BCG Promotes Cord Blood Monocyte-Derived Dendritic Cell Maturation with Nuclear Rel-B Up-regulation and Cytosolic I κ B α and β Degradation

ENMEI LIU, HELEN K.W. LAW, AND YU-LUNG LAU

Department of Pediatrics, Faculty of Medicine, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong, China [E.L., H.K.W.L., Y.L.L.]

ABSTRACT

Mycobacterium bovis bacillus Calmette-Guerin (BCG) is given to millions of neonates in developing countries as a vaccine against *Mycobacterium tuberculosis*; however, little is known about the initiation of response in neonatal dendritic cells (DCs) to BCG. To address this issue, the interaction of BCG with human cord blood monocyte-derived DCs was studied. We showed that BCG could promote cord blood monocyte-derived DC maturation by up-regulation of CD80, CD83, CD86, CD40, and MHC class II molecules and down-regulation of mannose receptor. BCG was able to induce similar levels of tumor necrosis factor- α and IL-10 but no bioactive IL-12p70 production from cord blood DCs as from adult blood DCs. Functionally BCG-treated cord blood DCs had higher ability to induce mixed lymphocyte reaction than non-BCG-treated cord blood DCs. Both non-BCG-treated and BCG-treated cord blood DCs efficiently induced a high level of IL-10, medium level of interferon- γ , but little IL-4 production by cord blood naïve CD4⁺ T cells. Heat shock protein 65, a key component of BCG, had no effect on cord blood DC maturation in terms of CD86, MHC

class II, and mannose receptor up-regulation. During the BCG-induced maturation process of cord blood DCs, nuclear transcription factor Rel-B was up-regulated and cytosolic Rel-B down-regulated with cytosolic I κ B α and β degradation. These results suggest that BCG can promote cord blood monocyte-derived DC maturation, and that the mechanism is through the up-regulation of nuclear Rel-B secondary to the degradation of cytosolic I κ B α and β . (*Pediatr Res* 54: 105–112, 2003)

Abbreviations

BCG, *Mycobacterium bovis* bacillus Calmette-Guerin
DC, dendritic cell
HSP, heat shock protein
IFN- γ , interferon- γ
MFL, mean fluorescence level
MLR, mixed lymphocyte reaction
MR, mannose receptor
NF- κ B, nuclear factor-kappa B

Dendritic cells (DCs) are professional antigen-presenting cells and critical initiators of primary immune response (1). Immature DCs can be differentiated *in vitro* from CD34⁺ stem cells or peripheral blood CD14⁺ monocytes under appropriate cytokine stimulation (2–5). Inflammatory mediators or microbial agents promote DC maturation, with up-regulation of CD80, CD83, CD86, CD40, and MHC II class molecules (6). Expression of nuclear transcription factor Rel-B correlates with activation of human DCs (7–10). Functionally, DCs are di-

vided into myeloid DCs termed type 1 DC (DC1) and lymphoid DCs termed type 2 DC (DC2) according to their ability to polarize Th1 or Th2 development (11).

BCG has been found to have strong nonspecific immunostimulatory properties (12). It has been noted that interaction of human adult peripheral blood DCs with BCG results in direct maturation and activation, with up-regulation of costimulatory molecules and increases in IL-12 and proinflammatory cytokine production (13, 14). BCG-infected DCs are more efficient than uninfected DCs in eliciting a potent T-helper 1 (Th1) response (15). As a strong inducer for Th1 polarization, BCG has the potential to be used in the treatment of atopic asthma (16).

Neonates are susceptible to infections because of immune deficiency, in particular Th1 deficiency, which results in the vulnerability to different intracellular pathogens such as mycobacteria and viruses (17). The Th2 bias in newborns would

Current address (E.L.): Children's Hospital, Chongqing University of Medical Sciences, Chongqing, 40014, China.

Received July 31, 2002; accepted November 19, 2002.

Correspondence: Yu-Lung Lau, MD, Department of Pediatrics, Faculty of Medicine, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong, China; e-mail: lauylung@hkucc.hku.hk

Partially supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region, China (RGC#HKU-7260/01M).

DOI: 10.1203/01.PDR.0000069703.58586.8B

impair their response to vaccines (18). It has been suggested that the Th1 response induced by mycobacteria could prevent the development of allergen-specific Th2 response and atopy during childhood (19). Because neonatal BCG vaccination represents one of the first infectious challenges during life, it could have a strong impact on the development of the immune system. Marchant *et al.* (20) found that BCG vaccination at birth induces a memory Th1-type response of similar magnitude to that when given later in life.

In our previous studies, we have reported that the proliferative response of T cells, cytokine production, and monocyte function of cord blood are significantly decreased compared with that of adult peripheral blood (21–23). Furthermore, we found that the ability of cord blood monocytes to differentiate into CD1a⁺ dendritic cells was significantly lower than that of adult peripheral blood monocytes and speculate that the dysfunction of cord blood monocytes to differentiate into professional DCs may affect the initiation of naïve T cells, especially Th1 development, and may be related to the susceptibility of neonates to different infections in particular intracellular pathogens, such as *Mycobacterium tuberculosis* (24).

