

Age-related changes in expression and function of Toll-like receptors in human skin

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SUMMARY

Toll-like receptors (TLRs) initiate innate immune responses and direct subsequent adaptive immunity. They play a major role in cutaneous host defense against micro-organisms and in the pathophysiology of several inflammatory skin diseases. To understand the role of TLRs in the acquisition of immunological competence, we conducted a comprehensive study to evaluate TLR expression and function in the developing human skin before and after birth and compared it with adults. We found that prenatal skin already expresses the same spectrum of TLRs as adult skin. Strikingly, many TLRs were significantly higher expressed in prenatal (TLRs 1-5) and infant and child (TLRs 1 and 3) skin than in adult skin. Surprisingly, neither dendritic cell precursors in prenatal skin nor epidermal Langerhans cells and dermal dendritic cells in adult skin expressed TLRs 3 and 6, whereas the staining pattern and intensity of both TLRs in fetal basal keratinocytes was almost comparable to those of adults. Stimulation of primary human keratinocytes from fetal, neonatal and adult donors with selected TLR agonists revealed that the synthetic TLR3 ligand poly (I:C) specifically, mimicking viral double-stranded RNA, induced a significantly enhanced secretion of CXCL8/IL8, CXCL10/IP-10 and TNF α in fetal and neonatal keratinocytes compared with adult keratinocytes. This study demonstrates quantitative age-specific modifications in TLR expression and innate skin immune reactivity in response to TLR activation. Thus, antiviral innate immunity already in prenatal skin may contribute to protect the developing human body from viral infections in utero in a scenario where the adaptive immune system is not yet fully functional.

KEY WORDS: Toll-like receptor, Skin, Keratinocyte, Innate immune system, Dendritic cell, Expression, Function, Human

INTRODUCTION

The skin is the first line of defense, protecting the body from infections caused by microbial pathogens by employing innate arms of the immune system that are linked to the initiation of adaptive immune responses. A major part of innate protection is the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) that in humans comprise Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG I)-like receptors (RLRs) and C-type lectin receptors (CLRs) (Kumar et al., 2009). The ligation of PRRs by PAMPs results in an inflammatory response leading to efficient destruction of the invading pathogens (Medzhitov and Janeway, 1997). To accomplish these functions in skin, resident immune system cells [e.g. epidermal Langerhans cells (LCs), dermal dendritic cells (DDCs) (Flacher et al., 2006; van der Aar et al., 2007) and mast cells (Kulka and Metcalfe, 2006)] as well as nonimmune system

cells [e.g. keratinocytes (Miller and Modlin, 2007), melanocytes (Yu et al., 2009), endothelial cells of the microvasculature (Fitzner et al., 2008), stromal cells such as fibroblasts (Proost et al., 2003) and adipocytes (Creely et al., 2007; Vitseva et al., 2008)] are endowed with an array of PRRs. Pathogenic micro-organisms in the skin can also be killed through antimicrobial peptides such as β -defensins and cathelicidins, which are either constitutively produced by keratinocytes or induced after an inflammatory response or direct activation of PRRs by microbial components (Hata and Gallo, 2008).

TLRs recognize pathogens at the cell surface (TLRs 1, 2, 4-6, 10) or within the endosome (TLRs 3, 7-9), whereas NLRs and RLRs act as intracellular surveillance molecules (Kumar et al., 2009). To date, ten functional human TLRs (Kawai and Akira, 2010) and 23 members of the human NLR protein family have been reported (Franchi et al., 2009). Recent findings suggest that NLRs not only function in pathogen recognition but also play a role in tissue homeostasis, apoptosis and graft-versus-host disease (Kufer and Sansonetti, 2011). The RLR family includes three members [RIG I, melanoma-differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2)] (Kawai and Akira, 2010). RLRs are crucial for host antiviral infections and sense double-stranded (ds) RNA. Viral infections are common in human skin, with keratinocytes as viral targets. Therefore, the capacity to induce a sufficient antiviral response seems to be crucial during the earliest phases of the innate immune response.

Keratinocytes in vitro respond very well to poly (I:C), a synthetic ds RNA analog, which occurs as a metabolite during viral infection. The corresponding receptors are TLR3 and MDA5, which are expressed in multiple cell types including keratinocytes (Kalali et al., 2008; Köllisch et al., 2005; Lebre et al., 2003; Mempel et al., 2003).

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Activation of TLR3 either directly leads to NF- κ B activation and upregulation of NF- κ B-responsive proteins such as CXCL8/IL8, or trigger TRIF activation that ultimately stimulates, independently of MyD88, the IRF3 transcription factor through TANK-binding kinase-1 (TBK1), followed by the production of type I interferons (IFNs) and the expression of proinflammatory cytokines (Jiang et al., 2004; Sankar et al., 2006; Yamamoto et al., 2003). In addition to MDA5/RIGI-mediated signaling, an antiviral defense status in keratinocytes can also be induced by Protein kinase R (PKR) in keratinocytes (Kalali et al., 2008). Thus, keratinocytes are equipped with a broad antiviral defense program enabling them to efficiently target viral infections of the skin.

The fetal innate immune system is fundamentally different from that observed in term neonates or adults (Strunk et al., 2004). The acquisition of immunological competence during ontogeny could partly be explained by the sequential expression of PRRs. Evidence for the validity of this assumption comes from a study demonstrating a distinct ontogeny in the responsiveness of fetal mouse lung to in utero administered lipopolysaccharide (LPS) which is recognized by TLR4, MD2 and CD14 (Harju et al., 2005). With regard to skin, quantification of PRR transcript expression revealed that fetal sheep skin expresses the same spectrum of PRRs as the adult but that the level of expression for most PRRs is age-dependent (Nalubamba et al., 2008), implying that the prenatal skin may in certain aspects be as capable as that of the adults to respond to TLR stimulation but responds in a qualitatively different manner. Indeed, data provided recently contradict the notion of a linear progression from an 'immature' neonatal to a 'mature' adult pattern. Corbett and colleagues provided evidence that age-specific regulatory mechanisms are in place governing the TLR response by human antigen-presenting cell subsets (Corbett et al., 2010). It is conceivable that these differences together with other factors may relate to the observed neonatal infections and suboptimal immune responses to vaccines when administered around birth (Levy, 2007; Philbin and Levy, 2009; Siegrist, 2001).

Although the incidence of infections is highest in the neonatal period, infants continue to remain at elevated risk for acute bacterial infections for several years (Donald et al., 2010). Besides, most vaccines are not administered around birth, but over the first few years of life (Siegrist, 2001). Thus, a better understanding of the ontogeny of the innate immune system is needed. In the current study we aimed to profile the TLR expression pattern of human skin cells during ontogeny as epithelial surfaces are engaged in the primary interactions between host and pathogen in various situations. We tested the human skin innate immune response to well-defined TLR ligands starting at a prenatal stage and continuing beyond the neonatal period and compared it to adults.

MATERIALS AND METHODS

Skin samples

Embryonic [estimated gestational age (EGA) of 9-11 weeks] and fetal (EGA 12-13 weeks) human trunk skin specimens were obtained after legal termination of pregnancy. Human foreskin samples from selected age groups [neonates, infants (3-10 months), children (6-12 years) and adults (18-31 years)] were obtained as discarded material from routine circumcisions. Healthy adult (18-45 years) abdominal, back and breast skin was collected after plastic surgery. Sample numbers per group are indicated in the respective figure legends. The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki principles. Participants/parents gave their written informed consent.

Skin preparation and dermal-epidermal separation

Skin samples were refrigerated and processed no longer than 4 hours after surgery/abortion. Embryonic and fetal skin was dissected using an inverted

binocular microscope (Wild M3Z, Heerbrugg, Switzerland). For certain experiments it was necessary to separate epidermis from dermis. After removal of subcutaneous tissue, skin (with the exception of prenatal skin, for which it is not possible to separate epidermis from dermis efficiently using common protocols) was cut into ($\sim 5 \times 5$ mm²) small pieces and incubated on 3.8% ammonium thiocyanate (Merck, Whitehouse Station, NJ, USA) as described previously (Trost et al., 2007) dissolved in Dulbecco's phosphate-buffered saline (PBS, pH 7.4; Invitrogen, Carlsbad, CA, USA) at room temperature for 60 minutes. Epidermal sheets were then peeled from the dermis using sterile forceps. Whole skin, epidermal and dermal sheets were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction or for immunostaining purposes immediately fixed in acetone for 10 minutes and subsequently stained as indicated below.

