

INVESTIGATIVE REPORT

Expression of Class II Cytokine Genes in Children's Skin

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Immune regulation of the skin plays an important role in susceptibility and development of illnesses. The aim of our study was to localise the interleukin (IL)-10 family of cytokines, in children's skin and to determine possible age-related differences in the expression level. The mRNA expression level of IL10, IL19, IL20, IL22, IL24, IL26, IL28B, IL29 and their receptors IL10RA, IL10RB, IL20RA, IL20RB, IL22RA1, IL22RA2, IL28RA was compared in skin biopsies of children and adults and in children's skin cells by quantitative real-time PCR (qRT-PCR). Immunohistochemistry was performed to confirm the qRT-PCR findings. We found age-related differences in the expression of IL10RB, IL20, IL20RA, IL22RA1, IL22RA2, IL26 and IL28RA genes. Cell type-dependent expression of IL10 family cytokines was apparent in the skin. In addition to previously known differences in systemic immunological response of adults and children, the present results reveal differences in immune profile of adult and juvenile skin. Key words: IL10 family cytokines; gene expression; skin; children's immune system.

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It is well known that during the first years of life, several changes occur leading to the formation of individual immune state. Transient fluctuations in the immunoglobulin levels are common until the end of the second year of life, but may be present even up to the 6th year of life (1, 2). The production level of cytokines such as interleukin (IL)1 α , IL1 β , and TNF α is lower, fluctuating or inadequate compared to adults (3), which explains the variability in the severity of infections in children and also the vulnerability to sepsis as a result of an overreaction of the immune system.

Comparative studies of the immune system of adults and children have usually been based on the analysis of blood. However, skin is the most effective barrier between the organism and the environment. During the first year of life, skin is in a state of active development. Compared to adults there are differences in microstruc-

ture density, cell size, epidermal layer thickness, dermal structure, and the density of papillae in the infant's skin (4). The features of immune mechanisms in the skin of children have so far been poorly studied and a few comparative gene expression studies have been made between the skin of adults and children (5, 6).

The IL10 family includes a number of cellular cytokines: IL10, IL19, IL20, IL22, IL24, IL26, IL28A, IL28B, and IL29. This classification is based on distinctive six-alpha-helix structure that is common to all mature IL10 family members. They also share up to 28% of their amino acid structure (7, 8). However, receptor-binding units of IL10 family cytokines are variable, defining interaction with different receptors (9, 10) (Table S1¹). The variability of binding sites may cause differences in the biological action of these ILs.

All receptors of IL10 family cytokines (IL10RA, IL10RB, IL20RA, IL20RB, IL22RA1, IL22RA2 and IL28RA) belong to class II cytokine receptor family (11, 12) commonly composed of ligand-binding alpha subunit and signal-transducing beta or gamma chain subunits.

The IL10 family of cytokines are responsible for host defense mechanisms, they can improve the healing process in injuries, limit infection-caused damages or modify inflammation by promoting innate immune responses in epithelial tissue. A number of IL10 family cytokines have both pro-inflammatory and anti-inflammatory roles (13, 14) making the investigation of their individual functions quite complicated.

Recent results suggest that the IL10 family of cytokines are involved in the function of skin as well as in the pathogenesis of major skin diseases (e.g. psoriasis) (15, 16). Our group has described the expression profile differences of the IL10 family of cytokines in whole skin and blood samples of vitiligo patients, compared to healthy controls (17). In order to describe differences in the mRNA expression of IL10 family interleukins in the skin of adults and children we compared gene expression levels in whole skin samples of healthy subjects. To confirm our results, we also analysed samples for the expression of respective proteins in adults' and children's whole skin. In addition, gene expression was

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analysed in each of the 3 main cell types (keratinocytes, melanocytes and fibroblasts) of the skin of healthy infants. Taken together, the aim of our study was to analyse the IL10 family cytokine expression in the skin and to determine possible age-related differences in the expression level.

MATERIALS AND METHODS

A written informed consent was obtained from all patients. All procedures were carried out in accordance with the ethical standards of the Research Ethics Committee of the University of Tartu.

