Thesis

Characterisation of the lymphocytic infiltrate in skin biopsies of scleroderma patients before and during longtime B-cell-depletion treatment

submitted by

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Declaration of Authorship

I declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. Where I have quoted from the work of others, the source is always given at their point of use.

Graz, September 3rd, 2018 *Verena Maria Kriso eh*

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Zusammenfassung

Ziel: Ziel dieser Diplomarbeit war es, eine Färbemethode zu etablieren, um die Anzahl der Th17 Zellen im lymphozellulären Infiltrat in Hautproben von SklerodermiepatientInnen vor und unter laufender CD20 – Depletionstherapie mittels Rituximab erheben zu können und die Ergebnisse anschließend mit Hautproben von Gesunden zu vergleichen.

Einleitung: Bei der systemischen Sklerodermie handelt es sich um eine seltene Bindegewebserkrankung, die zumeist an der Haut beginnt und im weiteren Verlauf auch die inneren Organe (Lunge, Herz, etc.) befällt. Dies kann unbehandelt in kurzer Zeit zum Tod führen. Pathogenetisch spielt die überschießende Aktivität des körpereigenen Immunsystems mit exzessiver Fibroseaktivierung eine wichtige Rolle. Daher werden immunsuppressive Therapeutika, wie z. B. Rituximab, eingesetzt. Die positive Wirkung dieses Medikaments wird in einer klinischen Besserung der Symptomatik sichtbar. Da in der Literatur nunmehr speziell die Th17 – Zellen als Auslöser für Autoimmunerkrankungen genannt werden, wollen wir uns die Hautproben der SSc-PatientInnen bzgl. dieser Zellen ansehen.

Material und Methoden: Die Durchführung einer immunhistochemischen Doppelfärbung wurde etabliert. PatientInnen, die bereits eine Hautprobe vor Beginn der Rituximab-Therapie im Rahmen der Studie (EK-No.: 24-184 ex 11/12) gespendet hatten, wurden gefragt, ob sie nochmals eine Probe, mit einem Abstand von mindestens einem Jahr, seit Therapiebeginn abgeben würden (EK-No.: 30-091 ex 17/18). Diese Proben wurden ins Labor der Abteilung für Rheumatologie gebracht, weiterverarbeitet und Schnitte davon immunhistochemisch doppel- und einfachgefärbt. Mögliche Einflussfaktoren (Unterform der systemischen Sklerodermie, zusätzliche immunsuppressive Therapie) wurden erfasst. Die Einzelfärbungen werden noch per Computer eingescannt und zur Interpretation übereinandergelegt werden.

Ergebnisse: 7 PatientInnen willigten ein an der Studie teilzunehmen. Durch die Überlappung des Oberflächen- (CD4) und zytoplasmatischen (IL-17) Markers, war eine objektivierbare und reproduzierbare Auswertung der Th17 Zellen in der Doppelfärbung nicht möglich. In der anschließenden Einzelfärbung der jeweiligen Marker, lies sich in der CD4-Färbung eine erhöhte Anzahl von T Helferzellen im Vergleich zur „gesunden“ Haut feststellen. Die IL-17 Färbung wird bezüglich der T Zellen erst in Kombination mit der

CD4-Färbung am Computer auswertbar sein. Als Nebenbefund ließen sich jedoch bereits IL-17 positive Mastzellen darstellen.

Diskussion: Trotz großer technischer Herausforderungen wurde die immunhistochemische Färbemethode etabliert. Wir konnten IL-17+ Mastzellen nachweisen, die dafürsprechen, dass die Einzelfärbung funktioniert und bald auch interessante Ergebnisse bezüglich der Th17 Zellen folgen werden. Die Verzögerung des ursprünglichen Zeitplanes ließ eine Interpretation im Rahmen dieser Diplomarbeit leider nicht mehr zu. Auf zukünftige Publikationen sei demnach hier verwiesen. Zusätzlich ist zu erwähnen, dass die angefärbten Mastzellen mit dieser Methodik sehr schön darstellbar sind und ein anschließendes Projekt mit Schwerpunkt auf diesen Zellen unter RTX-Therapie sowie mögliche Veränderungen des IL-17-Spiegels von Interesse wären.

Abstract

Objective: The aim of this thesis was to establish a staining technique in order to evaluate the amount of Th17 cells in the skin of patients suffering from Systemic Sclerosis before and during long time CD20 – depletion therapy with Rituximab and compare the results to healthy controls.

Introduction: Systemic Sclerosis is a rare, however, devastating connective tissue disease, which untreated, can lead to an involvement of the inner organs (lung, heart) and death. As the immune system plays an important part in the pathogenesis of the disease by overreacting and stimulating excessive fibrosis, immunomodulatory therapeutics such as Rituximab are being used and show promising improvement of the clinical state of the patients. It is discussed in the literature that also the Th17 cells of the immune system contribute to the disease and therefore we decided to take a closer (microscopical) look at the skin lesions before and during the treatment with Rituximab regarding the presence of these cells.

Material and Methods: The technique to perform a double immunohistochemistry on paraffin embedded skin sections for Th17 cells was established. Patients, having already provided a skin sample before the initiation of the CD20 – depletion treatment in the study (EK. number 24-184 ex 11/12), were asked to give another sample at the earliest one year after the first biopsy (EK-No.: 30-091 ex 17/18). The specimens were further processed in the laboratory of the department of Rheumatology and immunohistochemically double and single stained. Possible influencing factors (type of Systemic Sclerosis, additional immunosuppressive therapy) were assessed. The single stained specimens are going to be scanned and overlaid on the computer to find the double stained Th17 cells.

Results: 7 patients participated in this pilot study. Due to the overlay of the chromogens on the surface (CD4) and cytoplasmic (IL-17) working antibodies, it was not possible to reliably and objectively interpret the results of the double staining immunohistochemistry. The single staining of the CD4+ cells showed that the amount of the T helper cells is increased in SSc patients compared to healthy controls. Regarding a statement concerning the Th17 subgroup, the computer scans need to be awaited and interpreted. However, as an additional finding we noticed IL7+ mast cells.

Discussion: Despite great challenges, the immunohistochemical staining method was established. We found IL-17 expressing mast cells, which proves that the single staining worked and it is only a matter of time until interesting results regarding the Th17 cells will follow. However, the delay in schedule prevented possible findings from being presented in this thesis. They will be discussed in a separate scientific work. Additionally, it should be mentioned that it might be of interest to investigate the stained mast cells regarding their development during RTX-treatment in combination with the IL-17-levels.

Contents

Glossary and Abbreviations

Ab	antibody
ACA	Anti-centromere antibody
ACE	angiotensin converting enzyme
ACR	American College of Rheumatology
α-SMA	alpha smooth muscle actin
AMP	antimicrobial peptide
APAAP	Alkaline Phosphatase - Anti-Alkaline Phosphatase
APC	antigen presenting cell
AS	activity score
AT II	angiotensin II
AZA	azathioprine
BAFF	B cell activating factor
BHPR	British Health Professionals in Rheumatology
Breg cell	regulatory B cell
BSR	British Society of Rheumatology
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CLL	chronic lymphocytic leukaemia
CTGF	connective tissue growth factor
CYC	cyclophosphamide
dcSSc	diffuse cutaneous Systemic Sclerosis
D. n. c.	Data not collected
DM	dermatomyositis
ECM	extra cellular matrix
e. g.	exempli gratia
EScSG	European Scleroderma Study Group
EULAR	European League against Rheumatism
EUSTAR	European Scleroderma Trials and Research group
g	gram
GAG	glycosaminoglycan
GERD	gastroesophageal reflux disease
GI	gastrointestinal

GPA	granulomatosis with polyangiitis
h	hour
H2	histamine H2
HRP	horseradish peroxidase
HSCT	haematopoietic stem cell transplantation
ICAM-1	Intercellular Adhesion Molecule 1
i. e.	id est
Ig	Immunoglobulin
ICH	immunohistochemistry
IL	interleukin
ILD	interstitial lung disease
INF-γ	interferon gamma
IRR	infusion related reactions
IV	intravenous
KC	Keratinocyte chemoattractant
lcSSc	limited cutaneous Systemic Sclerosis
MCP	metacarpophalangeal joint; monocyte chemoattractant
Proteins	
MCTC cells	tryptase+ and chymase+ mast cells
mg	Milligramm
min	minutes
MIP	Macrophage Inflammatory Protein
ml	millilitre
mm	millimetre
µL	microlitre
µm	micrometre
MMF	mycophenolate mofetil
MMP	matrix metalloproteinase
MPA	microscopic polyangiitis
mRSS	modified Rodnan Skin Score
MTX	methotrexate
NHL	Non Hodgkin’s Lymphoma
NKC	natural killer cell
PAH	pulmonary arterial hypertension

PAP	peroxidase-antiperoxidase
PBS	Phosphate-buffered saline
PDE-5	phosphodiesterase type 5
PDGFR	platelet derived growth factor receptor
pg	picogram
PIP	proximal interphalangeal joint
PM	polymyositis
p.o.	per os
RA	rheumatoid arthritis
RNAP-III	RNA polymerase III
RNP	ribonucleoprotein
RP	Raynaud's Phenomenon
RTX	Rituximab
sFLC	serum free light chains of immunoglobulins
SLE	systemic lupus erythematosus
SMR	standardized mortality ratio
SRC	scleroderma renal crisis
SSc	Systemic Sclerosis
TBS	tris buffered saline
TGFβ1	Transforming growth factor beta 1
Th cell	T helper cell
TLCO	carbon monoxide transfer factor
TNFα	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
Treg cell	regulatory T cell

List of Figures

List of Tables

Introduction

1.1 Systemic sclerosis

Systemic sclerosis is a connective tissue disease of unknown origin which is defined by three abnormalities occurring in the human body: vascular anomalies leading to tissue ischemia, altered B- and T-lymphocyte function causing a faulty immune response and dysfunctional fibroblasts leading to a stronger deposition of extracellular matrix. (1)
The incidence rate is 1,8 new cases per 100.000 and year. (2) The prevalence varies geographically with a higher presence in the USA and Australia compared to Europe and Japan. Women are more frequently affected than men and blacks more than whites. (3) The average age of diagnosis is 46 years, however, it can also effect children and the elderly (4).
Depending on the involvement of the skin, the autoantibody association and the affected inner organs, two main forms can be distinguished:

Diffuse cutaneous systemic sclerosis (dcSSc)
strong and early skin involvement of the hands, forearms, upper arms, shoulder girdle and belly region combined with an early onset of progressive interstitial lung disease, frequent oesophageal dysmotility, small and large intestinal involvement, renal crisis, Anti-Scl-70- and Anti-RNA polymerase antibody, tendon friction rub

Limited cutaneous systemic sclerosis (lcSSc)
late peripheral skin involvement of the hands, forearms and face and in the course of the disease occurrence of isolated pulmonary arterial hypertension in 30% of the cases, telangiectasia and calcinosis, anticentromere antibodies (2,5)

Objective complications of the heart (conduction block, diastolic dysfunction and left ventricle ejection failure) occur with a similar frequency in both forms (6).
Because of the clinical heterogeneity of the disease, the American College of Rheumatology and the European League Against Rheumatism revised the classification criteria from 1980 to develop a new classification shown in Table 1. Patients with a total score of ≥ 9 are classified with the diagnosis SSc, whereas only the maximum score of each category gets added. (7)
Muriel Elhai et al. calculated the standardized mortality ratio (SMR) of the disease. This is the ratio between the observed number of deaths in a cohort of patients suffering from

systemic sclerosis to the expected amount of deaths of a comparable age- and sex-matched population and came to the result of 3.5. (8)

Elhai et al. compared the causes of death of all French patients with SSc to the international EUSTAR sample and combined the numbers to a report. Almost one third of the deaths resulted from cardiac complications, 17% were due to respiratory involvement, 9 – 11 % were infectious, malignancies - in particular lung cancer - contributed to 9 - 13%, atherosclerosis to 5-8%, gastro-intestinal complications to 4 – 6.6% and renal complications were responsible for 3 % of the deaths (9).

Table 1: American College of Rheumatology/ EULAR criteria for the classification of Systemic Sclerosis (7)

Item	Sub-item(s)	Score
Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints	-	9
Skin thickening of the fingers	Puffy fingers	2
	Sclerodactyly of the fingers (distal to the MCP-joints but proximal to the PIP-joints)	4
Fingertip lesions	Digital tip ulcers	2
	Fingertip pitting scars	3
Telangiectasia	-	2
Abnormal nailfold capillaries	-	2
Pulmonary arterial hypertension and/ or interstitial lung disease	Pulmonary arterial hypertension	2
	Interstitial lung disease	2
Raynaud's phenomenon	-	3
SSc-related autoantibodies (anticentromere, Anti-Scl-70, anti-RNA polymerase III)	Anticentromere	3
	Anti-topoisomerase [Anti-SSc-70]	
	Anti-RNA polymerase III	

1.1.1 Therapeutic guidelines

The clinical complexity of the disease and its complications are challenging aspects that make the treatment very difficult. Therefore, clinical experts from the European League against Rheumatism (EULAR) and the British Society of Rheumatology (BSR) and British Health Professionals in Rheumatology (BHPR) tried to develop treatment guidelines to improve the care of patients with SSc.

The BSR and BHPR divided the treatment into a general approach to SSc management, key therapy and organ-based therapy:

General approach

Just like the EULAR, they recommend methotrexate (MTX) and cyclophosphamide (CYC) for therapy in the early diffuse SSc. The evidence base for mycophenolate mofetil (MMF) or azathioprine (AZA) is weak. The BSR and BHPR also name Rituximab (RTX) as a possible treatment for skin involvement. (10,11)

Key therapy and organ-based therapy

Raynaud's Phenomenon

In case of Raynaud's-Phenomenon dihydropyridine-type calcium antagonists such as nifedipine and PDE-5 inhibitors were found to reduce the frequency and severity of attacks and are therefore highly recommended (12–14). Furthermore the Britons also name AT II-receptor antagonists as first line treatment (10). Regarding the effect of iloprost and cisaprost on RP, Pope et al. could not demonstrate an impressive efficacy whereas iloprost is highly recommended in the therapy of digital ulcers (15–17).

