Mycobacterium bovis bacillus Calmette-Guerin treated human cord blood monocyte-derived dendritic cells polarize naïve T cells into a tolerogenic phenotype in newborns

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Original article

Background: **As one of the first infectious challenges of life, the impact of neonatal *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccination on the polarization of neonatal T helper subset has not been well defined.**

Methods: **We investigated the effect of BCG-treated cord blood (CB) dendritic cells (DCs) on naïve CD4+ T cells polarization compared with that of adult blood DCs.**

Results: **BCG-treated CB DCs had significantly lower expression of CD83 and a higher ratio of CD47/Fas than BCG-treated adult blood DCs. BCG induced significantly lower IL-12 but relatively higher IL-10 production from CB DCs than adult blood DCs. Moreover, in comparison with BCG-treated adult blood DCs, BCG-treated CB DCs induced higher IL-10 production and cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression, and lower interferon-gamma (IFN-γ) production from naïve CD4+ T cells. On the other hand, lipopolysaccharide-treated CB DCs had similar capacity as prime naïve CD4+ T cells did to produce higher IFN-γ, lower IL-10 production, and CTLA-4 expression compared with their adult counterparts.**

Conclusion: **These results suggested that BCG-treated CB DCs might be semi-mature DCs which polarize naïve T cells into a tolerogenic T cell phenotype in newborns.**

World J Pediatr 2010;6(2):132-140

Key words: **bacillus Calmette-Guerin;**
cord blood;
dendritic cells;
newborns;
T cells;
tolerance

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doi:10.1007/s12519-010-0019-0

Introduction

Dendritic cells (DCs) function as sentinels at the front line of host defense and play an important role as antigen-presenting cells (APCs) in shaping these immune responses. In contrast to other types of APCs, DCs are potent activators of naïve T cells and are regarded as important initiators of primary specific immune responses.[1,2] Numerous factors induce and/or regulate the maturation of DCs. Pathogen-related molecules such as endotoxin of Gram-negative bacteria lipopolysaccharide (LPS)[3,4] and attenuated live vaccine bacillus Calmette-Guerin (BCG) can induce adult monocyte-derived DCs maturation by different pathways *in vitro*.[5,6] Mature DCs are capable of priming naïve T cells to become effector T cells, whereas immature or semi-mature DCs have a distinct role in regulating immune response and promoting tolerance rather than immunity.[7-9]

Neonates are vulnerable to different infections because of immature immunity. It has been shown that the dysfunction of CB T cells in terms of naïve phenotype, proliferation and IL-4 and IFN-γ production accounts for much of the immaturity of the neonatal immunity.[10] Since DCs play a critical role in primary T cell response, the dysfunction of CB DCs might partially account for the dysfunction of CB T cells.[11,12]

Infectious challenges during early life have strong impact on the development of neonatal immunity. There is increasing evidence suggesting that the predominant type of response, T helper (Th) 1-like or Th2-like cells, to a given antigen is determined at the time of the

primary encounter with the antigen. BCG vaccination at birth induces a memory Th1-type response of similar magnitude to that when given later in life.[13] Recently, it has been reported that DCs derived from BCG-infected precursors induced Th2-like immune response.[14] The pivotal role of APC to induce differentiation of Th1-type effectors and memory cells early in life is increasingly recognized. However, the effects of BCG on naïve T cells development and polarization have not been well defined. In particular, how the effects are mediated through DCs has not yet been defined.

We have previously demonstrated that NF-κB family proteins played an important role in the maturation of CB DCs induced by BCG.[15] In this study, we further determined the phenotypic expression and cytokine production of BCG-treated CB and adult blood DCs as well as their impact on naïve CD4+ T cells polarization, emphasizing the differential effects of BCG and LPS on the maturation of CB and adult blood DCs, and their subsequent effects on CB naive CD4+ T cells.

Methods

Isolation of monocytes

Human umbilical CB was obtained from the placentas of normal, full-term infants, after the placentas were delivered and separated from the infants, with prior written informed consent of their mothers (n=15). Adult peripheral blood was obtained from healthy blood donors of Hong Kong Red Cross (n=19). The protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster [EC1473-00].

CB mononuclear cells (MCs) and adult peripheral blood MCs were isolated from whole blood by density centrifugation (Ficoll-Hypaque, Pharmacia Biotech, Uppsala, Sweden). Monocytes were isolated by positive immunomagnetic selection (Miltenyi Biotec, Bergisch Gladbach, Germany).

Isolation of naïve CD4+ T cells

Naïve CD4+ T cells were isolated by positive immunomagnetic selection from CBMC by CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), with more than 90% of the isolated cells being CD4+CD45RA+ T cells.

