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Chronological maturation of the skin immune barrier is topographically different

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ABSTRACT

Adult skin varies across regions, with differences in chemical, physical, microbiota, and immune barriers. However, data on topographical immune differences in other age groups are limited. This study aimed to explore the chronological maturation of the immune barrier in various skin regions.

A TaqMan low-density array and immunohistochemical and immunofluorescence detection of various immune cells and mediators in sebaceous gland-rich (SGR) and gland-poor (GP) healthy skin were performed in children, adolescents, and adults.

The maturation of SGR skin showed a general upward trend in the mRNA levels of most Th17-related molecules with a significant increase in IL-1B from childhood to adulthood, but with only a slight elevation between childhood and adolescence. In addition, T cell, Treg, dendritic cell (DC) counts, as well as the levels of several Th17-related proteins (IL-17, IL-10, IL-23, CCL20, S100A8, sTSLP, LCN2), increased significantly with age. In GP skin, AHR mRNA levels decreased, while Th17-related protein levels increased, although only moderately. When comparing the two regions, SGR and GP skin were similar in childhood, with differences emerging in adolescence and becoming significant in adulthood, particularly in the IL-17 pathway, mainly produced by Th17 cells.

Our results show a similarly directed maturation process in GP and SGR regions, with more pronounced development of the SGR skin immune barrier (with more immune cell infiltration and cytokine production) during and after the adolescence. This is likely to be related to the significant changes in the chemical and microbiota barriers of the SGR skin during adolescence, and may explain the high incidence of inflammatory skin diseases on the SGR skin of adolescents, highlighting the need for targeted skin care in this region.

Introduction

A detailed understanding of the structure, function, and development of the skin barrier is essential for clinicians and researchers, as damage to any of the skin barrier elements, including the chemical, microbial, physical (permeability), and immunological barriers, can

cause inflammatory skin diseases. These four elements work closely together to form a complex barrier function in healthy human skin.^{1–4} Therefore, impairment of one barrier element, or even its natural development, can lead to changes in the other barriers.^{5–10} The interactions between the barrier units are reflected in recent studies showing that all four barrier elements exhibit topographical variations

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across the skin surface.^{11–23} The activity and composition of the microbial, chemical, permeability, and immune barriers are significantly different in at least 3 distinct skin regions, namely in sebaceous gland-rich (SGR) sebaceous areas, apocrine gland-rich (AGR) moist areas, and gland-poor (GP) dry areas.

Regional variations in the four barrier elements characterize healthy adult skin. However, limited human data are available on the development of the four barrier elements from childhood to adulthood, and no published data demonstrate how and when the topographical differences of these four barrier elements develop with age. The most detailed data currently available relate to the maturation of the microbiota barrier.^{24–26} In children, the skin microbiota is less diverse; Firmicutes (Streptococcaceae), Actinobacteria, Proteobacteria, and Bacteroidetes are present over the entire skin surface in this age group.²⁴ However, the microbiota diversifies with age. GP regions are colonized by similar microbiota in children and adults, whereas Corynebacteriaceae and Propionibacteriaceae are present in the SGR regions in adults.^{12–15} Adolescents exhibit an intermediate state with a significant shift in the microbiota during sexual maturation.^{26–27} Similar to the microbiota barrier, the chemical environment changes the most also in the SGR region; the lipid content, in particular, increases as the sebaceous glands mature and is especially high in adolescents.^{28–29} The physical barrier also appears to develop differently in different skin areas, as indicated by age-related changes in transepidermal water loss (TEWL), skin hydration, and natural moisturizing factor (NMF) levels between SGR and GP sites.^{30–31} These observations suggest that the microbiota, chemical, and permeability barriers develop differently in different skin regions.

Although the maturation of the skin's immune barrier from childhood to adulthood has been investigated,^{31–33} regional differences were not studied. Therefore, our investigation aimed to characterize changes in the immune barrier in different skin regions from childhood to adulthood. In addition, we aimed to determine whether the immunological characteristics of different body sites are as diverse in childhood as in adults, or whether immunotopographical differences only emerge in adulthood, similar to the chemical and microbiota barriers. Although three main regions have been identified based on immunological differences in adulthood, we did not study the AGR region separately due to its high similarity to the SGR region. We assume that findings in the SGR can be extended to the AGR.

Materials and methods

Skin biopsies

Whole skin biopsies were collected from healthy children (inclusion criteria: 1–8 years), adolescents (inclusion criteria: 12–18 years), and adult donors (inclusion criteria: ≥ 19 years). Infants under 1 year of age (to exclude the robust changes of the first year), and children between 9–11 years (to clearly distinguish children and adolescents) were not involved in the study (Table S1 and S2). The investigated individuals were age and gender matched for the GP and SGR regions. Healthy donors with no known skin disease and no other chronic medical conditions were included in the study. Skin samples originated from the margins of biopsy specimens removed for unrelated medical reasons, including the excision of benign lesions. We specifically focused on samples from the SGR and GP regions considering the challenges of collecting skin biopsies from vulnerable children and adolescents, especially from the AGR skin areas. Moreover, we chose to examine only the SGR and GP regions because these regions cover a much larger skin area than the AGR regions. The SGR and GP regions exhibit the most significant differences in adults, whereas the AGR region is similar to the SGR region.^{17–18,21} SGR samples were obtained from the facial regions (around the ears, cheek), neck and from the upper part of chest and back; while GP samples were collected from the upper and lower extremities (upper arm and forearm, thigh and shin); and from waist and abdomen. Besides the origin, SGR and GP skin samples were also

distinguished according to our previously introduced histological definitions connected to the number of sebaceous glands (samples containing $n \geq 3$ were defined as SGR skin, while samples with no sebaceous glands were considered as GP).^{17,21}

Written, informed consent was obtained according to the principles of the Helsinki Declaration, and the study was approved by the local ethics committee (Regional Institutional Research Ethics Committee, Clinical Center, University of Debrecen, Debrecen, Hungary). Due to the challenges in collecting samples from children and adolescents, for the TLDA analysis, the sample sizes were $N = 10$ for each of the SGR age groups (children, adolescent, and adult), and $N = 9$, $N = 7$, and $N = 8$ for the GP children, adolescent, and adult groups, respectively. Biopsies for the mRNA study were stored at -70°C in RNAlater (Qiagen, Hilden, Germany) for RNA isolation. Further skin biopsies ($N = 6$ per group) were formalin-fixed and paraffin-embedded for immunohistochemistry (IHC) and immunofluorescent staining (IF).

TaqMan Low-Density Array

Gene expression was analyzed using Custom TaqMan® Gene Expression Array Cards (Thermo Fisher Scientific, Waltham, MA, USA). The microfluidic cards contain 384 wells and 384 simultaneous real-time PCR reactions can be performed, enabling the parallel analysis of 96 pre-loaded TaqMan® Gene Expression Assay targets in two samples. The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), following the manufacturer's instructions. The total reaction mixture consisted of 400 ng of cDNA diluted in 200 μl of nuclease-free water and 200 μl of $2 \times$ TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). This mixture was evenly distributed across the four sample-loading ports (100 ng cDNA in each port) of the TaqMan Low-Density Array (TLDA); each port was connected to a set of 96 gene targets. The cards were centrifuged (1 min, 1300 RPM, room temperature) to ensure uniform sample distribution across all wells and sealed to prevent cross-well contamination. RT-qPCR amplification was performed using the Applied Biosystems Prism 7900HT sequence detection system under the following conditions: 2 min at 50°C , 10 min at 94.5°C , followed by 40 cycles of 30 s at 97°C and 1 min at 59.7°C . Relative mRNA levels were calculated using either the comparative Ct method and normalized to the expression of housekeeping gene RPLP0.

Immunohistochemistry

For IHC analysis, freshly prepared formalin-fixed paraffin-embedded (FFPE) sections of skin samples were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched using 3 % hydrogen peroxide (H_2O_2) for 15 min. After heat-induced antigen retrieval, the sections were blocked with a 1 % bovine serum albumin (BSA) solution and incubated with primary antibodies overnight at 4°C . The following antibodies were used: human CD4 (rabbit monoclonal IgG [ab133616]: Abcam, Cambridge, UK), human CD8 (mouse monoclonal IgG [MA513473]: Thermo Fisher Scientific); human FOXP3 (mouse, monoclonal IgG [ab20034]: Abcam), human CD11c (rabbit monoclonal IgG [ab52632]: Abcam), human CD163 (mouse monoclonal IgG [BM4041B]: Origene, Rockville, MD, USA), human IL-17 (rabbit polyclonal IgG [bs-2140R]: Bioss Antibodies, Woburn, MA, USA), human IL-10 (mouse monoclonal IgG [mab30207]: Covalab, Bron, France), IL-23 (rabbit polyclonal IgG [PA5-20239]: Thermo Fisher Scientific); human CCL20/MIP-3 α (rabbit polyclonal IgG [ab9829] Abcam), human S100A8 (rabbit polyclonal IgG [HPA024372]: Sigma-Aldrich, Budapest, Hungary), human lipocalin/NGAL (rabbit polyclonal IgG [PA5-32476]: Invitrogen, Carlsbad, CA, USA), human TSLP (rabbit polyclonal IgG [ab47943] Abcam). The following day, the sections were incubated with HRP-conjugated secondary antibodies (anti-mouse/rabbit, Dako, Santa Clara, CA, USA). The samples were washed three times with TBST for 5 min each between antibody incubations. Antibody binding was

visualized using the Vector® ImmPACT™ NovaRED™ Kit (VECTOR Laboratories, Burlingame, CA, USA), and the background was counterstained with methylene green. Each protein was detected in parallel on all sections to ensure comparability of protein expression levels. Positive, Ig, and isotype controls were used to standardize staining across all proteins.

