Human Beta-Defensin 3 Induces Maturation of Human Langerhans Cell–Like Dendritic Cells: An Antimicrobial Peptide that Functions as an Endogenous Adjuvant

Laura K. Ferris1,5, Yvonne K. Mburu2,3,5, Alicia R. Mathers3, Eric R. Fluharty1, Adriana T. Larregina1,3, Robert L. Ferris2,3,4 and Louis D. Falo Jr1,4

Human beta-defensins (hBDs) are antimicrobial peptides that have an important role in innate immune responses at epithelial barriers such as the skin. However, the role that hBDs have in initiating cellular immune responses that contribute to antigen-specific adaptive immunity is not well understood. Here we show that one member of the hBD family, hBD3, can induce maturation and T-helper type 1 skewing function in human Langerhans cell–like dendritic cells (LC-DCs). Specifically, hBD3 potently induces phenotypic maturation of LC-DCs, including increased expression of CCR7, which mediates functional chemotactic responses to CCL19 and CCL21. hBD3-stimulated LC-DCs induce strong proliferation of and IFN-γ secretion by naive human T cells. hBD3 also induces phenotypic maturation of primary human skin-migratory DCs derived from human skin explants. These results suggest an important role for hBD3 in inducing DC activation, migration, and polarization. Thus, hBD3 contributes to the integration of innate and adaptive immune responses in the skin, and may be a useful adjuvant for skin immunization and an important factor in the pathophysiology of inflammatory skin diseases.

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INTRODUCTION

Antimicrobial peptides such as human beta-defensins (hBD1-4) and cathelicidins are produced in a variety of epithelial tissues, with particularly high expression in inflamed skin. They are capable of killing pathogens that breach surface barriers, providing a primitive but critical function in host defense. Indeed, their expression is induced in the skin under conditions of inflammation, infection, and wound healing because of the secretion of various pathogenic stimuli and cytokines. For instance, increased expression of hBD2 has been directly associated with stimulation by tumor necrosis factor (TNF)-α, IL-1, lipopolysaccharide (Liu et al., 2002), flagellin (Gerstel et al., 2009), and IL-22 (Wolk et al., 2004). The increased expression of hBD3 is associated with stimulation by IL-22 (Wolk et al., 2004), transforming growth factor (TGF)-α alone, or in combination with insulin-like growth factor I (Sorensen et al., 2003), stimulation of Toll-like receptor (TLR)-2 (Menzies and Kenoyer, 2005, 2006), and IFN-γ (Joly et al., 2005).

In addition to their antimicrobial functions, BDs may contribute to the induction of acquired immune responses by recruiting monocytes, dendritic cells (DCs), and T cells to sites of inflammation (Territo et al., 1989; Oppenheim et al., 2003; Larregina and Falo, 2005; He et al., 2006). In previous studies, hBD2 and/or hBD3 have been implicated in the chemotaxis of immature DCs and T cells through their interactions with chemokine receptor 6 (CCR6) and the chemotaxis of monocytes through their interactions with CCR2 (Rohrl et al., 2010). In addition, hBD3 has been shown to induce the expression of various costimulatory molecules on monocytes and myeloid DCs through its interactions with TLRs 1 and 2 (Funderburg et al., 2007). Interestingly, antimicrobial peptides can mediate either induction or inhibition of DC activation, depending on experimental conditions. In mice, murine beta-defensin 2 (mBD2) has...
been shown to promote the maturation of DC via TLR4, the receptor for bacterial lipopolysaccharide (Biragyn et al., 2002). The human cathelicidin LL-37 has been shown to induce DC maturation in vitro (Davidson et al., 2004). Conversely, cathelicidins have been shown to block TLR4-mediated DC activation and inhibit allergic contact sensitization in mice (Di Nardo et al., 2007). Both BDs and cathelicidins have been implicated in the pathogenesis of inflammatory skin diseases. Indeed, the expression of hBD3 in particular has been shown to be increased in psoriatic skin (Nomura et al., 2003) and a higher copy number of BD loci is associated with increased susceptibility to psoriasis (Hollox et al., 2008). However, the influence of hBD3 on innate and adaptive immunity remains poorly understood.

