Cultured human melanocytes express functional Toll-like receptors 2–4, 7 and 9

Ning Yu a, Shujie Zhang b, Fuguo Zuo a, Kefei Kang a, Ming Guan c, Leihong Xiang a,⁎

a Department of Dermatology, Huaishan Hospital, Fudan University, Shanghai 200040, PR China
b Key Laboratory of Medical Molecular Virology, Shanghai Medical College, Fudan University, Shanghai 200032, PR China
c Central Laboratory, Huaishan Hospital, Fudan University, Shanghai 200040, PR China

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ABSTRACT

Background: Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs), which have been evolutionarily conserved in microbes. Human melanocytes are not simply pigment-producing cells but also have the phagocytic capacity and can produce pro-inflammatory mediators. However, the mechanisms of recognition of microbes by melanocytes have not yet been fully established.

Objective: We investigated the TLRs 1–10 expression profile in human epidermal melanocytes and assessed their functions after triggering by their specific ligands.

Methods: TLRs mRNA expression was determined by RT-PCR, and the TLR protein expression was measured by flow cytometry and immunofluorescence assays. After stimulation with various TLR ligands, the production of inflammatory cytokine IL-8 and IL-6 was measured by ELISA and the mRNA for chemokine CCL2, CCL3 and CCL5 was analyzed by real-time PCR. Phosphorylation of IkBα in TLR ligands-triggered melanocytes was determined by Western blot and the nucleus translocation of NF-κBp65 was analyzed by immunofluorescence.

Results: Human melanocytes constitutively expressed TLRs 1–4, 6, 7 and 9 mRNA. Ample amounts of TLRs 2–4, 7 and 9 were confirmed at protein level. Stimulation of melanocytes with TLRs ligands resulted in the release of cytokines (IL-8 and IL-6) and the mRNA accumulation of chemokines (CCL2, CCL3 and CCL5). Triggering of TLRs in melanocytes resulted in the up-regulation of phosphorylated IkBα and in the nucleus translocation of NF-κBp65.

Conclusion: Present study indicates human melanocytes express a panel of functional TLRs. The ligation of TLRs can turn these cells into active players of the skin innate immunity.

⁎ Corresponding author. Tel.: +86 21 62489999; fax: +86 21 62486388.
E-mail address: flora_xiang@vip.163.com (L. Xiang).

1. Introduction

The human epidermis provides a first defense barrier to a potentially hostile environment. It has been shown that within the epidermis, not only professional immune cells (e.g., Langerhans cells) but also non-immune cells (e.g., keratinocytes and melanocytes) are involved in the immune protection of the host [1–3]. Although originally identified as a professional producer of melanin, recent studies have revealed that melanocytes exhibit a variety of functions. For example, human melanocytes have the capacity to express HLA-DR, CD40 and adhesion molecules such as ICAM-1 and VCAM-1 [4–7]. In addition, they can produce various soluble mediators of inflammation such as IL-1, IL-6 and IL-8 [8,9]. These studies suggest that melanocytes are not simply pigment-producing cells but also immunocompetent cells. However, it is unclear whether melanocytes can recognize pathogens and plays an active role in the skin’s local immune defense system.

Recognition of pathogens by innate immune cells is mediated by pattern-recognition receptors that recognize conserved pathogen-associated molecular patterns (PAMPs). One major group of pattern-recognition receptors is the Toll-like receptors (TLRs), which transduce signals leading to the activation of NF-κB, which subsequently drive the induction of several pro-inflammatory cytokines and chemokines [10–12]. Apart from sensing exogenous ligands from microbial components, which is critical for pathogen elimination, TLRs are able to recognize endogenous ligands, such as heat shock proteins (HSPs) and extracellular matrix components, which may be called danger-associated molecular patterns (DAMPs), in analogy to PAMPs [13–17].

Thus far, more than 10 different TLRs with distinct ligand specificity have been identified [18]. In human skin, the distribution of TLRs is incompletely defined. Several studies demonstrated that keratinocytes display TLRs and respond to corresponding PAMPs by producing pro-inflammatory cytokines [19–22]. In contrast, the TLR expression pattern of primary human melanocytes has never been systematically examined.