Digital ulcera

In addition PDE-5 inhibitors should also be used in the healing process of digital ulcers (18). Bosentan can reduce the number of new ulcers during the treatment, however it has no effect on the healing of existing ulcers (19,20).

Pulmonary arterial hypertension (PAH)

In case of PAH Endothelin receptor antagonists like Bosentan, Ambrisentan and Macitentan, PDE-5 inhibitors like Sildenafil or Tadalafil, Riociguat, a soluble guanylate cyclase stimulator and prostacyclin analogues like Epoprostenol should be used (21–24).

Scleroderma related interstitial lung disease (ILD)

In case of interstitial lung disease (especially in progressive ILD), the EULAR recommends cyclophosphamide (CYC) as a therapy option, despite its toxicity. Haematopoietic stem cell transplantation (HSCT) can, next to the improvement of skin involvement, stabilize the lung function and its possible benefit and side effects should be weighed in patients with rapidly progressive SSc at risk of organ failure. The therapeutical effect of HSCT on the skin score and lung volumes can be superior to CYC treatment. The treatment-related mortality is elevated in the first year after HSCT compared to CYC,

whereas the long-term event-free survival and overall survival are significantly improved. (11)

Scleroderma renal crisis (SRC)

In the event of Scleroderma renal crisis, experts of the EULAR recommend ACE inhibitors (e. g. Captopril, Enalapril) in high doses. However, no preventive use to decrease the risk of developing SRC, as there is no supporting literature. (11) Because of the associated severe hypertension, the blood pressure should be monitored at least once a week (10). High dose Steroid therapy seems to contain a higher risk for SRC (25,26).

Scleroderma related GI-diseases

To prevent SSc-related GI reflux and oesophageal ulcers and strictures, it is recommended to use proton pump inhibitors and H2 receptor antagonists. In the event of symptomatic motility disturbances such as dysphagia, GERD, etc. prokinetic drugs (e. g. dopamine antagonists, short-time usage of Cisapride - side effect: QT syndrome) should be considered. Oral broad-spectrum antibiotics (e. g. quinolones, amoxicillin-clavulanic acid, metronidazole) can be used in case of symptomatic small intestine bacterial overgrowth. (10,11)

Scleroderma related cardiac disease

In case of systolic heart failure, the BSR and BHPR propose to consider immunosuppression with or without a pacemaker, an implantable cardioverter defibrillator, ACE inhibitors and carvedilol.

A diastolic heart failure with preserved left ventricular ejection fraction may be treated with diuretics like spironolactone and furosemide or calcium channel blockers. (10)

Rituximab (RTX)

In our project we study the effect of Rituximab on the lymphocellular infiltrate in the skin of SSc-patients. This drug is a chimeric immunoglobulin G1 (IgG1) monoclonal antibody that binds to the CD20 antigen (27). The antigen is expressed by B cell precursors and mature B cells. It then gets lost during the further differentiation into plasma cells. (28) The monoclonal antibody eliminates circulating B cells in the peripheral blood and the bone marrow at least for 3 months (29–31). Whereas the number of B cells in disease tissue gets depleted, however not eliminated (32).

Wilk et al. found out that about 1.6% of all T cells also co-express CD20 and can therefore be depleted by Rituximab (33).

Furthermore the T cell subset Th17 also partially co-expresses CD20 and can be inhibited by the anti-CD20 antibody (34,35).

At present, Rituximab is indicated in the therapy of Non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukaemia (CLL), rheumatoid arthritis (RA), granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) (36).

However, it is already found in the literature, that Rituximab can be also used as an effective off-label therapeutic in Systemic Sclerosis, especially with a progressive involvement of the lung. More precisely, Moazedi-Fuerst et al. and others were able to show that long-term RTX-therapy (2 years) in SSc patients significantly improves lung function and reduces skin fibrosis (37–39). In addition, it seems to reverse calcinosis cutis (40).

Regarding the side effects, the German Registry of Autoimmune Diseases evaluated in 2011 the safety and clinical outcomes of Rituximab in several autoimmune diseases apart from RA and NHL. Of the 370 patients with autoimmune diseases, 86% received more than 1 infusion with Rituximab. The majority received 2 (39.2%) and 4 (34.1%) infusions. 13.5% received only one infusion. Most patients (77.6%) had one course of RTX, 18.6% received two courses. The mean dose of 2,440 mg/ patient was received over a median period of 194 days. The overall rate of infections during RTX-therapy was 18.1 per 100 patient years. 3.8% of these infections were mild, 3.2% moderate and 3.7% severe infections. The majority of the infections with clinical relevance – mostly bacterial - took place within 7 months after the first infusion with Rituximab. In the course of the Rituximab-therapy, there occurred 5.9% infusion related reactions (IRR), 4.1% allergic reactions, 2.4% severe infusion related reactions which lead to a discontinuation of the treatment and 5.7% undifferentiated IRRs. 3% of the patients died after RTX-therapy (mean 11.6 months after first infusion) mainly due to infections. The majority of the patients, that died, were in an uncontrolled and highly active stage of their disease and received Rituximab as a final option during intensive care therapy. (41)

1.1.2 Immunopathogenesis

B Lymphocytes

B cells are part of the adaptive immune system in which they function not only as executors but also as powerful regulators. They produce antibodies, present antigens to T

cells and secrete cytokines. These cytokines determine whether the subset is a "regulatory" or "effector" subset. Furthermore, B cells are able to organize the structure of lymphoid tissue and play a role in the regulation of lymphangiogenesis. (42)

Naïve B cells get activated by antigens and helper T cells and differentiate into antibody-secreting cells (=plasmablasts[1], plasma cells[2]) and into long-lived memory cells.

In patients with Systemic Sclerosis B cells are hyperactivated and seem to contribute strongly to the pathogenesis of the disease (43). Sato et al. found out that the absolute amount of CD19+ B cells in the blood from SSc patients is about 30 % higher than in healthy controls. Naive B cells are increased, whereas the number of memory B cells and plasmablasts is reduced. These memory B cells express activation markers (e. g. CD80, CD86, CD95) more strongly. (44) Lanteri et al. assessed serum free light chains of immunoglobulins (sFLC) in the blood of SSc patients. These levels, which are a way to measure B cell activation, were significantly elevated and correlated with modified Rodnan skin score[3]. (45)

Immunohistochemical proof of B cells in the skin of SSc patients bears some difficulty, however gene expression in skin biopsies associated with B-lymphocytes is prominent, indicating B cell presence (46).

Also, the increased number of autoantibodies in the serum of SSc-patients suggests a further involvement of B lymphocytes in the pathogenesis of this connective tissue disease. Specific autoantibodies to Scleroderma are the Anti-centromere antibodies (ACAs) (in approx. 20 – 42% of patients), Anti-topoisomerase I (Scl70) antibodies (in 14 – 42%) and Anti-RNA polymerase III (RNAP-III) antibodies (in 16 – 20%). Furthermore there can occur Anti-Th/To (in 2 – 5%), Anti-U3-RNP antibodies (in 18.5% in African Americans) and Anti-U11/U12 RNP antibodies (in 3.2% of patients suffering from Systemic Sclerosis who had no other SSc-associated autoantibodies).[4] (47) Stimulatory autoantibodies against platelet derived growth factor receptor (PDGFR) are also present in the blood of SSc patients. These antibodies lead to type I collagen–gene expression and fibroblast activation. (48)

It has been proven that B lymphocytes are a source of IL-6 and TGFβ1 and can also induce collagen production by direct cell-cell contact with SSc dermal fibroblasts (49).

[1] secrete already antibodies, however, are still dividing and interacting with T cells over antigen-presentation on the surface

[2] Secrete antibodies at a higher rate, but are unable to respond to antigens

[3] used as a primary or secondary outcome measure of skin thickness in SSc (127); for further information see chapter 2.4.1

[4] quoted figures refer to North America

However, not all B cells enhance immune responses in inflammation or autoimmunity (50). Wang et al. proved that regulatory B cells are capable of suppressing Th17 and Th1 cells and promote T_{reg} expression (51). Furthermore, a certain subset of B_{regs}, the B10 cells, which produce IL-10, are functionally impaired and numerically decreased in patients with Systemic Sclerosis (52). The reduced level of IL-10 producing regulatory B cells negatively correlates with the autoantibody titer (anti-topo I Ab, anticentromere Ab) and the disease activity of SSc (53). IL-10 is also produced by T_{reg} cells and suppresses the expression of MHC- and co-stimulatory molecules by antigen presenting cells (APCs). In addition, it inhibits the cytokine production (e. g. IL-12 and IL-23) by APCs which leads to an impairment of their promoting effect on the differentiation and maintenance of Th1 and Th17 cells. (54)

T Lymphocytes

Prescott et al. examined the skin biopsies of patients with systemic sclerosis to evaluate the different stages in change in the dermal microvasculature and were able to show that a perivascular lymphocytic cell infiltrate precedes the fibrotic changes (55). This inflammatory infiltrate in the skin is rich in T lymphocytes, macrophages and mast cells, however contains no B cells (55–57).

Out of these subgroups of immune cells, that seem to contribute to the inflammation in the skin lesions, we are especially interested in the T lymphocytes, which are – just like the B lymphocytes - part of the adaptive immune system. Before having encountered their specific antigen, they are called naïve T cells and circulate through the bloodstream and the lymphatics. As soon as they have met the antigen specific to them, they proliferate and differentiate into effector T cells, which carry out their functions on the target cells without further differentiation.

These effector T cells can be subdivided into CD8+ cytotoxic T cells and CD4+ helper T cells. The cytotoxic T cells are responsible for the elimination of cells infected with intracellular pathogens. They release granzymes, perforin and granulysin which are antimicrobial and lead to the apoptosis of the infected target cell. The CD4+ helper T cells orchestrate different immune functions. They can further differentiate into specialized subsets called Th1, Th2, Th17 and $Th_{FH,}$ which can each activate distinct arms of the immune system:

- Th1: produce cytokines that activate macrophages
- Th2: produce cytokines that recruit and activate eosinophils, mast cells and basophils, promote enhanced barrier immunity at mucosal surfaces to eradicate helminths
- Th17: produce IL-17 family cytokines that lead in a further process to the recruitment of neutrophils to the site of infection
- $T_{FH:}$ promote germinal center response in B-cell follicles

In addition, about 5 – 10 % of the circulating CD4+ T cells are regulatory T cells ($T_{regs),}$ which help to limit and suppress excessive immune responses. (54)
In patients suffering from Systemic Sclerosis especially the CD4+ memory T cell phenotype of the T lymphocytes is elevated (58). For more than a decade now it has been known that the Th2 subgroup, which produces IL-4, a profibrotic Cytokine, is activated in SSc (59).
Within the last couple of years another subgroup of T helper cells - the Th17 subgroup - and its produced Interleukin-17 have received a lot of attention regarding autoimmunity. Undertaken studies with Scleroderma patients show contradictory results, some claiming higher levels of IL-17 in SSc patients, some indifferent and some even lower levels compared to healthy controls. (58,60–63)

Th17 cells and IL-17

In the present state of knowledge, the IL-17-cytokine-family consists of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F (64). Th 17 cells produce IL-17A and IL-17F (54).
This subgroup of T helper cells arises when IL-6 and TGFβ1 predominate during naive CD4+ T cell activation, whereas its expansion and survival is influenced by IL-23 (47,48). Olewicz-Gawlik et al. found decreased IL-23 levels in the sera of SSc patients (63). This contrasts with a study carried out by Komura et al., in which the IL-23 levels in the sera of 27 % of lcSSc and dcSSc patients were significantly elevated (cut-off value: 1.20 pg/ml) compared to healthy controls. Furthermore, the affected patients were suffering from pulmonary fibrosis (71 %) more often than patients without increased IL-23 levels (32 %). (67) The difference in the outcome of these studies might be due to the stronger immunosuppressive therapy in the study of Olewicz-Gawlik et al (63). However not only the IL-23 levels seem to be elevated in Systemic Sclerosis, Radstake et al. found an enhanced expression of the IL-23 receptor (IL-23R) on CD3+, CD45Ro+ (memory) and

CD45Ra+ (naïve) cells (68). The effect of IL-23 on the production of IL-17 is reinforced by IL-1α and IL-1β (69). IL-1α serum levels of SSc patients are also highly elevated in comparison to controls (68). Additionally, IL-1α is expressed intracellularly in all affected fibroblasts in SSc skin, which leads to an increased IL-6 and type I - collagen production and proliferation of these fibroblasts (70).

Serum IL-6 levels are increased in patients with Systemic Sclerosis and while this interleukin favours the development of Th2 and Th17 cells, its presence suppresses regulatory T cells, which are important for peripheral tolerance and the inhibition of autoimmunity (58,71,72).

Furthermore, Wei et al. discovered that IL-21 is also involved in the differentiation and generation of Th17 cells in mice and is secreted as an autocrine factor by the Th 17 cells (73). The IL-21 receptors are up-regulated in the keratinocytes of patients suffering from SSc, however, just slightly expressed in the fibroblasts and vascular endothelial cells (74). Zhou et al. found also out that the IL-21 and IL-21R levels in the patients correlate with IL-4 mRNA, enabling Th2 stimulation and consecutive fibrosis (74,75). Kuchen et al. demonstrated that in the CD4+ T cell mediated axis, IL-21 is essential for the activation and further differentiation of B cells into plasma cells (76). In addition, this cytokine collaborates with IL-15 to stimulate the generation and further expansion of cytotoxic T cells (77). Last but not least IL-21 effects the development and function of natural killer cells in different ways, depending on the dosage (78).