Generation of DCs *in vitro*

Isolated CD14+ cells were cultured at a density of 1×10^6 cells/ml in RPMI-1640 (Invitrogen Life Technologies, Grand Island, USA) plus 10% fetal bovine serum, supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin, in the presence of IL-4 (10 ng/ml; R&D, Minneapolis, USA) and GM-CSF (50 ng/ml; R&D, Minneapolis, USA) at 37ºC in a humidified atmosphere containing 5% CO_2 as in our previous studies.[11,12] The cultures were fed with fresh culture medium and cytokines every three days and cell differentiation was monitored by light microscopy. Immature DCs were harvested on day 7. For the generation of BCG and LPS-treated DCs, BCG (50 μl, Merieux Seed derived from strain 1077, Pasteur Merieux Connaugh, France) or LPS (10 μg/ml, Sigma Chemical Co., St. Louis, MO, USA) was added on day 5 for the last 2 days of the culture. The dose and time points were consistent with our previous studies.[11,12,15]

Immunofluorescence staining for flow cytometry analysis

After 7 days of culture, DCs were stained for flow cytometric analysis using FITC, PE and PC5 conjugated isotype controls and CD14-FITC, CD80-FITC, CD83-FITC, CD86-FITC, CD40-FITC, CD47-FITC, major histocompatibility complex (MHC) class II-FITC, CD11c-PE, mannose receptor (MR)-PC5 and CD1a-PC5 (BD PharMingen, San Diego, CA, USA) and Fas-FITC (Beckman Coulter, Miami, Florida, USA).

Measurement of IL-10, IL-12, IL-18 and TGF-β production from DCs

After 7 days of culture, the supernatants of immature and BCG/LPS-treated DCs were harvested and stored at -20ºC. The levels of IL-10, IL-12, IL-18 and TGF-β in the supernatants of immature DCs and BCG/LPS-treated DCs were measured by ELISA kits according to the manufacturer's instructions (R&D Systems Inc, Minneapolis, USA) with detection limits of 7.8 pg/ml, 7.8 pg/ml, 12.5 pg/ml and 31.2 pg/ml respectively.

Measurement of IL-4, IL-10 and IFN-γ production from naïve CD4+ T cells primed by DCs

Freshly isolated naïve CD4+ T cells from CB were co-cultured with allogeneic immature or BCG/LPS-treated CB DCs and adult blood DCs at a DC:T ratio of 1:10. After 7 days, supernatants were harvested and stored at -20ºC. The concentrations of IL-4, IL-10 and IFN-γ in the supernatants were assayed by ELISA kit (R&D Systems Inc, Minneapolis, USA) with detection limits of 31.2 pg/ml, 62.5 pg/ml and 15.6 pg/ml respectively.

Determination of surface CD40L+, intracellular CTLA-4+ and IFN+ cells in naïve CD4+ T cells primed by DCs

Primed by DCs for 7 days, naïve CD4+ T cells were harvested, resuspended at 1×10^6/ml in fresh medium

and re-stimulated by phorbol myristate acetate (PMA, 25 ng/ml, Sigma, Saint Louis, Missouri, USA) plus ionomycin (1 μg/ml, Sigma, Saint Louis, Missouri, USA) for 5 hours. The percentage of CD40L+ cells was measured by surface staining and the percentage of cytotoxic T-lymphocyte antigen 4 (CTLA-4)+ cells was determined by intracellular staining using FACS™ lysing and FACS™ permeabilizing solution (BD immunocytometry systems, San Jose, CA, USA). CD40L-PE and CTLA-4-PC5 antibodies were purchased from BD PharMingen (San Diego, CA, USA).

Statistical analysis

All data were expressed as mean ± SEM. To determine difference between the two groups, paired Student's *t* test or the nonparametric test was performed using the Instat software (GraphPad Software, Inc. San Diego, CA, USA). For all analyses, $P<0.05$ was considered statistically significant.

Results

Similar phenotypic expression of CD40, CD80, CD86, MR and MHC class II on maturation with BCG or LPS in CB DCs and adult blood DCs

Maturation of DCs is associated with profound changes in the surface phenotype, including up-regulated expression of MHC class II and co-stimulatory molecules and down-regulation of MR.[16] The expression levels of these phenotypic markers were normalized to the expression levels of the respective markers of the non-BCG/LPS treated immature CB or adult blood DCs and expressed as relative expression levels (Fig. 1). CB DCs and adult blood DCs maturation was induced by BCG (Fig. 1A) and LPS (Fig. 1B), with similar levels of up-regulated expression of CD40, CD80, CD86, MHC class II molecules and down-regulated expression of MR.

Lower CD83, lower up-regulation of Fas and higher CD47/Fas expressions on maturation with BCG or LPS in CB DCs than in adult blood DCs

Upon BCG stimulation, CB DCs expressed a lower mean fluorescence level (MFL) of CD83 than adult blood DCs (CB DCs: 64±13 MFL, $n=6$; adult blood DCs: 113 ±20 MFL, $n=10$; $P=0.0587$) (Fig. 2A). Similarly LPS induced a lower level of CD83 expression on CB DCs than that on adult blood DCs, but the difference was not statistically significant (Fig. 2A).

Fas (CD95) is a member of the TNF receptor family, and is expressed in mouse DCs and immature human CD34+-derived DCs after TNF-α and LPS induction.[17] In this study, we detected similarly low levels of Fas on immature CB DCs (25±5 MFL, $n=6$) and adult blood DCs (27±6 MFL, $n=7$) (Fig. 2B). Both BCG and LPS induced higher expression of Fas on CB and adult blood DCs; the up-regulation of Fas on adult blood DCs induced by BCG was higher than that on CB DCs ($P=0.0163$ and $P=0.0637$ for BCG and LPS treated DCs respectively).