In this study, we postulated that human epidermal melanocytes can respond to multiple TLR ligands of different sources through...
expression of various TLR family members. We here show that human melanocytes express functional TLRs 2–4, 7 and 9. Upon stimulation with TLRs 2–4, 7 and 9 ligands, melanocytes produce several pro-inflammatory cytokines and chemokines, with the activation of NF-κB signaling pathway. Thus, epidermal melanocytes can act as one of the early sensors of infection or cellular stress by up-regulating pro-inflammatory mediators upon TLRs ligation. This observation provides more evidences for identifying melanocytes as potential immunocompetent cells, establishing a new aspect of melanocyte biology.

2. Materials and methods

2.1. Primary human melanocyte cultures

Primary human melanocyte cultures were obtained from neonatal foreskin. All samples obtained were from surgical procedures with the patients’ informed consent following a protocol approved by the Huashan Hospital Fudan University Institutional Review Board. The epidermis was separated from the dermis after an overnight incubation of skin samples in a 0.25% Dispase solution (Sigma–Aldrich, USA) in PBS at 4 °C. In order to separate cellular elements, epidermal sheets were incubated at 37 °C in a solution of 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS, for 10 min. Cellular suspension was then filtered through 70 μm cell strainer (BD Falcon) and then centrifuged at 1500 rpm for 7 min to harvest cells. Then, melanocytes were selectively grown in a defined medium M254 with PMA-free human melanocyte growth supplements (Cascade Biologics, USA). The cells were maintained in a humidified incubator with 5% CO2 at 37 °C and were fed every 3–4 d, and further passaged at 1:2 when they became 80% confluent, with experiments being carried out from 3 to 4 passages.

2.2. Analysis of TLR mRNA expression in cultured human melanocytes by reverse transcription polymerase chain reaction (RT-PCR)

Melanocytes were plated at the concentration of 5 × 10^5 cells/well (6-well plates, Corning-Costar, USA), and cultured at least for 48 h before the cells were lysed. Melanocyte total RNA was purified by using the RNeasy Mini kit (Qiagen, German) according to the manufacturer’s instructions. Potential DNA contamination in the RNA sample was removed by RNase-Free DNase treatment. 1 μg of DNase-treated total RNA was reverse transcribed using the first-strand cDNA synthesis kit for RT-PCR (TaKaRa, Japan). TLRs 1–10, keratin 10, keratin 14, ASO2 and GAPDH were amplified using ExTaq DNA polymerase (Takara). The primer sequences used in this RT-PCR analysis were detailed in Table 1. PCR assay cycles were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The PCR products were visualized on 2% agarose gels and ethidium bromide staining.

2.3. Determination of TLR expression in human melanocytes by flow cytometry

Flow cytometry was performed to investigate the expression of TLRs 2–4, 7 and 9 in human melanocytes and the specific cellular localization of TLR3. For surface TLRs 2–4 staining, 2 × 10^6 human melanocytes were incubated with mouse anti-human TLR2 mAb (ebioscience, USA), mouse anti-human TLR3 mAb (ebioscience), mouse anti-human TLR4 mAb (ebioscience), or isotype control mouse IgG (ebioscience) for 1 h at 4 °C. For intracellular TLRs 3, 7 and 9 staining, 2 × 10^6 melanocytes were pretreated with fixation/ permeabilization solution (BD Pharmingen, USA) for 20 min at 4 °C, washed once with PBS, and then incubated with the mouse anti-human TLR3 mAb (ebioscience), mouse anti-human TLR7 mAb (Abcam, United Kingdom), mouse anti-human TLR9 mAb (Abcam) or isotype control mouse IgG for 1 h at 4 °C. For secondary antibody reaction, melanocytes were then washed twice and incubated with FITC-conjugated rat anti-mouse IgG (Jackson ImmunoResearch) for 1 h at 4 °C. Cells were then again washed twice and analyzed by a flow cytometer equipped with the manufacturer’s software (CellQuest; Becton Dickinson) for data acquisition and analysis.

