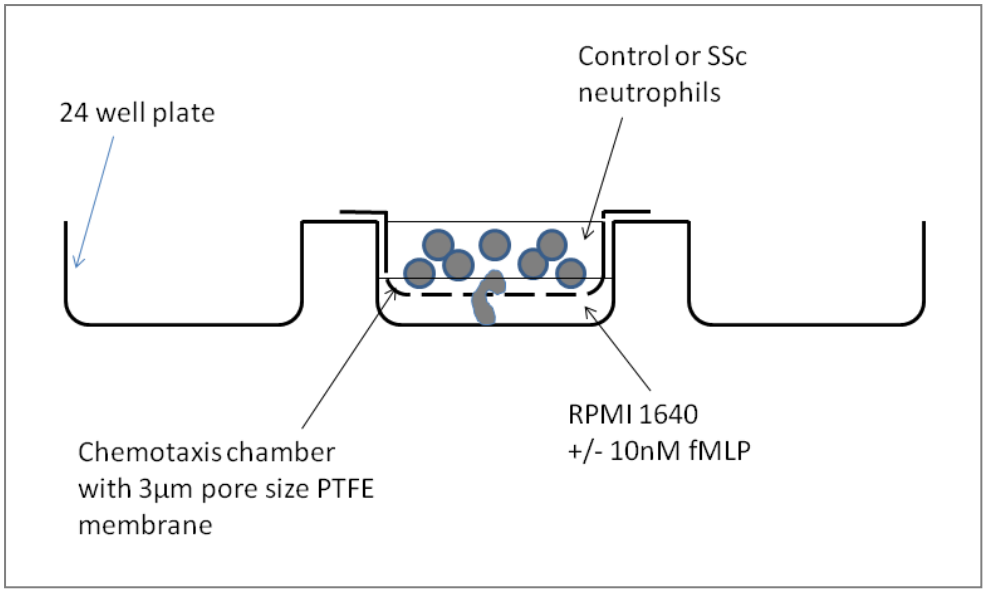


Fig. 8. Chemotaxis chamber set up.

3.2.4 Assessment of neutrophil apoptosis using annexin-V staining.

Neutrophils were isolated from control and SSc blood as previously described and resuspended at $5x10^6$ in HBSS. 100µl of neutrophil suspension was transferred to a FACs tube and incubated with 1µl of annexin V-FITC for 15min in the dark at room temperature. Annexin V labels phosphatidylserine residues exposed on the cell surface during apoptosis. Samples were made up to 1ml and analysed on the flow cytometer using the FITC channel, following excitation with the 488nm laser. Apoptosis was expressed as the percentage of annexin V labeled cells.

3.2.5 Labelling neutrophils for surface expression of CD16, CD11b and CD18 by flow cytometry.

Neutrophils were isolated from SSc and healthy control blood as previously described. Cells were resuspended in RPMI 1640 +25mM HEPES +2mM glutamine at $5x10^6$/ml. 100µl of cells were placed in a flow cytometry tube and excess PBS EDTA was added, cells were centrifuged at 1000*x*g for 3min and resuspended in 200µl of PBS 2%BSA. Cells were then incubated for 30min in the dark at 4˚C with 2µl of allophycocyanin (APC)-conjugated anti-

CD16 antibody, 2µl of APC-conjugated anti-CD11b antibody or 4µl of PE-conjugated anti-CD18 antibody. Cells were washed x1 with PBS and analysed on the flow cytometer. Cells were gated to include only neutrophils according to characteristic forward and side scatter characteristics. APC emission was measured at 660nm following excitation with the 635nm laser; PE was measured at 575nm following excitation with the 488nm laser. When labeling for CD16-APC a simultaneous assessment of eosinophil number was made measuring spontaneous fluorescence on the PE-Cy5 channel, which is characteristic of eosinophils. This assessment was made because eosinophils are reportedly increased in the circulation of patients with SSc [142] and would fall inside the neutrophil gate on forward and side scatter but would not express CD16.

3.2.6 The role of ROS in neutrophil: endothelial cell co-cultures.

HDMECs were cultured in 48 well plates in microvascular endothelial cell medium (Promocell). Cultures were used in passage 4-8 when they reached 90% confluence. Under these conditions, cell phenotype is guaranteed up to passage 14 (Promocell).

3.2.6.1 Preparation of serum samples.

Serum samples were collected in clot-activating tubes (Vacutainer) and centrifuged at 340*xg* for 15min to precipitate the clot. Serum was aspirated and flash frozen in liquid nitrogen. Samples were thawed and filtered through a 0.4µm filter to remove particulate material that could interfere with confocal microscopy. Serum samples were kept on ice until used.

3.2.6.2 Preparation of neutrophil: endothelial cell co-cultures.

Endothelial cell culture medium was removed and replaced with 450µl of fresh medium or neutrophil suspension, and 150µl of SSc or healthy control serum. 1.5µl of annexin V and 2.5µl of a 1:10 dilution of the E-selectin antibody were added to each well. Co-cultures were incubated for 24h at 37˚C, in a 5% CO_2, humidified atmosphere.

3.2.6.3 Confocal microscopy.

Confocal images were taken using an LSM-710 (Zeiss) confocal microscope using the Zen 2009 software. Annexin V-FITC was excited using the 488nm laser while APC was excited using the 633nm laser. Images were taken using the optimum separation of emission spectra mode. Duplicate images were taken for each experimental variable. A z-stack of 13 images was taken over a distance of 9.91µm, focusing from the endothelial cells to the neutrophils. Images were taken using a 10x fluar 0.5 NA objective.

Images were analysed using Image J software (NIH, USA) and those representing the maximal fluorescence values for red and green fluorescence were measured for total fluorescence intensity. Values were corrected for fluorescence intensity of cells cultured in media alone.

In order to assess the role of ROS in these co-cultures, 2000U/ml catalase was added to wells to catalyse the reaction $H_2O_2 \longrightarrow O_2+H_20$.

As previous experiments had shown a role for interleukin-6 (IL6) in endothelial cell activation and apoptosis in neutrophil-endothelial cell co-cultures (Chapter 7), AB serum spiked with 200ng/ml recombinant IL-6 was added to co-cultures instead of patient or control serum in the presence or absence of catalase, to determine if the observed effect of IL-6 was mediated by ROS.

In addition, co-cultures were incubated with 8µM DHR-123 for 24h. This probe is taken up freely by cells and is oxidised by H_2O_2 into the fluorescent product rhodamine-123. Fluorescence was then measured and localized by confocal microscopy following excitation with the 488nm laser.

3.3 Results.

3.3.1 Unstimulated neutrophil ROS generation is lower in patients with SSc compared to controls.

Total unstimulated chemiluminescence, calculated as the area under the curve, corrected to healthy controls was decreased on average by 25% in SSc neutrophils (p=0.0007, n=13). (Fig. 9.)

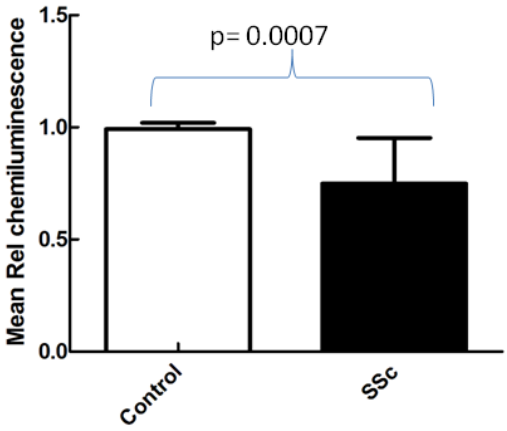


Fig. 9. Baseline reactive oxygen species generation, measured by luminol enhanced chemiluminescence, in neutrophils isolated from patients with SSc is significantly lower than in control neutrophils.

3.3.2 PMA stimulated neutrophil ROS generation is greater in patients with SSc compared to controls.

However, the total increase in chemiluminescence in response to PMA stimulation was greater in SSc neutrophils compared to healthy control neutrophils, both when unprimed (p=0.02) and when TNF-primed (p=0.01). Healthy control neutrophils increased total chemiluminescence 4.9 fold (unprimed) and 5.0 fold (TNF primed) as compared to SSc neutrophils which increased 6.3 fold (unprimed) and 6.7 fold (TNF primed)(Fig. 10.). This resulted in similar levels of PMA stimulated chemiluminescence in both patients and controls, given that unstimulated levels of chemiluminescence were lower prior to the addition of PMA.

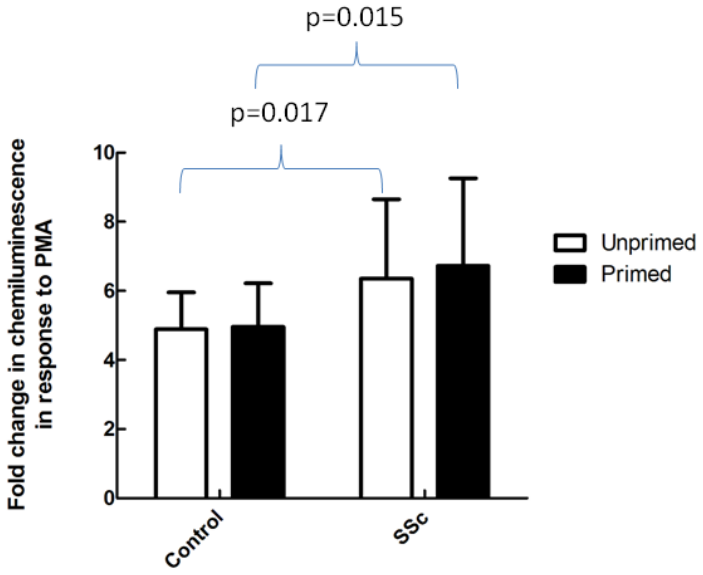


Fig. 10. PMA stimulation (100ng/ml) resulted in a greater increase in reactive oxygen species generation (measured by luminol enhanced chemiluminescence) in SSc neutrophils, in both unprimed and TNF primed cells, compared to controls. (N=13).

3.3.3 Formyl-met-leu-phe (fMLP) stimulated ROS generation is greater in unprimed SSc neutrophils compared to controls.

Neutrophil responses to fMLP usually require priming with agents such as TNF and GMCSF. However, the average increase in total chemiluminescence in response to fMLP in SSc neutrophils was 2-fold. This was significantly increased compared to healthy controls, which increased on average 1.7 fold (p=0.046) (Fig. 11). This suggests that neutrophils, isolated from SSc patients are primed *in vivo*. The response of TNF primed neutrophils from SSc patients was not significantly different to healthy controls.

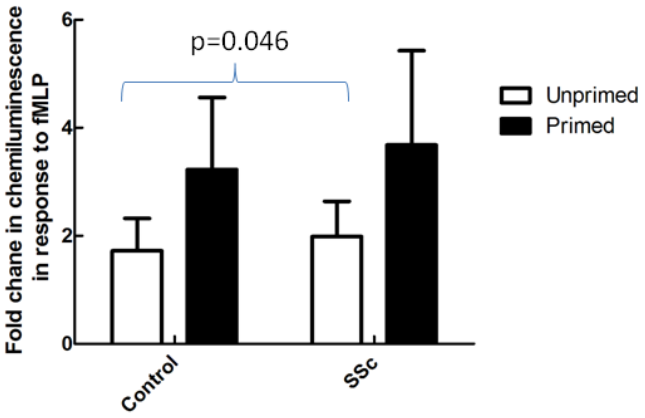


Fig. 11. Unprimed SSc neutrophils generate more reactive oxygen species (measured by luminol enhanced chemiluminescence), in response to fMLP (1µM) than control neutrophils. Following TNF priming, however, this effect was negated. N=13.

3.3.4 Soluble factors in SSc serum do not effect neutrophil ROS generation.

One possible explanation for the decreased ROS generation of SSc neutrophils is that neutrophils are activated *in vivo* and are therefore exhausted *ex vivo.* To explore this possibility, healthy control neutrophils were exposed to SSc or healthy control serum and ROS generation was measured by chemiluminescence. No differences were found in the total chemiluminescence of either unprimed or TNF primed neutrophils in response to SSc serum, compared to healthy control serum (Fig. 12).

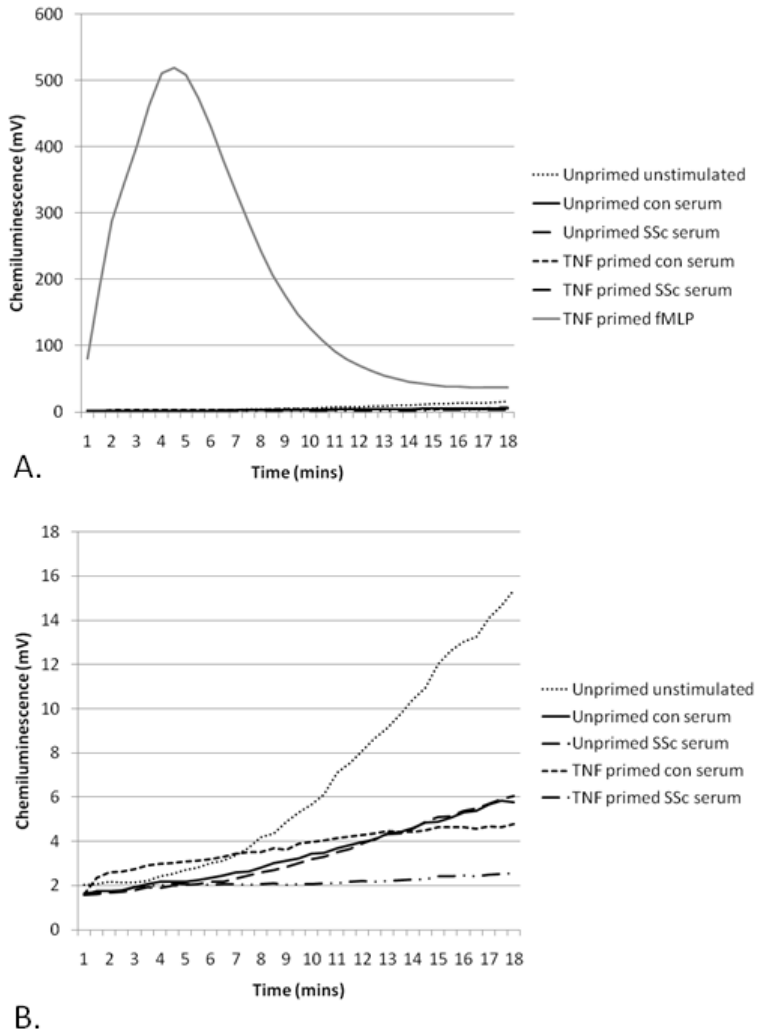


Fig. 12A. Healthy control neutrophils were primed with TNFα (10ng/ml) for 20min and then stimulated with healthy control serum, SSc serum or fMLP as a positive control. Reactive oxygen species were measured by luminol enhanced chemiluminescence. Neither control nor SSc serum were able to stimulate reactive oxygen species generation by neutrophils. A representative trace from 3 independent experiments is shown. B. Shows a close up of the patient and control serum samples.

3.3.5 ROS generation in neutrophil: endothelial cell co-cultures.

SSc serum increased endothelial cell apoptosis (annexin-V staining) and activation (E-selectin expression) in neutrophil: endothelial cell co-cultures at 24h (p=0.006 and 0.00004 respectively, n=17) (see Chapter 7). Addition of catalase to the co-culture media did not significantly decrease this effect (n=8) (Fig.13). However, for 3 individual patients with the greatest response to SSc serum, the addition of catalase to the media resulted in a marked decrease in the endothelial cell activation (Fig.14). There were no distinguishing clinical

features in these 3 patients. Of note, in single cell neutrophil cultures, sera from these individuals did not increase neutrophil ROS generation (data not shown).

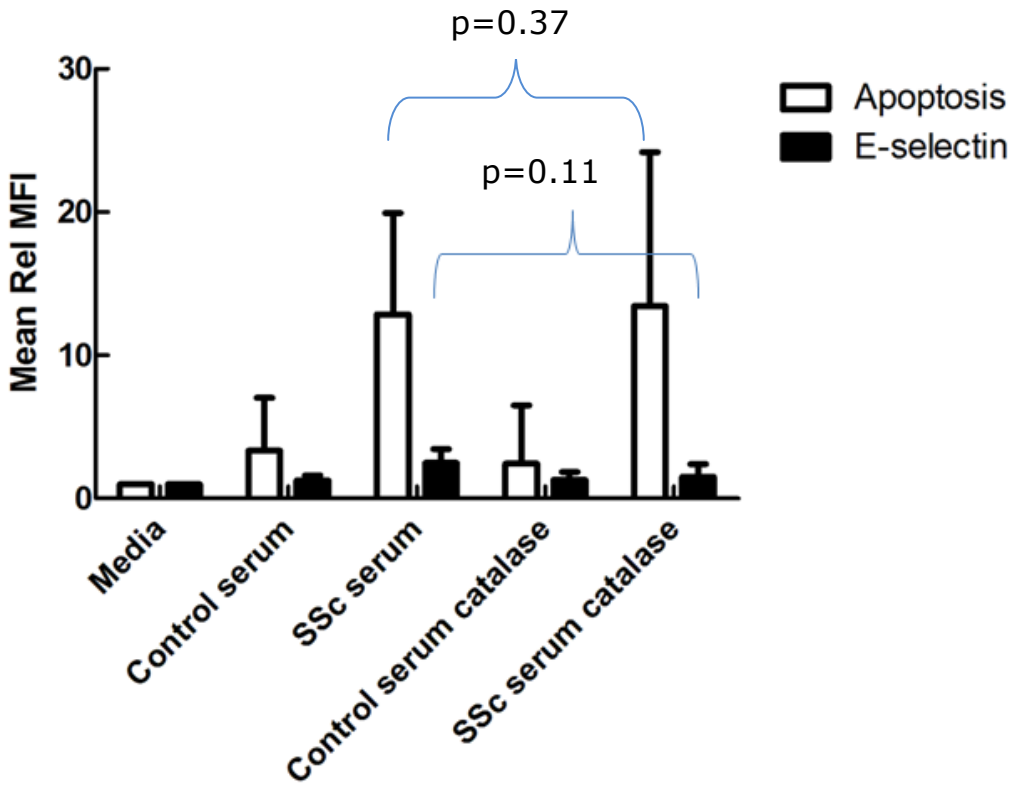


Fig. 13. Human dermal microvascular endothelial cells (HDMECS) were co-cultured with healthy control neutrophils for 24h in the presence of 25% healthy control or SSc serum. Apoptosis was measured using annexin V FITC and E-selectin expression was measured using an APC conjugated monoclonal antibody. Mean fluorescence was measured by confocal microscopy and expressed as a fold change in mean fluorescence corrected to media alone. Catalase was added to cultures to scavenge ROS. N=8.

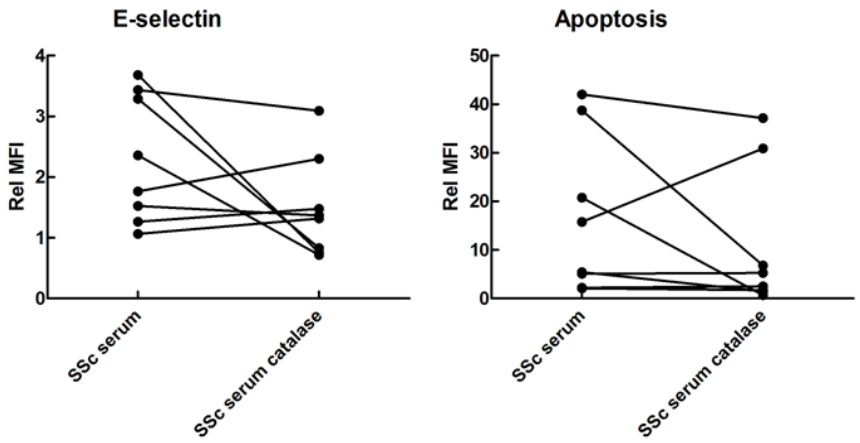


Fig. 14. Human dermal microvascular endothelial cells (HDMECs) were cultured with healthy control neutrophils in the presence of 25% control or SSc serum for 24h. E-selectin expression was measured using an APC conjugated monoclonal antibody. Mean fluorescence was measured by confocal microscopy and expressed as a fold change in mean fluorescence corrected to media alone. Each line represents single experimental repeats. N=8. E selectin expression was higher in the presence of SSc serum, but on average, catalase did not abrogate the effect of SSc serum. However, in three patients with the greatest increase in E-selectin expression, catalase markedly decreased the E-selectin expression.

3.3.6 Catalase does not abrogate IL-6 induced endothelial cell apoptosis and activation in neutrophil: endothelial cell co-cultures.

Studies (Chapter 7) have indicated that IL-6 has a role in the effect of SSc serum on increasing endothelial cell apoptosis and activation in neutrophil: endothelial cell co-cultures. However, catalase did not abrogate this effect, indicating that IL-6 does not mediate its effect by increasing ROS generation.

3.3.7 SSc serum increases intracellular ROS generation in neutrophils as measured by conversion of DHR-123 to rhodamine-123.

Of the 3 patient sera with a marked response to SSc and abrogation of the effect by catalase, one was used at 25% to stimulate neutrophil: endothelial cell co-cultures. Intracellular ROS generation was measured at 24h by the conversion of DHR-123 to rhodamine-123; fluorescence was quantified and localised by confocal microscopy. For this

serum sample, ROS generation was increased compared to healthy control serum, and the fluorescence increase was located to the neutrophils (Fig. 15).

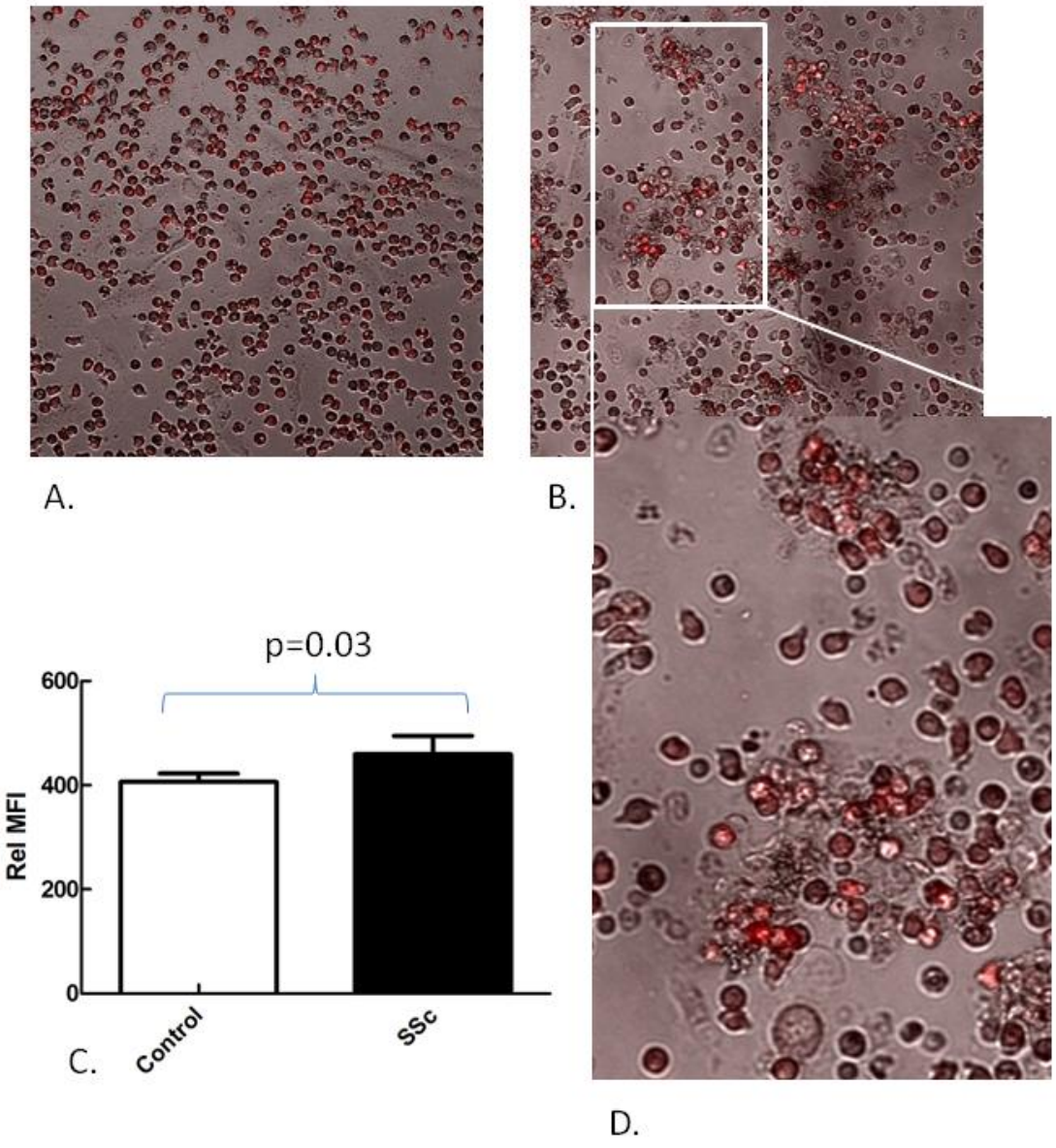


Fig. 15. Neutrophil: endothelial cell co-cultures in the presence of 25% A. control or B. SSc serum were incubated with DHR-123 for 24h. Conversion of DHR-123 to fluorescent rhodamine-123 (red) by ROS was measured as mean fluorescence intensity (MFI) using confocal microscopy. The results of 3 technical repeats are shown in C (mean ± SE). D. Rhodamine-123 was located within neutrophils. SSc serum resulted in more intracellular ROS generation in co-cultures than control serum.

3.3.8 Neutrophil chemotaxis.

Fewer neutrophils isolated from SSc patients migrated towards fMLP compared to control neutrophils. In addition, in the absence of a chemotactic gradient, fewer SSc neutrophils were found in the bottom chamber of the chemotaxis chamber compared to control neutrophils indicating a decrease in random motility. More SSc neutrophils were unaccounted for in either chamber, likely to be due to increased adhesion of neutrophils to the membrane or the plastic of the chemotaxis chamber (Fig. 16) via increased adhesiveness of unstimulated SSc neutrophils compared to controls. Increased adhesion to plastic surfaces was confirmed by inspection of the plastic ware following removal of the neutrophil suspension and washing of the plastic ware with excess PBS.

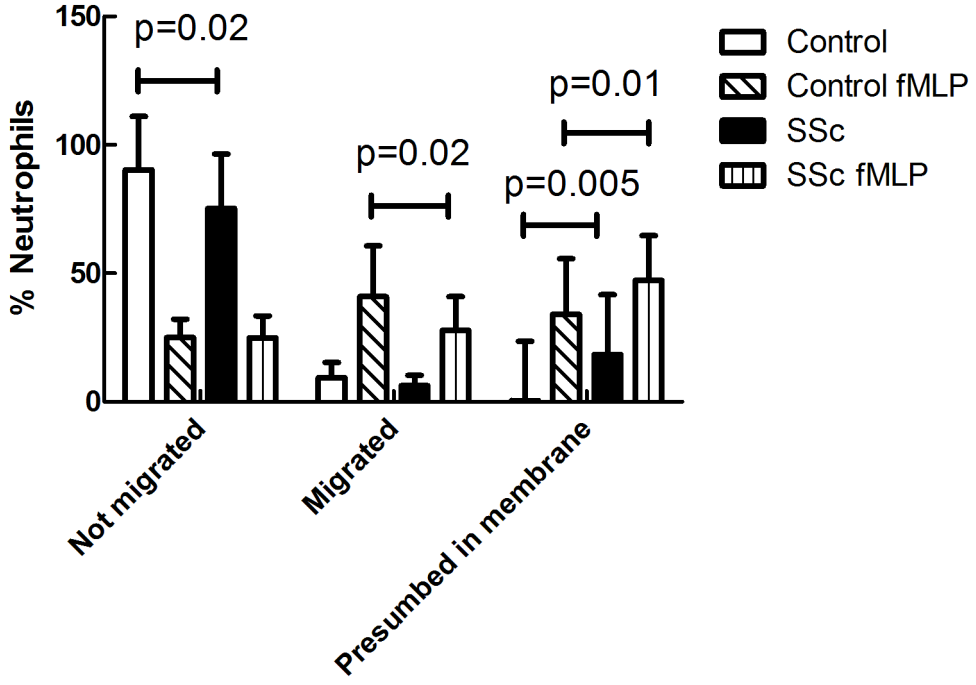


Fig. 16. SSc neutrophils showed lower random migration and chemotaxis down an fMLP gradient (10 nM) than control neutrophils. Random movement or chemotaxis was measured over 90min.

3.3.9 Neutrophil CD11b and CD18 expression

Given the suggestion above that SSc neutrophils are more adhesive than control neutrophils, cell surface expression of the neutrophil integrin proteins CD11b and CD18 was measured by flow cytometry. CD11b is increased on the neutrophil surface on priming, but there was no difference in CD11b expression between control or SSc neutrophils. CD18 expression changes very little upon priming, and so the ratio of CD11b to CD18 was calculated. There was no difference in the ratio of CD11b:CD18 in SSc neutrophils compared to controls (N=11) (Fig.17).

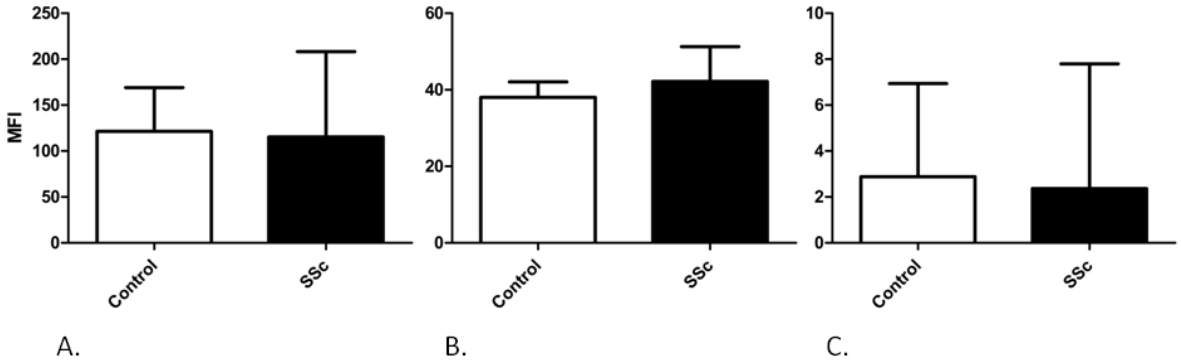


Fig. 17.There was no difference in the cell surface expression of (A) CD11b (B) CD18 or (C) the ratio of CD11b:CD18 in SSc neutrophils compared to controls as assessed by flow cytometry (N=11).

3.3.10 Neutrophil apoptosis.

There was no difference in neutrophil apoptosis between freshly isolated SSc and control neutrophils, as measured by annexin V FITC labeling of surface phosphatidylserine expression and flow cytometry (Fig. 18).

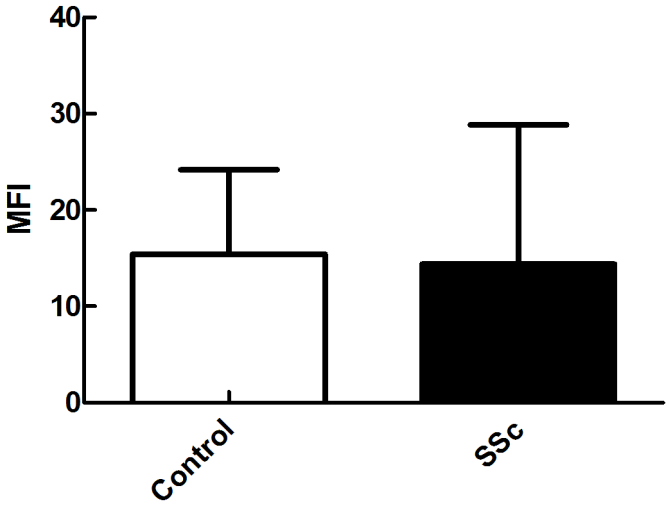


Fig. 18. Neutrophil apoptosis of freshly-isolated cell is no different in SSc compared to controls. Apoptosis was measured by annexin V-FITC binding to surface phosphatidylserine expression.

3.3.11 Neutrophil phagocytosis.

A dual phagocytosis: ROS production flow cytometry assay was used to correlate these two events. In control and SSc neutrophils, depending on the ratio of bacteria: neutrophils, a proportion of neutrophils were dim for phagocytosis and ROS generation. However, there were no differences in the proportion of dim neutrophils between SSc and controls at any concentration of bacteria (Fig. 19). There was no significant difference in mean phagocytosis of PI-labeled S. *aureus* or ROS generation at any ratio of bacteria: neutrophils between SSc and control neutrophils (Fig. 20 and Fig. 21). There was a tendency for SSc neutrophils to have a higher ROS generation in response to phagocytosis but this was not significant. At high ratios of bacteria: neutrophils this trend was reversed.

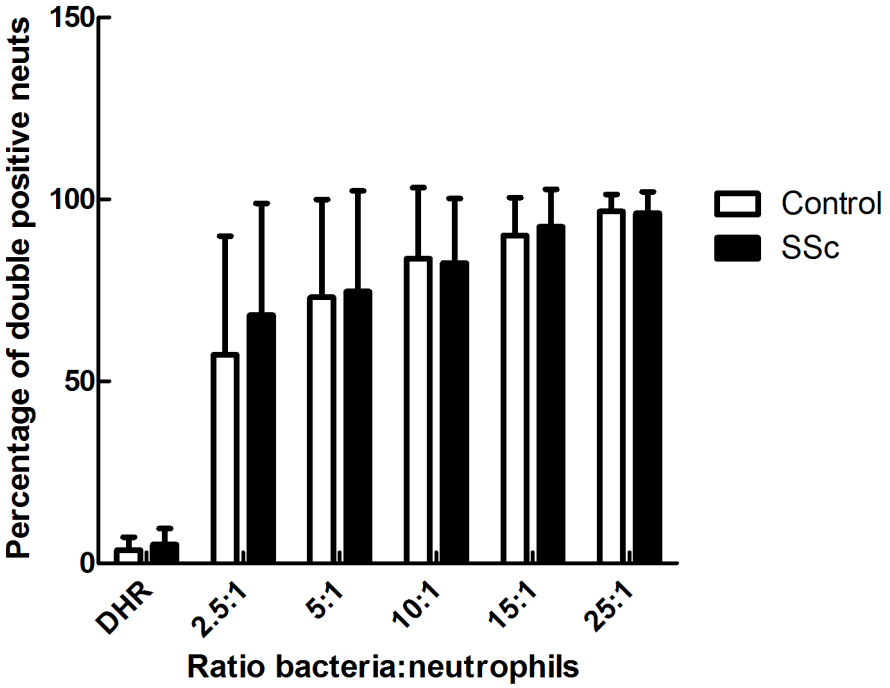


Fig. 19. The proportion of neutrophils which were dim for phagocytosis of S. *aureus* PI and for ROS generation (DHR) were no different between SSc and controls but did vary according to the ratio of bacteria: neutrophils. DHR staining of ROS generation was also measured at baseline in the absence of phagocytosis.

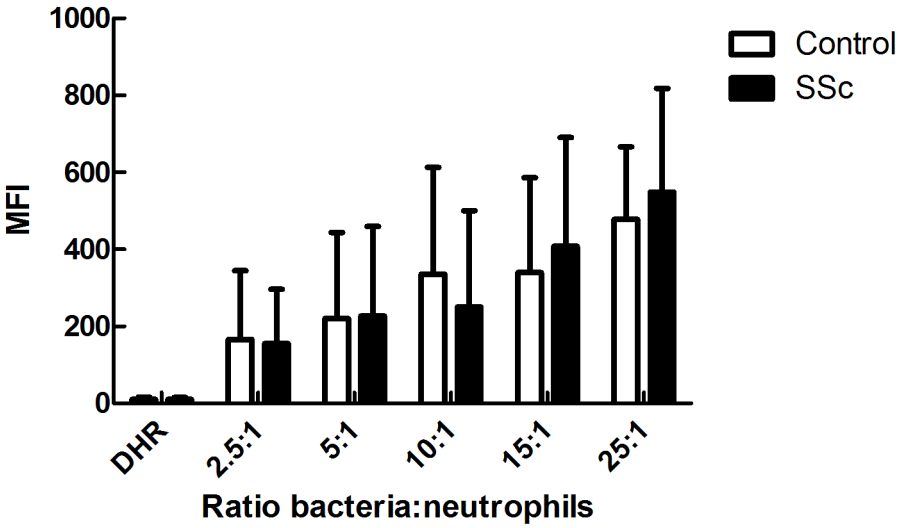


Fig. 20. Mean phagocytosis of PI labeled S. *aureus* did not differ between SSc and control neutrophils at any ratio of bacteria: neutrophils.

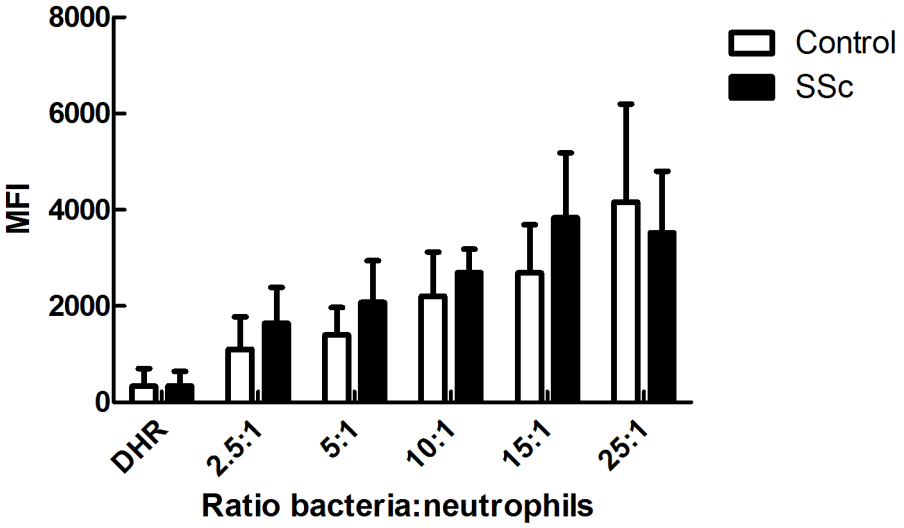


Fig. 21. Mean ROS generation in response to phagocytosis of PI labeled S. *aureus* did not differ between SSc and control neutrophils at any ratio of bacteria: neutrophils. However, at most bacterial concentrations there was a tendency for SSc neutrophils to produce more ROS. At high concentrations of bacteria (25:1) this was reversed.

There was no difference in the ratio of ROS generation to phagocytosis between control and SSc neutrophils at any bacterial concentration (Fig. 22).

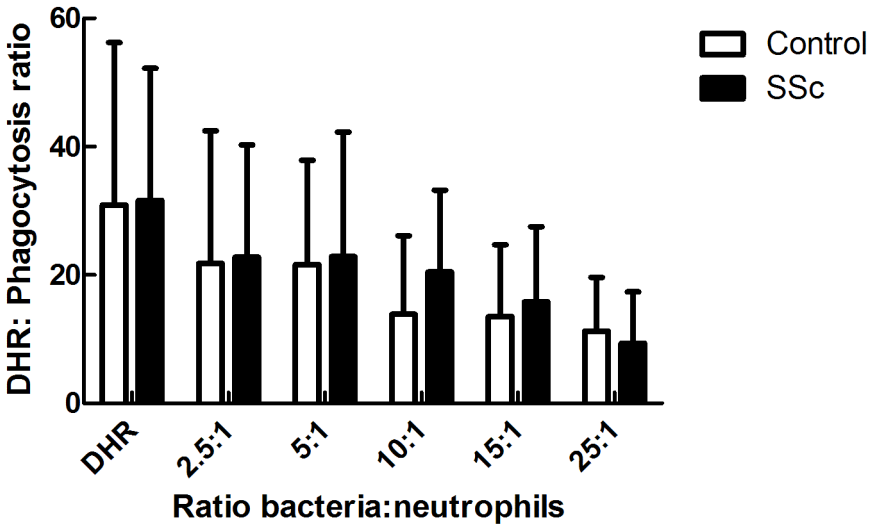


Fig. 22. There was no significant difference in the ratio of ROS generation to level of phagocytosis between control and SSc neutrophils at any ratio of bacteria: neutrophils.

3.3.12 Neutrophil CD16 expression.

There was no difference in mean or median CD16 expression on control or SSc neutrophils. CD16 expression on control and SSc neutrophils was divided into bright and dim expression. An increased proportion of SSc neutrophils were CD16 dim compared to control neutrophils (Fig. 23).

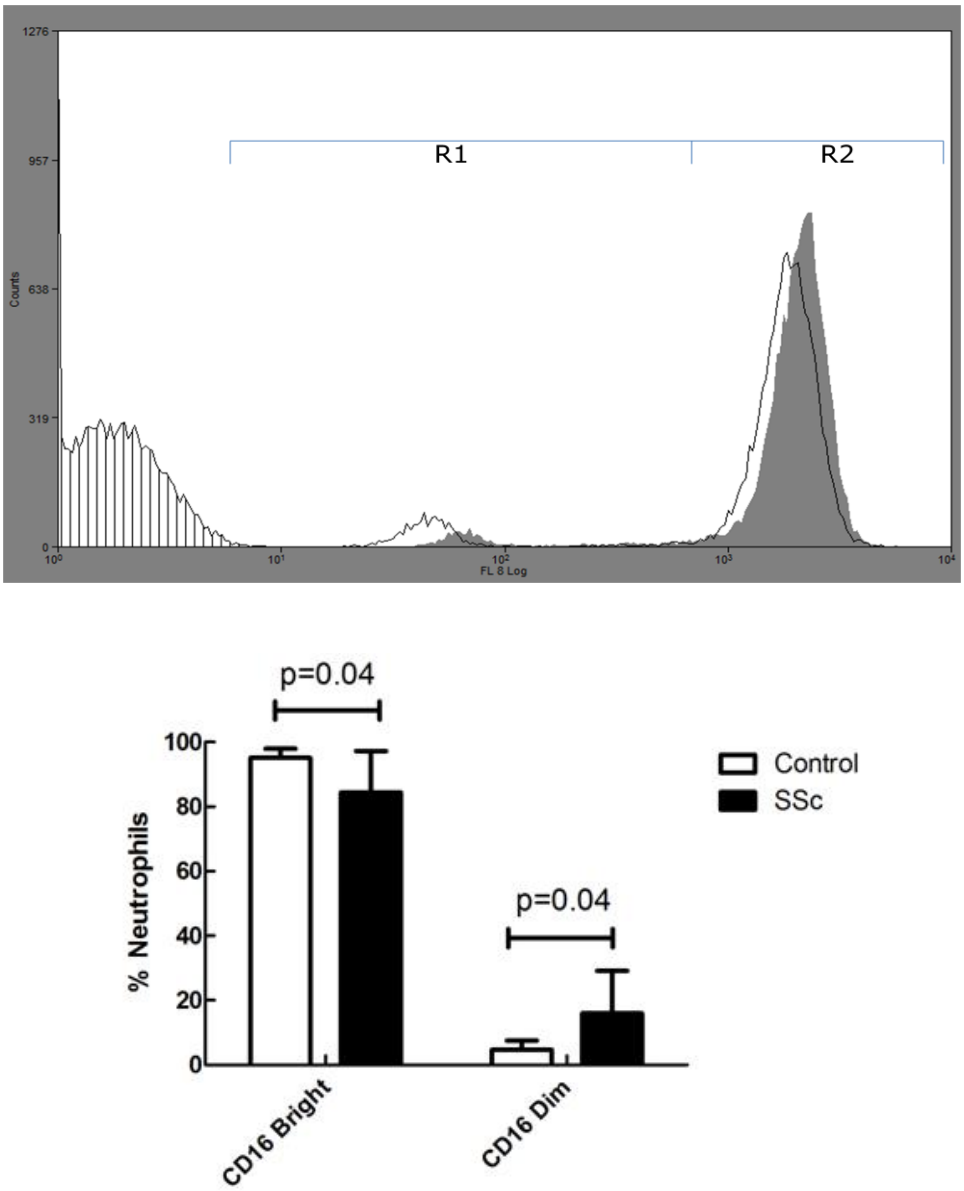


Fig. 23. Increased proportion of CD16 dim SSc neutrophils compared to controls. The top panel shows a representative flow cytometry trace. R1 contains CD16 dim neutrophils. R2 contains CD16 bright neutrophils. The unfilled trace represents SSc neutrophils. The filled trace represents control neutrophils. The hashed trace shows the isotype control. The bottom panel represents the mean and SD of 12 experimental repeats.