IL-17 is the pivotal cytokine produced by Th 17 cells and plays an important role in inflammation. Synergizing with IL-17F and IL-22, IL-17A enhances the expression of antimicrobial peptides (AMPs) to regulate the immune response against extracellular pathogens and seems to regulate local tissue inflammation (79). It induces the expression of other proinflammatory cytokines (e. g. IL-6, TNFα) and chemokines (e. g. KC, MCP-1, MIP-2, matrix metalloprotease) (58,80). IL-17F is able to stimulate endothelial cells to produce TGF-β. (58) Furthermore, IL-17 provokes epithelial, endothelial cells and fibroblasts to secrete cytokines (e. g. IL-8, granulocyte-colony-stimulating factor and prostaglandin E2), enhances the maturation of dendritic cells and takes part in the co-stimulation of T cells (61,81).

In addition, IL-17 has an important chemoattractant effect on neutrophils, which play a crucial role in pulmonary involvement in SSc (58).

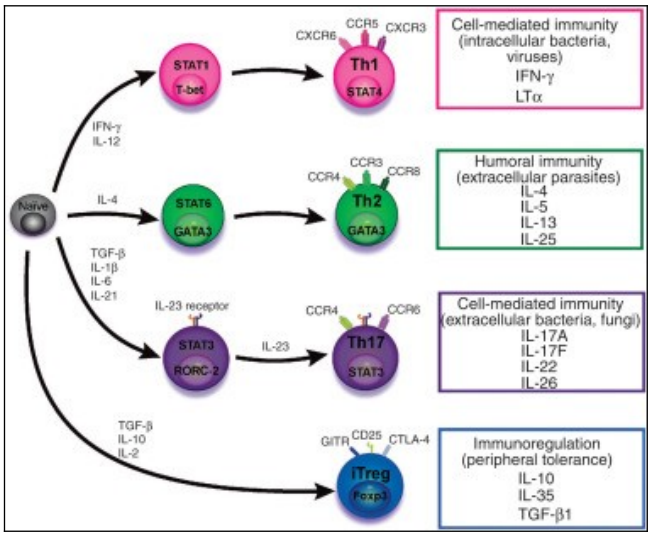

Figure 1: Model of the differentiation from naive T cells to the different types of helper T cells or regulatory T cells (82)**.** Illustration used with the permission of Elsevier and Copyright Clearance Center. (license number: 4372410850128)

Nakashima et al. found a significantly increased IL-17A expression in the skin and sera of SSc patients, however, no elevation of the IL-17F levels. Interestingly they showed that IL-17A reduces the protein expression of α1 collagen and connective tissue growth factor (CTGF), which implicates that this interleukin has also an antifibrotic regulatory function. (83)

Truchetet et al. confirmed the inhibitory effect of IL-17A on fibrosis. They showed an inhibition of α-SMA synthesis in both SSc and healthy dermal fibroblasts. (84) Furthermore, they found an increase in matrix metalloproteinase-1 (MMP-1) protein production in the fibroblasts of SSc patients and healthy controls, which cleaves collagens located in the extra cellular matrix (84,85).

In a consecutive study from Brembilla, Truchetet et al., they propagated the IL-17A concept of a triggered proinflammatory fibroblast response and simultaneously antifibrotic effect on the ECM turnover. The limiting effect on collagen synthesis seems to partially depend on a collaboration of IL-17, TNF and INF-γ. (86)

Regardless of their true effector function, the interleukin 17 - ligands need certain receptors to bind and induce further processes. At present, this interleukin 17 receptor family is made up of 5 members: IL-17RA (also called IL-17R), RB, RC, RD and RE (87). The two IL-17 cytokines produced by Th 17 cells – IL-17A and IL-17F, which are 50 % identical to one another – both bind to the IL-17RA and IL-17RC complex (87,88).

Yao et al. investigated the cellular and tissue distribution of IL-17R mRNA and concluded that the receptor is expressed ubiquitously in all cell lines (including fibroblasts, muscle cells, mast cells, splenic B cells and counting) (89). DcSSc fibroblasts show higher IL-

17RA mRNA relative levels compared to healthy controls and lcSSc, regarding the relative levels of IL-17RC mRNA, there was no difference found (86). However, they concluded just like Nakashima et al. in their study in 2012, that the expression of the IL17-RA in SSc fibroblasts is decreased in immunohistochemical investigation and Western blotting (74,83). The mRNA levels of IL-17RA do not correlate with the severity of the skin lesions (74).

The signalling of IL-17A can be suppressed through receptor-downregulation in SSc fibroblasts by TGFβ1 (83).

It is important to know that, regarding the general IL-17 levels, Th17 cells are not the only source. Happel et al. showed that also CD8+ T cells are capable of IL-17 production (90). The type ILC3 of the innate lymphoid cells also produce IL-17A like the TH17 cells (91). In a mouse model, when stimulated with IL-1β and IL-23, γδ T cells produced IL-17 (92). In addition, natural killer cells (NKC) and mast cells have also been identified as a source of this interleukin (93–95). De Boer et al also found IL-17 A/F+ neutrophils in complicated human atherosclerotic plaques (96). Last but not least, Alves de Lima Silva et al. conducted a study on chromoblastomycosis lesions and concluded that also Langerhans cells express IL-17A (97).

Concerning the IL-17A levels in SSc patients, NKC and γδ T cells don't seem to be the important source due to their decreased number in the blood of the patients (98). The number of mast cells, on the other hand is elevated in the early induration phase of progressive systemic sclerosis, however, not in the later stages of the disease. (99) Furthermore, mast cells not only produce IL-17, they can also enhance generation of Th17 cells (90).

The exact origin of the elevated IL-17 levels in SSc-patients is still not known very well. Just like Truchetet, our goal was to find out, how present the Th 17 cells are in the skin lesions in this certain disease and whether other IL-17A+ cell types are also involved.

To complete the Th17 cell picture, it is necessary to know that they – apart from IL-17 - also produce IL-22 and IL-26 (79,102). In host defence IL-22 is responsible for the enhancement of antimicrobial peptide expression. (79) The response of fibroblasts to TNF is IL-22 dependent and the favour of TNF-induced keratinocyte activation leads to an increase in proinflammatory fibroblast phenotypes (103). Mathian et al. found significantly higher IL-22 transcription levels in the skin of SSc patients than in healthy controls (104).

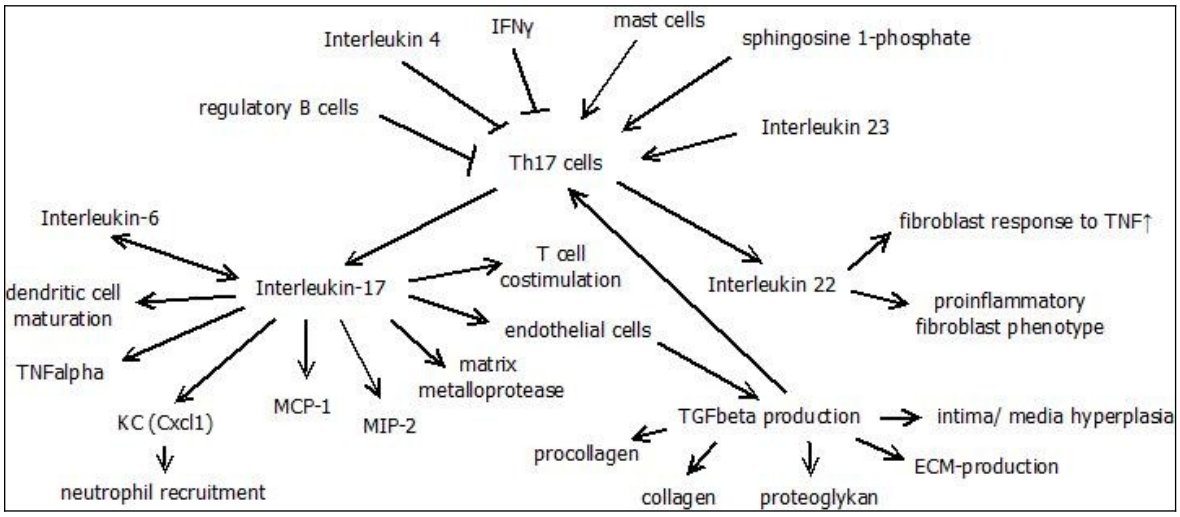

Figure 2: Overview Th17 cell modulation and pathways.

Fibrosis activation in systemic sclerosis

TGFβ

An excessive TGFβ-activity leads to an inordinate tissue fibrosis, which when induced in a mouse model, reassembles human SSc (105). Therefore, it is compelling to assume a link between aberrant TGFβ-signalling and pathologic fibrosis in Systemic Sclerosis.

As mentioned above, TGFβ is produced by B cells (49). However, the B cells are not the only cells capable of TGFβ-production, also many parenchymal cells, monocytes, macrophages and platelets are potential sources (106). Epithelial cells produce this cytokine when stimulated with IL-17F (58).

TGFβ 1 promotes extracellular matrix production, intimal and medial hyperplasia and reinforces procollagen, collagen and proteoglycan synthesis in the neointima (107).

In addition, TGFβ induces connective tissue growth factor (CTGF) and the serum levels of CTGFs are significantly elevated in patients with SSc compared to systemic lupus erythematosus (SLE) or polymyositis (PM) / dermatomyositis (DM). Within the SSc-subsets the levels were significantly higher in dcSSc than in lcSSc and the levels correlated with the severity of pulmonary fibrosis and the extent of skin sclerosis. (108,109)

Francois et al. found out that the promotion of fibrosis induced by B cells and BAFF in dermal fibroblasts isolated from patients with SSc is dependent on the presence of TGFβ1 suggesting a key mediatory role in B cell induced collagen overproduction (49).

TGFβ can also induce the release of MCP-3 in murine fibroblasts (110).

The expression of TGFβ- and IFN-regulated genes is up-regulated in patients with SSc (111).

TNFα

TNFα levels are elevated in the blood of patients suffering from Systemic Sclerosis (112). Additionally, also in the affected skin the lymphocytic infiltrate and the fibroblasts express a great amount of TNFα protein in the early stage of the disease (113). The synthesis of TNFα is inter alia induced by IL-17 (58). TNFα executes its effect through two receptors: TNF receptor I (TNFR-I), which is expressed on a wide range of cells, such as macrophages or endothelial cells, and TNF receptor II (TNFR-II), which is preponderantly expressed by lymphocytes (54). Hügle et al. showed that the expression of TNFR-I and TNFR-II is up-regulated on dermal T cells in dcSSc. These T cells, when activated, produced more IL-6 and Il-13 compared to the healthy control group, whereas their capability to produce IL-10 weakened. This led to an increase of type I collagen production in SSc fibroblasts. They additionally claimed that the expression of TNFR-II correlates with the skin thickening of the patients. (114) Gruschwitz et al. also proved that recombinant TNFα induces the transcription of ICAM-1[5] in SSc skin and normal skin fibroblasts (54,113).

B cells

In addition to the profibrotic TGFβ, B cells also produce IL-6, which enhances the collagen and glycosaminoglycan production in fibroblasts (49,115). CCL2 secretion is also significantly increased in the coculture of B cells and fibroblasts. This chemokine is part of the CC chemokine group and its main responsibility is to activate macrophages and basophil histamine release and to promote Th2 immunity. (54) The presence of B cell activating factor (BAFF) and anti-IgM enhances the release of these two profibrotic cytokines. Furthermore, fibroblasts secrete an increased amount of collagen when put in direct cell-cell contact with B cells and their cell membrane factors. (49)

IL-4

The activated Th2 cells in patients with Systemic Sclerosis secrete large amounts of IL-4 and the plasma protein levels are highly elevated. However, it can also be produced by natural killer T cells, mast cells, eosinophils, basophils and innate-type lymphoid cells (116). This interleukin promotes the production of TGF-β, collagen, fibronectin, glycosaminoglycan, and proteoglycan synthesis in vitro (59). Furthermore, fibroblast-proliferation and their differentiation into myofibroblasts is stimulated by IL-4 (117).

[5] important adhesion molecule for the binding of leukocytes to endothelial cells and thus enabling further migration of the cells into the tissue

IL-6

As mentioned above, IL-6 stimulates dermal fibroblasts to produce increased amounts of collagen and glycosaminoglycan (GAG). However, without elevating the levels of fibronectin or the general protein synthesis. (115) Sato et al. showed that serum levels of IL-6 are significantly higher in patients suffering from diffuse Systemic Sclerosis compared with those in patients with limited Systemic Sclerosis and healthy controls. In addition, the serum levels positively correlated with modified Rodnan Skin Score. (118)

IL-17

The effects of IL-17 have already been discussed above. (see page 8 and following)

Monocyte chemoattractant proteins (MCPs)

MCP-1 and MCP-3 are linked to the pathogenesis of SSc. They are inducible, pro-inflammatory chemokines and chemotactic for monocytes, eosinophils and basophils.

In the skin of SSc patients, MCP-1 is not only expressed by mononuclear cells, fibroblasts, endothelial cells and keratinocytes, but also abundantly on the mRNA and protein level. Furthermore, the serum levels of MCP-1 are elevated. High serum levels might correlate with an increased risk of major organ involvement.

CCR2 is the receptor for MCP-1 and its activation might play an important role for the migration of fibrocytes into affected tissues in fibrotic diseases. Moore et al. showed that murine fibrocytes isolated from fibrotic lungs express CCR2 and migrate towards the CCR2 ligands MCP-1 and -5 in vitro.

CCR2-positive fibroblasts express α-smooth muscle actin, indicating that it might be restricted to myofibroblasts to express this cytokine. In addition, myofibroblasts also overexpress MCP-1, which suggests an autocrine activation loop.

MCP-1 is also capable of regulating the expression of matrix-degrading enzymes and their inhibitors.

Furthermore, MCP-1 stimulates a Th2 differentiation in Th0 cells.

MCP-3 binds to CCR1, CCR2 and CCR3. It is released by dermal fibroblasts and has a chemoattractant effect on dendritic cells, granulocytes, monocytes, NK cells and lymphocytes. In SSc it is expressed by mononuclear cells, dermal fibroblasts and basal keratinocytes. (119)

1.2 Histological structure of the skin

Due to the fact, that we investigated the cells in skin biopsies, it is also important to get to know the general structure of human skin tissue.

