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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Contents

Abbreviations

Ab	Antibody
AD	Atopic dermatitis
ANOVA	One-way analysis of variance
AP	Activating protein
APC	Antigen presenting cell
AR	Allergic rhinitis
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Crohn's disease
DC	Dendritic cell
DC-LAMP	Lysosomal-associated membrane protein 3
EC	Epithelial cell
ELISA	Enzyme-linked immunosorbent assay
EoE	Eosinophilic esophagitis
ET	Epidermal thickness
FA	Field area
FFA	Free fatty acid
FLG	Filaggrin
FOXP3	Forkhead box P3
GATA3	GATA Binding Protein 3
GWAS	Genome-wide association study
H&E	Haematoxylin and eosin
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
iNKT	Invariant natural killer T cell
IRF	Interferon regulatory factor 3
KC	Keratinocyte
KLK7	Kallikrein 7

LC	Langerhans cell
MA	Mask area
MAPK	Mitogen-activated protein kinase
MDC	Macrophage-derived chemokine
MGG	May-Grünwald-Giemsa
MHC	Major histocompatibility complex
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEK	Normal human epidermal keratinocyte
NOD	Nucleotide-binding oligomerization domain-containing protein
OSCORAD	Objective score of atopic dermatitis
PPIA	Peptidylprolyl isomerase A
PPR	Papulopustular rosacea
ROI	Region of interest
RORC	RAR Related Orphan Receptor C
RT-PCR	Real-time quantitative polymerase chain reaction
RXR	Retinoid X receptor
SDF-1	Stromal cell-derived factor 1
SGP	Sebaceous gland poor
SGR	Sebaceous gland rich
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation-regulated chemokine
TBX21	T-box transcription factor 21
TGF	Tumor growth factor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
TSLPR	Thymic stromal lymphopoietin receptor
UC	Ulcerative colitis
VDR	Vitamin D receptor
Wt	Wild type

1. Introduction

As an outstanding discovery of recent years, the microbial community has been shown to exhibit remarkable differences on topographically distinct skin areas[1,2]. 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[1,2,3,4,5]. 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[6]. 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[7,8,9]. 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)[10,11]. 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[12,13]. 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[14].

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.

1.1. TSLP protein and its receptor

TSLP was first cloned and identified in the medium of a murine thymic stromal cell line, as a growth factor effecting B cell development[15,16,17]. The human form of TSLP was cloned independently by two workgroups[18,19] and it was proven that the sequence homology of human TSLP is only 34% with its mouse orthologue[19]. The human TSLP gene is localized in chromosome 5q22.1 next to the atopic cytokine cluster on 5q31[18]. This four-helix bundle short chain hematopoietic cytokine is characterized by strong structural and functional homology to IL-7[17] and shares an overlapping, but distinct, biologic profile[18].

According to recent studies a second, short TSLP isoform was also identified. The long form TSLP protein (described earlier) consists of 159 amino acids (1-28. signal peptide) with two potential N-glycosylation sites (on 64. and 119. amino acids) and three disulphide bonds (between 34 ↔ 110, 69 ↔ 75 and 90 ↔ 137 amino acids). The short form TSLP (recently discovered) 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)[20,21,22,23]. Between human and murine TSLPR low (39%) sequence similarity can be also found[23,24]. 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[14,19]. TSLPR is expressed only by a few cell types, namely DCs, monocytes and some T cell clones[19,25].

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[26,27]. 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 (Figure 1).

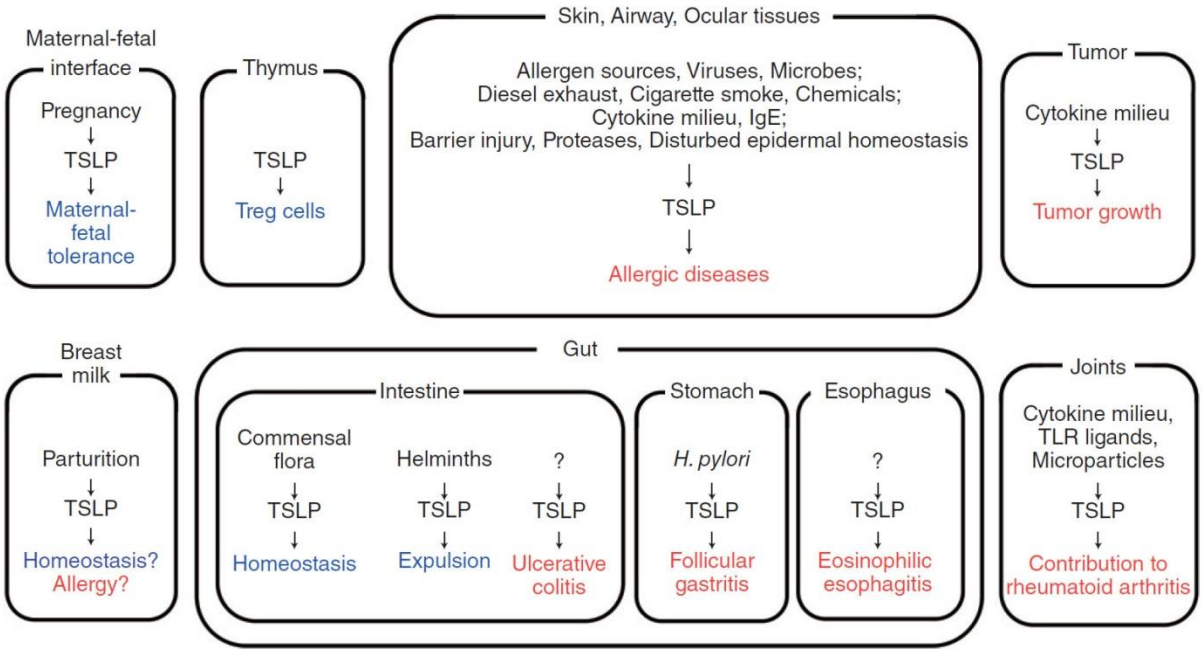


Figure 1. TSLP triggered by endogenous and environmental factors can contribute to disorders and homeostasis. *TSLP is predominantly produced by epithelial cells and keratinocytes at barrier surfaces and its constitutive expression was described in intestinal ECs and the thymus. TSLP in the skin, airways, and ocular tissues plays a critical role in the pathogenesis of allergic diseases. In the thymus, constitutive expression of TSLP leads to the differentiation of Treg cells, while in the presence of TSLP intestinal ECs interacting with commensal microbiota may result in intestinal homeostasis, a loss of which can be responsible for the pathologic events in Crohn's disease. TSLP expressed in trophoblasts may contribute to maternal-fetal tolerance. TSLP expression in the tumor microenvironment may lead to tumor growth and in synovial fluid it may have a role in rheumatoid arthritis. Red: Disorders. Blue: Homeostasis. IgE, Immunoglobulin E; TLR, Toll-like receptor; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin;*

Source: Takai, T., TSLP expression: cellular sources, triggers, and regulatory mechanisms. Allergol Int. 2012. 61:3-17[28]

1.2. Regulators of TSLP

TSLP expression can be promoted through either Toll-like receptor (TLR)3 ligands, Th2 cytokines, TSLPR or IL-7Rα[29,30] and multiple regulatory molecules can influence its expression both positively and negatively (Figure 2).

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[31,32], but may be also controlled by the mitogen-activated protein kinase (MAPK) pathway[33].

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[30,34,35,36].

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[37,38]. It is hypothesized that during repression RXR, NF-κB and vitamin D3-VDR complex are physically connected since knocking out RXR or blocking vitamin D3 binding to VDR terminate the repression of TSLP expression[37,39,40]. Glucocorticoids can also negatively regulate the expression of TSLP, probably by inhibiting AP-1 or NF-κB[30].

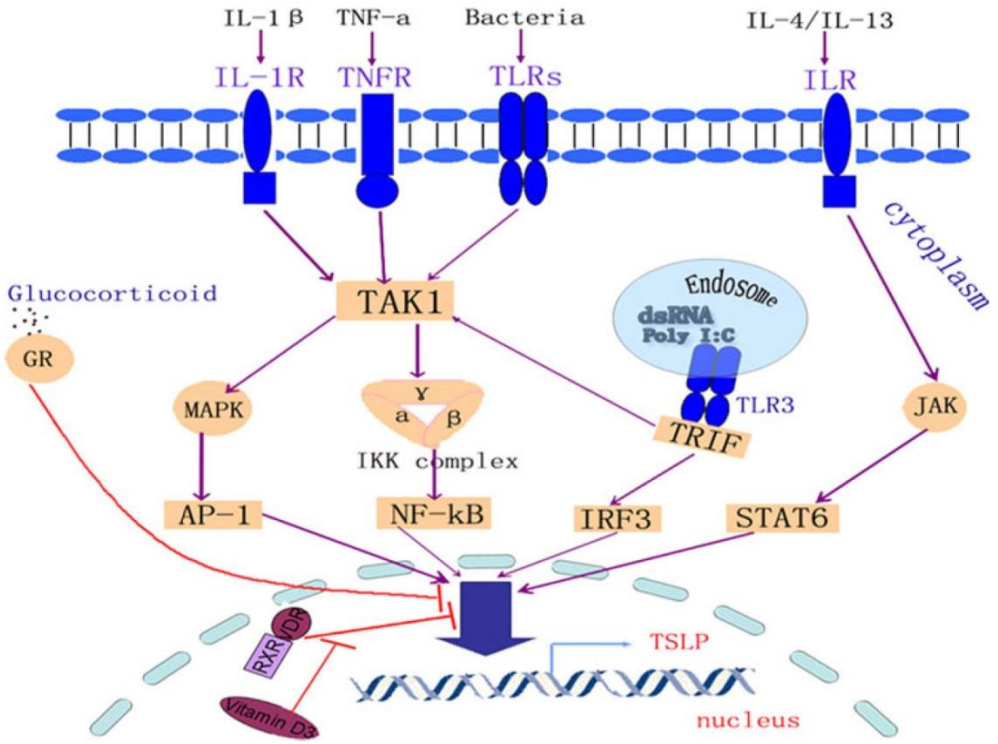


Figure 2. Regulation of TSLP gene expression. *NF-κB, IRF3, and AP-1 are the main regulators of TSLP gene expression, which factors can be induced by double-stranded RNA or poly(I:C). IL-4 and IL-13 induce TSLP via the JAK-STAT6 pathway, while NF-κB and AP-1 pathways can be activated by IL-1β and TNF-α upregulating TSLP expression. TLRs, by sensing bacteria, are also capable of activating NF-κB. RXR/VDR dimers and glucocorticoids are negative regulators of TSLP expression by blocking NF-κB or AP-1 activity, but vitamin D3 can inhibit the effect of RXR/VDR dimers. AP-1, activating protein-1; IL, interleukin; IRF3, interferon regulatory factor 3; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RXR, retinoid X receptor; STAT6, signal transducer and activator of transcription 6; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; TSLP, thymic stromal lymphopoietin; VDR, vitamin D receptor.*

Source: Li, M. et al., The regulation of thymic stromal lymphopoietin in gut immune homeostasis. Dig Dis Sci. 2011. 56(8):2215-20[41]

1.3. 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[42] and „represent the 'graveyard' for dead thymocytes[43,44], and also the 'privileged' area for the maturation of medullary thymocytes"[45,46]. Active cytokine or growth factor receptor-mediated cell signaling and cell metabolism are characteristic to Hassall's corpuscles[47], as transforming growth factor (TGF)-α, interleukin (IL)-7, stromal cell-derived factor 1 (SDF-1), CD30 ligand and macrophage-derived chemokine (MDC) were found to be expressed within them[48,49,50,51,52]. 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*[53].

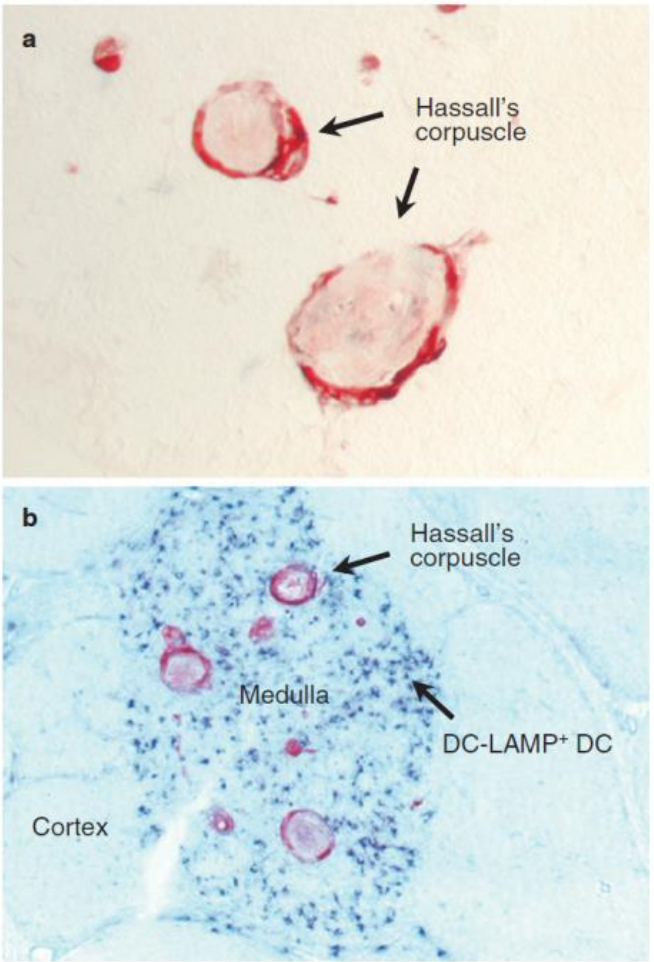


Figure 3. Epithelial cells in Hassall's corpuscles express TSLP. *Representative images of* ***(a)*** *TSLP staining in Hassall's corpuscles (red) and* ***(b)*** *co-localization of TSLP (red) and DC-LAMP (blue) in the human thymic medulla. DC-LAMP, Lysosome-associated membrane glycoprotein 3; TSLP, thymic stromal lymphopoietin.*
Source: Hanabuchi, S. et al., TSLP and immune homeostasis. Allergol Int. 2012. 61(1):19–25[45]

Immature CD11c+ myeloid DCs are strongly activated by human TSLP leading to the upregulation of their major histocompatibility complex (MHC) class II molecules, dendritic cell lysosome-associated membrane protein (DC-LAMP, which is only characteristic to activated DCs) and CD80 and CD86 co-stimulatory molecules[24,53].

CD11c+ DCs and TSLP expressing Hassall's corpuscles are present in the thymic medulla and the co-localization of CD11c+ DC-LAMP+ DCs and Hassall's corpuscles were also detected in the central part of the medulla. On the contrary, CD11c+ DC-LAMP- immature DCs are mainly present in the cortico-medullary junction and cortex of the thymus[54]. TSLP-activated DCs are suggested to have key role in the selection of self-reactive thymocytes and promoting them to differentiate into regulatory T cells (Treg), since parallel with the high expression levels of MHCII and the mentioned co-stimulatory molecules[55,56], which are necessary to Treg development, they can also induce homeostatic naïve T cell proliferation[53], and development of Treg cells can be inhibited by proinflammatory cytokines (IL-1, IL-6 and IL-12)[45]. Probably, TSLP activated DCs, by providing long-lasting and strong survival signal to self-reactive thymocytes, can promote a switch from negative to positive selection of Treg cells[45]. This hypothesis is also strengthened by a previous study showing that expansion and differentiation of CD4+CD8−CD25− thymocytes could be only induced by TSLP-activated DCs into CD4+CD8− CD25+FOXP3+ Treg cells, which was dependent on IL-2 and CD28 signals, but DCs stimulated with or without IL-7, CD40 ligands or poly (I: C), could not promote Treg development[54]. 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[54].

Three distinct DC populations were described in the thymus: plasmacytoid DCs (pDCs),

CD11c+CD11b− and the CD11c+CD11b+ myeloid DCs[57,58]. Approximately 20% of pDCs express TSLPR under steady-state conditions[59]. These pDCs, conditioned by TSLP, are able to secrete CCL-17 (TARC) and CCL-21 (MDC) chemokines and guide FOXP3+ T cells during their way to the medulla[54]. A previous *in vitro* study could prove that TSLP-conditioned pDCs can only promote the expansion of CD4+CD25+FOXP3+ Treg cells, but are not capable of doing that with naïve peripheral T cells[45]. Interestingly, different antigen presenting cells seem to have multiple roles on the development of functionally different Treg subsets, as TSLP-activated pDCs and mDCs can induce two distinct Treg populations. Treg cells activated by pDCs were found IL- 10^{high} TGF-β^{low}, while the other population was IL-10^{low} TGF-β^{high}[45]. These data were strengthened by another workgroup, as human thymus, secondary lymphoid tissues, as well as peripheral blood, consisted of two distinct CD25+FOXP3+ Treg cell subsets by investigating the expression of ICOS, TGF-β and IL-10[60].