SSc patients have been reported to have an elevated eosinophil count compared to controls [142]. Eosinophils are likely to be a contaminant of the neutrophil preparation procedure as polymorphprep does not separate neutrophils from eosinophils. Eosinophils do not express CD16 and autofluoresce in the PECy5 channel. Simultaneous examination for CD16 expression (APC) and autofluorescence in the PECy5 channel reveals that the CD16 dim population of cells did not autofluoresce and therefore are not eosinophils (Fig. 24).

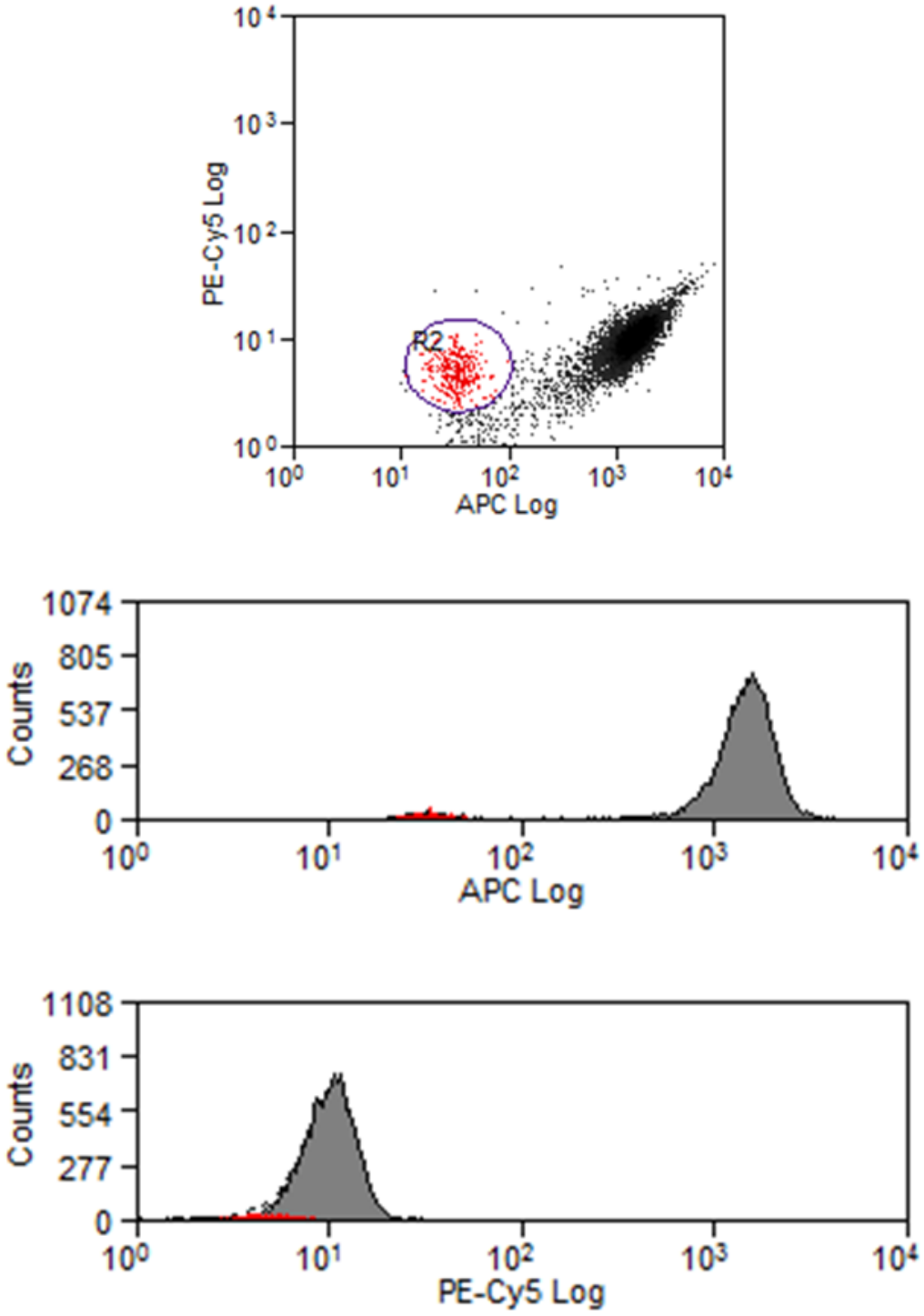


Fig. 24. CD16 dim cells in the SSc neutrophil samples are not eosinophils. The top panel plots CD16 expression (APC) against autofluorescence (PECy5). CD16 dim cells (R2) are marked in red. These cells did not have an increased autofluorescence in the PECy5 channel (bottom panel) showing that they do not represent eosinophils.

3.4 Discussion.

SSc neutrophils are hypofunctional in terms of spontaneous ROS generation and chemotaxis *in vitro.* This may represent a primary functional deficit in SSc neutrophils or may reflect *in vivo* activation and hence *in vitro* "exhaustion".

To explore this further, control neutrophils were exposed to SSc serum to see if this would stimulate ROS production. However, no such effect was seen. In addition, although unstimulated neutrophils were hypofunctional in terms of ROS production, they were capable of producing similar levels of ROS to control neutrophils in response to stimulants such as fMLP and PMA. This would suggest that the neutrophils are not fundamentally hypofunctional.

ROS generation in response to fMLP requires prior priming to increase the expression/affinity of the fMLP receptor on the neutrophil surface. SSc neutrophils produced more ROS in response to fMLP when unprimed suggesting that neutrophils are primed *in vivo*. This could be confirmed by measuring the surface expression of the fMLP receptor in SSc and control neutrophils by flow cytometry but time did not permit these experiments.

Priming can occur in response to a number of different stimuli including cytokines TNFα, GMCSF and IL-8. Interestingly, IL-8, which has been found in increased concentrations in SSc serum, is capable of priming ROS not only in response to fMLP but also in response to PMA unlike other priming agents [230]. Therefore, IL-8 exposure *in vivo* may explain the increased response to both fMLP and PMA of SSc neutrophils. It is however, evident that further priming *in vitro* can be achieved from the more powerful priming agent TNFα.

Luminol enhanced chemiluminescence was used here to measure ROS generation. This method measures both extracellular (secreted) and intracellular ROS generation due to the cell permeability of luminol. My data is in agreement with another recently published paper which uses an alternative and exclusively intracellular probe DHR-123, which demonstrated

a decrease in basal ROS production using a whole blood assay with neutrophil ROS generation measured by FACS gated for neutrophils only [217]. They also did not demonstrate a greater ROS generation in SSc neutrophils compared to controls in response to PMA, but, although the authors did not specifically report this, the data seems to suggest a greater fold change in ROS in SSc neutrophils in response to PMA [217].

The study described above used whole blood, therefore maintaining neutrophil interactions with serum cytokines and also leukocyte-leukocyte interactions. It is interesting, therefore, that they still demonstrated a hypoactive neutrophil phenotype, indicating perhaps that if *in vivo* neutrophil activation does occur, then it is not driven by a blood borne factor.

There are, however, other interactions that occur *in vivo*, including important interactions with activated endothelium that may cause neutrophil activation. My preliminary data suggests that over 24h neutrophils may produce more intracellular ROS in co-cultures with endothelial cells when exposed to SSc serum compared to control serum. This data needs to be repeated and if confirmed, the factors mediating this phenomenon will need to be systematically investigated.

The literature contains several reports of increased ROS generation by SSc neutrophils *in vitro* in direct contradiction to my results [215 216 228]. The discrepancies may be explained by different methodologies, as described below.

Czirjak *et al* used a whole blood assay using luminol to measure ROS generation. They were unable to discriminate which cells were producing the ROS but made the assumption that neutrophil ROS was so dominant that it would be most likely responsible for the increase [216]. However, the same authors also demonstrated an increase in mononuclear cell ROS production in SSc and therefore the validity of this assumption is questionable [231].

Maslen *et al* and Stevens *et al* described an increase in ROS generation in unstimulated SSc neutrophils compared to controls [215 228]. Both authors used Percoll gradients for

neutrophil separation, which can cause more neutrophil activation than Polymorphprep. Both of these studies measured only extracellular ROS generation. The luminol method used in my study measures both intra and extracellular ROS generation. It is possible that there is an increase in extracellular ROS production (as described by Maslen and Stevens) and a decrease in intracellular ROS production (as described by Foerster *et al* [217]) so the overall effect is a decrease in total ROS generation, as reported here.

My data also demonstrated that SSc neutrophils are hypofunctional in terms of chemotaxis towards fMLP, compared to control neutrophils. There was also a trend towards a decrease in SSc neutrophil random motility compared to control neutrophils, but this did not reach statistical significance. The method that I used, however, was able to measure not only the number of cells that had migrated but also the number that remained in the top chamber. This allowed me to calculate the number of cells that were unaccounted for. These cells were assumed to be either in the membrane or stuck to the plastic ware. The number of cells unaccounted for were significantly greater in SSc. This was taken to reflect an increase in cell adhesiveness.

Previous literature has also shown that neutrophils from SSc patients are hypofunctional in terms of chemotaxis [216 221]. Czirjak *et al* demonstrated a decrease in neutrophil chemotaxis towards zymosan conditioned media. Spisani *et al* found no significant difference in random motility or chemotaxis towards casein. However, there was a trend for SSc neutrophils to migrate a shorter distance in the leading front assay, at all concentrations of casein tested. The lack of a statistically significant result is likely to reflect the small number of samples tested and the assay technique used. In my hands the leading front assay is difficult to reproduce and somewhat subjective.

Because the migration assay suggested that SSc neutrophils were more adhesive I went on to explore the expression of the predominant leukocyte adhesion molecule Mac-1 (CD11b/CD18). Leukocyte adhesion is an essential part of leukocyte migration especially

through endothelial surfaces. The first stage in leukocyte migration involves the rolling of neutrophils along activated endothelial surfaces. This is mediated by transient leukocyte attachments to the endothelial cell surface mediated via L-selectin (neutrophil) and Sialyl-Lewis^{x}, E-selectin (endothelial cell). Following this, L-selectins are shed by a proteolytic mechanism and firm adhesion of the neutrophil to the endothelial surface is mediated by the binding of neutrophil integrins to their ligands. Thereafter, neutrophils undergo diapedesis, the process of deformation and movement between endothelial cells into tissues. This is mediated by cytoskeletal changes and interaction of neutrophils with endothelial cell adhesion molecules (ICAM 1,2, PECAM-1, JAMs) (Fig. 25) [232].

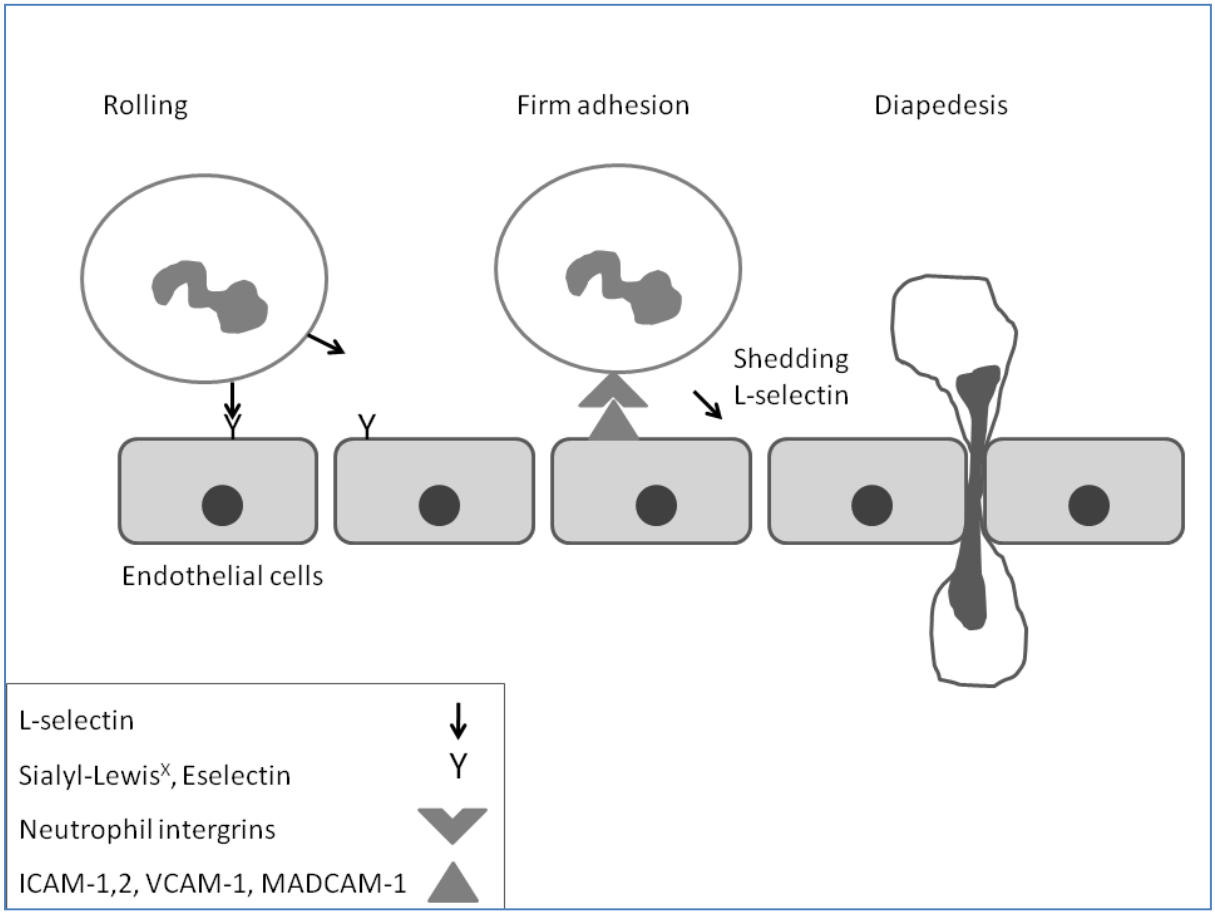


Fig. 25. Neutrophil migration. This process is triphasic involving initial rolling, followed by firm adhesion and then diapedesis.

The main neutrophil integrins are Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18). The up-regulation of neutrophil integrin expression is mediated by priming factors that enable the

translocation of intracellular integrin stores to the neutrophil surface. Expression is transient and localized to the leading front of the cell and activation may be mediated by the actin cytoskeleton [218].

Mac-1 is involved in the adhesion of complement-coated particles to phagocytes, neutrophil self-aggregation and binding to endothelial cells. It also binds fibrinogen. Ligation stimulates neutrophil activation and it constitutes the major neutrophil integrin. LFA-1 is involved in binding to cytotoxic T-cells. ICAM-1 is also a ligand for LFA-1 [218].

I did not demonstrate any difference in the expression of CD11b or CD18 in SSc neutrophils compared to controls nor in the ratio of CD11b to CD18. However, Mac-1 is only one of several molecules that can make neutrophils more adhesive. In addition Mac-1 activation does not only rely on an increase in cell surface expression but can be mediated by a change in configuration to increase the affinity of Mac-1 for its ligands. Mediators of such conformational change are incompletely defined but include cytoskeletal molecules that increase local concentrations of Mac-1 as well as increasing Mac-1 affinity. L-Plastin is one such example and I present evidence in Chapter 5 that L-plastin expression at the plasma membrane is increased in SSc neutrophils [233 234].

It is interesting to note that Mazzone *et al* reported that Iloprost (prostacyclin) infusions decrease the expression of Mac-1 on SSc neutrophils 6h after infusion [223]. Iloprost is known to improve Raynaud's phenomenon and digital ulceration in SSc patients not only during the infusion but for a prolonged period after the infusion. However, they reported similar changes in patients without SSc who had Iloprost for peripheral vascular disease.

I also noted in neutrophil: endothelial cell co-cultures (see Chapter 4) that cell-cell aggregates containing endothelial cells and neutrophils are increased in response to SSc serum. This may indicate that a soluble factor in SSc serum increases the adhesiveness of neutrophils either directly or indirectly via endothelial cell activation.

Finally, I have frequently observed that neutrophils isolated from patients with SSc often form cell aggregates, in common with previous studies. Lau *et al*. used cell aggregation as a surrogate marker for neutrophil activation [235]. They found that SSc neutrophils more readily underwent aggregation when stimulated with fMLP compared to control neutrophils, when read on a particle volume analyser.

To take these observations forward, I would ideally like to assess the adhesiveness of SSc neutrophils compared to control neutrophils using an adhesion assay. The most meaningful assay would be to assess neutrophil adhesion to TNFα activated endothelium. Similarly, I would like to formally quantify neutrophil aggregation in SSc as this may have pathological relevance by occluding microvessels and contributing to prolonged ischaemia. This could also be done by assessing neutrophil singlets, doublets and triplets by flow cytometry, using differences in forward and side scatter to separate and quantify the different sized cell aggregates.

Like previous authors, I did not observe any difference in the phagocytic activity of SSc neutrophils compared to controls [216 221].

I found that neutrophils isolated from patients with SSc had a higher percentage of CD16 dim cells compared to controls. CD16, also known as FcγIIIb, is a cell surface expressed member of the immunoglobulin gene superfamily. It is a low affinity receptor for IgG and is not a transmembrane receptor but is attached to the neutrophil surface by a GPI linkage. It exists not only on the cell surface but also preformed in intracellular stores. By virtue of the GPI-linkage, it can be cleaved from the surface by enzymes such as metalloproteinases, elastase, pronase and PI-phospholipase C [236 237]. The main function of CD16 ligation appears to be ROS generation in response to ligation by soluble immune complexes in primed neutrophils [238].

The control of surface expression is via a combination of proteolytic cleavage and mobilization of intracellular stores [236]. Different substances control surface expression by different mechanisms: GMCSF increases proteolytic cleavage but also increases mobilization to maintain cell surface expression whereas sodium butyrate decreases proteolytic cleavage with little effect on mobilization [239]. Loss of CD16 expression correlates with apoptosis and other cell surface markers of apoptosis like phosphatidylserine expression [240-242]. However, studies have shown that these events are controlled independently [243]. Proteolytic cleavage of CD16 from the cell membrane *in vitro* does not by itself increase apoptosis and does not appear to mark cells for phagocytosis [244]. A decrease in CD16 is not specific for apoptosis, as neutrophils incubated with LPS undergo delayed apoptosis but have decreased surface CD16 expression [241]. In addition, actin polymerization can induce shedding of CD16 [245].

As there were more CD16 dim neutrophils in SSc, I also quantified apoptosis at baseline by annexin-V binding to phosphatidylserine residues and found no increase in apoptosis. Therefore, the significance of the increase in CD16 dim cells is unknown. It maybe that SSc neutrophils have defective mobilization of intracellular stores or increased proteolytic cleavage of CD16. Interestingly, soluble CD16 (sCD16) is found in the circulation and proteolytic cleavage from neutrophils is thought to be the major source [246]. Serum levels of sCD16 are elevated in autoimmune inflammatory disorders such as Sjögrens syndrome, systemic lupus erythematosus (SLE) and rheumatoid arthritis [247-249]. This is thought to represent increased shedding of CD16 during increased neutrophil apoptosis in these diseases. However, although neutrophil apoptosis is increased in SLE, neutrophil apoptosis is delayed in rheumatoid arthritis [250-252]. Therefore, the increased shedding may serve some other purpose and may occur independently of apoptosis *in vivo.* Accordingly sCD16 has many attributed roles in immune function [253].

Future work could explore the levels of sCD16 in the serum of patients with SSc, could quantify the intracellular stores of CD16 in SSc neutrophils and assess the ability of intracellular stores to be mobilized by TNFα.

In conclusion, SSc neutrophils have an abnormal functional phenotype *ex vivo* compared to control neutrophils. They are hypofunctional in terms of unstimulated ROS generation but are stimulated to produce a greater increase in ROS in response to fMLP and PMA. The former observation may indicate exhaustion following *in vivo* activation and the latter may indicate *in vivo* priming. IL-8 could be a candidate priming agent. In support of this theory, SSc neutrophils are also hypofunctional in terms of chemotaxis but appear to be more adhesive *ex vivo*. This may indicate exposure to neutrophil chemotactins such as interleukin-8 *in vivo.* Although SSc neutrophils do not express more cell surface Mac-1 proteomic studies do suggest that they express more L-plastin (see Chapter 5), which can lead to Mac-1 activation by promoting focal accumulations of Mac-1 and by increasing its binding affinity. IL-8 can induce L-plastin function [254].

Neutrophil activation at the endothelial surface could have important ramifications for the pathogenesis of SSc. The release of cytokines, proteases and ROS could directly contribute to the endothelial cell injury and activation, which is thought to be central to the pathogenesis. ROS and proteases, in addition to endothelial cell activation, are all profibrotic phenomena and therefore could be enhancing and perpetuating the main pathological features of this disease. Finally, neutrophils have a role in promoting mononuclear cell recruitment, which may lead to the typical chronic inflammatory cell infiltrates that are typically seen in this disease.

Chapter 4: Neutrophil proteomics.

4.1 Introduction.

Functional studies (Chapter 3) have revealed that neutrophils have a distinct phenotype in patients with SSc. To shed further light on this phenotype a study of the neutrophil proteome was undertaken to look for changes in protein expression in these cells compared to healthy controls. It was anticipated that information would be gained from differences in the pattern of protein expression which would shed light on the underlying processes resulting in this change in phenotype.

Proteomic techniques are often limited by the fact that there is significant gel to gel variation making comparisons between samples (e.g. patient and control) very difficult. In order to overcome this problem, I used the DIGE technology (GE Healthcare) [255]. This uses 3 fluorescent, cyanine-based dyes, the first of which is used to label the patient sample and the second to label the healthy control sample. The third dye is used to label a mixture of equal volumes of the patient and healthy samples to act as an internal control to compensate for loading variance. All three samples are then run on the same gel in order to eliminate gel to gel variation. The fluorescence emission of each dye is read separately on a fluorescence scanner giving a highly sensitive measure of protein abundance (sensitivity 0.25-0.95ng, linear dynamic range 1-10000ng). Changes in expression as low as 10% can be accurately measured using this method [255].

4.2 Methods.

4.2.1 Protein extraction and preparation for 2D PAGE

5 x 10^6 neutrophils were lysed using 200µl of a hypertonic lysis buffer (7M urea, 2M thiourea, 30mM Tris, 1% Triton X-100, 4% CHAPS, 5mM MgAc, protease inhibitors 5mM PMSF). Samples were centrifuged at 14000*x*g for 10 min at 4ºC in order to remove insoluble

material. The protein concentration of the supernatant was quantified using the RCDC assay according to the manufacturer's instructions. Samples were made up to 320µl with rehydration buffer (8M urea, 2M thiourea, 4% CHAPS, 1.5mM DTT, 0.8% Bio-Lytes 3-10). 3.2µl ASB14 (5mg in 100µl H_2O) was added, and the mixture incubated at room temperature for 1h.

4.2.2 2D-PAGE of underivatised proteins.

Initially, the conditions for the 2D PAGE of neutrophil samples were optimised by running 2D-gels, and visualising spots by silver staining [256]. The optimised conditions were then used for running 6 replicate samples on DIGE gels (see below). Following analysis of the DIGE results, the differentially expressed proteins were identified by tandem MS following trypsin digestion of spots removed from comparator colloidal-Coomassie or silver-stained gels of underivatised protein [256] [257]. IEF was performed using a Protean IEF cell (Bio-Rad) with a 17cm IPG ReadyStrip pH 3-10 non-linear (BioRad).

For analytical gels, 100µg of protein sample in rehydration buffer, in a total volume 300µl, was applied to the IPG strip, whereas for gels to be Coomassie blue stained, 300µg-1mg of protein in 300µl of buffer was applied to yield sufficient protein for MS analysis. Proteins were applied using the cup loading technique to improve resolution of the basic proteins [258]. Strips were actively rehydrated at 50V for 12h and then proteins were focused at 250V for 30min (linear ramp), 10000V for 3h, 10000V to 40000V/h (linear ramp). Strips were equilibrated in equilibration buffer [50mM Tris, pH 6.8, 6M urea, 2% (w/v) SDS, 30% (w/v) glycerol, bromophenol blue] containing 20mM DTT for 15min, then in equilibration buffer containing 25mM IAA for 15min.

Strips were then loaded onto 12.5% SDS-PAGE gels and run at 15mA/gel for 30min and then 30mA/gel until the dye front had just run off the gel. Gels were scanned in a Bio-Rad GS-710 densitometer using PDQUEST 7.31 2D software package (Bio-Rad). Spots of

interest identified by DIGE electrophoresis (below) were matched to spots on comparator gels by eye.

4.2.3 DIGE electrophoresis.

Samples were adjusted to pH 8.5 and were labelled using the fluorescent cyanine dyes developed for DIGE (GE Healthcare), following the manufacturer's protocol (Fig.26). 50µg of neutrophil protein extracts from patients with SSc were labelled with Cy3 dye, 50µg of neutrophil protein extracts from healthy controls with Cy5 dye and an internal loading control (25µg of patient protein plus 25µg of healthy control protein) with Cy2 dye. In each case, 400pmol of amine-reactive cyanine dye, freshly dissolved in anhydrous dimethyl formamide was used and the reaction incubated in the dark at 4ºC for 30min. The reaction was stopped with lysine (1mM final concentration). The samples were then pooled and incubated with rehydration buffer, total volume 320µl plus ASB14 for 15min at 4ºC and then frozen overnight at -70ºC.

Samples were thawed and loaded onto IPG strips using the cup loading technique and subjected to 2D electrophoresis using the conditions given above. Cyanine-dye-labelled proteins were visualised on a Typhoon scanner (GE Healthcare). Protein profiles were analysed using DeCyder software (GE Healthcare) with spot filters set as slope >1.1, area <100, peak height <100 and peak volume<10000. Individual filtered proteins were then filtered manually to check that the spots were genuine. Relative protein abundance between patients and healthy controls was analysed using one-way ANOVA with correction for multiple analyses.

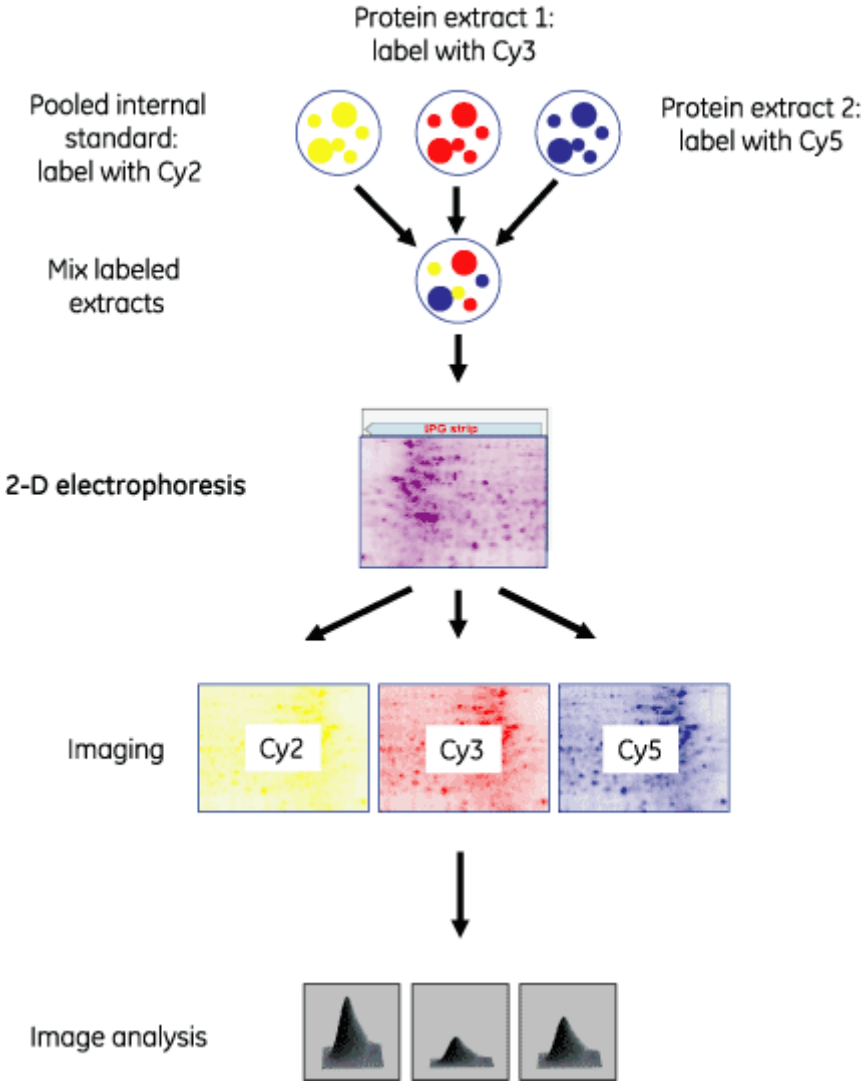


Fig. 26. DIGE workflow. On each gel, 2 protein samples can be compared. Each protein extract is labeled with a different cyanine based dye. An internal standard, comprising equal quantities of each protein sample, is labeled with the third dye. All 3 samples are then run simultaneously on one gel. Each dye is imaged separately and image analysis using DeCyder software assesses the differential protein expression in each sample compared to the internal standard.

4.2.4 Trypsin digestion and mass spectrometry.

In-gel trypsin lysis was performed on spots excised from comparator gels. Supernatants were analysed using an UltiMate nano-liquid chromatograph (LC Packings) connected to a Waters (Manchester, UK) Q-TOF Micro electrospray tandem mass spectrometer, operating in positive ion mode. Chromatography was performed using a µ-Precolumn C18 cartridge

(LC Packings) connected to a PepMap C18 column (3µm 100Á packing; 15cmx 75µm i.d.), using a linear gradient of 5% (v/v) solvent B [0.1% v/v formic acid in 80% (v/v) acetonitrile in water] in solvent A [0.1% (v/v) formic acid in 2% (v/v) acetonitrile in water] to 100% solvent B over 60min at a flow rate of 200nl/min. The spectrometer was operated in Data Directed Analysis (DDA) mode, where a survey scan was acquired from m/ζ 400-1500, with switching to MS/MS on multiply charged ions. MS/MS mass spectrum acquired over mass range 80-2000Da.

4.2.5 Database searching and protein identifications.

For the MS/MS data, peak lists were created using Masslynx (version 4), and necessary subtraction was performed with the following parameters: polynomial order 15, 50% below curve, tolerance 0.01. Smoothing of the peak list was performed using the Savitzky Golay method to 2 smooths. The data were centred using the centroid top method at 80%. MS/MS ion searches of the NCBI MSDB database (20060831) restricted to the *Homo sapiens* entries (total proteins searched 148148) were undertaken using the MASCOT search engine version 2.3 (http://www.matrixscience.com) to yield protein identifications. Searches were performed without restriction of protein Mr or pI. Searches were restricted to trypsin cleavage products and one trypsin miscleavage was allowed. Searches took into account the following post-translational modifications: fixed carbamidomethylation at cysteine residues, variable oxidation of methionine, and variable phosphorylation at serine, threonine and tyrosine. Peptide mass tolerance and fragment mass tolerance were set to 2.0Da and ±0.8Da, respectively. Peptide matches were assigned following statistically significant MASCOT probability scores (<0.05). Protein identifications were assigned if 2 or more peptide matches were made. False discovery rates were derived by searching against a decoy database and the false discovery rate was zero.

4.2.6 Staining of intracellular F-actin using phalloidin-FITC.

Actin exists in 2 major pools within the cell, G-actin (unpolymerised) and F-actin (polymerised). Many cellular functions involve increased actin polymerisation, which can be detected using Phalloidin, which binds to F-actin but not G-actin. Phalloidin can be conjugated to the fluorescent probe FITC which, when measured on the flow cytometer can give a relative quantification of the F-actin content of the cell.

$2x10^6$ neutrophils were placed in FACs tubes, spun down and resuspended in 3.7% formaldehyde with 200µg/ml phosphatidylcholine. Cells were incubated at 4°C for 15min to fix and permeabilise the cells. The cells were washed 3 times and resuspended in 500µl PBS. Cells were incubated with FITC-Phalloidin at 2.5ng/ml for 30mins in the dark at 4°C. Cells were washed 3 times and fluorescence detected by flow cytometry (FITC channel) or by confocal microscopy. On the confocal microscope Z-stacks of 30 images were taken focussing from the top to the bottom of the neutrophils.

As a positive control, patient and control neutrophils were stimulated with 1nM fMLP for 1min prior to pelleting the cells, and labelling with Phalloidin as above.

4.2.7 Western blotting.

Western blotting was undertaken as indicated in the General Methods Chapter.

4.2.8 Statistical methods.

Comparisons between means were assessed using the student t test. Multiple comparisons were made using ANOVA. Where appropriate, in the assessment of actin expression, the paired t test was used to compare differences between paired control and SSc samples from the same experimental repeat.

4.3 Results.

4.3.1 Differential protein expression by DIGE.

In this section of the work, protein expression in 6 SSc patients was compared by DIGE with 6 healthy controls. For patient characteristics please see Table 7. Equal amounts of protein from both types of extracts were combined and used as an internal control in the DIGE analysis. Up to 2136 different protein spots were resolved on the gels.

Characteristic	Patient (n=6)	Control (n=6)
Age/yrs (median, IQR)	47 (42,57)	42 (38,49)
Male: Female ratio	1:5	1:5
Limited disease (%)	5/6 (83)	N/A
Diffuse disease (%)	1/6 (17)	N/A
Pulmonary fibrosis (%)	2/6 (33)	N/A
Pulmonary artery hypertension (%)	1/6 (17)	N/A
Disease duration/yrs (median, IQR)	4 (3,6)	N/A

Table 7. Patient characteristics. Characteristics of patients and controls in whom neutrophil proteinexpression was compared.

42 proteins were differentially expressed (statistical significance <0.05) between patients with SSc and healthy controls. Positive protein identifications were made for 24 of these (57%) (Fig.27). This is in keeping with previous studies which found that only 60% of proteins identified by DIGE could be identified by matching with comparator gels stained with colloidal Coomassie, due to differences in the dynamic ranges of the two different stains [255]. It was possible to locate 70% of the 42 differentially expressed proteins by visual matching of the DIGE and the comparator gels. Of these, some spots did not yield identities due to the strict criteria set for protein identification, coupled with their low abundance. One of the 4 observed down-regulated proteins yielded identification (Table 8).

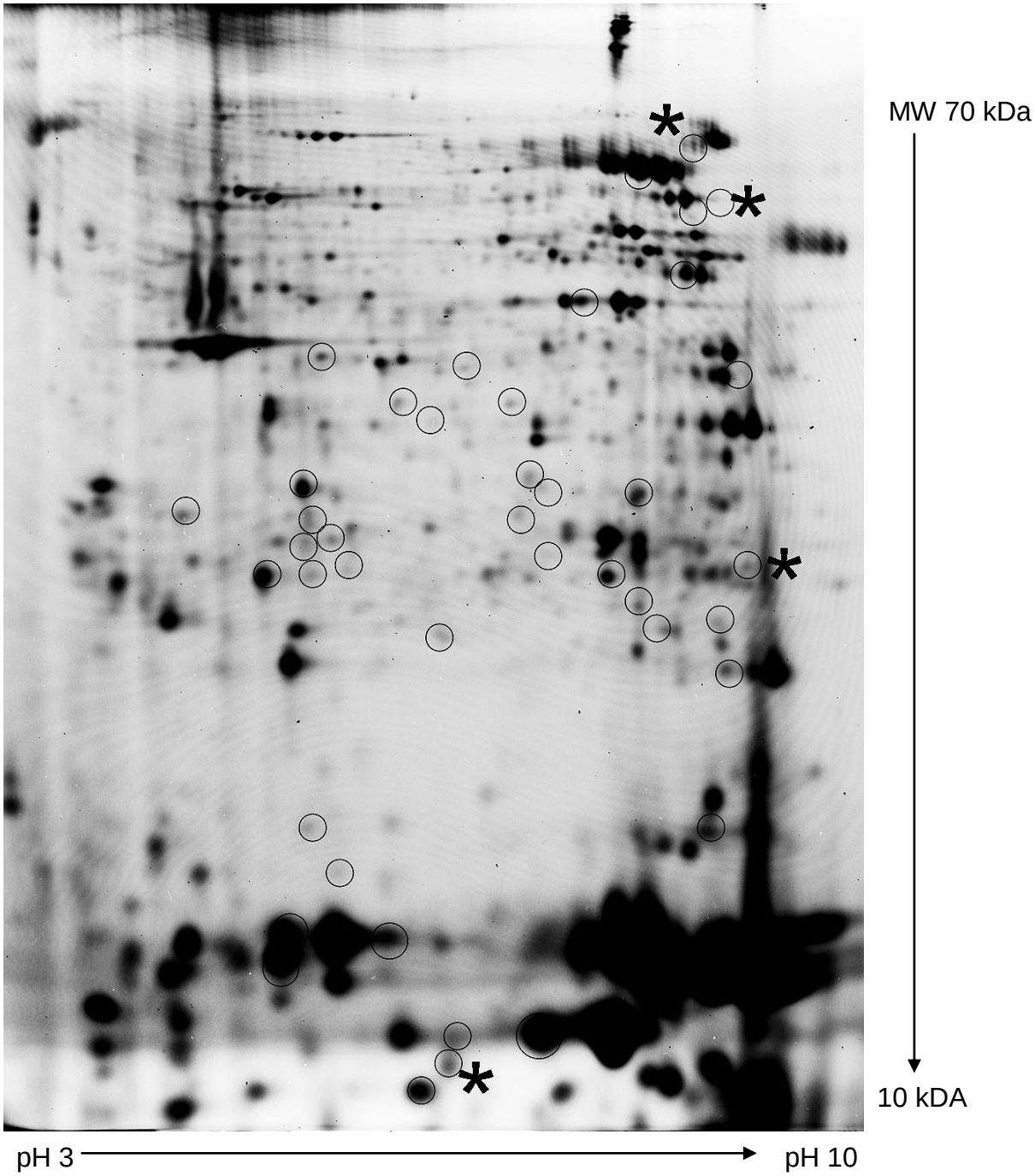


Fig.27. DIGE gel showing proteins that were differentially expressed between systemic sclerosis patients and healthy control samples. Protein (50µg) from the neutrophils of patients with systemic sclerosis, healthy controls, or mixtures of both protein extracts (internal loading control) were labelled with Cy dyes (see Methods) separated on a pH3-10 non-linear IPG strip in the first dimension and by 12.5% acrylamide SDS-PAGE in the second dimension. The resulting gel was scanned using a Typhoon fluorescence scanner and the results analysed using DeCyder software. 38 proteins were up-regulated (unmarked) whereas 4 proteins were down-regulated (marked *) in systemic sclerosis patients compared to healthy control samples.

					Fold change		Identification		
Protein ID	**Database source**	**Accession number**	**Theoretical mW (kDa)**	**Estimated mW (kDa)**	**Magnitude**	**Anova (p)**	**No. of peptides matched**	**Max MOWSE score**	**Sequence coverage (%)**
Lactoferrin	MSDB	Q5EK51	76	72	1.8	0.014	23	453	21
Glutathione disulfide reductase	MSDB	P00390	52	52	1.4	0.045	4	102	9
Actin gamma	MSDB	P63261	41	40	1.7	0.00077	7	211	12
Phosphoglycerate kinase	MSDB	P00558	44	40	1.5	0.014	4	149	10
Ficolin 1	MSDB	Q5VYV5	35	34	1.4	0.027	6	138	16
Annexin 3	MSDB	P12429	36	34	1.5	0.04	21	545	38
Bacteriocidal permeability-increasing protein fragment	MSDB	Q5JRQ5	34	34	1.5	0.0066	2	57	4
Actin related peptide 2/3 complex subunit 2	MSDB	Q53R19	34	33	1.7	0.0043	7	142	22
Purine nucleoside phosphorylase	MSDB	P00491	32	32	1.4	0.0092	11	310	27
Chloride intracellular channel protein 1	MSDB	O00299	27	32	1.5	0.0035	6	139	20
Actin capping protein	MSDB	Q5VV25	31	31	1.4	0.018	14	321	30
Elastase inhibitor	MSDS	P30740	42	30	2	0.023	4	87	5
Actin gamma fragment	MSDB	Q6PJ43	29	28	1.8	0.0018	21	289	22
Annexin 3	MSDB	P12429	36	28	1.8	0.0019	2	64	5
Triosephosphate isomerase	MSDB	P60174	26	28	1.8	0.034	4	240	21
Beta haemoglobin	MSDB	P68871	16	27	1.3	0.033	4	133	35
PYD and CARD domain containing protein	MSDB	Q9ULZ3	21	24	1.6	0.0095	10	506	45
Actin related peptide 2/3 complex subunit 5	MSDB	O15511	16	17	1.8	0.0006	4	122	15
GAPDH fragment	MSDB	P00354	36	17	1.6	0.0067	9	259	18
Allograft inflammatory factor 1	MSDB	P55008	16	15	1.8	0.033	2	74	12
Calgranulin B	MSDB	P06702	13	13	2.1	0.00025	4	111	32
Calgranulin B	MSDB	P06702	13	13	2.1	0.0069	3	92	25
Calgranulin A	MSDB	P05109	11	11	1.8	0.0043	5	161	45
Calgranulin A	MSDB	P05109	11	11	1.8	0.038	25	244	68
Beta haemoglobin fragment	MSDB	Q3LR79	11	11	0.2	0.037	6	72	32

Table 8. Differentially expressed proteins in SSc neutrophils.

Differentially expressed proteins fell into 2 main categories; neutrophil granular proteins and proteins involved in actin turnover. Table 8 also shows that some of the proteins, although securely identified, ran on the gels at lower than the predicted molecular mass, implying that they have been cleaved *in vivo* or *in vitro*. The fact that there was an increase in the abundance of such cleavage products in patients compared to healthy controls, despite the presence of excess protease inhibitors in the lysis buffer, may suggest that the proteins may have been cleaved *in vivo*. This may have resulted from an increase in the amount or activity of intracellular proteases or perhaps a functional deficit in antiproteinases.