Then, IHC sections were digitized using whole slide imaging (WSI) technology as previously described.^{20–21} Proteins exhibiting epidermal positivity were analyzed using the HistoQuant application of the CaseViewer software (3DHistech, Budapest, Hungary). The application was specifically trained for each molecule to distinguish between positive staining (identifying positive pixels) and background staining.

For each section, at least three representative regions of interest (ROIs) were selected, each corresponding to an epidermal area associated with an epidermal length of 500 µm. The pre-trained algorithm was then used to evaluate these ROIs. Finally, the total staining intensity was quantified and compared across sample groups.

The evaluation of dermal immune cells and cytokine-positive cells followed a similar approach. At least three representative ROIs per sample were selected, with each ROI defined as a 1000 µm diameter circular area in the WSI-digitized samples. The number of positive cells within these regions was then determined and compared between sample groups.

Immunofluorescent staining

IF staining was performed on FFPE sections using the same protocol as described for immunohistochemistry, except for the H₂O₂ blocking step, which was unnecessary. After incubation with primary antibodies against CD4 and IL-17A, Alexa Fluor™ 555 goat anti-mouse IgG (H + L) and Alexa Fluor™ 488 goat anti-rabbit IgG (H + L) secondary antibodies were applied (Thermo Fisher Scientific). At least 3 images were taken per section at 200 × magnification to evaluate the IF staining. The total intensity value per epidermal area was determined by Fiji (ImageJ) in 8-bit grayscale images.

Heatmap generation

The heatmaps were generated using Morpheus (Matrix Online Resource for Phenotype-Expression-Utility Studies), a web-based software developed by the Broad Institute (<https://software.broadinstitute.org/morpheus/>). First, the input data matrix containing the selected molecules and their expression values was uploaded. Next, hierarchical clustering was performed using the one minus Pearson correlation as the distance metric and average linkage as the clustering method. A relative color scheme was applied, where the minimum and maximum values in each row were used to convert the values to colors. Finally, the resulting heatmap was exported.

Enrichment analysis

To verify the prominent role of IL-17/Th17 signaling in the development and maturation of SGR and GP skin areas, molecules that changed significantly at the gene and/or protein levels during the development of the two regions were analyzed using bioinformatics. Two distinct sets of molecules characteristic of the SGR and GP regions were subjected to separate enrichment analyses using STRING (string-db.org).³⁴ We entered the input molecules under the *Multiple proteins* option, selected *Homo sapiens* as the species, and ran the analyses using the default settings. The results were examined based on the Gene Ontology Biological Process, KEGG, and WikiPathways databases, and we identified the significantly enriched pathways common to both SGR and GP datasets.

Statistical analysis

The distribution of the data was tested using the Shapiro-Wilk test. In case of normal distribution Two-way ANOVA was used with Tukey's post hoc test. If the data were not normally distributed, the data were transformed by Box-Cox transformation before the Two-way ANOVA. Fisher exact tests were applied to analyze the categorical variables. In cases of a normal distribution, independent t-tests (two-tailed) were used to compare the continuous variables, otherwise Mann-Whitney test was applied.

Data are presented as means with the corresponding 95 % confidence intervals (CI, boxes). The significance level was $p < 0.05$ in all cases. Statistically significant differences are denoted by *, **, and *** for $p < 0.05$, $p < 0.01$, and $p < 0.001$. Statistical analyses were performed using GraphPad Prism software version 8 (GraphPad Software Inc., San Diego, CA, USA).

Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Results

The development of skin regions at the mRNA and protein levels was investigated using whole skin biopsies from the GP and SGR regions of healthy children, adolescents, and adult donors. Since only small healthy skin samples could be obtained, especially from children and adolescents, TaqMan Low-Density Array (TLDA) card was employed to investigate the development of the two different skin regions at the mRNA level. This method allowed the simultaneous examination of several pre-selected genes. Based on previous studies that identified differences in dendritic cells (DCs) and T cell-related molecules between the SGR and GP regions, we specifically investigated genes involved in DC maturation and genes associated with the Th1, Th2, Th22/Treg, and Th17 pathways, including cytokines, chemokines, transcription factors, and antimicrobial peptides (AMPs) (see Fig. 1 for the list of genes and the corresponding heatmap).^{17–18,21} The key representatives of these pathways were confirmed at the protein level using IHC.

Age-related development of the SGR and GP skin regions at the mRNA level

As a first step, the maturation of different skin regions was investigated at the mRNA level. Hierarchical clustering of the TLDA data clearly distinguished samples from the SGR and GP skin regions (Fig. 1a).

In the SGR region, the mRNA expression of most of the Th1- and Th17-related molecules tended to increase with age. Generally, similar or slightly elevated levels could be detected in adolescents compared with children, and more pronounced increases were observed in adults compared to adolescents. Overall, the most prominent changes were observed when comparing children and adults. The Th1-related cytokine IL-12A and the Th17-related cytokine IL1B increased significantly in adults compared with children (Fig. 1a, results with prominent changes highlighted in Fig. 1b). Meanwhile, IL-17 and its associated transcription factor RORC showed a tendency to increase with age; however, their increase did not reach statistical significance. Among the Th17-related AMPs LCN2, S100A7, CAMP, and DEFB4B showed increased levels in adulthood, whereas S100A8 and S100A9 were highly expressed even in childhood, with no substantial increase over time (Fig. 1a). Hierarchical clustering confirmed the similarity between children and adolescents, while adult skin specimens were clearly distinguished due to the higher levels of Th17-related molecules (Fig. 1a).

