Clinical and Developmental Immunology

Immunity to *Mycobacterium tuberculosis*

Guest Editors: James A. Triccas, Nathalie Winter, Carl G. Feng, and Nicholas P. West

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Editorial

Immunity to *Mycobacterium tuberculosis*

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Tuberculosis (TB) remains a major cause of mortality and morbidity worldwide. Currently, a third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of TB, and annually there are 10 million new cases of clinical TB and approximately 2 million deaths [1]. TB kills more individuals each year than any other bacterial pathogen, and alarmingly, current control practices have not been able to significantly reduce the incidence of the disease over the past 15 years [1]. The current vaccine in use, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), has been unable to limit the transmission of the disease, and the problem is compounded by the HIV/AIDS pandemic and the emergence of multidrug resistant strains of *M. tuberculosis* including extensively drug-resistant strains in multiple countries [2]. There is thus an urgent need to better understand the host response to *M. tuberculosis* and develop more effective strategies to control TB.

This special issue contains original research reports and review articles covering a number of aspects of TB immunity. The first series of articles focuses on immune response to *M. tuberculosis* in humans. These studies range from the expression of cytolytic mediators following BCG vaccination in children (P. L. Semple et al.), control of antigen presentation function (A. Aquino et al.), changes in cellular makeup during postprimary TB (K. J. Welsh et al.), and treatment of disseminated infection in immunocompromised individuals (A. A. Alangari et al.). The reviews of S. Meraviglia et al. and H. Saiga et al. describe the role of immune effectors in mycobacterial infection, while articles describing *M. tuberculosis* strain diversity (E. Nava-Agliluera et al.), TB in myelitis (Y. Feng et al.), and humans T- and B-cell responses to immunodominant mycobacterial antigens (G. C. Macedo et al.) also form part of this special issue.

New insights on the interaction of *M. tuberculosis* with the host are also provided in this special issue. The reviews of M. Abebe et al., S. L. Sampson et al., and S. Ahmad describe pathogenic mechanisms and virulence factors expressed by *M. tuberculosis*, while the research articles of N. Sanarico et al. and E. Giacomini et al. investigate the transcriptional and cytokine response of host cells to *M. tuberculosis* infection. Reviews on TB transcriptomics (C. R. Zárate-Bladés et al.) and granuloma liquefaction (P.-J. Cardona) provide further insight into the disease process during *M. tuberculosis* infection.

The development of new vaccines is a major goal of TB research programs, and this special issue contains a number of articles investigating vaccine design and testing in animal models. G. G. Guerrero and C. Locht report on the use of recombinant antigens to boost BCG-induced immunity, while C. Wang et al. and M. Okada et al. similarly investigate prime-boost approaches to develop more effective TB vaccine regimens. The immune response following vaccination with *M. tuberculosis* lipoproteins is described in the article of C. Palma et al., and the use of cattle as a model to study TB immunity is the focus of the review article of W. R. Waters et al. The special issue closes with an overview of biosensing technologies for detection of *M. tuberculosis* by Z. Zhou et al.

In conclusion, the aspects of immunity to *M. tuberculosis* infection, host-pathogen interaction and vaccine development covered in this special issue may lead to future advances in the treatment and control of TB.

James A. Triccas
Nathalie Winter
Carl G. Feng
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Research Article

Induction of Granulysin and Perforin Cytolytic Mediator Expression in 10-Week-Old Infants Vaccinated with BCG at Birth

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Background. While vaccination at birth with *Mycobacterium bovis* Bacilli Calmette-Guérin (BCG) protects against severe childhood tuberculosis, there is no consensus as to which components of the BCG-induced immune response mediate this protection. However, granulysin and perforin, found in the granules of cytotoxic T lymphocytes and Natural Killer (NK) cells, can kill intracellular mycobacteria and are implicated in protection against *Mycobacterium tuberculosis*. *Methods*. We compared the cellular expression of granulysin and perforin cytolytic molecules in cord blood and peripheral blood from 10-week-old infants vaccinated at birth with either Japanese or Danish BCG, administered either intradermally or percutaneously. *Results*. In cord blood, only $CD56^+$ NK cells expressed granulysin and perforin constitutively. These cytolytic mediators were upregulated in $CD4^+$ and $CD8^+$ cord blood cells by *ex vivo* stimulation with BCG but not with PPD. Following BCG vaccination of neonates, both BCG and PPD induced increased expression of granulysin and perforin by $CD4^+$ and $CD8^+$ T cells. There was no difference in expression of cytolytic molecules according to vaccination route or strain. *Conclusions*. Constitutive expression of perforin and granulysin by cord blood NK-cells likely provides innate immunity, while BCG vaccination-induced expression of these cytolytic mediators may contribute towards protection of the neonate against tuberculosis.

1. Introduction

It is estimated that approximately one third of the world's population is infected with *Mycobacterium tuberculosis* (M.tb) resulting in about 1.7 million deaths from tuberculosis (TB) annually [1]. *Mycobacterium bovis* Bacille Calmette Guérin (BCG) is the most commonly used vaccine worldwide and the only vaccine available for the prevention of TB. The vaccine offers good protection against childhood miliary disease and TB meningitis [2, 3] and has been associated with a reduced risk of acute respiratory tract infections in neonates [4]. However, BCG affords very little protection against pulmonary TB and other manifestations of adult disease. It has been suggested that the strain of BCG and the route of administration may affect the efficacy of the vaccine [5–8] as well as determine the nature of the antigen-specific T-cell responses [9].

Both innate and adaptive cellular immune responses are required for an effective host defense against M.tb and individuals with defective cell-mediated immunity (CMI)

have a predisposition towards developing TB, or towards more severe disease [10]. T-cell derived interferon gamma (IFN-γ) is widely recognized as important in antimicrobial protection. This cytokine is essential for the activation of macrophages, a process required to limit the replication of the bacilli in these cells [11, 12]. However, despite having a full repertoire of helper and cytotoxic T-cells, B-cells, and dendritic cells (DC) [13], circulating neonatal lymphocytes are functionally distinct as compared with adults. Consequently, activation is submaximal [14] and IFN-γ production in response to mycobacterial antigens is significantly lower in neonates [15, 16]. However, the value of IFN-γ as the best correlate of protection against TB has been challenged [17, 18]. Lymphoid cell-derived cytotoxic molecules found in cytotoxic lymphocytes (CTL) and natural killer (NK) cells have also been implicated in contributing to protective immunity against intracellular pathogens. These cells contain granules rich in granulysin, perforin and granzyme, molecules that contribute to lysis of infected cells. Granulysin, a member of the saponin-like family of lipid binding proteins, has also been shown to directly kill extracellular bacilli and, together with perforin, was able to substantially reduce the viability of intracellular M.tb [19].

The aim of our study was to evaluate the expression of granulysin and perforin in NK cells and T-cells in cord blood mononuclear cells (CBMCs) and to compare this with peripheral blood mononuclear cells (PBMCs) from neonates after vaccination with BCG. PBMCs from healthy adults who were purified protein derivative of tuberculin (PPD) responsive *in vitro* were used as controls. In addition, we determined whether the route and strain of BCG vaccination affected the expression of these cytolytic mediators. The results show that while T-cells from CBMCs do not express granulysin or perforin constitutively, BCG vaccination at birth results in strong upregulation of these mediators in the T-cells of 10-week-old infants. NK cells from CBMCs and PBMC of vaccinated infants constitutively expressed both markers. Variation in route and strain of BCG vaccine did not have a significant effect on cytotoxic mediator expression in T-cells or NK cells of PBMC obtained from 10-week-old neonates.

2. Material and Methods

2.1. Human Subjects and Vaccination. Healthy HIV-negative pregnant women scheduled to undergo elective Ceasarian section, due to previous Ceasarian sections, were recruited for umbilical cord blood (UCB) collection. This population was selected to avoid the effects of labour on immune responses.

Healthy 10-week-old infants were enrolled at primary care clinics in the Cape Town region. At birth, the babies had received either Japanese BCG (strain 172: Japan BCG Laboratory) or Danish BCG (strain 1331: Statens Serum Institute). The Japanese vaccine was administered either intradermally (JID) or percutaneously (JPC), while the Danish vaccine was given intradermally (DID). Infants with any acute or chronic disease, born to an HIV+ mothers, or living with a person with active tuberculosis, were excluded. A positive ELISA test for HIV, performed on all infants, also resulted in exclusion.

A positive proliferative response to PPD stimulation in peripheral blood (as defined by both microscopic cell clustering and flow cytometric evaluation) was used to identify healthy adults with prior exposure to mycobacterial antigens, and potentially latently infected with M.tb. All volunteers were HIV negative and were clinically well. Human participation was according to the US Department of Health and Human Services and Good Clinical Practice guidelines. This included the protocol approval by the University of Cape Town Research Ethics Committee and by the UMDNJ Institutional Review Board and informed written consent from a parent of the neonate or from the adult volunteers.

2.2. Cord Blood Mononuclear Cells (CBMCs). After inserting a needle with collection tubing into the umbilical vein of the placenta, the blood was allowed to flow by gravity into a standard blood donation bag (Sabax, Johannesburg, South Africa) containing 2000 units of sodium heparin (Sigma- Aldrich, Steinham, Germany). In the laboratory, the UCB was diluted with equal volumes of Ca^+ and Mg^+ free phosphate buffered saline (PBS, Bio Whittaker, Walkersville, MD, USA), and mononuclear cells were isolated by density sedimentation using Ficoll-Hypaque (Sigma-Aldrich, Steinham, Germany). Due to the large number of erythroid precursor cells in UCB, the procedure was repeated [20]. After the final wash the CBMCs were suspended in RPMI (Bio Whittkaker) supplemented with 10% human AB serum (Western Province Blood Transfusion Service, South Africa).

2.3. BCG-Vaccinated Infant and PPD-Reactive Adult Peripheral Blood Mononuclear Cells (PBMCs). PBMC were separated as described for CBMCs, except that a single density gradient centrifugation was performed. PBMCs were cryopreserved until analysis: preliminary experiments showed no significant difference between cytolytic mediator expression in freshly isolated PBMCs and cryopreserved PBMCs from the same donors in both adults and 10-week-old infants (data not shown). Cell viability was determined by trypan blue exclusion dye (Sigma Aldrich).

2.4. Stimulation of CBMCs and PBMCs. 1×10^6 cells/ml were stimulated with 6 μg/ml of PPD (Statens Serum Institute, DM) or 100 IU interleukin-2 (Aldesleukin, Chiron) for 7 days at 37°C in a 5% CO_2 humidified incubator. Unstimulated cells served as a background control. The duration of lymphoid cell stimulation was validated in preliminary experiments where granulysin and perforin expression was determined ex vivo and 1, 4, 6, 7, and 8/days after PPD and BCG stimulation. Although granulysin and perforin were detected in NK cells ex vivo, little or no expression was seen in $CD4^+$ and $CD8^+$ T-cells until 4 days after stimulation and optimal expression peaked at day 7 (data not shown).

CBMCs and PBMC isolated from adults who were PPD-reactive were also stimulated with BCG (Danish BCG strain 1331, Statens Serum Institute) at a multiplicity of infection (MOI) of 1 : 1 and 5 : 1, respectively, shown to be optimal in preliminary experiments that evaluated cell death with the viability stain 7AAD. The 7-day duration of lymphoid cell stimulation was validated in preliminary experiments (data not shown).

2.5. Flow Cytometric Analysis. After stimulation with appropriate stimuli, the cells were washed with PBS containing 1% human AB serum and 0.1% sodium azide (Sigma Aldrich), resuspended in the same medium (wash buffer) and counted on a haemacytometer. Cell viability was determined by trypan blue exclusion (Sigma Aldrich). Approximately 0.1 $\times 10^6$ viable cells were stained with CD3 FITC (Caltag, Burlingame, CA) or CD3 PerCP (BD Biosciences) and either CD4 APC or CD8 APC (Caltag) or CD56 APC (BD Biosciences) for 15 minutes at room temperature. After washing, the cells were fixed and permeabilised with FACS Permeabilising Solution (BD Biosciences) then stained with either rabbit polyclonal granulysin antibody (which specifically recognises the 9 and 15-kDa forms of granulysin) [21–23] at a concentration of 1/1000 or Perforin-FITC (BD Biosciences) for 30 minutes at 4°C. For granulysin staining, $F(ab)_2$ goat antirabbit IgG (H+L)-PE conjugated secondary antibody (Caltag) was added for 30 minutes at 4°C. The cells were resuspended in the wash buffer for analysis on a FACSCalibur using *Cell Quest* software. Isotypic matched controls were used for all monoclonal antibodies while normal rabbit serum (Sigma Aldrich) served as a control for the granulysin polyclonal antibody. The flow cytometric gating strategy (shown in Figure 1(a)) was employed in order to define three distinct peripheral blood lymphocyte subsets for quantification of either granulysin or perforin expression. The lymphocyte gate was defined by forward and side scatter cell characteristics (R1). Within this population, $CD3^+$ T-cells were split into $CD3^+4^+$ or $CD3^+8^+$ subsets (i.e., cells that are in both gates R1 and R2), while NK cells were operationally defined as $CD3^-56^+$ (cells that are in both gates R1 and R3) (Figure 1).

2.6. Statistical Analysis. The primary outcomes were expression of granulysin or perforin in $CD4^+$ or $CD8^+$ T-cells, or NK cells. These outcomes were compared according to mycobacterial infection status (naïve cord blood, BCG-vaccinated infants, and healthy PPD-reactive adults) and according to antigen used in assays. The Mann-Whitney two-tailed test or Kruskal Wallis test was used to determine significance of differences, and results are stated throughout the text as medians with interquartile range. All statistical analysis was carried out using either *GraphPad Instat* (version 3.06) or *GraphPad Prism* software (version 4.0).

3. Results

3.1. Participants. Ten pregnant volunteers with a mean age of 26 years (range 19–34) consented to donating cord blood. Ten PPD-reactive healthy adults (PPD^+) with a mean age of 36 years (range 24–55) were enrolled in the study. The volunteers comprised of 5 males and 5 females. Forty five babies, vaccinated at birth with BCG, were enrolled in this study: 15 babies received DID whilst 31 babies were vaccinated with Japanese BCG: 15 with JID and 16 with JPC.

3.2. Effect of BCG Vaccination on PPD-Induced Granulysin Expression. CBMCs isolated from placental umbilical veins of 10 neonates were stimulated with PPD for 7 days. $CD4^+$ and $CD8^+$ T-cells and $CD56^+$ NK cells were analysed by flow cytometry for granulysin expression. IL-2 stimulated CBMCs acted as a positive control. Unstimulated and PPD stimulated cord blood $CD4^+$ T-cells were either negative or expressed very low levels of granulysin (Figure 2(a)). Similar results were seen in cord blood $CD8^+$ T-cells (Figure 2(b)). IL-2 stimulation of CBMCs *in vitro* induced an increase in the percentage of granulysin positive $CD4^+$ T cells of 23.5% (12.5–62, $P = .0001$) and $CD8^+$ T cells of 32.5% (10.5–55.5, $P = .0001$). In contrast to T-cells, unstimulated NK cells expressed high levels of granulysin and the percentage of positive cells was decreased following PPD stimulation although this was not statistically significant ($P = .09$, Figure 2(c)). Similarly, the level of granulysin in IL-2 stimulated $CD56^+$ cells was lower at 20.5% (11.8–32.5), compared to the unstimulated cells at 52% (34.7–59.8), and this was statistically significant ($P = .0025$, not shown).

When infants were vaccinated with BCG at birth and their PBMC evaluated for granulysin expression at 10 weeks postvaccination, a different pattern was seen. Granulysin was expressed in 2% (0.5–3.5) of unstimulated $CD4^+$ and 1% (1–2.5) of unstimulated $CD8^+$ T-cells (Figures 2(a) and 2(b)). After 7 days of *in vitro* PPD stimulation of PBMC, granulysin expression in both $CD4^+$ and $CD8^+$ T-cells increased significantly to a median of 61% (46–76, $P \leq .0001$) and 53% (9–72, $P \leq .0001$) of cells, respectively. IL-2 stimulation of PBMC induced similar strong expression with median of 34% (16.5–81, $P \leq .0001$) for $CD4^+$ T cells and 69% (27.5–87, $P \leq .0001$) for $CD8^+$T cell (not shown). Thus, in contrast to CBMCs, PBMC of vaccinated babies had significantly higher levels of granulysin expressing T lymphocytes after *in vitro* PPD stimulation. This high level of expression is comparable to that previously obtained in adults [23]. Similar to CBMCs, constitutive expression of granulysin was seen in unstimulated NK cells of PBMC from vaccinated infants.

Healthy PPD^+ adult volunteer controls were also evaluated for granulysin expression in NK^+ cells, and $CD4^+$ and $CD8^+$ T cells. Unlike both the CBMCs and PBMC from the 10-week-old infants, constitutive granulysin expression was seen in 8% (5.5–22.5) of $CD8^+$ T-cells (Figure 2). The median granulysin expression in PPD-stimulated adult PBMC was 39.5% (18–47.3) and 40.5% (28–48) for $CD4^+$ and $CD8^+$ T cells, respectively, and 57% (41.8–65.5) for NK cells. Compared to unstimulated cells, granulysin expression was significantly increased in PPD-stimulated $CD4^+$ ($P < .0001$) and $CD8^+$ ($P = .0015$) T-cells but there was no significant difference in granulysin expression between

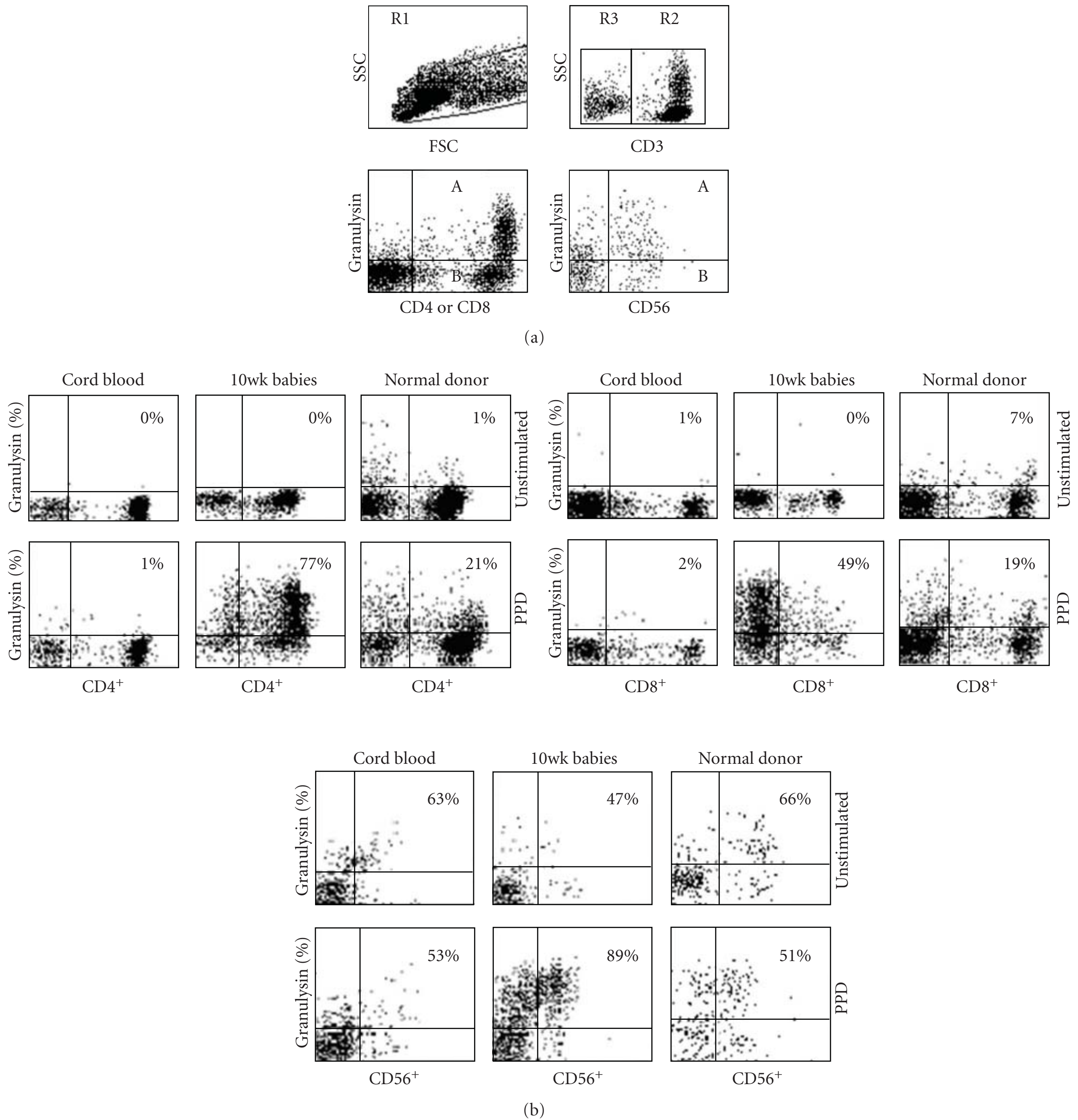

Figure 1: (a) Flow cytometric diagram illustrating the gating strategy for the expression of granulysin (or Perforin) in T-cells and NK cells. R1 represents a general lymphocyte gate including all lymphocytes in a PPD stimulated PBMC sample. R2 incorporates all $CD3^+$ T-cells within the R1 gate, and R3 includes all cells that are $CD3^-$. Percentage cells expressing granulysin (represented on the y-axis) are evaluated by dividing the number of cells in A by the number of cells in $A + B \times 100$. (b) Flow cytometric dotplots of granulysin in unstimulated or PPD-stimulated representative samples of cord blood mononuclear cells in the left column, 10-week-old infants after BCG vaccination in the middle column, and PBMC from healthy PPD^+ adult volunteers in the right column. Results shown represent T-lymphocytes which are $CD4^+/CD3^+$ or $CD8^+/CD3^+$ or NK cells which are $CD56^+/CD3^-$. See Section 2 for details.

unstimulated and PPD-stimulated NK cells ($P = .8$). However, similar to CBMCs and PBMC from vaccinated babies, constitutive granulysin expression was also seen in 54.5% (34.8–73) of unstimulated $CD56^+$ cells from healthy PPD+ adult controls.

3.3. Effect of BCG Vaccination on PPD-Induced Perforin Expression. A similar analysis was undertaken to determine levels of expression of perforin. Neither unstimulated $CD4^+$ nor $CD8^+$ T-cells from cord blood, nor unstimulated $CD4^+$ or $CD8^+$ T cells from 10-week-old vaccinated infants

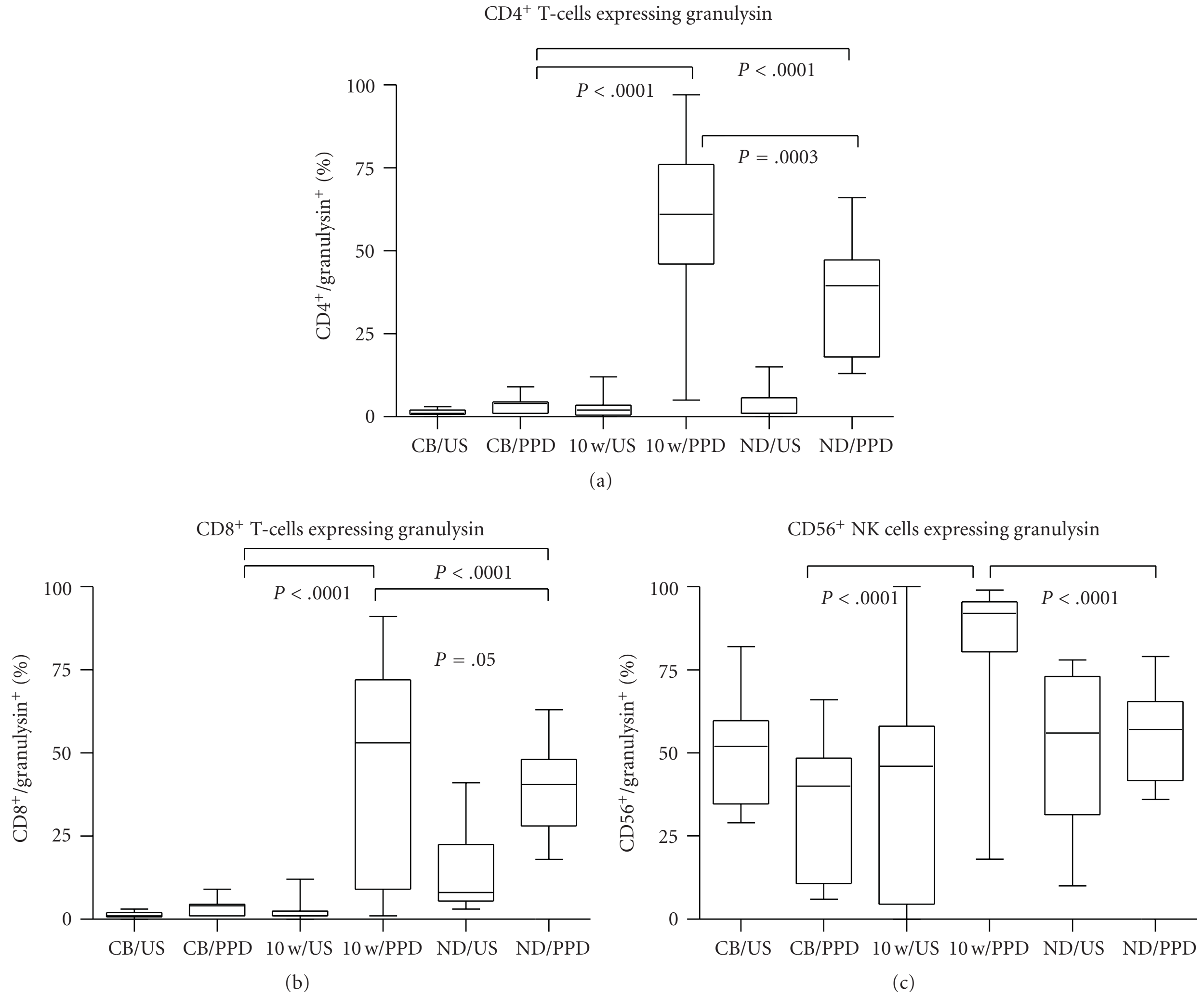

Figure 2: Granulysin expression in CD4$^+$ (a) and CD8$^+$ (b) T-cells and CD56$^+$ (c) NK cells in unstimulated (US) and PPD-stimulated mononuclear cells isolated from cord blood (CB, $n = 10$) or peripheral blood from BCG-vaccinated infants (10 w, $n = 45$) or healthy PPD$^+$ adult volunteers (ND, $n = 10$). Significance is shown as PPD stimulation in CB compared to 10-week-old infants and healthy adult controls, and 10-week-old infants compared to healthy adult controls. The box extends from the 25th to 75th percentile, the line represents the median, and the whiskers represent the maximum and minimum values.

expressed perforin (Figures 3(a) and 3(b)). In contrast, 38% (17–61.8) of NK-cells in CBMCs and 6% (0–48.3) of NK cells in 10-week-old infant PBMC constitutively expressed the cytolytic marker (Figure 3(c)). Again, PPD stimulation did not induce expression of perforin in CD4$^+$ or CD8$^+$ T-cells from cord blood (Figures 3(a) and 3(b)) while a reduction in the levels of expression in CD56$^+$ NK cells to 16.5% (8–33.2, $P = .02$) as compared to unstimulated CBMCs was noted. In contrast, PPD stimulation induced significantly increased expression of perforin in PBMC of vaccinated infants both in CD4$^+$ T-cells ($P = .04$) and CD8$^+$ T-cells ($P = .009$) (27% (13.8–44.3) and 38.5% (19.5–52.8), resp.) as well as in NK cells ($P < .0001$) (87.5% (66–95)). Compared to CBMCs, there was a significant difference in perforin expression after PPD stimulation in the 10-week-old vaccinated infants in CD4 and CD8 T-cells (P, 0.0001 for both T-cells, Figures 2(a) and 2(b)) and in NK cells ($P = .0003$, Figure 3(c)). After IL-2 stimulation, 18% (14–28.5, $P = .0015$) of CD4$^+$ T-cells and 36% (22–40, $P = .002$) of CD8$^+$ T-cells expressed perforin (not shown).

In PPD$^+$ adults, constitutive expression of perforin was seen in 3.5% (2–11.8) of CD8$^+$ T-cells and 76.5% (57.5–85.8) of NK cells; after PPD stimulation, 14.5% (8.8–16.8) of CD4$^+$ T-cells and 21% (17.3–25.3) of CD8$^+$ T-cells expressed perforin. PPD stimulation of NK cells resulted in a reduction of perforin expression (from 76.5% to 60%).

3.4. Effect of BCG Stimulation on Granulysin and Perforin Expression in CD4$^+$ and CD8$^+$ T Cells of CBMCs and in PBMC of PPD$^+$ Healthy Adults. PBMC from healthy PPD$^+$ adults and CBMCs were stimulated with Danish BCG, and granulysin and perforin expression was determined within

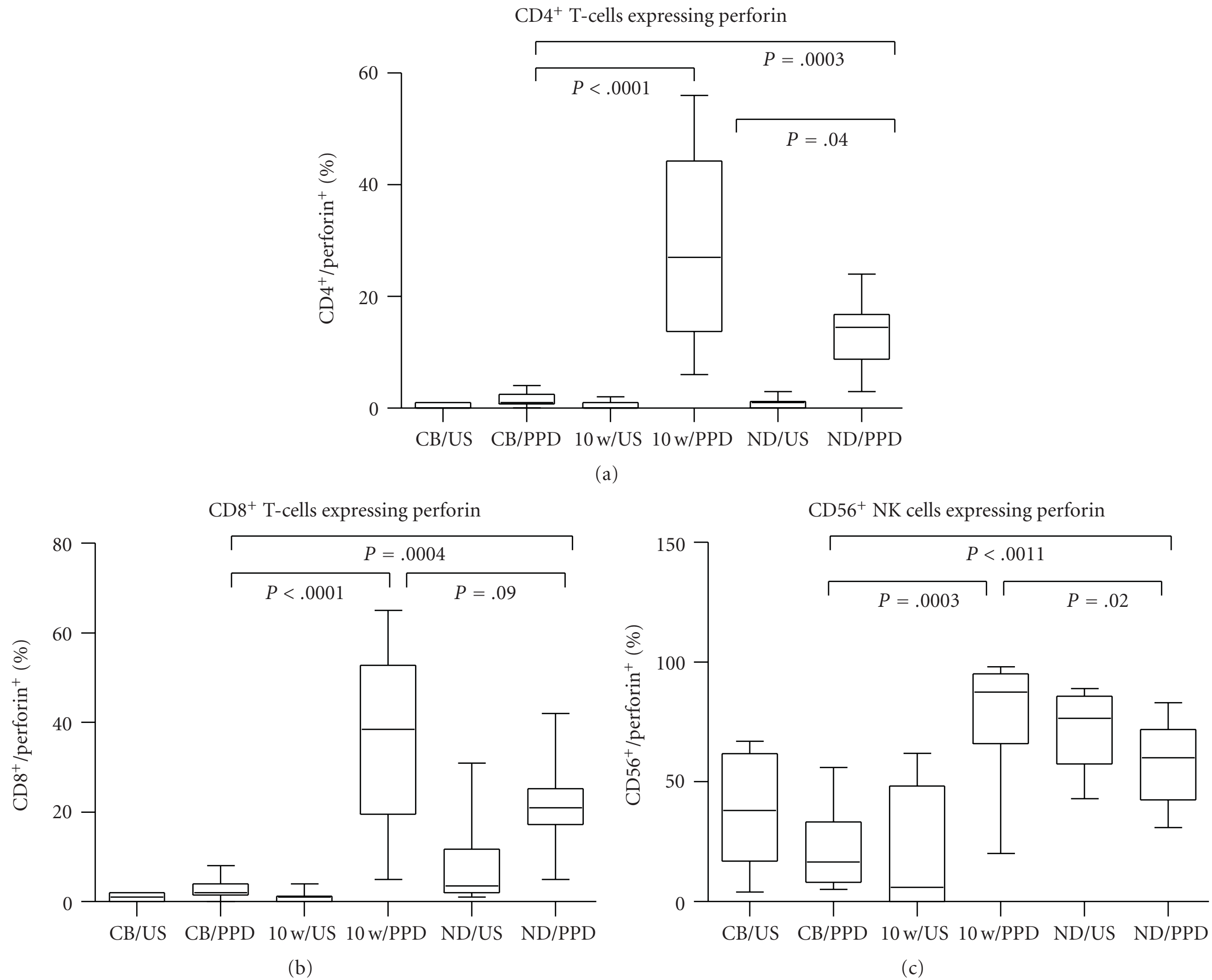

Figure 3: Perforin expression in $CD4^+$ (a) and $CD8^+$ (b) T-cells and $CD56^+$ (c) NK cells in unstimulated (US) and PPD-stimulated mononuclear cells isolated from cord blood (CB, $n = 10$) or peripheral blood from BCG-vaccinated infants (10 w, $n = 45$) or healthy PPD^+ adult volunteers (ND, $n = 10$). Significance is shown as PPD stimulation in CB compared to 10-week-old infants and healthy adult controls, and 10-week-old compared to healthy adult controls. The box extends from the 25th to 75th percentile, the line represents the median, and the whiskers represent the maximum and minimum values.

the respective $CD3^+$ cells. Unlike PPD, BCG stimulation did result in an increase in both granulysin and perforin expressions in CBMCs. There was a statistically significant difference in granulysin expression in BCG-stimulated T-cells compared to PPD-stimulated T-cells ($P \leq 0.0001$ for $CD4^+$ and $P = .0005$ for $CD8^+$, Figures 4(a) and 4(c)) in CBMCs. Similarly, perforin-expressing T-cells were also increased in BCG-stimulated compared to PPD-stimulated CBMCs ($P = .003$ for $CD4^+$ and $P = .004$ for $CD8^+$, Figures 4(b) and 4(d)). 10.5% (4–25) of $CD4^+$ T-cells and 11% (6–27) of $CD8^+$ T-cells expressed granulysin after BCG stimulation (Figure 4), and perforin expression was seen in 5.5% (2.5–17) and 8% (3.8–19) of $CD4^+$ and $CD8^+$ T-cells, respectively, after BCG stimulation. Thus, although the percentage of expression was considerably lower than in healthy PPD positive adults, significant expression was induced in both lymphocyte subsets compared to PPD stimulation. In contrast, in PPD^+ healthy adult T-cells, equally strong granulysin and perforin expression was obtained with no significant difference between PPD and BCG stimulation being noted (Figures 4(a)–4(d)).

3.5. Effect of Route and Strain of the BCG Vaccine on Granulysin and Perforin in T-Cells and NK Cells of PBMCs from 10-Week-Old Infants. To evaluate whether the strain of BCG or the route of administration of the vaccine affected the extent of cytotoxic molecule expression, PBMC from 10-week-old infants vaccinated with either Danish BCG administered intradermally ($n = 14$ granulysin and $n = 3$ for perforin) or Japanese BCG administered either intradermally ($n = 15$ for granulysin and $n = 4$ for perforin) or percutaneously ($n = 16$ granulysin, and $n = 7$ for perforin) were evaluated for granulysin and perforin expression. Although the number of patients evaluated for perforin expression was low, no significant differences were found in granulysin or perforin expression between vaccination with Japanese or Danish

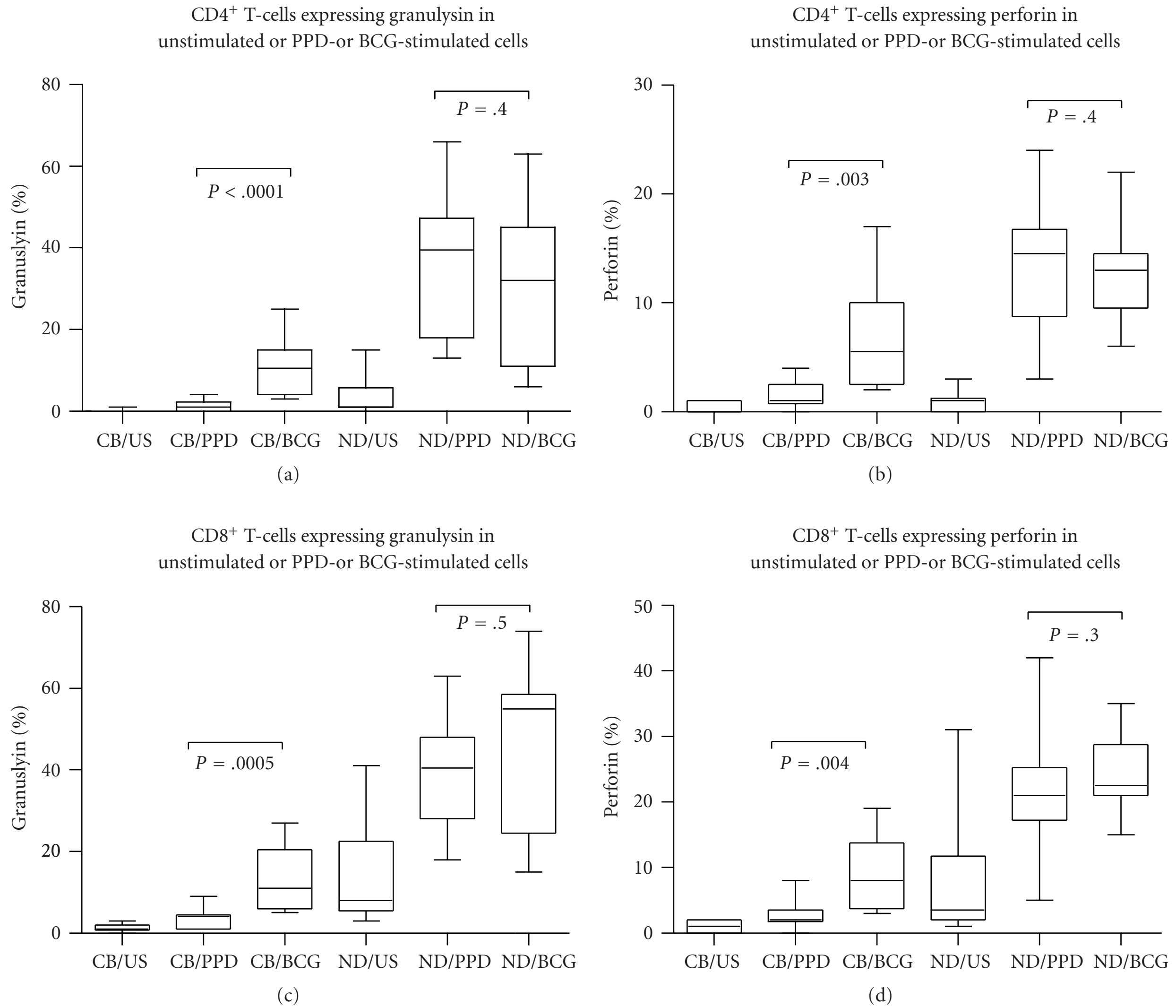

Figure 4: Percentage $CD4^+3^+$, $CD8^+3^+$ expressing Granulysin (a, c), or Perforin (b, d) in unstimulated (US) or PPD- or BCG-stimulated cord blood mononuclear cells (CB $n = 10$) or peripheral blood mononuclear cells from healthy PPD^+ adult volunteers (ND $n = 10$). Significance is shown between PPD and BCG stimulation in CB and adult volunteers. The box extends from the 25th to 75th percentile, the line represents the median, and the whiskers represent the maximum and minimum values.

BCG. Similarly, the route of administration of the vaccine also did not have any effect on expression of these markers (data not shown).

4. Discussion

In the current study, we have evaluated expression of the cytolytic mediators perforin and granulysin in both $CD56^+$ NK cells and in $CD4^+$ and $CD8^+$ T-cell subsets in CBMCs and observed that (i) intrinsic expression of cytolytic mediators was limited to $CD56^+$ cells and not found in $CD4^+$ and $CD8^+$ T-cells and (ii) induction of perforin and granulysin expression in cord blood T-cells occurred selectively in response to *in vitro* stimulation with BCG, but not PPD. In contrast, in 10-week-old BCG-vaccinated infants, upregulation of perforin and granulysin expression in $CD4^+$ and $CD8^+$ T-cell was demonstrable in response to both BCG and PPD stimulation (data not shown). In this study, the response was independent of variation in vaccine strain or route of vaccine delivery (data not shown), although the sample size was small and not statistically powered. To our knowledge, a comprehensive comparative evaluation of NK and $CD4^+$ and $CD8^+$ T subset cytolytic mediator expression in cord blood and in BCG vaccinated infants has not been documented before. Previous studies have reported that cord blood $CD4^+$ lymphocytes lack constitutive expression of perforin [24] while most $CD8^+$ T-cells of newborns have been shown to contain perforin and granzyme. In contrast, we observed that unstimulated cord blood $CD8^+$ T-cells did not express granulysin or perforin. This difference may be explained by the fact that in our

study the CBMCs were from mothers undergoing caesarean section. During normal vaginal delivery, cytokines including IL-15 may be induced which could activate $CD8^+$ T cells to acquire cytotoxic potential [25].

Considerable evidence supports the observation that cytotoxic T-cell activation occurs during induction of the host protective immune response to TB [26–28]. We have previously documented $CD4^+$ cytotoxic activity in BCG-vaccinated neonates using a chromium release assay [29]. It has previously been shown that the level of expression of cytotoxic mediators is directly related to functional cytotoxic activity of memory $CD8^+$ T-cells [30]. Using this approach, we have reported on $CD8^+$ cytotoxic molecule expression in infants who had received BCG vaccination at birth [31]. Here we demonstrate that cytotoxic cells expressing granulysin and perforin constitutively exist in neonatal blood even before BCG vaccination. Perforin and granulysin are important cytolytic effector molecules involved in lysis of infected macrophages. Granulysin, found in the granules of cytotoxic T-lymphocytes and NK cells, has a broad spectrum of antimicrobial activity [19]. The molecule has been shown to kill extracellular *M. tuberculosis* and, together with perforin, is bactericidal to intracellular organisms [19]. In addition, granulysin and perforin were significantly increased in lymphoid cells of cattle following vaccination with *M. bovis* BCG and *M. bovis* ΔRD1 compared with nonvaccinated animals [32].

IFN-γ production by CBMC NK cells is most needed after birth [33, 34], at a time when $CD4^+$ CD45RO- T-cells have down-regulated Th1 function by transcriptional regulation of the IFN-γ promoter gene due to hyper-methylation [35]. Intrinsic cytolytic mediator expression provides a second mechanism for NK cell mediated protective immunity during this critical period of heightened susceptibility to mycobacterial infection. Our observation that BCG upregulates cytotoxic molecules in cord blood $CD4^+$ and $CD8^+$ T-cells may also be secondary to activation of cells of the innate immune system. Since IL-2 treatment of the T-cells of cord blood directly upregulated perforin and granulysin expression, the effect of BCG could be via activation of NK cells to produce IL-2 which in turn could affect $CD4^+$ and $CD8^+$ T-cell activation. Support for a central role of NK cells in the host response against TB infection also comes from the bovine model where activated NK cells were shown to have increased granulysin and perforin expression and to lyse *Mycobacterium bovis* BCG-infected alveolar and monocyte-derived macrophages [36].

The role of IFN-γ producing $CD4^+$ T-cells in protection against TB is well described [37–39]. Support for a role for cytotoxic $CD4^+$ effectors in protective immunity against TB comes from studies carried out in BCG-vaccinated cattle. In the bovine model, memory $CD4^+$ T-cells expressing elevated levels of perforin and granulysin strongly lysed BCG-infected macrophages [40]. Cytotoxic $CD4^+$ T-cells have also been shown to use granulysin to kill Cryptococcus neoformans [41]. Furthermore, cytotoxic granulysin-expressing $CD4^+$ T-cells have been isolated from skin lesions of tuberculoid leprosy patients [42]. Recently, Murray et al. showed that BCG vaccination induced specific $CD8^+$ T-cells which produced IFN-γ and had increased expression of cytotoxic proteins in response to BCG stimulation *in vitro,* providing evidence of a role for $CD8^+$ T-cells as well [31]. This is supported by the finding of reduced perforin and granulysin coexpression in $CD8^+$ T-cells found at the site of infection in chronic TB [43].

In the present study, we observed that there was no statistical difference in granulysin or perforin expression in cells obtained from neonates vaccinated at birth with either JID or DID and no difference between vaccinations via the two routes (intradermal versus percutaneous) of administration of the Japanese BCG. Differences have been reported in a study using a similar but larger cohort of infants, where JPC induced significantly more BCG-specific IFN-γ producing $CD4^+$ and $CD8^+$ T-cells and greater Th1-specific immunity than JID or DID [9]. In addition, JPC induced greater $CD4^+$ and $CD8^+$ T-cell proliferation. Taken together, the data suggest that distinct factors are involved in promoting the development of the two pathways, that is, Th1 cytokine and T-cell proliferation and expansion of cytotoxic cells expressing granulysin and perforin. In support of this dichotomy, it has been shown that BCG-infected immature DCs selectively expanded perforin-positive $CD8^+$ T-cells with little contribution from cytokines, including IFN-γ, TNF-α, or IL-12 [44]. In contrast, IL-15 promotes granulysin expression [36, 45] while IL-21 has been shown to enhance lytic activity of cytotoxic T-cells and NK cells [46]. These findings may have implications for immunotherapeutic boosting of cytotoxic $CD8^+$ activity in TB and BCG vaccination protocols. In addition to therapeutic strategies aimed at inhibition of IL-4 and TGFβ [47], molecular engineering of BCG to incorporate IL-15 or IL-21 could result in enhanced cytotoxic activity of both NK cells and $CD8^+$ cytotoxic T-cells.

In conclusion, data presented here demonstrate (i) constitutive expression of granulysin and perforin cytolytic mediators in NK cells which could provide innate protective immunity for newborn infants and (ii) that BCG vaccination at birth can induce a cytolytic effector function in neonates, where $CD4^+$ and $CD8^+$ T-cells are all essentially naïve. By contrast, PPD antigenic stimulation could only induce cytolytic effector functions in memory $CD4^+$ and $CD8^+$ T-cells. While no currently available immunologic test can predict vaccine efficacy, BCG readily elicits a type 1 cytokine response [29, 48] and it has been proposed that additional factors may be important for vaccine efficacy. These include IL-4 and the Th1 cytokine balance [49, 50] and induction of cytolytic activity in memory T-cells [40, 51]. Further research is needed to establish if BCG-induced cytolytic mediator expression is sustained in memory CTL. It has been postulated that protection due to BCG vaccination wanes with time [52] which may be due to overattenuation of BCG strains with resultant gene deletions [53] and failure to stimulate a durable immune response.

Conflict of Interests

A. M. Krensky holds patents on granulysin as follows: US6485928 Use of granulysin as an antimicrobial agent, US11438563 Antimicrobial peptides, and US4994369 T cell

activation related gene (granulysin). P. L. Semple, M. L. V. Watkins, V. Davids, W. A. Hanekom, G. Kaplan, and S. R. Ress have no conflict of interests.

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Review Article

Exogenous Control of the Expression of Group I CD1 Molecules Competent for Presentation of Microbial Nonpeptide Antigens to Human T Lymphocytes

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Group I CD1 (CD1a, CD1b, and CD1c) glycoproteins expressed on immature and mature dendritic cells present nonpeptide antigens (i.e., lipid or glycolipid molecules mainly of microbial origin) to T cells. Cytotoxic CD1-restricted T lymphocytes recognizing mycobacterial lipid antigens were found in tuberculosis patients. However, thanks to a complex interplay between *mycobacteria* and CD1 system, *M. tuberculosis* possesses a successful tactic based, at least in part, on CD1 downregulation to evade CD1-dependent immunity. On the ground of these findings, it is reasonable to hypothesize that modulation of CD1 protein expression by chemical, biological, or infectious agents could influence host's immune reactivity against *M. tuberculosis*-associated lipids, possibly affecting antitubercular resistance. This scenario prompted us to perform a detailed analysis of the literature concerning the effect of external agents on Group I CD1 expression in order to obtain valuable information on the possible strategies to be adopted for driving properly CD1-dependent immune functions in human pathology and in particular, in human tuberculosis.

1. Introduction

Cell-mediated immunity involved in host resistance against *mycobacteria* and other infectious agents appears to rely to a large extent on classical HLA-restricted responses against microbial peptides [1] mediated mainly by interferon (IFN) γ-producing T-cells [2]. However, in recent years growing attention has been given to T-cell-mediated responses directed against lipid or glycolipid antigens presented by four relatively nonpolymorphic CD1molecules ([3–5], reviewed in [6]).

Two groups of CD1 isoforms expressed on the cell membrane of various antigen-presenting cells (APCs) have been identified in the course of the last 20 years. In particular, Group I (i.e., CD1a, CD1b, CD1c) and the isoform CD1e, that is confined to the intracellular compartment and is classified as Group III by some authors, are detectable in man but not in mice. On the contrary, Group II (i.e., CD1d, a biological entity outside the scope of the present review) is expressed in mice and men as well, and is involved in Invariant Natural Killer T-cell responses (specifically reviewed in [7]). The molecular structure of CD1 is similar to that of MHC class I. Both CD1 and MHC class I are comprised of heavy chains of similar length, which are organized into three extracellular domains ($\alpha1$, $\alpha2$, and $\alpha3$) and bind $\beta2$ microglobulin.

Group I CD1 molecules are expressed most prominently on APCs of the myeloid lineage, including dendritic cells (DCs) derived from circulating monocytes (MOs). Peripheral blood $CD1^-/CD14^+$ MOs can be activated by granulocyte-macrophage colony stimulating factor (GM-CSF) alone or more efficiently in combination with interleukin-4 (IL-4) (i.e., GM-CSF + IL-4, hereafter referred to as G4) to express Group I CD1 glycoproteins [9, 10]. These molecules are the products of the *CD1A*, *-B*, and *-C* genes and are known to be involved in the presentation of nonpeptide microbial antigens to T-cells [6, 10–12]. In particular, Beckman et al. in 1994 [13] discovered that the CD1b-presented antigens obtained from *Mycobacterium*

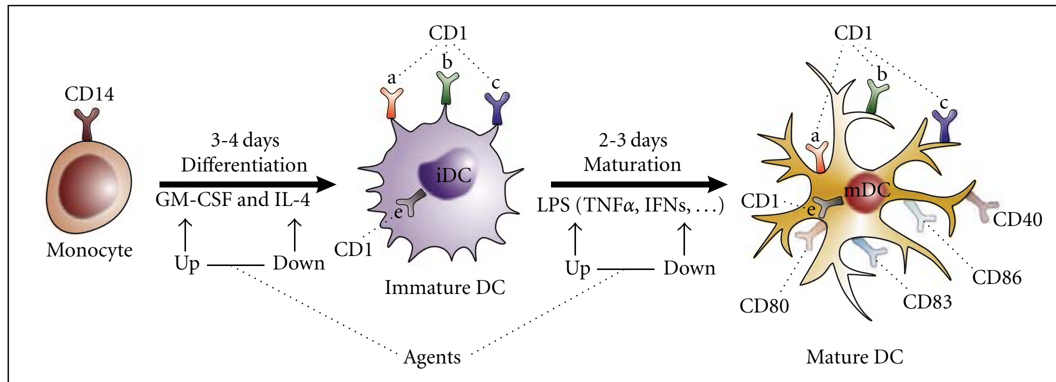

Figure 1: *Dendritic Cell (DC) generation and maturation.* Schematic drawing depicting the differentiation of monocytes to immature DC (iDC), generation of mature DC (mDC) and cytokines involved in these processes. Dotted lines point to the modulating effects of external agents.

tuberculosis were mycolic acids, that is, lipids associated with microbial cell wall. Later, it was demonstrated that CD1 molecules are competent for presentation of a great variety of microbial antigenic lipid structures to T-cells, so that CD1 could be tentatively considered a wide spectrum system of anti-infectious immune surveillance [6].

Particular attention of the present review is dedicated to the studies concerning the CD1 system predominantly engaged in antitubercular responses, and therefore involved in mycobacterial lipid presentation to CD1-restricted T-cells. A fraction of responder T-cells comes from the $CD4^-/CD8^-$ phenotypic subset of $CD3^+$ T-cell receptor (TCR) α/β T-cells. These cells, sometimes referred to as double-negative TCR α/β T lymphocytes [14], proliferate and generate cytotoxic clones following interaction with mycobacterial glycolipids, presented by $CD1b^+$ DCs-derived from G4-preactivated MOs. However, CD1-restricted $CD8^+$ or $CD4^+$TCR α/β T-cell clones [15, 16] and TCR γ/δ T-cells [3, 17] have also been demonstrated. Thus, responder cells that potentially play a role in CD1-restricted responses to nonpeptide antigens, have been found to belong to all of the major phenotypic subsets of T-cells. Noteworthy is the general observation that CD1-restricted recognition of bacteria-associated lipids results in killing of the infected cells as well as of the microorganism, thus providing presumably a way to prevent infection spreading in the host [15, 18].

The induction of effector T-cells against microbial antigens is accompanied by the presence of autoreactive CD1-restricted T-cells directed against self-lipid antigens [19]. These lymphocytes appear to cooperate in early suppression of invading microorganisms, in the induction of CD1-restricted memory T-cells and in the maturation of DCs able to produce substantial amounts of IL-12. In turn, IL-12 stimulates T-cells to produce IFNγ (reviewed in [20]) and plays an important role in antitubercular immunity [21]. Autoreactive CD1-restricted T-cells have also been accused to take part in the immune mechanisms underlying multiple sclerosis (MS) and Guillan-Barre syndrome [22, 23]. However, detection of autoreactive cytotoxic T lymphocytes in patients affected by autoimmune disease, does not necessarily mean that these cells play a role in the pathological events affecting target organs.

Up to now, it has not been definitely established whether tuberculosis prevention could be achieved through vaccinial procedures based on *M. tuberculosis*-associated lipids as sensitizing agents. Improvement in the course of the disease has been noted in guinea pigs sensitized with lipid extracts of *M. tuberculosis* [24, 25]. Moreover, a recent study published by Felio et al. [26] showed that human Group I CD1 transgenic mice are competent for mounting a CD1-restricted adaptive immune responses to *mycobacteria*, thus allowing further preclinical investigations on lipid-based antitubercular vaccines in mouse models.

In view of a potential role of Group I CD1 glycoprotein-dependent presentation of mycobacterial lipids to T-cells, it is reasonable to hypothesize that pharmacological or biological agents able to modulate CD1 expression could modify host's responses against infectious diseases, including infections caused by *M. tuberculosis.* Therefore, the aim of the present short survey is to illustrate the data presently available in the literature, relative to the influence that can be exerted by external agents on Group I CD1 molecule expression. In particular, the reported studies will consider human MOs driven *in vitro* or *in vivo* to differentiate into immature and thereafter mature DCs (Figures 1 and 2) competent for peptide or nonpeptide molecule presentation to T-cells.

2. In Vitro and In Vivo Assays of CD1 Induction

A classical experimental design to explore the functional pathways involved in the differentiation and maturation of human myeloid DCs *in vitro* system, starting from purified $CD14^+$ MOs obtained from peripheral blood mononuclear cells (PBMNC), can be described as follows (Figure 2):

Step 1. In vitro cultivation of MOs with G4 for 3–6 days (or, in some cases, for up to 7 days). This treatment is able to induce "immature DCs" (iDCs) showing high expression of CD1a, CD1b, and CD1c glycoproteins on cell membrane, competent for lipid antigen presentation to CD1-restricted T-cells.

Experiment design (ED) codes

n	Type of study	Code	Exposure to external agents (EA)	CD1 assay
1	*in vitro*	ED-1	Step 1 only (initial differentiation pathway)	on iDCs (day 3–6)
2	*in vitro*	ED-2	Step 2 only (iDCs maturation to mDCs)	on mDCs (day 7–10)
3	*in vitro*	ED-3	Step 1 only	on mDCs (day 7–10)
4	*in vitro*	ED-4	Step 1 + step 2	on mDCs (day 7–10)
5	*in vivo*	IvDC	EA *in vivo*	on tissue iDC or mDC
6	*in vitro* or *in vivo*	OED	Other experimental design (to be detailed in the "observations" section of the tables)	on iDC or mDC

MO, monocyte; iDC, immature dendritic cell; mDC, mature dendritic cell; CDI assay, evaluation of group I CDI glycoproteins; IvDC *in vivo* studies on DC induction

GM-CSF + IL-4
LPS (TNF, IFNs)
MO ($CD14^+$)
iDC ($CD1^+$)
mDC ($CD1^+$, $CD83^+$)
STEP 1
STEP 2
CD1 assay
CD1 assay
EA
EA
EA

Figure 2: Effect of external agents on group I CD1 glycoprotein expression.

Step 2. In vitro culture of iDCs with lipopolysaccharide (LPS) and/or various cytokines (e.g., TNFα, IFNα, TGFβ, etc.) for additional 2-3 days, leading to mature $CD83^+$ DCs (mDCs), fully competent to behave as classical APCs.

In a large number of studies published in more than 15 years, iDCs have been also generated from cord blood $CD34^+$ cells cultured *in vitro* with a cocktail of cytokines containing GM-CSF. In addition, several investigations have been conducted *in vivo* by evaluating the number of DCs in various organs, in different clinical and treatment conditions using immunohistochemical detection of mainly $CD1a^+$ cells.

All these methods, able to explore the functional pathways leading to mDCs, allowed to test the effect of a number of exogenous agents on the expression of Group I CD1 molecules induced in host's cell population involved in resistance against pathogens, including *mycobacteria*.

In order to offer a concise picture on the external control of CD1 expression, the present review provides information on the complex relationship between *mycobacteria* and CD1 levels, and four tables summarize schematically what we presently know on the regulation of CD1 expression by pharmacological and biological agents. Moreover, with the intent to provide a simplified information on the experimental strategy utilized for studying the influence exerted by exogenous agents on CD1 expression during myeloid DCs induction and maturation, we decided to adopt the codes that are illustrated in Figure 2.

3. CD1 Expression

It is generally agreed that transcriptional control of gene expression and posttranscriptional regulation of mRNA function are usually under the control of proteins targeting specific DNA sequences (i.e., transcription factors) and microRNAs, respectively. In particular, expression of Group I *CD1* genes is under the control of transcription factors, that have been described in detail for CD1a glycoprotein by Colmone et al. [27]. A minimal 1000-bp region upstream of the translation start site has been identified as necessary for proximal promoter activity required for *CD1A* transcription. This region contains multiple sites that were considered to be coordinatively involved in *CD1A* gene expression on the basis of a series of experiments performed by means of deletion and site-specific mutant analysis. In particular, a critical role appeared to be played by a potential cAMP response element (CRE), 965 bp upstream of the *CD1A* translation start site. It was found that the CRE-binding protein 1 (CREB-1) and the activating transcription factors-2 (ATF-2) that are enlisted among the ATF/CREB family members, are able to bind this site *in vitro* and *in vivo* in various cell types, including human MOs [27]. Moreover, the results of these studies speak in favour of ATF-2-induced inhibition counterbalanced by a stimulatory activity on gene transcription by CREB-1, possibly through a competition of CREB-1 and ATF-2 for CRE binding. The hypothesis of opposite control performed by two transcription factors acting on the same gene promoter appears to be supported by the studies published by Niwano et al. [28] who proposed a similar mechanism for endothelial nitric oxide synthase.

In the present survey of the literature, we noticed the emerging role played by miRNAs on hematopoiesis (reviewed in [29]). Therefore, we have considered the possibility that miRNAs could affect *CD1* expression. An *in silico* analysis was performed using the miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/) algorithms for miRNA target prediction. Under miRanda

analysis, miRNA list indicates conserved miRNAs with good mirSVR scores [8]. As illustrated in Table 1, this analysis revealed that mRNAs transcribed from all three Group I *CD1* genes can be targeted and potentially regulated at the 3′UTR region by a number of different miRNAs. In particular, 10 miRNAs have been found to share a potential capability of controlling the transcriptional activity of two CD1 genes. Six miRNAs (i.e., 33a, 33b, 421, 495, 590-3p, and 590-5p) could target both CD1a and CD1c, whereas miRNA-224 could be active on CD1a and CD1b, and 3 miRNAs (i.e., 129-5p, 185 and 203) appear to be theoretically competent to target CD1b and CD1c. However, up to now no study able to validate the *in silico* prediction patterns is available from the literature. Nevertheless, a number of miR genes have been found to be involved in the regulation of immune responses [30, 31] and acute inflammation [32]. Moreover, quite recently Kuipers et al. [33] described that microRNAs control maturation, function, and maintenance of DCs in the epidermis (i.e., Langerhans cells, LC) *in vivo*. In addition, exchange of genetic material between prokaryotic and eukaryotic multicellular organisms has been described [34]. Therefore, since pathogenic microorganisms, including *mycobacteria* contain a large amount of small noncoding RNA [35, 36], it is reasonable to hypothesize that invading microbes could control gene expression of host eukaryotic cell through their miRNA-like molecules to acquire a survival advantage.

Table 1: miRNAs with putative binding sites in the 3′UTR of *CD1A*, *CD1B*, and *CD1C* genes.

Gene	Algorithm	
	miRanda[a]	Target Scan[b]
CD1A	19a, 21, 28-5p, 31, 33a, 33b, 146a, 146b-5p, 214, 217, 361-5p, 383, 421, 448, 495, 590-3p, 590-5p, 708, 873	**21**, 28-5p, **31**, **33a**, **33b**, 125a-3p, **138**, **146a**, **146b-5p**, 197, **205**, 224, 421, 448, 495, **590-5p**, 708
CD1B	129-5p, 137, 185, 203, 224, 543	**129-5p**, **137**, **203**, 543
CD1C	26a, 26b, 33a, 124, 125a-5p, 125b, 129-5p, 181a, 181b, 181c, 181d, 190, 190b, 203, 216a, 216b, 218, 219-5p, 300, 326, 330-5p, 340, 367, 376a, 376b, 381, 410, 421, 433, 455-5p, 494, 495, 505, 506, 539, 543, 590-3p, 1297	**22**, **26a**, **26b**, **33a**, **33b**, **124**, **125a-5p**, **125b**, **129-5p**, **132**, 185, **190b**, **203**, **212**, **216a**, **216b**, **218**, **219-5p**, **221**, **222**, 300, 326, 330-5p, 376a, 376b, 410, 495, **425**, 433, **455-5p**, **489**, 494, 505, **506**, 539, 542-3p, 543, 590-5p, 599, **1297**

[a]Conserved miRNAs with good mirSVR scores [8].
[b]miRNAs broadly conserved among vertebrates (bold) or conserved only among mammals.

4. Mycobacteria and CD1 Expression

Anti-tubercular immunity relies on humoral and cell-mediated immune responses against *M. tuberculosis*-associated epitopes of various origin, and possibly includes CD1-presented lipid antigens recognized by dedicated T-cell subpopulations [37]. More than eighty years ago, attenuated strains of *M. bovis* (i.e., Bacillus Calmette-Guerin, BCG) were developed and utilized as antitubercular vaccine, since they share a variety of antigenic molecules with virulent pathogenic bacilli [38]. Although BCG vaccine reduces the risk of severe forms of tuberculosis in early childhood, unfortunately it is not very effective in preventing the pulmonary infection in adolescents and adults, the populations with the highest rates of tuberculosis disease. Moreover, *M. tuberculosis* is changing and evolving, making the development of new vaccines [39] more crucial to control the disease that is continuously expanding, favored, at least in part, by AIDS pandemia.

In the last years, a considerable amount of experimental studies has been dedicated to investigate the complex relationship between the infection with virulent *M. tuberculosis* or BCG and functional activity of the CD1 system. A number of studies confirm that lipid antigens recognized and presented by Group I CD1 glycoproteins include fatty acids isolated from *M. tuberculosis* cell wall [40]. Among others, they comprise the fatty-acid-derived mycolic acid, the lipopeptide didehydroxymycobactin [41], the isoprenoid-like structure mannosyl phosphomycoketide [42], and the acylated sulfoglycolipid Ac2SGL [43].

In this context, CD1b appears to play a particularly important role, since CD1b-restricted T lymphocytes recognize a large variety of mycobacterial lipids [44], including *M. tuberculosis* Ac2SGL antigens [45]. Moreover, CD1b groove is much larger than that associated with the other CD1 isoforms, so that it can adjust long chain foreign lipids, including long mycobacterial mycolates that are not presented by the other CD1 molecules [46]. On the basis of all these findings and taking into account additional information from the literature (reviewed in [6, 46]), it is reasonable to consider Group I CD1 as a relevant part of the complex antigen-presenting systems involved in the T-cell-dependent immune response machinery against *mycobacteria*. Actually, in human leprosy lesions CD1 expression correlates with host immunity as manifested by active cellular immunity to *M. leprae* [47]. A number of clinical and experimental data indicate that long-lived immunity to *M. tuberculosis* relies largely on antigen-specific $CD4^+$ and $CD8^+$ T-cells that could play consistent roles in vaccination strategies [48]. Therefore it is reasonable to hypothesize that CD1-restricted effector T lymphocytes, that show a limited repertoire but are able to recognize large amounts of lipid antigens based on antigenic cross-reactivity [49], would contribute to antitubercular immunity. Ulrichs et al. [50] collected PBMNC from patients with pulmonary tuberculosis, from asymptomatic individuals with known contact with *M. tuberculosis* documented by conversion of their tuberculin skin tests, and from healthy tuberculin skin test negative subjects. *In vitro*, in presence of autologous $CD1^+$ iDCs, the extent of CD1-restricted T-cell responses to a lipid extract of *M. tuberculosis* was tested by means

of proliferation and IFNγ production by effector T-cells. The results showed that T-cells from asymptomatic *M. tuberculosis*-infected donors were significantly more responsive than those obtained from uninfected healthy donors. Moreover, essentially no CD1-restricted T-cell response was detectable in lymphocytes collected from patients with active tuberculosis prior to chemotherapy. However, significant antilipid immune reactivity became detectable in blood samples drawn two weeks after the start of treatment, as a possible consequence of chemotherapy-induced relief of the inhibitory effect exerted by *mycobacteria* on cell-mediated immunity [51].

In order to better define the possible role that can be played by CD1-dependent antimycobacterial immunity, it is important to identify the target of CD1-restricted effector T-cells and the modality of target suppression. Of note are the findings illustrated by Vincent et al. [52] who used CD1-restricted human α/β T-cells generated by autologous DCs in presence of microbial detergent extracts from *M. tuberculosis*, *E. coli*, or *Y. enterocolitica*. Effector T-cells were found to be active in terms of proliferation and IFNγ release when tested against target cells presenting microbial lipid antigens via CD1a, CD1b, or CD1c molecules. However, similar activity, although to a lower extent, was detected in absence of foreign lipids, thus indicating that sensitized lymphocytes were also endowed with effector function against self-lipids. The authors propose that CD1-restricted T lymphocytes fit in two T-cell populations, that is, naive T lymphocytes able to mount an adaptive response to microbial lipids as well as memory/effector T-cells. The latter population, characterized by reactivity against self and foreign lipids, would be particularly dedicated to rapid initial immune responses against invading pathogens and yet able to undergo clonal expansion responsible for long-standing cellular memory to foreign lipid antigens. Actually, Nguyen et al. [53] have recently reported that upon experimental vaccination of cattle, CD1b-restricted memory T-cell response can be elicited by the mycobacterial glycolipid glucose monomycolate.

The effector function of T lymphocytes against microbial targets, including *M. tuberculosis* follows a rather complex pattern (reviewed in [20]). When primed T-cells interact with CD1$^+$ *mycobacteria*-infected target cells, they kill directly *mycobacteria* through granulysin/perforin-based mechanism release [54], or they induce Fas-dependent apoptotic death of target cells without killing the intracellular infectious agent. In this case *mycobacteria* are released and infect adjacent macrophages and DCs where invading bacilli are possibly killed, depending on microbial burden. In addition to direct cytotoxic effects, CD1-restricted T-lymphocytes release Th1 cytokines (i.e., IFNγ and TNFα) that activate the microbicidal functions of macrophages and DCs [20].

Recently, the role of IFNγ released by CD1-restricted effector T-cells has been subjected to detailed analysis by Lee and Kornfeld [55]. These authors reported that IFNγ released by T-cells inhibits bacterial replication in infected macrophages carrying low intracellular burden of *mycobacteria*, thus contributing to host defenses against tuberculosis. However, when macrophages are engulfed with high bacteria load, IFNγ facilitates host cell death, thus promoting necrosis and spreading of the infection, with potentially adverse effects on the course of the disease.

A large body of experimental data is presently available from the literature showing that *mycobacteria* have developed highly sophisticated strategies to escape host's resistance based either on innate or adaptive immunity (reviewed in [56]). Tuberculosis is predominantly a lung disease characterized by long chronic course due to persistent and sometimes dormant infection. It is well documented that upon contact with inhaled *M. tuberculosis,* both alveolar macrophages, that do not express CD1 molecules, and CD1$^+$ DCs phagocytose *mycobacteria.* But most of the microorganisms are taken up by macrophages that are by far more efficient than resident lung DCs in the ability to phagocytose and possibly kill bacteria [57]. However, the fate of *M. tuberculosis* within the infected alveolar macrophage depends on the state of activation of the phagocyte. Actually, the bacillus is able to survive preferentially within a macrophage subpopulation displaying an anti-inflammatory phenotype with a reduced oxidative burst. Moreover, phagocytosed *mycobacteria* end up in a phagosome, the maturation of which is arrested at an early stage [58], at least in part by *mycobacteria*-released glycolipids, such as lipoarabinomannan and phosphatidylinositol mannoside [59]. *M. tuberculosis* inhibits phagosomal acidification, prevents phagosome-lysosome fusion and survives within macrophages by avoiding lysosomal delivery thanks, at least in part, to coronin 1 that is actively recruited to mycobacterial phagosomes [60]. Since alveolar macrophages do not express CD1 molecules, and mycobacterial peptide antigens confined to phagosomes are excluded from the classical MHC-I presentation pathway, they cannot be targeted by MHC-I- or CD1-restricted cytotoxic lymphocytes. Therefore, in the lung environment, host's defenses against *mycobacteria* are mainly activated through apoptosis induction of infected alveolar macrophages followed by cross-priming of resident DCs endowed with the appropriate machinery for peptide and lipid/glycolipid antigen presentation to T-cells [61]. However, mycobacterial infection inhibits specifically macrophage apoptosis [62], thus preventing DC cross-priming and consequently providing an additional mechanism of impairment of host's T-cell defenses based on bacterial antigen recognition.

