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

Investigation of the keratinocyte-derived mediators in the healthy skin and in atopic dermatitis

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DEBRECEN, 2024

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The PhD Defense takes place at the Lecture Hall of the Institute of Internal Medicine “A” building, Faculty of Medicine, University of Debrecen, 25th October 2024, 13:00

INTRODUCTION

Our skin maintains an essential relationship between our body and the environment. In addition to its well-known *permeability barrier* function, our skin also forms an active *immunological barrier*, providing the first line of immunological defence against infections. There is an effective 'crosstalk' between epithelial cells and immune cells that ensures host protection and the maintenance or restoration of tissue homeostasis. In addition, the surface and appendages of the skin are populated by a highly diverse microbial community *(microbial barrier*), which is also in interaction with the epithelial and immune cells of the body and is able to influence the immunological functions. In addition to the microbial community, a tightly regulated chemical milieu *(chemical barrier*) also plays a role in the barrier function of the skin. A fine-tuned balance of well-regulated interactions between these four main barrier components (permeability, immunological, microbial, chemical barrier) is essential for the skin barrier to function, which must ensure the maintenance of the mechanical (permeability) barrier, the immunological barrier, the microbial community and the chemical milieu.

If any one of the four components is damaged, the balance between them can be disrupted, which may lead to infectious, cancerous or immune-mediated inflammatory skin diseases. A characteristic feature of these diseases is a region-specific appearance initiated by barrier damage, such as atopic dermatitis and rosacea.
In addition, it is a well-known fact that our skin is not uniform, since three main regions can be distinguished based on anatomical differences: sebaceous gland rich (SGR), apocrine gland rich (AGR) and gland poor (GP) skin areas. The SGR region includes the hairy scalp, face, the area behind the ears, chest and shoulders. The AGR region includes the axillae and gluteal region, while the GP region includes the limbs and lower trunk. Research in recent years has revealed that, in addition to anatomical regional differences, regional differences in barrier components may also be observed, and this may be important in explaining the characteristics of some inflammatory skin diseases, in particular those that are initiated by barrier damage.

The main research goal of our research group is to study the barrier units of healthy skin regions and to elucidate the molecular mechanisms underlying in the development of region-specific skin diseases in different skin regions. In the first study of my PhD thesis the main cytokine-type mediators produced by keratinocytes were investigated in the three different skin regions and in atopic dermatitis, which is localized to the GP region. The second study is connected to

atopic dermatitis (AD) , focusing on the investigation of antimicrobial peptide (AMP)-type mediators and comparing them with the expression levels measured in psoriasis vulgaris (PsV).

AIMS

Previous investigations by our research team have already revealed the pattern of the expression of AMPs and chemokines in the epidermis of the three healthy skin areas, but the keratinocyte-derived cytokines have not yet been investigated. In addition, no one has comprehensively investigated the expression of AMP-type epimmunome molecules in atopic dermatitis.

I. In the present study, we aim to compare the cytokine-type epimmunome profiles of healthy SGR, AGR and GP skin regions at **1)** the mRNA level by qRT-PCR, **2)** at the protein level by immunohistochemistry (IHC) and **3)** immunofluorescence (IF) methods.

II. Next, we aimed to investigate cytokine-type epimmunom molecules that are specific for the healthy GP skin in a region-specific immune-mediated inflammatory skin disease, atopic dermatitis at the protein level by IF and to compare the expression of these cytokines with two other inflammatory skin diseases, rosacea (PPR) and PsV.

III. Finally**,** a comprehensive analysis of the expression levels of AMP-type epimmunome molecules was performed **1)** in AD at the mRNA level by qRT-PCR, and **2**) at the protein level by IHC, and **3)** in PsV at the protein level by IHC, in order to obtain comparable protein expression levels.

MATERIALS AND METHODS

For the current studies, healthy skin samples were obtained from healthy individuals undergoing plastic surgery, while diseased skin biopsy samples were from patients of the Department of Dermatology, University of Debrecen. Informed consent forms in accordance with the guidelines of the Helsinki Declaration for the study were signed in all cases. The study was approved by the local ethics committee of the University of Debrecen (licence number IV/2072-2/2020/EKU).

During skin sample collection, each sample was cut into 2 pieces, one half was placed in *RNAlater* for subsequent RNA isolation and stored at -80°C, and the other half was embedded in paraffin after formalin fixation for protein level experiments. Healthy samples were grouped according to the number of sebaceous glands and apocrine glands after hematoxylin-eosin staining. At 10X magnification of a light microscope, if the number of sebaceous glands per unit area was greater than three, the sample was assigned as SGR sample; if the number of apocrine glands was above two, the sample was AGR; and if the number of apocrine and sebaceous glands was less than one, the sample was classified as GP. Patient skin samples were obtained from patients with moderate to severe condition, who were not on systemic treatment or treatment was stopped at least 3 weeks before sampling.