In contrast to SGR skin, where TLDA revealed a prominent increase

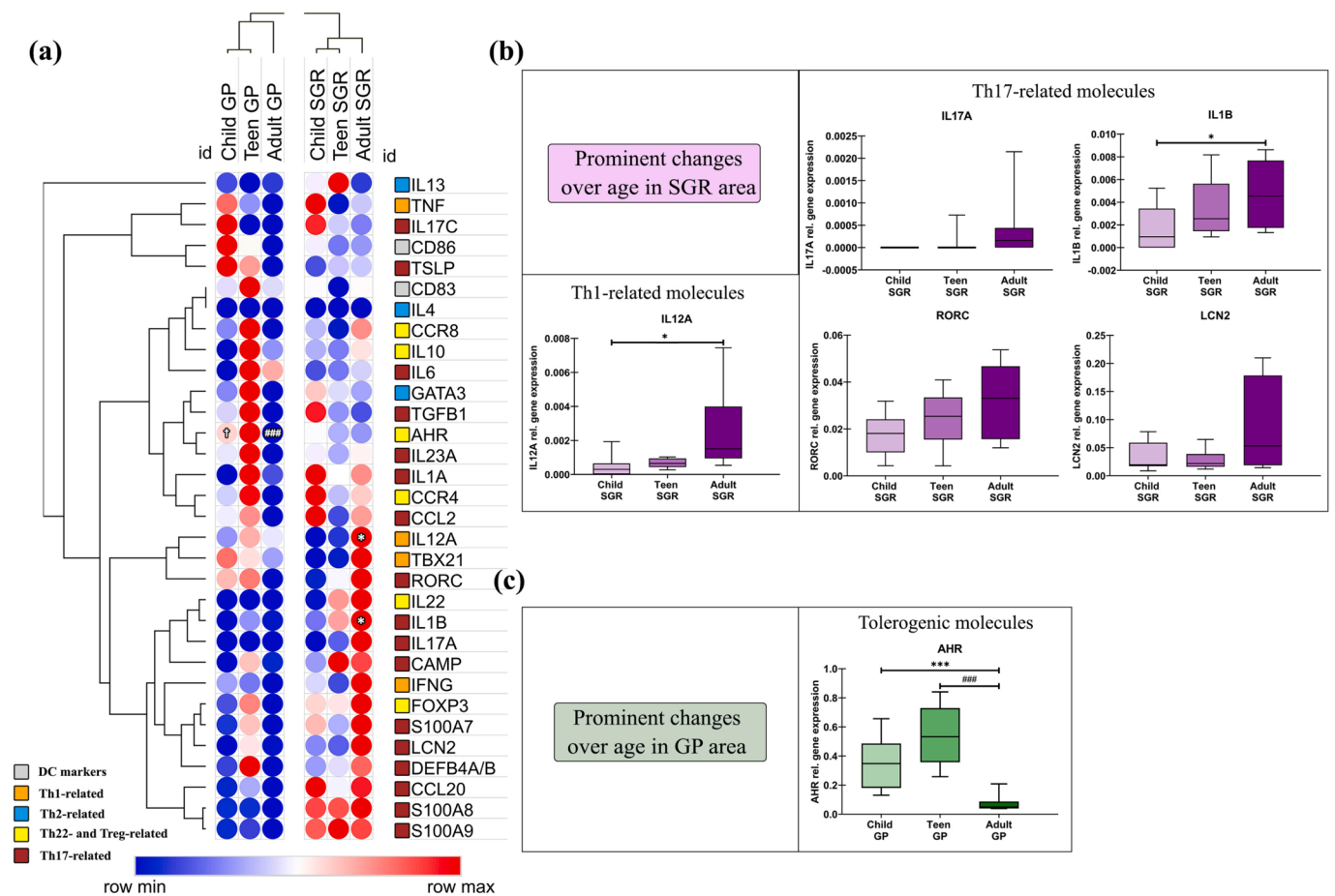


Fig. 1. SGR skin is characterized by a more robust, Th17-related maturation at the mRNA level than GP area (a) Heatmap of genes in different age groups of the 2 skin regions by k-means clustering (b) Prominent changes in the SGR area (c) Prominent changes in the GP area. The graphs show the median \pm 95 % confidence interval (* p < 0.05; ** p < 0.01; ***/### p < 0.001, determined by Two-way ANOVA followed by Tukey's post hoc test in case of normal distribution. If the data were not normally distributed, the data were transformed by Box-Cox transformation before the Two-way ANOVA.). Abbreviations: AHR, aryl-hydrocarbon receptor; CAMP, cathelicidin antimicrobial peptide; CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; DC, dendritic cell; DEFb, defensin beta; GP, gland poor; IL, interleukin; IFN, interferon; LCN2, lipocalin RORC, RAR-related orphan receptor C; S100, S100 calcium binding protein; SGR, sebaceous gland rich; TBX, T-box transcription factor; Th, T helper; TGFB, transforming growth factor b; TNF, tumor necrosis factor; Treg, regulatory T; TSLP, TSLP thymic stromal lymphopoietin;

in several Th17-related molecules from childhood to adulthood, only one molecule showed significant change during the maturation of the GP region (Fig. 1a). Specifically, the mRNA level of the homeostatic molecule AHR increased from childhood to adolescence but decreased significantly later in adulthood (Fig. 1a and c). No other significant differences in mRNA levels were detected between the child, adolescent, and adult groups. Notably, except for sTSLP, all AMPs examined remained consistently low across all age groups in the GP region (Fig. 1a.).

Age-related development of the SGR and GP skin regions at the protein level

At the protein level, epidermal and dermal cells and mediators were analyzed by IHC. First, the characteristics of the SGR region were studied, where most of the investigated immune cell counts increased significantly from childhood to adulthood (CD4 + and CD8 + T cells, Foxp3 + regulatory T cells (Tregs) and CD11c + dendritic cell (DCs); Fig. 2, Supplementary Fig. S1). From childhood to adolescence, the numbers of CD4 + and CD8 + T cells, Foxp3 + Tregs and CD11c + DCs in the dermis of SGR skin showed a slight increase. However, from adolescence to adulthood, their counts elevated significantly (CD4 + and CD8 + T cells, CD11c + DCs) or showed a tendential increase

(Foxp3 + Tregs). The number of CD163 + macrophages tended to increase from childhood to adolescence and from adolescence to adulthood, although the differences were not significant (Fig. 2, Supplementary Fig. S1). The numbers of Th17-related IL-17+, IL-10+, IL-23+, and CCL20 + cells were similar in children and adolescents but significantly increased in adults compared to adolescents. These immune markers increased significantly from childhood to adulthood (Fig. 2, Supplementary Fig. S1, S2).

In the epidermis of SGR skin, Th17-related mediators including CCL20, S100A8, LCN2, and the tolerogenic short isoform of thymic stromal lymphopoietin (sTSLP) were similar in children and adolescents. However, their levels were significantly increased in adults compared to both children (all 4 molecules) and adolescents (S100A8, sTSLP, LCN2) (Fig. 2, Supplementary Fig. S2) at the protein level. Regarding the dermal components, the numbers of CD4 + and CD8 + T cells, Foxp3 + Tregs, CD11c + DCs tended to increase with age, however, these differences were not significant when comparing the age groups (Fig. 3, Supplementary Fig. S1).

Among the Th17-related molecules in the dermis of the GP skin area IL-17 + cells tended to increase with age, while IL-10+, IL-23+, and CCL20 + cells were present in similar numbers in childhood and adolescence, however, their cell counts increased significantly in adults compared to both children (all of them) and adolescents (IL-10+, IL-23

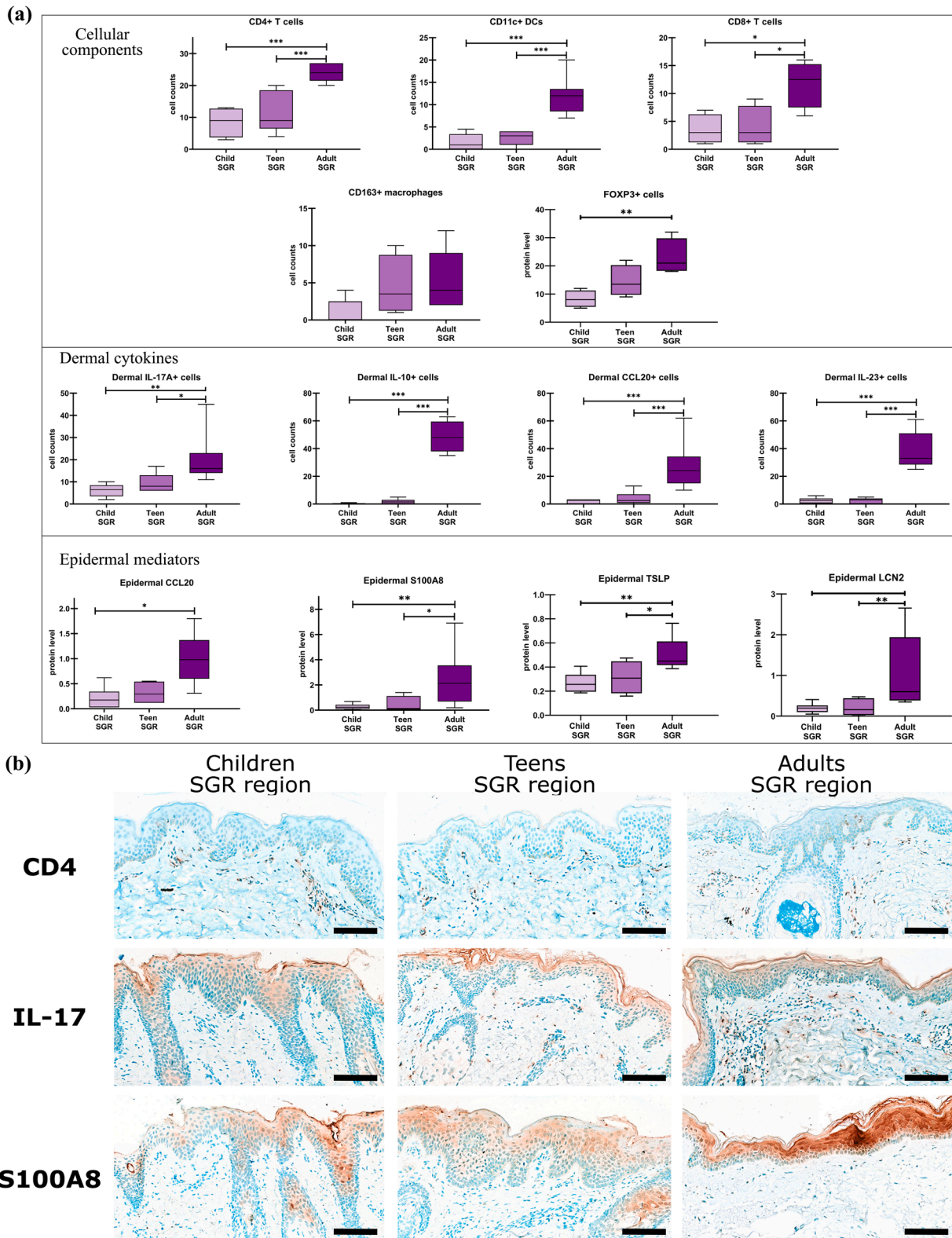


Fig. 2. Maturation of the sebaceous gland-rich (SGR) skin is characterized by significant increase in the number of cellular components, and in protein expression of Th17 related dermal and epidermal mediators after adolescence (a) Quantification of the IHC staining of cellular components, dermal cytokines, and epidermal mediators in SGR skin sections (b) Representative images for immunostaining (1–1 member of each group, see remaining in supplementary data), scale bar = 100 μ m. The graphs show the median \pm 95 % confidence interval (* p < 0.05; ** p < 0.01; * p < 0.001, determined by Two-way ANOVA followed by Tukey's post hoc test in case of normal distribution. If the data were not normally distributed, the data were transformed by Box-Cox transformation before the Two-way ANOVA.) Abbreviations: CCL, chemokine (C-C motif) ligand; IL, interleukin; LCN2, lipocalin; S100, S100 calcium-binding protein; SGR, sebaceous gland rich; TSLP, TSLP thymic stromal lymphopoietin;**