The proteins were increased 1.4 to 2-fold, (Table 8) in keeping with previously reported changes in protein expression on neutrophil activation [259]. Fig.28 shows the individual levels of four proteins in 6 patients and healthy controls. Each dotted line represents one DIGE gel and the continuous line represents the average of all 6 gels. Clearly, there was statistically significant up-regulation of the three proteins in the patients, although this was not apparently observed in all the individual patients. Also shown is a 3-D representation of the abundance of the protein spots on the DIGE gels.

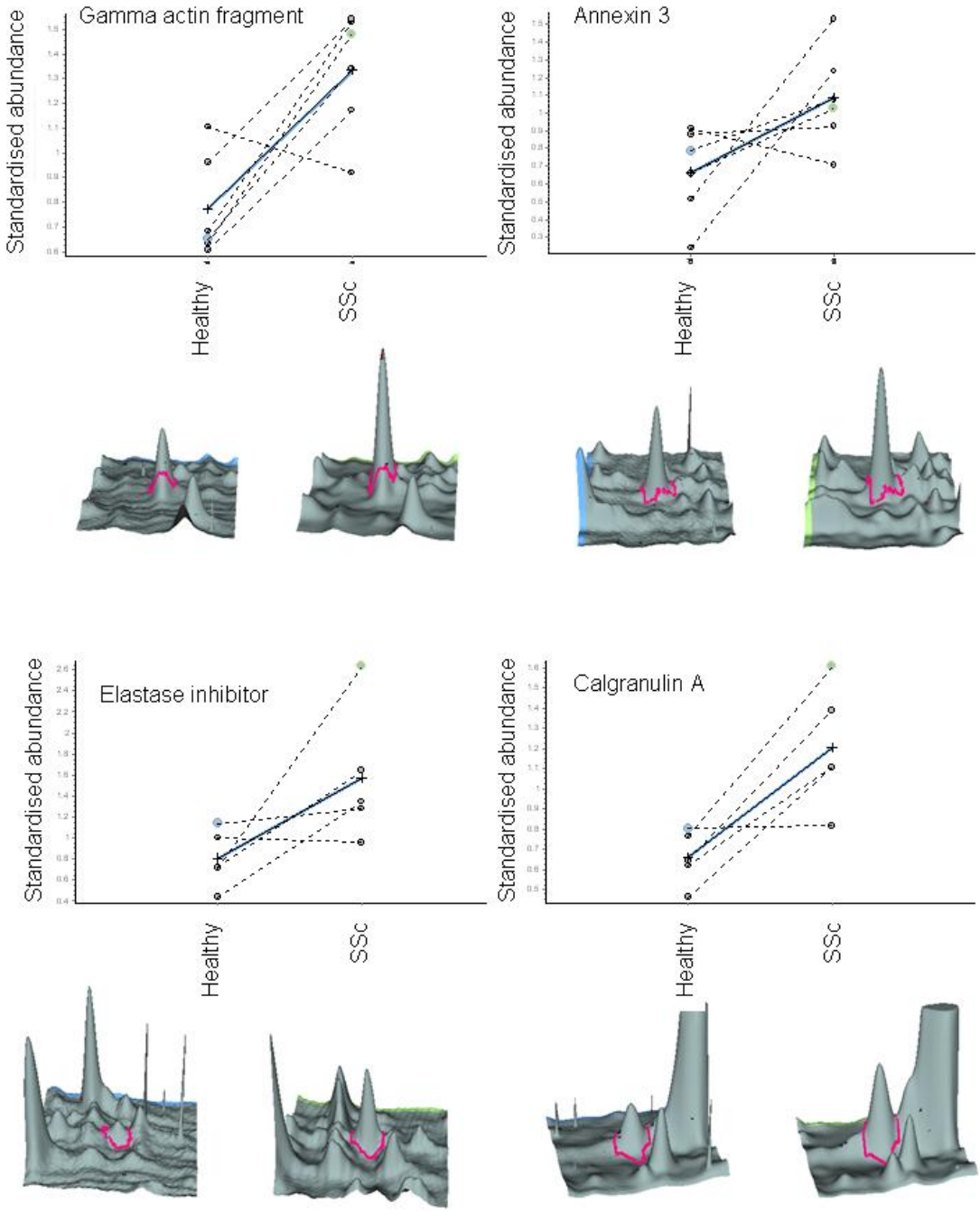


Fig.28. Standardised abundance of selected proteins in individual systemic sclerosis patients and healthy controls. The individual levels of four proteins in 6 patients and 6 healthy controls are shown. Each dotted line represents the standardised quantification from one DIGE gel and the continuous line represents the average of all 6 gels. Also shown is a 3-D representation of the abundance of the protein spots on the DIGE gels.

Attempts were made to validate the proteomic studies by assessment of F-actin turnover and western blotting of calgranulin B and SERPIN B1. Calgranulin was chosen for validation because it showed the greatest fold change by DIGE. SERPIN B1 was also chosen for validation because changes in protease activity especially neutrophil elastase may be relevant to tissue fibrosis.

4.3.2 F-actin turnover measured by Phalloidin-FITC staining.

F-actin turnover was assessed by measuring phalloidin-FITC staining before and after stimulation with fMLP by flow cytometry and by confocal microscopy. Flow cytometry experiments showed that there was no significant difference in F-actin expression between control and SSc neutrophils at baseline (Fig.29).

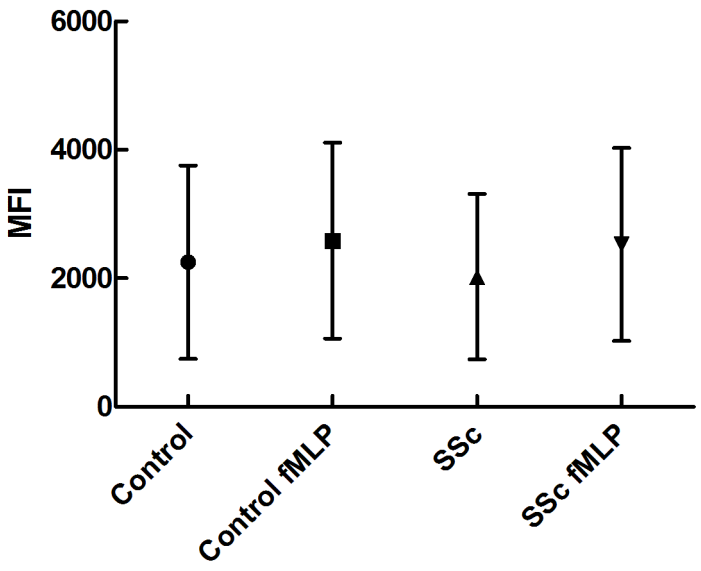


Fig.29. Neutrophils were isolated from patients with SSc or healthy controls. F-actin was labeled with Phalloidin-FITC. Neutrophils were stimulated with fMLP (1nM for 1min). Mean fluorescence intensity (MFI) was measured by flow cytometry. No significant differences in F-actin content were identified (n=7).

When imaged by confocal microscopy, less SSc neutrophils were weakly stained and SSc neutrophils appeared to have more focal accumulations of F-actin i.e. the cells were more frequently polarised indicating activation (Fig.30).

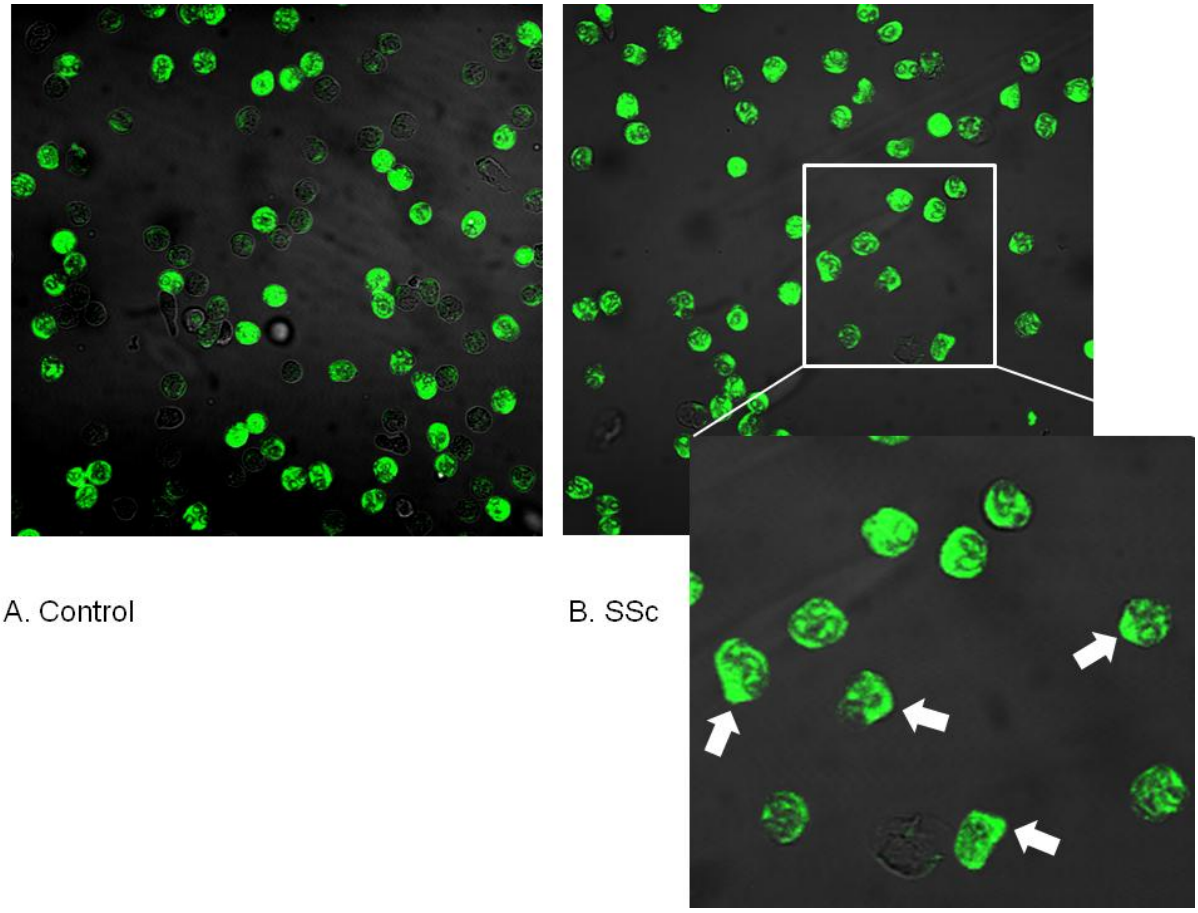


Fig.30. Healthy control (A) and SSc (B) neutrophils were stained with Phalloidin-FITC to label F-actin content and were visualized by confocal microscopy. A representative image is shown. There are less weakly stained SSc neutrophils and SSc neutrophils appear to have more focal concentrations of F-actin (arrows).

To further investigate this, stacks of 30 slices were taken through the neutrophils over 9.91µm by confocal microscopy. fMLP was used as a positive control. Control neutrophils are uniformly stained with Phalloidin-FITC and on treatment with fMLP they form focal actin aggregates. Untreated SSc neutrophils have focal actin aggregates similar to those seen on fMLP treatment (Fig. 31).

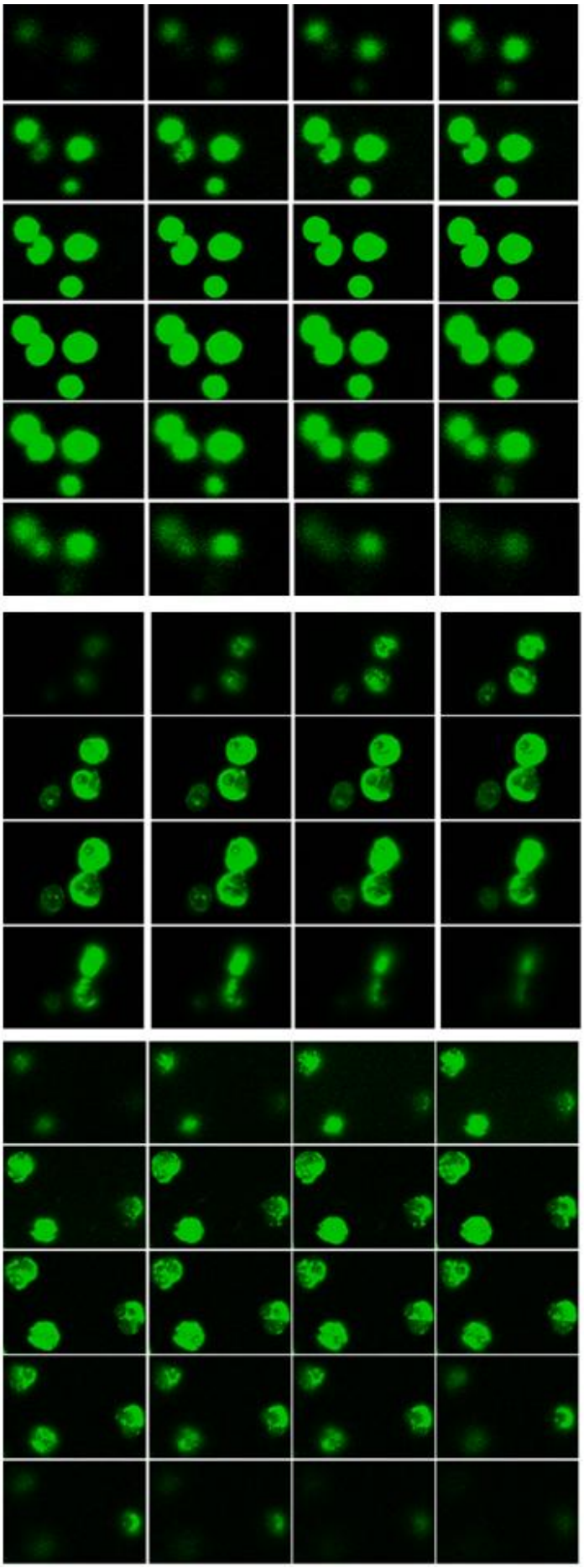


Fig. 31. Untreated control neutrophils (A) stain uniformly with Phalloidin-FITC which label F-actin. On treatment with fMLP (1nM 1min) control neutrophils form focal actin aggregates and cells polarize and decrease in size (B). Untreated SSc neutrophils (C) display a similar phenotype to fMLP treated control neutrophils. Images represent consecutive slices through the neutrophil taken by confocal microscopy.

4.3.3 Calgranulin B and SERPIN B1 expression by western blotting.

Western blots of calgranulin B showed an increase in calgranulin B expression in SSc neutrophils compared to controls, however this did not reach significance (Fig.32).

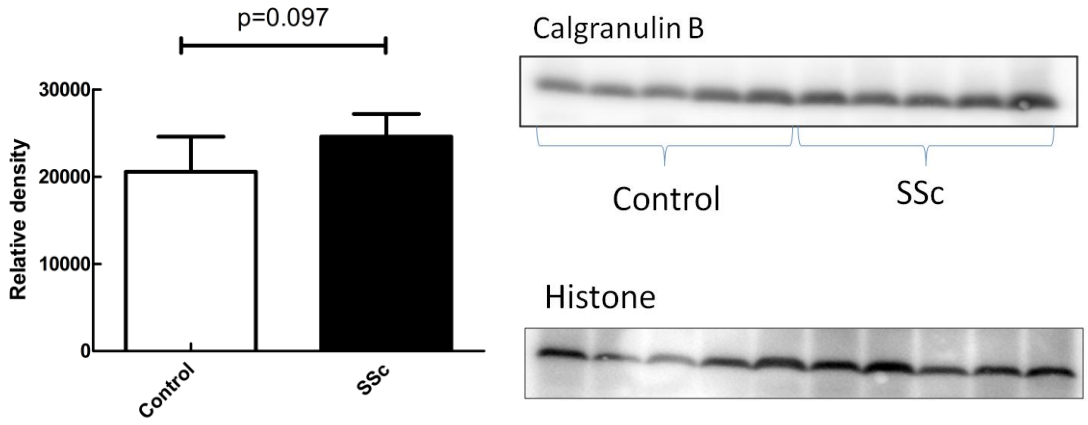


Fig.32. There was no significant increase in calgranulin B expression in SSc neutrophils compared to healthy controls. Neutrophil lysates were prepared for control and SSc neutrophils. These were analysed for calgranulin B expression by western blotting using Tris-tricene gels. Protein expression was quantified and corrected for histone expression. Histone was used for normalisation as gels were optimised for low molecular weight proteins. N=5 (p=0.097).

SERPIN B1 expression as assessed by western blotting, was decreased in SSc neutrophils compared to controls, with an increase in the 29kDa fragment. The 29kDa fragment is thought to arise by proteolytic cleavage of SERPIN B1 by neutrophil elastase and therefore this result may imply an increase in intracellular elastase activity in SSc neutrophils (Fig.33).

The western blot studies concur with the DIGE studies in that the direction of change in protein expression is similar. However, the magnitude of change is much smaller when assessed by western blotting and does not reach statistical significance.

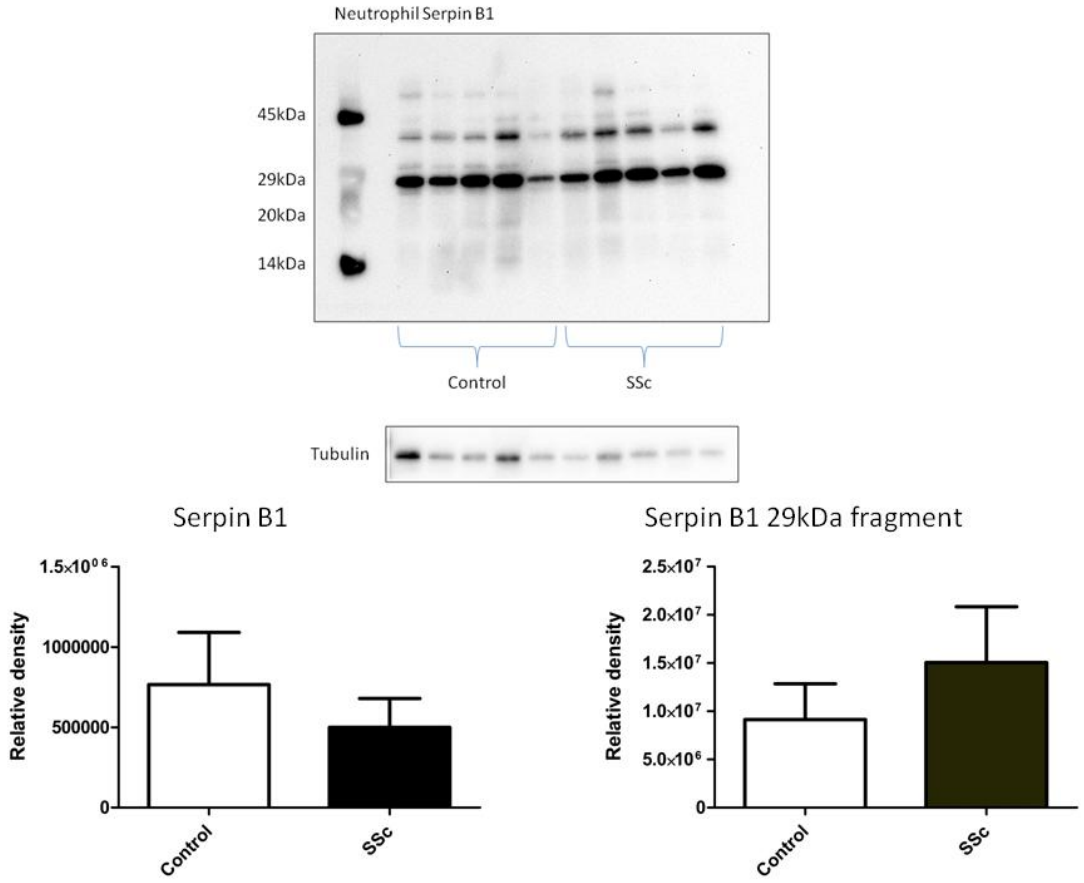


Fig.33. There was a non-significant decrease in SERPIN B1 expression in SSc neutrophils compared to healthy controls (p=0.15), and a non-significant increase in the 29kDa fragment of SERPIN B1 (p=0.09). Neutrophil lysates were prepared for control and SSc neutrophils. SERPIN B1 expression was quantified using conventional western blotting. Protein expression was corrected for tubulin expression. N=5.

Further investigations into neutrophil elastase expression and function are detailed in Chapter 6.

4.4 Discussion.

These data show that DIGE is a powerful technique to compare protein profiles of disease and control human samples and allow a novel insight into potential disease mechanisms. We have identified clear and statistically-significant changes in expression of key neutrophil proteins in a disease state (SSc), which may help uncover mechanisms whereby neutrophils are activated *in vivo* and contribute to pathology.

The actin cytoskeleton in the neutrophil is a dynamic cellular component which has roles in motility, phagocytosis receptor affinity, apoptosis, cell cycle and cell signalling [260]. Actin exists in the cell as both G-actin (unpolymerised) and F-actin (polymerised into fibrils). Neutrophil activation results in an increase in the cellular F-actin content [260]. Many associated proteins are involved in the regulation of actin polymerisation. The rate limiting step in actin polymerisation is nucleation, where monomers of G-actin form trimers which serve to seed F-actin formation.

Actin related peptide (ARP 2/3 complex) is involved in the promotion of actin nucleation thereby promoting actin polymerisation [261]. I found ARP 2/3 complex expression to be increased in SSc neutrophils compared to controls. Following nucleation, actin filaments grow by the addition of G-actin at the “barbed” end of the filament. However, in the resting state, most of the barbed ends are “capped” by capping proteins such as actin capping protein (CapZ). This serves to regulate actin polymerisation by preventing filament extension. On activation, capping proteins are cleaved from the barbed ends allowing filament extension [262]. I found an increase in the abundance of CapZ in SSc neutrophils compared to controls. This may indicate cleavage of CapZ from the barbed ends of actin filaments as a result of neutrophil activation. In SSc, these proteins are upregulated and may indicate increased neutrophil motility as a result of activation. Finally, G-actin forms complexes with actin-sequestering proteins that bind G-actin with high-affinity and prevent it

from polymerising. Sequestering proteins include thymosin β4 and profilin. G-actin is released from the binding proteins during activation (Fig.34) [260].

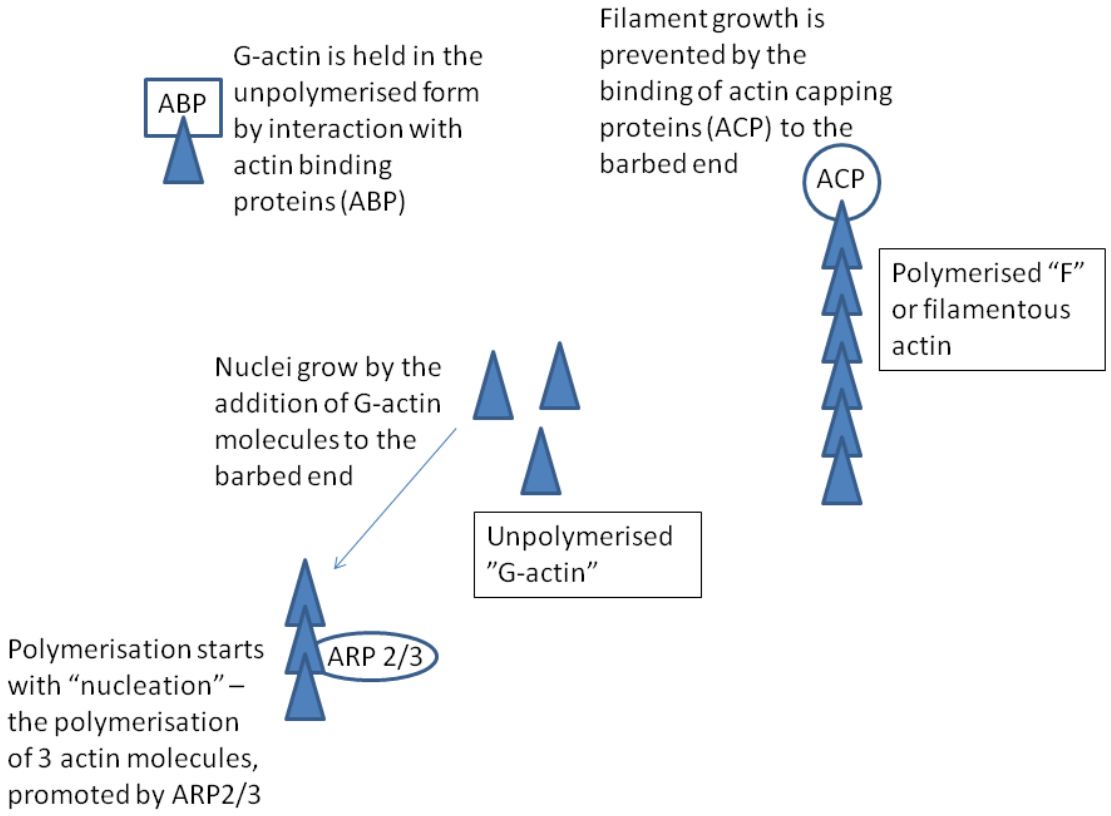


Fig.34. The control of actin polymerization. ARP 2/3 promotes nucleation of actin filaments. Actin capping proteins prevent filament growth. Actin binding proteins sequester G-actin.

We have found an increase in cellular actin in SSc neutrophils compared to controls. This may represent release of G-actin from the sequestering proteins during neutrophil activation as total cellular actin is not thought to vary. It is however, difficult to interpret the changes in actin levels as polymerised actin would be insoluble and would therefore not be loaded onto a 2D gel.

Experiments examining the F-actin concentration by Phalloidin-FITC staining do not confirm the hypothesis that there is increased F-actin content in SSc neutrophils at baseline. However, imaging studies do suggest an increase in neutrophil polarisation in SSc and

although this is not accompanied by an increase in Phalloidin-FITC staining, it does mimic the changes induced by neutrophil activation with fMLP.

The demonstrated increase in protein fragments including elastase inhibitor (SERPIN B1) may indicate an increase in neutrophil proteases or a deficit in anti-proteinases. In this study, I have not identified any change in the expression of the neutrophil proteases, elastase and cathepsin G. This is likely to be due to the basic pI of these proteins (8.7 and 11.37, respectively). The IEF strips used had a range of pH3-10, but they are expanded in a non-linear fashion with maximum resolution in the range pH4-7. Therefore, to optimally resolve these basic proteins, it would be necessary to use strips with a more basic pH range. I did conduct repeat experiments using pH7-10 strips, but no significant differences in protein abundance were detected. However, cathepsin G would obviously not be identified on such strips and even elastase would be towards the basic limit of resolution of these gels.

Basic proteins are notoriously difficult to resolve using 2D protein electrophoresis due to the difficulties in maintaining the solubility of such proteins. The 29kDa fragment of SERPIN B1 is the result of elastase dependent proteolytic cleavage, which occurs during binding of elastase to its endogenous inhibitor (SERPIN B1). Elastase expression and activity are further explored in Chapter 6.

Annexin 3, actin gamma fragment, elastase inhibitor and calgranulins A and B were increased in SSc neutrophils. In a previous proteomic study, an increase in the levels of annexin 3, actin gamma fragment and elastase inhibitor was found when neutrophils were exposed to lipopolysaccharide, a substance known to activate neutrophils *in vitro*. The magnitude of the proteomic changes seen in the latter study were similar to those found in my study [259]. There were some discrepancies between the two studies however; ficolin expression was decreased, but in my study is increased. The reason for this discrepancy is not known. In addition, unpublished proteomic studies from our laboratory have shown that

calgranulin levels are increased in neutrophils following *in vitro* exposure to TNF (personal communication J. Allen). Calgranulins will be further discussed in Chapter 5.

The inability to validate the findings of DIGE using western blotting to estimate protein expression is disappointing. Western blotting is a powerful technique for exploring changes in protein expression in one sample exposed to several different conditions. However, it has major limitations in assessing differences between samples from different donors taken at different times. It is extremely difficult under these circumstances to ensure equal protein loading. Although correction can be made to so called “house-keeping” genes, the variability in expression of these proteins in disease settings has not been explored. Finally, but most fundamentally, although western blotting is extremely sensitive, it is not very precise, that is it is semi-quantitative at best [263].

The direction of change in both calgranulin B expression and SERPIN B1 29kDa fragment were, however, the same as those seen in DIGE experiments and were approaching statistical significance ($p=0.09$) and therefore western blot data was consistent with evidence of neutrophil activation.

A limitation of this study has been the difficulty in matching DIGE gels. DIGE was designed to decrease the effects of gel-to-gel variation and, indeed, within one gel this can be very effective. However, in order to increase the statistical power of this study it was necessary to compare 6 DIGE gels. The number of spots resolved on the gels ranged from 1279 to 2136 and the number of spots matched on all 6 gels was 768. Gel spots included within the results were rigorously analysed on an individual basis to check that they represented true proteins and that the spot represented the same protein on all gels analysed. This will, however, have led to some spots being excluded from the analysis that may have been differentially expressed.

An even greater limitation is that comparator gels used for protein identification were stained with colloidal Coomassie or silver. The dynamic ranges of these stains are much lower than the dynamic range of DIGE and therefore matching of low abundance spots was often difficult. Further attempts were made to increase the number of protein identifications by preparing overloaded silver stained gels and overloaded lava Purple (GE Healthcare) stained gels following removal of the high abundance low molecular weight proteins by using a 10kDa centrifugal filter device (Millipore). The gels were however so different in overall appearance that it was difficult to match proteins by eye. Overloaded lava purple stained gels were then prepared from the complete neutrophil protein lysate as lava purple has a wider dynamic range than silver or Coomassie colloidal staining and the digital images of the lava purple stained gels could be scanned into and matched to the original DIGE images using the Decyder software. However, this approach did not lead to any further protein identifications.

These limitations will have led to missing proteins of interest, however, they do not detract from the positive results already discussed above.

Chapter 5 explores the use of cellular fractionation and gel-free shotgun techniques (iTRAQ) to further investigate the neutrophil proteome in SSc. This technique is able to overcome some of the limitations outlined above. In addition, iTRAQ, potentially, allows differences in post-translational modifications between SSc and control to be explored. Although, mass spectrometry is capable of detecting post-translational modifications to peptides, in this experiment a single sample loaded onto the comparator gel is used to generate the peptide identification and therefore differences between patient and control post-translational modifications will be missed.

DIGE technologies require spot matching for relative quantification and therefore if a protein is expressed, but is absent from one of the two samples being compared this will not be

picked up. iTRAQ however will still be able to quantify proteins which are present in at least one sample even if it is absent from the others.

In conclusion, circulating neutrophils from SSc patients exhibit an activated phenotype. It is possible that these activated neutrophils may release proteases and ROS that contribute to endothelial cytotoxicity in SSc. Neutrophils have a complex role in the immune system, being capable of antigen presentation, cytokine and chemokine release [264]. Therefore, if neutrophils are activated in the peripheral blood in SSc, they may drive ongoing inflammation and endothelial cytotoxicity, leading to fibrosis-the characteristic clinical feature of this disease.

Chapter 5: Membrane proteomics.

5.1 Introduction.

Functional studies of neutrophils isolated from patients with SSc indicate that the neutrophils may be primed *in vivo* (Chapter 3). Neutrophil priming is defined as the process of enhancing ROS generation in response to an activating agent by prior treatment with a priming agent (Fig.35). This two stage process increases control over neutrophil activation which can result in the production and release of potentially tissue damaging ROS [1 230].

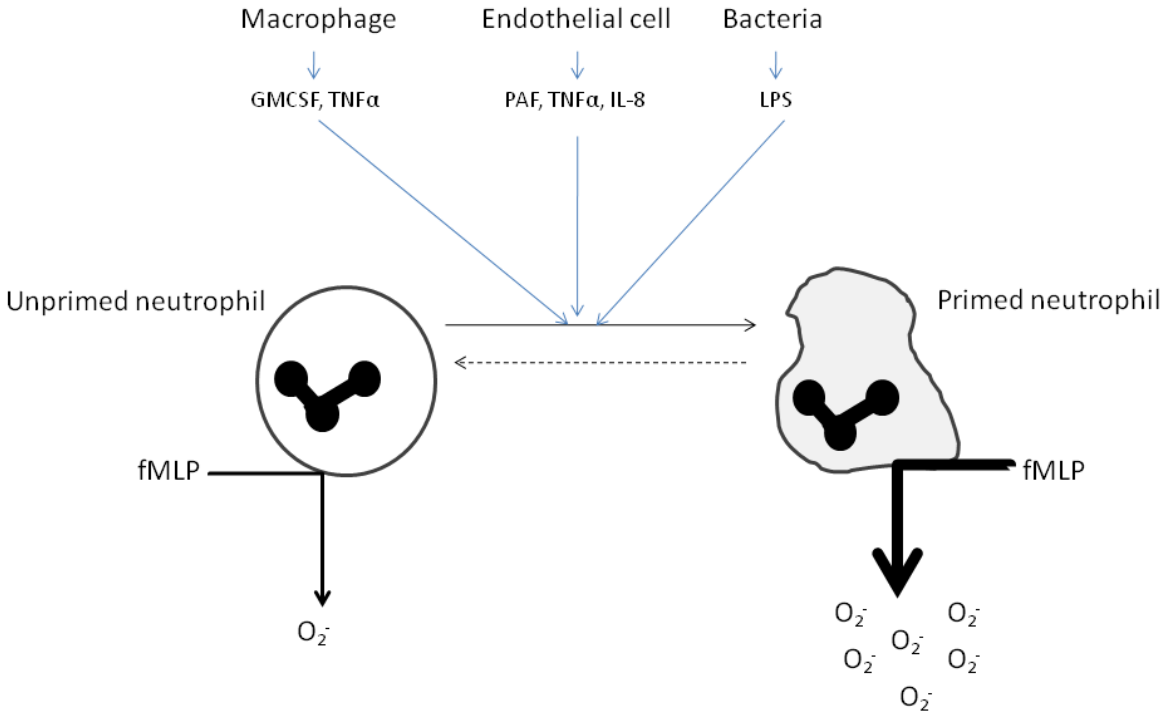


Fig.35. Neutrophil priming by inflammatory mediators results in enhanced superoxide production (reproduced from Condliffe et al 1998 [1]).

Neutrophil priming can be caused by substimulatory concentrations of cytokines which are known to activate neutrophils at higher concentrations. At these low concentrations, however, these cytokines are not capable of stimulating the neutrophil *per se*. However, incubation with these substimulatory concentrations of cytokines results in an enhanced

capacity for cellular functions, such as ROS generation in response to activating substances such as fMLP, C5a and LTB4 [218].

Neutrophil priming is not strictly limited to the potentiation of ROS production. It can also involve the *de novo* synthesis of many proteins and also changes in protein subcellular localization. For example, priming increases the plasma membrane expression of the fMLP receptors, CR1, CR3 and cytochrome b molecules necessary for ROS generation and adhesion. In addition, there are changes in cell shape and changes in cytoskeletal re-arrangements. The increased expression of proteins on the plasma membrane may not require *de novo* synthesis but instead involve the mobilisation of proteins from intracellular stores. This involves movement of the stores via changes in the cytoskeletal network and fusion of intracellular membrane bound stores with the plasma membrane [1 218 230].

Some changes in primed neutrophils however, do not reflect an increase in the number of membrane proteins but rather a change in ligand-binding affinity, for example binding to endothelial surfaces by integrin- dependent mechanisms [218].

Neutrophil priming is essential for, amongst other things, neutrophil mediated endothelial damage. In the presence of activating agents, fMLP, C5a or LPS, neutrophils cause only minimal damage to endothelial cells. However, if neutrophils are first primed, significant damage to endothelial cells ensued in response to the same activating substances [265].

Finally, priming increases neutrophil survival. Potentially injurious neutrophils usually undergo apoptosis after 12-18h in the circulation. This programmed cell death allows efficient removal without the release of potentially toxic substances from the cell. However, primed neutrophils are not only more active following stimulation but they have increased survival [1 218 230].

Priming is not a homogenous process. Different priming agents result in different effects on neutrophil function. Although, by definition, all priming agents increase ROS generation

following stimulation with agents such as fMLP, other priming functions differ. For example, interleukin-8, in contrast to TNFα and GMCSF, increases oxidative burst in response to PMA. Priming agents also have differing effects on degranulation and on membrane protein expression [230].

The proteomic studies undertaken previously (Chapter 4) using gel-based techniques are limited because membrane proteins which are highly hydrophobic proteins are poorly resolved in such systems. In this study, I undertook further proteomic experiments on the plasma membrane fraction of SSc and control neutrophils. The purpose was to; examine for differences in membrane protein expression between control and SSc neutrophils; identify patterns which are consistent with neutrophil priming *in vivo*; identify a cytokine or other priming agent signature.

5.2 Methods.

5.2.1 Neutrophil isolation.

Neutrophil isolation was performed as per Chapter 2. Cells were resuspended in RPMI 1640 +25mM HEPES and 2mM glutamine at a concentration of 1 x 10^7 cells per ml.

5.2.2 Neutrophil fractionation.

A nitrogen decompression chamber was used to disrupt the neutrophils as this conferred several advantages over alternative methods. Firstly, disruption could occur in any medium, allowing compatibility with down-stream processing including iTRAQ labelling and MS/MS. Secondly, the disruption of cells occurs without any heating of the sample and in the absence of oxygen, thereby decreasing degradation or oxidative damage of proteins. Finally, nitrogen disruption produces a highly reproducible and homogenous cell lysate.

Neutrophils were centrifuged (at 1000*x*g for 3min) to pellet the cells and resuspended in relaxation buffer (100mM KCl, 3mM NaCl, 3.5mM Mg_2Cl, 1mM $ATPNa_2$ (store aliquots at

50mM, -20°C and add just prior to use) 10mM PIPES, pH 7.2) containing complete protease inhibitor cocktail (Roche). Cells were placed on ice and transferred to a 45ml nitrogen disruption chamber (Parr). The cells were equilibrated at a pressure of 350Psi for 20min before releasing the cells into 100mM Na EGTA to give a final concentration of 25µM. Bomb pressures were optimized to give maximal cell disruption whilst giving minimal nuclear and granule disruption (determined experimentally).

Cell lysates were centrifuged at 1500*xg* for 10min to pellet unlysed cells and nuclei. The supernatants were centrifuged at 7000*xg* for 1h to pellet the microsomal fraction. Optimal centrifugation speeds were confirmed by western blot for myeloperoxidase (granule enzyme), histone (nuclear protein) and HLA (membrane protein marker). The presence of the membrane protein fraction in the supernatant was also confirmed by measuring latent and non-latent alkaline phosphatase activity in pellets and supernatant. The supernatants were centrifuged at 250000*xg* for 4h to pellet the membrane fraction. The pellet was washed with 50mM ambic pH 8 and re-centrifuged at 250000*xg* for 4h.

5.2.3 Measuring alkaline phosphatase activity.

The alkaline phosphatase activity in supernatants and resuspended post 7000*xg* pellets was assayed using the alkaline phosphatase dependent production of p-nitrophenol, from 2mg/ml nitrophenol phosphate (NPP). The coloured product was measured by absorption at 405nm. The assay was performed in a buffer at pH10 (100mM 2-amino-2-methyl-1 propanol, 1mM $MgCl_2$) at 37˚C and read after 30min. The assays were repeated in the presence and absence of 0.4% Triton X-100 to assess latent and non-latent activity.

5.2.4 iTRAQ.

5.2.4.1 Sample preparation.

The membrane pellet was resuspended in non-reducing sample buffer (3% SDS, 10% glycerol, 62.5mM Tris-HCl pH 6.8). Samples were quantified using the BCA assay (Pierce). 50µg aliquots were taken of 4 patient samples and 3 healthy control samples. An internal control was prepared by combining 12.5µl of each of the patient and control samples. The protein was precipitated overnight at -20˚C by adding 6 vol of ice cold acetone. The precipitate was pelleted by centrifugation at 1000*xg* for 5min at 4˚C. The supernatant was removed.

5.2.4.2 ITRAQ labeling.

The pellet was dissolved in labeling buffer (8M urea, 25mM TEAB, 2% Triton-X100, 0.1%SDS, pH8.5). The sample was reduced using 50mM TCEP (2µl per 20µl sample) at room temperature for 1h. Cysteine blocking was performed using MMTS 200mM in isopropanol (1µl per 20µl sample) at room temperature for 10min with agitation. Trypsin was added to the protein samples in a ratio of 1:20 and incubated at 37˚C overnight. Samples were centrifuged at 1000*xg* for 5min, and the supernatant was removed to a clean tube and dried in a Speedvac. The samples were resuspended in 25µl 1M TEAB and 60µl isopropanol pH 8.5 then each was transferred to a separate vial of iTRAQ label and incubated with agitation for 2h at room temperature. 100µl of H_2O was added and incubated at room temperature for 2h with agitation. All samples were combined, dried in a Speedvac and resuspended in 3ml of loading buffer/buffer A for cation exchange (10 mM KH_2PO_4 in 25% acetonitrile at pH 3.0). Cation exchange was performed over a gradient of 100% buffer A to 50% buffer A (50% acetonitrile). A total of 59 fractions were collected. Acetonitrile was evaporated off prior to loading peptides on to the mass spectrometer.

5.2.4.3 Mass spectrometry.

Mass spectrometry experiments were performed using a QTof Premier (Waters, Milford, Massachucetts) quadrupole time-of-flight hybrid instrument. The QTof was operated either in MS mode or MS/MS mode, in a data-dependent manner. Separation of peptides was performed by reverse-phase chromatography using a nanoAcquity HPLC pump, at a flow rate of 300nl/min and a Waters C18 nanoAcquity UPLC column (BEH 130 C18, 1.7µm particle size, 75µm i.d. x 150mm). Peptides were loaded onto a trap column (Waters Symmetry C18, 5µm particle size, 180µm i.d. x 20mm) from the autosampler using 0.1% formic acid for 5 min at a flow rate of 5µl/min. After this period the valve was switched to allow elution of the peptides onto the analytical column. Solvent A was 1% acetonitrile + 0.1% formic acid and solvent B was 99% acetonitrile + 0.1% formic acid. The gradient employed was 1-50% B in 112 min.

In MS mode, data were collected from *m/z* 350-1600. For data dependent mode, MS/MS data were collected between *m/z* 50-2000. Briefly, peptides were selected for MS/MS based on charge-state recognition (2+ and 3+), providing the ion counts were above 10 counts. MS/MS data was collected for a total of 3s, before the instrument was switched back to MS.

5.2.4.4 Data processing.

All data was processed in ProteinLynx Global Server 2.3. Text files (.pkl) were generated which contained precursor ion *m/z* values and associated fragment ion *m/z* values. Processed files were submitted to the Mascot search algorithm 2.2.0 (Matrix Science, London UK) and searched against forward and reverse ipi.HUMAN.v3.38.fasta database for protein identification, using fixed modification of MMTS (C) and variable modifications of oxidation (M), iTRAQ8plex (K),iTRAQ8plex (N-term) and iTRAQ8plex (Y).