2.4. Immunofluorescent staining

Human melanocytes were seeded at the concentration of 3 × 10^5 cells/well (6-well plates) onto gelatin-coated chamber slides, and cultured at least for 48 h before conducting experiments. Cells were fixed in PBS containing 3.7% paraformaldehyde and permeabilized by incubation with PBS containing 0.2% Triton X-100 and 10% PBS for 20 min at room temperature. The cells were then incubated overnight at 4 °C with primary antibodies: anti-TLR2 mAb, anti-TLR3 mAb, anti-TLR4 mAb, anti-TLR7 mAb and anti-TLR9 mAb. After several washes with PBS, FITC-conjugated rat anti-mouse IgG at a dilution of 1:100 was added and incubated for 1 h at 37 °C. The nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI). Then the slides were mounted and observed under fluorescence microscope (Nikon).

2.5. Determination of cytokine production by ELISA

Human melanocytes were plated at the concentration of 1 × 10^6 cells/200 μl (96-well plates; Costar), and cultured at least for 48 h before conducting experiments. To induce cytokine production, melanocytes were cultured in the presence of different concentrations of PGN (1, 10, 100 μg/ml), poly(I:C) (0.1, 1, 10 μg/ml), LPS (1, 10, 100 μg/ml), imiquimod (1, 10, 100 μg/ml) or CpG

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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</tr>
<tr>
<td>TLR2</td>
<td>5′-TCT GAA GTC CAG CTC ACC CT-3′</td>
</tr>
<tr>
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<td>5′-AAC CTT AGG GGA AAC ATC TCT-3′</td>
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<td>5′-GGA ATA TGC AGC CTC GGC AT-3′</td>
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<td>5′-GAA ATG GAG GCA CCC CTC-3′</td>
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<tr>
<td>β-Actin</td>
<td>5′-GCA TCC GTC CCT ACC GT-3′</td>
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Table 1

The sequence of primers used for RT-PCR and real-time PCR.
2006 (5′-GGG GGG AGC ATC GTC GGG GGG-3′) (0.5, 1, 5 μM; Invivogen, USA). After stimulation for 48 h with TLR ligands, the melanocytes culture supernatants were harvested and tested by ELISA (R&D Systems) for IL-8 and IL-6, following the manufacturer’s instructions.

2.6. Quantification of chemokine mRNA by real-time RT-PCR

Melanocytes were plated at the concentration of 5 × 10^5 cells/well (6-well plates), and cultured at least for 48 h before conducting experiments. To induce chemokine mRNA expression, melanocytes were cultured for 6 or 24 h in the presence of PGN (100 μg/ml), poly (I:C) (10 μg/ml), LPS (100 μg/ml), imiquimod (100 μg/ml) or CpG 2006 (5 μM). Then the cells were lysed and total RNA was purified using the RNeasy Mini kit. For real-time RT-PCR analyses, 1 μg of DNase-treated total RNA was reverse transcribed. The amplification of the cDNA was accomplished using the ABI Prism 7900HT sequence detection system (Applied Biosystems) in the presence of the commercially available SYBR Green PCR Master Mix (Takara) in a 40-cycle PCR. The denaturing, annealing and extension conditions of each PCR cycle were 95 °C for 5 s, 60 °C for 20 s and 72 °C for 34 s, respectively. The relative expression was calculated using the 2^−ΔΔCT method. The mRNA levels of each target gene were normalized to the levels of β-actin and were represented as fold induction. The primer sequences of for real-time PCR were shown in Table 1.

2.7. Western blotting

Human melanocytes were plated at the concentration of 1 × 10^6 cells/well (6-well plates), and cultured at least for 48 h before the cells were stimulated. 6 h after stimulation with PGN (100 μg/ml), poly (I:C) (10 μg/ml), LPS (100 μg/ml), imiquimod (100 μg/ml) and CpG 2006 (5 μM), melanocytes were washed twice with ice-cold PBS and then lysed in 2 × SDS-PAGE sample buffer. The melanocytes lysates were loaded on a 12% polyacrylamide gel and separated under reducing conditions, using Rainbow-colored protein molecular weight markers (Amersham, USA) as a reference. At the end of the electrophoresis, the gel was soaked in Western transfer solution (39 mM glycine, 48 mM Tris–base, 20% methanol, pH 8.0) for 10 min before electrophoretic transfer of the protein onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) at 125 V for 120 min. The membrane was blocked for 2 h in TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% dried skimmed milk powder. After three washes in TBST for 15 min, the membranes were incubated with 2 μg/ml rabbit anti-human phospho-IκBα (Abcam) primary antibody in TBST containing 5% dried skimmed milk powder for 1 h at room temperature followed by three washes with TBST. The membranes were then incubated with a 1:1000 dilution of HRP-conjugated goat anti-rabbit IgG antibody (eBioscience, USA) for 1 h at room temperature before being washed three times and developed with the ECL Western blotting system (Pierce, USA) according to the supplier’s recommendations. The membranes were then exposed to X-ray film which was subsequently developed.

