**Mycobacterium bovis** and BCG induce different patterns of cytokine and chemokine production in dendritic cells and differentiation patterns in CD4+ T cells

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Received 31 January 2012
Revised 2 September 2012
Accepted 3 December 2012

**INTRODUCTION**

One-third of the world’s population is infected with *Mycobacterium tuberculosis* (*M.tbc*). While most infections are persistent, only 5–10% of these infections develop progressive tuberculosis (TB). However, this represents about 8 million new TB cases and 2 million deaths each year. The outcome of infection depends on the response of both the innate and the adaptive immune systems (Gupta et al., 2012). Currently, *Mycobacterium bovis* bacille Calmette–Guérin (BCG) is the only TB vaccine available for use in humans.

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**Abbreviations:** AO, Acridine orange; DCs, dendritic cells; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MHC, major histocompatibility complex; *M.tbc*, Mycobacterium tuberculosis; PDTC, pyrrolidine dithiocarbamate; PE, phycoerythrin; PI, post-infection; TB, tuberculosis; TNF, tumour necrosis factor; Treg, regulatory T cell.

It is an attenuated strain of *M. bovis* developed by Calmette and Guérin which lost its virulence after 230 passages over a period of 13 years and has a deletion of five DNA regions including 38 ORFs (Seki et al., 2009). Although the efficacy of BCG in preventing active TB in adults has been challenged, it saves millions of lives by preventing severe, disseminated extra-pulmonary TB, including miliary and meningitis TB in young children (Parthasarathy, 2003). As the genome sequences of *M.tbc* and *M. bovis* show over 99.95% identity, and the pathological changes caused by *M. bovis* in cattle are also similar to those of human TB, *M. bovis* and its isogenic attenuated strain BCG are ideal model strains for studying *M.tbc* pathogenesis and immunity.

Although it is well known that establishment of a productive infection of *M.tbc* depends on its ability to invade the alveolar space and to survive within macrophages, it has been demonstrated that dendritic cells (DCs)
also contribute to the cellular immune response against mycobacterium infection (Giacomini et al., 2001; Tascon et al., 2000; Sanarico et al., 2011). However, unlike macrophages, the involvement of DCs in M.tb infection is poorly defined. This may result from the later recognition of DC participation in M.tb infection and the complexity of DCs and the procedures for studying them. Due to the absence of cell lines, DCs used in experiments are generated ex vivo from peripheral blood monocytes or bone marrow/circulating haematoipoietic stem cells, which are cultured in the presence of cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). During the immature stage, DCs can capture and degrade bacteria. After interaction with the antigens, DCs mature and migrate to lymphoid organs, where they present T cells with the antigens coupled within cell surface major histocompatibility complex (MHC) molecules and initiate an adaptive immune response specifically targeting the antigens (Banchereau & Steinman, 1998; Henderson et al., 1997). As these processes are orchestrated by a series of cytokines and chemokines induced by the captured antigens (Orme & Cooper, 1999), we investigated differential cytokine and chemokine production in DCs after infection with M. bovis and BCG.

In this study, we found that BCG infection produced higher concentrations of monocyte chemotactic protein-1 (MCP-1), RANTES, interleukin-12 (IL-12), tumour necrosis factor-z (TNF-z) and IL-6 in DCs, while M. bovis stimulated higher levels of IL-1b, IL-10 and IL-23. DC cultures infected by M. bovis directed naïve CD4+ T cells more effectively to differentiate into regulatory T cells (Tregs) and secrete more IL-17, a Th17 cytokine, while DC cultures infected by BCG stimulated CD4+ T cells to produce more interferon-c (IFN-c), a Th1-type response.

METHODS

Bacterial culture and counting. M. bovis (ATCC 19210) and BCG Tokyo strain (ATCC 35737), kindly provided by Dr Li Chuan-You from the Beijing Tuberculosis & Thoracic Tumor Research Institute, were grown to exponential phase in 250 ml Middlebrook 7H9 medium (Becton Dickinson) supplemented with 10% oleic acid, albumin, glucose and catalase (OADC) (Difco), and 0.05% Tween 80 (Amresco) at 37 °C in the biosafety level 3 laboratory (BSL-3) at Huazhong Agricultural University.

Bacteria were harvested from the culture medium by centrifugation at 5000 g for 10 min, and pellets were transferred to mortar-grinders, ground into homogenates and resuspended with 40 ml 7H9 medium. After standing for about 5 min, the supernatant was collected, mixed, aliquoted and then stored at −80 °C until used.

For the colony counting assay, bacteria were serially diluted 10-fold with Middlebrook’s 7H10 medium (Becton Dickinson) and 0.1 ml of each dilution was transferred to 7H10 plates. Bacteria were grown for 4–6 weeks until the colonies were suitable for counting by eye. Bacterial concentration was recorded as c.f.u. ml⁻¹.