Infection with *M. tuberculosis* can also adversely affect DC function by interfering with their expression pattern of antigen-presenting molecules. Therefore, among the different escape mechanisms operated by *mycobacteria*, of particular relevance for the present survey are the complex autocrine and paracrine devices that the microorganism uses to control the induction of Group I CD1 molecule expression in infected and adjacent noninfected MOs. In 1998 Stenger et al. [63] exposed *in vitro* MOs from healthy donors to G4 for 3 days, obtaining iDCs expressing high levels of Group I CD1 glycoproteins. Thereafter, iDCs were heavily infected with *M. tuberculosis* that was able to suppress entirely CD1 expression within 24 h independently from any cytokine intervention. On the other hand, Prete et al. [64] reported later that *in vitro* coculture of BCG with untreated MOs was able to induce

GM-CSF release by infected cells leading to limited CD1b expression. Modest upregulation of Group I CD1 antigen expression was also described by Roura-Mir et al. [65] in untreated MOs after *in vitro* infection with *M. tuberculosis* at 2 or 10 bacteria per cell. These authors report that their findings could be explained, at least in part, through Toll-like receptor-2 (TLR-2) signaling induced by mycobacterial cell wall lipids. A possible, although limited induction of CD1 expression by *mycobacteria* has also been described *in vivo*. Videira et al. [66] found that prophylactic administration of intravesical BCG to prevent tumor recurrence in bladder cancer patients, was followed by upregulation of *CD1A*, *CD1B*, *CD1C*, and *CD1E* gene transcripts in cells obtained from urothelium biopsies. This effect was significantly higher in patients with a more favorable response with respect to that observed in patients with early tumour recurrence [66]. Marked accumulation of CD1a$^+$ LC after mycobacterial stimuli was also described in leprosy skin lesions [67]. On the other hand, *in vitro* maturation of MOs to CD1a$^+$ DCs under the influence of G4 and LPS was found to be sensibly impaired when MOs were collected from patients with pulmonary tuberculosis [68]. The intriguing Janus-like behavior of *mycobacteria* relative to CD1 expression has been investigated in 2001 by Prete et al. [69] and Giuliani et al. [70], who found that BCG induced *in vitro* a limited expression of CD1 in untreated MOs from healthy donors, but inhibited markedly G4-induced CD1 upregulation in the same cells. Thereafter, further investigations confirmed that *in vitro* infection with *mycobacteria* downregulates CD1 expression [71, 72]. In particular, upon exposure to G4, MOs infected with *M. smegmatis* failed to express CD1a and evolved directly into CD83$^+$ mDCs [73]. In 2007, Prete et al. [74] provided direct experimental evidence that *in vitro* exposure of healthy MOs to BCG induced release of both GM-CSF and IL-10, and that the interplay between the two cytokines was presumably involved, at least in part, in the Janus-like behavior of BCG. Actually, early GM-CSF release was responsible for the limited autocrine and paracrine CD1 induction. On the other hand, slightly delayed appearance in culture medium of IL-10 produced by BCG-infected MOs contributed to the severe limitation of further increase of CD1 proteins, even in the presence of exceedingly high concentrations of added GM-CSF. More recently, Gagliardi et al. [75] reported that *mycobacteria* trigger phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) in human MOs, leading to CD1 expression impairment. In fact, pretreatment with a specific p38 MAPK inhibitor allows infected MOs to differentiate into CD1$^+$ DCs, which are fully capable of presenting lipid antigens to specific T-cells. Further studies have been conducted on the possible role of cytokines in restraining the GM-CSF-induced upregulation of Group I CD1 glycoproteins in *mycobacteria* infected MOs. Quite recently, Remoli et al. [76] confirmed the results of the studies described by Prete et al. [74] showing that IL-10 produced by MOs infected with *M. tuberculosis* is responsible for *in vitro* suppression of CD1. Moreover, consistently with the results obtained previously by the same group [75], they suggested that IL-10 release by infected MOs was induced by the activation of p38 MAPK signal transduction pathways. Several reports from the literature indicate that *mycobacteria* activate *IL-10* gene and promote IL-10 release from MOs, phagocytes, and DCs through different intracellular pathways, including PI3K/AKT and p38 MAPK [77–81], phosphorylation and activation of dsRNA-activated serine/threonine protein kinase [82] and glycogen synthase kinase 3 [83]. Noteworthy is the role of proline-glutamic acid/proline-proline-glutamic acid family of proteins of *M. tuberculosis* that can stimulate macrophages to secrete IL-10 via activation of the TLR-2 leading to an early and sustained activation of p38 MAPK, which is critical for IL-10 induction [84]. The role of MAPK in the impairment of CD1 expression by *mycobacteria* has been also confirmed and emphasized very recently by Balboa et al. [85] who found that *mycobacteria*-induced loss of CD1b molecules partially involves TLR-2/p38MAPK activation.

Several other molecular mechanisms distinct from those relative to impairment of CD1 gene transcription could be involved in *mycobacteria*-induced decrease of CD1 expression or of antigen presentation efficiency. The complex cycle of CD1 biosynthesis, cell surface expression, and lipid loading [12, 44, 86] highlights the several means by which *mycobacteria* can interfere with CD1 expression on cell membrane and antigen presentation to T-cells. After biosynthesis in the endoplasmic reticulum, CD1e remains in the cell, whereas all other CD1 molecules reach the cell surface through the Golgi and trans-Golgi network where they bind to self-lipids. Direct loading of lipids may occur at the plasma membrane, as described for glycosphingolipids that bind to CD1b on the cell surface at neutral pH. Thereafter, glycosphingolipids are recognized without internalization or processing and stimulate specific T-cells [87]. Moreover, various cell-surface CD1a proteins are stabilized by exogenous glycosphingolipids and phospholipids present in serum [88].

As a rule, processing and presentation of microbial CD1-bound lipid antigens require that CD1 molecules, loaded with self-lipids, undergo a recycle process. CD1-self lipid complexes are internalized, traffic through the endosomal compartments, where loading and/or exchange with exogenous lipid antigens occur, then the new CD1-nonself lipid complexes re-emerge on plasma membrane. This process resembles peptide sampling by MHC class II proteins, although MHC class II molecules may reach the endocytic compartment directly from the trans-Golgi-network, without first travelling to the cell membrane.

Cell surface CD1 molecules are internalized according to two distinct mechanisms. Specifically, CD1a molecules, which lack a tyrosine-based internalization motif, are internalized to the early endosomes [89] through a clathrin/dynamin-independent manner and recycle back to the plasma membrane through a mechanism that relies on small GTPases, such as Rab22 and ADP-ribosylation factor 6. Both CD1b and CD1c molecules, instead, have a tyrosine-based motif in their cytoplasmic tail and are internalized through clathrin-coated pits via the adaptor protein 2 (AP-2). Thereafter, CD1b is transported to the late endosomes and, after binding to AP-3, traffics to the lysosomes and then recycles to the plasma membrane. On the other hand, CD1c,

after reaching the sorting endosomes, routes to the early endosomes, and, although to a lesser extent, to the late endosomes and lysosomes, and then recycles to the plasma membrane. It follows that CD1c operates a comprehensive survey for lipid antigens throughout the endocytic system [90].

The entire CD1 recycling pattern reveals that a large variety of molecular targets could be affected by *M. tuberculosis.* In addition to that, it must be considered that intracellular lipid loading presumably requires the functional intervention of a number of helper and adaptor molecules, including saposins and apolipoproteins [91, 92] and CD1e itself [93, 94]. Moreover, acidic pH promotes lipid binding to CD1b proteins, thus suggesting that pH fluxes during endosomal recycling regulate the conformation of the CD1 heavy chain to control the size and rate of antigen capture [95]. Within this context, it is worth of note the finding that *mycobacteria* impair phagosome acidification [58] thus reducing the extent of mycobacterial lipids bound to CD1b for T-cell presentation.

5. HIV and CD1 Expression

Interestingly enough, not only the *mycobacterial* infection, but also HIV or HTLV-1 infection or intracellular presence of HIV products are able to interfere with CD1 expression. For example, HIV-1-Nef was found to interfere with the intracellular trafficking of CD1a [96], although recombinant Nef added to iDCs increases CD1a expression [97]. Moreover, it must be pointed out that viable HIV-1 particles infect target $CD4^+$ T-cells via $CD1b^+$ exosomes [98]. On the other hand, in 30 to 45% of HIV-infected white and African subjects, peripheral blood MOs exposed *in vitro* to G4 followed by LPS gave rise to $CD1a^-$ mDCs releasing IL-10 but not IL-12 [99]. In addition, DCs from HTLV-I-infected monocytes fail to present adequate amounts of CD1a glycoprotein [100].

Preliminary investigations of experimental design (ED)-1 type (see ED codes illustrated in Figure 2) performed in our laboratory, revealed also a possible link between HIV infection and CD1 system, presumably relevant to the increased susceptibility of HIV-infected individuals to *mycobacteria*. A vector expressing *tat* DNA (PCV-TAT, [101]) under the control of the major adenoviral late protein, and a control empty vector (PCV-0) were kindly provided by Barbara Ensoli MD of the Italian National Institute of Health. Peripheral blood MOs of healthy donors were incubated with G4 alone or with G4 + a supernatant obtained from the human T-cell leukemia line Jurkat transfected with PCV-0 (sup-PCV-0) or with PCV-TAT (sup-PCV-TAT). The results of a representative experiment demonstrated that *tat*-induced factors released by transfected cells are able to down-regulate CD1b expression. In fact, after 5-day exposure to G4 *in vitro*, iDCs generated in the absence of supernatants or in the presence of sup-PCV-0 showed 72% and 79% $CD1b^+$ cells, respectively. In contrast, when iDCs were generated in the presence of sup-PCV-TAT, the percentage of $CD1b^+$cells dropped significantly to 54% (Franzese et al., in preparation). Moreover, if monoclonal antibodies against IL-10 were added to G4 + sup-PCV-TAT at the onset of iDC generation, the percentage of $CD1b^+$ cells raised to 81%. These results along with previous findings indicating that TAT induces IL-10 in MOs [102] and that IL-10 downregulates CD1 expression [74–76, 103–106], are consistent with the hypothesis that IL-10, generated in the presence of TAT, plays a critical role in compromising CD1b expression.

6. Chemical, Biological, and Physical Agents Affecting CD1 Expression

6.1. Drugs. A number of natural and synthetic compounds of pharmacological interest are able to modulate the expression level of Group I CD1 proteins on immature and/or mature DCs, either *in vitro* and *in vivo*, as reported in Table 2.

As expected, most of the immunosuppressant and anti-inflammatory agents, including corticosteroids, nonsteroidal anti-inflammatory drugs (NSAID), and anti-asthma compounds, down-regulate cytokine-induced CD1 expression of MOs and impair their functional activity. However, local application of Pimecrolimus on skin in atopic dermatitis, is followed by increase in the number of $CD1a^+$ cells. Moreover, *in vitro* exposure of $CD34^+$ peripheral blood progenitor cells to Tacrolimus favors the expression of CD1a induced by 14-day treatment with cytokines. Notable exceptions to the inhibitory effects of anti-inflammatory drugs is also represented by Piceatannol (a stylbene compound similar to resveratrol) and terpenes that were found to increase CD1a expression after G4 treatment *in vitro* of MOs obtained from healthy donors. Of sensible relevance to the problem of MS therapy and identification of disease pathogenesis is the finding that Glatiramer acetate (GA), alone or in combination with IFNβ, is able to down-regulate CD1 expression *in vitro* or *in vivo*. Similar inhibitory effects have been described *in vitro* with vitamin D3 that shows beneficial effects in MS management. These observations appear to provide further support to the hypothesis that significant participation of CD1-restricted T-cell responses against self lipid antigens is involved in the neuronal damage occurring in MS.

Among chemotherapeutic agents, antitubercular (rifampicin) or antiretroviral (entecavir) drugs tend to up-regulate CD1 expression, whereas zidovudine (AZT), that inhibits iDC proliferation, diminishes the overall availability of $CD1a^+$ cells. In the area of antineoplastic therapy, reduction of cytokine-induced CD1 levels by various agents is the dominant finding, as shown *in vitro* by histone deacetylase (HDAC) inhibitors, tyrosin kinase inhibitors (i.e., imatinib and sorafenib) and antiestrogens, and *in vivo* by thalidomide in multiple myeloma (MM) patients.

More difficult to interpret is the activity of a classical agent largely utilized in mood disorders including bipolar affective disorders, such as lithium. The drug downregulates the *in vitro* cytokine-induced CD1a expression in MOs of healthy donors. However, limited CD1a expression is elicited by G4 in MOs collected from patients with bipolar disorders. In this case, *in vivo* treatment of donor patients with lithium restores full responsiveness of their MOs to G4 exposure *in vitro.*

6.2. Cytokines and Autacoids. Table 3 illustrates the limited information available from the literature on the effect

Table 2: Pharmacological modulation of CD1 molecule expression.

Drug class	Agent	Therapeutic use	CD1[a]	ED[b]	Ref[c]	Observations
Angiotensin receptor antagonists	Losartan (AT1-R) PD123319 (AT2-R)	Hypertension	D U	ED-1 ED-1	[107]	Assay performed on day 7.
Anti-asthma	Suplatast tosilate	Inhibitor of Th-2 responses	D	ED-1 ED-2	[108]	In ED-1 the assay was performed on day 7. DCs were obtained from pts with asthma.
Anti-depressant	Lithium	Bipolar disorders	D	ED-1	[109]	MOs, obtained from bipolar pts, were incubated with G4.
			U	IvDC	[109]	*In vitro* generated DCs from lithium-treated pts showed higher CD1a expression than DCs from untreated pts.
			D	ED-1	[110]	*Mechanism:* CD1 down-regulation is likely mediated through the GSK-3β pathway.
Anti-estrogens	Tamoxifen Toremifene	Breast cancer	D	ED-1 ED-2	[111]	Assay performed on day 7.
Anti-inflammatory corticosteroids	Beclomethasone dipropionate (BDP, inhaled)	Asthma	D	IvDC	[112]	In bronchial mucosa of asthmatic pts there is an increase of CD1a$^+$ DCs. Following long-term treatment with BDP the number of CD1a$^+$DCs went to normal levels of non-asthmatic pts.
	Dexamethasone (DEX)	Inflammatory diseases	D	ED-1 ED-4	[113]	Strong CD1a down-regulation. *Mechanism*: high IL-10 via Extracellular signal-regulated kinases (ERK) phosphorylation.
			D	OED	[114]	CD34$^+$ cord blood stem cells were cultured with SCS, Flt3-ligand and GM-CSF (Pre-DC). After 5 days, TNFα and IL-4 were added (differentiation stage). On day, 10 CD-40 ligand and anti-human CD40-ligand were also added (maturation stage). DEX, added during differentiation stage, suppresses CD1a at the end of the immature (day 10) and at the mature stage (day 12). On the contrary, CD1a was expressed at normal levels when DEX exposure was limited to the 2 day maturation stage.
				ED-1 ED-3 ED-4	[115]	The MO-derived DCs were obtained from neonatal cord and adult blood.
	Various including DEX		NC	OED	[116]	CD1a$^+$ cells derived from bronchoalveolar lavage showed lower APC function if treated with DEX *in vitro*.
Anti-tubercular agents	Rifampicin		U	ED-1	[117] [118]	Test on CD1b: the drug does not affect the functional activity of the T-cell clone capable of recognizing the mycolic acid of *M. tuberculosis* origin, presented by CD1b proteins. Test on CD1b: effect obtained at clinical concentration of the drug.
Antiviral	AZT Entecavir	HIV treatment Hepatitis B treatment	NC U	ED-1 ED-1	[119] [120]	AZT inhibits DC proliferation, thereby reducing the total number of DCs.
Bisphosphonates	Zoledronic acid	Osteoporosis	D	ED-4 ED-2	[121]	Mechanism: possibly via IL-10 induction, antagonized by geranylgeraniol.

Table 2: Continued.

Drug class	Agent	Therapeutic use	CD1[a]	ED[b]	Ref[c]	Observations
Disinfectants	Sodium Chlorate		D	ED-1	[122]	Sodium chlorate reduces GAG sulfation on MO surface. Reduction of sulfated CSB impairs IL-4 mediated DC differentiation and CD1a expression.
HDAC inhibitors	MS-275 Sodium valproate	Antitumor	D	ED-4 ED-1	[123]	Mechanism: NF-*k*B, IRF-3 and IRF-8 inhibition. Possible use in inflammatory and autoimmune disorders.
	Na butyrate		D	ED-1	[124]	The agent prevents CD1 upregulation induced by activation of TLR-2.
Immuno stimulant agents	Imiquimod (imidazo quinoline)	Topical use in squamous cell carcinoma	D	IvDC	[125]	In skin biopsies after topical treatment.
	OK-432	In cancer treatment	U	ED-2	[126]	The maturation step was performed with OK-432 which promotes a higher expression of CD1a in respect to that obtained with LPS.
Immuno suppressive agents	Gold sodium thiomalate (GST)	Rheumatoid arthritis (RA)	D	ED-1 ED-2 ED-4	[127]	DCs were obtained from healthy donors or RA pts. The suppression of DC differentiation and function might explain the *in vivo* effect of GST on RA patients.
	Glatiramer acetate (GA) + minocycline (MIN)	Multiple sclerosis (MS)	D	ED-1 ED-4	[128]	DCs were obtained from untreated and GA-treated MS pts. The possible additive effects of GA and MIN on MO-derived DCs, seem to support the use of such combination therapy in MS.
	GA + IFNβ	MS	D	IvDC	[129]	MOs were obtained from untreated or treated MS pts and from healthy donors. Combination therapy with IFNβ+ GA resulted in a more pronounced decrease of circulating CD1a compared to monotherapy with IFNβ.
		MS	D	ED-1	[130]	Assay was performed on day 7. DC were obtained from MS pts. Synergistic effects of GA and IFNβ.
	Monomethyl-fumarate (MMF)	Psoriasis	D	ED-1 ED-4	[131]	MMF interfered with the MO-derived DC differentiation, resulting in impaired maturation of these cells.
	Pimecrolimus	Atopic dermatitis	NC	ED-1	[132]	No interference with the function of DCs, whereas the activation of effector T-cells was inhibited.
			U	IvDC	[133]	In epidermal cells (biopsy) after topical treatment.
	Rapamycin	Immuno-suppressant	U	ED-1 ED-4	[134]	Reduction of MHC-I, MHC-II and Ag uptake.
	Sinomenine		U	ED-1	[135]	The drug prevents LPS-induced DC maturation.
	Tacrolimus (FK506)		D	ED-1 ED-2 ED-4	[136]	Effect on LPS-induced DCs *in vitro.*
			D	IvDC	[137]	Topical treatment of epidermal CD1a^{+}, in pts with atopic dermatitis.
			U	OED	[138]	Generation of DCs from CD34^{+} peripheral blood progenitors obtained by culturing the cells with GM-CSF, TNFα, stem cell factor for 14 days. FK506 was added throughout the culture starting on day 0.

Table 2: Continued.

Drug class	Agent	Therapeutic use	CD1[a]	ED[b]	Ref[c]	Observations
	Triptolide	Polycystic Kidney disease	D	ED-1 ED-2	[139]	Suppression of DC differentiation and maturation by triptolide may explain some of its immunosuppressive properties.
Insecticides	Rotenone		D	ED-1	[140]	*Mechanism:* increased levels of reactive oxygen species that seem to trigger the differentiation process of DC.
Multidrug resistance (MDR) protein antagonists	MK571[multi drug resistance protein 1 (MRP1) blocker] PSC833 (P-gp blocker)	Possible use in MDR	D	ED-4	[141]	MRP1 transporter activity is important for DC differentiation.
			NC	OED		Langerhans-like DCs were obtained from human acute myeloid leukemia cell line MUTZ-3, cultured with TGF-β1, GM-CSF and TNFα for 10 days, and MDR antagonists were added on day 4, 7, and 10.
Monoclonal antibodies	Infliximab	Anti-TNFα	D	ED-1 ED-4	[142]	MOs from psoriasis pts. Reduction of antigen-presenting capacity of DCs, proliferation and IFNγ release by psoriatic T-cells.
NSAID	Acetylsalicylic acid (ASA)	Inflammation	D	ED-1 ED-4	[143]	The new nitric oxide releasing-ASA (NCX-4040, NCX-4016) did not affect the expression of CD1a during maturation stage (ED-4).
	Niflumic acid (NFA)	Inflammation	D	ED-4	[144]	NFA inhibits LPS-induced DC maturation by inhibiting co-stimulatory molecule expression and IL-12p70 production.
Microsomal triglyceride transfer protein (MTP) inhibitors	BMS212122	Anti-lipid	D	ED-1	[145]	MTP inhibitors down-regulate self as well as exogenous lipid antigen presentation.
NO donors	DEA-NO, SIN-1, DETA-NO		U NC	ED-1 ED-2 ED-1	[146]	The drugs are TNFα receptor inhibitors.
Statins	Atorvastatin	Dyslipidemia	U	OED	[147]	MO-derived DCs were obtained from healthy donors. MOs exposed to atorvastatin in combination with IFNα, showed an increased levels of CD1a compared to IFNα alone.
	Lovastatin		D	ED-2	[148]	DCs were obtained from MS pts. Lovastatin was added after G4, simultaneously with TNFα.
TLR agonists	Pam CSK resiquimod (R848)	Immune response modifier	U	OED	[149]	Induction of CD1a$^+$ cells in freshly isolated BM CD34$^+$ progenitor cells cultured with TLR agonists without cytokines.
Tyrosine Kinase Inhibitors	Imatinib	Antitumor	D	ED-1 ED-4	[150]	In ED-1 the assay was performed on day 7. *Mechanism*: NF-*k*B and AKT inhibition.
			D	OED	[151]	*In vitro* effects of imatinib, added to the culture together with different cytokines, on the development of mobilized human CD34$^+$ peripheral blood progenitor cells into DCs.
	Sorafenib	Antitumor	D	ED-1	[152]	In ED-1 sorafenib was added on day 5.
				ED-2		*Mechanism*: P13MAPK and NF-*k*B inhibition.
Various	All trans-retinoic acid (ATRA)	Various	U	ED-1	[153]	In ED-1 ATRA was associated with GM-CSF without IL-4.

Table 2: Continued.

Drug class	Agent	Therapeutic use	CD1[a]	ED[b]	Ref[c]	Observations
	Retinoic acid (Am80)	Various	D	ED-1	[154]	Am80 treatment ameliorated macro- and microscopic damage in dextran sodium sulfate-induced colitis in mice, and suppressed the colitis induced elevation of IL-12.
	Thalidomide	Multiple myeloma (MM)	D	OED	[155]	MOs were obtained from peripheral blood of MM pts treated or not with thalidomide. For *in vitro* DCs generation standard cytokines were used.
		Sarcoidosis	U	IvDC	[156]	In skin biopsies of sarcoidosis pts treated or not with thalidomide.
	Trimethyl psoralen + PUVA	Psoriasis	D	IvDC	[157]	Biopsies of lesional skin were performed in pts with psoriasis, before treatment, after 2 weeks of treatment or at the end of treatment.
	Dehydro-epiandro-sterone		D	ED-1	[158]	Slight down-regulation. The assay was performed on day 7. Reduction of IL-10 (opposite effect respect to DEX).
	Terpenes: Calamenene T-cadinol	Anti-inflammatory, anti-septic	U	ED-2	[159]	
	Terpenes: Epicubenol, Ferruginol	Anti-septic	U	OED	[160]	MOs were cultured with G4, followed by another 2 days with the drugs. Surprisingly both induce IL-10 generating Treg.
	Piceatannol (stilbene derivative)	Anti-inflammatory, immunomodu-latory and anti-proliferative	U	OED	[161]	MOs were cultured with G4 for 6 days, followed by another 2 days in the presence of piceatannol alone. On the contrary high concentration of resveratrol, another stilbene derivative, markedly reduces CD1b expression on G4-induced iDCs (Fuggetta et al., in preparation).
Vegetal products	Ginseng saponins (M1 and M4)	Various	U	ED-2	[162]	After G4 DCs were treated on day 6 only with M1 or M4.
Vitamins	Alpha dihydroxy vitamin D3	MS	D	ED-1	[163]	1,25(OH)2D3 hampers the maturation of fully active immunostimulatory MHC-II$^+$, CD1a$^+$, CD80$^+$DCs from MOs.
			D	ED-1 OED	[164]	CD34$^+$ cells were collected by apheresis either from cancer pts after chemotherapy or from healthy donors after G-CSF treatment. For DC generation the cells were cultured with standard cytokines.
			D	ED-2	[165]	DCs were obtained from MS pts. Beneficial action of vitamin D in MS may be associated with its inhibition on both differentiation and maturation of DCs.
			D	ED-4	[166]	Accompanied by overexpression of miR-378 and low expression of miR-155 that could have a role in DC function.
			D	ED-1	[167]	D3 up-regulates colony stimulating factor 1 and downregulates its receptors.
			D	ED-1	[168]	Assay was performed on day 7. Inhibition of DC differentiation and maturation.
	Calcipotriol (vit. D3 analog)	Topical in psoriasis	D	OED	[169]	*In vivo* treated psoriatic skin.

[a]Evaluation of CD1a expression if not otherwise specified: U: upregulation; D: down-regulation; NC: no change.
[b]Experimental design code (see Figure 2).
[c]Reference number.

of prostaglandins and serotonin on CD1a expression in different experimental conditions *in vitro*. In all cases, the agents show suppressive activity.

When cytokines are considered, GM-CSF and IL-4 are not enlisted in Table 3. Actually, this cytokine combination is used by most of *in vitro* tests, to induce iDCs that express high levels of CD1 proteins (Figures 1 and 2). In particular, GM-CSF is the most potent inducer, whereas IL-4 reinforces the effect of GM-CSF but is scarcely active if used alone.

A number of data from the literature is presently available on IFNs that show predominant inhibitory effects on CD1 system. While IFNα can be involved in the transition from iDCs to mDCs (Figure 1), IFNβ downregulates CD1 protein expression either *in vivo* or *in vitro*. In addition this cytokine was found to reduce the functional activity of mDCs. Since IFNβ has acquired a definite role in MS treatment, these results add further support to the hypothesis of the involvement of CD1 system in MS pathogenesis.

Consistent inhibitory effects on CD1 expression are manifested by IL-6 and IL-10 in various experimental conditions. It must be pointed out that in many cases down-regulation of G4-induced CD1 expression provoked by various agents appears to be mediated by the release of IL-6 and more frequently by the release of IL-10 that operates according to an autocrine pattern.

Of interest, finally is the mechanism by which TGFβ appears to maintain CD1a expression on LC generated *in vitro* from purified CD34$^+$ cells. In this case, the expression of CD1a, that is normally found to be elevated in immature LCs, declines with LC maturation. Since TGFβ prevents LC maturation, it allows the long-term presence of high CD1a levels in LCs.

6.3. Biological and Physical Agents. With the exception of the placental growth factor, all biological and physical agents illustrated in Table 4 provoke down-regulation of cytokine-induced CD1 protein expression. The mechanism underlying the effect of various lipids including some contained in human serum, indicates a common target consisting in peroxisome proliferator-activated receptor (PPAR)γ that appears to be activated by these molecules in various experimental conditions. The observation that human serum, either for the presence of different lipoproteins or for the presence of IgG and β2-microglobulin (Table 4), provides inhibitory effects, poses undoubtedly the question of the efficiency of the CD1 system *in vivo* in infected patients.

Of considerable interest is the finding that various supernatants of human tumor cell cultures contain inhibitory factors. Although mycoplasma contamination of cultured cells could be, at least in part, responsible for these findings (see Table 5), it cannot be excluded that this type of suppression of antigen-presenting function could be of relevance in tumor-induced immune suppression.

The *in vivo* impairment of CD1a expression by ultraviolet light is not surprising, since the general immune-suppressive effects of this type of radiation has been demonstrated in different effector functions of the immune system.

6.4. Infectious Agents or Microorganism Products. In vitro and *in vivo* studies concerning modulation of CD1 system by bacterial and chlamydial infections generally demonstrated a CD1 upregulation (Table 5). It is reasonable to speculate that, in certain experimental conditions, TLR-2 activation by microorganisms could be involved [29]. Surprisingly, however, is the finding that antral biopsies performed in *H. pilori*-infected children reveal CD1a/b upregulation respect to normal subjects, whereas *in vitro* exposure of MOs to formalin-killed *H. pilori* prevents CD1 induction by G4.

Of particular note is the finding that CD1a is up-regulated *in vitro* by G4 more vigorously in MOs obtained from MS patients bearing an infectious disease, with respect to MOs obtained from noninfected MS patients. This observation has been put in relationship with the clinical finding that subjects affected by MS are at particular risk of relapse in the course of bacterial infections. Again, this seems to provide support to the hypothesis of a significant role that could be played by CD1 system in MS.

Differently from the *in vivo* and *in vitro* effect of the bacteria and chlamydia reported in Table 5, infections with various protozoa, with at least two types of helminthes, and viruses such as HHV-8 and Cytomegalovirus leads to impairment of CD1 expression in various types of experimental design. This is not surprising since the general immunodepressive activity of these infections has been known for several years.

When microorganism products are considered, only attenuated Dengue-2 live vaccine, malaria-associated AMA-1, and staphylococcus superantigen are able to up-regulate cytokine-induced CD1 expression. Toxins and malaria hemozoin provide opposite effects on the system. A particular feature that distinguishes the activity of pertussis toxin from the other microorganism products resides in its unusual property of suppressing CD1a expression selectively, without reducing the levels of the other components of the system (i.e., CD1b and CD1c). It is not excluded that this could allow selective analysis of *CD1A* gene regulation distinct from that of the other *CD1* genes.

Finally, of relevance is the finding that LPS is able to down-regulate G4-induced CD1a. LPS, that is considered the standard agent for generating mDCs from iDCs (Figures 1 and 2), is a common constituent of pathogenic or nonpathogenic microorganisms, being present in the cell wall of gram-negative bacteria. Therefore, it is reasonable to consider that this molecule could play a significant role in the clinic, possibly through its modulating activity on CD1 expression and DC maturation.

7. Conclusions and Perspectives

Fine tuning of biological functions governed by a complex signaling network is commonly seen in living organisms, and the CD1 system does not represent an exception to this rule. This opens up several options to intentionally manipulate the CD1 expression in order to enhance or depress antigenic lipid presentation according to the therapeutic needs. The results of the literature analysis presented here clearly demonstrate that a large variety of different externally acting agents,

Table 3: Effect of autacoids or cytokines on CD1 molecule expression.

Molecule	CD1[a]	ED[b]	Ref.[c]	Observations
Autacoids				
Prostaglandin PGE_2	D	ED-4	[170]	
	D	ED-1	[171]	Purified $CD14^+$ cells from PB of healthy donors mobilized with G-CSF for allogeneic transplantation.
	D	ED-2		
	LD	ED-3		
	D	ED-4		
	D	ED-1	[172]	
	D	ED-1	[173]	
	D	ED-1	[174]	
Cyclopentenone Prostaglandins (CP) (15d-PGJ2, 12-PGJ2, PGA2, PGD2, and PGE2)	D	ED-1	[175]	MOs + G4 for 7 days. CP were added during the last 24 h of culture without adding maturation factors. In these experimental conditions, CP induced apoptosis.
Serotonin (5-hydroxy-tryptamine, 5-HT)	D D	ED-1 ED-4	[176]	MOs. 5-HT effects mediated via $5\text{-HTR}_{1/7.}$ iDCs and mDCs exposed to 5-HT for 24 h did not show alteration of CD1a expression.
Cytokines				
IFNα2a	D	OED	[177]	MOs cultured for 7 days with (GM-CSF+IL-4+TNF-α) +/− IFNα2a.
IFNα2b	D	OED	[178]	MOs cultured with GM-CSF +/− IFNα2b for 5 days.
IFNα + IL-2 or IL-12 alone	NC	IvDC OED	[179]	MOs obtained from PB of pts with renal cell cancer before, during, and after therapy with the indicated cytokines, or from healthy subjects were cultured with G4 for 8 days. The yield of DCs from cancer pts was lower than that from healthy subjects. However, the phenotype of DCs generated from MO of pts was comparable to that of DCs generated from MO of healthy subjects.
IFNβ1a	D	ED-2	[148]	MOs from PB of untreated pts with MS.
	D	ED-1	[180]	MOs from PB of untreated or IFNβ1a-treated pts with MS.
	D	ED-4	[181]	MOs from PB of untreated pts with MS. Analysis was performed on CD1a/b/c molecules.
	D	ED-1	[182]	
	D	IvDC	[183]	Evaluation of % of $CD1a^+HLA\text{-}DR^+$ MNC in PB of MS pts, either untreated or treated with IFNβ1a, and in healthy subjects.
	D	ED-4	[184]	Purified $CD14^+$ cells from PB of healthy subjects.
IFNβ1b	D	ED-1	[185]	MOs from PB of untreated or IFNβ1a-treated pts with MS and from healthy subjects.
IFNγ	D	ED-1	[186, 187]	MOs + G4 for 12 days.
Il-1β	NC	ED-1	[188]	

Table 3: Continued.

Molecule	CD1[a]	ED[b]	Ref.[c]	Observations
IL-3	U	OED	[189]	$CD14^+$ osteoclast precursors from PB of healthy donors cultured with (M-CSF+ RANKL) +/− IL-3 for 7 days.
IL-6 sIL-6Rα/IL-6 fusion protein (FP6)	D D	OED	[190]	$GPA^-CD15^-CD14^-CD1a^-IL\text{-}6R^+$ myeloid progenitors (generated after incubation of cord blood-derived $CD34^+CD38^-$ cells with SCF+FLT3-L+TPO+IL-3 for 6-7 days) were cultured with (SCF+FLT3-L+TPO+IL-3) +/− IL-6 or FP6 for 11–14 days.
IL-6 sIL-6R/IL-6 fusion protein (FP6)	D D	OED	[191, 192]	$CD36^-CD15^-CD14^-CD1a^-IL\text{-}6R^+$ myeloid progenitors (generated after incubation of cord blood-derived $CD34^+CD38^-$ cells with SCF+FLT3-L+TPO+IL-3 for 7 days) were cultured with (SCF+FLT3-L+TPO+IL-3) +/− IL-6 or FP6 for 7 days.
IL-6	D	OED	[193]	Purified $CD34^+$hematopoietic progenitor cells from PB of G-CSF-treated Pts with MM were cultured with (FLT3-L+TNFα+GM-CSF+SCF+IL-4) +/− IL-6 (added on day 0 or day 7 of culture) for 14 days. CD1a evaluation on day 14.
	D	ED-1	[194]	MOs. *Mechanism,* Il-6-induced expression of G-CSF receptor.
IL-10	D	IvED	[195]	Psoriatic skin after systemic IL-10 administration.
	D	OED	[196]	MOs cultured with (GM-CSF+IL-13) +/− IL-10 for 7 days.
	D	OED	[197]	MOs cultured with (GM-CSF+IL-13) +/− IL-10 for 7 days.
IL-13	U	OED	[198]	MOs cultured with G4 or with GM-CSF+IL-13 for 7 days. Higher CD1a upregulation with GM-CSF+IL-13. MOs cultured with G4 for 7 d and then with IL-13 or TNFα or IL-4 for 48 h. IL-13 as affective as TNFαin inducing maturation of imDC.
Platelet factor 4	D	ED-1	[199]	
TGF-β1	U	OED	[200]	Purified $CD34^+$ hematopoietic progenitor cells from cord blood cultured with (FLT3-L+TNFα+GM-CSF+SCF) +/− TGF-β1 for 10–14 days. Cells generated in the presence of TGF-β1 resemble immature LC with high CD1a antigen expression. *Mechanism*: maturation of LC leading to CD1a down-regulation is prevented by elevated E-cadherin expression induced by TGF-β1.

[a]Evaluation of CD1a expression if not otherwise specified: D: down-regulation; U: upregulation; LD: limited down-regulation; NC: no changes.
[b]Experimental design code (see Figure 2).
[c]Reference number.

either of synthetic or natural origin, can affect profoundly the expression levels of CD1 glycoproteins, with a possible consequence on DC-mediated lipid presentation to T-cells. Actually, Group I CD1 glycoproteins are mainly involved in the presentation of *M. tuberculosis*-derived lipids to CD1-restricted T-cells. Pharmacological amplification of the system could provide a significant help for vaccination and treatment modalities concerning millions of subjects presently exposed to tuberculosis threat. In particular, the rapidly expanding area of small RNAs capable of controlling directly or indirectly the expression level of an extremely high numbers of genes, could be carefully considered for

Table 4: Effect of biological or physical agents on CD1 molecule expression.

Type of biological agents	Agent	CD1[a]	ED[b]	Ref.[c]	Observations
Growth factors	Placental growth factor (PLGF)	U	ED-2	[201]	Modest upregulation. PLGF antagonizes LPS-induced down-regulation of CD1a in iDCs. *Mechanism*: inhibition of NF-*k*B signal transduction pathway.
Heat-shock proteins	HSP-27	D	ED-1	[202, 203]	*Mechanism:* IL-10 induction.
Immuno-complexes	Anti-OVA rabbit IgG + OVA	D	ED-1	[204]	*Mechanism*: interaction with FcγRI and FcγRII.
Ligand proteins	Peptide ligand of melanocortin-4 receptor (NDP-MSH)	D	ED-4	[205]	mDCs from treated precursors show impaired ability to prime T-cells.
	sLAG-3 (CD223) soluble MHC-II ligand	D	ED-1 ED-3	[206]	CD1a down-regulation. *Mechanism* (hypothesis): phosphorylation of PLCγ2, p72syk, or AKT molecules.
Lipids	Lipids	D	ED-1	[207]	High individual variability of CD1a induction after G4. Lipoproteins (VLDL > LDL > HDL) and PPARγ activation reduce the number of G4-induced CD1a$^+$ cells.
	Lysophosphatidic acid (LPA)	D	ED-1	[208]	*Mechanism:* LPA is a potent natural ligand for PPARγ.
	Oxidized Phospholipids	D	ED-1	[209]	Oxidized phospholipids (generated during inflammation) down-regulate CD1a/b/c and block histone modifications required to activate mDCs.
Malignant cell products	Hepatoma cell supernatant	D	ED-1	[210]	CD1a down-regulation by hepatoma but not normal liver cell supernatants. Induction of Treg *Mechanism:* possibly IL-10-dependent.
	Human renal cell carcinoma lines	D	OED	[211]	From CD34$^+$ progenitor cells: severe inhibition of CD1a and APC function of induced CDs. *Mechanism*: possibly due, at least in part, to IL-6 and macrophage colony stimulating factor.
	Leukemia cell supernatant	D	*ED-1*	[212]	Supernatant of K562, HL-60 and DAUDI on CD1a expression. *Mechanism*: at least in part, due to IL-1β secreted by MOs in response to leukemic cell products.
	Melanoma cell supernatant	D	ED-1 IvDC	[213]	CD1a/b/c. *Mechanism:* IL-10 release induction *in vivo* reduced CD1-positive cells in metastatic melanoma.
		D	OED	[214]	LC generated *in vitro* from cord blood CD34$^+$ progenitors are CD1a-deficient when cultured with melanoma cells in a transwell design setting.
	Supernatant from primary or long-term cultured tumor cells	D	ED-1	[215]	Reduction of CD1a by supernatant of tumor cell lines was much less active respect to supernatants of primary tumors. Similar results obtained with CD34$^+$ progenitor-derived DCs. *Mechanism*: at least in part mediated by PGE2 released by primary tumor cells.

Table 4: Continued.

Type of biological agents	Agent	CD1[a]	ED[b]	Ref.[c]	Observations
Nucleotides	cAMP, cGMP	D	ED-1	[216]	cAMP increase was mimicked by the adenylate cyclase activator forskolin or cAMP analog 8 bromo-cAMP; cGMP increase was mimicked by 8 bromo-cGMP; increase of both was induced by PDE inhibitor IBMX. Down regolation of CD1a is followed by impairment of LPS-induced mDC function.
Serum and serum components	Human serum	D	ED-1	[217]	Human serum lipids: impairment of CD1a/b/c transcription. Reduced induction of CD1c-restricted T-cell responses. *Mechanism:* PPARγ activation.
				[218]	Human serum: *Mechanism:* PPARγ activation and IL-10 induction.
				[219]	Autologous serum (iDCs from MOs or from CD34^{+} precursors).
	IgG	D	ED-1	[220]	Down-regulation of CD1a/b/c and upregulation of CD-1d transcripts. *Mechanism*: IgG-mediated activation of Fcγ receptor FcγRIIa (CD32a).
		D	ED-1 ED-4	[221]	This study starts from the observation that intravenous immunoglobulin attenuates MS.
	β2-microblobulin	D	ED-1 ED-4	[222]	Down-regulation of CD1a and mDC function. *Mechanism*: inhibition of MAPK, ERK, MEK, and NF-*k*B, and activation of STAT3.
Physical agents					
Ultraviolet light	UVAI (340–400 nm)	D	IvDC	[223]	Decrease of CD1a^{+} LC in a epidermis 3 days after ultraviolet exposure.
	UVB	D	IvDC	[224]	UV irradiation induces CD1a^{+} LC down-regulation and IL-10 induction *in vivo* in skin. This is prevented *in vivo* by Zn-containing or octylmetoxy cinnamate sunscreen preparations.
		D	IvDC	[225]	CD1a^{+} Langerhans cell loss after exposure of human epidermis and dermis to UVB, accompanied by infiltration with IL-10 producing macrophages.
		D	IvDC	[226]	Organ culture *in vitro* of human cornea (immunohistochemistry): low-dose UVB (100 mJ/cm^{2}) decreases HLA-DR and CD1a expression of organ-cultured human corneas and induces moderate corneal injuries, and might be useful for preventing allograft rejection.

[a]Evaluation of CD1a expression if not otherwise specified: U: upregulation; D: down-regulation.
[b]Experimental design code (see Figure 2).
[c]Reference number.

Table 5: Influence exerted by infectious agents or microorganism products on group 1 CD1 antigen expression.

Infectious Agent	Agent	CD1[a]	ED[b]	Ref.[c]	Observations
Bacteria	*Helicobacter pilori* *E. coli*	D	ED-1	[227]	*In vitro* exposure to paraformaldehyde-fixed bacteria. IL-10 independent.
	Helicobacter pilori (*in vivo*)	U	IvDC	[228]	*In vivo* detected by antral biopsies (lamina propria): increased CD1a and CD1b in infected children with respect to normal subjects. Expression of local immune responses.
	Propionibacterium acnes	U	ED-2	[229]	Heat-killed bacteria added on day 6 to iDCs of pts with acne vulgaris.
	Various	U	OED	[230]	Increased CD1a expression in *in vitro* generation of mDCs from MS pts with bacterial infections versus MS pts without infections.
Chlamydia	*Chlamydia trachomatis*	U	IvDC	[231]	Myeloid DCs collected from cervical mucosa of chlamydia-infected woman show myeloid DCs with increased CD1a expression with respect to that of healthy women.
Mycoplasma	*Mycoplasma* present in cell culture supernatant	D	ED-1	[232]	If mycoplasma is removed, culture supernatants are no more able to down-regulate CD1a.
Protozoa	*Leishmania donovani*	D	ED-1	[233]	*L. donovani* infection *in vitro* impairs induction of CD1a/b/c expression in terms of gene transcript and protein.
	Leishmania amazonensis	D	ED-1 ED-4	[234]	Leishmania or soluble Leishmania antigen inhibited CD1a expression, but did not prevent further DC maturation toward $CD83^+$ mDCs.
	Leishmania donovani and Leishmania major	D	OED	[235]	*In vitro* G4-induced iDCs were infected with *L. donovani* or *L. major* on day 7 and tested for CD1a/b/c/d expression 8 h later. Down-regulation of mainly Group I CD1 molecules at the transcriptional (qRT-PCR) and surface expression levels was detected.
	Toxoplasma gondii	NC	OED	[236]	*In vitro* infection of untreated MOs with *T. gondii* does not induce CD1a.
Helminthes	*Necator Americanus*	D	OED	[237]	*In vitro* G4-induced CD1a in mDCs is lower when MOs were obtained from infected pts versus normal donors.
	Echinococcus granulosus	D	ED-1 ED-4	[238, 239]	Hydatid cyst components (AgB- and SHF) down-regulate CD1a and further prevent IL-12 production, increasing IL-10 release.
Viruses *(for HIV& HTLV-I see text)*	Human Herpes Virus-8 (alive or UV-inactivated)	D	ED-1	[240]	Reduced mDC activity and sixfold reduction in IL-12 (p70) production with consequent impairment of T-cell-mediated responses.
	Cytomegalovirus	D	ED-1	[241]	CD1a assay at day 7, before adding LPS (to evaluate iDC CD1a expression). Down-regulation of CD1a occurs also with UV-inactivated virus. Moreover, HCMV-infected mDCs were unable to induce a T-cell response, in line with the immunodepressive effects of HCMV infection.

Table 5: Continued.

Infectious Agent	Agent	CD1[a]	ED[b]	Ref.[c]	Observations
Micro-Organism products					
Bordetella pertussis	Pertussis toxin	D	ED-1	[242]	Selective suppression of CD1a (mRNA and protein) but not of CD1b and CD1c. LPS-induced mDCs are functionally normal.
Candida albicans	Supernatant	D	ED-1	[243]	*C. albicans* supernatants contain a glycoprotein termed "Secretory IL-12 Inhibitory Factor", able to down-regulate CD1a expression and IL-12 production by iDCs and DCs.
Dengue	Dengue-2 (live attenuated vaccines)	U	ED-2	[244]	DC maturation step 2 was attained with live attenuate vaccine LAV2 or DEN2 without adding LPS. Heat-inactivated virus was used as a negative control for virus infection. CD1a assay was performed after incubation at 32°C for 48 h.
Malaria	Hemozoin (malaria pigment)	D	ED-1 ED-4	[245]	*Mechanism*: increased PPARγ expression (qRT-PCR) following hemozoin-induced activation in MOs.
	Atypical Membrane Antigen-1 (AMA-1)	U	ED-4	[246]	mDCs of *P. vivax* infected pts show lower CD1a expression than that of mDCs developed *in vitro* from MOs of noninfected controls. *In vitro* exposure to AMA-1 increases CD1a levels in mDCs developed *in vitro* from MOs of infected donors.
Mycotoxins	T-2 toxin	D	ED-1	[247]	Strong inhibition.
Staphylococcus aureus	Staphylococcus superantigen	U	IvDC	[248]	CD1a$^+$ cell number in the epidermis was significantly higher in the lesional skin with respect to that in non-lesional skin from atopic dermatitis pts or to that in the skin from normal donors.
Various bacteria	LPS	D	ED-1	[249]	LPS (from *Salmonella*) was added to MOs+G4 culture on day 0 instead of on day 5–9, as usually used for inducing iDC maturation to mDCs. *Mechanism:* in part by induction of IL-10, and mostly by MAPKp38 activation followed by ERK and NF-*k*B inactivation.

[a]Evaluation of CD1a expression if not otherwise specified: U: upregulation; D: down-regulation.
[b]Experimental design code (see Figure 2).
[c]Reference number.

planning new types of antimycobacterial vaccines. It is reasonable to predict that properly designed siRNA(s) could be combined in a near future, with BCG or BCG-like vaccines in order to obtain *gene silencing vaccines* able to inactivate the intracellular signals responsible of Group I CD1 protein suppression.

Abbreviations

Ac2SGL: Acylated sulfoglycolipid
APC: Antigen-presenting cells
ASA: Acetylsalicylic acid
ATF-2: Activating transcription factor

ATRA: All trans-retinoic acid
AZT: Zidovudine
BCG: Bacillus Calmette-Guerin
BDP: Beclomethasone dipropionate
CRE: cAMP response element
CREB-1: CRE-binding protein
DC: Dendritic cells
DEX: Dexamethasone
ERK: Extracellular signal-regulated kinases
G4: GM-CSF + IL-4
GA: Glatiramer acetate
GM-CSF: Granulocyte-macrophage colony stimulating factor
GST: Gold sodium thiomalate
HDAC: Histone deacetylases
iDC: Immature dendritic cells
IFN: Interferon
IL: Interleukin
LC: Langerhans cells
LPS: Lipopolysaccharides
MAPK: Mitogen-activated protein kinase
mDC: Mature dendritic cells
MDR: Multidrug resistance
MIN: Minocycline
MM: Multiple myeloma
MMF: Monomethyl-fumarate
MOs: Monocytes
MRP1: Multidrug resistance protein 1
MS: Multiple sclerosis
MTP: Microsomal triglyceride transfer protein
NFA: Niflumic acid
NSAID: Nonsteroidal anti-inflammatory drugs
PBMN: Peripheral blood mononuclear cells
PPAR: Peroxisome proliferator-activated receptor
pts: Patients
RA: Rheumatoid arthritis
TCR: T-cell receptor
TLR: Toll-like receptors
TNF: Tumor necrosis factor.

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Research Article

Immunopathology of Postprimary Tuberculosis: Increased T-Regulatory Cells and DEC-205-Positive Foamy Macrophages in Cavitary Lesions

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Postprimary tuberculosis occurs in immunocompetent people infected with *Mycobacterium tuberculosis*. It is restricted to the lung and accounts for 80% of cases and nearly 100% of transmission. Little is known about the immunopathology of postprimary tuberculosis due to limited availability of specimens. Tissues from 30 autopsy cases of pulmonary tuberculosis were located. Sections of characteristic lesions of caseating granulomas, lipid pneumonia, and cavitary stages of postprimary disease were selected for immunohistochemical studies of macrophages, lymphocytes, endothelial cells, and mycobacterial antigens. A higher percentage of cells in lipid pneumonia (36.1%) and cavitary lesions (27.8%) were positive for the dendritic cell marker DEC-205, compared to granulomas (9.0%, $P < .05$). Cavities contained significantly more T-regulatory cells (14.8%) than found in lipid pneumonia (5.2%) or granulomas (4.8%). Distribution of the immune cell types may contribute to the inability of the immune system to eradicate tuberculosis.

1. Introduction

Mycobacterium tuberculosis (MTB) is endemic in every part of the world. This organism accounts for nearly 2 million deaths annually, making it the leading bacterial cause of death worldwide [1]. Once thought to be controlled, tuberculosis (TB) incidence is rising in many areas caused in part by the emergence of drug-resistant strains and the HIV epidemic. Furthermore, nearly one-third of the world is latently infected with MTB, making eradication of the organism difficult.

MTB infection begins as primary TB with the deposition of bacilli in the alveoli, which are phagocytosed by alveolar macrophages [2]. Numerous animal models of primary TB are available and much is known about the immune responses that drive granuloma development [3]. Macrophage production of TNF-α and chemokines recruits systemic monocytes which form the nascent granuloma. Adaptive immunity develops after infected dendritic cells (DCs) migrate to a draining lymph node [4, 5]. Neutrophils may also facilitate the presentation and migration of mycobacterial antigens to draining lymph nodes [6]. IL-12 production by DCs promotes the development of IFN-γ Th1 cells that activate macrophages, thereby stabilizing bacterial growth [3]. The development of an acquired immune response results in the formation of a mature caseating granuloma that consists of central necrosis and infected macrophages surrounded by epitheliod and foamy macrophages and an outer layer of fibrosis associated with lymphocytes [7]. Most of such lesions heal, but viable organisms persist. Patients at this stage of infection are considered latently infected because they lack clinical symptoms and have stable chest X-rays, and sputums are negative for acid-fast bacilli [8]. The TB bacilli persist, despite the strong immune response needed to generate granulomas. This is possibly explained by the finding that the acquired immune response to MTB is delayed in comparison to other infectious diseases [9].

A fraction of latently infected individuals develop reactivation of infection or are reinfected from the environment [10]. Such infection of a previously immune

or sensitized person produces a very different type of disease known as postprimary TB (also referred to as adult type or secondary infection). Postprimary TB is typically restricted to the lung where it produces cavities that are responsible for 80% of all TB disease and virtually 100% of transmission of the bacilli from person to person [11]. Postprimary TB occurs characteristically in young immunocompetent adults with sufficient immunity to control primary TB [12].

It is a paradigm of contemporary research that caseating granulomas are the characteristic lesion of both primary and postprimary TB and that cavities form by expansion of caseating granulomas with softening and liquefaction of their contents that are discharged as they erode into bronchi. We previously reported studies of untreated primary and postprimary TB that contradicted this paradigm [13]. Based on our observations from preantibiotic era derived samples, we hypothesized that postprimary TB begins as a lipid pneumonia in which infection is restricted to foamy alveolar macrophages. Therefore, resultant cavities appeared to result from necrosis of tuberculous pneumonia in individuals who had no histologic evidence of caseating granulomas. The present studies were undertaken to characterize the cellular makeup of each of the characteristic stages of human pulmonary TB in immunocompetent adults. We examined slides of the lungs of 30 people who died of pulmonary TB and selected representative sections of each of the major stages of the disease. These were (1) caseating granulomas of primary TB, (2) lipid pneumonia, and (3) cavitary lesions. These sections were studied with immunohistochemical markers of T-cell subsets, macrophages, DCs, and endothelial cells based on published studies of these markers in animal models of TB. Immunohistochemical analysis of MTB antigens and acid fast bacilli (AFB) staining was included to explore our hypothesis that a progressive buildup of mycobacterial antigens precedes and contributes to the rapid necrosis of caseous pneumonia [11]. These studies were accomplished with a multispectral imaging microscope and image analysis software that facilitates accurate differentiation and quantitation of multiple cell types with precise quantitation of immunohistochemical markers.

The results demonstrate distinct patterns of immune cells in each of the stages of pulmonary TB. Foamy macrophages (FMs) found in lipid pneumonia and in the walls of cavitary lesions were frequently positive for the DC marker DEC-205. An increased percentage of T-regulatory (Treg) cells in the cavity walls of TB provide evidence of an active role in the disease process. It is hypothesized that the local accumulation of immune cells with suppressive properties may contribute to the chronic nature of MTB infection.

2. Methods

2.1. Tissue Specimens. Formalin-fixed, paraffin-embedded tissue blocks from patients with postprimary TB were obtained from the First Infectious Disease Hospital, St. Petersburg, Russian Federation. Specimens were from archived cases of autopsies of patients who had died of pulmonary TB and were culture positive for MTB or had positive stains for acid-fast bacilli. Thirty cases of postprimary TB patients were available for investigation. Although detailed information is not available, all patients were HIV negative. All cases were stained with hematoxylin and eosin and acid fast stained by the Ziehl-Neelsen method per standard protocols.

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of UT-Houston Medical School IRB protocol number HSC-MS-10-0109, Immunopathology of Tuberculosis. We studied microscope slides of tissues from patients with tuberculosis. All materials were byproducts of regular autopsy practice and were obtained after completion of all medical needs. The specimens were deidentified for the study.

2.2. Immunohistochemistry Staining for Immune Cell Markers. Five-μm tissue sections were deparaffinized and stained with monoclonal antibodies for CD4 (Leica-Microsystems, Bannockburn, IL), CD8 (Dako, Carpinteria, CA), CD20 (Dako), CD31 (Leica-Microsystems), CD68 (Dako), DEC-205 (Santa Cruz Biotechnology, Santa Cruz, CA), and Foxp3 (Abcam, Cambridge, MA). Mouse monoclonal antibodies were used for all immune cell markers. A rabbit polyclonal antibody to purified protein derivative (Abcam) was used for the detection of MTB. The pretreatments, dilutions, and incubation times are shown in Table 1. After washing, sections were incubated for one hour with antimouse (Dako) or antirabbit (Biocare Medical, Concord, CA) polyclonal antibodies labeled with HRP.

2.3. Photography and Image Analysis. All images were taken using the Nuance multispectral imaging system (CRI, Woburn, MA), which allowed enumeration of cellular phenotypes in defined areas of pathology. Three distinct pathologic manifestations of TB were analyzed that included granulomas, lipid pneumonia, and cavitary lesions. Images from at least 5 cases of the defined pathological manifestations were captured with the 10x objective. Quantification of immune cells expressing specific markers was performed with the tissue and cell segmenting functions of Inform software (CRI), according to the manufacturer's instructions. The total number of cells in an image was determined using the hematoxylin stain.

3. Results

3.1. Granulomatous Pathology. Three distinct patterns of pathology of pulmonary TB were analyzed. The first type is the caseating granuloma characteristic of primary TB. Similar caseating granulomas were observed in primary TB, miliary TB, and chronic fibrocaseous postprimary TB. A representative image is shown in Figure 1(a). A central region of caseous necrosis is surrounded by foamy and epitheliod macrophages, often presenting with Langhan's giant cells (Figure 1(b)). The percentages of different immune cell markers obtained by quantitative image analysis are shown in Table 2. Granulomas contained an abundance of CD68+

Table 1: Conditions for immunohistochemistry staining of immune cell markers and MTB.

Marker	Pretreatment	Dilution	Incubation time[a]
CD4	steam for 20 minutes in EDTA, pH 8.0	1 : 25	45
CD8	steam for 20 minutes in EDTA, pH 8.0	1 : 125	30
CD20	5 minutes under pressure in citrate buffer, pH 6.0	1 : 800	20
CD31	5 minutes under pressure in citrate buffer, pH 6.0	1 : 100	30
CD68	5 minutes under pressure in citrate buffer, pH 6.0	1 : 2000	20
DEC-205	steam for 20 minutes in EDTA, pH 8.0	1 : 50	overnight
Foxp3	5 minutes under pressure in citrate buffer, pH 6.0	1 : 100	30
MTB	Proteinase K	1 : 100	60

[a] minutes.

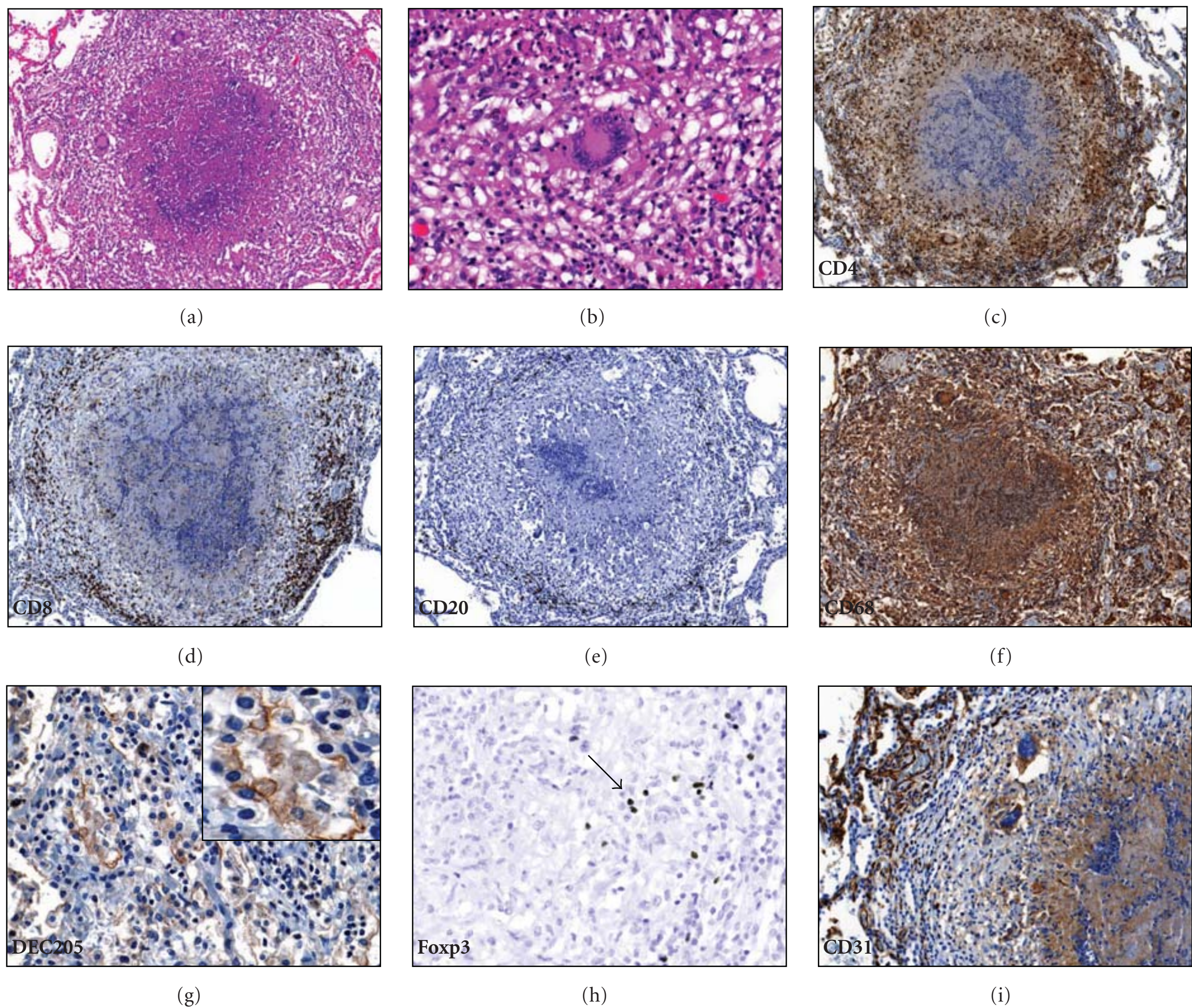


Figure 1: Granuloma histopathology. (a) Typical caseating granuloma, with central necrosis flanked by epitheliod cells and foamy macrophages. The periphery of the granuloma consists of lymphocytes, 100x. (b) Langhan's giant cell, 400x. (c) Abundant CD4+ cells in the granuloma include both lymphocytes and macrophages, 100x. (d) CD8+ cells were mainly located in the periphery of the granuloma, 100x. (e) B-cells, identified by the CD20 stain, were noted in the outer layer of the granuloma. (f) Granulomas stained heavily for the CD68 marker, 100x. (g) Macrophages at the periphery of the granuloma stain positive for the dendritic cell marker DEC-205, 400x. The insert demonstrates a digitally zoomed region to detail the DEC-205 staining. (h) T-regulatory cells, identified by the Foxp3 marker (arrow), were found in the lymphocytic layer of the granuloma, 400x. (i) CD31 stain shows well-vascularized tissue at the periphery of the granuloma, 200x.

macrophages and CD4+ lymphocytes (Figures 1(f) and 1(c)). CD8+ lymphocytes, as well as B-cells identified by the CD20 marker, were found in the lymphocytic outer region of the granuloma (Figures 1(d) and 1(e)). The outermost layer of the granuloma near the alveoli consisted of FMs that were frequently positive for the DC marker DEC-205 (Figure 1(g)). Foxp3, a marker for Treg cells, were occasionally noted interspersed between other stained lymphocytes (Figure 1(h)). The periphery of granulomas was well-vascularized as assessed by the CD31 stain (Figure 1(i)).

3.2. Lipid Pneumonia. The alveoli were filled with FMs positive for the CD68 marker with lymphocytes present in the alveolar walls (Figures 2(a)–2(c)). Cells in the alveoli occasionally had a "dry" appearance with areas of fibrosis (Figure 2(a)) or a homogenous consistency of necrotic material (Figure 2(c)). Bronchial obstruction was frequently present [13]. CD4+ cells were present at significantly less frequency compared to numbers seen in granulomas ($P < .05$, Table 2 and Figure 2(d)). CD8+ cells were frequently observed in the alveolar walls as well as the alveoli (Figure 2(e)). B-cells were rarely noted in the regions of lipid pneumonia but were observed to occur in large clusters in regions that bordered the lipid pneumonia (not shown). The FMs in the alveoli were frequently positive for the DC marker DEC-205 (Figure 2(g)). Treg cells were noted in the alveoli but were scarce elsewhere (Figure 2(h)). Disrupted lung architecture of developing caseaous necrosis was readily apparent on the CD31 stain of vascular endothelial cells (Figure 2(i)).

3.3. Cavitary Lesions. An example of a cavity that appears to be developing by necrosis and fragmentation of lipid pneumonia is shown in Figure 3(a). The cavity wall is made up of necrotic debris, foamy macrophages, and lymphocytes. Abundant fibrosis develops as the lesion becomes chronic (Figure 3(b)). The lymphocytes lining the walls of mature cavities were a mixture of CD4, CD8, and CD20 positive cells (Table 2 and Figures 3(c)–3(e)). The FMs lining the cavity wall were strongly positive for the DC marker DEC-205 (Figure 3(g)). Numerous Treg cells were present around the cavities (Figure 3(h)). The walls of the cavitary lesions contained abundant granulation tissue (Figure 3(i)). None of the cavitary lesions were directly associated with caseous granulomatous pathology.

3.4. Localization of MTB Antigens and Acid-Fast Bacilli. MTB were rarely observed in granulomas by either Zhiel-Neelsen staining or immunohistochemistry for MTB. Representative images from cases of lipid pneumonia and cavitary lesions are shown in Figure 4. Early lipid pneumonia demonstrated evidence of mycobacterial antigens throughout alveoli, but very few acid-fast bacilli were seen (Figures 4(a)–4(c)). As the lipid pneumonia undergoes necrosis, both abundant tuberculin antigens and bacilli are evident (Figures 4(d)–4(f)). Cavities had roughly equal amounts of antigen and bacteria, most of which were localized to regions representing the cavity wall (Figures 4(g)–4(i)).

4. Discussion

A central question in TB research is why the immune system successfully controls primary TB but fails to clear a postprimary TB cavity and thus halt transmission of infection. A problem is that investigators have little to study since few specimens of human pulmonary TB are available to them. Recent descriptions of specimens recovered from surgical cases of patients with cavitary TB are available [14]. This project was built on earlier studies that described the histopathology of the stages of postprimary TB [13]. To our knowledge, this study is the first recent description of postprimary TB that includes Tregs and cells with the DEC-205 marker.

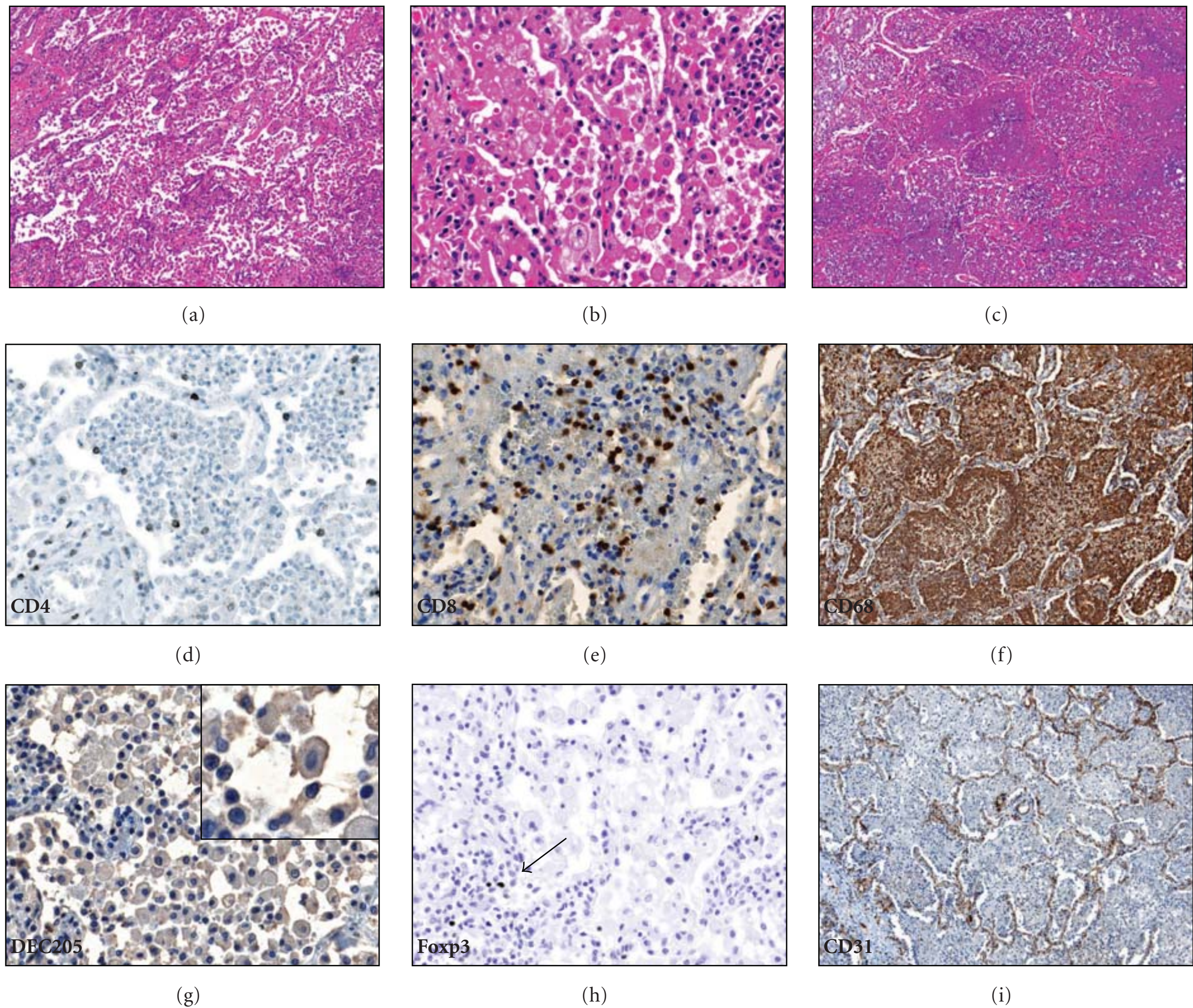
Histopathologic examination of the 30 cases in this study supported our previous hypothesis that postprimary TB begins as a lipid pneumonia [13]. Other authors suggest that cavities develop when granulomas rupture into the airways and that lipid pneumonia is a complication of cavitary TB [2, 3, 15]. Analysis of the development of cavities is beyond the scope of this paper. Nevertheless, a key question of postprimary TB is how MTB survive and proliferate in alveolar macrophages in restricted areas of lung while the entire rest of the body remains highly immune. The present studies provide some clues. Foamy macrophages positive for the DC marker DEC-205 accumulate in the alveoli with CD8+ lymphocytes in the alveolar walls and alveoli. Ordway and colleagues [16] reported that FMs from murine granulomas were strongly positive for the DC marker DEC-205. Interestingly, a high percentage of FMs found in lipid pneumonia and those lining the cavity walls were positive for DEC-205. The FMs in mice were reported to have additional markers of DCs such as CD11c, MHC class II, and CD40 as well as high levels of antiapoptotic markers [16]. The significance of FMs expressing DC markers is not yet clear. However, MTB-infected DCs can harbor the bacilli for extended periods of time and do not have efficient mechanisms of eliminating MTB [17–19]. FMs with characteristics of DCs may therefore provide a permissive environment for the growth of mycobacteria. Furthermore, FMs may be a tissue source of immunosuppression that inhibits the cell-mediated immunity necessary to clear MTB. For example, FMs secrete high levels of transforming growth factor-beta (TGF-β) that can cause apoptosis of immune effector cells [20]. Additionally, FMs produce high levels of inducible nitric oxide synthetase, which has been associated with suppression of T-cells in murine MTB infection [21]. Therefore, FMs may be a contributor to the local immunosuppression responsible for the inability of the immune system to eliminate postprimary TB.