We therefore investigated in this study whether BCG could promote maturation of the cord blood monocyte-derived DCs as in adults, and furthermore whether these mature monocyte-derived DCs could support Th1 development. In addition, we studied the role of NF- κ B pathway in the BCG-induced maturation of cord blood monocyte-derived dendritic cells.

METHODS

Sample collection. Human umbilical cord blood was obtained from the placentae of normal, full-term infants, after the placentae were delivered and separated from the infants, with prior written informed consent of their mothers. All samples were collected in heparinized flasks. Adult blood was collected from normal volunteers with oral consent. The ethics committee of the University of Hong Kong approved the protocol.

Isolation of monocytes. Blood mononuclear cells were isolated from whole blood by centrifugation, using Ficoll-Hypaque gradients (Pharmacia Biotech, Uppsala, Sweden). Mononuclear cells at the interface were collected, washed three times, and resuspended at 1×10^8 /mL, pH 7.2, Hanks/HEPES buffer. Monocytes were isolated from mononuclear cells by positive selection using anti-CD14–conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells from the positive fraction were cultured at a density of 1×10^6 cells/mL in a serum- and hormone-free medium, Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM-F12; Sigma Chemical Co, St. Louis, MO, U.S.A.), which did not contain insulin, IGF-I, or other hormones, supplemented with 50 IU/mL penicillin and 50 μ g/mL streptomycin. Cell viability, as measured by trypan blue exclusion, was more than 95%. The purity of monocytes was measured by flow cytometry with 85% to 95% of the cells being CD14⁺.

Isolation of cord blood CD3⁺ T cells and naïve CD4⁺ T cells. Cord blood CD3⁺ T cells were isolated from cord blood mononuclear cells by positive selection using anti-CD3–

conjugated magnetic microbeads (Miltenyi Biotec). The purity of the isolated CD3⁺ T cells was consistently within the range of 95% to 99%. CD4⁺ naïve T cells were isolated from cord blood monocytes by CD4⁺ T cell isolation kit (Miltenyi Biotec), with more than 90% of the isolated cells being CD4⁺CD45RA⁺ T cells.

Generation of immature DCs in vitro. A total of 1×10^6 monocytes were cultured in the presence of IL-4 (10 ng/mL; R&D Systems Inc, Minneapolis, MN, U.S.A.) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL; R&D Systems Inc) for 7 d at 37°C in a humidified atmosphere containing 5% CO₂ as in our previous study (24). The cultures were fed with fresh serum-free DMEM-F12 medium and cytokines every 3 d, and cell differentiation was monitored by light microscopy. This constituted the non-BCG-treated DC group. For the BCG-treated DC group, BCG (50 μ L, Danish strain 1331; Statens Serum Institute, Copenhagen S., Denmark, or Merieux Seed derived from strain 1077; Pasteur Merieux Connaught, Lyon, France) was added for the last 2 d of the culture. Dendritic cells were harvested, centrifuged, and washed before further assays.

Immunofluorescence staining. After 7 d in culture, cells were collected, transferred to microcentrifuge tubes, diluted with 1 mL PBS, and centrifuged at $13,000 \times g$ for 10 s. The supernatant was removed, and the cell pellet was resuspended in the small amount of remaining PBS. Fluorochrome-conjugated antibodies at concentrations of 4 μ g/ 10^6 cells were added, and then the cell suspension was incubated for 15 min at 20°C. After incubation, the cells were diluted in 1 mL of wash solution (PBS + 0.1% fetal bovine serum + 0.1% azide), and after centrifugation and removal of supernatant, the cells were resuspended in 300 μ L of the same solution for flow cytometric analysis. FITC-, Phycoerythrin (PE)-, and Phycoerythrin-cyanin 5.1 (PC5)-conjugated isotype controls and CD14-FITC, CD80-FITC, CD83-FITC, CD86-FITC, CD40-FITC, MHC class II-FITC, CD11c-PE, MR-PE, and CD1a-PC5 were purchased from PharMingen (San Diego, CA, U.S.A.).

Signal analysis. Flow cytometric analysis was performed with a Coulter Epics Elite Flow Cytometer (Coulter Corporation, Miami, FL, U.S.A.). The machine was optimized daily using standard Flow Check fluorescence beads (Coulter Corporation), and linearity was checked by Quantum25 beads (Flow Cytometry Standards Corp, San Juan, PR, U.S.A.). Ten thousand events per sample were collected into listmode files and analyzed by the WINMDI 2.8 analysis software (Purdue University, West Lafayette, Indiana, USA). The DC analysis was gated to the standard forward scatter and side scatter profile for large cells.

