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Human Beta Defensin 3 induces maturation of human langerhans cell like dendritic cells: An antimicrobial peptide that functions as an endogenous adjuvant

Laura K. Ferris[1,5,*], **Yvonne K. Mburu**[2,3,*], **Alicia R. Mathers**[1], **Eric R. Fluharty**[1], **Adriana T. Larregina**[1,3], **Robert L. Ferris**[2,3,4], and **Louis D. Falo Jr.**[1,4]

[1]Department of Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, PA

[2]Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA

[3]Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA

[4]University of Pittsburgh Cancer Institute, Pittsburgh, PA, 15213

Abstract

Human beta defensins (hBDs) are antimicrobial peptides that play an important role in innate immune responses at epithelial barriers such as the skin. However, the role that hBDs play 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 (Th1) skewing function in human Langerhans cell-like DCs (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 and IFN-γ secretion by naïve human T cells. HBD3 also induces phenotypic maturation of primary human skin-migratory dendritic cells 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.

Keywords

Langerhans cells; Skin-derived dendritic cells; Human beta-defensin 3

[5]To whom correspondence should be addressed: University of Pittsburgh School of Medicine, Department of Dermatology, 145 Lothrop Hall, 190 Lothrop Street, Pittsburgh, PA 15213.

*These authors contributed equally to this work

Conflict of Interest

The authors declare no conflicts of interest.

Introduction

Antimicrobial peptides such as the human beta defensins (hBD1-4) and cathelicidins are produced in a variety of epithelial tissues, with particularly high expression in inflammed 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 due to the secretion of various pathogenic stimuli and cytokines. For instance, increased expression of hBD2 has been directly associated with stimulation by TNF-α, IL-1, LPS (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), TGF-α alone or in combination with insulin-like growth factor I (IGF-I) (Sorensen *et al.*, 2003), stimulation of toll-like receptor (TLR)-2 (Menzies and Kenoyer, 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 and T cells to sites of inflammation (He *et al.*, 2006; Larregina and Falo, 2005; Oppenheim *et al.*, 2003; Territo *et al.*, 1989). 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). Additionally, hBD3 has been shown to induce the expression of various costimulatory molecules on monocytes and myeloid DCs through its interactions with toll like receptors (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, mBD2 has been shown to promote the maturation of DC via TLR4, the receptor for bacterial lipopolysaccharide (LPS) (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 pathogensis 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 beta defensin 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 the psoriatic skin flakes and retains antimicrobial activity at physiologic salt concentrations (Harder *et al.*, 2001). Because hBD3 is made primarily by keratinocytes, we specifically studied its effect on human Langerhans cells like DCs (LC-DC) and skin-derived DCs. LC play 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, LC migrate rapidly out of the skin in a CCR7-dependent fashion 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; Guironnet *et al.*, 2002; Yang *et al.*, 1999b). LC-DCs differ from classic monocyte derived DCs generated by culture with GM-CSF and IL-4 and uniquely express molecules typical of Langerhans cells (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 naïve CD4+CD45RA+ T cells. HBD3 also induced maturation/activation of primary human skin-migratory DC, 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 PBMC 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).

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 5μM hBD3. For comparison, we evaluated the activation effects of TNF-α, a well-established LC activation signal which is present at increased levels in inflamed skin, by stimulating immature LC-DCs with 10ng/ml 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 CD40 (Figure 2A). In contrast, TNF-α induced increased expression of HLA-DR on LC-DCs, but did not significantly up regulate the other maturation molecules tested. Notably, hBD3 strongly upregulated CCR7, a crucial lymph node homing receptor, while 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 dose-response analysis using 500nM, 1μM, 2 μM 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 co-stimulatory 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-DC 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 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 or hBD3-treated LC-DCs to activate naïve 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 Th1-type immune response.

hBD3 induced maturation of LC-DCs is not MyD88 or G_iPCR dependent, but is dependent on NF-κB and 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-DC by hBD3, we pretreated LC-DC with a MyD88 peptide inhibitor (100μM, 18hr) prior to 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 LPS-induced maturation of LC-DC at similar concentrations (Figure 5B).

In addition, other studies have shown that hBDs can signal through G_i-protein coupled receptors (G_iPCRs) such as CCR2, CCR6 or CXCR4 (Feng *et al.*, 2006; Rohrl *et al.*, 2010; Yang *et al.*, 1999a). To determine whether any of these G_iPCRs were involved in the maturation of LC-DCs by hBD3, we pretreated day 6 LC-DCs with pertussis toxin for 2hr prior to maturation with hBD3, and then examined HLA-DR, CD83, CD86 and CCR7 upregulation as markers of overall LC-DC maturation. While there was a notable reduction in CD86, our findings show that the blocking of G_iPCRs 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; Mburu *et al.*, 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 IκBαAA 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). Secondly, to determine whether MAPK/AP1 activation was necessary for hBD3-mediated LC-DC maturation and CCR7 upregulation, we pre-treated day 6 LC-DC with the MAPK inhibitor PD98059 (100μM, 2hr) followed by overnight stimulation with 5μM hBD3. MAPK/AP1 inhibition resulted in a reduction in hBD3-mediated CD86 and CCR7 upregulation in LC-DCs. Together, these data suggest that hBD3 stimulates the maturation of LC-DCs through NF-κB and MAPK/AP1 dependent pathways.

