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Eosinophil-associated inflammation, tissue damage and remodelling in skin diseases

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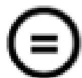 **Keine Bearbeitung**. Dieses Werk darf nicht bearbeitet oder in anderer Weise verändert werden.

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1 Abstract

Background: Eosinophils are multifunctional leukocytes. Their primary role attributes to host defence, in particular against helminth infection. Furthermore, they have a function in immunoregulation and play a role in the development of fibrosis and tissue remodelling. Effector molecules secreted by eosinophils include granule proteins, leukotrienes, enzymes and a broad spectrum of cytokines. Eosinophils are found among infiltrating cells in different skin disorders including allergic, autoimmune and infectious diseases, as well as malignancies. However, their precise functional role in the pathogenesis of various skin diseases is not fully understood yet.

Objective: This doctoral thesis is part of a research project aiming at the investigation of the potential functional roles of eosinophils in skin diseases. We were interested in the cytokine expression by eosinophils infiltrating the skin, including IL-6, IL-11, IL-17 and IL-25, and the expression of cytokines known to activate eosinophils, including IL-33 and TSLP. A further objective was to study the association of tissue eosinophils and fibrosis/remodelling and tissue damage in skin diseases. Cleaved caspase-3 was used as a marker for apoptosis. IL-6, IL-11, IL-17, MMP-9 and procollagen 3 were investigated as markers for fibrosis and/or tissue remodelling.

Methods: Skin sections of twenty-seven eosinophilic skin diseases with different pathogenesis, categorised as autoimmune, infectious and allergic/reactive diseases as well as tumours/lymphomas, were stained by immunofluorescence techniques using primary antibodies directed to cleaved caspase-3, IL-6, IL-11, IL-17, IL-25, IL-33, MMP-9, procollagen-3 and TSLP. All slides were analysed by confocal laser scanning microscopy.

Results: The expression of the effector molecules IL-6, IL-11 and MMP-9 differed significantly between disease groups. The highest number of eosinophils expressing IL-6 was found in tumours/lymphomas. MMP-9+ eosinophils were predominant in autoimmune diseases. Eosinophils in allergic/reactive diseases mainly expressed IL-11. Large numbers of eosinophils were seen in proximity to cleaved caspase-3+ epidermis cells covering blisters in bullous pemphigoid.

Conclusion: In this study we observed that eosinophils are able to express a wide spectrum of effector molecules. Interestingly, the expression varied between skin disease groups, suggesting that eosinophils play distinct functional roles. Depending on their cytokine expression patterns, eosinophils might contribute to host defence, immunomodulation, fibrosis and/or tissue remodelling in skin diseases. Apoptotic keratinocytes next to large numbers of eosinophils let assume that eosinophils might be involved in tissue damage including blister formation.

2 Abbreviations

ACD	Atopic contact dermatitis
AD	Atopic dermatitis
ALH	Angiolymphoid hyperplasia
APT	Atopy-patch-test induced allergic reaction
BCL	B cell lymphoma
BP	Bullous pemphigoid
BSA	Bovine serum albumin
CLSM	Confocal laser scanning microscopy
CM	Cutaneous mastocytosis
DH	Dermatitis herpetiformis
DHS	Drug hypersensitivity
DM	Dermatomyositis
ECTOP	Ectoparasitosis
EF	Eosinophilic fasciitis
EPF	Eosinophilic pustular folliculitis
HES	Hypereosinophilic syndrome
IF	Immunofluorescence
Ig	Immunoglobulin
IL	Interleukin
KA	Keratoacanthoma
LCH	Langerhans cell histiozytosis
LCs	Langerhans cells
LM	Larva migrans
MMP	Matrix metalloproteinase
MOR	Morphea
NS	Normal skin
PAN	Polyarteriitis nodosa
PBS	Phosphate buffered saline
PF	Pemphigus foliaceus
PI	Propidium iodide
PLY	Pseudolymphoma
PN	Prurigo nodularis
SEM	Standard error of the mean
TCL	T cell lymphoma
T_H	T helper cells
TSLP	Thymic stromal lymphopoietin
TU	Tumour
URT	Urticaria
WG	Wegener’s granulomatosis
WS	Wells’ syndrome

3 Preface

Here, I present the results of my research project, which consists of two parts, the master thesis and the doctoral thesis. Chapters of the master thesis (submitted September 19, 2010) required for the understanding and presentation of the results are cited in this manuscript (1).