2.8. Detection of NF-κBp65 nuclear translocation by immunofluorescence analysis

Human melanocytes were seeded at the concentration of 3 × 10^5 cells/well onto gelatin-coated chamber slides, and cultured at least for 48 h before conducting experiments. To induce nuclear translocation of NF-κB, melanocytes were cultured for 4 h in the presence of PGN (100 μg/ml), poly (I:C) (10 μg/ml), LPS (100 μg/ml), imiquimod (100 μg/ml) or CpG ODN 2006 (5 μM). After removal of the culture medium, cells were then washed twice with PBS, and fixed in PBS containing 3.7% paraformaldehyde for 10 min at room temperature. Cells were washed again with PBS and permeabilized by incubation with PBS containing 0.2% Triton X-100 and 10% fetal calf serum for 10 min at room temperature. After washing in PBS, the slides were incubated (30 min at room temperature) with a rabbit anti-human NF-κBp65 antibody (2 μg/ml, sc-109, Santa Cruz Biotechnology) diluted in PBS/1% fetal calf serum. The cells were then washed with in PBS, followed by 30 min incubation with a Cy3-conjugated goat anti-rabbit IgG (Abcam) at room temperature in the dark. After washing in PBS, the slides were counter-stained with DAPI in order to visualize nuclei, and then the cells were observed and photographed with a fluorescence microscope.

2.9. Statistical analysis

Results are expressed as the mean ± (S.D.). A Student’s t-test was used to determine significance among the groups. A value of p < 0.05 was considered significant. Analyses and graphical representation were performed using GraphPad Prism 5.01 software (Graphpad).

3. Results

3.1. Human melanocytes constitutively express mRNA for TLRs 1–4, 6, 7 and 9, but not TLRs 5, 8 and 10

To investigate the expression profile of TLRs in human melanocytes, in vitro-expanded melanocytes obtained from healthy subjects were assessed by reverse transcription (RT)-PCR for TLR mRNA using a panel of specific primers for TLRs 1–10. We found the constitutive mRNA expression of TLRs 1–4, 6, 7 and 9 in human melanocytes but not TLRs 5, 8 and 10 (Fig. 1A). As a positive control, human PBMC were shown to express all mRNA of TLRs 1–10 (Fig. 1B). The purity of the cultured human melanocytes was verified by RT-PCR analysis for the keratinocyte markers, such as keratin 10 and keratin 14, and the fibroblast marker ASO2, showing that contamination from keratinocytes or fibroblasts was negligible (Fig. 1C).

3.2. Human melanocytes are immunopositive for TLRs

To measure the TLR protein expression in human melanocytes, flow cytometry and immunofluorescence assays were performed with specific antibodies to human TLR proteins. Ample amounts of TLRs 2, 4, 7 and 9 were detected in human melanocytes indicated by flow cytometry analysis (Fig. 2A). TLR3 is generally recognized as an intracellular receptor, but a recent study showed a surface expression of TLR3 on human skin and lung fibroblasts [23]. Thus, we analyzed the specific location of TLR3 expression in human melanocytes. Using the same mAb specific to TLR3, we demonstrated the expression of intracellular TLR3 in human melanocytes but not on the cell surface (Fig. 2B). The protein expression of TLRs on in vitro-cultured human melanocytes was also demonstrated by immunofluorescence assays, and consistent findings were noted. Compared to the isotype control, a clear positive staining for TLRs 2–4, 7 and 9 was obtained (Fig. 2C). These results paralleled our RT-PCR findings. Thus human melanocytes not only expressed TLRs mRNA but also expressed TLRs protein.