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[61,62].

1.4. 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.

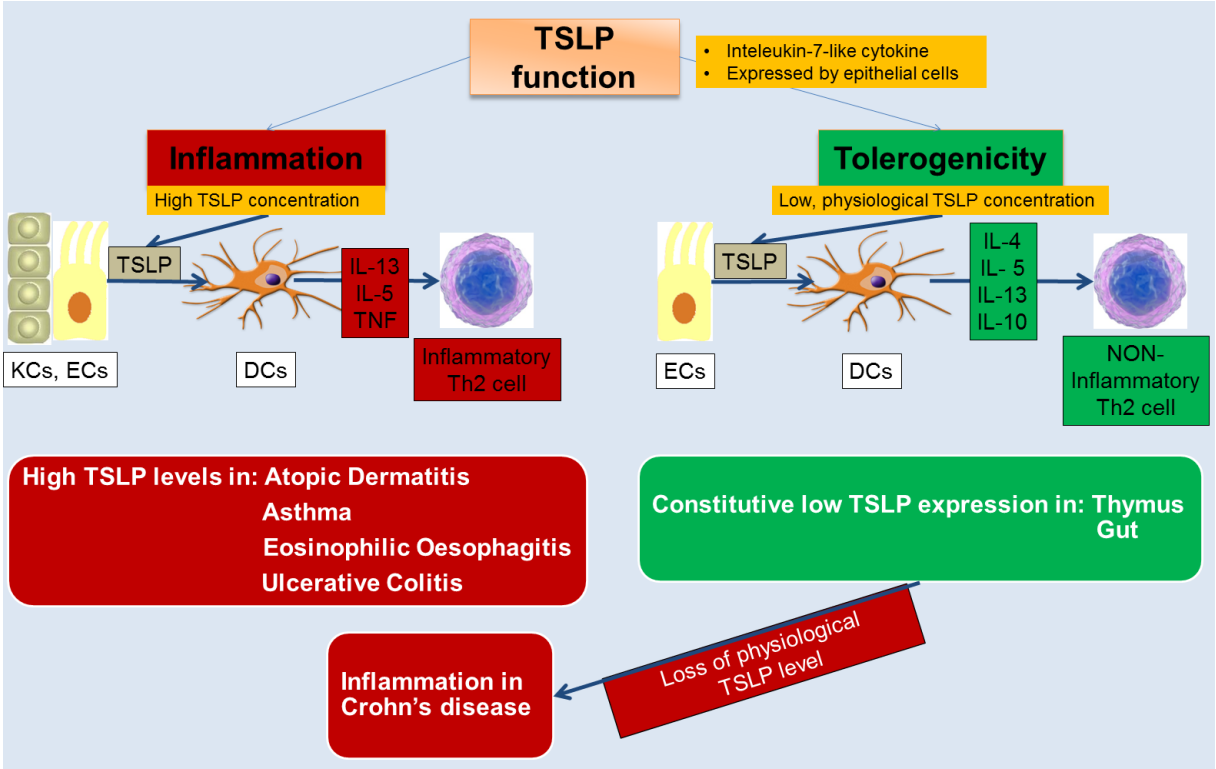


Figure 4. Dual role of TSLP in immune modulation. *Beside thymic ECs, intestinal ECs express low levels of TSLP in the lower gastrointestinal tract in steady-state. Parallel to atopic diseases (AD and asthma) and eosinophil esophagitis, highly elevated TSLP levels have been described in ulcerative colitis, being associated with inflammation. On the contrary, loss of TSLP has been demonstrated in Crohn's disease, another inflammatory bowel disease, which thought to be crucial in its pathogenesis.*

Intestinal ECs constitutively express TSLP in the lower gastrointestinal tract and its highest levels have been detected in the colon[10,63,64]. Constitutive production of TSLP was also described in cultured human intestinal ECs *in vitro* in response to and without stimulation[10,29]

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[65,66]. 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[10,66].

These DCs also have the ability to induce FOXP3+ Treg cells, thus they can indirectly suppress excessive responses of the immune system and maintain self-tolerance[59,67]. These data are also supported by an experiment performed on mouse model where disrupted TSLP-TSLPR pathway led to the loss of noninflammatory Th2 cell polarization[64]. These results together 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 and with a proven genetic predisposition of the patients[68]. A growing number of evidence has strengthened that TSLP is a key player in the development of IBDs[10,14,29,69,70].

Elevated TSLP levels were observed in the mucosal lesions of UC patients and inflammatory Th2 cytokines were proven to be responsible for this enhancement[29]. It is also known from the literature 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[69].

In contrast, in CD, a chronic autoimmune disorder, colonic ECs express lower levels of TSLP compared to healthy controls and primary IECs from patients with CD do not produce it[10,31], subsequently, IL-12 release by commensal-activated DCs cannot be inhibited[10]. In response to these events Th1/Th17 type inflammation, characterized by IFN-γ and IL-17 production, is initiated[69].

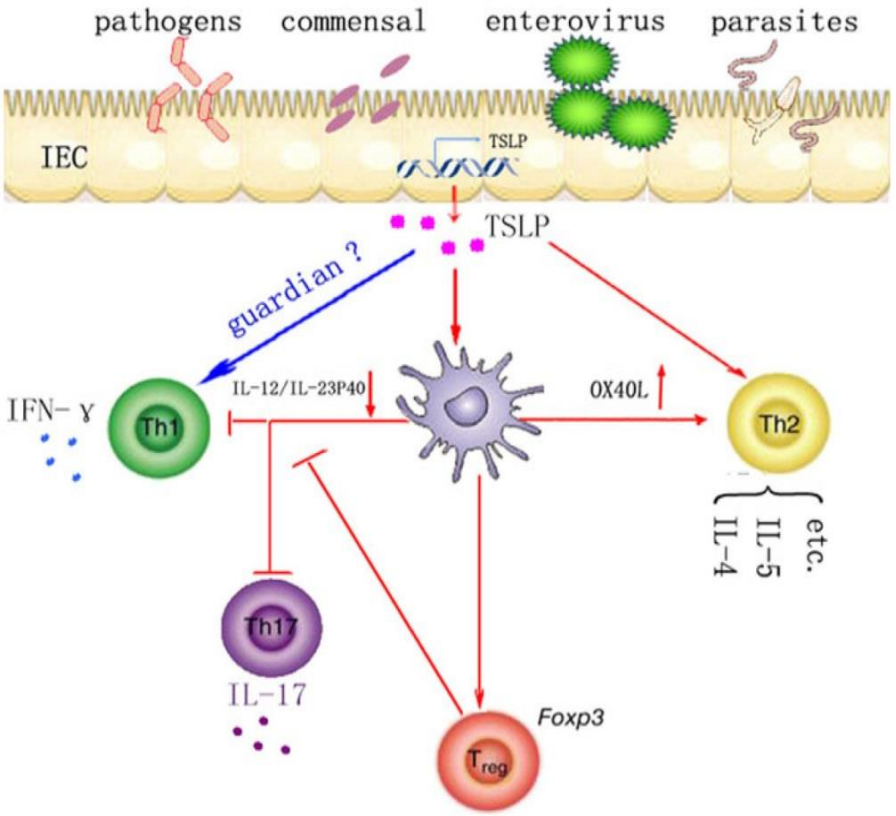


Figure 5. Key role of TSLP in intestinal immune homeostasis. *Intestinal ECs produce TSLP in response to commensal microflora in the gut. TSLP-conditioned DCs can promote Treg and Th2 cell differentiation (in the latter case by OX40L upregulation). They may also responsible for the inhibition of IL-12/23 p40 subunit and therefore attenuation of proinflammatory IL-17 and IFN-γ expression. TSLP can also directly promote Th2 cell differentiation. In case of the infections (enteric bacteria, viruses or parasites), IECs up-regulate their TSLP expression and maintain the balance between immune clearance and inflammatory response. In Salmonella-infected intestinal ECs, TSLP may also function as a 'guardian' to promote Th1 cells.*

Source: Li, M. et al., The regulation of thymic stromal lymphopoietin in gut immune homeostasis. Dig Dis Sci. 2011. 56(8):2215-20[41]

The important role of TSLP has been revealed in other organs of the gastrointestinal tract, such as in the esophagus. In eosinophilic esophagitis (EoE), a non-IgE mediated allergic disease of this organ, ECs overexpress TSLP mRNA and a prominent influx of basophils is also a characteristic feature[70,71,72,73]. In EoE Th2 type inflammation may be promoted by TSLP via basophils, which cells are responsible for type I allergic responses as they are capable of secreting histamine, as well as Th2 cytokines and chemokines such as IL-4, IL-13, CCL3, CCL4 and CCL12[73,74,75,76].

TSLP has not only effect on basophils in EoE, but also on DCs, T cells, Tregs and invariant natural killer T cells (iNKTs). TSLP activates DC maturation and activation, which promotes the polarization of naïve CD4+ T cells into Th2 cells[24]. It is hypothesized that the dysfunctional barrier of the esophagus in EoE allows APCs to process antigens, which promotes Th2 T cells in genetically predisposed individuals[77]. Moreover, TSLP can directly influence T cells to secrete Th2 cytokines[73,78,79].

On the other hand, increased Treg counts, but decreased Treg-derived anti-inflammatory IL-10 levels were detected in the esophagus of EoE patients compared to controls. These findings can be explained by immune compensatory mechanisms and by the well-known inhibitory effect of TSLP on the function of Tregs[80,81].

iNKTs are capable of recognizing self and foreign lipids and are supposed to link cow milk allergy with the pathogenesis of EoE. iNKTs from EoE patients secreted higher IL-13 levels than controls after milk sphingolipid treatment and their IL-13 production could be increased in the presence of TSLP[80,81,82].

1.5. 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 mRNA in low levels[24], in in the airway epithelium of patients suffering from asthma its mRNA and protein levels were detected to be increased and correlated with the levels of Th2 characteristic cytokines and disease severity. In contrast, inverse correlation was found between TSLP levels and lung function[14,73,83,84,85]. Genome-wide association studies (GWAS) revealed that TSLP is a susceptibility factor in the development of asthma[86,87]. 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[83].

Together with asthma and AD, AR makes up the so-called "allergic triad"[70]. GWAS studies demonstrated the association of TSLP polymorphism with AR in patients with asthma[88]. 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[87,89,90,91,92].

Nasal polyposis is another inflammatory disease of the upper airways with known association with AR and asthma. In nasal polyps increased TSLP expression was detected with the highest levels in patient suffering also from AD or rhinitis[70,90,93]. TSLP levels were found to be correlated with IgE levels and eosinophil counts indicating a crucial role of TSLP

in the background of prominent eosinophil influx. Moreover, TSLPR and OX40L expression of DCs were also highly upregulated in nasal polyps[90].

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 (e.g. CCL17 and CCL21) secretion, which finally leads to the promotion of inflammatory Th2 cells accompanied by the production of Th2 cytokines[24,94,95,96,97].

Literature data suggest that TSLP can directly induce the cytokine production ofTh2 cells in the challenge stage of this allergic diseases[96,98,99,100]. Similar to IBDs, impaired Treg development was also described[101,102]. Treg dysfunction may be mediated via nucleotide-binding oligomerization domain-containing protein (NOD) 2 and NOD1 activation. NOD1 and NOD2 upregulate the expression of TSLP (most likely via NF-κB), OX40L and TH2 cytokines[24,32,33,93,94,103,104]; on the contrary, activation of NODs inhibit antigen-specific FOXP3+ Treg cells[103]. Nevertheless, 9-cis-retinoic acid can inhibit TSLP expression via RXRs[39]. These findings suggest the existence of complex regulatory mechanisms of TSLP in the airways.

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[104].

1.6. Role of TSLP in the skin

Although TSLP mRNA expression has already been detected in healthy skin[26, 27], 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[24,28,105,106]. Till our study, no data was available regarding the possible homeostatic role of TSLP in the skin.

1.6.1. 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, hair shaft defects, and stratum corneum detachment via epidermal protease hyperactivity[107,108,109]. As LEKTI acts as an inhibitor of serine proteases such as kallikrein (KLK) 5, KLK7, and KLK14, loss of LEKTI causes permanent activation of these proteins[110]. 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[111].

A study performed on Spink5^{-}Par2^{-} double knockout mice could confirm the tight connection between KLK5 and PAR2 in initiating TSLP since in this condition TSLP levels could be dramatically decreased[112].

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[28,106].

1.6.2. 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[113].

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[113]. 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.

1.6.3. 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[114,115,116,117,118,119]. Over-reactive adaptive, dysregulated innate immune responses, and impaired skin barrier functions together lead to the manifestation of the disease[120]. 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[121]. Besides the altered adaptive immune functions, dysregulated innate immune and skin barrier mechanisms have also been studied[122,123,124]. A growing number of evidence supports the hypothesis that KCs can enhance the inflammatory responses in AD[125,126,127,128] by producing a unique profile of cytokines and chemokines (TSLP, IL-33, and CCL27)[123].

In the last few years, the importance of TSLP in AD has been highlighted. 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[129,130], epidermal[14,24] and stratum corneum[131] 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[131], on the contrary, the relationship between serum and epidermal TSLP levels and OSCORAD were highly controversial[129,130,132].

In the last decade, the role of KCs in the background of skin barrier dysfunction has also been highly emphasized[123]. FLG is a crucial skin barrier structural protein in the granular and corneal layers 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[133,134,135]. Besides genetic mutations of FLG gene, its copy number variations and other less studied barrier gene mutations (KLK7, SPINK5, and Claudin-1) were also described in AD[124].

On the other hand, several previous investigations have indicated that inflammatory cytokine and chemokine milieu, as well as frequent usage of detergents and exposure to allergens and *Staphylococcus,* 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[118,136,137,134,135]. Until now, the question of whether genetic or acquired skin barrier dysfunctions can alter KC immune function differently has not been raised.

1.6.4. Possible role of TSLP in healthy skin and papulopustular rosacea

Our skin provides an effective first line protection against pathogens and physico-chemical insults[138], on the other hand, harmless environmental agents and commensal microbiome are tolerated[1,2]. 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[139].

The ultrastructure of the skin surface is riddled with invaginations, including sweat glands, hair follicles, and sebaceous glands (Figure 6). These appendages go through the barrier into the dermis becoming a channel for external agents to reach inner tissues.

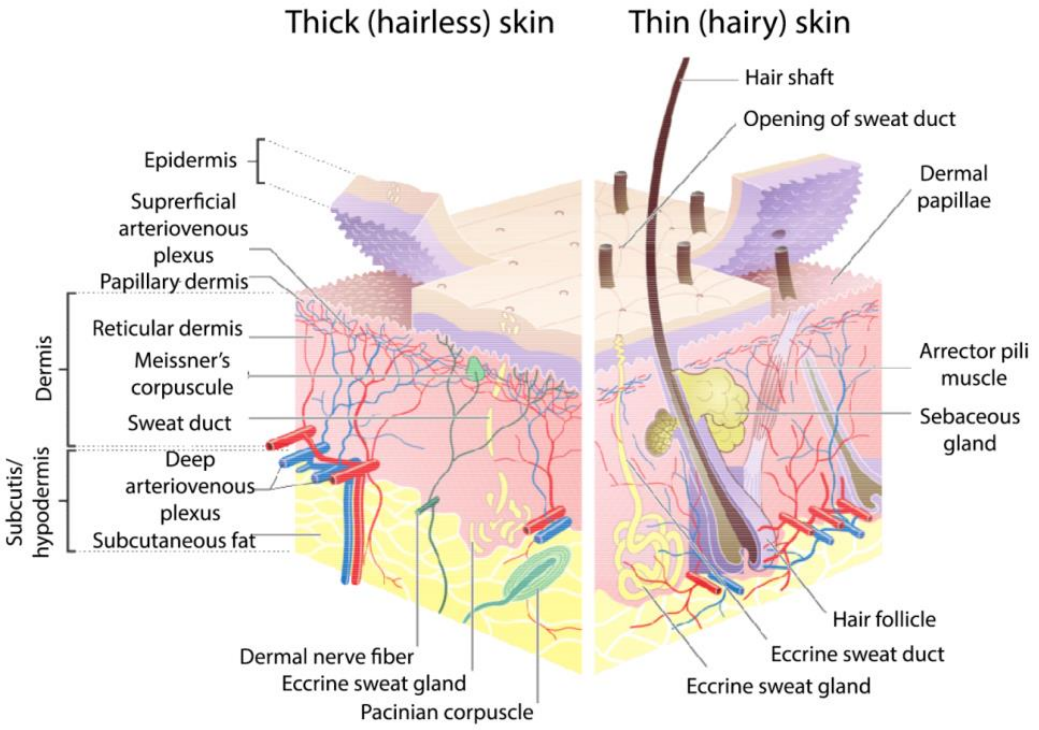


Figure 6. Illustration of the structures found within the skin

Source: https://www.boundless.com/physiology/textbooks/boundless-anatomy-and-physiology-textbook/integumentary

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[139]. Apocrine sweat glands are found mainly in sites such as the axilla, genitalia and perianal regions, and start their activity at puberty[139].