My doctoral thesis is a part of a research project analysing the cytokine expression of eosinophils in various skin diseases in order to evaluate potential functions. In this sub-project, skin sections were analysed by confocal microscopy following immunofluorescence staining with nine different antibodies directed to cleaved caspase-3, interleukin (IL) -6, IL-11, IL-17, IL-25, IL-33, matrix metalloproteinase (MMP)-9, procollagen 3 and thymic stromal lymphopoietin (TSLP). The results of the expression of cleaved caspase-3 have been published in a first paper with the title “Eosinophil extracellular DNA traps in skin diseases” (2). The findings of the analysis of the other eight antibodies have been published in a second paper entitled “Distinct eosinophil cytokine expression patterns in skin diseases – the possible existence of functionally different eosinophil subpopulations” (3). These two publications are attached in chapter 8.

4 Objectives

4.1 Introduction

The research on skin disorders has not yet provided the answer to the precise functional role of eosinophilic cells in the pathogenesis of various skin diseases (4). Eosinophils are multi-functional leukocytes identifiable by their characteristical bilobar nucleus (5,6). They are found among infiltrating cells in a broad spectrum of skin disorders including allergic diseases, autoimmune diseases, infections (parasitic helminths, bacterial and viral) and malignancies (4,5,7).

4.1.1 Functions of eosinophils

Eosinophils appear under different physiologic and pathologic conditions (Fig. 1). They have an important function in host defence and immunoregulation (8,9). Furthermore, they seem to play a role in tissue remodelling and to have the potential to cause tissue damage and fibrosis (10–12).

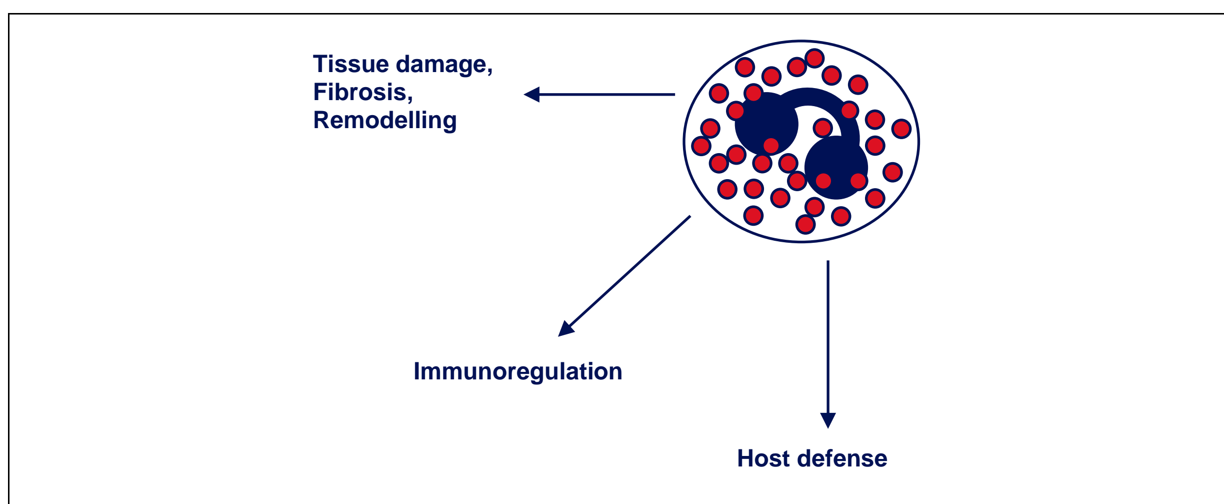

Fig. 1: Scheme of an eosinophilic cell with its functions.
Granules are shown in red, the bilobar nucleus is dark blue. Illustration adapted from Simon et al. (13) and Hogan et al. (14).

