Interleukin-32 promotes detachment and activation of human Langerhans cells in a human skin explant model

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Conflicts of interest
None to declare.

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Summary

Background
Cross-talk between skin keratinocytes (KCs) and Langerhans cells (LCs) plays a fundamental role in the body’s first line of immunological defences. However, the mechanism behind the interaction between these two major epidermal cells is unknown. Interleukin (IL)-32 is produced in inflammatory skin disorders. We questioned the role of IL-32 in the epidermis.

Objectives
We aimed to determine the role of IL-32 produced by KCs on surrounding LCs.

Methods
We used an ex vivo human explant model from healthy donors and investigated the role of IL-32 on LC activation using imaging, flow cytometry, reverse transcriptase quantitative polymerase chain reaction and small interfering (si)RNA treatment.

Results
Modified vaccinia virus ankara (MVA) infection induced KC death alongside the early production of the proinflammatory cytokine IL-32. We demonstrated that IL-32 produced by MVA-infected KCs induced modest but significant morphological changes in LCs and downregulation of adhesion molecules, such as epithelial cell adhesion molecule and very late antigen-4, and CXCL10 production. The treatment of KCs with IL-32-specific siRNA, and anti-IL-32 blocking antibody significantly inhibited LC activation, demonstrating the role of IL-32 in LC activation. We also found that some Toll-like receptor ligands induced a very high level of IL-32 production by KCs, which initiated LC activation.

Conclusions
We propose, for the first time, that IL-32 is a molecular link between KCs and LCs in healthy skin, provoking LC migration from the epidermis to the dermis prior to their migration to the draining lymph nodes.

What’s already known about this topic?

- The shortening of dendrites in Langerhans cells (LCs), morphological changes and migration to the draining lymph nodes is a major step allowing for the initiation of adaptive immunity.
- LC and keratinocyte interactions involve the regulation of adhesion molecule expression.
- Keratinocytes produce interleukin (IL)-32 in pathological inflammatory disorders such as atopic dermatitis.
What does this study add?

- We questioned the role of IL-32 in LC behaviour in response to external stimuli.
- Keratinocytes produce IL-32 in response to danger signals in nonpathological skin. The level of IL-32 production is dependent on Toll-like receptor ligand stimulation pathways.
- IL-32 produced by keratinocytes promotes the initial steps of LC migration from the epidermis to the dermis.
- This step contributes to LC activation, decreased surface expression of adhesion molecules and a slight but significant increase in CXCL10 production.

What is the translational message?

- Our work proposes that IL-32 is a molecular link between keratinocytes and LCs in healthy skin, which could be exacerbated in pathological conditions during skin barrier disruption such as atopic dermatitis, hidradenitis suppurativa and alopecia areata.

Epidermal cells play a fundamental role in cutaneous innate immunity, providing both a physical barrier via tight junction formation and a chemical barrier via the production of antimicrobial peptides and proteases. CD45+ keratinocytes (KCs) comprise up to 90% of this epidermal cell population, where they exist alongside the more scarce Langerhans cell (LC) population (1–5% of total epidermal cells), which are specialized antigen-presenting cells characterized by CD45, CD1a, CD1c and human leucocyte antigen (HLA)-DR expression. Together, KCs and LCs form a strong barrier to fight pathogen entry. Skin barrier disruption provokes the local production of proinflammatory cytokines and chemokines by KCs, bridging innate immunity to adaptive immunity via LCs. In this regard, the production of cytokines and chemokines by KCs and LCs has been observed in many skin disorders and more particularly, the production of interleukin (IL)-32, a proinflammatory cytokine produced by KCs. However, the impact of local production of IL-32 on LCs has not been explored. Here, we questioned whether IL-32 produced by KCs could impact the behaviour of LCs during microbial insults.

LC activation is characterized by the upregulation of costimulatory molecules such as CD80 and HLA-DR in addition to the release of proinflammatory cytokines and chemokines including CXCL10. Furthermore, activated LCs change their morphology by the shortening of dendrite size in response to modified vaccinia virus ankara (MVA) intradermal administration. So-called ‘en route’ LCs start migrating to the draining lymph nodes. They also exhibit an altered expression of adhesion molecules, including very late antigen (VLA)-4, epithelial cell adhesion molecule (EpCam) and E-cadherin. We recently demonstrated that LC migration from skin to the draining lymph nodes allows for the priming of CD4 and CD8 responses observed after intradermal and transcutaneous application of vaccines. However, local epidermal events initiating the activation of LCs remain to be studied.