Organisms in cavities typically grow in massive numbers on the inner surface of a cavity but nowhere else in the body even though other parts of the body, particularly the lung, are continually exposed to large numbers of virulent organisms being coughed into the environment. The surface of a cavity has been described as an area of "failed immunity" [22]. This study found a significantly increased percentage of Treg cells in the cavitary wall compared to other tissue manifestations of TB. Treg cells, identified by

Table 2: Percentage of cells positive for immunological markers.

	Primary TB Granuloma	Postprimary TB Lipid pneumonia	Postprimary TB Cavitary lesion
Marker			
CD4	30.7 ± 4.1	10.1 ± 3.1[a]	23.1 ± 4.6[a,b]
CD8	17.8 ± 4.2	19.6 ± 3.8	27.7 ± 7.1
CD20	2.0 ± 0.5	2.3 ± 0.7	7.8 ± 1.2[a,b]
CD68	49.4 ± 5.0	64.0 ± 12.5	55.1 ± 14.8
DEC-205	9.0 ± 3.5	36.1 ± 8.5[a]	27.8 ± 8.5[a]
Foxp3	4.8 ± 1.1	5.2 ± 1.2	14.8 ± 4.3[a,b]

Images of the different tissue manifestation of TB were taken using the 10x objective from at least 5 regions of multiple cases. The total number of cells was determined using the hematoxylin stain. The data are presented as the percentage of cells positive for a given marker, ±SD.
[a] $P < .05$, comparison to granuloma.
[b] $P < .05$, comparison to lipid pneumonia.



(a) (b) (c) (d) (e) (f) (g) (h) (i)

Figure 2: Lipid pneumonia. (a) Alveoli are filled with macrophages and the alveolar walls have high numbers of lymphocytes, 100x. (b) Macrophages in the lipid pneumonia have a foamy appearance, 400x. (c) The cells in the alveoli may undergo necrosis to produce a homogenous appearance, 100x. (d) Relatively few CD4+ cells are present in lipid pneumonia, 400x. (e) Abundant CD8+ cells are noted in the alveoli as well as in the alveolar walls, 400x. (f) Foamy cells stain strongly with the CD68 marker. (g) Foamy macrophages in the lipid pneumonia are frequently positive for the dendritic cell marker DEC-205, 400x. The insert shows a detailed image of a DEC-205-positive macrophage. (h) T-regulatory cells were observed in regions of lipid pneumonia (arrow), 400x. (i) The CD31 stain highlights the disrupted lung architecture, 100x.

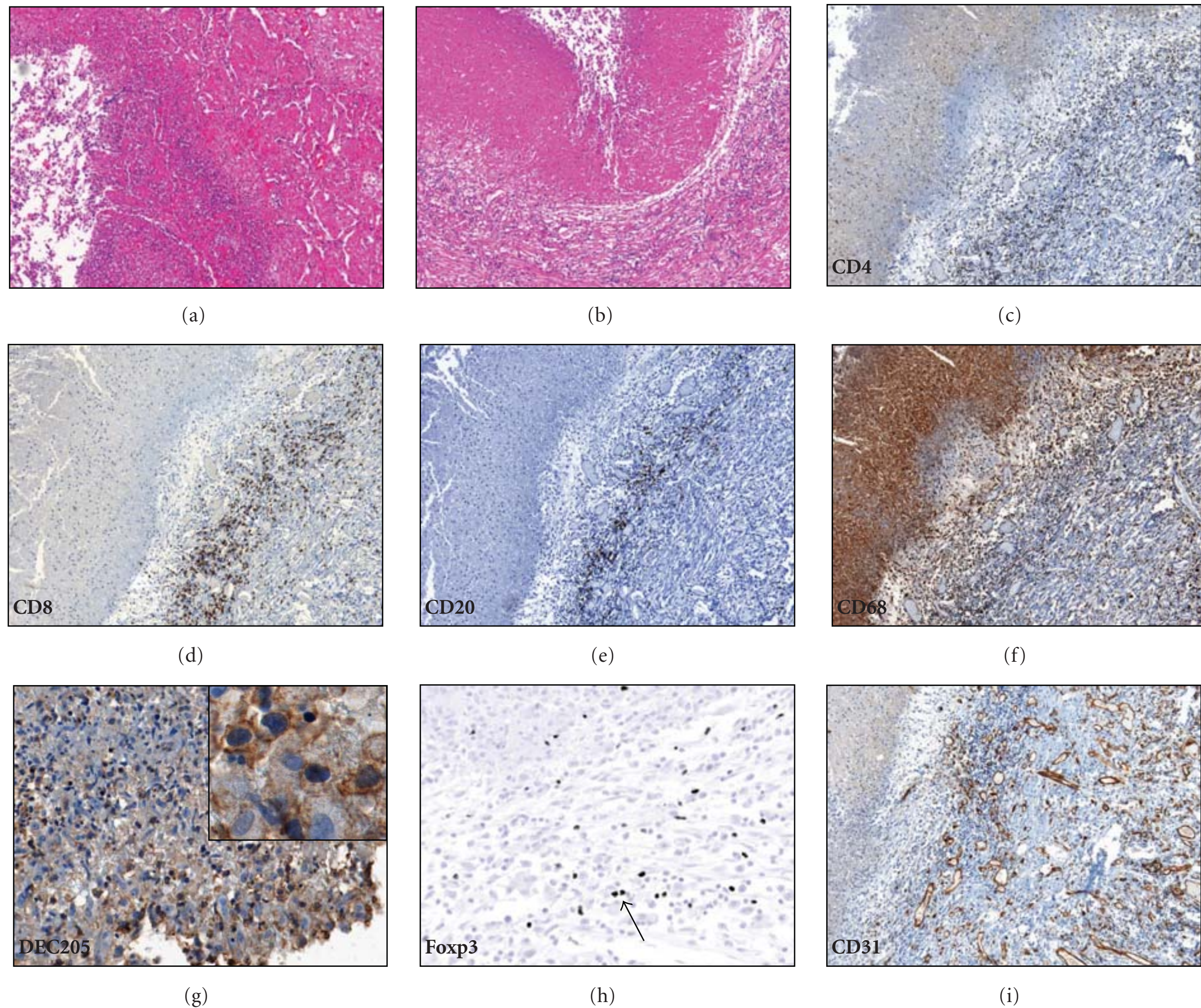


Figure 3: Immunopathology of cavitary lesions. (a) Early cavitary lesion, surrounded by lipid pneumonia, 100x. (b) Chronic cavitary lesion. The cavity wall consists of necrotic material, foamy macrophages, and fibrous tissue with abundant lymphocytes, 100x. (c) Numerous CD4+ cells make up the lymphocytic portion of a cavity wall, 100x. (d) CD8+ cells in the wall of a cavity, 100x. (e) CD20+ cells in the wall of a cavitary lesion, 100x. (f) The cells lining the cavity are CD68+ macrophages, 100x. (g) Foamy macrophages lining the cavity wall are positive for the dendritic cell marker DEC-205, 400x. Insert demonstrates a region detailing the staining for DEC-205. (h) Numerous Foxp3+ cells (arrow) were found in the cavity wall, 400x. (i) Abundant granulation tissue in the wall of a cavitary lesion noted with the CD31 stain, 100x.

expression of the transcription factor Foxp3, are essential for preventing self-reactive immune responses and limiting immune-mediated tissue damage during infection [23, 24]. However, the role of Tregs in infectious diseases such as TB is complex. Tregs produce inhibitory cytokines, such as IL-10 and TGF-β, that protect host tissue by limiting excess inflammation but may conversely limit clearance of pathogens [25]. Peripheral blood Treg cells are decreased in newly infected contacts of TB patients, possibly due to accumulation in infected lung [26]. As the infection progresses, Tregs expand to regions of disease sites, as well as in blood [27–30]. Differential expression of Foxp3 in PBMCs was predictive of active TB versus latent infection [31]. Of significance, Foxp3 cells decreased T-cell-mediated responses to mycobacterial antigens in human TB [32]. Depletion of Treg cells during a murine model of infection enhanced MTB elimination [33, 34], providing further evidence that Treg cells inhibit the clearance of MTB. A recent study demonstrated that small numbers of MTB-specific Tregs inhibit the accumulation of CD4 and CD8 T-cells in the lungs of infected mice [35]. The findings from our study demonstrate high numbers of Treg cells in a tissue manifestation of TB from which the bacilli are frequently not eradicated, providing further support to the hypothesis that accumulation of Tregs at the sites of MTB infection suppresses the activation of protective immune responses. However, it should be noted that Treg activity was not the limiting factor in the effectiveness of BCG vaccination of mice [36]. However, since mice do not develop cavities, the interpretation of such studies for the human host response to cavities must be taken with caution.

These studies also confirmed earlier observations that large amounts of antigens of MTB demonstrable by immunohistochemistry are present in alveoli of the lipid

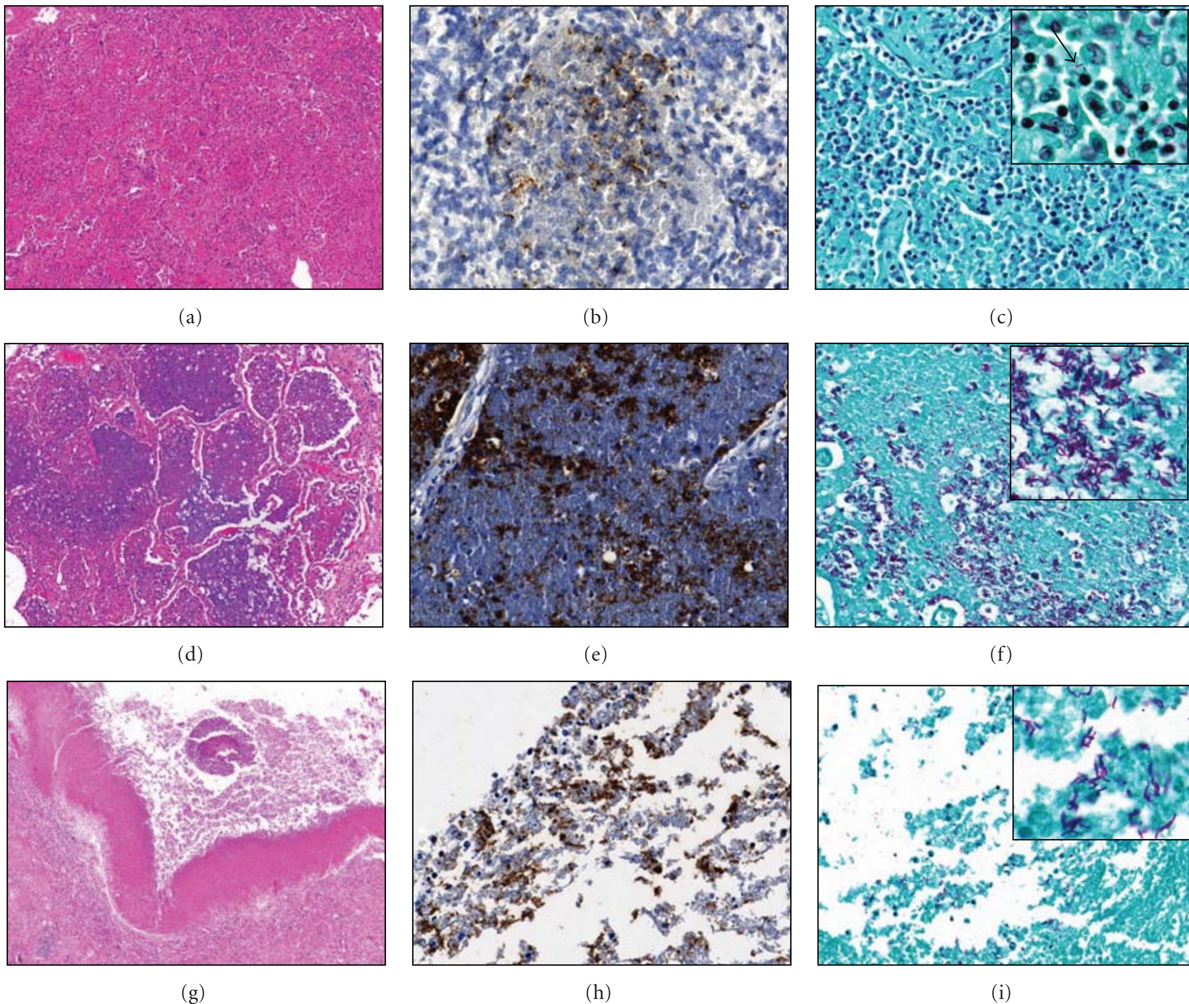


Figure 4: Comparison of immunohistochemistry (IHC) for MTB and Zhiel-Neelsen acid fast staining. Early lipid pneumonia (a)–(c). (a) H&E stain demonstrates macrophages and lymphocytes within the alveoli, 100x. (b) MTB are noted on the IHC, 400x. (c) Few AFB are observed by Zhiel-Neelsen staining, 400x. Lipid pneumonia undergoing abrupt necrosis (d)–(f). (d) Cells in alveoli undergo necrosis and have a homogenous appearance, 100x. (e) Abundant mycobacterial antigens noted on the IHC stain for MTB, 400x. (f) Numerous acid-fast bacilli are observed, 400x. Cavitary TB (g)–(i). (g) H&E stain showing a cavity with a piece of detached lung and necrotic debris, 40x. (h) The wall of the cavity is strongly positive for MTB antigens, 400x. (i) Acid-fast bacilli are found lining the cavity wall, 400x.

pneumonia [13]. In this stage, there was far more MTB antigen in alveoli than organisms observed by acid-fast staining. Much of it was present in structures that were distinct from aggregates of AFB. We hypothesize that mycobacterial antigens accumulate progressively in alveoli during this stage of disease. This is similar to the accumulation of MTB antigens in alveoli of slowly progressive pulmonary TB in mice [13]. We have proposed that this build up of mycobacterial antigens including glycolipids is an essential precursor to the abrupt necrosis that produces cavities [7, 37, 38].

This study has a number of important limitations. Tissues from autopsy cases of patients who died of pulmonary TB were received as deidentified paraffin-embedded blocks. While we were told that the tissues came from untreated or inadequately treated patients, detailed clinical information on a case-by-case basis is not available. Nonetheless, this study provides important details of the cellular phenotypes in characteristic tissue manifestations of postprimary TB.

5. Conclusions

In summary, better understanding of the pathology of the multiple stages of postprimary disease has facilitated investigations of manifestations of the disease that have heretofore been unapproachable by modern science. The key questions are how does MTB establish and survive in two privileged sites in the upper lobes of lungs of hosts who are strongly immune in all other parts of the body. The first site is an endogenous lipid pneumonia in which MTB survive only in foamy alveolar macrophages in parts of the lung. The second is a mature cavity in which MTB proliferate in massive numbers on the cavitary surface, but nowhere else. The present studies demonstrate

that these questions can be approached with modern quantitative immunohistochemical technologies and that different host responses are operative at each stage of the disease. The results demonstrate that both types of privileged sites are characterized by an increased percentage of cell phenotypes with immunosuppressive properties that may be a critical component responsible for the inability of the immune system to clear MTB. It is anticipated that a better understanding of the immunopathology of MTB, and the molecular mechanisms governing the transition from latent to active disease will ultimately lead to more effective control of TB.

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Research Article

Treatment of Disseminated Mycobacterial Infection with High-Dose IFN-γ in a Patient with IL-12Rβ1 Deficiency

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IFN-γ has been used in the treatment of IL-12Rβ1 deficiency patients with disseminated BCG infection (BCGosis), but the optimal dose to reach efficacy is not clear. We used IFN-γ in the treatment of a 2.7-year-old patient with IL-12Rβ1 deficiency and refractory BCG-osis. IFNγ was started at a dose of 50 μg/m^2 3 times per week. The dose was upgraded to 100 mcg/m^2 after 3 months, then to 200 mcg/m^2 6 months afterwards. Serum mycobactericidal activity and lymphocytes number and function were evaluated throughout the study. There was no clinical response to IFN-γ with 50 or 100 μg/m^2 doses. However, there was some response to the 200 μg/m^2 dose with no additional adverse effects. The serum mycobactericidal activity was not significantly different during the whole treatment period. Lymphocytes proliferation in response to PHA was significantly higher after 3 months of using the highest dose as compared to the lowest dose. The tuberculin skin test reaction remained persistently negative. We conclude that in a patient with IL-12Rβ1 deficiency, IFN-γ at a dose of 200 μg/m^2, but not at lower dosages, was found to have a noticeable clinical effect with no additional adverse effects.

1. Introduction

Investigation of a human syndrome known as Mendelian susceptibility to mycobacterial diseases (MSMD) (OMIM 209950) has, in the past 15 years, led to the identification of a series of genetic defects in the IL-12/IFN-γ axis. These include defects in three autosomal genes controlling the response to IFN-γ: *IFNGR1*, encoding the ligand binding, first chain of the IFN-γ receptor; *IFNGR2*, encoding the signaling, second chain of the IFN-γ receptor; *STAT1*, encoding the signal transducer and activator of transcription 1 downstream from IFN-γ receptor. They also include defects in two other autosomal genes controlling the production of IFN-γ: *IL12B*, encoding IL-12p40 shared by IL-12 and IL-23; *IL12RB1*, encoding the first chain of the IL-12 and IL-23 receptor (IL-12Rβ1). In addition, there is one X-linked gene that encodes nuclear factor-kB essential modulator (*NEMO*) [1–3].

IL-12Rβ1 defect was first described in 1998 [4] and is the most common among the known genetic disorders that predispose to mycobacterial infections. The spectrum of infections reported in these patients is, however, surprisingly narrow. These patients display selective susceptibility to weakly virulent *Mycobacteria*, such as environmental mycobacteria (EM) and live bacille Calmette-Guérin (BCG) vaccine (an attenuated substrain of *Mycobacterium bovis*), and *Salmonella*. They showed almost no increased susceptibility to other pathogens including other bacteria

and ubiquitously distributed viruses and fungi [5]. Evidence suggests that the immune system is redundant in its response to many intracellular microorganisms. For other organisms like varicella zoster virus, Th1 activation and IFN-γ production was found to be stimulated by IFN-α not IL-12 [6].

In a very recent report, that included the largest cohort of patients with IL-12Rβ1 deficiency (141 patients) [7], the mean age of onset of the first infection was 2.4 years. The severity of the disease varied significantly from subjects who were asymptomatic until adulthood to patients who died in early childhood from complications of the disease. The mortality rate was 32% among symptomatic subjects and the mean age of death was 7.5 years, mostly secondary to BCGosis or EM disease.

In patients with IL-12Rβ1 deficiency who do not respond to prolonged therapy with multiple anti-mycobacterial drugs, the mycobacteria frequently develop resistance to some of these medications. The management of those patients subsequently becomes very difficult and frequently they will not recover using antimycobacterials alone. Since the most important reason of those patients' failure to control mycobacterial infections is the inability of their T cells and NK cells to produce IFN-γ in response to IL-12 stimulation, IFN-γ is an attractive candidate therapy that can be tried in those patients [8]. However, the optimal dose and duration of using IFN-γ are not clear.

2. Subject and Methods

2.1. Study Patient. Our patient was referred to us at 6 months of age with left axillary lymphadenitis that grew the vaccine strain of *Mycobacterium bovis* (BCG), a routine vaccine given in day one of life in Saudi Arabia. There was no granuloma formation on the lymph node biopsy. She was otherwise well apart from intermittent fever and night sweats. Her parents were not consanguineous but from the same tribe. She had a 5-years-old brother who had left axillary BCGitis in infancy that resolved promptly after treatment with isoniazid alone for 6 months. She also had a 7-years-old sister who has been completely healthy. She did not respond to isoniazide and rifampicin. Sensitivity results then showed that the organism was resistant to both, so she was started on ethambutol 300 mg OD, cycloserine 250 mg OD, and moxifloxacin 200 mg OD for which the organism was sensitive. Meanwhile, immunological investigations revealed that our patient's lymphocytes have no IFN-γ production in response to IL-12+BCG as compared to controls (those investigations were performed in Dr. Casanova's lab in Paris. Her parents were travel controls). Her brother had a similar cellular phenotype (i.e., no IFN-γ production after BCG and BCG+IL-12 stimulation in whole-blood assay) (Table 1). In addition, no cell surface-expressed IL12RB1 could be detected from their PHA-T cell blast (data not shown). Genetic testing confirmed that she and her brother have homozygous 1336delC mutation in the *IL12RB1* gene, leading to complete IL12Rβ1 deficiency. Her sister was functionally and genetically normal. To our knowledge this is the only family with this specific type of mutation to be reported [7].

Table 1: In vitro Lymphocytes' IFN-γ production in different conditions.

Stimulant	IFN-γ (pg/ml)					
	Control	Father	Mother	Sister	Brother	Patient
Medium	0	62	62	0	0	0
BCG	738	202	68	47	35	0
BCG+IL12	44461	6000	4233	988	13	0

2.2. Study Plan. After parental consent, baseline and followup investigations were obtained including CBC, ESR, liver enzymes, lymphocytes subsets, and tuberculin skin test (TST) every 3 months. Immunoglobulin levels, serum mycobactericidal activity, and lymphocytes proliferation in vitro were performed every 6 months.

The patient was seen in the clinic every 6 weeks. IFN-γ-1b (Imukin, Boehringer Ingelheim) was started at a dose of 50 μg/m^2 subcutaneously 3 times/week. If there is no significant response within 3 months the dose will be doubled to 100 mcg/m^2, at the same frequency.

2.3. Cellular Studies. Blood samples were taken from the patient before starting treatment with IFNγ (S1), 6 months (S2) and 12 months (S3) after starting treatment. PBMCs were isolated from whole blood by density centrifugation (Lymphoprep, Nycomed, Oslo, Norway) and assayed for in vitro proliferative responses by the thymidine incorporation method against phytohemagglutinin (PHA) (Sigma, St. Louis, Mo.) and IL2 (R&D System, Abington, UK).

The proliferation results are expressed as mean count per minute of triplicate cultures for the antigen concentration giving maximum response minus the mean count-per-minute values for 12 wells without antigen (medium only).

2.4. Serum Mycobactericidal Assay. Two specimens of the patient's serum were collected at 6 months and 12 months of the study period, 2 hours after anti-mycobacterial drug administration, to determine the serum inhibitory and bactericidal titers against the patient's mycobacterial isolate as well as mycobacterium bovis BCG vaccine strain (Statens Serum Institute, Copenhagen S, Denmark) and mycobacterium tuberculosis H37RV reference strain (ATCC, Atlanta, GA, USA), as previously described [9].

3. Results

Our patient was started on IFN-γ at a dose of 50 μg/m^2 three times a week in addition to her anti-mycobacterial medications as above (Figure 1). Three months later, the patient's condition was slightly worse, so ethionamide 250 mg OD was added to her drug regimen and IFN-γ dose was upgraded

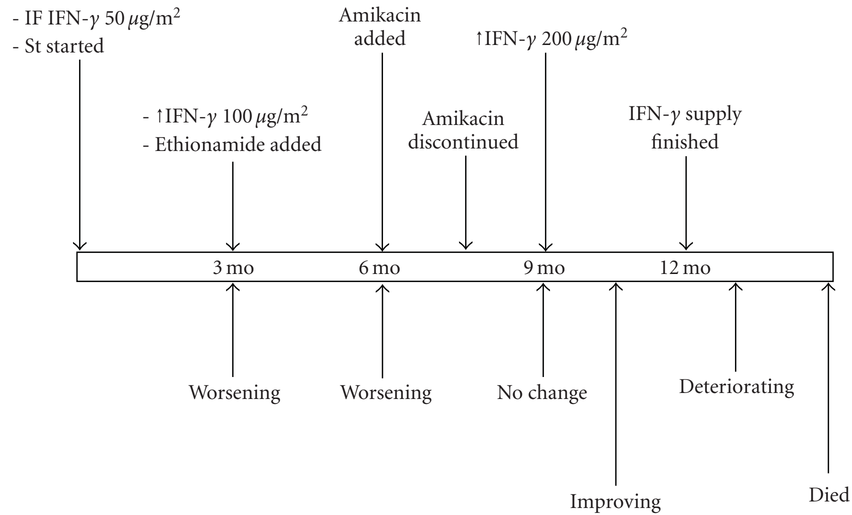

Figure 1: Time line of the patient's course. Above are the changes in medications and below are the changes in clinical condition from the beginning of IFN-γ introductions till the patients' death 15 months later.

to 100 μg/m^2 three times a week. Six months from the beginning of the study her clinical condition continued to worsen and she developed a left chest wall abscess. At that time amikacin was added to her drug regimen for 6 weeks only. She was continued on the same dose of IFN-γ. Nine months from the beginning of the study there was no clinical improvement. She was on four anti-mycobacterial medications as mentioned above. At this point the dose was upgraded to 200 μg/m^2. One month later the enlarged lymph nodes and the chest wall abscess started to discharge pus that was positive for acid-fast bacilli on ZN stain and the culture grew *Mycobacterium bovis*. The patient felt better and the abscesses and the discharging lymph nodes healed. One month later she developed right pleural effusion, but remained clinically stable. Cultures from the effusion were negative. After 12 months from the beginning of the study we ran out of IFN-γ and the patient continued on her usual anti-mycobacterial medications. She then started to deteriorate gradually. Three months later she developed massive pneumonia and died. The only adverse effect from IFN-γ therapy that was noted throughout the study at the different doses was fever (up to 39°C) and lethargy up to 8 hours from giving the injection. Her CBC, liver enzymes, lymphocyte subsets, and immunoglobulin levels were not significantly changed and her ESR fluctuated between 80–107 mm/hr throughout the study period.

The spectrum of proliferative responses of T cell following stimulation with PHA and IL-2 is shown in Figure 2. The percent increase of T cell proliferation in response to PHA (1 : 10 dilution) rose from 17% at baseline to 26% at 6 months of the study, reaching 32% at 12 months ($P = .02$) after increasing the dose of IFN-γ to 200 μg/m^2. There was, however, no change after IL-2 stimulation, where percent increase of T cell proliferation was 20%, 18%, and 17% at baseline, 6 months, and 12 months, respectively.

There was no significant difference between the serum mycobactericidal activity at 6 months and at 12 months.

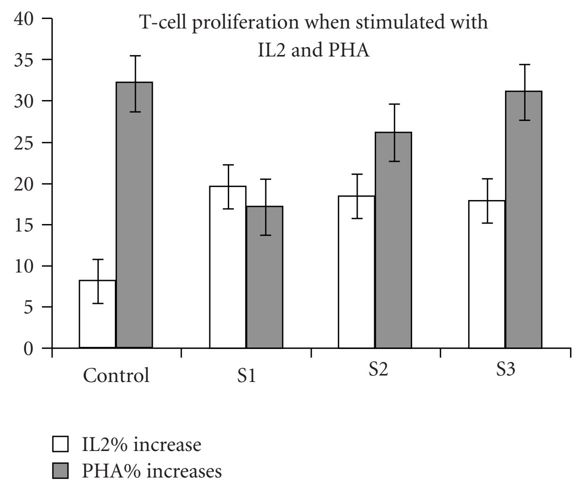

Figure 2: The percent increase in T cell proliferation stimulated with PHA and IL2 in control and the three samples from the patient: (S1) before treatment, (S2) 6 months after treatment (S3) 12 months after treatment. The percent increase in Sample, (S3) stimulation with PHA was significantly higher than the response before treatment (S1) ($P = .02$), while no significant difference with IL2.

4. Discussion

Our patient had a severe phenotype of IL-12Rβ1 deficiency. In addition, the organism was resistant to anti-BCG antibiotics. Therefore, it was more stringent to try IFN-γ as a therapeutic agent. IFN-γ was tried initially at a dose of 50 μg/m^2 3 times a week based on the experience of using it in other conditions like chronic granulomatous disease (CGD) [10]. However, it was not surprising that it did not work at this concentration with our patient since, unlike patients with CGD, she is not able to produce IFN-γ and the pathophysiology of the disease is completely different. The clinical effect noted after using the high-dose IFN-γ

(200 μg/m^2) may be attributed to this medication. This could be supported by the deterioration in the patient's condition after cessation of IFN-γ. Since there was no difference in the mycobactericidal activity between 6 months and 12 months of the study period, the changes in the patient's clinical condition are unlikely to be due to the anti-mycobacterial medications.

The increase in the lymphocyte proliferation in response to PHA at 12 months as compared to 0 and 6 months may be secondary to indirect stimulation of the patient's lymphocytes with the high-dose IFN-γ. However, the persistently negative tuberculin skin test suggests that IFN-γ therapy did not improve specific DTH to mycobacterial PPD antigens. Since there were no additional adverse effects with the 200 μg/m^2 dose of IFN-γ, our experience with this patient may encourage physicians to start directly with this dose especially when there is no good response to maximum anti-mycobacterial therapy after few months of treatment.

Achieving clinical resolution is very important in the management of patients with IL-12Rβ1 deficiency and BCGosis. Patients with BCG disease rarely have recurrence or develop EM disease, if they responded to multidrug antibiotic treatment [7]. On the other hand, patients frequently develop recurrence of salmonellosis, sometimes involving the same serotype. Interestingly, more than a quarter of the genetically affected siblings remain asymptomatic. This strongly suggests that the IL-12 pathway is redundant for the primary immune response against *Mycobacteria* and *Salmonella* in a substantial proportion of patients.

IFN-γ was tried in some patients with IL-12Rβ1 deficiency and BCGosis. There are only two reports of three patients where IFN-γ was tried. The first report includes 2 patients with BCGosis treated with IFN-γ for 18 months in addition to antimycobacterials [11]. One patient survived and the other died. The patient who responded had maintained a normal number of Th cells during treatment and had good proliferative response to mycobacterial antigens in vitro compared to the other patient. The second report included one patient who had BCGosis for 13 years and failed multiple first- and second-line antimycobacterials. She was having chronic diarrhea and her serum showed no in vitro bactericidal nor bacteriostatic activity against BCG. She fully recovered when IFN-γ was used for one year plus the addition of intravenous anti-mycobacterial treatment to her regimen [9]. Two other patients with similar presentation were treated similarly by the same group and did well (direct communication with Dr. Rosenzweig).

In many countries around the world BCG vaccine is given at birth or few months afterword. In Saudi Arabia, BCG vaccine is given in day 1 of life. This was mainly because of high incidence (243/100,000 in 1978) and problems with compliance with vaccination schedule. Subsequently, the incidence dropped to 90/100,000 in 1990 and now 11/1000,000 (Ministry of Health statistics). Recent studies showed that giving BCG vaccine at birth induces significant mycobacterial immune response as early as 2 months of life. When this response was tested at about 9 months of age it was found to be maintained and comparable to infants who received the vaccine at 2 or 4.5 months of life [12, 13].

In conclusion, in one patient with IL-12Rβ1 deficiency, IFN-γ at a dose of 200 μg/m^2, but not at lower dosages, was found to have a positive clinical effect with no additional adverse effects. This medication holds promise in the management of such patients especially if used early in the course of disease. Multicenter studies are needed to establish the effectiveness, dose, and duration of IFN-γ treatment on a large number of patients.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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Review Article

$\gamma\delta$ T Cells Cross-Link Innate and Adaptive Immunity in *Mycobacterium tuberculosis* Infection

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Protective immunity against mycobacterial infections such as *Mycobacterium tuberculosis* is mediated by interactions between specific T cells and activated antigen presenting cells. To date, many aspects of mycobacterial immunity have shown that innate cells could be the key elements that substantially may influence the subsequent adaptive host response. During the early phases of infection, innate lymphocyte subsets play a pivotal role in this context. Here we summarize the findings of recent investigations on $\gamma\delta$ T lymphocytes and their role in tuberculosis immunity.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is one of the most prevalent and commonest serious infectious diseases worldwide, afflicting almost 10 million people annually [1]. The disease, fuelled by Human Immunodeficiency virus (HIV) infection and poverty, is out of control in developing countries, and the emergence of drug-resistant strains threatens TB control in several other regions of the world [1, 2]. The current available vaccine, Bacillus Calmette-Guerin (BCG) as well as existing therapeutic interventions for TB, are at present suboptimal. Thus, new vaccines and immunotherapeutic strategies are urgently required to improve TB control efforts [3]. A better understanding of the immunopathogenesis of TB could facilitate the identification of correlates of immune protection, the design of effective vaccines, the rational selection of immunotherapeutic agents, and the evaluation of new drug or adjuvant candidates [3, 4].

Generally, effective immune responses to pathogenic and commensal microorganisms require T lymphocytes be endowed with effector properties appropriate to each challenge. In this context, CD4 T lymphocytes differentiate in the peripheral tissues to adopt a variety of fates such as the T helper (Th)-1 cells, which produce interferon (IFN)-γ and Th-2 cells, which produce interleukin (IL)-4. Specific cell-mediated immunity is critical in the host defense against mycobacteria, but many aspects of mycobacterial immunity involve other levels of responses. TB is primarily a disease of the lung, and dissemination of the disease depends on productive infection of this critical organ. Upon aerosol infection with MTB, the acquired cellular immune response is slow to be induced and to be expressed within the lung. MTB has a variety of surface molecules and soluble products that interact with the innate immune compartment, and this interaction along with the autoregulation of the immune response by several mechanisms results in less-than-optimal control of bacterial growth. Antigen-specific $\gamma\delta$ T cells represent an early innate defense that may play a role in antimycobacterial immunity. Studies done in humans and animal models have demonstrated complex patterns of $\gamma\delta$ T cell immune responses during early mycobacterial infections and chronic TB. In this paper, we focus on the role of $\gamma\delta$ T cells in the innate defense and the immune regulation

of mycobacterial immunity, as well as on their possible involvement in the new immunotherapies.

2. $\gamma\delta$ T Cells: An Overview

The concept of a strict dependent relationship between cells from innate and adaptive immunity changed the point of view about the regulation of immune system. During the most part of host reactions, both adaptive and innate sections cooperate in the host's protection and tissue damage. However, the infection by microbial agents often occurs in the peripheral tissues whereas specific naïve T lymphocytes are confined to lymphoid areas. Thus, the innate cells recruited or resident in the tissues play a crucial role in the containment of infection and the deployment of adaptive immune response [5]. Like $\alpha\beta$ T lymphocytes, $\gamma\delta$ T cells carry antigen T-cell receptors (TCR) that vary in the physical properties of their ligand-binding sites [6]. Indeed, $\gamma\delta$ TCR have a great potential of diversity at their putative ligand-binding sites as well as $\alpha\beta$ T and B cells. This means that $\gamma\delta$ T cells have a potential to recognize different pathogenic agents through the recognition of common molecular patterns.

$\gamma\delta$ T cells constitute a whole system of functionally specialized subsets that have been implicated in the innate responses against tumors and pathogens, the regulation of immune responses, cell recruitment and activation, and tissue repair [7]. The concept of $\gamma\delta$ T cells as "first line of defense" has been recently reviewed as a nonredundant system of responses based on an innate immunity program involved in systemic and specific responses depending on the inflammatory microenvironment, on microbes features and on signals that are engaged. This concept address facets of a complex behaviour where several enigmas started to be resolved. In humans and other primates, $\gamma\delta$ T cells represent a small percentage among peripheral blood lymphocytes (1–5%) and represent a special case of CD3+ T cells relying on their known separate set of receptor genes [8]. Thus, $\gamma\delta$ T cells are a specialized and independent population of lymphocytes, and basing on TCR recombination different settings of $\gamma\delta$ T cells may be now classified. The first $\gamma\delta$ T cell lineage appearing in the human foetal thymus uses the Vδ1 chain paired with different Vγ chains, and these preferentially home in epithelial tissues as the intestine [9]. Vδ1 T cells constitute only a minor proportion of human blood while they are a large population of the human intraepithelial cells and have been found to enrich various human epithelial tumors and lymphomas [10]. Vδ1 T cells recognize stressed cells via presentation of self-lipids by CD1 and/or expression of stress-induced molecules through the NKG2D receptor. In contrast, Vγ9Vδ2 T cells are the major subset of the adult peripheral blood of humans, ranging from 80/90% of $\gamma\delta$ T cell pool. They typically recognize phosphomonoester molecules synthesized in the mevalonate (MVA) and 1-deoxy-D-xylulose 5-phosphate (DOXP) metabolic pathway [11]. These lymphocytes have been defined "nonconventional" T cells, owing to several distinguishing features that are shared with both innate and adaptive immune cells. Since their discovery, $\gamma\delta$ T cells have been shown to play a significant role against pathogens and tumors and they were placed in the innate immunity as cells of immunosurveillance. In antitumor response, $\gamma\delta$ T cells show a high production of IFN-γ in the early development of tumor [12, 13]. $\gamma\delta$ T cells may recognize and kill tumor cells through the engagement of NKG2D expressed on their surface which binds MICA and MICB or retinoic acid early-1, respectively, in humans and in mice. These ligands are up-regulated on several tumor cells including melanoma cells acting as target for their destruction by locally resident intraepithelial lymphocytes (IEL) as well as other cytotoxic lymphocytes. The binding can provide a costimulatory signal for cell lysis [14–16].

Several studies have reported *in vitro* reactivity of both human Vδ2 and Vδ1 against a broad range of tumor cell lines, normal cells infected with viruses, parasites, and bacteria [7]. In respect to transformed cells, the range of tumor cell lines recognized by Vγ9Vδ2 T cells is now extended to either haematopoietic or solid tumors [16–18]. Both subsets of human $\gamma\delta$ T cells are able to recognize and destroy tumor cells as well as produce proinflammatory and Th1 cytokines through different mechanisms. A direct implication of these cells *in vivo* diseases is well accepted. However, distinct mechanisms in the recognizing and the functions of Vδ2 and Vδ1 T cells have been demonstrated, according also to different tumors, infections, and environments.

The highly restricted T cell receptor V region repertoire of $\gamma\delta$ T cells is certainly one of the most salient features distinguishing these lymphocytes from conventional MHC restricted $\alpha\beta$ T cells. Most Vγ9Vδ2 T cells react against the same related set of nonpeptidic, phosphorylated antigens [15, 16] that are produced by both microbial and endogenous metabolites, whereas Vγ9Vδ1 T cells seem to recognize heterogeneous yet undefined antigens, presumably unrelated to Vγ9Vδ2 agonists. Vγ9Vδ2 T cell antigens are recognized in a TCR-dependent manner and are referred to as phosphoantigens. The most potent antigen appears to be the hydroxydimetihyl-allyl-pirophosphate (HDMAPP), an intermediate of DOXP pathway, restricted to plant cells and some microorganisms. Metabolic intermediates as isopentenyl pyrophosphate (IPP) can also activate Vγ9Vδ2 T cells although at concentrations of 100000-fold higher than those for microbial agonists. These compounds derive from the MVA pathway used by mammalian cells and some bacteria and are essential for sterol synthesis, cell growth, and membrane integrity. Aminobisphosphonate (ABPs) compounds may also stimulate Vγ9Vδ2 T cells through their ability to inhibit farnesyl pyrophosphate synthase, an enzyme acting downstream of IPP synthesis along MVA pathway, promoting intracellular accumulation of IPP. Finally, the alkylamines remain a debated class of antigens. Some studies strongly suggest that like ABPs, alkylamines promote intracellular accumulation of Vγ9Vδ2 agonists derived from MVA pathway [19]. Although phosphoantigen-mediated activation of Vγ9Vδ2 T cells clearly requires the expression of TCR, as indicated by gene transfer approach, how precisely this occurs remains unclear to date. The cell-cell contact required for the activation implicates either that phosphoantigens induce the structural modification of

TCR or that are presented by surface molecules at present undefined. Interestingly, Vγ9Vδ2 T cells have been recently shown to recognize a complex formed between apolipoprotein A1 and ATP synthase, a mitochondrial enzyme that is translocated on the surface of normal hepatocytes and some tumor cell lines, in a TCR-dependent fashion [20]. Among other costimulatory factors, human Vγ9Vδ2 T cells express frequently activating or inhibitor NK receptors such as NKG2D or CD94/NKG2A that seem to be the major contributing receptor to Vγ9Vδ2 T cell activation/inhibition [21]. In the plethora of activation signals of human $\gamma\delta$ T cells, an important pathway is represented by Toll-like receptors (TLR). TLRs have emerged as central regulators of innate immunity being receptors specifically sensing molecular patterns of microbes, leading to immediate cellular responses through the activation of transcription factors, notably NF-κb, AP-1, and IRF [22]. Although certain TLRs are expressed on myeloid cells, several reports have shown functional expression on B, $\alpha\beta$ and $\gamma\delta$ T cells [23]. It has been reported that TLR ligands including TLR3 ligand poly (I:C) and TLR9 ligand CpG enhance the activation of $\gamma\delta$ T cells *in vitro* via promoting type I IFN production in myeloid and plasmacytoid dendritic cells (DCs), respectively [22]. More recently, it has been shown that highly purified $\gamma\delta$ T cells expressed more TLR3 mRNA than $\alpha\beta$ T cells, thus opening the possibility that $\gamma\delta$ T cells might respond directly to TLR3 ligands in the absence of APC [24]. Taken together, these results confirm that $\gamma\delta$ T cells may play a crucial role in innate immune response and studies on the different signals activating or enhancing their functions may help to improve the understanding of these cells and their usage in the immunotherapy.

Although immunological memory is a hallmark of adaptive immune response, Vγ9Vδ2 T cells seem to show some features of memory cells. Studies in monkeys suggested that phosphoantigen-specific Vγ9Vδ2 T cells, expanded during a primary TB vaccination, showed an accelerated response after a secondary challenge [25]. The ubiquitous nature of exogenous and endogenous phosphoantigens for Vγ9Vδ2 T cells also suggests that the development of memory state may be quite different from conventional cells, which are programmed to respond to foreign peptide antigens. After antigen exposure, Vγ9Vδ2 T cells undergo the same change of CD8 T cells. Basing on the expression of CD45RA and CD27 molecules on their surface, it is possible to distinguish 4 subsets of Vγ9Vδ2 T cells as naïve, central memory (T_{CM}), effector memory (T_{EM}), and terminal differentiated effector cells (T_{EMRA}). Vγ9Vδ2 T cells acquire CD45RO expression like early memory CD8 T cells and are termed central memory Vγ9Vδ2 T cells. They lose CD27 and CD28 expression and re-express CD45RA becoming terminally differentiated cells. Approximately 90% of Vγ9Vδ2 T cells in the adult have a memory phenotype [26]. Effector memory Vγ9Vδ2 T cells represent a readily available pool of antigen-primed Vγ9Vδ2 T cells which enter the peripheral tissues, where they can eventually further differentiate into CD45RA+CD27− cells, produce cytokines, and exert cytotoxicity contributing to the containment of invading microbial pathogens.

In TB, the establishment of the disease and its clinical manifestations are closely linked to host's immune response. The spectrum of the immune response in TB ranges from a protective response in latent TB, to the absence of response and the dissemination of mycobacteria in miliary TB. In pulmonary TB, there is an effective antimycobacterial response with a clinically progressive disease involving innate and adaptive immune compartment [27]. MTB cannot evade the induction of cell-mediated immunity; MTB has evolved to survive it, and survive it does—even if the initial infection is successfully controlled, many infected individuals develop a latent infection that can persist for decades. On the other hand, some heavily MTB exposed individuals show no signs of infection: no pathology, no symptoms, and no apparent adaptive immune response. It is possible that in these cases, the innate immune response has eliminated the pathogen at the earliest stage [28]. Indeed, early immune mediators as IFN-γ are produced initially by NK cells and $\gamma\delta$ T cells before adaptive T cells are instructed by IL-12 and IL-18 secreted by antigen presenting cells (APC) as DCs and macrophages. For this reason, we will focus the attention on $\gamma\delta$ T cells that represent an optimal bridge between innate and adaptive immune response [29, 30]. Interestingly, the activity of a subset of human $\gamma\delta$ T cells *in vitro* and *in vivo* can be stimulated by many nonpeptidic molecules (some drugs are currently being tested in Phase I cancer trials). The relatively low *in vivo* toxicity of many of these drugs makes possible novel vaccine and immune-based strategies for infectious diseases. Collectively this scenario indicates that different pathways and cell types interact to mediate innate immunity against MTB providing mechanisms that could likely be target for future therapeutic interventions in TB.

3. $\gamma\delta$ T Cell Response in MTB Infection

In infections, responses of $\gamma\delta$ T cells to MTB were described as early as in 1989 [31]. Later, a range of studies described a marked expansion of this subset in the blood of (TB) patients and also with a range of other infections as leprosy, malaria, salmonella, and *Streptococcus pneumoniae*. Mycobacterial phosphoantigens were identified as potent stimulators of Vγ9Vδ2 T cell functions [15]. Specifically, Vγ9Vδ2 T cells predominate in mycobacterial infections whereas Vδ1 T cells are preferentially expanded in HIV patients and in immunocompromised subjects probably undergoing CMV reactivation [32, 33]. Parallel to the studies in murine models, an association between mycobacterial and human Vγ9Vδ2 T-cell responses was rapidly established. $\gamma\delta$ T-cell clones were isolated from synovial fluid of rheumatoid arthritis patients, which had been stimulated with mycobacterial antigens and were found to proliferate to mycobacterial antigens [34]. *In vivo*, $\gamma\delta$ T cells were observed in granulomatous skin reactions of leprosy patients, and $\gamma\delta$ cell lines derived from these persons proliferated to mycobacterial extracts [35]. Direct evidence for the ability of MTB to activate $\gamma\delta$ T cells was provided by studies of Kabelitz et al. which determined by limiting dilution analysis that the majority of peripheral blood $\gamma\delta$ T cells proliferated

in response to a killed preparation of MTB bacilli [36]. Subsequently, the predominance of Vγ9Vδ2 T cells in TB infection was confirmed [37]. Being MTB an intracellular pathogen residing within mononuclear phagocytes, many studies on the role of these cells in the activation of $\gamma\delta$ T cells following the infection of MTB rapidly appeared. Monocytes infected with MTB were found to be efficient accessory cells for $\gamma\delta$ T cells in a non-MHC restricted manner [38]. *In vitro*, Havlir et al. demonstrated that monocytes infected with live MTB bacilli were particularly effective in expanding Vγ9Vδ2 T cells, compared to heat-killed bacteria and soluble protein antigens of MTB [39]. Similarly, MTB-infected alveolar macrophages, the first target of inhaled MTB, served as non-MHC-restricted accessory cells for $\gamma\delta$ T cells. There were differences, however, between alveolar macrophages and monocytes. At the high alveolar macrophage to T-cell ratios normally present in alveolar spaces, expansion of resting $\gamma\delta$ T cells was inhibited by alveolar macrophages in a dose- and cell-contact-dependent manner. However, upon invasion by MTB, alveolar macrophages are certainly capable of serving as accessory cells for $\gamma\delta$ T cells, providing a mechanism for $\gamma\delta$ T-cell activation in the lung. $\gamma\delta$ T cells are dependent on costimulators for proliferation, cytokine secretion, and expression of cytolytic effector function. Like their $\alpha\beta$TCR+ counterparts, $\gamma\delta$ T cells are dependent upon interactions of CD40-CD40L, CD28-B7.1/7.2, and CD2, as well as adhesion molecules (CD2-LFA-3, LFA-ICAM) for co-stimulation, and these second signals are readily provided by accessory cells such as monocytes and alveolar macrophages [40]. Whether accessory cells process and present mycobacterial antigens to MTB activated $\gamma\delta$ T cells, and thus serve as true antigen-presenting cells, has yet to be determined.

The major effector functions of T cells in the immune response to MTB are cytokine secretion, cytotoxic effector function (CTL), and cell-contact-dependent "help". The goal of these effector functions is to help to contain mycobacterial growth and to stimulate memory immunity. Studies with MTB antigen-activated $\gamma\delta$ T-cell clones or primary cells determined that there was some heterogeneity in cytokine profile among clones [41]. There was no clear-cut Th-1 versus Th-2 dichotomy, nor there were major differences found in cytokine patterns between ⟨$\alpha\beta$ TCR+ and $\gamma\delta$ T-cell clones⟩. In general, most $\gamma\delta$ T-cell clones produced IFN-γ, a cytokine associated with protective immunity to MTB, and a marker of the proinflammatory cytokine environment characteristic of the cellular immune response to intracellular bacteria. Some studies have used intracellular staining for cytokines and determined that in response to phosphoantigens, $\gamma\delta$ T cells produce both TNF-α and IFN-γ [42]. When MTB-activated CD4+ and $\gamma\delta$ T-cell populations from healthy tuberculin-positive donors were analyzed for patterns of cytokine production in response to MTB-infected monocytes, both subsets secreted large amounts of IFN-γ [43]. Intracellular IFN-γ levels were similar between CD4+ and $\gamma\delta$ T cells, suggesting more efficient IFN-γ release by $\gamma\delta$ T cells. In contrast, CD4+ T cells produced more IL-2 than $\gamma\delta$ T cells, which correlated with diminished T-cell proliferation of $\gamma\delta$ T cells compared with CD4+ T cells. CD4+ and $\gamma\delta$ T cells from some healthy donors produced IL-4, reemphasizing the absence of a Th-1 versus Th-2 dichotomy among these two T-cell subsets.

Although activated $\gamma\delta$ T cells produce IL-2, they produce far less IL-2 than CD4+ T cells, which accounts for their poor proliferative ability and need for exogenous IL-2 to induce $\gamma\delta$ T-cell expansion [44]. Furthermore, IL-15 is a T-cell growth factor for $\gamma\delta$ T cells, and is produced by mononuclear phagocytes, thus providing a link between MTB-infected macrophages and $\gamma\delta$ T-cell activation in the absence of CD4+ T-cell responses [42]. The balance between these two factors (CD4 versus macrophage "help") may account for the variability one observes in $\gamma\delta$ physiology. Finally, contribution of Vγ9Vδ2 T lymphocytes to immune protection against MTB is based also on the cytotoxic effector functions of these cells. It was reported earlier that Vγ9Vδ2 T lymphocytes kill macrophages harboring live MTB through a granule-dependent mechanism, resulting in killing of intracellular bacilli; moreover, it has been reported that these cells reduce the viability of both extracellular and intracellular MTB through granulysin and perforin, both detected in Vγ9Vδ2 T lymphocytes. These findings have suggested that Vγ9Vδ2 T lymphocytes directly contribute to a protective host response against MTB infection [45].

A controversial feature of Vγ9Vδ2 T lymphocytes is based on their capability to mount a memory response against a microbial reinfection or reactivation. Vγ9Vδ2 T lymphocytes mount a response against MTB infection during the early phases of infection, and a strong expansion of this T cell subset has been observed in different reports, but their functional response against mycobacterial infection seems not limited to an innate reaction. As mentioned above, Vγ9Vδ2 T cells follow a phenotype differentiation similar to $\alpha\beta$ T cells and probably a certain memory response is generated. Relevant studies in mice cannot be performed because murine $\gamma\delta$ T cells do not express the homolog of Vγ9Vδ2 TCR, and there is no functional equivalent for these cells, so far, identified in mice. A pioneering study showed the capability to mount a memory response after microbial reinfection or reactivation [25]. Indeed, to examine a primary role of $\gamma\delta$ T cells during mycobacterial infection, macaques inoculated with BCG were analysed for the change in the $\gamma\delta$ T cell repertoire. Striking expansion of Vγ9Vδ2 T cells were detected in the blood after BCG inoculation, whereas there was no apparent increase in other $\gamma\delta$ T cell subsets. This expansion indicated the development of primary response of these cells during mycobacterial infection. Apart systemic Vγ9Vδ2 T cells, other pools of expanding Vγ9Vδ2 T cells have been observed in pulmonary and intestinal tissues after intravenous BCG inoculation and only a small amount of these cells were observed in lymph nodes suggesting that tissues but not peripheral lymph node tissues could expand in response to mycobacterial infection. Of note, after a second inoculation of BCG, a marked re-expansion of Vγ9Vδ2 T cells appeared in the blood. The expansion of these cells after a reinfection was 2–9 times larger than those seen during the primary infection. Furthermore, the expansion was persistent for as long as 7 months after the second BCG challenge [25]. This evidence provide that Vγ9Vδ2 T cells underwent polyclonal expansion during

a primary mycobacterial infection can mount a memory recall response after a secondary challenge. Another important result has been reported by other reports suggesting that the route and the dose of mycobacterial infection related to the expansion of $\gamma\delta$ T cells. Systemic BCG inoculation induced a dose-dependent expansion of circulating $\gamma\delta$ T cells as well as CD4 and CD8 T cells whereas, in the pulmonary compartment, the systemic infection resulted in a predominant increase in numbers of $\gamma\delta$ T cells. In contrast, pulmonary exposure to mycobacteria induced a detectable expansion of CD4, CD8, and $\gamma\delta$ T cells only in the lung but not in the blood [46, 47]. The pattern and kinetics of $\gamma\delta$ T cell responses during mycobacterial infection might contribute to characterizing immune protection against TB and testing new TB vaccines in primates.

4. $\gamma\delta$ T Cells Cross-Talk with DCs during Mycobacterial Infections

Recent knowledge about the interaction between apparently different compartments of immune system changed the way to consider the immune response and its regulation. Studies in animal models suggest that even the smallest population of immune cells in a site of infection can exert large biological effects up to systemic level. This recent concept is due to the continuous interaction between local and recruited innate immune cells with APCs. Indeed, $\gamma\delta$ T cells and DCs participate in early phases of immune response against MTB. Continuous cross-talk between $\gamma\delta$ T cells and myeloid cells is evident in histological studies, *in vitro* culture experiments, and in animal models. Indeed, $\gamma\delta$ T cells participate in early immune response against MTB producing cytokines (IFN-γ and TNF-α) and chemokines, prompting cytotoxicity or modulating other cell in mice [48].

The first evidence of an influence exerted by $\gamma\delta$ T cells on DCs system came from studies by Ismaili et al., showing that human $\gamma\delta$ T cells activated *in vitro* by phosphoantigens are capable of inducing maturation of monocyte-derived DCs [49] and this process involved both membrane-bound (i.e., CD40L) and soluble (i.e., TNF-α and IFN-γ) T cell-derived signals [49–51]. Recent studies support the notion that DCs strengthen the cellular immune response against mycobacterial infection [52–56]. Even if the critical role of DCs in the initiation of immune response has been firmly established [57], their involvement in immune responses occurring at sites of MTB infection needs further elucidations. DCs are highly represented at sites of MTB infection at the onset of the inflammatory response [58], and it is conceivable that immature DCs present in the lung mucosa are specialized for antigen uptake and processing. After interaction with pathogens, they mature and migrate to lymphoid organs where they prime naïve T cells through cell surface expression of MHC and costimulatory molecules and the secretion of immunoregulatory cytokines such as IL-12.

Although infection with mycobacteria has been reported to induce maturation of DCs, *in vitro* infection of human mDC by virulent MTB strain H37Rv has been shown to impair their maturation, reduce their secretion of interleukin (IL)-12, and inhibit their ability to stimulate T cell proliferation [51, 59]. *In vivo* experiments demonstrated that MTB affects DC migration and antigen presentation, promoting persistent infection in mice [60]. These findings suggest that MTB can interfere with the host immune response by hampering several functions of DCs, and in particular suppressing the migration of mDCs and modulating its cell trafficking ability, and these mechanisms may encourage the long-term persistence of the bacilli in the host during chronic infections. However, in more physiological situation, such as during infection by a Vγ9Vδ2-stimulating pathogen unable to promote complete DC maturation, it is easy to hypothesize that many different stimuli, besides microbial-derived phosphoantigens, may influence the activation state of DCs and Vγ9Vδ2 T cells. Accordingly, in a recent paper, Meraviglia et al. demonstrated that Vγ9Vδ2 induce full maturation of MTB-infected immature DCs, that were otherwise unable to complete maturation. In detail, MTB infection caused up-regulation of CD86 and HLA-DR molecules, but not of CD80 and CD40, while the co-culture of MTB-infected DCs with Vγ9Vδ2 T cells determined up-regulation of CD80 and CD40 expression, no changes of HLA-DR and CD86 expression, and a significant up-regulation of IL-12p70 production, suggesting that Vγ9Vδ2 T cells mediate full maturation of MTB-infected DC [61]. On the other hand, MTB infected DCs lead to a rapid and strong activation of co-cultured Vγ9Vδ2 T cells without requirement for any additional stimulations. The MTB infected DC-mediated potentiation of Vγ9Vδ2 T cell responses could be explained at least in part by up-regulation and/or presentation of MTB derived phosphoantigens to Vγ9Vδ2 T cells. However, and most surprisingly, MTB infected DCs selectively induced proliferative, but not cytokine or cytolytic responses by Vγ9Vδ2 T cells and this was associated to the expansion of phenotypically "immature", central memory-type Vγ9Vδ2 T cells. Similar results have been obtained using BCG infected DCs co-cultured with Vγ9Vδ2 T cells [62]. Possible explanation for the incomplete phenotypic and functional differentiation of Vγ9Vδ2 T cells include the lack of IL-15 production by MTB infected DCs; IL-15 is a relevant cytokine for the differentiation of $\gamma\delta$ cells [63], and its main effect in the pathway leading to differentiation of Vγ9Vδ2 T cells towards effector memory cells was associated with induction of Bcl-2 expression and resistance to cell death [63]. Indeed, adding IL-15 to co-cultures of MTB infected DCs and Vγ9Vδ2 T cells caused efficient differentiation of $\gamma\delta$ T cells with maintenance of the central memory pool and with generation of effector-memory and terminally differentiated effector memory cells, which displayed potent antimycobacterial function, as demonstrated by their ability to efficiently reduce the viability of intracellular MTB. We therefore conclude that mechanisms fine-tuning the DC-$\gamma\delta$ T cells cross talk are still not clear, including identification of the critical receptor/ligand interactions, as well as the underlying molecular mechanisms; therefore, further studies are needed such as the analysis of the effect of inhibitors of various signalling cascades coupled with transcriptome analysis of maturing DCs at various time points after Vγ9Vδ2 T cell incubation.

5. Incomplete Maturation of $\gamma\delta$ T Cells in TB Patients

A number of studies have attempted to determine the *in vivo* role of $\gamma\delta$ T cells in the human immune response to MTB. Barnes et al. established that patients with pulmonary or miliary TB had a diminished ability to expand $\gamma\delta$ T cells *in vitro* in response to heat-killed MTB and IL-2, although there was quite a range of $\gamma\delta$ T-cell expansion among the different groups [64]. Some investigators have suggested an increase in peripheral $\gamma\delta$ T cells in patients with TB or among hospital workers with contact with TB patients but this has not been a consistent finding [65, 66]. Studies of T-cell phenotype in bronchoalveolar cells from healthy PPD^+ subjects and from affected and unaffected lungs of patients with pulmonary TB found a T lymphocytic alveolitis in the affected tuberculous lung [67]. $\gamma\delta$ T cells were found among the lymphocytes in this alveolitis, but their proportion was not increased relative to $\alpha\beta$ T cells. Thus $\gamma\delta$ T cells are present *in situ* in pulmonary TB but are not expanded compared to $\gamma\delta$ T cells in peripheral blood or unaffected lung. The presence of $\gamma\delta$ T cells in the tuberculous lung is consistent with the findings that alveolar macrophages can serve as APCs for $\gamma\delta$ T cells. In the study by Schwander et al., monocytes were markedly increased in alveolar spaces of tuberculous lung [67]. Monocytes also are efficient APCs of $\gamma\delta$ T cells, and hence in TB adequate accessory cell populations are available for $\gamma\delta$ T-cell activation.

More recently, dramatic expansion of Vγ9Vδ2 T cells has been found after BCG vaccination in infants, and several phosphorylated antigens derived from mycobacteria have been defined [68]. It is already known in the context of a natural infection the consistent expansion of Vγ9Vδ2 T cells with a T_{CM} phenotype in the peripheral blood of patients with active TB, which was accompanied by the dramatic reduction of the pool of Vγ9Vδ2 cells with immediate effector functions (T_{EM} and T_{EMRA} cells). However, this skewed representation of circulating Vγ9Vδ2 T cell phenotypes during active TB was transient and completely reversed after successful antimycobacterial therapy. This explains previous findings from Dieli's group, showing that Vγ9Vδ2 T cells from children affected by active TB have an increased proliferative activity, but decreased IFN-γ production and granulysin expression [69]. After successful chemotherapy, the Vγ9Vδ2 T cell proliferative response strongly decreased, whereas IFN-γ and granulysin production consistently increased.

Other previous observations have indicated an increased proliferative activity of Vγ9Vδ2 T cells from patients with TB [70, 71] but reduced production of IFN-γ, compared with that of healthy tuberculin reactors [72]. Additionally, Dieli et al. reported that decrease of Vγ9Vδ2 T cell effector functions involves not only IFN-γ production but also expression of granulysin, a molecule known to be responsible for the killing of MTB [69]. The reason for the loss of Vγ9Vδ2 T cell effector functions during TB is unknown. One possibility is that sustained *in vivo* mycobacterial stimulation of Vγ9Vδ2 T cells causes their apoptosis [73]. For example, high levels of bacteria (such as those occur in patients with TB), resulting from the inability to contain and prevent their spread, would presumably result in chronic stimulation of effector Vγ9Vδ2 T cells by mycobacterial antigens and in their apoptosis, thus providing an explanation for why this population of $\gamma\delta$ T cells is lost in patients with active disease but recovers after drug therapy. Alternatively, it is possible that reduced IFN-γ and granulysin expression in children with TB, which recovers after disease improvement, could be the consequence of generalized illness. The finding that IFN-γ and granulysin production are restored by successful chemotherapy, which is suggested to induce the generation of a protective immune response, strongly supports this possibility. Another possible explanation for the incomplete phenotypic and functional differentiation of Vγ9Vδ2 T cells could be explained by the lack of relevant cytokines secreted by MTB infected DCs. In fact, it has been previously shown that, differentiation of Vγ9Vδ2 T_{CM} cells into T_{EM} and T_{EMRA} cells occurs upon antigen stimulation in the presence of IL-15, while any other tested cytokine, including IL-7, had no such effect [63]. The main effect of IL-15 in the pathway leading to differentiation of Vγ9Vδ2 T cells towards effector memory cells was associated with resistance to cell death and Bcl-2 expression. Meraviglia et al. demonstrated that the lack of IL-15 production by MTB infected DCs was not due to the fact that MTB simply does not induce synthesis of this cytokine, rather it actively inhibits IL-15 secretion. Additionally, and similar to the *in vitro* data, their analyses of IL-15 serum levels in healthy contact (HC) subjects and TB patients showed that IL-15 production is not induced in patients with active TB, but increases after completion of chemotherapy [61]. However, the analysis of Vγ9Vδ2 T cell functions in TB patients and especially in the site of infection needs further investigations.

6. $\gamma\delta$ T Cells Producing IL-17: Possible Involvement in Mycobacterial Infection

The cytokine IL-17 has received considerable attention since the discovery of a distinct CD4+ T helper cell subset producing it, known as Th-17 profile. This discovery provided compelling reasons to explore outside the Th-1/Th-2 paradigm, searching new answers to explain independent effector T cell responses. A rapid succession of studies defined the Th-17 cell paradigm, in which IL-6/STAT3 activation of the transcriptional regulator retinoic acid receptor-related orphan receptor-γt (RORγt) controls the lineage fate of IL-17A-, IL-17F-, IL-21-, and IL-22-producing T cells (collectively known as Th-17 cells) that are highly responsive to IL-1 receptor 1 (IL-1R1) and IL-23R signaling [74]. IL-17A has been reported to participate in host defense against various types of pathogen [75, 76] and estimated to be an important cytokine in the immune response against mycobacterial infection [77]. Indeed, IL-17 is produced immediately after pulmonary BCG infection and was also detected at later stages of MTB infection in mice [78]. Interestingly, IL-17-expressing cells in the mycobacterial infected lungs in murine models are $\gamma\delta$ T cells rather than CD4+ T cells. As mentioned, $\gamma\delta$ T cells may play an important role in

the effector functions and regulation of immune responses to infection of MTB, but the precise role of IL-17 producing $\gamma\delta$ T cells remains unclear. In a study performed on 27 patients with active pulmonary TB and 16 healthy donors, it has been found that proportion of IL-17-producing cells among lymphocytes was similar between TB patients and HD, whereas the proportions of $\gamma\delta$ T cells in IL-17-producing cells (59.2%) in peripheral blood were markedly increased in TB patients when compared to those in HD. In addition, the proportions of IFN-γ producing $\gamma\delta$ T cells in TB patients were obviously lower than that in HD. Upon re-stimulated with MTB heat-treated antigen *in vitro*, fewer IL-17-producing $\gamma\delta$ T cells were generated from HD than TB patients [79]. These findings were consistent with murine investigations showing that the IL-17-producing $\gamma\delta$ T cells were main source of IL-17 in mouse model of BCG infection, suggesting that $\gamma\delta$ T cells might be involved in the formation of tubercular granuloma in pulmonary TB patients [80], but these investigations need further identification in humans. Another study showed the differentiation and distribution of human IL-22-producing T cells in a nonhuman primate model of MTB infection. Since IL-22-producing T cells also produce IL-17, knowledge about this cytokine profile may help the understanding of the Th-17 profile. An apparent increase in the number of T cells capable of producing IL-22 de novo without *in vitro* Ag stimulation has been observed in lungs compared to blood and lymphoid tissues. Consistently, IL-22-producing T cells were visualized *in situ* in lung TB granulomas, indicating that mature IL-22-producing T cells were present in TB granuloma. Surprisingly, phosphoantigen HMBPP activation of Vγ9Vδ2 T cells down-regulated the capability of T cells to produce IL-22 *de novo* in lymphocytes from blood, lung/BAL fluid, spleen and lymph node. Up-regulation of IFN-γ-producing Vγ9Vδ2 T effector cells after phosphoantigen stimulation coincided with the down-regulated capacity of these T cells to produce IL-22 *de novo* [81]. These findings raise the possibility to ultimately investigate the function of IL-22 producing T cells and to target Vγ9Vδ2 T cells for balancing potentially hyper-activating IL-22 producing T cells in severe TB.

7. $\gamma\delta$ T Cell-Specific Phosphoantigen Based-Immunotherapy in TB: Lesson from the Cancer

As mentioned above, $\gamma\delta$ T cells have antimicrobial as well as antitumor activity through the production of proinflammatory cytokines, chemokines, and cytotoxic molecules such as perforins and granzymes. This suggests their involvement in the control of infections *in vivo* and could be considered as target for new intriguing therapeutic approaches. Moreover, the capacity of $\gamma\delta$ T cells to interfere in DC functions would allow their use in specific immunotherapy. Although other nonclassical lymphocytes may support DC maturation and contribute to the antigen presentation, $\gamma\delta$ T cells in humans represent an easy model to amplify the DC system. Given that the different classes of pharmacological agents are used in therapies for different diseases, the possibility to make new vaccines or adjuvants based on these compounds is very close. A variety of natural and synthetic nonpeptidic antigens have been demonstrated to activate $\gamma\delta$ T cells such as IPP, dimethylallyl diphosphate (DMAPP), geranylgeranyl pyrophosphate (GGPP) including Nitrogen containing bisphosphonates (N-Bps). At present two approaches showed exciting results. Tumor immune evasion mechanisms are common and include the down-regulation of tumor-associated antigens, MHC, and costimulatory molecules. By contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells are not MHC restricted and show less dependence on costimulatory molecules such as CD28. Moreover, $\gamma\delta$ T cells are involved in the resistance of cutaneous carcinogenesis in mice and display potent cytotoxicity against various human tumor cell lines *in vitro*. Indeed, human Vγ9Vδ2 T cells expanded *in vitro* and transferred to immunodeficient mice, xenografted with tumor cells, showed efficacy against B cell lymphoma, melanoma, and renal carcinoma [82]. On this ground, in patients with multiple myeloma or with low-grade non-Hodgkin lymphoma, occurrences of acute phase reaction to intravenously injection of an aminobisphosphonate, called Pamidronate (PAM), were attributed to the systemic activation of $\gamma\delta$ T cells [83], and this provoked the deliberate treatment of lymphoma patients with PAM and IL-2. Promising results were achieved after the patients were prescreened for substantively response to PAM and IL-2 of $\gamma\delta$ T cells *in vitro*. By several criteria, zoledronate is more potent and efficacious than PAM. Previous studies in patients with breast and prostate tumors showed that zoledronate induced *in vivo* activation of peripheral $\gamma\delta$ T cells into more potent cytotoxic and IFN-γ producing cells. Recently, a phase I clinical trial in metastatic HRPC has been conducted by Dieli et al. to determine the safety, feasibility, and response induced by Vγ9Vδ2 T cells *in vivo*, using zoledronate alone or in combination with low-doses of IL-2 [84]. The encouraging prospect that the activation of peripheral blood Vγ9Vδ2 T cells can be efficacious against solid tumors could be explained by the double role played by these cells; activated $\gamma\delta$ T cells can infiltrate tumor sites and display cytotoxic activity against tumor cells or they help other cells as DCs to trigger an adequate specific CD8 T cell immune response.

Different interesting results have been shown in animal models aimed to improve the effectiveness of vaccination against TB. As known, although the vaccination with BCG protects children against disseminated TB, it is now clear that it does not protect efficiently against pulmonary disease. Therefore, the ever-increasing incidence of TB worldwide urges to improve this vaccine. It is widely accepted that one of the best immunological predictors of protective and long-lasting immunity to TB is a high frequency of MTB-specific IFN-γ-secreting cells (ISCs) in the peripheral blood [85]. A quantitatively sizeable population of effector T cells able to release IFN-γ seems to promote the protective bioactivity of infected macrophages. Therefore, most of current TB vaccine candidates and injection regimens aim to increase the frequency of these MTB-specific ISC. These candidates comprise recombinant BCG, attenuated MTB, modified vaccinia virus, naked DNA, and subunit combinations of either MTB protein antigens or recombinant fusion proteins [86].