Mixed lymphocyte reaction. *In vitro*–generated DCs were washed with PBS, counted, resuspended at different concentration, and irradiated (30 Gy). Allogeneic cord blood CD3⁺ T cells (1×10^5 responder cells/wells) were cultured in triplicate in a 96-well flat-bottom microplate with different numbers of stimulator DCs (DC-to-T cell ratios were 1:1000, 1:100, and 1:10). Cultures were maintained in a humidified atmosphere at 37°C and 5% CO₂. Twenty microliters of bromodeoxyuridine (BrdU 100 μ M) was added into the wells 16–18 h before the

end of 5 d culture, and its incorporation was then measured by Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Molecular Biochemicals, Mannheim, Germany).

IL-12p70, IL-10, and TNF- α production by DCs. After 7 d culture, the supernatants of non-BCG-treated and BCG-treated DCs were harvested and stored at -20°C . The levels of IL-12p70, IL-10, and TNF- α in supernatants of non-BCG-treated and BCG-treated DCs were measured by ELISA kits (R&D Systems Inc). The detection ranges of IL-12p70, IL-10, and TNF- α were 7.8–500 pg/mL, 62.5–4000 pg/mL, and 15.6–1000 pg/mL, respectively.

Cocultures of non-BCG-treated DCs or BCG-treated DCs with cord blood naïve CD4 $^{+}$ T cells. Freshly isolated cord blood naïve CD4 $^{+}$ T cells were cocultured with non-BCG-treated cord blood DCs or BCG-treated cord blood DCs (DC-to-T cell ratio was 1:10) for 7 d. Supernatants were harvested and stored at -20°C . The concentrations of IL-4, IL-10, and IFN- γ in the supernatants were assayed by ELISA kit (R&D Systems Inc). The detection ranges of IL-4, IL-10, and IFN- γ were 31.2–2000 pg/mL, 62.5–4000 pg/mL, and 15.6–1000 pg/mL, respectively.

Effects of HSP65 on cord blood monocyte-derived DC maturation. Different concentrations of HSP65 (10, 100, and 1000 ng/mL, Calbiochem, San Diego, CA, U.S.A.) were added after d 5 of culture with IL-4 + GM-CSF for 2 more days. The phenotypic analysis of DCs was performed by flow cytometry.

Neutralization of TNF- α in DC cultures. Mature DCs are known to produce high levels of TNF- α . To determine whether TNF- α played a role in mediating the effects of BCG, neutralizing antibodies for TNF- α (R&D Systems Inc) at 10 $\mu\text{g/mL}$ were added to the CD14 $^{+}$ cell culture on d 5 together with BCG and cultured for 2 more days. The phenotypic analysis of DCs was performed by flow cytometry.

Preparation of protein extracts and Western blot. Nuclear and cytosolic protein extracts from all cells were prepared according to Schreiber *et al.* (25). Protein concentrations were determined by a Coomassie Plus Protein Assay Reagent kit (Pierce, Rockford, IL, U.S.A.). For Western blot assays, 5 μg of nuclear protein and 30 μg of cytosolic protein were separated on 8% to 12% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The following polyclonal antibodies were used for immunodetection: anti-NF κ B p65 and anti-I κ B α (Calbiochem), and anti-RelB and anti-I κ B β (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Statistical analysis. All data were expressed as mean $\pm$ SEM. To determine difference between two groups, the paired *t* test or nonparametric test was used.

RESULTS

BCG promoted cord blood DC maturation by up-regulation of MHC class II and costimulatory molecules and down-regulation of MR. We investigated whether BCG could promote cord blood monocyte-derived DC phenotype maturation as reported in adult blood. In cord blood monocyte-derived DCs, there was no difference in the percentages of CD11c $^{+}$ and CD1a $^{+}$ cells between the non-BCG-treated and BCG-treated

Figure 1. Percentages of CD11c $^{+}$, CD1a $^{+}$, and MR $^{+}$ cells in non-BCG-treated and BCG-treated cord blood DCs. No difference was found in the percentages of CD11c $^{+}$ ($n = 8$, $p > 0.05$) and CD1a $^{+}$ ($n = 11$, $p > 0.05$) cells between non-BCG-treated and BCG-treated cord blood DCs. The percentage of MR $^{+}$ cells was significantly decreased in the BCG-treated cord blood DCs ($n = 11$, $p < 0.0001$). Results are shown as mean $\pm$ SEM.

cord blood DCs. The percentage of MR $^{+}$ cells in BCG-treated cord blood DCs was significantly lower than that in non-BCG-treated DCs (Fig. 1). Compared with non-BCG-treated cord blood DCs, BCG-treated cord blood DCs were found to have significantly increased intensity in the expression of CD80, CD83, CD86, CD40, and MHC class II (Fig. 2).