Sample collection

For the cytokine-type epimmunome molecule-investigation (first project), healthy skin samples (n=10-10-10) were obtained from SGR (mean age ± SD: 63.4 ± 15.43 years), AGR (mean age ± SD: 63.3 ± 10.48 years) and GP (mean age ± SD: 48.1 ± 15.94 years) skin areas. In addition, 7 lesional AD (mean age ± SD: 29 ± 8.25) and PPR (mean age ± SD: 53.3 ± 14.70) samples and 6 PsV (mean age ± SD: 68 ± 15.52) and Scalp Ps (mean age ± SD: 57 ± 18.12) patient samples were collected.

To investigate the expression of AMP-type epimmunome molecules, skin samples were collected from lesional (L) and non-lesional (NL) areas of 10 patients with severe AD (mean age ± SD 34.3 ± 10.06) and 5 patients with severe PsV (mean age ± SD 47.8 ± 14.3). As healthy controls, 10 GP (mean age ± SD 46.90 ± 7.95) skin samples were used.

RNA isolation, reverse transcription reaction and real-time quantitative PCR (qRT-PCR)

All samples were homogenized in TriReagent (Sigma-Aldrich, Dorset, UK) solution using Tissue Lyzer (QIAGEN, Germany) with metal bead-filled innuSPEED lysis tubes (Analytik Jena, Germany). Total RNA was isolated from the skin biopsies, RNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Bioscience, Waltham, MA), and RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent

Technologies, Santa Clara, CA, USA). RNA was converted to complementary DNA (cDNA) using the High Capacity cDNA Archive Kit (Invitrogen, Life Technologies, San Francisco, CA, USA) following the manufacturer's instructions. Samples were pretreated with DNase I (Applied Biosystems, Foster City, CA, USA). Reverse transcription real-time quantitative PCR (qRT-PCR) measurements were performed in triplicate using predesigned FAM-MGB assays and TaqMan® Gene Expression Master Mix (Applied Biosystems, Life Technologies). All measurements were performed using the LightCycler® 480 (Roche) instrument. Relative mRNA level was calculated using the 2-ΔΔCt method normalized to PPIA mRNA expression.

Immunohistochemistry (IHC)

Samples sectioned from paraffin-embedded samples were used for IHC studies. After deparaffinization and rehydration, endogenous peroxidase inhibition was performed for 15 min_{with}3% $H2O2$. This was followed by heat-induced antigen retrieval. After blocking with 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich Ltd.) (1 h), sections were incubated with the eluted primary antibodies in a humidity chamber overnight at 4°C. Samples were then incubated the next day with anti-mouse/anti-rabbit HRP conjugated secondary antibody (Dako) for 45 min. Before and after incubation with antibodies, samples were washed three times for 5 min with TBST. Signals were detected using Vector® VIP and ImmPACT™NovaRED™Kit (VECTOR Laboratories, Burlingame, CA, USA), and methyl green was used for background staining. Detection of each protein was performed simultaneously in parallel on all sections to allow for comparison of detected protein expression levels when evaluating the results. Positive, negative and isotype controls were used to normalize staining.

IHC quantification

All stainings were digitized by Whole Slide Imaging using a Zeiss Mirax Midi scanner (Zeiss, Oberkochen, Germany)after staining. Then, the sections were evaluated using the Pannoramic Viewer software (3DHistech, Budapest, Hungary) with HistoQuant. The application was trained to separate the positive area (positive pixel) and the background for each molecule. At least 3 regions of interest (ROI) containing 500 μm epidermal area were selected in each section. This trained algorithm was used to evaluate the ROI for all sections. Finally, we determined the total staining intensity and compared the values between the sample groups.

Scoring system

For the investigation of the cytokine-type epimmunome molecules, we aimed to determine the epimmunome pattern in each region after the evaluation of the sections, using a simplified scoring system approach. For each molecule, the individual scores (measured with the Pannoramic Viewer) for all sample groups were sorted in ascending order and the linear scale between the smallest and the largest value was divided into 3 equal ranges. These predefined ranges were marked with (+), (++) or (+++). For each molecule, the median value of the data representing each range was ranked into these ranges. Finally, those molecules were considered representative of a given region that were assigned three (+++) marks.

Immunofluorescence (IF) staining and quantification

For the investigation of the cytokine-type epimmunome molecules, IF staining was performed as described for IHC staining up to the point of secondary antibody application. After incubation with primary antibodies, Alexa FluorTM 555 goat anti-mouse IgG (H + L) and Alexa FluorTM 555 goat anti-rabbit IgG (H + L) secondary antibodies (ThermoFisher Scientific) were used. To evaluate IF staining, at least 3 images per section were taken at 200× magnification. Fiji (ImageJ) software was used to determine the total intensity value per epidermal area in 8-bit grayscale images. Since the IL-33 molecule is localized in the nucleus, positivity was measured only in the nuclear area for this cytokine.