It exists now a consensus on the ability of heterologous prime-boosts regimens to induce high titers of MTB-specific ISC. The priming with an optimized "starter" such as BCG or improved BCG could likely induce a broad diversity of memory cells. The further boost with antigens common to the priming would expand and differentiate into effector memory, MTB-specific ISC. The immunodominant protein antigens from MTB include members of the "proline-proline-glutamic acid family" proteins (Mtb39a-e), Mtb9.9, TB10.4, so-called "6-kDa early secretory antigenic target" (ESAT-6), and mycolyl transferase complex Ag85A, B, C [86]. Despite their good specificity, these purified antigens were weakly immunogenic when injected alone, and therefore needed to be combined either to other antigens (hybrid proteins) or to adjuvants. Hybrids of the most promising proteic antigens, namely, Mtb72F and H-1 have been generated: they corresponded to fusions Mtb39 to Mtb32 and ESAT-6 to mycolyl transferase complex antigen 85B (Ag85B), respectively. Hybrid H-1 is highly specific of MTB and induces a detectable ISC population but its immunogenicity was quite low, even after several boosts. Therefore, H-1 was combined with adjuvants such as Lipovac or IC31. Nonhuman primates $\gamma\delta$ T cells like those from rhesus macaques present a TCR with similarity of 90% to the human Vγ9Vδ2 TCR sequence, and the same pattern of specificity for phosphoantigens [44]. Therefore, these animals represent a model suited to investigate the role of phosphoantigen-induced $\gamma\delta$ T cell responses in immunity to TB. A pioneering analysis of rhesus infected with MTB demonstrated that rhesus $\gamma\delta$ T lymphocytes mounted memory responses to mycobacteria. This adaptive response correlated with a faster $\gamma\delta$ T cell expansion in the secondary respect to primary exposure to mycobacteria and was associated with a reduced bacteremia and protection against fatal TB. Furthermore, two studies have independently shown that blood $\gamma\delta$ T cells from several monkey species could be monitored using mAb reagents for human T cells. These studies confirmed that phosphoantigen-induced proliferation of naïve, central memory CD27+, and effector memory CD27− $\gamma\delta$ T cells require IL-2 *in vivo* [87]. Since macaque $\gamma\delta$ T cells seem to react as human Vγ9Vδ2 T cells during BCG vaccination or TB infection and to phosphoantigen stimulation, the bioactivity of a synthetic phosphoantigen combined to a subunit vaccine candidate for TB has been assessed *in vivo*. Since TB mainly alters cytokine production and cytotoxic activity but not proliferation of human $\gamma\delta$ T cells, this study focused on effector functions in defence against TB: secretion of Th-1 cytokines, most notably IFN-γ, and perforin [88]. In this paper, an efficient immunogenicity against MTB antigens in naïve cynomolgus after a prime-boost with the hybrid H-1 solubilized in Lipovac adjuvant with or without the synthetic phosphoantigen Picostim has been reported. Although the IC31 adjuvant was selected for clinical trial of the H-1 subunit vaccine, in this work the adjuvant Lipovac was preferred for its lower bioactivity, in order to be able to detect additional adjuvant effect on phosphoantigens. However, Picostim, a new generation of synthetic phosphoantigens, induced immediate cytokine production by $\gamma\delta$ T cells (IL-2, IL-6, IFN-γ, and TNF-α), but a subsequent anergy up to 4 months after the initial administration. This phenomenon could be related to the TCR down-modulation/regulation or apoptosis induced cell death [73, 89]. However, this early $\gamma\delta$ response translates into differential induction of recall response eliciting the H-1-specific $\alpha\beta$ T cell responses, which essentially comprised recall of cytotoxic $\alpha\beta$ T lymphocytes specific for Ag85B and few ISC $\alpha\beta$ T lymphocytes in both groups of animals. So this study demonstrated that a prime-boost regimen with the H-1/phosphoantigen combination added a primary wave of adaptive immune responses from phosphoantigen-specific $\gamma\delta$ T cells to the secondary wave of H-1-specific $\alpha\beta$ T cells. In summary, nonhuman primates vaccinated with phosphoantigens associated to a subunit of antituberculosis vaccine, mounted a differential immune response by $\alpha\beta$ or $\gamma\delta$ T cells, where boosts anergized $\gamma\delta$ T cells but promoted $\alpha\beta$ recall responses. Finally, these models of usage of phosphoantigens against tumors and infections may allow to design subunit combinations promoting memory by both classes of lymphocytes in order to improve TB therapy.

8. Concluding Remarks

$\gamma\delta$ T cells appear to combine properties of both adaptive and innate immunity. The identification of unusual compounds that are recognized by human $\gamma\delta$ T cells but not by $\alpha\beta$ T cells has recently stimulated great interest in the development of $\gamma\delta$ T cell-based therapies. In contrast to other potential effector cells, it is possible to envisage combined *in vivo* activation and adoptive cell therapy with *ex vivo* expanded $\gamma\delta$ T cells, because several drugs as ABPs and synthetic phosphoantigens are licensed for clinical application and in clinical trials, respectively. Recent advances on their multipotent functions, not only to the innate immune response, but to DC and antigen presentation system, increase the interest in a possible usage in clinical treatments. The effectiveness of these compounds in stimulating a cytotoxic response against tumors, as well as amplifing the antigen presentation of soluble specific peptides through DCs, represents a new possibility in the approaches based on immune cells.

Furthermore, the intriguing capacity of $\gamma\delta$ T cells to naturally respond to particular infections, such as TB, put these cells in a central place mainly in those pathologies where the classical presentation of antigens is compromised. However, $\gamma\delta$ T cells are part of the multicellular immune system that is tightly regulated by multiple pathways and cells including the regulatory cells. We still know very little about the nature of $\gamma\delta$ T cell antigens, their precise recognition mechanism, and their therapeutic relevance. Also the mechanisms regarding DC/$\gamma\delta$ T cells cross talk are still not clear, as the receptors and ligands involved in this interaction, the molecular factors, and the possibility to verify this interaction in a model *in vivo*. Future studies should also address the possible advantage of combining $\gamma\delta$ T cell therapy with conventional therapy or other therapeutical approaches.

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Review Article

Innate Immune Effectors in Mycobacterial Infection

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Tuberculosis, which is caused by infection with *Mycobacterium tuberculosis* (Mtb), remains one of the major bacterial infections worldwide. Host defense against Mtb is mediated by a combination of innate and adaptive immune responses. In the last 15 years, the mechanisms for activation of innate immunity have been elucidated. Toll-like receptors (TLRs) have been revealed to be critical for the recognition of pathogenic microorganisms including mycobacteria. Subsequent studies further revealed that NOD-like receptors and C-type lectin receptors are responsible for the TLR-independent recognition of mycobacteria. Several molecules, such as active vitamin D_3, secretary leukocyte protease inhibitor, and lipocalin 2, all of which are induced by TLR stimulation, have been shown to direct innate immune responses to mycobacteria. In addition, Irgm1-dependent autophagy has recently been demonstrated to eliminate intracellular mycobacteria. Thus, our understanding of the mechanisms for the innate immune response to mycobacteria is developing.

1. Introduction

In humans, tuberculosis is one of deadly infectious diseases. Indeed, approximately 2 million tuberculosis patients die every year. The risk of disease is also increased by emergence of acquired immune deficiency syndrome and development of multidrug-resistant mycobacteria [1]. Therefore, it is important to understand the host defense mechanisms against mycobacteria. Inhalation of aerosols containing *Mycobacterium tuberculosis* (Mtb) causes tuberculosis. After inhalation, Mtb invades alveolar macrophages to enter into the host and establish the infection. The host, in turn, ignites defense responses through sequential activation of immunity, a combination of innate and adaptive immune systems. In the adaptive phase of immune responses, the importance of Th1/IFN-γ-mediated responses in mycobacterial infection has been well established [2]. In contrast, although macrophages are the major target of invasion by Mtb, how the innate arm of immunity mediates host defense against mycobacteria had long remained unknown. However, the mechanisms behind innate immune responses have been revealed in the past 15 years following the identification and characterization of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) [3]. Furthermore, it has been elucidated that TLR-dependent activation of innate immunity controls the development of adaptive immune responses [4]. The involvement of PRRs other than TLRs in the recognition of mycobacteria has also been revealed. In addition to the induction of adaptive immune responses, the PRR recognition of mycobacteria induces expression of several effector molecules participating in the innate host responses. The role of these innate effector molecules in mycobacterial infection is being elucidated. PRR-independent mechanisms for mycobacterial killing, such as autophagy, have also been revealed. In this paper, we will describe recent advances in our understanding of effectors that mediate innate immune responses against mycobacteria.

2. Toll-Like Receptors in Mycobacterial Infection

Innate immune responses after mycobacterial infection are initiated by recognition of mycobacterial components by PRRs, with mycobacterial components activating several

TLRs (Figure 1). Genomic DNA from a *Mycobacterium bovis* strain, bacillus Calmette–Guérin (BCG), have an ability to augment NK cell activity and induce type I IFNs from murine spleen cells and human peripheral blood lymphocytes. The immunostimulatory activity of mycobacterial DNA was ascribed to the presence of palindromic sequences including the 5′-CG-3′ motif, now called CpG motif [5], and now known to activate TLR9 [6]. The mycobacterial cell wall consists of several glycolipids. Among these, lipoarabinomannan (LAM) lacking mannose end capping, lipomannan (LM), and phosphatidyl-*myo*-inositol mannoside (PIM) are recognized by TLR2 [7, 8]. The 19-kDa lipoprotein of Mtb also activates macrophages via TLR2 [9, 10]. TLR4 is also presumed to recognize mycobacterial components.

The *in vivo* importance of the TLR-mediated signal in host defense to Mtb was highlighted in studies using mice lacking MyD88, a critical component of TLR signaling. MyD88-deficient mice are highly susceptible to airborne infection with Mtb [11–13]. In contrast to mice lacking MyD88, mice lacking individual TLRs are not dramatically susceptible to Mtb infection. Susceptibility of TLR2-deficient mice to Mtb infection varies between different studies [14, 15], while TLR4-deficient mice do not show high susceptibility to Mtb infection [16, 17]. A report demonstrates that TLR9-deficient mice are susceptible to Mtb infection and mice lacking both TLR2 and TLR9 are more susceptible [18]. These findings indicate that multiple TLRs might be involved in mycobacterial recognition. However, a recent report using mice lacking TLR2/TLR4/TLR9 indicated that these triple KO mice show a milder phenotype than MyD88-deficient mice [12]. Therefore, more intensive examination is required to reveal whether TLRs or molecules other than TLRs activating MyD88 mediate innate immune responses to mycobacterial infection. This study also demonstrated that Th1-like adaptive immune responses are induced even in Mtb-infected MyD88-deficient mice [12]. Therefore, the TLR/MyD88-independent component of innate immunity is involved in the induction of adaptive immune responses during mycobacterial infection. The TLR/MyD88-independent response might be induced by other PRRs described below.

3. Non-TLRs in Mycobacterial Infection

Several recent findings have indicated that PRRs other than TLRs evoke innate immune responses [19]. These include RIG-I-like receptors, NOD-like receptors (NLRs), and C-type lectin receptors. Among these PRRs, NOD-like receptors and C-type lectin receptors have been implicated in the innate recognition of mycobacteria (Figure 2).

NOD2 is a member of NLRs that recognize muramyl dipeptide (MDP), a core component of bacterial peptidoglycan, in the cytoplasmic compartment. Macrophages from NOD2-deficient mice show a defective cytokine production after Mtb infection [20]. Similarly, mononuclear cells of individuals homozygous for the *3020insC NOD2* mutation show a defective cytokine response after stimulation with Mtb [7]. Activation of the NOD2-mediated pathway is induced by stimulation with live Mtb, but not by heat-killed Mtb [8]. Live Mtb, which is localized in the phagosomal compartment within macrophages, stimulates the cytosolic NOD2 pathway by inducing phagosomal membrane damage [21]. The NOD2 ligand MDP is N-acetylated in most bacteria. However, MDP is N-glycolylated by N-acetyl muramic acid hydroxylase (NamH) in mycobacteria. Analyses using *M. smegmatis* namH mutant and NOD2-deficient mice showed that N-glycolyl MDP is recognized by NOD2. In addition, N-glycolyl MDP is the more potent NOD2 activator than N-acetyl MDP [22]. Thus, NOD2 contributed to the recognition of mycobacteria.

Several members of the NLR family, such as NLRP1, NLRP3, and IPAF, induce assembly of the inflammasome, which leads to caspase-1-dependent secretion of IL-1β and IL-18 [23]. The involvement of IL-1β and IL-18 in mycobacterial infection was demonstrated in studies using knockout mice [24–27]. A recent study demonstrated that mycobacteria inhibit the inflammasome-dependent casapase-1 activation leading to defective IL-1β production [28]. The inhibition of caspase-1 activation has further been shown to be mediated by an Mtb gene, *zmp1*, which encodes a putative Zn^{2+} metalloprotease. Thus, Mtb has a strategy that evades the inflammasome-mediated innate immune responses.

C-type lectin receptors, such as mannose receptor, were originally reported to mediate phagocytosis of mycobacteria [29]. Another C-type lectin receptor, DC-SIGN, has been shown to recognize mycobacteria, and thereby modulate the function of dendritic cells [30–32]. Recognition of mycobacteria by dectin-1 has been shown to induce gene expression such as TNF-α, IL-6, and IL-12 [33, 34]. In addition, macrophage inducible C-type lectin (Mincle) has recently been shown to recognize trehalose-6,6′-dimycolate (TDM: also called cord factor), a mycobacterial cell wall glycolipid that is the most studied immunostimulatory component of Mtb [35, 36], thereafter modulating macrophage activation. Thus, several C-type lectin receptors are involved in the recognition of mycobacteria.

CARD9 is involved in the signaling pathways of several PRRs including TLRs, NOD-like receptors, and FcRγ-associated C-type lectin receptors through association with Bcl-10 and MALT. Therefore, it is not surprising that CARD9-deficient mice are highly susceptible to Mtb infection. However, interestingly the high susceptibility of CARD9-deficient mice to the infection has been shown to be excessive inflammatory responses due to defective production of the immunosuppressive cytokine IL-10 [37]. Mincle is a member of C-type lectin receptors associated with FcRγ [38]. Accordingly, TDM-induced immune responses are mediated by the signaling pathway activating CARD9 [36, 39].

TLRs and C-type lectin receptors are expressed on the plasma membrane or the endosomal/phagosomal membrane, whereas NOD-like receptors are expressed within the cytoplasm. Indeed, distinct patterns of TLR- and NOD-like receptor-mediated gene expression profiles have been demonstrated in infection with intracellular bacteria [40]. Thus, several PRRs recognize mycobacteria in distinct sites within the host cells (macrophages) to synergistically induce effective host defense responses.

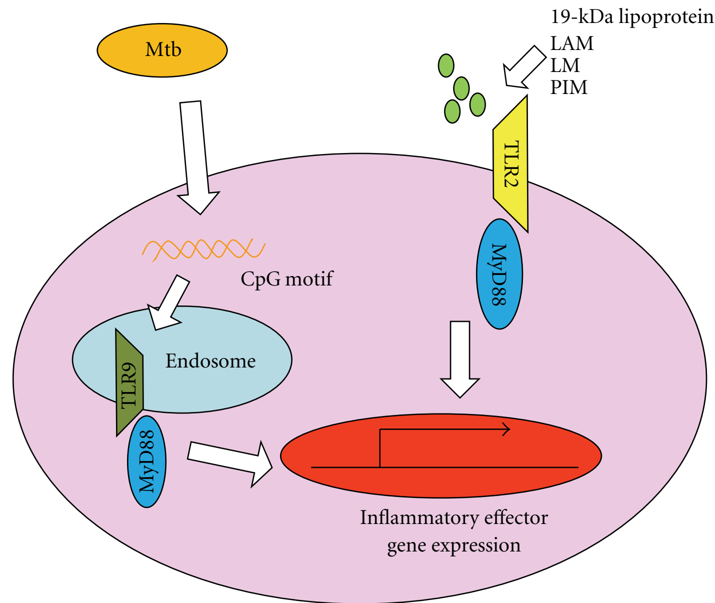

FIGURE 1: Recognition of mycobacteria by Toll-like receptors. TLR2 recognizes several mycobacterial-derived components. TLR9 recognizes mycobacterial DNA including the CpG motif within endosomal compartments. TLR-dependent recognition of mycobacteria induces activation of signaling pathways via the adaptor molecule MyD88, leading to activation of gene expression.

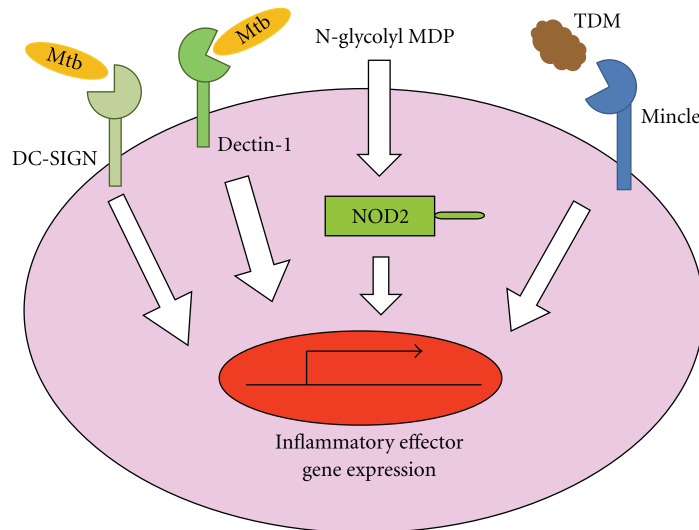

FIGURE 2: Recognition of mycobacteria by pattern recognition receptors. Several pattern recognition receptors, such as NOD-like receptors and C-type lectin receptors, mediate the TLR-independent recognition of mycobacteria. NOD2, a member of NOD-like receptors, recognizes mycobacterial N-glycolyl MDP within the cytoplasm. DC-SIGN and dectin-1 are members of C-type lectin receptors, which are implicated in the recognition of mycobacteria. In addition, Mincle has been shown to recognize TDM (a mycobacterial cell wall glycolipid).

4. Effectors for Mycobacterial Killing

The recognition of mycobacteria by several PRRs induces the expression of several genes that mediate host defense (Figure 3). Among these gene products, vitamin D receptor (VDR) and Cyp27b1, a 25-hydroxyvitamin D_3 1-α-hydroxylase that catalyzes inactive provitamin D into the bioactive form of vitamin D (1, 25 $(OH)_2D_3$), have been shown to be induced by TLR2 ligands in human macrophages [41]. Stimulation of macrophages with 1, 25 $(OH)_2D_3$ induces the expression of the antimicrobial peptide cathelicidin, and thereby enhances the antimycobacterial killing activity [42]. In addition to cathelicidin, the small cationic antimicrobial peptide defensin mediates innate immune responses to Mtb [43, 44]. Experimental infection of the lung epithelial cell line A549 with Mtb strongly induces production of human β-defensin HBD-2, which leads to Mtb killing [43]. HBD-2 expression has also been shown to be induced by TLR2 [45].

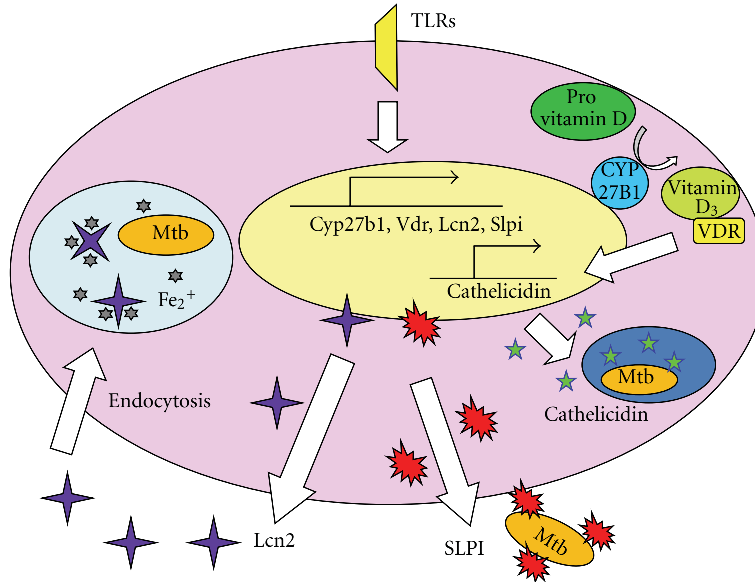

Figure 3: TLR-dependent innate response to mycobacteria. Several TLR-dependent gene products mediate innate immune responses to mycobacteria. Mycobacterial stimulation of TLR2 induces expression of Cyp27b1 and vitamin D receptor (VDR), both of which are involved in vitamin D_3-dependent induction of cathelicidin which directly kills mycobacteria. TLR-dependent induction of SLPI mediates disruption of the mycobacterial cell wall. Lcn2, which is also induced by TLR stimulation, is internalized into the alveolar epithelial cells and inhibits mycobacterial growth by sequestering iron uptake.

Gene expression analyses of the lung of mycobacteria-infected mice have identified several TLR-dependent genes that are involved in innate immune responses during mycobacterial infection. These genes include *Slpi*, encoding secretory leukocyte protease inhibitor (SLPI), and *Lcn2*, encoding lipocalin 2 (Lcn2). SLPI is a secreted protein composed of two cysteine-rich whey acidic protein (WAP) domains [46–48]. SLPI was named after its presence in secretions and its function as a serine protease inhibitor. SLPI was originally shown to mediate wound healing [49, 50]. SLPI is produced by bronchial and alveolar epithelial cells as well as alveolar macrophages and is secreted into the alveolar space at the early phase of mycobacterial respiratory infections. Recombinant mouse SLPI effectively inhibits the *in vitro* growth of BCG and Mtb through disruption of the mycobacterial cell wall structure. Cationic residues within the WAP domains of SLPI are essential for the disruption of mycobacterial cell walls. Moreover, SLPI-deficient mice are highly susceptible to mycobacterial infection [51]. The mechanism by which SLPI attaches to the membrane of mycobacteria has been elucidated. SLPI recognizes mannan-capped lipoarabinomannans and phosphatidylinositol mannoside, which are conserved in mycobacteria. Thus, SLPI might act as a PRR in order to bind to the mycobacterial membrane [52].

Lcn2 (also known as neutrophil gelatinase-associated lipocalin, 24p3, or siderocalin) was originally identified in the granules of human neutrophils. Lcn2 is a member of the lipocalin protein family and able to bind to small hydrophobic molecules, siderophore. It is a bacterial molecule made in iron-limited environment and facilitates iron uptake by bacteria [53–58]. The expression of Lcn2 is increased in macrophages of LPS-treated mice [59]. In addition, it is secreted into the alveolar space by alveolar macrophages and epithelial cells during the early phase of respiratory mycobacterial infection. Lcn2 inhibits *in vitro* growth of Mtb by binding the mycobacterial siderophore carboxymycobactin, thereby sequestering iron uptake. Moreover, Lcn2-deficient mice are highly susceptible to intratracheal infection with Mtb. Lcn2 is internalized into alveolar epithelial cells by endocytosis and colocalized with mycobacteria within the cells. Therefore, Lcn2 presumably sequesters iron uptake of mycobacteria within epithelial cells and thereby inhibits their intracellular growth. Within macrophages, the endocytosed Lcn2 and mycobacteria show distinct patterns of subcellular localization, which might allow growth of mycobacteria within macrophages [60]. Thus, Lcn2, which is secreted into the alveolar space during the early phase of mycobacterial infection, is endocytosed into alveolar epithelial cells, thereby inhibiting mycobacterial growth [61].

5. Autophagy in Mycobacterial Infection

Phagocytosis of myobacteria and PRR-dependent recognition of mycobacteria activate several effector functions in macrophages (Figure 4). Maturation of phagosomes is a crucial step in the elimination of intracellular bacteria. The natural-resistance-associated macrophage protein (Nramp1), which is encoded by Slc11a1, is thought to mediate transportation of divalent cations in the phagosomal membrane and thereby sequesters iron (Fe^{2+}) from mycobacteria to enhance bacterial killing by macrophages [62]. Polymorphisms of the *SLC11A1* gene have been associated with susceptibility to several infectious diseases,

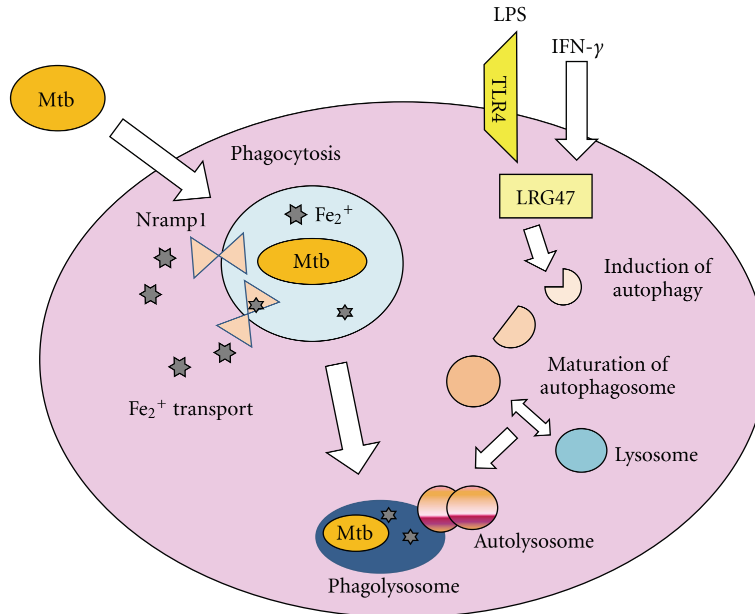

Figure 4: Effectors that mediate mycobacterial killing in macrophages. Macrophages eliminate invading mycobacteria by activating several effector functions, such as phagosomes and autophagy. Nramp1 is expressed in the phagosomal membrane and presumably mediates mycobacterial killing by sequestering iron uptake. IFN-γ and the TLR4 ligand induce expression of LRG47, which in turn stimulates autophagy in macrophages. Autophagy is responsible for mycobacterial killing by promoting fusion of mycobacterial phagosomes to lysosomes.

including tuberculosis [63, 64]. However, *in vivo* studies have shown that Nramp1-deficient mice are not more susceptible than wild-type mice to infection with virulent Mtb [65]. Thus, the role of Nramp1 in mycobacterial infection is still controversial. This might be due to the presence of other killing mechanisms for mycobacteria in macrophages. Indeed, autophagy has recently been shown to be involved in host defense against several intracellular pathogens that reside within phagosomes [66]. Autophagy was originally identified as a homeostatic mechanism for the catabolic reaction of cellular constitutes [67, 68]. It has been demonstrated that autophagy mediates innate immune responses against mycobacteria by promoting phagolysosomal maturation within macrophages [69, 70]. Autophagy is induced by IFN-γ-dependent induction of a member of the immunity-related p47 guanosine triphosphatases (IRG) family, LRG47 (also known as Irgm1) in murine macrophages [69]. The importance of LRG47 in resistance to Mtb infection was demonstrated in LRG47-deficient mice, which show high susceptibility to infection [71]. A subsequent study demonstrated that stimulation of macrophages with the TLR4 ligand LPS leads to the MyD88-independent induction of autophagy, which enhances mycobacterial colocalization with the autophagosomes. Since LPS stimulation induces expression of LRG47, the TLR signaling establishes a close relationship between innate immunity and autophagy in mycobacterial infection [72]. In humans, the most equivalent gene to murine Irgm1 is IRGM. IRGM has also been implicated in the induction of autophagy in mycobacteria-infected human macrophages [73]. Irgm1 has been shown to associate with the mycobacterial phagosome by interacting with phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P(2)) and PtdIns(3,4,5)P(3) [74]. The connection of the IRG family of proteins with autophagy has been further demonstrated in an alternative intracellular infection model. In this study, Irgm3 (also known as IGTP) has been implicated in autophagy induction in macrophages infected with *Toxoplasma gondii* [75].

p62 (also called A170 or SQSTM1) directly binds to cytosolic polyubiquitinated proteins and thereby induces their autophagic clearance [76, 77]. It has also been shown that p62 targets intracellular *Salmonella typhimurium* decorated by ubiquitinated proteins to induce autophagy [78]. In the case of mycobacteria residing in the phagosome, p62 delivers cytosolic ubiquitinated proteins to autophagolysosomes where they are proteolytically processed to products that are able to kill mycobacteria [79]. In accordance with this finding, it has been shown that mycobacterial killing by ubiquitin-derived peptides is enhanced by autophagy [80].

As described above, 1, 25 $(OH)_2D_3$ mediates antimycobacterial activity via induction of cathelicidin. A recent report demonstrated that 1, 25 $(OH)_2D_3$-mediated expression of cathelicidin induces autophagy [81]. Thus, several innate immune effectors are closely interacted.

6. Human Genetics in Tuberculosis

In addition to the intensive studies using murine models, considerable advances have been made in our understanding of the susceptibility to Mtb infection in humans through the identification of mutations and polymorphisms of

innate immunity-related genes in tuberculosis patients. As described above, polymorphisms of the *SLC11A1* gene are associated with tuberculosis. Subsequent studies identified a significant distinction between tuberculosis patients and healthy controls in *TLR2* Arg753Gln polymorphism genotype, indicating that the *TLR2* polymorphism influences the susceptibility of Mtb infection [82]. *VDR* polymorphisms have also been implicated in the susceptibility of Mtb infection [83]. These studies suggest that several genes, which have been revealed to be critical in innate responses in mouse models of Mtb infection, regulate Mtb infection in humans.

7. Conclusion

Since the discovery of TLRs at the end of the 20th century, rapid advances have been made in our understanding of the mechanisms for activation of innate immunity. Accordingly, innate immunity has been revealed to have a pivotal role in host defense against mycobacteria. The TLR-independent mechanisms for the innate immune response to mycobacteria have also been elucidated. The emergence of multidrug-resistant Mtb is now a major public health problem all over the world. In this context, it is highly critical to develop a new strategy for the treatment of Mtb-infected patients that supplements the conventional antimycobacterial chemotherapeutic drugs. More precise understanding of the innate immune response to Mtb will pave the way for the development of an effective drug that targets the host innate immunity for the treatment of tuberculosis.

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Research Article

Clustering of *Mycobacterium tuberculosis* Cases in Acapulco: Spoligotyping and Risk Factors

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Recurrence and reinfection of tuberculosis have quite different implications for prevention. We identified 267 spoligotypes of *Mycobacterium tuberculosis* from consecutive tuberculosis patients in Acapulco, Mexico, to assess the level of clustering and risk factors for clustered strains. Point cluster analysis examined spatial clustering. Risk analysis relied on the Mantel Haenszel procedure to examine bivariate associations, then to develop risk profiles of combinations of risk factors. Supplementary analysis of the spoligotyping data used SpolTools. Spoligotyping identified 85 types, 50 of them previously unreported. The five most common spoligotypes accounted for 55% of tuberculosis cases. One cluster of 70 patients (26% of the series) produced a single spoligotype from the Manila Family (Clade EAI2). The high proportion (78%) of patients infected with cluster strains is compatible with recent transmission of TB in Acapulco. Geomatic analysis showed no spatial clustering; clustering was associated with a risk profile of uneducated cases who lived in single-room dwellings. The Manila emerging strain accounted for one in every four cases, confirming that one strain can predominate in a hyperendemic area.

1. Introduction

Tuberculosis (TB) remains a global health problem, mainly related to poverty and concomitant diseases [1]. The reported annual incidence of pulmonary TB in Mexico is 14.27 cases per 100,000 people; in Guerrero state, the rate is 34.56 per 100,000 [2]. Acapulco is of particular concern as a centre for internal migration in Guerrero state, in addition to its role in international tourism.

TB recurrence and reinfection have quite different implications for prevention. A key issue among migrants to a city like Acapulco is whether TB infection contracted earlier in life is reactivated, to be detected in the city as clinical cases, or whether these are new infections contracted in the city. One research device to help differentiate between recurrence and reinfection is to establish whether contemporary cases are clustered; unless they all came from the same distant place of original infection, clustering suggests a shared source of infection in the locality of the study.

Molecular typing is a well-recognized tool for identifying clustering. The sequence insertion of IS6110 is often the basis for molecular fingerprinting, as the number of copies of this insertion and its localization in the chromosome varies among different strains [6]. Another sequence targeted for molecular typing is the 36-base pair Direct Repeat (DR) locus. Spoligotyping is a PCR-based typing method that

generates distinct patterns based on the hybridization of 43 different oligonucleotides to amplified spacer sequences that lie between DR loci in the *M. tuberculosis* chromosome [7]. This estimates the frequency of recent infection with *M. tuberculosis* estimated by identification of clusters of a shared spoligotype.

This paper studies spoligotypes isolates of *M. tuberculosis* from a series of patients in Acapulco, to determine the level of clustering and to identify risk factors for clustering.

2. Material and Methods

2.1. Study Location and Population. A total of 330 patients with pulmonary TB presented a positive diagnosis between February 2001 and September 2002 in the municipality of Acapulco. Receipt of funding set the start of the series which closed with 330 consecutive participants recruited through a review of recent medical records. The series excluded only 30 who had left the city and who could not be localised. Using addresses from clinical records, researchers approached each participant and explained the study, obtaining written consent prior to administering a pretested questionnaire. The study provided an opportunity to follow patients with a preliminary sputum positive result; the Ministry of Health offered directly observed therapy, short course strategy (DOTS) [8] for all cases.

2.2. Bacterial Strains. The 300 participants contacted yielded 273 isolates of *M. tuberculosis* from sputum samples collected by the Acapulco General Hospital, the Donato G. Alarcón Hospital, the State Public Health Laboratory and the Clínica Avanzada de Atención Primaria a la Salud. Processing began with the Petroff method for preparation and decontamination of sputum samples, followed by inoculation of 0.5 mL of each sample onto Löwenstein-Jensen media, incubated at 37°C for eight weeks.

2.3. Genotyping. Extraction of DNA required resuspension of *M. tuberculosis* colonies in 1 mL of sterile distilled water, with samples lysed at 90°C for 10 minutes and frozen at −70°C for 15 minutes. Samples were then thawed and incubated at 90°C for 10 minutes. Centrifugation for three minutes concentrated bacterial contents, and the supernatant was transferred to a new tube. Spoligotyping relied on a commercially available kit (Isogen Bioscience BV Maarssen, The Netherlands) for amplification of DNA from the DR locus, the region with the highest level of polymorphism in the *M. tuberculosis* chromosome [7]. PCR amplification used 5 uL of extracted DNA from each sample combined with 1.5 uL $MgCl_2$, 2.5 mM of each dNTP, 5 μl 10X buffer, 0.5 U Taq polymerase, and 20 pmol of each primer (DRa: biotin-5′-CCG AGA GGG GAC GGA AAC-3′ and DRb: 5′-GGT TTT GGG TCT GAC GAC-3′) in a final volume of 50 uL. The amplification protocol required 5 minutes at 94°C followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C, and 30 seconds at 72°C, with a final extension of 10 minutes at 72°C. The assay included two positive controls (chromosomal DNA from *M. tuberculosis* H37Rv and from *M. bovis* BCG P3) and a negative control (sterile H_2O).

We hybridized the amplified DNA with 43 oligonucleotides covalently linked to a nylon membrane (Isogen Bioscience BV, Maarssen, The Netherlands) at 60°C for 1 hour in a blotter with 45 lines (Miniblotter 45; Immunetics, Cambridge, Mass). Afterwards, hybridized DNA fragments were incubated with streptavidin, conjugated to peroxidase (Boehringer Mannheim), and then analyzed by chemiluminescence by incubating for 1 minute in 20 mL of ECL detection reagent (Amersham, Buckinghamshire, England) and exposing to X-ray film for 20 minutes.

2.4. Data Capture and Analysis. Data capture from the questionnaires relied on Epi-Info (CDC, version 6.04). We compared spoligotyping results with the SpolDB4 spoligotyping database from the Institute Pasteur de Guadeloupe which, at the time of the analysis, contained 1,939 types from 39,295 strains contributed by 122 countries [3, 9]. The analysis defined a cluster as two or more isolates with identical genetic patterns. We used the preexisting code for those already identified matching patterns. When the spoligotype was not found in SpolDB4, we labeled it as "Mx" with a number (for example, Mx1).

We estimated the Recent Transmission Index (RTI) using the formula of RTI$n - 1$ [10] and RTIn, which takes into account the number of patterns with unique genotype (singletons) described by Luciani et al. [11]. Supplementary analysis of the spoligotyping data used SpolTools [12]: DESTUS (Detecting Emerging Strains of Tuberculosis Using Spoligotypes) focused on the detection of rapidly propagating strains; spoligoforests allowed visualization of probable relations between the spoligotypes based on a plausible history of mutation events. The clusters of strains sharing the same spoligotype in the diagram are nodes, labeled with shared type (ST) numbers in SpolDB4 [3].

2.5. Risk and Point Cluster Analysis. Potential risk factors for clustering included age (less than or older than 30 years), sex, marital status, education, employment, ethnicity, area of residence (urban or rural), duration of residence, number of people in the dwelling, use of alcohol, concomitant illness, or spouse with TB. When simple bivariate risk analysis revealed no statistically significant contrasts, we derived risk profiles of factor combinations associated with clustering. We examined these risk profiles in a multivariate analysis, beginning with a saturated model and eliminating the weakest association stepwise until only significant associations remained in the final model. We report this as an adjusted odds ratio and 95% confidence interval.

For the spatial analysis, we geoindexed cases on a map of Acapulco at a topographic chart scale of 1 : 50,000 in DXF vector data format (Instituto Nacional de Estadística, Geografía e Informática; 2001). Point cluster analysis of quadrants relied on QUADRAT (IDRISI 32, Clark Labs, Worcester, MA), which determines the total number, mean number or density, variance and variance/mean ratio of points in cases where each grid cell measures the total point count found in that cell. The variance/mean ratio describes the pattern of a point set, with values close to 1.0 suggesting a random point pattern, values significantly smaller than

1.0 suggesting a regularly distributed pattern, and values greater than 1.0 suggesting a clustered pattern. The location of each case was compared with the locations of the reported TB cases. Final display relied on ArcView GIS software (ArcView GIS 3.2, Environmental Systems Research Institute Inc., Redlands, CA).

This study was approved by the Committee of Research Ethics at the Centro de Investigación de Enfermedades Tropicales of the Universidad Autónoma de Guerrero.

3. Results

3.1. Spoligotyping Patterns of M. tuberculosis Isolates. Spoligotyping the 267 isolates produced 85 distinct genotypes, 59 of them with a unique pattern. The 208 (77.9%) remaining isolates were grouped into 26 clusters that were shared with at least one patient. The cluster size varied from 2 to 70 patients, however, most (21/26) included 2 to 5 patients. The Recent Transmission Index, or RTIn, taking into account the number of cases with unique genotypes (singletons), was 0.78, and the RTI$n - 1$ was 0.68. Table 1 presents a comparison of the Acapulco spoligotypes with data from the global spoligotype database of the cluster genotypes; four were new and 22 had been previously identified by this database.

Approximately, 59% of the 85 Acapulco spoligotypes, involving 58 (21.7%) of the 267 *M. tuberculosis* isolates, were previously unreported. The main SITs (Spoligo International Type Number)—19, 8, 53, 42, and 342—permitted the classification of isolates in representative patterns described as families or groups [3, 13, 14]. The largest single cluster of 70 patients included 26.2% (70/267) of *M. tuberculosis* isolates of Acapulco; the spoligotype pattern was the East African Indian group (EAI2), previously reported as *M. tuberculosis* Manila family [15]. Approximately 44.6% (119/267) of the isolates descended from East African Indian family (EAI) subgroups 1, 2, 3, and 5. Another 11.6% (31/267) came from Latin American and Mediterranean families (LAM) subgroups 1, 2, 3, 4, 6, and 9. A further 11.2% (30/267) belonged to the "T" family (strain of modern TB) subgroups 1 and 2. Another 3.7% (10/267) were of the "Manu" family, subgroups 1 and 2, and 3.0% (8/267) consisted of the Haarlem family, subgroups 1, 2, and 3. A further 1.9% (5/267) were group "S", and 1.1% (3/267) were "X" family subgroup 3 and 3 variant 1. Two cases (0.8%) were not classified ("U"), and 0.4% (1/267) pertained to the Bovis group, subgroup 1. We identified no isolate of the Beijing genotype.

3.2. Detection of an Emerging Strain and Visualization of the Relations between Spoligotypes. The S19 strain demonstrates an elevated rate of transmission, independent of the sampling fraction f (Table 2), behaving as an emerging strain according to the method proposed by Tanaka and Francis [16]. According to the SpolDB4 database, S19 corresponds to the EAI2-Manila strain.

Figure 1 shows the Spoligoforest hierarchical layout, with the lines between the nodes denoting the weights calculated using this model. The spoligoforest contains three trees with connected components and 26 unconnected nodes. The biggest tree has the ST 100 strain as a root, suggesting that it is the oldest spoligotype in this tree. Seven spoligotypes descend from ST 100, four of which are in small clusters (ST 54, ST 1193, ST 236, and Mx 3, with 4, 3, 2, and 2 isolates, resp.). The ST 54 and ST 236 spoligotypes form two lineages distinct from ST 100. Comparing these spoligotypes with the families in the SpolDB4 database, these lineages are classified as Manu Family and EAI (East African Indian). The Mx 45 genotype, not registered in the SpolDB4 database, also descending from ST 100, originates another lineage. ST 53 and its descendents ST 42, ST 34, ST 47, ST 52, ST 50, ST 51, ST 291, and ST 118 belong to the LAM (Latin American Mediterranean), Haarlem, cluster T and Clade S family of strains. ST 342 and its descendents ST 19 and Mx 44, ST 19 belongs to the Manila family (EAI2-Manila) and is the biggest node, representing 70 isolates and Mx 44 are not registered in SpolDB4. Finally, ST 8 is from the EAI (East African Indian) family, and its descendents Mx 1, Mx 2, and Mx 46 are not registered in SpolDB4.

3.3. Epidemiological and Clinical Characteristics of the Tuberculosis Cases. Of 330 diagnosed TB cases in the series, 30 (9.1%) could not be found; 273 (91.0%) of the 300 contacted yielded a positive culture of *M. tuberculosis:* 190 (63.3%) of them were male, and the average age was 41.0 years (standard deviation: 15.5, range: 15–86 years). Most (90.7%, 272/300) were from Guerrero state, and 55.1% (150/272) of these were from the municipality of Acapulco (Table 3). Some 59% (172/293) lived less than 16 years in their community of origin. The average duration of residence in Acapulco was 2.6 years (SD: 1.66, range: 1–10 years). Household had an average of 5.1 occupants (SD: 2.83, range: 1–20 people).

Data on the start date of symptoms permitted identification of the "first case" (index case) in the largest clusters. The "first case" of the Manila cluster, for example, was a 35-year-old adult addicted to drugs and alcohol who had symptoms for 10 years and, on examination, had advanced pulmonary TB (bacilloscopy positive, grade 3). Thirty of the 70 cases in this cluster reported a concomitant disease, diabetes being the most common (17/70).

The largest five clusters included 70, 31, 22, 15, and 8 cases, together contributing 70% (146/208) of the case series (Table 4). Figure 2 shows a random distribution pattern of spatial variances. There was no evidence of spatial grouping, consistent with recent infection associated with factors other than residence.

Table 5 shows the list of conventional TB risk factors, none of which on their own showed a statistically significant association with clustering on univariate analysis. Seven risk profiles, combining two risk factors, did show significant differences between clustered and nonclustered TB cases (Table 6): males who consumed alcohol, unmarried men under the age of 30 years, unemployed single cases, indigenous cases without remunerated employment, young people in urban areas, and uneducated people living in one-roomed dwellings. The size of the study did not permit combining all of these profiles in a single multivariate model. Each was therefore tested to see if it was explained by each other profile

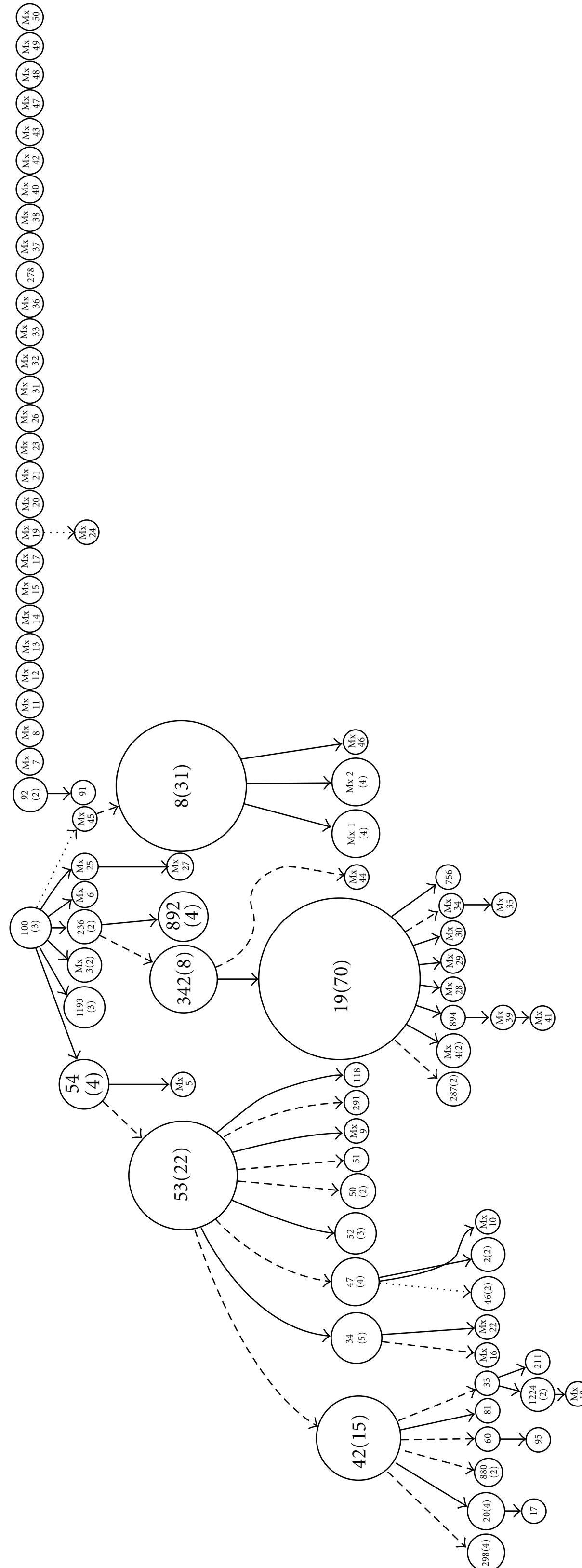
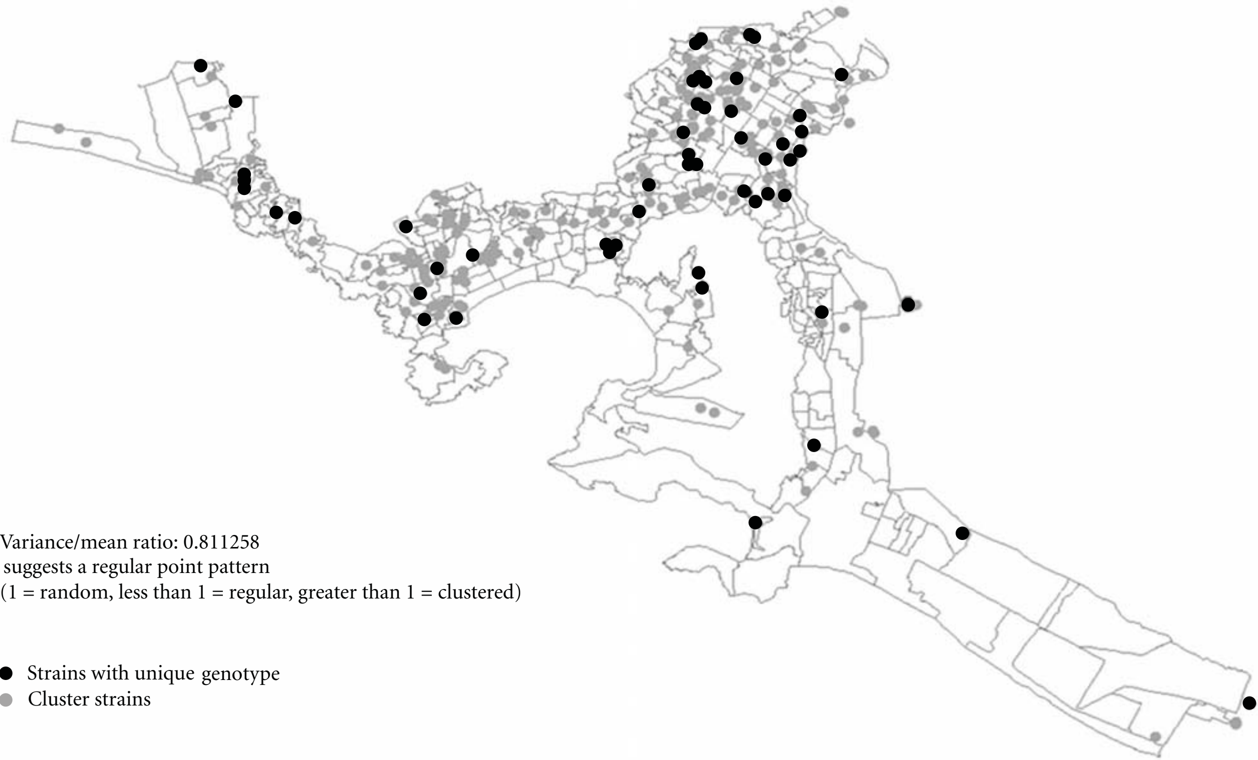
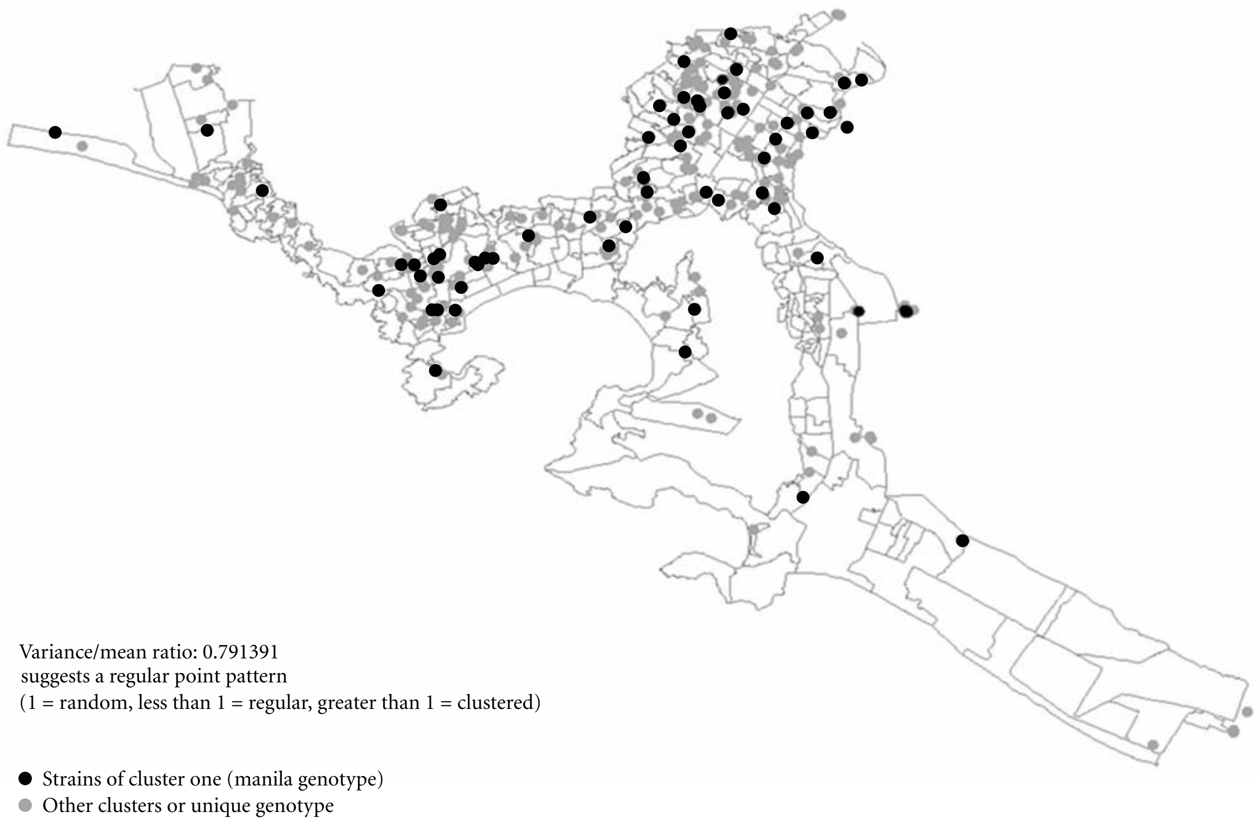


Figure 1: Spoligoforest of tuberculosis cases in Acapulco, Mexico. Nodes are labeled with the ST identifier as indicated in SoplDB4 [3], with the cluster size enclosed in parentheses. Spoligotype not in SpolDB4 was labeled as "Mx" with a number. The size of each node increases as a function of the number of isolates (size of the cluster); the lines among the nodes reflect the evolutionary relationships among spoligotypes with arrows that denote the descendent. If the weight of the line was equal to 1, we drew a solid line; if the weight was greater than or equal to 0.5 but less than 1, we used a dashed line; if the weight was less than 0.5, we used a dotted line.



(a) Comparison of *Mycobacterium tuberculosis* strains with unique patterns versus cluster spoligotypes.



(b) Comparison of *Mycobacterium tuberculosis* strains of cluster one (Manila genotype) with other clusters or unique spoligotype patterns.

Figure 2: Geographical distribution of tuberculosis cases in Acapulco, Mexico. Mapping and georeferencing of tuberculosis cases used a scale of 1 : 50000. Point cluster analysis relied on the IDRISI QUADRAT module. The variance/mean ratio significantly smaller than 1.0 suggests a regular distribution, while values greater than 1.0 suggest spatial clustering.

TABLE 1: Acapulco patterns of *Mycobacterium tuberculosis* spoligotypes.

SIT	Spoligotyping pattern	n	%	Clade
19		70	26.2	EAI2-Manila
8		31	11.6	EAI-3
53		22	8.2	T-1
42		15	5.6	LAM-9
342		8	3.0	EAI-5
34		5	1.9	S
54		4	1.5	MANU-2
47		4	1.5	H-1
398		4	1.5	LAM-9
892		4	1.5	EAI-5
20		4	1.5	LAM-1
New		4	1.5	MX-1
New		4	1.5	MX-2
100		3	1.1	MANU-1
52		3	1.1	T-2
1193		3	1.1	MANU-1
New		2	0.7	MX-3
50		2	0.7	H-3
236		2	0.7	EAI-5
46		2	0.7	U
880		2	0.7	T-1
1224		2	0.7	L1M-3
92		2	0.7	X-3
287		2	0.7	EAI-2
New		2	0.7	MX-4
2		2	0.7	H-2
New		1	0.4	MX-5
51		1	0.4	T-1
New		1	0.4	MX-6
New		1	0.4	MX-7
New		1	0.4	MX-8
New		1	0.4	MX-9
New		1	0.4	MX-10
291		1	0.4	T-1
60		1	0.4	LAM-4
95		1	0.4	LAM-6
New		1	0.4	MX-11
New		1	0.4	MX-12
118		1	0.4	T-2
New		1	0.4	MX-13
New		1	0.4	MX-14
New		1	0.4	MX-15
81		1	0.4	LAM-9
New		1	0.4	MX-16
New		1	0.4	MX-17
33		1	0.4	LAM-3
211		1	0.4	LAM-3
New		1	0.4	MX-18
New		1	0.4	MX-19

Table 1: Continued.

SIT	Spoligotyping pattern	*n*	%	Clade
New		1	0.4	MX-20
New		1	0.4	MX-21
New		1	0.4	MX-22
New		1	0.4	MX-23
91		1	0.4	X-3 Variant 1
New		1	0.4	MX-24
New		1	0.4	MX-25
New		1	0.4	MX-26
New		1	0.4	MX-27
894		1	0.4	EAI-1
New		1	0.4	MX-28
New		1	0.4	MX-29
New		1	0.4	MX-30
New		1	0.4	MX-31
New		1	0.4	MX-32
17		1	0.4	LAM-2
New		1	0.4	MX-33
New		1	0.4	MX-34
New		1	0.4	MX-35
New		1	0.4	MX-36
778		1	0.4	Bovis 1
New		1	0.4	MX-37
756		1	0.4	EAI-2
New		1	0.4	MX-38
New		1	0.4	MX-39
New		1	0.4	MX-40
New		1	0.4	MX-41
New		1	0.4	MX-42
New		1	0.4	MX-43
New		1	0.4	MX-44
New		1	0.4	MX-45
New		1	0.4	MX-46
New		1	0.4	MX-47
New		1	0.4	MX-48
New		1	0.4	MX-49
New		1	0.4	MX-50
Total		267	100	

SIT, designation of the spoligotype in the international database. Spoligotyping patterns, binary description: (■) hybridization and (□) no hybridization. *n*, number of strains; clade, defined mainly as described [4, 5]: EAI-2/Manila: East African Indian 2/Manila, T (ill-defined T cluster), LAM: Latin American and Mediterranean, S: S clade, MANU: Manu family, H: Haarlem, U: Undesignated, X: X cluster, Bovis. New clusters designated as MX (Mexican).

Table 2: Emerging strains detected in the pulmonary TB by using spoligotypes in Acapulco.

f							
0.95		0.5		0.1		0.01	
Strain	q value	Strain	q value	Strain	q value	Strain	q value
S19	0.00000000	S19	0.00000006	S19	0.00000477	S19	0.00001087
S8	0.03205437	S8	0.08223924	S8	0.17184278	S8	0.20014382

f = sampling fraction.

Table 3: Characteristics of the pulmonary TB case series in Acapulco.

Variable	$n = 300$	%
*Sex**		
Male	190	63.3
Female	110	36.7
*Age (in years)***		
15–24	51	17
26–34	72	24
35–44	58	19.3
45–54	55	18.3
55–64	40	13.3
65 +	24	8
Signs and symptoms		
Duration of the cough >3 months**	192	64
Lost weight**	271	90.3
Fever**	227	75.7
Hemoptysis**	151	50.3
Associated illness**	132	44
*Type of associated illness** (n = 132)*		
Diabetes	77	58.3
Drug user	20	15.2
AIDS	12	9.1
Alcoholism	7	5.3
Undernutrition	3	2.3
Others	13	9.8
*Prior TB***	26	8.7
*Husband/wife with prior TB** (n = 190)*	22	11.6
*Recent contact with someone with TB***		
Yes	112	37.3
No	150	50
Do not know	37	12.3
*Indigenous***	27	9
*Indigenous group** (n = 27)*		
Náhuatl	16	59.3
Mixteco	9	33.3
Amuzgo	2	7.4
*State of birth***		
Guerrero	272	90.7
Oaxaca	11	3.7
Estado de México	4	1.3
Michoacán	3	1
Other state	10	3.3
*Region of birth** (n = 272)*		
Acapulco	150	55.1
Zone Centro	25	9.2
La Montaña	7	2.6
Costa Chica	46	16.9
Costa Grande	21	7.7
Zone Norte	8	2.9
Tierra Caliente	12	4.4

*P value =.05,
**P value <.05.

Table 4: Characteristics of the largest clusters detected by spoligotyping in Acapulco.

No. Cluster	Cluster size (Number of male)	Age (range)	Place of birth (*n*)	Indigenous group (*n*)	Associated illness % (*n*)	Immune-compromised illness (*n*)	Previous TB % (*n*)	Contact/ TB case % (*n*)	Husband/ wife with TB % (*n*)	BAAR diagnostic %
1	70 (45)	41.6 (15–86)	Guerrero (62) Oaxaca (4) Mexico (1) DF (1) Gto (1) Tabasco (1)	Nah (4)	43% (30)	Diabetes (17) AIDS (2) Alcoholism (2) Drug user (2) undernourished (2)	10% (7)	39% (27)	8% (5)	+ 47 ++ 23 +++ 30
2	31 (21)	41.2 (21–73)	Guerrero (28) Oaxaca (1) EdoMex (1) Veracruz (1)	Nah (2)	45% (14)	Diabetes (10) AIDS (1)	13% (4)	26% (8)	17% (5)	+ 36 ++ 19 +++ 45
3	22 (19)	44.0 (22–76)	Guerrero (20) Oaxaca (1) EdoMex (1)	Nah (3) Mix (1)	27% (6)	Diabetes (4) Alcoholism (1) Drug user (1)	14% (3)	41% (9)	0	++ 36 +++ 64
4	15 (7)	45.4 (19–75)	Guerrero (13) Oaxaca (1) Michoacán (1)	Nah (1) Mix (2)	60% (9)	Diabetes (3) AIDS (1) Alcoholism (1) Drug user (3)	0	53% (8)	0	+ 33 ++ 27 +++ 40
5	8 (4)	43.6 (23–68)	Guerrero (8)	Mix (2)	50% (4)	Diabetes (1) Drug user (2)	0	63% (5)	0	+ 12.5 +++ 88

Place of birth: DF= Federal district, Mexico; Gto= Guanajuato state, EdoMex= State of México. Indigenous group: Nah= Náhuatl, Mix= Mixteco.

in turn. Only one risk profile remained that could not be explained by any others—uneducated cases living in single-room dwellings.

4. Discussion

Reflecting the still very partial documentation of spoligotypes worldwide, 58% of our spoligotypes were unique to Acapulco; 21.7% of cases were of a type not previously registered in the global database.

Our five most frequent spoligotypes included more than one half (54.7%) of the cases; these types are commonly recognized worldwide [3, 13, 14]. The T1 Spoligotype (shared type, 53), EAI (shared type 19, 8, and 342), and LAM9 (shared type 42) currently represent 28.75% of *M. tuberculosis* isolates in the global spoligotype database, being prevalent in Europe, Africa, India, and other countries [3].

The largest cluster, including 33.6% (70/208) of the isolates, was the Manila family. This demonstrates the public health impact of this strain in Acapulco. This relates to Mexico's historic ties with Asia, as the Manila strain is found throughout South East Asia, particularly in the Philippines (73%), Myanmar and Malaysia (53%), and Vietnam and Thailand (32%) [3, 15, 17]. In a study in the Philippines, 90% (43/48) of isolates were of the Manila genotype [15]. Phylogenetic studies of related spoligotype strains demonstrated that the EAI (East African Indian) genotype has shared ancestral relations [17]. This group includes the Manila or EAI2-Manila strain [3].

Internationally, certain *M. tuberculosis* strains are linked with a large proportion of recent infection, suggesting these strains might have greater transmissibility or higher probability to cause disease soon after transmission. These strains are associated with families or groups of related isolates, such as the genotypes of the Beijing family strain W, Haarlem, and Africa [3, 18, 19]. These "cluster strains" come from the Latin American Mediterranean families (LAM), Haarlem (H), and *M. Bovis* [20]. The Manila strain, responsible for a third of our cluster strain cases, might share this greater transmission or progress to active disease—at least in Acapulco.

The method used for detection of rapidly propagating strains [16] allowed identification of an emerging strain (strain S19), previously classified as EAI2-Manila [3]. This result coincides with the spoligoforest result, where node S19 ($n = 70$) has the most rapid transmission. Spoligoforest demonstrates all of the possible relations between spoligotypes under the assumption of spoligotype mutation [12]. In our data set, the largest root of the tree was the ST 100 strain, which corresponds to the MANU family. Among the principal descendants, we identified strains ST 54, ST 236, and ST 1193. However, the majority of the 26 unconnected nodes had not been previously registered in the SpolDB4 database.

Our proportion of clustered strains (77.9%) is higher than in other studies. A six-year study in two urban communities in South Africa identified 72% of the cases as belonging to clusters [21]. Similar results came from the Grand Canary Islands of Spain [4]. Other studies in South Africa and in Equatorial Guinea identified a level of grouping of 67 and 61.6%, respectively [5, 22]. A range of clustering rates (31 to 67.7%) have been reported in cities from industrial countries such as Spain, Italy, Holland, Denmark, Slovenia, Canada, and United States [23–29], while France, London, and Switzerland reported a minor portion of 18 to 27.6% cluster strains [30–32].

Table 5: Bivariate analysis of conventional TB risk factors, contrasting clustered and nonclustered cases.

Variable		Clustered		Not clustered		OR	95% CI
		N	%	*n*	%		
Sex	Male	135	80.6	32	19.4	1.60	0.87–2.2
	Female	72	72.7	27	27.3	1	
Age	<30 years	55	76.4	17	23.6	0.91	0.48–1.72
	≥30 years	150	78.1	42	21.9	1	
Marital status	Single	65	83.3	13	16.4	1.64	0.83–3.2
	Married	140	75.3	46	24.7	1	
Education	No formal studies	39	72.2	15	27.8	0.67	0.34–1.33
	Formal study	166	79.4	43	20.6	1	
Occupation	Unemployed	70	74.5	24	25.5	0.76	0.40–1.43
	Employed	135	79.4	35	20.6	1	
Ethnicity	Indigenous	19	73.1	7	26.9	0.75	0.28–2.08
	Mestizo	189	78.4	52	21.6	1	
Migration	Yes	99	76.2	31	23.8	0.82	0.45–1.47
	No	105	79.5	27	20.5	1	
Area of residence	Urban	153	78.5	42	21.5	1.19	0.59–2.37
	Rural	52	75.4	17	24.6	1	
Duration of residence	≥17 years	123	79.4	32	20.6	1.09	0.59–2.0
	<17 years	74	77.9	21	22.1	1	
Number of residents per household	≥6 persons	70	72.9	26	27.1	0.66	0.37–1.19
	<6 persons	135	80.4	33	16.6	1	
Use of alcohol	Yes	67	75.3	22	24.7	0.82	0.45–1.50
	No	137	78.7	37	21.3	1	
Concomitant illness	Yes	94	77.7	27	23.3	1.02	0.55–1.91
	No	111	77.6	32	23.4	1	
Spouse with TB	Yes	15	71.4	6	28.6	0.66	0.24–1.85
	No	113	79.0	30	21.0	1	

Table 6: Bivariate analysis of risk profiles associated with clustering.

Variable		Cluster		No cluster		OR	95% CI
		N	%	*n*	%		
Among cases who used alcohol	Male	71	85.5	12	14.5	2.1	1.05–4.03
	Female	68	73.1	25	26.9	1	
Among cases over the age of 29 years	Single	36	92.3	3	7.7	4.1	1.29–13.1
	Married or cohabiting	114	74.5	39	23.5	1	
Among cases single/unmarried cases	Unemployed	7	87.5	1	12.5	13.7	1.81–104
	Employed	8	40.0	12	60.0	1	
Among cases with remunerated employment	Mestizo	132	81.0	31	19.0	3.2	1.07–9.1
	Indigenous	8	57.1	6	42.9	1	
Among cases who live in one place for less than 15 years	Urban	66	78.6	18	21.4	3.0	1.22–10.0
	Rural	11	55.0	9	45.0	1	
Cases younger than 30 years of age	Urban	43	82.7	9	17.3	3.2	1.03–9.82
	Rural	12	60.0	8	40.0	1	
Among cases without formal education	1 room	22	91.7	2	8.3	8.9	2.0–39.1
	2 or more rooms	16	55.2	13	44.8	1	

OR= Odds Ratio, CI= Confidence Interval.

Considering the probable existence of an index case in each cluster, we estimated that 68% (208–26) of our 267 cases could have been due to recent infection [10]. Taking into account the number of singletons, the estimate of RTI was greater (78%). Migration, duration of the study [33], predominance of a local strain, simultaneous reactivation of an infection acquired remotely, and laboratory error can all influence the reliability of this inference [34]. In our study, other factors support a high proportion of recent transmission, including the high prevalence rate, the mobility of population [29], the bacillary load of the cases, and the duration of their symptoms.

Although one cluster included 70 cases, most clusters had five or fewer cases. Similar results come from the Grand Canary Islands [4]. A study in San Francisco in 1996-97 found 73 of 221 (33%) cases in multiple clusters, implying multiple points of infection in the community.

Analysis of why certain strains of *M. tuberculosis* propagated effectively points mostly to delayed diagnosis [35]. The index cases in five of our clusters were young adult males with at least one risk factor, such as drug use or alcohol addiction [4, 10, 25, 36]. Cases with an associated pathology have a higher tendency to acquire a new infection of *M. tuberculosis* [37] or to develop tuberculosis [38].

Our analysis of potential risk factors for clustering did not produce clearly actionable results. The notable absence of spatial clustering implies that place of residence is not a useful risk indicator. Type of residence (single room) combined with lack of formal education, on the other hand, was the single enduring risk profile.

SpolTools permitted the analysis of *M. tuberculosis* spoligotyping data to identify emerging strains and visualization of the probable evolutionary relationships between the spoligotypes in our series. Considering that a single emerging strain, the EAI2-Manila genotype, can account for so many cases, an evolution of spoligotyping could conceivably be used to evaluate the impact of TB prevention and early diagnosis efforts. Further studies of the virulence and drug sensitivity of the Manila genotype may be warranted.

Conflicts of interests

The authors do not have funding, commercial or other associations that might pose a conflict of interest.

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Clinical Study

Mycobacteria Infection in Incomplete Transverse Myelitis Is Refractory to Steroids: A Pilot Study

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Incomplete transverse myelitis (ITM) of unknown origin is associated with high rates of morbidity and mortality. This prospective, open-label study was undertaken to determine whether antituberculous treatment (ATT) might help patients with ITM whose condition continues to deteriorate despite receiving IV methylprednisolone treatment. The study consisted of 67 patients with steroid-refractory ITM in whom *Mycobacterium tuberculosis* (MTB) was suspected clinically and in whom other known causes of myelopathy were excluded. The study occurred from January 2003 to June 2010. Patients underwent trial chemotherapy with ATT. Efficacy was assessed by the American Spinal Injury Association (ASIA) scoring system, the Barthel Index (BI) and the Hauser Ambulation Index (AI) at baseline, 12 months, and 24 months, using magnetic resonance imaging (MRI). Of the 67 patients enrolled, 51 were assessed and 16 withdrew. At 24 months, 49 patients experienced benefits as indicated by significantly increased ASIA and BI scores. The Hauser AI index also improved with markedly decreased abnormal signals in spinal cord MRI over time. The results from this prospective study provide beneficial clinical and MRI data on the efficacy of ATT in ITM patients and suggests mycobacteria may be an important and neglected cause of myelitis.