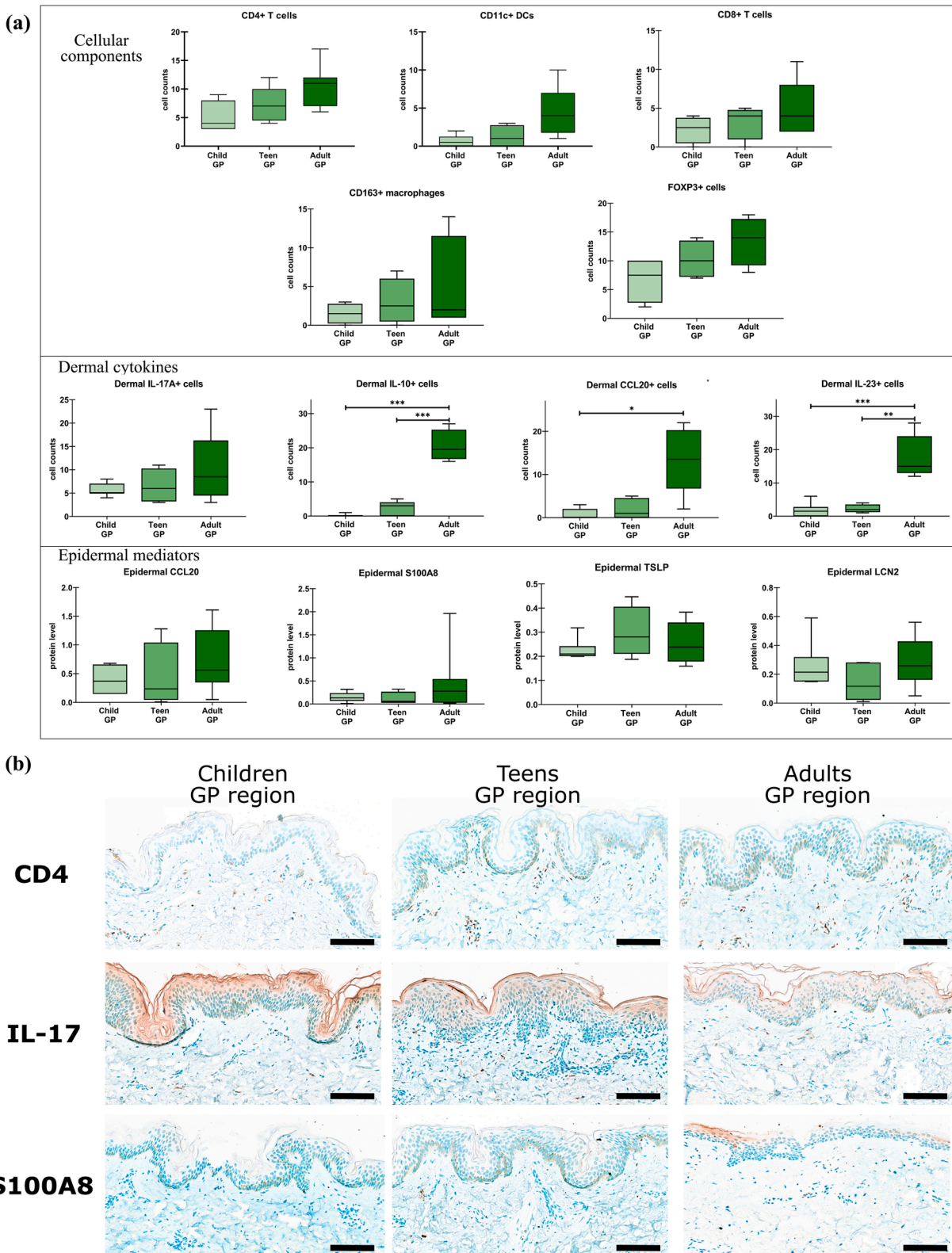


Fig. 3. Maturation of the gland-poor (GP) skin region is characterized by moderate increase in the number of cellular components, and protein expression of Th17 related dermal and epidermal mediators (a) Quantification of the IHC staining of cellular components, dermal cytokines and epidermal mediators in GP skin sections (b) Representative images for immunostaining (1-1 member of each group, see remaining in supplementary data), scale bar = 100 μ m. The graphs show the median \pm 95 % confidence interval (* p < 0.05; ** p < 0.01; *** p < 0.001, determined by Two-way ANOVA followed by Tukey's post hoc test in case of normal distribution. If the data were not normally distributed, the data were transformed by Box-Cox transformation before the Two-way ANOVA). Abbreviations: CCL, chemokine (C-C motif) ligand; IL, interleukin; LCN2, lipocalin; S100, S100 calcium-binding protein; SGR, sebaceous gland rich; TSLP, TSLP thymic stromal lymphopoietin;

+) (Fig. 3, Supplementary Fig. S1, S2).

Regarding the epidermal expression of CCL20, S100A8, sTSLP, and LCN2 no significant difference could be observed across the different age groups (Fig. 3, Supplementary Fig. S2).

Comparison of the two skin regions across age groups

After examining the development of each skin region across different ages, we aimed to directly compare the two regions within each specific age group (childhood, adolescence, and adulthood) using the previously presented data. Since both regions showed signs of immune maturation with the same orientation, but the extent of changes was different, we aimed to find out, when the immunological characteristics of two skin areas become significantly different, i.e. when the regionally different immune characteristics of adult skin develop.

In childhood, no significant differences in the expression of any of the investigated molecules, either at the mRNA or protein level, were detected between the two skin regions (Fig. 4a, b). In adolescents, some slight diversification was detected between the two skin regions. Significantly higher AHR mRNA level could be detected in GP skin compared to SGR. On the other hand, notably but insignificantly higher mRNA levels of S100A8 and a slight increase in IL-17 + expression were detected in SGR skin compared with GP skin (Fig. 4a). At the protein level, slight but insignificant differences were detected between the two regions at this age groups (Fig. 4b).

In adulthood, the presence of some Th17-related molecules (IL-1B cytokine, FOXP3 transcription factors, and LCN2 AMP) were significantly higher in the SGR region compared with the GP region even at the mRNA levels (Fig. 4a). However, even more pronounced differences could be detected at the protein level. Specifically, CD4 + and CD8 + T cells and CD11c + DC counts were significantly higher in the SGR region compared with the GP region. In addition, dermal IL-10+, IL-23 + and CCL20 + cell counts and epidermal S100A8, sTSLP and LCN2 increased significantly in SGR skin compared with the GP region. Although the number of FOXP3 + Tregs and dermal IL-17 + cells tended to increase in SGR skin, these differences did not reach the level of significance (Fig. 4b).

The possible discrepancies between mRNA and protein expressions can be explained by posttranscriptional regulatory mechanisms,³⁵ or by methodological reasons (mRNA-level analyses assessed whole skin biopsies, whereas IHC staining allows for separate evaluation of epidermal and dermal expression patterns).

Validation of the Th17 characteristics in skin development

To gain a deeper understanding of the molecular characteristics of skin development in the SGR and GP regions, we performed an enrichment analysis using String (string-db.org). This analysis focused on two distinct sets of molecules showing significantly different expression at the mRNA and/or protein levels during the maturation process. We used STRING not for traditional gene set enrichment analysis but as an unbiased tool to explore molecular interactions and pathways, while acknowledging its limitations with small gene sets. By integrating data from the Gene Ontology Biological Process, KEGG, and WikiPathways databases, we identified overlapping significantly enriched pathways between the two regions (Fig. 5a). This in silico analysis confirmed our previous findings and suggested that both regions share similar developmental characteristics, but the SGR region undergoes a more pronounced transformation over time than the GP region.

