SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the role of Thymic Stromal Lymphopoietin in healthy skin and in immune-mediated skin inflammation

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The Examination took place at the Conference Room of Building C, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 23rd June 2016 at 11:00 a.m.

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 6th October 2017 at 1 p.m.

INTRODUCTION

As an outstanding discovery of recent years, the microbial community has been shown to exhibit remarkable differences on topographically distinct skin areas. It has been demonstrated that colonization of these bacteria is dependent on the physiology of the skin site, as specific bacteria are being associated with moist, dry or sebaceous microenvironments, and the diversity of the chemical milieu in which these microbial communities live was also described. High-scale diversity of the microbiota was not only described on the skin barrier surface, but distinct sections of the gut are also known to be colonized by heterogeneous microbiota, which is associated with the different anatomical and physiological features of these sites. Besides the diversity of microbiota, recent studies indicated a mutual relationship between the host and these microorganisms, since they play important role in tissue homeostasis and local immunity. These assume the possibility that the level of immune activation may differ in distinct barrier surfaces, which has been already indicated in the gut. For example, thymic stromal lymphopoietin (TSLP), one of the major epimmunomes (epithelial cell-derived molecules which can instruct immune cells), was detected only in particular gut sections, with its highest, constitutive expression in colonic epithelial cells (ECs). This protein is involved in the development of tolerance to commensal microflora through modulation of dendritic cell (DC) functions in the gut. The tolerogenic role of TSLP is supported by recent studies where decreased TSLP level and altered microbial composition were found in Crohn's disease. Until now, TSLP in the skin was only described under inflammatory conditions, such as atopic dermatitis (AD) and psoriasis, and its only known function in this organ so far is the promotion of T helper (Th)2 polarizing DCs.

In our first study, we asked the question whether the above topographical differences in skin microbiota and physiology can also be accompanied by topographical differences in skin immune activity and TSLP production. The possibility that the skin immune system is characterized by distinct functional tuning on different skin regions was not challenged until now in the literature.

In our second study, we aimed to determine whether TSLP production and other components of the immune-mediated skin inflammation (KC function, T cell and DC count) differ between severe AD patients with or without common R501X and 2282del4 filaggrin (*FLG)* mutations. The T helper (Th) 2 promoting capacity of TSLP is well-known in AD skin, but until now no data can be found in the literature which distinguishes and compares KCs' TSLP production and other innate immune functions, and T cell and DC counts in the lesional

skin of severe AD patients with genetic or acquired FLG loss.

TSLP protein, its receptor and regulators

TSLP was first cloned and identified in the medium of a murine thymic stromal cell line, as a growth factor effecting B cell development. After cloning the human form of TSLP it was proven that its sequence homology is only 34% with its mouse orthologue. The human TSLP gene is localized in chromosome 5q22.1 next to the atopic cytokine cluster on 5q31. This four-helix bundle short chain hematopoietic cytokine is characterized by strong structural and functional homology to IL-7 and shares an overlapping, but distinct, biologic profile.

According to recent studies a second, short TSLP isoform was also identified. The long form TSLP protein (described earlier) consists of 159 amino acids and the short form TSLP has the same amino acid sequence, but the first 96 amino acids are missing.

Biological activity of TSLP is exerted by binding to its heterodimer receptor, which consists of IL-7 receptor α chain and TSLP receptor (TSLPR). The affinity of human TSLPR alone for TSLP is low, but after forming a high-affinity complex with IL-7Rα, dimerization can trigger TSLP signaling. TSLPR is expressed only by a few cell types, namely DCs, monocytes and some T cell clones. Regarding the function of the two TSLP isoforms Fornasa et al. and Bjerkan et al. found that short form TSLP may have anti-inflammatory and antimicrobial properties and was also stated as homeostatic, while the long form TSLP could be connected to the initiation of inflammation. Nevertheless, until now, only a few workgroups investigated the exact function of the two TSLP isoforms. Further experiments are necessary to clarify their specific role in steady state, as well as in inflammatory conditions. In the recent years, since TSLP was described in numerous homeostatic and diseased conditions, its role was highly emphasized. TSLP expression can be promoted through either Toll-like receptor (TLR)3 ligands, Th2 cytokines, TSLPR or IL-7R and multiple regulatory molecules can influence its expression. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activated by IL-1β and tumor necrosis factor (TNF-α) is one of the positive regulators of TSLP gene expression, but may be also controlled by the mitogen-activated protein kinase (MAPK) pathway. Double-stranded RNA and poly(I:C), a well-known TLR3 ligand and agonist, can also induce TSLP via the activation of NF-κB, interferon regulatory factor 3 (IRF3) and activating protein (AP)-1, while IL-4 and IL-13 can signal transducer and activator of transcription 6 (STAT6)-dependently promote TSLP expression. Retinoid X receptor (RXR)-α and/or RXR-β dimers in the presence of co-repressors such as free vitamin D receptor (VDR) or retinoic acid receptor-γ can efficiently

inhibit TSLP expression. Glucocorticoids can also negatively regulate its expression, probably by inhibiting AP-1 or NF-κB.

Role of TSLP in the thymus

The corpuscular bodies of ECs, namely Hassall's corpuscles, located within the thymic medulla, were described first time by Arthur Hill Hassall in 1849. These structures are well developed in human thymus and „represent the 'graveyard' for dead thymocytes, and also the 'privileged' area for the maturation of medullary thymocytes". Active cytokine or growth factor receptor-mediated cell signaling and cell metabolism are characteristic to Hassall's corpuscles, as transforming growth factor (TGF)-α, interleukin (IL)-7, stromal cell-derived factor 1, CD30 ligand and macrophage-derived chemokine (MDC) were found to be expressed within them. These findings suggest active communication between thymus and antigen-presenting cells as well as developing T cells. Moreover, thymic ECs express TSLP within the human thymic medulla.

TSLP-activated DCs are suggested to have key role in the promotion of thymic T cells to differentiate into regulatory T cells (Treg), since parallel with the high expression levels of MHCII and the mentioned co-stimulatory molecules, which are necessary to Treg development, they can also induce homeostatic naïve T cell proliferation, and development of Treg cells can be inhibited by proinflammatory cytokines. The localization of CD4+CD25+ thymocytes is restricted to the thymic medulla and they are in close connection with activated DC-LAMP+ CD86+ DCs and Hassall's corpuscles, thus these finding suggest that Treg cells are developed in the thymic medulla in association with DCs activated by TSLP, which is originated from the ECs of Hassall's corpuscles.

Several transcription factors are responsible for the development of different lymphoid cell lineages, not only determining the fate of T cells in the early stage, but also have a crucial role in repressing alternative pathways of their differentiation. As a specific example, IL-12 and IL-4, the well-known Th1 and Th2-promoting cytokines, can override FOXP3 pathway and actively inhibit Treg cell development from CD4+ thymocytes. Although a unique niche is characteristic to the thymus, its exact nature remains to be determined.