3.3. TLR stimulation in human melanocytes triggers significant increases in pro-inflammatory cytokines and chemokines

To characterize the functional relevance of TLRs in human melanocytes, the production of inflammatory cytokines by melanocytes in response to TLR ligands were determined. Human melanocytes were stimulated with various TLR ligands, for example, The TLR2 ligand, peptidoglycan (PGN), the TLR3 ligand,
CCL2 and CCL5 were significantly induced by all the TLR ligands. mRNA for CCL3 was induced in 6-h stimulated melanocytes, whereas mRNA for IL-6 was only induced by PGN and poly (I:C) (Fig. 3A). Melanocytes, like keratinocytes, are known to produce IL-6 in response to TLR ligands. IL-6 production was observed at 1 h, with maximal secretion being observed at 8 h, indicating the involvement of a cytokine network in melanocyte activation.

Accumulation of mRNAs of inflammatory chemokines such as CCL2, CCL3, and CCL5 was observed in unstimulated melanocytes. The expression of these chemokines was significantly induced by all the TLR ligands, with maximum induction observed at 6 h (Fig. 4). All the TLR ligands induced significant amounts of CCL2 mRNA in 6-h stimulated melanocytes, whereas mRNA for CCL3 and CCL5 was induced by the TLR ligands. Although basal expression of CCL2, CCL3, and CCL5 could be observed in nonstimulated cells, there was at least 1-fold increase in their mRNA levels after treatment with TLR ligands.

Collectively, these data demonstrated that TLR ligand pretreatment of epidermal melanocytes created a pro-inflammatory milieu by elevating cytokines and chemokines.

3.4. TLRs 2–4, 7 and 9 triggering results in phosphorylation of IκBα and nuclear translocation of the NF-κB subunit p65

Activation of NF-κB plays a central role in TLR-mediated cellular activation and gene expression in a variety of cell types. Therefore, we tested whether triggering TLRs in human melanocytes can give rise to the activation of NF-κB signaling pathway. TLRs within human melanocytes were stimulated for 6 h by various ligands and assessed by Western blot analysis to examine phosphorylation of IκBα, which is one of the markers of NF-κB activity. No phosphorylated-IκBα was observed in unstimulated melanocytes. On the contrary, the clear bands of phosphorylated-IκBα could be induced by PGN (100 μg/ml), poly (I:C) (10 μg/ml), LPS (100 μg/ml), imiquimod (100 μg/ml) or CpG 2006 (5 μM) to different degrees, indicating that NF-κB pathway participated in TLRs 2–4, 7 and 9 signaling in response to the respective PAMPs (Fig. 5A).

To further substantiate the activation of NF-κB pathway in TLR ligand-treated melanocytes, translocation to the nucleus of the NF-κB p65 was analyzed. Human melanocytes were cultured in slide chambers in the presence of TLR ligands and stained for the NF-κB p65 as described in Section 2. The slides were then analyzed for nuclear localization by fluorescence microscopy. In unstimulated melanocytes, the stainings for the NF-κB p65 were clearly visible in the cytoplasm. In contrast, when melanocytes were stimulated with PGN (100 μg/ml), poly (I:C) (10 μg/ml), LPS (100 μg/ml), imiquimod (100 μg/ml) or CpG 2006 (5 μM), a clear nuclear translocation of the p65 subunit was observed in all the stimulated groups (Fig. 5B). These data indicated that TLRs 2–4, 7 or 9 ligand engagement led to the activation of NF-κB, which explained the numerous pro-inflammatory genes being activated.