Mascot search results were processed using Mascot Percolator (Markus Brosch, Sanger Institute) to calculate q-values (estimated false discovery rate). Quantitation of reporter ions

was calculated in centroided mode using iSPY (Phil Charles, PhD student, unpublished). Protein identifications and corresponding reporter ion quantitation were then coupled using iSPY with an ion count threshold of 15 and application of ratiometric normalistion. Finally, results were filtered by q-value using a false discovery rate of 1% per run.

5.3 Results.

5.3.1 Optimisation of bomb pressure.

Well-validated markers of membrane subfractions were used to optimise the bomb pressure used for nitrogen cavitation. In keeping with previously published data[266], a bomb pressure of 350Psi resulted in the optimal recovery of the membrane fraction while limiting contamination with granular or nuclear proteins (Fig.36).

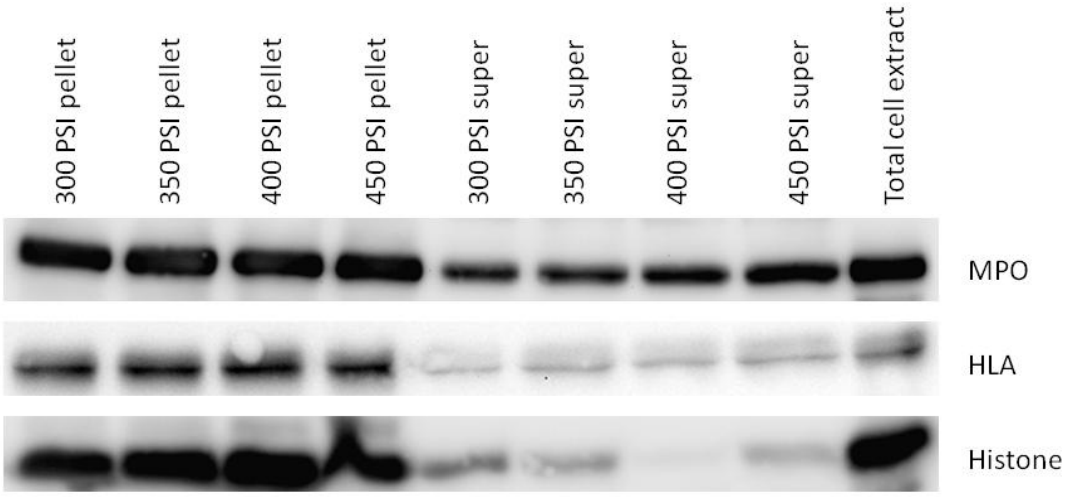


Fig.36. The amount of MPO in the supernatant progressively increases with increasing bomb pressure. The lower the bomb pressure the greater the number of unlysed cells (larger pellet). Therefore, a bomb pressure of 350PSI was chosen to maximise the number of cells lysed but minimise the granule disruption.

5.3.2 Optimisation of centrifugation steps.

The same markers of neutrophil subcellular fractions were used to optimize the centrifugation steps to recover the membrane fraction with minimal contamination from the

granular and nuclear proteins. Protein markers by western blot and enzymatic assays of alkaline phosphatase activity revealed that an initial centrifugation step of 7000*x*g for 30min (Fig.37 and 38) followed by a second centrifugation step of 250000*x*g for 4h (Fig.39) gave the optimal separation of the plasma membrane fraction. This is in agreement with previously published data [266].

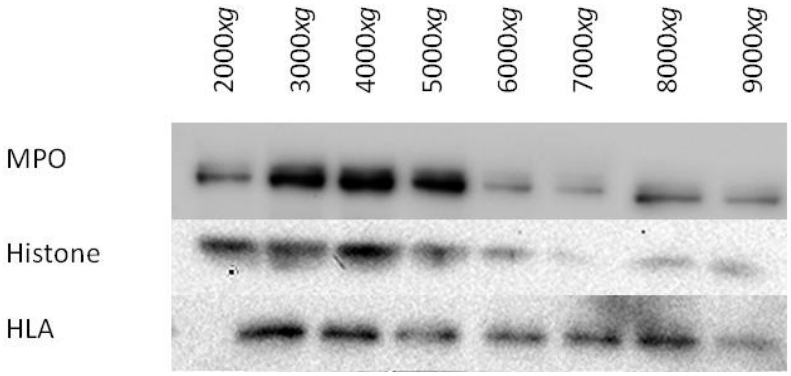


Fig.37. Centrifugation of the supernatant at speeds of greater than or equal to 6000*x*g resulted in the removal of granular proteins (MPO) and centrifugation at speed greater than or equal to 7000*x*g resulted in the optimal removal of nuclear proteins (histone). Although some membrane proteins (HLA) were lost at these speeds there was no difference in HLA recovery between 6000 and 7000*x*g. Therefore 7000*x*g was chosen as the appropriate centrifugation speed.

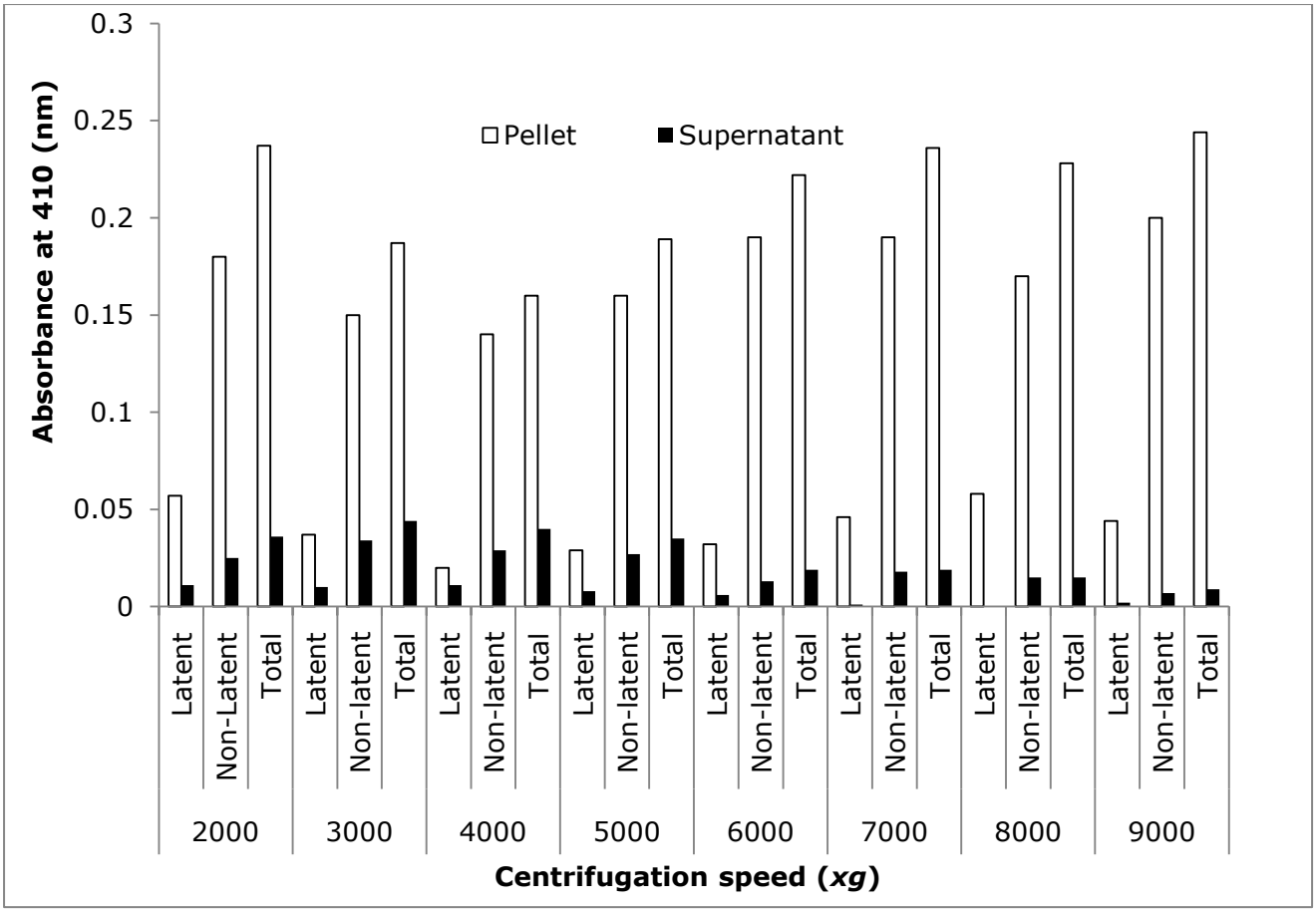


Fig.38. Alkaline phosphatase activity is a marker for membrane proteins. Latent activity represents secretory vesicle alkaline phosphatase activity whilst non-latent alkaline phosphatase activity represents plasma membrane protein levels. Latent activity is revealed in the presence of 0.4%TritonX-100 and non-latent activity is calculated by subtracting the latent activity from the total activity. Whilst lower centrifugation speeds give greater non-latent (membrane) activity there is in addition, latent activity indicating contamination with intracellular membrane bound organelles (secretory vesicles). Centrifugation at 7000*x*g gives the optimal recovery of non-latent alkaline phosphatase in the supernatant with no latent contamination.

Fig.39. Centrifugation of the 7000*x*g supernatant for 250000*x*g for 4h results in pelleting of the membrane fraction (HLA-I) with no detectable contamination of the supernatant by western blot. This pellet was therefore used for membrane proteomics.

5.3.3 Patient characteristics.

The characteristics of the 4 patients used in the iTRAQ experiments are summarised in Table 9.

Male: Female	0:4
Median disease duration (mths)	42
Median Raynaud's duration (yrs)	5
Median Rodnan skin score	14.5
Clinical feature	Number (%)
Limited subtype	3/4 (75)
Diffuse subtype	1/4 (25)
Lung involvement	0/4 (0)
Pulmonary artery hypertension	1/4 (25)
Renal involvement	0/4 (0)
ANA positive	1/4 (25)
Anticentromere positive	1/4 (25)
Anti Scl70 positive	1/4 (25)
Anti RNP positive	0/4 (0)
DMARDs	2/4 (50)
Mycophenolate mofetil	1/4 (25)
Sildenafil	1/4 (25)

Table 9. Patient characteristics.

							Control		SSc		
Protein description	**Gene name**	**Accession no.**	**No. of peptides†**	**Internal control‡**	**Average protein∆**	**% Difference⌽**	**Average**	**SD**	**Average**	**SD**	**p value**
Myosin-9	MYH9	http://www.uniprot.org/uniprot/P35579	29	1.20	1.23	1.77	1.13	0.15	1.30	0.49	0.61
Keratin	KRT1	http://www.uniprot.org/uniprot/P04264	16	0.99	1.18	19.35	0.97	0.69	1.34	0.76	0.54
Keratin	KRT9	http://www.uniprot.org/uniprot/P35527	13	1.40	2.86	104.53	1.17	0.58	4.14	6.20	0.46
Glycogen phosphorylase	PYGL	http://www.uniprot.org/uniprot/P06737	13	1.36	1.76	29.21	1.33	0.78	2.09	0.72	0.24
Major vault protein	MVP	http://www.uniprot.org/uniprot/Q14764	13	0.90	1.11	23.30	0.69	0.11	1.43	0.56	0.08
Matrix metalloproteinase 9	MMP9	http://www.uniprot.org/uniprot/P14780	12	0.83	0.89	7.73	0.60	0.34	1.11	0.41	0.14
Cathepsin G precursor	CTSG	http://www.uniprot.org/uniprot/P08311	10	4.90	4.37	10.75	4.77	0.63	4.08	2.09	0.61
Ras GTPase-activating-like protein	IQGAP1	http://www.uniprot.org/uniprot/P46940	9	2.55	1.37	46.04	1.98	1.10	0.92	0.57	0.15
Keratin 10	KRT10	http://www.uniprot.org/uniprot/P13645	9	1.12	0.69	38.55	0.77	0.39	0.63	0.18	0.53
Histone	HIST2H4A	http://www.uniprot.org/uniprot/P62805	7	2.10	2.65	26.10	1.68	1.22	3.37	2.75	0.37
Plastin-2	LCP1	http://www.uniprot.org/uniprot/P13796	7	1.14	0.86	24.24	0.40	0.08	1.21	0.48	**0.04**
Calgranulin A	S100A8	http://www.uniprot.org/uniprot/P05109	6	1.84	2.07	12.88	2.43	0.31	1.80	0.79	0.26
Talin-1	TLN1	http://www.uniprot.org/uniprot/Q9Y490	6	0.95	1.34	40.88	1.38	0.25	1.32	0.23	0.77
Leukocyte elastase precursor	ELA2	http://www.uniprot.org/uniprot/P08246	6	5.46	5.40	1.13	5.45	0.87	5.35	2.07	0.94
Phosphoglycerate kinase 1	PGK1	http://www.uniprot.org/uniprot/P00558	5	1.68	1.50	10.44	1.00	0.20	1.88	1.76	0.44
Actin-related protein 3	ACTR3	http://www.uniprot.org/uniprot/P61158	5	2.65	1.73	34.76	0.60	0.04	2.58	1.88	0.14
Brain acid soluble protein 1	BASP1	http://www.uniprot.org/uniprot/P80723	5	1.07	2.80	160.31	2.03	1.32	3.37	5.11	0.68
Protein-arginine deiminase type-4	PADI4	http://www.uniprot.org/uniprot/Q9UM07	5	0.92	1.50	63.51	1.04	0.51	1.85	1.10	0.29
Adenylosuccinate synthetase isozyme 2	ADSS	http://www.uniprot.org/uniprot/P30520	5	0.88	1.11	26.77	1.09	0.48	1.13	0.66	0.93
Keratin	KRT2	http://www.uniprot.org/uniprot/P35908	5	0.77	1.20	56.61	0.63	0.31	1.63	2.18	0.48
Bactericidal permeability-increasing protein precursor	BPI	http://www.uniprot.org/uniprot/P17213	4	1.29	2.18	68.13	1.33	0.44	2.81	2.66	0.39
Isoform 1 of Dedicator of cytokinesis protein 2	DOCK2	http://www.uniprot.org/uniprot/Q92608	4	1.05	1.08	2.38	0.47	0.16	1.53	0.85	0.09
Leukocyte elastase inhibitor	SERPINB1	http://www.uniprot.org/uniprot/P30740	4	0.90	0.69	23.02	0.44	0.18	0.89	0.46	0.17
T-complex protein 1 subunit epsilon	CCT5	http://www.uniprot.org/uniprot/P48643	4	0.67	1.51	123.86	1.18	0.86	1.76	1.54	0.59
Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	http://www.uniprot.org/uniprot/O75874	4	2.48	1.53	38.22	1.17	0.62	1.81	1.37	0.50
Cofilin-1	CFL1	http://www.uniprot.org/uniprot/P23528	4	0.80	2.09	159.61	1.62	1.23	2.44	2.47	0.63
Myeloblastin precursor	PRTN3	http://www.uniprot.org/uniprot/P24158	3	1.10	1.01	7.85	1.10	0.06	0.95	0.66	0.70
Lysozyme C	LYZ	http://www.uniprot.org/uniprot/P61626	3	0.82	1.03	24.96	0.51	0.14	1.42	0.43	**0.02**
Cathelicidin antimicrobial protein	CAMP	http://www.uniprot.org/uniprot/P49913	3	1.71	1.46	14.40	0.78	0.14	1.97	1.25	0.17
Catalase	CAT	http://www.uniprot.org/uniprot/P04040	3	2.13	1.94	9.09	0.85	0.89	2.75	0.72	**0.03**
Calgranulin B	S100A9	http://www.uniprot.org/uniprot/P06702	3	0.42	1.66	297.96	2.58	0.22	0.96	1.35	0.10

Vacuolar ATP synthase catalytic subunit A	ATP6V1A	http://www.uniprot.org/uniprot/P38606	2	0.75	0.51	32.35	0.47	0.06	0.53	0.40	0.80
Alpha-centractin	ACTR1A	http://www.uniprot.org/uniprot/P61163	2	0.72	1.98	175.30	1.25	0.10	2.53	3.20	0.53
Coronin-1A	CORO1A	http://www.uniprot.org/uniprot/P31146	2	1.75	2.44	39.14	2.00	0.85	2.77	2.01	0.57
T-complex protein 1 subunit beta	CCT2	http://www.uniprot.org/uniprot/P78371	2	0.56	2.18	290.12	1.52	1.45	2.67	1.43	0.35
Aminopeptidase N	ANPEP	http://www.uniprot.org/uniprot/P15144	2	0.74	1.04	40.70	0.62	0.02	1.35	1.57	0.46
1,4-alpha-glucan-branching enzyme	GBE1	http://www.uniprot.org/uniprot/Q04446	2	1.29	3.41	164.69	2.39	0.51	4.18	5.12	0.58
Synaptic vesicle membrane protein VAT-1 homolog	VAT1	http://www.uniprot.org/uniprot/Q99536	2	3.92	1.53	60.91	0.42	0.02	2.36	2.49	0.24
Cytochrome b-245 light chain	CYBA	http://www.uniprot.org/uniprot/P13498	2	1.36	3.52	158.60	1.95	0.40	4.70	3.38	0.23
L-lactate dehydrogenase B chain	LDHB	http://www.uniprot.org/uniprot/P07195	2	3.06	1.79	41.65	0.82	0.09	2.51	1.60	0.13
Cytochrome b-245 heavy chain	CYBB	http://www.uniprot.org/uniprot/P04839	2	1.20	1.38	15.28	1.65	1.00	1.18	0.15	0.38
Myeloid cell nuclear differentiation antigen	MNDA	http://www.uniprot.org/uniprot/P41218	2	1.07	1.58	47.77	1.78	1.74	1.43	1.21	0.76
Aspartyl-tRNA synthetase	DARS	http://www.uniprot.org/uniprot/P14868	2	3.68	2.03	44.98	0.95	0.32	2.83	1.55	0.10
Copine-3	CPNE3	http://www.uniprot.org/uniprot/O75131	2	0.41	1.47	263.60	0.67	0.25	2.08	2.51	0.39
Importin subunit beta-1	KPNB1	http://www.uniprot.org/uniprot/Q14974	2	3.54	1.39	60.67	0.30	0.11	2.21	2.51	0.26
Isoform 1 of L-lactate dehydrogenase A chain	LDHA	http://www.uniprot.org/uniprot/P00338	2	4.60	2.81	38.92	1.35	0.31	3.90	2.22	0.11
Integrin-linked protein kinase	ILK	http://www.uniprot.org/uniprot/Q13418	2	2.81	1.33	52.84	0.82	0.41	1.71	1.66	0.41
Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	http://www.uniprot.org/uniprot/P20292	2	2.68	3.35	24.95	1.78	0.63	4.52	4.68	0.37

Table 10. Membrane proteomics. Shows proteins identified from the membrane fraction of neutrophils isolated from SSc patients and healthy controls based on >1 matched peptide. †Indicates the number of unique peptides matched to each protein. ‡Indicates the relative abundance of the protein in the internal control. Δ Indicates the average relative protein abundance in all samples. ϕ Indicates the difference between Δ and ϕ as a percentage of Δ. P value is calculated for difference between mean protein abundance in SSc neutrophils and healthy controls using the student T test. ANOVA analysis with Bonferroni correction revealed no significant differences between SSc and control.

						Control		SSc		
Protein description	**Gene name**	**Accession no.**	**Internal control**	**Calculated**	**% Difference**	**Average**	**SD**	**Average**	**SD**	**p value**
fMet-Leu-Phe receptor	FPR1	http://www.uniprot.org/uniprot/P21462	0.23	0.79	315.00	1.20	0.90	0.48	0.73	0.30
Dynein heavy chain, cytosolic	DYNC1H1	http://www.uniprot.org/uniprot/Q14204	1.41	0.64	95.65	0.16	0.09	1.00	0.89	0.17
Proteasome subunit alpha type-2	PSMA2	http://www.uniprot.org/uniprot/P25787	0.40	1.64	364.00	2.84	2.24	0.73	0.31	0.11
Zinc finger protein 677	ZNF677	http://www.uniprot.org/uniprot/Q86XU0	1.66	1.17	95.20	1.45	0.94	0.96	0.81	0.49
Peptidoglycan recognition protein	PGLYRP1	http://www.uniprot.org/uniprot/O75594	0.26	0.88	315.00	0.76	0.54	0.97	0.68	0.68
Protein unc-13 homolog D	UNC13D	http://www.uniprot.org/uniprot/Q70J99	0.69	1.06	85.00	0.87	0.12	1.20	1.22	0.66
N-acetylglucosamine kinase	NAGK	http://www.uniprot.org/uniprot/Q9UJ70	0.30	1.27	387.00	1.76	1.30	0.90	0.98	0.36
HSPA5 protein	HSPA5	http://www.uniprot.org/uniprot/Q2KHP4	0.51	2.72	487.00	2.70	0.87	2.74	2.12	0.98
Zinc finger protein 428	ZNF428	http://www.uniprot.org/uniprot/Q96B54	1.68	7.27	263.00	1.71	0.86	11.44	19.35	0.43
NULL			0.00	0.17	NA	0.03	0.06	0.27	0.54	0.49
26S proteasome non-ATPase regulatory subunit 2	PSMD2	http://www.uniprot.org/uniprot/Q13200	0.51	0.74	93.00	0.53	0.22	0.90	0.63	0.38
Immunoglobulin superfamily member 2	IGSF2	http://www.uniprot.org/uniprot/Q93033	2.09	2.01	113.11	1.76	0.26	2.19	0.67	0.34
Integrin beta chain, beta 2 variant	ITGB2	http://www.uniprot.org/uniprot/P05107	0.35	0.98	243.00	1.08	0.80	0.90	0.91	0.79
Uncharacterised	C6orf10	http://www.uniprot.org/uniprot/Q5SRN2	0.00	0.39	NA	0.37	0.34	0.39	0.48	0.95
Ret finger protein-like 4B	RFPL4B	http://www.uniprot.org/uniprot/Q6ZWI9	1.08	3.34	200.00	1.26	0.05	4.90	7.48	0.45
cAMP-dependent protein kinase type I-alpha regulatory subunit	PRKAR1A	http://www.uniprot.org/uniprot/P10644	0.25	1.09	407.00	1.88	1.46	0.49	0.54	0.13
Heat shock 70kDa protein 12A	HSPA12A	http://www.uniprot.org/uniprot/O43301	0.53	2.94	496.00	1.48	2.29	4.04	4.44	0.41
Epiplakin	EPPK1	http://www.uniprot.org/uniprot/P58107	4.26	8.28	231.44	3.84	0.34	11.62	10.83	0.28
Puromycin-sensitive aminopeptidase	NPEPPS	http://www.uniprot.org/uniprot/P55786	0.62	2.63	360.00	4.23	3.09	1.44	2.03	0.20
Vasodilator-stimulated phosphoprotein	VASP	http://www.uniprot.org/uniprot/P50552	0.84	2.45	205.00	4.31	3.29	1.05	0.62	0.10
Coatomer subunit beta	COPB1	http://www.uniprot.org/uniprot/P53618	0.55	0.74	80.00	0.45	0.13	0.95	0.87	0.38
Actin-like protein (Fragment)	P704P	NA	0.43	1.55	318.00	2.81	2.27	0.61	0.60	0.11
T-complex protein 1 subunit beta	TCP1	http://www.uniprot.org/uniprot/P17987	1.90	4.24	34.00	1.70	0.14	6.15	8.52	0.42
Annexin A1	ANXA1	http://www.uniprot.org/uniprot/Q05BR2	8.31	2.59	799.59	0.76	0.17	3.97	3.72	0.21
Calpain-1 catalytic subunit	CAPN1	http://www.uniprot.org/uniprot/P07384	0.50	0.74	97.00	0.49	0.10	0.92	1.06	0.52
Eukaryotic initiation factor 4A-III	EIF4A3	http://www.uniprot.org/uniprot/P38919	1.10	1.59	34.00	0.95	0.09	2.08	2.24	0.43
Regulator of G-protein signalling 19	RGS19	http://www.uniprot.org/uniprot/P49795	0.39	0.75	151.00	0.35	0.09	1.04	1.42	0.45
Keratin, type II cytoskeletal 5	KRT5	http://www.uniprot.org/uniprot/P13647	0.61	0.80	69.00	0.82	0.06	0.78	0.53	0.91
Proteasome 26S non-ATPase subunit 8	PSMD8	http://www.uniprot.org/uniprot/P48556	0.47	0.79	121.00	0.43	0.11	1.07	1.38	0.47
Short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor	C20orf70	http://www.uniprot.org/uniprot/Q96DR5	7.49	2.87	710.56	0.67	0.42	4.52	6.93	0.39
Cytidine deaminase	CDA	http://www.uniprot.org/uniprot/P32320	10.21	4.78	973.95	1.05	0.24	7.58	8.40	0.25

Glucose-6-phosphate isomerase	GPI	http://www.uniprot.org/uniprot/P06744	3.32	0.48	317.66	0.17	0.02	0.71	0.61	0.20
Osteoclast-stimulating factor 1	OSTF1	http://www.uniprot.org/uniprot/Q92882	1.34	0.60	89.70	0.22	0.05	0.89	0.79	0.21
Retinoblastoma-associated protein	RB1	http://www.uniprot.org/uniprot/P06400	0.14	0.98	662.00	1.86	1.57	0.31	0.35	0.11
CDNA FLJ34945 fis, clone NT2RP7008454	NA	NA	0.68	0.48	3.00	0.43	0.37	0.51	0.60	0.84
Actin-related protein 2/3 complex subunit 2	ARPC2	http://www.uniprot.org/uniprot/O15144	2.09	0.97	162.06	1.20	0.78	0.80	0.67	0.50
55kDa protein	NA	NA	1.10	1.06	13.44	1.17	0.39	0.98	0.28	0.48
CDNA FLJ36836 fis, clone ASTRO2011149	NA	NA	2.24	8.77	167.00	2.70	0.56	13.32	19.79	0.41
MYL6 11kDa protein	MYL6	http://www.uniprot.org/uniprot/P60660	0.08	0.20	241.00	0.19	0.27	0.21	0.31	0.96
Short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor	C20orf70	http://www.uniprot.org/uniprot/Q96DR5	1.32	1.58	12.90	1.23	0.01	1.84	0.46	0.08
Calnexin precursor	CANX	http://www.uniprot.org/uniprot/P27824	0.68	0.04	62.23	0.00	0.00	0.07	0.09	0.30
Serine/threonine-protein kinase OSR1	OXSR1	http://www.uniprot.org/uniprot/O95747	0.47	0.69	97.00	0.51	0.14	0.82	0.78	0.53
MET 24 kDa protein	MET	http://www.uniprot.org/uniprot/P08581	2.00	1.56	121.83	0.86	0.75	2.08	3.19	0.55
IGHV4-31 Immunoglobulin heavy variable 4-31	IGHV4-31	NA	0.00	6.03	NA	7.46	12.92	4.97	9.93	0.78
Heat shock protein HSP 90-beta	HSP90AB1	http://www.uniprot.org/uniprot/P08238	0.27	0.92	311.00	0.31	0.06	1.38	1.88	0.38
Proteasome subunit alpha type-5	PSMA5	http://www.uniprot.org/uniprot/P28066	0.51	0.59	65.00	0.17	0.00	0.91	1.38	0.41
Macrophage migration inhibitory factor	MIF	http://www.uniprot.org/uniprot/P14174	4.26	2.15	375.65	0.52	0.11	3.38	4.77	0.36
Uncharacterized protein ENSP00000382129	NA	NA	0.61	1.72	223.00	2.89	2.62	0.84	0.73	0.19
Eukaryotic translation elongation factor 1 epsilon-1	EEF1E1	http://www.uniprot.org/uniprot/O43324	1.53	1.24	73.00	1.32	0.58	1.17	0.61	0.76
Isoform 1 of NLR family CARD domain-containing protein 4	NLRC4	http://www.uniprot.org/uniprot/Q9NPP4	0.09	0.10	106.00	0.11	0.02	0.10	0.02	0.56
Annexin A11	ANXA11	http://www.uniprot.org/uniprot/P50995	0.41	1.67	364.00	1.59	0.41	1.74	0.76	0.77
Profilin-1	PFN1	http://www.uniprot.org/uniprot/P07737	1.05	0.32	75.05	0.08	0.01	0.49	0.50	0.22
Cytokine receptor-like factor 3	CRLF3	http://www.uniprot.org/uniprot/Q8IUI8	0.35	1.42	374.00	2.27	1.54	0.79	1.09	0.19
Protein phosphatase 1 regulatory subunit 3D	PPP1R3D	http://www.uniprot.org/uniprot/O95685	1.08	2.71	142.00	3.02	2.07	2.47	0.79	0.64
60S ribosomal protein L18	RPL18	http://www.uniprot.org/uniprot/Q07020	1.19	1.50	6.00	0.94	0.15	1.92	1.56	0.34
Olfactomedin-4 precursor	OLFM4	http://www.uniprot.org/uniprot/Q6UX06	0.37	0.44	80.00	0.30	0.16	0.54	0.52	0.47
Threonyl-tRNA synthetase, cytoplasmic	TARS	http://www.uniprot.org/uniprot/Q5M7Z9	0.29	0.65	200.00	0.35	0.08	0.88	0.72	0.28
Hexokinase-3	HK3	http://www.uniprot.org/uniprot/P52790	4.01	1.55	362.63	0.50	0.03	2.33	2.16	0.21
Galectin-3	LGALS3	http://www.uniprot.org/uniprot/Q6FGL0	0.64	1.49	166.00	0.59	0.07	2.16	2.99	0.42
Uncharacterized protein ENSP00000382574	NA	NA	0.23	0.31	111.00	0.21	0.08	0.39	0.22	0.25
Myosin IG	MYO1G	http://www.uniprot.org/uniprot/B0I1T2	0.69	1.13	94.00	0.60	0.07	1.53	1.90	0.45
7 kDa protein	CALCOCO1	http://www.uniprot.org/uniprot/Q9P1Z2	1.75	5.04	112.00	1.50	0.20	7.69	8.81	0.29
High mobility group protein B2	HMGB2	http://www.uniprot.org/uniprot/P26583	1.71	4.83	111.00	2.23	1.16	6.77	11.18	0.52
Uncharacterized protein ENSP00000368456	NA	NA	1.42	2.01	1.45	0.98	0.34	2.77	3.33	0.41
Inosine-5'-monophosphate dehydrogenase 2	IMPDH2	http://www.uniprot.org/uniprot/P12268	0.09	0.38	403.00	0.59	0.45	0.23	0.29	0.25

Leukocyte surface antigen CD53	CD53	http://www.uniprot.org/uniprot/P19397	0.87	0.96	24.00	0.70	0.18	1.16	0.40	0.13
Neutrophil collagenase precursor	MMP8	http://www.uniprot.org/uniprot/P22894	2.51	0.74	221.31	0.22	0.06	1.12	1.15	0.24
Exportin-1	XPO1	http://www.uniprot.org/uniprot/O14980	0.63	2.15	281.00	3.65	2.96	1.02	0.91	0.15
Isoform 1 of Spatacsin	SPG11	http://www.uniprot.org/uniprot/Q96JI7	1.02	1.07	3.00	0.62	0.44	1.41	1.10	0.29
40S ribosomal protein S13	RPS13	http://www.uniprot.org/uniprot/P62277	0.35	1.08	274.00	1.71	1.42	0.60	0.83	0.25
Pentraxin-related protein PTX3 precursor	PTX3	http://www.uniprot.org/uniprot/P26022	0.00	0.19	NA	0.04	0.06	0.30	0.61	0.49
Centromere protein F	CENPF	http://www.uniprot.org/uniprot/P49454	1.26	0.52	85.33	0.18	0.01	0.77	0.68	0.21
Ficolin-1 precursor	FCN1	http://www.uniprot.org/uniprot/O00602	2.14	1.30	152.79	1.49	1.06	1.16	1.32	0.74
Receptor-type tyrosine-protein phosphatase eta precursor	PTPRJ	http://www.uniprot.org/uniprot/Q12913	0.62	2.10	280.00	3.52	2.55	1.04	0.69	0.11
Talin-related protein (Fragment)	NA	http://www.uniprot.org/uniprot/Q9Y4A1	1.11	2.26	92.00	2.34	0.26	2.20	0.67	0.76
Isoform alpha-enolase of Alpha-enolase	ENO1	http://www.uniprot.org/uniprot/P06733	0.98	5.35	450.00	3.30	3.75	6.88	3.43	0.24
26S proteasome non-ATPase regulatory subunit 3	PSMD3	http://www.uniprot.org/uniprot/O43242	0.38	1.34	312.00	2.06	1.48	0.81	1.12	0.25
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor	RPN1	http://www.uniprot.org/uniprot/P04843	0.82	2.99	284.00	5.32	4.95	1.24	0.64	0.15
Uncharacterized protein ENSP00000381431	NA	NA	0.71	1.54	147.00	1.35	1.08	1.69	0.93	0.68
Isoform 1 of Immunoglobulin superfamily member 10 precursor	IGSF10	http://www.uniprot.org/uniprot/Q6WRI0	2.26	4.63	21.02	2.20	0.85	6.45	8.90	0.46
Glial maturation factor gamma	GMFG	http://www.uniprot.org/uniprot/O60234	1.46	1.38	51.41	1.43	0.15	1.35	0.46	0.79
60S acidic ribosomal protein P2	RPLP2	http://www.uniprot.org/uniprot/Q6FG96	1.67	0.58	132.66	0.15	0.01	0.91	0.81	0.18
40S ribosomal protein S5	RPS5	http://www.uniprot.org/uniprot/P46782	3.21	0.91	293.12	0.23	0.02	1.42	1.34	0.19
CDNA FLJ32372 fis, clone SALGL1000005	RILPL2	http://www.uniprot.org/uniprot/Q969X0	0.35	1.36	350.00	2.11	1.59	0.80	1.15	0.26
CD59 glycoprotein precursor	CD59	http://www.uniprot.org/uniprot/P13987	0.42	0.43	60.00	0.26	0.03	0.56	0.59	0.42
LOC442501 Uncharacterized protein ENSP00000382470	NA	NA	1.74	4.63	91.00	2.73	0.62	6.04	8.26	0.53
FACT complex subunit SPT16	SUPT16H	http://www.uniprot.org/uniprot/Q9Y5B9	1.07	1.67	49.00	1.87	0.23	1.52	0.44	0.27
Adenosylhomocysteinase	AHCY	http://www.uniprot.org/uniprot/P23526	0.27	1.51	538.00	0.39	0.19	2.35	2.58	0.26
Ras-related protein Rab 8B	RAB8B	http://www.uniprot.org/uniprot/Q92930	0.58	1.98	282.00	3.35	2.48	0.96	0.73	0.12
Azurocidin precursor	AZU1	http://www.uniprot.org/uniprot/P20160	0.49	0.74	101.00	0.37	0.18	1.02	1.03	0.34
Eosinophil peroxidase	EPX	http://www.uniprot.org/uniprot/P11678	5.52	2.38	508.44	0.32	0.03	3.92	4.87	0.27
Alpha-actinin-4	ACTN4	http://www.uniprot.org/uniprot/O43707	0.57	0.98	112.00	0.62	0.06	1.25	1.25	0.44
U4/U6.U5 tri-snRNP-associated protein 1	SART1	http://www.uniprot.org/uniprot/O43290	1.85	0.97	132.14	1.31	0.96	0.71	0.73	0.38
14-3-3 protein epsilon	YWHAE	http://www.uniprot.org/uniprot/P62258	0.00	0.09	NA	0.00	0.00	0.16	0.33	0.44
Tubulin alpha-4 chain	TUBA4B	http://www.uniprot.org/uniprot/Q9H853	0.74	1.02	65.00	0.79	0.07	1.20	0.66	0.34
26S protease regulatory subunit 6A	PSMC3	http://www.uniprot.org/uniprot/P17980	1.24	2.05	42.00	1.25	0.28	2.65	2.64	0.41
Glycogen phosphorylase	PYGB	http://www.uniprot.org/uniprot/P11216	2.30	3.43	80.62	1.94	0.46	4.55	5.66	0.47
Resistin precursor	RETN	http://www.uniprot.org/uniprot/Q9HD89	0.50	1.06	160.00	0.42	0.01	1.54	1.80	0.34

FMLP-related receptor I	FPRL1	http://www.uniprot.org/uniprot/P25090	0.60	2.40	338.00	3.58	2.59	1.52	2.28	0.31
Alpha-actinin-2	ACTN2	http://www.uniprot.org/uniprot/P35609	1.00	1.66	65.00	1.01	0.16	2.14	2.14	0.41
Phosphorylase b kinase regulatory subunit alpha	PHKA2	http://www.uniprot.org/uniprot/P46019	0.17	0.93	542.00	1.59	1.13	0.43	0.55	0.13
Protein-arginine deiminase type-2	PADI2	http://www.uniprot.org/uniprot/Q96DA7	0.99	0.63	34.45	0.43	0.07	0.79	0.43	0.22
Ras GTPase-activating-like protein IQGAP3	IQGAP3	http://www.uniprot.org/uniprot/Q86VI3	0.81	0.72	8.00	0.48	0.46	0.90	0.35	0.22
Interferon gamma precursor	IFNG	http://www.uniprot.org/uniprot/P01579	0.99	4.39	345.00	8.18	6.87	1.55	1.41	0.11
Laminin subunit alpha-5 precursor	LAMA5	http://www.uniprot.org/uniprot/O15230	0.74	2.96	324.00	0.84	0.31	4.54	6.70	0.39

Table 11. Membrane proteomics. Shows proteins identified from the membrane fraction of neutrophils isolated from SSc patients and healthy controls based on only 1 matched peptide. No significant differences were found in protein expression between SSc patients and controls.

5.3.4 iTRAQ.

iTRAQ identified 48 proteins using more than one peptide (Table 10) and 105 proteins by 1 peptide identification in the neutrophil membrane fraction (Table 11). Proteins based on only 1 peptide identification were discounted in further analyses. An internal control was included which contained equal volumes of each protein sample. Therefore, assuming correct quantification, the relative quantity of each protein in the internal control should equal the sum of the quantity of that protein in each sample divided by the number of samples (N=7). The percentage difference between these 2 quantities was calculated and reflects the accuracy of the quantification in each case. The variation ranged from 1.13% (leukocyte elastase) to 297.96% (calgranulin B). Proteins that varied from the control more than 63% (75th centile) were disregarded in further analysis (Fig.40).

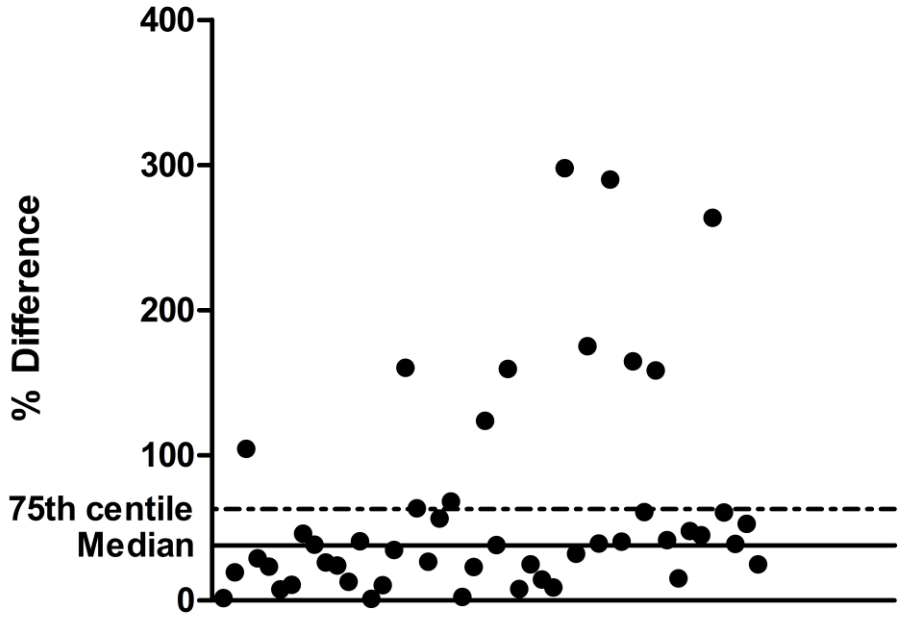


Fig.40. Assessment of the reliability of protein quantification using iTRAQ. Each point on the scatter graph represents one protein identified in the neutrophil membrane proteome. The difference between the average protein abundance across samples compared to the internal control abundance is plotted as a percentage of the internal control abundance. The smaller the difference the more reliable the quantification data for that protein. Proteins that lie above the 75th percentile for % difference (63%) are regarded as too unreliable and quantification data for these proteins is discarded (n=11).

Membrane proteomic data shows no membrane proteins are differentially expressed in SSc neutrophils compared to controls with statistical significance. There is a trend to significance for the expression of several proteins but due to the multiple comparisons made, the application of the stringent Bonferroni correction meant that no changes were significant. iTRAQ is also limited by the fact that a maximum of 7 samples can be compared in one experiment (the 8th being reserved for internal control, a mixture of equal quantities of all 7 samples used for validation of quantification (see above)). Therefore only 3 control and 4 SSc samples could be compared. Therefore, it is unsurprising that results did not reach statistical significance. However, the study served its purpose by indicating potential differentially expressed proteins which can be explored further using other techniques such as flow cytometry which can be carried out in greater numbers of patients. As has often been the case in patients with SSc, the protein abundance is often highly variable (more variable than abundance in healthy controls), likely to reflect the heterogeneity of the disease.

5.3.5 Calgranulin expression is decreased in the plasma membrane fraction of SSc neutrophils compared to controls.

Proteins of interest include membrane-expressed calgranulin A (S100A8), which is down regulated in 3 of the patients with SSc (Fig.41). Total neutrophil calgranulin was elevated in SSc compared to controls (Chapter 4), therefore it is interesting to find a down regulation in its localisation within membranes. N.B. The membrane fraction may contain intracellular membrane proteins in addition to plasma membrane proteins. Proteins associated with the plasma membrane are not necessarily orientated to the outside of the membrane, as many are associated with the intracellular surface of the plasma membrane where they are involved in signal transduction and cytoskeletal organisation.

Calgranulin B (S100A9), the heterodimer partner of calgranulin A, was also found to be down-regulated in 3 of the SSc neutrophil membrane samples (Fig.42) However, the average protein abundance varied widely from the internal control abundance (>63%) and

so this apparent down-regulation is questionable. However, the 2 proteins are most stably expressed as a heterodimer which is responsible for many of the protein's functions. As both proteins were found to be down-regulated in the same patients when the majority of other proteins were upregulated or remained constant in SSc, the down-regulation may be genuine.