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (RT-PCR)

Tissue was minced using a homogenizer (Ultra Turex T25, IKA Works, Inc., Wilmington, NC, USA). Subsequently, total RNA was extracted using a Trizol reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. RNA was solubilized in diethyl pyrocarbonate-treated water (Invitrogen), incubated at 55°C for 10 minutes and stored at -80°C until further use. Optical density was measured using a UV/Vis scanning spectrophotometer (DU series 700 Beckman Coulter Inc., Fullerton, CA, USA) and confirmed using a NanoDrop Spectrophotometer 2000c (Thermo Scientific, Waltham, MA, USA) indicating RNA yield and quality in each sample. Total RNA (3 μg) was reverse transcribed into cDNA with a superscript first-strand synthesis system for RT-PCR using Oligo (dt)¹²⁻¹⁸ primers (0.5 $\mu\text{g}/\mu\text{l}$) according to the manufacturer's instructions (Invitrogen). Quantitative RT-PCR was performed with Applied Biosystems Step One Plus Systems using predesigned exon-overlapping TaqMan gene expression assays (Applied Biosystems, Waltham, MA, USA) specific for human TLR1 (Hs00413978_m1), TLR2 (Hs00610101_m1), TLR3 (Hs00152933_m1), TLR4 (Hs00152937_m1), TLR5 (Hs00152825_m1), TLR6 (Hs00271977_s1), TLR7 (Hs00152971_m1), TLR8 (Hs00152972_m1), TLR9 (Hs00152973_m1) and MD2 (Hs00209771_m1). As a control, RNA samples not subjected to reverse transcriptase were analysed to exclude unspecific signals arising from genomic DNA. Input cDNA in each well was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905_m1) as an internal control in each reaction for all foreskin samples and Beta-2 microglobulin (B2M, Hs00187842_m1) as housekeeping gene for embryonic, fetal and adult skin samples. The final concentration of primers and minor groove binder probes (MGB) in the TaqMan Universal PCR Master Mix (Applied Biosystems) for each gene was 900 nM and 250 nM, respectively. Amplification was performed using the following cycling conditions: 2 minutes at 50°C , 10 minutes at 95°C and 40 two-step cycles of 15 seconds at 95°C and 60 seconds at 60°C . The specificity of each primer was confirmed by running PCR products on 1.5% agarose gel (Invitrogen). Data were analysed using step one plus 2.1 software (Applied Biosystems). Relative mRNA expression was calculated using the formula $2^{-\Delta\Delta\text{Ct}}$ as described previously (Pfaffl, 2001).

Immunofluorescence

Staining of cryostat sections

Whole skin specimens were embedded in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek, Zoeterwoude, The Netherlands), snap frozen in liquid nitrogen and stored at -80°C until further processing. Sections (5 μm) were cut, transferred onto capillary gap microscope slides (Dakocytomation, Glostrup, Denmark), fixed in ice-cold acetone for 10 minutes and air dried. Subsequently, sections were stained with the unconjugated primary antibodies anti-CD1c (AbD Serotec, Oxford, UK) or anti-keratin 10 (K10; Thermo Fisher Scientific, Manor Park, Runcorn, UK) overnight at 4°C . To visualize CD1c, sections were incubated with biotin-conjugated goat anti-mouse IgG (Elite mouse IgG Vectastain kit, Vector Laboratories, Burlingame, CA, USA) followed by Streptavidin-FITC (BD Biosciences, Franklin Lakes, NJ, USA) (each 60 minutes at room temperature). K10 was detected

with goat anti-mouse Alexa Fluor 488 (Invitrogen). Subsequently, sections were blocked with 10% goat serum (Vector Laboratories) for 60 minutes at room temperature and stained with anti-TLR 3 and 6 antibodies (Promokine, Heidelberg, Germany) overnight at 4°C. Goat anti-rabbit Alexa Fluor 546 (Invitrogen) was used to visualize TLR staining.

Staining of skin equivalents

Formalin-fixed, paraffin-embedded sections were deparaffinized and stained with anti-K10 (Thermo Fisher Scientific) and anti-Loricrin (Covance, Berkeley, CA, USA) antibodies essentially as described (Mildner et al., 2010).

Staining of epidermal sheets

Fixed epidermal sheets were incubated overnight at 4°C with the primary anti-TLR 3 and 6 antibodies (Promokine) and Alexa Fluor 488 anti-CD207/Langerin (Dendritics, Lyon, France) and subsequently counterstained with goat anti-rabbit Alexa Fluor 546 (Invitrogen) at room temperature for 2 hours.

Appropriate isotype controls (BD Biosciences) and rabbit control serum (Vector Laboratories) were included. Nuclear counterstain was performed either with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) or Hoechst dye (Dako, Glostrup, Denmark) followed by mounting with Fluorep (BioMerieux, Marcy l'Etoile, France). Images were recorded using conventional immunofluorescence microscopes (Olympus AX70, Hamburg, Germany; Axio Imager, Carl Zeiss, Göttingen, Germany).

Cell culture and cytokine analysis

Human dermal fibroblasts were obtained from Cascade Inc. (Portland, OR, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, PAA, Linz, Austria) supplemented with 10% fetal bovine serum (FBS, PAA) and 1% penicillin/streptomycin (Gibco). Second passage primary keratinocytes from fetal (20-23 weeks EGA; $n=3$; tebu-bio, Offenbach, Germany), neonatal (3-4 days after birth; $n=3$; Lonza, Basel, Switzerland) and adult skin (19-37 years; $n=3$; Cell Systems, Troisdorf, Germany) were cultured in 12-well-plates (Costar, Cambridge, MA, USA) and grown to approximately 75% confluence in a serum-free, keratinocyte growth medium (KGM, Lonza) at 37°C and 5% CO₂. Subsequent stimulation of cells was performed with the following heat-inactivated bacterial strains and TLR ligands (Invivogen, San Diego, CA, USA): *Escherichia coli* (*E. coli*; NK 9373) and *Streptococcus pyogenes* (*S. pyogenes*; clinical isolate), Pam3CSK4 (20 µg/ml), LTA-SA (10 µg/ml), poly (I:C) (low molecular weight; 20 µg/ml), native flagellin from *Salmonella typhimurium* and FSL-1 (10 µg/ml each). IL1 α (10 ng/ml, Peprotech, Rocky Hill, NJ, USA) was used as a positive control, cell culture medium (untreated) and tryptic soy broth medium (TSB, 1:100 in KGM, Fluka, Buchs, Switzerland) as negative controls. The optimal ligand concentrations and bacterial density were obtained in preliminary experiments according to a published protocol (Abtin et al., 2010). After 24 hours, cell culture supernatants were harvested and frozen at -80°C until further analyses. The concentrations of secreted CXCL8/IL8, CXCL10/IP-10 and TNF α were measured with a DuoSet enzyme-linked immunosorbent assay (ELISA) Development Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Optical density at 450 nm was measured using a microplate reader (Floustar Optima, BMG Labtech, Offenburg, Germany).

SDS-PAGE and western blot

Western blot analysis was performed as described (Mildner et al., 2002). Briefly, untreated and poly (I:C)-stimulated keratinocytes were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, sonicated, centrifuged, and denatured before loading on a gel. SDS-PAGE was conducted on 8-18% gradient gels (GE Amersham Pharmacia Biotech, Uppsala, Sweden). The proteins were then electro-transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and incubated with primary antibodies against TLRs 3 and 6 (Promokine) and GAPDH (Acris Antibodies, San Diego, CA, USA). Signals were detected using the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) and reaction products

were visualized by chemiluminescence with the ChemiGlow reagent (Biozyme Laboratories Limited, South Wales, UK) according to the manufacturer's instructions.

Protein microarray analyses

Keratinocytes (1×10^5) from fetal, neonatal and adult human donors were cultured in KGM and stimulated with poly (I:C) or left untreated for 24 hours. Supernatants were screened for cytokines, chemokines and angiogenic factors using commercially available protein array systems (Proteome Profiler Arrays, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Skin equivalents

In vitro skin equivalents were generated as described previously with minor modification (Mildner et al., 2010). Briefly, a suspension of collagen type I (PureCol, Advanced Biomatrix, San Diego, CA, USA) containing 1×10^5 fibroblasts per ml was poured into cell-culture inserts (3 µm pore size; BD Biosciences) and allowed to gel for 2 hours at 37°C. After equilibration with KGM for 2 hours, 1.5×10^6 keratinocytes, in a total volume of 2 ml KGM, were placed on the collagen gel. After overnight incubation the medium was removed from both the inserts and external wells, and replaced in the external wells only by serum-free keratinocyte-defined medium consisting of KGM but without bovine pituitary extract and supplemented with 1.3 mM calcium (Sigma, Vienna, Austria), 10 µg/ml transferrin (Sigma), 50 µg/ml ascorbic acid (Sigma), and 0.1% bovine serum albumin (Sigma). The volume of culture medium was chosen so that keratinocytes were positioned at the air-liquid interface. Serum-free keratinocyte-defined medium was changed every second day for 6 days.