4. Discussion

As TLRs are key players in the innate response to pathogens, the expression and function of TLRs at sites of host–pathogen interaction is critical for host defense. Given that the skin is another crucial interface and physical barrier where host encounters with microbial invaders, it seems appropriate that the skin express TLR to accomplish its job as a barrier to infection. Previous studies have demonstrated that human keratinocytes express TLRs 1–6 and 9. In addition, some of these studies have demonstrated that TLRs are not merely present on keratinocytes but may be active participants in cutaneous defense through triggering NF-κB activation and thus production of cytokines and chemokines. Induction of chemokines and cytokines through TLR activation promotes the recruitment of immune cells out of the circulation to sites of infection, such as the skin, and the modulation of immune cell behavior. Contrary to keratinocytes, the possible role of melanocytes in the overall skin defense system is far from being clarified. Both largely anecdotal medical opinions and published studies noted that darkly pigmented individuals are less susceptible to skin infections than their lightly pigmented counterparts. This phenomenon implied a strategic role played by melanocytes in skin defense system. It was reported that darkly pigmented melanocytes could further acidify the outer epidermis, contributing to enhanced cutaneous antimicrobial defense of darkly pigmented skin. Melanocytes, like Langerhans cells, are dendritic cells with a large surface area, which could possibly serve...
both in antigen-processing and as microbial scavengers. Melanocytes synthesize primary cytokines, and are thus capable of eliciting a local immune response. Moreover, radicals and other compounds produced during melanogenesis are believed to exert strong antimicrobial activity. In this study, we found melanocytes grown in vitro expressed a panel of functional TLRs, it is possible now that many of these immunological properties of melanocytes are related to the activation of TLRs.

TLR2 participates in recognizing a broad range of PAMPs including PGN and phenol-soluble modulin from Gram-positive bacteria, bacterial lipoprotein/lipopeptide, yeast cell wall particle zymosan, and mycobacterial lipoarabinomannan [28–31]. Whereas TLR2 alone recognizes PGN, TLR1 and TLR6 were shown to interact functionally with TLR2 in the recognition of certain TLR2 ligands. The primary lesion of pityriasis versicolor caused by Malassezia is hypopigment and/or hyperpigment. The mechanisms by which the yeasts cause these abnormalities, however, are not yet clear. Components of lipophilic yeast Malassezia furfur can serve as the ligands for TLR2 [32], so it will be intriguing to explore the relation between the Malassezia infection and melanocyte biology especially melanogenesis.

TLR3 constitutes a separate subfamily within the mammalian TLRs that is characterized by intracellular expression. TLR3 activation was achieved by the TLR3 ligand poly (I:C) a synthetic analogue of double-stranded viral RNA. It will be interesting to differentiate whether the poly (I:C) mediated effects can solely be attributed to TLR3 or are also dependent on double-stranded RNA-activated protein kinase, an interferon-γ (IFN-γ)-induced protein constitutively expressed in some cell types, such as monocytes and keratinocytes [33,34]. Human melanocytes might possibly sense

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**Fig. 2.** Expression of TLRs in human melanocytes at the protein level. (A) Melanocytes were incubated with anti-TLR2, anti-TLR4, anti-TLR7 or anti-TLR9 mAb (solid line), with shaded histograms indicating cells stained with isotype control mouse IgG. After incubation with FITC-conjugated rat anti-mouse IgG, the cells were then detected by flow cytometric analysis. Representative data from three independent experiments are shown. (B) To localize the TLR3 in human melanocytes by flow cytometric analysis, melanocytes were stained for cell surface or intracellular TLR3 with mAb against TLR3 (solid line), and then detected using FITC-conjugated rat anti-mouse IgG. Shaded histograms represent cells stained with isotype control mouse IgG as the primary antibody. Representative data from three independent experiments are shown. (C) TLRs 2, 4, 7 and 9 were detected by indirect immunofluorescence, using anti-TLR2, anti-TLR3, anti-TLR4, anti-TLR7, anti-TLR9 or isotype control mouse IgG as primary antibody, and FITC-conjugated rat anti-mouse IgG as secondary antibody. The nuclei were counter-stained with DAPI. Melanocytes were then analyzed by fluorescence microscopy. Original magnification 200×. The data shown are representative of four experiments.
viral infections through their functionally active TLR3 receptors which enable the initiation of an immediate innate and delayed adaptive immune response to viruses infecting the skin. Vitiligo results from a progressive destruction of melanocytes by an autoimmune process. Although a genetic susceptibility is important for disease development, environmental factors may also be indispensable for the initiation and/or progression of vitiligo. Viral infections have been implicated in the pathogenesis of vitiligo [35,36]. It can be speculated that viruses may directly infect and destroy melanocytes or they may trigger (or contribute to) melanocyte-specific autoimmunity with or without melanocyte infection. In line with this hypothesis, TLR3 in melanocytes may sense viral infection and thus trigger apoptosis of melanocytes and local production of cytokines and chemokines, which will contribute to the development of vitiligo.