Statistical analysis

In all cases, statistical significance was calculated using one-way, ANOVA, followed by Tukey's post hoc test (if the data distribution was normal) or Kruskal-Wallis test followed by Dunn's post hoc test (if the data distribution was not normal). The graphs show the means of the measured mRNA and protein levels and the min and max values of the 95% confidence intervals. For our IF analyses, we used an unpaired t-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) to determine statistical significance between groups if the data distribution was normal, or Mann Whitney test if the data distribution was not normal. Statistical analyses were performed using GraphPad Prism software version 8 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

1. Analysis of the cytokine-type epimmunome molecules in topographically distinct healthy skin areas

1.1 At the mRNA level, different skin areas are characterized by different sets of epimmunome molecules

First, we investigated the cytokine-type epimmunome mediators known from the literature at the mRNA level by RT-qPCR*:* *IL25*, *IL33*, *IL23A*, *IL17C*, *IL36RA*, *IL37*, *IL38*, *IL18*, *IL1B*, *IL24*, *IL6*, *IL1A*, *C-X-C Motif Chemokine Ligand [CXCL]8* and *IL36A*. Since regional differences in AMP- and chemokine-type epimmunome molecules have been investigated in our previous studies, they were not included in this current study. Our results showed that levels of *IL25*, *IL36RA*, *IL37*, *IL38* and *IL18* were significantly higher in the GP region compared to the SGR region. However, *IL1B* showed significantly elevated levels in SGR skin. When comparing the AGR region with GP region, the mRNA levels of *IL36RA*, *IL38* and *IL18* molecules showed significantly lower expression in AGR skin. When comparing AGR and SGR regions, the mRNA levels of *IL33* and *IL6* molecules were significantly higher and *IL1B* mRNA levels were significantly lower in AGR area compared to SGR skin.

1.2 Different skin areas are characterised by different levels of epidermal epimmunome protein levels

As a next step, we aimed to investigate the cytokine-type epimmunome molecules (IL-25, IL-33, IL-23, IL-17C, IL-36RA, IL-38, IL-18, IL-1α, IL-1β, IL-6, IL-8 and IL-24) at the protein level by IHC in the three healthy skin areas. Based on their staining pattern, IL-18, IL-1β, IL-23, IL-25, IL-33 and IL-17C epimmunome molecules showed a homogeneous distribution in the epidermis, IL-33 and IL-18 molecules were expressed in the nucleus, while the other cytokines were observed in the cytoplasm of KCs. The intensity of staining for IL-1α, IL-24, IL-36RA and IL-38 was the strongest in the stratum granulosum layer, with decreasing intensity towards the basal layer. IL-6 and IL-8 staining was weaker but showed a similar decreasing pattern towards the basal KCs. The staining pattern was similar for all molecules in the different

regions. When comparing SGR and GP skin regions, protein levels of IL-25, IL-33, IL-36RA and IL-38 were significantly higher in the GP region. In contrast, the protein levels of IL-17C and IL-23 molecules were significantly higher in the SGR region compared to the GP. When comparing the AGR and GP regions, significantly higher IL-17C protein expression was measured in the AGR region. And when comparing the AGR and SGR skin regions, IL-1β, IL-24, IL-25 and IL-33 protein levels were significantly higher in the AGR region.

The discrepancy between mRNA and protein level studies can be explained by the fact that there are several steps between transcription and protein synthesis (RNA processing, transcriptional and translational modifications) that can affect the amount of protein produced. However, the discrepancy may also be due to the fact that we used whole skin biopsy samples for our mRNA level studies, whereas we only considered epidermal staining when analysing protein level results, as the focus of our present study is on epimmunome molecules produced by epidermal KCs, and thus cytokine production by gland cells in the dermis was not quantified.

To further confirm the results of our IHC measurements, we performed IF staining for molecules that differed significantly in the three dermis regions, as the intensity of fluorophores is linearly proportional to protein content. Using this approach, we found that the protein levels of epidermal IL-17C, IL-23, IL-25, IL-33, IL-36RA and IL-38 were all consistent with IHC measurements.

1.3 Functional characterisation of different epimmunome profiles in different skin areas

Since we investigated healthy skin regions in the present study, we could not use control skin samples in the traditional way. Therefore, we aimed to determine the unique epimmunome pattern of each region by developing a simplified scoring system. For each molecule, the median value of the data representing each region was ranked according to three predefined ranges, represented by (+), (++) or (+++). Molecules were considered to be representative of a region if they were assigned three (+++) marks. Our results show that the SGR region is characterized by the presence of IL-23, IL-17C and IL-18, the GP region by the presence of IL-25, IL-33, IL-36RA, IL-38 and IL-18 molecules, and the AGR region is represented by the presence of IL-25, IL-33, IL-23 and IL-18, thus carrying the features of both SGR and GP regions.