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[139].

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[2]. Skin microbial community exhibits remarkable differences on sebaceous, dry and moist regions probably connected to the different physiology of these sites[1,2] (Figure 7).

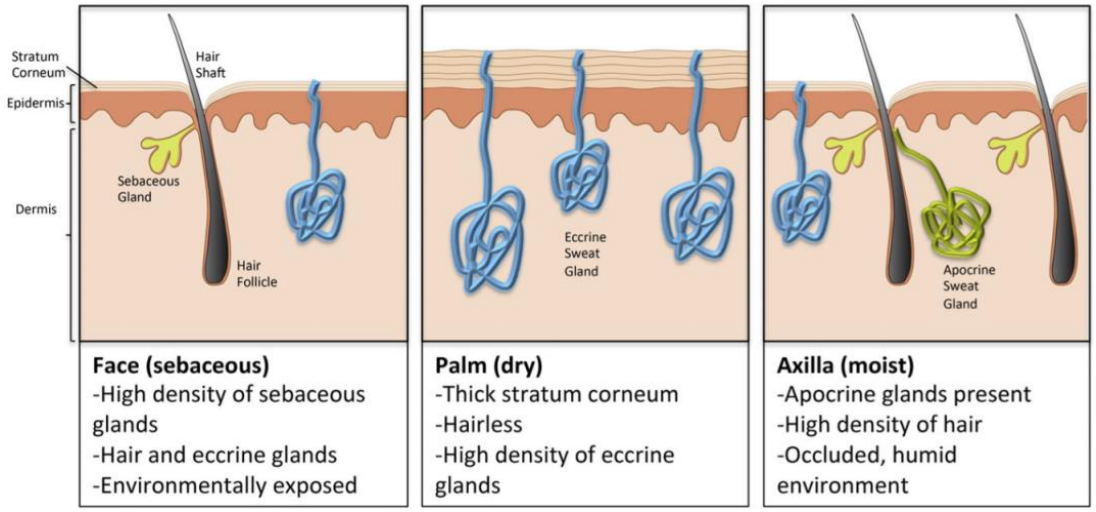


Figure 7. Anatomically different features of sebaceous, dry and moist sites. *Anatomic and environmental factors alter surface conditions contributing to microbial diversity.*
Source: Sanford, JA. & Gallo, RL., Semin Immunol. 2013. 30;25(5):370-7[139].

Since skin microbiota has a mutualistic connection with the skin immune system[2,8,9,140], 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[7,8,9].

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[26,27].

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[141], elevated cutaneous protease activity and LL-37 mRNA and protein expression were detected despite the absence of an obvious infectious or dangerous trigger[142,143,144]. 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[145,146,147]. Until our investigation, the possible role of TSLP in the background of PPR pathogenesis has not been revealed.

1.7. Role of TSLP in other conditions

1.7.1. Ocular tissues

The expression of TSLP has been described in conjunctival ECs, corneal ECs and corneoscleral tissues from patients with chronic allergic keratoconjunctivitis (atopic or vernal keratoconjunctivitis), furthermore, TSLP production could be triggered by *in vitro* stimulation[148,149,150].

1.7.2. Brest milk and maternal-fetal interface

TSLP is 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[151].

A recent study has been revealed the possible role of TSLP in maternal-fetal tolerance since decidual ECs and trophoblasts of the maternal-fetal interface in early placenta were expressed TSLP mRNA, but TSLP protein was found to be secreted only by trophoblasts[152].

1.7.3. Autoimmune diseases

An increasing number of evidence suggests the importance of TSLP in autoimmune diseases, but its exact role in their pathophysiology is still unclear and these studies particularly were performed on animal models[153,154].

For the present time, rheumatoid arthritis is the only autoimmune disease where the direct role of TSLP has been proven in disease pathogenesis. Increased TSLP and TNF-α concentrations were reported in synovial fluid and synovial fibroblasts of patients suffering from rheumatoid arthritis. Moreover, in synovial fibroblasts of patients with rheumatoid arthritis TSLP production could be induced by TNF-α and by isolated, synovial fluid-derived microparticles[155,156,157].

1.7.4. Infections

Besides the role of TSLP in the aforementioned diseases, it 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[10]. A recent study demonstrated that interactions between TSLP and its receptor are necessary for the initiation of protective Th2 immunity against Trichuris in a mouse model[64].

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, exceeding this interval DCs gain the ability to produce IL-12 and to promote protective Th1 immune responses, which are essential against intracellular pathogens[10].

In patients with *Helicobacter pylori*-infected follicular gastritis, TSLP protein production was described in mucosal lesions[158] and in response to *in vitro* stimuli, human primary gastric ECs produced TSLP[71,158].

At the present time, the exact role of TSLP in viral infections is unrevealed. KCs and airway ECs were detected to upregulate their TSLP expression in response to viral infections and double-stranded RNA[30,32,94,159,160,161]. TSLP secreted by respiratory syncytial virus-infected airway ECs could promote DC maturation and activation by elevating MHC II, OX40L and CD86 expression of DCs[70,159].

1.7.5. Cancers

The role of TSLP in cancer development has been recently reported[162,163]. TSLP is highly expressed in various cancers and melanoma as well as breast cancer cell lines[164,165]. TSLP has been described to promote intratumoral Th2 differentiation which led to tumor growth[164,166].

In vitro, human breast cancer cell-derived TSLP induced OX40L expression on the surface of DCs leading to an amplified TNF and IL-13 production by Th2 cells. Thus TSLP seems to be a key factor in establishing inflammatory Th2 microenvironment that initiates the development of breast cancer[164].

Human pancreatic cancers were also found to be associated with TSLP. Similar to human breast cancer, prominent Th2 infiltration was reported in patients with pancreatic cancer. 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. These CD11c+TSLPR+ DC could be described not only in the tumor stroma but also in tumor-draining lymph nodes[166].

Increased serum TSLP levels were also reported in patients with cutaneous T cell lymphomas[167].

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[168]. Moreover, in lung cancer tissues TSLP expression correlated with the number of Treg cells[169].

2. 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:

1. 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.
2. To investigate the effect of SGR skin-specific factors such as chitin and sebum components on TSLP expression.
3. 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).
4. 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.

3. Materials and Methods

3.1. 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; Table I) were obtained. After haematoxylin and eosin (H&E) staining, samples were sorted according to the number of sebaceous glands and were defined as SGP skin when containing $n \leq 1$ sebaceous glands and as SGR skin when containing $n \geq 3$ sebaceous glands in the field of view on 10 x magnification in the microscope (Table I).

Healthy individuals (HI)	Sex	Age	Localization	Count of Sebaceous Glands	Intensity of TSLP staining (visual scoring)	Intensity of TSLP staining (Pannoramic Viewer software)
SGP skin (n=8)						
HI 1	M	77	Shin	-	+	1,50E-03
HI 2	M	85	Shin	-	-	7,01E-04
HI 3	F	72	Lower arm	-	-	6,43E-04
HI 4	F	81	Lower arm	-	-	5,70E-04
HI 5	M	40	Lower arm	-	-	6,86E-04
HI 6	F	72	Lower arm	-	+	1,30E-03
HI 7	F	86	Hand	-	-	8,90E-04
HI 8	F	56	Shin	-	-	2,70E-04
MEAN AGE ± SD		71,1 ± 15,8				
SGR skin (n=10)						
HI 9	F	77	Heary scalp	+	++	5,66E-03
HI 10	M	62	Mandibula	++	+++	6,72E-03
HI 11	F	57	Nose	+++	+++	9,18E-03
HI 12	F	61	Nose	+++	+++	6,72E-03
HI 13	F	42	Scapula	++	++	5,51E-03
HI 14	F	38	Chin	++	++	4,51E-03
HI 15	M	56	Shoulder	+++	++	4,91E-03
HI 16	M	47	Heary scalp	++	+++	9,90E-03
HI 17	F	19	Face (central part)	+++	+++	8,01E-03
HI 18	M	66	Face (lateral part)	+++	+++	7,07E-03
MEAN AGE ± SD		52,5 ± 16,8				

Patients (P)	Sex	Age	Localization	Count of Sebaceous Glands	Intensity of TSLP staining (visual scoring)	Intensity of TSLP staining (Pannoramic Viewer software)
PPR skin (n=10)						
P 1	F	65	Face	+++	++	6,78E-03
P 2	F	71	Face	+++	-	9,18E-04
P 3	M	70	Nose	+++	-	1,03E-04
P 4	F	68	Face	+++	-	1,01E-03
P 5	F	57	Nose	+++	+	3,01E-03
P 6	M	69	Nose	+++	+	3,83E-03
P 7	M	66	Face	++	+	4,25E-03
P 8	M	67	Eyebrow	+++	++	6,41E-03
P 9	M	65	Forehead	++	+	3,79E-03
P 10	M	72	Eyebrow	++	+	4,56E-03
MEAN AGE ± SD		67,0 ± 4,3				
AD skin (n=6)						
P 11	F	18	Lower arm	-	+++	2,65E-02
P 12	F	29	Lower arm	-	+++	1,56E-02
P 13	M	35	Lower arm	-	+++	1,36E-02
P 14	F	21	Lower arm	-	+++	1,43E-02
P 15	F	19	Lower arm	-	+++	1,79E-02
P 16	M	38	Lower arm	-	+++	1,76E-02
MEAN AGE ± SD		26,7 ± 8,6				

Table I. Characteristics and TSLP protein expression in the skin of the studied individuals. *Scoring of sebaceous glands' count was performed according to the number and size of sebaceous glands in the field of view on 10 x magnification: samples containing $n \leq 1$ sebaceous gland were defined negative (-), containing $n \geq 3$ were defined positive and scored in accordance with the area of sebaceous glands in percentage of dermal surface: (+): 5-15%; (++): 15-30% and (+++): more than 30%. Visual scoring of TSLP staining was performed by a professional pathologist according to the percentage of the epidermal surface positively stained for TSLP: (-): 0-5%; (+): 5-15%; (++): 15-30% and (+++): more than 30%.*

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)[170] 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 characteristics of both AD patient groups are shown in Table II.

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, mean OSCORAD: 44.8) and patients with severe symptoms with FLG mutation (n=12, all were heterozygotes for one of the 2 alleles [2282del4, R501X], mean OSCORAD: 42.6). Biopsies were taken from all 24 patients with AD, 5–5 samples in both groups were used for immunohistochemistry (IHC) and 12–12 samples were analyzed by RT-PCR. Biopsies from 5 healthy controls were investigated in all experiments.

	FLG Wt severe AD n=12		FLG Mutant severe AD n=12	
FLG mutation	2282del4	0/12	2282del4	12.szept
	R501x	0/12	R501x	12.márc
	Mean	**±SD**	**Mean**	**±SD**
Age (year)	21	9,98	12,8	7,85
Age at onset (month)	37	41,8	5	7,7
SCORAD	44,8	8,28	42,6	6,3
Blood Eosinophil count (%)	0,87	0,57	0,82	0,53
TEWL nonlesional skin (g/m2/h)	29,76	10,59	26,2	12,93
TEWL lesional skin (g/m2/h)	43,31	4,52	42,39	9,97
Serum Total IgE (kU/L) *	2813,9	1750,3	8313,7	6624,8
Sensitization proven by Prick test*	6/12		12/12	

Table II. Clinical characteristics of AD patients in our second study. *There were no AD patients with a compound heterozygous mutation of FLG. Significant differences were found between the two AD groups in total IgE levels and frequency of sensitization (*p < 0,05). Other parameters did not differ significantly. AD, atopic dermatitis; IgE, Immunoglobulin E; SCORAD, SCORing Atopic Dermatitis; SD, standard deviation; TEWL, transepidermal water loss.*

3.2. Immunohistochemistry

For IHC analyses, paraffin-embedded sections from patients and healthy controls were deparaffinized. Heat-induced antigen retrieval was performed and sections were pre-processed with H_2O_2 for 10 minutes. Sections were stained with antibodies (Ab) against human TSLP

(TSLP Ab 1: rabbit IgG [ab47943]: Abcam, Cambridge, UK; TSLP Ab 2: sheep polyclonal IgG [AF1398] (R&D Systems, MN, USA); TSLP Ab 3: mouse monoclonal IgG [MAB1398]: R&D Systems), human CD3 (rabbit polyclonal IgG [bs-0765R]: Bioss, MA, USA), human CD4 (rabbit monoclonal IgG [ab133616]: Abcam), human CD11c (rabbit monoclonal IgG [ab52632]: Abcam), CD1a (rabbit monoclonal IgG [ab108309]: Abcam), CD163 (rabbit monoclonal IgG [ALX-810-213]: Enzo, Farmingdale, NY, USA), CD83 (mouse monoclonal IgG [ab123494]: Abcam), TARC (goat polyclonal IgG [AF364]: R&D Systems), human IL-10 (mouse monoclonal IgG [mab71148]: Covalab, Budapest, Hungary), human IL-13 (rabbit polyclonal IgG [bs-0560R]: Bioss), human IL17A (rabbit polyclonal IgG [pab70016]: Covalab), human IFN-γ (mouse monoclonal IgG [mab30200]: Covalab), human FLG (mouse monoclonal IgG [ab218862]: Abcam), human Ki67 (mouse monoclonal IgG [AMAB90870]: Sigma-Aldrich, Dorset, UK), human IL-33 (mouse monoclonal IgG [ab54385]: Abcam) and human CCL27 (mouse polyclonal IgG [SAB1410133]: 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, Burlingame, CA, USA) or 3,3'-Diaminobenzidine (DAB) (Dako). Sections were counterstained with methylene green or haematoxylin, dehydrated and covered with a glass coverslip. The detection of one protein was carried out on all sections in parallel at the same time to enable us to evaluate comparable protein levels. Positive, Ig, and isotype controls were also used to normalize staining against all proteins (mouse IgG2a Kappa [Covalab], sheep serum [Sigma-Aldrich], rabbit immunoglobulin fraction and goat serum [Dako]).

3.3. Haematoxylin and eosin and May–Grünwald–Giemsa stainings

Skin specimens were also stained with haematoxylin and eosin (H&E) in order to determine the number and size of sebaceous glands as well as to measure the epidermal thickness of AD skin samples as the quotient of the epidermal area and epidermal length in each specimen. To perform H&E staining the deparaffinized samples were incubated in haematoxylin solution for 5 min at room temperature, flushed with tap water for 15 min), then counterstained for 1 min in 0.1% ethanol solution of eosin acetified with a few drops of acetic acid, dehydrated and covered with a glass coverslip.

May–Grünwald–Giemsa (MGG) staining was also performed to detect mast cells, eosinophil and neutrophil granulocytes in SGP, SGR, and PPR samples. To complete MGG staining deparaffinized samples were incubated in MG working solution for 5 min and then, without flushing, stained with Giemsa working solution for 15 min at room temperature, washed in distilled water, dehydrated and covered with a glass coverslip.

3.4. 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 Ltd., Budapest, Hungary), 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.

Measuring TSLP levels ROIs were selected according to two different methods. Relative (MA/FA) TSLP level was quantified as described above. Absolute TSLP level was measured as the quotient of FA and the epidermal length of the FA in each specimens. Important to mention that both methods showed the same result with smaller differences in relative TSLP

levels due to the fact that acanthotic and thicker epidermis is characteristic to AD skin (smaller FA/MA value). Comparing the TSLP staining in the upper layer (approximately 50 μm which corresponds to the thickness of healthy epidermis) of AD epidermis to healthy SGR epidermis our result was similar that we found in absolute TSLP levels (not shown).