The skin can be divided in two main layers called the epidermis and dermis (120). Underneath lies the subcutis (121). The superficial epidermis is made of a stratified squamous epithelium consisting mainly of keratinocytes in different stages of differentiation. When the differentiation is complete, they built a thin layer of keratinized cells. (120,121) The different layers of the epidermis formed by the different stages are called:

- Stratum basale

  Consists of a few rows of columnar cells in contact with the basal membrane via hemidesmosomes. The majority of the mitoses is occurring in this layer.

- Stratum spinosum

  Consists of a couple of rows of polygonal cells which flatten towards the surface. They keep in contact with each other via desmosomes.

- Stratum granulosum

  Consists of one or more rows of flat cells, containing granules filled with keratohyalin (only visible in the electron microscope).

- Stratum corneum

  Consists of polygonal, platelet-like cells without a nucleus, which desquamate on the surface.

Furthermore, melanocytes, Merkel cells and Langerhans cells are found in the Epidermis. The dermis is made of a papillary and a reticular layer:

- Papillary layer

  The narrow papillary layer is rich in cells and capillaries and consists of loose connective tissue. The papillae contain Meissner's corpuscles.

- Reticular layer

  The reticular layer is a taut, irregular connective tissue poor in cells. (121)

Skin appendages such as sweat glands and hair follicles are located in the dermis. The elongated, excretory ductal portions of the sweat glands continue to the surface, whereas the more circular secretory portions in the deeper layers occur. (120)

Thesis Background

This thesis is part of the project "Characterization of the lymphocellular infiltrate in the skin of SSc patients before and after treatment with B cell depletion therapy" approved by the ethical review committee of the Medical University of Graz (EK-No.: 30-091 ex 17/18).

At our department of rheumatology, we have been applying the "Grazer schema" – consisting of 500 mg MabThera® (=Rituximab) IV at day 0 and 14 and after that every 3 months, optionally combined with mycophenolate mofetil 500 mg 1-0-1 p. o. - as a therapy option since 2010. So far, we have been able to report a clinical remission (decrease in modified Rodnan Skin Score and an increase in lung function) in the patients treated with Rituximab (37). The positive effect of the treatment was independently confirmed by others (122–124). Lafyatis et al. however, recruited 15 patients suffering from dcSSc and that had received two intravenous doses of 1000 mg Rituximab two weeks apart and could not determine a significant beneficial effect on skin disease after 6 months (125).

Regarding the effect of Rituximab on the immune system, it is well understood that it suppresses B cells, which has also been observed in the patients suffering from SSc (27,37). However, the influence on other parts of the immune defence in SSc patients, such as the T cells in the skin, has so far not been investigated.

The aim of the research study is to evaluate the influence of this monoclonal antibody on Th 17 cells in the skin and simultaneously learn more about the involvement of Th 17 cells in the pathogenesis of this disease. Within this thesis, the main focus lied on the establishment of the right immunohistochemical staining of the specimens, as it was not possible to meet the schedule and the interpretation of the results still lies ahead. At first we collected skin samples of 7 patients undergoing long time CD20 - depletion therapy, which had already kindly provided a skin sample for the research study with the ethical number: 24-184 ex 11/12 before initiation of treatment. These specimens were further processed in the laboratory of the department of rheumatology and immunostained.

Due to the still ongoing processing of the samples, we were not yet able to prove our hypothesis - i. e. the Rituximab-therapy effects the Th 17 cells. However, we managed to establish the single staining and detected an enhanced Il-17A staining in mast cells. The

final step of the method, in which the single staining specimens are scanned and overlaid on the computer to be evaluated regarding the occurrence of the Th17 cells, is still ongoing but promises interesting results.

2 Material and Methods

The Pilot study was carried out at the department of Rheumatology of the Medical University in Graz and was approved by the local ethics committee. It was conducted in accordance to the Declaration of Helsinki.

The recruitment took place in the outpatient department and/ or the ward of the department of Rheumatology. Each participating patient had to sign a written informed consent and patient information sheet.

2.1 Study population

Inclusion Criteria:

Group 1: Patients classified as having Systemic Sclerosis by the ACR/ EULAR classification criteria for SSc and undergoing a long time Rituximab-therapy. In the course of the project "Laboruntersuchung der Wirkung von Rituximab auf Zellen von SklerodermiepatientInnen in der Zellkultur – eine Pilotstudie (EK. number 24-184 ex 11/12)" these patients have already provided a skin sample before the start of the therapy.

Group 2: Control group; Patients from the department of plastic and cosmetic surgery of the Medical University in Graz, whose excessive healthy skin, which was removed and would normally have been thrown away within the cosmetic surgery.

Exclusion criteria:

Patients suffering from other kinds of autoimmune diseases, malignancies, sepsis, psychiatric disorders, under age and/ or not empowered to sign were excluded.

2.2 Sampling

Biopsy number 1 (before initiation of Rituximab-therapy):

All the participating patients suffering from Systemic Sclerosis had already provided a skin sample during the study: ethical number 24-184 ex 11/12. This sample taking occurred before the start of the B cell depletion-therapy.

Biopsy number 2 (during long time Rituximab-therapy > 1 year):

These patients were now, after signing the declaration of consent and information sheet, called into the outpatient department of the Rheumatology, Medical University of Graz.

Before the biopsy, they received questions regarding possible allergies and/ or incompatibilities to local anaesthesia in the past. Subsequently the biopsy area in the upper part of the forearm was disinfected and hypodermically injected with approx. 0.5 ml Xylocain 1%. Then the specimen was taken with a punching cylinder of 5 mm depth and a scalpel, put into a Falcon tube with PBS and further processed in the laboratory of Rheumatology within the following hour. The wound was taken care of with steristrips and an adhesive plaster. The patients were asked to avoid contact with any kind of dirt until the lesion was fully healed to prevent infections. An inspection of the wound occurred at the next routine control in the clinic or if desired by the patient, at the family doctor. (see Figure 3)

In the control group, the sampling took place on the surplus tissue, that would have been thrown away during the cosmetic surgery at the department of plastic and cosmetic surgery. Therefore, the tissue was cleaned, disinfected and subsequently the biopsy taken with a 5 mm punching cylinder.

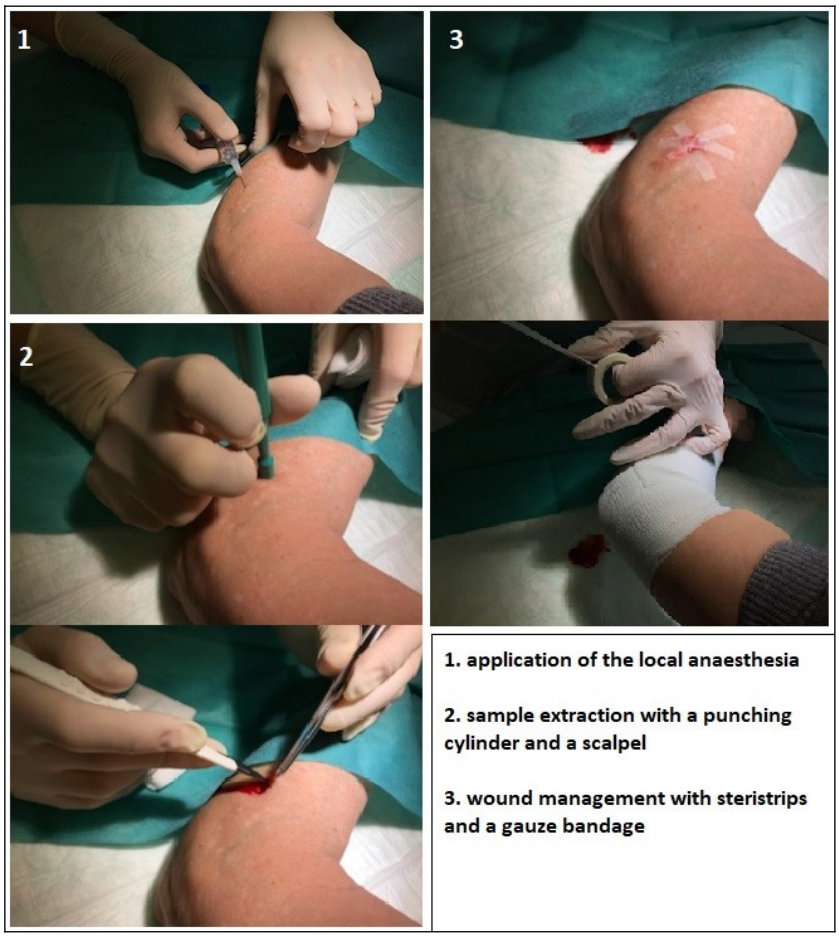

Figure 3: example of the 2nd biopsy of a patient suffering from Systemic Sclerosis

2.3 Sample processing

2.3.1 Paraffin embedding and sectioning

Paraffin embedding

The skin samples taken were washed in phosphate-buffered saline (PBS), the subcutaneous fat was removed and the specimen split in half with a scalpel. Subsequently the punches got fixed in 4% formaldehyde for 24 hours.

The next day the specimens were put into the embedding station overnight, in which they got dehydrated through an ascending alcohol series, saturated with xylene and embedded in hot and liquid paraffin in accordance to this routine:

Table 2: embedding procedure

Step	*Solution*	*Concentration (%)*	*Time (h)*	*Temperature (C°)*
1	Formalin	4.0	1:00	40
2	Formalin	4.0	1:00	40
3	Ethanol	70.0	1:00	40
4	Ethanol	80.0	1:00	40
5	Ethanol	96.0	1:00	40
6	Ethanol	96.0	1:00	40
7	Ethanol	100.0	1:00	40
8	Ethanol	100.0	1:00	40
9	Tissue clear		1:00	40
10	Tissue clear		1:00	40
11	Paraffin		1:00	53
12	Paraffin		1:00	53
13	Paraffin		2:00	53
14	Paraffin		2:00	53

The following day the paraffin blocks were produced by allowing the paraffin to cool down.

Sectioning tissues

The tissue blocks were sectioned using a sledge microtome (Microm HM430). Before the sectioning process, the blocks were cooled down to -20°C to achieve a better cutting quality. The thickness of the sections was set on 1-2 µm.

2.3.2 Double staining Immunohistochemistry (double staining IHC)

Background

An immunohistochemical examination takes place on cryo – or paraffin sections of the specimens. Unmarked primary antibodies are made visible by adding peroxidase- or alkaline phosphatase- marked secondary antibodies with consecutive enzyme-catalysed reaction (e. g. APAAP-technique, PAP-method). (126)

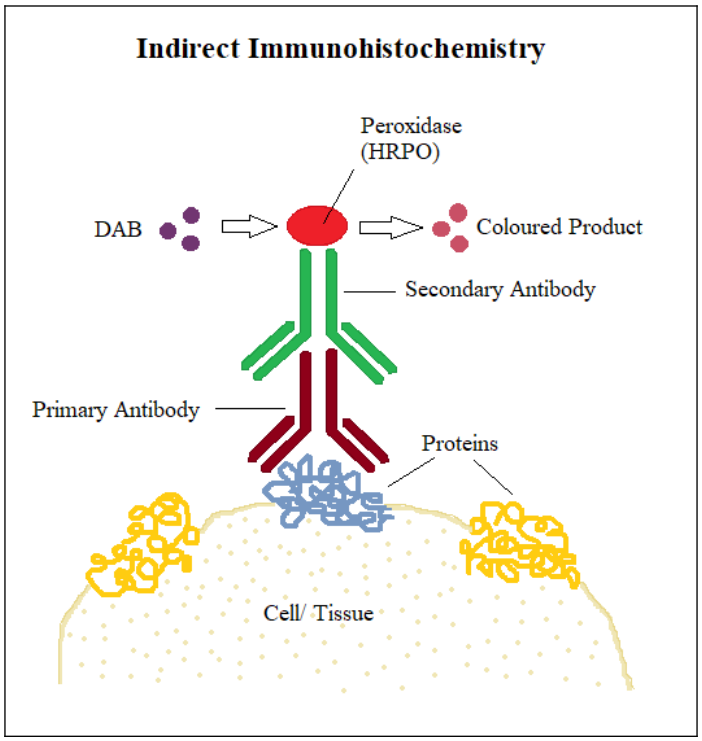

Figure 4: Overview Indirect Immunohistochemistry, modified from (126)

Protocol – Th17 – double staining immunohistochemistry

Primary Antibodies

Anti-CD4 antibody: a recombinant rabbit monoclonal [EPR6855] antibody to human CD4 from Abcam (ab133616)

Anti-IL17 antibody: a synthetic rabbit polyclonal antibody to human IL-17 from Abcam (ab79056)

Positive Control Tissue

A sample of human lung was ordered at the biobank Graz to be used as a known positive control tissue.

CD4/ IL-17 double IHC Assay

Day 1

1. Pre-treatment (dewaxing)

As a first step, we incubated the sections at 60°C for 60 minutes. Subsequently they were put in xylene for 15 minutes to dissolve the paraffin out of the tissue. Then, the rehydration in a descending alcohol series followed. Starting with 3 minutes in 100% ethanol, followed by 3 minutes in 50% ethanol and a short bath in tap water. After that, the specimens were put in distilled water.

2. Antigen retrieval

For the antigen retrieval, we mixed 20 mL of the Dako-Retrieval Solution, 10x concentrated, S1699 with 180 mL distilled water and warmed it up in a cuvette. We put the sections in the cuvette and placed it in a preheated water bath (GFL 10483805K) at 96°C for 10 minutes. Subsequently the cuvette was cooled down under running tab water for 10 minutes.

3. Washing buffer

Then, the slides were rinsed with custom-built tris buffered saline (TBS) for 5 minutes:

Ingredients:

- 30.3 g Trizma Hydrochloride (T5941 Sigma)
- 6.9 g Tris (Hydr.Meth.) Amin PA (Merck 8382)
- 43.85 g sodium chloride (Merck 6404)
- 2.5 g Tween-20 (Art. Nr. S6536884235)

  + 100 mL distilled water

  → 50x Stock

Before using the TBS, the washing buffer needs to be diluted to 1x.