To distinguish live and dead bacteria, Acridine orange (AO) staining was performed as previously described (Yuan et al., 1989). The AO stain was made by mixing 1 % AO (AMRESCO) and 1 % Auramine (Sinopharm Chemical Reagent Co., Ltd) at a ratio of 6:1 and further diluted 10 times with 0.15 M PBS (pH 6.5) immediately before use. Counter-staining was performed with 0.5% Amino black (Sinopharm). The smears of bacterial samples were air-dried, stained with 100 μl AO stain at room temperature for 3 min, then washed under flowing water. Slides were then counter-stained with 100 μl Amino black in the dark at room temperature for 30 s, washed again and air-dried. The stained smears were observed under a fluorescence microscope (Olympus, IX70). Dead bacteria stained red, while live bacteria were green or yellow-green.

When it was necessary to use dead mycobacteria as a control, the bacteria were heat-inactivated in a 90 °C water bath for 30 min and evaluated with AO staining as described above.

Conventional acid-fast staining (Ziel–Neelsen staining) was performed with an acid-fast staining kit (Shanghai Yuanye Bio-Technology Co. Ltd) according to the manufacturer’s instructions. The air-dried bacterial smears were heat-fixed by rapidly passing the slide through a flame. Fifty microlitres of reagents 1 and 2 were simultaneously added to the slides, which were stained for 2 min at room temperature and washed under flowing water. Fifty microlitres of reagent 3 was then added and stained for 2 min. After washing, the slides were air-dried and observed under a light microscope (Zeiss, AX10). Mycobacteria stained red.

DC preparation and infection. Preparation of murine bone marrow-derived DCs and characterization of immature DCs were performed as described previously (Guo et al., 2007). Six–to 8-week-old C57BL/6N female mice were purchased from Zhongnan Hospital, Wuhan University, and temporarily housed in the animal facility at Huazhong Agricultural University. Animal treatment was carried out in strict accordance with the recommendations in the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals, 2005. The protocol was approved by the China Hubei Province Science and Technology Department [permitt no.: SYXK(ER) 2010-0029].

Cells were cultured in complete RPMI 1640 medium supplemented with 10% heat-inactivated FBS and recombinant murine granulocyte-macrophage colony stimulating factor (rGM-CSF; 20 ng ml⁻¹) (Peprotech). Non-adherent cells were collected on days 6–8 and assayed for the expression of surface markers (CD11c, CD40, CD80, CD86 and MHC-II) by flow cytometry (FACS Calibur). Fluorescent antibodies (Abs) included either red phycoerythrin (PE)-conjugated antibody (PE-Ab) to CD11c or green fluorescein isothiocyanate (FITC)-labelled antibodies (FITC-Ab) to CD40, CD80, CD86 and MHC-II. Corresponding PE- and FITC-labelled isotypes (eBioscience) were used as controls. The percentage of immature DCs (CD11c⁺, low-to intermediate-level expression of MHC-II, low-level expression of CD80 and CD86, and no expression of CD40) was generally greater than 80%.

Non-adherent cells were collected by centrifugation before infection, washed once with incomplete RPMI 1640 medium without FBS and then resuspended in complete RPMI 1640 with 10% FBS without GM-CSF at a density of 1 x 10⁶ cells ml⁻¹. The cell suspension was distributed into 24-well plates at 1 ml per well (Costar). Bacteria were then added to the wells at an m.o.i. of 10. The infected DCs were cultured in complete RPMI 1640 medium for various lengths of time and were subjected to further assays. An equal amount of heat-killed bacteria was added in parallel to the control when it was necessary to evaluate the specific effect of live mycobacteria. All manipulations were performed in the BSL-3 laboratory.

To check for the presence of mycobacteria in the DC-infection cultures, Ziel–Neelsen staining of DCs was performed at 24 h after infection, and the ratio of DCs associated with mycobacteria was analysed using a light microscope.
Detection of cytokines and chemokines by ELISA. Supernatants were collected from DC cultures with or without *M. bovis* and BCG infection at 6, 12 and 24 h post-infection (PI). Ten cytokines and chemokines were measured in the culture supernatants using commercial ELISA kits according to the manufacturer’s instructions. These were MCP-1 (Bender Medsystems), RANTES (Invitrogen), IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17 and TNF-α (R&D Systems), and IL-23 (Biolegend).

Chemokine and cytokine blocking assay. Pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich) was used to specifically block the activation of NF-κB. Briefly, 18 wells of DCs in a 24-well cell culture plate were divided into three groups, each of six wells: mock-infection, BCG-infected and *M. bovis*-infected. Each group was subdivided into two subgroups, each of three wells, for blocked and unblocked treatments. Each blocked subgroup was incubated with PDTC (100 μM), while the unblocked subgroup was treated with PBS. One hour later, the two infection groups (four subgroups) and two subgroups in the mock-infection group were infected with either BCG or *M. bovis*, while another two subgroups in the mock-infection group were treated with PBS. All the groups were incubated for a further 24 h. The culture supernatant in each well was then collected for detection of chemokines and cytokines by ELISA as described above. Each treatment was carried out in triplicate.