Epidermal thickness as a well-accepted method for the measurement of the severity of skin inflammation in AD 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 (a dermatopathologist and a biologist) by using Pannoramic Viewer 1.15.2 (3DHistech Ltd., Budapest, Hungary) software's HistoQuant and NuclearQuant applications. The observers did not have information about the FLG genotype of patients with AD. Visual scoring of TSLP, May-Grünwald-Giemsa staining, and count of sebaceous glands was performed by a dermatopathologist.

3.5. Stratum corneum samples and TSLP immunocytochemistry

Tape-stripping method was used to collect stratum corneum samples from the forearm of healthy and AD individuals and from the face of healthy and PPR individuals according to the method described in a previous report[171]. Until analysis, the tapes containing stratum corneum samples were stored at −20°C. The tapes were attached to silane coated microscope slides (Sigma-Aldrich), and then incubated overnight in n-hexane (Sigma-Aldrich), which allowed the tapes to remove spontaneously. 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 (rabbit polyclonal IgG: Abcam) at 4°C. The cells were then incubated with Alexa-Fluor®-488-conjugated anti-rabbit IgG secondary antibody (goat; 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 photographed, and TSLP levels were determined as the mean values of the quotient of

fluorescent intensity and the area of the stratum corneum in five different fields.

3.6. Cell culture experiments

HaCaT KCs were cultured at 37°C in a humidified atmosphere containing 5%(v/v) CO_2, in Dulbecco Modified Eagle Medium (DMEM) (Thermo Scientific, Bioscience, Budapest, Hungary) supplemented with 10% Fetal Bovine Serum, 1% Antibioticum Mixture (Penicillin, Streptomycin, Neomycin) and 2 mM glutamine (Sigma-Aldrich, Dorset, UK). Cells were seeded at 50 000 cells/well in 12-well plates for RT-PCR and cytokine ELISA measurements, and cultured until they reached 80% confluence, then the medium was changed. After further 24 hours of incubation, cells were treated for 6h or 24 h with different materials (Sebomed with or without SZ95 supernatant, chitin, free fatty acids (FFAs) (squalene, palmitic acid, stearic acid, oleic acid and linoleic acid).

NHEK cells were cultured at 37°C in a humidified atmosphere containing 5%(v/v) CO_2, in EpiLife® medium supplemented with HKGS (all from Gibco™, Thermo Fisher Scientific, Budapest, Hungary). Cells were seeded at 50 000 cells/well in 12-well plates for RT-PCR and cytokine ELISA measurements, and cultured until they reached preconfluency (70-80%) or postconfluency, then the medium was changed. After incubating preconfluent cells for 24 hours and postconfluent cells for 72 hours, cells were treated for 6h or 24 h with different free fatty acids (FFAs): with squalene, palmitic acid, stearic acid, oleic acid and with linoleic acid.

Human SZ95 sebocytes[172] were cultured at 37°C in a humidified atmosphere containing 5%(v/v) CO_2, in Sebomed medium (Biochrom, Berlin, Germany) supplemented with 10% Fetal Bovine Serum, 1 mM $CaCl_2$ solution, 1% penicillin/streptomycin and 5 µg/ml Epidermal Growth Factor (EGF) (all from Sigma-Aldrich). Cells were kept in culture until reaching approximately 80% confluence. Prior to supernatant collection, the used medium was replaced with Sebomed medium containing 0.5% Fetal Bovine Serum, 1 mM $CaCl_2$ solution, with or without 1% penicillin/streptomycin, lacking EGF. 24h supernatants were

collected and filtered using 0,2-µm syringe filters (Sarstedt, Nümbrecht, Germany) and used for experiments.

3.7. 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).

3.8. RNA isolation and cDNA synthesis

All samples were homogenized in Tri reagent solution (Sigma-Aldrich, Dorset, UK) with Tissue Lyser (QIAGEN) using previously autoclaved metal beads (QIAGEN), and total RNA was isolated from the human skin tissues and HaCaT and treated with DNase I (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentration and purity of the RNA were measured by means of NanoDrop spectrophotometer (Thermo Scientific, Bioscience, Budapest, Hungary), and its quality was checked using Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For RT-PCR, cDNA was synthesized from the isolated RNA using the High Capacity cDNA Archive Kit (Invitrogen, Life Technologies, San Francisco, CA).

3.9. Quantitative real-time PCR

RT-PCR was carried out in triplicate using pre-designed MGB assays ordered from Applied Biosystems (Life Technologies). The following TaqMan Gene Expression assays were used: Peptidylprolyl isomerase A (PPIA) (Hs99999904_m1), total TSLP (Hs00263639_m1), CD80 (Hs01045163_m1), CD83 (Hs00188486_m1), CD86 (Hs01567026_m1), LAMP3 (Hs00180880_m1), IL-13 (Hs00174379_m1), IL-10 (Hs00174086_m1), IL-17A (Hs00174383_m1), IFN-γ (Hs00174143_m1), T-box transcription factor 21 (TBX21) (Hs00203436_m1), GATA Binding Protein (GATA) 3 (Hs00231122_m1), RAR Related

Orphan Receptor C (RORC) (Hs01076112_m1), Forkhead box P3 (FOXP3) (Hs01085834_m1), C-C chemokine receptor (CCR) 4 (Hs00747615_s1) and CCR8 (Hs_00174764_m1), IL-33 (Hs00369211_m1) and CCL27 (Hs00171157_m1).

All reactions were performed with an ABI PRISM® 7000 Sequence Detection System. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method[173] normalized to the expression of PPIA mRNA. In our second study relative gene expression to healthy controls was calculated as a quotient of the normalized gene expression in Wt AD or FLG mutant AD skin and normalized gene expression in healthy skin.

3.10. Filaggrin genotyping

Analyses of the FLG mutations R501X and 2282del4, responsible for 80–99% of all FLG mutations in white European patients with AD[174,175], were performed for all patients. DNA isolated from peripheral blood mononuclear cells with GenElute Blood Genomic DNA Kit (Sigma, Chemical Co., St Louis, MO, USA) was subjected to PCR amplification. Primers for genotyping were: ACG TTC AGG GTC TTC CCT CT and ATG GGA ACC TGA GTG TCC AG for R501X; and CAG TCA GCA GAC AGC TCC AG and AAA GAC CCT GAA CGT CGA GA for 2282del4. PCR amplification conditions were as follows: 1 cycle of 95°C for 5 min; 35 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 10 min. All PCR products were purified with QIAquick PCR purification Kit (Qiagen Inc., Hilden, Germany) and bidirectionally sequenced on an ABI Prism 3100 automated sequencer with Big-Dye terminator cycle sequencing reagents (Applied Biosystems, Foster City, CA, USA).

3.11. 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, Cologne, Germany) 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, Cologne, Germany) on the flexural forearm and the face of healthy individuals (n=50).

3.12. 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. Two-tailed P values <0.05 were considered statistically significant (*p<0.05, **p<0.01). Each experiment was performed in triplicate obtained from three independent biological replicates.

4. Results

4.1. Characterization of the special immune milieu of sebaceous and dry healthy skin regions and their alterations in region-specific skin diseases

4.1.1. 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 (Figure 8a). 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 (Table 1.). 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 (Figure 8a and b). 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 (Figure 8c). Interestingly, RT-PCR analysis detected nearly similar total TSLP mRNA expression in all skin types (SGR, SGP, and AD skin) (Figure 8d).

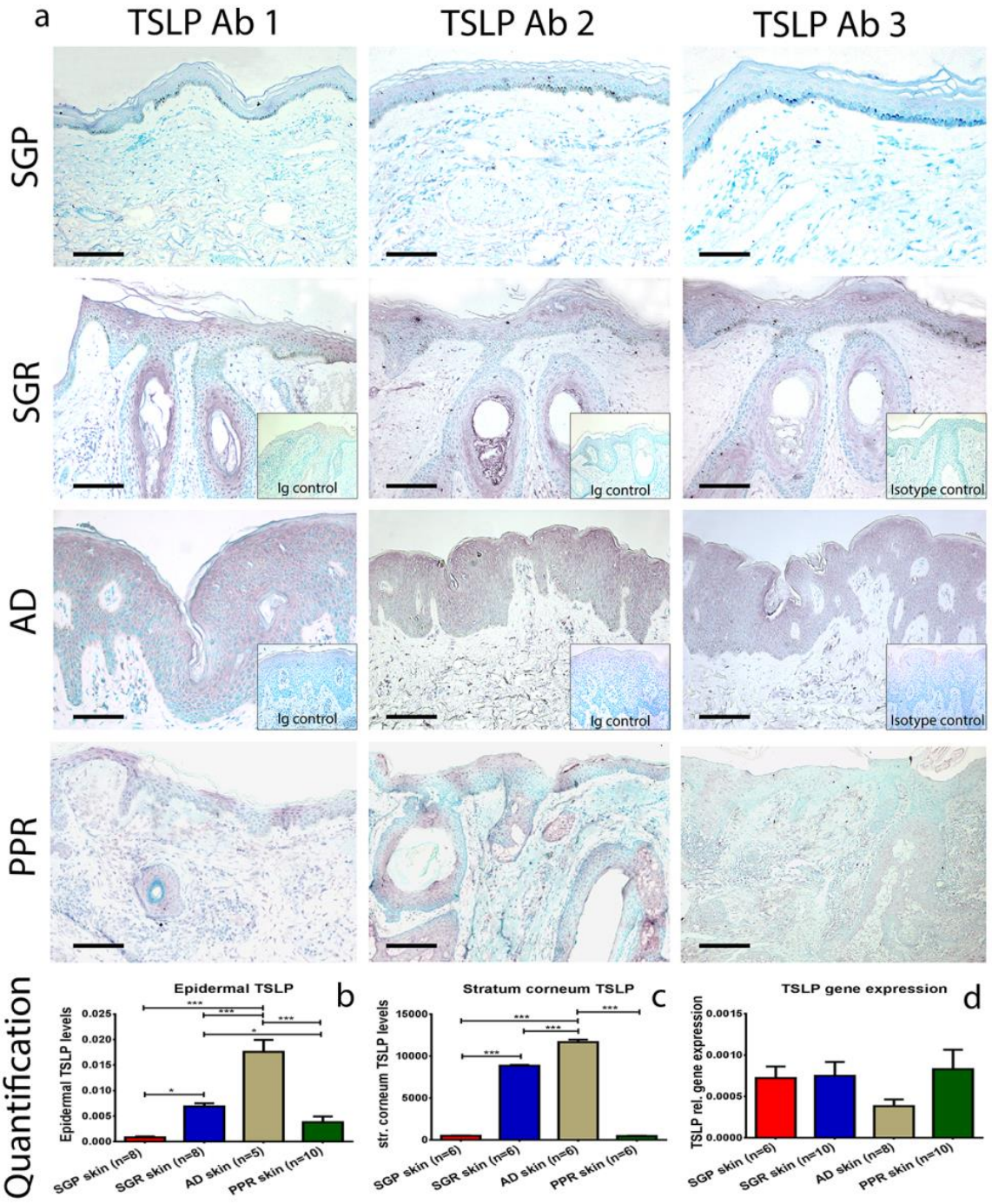


Figure 8. TSLP is absent from SGP skin, but constitutively expressed in SGR skin and attenuated in PPR skin. *(**a**) Representative images for immunostaining of TSLP with 3 different TSLP antibodies (TSLP Ab 1: rabbit polyclonal anti-human TSLP Ab; TSLP Ab 2: sheep polyclonal anti-human TSLP Ab; TSLP Ab 3: mouse monoclonal anti-human TSLP Ab) in SGP, SGR, AD and PPR skin sections. Size bars = 100 µm. Ig or isotype controls are presented in the bottom right corner of SGR and AD samples. Quantification of (**b**) epidermal TSLP protein levels; (**c**) Stratum corneum TSLP protein levels and (**d**) TSLP mRNA levels. Graphs show the mean ± standard error of the means of measured protein and mRNA levels (*P < 0.05; **P < 0.01; ***P < 0.001, as determined by one-way ANOVA followed by Newman-Keuls test). Ab, antibody; AD, atopic dermatitis; ANOVA, analysis of variance; PPR, papulopustular rosacea; SGP, sebaceous gland poor; SGR, sebaceous gland rich; TSLP, thymic stromal lymphopoietin.*

4.1.2. 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) biopsy samples (Table 1), we did not investigate further the effect of UV.

To study the effect of chitin – a major component of *Demodex folliculorum*, which is part of the normal skin flora in SGR skin – and sebum, HaCaT KCs were treated with chitin (Figure 9a), with supernatant of cultured human SZ95 sebocytes (Figure 9b) and with different lipid components of sebum (Figure 9c). After chitin and sebocyte supernatant treatment, induction of TSLP mRNA could be non-significantly triggered. Of the used lipid components, palmitic acid, oleic acid 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 (Figure 9d and e). On the other hand, the basal TSLP protein levels could not be elevated by any of the aforementioned agents (Figure 9a-c). 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 (Figure 9f and g). No TSLP protein secretion by NHEKs could be detected (not shown). It has previously been found in AD skin that barrier damage can also lead to TSLP production by KCs [132]; 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 (not shown).

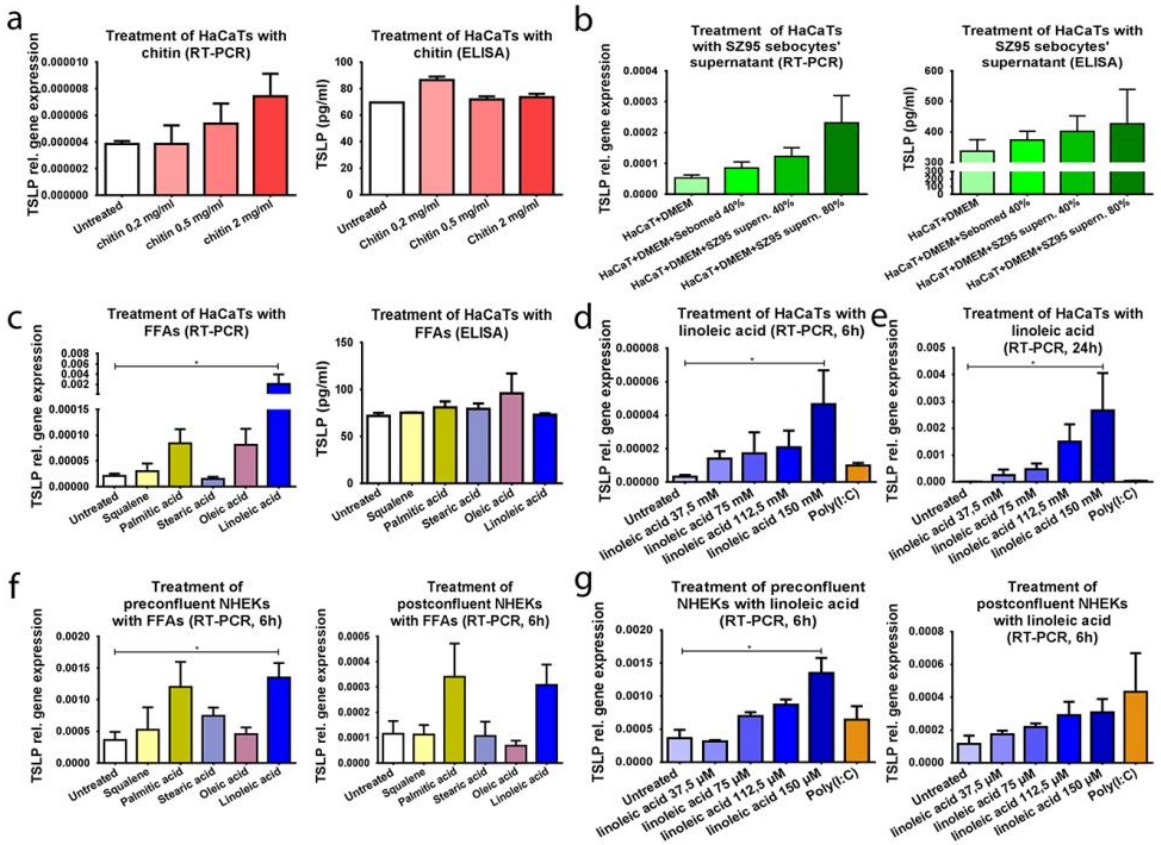


Figure 9. Linoleic acid upregulates TSLP gene expression in HaCaT keratinocytes. *HaCaT KCs were incubated with (**a**) different chitin concentrations (0,2; 0,5 and 2 mg/ml), with (**b**) SZ95 sebocyte culture medium and SZ95 sebocyte supernatant (40% and 80%) and with (**c**) different sebum components, for 24 hours. Concentration-dependent effect of linoleic acid (37,5; 75; 112,5 and 150 µM) and Poly (I:C) after (**d**) 6h and **(e)** 24h treatment. TSLP mRNA levels were detected after treating pre- and postconfluent NHEK cells with different sebum components (**f**) and with different concentrations of linoleic acid (**g**) for 6 hours. No TSLP secretion of NHEKs could be detected by ELISA (not shown). Higher concentrations of linoleic acid than 150 µM highly decreased the viability of both cell types and incubation of NHEKs with linoleic acid for 24h had toxic effect. TSLP mRNA and protein levels were quantified by RT-PCR and ELISA. Graphs show the mean ± standard error of the means of measured protein and mRNA levels (*P < 0.05, as determined by one-way ANOVA followed by Newman-Keuls test). ANOVA, analysis of variance; ELISA, Enzyme-Linked Immunosorbent Assay; FFA, free fatty acid; RT-PCR, quantitative real-time PCR.*

4.1.3. 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 in SGR and SGP skin samples. IHC revealed no significant difference between the LC counts of SGP and SGR skin samples (Figure 10).