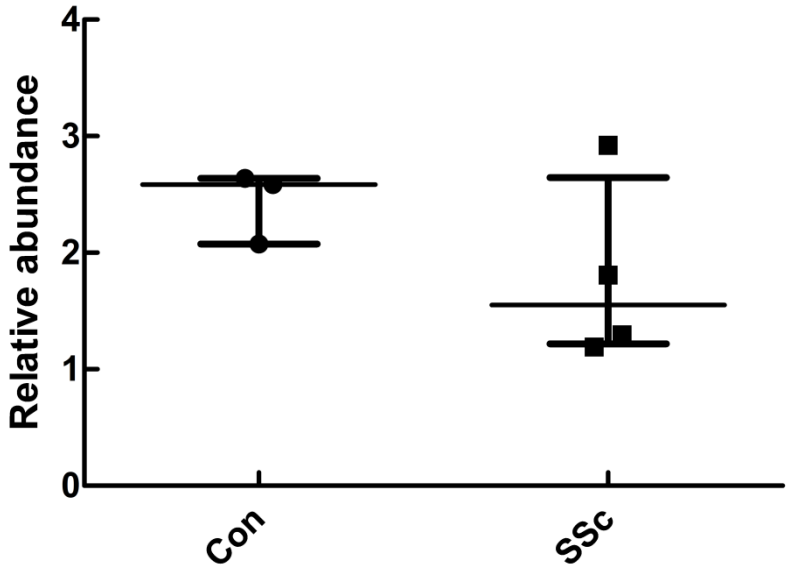


Fig.41. Calgranulin A (S100A8) abundance in 3 control neutrophil and 4 SSc neutrophil membrane samples. ■ and ● represent individual patient and control samples respectively. In addition, median and interquartile range are shown.

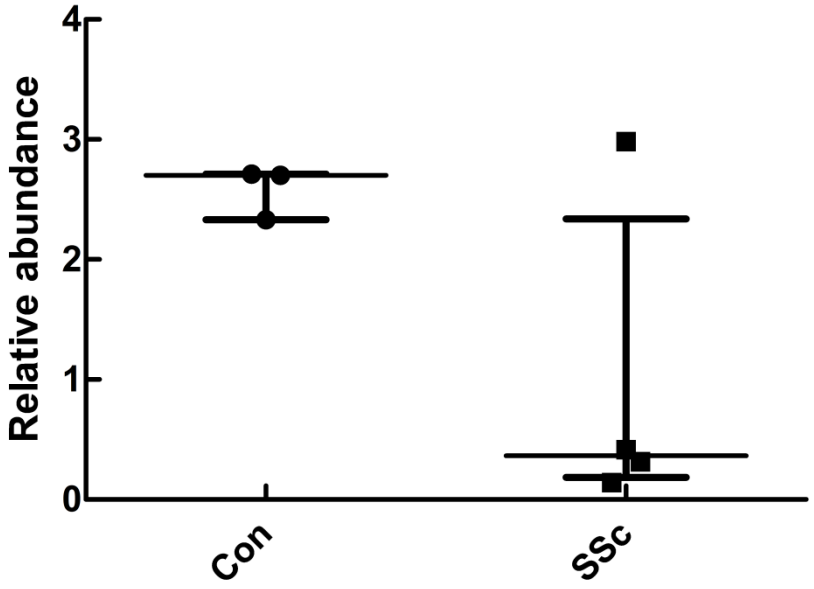


Fig.42.Calgranulin B (S100A9) abundance in 3 control neutrophil and 4 SSc neutrophil membrane samples. ■ and ● represent individual patient and control samples retrospectively. In addition, median and interquartile range are shown.

5.3.6 Specific granule proteins are upregulated in the plasma membrane of SSc neutrophils compared to controls.

Lysozyme C is also upregulated (Fig.43), it is consistently upregulated in all patient samples compared to control levels.

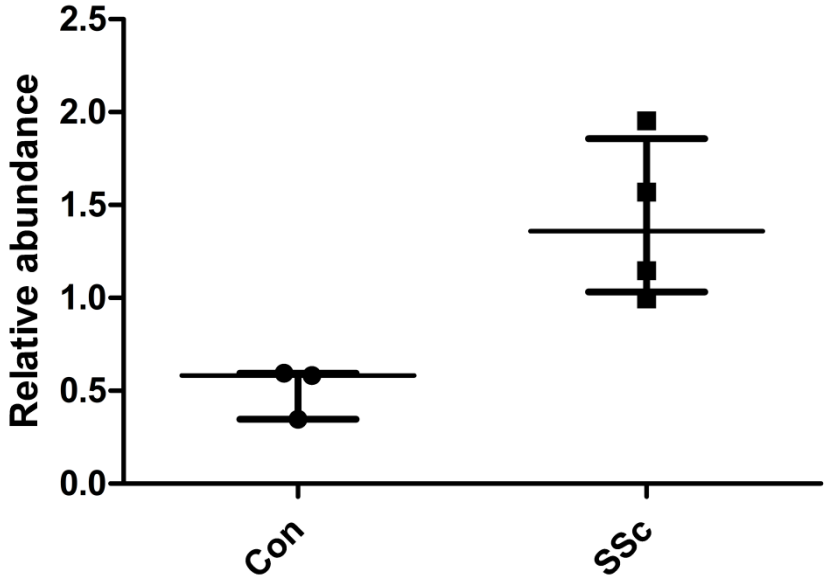


Fig.43. Lysozyme C protein abundance in 3 control and 4 SSc neutrophil membrane samples. ■ and ● represent individual patient and control samples retrospectively. In addition, median and interquartile range are shown.

5.3.7 The anti-oxidant enzyme catalase is increased in the plasma membrane fraction of SSc neutrophils compared to controls.

Median catalase expression in the plasma membrane fraction is also increased in patients compared to controls (Fig.44).

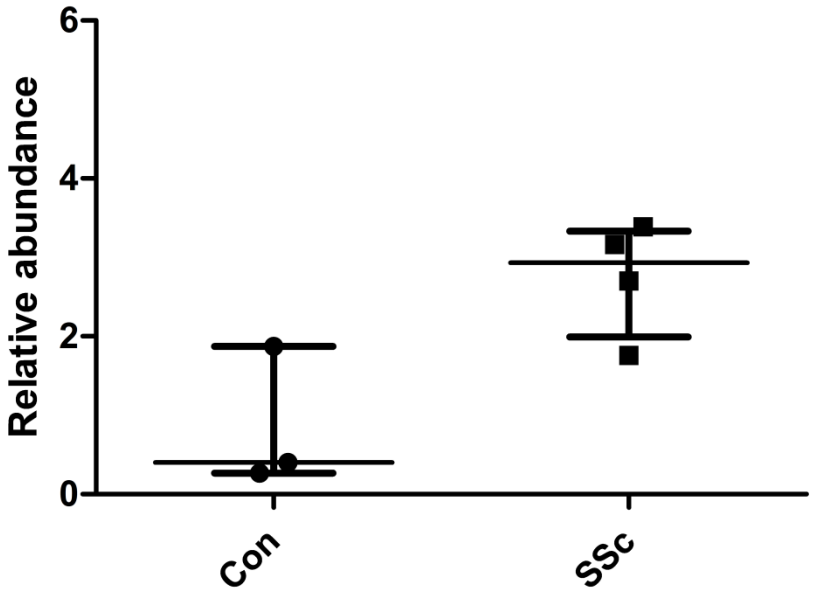


Fig.44. Catalase protein abundance in 3 control and 4 SSc neutrophil membrane samples. Symbols ■ and ● represent individual patient and control samples retrospectively. In addition, median and interquartile range are shown.

5.3.8 Proteins involved in the control of actin polymerisation are increased in the plasma membrane fraction of SSc neutrophils compared to controls.

L-plastin, otherwise known as actin-bundling protein, is increased in the plasma membrane fraction of 3/4 SSc neutrophils compared to controls (Fig.45). L-plastin is a multifunctional protein, involved not only in the control of actin polymerisation but is also an important signalling molecule.

Actin-related peptide is involved in the control of actin polymerisation. It is important in the nucleation of actin monomers necessary for the subsequent propagation of polymerisation. Actin-related peptide was increased in the plasma membrane fraction of 3/4 SSc patients compared to controls (Fig.46).

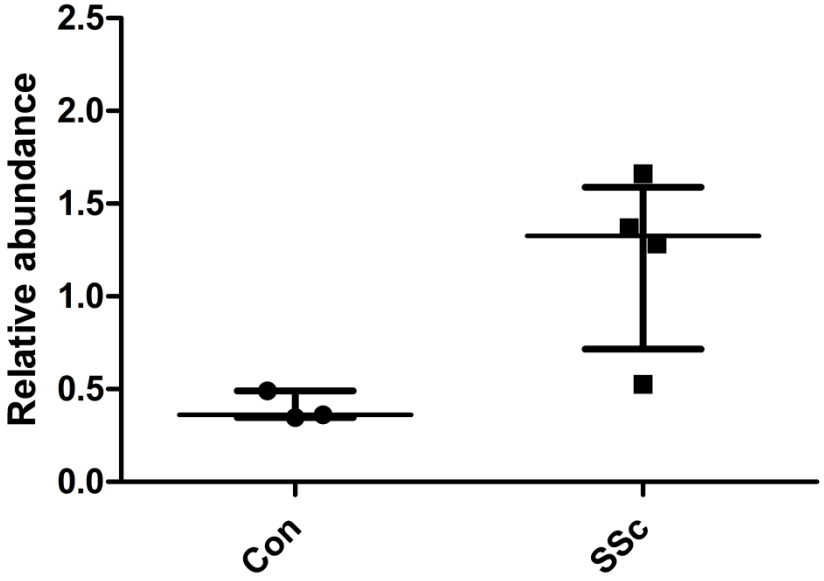


Fig.45. L-plastin protein abundance in 3 control and 4 SSc neutrophil membrane samples. Symbols ■ and ● represent individual patient and control samples retrospectively. In addition, median and interquartile range are shown.

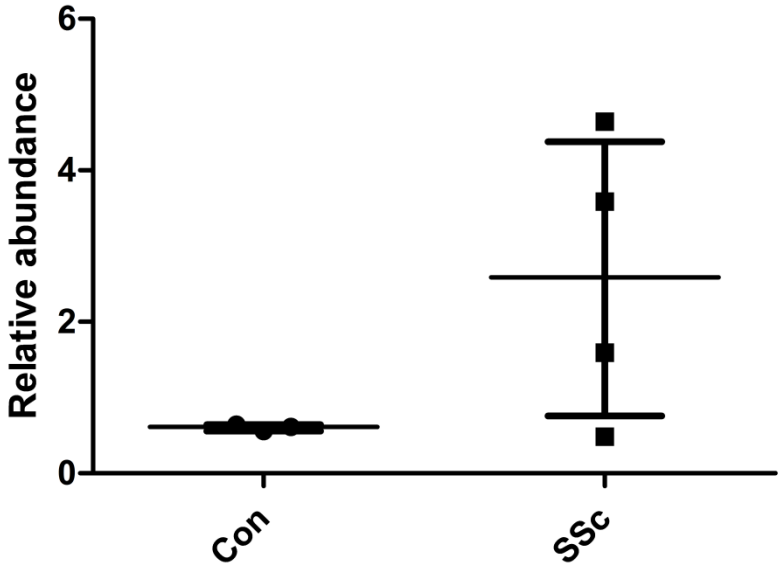


Fig.46. Actin-related peptide abundance in 3 control and 4 SSc neutrophil membrane samples. ■ and ● represent individual patient and control samples, retrospectively. In addition, median and interquartile range are shown.

It is interesting to note that in many cases one of the patient samples did not fit with results seen in the rest of the disease group but had protein expression levels more similar to the control group. This patient is a patient with long standing limited SSc, has pulmonary artery hypertension and is on Sildenafil. The other patients had much shorter disease duration and have progressive symptoms.

5.4 Discussion.

5.4.1 Calgranulin.

It is potentially interesting to find differential expression in both the whole cell and membrane levels of calgranulin A (S100A8) and B (S100A9) and that similar changes were seen in both proteins. The functions of S100A8 and S100A9 have been the subject of a great deal of recent research. S100A8 and S100A9 are members of the S100 family of proteins, are expressed by monocytes and neutrophils and also can be induced in keratinocytes during inflammation. Their expression is up-regulated in a wide range of malignancies and also in inflammatory disease [267]. They can be found as monomers but frequently form homo- and hetero-dimers and higher order oligomers. They are found in high abundance within the neutrophil cytoplasm constituting up to 45% of the neutrophil cytoplasmic protein [268] but they can also be found associated with the plasma membrane on activation [269]. They may be secreted into the extracellular space in an energy-dependent, microtubule mediated process, separate from the classical Golgi dependent secretion process [270]. S100A8 and S100A9 appear to have distinct functions in all 3 locations and function is also dictated by dimerisation and post-translational modification [271]. The complex functions of these proteins have been difficult to untangle, not least because the mouse homologues do not always function in an analogous fashion [270 271]. Therefore, extensive studies using mouse knock-out models have sometimes given results that are contradictory to human *in vitro* investigations.

5.4.1.1 Effects on the endothelial cell.

Treatment of endothelial cell lines with the heterodimer of S100A8 and S100A9, calprotectin results in an increase in endothelial cell ICAM, VCAM and thrombospondin expression and an increase in the production of chemokines IL-8, MCP-1[272]. This phenotype is reflected in SSc, where there is evidence of increased serum levels of soluble ICAM and soluble VCAM, which correlate with increased endothelial expression of these molecules [16]. There is also, in SSc serum, an increase in the serum levels of thrombospondin and serum levels of all 3 markers of endothelial activation correlate with disease severity [16 19]. MCP-1 and IL-8 levels are also increased in SSc serum and an increased expression of MCP-1 is found in histological samples from SSc skin and in explanted SSc endothelial cells grown *in vitro* [33 80 82 105 108 110 273].

Calprotectin can also result in functional changes at the endothelial surface resulting in a prothrombogenic phenotype with increased endothelial cell permeability and a decrease in monolayer integrity, all changes which are mirrored in SSc [272]. Calprotectin may increase endothelial cell detachment and initiate caspase-dependent and -independent endothelial cell apoptosis, also features of the SSc endothelium [274].

Extracellular calprotectin binds to heparan sulphate proteoglycans and carboxylated glycans on endothelial cells [275]. Calprotectin also binds arachidonic acid and may act as carrier protein to translocate arachidonic acid to the endothelial cell. It is of interest to note that endothelial cells utilise endogenous and exogenous arachidonic acid for transcellular production of thromboxane, an eicosanoid which acts as a powerful vasoconstrictor [276]. Thromboxane A_2 is elevated in SSc and levels correlate with disease severity [19]. It is tempting to hypothesise that locally-generated thromoboxanes may be acting in a paracrine fashion to cause vasoconstriction resulting in Raynaud's phenomenon which is a prominent symptom in SSc.

5.4.1.2 Effects on leukocytes.

S100A8/9 (calprotectin) increases the activity of neutrophil NAPDH oxidase, increasing the production of ROS [277]. This involves intracellular interaction with components of the oxidase and the binding of arachidonic acid is essential for oxidase assembly [278-280]. Arachidonic acid has previously been established as a cofactor for NAPDH oxidase and it may be that calprotectin with its arachidonic-binding capability provides this by interacting with the NADPH oxidase complex.

Murine S100A8 is chemotactic for neutrophils and monocytes, but the data for human S100A8 and 9 in neutrophil adhesion and chemotaxis is somewhat confused. In part, this may be due to the improper folding of recombinant forms of human S100A8 and A9 used in these experiments. Also, posttranslational oxidation and nitrosylation of S100A8 and A9 have profound effects on their function and if not controlled could yield disparate results in *in vitro* systems. S100A8, A9 and the heterocomplex have been shown to increase neutrophil adhesion to fibrinogen, by increasing the activity and expression of Mac-1 (CD11b/CD18) [281]. Others, however, have shown that human S100A9 increased neutrophil adhesion but that this was inhibited in the presence of S100A8, presumably due to heterocomplex formation [282]. Others have confirmed that S100A9 and the heterodimer have a role in increasing neutrophil adhesion to fibronectin but failed to show any inhibitory effect of S100A8 [283].

Sroussi *et al* [284] found that S100A8 and A9 had no chemotactic activity for neutrophils; in fact they found the proteins to cause fugetaxis (repulsion of cells). This activity was abolished by various post-translational modifications of S100A8 and A9 [284]. Others however, have shown that S100A8, A9 and the heterodimer are chemotactic to neutrophils [281].

S100A9 and the heterodimer, promote transendothelial migration of monocytes and monocytes expressing the heterodimer on their cell surface represent a cohort of monocytes which preferentially migrate across endothelial cell layers [285].

In summary, there is contradictory evidence for the role of S100 proteins in leukocyte recruitment, but studies have consistently shown that they can increase neutrophil adhesion via activation of Mac-1 and that they promote the transendothelial cell migration of monocytes. In addition, the effect of S100 proteins on endothelial cells is likely to further promote leukocyte recruitment as a result of an increase in the expression of ICAM and VCAM. A monocytic infiltrate in lesional skin is typically found in histologic samples from SSc patients.

5.4.1.3 S100 proteins in the innate immune system and amplification of inflammation.

S100A8 and A9 are specifically expressed in leukocytes of the innate immune system (neutrophils and monocytes) but not by lymphocytes. The innate immune system comprises the first line of defence in response to invading pathogens. Monocytes and neutrophils respond non-specifically to a range of invading pathogens through receptor systems that are ligated by pathogen associated molecular patterns (PAMPs) e.g. lipopolysaccharide (LPS). Engagement of these receptor systems e.g. Toll like receptors (TLRs) results in the release of cytokines which amplify the inflammatory response and recruit cells of the adaptive immune system [267].

In addition to responding to invading pathogens, the innate immune system can respond to cell damage in a similar way. In this case, damage associated molecular patterns (DAMPs) are released and can engage with similar receptors to amplify the inflammatory response. In fact, TLR4 seems to respond to PAMPs and DAMPs. S100A8 and A9 appear to function as DAMPs and can ligate TLR4 and possibly RAGE expressed on monocytes [267].

5.4.1.4 S100 proteins and autoimmunity.

Elevated S100A8 and A9 serum levels are associated with several autoimmune disorders including rheumatoid arthritis, SLE and systemic onset juvenile idiopathic arthritis [267].

A recent study has shown that, Quinoline-3-carboxamides, which are in development for the treatment of autoimmune diseases e.g. MS, SLE and type I diabetes, may exert their actions by binding to S100A9 and preventing ligation of TLR4 and RAGE by S100A9 [286].

In a recent study in rheumatoid arthritis, elevated serum S100A8 and A9 concentrations were associated with the presence of anti-CCP and RhF [287].

5.4.1.5 S100 proteins role in oxidative stress.

S100A8 and A9 have interesting roles in oxidative stress, acting as scavengers for reactive oxygen and nitrogen species. It is thought that in this way, they may contribute to protecting phagocytes from intracellular ROS. However, as mentioned above, they can also interact with the intracellular portion of NADPH oxidase of phagocytes and increase ROS production. Extracellular S100A8 and A9 can act as ROS scavengers and therefore may decrease oxidative stress [271 288].

S100A8 and A9 are susceptible to cysteine oxidation and s-nitrosylation. These post-translational modifications can lead to completely different protein functions and can promote dimerisation, which in turn can alter the functionality. In fact, most known functions of S100 proteins are mediated by homo- or hetero-dimers [271]. There is evidence of increased oxidative stress in SSc; this may be as a result of inflammation or recurrent hypoxia-reperfusion [3 11 198 289]. Whatever the cause, oxidative stress may result in post-translational modification of S100A8 and A9 resulting in increased dimerisation and a specific functional repertoire.

5.4.2 Specific granule proteins.

Lysozyme C is found in the primary and secondary neutrophil granules. Other granule proteins are found expressed on the plasma membrane surface at increased concentrations after degranulation, reflecting the fusion of granule membrane with the plasma membrane

(e.g. CD11b, CD63, cytochrome b245, lactoferrin, fMLP receptor). One hypothesis for the increased membrane expression of lysozyme C in SSc is that this may represent a similar insertion of granule protein into the membrane and may reflect degranulation. Further experiments would be required to confirm this.

Neutrophil granules tend to discharge in a predefined order with increasing concentrations of activating or priming stimuli: secretory vesicles are mobilized first, followed by gelatinase-containing granules, secondary granules and finally primary granules (Fig.47) [218].

It is also interesting to note that different priming agents have differential effects on neutrophil degranulation. For instance TNFα, PAF and LPS all cause degranulation of secretory vesicles, secondary and gelatinase-containing granules, whereas GMCSF is capable of mobilising secretory vesicles only. Interleukin-8 results in degranulation of only secretory vesicles and secondary granules at priming doses [230].

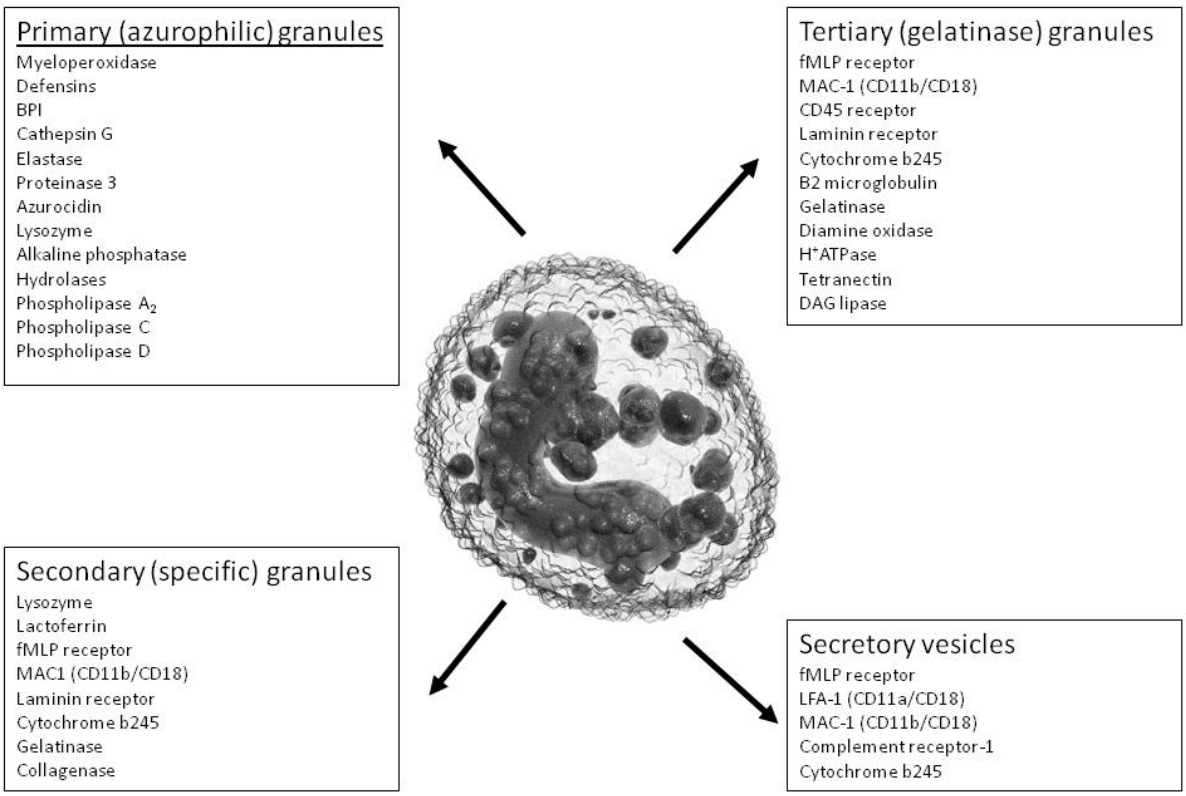


Fig.47. Neutrophil granules. Granules are mobilised hierarchically with increasing stimulant concentrations: secretory vesicles first, followed by gelatinase-containing granules, secondary granules, and finally primary granules.

Other markers of specific granule degranulation were identified in this study but were excluded from the analysis due to the unreliability of the data. Cytochrome b-245 light chain was increased in 3/4 patient samples but, it was excluded from analysis because variability compared to the internal control was greater than 63% (158%). In addition, the cytochrome b-245 heavy chain was not increased in SSc samples and the data was more reliable as the variation from internal control was only 15%. β-integrin and the fMLP receptor were both detected in the sample but only 1 peptide of each were detected and the information available was insufficient to allow meaningful quantification.

5.4.3 Cytoskeletal proteins.

L-plastin (LCP-1) is an actin-bundling protein that consists of two actin-binding domains and a head-piece that contains two calcium-binding EF hand motifs (Fig.48).

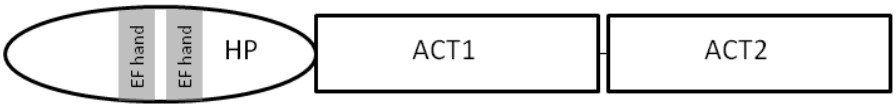


Fig.48. Structure of L-plastin. ACT=actin-binding domains. HP=head-piece. Shaded areas in head-piece represent calcium binding EF hand motifs.

L-plastin is calcium regulated and its actin cross linking functions are inhibited by rises in calcium in the physiological range. However, in addition to its actin-bundling activity, it is also involved in signal transduction. L-plastin is phosphorylated in response to stimulation with inflammatory cytokines, PMA, chemotactic peptides and immune complex ligation of FcγRII when bound to solid surfaces [254 290-293].

An intact cytoskeleton is essential for many signal transduction pathways, likely providing a scaffold for signal transduction molecules, keeping them within the vicinity of receptors. Focal adhesions are aggregates of actin, actin-binding proteins and signal transduction molecules. In motile leukocytes, these foci are called podosomes [294].

L-plastin phosphorylation occurs at a serine residue (Ser5) in the head-piece. Cell permeant peptides derived from the head-piece and containing the phosphorylated serine are capable of inducing neutrophil adhesion through activation of $\alpha_M\beta_2$ integrins. This can be abrogated by blocking the actions of phosphoinositol 3-kinase and protein kinase C. However, if the peptide was exogenously phosphorylated at serine 5, the peptide induced adhesion and this action was not blocked by kinase inhibitors. This integrin activation however, is not accompanied by an increase in calcium mobilisation or ROS generation [233].

Activation of integrins involves two distinct processes; first, there is an increase in integrin diffusion as a result of release from cytoskeletal constraints allowing integrin clusters to form; secondly, there is a conformational change within the integrin resulting in increased affinity for the ligand. L-plastin has been implicated in both these roles. The cell permeant L-plastin peptides described above were shown to mediate their effects on $\alpha_v\beta_3$ integrins through actin depolymerisation, presumably to increase integrin diffusion, and by co-operating with Arg-Gly-Asp ligand to change the conformation of the integrin to a higher affinity form [234].

This is interesting in the light of data presented in Chapter 4 which describes a decrease in F-actin content but an increase in focal adhesions in SSc neutrophils compared to controls. Although other activating stimuli are associated with an increase in F-actin, L-plastin-dependent signalling results in a decrease in F-actin but an increase in focal adhesions as seen in SSc neutrophils. Taken together with the increase in L-plastin expression, this may imply that SSc neutrophils have been activated by a stimulus that acts through L-plastin and as a result is likely to increase integrin function and adhesion, though not necessarily through an increase in integrin expression. This reflects the SSc neutrophil phenotype which I have observed to be more “sticky” (see Chapter 3) and the effect of SSc serum on healthy neutrophils which induces cell-cell interactions and the formation of multi-cellular aggregates (see Chapter 7).

An upregulation of membrane L-plastin has not previously been described and therefore the functional implications are not fully known. However, it is speculated that phosphorylation of L-plastin may regulate its molecular location, specifically, localisation to podosomes on the cell membrane [291].

Neutrophils of L-plastin knock-out mice have defective adhesion-dependent respiratory burst and hence a decreased capacity to kill *S. aureus* despite normal phagocytosis. This effect was due to markedly decreased integrin dependent syk activation. However, unlike β_2 integrin knockouts, L-plastin knockout murine neutrophils have normal adhesion to fibrinogen, fibronectin and vitronectin [295]. This result is, however, somewhat confusing since β_2 integrins do not bind to fibronectin or vitronectin. A recent study showed that engagement of β_2 integrins induce surface expression of β_1 integrins, which do bind to extracellular matrix components, but this does not explain the findings outlined above [296]. Knockout models may have their limitations since murine integrins may not faithfully represent the functions of human integrins and knock-outs may also disrupt epigenetic phenomena. The normal adhesion and spreading of L-plastin knockout neutrophils, does not rule out an important role for L-plastin in the regulation of integrin function, as many other interactions contribute to neutrophil adhesion. The neutrophils in this study were also stimulated with TNFα (1nM for 30min).

Phosphorylation of L-plastin has also been implicated in activation of neutrophil NADPH-oxidase but further studies are needed to confirm this [297].

It is interesting to note that integrin-linked protein kinase is increased in 2/4 patients with SSc. This protein is found in the podosomes and is involved in integrin-dependent signalling. It is implicated in cytoskeletal reorganization and inside-out integrin signalling. It phosphorylates the cytoplasmic domain of the β-integrins and may be implicated in integrin ligand avidity.

5.4.4 Catalase expression.

Catalase is an important anti-oxidant in neutrophils. It catalyses the decomposition of hydrogen peroxide into water and oxygen. It is predominantly found in the organelles of neutrophils. To my knowledge, there are no reports of plasma membrane expression of catalase.

There are two important mechanisms in the neutrophil that protect them from oxidative damage. The first is catalase, the second is the cytosolic glutathione reductase system which reduces hydrogen peroxide by oxidising NADPH, itself regenerated by the hexose monophosphate shunt. Neutrophils express less glutathione and glutathione peroxidise than monocytes and macrophages but higher concentrations of catalase [298]. Experiments on neutrophils from acatalasemic patients have revealed that the glutathione reductase system is sufficient to protect neutrophils from endogenous ROS, but insufficient to protect from exogenous oxidative stress [299]. Catalase in neutrophils affords them protection against exogenous oxidative stress and the higher levels of catalase found in neutrophils compared to other inflammatory cells means that they are more resistant to extracellular oxidative damage [298].

In other cell types such as liver and endothelial cells, oxidative stress can induce the expression of catalase [300 301], but it is not known whether this is also the case in neutrophils. This could explain the apparent upregulation found in this proteomic study. An alternative explanation is that catalase is translocated to the plasma membrane under conditions of exogenous oxidative stress, but this remains to be confirmed. Finally, although my experiments would imply an increase in the plasma membrane expression of catalase, the possibility cannot be excluded that there is contamination of the membrane sample. Catalase is primarily located in the cytoplasmic compartment and is co-localised with lactate dehydrogenase (LDH) [302]. LDH was also found in the membrane fraction, indicating some contamination from the cytoplasmic fraction is possible and there was a tendency for this

contamination to be greater in the SSc neutrophils. All samples were treated in exactly the same manner so there is no reason to suspect that the increased contamination with catalase and LDH occurred for methodological reasons. It is more likely that these proteins have an increased association with the plasma membrane fraction in SSc neutrophils either due to a specific increase in plasma membrane expression or due the increased expression of other membrane proteins which lead to increased adhesion of LDH and catalase indirectly. Other cytoplasmic proteins are not differentially expressed between SSc and controls in the plasma membrane samples.

The overall signature of the neutrophil phenotype in SSc cannot be confirmed from these data, predominantly due to the small number of samples examined. However, the data do point to some membrane proteins of potential interest for examination using other techniques (e.g. flow cytometry) which will allow examination of a larger number of patients. There is insufficient evidence from these proteomic studies to confirm or refute *in vivo* neutrophil priming in SSc. However, if further work can confirm that lysozyme C is a marker of degranulation and that lysozyme C is increased on the plasma membrane of SSc neutrophils, this will provide tantalising evidence of neutrophil priming in SSc. Priming is not an all or nothing response but rather a continuous spectrum of responses depending on the concentration of the priming agent. In addition, different priming agents have different effects on neutrophil phenotype. These factors, coupled with the heterogeneity of SSc, may go some way to explain the high degree of variation seen in SSc neutrophil protein expression.

Despite the obvious limitations of this approach, it does have some great strengths. It allows for an unbiased examination of the changes in membrane protein expression, and it has revealed important but unexpected changes, such as the down-regulation of membrane calgranulins and the up-regulation of L-plastin and catalase. These changes may give important clues to the factors in SSc which give rise to the change in neutrophil phenotype. For instance, the increase in catalase expression, if confirmed, could imply activation by

exogenous oxidative stress which is well established in SSc. The increase in membrane L-plastin may reflect translocation to the membrane following phosphorylation. L-plastin is phosphorylated in response to interleukin-8, which also acts as a neutrophil priming agent resulting not only in specific granule degranulation but in the priming of ROS generation not only in response to fMLP but also to PMA which may tie in the functional changes seen in chapter 3. Interleukin-8 is raised in the serum and in lesional skin of patients with SSc and is associated with early disease [34 79 224].

Chapter 6: Neutrophil elastase.

6.1Introduction.

Neutrophil elastase is a serine protease that is stored in the azurophilic granules of neutrophils. It predominantly functions as an intracellular anti-microbial protein and is released into the phagolysosome following phagocytosis to mediate bacterial digestion. However, in addition it is secreted by the cell. Azurophilic granules are the most resistant to exocytosis and therefore release of elastase into the extracellular environment is usually limited [303]. Secretion of elastase is thought only to be significant in frustrated phagocytosis or when excess neutrophil apoptosis overwhelms the phagocytic capacity to remove neutrophils, leading to secondary necrosis and the release of intracellular proteins [304 305]. In the extracellular environment it has anti-microbial activities and can break down extracellular matrix.

It is apparent however, that neutrophil elastase has additional functions in the regulation of inflammation and it is implicated in inflammatory and fibrotic conditions, including fibrotic lung disease [306 307]. Elevations in serum elastase have previously been reported in patients with SSc and were associated with lung disease [222].

Serum elastase activity is regulated by serine protease inhibitors. The main intracellular inhibitor of elastase is SERPINB1, while the main extracellular inhibitor of neutrophil elastase is α_1-protease inhibitor (α_1PI) and additional inhibition is mediated by α_2-macroglobulin, elafin and secreted leukocyte proteinase inhibitor (SLPI) [308].

Neutrophil elastase can also be expressed on the neutrophil membrane, where it occupies low and high affinity binding sites. Membrane expression is increased by neutrophil activation with cytokines such as TNFα and IL-8 [303]. The increase in membrane expression is more significant than the amount secreted extracellularly during typical activation. Plasma

membrane bound elastase has the same catalytic functions as soluble elastase but there is some evidence that the membrane bound enzyme is relatively resistant to α_1PI [309 310].

Previous work in my thesis has implied neutrophil activation in SSc. Since, neutrophil elastase has established roles in other inflammatory and fibrotic disorders I hypothesised that neutrophil elastase could be an important mediator in the pathogenesis of SSc.

To explore this hypothesis, I measured the concentration and catalytic activity of neutrophil elastase in SSc serum compared to controls. I also examined the levels of membrane expression of elastase in SSc neutrophils compared to controls and studied clinical correlates.

6.2 Methods.

6.2.1 Neutrophil elastase concentration.

Neutrophil elastase concentrations were assessed in SSc and control whole cell lysates by western blotting, as outlined in Chapter 2.

6.2.2 Serum elastase concentrations.

Neutrophil elastase levels were measured in SSc patient and control serum using a PMN elastase ELISA (Bender, Vienna) according to the manufacturer's instructions.

6.2.3 Neutrophil elastase concentrations in neutrophil culture supernatants.

Neutrophils were isolated from SSc and healthy control blood as previously described, and were cultured for 6h with gentle agitation at 37˚C at a density of 10^7cells/ml in RPMI 1640 +25mM HEPES +2mM glutamine. Cells were precipitated by centrifugation (1000*x*g for 5min) and the neutrophil elastase concentration in the supernatants was measured by ELISA (Bender, Vienna).

6.2.4 Serum neutrophil elastase enzymatic activity.

Following experiments to optimise substrate concentration and length of incubation, serum elastase activity was measured using a colorimetric assay. In 96 well clear plastic plates, 150µl of elastase buffer (0.1M HEPES, 0.5M NaCl, pH7.5) was added to 50µl serum samples from patients with SSc and healthy controls. The substrate (15mM methoxy-succinyl- alanyl-alanyl-prolyl-valyl-p-nitroanilide) was added to a final concentration 750µM. The reaction was incubated in the dark at 37˚C for 6h. The elastase dependent conversion of colourless methoxy-succinyl- alanyl-alanyl-prolyl-valyl-p-nitroanilide to yellow p-nitroanilide was measured as a change of absorbance at 405nm on a plate reader. Standards of known concentration were used to generate a calibration curve (data not shown).

6.2.5 Clinical data.

Clinical data was collected on all patients as a part of routine clinical care and included: Rodnan skin score; neutrophil count; autoantibody profile; medications; major organ involvement and disease duration (defined as time since onset of first non-Raynaud's symptom).

6.2.6 Serum SERPINB1 levels.

As no ELISA kits were available, serum SERPINB1 concentrations were estimated by western blotting as detailed earlier. Serum samples were depleted of the albumin and IgG using an Albumin/IgG dye based depletion kit (Pierce, Rockford, USA) in order to minimise distortion of the western blot.

6.2.7 Neutrophil membrane elastase expression.

Neutrophil membrane elastase expression was measured by flow cytometry. Neutrophils were isolated from SSc and healthy control blood as previously described. Cells were resuspended in RPMI 1640 +25mM HEPES +2mM glutamine at $5x10^6$/ml. 100µl of cells

were placed in a flow cytometry tube and excess PBS EDTA was added. Cells were then centrifuged at 1000*x*g for 3min and resuspended in 200µl of PBS 2%BSA, and incubated for 30min in the dark at 4˚C with 2µl elastase monoclonal antibody raised in rabbit (Calbiochem), following experiments to find the optimal antibody dilution (data not shown). The cells were washed 3x in PBS and resuspended in PBS 2%BSA and 2µl FITC-conjugated anti-rabbit IgG secondary antibody. Cells were incubated for 30min at 4˚C in the dark. Cells were washed 3x in PBS, resuspended in PBS and analysed on the flow cytometer on the FITC channel. Mean fluorescence readings were corrected for secondary antibody controls.

6.2.8 Statistical methods.

The data was non-normally distributed and so means were compared using the Mann-Whitney U test. The strength and significance of bivariate correlations were made using Spearman's Rho.

6.3 Results.

6.3.1 Clinical characteristics.

	Median (IQR)
Disease duration/mths	40 (21-96)
Neutrophil count x10^9/l	4 (2.4-4.8)
Rodnan skin score	6 (3-10)
	Number (%)
Limited SSc	29/33 (88)
Diffuse SSc	4/33 (12)
ANA	26/31 (84)
Anticentromere	13/31 (42)
Anti RNP	9/31 (29)
Anti Scl70	9/31 (29)
Lung involvement	10/33 (30)
PAH	5/33 (15)
DMARDs	14/33 (42)
HCQ	3/33 (9)
MMF	4/33 (12)
MTX	1/33 (3)
CYC	1/33 (3)
Pred	3/33 (9)
Aza	1/33 (3)
Bosentan	1/33 (3)
Sildenafil	3/33 (9)

Table 12. Patient characteristics. HCQ=hydroxychloroquine, MMF= Mycophenolate mofetil, MTX= methotrexate, CYC= cyclophosphamide, Pred= prednisolone, Aza= azathioprine.

6.3.2 Neutrophil elastase is not increased in SSc neutrophils compared to controls.

Neutrophil elastase expression was unchanged in SSc neutrophils compared to controls in neutrophil whole cell lysates, by western blotting (Fig.49).

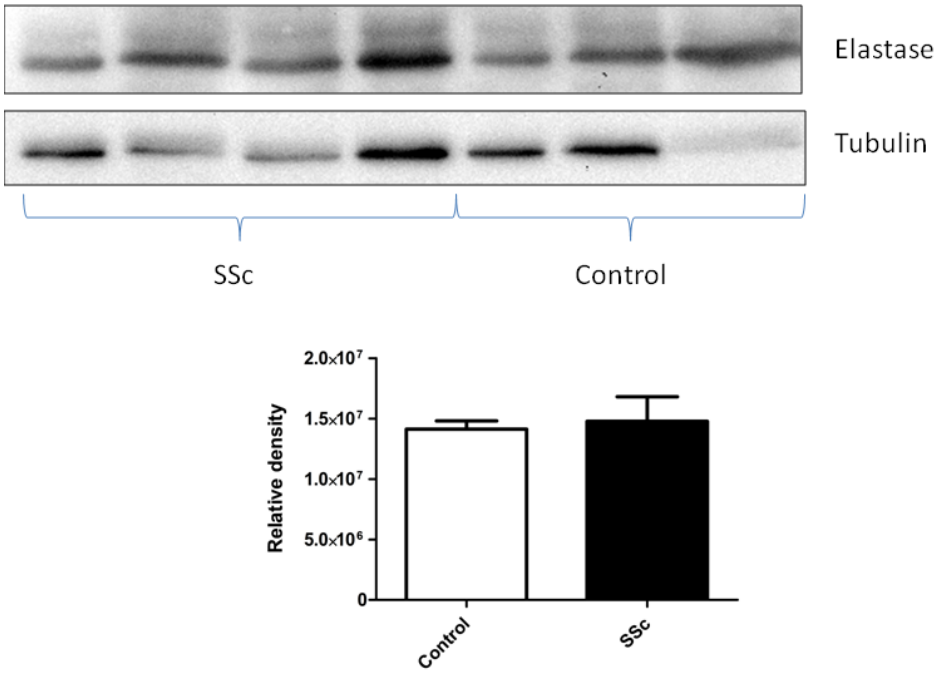


Fig.49. Neutrophil elastase expression is equivalent in SSc neutrophils compared to controls by western blotting corrected for tubulin expression. The graph indicates the mean ±SD.

6.3.3 Serum neutrophil elastase levels were equivalent in patients with SSc compared to controls.

There was no significant difference between SSc patient and control serum levels of neutrophil elastase (p=0.11) (Fig.50).

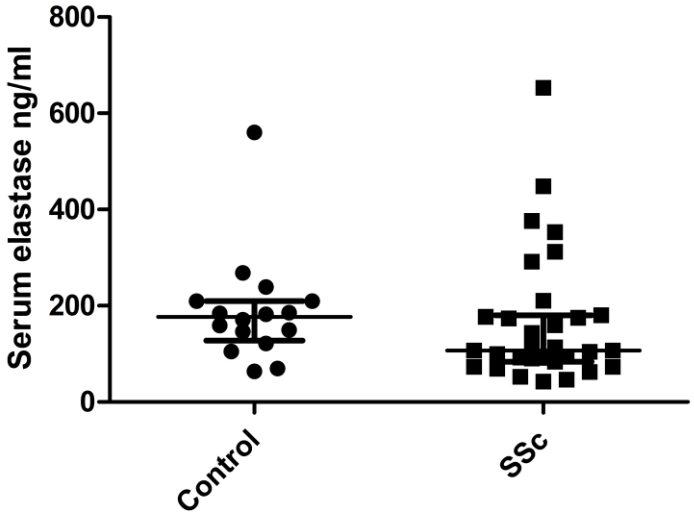


Fig.50. No differences were found in serum elastase concentrations between SSc patients and controls. Serum neutrophil elastase concentrations were measured by ELISA. Graph indicates median and IQR (p=0.11).

6.3.4 Neutrophil elastase concentrations in control and SSc neutrophil culture supernatants are equivalent.

There was no difference in the levels of neutrophil elastase in supernatants following 6h culture of control and SSc neutrophils in RPMI1640 +25mM HEPES +2mM glutamine (p=0.53) (Fig.51).