4. Hydrophobic border

To prevent any run-off of the components used in the following steps of the staining process, a pen (Dako Pen S2002) was used to draw a circular border around the specimens.

5. Primary antibody CD4

The primary antibody against CD4 was diluted 1:500 with the Dako Antibody Diluent S0809 and depending on the size of the specimen 100 - 200 µL of the mixture were put on every section. An incubation time of 1 hour followed.

6. Washing buffer

Afterwards the sections were rinsed with TBS for 10 minutes.

7. Peroxidase blocking

Following the primary antibody incubation, we blocked the endogenous peroxidase by adding Dako REAL Peroxidase Blocking Solution for 5 minutes.

8. Washing buffer

Then the slides were rinsed with TBS for another 5 minutes.

9. Protein block

3 to 4 drops of the UltraVision Protein Block from Thermo Fisher Scientific were put on every section for 8 minutes to avoid non-specific binding of antibodies to the tissue.

10. Washing buffer

A washing period of 5 minutes with TBS followed.

11. Secondary antibody

Subsequently Dako REAL EnVision/HRP, Rabbit/ Mouse (K 5007, bottle A) was added with an incubation time of 30 minutes.

12. Washing buffer

Then the slides were again rinsed with TBS for 10 minutes.

13. Chromogen

For the chromogenic detection we used the Dako REAL EnVision Detection System. At first, we had to prepare a substrate-chromogen-solution. Therefore, we mixed 1 mL of the substrate buffer (K 5007, bottle B) with 20 µL of the chromogen (K 5007, bottle C). The mixture was then put on the sections for 6 minutes.

14. Washing buffer

Subsequently the sections were rinsed with TBS for 10 minutes.

15. Endogenous alkaline phosphatase block

To prevent non-specific binding from antibodies to the endogenous alkaline phosphatase, the specimens were incubated with Dako Dual Endogenous Enzyme Block Code S2003 for 10 minutes at room temperature.

16. Washing buffer

A washing period of 10 minutes with TBS followed.

17. Primary antibody IL-17

Afterwards the primary antibody against IL-17 was diluted 1+2000 with the Dako Antibody Diluent S0809 and depending on the size of the specimen 100-200 µL of the mixture were put on each section.
Then, the slides were put in the fridge at 4°C over night.

Day 2

1. Washing buffer

To start, we rinsed the sections for 15 minutes with TBS.

2. Secondary antibody

Subsequently the Dako REAL™ Link, Biotinylated Secondary Antibodies (K 5005, bottle A), containing biotinylated goat anti-mouse and anti-rabbit immunoglobulins in a buffered solution with stabilizing protein and sodium azide, were added for an incubation period of 20 minutes.

3. Washing buffer

Then the slides were rinsed with TBS for 15 minutes.

4. Streptavidin Alkaline Phosphatase AP

Afterwards we put Dako REAL™ Streptavidin Alkaline Phosphatase AP (K 5005, bottle B) for 20 minutes on the sections.

5. Washing buffer

A washing period of 15 minutes with TBS followed.

6. Chromogen

For the chromogenic detection we had to mix:

- 1 drop of Dako REAL™ Levamisole (Bottle G) with 750 µL of Dako REAL™ AP Substrate Buffer (Bottle F)
  + 30 µL of Dako REAL™ Chromogen Red 1 (Bottle C)
  + 30 µL of Dako REAL™ Chromogen Red 2 (Bottle D)
  + 30 µL of Dako REAL™ Chromogen Red 3 (Bottle E) in exactly this sequence.

  (The mixture must be used up within 20 min to be properly working.)

It was put on the slides and rinsed off when the tissue started to turn orange (approx. after 3 minutes).

7. Washing buffer

Subsequently we flushed the sections with TBS for 5 minutes

8. Counterstaining

To enable the counterstaining, we rinsed the slides with distilled water and then put them into Mayer's hemalum solution for 5 seconds. Afterwards the sections were placed under warm running tap water (~4min) for bluing and then again rinsed with distilled water. Subsequently they were put into an ascending alcohol series, beginning with 90% ethanol for 2 minutes, followed by 2 minutes oft 100% ethanol and finally 2 minutes in xylene.

9. Coverslipping the slides

The slides were tapped onto a tissue to minimize the residue of xylene and afterwards a small amount of mounting medium was applied to the surface of the slide. The coverslip was slowly tipped onto the mounting medium and pushed down to get rid of possible bubbles.

However, the interpretation of the double staining proved great difficulty. Therefore, we decided to separately stain for the two antigens in two histological slices (1 µm), taken so close to each other, that they should contain mostly the same cells. Additionally, we increased the concentration of the IL-17 antibody to receive a stronger signal of the IL-17+ cells.

To decide, which concentration would give us the best result, 4 different dilutions were tested (1:200, 1:500, 1:100, 1:1500) on a human tonsil. The ensuing CD4 and IL-17 stainings were conducted as followed:

Protocol – CD4 and Th17 – single staining immunohistochemistry

CD4 IHC Assay

1. Pre-treatment (dewaxing)

As a first step, we incubated the sections at 60°C for 60 minutes. Subsequently they were put in xylene for 15 minutes to dissolve the paraffin out of the tissue. Then, the rehydration in a descending alcohol series followed. Starting with 3 minutes in 100% ethanol, followed by 3 minutes in 50% ethanol and a short bath in tap water. After that, the specimens were put in distilled water.

2. Antigen retrieval

For the antigen retrieval, we mixed 20 mL of the Dako-Retrieval Solution, 10x concentrated, S1699 with 180 mL distilled water and warmed it up in a cuvette. We put the sections in the cuvette, which we placed in a preheated water bath (GFL 10483805K) at 96°C for 10 minutes. Subsequently the cuvette was cooled down under running tab water for 10 minutes.

3. Washing buffer

Then, the slides were rinsed with custom-built tris buffered saline (TBS) for 5 minutes:

Ingredients:

- 30.3 g Trizma Hydrochloride (T5941 Sigma)
- 6.9 g Tris (Hydr.Meth.) Amin PA (Merck 8382)
- 43.85 g sodium chloride (Merck 6404)
- 2.5 g Tween-20 (Art. Nr. S6536884235)
  + 100 mL distilled water

  → 50x Stock

Before using the TBS, the washing buffer needs to be diluted to 1x.

4. Hydrophobic border

To prevent any run-off of the components used in the following steps of the staining process, a pen (Dako Pen S2002) was used to draw a circular border around the specimens.

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6. Washing buffer

Afterwards the sections were rinsed with TBS for 10 minutes.

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Following the primary antibody incubation, we blocked the endogenous peroxidase by adding Dako REAL Peroxidase Blocking Solution for 5 minutes.

8. Washing buffer

Then the slides were rinsed with TBS for another 5 minutes.

9. Protein block

3 to 4 drops of the UltraVision Protein Block from Thermo Fisher Scientific were put on every section for 8 minutes to avoid non-specific binding of antibodies to the tissue.

10. Washing buffer

A washing period with TBS of 5 minutes followed.

11. Secondary antibody

Subsequently Dako REAL EnVision/HRP, Rabbit/ Mouse (K 5007, bottle A) was added with an incubation time of 30 minutes.

12. Washing buffer

Then the slides were again rinsed with TBS for 10 minutes.

13. Chromogen

For the chromogenic detection we used the Dako REAL EnVision Detection System. At first, we had to prepare a substrate-chromogen-solution. Therefore, we mixed 1 mL of the substrate buffer (K 5007, bottle B) with 20 µL of the chromogen (K 5007, bottle C). The mixture was then put on the sections for 6 minutes.

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To enable the counterstaining, we rinsed the slides with distilled water and then put them into Mayer's hemalum solution for 5 seconds. Afterwards the sections were placed under warm running tap water (~4min) for bluing and then again rinsed with distilled water. Subsequently they were put into an ascending alcohol series, beginning with 90% ethanol for 2 minutes, followed by 2 minutes oft 100% ethanol and finally 2 minutes in xylene.

16. Coverslipping the slides

The slides were tapped onto a tissue to minimize the residue of xylene and afterwards a small amount of mounting medium was applied to the surface of the slide. The coverslip was slowly tipped onto the mounting medium and pushed down to get rid of possible bubbles.

IL-17 IHC Assay

Day 1

1. Pre-treatment (dewaxing)

As a first step, we incubated the sections at 60°C for 60 minutes. Subsequently they were put in xylene for 15 minutes to dissolve the paraffin out of the tissue. Then, the rehydration in a descending alcohol series followed. Starting with 3 minutes in 100% ethanol, followed by 3 minutes in 50% ethanol and a short bath in tap water. After that, the specimens were put in distilled water.

2. Antigen retrieval

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3. Washing buffer

Then, the slides were rinsed with custom-built tris buffered saline (TBS) for 5 minutes:

Ingredients:

- 30.3 g Trizma Hydrochloride (T5941 Sigma)
- 6.9 g Tris (Hydr.Meth.) Amin PA (Merck 8382)
- 43.85 g sodium chloride (Merck 6404)
- 2.5 g Tween-20 (Art. Nr. S6536884235)

  + 100 mL distilled water

  → 50x Stock

Before using the TBS, the washing buffer needs to be diluted to 1x.

4. Hydrophobic border

To prevent any run-off of the components used in the steps to be followed in the staining process, a pen (Dako Pen S2002) was used to draw a circular border around the specimens.

5. Endogenous alkaline phosphatase block

To prevent non-specific binding from antibodies to the endogenous alkaline phosphatase, the specimens were incubated with Dako Dual Endogenous Enzyme Block Code S2003 for 10 minutes at room temperature.

6. Washing buffer

A washing period of 10 minutes with TBS followed.

7. Primary antibody IL-17

Afterwards the primary antibody against IL-17 was diluted 1+200 with the Dako Antibody Diluent S0809 and depending on the size of the specimen 100-200 µL of the mixture were put on each section.

Then, the slides were put in the fridge at 4°C over night.

Day 2

1. Washing buffer

To start, we rinsed the sections for 15 minutes with TBS.

2. Secondary antibody

Subsequently the Dako REAL™ Link, Biotinylated Secondary Antibodies (K 5005, bottle A), containing biotinylated goat anti-mouse and anti-rabbit immunoglobulins in a buffered solution with stabilizing protein and sodium azide, were added for an incubation period of 20 minutes.

3. Washing buffer

Then the slides were rinsed with TBS for 15 minutes.

4. Streptavidin Alkaline Phosphatase AP

Afterwards we put Dako REAL™ Streptavidin Alkaline Phosphatase AP (K 5005, bottle B) for 20 minutes on the sections.

5. Washing buffer

A washing period of 15 minutes with TBS followed.

6. Chromogen

For the chromogenic detection we had to mix:

- 1 drop of Dako REAL™ Levamisole (Bottle G) with 750 µL of Dako REAL™ AP Substrate Buffer (Bottle F)
  + 30 µL of Dako REAL™ Chromogen Red 1 (Bottle C)
  + 30 µL of Dako REAL™ Chromogen Red 2 (Bottle D)
  + 30 µL of Dako REAL™ Chromogen Red 3 (Bottle E) in exactly this sequence.

  (The mixture must be used up within 20 min to be properly working.)

It was put on the slides and rinsed off when the tissue started to turn orange (approx. after 3 minutes).

7. Washing buffer

Subsequently we flushed the sections with TBS for 5 minutes

8. Counterstaining

To enable the counterstaining, we rinsed the slides with distilled water and then put them into Mayer's hemalum solution for 5 seconds. Afterwards the sections were placed under warm running tap water (~ 4min) for bluing and then again rinsed with distilled water. Subsequently they were put into an ascending alcohol series, beginning with 90% ethanol for 2 minutes, followed by 2 minutes oft 100% ethanol and finally 2 minutes in xylene.

9. Coverslipping the slides

The slides were tapped onto a tissue to minimize the residue of xylene and afterwards a small amount of mounting medium was applied to the surface of the slide. The coverslip was slowly tipped onto the mounting medium and pushed down to get rid of possible bubbles.

Scanning of the microscope slides

Last but not least, the sections will be scanned and put on top of each other on the computer to make the double positive cells visible.

2.4 Acquisition of influencing variables and additional information about the patients

Additionally, to the staining in the laboratory, potential influencing factors, that could affect the outcome were taken into account: Further information regarding the participating patients such as the type of Systemic Sclerosis, the disease duration, the amount and time period of received infusions of Rituximab and additional immunosuppressive medication were also acquired.

Furthermore, the modified Rodnan Skin Score (mRSS) and activity score were assessed before the initiation of the RTX-treatment and by the time of the 2nd biopsy, to allow a better evaluation of the therapy effect on the clinical manifestations of the disease.