Role of TSLP in the gastrointestinal tract

In contrast to the thymus, a dual role of TSLP has been described in the gut, since previous studies revealed its homeostatic function in low concentration, as well as, its capability of promoting inflammation in both increased and decreased levels. Intestinal ECs constitutively express TSLP in the lower gastrointestinal tract and its highest levels have been

detected in the colon. Constitutive production of TSLP was also described in human intestinal ECs *in vitro*. Previous studies suggested the role of interactions between gut microbiota and intestinal ECs' basal TSLP production, which may promote tolerance of DCs in the mucosa to commensal microbiota. These DCs conditioned by intestinal ECs can upregulate their OX40 ligand expression promoting the polarization of T cells into noninflammatory Th2 type and downregulate p40, a subunit shared between IL-12 and IL-23 heterodimer cytokines. These DCs also have the ability to induce FOXP3+ Treg cells. These results support the crucial role of TSLP in the maintenance of intestinal immune homeostasis. The role of TSLP was also described in diseased conditions of the gut. Inflammatory bowel diseases (IBD), namely Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial diseases with abnormal immune responses to commensal microbiota in the gut. Elevated TSLP levels were observed in the mucosal lesions of UC patients and inflammatory Th2 cytokines were proven to be responsible for this enhancement. It is also known that Th2 associated inflammation can be promoted by TSLP, thus a vicious circle may lie in the background of UC's pathogenesis as a result of an allergic condition. In contrast, in CD, colonic ECs express lower levels of TSLP compared to healthy controls and primary IECs from patients with CD do not produce it, subsequently, IL-12 release by commensal-activated DCs cannot be inhibited. In response to these events Th1/Th17 type inflammation, characterized by IFN-γ and IL-17 production, is initiated. The important role of TSLP has been revealed in other organs of the gastrointestinal tract, such as in the esophagus. In eosinophilic esophagitis (EoE), an allergic disease of this organ, ECs overexpress TSLP mRNA and a prominent influx of basophils is also a characteristic feature. In EoE Th2 type inflammation may be promoted by TSLP via basophils' histamine and Th2 cytokine and chemokine secretion. TSLP also activates DC maturation and activation, promoting Th2 cells. Moreover, TSLP can directly influence T cells to secrete Th2 cytokines.

Role of TSLP in the airways

Regarding the role of TSLP in the airways, the most data in the literature is connected to its inflammatory characteristic, less is known about its action in homeostatic conditions. Previous studies revealed TSLP as a key molecule in the initiation of allergic airway inflammations such as asthma, allergic rhinitis (AR) and nasal polyposis. Although healthy human bronchial epithelial and smooth muscle cells, as well as lung fibroblasts, express TSLP, in the airway epithelium of asthma patients its mRNA and protein levels were detected to be increased and correlated with the levels of Th2 characteristic cytokines and disease severity. Genome-wide association studies (GWAS) revealed that TSLP is a susceptibility factor in the development of asthma. Similar findings were found in individuals with chronic obstructive pulmonary disease, proposing that dysfunctional epithelium may have a role in initiating TSLP upregulation in the lung of asthma patients. Together with asthma and AD, AR makes up the so-called "allergic triad". GWAS studies demonstrated the association of TSLP polymorphism with AR in patients with asthma. TSLP seems to be a key player in AR pathophysiology as in the nasal epithelium of patients suffering from AR TSLP levels were detected to be increased compared to controls, correlated with disease severity and IL-4 levels and associated with Th2-type inflammation by promoting Th2 cells and by inhibiting Tregs.
Nasal polyposis is another inflammatory disease of the upper airways. In nasal polyps increased TSLP expression was detected. TSLP levels were found to be correlated with IgE levels and eosinophil counts. TSLPR and OX40L expression of DCs were also upregulated in nasal polyps.

It is hypothesized, that TSLP acts through similar pathways in the members of the "allergic triad. TSLP takes effect on DCs by upregulating their OX40L expression and boosting Th2 chemokine secretion, which finally leads to the promotion of inflammatory Th2 cells and Th2 cytokine production. It is important to note that TSLP alone is not capable of initiating a fully developed allergic airway disease since the presence of foreign antigens and CD4+ T cells is also required. TSLP is most likely a crucial susceptibility factor in the airways to the promotion of altered Th2 responses in allergy.

Role of TSLP in the skin

Although TSLP mRNA expression has already been detected in healthy skin, its protein expression and exact role were described only in the inflamed epidermis of AD and psoriatic patients and in Netherton syndrome, a severe genetic skin disease, until now. Till our study, no data could be available regarding the possible homeostatic role of TSLP in the skin.

Netherton syndrome

Netherton syndrome is an autosomal recessive skin disorder caused by a loss-of-function mutation in serine protease inhibitor of kazal type 5 (SPINK5) gene encoding lympho-epithelial kazal type related inhibitor (LEKTI) and characterized by constant atopic manifestations. The loss of LEKTI causes permanent activation of serine proteases. In turn, protease-activated receptor-2 (PAR2) can be directly activated by KLK5 leading to the upregulation of TSLP expression and to the induction of TSLP production in KCs. Parallel to PAR2, KLK7 and neutrophil elastase (ELA2) can be also activated by KLK5 promoting the formation of the dysfunctional skin barrier. In consequence of barrier alterations, microbes and allergens can penetrate it leading to IL-1β production via caspase 1 activation which further enhances inflammation.

Psoriasis

Interestingly, in a recent study, TSLP has been found to be highly expressed in the epidermis of patients with psoriasis. These results were unexpected as previously the role of TSLP has been described only in the pathogenesis of Th2 diseases, but not in psoriasis, an autoimmune disease with well-known Th1/Th17 characteristics. Volpe et al. reported that TSLP and OX40 ligand could synergistically induce IL-23 production of DCs. Furthermore, the authors found that IL-4, a Th2 promoting cytokine could STAT6-independently inhibit the production of IL-23 in DCs triggered by TSLP and OX40 ligand together. These results suggest that TSLP can act in different ways depending on the type of inflammation and propose TSLP as a potential therapeutic target in the treatment of psoriasis.