The effects of hBD on human DCs have not been well documented. One human defensin, hBD3, was originally isolated from psoriatic skin flakes and retained antimicrobial activity at physiological salt concentrations (Harder et al., 2001). As hBD3 is produced primarily by keratinocytes, we specifically studied its effect on human Langerhans cell-like DCs (LC-DC) and skin-derived DCs. LCs have an important role in protecting the skin against pathogens and are remarkably plastic and responsive to environmental signals, enabling them to functionally bridge innate and adaptive immune responses. Under inflammatory conditions, LCs migrate rapidly out of the skin in a CCR7-dependent manner and move toward the draining lymph node where they can present antigen to T cells and initiate antigen-specific immune responses (Ohl et al., 2004). DCs with features of LCs (LC-DCs) can be generated in vitro from human monocytes by culture in the presence of GM-CSF and TGF-β (Geissmann et al., 1998; Yang et al., 1999b; Guironnet et al., 2002). LC-DCs differ from classic monocyte-derived DCs generated by culture with GM-CSF and IL-4 and uniquely express molecules typical of LCs, including langerin, CCR6, and E-cadherin.

In this study, we demonstrate that hBD3 induces phenotypic and functional maturation of immature human LC-DCs, and this is mediated in part by NF-κB activation. Furthermore, hBD3-stimulated LC-DCs induce strong proliferation and IFN-γ production by naive CD4+CD45RA+ T cells. hBD3 also induced maturation/activation of primary human skin-migratory DCs, suggesting that hBD3 is an important contributor to cutaneous immune regulation in vivo.

RESULTS
hBD3 induces phenotypic maturation of human LC-DC
DCs with features of LCs (LC-DCs) were generated from peripheral blood mononuclear cells by culture with GM-CSF and TGF-β using well-described methods (Geissmann et al., 1998; Guironnet et al., 2002). The identity of these LC-DCs was confirmed by flow-cytometric staining for the LC markers CD1a, E-cadherin, CCR6, and CD207 (Langerin) (Figure 1). The LC-DCs also expressed HLA-DR, characteristic low to moderate levels of the costimulatory molecules CD40, CD80, CD83, and CD86, and lacked expression of the monocyte/macrophage marker CD14 (not shown).

Figure 1. Culturing human monocytes with GM-CSF and transforming growth factor (TGF)-β1 generates Langerhans cell-like dendritic cells (LC-DCs) that are distinctly different from DCs generated in the presence of GM-CSF and IL-4. Human monocytes were isolated from the blood of healthy donors by density gradient centrifugation and cultured in serum-free AIM-V medium with rhGM-CSF and rhIL-4 or with rhGM-CSF and rhTGF-β1 for 6 days. Cell surface expression of HLA-DR, E-cadherin, CD1a, CCR6, and langerin was assessed by flow cytometry. In each case, the percentage of cells considered positive for each marker is shown. Results are representative of three separate experiments with cells generated from different donors.

Maturation stimuli such as inflammatory cytokines or TLR ligands induce the maturation and activation of DCs, including increased cell surface expression of costimulatory
and adhesion molecules. To evaluate the potential effect of hBD3 stimulation on the maturation/activation of LC-DCs, day 6, human immature LC-DCs were stimulated for 18 hours with medium alone or with 5 μM hBD3. For comparison, we evaluated the activation effects of TNF-α, a well-established LC activation signal that is present at increased levels in inflamed skin, by stimulating immature LC-DCs with 10 ng ml⁻¹ TNF-α. The LC-DCs were then stained with antibodies against HLA-DR, CD83, CD86, CD40, and CCR7, and surface expression was evaluated by flow cytometry. Our findings show that hBD3 consistently induced increased surface expression of HLA-DR, CD83, CD86, and CCR7, but not of CD40 (Figure 2a). In contrast, TNF-α induced increased expression of HLA-DR on LC-DCs but did not significantly upregulate the other maturation molecules tested. It is noteworthy that hBD3 strongly upregulated CCR7, a crucial lymph node-homing receptor, whereas TNF-α had no measurable effect on CCR7 expression in LC-DCs. Furthermore, the observed LC-DC maturation phenotype was specific to hBD3 because neither hBD2 nor hBD4 matured LC-DC (Figure 2b). To determine the optimal concentration of hBD3, we performed a dose–response analysis using 500, 1, 2, and 5 μM concentrations of hBD3 (Figure 2c). The results show that increasing the concentrations of hBD3 resulted in enhanced maturation of LC-DCs as measured by the upregulation of several costimulatory markers. These data show that hBD3 induces phenotypic changes in LC-DCs consistent with DC activation.

