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Human Langerhans Cells Express a Specific TLR Profile and Differentially Respond to Viruses and Gram-Positive Bacteria[1]

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Dendritic cells (DC) are APCs essential for the development of primary immune responses. In pluristratified epithelia, Langerhans cells (LC) are a critical subset of DC which take up Ags and migrate toward lymph nodes upon inflammatory stimuli. TLR allow detection of pathogen-associated molecular patterns (PAMP) by different DC subsets. The repertoire of TLR expressed by human LC is uncharacterized and their ability to directly respond to PAMP has not been systematically investigated. In this study, we show for the first time that freshly purified LC from human skin express mRNA encoding TLR1, TLR2, TLR3, TLR5, TLR6 and TLR10. In addition, keratinocytes ex vivo display TLR1–5, TLR7, and TLR10. Accordingly, highly enriched immature LC efficiently respond to TLR2 agonists peptidoglycan and lipoteichoic acid from Gram-positive bacteria, and to dsRNA which engages TLR3. In contrast, LC do not directly sense TLR7/8 ligands and LPS from Gram-negative bacteria, which signals through TLR4. TLR engagement also results in cytokine production, with marked differences depending on the PAMP detected. TLR2 and TLR3 ligands increase IL-6 and IL-8 production, while dsRNA alone stimulates TNF-α release. Strikingly, only peptidoglycan triggers IL-10 secretion, thereby suggesting a specific function in tolerance to commensal Gram-positive bacteria. However, LC do not produce IL-12p70 or type I IFNs. In conclusion, human LC are equipped with TLR that enable direct detection of PAMP from viruses and Gram-positive bacteria, subsequent phenotypic maturation, and differential cytokine production. This implies a significant role for LC in the control of skin immune responses. *The Journal of Immunology,* **2006, 177: 7959–7967.**

Dendritic cells (DC)[5] are APCs that are required for initiation of specific T cell-driven immune responses (1). DC residing in nonlymphoid tissue, such as Langerhans cells (LC) in the epidermis, are immature cells whose primary function is to capture Ag. This function is principally achieved through specialized surface-membrane endocytic structures. Concomitant with Ag processing in specialized organelles of the endocytic pathway, DC, upon exposure to pathogen-associated molecular patterns (PAMP) (2), undergo a series of phenotypic modifications, termed maturation, and migrate to secondary lymphoid tissue. The maturation of DC results in expression of surface MHC class II and costimulatory molecules, which eventually translates into highly efficient presentation, by the appropriate MHC molecules, of processed Ag to T cells (1).

The TLR represent a family of germline-encoded type I transmembrane proteins which are essential to the recognition of PAMP by the innate immune system (3). Ten human TLR have been described so far. Upon engagement of TLR, signal transduction events are initiated by the cytoplasmic Toll/IL-1R domain. Depending on which TLR is engaged, a specific set of transcription factors (including AP-1, IFN response factors, and NF-κB) is activated, subsequently inducing expression of proinflammatory cytokines, chemokines, and costimulatory molecules (4, 5).

TLR recognize a wide array of molecules whose origin can be bacterial, viral, or parasitic. TLR2 and its associated receptors TLR1 and TLR6 are mainly involved in the detection of molecules from mycobacteria and Gram-positive bacteria (6, 7). Some PAMP, such as lipoteichoic acid (LTA), only require TLR2 (7). In contrast, TLR1 and TLR2 together detect triacylated lipoproteins (8), whereas TLR2 associates with TLR6 to recognize diacylated lipoproteins and peptidoglycans (PGN) (9, 10). Like TLR1 and TLR6, TLR10 is highly homologous to TLR2 and is probably another TLR2-associated receptor, but its function is still unknown (11). TLR4 is the receptor for LPS, a characteristic component of the cell wall of Gram-negative bacteria (12). Flagellin, a protein typical of flagellated bacteria, is specifically identified by TLR5 (13). Finally, TLR3, TLR7, TLR8, and TLR9 are intracellular receptors for nucleic acids (4). Indeed, when detected in vesicular compartments where these TLR reside, RNA and DNA characterize bacterial uptake or viral infection. TLR3 recognizes dsRNA which constitute a viral genome or are generated during viral replication. TLR7 and TLR8 are engaged by viral ssRNA and by synthetic small molecules mimicking features of nucleic acids, such as imiquimod and R-848. Finally, TLR9 recognizes oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (CpG

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[5] Abbreviations used in this paper: DC, dendritic cell; LC, Langerhans cell; PAMP, pathogen-associated molecular pattern; LTA, lipoteichoic acid; PGN, peptidoglycan; ODN, oligodeoxynucleotide; mDC, myeloid DC; pDC, plasmacytoid DC; rRNA, ribosomal RNA; CBA, cytometric bead array; PI, propidium iodide.

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ODN), which are underrepresented in mammalian genomes, but abundant in viral and bacterial DNA.

Hence, TLR have the capacity to directly initiate maturation of DC exposed to potentially pathogenic microorganisms (2). Interestingly, in humans, striking differences exist in TLR expression from one DC subset to another. In the blood, DC of myeloid origin (mDC) display TLR1–6, TLR8, TLR10, and low levels of TLR7, whereas plasmacytoid DC (pDC), which are the main producers of type I IFNs following viral challenge, only express TLR1, TLR6, TLR10, and very high levels of TLR7 and TLR9 (14–17). This restricts their reactivity to a defined pattern of PAMP and, consequently, pathogens.

The distribution of TLR in human skin is incompletely defined. Several studies demonstrated that keratinocytes differentiated in vitro display TLR and respond to corresponding PAMP by producing proinflammatory cytokines (18–22). Some discrepancies remain, though, which likely result from differences in culture conditions. In contrast, the TLR expression pattern of freshly isolated human LC has never been systematically examined (23). Moreover, because ex vivo purification of LC by positive selection of $CD1a^+$ cells from human skin is sufficient to drive a significant spontaneous maturation (24), it has been proven difficult to monitor PAMP-induced maturation of fresh LC (25). In this report, we analyze the expression pattern of the 10 identified human TLR in freshly isolated LC and keratinocytes. Furthermore, an extraction procedure based on gradient density centrifugation allowed us to avoid spontaneous phenotypic maturation and to address the issue of maturation and cytokine production of LC exposed to defined PAMP known as TLR agonists.