Atopic dermatitis

AD is a chronic inflammatory skin disease, which is often accompanied by other allergic diseases and impaired quality of life and is driven by interactions between genetic and environmental factors. Over-reactive adaptive, dysregulated innate immune responses, and impaired skin barrier functions together lead to the manifestation of the disease. Previous studies have shown that AD is a Th2-mediated disease and the simultaneous presence of Th1 and Th22 cells in the chronic phase of skin inflammation was also detected. Besides the altered adaptive immune functions, dysregulated innate immune and skin barrier mechanisms have also been studied. A growing number of evidence supports the hypothesis that KCs can enhance the inflammatory responses in AD by producing a unique profile of cytokines and chemokines (e.g. TSLP, IL-33, and CCL27). In AD skin, TSLP is produced by KCs and is known for its capacity to induce CD11c+ myeloid DCs to promote Th2-skewed inflammatory

responses. Previous studies have shown significantly elevated serum, epidermal and stratum corneum TSLP levels in AD compared to controls. Its expression in the stratum corneum correlated with clinical severity. In the last decade, the role of KCs in the background of skin barrier dysfunction has also been highly emphasized. FLG is a crucial skin barrier structural protein of the skin and previous investigations have demonstrated that common (R501X and 2282del4), as well as rare (S3247X, R2447X and 3702delG) *FLG* null mutations, are crucial predisposing factors for AD. On the other hand, several previous investigations have indicated that inflammatory cytokine and chemokine milieu can similarly impair skin barrier in severe AD leading to acquired FLG loss by down-regulating the gene expression of *FLG* and profilaggrin processing enzymes. Until now, the question of whether genetic or acquired skin barrier dysfunctions can alter KC immune function differently has not been raised.

Possible role in healthy skin and papulopustular rosacea

Our skin provides an effective first line protection against pathogens and physico-chemical insults, on the other hand, harmless environmental agents and commensal microbiome are tolerated. Different layers of this barrier have been distinguished: the physical, the chemical/biochemical (antimicrobial, innate immunity) and the adaptive immunological barriers. The physical barrier consists mainly of the stratum corneum, but the nucleated epidermis, the cell-cell junctions and associated cytoskeletal proteins also contribute to this function. The chemical/biochemical barrier is formed by lipids, acids, hydrolytic enzymes, antimicrobial peptides. The immunological barrier is composed of humoral and cellular constituents of the immune system, both in the epidermis and the dermis.

The ultrastructure of the skin surface is riddled with invaginations, including sweat glands, hair follicles, and sebaceous glands. These appendages go through the barrier into the dermis becoming a channel for external agents to reach inner tissues.

Eccrine sweat glands are distributed across nearly the entire skin surface, and contribute to maintain a cool, dry and slightly acidic environment. Furthermore, they constitutively secrete antimicrobial peptides, limiting the composition of microbes. Apocrine sweat glands are found mainly in sites such as the axilla, genitalia and perianal regions, and start their activity at puberty.

Sebaceous glands are mainly found in hairy areas of the skin and they are connected to hair follicles, forming the pilosebaceous unit. They secrete sebum, the lipid-rich substance which lubricates the hair and skin creating an anoxic, lipid-rich milieu. The breakdown of sebum generates free fatty acids, which work to control microbial colonization along with

sebocyte derived cathelicidin, defensins, and antimicrobial histones.

Based on the density of apocrine sweat glands and sebaceous glands we can distinguish three types of skin regions: sebaceous, moist and dry areas. Moisturized sites like the scalp or axilla may support dense hair growth, and sebaceous locations produce more oil, such as the face, back, and chest. The driest sites are the volar forearm and the hypotenar palm. Skin microbial community exhibits remarkable differences on sebaceous, dry and moist regions probably connected to the different physiology of these sites.

Since skin microbiota has a mutualistic connection with the skin immune system, possible immunological distinctions between topographically different healthy skin sites can be postulated since in the gut it plays important role in tissue homeostasis and local immunity.

According to recent literature data in healthy skin TSLP mRNA expression was detected, but its protein expression and its exact role were not investigated in details.

On the basis of these, above mentioned findings in our investigations, we asked the question whether topographically different skin areas bear distinct immune characteristics. In order to answer our questions we aimed to compare the immune milieu of healthy sebaceous gland rich (SGR) and sebaceous gland poor (SGP) skin areas, and of two inflammatory skin diseases characteristically localized on SGP and SGR skin sites (AD and Papulopustular rosacea [PPR], respectively).

PPR is a Th1/Th17-mediated inflammatory skin disease, exclusively localized to SGR skin part. In PPR TLR2 and NALP3 up-regulation, elevated cutaneous protease activity and LL-37 mRNA and protein expression were detected despite the absence of an obvious infectious or dangerous trigger. It is suggested in the literature that, although the well-known rosacea triggers do not activate TLRs or NLRs under normal conditions, decreased tolerance could explain the increased skin sensitivity and the triggering of inflammatory pathways by rosacea associated otherwise harmless agents. Until our investigation, the possible role of TSLP in the background of PPR pathogenesis has not been revealed.

Role of TSLP in other conditions

The expression of TSLP has been described in conjunctival ECs, corneal ECs and corneoscleral tissues from patients with chronic allergic keratoconjunctivitis.

TSLP is also detectable in human breast milk, most likely produced by mammary ECs. Its role and possible contribution to the development of allergic conditions and of the gastrointestinal tract in the fetus is unknown in the present time. A recent study has been revealed the possible role of TSLP in maternal-fetal tolerance.

An increasing number of evidence suggests the importance of TSLP in autoimmune diseases, but its exact role in their pathophysiology is still unclear. For the present time, rheumatoid arthritis is the only autoimmune disease where the direct role of TSLP has been proven. Increased TSLP and TNF-α concentrations were reported in synovial fluid and fibroblasts of patients.

Besides the role of TSLP in the mentioned homeostatic and diseased conditions, TSLP is also a key player in response to different infections. Large intestinal ECs subjected to infection with the nematode pathogen *Trichuris* rapidly upregulate TSLP mRNA expression, indicating that the composition of commensals can be sensed in the gastrointestinal tract by ECs. In response to *Salmonella typhimurium* infection ECs also produce high amounts of TSLP, but as only a narrow interval of TSLP concentration has been detected to attenuate IL-12 release and promote non-inflammatory Th2 polarization. In *Helicobacter pylori*-infected follicular gastritis, TSLP protein production was described in mucosal lesions. KCs and airway ECs were detected to upregulate their TSLP expression in response to viral infections, but the exact role of TSLP is unrevealed until now.

The role of TSLP in cancer development has been also recently reported. TSLP is highly expressed in various cancers and melanoma as well as breast cancer cell lines. TSLP has been described to promote intratumoral Th2 differentiation which led to tumor growth. Human pancreatic cancers were also found to be associated with TSLP. Similar to human breast cancer, prominent Th2 infiltration was reported. Cancer-associated fibroblasts in the presence of TNF-α and IL-1β could secrete TSLP, which upregulated the expression of TSLPR on DCs allowing them to acquire Th2-polarizing capability. Increased serum TSLP levels were also reported in patients with cutaneous T cell lymphomas. Tregs are described to be present in elevated numbers in tumors and peripheral blood of patient which may explain how tumor cells can escape immune surveillance of the host. Moreover, in lung cancer tissues TSLP expression correlated with the number of Treg cells.

OBJECTIVES

It is well known that TSLP is a principal factor both in mediating homeostatic and pathologic conditions in distinct organs, but its role in the skin was described only in diseased conditions in details. However, the question whether the level of TSLP and other components of immune activation may differ in topographically different healthy skin surfaces, which could explain the characteristic localization of inflammatory skin diseases such as AD and PPR, has not been arisen up to the present.