The primary role of eosinophils is regarded to be a defence against helminth infection (8). A study investigating the gastrointestinal immune system showed that eosinophils are able to release mitochondrial DNA in a catapult-like manner. The mitochondrial DNA is secreted together with granule proteins. It is able to bind and kill bacteria and therefore participates in antibacterial defence mechanisms (15). In addition to the host defence against helminth infections and bacteria, eosinophils play a role in host defence against viruses (16).

Eosinophil infiltration and degranulation has also been reported in association with fibrosis of various organs (17). In eosinophilic esophagitis remodelling and fibrosis have been observed in association with eosinophils (10). In the context of inflammation, eosinophils can cause

cell and tissue damage by releasing granule associated basic proteins, lipid mediators and oxygen species (18,19). The influence of eosinophilic inflammation on fibrosis, apoptosis, tissue remodelling and tissue damage is not very clear in the context of several skin diseases.

Eosinophils have been shown to play a role in immune regulation. They can act as antigen-presenting cells (20) and there is evidence that eosinophils can be the source of numerous effector molecules (see chapter 4.1.2).

4.1.2 Eosinophil effector molecules

Effector molecules of eosinophils include granule proteins, leukotrienes, enzymes and different cytokines, for example interleukins (IL) (Fig. 2) (5,18,21,22).

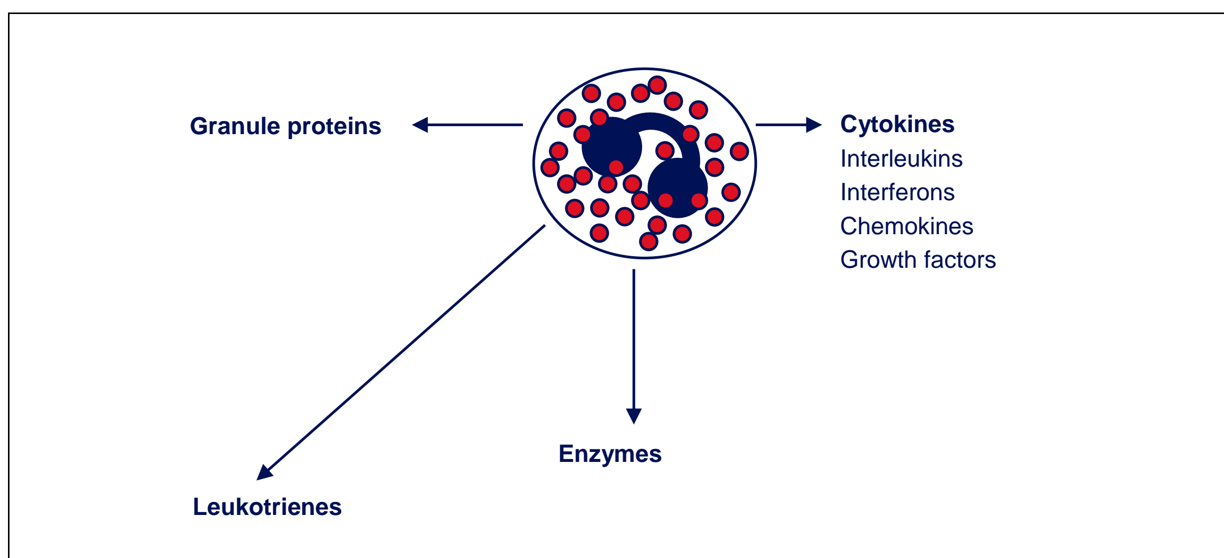

Fig. 2: Effector molecules of eosinophils.
Eosinophils can secret granule proteins, leukotrienes, enzymes and cytokines. Illustration based on Simon et al. (13) and Hogan et al. (14).

Eosinophils are able to produce, store and secret a wide range of interleukins (4,9,23,24). For instance, it has been reported that eosinophils can be the source of IL-11 (23), IL-6 (24), IL-17 (23) and IL-25 (9). Eosinophils can degranulate and release several enzymes such as the gelatinase matrix metalloproteinase-9 (MMP-9) (22).

4.1.3 Eosinophilic skin diseases

Eosinophils are present in various skin diseases (4). The classification of eosinophilic disorders in general differentiates between intrinsic eosinophilic disorders and extrinsic eosinophilic disorders. The same classification can also be utilised for eosinophilic diseases of the skin (7). In intrinsic eosinophilic disorders the primary cause of eosinophilia is inside the eosinophil linage itself. Mutations of pluripotent hematopoietic stem cells and multipotent myeloid stem cells are the two groups that belong to the intrinsic eosinophilic disorders (7).