CD47, an integrin-associated protein, is a widely expressed multispan transmembrane protein that is physically and functionally associated with $\alpha_v\beta_3$ integrin.[18] Ligation of CD47 by monoclonal antibody or its natural ligand inhibits IL-12, TNF-α, IL-6 and GM-CSF production and reduces the maturation of DCs in response to bacterial stimulation.[19] CD47 expression was higher on immature, BCG-treated and LPS-treated CB DCs ($n=6$) than their adult blood DCs counterparts ($n=9$), but the difference was not statistically significant

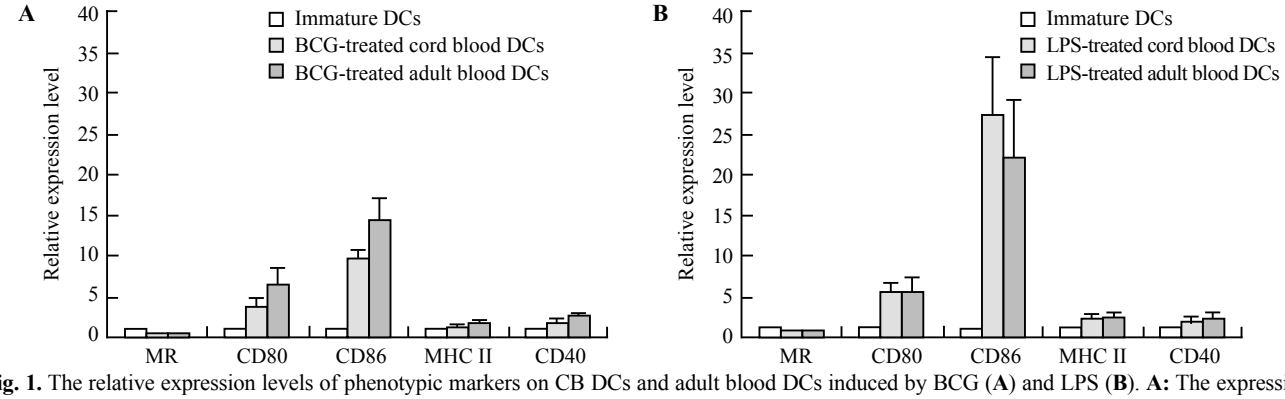

Fig. 1. The relative expression levels of phenotypic markers on CB DCs and adult blood DCs induced by BCG (**A**) and LPS (**B**). **A:** The expression levels of phenotypic markers of BCG-treated CB and adult blood DCs were normalized to the expression levels of the respective non-BCG-treated immature CB DCs or adult blood DCs respectively. The up-regulated expression levels of CD80, CD86, MHC class II, CD40, down-regulated expression of MR were similar on CB ($n=6$) and adult DCs ($n=10$). **B:** Similarly, LPS up-regulated the expression levels of markers on CB and adult blood DCs.