The “Th17 cell differentiation pathway” was one of the most significantly enriched pathways in both the SGR and GP regions, indicating that Th17-related immune responses play a crucial role in skin maturation in both skin areas (Fig. 5a). In addition, other immune-related pathways, including “Cytokine-cytokine receptor interaction” and “Defense response to bacterium”, were enriched in both regions. However, the magnitude of enrichment and the number of genes

observed were generally higher in the SGR region compared with the GP region, highlighting the more dynamic immunological barrier-related maturation process in the SGR region (Fig. 5a.). Overall, these results suggest that the SGR and GP regions develop in the same direction, but the SGR region develops stronger immune activation over time.

The molecular analyses highlighted the importance of IL-17 in the development of both regions and the in-silico analysis confirmed the significant enrichment in the Th17 pathway. Therefore, we aimed to identify the cellular sources of IL-17. IL-17 is produced by many adaptive, innate, and innate-like cell populations, although helper T cells and CD8 + cytotoxic T cells are the most abundant. To determine the source of IL-17, first we performed double immunofluorescence staining for IL-17 and CD4, a marker for helper T cells. The IF staining revealed that the number of CD4 + cells and IL-17 + cells highly increased in the SGR region and moderately increased in the GP regions during development from childhood to adulthood. In addition, almost all of the IL-17 positivity was associated with CD4 + T cells, suggesting that CD4 + helper T cells were the primary source of IL-17 in both skin regions (Fig. 5b.). Due to the extremely high rate of CD4-IL-17 colocalization, other cell types were not investigated as potential sources of IL-17. As only healthy skin regions exhibiting non-inflammatory immune milieu were examined in our study (with elevated numbers of Tregs and non-activated DCs and increased IL-10 levels), the Th17 cells were probably non-pathogenic Th17(β) cells.

Discussion

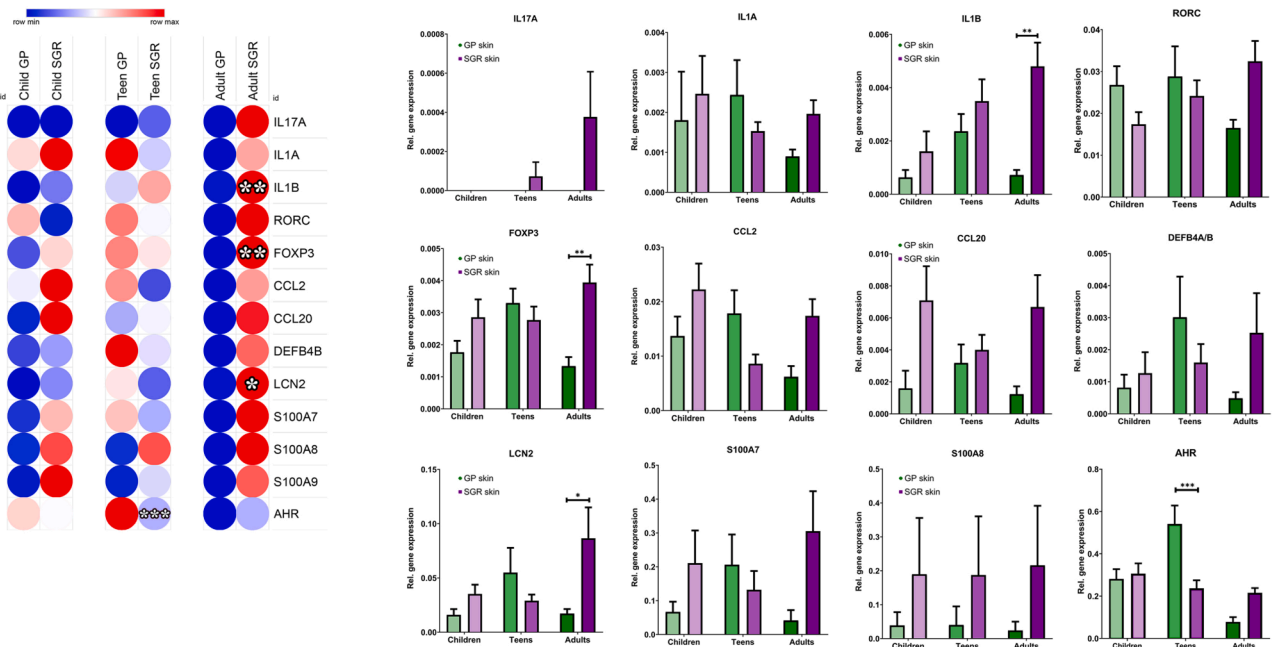
In most barrier organs, such as the gastrointestinal and respiratory tracts, distinct regions can be easily distinguished. In contrast, microscopic or molecular analyses are usually required to detect regional differences in the skin. The chemical composition, permeability, microbiota, and also the immune barrier varies across different regions of healthy adult skin.^{12–22} In contrast to GP areas, gland-rich skin regions (SGR and AGR) of adults are characterized by a non-inflammatory IL-17/IL-10-rich environment.^{17–18,21} The immune barrier of these regions contains more non-inflammatory DCs and Th17(β) cells and increased Th17-related cytokines, chemokines, and AMPs. The most prominent differences are observed between SGR and GP regions; AGR regions are similar to SGR regions, but the differences are slightly less pronounced.^{17–18,21} Whether the differences in the skin immune system in different skin regions are present from birth or acquired over time was unclear. Thus, we investigated the immune maturation of different skin areas separately. Understanding the complex development of the skin immune barrier across the child-adolescent-adult spectrum and comparing the regional maturation programs is essential for understanding the pathogenesis of region- and age-specific skin diseases.

Our findings revealed that the relatively small number of dermal infiltrates of the SGR region in childhood significantly expands during development into adulthood, including CD4 + and CD8 + T cells and CD11c + DCs. The number of Tregs also increases significantly with age. In addition, the number of cells positive for Th17-related cytokines (IL-17, IL-10, IL-23, and IL-1B), chemokines (CCL20), and AMPs (LCN2, S100A8, and homeostatic sTSLP) increased significantly during maturation. The degree of changes was negligible between childhood and adolescence, and larger between adolescence and adulthood. Thus, significant differences in immune-related cells and molecules were detected between childhood and adulthood.

In GP skin, the direction of the immune barrier development was similar to SGR skin, but the extent of changes during development was lower. The expression of a homeostatic molecule, AHR decreased at the gene level, but this change was not confirmed at the protein level. On the other hand, the numbers of cellular components (T cells, Tregs and DCs) as well as of dermal IL-17 + cells tended to increase with age, while the Th17-related IL-10+, IL-23+, and CCL20 + cell counts increased significantly.

Our results suggest that a predominantly IL-17/IL-10 immune milieu

(a)



(b)

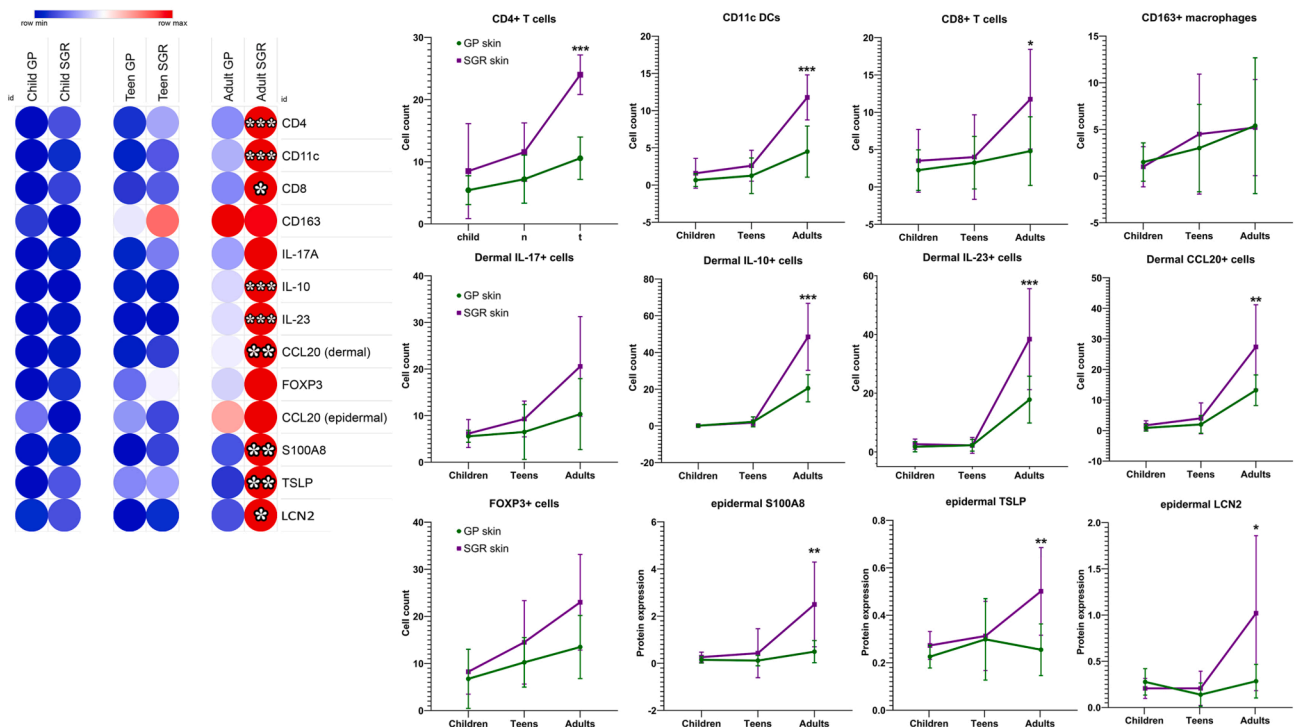
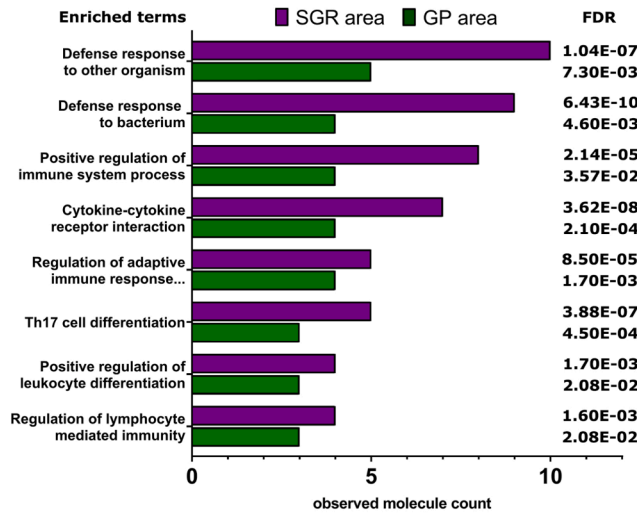