Histochemistry and immunohistochemistry

A standard Hematoxylin and Eosin (H&E) staining technique (Merck) was used for histological analyses of paraffin-embedded skin equivalents. For immunohistochemistry, skin sections from cryopreserved skin equivalents or whole skin specimens were stained with the unconjugated primary anti-TLR3 and 6 antibodies (Promokine) overnight at 4°C, followed by blocking of endogenous peroxidase activity by incubating sections with 0.03% hydrogen peroxide/methanol (Merck) for 10 minutes at room temperature. Subsequently, sections were incubated with biotin-conjugated anti-rabbit IgG antibody (Elite rabbit IgG Vectastain kit, Vector Laboratories) for 60 minutes at room temperature. Biotinylated antibodies were detected with HRP-streptavidin and staining was visualized with amino-ethyl-carbazole (AEC; Dako, Glostrup, Denmark). Finally, sections were counterstained with hematoxylin (Merck) and mounted with Aquatex (Merck) and examined using a microscope (Eclipse 80; Nikon). Rabbit serum (Vector Laboratories) was used as control.

Statistical analysis

Data are given as means, and error bars represent the standard error of the mean. Nonparametric paired (Wilcoxon signed-rank test Mann-Whitney U-test) and unpaired (Mann-Whitney U-test Wilcoxon signed-rank test) *t*-test group comparisons were used when appropriate (Graph Pad Software, La Jolla, CA, USA). For comparison of cytokine concentrations, one-way ANOVA testing was applied and data were corrected for multiple testing in post-hoc analyses (Dunnett-T or Bonferroni, where appropriate). A *P*-value of less than 0.05 was considered significant. Statistical testing was performed using the SPSS 15.0 software package (SPSS, Chicago, IL, USA).

RESULTS

Many TLRs are significantly higher expressed in human skin specimens before and after birth compared with adults

To examine at which time point during ontogeny skin cells acquire the prerequisites to warrant adequate immune responses, we evaluated the mRNA expression of TLRs in human skin specimens dissected from embryos, fetuses, infants and children and compared them with those of adults using quantitative RT-PCR analyses. We found that prenatal whole skin specimens at all time

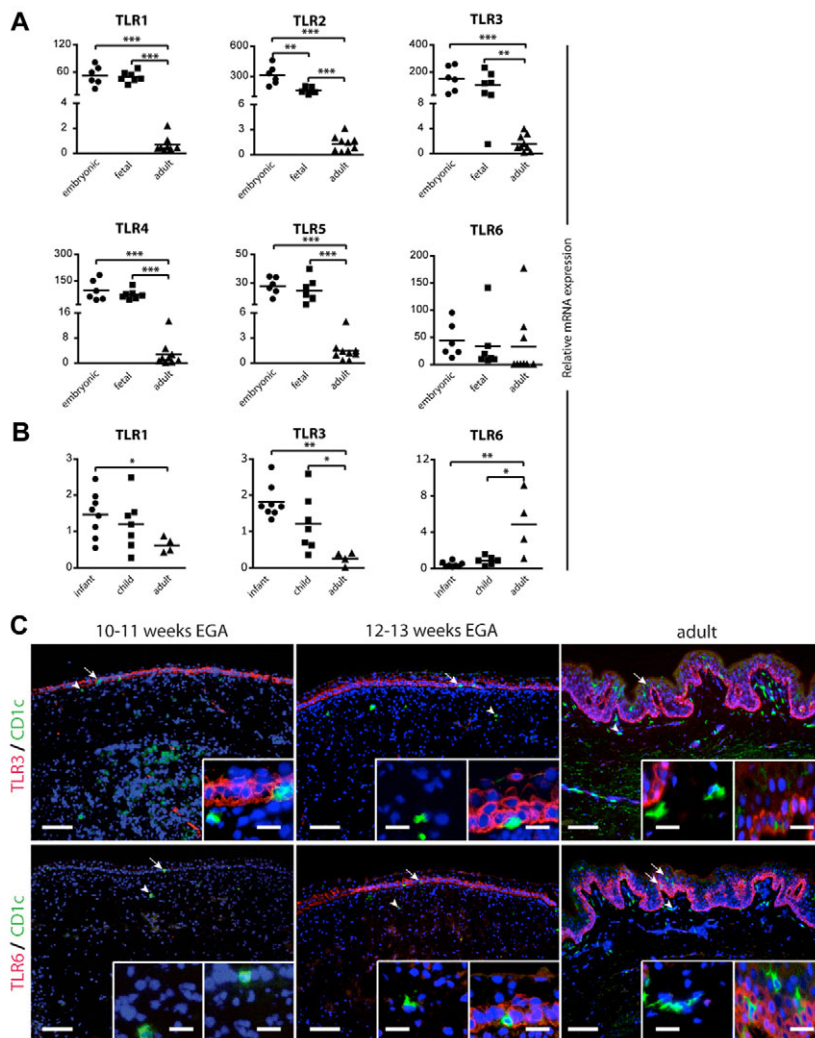


Fig. 1. TLR expression profile of human skin before and after birth. (A) Embryonic (9-11 weeks EGA), fetal (12-13 weeks EGA) and adult (18-45 years) skin specimens were investigated ($n=6-9$ donors/group) for the indicated TLR expression levels using quantitative RT-PCR. Reactions were executed in duplicates and data were normalized to the housekeeping gene B2M. (B) Epidermis was separated from the dermis of skin samples from infants (3-10 months), children (5-12 years) and adults (18-31 years) ($n=4-8$ donors/group) using ammonium thiocyanate. Indicated TLR expression levels were determined using quantitative RT-PCR. Reactions were executed in duplicates and data were normalized to the housekeeping gene GAPDH. The x - and y -axis indicate selected age groups and relative mRNA expression, respectively. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. (C) Immunofluorescence double labeling of cryostat sections from embryonic, fetal and adult skin samples ($n=3$ donors/group) revealed CD1c⁺TLR3⁻TLR6⁻ dendritic cells in prenatal and adult skin (inserts). The arrows denote epidermal LCs and the arrowheads identify DDCs. Nuclear counterstain was performed with DAPI. Scale bars: 100 μ m in C; 20 μ m in inserts.

points tested already expressed the same spectrum of TLRs as adult skin (data not shown). Interestingly, significantly higher mRNA expression levels of TLRs 1-5 and the TLR4 co-factor MD2 were identified in embryonic and fetal skin when compared with adult skin (Fig. 1A and data not shown). TLR6 was essentially equally expressed in embryonic and fetal skin (Fig. 1A) and TLRs 7-9 were absent in adult skin but weakly detectable in prenatal skin (data not shown).

With the exception of marginal but significantly higher expression of TLRs 1 and 3, most of the TLRs investigated were comparable in their magnitude of expression in the epidermis after birth (infants and children) when compared with adults (Fig. 1B, and data not shown). Conversely, TLR6 mRNA had significantly lower expression in all skin specimens after birth than in adults. However, the differences were minute and not obvious by immunohistochemistry (Fig. 1B; supplementary material Fig. S1B).

Keratinocytes in human embryonic skin express TLR3 but not TLR6 protein in situ

As skin is composed of heterogeneous cell populations, we attempted to characterize the exact cellular source for the differentially expressed TLRs 3 and 6 in prenatal skin using immunofluorescence and immunohistochemistry. Although the staining intensity was variable between the antibodies and the age

groups investigated, both gave a similar staining pattern and showed a predominant staining in the epidermis and negative or rare positive cells in the dermis. We found TLR3 expression in the epidermal basal layer already at 9-11 weeks EGA, faint expression in the periderm, which is a protective development-specific cell layer covering the basal layer, and few cells in the dermis of embryonic skin. The staining pattern remained similar until 12-13 weeks EGA (Fig. 1C; supplementary material Fig. S1A). The staining intensity of predominantly basal keratinocytes for TLR3 appeared comparable in fetal and adult skin (Fig. 1C, upper panel, inserts; supplementary material Fig. S1A). By contrast, no TLR6⁺ cells were detected in embryonic skin at 9-11 weeks EGA (Fig. 1C, lower panel, inserts; supplementary material Fig. S1A). However, at 12-13 weeks EGA all basal keratinocytes were positive for TLR6 and the staining pattern resembled very much the pattern observed in adult skin (Fig. 1C, lower panel, inserts; supplementary material Fig. S1A).

It has previously been reported that freshly isolated and purified epidermal LCs and DDCs express TLRs 3 and 6 at the mRNA and protein level (Flacher et al., 2006; van der Aar et al., 2007). To test whether some of the TLR3 and 6⁺ skin cells may represent dendritic cells, prenatal and, for comparison, adult skin sections and epidermal sheets that had been stained with antibodies directed against TLRs 3 and 6, were counterstained with CD1c, the earliest dendritic cell marker expressed in human skin during ontogeny

(Schuster et al., 2009; Schuster et al., 2012) or CD207. We identified CD1c⁺ epidermal (Fig. 1C, arrows) and dermal (Fig. 1C, arrowheads) dendritic cells in embryonic and fetal skin and higher frequencies of these cells in adult skin, thus confirming our previous reported results (Schuster et al., 2009; Schuster et al., 2012). Surprisingly, neither LCs and DDCs in adult skin, nor LC precursors and DDCs in prenatal skin expressed TLRs 3 and 6 at detectable levels (Fig. 1C, inserts; supplementary material Fig. S2).