Next, to explore the role of epimmunome molecules specific to each region, we reviewed the literature on the homeostatic and inflammatory functions of the identified molecules. Literature data suggest that GP-specific epimmunome molecules induce Treg cells under homeostatic conditions and promote Th2 and/or inhibit Th17 responses under inflammatory conditions. Mediators specific to the SGR region help to maintain a balance with skin colonizing microbes under homeostatic conditions by maintaining a non-inflammatory Th17 immune milieu, while in inflammation they induce inflammatory Th17 responses. The AGR region has features of both the SGR and GP regions.

2. Analysis of cytokine-type epimmunome molecules in atopic dermatitis compared with rosacea and psoriasis

Based on the literature analysis, the different regions are characterized by either a pro-Th2 or pro-Th17 epimmunome profile. Therefore, we next aimed to investigate how the expression of epimmunome molecules changes under inflammatory conditions. Our studies investigated the major Th2- and Th17-associated cytokines produced by KC (Th17: IL-23 and IL-17C; Th2: IL-33, IL-25, IL-36RA and IL-38) by immunofluorescence staining in AD and PPR, which are considered as outside-in skin diseases induced by "epidermal challenge" in GP and SGR regions. In addition, as a disease control, we also examined PsV and Scalp Ps samples specific to the same GP and SGR regions to also detect changes associated with epimmunome molecules in an inside-out skin disease.

In AD, the epidermal fluorescence intensity of the GP region-specific cytokines IL-33, IL-25, IL-36RA and IL-38 was significantly increased, whereas the levels of IL-23 and IL-17C were unchanged. In contrast, in PPR, the intensity of the cytokines IL-23 and IL-17C, which characterize the healthy SGR region, was strongly increased, whereas the expression of IL-33, IL-25, IL-36RA and IL-38 was not increased. These results suggest that in outside-in skin diseases (AD and PPR) caused by external epidermal challenge, inflammatory epimmunome production closely resembles the homeostatic region-specific epimmunome patterns, but to an exaggerated extent. In contrast, in psoriasis, which is an inside-out skin disease, the inflammatory epidermal cytokine milieu is independent of the homeostatic epimmunome milieu of the specific skin region.

3. Analysis of antimicrobial peptide-type epimmunome molecules in atopic dermatitis

3.1 Analysis of mRNA expression of antimicrobial peptide-type epimmunome molecules in lesional (AD L) and non-lesional atopic dermatitis (AD NL) skin

First, we compared the mRNA expression levels between healthy control (HC) skin samples and samples from the AD NL area. Significant changes were hardly detectable; only for 2 AMPs we found significantly different expression levels, namely, the RNASE7 was reduced, while SLPI was expressed at higher levels in AD NL samples compared to healthy controls. Other AMPs were expressed at similar levels in these two sample groups. Next, the AD L samples were compared with the HC sample group. Among the classical AMPs, the expression of the molecules encoding hBD-2, beta defencin (DEFB)4B, and DEFB104A, encoding hBD-4, was significantly higher in AD L skin compared to control skin. In contrast, DEFB1 encoding hBD-1 showed an opposite trend with significant differences between AD L and control skin.

The gene expression levels of DEFB103A/DEFB103B encoding hBD-3 and CAMP encoding cathelicidin/LL-37 were not significantly different between the sample groups. For AMPs with protease inhibitory and enzymatic activity, we found that PI3 and LYZ were significantly higher, while RNASE5/ANG and RNASE7 mRNA levels were significantly lower in AD L skin compared to the control group. SLPI gene expression levels were similar in AD L and healthy control skin. As for AMPs with chemocine activity, the mRNA expression level of S100A molecules was significantly higher in AD L skin compared to control skin. There was no significant difference between the sample groups regarding to CCL20. The gene expression level of ADM with neuropeptide activity was significantly lower in AD L skin compared to controls, while the level of LCN2 was significantly higher in AD L skin.

3.2 Protein expression of antimicrobial peptides in AD L and AD NL skin

Subsequently, the expression of AMPs belonging to different functional groups was determined at protein level by IHC and then evaluated. When comparing the AD NL and control groups, similar protein levels were detected for almost all AMPs (hBDs, RNase7, CCL20, S100A8, ADM, LCN2) in agreement with the mRNA level results, except for LL-37, where significantly lower levels were measured in AD NL skin compared to control skin. When comparing AD L

and healthy controls, most AMPs tested (6 out of 9) were significantly higher expressed in AD L skin compared to healthy control skin, consistent with the mRNA level results. Two AMPs were expressed at similar levels, while only one AMP was significantly lower in AD L skin compared to control skin. As for the classical AMPs, hBDs were present at significantly higher levels, while a decreasing trend regarding LL-37 was observed in AD L skin compared to control. Protein levels of CCL20 and S100A8 molecules were significantly higher in AD L skin compared to healthy control skin. No significant differences were detected in the expression of RNase7 and ADM molecules between AD L and healthy skin samples, but LCN2 levels were significantly higher in AD L skin compared to control.