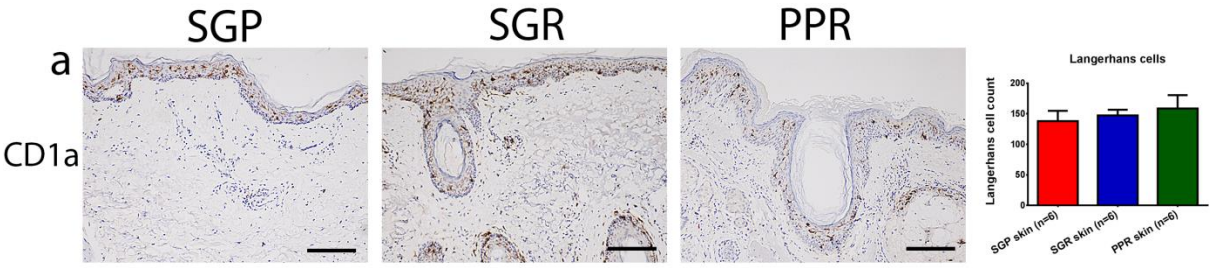


Figure 10. Langerhans cell counts are similar in SGP, SGR and PPR skin samples. *Representative images of CD1a immunostaining and cell counts of Langerhans cells. Size bars = 100 µm. Graphs show the mean ± standard error of the means of cell counts. PPR, papulopustular rosacea; SGP, sebaceous gland poor; SGR, sebaceous gland rich.*

In contrast, CD11c+ DCs were present in significantly higher numbers (Figure 11a and d) 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 (Figure 11a and d).

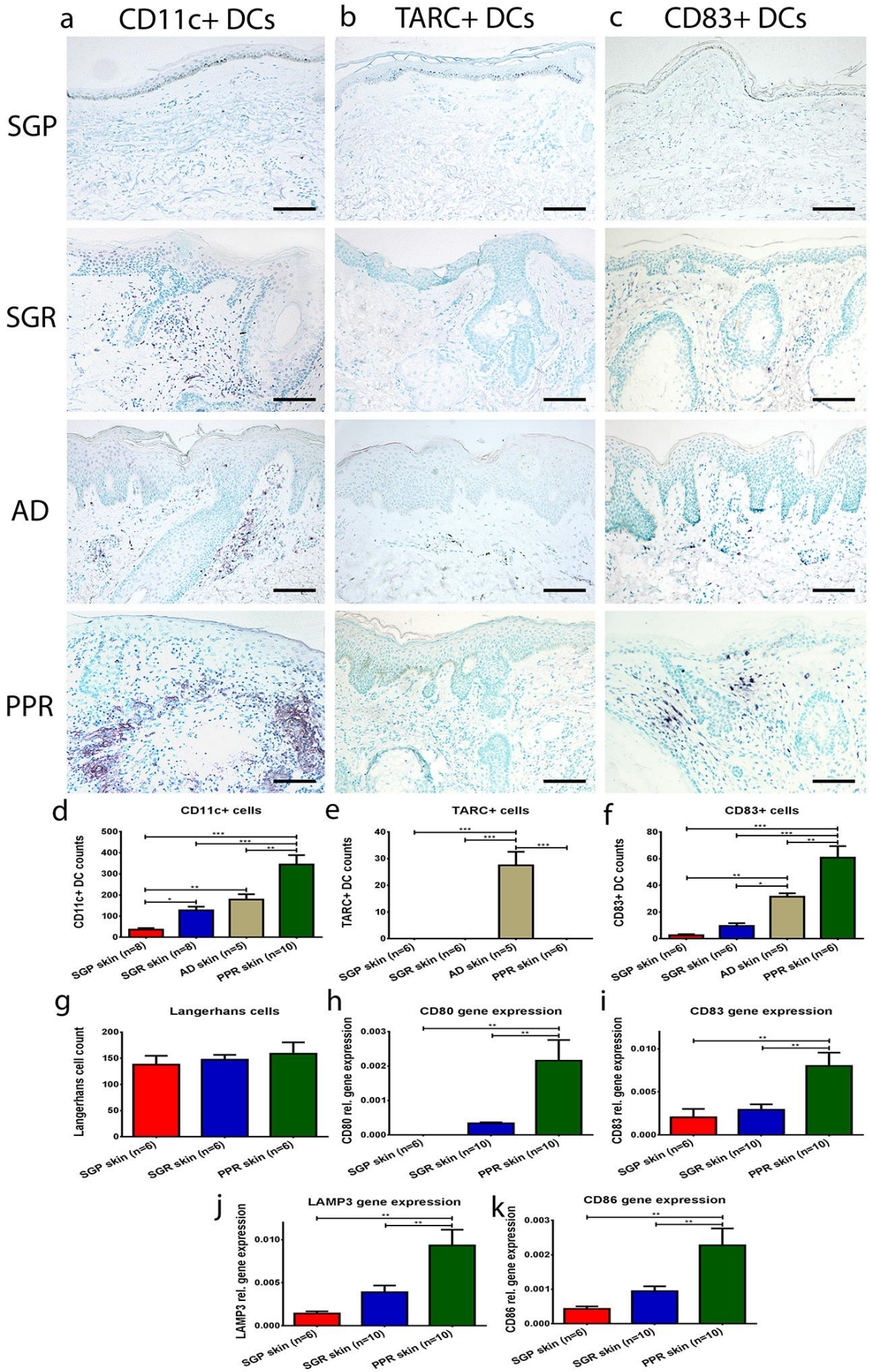

a CD11c+ DCs
b TARC+ DCs
c CD83+ DCs
SGP
SGR
AD
PPR
d
CD11c+ cells
CD11c+ DC counts
SGP skin (n=8)
SGR skin (n=8)
AD skin (n=5)
PPR skin (n=10)
e
TARC+ cells
TARC+ DC counts
SGP skin (n=6)
SGR skin (n=6)
AD skin (n=5)
PPR skin (n=6)
f
CD83+ cells
CD83+ DC counts
SGP skin (n=6)
SGR skin (n=6)
AD skin (n=5)
PPR skin (n=6)
g
Langerhans cells
Langerhans cell count
SGP skin (n=6)
SGR skin (n=6)
PPR skin (n=6)
h
CD80 gene expression
CD80 rel. gene expression
SGP skin (n=6)
SGR skin (n=10)
PPR skin (n=10)
i
CD83 gene expression
CD83 rel. gene expression
SGP skin (n=6)
SGR skin (n=10)
PPR skin (n=10)
j
LAMP3 gene expression
LAMP3 rel. gene expression
SGP skin (n=6)
SGR skin (n=10)
PPR skin (n=10)
k
CD86 gene expression
CD86 rel. gene expression
SGP skin (n=6)
SGR skin (n=10)
PPR skin (n=10)

Figure 11. Elevated DC count with low activation state and without TARC positivity is detected in SGR skin. A robust influx of CD83+, TARC negative DCs is characteristic to PPR. *Representative images for immunostaining of (**a**) CD11c, (**b**) TARC and (**c**) CD83 in SGP, SGR, AD and PPR skin sections. Size bars = 100 μm. Cell counts of (**d**) CD11c+ DCs, (**e**) TARC+ DCs, (**f**) CD83+ DCs and (**g**) Langerhans cells were blindly analyzed by Pannoramic Viewer software. Quantification of (**h**) CD80, (**i**) CD83, (**j**) LAMP3, and (**k**) CD86 mRNA levels by RT-PCR. Graphs show the mean ± standard error of the means of measured protein and mRNA levels (*P < 0.05; **P < 0.01; ***P < 0.001, as determined by one-way ANOVA followed by Newman-Keuls test). ANOVA, analysis of variance; AD, atopic dermatitis; LAMP3, lysosome-associated membrane glycoprotein 3; PPR, papulopustular rosacea; RT-PCR, quantitative real-time PCR; SGP, sebaceous gland poor; SGR, sebaceous gland rich; TARC, thymus and activation regulated chemokine.*

To further analyze the characteristics of DCs, their classical maturation and/or 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 (CCL17)], an atopic eczema specific, DC secreted chemokine, and CD83 were also assessed by IHC.

Although the number of CD83 positive cells (Figure 11c and f) and mRNA levels of CD80 (Figure 11h), CD83 (Figure 11i), CD86 (Figure 11k) and LAMP3 (CD208) (Figure 11j) 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 (Fig 11c and f). TARC was completely absent from both types of healthy skin but was present in AD samples (Figure 11b and e).

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

Next, CD3+ and CD4+ cells were stained in SGR and SGP skin samples. CD3+ (Figure 12a and d) and CD4+ (Figure 12b and e) 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.

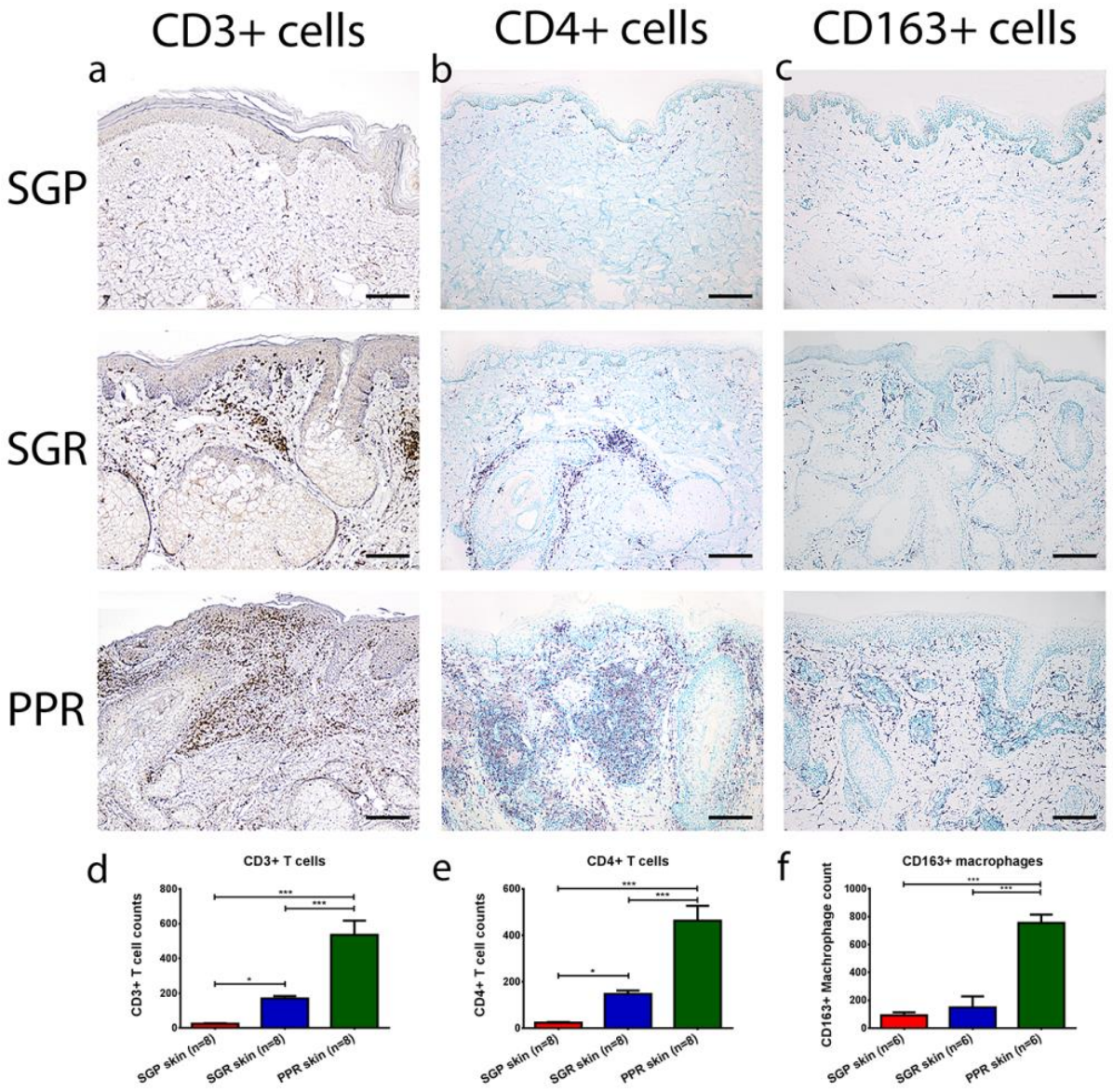


Figure 12. SGR skin sites are characterized by remarkable T cell presence and similar macrophage count compared to SGP skin. In PPR skin, robust influx of both cell types was observed. *Representative images for immunostaining of (**a**) CD3, (**b**) CD4 and (**c**) CD163 in SGP, SGR and PPR skin sections. Cell counts of (**c**) CD3+, (**d**) CD4+ T cells and (**e**) CD163+ macrophages were blindly analyzed by Pannoramic Viewer software. Size bars = 100 μm. Graphs show the mean ± standard error of the means of measured protein levels (*P < 0.05; **P < 0.01; ***P < 0.001, as determined by one-way ANOVA followed by Newman-Keuls test). ANOVA, analysis of variance; PPR, papulopustular rosacea; SGP, sebaceous gland poor; SGR, sebaceous gland rich.*

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 (Figure 13a-d). RT-PCR analyses of the aforementioned cytokines were also performed and showed a similar pattern to that found at the protein levels, although the differences were not significant (Figure 14a-d). In SGP skin the cytokine content was very low, in contrast to the characteristic IL-17/IL-10 cytokine milieu of SGR skin.