1. Introduction

Transverse myelitis is a focal inflammatory disorder of the spinal cord, resulting in motor, sensory, and autonomic dysfunction. It is a poorly defined condition and displays heterogeneous etiopathogenesis [1, 2]. Incomplete transverse myelitis (ITM) is a rare syndrome easily recognized in clinical practice, with symptoms and signs producing bilateral partial impairment of motor, sensory, and autonomic functions, usually with an acute or subacute onset. Inflammation is not only the commonest basis of ITM but is also the most difficult to identify [3]. This clinical picture can be shared by variety of etiologies including different bacterial or viral infections. Clinical presentations of infectious and demyelinating diseases of the spinal cord are protean and often nonspecific. Magnetic resonance imaging (MRI) is sensitive but lacks specificity and most of the lesions feature high signal intensity on T2-weighted images. There does not seem to be any clinical, immunological, or radiological strategies that reliably distinguish between demyelinating, infectious, inflammatory, vascular, neoplastic, or paraneoplastic etiologies. Therefore, physicians must be aware of the many potential treatable etiologies for ITM which might mean there exists an efficient approach for the patients.

ITM is a well-recognized but rare symptom of *Mycobacterium tuberculosis* infection. However, in developing countries, mycobacteria infection remains a significant and threatening problem. Most mycobacteria infections in humans result in asymptomatic, latent infections [4, 5]. So the index of suspicion for these infections must therefore be raised to a level in ITM. Open-label use of antituberculous treatment (ATT) was reported as beneficial in neuromyelitis optica (NMO) patients' refractory to immunotherapy [6]. Given the lack of proven efficacious treatments, this prospective, uncontrolled trial was designed to test ATT efficacy for treating ITM. We assessed patient's disability using a panel of clinical scales at baseline and after 1 or 2 years of ATT. Disease progression was followed by sequential MRI studies.

2. Patients and Methods

This prospective, open-label study was conducted at the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China), from Jan 2003 to Jun 2010. The Neurology Department in this hospital is a referral center focused on CNS diseases and accepts patients from other hospitals in southern china. Sixty-seven patients qualified for inclusion in the study, according to the inclusion and exclusion criteria (see below). There were 24 males and 43 females. The male to female ratio was 6 : 11. The median patient age was 39.07 ± 18.33 years. A detailed neurological examination was carried out, including evaluation of the motor, sensory, visual, and sphincter systems. A Mantoux test, chest X-ray, serum biochemistry, serum and cerebral spinal fluid (CSF) Borrelia, and human immunodeficiency virus (HIV) testing were performed to exclude other diseases. The CSF cell count, glucose, and protein levels were obtained from lumbar punctures performed prior to the onset of ATT. A direct smear for acid-fast bacillus and a *M. tuberculosis* antibody test (PPD-lgG) were also conducted. The presence of oligoclonal bands was recorded in 26 of the 67 patients.

Upon admission, a spinal MRI was performed on all patients. The number of levels affected in the sagittal plane in the T2 sequence was measured. A brain MRI was also performed and if the MRI scan was not normal, the patient was excluded.

2.1. Inclusion and Exclusion Criteria. This study was conducted with patients in whom MTB was suspected clinically but not proven based on conventional measures. The inclusion criteria were (i) development of sensory, motor, or autonomic dysfunction attributable to the spinal cord, (ii) varying degrees of motor, sensory, and sphincter dysfunction (although not necessarily symmetric), but without complete paraplegia, (iii) exclusion of extra-axial compressive etiology by MRI, (iv) worsening symptoms despite at least one 5-day course of IV methylprednisolone (0.5–1 g/day), and (v) negative CSF MTB cultures with a cell count $<50/mm^3$ and a total protein <1.5 g/L. The exclusion criteria were (i) sudden onset, (ii) history of previous radiation to the spine within the last 10 years, (iii) CNS manifestations of syphilis, Lyme disease, or HIV infection, (iv) clear arterial distribution and clinical deficit consistent with thrombosis of the anterior spinal artery, (v) history of clinically apparent optic neuritis, (vi) brain MRI abnormalities suggestive of MS or clinically definitive MS, and (vii) serologic or clinical evidence of connective tissue disease, such as, sarcoidosis, Behcet's disease, Sjögren's syndrome, systemic lupus erythematosus (SLE), or mixed connective tissue disorder.

The university ethics committee approved the study. All patients were informed of the potential short- and long-term drug complications of ATT. Written informed consent was obtained from patients who agreed to participate in the study. Patients were instructed to contact the study neurologist in the event of neurological symptoms. Before the start of ATT treatment, all patients received a baseline evaluation.

2.2. Study Drug Administration. Prior to ATT initiation, all treatments with corticosteroids and other systemic immunosuppression therapies were discontinued. Treatment protocols consisted of three antituberculous drugs (isoniazid, rifampicin, and pyrazinamide for 9 months), followed by a combination of isoniazid and rifampicin for 24 months. The doses were isoniazid at 10 mg/kg/day, rifampicin at 10 mg/kg/day, and pyrazinamide at 25 mg/kg/day. Treatment was under extensive observation. All patients had weekly liver function tests for the first one month of therapy and subsequently every 3 monthly (serum bilirubin, serum transaminases (SGOT/SGPT), and alkaline phosphatase).

2.3. Assessments. Eligible patients underwent complete physical and neurological examinations at entry, at 12 and 24 months, and as needed for assessment of acute relapses or safety. The American Spinal Injury Association (ASIA) standards [7] were adopted to assess subjects' neurological status. We used the ASIA Impairment Scale to evaluate sensory and motor function of the degree of spinal injury. Activities of daily living were assessed by the Barthel Index (BI) (0–100 scale, with lower scores denoting less independence in activities of daily living). Mobility was scored by the Hauser Ambulation Index (AI) [8] (0–9 scale designed to assess mobility). All patients were followed for at least 2 years after treatment. Quality of life changes were measured by the ASIA, BI and AI indices at baseline and at 24 months, along with evaluation by MRI. Each patient was followed up and assessed by the same physician during the study.

Multiple sclerosis was diagnosed according to McDonald et al.'s criteria [9]. Patients that subsequently developed optic neuritis with or without recurrent ATM, but had no clinical manifestations or involvement of other parts of the CNS, were classified as NMO according to Wingerchuk et al.'s criteria [10].

2.4. Statistical Methods. Statistical analysis was performed using Student's *t*-test on paired samples. SPSS 13.0 statistical Analysis System software was used for statistical analysis.

3. Results

3.1. Demographic and Clinical Characteristics. The median interval from onset to treatment with ATT was 7.5 months (range, 1–57 months). The most common modality of presentation was in the form of sensory-motor involvement (59 patients, 88%). Motor impairment presented as asymmetric weakness, paraparesis or even quadriparesis. Sensory symptoms included numbness or paresthesias. Eight patients presented with just motor involvement (12%), whereas 26 patients (38.8%) had some form of urinary symptoms in the form of absent bladder sensations, incontinence, or increased urination frequency. Three patients had definitive TB evidence upon chest radiography (Mantoux test ≥ 10 mm in 48 hours). Baseline demographic and clinical information is reported in Table 1.

The CSF findings were as follows. Elevated opening pressure (>200 mm H_2O) was present in eight patients, and

Table 1: Baseline demographic and clinical information at inclusion.

Characteristic	Treatment group
Number	67
Female/male	43/24
Age of study entry (years) mean ± SD	39.07 ± 18.33
Disease duration (months)	7.55 (range, 1–57)
Drop-out	16
Mantoux test ⩾ 10 mm.	48
Patients with CSF normal finding	12
Patients with abnormal MR change	43

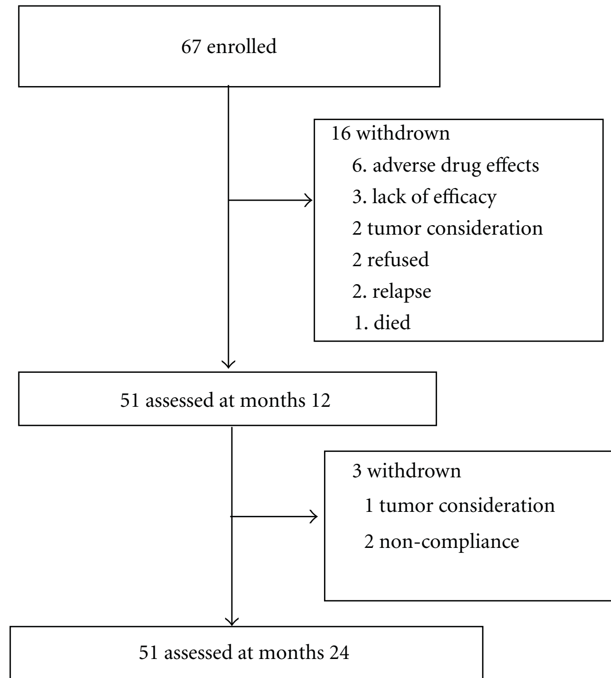


Figure 1: Trial profile.

pleocytosis was found in 29 patients (43.3%) with lymphocyte predominance. Protein level was increased (>0.45 g/L) in 42 patients, 5 patients (7.5%) had decreased glucose concentration, and eight patients (11.9%) revealed a low CSF chloride level. Overall, CSF was abnormal in 55 of 67 patients, with 12 (17.9%) completely normal.

Spinal MRI lesions were visible in 43 patients (64.2%). Lesions were usually hypointense or isointense on T1-weighted and hyperintense in T2-weighted sequences. In 14 cases, we observed obvious spinal cord swelling. DTPA-enhanced MRI scans were performed for 19 patients, of which 13 showed Gd-enhancement. Ring enhancement and/or heterogeneous enhancement were common findings. Two patients had associated syrinx. Brain MRI was performed for all patients but did not reveal any infectious or demyelinating lesions.

3.2. Followup. Sixteen (23.9%) of 67 patients discontinued ATT within the first year. Another three patients dropped out during the second year medication but were still followed (Figure 1). Thus, complete 24-month data were available for 51 (76.1%) patients. The principle reasons for discontinuation were either side-effects or absence of perceived efficacy. Six patients withdrew early due to adverse drug effects. Three patients dropped out because of physician or participant's perception of therapy ineffectiveness. Two patients dropped out due to intramedullary spinal cord tumor considerations that required visiting another hospital. Two patients stopped treatment because of relapse, and two patients exhibited poor compliance. One patient died following an undiagnosed high fever and cerebral salt-wasting syndrome. The median follow-up interval after initial ATT was 32.8 months (range, 24–92 months).

3.3. Treatment Efficacy. Treatment was halted for patients experiencing deteriorating clinical symptoms since 43/51 (84%) patients experienced good response in the early course of ATT treatment. During the first week, 18/51 (35%) patients had considerable improvement in limb weakness. Sphincter symptoms gradually ameliorated in 21/26 (81%) patients. The clinical benefit was sustained and continued to improve in most cases. After one year, 49/51 (96%) of treated patients had achieved marked improvement of neurological signs, and five had experienced a full recovery without any motor or sensory sequelae. Spinal cord evaluation showed improved ASIA scores, significantly increased BI ratings, and an improved Hauser AI. The benefit was sustained and continued to improve over the second year (Table 2). However, the major improvement occurred during the first year of ATT treatment.

Neurological relapses were experienced by 9/51 (18%) patients, although they improved upon active treatment. During the first year followup, 13 relapses occurred, primarily in the initial three months (six relapses), with six more occurring in the second year of ATT treatment. Four patients who continued ATT treatment in relapses periods, and did not receive other immunosuppressive treatment, recovered, whereas five patients that received IVIG treatment responded well. Two patients experienced relapse of visual decline (at 6 months and 15 months, resp.) as limb weakness greatly improved, although both patients repeatedly showed a normal brain MRI. NMO diagnosis was established according to Wingerchuk's criteria. Similar to our previous study [6], visual acuity gradually recovered without change to our regimen. Eighteen relapses occurred in patients with longitudinal extensive lesions, and one relapse occurred in a patient involving less than two segments.

3.4. MRI Changes. Two-year observations based on MRI imaging were completed for 33/51 (65%) patients who had cord lesions. Abnormal symptoms and swollen spinal cords progressively decreased in 32 patients after therapy initiation, and T2 signal abnormalities decreased markedly in size. This happened in poorly defined, extensively swollen cord lesions (Figure 2), with spinal cord demyelination involving less than two segments (Figures 3 and 4), and with longitudinal

Table 2: The changes of spinal function scores on baseline and after 1 year and 2 years of ATT (51 patients).

Years	ASIA sensory scores	ASIA motor scores	Barthel index	Ambulation index
Baseline	184.02 ± 23.57	78.86 ± 14.51	74.42 ± 22.40	3.92 ± 2.06
Year 1	209.33 ± 21.42*	93.75 ± 8.03*	93.43 ± 10.61*	1.63 ± 1.72*
Year 2	211.10 ± 19.96*	95.10 ± 8.34*	95.47 ± 6.25*	1.09 ± 1.36*

Each value represents mean ± SEM. *Improvement in values is statistically significant ($P < .05$) from baseline (paired sample t-test).

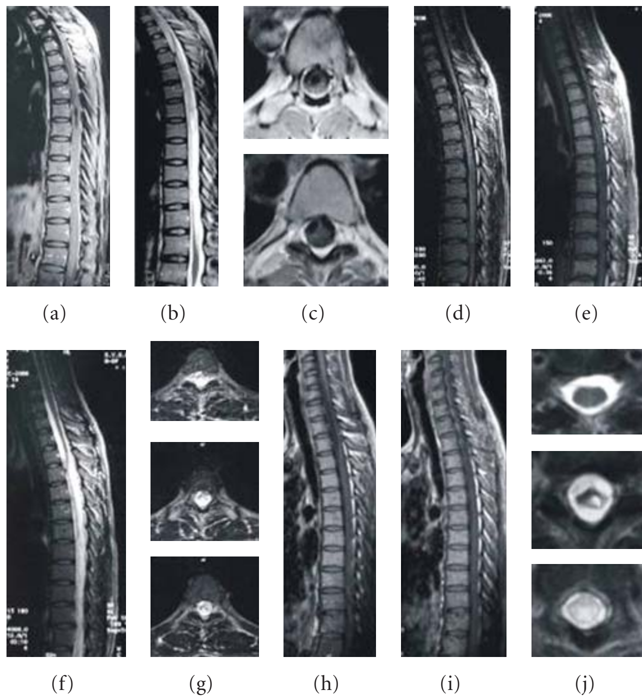


Figure 2: A 25-year-old woman was admitted due to the progressive weakness and parasthesia in both legs with voiding problem for 3 months. After 2-year ATT, her situation improved with good recovery of spinal function. The MRI changes are as follows: (a) first MRI performed in August 2005, T1 weighted image showing cord edema from T1 to bottom in the sagittal plane, with focal atrophy and syrinx cavity, (b) T2-weighted image showing a contiguous area of increased signal intensity, (c) cavity and band-like structure on axial T1-weighted images, (d, e) after one year of treatment, the swelling of cervical spinal cord greatly dissolved on sagittal T1-weighted image; the syrinx cavity is obviously diminished, (f) contiguous abnormal signal still left but diminished on the sagittal T2-weighted image, (g) the lesion is still obvious on axial T2-weighted images, (h, i) three years after treatment, the syrinx cavity is not obvious, and (j) some parts of thoracic cord restore normal signal on T2-weighted axial cord images. A small cavity is visible on T8, and increased signal intensity still exists below T9.

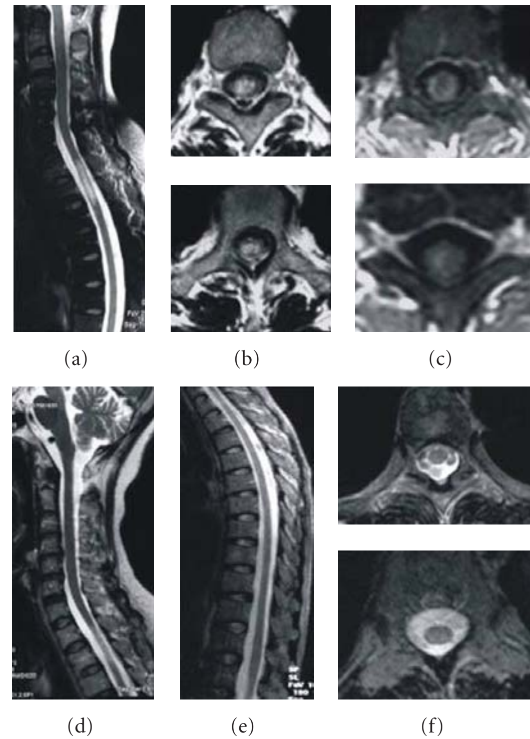


Figure 3: A 58-year-old female developed paraplegia and paresthesias in August 2007; weakness slowly worsened. Pulse methylprednisolone and IVIG were initiated with no resolution; she continued to deteriorate. After 2-year ATT, her situation fully recovered. The MRI changes are as follows: (a) sagittal T2-weighted image showing a contiguous area of increased signal intensity spanning T2 to T3 level, (b) axial T2-weighted images reveal the strong signals, (c) axial MRI images showing focal cord enhancement, (d) after 5 months of ATT, the lesion decreased, (e) following 1-year ATT, sagittal T2-weighted MRI of the thoracic cord reveals no abnormal signals, and (f) no lesion is visible on cross-sectional T2-weighted images.

lesions involving more than three segments (Figures 5, 6, and 7). Additionally, regions where well-defined signals and poorly defined enhancements had been seen on pretreatment resolved with treatment. During the observational period, however, a decreased intramedullary lesion burden was not detected in two patients. One patient received an operation at 19 months due to lesion expansion, and pathological examination revealed an astrocytoma. The lesion remained in another patient with improved condition after 24-month followup (Figure 8).

3.5. Adverse Events. Adverse events occurred in 16/67 patients (23.9%). Two patients had somnolence and weakness due to hyponatremia during the first months of ATT treatment and quickly recovered on active therapy. No other serious adverse effects were seen but six patients withdrawn early because of lethargy, nausea, and vague ill health. Eight patients had mild gastrointestinal syndrome on active therapy but were able to continue the trial and resolved quickly. One death that occurred during the study was considered unrelated to the trial.

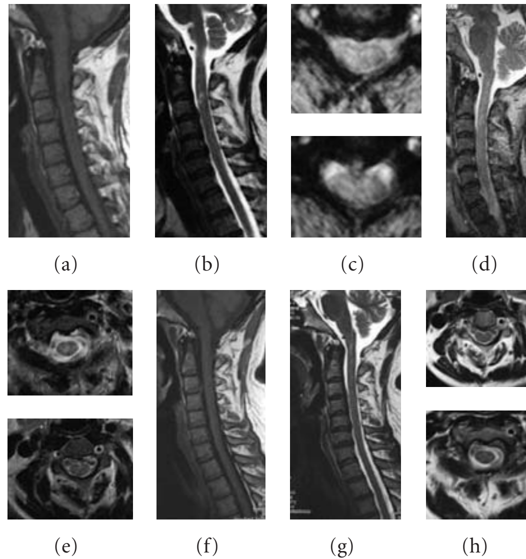


Figure 4: A 51-year-old female suffered from tetraparesis and frequently paroxysmal tics of extremities for 3 months. ATT treatment quickly improved her symptoms. After 2-year ATT, she fully recovered without visible symptoms. The MRI changes are as follows: (a) sagittal T1-weighted MRI showing focal thickening of the cord at C2-C3 level, (b) sagittal T2-weighted MRI showing a focal demyelinating lesion, (c) axial T2-weighted MRI showing the lesion to involve the whole cord, (d) sagittal T2-weighted MRI after 6 months of treatment, showing the swelling of the cervical spinal cord and patchy abnormal signal have resolved, (e) axial T2-weighted MRI showing an abnormal signal remains, (f) sagittal T1-weighted MRI after 2 years of treatment is normal, (g) sagittal T2-weighted MRI after 2 years of treatment revealing no abnormal signals, and (h) axial T2-weighted cord MRI after 2 years of treatment.

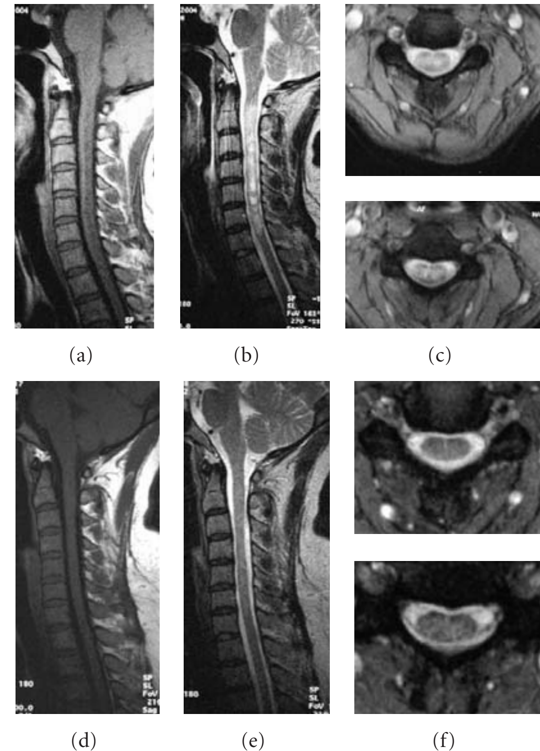


Figure 5: A 40-year-old woman had tetraparesis, C3 sensory level, and urinary incontinence for five months. Pulse methylprednisolone and IVIG were initiated with no resolution. However, ATT greatly improved her condition. The MRI changes are as follows: (a) sagittal T1-weighted MRI showing thickening of the cervical cord, (b) sagittal T2-weighted MRI showing a contiguous area of increased signal intensity spanning C2 to C7, (c) axial T2-weighted MRI showing the strong signal derived from the whole cord, (d) sagittal T1-weighted MRI after 2 years of treatment is normal, (e) sagittal T2-weighted MRI after 1 year of treatment, showing a normal spinal cord, and (f) axial T2-weighted MRI, showing no lesions.

4. Discussion

We report our experience with a select group of myelopathy patients treated with ATT. This is the first clinical study to evaluate the therapeutic effects of ATT on steroid-refractory ITM. Sustained increases in ASIA scores and BI, with decreased AI scores, show that treatment is effective in most cases. This study used only pure antituberculosis chemotherapy, without adopting steroid and other immune suppressing drugs. The encouraging results are not only associated with significantly improving the neurological status of these patients, but also with beneficial changes to spinal lesions as determined by MRI. This study suggests ATT has beneficial effects in some ITM patients, and that *M. tuberculosis* infection may be an important cause of this disease.

Tuberculosis is still a major health problem in many parts of the world, especially Asia and Africa. However, tuberculous intramedullary involvement is considered to be very rare compared with tuberculous spondylitis or arachnoiditis [11]. Since its first description by Abercrombie in 1829 [12], there have only been some isolated cases reported. A true epidemiological and clinical profile has been elusive. This study shows mycobacterial infections might be more common than usually suspected in myelitis. However, due to the absence of a sensitive laboratory test, many cases may have been considered as "idiopathic" with a presumed immune mediated pathophysiological mechanism, despite extensive diagnostic workup.

The etiology of the MRI signal abnormalities remains speculative. In the current series, the MRI signal changes in ITM vary from a small single lesion to extended longitudinal lesions over several spinal segments. In the literature, the majority of ITM cases with a single lesion is diagnosed as a clinically isolated syndrome (CIS), which later may be converted to multiple sclerosis (MS) [13, 14]. However, not all CIS patients develop MS. Another diagnostic problem is posed by patients with long hyperintense MRI lesions, involving three or more segments of the spinal cord, or the so-called longitudinal extensive transverse myelitis (LETM). Today, such patients are designated as part of the NMO spectrum [15]. In this study, CIS and LETM lesions were revealed gradually as the imaging resolution of the abnormalities improved after ATT therapy was initiated.

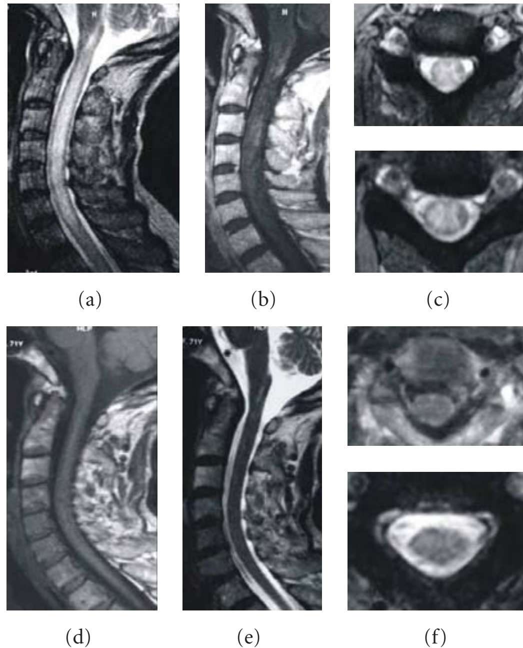


Figure 6: A 70-year-old female was admitted due to progressive paraparesis, paresthesias, and urinary retention for 2 months. After 2-year of ATT, her situation improved. The MRI changes are as follows: (a) sagittal T2-weighted image reveals a long, high intensity signal from C1 to T1 in the spinal cord before treatment regimen, (b) T1W contrast image showing patchy enhancement and thickening of the cord, (c) the diffuse lesions on axial T2-weighted images, (d) a year later the swelling of the cervical spinal cord disappeared on sagittal T1-weighted images, (e) the hyperintensity resolved following 1 year of ATT, and (f) no lesion is visible on axial T2-weighted images.

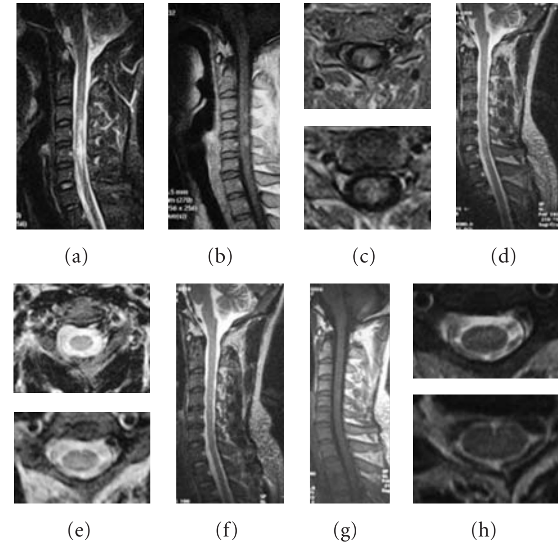
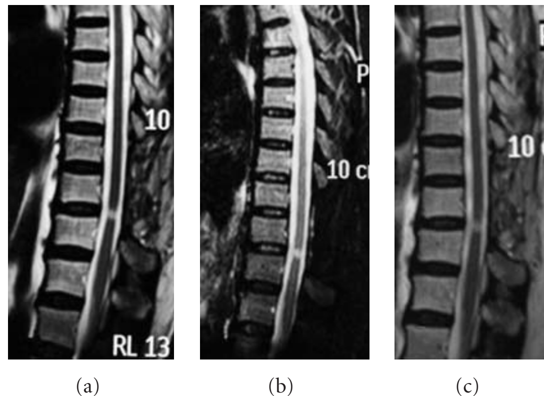


Figure 7: A 30-year-old male suffered from left paraparesis and paresthesias for 1 month. MRI of the cervical spinal cord revealed a demyelinating lesion in T2-weighted images extending from C4 to C7. Pulse methylprednisolone and IVIG were initiated with no resolution. The MRI changes are as follows: (a) sagittal T2-weighted image reveals a high intensity signal from C4 to C7 before ATT regimen, (b) sagittal T1-weighted MRI showing light thickening of the cord with subtle intraparenchymal hyperintensity, (c) the left local lesions on axial FLAIR sequences, (d) the hyperintensity resolved following 6 months of ATT, (e) no lesion is visible on axial T2-weighted images, and (f)–(h) no lesion is visible on follow-up MRI after 2 years of treatment.

The results of the present study suggest that lesion length may not correlate with different pathological findings. It is possible that aspects of LETM and CIS are based on the same mycobacteria etiology. However, this hypothesis may serve as a basis for further studies since this small series of patients and study design was not designed to address this important question.

In this study, 24 patients (38.2%) develop ITM without signs of demyelination or other abnormalities of the spinal cord as determined by MRI. Previous reports [16] show patients in this group usually have worsening symmetrical clinical symptoms of transverse spinal cord syndrome, in which spinal MRI may show atrophic changes later. There is no evidence in the literature that any effective treatment exists. However, ATT shows an excellent treatment effect for 17 patients of this trial. It may be that these puzzling results are associated with mycobacteria infection. It is noteworthy that patients in this category may present with clinical symptoms similar to that of tropical spastic paraparesis (TSP), which has been associated with human T-cell lymphotropic virus type 1 (HTLV-1) [17]. TSP diagnosis may require exclusion by laboratory tests for HTLV-1 antibodies. Nevertheless, trial ATT treatments may be beneficial in tuberculosis endemic areas where diagnosis is not definite, but where no treatment is available to treat TSP patients.

We found that subsequent neurological relapses during ATT treatment usually occur in at least some patients, potentially through a mechanism similar to paradoxical deterioration in tuberculosis. Paradoxical deterioration during ATT is usually defined as the clinical or radiological deterioration of pre-existing tuberculous lesions or the development of new lesions in a patient who initially improved [18]. In three reported cases of acute myelopathy associated with pulmonary tuberculosis that have undergone postmortem examination [19], the myelopathy had been progressive or relapsing, and demyelination of the white and grey matter was found in the spinal cord of all three patients. Relapses in tuberculous myelopathy can recur several times during treatment [20]. Reid and Bone reported that clinical improvement can be achieved after steroid therapy [21]. However, disease progression despite adequate ATT treatment is well documented [22]. Although relapses occurred in some patients, there was a tendency for the relapse rate to decrease as treatment continued. In this trial, two LETM patients experienced optica neuritis, and hence satisfied criteria for a diagnosis of NMO. However, both patients successfully recovered without changing the regimen.



(a) (b) (c)

Figure 8: A 67-year-old female suffered from progressive paraparesis and paresthesias for 1 month. There is a small demyelinating lesion at the level between T9 and T10. Steroid pulsed treatment worsened her condition. After ATT, her weakness quickly improved. She tried to stop ATT two times during the early stage of treatment due to drug side-effects, but failed because her condition grew worse. Although she got better, the lesion seems to have not changed after 18 month treatment. (a) Sagittal T2-weighted image reveals a small high intensity signal between T9 and T10 before our regimen; (b) sagittal T2-weighted image after 6 months of ATT; (c) the lesion still exists on sagittal T2-weighted images after 18 months of treatment.

There are still few reports describing the features of tuberculous myelopathy detected by MRI. The first documented description of using MRI to determine intramedullary spinal tuberculosis was published by Rhoton et al. in 1988 [23]. Lesions are usually hypointense on T1W images and iso-to-hyperintense on T2W images with cord expansion. Ramdurg et al. reported the largest series of 15 spinal intramedullary tuberculosis with varied MR changes [24]. This study shows excellent clinical outcomes can be obtained with a combination of medical treatment and surgical management, although patients presenting late had a poorer outcome. In our study, there was a significant effect of ATT treatment on T2-lesion volume over time. It is impressive that abnormal signals and expanded spinal cords progressively decreased with ATT and even completely disappeared in some cases. All these changes may just reflect that the infection subsided. Perhaps a more fundamental problem is related to the interpretation of results from ATT trials. Further studies should investigate whether this syndrome is due to an immune reaction to TB or a primary TB infection.

There are methodological limitations to the current study. This is an uncontrolled study, the number of patients in the trial was small, and relapses were less well defined. Overall, the trial provides some inspiring data and indicates ATT may be an efficient, cost-effective approach to some patients. Based on this study, a double-blind controlled study of ATT efficacy will need to be performed, preferably over a longer duration and with a larger sample size.

5. Conclusion

We have presented a prospective pilot study on steroid-refractory ITM. This study has identified, for the first time, the long-term clinical efficacy of ATT in some steroid-refractory ITM patients. Our results suggest that ATT treatment can not only stop disease activity and progression but may also result in a significant recovery of fixed neurological deficits. On the other hand, the study also suggests that tuberculosis infection might be an important and still neglected cause of myelitis. Additional efforts must be made to conduct well-designed trials since no other effective treatments currently exist.

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Research Article

Human T Cell and Antibody-Mediated Responses to the *Mycobacterium tuberculosis* Recombinant 85A, 85B, and ESAT-6 Antigens

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Tuberculosis remains a major health problem throughout the world causing large number of deaths. Effective disease control and eradication programs require the identification of major antigens recognized by the protective responses against *M. tuberculosis*. In this study, we have investigated humoral and cellular immune responses to *M. tuberculosis*-specific Ag85A, Ag85B, and ESAT-6 antigens in Brazilian patients with pulmonary (P, $n = 13$) or extrapulmonary (EP, $n = 12$) tuberculosis, patients undergoing chemotherapy (PT, $n = 23$), and noninfected healthy individuals (NI, $n = 7$). Compared to NI, we observed increased levels of IgG1 responses to Ag85B and ESAT-6 in P and PT groups. Regarding cellular immunity, Ag85A and ESAT-6 were able to discriminate P, PT, and EP patients from healthy individuals by IFN-γ production and P and PT groups from EP individuals by production of TNF-α. In summary, these findings demonstrate the ability of Ag85A, Ag85B, and ESAT-6 to differentiate TB patients from controls by IgG1, IFN-γ and TNF-α production.

1. Introduction

Tuberculosis (TB) remains the largest single infectious cause of death globally. It is estimated that 30% of the word population is infected with *Mycobacterium tuberculosis* resulting in approximately 2-3 million deaths each year [1]. Further, the AIDS epidemic and the appearance of multidrug resistant strains of *M. tuberculosis* have contributed to the reemergence of TB in developing countries; however, this disease continues to be a devastating entity in the developing world [2]. At present, the only registered vaccine against tuberculosis, *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), was introduced in 1921 and has been widely used; however, its effectiveness remains controversial because their protection levels are extremely variable in different population [3–5]. Furthermore, vaccination with *M. bovis* BCG is contraindicated in immunocompromised subjects, including acquired immunodeficiency syndrome patients, who are usually at a very high risk of developing TB [6]. In addition, the diagnostic value of the presently used skin test reagent, purified protein derivative (PPD) of *Mycobacterium tuberculosis*, is low, owing to cross-reactivity with environmental mycobacteria and vaccine strains of *M. bovis* BCG [7, 8]. Thus, the effective control and eradication of TB is dependent upon the availability of effective vaccines and reagents for specific diagnosis. For this purpose, the identification

of major antigens recognized by the protective immune response against *M. tuberculosis* remains a critical step.

Among *M. tuberculosis* antigens studied, the 30/32 KDa antigen 85 (Ag85) complex has been the focus of intense research over the past several years and comprises three closely related proteins, 85A (32 KDa), 85B (30 KDa), and 85C (32.5 KDa) that possess enzymatic mycolyl-transferase activity [9–11]. The Ag85 complex induces protective immunity against TB in guinea pigs [12], and strong proliferation and IFN-γ production in peripheral blood mononuclear cells (PBMC) from healthy tuberculin reactors [13]. Regarding, ESAT-6, the early secreted antigenic target is a low-molecular-weight protein essentially present in pathogenic mycobacteria including members of the mycobacterium complex (*M. tuberculosis, M bovis,* and *M. africanum*) and *M. leprae* [14]. Analysis of T-cell responses to *M. tuberculosis* ESAT-6 showed an elevated range of recognition from many tuberculosis patients [15]. Consequently, the possible use of ESAT-6 as a marker of *M. tuberculosis* infection has been proposed. Moreover, other studies have demonstrated the ability of this protein to discriminate tuberculosis patients from health donors in a high endemic area [16]. Additionally, ESAT-6 is able to differentiate tuberculosis patients from both BCG-vaccinated individuals and *M. avium* infected patients [17].

The main goal of this study was to evaluate the cellular and humoral immune responses to the recombinant proteins Ag85A, Ag85B, and ESAT-6 in Brazilian pulmonary and extra-pulomary tuberculosis patients and individuals undergoing chemotherapy. The recombinant proteins were produced in *E. coli* and purified by affinity chromatography. Cellular proliferation and cytokine production were evaluated in peripheral blood mononuclear cells (PBMC) and specific antibody isotypes to Ag85A, Ag85B and ESAT-6 were measured in serum of TB patients and controls. In this study, we have shown the ability of Ag85B and ESAT-6 to differentiate TB patients from controls by IgG1 production. Additionally, the results here demonstrated that Ag85A and ESAT-6 were able to discriminate P, PT, and EP patients from healthy individuals by IFN-γ production and P and PT groups from EP individuals by production of TNF-α.

2. Materials and Methods

2.1. Study Population. Patients with active pulmonary TB (P, $n = 13$) or active extra-pulmonary TB (EP, $n = 12$), and pulmonary TB patients with 1–3 months of anti-TB chemotherapy (PT, $n = 23$), diagnosed at the outpatient unit of the Oswaldo Cruz Health Center, Belo Horizonte, Minas Gerais, Brazil, were enrolled in this study. All TB patients had sputum-positive bacilloscopy or culture-confirmed disease. The EP-TB group comprised six pleural TB, five miliary TB and one intestinal TB as shown in Table 1. Seven healthy non-BCG vaccinated individuals (all PPD-) without prior history of mycobacterial infection were included as control group. All enrolled patients tested negative by ELISA for HIV. None of the individuals had evidence of acute infections (other than TB) at the time of sample collection. Twenty ml of blood was taken from each patient.

2.2. Ethics Committee. All patients gave permission for blood sampling after written consent, and the Ethics Committee of the Santa Casa Hospital at Belo Horizonte, Minas Gerais, Brazil approved the research protocol.

2.3. Mycobacterial Antigens. The recombinant Ag85A, Ag85B e ESAT-6 was produced using the pMAL-c2 expression system. Briefly, the pMAL-85A, pMAL-85B, or pMAL-ESAT-6 construct was used to transform *Escherichia coli* DH5α strain as previously described [18]. Bacterial cells were induced using 0.42 mM IPTG (isopropyl-β-D-thiogalactoside) and recombinant proteins fused to the maltose binding protein (MBP) were produced. Three hours after gene expression, the cells were harvested and lysed using thermal shock, sonication, and lysozyme treatment. The fusion protein recovered in the supernatant was then purified by affinity chromatography using an amylose resin (New England BioLabs). Residual endotoxin levels were removed from recombinant proteins by using Triton X-114 and measured to be <50 EU/mg recombinant protein by the LAL assay (Limulus amoebocyte lysate). Purified protein derivative (PPD-RT50) was obtained from Statens Serum Institute, Copenhagen, Denmark.

2.4. T Cell Proliferation Assays. Heparinized venous blood was obtained from all patients and controls, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Ficoll 6,42% (SIGMA) and Hypaque 50% (Sanofi Synthelab)) density centrifugation. Cells were washed in RPMI 1640 fresh medium and cultured ($2,5 \times 10^5$ cells/well) in flat-bottom 96-well plates (Nunc Brand Products) in 200 μL of RPMI 1640 medium supplemented with 10% AB + heat-inactivated human serum and 1% antibiotic/antimycotic (Gibco-BRL) and incubated at 37°C in a humidified 5% CO_2 incubator with recombinant antigens or medium alone (control). The recombinant antigens were titered to determine the optimal protein concentration for proliferation assays. Ag85A, Ag85B, and PPD was used at 25 μg/ml, ESAT-6 at 50 μg/ml, and phytohaemagglutinin (PHA) was used at 10 μg/ml. The concentration of the recombinant antigens tested here was higher compared to the used by other authors because we produced the mycobacterial antigens fused to MBP that has itself a molecular mass of approximately 42.6 kDa. All antigens were plated in triplicate. After 3 days (mitogen) or 5 days (antigens) of incubation, [^{3}H] thymidine (0.5 μCi/well) was added to the cultures. Eighteen hours later, the supernatants were collected, and the cells were harvested. The incorporated radioactivity in the cells was evaluated by liquid scintillation spectroscopy as a measure of cellular proliferation. Mean counts per minute for triplicate cultures and stimulation index (SI) were obtained for each patient. The SI was the ratio of mean counts per minute in the presence of antigen to means counts per minute in medium alone.

2.5. Cytokine Measurement. Concentration of human IFN-γ, TNF-α, IL-10, and IL-4 in cell culture supernatants of proliferation assays was determined by enzyme-linked immunosorbent assay (ELISA) using kits Duoset from R&D

Table 1: Clinical characteristics of TB patients and controls in this study.

Groups	No. of subjects	TST	Males/females	Age (mean ± SD)	Age range
Non-infected (NI)	7	−	05/02	40.1 ± 9.5	28–55
TB patients under treatment (PT)*	25	+	14/11	39.8 ± 14.6	22–76
Pulmonary TB untreated (P)	13	+	09/04	41.0 ± 15.7	19–69
Extra-pulmonary TB (EP)	12	+	08/04	41.1 ± 16.5	21–76
(i) Pleural	6				
(ii) Miliary	5				
(iii) Intestinal	1				

*The treatment consisted of Rifampicin (10–20 mg/kg/day), Isoniazide (10–20 mg/kg/day), and Piraminazide (30–50 mg/kg/day). PT patients were undergoing 1–3 months of chemotherapy. TST tuberculin skin test.

Systems (Minneapolis, MN, USA) according to manufacturer's directions.

2.6. Detection of Antibody Responses. Detection of antibody against recombinant Ag85A, Ag85B, and ESAT-6 antigens in TB patients and healthy individual sera was performed by a modified ELISA [19]. Briefly, ninety-six-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μL of each recombinant antigen separately at a concentration of 5 μg/ml in 0.1 M carbonate bicarbonate buffer (pH 9.6) per well. The plates were then blocked with 10% bovine fetal serum in PBS (pH 7.4) for 2 h at room temperature. Subsequently, the plates were washed three times with PBS plus 0.05% Tween-20 (PBS-T20). Serum samples diluted 1 : 100 in PBS-T20 (100 μL/well) were added in duplicate, and the plates were incubated for 1 h at room temperature. Peroxidase-labeled anti-IgG (Sigma Chemical Co., St. Louis, MO), anti-IgM (Sigma), and anti-IgA (Sigma) were added at dilutions of 1 : 2,000, 1 : 2,000, and 1 : 10,000 (100 μL/well), respectively. After 1 h at 37°C, the plates were washed, and OPD (orthophenyl-diaminobenzidine) plus 0.05% hydrogen peroxide in phosphate citrate buffer (pH 5) was added (100 μL/well). This mixture was then incubated for 30 min at room temperature, and the reaction was stopped by addition of 5% H_2SO_4 (50 μL/well). Absorbance was read at 492 nm using a microplate reader (BioRad, Hercules, CA). To determine IgG subclasses levels, the previous protocol was slightly modified. The serum dilution was changed to 1:80 for IgG1 and IgG3 and 1:20 to IgG2 and IgG4. Diluted sera were added to the plates, and they were incubated for 2 h at 37°C. After washing, peroxidase-labeled antihuman antibody was dispensed in each well at concentrations of 1 : 1,000 (IgG1, IgG3) or 1 : 500 (IgG2, IgG4), and the plates were incubated for 12–16 h at 4°C. The next steps were identical to those described above.

2.7. Statistical Analysis. Results are reported as means ± standard errors. Differences between responses from TB patient groups and control groups were analyzed with non-parametric Kruskall-Wallis test. Correlation between ESAT-6 induced IFN-γ and proliferation responses from patients WAS identified using Spearman Correlation. Statistical analysis was performed using the GraphPad Prism software version 5.0 (GraphPad software incorporated). Statistical differences were considered significant at $P < .05$.

3. Results

3.1. IgG1 Is the Predominant Antibody Isotype Present in Sera of TB Patients. To investigate the presence of specific anti-Ag85A, -Ag85B or -ESAT-6 antibodies in sera of TB patients with different clinical forms of the disease, ELISA were performed. Figure 1 shows the levels of specific IgG, IgM and IgA to mycobacterial antigens in sera of TB patients and healthy donors. The levels of anti-PPD IgG were significantly elevated in all tuberculosis patients compared to NI group. Furthermore, increased levels of IgG anti-Ag85B and anti-ESAT-6 were detected in P and PT groups compared to NI individuals. Interestingly, no significant titers of IgG anti-Ag85A were detected in studied patients. Levels of specific IgA antibodies to all antigens were very low and did not differ between the studied groups. In addition, only marginal anti-Ag85B and anti-PPD IgM levels were observed in the P and PT groups.

Having observed elevated IgG levels to Ag85B and ESTA-6 antigens in patient sera, we decided to determine the IgG subclasses involved. The IgG subclass profile of TB patients was characterized predominantly by IgG1 responses to rAg85B and rESAT-6 on P and PT groups (Figure 2). Additionally, statistically significant levels of IgG3 to rAg85B, ESAT-6 and Ag85A were also detected in sera of PT and P group, however at lower levels. These results demonstrate the better performance of the Ag85B and ESAT-6 antigens compared to Ag85A to determine humoral responses in patients with active TB.

3.2. Proliferative Responses to Mycobacterial Antigens. In order to determine T cell-reactivity to the mycobacterial antigens tested, lymphoproliferative responses were measured in cells from tuberculosis patients and healthy individuals. As shown in Figure 3, proliferative responses upon stimulation with rESAT-6 were able to discriminate P, EP, and PT tuberculosis patients from healthy individuals. Regarding Ag85A and Ag85B, they were able to differentiate PT and P tuberculosis patients from noninfected individuals. Only when PPD was used as antigen, this assay was able to discriminate PT and P patients from individuals with

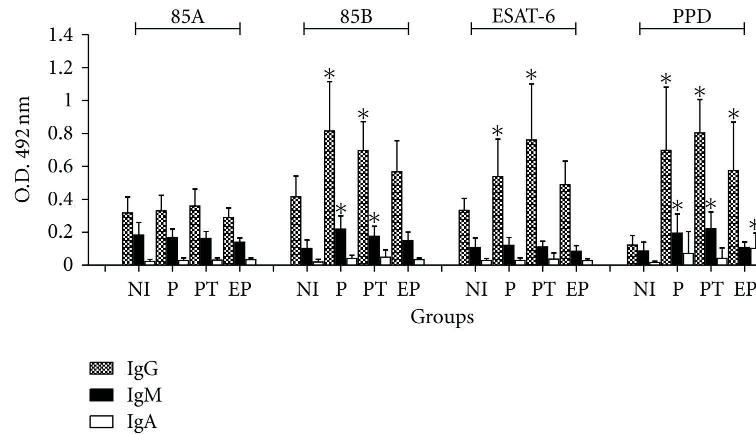


FIGURE 1: Levels of serum IgG (hatched box), IgM (black box) and IgA (empty box) to Ag85A, Ag85B, ESAT-6, and PPD were determined in serum of non-infected individuals (NI), pulmonary tuberculosis patients (P), pulmonary tuberculosis patients undergoing treatment (PT) and extra-pulmonary tuberculosis (EP). Results are presented as mean ± standard deviation. Differences between responses from TB patient groups and control groups were analyzed with nonparametric Kruskall-Wallis test. *Statistically significant differences in relation to NI group ($P < .05$).

extrapulmonary TB. PBMC of all individuals showed high cell proliferation after stimulation with PHA as a positive control (data not shown).

3.3. Cytokine Profile in Response to Recombinant Mycobacterial Antigens. In order to evaluate the cytokines produced by P, EP, and PT patient cells to the mycobacterial recombinant antigens, IFN-γ, TNF-α, IL-10 and IL-4 were measured. Figure 4 shows a significant production of IFN-γ after stimulation with Ag85A and ESAT-6 in the PT group (1285 ± 1571 and 1355 ± 976, resp.), P group (1268 ± 1722 and 974 ± 218, resp.), and EP group (903 ± 785 and 920 ± 2477, resp.) in comparison to the non-infected control group. This elevated production of IFN-γ in ESAT6-stimulated PBMC of TB patients correlated with positive response to ESAT6 in the proliferation assays (Spearman test; PT group $r = 0.5158$, $P = .0118$; P group $r = 0.5659$, $P = .0438$; EP group $r = 0.6224$, $P = .0307$). Moreover, in response to Ag85B, only PT (1206 ± 2087) and P (1227 ± 799) patients produced significant levels of IFN-γ compared to healthy individuals. The PT groups were the higher producers of IFN-γ in response to all recombinant antigens; however no difference was observed within this group among the different antigens used. All TB patients produced high levels of IFN-γ to PPD, and this result is in agreement with the complex antigenic mixture of PPD.

Regarding TNF-α production, we observed that PT and P groups produced significant levels of this cytokine when stimulated with Ag85A, Ag85B or ESAT-6. However, no recombinant antigens tested were able to induce greater levels of TNF-α in EP group compared to healthy individuals. Furthermore, only Ag85A or ESAT6 antigens induced production of higher amounts of TNF-α by PT and P cells that allowed discrimination between these groups from EP group. Additionally, all patients produced high levels of TNF-α to PPD.

As for IL-10, a regulatory cytokine, we observed that the group of TB patients who had initiated chemotherapy (PT) produced higher amounts of IL-10 compared to P or EP groups when Ag85A, Ag85B and ESAT-6 were tested. These values were significant to differentiate PT from P and EP groups. None of the tested recombinant antigens or PPD induced detectable production of IL-4 by PBMC of all individuals tested (data not shown).

4. Discussion

For the development of new vaccines and diagnostic reagents, there is an urgent need for assessment of immune responses to *M. tuberculosis* antigens in areas of TB endemicity. In the present study T-cell and antibody responses to recombinant Ag85A, Ag85B or ESAT-6 were investigated in Brazilian patients with pulmonary or extra-pulmonary TB and patients undergoing treatment compared to non-infected individuals. Several studies have detected antibodies in sera of patients with active TB against a variety of *M. tuberculosis* antigens [20–22]. Herein, patients with active disease or undergoing >2 months of treatment presented elevated levels of IgG anti-ESAT-6 and anti-Ag85B but not to Ag85A. High levels of antibodies against filtered *M. tuberculosis* antigens in the first two months of chemotherapy have been associated with intense stimulation of the humoral response by antigens released from killed bacteria combined with the disappearance of circulating mycobacterial antigens so that specific antibodies are no longer trapped in the immune complexes [23]. Therefore, large amounts of IgG antibodies against secreted Ag85B and ESAT-6 antigens appear to be associated with viable and metabolically active bacilli. Little attention has been given to the subclasses involved in TB [24]. In our study, the analysis of IgG subclasses to the mycobacterial recombinant antigens revealed the predominance of IgG1 but not IgG2 and IgG4 to ESAT-6 and Ag85B in sera of patients of the P or PT group. However, we also detected significant levels of IgG3 against Ag85A, Ag85B and ESAT-6 in these groups of patients. Our results are consistent

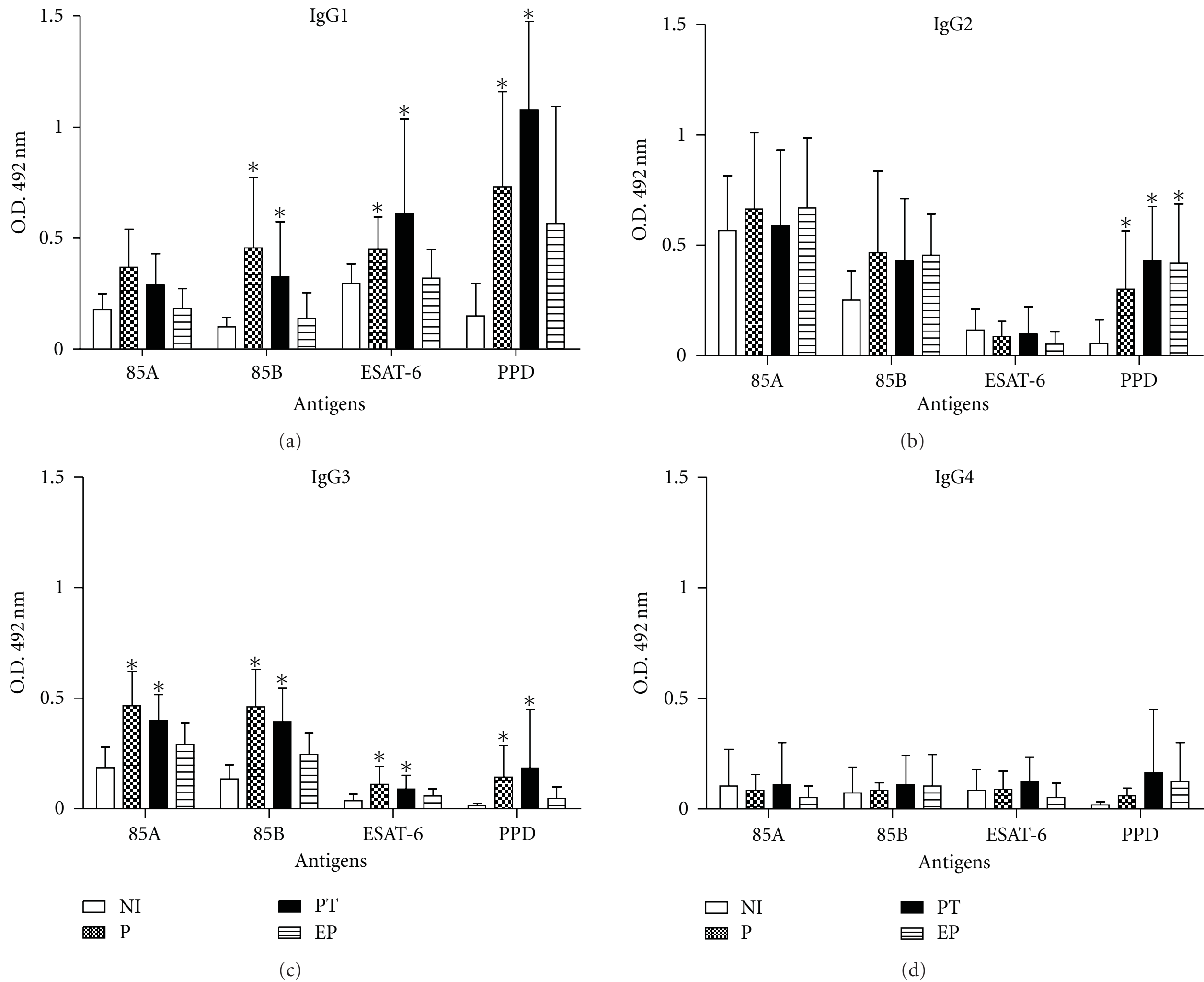


Figure 2: Levels of serum IgG1 (a), IgG2 (b), IgG3 (c) and IgG4 (d) to Ag85A, Ag85B, ESAT-6, and PPD were determined in serum of non-infected individuals (NI), pulmonary tuberculosis patients (P), pulmonary tuberculosis patients undergoing treatment (PT) and extra-pulmonary tuberculosis (EP). Results are presented as mean ± standard deviation. Differences between responses from TB patient groups and control groups were analyzed with nonparametric Kruskall-Wallis test. *Statistically significant differences in relation to NI group ($P < .05$).

with other studies that also observed predominance of IgG1 antibodies in the sera of patients with active TB [19, 24, 25]. Since Ag85A and Ag85B share around 77% of amino acids identity, one could expect them to have common immunodominant epitopes. Despite pronounced sequence homology among these Ag85 members, D'Souza et al. [26] have shown that different Ag85-specific immunodominant T-cell epitopes were identified in BALB/c and C57BL/6 mouse strains. These differences in MHC-restriction during Ag85A and Ag85B epitope mapping might be one of the reasons why we did not observed significant levels of anti-Ag85A IgG in our TB patients. Similarly, Van Vooren et al. [27] suggested that Ag85B was the most useful component of the Ag85 complex for serodiagnosis of the active form of TB.

Recently, commercial immunodiagnostic tests for TB have been introduced. These tests are based on the *M. tuberculosis* ESAT-6 and culture filtrate protein 10 (CFP-10) and include a whole-blood IFN-γ ELISA (QuantiFERON-TB Gold, Cellestis Ltd, Victoria, Australia) and an ELISPOT assay (T-SPOT.TB, Oxford Immunotec, Oxfordshire, UK). Both tests have shown promising results in the detection of latent TB and the potential use for differential diagnoses of active tuberculosis [28, 29]. However, the sensitivities and specificities of these assays vary among the different populations studied, due mostly to the different HLA genetic backgrounds, the prevalence of TB infection, and the coverage of *M. bovis* BCG vaccination [30]. Furthermore, there is a need to develop new diagnostic tools to detect extra-pulmonary TB and sputum negative cases. In our study, pulmonary or extra-pulmonary TB patients and individuals undergoing chemotherapy responded to ESAT-6 as evaluated by lymphoproliferative responses or by IFN-γ production determined in the supernatants of stimulated

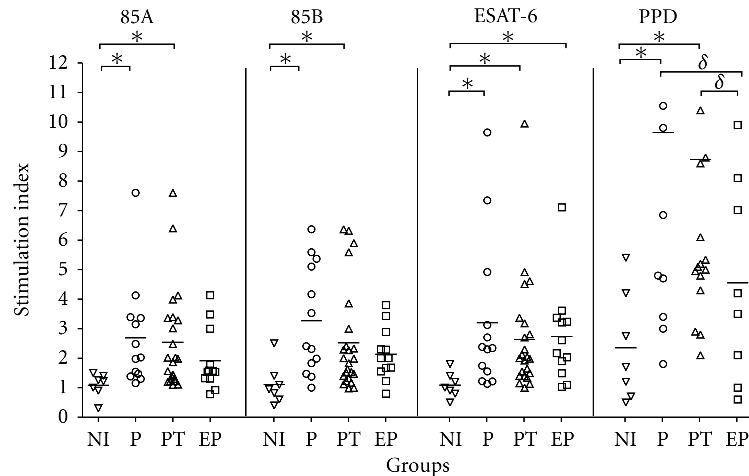
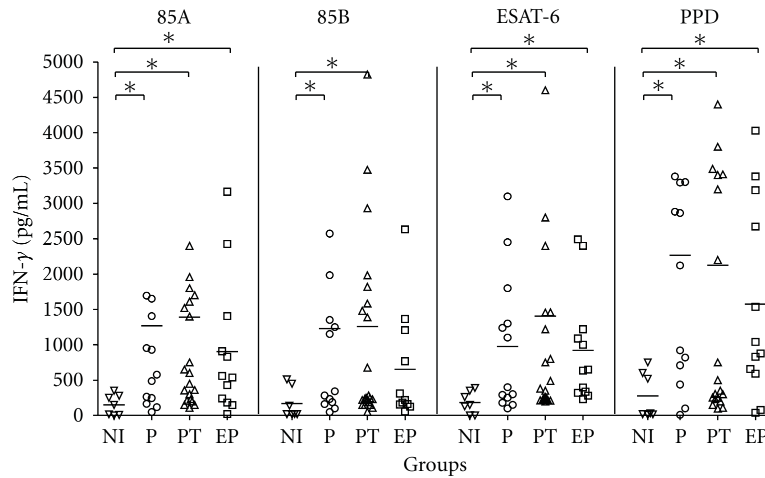


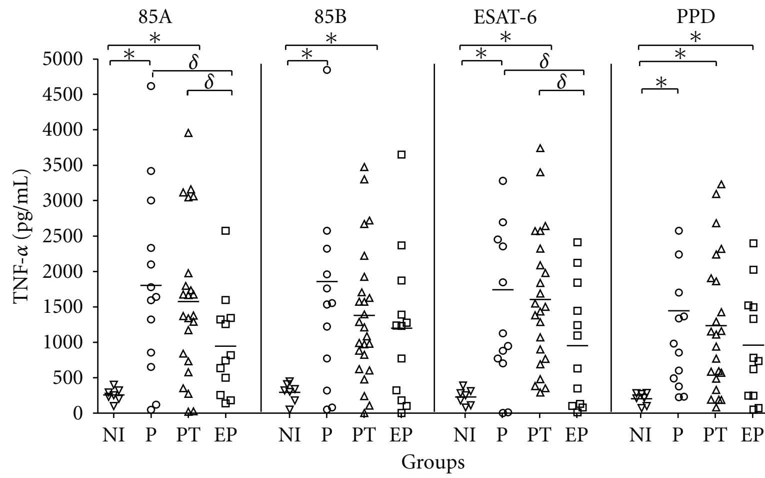
Figure 3: Lymphocyte proliferation in response to recombinant Ag85A, Ag85B and ESAT-6. Freshly isolated PBMC from non-infected individuals (NI), pulmonary TB patients (P), pulmonary TB patients in treatment (PT) and extra-pulmonary TB (EP) were cultured in the presence of Ag85A (25 μg/ml), 85B (25 μg/ml), PPD (25 μg/ml) or ESAT-6 (50 μg/ml) for 5 days and incorporation of [^{3}H] thymidine was measured. Results are expressed as stimulation index (SI) mean of triplicate cultures. Horizontal bars indicate mean values. Differences between responses from TB patient groups and control groups were analyzed with nonparametric Kruskall-Wallis test. (*) Statistically significant differences in relation to NI group and (δ) in relation to EP group ($P < .05$).

PBMC. Additionally, Ag85A was also recognized by all TB patient cells compared to non-infected individuals as measured by IFN-γ secretion. This data demonstrates that ESAT-6 and Ag85A are recognized by T cell from many tuberculosis patients undergoing distinct periods of clinical disease and is consistent with Ulrichs et al. [15] findings by which PBMCs from tuberculosis patients, but not healthy donors, respond to ESAT-6. Furthermore, as described previously by Antas et al. [31], the Ag85A and Ag85B proteins were also recognized by PBMC of pulmonary tuberculosis patients and individuals undergoing treatment measured by elevated proliferation and IFN-γ production. Regarding TNF-α, clinical studies have associated the use of TNF-blockers with progression from latent tuberculosis infection to disease [32]. Further, Caccamo et al. [33] have reported high percentage of CD4+ T cells expressing IFN-γ/IL-2/TNF-α in active TB patients and it seems to be associated with live bacterial loads, as indicated by the decrease in frequency of multifunctional T cells in TB patients after completion of antimycobacterial therapy. In our study, we have observed elevated levels of TNF-α to Ag85A, Ag85B, and ESAT-6 in patients with pulmonary tuberculosis or undergoing treatment but not in extra-pulmonary TB patients. However, only Ag85A and ESAT-6 antigens were able to discriminate PT and P patients from EP. This data might be associated to differential production of TNF-α by CD4+, and CD8+ T cells and the compartmentalization of immune response at site of disease. Marei et al. [34] demonstrated a differential expression of IFN-γ and TNF-α in CD4+ T cells and CD8+ T cells after stimulation with ESAT-6. In their study, CD4+ T cells are the main producer of TNF-α while IFN-γ was produced by either CD4+ or CD8+ T cells. In addition, other studies have provided evidence for compartmentalization of Th1 cytokines at the site of disease in humans [35, 36]. We suggest that compartmentalization of the immune response in EP patients can lead to sequestration of TNF-α producing cells at the disease site and it contributes to reduced production of this cytokine in peripheral blood in response to Ag85A, Ag85B and ESAT-6. Herein, our results suggest that Ag85A and ESAT-6 are able to differentiate P, PT and EP patients from healthy individuals by IFN-γ production and from P and PT groups to EP individuals by production of TNF-α. In a recent study, it was reported that similar levels of cytokine and antibody responses to *M. tuberculosis* ESAT-6/CFP-10 fusion protein were detected in PPD+ and PPD− groups from an endemic area of Juiz de Fora, Minas Gerais, Brazil [19]. Herein, the NI group (PPD−) used also lives in a TB endemic area in Brazil and probably has been exposed to multiple forms of environmental mycobacteria. However, to confirm the diagnostic potential of these antigens further studies using BCG vaccinated controls (PPD+) are required.

Antibodies conventionally are considered to play little role in defence against mycobacteria, and the function of antibodies in pathogenesis is yet to be determined. Macrophages in which mycobacteria resides, and multiply have high affinity receptors (Fcγ1 and Fcγ3) for IgG1 and IgG3 antibodies, and the presence of IgG1 and IgG3 antibodies may enhance bacterial uptake and clearance of pathogen via the Fc receptor [37]. Hussain et al. [38] reported that opsonizing antibodies upregulate macrophage proinflammatory cytokines TNF-α and IL-6 in mycobacterial-stimulated macrophages thus suggesting a role for this isotype in TB, since TNF-α synergizes with IFN-γ in its tuberculostatic activity. Since IgG responses against proteins are T cell dependent, antigen recognition by IgG isotypes implies that helper T cells also recognize these mycobacterial antigens. Further, IFN-γ produced by Th1 cells induces murine IgG2a and IgG2b and human IgG1 and IgG3 [39, 40].



(a)



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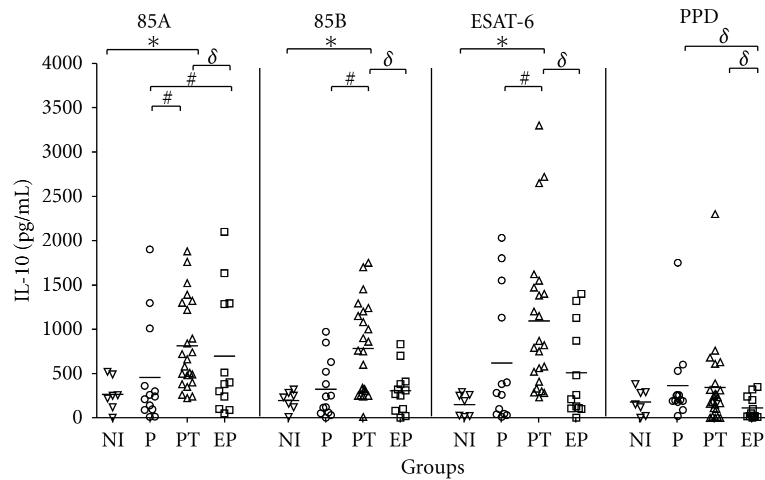

(c)

Figure 4: Individual IFN-γ (a), TNF-α (b), and IL-10 (c) production in response to recombinant Ag85A, Ag85B, and ESAT-6. Freshly isolated PBMC from non-infected individuals (NI), pulmonary TB patients (P), pulmonary TB patients in treatment (PT), and extra-pulmonary TB (EP) were cultured in the presence of Ag85A (25 μg/ml), Ag85B (25 μg/ml), PPD (25 μg/ml), or ESAT6 (50 μg/ml). Supernatants were harvested after five days and the cytokines measured by ELISA. Horizontal bars represent mean values. Differences between responses from TB patient groups and control groups were analyzed with nonparametric Kruskall-Wallis test. *Statistically significant differences in relation to NI group, (δ) in relation to EP group, and (#) in relation to P group ($P < .05$).

Human IgG1 and IgG3 counterparts of murine IgG2a and IgG2b share the ability to fix complement and function as opsonins. In this study, we observed that the increased IgG1 was coincident with augmented levels of IFN-γ and TNF-α detected in PBMCs of patients with active TB and individuals undergoing treatment stimulated with Ag85B and ESAT-6. These results suggest a possible correlation of IgG1 production with Th1 and inflammatory response in TB.

Interestingly, IL-10 production was elevated only in patients under treatment in response to Ag85, Ag86, and ESAT-6. In this study, IL-10 levels could differentiate individuals undergoing chemotherapy from pulmonary or extra-pulmonary patients. Priya et al. [41] have shown that TB patients from India at the beginning of chemotherapy produced similar levels of IFN-γ and IL-10 to Ag85A and the ratio of IFN/IL-10 increases after successful treatment. Furthermore, Meyaard et al. [42] demonstrated that IL-12 is able to induce T cells to produce IL-10 and suggest that IL-10 is a negative regulator of IL-12- induced T cell response. In another study testing TB patients, Priya et al. [43] observed that high levels of IL-10 detected in active TB decreased in patients considered cured. These results and our data led us to hypothesize that elevated production of IL-10 encountered in PT group is probably a modulatory effect in response to IL-12 and IFN-γ production and can be associated to the regulation of immune response.

Th2 responses characterized by inteleukin-4 (IL-4) production have been associated with a lack of protection in TB [44]. In our study, IL-4 levels in response to mycobacterial recombinant antigens were not detected in all groups analyzed. These results are in accordance with others that show that PBMC from TB patients do not produce significant amounts of IL-4 [45–47]. These results confirm the polarization of immune response to recombinant antigens to Th1 profile characterized by the production of high levels of IFN-γ and TNF-α and no IL-4.

Finally, TB causes a staggering burden of mortality worldwide, killing an estimated 1.9 million persons annually. Effective treatment of tuberculosis in developing countries is hampered by the cost of antituberculosis drugs, inability to ensure completion of therapy, and rising drug resistant rates. Vaccination is the most cost-effective strategy to control and eventual elimination of tuberculosis. The current BCG vaccine provides some degree of protection against the most severe manifestations of childhood tuberculosis. However, protection is incomplete, and BCG vaccine does not reduce TB rates in adults. In fact, MVA85A, a recombinant modified vaccinia virus Ankara expressing Ag85A, is the first candidate TB subunit vaccine to enter human trials since BCG was first introduced over 80 years ago. More recently, Dissel et al. [48] demonstrated that vaccination of human naïve volunteers with adjuvanted Ag85B-ESAT-6 subunit vaccine elicited strong antigen-specific T-cell responses. Since a basic principle for selecting novel antigen candidates for designing a TB subunit vaccine is based on their ability to induce a protective Th1 response [16], our study also confirmed the value of Ag85A, Ag85B and ESAT-6 as potential vaccine candidates based upon specific T cell responses measured by IFN-γ and TNF-α production in all studied patients.

5. Conclusions

Currently, there are no accurate surrogate biomarkers of protective immunity and diagnoses in TB but clearly host defense against TB depends critically on Th1 responses and IFN-γ production. In this study, we have shown that Ag85A and ESAT-6 are antigens able to differentiate pulmonary, extra-pulmonary and tuberculosis patients undergoing chemotherapy from healthy individuals by IFN-γ production and pulmonary and under treatment patients from extra-pulmonary TB by TNF-α. Therefore, not only IFN-γ production but also TNF-α to Ag85A and ESAT-6 could be used as biomarkers for the clinical status of TB patients while IL-10 could be useful monitoring TB successful treatment. Finally, the Th1 cytokine profile induced in PBMC of TB patients by all tested antigens reinforces their position as potential vaccine candidates.

Acknowledgments

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Review Article

Modulation of Cell Death by *M. tuberculosis* as a Strategy for Pathogen Survival

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It has been clearly demonstrated that *in vitro*, virulent *M. tuberculosis* can favor necrosis over apoptosis in infected macrophages, and this has been suggested as a mechanism for evading the host immune response. We recently reported that an effect consistent with this hypothesis could be observed in cells from the blood of TB patients, and in this paper, we review what is known about evasion strategies employed by *M. tuberculosis* and in particular consider the possible interaction of the apoptosis-inhibiting effects of *M. tuberculosis* infection with another factor (IL-4) whose expression is thought to play a role in the failure to control *M. tuberculosis* infection. It has been noted that IL-4 may exacerbate TNF-α-induced pathology, though the mechanism remains unexplained. Since pathology in TB typically involves inflammatory aggregates around infected cells, where TNF-α plays an important role, we predicted that IL-4 would inhibit the ability of cells to remove *M. tuberculosis* by apoptosis of infected cells, through the extrinsic pathway, which is activated by TNF-α. Infection of human monocytic cells with mycobacteria *in vitro*, in the presence of IL-4, appears to promote necrosis over apoptosis in infected cells—a finding consistent with its suggested role as a factor in pathology during *M. tuberculosis* infection.