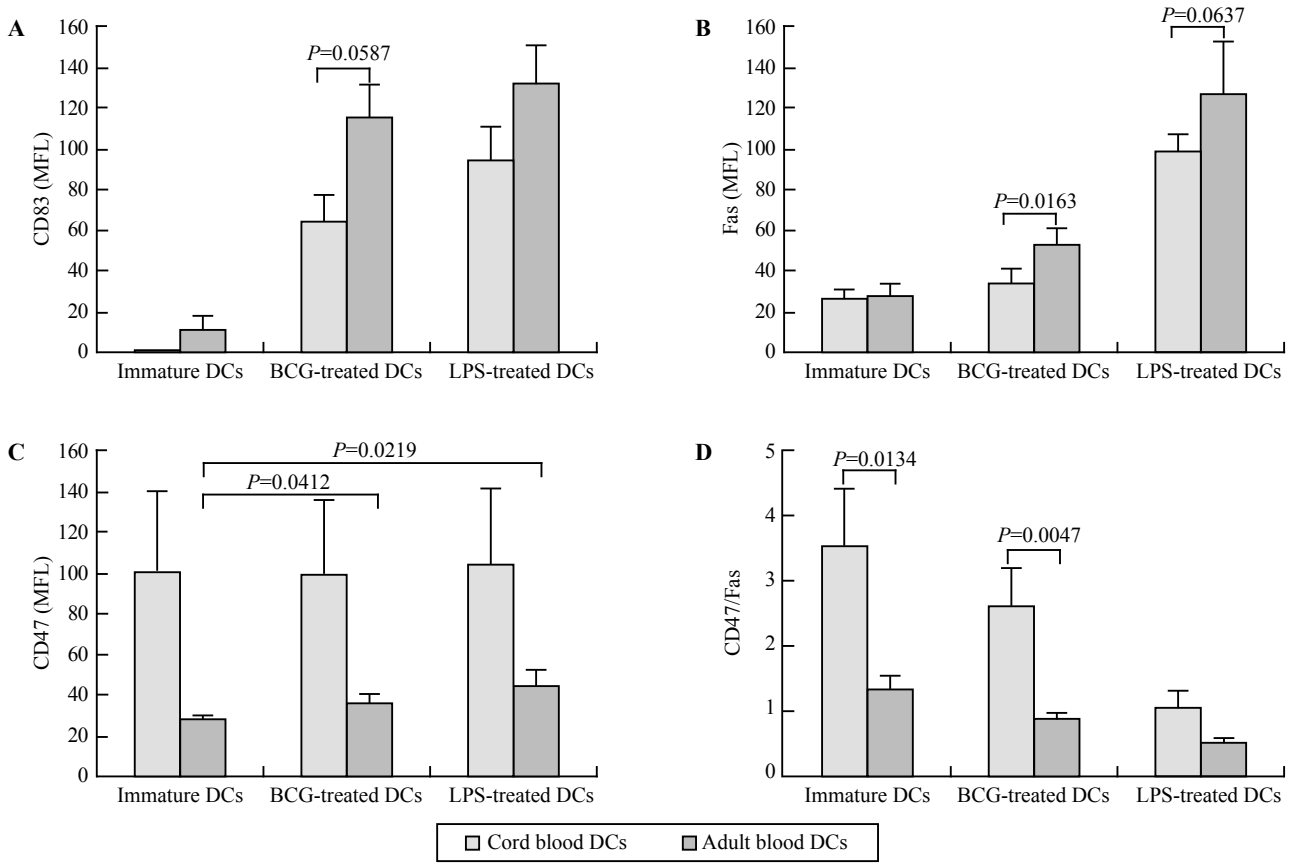

Fig. 2. Comparison of the expression levels of CD83 (**A**), Fas (**B**), CD47 (**C**) and the ratio of CD47/Fas (**D**) on CB and adult blood DCs. **A:** BCG induced lower mean fluorescence level (MFL) of CD83 expression on CB DCs (n=6) than that on adult blood DCs (n=9, P=0.0587). **B:** The expression of Fas in BCG/LPS treated adult blood DCs (n=7) was higher than that in CB DCs (n=6). **C:** The expression level of CD47 on CB DCs was slightly higher than that on adult blood DCs. BCG and LPS significantly up-regulated the expression of CD47 on adult blood DCs but not on CB DCs. **D:** Immature and BCG-treated CB DCs (n=6) had a significantly higher ratio of CD47/Fas than their adult counterparts (n=7, P=0.0134 vs P=0.0047).

(Fig. 2C). Both BCG and LPS induced higher expression of CD47 on adult blood DCs (P=0.0412 and P=0.0219 for BCG and LPS treated DCs respectively). A higher ratio of CD47/Fas was found on immature and BCG-treated CB DCs (n=6) than that on immature and BCG-treated adult blood DCs respectively (n=7, P=0.0134 and P=0.0047 respectively) (Fig. 2D).

Significantly lower levels of IL-12 production induced by BCG and LPS from CB DCs than from adult blood DCs

We determined the capacities of CB and adult blood DCs to release IL-10, IL-12, IL-18, and TGF-β in response to BCG and LPS. Immature CB DCs and adult blood DCs produced low levels of IL-10, IL-12, IL-18 and TGF-β (Fig. 3). Compared to the immature DCs, BCG and LPS induced CB and adult blood DCs to produce higher level of IL-10 and IL-12 (Table 1), but had no effect on IL-18 and TGF-β production. CB DCs primed by BCG and LPS produced significantly lower levels of IL-12 than adult blood DCs (Fig. 3B; P=0.0278 and P=0.0227 respectively).

BCG-treated CB DCs not as efficient as BCG-treated adult blood DCs in up-regulating IFN-γ production and down-regulating IL-10 production by naïve CD4+ T cells

We also studied the cytokine production of naive CD4+ T cells after co-culturing with CB or adult blood DCs. Immature CB DCs induced naïve CD4+ T cells to produce a significantly higher level of IFN-γ than immature adult blood DCs (P=0.0152), while BCG/LPS-treated CB DCs and adult blood DCs induced similar levels of IFN-γ production from naïve CD4+ T cells (Fig. 4A). BCG-treated DCs did not up-regulate IFN-γ production by naïve CD4+ T cells as compared with immature DCs (Table 2). However, both LPS-treated CB and adult DCs were able to induce high IFN-γ production in naïve CD4+ T cells (P<0.001, Table 2).