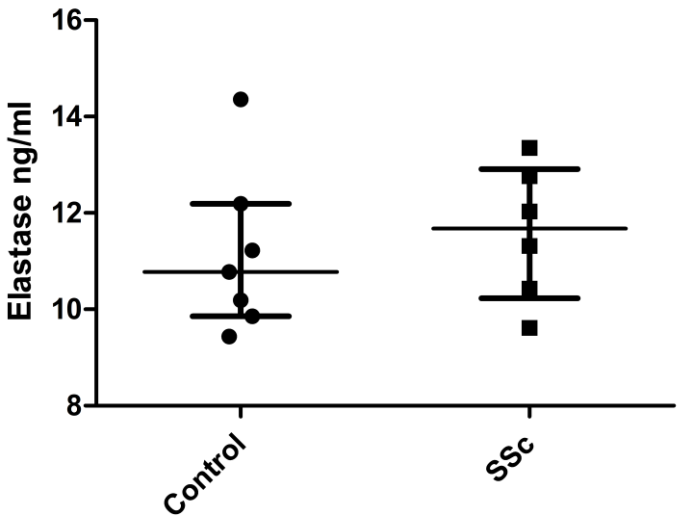


Fig. 51. No differences were found in the levels of neutrophil elastase secreted by control and SSc neutrophils in 6h. Neutrophils isolated from SSc patients and healthy controls were cultured for 6h and elastase concentrations in the culture supernatant were measured by ELISA. Graph indicates median and IQR (p=0.53).

6.3.5 Serum neutrophil elastase activity is equivalent in SSc patients and controls.

The serum neutrophil elastase activity was measured in SSc patients and controls by the elastase dependent conversion of methoxy-succinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide to p-nitroanilide. No difference in serum elastase activity was found between SSc patients and controls (p=0.91) (Fig. 52). However, there was a proportion of patients 6/18 (33%) which had high serum elastase activity (>0.15U/ml) compared to 1/9 (11%) controls. These patients had either early disease <36mths or a higher Rodnan skin score >9.

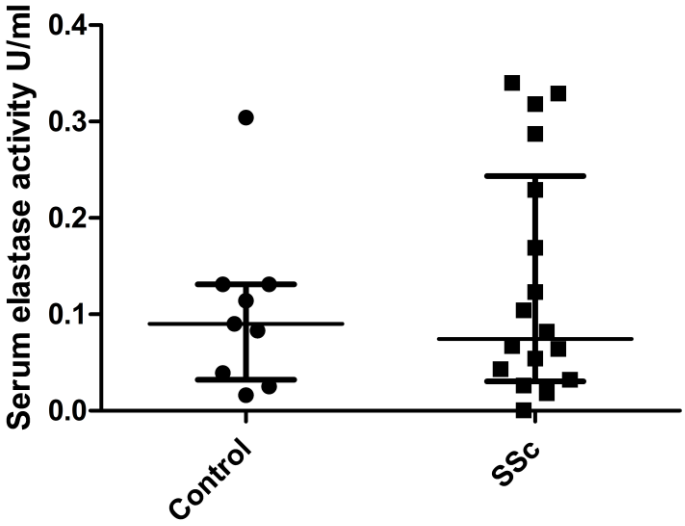


Fig. 52. There was no difference in serum elastase activity in SSc patients compared to controls. Elastase activity was measured by the conversion of methoxy-succinyl- alanyl-alanyl-prolyl-valyl-p-nitroanilide to p-nitroanilide. Graph indicates median and IQR (p=0.91).

6.3.6 Serum neutrophil elastase concentration correlates with serum neutrophil elastase activity: this relationship is stronger in controls than in SSc.

As expected the serum neutrophil elastase concentrations correlated closely with the serum neutrophil elastase activity (Spearman's Rho= 0.62, p=0.0006). However, in SSc patients

the relationship was weaker (Spearman's Rho =0.67, p=0.003) compared to controls (Spearman's Rho= 0.85, p=0.006) despite the larger number of patients (Fig. 53). This indicates a significant difference between the ratio of serum elastase concentration: activity (p=0.03) (Fig. 54). There were no distinguishing clinical features in the patients with a low serum elastase concentration: activity ratio (<50000).

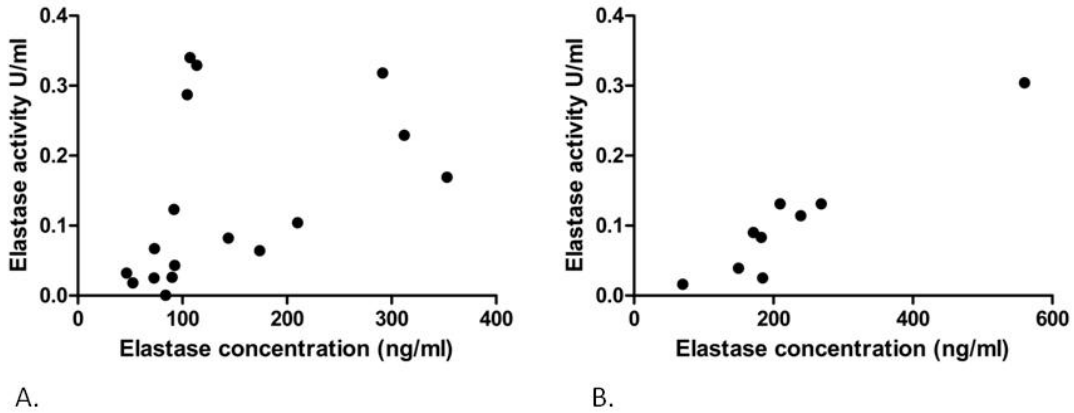


Fig.53. The correlation between serum elastase concentration and serum elastase activity is stronger in (B) controls (Rho=0.85 p=0.006) compared to (A) SSc patients (Rho=0.67, p=0.003).

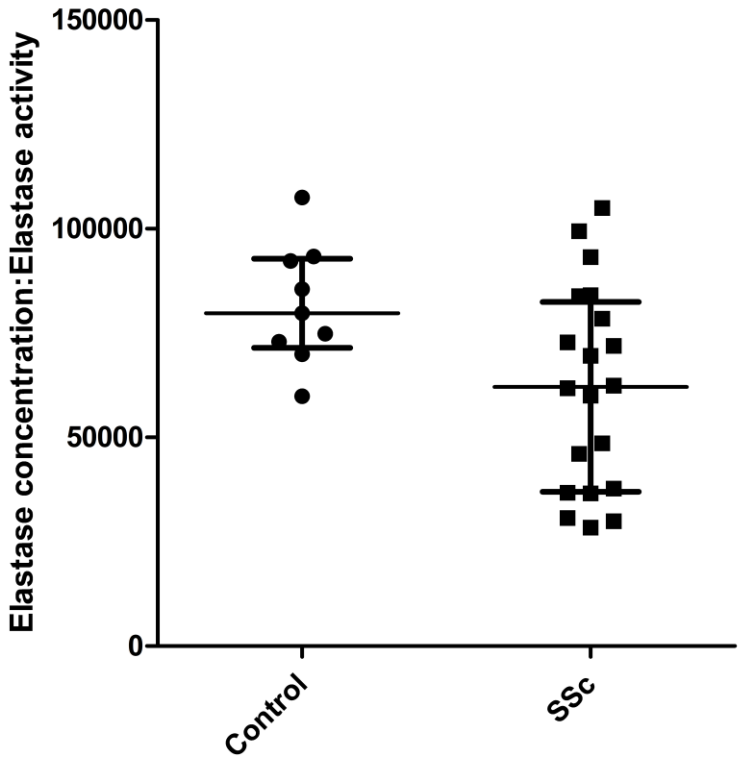


Fig.54. There is a lower ratio of serum elastase concentration to serum elastase activity in SSc patients compared to controls. This may indicate a decrease in elastase inhibitors in SSc serum compared to controls (p=0.03).

6.3.7 Serum SERPIN B1 levels are not different between SSc and control serum.

Serum SERPIN B1 levels are not significantly different between SSc patients and controls; neither is the 66kDa complex of SERPINB1 with elastase or the 29kDa fragment of SERPINB1 caused by elastase dependent cleavage (Fig.55-57).

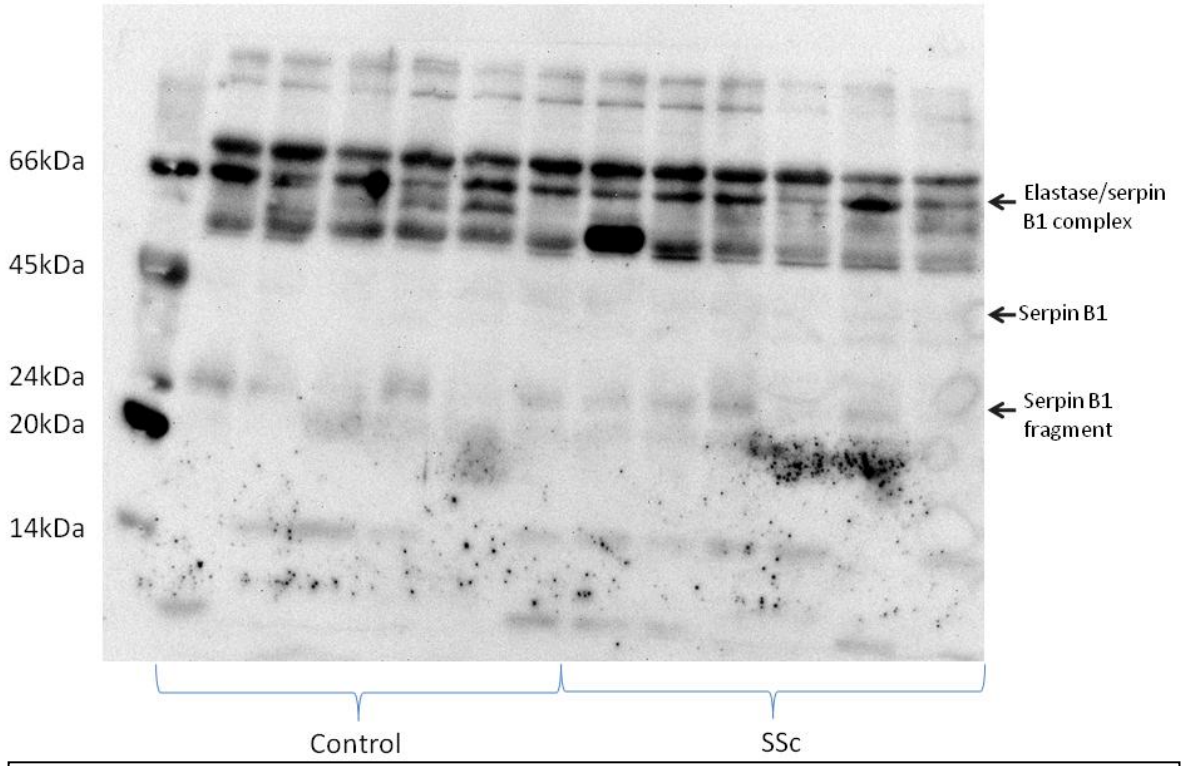


Fig.55. Serum SERPIN B1 levels are not different in SSc patients compared to controls. Measured by western blot on albumin/IgG depleted serum samples.

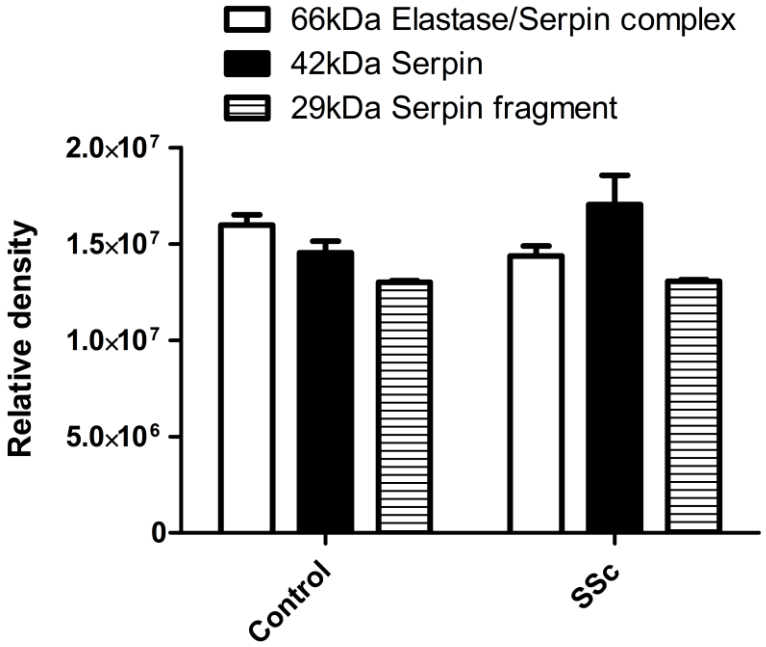


Fig.56. Serum SERPIN B1 levels, the 66kDa complex of SERPIN B1 and the 29kDa fragment caused by elastase dependent cleavage of SERPIN B1 are not significantly different between SSc patients and controls as measured by western blot of albumin/IgG depleted serum samples. Graphs show mean±SD. N=6.

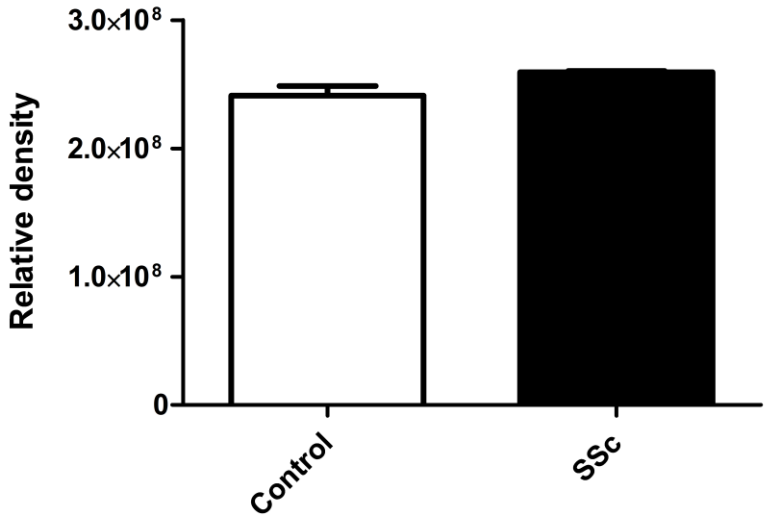


Fig.57. Total serum SERPINB1 concentration is not different between SSc patients and controls, as measured as the total lane density on western blot of albumin/IgG depleted serum. Graphs show mean±SD. N=6.

6.3.8 Clinical correlations with serum neutrophil elastase concentration and activity.

Disease subtype, disease duration, major organ involvement, neutrophil count or DMARD use did not correlate with either serum neutrophil elastase concentration or activity. Serum elastase concentration was significantly lower in RNP and ANA positive patients compared to antibody negative patients (p=0.03 and p=0.003 respectively) (Fig. 58-59). In addition, serum elastase activity was significantly lower in RNP antibody positive patients (p=0.02) (Fig. 60).

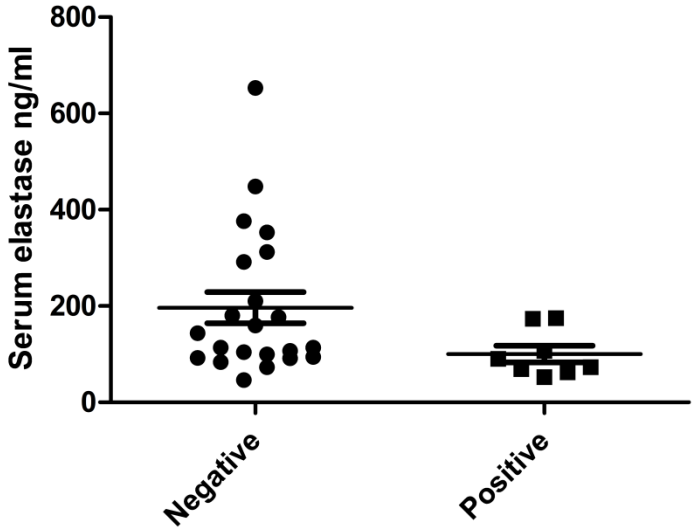


Fig.58. Serum elastase concentrations are lower in RNP positive patients compared to RNP negative patients. Serum elastase concentrations were measured by ELISA. Graphs indicate median and IQR (p=0.03).

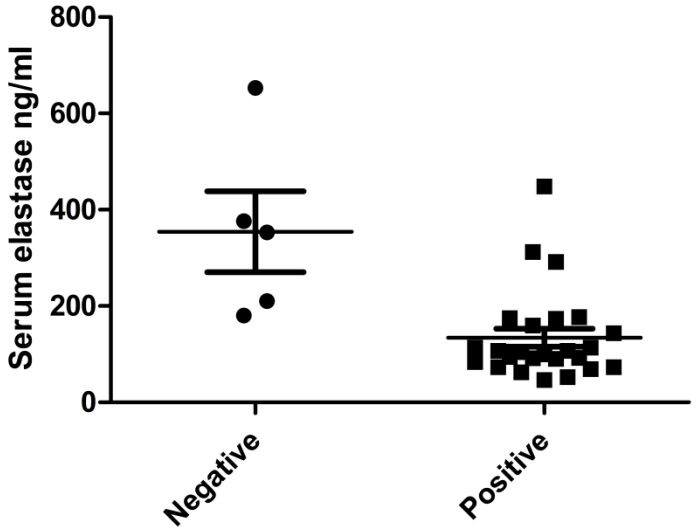


Fig.59. Serum elastase concentrations are lower in ANA positive patients compared to ANA negative patients. Serum elastase concentrations were measured by ELISA. Graphs indicate median and IQR (p=0.003).

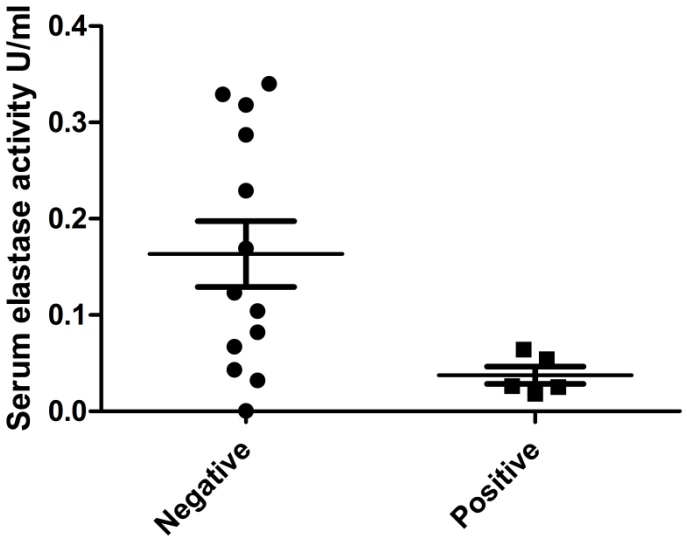


Fig.60. Serum elastase activity was lower in RNP positive patients compared to RNP negative patients. Serum neutrophil elastase activity was measured in SSc patients and controls by the elastase dependent conversion of methoxy-succinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide to p-nitroanilide. Graphs indicate median and IQR (p=0.02).

6.3.9 Neutrophil count correlates with Rodnan skin score.

Peripheral blood neutrophil count correlated with Rodnan skin score in SSc patients (Spearman's Rho=0.39, p=0.04) (Fig.61). The 3 outliers with low Rodnan skin score and a relatively high neutrophil count were either antibody negative (1/3) or RNP positive (2/3), No other clinical correlations were however noted.

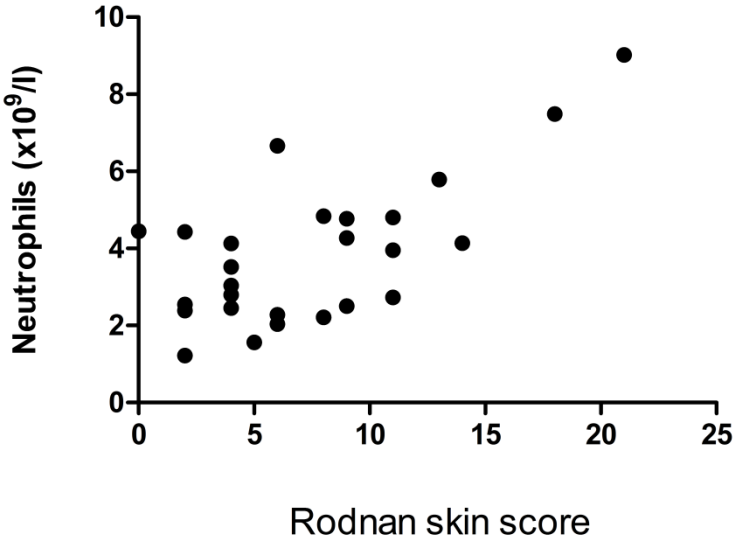


Fig.61. Peripheral neutrophil count correlates with Rodnan skin score in SSc patients. Rho=0.39, p=0.04.

6.3.10 Membrane expression of neutrophil elastase.

No difference was found in the membrane expression of neutrophil elastase between SSc and control neutrophils (Fig. 62).

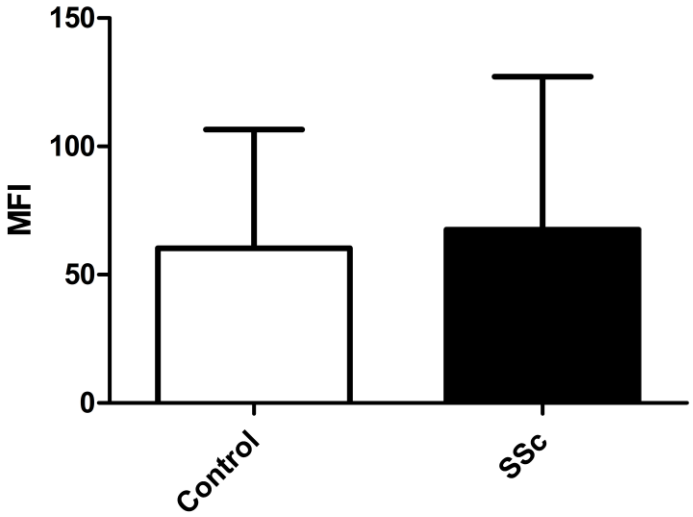


Fig. 62. Membrane expression of neutrophil elastase was not different between controls and SSc neutrophils. Membrane expression of neutrophil elastase was measured by flow cytometry. Mean±SD shown. n=9 (p=0.63).

6.4 Discussion.

In this study I indentified no difference in serum elastase concentration or catalytic activity in SSc patients compared to controls. This contradicts a previous study by Hara *et al* which observed an increase in serum elastase concentration in both limited and diffuse SSc patients [222]. However, an examination of their data reveals that the serum elastase levels measured in my study were similar in magnitude and variation to their observations in SSc patients. In my study however, I found some higher concentrations and greater variance in concentration in the control cohort whereas the previous study showed consistently low levels in all controls. The previous study did not examine elastase activity.

Hara *et al* also reported that serum elastase levels were more likely to be outside the normal range in patients with joint involvement, and they observed that most patients who were anti-centromere antibody positive were likely to have normal levels of elastase [222]. I did not record joint involvement as a clinical outcome in my cohort. It is interesting to note, however, that I observed lower serum elastase levels in RNP positive patients, since these patients would be expected to have higher rates of joint involvement. Hara *et al*, did not record RNP antibody status in their study.

It may seem somewhat surprising that Hara *et al* described such an increase in serum elastase levels since only 2% of serum elastase is released during neutrophil activation and raised serum levels are usually only found in situations where there is pronounced neutrophil infiltration, frustrated phagocytosis or excess neutrophil apoptosis which overwhelms phagocytic clearance [303-305]. As none of these are implicated in SSc, I would not expect to find elevated serum levels, an expectation confirmed in my studies. Certainly, direct observations do not show significant neutrophilic infiltration and there is no evidence of excessive neutrophil apoptosis. Therefore, the most likely explanation for increased secretion would be frustrated phagocytosis and a search for a potential stimulus should be undertaken. An alternative explanation for the data reported by Hara *et al* could be that

serum elastase is protected from degradation in SSc patients leading to increased serum levels.

I did not find any change in the neutrophil elastase concentration in SSc neutrophils. Since elastase is only transcribed at the promyelocytic stage of neutrophil development, I would not have expected to find an increase in cellular content of neutrophil elastase [311]. The fact that neutrophil elastase concentrations were not decreased in SSc neutrophils could be used to argue against *in vivo* secretion, but western blot analysis is relatively insensitive to small changes in large concentrations.

I did not observe any increase in the membrane expression of elastase on SSc neutrophils. Secretion of azurophilic granule contents during degranulation or activation by cytokines results in the increased expression of neutrophil elastase at the membrane. Therefore, this observation once again argues against significant degranulation of the azurophilic granules in SSc neutrophils [303].

I did observe an increase in the ratio of elastase activity: concentration in SSc serum. This may imply a decrease in the serum concentration or activity of neutrophil elastase inhibitors. SERPIN B1 was not decreased in the serum of patients with SSc, but this does not constitute the main capacity for elastase inhibition in the serum. In future experiments I would wish to explore the serum concentrations of α1-PI and α2-macroglobulin in SSc patients to see if these were decreased. I would measure these by ELISA. In addition however, it is important to note that serum elastase inhibitors can be oxidised in the presence of ROS and this decreases their affinity for elastase, reducing their inhibitory capacity [312] [313]. There is evidence for increased oxidative stress in SSc and therefore a normal concentration of elastase inhibitors does not necessarily equate to normal activity [11]. Additional studies should therefore be performed to explore the elastase inhibitory capacity of SSc serum.

Although there was no increase overall in serum elastase activity in patients with SSc the apparent decrease in inhibitory capacity could have important consequences at a local level since elastase is likely to be released locally at the site of inflammation, but, α_1-PI the predominant inhibitor, is produced by the liver [308].

Neutrophil elastase has pleiotropic roles in the extracellular environment including profound effects on inflammation [306 307], such as it:

1) Activates and degrades inflammatory cytokines [314]
2) Binds to the neutrophil integrin Mac-1=CD11b/CD18 thereby regulating neutrophil attachment and detachment [315]
3) Activates protein activated receptor 2 (PAR-2) leading to the release of IL-8 and MCP-1, though at higher concentrations in can inactivate the PAR-2 by a proteolytic mechanism [316 317].
4) Activates TLR-4 leading to the release of IL-8, cathepsin B and MMP-2 [318 319].
5) Cleave the TNF p75 receptor and the IL-6R from cells leading to an increased pool of soluble receptor [320 321]. This leads to inactivation of soluble TNFα but enhances IL-6 trans signalling.
6) Cleaves the phosphatidylserine receptor leading to defects in the phagocytic clearance of apoptotic cells [322].
7) Enters endothelial cells and cleaves NF-κB leading to endothelial cell apoptosis [323].

In co-cultures of control neutrophils with HDMECs and SSc serum, increased levels of endothelial cell activation and apoptosis were seen compared to control serum. The serine protease inhibitor AEBSF abrogated the effect on apoptosis but not on endothelial cell activation (Chapter 7). This implies that serine proteases contribute significantly to the ability of SSc serum to induce apoptosis in this context. Experiments using specific neutrophil elastase inhibitors could define whether neutrophil elastase is the serine protease responsible for this effect.

Whilst the role of neutrophil elastase in fibrosis is well established, the mechanism remains unclear. Certainly, elastase deficient mice are resistant to bleomycin induced fibrosis and treatment with elastase inhibitors also abolished fibrosis in this model [324-327].

Neutrophil elastase can cleave TGFβ binding protein leading to release of latent TGFβ from extracellular matrix stores [328]. It has also been shown to release PDGF and VEGF from stores by a similar mechanism [329]. All of these cytokines are implicated in the pathogenesis of SSc; TGFβ and PDGF are profibrotic and VEGF is proangiogenic. Neutrophil elastase has other roles in promoting the activity of TGFβ. Intra-tracheal instillation of elastase in mice leads to a time-dependent increase in the TGFβ content of the bronchoalveolar lavage fluid [330]. In elastase deficient mice, the resistance to bleomycin is associated with an inability to activate TGFβ [324].

It is interesting that serum elastase concentrations and activity are lower in RNP positive patients. This may imply that there is a different pathological process involved in RNP positive patients and that neutrophil elastase is unlikely to be a significant mediator in these patients. In fact, serum elastase concentrations were lower in RNP positive patients than controls (p=0.008). This may represent an accelerated loss of neutrophil elastase in RNP positive patients.

Peripheral neutrophil cell count strongly correlated with Rodnan skin score, a measure of the extent of skin fibrosis in SSc. The reason for this is unclear, but it is possible that patients with the higher skin score tend to have higher levels of inflammation and therefore develop a relative neutrophilia. Conversely, if neutrophil elastase or another neutrophil derived mediator was instrumental in the development of fibrosis the higher neutrophil counts may lead to an increase in the levels of these mediators. Some patients had a neutropenia, and this may have protected them from neutrophil mediators that can promote fibrosis, including neutrophil elastase.

Patients whose neutrophil count did not correlate so well with the Rodnan skin score (low skin score despite high neutrophils) were frequently RNP positive but had no other distinguishing disease manifestations. In particular, they were not on steroids, and did not uniformly have a short disease duration. Of the 3 neutropenic patients (neutrophil count <2), all were on disease modifying therapies: 1 on oral prednisolone, 1 on hydroxychloroquine and 1 on methotrexate. 2 of these patients were RNP antibody positive. All 3 had a low skin score (≤5).

Data on neutrophil count was only obtained at one single time point. It would be interesting to follow the neutrophil count longitudinally in SSc patients and see whether some patients had a consistently higher neutrophil count and whether this predisposed to the development of greater fibrosis.

Analysis of the literature shows that neutrophil elastase could be an attractive mediator in SSc. It can promote chronic inflammation, cause endothelial cell apoptosis and it can promote TGFβ dependent fibrosis. Serum deficiency in elastase inhibitors, if confirmed, could lead to a localised excess of elastase activity despite normal serum concentrations. This could have potential therapeutic implications as neutrophil elastase inhibitors already exist in clinical practice and could be used in SSc patients if a systemic deficiency is confirmed.

Chapter 7. Neutrophil: endothelial cell co-cultures.

7.1 Introduction.

Previous studies have demonstrated an abnormal neutrophil phenotype in patients with SSc. There is a tendency for neutrophils from SSc patients to be hypoactive *ex vivo* in terms of ROS generation. It is postulated that this may indicate *in vivo* neutrophil activation prior to isolation and hence "exhaustion". It is, however, difficult to directly test this hypothesis, partly because isolating neutrophils from blood removes the cells from the soluble mediators and cellular interactions which may have stimulated them *in vivo.* In addition, neutrophils *in vivo* are often exposed to hypoxia, acidosis and intermittent episodes of ischaemia, in addition to shear forces as they are under flow. These conditions are difficult to reproduce *ex vivo*. Further, the factors causing the postulated *in vivo* activation are unknown.

Endothelial cells have been proposed to be the primary lesional target in this disease [5 331 332]. Evidence for endothelial cell activation in SSc has often been observed, and there is some evidence to suggest that this may represent the earliest lesion that leads to secondary fibrosis and immune cell infiltration [6 7 15 19 24 333]. It is postulated that, as a result of chronic inflammation and endothelial cell activation, there is fibroblast stimulation resulting in fibrosis, the phenotypic hallmark of this disease.

It is essential therefore, when examining the phenotype and functionality of any cell type in SSc to make an attempt to simulate the *in vivo* conditions that may exist. One method that permits such modeling is live cell confocal microscopy which allows the simultaneous measurement of many cellular parameters in a co-culture system and can, in addition, be used to study cellular interactions. It also allows cells to remain intact and functional during assessment thereby providing dynamic measurements in real-time.

In this Chapter, I have used non-invasive live cell imaging to study the dynamic interactions between neutrophils and endothelial cells that may occur in SSc. The data presented highlight the usefulness of this experimental approach and uncover a novel role for IL-6 in promoting changes in cell phenotype that resemble those that occur in SSc.

7.2 Methods.

7.2.1 Human dermal microvascular endothelial cell (HDMEC) culture.

HDMECs were used as the source of endothelial cells in culture as they are most representative of the lesional endothelial cells in SSc, being microvascular and dermal in origin. There is extensive evidence in the literature to support the argument that other endothelial cell cultures, especially HUVECs (human umbilical vein endothelial cells), are not phenotypically representative of all endothelial cells [334-336].

HDMECs were cultured in 48 well plates seeded at 10,000 cells per well if cultured for 48h or 20,000 cells per well if cultured for 24h. Cultures were used at passage 4-8, and once they had reached 90% confluence. The cell phenotype was guaranteed by Promocell up to passage 14. Cells were cultured in Promocell's microvascular endothelial cell medium.

7.2.2 Neutrophil isolation.

Neutrophils were isolated from patients or healthy controls as described in Chapter 2 and were centrifuged at 1000*x*g for 3 min to pellet the cells. Neutrophils were resuspended in microvascular endothelial cell medium supplemented with 2mM glutamine at a concentration of $1x10^6$ cells/ml or at $2x10^6$ cells/ml when cultured with IL-6 immunodepleted serum.

7.2.3 Neutrophil: endothelial cell co-cultures.

The culture medium was aspirated at time 0 and 450µl of neutrophil suspension and 150µl of serum (or media in negative controls) were added to each well. 1.5µl of Annexin V FITC (to

detect apoptosis) was added to each well following initial experiments to optimise the concentration. In addition, monoclonal antibodies to ICAM-1 (conjugated to PE) or E-selectin (conjugated to APC) were added at time 0 to detect endothelial cell activation. Antibodies were diluted 1:10 and then 2.5µl of each was added to each well, again following optimisation experiments.

In initial experiments, images were taken continuously over a 6h time period, however after this time excessive evaporation of media led to decreased cell viability. Therefore co-cultures were incubated in a standard CO_2 incubator for 24h and a single confocal image was taken at the end of the culture period.

Confocal images were taken using a LSM-710 (Zeiss) confocal microscope using the Zen 2009 software. Annexin V FITC and PE were excited using the 488nm laser whereas APC was excited using the 633nm laser. Images were taken using the optimum separation of emission spectra mode. Duplicate images were taken for each experimental variable. For each experiment a z-stack of 13 images was obtained over a distance of 9.91µm, focusing from the endothelial cells to the neutrophils using the 10x Ph2 objective.

Images were analysed using the Image J software (NIH, USA). Total fluorescence values presented in the figures are those of the slice with the maximal fluorescence intensity corrected for the fluorescence intensity of cells cultured in media alone. Statistical analysis was performed using the paired T test.

Initial experiments were performed comparing the effects of control and SSc neutrophils, and control and SSc serum on both neutrophil apoptosis and endothelial cell apoptosis and activation in co-cultures. Control experiments were conducted without neutrophils or without serum.

Further experiments were conducted to explore the role of monocytes and lymphocytes under identical experimental conditions. Monocytes and lymphocytes were separated from

the PBMC layer (removed during neutrophil preparation using polymorph prep) using CD15 positive selection Easysep beads and CD3 positive selection Dynabeads respectively, according to the manufacturers protocol.

7.2.4 Labeling of neutrophils and endothelial cells for 24h time lapse imaging.

Endothelial cells were seeded onto 3cm glass-bottomed culture dishes (iWAKI) and cultured until 90% confluence. Endothelial cells were incubated with calcein-AM (2.5µM) for 1h. Freshly isolated neutrophils were incubated with 100nM mitotracker red for 15min. Neutrophils were washed in PBS then resuspended in microvascular endothelial cell medium supplemented with 2mM glutamine, at 5 x 10^5 cells/ml. The endothelial cells were washed in PBS and medium was replaced with 1.5ml of the neutrophil suspension described above and 500µl of either control or SSc serum. 24h time-lapse movies were taken using a Pascal confocal microscope (Zeiss). FITC and Mitotracker Red were excited using the 488nm and 543nm lasers respectively. Images were taken continuously from 6 separate fields over a period of 24h, and processed using the LSM510 software.

7.2.5 Luminex cytokine assay.

The Luminex multiplex assay allows simultaneous detection of up to 100 different analytes in a single sample e.g. plasma, serum, culture supernatant. Cytokine assay kits are available which can measure the concentration of up to 35 different cytokines. The assay is an immunoassay; antibodies for each cytokine are attached to different beads with unique fluorescent properties when excited by a 635nm laser. Cytokines bound to the bead-mounted antibodies are detected using a streptavidin-PE labeled secondary antibody. The amount of PE fluorescence is then measured when excited by a 532nm laser. The assay has a high sensitivity (10pg/ml of cytokine).

A 13-plex luminex cytokine profiling assay was used to explore the cytokine profile of co-culture supernatants and control and SSc serum in order to investigate what might be

responsible for the increased endothelial cell activation in initial co-culture experiments. The cytokines measured were: TNF-α, GCSF, interferon-γ, GMCSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, IL-1Ra.

7.2.6 Exploration of potential mediators of endothelial cell activation in neutrophil: endothelial cell co-cultures.

In further neutrophil: endothelial cell co-cultures, healthy control or SSc serum was substituted for heat-inactivated pooled human AB serum filtered through a 0.45µm filter and spiked with 200pg/ml of rIL-6 (Sigma), which was a similar concentration to the levels found in 2 of the patient sera samples tested above.

In addition, healthy control and patient sera were complement-inactivated by heating to 56˚C for 30mins with intermittent vortexing. A polyclonal IL-6 antibody (Abcam) was added to the co-cultures in quantities sufficient to neutralise any IL-6 in the culture (1:400). A serine protease inhibitor, AEBSF (0.2mM), was added to the co-cultures, higher concentrations were toxic to neutrophils. Catalase (2000U/ml) was added to co-cultures in order to remove ROS. Healthy and control sera were immunodepleted of IL-6 using a direct immunodepletion kit (Pierce) using a polyclonal IL-6 antibody (Abcam) (see below). As sera required 2:1 dilution prior to immunodepletion, 225µl of neutrophil suspension ($2x10^6$ per ml) was used, plus 75µl of media and 300µl of diluted, immunodepleted serum.

7.2.7 IL-6 immunodepletion.

A polyclonal mouse IL-6 antibody (1 µl) was conjugated to the direct immunodepletion beads (Pierce) according to the manufacturer's protocol. This amount was chosen following optimization experiments using AB serum spiked with 200pg/ml rIL-6, which was subsequently subjected to immunodepletion. The depleted serum was retained. In addition as a control a portion of the sample was also submitted to a dummy procedure using resin which was not conjugated to IL-6 antibody. IL-6 concentrations in all samples were then

quantified by western blotting. The immunoprecipitation procedure resulted in a 60% reduction in IL-6 whereas the unconjugated resin led to a non-specific reduction of 15% (Fig. 63).

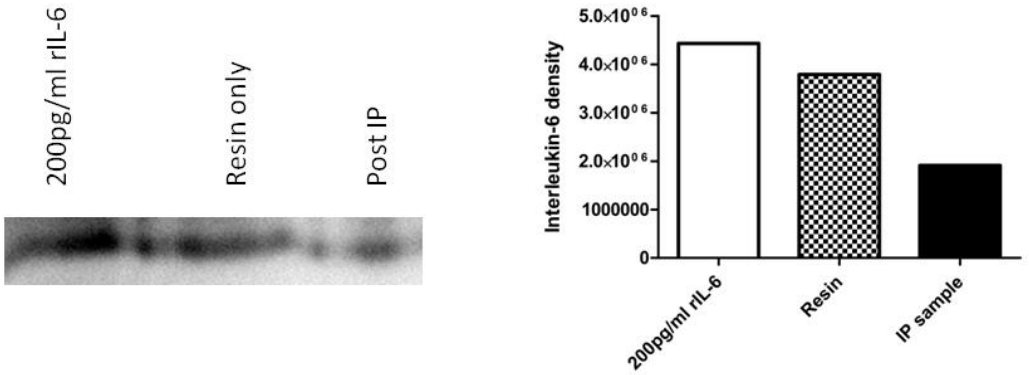


Fig. 63. IL-6 immunoprecipitation results in a 60% reduction in IL-6 concentration. AB serum was spiked with 200pg/ml rIL-6. The sample was submitted to immunopreciptation against unconjugated resin (resin) and resin conjugated to a polyclonal IL-6 antibody (IP).

7.2.8 ELISA of sICAM-1 and sIL-6R in serum and culture supernatants.

Serum samples from controls and SSc patients and 24h culture supernatants from co-cultures incubated in the presence of 25% control or SSc serum were measured for their sICAM (Bender) and sIL6R (Invitrogen) content by ELISA according to manufacturer's instructions. In addition, single cell cultures of neutrophils (10^7/ml) were incubated with control and SSc serum for 24h and sIL-6R concentrations were measured in the supernatants by ELISA.

7.2.9 Statistics

Apoptosis data from co-cultures was not normally distributed and so comparisons between samples were made using non-parametric tests (Wilcoxon signed rank test) E-selectin and ICAM expression data were however normally distributed and therefore comparisons between means were made using the paired T-test.

7.3 Results.

7.3.1 Patient characteristics.

Patient characteristics are summarized in table 13.

Male: Female	1:16
Median disease duration/mths (IQR)	37 (25.5,58)
Median Raynaud's duration/yrs (IQR)	6.5 (4, 10.5)
Median Rodnan skin score (IQR)	6 (4,11)
Clinical feature	Number (%)
Limited subtype	13/17 (76)
Diffuse subtype	4/17 (24)
Lung involvement	6/17 (35)
Pulmonary artery hypertension	1/17(6)
Renal involvement	0/17 (0)
ANA positive	12/17 (71)
Anticentromere positive	6/17 (35)
Anti Scl70 positive	5/17 (29)
Anti RNP positive	3/17 (18)
DMARDs	6/17 (35)
Mycophenolate mofetil	5/17 (29)
Sildenafil	2/17 (12)
Methotrexate	1/17 (6)

Table 13. Patient characteristics.

7.3.2 Effects of SSc neutrophils on endothelial cell activation and apoptosis in co-cultures.

Freshly isolated SSc neutrophils, when incubated with HDMECs in the absence of serum for 24h did not induce any change in the levels of endothelial cell activation (ICAM-1 expression) or apoptosis (Fig.64).

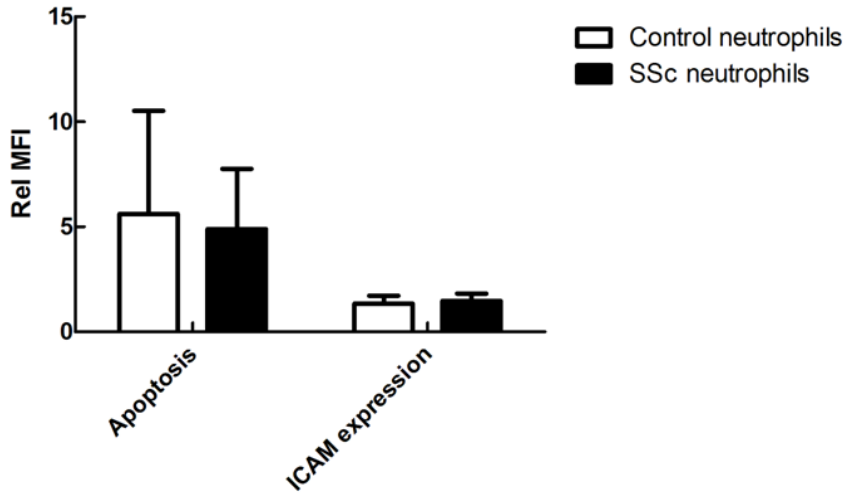


Fig.64. SSc neutrophils did not increase endothelial cell apoptosis or ICAM-1 expression in co-cultures compared to control neutrophils. MFI=mean fluorescent intensity.

