Molecular Immunology 109 (2019) 99–107

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Immunoregulatory effect of human β-defensin 1 on neonatal cord blood monocyte-derived dendritic cells and T cells

Jing Wu[b], Ruo-Lan Gong[b], Qing-Feng Hu[b], Xu-Ting Chen[b], Wei Zhao[c], Tong-Xin Chen[a,b,*]

[a] Department of Rheumatology/Immunology, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China
[b] Division of Immunology, Institute of Pediatric Translational Medicine, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China
[c] Division of Allergy and Immunology, Department of Pediatrics, Virginia Commonwealth University, Richmond, VA, 23298, USA

ARTICLE INFO

Keywords:
Immunoregulatory effect
hBD-1
Cord blood
Dendritic cells
T cells

ABSTRACT

The relationship between breastfeeding and infant health has been well elucidated in past decades. Our previous study has shown that human β-defensin 1 (hBD-1) in human breast milk plays a protective role in reducing the incidence of upper respiratory infection in infants younger than 6 months. In the present study, we aim to reveal the mechanism underlying the protective role of hBD-1 by focusing on its immunoregulatory function in neonates. Cord blood (CB) from newborns' umbilical cords, which can simulate many of the neonatal symptoms, was used to study the immunomodulatory role of hBD-1 in neonates *in vitro*. Our results showed that hBD-1 promotes the GM-CSF- and IL-4-driven differentiation of neonatal umbilical CB monocytes to immature dendritic cells (DCs) and the final maturation of CB monocyte-derived DCs (moDCs) induced by LPS but not inflammatory cytokine production. In addition, hBD-1 inhibits apoptosis in neonatal moDCs through CCR6, which might be a possible mechanism of the hBD-1-induced phenotypes in moDCs. Furthermore, we found that hBD-1 promotes the proliferation and activation, but not the maturation, of neonatal CB CD4 + T cells. These results extend the immunoregulatory effects of hBD-1 and provide a potential mechanism for the protective role of hBD-1 in early infants, which will inform the development of infant nutrition, novel vaccines and anti-infective strategies in the future.

1. Introduction

Neonates are reported to be vulnerable to infections, and their sensitivity to infectious agents is largely due to their immature immunity (Adkins et al., 2004). Indeed, mounting studies have demonstrated that there are both quantitative and qualitative differences in innate and adaptive immunities between newborns and adults (Basha et al., 2014; Das et al., 2017). For example, compared to adult monocytes, neonatal monocytes have impaired chemotactic, phagocytic and bactericidal properties (Dowling and Levy, 2014). Fewer conventional dendritic cells were reported in newborn cord blood (CB) than in adult peripheral blood (PB) (Kollmann et al., 2009). Immature monocyte-derived dendritic cells (moDCs) are less abundant and have reduced phenotypic expression and function in CB compared to PB, and *in vitro* differentiated neonatal CB moDCs are less responsive to TLR stimulation (Kollmann et al., 2009; Liu et al., 2001b). Furthermore, the impairment of DC function reportedly leads to T-cell dysfunction in neonates, and early infants have fewer memory-effector T cells ($CD45RA^-$, $CD45RO^+$) and B cells ($CD27^+$) (Dowling and Levy, 2014). These immature and dysfunctional features of the neonatal innate and adaptive immunities contribute to their susceptibility to intracellular pathogens.

In spite of their immature immunity, many studies have demonstrated that breastfed infants have reduced incidence of infections and allergies (Gdalevich et al., 2001; Oddy, 2001, 2004; Van et al., 2015). Human breast milk contains numerous immunological components, including antimicrobial substances, immune cells and immunoregulatory constituents (Field, 2005). Human β-defensins (hBDs), which comprise of a group of host defense peptides, have been identified in human breast milk by our own and other studies (Murakami et al., 2005; Wang et al., 2014). Our previous study also demonstrated that hBD-1 abundance in the colostrum reduces the incidence of upper

Abbreviations: hBD1, human β-defensin 1; CB, cord blood; PB, peripheral blood; CBMC, cord blood mononuclear cells; DCs, dendritic cells; MoDCs, monocyte-derived dendritic cells; MR, mannose receptor; LPS, lipopolysaccharide; MLR, mix lymphocyte reaction

* Corresponding author at: Department of Rheumatology/Immunology, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, 1678 Dongfang Road, Shanghai, 200127, China.
E-mail address: tongxinc@sjtu.edu.cn (T.-X. Chen).

https://doi.org/10.1016/j.molimm.2019.03.007
Received 31 October 2018; Received in revised form 11 March 2019; Accepted 15 March 2019
Available online 26 March 2019

respiratory infection in infants younger than 6 months, suggesting that hBD-1 plays a protective role against infections (Wang et al., 2014). The antimicrobial activity of hBD-1 presumably contributes to its protective function. However, the exact mechanism is still unknown.

Defensins, which are considered important components of the human innate immune system, comprise a group of antimicrobial peptides. In humans, there are two types of defensins: α-defensins (HNP-1-4, HD5, and HD6) and β-defensins (hBD-1-6). It has become increasingly clear that these peptides have numerous functions, of which antimicrobial activity is just one (Kohlgraf et al., 2010; Zhao and Lu, 2014). In recent years, immunoregulatory functions of defensins has been reported (Yang et al., 2015; Semple and Dorin, 2012a; 2012b). However, there have been few reports about the immunoregulatory functions of hBD-1. Yang et al. (Yang et al., 1999) reported that hBD-1 induces chemoattraction of CD4+ memory T cells and immature DCs by binding to CCR6. hBD-1 was also shown to activate adult moDCs and promote the production of proinflammatory cytokines (Presicce et al., 2009). However, these studies on hBD-1 were conducted in adults, and there have been no studies in neonates. Therefore, the immunomodulatory functions of hBD-1 in neonates still needed to be determined.

In the present study, we aim to determine the immunoregulatory function of hBD-1 in neonatal CB. CB from newborns' umbilical cords, which can simulate many of the neonatal symptoms, was used here to study the immunomodulatory role of hBD-1 in neonates *in vitro*. We demonstrated, for the first time, that hBD-1 plays an immunoregulatory role in neonates, which will further inform our understanding of the protective role of hBD-1 against infections in neonates.

2. Material and methods

2.1. Isolation of monocytes and T cells

This study was conducted according to the guidelines in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of Shanghai Children's Medical Center, which is affiliated with Shanghai Jiao Tong University School of Medicine. Human umbilical CB was collected from the placentae of normal, full-term infants after delivery of the placentae. Written informed consent was obtained from all subjects.

Cord blood mononuclear cells (CBMCs) were isolated by using Ficoll-Hypaque gradients (Lymphoprep, Alexis-Shield, Oslo, Norway). Highly enriched monocytes (> 80% $CD14^+$ cells) were obtained by adherence. Briefly, the isolated CBMCs were seeded on plastic plates at a density of 5×10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin and 100 U/mL streptomycin. After an incubation at 37 °C and 5% CO_2 for 2 h, nonadherent cells were removed, and the purity of $CD14^+$ cells was detected by fluorescence staining and subsequently flow cytometry analysis. T cells were isolated from CBMCs by positive selection using anti-CD4-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.2. Generation of immature moDCs in vitro

Purified monocytes (1×10^6 cells/mL) from different donors were cultured with GM-CSF (100 ng/mL, PeproTech, USA) and IL-4 (50 ng/mL, PeproTech, USA) for 5 days in the presence or absence of hBD-1 (50 ng/mL, ProSpec, East Brunswick, NJ). The cultures were fed with fresh medium and cytokines after 3 days. On day 5, cells were harvested, centrifuged, washed, and collected before further experiments.

2.3. moDC maturation in vitro

Purified monocytes (1 × 106 cells/mL) from different donors were cultured with GM-CSF (100 ng/mL, PeproTech, USA) and IL-4 (50 ng/mL, PeproTech, USA) for 5 days. On day 5, the differentiated immature moDCs were further stimulated with lipopolysaccharide (LPS; 100 ng/ml, Sigma) in the presence or absence of hBD-1 (50 ng/mL) for 48 h. Then, cells were harvested, centrifuged, washed, and collected before further experiments.