In extrinsic eosinophilic disorders the primary cause of eosinophilia is outside the eosinophil lineage. This means, cells other than eosinophils release the cytokines that trigger eosinophilia (7).

In the following chapters, five groups of skin diseases with eosinophilia are specified: Allergic diseases, tumours, autoimmune diseases, eosinophil dermatoses and infectious diseases.

4.1.3.1 Allergic diseases

In allergic diseases cytokine-mediated increased production and/or survival of eosinophils causes eosinophilia. Therefore, allergic diseases were assigned to the group of extrinsic eosinophilic disorders (7). T helper (T_H)2 cells are important in the pathogenesis of allergic diseases. They are activated by antigen presenting cells following allergen contact. The activated T_H2 cells secrete a range of cytokines (e.g. IL-4, IL-5 and IL-13). IL-5 is the most important cytokine causing eosinophilia, because IL-5 is able to increase eosinophil activation and survival (25). In diverse allergic diseases it has been shown that eosinophilia correlates with the disease activity (26,27).

A typical example of an allergic skin disease with eosinophilia is atopic dermatitis (AD). In AD peripheral blood eosinophilia can be found associated with tissue eosinophilia. However, the exact pathogenic role of eosinophils in AD is not yet clear (4). Activated eosinophils are seen in acute and chronic AD. Pronounced tissue eosinophilia is found in chronic AD suggesting that eosinophils play a role in the development or maintenance of chronicity (28).

4.1.3.2 Tumours

Tumours from hematopoietic cells as well as solid tumours can produce cytokines and therefore are able to initiate skin eosinophilia. The primary cause of eosinophilia is outside the eosinophil lineage. Consequently these diseases belong to the group of extrinsic eosinophilic disorders (4,7).

An example for tumours associated with skin eosinophilia is Langerhans cell histiocytosis (LCH). LCH is characterized by a clonal proliferation of Langerhans cells (LCs) (29). LCs are derived from bone marrow. They migrate into the epidermis where they act as cutaneous antigen presenting cells (30). In LCH the infiltrate is not only composed of LCs but also of eosinophils, lymphocytes and neutrophils (31,32). Eosinophils in LCH were associated with the expression of diverse interleukins (e.g. IL-3, IL-5, IL-7 and IL-10) (33). The exact function of eosinophils in LCH remains poorly understood (34).

4.1.3.3 Autoimmune diseases

Autoimmune diseases represent a further group of extrinsic eosinophilic disorders. Eosinophilia can be found in the dermis, epidermis and also in the peripheral blood of diverse autoimmune skin diseases (7).

Bullous pemphigoid (BP) and eosinophilic fasciitis (EF) are two examples of autoimmune skin diseases with eosinophilia. In BP peripheral blood eosinophilia can be found associated with tissue eosinophilia (4). BP is a skin disorder characterised by subepidermal blister formation (35). Eosinophils seem to play a role in the development of the blisters by releasing toxic granule proteins and proteolytic enzymes. However the molecular mechanisms are not yet fully clear (4,22). In the histology of EF an inflammatory infiltrate composed of lymphocytes, eosinophils, plasma cells, and histiocytes can be found in the dermis. EF is associated with fibrosis (7,36).

4.1.3.4 Eosinophil dermatoses

Wells' syndrome (WS) and hypereosinophilic syndrome (HES) are two typical eosinophil dermatoses. In WS the histology shows oedema and an infiltrate of eosinophils, lymphocytes and histiocytes in the dermis (37,38). Eosinophils are the predominant infiltrating cells (7). HES includes diverse disorders with persistent blood eosinophilia. Different underlying aetiologies describe different subtypes of HES (39–41). HES does not only affect the skin, but also the heart, the nervous system, the lung and the blood (40).

4.1.3.5 Infectious diseases

In the classification of eosinophilic disorders, infectious diseases belong to the group of extrinsic eosinophilic disorders (7). Eosinophilia can be found in infections with helminths, bacteria and viruses (see chapter 4.1.1) (5).