Subjects

We had 5 different sets of samples to measure gene expression: adults' whole skin, children's whole skin, and children's keratinocytes, fibroblasts and melanocytes.

We isolated keratinocytes, melanocytes and fibroblasts from 15 paediatric foreskins of healthy circumcised patients (aged 5 months to 10 years) and measured gene expression from the established cell cultures. The same paediatric samples were used to measure gene expression in children's whole skin. The children were recruited from elective patients present at the Department of Pediatric Surgery, Tallinn Children's Hospital, with no concurrent diseases and signs of infection. Skin tissue was divided into two parts. A tissue sized 4 mm was transferred into RNALater (Life Technologies Co, USA) for RNA extraction and the remaining tissue part into 0.9% NaCl solution for cell culture. Existing protocols of isolating and growing skin cells were used (18).

Punch-biopsies of 4 mm were taken from 15 healthy adult volunteers (aged 19 to 79 years) without known skin diseases from non sun-exposed areas. Healthy adult volunteers were recruited from patients attending the outpatient clinic of the Department of Dermatology of the University of Tartu. Biopsies were instantly snap-frozen in liquid nitrogen and stored at -80°C until used for RNA extraction.

Cell culture

Skin pieces from juvenile skin were rinsed in phosphate-buffered saline (PBS w/o Ca, Mg, PAA Laboratories GmbH, Pasching, Austria). Subcutaneous fat was removed and tissue was incubated in dispase II diluted in PBS (2.4 U/ml, Sigma-Aldrich, Munich, Germany) at $+4^{\circ}\text{C}$ overnight. Epidermis was peeled off from the dermis, transferred into 0.05% trypsin/0.02% EDTA (Life Technologies Co, USA) for 3 min at 37°C . Enzymatic process was stopped with trypsin inhibitor (Sigma-Aldrich). We used cell-specific selective media to isolate a particular cell type culture: EpiLife[®] basal medium with human keratinocyte growth supplement (Life Technologies Co, USA) and melanocyte growth medium M2 with supplement mix (PromoCell, Heidelberg, Germany). Melanocyte culture dishes were precoated with gelatin.

A piece of dermis was used for isolation of fibroblasts by migration method. The dermis was rinsed in PBS, cut into 4×4 mm pieces and attached onto a culture dish, covered with 10 ml of DMEM (PAA Laboratories GmbH, Austria) with 10% foetal bovine serum (Sigma-Aldrich), penicillin (100 UI/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) (PAA Laboratories GmbH, Cölbe, Germany) and amphotericin B 250 ng/ml. The medium was changed every 2nd day throughout the study.

Subculture (passage) of cells was done with 65–90% cellular confluence in the Petri dish maximum 3 times. Cells with passage No. 2–3, were used for subsequent isolation of RNA.

Lipopolysaccharide stimulation

Reaching approximately 90% confluence, melanocytes and fibroblasts were incubated with media alone (controls) and lipopolysaccharide (LPS) (*E. coli* 0111:B4, Sigma-Aldrich) with 10 ng/ml for 12 h. After treatment, the cells were washed with PBS and used for isolation of RNA for qRT-PCR.

RNA extraction

The skin biopsies were homogenised using T10 basic homogeniser (IKA Labortechnik, Staufen, Germany) and total RNA was isolated from tissues with RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia CA, USA). The RNA from cells was extracted using Trizol[®] reagent (Life Technologies Co, USA) RNeasy mini columns (Qiagen) combined protocol. The RNA content was determined by spectrophotometry and 500 ng of each sample was used to synthesise cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies Co) according to the manufacturer's protocol.