2.4. T cell proliferation, activation and maturation

Purified CD4 + T cells from different donors were stimulated with vehicle or PHA (5 μg/mL) in the presence or absence of hBD-1 (5 ng/ml) for 36 h, and then, cell proliferation was assessed with a Cell Counting Kit-8 (CCK-8). For T-cell activation and maturation assays, CBMCs were stimulated with vehicle, PHA or PMA/Ionomycin in the presence or absence of hBD-1(5 ng/ml) for 36 h. Activation markers of CD25, CD69, and CD40L and phenotypic changes of CD45 isoforms in CD4 + T cells were detected by flow cytometry analysis.

2.5. Phenotypic analysis

Cell phenotypic analysis of different donors was performed using flow cytometry. The following mAbs were used: CD14-FITC, CD1a-PE, MR-PE-Cy7, CD11c-APC (eBioscience, USA), CD40-APC-H7, CD80-PE, CD86-APC, MHCII-APC-Cy7, CD19-FITC, CD4-FITC, CD3-FITC, CD8-PE-CY7, CD69-PE, CD25-APC, CD40L–APC, CD45RA-PE, and CD45RO-APC (BD PharMingen, USA). Cells were stained and analyzed using a FACSCanto II (Becton Dickinson) flow cytometer, and data analysis was performed by using FACSDiva (Becton Dickinson) and FlowJo software.

2.6. Endocytosis

Mannose receptor (MR)-mediated endocytosis and fluid-phase endocytosis were measured by the uptake of FITC-dextran and Latex beads (Sigma), respectively. Before the experiment, latex beads were opsonized in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum for 30 min at 37 °C. Then, approximately 2×10^5 DC cells from different donors were incubated with FITC dextran (1 mg/ml, Sigma) or Latex beads for 0, 60, or 120 min. After that, the cells were washed with PBS twice and quantified by flow cytometry.

2.7. Mixed leukocyte reaction

moDCs from different donors were extensively washed, treated with mitomycin C for 30 min, and then labeled as stimulatory cells. The responder cells were allogeneic monocyte-depleted CBMCs; they were labeled with CFSE at day 0. Next, 5×10^5 CFSE-labeled responder cells were seeded in 96-well round-bottom plates, and then, stimulatory cells were added in graded doses of 5×10^4, 5×10^3, and 5×10^2 cells. After 5 days, the proliferation of alloreactive lymphocytes was determined by flow cytometry as the percentage of $CFSE^{low}$ CD4+ lymphocytes.

2.8. Cytokine production

Monocytes from different donors were cultured with GM-CSF and IL-4 for 5 days and then further induced with LPS in the presence or absence of hBD-1 for 48 h. Supernatants were harvested and stored at −80 °C. The levels of IL-6, IL-10, TNFα and IL12p70 were measured by commercially available enzyme-linked immunosorbent assays (ELISA; R&D systems, Minneapolis, MN, USA).

2.9. Apoptosis assay with AV/PI

The moDCs from different donors were pretreated with 5 g/ml CCR6 neutralizing antibody or 5 g/ml isotype control antibody (R&D Systems) at 37 °C and 5% CO2 for 30 min and were then cultured in the

presence or absence of hBD-1, as described above. Apoptotic cells were detected by with the Annexin V/PI apoptosis kit (BD PharMingen, USA). According to the manufacturer's instructions, cells were re-suspended in 100 μL of binding buffer and incubated with 5 μL of APC-conjugated Annexin-V and 5 μL of PI for 15 min at room temperature in the dark. After this incubation, 200 μL of binding buffer was added to each tube, and the cells were immediately analyzed by flow cytometry.

2.10. Western blot assay

MoDCs from different donors were obtained as described above, and total protein was extracted with ice-cold RIPA Lysis Buffer (Biyuntian, China). The total protein was separated with 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and it was subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. After that, the PVDF membrane was blocked, washed, and incubated with antibodies against beta actin (Sigma, USA) and BCL-xL (Cell Signal, USA) and then secondary antibodies. The membranes were then viewed on the Licor Odyssey imager.

2.11. Statistical analysis

Data are expressed as the mean ± SD. Statistical analyses were conducted in the SPSS 17.0 software. Significant differences were determined by paired t tests. P values < 0.05 were considered significant.

3. Results

3.1. hBD-1 interferes with the expression of surface antigens during the differentiation of immature moDCs from human CB monocytes

To investigate the effect of hBD-1 on immature DC differentiation from human CB monocytes, cell surface antigen expression was assessed by flow cytometry. CB monocytes were cultured with GM-CSF and IL-4 for 5 days in the presence (hBD-1 group, hBD-1) or absence of hBD-1 (Control group, CTR). The optimal concentration of hBD-1 was tested by evaluating the expression of CD40, and the concentration of hBD-1 used in this study is 50 ng/ml (data not shown). As shown in Fig. 1A and B, compared to the control group, cells in the hBD-1 group had similar expression levels of CD14 and CD1a. However, hBD-1 significantly upregulated CD11C, the costimulatory molecules (CD40, CD80 and CD86) and MHC class II molecules, while it downregulated mannose receptor (MR) expression. These results indicated that hBD-1 affects the expression of surface antigens during the differentiation of immature moDCs from human CB monocytes.

3.2. hBD-1-treated CB immature moDCs show decreased endocytic activity

In vitro, immature moDCs have high endocytic activity, mainly via two different mechanisms: a fluid-phase uptake (micropinocytosis) and a receptor-mediated uptake. As moDCs mature, phagocytic activity decreases. In this study, latex beads and FITC-dextran were used to evaluate the fluid-phase endocytic activity and the MR-mediated endocytic activity of immature moDCs. The results showed that, compared to the control group, the hBD-1 group had reduced endocytosis of latex beads (Fig. 2A). Similarly, the hBD-1 group also exhibited decreased MR-mediated endocytosis of FITC-dextran (Fig. 2B).

3.3. hBD-1-treated CB immature moDCs stimulated allogeneic lymphocyte proliferation

DCs were reported to be potent stimulators of allogeneic T cells. Here, we investigated whether hBD-1 was able to stimulate the proliferation of allogeneic CB T lymphocytes in mixed lymphocyte reactions (MLRs). As shown in Fig. 2D, as the ratio of DCs to T cells increased, immature moDCs in the CTR group and hBD-1 group both had an increased ability to stimulate $CD4^+$ T cells. When the DC to T cell ratio is 1:10, the immunostimulatory capacity of immature moDCs in the MLR was significantly higher in the hBD-1 group (Fig. 2C and D).

3.4. hBD-1 interferes with the expression of surface antigens during the maturation of CB moDCs induced by LPS

We further attempted to determine whether hBD-1 affects the maturation of CB moDCs. CB immature moDCs were induced with LPS in the presence or absence of hBD-1 for 48 h. As shown in Fig. 3, compared to the control group, hBD-1 upregulated the expression of CD83, the costimulatory molecules (CD40, CD80 and CD86) and the MHC class II molecules. These results indicate that hBD-1 affects the expression of surface antigens during the maturation of CB moDCs induced by LPS.

3.5. hBD-1-treated mature moDCs promote allogeneic lymphocyte proliferation

As shown in Fig. 4A and B, compared to the non-LPS-treated group, the percentage of proliferative CD4 + T cells was significantly higher in the LPS-treated groups. Moreover, in the two LPS-treated groups, the percentage of proliferative CD4 + T cells was significantly higher in the hBD-1 treated group at DC to T cell ratios of 1:10 and 1:100.

3.6. hBD-1 does not affect cytokine production of moDCs induced by LPS

To investigate the capacity of hBD-1 to interfere with cytokine production of CB moDCs induced by LPS, immature moDCs were induced with or without LPS in the presence or absence of hBD-1. As shown in the Fig. 5, TNFα, IL-6 and IL-10 were significantly induced by LPS, but hBD-1 treatment did not promote their production. However, IL-12p70 was not be induced by LPS, and hBD-1 also did not affect IL-12p70 production (data not shown).