hBD3-stimulated LC-DCs migrate toward CCL19 and CCL21
To determine the functional significance of hBD3-induced CCR7 expression, we examined the ability of hBD3-treated
LC-DCs to migrate toward the CCR7 ligands CCL19 and CCL21. Again, day 6 immature LC-DCs were stimulated for 18 hours with medium alone, hBD3, or with TNF-α. Chemotactic migration toward CCL19 and CCL21 was measured using an in vitro transwell system. Stimulation of LC-DCs with hBD3, but not with TNF-α or medium, enabled significant migration toward both CCR7 ligands across a transwell micropore membrane (P < 0.02; Figure 3). This effect appears to be CCR7-specific, as inclusion of CCL19 with the cells in the upper well abolishes migration toward CCL21. These data suggest that hBD3 can promote migration and lymph node localization of LC-DCs.

hBD3-stimulated LC-DCs polarize T cells to produce IFN-γ
To determine the effect of hBD3 on the T-cell stimulatory function of LC-DCs, we compared the ability of untreated, TNF-α-treated, or hBD3-treated LC-DCs to activate naive CD4+ T cells in a mixed lymphocyte reaction. Day 6 immature LC-DCs were treated for 18 hours with medium, TNF-α, or hBD3, and then washed and incubated with allogeneic CD4 + CD45RA+ T cells for 5 days. T-cell proliferation was determined by measuring the incorporation of tritiated thymidine. IFN-γ secretion was also evaluated by determining the concentration of IFN-γ in culture supernatants by ELISA. Stimulation with either hBD3 or TNF-α enabled LC-DCs to induce potent T-cell proliferation, consistent with the well-established antigen presentation function of activated DCs (Figure 4a; *P < 0.05). However, LC-DCs stimulated with hBD3 uniquely induce high-level production of IFN-γ by responding T cells (Figure 4b; *P < 0.031). Taken together, these data demonstrate that hBD3 exposure induces potent antigen presentation capacity in LC-DCs, and unlike TNF-α, hBD3 induces high levels of IFN-γ production by primed T cells, suggesting that hBD3 skews T-cell activation toward a T-helper type-1 immune response.

hBD3-induced maturation of LC-DCs is not MyD88- or Gαi protein-coupled receptor-dependent, but is dependent on NF-kB and mitogen-activated protein kinase (MAPK) activation
Studies have shown that hBD3 can signal through TLR1 and TLR2 in a MyD88-dependent manner (Funderburg et al., 2007). To determine whether MyD88-dependent TLR
signaling was required for the maturation of LC-DCs by hBD3, we pretreated LC-DCs with an MyD88 peptide inhibitor (100 μM, 18 hours) before hBD3 treatment. The inhibition of MyD88 signaling had no detectable effect on the upregulation of CD86 and CCR7 in LC-DCs (Figure 5a). We confirmed the ability of the MyD88 peptide to inhibit lipopolysaccharide-induced maturation of LC-DCs at similar concentrations (Figure 5b).