Transcription of NF-κB signal pathway-related genes. Total RNA was isolated from DCs at 6, 12 and 24 h PI using TRIzol reagent (Invitrogen). The RNA concentration was calibrated using a NanoDrop Spectrophotometer to an OD260/OD280 ratio of 1.8–2.0, and RNA integrity was evaluated by agarose gel electrophoresis.

Quantitative RT-PCR (qRT-PCR) was used to determine the transcription of the NF-κB p50 and p65 subunits using β-actin as the internal reference gene. Primer sequences used are listed in Table 1. All primer sets had a calculated annealing temperature of 58 °C. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Total RNA from each sample was reverse-transcribed into cDNA using a First Strand cDNA Synthesis kit (Toyobo) according to the manufacturer’s instructions. Subsequently, real-time qPCR was performed using THUNDERBIRD SYBR qPCR mix (Toyobo). The volume of each reaction was 25 μl, including 100 ng cDNA, 200 nmol of each primer and 12.5 μl 2 × SYBR Green dye. Reactions were programmed in an ABI 7500 Real-Time PCR System (Applied Biosystems) as follows: 95 °C for 10 min (hot start), followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s. The fluorescence signal was collected at the end of each elongation step. β-Actin was used as the internal standard and all reactions were performed in triplicate. Relative gene transcription was obtained using the formula 2−ΔΔCt, where ΔΔCt=ΔCtarget gene−ΔCβ-Actin, and ΔΔCt=ΔCtreatment 1−ΔCtreatment 2. The transcription of different related genes was normalized against the level of the gene with the lowest transcription rate.

Western blotting of phospho-NF-κB p65 and IκB. As the activation of NF-κB is followed by the degradation of IκB, repressors of NF-κB, both NF-κB p65 and IκB, were subjected to Western blotting using β-actin as an internal reference. RIPA lysis buffer P0013 (Beyotime) was used to lyse DCs mock-infected or infected with either *M. bovis* or BCG. The concentration of total protein was determined with a bicinchoninic acid (BCA) protein assay kit according to the manufacturer’s instructions (Thermo Fisher Scientific and Pierce Biotechnology). Proteins were separated by SDS-PAGE and then semi-dry-blotted onto a nitrocellulose membrane. After blocking with 5% non-fat milk powder, the probing antibodies, including rabbit monoclonal IκG against mouse phospho-NF-κB p65 (1:1000) (Cell Signaling Technology), IκB (1:10 000) (Epitomics) and β-actin (1:500) (Santa Cruz), were added as appropriate and incubated overnight at 4 °C. Horseradish peroxidase-labelled goat antibody against rabbit IgG was then added and incubated for 1 h. The NC membrane was washed with TBST after each step. Finally, 1–2 ml of ECL detection solutions A and B was slowly added and incubated for 2 min at room temperature. A Kodak Image Station was used to detect chemiluminescence signals. The band image was converted to greyscale and analysed with ImageJ.

Isolation of CD4+ T cells and detection of cytokine secretion. Mouse splenocytes and lymph node cells were pooled, and naive CD4+ T cells were labelled with a biotin–antibody cocktail. Cells were further incubated with anti-biotin magnetic beads (Miltenyi Biotec) and separated over LS columns (Miltenyi Biotec) in accordance with the manufacturer’s instructions to obtain naive CD4+ T cells. The purity of the sorted cells was over 95%.

Whole DC cultures (hereafter referred to simply as ‘SC’, including DCs, intracellular and extracellular mycobacteria, and supernatant) were passed through a 0.22 μm pore-size filter to separate bacteria-infected DCs (hereafter referred to as ‘C’, containing DCs, and their associated intracellular and extracellular mycobacteria) from the supernatant (hereafter referred to as ‘S’, free of DCs and mycobacteria). Naïve CD4+ T cells were plated onto a 24-well plate at a density of 2 × 10^6 cells ml⁻¹ and incubated for 24 h with 100 μl supernatant (S) or 100 μl of whole culture (SC) containing 2 × 10^6 DCs, or only 2 × 10^6 DCs (C) with or without live or heat-killed *M. bovis* and BCG. The live and heat-killed BCG or *M. bovis* were added to naive CD4+ T cells at an m.o.i. of 10 as controls to detect the direct effect of mycobacteria on these T cells. Supernatants were collected to assay cytokine levels (IL-4, IL-10, IL-12, IL-17A and IFN-γ), while CD4+ T cells were subjected to qRT-PCR for detection of foxp3.

Table 1. Primers used for qRT-PCR in this study

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<th>Gene</th>
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<tr>
<td></td>
<td>R</td>
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<td></td>
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transcription as described above by using the primer set in Table 1, and to FACS assays for the detection of Tregs (CD4+CD25+FoxP3+) after staining with a mouse regulatory T-cell staining kit (#1, Ebioscience) according to the manufacturer’s instructions.