Regarding the staining patterns of the AMPs studied, we found that the expression of LCN2, hBD-4, ADM molecules in the epidermis showed a homogeneous distribution. The staining of LCN2 and hBD-4 was strong, whereas the staining of ADM was weak in AD L samples, whereas the staining of LL-37 was barely detectable in AD groups. The hBD-1, hBD-2, S100A8 and LCN2 molecules were mainly localized in the apical part of the epidermis in all sample groups, with a decreasing trend towards the basal keratinocytes. RNase7 was observed in the stratum corneum and in the upper granular epidermal layer in all sample groups.

In summary, only LL-37 levels were significantly reduced in AD NL skin compared to control. AMPs showing a significant decrease at mRNA level did not show a decrease at protein level in AD L samples. At the protein level, the expression of AMPs showed a significant increase in most cases, similar expression was measured in two cases, while a decrease was detected for one molecule, LL-37, in AD L skin compared to control.

3.3 Comparison of antimicrobial peptide protein levels between AD L and lesional psoriasis vulgaris (PsV L) skin samples

We hypothesize that the conflicting results in the literature may be due to the fact that AMP levels were compared with PsV skin instead of healthy skin. Therefore, we aimed to detect AMP molecules at the protein level in PsV L samples to clarify this discrepancy. For most AMPs, we detected significantly higher protein expression in PsV L skin compared to healthy

skin. ADM and RNase7 showed prominent staining, but their levels were not significantly different in PsV L skin compared to healthy control skin. Interestingly, when comparing AD L and PsV L groups, expression was similar for most AMPs between AD L and PsV L groups. In contrast, LL-37 and CCL20 AMPs showed significantly higher levels in PsV L skin compared to the AD L group, while ADM showed a similar trend, but a slight increase was also observed for this molecule in the PsV L sample group.

DISCUSSION

1. Analysis of cytokine-type epimmunome molecules in topographically distinct healthy skin areas

In our study, we performed a comparative analysis of cytokine-type epimmunome mediators at the mRNA (RT-qPCR) and protein (IHC and IF) levels between three healthy skin sites. Furthermore, we also determined the absolute homeostatic epimmunome profiles of these areas using a simplified scoring system. Our results show that different healthy skin areas have different homeostatic epidermal epimmunomic profiles. Based on our protein level results, the GP skin is characterized by an IL-25/IL-33/IL-36RA/IL-38/IL-18 epidermal epimmunome milieu, the SGR region has an IL-23/IL-17C/IL-18 epimmunome pattern, and the AGR region has an IL-25/IL-33/IL-23/IL-18 milieu. Next, to better understand the role of different epimmunome profiles in different healthy skin regions, a comprehensive literature analysis was performed.

Based on the literature data, we hypothesize that the characteristic epimmunome sets of the three skin areas differ in the mechanisms of homeostasis maintenance, as GP area-specific mediators appear to support Treg cell function, SGR area-specific cytokines induce non-inflammatory Th17 cells, whereas the AGR area carries both SGR and GP area characteristics. Under inflammatory conditions, epimmunome-specific molecules in the SGR region appear to support type 3 (Th17-related) and GP region-specific molecules support type 2 (Th2-related) adaptive immune processes, while the AGR region carries features of both.

Consistent with these results, *Del Duca et al.* reported higher levels of CXCL8, IL1B, IL6 and IL23 mRNA in the SGR region (back), while the GP region (arm, outer thigh and abdomen) was characterized by higher levels of IL33 and IL37 mRNA levels. In another study, differences

in Treg numbers were found in different skin regions, and the authors suggest that different Treg levels may be responsible for the different likelihood of developing skin metastases in different regions. These results are also in agreement with our previous studies, where we described a prominent role of the non-inflammatory Th17(β) cells in the presence of Th17-related AMPs (LCN2 and S100A8) and chemokines (CCL2) in healthy SGR and AGR regions.

Several factors, such as microbiota, lipid composition, moisture and pH, may all be responsible for differences in the differential expression of epimmunome molecules detected in healthy regions. However, we believe that among these, the diversity of the skin microbiota is a very important factor and contributes greatly to these observed differences, as the microbiota is in continuous direct contact with KC and may affect these cells differently due to their regional variation. In addition to the unique bacterial composition, the SGR and AGR skin areas are also favoured for colonisation by different fungal communities. Recent sequencing studies have shown that the Malassezia fungal species is dominant on the human skin surface, with an extremely high absolute abundance in the SGR region, but also a prominent presence in the AGR region. More importantly, Malassezia species have been found to elicit Th17 responses, which may play a role in triggering Th17 pathway-related epimmunome production in the epidermis of the SGR and AGR regions.