In addition, other studies have shown that hBDs can signal through Gi protein–coupled receptors such as CCR2, CCR6, or CXCR4 (Yang et al., 1999a; Feng et al., 2006; Rohrl et al., 2010). To determine whether any of these Gi protein–coupled receptors were involved in the maturation of LC-DCs by hBD3, we pretreated day 6 LC-DCs with pertussis toxin for 2 hours before maturation with hBD3, and then examined HLA-DR, CD83, CD86, and CCR7 upregulation as markers of overall LC-DC maturation. Although there was a notable reduction in CD86, our findings show that the blocking of G protein–coupled receptors by pertussis toxin had no significant effect on hBD3-induced HLA-DR, CD83, and CCR7 upregulation of LC-DCs (Figure 5c).

Our recent studies have shown that the hBD3-mediated upregulation of CCR7 in human tumors is dependent on NF-κB and AP1 transcription factors (Mburu et al., 2011, 2012). Indeed, these transcription factors are activated by various inflammatory stimuli and are associated with the maturation and survival of antigen-presenting cells. First, to determine whether NF-κB activation was required for hBD3-induced maturation and CCR7 upregulation, we transduced the LC-DCs with an adenovirus vector encoding the IκBAA dominant-negative super repressor, or with a control, blank adenovirus. NF-κB blockade resulted in significant inhibition of hBD3-induced CD86 and CCR7 upregulation in LC-DCs (Figure 5d). Second, to determine whether MAPK/AP1 activation was necessary for hBD3-mediated LC-DC maturation and CCR7 upregulation, we pretreated day 6 LC-DCs with PD98059 (100 μM, 2 hours), followed by hBD3 (5 μM, 18 hours). Results shown are representative of two to three experiments with cells from different donors.

Figure 5. Human beta-defensin 3 (hBD3) stimulation of Langerhans cell-like dendritic cells (LC-DCs) is not MyD88 or Gi protein-coupled receptor (GPCR) dependent but is dependent on NF-κB and mitogen-activated protein kinase (MAPK) activation. (a, b) Day 5 LC-DCs were treated overnight with MyD88 inhibitor (100 μM), followed by stimulation with (a) hBD3 (5 μM, 18 hours) or (b) lipopolysaccharide (LPS; 100 ng ml⁻¹, 18 hours), and analyzed using flow cytometry. Values represent mean fluorescence intensity in treated cells. (c) Day 6 LC-DCs were treated with pertussis toxin (200 ng ml⁻¹, 2 hours), followed by hBD3 (5 μM, 18 hours). (d) Day 5 LC-DCs were transduced with control (solid line) or IκBAA (dotted line) containing adenovirus over 24 hours, followed by treatment with hBD3 (5 μM, 18 hours). (e) Day 6 LC-DCs were treated with PD98059 (100 μM, 2 hours), followed by hBD3 (5 μM, 18 hours). Results shown are representative of two to three experiments with cells from different donors.
with the MAPK inhibitor PD98059 (100 μM, 2 hours), followed by overnight stimulation with 5 μM hBD3. MAPK/ AP1 inhibition resulted in a reduction in hBD3-mediated CD86 and CCR7 upregulation in LC-DCs (Figure 5e). Together, these data suggest that hBD3 stimulates the maturation of LC-DCs through the NF-κB- and MAPK/AP1-dependent pathways.

Skin-migratory DCs acquire a mature phenotype following exposure to hBD3

We focused our studies on LC-DCs as they can be reliably generated in sufficient quantities to allow for both phenotypic and functional studies to be conducted. However, because cultured cells can behave differently from primary human cells, we collected primary human DCs that had migrated out of skin explants (Larregina et al., 2001) to determine whether hBD3 stimulates the maturation of LC-DCs through the NF-κB- and MAPK/AP1-dependent pathways.

Skin-migratory DCs were cultured for 18 hours in the presence or absence of hBD3 (5 μM). They were then assessed for cell surface expression of maturation markers. (a) CD86 and (b) CCR7 expression is shown in cells cultured in medium alone (solid line) and in the presence of hBD3 (dashed line). Solid histograms show staining of cells with an isotype-matched control antibody.