Herein, we used a human skin explant model to investigate the role of a proinflammatory cytokine, IL-32, as a molecular link between stimulated KCs and LCs. We demonstrated that IL-32 leads to LC activation as shown by morphological changes, detachment from the epidermal layer and the production of chemotactic CXCL10.

Materials and methods

Human skin explant collection and preparation

Human skin samples were obtained from healthy volunteers undergoing plastic surgery for breast, abdomen or facelift (Service de Chirurgie Plastique, Reconstructrice et Esthétique – Centre de Traitement des Brûlés, Saint-Louis Hospital, Paris, France and Centre de Chirurgie Plastique et Reconstructrice, Tenon Hospital, Paris, France). All skin samples were taken after informed consent was provided according to the local Institutional Ethics Committee guidelines (IRB 00003835) and ethical rules stated in the Declaration of Helsinki principles. Immediately after surgical excision, skin samples were conserved in NaCl and processed rapidly.

Skin administration

Skin explant intradermal injections were performed using the Mantoux method. For transcutaneous immunization, cyanoacrylate skin surface stripping was performed as previously described. Wild-type MVA was obtained from R. Wagner (University of Regensburg, Regensburg, Germany) and was used at 10⁷ plaque-forming units (PFU) per cm² of skin. Pam3CSK4 and imiquimod Toll-like receptor ligands (Invivogen, San Diego, CA, U.S.A.), human IL-32 blocking antibody
at least 5 and LSRFortessa (BD Biosciences). Samples were analysed on CXCL10-PE were used. Cells were run using BD FACSCanto II (BioLegend) and streptavidin APC (BD Biosciences) and anti-Sigma-Aldrich). Then, a biotinylated antihuman IL-32/C0 blocked antibody (1 nmol L−1) at one-sided signaling and decreased adhesion markers expressed on LCs, and untreated samples. Our hypotheses regarding the increased activation and decreased adhesion markers expressed on LCs, and also regarding the changing morphology to activated round LCs, were tested in one direction. As we had a limited number of human samples and we considered the consequences of missing an effect in the untested direction to be negligible, we used one-tailed tests. For comparisons of more than two groups, one-way Friedman test or Kruskal-Wallis test with Dunn’s correction was applied before paired (Figs 1d, 3a, d) or unpaired tests (Figs 2b, 3b), respectively. Results are presented as median with interquartile range. Significance was set at one-sided P-value < 0.05. Prism 6 software was used for data handling, analysis and graphic representations.

Results

Interleukin-32 produced by modified vaccinia virus ankara-infected keratinocytes contributes to rapid activation of Langerhans cells promoting CXCL10 production

We have previously demonstrated that epidermal exposure to MVA resulted in dendrite retraction on CD1a+ LCs and their activation.15 We hypothesized that cytokines released by KCs could impact LC activation. To explore the involved mechanism further, we sorted KCs from the epidermal layer of fresh human skin and infected them with MVA (1 PFU per cell) 3 h prior to the addition of LCs (Fig. 1a). We investigated the expression of
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CD80 costimulatory molecules, HLA-DR^{high} in addition to CXCL10 production, which are indicators of LC activation in response to danger signals as previously described.\(^1\),\(^2\),\(^4\),\(^24\),\(^25\) We observed a significant increase in the percentage of CXCL10-producing LCs along with a slight but significant increase in CD80 and HLA-DR^{high} expression at 24 h after coculture of LCs with MVA-treated KCs compared with controls (Fig. 1a, \( n = 6 \) donors; \( P < 0.05 \)). These data were confirmed by reverse transcriptase quantitative polymerase chain reaction and revealed that among CXCR3-binding chemokines (CXCL4, CXCL9, CXCL10 and CXCL11) only CXCL10 was significantly higher in the epidermis (data not shown).

KCs are known to produce proinflammatory factors, including IL-32, in atopic dermatitis (AD).\(^1\) Using immunofluorescence microscopy, we found that CD1a^{+} KCs produced IL-32 at 4 h post-MVA infection compared with control medium (Fig. 1b). We did not detect any IL-32 in CD1a^{+} LCs in these conditions (Fig. 1b). The frequency of IL-32-producing KCs was counted and represented in Figure 1c. We found an approximatively twofold increase in IL-32 production by KCs following MVA stimulation (Fig. 1c, \( n = 5 \) donors; \( P < 0.05 \)).

In order to block IL-32 production, epidermal cells were treated with siRNA targeting IL-32 or scrambled control siRNA (10 nmol L^{-1}) analysed by flow cytometry analysis. Data are represented using box and whisker plots with median (min/max). For comparisons of more than two groups, Friedman test was applied before one-tailed Wilcoxon matched paired signed-rank test. \( * P < 0.05 \).