7.3.3 Effects of SSc serum on endothelial cell activation and apoptosis.

SSc serum (in the absence of neutrophils) increased endothelial cell activation (ICAM-1 expression) but not apoptosis after 24h. Serum from patients with newly-diagnosed, untreated rheumatoid arthritis did not increase either endothelial cell activation or apoptosis (Fig.65). The increase in ICAM-1 expression was lower in magnitude than the changes seen in neutrophil: endothelial cell co-cultures (Fig.66) and was not mirrored by an increase in E-selectin expression (Fig.72A).

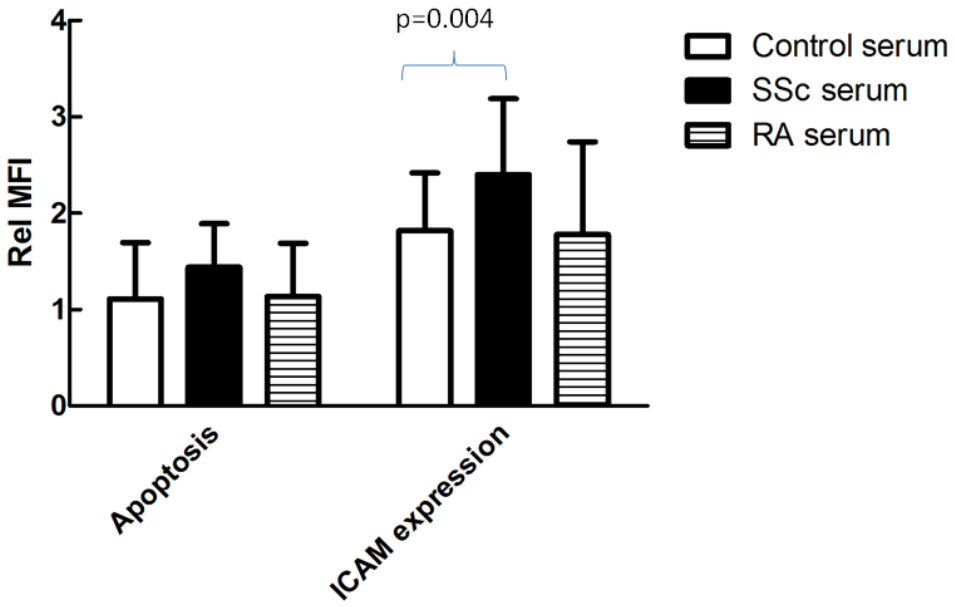


Fig.65. SSc serum increased endothelial cell ICAM-1 expression but not apoptosis in single cell HDMEC cultures compared to control serum. Rheumatoid arthritis (RA) serum did not increase either endothelial cell ICAM-1 expression or apoptosis compared to control serum.

7.3.4 Effects of SSc serum on neutrophil: endothelial cell co-cultures.

SSc serum increased apoptosis (p=0.02) and endothelial cell activation (p=0.007) as measured by ICAM-1 expression in neutrophil: endothelial cell co-cultures compared to control serum (Fig.66). Sera from patients with untreated, newly diagnosed, rheumatoid arthritis were used as an autoimmune, inflammatory control.

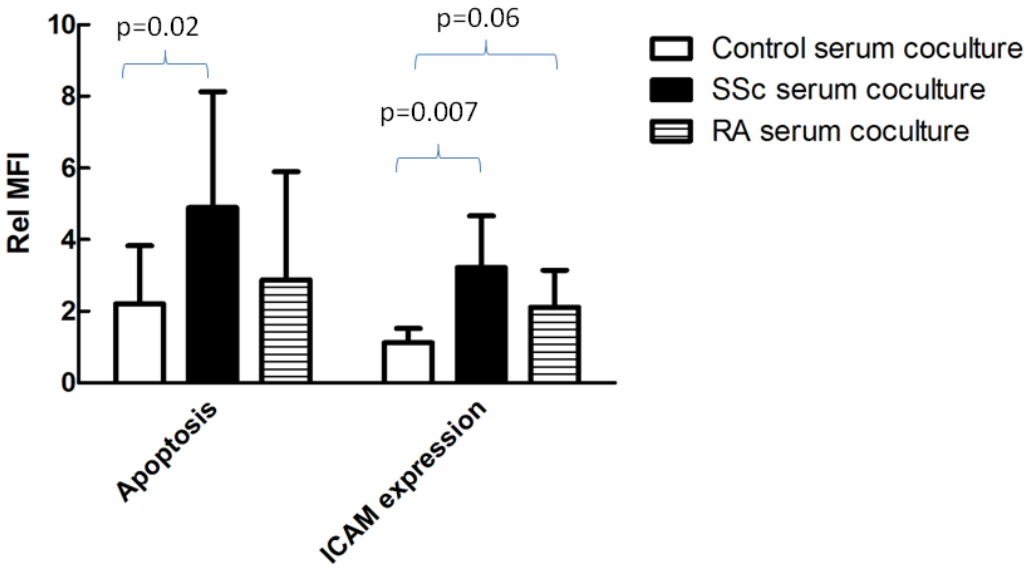
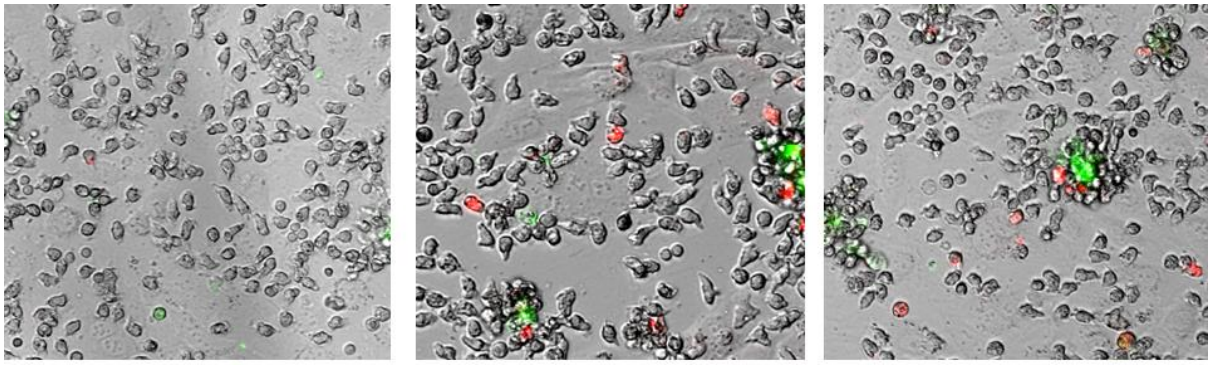


Fig.66. SSc serum increased apoptosis and ICAM-1 expression in neutrophil: endothelial cell co-cultures compared to control serum. RA serum also increased ICAM-1 expression, though to a lesser extent, but did not increase apoptosis.

Serum from untreated early rheumatoid arthritis patients (RA) gave a similar increase in ICAM-1 expression in co-cultures but the magnitude of the changes was much smaller (Fig.66) and this did not reach statistical significance. RA serum did not increase ICAM-1 expression in single cell endothelial cell cultures (Fig.65). RA serum had no significant effect on apoptosis (Fig.65 and 66).

Confocal images demonstrated that ICAM-1 expression was increased on neutrophils as well as endothelial cells on exposure to SSc and RA sera (Fig.67). Furthermore, it has previously been shown that ICAM-1 expression in neutrophils can be increased by inflammatory cytokines [337]. Therefore, further experiments were conducted using the more specific endothelial cell activation marker E-selectin.



A. B. C.

Fig.67. SSc serum-induced apoptosis and ICAM-1 expression in neutrophil: endothelial cell co-cultures. Human dermal microvascular endothelial cells (HDMECs) were cultured with healthy control neutrophils in the presence of 25% control (A) rheumatoid arthritis serum (B) or SSc serum (C). Apoptosis was measured by annexin V-FITC binding (green) and ICAM-1 was measured using a PE-conjugated monoclonal antibody to ICAM-1 (red). Fluorescence intensity was quantified and located by confocal microscopy. Patient sera increase ICAM-1 expression on neutrophils as well as endothelial cells.

SSc serum resulted in greater annexin-V binding ($p=0.006$) and E-selectin ($p=0.00004$) expression compared to control serum when added to neutrophil: endothelial cell co-cultures (Fig.68). When corrected for background staining in serum-free media, control serum did not increase E-selectin expression, whereas SSc serum resulted in a 2.5 fold increase in E-selectin expression. Control serum resulted in a 2.5 fold increase in annexin-V staining, whereas SSc serum resulted in a 7-fold increase, compared to media alone.

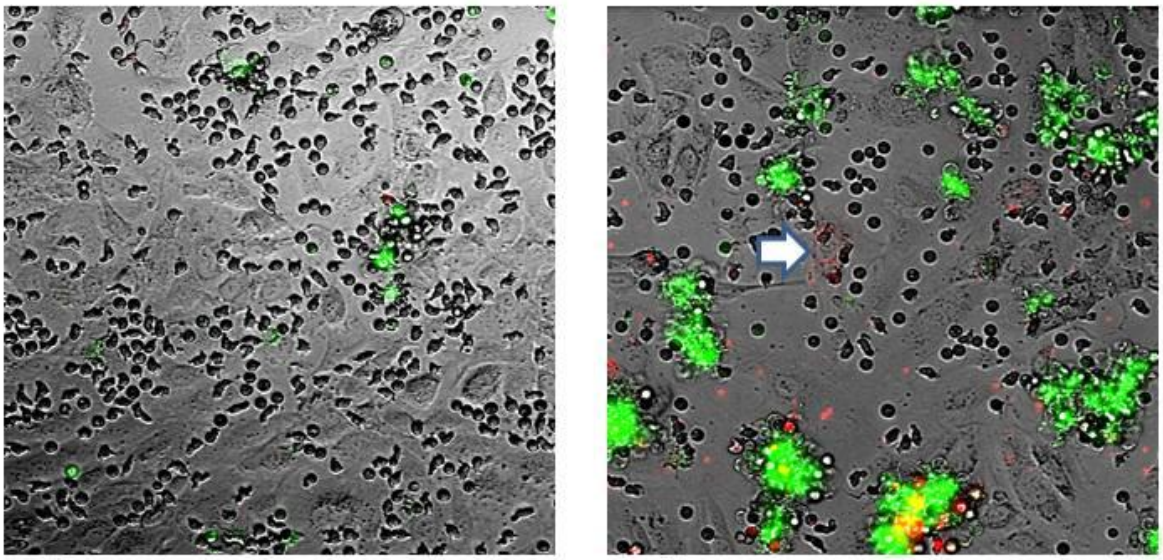


Fig.68. SSc serum-induced apoptosis and E-selectin expression in neutrophil: endothelial cell co-cultures. Human dermal microvascular endothelial cells (HDMECs) were cultured with healthy control neutrophils in the presence of 25% control (A) or SSc serum (B). Apoptosis was measured by annexin V-FITC binding (green) and E-selectin was measured using an APC-conjugated monoclonal antibody to E-selectin (red). Fluorescence intensity was quantified and located by confocal microscopy. Arrow shows E-selectin stained endothelial cell. E-selectin staining also exists in microparticles.

There was wide variation in the effects the control sera and, in particular SSc sera (Fig.69), but pooled data from experiments using 17 separate samples of each type of serum, showed significantly enhanced apoptosis ($p=0.006$) and E-selectin expression ($p=0.00004$) by the SSc serum.

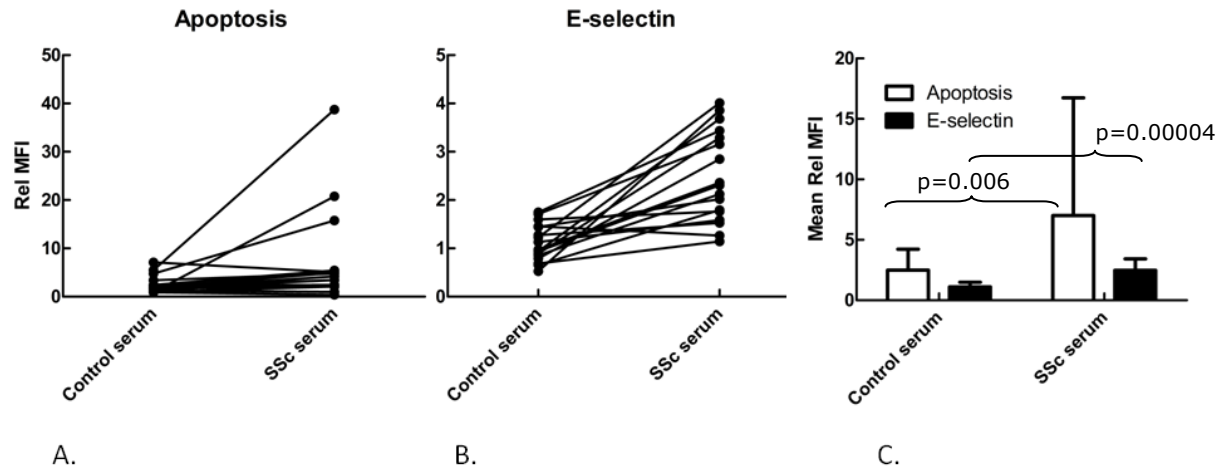


Fig.69. There was a large amount of variability in apoptosis and E-selectin expression between experimental repeats. Each line in A and B represents an individual experimental repeat (n=17). C shows the pooled data.

Annexin-V binding was detected on both neutrophils and endothelial cells (Fig.68,70). E-selectin is a specific endothelial cell marker and reflects endothelial cell activation. However, E-selectin expression in co-cultures was predominantly associated with cellular aggregates where it was difficult to distinguish between neutrophils or endothelial cells morphologically. High magnification z-stacks through the cellular aggregates were performed, but these could not unambiguously assign E-selectin staining to a particular cell type in these co-cultures. Therefore, cell-specific counter-staining was used. 24h time-lapse image series of co-cultures, where endothelial cells were stained with calcein-AM (green) and neutrophils with Mitotracker red (red) showed that both endothelial cells and neutrophils were present in the cellular aggregates (Fig.71C). Such time lapse analyses also enabled us to show that endothelial cell apoptosis (as evidenced by membrane blebbing and cell detachment) (Fig.71B) was the initiating factor for the formation of the aggregates.

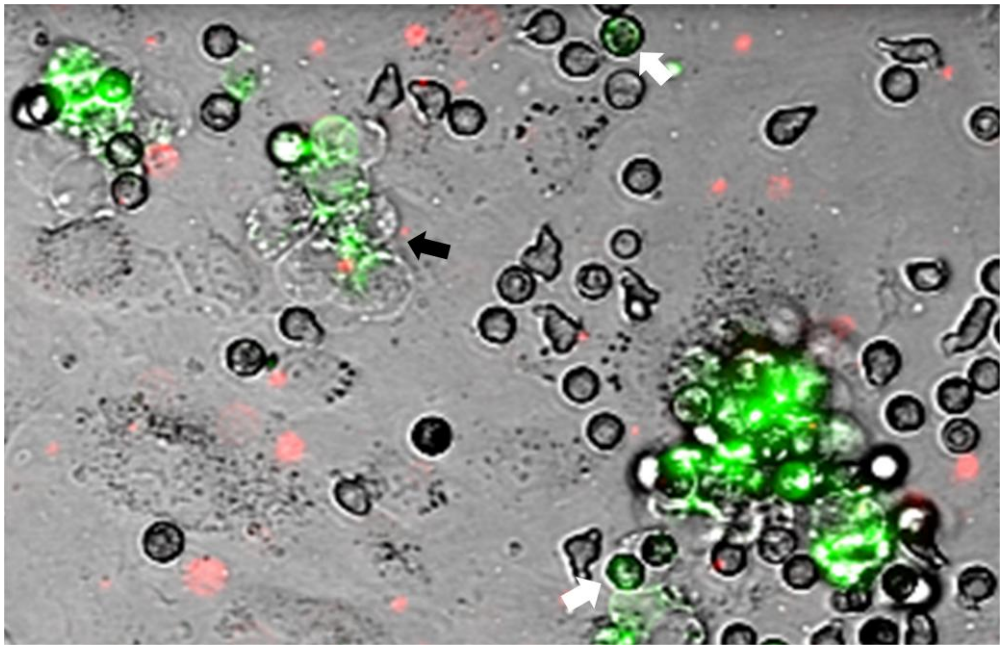


Fig.70. Annexin V-FITC (green) binding was found on neutrophils (white arrows) and endothelial cells (black arrows) but was predominantly associated with cell aggregates.

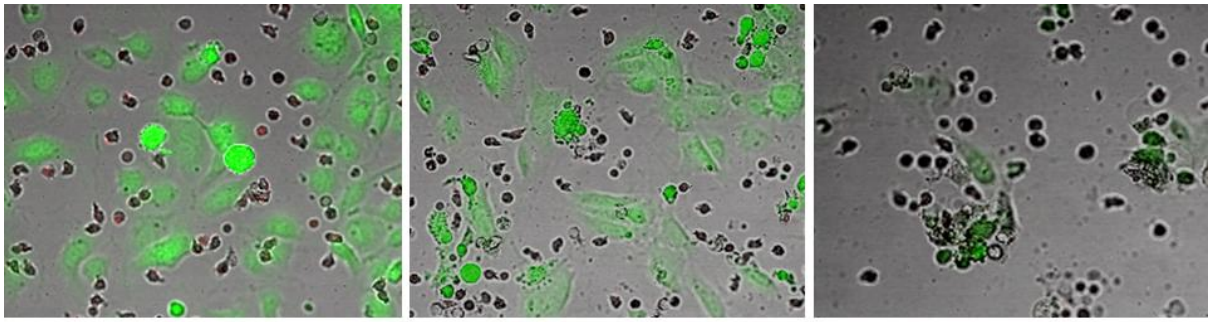


Fig.71. Time-lapse movies demonstrated that in the presence of SSc serum endothelial cells undergo apoptosis and form cellular aggregates with neutrophils in neutrophil: endothelial cell co-cultures. HDMECs were cultured with healthy control neutrophils for 24h in the presence of 25% SSc serum. Endothelial cells were labeled with calcein-AM (green) and neutrophils were labeled with mitotracker red (red). A is an image taken at baseline. B is an image taken at 1h, endothelial cells are undergoing apoptosis as evidenced by cell blebbing and detachment. C demonstrates that both endothelial cells (green) and neutrophils form the cellular aggregates.

SSc sera did not induce apoptosis or endothelial cell activation in the absence of neutrophils (Fig.72A). Somewhat surprisingly, although the effect of SSc serum on apoptosis was not seen in neutrophil single cell cultures there was a small but significant (p=0.02) apparent increase in E-selectin expression (Fig.72B). The increase was however much smaller than that seen in neutrophil: endothelial cell co-cultures. To explore this further, neutrophils from co-cultures were aspirated from the co-cultures after 24h following gentle agitation and analysed by flow cytometry. Neutrophils were gated according to typical forward and side scatter properties. No E-selectin expression was seen on these neutrophils. The proportion of Annexin V positive neutrophils was also unchanged between co-cultures with SSc and control serum (Fig.73). Much of the E-selectin seems to be associated with microparticles and indeed an increase in endothelial cell microparticles has been described in SSc and indicates endothelial cell activation[338].

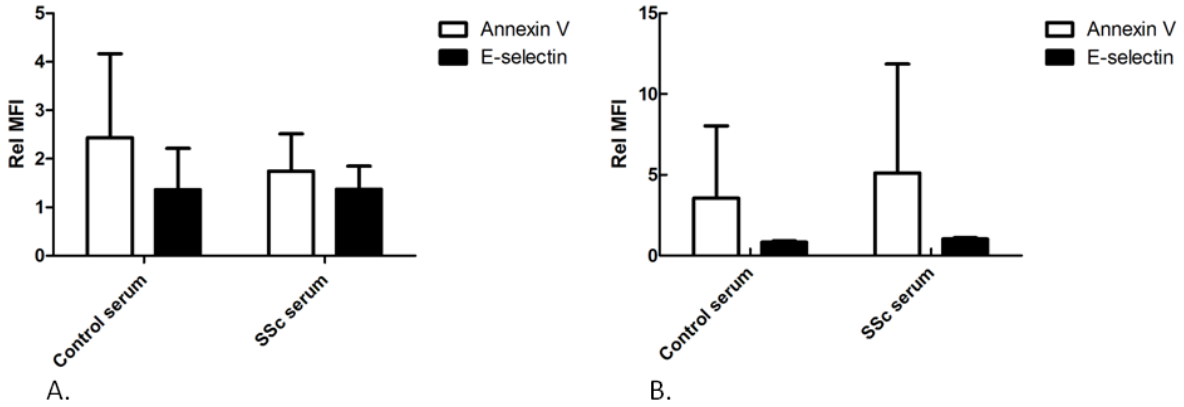


Fig.72. Apoptosis and E-selectin expression in endothelial cell (A) and neutrophil (B) single cell cultures after 24h. There was no difference in apoptosis or E-selectin expression in endothelial cell cultures exposed to control or SSc serum (25%). There was no difference in neutrophil apoptosis on exposure to SSc or control serum however, there was a small but significant increase in E-selectin expression (p=0.02).

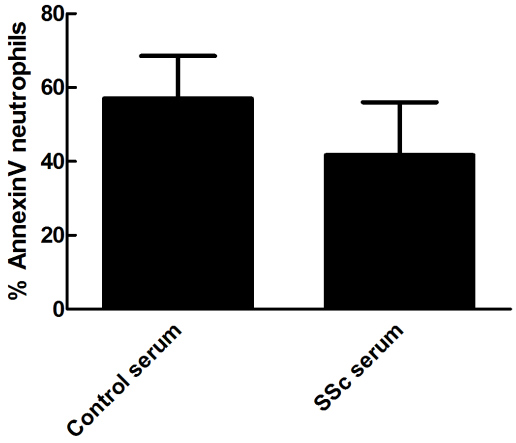


Fig.73. Neutrophils co-cultured with endothelial cells for 24h with control or SSc serum (25%) were aspirated from the co-cultures following gentle agitation. % Annexin V positive neutrophils were measured by flow cytometry.

Further control experiments were performed to ascertain whether the effect of SSc serum was specific to endothelial cell co-cultures with neutrophils, or if other leukocytes would also give rise to a similar effect. Co-cultures with lymphocytes did not lead to increases in apoptosis or endothelial cell activation, but co-cultures with monocytes did (n=2)(Fig.74).

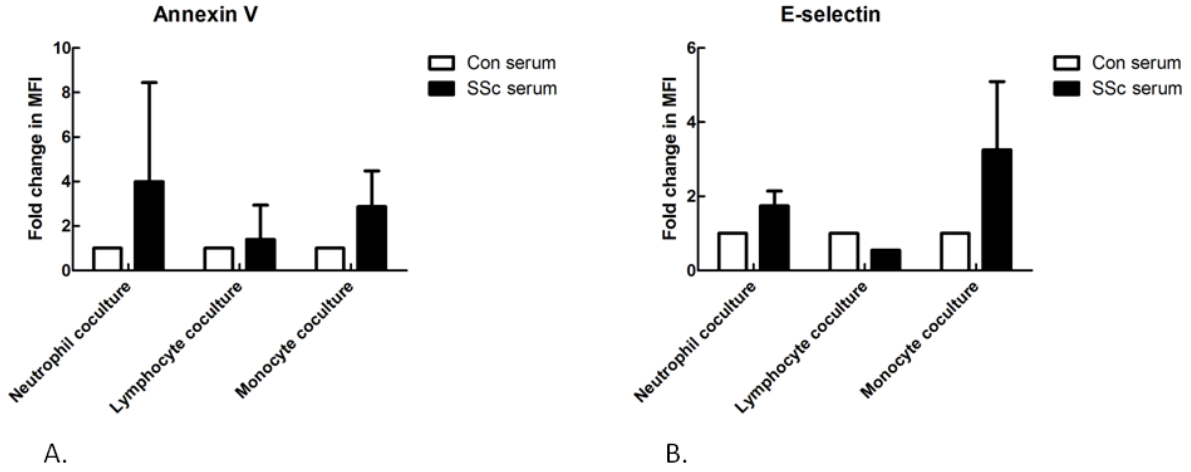


Fig.74. Phagocytes increased apoptosis and endothelial cell activation in co-culture with endothelial cells in the presence of SSc serum, whereas lymphocytes did not. Neutrophils, lymphocytes and monocytes isolated from healthy controls were co-cultured with HDMECs in the presence of control or SSc serum (25%). Apoptosis was measured by annexin V binding (A) and endothelial cell activation was measured by an APC-conjugated monoclonal antibody to E-selectin (B) at 24h. Fluorescence was quantified and located by confocal microscopy.

Serum interleukin-6 (IL-6) concentrations were increased in patients with SSc and these correlated with the relative annexin V staining and ICAM-1 expression in neutrophil:

endothelial cell co-cultures (Fig.75). The serum concentrations observed were in agreement with the levels found in plasma by Radstake *et al* who used a multiplex platform similar to the one used here in a large cohort of patients [339] but were higher than levels reported by ELISA on serum samples from a smaller cohort [81].

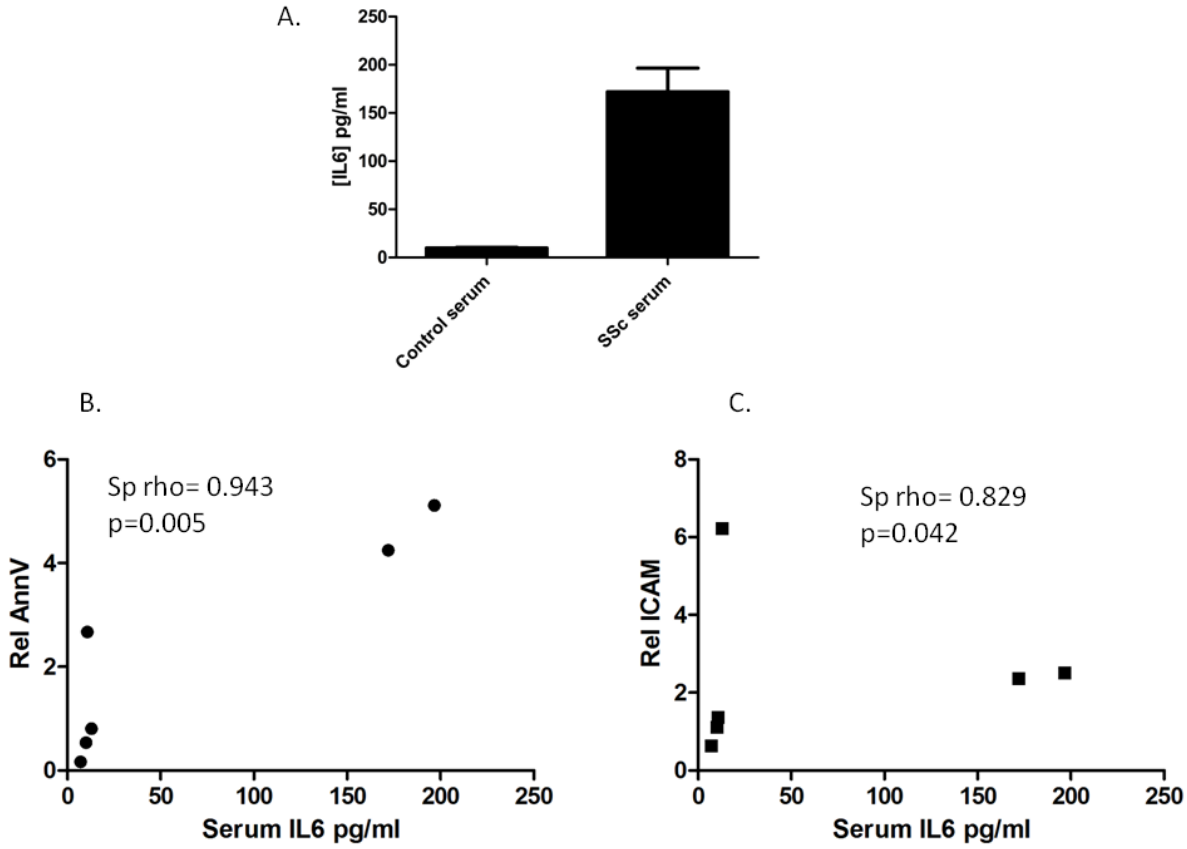
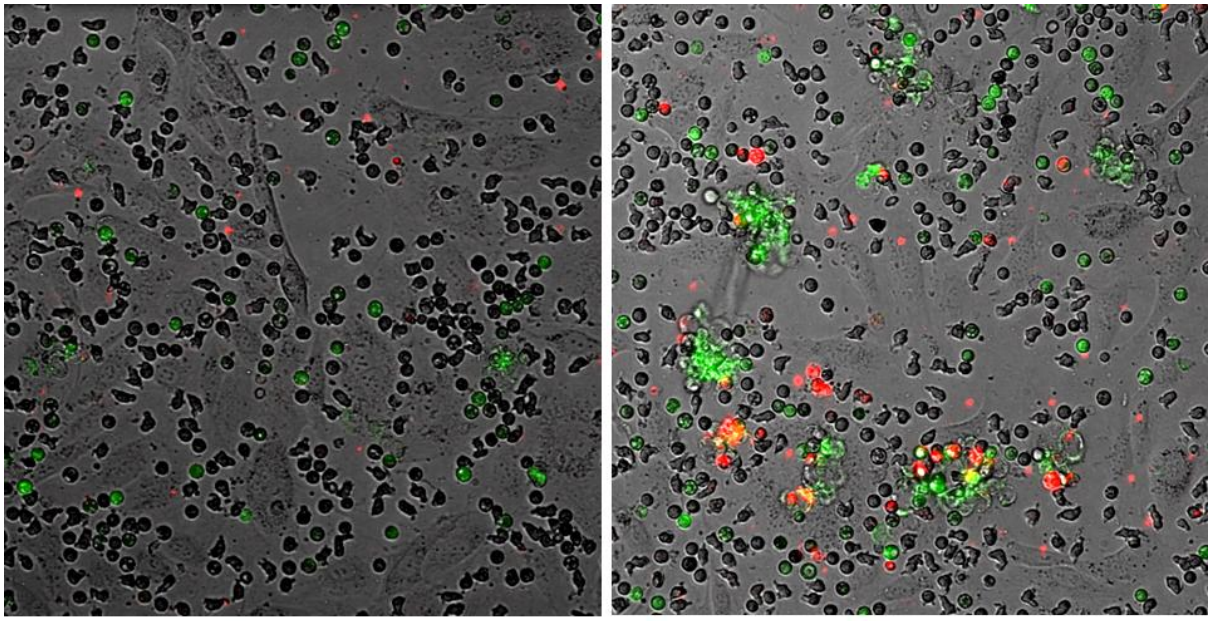


Fig.75. Serum IL-6 concentrations were elevated in SSc (median and interquartile range shown in A. p=0.1, N=3) and correlated with apoptosis (B) and endothelial cell activation (C) in neutrophils: endothelial cell co-cultures.

Control serum spiked with rIL-6 200pg/ml increased annexin V staining and E-selectin expression in neutrophil: endothelial cell co-cultures (Fig.76). Neutrophils were essential in the co-culture for this effect (Fig.77).



A B

Fig.76. Recombinant IL-6 increased apoptosis and E-selectin expression in neutrophil endothelial cell co-cultures. HDMECs were co-cultured for 24h with neutrophils in the presence of AB serum (A) or AB serum spiked with 200pg/ml rIL-6. Apoptosis was measured using annexin V staining and E-selectin expression was measured using an APC-conjugated monoclonal antibody. Fluorescence was quantified and located by confocal microscopy. In addition to increasing annexin V staining and E-selectin expression, rIL-6 also increased the formation of cellular aggregates.

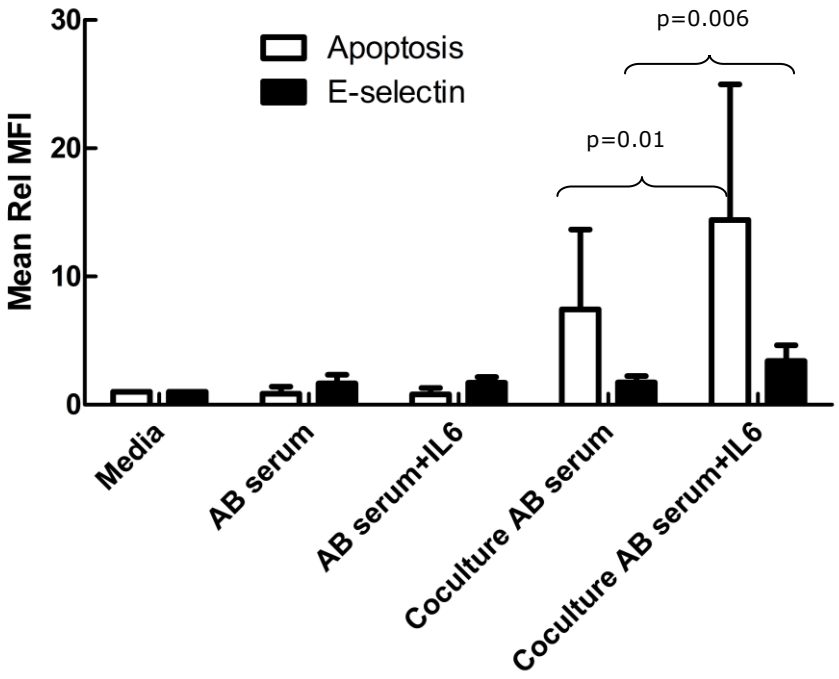


Fig.77. Recombinant IL-6 significantly increased apoptosis and endothelial cell activation. Neutrophils were essential for this effect. HDMECs were cultured for 24h in the presence or absence of neutrophils with AB serum or AB serum spiked with 200pg/ml rIL-6.

The addition of a polyclonal IL-6 neutralising antibody to neutrophil: endothelial cell co-cultures decreased the effect of SSc serum on E-selectin expression and apoptosis (p=0.005, p=0.001 respectively). (Fig.78).

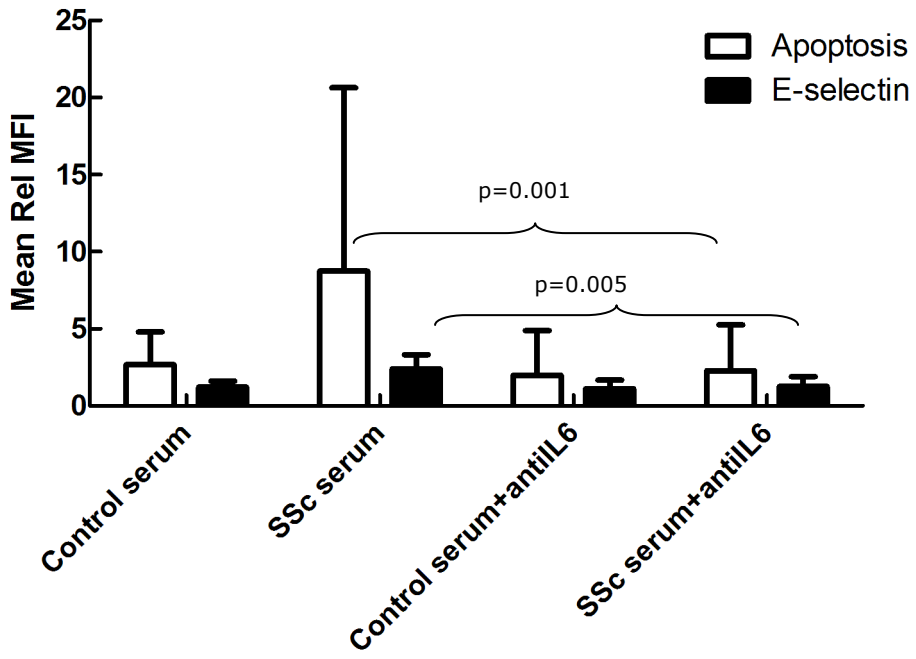


Fig.78. Neutralisation of IL-6 decreased the effect of SSc serum on endothelial cell activation. HDMECs were co-cultured with control neutrophils for 24h in the presence of control or SSc serum (25%). A polyclonal IL-6 antibody was added in neutralizing concentrations (1:400). Apoptosis was measured by annexin V staining, E-selectin expression was measured by an APC conjugated anti E-selectin antibody. Fluorescence was quantified by confocal microscopy. N=11.

Immunodepletion of IL-6 in SSc serum also decreased E-selectin expression in neutrophil: endothelial cell co-cultures (p=0.01). Apoptosis was also decreased but this did not reach statistical significance due to the high variability of the samples (Fig.79).

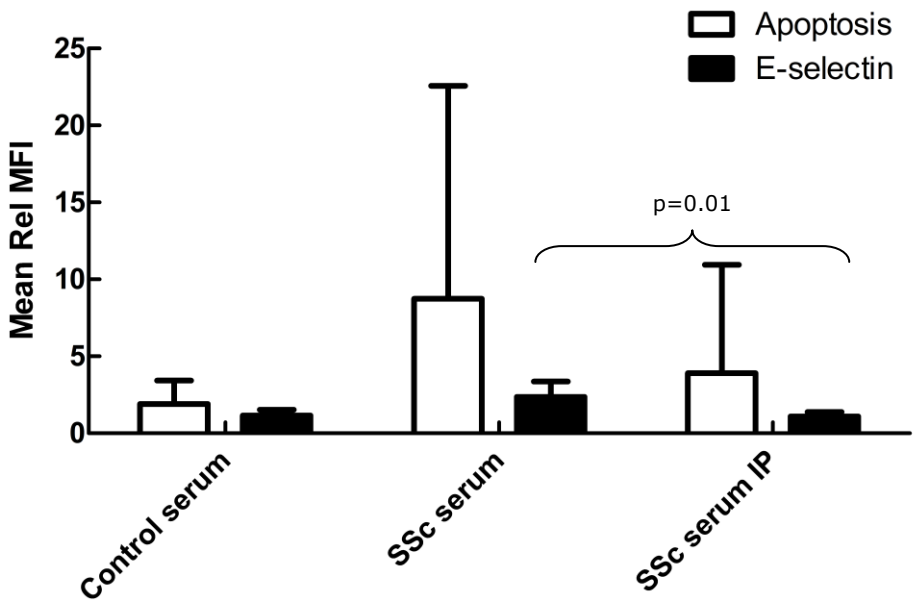


Fig.79. Immunodepletion of IL-6 abrogated the effect of SSc serum on endothelial cell activation. HDMECs were co-cultured with control neutrophils for 24h in the presence of control or SSc serum (25%). SSc serum was either incubated with antiIL-6 conjugated beads (IP) or unconjugated beads. Apoptosis was measured by annexin V staining, E-selectin expression was measured by an APC conjugated anti E-selectin antibody. Fluorescence was quantified by confocal microscopy. N=8.

Soluble gp130, which specifically blocks IL-6 trans signalling, abrogated the effect of SSc serum on E-selectin expression (p=0.03). There was a decrease in apoptosis (p=0.06) in neutrophil: endothelial cell co-cultures but this did not reach significance (Fig.80).

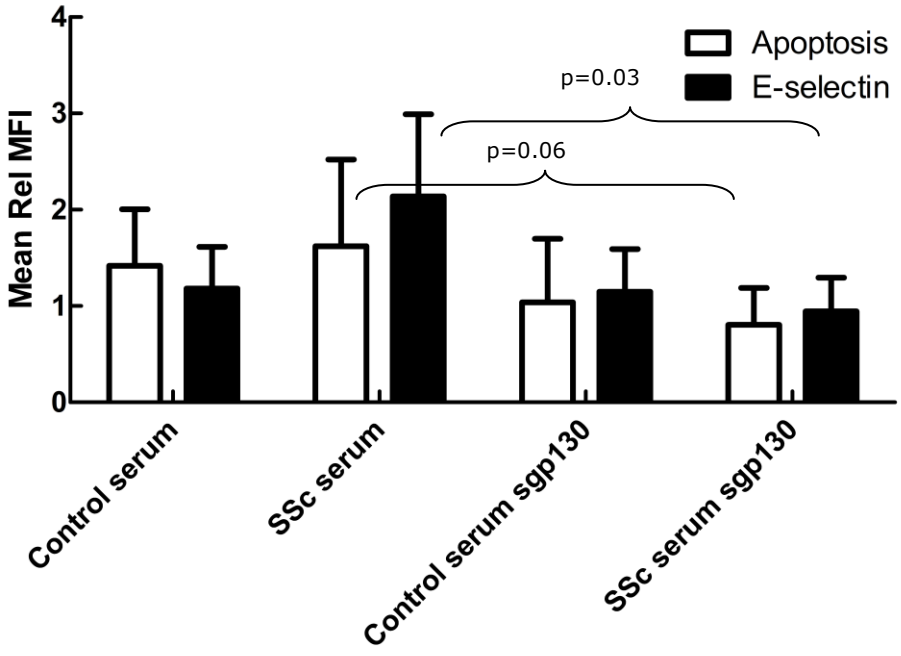


Fig.80. Soluble gp130 (500ng/ml) decreased the effect of SSc serum on apoptosis and endothelial cell activation in neutrophil: endothelial cell co-cultures. HDMECs were co-cultured with control neutrophils for 24h in the presence of control or SSc sera (25%). Soluble gp130 (500ng/ml) was added to block IL-6 trans-signalling. Apoptosis was measured by annexin V staining, E-selectin expression was measured by an APC conjugated anti E-selectin antibody. Fluorescence was quantified by confocal microscopy. N=5.

Inactivation of complement in SSc serum by heating to 56˚C for 30mins did not significantly change the effect of SSc on apoptosis or endothelial cell activation in neutrophil: endothelial cell co-cultures (Fig.81). This implies that complement does not play a significant role in this effect.