The capacity of naïve CD4+ T cells to produce

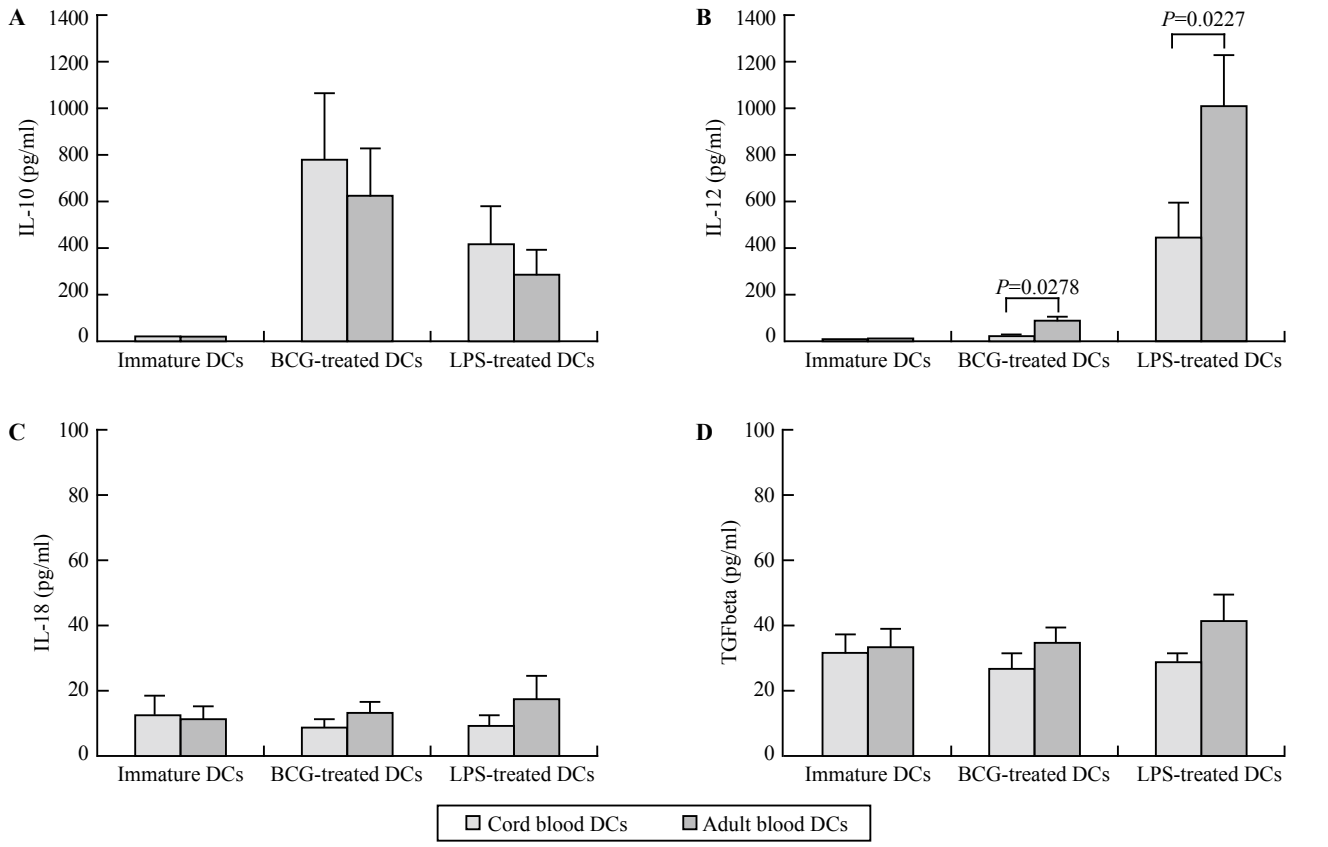

Fig. 3. Comparison of IL-10 (**A**), IL-12 (**B**), IL-18 (**C**) and TGF-β (**D**) production between CB and adult blood DCs induced by BCG and LPS. **A:** BCG and LPS induced a similar level of IL-10 production from CB DCs (n=7) and adult blood DCs (n=7). **B:** BCG and LPS induced a significantly higher level of IL-12 production from adult blood DCs (n=12) than that from CB DCs (n=9) (P=0.0278 and 0.0277 respectively). **C:** The levels of IL-18 were very low in both CB and adult blood DCs. BCG and LPS did not induce IL-18 secretion from either CB DCs (n=7) or adult blood DCs (n=7). **D:** No difference of TGF-β was found between CB (n=7) and adult blood DCs (n=7). BCG and LPS had no effect on TGF-β production.

Table 1. Comparison of effects of BCG/LPS on IL-10 and IL-12 production in CB and adult DCs

		BCG vs immature DCs	LPS vs immature DCs	BCG vs LPS
IL-10	CB	0.0156	0.0156	>0.05
	Adult	0.0006	0.0012	0.0313
IL-12	CB	0.0012	0.0002	<0.0001
	Adult	<0.0001	<0.0001	<0.0001

For all analyses, P<0.05 was considered statistically significant. BCG: bacillus Calmette-Guerin; LPS: lipopolysaccharide; CB: cord blood; DCs: dendritic cells.

Table 2. Comparison of effects of BCG/LPS treated DCs on IFN-γ and IL-10 production by naïve CD4+ T cells

		BCG vs immature DCs	LPS vs immature DCs	BCG vs LPS
IFN-γ	CB	>0.05	<0.0001	<0.0001
	Adult	0.0006	<0.0001	<0.0001
IL-10	CB	>0.05	<0.0001	0.0189
	Adult	0.0126	0.0025	0.0316

For all analyses, P<0.05 was considered statistically significant. BCG: bacillus Calmette-Guerin; LPS: lipopolysaccharide; CB: cord blood; DCs: dendritic cells.

IL-10 was higher when co-cultured with immature and BCG-treated DCs than with LPS-treated DCs (Table 2). Immature CB DCs were slightly more potent than immature adult DCs in inducing IL-10 production by CB T cells, but the difference was not statistically significant. BCG-treated adult blood DCs were significantly more efficient than the BCG-treated CB DCs in down-regulating IL-10 production by naïve CD4+ T cells (Fig. 4B, P=0.0018). Both the LPS-treated adult blood DCs and LPS-treated CB DCs had similarly strong capacity in down-regulating IL-10 production by naïve CD4+ T cells.