Quantitative real-time PCR

RNA expression was observed using qRT-PCR (7900 Fast QRT-PCR, Life Technologies Co). Two primers and labelled probe were used to detect the mRNA expression level of the reference gene hypoxanthine phosphoribosyl-transferase-1 (HPRT-1) (primer sequences available upon request). Expression levels of other genes under investigation were detected using following 20x probe assays (Life Technologies Co): IL10 (Hs00174086_m1), IL10RA (Hs00155485_m1), IL10RB (Hs00175123_m1), IL19 (Hs00203540_m1), IL20 (Hs00218888_m1), IL20RA (Hs00205346_m1), IL20RB (Hs00376373_m1), IL22RA1 (Hs00222035_m1), IL22RA2 (Hs00364814_m1), IL24 (Hs00169533_m1), IL26 (Hs00218189_m1), IL29 (Hs00601677_g1), IL28RA (Hs00417120_m1).

Statistical analysis

The relative gene expression levels were calculated using $2^{-\Delta\text{CT}}$ method utilising the housekeeping gene *HPRT-1* as an internal control (19).

The data of all studied genes that followed normal distribution (using D'Agostino & Pearson omnibus normality test) were parametrically tested by unpaired *t*-test and the data not following the normal distribution by Mann-Whitney *t*-test.

Immunohistochemistry

Skin pieces from adults and children ($n=5$ in both group) for immunohistochemistry (IHC) were fixed immediately after excision in 10% formaline for 24 h and paraffinised. Deparaffinised sections were treated with 3% H_2O_2 followed by Dako REAL Antibody Diluent (Dako Denmark A/S, Glostrup, Denmark) to block non-specific binding. After blocking, sections were incubated with rabbit polyclonal antibody to IL29 (ab38569) 1:100, IL28 receptor alpha (ab83865) 1:200, IL22 RA2 (ab96341) 1:500, IL26 (ab102977) 1:200 or IL10RB (ab106282) 1:200 overnight at 4°C (all antibodies were purchased from Abcam Ltd., Cambridge, UK). Visualisation of the primary antibodies was performed by using Dako REAL[™] EnVision[™] Detection System (Dako Denmark A/S). The washing steps were carried out with PBS containing 0.07% of Tween 20. Thionine blue (Sigma-Aldrich) was used for background staining. No immunohistochemical staining was noted in negative controls where the primary antibody was omitted.

RESULTS

We discovered that IL10 is expressed similarly in children's and adults' skin (Fig. 1a), but the expression in the juvenile skin cell cultures was undetectable (data not shown). IL10RA was equally detectable in children's as well as in adults' skin (Fig. 1b). In cell cultures IL10RA expression was similar in the melanocytes and fibroblasts of adults and children while in keratinocytes the expression level was below the detection limit (Fig. 2a). IL10RB expression analysis revealed a statistical difference between children's and adults' skin, the latter being much higher (Fig. 1c). This was also confirmed by IHC, which revealed clearly higher immunoreactivity in adults' skin (Fig. 3 a, g). High IL10RB immunoreactivity was found in all layers of adults' skin. These findings were corroborated by mRNA expression studies in juvenile cell culture demonstrating similar expression level of IL10RB in all studied cell types (Fig. 2b).

In children, IL20 expression was highly variable, whereas in adults' skin it was undetectable (Fig. 1d). In cell cultures, IL20 expression could be observed only in keratinocytes (Fig. 2c).

IL20RA was expressed higher in adults' skin compared with children's whole skin ($p=0.0015$, Mann Whitney test) (Fig. 1e). The expression level of IL20RA was significantly higher in juvenile keratinocytes than in fibroblasts and absent in melanocytes (Fig. 2d). IL20RB

expression level was similarly high in children's and adults' skin (Fig. 1f). In cell culture from children, IL20RB expression was the highest in keratinocytes compared to being barely detectable in melanocytes and fibroblasts (Fig. 2e).

Both IL22RA1 and IL22RA2 expression levels were significantly higher in children's skin ($p<0.0001$, unpaired *t*-test) (Fig. 1g, h). In cell culture, the expression level of IL22RA1 was similar in keratinocytes and fibroblasts, whereas no expression could be detected in melanocytes (Fig. 2f). No expression of IL22RA2 (as well as IL10) could be detected in these 3 cell cultures (data not shown). No IL22RA2 antibody staining was detected by IHC (Fig. 3b, h).