2. Exploring the role of the cytokine-type epimmunome molecules in inflammatory skin diseases

Next, we determined the changes in epimmunome production in GP and SGR regions in their respective outside-in inflammatory skin diseases (AD and PPR). We examined the expression of the most important KC-derived epimmunome molecules, including IL-17C, IL-23, IL-25, IL-33, IL-36RA and IL-38, in lesional AD and PPR skin samples. In addition, we also examined lesion skin samples from the SGR (Scalp Ps) and GP (PsV) regions of psoriatic patients to determine changes in the expression of epimmunome molecules in an inside-out type of skin disease. Our results show that when an outside-in, i.e. epidermal challenge-induced skin disease develops in the GP region, the levels of IL-33, IL-25, IL-36RA and IL-38 (homeostatic cytokines of the GP) molecules are significantly increased, whereas the levels of IL-23 and IL-17C (low levels in the GP during steady-state) are not altered, and consequently a type 2 (Th2-mediated) inflammation develops, as in AD. In the SGR region, when epidermal challenge from the outside induces inflammation, the levels of the mediators IL-23 and IL-17C

(homeostatic cytokines of the SGR) are significantly increased, whereas IL-33, IL-25, IL-36RA and IL-38 (low levels in the SGR under homeostatic conditions) are not induced, and this may promote the development of type 3 (Th17 mediated) inflammation, as in PPR.

Consistent with our present findings, *De Benedetto and colleagues* have described that the epidermal milieu induced by barrier damage in certain tissue environments can promote different types of adaptive immune activation, thereby inducing type 2 or type 3 immune responses. In contrast, in psoriasis, both GP (PsV) and SGR (Scalp Ps) areas behave similarly, with a highly upregulated IL-23 and IL-17 milieu present, which may be due to autoantigens presented by DCs rather than "epidermal challenge". These results therefore suggest that in skin diseases induced by epimmunome molecules, the expression of those KC-derived epimmunome mediators is elevated, which were already present at higher levels in the skin area under homeostatic conditions. It is important to note that region-specific epimmunome production under homeostatic and inflammatory conditions is not unique to the skin. Differential levels of TSLP, IL-25 and IL-23 were detected in the small intestine and colon, which may influence the characteristic localization of certain inflammatory bowel diseases such as Crohn's disease (Th1/17-driven) and ulcerative colitis (Th2-driven).

In conclusion, we believe that, under homeostatic conditions, different skin regions are "specialised" to produce specific epimmunome molecules due to the specific microbiota composition of different skin areas and thus due to different immune "training". This may influence the mediator production of KCs under pathological conditions and may determine the complex immune nature and localisation of inflammatory skin diseases where an external epidermal challenge is involved in disease initiation, such as in AD (Th2 inflammation on GP) and PPR (Th17 inflammation on SGR). In contrast, in an inside-out skin disease such as psoriasis, inflammation is independent of the homeostatic epimmunome profile of the region. Based on our results, we suggest that specific differences in skin regions should be considered when choosing barrier repair therapies for specific skin regions.

3. Analysis of the expression of AMP-type epimmunome molecules in inflammatory skin diseases

AMPs play a prominent role in the pathogenesis of many immune-mediated skin diseases. In PsV and PPR, the production of these molecules is strongly induced in keratinocytes and some

AMPs are known to play an initiating role in disease pathogenesis. In AD, damage to the skin permeability barrier has been shown to be a major factor in the pathogenesis of the disease. The literature also suggests that skin permeability and antimicrobial barrier regulation are closely linked. However, studies on AMPs in AD are incomplete and there are many controversies regarding the extent of AMP expression. These may be partly because AMPs expression has often been compared with PsV rather than normal controls. In addition, in many cases AMPs have only been examined at the mRNA level, which can be misleading as proteins are functional forms of molecules. In most cases, the studies did not include AD NL skin samples, which could provide critical data on the initial steps of disease pathophysiology. Finally, no study to date has examined AMPs in AD by considering to which functional subset each AMP belongs. In this study, we comprehensively investigated the 5 functional AMP subgroups at both mRNA and protein levels.