Skin-migratory dendritic cells acquire a mature phenotype following exposure to hBD3

We focused our studies on LC-DC since they can be reliably generated in sufficient quantities to allow for both phenotypic and functional studies to be performed. 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 if hBD3 had a similar effect on the activation of these cells. Skin migratory DC were cultured for 18 hours with medium alone or with hBD3 (5μM, 37°C) and expression of CD86 and CCR7 was measured by flow cytometry (Figure 6). As seen in our LC-DC experiments, primary skin-migratory DC, although already in a more mature state at baseline, showed further phenotypic maturation following treatment with hBD3, extending the relevance of our findings to human cutaneous DCs.

Discussion

Antimicrobial peptides, including BDs and cathelicidins, are made 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, 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 (Berthier-Vergnes *et al.*, 2005; De Smedt *et al.*, 1996; Flacher *et al.*, 2006; Rieser *et al.*, 1997). While the role of BDs in innate immunity is 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 homologue to hBD3) which show that BDs can have some anti-inflammatory properties as well, through the induction of T

regulatory cells (Navid *et al.*, 2012; Semple *et al.*, 2011; Semple *et al.*, 2010). However, the effects of hBD3 in skin-resident DCs has not been specifically examined. We chose to address this issue by investigating BD effects on dendritic cells, critical antigen presenting cells capable of priming antigen-specific T-cell responses and skewing acquired immunity in response to environmental stimuli. Since defensins are made 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 Langerhans cell 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 plays an important role in stimulating acquired immune responses by inducing phenotypic and functional maturation of LC-DC *in vitro*. Consequently, matured LC-DCs achieve the capacity to induce proliferation and IFN-γ production by naïve T cells. As part of the maturation process, hBD3-treated LC-DC 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 dendritic cells 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.* using mBD2 and murine DCs found that mBD2 induced phenotypic maturation and improved antigen presentation function in MLRs (Biragyn *et al.*, 2002). Results from Biragyn *et al.* were reported as consistent with a mechanism whereby mBD2 induced DC maturation via TLR4 (Biragyn *et al.*, 2002). In contrast, cathelicidin peptides have been shown to block TLR4 mediated activiation 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.* showed that activation of TLR1/2 heterodimers was required for hBD3-induced maturation, while 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) while hBD3 has been suggested to bind to CXCR4 (inducing downregulation of this receptor and preventing its use by HIV as a co-receptor for T cell infection) (Feng *et al.*, 2006). Our findings suggest that LC-DCs do not use these pathways since the use of a MyD88 peptide inhibitor or pertussis toxin (an inhibitor of G_i-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 (since 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 sum, 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 is well documented, since 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 in order 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; Mburu *et al.*, 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 Th1-mediated disease, associated with high levels of T cell production of IFN-γ (Liu *et al.*, 2006). Further, infection of skin or other soft tissue has been implicated in the pathogenesis of psoriasis (Teravert WC, 1970). This could be due in part to increased production of hBD3 in response to microbial infection which in turn induces cutaneous langerhans cell 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-DC and primary human cutaneous DC *in vitro*. Activated LC-DCs upregulate CCR7, become responsive to the lymph node homing chemokines CCL19 and CCL21, prime naïve 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 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 2331), mouse anti-human CCR6-PE (clone 11A9), rat anti-human CCR7-FITC (clone 3D12), mouse anti-human HLA-DR-FITC (clone L243). PE-conjugated mouse anti-human langerin (CD207) (clone DCGM4) was purchased from Immunotech (Fullerton, CA). Synthetic hBD3 containing the proper disulfide bridges was purchased from Peptides International (Louisville, KY). The MyD88 peptide inhibitor was purchased from Imgenex (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 (PBMC) were separated over a Ficoll gradient by centrifugation at 1400 rpm for 30 minutes at room temperature. Cells were then washed 3 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 (10ng/ml) + rhTGF-β1 (5ng/ml) for LC-DCs, or rhGM-CSF (10ng/ml) + rhIL-4 (10ng/ml) for mDCs. Fresh cytokines were added on culture day 3. Cells were harvested by placing at 4°C and resuspending in cold 1× PBS and were used on day 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% FBS in PBS and the manufacturer's recommended concentration of antibody (or if no recommendations were given a concentration of 5μg/ml was used) to stain cells for 25 minutes. Cells were washed twice in wash buffer and fixed in 2%PFA in PBS prior to data acquisition.