Our aims were:

- To compare the immune milieu of healthy sebaceous gland poor (SGP) and sebaceous gland rich (SGR) skin areas by detecting TSLP, immune cell counts, cytokine milieu, and transcription factors.
- To investigate the effect of SGR skin-specific factors such as chitin and sebum components on TSLP expression.
- To detect how the special immune surveillance of healthy SGP and SGR skin sites may change in skin diseases exclusively localized on SGP and SGR skin sites (AD and PPR).
- To determine whether immune-mediated skin inflammation (TSLP level, other KC functions, and immune cell counts) differ between severe AD patients with FLG haploinsufficiency or acquired FLG loss.

MATERIALS AND METHODS

Patients and healthy controls

Skin punch biopsies (0.5-1 cm^2) were taken from patients and from healthy individuals. All participants provided written informed consent according to the Declaration of Helsinki principles. Our studies were approved by the local ethics committee of University of Debrecen, Hungary. All biopsies were cut into two pieces. For immunohistochemistry (IHC), samples were formalin-fixed and paraffin-embedded and for quantitative real-time PCR (RT-PCR) samples were stored in RNAlater (Qiagen, Hilden, Germany) at -70°C until RNA isolation. In our first study skin biopsies from lesional skin of 8 AD patients, from 10 patients with PPR, and from normal skin of 18 healthy individuals (8 from sebaceous gland poor (SGP) and 10 from SGR skin sites) were obtained. In our second study patients with severe extrinsic type of AD (associated with high serum IgE levels, allergen-specific IgE and positive skin-prick test reactions and healthy controls were involved. Patients with AD did not have any concomitant skin diseases at the time of examination and had not been treated with any moisturizers for one day, with topical corticosteroids for 3 days and with systemic immunosuppressants for 28 days prior to the examination. The severity of AD was determined using OSCORAD as well as epidermal thickness (ET) and Ki67 expression measurements on biopsies. Two groups were formed according to their FLG genotype: patients with severe symptoms without FLG mutations (Wt) (n = 12) and patients with severe symptoms with FLG mutation (n=12 [2282del4, R501X]).

Immunohistochemistry, haematoxylin and eosin and May–Grünwald–Giemsa stainings

For IHC analyses, paraffin-embedded sections were deparaffinized. Heat-induced antigen retrieval was performed and sections were pre-processed with H_2O_2 for 10 minutes. Sections were stained with the following anti-human antibodies (Ab): TSLP (Ab 1, Abcam; Ab 2 and 3, R&D Systems), CD3 (Bioss), CD4 (Abcam), CD11c (Abcam), CD1a (Abcam), CD163 (Enzo), CD83 (Abcam), TARC (R&D Systems), IL-10 (Covalab), IL-13 (Bioss), IL17A (Covalab), IFN-γ (Covalab), FLG (Abcam), Ki67 (Sigma-Aldrich), IL-33 (Abcam) and CCL27 (Sigma-Aldrich). Subsequently, the following HRP-conjugated secondary Abs were employed: anti-mouse/rabbit (Dako), anti-sheep and anti-goat (R&D Systems). Before and after incubating with Abs, washing of samples was performed for 5 minutes, 3 times in each step. Staining was detected with the Vector VIP Kit (VECTOR Laboratories) or 3,3'-Diaminobenzidine (DAB) (Dako). Sections were counterstained with methylene green or haematoxylin, dehydrated and covered with a glass coverslip. Skin specimens were also

stained with haematoxylin and eosin (H&E) and May–Grünwald–Giemsa (MGG).

Whole-slide imaging

The slides were digitalized using a Pannoramic SCAN digital slide scanner with a Zeiss plan-apochromatic objective and Hitachi 3CCD progressive scan color camera. Immunostainings were analyzed with Pannoramic Viewer 1.15.2 (3DHistech), using the HistoQuant and NuclearQuant applications. Regions of interest (ROIs) (n=20/slide) were selected and then the Field area [FA (mm^2)] and the Mask area [MA (mm^2)] were measured by the software. The FA shows the whole area of the ROI and the MA represents the positive area. The MA/FA values were counted for all ROIs. Epidermal thickness in AD samples was calculated as the quotient of the field area (FA) of the region of interests (ROI) and the length of the epidermis in each ROIs. The protein levels were analyzed by 2 independent observers by using Pannoramic Viewer 1.15.2) software.

Stratum corneum samples and TSLP immunocytochemistry

Tape-stripping method was used to collect stratum corneum samples according to the method described in a previous report. Until analysis, the tapes were stored at −20°C. The tapes were attached to silane coated microscope slides (Sigma-Aldrich), then incubated overnight in n-hexane (Sigma-Aldrich). Then samples were fixed in cold acetone for 10 min and blocked with 1.0% bovine serum albumin. After washing with PBS, the cells were incubated overnight with anti-human TSLP antibody (Ab 1) at 4°C. The cells were then incubated with Alexa-Fluor®-488-conjugated anti-rabbit IgG secondary antibody (Life Technologies) for 2 h at room temperature while being protected from light. After mounting, the cells were observed under a fluorescence microscope. The fluorescent images were taken, and TSLP levels were determined as the mean values of the quotient of fluorescent intensity and the area.

Cell culture experiments

HaCaT KCs were seeded in DMEM at 50 000 cells/well in 12-well plates for RT-PCR and cytokine ELISA measurements, and cultured until they reached 80% confluence. Then, cells were treated for 6h or 24 h with the following materials: Sebomed with or without SZ95 supernatant, chitin and free fatty acids (FFAs) (squalene, palmitic acid, stearic acid, oleic acid and linoleic acid). NHEK cells were cultured similarly to HaCaT cells in EpiLife® medium until they reached preconfluency (70-80%) or postconfluency for RT-PCR and cytokine ELISA measurements. Cells were treated by the mentioned factors for 6h or 24h. Human SZ95 sebocytes were cultured in Sebomed medium until reaching 80% confluence. Prior to

supernatant collection, medium was replaced with Sebomed lacking EGF. 24h supernatants were collected and filtered using 0,2-µm syringe filters and used for experiments.

Enzyme-linked immunosorbent assay

The concentration of TSLP in the supernatant was quantified in triplicates by using anti-human TSLP Quantikine® enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

RNA isolation and cDNA synthesis

After homogenizing the samples in Tri reagent solution (Sigma-Aldrich) with Tissue Lyser (Qiagen) total RNA was isolated from the human skin tissues and HaCaT and NHEK cells, then treated with DNase I (Applied Biosystems) according to the manufacturer's instructions. The concentration and purity of the RNA were measured by NanoDrop spectrophotometer (Thermo Scientific), and Agilent 2100 bioanalyzer (Agilent Technologies). For RT-PCR, cDNA was synthesized from the isolated RNA using the High Capacity cDNA Archive Kit (Life Technologies).