Materials and Methods

Media and reagents

The medium used was RPMI 1640 with Glutamax (Invitrogen Life Technologies), supplemented with 10% heat-inactivated FBS (Flow Laboratories), penicillin, and streptomycin (referred to as complete medium). *Staphylococcus aureus* PGN was obtained from Fluka. Poly(I:C), *Escherichia coli* LPS (strain 011:B4), and *Bacillus subtilis* LTA were obtained from Invivogen Life Technologies. Imiquimod was purchased from Sequoia Research Products. CpG ODN 2216 was obtained from MWG Biotech. R-848 was synthesized at the Schering-Plough Research Institute. PGN, LTA, imiquimod, and R-848 were shown to be free of endotoxin, as determined by a *Limulus*-Amoebocyte Assay (BioWhittaker).

LC isolation from human skin

Human epidermal cell suspensions were obtained from normal skin of patients undergoing abdominal reconstructive plastic surgery after patients' informed consent and according to institutional guidelines. Skin was split-cut with a keratome set and the dermoepidermal slices were treated for 18 h at 4°C with 0.05% trypsin (Sigma-Aldrich) in HBSS without Ca^{2+} and Mg^{2+} (Seromed; Biochrom). The epidermis was detached from the dermis with fine forceps. Epidermal cell suspensions were obtained by subsequent tissue dissociation and filtration through sterile gauze. Basal keratinocytes were removed by adhesion on collagen-coated plates (Corning-Iwaki Glass). Enrichment was obtained by two consecutive density gradient centrifugations on Lymphoprep (Nycomed Pharma), which yielded 75.0 ± 8.4% $CD1a^+$ LC based on 10 experiments. For quantitative PCR and some activation experiments, LC were further purified by positive selection using anti-CD1a MACS (Miltenyi Biotec). The CD1a-negative fraction contained suprabasal keratinocytes. The LC fraction obtained by this technique was >95% positive for CD1a expression.

DC isolation from human blood

Blood samples of healthy donors were obtained according to institutional guidelines. PBMC were isolated by Ficoll-Hypaque centrifugation (Eurobio). The $CD19^+$ B cells and $CD14^+$ monocytes were removed from the sample by CD19 and CD14 MACS purification using MiniMACS separation columns (Miltenyi Biotec). The remaining cells were further enriched in MiniMACS columns using microbeads coated with anti-BDCA-1 or anti-BDCA-4 mAbs (BD Biosciences), thereby selecting mDC and pDC, respectively. Reanalysis of the sorted populations showed purity higher than 98%.

Analysis of TLR expression by real-time quantitative PCR

Total RNA was extracted using an RNeasy Mini kit (Qiagen) and treated with DNase I (Qiagen) for 15 min at room temperature. Then RNA was reverse transcribed using a mix of random hexamer primers (Invitrogen Life Technologies), $oligo(dT)_{15}$ (Promega), and Superscript II RNase-H reverse transcriptase (Invitrogen Life Technologies). Real-time quantitative PCR was set up in an Icycler IQ (Bio-Rad), using TaqMan Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems), in a final volume of 25 μl (0.4 μM of each primer, 0.2 μM TaqMan probe, and 25 ng of cDNA). Experiments were performed in triplicates. Primers and probes of TLR and the CD4 promoter were designed using Primer 3 software (Whitehead Institute), with a melting temperature of ~60°C for primers and ~70°C for probes. Sequences and dyes are detailed in Table I. The absence of genomic DNA contamination was controlled using human CD4 promoter probe. cDNAs were normalized to 18S ribosomal RNA (rRNA) with the TaqMan Ribosomal RNA Control Reagents (Applied Biosystems). Real-time data were acquired and analyzed using Icycler IQ Optical System software (Bio-Rad) with automatic adjustment of the baseline and threshold parameters. Gene expression levels were determined using cycle threshold values, directly transferred on the PCR standard curve, and transformed into copy numbers. Results are expressed as copy numbers of a specified gene for 10^5 copies of 18S rRNA.

Activation of LC by TLR agonists

Freshly extracted LC were incubated for 24 or 48 h with TLR agonists or with CD40L transfected L cells. Briefly, 10^5 murine Ltk^- fibroblastic L cells stably transfected with the human *CD40L* gene (26) were irradiated at 7500 rad, then seeded together with 5×10^5 LC. Alternatively, LPS (1 μg/ml), PGN (10 μg/ml), LTA (10 μg/ml), poly(I:C) (25 μg/ml), R-848 (10 μM), imiquimod (25 μM), or CpG ODN 2216 (10 μg/ml) were added to the culture medium.

Cytofluorimetric analysis

Cells were labeled with FITC-conjugated anti-CD1a, -CD86, -CD80 (DakoCytomation), -CD25, -CD83 (Beckman Coulter). Indirect stainings with anti-DC-LAMP/CD208 (Beckman Coulter) or anti-TLR3 (clone 713E4; Abcys) were revealed by goat anti-mouse IgG-FITC (DakoCytomation). For intracytoplasmic phenotyping (DC-LAMP and TLR3), cells were stained in Fix&Perm reagent (DakoCytomation), following manufacturer's instructions. Negative controls were performed with isotype-matched control Abs. Fluorescence was determined with a FACScan flow cytometer (BD Biosciences) and data were analyzed using the CellQuest software (BD Biosciences).

Cytokine production

Freshly purified LC (10^6 cells/ml) were cultured for 24 or 48 h with TLR agonists at the indicated concentrations. As a control for IFN-α production, blood pDC (5×10^5 cells/ml) were incubated for 24 h with 20 ng/ml IL-3 (R&D Systems), or formaldehyde-inactivated influenza virus (five cases, 1 hemagglutinin unit/ml; strain A/New Caledonia/20/99 IVR116; Aventis-Pasteur). TNF-α, IL-6, IFN-α, and IFN-β levels were evaluated in the cell culture supernatants using commercially available ELISA kits (TNF-α and IL-6, R&D Systems; IFN-α, Abcys; IFN-β, Fujirebio). Cytokines were quantified relative to a standard curve representing a range of dilutions of recombinant cytokines. IL-10 and IL-8 levels were evaluated using cytometric bead array (CBA) assays (BD Biosciences). Following acquisition of sample data by flow cytometry, results were analyzed using the BD Biosciences CBA analysis software. Standard curves, plotted using the mean fluorescence intensity of the beads for cytokine values ranging from 0 to 5000 pg/ml, were used for quantifying cytokines.