Chemotaxis assay

Chemotaxis assays were performed using disposable 96 well chemotaxis plates (Neuro Probe Inc., Gaithersburg, MD). 30μl of serum-free AIM-V medium alone or media containing the respective chemokine ligands CCL19 or CCL21 (250ng/ml) was added to the lower wells of a 96-well plate. An 8μm pore membrane was placed over the wells using care not to trap any air bubbles. To the top of the membrane, a 50μl cell suspension containing 2.5×10^4 LC-DCs in AIM-V was then 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 done in duplicate. Chemotactic index, a measure of relative migration, was obtained by calculating number of cells migrating into chemokine-containing wells relative to that migrating in 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 then washed and 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 per manufacturer's instructions (Miltenyi, Auburn, CA). For stimulation assays, various dilutions of LC-DCs and 10^5 T cells were added in AIM-V in a total volume of 200μl 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) per 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 DC

The generation of primary human skin-migratory DC has been described and these cells have been characterized previously (Larregina *et al.*, 2001). Briefly, residual human skin generated following abdominoplasty 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 steel meshes with RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 20 mM Hepes, 2 mM l-glutamine, 200 U/ml of penicillin-streptomycin (Gibco-BRL, Grand Island, NY) and 20 μg/ml of gentamicin (Sigma, St Louis, MO) at 37 °C in 5% CO_2. After 72 h, 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 ± S.E. of 2–3 repeats. A two-tailed student's *t* test was used to calculate whether the observed differences were statistically significant. The threshold for significance was $p<0.05$.

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Abbreviations

HBD3	human beta-defensin 3
LC-DC	langerhans cell like dendritic cells
TLR	Toll like receptor
PBMC	peripheral blood mononuclear cells

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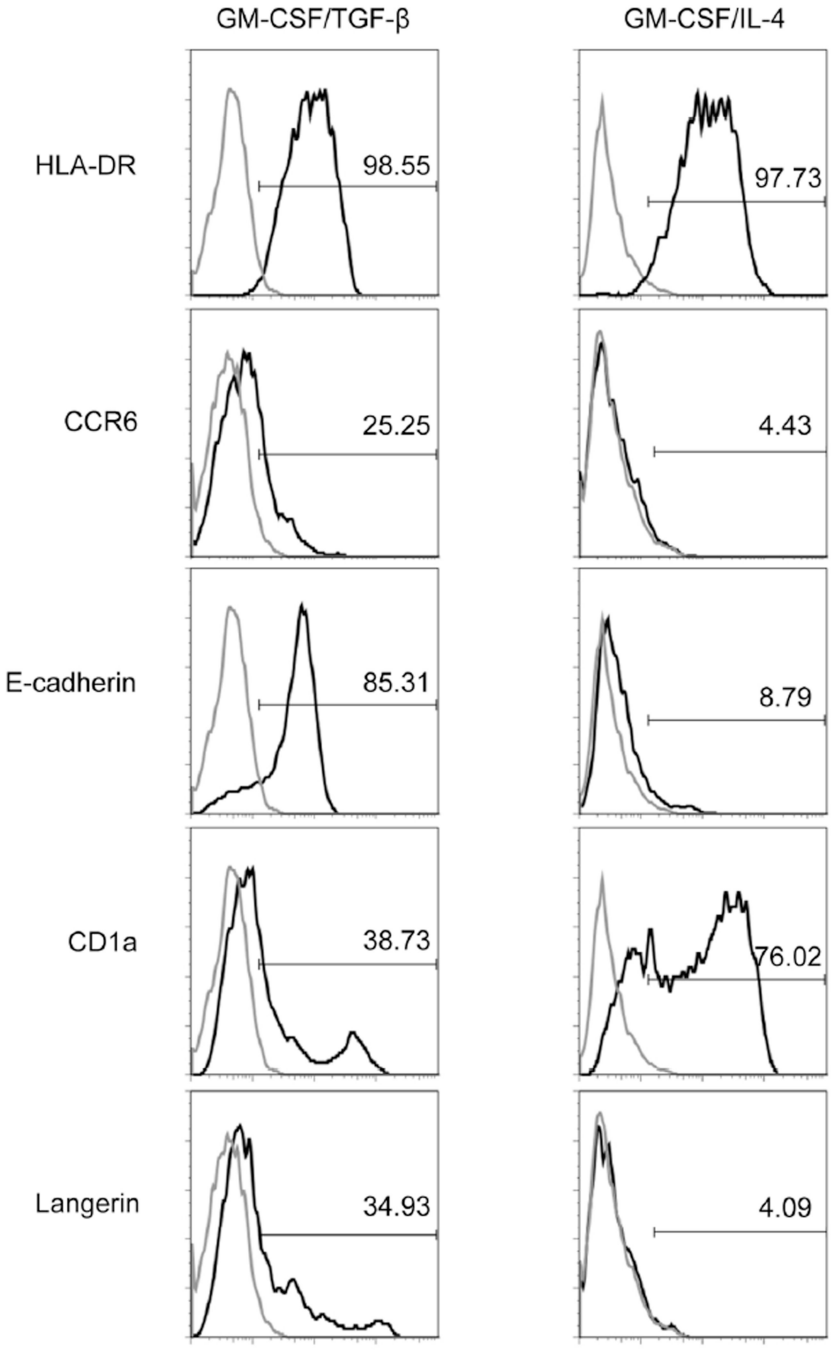