3.7. hBD-1 inhibits early but not late apoptosis in CB immature moDCs through CCR6

We further attempted to determine whether hBD-1 is a pro-survival factor for CB immature moDCs. The monocytes were pretreated with 5 g/ml neutralizing CCR6 antibody (anti-CCR6) or an IgG isotype control and then cultured with GM-CSF and IL-4 for 5 days in the presence or absence of hBD-1. The results showed that the percentage of early apoptotic cells (Annexin V + / PI-) significantly decreased in the hBD-1 group, while blocking CCR6 significantly reversed the hBD-1-induced suppression of early apoptosis in immature moDCs. These results indicated that hBD-1 might affect apoptosis of CB immature moDCs through CCR6. However, the percentage of late apoptotic cells (Annexin V + / PI +) was similar between groups (Fig. 6A).

Furthermore, we evaluated the effect of hBD-1 on the expression of Bcl-xL, an antiapoptotic protein. The results showed that hBD-1 significantly upregulated the expression of Bcl-xL in immature moDCs, while neutralizing CCR6 reversed the hBD-1-upregulated expression of Bcl-xL. These results suggest that hBD-1 exerts its antiapoptotic effect through CCR6 (Fig. 6C).

3.8. hBD-1 inhibits apoptosis of mature DCs induced by LPS through CCR6

We detected apoptosis to determine whether hBD-1 is a survival factor during the maturation of CB moDCs induced by LPS. The results showed that the percentages of both early apoptotic and late apoptotic cells significantly decreased in the hBD-1 group, while blocking CCR6 significantly reversed the hBD-1-induced suppression of apoptosis in moDCs induced by LPS (Fig. 6B). Moreover, hBD-1 significantly upregulates the expression of Bcl-xL in moDCs induced by LPS, while neutralizing CCR6 reverses the hBD-1-upregulated expression of Bcl-xL (Fig. 6D).

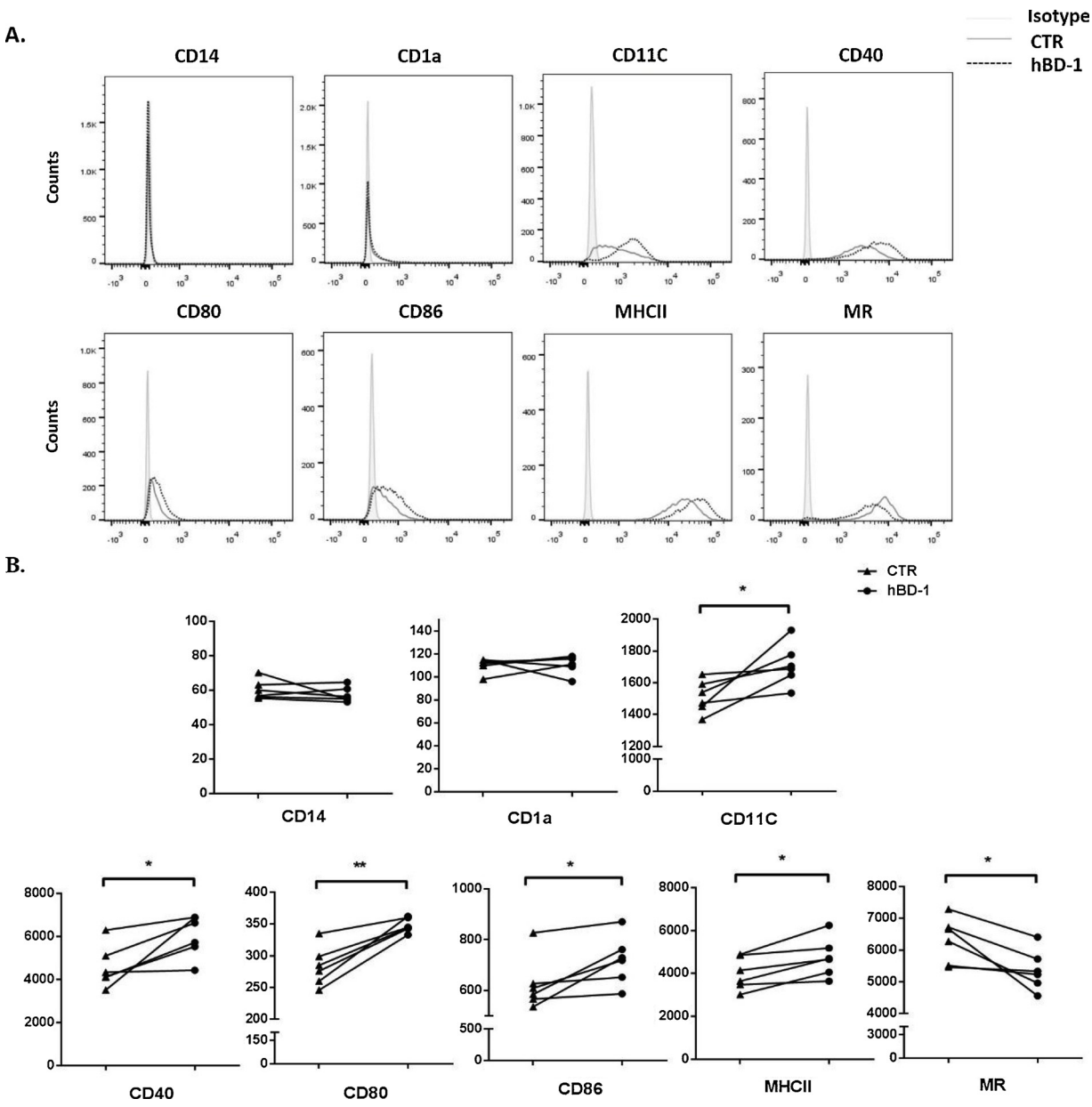

Fig. 1. The expression of surface markers on hBD-1–treated human cord blood (CB) immature monocyte-derived dendritic cells (moDCs). Monocytes were cultured with 100 ng/ml GM-CSF and 50 ng/ml IL-4 for 5 days in the presence (hBD-1) or absence of hBD-1 (CTR), and then, the expression of surface markers was assessed by flow cytometry. Representative flow cytometric analysis before and after hBD-1 treatment is shown (A), and mean fluorescence intensities (MFIs) of immature moDCs from 6 different donors are given (B). * $P < 0.05$ and ** $P < 0.01$.

3.9. hBD-1 promotes the proliferation and activation, but not maturation, of human CB T cells

To investigate the effect of hBD-1 on human CB T cells, we assessed the proliferation, activation and maturation of human CB T cells following hBD-1 treatment. In the proliferation experiment, purified CB CD4 + T cells were stimulated with PBS or PHA in the presence or absence of hBD-1. The concentration of hBD-1 used was 5 ng/ml, which was demonstrated to promote the highest proliferation of purified CB CD4 + T cells in our preliminary experiments (data not shown). As shown in Fig. 7A, compared to the control group, augmented CD4 + T cell proliferation was observed in both groups stimulated with hBD-1 alone and groups costimulated with PHA and hBD-1. Furthermore, T-cell activation markers of CD25, CD69 and CD40 L were also upregulated in the hBD-1-stimulated group and the PHA (PMA/Ionomycin) plus hBD-1 costimulated group (Fig. 7B). However, neither the hBD-1-stimulated group nor the PHA plus hBD-1-costimulated group exhibited altered CD45 isoform expression in CB T cells (changing from naïve CD45RA + to memory CD45RO + T cells) (Fig. 7C). These results indicated that hBD-1 promotes the proliferation and activation, but not maturation, of human CB T cells. In addition, activation markers of CD80 and CD86 in CD19 + B cells were also upregulated in the hBD-1 groups in this study (Fig. 7D).