Quantitative real-time PCR

RT-PCR was carried out in triplicate using pre-designed MGB assays ordered from Applied Biosystems (Life Technologies). TaqMan Gene Expression assays were used to detect PPIA, total TSLP, CD80, CD83, CD86, LAMP3, IL-13, IL-10, IL-17, IFN-γ, TBX21, GATA3, RORC, FOXP3, CCR4, CCR8, IL-33 and CCL27 mRNA levels (all from ThermoFisher). All reactions were performed with an ABI PRISM® 7000 Sequence Detection System. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method normalized to PPIA expression.

Filaggrin genotyping

Analyses of the FLG mutations R501X and 2282del4 were performed for AD patients. DNA isolated from peripheral blood mononuclear cells with GenElute Blood Genomic DNA Kit (Sigma-Aldrich) was subjected to PCR amplification for genotyping R501X; and 2282del4 muations. All PCR products were purified with QIAquick PCR purification Kit (Qiagen) and bidirectionally sequenced on an ABI Prism 3100 automated sequencer.

Measurement of transepidermal water loss and skin pH

Measurements were performed under standardized laboratory conditions at a temperature of 22–25°C and a humidity level of 40–60%. Before the measurements, individuals were allowed to adapt to the room conditions for 5 min. Transepidermal water loss (TEWL) measurements (g/hm^2) were carried out with Tewameter TM300 (Courage and Khazaka) on

the flexural forearm and on the face of individuals (n=50). The duration of the measurements, performed in triplicates, was 30 s. Skin pH measurements were carried out with pH 905 (Courage and Khazaka) on the flexural forearm and the face of healthy individuals (n=50).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). To determine the statistical significance between the groups, one-way analysis of variance (ANOVA) test and Newman-Keuls post test were used. Differences between the groups were demonstrated using MEAN ± SEM. P-values <0.05 were considered statistically significant (*p<0.05; **p<0.01; ***p<0.001). Analysis of correlations was performed by Pearson r test. When two groups were compared two-tailed P values <0.05 were considered statistically significant (*p<0.05, **p<0.01).

RESULTS

TSLP protein is constitutively expressed in SGR healthy skin, but almost absent from SGP healthy skin

To detect TSLP protein in topographically different skin regions, biopsies from sebaceous gland poor (SGP; representing dry areas) and sebaceous gland rich (SGR; representing seborrheic areas) healthy skin were obtained. Lesional skin of severe atopic dermatitis (AD) patients was used as positive controls for TSLP staining. To confirm immunohistochemistry (IHC) results three different antibodies (Abs) against TSLP were used. In AD samples, strong TSLP positivity was detected in the granular and corneal but not in the basal and suprabasal layers of the epidermis. In all SGR skin biopsies, high TSLP expression was detected with all three anti-TSLP Abs in the epidermal keratinocytes (KCs), mainly in the upper epidermal layers, and in sebocytes of sebaceous glands. In contrast, in SGP samples, TSLP was completely or almost completely absent. Importantly, the intensity of TSLP staining (as assessed by Pannoramic Viewer software) was found to be significantly higher in SGR skin compared to SGP skin. However, TSLP expression in SGR skin was significantly lower than in AD skin. TSLP protein levels were also measured in the stratum corneum by immunocytochemistry and were also significantly elevated in SGR skin compared to SGP skin, but did not reach the level found in AD skin. Interestingly, RT-PCR analysis detected nearly similar total TSLP mRNA expression in all skin types (SGR, SGP, and AD skin).

Linoleic acid induces TSLP expression in keratinocytes

Sebum content, composition of commensal microbiota and UV radiation are able to influence SGR and SGP skin differently; therefore, the effects of these factors on TSLP production in HaCaT and NHEK cells were analyzed by using RT-PCR and ELISA. As similar TSLP protein levels were detected in the hairy scalp (UV-protected) and face (UV-exposed) biopsies, we did not investigate further the effect of UV.

To study the effect of chitin and sebum, HaCaT KCs were treated with chitin, with supernatant of cultured human SZ95 sebocytes and with different lipid components of sebum. After chitin and sebocyte supernatant treatment, induction of TSLP mRNA could be non-significantly triggered. Of the used lipid components, palmitic, oleic and linoleic acid upregulated TSLP gene expression, but only linoleic acid could elevate it significantly. Further, we showed that linoleic acid induces TSLP mRNA expression in a concentration-dependent manner, reaching its maximum and significantly higher level at 150 μM. On the other hand, the basal TSLP protein levels could not be elevated by any of the aforementioned agents. As sebum components influenced prominently TSLP expression in HaCaT cells, these experiments were repeated in NHEKs and similarly linoleic acid could dose-dependently elevate TSLP mRNA levels. No TSLP protein secretion by NHEKs could be detected. It has been previously found in AD skin that barrier damage can also lead to TSLP production by KCs; therefore, transepidermal water loss and skin pH, representing barrier functions, were measured on SGP and SGR skin regions. No differences were detected, indicating that barrier damage is most probably not the cause of distinct TSLP production in SGR and SGP skin.

SGR skin is characterized by an elevated number of DCs without prominent activation and maturation compared to SGP skin

The significantly higher TSLP level of SGR skin suggested that differences in other immune surveillance factors may also exist. Since DCs are the major target cells of TSLP, CD11c+ dermal myeloid DCs and CD1a+ Langerhans cells (LCs) were immunolabeled and quantified. IHC revealed no significant difference between the LC counts of SGP and SGR skin samples. In contrast, CD11c+ DCs were present in significantly higher numbers in SGR skin compared to SGP skin and the majority of these cells were characteristically localized near to sebaceous glands or the duct of the glands. In AD skin DC count was higher compared to SGR skin and DCs were found to be diffusely infiltrated in the dermis.

To further analyze the characteristics of DCs, their classical maturation/activation markers CD80, CD83, CD86 and DC-LAMP were investigated on mRNA level. As the

classical proinflammatory effect of TSLP is to boost Th2 polarizing DCs in allergic diseases, TARC [also known as Chemokine (C-C motif) ligand 17], an atopic eczema specific, DC-secreted chemokine, and CD83 were also assessed by IHC. Although the number of CD83 positive cells and mRNA levels of CD80, CD83, CD86 and LAMP3 could be found in somewhat higher amounts in SGR skin compared to SGP, none of the investigated markers' expression differed significantly; while significantly higher numbers of CD83+ cells were detectable in AD samples. TARC was completely absent from both types of healthy skin but was present in AD samples.

Elevated T cell number and noninflammatory IL-10/IL-17 cytokine milieu features SGR skin

Next, CD3+ and CD4+ cells were stained. These T cells were present in significantly higher numbers in SGR skin compared to SGP skin. The localization of T cells was similar to that of DCs and the clear majority of T cells were Th cells. As a next step, representative cytokines of Th subsets [IL-10: regulatory T cell (Treg); IL-13: Th2; IL-17: Th17 and interferon-γ (IFN-γ): Th1] were immunostained. IHC revealed that no IL-13+ and IFN-γ+ cells could be detected in either of the healthy skin types. IL-10+ and IL-17+ cells showed similar patterns; they were detected at very low levels or absent from SGP skin, but were found at significantly higher levels in SGR skin. RT-PCR analyses of the aforementioned cytokines were also performed and showed a similar pattern to that found at the protein levels. In SGP skin the cytokine content was very low, in contrast to the characteristic IL-17/IL-10 cytokine milieu of SGR skin.