Figure 1. Culturing human monocytes with GM-CSF and TGF-β1 generates Langerhans cell-like dendritic cells (LC-DC) that are distinctly different from dendritic cells generated in the presence of GM-CSF and IL-4

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 six days. Cell surface expression of HLA-DR, E-cadherin, CD1a, CCR6, and langerin was assessed by flow cytometry. In each case, the percentage cells considered positive for each marker is shown. Results are representative of three separate experiments with cells generated from different donors.

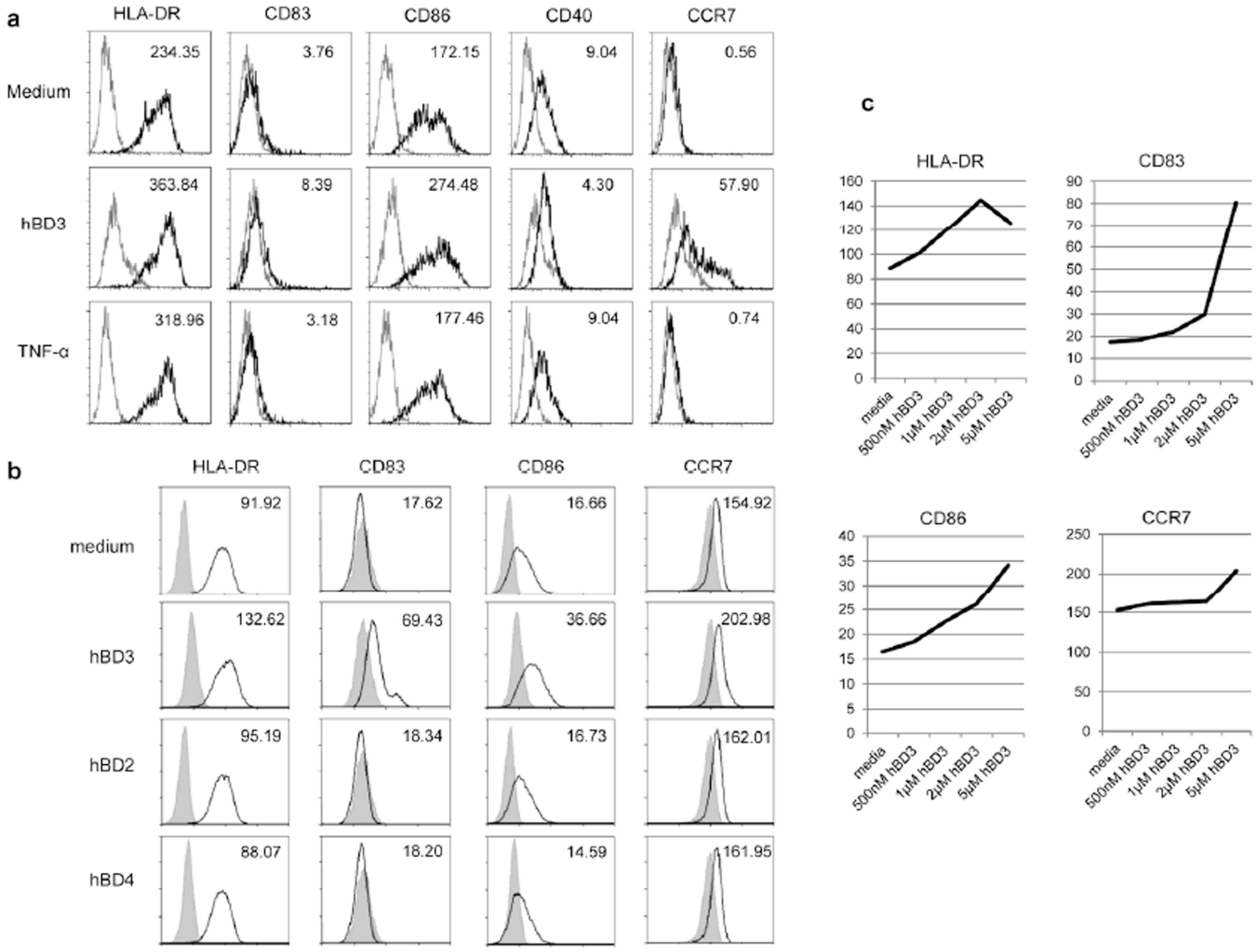

Figure 2. hBD3 induces phenotypic maturation of LC-DC

(A) Day 6 LC-DC were cultured for 18hr with medium, TNF-α (10ng/ml), or hBD3 (5μM). The surface expression of HLA-DR, CD83, CD86, CD40, and CCR7 was assessed by flow cytometry. The shaded gray histogram indicates the isotype-matched irrelevant antibody, while the black line represents the antibody to the indicated protein. Numbers represent the MFI of each experiment. (B and C) Flow cytometric analysis following overnight culture of day 6 LC-DC with (B) 5μM hBD2, hBD3 or hBD4 or (C) varying concentrations of hBD3 (500nM, 1μM, 2μM, 5μM). The surface expression of various markers was assessed and the MFI values plotted graphically. Results are representative of 3–5 experiments using cells from different donors.