4. Discussion

The relationship between breastfeeding and infant health has been well elucidated in the past decades (Oddy, 2001, 2004). Antimicrobial peptides in human milk, such as defensins and cathelicidins, are thought to play an important role in protection against infections (Levy, 2007). In addition to their potent antimicrobial properties, these peptides also play a role in regulating the infant immune system (Yang

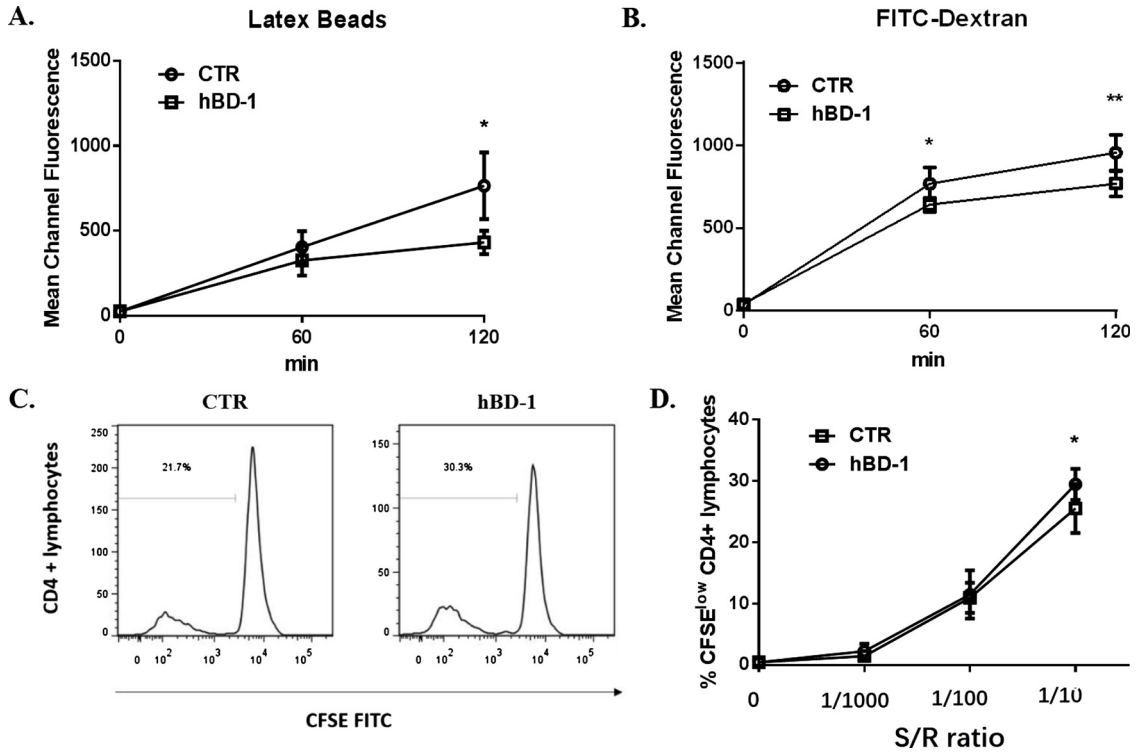

Fig. 2. Endocytic activity and immunostimulatory capacity of hBD-1–treated human CB immature moDCs. Monocytes were cultured with GM-CSF and IL-4 for 5 days in the presence or absence of hBD-1. Fluid-phase endocytosis and receptor-mediated endocytosis of immature moDCs from different donors was evaluated by the uptake of Latex beads (A) (n = 3) and FITC-Dextran (B) (n = 6) and subsequent measurement with flow cytometry. The results are shown as the mean channel fluorescence ± SD. Mixed lymphocyte reactions (MLRs) were used to evaluate the stimulatory activity of moDCs. Immature moDCs cultured in the presence or absence of hBD-1 (stimulatory cells, S) were extensively washed, treated with mitomycin C, and added in graded doses (0, 1/1000, 1/100 and 1/10) to 5×10^5/well allogeneic monocyte-depleted CBMCs (Responder cells, R); they were then assessed by flow cytometry. A representative flow cytometric analysis before and after hBD-1 treatment is shown, and the ratio of S to R was 1:10 (C). The results were expressed as the mean percentage of proliferative CD4+ lymphocytes ± SD from 8 different donors (D). * $P < 0.05$ and ** $P < 0.01$.

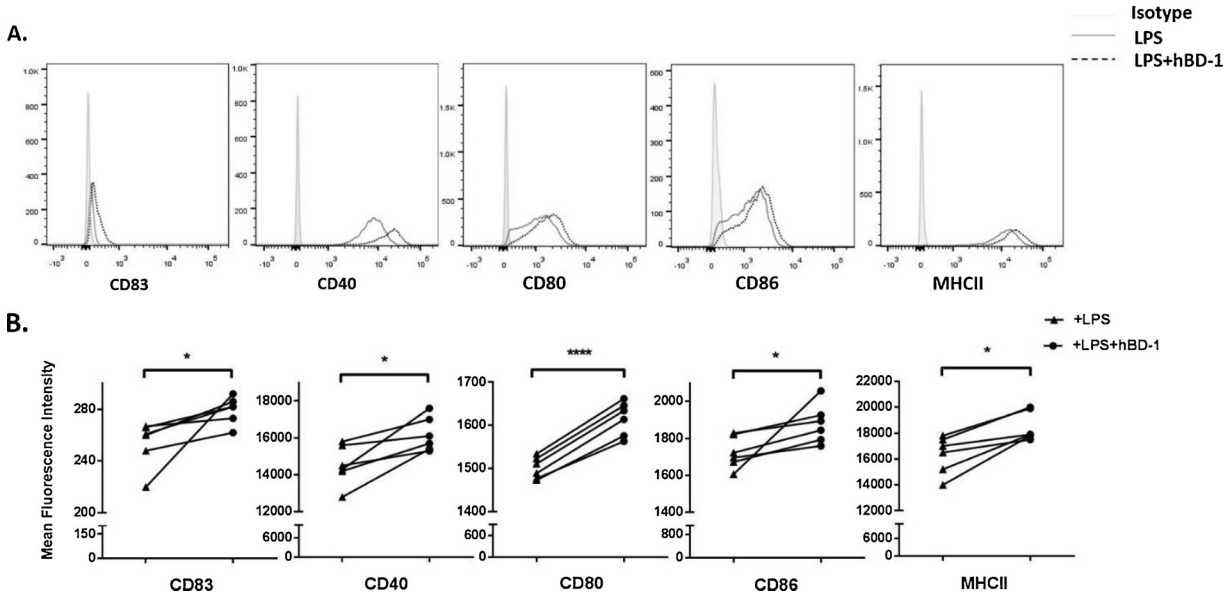

Fig. 3. The expression of surface markers on hBD-1–treated CB moDCs induced by LPS. Immature moDCs were induced with LPS in the presence or absence of hBD-1 for 48 h. The expression of surface markers was measured by flow cytometry. Representative flow cytometric analysis before and after hBD-1 treatment is shown (A), and MFIs of moDCs from 6 different donors are given (B). * $P < 0.05$ and **** $P < 0.001$.

et al., 2015; Semple and Dorin, 2012a; 2012b). In our previous studies, a significantly high concentration of hBD-1 ranging from 1.04–12.81 mg/ml was detected in colostrum from Chinese mothers. Furthermore, we reported that infants who received a relatively high concentration of hBD-1 from colostrum had reduced incidence of upper respiratory infection (Wang et al., 2014). Although hBD-1 has been well demonstrated to be involved in antimicrobial clearance, studies on its immunomodulatory properties are still limited (Semple and Dorin,