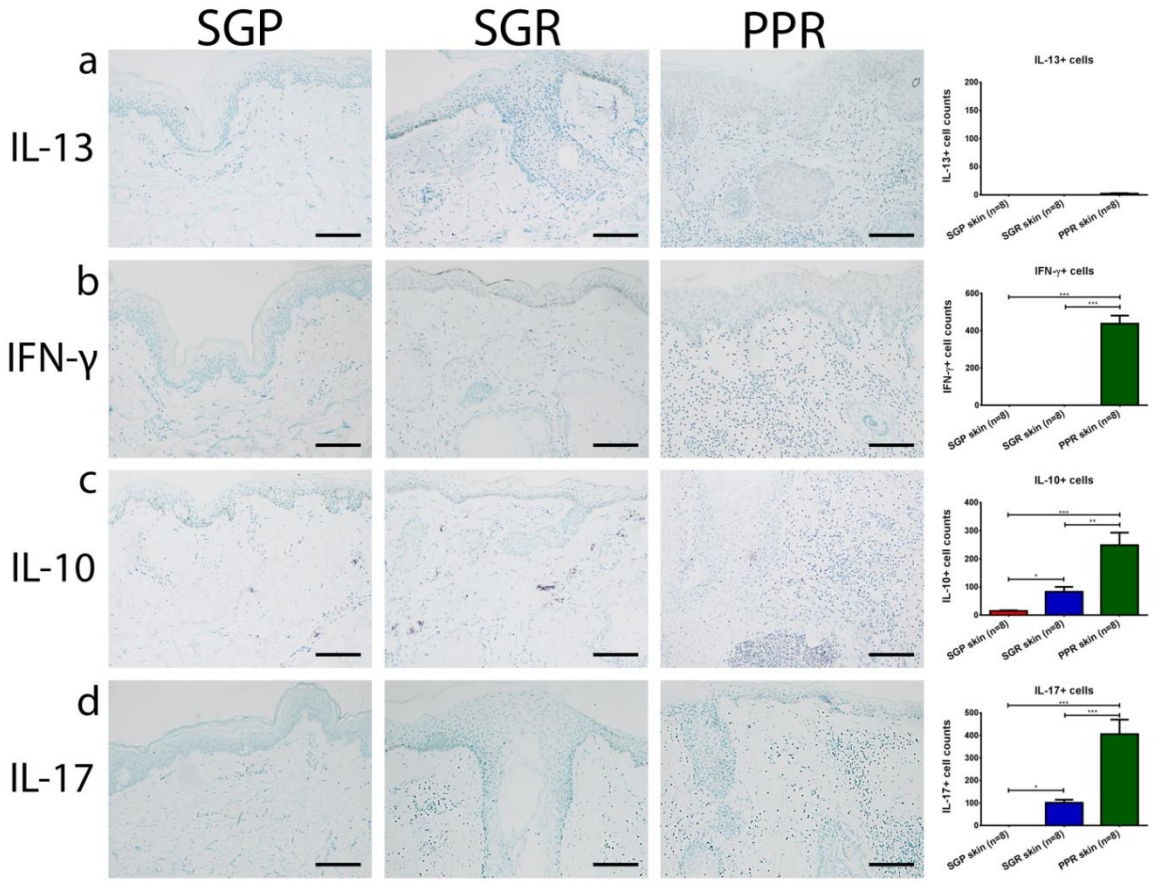


Figure 13. SGR skin sites, but not the SGP skin areas, are characterized by non-inflammatory IL-17/IL-10 milieu. In PPR skin, inflammatory IFN-γ/IL-17 cytokine milieu was observed. *Representative images and cell counts of (**a**) IL-13+, (**b**) IFN-γ+, (**c**) IL-10+ and (**d**) IL-17+ cells. Graphs show the mean ± standard error of the means of measured protein and mRNA levels. (*P < 0.05; **P < 0.01; ***P < 0.001, as determined by one-way ANOVA followed by Newman-Keuls test). ANOVA, analysis of variance; IL, interleukin; IFN-γ, interferon-gamma; PPR, papulopustular rosacea; SGP, sebaceous gland poor; SGR, sebaceous gland rich.*

Then, the mRNA levels of transcription factors characteristic of different Th cell subsets were investigated. Expression of T-bet (TBX21 gene), mediating inflammatory Th17 [Th17(23)] and Th1 cell responses (Figure 14e) and GATA3, mediating Th2 responses (Figure 14f), were detected at similar levels in SGP and SGR skin. On the other hand, RORγt (RORC gene), mediating non-inflammatory Th17 [Th17(β)] and Th17(23) development (Figure 14g) and FOXP3, characteristic of Tregs (Figure 14h), showed notably higher expression levels in SGR compared to SGP skin. CCR4 (Figure 14i) and CCR8 (Figure 14j) mRNA levels, typical skin homing receptors of Tregs, were also detected in notably, but non-significantly higher levels in SGR skin compared to SGP [176, 177].

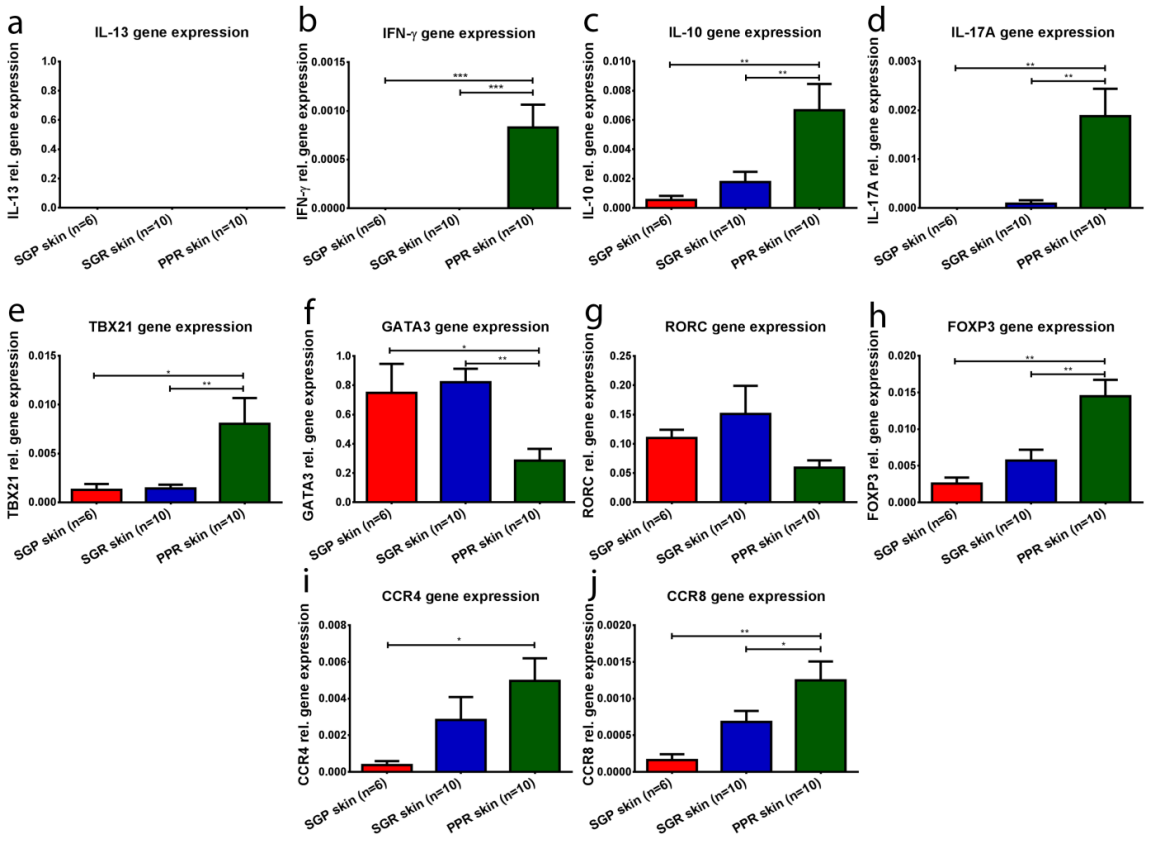
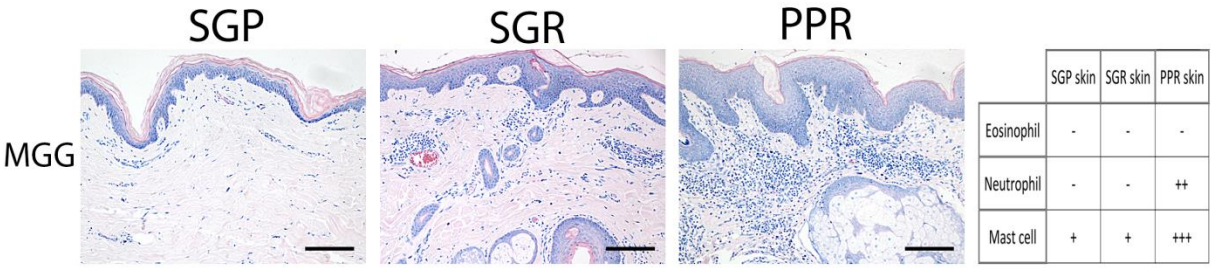


Figure 14. Gene expression profile of cytokines, transcription factors and Treg homing receptors in SGP, SGR and PPR skin. *Gene expression levels of* ***(a)*** *IL-13,* ***(b)*** *IFN-γ,* ***(c)*** *IL-10,* ***(d)*** *IL-17A cytokines and* ***(e)*** *TBX21,* ***(f)*** *GATA3,* ***(g)*** *RORC and* ***(h)*** *FOXP3 transcription factors and* ***(i)*** *CCR4 and* ***(j)*** *CCR8 Treg homing receptors detected by RT-PCR. Graphs show the mean ± standard error of the means of measured protein and mRNA levels. (*P < 0.05; **P < 0.01; ***P < 0.001, as determined by one-way ANOVA followed by Newman-Keuls test). ANOVA, analysis of variance; IL, interleukin; IFN-γ, interferon-gamma; PPR, papulopustular rosacea; SGP, sebaceous gland poor; SGR, sebaceous gland rich.*

4.1.5. 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 (macrophage labeling) and May-Grünwald-Giemsa (MGG) staining were performed. Examining the overall view of the skin sections, no significant differences 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 (Figure 12c and f). Neither neutrophils nor eosinophils were present in healthy skin regions, whereas mast cells were found in low numbers in both SGR and SGP skin samples (Figure 15).



	SGP skin	SGR skin	PPR skin
Eosinophil	-	-	-
Neutrophil	-	-	++
Mast cell	+	+	+++

Figure 15. PPR skin is characterized by prominent inflammatory infiltrate of mast cells and neutrophils. *Representative images of May-Grünwald-Giemsa staining and cell counts of eosinophils, neutrophils and mast cells. Visual scoring of May-Grünwald-Giemsa staining was performed by professional pathologist. Scoring system: (-) no cell observed; (+): low cell count; (++): moderate cell count; (+++): high cell count. Size bars = 100 μm. MGG, May-Grünwald-Giemsa; PPR, papulopustular rosacea; SGP, sebaceous gland poor; SGR, sebaceous gland rich.*

4.1.6. 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 (Figure 8a and b) and stratum corneum (Figure 8c) 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 (Figure 8d). Infiltrating CD11c+ DCs (Figure 11a and d), CD3+ and CD4+ T cells (Figure 12a, b, d and e) 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 and/or maturation markers of DCs (Figure 11h-k) were all significantly upregulated on mRNA levels compared to SGR skin. Although CD83+ DCs were present in significantly elevated numbers (Figure 11c and f), TARC positivity was almost undetectable in PPR skin (Figure 11b and e). Moreover, strong, but non-significant correlation was detected between the increase of DC count and the decrease of TSLP level in PPR samples (Figure 16a). No difference was found between SGR and PPR skin regarding the number of LCs (Figure 10). 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 (Figure 12c and f, Figure 15).

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 (Figure 13a-d). The mRNA levels of cytokines corresponded to their protein levels (Figure 14a-d). Gene expression levels of TBX21 (Figure 14e) and FOXP3 (Figure

14h) were significantly higher in PPR compared to SGR skin, while RORC (Figure 14g) and GATA3 (Figure 14f) gene expression levels were lower than in healthy SGR skin. Expressions of both Treg homing receptors (CCR4 and CCD8) were significantly higher in PPR samples than in SGR skin (Figure 14i and j).

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 (Figure 16a), while statistically significant correlation was detected between T cell count and IFN-γ+ cell count in PPR skin (Figure 16b).

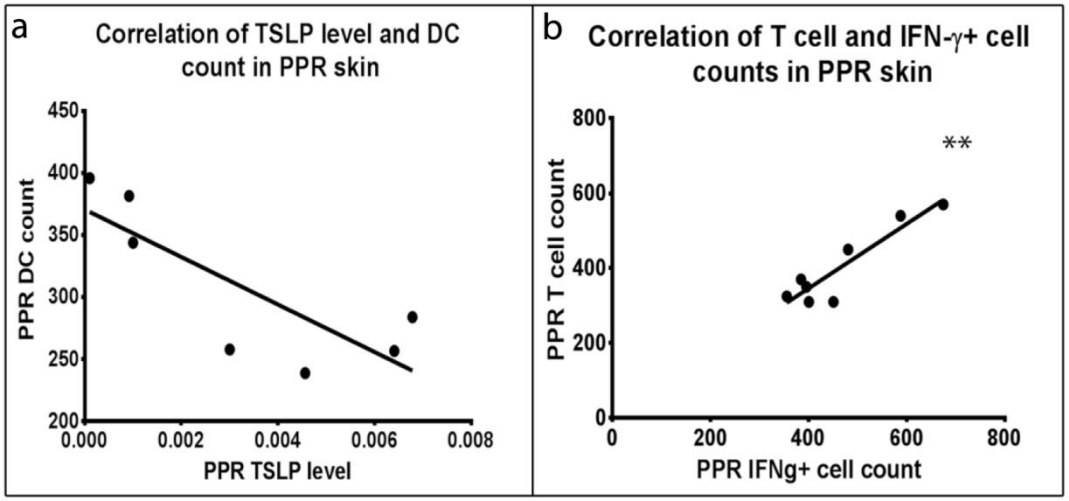


Figure 16. Inverse correlation between TSLP level and DC count and direct correlation between T cell count and IFN-γ+ cell count were detected in PPR skin. *Strong, but not significan inverse correlation was found between* ***A,*** *TSLP level and DC count (P=0.0526; Pearson r = -0.7219) and significant direct correlation between T cell and IFN-γ+ cell count (P=0.0025; Pearson r = 0.9289). (**P < 0.01, as determined by Pearson r test).*

Protein levels/cell counts	SGR vs SGP skin	PPR vs SGR skin	AD vs SGP skin
TSLP	⇑⇑	⇓⇓	⇑⇑
CD1a+ LCs	∅	∅	∅
CD11c+ DCs	⇑⇑	⇑⇑	⇑⇑
TARC+ DCs	∅	∅	⇑⇑
CD83+ DCs	⇑	⇑⇑	⇑⇑
CD3+ T cells	⇑⇑	⇑⇑	not examined in our study
CD4+ T cells	⇑⇑	⇑⇑	
CD163+ macrophages	∅	⇑⇑	
IL-13+ cells	∅	∅	
IFN-γ+ cells	∅	⇑⇑	
IL-10+ cells	⇑⇑	⇑⇑	
IL-17+ cells	⇑⇑	⇑⇑	
Eosinophils	∅	∅	
Neutrophils	∅	⇑⇑	
Mast cells	∅	⇑⇑	

∅: similar level
⇑: elevated level
⇑⇑: significantly elevated level
⇓: decreased level
⇓⇓: significantly decreased level

Gene expression levels	SGR vs SGP skin	PPR vs SGR skin	AD vs SGP skin
TSLP	∅	∅	∅
CD80	⇑	⇑⇑	not examined in our study
CD83	⇑	⇑⇑	
CD86	⇑	⇑⇑	
LAMP3	⇑	⇑⇑	
IL-13	∅	∅	
IFN-γ	∅	⇑⇑	
IL-10	⇑	⇑⇑	
IL-17	⇑	⇑⇑	
TBX21	∅	⇑⇑	
GATA3	∅	⇓⇓	
RORC	⇑	⇓	
FOXP3	⇑	⇑⇑	
CCR4	⇑	⇑⇑	
CCR8	⇑	⇑⇑	

Table III. Summary of the protein and gene expression of the investigated parameters. *The protein and gene expression levels of the following groups were compared: SGR vs SGP, PPR vs SGR and AD vs SGP.*

4.2. Comparison of the immune-mediated skin inflammation in the lesional skin of severe AD patients with or without filaggrin mutation

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

The quantification of two histological severity markers, namely the measurement of ET (not shown) and the detection of Ki67 positive cells (Figure 17b), was performed. No differences were found in the levels of these parameters between the two 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 (OSCORAD) of the patients.

4.2.2. 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 (Figure 17a). 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 the two proinflammatory cytokines and chemokines were significantly higher in the skin of AD patients than control group, but no differences were found between the two AD groups (Figure 17c, d and e). It is important to note that TSLP was slightly or not detectable in control skin. Cytoplasmic positivity of TSLP in AD skin showed decreasing intensity from the granular layer towards the basal membrane in the epidermis. Strong IL-33 nuclear positivity was observed above the basal membrane in

two to five cell layers in AD skin. It was expressed moderately in the normal skin of healthy control subjects, but only in basal KCs. CCL27 expression two to three cell layers below the granular layer was detectable in control skin samples but was significantly higher in the AD groups.

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

$CD3^+$ T cells (Figure 17f) and $CD11c^+$ DCs (Figure 17g) 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.