2.4.1 Modified Rodnan Skin Score (mRSS)

The modified Rodnan Skin Score is used as a primary or secondary outcome measure of skin thickness in Systemic Sclerosis (127). The surface of the patient's body gets divided into 17 different areas and each area is rated by clinical palpation using a scale from 0-3 (0=normal skin, 1=mild thickness, 2=moderate thickness, 3=severe thickness with the inability to pinch the skin into a fold). The sum of the individual values is defined as the mRSS. (128) (see Figure 5)

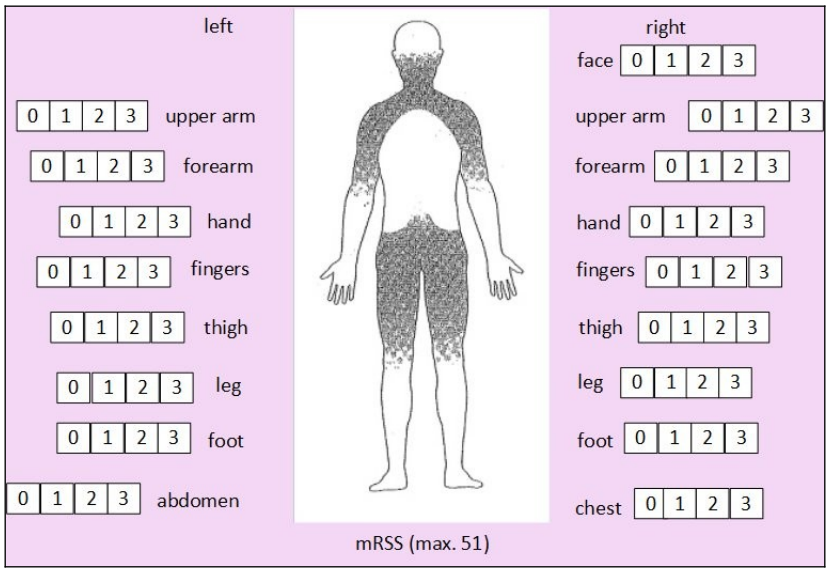


Figure 5: Modified Rodnan Skin Score (mRSS) adapted from (129) and the mRSS-sheet from the outpatient department of the Rheumatology in Graz with the permission from the Copyright Clearance Center (license number: 4392471044844) and OA Moazedi-Fürst

2.4.2 SSc Disease Activity Score (AS)

At our department the disease activity of patients suffering from Systemic Sclerosis is assessed based on the revised EScSG (European Scleroderma Study Group) index from

2003, consisting of 10 criteria with different index values adding up to a sum that is defined as the activity score. The total maximum disease activity index can vary between 0 and 10. (see Table 3)

Table 3: EScSG criteria 2003 to evaluate disease activity in SSc patients (130)

Criteria			Indices	
Rodnan Skin Score ≥ 14	yes	no	1	Assessment of the skin in 17 defined anatomic areas from 0 (normal) to 3 (strong thickening); values from 0 – 51
Scleredema	yes	no	0.5	Increase of the soft tissue mass (in particular on the fingers) due to thickening of the skin
Skin	yes	no	2	Patient states a deterioration of the skin lesions within the last month
digital necrosis	yes	no	0.5	Active digital ulceration – ranging from small infarcts to complete gangrene
Peripheral-vascular System	yes	no	0.5	Patient states a deterioration in the blood circulation of the fingers within the last month
Arthritis	yes	no	0.5	Symmetric swelling and pressure pain in peripheral joints
TLCO (carbon monoxide transfer factor)	yes	no	0.5	TLCO < 80% of the age-appropriate standard
Heart/ Lung	yes	no	2.0	Patient states a deterioration regarding his cardiopulmonary status within the last month
ESR (erythrocyte sedimentation rate) ≥ 30 mm/ 1st h	yes	no	1.5	
Hypocomplementaemia	yes	no	1.0	Either low C3 or C4
Total maximum disease activity index			10	

2.4.3 Disease Duration

Existing literature on the topic suggests, that the amount of the Th17 cells varies depending on the time that has passed since the first onset of the disease (60,63). Therefore, we also wanted to consider this variable in our investigation, dividing our patients into two groups – i. e. early and late SSc.

2.4.4 Additional immunosuppressive medication

Due to the fact, that our target variables – the Th17 cells – are part of the immune system, it deemed necessary to evaluate the full immunosuppressive treatment, that could have an influence on the amount of the exiting Th17 cells in the affected skin.

3 Results

In the beginning of this study, 9 patients having already participated in the study: ethical number 24-184 ex 11/12 were asked whether they would be willing to give another sample. 1 patient declined, another had to be excluded from the study due to ongoing haemodilution and therefore was not suited for a sample extraction by biopsy.

So overall 7 patients participated, consisting of 6 female and one male, all Caucasian. The age ranged from 51 – 72 years with a median age of 62.43 years. (see Table 4) Among the participating patients, 5 (71%) are suffering from diffuse cutaneous systemic sclerosis and 2 (29%) from limited cutaneous systemic sclerosis. (see Table 5)

Table 4: age of the participants

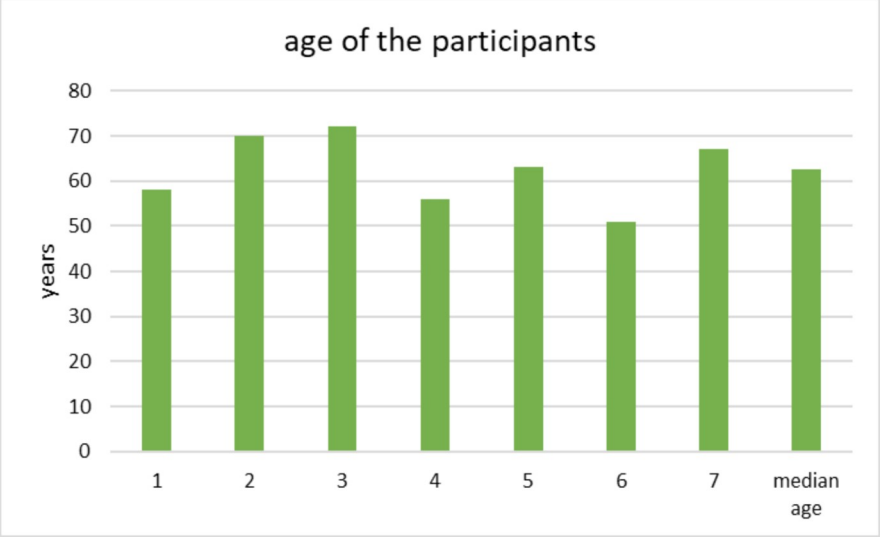
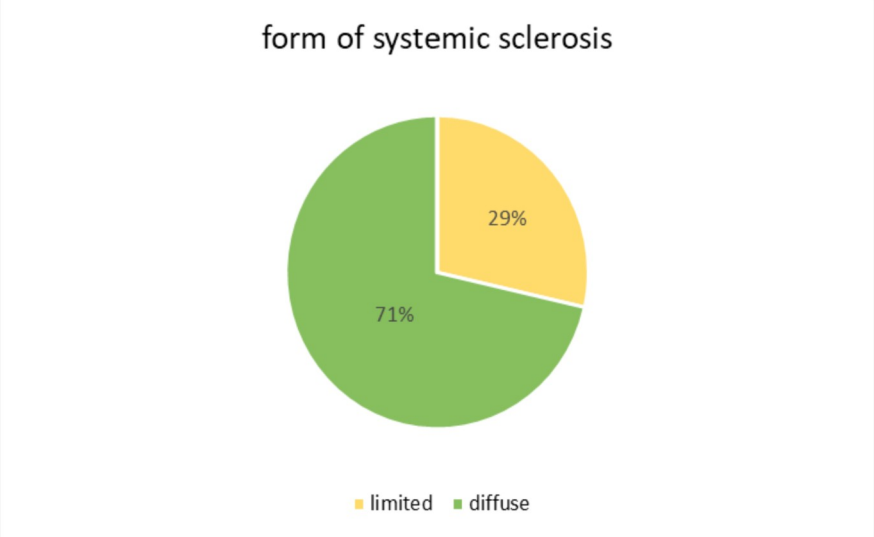


Table 5: types of systemic sclerosis in the participating patients



The disease duration was estimated depending on the first onset of the clinical manifestation complying with the ACR/ EULAR classification criteria (e. g. Raynaud's phenomenon, puffy fingers, etc.). 4 patients were considered to have early SSc with a disease duration ≤ 3 years and the other 3 late SSc with a disease duration > 4 years. (see Table 6)

Table 6: disease duration in the patients suffering from systemic sclerosis

Specimens	Disease duration before initiation of RTX-therapy
SKL42 + SKL52	9 years → late SSc
SKL44 + SKL51	1 year → early SSc
SKL47 + SKL50	19 years → late SSc
SKL40 + SKL49	4 years → late SSc
SKL31 + SKL55	6 months → early SSc
SKL43 + SKL56	3 years → early SSc
SKL46 + SKL54	2 years → early SSc

Out of the 7 participating patients, 5 received an additional immunosuppressive medication at some point during the time period between the two biopsies.

4 of these received mycophenolate mofetil 500 mg 1-0-1 p. o. in accordance with the "Grazer Schema". In one case, this medication had to be withdrawn after 5 weeks due to nausea and diarrhoea.

One patient continued her preceding therapy with Ebetrexat 15 mg 1x/week p.o. and was reduced to 7.5 mg 1x/week p o. during the RTX-therapy. (for further details see Table 7)

Table 7: additional immunosuppressive medication of the SSc-patients

specimens	additional medication
SKL42 + SKL52	Cellcept 500 mg 1-0-0 for 14 d, then 1-0-1 for 7 d, 14 d before the 2nd biopsy it was withdrawn because of nausea and diarrhoea
SKL44 + SKL51	Cellcept 500 mg 1-0-1 for 860 d before the 2nd biopsy
SKL47 + SKL50	-
SKL40 + SKL 49	Cellcept 500 mg 2-0-2 at the beginning of the RTX-therapy for 120 d, then reduction to 1-0-1 for 14 d, subsequently re-increase to 2-0-2 for 16 d, reduction to 1-0-1 for the remaining 699 d till the 2nd biopsy
SKL31 + SKL55	Ebetrexat 15 mg 1x/week p.o at the beginning of the RTX-therapy for 447 d, then reduction to 7.5 mg 1x/week p.o. for 442 d, further reduction to 5 mg 1x/week p.o. for 98 d, increase to 7.5 mg 1x/week p.o. for 565 d until the 2nd biopsy
SKL43 + SKL56	-
SKL46 + SKL54	Cellcept 500 mg 1-0-1 since the beginning of the RTX-therapy for 707 d

3.1 Modified Rodnan Skin Score (mRSS) and SSc Disease Activity Score (AS)

3.1.1 Modified Rodnan Skin Score

In 6 out of 7 patients the modified Rodnan Skin Score (mRSS) decreased significantly during the RTX-treatment. In one case however, the mRSS increased by 3 points, interestingly this was the only male participant in this study. (see Table 8)

Table 8: modified Rodnan Skin Score (mRSS) during the course of the RTX-therapy

specimens	mRSS before RTX-treatment initiation	mRSS at the 2nd biopsy
SKL42 + SKL52	15	2
SKL44 + SKL51	6	9
SKL47 + SKL50	28	3
SKL40 + SKL49	22	10
SKL31 + SKL55	36	2
SKL43 + SKL56	19	2
SKL46 + SKL54	38	3

3.1.2 SSc Disease Activity Score

In two patients the Disease Activity Score (AS) was not assessed around the 2nd biopsy and in one patient not before the initiation of the RTX-treatment. In the other 4 participants the AS decreased significantly below 0.15. (see Table 1Table 9)

Table 9: SSc disease activity score before and during RTX long time treatment

Specimen	AS before RTX-treatment initiation	AS around (+/- 3 months) the 2nd biopsy
SKL42 + SKL52	0.5	Data not collected
SKL44 + SKL51	6.5	0.0
SKL47 + SKL50	3.0	0.0
SKL40 + SKL49	D. n. c.	
SKL31 + SKL55	4.5	D. n. c.
SKL43 + SKL56	1.0	0.0
SKL46 + SKL54	7.0	0.15

3.2 Skin histology

3.2.1 Double staining Immunohistochemistry (double staining IHC)

Initially, we performed a double staining immunohistochemistry on the paraffin embedded skin sections of healthy controls and patients suffering from Systemic Sclerosis before and during long time CD20 depletion – therapy. In our study, we wanted to concentrate on the lymphocytic infiltration of the epidermis and the dermis. The CD4 expressing T helper cells (brown) partly infiltrated the epidermis (in 4 of the 7 cases). Additionally, in all cases they concentrated in nests around the vessels and occurred sporadically throughout the whole dermis. (see Figure 6)

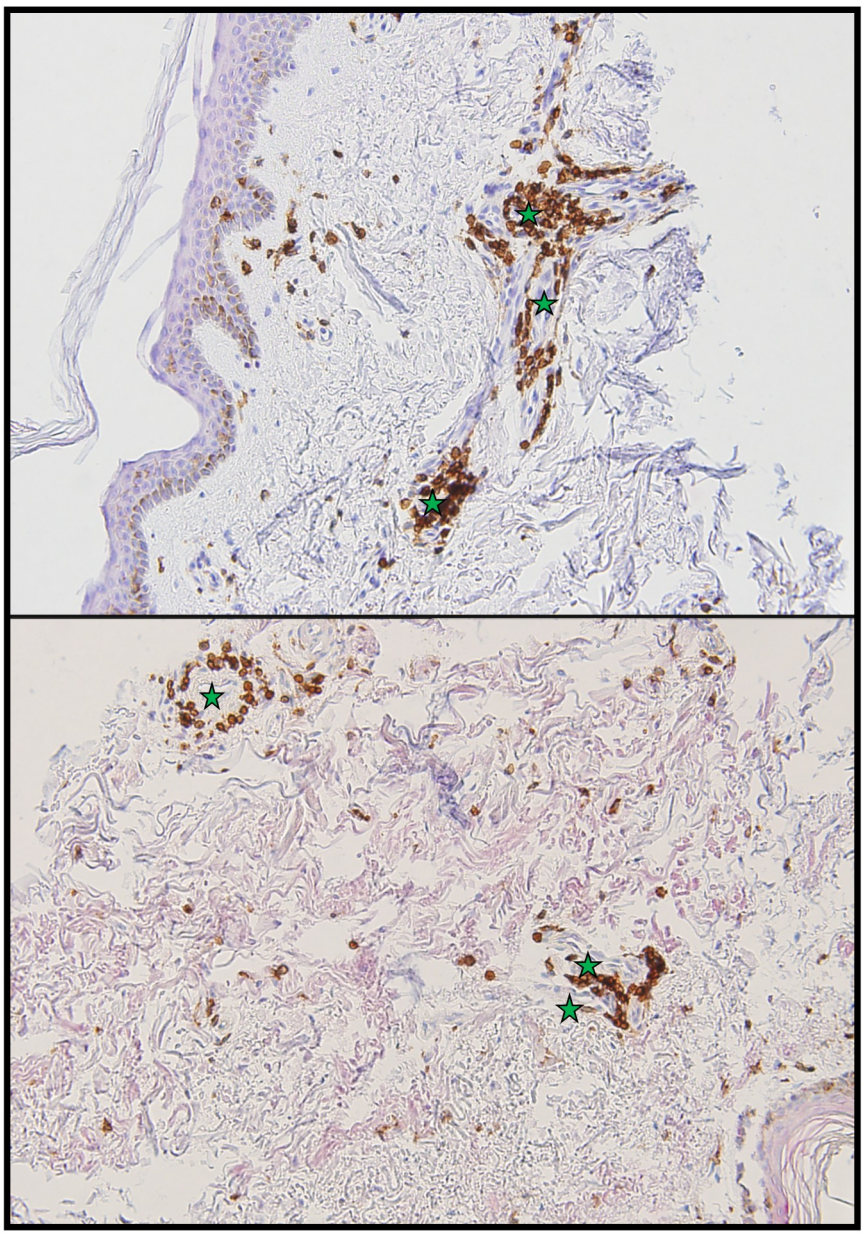


Figure 6: „nest-like“ Arrangement of the T helper cells (brown) in the areas close to the vessels (★)
T helper cells seem to gather especially around the vessels in the skin of SSc patients