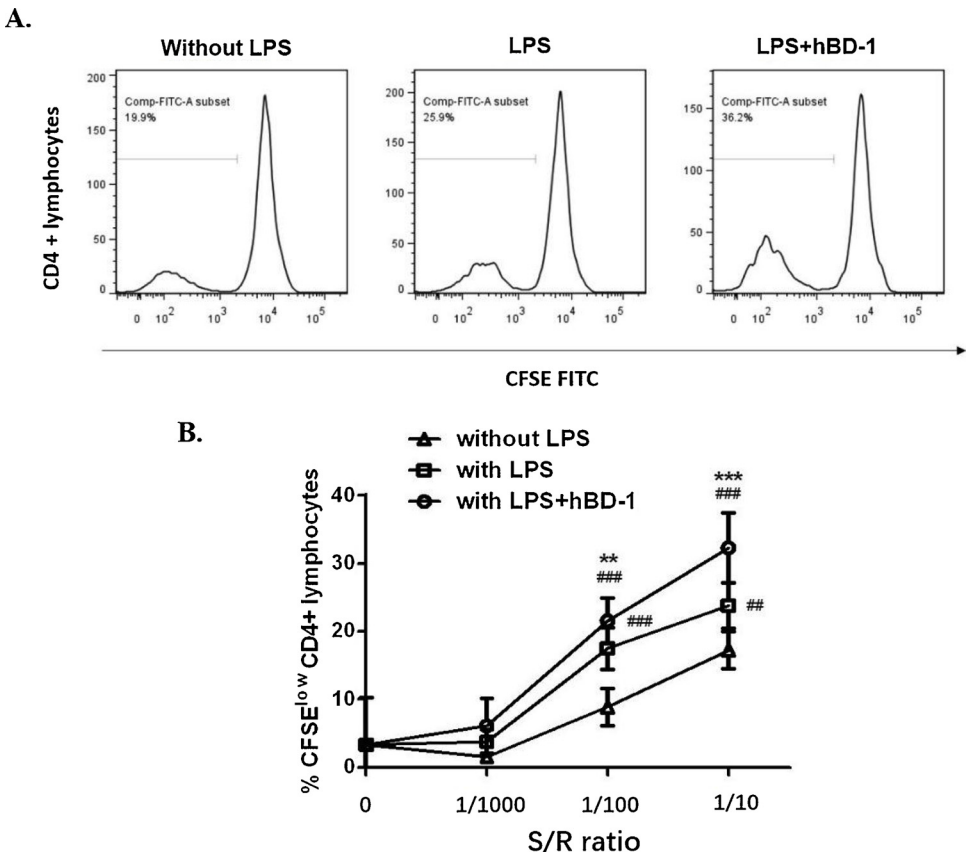

Fig. 4. Immunostimulatory capacity of hBD-1–treated CB mature moDCs. Human CB moDCs were induced with/without LPS in the presence or absence of hBD-1 for 48 h. Then, MLRs was used to evaluate the stimulatory activity of moDCs. A representative flow cytometric analysis is shown, and the ratio of S to R was 1:10 (A). The results are expressed as the mean percentage of proliferative CD4 + lymphocytes ± SD from 8 different donors (B). ** $P < 0.01$ and *** $P < 0.005$ compared with the LPS-induced group. ## $P < 0.01$ and ### $P < 0.005$ compared with the non-LPS-induced group.

2012a; 2012b). In the present study, we first demonstrated that hBD-1 promotes the GM-CSF- and IL-4-driven differentiation of neonatal CB monocytes to immature DCs and the final maturation of CB moDCs induced by LPS. In addition, hBD-1 inhibits apoptosis in neonatal moDCs through CCR6, which might be a possible mechanism of the hBD-1-induced phenotypes in moDCs. Furthermore, we reported that hBD-1 promotes the proliferation and activation, but not maturation, of neonatal CB CD4 + T cells. These results suggest that hBD-1 is not only a directly acting antimicrobial peptide but also an immunoregulator in infants.

DCs, known as professional antigen-presenting cells (APCs), critically link innate and adaptive immunity (Banchereau et al., 2000; Marodi, 2006; Velilla et al., 2006). Numerous studies have demonstrated both qualitative and quantitative differences between neonatal and adult DCs. For example, the total number of plasmacytoid DCs contained in human term neonatal CB are comparable to adult PB, but their phenotypes and subset compositions are different (Dowling and Levy, 2014). Fewer conventional DCs (cDCs) are found in newborn CB than in adult PB (Basha et al., 2014). Further, the neonatal moDCs, which are differentiated from blood monocytes by *in vitro* culturing with GM-CSF and IL-4, were reported to be less abundant and have reduced phenotypic expression and function compared to adult moDCs (Liu et al., 2001a, 2001b). Moreover, neonatal moDCs stimulated by LPS have been reported to have decreased expression of CD83 (maturation marker of DCs) (Liu et al., 2001b). The DC defects in neonates leads to T cells dysfunctions and furthers their susceptibility to infection.

Immature DCs, which express low level of costimulatory molecules (CD80, CD86 and CD40) and MHC molecules, have a high capacity for Ag uptake and processing but a modest ability to activate T cells (Lutz and Schuler, 2002). MHC class II molecules play a vital role in the crosstalk between DCs and T cells, and the costimulatory molecules CD40, CD80 and CD86 provide important accessory signals for T cell activation (Hivroz et al., 2012). Furthermore, the antigen-presenting molecule CD1a is also used for presenting unusual antigens, such as lipids and glycolipids (Porcelli and Modlin, 1999). Our study showed

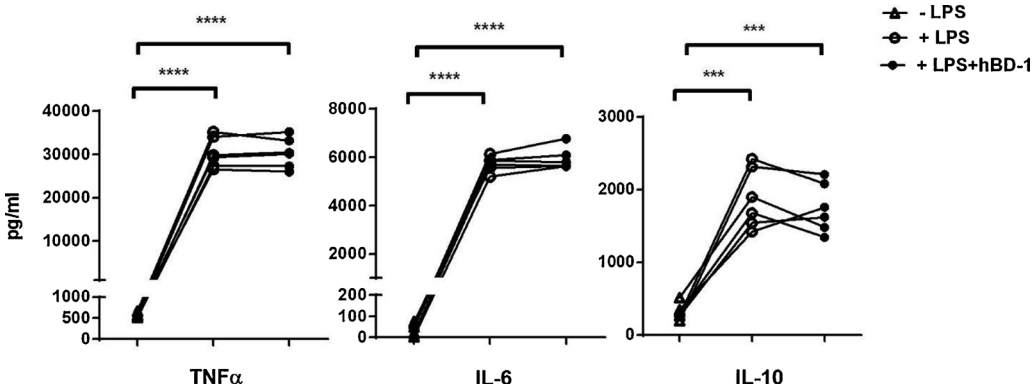

Fig. 5. Cytokine production from hBD-1–treated CB moDCs induced by LPS. Human CB immature moDCs induced with/without LPS in the presence or absence of hBD-1. Supernatants were harvested 48 h later and tested for the concentration of TNFα, IL-6 and IL-10. The concentrations of these cytokines from 6 different donors are given. *** $P < 0.005$ and **** $P < 0.001$.