**DISCUSSION**

Antimicrobial peptides, including BDs and cathelicidins, are produced by epithelial cells under conditions of inflammation or infection. Their production is stimulated by exogenous microbial danger signals, such as TLR agonists, and by endogenous mediators of inflammation, such as TNF-α, IL-1, IFN-γ, and IL-17 (Biragyn et al., 2002; Chadebech et al., 2003; Joly et al., 2005; Kolls et al., 2008). Indeed, such inflammatory mediators can induce skin-resident DC maturation by directly promoting the maturation of DCs, or by inducing DC activation indirectly by stimulating keratinocytes to produce BDs (De Smedt et al., 1996; Rieser et al., 1997; Berthier-Vergnes et al., 2005; Flacher et al., 2006). Although the role of BDs in innate immunity has been well established, their influence on acquired immune responses is less clear. As described in the introduction, several studies have shown that BDs can potentiate an inflammatory response by recruiting monocytes, DCs, and T cells. However, there are recent studies using mBD14 (the mouse homolog to hBD3) that show that BDs can have some anti-inflammatory properties as well, through the induction of T-regulatory cells (Semple et al., 2010, 2011; Navid et al., 2012). However, the effects of hBD3 in skin-resident DCs have not been specifically examined. We chose to address this issue by investigating BD effects on DCs, critical antigen-presenting cells capable of priming antigen-specific T-cell responses and skewing-acquired immunity in response to environmental stimuli. As defensins are produced primarily in skin, we chose to study the effects of hBD3 on cultured DCs that most closely resemble those found in skin. Using well-established techniques, we were able to generate and manipulate sufficient numbers of human LC-DCs from peripheral blood precursors obtained from normal donors. The immature LC-DCs used in our studies express the LC markers CD1a, langerin, and E-cadherin.

Human BD3 has been shown to be chemotactic for monocytes and memory T cells (Wu et al., 2003). We now show that this molecule has an important role in stimulating acquired immune responses by inducing phenotypic and functional maturation of LC-DCs in vitro. Consequently,
matured LC-DCs achieve the capacity to induce proliferation and IFN-γ production by naive T cells. As part of the maturation process, hBD3-treated LC-DCs upregulate expression of CCR7 and become responsive to the lymph node-homing CCR7 ligands CCL19 and CCL21. Together, our findings suggest that hBD3 may act as an endogenous danger signal that alerts the immune system to possible infection and mobilizes, activates, and polarizes DCs to become effective activators of T-cell responses. Interestingly, the extent of this hBD3-mediated maturation of LC-DCs was much greater than that observed with TNF-α treatment.

In a mouse model, Biragyn *et al.* (2002) using mBD2 and murine DCs found that mBD2 induced phenotypic maturation and improved the antigen presentation function in mixed lymphocyte reactions. Results from Biragyn *et al.* (2002) study were reported as being consistent with a mechanism, whereby mBD2 induced DC maturation via TLR4. In contrast, cathelicidin peptides have been shown to block TLR4-mediated activation of DCs in a murine model (Di Nardo *et al.*, 2007). Interestingly, studies now suggest that hBD3 binds promiscuously to several receptors, depending on the cell type and function. For instance, in studying the response of human monocytes to hBD3 maturation, work by Funderburg *et al.* (2007) showed that activation of TLR1/2 heterodimers was required for hBD3-induced maturation, whereas a different group (Rohrl *et al.*, 2010) showed that hBD3-mediated monocyte chemotaxis is dependent on the CCR2 receptor. In other studies, hBD1 and hBD2 have been reported to bind to CCR6 (inducing chemotaxis; Yang *et al.*, 1999a), whereas hBD3 has been suggested to bind to CXCR4 (inducing downregulation of this receptor and preventing its use by HIV as a coreceptor for T-cell infection; Feng *et al.*, 2006). Our findings suggest that LC-DCs do not use these pathways as the use of a MyD88 peptide inhibitor or pertussis toxin (an inhibitor of G protein–coupled receptor signaling) had no significant effects on the maturation of LC-DCs by hBD3. In separate studies, we investigated whether TLR5 might be involved in the hBD3-mediated effects (as flagellin-mediated signaling is a strong inducer of hBD3 induction in skin cells (Gerstel *et al.*, 2009)). We found that hBD3 does not require TLR5 for LC-DC maturation (unpublished observations). In summary, these findings suggest that these receptors are not involved in mediating the observed hBD3 maturation of LC-DCs. However, further work is needed to determine which receptor(s) is/are utilized by hBD3 to mediate LC-DC maturation.