In 4 cases, they seemed to decrease in their intensity and number (see Figure 7). In the other 3 cases, their appearance stayed the same or even increased (see Figure 8). In the skin of the healthy controls, there were also CD4+ cells present. Mainly around the vessels close to the epidermis, however less concentrated and there was almost no diffuse distribution in the rest of the dermis. (see Figure 9)

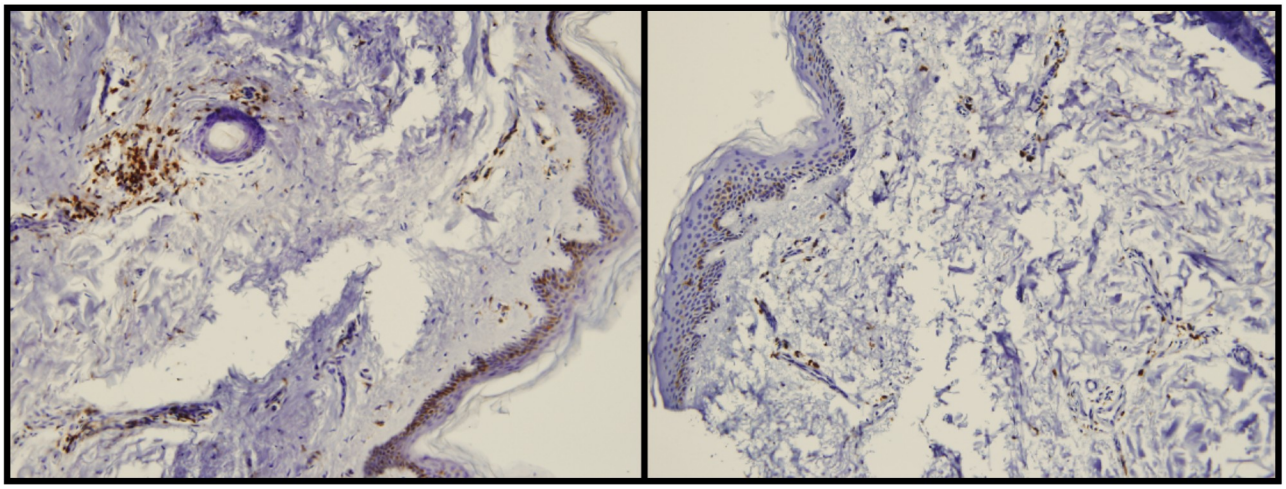


Figure 7:example of a decrease of T helper cells during long time CD20 – depletion therapy
Left picture: 1st biopsy, right picture: 2nd biopsy

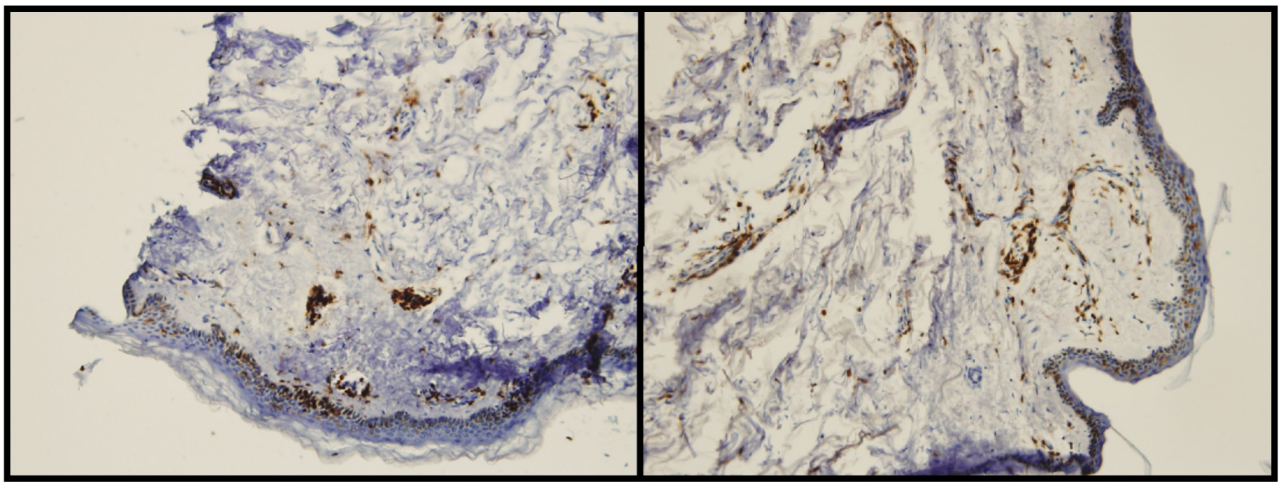


Figure 8: example of one of the three cases with still increased T helper cells during long time CD20 – depletion therapy
Left picture: 1st biopsy, right picture: 2nd biopsy

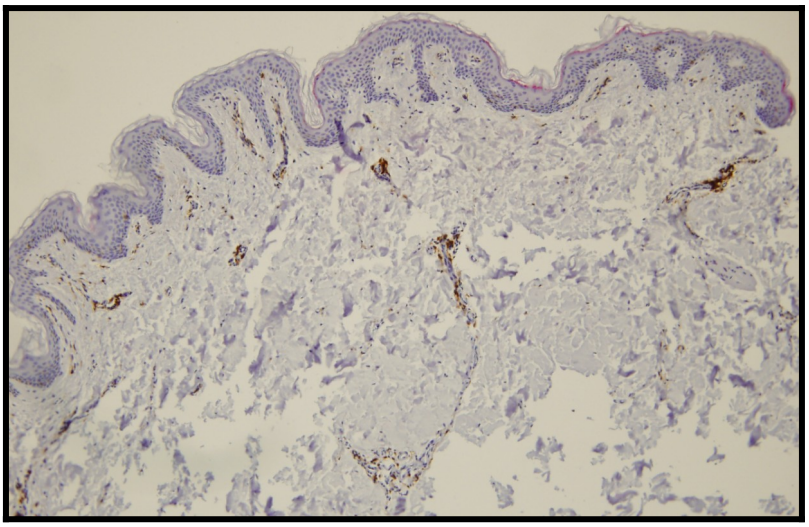


Figure 9: example of healthy skin

The further differentiation of the T cell subgroups with the help of the IL-17A staining proved to be difficult, due to the intense CD4 staining, overlaying the IL-17A - antibody (pink) in the double staining for the most part. (see Figure 10) Therefore, we were unable to interpret the results in this direction in a scientifically reliable way.

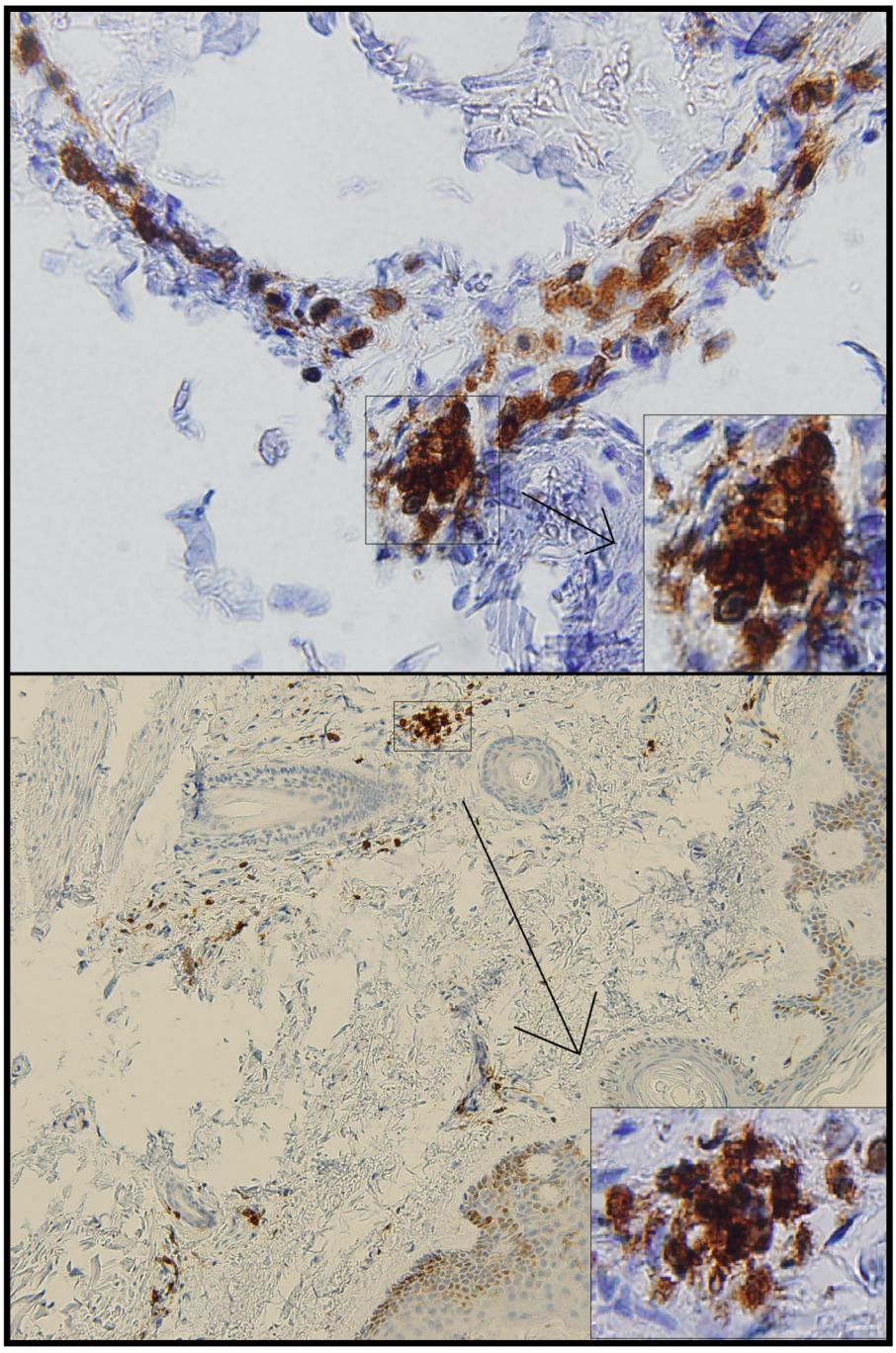


Figure 10: example of the double stained T helper cells
The brown CD4 - staining overlays the pink IL-17A - staining almost completely, making a differentiation between pure brown T helper cells and double stained Th 17 very difficult

3.2.2 Single staining immunohistochemistry (single staining IHC)

Due to the difficulties with the double immunohistochemistry we decided to stain two slides of the same specimen separately for CD4 and IL-17A, scan them after and overlay them on the computer. The CD 4 single staining IHC worked just fine and for the IL-17A staining we decided to try out some stronger concentrations on a human tonsil to reach a stronger colour signal. The dilution 1:200 provided the best result with clearly visible IL-17A+ lymphocytes. (see Figure 11)

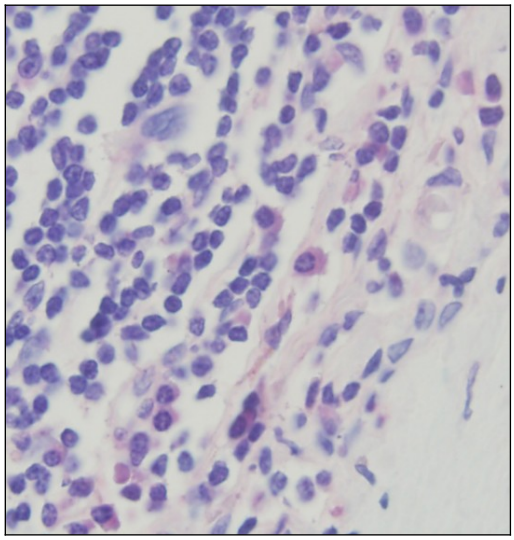


Figure 11: IL 17+ lymphocytes in the human tonsil at an antibody dilution 1:200 during the re-establishment of the IL 17 – staining

Therefore, we performed another single staining on the study specimens using this antibody concentration. In this staining, the mast cells, which are known to produce IL-17A (84), stained strongly positive in the SSc skin (see Figure 12). Unfortunately, the unspecific background staining was too strong in the skin sections (see Figure 13) to interpret the staining of the IL-17A positive lymphocytes, preventing us from interpreting the occurrence of the Th17 subgroup without the overlay on the computer with the CD4 stained slides.