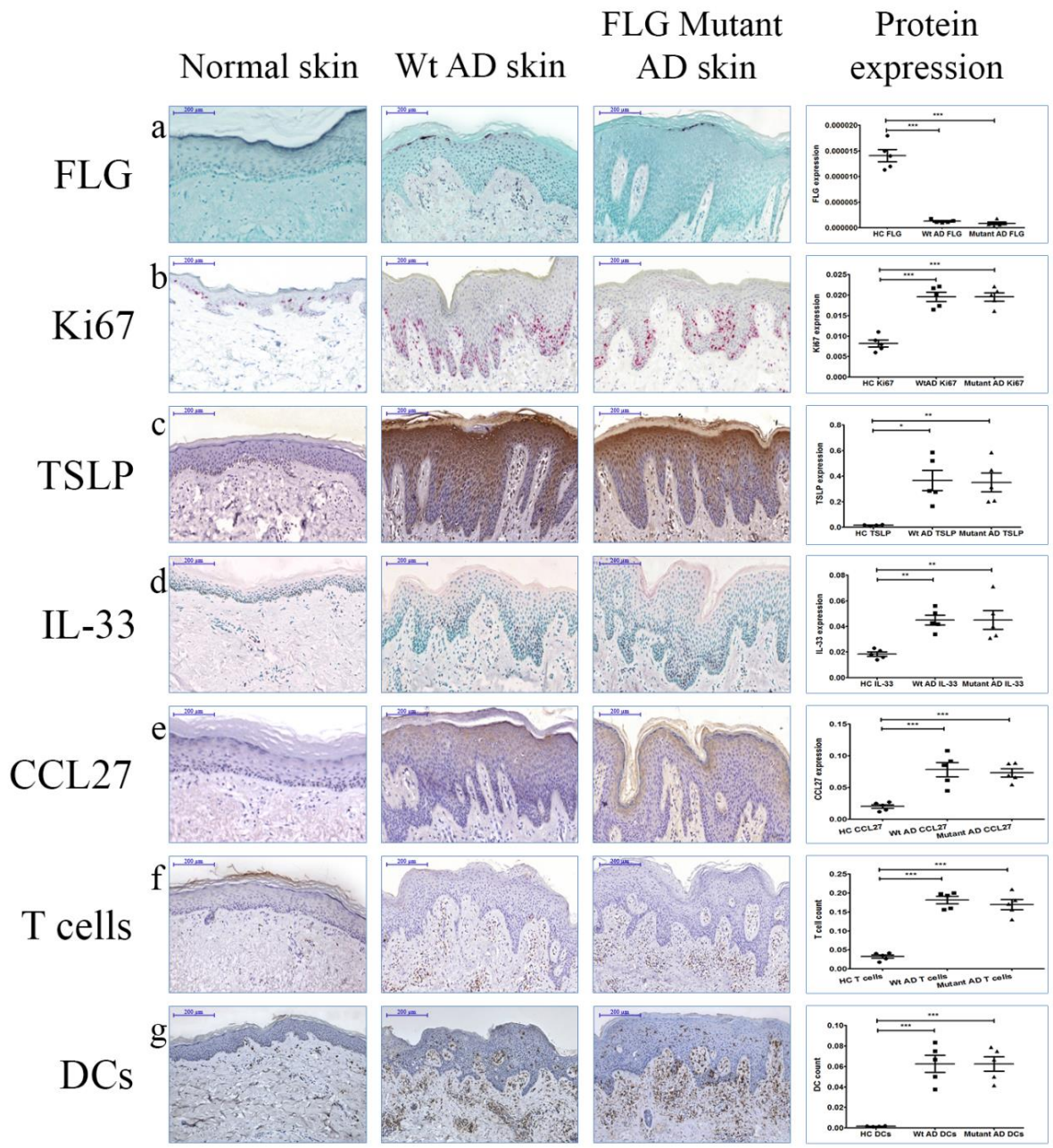


Figure 17. Expression of FLG (a), Ki67 (b), TSLP (c), IL-33 (d), CCL27 (e), CD3 (f) and CD11c (g) in normal, WT severe AD and FLG mutant severe AD skin. *No difference was found between the severe AD groups in regards of all measured parameters. FLG (a) level was found to be significantly decreased in AD groups compared to controls* ***(a)****; all of the other investigated parameters (Ki67* ***(b)****, TSLP* ***(c)****, IL-33* ***(d)****, CCL27* ***(e)****, CD3+ T cells* ***(f)****, CD11c+ DCs* ***(g))*** *expressed in significantly higher amounts in the skin of AD patients. Graphs show the MEAN ± 95% confidence interval of measured protein levels. P-values <0.05 were considered statistically significant (*p<0.05; **p<0.01; ***p<0.001)).*

4.2.4. Detection of TSLP, IL-33 and CCL27 mRNA levels in the skin of wild type and FLG mutant severe AD patients

Regarding all the three investigated parameters no significant differences could be detected on mRNA levels between the two AD groups (Table IV). Comparing the healthy control group to the AD groups, difference could be found only regarding IL-33 mRNA levels.

	FLG Wt severe AD n=12		FLG Mutant severe AD n=12	
RT-PCR results	**Mean**	**±SEM**	**Mean**	**±SEM**
TSLP	0,51	0,21	0,55	0,14
IL-33	0,1	0,049	0,06	0,008
CCL27	1,87	0,57	1,76	0,64

Table IV. Quantitative real-time PCR data of patients with AD. *RT-PCR data was presented as gene expression relative to controls. No significant differences were detected between the 2 AD groups. AD, atopic dermatitis; CCL27, chemokine (C-C motif) ligand; FLG, filaggrin; IL, interleukin; RT-PCR, Quantitative real-time PCR; TSLP, thymic stromal lymphopoietin; SEM, standard error of the mean; Wt, wild type.*

4.2.5. 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 (Figure 18a), between Ki67 and CCL27 levels (Figure 18b), between ET and IL-33 levels (Figure 18c) and between ET and $CD3^+$ cell count (Figure 18d). 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 (not shown).

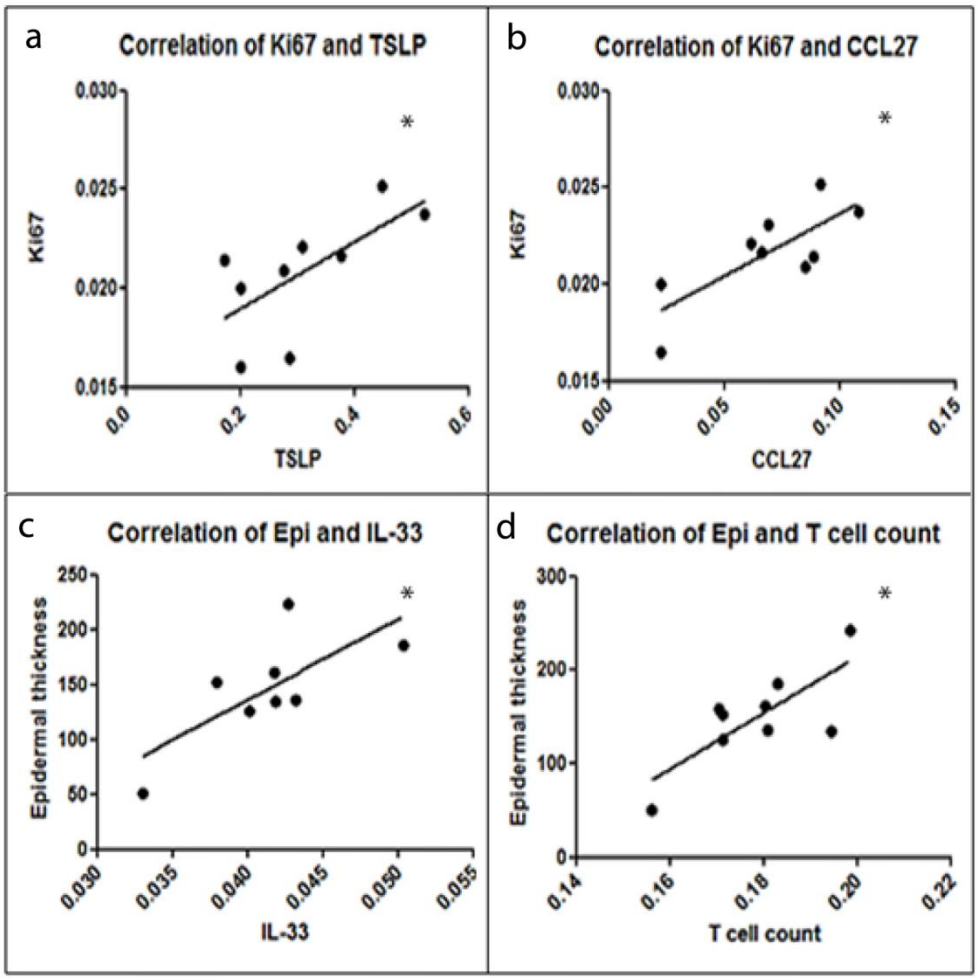


Figure 18. Correlation between the histological severity markers (Ki67, epidermal thickness (ET)), T-cell count and the levels of keratinocyte-derived cytokines and chemokines. *Statistically significant correlations were found* ***(a)*** *between Ki67 and thymic stromal lymphopoietin (TSLP) levels (p = 0.023; Pearson r = 0.706),* ***(b)*** *between Ki67 and CCL27 levels (p = 0.014; Pearson r = 0.777),* ***(c)*** *between ET and interleukin 33 (IL-33) levels (p = 0.043; Pearson r = 0.722) and* ***(d)*** *between ET and CD3+ cell count (p = 0.016; Pearson r = 0.765). *P < 0.05, as determined by Pearson r test.*

5. Discussion

While our skin provides an effective first line protection against pathogens and physico-chemical insults[138], harmless environmental agents and commensal microbiome are tolerated. Therefore both active defense mechanisms and tolerogenic functions are needed to be fulfilled by the skin immune system, although much less is known about these later events.

According to recent literature data in healthy skin TSLP mRNA expression was detected, but its protein expression and role was not investigated in details[26,27]. In this study, for the first time a constitutive TSLP protein expression was detected by two methods (IHC and immunocytochemistry) in healthy SGR skin areas, while in healthy SGP areas TSLP was practically absent. In previous dermatological studies TSLP protein was detected in inflamed epidermis (AD, psoriasis), but not in healthy skin samples[24,105], but these earlier investigations used healthy SGP skin samples and never SGR samples as controls of AD or psoriasis. Although the protein expression of TSLP showed remarkable differences between healthy SGP, SGR and AD skin, mRNA levels were nearly the same in all samples. After these findings two questions may occur. Why can we detect TSLP mRNA at a nearly same level in healthy and inflamed skin and why does TSLP mRNA expression not always reflect the following protein expression? According to the results of Penna et al.[26], we hypothesized that different ratios of the two TSLP mRNA isoforms (short and long) are responsible for the nearly similar total mRNA levels in different skin tissue samples, although the expression of the long form in skin samples seems to be very low according to published studies[26] and was probably under the detection limit in our study. Discrepancy between TSLP protein and mRNA expression can be explained by important, but presently uncovered posttranscriptional modifications during KC differentiation. Bogiatzi and colleagues[178] detected a basal TSLP mRNA expression without the presence of the protein in 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 were used, TSLP protein could be measured after cytokine incubation[178]. Posttranscriptional modification of the two mRNA isoforms can be even different, as the roles of the coded proteins are suggested to be opposite[26]. The importance of the posttranscriptional modifications can also explain our observations, that those agents (sebocytes' supernatant, FFAs, chitin) which are characteristic to SGR skin could elevate TSLP mRNA levels, but TSLP protein could not always be induced in cultured KCs, however in a previous study chitin could elevate TSLP protein levels in concentration dependent manner[179].

The possible role of TSLP in healthy SGR skin required further analysis. Numerous studies now implicate that TSLP has several functions, although the best known is that this molecule is a master regulator of type 2 allergic inflammation through a myriad of different pathways. On the other hand TSLP has a potent immunoregulatory effect on DCs, it is responsible for the differentiation of regulatory T cells in the human thymus[54] and plays an important role in intestinal homeostasis and modulation of Th1/Th17 inflammation in the gut[63,65,67]. Rimoldi and colleagues indicated that in the gut the effect of TSLP depends on its concentration and that physiological amounts of TSLP do not induce DC maturation[10]. Our present results suggest the homeostatic role of constitutive TSLP in SGR skin, since the amount of TSLP protein was significantly lower compared to AD, and although the number of CD11c+ DCs, known as the primary target cells of TSLP in human, was significantly elevated compared to SGP areas, these cells did not express those surface markers (TARC, CD83) which are characteristic to AD specific inflammatory DCs[180]. Noninflammatory function of TSLP in SGR skin is also supported by the fact that we detected only the short isoform's mRNA, which has homeostatic role according to recent data[26].

The number of T cells was also significantly higher in SGR skin, than in SGP skin and T cell characteristic cytokines represented a noninflammatory IL-17/IL-10 cytokine milieu.

Parallel with these findings no difference between, macrophage, neutrophil, eosinophil and mast cell counts were found between SGR and SGP skin. Recently Th17 cells were divided in nonpathogenic and pathogenic Th17 cells. Nonpathogenic Th17 cells produce IL-17 and IL-10, while pathogenic Th17 cells play an important role in the development of inflammatory and autoimmune diseases and produces IL-17, IL-22, INF-γ and GM-CSF[176,177]. Since in our study in SGR skin high expression of RORγt transcription factor mediating nonpathogenic and also pathogenic Th17 responses parallel with low expression levels of T-bet transcription factor, which is a key player of pathogenic Th17 and Th1 cell responses[176,177] were found, and also taking into consideration the cytokine milieu, we suggest that in SGR skin nonpathogenic Th17 cells were detected.

According to our results sebocytes, FFAs and microbiome can have role in the initiation of TSLP production in SGR skin and this homeostatic TSLP production can support tonic signals to DCs in SGR skin parts, like it was published in the gut epithelium[181]. Based on these findings the activity of the skin immune system under steady-state conditions seems to be not equal on the human skin surface, but bears topographical distinctions. This finding correlates with observations that skin microbial community exhibits remarkable differences on sebaceous, dry and moist regions and that skin microbiome has mutualistic connection with skin immune system[2].

After detecting homeostatic TSLP production accompanied by special immune surveillance in healthy SGR skin, we wondered how this can change in an immune mediated skin disease, like PPR, which is exclusively localized to SGR skin part. In PPR TLR2 and NALP3 up-regulation[141], elevated cutaneous protease activity and LL-37 mRNA and protein expression were detected despite the absence of an obvious infectious or dangerous trigger[142,143,144]. 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[145,146,147].

According to our findings PPR compared to SGR skin is characterized by a significant reduction of homeostatic TSLP production leading to the loss of the tolerogenic tonic signal to DCs, which cells after an increase in number and activity (correlation between TSLP loss and DC number also supports this) can initiate T cell infiltration, activation and also the significant influx of macrophages, neutrophils and mast cells. Parallel with the high T cell number a prominent shift in the cytokine milieu from IL-17/IL-10 type to IL-17/IFN-γ/IL-22 type develops, produced by pathogenic Th17 and Th1 cells. Up-regulation of Th1 and pathogenic Th17 cell specific T-bet transcription factor in PPR skin also supported this. It is known from literature data that IL-17/IFN-γ type responses are attenuated by TSLP[182], so decreased TSLP expression detected in PPR, can also result in the increased IL-17/IFN-γ type inflammation which can further destroy TSLP production, leading to a vicious circle (Figure 19). Moreover, increased IL-17/IFN-γ/IL-22 level can further induce antimicrobial peptide and TLR2 expression and neutrophil influx, which are the main pathogenic characteristics of PPR skin[183,184,185,186]. This IL17/IFN-γ type cytokine milieu in rosacea was detected by another recent study[187]. TSLP loss and induction of inflammation is also known in Crohn's disease where colonic ECs show lower expression of TSLP[188]. Since sebocytes and FFAs seem to initiate TSLP production in healthy SGR skin, literature data on altered sebum composition and on dry facial skin[189,190] in rosacea patients indicate, that alteration of these factors can have a role in TSLP loss of PPR patients.