Then, the mRNA levels of transcription factors characteristic of different Th cell subsets were investigated. Expression of T-bet, mediating inflammatory Th17 [Th17(23)] and Th1 cell responses and GATA3, mediating Th2 responses, were detected at similar levels in SGP and SGR skin. On the other hand, RORγt, mediating non-inflammatory Th17 [Th17(β)] and Th17(23) development and FOXP3, characteristic of Tregs, showed notably higher expression levels in SGR compared to SGP skin. CCR4 and CCR8 mRNA levels, typical skin homing receptors of Tregs, were also detected in notably higher levels in SGR skin compared to SGP.

Macrophage, neutrophil, eosinophil and mast cell counts are similar in SGR and SGP skin

To determine whether the numbers of macrophages, neutrophils, eosinophils and mast cells differ in SGP and SGR skin, anti-CD163 and May-Grünwald-Giemsa (MGG) staining were performed. No significant difference could be detected in the above mentioned cell counts between SGR and SGP skin areas, although CD163+ macrophages were found in higher numbers in SGR skin. Neither neutrophils nor eosinophils were present in healthy skin regions, whereas mast cells were found in low numbers in both SGR and SGP samples.

Papulopustular rosacea is characterized by significantly decreased TSLP level, elevated DC count and activity, robust influx of T cells and innate immune cells and an inflammatory IL-17/IFN-γ cytokine profile

To investigate the alterations of the characteristic immune surveillance of SGR skin in an inflammatory disease typically occurring in that skin region, papulopustular rosacea (PPR) samples were analyzed. Epidermal and stratum corneum TSLP protein levels were significantly decreased in PPR samples compared to SGR skin. The loss of the protein was not homogenous, but discontinuous through PPR epidermis. In contrast, no differences in its mRNA levels were found. Infiltrating CD11c+ DCs, CD3+ and CD4+ T cells were detected in significantly higher numbers in PPR compared to SGR skin and were present diffusely through the dermis. CD80, CD83, DC-LAMP and CD86 activation/maturation markers of DCs were all significantly upregulated on mRNA levels compared to SGR skin. Although CD83+ DCs were present in significantly elevated numbers, TARC positivity was almost undetectable in PPR skin. Moreover, strong, but non-significant correlation was detected between the increase of DC count and the decrease of TSLP level in PPR samples. No difference was found between SGR and PPR skin regarding the number of LCs. Significantly higher numbers of macrophages, mast cells and neutrophils could be detected in PPR skin compared to SGR, while eosinophils were absent from both SGR and PPR samples.

The characterization of cytokine milieu was also performed in PPR skin samples. Parallel to the prominent increase in the number of IL-10+ and IL-17+ cells in PPR compared to SGR skin samples, an especially robust IFN-γ+ cell presence was detected, while IL-13+ cells were absent. The mRNA levels of cytokines corresponded to their protein levels. Gene expression levels of TBX21, FOXP3, CCR4 and CCR8 were significantly higher in PPR compared to SGR skin, while RORC and GATA3 gene expression levels were lower than in healthy SGR skin.

As the main characteristics of skin inflammation in PPR were decreased TSLP level, elevated DC and T cell count and robust IFN-γ appearance, correlations between these factors were calculated. Statistically not significant inverse correlation was found between TSLP level and DC count.

Detection of severity markers in the skin of wild type and FLG mutant severe AD patients

To compare the two AD groups, the quantification of histological severity markers (ET and Ki67, was performed. No differences were found in the levels of these parameters between filaggrin mutant and wild type AD groups, but compared to controls ET and Ki67 expression levels were significantly higher in both AD groups. These data corresponded to an almost identical clinical severity of the patients.

IHC analyses of KC-derived cytokines, chemokine and FLG in the skin of wild type and FLG mutant severe AD patients

To demonstrate FLG loss in the skin of the patient groups, immunostaining of FLG was performed. No difference was found between the levels of FLG in the skin of the two patient groups and the protein levels were significantly lower compared to controls. In AD skin, FLG could be detected discontinuously with mild positivity; in contrast, FLG was found continually with strong positivity in the granular layer of normal skin.

Quantification of KC-derived proinflammatory cytokines TSLP and IL-33 and chemokine CCL27 was also carried out. The levels of these proinflammatory molecules were significantly higher in the skin of AD patients than the control group, but no differences were found between the two AD groups. It is important to note that TSLP was slightly or not detectable in control skin. Strong IL-33 nuclear positivity was observed in AD skin. It was expressed moderately in healthy skin. CCL27 was also detectable in healthy skin but was present in significantly higher levels in the AD groups. Regarding all the three investigated parameters no significant differences could be detected on mRNA levels between the two AD groups. Comparing the healthy control group to the AD groups, difference could be found only regarding IL-33 mRNA levels.

T cells and DCs in the skin of wild type and FLG mutant severe AD patients

$CD3^+$ T cells and $CD11c^+$ DCs were also immunostained. The number of T cells and DCs were significantly higher in the skin of AD patients compared to the skin of healthy controls, but showed no differences between the skin samples of the two AD groups.

Correlations between histological severity markers and KC-derived cytokines and chemokine, T cell and DC counts

Since no differences were found with regard to the measured parameters between the two AD groups, all data for AD patients were pooled together and immune cell counts and levels of KC-derived cytokines and chemokine were correlated with ET, Ki67 expression and FLG contents. Statistically significant correlations were found between Ki67 and TSLP levels, between Ki67 and CCL27 levels, between ET and IL-33 levels and between ET and $CD3^+$ cell count. No correlations were observed between histological severity markers and DC count and between FLG levels and severity markers or T cell and DC counts and between OSCORAD and our investigated parameters.