In this system, the IL-4 production was extremely low (<40 pg/ml) for naïve CD4+ T cells primed by either immature or BCG/LPS-treated CB or adult blood DCs (Fig. 4C).

Similar effects of CB DCs and adult blood DCs on the naive CD4+ T cells expression of surface CD40L and intracellular CTLA-4

Whether primed by immature, BCG or LPS-treated

CB or adult blood DCs, naïve CD4+ T cells had similar percentages of CD40L+ cells or CTLA-4+ cells (Fig. 5). Compared with their corresponding immature DCs, BCG-treated CB and adult blood DCs down-regulated CTLA-4+ cells in naïve CD4+ T cells, but the difference was not statistically significant. LPS-treated CB and adult blood DCs significantly down-regulated CTLA-4+ cells in naïve CD4+ T cells (Fig. 5B; P=0.0287 and 0.0134 for CB and adult blood LPS treated DCs respectively).

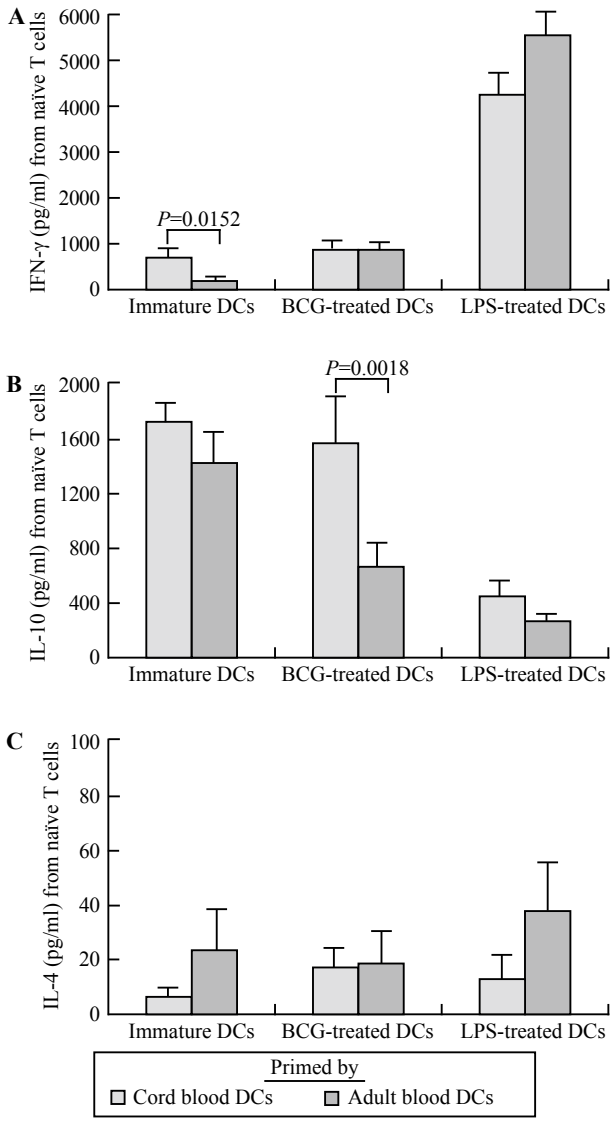

Fig. 4. Comparison of IFN-γ (**A**), IL-10 (**B**), IL-4 (**C**) production of naïve CD4+ T cells primed by CB and adult blood DCs for 7 days. **A:** Immature CB DCs had higher capacity than immature adult blood DCs to induce IFN-γ production from naïve CD4+ T cells (P=0.0152). BCG-treated CB DCs did not up-regulate IFN-γ production. LPS-treated CB and adult blood DCs had similar capacity to up-regulate IFN-γ. **B:** BCG-treated adult blood DCs were more efficient than the BCG-treated CB DCs in down-regulating IL-10 production by naïve CD4+ T cells (P=0.0018). Both the LPS-treated adult blood DCs and LPS-treated CB DCs have similarly strong capacity in down-regulating IL-10 production by naïve CD4+ T cells. **C:** Little IL-4 was produced from naïve CD4+ T cells after being primed by immature or BCG/LPS-treated CB (n=7) and adult blood DCs (n=7).

Discussion

In this study, we investigated the phenotypic expression and function of CB and adult blood DCs induced by BCG and LPS. Our results showed that upon BCG and LPS stimulation, both immature CB DCs and adult blood DCs had similar levels of up-regulated expression of MHC class II and co-stimulatory molecules, such as CD80, CD86, CD40, and down-regulated expression of MR.