IL24 was not detectable in children's and adults' whole skin (data not shown), however, it became detectable after seeding the cells into cell cultures. The expression level of IL24 was the highest in melanocytes compared to keratinocytes and the weakest in fibroblasts (Fig. 2g). Notably, IL24 was the only studied gene induced by LPS stimulation in cell culture, increasing up to 10 times in melanocytes (Fig. 2g).

The expression of IL26 was low and relatively variable in children's skin, whereas in adults, no IL26 gene expression could be detected (Fig. 1i), nor was IL26 detected in children's skin cells (data not shown). IHC revealed no observable IL26 antibody staining (Fig. 3c, i).

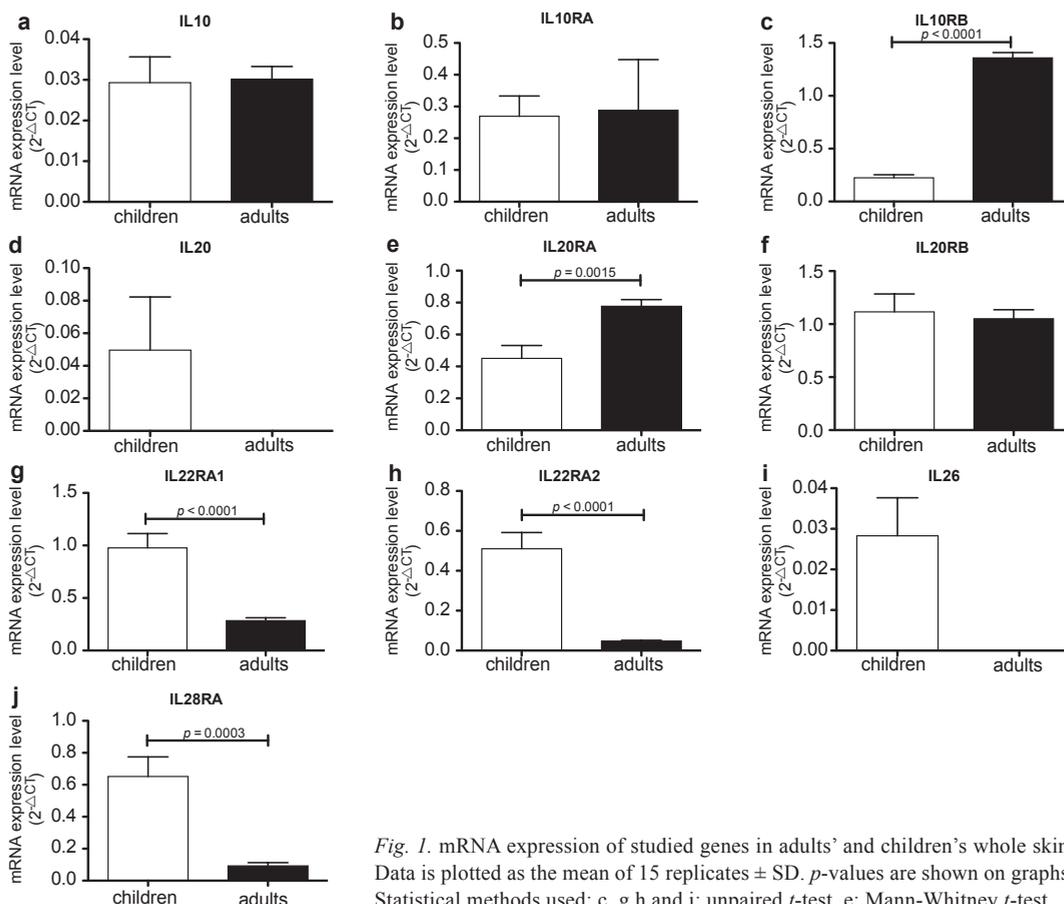


Fig. 1. mRNA expression of studied genes in adults' and children's whole skin. Data is plotted as the mean of 15 replicates \pm SD. *p*-values are shown on graphs. Statistical methods used: c, g and j: unpaired *t*-test. e: Mann-Whitney *t*-test.