**Statistical analysis.** Data were analysed with the statistical package SPSS. Multiple comparisons of cytokine concentrations and ratios of DCs associated with mycobacteria were performed with one- or two-way ANOVA, and FACS data on Treg cell proportions were compared using the chi-squared test. Values were expressed as means ± SD. *P* values <0.05 were considered to be significantly different (*) and *P* values <0.01 very significantly different (**).

**RESULTS**

**BCG and M. bovis induced different cytokine and chemokine profiles in DCs**

Before infecting DCs, we used AO staining to determine the proportion of live and heat-killed bacteria. Over 99% of the bacilli in the live mycobacterial preparation used in this study were living, while heat-killed bacilli were dead. Concentrations of IL-1β, IL-4, IL-6, IL-10, IL-12, IL-23, TNF-α, MCP-1 and RANTES were then measured by ELISA at three time points (6, 12 and 24 h PI) in supernatants from DC cultures with or without infection with M. bovis or BCG. In general, compared with mock-infected controls, both M. bovis and BCG stimulated significant production of IL-12, IL-23, IL-6, IL-10, TNF-α, MCP-1 and RANTES at most of the time points, although there were apparent differences in cytokine and chemokine profiles after BCG and M. bovis stimulation (Fig. 1).

BCG infection caused significantly higher production of IL-12 and TNF-α than M. bovis infection at all three time points (6, 12 and 24 h PI) (P<0.01), of IL-6 and MCP-1 at 12 and 24 h PI (P<0.01), and of RANTES at 6 and 12 h PI (P<0.01). The largest difference observed was in IL-12 production, and average IL-12 concentrations in BCG-infected DCs at the three time points were, respectively, 4.1-, 5.0- and 4.7-fold higher than those in M. bovis-infected DCs, while the production of TNF-α, IL-6, MCP-1 and RANTES in BCG-infected DCs was about 2-fold higher than that in M. bovis-infected DCs at 12 and 24 h PI (Fig. 1).

In contrast, M. bovis infection stimulated 2.8-, 2.7- and 2.4-fold higher production of IL-23 in DCs at 6, 12 and 24 h PI (P<0.01), respectively, than did BCG infection. Similarly, the production of IL-10 at the three time points in M. bovis-infected DCs was significantly higher than that in BCG-infected DCs (P<0.01). IL-1β generation was also higher in M. bovis, but only at 24 h PI (P<0.01). However, the absolute concentrations of IL-4 and IFN-γ were very low, as expected, because DCs are not efficient at secreting these two cytokines (Fig. 1).

To further determine whether the differences in cytokine expression in DCs were specifically due to infection with live mycobacteria, we compared the proportions of DCs associated with the heat-killed and live mycobacteria and cytokine expression by DCs between equal doses of the heat-killed and live bacterial infections. Fifteen fields of 87–99 DCs were observed for each group after acid-fast staining at 24 h PI. According to microscopy findings, the proportion of DCs associated with the live and dead mycobacteria was similar for each strain (P>0.05). However, the proportion of DCs associated with M. bovis was significantly higher [84.64 ± 19.16% (live M. bovis) and 95.63 ± 13.15% (heat-killed M. bovis)] than that associated with either live (62.75 ± 18.45%) or heat-killed BCG (60.09 ± 27.60%) (P<0.01). Although the heat-killed bacteria stimulated the production of cytokines to some extent, infection with live mycobacteria significantly promoted cytokine production by DCs to a much greater extent (P<0.05) (Fig. 2).

**Activation of NF-κB in DCs after infection with BCG or M. bovis**

NF-κB p50 was transcribed at significantly higher levels in DCs infected by BCG than in those infected by M. bovis at 6 h PI (P<0.01). The difference between treatments became smaller as infection time increased to 12 h (P<0.05), and was non-existent at 24 h PI (Fig. 3a). Unlike NF-κB p50, the difference in NF-κB p65 transcription between BCG- and M. bovis-infected DCs was significant only at 24 h PI (P<0.01) (Fig. 3b).

To further confirm the involvement of NF-κB, expression of NF-κB p65 and its repressor IκB (Hoffmann et al., 2002) was examined by Western blotting (Fig. 3c, d). Using β-actin as an internal reference, results indicated that IκB expression was higher in M. bovis-infected DCs than in BCG-infected DCs. When gel electrophoresis images were quantitatively compared, the band intensity (expressed as greyscale) for M. bovis infection was 2.9-fold higher than that for BCG infection (Fig. 3c). Conversely, expression of phospho-NF-κB p65 (P-p65) in DCs with BCG infection was higher than that for M. bovis infection, and the difference in greyscale was 1.72-fold higher (Fig. 3d).