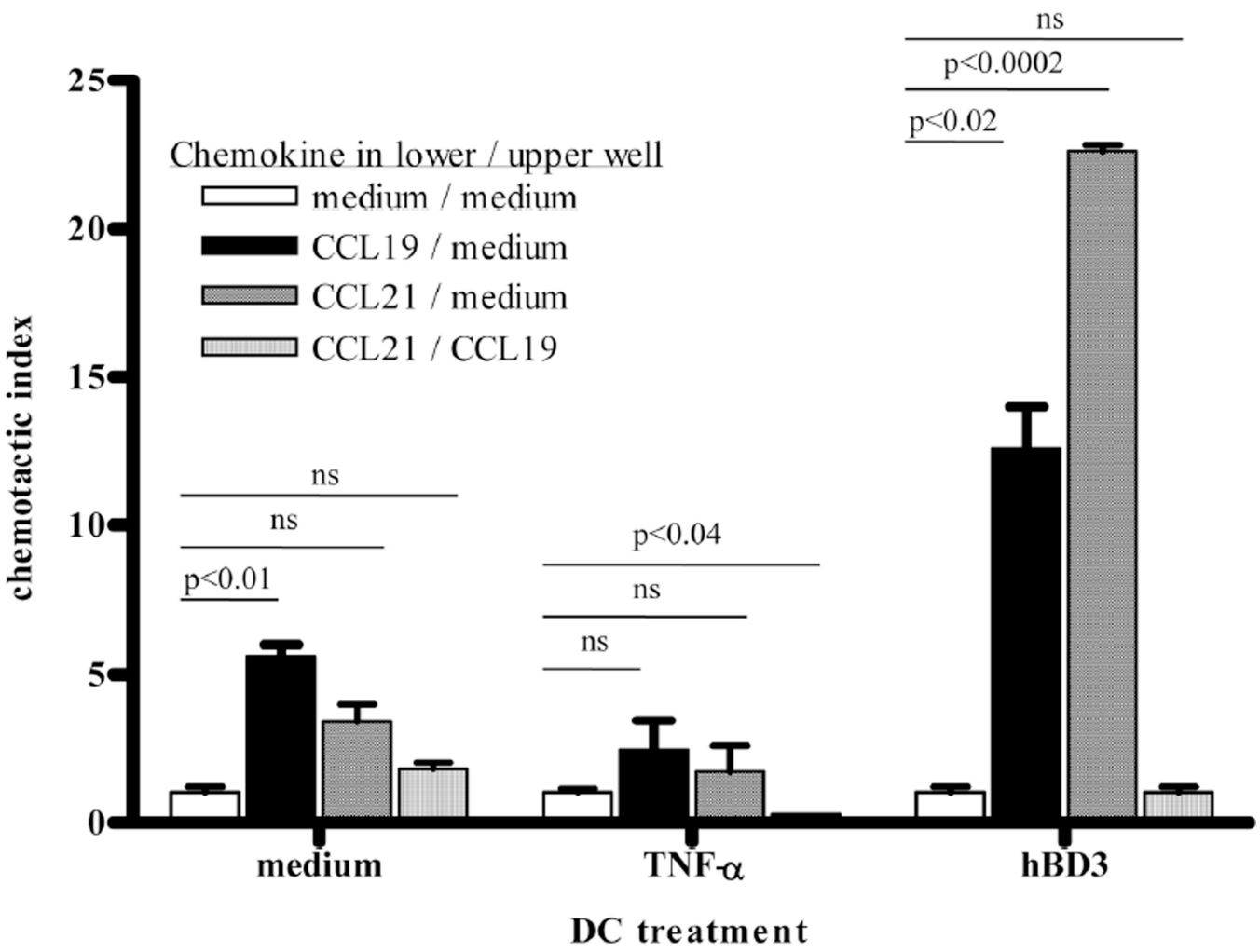

Figure 3. hBD3-matured LC-DCs are chemotactic for the CCR7 ligands

Day 6 LC-DCs were treated for 18 hours with medium alone, TNF-α (10ng/ml) or hBD3 (5μM). The cells were collected and added to the upper chamber of a chemotaxis plate containing medium, CCL19 (250ng/ml) or CCL21 (250ng/ml) in the lower wells. Migration of cells toward the chemokine was measured by counting the number of cells migrated into the lower wells after 90 minutes. Chemotactic index = cells migrating toward