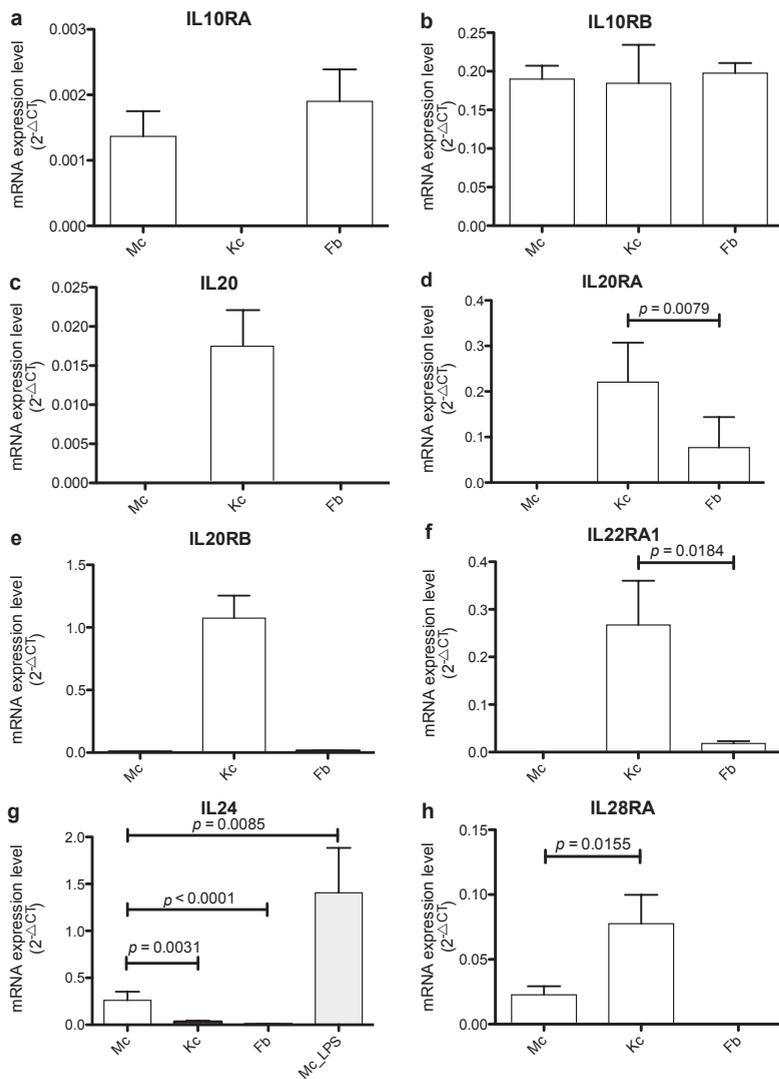


Fig. 2. mRNA expression of studied genes in children's skin cells. Melanocytes (Mc), keratinocytes (Kc), fibroblasts (Fb), LPS stimulated melanocytes (Mc_LPS). Data is plotted as the mean \pm SD of 15 replicates. *p*-values are shown on graphs. Statistical methods used: d, g, h: Mann-Whitney *t*-test, f: Unpaired *t*-test.

The expression of *IL28RA* gene was much higher in children's skin than in adults' skin (Fig. 1j). *IL28RA* protein staining was not significant in children's skin (Fig. 3d). In adults' skin IHC analysis revealed a *IL28RA* positive staining in epidermis (Fig. 3j). In the juvenile cell culture, *IL28RA* gene expression was the highest in keratinocytes but no expression was detectable in fibroblasts (Fig. 2h).

An overview of the expression levels of *IL10* family genes in adults' and children's whole skin and in children's skin cells is presented in Table I. Expression of *IL19*, *IL22*, *IL28B* and *IL29* could not be detected in neither whole skin nor in cell culture.