**Effect of blocking NF-κB on cytokine production**

The IL-1β, IL-4, IL-6, IL-12, IL-23, TNF-α, MCP-1 and RANTES cytokines and chemokines secreted by DCs after infection with BCG and M. bovis for 24 h were detected respectively in the presence of the NF-κB inhibitor PDTC. PDTC pretreatment completely blocked the production of IL-1β, RANTES and MCP-1, and greatly inhibited the secretion of IL-6, IL-10 and TNF-α (P<0.01) (Fig. 4). However, PDTC could not suppress the production of IL-12 and IL-23, but the reasons for this remain to be investigated.

**Differential activation of naïve CD4+ T cells**

After infection with BCG or M. bovis for 24 h, cell-free supernatants (S), whole DC cultures (SC) and DCs alone (C) were used separately to stimulate naïve CD4+ T cells
Cytokine concentration (pg ml\(^{-1}\))

- TNF-\(\alpha\)
- IL-12
- IL-10
- IL-6
- IL-23
- IL-1\(\beta\)
- MCP-1
- RANTES
- IFN-\(\gamma\)
- IL-4

Time post infection (h)

** X. Zhang and others

Microbiology 159
Fig. 1. Differential production of cytokines and chemokines in DCs after infection with BCG and \textit{M. bovis}. DCs were infected with BCG and \textit{M. bovis} at an m.o.i. of 10. Cytokine and chemokine levels were measured with commercial ELISA kits at 6, 12 and 24 h PI. The data shown are the mean ± SD of three replicates. *Significant difference (P<0.05); **very significant difference (P<0.01).

for 24 h, and cytokine production was subsequently measured (Fig. 5a). Of all the cytokines tested, IL-10 showed the strongest response to stimulation in all the samples tested. A large increase in CD4\(^+\) T-cell IL-10 secretion was observed for both the supernatant (S) and the whole culture (SC) from \textit{M. bovis}- but not from BCG-infected DCs (P<0.01). The whole DC culture had a stronger ability than the supernatant alone to enhance IL-10 production by CD4\(^+\) T cells (P<0.01). IL-10 concentration reached 1086.96 ± 54.35 pg ml\(^{-1}\) when stimulated by the supernatant, but 1781.21 ± 89.06 pg ml\(^{-1}\) when stimulated by the whole DC culture, suggesting that both the contact of DCs/T cells and soluble factors are necessary to activate naive CD4\(^+\) T cells. In addition, \textit{M. bovis}-infected DC cultures induced significantly more IL-17 production, while BCG-infected DC cultures stimulated significantly more IFN-\(\gamma\) production by CD4\(^+\) T cells (P<0.01) (Fig. 5a).

Differentiation of Tregs from naïve CD4\(^+\) T cells after stimulation by activated DCs after infection with either BCG or \textit{M. bovis} was monitored by examining foxp3 transcription by qRT-PCR. Transcription of foxp3 was significantly enhanced in CD4\(^+\) T cells after stimulation by DC culture supernatants from infections with \textit{M. bovis}, and the relative transcription of foxp3 in CD4\(^+\) T cells was 2.2-fold higher than when the DC culture supernatants were from BCG infections.

To check the direct effect of mycobacteria on the development of naïve CD4\(^+\) T cells, we performed further experiments by directly adding mycobacteria (live or heat-killed BCG and \textit{M. bovis}) to naïve CD4\(^+\) T cells. In addition, DC cultures infected with either heat-killed or live BCG or \textit{M. bovis} for 24 h were further fractionated into three parts, S, SC and C, to investigate the individual effect of each part on activating naïve CD4\(^+\) T cells. Similarly, supernatants were collected for assaying the levels of three cytokines (IL-10, IL-17A and IFN-\(\gamma\)), and cells were subjected to FACS assays to monitor the development of Tregs (CD4\(^+\)CD25\(^+\)Foxp3\(^+\)). Neither BCG nor \textit{M. bovis} could directly stimulate CD4\(^+\) T cells to secrete IL-10, IL-17 and IFN-\(\gamma\) cytokines. In agreement with previously reported results, BCG-infected DCs significantly stimulated production of IFN-\(\gamma\), while \textit{M. bovis}-infected DC cultures significantly stimulated secretion of IL-10 and IL-17 by CD4\(^+\) T cells. Compared with DC cultures infected by live mycobacteria, DC cultures infected with heat-killed bacteria induced CD4\(^+\) T cells to yield these three cytokines, at significantly lower levels (P<0.05 or P<0.01) (Fig. 5b–d).

Whole DC cultures stimulated the highest production of cytokines by CD4\(^+\) T cells, while bacteria- and cell-free supernatants induced significantly lower (but still significantly higher than the control group, P<0.01) secretion of cytokines (P<0.01). However, DCs alone did not appreciably stimulate the expression of these three cytokines (Fig. 5b–d). We therefore concluded that combining the supernatant with DCs is essential for activating naïve CD4\(^+\) T cells.