Enhanced CXCL8, CXCL10 and TNF α induction by several TLR ligands in fetal and neonatal primary keratinocytes compared with adult keratinocytes

To address whether enhanced TLR mRNA expression in keratinocytes before and after birth correlates with enhanced function, primary keratinocytes from respective age groups were

used and compared in functional experiments. A side-by-side culture revealed that neonatal but not fetal keratinocytes were smaller in size compared with adult keratinocytes (Fig. 2A). Keratinocytes from these age groups were stimulated with heat-inactivated micro-organisms (*E. coli*, *S. pyogenes*) and synthetic TLR agonists [TLR1/2 (Pam3CSK4), TLR2 (LTA-SA), TLR3 (poly I:C), TLR5 (Flagellin) and TLR6 (FSL-1)]. Supernatants were harvested 24 hours after stimulation and induction of chemokines and cytokines was quantified by ELISA. Comparable to the mRNA expression profile, we repeatedly observed a trend for higher induction of CXCL8 and CXCL10 as well as the proinflammatory cytokine TNF α in fetal and neonatal keratinocytes in comparison with those from adults irrespective of the stimulus chosen (Fig. 2B-D). Notably, poly (I:C) induced robust and significantly higher secretion of CXCL8, CXCL10 and TNF α

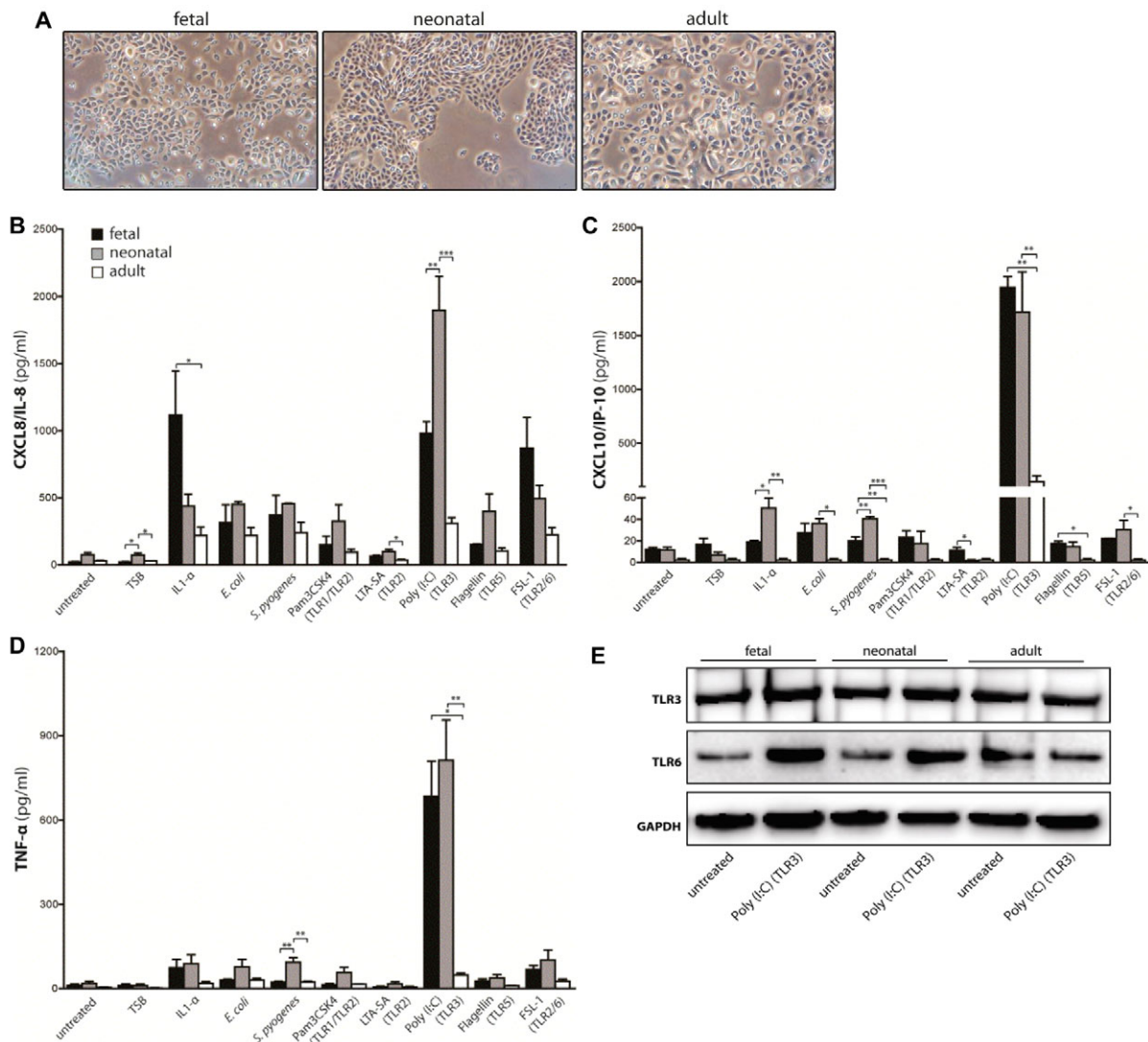


Fig. 2. Bacterial stimuli and TLR ligands induce distinct immune responses in fetal, neonatal and adult keratinocytes. (A) Primary keratinocytes from fetal, neonatal and adult skin were cultured in 12-well plates to ~75% confluence and stimulated in triplicates for 24 hours with the indicated bacterial stimuli and ligands at concentrations described in the Materials and methods. IL1 α was used as positive control, cell culture medium (untreated) and TSB as negative controls. (B) CXCL8 (C) CXCL10 and (D) TNF α levels in supernatants were quantified by ELISA. Experiments were performed as three biological replicates/age group (different donors) and the data represent the average (mean \pm s.e.m.) of these donors. * P <0.05, ** P <0.01, *** P <0.001. (E) Western blot analysis of untreated primary keratinocytes showed an almost equal expression of TLR3 and TLR6 proteins in all indicated age groups. Stimulation of keratinocytes with poly (I:C) did not affect TLR3 expression, whereas TLR6 expression was upregulated in fetal and neonatal, but not in adult, keratinocytes.

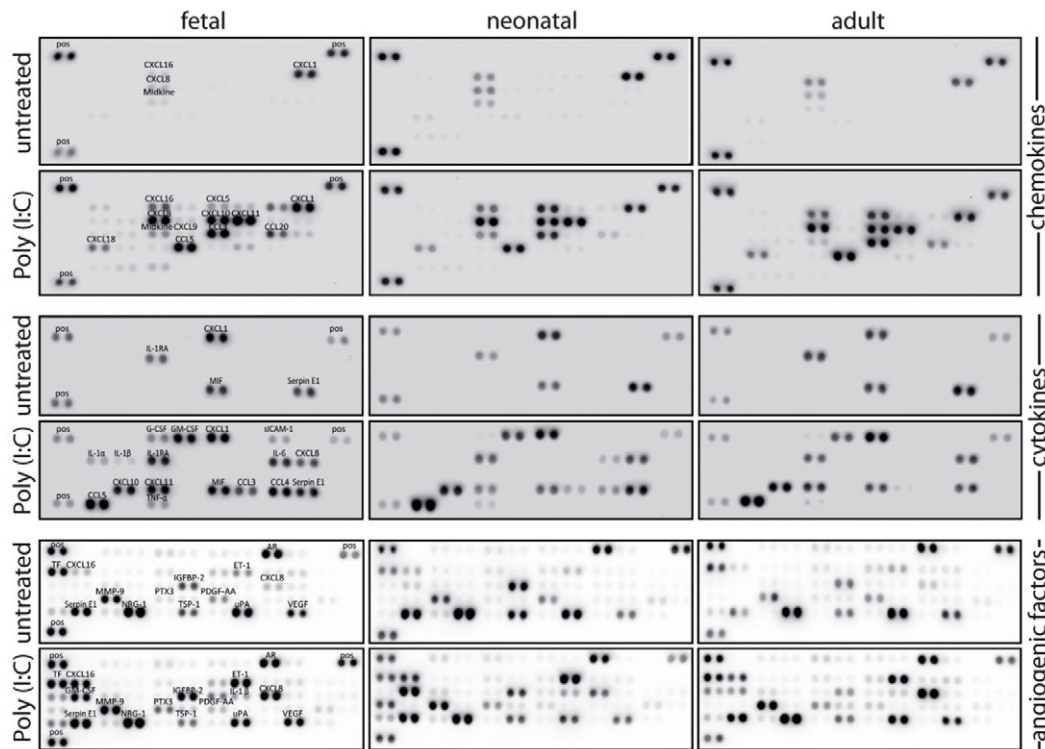


Fig. 3. Poly (I:C) activates differential expression of chemokines, cytokines and angiogenic factors in fetal keratinocytes in comparison with neonates and adults.