BCG-treated cord blood DCs significantly increased MLR. Freshly harvested cord blood DCs were investigated for their ability to stimulate the proliferation of allogeneic cord blood CD3 $^{+}$ T cells in MLR. Background BrdU uptake by either CD3 $^{+}$ T cells (1×10^5) or irradiated monocyte-derived DCs (1×10^4) alone was undetectable. Compared with non-BCG-treated cord blood DCs, BCG-treated cord blood DCs had a higher ability to stimulate CD3 $^{+}$ T cells at DC-to-T ratio of 1:10 (non-BCG-treated DCs, 0.37 ± 0.07 ; BCG-treated DCs, 0.61 ± 0.08 ; $n = 9$; $p < 0.0001$; Fig. 3).

Comparing the phenotypic maturation between cord blood DCs and adult blood DCs treated by BCG. When normalized to the respective expression levels of non-BCG-treated DCs, the relative expression levels of MR, CD83, CD40, and MHC class II for cord blood and adult blood DCs were not significantly different (Fig. 4).

Both BCG-treated cord blood DCs and adult blood DCs produced high levels of IL-10 and TNF- α but not IL-12p70. To evaluate the ability of cytokine production induced by BCG, we compared the IL-12p70, IL-10, and TNF- α levels between cord blood DCs and adult blood DCs. The levels of IL-10 and TNF- α were similar in non-BCG-treated cord blood and adult blood DCs. BCG induced high levels of IL-10 and TNF- α production from cord blood and adult blood DCs, but no differences were found between the two groups (Fig. 5). IL-12p70 production was not detected in cord blood and adult blood DCs whether treated with BCG or not.

Cord blood DCs induced naïve CD4 $^{+}$ T cells to produce high levels of IL-10 and medium levels of IFN- γ but little

Figure 2. Expression levels of CD14, CD80, CD83, CD86, CD40, and MHC class II molecules on non-BCG-treated and BCG-treated cord blood DCs. *a*, Analysis of the DCs in the gated region reveals that expression levels of CD80, CD83, CD86, CD40, and MHC class II molecules were higher in the BCG-treated cord blood DCs compared with that of non-BCG-treated cord blood DCs (representative histograms). *b*, Statistical significance was confirmed in $n = 8, 9, 8, 8$, and 11 experiments, respectively. Results are shown as mean $\pm$ SEM.

IL-4. To investigate the ability of BCG-treated cord blood DCs to skew naïve CD4⁺ T cell polarization to Th1 or Th2, we measured the levels of IL-4, IL-10, and IFN- γ production from naïve CD4⁺ T cells after priming by either non-BCG-treated or BCG-treated cord blood DCs for 7 d. The level of IL-4 production was very low for both (data not shown). Compared with non-BCG-treated cord blood DCs, BCG-treated cord blood DCs did not significantly down-regulate IL-10 and up-regulate IFN- γ production from naïve CD4⁺ T cells (Fig. 6).

HSP65 had no effect on cord blood monocyte-derived DC maturation. To investigate for the active component of BCG responsible for promoting DC maturation, we have substituted HSP65 for BCG in our experimental system. HSP65 is a 65-kD member of the heat shock protein family, which has been identified as the immunodominant antigen in mycobacterial diseases (26, 27). In our system, HSP65 at three different concentrations had no effect on MR, CD86, and MHC class II expression of immature DCs (Fig. 7).

Figure 3. Proliferative response of cord blood CD3⁺ T cells (1×10^5) cultured with irradiated allogeneic non-BCG-treated or BCG-treated cord blood DCs at different ratios. Higher ability of BCG-treated cord blood DCs to stimulate proliferation of CD3⁺ T cells was observed at a DC-to-T ratio of 1:10 compared with that of non-BCG-treated cord blood DCs ($n = 9, p < 0.001$). Results are shown as mean $\pm$ SEM.

Figure 4. Relative expression levels of MR, CD83, CD40, and MHC class II molecules on BCG-treated cord blood and adult blood DCs. For the non-BCG-treated cord blood DCs, the expression level of MR was 69% ($n = 15$); CD83 was 16 ± 3 MFL ($n = 11$); CD40 was 14 ± 6 MFL ($n = 8$); and MHC class II was 282 ± 56 MFL ($n = 10$). For non-BCG-treated adult blood DCs, the expression level of MR was $72 \pm 7\%$ ($n = 7$); CD83 was 17 ± 5 MFL ($n = 5$); CD40 was 57 ± 25 MFL ($n = 5$); and MHC class II was 308 ± 48 MFL ($n = 6$). After BCG treatment, the relative down-regulation of MR and up-regulation of CD83, CD40, and MHC class II molecules was similar between cord blood DCs and adult blood DCs. Results are shown as mean $\pm$ SEM.