### Fig 1.

Modified vaccinia virus ankara (MVA)-infected keratinocytes (KCs) producing interleukin (IL)-32 induce rapid activation of Langerhans cells (LCs). (a) KCs and LCs were separately analysed by cell sorting. KCs were treated with MVA (1 plaque-forming unit per cell) or medium as indicated for 3 h and washed before being cocultured with LCs for an additional 24 h. The percentages of CXCL10^{+}, CD80^{+} and human leucocyte antigen (HLA)-DR^{high} LCs (ViViD^{+} CD45^{+} CD1c^{+} live cells) were measured by flow cytometry. Each donor is represented (\( n = 5 \)). One-tailed Wilcoxon matched paired signed-rank test was used. \( * P < 0.05 \). (b) Immunofluorescence staining of epidermal cells. IL-32 (red), CD1a (green) and DAPI (4′,6-diamidino-2-phenylindole) nuclear stain (blue) on skin cell suspension following MVA or medium treatment at 4 h (scale bars = 10 μm, representative 10 fields for each donor, \( n = 5 \) donors). (c) Mean percentage of IL-32^{+} KCs counted in 10 fields for five donors. One-tailed Wilcoxon matched paired signed-rank test was used. \( * P < 0.05 \). (d) Percentage of CXCL10^{+} LCs (ViViD^{+} CD45^{+} CD1c^{+} live cells) at 24 h post-MVA infection of epidermal cells treated with IL-32 specific small interfering (si)RNA (10 nmol L^{-1}) or scrambled control siRNA (10 nmol L^{-1}) analysed by flow cytometry analysis. Data are represented using box and whisker plots with median (min/max). For comparisons of more than two groups, Friedman test was applied before one-tailed Wilcoxon matched paired signed-rank test. \( * P < 0.05 \).

| Conditions | Percentage CD80^{+} LCs | Percentage CXCL10^{+} LCs | Percentage HLA-DR^{high} LCs |
|------------|-------------------------|---------------------------|-------------------------------|
| KCs        | -                       | -                         | -                             |
| MVA + KCs  | +                       | *                         | *                             |
| LCs        | -                       | +                         | -                             |

| Conditions | Percentage IL-32^{+} KCs |
|------------|--------------------------|
| Medium     | -                        |
| MVA        | +                        |
| scrambled RNA | -  +  +  -           |
| siRNA IL-32 | -  +  +  +        |

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that KCs (Fig. S1c, left panel, six donors) significantly increased IL-32 expression following MVA stimulation, but LCs did not (Fig. S1c right panel, six donors). This expression was inhibited by IL-32-specific siRNA treatment compared with scrambled siRNA. Thus, compared with scrambled siRNA, IL-32-specific siRNA treatment significantly decreased the proportion of IL-32+ KCs in a dose-dependent manner.

Finally, we then examined the effect of IL-32-specific siRNA treatment of epidermal cells on CXCL10 production by LC activation (Fig. 1d). We found a significant decrease of CXCL10-producing LCs after IL-32-specific siRNA treatment at 24 h following MVA infection compared with scrambled siRNA by flow cytometry analysis (Fig. 1d, n = 6 donors; P < 0.05).

Thus, the modest IL-32 production by KCs induced LC activation as assessed by CXCL10 production, which is abrogated by IL-32 siRNA treatment.

**Interleukin-32 induces detachment of Langerhans cells from the epidermal layer allowing for their migration**

We then evaluated the impact of rhIL-32γ protein and MVA treatment compared with PBS treatment of epidermis on LC morphology using immunofluorescence microscopy (Fig. 2a,
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(a) Percentage of IL-32pos CD45neg KCs

(b) Percentage of round CD1apos cells

(c) PBS, Pam3CSK4, Imiquimod, Anti-IL-32 + Pam3CSK4, Anti-IL-32 + Imiquimod

(d) Percentage of CXCL10pos CD1apos LCs

KC, Pam3CSK4, Imiquimod, PBS, POLY IC LMW, POLY IC HMW, LPS, Flagellin, ODN 2006
representative immunofluorescence imaging). As depicted in Figure 2b, both rhIL-32γ and MVA stimulation of epidermis, compared with PBS treatment, significantly induced shortening of the dendrite size of CD1a+ LCs at 4 h after transcutaneous application or intradermal injection (Fig. 2b, three donors, five fields). Skin treatment with anti-IL32 antibody (0.1 µg mL−1) (Fig. 2a, b) significantly inhibits LC activation, but treatment with isotype control antibody does not (not shown; see Supporting Information). We have previously demonstrated that such shape changes represent an early activation step prior to the migration of LCs to the dermis and draining lymph nodes for CD8+ T-cell priming. 15