The skin of AD NL (clinically asymptomatic) and AD L (clinically symptomatic) patients was compared to GP healthy control (HC) samples. PsV is a chronic inflammatory skin disease with clinical and immunological features completely different from AD; barrier damage is presumably not the underlying cause of the disease, therefore PsV L samples were also tested as disease controls. When comparing AMP levels in AD NL and control samples, no striking differences were found at the mRNA level, and only 2 AMPs showed significant changes. At protein level, only the expression of LL-37 changed, which showed significantly reduced levels in AD NL skin. Limited data are available in the literature regarding protein expression of AMPs in AD NL skin, and no study has examined most AMPs simultaneously in a single study. Consistent with our present results, no striking differences were detected between AD NL and control samples. Expression of several AMPs was significantly altered in AD L skin at the mRNA level, and most AMP levels were elevated compared to controls. At the protein level, the expression of AMPs was significantly increased in AD L skin compared to controls, while the expression of 2 AMPs was unchanged, and LL-37 was the only AMP with strongly decreased levels in AD L skin.

Despite a relatively large number of studies, the published data are contradictory. The summary articles emphasise that the expression of AMPs is generally reduced in AD L skin; however, the original studies predominantly reported elevated or similar AMP levels. However, most data are only available at the mRNA level, and quantification of IHC results was absent or subjective in most cases. There are limited studies available for the study of acute AD, but the

results suggest that the expression of AMPs (S100A7, hBD-2, RNase7) is already strongly induced in acute AD and that their expression does not increase significantly during the acute-chronic transition.

When comparing AD L and PsV L samples, we found that most AMPs were highly induced in both diseases, with similar expression patterns; however, in many cases, the levels of AMPs in AD were below to those measured in PsV. LL-37 showed the most striking difference between the two diseases, as its levels were significantly increased in PsV lesions and decreased in AD compared to controls. Consistent with the current findings, elevated levels of most AMPs have been shown in both diseases in previous studies. However, a review of the literature reveals conflicting data on LL-37 in AD. So far, only one study has described higher levels of LL-37 in AD L skin compared to controls; however, the authors were unable to detect the molecule in several AD L samples by IHC, which in turn is in agreement with our own results. In addition, some other studies have not detected significant differences in LL-37 protein levels between AD L and controls. Finally, in several studies LL-37 was below the limit of detection, which is also consistent with our present results.

The fact that LL-37 was the only AMP to show reduced levels raises the question of its role in the pathogenesis of AD. The literature data suggest that LL-37 may be associated with all three major pathogenetic features of AD, including barrier damage, Staphylococcal hypercolonization and Th2 inflammation. Under healthy conditions, there is a homeostatic balance between LL-37, the permeability barrier and the microbiota, which maintains an anti-inflammatory T cell (effector and resident memory) and Treg environment. However, literature data suggest that permeability barrier impairment is closely associated with LL-37 reduction and Staphylococcal overgrowth. A decrease in LL-37 levels leads to a weakening of the tight junction and impaired skin barrier function, as LL-37 is known to increase the expression of tight junction molecules (e.g. claudins) that maintain the skin permeability barrier. In addition, the overexpression of Staphylococcus bacteria can damage both antimicrobial and permeability barriers, as the cysteine protease activity of S. epidermidis has been shown to cleave LL-37 and one of the major desmosome components, DSG1, in vitro. These results are also very important because Staphylococcus density is already significantly increased on AD NL skin. In addition, LL-37 decrease may further enhance susceptibility to Staphylococcal hypercolonization in AD, as LL-37 is highly effective against Staphylococcal species and biofilms, unlike other AMPs such as hBD. Furthermore, during barrier damage, KCs produce

alarmins; mainly mediators that trigger Th2 cell promotion. The AD-specific Th2 cytokine milieu is known to inhibit the induction of LL-37 in vitro, which is consistent with the reduced LL-37 levels observed in our study.

In summary, the expression of AMPs is generally unchanged or increased in AD lesion areas. The lack of LL-37 induction was the only decrease observed in AD at the protein level. The pattern and level of expression of AMP molecules showed remarkable similarity between AD and PsV samples, with the only exception being LL-37. The prominent role of LL-37 in the pathogenesis of AD is easily conceivable, as LL-37 has been associated with all three major pathogenetic features of AD, including barrier damage, Staphylococcal hypercolonization and Th2 inflammation. Significantly decreased levels of LL-37 in AD NL skin indicate that this molecule may play a leading role in the pathogenesis of AD and raise the possibility of LL-37 as a therapeutic target in the treatment of AD.