Fig. 4. SGR and GP skin regions are similar in childhood with differences emerging after adolescence and becoming significant in adulthood (a) The heatmap (left panel) and graphs (right panel) show molecules with prominent gene expression differences between the two regions, as identified by TLDA, across all ages examined. (b) The heatmap (left panel) and the graphs (right panel) illustrate molecules with significant and trending differences in protein expression between the two regions, as detected by immunohistochemistry across all ages. In the heatmaps, red indicates higher expression, and blue indicates lower expression. In the graphs, purple denotes the SGR region, while green represents the GP region. The graphs show the median \pm 95 % confidence interval (* p < 0.05; ** p < 0.01; *** p < 0.001, determined by Two-way ANOVA followed by Tukey's post hoc test in case of normal distribution. If the data were not normally distributed, the data were transformed by Box-Cox transformation before the Two-way ANOVA.) Abbreviations: CCL, chemokine (C-C motif) ligand; DEFB, defensin beta; FOXP3, forkhead box P3; GP, gland-poor; IL, interleukin; LCN2, lipocalin; RORC, RAR-related orphan receptor C; S100, S100 calcium binding protein; SGR, sebaceous gland rich; TSLP, thymic stromal lymphopoietin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(a)



(b)

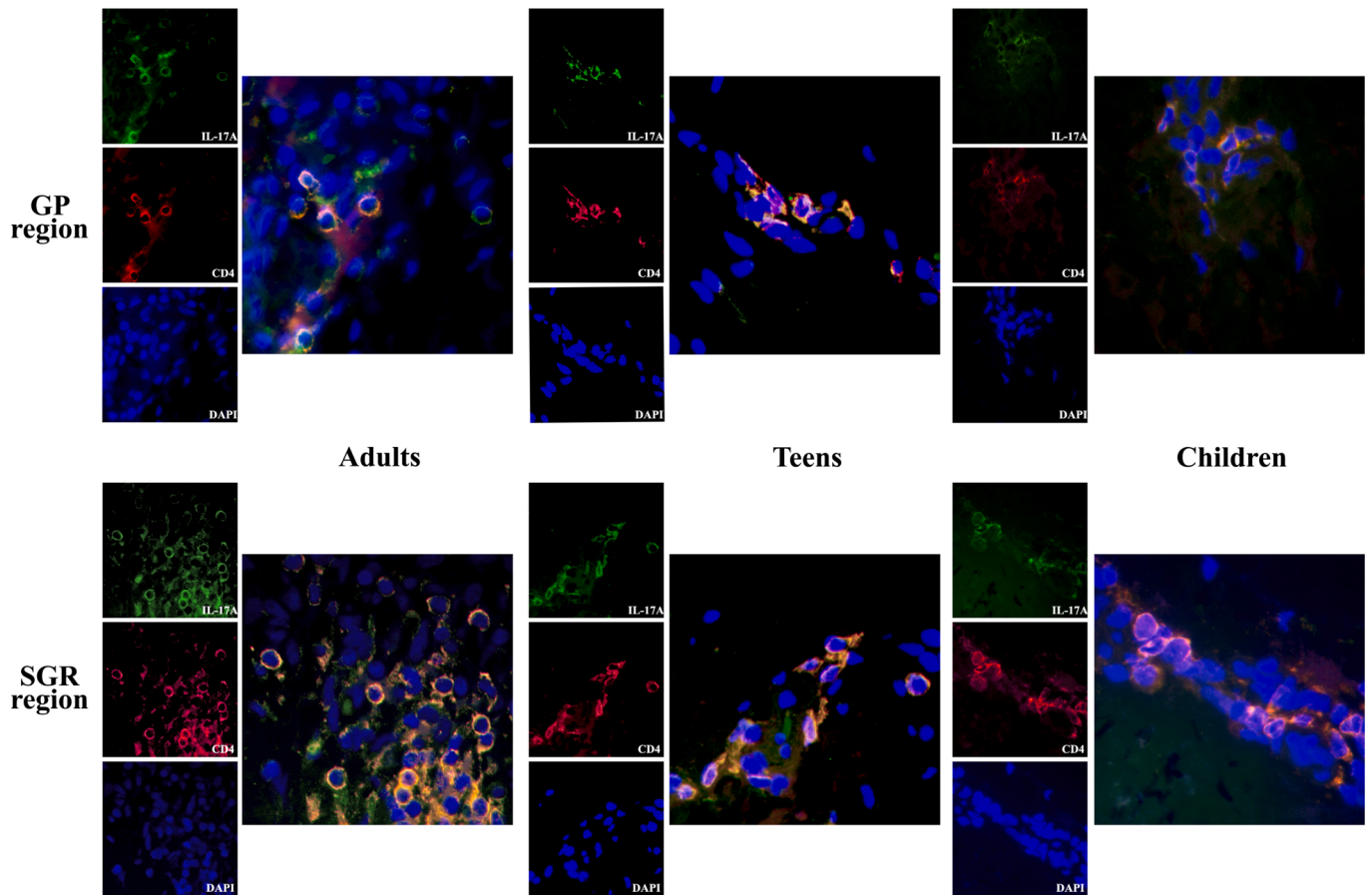


Fig. 5. Non-inflammatory Th17 dominance characterizes the maturation of GP and SGR skin areas (a) Common, significantly enriched pathways related to the maturation of both GP and SGR skin regions revealed by the STRING platform. (b) Representative images for double immunofluorescence staining of CD4 and IL-17A. Blue: DAPI, red: CD4, green: IL-17A. The orange color represents the co-localization of CD4 and IL-17A (Th17 cells). Abbreviations: FDR, false discovery rate; GP, gland-poor; SGR, sebaceous gland rich. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

develops in both regions over time, with a significantly more pronounced effect in the SGR skin region, represented by significantly higher immune cells infiltration and Th17 related cytokine production. Furthermore, immunofluorescence staining identified T helper cells as

the source of elevated IL-17 in these regions. Enrichment analysis confirmed the involvement of the Th17 pathway in the maturation of both the SGR and GP regions. However, the Th17 milieu appears to be non-inflammatory, as evidenced by the high levels of IL-10, the presence

of Tregs and non-activated dendritic cells (indicated by low levels of the maturation markers CD83 and CD86 measured by TLDA), and the absence of inflammatory cytokines such as IFN- γ shown previously.¹⁷ This non-pathogenic Th17 milieu may be crucial in maintaining barrier homeostasis and integrity and promoting coexistence with commensal microbiota.