Results

Isolation of immature human LC

To analyze LC activation, we used an isolation technique based on two consecutive density gradients, as previously described (24). This procedure permitted to obtain 75.0 ± 8.4% purity as revealed by CD1a staining. Contaminant cells appeared as large, CD1a-negative cells (Fig. 1*A*). Pluristratified epithelia such as the epidermis are constituted of basal, dividing keratinocytes and suprabasal, differentiated keratinocytes. As basal keratinocytes were

Table I. *Sequences of primers and probes used for real-time quantitative PCR*[a]

Gene	Forward Primer (5′-3′)	TaqMan Probe	Reverse Primer (5′-3′)
TLR1	CCC ATT CCG CAG TAC TCC AT	[5′FAM] AGC TCA AAA GTC TCA TGG CCA GGA GGA [3′TAM RA]	TTT TCC TTG GGC CAT TCC A
TLR2	CCC ATT GCT CTT TCA CTG CT	[5′FAM] GTA GTT GTG GGT TGA AGC ACT GGA CAA T [3′TAM RA]	CTT CCT TGG AGA GGC TGA TG
TLR3	TGG TTG GGC CAC CTA GAA GTA	[5′FAM] ACC TGG GCC TTA ATG AAA TTG GGC AA [3′TAM RA]	TCT CCA TTC CTG GCC TGT G
TLR4	CTG CAA TGG ATC AAG GAC CA	[5′FAM] AGG CAG CTC TTG GTG GAA GTT GAA CG [3′TAM RA]	TTA TCT GAA GGT GTT GCA CAT TCC
TLR5	TGC CTT GAA GCC TTC AGT TAT G	[5′HEX] CCA GGG CAG GTG CTT ATC TGA CCT TAA CA [3′TAM RA]	CCA CCA CCA TGA TGA GAG CA
TLR6	CCC TCA ACC ACA TAG AAA CG	[5′HEX] ACC GAC TTG GAA ATG CCT GGT CAG [3′TAM RA]	GAG ATA TTC CAC AGG TTT GG
TLR7	TTA CCT GGA TGG AAA CCA GCT ACT	[5′HEX] AGA TAC CGC AGG GCC TCC CGC [3′TAM RA]	TCA AGG CTG AGA AGC TGT AAG CTA
TLR8	AAC TTT CTA TGA TGC TTA CAT TTC TTA TGA C	[5′HEX] CCA AAG ATG CCT CTG TTA CTG ACT GGG TG [3′TAM RA]	GGT GGT AGC GCA GCT CAT TT
TLR9	TGA AGA CTT CAG GCC CAA CTG	[5′FAM] AGC ACC CTC AAC TTC ACC TTG GAT CTG TC [3′ TAM RA]	TGC ACG GTC ACC AGG TTG T
TLR10	TTT GAT CTG CCC TGG TAT CTC A	[5′HEX] AGG TCA ATG CAC ACA AAC ATG GCA CA [3′TAM RA]	AGT TGT TCT TGG GTT GTT TTC CTA AC
CD4 promoter	TTC CAC ACT GGG CCA CCT AT	[5′FAM] CAC TGG ACA CAA TTG CCC TCA GG [3′TAM RA]	TTG TGG GCT TAC CAC TGC TG

[a] The quencher dye, TAMRA, was placed at the 3′ end of probes. The reporter dyes, linked to the 5′ end, were HEX, FAM, or VIC (18S ribosomal RNA).

previously excluded by a collagen adhesion step, contaminant cells therefore represent differentiated suprabasal keratinocytes.

Propidium iodide (PI) stainings showed that 69.1 ± 10.1% of LC cultured for 24 h were viable, whereas <50% of LC were still viable after 48 h of culture (Fig. 1*A* and data not shown). In this context, we used 24 h of maturation and FACS results were gated on PI-negative cells.

Gradient-purified LC had an immature phenotype, as demonstrated by the lack of CD25, CD83, or CD80, and low CD86 staining at day 0. The intracellular maturation marker DC-LAMP was also absent (Fig. 1*B*). During extraction from the skin, disruption of critical interactions of LC with keratinocytes and degradation of the extracellular matrix may be directly or indirectly sensed by LC (27). These events are probably sufficient to initiate a spontaneous maturation, which is observed after 1 day of culture as judged by a limited up-regulation of LC surface markers. Indeed, following a 24 h incubation in medium alone, expression of the maturation markers CD83 and DC-LAMP, and the costimulatory molecules CD80 and CD86 was increased (Fig. 1*B*). Interestingly, CD25 was only slightly increased by the spontaneous maturation and was therefore of particular interest. Considering that CD86 was rapidly up-regulated in the majority of LC, we based our analysis of this marker on mean fluorescence intensity rather than percentages of positive cells. Due to biological variability, spontaneous maturation judged by the percentage of $CD83^+$ cells was observed on 51.8 ± 9.2% of LC after 24 h of culture. However, engagement of CD40 by CD40L-expressing fibroblasts resulted in an increased expression of costimulatory and maturation markers, showing that this modest spontaneous maturation does not preclude further activation of LC (Fig. 1*B*).

Freshly isolated LC are devoid of TLR4 and do not directly respond to LPS

Expression of TLR by DC is a key element to explain their capacity to respond to many danger signals. The constitutive low expression of TLR proteins (28) and the poorly defined specificity of most of the commercially available Abs against TLR proteins (personal observations) currently make PCR the most reliable way to systematically analyze expression of these receptors. Therefore, we evaluated mRNA expression of the 10 known TLR on LC by real-time quantitative PCR, using specific primers and probes (Table I). Of note, the high sensitivity of this technique enables the detection of very low levels of TLR mRNA, which may not be sufficient to express adequate amounts of functional protein. Because the pattern of expression of functional TLR was thoroughly described in human blood mDC and pDC (14–17), we used these DC populations as a reference to determine the level of significance of PCR results. Our data regarding TLR displayed by blood DC was consistent with the literature (see below).

Because primary keratinocytes also display TLR (18, 22, 29, 30), we used magnetic beads purification of $CD1a^+$ LC and investigated TLR expression of suprabasal keratinocytes (i.e., $CD1a^-$ fraction) in parallel. To avoid the strong spontaneous maturation that results from this isolation technique (Ref. 24 and our unpublished data), RNA extraction was performed immediately after purification.