DISCUSSION

We found that the majority of the genes of *IL10* family and their receptors were detectable in children's

skin (*IL10*, *IL10RA*, *IL10RB*, *IL20*, *IL20RA*, *IL20RB*, *IL22RA1*, *IL22RA2*, *IL26*, *IL28RA*). On the other hand, 50% of the genes expressed in children's skin were downregulated (*IL22RA1*, *IL22RA2*, *IL28RA*) or absent (*IL20*, *IL26*) in adults (Table I). The results refer to the immaturity of the children's immune system. Differences in innate immunity function, an amount of antibody production (20) and qualitative differences in blood cytokine profile (21–24) during early life versus adulthood have been described previously. Our present study demonstrates an age-dependent effect on cytokines also in skin tissue, based on RNA quantification and protein (IHC) staining levels. Gene expression data were verified by whole transcriptome sequencing analysis, SOLiD™ (Reemann et al., unpublished data). In some cases RNA and protein expressions did not correlate. The reason for this discrepancy is not known, but different regulatory mechanisms for RNA and protein expression may play a potential role. For several genes, for example *IL10RB*, we observed a significantly weaker or missing antibody reaction in children's samples compared to adults' (Fig. 3a). Together with *IL10RA*, *IL10RB* has been shown to be required for *IL10*-induced signal transduction and therefore may regulate susceptibility to different kinds of illnesses. For example mutations in *IL10R* genes cause severe and early childhood onset of the inflammatory bowel disease (25).

We found that interleukin receptors are more prominently expressed in the skin than interleukins (Fig. 1). This indicates that skin cells are targets for circulating cytokines. There were also differences in expression of receptor subunits. Interestingly, the expression level of receptor genes correlated with the overall prevalence and selectivity of respective subunits. For example *IL10RA* is unique to *IL10R* whereas *IL10RB* is shared by several other cytokines, including *IL22*, *IL26*, and *IL28A*, *IL28RB* and *IL29* (Table SI¹).

Concurrently, there was a group of genes of the *IL10* family (*IL19*, *IL22*, *IL28B*, *IL29*), which have been shown to play a role in different skin disorders, such as psoriasis and infections (26–28), but in healthy adults' and children's skin and cells their expression was below the detection limit (Table I). It indicates that these cytokines are significantly expressed only in pathological conditions. For example *IL28B* and *IL29*,