To analyse our FACS data, we gated CD4\(^+\) T cells and calculated the proportion of Tregs (CD25\(^+\)Foxp3\(^+\)). Stimulation of \textit{M. bovis}-infected DCs (whole culture SC and supernatant S) yielded a significant increase in Tregs; the proportion of Tregs (CD4\(^+\)CD25\(^+\)Foxp3\(^+\)) was 7.85 % for supernatant stimulation and 7.94 % for whole DC culture stimulation. BCG-infected DCs possessed a significantly weaker ability to induce the differentiation of Tregs than \textit{M. bovis} (P<0.01); the resultant proportion of Tregs was 3.78 % for supernatant stimulation of DC cultures and 3.95 % for stimulation of whole DC cultures. There was no statistical difference between stimulation with the supernatant and stimulation with whole DC cultures. However, DCs alone, and live or heat-killed mycobacteria alone could not appreciably stimulate the development of Tregs (Fig. 6). These findings are similar to those for cytokine production (Fig. 5).

Together, these results demonstrate that a combination of the culture supernatant and DCs associated with \textit{M. bovis} stimulates the differentiation of CD4\(^+\) Tregs and the production of IL-17, a Th17 cytokine, while combining the culture supernatant and DCs associated with BCG stimulates production of IFN-\(\gamma\), a Th1 cytokine.

**DISCUSSION**

Although DCs function as an in vivo bridge linking innate and acquired immunity and therefore may play an important role in pathogenesis or protective immunity during \textit{M. bovis} infection, there is no evidence to affirm that DCs are involved in the pathogenesis of \textit{M. bovis}. As both the cytokine environment produced by DCs and activated DCs themselves determine T-cell development, we compared differential production of cytokines and chemokines in DCs after BCG and \textit{M. bovis} infection, and its possible effects on CD4\(^+\) T-cell differentiation. Differences revealed in this study between these two strains partially explain the pathogenic behaviour of \textit{M. bovis} during infection and the protective immune response of BCG as a vaccine.

**\textit{M. bovis} has a stronger ability than BCG to induce proinflammatory factors in DCs and cytokines in CD4\(^+\) T cells related to Th17 cell development**

Inflammation is a fundamental defence response, and is related to the secretion of proinflammatory and anti-inflammatory factors.
Fig. 2. Differential production of cytokines and chemokines in DCs on stimulation with live or heat-killed mycobacteria for 24 h. Live or heat-killed BCG and *M. bovis* were added to DCs at a ratio of 1 : 10 (DCs/mycobacteria) and co-incubated for 24 h. The cytokine and chemokine levels in the culture were measured with commercial ELISA kits. Data shown are the mean ± SD of three replicates. *Significant difference (P<0.05); **very significant difference (P<0.01).
cytokines. When it goes awry, however, it becomes a major cofactor in the pathogenesis of chronic human and animal diseases such as TB. In this study, we found that, contrary to BCG, the cytokine profile of DCs induced by M. bovis was characterized by significantly higher levels of IL-23, IL-1β and IL-10 but lower levels of IL-12, and that of CD4<sup>+</sup>T cells activated by M. bovis-infected DC cultures was characterized by significantly higher levels of IL-17 and IL-10. As Th17 cells are IL-17-producing effector T helper cells, and IL-23 is a growth and stabilization factor of Th17 cells (Korn et al., 2009), our results suggest that Th17 cells may be involved in chronic TB inflammatory and pathogenic processes. Furthermore, IL-1β is another proinflammatory cytokine and plays an important role in promoting the secretion of IL-17 (Masters et al., 2010). The current results are in agreement with results from our previous genome microarray assay, which showed that transcription of the IL-1α gene in DCs is enhanced 3.1-fold and that of the IL-1β gene 2.5-fold more by M. bovis infection than by BCG infection (Cao, 2008).

**Fig. 3.** Relative transcription of NF-κB p50 and p65 subunits, and expression of phospho-NF-κB p65(P-p65) and repressor I-κB. DCs were infected with BCG and M. bovis at an m.o.i. of 10 and the relative transcription of NF-κB p50 (a) and p65 (b) subunits at 6, 12 and 24 h PI was measured by qRT-PCR. The transcription activity of infected DCs was normalized against the uninfected DC control whose transcription activity was taken as 1. Data shown are the mean±SD of three replicates. The expression of phospho-NF-κB p65(P-p65) and repressor I-κB in DCs was detected with Western blotting at 24 h after infection with BCG and M. bovis (c, d). β-Actin was used as an internal reference. Upper panels show the ratio of the greyscales of the I-κB (c) and P-p65 (d) bands to β-actin and were quantified using densitometry measurements of the Western blot bands of I-κB (c) and P-p65 (d) shown in the bottom panel of the figure. *Significant difference (P<0.05); **very significant difference (P<0.01).
IL-23, mainly produced by DCs and macrophages, is a heterodimeric cytokine which shares the p40 subunit in common with IL-12, but is covalently linked to a specific p19 subunit. However, unlike IL-12, IL-23 functions in mediating inflammatory and pathological processes either by activating Th17 cells or by directly stimulating macrophages to secrete IL-1, TNF and IL-6 to promote inflammation (Cua et al., 2003; Yen et al., 2006). In agreement with this, the activation assay of CD4+ T cells in this study confirmed that the CD4+ T cells stimulated by M. bovis-infected DC cultures produce higher levels of IL-17 than BCG-infected DC cultures. Our findings on IL-23 are in agreement with an earlier study with macrophages, which revealed that the virulent strain H37Rv markedly increases expression of IL-23 p19 in alveolar macrophages compared with the avirulent strain H37Ra (Silver et al., 2009).