Direct recognition of LPS by a given cell type requires expression of TLR4 (12). Keratinocytes and mDC expressed comparable levels of TLR4 transcripts (Fig. 2*A*), which suggests that keratinocytes, like mDC, respond to LPS (16, 31). In contrast, no TLR4 mRNA was detected in LC and pDC (Fig. 2*A*). To further demonstrate the absence of functional TLR4, gradient-purified LC were incubated for 24 h with LPS. Even in the presence of a high dose (1 μg/ml) of LPS, only marginal increases of maturation

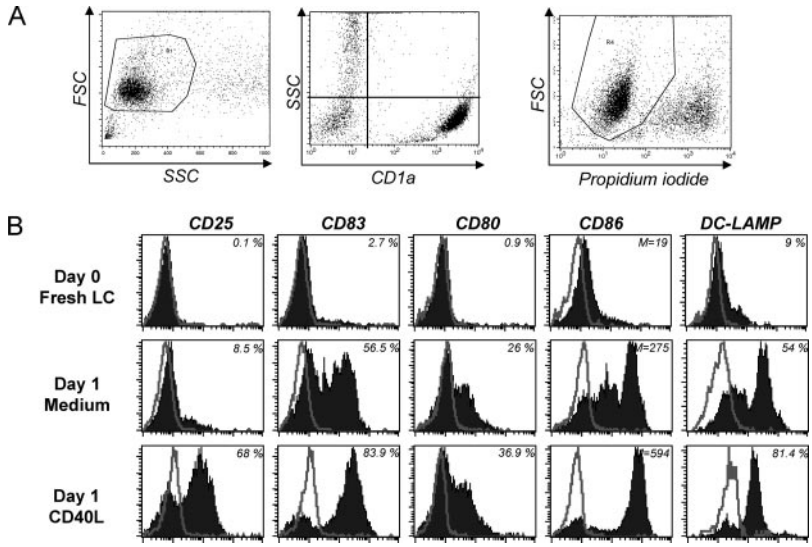

FIGURE 1. Immature LC purified ex vivo exhibit an immature phenotype and mature following CD40 triggering. LC were enriched by gradient purification as described in *Materials and Methods*, then analyzed by flow cytometry. *A*, Freshly isolated LC were gated as PI-negative, CD1a-positive cells. *B*, Freshly isolated LC were stained with anti-CD25, -CD83, -CD80, -CD86, or -DC-LAMP mAbs (filled histograms) or isotype-matched negative control Abs (open histograms). LC were also stained after 24 h of culture (day 1) in medium alone or in the presence of CD40L-expressing fibroblasts. Percentages of positive cells or mean fluorescence intensity (M) are shown in the *upper right corner* of the histograms. Results are representative of at least eight independent experiments.

markers (CD25, CD83, DC-LAMP) and of costimulatory molecules (CD80, CD86) were consistently observed (Fig. 2*B*). Therefore, we conclude that freshly isolated human LC are unable to directly sense LPS.

LC display TLR1, TLR2, and TLR6, and TLR2 agonists trigger LC maturation

We next investigated expression of TLR2 and its associated receptors, TLR1, TLR6, and TLR10, which are notably involved in the detection of Gram-positive bacteria (7). Real-time PCR analysis showed that LC expressed TLR1, TLR2, TLR6, and TLR10 mRNA, whereas keratinocytes displayed mRNA for TLR1, TLR2, and TLR10. It is noteworthy that LC had particularly high levels of TLR1 mRNA as compared with pDC and mDC (Fig. 3*A*), while TLR10 mRNA expression was much weaker in keratinocytes and LC than in both blood DC subsets. Expression of TLR2 mRNA in LC was weak, as compared with blood mDC, but was confirmed using different primer sets (data not shown and Ref. 23). Moreover, in line with the literature, TLR2 was not detectable in pDC (14–16). Therefore, we considered the low levels of TLR2 mRNA expressed by LC as significant. This was confirmed by exposure of LC to TLR2 agonists. Indeed, we cultured gradient-purified LC for 24 h in the presence of PGN and LTA and found that TLR2 agonist PGN reproducibly up-regulated expression of maturation markers, while LTA induced weaker activation, based on CD25 staining (Fig. 3*B*).

TLR3 is functionally expressed on purified LC

TLR3 is a receptor for dsRNA and is therefore thought to play a critical role in the detection of many viruses (4). Similar to Kadowaki et al. (15) who demonstrated that responsiveness of blood mDC to dsRNA correlates with TLR3 expression, we found that mDC, but not pDC, expressed high levels of this receptor. Strikingly, keratinocytes presented constitutive levels of TLR3 mRNA that were even higher than in mDC (Fig. 4*A*).

TLR3 mRNA expression in highly purified LC was 10-fold higher than in the dsRNA unresponsive pDC (Fig. 4*A*) (15). FACS analysis confirmed that TLR3 is expressed intracellularly in human LC (Fig. 4*B*). Finally, activation assays definitively stated that LC display functional TLR3: a particularly strong increase of CD25, CD83, CD80, CD86, and DC-LAMP expression was observed following 24 h of incubation with poly(I:C), a synthetic dsRNA analog (Fig. 4*C*). Thus, LC can be directly and potently activated by viral dsRNA.

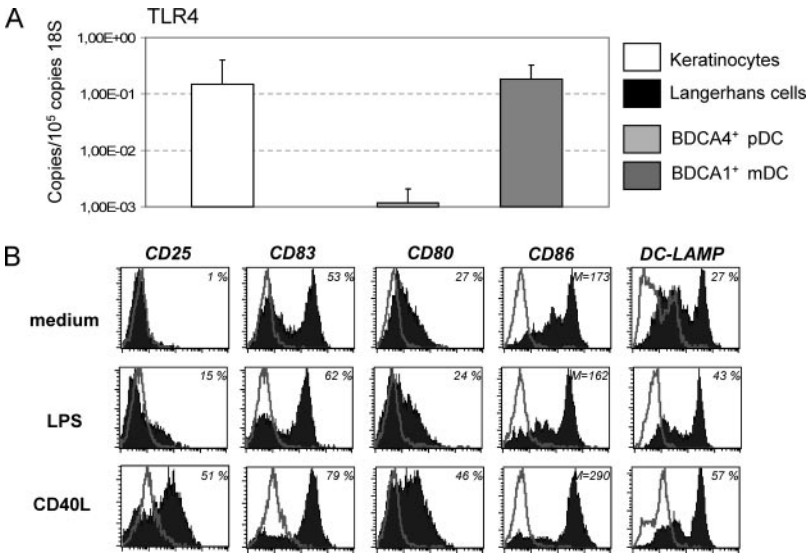

FIGURE 2. Human LC do not express TLR4 and are unresponsive to LPS. *A*, Real-time quantitative PCR for TLR4 was performed on cDNA obtained from keratinocytes, MACS-purified CD1a$^+$ LC, BDCA-4$^+$ blood pDC, and BDCA-1$^+$ blood mDC. Results were expressed as copy numbers for 10^5 copies of 18S rRNA. *B*, Gradient-purified LC were cultured in medium alone with 1 μg/ml LPS or with CD40L-transfected L cells. Twenty-four hours later, cells were harvested and stained with anti-CD25, -CD83, -CD80, -CD86, and -DC-LAMP mAbs (filled histograms) or isotype-matched negative control Abs (open histograms). Percentages of positive cells or mean fluorescence intensity (M) are shown in the *upper right corner* of the histograms. Results are representative of three independent experiments.