Larva migrans (LM) and ectoparasitosis (ECTOP) are two infectious diseases associated with eosinophilia (7). Two examples for ECTOP are scabies and arthropod bites. Eosinophils were seen in scabies in skin lesions with bullae and in the dermis of arthropod bites (42,43).

4.2 Aim of the doctoral thesis

As mentioned above, my research project is composed of both a master thesis and a doctoral thesis. The aim of the general project was to contribute to the understanding of the function of eosinophils in skin diseases.

The master thesis aimed at capturing the results of the master project consisting of laboratory work and a preliminary analysis of the results. It served as an intermediate step for further analysis in a next phase.

The aim of the doctoral thesis was to perform an in-depth analysis and thorough interpretation of the interim results. In particular, we were interested in:

1. The cytokine expression by eosinophils.
2. Fibrosis and tissue remodelling.
3. Tissue damage including apoptosis in association with tissue eosinophilia.
4. The composition of the inflammatory infiltrates.
5. The expression of cytokines known to activate eosinophils in a broad spectrum of different eosinophilic skin diseases.

These findings might provide information on a possible role of eosinophils under certain pathologic conditions.

5 Materials and methods

5.1 Antibodies used in this study

Skin sections of twenty-seven different eosinophilic skin diseases were stained by nine distinct primary antibodies. The following table shows the primary and compatible secondary antibodies used in this study, their clonality, their immunoglobulin isotype and the host animals that were used for their fabrication (Table 1).

In chapter 5.1.1 to chapter 5.1.9 some already known functions of the nine antibodies used in this study are described. The main focus is on the connection between the diverse antibodies and eosinophils, fibrosis, apoptosis, tissue remodelling and tissue damage.

Primary Antibody	**Company Country Product / Catalogue No Lot ID**	**Secondary Antibody**	**Company Product / Catalogue No Lot ID**
Mouse			
Mouse Anti-Human IL-25 (IL-17E) Monoclonal IgG	LifeSpan BioSciences USA LS-B123 Lot: 13861	Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-mouse IgG	Invitrogen Molecular Probes A11017 Lot: 727755
Mouse Anti IL-33 Monoclonal IgG	Enzo Life Science USA ALX-804-840-C100 Lot: L23118/b	Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-mouse IgG	Invitrogen Molecular Probes A11017 Lot: 727755
Mouse Anti IL-6 Monoclonal IgG	Santa Cruz Biotechnology USA Sc-130326 Lot: C0909	Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-mouse IgG	Invitrogen Molecular Probes A11017 Lot: 727755
Mouse Anti MMP-9 (2C3) Monoclonal IgG	Santa Cruz Biotechnology USA Sc-21733 Lot: G1808	Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-mouse IgG	Invitrogen Molecular Probes A11017 Lot: 727755
Rabbit			
Rabbit Anti TSLP (FL-159) Polyclonal IgG	Santa Cruz Biotechnology USA Sc-33791 Lot: K1907	Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-rabbit IgG	Invitrogen Molecular Probes A11070 Lot: 486513
Rabbit Anti IL-17 (H-132) Polyclonal IgG	Santa Cruz Biotechnology USA Sc-7927 Lot: I1608	Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-rabbit IgG	Invitrogen Molecular Probes A11070 Lot: 486513
Rabbit Anti Procollagen Type III (PIIINP) Polyclonal Ig*	Chemicon (Millipore) USA AB764P Lot: LV1471024	Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-rabbit IgG	Invitrogen Molecular Probes A11070 Lot: 486513
Rabbit Anti Cleaved Caspase-3 (Asp175) Polyclonal IgG	Cell Signaling Technology USA # 9661 S Lot: 23	Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-rabbit IgG	Invitrogen Molecular Probes A11070 Lot: 486513
Goat			
Goat Anti-Human IL-11 Polyclonal IgG	R&D Systems USA AB-218-NA Lot: DX06	Alexa Fluor® 488 F(ab')$_2$ fragment of rabbit anti-goat IgG	Invitrogen Molecular Probes A21222 Lot: 56569A

Table 1: Primary and secondary antibodies used in this study (1)**.**
* There is not only one isotype present in the product but the whole range of antibodies that recognize the antigen.