1. Introduction

It is generally accepted that tuberculosis (TB) is responsible for 2-3 million deaths and more than 8 million new cases annually [1]. The majority of these occur in developing countries, especially in Sub-Saharan Africa [2], where a substantial proportion of the population (perhaps as much as a third) is thought to be latently infected. Though they are able to control the initial infection, they may later reactivate their disease if they become immunocompromised [3]. Infection with *M. tuberculosis* is associated with an active inflammatory immune response, characterized by elevated expression of both TNF-α [4–7] and IFN-γ [8–10]. These two cytokines are essential for controlling mycobacterial infections [11–14] but it is clear that in many cases, *M. tuberculosis* is able to survive this inflammatory process. Indeed, *M. tuberculosis* depends on the induction of an inflammatory response and the subsequent tissue damage for cavitation and dissemination via pulmonary disease to new hosts. It is probably for this reason that it expresses multiple molecules on its surface to promote inflammatory responses by the host.

It is therefore no surprise that *M. tuberculosis* has evolved a number of mechanisms by which it interacts with, and modulates, the host's immune response. In addition to inflammation-promoting molecules [15], *M. tuberculosis* also expresses surface antigens that can induce IL-10 and IL-4, [16–18] that typically have an anti-inflammatory effect. Elevated expression of IL-4 (a cytokine with pleiotropic activity) has been implicated as a potential virulence factor, both for its anti-inflammatory capacity and apparent ability to promote tissue damage in association with TNF-α [19].

Higher levels of IL-4 expression also correlate with heightened immune responsiveness to ESAT-6, a proxy marker for infection in TB contacts [20–22] and for bacterial load. Finally, the ratio of IL-4 to IFN-γ or the IL-4 antagonistic splice variant, IL-4δ2, appears to be correlated with clinical status and in particular, with TB-related pathology [23–26] rather than of infection.

These studies suggest that IL-4 (alone or together with TNF-α) may play a role in tissue destruction and/or cell death during *M. tuberculosis* infection. Since cell death (by apoptosis) is a mechanism by which the host can remove infected cells [27, 28] while minimizing cell death and tissue destruction in adjacent, uninfected cells [29], this has obvious relevance for the control of *M. tuberculosis* infection. Indeed, there is a substantial body of literature suggesting that *M. tuberculosis* can directly interfere with the apoptosis of infected cells *in vitro* [30, 31] and that this appears to be directly related to virulence [32, 33]. In contrast, nonvirulent mycobacteria have a much weaker effect and, being dependant on dose, may even promote apoptosis [30].

This question has come under increasing scrutiny in the last few years, and the mechanisms by which *M. tuberculosis* can inhibit apoptosis are being rapidly identified [34]. However, the relative importance of apoptosis as a virulence mechanism *in vivo* and interaction of apoptotic mechanisms with the host cytokine response have until recently been largely unexplored and it is only recently that this area has come into focus [35].

2. *M. tuberculosis* and the Generation of Pathology

M. tuberculosis normally enters the host though the mucosal surfaces—via the lung after inhalation of exhaled droplets containing bacteria or less frequently through the gut after ingestion of bacteria (e.g., in milk from an infected animal). Although some *M. tuberculosis*-exposed individuals show no signs of infection or T cell memory—having possibly eliminated the pathogen via the innate immune response—the majority of exposed persons display the induction of a rapid inflammatory response. Cytokine and chemokine release triggers the swift accumulation of a variety of immune cells and, with time, the formation of a granuloma, characterized by a relatively small number of infected phagocytes, surrounded by activated monocyte/macrophages and lymphocytes [36]. Traditionally, the granuloma has been thought of as a containment mechanism of the host, but recent work suggests that granulomas are dynamic entities, growing and shrinking as cells are recruited and die [37]. The granuloma may eventually disappear, leaving a small scar or calcification, and the patient's T cells become responsive to *M. tuberculosis*-derived antigens. However, if bacterial replication is not successfully controlled, the granuloma can increase in size and cellularity. The end point of this process is necrosis, and the tissue destruction caused by necrosis, can breach the mucosal surface allowing the granuloma contents to leak into the lumen of the lung or allowing *M. tuberculosis* to escape into the blood vessels of the lung, leading to further dissemination. Destruction of the lumen of the lung—a process referred to as cavitation—gives rise to the prototypic symptom of TB, a persistent cough with blood in the sputum. At this point the patient is infectious, spreading the bacteria by aerosol.

3. Inhibition of Early Host Responses

M. tuberculosis's ability to persist within the host is directly linked to the fate of the immune cells which phagocytose it. The macrophage/monocyte thus occupies a pivotal place, as the prototypic host cell for *M. tuberculosis*, and also as the cell responsible for both killing the bacteria directly and priming immune responses by antigen presentation. *M. tuberculosis* interferes with immune activation at virtually every stage. The processes involved in the pathogen's interference with vesicle trafficking and intracellular killing have been well described [38]. The processes involved in large-scale tissue destruction and cell death, however, remain to be mapped out.

Tissue destruction is not mediated directly by *M. tuberculosi*; the bacterium has little or no lytic activity; it is primarily an immunopathological process. Unlike pathogens such as *Leishmania spp*, which can establish chronic infection by evading the host immune response, *M. tuberculosis* actively provokes it. The pathogen expresses a number of molecules that bind to the host's pathogen-associated molecular pattern (PAMP) receptors, such as the Toll-like Receptor (TLR) family [39]. Interestingly, despite *M. tuberculosis*'s long coevolutionary history with humanity, these molecules are largely conserved, even though most of them do not appear to be essential for the pathogen's growth or invasive ability [40, 41]. The simplest explanation is that *M. tuberculosis* depends on the immunopathology that promotes necrosis both for dissemination within the host and for spread to new hosts, but also subverts this response, to allow it to persist in the host. Moreover, the ability of *M. tuberculosis* to rapidly alter its pattern of gene expression in response to stress [42] suggests that the pathogen may do both: in response to the local microenvironment, it may manipulate immune responses so as to favor apoptosis (reducing inflammation, thus allowing persistent infection) or necrosis (promoting tissue destruction, cavitation, and spread to new hosts).

Inhibition of inflammation at early stages may give *M. tuberculosis* a breathing space to initiate a productive infection. It has been suggested that invasion of phagocytes which are not yet activated is important for the bacteria's survival since exposure of macrophages to IFN-γ and/or TNF-α before—but not after—infection decreases the ability of pathogenic mycobacteria to inhibit phagosome maturation and function [43] at least partially by upregulating the production of reactive oxygen and nitrogen derivatives [44–48]. Mannose derivatives on the pathogen's surface molecules from pathogenic (but not nonpathogenic) mycobacteria inhibit phagocytosis by activated macrophages [49] perhaps targeting the pathogen to cells less prepared to contain it and inhibiting the initiation of inflammatory responses.

It does this in part, by targeting the very mechanisms involved in activating cell-mediated immunity. Though TLR2/4 ligation can initiate the inflammatory cascade in

response to mycobacterial infection [50–53], it appears that interference in IFN-γ-signaling via TLR signaling is also a potential virulence mechanism [54]. The 19 kDa lipoprotein of *M. tuberculosis* appears to be a virulence factor [55] that reduces overall immunity to the bacterium in mice [56]. It is known to bind to TLR1/2 on host cells [57, 58] with resulting inhibition of inflammatory cytokine production (reducing expression of over a third of the IFN-γ-activated genes [59]), and reduced antigen-processing and MHC II expression [59–61]. The virulence factor ESAT-6 has a similar effect, also operating through TLR-2 [62], apparently by modulating TCR signaling pathways downstream of the proximal TCR signaling molecule, ZAP70 [63]. And other factors such as phosphoglycolipids bind to other PAMPs to induce IL-4 and IL-13, apparently contributing to virulence [16, 17, 64], and modulating cytokine expression in concert with other factors [65].

Indeed, *M. tuberculosis* appears to actively modulate cytokine expression at multiple levels. The mannose derivative lipoarabinomannan (LAM), which is expressed by pathogenic (but not nonpathogenic) mycobacteria, can bind to the DC-SIGN molecule, expressed on the surface of dendritic cells. The binding of LAM to DC-SIGN inhibits maturation and induces dendritic cells to secrete IL-10 [18, 66]. This inhibits antigen presentation, expression of MHC molecules, and expression of costimulatory receptors. Consistent with this, recent studies have found that expression of IL-10 is significantly elevated in TB patients with active disease [67–69]. LAM binding to DC-SIGN also inhibits the production of IL-12 by affected antigen-presenting cells. IL-12 is crucial to immunity to *M. tuberculosis*, as indicated by the effect of gene polymorphisms on susceptibility to TB, and the extreme susceptibility to mycobacterial disease of individuals with lesions in genes of the IL-12 and IL-12R pathways [70, 71]. Control of IL-12 expression is key to the expansion and activation of IFN-γ—secreting CD4 T cells which are crucial for immunity to TB, as shown by the susceptibility of animals or patients defective in CD4 T cell function or IFN-γ expression or recognition [72–76].

4. Activating and Modulating the Adaptive Immune to *M. tuberculosis*

Both CD4 and (to a lesser extent) CD8 T cells are thought to be crucial to containing *M. tuberculosis* infection via IFN-γ production and possibly cytotoxicity [77–79]. As discussed above, *M. tuberculosis* appears to subvert the host's immune response, in part by directly countering the activation of T cell—particularly Th1-responses.

Consistent with this, IFN-γ recall responses are generally reduced in patients with advanced TB [80], while IL-4 is elevated [81–83]. The level of IL-4 gene expression appears to correlate with both disease severity in TB patients [81, 82] and risk of subsequent disease in TB-exposed individuals [23, 25]. The IFN-γ/IL-4 ratio increases in most patients during therapy, but decreases in contacts that become ill, suggesting that this state is directly related to the disease [25]. This is supported by reports that increased production of the IL-4 antagonist IL-4δ2 is seen in individuals who are controlling TB in its latent stage [20] and that the IL-4δ2/IL-4 ratio increases during treatment of TB patients [25] and in those TB patients who respond most rapidly to therapy [84]. Similar observations have also been made in animal models of TB [85, 86]. A poor prognosis in TB is associated with a low IFN-γ/IL-10 ratio just as is seen for IFN-γ/IL-4 [8, 25, 87]. Altering the balance between IFN-γ and IL-4 or IL-10 production and function thus seems to be a second major survival strategy for *M. tuberculosis,* and the studies above suggest that when this balance is shifted towards IL-4, the result is increased pathology.

Although IL-4 can inhibit the effect of IFN-γ by decreasing the production of IFN-γ response factor-1 (IRF-1), a transcriptional element that enhances expression of IFN-γ-inducible genes such as iNOS [88], high levels of IL-4 are not associated with an absence of inflammatory factors. The proinflammatory cytokine TNF-α is a crucial component for protection against *M. tuberculosis*, as shown by the rapid reactivation of latent *M. tuberculosis* infection in people treated with TNF-α receptor antagonists [89, 90] and the susceptibility of TNF-α-deficient animals to *M. tuberculosis* [5, 7]. Nonetheless, TNF-α mRNA is elevated in TB patients [4] and in TB/HIV-infected patients elevated levels of TNF-α were associated with necrosis [91]. It has been suggested that while it is essential for protection, that in the presence of elevated levels of IL-4, TNF-α appears to promote tissue damage rather than protection [19, 92], possibly by a cooperative effect of transcription [93, 94]. These studies indicate that *M. tuberculosis* seems to have multiple mechanisms devoted to inhibiting both IFN-γ and TNF-α function and that the pathogen can evade killing by the immune system while still generating the pathology it needs for dissemination—and suggest that IL-4 may play a crucial role.

5. Cytokines, Cell Death, and Pathology

One clue to the mechanisms possibly involved is reports showing that resolving granulomas are rich in apoptotic cells and that inhibition of apoptotic capacity leads to reduced ability to control *M. tuberculosis* [95]. Granulomas are metabolically active sites, with cells being continually recruited and eliminated [37]. This can occur by several processes—but apoptosis or necrosis feature prominently. It has been suggested that apoptosis is a "silent" method whereby the host can remove infected cells [27, 28] while minimizing cell death in adjacent, uninfected cells, thus decreasing tissue destruction [29]. Antigens from engulfed apoptotic cells are presented, thus enabling cross-priming of the immune response [96]. Modeling studies suggest that TNF-α is one of the strongest factors controlling monocyte recruitment to the granuloma and that TNF-α-driven apoptosis is the strongest negative factor [97]. This is not surprising: TNF-α is a potent inducer of cell death by apoptosis [98]. Necrosis, on the other hand, is associated with the lysis of the infected cell, release of viable *M. tuberculosis,* and damage to the surrounding tissue [29] and TNF-α is also a major player here [91]. The centre of large unresolved granulomas often becomes necrotic and

as mentioned above, this tissue destruction is an essential feature in the spread of *M. tuberculosis*.

There is now a substantial body of evidence from both *in vitro* and *in vivo* studies indicating that virulent *M. tuberculosis* (but not avirulent mycobacteria) can inhibit apoptosis, and that this may represent an escape mechanism whereby the pathogen can avoid the death of its host cell by apoptosis (and the internalized bacteria along with it as the apoptotic cell is digested) [32, 99–105]. Recent work suggests that *M. tuberculosis* can actively promote necrosis over apoptosis, consistent with the idea that this is a survival/virulence mechanism for the bacteria [106–109]. Supporting this hypothesis, studies indicate that elevated levels of necrosis are associated with genetic susceptibility to *M. tuberculosis* in mice [110] or virulence of human-derived clinical isolates [111] and that control of apoptosis via CD43/TNF-α inflammatory responses is important for control of *M. tuberculosis* [112]. Some of the genes involved such as *nuoG* have already been identified [113].

6. Interplay between TNF-α, IL-4, and Cell Death *In Vivo*

We therefore have started to examine the significance of TNF-α-mediated apoptosis in human TB. Recently published data [4] indicates that there is a strong upregulation of genes for factors that promote apoptosis in PBMC from individuals with active disease, including TNF-α and its receptors, *Fas* and *FasL* and pro-Caspase 8, when compared to exposed individuals without active disease. This is consistent with an important role for apoptosis in human TB. The fact that expression of these molecules are elevated in those with overt disease and also that the degree of expression of TNF-α correlated with severity of pathology in humans (author's unpublished data and [91, 114]) suggests that TNF-α is directly involved in the generation of immunopathology. However, it is hard to reconcile inhibition of apoptosis as a mechanism for pathology if expression of apoptotic genes is highest in those with the worst pathology. A possible explanation for this is the observation that while expression of proapoptotic markers was elevated in PBMC from TB patients, when the CD14+ monocytic fraction was examined, the reverse was true [4]. Our conclusion was that monocytes from TB patients—but not monocytes from those infected with *M. tuberculosis* but asymptomatic, such as individuals with latent TB—were likely less responsive to extrinsic stimuli promoting apoptosis such as TNF-α. Further, we hypothesized that since it was highly unlikely that the majority of CD14+ cells in the blood were infected with *M. tuberculosis*, this effect was likely modulated by soluble factors. IL-4 is an obvious candidate, given that it is also the most elevated in these patients, it declines as symptoms abate during treatment [25], and its modulation of necrosis induced by TNF-α has been suggested in the past [115, 116]. Increased IL-4 and TNF-α expressions are also apparently associated with severity of pathology in mouse model [117], but interestingly, the only study to look at these factors in granulomas from human disease found no association between IL-4 and necrosis—though as the authors note, they could not distinguish between IL-4 and the IL-4 antagonistic splice variant IL4δ2 [91] which renders this difficult to interpret.

7. Interplay between TNF-α, IL-4, and Cell Death *In Vitro*

In the current absence of more data from human studies, we have examined this hypothesized interaction *in vitro*, infecting the human monocytic cell line THP-1 to observe what, if any, effect IL-4 had on the expression of genes that have been shown to be differentially regulated by mycobacterial infection [118–122] particularly those involved in activation of the extrinsic (inflammation-induced) pathway of apoptosis (TNF-α, TNFR1, TNFR2, *Fas, FasL*, and Caspase 8). Although not a perfect substitute, THP-1 cells have been frequently used as proxies for alveolar macrophages and have been used in many prior studies of mycobacteria-induced apoptosis [123]. We therefore infected these cells with the virulent *M. tuberculosis* Erdman strain (a clinical isolate) as a prototypical virulent mycobacteria, while the TB vaccine strain BCG Danish 1337 was used as the prototypical avirulent strain. Pilot experiments with the H37Rv and H37Ra virulent and avirulent strains were also done, with similar results to those reported here (data not shown). Bacterial infections were titrated down from a high dose (MOI of 50) down to a low dose (MOI of 5) based on previous publications [124]. We chose the higher dose, based on previous work, which suggested that a higher MOI was needed to induce rapid and detectable apoptosis *in vitro* [30, 123, 125].

To ensure that the infection protocol used induced significant levels of apoptosis, we infected THP-1 cells in the presence or absence of IL-4 at 10 ng/ml and monitored apoptosis and necrosis with a cell death ELISA kit, optimized to detect both apoptosis and necrosis. As can be seen in Table 1, after 24 hours, infection with BCG led to a major increase in the amount of apoptosis. Interestingly, the level of apoptosis was slightly reduced by IL-4 treatment alone, though this did not significantly decrease the increase of apoptosis induced by BCG. However, supernatants from the same cultures were assayed for total cell death to assess necrosis and this revealed that IL-4 had a significant effect on the balance of cell death. While IL-4 alone did not significantly affect the level of necrosis, in combination with BCG infection, it had a clear pronecrotic effect. Thus, IL-4 appears to bias cell death slightly towards necrosis over apoptosis, and this effect was enhanced by BCG infection (Table 1).

The results with *M. tuberculosis* Erdman were strikingly different from BCG. At 24 hours, *M. tuberculosis* infection slightly reduced apoptosis and this effect was marginally (but not significantly) augmented by IL-4 (Table 1). But either *M. tuberculosis* infection or IL-4 treatment led to an increased ratio of cell death by necrosis compared to apoptosis.

This effect required living bacteria, as heat killed bacteria had no significant effect on apoptosis or necrosis after 24, 48, or 72 hours of culture (data not shown). We also looked

Table 1: Alteration in cell death by apoptosis or necrosis in THP-1 cells infected with BCG or *M. tuberculosis* Erdmann (MOI 50) assessed at 24 hours by the Cell Death Detection ELISA^{PLUS} photometric enzyme immunoassay (Roche Diagnostics, Lewes, UK) which measures cell death by both apoptosis and necrosis on fractionated samples. Associated changes in mRNA for the major host genes involved in the activation of the extrinsic pathway of apoptosis were assessed by quantitative Real-Time PCR, using HuPO as a housekeeping gene for normalization. Results shown are relative to untreated cells, of the means of assays from a single experiment (representative of 4) performed in triplicate (ELISA) or quadruplicate (RT-PCR). Values marked in bold text represent a significant increase, those in italics a significant decrease. The ANOVA test (with Dunnett's multiple comparison posttest for all groups against untreated controls) was used for analyses between groups. In all instances, a P value $< .05$ was considered significant. A value of "<.1" indicates below the limit of detection, with "ND" indicates that the experiment was not done.

Infected with:		Fold change over uninfected THP-1 cells							
		Apoptosis	Necrosis	TNF-α	TNFR1	TNFR2	Fas	FasL	Caspase 8
null	+ IL-4	*0.67 ± 0.13*	*0.79 ± 0.19*	0.51	0.68	0.57	*0.66*	0.96	0.67
BCG	− IL-4	**3.38 ± 0.04**	1.06 ± 0.18	**2.15**	**15.59**	**18.99**	*0.13*	ND	**4.35**
BCG	+ IL-4	**3.29 ± 0.08**	**2.68 ± 0.14**	*0.05*	2.27	3.00	*<0.1*	ND	0.61
Erdmann	− IL-4	*0.44 ± 0.22*	1.13 ± 0.30	**17.43**	**4.96**	**5.20**	*0.65*	1.30	*0.36*
Erdmann	+ IL-4	*0.37 ± 0.12*	**1.34 ± 0.07**	**4.21**	1.38	**1.89**	*0.34*	1.15	*0.35*

at the effect of BCG and *M. tuberculosis* infection (with or without IL-4) after 72 hours of culture and obtained very similar results (data not shown). Finally, we confirmed the ELISA data by FACS analysis for annexin V, which was expressed by 16.28% after 24 hours of culture with BCG, while only 7.26% of control cells were positive. In the presence of IL-4, only 7.06% of BCG-exposed cells were annexin-V positive, confirming the ELISA data. These data are thus consistent with earlier studies suggesting that virulent (but not avirulent) mycobacteria are capable of inhibiting apoptosis, possibly as a defence mechanism against clearance by the host [32, 33]. In addition, the data suggest that IL-4 can also have a mild antiapoptotic effect—though it appears in this *in vitro* model that this inhibition of apoptosis by IL-4 does not prevent cell death, so much as renders host cells more susceptible to death by necrosis—potentially releasing bacteria which could reinfect adjacent cells, thus further promoting inflammation and immunopathology.

8. Effect of IL-4 and Mycobacterial Infection on Expression of Apoptosis-Modulating Genes

To examine the mechanism behind the IL-4 effect, we examined expression of multiple genes involved in activating pathways of induced cell death. It was clear from the apoptosis data (Table 1) that the processes driving apoptosis had already started by 24 hours. We thus performed the PCR analyses after 24 hours of culture, using quantitative PCR to compare the mRNA expression in infected and uninfected cells with or without IL-4 added to the cultures. As shown in Table 1, mycobacterial infection induced a strong TNF-α response at 24 hours, and strongly activated expression of the genes for the two TNF-α receptors. All of these activating effects were antagonized by IL-4. We also analyzed the supernatants from these cultures and found that in parallel with the induction of TNFR2 mRNA by BCG and *M. tuberculosis*, there was a significant increase ($P < .01$) in the amount of soluble TNFR2 protein detectable in culture supernatants 24 hours after infection (data not shown). This increase was identical for BCG and *M. tuberculosis* and was not inhibited in the presence of IL-4, suggesting that in the presence of IL-4, infected cells continue to shed the TNFR2 receptor at increased levels (compared to uninfected cells), at the same time in which mRNA production is downregulated by IL-4, potentially leading to reduced surface expression and further decreasing the responsiveness of these cells to TNF-α. This is consistent with the picture we drew from patient PBMC [4].

Gene expression for the proapoptotic molecule *Fas* was not affected by BCG infection, although it was significantly decreased by IL-4. In *M. tuberculosis*-infected cells, however, *Fas* expression declined significantly, (Table 1). Since this is likely to render *M. tuberculosis*-infected cells more resistant to Fas-mediated death, we also assessed expression of *FasL* in these cells, to gain an idea of what effect they might have on sensitized cells that came into contact with them. However, despite some variability, no significant differences in *FasL* expression were seen that could be attributed to IL-4 or *M. tuberculosis* infection (Table 1).

Downstream of both *Fas* and the TNF-α receptor complexes lies one of the major activating molecules of the extrinsic death pathway, Caspase 8. In BCG-infected THP-1 cells, pro-Caspase 8 transcription increased dramatically and this increase was inhibited by IL-4 consistent with the effects seen on apoptosis. In contrast, in *M. tuberculosis*-infected cells, the opposite was seen, with falling pro-Caspase 8 expression. IL-4 also reduced pro-Caspase 8 expression by itself, but this effect was not significantly different from that induced by *M. tuberculosis* infection. To determine if the decrease in Caspase 8 induced by *M. tuberculosis* infection could be countered by falling levels of apoptosis-antagonising molecules, we also assessed the levels of gene expression for the antiapoptotic molecule FLIPs. Here, however, we found significantly increased expression

induced by *M. tuberculosis* infection ($P < .01$), suggesting that if anything, the antiapoptotic effect of decreased Caspase 8 would be amplified. Neither IL-4 nor BCG had a significant effect on FLIPs (data not shown).

In total, these data are consistent with prior findings that *M. tuberculosis* has an apoptosis-blocking effect and indicate that this affects not just the intrinsic pathway but also extrinsic activation of apoptosis mediated through the pro-Caspase 8 molecule, which avirulent mycobacteria do not share. In addition, they suggest that this is potentiated by IL-4, which promotes necrosis instead, supporting a role in the virulence of *M. tuberculosis*. The data also indicate that this antiapoptotic effect occurs at the gene transcription level and affects multiple gene pathways—though the simple experiments presented here are indicative, not definitive.

9. A Model for *M. tuberculosis* Pathogenesis

There is a significant body of evidence from both *in vitro* and *in vivo* studies indicating that virulent *M. tuberculosis* can inhibit apoptosis and that this may represent an escape mechanism whereby the pathogen can avoid the death of its host cell—and the internalized bacteria along with it [32, 99–105]. Knock-in studies using the *nuoG* gene of *M. tuberculosis* showed that this gene conferred the ability to inhibit apoptosis and increased virulence in mice to avirulent mycobacteria, while its deletion rendered *M. tuberculosis* less able to inhibit apoptosis of infected THP-1 cells [113]. A number of genes involved in membrane repair and lipid biosynthesis have also been identified [34]. All of these studies indicate that *M. tuberculosis* actively interferes with the intrinsic pathway of apoptosis in the infected host cell as a means of virulence and that disregulation of the host's lipid metabolism is a major pathway for generating pathology [126] and promoting necrosis over apoptosis [34].

The picture for inhibition via the extrinsic apoptotic pathway is also rapidly becoming filled in. Knockout studies of the OppA and OppD genes have implicated the peptide transporters encoded by Rv3665c-Rv3662c and Rv1280c-Rv1283c as inhibitors of apoptosis and this is associated with decreased production of cytokines, including TNF-α [127]. Likewise, the hypothetical proteins Rv3654c and Rv3655c appear to interfere with the extrinsic pathway by diminishing the availability of active Caspase 8 through post-transcriptional modification [128]. Inhibition of signaling via members of the TNF receptor superfamily (TNF-α and Fas) has long been suggested as a major factor [32, 129, 130] for modulating pathology and more of the genes apparently involved in this process are being identified [131–133]. Interestingly, these findings are tying identified genotypes (such as *nuoG* mutants) to the same mechanisms—production of TNF-α and reactive oxygen species—already associated with defence and immunopathology in TB [113, 131, 132].

M. tuberculosis infection is known to induce TNF-αproduction, but *in vivo*, infection of host cells does not occur in a vacuum, but in the presence of a variety of immunomodulating factors. We hypothesize that one such factor, IL-4, a cytokine whose expression appears to correlate with a poorer prognosis after *M. tuberculosis* infection [21, 23–25, 92, 134] when combined with TNF-α, may worsen TB-related pathology, possibly by biasing cell death towards necrosis instead. If this effect is replicated *in vivo*, (and our data in clinical studies suggest it is [4]) it might help explain why a bias toward IL-4 expression can lead to aggravated pathology in TB [20, 26, 134, 135]. In addition, IL-4 strongly inhibits the expression of the pro-apoptotic molecule TNF-α and its two receptors, which are otherwise increased by mycobacterial infection—an effect which may be exacerbated since mycobacterial infection appears to promote the shedding of the soluble form of the receptors [4] that can act as competitive inhibitors. Inhibiting TNF-α in primate studies appears to promote pathology [136]. All of this supports the hypothesis that control of apoptosis via CD43/TNF-a inflammatory responses is important for control of *M. tuberculosis* [106, 108, 112]. Finally, IL-4 appears to play a role in the differentiation of M2 (or anti-inflammatory) macrophages, [137–139], which not only promote IL-4 and IL-10 production, but also handle arginine and iron—two important resources for *M. tuberculosis*—differently from M1 macrophages [140, 141]. We suggest expanding the mechanisms by which *M. tuberculosis* actively interferes in this process to suggest that the induction of IL-4, which has been linked to virulence, does so via multiple pathways, and at least partially by promoting cell death by necrosis instead of apoptosis. Identifying the mycobacterial factors which drive this process could offer potential new targets for vaccine and drug development and we are thus investigating *M. tuberculosis* factors that may be involved.

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Review Article

Mycobacterial PE/PPE Proteins at the Host-Pathogen Interface

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The mycobacterial PE/PPE proteins have attracted much interest since their formal identification just over a decade ago. It has been widely speculated that these proteins may play a role in evasion of host immune responses, possibly via antigenic variation. Although a cohesive understanding of their function(s) has yet to be established, emerging data increasingly supports a role for the PE/PPE proteins at multiple levels of the infectious process. This paper will delineate salient features of the families revealed by comparative genomics, bioinformatic analyses and genome-wide screening approaches and will summarise existing knowledge of subcellular localization, secretion pathways, and protein structure. These characteristics will be considered in light of findings on innate and adaptive host responses to PE/PPE proteins, and we will review the increasing body of data on B and T cell recognition of these proteins. Finally, we will consider how current knowledge and future explorations may contribute to a more comprehensive understanding of these intriguing proteins and their involvement in host pathogen interactions. Ultimately this information could underpin future intervention strategies, for example, in the area of new and improved diagnostic tools and vaccine candidates.

1. Introduction

Tuberculosis (TB) represents an ongoing threat to global health, with the current epidemic fuelled by HIV-coinfection and an increasing incidence of drug resistant strains of *Mycobacterium tuberculosis* [1]. Effective new interventions are urgently needed, and genes that are unique to mycobacteria may provide a starting point for developing these. The intriguing *pe/ppe* genes first attracted attention due to their genetically hypervariable nature [2, 3] and were initially exploited as informative molecular markers for mycobacterial strain typing [2, 4]. Shortly thereafter, the first *M. tuberculosis* genome sequence was completed, and it was revealed that these variable regions were in fact part of two extensive families encoding almost 200 putative proteins [5]. It is now known that these genes are unique to mycobacteria and are particularly abundant in pathogenic mycobacteria, such as *M. tuberculosis*. Naturally, the PE/PPE families have provoked much speculation, although we have yet to establish a complete understanding of their function. However, the advent of the mycobacterial genomic age, together with improved molecular tools and a deeper understanding of the immunopathogenesis of TB, has advanced our knowledge of these gene families and the potential functions of their encoded proteins.

2. PE/PPE Genomics

Analysis of the *M. tuberculosis* H37Rv genome sequence revealed the presence of two novel gene families that comprise almost 10% of the coding capacity of the genome [5]. These were designated the *pe* and *ppe* genes, after highly conserved Proline-Glutamate and Proline-Proline-Glutamate residues near the start of their encoded proteins. The proteins can be categorized into subgroups, encompassing members with highly variable length and sequence features (Figure 1) [5]. The relatively conserved N-terminal is approximately 110 amino acids (aa) and 180 aa in the PE and PPE families, respectively. The smallest members of both families consist of just this conserved domain, while other subclasses have additional C-terminal regions. The PE_PGRS (polymorphic GC-rich sequence) and PPE_MPTR (major polymorphic tandem repeat) subgroups possess C-terminal

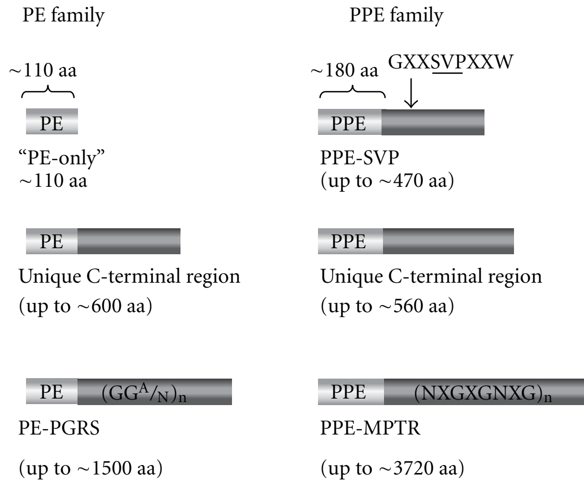

Figure 1: Schematic representation of PE and PPE family subgroups. PE and PPE proteins possess relatively conserved N-terminal domains of approximately 110 aa and 180 aa, respectively. One subgroup of the PPE family incorporates a characteristic "SVP" motif at approximately 350 aa. Both PE and PPE families can be divided into distinct subgroups on the basis of their variable C-terminal domains. The regions encoded by the Polymorphic GC-Rich Sequence (PGRS) of the *pe* family and the Major Polymorphic Tandem Repeats (MPTR) of the *ppe* family are major contributors to genome polymorphism.

regions of enormously variable size—these can reach over 3700 aa in length; they are also the family members which exhibit the most sequence variation.

The remarkable length and extensive sequence variation of the PE_PGRS and PPE_MPTR proteins appear to be primarily associated with lengthy stretches of GC-rich, imperfect triplet repeats within their associated genes. These are thought to be hotspots for recombination events and other mutations, including insertion of transposable elements [5, 6]. Other *pe/ppe*-associated sequence variation includes large sequence polymorphisms which appear to be mediated by highly homologous sequences in the conserved 5′ regions of *pe/ppe* genes [6]. Given the diverse mechanisms whereby *pe/ppe* polymorphisms can arise, together with the high potential for redundancy within the gene families, it is perhaps unsurprising that some *pe/ppe* genes vary extensively in clinical isolates [7–9]. Consequently, it has been speculated that these proteins represent a source of antigenic variation which allows the organism to escape antigen-specific host responses [5, 7, 8]. However, this hypothesis is as yet unproven, and there is little evidence to support rapid within-host diversification of these genes. Therefore if the observed sequence variation is providing a source of antigenic diversity, the benefits of this are likely to operate on a population-wide scale, rather than within individual hosts. One study has demonstrated a moderately significant association of large sequence variation in *pe_pgrs33* with noncavitary TB and case clustering [10]. However, further experimentation will be required to establish whether there is a causal link between these observations, and if so, to determine the underlying mechanistic basis. It is worth noting that while the *pe_pgrs* and *ppe_mptr* genes represent some of the most variable regions of the *M. tuberculosis* chromosome, it is an oversimplification to extend this to all *pe/ppe* family members, as some are in fact conserved across strains and species [11]. It is important to bear this distinction in mind when considering potential functional roles and to extrapolate experimental results with caution.

An increasing wealth of mycobacterial genome sequence data has advanced our knowledge of the sequence diversity and evolutionary history of the *pe/ppe* gene families. One comparative genomics study revealed that the evolution and major expansion of the *pe/ppe* families is closely associated with the *esx* regions [12]. These encode the so-called Type VII or ESX secretion systems, of which there are 5 in *M. tuberculosis* [13]. The best characterized of these is ESX-1, which has been implicated in mycobacterial virulence through the secretion of effectors such as EsxA and EsxB [14–16] (otherwise known as ESAT-6 (early secreted antigenic target of 6 kDa) and CFP-10 (culture filtrate protein of 10 kDa), resp.). Gey van Pittius and colleagues further showed that the most repetitive and variable family members represented by the PE_PGRS and PPE_MPTR subgroups are also the most phylogenetically recent. These subgroups are restricted to the pathogenic mycobacteria, and their massive expansion is closely associated with the most recently duplicated *esx* gene cluster, *esx-5* [12]. These findings were suggestive of a functional link between the PE/PPE families and the ESX secretion systems. Indeed, it has since been demonstrated that the secretion of multiple PE/PPE family members is ESX-mediated [17–19].

3. Transcriptomics, Proteomics, Structure, and Subcellular Location

In tandem with burgeoning mycobacterial genome data, other "-omics" undertakings have shed light on certain aspects of the PE/PPE families. An exhaustive account of *pe/ppe* transcriptomics is beyond the scope of this paper. However, it is worth mentioning that a study of *M. tuberculosis pe/ppe* gene expression under 15 different conditions revealed that 128/169 *pe/ppe* genes analysed were differentially regulated [20]. Other groups have demonstrated that several *pe/ppe* genes are upregulated upon macrophage infection and in host tissues [21–24]. For example, Rachman et al. demonstrated that two *pe* genes (*pe11, pe34*), four *pe_pgrs (pe_pgrs14, 33, 55* and *57),* and three *ppe-mptr* genes (*pe_pgrs54, 55 and 62)* were all upregulated in human lung granulomas compared to *in vitro* grown bacteria [24]. Together with other published expression data, these results lend support to the idea that the *pe/ppe* genes play important functional roles *in vivo*.

Characterisation of regulatory and other mutants has revealed that subsets of *pe/ppe* genes are frequently among the genes whose expression levels are altered [25–27]. In one example, disruption of the virulence-associated PhoPR two-component regulator resulted in altered expression of at least 14 *pe/ppe* genes. These include *pe_pgrs41*, which demonstrated a striking 143-fold higher expression level in

wild type *M. tuberculosis* compared to the *phoP* mutant [26]. It has been demonstrated that selected *pe/ppe* genes may be differentially regulated in genotypically diverse clinical isolates [28–30]. For instance, Gao et al. examined gene expression in 10 clinical isolates of *M. tuberculosis* and found that 28 of the 77 *pe/ppe* genes included in the analysis showed variable expression [29]. There is also evidence for divergent regulation of specific family members within individual strains [31, 32]. This is exemplified by *pe_pgrs16* and *pe_pgrs26*, which are thought to be inversely regulated *in vitro* [31], although the mechanistic basis for this apparently coordinated expression is unclear. Taken together, available data does not support global regulation of *pe/ppe* gene expression, suggesting that there is likely to be a relatively high degree of plasticity in the *pe/ppe* expression repertoire. Although this may suggest that some *pe/ppe* genes are not required at all, this could also indicate that members within different subgroups of the family play subtly different functional roles and are required at different stages of disease.

The existence of multiple highly similar *pe/ppe* family members suggests a high potential for functional redundancy within subgroups. It is therefore not surprising that very few of these genes are essential for *in vitro* or *in vivo* growth, as demonstrated in a series of studies by Rubin and colleagues [33–35], Table S1 (see Table S1 in supplementary material available online at doi:10.1155/2011/497203). This high degree of redundancy contributes to one of the experimental challenges presented by this family, that is, that mutations in single *pe/ppe* genes may lack measurable phenotypes. However, there are some examples where this is not the case, and analysis of selected *pe/ppe* mutants has yielded important functional insights, as will be described below.

High-throughput proteomics-based studies have highlighted further challenges of working with PE/PPE proteins. The proteins are often very large, may be tightly associated with the cell wall, and can have limited proteolytic cleavage sites. These inherent properties may restrict the utility of some high-throughput proteomics approaches [18]. Nonetheless, using optimized growth conditions and sample preparation methods in conjunction with sensitive detection methodologies, some studies have successfully identified the subcellular localization of selected PE/PPE proteins in different mycobacterial strains and species (Table S1). For example, using proteolytic shaving of intact *Mycobacterium avium subsp. paratuberculosis* followed by LC-MS/MS, Newton et al. identified 2 PPE orthologues associated with the cell wall [36]. PE_PGRS and PPE orthologues were also identified in the cell membrane fraction of *Mycobacterium immunogenum* [37], and a recent detailed analysis of the *Mycobacterium marinum* capsule using cryoelectron microscopy in conjunction with LC-MS/MS demonstrated that 6/25 major cell surface proteins were members of the PE/PPE families [38]. Similarly, Målen et al. utilized MALDI-MS and LC-MS/MS to identify at least 7 and 16 PE/PPEs in the *M. tuberculosis* culture filtrate and envelope fractions, respectively [39, 40]. High-throughput proteomics approaches therefore suggest that cell wall/surface localization is a characteristic of several PE/PPE proteins. This has been corroborated by more focused studies which provide further substantial evidence indicating that numerous PE/PPE family members are associated with the bacterial cell wall [37, 38, 40–43]. These include PPE36 [42], PPE68 [41], PE_PGRS33 [44], PE_PGRS63 [43], and PPE_MPTR34, with further examples listed in Table S1. In some cases, results indicate surface exposure of these proteins [17, 44–47]. Although it has been proposed that PE/PPE proteins could form complex surface structures [48], this hypothesis has yet to be addressed experimentally.

To date, only one PE/PPE protein structure has been solved, perhaps reflecting the difficulty experienced with recovering stable, soluble recombinant PE/PPE proteins. Strategies used to successfully overcome this challenge include on-column refolding [49], use of mycobacterial host strains for expression [50], and coexpression of cognate PE/PPE pairs [51, 52]. Using the latter approach, Strong and coworkers successfully purified recombinant PE25/PPE41 and subsequently determined the crystal structure, which showed that the protein pair forms a stable 1 : 1 heterodimer [52]. The PE/PPE complex is reminiscent of the EsxA/EsxB complex, which could suggest common secretion pathways. Intriguingly, the PE25/PPE41 complex displays an apolar stripe on one face, which could represent a docking site for an as-yet unidentified bacterial or host target [52]. An attempted structure/function analysis revealed that the heterodimeric complex shares some features of signal transduction molecules [52], although this has yet to be explored experimentally. It is unknown whether the PE25/PPE41 structure is truly representative of the rest of the protein family. Indeed, it is unlikely that this structure can be extrapolated to family members which are not predicted to form heterodimers [53] or which demonstrate substantial variation in sequence length and content. The structural biology of PE/PPE proteins is therefore an area in much need of further development, as characterization and comparative analysis of additional PE/PPE structures could perhaps provide clues to their possible function. Another crucial step towards determining the function of the PE/PPE proteins will be identifying their interaction partners. In particular, determining host targets of these proteins could provide critical insights into their impact on host responses.

As mentioned above, the PE/PPE proteins are intimately associated with the ESX systems [12]. A functional link has been elucidated by the work of Abdallah and colleagues, who demonstrated that the ESX-5 apparatus mediates the secretion of multiple PE/PPE proteins, including members of the PE_PGRS and PPE_MPTR subsets [17, 18]. The ESX-5 locus is implicated in *M. marinum* virulence [17, 54], although it remains to be seen whether the secreted PE/PPE proteins play a central role in this. In addition, ESX-5-mediated PE/PPE secretion has yet to be demonstrated for *M. tuberculosis*. Of course, given the parallels with the EsxA/EsxB complex and the close association with the ESX secretion apparatus, it is tempting to speculate that PE/PPE complexes (or the individual constituent proteins) could be virulence effectors secreted by ESX-5. Once again, caution should be exercised when extrapolating these results

to other family members; it is worth noting that some PE/PPE proteins in fact possess functional N-terminal signal peptide cleavage sites [39]. It is therefore plausible that their secretion/localization may occur via other mechanisms, for example, the Sec-dependent export pathway. In support of this, several studies have reported cell-wall localization of PE/PPE proteins in *Mycobacterium smegmatis,* which lacks the ESX-5 region [44, 46, 47, 55]. In addition to the possible role of ESX-5, there is some evidence implicating ESX-1 in PE/PPE secretion. For example, the genes encoding PE35/PPE68 are situated within the *esx-1* region, and one study has suggested that PE35 secretion is ESX-1-dependent [19]. Others have demonstrated cell-wall association of PPE68 in *M. tuberculosis* [40, 41], and this protein also interacts with multiple components of the ESX-1 machinery [56, 57]. Interestingly, disruption of *ppe68* is associated with increased secretion of ESAT-6, leading to the suggestion that PPE68 may act as an ESX-1 gating protein [58], although this has yet to be experimentally verified.

Regardless of their secretion mechanism, several lines of evidence support surface localization of PE/PPE proteins (Table S1). Some PE/PPEs may be actively secreted or passively shed from the bacterium into the host cell milieu, and could even be released from host cells via exosomes [59]. Thus, they are ideally positioned to interact with the host immune system. Indeed, there is extensive evidence that they do so in ways that could have important consequences for the outcome of infection.

4. PE/PPEs Modulate Innate Immune Responses

There is mounting evidence that PE/PPE proteins interact with host components and thereby modulate and possibly subvert critical innate immune pathways. Ramakrishnan et al. provided the first definitive evidence that (at least some) PE_PGRS proteins are virulence factors, when they showed that two *M. marinum pe_pgrs* genes were required for survival in macrophages and granulomas [60]. Since then, the most extensively studied "examplar" member of the *M. tuberculosis* PE_PGRS family, namely PE_PGRS33, has provided some insight into the numerous ways in which these proteins could modulate host immune responses. Detailed examination revealed that PE_PGRS33 interacts directly with TLR-2, thereby mediating apoptosis and cytokine secretion [61]. Others have confirmed that PE_PGRS33 promotes host cell apoptosis [59], and, in one study, necrosis [62]. Interestingly, sequence variants of PE_PGRS33 and other PE_PGRS family members elicited differential effects [61]. PE_PGRS11 and PE_PGRS17 were also shown to interact with TLR-2, prompting maturation and activation of dendritic cells [63]. Independent studies have subsequently shown that PPE18 and an MPTR-containing domain of PPE_MPTR34 can also modulate host responses through TLR-2 [64, 65]. Although it has yet to be established if TLR-2 engagement is a common property of PE/PPE proteins, these results suggest that the additive host response to interaction with multiple PE/PPE proteins may be very complex, and could profoundly impact on the course of disease.

PE/PPE proteins can also influence macrophage function by other means. For example a BCG *pe_pgrs33* transposon mutant demonstrated reduced entry into macrophages [66], suggesting that PE_PGRS33 may play a role in promoting macrophage uptake. In contrast to other bacteria, this may be advantageous for *M. tuberculosis* at the very early stage of infection, as it could promote dissemination and colonization of multiple organs. This strategy is only effective because the pathogen has evolved multiple strategies to overcome the hostile environment encountered within host cells. In this context, several PE/PPE proteins have been shown to facilitate, or be required for, *in vivo* survival [35, 60, 67, 68]. A key macrophage defence mechanism is phagosome maturation and acidification, which normally results in killing of intraphagosomal pathogens. However, *M. tuberculosis* is adept at subverting this pathway, and there is evidence that different PE/PPE family members may contribute to this. Selected PE/PPE family members have been implicated in the modulation of vacuole acidification [69–71]. For example, a high-throughput analysis of a *Mycobacterium bovis* BCG tranposon mutant library identified four *pe_pgrs* mutants (*pe_pgrs5, 28, 44, 59*) and three *ppe_mptr* mutants (*ppe_mptr10, 16, 21*) that were enriched in acidified phagosomes. A more recent study showed that a *M. tuberculosis ppe54* transposon mutant was impaired in its ability to arrest phagosome maturation and trafficked rapidly into acidified compartments [72].

Available data therefore indicates that selected PE/PPE family members play important roles in subverting innate immune responses, and together with other bacterial mediators, may assist the pathogen in establishing itself within the host. Once this has occurred, PE/PPEs may then contribute to processes which allow the pathogen to persist within host tissues. Intriguingly, enzymatic functions have been assigned to 3 PE proteins: PE_PGRS11 has been reported to be a functional phosphoglycerate mutase [55], while both PE_PGRS63 (LipY, [43]) and PE11 (LipX, [73]) demonstrate lipase activity. The latter 2 proteins may therefore play a role in energy provision. It should be noted though that these are exceptional examples; they appear to have arisen as a result of recombination events which lead to functional divergence, and enzymatic activity is not predicted to be a general feature of the PE/PPE proteins.

Once established within their intracellular niche, there are a number of ways in which mycobacteria can limit engagement of the adaptive immune response, and the PE/PPE proteins may contribute to some of these mechanisms. The observation that PE_PGRS proteins bear some resemblance to the Epstein-Barr virus nuclear antigen (EBVNA) gave rise to speculation that, akin to the EBVNA, the PE_PGRS proteins may inhibit antigen processing [5]. This has subsequently been demonstrated for PE_PGRS33 [48] and PE_PGRS17 [74], supporting the idea that these proteins may assist in immune evasion by limiting antigen presentation, thereby preventing recognition and killing of mycobacteria-infected host cells.

Taken together, the observations described above suggest that different PE/PPE proteins may play distinct, but complementary roles as the infection progresses, acting in concert

to facilitate adaptation to the hostile host environment. Members of these families may be critical mediators of host responses which ultimately determine the outcome of infection. Understanding their individual and cumulative impact on host responses is an important undertaking which could provide the starting point for novel interventions. The large number and diverse effects of these proteins present some experimental challenges. Future explorations may therefore benefit from systems-level approaches to help unravel their biological role.

5. B and T Cell Recognition of PE/PPE Proteins

5.1. Humoral Immunity. The contribution of humoral responses to controlling *M. tuberculosis* infection has long been underappreciated. However, it is known that mycobacteria-specific antibodies can both influence mycobacterial dissemination and modulate potentially detrimental inflammatory tissue responses [75, 76]. It is increasingly recognized that B cells can exert an influence on T cells [77, 78] and are an important constituent of granuloma architecture [79]. B cells are thus likely to be more important in determining the outcome of infection with *M. tuberculosis* than previously supposed, and ignoring this aspect of the host immune response may restrict our understanding of TB immunopathology. In this context, PE/PPE family members are a potentially rich source of B cell epitopes, and a number of PE/PPE proteins may be surface exposed, increasing the likelihood that they could be targeted by the humoral response. Accordingly, numerous PE/PPE proteins have been shown to elicit B cell responses (Table S1). There is evidence for both members of the PE_PGRS and PPE_MPTR families that the highly repetitive domains are chiefly responsible for eliciting antibody responses. Delogu and Brennan were the first to demonstrate differential B and T cell targeting of the conserved N-terminal and highly repetitive, more variable, C-terminal of PE_PGRS33; DNA vaccination of mice with only the PE domain elicited predominantly cell-mediated immunity and subsequent protection against challenge, whereas a full-length PE_PGRS33 construct promoted a nonprotective, B cell-skewed response [80]. In agreement with these results, PPE_MPTR42 was found to elicit a primarily humoral response, directed toward the glycine and asparagine-rich repeat domain [81]. In other work, it was shown that the full-length PE_PGRS17 and PE_PGRS62 proteins were preferentially recognized in comparison to the PE-only versions [82]. Further elucidation of differential immune targeting of PE/PPE proteins could enable fine-tuning of new vaccine candidates.

5.2. PE/PPE Proteins as T Cell Immunogens. Cell-mediated immunity is especially important in the control of *M. tuberculosis* infection [83, 84]. Therefore, T cell antigens are of great interest for vaccine development and could form useful components of subunit DNA or protein vaccines or engineered into live recombinant vectors. Consequently, numerous studies have investigated the ability of PE/PPE proteins to elicit T cell responses (Table S1). For example, T cell expression cloning was successfully exploited to identify T cell immunogens in *M. tuberculosis*-sensitized individuals [85]; this relatively high-throughput approach resulted in the identification of the potent T cell antigen Mtb39a, which is encoded by *Rv1196/ppe18*. In subsequent work, this antigen has been evaluated as part of the polyprotein subunit vaccine candidate, Mtb72f, as described further below (Section 6.2). In addition to PPE18, at least 20 PE/PPE proteins have been reported to elicit CD4 and/or CD8 responses, either in the form of whole recombinant proteins or as individual peptides (Table S1). Together, these results suggest that PE/PPE proteins are worthy of further evaluation as potentially protective antigens for inclusion in new TB vaccine candidates.

Aside from the possible practical applications of PE/PPE-derived antigens (see Section 6), their high degree of immunogenicity could also provide some clues regarding their biological function. A recent study which combined multiple genome sequence comparisons with analysis of published immunogenicity data reported an unexpectedly high level of conservation of human T cell epitopes [86]. This runs counter to the existing dogma that antigenic targets will exhibit a relatively high degree of variation due to selection pressures imposed by host immune responses. Unfortunately, the high-throughput sequencing methodology used in the study imposes inherent technical limitations, which impact substantially on *pe/ppe* genes; due to the short sequence reads, highly repetitive, multicopy sequences are difficult to accurately assemble. As a result, PE/PPE proteins were excluded from their analysis. It is possible that some members of these protein families, in particular within the PE_PGRS and PPE_MPTR subgroups, could be exceptions to the described finding. However, our own recent work suggests that a large subset of the PE/PPE family, which is relatively well conserved across multiple clinical isolates, is also highly immunogenic (manuscript in preparation). The most conserved regions of the proteins are also the most well recognized, and even when PE/PPE amino acid sequences do vary, this does not necessarily impair epitope recognition by cross-reactive T cells (manuscript in preparation). Taken together, this suggests that at least some PE/PPE family members will fit the paradigm revealed by Comas et al. [86]. This raises the question of whether the high level of immunogenicity displayed by proteins such as the PE/PPEs confers a selective advantage on the pathogen. As speculated by others [87], it is possible that robust inflammatory responses lead to lung damage which could promote *M. tuberculosis* transmission. An alternative hypothesis is that these potent T cell antigens could provide a further means of immune evasion by overwhelming and misdirecting the adaptive immune response.

To date, there is little experimental data to support the contention that PE/PPE proteins contribute to antigenic variation in the conventional understanding of the phenomenon. However, there is substantial evidence that these proteins contribute to immune evasion via other important mechanisms, as described above.

6. Translational Applications

The findings described in the preceding sections have implications for both vaccine development and diagnostic tools.

6.1. Diagnostic Tools. In terms of new diagnostic tools, several highly immunogenic PE/PPEs have been identified, which could contribute to the development of antigen cocktails to be used in IFN-γ release assays or antibody-based tests. However, there are some caveats. Firstly, there is the issue of intraspecies cross-reactivity—a number of PE/PPE proteins are present in mycobacteria outside of the *M. tuberculosis* complex [12] and are recognised immune targets [36, 81, 88, 89]. Therefore, antigens would need to be rigorously evaluated to ensure species-specificity. Secondly, although there are some indications that some PE/PPE-derived antigens may distinguish between vaccinated and nonvaccinated individuals, or between different forms of disease and/or stages of infection, [55, 82, 90–95], existing data suggests that differential diagnostic capability does not apply to all PE/PPE proteins [90, 91, 96]. Therefore their utility in distinguishing between active disease, latent infection and vaccinated individuals will likely be limited to selected family members, and would need rigorous validation in larger patient cohorts prior to clinical implementation.

6.2. Vaccines. PE/PPE proteins are a potentially rich source of T-cell antigens, and several of these have been assessed for possible inclusion in new vaccines [80, 97–102], Table S1. As mentioned above, the PPE18-based subunit vaccine candidate Mtb72f, formulated with the adjuvant AS02A, has yielded promising results in multiple animal models [103, 104] and is now undergoing Phase I clinical trials [105, 106]. Similarly, evaluation of 2 polyprotein vaccine candidates which incorporate PPE_MPTR42 is at a relatively advanced stage. PPE_MPTR42 was shown to confer partial protection in mice when formulated with the TLR-9 agonist CpG [97]. The fusion protein ID83 (which incorporates Rv1813, Rv3620, and PPE_MPTR42) elicits protective immunity in mice [97], which can be modulated by use of different adjuvants and/or route of immunization [107]. In recent work, a similar fusion protein (ID93, which incorporates PPE_MPTR42, Rv1813, Rv3620 as well as a fourth antigen, Rv3619) has been shown to elicit protection against challenge with virulent *M. tuberculosis* in both mice and guinea pigs [108]. As it is likely that subunit vaccine candidates (either DNA or protein-based) will be introduced in the context of a BCG-prime/subunit boost regimen, it is appropriate that these be tested in this setting; importantly, ID93 offers enhanced protection in a BCG-prime/ID93-boost regimen in guinea pigs [108]. Furthermore, ID93 is immunogenic in cynomolgous macaques and elicits polyfunctional CD4 and CD8 responses in peripheral blood mononuclear cells from BCG-vaccinated humans [108]. Together, these promising preclinical results support further evaluation of ID93 in humans in Phase I clinical trials.

The protective capacity of additional PE/PPE-derived subunit candidates is also under investigation. For example, DNA vaccines based on PPE44 [99], PPE41 [100], and the PE domain of PE_PGRS33 [80] have all been shown to elicit protection in mice. In addition, PE_PGRS62- and PE20-derived DNA vaccines have been shown to reduce guinea pig bacterial lung burden by >0.5 log [101]. An alternative to the BCG prime/subunit boost approach is to heterologously express candidate antigens in a live vaccine vehicle, and thereby appropriately supplement/modulate immune responses. Wang et al. have explored this approach by coexpressing PPE57 and Ag85B in BCG and have demonstrated that the recombinant BCG strain elicits enhanced immunogenicity in mice [102]. However, it has yet to be determined whether this approach will result in improved protection.

In addition to their immunogenicity, other features of PE/PPE proteins may be beneficial to vaccine development efforts. For example, the PE domain is thought to be responsible for protein localization to the cell wall [44], and this property could be exploited to ensure optimal localization of PE/PPE-derived or other heterelogous antigens in live recombinant vaccines. Secondly, selected PE/PPE proteins may be potent immune-modulators that could be exploited to fine-tune the immune response to enhance protective capabilities of vaccine antigens.

Although PE/PPE proteins show potential for exploitation in vaccine development efforts, several issues need to be considered. Firstly, many of the known PE/PPE immunogens have been identified on the basis of their ability to stimulate IFN-γ production. While this cytokine is clearly important for the control of TB, it is not necessarily the best predictor of protection against subsequent challenge [109]. The simultaneous production of other cytokines, such as IL-2, has been shown to be a significant component of a protective immune response [110]. Likewise, the phenotypic makeup of the responding cell population is also important; for example, the presence of antigen-specific central memory T cells correlates with protection [111]. Future antigen discovery and evaluation exercises therefore need to consider a broader cytokine profile, as well as the phenotype of the responding cell populations. A second point of caution is that the many of the immunogenic PE/PPE epitopes which have been identified are recognized during active infection, suggesting that the ability to mount an immune response against them does not necessarily confer protection. However, with a more comprehensive understanding of what constitutes a protective immune response, it may be possible to fine-tune factors such as vaccination dose, timing, and formulation to accordingly modulate responses to vaccination. Finally, it will also be important to have a clearer understanding of PE/PPE function and possible adverse effects on host responses. For example, at least 3 PE_PGRS proteins, PPE_MPTR34, and PPE18 have all been shown to interact directly with surface receptors on host macrophages eliciting responses such as apoptosis and cytokine production [61, 63–65] that may not necessarily be desirable outcomes. Until we have a better understanding of the nuances of PE/PPE-host interactions, the use of these proteins as vaccine candidates should be cautiously approached.

Importantly, consideration is increasingly being given to the "real-world" setting in which new TB vaccine candidates will be tested and utilized. For example, epitope prediction tools can be used to assess the prevalence of peptides which are likely to bind MHC alleles found in the target population [112]. Also, multiple genome sequence comparisons can help to determine the degree of conservation of predicted MHC binding peptides in circulating mycobacterial strains [113]. Using Mtb72f as an example, McNamara et al. applied a combination of epitope prediction software packages to demonstrate that several MHC DRB1 alleles would have limited binding to predicted PPE18 epitopes [114]. They further showed that predicted binding peptides were relatively unconserved [114], in accordance with an earlier report [113]. On the basis of these results, it was suggested that Mtb72f might have limited efficacy in selected populations [114]. Although epitope prediction tools are still in need of refinement, *in silico* approaches such as these can be applied to prioritize antigen screening. They could also be used to predict the likely efficacy of selected vaccine candidates prior to undertaking lengthy and resource-intensive clinical trials.

7. Conclusions

More than a decade after their discovery, a cohesive understanding of the function of the PE/PPE proteins remains elusive. The large and complex families pose a number of experimental challenges. However, some interesting themes are emerging: several members of the family trigger a range of innate immune responses, and many are targets of the adaptive immune system. These families are a potentially rich source of diagnostic and vaccine antigens and could even find application as immunomodulatory agents. However, it will be critical to improve our understanding of their function and potential effects of these proteins on the host. Fundamental areas in need of attention include PE/PPE structural biology, identification of PE/PPE host targets, and bacterial interaction partners and an expanded understanding of the mechanisms whereby these proteins modulate host immune responses. Some of the experimental challenges posed by these large and complex proteins families could be addressed using approaches informed by the field of systems biology. Future investigations which exploit the wealth of mycobacterial genome sequence data in the context of our increasing understanding of host and pathogen biology may provide a platform for answering some of the intriguing questions surrounding these enigmatic proteins.

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Research Article

Different Transcriptional Profiles of Human Monocyte-Derived Dendritic Cells Infected with Distinct Strains of *Mycobacterium tuberculosis* and *Mycobacterium bovis* Bacillus Calmette-Guérin

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In order to analyze dendritic cells (DCs) activation following infection with different mycobacterial strains, we studied the expression profiles of 165 genes of human monocyte-derived DCs infected with H37Rv, a virulent *Mycobacterium tuberculosis* (MTB) laboratory strain, CMT97, a clinical MTB isolate, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), Aventis Pasteur, and BCG Japan, both employed as vaccine against tuberculosis. The analysis of the gene expression reveals that, despite a set of genes similarly modulated, DCs response resulted strain dependent. In particular, H37Rv significantly upregulated EBI3 expression compared with BCG Japan, while it was the only strain that failed to release a significant IL-10 amount. Of note, BCG Japan showed a marked increase in CCR7 and TNF-α expression regarding both MTB strains and it resulted the only strain failing in exponential intracellular growth. Our results suggest that DCs display the ability to elicit a tailored strain-specific immune response.

1. Introduction

How organisms respond appropriately to the wide variety of pathogens and antigens they encounter, and how pathogens can subvert the host immune response, has not been fully analyzed. The immune response to infectious agents involves a complex interaction of different cell types, and two defense arms have evolved to protect the host from microbial attack: a rapidly responding innate immune response to sequester and eliminate pathogens followed by a highly specific adaptive immune response. Dendritic cells (DCs) represent the bridge between the innate and adaptive immune response [1], and several studies support the hypothesis that DCs specifically strengthen the cellular immune response against mycobacterial infections [2, 3]. Even though the critical role of DCs in the initiation of immune response has been established [4], their involvement in *Mycobacterium tuberculosis* (MTB) infection is not completely characterized. Following pulmonary infection with MTB, DCs are activated rapidly to produce a specific pattern of chemokines and cytokines, key participants in the early immune response, and to express maturation markers that allow them to migrate to the draining lymph nodes. DCs become fully competent antigen presenting cells (APCs) and participate to the development of T helper 1 (Th-1) cells, required for the elimination of intracellular pathogens [4–6]. In particular, interleukin 12 (IL-12) produced by activated DCs induces Th-1 cells that, in turn, release interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α). These cytokines activate intracellular microbicidal mechanism and initiate a protective granulomatous response [7]. The magnitude of host immune response depends, to a large extent, on the presence of costimulatory molecules and signaling lymphocytic activation molecules on DC surface, as well as local production of cytokines [8].

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is a widely used vaccine against tuberculosis (TB) but comparative genetic analyses of BCG around the globe have revealed that each vaccine currently in use has different traits [9]. For decades, a number of factors have been considered responsible for the variable efficacy of BCG, including the type of strains used. In general, different kinds of stimuli result in differently activated DCs that induce qualitatively different T cell responses. Recently, it has been described that DCs are able to discriminate between phylogenetically diverse pathogens. In fact, the analysis of the DCs responses to *E. coli* and *C. albicans* showed that a unique number of genes, were regulated by each pathogen [10, 11]. However, the downstream target genes induced in DCs by the different BCG strains have not yet been fully determined.

The importance of DCs in initiating an immune response against mycobacterial infections led us to investigate the activation induced on these APCs following stimulation with two widely employed and different BCG strains. The goal of the present study was to determine whether the strains divergences may influence their relative immunogenicity [12, 13], virulence [14, 15], and viability [16], factors that must be considered for the design and improvement of a vaccine against TB.

We also analyzed the DCs' response to the commonly used MTB virulent laboratory strain (H37Rv) and to an MTB clinical isolate (CMT97), previously reported to behave differently from H37Rv in human macrophages [17], in order to understand if the laboratory strain could be considered a real model of DCs-MTB interaction.

We aimed to understand whether the maturation reprogramming occurring on DCs, following infection with MTB H37Rv, MTB CMT97, BCG Aventis and BCG Japan, could be different as a consequence of the ability of DCs to discriminate between these mycobacterial strains.

We used oligonucleotide macroarrays to characterize DCs gene expression profile and we found that although all infecting mycobacteria induced a core response, a strain-specific program emerged. The data obtained showed that BCG Japan was more effective than both MTB strains at inducing the expression of TNF-α, a gene involved in inflammation, as well as CCR7, responsible for DCs migration to lymph nodes. Furthermore, MTB H37Rv displayed, as compared to BCG Japan, an improved induction of EBI3, a IL-12p40-related polypeptide of IL-27 that may play a role in regulating cell-mediated immune response [18, 19], but, on the other hand, it resulted to be the only mycobacterial strain unable to promote a statistically significant IL-10 release as compared to uninfected cells. Moreover, IL-12 was significantly released only upon BCG Aventis infection. Finally, we also observed that BCG Japan displayed the lowest rate of intracellular replication as compared to BCG Aventis and both MTB strains.

2. Materials and Methods

2.1. Mycobacteria. Two pathogenic strains of MTB were chosen to infect DCs: the laboratory H37Rv strain (ATCC N° 27294) and the clinical isolate CMT97. CMT97 was isolated at the Monaldi Hospital, Naples, Italy, from a TB patient's sputum [20]. Both strains were transferred every two months to Sauton's medium, allowing them to grow as a layer on the medium surface. Mycobacterial layers were harvested every two months, spun down, and resuspended in phosphate-buffer saline (PBS). To get an homogeneous suspension, mycobacteria were placed in glass tubes and sonicated in a bath sonicator (UST; 20 kHz) at the maximum power of 50 W. Samples were than aliquoted and stored at −80°C. To titrate mycobacteria, few aliquots were thawed and grown on 7H10 Middlebrook plates (Becton Dickinson, Franklin Lakes, N.J.). The same frozen master batch was used for each infection experiment. As regards to BCG strains employed, we chose BCG Aventis Pasteur seed Merieux (BCG Aventis), derived from strain 1077 (Aventis Pasteur SA 2 Avenue Pont Pasteur, F-69007 Lyon), and 3-1-5, Japan BCG Laboratory Matsuyama, Kiyose-shi, Tokyo (BCG Japan). Both of them were supplied freeze-dried and intended for live inoculation. They were reconstituted according to the manufacturer's instructions.

2.2. Generation and Infection of DCs. Peripheral blood mononuclear cells (PBMC) were isolated, by Lympholyte-H (Cederlane, Canada) gradient centrifugation, from peripheral blood of healthy donors drawn from healthy volunteers. Monocytes were selected by anti-CD14-coated magnetic beads (MACS, Milteny Biothec, Germany). The purity was >98%, as verified by flow cytometry analysis. Cells were plated at 1.5×10^6 cell/mL in RPMI-1640 medium (EuroClone, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, UT), L-Glutammin 2 mM, HEPES buffer 10 mM, sodium piruvate 1%, gentamicin 5 μg/mL (all EuroClone, UK) adding GM-CSF 50 ng/mL and IL-4 10 ng/mL (all EuroClone, UK) to allow them to differentiate into DCs. After 5 days of differentiation, immature DCs derived from the same donor were exposed to MTB H37Rv, MTB CMT97, BCG Aventis Pasteur, and BCG Japan at a multiplicity of infection (MOI) of 1 bacterium per cell or left uninfected. Before infection bacilli were sonicated to disrupt small aggregates of bacteria. After 3 h of incubation at 37°C all DCs (both infected and uninfected ones) were harvested and centrifuged at 800 rpm for 10 min to spin down the DCs and leave extracellular bacteria in the supernatant. DCs were resuspended in fresh complete and, at the indicated time points, cells were collected and supernatants were stored at −80°C. For all the infections we chose a MOI of 1 since this condition did not result in rapid cell death and allowed us to culture DCs for at least 7 days after infection.

2.3. Flow Cytometry and Monoclonal Antibodies. The following monoclonal antibodies (mAbs), directly fluorochrome conjugated, were used for flow-activated cell sorting (FACS) analysis: anti-CD1a, anti-CD25, anti-CD80, anti-CD86, anti-CD83, anti-HLA-DR, anti-CD14, anti-CD11c, and anti-HLA-A,B,C. Negative controls were isotype-matched mAb (all from Becton Dickinson Biosciences). To determine surface cell phenotype, 24 h after infection, uninfected and infected DCs were washed in assay buffer (PBS, 0.5% BSA

and 0.1% sodium azide), incubated with the above described mAbs for 15 min at +4°C, washed and then analyzed by flow cytometry.

2.4. Mycobacterial Enumeration by CFU Determination. Following infection, cells were washed and resuspended in fresh complete medium. At the indicated time points infected DCs were incubated for 30 min with 500 μL of lysis buffer (PBS, 0.1% Saponin), diluted in PBS, 0.01% Tween 80, sonicated and plated as 50 μL droplet on 7H10 plates in triplicates, at different dilutions. The CFU were checked after 21 days of dish culture at +37°C in a 5% CO_2 incubator.

2.5. cDNA Arrays. Total RNA was extracted from both the uninfected DCs and the infected cells, 24 h after infection. RNA was extracted with 4 M Guanidine iso-thio-cyanate single-step method [21]. The extraction was performed on an RNase-free bench, in a separate room. Absorption spectroscopy was used to measure the purity and concentration of RNA with an $A_{260/280}$ ratio of 2.0 indicating highly purified RNA. A total RNA sample (1.5 μg) was reverse transcribed using the Ampolabellig Kit (Superarray Bioscience Corporation) according to the manufacturer's instruction in the presence of [α-^{33}] P for the generation of radio-labeled cDNA probes. The probes were used to hybridize human Dendritic and Antigen Presenting Cell Gene Arrays (GEArray, S series; Supearray Bioscience Corporation) according to the manufacturer's instruction. Hybridization signals were detected by Phosphor-imager Thyphoon (Molecular Dynamics) and analyzed by Array Vision 7.0 software (Imaging Research Inc., Canada). Pathogen stimulations were repeated in three donors. Our data were expressed as normalized density (nDens) of each spot, corresponding to the density value of the spot minus background density and expressed as a multiple of the reference density value. The threshold of 0.001 was attributed to any value ≤0.001. We considered upregulated (+) and downregulated genes (−) only those ones that showed at least a twofold change in the level of RNA expression of the infected *versus* uninfected DCs in two of three independent experiments (fold change ≥2). Differences in the expression were calculated by dividing the gene nDens of infected cells by uninfected cells nDens.