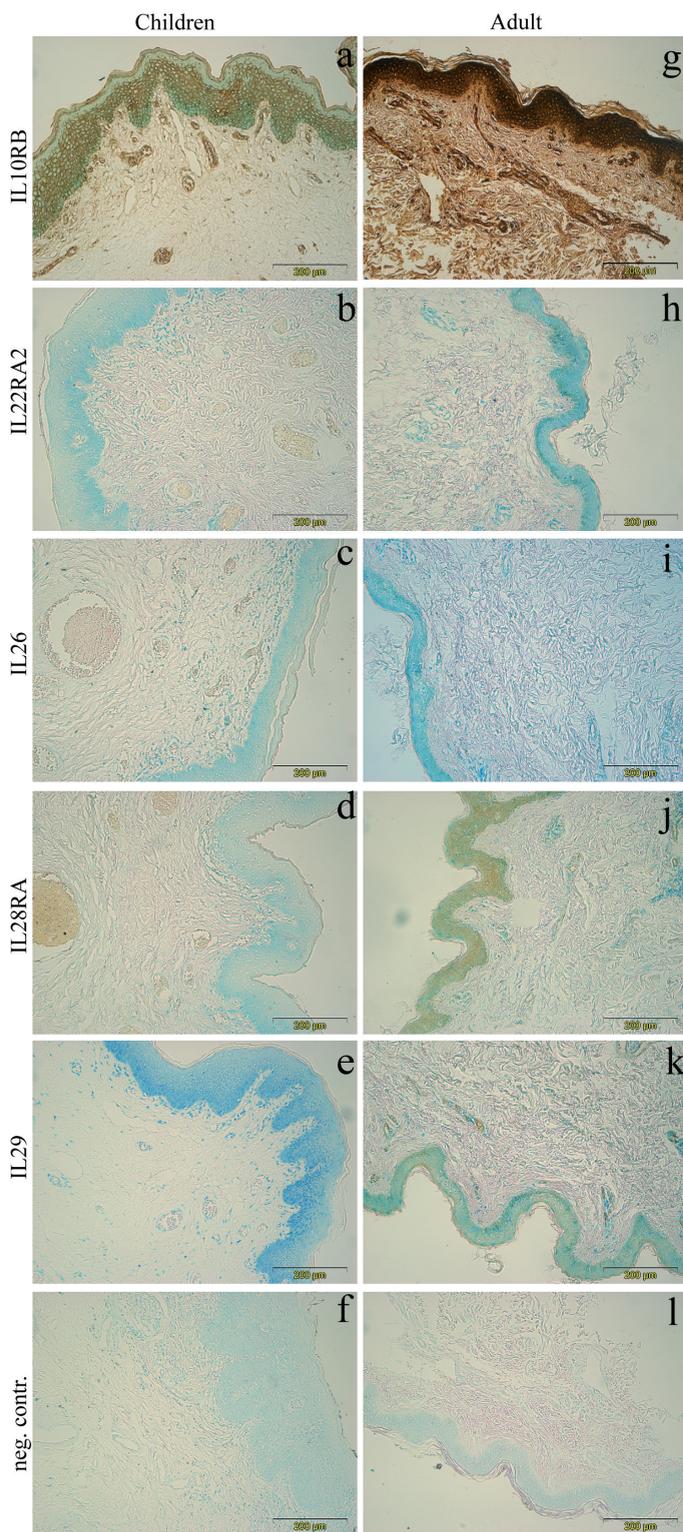


Fig. 3. Immunohistochemistry (IHC) staining of children's and adult's whole skin sample. Skin specimens ($n=5$, both in children's (a–f) and adult's (g–l) group) were stained with (blue background staining) and the antibodies for IL10RB, IL22RA2, IL26, IL28RA, IL29 (brown colour indicates positive antibody reaction); IL10RB (a, g) antibody staining was present in all layers of adults' and juvenile skin, but a significantly weaker antibody reaction in children's samples were observable. We detected IL28RA positive antibody reaction (d, j) in adults' epidermis, but no significant staining in children's skin. IHC revealed no observable antibody staining of IL22RA2, IL26 and IL29. Negative control for antibody staining shown in (f, l). Scale bars 200 μ m.

Table I. Overview of the expression levels of interleukin (IL)-10 family genes in adults' and children's whole skin, as well as in children's skin cells

Genes	Children	Adults	Kc	Mc	Fb
<i>IL10</i>	++	++	-	-	-
<i>IL10RA</i>	+++	+++	-	+	+
<i>IL10RB</i>	+++	++++	+++	+++	+++
<i>IL19</i>	-	-	-	-	-
<i>IL20</i>	++	-	++	-	-
<i>IL20RA</i>	+++	+++	+++	-	++
<i>IL20RB</i>	++++	++++	++++	+	++
<i>IL22</i>	-	-	-	-	-
<i>IL22RA1</i>	++++	+++	+++	-	++
<i>IL22RA2</i>	+++	++	-	-	-
<i>IL24</i>	-	-	++	+++	+
<i>IL26</i>	++	-	-	-	-
<i>IL28RA</i>	+++	++	++	++	-
<i>IL28B</i>	-	-	-	-	-
<i>IL29</i>	-	-	-	-	-

Kc: keratinocytes; Mc: melanocytes; Fb: fibroblasts; -: undetectable expression; +: mean $2^{-\Delta\text{CT}}$ 0.001–0.009; ++: mean $2^{-\Delta\text{CT}}$ 0.01–0.09; +++: mean $2^{-\Delta\text{CT}} \geq 0.1$ –0.9; ++++: mean $2^{-\Delta\text{CT}} \geq 1$.

the new interferon-like cytokines in the IL10 family, have been shown to exert their anti-viral, anti-proliferative, anti-tumour activity via the IL28RA and IL10RB receptor complex (9, 29). IL28 and IL29 can act synergistically with IL20 (28), which is one of the most investigated potential target cytokines for psoriasis treatment (30). Correlating with previous studies (31), we found IL20 to be absent in healthy adults' skin (Fig. 1d). The reason why IL20 expression was quite fluctuating but still measurable in children's whole skin and keratinocytes (Fig. 2c), might be connected to higher cell proliferation capacity of cell culture as well as juvenile skin.