SUMMARY

As a first step, the cytokine-type epimmunome molecules were analyzed in healthy SGR, AGR and GP skin regions at the mRNA (with RT-qPCR) and protein (with IHC) levels. Our results show that the GP region is characterized by the presence of IL-25, IL-33, IL-36RA, IL-38, and IL-18 molecules, while the SGR region is characterized by higher levels of IL-23, IL-17C, and IL-18 molecules. The AGR region is characterized by the presence of IL-25, IL-33, IL-23, and IL-18 molecules, thus having the features of both SGR and GP skin regions. Based on the literature analysis, we concluded that the different regions are characterized by a pro-Th2 or pro-Th17 epimmunome profile. As a next step, we also investigated the presence of the main Th2- and Th17-associated cytokines produced by KCs under inflammatory conditions (AD and PPR). In AD, high expression of the GP region-specific cytokines, like IL-33, IL-25, IL-36RA and IL-38 was observed, whereas in PPR the intensity of the healthy SGR region-specific cytokines IL-23 and IL-17C was strongly elevated. These results suggest that in outside-in skin diseases (AD and PPR), which are caused by external epidermal challenge, the expression of cytokines that were already characteristic cytokines of the given skin area under homeostatic conditions was elevated. In contrast, in psoriasis, which is an inside-out skin disease, the inflammatory epidermal cytokine milieu is independent of the homeostatic epimmunome milieu of the specific skin area.

In my second work, we analyzed the AMP-type epimmunome molecules (in five functional groups) in AD and PsV samples at the mRNA (RT-qPCR) and protein (IHC) levels, compared to healthy GP skin. Based on our results, when comparing AD L and PsV L samples, most AMPs were induced in both diseases, showing similar expression patterns; however, in many cases, the levels of these AMPs in AD were lower than in PsV. The greatest difference between the two diseases was found regarding the AMP LL-37, since its levels were significantly elevated in PsV lesions, but they were decreased in AD compared to the control group. The literature data is consistent with our current findings, as elevated levels of most AMPs were detected in both diseases, but in AD the literature data on LL-37 are conflicting. A prominent role for this molecule in the pathogenesis of AD is very likely, as LL-37 has been associated with all three major pathogenetic features of AD (barrier damage, Staphylococcal hypercolonization, Th2 inflammation). Significantly decreased levels of LL-37 in AD NL skin may also indicate that the molecule has an initiating role in the pathogenesis of AD, thus raising the possibility of this molecule as a therapeutic target in the treatment of this disease.

PUBLICATION LIST

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Registry number: DEENK/116/2024.PL
Subject: PhD Publication List

Candidate: Lilla Szabó
Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology

List of publications related to the dissertation

1. **Szabó, L.**, Kapitány, A., Somogyi, O., Alhafez, I., Gáspár, K., Palatka, R., Soltész, L., Törőcsik, D., Hendrik, Z., Dajnoki, Z., Szegedi, A.: Antimicrobial Peptide Loss, Except for LL-37, is not Characteristic of Atopic Dermatitis.
   Acta Derm.-Venereol. 103, adv9413, 2023.
   DOI: http://dx.doi.org/10.2340/actadv.v103.9413
   IF: 3.6 (2022)
2. **Szabó, L.**, Dajnoki, Z., Somogyi, O., Gáspár, K., Hendrik, Z., Szabó, I. L., Szöllősi, A. G., Dinya, T., Törőcsik, D., Kapitány, A., Szegedi, A.: Cytokine profile of the epidermis is region specific and may determine the characteristics of inflammation.
   Exp. Dermatol. 32 (7), 1120-1131, 2023.
   DOI: http://dx.doi.org/10.1111/exd.14820
   IF: 3.6 (2022)

List of other publications

3. Somogyi, O., Dajnoki, Z., **Szabó, L.**, Gáspár, K., Hendrik, Z., Zouboulis, C. C., Dócs, K., Szűcs, P., Dull, K., Törőcsik, D., Kapitány, A., Szegedi, A.: New Data on the Features of Skin Barrier in Hidradenitis Suppurativa.
   Biomedicines. 11 (1), 1-12, 2023.
   DOI: http://dx.doi.org/10.3390/biomedicines11010127
   IF: 4.7 (2022)
4. Dajnoki, Z., Somogyi, O., Retzlerné Medgyesi, B., Jenei, A., **Szabó, L.**, Gáspár, K., Hendrik, Z., Gergely, P., Imre, D., Póliska, S., Törőcsik, D., Zouboulis, C. C., Prens, E. P., Kapitány, A., Szegedi, A.: Primary alterations during the development of hidradenitis suppurativa.
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   DOI: http://dx.doi.org/10.1111/jdv.17779
   IF: 9.2

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5. Kapitány, A., Retzlerné Medgyesi, B., Jenei, A., Somogyi, O., **Szabó, L.**, Gáspár, K., Méhes, G., Hendrik, Z., Dócs, K., Szűcs, P., Dajnoki, Z., Szegedi, A.: Regional Differences in the Permeability Barrier of the Skin: implications in Acantholytic Skin Diseases.
Int. J. Mol. Sci. 22 (19), 1-15, 2021.
DOI: http://dx.doi.org/10.3390/ijms221910428
IF: 6.208

Total IF of journals (all publications): 27,308
Total IF of journals (publications related to the dissertation): 7,2

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

25 March, 2024

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