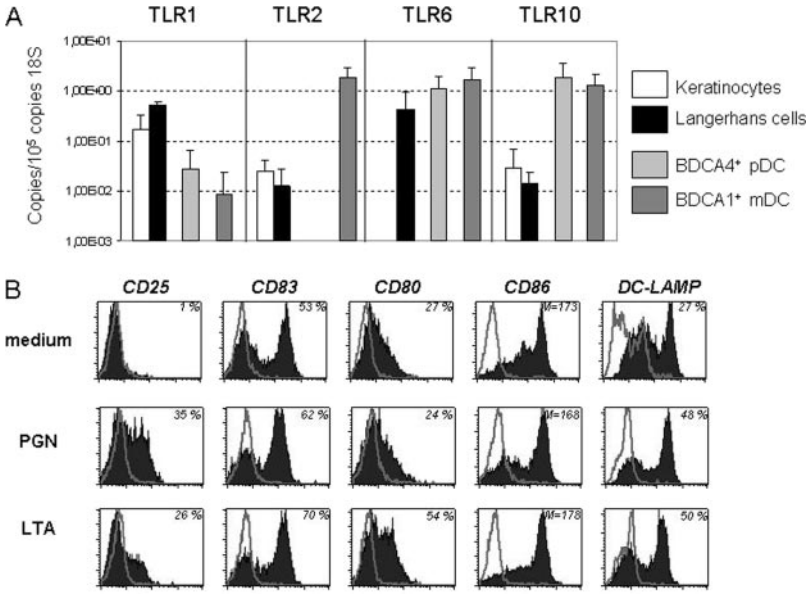

FIGURE 3. Human LC express TLR2 as well as accessory receptors TLR1 and TLR6 and mature in the presence of PGN and LTA. *A*, Real-time quantitative PCR for TLR1, TLR2, TLR6, and TLR10 were performed on cDNA obtained from keratinocytes, MACS-purified CD1a$^+$ LC, BDCA-4$^+$ blood pDC, and BDCA-1$^+$ blood mDC. Results were expressed as copy numbers for 10^5 copies of 18S rRNA. *B*, Gradient-purified LC were cultured in medium alone or with 10 μg/ml PGN or 10 μg/ml LTA. Twenty-four hours later, cells were harvested and stained with anti-CD25, -CD83, -CD80, -CD86, or -DC-LAMP mAbs (filled histograms) or isotype-matched negative control Abs (open histograms). Percentages of positive cells or mean fluorescence intensity (M) are shown in the *upper right corner* of the histograms. Results are representative of three independent experiments.

TLR7, TLR8, and TLR9 are absent from LC, while keratinocytes express TLR7

TLR7, TLR8, and TLR9 belong to a subgroup of the TLR family (32). This classification is primarily based on sequence similarity and closely related signal transduction pathways. Moreover, TLR7/8/9 are intravesicular receptors of nucleic acids (DNA or RNA oligonucleotides) or structurally related synthetic compounds (imiquimod, R-848).

Real-time PCR analysis revealed very low signals for TLR7 in LC; in contrast, keratinocytes exhibited ~10 times more mRNA for this receptor, which was present at levels comparable to mDC (Fig. 5*A*) (17). Consistent with this, the response of mDC and keratinocytes to the specific TLR7 agonist imiquimod was previously established (17, 33). TLR8 mRNA was detected in keratinocytes, LC, and pDC at comparable intensities (Fig. 5*A*). Nevertheless, pDC were frequently demonstrated to be devoid of TLR8 (14–17) and a TLR8-specific agonist is unable to activate pDC (34). Consequently, we considered the low levels of TLR8 expressed by keratinocytes and LC as nonsignificant. Finally, the last member of this subgroup, TLR9, was not detected in LC by real-time PCR and keratinocytes expressed TLR9 mRNA at levels similar to mDC, which are known to be unresponsive to CpG ODN (14–17) (Fig. 5*A*).

We next used activation assays to further establish the absence of functional TLR7, TLR8, and TLR9 in LC. For this purpose, we used synthetic agonists specific for TLR7 (imiquimod), for both TLR7 and TLR8 (R-848), or for TLR9 (CpG ODN 2216). As expected, expression of maturation markers was equivalent in LC cultured in medium alone or incubated with imiquimod, R-848, or CpG (Fig. 5*B*), thereby revealing that these cells do not express functional TLR7, TLR8, or TLR9 proteins.