5.1.1 Cleaved caspase-3

Cleaved caspase-3 is the last caspase in a signalling pathway called caspase cascade. The signalling pathway plays an important role in the induction of apoptosis. Therefore, cleaved caspase-3 can be used as marker for apoptosis. In a study about acute AD, caspase-3 cleavage was detected in normal skin (NS) as well as in acute lesional AD skin. In NS cleaved caspase-3 was expressed only by single cells of the basal layer. In acute AD skin an increased presentation of cleaved caspase-3 was observed in the basal layer and additionally cleaved caspase-3 was found in the spinous cell layer, mainly in spongiotic areas. Those observations led to the opinion that apoptosis in the basal cells could be in the course of physiologic epidermal renewal, whereas the secretion of cleaved caspase-3 in the spinous layer could be a sign of a pathologic event leading to spongiosis in AD (44).

5.1.2 IL-6

Different cells are able to produce IL-6. In systemic fibrosis for example it has been described that macrophages and dendritic cells are able to secrete IL-6. Together with other mediators IL-6 might lead to inflammation and fibrosis (45). In BP the release of IL-6 from human keratinocytes was described (46). Furthermore, IL-6 can also be produced by human eosinophils (24).

5.1.3 IL-11

IL-11 is a cytokine produced by different cells including epithelial cells, fibroblasts and eosinophils (23,47). A significant correlation between IL-11 and type I collagen deposition as well as IL-11 and the number of eosinophils was observed in AD. Therefore, the following two implications were suggested. First, eosinophils might be the major producers of IL-11 in chronic lesions. Second, IL-11 is probably involved in the remodelling of skin lesions in AD (23). Furthermore, IL-11 was observed to be increased in asthma, suggesting a crucial role of IL-11 in allergic diseases (23).

5.1.4 IL-17

IL-17 is a proinflammatory cytokine mainly released by T lymphocytes (48,49). Eosinophils can also be a source of IL-17 (23). IL-17 helps to protect against infections, plays a role in autoimmune diseases and it is involved in the induction and maintenance of chronic inflammatory diseases (48,50). In addition it was suggested that IL-17 is involved in the remodelling of skin lesions. It was observed that IL-17 is able to initiate tissue fibrosis in an indirect manner through the release of IL-11 in AD (23).

5.1.5 IL-25

Different cells are able to produce IL-25. For example T_H2 cells, basophils and epithelial cells can be a source of IL-25 (9,51,52). Eosinophils are also able to secret IL-25. IL-25 can lead to an enhanced function of adaptive T_H2 memory cells and consequently augments allergic inflammation. Evidence for a possible role of IL-25 in allergic diseases is the elevated expression of IL-25 observed in allergic disorders such as AD (9). In helminth infections IL-25 seems to be an important regulatory molecule inducing a potent response for the elimination of the parasite infection (53).

5.1.6 IL-33

IL-33 is produced by various cells including endothelial cells, epithelial cells and smooth muscle cells (54). IL-33 is able to affect eosinophils. There is indication that eosinophil adhesion and enhanced eosinophil survival can be due to IL-33 (55). IL-33 can act as potential direct activator of eosinophils and basophils. By acting on these two effector cells and on lymphocytes IL-33 may be an important cytokine leading to a T_H2 dominant inflammation (56,57).

5.1.7 MMP-9

MMP-9 is an enzyme produced by T cells, mast cells and eosinophils (58). MMP-9 can be found at the site of blister formation in bullous pemphigoid. It degrades various components of the basement membrane, for example type IV collagen, proteoglycans and laminin (22,59). Diverse studies suggested that MMP-9 might be involved in the formation of blisters in BP (22,58). Furthermore, MMP-9 was described as serum marker for skin fibrosis in a study about burn trauma (60).

5.1.8 Procollagen 3

Procollagen 3 is the precursor structure of collagen 3. It is synthesised and afterwards secreted into the extracellular space. Proteinases convert the procollagen 3 into the functional collagen 3 molecule (61,62). In a study on severe burn trauma, procollagen 3 was described as serum marker for skin fibrosis. Increased procollagen 3 was an indication for fibrogenic components (60).