Anti-TNF- α partially neutralized maturation effects of BCG on cord blood monocyte-derived DCs. Anti-TNF- α (10 μ g/mL) partially neutralized the maturation effects of BCG on the down-regulation of MR and up-regulation of CD83 and

Figure 5. IL-10 and TNF- α production by non-BCG-treated and BCG-treated DCs from cord blood and adult blood. BCG induced significant production of IL-10 and TNF- α from cord blood DCs ($n = 3, 9$) and adult blood DCs ($n = 5, 9$), but no significant difference was detected between cord blood and adult blood DCs. Results are shown as mean $\pm$ SEM.

MHC class II expression. However, it did not neutralize the effects of BCG on the up-regulation of CD86 and CD40 expression (Fig. 8).

Generation of mature DCs by BCG led to up-regulation of nuclear Rel-B and down-regulation of cytosolic Rel-B. In immunoblot assays using nuclear protein extracts from purified monocytes, non-BCG-treated DCs and BCG-treated DCs, the expression levels of p65/Rel-A and nuclear Rel-B were significantly higher in both non-BCG-treated DCs and BCG-treated DCs than purified monocytes (Fig. 9). The expression of p65/Rel-A in non-BCG-treated DCs and BCG-treated DCs was similar, but there was a striking increase in the expression of nuclear Rel-B in BCG-treated DCs accompanied with cor-

Figure 6. IL-10 and IFN- γ production by naïve CD4⁺ T cells after coculturing with non-BCG-treated or BCG-treated cord blood DCs. BCG-treated cord blood DCs induced less IL-10 and IFN- γ production by naïve CD4⁺ T cells than non-BCG-treated cord blood DCs, but the difference did not reach statistical significance.

Figure 7. Effects of HSP65 on expression of MHC-II, CD86, and MR on cord blood DCs. In the range of 10–1000 ng/mL, HSP65 had no effect on expression levels of CD86 or MHC class II molecules or the percentage of MR⁺ cells. This is a representative experiment of four different experiments with the same trend.

responding down-regulation of the cytosolic Rel-B expression (Fig. 9).

Maturation of BCG-treated DCs was associated with the degradation of I κ B α and β in the cytoplasm. The inducible degradation of I κ Bs is crucial for NF- κ B activation. We investigated whether the increase in the nuclear concentrations of Rel proteins during DC maturation induced by BCG might be mediated through a sustained degradation of I κ Bs, which is critical for NF- κ B activation. Western blot analysis of cytosolic proteins showed that the concentrations of I κ B α and β

Figure 8. Effects of anti-TNF- α neutralizing antibody (10 μ g/mL) on phenotypic expression of BCG-treated cord blood DCs ($n = 5$). Absolute basal levels of phenotypic expression on non-BCG-treated cord blood DCs were $65 \pm 3\%$ (MR), 18 ± 5 MFL (CD83), 142 ± 32 MFL (CD86), 42 ± 12 MFL (CD40), and 435 ± 158 MFL (MHC class II) against which the levels on BCG-treated cord blood DCs with or without anti-TNF- α antibody were compared. Anti-TNF- α antibody partially neutralized the down-regulation of MR and up-regulation of CD83 and MHC class II molecules induced by BCG, but it had no significant effect on CD86 or CD40 expression. Results are shown as mean $\pm$ SEM.

Figure 9. Immunoblots were performed with nuclear proteins and cytosolic proteins from d 0 monocytes (lane 1), d 7 non-BCG-treated cord blood DCs (lane 2), and d 7 BCG-treated cord blood DCs (lane 3). Rel proteins and cytosolic I κ Bs were differentially expressed during the development of non-BCG-treated DCs and BCG-treated DCs from cord blood monocytes. This is a representative experiment of five different experiments with the same trend.

were increased in non-BCG-treated DCs, and both I κ B α and β were degraded with further maturation induced by BCG (Fig. 9).

DISCUSSION

As one of the first antigenic challenges for millions of neonates in developing countries, BCG vaccination has been considered to promote Th1 development of the neonatal immune system (20). Even though the mechanism involved is

unclear, it is likely to be dependent on activation of DCs. To clarify the mechanism of BCG on neonatal immunity, we investigated the effects of BCG on cord blood monocyte-derived DCs and the interaction of BCG-treated cord blood DCs and naïve CD4⁺ T cells *in vitro*. First, our results showed that BCG could promote cord blood monocyte-derived DC maturation with up-regulated expression of CD83, CD40, and MHC class II and down-regulated expression of MR. Furthermore, the ability of BCG-treated cord blood DCs to stimulate CD3⁺ T cell proliferation as revealed by MLR was significantly higher than that of non-BCG-treated cord blood DCs.

Comparing the effects of BCG on cord blood and adult blood DCs, the up-regulated levels of CD83, CD40, and MHC class II and the down-regulated level of MR induced by BCG were similar for both neonates and adults. In our system, BCG could significantly augment the production of TNF- α and IL-10, but not IL-12p70 secretion from both cord blood monocyte-derived DCs and adult blood monocyte-derived DCs. These results indicated that BCG was efficient in inducing maturation of both cord blood and adult blood monocyte-derived DCs.