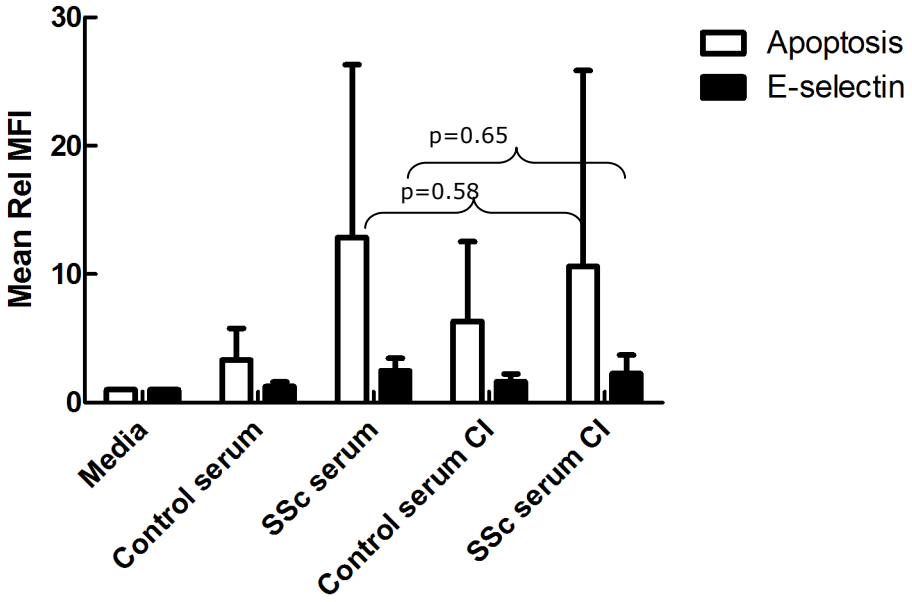


Fig.81. Complement inactivation did not change the effect of SSc serum on apoptosis or endothelial cell activation in neutrophils: endothelial co-cultures. HDMECs were co-cultured with control neutrophils for 24h in the presence of control or SSc serum (25%). Complement inactivation (CI) was performed by heating serum to 56˚C for 30mins. Apoptosis was measured by annexin V staining, E-selectin expression was measured by an APC conjugated anti E-selectin antibody. Fluorescence was quantified by confocal microscopy. N=7.

The addition of the serine protease inhibitor (AEBSF) decreased the pro-apoptotic effect of SSc serum (p=0.04) and endothelial cell activation (p=0.06) in neutrophil: endothelial cell co-cultures the effect on E-selectin expression did not reach statistical significance (Fig.82).

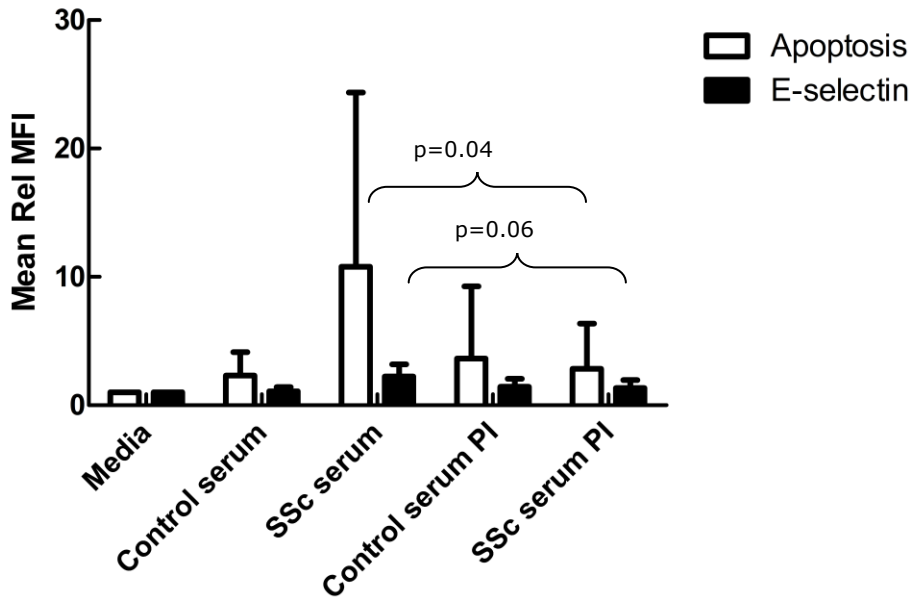


Fig.82.The serine protease inhibitor AEBSF decreased apoptosis and endothelial activation in neutrophil: endothelial cell co-cultures but this did not reach statistical significance. HDMECs were co-cultured with control neutrophils for 24h in the presence of control or SSc serum (25%). The serine protease inhibitor AEBSF (PI) was added (0.2mM). Apoptosis was measured by annexin V staining, E-selectin expression was measured by an APC conjugated anti E-selectin antibody. Fluorescence was quantified by confocal microscopy. N=8.

Removal of hydrogen peroxide from co-cultures by the addition of an excess of catalase, did not significantly change the effect of SSc serum on apoptosis and endothelial cell activation in neutrophil: endothelial cell co-cultures (Fig.83). However, in 3 individual patients the addition of catalase did significantly abrogate the effect of SSc serum (Fig.84). This was explored further in Chapter 3.

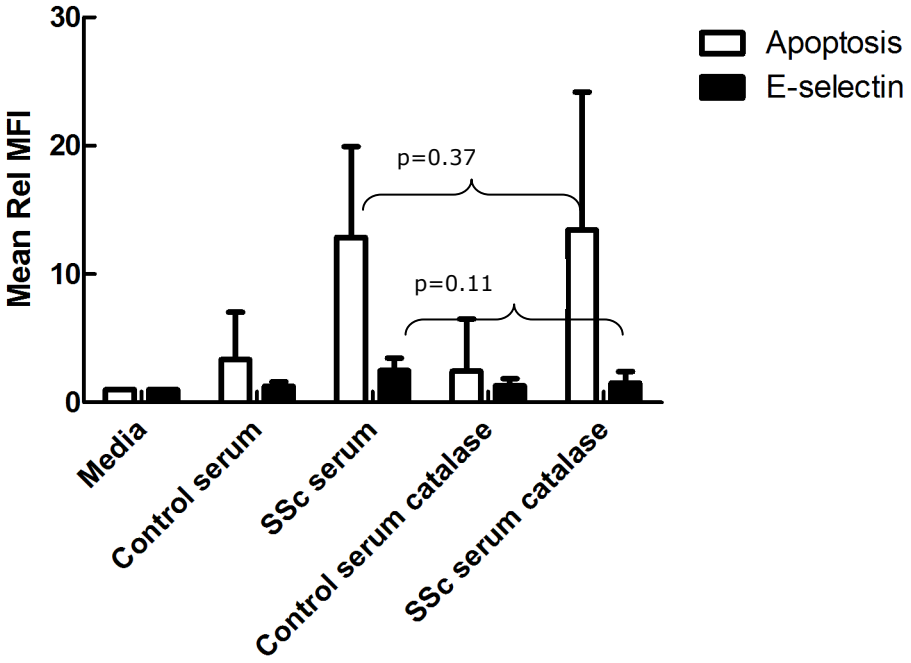


Fig.83. Catalase did not significantly alter the effect of SSc serum on apoptosis and endothelial cell activation in neutrophil: endothelial cell co-cultures. HDMECs were co-cultured with control neutrophils for 24h in the presence of control or SSc serum (25%). Catalase was added (2000U/ml). Apoptosis was measured by annexin V staining, E-selectin expression was measured by an APC conjugated anti E-selectin antibody. Fluorescence was quantified by confocal microscopy. N=7.

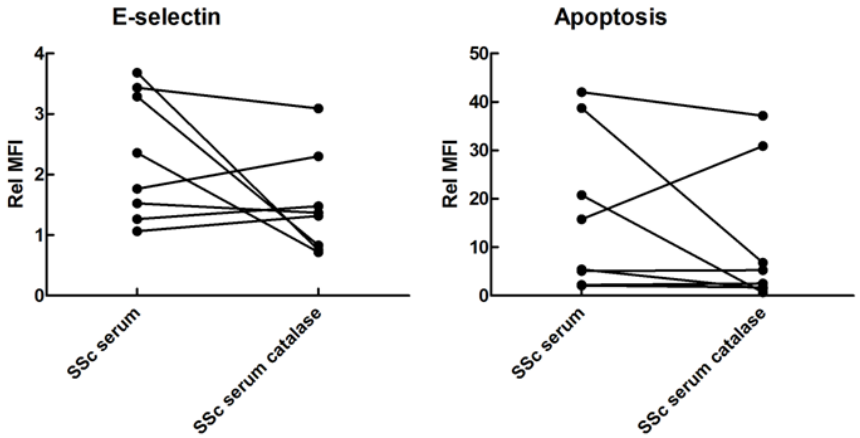


Fig.84. In 3 patients, the addition of catalase significantly decreased E-selectin expression in response to SSc serum in neutrophil: endothelial cell co-cultures. In addition, catalase decreased apoptosis in 2 of these cases.

Analyses were performed to explore whether this unusual response to catalase was associated with any clinical features including: disease duration, Raynaud's duration, use of DMARDs, organ involvement, auto-antibody profile, skin score, organ involvement or disease subtype (limited vs. diffuse). No clinical correlates were found.

7.3.5 Soluble IL-6R concentrations in serum and culture supernatants.

Soluble IL-6R concentrations were measured by ELISA, performed on serum, co-culture supernatants and neutrophils cultured with SSc serum. No difference was found between patient and control serum levels of sIL-6R, and no difference was found in the levels of sIL-6R when neutrophils were incubated with SSc serum. There was an increase in sIL6R levels in co-culture supernatants in the presence of SSc serum compared to control, but this did not reach statistical significance (Fig.85).

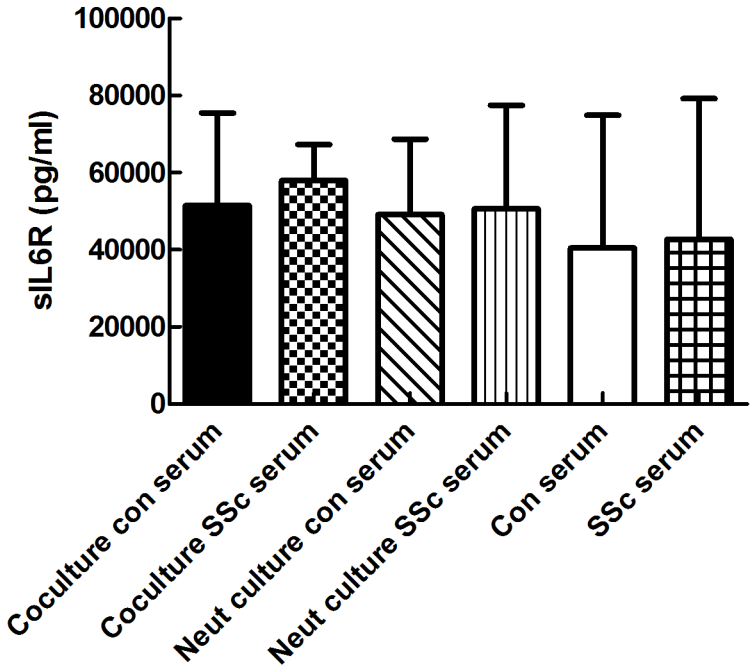


Fig.85. Soluble IL-6R levels were not significantly different between control or SSc serum, or between cultures containing control or SSc serum. There was a tendency for higher levels of sIL-6R in co-cultures containing SSc serum but this was not statistically significant. sIL-6R concentrations were measured by ELISA in control and SSc serum (N=9), in supernatants from 24h neutrophil cultures containing 10^6 neutrophils/ml and 25% control or SSc serum (N=6), or in supernatants from 24h cultures of neutrophils and endothelial cells in the presence of 25% control or SSc serum (N=5).

7.3.6 Soluble ICAM-1 concentrations in serum and culture supernatants.

Soluble ICAM-1 (sICAM-1) levels were also measured in serum and in the supernatants from 24h neutrophil: endothelial cell co-cultures in the presence of 25% control or SSc serum, and in supernatants from endothelial cell co-cultures in the presence of 25% control or SSc serum. We confirmed an increase in serum concentrations of sICAM-1 in SSc compared to controls. However, when corrected for added serum concentrations, there was no difference in sICAM-1 concentrations in culture supernatants (Fig.86). It is likely that there are several factors dictating the concentrations of sICAM-1 in the cultures, not just cleavage from the cell surface but also degradation of cleaved sICAM-1. In addition, given the relatively high levels in serum, small changes in the release of sICAM-1 from cells in cultures containing sera are likely to be swamped, and therefore undetectable.

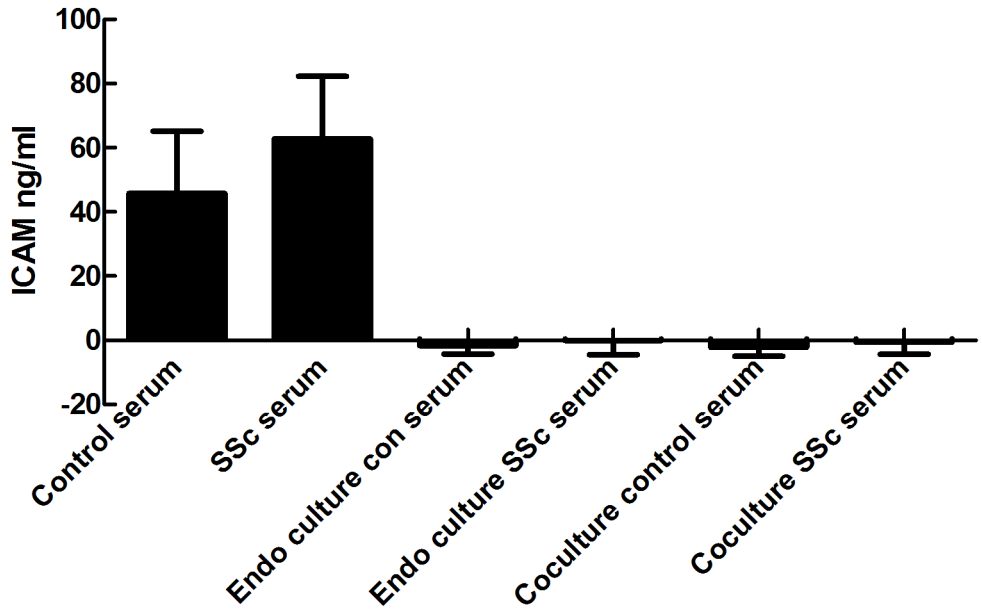


Fig.86. Soluble ICAM-1 concentrations were elevated in SSc serum but not in culture supernatants containing SSc serum compared to control serum. sICAM-1 levels were measured by ELISA in serum, supernatants from 24h endothelial cell cultures containing 25% control or SSc serum and in supernatants from 24h neutrophil: endothelial cell co-cultures containing 25% control or SSc serum. In supernatants, levels were corrected for the amount already present in the added serum, to try to detect increases in sICAM-1 in the cultures. No significant differences were found. N=5.

7.4 Discussion.

The results of this study show that SSc serum causes activation of endothelial cells as evidenced by an increase in E-selectin and ICAM-1 expression. SSc serum also causes an increase in neutrophil ICAM-1 expression, which has previously been reported in response to inflammatory sera [337]. E-selectin however represents a much more specific marker of endothelial activation. E-selectin is not usually expressed in unstimulated endothelial cells and has to be generated *de novo* which takes several hours, unlike ICAM-1 which is constitutively expressed by endothelial cells on the cell membrane, and also exists in intracellular stores that can be rapidly mobilised following endothelial cell activation.

Neutrophils aspirated from the co-cultures do not express any E-selectin, confirming the specificity of this marker, although there is the possibility that some neutrophils may be adherent to the endothelial cell layer, and E-selectin expression in those cells cannot be excluded.

In neutrophil: endothelial cell co-cultures there is, in addition, an increase in apoptosis of both neutrophils and endothelial cells in response to SSc serum compared to control serum, as assessed by confocal microscopy. However, when the neutrophils were removed and annexin V binding measured by flow cytometry, there was no increase in the percentage of apoptotic cells in response to SSc serum. An explanation for this may be that during the 24h culture, apoptotic neutrophils may have undergone secondary necrosis decreasing the number of recovered neutrophils, and leading to an underestimation of the total levels of apoptosis. However, when neutrophils were cultured in the absence of endothelial cells, there was no increase in neutrophil apoptosis with SSc serum compared to control serum. Therefore, it seems most likely that the observed increase in apoptosis is predominantly in the endothelial cell population.

During time-lapse videos of neutrophil: endothelial cell co-cultures endothelial cell apoptosis was observed, and these apoptotic cells then appear to attract neutrophils, forming the cellular aggregates observed in the presence of SSc serum. However, overall levels of apoptosis in the co-cultures incubated on the confocal microscope were higher than expected when compared to cultures that were performed in a standard incubator (37˚C, 5% CO_2, humidified). This is likely to be due to technical difficulties in maintaining uniform CO_2 concentrations and ample humidification on the confocal microscope.

IL-6 is considered as an anti-apoptotic cytokine but, its effects are dependent on the cell type. Previous studies have shown a decrease in endothelial cell apoptosis in response to IL-6 in cultured human umbilical vein endothelial cells (HUVECs) [340 341]. However, no studies are reported using HDMECs and endothelial cell heterogeneity may explain the effects reported here . Other explanations for the apoptosis seen in these experiments may include an indirect effect of IL-6 or an interaction with other cytokines. Indeed IL-6 pretreatment has been reported to cause increased hepatocyte apoptosis in response to TNFα [342]. Deficiency of the apoptosis protective protein, Tie-2, rendered endothelial cells sensitive to IL-6 induced apoptosis [343]. The balance of effects may be concentration dependent, as described for TNFα, and the concentrations of IL-6 in the lesional tissue are unknown. Finally, the effect may be dependent on direct contact between neutrophils and endothelial cells. To further explore this effect, future experiments should be performed using cell culture inserts with semi-permeable membranes. Neutrophils would be cultured in these chambers, suspended above cultured HDMECs The semi-permeable membranes would allow free diffusion of soluble mediators but prevent direct cell-cell contact.

Endothelial cell apoptosis and activation are thought to be central to the pathogenesis of SSc. In animal models of SSc, endothelial cell activation is seen early in the disease, preceding the fibrotic changes that are the hallmark of this disease [6 7]. Soluble markers of endothelial activation, sICAM-1 and sE-selectin, as well as other markers of endothelial cell

activation, vWF and ET-1 are increased in the serum of patients with SSc and correlate with clinical manifestations and clinical markers of severity [15 19 333]. Treatments that ameliorate the clinical features of SSc e.g. Iloprost, decrease markers of endothelial cell activation [344].

The mechanism for endothelial cell activation, however, is not clear. Pro-inflammatory cytokines interleukin-1 (IL-1), tumour necrosis factor α (TNFα) and interleukin-6 (IL-6) are capable of activating endothelial cells *in vitro*. 13-plex luminex studies of serum from SSc patients revealed a tendency for an increase in these inflammatory cytokines, and indeed there are many, though conflicting, reports in the literature of increases in serum levels of pro-inflammatory cytokines [78 80-82 142]. Within my data, IL-6 serum concentrations correlate with the degree of endothelial cell activation and apoptosis seen within co-cultures.

Endothelial cells, however, do not express the IL-6 receptor (gp80, IL6R) and are thought to respond to IL-6 through a process known as trans signalling [93]. Soluble IL-6 receptors (sIL-6R) exist in the serum and bind to IL-6 forming an IL-6-sIL-6R complex. This complex can bind to the gp130 receptor, which is expressed ubiquitously on cells including endothelial cells, which activates the STAT3 signalling pathway [98 99]. Endothelial cell activation via trans signalling results in an increase in the expression of adhesion molecules (ICAM-1, E-selectin), the release of chemokines (IL-8 and MCP) and the release of IL-6 [93 96] (Fig.87).

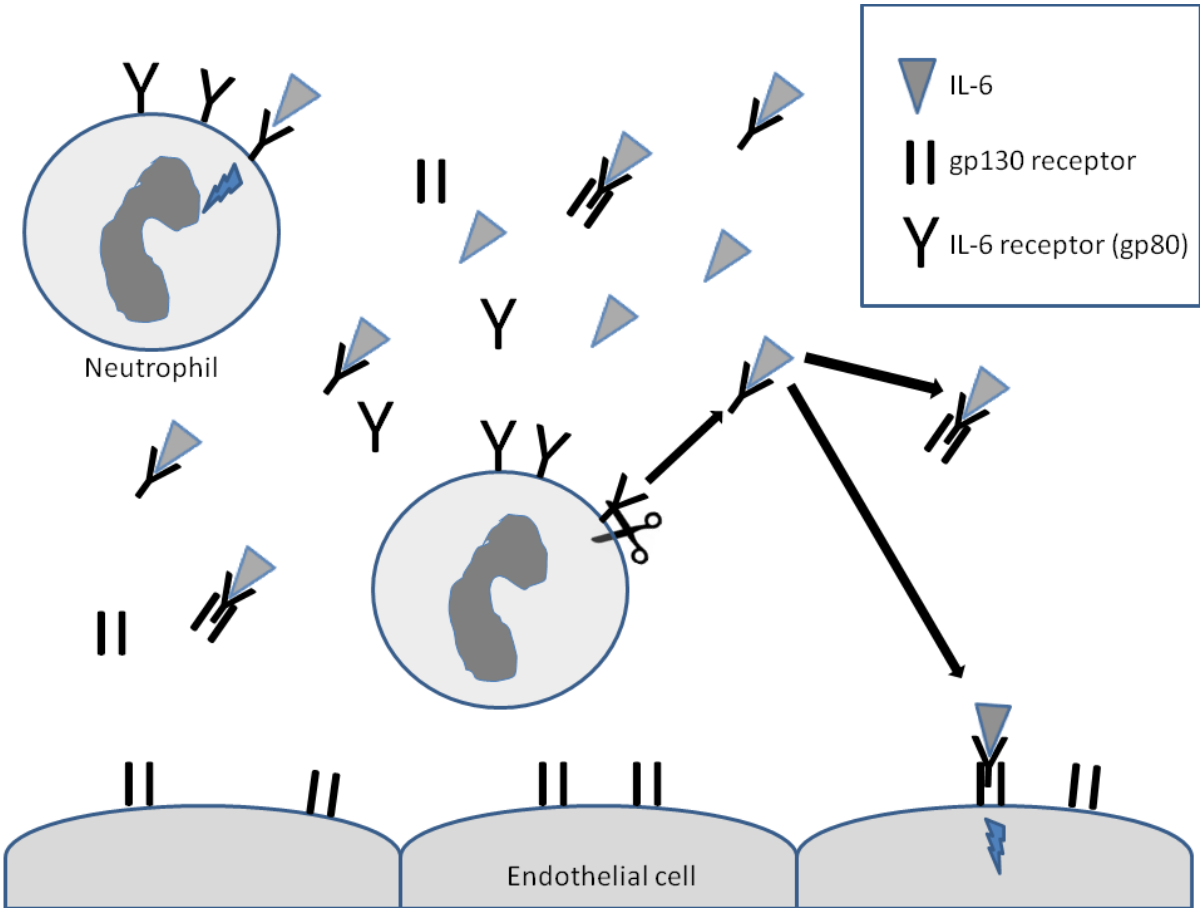


Fig.87. Interleukin-6 trans signalling.
IL-6 receptors are expressed on leukocytes including neutrophils but they are not expressed on tissue resident cells e.g. Endothelial cells. Endothelial cells can respond to IL-6 through the gp130 receptor only when the IL-6 is bound to a soluble IL-6 receptor (sIL-6R). sIL-6Rs are formed by secretion of an alternatively spliced version of the receptor or proteolytic cleavage from the surface of neutrophils. There is also a pool of sgp130 (sgp130) which can bind IL-6/sIL6R complexes and prevent them binding to cellular gp130. Therefore, the local concentrations of IL-6, sIL-6R and sgp130 regulate IL-6 signalling.

The IL-6R is expressed on hepatocytes, monocytes, B-cells and neutrophils in humans. It is also found on a subset of T-cells, but there is evidence that T-cells respond to IL-6 predominantly through trans signalling [98]. sIL-6R is produced by two separate mechanisms, firstly by proteolytic cleavage from the surface of neutrophils and secondly by secretion of an alternatively spliced version [345-348].

The regulation of the proteolytic cleavage has been partially elucidated and is stimulated by C-reactive protein (CRP). Cleavage from the surface of neutrophils, but not monocytes, is also stimulated by chemoattractants (IL-8, C5a, LTB4 and PAF) [349]. Proteolytic cleavage can occur via a TACE-like enzyme though this does not account for all of the proteolytic cleavage.

Levels of IL-8 were elevated in the SSc serum, as measured by luminex, in keeping with previous reports in the literature, and this may stimulate the release of sIL-6R from neutrophils within the co-cultures [224 350]. In addition there are reports in the literature that LTB_4 levels are elevated in the broncheoalveolar lavage fluid of patients with SSc lung disease [226], and while this was not measured within this study it may also contribute to the generation of sIL-6R.

SSc serum alone, in the absence of neutrophils, increased endothelial cell ICAM-1 expression after 24h, but the magnitude of the change was lower than that observed in the presence of neutrophils. SSc serum alone did not increase endothelial cell apoptosis or E-selectin expression. The fact that neutrophils are required in the co-culture for this effect may be explained by the fact that they are required as a sIL-6R donor.

IL-6 is a pleiotropic cytokine, and several observations support a role for this cytokine in SSc. Fibroblasts from patients with SSc are phenotypically unique. When isolated and cultured *in vitro* they continue to produce an excess of collagen [27 91]. IL-6 has been shown to either increase or decrease fibroblast proliferation, increase fibroblast collagen, glycosaminoglycan and TIMP-1 synthesis and increase MCP-1 and IL-6 production [84 85 89 91 351 352]. IL-6 regulates the expression of VEGF, an important mediator of angiogenesis and fibrosis which is elevated in patients with SSc [353].

IL-6 transcription is under the control of a hypoxic response element via HIF-1-α. Measurements taken from the lesional skin of patients shows that there is a persistent

decrease in oxygen tension [60]. Levels of oxygen equivalent to 3% have been found in the lesional skin, which reflect levels sufficient to induce HIF-1α signalling [60]. However, RT-PCR results show that there is no increase in IL-6 mRNA in neutrophils in response to hypoxia (data not shown). Indeed, the low levels of IL-6 mRNA detected in these experiments are likely to represent monocyte or eosinophil contamination and no enhancement due to hypoxia. Previous reviews of the literature support the fact that neutrophils do not transcribe or secrete IL-6 [229].

Fibroblasts isolated and cultured from the lesional skin of patients with SSc constitutively produce higher levels of IL-6 than non-lesional or healthy donor fibroblasts [32]. This demonstrates the importance of considering local concentrations of cytokines in disease. Serum concentrations may not necessarily reflect local levels of a relevant cytokine at the lesional site. Local interactions between fibroblasts, endothelial cells and immune cells in the presence of locally elevated levels of cytokines may be more important in *in vitro* models. Stimulated and unstimulated fibroblasts from lesional skin have also been shown to produce increased levels of IL-8 which may be implicated in local release of sIL6R from neutrophils [34].

It is important to note that hemodynamic flow may affect IL-6 induced signalling in endothelial cells [354]. Hemodynamic flow is dysregulated in patients with SSc and this may play an important role in modeling the effects of IL-6 on endothelial cells in this disease. Flow was not incorporated in the model used in this study, representing a limitation of this system. I would wish to consider this in further experiments.

IL-6 also has a profound effect on B-cells giving rise to plasma cell differentiation and antibody production. This may explain the polyclonal B cell expansion and hypergammaglobulinaemia which is frequently seen in SSc [99].

IL-6 has been implicated in the generation and propagation of chronic inflammation. Initially in acute inflammation, pro-inflammatory cytokines lead to neutrophil accumulation and the release of IL-6. Neutrophils then shed their IL-6Rs in response to chemokines e.g. IL-8. This promotes differential regulation of chemokine production by endothelial cells, promoting MCP production and decreasing IL-8 production therefore favouring monocyte accumulation. IL-6 trans signalling also increases the expression of endothelial leukocyte adhesion molecules (VCAM-1, ICAM-1 and E-selectin) therefore further promoting leukocyte accumulation [96 97]. In addition, IL-6 may have a role in promoting neutrophil apoptosis and therefore the resolution of acute (non-specific) inflammation [95 100]. Others however have reported an anti-apoptotic effect of interleukin-6 on neutrophils [102] while Biffl *et al* have shown that the effect depends on the neutrophil concentration [101]. We have been unable to reproduce any IL-6 specific effect on neutrophil apoptosis in our laboratory at concentrations of IL-6 ranging from 0.1-100ng/ml (personal communication Helen Wright).

Conversely, IL-6 reportedly rescues T-cells from apoptosis, which promotes a chronic inflammatory cell infiltrate [355-358]. IL-6 trans signalling also promotes the release of IL-6 from fibroblasts and endothelial cells in a positive autocrine feedback system. Therefore, it can be envisaged that IL-6 may have a role in propagating chronic inflammation, such as that seen in SSc. This is in keeping with immunocytochemical experiments which demonstrate that IL-8 and IL-6 are over-expressed in the lesional skin of patients with SSc, though in different patterns. The over-expression of IL-8 is associated with early disease (<1yr) whereas IL-6 over-expression is associated with later disease [79].

IL-6 has also been implicated in autoimmunity. Evidence from patients with Crohn's disease indicates that autoreactive T-cells are resistant to apoptosis due to protection by IL-6 trans signalling via the STAT3 signalling pathway [359]. IL-6 inhibits a Na^{2+}/K^{+} ATPase which regulates endosmosis and antigen presentation by dendritic cells to T-cells, which may promote presentation of autoantigens [360 361]. Finally, according to Matzinger's " danger

theory", naïve T-cells die if they receive a signal from proper antigen presentation but this is not followed up by ligation of CD40 [362]. There is evidence that IL-6/sIL-6R complex can inappropriately substitute for this second signal and therefore lead to the persistence of autoreactive T-cells [363]. Incidentally, autoimmune phenomena increase with age, in concert with an age-related increase in sIL-6R shedding [364]. SSc is a disease associated with autoimmune phenomena. Many different auto-antibodies are found in SSc (see Table 14.) and the auto-antibody profile in many cases correlates with clinical manifestations. There is however, no convincing evidence for a direct relationship with pathogenesis, though some investigators have reported that anti-endothelial cell antibodies, found in a proportion of patients are associated with endothelial cell activation [9 365].

A.

Autoantibody	Frequency %	Clinical association
Anti-centromere	32	Ltd, PHT
Anti-topoisomerase	13	Diffuse, renal involvement, ILD, disease activity/severity
Anti-RNA polymerase III	23	Diffuse, renal involvement
Anti-U1 RNP	5	Ltd, severe GI involvement
Anti-U3 RNP	4	MCTD, ILD, severe GI involvement
Anti-PMScl	3	Polymyositis overlap, ILD, severe GI involvement
Anti Th/To	4	Ltd, PHT

Ltd=lcSSc, PHT= pulmonary artery hypertension, ILD= interstitial lung disease, GI= gastro-intestinal, MCTD= mixed connective tissue disease.

B.

Autoantibody	In vitro activity
Anti-endothelial cell	Endothelial cell apoptosis.
Anti-fibrillin 1	Fibroblast activation, increased ECM production.
Anti-matrix metalloproteinase	Prevent degradation of the ECM.
Anti- PDGFR	Induce collagen 1 production. Convert fibroblasts to myofibroblasts.
Anti-fibroblast	Increased expression of ICAM and IL-6.
Anti-HSP47	Not known.

Table 14. Systemic sclerosis associated auto-antibodies. Panel A outlines the systemic sclerosis associated antibodies, their frequency in systemic sclerosis patients and their disease associations. Panel B outlines potentially pathogenic antibodies which have been described in a proportion of patients with systemic sclerosis. Reviewed in [4].

In this study, monocytes triggered similar levels of endothelial cell activation in co-cultures to neutrophils. Monocytes are also capable of donating sIL-6R, though evidence implies that they do this most frequently by an increase in the secreted, alternatively spliced form [366]. Certainly, in monocytes, cleavage of the IL-6R from the cell surface is not mediated by chemoattractants, as it is for neutrophils. A monocytic infiltrate is more commonly seen in the

lesional skin of SSc patients reflecting the chronic inflammatory phase of the disease. Therefore, monocyte derived sIL-6R may mediate a continuation of IL-6 trans signalling and hence fibroblast and endothelial cell activation in this disease. However, during this study I have only explored the role of trans signalling in neutrophil: endothelial cell co-cultures. In future, I would like to perform confirmatory experiments to show that IL-6 trans signalling is also responsible for endothelial cell activation in monocyte-endothelial cell co-cultures. This would further confirm that blockade of IL-6 trans signalling would be an appropriate therapeutic target in SSc. Previous research has shown that PBMCs from SSc patients, when cultured *in vitro,* produce higher levels of IL-6 and sIL-6R in the culture supernatants than control PBMCs, though levels of sgp130 were equivalent [169]. As monocytes form a large part of the immune cell infiltrate in the tissue, they may play a significant role in disease, but at the endothelial cell surface, the high number of neutrophils in the circulation, together with the presence of increased endothelial cell adhesion molecules and the release by endothelial cells of neutrophil chemoattractants, would argue for a significant role for neutrophil derived IL-6R.

IL-6 concentrations measured by luminex were not elevated in the supernatants from 24h neutrophil: endothelial cell co-cultures. This is in keeping with the previously reported inability of neutrophils to express IL-6 [229]. sIL-6R levels measured by ELISA were elevated in co-culture supernatants containing SSc serum compared to those containing control serum, although the difference was not significant. There was no difference in serum sIL-6R concentrations between SSc patients and healthy controls. In addition, there was no increase in sIL-6R concentration in the supernatants of neutrophils cultured with SSc serum for 24h in the absence of endothelial cells compared to control serum. These results demonstrate that local levels of sIL-6R are the important factor in controlling IL-6 signalling, and that overall serum levels are not likely to reflect what might be pathogenically significant changes in cytokine signalling at the cellular level. Secondly, the fact that neutrophils do not appear to shed sIL-6R in culture with SSc serum in the absence of endothelial cells implies

that the endothelial cell: neutrophil interactions may be even more complex. This may suggest that an unidentified factor in the SSc serum induces endothelial cell production of a further factor which in turn stimulates the proteolytic cleavage of sIL-6R from the neutrophil membrane. Interleukin-8 (IL-8) would be a good candidate for the intermediary produced by the endothelial cell. Endothelial cells can be stimulated to produce IL-8 in response to the proinflammatory cytokines IL-1 and TNF. Interestingly these were increased in the serum of patients compared to controls, though low sample size meant that this difference was not statistically-significant. Future work would include an assessment of the effect of blocking interleukin-8 in my co-culture system. In addition, measurement of SSc neutrophil IL6R expression by flow cytometry compared to controls may help to show whether there has been cleavage of the surface receptor. However the situation may be complicated as the regulation of cell surface expression of IL6R is not fully elucidated and may involve dynamic replacement by mobilisation of intracellular stores, in keeping with other neutrophil cell surface receptors.

Tocilizumab, an IL-6 receptor monoclonal antibody is licensed for the treatment of rheumatoid arthritis (RA) and is extremely effective [367-369]. I propose that IL-6 blockade is a potentially novel therapeutic strategy in SSc, targeting multiple aspects of the disease including endothelial dysfunction and fibrosis. There is evidence that IL-6 is important in early disease but is also likely to be important in continuing and progressive disease activity. It could represent an important intervention in a large number of patients suffering with this disabling and difficult to treat disease.

Chapter 8: Conclusion

8.1 Summary of results.

In Chapter 3 the functional phenotype of SSc neutrophils was explored. Neutrophils were found to be hypofunctional in terms of unstimulated ROS generation and chemotaxis along an fMLP gradient. Data also implied (though did not quantitatively prove) an increase in neutrophil adhesion both to inert materials and to each other. The hypofunctionality in terms of unstimulated ROS production was overcome using neutrophil stimulants e.g. fMLP and PMA implying that the neutrophils were not inherently dysfunctional but rather, activated *in vivo*. SSc neutrophils, in fact, produced significantly more ROS in response to fMLP than control neutrophils when unprimed, implying some *in vivo* priming of SSc neutrophils.

Proteomic studies, both whole cell and plasma membrane, showed evidence of dynamic changes in the actin cytoskeleton and actin-associated machinery. The changes observed in SSc neutrophils mimicked those seen in response to chemoattractant doses of fMLP and are also likely, again, to reflect *in vivo* neutrophil activation. L-plastin, an actin-associated protein that is activated by IL-8 and activates, in turn, neutrophil integrin function, was increased in the plasma membrane fraction of SSc neutrophils. In addition, proteomic studies highlighted differential expression of S100A8 and S100A9 in SSc neutrophils with increased intracellular concentrations and decreased plasma membrane concentrations of these proteins.

Neutrophil elastase was not increased in the serum of patients with SSc nor was there greater serum neutrophil elastase activity overall. However, in SSc serum there was more elastase activity detected for each unit of elastase protein detected. This may imply a deficit in neutrophil elastase inhibition, which may lead to important local effects of neutrophil elastase, which has powerful roles in the modulation and propagation of inflammation and the promotion of fibrosis.

There was an interesting observation that peripheral blood neutrophil count correlated strongly with the extent of skin thickening.

Finally, modelling the effects of neutrophil endothelial cell interactions revealed that SSc serum increased the amount of E-selectin expression and apoptosis compared to control serum, as well as stimulating the formation of cellular aggregates. Neutrophils were essential for this effect which appears to be mediated by interleukin-6. Serine proteases also play an important role in the induction of apoptosis in this *in vitro* model and reactive oxygen species may play a role in some individual patients.

8.2 Interpretation.

Neutrophils may be hypofunctional *ex vivo* for two reasons: either the neutrophils are hypofunctional *per se*, or they have decreased function *in vitro* following previous *in vivo* activation. It seems unlikely that SSc neutrophils are hypofunctional *per se*, since they produced normal responses to *in vitro* activating agents. In fact, given that they are hypofunctional at baseline *in vitro*, the magnitude of the response to activating agents was greater in SSc than in control neutrophils.

Proteomic studies have added important additional information in support of this proposal of *in vivo* activation. The proteomic profile of freshly-isolated neutrophils is likely to reflect the *in vivo* activation state of the neutrophil. Indeed, the proteomic profile of neutrophils mimicked that found in response to activating agents TNF and LPS, implying that neutrophils are in fact activated *in vivo*. Further evidence comes from the proteomic and confocal evidence that there are changes in the actin cytoskeleton in SSc neutrophils. The actin cytoskeleton is involved in many active cellular processes including, adhesion, degranulation, chemotaxis, signal transduction and neutrophil priming. All of these events reflect neutrophil activation.

There is evidence that SSc neutrophils may be primed for ROS generation. Substances that cause activation often lead, at lower concentrations, to neutrophil priming.

The fact that peripheral blood neutrophil counts correlate with Rodnan skin score provides some circumstantial evidence to imply that neutrophils or neutrophil mediators may eventually lead to fibrosis. This may or may not be a direct effect and may be a surrogate marker for the increased inflammation in these patients. However, it would be important to explore whether this relationship exists for other cells of the immune system in the peripheral blood and to examine the neutrophil counts longitudinally to see whether this relationship still exists.

8.3 Significance.

Although neutrophils are traditionally thought of as immune cells with a limited repertoire of functions, there is increasing evidence that they have diverse and important roles in not only initiating, but perpetuating inflammation and modulating the inflammatory response. A good example of this is through the shedding of its interleukin-6 receptor, to induce trans signalling on those cells that do not normally express this receptor. This can amplify IL-6 mediated events, leading to the promotion of chronic inflammation, endothelial cell activation and fibrosis.

As an abundant source of the S100 proteins S100A8 and S100A9, neutrophils can trigger profound effects on inflammatory events by; mediating responses to reactive oxygen species; promoting endothelial cell activation and apoptosis; amplifying the immune response and recruiting cells of the adaptive immune system by the activation of TLR4 receptors. Neutrophil elastase also has well established roles in the promotion of inflammation and fibrosis, and in endothelial cell apoptosis.

Neutrophil activation can lead to the release of S100 proteins and elastase, in addition to reactive oxygen species. Therefore, neutrophil activation could have profound effects at the endothelial surface that could give rise to pathogenically-relevant consequences. Neutrophil activation seems very likely given the fact that neutrophil chemotactins (IL-8) are elevated in

SSc serum and in the tissue of SSc patients, that endothelial cells express increased quantities of cell surface leukocyte adhesion molecules and that binding to these molecules activates neutrophils. In addition, increased concentrations of neutrophil activating cytokines e.g. TNFα, IL-1 and IL-8 are also reported in SSc serum and are produced by SSc tissue.

Neutrophils do not constitute a large part of the skin infiltrate in SSc, and the reason for this is not known. It may reflect the absence of a chemotactic gradient into the fibrotic tissue or it may reflect a defect in neutrophil diapedesis, due to either neutrophil or endothelial cell factors. However, the lack of neutrophilic infiltrate does not rule out interaction between neutrophils and endothelial cells in the lumen of blood vessels.

Co-culture models, in fact, imply that neutrophils are likely to interact with endothelial cells and each other in the presence of factors in SSc serum, and that this interaction results in increased endothelial cell activation and apoptosis. It is not clear from this model whether neutrophil activation forms part of this interaction leading to the release of mediators that then cause endothelial cell activation and apoptosis, but this seems likely. Mediators may include those outlined above.

Neutrophils appear to be more likely to form cell aggregates in response to SSc serum and when isolated from SSc patients. This may be of great pathological relevance as these may form microemboli, which could contribute to the ischaemic phenomena which occur in SSc patients.

The conclusions drawn from these studies are weakened due to the heterogeneity of the patients studied. Small numbers precluded disease subtype analysis and therefore important information was lost regarding the precise relevance of neutrophils in these different disease manifestations. More importantly, patients were not recruited exclusively early in the disease, when neutrophils are most likely to relevant to the pathogenesis. Small numbers of

such early patients prevented stratified recruitment according to disease duration and also precluded meaningful subgroup analyses of such patients.

8.4 Addressing the hypothesis.

The hypothesis "*Neutrophils are activated in SSc and contribute to endothelial cell activation and injury"* has been addressed in this thesis.

Proteomic evidence supports neutrophil activation in SSc and functional studies support neutrophil priming and also imply *in vivo* neutrophil activation. Confocal studies show dynamic changes in the actin cytoskeleton and observations suggest increased neutrophil adhesiveness. Together, these strongly point towards neutrophil activation in SSc.

Neutrophil: endothelial cell co-cultures suggest that SSc serum can induce endothelial cell activation and injury (apoptosis) and that neutrophils are required for this effect. Evidence supports a role for IL-6 trans signalling in this effect and neutrophils may be required to donate the interleukin-6 receptor. Although there is no direct evidence that neutrophil activation is required for the effect of SSc serum, serine proteases appear to have a role in endothelial cell apoptosis in these experiments and neutrophil activation may lead to the release of serine proteases e.g. neutrophil elastase. In addition, in some but not all patients, the effect of sera is abolished by catalase which scavenges ROS. Again, activated neutrophils are the likely, but not only potential, source of ROS in this system.

These studies, taken together, show that neutrophil activation does indeed occur in SSc and uniquely, *in vitro* models demonstrate that this is likely to lead to endothelial cell activation and injury. This may lead to a paradigm change in the design of therapeutic targets in SSc.

Appendix A:

Standard SDS-PAGE:

a) Reducing (sample) buffer

- 1M Tris pH 6.8
- 10% glycerol (v/v)
- 3% SDS (w/v)
- 100mM DTT
- 0.001% Bromophenol blue (w/v)

b) Wash buffer

- 10mM Tris pH 8.0
- 150mM NaCl
- 0.075% Tween 20

c) Running buffer

- 25mM Tris
- 250mM Glycine
- 0.1% SDS

d) Transfer buffer

- 25mM Tris pH 8.0
- 0.2M Glycine
- 20% Methanol

Tris-Tricene SDS-PAGE.

a) Inner chamber buffer

- 200mM Tris
- 200mM Tricene
- 0.2% SDS
- pH 8.5

b) Outer chamber buffer

- 400mM Tris pH 8.8

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