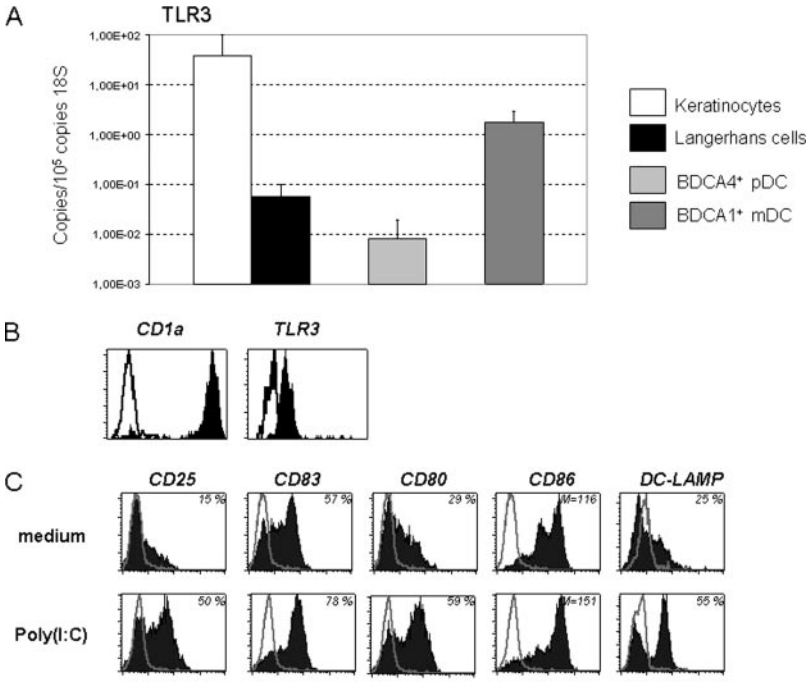

FIGURE 4. Human LC express TLR3 and mature in the presence of poly(I:C). *A*, Real-time quantitative PCR for TLR3 was performed on cDNA obtained from keratinocytes, MACS-purified CD1a$^+$ LC, BDCA-4$^+$ blood pDC, and BDCA-1$^+$ blood mDC. Results were expressed as copy numbers for 10^5 copies of 18S rRNA. *B*, Fresh, gradient-purified LC were stained with anti-CD1a or were permeabilized and stained with anti-human TLR3 (filled histograms). Open histograms represent isotype-matched negative control Abs. *C*, Gradient-purified LC were cultured in medium alone or with 25 μg/ml poly(I:C). Twenty-four hours later, cells were harvested and stained with anti-CD25, -CD83, -CD80, -CD86, or -DC-LAMP mAbs (filled histograms) or isotype-matched negative control Abs (open histograms). Percentages of positive cells or mean fluorescence intensity (M) are shown in the *upper right corner* of the histograms. Results are representative of at least five independent experiments.

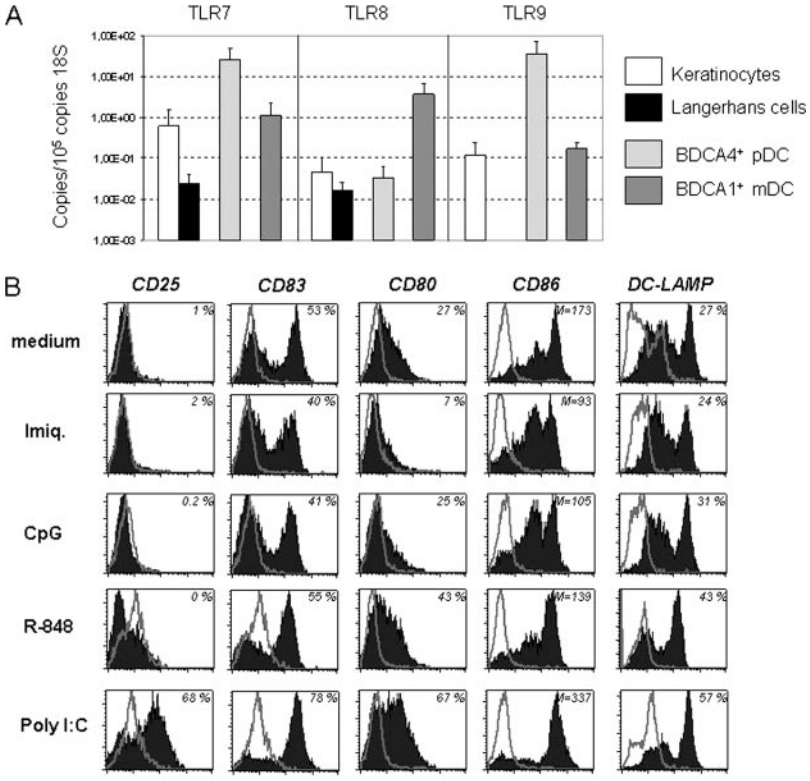

FIGURE 5. Human LC are devoid of TLR7, TLR8, and TLR9. *A*, Real-time quantitative PCR for TLR7, TLR8, and TLR9 were performed on cDNA obtained from keratinocytes, MACS-purified CD1a$^+$ LC, BDCA-4$^+$ blood pDC, and BDCA-1$^+$ blood mDC. Results were expressed as copy numbers for 10^5 copies of 18S rRNA. *B*, Gradient-purified LC were cultured in medium alone or with 25 μM imiquimod (Imiq.), 10 μM R-848, 10 μg/ml CpG ODN 2216 (CpG), or 25 μg/ml poly(I:C). Twenty-four hours later, cells were harvested and stained as indicated with anti-CD25, -CD83, -CD80, -CD86, or -DC-LAMP mAbs (filled histograms) or isotype-matched negative control Abs (open histograms). Percentages of positive cells or mean fluorescence intensity (M) are shown in the *upper right corner* of the histograms. Results are representative of at least five independent experiments.

LC produce cytokines following activation through TLR2 and TLR3

PAMP-driven maturation of DC is usually concomitant with the production of cytokines and chemokines (1, 2). Therefore, we examined the production of soluble factors induced by TLR ligands in gradient-purified LC to address possible differences in their respective activation capacity.

LC released substantial amounts of IL-6 when stimulated with PGN, LTA, and, even more potently, poly(I:C) (Fig. 6*A*). A modest secretion of IL-6 was detected following treatment of gradient-purified LC with LPS and imiquimod. As LC do not undergo noticeable maturation in response to these ligands, this low IL-6 production might be attributable to the few contaminating keratinocytes, which