The role of inflammatory NF-κB and MAPK/AP1 signals in promoting the maturation of various antigen-presenting cells has been well documented, as these transcription factors control several genes involved in DC maturation (see Li and Verma 2002 for review). Our studies demonstrate that activated NF-κB is required for hBD3-induced maturation in LC-DCs. In the presence of an IκB super-repressor, there is significant inhibition of CD86 and CCR7 upregulation in LC-DCs. It is interesting to note, however, that despite the well-documented potent NF-κB-inducing capability of TNF-α, this cytokine resulted in only a marginal upregulation of the costimulatory markers examined in our study. This suggests that NF-κB may well be required but is not sufficient to fully activate the LC-DC maturation pathway, and that other factors in the hBD3 signaling pathway are necessary to mature LC-DCs. Indeed, these factors may include the MAPK/AP1 pathway, which appears to be involved in the maturation of LC-DCs based on our results using PD98059 (an inhibitor of Erk-dependent MAPK/AP1 activation). Relevant to this, our group has recently identified cooperative transcriptional control of CCR7 expression in tumors by hBD3, which is mediated by NF-κB and AP1 transcription factors (Mburu *et al.*, 2011, 2012).

Our findings have important implications for understanding how immune responses are generated and regulated in the skin, particularly in understanding inflammatory skin diseases such as psoriasis. hBD3 has been shown to be upregulated in psoriatic skin. Psoriasis is a classic T-helper type 1-mediated disease, associated with high levels of T-cell production of IFN-γ (Liu *et al.*, 2007). Further, infection of skin or other soft tissue has been implicated in the pathogenesis of psoriasis (Teravert, 1970). This could partly be because of increased production of hBD3 in response to microbial infection, which in turn induces cutaneous LC maturation and subsequent T-cell activation and cytokine production. hBD3 appears to be a natural adjuvant capable of stimulating innate immunity and enhancing specific immune responses against antigens encountered by the skin.

In summary, we have shown that hBD3 induces activation of human LC-DCs and primary human cutaneous DCs *in vitro*. Activated LC-DCs upregulate CCR7, become responsive to the lymph node-homing chemokines CCL19 and CCL21, prime naive T cells, and stimulate T-cell production of IFN-γ. Furthermore, the observed hBD3-induced maturation of LC-DCs is dependent on NF-κB and MAPK/AP1 activation. These results contribute to our understanding of the potential role of hBD3 in the pathogenesis of inflammatory skin diseases and demonstrate the adjuvant function of hBD3. Our findings should be useful for the development of novel immunogens that incorporate hBD3 and specific antigens to enhance the immunogenicity of DC-based immunotherapies and skin-targeted vaccines.

**MATERIALS AND METHODS**

**Antibodies and reagents**

The following cytokines and chemokines were purchased from R&D Systems (Minneapolis, MN): rhGM-CSF, rhIL-4, rhTGF-β1, rhTNF-α, rhCCL19, and rhCCL21. The following antibodies were used for flow cytometric analysis were purchased from BD Biosciences (San Jose, CA): mouse anti-human CD40-PE (clone 5C3), mouse anti-human CD83-FITC (clone HB15e), mouse anti-human CD86-PE (clone L243), Phycoerythrin-conjugated mouse anti-human langerin (CD207; clone DCGM4) was purchased from Immunotech (Fullerton, CA). Synthetic hBD3 containing the proper disulfide bridges was purchased from Immunotech (San Diego, CA). Adenoviral constructs were obtained from the vector core facility at the University of Pittsburgh.
Cell culture
BUFFY coats from healthy donors were obtained from the Pittsburgh Central Blood Bank through an IRB-exempt protocol. Peripheral blood mononuclear cells were separated over a Ficoll gradient by centrifugation at 1400 r.p.m. for 30 minutes at room temperature. Cells were then washed three times in RPMI, and monocytes were separated from lymphocytes using 60, 45, and 34% percoll gradients. The monocyte layer was removed, washed in RPMI, and then plated at a density of 10^6 cells per ml in serum-free AIM-V medium supplemented with rhGM-CSF (10 ng ml⁻¹) + rhTGF-β1 (5 ng ml⁻¹) for LC-DCs or with rhGM-CSF (10 ng ml⁻¹) + rhIL-4 (10 ng ml⁻¹) for mDCs. Fresh cytokines were added on culture day 3. Cells were harvested by placing at 4°C and resuspending in cold 1 x PBS and used on days 6-7 of culture.