Keratinocytes from fetal, neonatal and adult donors ($n=3$ donors/group) were cultured in KGM and stimulated with poly (I:C) or left untreated (negative control) for 24 hours.

Supernatants were collected, pooled and analysed for the indicated chemokines, cytokines and angiogenic factors using commercially available protein arrays.

in fetal and neonatal keratinocytes compared with adult keratinocytes (Fig. 2B-D). In parallel, we performed western blot analysis with the same keratinocytes and stimulation protocol as used in our functional studies. No remarkable differences in TLR3 and 6 expression were observed in untreated keratinocytes, showing that the functional diversities between age groups are not due to different protein expression levels. Whereas stimulation of fetal, neonatal and adult keratinocytes with poly (I:C) did not affect TLR3 expression, TLR6 expression was upregulated in fetal and neonatal but not adult keratinocytes upon poly (I:C) stimulation compared with untreated controls (Fig. 2E).

Activation of TLR3 with poly (I:C) induces secretion of IFN-inducible chemokines in fetal keratinocytes

To further compare the responses of poly (I:C) ligation in fetal, neonatal and adult keratinocytes we performed protein microarray analyses. Untreated keratinocytes from all age groups constitutively secreted chemokines/cytokines (CXCL1, CXCL8, CXCL16, IL1RA) and factors such as Midkine, MIF, serpin E1, MMP9, etc. (Fig. 3). In analogy to our ELISA data, poly (I:C) stimulation of keratinocytes showed upregulation and/or induction of CXCL8, CXCL10 and TNF α secretion in all three age groups (Fig. 3). Similarly, secretion of several chemokines (CXCL16, CXCL1/GRO α , CXCL11/I-TAC, CCL3/MIP1 α , CXCL18), cytokines (IL1RA, MIF) and angiogenic factors (AR, TF, ET1, MMP9, PTX3, NRG1, TSP1, UPA, VEGF) were found in fetal, neonatal and adult keratinocytes (Fig. 3). Remarkably, however, many cytokines and chemokines (CXCL16, Midkine, CXCL9/MIG, CCL20/MIP-3 α , G-CSF, GM-CSF, sICAM1, IL1 α , IL1 β , IL6/IFN β 2, CCL3/MIP1 α and CCL4/MIP1 β , serpin E1, IGFBP2, UPA) were intensely activated in fetal donors but were less abundantly induced or even absent in neonatal and adult donors, whereas angiogenic factors with few exceptions were fairly equally expressed upon activation in all three age groups (Fig. 3).

In particular, we found that in response to TLR3 activation, fetal keratinocytes produce CCL3-5 and IFN-inducible chemokines such as CXCL9-11, which are associated with an antiviral innate defense program (Nakayama et al., 2006). Confirmation of these findings by ELISA is likely to yield important insights and constitutes the necessary next step in investigating innate skin immune ontogeny.

TLRs 3 and 6 are distinctly expressed in skin equivalents from fetal, neonatal and adult donors

As shown above, prenatal and neonatal human primary keratinocytes are even superior in many aspects to adult keratinocytes. We therefore attempted to also explore their differentiation capacity and have established an in vitro differentiation model, which was initiated according to a previously published protocol (Mildner et al., 2010). H&E staining revealed a similar differentiation potential of fetal and adult keratinocytes, whereas in neonatal equivalents more suprabasal layers were detectable, indicating a higher capability of neonatal keratinocytes to contract on the collagen matrix (supplementary material Fig. S3). Accordingly, differentiation marker analysis showed a similar staining pattern for K10 and Loricrin in fetal and adult keratinocyte-derived equivalents. However, they differed somewhat from adult control skin. Whereas TLRs 3 and 6 showed the strongest expression in basal keratinocytes and similar (TLR3) or weaker (TLR6) expression in suprabasal cell layers in equivalents (Fig. 4A), only K10⁻ basal keratinocytes were positive for these markers in fetal and adult control skin (Fig. 4B; supplementary material Fig. S4). Interestingly, even though thicker, the staining pattern for TLRs 3 and 6 in neonatal skin resembled the staining pattern in adult skin (Fig. 4A).

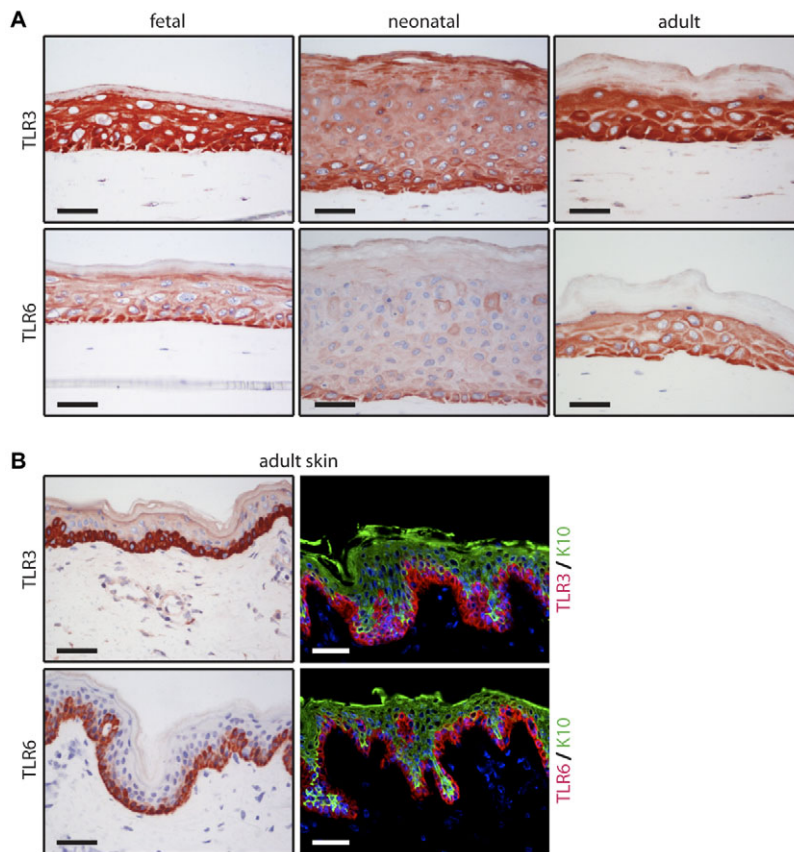


Fig. 4. Expression pattern of TLRs 3 and 6 in skin equivalents generated from fetal, neonatal and adult keratinocytes. (A) Cryostat sections of skin equivalents generated with keratinocytes from the indicated age groups ($n=3$ donors/group), and adult breast skin (positive control), were stained for TLR 3 and 6 protein expression using immunohistochemistry. (B) Immunofluorescence double labeling revealed $K10^{-}TLR3^{+}TLR6^{+}$ basal keratinocytes (red) and $K10^{+}TLR3^{-}TLR6^{-}$ suprabasal keratinocytes (green). Nuclei were stained with Hoechst dye (blue). Scale bars: 50 μm in A,B (immunohistochemistry); 40 μm in B (immunofluorescence).

DISCUSSION

In this study we investigated when during development the human skin innate immune system mechanisms start their protection program. A key element of innate protection is the recognition of PAMPs by PRRs, which is linked to the initiation of adaptive immune responses. We compared the expression and function of TLRs in the developing human skin and found that prenatal skin already expresses a similar spectrum of TLRs as the adult. However, some TLRs, most notably TLR3, exhibited enormous differences in the magnitude of expression and function in skin cells before and after birth when compared with adults, suggesting the existence of age-specific responses rather than a global, linear progression from a prenatal to an adult pattern (i.e. the relationships are not simply one of a higher level of TLR expression in adult skin).