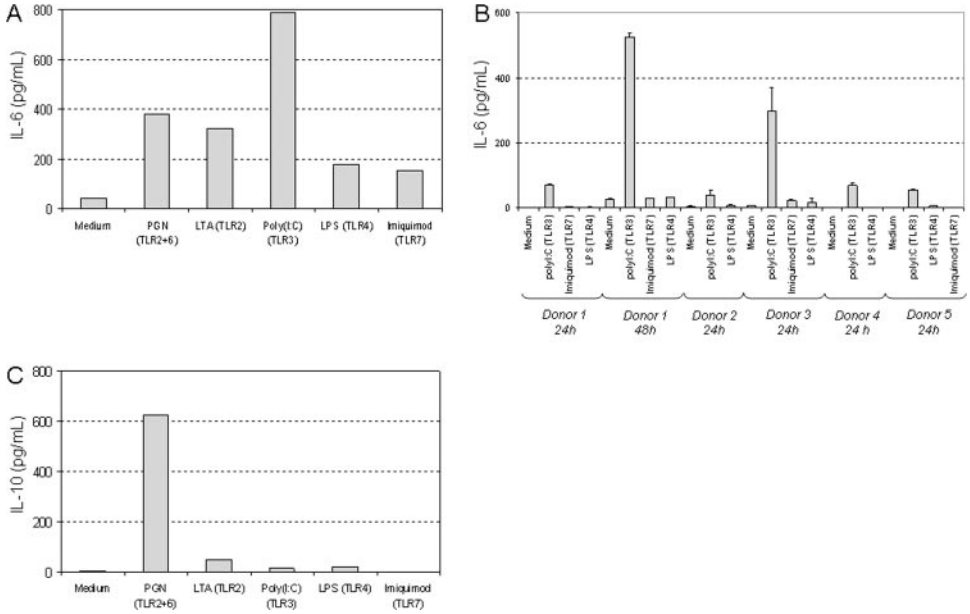

FIGURE 6. Production of IL-6 and IL-10 by human LC activated by TLR agonists. *A* and *C*, Gradient-purified LC were cultured in medium alone or with 10 μg/ml PGN, 10 μg/ml LTA, 25 μg/ml poly(I:C), 1 μg/ml LPS, or 25 μM imiquimod. Twenty-four hours later, supernatants were harvested and cytokine production was quantified by CBA assay (*A*, IL-6; *C*, IL-10). Note that, due to low numbers of LC yielded, CBA experiments were not duplicated within each skin sample, but results are representative of at least four independent experiments. *B*, Production of IL-6 by MACS-purified LC activated by TLR agonists was also analyzed on five independent donors. CD1a$^+$ LC were cultured in medium alone or with 25 μg/ml poly(I:C), 1 μg/ml LPS, or 25 μM imiquimod. Twenty-four or 48 hours later, supernatants were harvested and IL-6 production was quantified by ELISA.

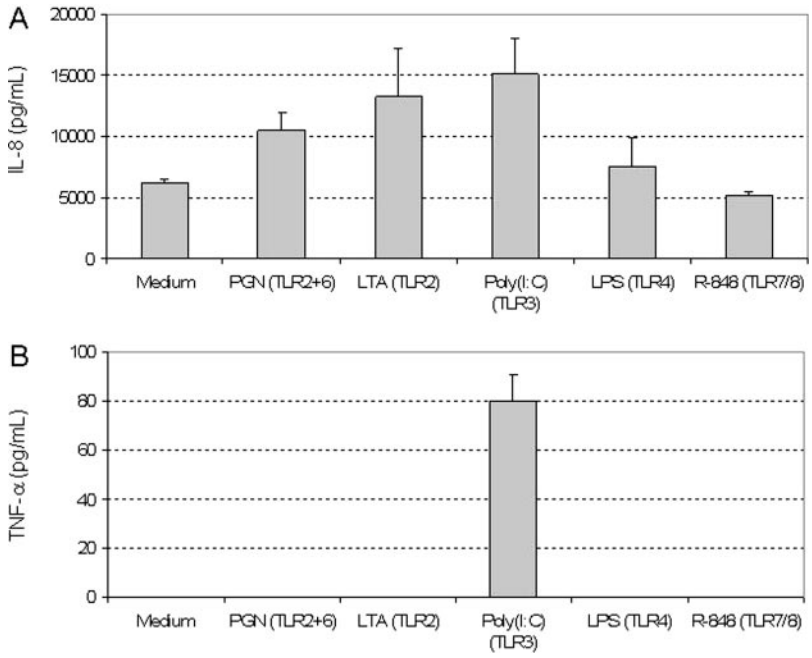

FIGURE 7. Production of IL-8 and TNF-α by human LC activated by TLR agonists. MACS-purified CD1a$^+$ LC were cultured in medium alone or with 10 μg/ml PGN, 10 μg/ml LTA, 25 μg/ml poly(I:C), 1 μg/ml LPS, or 10 μM R-848. Twenty-four hours later, supernatants were harvested and cytokine production was quantified by ELISA (*A*, IL-8; *B*, TNF-α). Results are representative of three independent experiments.

express high levels of TLR4 and TLR7. Indeed, no more IL-6 production could be observed in response to LPS and imiquimod when using MACS-purified LC (Fig. 6*B*). Treatment with poly(I:C), LPS, or imiquimod did not stimulate IL-10 production (Fig. 6*C*). Interestingly, production of IL-10 was induced by PGN, but not LTA, although recognition of both compounds relies on TLR2.

IL-8 and TNF-α can be secreted by LC, but they are also strongly induced in activated keratinocytes (18–22, 31, 33, 35). We showed that both keratinocytes and LC express TLR2 and TLR3 and might therefore secrete cytokines in response to the same agonists. Thus, evaluation of IL-8 and TNF-α secretion was performed on LC enriched by CD1a MACS rather than gradient purification. MACS-purified LC produced high constitutive levels of IL-8 when cultured in medium alone (Fig. 7*A*), which is most likely related to their spontaneous maturation. Yet, a notable increase in IL-8 could be induced upon PGN, LTA, or poly(I:C) exposure. We also noticed that only poly(I:C) stimulated production of TNF-α (Fig. 7*B*).

In addition, neither CD40 triggering nor TLR agonists were able to drive production of IL-1β and, most interestingly, bioactive IL-12p70 (data not shown). Finally, as type I IFNs are of critical importance in the context of viral infections, we evaluated the production of IFN-α and IFN-β by LC exposed to poly(I:C) (Table II). However, we were unable to detect significant concentrations of these mediators in the supernatants of activated LC.

Table II. *Production of type I IFN by human LC activated by poly(I:C)*[a]

	IFN-α (pg/ml)	IFN-β (pg/ml)
Langerhans cells (n = 4)		
Medium	<31, 25	<50
Poly(I:C)	<31, 25	<50
Blood pDC (n = 3)		
IL-3	<31, 25	ND
Influenza virus	26,219.4 ± 9,830.4	ND

[a] Gradient-purified LC were cultured in medium alone or with 25 μg/ml poly(I:C). Purified blood pDC were cultured with IL-3 (20 ng/ml) or formaldehyde-inactivated influenza virus (1 HAU/ml). Twenty-four hours later, supernatants were harvested and quantified for IFN-α and IFN-β production by ELISA. n represents the number of independent donors tested.

Discussion

To date, pathogen-derived molecules that directly drive maturation of human LC through activation of TLR are insufficiently characterized (25). Our systematic PCR analysis of LC freshly isolated from human skin reveals expression of TLR1, TLR2, TLR3, TLR6, and TLR10, as well as low levels of TLR5 mRNA (data not shown). In parallel, human suprabasal keratinocytes display TLR1–5, TLR7, and TLR10 mRNA. These TLR expression patterns, extended by LC activation assays performed with defined TLR agonists, shed light on the role of LC and keratinocytes in human skin exposed to potentially pathogenic microorganisms.