2.6. Quantitative Real-Time Reverse Transcriptase-PCR (q-rt RT-PCR). One μg of total RNA, treated with DNAse I Amplification Grade (Invitrogen, Paisley, UK) was reverse transcribed using random examers and SuperScript III Reverse Transcriptase (Invitrogen, Paisley, UK), according to the manufacturer's instruction. For quantification of PCR products ABI PRISM 7000 SDS was used (Applied Biosystem, Foster City, USA). The RealMasterMix SYBR ROX (Eppendorf AG, Germany) was used to produce fluorescently labeled PCR products, and we monitored increasing fluorescence during repetitive cycling of the amplification reaction. For all primers, the following temperature cycling profile was used: 2 min a +50°C and 10 min at +95°C followed by 30 sec at Ta, and 1 min at +68°C for 40 cycles. Primer sequences are reported in Table 1. L34 were used as an internal control to normalize total RNA amounts.

2.7. Cytokine Determination by ELISA. DCs supernatants were collected at the end of the 24 h of culture and stored at −80°C. The amount of IL-10, IL-12 and TNF-α levels was evaluated by ELISA (Pierce Endogen, Woburn, MA) according to manufacturer's instruction.

2.8. Graphical, Statistical and Cytofluorimetric Analysis. The cytometric analysis was performed on a FACScalibur flow cytometer (BD Biosciences) and data were analyzed using CellQuest software (BD Biosciences). GraphPad Prism 4 (Graphpad software, San Diego, CA) was used for graphical and statistical analysis. Statistical significance was assessed by using analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test; differences were considered significant at $P < .05$.

3. Results

3.1. Phenotype Analysis of Monocyte-Derived DCs Infected with Different Mycobacteria. In order to analyze DCs activation following infection with different mycobacterial strains, we evaluated the ability of MTB H37Rv, MTB CMT97, BCG Aventis and BCG Japan to induce the expression of maturation markers on DCs membrane. MTB H37Rv and MTB CMT97 were selected as both pathogenic: the first is the commonly used laboratory strain of MTB, while the second is a clinical isolate from a TB patient's sputum, displaying a peculiar clinical picture [20] and a specific ability to activate human mcrophages in comparison with MTB H37Rv [17]. We also chose two commonly employed vaccine strains: BCG Aventis and BCG Japan. DCs derived from the same donor were exposed to the different mycobacterial strains for 3 hours at a MOI of 1 bacteria per cell or left untreated, and, after 24 hours, infected and uninfected DCs were harvested, washed, and analyzed by flow cytometry for typical marker profiles. As shown in the representative histogram plots of Figure 1, MTB H37Rv and MTB CMT97 as well as BCG Aventis and BCG Japan were all efficient in stimulating DCs to undergo maturation when compared to uninfected DCs. This was assessed by the upregulation of activation markers such as HLA-ABC, HLA-DR, CD80, CD86, CD83, and CD25. The results obtained revealed that all the selected mycobacteria were found to induce phenotype maturation of DCs with a comparable efficacy.

3.2. Cell Vitality of DCs Following Stimulation with Different Mycobacterial Strains. We further examined whether cell vitality, could be affected when comparing DC cells infection with the two pathogenic MTBs and the BCG vaccine strains. The viability of the DCs was evaluated, at different time points, in terms of percentage of living cells. In Figure 2 we showed that the cell yield, 1-day-culture after mycobacterial exposure, appeared diminished as an effect of the infection and that the reduction was comparable in all the infections. Recovery of infected DCs remained almost stable for the following 4 days, when alive cells started to decrease with a comparable trend. On the contrary, the number of alive, uninfected DCs remained almost stable for 1 day, then, as expected, unstimulated immature DCs showed a reduction

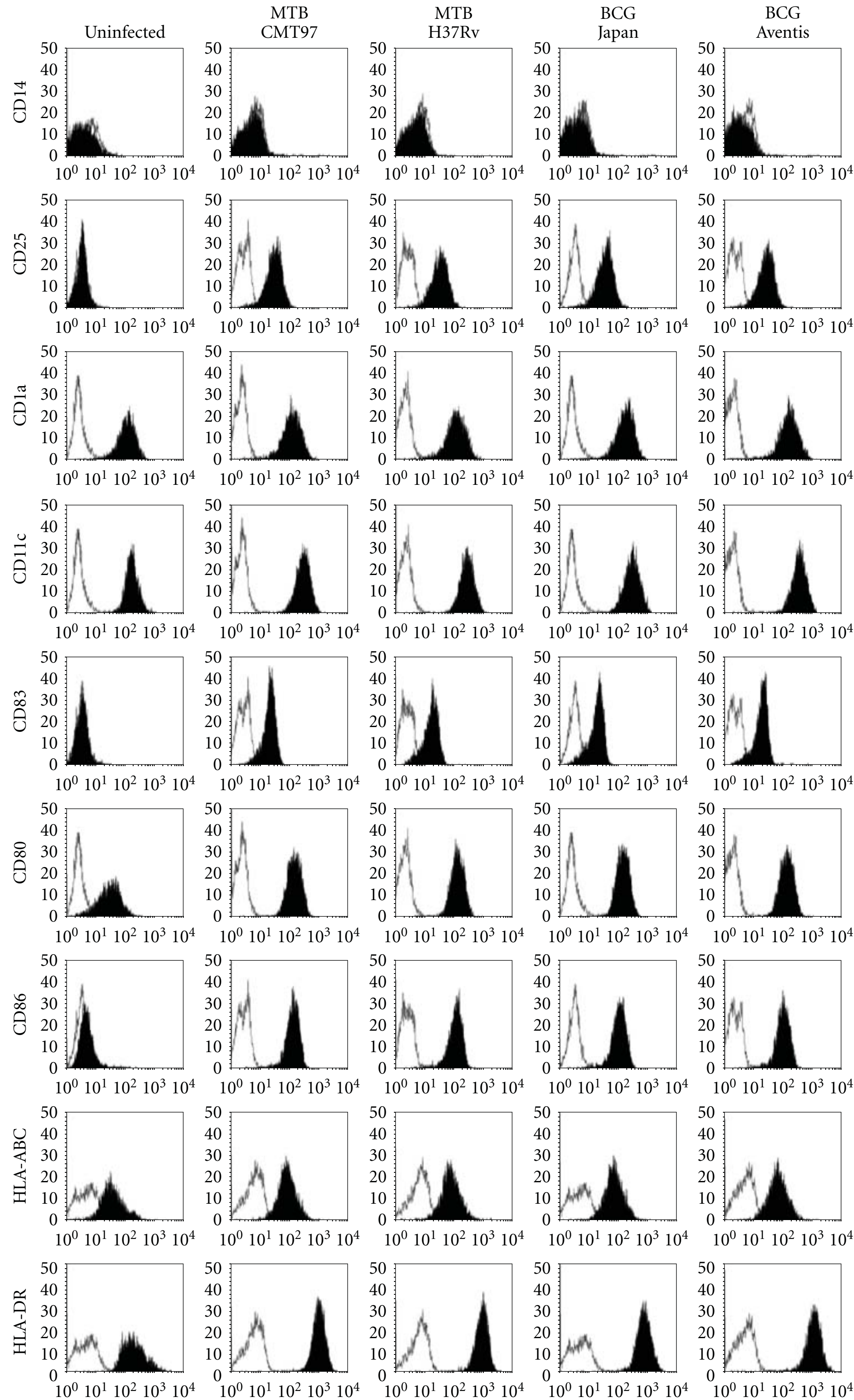

Figure 1: *Flow cytometry analysis of phenotype markers on DCs infected with different mycobacterial strains.* DCs, obtained from the same donor, were exposed to MTB H37Rv, MTB CMT97, BCG Aventis and BCG Japan for 3 h (MOI, 1) or left uninfected. Flow cytometric analysis of typical membrane molecule expression was performed 24 h after exposition to mycobacteria. Fluorescence histograms for each surface molecule (filled histograms) in comparison with isotype controls (empty histograms) are reported. Data are from a single donor, representative of 7, all with similar results.

Table 1: Primer sequences used in the q-rt RT-PCR.

Gene	Sense	Antisense	Annealing temp. (°C)
L34	5′-GGCCCTGCTGCATGTTTCTT-3′	5′-GTCCCGAACCCCTGGTAATAGA-3′	64
EBI3	5′-AGAGCACATCATCAAGCCCGAC-3′	5′-TCCCTGACGCTTGTAAGCGCATC-3′	64
CCR7	5′-AAAAGCGTGCTGGTGGTGGC-3′	5′-ATGATAGGGAGGAACCAGGC-3′	64
TNF-α	5′-AGGCGGTGCTTGTTCCTCA-3′	5′-GTTCGAGAAGATGATCTGACTGCC-3′	60
CCL19	5′-CCAATGATGCTGAAGACTGC-3′	5′-CTGGATGATGCGTTCTACCC-3′	62
CCL20	5′-TGAAGGCTGTGACATCAATGC-3′	5′-TGTTTTGGATTTGCGCACAC-3′	60
IL-12	5′-GCTGCTGAGGAGAGTCGTCCC-3′	5′-CCAGCTGACCTCGACCTGCC-3′	62
IL-10	5′-AGGCGCATGTGAACTCCCT-3′	5′-CACGGTCTTGCTCTTGTTTT-3′	64
IFN-γ	5′-GGCTGTTACTGCCAGGACCCCATATGT-3′	5′-GATGCTCTTGCACCTCGAAACAGCCAT-3′	64
IL-32	5′-GACATGAAGAAGCTGAAGGCC-3′	5′-ATCTGTTGCCTCGGCACCG-3′	62
IL-18	5′-GACAATTGCATCAACTTTGTGG-3′	5′-ATAGAGGCCGATTTCCTTGG-3′	62
c-FLIP	5′-TTCATGGGAGATTCATGCCC-3′	5′-AAGAGGCTGCTGTCCTCCA-3′	60
CD83	5′-AGGTTCCCTACACGGTCTCC-3′	5′-TTGAAGCTGGTAGTGTTTCG-3′	60

in viability as compared to all the infected DCs at 3, 5, and 7 days. Taken together, these data indicate that all the mycobacterial strains did not produce cell death differently in 7-day-culture after infection.

3.3. Mycobacterial Intracellular Growth. To ascertain whether bacterial burden could be comparable when DCs were infected with different BCG and MTB strains, we monitored the number of intracellular mycobacteria over 7-day culture following infection. For the laboratory strain MTB H37Rv and the clinical isolate MTB CMT97, we found an increase in mycobacterial counts, and a comparable rate of growth was assessed in DCs infected with BCG Aventis (Figure 3). Specifically, enumerated colony forming units (CFU) displayed a 4-fold increase after seven days of infection. On the contrary, the intracellular ability to replicate turned out to be greatly impaired when counting the intracellular BCG Japan CFU. In fact, the mycobacterial number remained almost steady over all the monitored period and resulted statistically reduced at the 7th day of infection, as compared to the other three mycobacteria. These results suggest that DCs differently contain the intracellular growth of the mycobacteria strains analyzed.

3.4. Gene Expression Profile of DCs Infected with Different MTB and BCG Strains. To test whether the mycobacterial strains we selected could differently modulate the overall gene expression of DCs, we analyzed the expression of 165 genes involved in DC activation and maturation using macroarrays. We analyzed and compared the expression profile of DCs exposed for 3 h to MTB H37Rv, MTB CMT97, BCG Aventis and BCG Japan, 1 day-culture after the infection. The gene modulation, showed in Table 2, was confirmed in at least two of three independent assays, where each assay was performed on DCs derived from the same donor. In general, 30 genes were detected as upregulated (+) (fold change ≥2 in infected DCs *versus* uninfected DCs), corresponding to 18% of the 165 spotted onto the membrane (Table 2(a)), and 33 genes out of 165 (20%) were detected as downregulated (−) (Table 2(b)). In more detail, only 17 out of 30 genes proved to be comparably upregulated in all infected DCs while the other 13 genes resulted induced upon the encounter of DCs with some of the four mycobacteria. Analogous results were obtained when analyzing the downregulated genes. In particular, despite the fact that all the different mycobacteria decreased the expression of 25 gene, other 8 genes resulted repressed only following some of the infection performed. As expected, among the commonly upregulated genes we found cytokines involved in proinflammatory immune response such as IL-1β, IL-6, IL-12 and TNF-α, the regulatory IL-10 and chemokines and their receptors able to trigger DCs migration to lymph nodes (ADAM19, CCR7 and CCL20). On the other hand, we found, as commonly downregulated, genes that are substantially involved in antigen capture, loading and presentation as DC-SIGN, that is known to be responsible for DCs-T interaction. In this group we also found IL-18, which plays an important role in enhancing IFN-γ production by T cells [22], which proved to be preferentially downregulated in BCG Japan-infected DCs. In order to exactly appreciate gene expression and modulation we showed, in Table 3, nDens of all genes differentially regulated, following mycobacteria exposure. Moreover, we indicated nDens of 2 genes induced and 2 genes repressed in all infection performed and nDens of all genes discussed above (IL-1β, IL-6, IL-12, TNF-α, IL-10, ADAM19, CCR7, CCL20 and IL-18). In conclusion, the macroarray analysis suggests that different mycobacterial strains such as MTB H37Rv, MTB CMT97, BCG Aventis and BCG Japan induce, on DCs, a pathogen-specific response, both in upregulated and downregulated genes, in the face of a common set of gene that appear to be similarly modulated.

3.5. Expression of Selected Genes by q-rt RT-PCR. The mRNA levels of 12 selected genes were further analyzed by quantitative real-time PCR using gene specific primers (Figure 4). Six of these genes (CD83, CCL20, CCR7, IL-10, IL-12, and TNF-α) proved to be similarly upregulated in response to all the four mycobacterial strains but, due to their

Table 2: (a) Upregulated genes of DCs infected with different MTB and BCG strains. (b) Downregulated genes of DCs infected with different MTB and BCG strains.

(a)

Gene	MTB H37Rv	MTB CMT97	BCG Aventis	BCG Japan	Common name	Classification	Function
ADAM19	+	+	+	+		Metalloproteinase	Migration/Inflammation
BASP1	+	+	+	+	CAP-23, NAP-2	Cell surface protein	Prot-prot interaction
CCL5	+	+	+	+	RANTES	Chemokine ligand	Migration
CCL20	+	+	+	+	MIP-3α	Chemokine ligand	Inflammation
CCL22	+	+	+	+	MDC	Chemokine ligand	Inflammation
CCR7	+	+	+	+		Chemokine receptor	Migration
CD83	+	+	+	+		Cell surface protein	Ag presentation
IL-1β	+	+	+	+		Cytokine	Inflammation
IL-6	+	+	+	+		Cytokine	Inflammation
IL-10	+	+	+	+		Cytokine	Immune regulation
IL-12p40	+	+	+	+		Cytokine	T cell stimulation
LY75	+	+	+	+	DEC-205	Cell surface receptor	Ag presentation
NFKB1	+	+	+	+		Trascriptional factor	Signal transduction
SOD2	+	+	+	+		Mytocondrial protein	Oxidative stress
TAP2	+	+	+	+		Ag transporter 2	Ag presentation
TLR2	+	+	+	+		Toll-like receptor	Pathogen assoc. receptor
TNF-α	+	+	+	+		Cytokine	Inflammation
ADAR	+			+		Adenosine deaminasi	RNA modification
CCL3	+	+	+		MIP-1α	Chemokine ligand	Inflammation
CCL19			+	+		Cell surface receptor	Migration
CD80				+		Cell surface protein	Ag presentation
CD86		+	+	+		Cell surface protein	Ag presentation
CFLAR			+	+	C-Flip	Apoptosis regulator	Apoptosis inhibitor
CRF			+			C1q related factor	
EBI3	+	+				Secreted glicoprotein	Immune-regulation
IL-1a		+	+	+		Cytokine	Inflammation
LAMP3	+	+		+	CD63	Lysosomal associated protein	Ag capture
MT2A		+	+			Metallothionein	Oxidative stress
PLAUR		+	+		CD87	Cell surface receptor	Migration
TNFRSF6			+	+	CD95, FAS	Cell surface receptor	Maturation

(b)

Gene	MTB H37Rv	MTB CMT97	BCG Aventis	BCG Japan	Common name	Classification	Function
ARHGDIB	–	–	–	–		RhoGDP dissociation inhibitor 2	Cell motility and adhesion
CD1A	–	–	–	–		MHC I like protein	Ag presentation
CD1B	–	–	–	–		MHC I like protein	Ag presentation
CD1C	–	–	–	–		MHC I like protein	Ag presentation
CD36	–	–	–	–		Cell surface protein	Ag capture
CD68	–	–	–	–		Cell surface protein	Ag capture
CD74	–	–	–	–	Invariant chain	MHC II assoc. protein	Ag loading
CD209	–	–	–	–	DC-SIGN	C-type lectin receptor	T/DCs interaction
CLECSF12	–	–	–	–	DECTIN-1	Cell surface receptor	Pattern recog. receptor
CLECSF6	–	–	–	–	DCIR	C-type lectin receptor	Ag capture
CST3	–	–	–	–		Proteinase inhibitor	
CXCL16	–	–	–	–		Chemokine	T cell stimulation
FCER1A	–	–	–	–		Fc receptor	Inflammation
FCER2	–	–	–	–		Fc receptor	Inflammation
GIP3	–	–	–	–		IFN-α inducible protein	

(b) Continued.

Gene	MTB H37Rv	MTB CMT97	BCG Aventis	BCG Japan	Common name	Classification	Function
HLA-DMA	–	–	–	–		MHC II accessory protein	Ag loading
HLA-DMB	–	–	–	–		MHC II accessory protein	Ag loading
IFI16	–	–	–	–		IFN-γ inducible protein	Cell cycle regulator
IFITM3	–	–	–	–		IFN inducible protein	Immune response
ITGB2	–	–	–	–		Integrin	Migration
LANGERIN	–	–	–	–		Cell surface protein	Ag capture
LIPA	–	–	–	–	Lipase A	Acid lipase	Lipidic metabolism
MX1	–	–	–	–		IFN-α inducible p78	Antiviral response
RNASE6	–	–	–	–		Ribonuclease	Ag presentation
TLR4	–	–	–	–		Toll-like receptor	Pathogen assoc. receptor
CSF1R	–	–		–	CD115	Cell surface receptor	M-CSF receptor
DCP1B	–			–		Decapping enzyme hDcp1b	RNA degradation
DCSTAMP	–		–			Cell trasmemb. protein	
GBP3				–		Guanilate binding protein	
GIP2	–			–		IFN-α inducible protein	
IL-18				–		Cytokine	Inflammation
PFN1				–			
TLR6	–			–		Toll-like receptor	Pathogen assoc. receptor

Upregulated (Table 2(a)) and downregulated (Table 2(b)) genes of DCs exposed for 3 h to MTB H37Rv, MTB CMT97, BCG Aventis and BCG Japan as compared to uninfected DCs were assayed by macroarray 1-day culture after infections. For genes to be referred as upregulated or downregulated we considered only those that showed at least a twofold change in the level of RNA as compared to uninfected DCs expression, in at least 2 independent experiments of the 3 performed.

important role in the modulation of immune response, we further analyzed their expression in order to appreciate some possible quantitative difference. We also chose CCL19, c-FLIP (CFLAR), EBI3, and IL-18, which appeared differently modulated following the four infections, in order to verify such dissimilarity. For its well-documented pivotal role in MTB host response, we also decided to include in this analysis IFN-γ, even if it was present in the array and did not show any significant modulation in DCs following infection. We also included in q-rt RT-PCR analysis IL-32, absent in macroarray, since this has been recently shown to be involved in antitubercular immunity [23], Moreover, it has been described that mycobacterial species such as *M. tuberculosis* or *M. bovis* BCG are potent stimuli for the production of the proinflammatory cytokine IL-32 and its production is dependent on endogenous IFN-γ [24]. The result obtained confirmed the upregulation of CCL20, IL-10, IL-12, previously observed with the macroarray assay, in DCs exposed to the MTB as well as the BCG strains. Also the similar upregulation of CD83, already described at the protein level in Figure 1, was confirmed. Interestingly, the q-rt RT-PCR pointed out a significantly higher mRNA transcript of CCR7 and TNF-α in BCG Japan-infected-DCs, as compared to MTB H37Rv- and MTB CMT97-infected DCs ($P < .01$ and $P < .05$, resp.).

Concerning CCL19, c-FLIP and IL-18, whose expression resulted different in DCs after the encounter with the two MTB and two BCG strains (Table 2), when we reanalyzed the mRNA levels by quantitative real-time PCR, the overall expression of these genes was comparable among the different infections although a certain degree of variability was observed.

In agreement with macroarray analysis, EBI3 expression resulted clearly induced in MTB H37Rv- and MTB CMT97-infected DCs, but a lower RNA induction was observed also in BCG infected DCs. In addition, a statistical analysis of EBI3 modulation underlined a consistent upregulation of this gene only in MTB H37Rv infected-DCs as compared to BCG Japan infected-DCs. In line with the modulation of EBI3, the upregulation of IFN-γ and IL-32 resulted to be stronger in MTB H37Rv and MTB CMT97 infected-DCs but no statistically significant differences can be described.

Collectively, these findings, consistent with the macroarray data, allow us to describe a different modulation of the selected genes, thanks to the higher sensitivity of the quantitative real-time PCR as compared to the macroarray assay. Furthermore these data suggest that the maturation of DCs, following the infection with different MTB and BCG strains, may result in a different modulation of some genes importantly involved in the response against mycobacteria.

Table 3: (a) nDens of upregulated genes of DCs infected with different MTB and BCG strains. (b) nDens of downregulated genes of DCs infected with different MTB and BCG strains.

(a)

Gene	MTB H37Rv	MTB CMT97	BCG Aventis	BCG Japan	Uninfected DCs
ADAM19	0.3; 0.4; 1.5	0.3; 0.2; 1.8	0.2; 0.7; 0.1	0.3; 0.6; 1	0.2; 0.07; 0.5
CCL20	0.4; 1; 0.8	0.4; 2.5; 1.4	0.9; 2.3; 0.8	0.5; 0.8; 1.1	0.04; 0.001; 0.4
CCL22	8.5; 1.8; 11.1	6.9; 3.7; 12.4	4.3; 1.8; 12.1	8.1; 1.5; 9.8	0.3; 0.5; 0.2
CCR7	3.1; 0.001; 1.7	3.2, 0.9; 1.9	0.5; 1.8; 1.6	2.4; 0.9; 2.2	0.1; 0.2; 0.05
IL-1β	2.1; 2.9; 14.4	1.7; 10.5; 19.0	0.3; 6.5; 10.8	1.9; 4.8; 14	0.09; 0.9; 0.8
IL-6	0.5; 0.02; 2.9	0.1; 0.4; 3.4	0.001; 0.6; 2.3	0.1; 0.3; 3.2	0.01; 0.01; 0.1
IL-10	1.8; 3.7; 6.6	1.0; 1.2; 8.9	1.2; 1.3; 5.8	1.0; 0.9; 4	0.4; 0.3; 4.4
IL-12p40	0.6; 0.3; 0.9	0.6; 0.7; 0.7	0.7; 0.1; 1.0	0.4; 0.01; 1.5	0.1; 0.1; 0.004
SOD2	0.8; 1.6; 5.5	0.8; 3.4; 7.6	0.9; 1.5; 3.7	0.5; 1.5; 5.4	0.1; 0.7; 0.7
ADAR	0.4; 0.2; 0.2	0.2; 0.1; 0.1	0.3; 0.1; 0.1	0.4; 0.3; 0.2	0.2; 0.1; 0.1
CCL3	4.1; 7.0; 11.0	3.6; 6.5; 5.6	7.0; 4.6; 10.8	2.5; 3.7; 1.2	1.7; 3.4; 0.6
CCL19	0.1; 0.1; 0.001	0.3; 0.007; 0.001	0.2; 0.3; 0.4	0.2; 0.1; 0.5	0.001; 0.1; 0.001
CD80	3.3; 0.4; 0.2	3.2; 0.5; 0.2	3.9; 0.9; 0.2	5.8; 0.7; 0.9	2.1; 0.3; 0.2
CD86	0.3; 0.001; 0.1	0.2; 0.02; 0.4	0.1; 0.1; 0.4	0.2; 0.01; 0.4	0.05; 0.02; 0.2
CFLAR	0.4; 0.5; 0.3	0.5; 0.5; 0.3	1.1; 1.3; 0.2	0.8; 0.8; 0.4	0.5; 0.3; 0.2
CRF	0.3; 0.3; 0.001	0.3; 0.1; 0.01	1.1; 0.2; 0.001	0.1; 0.02; 0.001	0.3; 0.1; 0.001
EBI3	0.1; 0.2; 0.5	0.1; 0.001; 0.7	0.01; 0.001; 0.2	0.01; 0.001; 0.3	0.01; 0.001; 0.008
IL-1α	0.02; 0.01; 0.4	0.1; 0.6; 0.5	0.3; 0.6; 0.3	0.4; 0.3; 0.9	0.02; 0.01; 0.001
LAMP3	5.1; 24.0, 1.6	10.6; 31.3; 2.1	13.4; 12.9; 2.3	21.8; 26.0; 1.7	9.3; 11.8; 0.7
MT2A	0.2; 0.1; 11.1	0.4; 0.5; 11.5	0.4; 0.2; 7.1	0.08; 0.1; 8.4	0.2; 0.2; 0.1
PLAUR	0.06; 0.1; 1.5	0.1; 0.7; 0.7	0.2; 0.4; 1.2	0.06; 0.1; 1.2	0.1; 0.1; 0.1
TNFRSF6	0.6; 0.1; 0.001	0.7; 0.006; 0.004	1.6; 0.4; 0.001	1.4; 0.2; 0.001	0.6; 0.001; 0.001

(b)

Gene	MTB H37Rv	MTB CMT97	BCG Aventis	BCG Japan	Uninfected DCs
CD1A	0.1; 0.2; 0.001	0.1; 0.001; 0.02	0.06; 0.001; 0.05	0.2; 0.001; 0.001	5.2; 0.3; 1.1
CD1B	0.04; 0.4; 0.001	0.05; 0.001; 0.02	0.05; 0.001; 0.06	0.008; 0.001; 0.07	1.3; 0.001; 0.7
CD209	0.05, 0.1; 0.08	0.02; 0.03; 0.001	0.04; 0.001; 0.03	0.01; 0.2; 0.03	0.3; 0.1; 0.4
CSF1R	0.05; 0.4; 0.1	0.1; 0.5; 0.001	0.5; 0.2; 0.04	0.1; 0.1; 0.3	0.3; 0.2; 0.5
DCP1B	0.2; 0.001; 0.04	0.3; 0.001; 0.1	0.3; 0.1; 0.07	0.1; 0.07; 0.01	0.4; 0.1; 0.1
DCSTAMP	1.3; 0.001; 0.001	1.6; 0.001; 0.3	0.2; 0.05; 0.02	2.1; 0.01; 0.001	3.0; 0.1; 0.05
GBP3	0.3; 0.4; 0.1	0.08; 0.5; 0.03	0.1; 0.8; 0.05	0.1; 0.3; 0.04	0.1; 0.7; 0.1
GIP2	0.05; 0.001; 0.9	0.08; 0.3; 0.6	0.07; 0.4; 0.4	0.001; 0.1; 0.7	0.3; 0.3; 0.7
IL-18	0.1; 1.9; 0.2	0.1; 1.6; 0.2	1.1; 1.8; 0.2	0.1; 0.6; 0.2	0.2; 2.7; 0.2
PFN1	0.9; 0.5; 3.1	0.6; 0.4; 5.0	0.7; 0.6; 2.3	0.7; 0.2; 3.5	0.8; 0.5; 8.5
TLR6	0.05; 0.09; 0.001	0.08; 0.3; 0.03	0.1; 0.3; 0.001	0.001; 0.1; 0.001	1.4; 0.3; 0.02

RNA expression of infected and uninfected DCs of three independent experiments. Values are reported as nDens.

3.6. Release of IL-10, IL-12 and TNF-α from Infected and Uninfected DCs. DCs have the unique capacity to stimulate naïve T lymphocytes driving these cells into a distinct class of effector cells. To investigate whether the infection with different mycobacterial strains could result in a different cytokine release from DCs, cell supernatants were collected 24 h after infection and the presence of IL-10, IL-12 and TNF-α was quantified by ELISA. We chose IL-10 and IL-12 since they are crucial for driving Th-1/Th-2 response and TNF-α, that already proved differently modulate at the RNA level, for its well-documented protective role against MTB and for its ability to mature DCs [25, 26]. As shown in Figure 5, a significantly higher release of IL-10 was observed from both BCG-infected and MTB CMT-infected DCs as compared to uninfected cells even though DCs exposed to MTB H37Rv also release a discrete amount of this regulatory cytokine.

Concerning IL-12, a consistent and significant production was found only in BCG Aventis-infected DCs while, in the other 3 infections, only a mild increase in the level of IL-12 was observed as compared to control DCs. Finally, the TNF-α released and accumulated in the supernatant for 24 h after infection resulted high and similar in all the infections, as compared to uninfected DCs ($P < .001$).

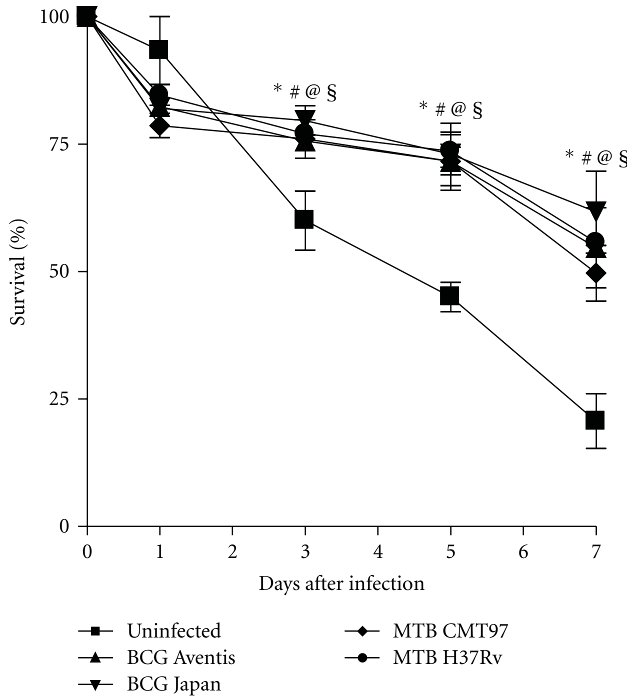

Figure 2: *Cell recovery of DCs following infection with different mycobacterial strains.* DCs infected or uninfected were enumerated by trypan blue at the indicated time points. Time 0 is referred to DCs before infection; following time points indicate days after infection. Data are shown as mean ± SEM from 3 independent experiments. Statistical significance was assessed by using two-way ANOVA. (Significant differences at 3 days after infection: *MTB H37Rv *versus* uninfected, $P < .05$; #MTB CMT97 *versus* uninfected, $P < .05$; @BCG Aventis *versus* uninfected, $P < .05$; §BCG Aventis *versus* uninfected, $P < .05$. significant differences at 5 and 7 days after infection: *$P < .001$; #$P < .001$; @$P < .001$; §$P < .001$.)

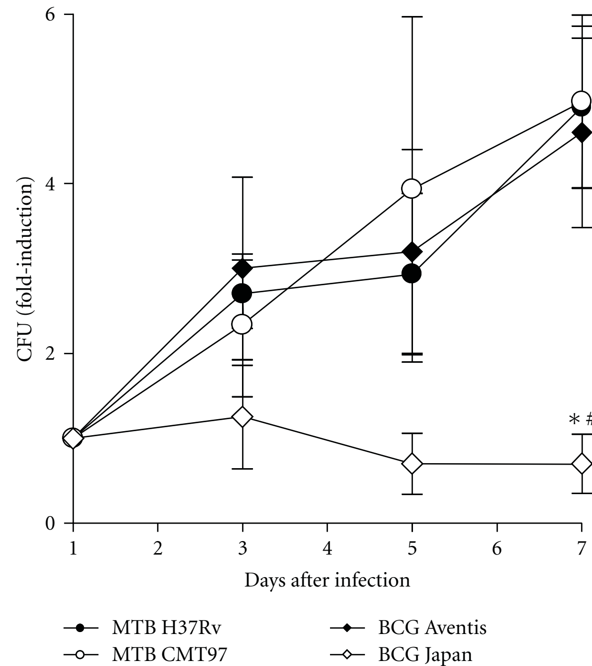

Figure 3: *Bacterial loads in DC infected with MTB H37Rv, MTB CMT97, BCG Aventis and BCG Japan.* DCs obtained from the same donor were exposed to different mycobacteria (MOI, 1). After 3 h, cells were washed then, part of them was checked for intracellular mycobacteria while the rest were resuspended in fresh medium and left in culture. Total bacterial load was assessed over the first 7 days and it is indicated as fold induction compared to the intracellular bacteria after 1 day from infection. The BCG Japan CFU were found significantly different at day 7 as compared to MTB H37Rv (*$P < .01$), MTB CMT97 (*$P < .01$) and BCG Aventis (#$P < .05$). Data shown as mean ± SEM from 3 independent experiments performed in triplicates. Statistical significance was assessed by using two-way ANOVA.

4. Discussion

MTB is an extremely well adapted pathogen that coexisted with the human host for thousands of years and during this period it has learnt how to modulate potentially protective host responses, to ensure its own survival. H37Rv is the currently used MTB laboratory strain and, considering how important it is to have a good *in vitro* model, it could be necessary to assess if H37Rv is able to induce a host response at least comparable to a clinical isolate. In fact, it has been clearly shown that different MTB clinical isolates have distinct effects and produce a different response that depends on their specific virulence [27, 28]. We decided to include in our study the strain CMT97, a clinical isolate previously characterized in infected cells of bronchial lavage fluid and in human infected macrophages [17, 20].

In parallel, BCG has emerged as a vaccine that changed from the original Pasteur strain into a range of strains that immunized many people around the world, with variable results. In this context, a characterization of a strain-specific host response and in particular a critical analysis and comparison between two widely used BCG strains could lead to a more rational approach towards the improvement of the BCG vaccine. In the present study, we investigated the DCs response to mycobacterial infections and we chose two different MTB and BCG strains: the laboratory strain H37Rv, CMT79, a clinical isolate from a TB patient, and two vaccine frequently used nowadays (BCG Aventis and BCG Japan). Several studies have been published dealing with the DCs response to different mycobacteria [10, 27, 28] but this is the first time that two different BCGs, an MTB clinical isolate and a laboratory MTB strain are compared simultaneously for the activation induced on human monocyte-derived DCs coming from the same donor.

First, we compared the selected mycobacteria for their ability to replicate inside DCs and, interestingly, it emerged that only BCG Japan was unable to enlarge the intracellular bacterial population in infected DCs. This suggests, in accordance with *in vivo* previous studies [29], that during the initial phase of infection with MTB H37Rv, MTB CMT97 and BCG Aventis, the growth of the bacterial population could be accompanied by an increment of the number of infected cells, which implies a high rate of cell-to-cell spread of the mycobacteria, while the cell-to-cell spread outcome

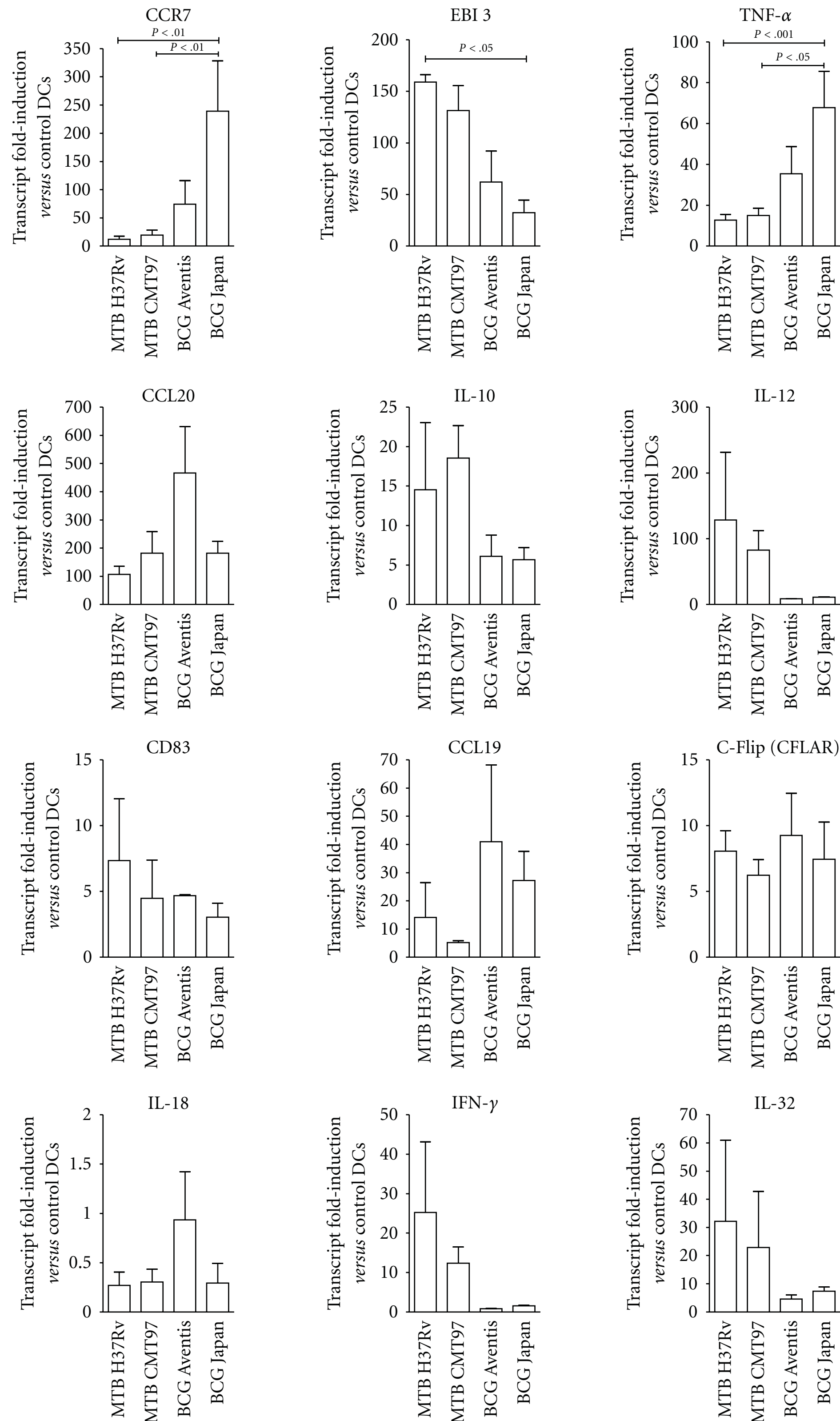

Figure 4: *Analysis of RNA modulation by q-rt RT-PCR*. Total RNA from DCs exposed for 3 h to MTB H37Rv, MTB CMT97, BCG Aventis and BCG Japan was assayed for gene expression by q-rt RT-PCR, 1-day culture after the infections. Gene expression was normalized to L34 and fold change was calculated with respect to uninfected DCs. Data are from 3 individual experiments and are expressed as mean ± SEM. The presence of significant differences in gene expression between DCs was calculated by a one-way ANOVA.

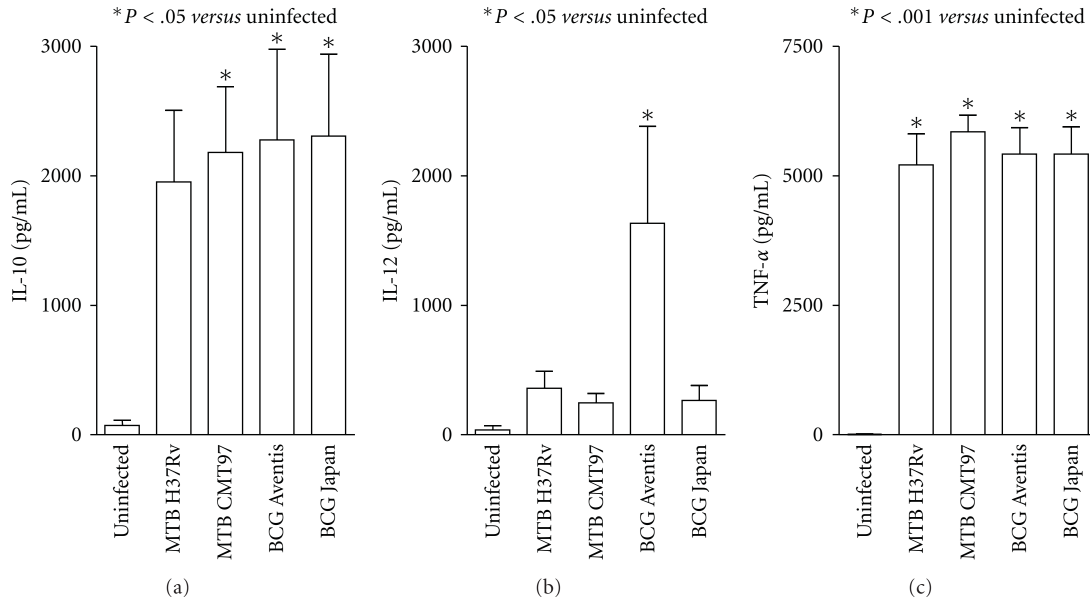

Figure 5: *Evaluation of IL-10, IL-12 and* TNF-α *released from infected DCs.* Cell supernatants derived from DCs uninfected or exposed to different mycobacteria strains as described before were collected at the end of the 24 h of culture. IL-10, IL-12 and TNF-α were measured by ELISA. Data are represented as mean ± SEM from 3 independent experiments. The presence of significant differences in the amount of cytokine released in the supernatant was calculated by a one-way ANOVA.

following the infection with BCG Japan may possibly occur to a lesser extent. Of note, although DCs contained differently the intracellular growth of BCG Japan compared to the other three mycobacteria, cell expression of activation markers as well as cell recovery proved to be comparable unrelated to the infecting mycobacteria. This supports the fact that DCs differently contain the intracellular growth of the mycobacteria considered, while cells similarly survive after all the infections performed.

It's important to keep in mind that, since 1921, the *in vitro* attenuation of *M. bovis* gave rise to a large number of BCG daughter strains, which have been classified as "early strains" and "later strains". The BCG Japan is an "early strain" while BCG Aventis Pasteur belongs to the "later strains", obtained before the loss of the RD14 region [9, 30]. Considering this, it is not surprising that different BCG strains could have such a different adaptive feature and that the differences existing between the BCG daughter strains may influence the activation of infected DCs. To explore globally the host gene expression differences we performed a DCs comparative *in vitro* transcriptome analysis across the two MTB and two BCG strains. The analysis of 165 genes involved in cell maturation and activation showed that 18% of these proved to be upregulated and 20% were detected as downregulated. More in detail, 17 out of 30 genes resulted to be comparably upregulated in all infected DCs and resulted to be involved in proinflammatory immune response (Il-1β, IL-6, IL-12 and TNF-α), immune regulation and migration (IL-10, ADAM19, CCR7 and CCL20) showing that all mycobacteria were able to elicit the expression of many genes already described as upregulated in common pathogen response [10]. The other 13 genes resulted differently induced and among these we chose CCL-19, C-Flip and EBI3 to be further investigated by q-rt RT-PCR.

Accordingly with the macroarray analysis, EBI3 resulted preferentially induced in the DCs infected with MTB virulent species than BCG a-virulent strains. Interestingly, when comparing the gene-fold induction across the four mycobacteria, H37Rv displayed a significantly higher EBI3 transcription as compared to BCG Japan. EBI3 is a subunit of IL-27 that, together with IL-12, is involved in driving commitment of naïve T cells to a Th-1 phenotype [31, 32]. Intriguingly, it has been recently published that neutralization of IL-27 reduced, even if modestly, viable MTB recovered from macrophages [33]. In this scenario, the lower EBI3 induction in BCG Japan-infected DCs might be involved in BCG Japan failing to grow exponentially in DCs.

The comparison, by q-rt RT-PCR, of gene-fold induction across the four mycobacteria also revealed that the proinflammatory TNF-α and the migratory chemokine receptor CCR7 were strongly induced in BCG Japan-infected DCs as compared to cells infected with both MTB strains. This suggests that BCG Japan might prove particularly efficient at promoting DCs migration into T cell-enriched areas of lymphoid tissue, where DCs are able to present Ag-derived peptides, associated with either class I or class II MHC molecules to naïve CD4 and CD8 T cells, respectively [34]. Moreover, previous studies have shown that TNF-α is fundamental in granuloma formation and maintenance and also affects cell migration upon MTB infection, since it influences the expression of adhesion molecules as well as chemokines and chemokine receptors such as MIP-1α, MIP-1β, RANTES, and CCR5 [35–38]. When we assayed

the release of TNF-α from supernatants, we observed that this proinflammatory cytokine was found to be abundantly released in all infected DCs. This result strongly supports the evidence that initial interaction of MTB and BCG induces proinflammatory cytokine production [27, 39].

As previously shown [40], we observed a consistent release of IL-10 from BCG-infected DCs even if these cells resulted to be well activated following mycobacteria exposure, as assessed by the upregulation of maturation markers. Also, MTB CMT97-infected DCs produced significantly higher levels of IL-10 as compared to uninfected cells while, MTB H37Rv induced the release of a discrete but not significantly amount of this cytokine. When we measured the release in the supernatant of IL-12, a cytokine pivotal in directing the polarization of immune response, we observed that despite the fact that a RNA upregulation was described in all MTB and BCG infections, only BCG Aventis induced a significant IL-12 production, while BCG Japan and both MTB strains turned out to be a stimulus insufficient for the induction of IL-12 releasing DCs. The data obtained, at a protein level, confirm the previously reported ability of MTB H37Rv and different MTB clinical isolates to suppress the secretion of IL-12 by monocyte-derived DCs [27] and, also suggest another possible difference in the immune activation induced by the two different BCG analysed.

Of note, even if there is an evident discrepancy between RNA analysis and protein detection from supernatants, it should be remembered that several experimental studies have shown that mRNA changes do not necessarily correlate with changes in the corresponding proteins, which are the ultimate determinants of cellular function [41]. The invalid assumption of the one-to-one correlation between the ratios of protein levels and the corresponding mRNAs [42, 43] suggests that the relation between transcription and translation, and consequently between mRNA and protein, is complex. Moreover, qRT-PCR shows what is happening in a particular moment, while the ELISA assay we performed gives us the total amount of protein, released in the course of 24 hours following infection.

In conclusion, the importance of DCs in initiating immune response gave reason to investigate if these cells could discriminate between a clinical and a laboratory MTB strain and also if DCs could sense differently two distinct BCG strains. The analysis of the individual response showed that DCs exhibit stimulus-specific maturation and activation. In fact, besides a shared core reprogramming, DCs are able to modulate the expression of exclusive genes, proving that these immune regulating cells are able not only to discriminate between phylogenetically distinct pathogens [10] but also, elicit a specific response as respect to diverse MTB and BCG strains. The results obtained describe the contribution to pathogen-host interaction of strictly correlated mycobacterial strains. In particular, our data indicate that the specific differential response to MTB H37Rv and MTB CMT97 mimics the previously reported different cellular response to distinct clinical MTB isolate, dependently to their specific virulence [27, 28] supporting the validity of MTB H37Rv as an *in vitro* model for mycobacterial-DCs interaction. On the other side, the strain-specific modulation observed in response to BCG Aventis and BCG Japan as well as the different ability of these mycobacteria to growth in infected DCs lead to important physiological consequence that must be considered and further studies of BCG-reg ulated genes may thus enhance our understanding of DCs maturation and provide future indication for the design and improvement of a vaccine against tuberculosis.

Acknowledgments

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Review Article

Pathogenesis, Immunology, and Diagnosis of Latent *Mycobacterium tuberculosis* Infection

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Phagocytosis of tubercle bacilli by antigen-presenting cells in human lung alveoli initiates a complex infection process by *Mycobacterium tuberculosis* and a potentially protective immune response by the host. *M. tuberculosis* has devoted a large part of its genome towards functions that allow it to successfully establish latent or progressive infection in the majority of infected individuals. The failure of immune-mediated clearance is due to multiple strategies adopted by *M. tuberculosis* that blunt the microbicidal mechanisms of infected immune cells and formation of distinct granulomatous lesions that differ in their ability to support or suppress the persistence of viable *M. tuberculosis*. In this paper, current understanding of various immune processes that lead to the establishment of latent *M. tuberculosis* infection, bacterial spreading, persistence, reactivation, and waning or elimination of latent infection as well as new diagnostic approaches being used for identification of latently infected individuals for possible control of tuberculosis epidemic are described.

1. Introduction

Tuberculosis (TB) has afflicted mankind from the time immemorial. Evidence of spinal disease has been found in Egyptian mummies of several thousand years BC and references to TB are found in ancient Babylonian and Chinese writings. Recent molecular genetic studies have shown that *Mycobacterium tuberculosis*, the most common cause of TB in humans worldwide, has a progenitor ~3 million years old [1]. *Mycobacterium tuberculosis* is a member of the *M. tuberculosis* complex (MTBC) which includes six other closely related species: *M. bovis, M. africanum, M. microti, M. pinnipedii, M. caprae,* and *M. canetti*. Although all MTBC members are obligate pathogens and cause TB, they exhibit distinct phenotypic properties and host range. The MTBC members are genetically extremely closely related, the genome of *M. tuberculosis* shows <0.05% difference with *M. bovis,* the latter species primarily infects cattle but can also cause TB in other mammals including humans [2, 3].

Tuberculosis is one of the most prevalent infections of human beings and a formidable public health challenge that shows little sign of abating. The disease contributes considerably to illness and death around the world, exacting a heavy toll on the world's most vulnerable citizens. The current TB epidemic is being sustained and fuelled by two important factors: the human immunodeficiency virus (HIV) infection and its association with active TB disease and increasing resistance of *Mycobacterium tuberculosis* strains to the most effective (first-line) anti-TB drugs [4]. Other contributing factors include population expansion, poor case detection and cure rates in impoverished countries, active transmission in overcrowded hospitals, prisons, and other public places, migration of individuals from high-incidence countries due to wars or famine, drug abuse, social decay, and homelessness. Active disease patients with sputum smear-positive pulmonary TB are the main source of infection in a community. Primary infection with *M. tuberculosis* leads to clinical disease in only ~10% of individuals. In the remaining cases, the ensuing immune response arrests further growth of *M. tuberculosis*. However, the pathogen is completely eradicated in only ~10% people, while the immune response in the remaining ~90% individuals only succeeds in containment of infection as some bacilli escape killing by blunting the microbicidal mechanisms of immune cells (such as phagosome-lysosome fusion, antigen presentation by MHC class I, class II, and CD1 molecules, production of nitric oxide, and other reactive nitrogen intermediates) and remain in nonreplicating (dormant or latent) state in old lesions.

The process is termed as latent tuberculosis infection (LTBI), and the dormant bacilli retain the ability to resuscitate and to cause active TB if a disruption of immune response (as in HIV infection) occurs. The World Health Organization (WHO) has estimated that one-third of the total world population is latently infected with *M. tuberculosis* and 5%–10% of the infected individuals will develop active TB disease during their life time [4, 5]. However, the risk of developing active disease is 5%–15% every year and lifetime risk is ~50% in HIV coinfected individuals [4, 6]. Most of the active disease cases in low TB incidence countries arise from this pool of latently infected individuals.

According to WHO estimates, 9.27 million new active disease cases corresponding to an estimated incidence of 139 per 100,000 population occurred throughout the world in 2007 [4]. Only 5.5 million of 9.27 million cases of TB (new cases and relapse cases) were notified to national tuberculosis programs of various countries, while the rest were based on assessments of effectiveness of surveillance systems. The highest number of TB cases occurred in Asia (55%) followed by Africa (31%). The highest incidence rate (363 per 100,000 population) was recorded for the African region, mainly due to high prevalence of HIV infection. The six most populous countries of Asia (China, India, Indonesia, Pakistan, Bangladesh, and Philippines) accounted for >50% of all TB cases worldwide. An estimated 1.37 million (15%) of incident TB cases in 2007 were coinfected with HIV. Nearly 80% of the HIV-infected TB patients were living in the African region [4]. Globally, 13.7 million total prevalent TB cases were recorded in 2007 corresponding to 206 cases per 100 000 population that resulted in 1.756 million deaths (including 456 000 among TB patients coinfected with HIV) [4]. Nearly 500 000 cases of multidrug-resistant TB (MDR-TB, defined as infection with *M. tuberculosis* strains resistant at least to the two most important first-line drugs, rifampin and isoniazid) occurred in 2007 [4]. By the end of 2008, extensively drug-resistant TB (XDR-TB; defined as MDR-TB strains additionally resistant to a fluoroquinolone and an injectable agent such as kanamycin, amikacin, viomycin, or capreomycin) has been found in 55 countries and territories of the world [4]. While MDR-TB is difficult and expensive to treat, XDR-TB is virtually an untreatable disease in most of the developing countries [7].

Population-based studies have shown that some individuals are more at risk of acquiring infection and developing active disease than others. Active transmission also occurs more frequently in small households and crowded places in countries with a high incidence of TB [8, 9]. Molecular epidemiological studies have shown that there are distinct differences in the disease presentation and population demographics in low TB incidence and high TB incidence countries. In several African and Asian countries, where the transmission of *M. tuberculosis* has been stable or increased in the last few years, the incidence rate is highest among young adults with most cases resulting from recent episodes of infection or reinfection. On the contrary, in low TB incidence countries of Western Europe and North America, a higher proportion of cases occur in older patients or among immigrants from high TB incidence countries [8, 10]. Pulmonary TB accounts for >85% of active TB cases in high TB incidence countries due to higher rates of active transmission, while extrapulmonary TB is also common in low TB incidence countries of the developed world, particularly among HIV-infected individuals and immigrants originating from TB endemic countries [11, 12].

2. Transmission of *M. tuberculosis* Infection

Tuberculosis is a communicable disease and patients with pulmonary TB are the most important source of infection. Infection is initiated by inhalation of droplet nuclei, which are particles of 1–5 μm in diameter containing *M. tuberculosis*, expectorated by patients with active pulmonary TB (open TB), typically when the patient coughs. The droplet nuclei, due to their small size, can remain suspended in the air for several minutes to hours. The risk of infection (Figure 1) is dependant on several factors such as the infectiousness of the source case, the closeness of contact, the bacillary load inhaled, and the immune status of the potential host [8–10]. The primary route of infection involves the lungs. Inhaled droplet nuclei avoid the defenses of the bronchi due to their small size and penetrate into the terminal alveoli where they are engulfed by phagocytic immune cells (macrophages and dendritic cells). *M. tuberculosis* can also infect nonphagocytic cells in the alveolar space including M cells, alveolar endothelial, and type 1 and type 2 epithelial cells (pneumocytes) [13–19]. In the early phase of infection, *M. tuberculosis*, internalized by phagocytic immune cells, replicates intracellularly, and the bacteria-laden immune cells may cross the alveolar barrier to cause systemic dissemination [14, 15]. The intracellular replication and simultaneous dissemination of the pathogen to the pulmonary lymph nodes and to various other extrapulmonary sites occur prior to the development of the adaptive immune responses. This exemplifies the extraordinary ability of *M. tuberculosis* to establish a protected niche where they can avoid elimination by the immune system and to persist indefinitely [20, 21].

In the vast majority of the infected individuals, an effective cell-mediated immune response develops 2–8 weeks after infection that stops further multiplication of the tubercle bacilli (Figure 1). The activated T lymphocytes, macrophages, and other immune cells form granulomas that wall off the growing necrotic tissue limiting further replication and spread of the tubercle bacilli. Most of the *M. tuberculosis* are killed in the caseating granulomas, and disease progression is arrested. However, the pathogen is not completely eradicated in some individuals as *M. tuberculosis* has evolved effective strategies to evade the immune response resulting in survival and persistence of some bacilli in a nonreplicating state in the host (LTBI) [8, 21, 22]. In support of this hypothesis, *M. tuberculosis* has been cultured and presence of *M. tuberculosis* DNA has been demonstrated from lung tissues of individuals who died from other diseases and who did not exhibit any pathological sign of TB disease [23, 24]. Furthermore, a recent report showing transmission of infection from father to son in Denmark in 1961 and

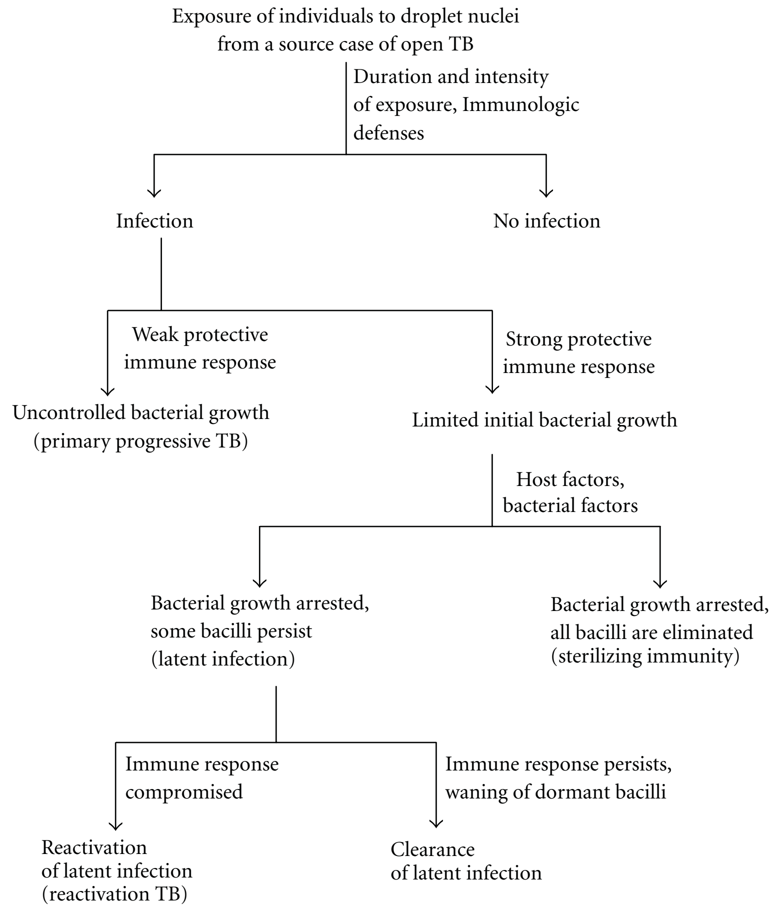

Figure 1: Progression of natural course of events and outcome in an immunocompetent individual following exposure to droplet nuclei containing *M. tuberculosis* expectorated by a source case of sputum smear-positive pulmonary (open) TB.

reactivation of latent infection in the son more than 30 years later (documented by molecular fingerprinting of their respective *M. tuberculosis* isolates) has indicated that the surviving bacilli may remain dormant for a long time (lasting up to a lifetime) [25]. A subsequent defect in cell-mediated immunity may result in reactivation of dormant bacilli causing active disease many years after the infection (reactivation TB). The current understanding of the mechanisms leading to the establishment of latent infection and the transition back to active growth in reactivation of latent disease is described below.

3. Entry Mechanisms of *M. tuberculosis*

Infection with *M. tuberculosis* starts with phagocytosis of the bacilli by phagocytic antigen-presenting cells in the lung including alveolar macrophages and dendritic cells. The recognition of pathogen-associated molecular patterns (PAMP) by specific pathogen recognition receptors (PRRs) is central to the initiation and coordination of the host innate immune response [26]. *M. tuberculosis* internalized through different receptors may also have different fate.

The *M. tuberculosis* or *M. tuberculosis* components are recognized by host receptors that include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-(NOD-) like receptors (NLRs), and C-type lectins. The C-type lectins include mannose receptor (CD207), the dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) and Dectin-1 [27, 28]. Other potential receptors include complement receptors, scavenger receptors, surfactant protein A receptors (Sp-A), and cholesterol receptors [29]. Some of these receptors (such as TLRs) are expressed on both, immune cells (such as macrophages, dendritic cells, B cells, and specific types of T cells) and nonimmune cells (like fibroblasts and epithelial cells). The interaction of *M. tuberculosis* with TLRs initiates an intracellular signaling cascade that culminates in a proinflammatory response (beneficial to the host); however, the bacterium has also evolved strategies that can trigger signals that dampen or modulate the innate immune response (beneficial to the pathogen). Other membrane bound PRRs (CD207, DC-SIGN, and Dectin-1) contribute to the relay of inflammatory signals while cytosolic PRRs (such as Nod-like receptor) modulate host recognition of the pathogen [27, 28].

The TLR engagement, particularly TLR2 and TLR4, with *M. tuberculosis*/*M. tuberculosis* component is an early event in the interaction of the pathogen with host cells and TLR signaling is the main arm of the innate immune response during *M. tuberculosis* infection [27, 28, 30]. The TLR polymorphisms regulate the innate immune response to mycobacterial lipopeptides and clinical susceptibility to pathogens [31]. Typically, signals generated by the interactions of TLRs with ligands on *M. tuberculosis* induce the activation of proinflammatory and antimicrobial innate immune response.

The *M. tuberculosis* cell envelope is composed of a cell wall that is covered with a thick waxy mixture of lipids and polysaccharides and is characterized by a high content of mycolic acids. The most important *M. tuberculosis* cell surface ligands that interact with TLRs and other receptors include the 19 and 27 kDa lipoproteins, 38 kDa glycolipoprotein, the lipomannan (LM) and mannose-capped lipoarabinomannan (ManLAM) [32–34]. Other ligands include LprA and LprG lipoproteins [35, 36] and, perhaps also, surface-exposed mammalian cell entry (Mce) proteins encoded by *mce1* and *mce3* operons [37–39]. The interaction of *M. tuberculosis* ligand(s) with TLRs eventually results in activation of nuclear transcription factor (NF)-κB and production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-12, chemokines, and nitric oxide through either myeloid differentiation primary response protein 88 (MyD88)-dependant or MyD88-independent pathway [27, 34, 40, 41].

Restricting TLR-induced proinflammatory signals is essential to avoid the risk of producing excessive inflammation that could damage host tissues. A family of receptor tyrosine kinases termed Tyro3/Axl/Mer (TAM) provide a negative feedback mechanism to both TLR-mediated and cytokine-driven proinflammatory immune responses [42]. This property has been exploited by *M. tuberculosis* to its advantage. The 19 kDa lipoprotein of *M. tuberculosis* is an agonist of the TLR2 and modulates the innate immunity and antigen presenting cell function [32]. Studies have shown that prolonged TLR2 signaling by lipoproteins of *M. tuberculosis* inhibits major histocompatibility complex (MHC)-II expression and processing of antigens by macrophages [43, 44]. Thus, a subset of infected macrophages with decreased antigen-presenting cell function may be unable to present *M. tuberculosis* antigens to $CD4^+$ T cells resulting in insufficient activation of effector T cells leading to evasion of immune surveillance and creation of niches where *M. tuberculosis* survives and persists [27, 32].

The mannose receptors interact with ManLAM present in the cell envelop of *M. tuberculosis*. The phagocytosis of tubercle bacilli by macrophages through mannose receptor is associated with an anti-inflammatory response as ManLAM inhibits mannose receptor-dependant IL-12 production. This inhibition of macrophage response to *M. tuberculosis* promotes infection and subsequent survival of *M. tuberculosis* in macrophages. The ManLAM exerts its effects on phagolysosome maturation by limiting phagosome-lysosome fusion [45, 46].

4. Immune Response of the Host to *M. tuberculosis*

The alveolar macrophages, after entry of *M. tuberculosis*, produce inflammatory cytokines and chemokines that serve as a signal for infection. The monocytes, neutrophils, and lymphocytes migrate to the focal site of infection, but they are unable to kill the bacteria efficiently. During this time, the bacilli resist the bactericidal mechanisms of the macrophage (phagolysosome) by preventing phagosome-lysosome fusion, multiply in the phagosome, and cause macrophage necrosis [47]. The released bacilli multiply extracellularly, are phagocytosed by another macrophage that also fails to control the growth of *M. tuberculosis,* and likewise are destroyed. In the meantime, dendritic cells with engulfed bacilli mature, migrate to the regional lymph node, and prime T cells (both $CD4^+$ and $CD8^+$) against mycobacterial antigens [48]. The specific immune response produces primed T cells which migrate back to the focus of infection, guided by the chemokines produced by the infected cells. The accumulation of macrophages, T cells, and other host cells (dendritic cells, fibroblasts, endothelial cells, and stromal cells) leads to the formation of granuloma at the site of infection [49].

The granuloma formation walls off tubercle bacilli from the rest of the lung tissue, limits bacterial spread, and provides microenvironment for interactions among macrophages and other cells of the immune system and the cytokines produced by these cells. The $CD4^+$ T cells producing interferon-γ (IFN-γ) recognize infected macrophages presenting antigens from *M. tuberculosis* and kill them [50]. The infection progression is halted; however, some resistant bacilli capable of surviving under the stressful conditions generated by the host escape killing and enter a state of dormancy and persist by avoiding elimination by the immune system [22, 51, 52]. Recent studies have shown that differences exist in the immunological response mounted by different individuals that lead to the formation of physiologically distinct granulomatous lesions in individuals exposed to *M. tuberculosis*. Some of these lesions suppress (sterilizing immunity) while others promote the persistence of viable *M. tuberculosis* in the microenvironment [53]. Low-dose infection of cynomolgus macaques that reproduce the clinical characteristics of human latent TB leads to the formation of at least two types of tuberculous granuloma [54, 55]. Histopathological studies have shown that the classic caseous granuloma are composed of epithelial macrophages, neutrophils, and other immune cells surrounded by fibroblasts. The central caseous necrotic region in this type of granuloma consists of dead macrophages/other cells and is hypoxic with *M. tuberculosis* residing inside macrophages in the hypoxic center [55, 56]. The other kind of granulomas seen in latent tuberculosis in both humans and cynomolgus macaques are fibrotic lesions, composed almost exclusively of fibroblasts that contain very few macrophages [55]. However, it is not known at present whether *M. tuberculosis* is located inside macrophages or in the fibrotic area in these lesions.

The microenvironment of the granuloma (hypoxia, low pH, presence of nitric oxide and carbon monoxide, etc.) increases the expression of several *M. tuberculosis* genes involved in dormancy induction [57–60]. Recent findings of formation of spore-like structures in *M. bovis* BCG, *M. marinum,* and *M. smegmatis* in response to prolonged stationary phase or nutrient starvation suggest that sporulation may be a general mechanism for mycobacterial dormancy [60–62]. The dormant bacilli can inhabit the granuloma during the lifetime of the host, but are able to resuscitate (or germinate) in the event of local immunodepression. The latent infection in a person without overt signs of the disease is indicated by the delayed-type hypersensitivity (DTH) response to purified protein derivative (PPD) prepared from culture filtrates of *M. tuberculosis* (tuberculin skin test) [8].

5. Specific Roles of Immune Cells and Cytokines in *M. tuberculosis* Infection

Studies in animal models and in humans have demonstrated that a wide range of immune components are involved in an effective immune response against *M. tuberculosis*. These include, beside macrophages and dendritic cells, $\alpha\beta$-T cells (both $CD4^+$ and $CD8^+$), CD1 restricted T cells, $\gamma\delta$-T cells, and cytotoxic T cells, as well as the cytokines produced by these immune cells [22, 63, 64]. The most important among these are $CD4^+$ T cells and the cytokine IFN-γ. Although $CD4^+$ T cells along with $CD8^+$ T cells and the natural killer (NK) cells are the major producers of IFN-γ, studies carried out in $CD4^+$ deficient mice have shown that it is the early production of IFN-γ by $CD4^+$ T cells and subsequent activation of macrophages that determine the outcome of infection [65, 66]. The $CD4^+$ T cells also play other roles in the defense against infection that is independent of IFN-γ production. Depletion of $CD4^+$ T cells was associated with the reactivation of infection in a chronically infected mice and resulted in increasing pathological features and death, even though IFN-γ levels were still high due to a strong response from $CD8^+$ T cells and normal levels of inducible nitric oxide synthase (iNOS) [67].

The $CD4^+$ T cells carry out several functions that are important to control infection in the granuloma. These include apoptosis of infected macrophages through Fas/Fas ligand interaction, production of other cytokines (such as IL-2 and TNF-α), induction of other immune cells (macrophages or dendritic cells) to produce other immunoregulatory cytokines such as IL-10, IL-12, and IL-15, and activation of macrophages through direct contact via CD40 ligand [63, 66, 68, 69]. The $CD4^+$ T cells also appear to be critical for the cytotoxic function of $CD8^+$ T cells that is mediated by IL-15 [66, 70]. It has also been shown that $CD4^+$ T cells can control the intracellular growth of *M. tuberculosis* by a nitric oxide-dependent mechanism that is independent of IFN-γ production [66, 71]. Thus, $CD4^+$ T cells, in addition to early production of IFN-γ appear to have several other secondary functions that are critical in the control of *M. tuberculosis* infection.

The $CD8^+$ T-cells, in addition to producing IFN-γ and other cytokines, may also be cytotoxic for *M. tuberculosis*-infected macrophages, and thus play an important role in providing immunity to TB. The $CD8^+$ T-cells can directly kill *M. tuberculosis* via granulysin, and facilitate the control of both the acute as well as chronic infection [66, 72]. The abundant presence of *M. tuberculosis*-specific $CD8^+$ T cells in latently infected individuals shows that the $CD8^+$ T cells also have a role in the control of latent infection. This is also supported by reactivation of latent infection following depletion of $CD8^+$ T cells in the Cornell model of latent TB [73].

Studies in primate models of TB have shown that unconventional T cells such as CD1 restricted T cells, and $\gamma\delta$-T cells also contribute to the protection against TB [64, 74, 75]. The CD1 restricted T cells recognize the glycolipids such as LAM that are abundant in the mycobacterial cell wall while $\gamma\delta$-T cells recognize small metabolites containing phosphate (phospholigands) [74]. Although it is well established that mycobacterial antigens in the phagosome of macrophages or dendritic cells are picked up by the MHC class II molecules and presented to $CD4^+$ T cells, studies have shown that the phagosomal membrane is also equipped with the MHC class I processing machinery [76, 77]. Also, CD1 proteins have the capability to present lipid antigens and lipopeptides to T cells, and thus play important roles in the immune response against lipid-rich *M. tuberculosis* [75, 78, 79]. Further, the vesicles formed due to apoptosis of *M. tuberculosis*-infected macrophages and containing mycobacterial antigens such as ManLAM, lipoproteins, and so forth are taken up by dendritic cells and presented to the T cells through the MHC class I and CD1 molecules [75, 78, 80].