To the best of our knowledge, only two studies have investigated changes in the immune environment of normal skin from childhood to adulthood, and both of these studies focused exclusively on the maturation of the GP region.^{32,36} Consistent with our findings, Renert-Yuval et al. did not observe significant immune-related differences in the GP regions between children and adults at the gene level, and no comprehensive analysis was performed at the protein level in this study.³² Visscher et al. examined the GP region across three age groups; similar gene expression patterns were observed between adults in their 20s and 60s at the whole transcriptome level, but the skin of newborns showed a markedly different transcriptome profile.³⁶

In the second phase of our study, we investigated when the different immune characteristics of the SGR and GP areas emerged. Our results indicate that the topographical immune barrier differences characteristic of adult skin are not detectable in childhood. Although some regional differences begin to emerge during adolescence (increased cell numbers and Th17/Treg-related molecules in the SGR skin), these differences only become pronounced in adulthood. Thus, adolescence appears to be the turning point; the first distinct regional immunological differences appear in these ages, eventually leading to the development of immunotopographical differences in adult skin (Fig. 6.). According to

previous literature data, other barrier elements also show no significant regional differences in childhood. For example, the chemical environment is consistent in childhood due to the uniformly low glandular secretions and sebum levels in various skin areas. Similarly, only minor regional variations in microbiota composition have been observed in children.²⁴

Our results show that the immunotopographical differences of adult skin are not innate but acquired. Although the skin regions develop similarly, the SGR regions undergo more significant changes due to hormonal maturation between adolescence and adulthood. The higher density and increased activity of sebaceous glands in SGR regions leads to increased sebum production, and may also alter the composition of sebum. This, in turn, alters the local chemical environment (higher sebum level and reduced pH). Sebum components, especially lipids are known to have immunomodulatory effects, with some lipids being pro-inflammatory and others anti-inflammatory.^{21,37} On the other hand, changes in the chemical environment also lead to a significant shift in the skin microbiota towards lipophilic species such as *Cutibacterium* (e.g., *Cutibacterium acnes*) and *Corynebacterium* (e.g., *Corynebacterium simulans*) in adolescence. compared to childhood.²⁵⁻²⁶ Based on experiments in murine models, this shift in microbiota content may induce a robust accumulation of effector T cells,³⁸ resulting in significant regional differences in the immune barrier in adulthood. The combined effects of these changes may explain why, after adolescence, the immune system of SGR skin undergoes more pronounced shift compared to GP regions.

Literature on whether barrier elements develop differently in the two genders owing to the different hormonal influence on sebaceous activity

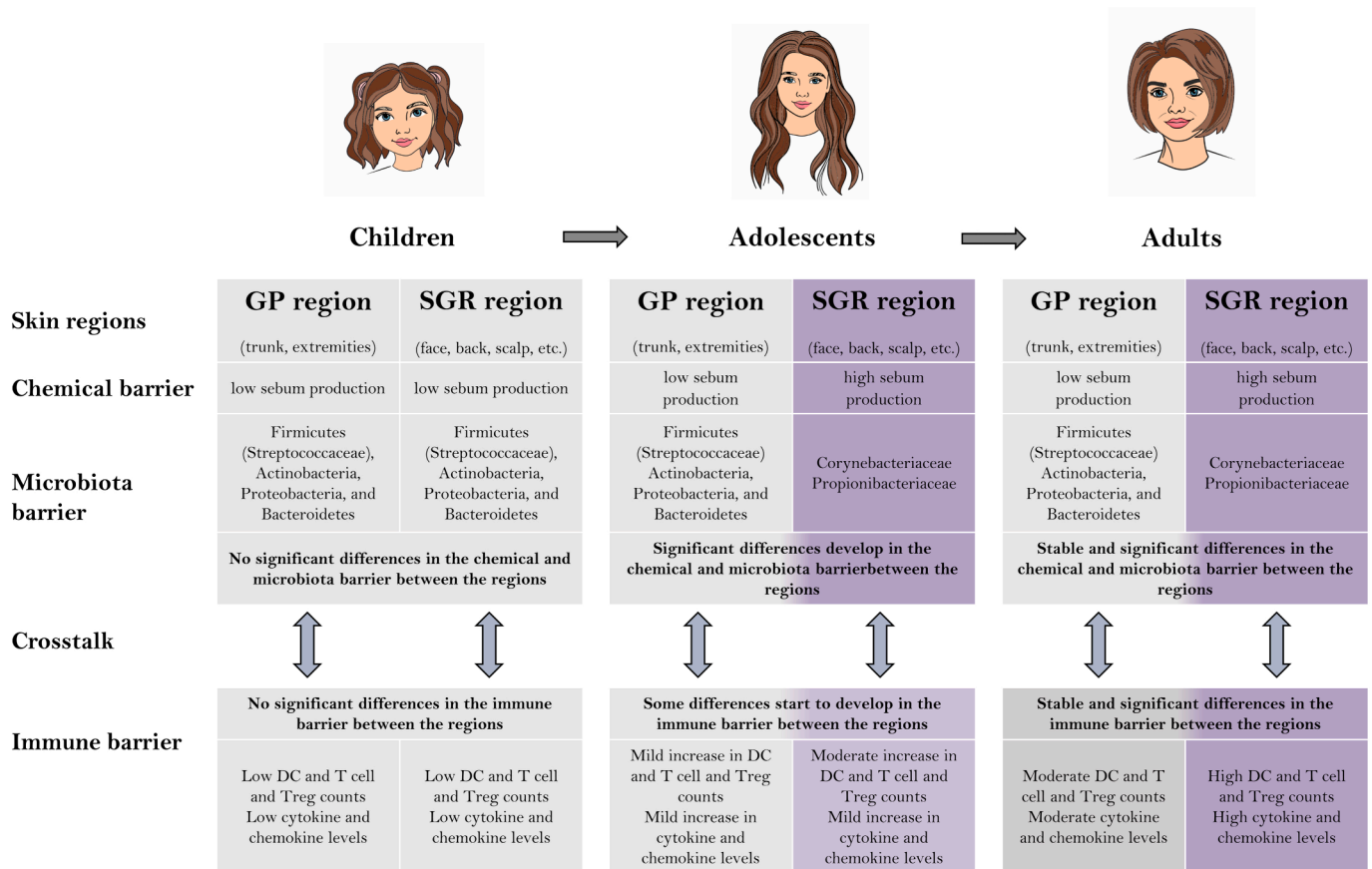


Fig. 6. Prominently distinct chronological maturation characterizes different skin regions In childhood, the chemical and microbiota barriers of the different skin regions are similar and, like them, the immune barrier does not differ significantly. In adolescence, significant differences develop between the SGR and GP regions in the chemical and microbiota barriers, while consequently, some differences begin to develop in the immune barrier of the different regions. After adolescence, SGR skin is characterized by a more robust development, resulting in stable and significant differences also in the immune barrier of the different skin regions in adulthood. Changes in the colors of the background (grey and purple) symbolize the maturation of the barriers in different skin regions. Abbreviations: GP, gland-poor; SGR, sebaceous gland rich. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are contradictory and incomplete. It has been shown that different sex hormones lead to different chemical milieu in SGR skin, like different amounts and compositions of sebum production, between males and females,^{39–40} in parallel, Park et al. found sex-dependent differences also in bacterial and fungal communities.²⁶ On the opposite, Oh et al. did not observe significant gender-related differences in microbiota composition.²⁵ During our current investigation on immune barrier maturation we could not detect significant gender-related differences, however to draw the right conclusions would have required a much larger sample size. The above mentioned two research groups, who also studied the immune barrier in different age groups did not examine gender differences et al.^{32,36} Although in our previous studies on adults we could not observe differences between females and males as well,^{17,21} further studies are needed with high sample-size to reveal the possible gender-related differences in the maturation of immune barrier as well.

The significance of our findings is that they may explain the frequent development of age- and region-specific diseases such as acne, since during the currently presented rapid immunological maturation of the SGR region inflammatory characteristics may temporarily exist, contributing to the onset of acne during adolescence as previously hypothesized⁴¹. Further importance of our study is that regional differences in the adult skin should be considered when developing barrier-restoring therapies or therapies for region-specific skin diseases. On the other hand, the development of biomarkers also requires the use of appropriate region-specific skin controls. In summary, the skin's immune barrier is uniform during childhood; the regional differences characteristic of adult skin are not innate but develop after puberty. Although the developmental trajectories of the GP and SGR regions are similar, changes in the SGR region are much more pronounced.

Our study had several limitations, firstly the sample size was small, however it is important to keep in mind that it was extremely difficult to gain biopsies from healthy children and teenagers. Due to relatively small sample size, the investigation of gender differences was not profound. Furthermore, the Tanner stage determination was not performed in the case of children and teenagers during the biopsy sampling, so its examination in this form could not be included in our study.

CRediT authorship contribution statement

Anikó Kapitány: Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Lilla Soltész:** Writing – original draft, Investigation. **Vivien Stercel:** Writing – review & editing, Resources. **Lilla Szabó:** Writing – original draft, Investigation. **Orsolya Somogyi:** Writing – original draft, Investigation. **Eszter Anna Janka:** Writing – review & editing, Investigation, Formal analysis. **Viktória Nagy:** Writing – original draft, Investigation. **Szilárd Póliska:** Writing – review & editing, Formal analysis. **Krisztián Gáspár:** Writing – review & editing, Resources. **Zoltán Hendrik:** Writing – review & editing, Resources. **Dániel Töröcsik:** Writing – review & editing, Resources. **Zsolt Dajnoki:** Writing – review & editing, Visualization, Funding acquisition, Formal analysis, Conceptualization. **Andrea Szegedi:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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implementation.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mucimm.2025.03.004>.