However, two consequences may arise from Th17 cell involvement in TB. BCG has lost its virulence at the price of the deletion of five DNA regions called regions of difference (RDs) covering 38 ORFs (Seki et al., 2009), of which region of difference 1 (RD1) is the most prominent. Some previous reports have demonstrated that RD1 protein ESAT-6 promotes differentiation of Th17 cells, which contributes to improved protection against M.tb challenge (Chatterjee et al., 2011). On the other hand, Majlessi et al. (2005) reported that BCG complemented with RD1 (BCG::RD1) can recover virulence in immune-competent mice, as shown by its persistence in vivo, similar

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**Fig. 4.** Blocking of cytokine and chemokine production in DCs after infection with BCG and M. bovis. DCs were pretreated with or without the NF-κB inhibitor PDTC, and then infected with BCG or M. bovis at an m.o.i. of 10. Cytokine and chemokine levels for groups of blank (without infection and blocking), unblocked and PDTC-blocked groups infected by either BCG or M. bovis were measured with commercial ELISA kits at 24 h PI. Data shown are the mean±SD of three replicate experiments. *Significant difference (P<0.05); **very significant difference (P<0.01).
to the virulent strain H37Rv. In addition, induction of chronic granulomatous inflammation is similar to that caused by H37Rv. To address these issues, Ernst (2012) proposed the concept of the immunological life cycle of TB. For instance, at the early stages of TB, Th17 cells enhance host protection against *M. tuberculosis* infection by promoting inflammation and decreasing the bacterial load in target tissues more effectively. However, this inflammation cannot clear the mycobacteria sufficiently, and furthermore it might be harmful at the late stage of infection in mice by maintaining the persistence of *M. tuberculosis* and causing excessive inflammation leading to inflammation-related tissue damage (Ernst, 2012). In fact, other evidence has shown that Th17 cells are related to inflammatory diseases such as the human autoimmune diseases rheumatoid arthritis, inflammatory bowel disease, psoriasis, multiple sclerosis and systemic lupus erythematosus (Cua et al., 2003; Hue et al., 2006). Although Th17 cells can enhance the protective capacity provided by both innate and acquired immunity against *M. tuberculosis* infection, they may not be sufficient to clear these bacteria, and the resultant overinflammation leads to formation of a granuloma in a chronic pathological process which can shelter *M. bovis* and maintain its persistence.

Chemokines are very important in the initiation of immune responses and inflammation. Here, we discovered that BCG induces DCs to produce significantly higher concentrations of MCP-1 and RANTES than *M. bovis*. MCP-1 is known to be a T-lymphocyte chemoattractant (Shimizu et al., 2005), and MCP-1 gene variants are associated with susceptibility or protection against mycobacterial infection (Intemann et al., 2011). Similarly, RANTES plays an important role in mediating T-cell...
migration to M.tb-infected lungs for optimal granuloma formation and contributes to early protection against M.tb (Vesosky et al., 2010). Furthermore, compared with macrophages, DCs secrete more MCP-1 in response to M.tb infection (Jang et al., 2008). Therefore, the higher production of MCP-1 and RANTES induced in BCG-infected DCs observed in this study should be significant for protection against TB after vaccination with BCG.
M. bovis and BCG induce various cytokines in DCs

Activation of DCs by M. bovis and the resulting cytokine environment induce proliferation of Tregs more effectively

To show that both activated DCs and the soluble cytokines produced by activated DCs contribute to the differentiation of naïve CD4+ T cells, we compared the effect of the supernatant (S), DCs alone (C) and whole DC cultures (SC) on naïve CD4+ T cells. Foxp3 gene transcription, a specific marker of CD4+ Tregs, was measured when the supernatant was added to naïve CD4+ T cells, while cytokine production by CD4+ T cells and development of CD4+ Tregs was compared when the three DC culture fractions (S, C, SC) were added respectively to naïve CD4+ T cells. The results showed that there is greater promotion of foxp3+ transcription. The supernatant and whole DC culture after M. bovis infection stimulated significantly higher development of CD4+ Tregs (CD4+CD25+Foxp3+) and secretion of very high levels of IL-10 by CD4+ T cells, inhibiting the protective immune response to M. bovis (in agreement with Kochetkova et al., 2011) by preventing hosts from clearing M. bovis, and thereby maintaining persistent infections. Moreover, CD4+ Tregs and Th17 cells may function synergistically to regulate M.tb pathogenesis. For instance, CD4+ Tregs may inhibit the immune response to bacilli protected by granuloma whose formation may be regulated by Th17 cells.