A critical step in this process is the regulation of adhesion molecules on the surface of the LCs, permitting their detachment from the KC layers. As shown in Figure 2c, IL-32γ-induced a significant downregulation of LC surface expression of EpCam (Fig. 2c, left panel, n = 5 donors; P < 0.05) and VLA-4 expression (Fig. 2c, right panel, n = 5 donors; P < 0.05) at 4 h post-rhIL-32γ treatment of epidermal cells. We did not observe any modulation of these adhesion molecules on KCs (data not shown). E-cadherin expression on KCs and LCs was also analysed, but did not exhibit any significant changes (data not shown).

Collectively, we demonstrated that IL-32γ plays a crucial role in the activation of LCs, expression of adhesion molecules and morphological modifications allowing for the acquisition of the ‘en route’ LC phenotype.

Innate sensors induce interleukin-32 production by keratinocytes together with morphological changes of Langerhans cells and CXCL10 production

We found that MVA treatment had a significant but modest effect on IL-32 production. Thus, we examined the effect of TLR agonists on IL-32 production by KCs (Fig. 3a) and consequently on LC morphological modification (Fig. 3b, c). Surprisingly, TLR-1/2 (Pam3CSK4), TLR-7 (imiquimod) and TLR-4 (LPS) agonist treatment induced a significant increased expression of IL-32 by KCs, shown by flow cytometry analysis (Fig. 3a, n = 6 donors; P < 0.05). These TLR agonists induced an average of a threefold to 16-fold increase in IL-32 production by KCs. IL-32 production was lower after flagellin, ssRNA and Poly(I:C) stimulation (Fig. 3a).

We also showed that both Pam3CSK4 and imiquimod induced a significant increase in the number of round-shaped LCs (Fig. 3b, three donors, five fields). However, one cannot exclude the direct effect of TLR agonists on LCs during epidermal layer treatments. In order to confirm the effect of IL-32 on LCs during TLR ligand stimulation, skin explants were treated with anti-IL-32 blocking antibodies together with TLR ligand treatment (Pam3CSK4 and imiquimod). Representative immunostaining is shown for each condition in Figure 3c. Anti-IL-32 blocking antibodies significantly restored dendritic LC morphology following either intradermal or transcutaneous administration of MVA compared with controls (Fig. 3c). The fact that IL-32γ-treated epidermis also induced morphological changes in LCs (as shown in Fig. 2a) strongly suggests that the TLR ligand effect on IL-32 production on KCs would, in turn, activate LCs.

In order to test the impact of KCs treated with TLR agonists on LC activation (production of CXCL10), we sorted KCs and LCs prior to treatment (Fig. 3d). CXCL10 production by LCs was modest but significantly higher when KCs were treated with Pam3CSK4 compared with untreated controls (Fig. 3d, seven donors; P < 0.05). The percentage of LCs expressing CXCL10 is similar to that seen in MVA treatment (Fig. 1). These results indicate that IL-32 production by KCs and subsequent LC activation may be elicited by specific pathogen-associated molecular pattern receptor activation in addition to MVA infection and could be a feature of the skin’s reaction to innate sensors.

Discussion

IL-32 production in the skin has been observed in inflammatory skin disorders such as AD, alopecia areata and hidradenitis suppurativa, suggesting that IL-32 production plays a critical role in skin diseases. 10, 11, 26 To improve the understanding of molecular mechanisms that allow LC activation in the epidermal layers, we have investigated the role of IL-32 in the epidermis. We first showed that KCs are the major cell population that produces IL-32 in the skin. We demonstrated,
for the first time, that the stimulation of KCs by MVA and several TLR agonists induces IL-32 production that, in turn, promotes LC activation as assessed by CXCL10 production, morphological changes and downregulation of cellular adhesion molecules. We showed that IL-32-specific siRNA in KCs strongly inhibited IL-32 expression and consequently LC activation. While our manuscript was under review, Gorvel et al.\textsuperscript{27} showed that LCs expressed IL-32 while in a ‘steady state’. However, the method they used for LC isolation is quite different from ours. Gorvel et al.\textsuperscript{27} isolated LCs following 48 h of migration outside the skin, in contrast to the enzymatic digestion of skin cells used in our model. Their method allows LC migration from the skin, which is often associated with activation and the IL-32 release can be observed. In our skin explant model, which reflects a condition close to steady state, KCs are the main producer of IL-32 at an early time point following stimulation.