5.1.9 TSLP

Thymic stromal lymphopoietin (TSLP) is a cytokine produced by different cells such as keratinocytes (63), epithelial cells (64) and smooth muscle cells (65). The high expression of TSLP in AD and in asthma indicated the importance of TSLP in allergic diseases (66,67). It was observed that eosinophils express functional TSLP receptors. Therefore, TSLP can

have direct effects on eosinophils. It is able to induce chemotactic and prosurvival effects in eosinophils. Due to TSLP the adhesion of eosinophils on fibronectin is enhanced, the trans-endothelial migration is facilitated and finally tissue eosinophilia is advanced. In addition TSLP can trigger the eosinophils to release inflammatory cytokines (68).

5.2 Skin sections

The skin sections used in this study were prepared and provided by the Department of Dermatology, Inselspital, University of Bern. Skin biopsies were fixed with paraformaldehyde and embedded in paraffin. Afterwards they were cut into thin slices and put onto glass object plates.

Samples of allergic, infectious and autoimmune skin diseases were chosen for the purpose of the present study. Further, specimens of eosinophil dermatoses and tumours affecting the skin as well as sections of normal skin were chosen. Table 2 shows the spectrum of selected diseases.

Allergic Diseases	**Autoimmune Diseases**
Atopic dermatitis (AD)	Bullous pemphigoid (BP)
Atopy-patch-test induced allergic reaction (APT)	Pemphigus foliaceus (PF)
Atopic contact dermatitis (ACD)	Dermatitis herpetiformis (DH)
Urticaria (URT)	Eosinophilic pustular folliculitis (EPF)
Drug hypersensitivity (DHS)	Polyarteriitis nodosa (PAN)
Prurigo nodularis (PN)	Dermatomyositis (DM)
	Morphea (MOR)
	Eosinophilic fasciitis (EF)
Tumours	Wegener's granulomatosis (WG)
Tumour (TU)	**Eosinophil Dermatoses**
Keratoacanthoma (KA)	Wells' syndrome (WS)
T cell lymphoma (TCL)	Hypereosinophilic syndrome (HES)
B cell lymphoma (BCL)	
Langerhans cell histiozytosis (LCH)	**Infectious Diseases**
Pseudolymphoma (PLY)	Ectoparasitosis (ECTOP)
Angiolymphoid hyperplasia (ALH)	Larva migrans (LM)
Cutaneous mastocytosis (CM)	

Table 2: Diseases observed in this study (1).
Abbreviations in brackets (Abbr.).

5.3 Staining procedure

Samples of skin diseases and samples of healthy skin were processed with immunofluorescence (IF) procedure. The paraffin-embedded skin sections were stained using nine different antibodies for indirect staining method. For indirect IF, two antibodies are used. The primary antibody is directed towards the molecule of interest on the skin sample and is unlabelled. The secondary antibody reacts with the first antibody and is chemically conjugated to a fluorochrome, which serves as fluorescent dye (69).

On day one, the whole batch of slides was incubated in the oven for at least two hours at 52°C. Afterwards the slides were deparaffinised in Neo-Clear solution. Neo-Clear is not water soluble, therefore the dewaxed samples were rehydrated in a series of decreasing concentrations of alcohol. After washing the slides with distilled water they were immersed in phosphate buffered saline (PBS) pH 7,4.

The tissue sections were then transferred into heat resistant polypropylene boxes containing sodium-citrate buffer pH 6 and subsequently microwaved at defrost and afterwards cooled at room temperature. The microwave/cooling cycle was performed two more times. For cleaved caspase-3 and anti human IL-6 staining, the samples were heated in the autoclave instead of performing the microwave/cooling cycle.

Next, the slides were washed with distilled water and PBS buffer and then treated with blocking solution. Thereafter, the skin sections with applied blocking solution were incubated in a moist chamber to prevent drying of the tissue sections (70). Afterwards the blocking solution was removed and replaced with the primary antibody diluted in blocking solution. The tissue sections with primary antibody applied were put into a moist chamber and were incubated overnight at 4°C.