It has been well known that mycobacterial or BCG infection of DCs results in the up-regulation of proinflammatory cytokines such as IL-1, IL-6, and TNF- α and regulatory cytokine IL-12 (13–15). These cytokines play a major role in the protective immunity against mycobacterial infection, because they contribute to delayed hypersensitivity. In mice, it has been reported that IL-12 production is crucial to the development of protective immunity against *M. tuberculosis* (28, 29). Because IL-10 has been shown to inhibit IL-12 production (30) and neutralization of IL-10 restores IL-12 secretion from mycobacterial-infected monocyte-derived macrophages (31), no production of IL-12p70 from cord blood and adult blood DCs induced by BCG in our study might be related to high levels of IL-10 production induced by BCG. The mechanism of no IL-12p70 production from BCG-treated DCs needs to be studied further.

The ability to prime naïve CD4⁺ T cells constitutes a unique and critical function of DCs (1). DC subsets may provide T cells with different cytokines that determine the classes of immune response, such as type 1 *versus* type 2 CD4⁺ helper cell profile. In humans, monocyte-derived CD11c⁺ DCs polarize naïve T cells predominantly toward a Th1 profile, whereas the CD11c[−] DCs induce T cells to a predominantly Th2 response (32). However, the extent of polarization by these cells may differ according to their method of isolation and maturation (33), the ratio of DCs to T cells (34), or the duration of DC activation (35). In our experiments, we used monocyte-derived DCs, which phenotypically and functionally belong to DC1, at a ratio of 1:10 (DC:T), which should favor Th1 development (33, 34). However, after priming with non-BCG-treated or BCG-treated cord blood DCs for 7 d, cord blood naïve CD4⁺ T cells produced high levels of IL-10, medium levels of IFN- γ , and little IL-4, resembling the cytokine profile of regulatory T cells (Tr1) (36–38). Different from immature non-BCG treated DCs, BCG-treated DCs were phenotypically mature, had high allogeneic antigen presentation capacity, and released high levels of TNF- α . Yet, unlike typical mature DC1, they released high levels of IL-10 and

maintain T cells as Tr1. These special properties render BCG-treated DCs more suitable than immature DCs for induction of tolerance. Moreover, this ability for BCG-treated DCs to maintain T cells as Tr1 may explain the negative association of BCG vaccination with atopy development. It opened up the possibility of using BCG-treated DCs for therapeutic intervention of atopy and other allergic diseases.

Mycobacterial HSP65, which has been identified as the immunodominant antigen in mycobacterial diseases that can confer protection equal to that from live BCG vaccine in mice, is a candidate vaccine against tuberculosis (39). In our system, even high concentrations of HSP65 (up to 1000 ng/mL) had no effect on CD86, MHC class II, and MR expression in cord blood monocyte-derived DCs. These results implied that the effects of BCG on the maturation of cord blood monocyte-derived DCs is either not mediated through HSP65 or that HSP65 has to be packaged in the context of BCG for its immunogenicity when presented to monocyte-derived DCs (40).

NF- κ B is a family of dimers composed of any of the five mammalian Rel proteins (p65/Rel-A, c-Rel, Rel-B, p50/NF- κ B1, and p52/NF- κ B2), which are structurally characterized by the Rel homology domain (41, 42). The Rel homology domain mediates important functions, such as specific DNA binding, nuclear translocation, and protein-protein interactions. The nuclear translocation and activity of NF- κ B factors are controlled by a family of cytosolic inhibitory proteins, the I κ Bs (43, 44). Many external stimuli lead to a site-specific phosphorylation of I κ Bs followed by their proteolytic degradation and release of active NF- κ B, which is then translocated into the nucleus (45). The genes controlled by NF- κ B factors encode proteins of principal importance for the immune system, such as MHC I and II molecules, cytokines and their receptors, or cell adhesion molecules. Most of these proteins are also synthesized by DCs and up-regulated with DC maturation. It is known that nuclear Rel-B plays a critical role in DC maturation (7, 8, 10). Neumann *et al.* (10) reported that monocyte-conditioned medium or cytokines induced maturation of DCs and resulted in an increase in nuclear Rel-B, p50, p52, and c-Rel. This up-regulation of NF- κ B factors did not correlate with lower levels of cytosolic I κ Bs (10). In contrast, we demonstrated that in the BCG-induced maturation of DCs, nuclear Rel-B was strongly up-regulated with cytosolic Rel-B down-regulation, with corresponding degradation of cytosolic I κ B α and β . This observation indicated Rel-B activation is an important signaling pathway for BCG-induced maturation of DCs.