High constitutive expression levels of many TLRs in prenatal human skin but similar keratinocyte differentiation in skin equivalents generated with keratinocytes from fetal and adult skin

Microbial exposure may lead to an increased expression of specific PRRs on immune and non-immune cells. As the prenatal human skin is not exposed to micro-organisms, our finding of relatively high constitutive expression levels of many TLRs was unexpected and may reflect their relative expression by predominant cell types rather than exposure to micro-organisms. Among all abundantly expressed TLRs, one of the most striking findings in our study was that TLR3 had significantly higher expression in prenatal human skin. TLRs are expressed on a variety of cell types found in the skin (reviewed by Miller and Modlin, 2007). Most contributions on microbial and non-microbial stress to epithelial-immune cell

interactions have focused on dendritic cells and macrophages, rather than on epithelial cells (Swamy et al., 2010). As human skin at a certain gestational age has variable ratios of these cells (Schuster et al., 2009; Schuster et al., 2012), we hypothesized that the higher TLR expression in prenatal skin could be due to hematopoietic precursors/stem cells and a high cellularity of dermal cells compared with adults, all of which potentially express TLR3. Surprisingly, $CD1c^{+}$ LCs/precursors and DDCs, neither in fetal nor adult skin, expressed TLR3 in situ, but it has been reported that hematopoietic stem cells express TLRs 1-9 (Dorner et al., 2009) and that LCs as well as DDCs express TLR3 at the mRNA, protein and functional level when isolated from adult skin (Flacher et al., 2006). This discrepancy may be explained by the long isolation procedure leading to an upregulation of TLR3 in LCs and DDCs and/or a contamination of the reported skin dendritic cell preparations with other TLR3⁺ skin cells such as keratinocytes. A constitutive low expression of TLRs in these cells may be below the detection by immunofluorescence or, alternatively, indeed reflect the situation in unperturbed skin. It can also be excluded that the high mRNA expression in prenatal skin may be due to a reflection of higher numbers of TLR3⁺ keratinocytes in prenatal skin, as we found that only basal keratinocytes express TLR3 with similar intensity in both fetal and adult skin, as demonstrated by our immunofluorescence and immunohistochemistry staining. Nonetheless, we found that all cells of the periderm and some scattered dermal cells in prenatal skin expressed TLR3. Their abundance could at least partly explain the higher TLR3 mRNA expression levels in prenatal human skin. When stimulating primary keratinocytes at the same cell densities, from different age groups with poly (I:C), we found significantly higher secretion of the neutrophil chemoattractant CXCL8, the Th1-attracting

chemokine CXCL10 (Lebre et al., 2007) and the proinflammatory cytokine TNF α in prenatal keratinocytes. From these data we can conclude that prenatal but not adult keratinocytes on a per cell basis do respond superior to viral ligands in vitro. Now it will be essential to investigate potential mechanisms underlying these differences in greater detail. An altered DNA methylation in fetal and adult skin may be one possibility. Indeed, it has been reported only recently that most fetal-tissue-specific differentially methylated regions seem to reflect transient DNA methylation changes during development rather than permanent epigenetic signatures (Byun et al., 2009; Yuen et al., 2011).

As pathogens first get into contact with skin in the upper, more differentiated layers one would expect a gradient of TLR expression in the adult but not in prenatal skin, which is not yet exposed to micro-organisms (Pivarcsi et al., 2004). We found that TLR 3 and 6 immunoreactivity is restricted to basal keratinocytes. Surprisingly, we found that the TLR6 mRNA expression pattern in prenatal and adult skin was similar, even though no TLR6⁺ cells were found in early embryonic skin. Thus, the relative quantities of the TLR6 receptor and mRNA do not seem to be linked and might reflect distinct transcriptional regulation of TLR6.

Keratinocyte differentiation involves a complex cellular program that has been extensively studied during the past decades using knockout or transgenic animal models and in vitro differentiation models. Here we used a skin-equivalent culture model to investigate the differentiation capacity of fetal and neonatal keratinocytes in comparison to adult keratinocytes. We found that already fetal and neonatal keratinocytes revealed a similar differentiation potential to adult keratinocytes with regard to histology and differentiation marker expression. Also the immunoreactivity of TLRs 3 and 6 was comparable to the respective controls. Further detailed investigations using skin equivalents established from fetal keratinocytes could lead to the discovery of mechanisms important in the ontogeny of skin innate immunity.

Prenatal human keratinocytes exhibit effective skin innate immunity

Most studies examining early life innate immune function have focused on cord blood (Philbin and Levy, 2009), and only a few have analysed postnatal innate immune development (Angelone et al., 2006; Belderbos et al., 2009; Nguyen et al., 2010). Recently a unique study was published which followed innate immune responses in the same subjects for over 2 years after birth (Corbett et al., 2010) describing qualitative and quantitative age-specific changes in human blood mononuclear cells in response to TLR stimulation. At the quantitative level, this is in line with our observations with skin cells, as neonatal keratinocytes secrete remarkable high levels of CXCL8, CXCL10 and TNF α upon ligation with poly (I:C) when compared with adults even though keratinocytes from both age groups express comparable levels of TLR3, as shown by western blot analysis. Moreover, we found that fetal skin cells were already able to respond to poly (I:C) with a similar strength to neonatal keratinocytes. Our current observation not only complements recent studies (Hau et al., 2011; Köllisch et al., 2005; Lebre et al., 2003) but also expands the age range, as experiments have previously been carried out either in adult or neonatal keratinocytes and a direct comparison of the response between these three age groups in one experiment has not been performed. It remains to be investigated whether these quantitative differences also correlate with qualitative differences. Some evidence for the validity of this assumption comes from our

observation of upregulated IL6 in fetal and neonatal but not adult keratinocytes upon poly (I:C) stimulation and significantly higher spontaneous IL6 production by prenatal skin cells when compared with adult skin cells in vitro (Schuster et al., 2009). Polarization in support of IL6, a cytokine with Th2-polarizing (Diehl and Rincón, 2002) and anti-inflammatory properties (Jones, 2005) may protect the fetus in utero from potentially harmful Th1 responses that can initiate spontaneous abortion (Makhseed et al., 2001) as well as premature delivery and its consequences (Raghupathy et al., 2001). Given the importance of Th1 polarizing cytokines in protection against multiple pathogens, IL6 may also contribute to a Th2 bias that can render the newborn more susceptible to microbial infection.

Activated keratinocytes, macrophages and monocytes are the most prominent producers of the IFN-inducible chemokines CXCL9-11 (Flier et al., 2001). Recently, it has been shown that CXCL9 and CXCL11 are potently induced in epidermal LC-like dendritic cells after TLR3 ligation, and that CXCL9 is expressed by epidermal LCs and keratinocytes in adult skin in vivo in two different viral skin diseases, such as molluscum contagiosum and verruca vulgaris (Renn et al., 2006). In support of this it has been demonstrated that epidermal LCs indeed can stimulate effective CD70-mediated CD8⁺ T cells in response to live FLU virus and poly (I:C) stimulation in vitro (van der Aar et al., 2011). Further evidence for IFN-mediated antiviral effects of CXCL9 in vivo has been provided by observations with SIV and vaccinia virus infections demonstrating CXCL9 at sites of viral replication (Mahalingam et al., 1999; Reinhart et al., 2002). In addition, CXCL9-11 are important in innate immunity because they also exhibit defensin-like, antimicrobial function (Cole et al., 2001). We found that fetal keratinocytes are able to produce CXCL9-11 and CCL3-5 upon TLR3 ligation. Evidence has been provided that the chemokines CCL3-5 may have a potential role in host defense against HSV-1 as direct antiviral agents (Nakayama et al., 2006). Given that TLR3 is a receptor involved in detection of RNA and DNA virus replication (Colby and Duesberg, 1969; Xiang et al., 1998), our data provide evidence that prenatal skin cells are already able to respond to both RNA and DNA viruses. This appears to be essential because many viruses can attack the epidermis, such as: HSV-1 (Sprecher and Becker, 1988); *Poxviridae* (molluscum contagiosum virus) (Renn et al., 2006); human papillomavirus (Renn et al., 2006); and HIV (Blauvelt et al., 2000; Compton et al., 1996; Rappersberger et al., 1988). It remains to be determined whether epidermal LC precursors in prenatal human skin can already cooperate with keratinocytes in the local antiviral response in the epidermis as shown for adult LCs (Renn et al., 2006; van der Aar et al., 2011).

Our in vitro findings with fetal skin cells upon exposure to TLR ligands may reflect the response to micro-organisms found in vivo. Evidence that TLRs are indeed involved in response to specific microbes in utero in humans has been provided by Kim and coworkers (Kim et al., 2006). They showed significantly higher expression of TLR2 with concomitant upregulation of cytokines/chemokines (IL1 α , TNF α , CXCL8) and antimicrobial factors (β -defensins) in keratinocytes of fetuses with histological chorioamnionitis, compared with cases without chorioamnionitis, suggesting that fetal keratinocytes can already be actively involved in the fetal inflammatory response (Kim et al., 2006). In line with this observation is the finding that exposure of fetal ovine keratinocytes or fetal skin in vitro and in utero to LPS consistently induced significant increases in IL1 β , IL6, TNF α and CXCL8 expression, providing further evidence that inflammation is

triggered directly in the skin and involves TLR regulation in utero (Kemp et al., 2011).