However, BCG induced lower CD83 expression on CB DCs than that on adult blood DCs, indicating that following BCG signal, CB DCs were not as efficient as adult blood DCs to up-regulate CD83. CD83 is

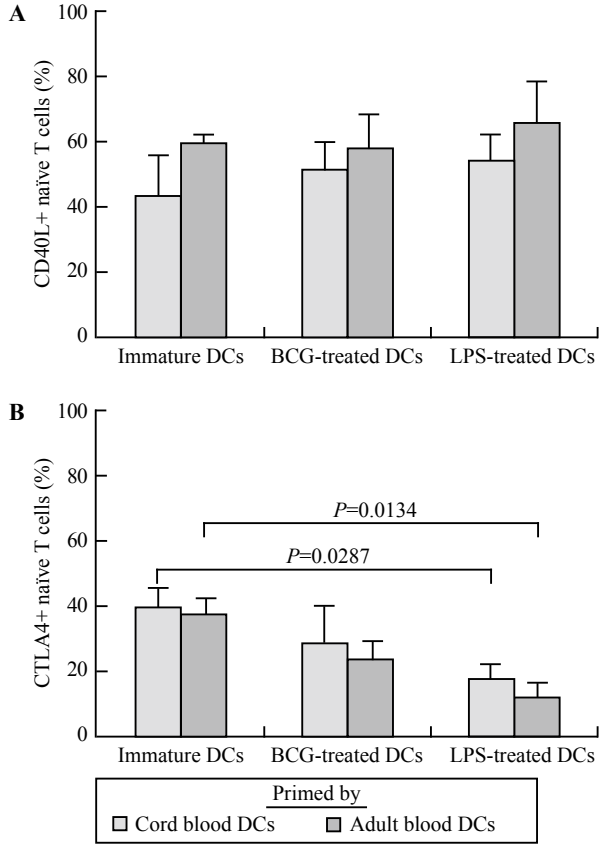

Fig. 5. Comparison of the percentages of CD40L+ (**A**), CTLA-4+ (**B**) in naïve CD4+ T cells primed by CB and adult blood DCs and re-stimulated by PMA plus ionomycin. **A:** The percentages of CD40L+ cells in naïve CD4+ T cells primed by either immature or BCG/LPS treated CB and adult blood DCs were similar. **B:** Compared with their corresponding immature DCs, BCG-treated CB and adult blood DCs down-regulated CTLA-4+ cells in naïve CD4+ T cells, but did not reach statistical significance. LPS-treated CB and adult blood DCs significantly down-regulated CTLA-4+ cells in naïve CD4+ T cells (P=0.0287 and 0.0134 respectively).

one of the best known markers for mature DCs. So far, the precise function of CD83 has not been clearly identified. Recent studies suggest that CD83 binding to its unknown ligand plays a critical role in the interaction between DCs and T cells.[20,21] Hence, the slightly lower expression level of CD83 induced by BCG on CB DCs than that on adult blood DCs implied that BCG-treated CB DCs would be not as efficient as BCG-treated adult DCs to stimulate T cell response.

Our results also indicated that similar levels of Fas and relatively higher levels of CD47 expressed on immature CB and adult blood monocyte-derived DCs. With the maturation of DCs induced by BCG and LPS, the expression level of Fas was up-regulated on both CB and adult blood DCs, but the up-regulation level of Fas induced by BCG and LPS on adult DCs was higher than that on CB DCs. Moreover, BCG-treated CB DCs maintained a significantly higher ratio of CD47/Fas than BCG-treated adult blood DCs. Fas is a positive signal in the maturation of DCs and Th1 polarization, whereas CD47 is a negative signal in DCs maturation and IL-12 production. Our results further suggested that BCG-treated CB DCs were not as mature as BCG-treated adult blood DCs in the induction of Th1 polarization and IL-12 production.

Upon stimulation by bacterial products, DCs undergo maturation processes and release several cytokines that mediate the inflammatory response.[2,22] The cytokine profile expressed by human DCs is dependent on different lineages and mode of activation. CB CD34+ progenitors-derived and adult CD14-derived DCs spontaneously expressed IL-1α, IL-1β, IL-6, IL-7, IL-12, IL-15, IL-18, TNF-α and TGF-β. After CD40 activation, both subpopulations secreted IL-12, but only CD14-derived DCs secreted IL-10.[23] *Mycobacterium tuberculosis* infected human monocyte-derived macrophages mainly secreted TNF-α, IL-1, IL-6 and IL-10. However, human monocyte-derived DCs infected with *Mycobacterium tuberculosis* produced IL-12, IL-18 and IFN-α.[24] In the presence of LPS, CD40L transfectants or poly (I:C), CB DCs secrete decreased IL-12 p70, similar levels of TNF-α, IL-8, IL-6 and IL-10 as compared with adult blood DCs.[25] Our results showed the production of IL-10, IL-18 and TGF-β induced by BCG and LPS was similar in CB and adult blood DCs. Consistent with previous reports, LPS induced less IL-12 production from CB DCs. Moreover, we further confirmed that the capacity of BCG treated CB DCs in producing IL-12 was reduced as well. Since IL-12 plays a central role in inducing IFN-γ production from T cells and NK cells,[26] lower capacity to produce IL-12 from CB DCs primed by different factors might interfere their interaction with naïve T cells.