The mechanism of BCG-induced maturation of DCs seems to differ from that induced by cytokines. In fact, we found that BCG could induce high levels of TNF- α production by cord blood monocyte-derived DCs, but anti-TNF- α could only partially neutralize the effects of BCG on the up-regulation of CD83 and MHC class II molecules and the down-regulation of MR. Anti-TNF- α , however, had no significant effect on BCG-induced CD86 and CD40 expression in cord blood DCs. These results suggested that the effects of BCG on CD83 expression of DCs are mediated partially through TNF- α . Other alternative pathways, such as the Toll-like receptors, which also

promote nuclear protein translocation (46), may also be involved.

CONCLUSIONS

In conclusion, BCG promoted cord blood monocyte-derived DC maturation to a similar extent as adult blood DCs. In the maturation process, nuclear Rel-B up-regulation and cytosolic Rel-B down-regulation were correlated with degradation of cytosolic I κ B α and β . Native HSP65 had no effect on cord blood monocyte-derived DC maturation, whereas anti-TNF- α could only partially neutralize the effects of BCG on cord blood DC maturation. Further elucidation of the mechanism of interaction between cord blood DCs and T cells, such as that underlying deficient IL-12p70 production, may lead to a novel strategy for developing a more effective vaccine against *M. tuberculosis*. In addition, the therapeutic use of BCG-treated DCs worth further exploration.

Acknowledgment. The authors thank the staff of the labor ward, Tsan Yuk Hospital, in facilitating the collection of cord blood.

REFERENCES

1. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K 2000 Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767–811
2. Chapuis F, Rosenzweig M, Yagello M, Ekman M, Biberfeld P, Gluckman JC 1997 Differentiation of human dendritic cells from monocytes *in vitro*. *Eur J Immunol* 27:431–441
3. Fisher GM, Iqbal S, Knight SC 1999 Gene expression during differentiation of human dendritic cells from cord blood CD34 stem cells. *Cytokine* 11:111–117
4. Palucka KA, Taquet N, Sanchez-Chapuis F, Gluckman JC 1998 Dendritic cells as the terminal stage of monocyte differentiation. *J Immunol* 160:4587–4595
5. Saraya K, Reid CD 1996 Stem cell factor and the regulation of dendritic cell production from CD34⁺ progenitors in bone marrow and cord blood. *Br J Haematol* 93:258–264
6. Zhou LJ, Tedder TF 1996 CD14⁺ blood monocytes can differentiate into functionally mature CD83⁺ dendritic cells. *Proc Natl Acad Sci USA* 93:2588–2592
7. Ammon C, Mondal K, Andreesen R, Krause SW 2000 Differential expression of the transcription factor NF- κ B during human mononuclear phagocyte differentiation to macrophages and dendritic cells. *Biochem Biophys Res Commun* 268:99–105
8. Clark GJ, Gunningham S, Troy A, Vuckovic S, Hart DN 1999 Expression of the RelB transcription factor correlates with the activation of human dendritic cells. *Immunology* 98:189–196
9. Lyakh LA, Koski GK, Telford W, Gress RE, Cohen PA, Rice NR 2000 Bacterial lipopolysaccharide, TNF- α , and calcium ionophore under serum-free conditions promote rapid dendritic cell-like differentiation in CD14⁺ monocytes through distinct pathways that activate NK- κ B. *J Immunol* 165:3647–3655
10. Neumann M, Fries H, Scheicher C, Keikavoussi P, Kolb-Maurer A, Brocker E, Serfling E, Kampgen E 2000 Differential expression of Rel/NF- κ B and octamer factors is a hallmark of the generation and maturation of dendritic cells. *Blood* 95:277–285
11. Reid SD, Penna G, Adorini L 2000 The control of T cell responses by dendritic cell subsets. *Curr Opin Immunol* 12:114–121
12. Patard JJ, Saint F, Velotti F, Abbou CC, Chopin DK 1998 Immune response following intravesical bacillus Calmette-Guerin instillations in superficial bladder cancer: a review. *Urol Res* 26:155–159
13. Kim KD, Lee HG, Kim JK, Park SN, Choe IS, Choe YK, Kim SJ, Lee E, Lim JS 1999 Enhanced antigen-presenting activity and tumour necrosis factor- α -independent activation of dendritic cells following treatment with *Mycobacterium bovis* bacillus Calmette-Guerin. *Immunology* 97:626–633
14. Thurnher M, Ramoner R, Gastl G, Radmayr C, Bock G, Herold M, Klocker H, Bartsch G 1997 Bacillus Calmette-Guerin mycobacteria stimulate human blood dendritic cells. *Int J Cancer* 70:128–134
15. Demangel C, Bean AG, Martin E, Feng CG, Kamath AT, Britton WJ 1999 Protection against aerosol *Mycobacterium tuberculosis* infection using *Mycobacterium bovis* bacillus Calmette-Guerin-infected dendritic cells. *Eur J Immunol* 29:1972–1979
16. Scanga CB, Le Gros G 2000 Development of an asthma vaccine: research into BCG. *Drugs* 59:1217–1221
17. Wilson CB, Lewis DB 1990 Basis and implications of selectively diminished cytokine production in neonatal susceptibility to infection. *Rev Infect Dis* 12(suppl 4):S410–S420
18. Barrios C, Brawand P, Berney M, Brandt C, Lambert PH, Siegrist CA 1996 Neonatal and early life immune responses to various forms of vaccine antigens qualitatively

- differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. *Eur J Immunol* 26:1489–1496
19. Shirakawa T, Enomoto T, Shimazu S, Hopkin JM 1997 The inverse association between tuberculin responses and atopic disorder. *Science* 275:77–79
 20. Marchant A, Goetghebuer T, Ota MO, Wolfe I, Ceesay SJ, De Groot D, Corrah T, Bennett S, Wheeler J, Huygen K, Aaby P, McAdam KP, Newport MJ 1999 Newborns develop a Th1-type immune response to *Mycobacterium bovis* bacillus Calmette-Guerin vaccination. *J Immunol* 163:2249–2255
 21. Liu E, Tu W, Law HK, Lau YL 2001 The changes of CD14 and CD1a expression are different between cord blood monocyte and adult peripheral blood monocytes. *Pediatr Res* 50:184–189
 22. Tu W, Cheung PT, Lau YL 1999 IGF-I increases interferon-gamma and IL-6 mRNA expression and protein production in neonatal mononuclear cells. *Pediatr Res* 46:748–754
 23. Tu W, Cheung PT, Lau YL 2000 Insulin-like growth factor I promotes cord blood T cell maturation and inhibits its spontaneous and phytohemagglutinin-induced apoptosis through different mechanisms. *J Immunol* 165:1331–1336
 24. Liu E, Tu W, Law HK, Lau YL 2001 Decreased yield, phenotypic expression and function of cord blood immature monocyte-derived dendritic cell. *Br J Haematol* 113:240–246
 25. Schreiber E, Matthias P, Muller MM, Schaffner W 1989 Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 17:6419
 26. Shinnick TM, Sweetser D, Thole J, van Embden J, Young RA 1987 The etiologic agents of leprosy and tuberculosis share an immunoreactive protein antigen with the vaccine strain *Mycobacterium bovis* BCG. *Infect Immun* 55:1932–1935
 27. Thole JE, Dauwerse HG, Das PK, Groothuis DG, Schouls LM, van Embden JD 1985 Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. *Infect Immun* 50:800–806
 28. Britton WJ, Roche PW, Winter N 1994 Mechanisms of persistence of mycobacteria. *Trends Microbiol* 2:284–288
 29. Cooper AM, Magram J, Ferrante J, Orme IM 1997 Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. *J Exp Med* 186:39–45
 30. Isler P, de Rochemonteix BG, Songeon F, Boehringer N, Nicod LP 1999 Interleukin-12 production by human alveolar macrophages is controlled by the autocrine production of interleukin-10. *Am J Respir Cell Mol Biol* 20:270–278
 31. Giacomini E, Iona E, Ferroni L, Miettinen M, Fattorini L, Orefici G, Julkunen I, Coccia EM 2001 Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J Immunol* 166:7033–7041
 32. Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, Liu YJ 1999 Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283:1183–1186
 33. Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A 1999 Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* 189:821–829
 34. Tanaka H, Demeure CE, Rubio M, Delespesse G, Sarfati M 2000 Human monocyte-derived dendritic cells induce naive T cell differentiation into T helper cell type 2 (Th2) or Th1/Th2 effectors. Role of stimulator/responder ratio. *J Exp Med* 192:405–412
 35. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F 2000 Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol* 1:311–316
 36. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG 1997 A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737–742
 37. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH 2000 Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 192:1213–1222
 38. Asseman C, Powrie F 1998 Interleukin 10 is a growth factor for a population of regulatory T cells. *Gut* 42:157–158
 39. Silva CL 1999 The potential use of heat-shock proteins to vaccinate against mycobacterial infections. *Microbes Infect* 1:429–435
 40. Cho BK, Palliser D, Guillen E, Wisniewski J, Young RA, Chen J, Eisen HN 2000 A proposed mechanism for the induction of cytotoxic T lymphocyte production by heat shock fusion proteins. *Immunity* 12:263–272
 41. Baeuerle PA, Henkel T 1994 Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* 12:141–179
 42. Thanos D, Maniatis T 1995 NF-kappa B: a lesson in family values. *Cell* 80:529–532
 43. Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U 1995 Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* 267:1485–1488
 44. Wulczyn FG, Krappmann D, Scheidereit C 1996 The NF-kappa B/Rel and I kappa B gene families: mediators of immune response and inflammation. *J Mol Med* 74:749–769
 45. Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA 1995 Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. *EMBO J* 14:2876–2883
 46. Tsuji S, Matsumoto M, Takeuchi O, Akira S, Azuma I, Hayashi A, Toyoshima K, Seta T 2000 Maturation of human dendritic cells by cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guerin: involvement of toll-like receptors. *Infect Immun* 68:6883–6890