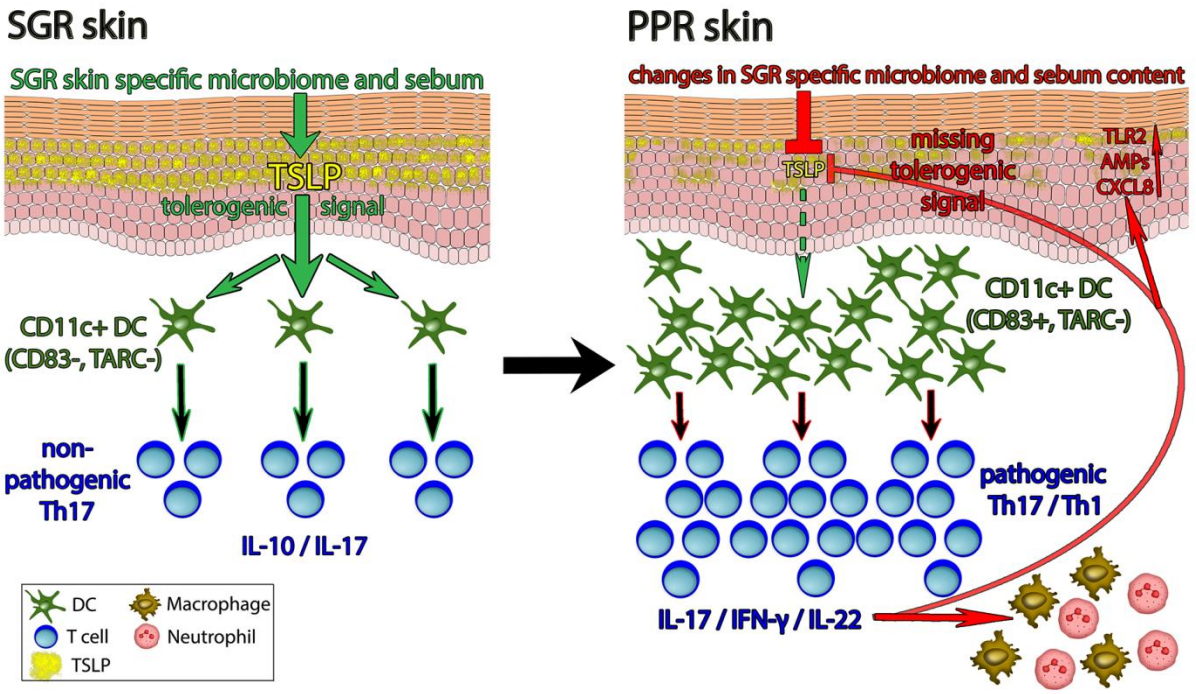


Fig. 19. Loss of TSLP induced tolerance may lead to the development of rosacea specific inflammation. *The appearance of sebum and SGR skin specific microbiome can initiate inflammation (influx of DCs and T cells), but realizing their nonpathogenic nature production of tolerogenic factors (TSLP) is also triggered. In the presence of homeostatic TSLP produced by KCs, SGR skin infiltrating DCs are noninflammatory and T cells are nonpathogenic Th17 cells. The significant decrease of TSLP can result in the loss of the tolerogenic signal to DCs, which upon activation can initiate T cell infiltration, activation and a prominent shift in the cytokine milieu from noninflammatory IL-17/IL-10 type to inflammatory IL-17/IFN-γ/IL-22 type, probably produced by pathogenic Th17 and Th1 cells. Increased IL-17/IFN-γ/IL-22 level can further induce antimicrobial peptide (LL-37) and TLR2 expression and neutrophil influx (via CXCL8 produced by KCs), which are the main pathogenic characteristics of PPR skin. Parallel with the robust increase in T cell number, Increased IL-17/IFN-γ type inflammation can further destroy TSLP production, leading to a vicious circle. The importance of missing TSLP hypothesis can explain not only the pathogenesis of PPR, but also observations that PPR can develop only on SGR skin and not on SGP skin.*

In conclusion our results suggest that similar to skin microbiome, a fine topographical difference seems to exist in the activity of human skin immune system, although in this present study the immune characteristics of moist skin are not investigated (manuscript under preparation). The significant difference in the immune surveillance of healthy SGR and SGP skin can give explanation on the characteristic localization of some immune-mediated skin diseases on special topographical skin areas, emphasize the importance of correctly used topologically identical control skin samples in scientific studies and can influence our future barrier repair therapeutic approaches. We are convinced that our present results allow

studying skin immune system and inflammatory skin diseases from new perspectives since a lot of unanswered questions remained. We did not analyze all elements of the skin immune system in this study, neither investigated moist skin parts. We do not understand exactly the posttranscriptional regulation of homeostatic TSLP expression in stratified squamous epithelium and until revealing these mechanisms, investigation of TSLP in KC cell culture may not reflect the complexity of in vivo environment. Answering these questions could be a breakthrough as TSLP seems to be a good therapeutic target to maintain the normal immune homeostasis of SGR skin.

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[114]. Over-reactive adaptive, dysregulated innate immune responses and impaired skin barrier functions together lead to the manifestation of the disease[120]. FLG, a crucial component of the physicochemical skin barrier, shows several genetic alterations (e.g. copy number variations and *FLG* null mutations) and together with other less studied barrier gene mutations (KLK7, SPINK5 and Claudin-1) can predispose to AD[124]. On the other hand, acquired barrier dysfunctions can be caused by the frequent usage of detergents and exposure to allergens and *Staphylococcus*[137], as well as by local skin inflammation[132]. 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 this study, our aim was to determine whether immune-mediated skin inflammation

(KC function, T cell and DC count) differ between severe AD patients with or without *FLG* mutations. We also investigated the correlations between histological severity markers, FLG content, KC-derived cytokine and chemokine levels and T cell and DC counts. In order to answer our questions, two patient groups were created: *FLG* Wt patients and *FLG* mutant patients with severe symptoms and matching OSCORAD. In our study, two parameters, the ET and Ki67 expression were investigated to score histological severity[137,191,192,193,194]. 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 irrespective of their *FLG* genotype.

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[132].

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 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[129,130], epidermal[14,24] and stratum corneum[131] 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[131], on the contrary relationship between serum and epidermal TSLP levels and OSCORAD were highly controversial[129,130,132]. In parallel with previous data in the literature[24,130], 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. In our study, epidermal TSLP levels significantly correlated with the level of the histological severity marker 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[195]. Our results showed that IL-33 protein expression was significantly elevated in the AD groups compared to controls and no significant difference was found in IL-33 protein levels between the Wt and *FLG* mutant AD patients. These data correspond to a previous investigation which found IL-33 protein expression to be up-regulated in the lesional skin of patients suffering from AD[195], 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[196]. 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[197]. A previous study described strong CCL27 expression in lesional keratinocytes of patients with AD by using IHC[198]. Similarly, we also found significantly elevated protein levels of CCL27 in AD skin, but when Wt and *FLG* mutant AD groups were compared, no difference was detected. Serum CCL27 level was found to correlate significantly with OSCORAD in patients with AD by a Japanese workgroup[198], but no data has been published about the relationship between the epidermal levels of CCL27 and disease severity. 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.

Our IHC results were also confirmed by RT-PCR analyses, since TSLP, IL-33 and CCL27 mRNA levels were similar in the two AD groups. In the literature no RT-PCR data can be found comparing FLG mutant and WT AD groups regarding these parameters, but a recent RNA sequencing investigation could indirectly strengthen our results, as these cytokines were not published in the list of differentially expressed (Fold change ≥ 2 and $p < 0,05$) genes[199]. Comparing healthy controls to AD patients, 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[130,195] 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 cytokines (TSLP, IL-33) and chemokine (CCL27) 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[125], their cell counts were assayed. In our investigation, similar to a previous study[200], 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. On the other hand, DC count showed no direct connection to AD histological severity markers and our workgroup also failed to find any correlation between FLG content and all of the

investigated parameters.

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.

6. Novel findings and clinical relevance of the dissertation

- Sebaceous gland rich healthy skin, which constitutively expresses TSLP protein, is characterized by a distinct, noninflammatory immune surveillance.
- The homeostatic TSLP protein content is significantly reduced in papulopustular rosacea in parallel with the influx of inflammatory DCs and T cells and a shift from IL-17/IL-10 type cytokine milieu to IL-17/IFN-γ type.
- The described fine topographical difference in the activity of the healthy skin immune system should be taken into account regarding the pathogenesis of inflammatory skin diseases, and in therapeutic approaches of barrier repair and immune modulation.
- These finding may explain the characteristic localization of inflammatory skin diseases.
- 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.

7. 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.

8. Összefoglalás

Az egészséges bőr immunaktivitását vizsgálva a thymic stromal lymphopoietin (TSLP) termelés, a dendritikus sejt (DCk) és a T sejt számot és funkciót tekintve jelentős topográfiai eltéréseket sikerült kimutatnunk. Konstitutív TSLP fehérje expressziót detektáltunk faggyúmirigyben gazdag (FMG) bőrterületeken; míg a faggyúmirigyben szegény (FMSZ) területeken nem volt kimutatható TSLP jelenlét. A linolsav, egy fontos szébum összetevő képes volt dózisfüggően növelni a TSLP mRNS expresszióját HaCaT és NHEK sejtekben. Úgy véljük, hogy a FMG bőrben jelen lévő TSLP-nek hasonló szerepe lehet, mint azt kimutatták a bél homeosztázisban, hiszen a vizsgált FMG bőrminták klinikailag egészségesek, gyulladásmentesek voltak; a TSLP mennyisége kisebb volt, mint az AD mintákban; és a DC-k TARC negatívak voltak és alacsony aktivitási státuszt mutattak, valamint a T sejtek sem bírtak gyulladásos jellemzőkkel. FMG bőrben mind a DC-k, mind a T sejtek nagyobb számban voltak jelen. A T sejtek elsősorban regulatórikus T sejtek és valószínűleg nem patogén T helper (Th) 17(β) sejtek voltak. Az FMG bőrben emelkedett mennyiségben detektáltunk IL-17+ és IL-10+ sejteket, míg az IFN-γ+ és IL-13+ sejtek teljesen hiányoztak. Eredményeink szerint az FMG bőrterületeket egy nem gyulladásos IL-17/IL-10 citokin miliő jellemzi. Papulopusztulózus rosaceában (PPR), mely bőrbetegség kizárólag FMG területeken alakul ki, a TSLP mennyisége csökkent, a DC-k aktiválódtak, a T sejtek gyulladásos típusúvá [Th1 and Th17(23)] váltak, és számuk jelentősen megnőtt. Szintén kimutattuk, hogy a megjelenő immun-mediált bőrgyulladás (keratinocyta-eredetű faktorok, T sejt és DC szám) filaggrin mutációval rendelkező, illetve vad típusú súlyos AD betegek bőrében azonos; és az AD-ra jellemző immunaktiváció sokkal inkább a betegség súlyosságával áll kapcsolatban, mint a barrier károsodás eredetével. Eredményeink magyarázatot adhatnak arra, hogy bizonyos bőrbetegségek miért mindig adott topográfiai bőrterületre lokalizálódnak (FMSZ bőrön AD, míg FMG bőrön PPR); rámutatathatnak a topológiailag megfelelő kontroll bőrminták használatára a tudományos kutatásokban; és végül a jövőben akár terápiás lehetőségekkel is kecsegtethetnek.

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10. Key words

thymic stromal lymphopoietin, skin immune system, dendritic cells, T cells, rosacea, atopic dermatitis, immunohistochemistry, innate immunity, filaggrin

11. Kulcsszavak

thymic stromal lymphopoietin, bőr immunrendszer, dendritikus sejtek, T sejtek, rosacea, atópiás dermatitisz, immunhisztokémia, innate immunitás, filaggrin

12. Publications related to dissertation

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Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology
MTMT ID: 10054954

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.
   J. Invest. Dermatol. 137 (5), 1114-1125, 2017.
   DOI: http://dx.doi.org/10.1016/j.jid.2016.12.025
   IF: 6.915 (2015)
2. **Dajnoki, Z.**, Béke, G., Mócsai, G., Kapitány, A., Gáspár, K., Hajdu, K., Emri, G., Nagy, B., Kovács, I., Beke, L., Dezső, B., Szegedi, A.: Immune-mediated Skin Inflammation is Similar in Severe Atopic Dermatitis Patients With or Without Filaggrin Mutation.
   Acta Derm.-Venereol. 96 (5), 645-650, 2016.
   DOI: http://dx.doi.org/10.2340/00015555-2272
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List of other publications

3. Kapitány, A., Béke, G., Nagy, G., Doan-Xuan, Q. M., Bacsó, Z., Gáspár, K., Boros, G., **Dajnoki, Z.**, Bíró, T., Rajnavölgyi, É., Szegedi, A.: CD1c+ Blood Dendritic Cells in Atopic Dermatitis are Premature and Can Produce Disease-specific Chemokines.
   Acta Derm.-Venereol. 97 (3), 325-331, 2017.
   DOI: http://dx.doi.org/10.2340/00015555-2540
   IF: 3.638 (2015)

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13. List of other publications

4. Khasawneh, A., Baráth, S., Medgyesi, B., Béke, G., **Dajnoki, Z.**, Gáspár, K., Jenei, A., Pogácsás, L., Pázmándi, K. L., Gaál, J., Bácsi, A., Szegedi, A., Kapitány, A.: Myeloid but not plasmacytoid blood DCs possess Th1 polarizing and Th1/Th17 recruiting capacity in psoriasis.
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5. Szegedi, A., **Dajnoki, Z.**: A rosacea pathomechanizmusa.
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6. Béke, G., Kapitány, A., **Dajnoki, Z.**, Hajdu, K., Gáspár, K., Bíró, T., Szegedi, A.: A bőr immunrendszerének felépítése és működése.
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7. Mócsai, G., Gáspár, K., **Dajnoki, Z.**, Tóth, B., Gyimesi, E., Bíró, T., Maródi, L., Szegedi, A.: Investigation of Skin Barrier Functions and Allergic Sensitization in Patients with Hyper-IgE Syndrome.
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8. Mócsai, G., **Dajnoki, Z.**, Irinyi, B., Gáspár, K., Szegedi, A.: A bőr barrierkárosodások non-invazív mérési lehetőségei = The non-invasive measurements of skin barrier disruption.
   Bőrgyógyász. Venerol. Szle. 90 (3), 89-93, 2014.
   DOI: http://dx.doi.org/10.7188/bvsz.2014.90.3.4

Total IF of journals (all publications): 19,768
Total IF of journals (publications related to the dissertation): 10,553

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

27 April, 2017

14. Presentations

1. **45rd Congress of Hungarian Society for Immunology, Velence, Hungary, 19-21 October 2014;** *Examination of the immune status of healthy apocrine glad rich skin regions* (poster walk)
2. **45rd Congress of European Society for Dermatological Research, Rotterdam, The Netherlands, 9-12 September 2015,** *IFNg/IL-17 cytokine milieu is characteristic of papulopustular rosacea* (poster presentation)
3. **Congress of European Academy of Allergy and Clinical Immunology, Barcelona, Spain, 6-10 June 2015;** *Has genetic or acquired filaggrin loss influenced the immune-mediated inflammation in severe atopis dermatitis?* (first author)
4. **43rd Congress of Hungarian Society for Immunology, Velence, Hungary, 15-17 October 2014;** *Investigation of skin immune system in rosacea* (oral presentation)
5. **44rd Congress of European Society for Dermatological Research, Copenhagen, Denmark, 10-13 September 2014;** *No difference in skin inflammation between atopic dermatitis patients with or without filaggrin mutation* (poster walk)
6. **2nd Experimental Dermatological Conference, Szeged, Hungary, 26-28 June 2014;** *Investigation of skin immune system in rosacea* (oral presentation)
7. **2nd Meeting of Middle-European Societies for Immunology and Allergology, 10-13 October 2013, Opatija, Croatia;** *Immunohistochemical evaluation of skin lesions of filaggrin mutant and wild type atopic dermatitis patients* (poster presentation)
8. **Immune-related Pathologies: Understanding Leukocyte Signaling and Emerging therapies Conference, 31 August – 3 September 2013, Mátraháza, Hungary;** *The atopic skin like microenvironment modulates the T cell-polarising cytokine production of myeloid DCs as determined by laser scanning cytometry* (oral presentation)
9. **43rd World Congress of International Investigative Dermatology, Edinburgh, Scotland, 8-11 May 2013;***The prevalence of obesity is increased in patients with late compared to early onset psoriasis after adjustment for age* (poster presentation)

Acknowledgments

I would like to thank to my PhD supervisor, Professor Andrea Szegedi, for supporting and motivating me during the past five years and for the scientific discussions she offered within this study. Her timely advices with kindness and enthusiasm have helped me to complete this PhD thesis.

I have to thank especially Professor Tamás Bíró for his scientific knowledge and constructive suggestions. I would like to express my deep appreciation to my colleagues and friends, Anikó Kapitány, Gabriella Béke, Krisztán Gáspár, Eszter Anna Janka, Gábor Boros and all the others for giving me their valuable time, advices and support. Many thanks I owe Tünde Toka-Farkas, Józsefné Kertész, Csapóné Ildikó Sandrai for excellent technical assistance.

In this very special moment, I would like to express my deepest thanks to my wife, Vica for her unconditional love, encouragement and supports mentally and comforted me in difficult times that made me possible to finish my study. My thanks are also addressed to my parents for their love, patience and encouragement in any situation of my life and supports financially.

My work was supported by the Hungarian Scientific Research Grant OTKA K108421 and OTKA-PD 112077, the project of TÁMOP-4.2.2.A-11/1/KONV-2012-0023-"DEFENSE-NET" (implemented by the New Hungary Development Plan co-financed by European Social Fund and European Regional Development Fund) and University of Debrecen RH-885/2013). The work is supported by the GINOP-2.3.2-15-2016-00050 project. The project is co-financed by the European Union and the European Regional Development Fund.