Gram-positive bacteria represent the majority of commensal microorganisms that colonize the skin of healthy individuals, but also the bulk of virulent species developing on wounded skin and causing severe skin infections (36). PGN and LTA, which are characteristic PAMP shared by all Gram-positive bacteria, trigger maturation of fresh human LC and enhance IL-6 and IL-8 release. Interestingly, PGN is the only TLR agonist inducing IL-10 production. This differential effect might be explained by the fact that, while both PGN and LTA engage TLR2, activation by PGN requires cooperation of TLR6 (9, 10), which is highly expressed in LC. In line with our results, a strong IL-10 production was found to be a hallmark of human DC stimulated with PGN (37). IL-10 inhibits APC function and migration of LC, most likely because it modulates proinflammatory cytokines secretions that enhance LC maturation (37–39). For this reason, we hypothesize that IL-10 could be required to avoid immune responses to commensal bacteria. Discrimination between unharmful and pathogenic Gram-positive bacteria may result from the level of PGN detection. Indeed, PGN from commensal bacteria is only available to extracellular TLR2 and TLR6 (10, 23), similar to our in vitro assays. In contrast, virulent *S. aureus* have an invasive phenotype and release PGN intracellularly, where it could engage the cytoplasmic NOD receptors, present in other DC subsets (40). Alternatively, IL-10 may keep immune responses under control, thereby preventing organ injuries. Indeed, while murine DC activated by *S. aureus* PGN produce high amounts of IL-10 (41),

resistance of TLR2$^{-/-}$ mice to *S. aureus* infections is severely impaired (42).

Human skin may also be exposed to Gram-negative bacteria, although they are not resident and are rarely responsible for infections (36). Murine LC migrate following skin exposure to LPS and have been suggested to express TLR4 (43, 44), but in vitro activation assays show poor correlation with these findings (43). Accordingly, and in line with earlier observations (23, 25), no TLR4 transcripts are present in human LC, and only negligible maturation is achieved upon treatment with high doses of LPS in vitro.

To determine a role of human LC in the detection of viruses, we investigated TLR3, TLR7/8, and TLR9, which are receptors for viral nucleic acids, and have been frequently highlighted for their capacity of inducing production of type I IFNs, a critical set of cytokines with direct effects on viral replication. We did not study expression of the cytoplasmic dsRNA receptors retinoic acid-inducing gene-I and protein kinase R, whose expression pattern in the skin is unknown so far (4). Freshly purified human LC displayed TLR3 mRNA, similar to murine LC (43, 45), as well as intracellular TLR3 protein. Consequently, poly(I:C) very strongly matured gradient-purified LC and augmented IL-6 secretion more potently than other TLR agonists. This is probably due to the unique ability of dsRNA to induce TNF-α, which likely amplifies LC response. Nevertheless, poly(I:C)-stimulated LC do not release type I IFNs. Hence, in contrast to pDC which produce high amounts of type I IFNs, human LC do not act as innate effector cells in viral infections.

Epicutaneous application of imiquimod, a specific agonist of TLR7, induces emigration of LC (46, 47) and strong immune responses, which have been proven efficient for the treatment of viral infection and cancers (48). Yet, we provide the first report of the absence of TLR7 and TLR8 in freshly isolated human LC, in line with lack of TLR7 in murine LC (43). Our findings explain and extend previous observations showing that human LC do not mature after exposure to imiquimod and R-848 in vitro (49). Regarding TLR9, mRNA has been described in purified murine LC (43), and CpG ODN trigger migration and activation of these cells in vivo (50, 51). However, activation of purified murine LC by CpG ODN could not be achieved in vitro (43). Likewise, freshly isolated human LC show no direct response to CpG ODN, consistent with their lack of TLR9, and keratinocytes do not express TLR9 either. Thus, we propose that, in vivo, deep penetration of imiquimod cream into the dermis and s.c. injections of CpG ODN activates nonepidermal cells, such as dermal DC, macrophages, and fibroblasts, the migration of LC being secondary to a release of proinflammatory cytokines. However, this hypothesis remains to be specifically evaluated.

Thus, the detection of pathogen invasion through LPS and ssRNA is not a primary function of LC, thereby suggesting a role for keratinocytes in the recognition of these PAMP. Earlier publications describing TLR in keratinocytes mainly relied on undifferentiated basal epidermal cells, isolated from foreskin, and differentiated after long periods of in vitro culture, which resulted in discrepancies (18–22). Here, we definitively state that suprabasal keratinocytes directly isolated from human skin notably display TLR4 and TLR7. Although other investigators could not detect the latter (22, 35), in vitro-cultured keratinocytes stimulated by TLR4 or TLR7 ligands increase transcription and release of proinflammatory mediators (22, 31, 33, 35). In line with this, LPS and imiquimod triggered IL-6 release only in keratinocyte-contaminated preparations of gradient-enriched LC. Yet, isolated suprabasal keratinocytes did not produce TNF-α when exposed to TLR agonists, presumably because their advanced state of differentiation precludes full activation in vitro (data not shown). Still, keratinocyte-induced inflammation in vivo probably results in maturation and migration of LC, as well as attraction of other immune cells (52, 53). Keratinocytes could therefore substitute to LC for the primary detection of LPS and ssRNA, and also, because they express high levels of TLR3, enhance dsRNA-induced LC maturation.

In summary, freshly purified human LC express a restricted set of functional TLR, namely TLR2, TLR6, and TLR3, and are directly activated by their respective ligands, while cooperation of keratinocytes and other cell types is probably essential for response to Gram-negative bacteria and viruses in vivo. In particular, lack of type I IFN production precludes a direct effector role of LC in viral infections and further argues that the full development of an immune response in the skin does not solely rely on these cells. Of interest, activation of LC by different TLR agonists leads to specific cytokine secretion profiles. Although the exact physiological relevance of PGN-specific IL-10 secretion remains to be determined, LC may contribute to the tolerance of commensal Gram-positive bacteria that colonize the skin of healthy individuals without causing inflammation. As no bioactive IL-12p70 was observed following TLR engagement alone, the release of this key Th1-polarizing cytokine may require additional signals provided by PAMP combinations or engagement of CD40 by T cells, as previously suggested for LC and other DC subsets (25, 54, 55). In this context, it will be of critical importance to decipher the functional influence of differential PAMP-driven activation of LC on the tuning of skin immune responses and particularly on T cell responses.

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Disclosures

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