DCs activated by BCG have a greater ability than M. bovis to induce Th1-type cytokines

IL-12 is produced naturally by DCs, macrophages and B cells in response to antigenic stimulation, and is critical for directing the differentiation of naïve T cells into Th1 cells which participate in defending against the invasion of pathogens (Trinchieri, 2003). Importantly, IL-12 production is related to effective protection against M.tb, and to a decrease in lung bacillary loads (Flynn et al., 1995). Inconsistencies in previous reports on IL-12 production in cell infection models after mycobacterial infection may be due to the use of different infection models. Silver et al. (2009) discovered that neither H37Rv nor H37Ra could induce IL-12 production in macrophages. Chatterjee et al. (2011) demonstrated that both H37Rv and BCG induce comparable levels of IL-12p40, a subunit common to IL-12 and IL-23. Here, using a DC model, we validated previous studies by showing that M. bovis and BCG induced either IL-12 or IL-23, sharing the increase of IL-12p40. However, we found that M. bovis induces significantly higher IL-23 but less IL-12 than BCG in DCs at 6, 12 and 24 h PI. We also demonstrated that BCG-infected DC cultures activate CD4+ T cells to generate significantly higher levels of IFN-γ. We therefore reason that, compared with M. bovis, BCG induces a better cytokine environment for DCs and CD4+ T cells and leads to a higher Th1 response. As is well known, vaccination with BCG produces Th1 cell-mediated immune responses, even though it is only moderately effective in protecting against disseminated TB and against meningitis in children (Chatterjee et al., 2011). In addition, BCG stimulates significantly higher production of IL-6 than M. bovis. IL-6 has been shown to be essential for the activation of T cells in the defence against M.tb by strengthening the effect of INF-γ (Leal et al., 1999).

NF-κB regulation of cytokine and chemokine secretion acts as a common switch during BCG and M. bovis infection

As NF-κB transcription factors are critical regulators of immunity and inflammation, we investigated the involvement of NF-κB. PDTC is a relatively selective inhibitor of NF-κB and inhibits its activation by a mechanism probably related to its antioxidant and metal-ion chelating activities that prevents degradation of I-κB, the NF-κB p65 suppressor (Hayden & Ghosh, 2011). In this study, PDTC completely blocked the secretion of IL-1β, RANTES and MCP-1, and partially inhibited TNF-α, IL-6 and IL-4. This is in agreement with previous reports that the production of TNF-α, IL-1β, IL-4, IL-6, MCP-1 and RANTES is mediated by NF-κB activation (Ghosh et al., 1998). Therefore, although these cytokines and chemokines are differentially induced, NF-κB is the common regulatory factor active in DCs. However, our results show that NF-κB is not the only transcription regulatory factor in DCs, as PDTC failed to block IL-12 and IL-23 production. Because these two
cytokines share a common subunit, IL-12p40, it is possible that they share transcription regulators other than NF-κB. In fact, previous reports have shown that in addition to NF-κB, IL-12p40 can be regulated by transcription factors such as the Ets family (Ets-2 and Pu.1), AP-1 and C/EBPB (Oeckinghaus et al., 2011). Further study is needed to understand the details of this regulation.

In summary, we have shown that M. bovis infection stimulates DCs to produce higher levels of IL-23, IL-1β and IL-10, and that DC cultures expand differentiation of CD4+ Tregs (CD25+Foxp3+IL-10+T) and create a high-IL-17 environment, leading to the development of Th17 CD4+ T cells. By contrast, BCG infection stimulates DCs to produce higher levels of IL-12, and these DC cultures induce CD4+ T cells to express more IFN-γ, a cytokine that stimulates the development of Th1 CD4+ T cells.

ACKNOWLEDGEMENTS

This work was supported by the China National Basic Research (973) Program (2012CB518801), the Key Special Science and Technology Program for Important Infectious Diseases such as AIDS and viral hepatitis (2012ZX1000302-016; 2012ZX10004214) and the National Natural Science Foundation of China (31172337). We thank Dr Li Chuan-You from the Beijing Tuberculosis & Thoracic Tumor Research Institute for kindly providing the M. bovis (ATCC 19210) and BCG Tokyo (ATCC 35737) strains, Mr Hang Yao for his assistance with flow cytometry techniques, and Mr Liang Zhen-Guang for use of BSL-3 facilities. The authors have no financial conflicts of interest.

REFERENCES


Edited by: G. R. Stewart