DISCUSSION

Skin microbial community exhibits remarkable differences on seborrheic, dry and moist regions probably connected to the different physiology of these sites. Since skin microbiota has mutualistic connection with the skin immune system, possible immunological distinctions between topographically different healthy skin sites can be postulated but have not been revealed. Previous investigations on the immune activity of intestinal mucosa indicated distinct presence of TSLP in particular gut sections, thus, in our study, TSLP, also an important cytokine of KCs, was studied first. A constitutive TSLP protein expression was detected in healthy SGR skin areas; in contrast, in healthy SGP areas TSLP was practically absent. Although TSLP mRNA expression has already been detected in healthy skin, its protein expression was found only in inflamed epidermis until now. The conflicting data between the previous publications and our current study might be explained by that the other investigators most probably used healthy SGP skin and no SGR samples as controls of AD or psoriasis. Although the protein expression of TSLP showed remarkable differences between healthy SGP and SGR skin, mRNA levels were similar in all samples. The discrepancy between the protein and mRNA expressions of TSLP can be explained by important, but presently unknown posttranscriptional modifications during KC differentiation. Bogiatzi et al. detected a basal TSLP mRNA expression without the presence of the protein in cultured KCs, and this mRNA content was not upregulated in the presence of proallergic cytokines. On the other hand, when whole skin explant, a model that preserves the differentiation of KCs, was used, TSLP protein could be measured after cytokine incubation. The importance of the posttranscriptional modifications can also explain our observations that, although linoleic acid could significantly and dose-dependently elevate TSLP mRNA levels, protein production was

not increased. Until now, no study investigated the effect of sebum components and SZ95 sebocyte supernatant on TSLP mRNA and protein expression of cultured or HaCaT KCs and only one research group examined the outcome of chitin treatment on primary and HaCaT KCs' TSLP protein production. Although mRNA levels were not investigated, the authors detected significantly elevated TSLP protein levels in a concentration-dependent manner, which observation could not be confirmed by us. Nevertheless, according to our results sebum lipid content may have a role in the initiation of TSLP production in SGR skin.

Since DCs are the major target cells of TSLP action, the significantly higher TSLP levels of SGR skin proposed that differences in other immune surveillance factors may also exist between SGR and SGP areas. Although no difference was found in the LC count, the number of CD11c+ DCs was significantly elevated in SGR skin compared to SGP areas. Despite their high number, DCs did not exhibit noticeable activity. Moreover, the complete absence of TARC+ cells indicates that TSLP expressed by SGR skin does not act as it is described in AD skin, where TSLP induces TARC+ DCs promoting Th2 responses. The fact that TSLP has not only an inflammatory, but also a tolerogenic function on DCs is known from immunological studies of the gut. A plethora of evidence supports the crucial role of TSLP in the maintenance of intestinal immune homeostasis and tolerance to commensal flora. We propose that TSLP found in SGR skin might have a similar role, since i) SGR skin samples were clinically healthy without any signs of inflammation; ii) the amount of TSLP was lower than found in AD samples; and iii) DCs were TARC negative without noticeable activation.

We also examined and found differences between SGR and SGP skin sites regarding the number of T cells and levels of cytokines, as well as transcription factors characteristic to different Th subsets. Significantly more T cells were present in SGR skin, which cells were dominantly Tregs and probably also Th17(β) cells. The presence of Tregs was proven by the higher expression of FOXP3 and CCR4 as well as CCR8 Treg homing receptors accompanied by significantly higher IL-10+ cell counts. According to recent data, about 20% of CD4+ cells in healthy human skin express FOXP3 and these Tregs are effector memory cells being associated with hair follicles; however, these authors did not compare different topographical skin sites. The higher Treg content detected in our study was also supported by a recent clinical investigation; i.e. skin metastases from solid-organ tumors were found most commonly on head and neck areas, and the authors also hold the higher number of Tregs responsible for the higher probability of metastases on these sites.

Recently Th17 cells were divided in non-pathogenic Th17(β) and pathogenic Th17/Th23(23) cells. Th17(β) cells are characterized by IL-17 and IL-10 production and the

expression of RORγt, while Th17(23) cells play an important role in the development of inflammatory and autoimmune diseases, produce IL-17, INF-γ and GM-CSF and express T-bet and RORγt transcription factors. Although significantly more IL-17+ and IL-10+ cells were present in SGR skin, IFN-γ+ cells were completely absent, and besides the higher RORγt expression, T-bet mRNA levels did not differ compared to SGP skin. Therefore, we suppose that non-pathogenic Th17(β) cells were detected in SGR skin. Innate lymphoid cells and γδT cells must also be taken into consideration as they are known to produce IL-17. The non-inflammatory T cell milieu of SGR skin was also supported by the similarly low appearance of innate immune cells in both healthy skin areas.

In conclusion, our results suggest that similar to skin microbiota and chemical milieu, a fine topographical difference does exist in the activity of the human skin immune system regarding an epidermal factor (TSLP), DCs and T cells, although in this study moist skin regions were not investigated (manuscript under preparation). These results hence may provide explanation of the characteristic localization of certain immune-mediated skin diseases in special topographical skin areas (i.e. AD on SGP and PPR on SGR skin sites). Moreover, our data highlight the importance of correctly used topologically identical control skin samples in scientific studies. Further, our study may influence future barrier repair therapeutic approaches.

After detecting this special immune surveillance in healthy SGR skin, we wondered how this can be changed in an immune mediated skin disease like PPR, which is exclusively localized to SGR skin regions. In PPR, special activation of both the innate and adaptive immune mechanisms were previously described despite the absence of an obvious infectious or dangerous trigger, and literature suggests that decreased tolerance could be responsible for this increased skin sensitivity. According to our findings, in PPR skin, TSLP was lost, DCs became activated, T cells turned to inflammatory type [Th1 and Th17(23)] and their numbers were highly elevated, similarly to a recent study, resulting in the disruption of the non-inflammatory immune milieu of SGR skin. Although Treg presence was also higher, this is not a contradiction, as their accumulation was usually detected in inflammatory skin diseases. These changes were accompanied by the significant influx of macrophages, neutrophils and mast cells. We were also able to show that linoleic acid, an important component of sebum, exerted an effect on TSLP expression; moreover, literature data described altered sebum composition in rosacea patients. Based on these findings we hypothesize that this altered sebum production in rosacea and the consequently occurring loss of tolerogenic TSLP may be one of the main events during PPR development. This change in TSLP level may influence

DC and T cell activation. At the same time, since DCs can potentially be exposed directly to changes of sebum, chemical milieu and microbiota of the skin, the disruption of the non-inflammatory milieu can also be initiated by DCs or T cells.

After revealing the differences between the immune surveillance of healthy SGP and SGR skin regions and characterizing the alterations of SGR skin specific microenvironment in PPR, we aimed to investigate whether immune-mediated skin inflammation (expression of TSLP and other Th2 characteristic factors, DC and T cell counts) differs in the skin of AD patients with or without *FLG* mutation.