In conclusion, we present the results of a comprehensive TLR expression and functional profiling study comparing human skin from selected age groups before and after birth. We provide evidence of quantitative age-specific changes in innate immune reactivity in response to TLR stimulation that contradicts the notion of a linearly progression of the innate immune response to TLR stimulation from fetus to adult. The characterization of TLR3 activation in prenatal and infant skin furthers our understanding of the complex mechanisms that initiate innate immune responses to viral infection, indicating at the same time that it may provide a powerful target for the development of therapeutic agents for the treatment of viral infections in the skin. Lastly, our data imply that fetal keratinocytes are already engendered with specific immune functions to tackle local invading pathogens.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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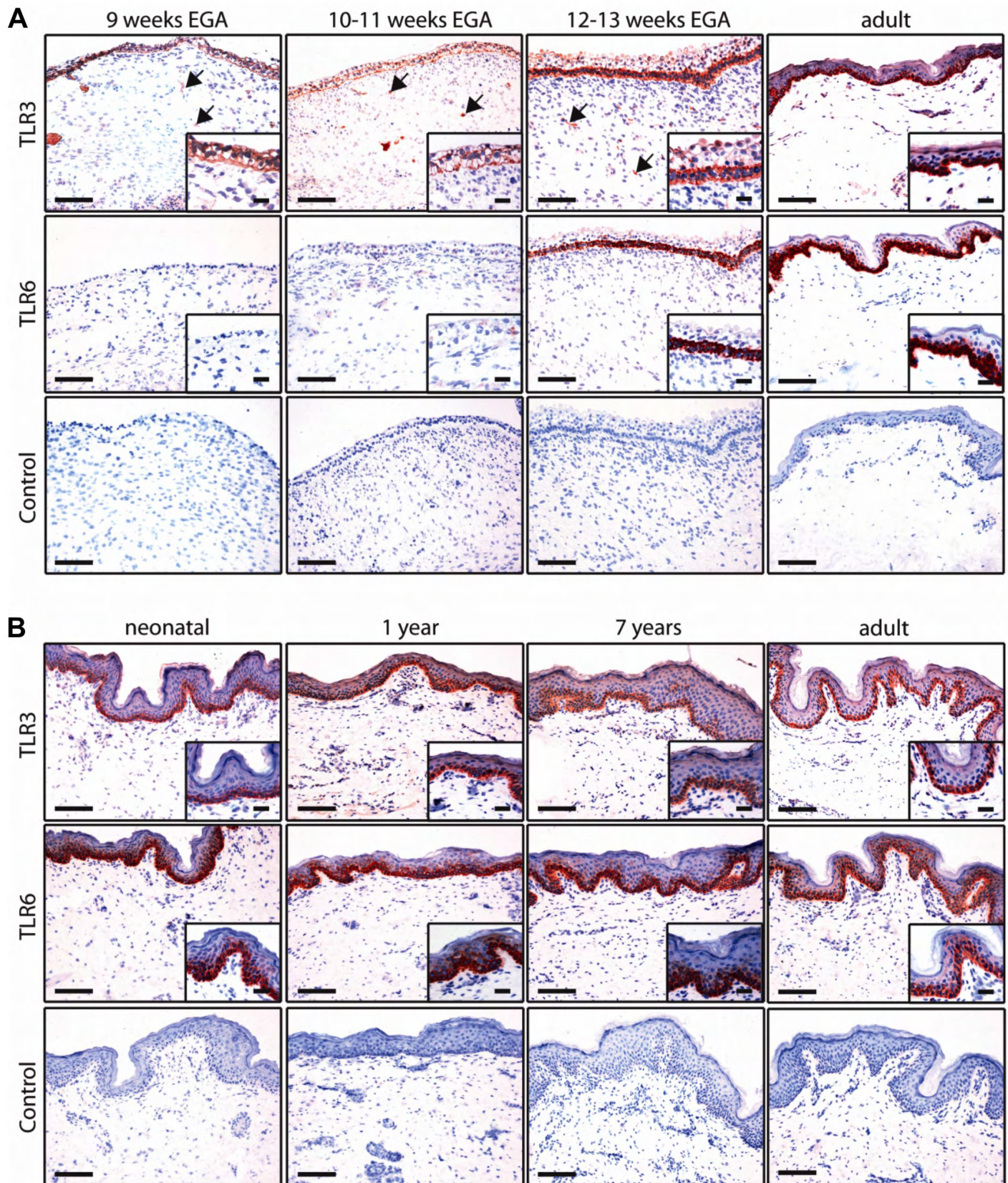


Fig. S1. Acquisition and kinetics of TLR3 and TLR6 expression in human prenatal skin and skin after birth. (A,B) Immunohistochemical staining was performed on cryostat sections of embryonic, fetal (A), neonatal, infant, child (B) and adult skin (A,B) ($n=2$ or 3 donors/group). TLR3 and TLR6 were visualized with AEC (red) and counterstained with Hematoxylin. Arrows indicate TLR3⁺ cells in the dermis. Scale bars: 100 μ m in A,B; 50 μ m in insets.

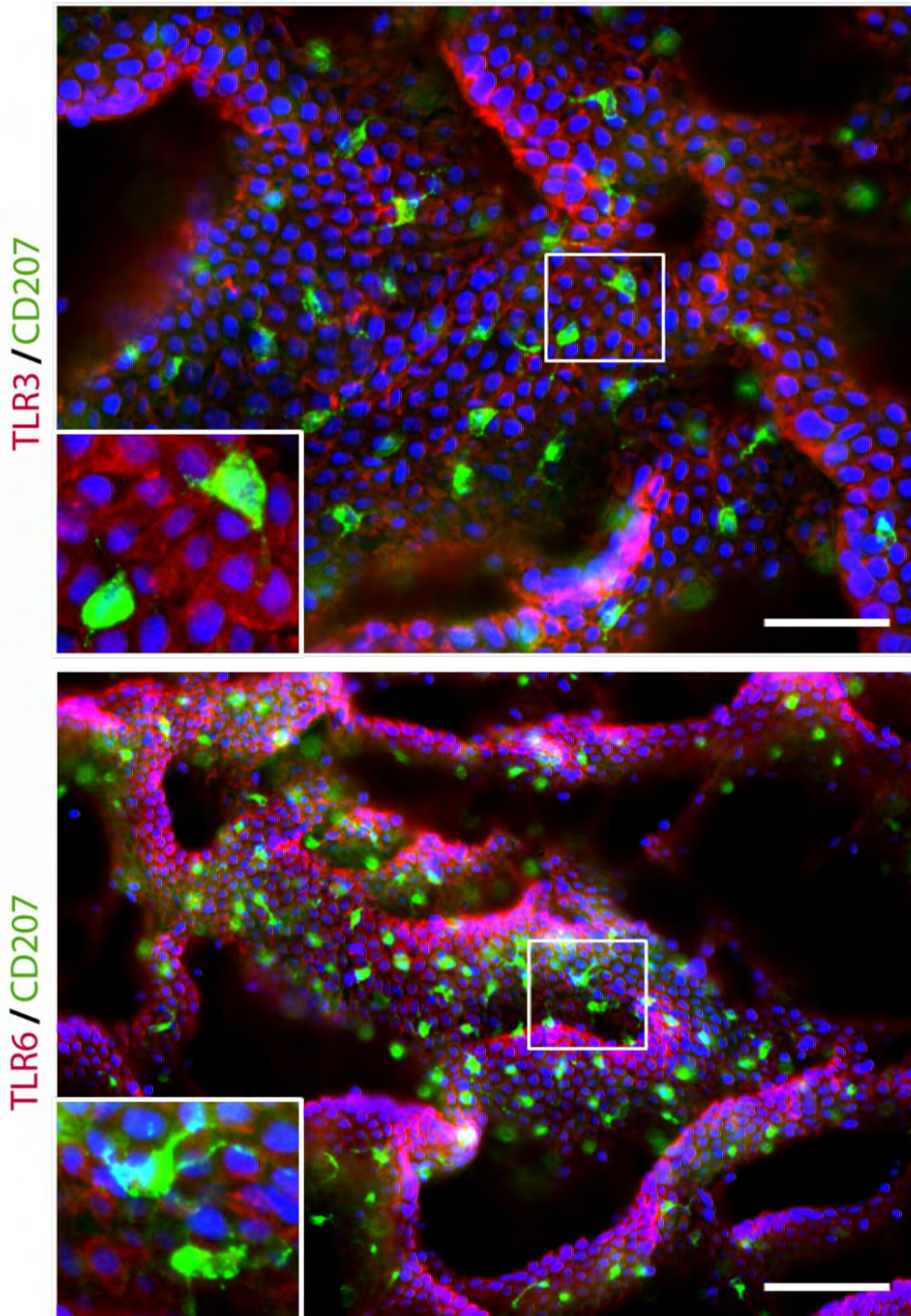


Fig. S2. Depiction of TLR3⁻TLR6⁻ LCs in epidermal sheets. Immunofluorescence double labeling of epidermal sheets from adult skin revealed CD207⁻TLR3⁺TLR6⁺ cells (red), presumably keratinocytes, and CD207⁺TLR3⁻TLR6⁻ LCs (green). Nuclear counterstain was performed with Hoechst dye (blue). One of two representative experiments is shown. Scale bars: 40 μ m.