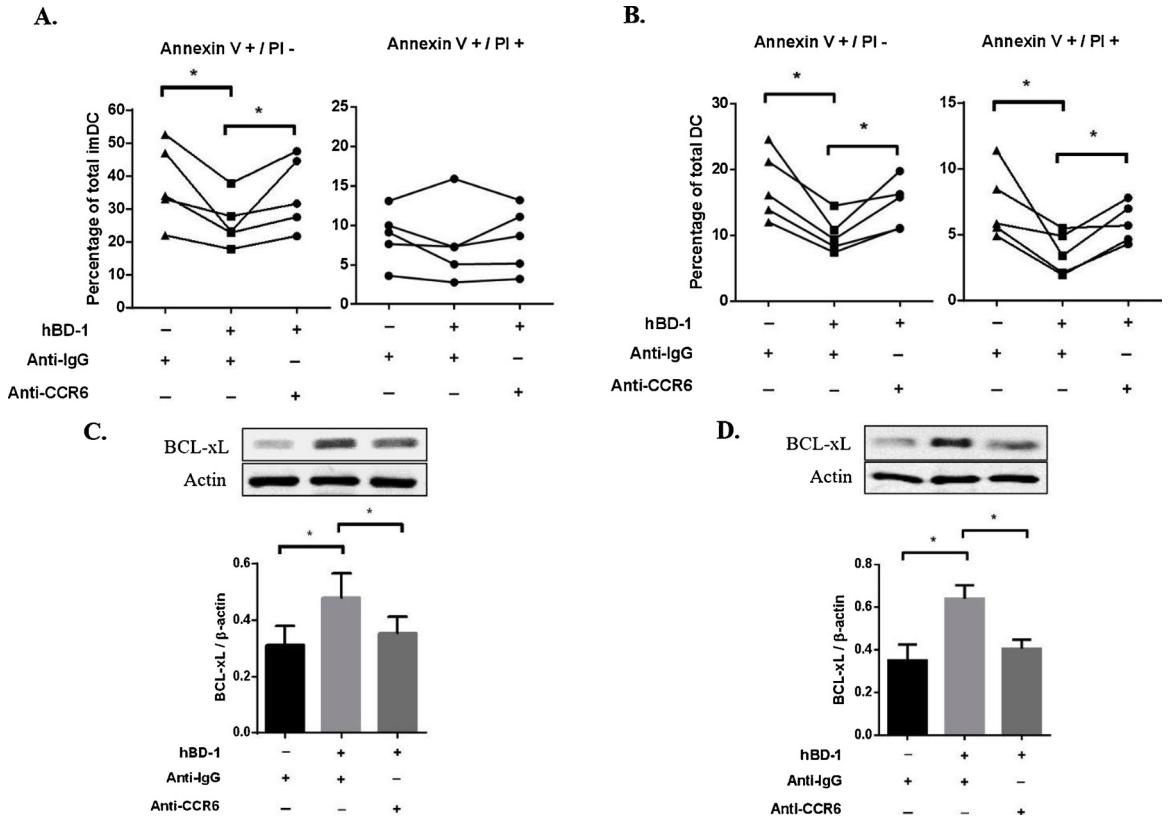

Fig. 6. Early and late apoptosis in hBD-1–treated CB moDCs. In the first experiment (A and C), the monocytes were pretreated with 5 g/ml neutralizing CCR6 antibody (anti-CCR6) or an IgG isotype control and then cultured with GM-CSF and IL-4 for 5 days in the presence or absence of hBD-1. In the second experiment (B and D), monocytes were cultured with GM-CSF and IL-4 for 5 days and then treated with 5 g/ml neutralizing CCR6 antibody (anti-CCR6) or an IgG isotype control. The treated cells were further cultured with/without LPS in the presence or absence of hBD-1 for 48 h. Early and late apoptosis was determined by Annexin V and PI labeling (n = 5), and the expression of Bcl-xL was determined by Western blot (n = 3). *P $<$ 0.05.

that hBD-1 promotes the differentiation of human CB immature moDCs with upregulated expression of CD40, CD80, CD86, and MHC class II molecules; this positively correlated with their enhanced immunostimulatory capacity to stimulate allogeneic lymphocyte proliferation. In addition, hBD-1 also significantly upregulates the expression of the myeloid-associated marker CD11C, a key surface marker of moDCs. However, hBD-1 does not affect the expression of CD1a in our study, which may be due to defective CD1a expression on moDCs in neonates. In a previous study, 63% of CD1a + cells were generated from adult peripheral blood monocytes, and only 18% were generated from cord blood monocytes (Liu et al., 2001b).

High endocytic capacity is a characteristic of immature DCs. As DCs mature, endocytic capacity decreases. Immature moDCs generated by culturing monocytes with GM-CSF and IL-4 for 5 days possess a potent ability to uptake external molecules, mainly through receptor-mediated and fluid phase endocytosis. In this study, we showed that hBD-1-treated CB immature moDCs have decreased endocytic activity and receptor-mediated endocytosis, which agrees with the reduced MR expression in CB immature moDCs in the hBD-1 group. These above results extend the known immunomodulatory effects of hBD-1 in the differentiation of neonatal moDCs.

In addition to promoting the differentiation of DCs from neonatal CB monocytes, hBD-1 has also been demonstrated to promote the final maturation of immature moDCs (Presicce et al., 2009). hBD-1 treatment was reported to activate adult immature moDCs with upregulated CD83, CD40, CD80, CD86 and MHC II molecules and to promote the production of proinflammatory cytokines (Presicce et al., 2009). However, in our study, we did not detect upregulation of CD83 or the costimulatory molecules CD40, CD80 and CD86 in response to hBD-1 alone (data not shown), which suggests that hBD-1 alone does not activate neonatal immature moDCs. Instead, we observed that hBD-1 significantly increases the expression of CD83, CD40, CD80, CD86 and MHC II molecules and consequently enhances the stimulatory capacity of CD4 + T lymphocytes after LPS stimulation. The differential response of immature moDCs following hBD-1 stimulation may be partially due to the immature or defective DC function in neonates.

Accumulating evidence has indicated that defective DC function is responsible for the decreased capability of human newborns to induce a protective Th1 immune response (Velilla et al., 2006). In particular, IL-12, which is a key stimulator in Th1 differentiation, is not be induced by LPS in neonatal DCs (Krumbiegel et al., 2007). In this study, with LPS stimulation, IL-12p70 is absent in neonatal DCs, and hBD-1 does not promote its production. Similarly, hBD-1 also does not affect the production of IL-6, IL-10 and TNF-α in response to LPS. However, a previous study showed that hBD-1 alone enhances the production of TNF-α, IL-6, and IL-12p70 but IL-10 in adult immature moDCs (Presicce et al., 2009). These results further suggest difference between adult and neonatal DCs.

In previous studies, HBDs were shown to have different roles in apoptosis (Semple and Dorin, 2012a; 2012b). Interestingly, one peptide was found to have opposite effects on apoptosis in different cell types. For example, HBD3 suppresses neutrophil apoptosis by binding to CCR6 at the neutrophil cell surface, initiating an increase of the antiapoptotic protein Bcl-xL and inhibition of caspase 3 expression (Nagaoka et al., 2008). In contrast, HBD3 induces human airway smooth muscle cell apoptosis via activating the ERK1/2 MAPK pathway (Wang et al., 2017). hBD-1 has previously been shown to cause caspase-mediated apoptosis in DU145 and PC3 prostate cancer cells and play a potential role in the suppression of prostate cancer development (Bullard et al., 2008). In this study, our results indicated that hBD-1 may be a survival