AD is a multifactorial immune-mediated inflammation of the skin that is driven by interactions of genetic and environmental factors. Over-reactive adaptive, dysregulated innate immune responses and impaired skin barrier functions together lead to the manifestation of the disease. FLG, a crucial component of the physicochemical skin barrier, shows several genetic alterations (e.g. copy number variations and *FLG* null mutations) and can predispose to AD. On the other hand, acquired barrier dysfunctions can be caused by the frequent usage of detergents and exposure to allergens and *Staphylococcus*, as well as by local skin inflammation. Although the role of TSLP is well-known in AD pathogenesis, the question of whether genetic or acquired skin barrier dysfunctions can alter KC immune function differently has not been raised. In our study, our aim was to determine whether immune-mediated skin inflammation differ between severe AD patients with or without *FLG* mutations. We also investigated the correlations between the detected parameters. In order to answer our questions, two patient groups were created: *FLG* Wt patients and *FLG* mutant patients with severe symptoms and matching OSCORAD. Two parameters, the ET and Ki67 expression were investigated to score histological severity. Significantly thickened epidermis and elevated Ki67 levels were found in the two AD groups compared to controls, whereas no differences were observed between the two severe AD groups. Although serum IgE levels and frequency of sensitization were significantly higher in the *FLG* mutant AD group, the clinical and histological severities were the same, and no difference was found in the epidermal FLG content. These findings are in good concordance with our previous results, as the level of FLG loss is connected to the severity of the skin inflammation, rather than to the cause of FLG loss, while IgE level and sensitization seem to be connected to *FLG* genotype with significantly increased levels in *FLG* mutant AD.

To study whether immune functions of KCs differ between severe AD patients with or without *FLG* mutations, TSLP, IL-33 and CCL27 tissue levels were compared. In the last few years, the importance of TSLP in AD has been highlighted. TSLP is known for its capacity to

induce CD11c+ myeloid DCs to promote Th2-skewed inflammatory responses. Previous studies have shown significantly elevated serum, epidermal and stratum corneum TSLP levels in AD compared to controls, while other workgroups failed to detect higher serum TSLP levels in these patients. The intensity of expression in the stratum corneum correlated with clinical severity, on the contrary relationship between serum and epidermal TSLP levels and OSCORAD were highly controversial. In parallel with previous data, we found significantly higher epidermal TSLP levels in AD patients than in controls; according to our results, TSLP protein levels did not differ between Wt and *FLG* mutant AD groups. Epidermal TSLP levels significantly correlated with the level of Ki67, but no relationship was found between TSLP levels and clinical severity. IL-33, a newly discovered AD specific cytokine, is expressed by ECs and activates Th2 lymphocytes, mast cells and eosinophils. Our results showed that IL-33 protein expression was significantly elevated in the AD groups compared to controls and no significant difference was found between the two AD groups. These data correspond to a previous investigation, although the comparison of *FLG* mutant and Wt AD groups was not performed in that study. In another investigation, a correlation was found between serum IL-33 levels and disease severity of AD. Our workgroup found for the first time a strong correlation between ET and levels of epidermal IL-33 protein, but failed to detect any relationship between clinical disease severity and epidermal IL-33 levels. CCL27 is a skin-specific CC chemokine produced by KCs, which contributes to tissue-restricted leukocyte trafficking and can induce inflammation by promoting the migration of Th2 cells into the skin. A previous study described strong CCL27 expression in lesional keratinocytes of AD patients. Similarly, we found significantly elevated protein levels of CCL27 in AD skin, but when Wt and *FLG* mutant AD groups were compared, no difference was detected. Significantly correlated expression levels of CCL27 and Ki67 were found by our workgroup, but no relationship could be detected between tissue CCL27 levels and OSCORAD.

In the literature no RT-PCR data can be found comparing FLG mutant and WT AD groups regarding these parameters. Comparing healthy controls to AD patients in our study, mRNA levels did not reflect the detected protein levels. This contrast can be explained by posttranscriptional modification, namely the regulation of mRNA degradation and translation by enzymes and micro RNAs which depends on the actual state of the keratinocytes' and systemic needs. Until now only two articles were published on the mRNA levels of TSLP and IL-33 and none on CCL27 gene expression in AD patients. The differences found in the relationship between the clinical and histological severity markers and KC-derived proinflammatory molecules draws attention to the fact that using OSCORAD is not always

parallel to the degree of inflammation in a given plaque; therefore, the local immune markers of inflammation presumably show better correlation with a local severity marker than a complete skin severity marker. Since the aforesaid cytokines and chemokine produced by KCs have an effect on T cells and DCs, their cell counts were assayed. In our investigation, similar to a previous study, the number of T cells and DCs were found to be significantly higher in the skin of severe AD patients compared to the skin of healthy controls Between the immune cell numbers of *FLG* mutant and Wt AD patient groups, no significant differences were found by our workgroup. We also detected a strong correlation between ET and $CD3^+$ T cell count.

To summarize our findings, our results suggest that immune-mediated skin inflammation represented by innate and adaptive immune cell counts and KC-derived cytokine and chemokine content does not differ between severe AD patients with acquired or genetically determined *FLG* loss, which may indicate that genetic *FLG* mutation in KCs does not influence the immune function of these cells in a different manner. Results of the correlations demonstrated that immune activation in the skin is connected to the severity of the disease rather than to the origin of barrier alterations.

SUMMARY

We could demonstrate that fine topographical difference exists in the activity of the human skin immune system regarding thymic stromal lymphopoietin (TSLP) production, dendritic cell (DCs) and T cell counts and functions. A constitutive TSLP protein expression was detected in healthy sebaceous gland rich (SGR) skin areas; in contrast, in healthy sebaceous gland poor (SGP) areas TSLP was absent. Linoleic acid, an important sebum component could dose-dependently elevate TSLP mRNA levels in HaCaT and NHEK cells. We propose that TSLP found in SGR skin might have a similar role to that found in gut homeostasis since SGR skin samples were clinically healthy without any signs of inflammation; the amount of TSLP was lower than found in AD samples; and DCs were TARC negative without noticeable activation. In SGR skin, DC and T cell counts were higher. T cells were dominantly regulatory T cells and non-pathogenic T helper (Th)17(β) cells. The presence of IL-17+ and IL-10+ cells was elevated in SGR skin compared to SGP, while IFN-γ+ and IL-13 cells were completely absent. These findings are indicating that a non-inflammatory IL-17/IL-10 milieu is characteristic to SGR skin. In papulopustular rosacea (PPR), which disease is exclusively localized on SGR skin, TSLP was lost, DCs became activated, T cells turned to inflammatory type [Th1 and Th17(23)] and their numbers were highly elevated, resulting in the disruption of the non-inflammatory immune milieu of SGR skin. We also revealed that immune-mediated skin inflammation (represented by keratinocyte-derived factors, T cell and DC counts) is similar in severe AD with or without filaggrin mutations and AD immune activation is connected to the severity of the disease rather than to the origin of barrier alterations. These results may provide an explanation of the characteristic localization of certain immune-mediated skin diseases in special topographical skin areas (i.e. AD on SGP and PPR on SGR skin sites). Moreover, our novel data highlight the importance of correctly used topologically identical controls in scientific studies. Further, our study may influence future barrier repair therapeutic approaches.

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List of publications related to the dissertation

1. **Dajnoki, Z.**, Béke, G., Kapitány, A., Mócsai, G., Gáspár, K., Rühl, R., Hendrik, Z., Juhász, I., Zouboulis, C. C., Bácsi, A., Bíró, T., Törőcsik, D., Szegedi, A.: Sebaceous gland rich skin is characterized by TSLP expression and distinct immune surveillance which is disturbed in rosacea.
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