On day two, the samples were washed again with PBS buffer. The secondary antibody diluted in 7,5% bovine serum albumin (BSA) was pipetted onto each skin section. Afterwards the slides were placed into the moist chamber and incubated at room temperature for one hour. At this point, two additional staining steps had to be performed in the case of anti TSLP (thymic stromal lymphopoietin) and anti IL-6 antibody. To make the cell nucleus visible each tissue section was incubated with propidium iodide (PI) in PBS. Afterwards slides were washed with PBS. Later on, a coverslip was fixed on every slide. The tissue sections were now ready to be analysed with the confocal laser scanning microscope.

Samples of healthy skin served a twofold purpose. On the one hand as reference to samples of skin diseases on the other hand to exclude random binding of secondary antibodies.

In order to have the possibility to compare skin from diseased subjects with skin from healthy subjects, normal skin (NS) sections were stained simultaneously with the samples of skin diseases. The fixation and staining procedures were identical and primary as well as secondary antibodies were applied.

In order to exclude random binding of secondary antibodies an approach called negative tissue controls was used. Negative tissue controls are specimens processed identically to the skin diseases, but not containing the relevant tissue marker (70). Therefore, NS samples were stained without the primary antibody to make sure that the secondary antibody did not bind to tissue antigens without the primary antibody.

5.4 Tissue section analysis using confocal laser scanning microscopy (CLSM)

In this study all skin sections (normal skin and skin diseases) were analysed with CLSM. The excitation wavelength 488 nm of the argon laser was selected in order to detect the fluorescent dye Alexa Fluor® 488, which was attached to every secondary antibody used in this study.
In the case of the slides stained by anti TSLP or anti IL-6 antibody an additional laser was necessary to scan the light emitted from PI. Therefore, the helium/neon laser with the excitation wavelength 543 nm was utilised.

Every sample was first viewed 400-times magnified. A magnification of 1000x was used for close scrutiny. Positive cells were counted with a magnification of 1000x in ten consecutive fields in representative areas of the skin section. In the stainings with the nine different antibodies the attention was not always directed towards the same positive cells. The exact number of positive eosinophils was evaluated in specimens stained by antibodies against IL-6, IL-25, IL-11, MMP-9 and procollagen 3. In the staining with antibody against IL-17 positive lymphocytes were counted. The total of positive cells in ten fields was determined for IL-6, cleaved caspase-3, IL-25, MMP-9 and procollagen 3.

A further point of interest was the epidermis. For the stainings with antibodies against IL-6, cleaved caspase-3, IL-25, IL-11, IL-33 and TSLP the distribution of positive cells in the epidermis was examined. IL-6+ dendritic cells were searched in the epidermis of all diseases.

In some stainings additional examinations were performed. In specimens stained by antibodies against IL-6 and IL-33, we evaluated the cytokine expression by epithelial cells and/or endothelial cells of the blood vessels. Procollagen 3+ extracellular material was evaluated in all skin diseases. Finally, in all sections stained by antibodies against cleaved caspase-3, positive extracellular structures containing DNA (so-called eosinophil extracellular traps (2)) were searched for.

5.5 Statistical analysis

In this study, the results were shown in diagrams and statistically analysed using GraphPad Prism® (version 5.01). Graphs depict the following four groups of eosinophilic skin diseases: Autoimmune diseases, infectious diseases, tumours/lymphomas and allergic/reactive diseases. The group of allergic/reactive diseases is composed of all allergic diseases plus all eosinophil dermatoses (see Table 2). Lines were drawn for means ± SEM. One-way ANOVA was applied in order to determine whether the means among groups are significantly different. Furthermore, graphs were drawn demonstrating the correlation between two variables and Pearson's coefficient was determined. A p value of <0.05 was considered statistically significant.

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8 Publications

The following two papers containing the results of my doctoral thesis are an integral part of this work:

1. Simon D, Hoesli S, Roth N, Staedler S, Yousefi S, Simon H-U. Eosinophil extracellular DNA traps in skin diseases. J. Allergy Clin. Immunol. 2011;127:194–199.
2. Roth N, Städler S, Lemann M, Hösli S, Simon H-U, Simon D. Distinct eosinophil cytokine expression patterns in skin diseases - the possible existence of functionally different eosinophil subpopulations. Allergy. 2011;66:1477–1486.