In order to localise gene expression of studied cytokines we analysed 3 cell types in children's skin, forming the majority of cellular component in the epidermis and dermis (melanocytes, keratinocytes and fibroblasts). We found that genes, which were not detectable in whole skin of both children and adults (*IL19*, *IL22*, *IL28B* and *IL29*) (Fig. 1), were also not detectable in cell cultures (Fig. 2). *IL24* can be pointed out as an exception. *IL24*, which was highly expressed in melanocytes, was not detectable in the children's and adults' whole skin. Compared to fibroblasts and keratinocytes, the number of melanocytes is very small in a skin biopsy and therefore in monoculture their characteristics are more easily observed. *IL24* has been shown to induce growth arrest and apoptosis in melanoma cells (32), but is also considered an important mediator for chronic inflammatory conditions, e.g. psoriasis (33).

From a methodologic point of view we have to take into account that despite using low-passage cell culture, gene expression could still be influenced by *in vitro* culture conditions (34, 35). This could be an issue in the case of *IL10*, *IL22RA2* and *IL26*

where gene expression was observed in whole skin, but absent in cell culture. Also, mRNA synthesis could occur somewhere else than in keratinocytes, melanocytes and fibroblasts. For instance, *IL26* and a soluble receptor *IL22RA2*, which is considered to be stored in the extracellular matrix for on demand releasing (36) are produced mainly by resident T cells (37, 38). Although both subunits of *IL-22R* complex and *IL10RB* are required to form the functional receptor (Table S1¹), *IL22RA2* is able to bind alone to *IL22* (39). Unlike *IL22RA2*, which is strictly an antagonist for *IL22* activity, *IL22RA1* regulates both *IL22* and *IL20* activity (Table S1¹) and its expression was detectable especially in keratinocytes (Fig. 2f).

To see whether we could upregulate *IL10* family cytokines genes and cause an inflammation-like state also *in vitro*, we performed LPS stimulation. LPS, a major component of the outer membrane of Gram-negative bacteria (40) and a strong inflammatory agent, promotes the secretion of proinflammatory cytokines such as *IL1 β* , tumour necrosis factor α (41) and proinflammatory cytokines limiting *IL10* (42) from cells. An interesting exception came out in our study, as *IL24* was the only studied gene the expression level which changed statistically significantly in response to stimulation with LPS and that occurred only in children's melanocyte culture (Fig. 2 g). This finding suggests that melanocytes can react to the inflammatory signals and are able to produce cytokines, which in turn suggests they play a role in immune defense mechanisms. The reason why other studied interleukins did not respond to stimulation could be explained by the previous finding that neonatal cells tend to have notably lower cytokine production in response to LPS compared to adults (21, 43).

We believe that a major value of this study lies in finding that gene expression differs in adults' and children's skin and the reason for this could be the immaturity of the organism's defense mechanisms as a result of which the reaction to harmful influences, such as inflammation and infection, differs. This could also explain why several childhood skin diseases go into remission, disappear or acquire a different appearance in adult age (e.g. atopic dermatitis, juvenile dermatomyositis and cutaneous mastocytosis).

From a clinical point of view the comprehension of the physiological state of the *IL10* family of cytokines would be helpful in developing routine laboratory techniques for both diagnostics as well as for evaluating the effectiveness of treatments. It is also important for establishing normal ranges of tests for each age group.

Analysing skin cells *in vitro*, we found cell-specific cytokine production, which helps to explain the roles of different cells in inflammatory system. Juvenile skin cells, due to their excellent proliferative capacity, are mostly used for tissue-engineered products. Thus, from a future research perspective it is crucial to understand

cell behaviour in an artificial environment, especially pro- and antiinflammatory markers, which play a role in tissue rejection process.

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The authors declare no conflict of interest.

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