chemokine / cells migrating toward medium. To show specificity of chemotactic response, LC-DCs were resuspended in medium containing CCL21 (250ng/ml) and their migration toward CCL19 (250ng/ml) chemoattractant in the lower wells was assessed (bars labeled CCL21/CCL19). Results are representative of 3 separate experiments performed in duplicate.

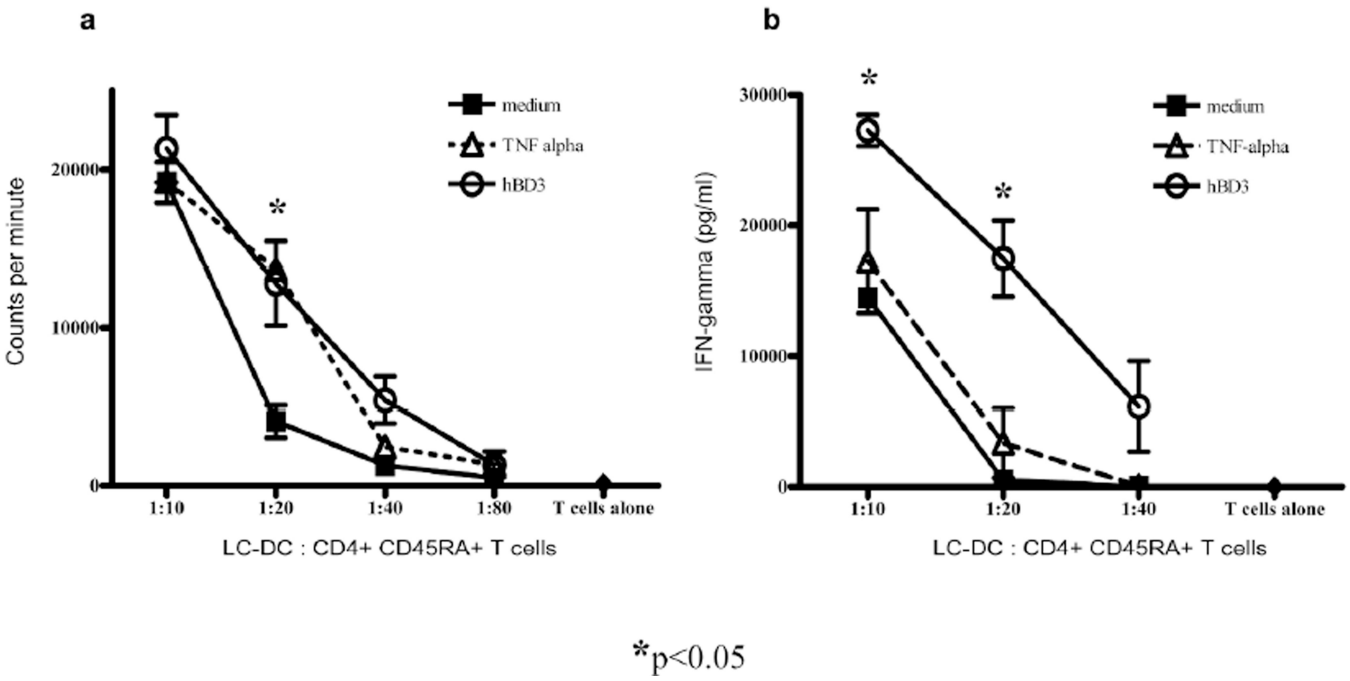

Figure 4. hBD3-matured LC-DCs activate naïve T cells and enhance the proliferation and IFN-γ secretion of T cells in a mixed lymphocyte reaction