As proposed by others,\textsuperscript{28} LC production of CXCL10 owing to IL-32 stimulation may condition the skin tissue positioning of the local microenvironment skin-resident T cells. Other proinflammatory cytokines, including TNF-\textgreek{a}, IL-12p70 and interferon-\textgreek{y}, have been observed at latter time points in Vaccinia virus infection of human primary KCs.\textsuperscript{12,29} Thus, we proposed IL-32 as the initial link between KCs and LCs in the response to pathogens that could be exacerbated in pathological skin disorders.

In addition, IL-32 is known to be produced but not secreted by KCs.\textsuperscript{10,30} Under these circumstances, induced cell death may serve to release IL-32 into the local microenvironment.\textsuperscript{31} Interestingly, we found that MVA infection of KCs upregulates ASC, which could contribute to the release of IL-32 (Fig. S3a, b, five donors; \( P < 0.05\); see Supporting Information). We also observed a slight increase in annexin V staining on KCs at 4 h following in vitro MVA (1 PFU per cell) stimulation (1.4–1.6-fold change) (Fig. S3c) or control TNF-\textgreek{a} stimulation (1.3–1.6-fold change) compared with unstimulated cells (Fig. S3c) in three donors. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL) immunofluorescence staining has been performed on human skin cryosections treated for 4 h with either PBS or MVA (1 PFU per cell) following intradermal or transcutaneous administration. We found an increase in TUNEL\textsuperscript{3} positivity of KCs (CD1a\textsuperscript{3}) in the epidermal layer as shown in Figure S3d. In conclusion, three methods of analysis of apoptotic cells, i.e. ASC, annexin V and TUNEL, using in vitro or ex vivo skin epidermal cells, showed that MVA treatment induced apoptosis of KCs. Measurement of IL-32 by enzyme-linked immunosorbent assay showed slight cytokine production under these conditions, which suggests its release in the skin microenvironment (Fig. S3c).

Through the expression of innate receptors, KCs and LCs are able to sense pathogen penetration and danger signals.\textsuperscript{32} MyD88 deficiency in KCs directly impairs LC migration after antigen exposure,\textsuperscript{33} demonstrating that LC migration to the dermis is directly dependent on the MyD88/nuclear factor-\textgreek{B} pathway in KCs. Accordingly, we found that several TLR agonists (such as Pam3CSK4/TLR1-2 and imiquimod/TLR7) induced IL-32 production by KCs and LC activation including LC morphological modification (Fig. S4; see Supporting Information).

Finally, IL-32 exists in four main isoforms that originate from the splicing of the \textgreek{g} isoform.\textsuperscript{31,32} Most studies on IL-32 have been performed on blood cells as illustrated by the effect of IL-32 upon blood-derived dendritic cell populations in upregulation of costimulatory molecules.\textsuperscript{34} Although the \textgreek{g} and \textgreek{b} isoforms have been associated with anti-inflammatory functions including reduced cell activation and proliferation, cytotoxic function\textsuperscript{35} and increased IL-10 production,\textsuperscript{36} the majority of studies define a proinflammatory character for IL-32.\textsuperscript{34,37,38} Finally, IL-32 has been proposed as a biomarker of skin inflammation in AD, alopecia areata and hidradenitis suppurativa.\textsuperscript{10,11,34} The analysis of IL-32 isoforms mRNA expression in skin biopsies from three skin disorders (hidradenitis suppurativa, psoriasis and AD) compared with skin from healthy donors, revealed that all main IL-32 isoforms are expressed in skin, but at different levels depending on the origin of skin disorder.\textsuperscript{11} Further studies are required to analyse the contribution of each isoform in LC activation. Meyer et al.\textsuperscript{27} also showed that high serum levels of IL-32 correlate with disease severity, highlighting its potential relevance in skin immunity.\textsuperscript{10} Here, we demonstrated the role of IL-32 in LC activation, bringing new insights into the cross-talk between KCs and LCs and linking innate to adaptive immunity.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Fig S1. Inhibition of interleukin (IL)-32 expression by IL-32 specific small interfering RNA treatment.

Fig S2. Anti-interleukin (anti-IL)-32 blocks IL-32 biological effect on Langerhans cell activation following modified vaccinia virus ankara (MVA) stimulation of epidermal cells.

Fig S3. Modified vaccinia virus ankara (MVA) epidermal cell infection induces apoptosis in keratinocytes and interleukin (IL)-32 release.

Fig S4. Step-by-step events leading to the detachment of Langerhans cells from the epidermal layer.

File S1 Supplementary materials and methods.