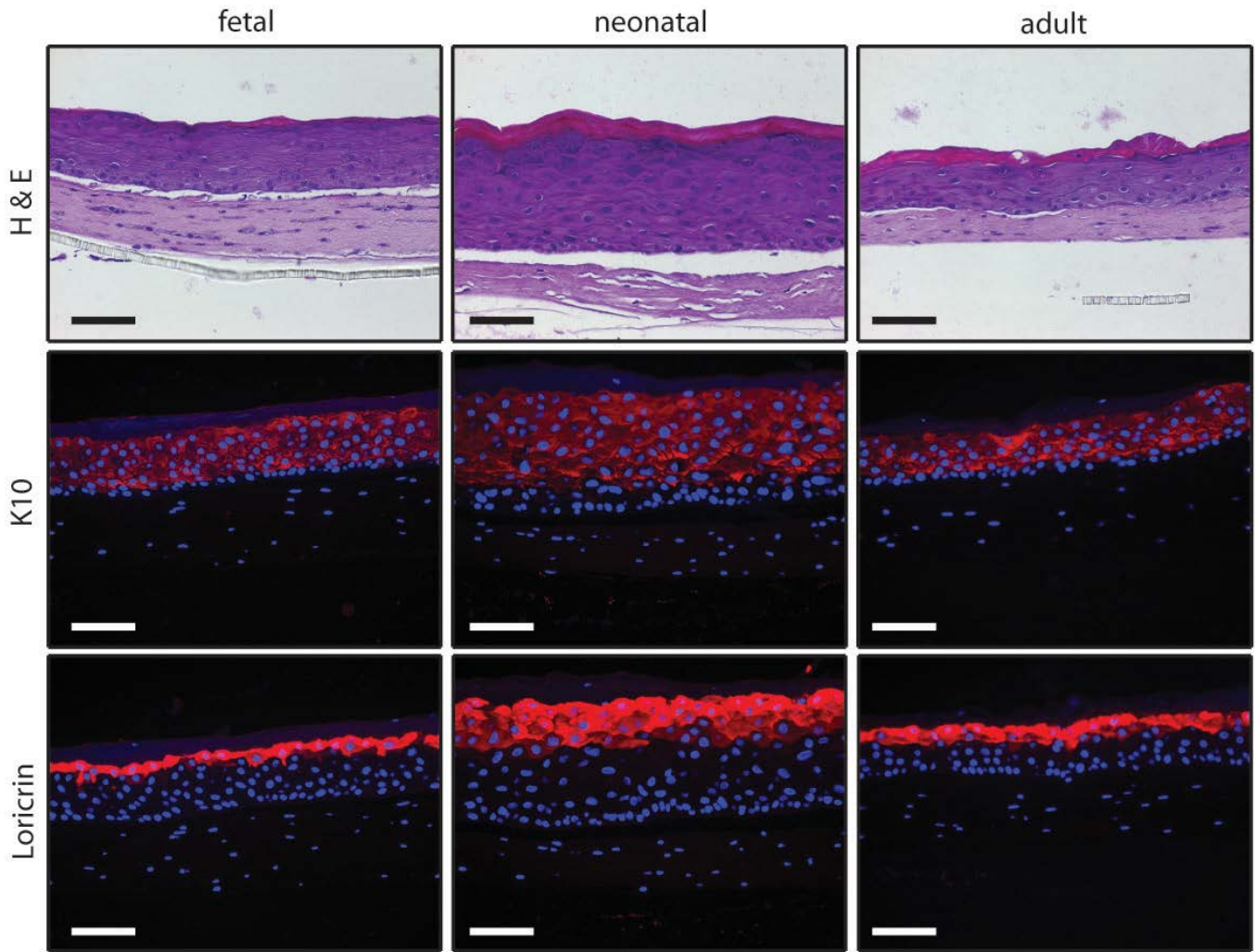


Fig. S3. Keratinocytes from fetal, neonatal and adult donors show a comparable differentiation potential in a skin equivalent model. Hematoxylin and Eosin staining of paraffin-embedded skin equivalents show a comparable keratinocyte differentiation and stratum corneum formation with keratinocytes from fetal, neonatal and adult donors. Immunofluorescence labeling revealed similar staining intensities and patterns of the differentiation-associated proteins K10 and Loricrin (red) in skin equivalents of all donors. Nuclei were visualized with Hoechst dye (blue). One representative skin equivalent model of 2 is shown. Scale bars: 100 μm .

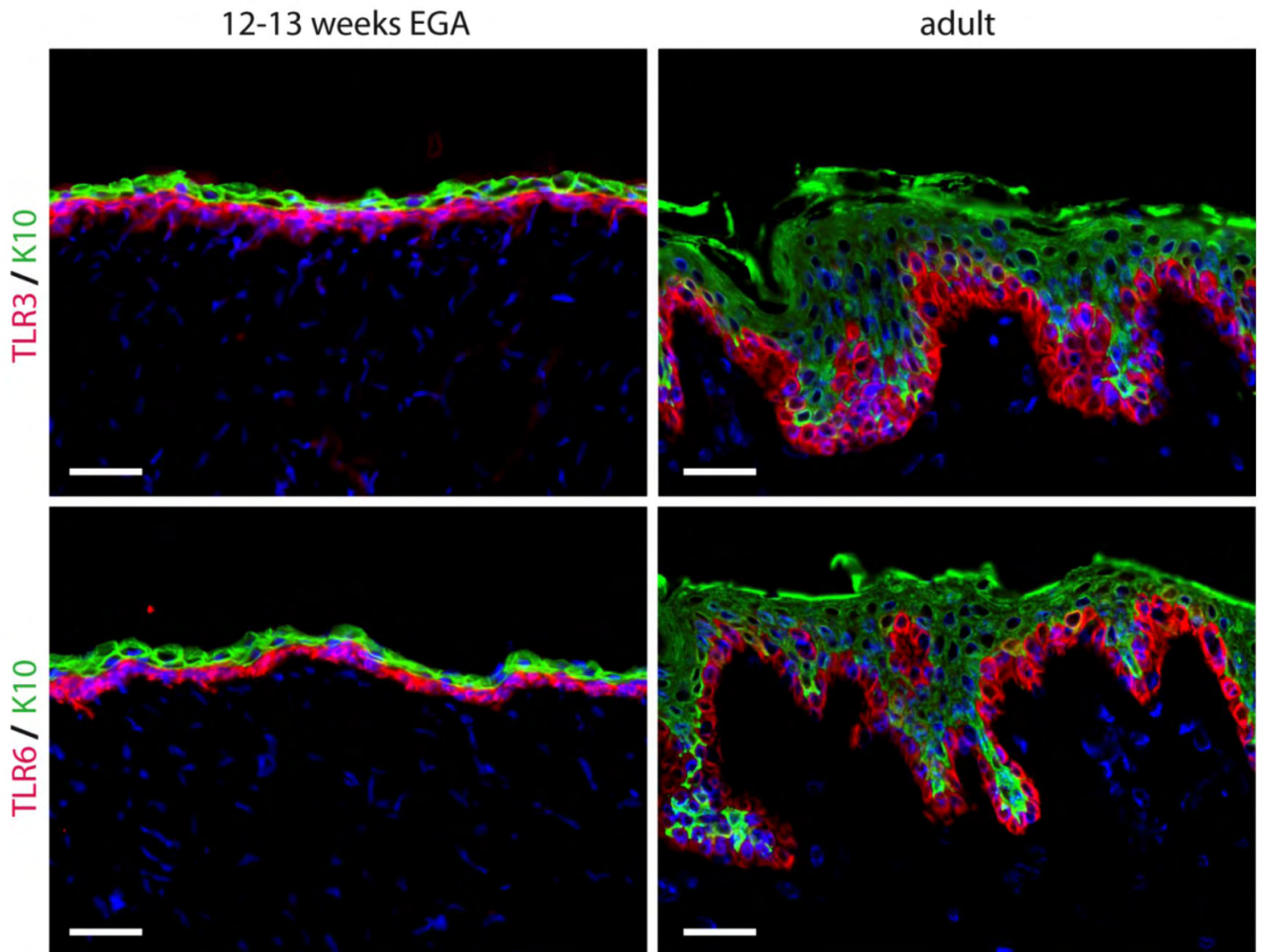


Fig. S4. Basal but not suprabasal keratinocytes express TLR3 and TLR6. Immunofluorescence double labeling was performed on cryostat sections and revealed that basal keratinocytes express TLRs 3 and 6 (red), while K10⁺ suprabasal keratinocytes (green) fail to express these markers in fetal and adult epidermis. Nuclei were counterstained with Hoechst dye (blue). Scale bars: 40 μ m.