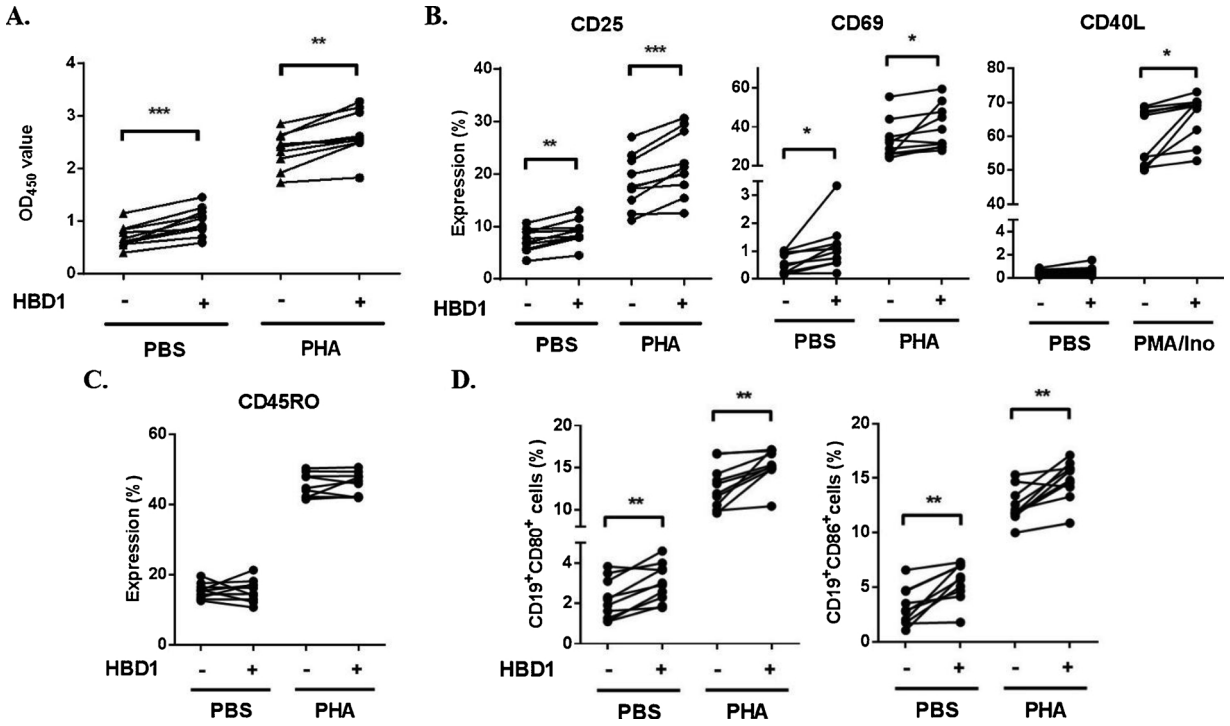

Fig. 7. Proliferation, activation and maturation of Human CB T cells following hBD-1 addition. Purified CD4 + T cells were stimulated with PBS or PHA in the presence or absence of hBD-1, and then, cell proliferation was assayed with CCK-8 (A). CBMCs were stimulated with PBS or PHA (PMA/Ionomycin) in the presence or absence of hBD-1. Activation markers of CD25, CD69 and CD40L in CD4 + T cells (B) and phenotypic changes of CD45 isoforms in CD4 + T cells (C) were detected by flow cytometry. Additionally, CD80 and CD86 in CD19 + B cells were also detected (D). The responsiveness to hBD-1 stimulation was tested in 10 different donors. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$.

factor for CB moDCs, which extends the known immunoregulatory effects of hBD-1 on DCs. The antiapoptotic effect of hBD-1 might in part contribute to other immunoregulatory effects of hBD-1 on CB moDCs. However, the exact mechanism is yet to be determined. Furthermore, CCR6, which has been implicated in hBD-1-induced chemoattraction of CD4+ memory T cells and immature DCs (Yang et al., 1999), is also involved in hBD-1-induced suppression of apoptosis in this study. The neutralization of CCR6 significantly reversed the hBD-1-induced suppression of apoptosis in moDCs, indicating that hBD-1 inhibits apoptosis of CB moDCs through CCR6.

According to the results described above, hBD-1 acts at two different steps of the DC lifecycle. On the one hand, it promotes CB moDC differentiation from monocytic precursors; on the other hand, it promotes the final maturation of DCs into potent APCs. Moreover, hBD-1 is a survival factor for CB moDCs and may play a protective role in DC development.

As a professional APC, DC connects the innate and adaptive immune responses by activating T and B cells. hBD-1 has also been shown to induce chemoattraction of CD4+ memory T cells and immature DCs by binding to CCR6 (Yang et al., 1999), suggesting that hBD-1 might affect both the innate and adaptive immune responses. As described above, enhanced T-cell proliferation was observed in hBD-1-treated and DC-stimulated MLRs. To determine whether hBD-1 stimulates T-cell proliferation directly, isolated neonatal CD4 + T cells were stimulated with hBD-1 alone or costimulated with PHA and hBD-1. The results showed that hBD-1 augments neonatal T-cell proliferation, suggesting a direct effect of hBD-1 on CB T cells.

CD40L is thought to be a T-cell growth factor that plays a vital role in promoting T-cell proliferation (Armitage et al., 1993). In response to stimulation, CB T cells are activated, and CD40L and other T-cell activation makers (CD25 and CD69) are greatly upregulated. In this study, hBD-1 treatment alone and costimulation with PHA both upregulate the expression of CD25 and CD69. Further, hBD-1 upregulates the expression of CD40L in CB T cells stimulated with PMA/ionomycin. These results suggest that hBD-1 promotes cord blood T-cell activation. However, hBD-1 treatment alone does not promote upregulation of CD40L expression, which might be partially due to defective expression of CD40L in cord blood T cells (Brugnoni et al., 1994).

T-cell responses can be at least partially determined by the ratio of naïve CD45RA + to memory/mature CD45RO + T cells(Tu et al., 2000). However, the majority of T cells in newborns are naïve CD45RA + T cells, which greatly contribute to neonatal immune immaturity (Cossarizza et al., 1996). Previous studies have shown that CB T cells with PHA stimulation gradually lose CD45RA and gain CD45RO expression, which suggests the maturation of neonatal T cells (Tu et al., 2000). However, in our study, compared to control groups, hBD-1 does not reduce the ratio of naïve CD45RA + to memory/mature CD45RO + T cells in nonstimulated and PHA-stimulated CB T cells; this indicates that hBD-1 does not promote cord blood T cell maturation. Unfortunately, there are no reports on the effects of hBD-1 in adult lymphocytes, and comparing the responsive difference of adult and neonatal lymphocytes to hBD-1 stimulation would help us to have a better understanding the immunoregulatory effect of hBD-1.

Despite these interesting findings described above, there are some limitations to this study. For example, we only demonstrated the immunoregulatory function of hBD-1 on neonatal CBs *in vitro*, and its function and optimal concentration *in vivo* is still unknown. Moreover, we did not determine the molecular mechanism underlying the immunoregulatory function of hBD-1 in this study, and this is an area needing investigation in future studies.

5. Conclusion

In conclusion, we first demonstrated that hBD-1 promotes the GM-CSF- and IL-4-driven differentiation of neonatal cord blood (CB) monocytes to immature dendritic cells (DC). It also promotes the final maturation of CB monocyte-derived DCs (moDCs) induced by LPS but not inflammatory cytokine production. In addition, hBD-1 inhibits

apoptosis in neonatal moDCs through CCR6, which might be a possible mechanism of the hBD-1-induced phenotypes in moDCs. Furthermore, we found that hBD-1 promotes the proliferation and activation, but not maturation, of neonatal CB CD4 + T cells. These results extend the known immunoregulatory effects of hBD-1 and provide a potential mechanism for the protective role of hBD-1 in early infants.

Conflict of interest statement

None of the authors have any potential financial conflicts of interest related to this manuscript.

Funding sources

This research was supported by grants from the National Natural Science Foundation of China (81571605, 81701626, and 81871303) and Medical-Engineering Cross Fund of Shanghai Jiaotong University, China (YG2017QN34).

Acknowledgements

We thank all the donors and the nursing staff for specimen collection.

References

Adkins, B., Leclerc, C., Marshall-Clarke, S., 2004. Neonatal adaptive immunity comes of age. Nat. Rev. Immunol. 4 (7), 553–564. https://doi.org/10.1038/nri1394.

Armitage, R.J., Tough, T.W., Macduff, B.M., Fanslow, W.C., Spriggs, M.K., Ramsdell, F., Alderson, M.R., 1993. CD40 ligand is a T cell growth factor. Eur. J. Immunol. 23 (9), 2326–2331.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B., Palucka, K., 2000. Immunobiology of dendritic cells. Annu. Rev. Immunol. 18, 767–811. https://doi.org/10.1146/annurev.immunol.18.1.767.

Basha, S., Surendran, N., Pichichero, M., 2014. Immune responses in neonates. Expert Rev. Clin. Immunol. 10 (9), 1171–1184. https://doi.org/10.1586/1744666X.2014.942288.

Brugnoni, D., Airò, P., Gralfu, D., Marconiu, M., Lebowitzu, M., Plebaniu, A., Gilianiu, S., Malacarne, F., Cattaneo, R., Ugaziou, A.G., 1994. Ineffective expression of CD40 ligand on cord blood T cells may contribute to poor immunoglobulin production in the newborn. Eur. J. Immunol. 24 (8), 1919–1924.