Day 6 LC-DCs were cultured for 18 hours with medium alone, TNF-α (10ng/ml) or hBD3 (5μM). Cells were then co-cultured with allogeneic purified CD4+CD45RA+ T cells. (A) T cells were cultured for 3 days at LC-DC:T cell ratios ranging from 1:10 to 1:80. T cell proliferation was measured by uptake of tritiated thymidine during the last 18 hours of culture. (B) Production of IFN-γ was measured by ELISA after 5 days of culture and is also shown for a range of LC-DC:T cell ratios. Results are representative of three separate experiments. (*p<0.05)

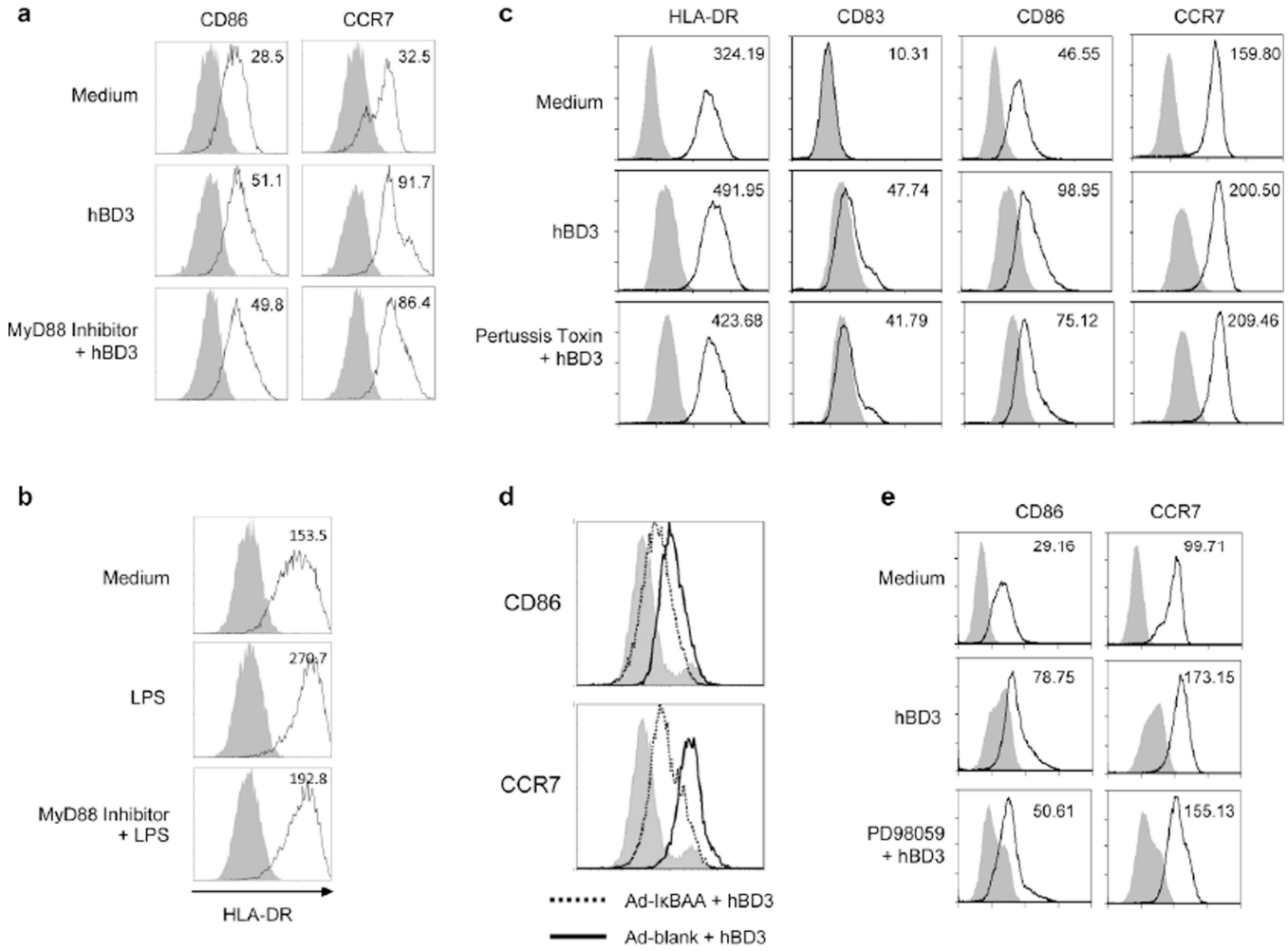

Figure 5. hBD3 stimulation of LC-DCs is not MyD88 or G_iPCR-dependent but is dependent on NF-κB and MAPK activation

(A and B) Day 5 LC-DCs were treated overnight with MyD88 inhibitor (100μM) followed by stimulation with (A) hBD3 (5μM, 18hr) or (B) LPS (100ng/ml, 18hr) and analysis by flow cytometry. Values represent MFI in treated cells. (C) Day 6 LC-DC were treated with pertussis toxin (200ng/ml, 2hr) followed by hBD3 (5μM, 18hr). (D) Day 5 LC-DCs were transduced with control (solid line) or IκBAA (dotted line) containing adenovirus over 24hr, followed by treatment with hBD3 (5μM, 18hr). (E) Day 6 LC-DC were treated with PD98059 (100μM, 2hr) followed by hBD3 (5μM, 18hr). Results shown are representative of 2–3 experiments with cells from different donors.