It has been reported functionally active LPS receptor proteins TLR4 and CD14 were expressed by human melanocytes [37,38]. However, these reports mainly focused on the role of TLRs in melanogenesis of melanocytes, and they did not focus on the role of TLRs in the context of local innate inflammatory responses in epidermis. In addition to LPS, TLR4 also recognizes other ligands such as stress-induced members of the endogenous heat-shock protein family, for example, HSP70. In vitro findings show that when released by distressed or necrotic cells, HSP70 acts like a danger signal by enhancing maturation of DCs, showing that HSP70 can be a trigger for immune activation [39]. It was

Fig. 3. Production of IL-8 and IL-6 by melanocytes after stimulation with various TLR ligands. Melanocytes were cultured in the presence of different concentrations of PGN (1, 10, 100 μg/ml), poly (I:C) (0.1, 1, 10 μg/ml), LPS (1, 10, 100 μg/ml), imiquimod (1, 10, 100 μg/ml) or CpG 2006 (0.5, 1, 5 μM) for 48 h, then the supernatants were collected and measured for IL-8 (A) and IL-6 (B) by ELISA. Results, expressed as mean ± (S.D.) of triplicate cultures, are from one experiment representative of five. Statistical significance analyzed for samples compared with the untreated control samples (*p < 0.05, **p < 0.01).

Fig. 4. Kinetics of CCL2, CCL3 and CCL5 mRNA accumulation in melanocytes stimulated with different TLR ligands. Melanocytes were cultured for 6 or 24 h in the presence of PGN (100 μg/ml), poly (I:C) (10 μg/ml), LPS (100 μg/ml), imiquimod (100 μg/ml) or CpG 2006 (5 μM), total RNA was then interrogated in real-time RT-PCR analyses for the chemokine CCL2 (A), CCL3 (B) and CCL5 (C) expression. The level of each chemokine mRNA was adjusted by the amount of β-actin mRNA. Chemokine/β-actin mRNA levels in unstimulated melanocytes were defined as 1. Experiments were done in triplicate and data shown are means ± (S.D.). Statistical significance analyzed for samples compared with the untreated control samples (*p < 0.05, **p < 0.01).
previously reported that vitiligo melanocytes could release HSP70 in response to 4-TBP [40]. In an autocrine manner, HSP70 may enhance the antigen-presenting and cytokine-producing ability of melanocytes by activating the TLR4. This hypothesis supports and expands the so-called 'Danger Model' proposed by Matzinger [41]. In this light, it is tempting to speculate that an innate immune response of melanocytes in response to endogenous TLR ligands plays a role in the initiation of autoimmune melanocyte destruction in vitiligo.

It has been shown that normal human melanocytes express TLR7 and imiquimod, a TLR7 agonist, inhibits melanogenesis and proliferation of the cells [42]. Our data confirmed the expression of TLR7 in human melanocytes and the corresponding ligand, imiquimod, induced NF-κB activation and subsequent gene transcription of NF-κB-sensitive genes.

As for TLR9 and reactivity to immunostimulatory CpG ODNs, the vast majority of studies have focused on the expression and function of TLR9 in professional immune cells such as B cells and plasmacytoid dendritic cells [43]. To date, relatively few studies have reported activation of nonimmune cells by CpG ODNs via TLR9. Recently, it has been reported the known stimulatory effect of CpG ODNs on immune cells did not apply to human keratinocytes, which have been found to express TLR9. The authors demonstrated that ODNs had a suppressive effect on IL-8 production of keratinocytes in vitro and offer anti-inflammatory properties in vivo [44]. It appears that keratinocytes may possess a TLR9-independent, as-yet-undefined intracellular recognition machinery of DNA that induces distinct signaling pathways. However, our data demonstrated that melanocytes seemed to share more similarities with professional antigen-presenting cells than keratinocytes in the responsiveness to CpG ODN.

In summary, this study provides evidence for a TLR expression and response profile of normal human melanocytes, which stress the importance of the melanocytes not only as pigment cells but also as sentinels of skin homeostasis. Further studies will have to elucidate the particular role and signaling response mediated by the various TLRs expressed by human melanocytes for the innate cutaneous immune response.

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