The induction of polarized T cell phenotype occurs at an early stage of the immune response and is influenced by the cytokine milieu during the priming process. The capacity of DCs to initiate or modulate immune responses appears to depend not only on their lineages,[27] but also on their method of isolation and maturation, the ratio of DCs to T cells,[28] or the duration of DC activation.[29] In the present study, we used immature or BCG/LPS-treated DCs at a relatively high ratio to T cells (DCs:T cells = 1:10) to prime naïve CD4+ T cells. Our result indicated that naïve CD4+ T cells primed by immature DCs maintained a higher level of IL-10, but a lower level of IFN-γ production than BCG/LPS-treated DCs. This cytokine profile of naïve CD4+ T cells matched with the reported properties of regulatory T cells.[30] Further investigations are needed to further characterize the functions of these putative regulatory cells. We also found that BCG-treated CB DCs were less efficient than BCG-treated adult DCs to down-regulate IL-10 and up-regulate IFN-γ production in naïve CD4+ T cells. These results may be due to the immature properties of CB DCs.

BCG-treated CB DCs represent a distinct subtype of semi-mature DCs with enhanced expression of CD80, CD86 and CD40 but secreted high levels of IL-10. This phenotype is similar to that of filamentous hemagglutinin (FHA) activated DCs described previously.[31] More recently, semi-mature DCs have been described as regulatory DCs or tolerogenic DCs which play an important role in regulatory T cells development and the induction of tolerance.[9] These semi-mature DCs can still develop into fully immunogenic IL-12 producing mature DCs, and then drive effector Th-1 responses, as exemplified by the LPS-treated CB DCs in this study.

Comparing the effects of BCG and LPS on the maturation of CB or adult blood DCs, we found that LPS had higher capacities to promote CD86 and Fas expression and a down-regulation ratio of CD47/Fas on both CB and adult blood DCs. Furthermore, LPS induced significantly higher IL-12 and lower IL-10 production from CB and adult blood DCs than BCG. The differences in cytokine production induced by BCG and LPS are consistent with the results that LPS-treated DCs strongly supported CB naïve T cells in skewing to Th1 producing a higher level of IFN-γ, a lower level of IL-10 and less IL-4 compared to BCG-treated DCs. It would be of interest to further study other Th2 cytokines, such as IL-5 and IL-13 in defining the naïve phenotype of the CB T cells.

CD40L (also known as CD154) and CTLA-4 (also known as CD152) are important regulatory proteins that are expressed on the surface of activated T cells. They play a role in the immune system. Only T cells stimulated by mature DCs result in an up-regulation of

CD40L, CD69, and CD70, whereas T cells activated with immature DCs show early up-regulation of negatively regulatory CTLA-4. These CTLA-4 positive T cells lose their ability to produce IFN-γ IL-2 and IL-4 and differentiate into non-proliferating IL-10 producing T cells.[30] Intracellular CTLA-4 has also been reported in regulatory T cells. Similar to previous reports, we detected higher intracellular CTLA-4 in naïve T cells primed by immature and BCG-treated DCs than those primed by LPS-treated DCs. This result provides evidence that BCG-treated DCs have semi-mature DCs properties that skewed naïve T cells to tolerogenic phenotype.

It is well-known that LPS induces DCs maturation through toll-like receptor (TLR) 4,[32] whereas TLR-2 has been shown to mediate mycobacterium or BCG induced intracellular signaling *in vitro*.[33] More recently, the cross talk between TLRs and c-type lectins on DCs has been studied. It has been reported that the recognition of *Mycobacterium tuberculosis* by DC-SIGN and TLR-4 inhibits each other and leads to reduced DC maturation and enhances IL-10 production favoring a Th2 responses for the survival of the pathogen.[34,35] Whether the cross talk between receptors is responsible for the semi-mature phenotype in BCG-treated CB DCs needs further investigation.

In addition to TLR cross talks, there may be intrinsic difference in the expression of TLRs on CB and adult DCs that may lead to differential response to the BCG/LPS stimuli. The BCG/LPS treatment may also stimulate different expression of TLRs. It is important to determine the functional significance of the differential expression of TLRs on CB and adult DCs.

The engagement of CR3[36] or CD47[37] on macrophages or DCs with specific antibodies or natural ligands has been shown to suppress IL-12 production, and this may represent a physiological phenomenon for normal control of immune response *in vivo*. *B. pertussis* FHA can exploit this function by binding to these intergrins on DCs, inducing the production of IL-10, and inhibiting inflammatory chemokine and IL-12 production and other aspects of DC maturation.[31] The interaction of BCG with these intergrins on DCs might be another important point to interpret the discrepancy between the impact of LPS and BCG on the maturation of DCs.

In conclusion, our results suggested that BCG-treated CB DCs might be semi-mature DCs which may promote naïve T cells skewing to tolerogenic phenotype.

Acknowledgments

The authors thank the staff of labor ward in facilitating the collection of CB and the blood donors for their donations.

Funding: The work described in this paper was partially supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region, China (RCG#HKU-7260/01M) and Edward Ho Tung Education & Research Fund of the Department of Pediatrics and Adolescent Medicine, The University of Hong Kong.

Ethical approval: The protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster [EC1473-00].

Competing interest: All authors do not have commercial or other association that might pose a conflict of interest.

Contributors: Liu EM, Law HKW and Lau YL designed research. Liu E and Law HKW performed the research. Liu EM, Law HKW and Lau YL analyzed data. Liu EM, Law HKW and Lau YL wrote the paper.

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Received October 27, 2008
Accepted after revision May 4, 2009