Bullard, R.S., Gibson, W., Bose, S.K., Belgrave, J.K., Eaddy, A.C., Wright, C.J., Hazen-Martin, D.J., Lage, J.M., Keane, T.E., Ganz, T.A., Donald, C.D., 2008. Functional analysis of the host defense peptide Human Beta Defensin-1: new insight into its potential role in cancer. Mol. Immunol. 45 (3), 839–848. https://doi.org/10.1016/j.molimm.2006.11.026.

Cossarizza, A., Ortolani, C., Paganelli, R., Barbieri, D., Monti, D., Sansoni, P., Fagiolo, U., Castellani, G., Bersani, F., Londei, M., Franceschi, C., 1996. CD45 isoforms expression on CD4+ and CD8+ T cells throughout life, from newborns to centenarians: implications for T cell memory. Mech. Ageing Dev. 86 (3), 173–195.

Das, A., Rouault-Pierre, K., Kamdar, S., Gomez-Tourino, I., Wood, K., Donaldson, I., Mein, C.A., Bonnet, D., Hayday, A.C., Gibbons, D.L., 2017. Adaptive from innate: human IFN-gamma(+)CD4(+) T cells can arise directly from CXCL8-producing recent thymic emigrants in babies and adults. J. Immunol. 199 (5), 1696–1705. https://doi.org/10.4049/jimmunol.1700551.

Dowling, D.J., Levy, O., 2014. Ontogeny of early life immunity. Trends Immunol. 35 (7), 299–310. https://doi.org/10.1016/j.it.2014.04.007.

Field, C.J., 2005. The immunological components of human milk and their effect on immune development in infants. J. Nutr. 135 (1), 1–4.

Gdalevich, M., Mimouni, D., David, M., Mimouni, M., 2001. Breast-feeding and the onset of atopic dermatitis in childhood: a systematic review and meta-analysis of prospective studies. J. Am. Acad. Dermatol. 45 (4), 520–527.

Hivroz, C., Chemin, K., Tourret, M., Bohineust, A., 2012. Crosstalk between T lymphocytes and dendritic cells. Crit. Rev. Immunol. 32 (2), 139–155.

Kohlgraf, K.G., Pingel, L.C., Dietrich, D.E., Brogden, K.A., 2010. Defensins as anti-inflammatory compounds and mucosal adjuvants. Future Microbiol. 5 (1), 99–113. https://doi.org/10.2217/fmb.09.104.

Kollmann, T.R., Crabtree, J., Rein-Weston, A., Blimkie, D., Thommai, F., Wang, X.Y., Lavoie, P.M., Furlong, J., Fortuno 3rd, E.S., Hajjar, A.M., Hawkins, N.R., Self, S.G., Wilson, C.B., 2009. Neonatal innate TLR-mediated responses are distinct from those of adults. J. Immunol. 183 (11), 7150–7160. https://doi.org/10.4049/jimmunol.0901481.

Krumbiegel, D., Zepp, F., Meyer, C.U., 2007. Combined Toll-like receptor agonists synergistically increase production of inflammatory cytokines in human neonatal dendritic cells. Hum. Immunol. 68 (10), 813–822. https://doi.org/10.1016/j.humimm.2007.08.001.

Levy, O., 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat. Rev. Immunol. 7 (5), 379–390. https://doi.org/10.1038/nri2075.

Liu, E., Tu, W., Law, H.K., Lau, Y.L., 2001a. Changes of CD14 and CD1a expression in response to IL-4 and granulocyte-macrophage colony-stimulating factor are different in cord blood and adult blood monocytes. Pediatr. Res. 50 (2), 184–189. https://doi.org/10.1203/00006450-200108000-00004.

Liu, E., Tu, W., Law, H.K., Lau, Y.L., 2001b. Decreased yield, phenotypic expression and function of immature monocyte-derived dendritic cells in cord blood. Br. J. Haematol. 113 (1), 240–246.

Lutz, M.B., Schuler, G., 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol. 23 (9), 445–449.

Marodi, L., 2006. Innate cellular immune responses in newborns. Clin. Immunol. 118 (2-3), 137–144. https://doi.org/10.1016/j.clim.2005.10.012.

Murakami, M., Dorschner, R.A., Stern, L.J., Lin, K.H., Gallo, R.L., 2005. Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk. Pediatr. Res. 57 (1), 10–15. https://doi.org/10.1203/01.PDR.0000148068.32201.50.

Nagaoka, I., Niyonsaba, F., Tsutsumi-Ishii, Y., Tamura, H., Hirata, M., 2008. Evaluation of the effect of human beta-defensins on neutrophil apoptosis. Int. Immunol. 20 (4), 543–553. https://doi.org/10.1093/intimm/dxn012.

Oddy, W.H., 2001. Breastfeeding protects against illness and infection in infants and children: a review of the evidence. Breastfeed. Rev. Profession. Publ. Nurs. Mothers Assoc. Austr. 9 (2), 11–18.

Oddy, W.H., 2004. A review of the effects of breastfeeding on respiratory infections, atopy, and childhood asthma. J. Asthma 41 (6), 605–621.

Porcelli, S.A., Modlin, R.L., 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. Annu. Rev. Immunol. 17, 297–329. https://doi.org/10.1146/annurev.immunol.17.1.297.

Presicce, P., Giannelli, S., Taddeo, A., Villa, M.L., Della Bella, S., 2009. Human defensins activate monocyte-derived dendritic cells, promote the production of proinflammatory cytokines, and up-regulate the surface expression of CD91. J. Leukoc. Biol. 86 (4), 941–948. https://doi.org/10.1189/jlb.0708412.

Semple, F., Dorin, J.R., 2012a. beta-Defensins: multifunctional modulators of infection, inflammation and more? J. Innate Immun. 4 (4), 337–348. https://doi.org/10.1159/000336619.

Semple, F., Dorin, J.R., 2012b. β-defensins: multifunctional modulators of infection, inflammation and more? J. Innate Immun. 4 (4), 337–348.

Tu, W., Cheung, P.T., Lau, Y.L., 2000. Insulin-like growth factor 1 promotes cord blood T cell maturation and inhibits its spontaneous and phytohemagglutinin-induced apoptosis through different mechanisms. J. Immunol. 165 (3), 1331–1336.

Van, O.J., Kull, I., Borres, M.P., Brandtzaeg, P., Edberg, U., Hanson, L.A., Hã¸St, A., Kuitunen, M., Olsen, S.F., Skerfving, S., 2015. Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966-2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. Allergy 58 (9), 833–843.

Velilla, P.A., Rugeles, M.T., Chougnet, C.A., 2006. Defective antigen-presenting cell function in human neonates. Clin. Immunol. 121 (3), 251–259. https://doi.org/10.1016/j.clim.2006.08.010.

Wang, X.F., Cao, R.M., Li, J., Wu, J., Wu, S.M., Chen, T.X., 2014. Identification of sociodemographic and clinical factors associated with the levels of human beta-defensin-1 and human beta-defensin-2 in the human milk of Han Chinese. Br. J. Nutr. 111 (5), 867–874. https://doi.org/10.1017/S0007114513003292.

Wang, W., Qu, X., Dang, X., Shang, D., Yang, L., Li, Y., Xu, D., Martin, J.G., Hamid, Q., Liu, J., Chang, Y., 2017. Human beta-defensin-3 induces IL-8 release and apoptosis in airway smooth muscle cells. Clin. Exp. Allergy 47 (9), 1138–1149. https://doi.org/10.1111/cea.12943.

Yang, D., Chertov, O., Bykovskaia, S.N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schroder, J.M., Wang, J.M., Howard, O.M., Oppenheim, J.J., 1999. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 286 (5439), 525–528.

Yang, D., Lw Kwak, A.B., Oppenheim, J.J., 2015. Mammalian defensins in immunity: more than just microbicidal. Trends Immunol. 23 (6), 291–296.

Zhao, L., Lu, W., 2014. Defensins in innate immunity. Curr. Opin. Hematol. 21 (1), 37–42. https://doi.org/10.1097/MOH.0000000000000005.