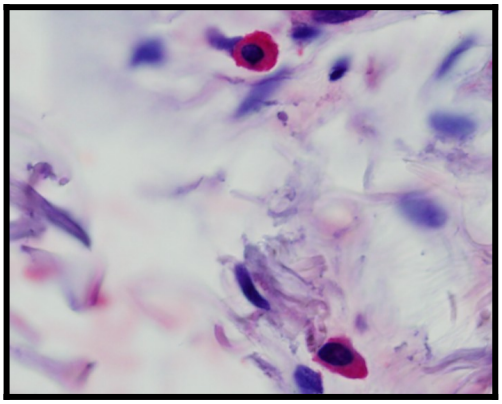


Figure 12: IL-17A+ mast cells in SSc skin

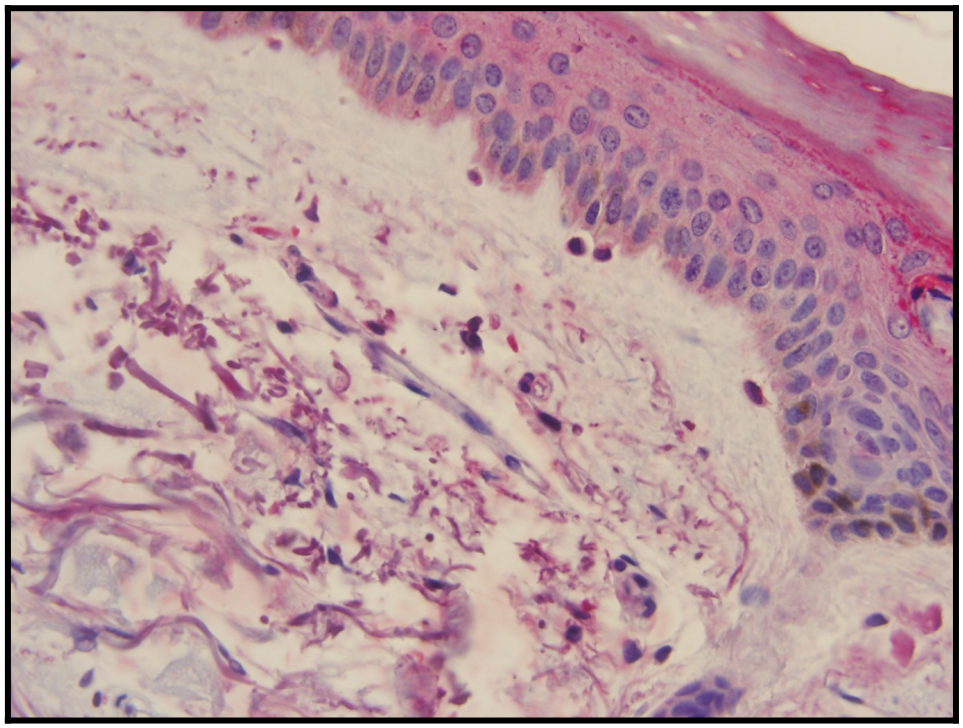


Figure 13: IL-17 staining with an antibody dilution 1:200
In the skin samples, the background colour was quite strong

4 Discussion

In this pilot study we immunohistochemically stained the skin samples of SSc patients before and during long time B cell-depletion-therapy in order to see whether a CD20-depletion can also have an effect on Th17 cells. Conducted off-label use of Rituximab in our department over the last couple of years has clinically proven to be effective in patients suffering from Systemic Sclerosis. As mentioned above, the positive effect can be seen in an improvement of lung function and a reduction of skin fibrosis and calcinosis cutis (37–39).

The main mode of action, namely the elimination of B cell precursors and mature B cells, is well known (29–31).

Due to the rising interest and implication of the Th17 cell and its pivotal Interleukin in the pathogenesis of the disease, we wanted to investigate, whether the amount of Th17 cells would differ between the skin of healthy controls and florid lesions of SSc patients before therapy and after treatment with Rituximab.

So far, most of the studies on the role of Th17 cells in the pathogenesis of systemic sclerosis focused mainly on the levels of Interleukin-17 in the blood and skin of the patients, however not the Th17 cells per se or if they are the main source of IL-17A or not.

We could not yet demonstrate whether the number of Th17 cells in the skin of patients suffering from scleroderma differs from healthy controls.

One limitation of our study was the small number of the participating patients (n=7). Of course, we know, that this is not a sufficient number of study participants to reach a satisfactory and significant result. Nevertheless, we wanted to do a pilot study on this subject to see, whether the Th17 cells are of a pathogenetic relevance and depending on the outcome, let a bigger study follow. Our department of rheumatology in Graz recruits approximately 6 new patients suffering from Systemic Sclerosis per year. Due to this fact, and the pre-existing biopsy samples from another study (EK. number 24-184 ex 11/12), we decided to ask the same patients for a second sample.

The pre-existing tissue of these patients had been fixed in paraffin. Therefore, we chose the immunohistochemistry as an analysis technique, instead of a FISH, in which the hybridization on formalin fixed and paraffin embedded tissue can bear a challenge (131).

The immunohistochemistry is an important diagnostic tool in the pathological anatomy laboratories and within the last decades has also become more and more commonly used as a means of scientific research (132). Single stainings are performed regularly in the

laboratory of Rheumatology, however this was the first attempt of a double staining IHC. Just like Luongo de Matos et al., we came to the conclusion that the usefulness and input of this analysis technique is direct proportionate to the experience of the performer of the reaction and additionally of the interpreter of the findings. (132)

Concerning the interpretation, it is important to know that, in light microscopy, a small lymphocyte (10 µm or smaller) consists mainly of the nucleus surrounded by the cytoplasm forming a narrow rim. In bigger cells, the cytoplasm can also be abundant (see Figure 14) (133), however the majority of our lymphocytes seemed to consist of the small type.

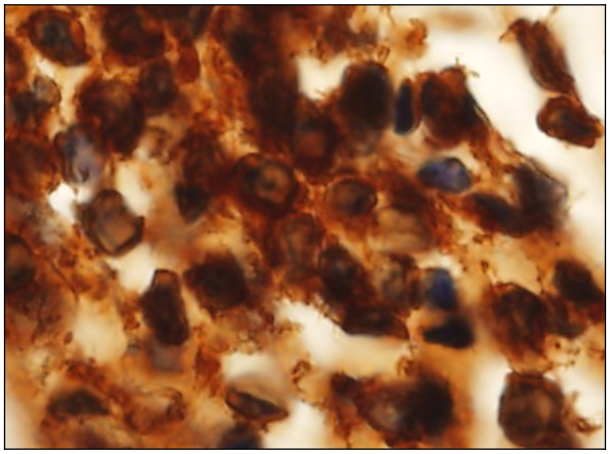


Figure 14: different sizes of lymphocytes in the SSc skin

So, with this analysis technique the challenge was that, at the present time, two markers are needed to correctly identify the Th17 cells immunohistochemically. Firstly, the CD4 surface antigen as an overall T helper cell marker and secondly, Interleukin-17A as the pivotal cytokine produced by the Th17 cells. The used IL-17A-antibody to further differentiate between the T cell subgroups, docks with the IL-17A which is located in the cytoplasm and transiently also on the surface of these lymphatic cells (134). Therefore, the area of reaction was quite small. Additionally, the chromogen we used was a Fast Red-type chromogen, which was supposed to form a crisp red end-product at the site of a target antigen (135). In our case however, the product in the double staining IHC was more of a bright deep pink, providing great difficulty in the light microscopy to be distinguished from the counterstaining with the blue-violet Mayer's hemalum and the chocolate/ saddle-brown CD4 staining. Because of the location of the CD4 antigens on the surface of the T cells, the brown staining coloured almost the whole cell and overlaid the pink staining of the IL-17A antigens (see Figure 15)

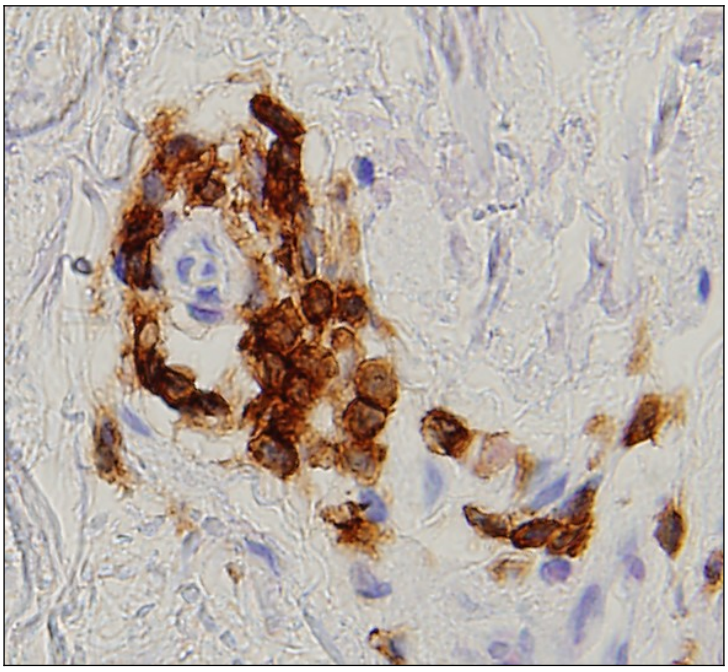


Figure 15: double staining of the Th 17 cells in the SSc skin
The overlap of the two stainings, due to the antigen location on the surface (CD4) and the cytoplasm (IL-17) made a reliable interpretation of the results very difficult

Therefore, we decided to do two separate single stainings, scan the slides and overlay them on the computer. Due to the thin slicing of the paraffin block, we hoped to have the same cells for the most part on both stainings. As mentioned above, we used a lower dilution of the IL-17A antibody in the single staining to reach a stronger signal, as this had worked fine during the establishment-phase on the human tonsil. The resulting unspecific background staining in the skin sections rose the question though, whether those specimens can be used for interpretation or not. It suggests, that either the dilution was too low, or the exposure time has to be reduced when using a higher concentration in skin samples. Scanned and overlaid with the CD4+ slides, it should be still possible to recognize the Th17 cells, otherwise a lower concentration or shorter exposition time should be used.

Nevertheless, we observed in the CD4 single staining that most of the T helper cells concentrated around the vessels. In 4 cases we could observe an infiltration of the epidermis during long time CD20 – depletion therapy, which points to an active inflammation, however the missing infiltration in three cases before treatment initiation can be due to the choice of the biopsy area.

Furthermore, we noticed that many CD4−IL-17+ cells in the dermis were mast cells. In order to confirm our impression, further investigations regarding the phenotype and location of the IL-17+ cells would have to be made.

Punt et al. examined the phenotype of IL-17 expressing cells in cervical cancer and concluded that the majority was made up of neutrophil granulocytes (66%), followed by mast cells (23%) and innate lymphoid cells (ILCs) (8%). Th 17 cells were only responsible for 4% of the IL-17+ cells in the malignancies. The total number of IL-17+ cells, neutrophils and the lowest quartile of mast cells correlated with poor survival in the early stage of the carcinoma, while a high number of Th 17 cells seemed to be a positive prognostic factor. Suggesting that the different types of IL-17+ cells might have diverse effects on the disease progression. (136) It would be of interest to know, whether this is also the case in Systemic Sclerosis.

In patients suffering from psoriasis, Lin et al. found that a major part of the elevated IL-17 levels is attributable to dermal tryptase$^+$ and chymase$^+$ mast cells (MC_{TC} cells), regardless of the status of the disease. In addition, the number of IL-17A+ neutrophils was also higher. In a next step, they evaluated the effect of IL-23 and IL-1β on these MC_{TC} cells and just like their effect on Th 17 cells, the cytokines led to an MC degranulation and the formation of mast cell extracellular traps (=MCETosis[6]) in the human skin, which was followed by the release of IL-17 and other mast cell products. (137)

Truchetet et al. immunohistochemically examined skin samples of 8 patients suffering from Systemic Sclerosis and healthy controls to answer the same question regarding IL-17A+ cells in this autoimmune disease. They concluded that the number of Th17 cells was, just like in the psoriatic lesions, elevated compared to healthy skin controls. The majority of the overall IL-17A+ cells was also formed by mast cells, followed by Th17 cells and neutrophils and macrophages (10-20%). In addition, the amount of these cells negatively correlated with the extent of skin fibrosis as assessed by the modified Rodnan skin thickness score (mRSS). The sample-taking in Truchetet's study was performed on patients without undergoing immunosuppressive therapy, apart from low dose glucocorticoids (≤6 mg per day). (84)

The elevated number of mast cells in Systemic Sclerosis has already been described in the 80s and 90s and is restricted to the early (scleroedematous) stage of the disease, whereas their amount is normal or even decreased in the later (sclerotic, atrophic) stages. (99,138).

Neither Nakashima et al., nor Truchetet et al. found a relation between the IL-17 and/ or Th17 levels and the stage of the disease (83,84). While Olewicz-Gawlik et al. concluded that the IL-17 levels negatively correlate with the disease duration and are significantly increased in SSc patients with a disease duration < 2 years (63). Concordantly Kurasawa et

[6] Active and controlled apoptosis of the MC by mixing the nuclear and the granular components with formation of an extracellular trap (140)

al. noticed that 73 % of the patients with a disease duration < 4 years (n=15) showed an overproduction of IL-17. Additionally, they associated the increased IL-17 production with a shorter disease duration from the first manifestation of clinical symptoms. They further concluded that the subtype of scleroderma (n=24 patients (7 dcSSc, 17 lcSSc)) does not impact the IL-17 mRNA levels in the skin and sera. (60) Rodriguez-Reyna et al. enrolled 135 patients and using a flow cytometry and found the number of IL-17A expressing CD4+ T cell to be higher in patients suffering from dcSSc (7.1%) compared to lcSSc (5.0%) and healthy controls (1%) (134).

In conclusion the scan and overlay of the two single stainings to interpret the occurrence of the Th17 cells still needs to be done. If the background staining of the IL-17A staining is too strong, there is still the possibility of an increase of the dilution and/ or reduction of the exposition time during the staining process. Regarding the IL17A+ mast cells, the established IL-17A single staining provides strong results. It would be of great interest to evaluate the effect of the Rituximab-therapy on this cells and also the IL-17A level in the sera of the SSc patients, as it is known in the literature, that mast cells are elevated in the early phase of Systemic Sclerosis (99,134). In addition, it would be appealing to investigate whether the IL-17+ cells involved in Systemic Sclerosis have different effects on the disease progression just like Punt et al. found out in cervical cancer (136). Unfortunately, this goes beyond the scope of this thesis and will have to be part of future papers.

5 References

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