Data availability

Data will be made available on request.

References

- Harris-Tryon TA, Grice EA. Microbiota and maintenance of skin barrier function. *Science (New York, NY)*. 2022;376(6596):940–945.
- Eyerich S, Eyerich K, Traidl-Hoffmann C, Biedermann T. Cutaneous Barriers and Skin Immunity: Differentiating A Connected Network. *Trends Immunol*. 2018;39(4):315–327.
- Szabó K, Bolla BS, Erdei L, Balogh F, Kemény L. Are the Cutaneous Microbiota a Guardian of the Skin's Physical Barrier? The Intricate Relationship between Skin Microbes and Barrier Integrity. *Int J Mol Sci*. 2023;24(21).
- Dajnoki Z, Kapitány A, Eyerich K, Eyerich S, Töröcsik D, et al. Topographical variations in the skin barrier and their role in disease pathogenesis. *J Europ Acad Dermatol Venereol JEADV*. 2024;00:1–11.
- Lovászi M, Szegedi A, Zouboulis CC, Töröcsik D. Sebaceous-immunobiology is orchestrated by sebum lipids. *Dermato-endocrinology*. 2017;9(1), e1375636.
- Mattii M, Lovászi M, Garzorz N, Atenhan A, Quaranta M, et al. Sebocytes contribute to skin inflammation by promoting the differentiation of T helper 17 cells. *Br J Dermatol*. 2018;178(3):722–730.
- Baurecht H, Rühlemann MC, Rodríguez E, Thielking F, Harder I, et al. Epidermal lipid composition, barrier integrity, and eczematous inflammation are associated with skin microbiome configuration. *J Allergy Clin Immunol*. 2018;141(5):1668–1676.e16.
- Pan Y, Tian T, Park CO, Lofthus SY, Mei S, et al. Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nature*. 2017;543(7644):252–256.
- Ohnemus U, Kohrmeyer K, Houdek P, Rohde H, Wladykowski E, et al. Regulation of epidermal tight-junctions (TJ) during infection with exfoliative toxin-negative *Staphylococcus* strains. *J Invest Dermatol*. 2008;128(4):906–916.
- Bäslér K, Galliano MF, Bergmann S, Rohde H, Wladykowski E, et al. Biphasic influence of *Staphylococcus aureus* on human epidermal tight junctions. *Ann N Y Acad Sci*. 2017;1405(1):53–70.
- Bouslimani A, Porto C, Rath CM, Wang M, Guo Y, et al. Molecular cartography of the human skin surface in 3D. *PNAS*. 2015;112(17):E2120–E2129.
- Byrd AL, Belkaid Y, Segre JA. The human skin microbiome. *Nat Rev Microbiol*. 2018;16(3):143–155.
- Findley K, Oh J, Yang J, Conlan S, Deming C, et al. Topographic diversity of fungal and bacterial communities in human skin. *Nature*. 2013;498(7454):367–370.
- Grice EA, Kong HH, Conlan S, Deming CB, Davis J, et al. Topographical and temporal diversity of the human skin microbiome. *Science (New York, NY)*. 2009;324(5931):1190–1192.
- Grice EA, Segre JA. The skin microbiome. *Nat Rev Microbiol*. 2011;9(4):244–253.
- Oh J, Byrd AL, Deming C, Conlan S, Kong HH, et al. Biogeography and individuality shape function in the human skin metagenome. *Nature*. 2014;514(7520):59–64.
- Béke G, Dajnoki Z, Kapitány A, Gáspár K, Medgyesi B, et al. Immunotopographical differences of human skin. *Front Immunol*. 2018;9:424.
- Jenei A, Dajnoki Z, Medgyesi B, Gáspár K, Béke G, et al. Apocrine gland-rich skin has a non-inflammatory IL-17-related immune milieu, that turns to inflammatory IL-17-mediated disease in hidradenitis suppurativa. *J Invest Dermatol*. 2019;139(4):964–968.
- Del Duca E, Pavel AB, Dubin C, Song T, Wallace EB, et al. Major differences in expression of inflammatory pathways in skin from different body sites of healthy individuals. *J Invest Dermatol*. 2019;139(10):2228–2232.e10.
- Szabó L, Dajnoki Z, Somogyi O, Gáspár K, Hendrik Z, et al. Cytokine profile of the epidermis is region specific and may determine the characteristics of inflammation. *Exp Dermatol*. 2023;32(7):1120–1131.
- Dajnoki Z, Béke G, Kapitány A, Mócsai G, Gáspár K, et al. Sebaceous Gland-Rich Skin Is Characterized by TSLP Expression and Distinct Immune Surveillance Which Is Disturbed in Rosacea. *J Invest Dermatol*. 2017;137(5):1114–1125.
- Kapitány A, Medgyesi B, Jenei A, Somogyi O, Szabó L, et al. Regional Differences in the Permeability Barrier of the Skin-Implications in Acantholytic Skin Diseases. *Int J Mol Sci*. 2021;22(19).
- Komatsu N, Tsai B, Sidiropoulos M, Saijoh K, Levesque MA, et al. Quantification of eight tissue kallikreins in the stratum corneum and sweat. *J Invest Dermatol*. 2006;126(4):925–929.
- Oh J, Byrd AL, Park M, Kong HH, Segre JA. Temporal Stability of the Human Skin Microbiome. *Cell*. 2016;165(4):854–866.
- Oh J, Conlan S, Polley EC, Segre JA, Kong HH. Shifts in human skin and nares microbiota of healthy children and adults. *Genome Med*. 2012;4(10):77.
- Park J, Schwardt NH, Jo JH, Zhang Z, Pillai V, et al. Shifts in the Skin Bacterial and Fungal Communities of Healthy Children Transitioning through Puberty. *J Invest Dermatol*. 2022;142(1):212–219.

27. Jo JH, Deming C, Kennedy EA, Conlan S, Polley EC, et al. Diverse Human Skin Fungal Communities in Children Converge in Adulthood. *J Invest Dermatol.* 2016; 136(12):2356–2363.
28. McGinley KJ, Webster GF, Ruggieri MR, Leyden JJ. Regional variations in density of cutaneous propionibacteria: correlation of *Propionibacterium acnes* populations with sebaceous secretion. *J Clin Microbiol.* 1980;12(5):672–675.
29. Pochi PE, Strauss JS, Downing DT. Age-related changes in sebaceous gland activity. *J Invest Dermatol.* 1979;73(1):108–111.
30. McAleer MA, Jakasa I, Raj N, O'Donnell CPF, Lane ME, et al. Early-life regional and temporal variation in filaggrin-derived natural moisturizing factor, filaggrin-processing enzyme activity, corneocyte phenotypes and plasmin activity: implications for atopic dermatitis. *Br J Dermatol.* 2018;179(2):431–441.
31. Stamatias GN, Roux PF, Boireau-Adamezyk E, Lboukili I, Oddos T. Skin maturation from birth to 10 years of age: Structure, function, composition and microbiome. *Exp Dermatol.* 2023;32(9):1420–1429.
32. Renert-Yuval Y, Del Duca E, Pavel AB, Fang M, Lefferdink R, et al. The molecular features of normal and atopic dermatitis skin in infants, children, adolescents, and adults. *J Allergy Clin Immunol.* 2021;148(1):148–163.
33. Trompette A, Ubags ND. Skin barrier immunology from early life to adulthood. *Mucosal Immunol.* 2023;16(2):194–207.
34. Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, et al. The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res.* 2023;51(D1): D638–D646.
35. Bogiatzi SI, Fernandez I, Bichet JC, Marloie-Provost MA, Volpe E, et al. Cutting edge: proinflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes. *J Immunol.* 2007;178(6): 3373–3377.
36. Visscher MO, Hu P, Carr AN, Bascom CC, Isfort RJ, et al. Newborn infant skin gene expression: Remarkable differences versus adults. *PLoS One.* 2021;16(10), e0258554.
37. Lovászi M, Mattii M, Eyerich K, Gácsi A, Csányi E, et al. Sebum lipids influence macrophage polarization and activation. *Br J Dermatol.* 2017;177(6):1671–1682.
38. Naik S, Bouladoux N, Linehan JL, Han SJ, Harrison OJ, et al. Commensal-dendritic-cell interaction specifies a unique protective skin immune signature. *Nature.* 2015; 520(7545):104–108.
39. Cotterill JA, Cunliffe WJ, Williamson B, Bulusu L. Age and sex variation in skin surface lipid composition and sebum excretion rate. *Br J Dermatol.* 1972;87(4): 333–340.
40. Giacomoni PU, Mammone T, Teri M. Gender-linked differences in human skin. *J Dermatol Sci.* 2009;55(3):144–149.
41. Szegei A, Dajnok Z, Bíró T, Kemény L, Töröcsik D. Acne: transient arrest in the homeostatic host-microbiota dialog? *Trends Immunol.* 2019;40(10):873–876.