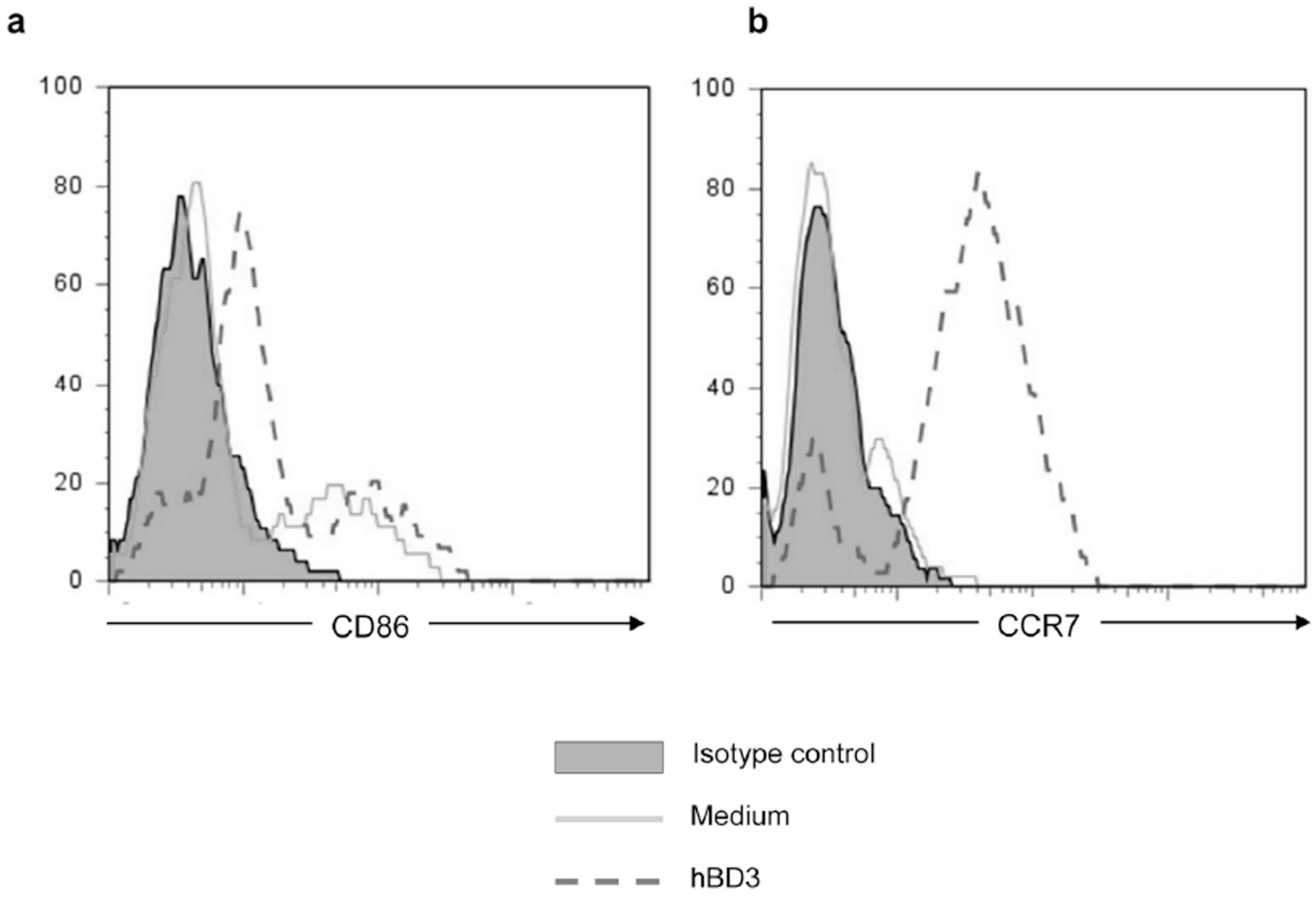

Figure 6. hBD3 induces phenotypic maturation of skin-migratory DC

Skin migratory DC were cultured for 18 hours in the presence or absence of hBD3 (5μM). 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 isotype-matched control antibody.