Flow cytometry
Flow cytometric analysis was performed using a Becton-Dickinson FACS calibur. Staining was performed using a wash buffer of 1% fetal bovine serum in PBS and the manufacturer’s recommended concentration of antibody (or if no recommendations were given, a concentration of 5 μg ml⁻¹ was used) to stain cells for 25 minutes. Cells were washed twice in wash buffer and fixed in 2% paraformaldehyde in PBS before data acquisition.

Chemotaxis assay
Chemotaxis assays were performed using disposable 96-well chemotaxis plates (Neuro Probe, Gaithersburg, MD). A volume of 30 μl of serum-free AIM-V medium alone or medium containing the respective chemokine ligands CCL19 or CCL21 (250 ng ml⁻¹) was added to the lower wells of a 96-well plate. An 8-μm-pore membrane was placed over the wells taking care not to trap any air bubbles. To the top of the membrane, a 50 μl cell suspension containing 2.5 × 10⁴ LC-DCs in AIM-V was added. Cells were allowed to migrate for 90 minutes at 37°C. The number of migrated cells in the lower wells was determined by counting on a hemacytometer under trypan blue staining. All assays were performed in duplicate. Chemotactic index, a measure of relative migration, was obtained by calculating the number of cells migrating into chemokine-containing wells relative to that migrating into wells containing medium only.

T-cell cytokine production and proliferation assays
LC-DCs were matured by incubation with indicated maturation inducer(s) for 18 hours in AIM-V medium containing 5 % human AB serum at 37°C, washed, and then counted. T cells from an allogeneic donor (from buffy coats purchased from the Pittsburgh Central Blood Bank) were isolated using a Percoll gradient as described above. CD4 + CD45RA + naïve T cells were then isolated by negative selection using magnetic beads as per the manufacturer’s instructions (Miltenyi, Auburn, CA). For stimulation assays, various dilutions of LC-DCs and 10⁵ T cells were added in AIM-V at a total volume of 200 μl per well of a 96-well plate. Plates were incubated for 5 days at 37°C, after which supernatants were harvested and stored at -20°C for later use or immediately and tested for IFN-γ using commercially available ELISA-matched antibody pairs (BD Pharmingen, San Jose, CA) as per the manufacturer’s protocol. In the T-cell proliferation assays, T cells were stimulated as described for cytokine production using LC-DCs that were irradiated at 2500 rads.

Proliferation was measured as a function of tritiated thymidine uptake during the final 16 hours of a 3-day culture.

Generation of skin-migratory DCs
The generation of primary human skin-migratory DCs has been described, and these cells have been characterized previously (Larregina et al., 2001). In brief, residual human skin generated following abdomenoplasty was excised with a skin graft knife (Padgett Instruments, Kansas City, MO) to generate skin explants with a thickness of 0.3-0.5 mm. These explants were then cultured, epidermal side up, on 1-mm-pore meshes with RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 20 μg ml⁻¹ gentamycin (Sigma, St Louis, MO) at 37°C in 5% CO₂. After 72 hours, nonadherent cells that had migrated out of the explants were collected and exposed to hBD3 or medium for 18 hours. Analysis for markers of maturation was performed as described above.

Statistical analysis
The data are expressed as mean ± SE of two to three repeats. A two-tailed Student’s t-test was used to calculate whether the observed differences were statistically significant. The threshold for significance was P<0.05.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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