The IFN-γ is the key cytokine for a protective immune response against *M. tuberculosis*. Humans and mice defective in IFN-γ or IFN-γ receptor genes are more susceptible to *M. tuberculosis* infection [63, 66, 81]. The IFN-γ, produced mainly by $CD4^+$, $CD8^+$ T cells, and the NK cells, synergizes with TNF-α and activates macrophages to kill intracellular bacilli. The IFN-γ also augments antigen presentation, leading to recruitment of $CD4^+$ T-cells and/or cytotoxic $CD8^+$ T-cells, which participate in mycobacterial killing and also prevents exhaustion of memory T cells [63, 82]. Furthermore, IFN-γ induces the transcription of more than 200 genes in macrophages including the upregulation of MHC class II expression and the production of antimicrobial effectors such as oxygen radicals and nitric oxide. A major effector mechanism responsible for the antimicrobial activity of IFN-γ in association with TNF-α is the induction of the production of nitric oxide and other reactive nitrogen intermediates (RNI) by macrophages via iNOS [63, 66, 83]. However, some *M. tuberculosis* factor(s), such as the 19-kDa lipoprotein, have the potential to attenuate the response of macrophages to IFN-γ by blocking the transcription of a subset of IFN-γ-responsive genes (Table 1) [44, 84, 85].

TNF-α, produced by macrophages, dendritic cells, and T-cells, is another cytokine that has a major protective role against *M. tuberculosis* infection both in mice and humans [104, 105]. Paradoxically, TNF-α also contributes significantly to the development of immunopathology associated

Table 1: Some important *M. tuberculosis* factors that modulate the innate immune response and promote persistence of the pathogen leading to latent tuberculosis infection.

M. tuberculosis component	Immune cell process inhibited/affected	Reference(s)
19 kDa Lipoprotein (LpqH)	MHC class II expression and antigen presentation	[32, 44, 85–87]
19 kDa Lipoprotein (LpqH)	Phagosomal processing by MHC class I pathway	[88]
Mannose capped lipoarabinomannan	Phagolysosome biogenesis	[46, 89, 90]
Mannose capped lipoarabinomannan	MHC class II expression and antigen presentation	[85, 90]
Mannose capped lipoarabinomannan	IL-12 secretion of dentritic cells/macrophages	[45, 90]
Mannose capped lipoarabinomannan	Apoptosis of macrophages	[90, 91]
Trehalose dimycolate (cord factor)	Phagolysosome biogenesis	[92, 93]
Trehalose dimycolate (cord factor)	MHC class II expression and antigen presentation	[94]
6-kDa early secreted antigenic target (ESAT-6)	Pathogen containment in phagolysosome/macrophage	[95–97]
ESX-1 secreted proteins	Macrophage proinflammatory cytokine response	[98]
Serine/threonine protein kinase G (PknG)	Phagolysosome biogenesis	[99, 100]
Lipid phosphatase (SapM)	Phagolysosome biogenesis	[101]
Lipoprotein LprA	MHC class II expression and antigen presentation	[36]
Lipoprotein LprG	MHC class II expression and antigen presentation	[35]
Secretion system SecA2	Apoptosis of macrophages and dendritic cells	[102]
Superoxide dismutase (SodA)	Apoptosis of macrophages and dendritic cells	[102]
NADH dehydrogenase (NuoG)	Apoptosis of macrophages and dendritic cells	[103]

with TB [52]. Mice deficient in TNF-α or TNF-α receptors are more susceptible to mycobacterial infections [104]. This cytokine is involved in both immune and immunomodulatory responses and acts in synergy with IFN-γ to enhance the expression of iNOS and the antimycobacterial activity of macrophages [63, 83]. TNF-α also initiates cell migration and formation of microbicidal granulomas while disruption of TNF-α responses leads to overgrowth of the mycobacterial pathogens [63, 66]. The TNF-α produced by the infected macrophages induces the expression of chemokines, such as IL-8, MCP-1, and RANTES which provide signals for migration of immune cells to the sites of *M. tuberculosis* infection [106]. Both T cell- and macrophage-derived TNF-α are required for sufficient and long-term protection against *M. tuberculosis* infection [107]. The phenolic glycolipid, a virulence factor in the cell wall of a hypervirulent strain of *M. tuberculosis* (W-Beijing family) inhibits the release of pro-inflammatory cytokines TNF-α, IL-6 and IL-12 by macrophages [108].

The importance of IL-12 is also evident from increased susceptibility of mice and humans deficient in IL-12 responses to mycobacterial infections [109]. Individuals with defects in the production of IL-12 or its receptor are highly susceptible to active TB disease [110]. The T-cell-derived cytokines, IFN-γ and TNF-α, are produced abundantly by activated $CD4^+$ T cells under the influence of IL-12, and the role of IFN-γ and TNF-α in activating and augmenting the microbicidal effector functions of phagocytic cells during a protective immune response against *M. tuberculosis* infection is well established [63, 66, 83].

6. Antigen Presentation Pathways and Their Modulation by *M. tuberculosis* Components

The tubercle bacilli reside in the phagosome soon after their entry inside alveolar macrophages and dendritic cells. The priming of $CD4^+$ T cells for a protective immune response requires the presentation of *M. tuberculosis* antigens through MHC class II pathway. The phagosomal membrane is also equipped with the MHC class I processing machinery [74, 77]. Further, mycobacterial glycolipids, lipids, and other phospholigands may also be presented to the T cells [78, 80]. Some *M. tuberculosis* factors particularly those associated with the cell wall modulate antigen-processing pathways by MHC class I, MHC class II, and CD1 molecules [28, 63, 111]. The ManLAM, trehalose $6,6'$-dimycolate (TDM, also known as cord factor), and the 19 kDa lipoprotein downregulate IFN-γ-inducible genes including those involved in antigen presentation by MHC class II machinery (Table 1) [32, 86, 94, 111, 112]. Other mechanisms that modulate antigen presentation include antigen processing and binding of peptides to MHC class II molecules [32, 87]. The 19 kDa lipoprotein also inhibits MHC class I antigen processing via Toll-like receptor signaling [88]. It is probable that continuous attenuation of antigen presentation through multiple mechanisms is advantageous for slowly growing pathogens like *M. tuberculosis* [111, 112]. Inhibition of antigen presentation by these mechanisms results in persistence of *M. tuberculosis* inside macrophages [112].

7. Dampening of Other Macrophage Functions by *M. tuberculosis* Components

The two major antimycobacterial mechanisms of macrophages include the generation of nitric oxide and other RNI which exert toxic effects on the bacilli and fusion of the phagosomes containing mycobacteria with lysosomes that is bactericidal [83, 92, 113]. The T cell-derived cytokines, mainly IFN-γ and TNF-α, activate macrophages, which then generate nitric oxide and other RNI by iNOS and are mycobactericidal [83]. Direct demonstration of the presence of nitrotyrosine, an RNI derived from tyrosine and peroxynitrite in the lungs of infected mice, has shown that RNI are formed in tuberculous granuloma. Furthermore, inhibition of iNOS activity or disruption of iNOS gene, required for the production of RNI, not only abolished the protective effect of RNI but also led to reactivation of latent infection in mice [83]. Although these studies point towards an essential role for iNOS in the control of both acute and chronic persistent infection, the RNI generated through these mechanisms is not sufficient to eliminate the bacterium completely.

The protective role of RNI in human TB has also been suggested [92, 114]. Studies have shown that *M. tuberculosis* has evolved several strategies to evade the RNI toxicity. It has been shown that iNOS, a cytoplasmic protein, may be recruited to the phagosomes and this recruitment may be inhibited by *M. tuberculosis* [115]. The *M. tuberculosis* gene, alkyl hydroperoxide reductase subunit C (*ahpC*), detoxifies, in conjunction with some other proteins, the highly reactive peroxynitrite anion (formed by the reaction of nitric oxide with superoxide) [116]. Another potential mechanism for blunting the toxic effects of RNI is the presence of two haemoglobin-like proteins encoded by *glbN* and *glbO* in *M. tuberculosis*. The *glbN* knockout mutant of *M. bovis* BCG was highly attenuated, and its growth, *in vitro*, was also inhibited by nitric oxide under aerobic conditions [117]. Microarray analyses have shown that more than 30 *M. tuberculosis* genes are induced by RNI and hypoxia [59, 118]. Furthermore, hypoxia and inhibition of respiration by nitric oxide induce a dormancy program in *M. tuberculosis* that leads to increased survival and persistence of the pathogen in immune cells [59, 119].

8. Phagolysosome Maturation and Its Inhibition by *M. tuberculosis* Components

The phagocytosis of *M. tuberculosis* by macrophages is followed by the maturation of phagosomes containing the pathogen through a series of fusion and fission events with several endocytic vesicles that culminate in a phagolysosome [120, 121]. The fission-fusion events remodel the phagosomal membrane, and the recruitment of vacuolar-proton transporting ATPase (vH$^+$-ATPase) lowers the internal pH that allows lysosome-derived acid hydrolases to function efficiently for their microbicidal effect [122, 123]. Furthermore, phagosome maturation is dependant on Ca^{+2} signaling cascade that begins with phosphorylation of sphingosine to sphingosine 1-phosphate by sphingosine kinase resulting in elevation of cytosolic [Ca^{+2}] inside macrophages due to release of Ca^{+2} from intracellular stores in the endoplasmic reticulum and continues through Ca^{+2}-calmodulin complex-dependant activation of protein kinase II and phosphatidyl inositol 3-kinase (PI-3K). The cascade culminates in phosphorylation of phosphatidyl inositol to phosphatidyl inositol 3-phosphate (PI-3P) by PI-3K in the phagosome membrane and maturation of phagosome to an acidic bactericidal compartment (phagolysosome) after binding of early endosomal antigen-1 (EEA-1) to PI-3P [89, 124–126].

M. tuberculosis has also evolved several strategies to avoid the destruction by lysosomal enzymes by disrupting the maturation of bacilli-containing phagosomes into phagolysosmes [46, 90, 92, 127, 128]. Exclusion of vH$^+$-ATPase during maturation of phagosomes contributes to the acidification defect that prevents the fusion of phagosomes with lysosomes [122]. Similarly, modulation of Ca^{+2} signaling cascade, such as SapM-mediated hydrolysis and inactivation of PI-3P, inhibits phagosome maturation leading to enhanced intracellular survival of *M. tuberculosis* (Table 1) [101, 126–128].

Other *M. tuberculosis* components such as ManLAM and TDM (Table 1) also affect phagosome maturation by interfering with the tethering and fusion machinery of vesicular transport in mammalian cells and promote persistence of the bacterium inside macrophages [46, 89, 90, 93, 101]. The targets include the soluble N-ethylmaleimide-sensitive factor-attachment protein receptors (SNAREs), the tethering proteins (such as EEA-1), and the Rab family of GTPases [89, 120, 126, 128]. Some of the membrane trafficking processes affected by mycobacterial factors are also affected by HIV during viral budding and this overlap partially contributes towards the synergism observed between AIDS and active TB [129]. Another mechanism by which mycobacteria interfere with phagosome maturation is by retention of the host tryptophan aspartate rich coat protein (TACO) (homolog of coronin-1) on the cytoplasmic side of their phagosomes that likely inhibits the normal process of phagosome-lysosome fusion [99]. The serine/threonine protein kinase G encoded by *pknG* of *M. tuberculosis* (Table 1) is implicated as the potential effector of the inhibition of phagosome-lysosome fusion [100, 111].

Another component of the antimicrobial repertoire of macrophages includes lysosomal killing of *M. tuberculosis* mediated by ubiquitin-derived peptides [130]. The ubiquitination destroys tubercle bacilli by autophagy as a ubiquitin-derived peptide impairs the membrane integrity of *M. tuberculosis* that allows nitric oxide to kill more efficiently. On the contrary, decreased outer membrane permeability protects *M. tuberculosis* from killing by ubiquitin-derived peptides [131].

9. Apoptosis of Infected Macrophages and Its Inhibition by *M. tuberculosis* Components

The apoptosis of infected macrophages participates in host defense against infection as apoptotic vesicles containing

mycobacterial antigens are taken up by dendritic cells for $CD8^+$ T cell activation by phagosome-enclosed antigens [79, 80]. The $CD8^+$ T cells activated by apoptotic vesicles from *M. tuberculosis*-infected cells produce IFN-γ, which causes uninfected macrophages to produce RNI to effectively kill intracellular *M. tuberculosis*. Several *M. tuberculosis*-derived factors are capable of modulating (activating as well as inhibiting) the apoptosis of infected macrophages through differential expression of proapoptotic and antiapoptotic genes [90]. The mycobacterial components modulating apoptosis of macrophages usually target the caspase cascade or the one involving TLRs. The *M. tuberculosis* components that inhibit apoptosis include cell wall components, ManLAM, virulence-related secretion system encoded by *secA2* that transports superoxide dismutase (encoded by *sodA*) to control reactive oxygen intermediates, and NADH dehydrogenase (encoded by *nuoG*) (Table 1) [91, 102, 103]. Two secretory proteins of *M. tuberculosis* encoded by Rv3654c and Rv3655c that inhibit apoptosis of macrophages have also been identified recently [132]. By inhibiting apoptosis of macrophages, *M. tuberculosis* avoids host defenses and escapes from infected cells by causing necrotic cell death [133].

10. Escape of *M. tuberculosis* from Phagosome/Phagolysosome

Although it has been known for quite some time that *M. tuberculosis* survives in the phagosome by blocking (or slowing down) its maturation into phagolysosome and persists, one of the mechanism by which it escapes from phagosome/phagolysosome to infect other macrophages and other immune/alveolar cells has been elucidated recently. Initial subtractive hybridization-based studies identified a genomic region, termed region of difference 1 (RD1), that was present in all virulent *M. tuberculosis* and *M. bovis* strains but was absent in the vaccine strain *M. bovis* BCG [2, 134, 135]. Subsequently, it was shown that RD1 is crucial for the virulence of *M. tuberculosis* as it encoded proteins that formed a novel protein secretion system (ESX-1). ESX-1 (type VII secretion system) is involved in the export of several *M. tuberculosis* proteins including two potent T cell antigens encoded by RD1 itself, the 6-kDa early secreted antigenic target (ESAT-6) (encoded by *esxA*) and 10-kDa culture filtrate protein (CFP-10) (encoded by *esxB*) that lack signal sequences for their export [2, 134, 136–140].

The importance of ESX-1 secreted proteins in virulence of *M. tuberculosis* has been shown by deletion of RD1 or disruption of ESAT-6 from *M. tuberculosis* genome that resulted in reduced virulence (spreading) both, in cultured macrophages and in mice [140, 141]. Furthermore, the introduction of RD1 genes in *M. bovis* BCG resulted in altered colonial morphology, increased virulence in severely combined immune deficient mice including the formation of granuloma, and longer persistence in immunocompetent mice [142]. In *M. tuberculosis*, ESAT-6 complexes with CFP-10 in 1 : 1 ratio before its export outside the cell but can dissociate from its partner (CFP-10) at lower pH. Individually, ESAT-6, but not CFP-10, can cause disruption of artificial membranes as well as cytolysis [143–145]. ESAT-6 alone has also been shown to associate strongly with liposomes containing dimyristoylphosphatidylcholine and cholesterol (constituents of mammalian cell membranes) and causing destabilization and lysis of liposomes [95, 96, 144].

The studies carried out by de Jonge et al. [96] have shown that ESAT-6:CFP-10 complex secreted by live *M. tuberculosis* inside phagosome splits apart when tubercle bacilli are stressed following acidification of phagosome, and ESAT-6 inserts itself into lipid bilayer, causing lysis and escape of *M. tuberculosis* from phagosome. Further studies have shown that ESAT-6 also induces apoptosis of macrophages via the extrinsic (caspase-dependent) pathway by formation of pores in cell membrane [146] and contributes (or helps) in the translocation of *M. tuberculosis* from the phagolysosomes to the cytoplasm in myeloid cells [97]. More recently, ESAT-6 has also been shown to cause cytolysis of type 1 and type 2 alveolar epithelial cells. This ESAT-6-mediated cytolysis was shown to help in the dissemination of *M. tuberculosis* through alveolar wall [19]. The ESAT-6 homolog from *Mycobacterium marinum*, the bacterium that causes tuberculous granuloma in zebrafish, has also been demonstrated to cause lysis of red blood cells and macrophages by forming pores in their membranes [147, 148]. The presence of a capsular layer containing high amounts of proteins that are secreted via the ESX-1 secretion system including ESAT-6 has also been demonstrated in pathogenic mycobacteria recently [98]. Furthermore, ESX-1-associated proteins in the capsular layer enhanced the interaction of mycobacteria with macrophages and also dampened proinflammatory cytokine response of macrophage. These studies have established the role of ESX-1 secretion system and ESAT-6 protein of *M. tuberculosis* in facilitating macrophage infection and subsequent bacterial escape to infect other nearby cells (Table 1).

11. Persistence and Reactivation of Latent TB Infection

The hallmark of *M. tuberculosis* infection in humans is the inability of an otherwise effective immune response to completely eliminate the pathogen. The tubercle bacilli have evolved multiple strategies to manipulate infected host cells in order to evade or modify the ensuing immune response so as to avoid elimination and thus persist in the host. As described above, several *M. tuberculosis* factors, ManLAM and 19-kDa lipoprotein notable among them, have been identified that modulate antigen presentation pathways and either blunt the microbicidal functions of macrophages and other immune cells (such as RNI) or prevent their maturation (phagolysosome) (Table 1).

Two experimental strategies have been employed to identify other *M. tuberculosis* factors, which promote persistence of the pathogen in immune cells including macrophages. One approach involves cloning of *M. tuberculosis* genes in nonpathogenic mycobacteria and studying their increased

survival in macrophages or other mammalian cells while the other approach uses knockout mutants of *M. tuberculosis* for selected genes for persistence of the pathogen in macrophages and other immune cells. Several additional *M. tuberculosis* factors promoting persistence or increased survival in mammalian cells have been identified. Some of these factors include phospholipases encoded by *plcA, plcB, plcC,* and *plcD* [149], the two *PhoP* and *PhoQ* regulatory proteins [21], phosphate-binding proteins PstS1 and PstS2 [150], and proteins encoded by *mce* operons [151]. Thus, *M. tuberculosis* has devoted a large part of its genome towards functions that promote its intracellular survival in mammalian cells including macrophages.

Reactivation of latent infection requires latent *M. tuberculosis* cells to exit dormancy. Several factors can trigger the development of active disease from the reactivation of remote infection, and this typically involve the weakening of the immune system. HIV infection is the most important single risk factor for progression to active disease in adults as it causes depletion of $CD4^+$ T cells and functional abnormalities of $CD4^+$ and $CD8^+$ T-cells which play an important role in providing protection against active TB disease [4, 6]. Likewise, *M. tuberculosis* infection accelerates the progression of asymptomatic HIV infection to acquired immunodeficiency syndrome (AIDS) and eventually to death. This is recognized in the current AIDS case definition as pulmonary or extrapulmonary TB in HIV-infected patient is sufficient for the diagnosis of AIDS. Old age, malnutrition, and medical conditions that compromise the immune system such as poorly controlled diabetes mellitus, renal failure, and therapy with immunosuppressive drugs are other factors that lead to immunodepression and reactivation of a dormant infection [6, 8, 152, 153]. The reactivation TB can occur in any organ system in which the tubercle bacilli were seeded during the primary infection; however, in immunocompetent individuals, the reactivation usually occurs in the upper lobes, where higher oxygen pressure supports good bacillary growth. The lytic transglycosylases known as resuscitation promoting factors (RPFs) and an endopeptidase (RipA) of *M. tuberculosis* have recently been recognized as vital components for revival from latency [154–156].

12. Current Dynamic Model of Latent Infection

The LTBI has traditionally been defined as infection with *M. tuberculosis* in foci within granuloma that remain in nonreplicating state but retain their ability to come out of latency and cause active TB if and when a disruption of the immune response occurs [57]. However, recent experimental data supports a dynamic model of LTBI where endogenous reactivation as well as damage response occurs constantly in immunocompetent individuals [157]. The model suggests that during infection, *M. tuberculosis* grows well inside phagosomes; however, some bacilli released from necrotic macrophages in extracellular milieu in developing granulomas stop replicating. The arrest of bacterial growth occurs even before an effective immune response has fully been developed due to hypoxic and acidic environment (conditions that mimic stationary bacterial cultures) in the extracellular milieu and release of bactericidal enzymes from dead macrophages and neutrophils. The actively growing bacillary population is eventually killed due to the development of an effective immune response; however, nonreplicating bacilli resist killing and survive [158, 159].

The model further suggests that some macrophages (foamy macrophages) also emerge during the chronic inflammatory process, as they have phagocytosed the cellular debris rich in fatty acids and cholesterol derived from cellular membranes [160, 161]. The foamy macrophages also phagocytose extracellular nonreplicating lipid-rich *M. tuberculosis*; however, the bacilli do not grow in the intracellular environment of activated macrophages but are also not killed due to the nonreplicating state of the bacilli [162]. The nonreplicating bacilli-laden foamy macrophages drain from lung granuloma towards the bronchial tree and return to a different region of lung parenchyma due to aerosols generated by inspired air and begin the infection process at this new location once again [157, 160, 161, 163]. In this dynamic process, reinfection in the upper lobe may have the chance to cause cavitary lesion. This is aided by higher oxygen pressure in the upper lobes that can support rapid extracellular bacillary growth resulting in bacillary concentration that can not be controlled by the optimum immune response by the host. The subsequent much stronger inflammatory response leads to tissue destruction, liquefaction, and extracellular bacillary growth which amplifies the response further and causes cavitation [157, 158].

The dynamic infection process leading to active disease in the upper lobes has some parallels with immune reconstitution inflammatory syndrome and the active TB disease that occurs in HIV-infected patients. The presence of bacilli is tolerated by the HIV-infected patients with low $CD4^+$ cell counts as the host is unable to mount an inflammatory response needed to control their growth. However, the sudden increase in $CD4^+$ T cells in AIDS patients receiving highly active antiretroviral treatment causes an aggressive granulamatous response and active TB disease [164, 165]. The possibility of slow clearance of latent infection proposed by the dynamic infection model has also been supported by a recent study from Norway comprising a population of individuals exposed to a minimal risk of active transmission of infection. Cohort analysis of data from National Tuberculosis Registry to calculate rates and changes in rates of active TB disease, among patients previously exposed to *M. tuberculosis*, has shown that the rate of reactivated TB has progressively decreased over time [166]. The study further suggested that the number of individuals with latent infection could be reduced in half in approximately 9 years in populations in which new infections are effectively prevented. The dynamic infection model also explains how therapy for a relatively short time (9 months) with a single drug (isoniazid), active only against actively dividing bacilli [167], is highly effective for a latent infection that can possibly remain dormant for the entire lifetime of the host. As isoniazid will prevent episodes of reinfection by bacilli resuscitated from dormancy, slow drainage and destruction of nonreplicating bacilli in

the stomach will eventually lead to clearance of latent infection [157, 166].

13. Diagnosis of Latent *M. tuberculosis* Infection

The persons infected with *M. tuberculosis* may be identified by tuberculin skin test six to eight weeks after exposure to the bacilli. The test is based on a delayed-type hypersensitivity (DTH) response to a complex cocktail of *M. tuberculosis* antigens, known as purified protein derivative (PPD). The induration of more than 5 mm, recorded 48 to 72 hours after injection of PPD, is considered as positive. Surveys conducted with PPD skin test suggest that nearly a third of the world's and half of Asia's population is infected with *M. tuberculosis* [5]. Skin test reaction over 20 mm is usually due to active disease; however, a negative skin test in an active TB patient may also result from anergy or incorrect administration of the test. The tuberculin skin test lacks sensitivity and specificity as it can not differentiate between infection with *M. tuberculosis* and sensitization with other environmental mycobacteria [5, 8]. Also, BCG vaccination may cause false-positive reactions, but these generally last only a few years after vaccination and are in the moderate range (5 to 10 mm).

More sensitive and specific tests such as cell-mediated immunity-based interferon-gamma (IFN-γ) release assays (IGRAs) have also been developed that detect T cell responses after stimulation by two *M. tuberculosis*-specific antigens, early secreted antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) [168–173]. The IGRAs have excellent specificity as the antigens (ESAT-6 and CFP-10) used in these assays are encoded by genes deleted in the vaccine strain *M. bovis* BCG and majority of environmental mycobacteria of clinical relevance [134, 135, 170]. Another variation of conventional IGRAs has also been developed by using flow cytometry [174]. Although flow cytometric approach has an advantage over conventional IGRAs as a smaller blood volume (<1 ml) is required for testing, the assay has limited utility in much of the developing world due to the requirement of technical expertise and the high cost of flow cytometers. The detection of significant levels of antibodies to some *M. tuberculosis*-specific proteins has also been noted in latently infected individuals as well as in patients with active TB disease but not in healthy subjects [175–178]. However, antibody-based tests have not been used so far for the detection of LTBI.

Two commercial IGRAs, whole blood, ELISA-based QuantiFERON (QFN)-TB Gold assay (Cellestis Ltd., Carnegie, Australia) and peripheral blood mononuclear cell (PBMC) and enzyme-linked immunospot (ELISPOT) technology-based T SPOT-TB (Oxford Immunotec, Oxford, UK) test have also been developed and approved by Food and Drug Administration (FDA) for detecting LTBI. The tests were initially based on stimulation of T lymphocytes with ESAT-6 and CFP-10 proteins and measurement of IFN-γ production (QFN-TB Gold) or detection of T-cells themselves (T SPOT-TB). These tests have undergone further improvement. The newer version of the QFN-TB Gold is called QuantiFERON-TB-Gold-In-Tube (QFT-G-IT) (Cellestis Ltd., Carnegie, Australia) that uses ESAT-6 and CFP-10 and TB7.7 (corresponding to Rv2654 [2]) peptides as antigens. The newer version of T-Spot-TB also uses peptides of ESAT-6 and CFP-10 instead of whole ESAT-6 and CFP-10 proteins as antigens (Oxford Immunotec, Oxford, UK). The performance of both QFT-G-IT and T-Spot-TB tests have recently been evaluated extensively, and several systematic reviews are available for a more detailed description [173, 179–182]. Although IGRAs can not distinguish between LTBI and active TB disease in immunocompetent adults [173], in high-risk individuals with immunosuppressive conditions and children, IGRAs may help in the diagnosis of active disease as adjunctive diagnostic tests, particularly if specimens from the suspected site of infection (such as bronchoalveolar lavage, cerebrospinal fluid) rather than blood is used [183, 184]. While the results of IGRAs exhibit better correlation with surrogate measures of exposure to *M. tuberculosis* in low TB incidence countries; however, their performance is suboptimal in countries with a high TB incidence [173, 180–182, 185].

In low TB incidence countries of North America and Western Europe, the majority of active TB disease cases occur in foreign-born persons. Previous studies have shown that majority of active disease cases in immigrants/expatriates originating from TB endemic countries occur as a result of reactivation of previously acquired infection mostly within two years of their migration [8, 12, 171, 172]. Application of IGRAs to identify latently infected individuals and their treatment for LTBI has greatly helped in lowering the incidence of TB in rich, advanced countries [171, 172, 182, 186, 187]. Some other low-intermediate TB incidence countries which contain large expatriate populations originating from TB endemic countries [188–192] are also evolving similar strategies for controlling TB [12, 193].

14. Conclusions

With nearly 9 million new active TB cases and 2 million deaths occurring every year, TB remains a major infectious disease of global proportion. Active disease patients with sputum smear-positive pulmonary TB are the main source of infection. Primary infection with *M. tuberculosis* leads to clinical disease in ~10% of individuals. In the remaining cases, the ensuing immune response arrests further growth of *M. tuberculosis*. However, the pathogen is eradicated completely in ~10% people while the immune response in the remaining ~90% individuals only succeeds in containment of infection as some bacilli escape killing by blunting the microbicidal mechanisms of immune cells and remain in nonreplicating (dormant or latent) state in old lesions. The dormant bacilli retain their ability to induce reactivation and to cause active TB if a disruption of immune response occurs. While active transmission is a significant contributor of active disease cases in high TB burden countries, most cases in low TB incidence countries arise from this pool of latently infected individuals. The positive tuberculin skin

test or more recent and specific T cell-based IGRAs in a person without overt signs of the disease indicates LTBI. Two commercial IGRAs, QFT-G-IT and T-Spot-TB, are also available. Application of IGRAs to identify latently infected individuals and their treatment for LTBI has greatly helped in lowering the incidence of TB in rich, advanced countries. Similar approaches also hold great promise for other countries with low-intermediate rates of TB incidence.

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Research Article

Expression of Proinflammatory and Regulatory Cytokines via NF-κB and MAPK-Dependent and IFN Regulatory Factor-3-Independent Mechanisms in Human Primary Monocytes Infected by *Mycobacterium tuberculosis*

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Knowledge of the molecular events regulating the innate response to *Mycobacterium tuberculosis* (Mtb) is critical for understanding immunological pathogenesis and protection from tuberculosis. To this aim, the regulation and the expression of regulatory and proinflammatory cytokines were investigated in human primary monocytes upon Mtb infection. We found that Mtb-infected monocytes preferentially express a proinflammatory cytokine profile, including IL-6, TNF-α, and IL-1β. Conversely, among the regulatory cytokines, Mtb elicited IL-10 and IL-23 release while no expression of IL-12p70, IL-27, and IFN-β was observed. The analysis of the signalling pathways leading to this selective cytokine expression showed that in monocytes Mtb activates MAPK and NF-κB but is unable to stimulate IRF-3 phosphorylation, a transcription factor required for IL-12p35 and IFN-β gene expression. Thus, by inducing a specific cytokine profile, Mtb can influence the immunoregulatory properties of monocytes, which represent important target of novel vaccinal strategies against Mtb infection.

1. Introduction

Mortality and morbidity caused by the pulmonary pathogen *Mycobacterium tuberculosis* (Mtb) remain alarmingly high [1]. Although much is known about the immunology of tuberculosis (TB), the precise nature of the protective immune mechanisms against Mtb has not been completely defined. Indeed, it has been estimated that 30% of the exposed individuals will become tuberculin-positive patients and, among the infected individuals, only 5%–10% will develop clinical manifestations of active TB, while the majority controls the infection but not completely eradicates the bacteria developing into a latent TB [2]. Therefore, the characterization and comprehension of the immune mechanisms leading to the control of initial infection and to the prevention of reactivation of latent infection are crucial to contain this disease. A complex series of interactions among various cell populations is involved in the containment of Mtb infection [2]. In particular, macrophages and dendritic cells (DCs) are essential players against Mtb given their involvement in phagocytosis, antigen processing and presentation, and cytokine production [3, 4]. After the interaction with Mtb in the alveoli, macrophages and DC become activated and participate to the immune response against Mtb infection playing different roles [5]. DCs are engaged in inducing T cells in virtue of their production of Th1/IFN-γ-inducing cytokines and expression of costimulatory molecules, while macrophages are primarily involved in the formation of the granuloma, where tissue macrophages harboring tubercle bacilli are surrounded by and interact with natural killer (NK) and effector T lymphocytes. Indeed, NK and T cells are recruited after Mtb infection and produce IFN-γ and TNF-α,

which play a central role in the host defense against Mtb given their capacity to activate antimicrobial mechanisms in monocytes and macrophages [6]. Besides macrophages and DC, circulating monocytes are increasingly implicated in defense against a range of microbial pathogens by supplying tissues with macrophage and DC precursors [7]. We have identified new mechanisms of Mtb immune evasion that relies on the capacity of Mtb to interfere with monocyte differentiation into DC [8, 9]. In particular, our studies provided evidences that CR3 engagement on monocytes by mycobacteria leads to p38 mitogen-activated protein kinase (MAPK) and ATF-2 phosphorylation and that antibody-dependent CR3 blockade or treatment with a specific p38 MAPK inhibitor caused a notable increase in CD1 molecule expression in DC derived from mycobacteria-infected cells [9]. In addition, more recently the involvement of p38 MAPK and Signal Transducer and Activator of transcription pathways has been described in the expression of Interleukin-10 (IL-10), which inhibits the differentiation of bystander noninfected monocytes into DC [8]. Thus, by limiting the generation of functionally active DC, Mtb could block the expansion of T lymphocytes lacking effector function and, in turn, reducing the T cell help provided to infected alveolar macrophages for killing the intracellular pathogens.

To further investigate the response of monocyte to Mtb infection, we sought to investigate the profile of proinflammatory and regulatory cytokines and the molecular mechanisms underlying this expression. Our results indicate that human monocytes produce preferentially proinflammatory cytokine profile, including IL-6, TNF-α, IL-1β. Interestingly, we found that although monocytes activated IFN regulatory factor (IRF)-3 and synthesized IFN-β in response to LPS, they failed to do this upon Mtb challenge. Accordingly, the IFN-β-mediated expression of IL-27p28 did not occur in Mtb-infected monocytes as well as the release IL-12p70, a cytokine partially dependent on IRF-3 activation. Conversely, the robust production of IL-23, another IL-12 family member, was regulated by MAPK pathway. Collectively, our results highlight the capacity of Mtb to stimulate in monocytes a specific cytokine profile whose regulation was mainly dependent on MAPK and NF-κB signalling.

2. Materials and Methods

2.1. Reagents. LPS from Escherichia coli 0111:B4 (Sigma-Aldrich, St. Louis, MO, USA) was used at 1 μg/ml. The p38 MAPK inhibitor SB203580 and the ERK inhibitor PD98059 (Calbiochem Biochemicals, San Diego, CA, USA), at an optimal concentration of 3 μM, were added 30 min before Mtb infection.

2.2. Bacterial Strains, Media, and Growth Conditions. Mtb H37Rv (ATCC 27294) was grown and prepared as previously described [5]. Mtb was harvested at midlogarithmic growth phase. Bacterial viability was determined by counting the number of colony-forming units (CFUs) on Middlebrook 7H10 agar plates. All bacteria preparations were analyzed for LPS contamination by the Lymulus lysate assay (BioWhittaker, Verviers, Belgium) and contained less than 1 EU/ml.

2.3. Monocyte Preparation and Infection. Monocytes were prepared as previously described [5]. Monocytes were cultured at 1×10^6 cells/ml in RPMI 1640 (BioWhittaker) supplemented with 2 mM L-glutamine and 15% FCS (BioWhittaker). No antibiotics were added to the cultures. Monocytes were infected with a multiplicity of infection (MOI) of one bacterium/cell, which was previously found to be optimal for the infection of primary cells [5]. Mtb preparations were sonicated prior to infection of monocyte cultures. The infection was evaluated at various time points (0, 4, and 24 hr) by CFU assay to determine the number of intracellular bacteria, as previously described [10]. Briefly, 2 hr after infection, the cell cultures were gently washed (three times) with RPMI 1640 and centrifuged at $150 \times g$ for 10 min to selectively spin down cells while extracellular bacteria remain in the supernatants. Cells were resuspended in complete medium and cultured for the times indicated. T0 refers to cell cultures washed after 2 hr of infection, where a 20% reduction of the original MOI was observed. However, we found that Mtb was able to infect and to survive inside the monocytes as the number of CFU remained constant over a 24-hr period of examination (data not shown).

2.4. Cytokine Determinations. Supernatants from monocyte cultures were harvested 24 hr after infection or treatments, filtered by 0.2 μm low protein-binding syringe filters (Millex-GV, Millipore, Bedford, MA, USA), and stored at -80°C. IL-6, IL-10, IL-12 p70, TNF-α, and IL-1β were measured with the human inflammation cytometric bead assay (CBA) (BD Bioscience, Pharmingen, San Diego, CA, USA). The sensitivity of this assay varies from 1.9 pg/ml for IL-12p70 to 7.2 pg/ml for IL-1β. IL-23 was assayed with ELISA kit (Bender MedSydtem, Burlingame, CA, USA). The sensitivity is 20 pg/ml for IL-23. All assays were conducted according to manufacturers' instructions.

2.5. RNA Isolation and Real-Time PCR Quantification. RNA was extracted from DC with RNeasy kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. A phenol/chloroform extraction was performed to inactivate residual mycobacterial particles. Reverse transcriptions were performed as previously described [11]. Quantitative PCR assays were performed in triplicate using the Platinum Taq DNA Polymerase (Invitrogen Life Technologies Frederick, MD) and the SYBR Green I (BioWittaker Molecular Applications, Rockland, ME) on a LightCycler (Roche Diagnostics, Basel, CH). The sequences of primer pairs have been previously described [12, 13]. A calibration curve of a purified positive control RT-PCR product, to whom arbitrary values were assigned, was used to calculate the value of a target gene. The quantification standard curves were obtained using dilutions (4-log range) of the purified positive control RT-PCR product in 10 μg/ml sonicated salmon sperm DNA. Quantification data are presented as a ratio to the GAPDH mRNA level present in the same sample

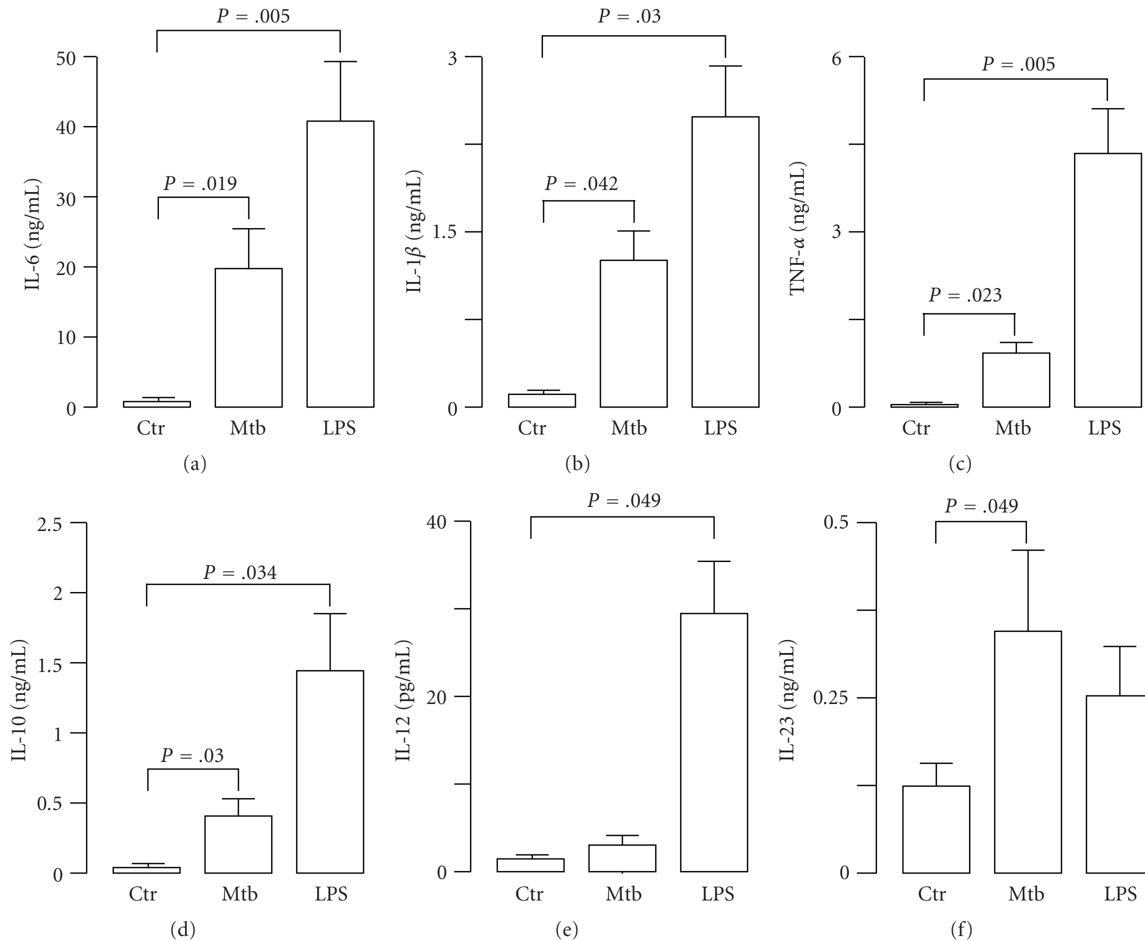

Figure 1: Cytokine production in Mtb-infected or LPS-treated human monocytes. Monocytes were infected with Mtb (MOI = 1) or treated with LPS (1 μg/ml). After 24 hr cell culture supernatants were collected and determination of cytokine content was performed by CBA or ELISA. The results represent the mean ± SE of four separate experiments performed with different donors.

and represent the mean ± SE of triplicate values. Only ratios with a SE 0.2 log (95% confidence limits) were considered for the determination of induction levels. The standard errors (95% confidence limits) were calculated using the Student's t-test.

2.6. Western Blot Analysis. Western blot was performed as previously described [13]. Briefly, 30 μg of total cell extracts were separated by SDS-PAGE gel and blotted onto nitrocellulose membranes. Blots were incubated with 1 μg/ml of rabbit polyclonal antibodies against IRF-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and reacted with anti-rabbit HRP-coupled secondary antibody (dilution 1 : 2000; Amersham Pharmacia Biotech, Little Chalfont, UK) using an ECL system. Western blots were performed on 7% SDS-PAGE gel to detect the slower migrating phosphorylated form (IRF-3P), while the IRF-3 content was evaluated in a shorter run on 10% SDS gels.

2.7. Electrophoretic Mobility Shift Assay (EMSA). Synthetic double-stranded oligonucleotides were end labeled with [γ-32P]ATP by T4 polynucleotide kinase. Binding reactions mixture (20 μl final volume) contained labeled oligonucleotide probes (30,000 cpm) in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 M Tris-HCl (pH 7.5), and 1 μg poly (dI)-poly(dC)). Nuclear lysates (5 μg) were added, and the reaction mixture was incubated for 30 min at room temperature. For supershift analysis, 1 μg of anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the reaction. Glycerol was added to 13% (v/v), and samples were analyzed on 5% polyacrylamide gels with 0.5x TBE (1x TBE is 50 mM Tris-borate (pH 8.2) and 1 mM EDTA) for 1.5 hr at 200 V at 18°C. The oligonucleotide used to monitor the NF-κB binding to κB sequences within the IFN-β promoter was 5′-AGTGGGAAATTCCTCT-3′.

2.8. Statistical Analysis. Statistical analysis was performed by a two-tailed Student t-test for paired data using Java Applets & Servlets for Biostatistics. A P-value <.05 was considered statistically significant.

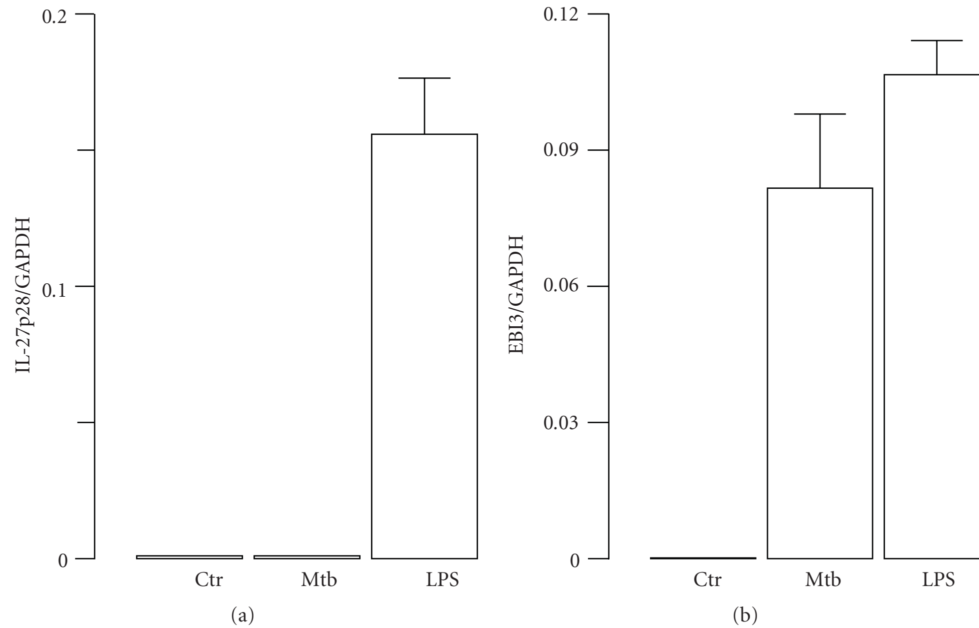

Figure 2: Expression of IL-27 subunits in Mtb-infected or LPS-treated human monocytes. RNA was extracted from monocytes infected with Mtb (MOI = 1) or LPS (1 μg/ml) for 8 hr. Real-time reverse transcription-polymerase chain reaction was performed to measure the expression of IL-27p28 and EBI3 subunits. The results are shown as a ratio to the GAPDH level and represent the mean ± SE of triplicate values. The results shown are from one out of three experiments performed with different donors that yielded similar results.

3. Results and Discussion

3.1. Proinflammatory and Regulatory Cytokine Release from Mtb-Infected Monocytes. Given the importance of cytokines in orchestrating the immune response against Mtb [3], we sought to investigate how Mtb stimulates the expression profile of proinflammatory and regulatory cytokines in human primary monocytes, whose role in the immune response against pathogens is critical in supporting the development of tissue macrophages and resident DC. To this aim, supernatants were harvested from monocyte cultures infected for 24 hr with Mtb (MOI 1 : 1), and the release of proinflammatory and regulatory cytokines was analyzed (Figure 1). As control, the cultures were stimulated for 24 hr with LPS (1 μg/ml). Monocytes responded to Mtb or LPS stimulation by producing IL-6, TNF-α, and IL-1β and the regulatory cytokines IL-10 and IL-23, although at different extent. Conversely, the weak secretion of IL-12p70 from LPS-treated monocytes was absent in monocyte cultures infected with Mtb. These results indicated that Mtb differentially induces the release of IL-12p70 and IL-23 by infected monocytes and, in turn, modulates the T cell-dependent inflammatory responses by acting on the balance between Th1 and Th17 cells [14]. Interestingly, our observations are in line with recent findings showing the involvement of IL-23 in mediating protection against intracellular pathogens [15, 16].

3.2. Expression and Regulation of IL-12 Family Members in Mtb-Infected Monocytes. To further investigate the expression of IL-12 family member in Mtb-infected monocytes, we extended our analysis to another member of IL-12 family, such as IL-27, a heterodimeric protein composed of IL-27p28 and EBI3 [17]. To this aim, total RNA was extracted 8 hr after Mtb infection or LPS treatment, and the transcripts for EBI3 and IL-27p28 subunits were evaluated by real-time RT-PCR (Figure 2). The data showed that LPS induced the transcription of both IL-27 subunits while Mtb promoted only the expression of EBI3 subunit. These findings suggested that signalling pathways regulating the expression of IL-12 and IL-27 mediated by the IRF-3/IFN-β axis are not operative in Mtb-infected monocytes. Indeed, evidences indicating the involvement of both NF-κB and IRF-3 in the expression of IL-12p35 have been provided by Goriely and collaborators [18, 19]. In addition, our previous study showed that the expression of IL-27p28 subunit was induced by IFN-β alone or during LPS-induced maturation of DC in a type I IFN-dependent manner through IRF-1 activation [12]. Therefore, we sought to investigate the expression of IFN-β and the related IRF-3 activation in monocytes challenged with Mtb (Figure 3). IRF-3 is a transcription factor present in the cytoplasm in a latent form, which is activated by two kinases known as IKK-ε and TBK-1 at the level of specific serine residues (Ser385 and Ser386) present at the C-terminal [20]. In contrast with previous data showing the ability of Mtb to promote IFN-β expression in human DC via NF-κB and IRF-3 activation [13], neither IFN-β nor IRF-3 induction was observed in monocytes infected with Mtb in spite of a strong NF-κB binding activity to kB sequences within IFN-β promoter (Figure 3). In particular, to investigate IRF-3 activation, immunoblots were performed with whole cell extracts prepared at different time

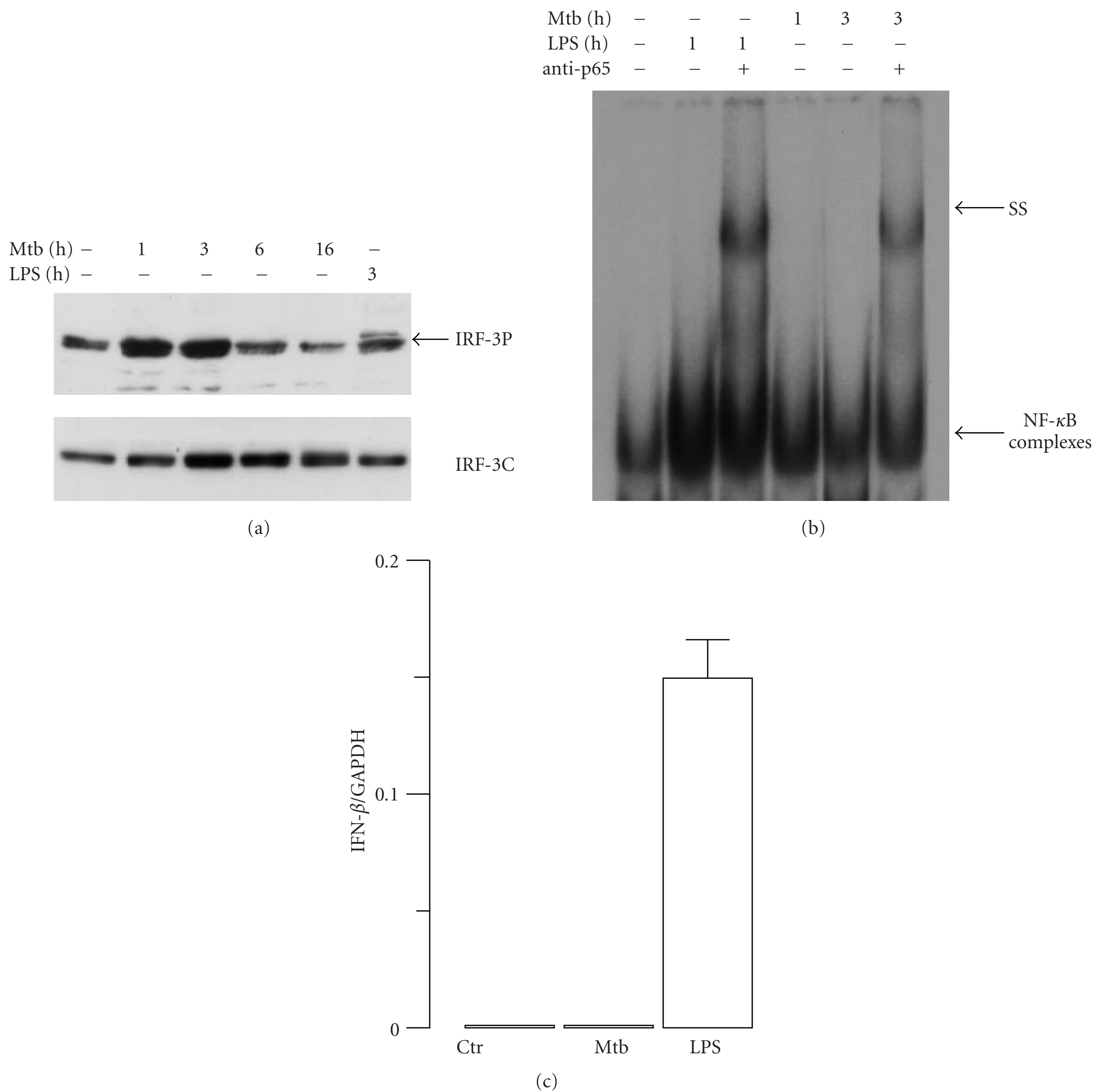

Figure 3: Analysis of IFN-β gene regulation in Mtb-infected or LPS-treated human monocytes. (a) Total cell extracts were prepared at different times following infection with Mtb or LPS treatment. Whole cell extracts (30 μg) were analyzed on an SDS-7% PAGE gel and subjected to immunoblot analysis with anti-IRF-3 antibody to detect the phosphorylated IRF-3 isoform (IRF-3P; upper panel). The total content of IRF-3 was evaluated as an internal loading control (IRF-3C; lower panel). The results shown are from one of three experiments performed with cell extracts from different monocyte cultures that yielded similar results. (b) Nuclear extracts were prepared from Mtb-infected at different time points or from cells treated for 1 hr with LPS. Five μg of nuclear proteins were subjected to EMSA analysis using as oligonucleotide the κB-IFN-β sequence. Supershift assays were performed after incubation with anti-p65 Abs as indicated (ss). (c) RNA was extracted from monocytes infected with Mtb (MOI = 1) or treated with LPS (1 μg/ml) for 8 hr. Real-time reverse transcription-polymerase chain reaction was performed to measure the expression of IFN-β. The results are shown as a ratio to the GAPDH level and represent the mean ± SE of triplicate values. The results shown are from one out of three experiments performed with different donors that yielded similar results.

points following infection of monocytes with Mtb or after 3 hr of LPS treatment (Figure 3(a)). To study whether Mtb infection was able to activate NF-κB complex, monocytes were infected with Mtb for 1 or 3 hr or treated with LPS for 1 hr. NF-κB DNA binding was detected 3 hr after Mtb infection and 1 hr after LPS treatment. We confirmed the activation of NF-κB by supershift experiments using Abs raised against the p65 subunit (Figure 3(b)). In addition, the expression of IFN-β was investigated by real-time RT-PCR performed on mRNA extracted from Mtb-infected or LPS-treated monocytes (Figure 3(c)). While LPS-treated monocytes expressed IFN-β mRNA, Mtb failed to stimulate IFN-β transcription at 8 hr after infection (Figure 3(c)) and at later time points (data not shown). Collectively, these data showed that although monocytes possess the machinery to activate IRF-3 in response to LPS (Figure 3), Mtb does

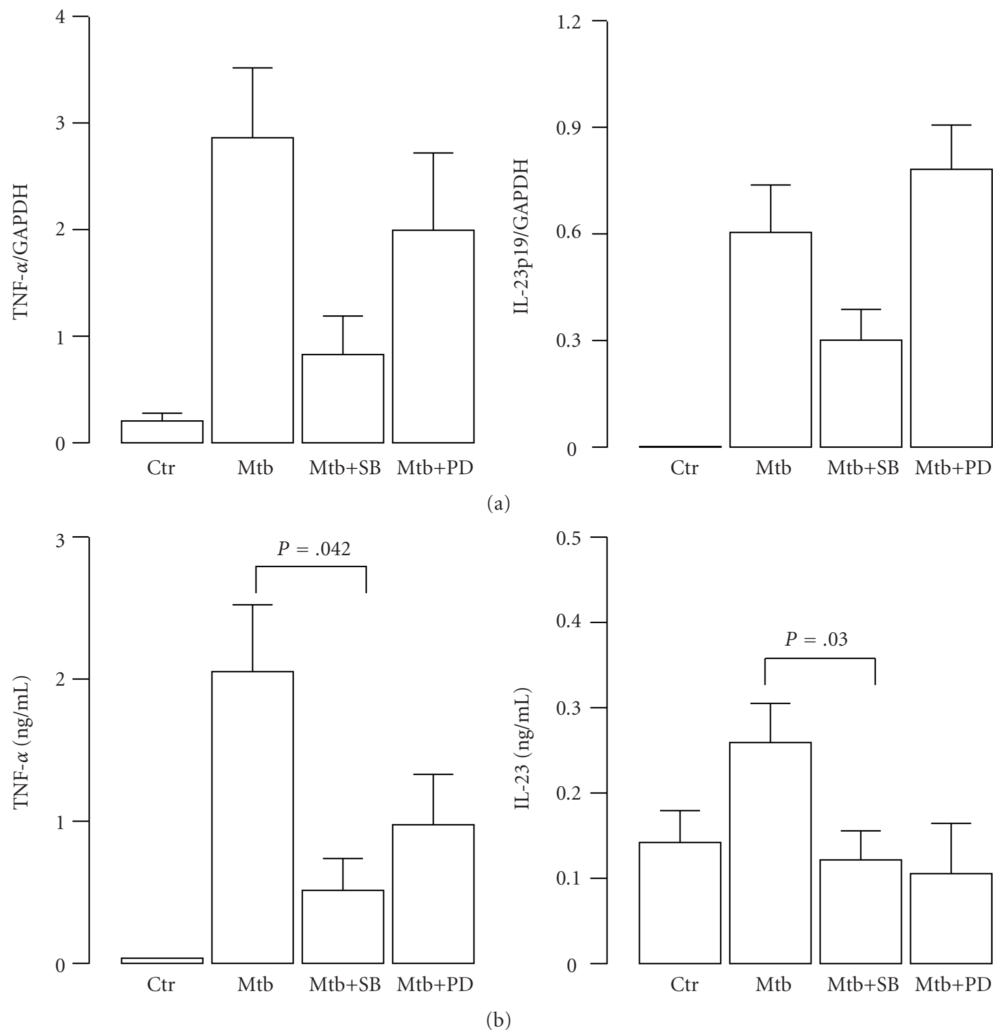

Figure 4: Regulation of IL-23 expression by MAPK in Mtb-infected human monocytes. Monocytes were pretreated or not with p38 (SB203580) or ERK (PD98059) inhibitors for 30 min and afterwards were infected with Mtb as indicated. (a) After 8 hr, RNA was extracted and real-time RT-PCR was performed to measure the expression of TNF-α and IL-23p19 subunit transcripts. The results shown are from one out of three experiments performed with different donors that yielded similar results. (b) After 24 hr, the cell culture supernatants were collected, and the production of TNF-α and IL-23 was measured by CBA or ELISA assay. The results represent the mean ± SE of four independent experiments.

not trigger such pathways and, therefore, does not elicit the expression of IL-27 and IL-12p70. The TLR-4-mediated activation of IRF-3, occurring in Mtb-infected DC, seems to be impaired in monocytes through a mechanism not yet identified. We can also envisage that Mtb triggers different receptors on monocytes and DC leading to the activation of distinct intracellular pathways and, in turn, to the secretion of specific cytokine profiles.

3.3. Regulation of TNF-α and IL-23 Expression via MAPK in Mtb-Infected Monocytes. Next, we explored other possible pathways activated in infected monocytes, which could account for the production of proinflammatory and regulatory cytokines. In particular, we focused on MAPK signalling that has been described to be involved in the production of inflammatory cytokines (such as TNF-α, IL-6, and IL-12p40) and therefore considered important for the initiation of an effective immune response against Mtb [14, 21–24]. Having found that Mtb induced rapidly both ERK phosphorylation and p38 phosphorylation [9], we investigated the involvement of these pathways on IL-23 expression given its key role in controlling the inflammatory response directed against Mtb [14, 25]. As control, we analyzed the effects induced by the addition of these MAPK inhibitors on the Mtb-induced expression of TNF-α, which has been previously demonstrated to be regulated by MAPK [22, 26]. Total RNA was extracted in monocyte cultures pretreated for 30 min with MAPK inhibitors and afterward infected with Mtb for additional 8 hr. We observed that p38 was likely involved in Mtb-induced IL-23 and TNF-α expression since a strong reduction of transcripts coding for TNF-α and

IL-23p19 subunit was observed in Mtb-stimulated cultures pretreated with SB203580. On the contrary, the Mtb-stimulated expression of IL-23p19 was slightly reinforced while that of TNF-α was only partially reduced by the Erk inhibitor PD98059 (Figure 4(a)). On the other hand, the release of TNF-α and IL-23 was reduced when monocytes were stimulated with Mtb for 24 hr in presence of MAPK pharmacological inhibitors (Figure 4(b)). Taken together these results indicate that Mtb utilizes the MAPK signalling to promote the expression of the regulatory cytokine IL-23, which is in turn involved in the fine regulation of Th1/Th17 balance [16].

4. Conclusions

The importance of type-1 cytokines (IL-12, IL-23, and IFN-γ) in the regulation of innate and adaptive immunity against different intracellular pathogens, including Mtb, has been largely demonstrated both in animal models as well as in individuals with deficiencies in type-1 cytokine signalling pathways, which have an enhanced susceptibility to environmental mycobacteria or to the vaccine strain BCG [3, 14, 27]. The emerging picture from these findings indicates that the interplay between Mtb and phagocytes is crucial for the final outcome of Mtb infection.

Within this scenario, the role of monocytes has been underestimated since the majority of the analysis on the effect induced by Mtb infection in phagocytes has been performed in macrophages and DC. In this study, we have characterized the signalling pathways and the expression of regulatory and proinflammatory cytokines in human primary monocytes infected by Mtb. Interestingly, a selective expression of cytokines known to be involved in the establishment and maintenance of an inflammatory state was observed. Indeed, the simultaneous release of IL-23, IL-6, IL-1-β, and TNF-α and the lack of IL-12, IFN-β, and IL-27 production from infected monocytes, likely recruited in the lung, might impact on both innate and adaptive immune response directed against Mtb. It has been shown that IL-12 family cytokines are involved in the autocrine/paracrine regulation of antimycobacterial activity of macrophages [28, 29]. Indeed, the treatment with IL-12 and a soluble receptor for IL-27 during infection reduced the growth of bacteria recovered from macrophages. Moreover, IL-12 and IL-27 balance influenced also the profile of proinflammatory cytokines and chemokines produced by macrophages. In particular, the neutralization of IL-27 by soluble receptor has been shown to augment the levels of TNF-α and IFN-γ during early infection and may allow macrophages to better combat Mtb at a critical time. Based on these evidences, we can envisage that the absence of IL-27 production from Mtb-infected monocytes could lead to a more efficient elimination of mycobacteria, which is crucial for the containment of the infection.

In addition to the modulation of antimicrobial activity, the cytokines released by Mtb-infected monocytes may also influence the induction of T-cell immunity acting on the balance between Th1/Th17 memory responses. Indeed, IL-12p70 is required for the optimal IFN-γ T-cell response, which is crucial for control of Mtb growth while IL-23 can induce IFN-γ responses in the lung if IL-12 is not present. However, the major property of IL-23 is the ability to sustain the function of IL-17-producing $\gamma\delta$ T [30] and the Th17 expansion [15, 16], which are, respectively, essential component of the early and late protective immune response directed against intracellular pathogens within the lung, respectively.

Although the role of Th17 in the immune response to Mtb is still controversial [31], future vaccine strategies have to consider also a correct stimulation of monocytes, which participate to the immune response against Mtb not only by supplying the infected lung tissues with precursors of macrophages and DC, but also providing a cytokine milieu favouring the IL-17 response.

Acknowledgments

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Review Article

The Impact of Transcriptomics on the Fight against Tuberculosis: Focus on Biomarkers, BCG Vaccination, and Immunotherapy

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In 1882 Robert Koch identified *Mycobacterium tuberculosis* as the causative agent of tuberculosis (TB), a disease as ancient as humanity. Although there has been more than 125 years of scientific effort aimed at understanding the disease, serious problems in TB persist that contribute to the estimated 1/3 of the world population infected with this pathogen. Nonetheless, during the first decade of the 21st century, there were new advances in the fight against TB. The development of high-throughput technologies is one of the major contributors to this advance, because it allows for a global vision of the biological phenomenon. This paper analyzes how transcriptomics are supporting the translation of basic research into therapies by resolving three key issues in the fight against TB: (a) the discovery of biomarkers, (b) the explanation of the variability of protection conferred by BCG vaccination, and (c) the development of new immunotherapeutic strategies to treat TB.

1. Introduction

The challenge of the World Health Organization Stop TB Strategy (WHO-STOP TB) and the Global Plan to Stop TB [1, 2] is to eradicate tuberculosis (TB) by 2050, while the United Nations Millennium Development Goals aim to halve TB prevalence and deaths by 2015, as compared to their levels in 1990. These are important objectives for public health considering the high burden of the disease, with almost 4 deaths each minute [1]. Moreover, the WHO estimates that 90% of multidrug resistant tuberculosis (MDR-TB) cases are not diagnosed and treated according to international guidelines. Extensively drug-resistant cases of TB (XDR-TB) have been reported in 59 countries since the first description of XDR-TB in 2006 [1].

Since the development of the Bacillus Calmette-Guérin vaccine (BCG) in 1921 and the discovery of the principal anti-TB drugs between 1940 and 1960, essentially no new vaccines or drugs specific for this infection have become available on the market; nonetheless, the use of "-omics" approaches to better understand the host-bacillus interaction, pathogen biology, and host resistance/susceptibility has provided a new impulse for the development of anti-TB strategies. These high-throughput methodologies have recently been applied to delineate the bacillus infective process and to understand the molecular responses of different immune mechanisms. These methods will ultimately lead to a more rational design of novel prophylactic and therapeutic tools against TB.

Microarray technology has been demonstrated to be an especially important tool in these studies and has been applied by different laboratories around the world that are trying to identify specific molecules responsible for key aspects of the disease. Microarray studies have evaluated a myriad of elements of the bacillus and host during the infection. Most studies have focused on identifying novel

drug targets and analyzing the gene expression of the host during the course of cell invasion and subsistence [3, 4]. A considerable number of articles have attempted to identify the genetic bases of different grades of virulence among *M. tuberculosis* strains [5, 6], as well as among *M. bovis* [7], that cause TB in bovines and also in humans [8].

A number of studies have evaluated the response of the innate immune cells that the pathogen confronts when it invades the lungs. Special attention has been devoted to macrophages [9] and also to the response of dendritic cells to the infection [10, 11]. These studies underline the importance of these cells as the main orchestrators of the priming of the adaptive immune responses; in TB the Th1 response is essential to effectively clear the infection [12]. These studies were performed with a variety of cell sources (i.e., Different cell lineages, bronchoalveolar cells, and bone marrow-derived cells), microbe strains and culture conditions [9–11, 13]. They indicated that several genes are involved in the cell response to invasion from mycobacteria, and some of them are possible targets for intervention strategies against the bacillus. Although these studies are important for understanding the close interaction between the bacilli and the invaded cells, they produced highly variable results [13]. This makes it difficult to extrapolate the results to the more complex situation in the lungs, where a highly diverse set of cells are present when the mycobacteria arrives and triggers the innate and adaptive arms of the immune response in a multifaceted manner.

Moreover, as will be mentioned later, novel evidence from the immune response during infection, vaccination, and therapy indicates that a complex array of different players takes part in these processes and engages several mechanisms to effectively clear the infection. This means that the immune response functions through the activation of different types of responses that act at various levels to trigger a well-balanced response, rather than the unique activation of the Th1 mediators. This contradicts the previous perception of the immune system that led to the idea that the quantity of the Th1 mediators could reflect the subversion of the pathogen and correlate with the protection by vaccines, and the response to therapeutic regimens [14, 15].

Taking into account the availability of published material on the immune response to TB, this review will focus on the studies that used high-throughput technology, such as microarrays, to examine three currently important issues in the battle against TB: (1) the development of biomarkers for TB and response to treatment, (2) elucidation of BCG vaccine efficacy/failure, and (3) the development of novel therapeutic strategies against the infection by boosting the host immune response.

2. Biomarkers of TB

We define biomarkers as molecular features that indicate a defined status of the host in relation to any process or intervention. For TB, biomarkers for different situations are needed. These situations include protection by vaccination, discrimination of latent and active disease to facilitate rapid diagnosis, and treatment outcome or assess relapse risk [16, 17]. Therefore, these biomarkers would be of enormous value to evaluate new candidate vaccines, drugs or any other interventional strategy by accelerating their translation to the clinic [16, 17].

The power of transcriptomics to identify transcriptional biomarkers was previously demonstrated in classical studies on breast cancer, where, through the analysis of the gene expression signatures of primary tumors, identification of a predictive outcome profile was possible [18]. Along these lines, Jacobsen et al. [19] studied the gene expression signature of peripheral blood mononuclear cells (PBMCs) of patients with active TB compared to infected (latent TB) patients and nonsinfected healthy individuals. The evaluation, which combined microarrays, real-time PCRs and a linear discrimination analysis approach, allowed the authors to identify a set of genes including Rab33A, lactoferrin and CD64, that could differentiate between these three groups of individuals. More importantly, the different gene expression patterns of PBMCs allowed for discrimination between individuals with latent TB and patients with active disease and also between patients with recurrent disease and cured TB patients [20]. In this work, the authors used microarray chips to test 50,000 gene sequences, and after discriminatory computational-based analysis, they claimed that they could differentiate the four groups of individuals using a set of 9 genes (RIN3, LY6G6D, TEX264, C14orf2, SOCS3, KIAA2013, ASNA1, ATP5G1, and NOLA3).

Although no studies have been published, to our knowledge, reporting the clinical validation of these biomarker candidates, the studies mentioned above are important because the rationale for the approach rests on the study of PBMCs as a surrogate for lung tissue. These cells are considered a complex sampling that better resemble the environment at the site of the infection, as opposed to the evaluation of one specific cell type. In our view, this approach is appropriate as a starting point for further studies of the host response during the course of the infection and for distinguishing between good and poor responders that are subjected to interventional strategies (vaccines and therapies).

More recently, Berry et al., [21] reported the analysis of the transcriptome of patients with latent TB and active TB and compared them to healthy subjects. The identification of a 393 gene set enable the authors to identify a gene expression signature that differentiates between latent and active TB. These results were confirmed comparing the transcriptome of patients of different parts of the world demonstrating the power of the strategy adopted. Since this patients were included into the groups analyzed with no other criteria than the clinical data in order to define them as patients with active or latent TB, and that 10%–20% of the patients classified as with latent TB were grouped into the active TB group by their gene expression profiles, the authors suggested that these patients could represent those who will develop active disease in the future [21]. Although this assumption needs to be confirmed by a longitudinal analysis, this study is of outstanding importance not only for giving support to

the notion that a transcriptomic approach for TB biomarker development is feasible, but mainly because obtaining such biomarkers seems to be a matter of time

3. Assessment of BCG Vaccination Efficacy/Failure

Another important issue confounding the TB epidemic is why BCG vaccination exhibits high variability in conferring protection among different populations. Although BCG is the most commonly used vaccine in the world with more than two billion doses administered to humans since its development by Albert Calmette and Camile Guérin almost 90 years ago, it only confers good protection against TB in infants and not adults [22]. In several murine models, BCG confers good protection against experimental challenge with *M. tuberculosis* and therefore is commonly used as a "gold standard" for comparing the performance of new vaccine candidates. For this reason, several articles have described the effects of BCG immunization on the immune response in different experimental models and also in humans [23], but the exact immunological mechanisms that leads to BCG protection or to its failure remain poorly understood.

With regard to transcriptomic approaches, the study of Behr et al. [24] is exceptional. These authors evaluated BCG samples used in different parts of the world using DNA microarrays and found a surprisingly high variability between these vaccine samples in specific genome regions associated with virulence. This provided the first explanation for the global divergence in BCG vaccination effectiveness from the microbial perspective; however, this phenomenon might not only be due to bacilli variability. It may also be due to variability in the protection responses among individuals [23]. This has been noted in other vaccination strategies and treatments.

Understanding BCG failure or efficacy is important because it represents a way to discover the mechanisms underlying protection, which could be useful in the formulation of more efficient vaccines. In this context, host transcriptome analysis following BCG vaccination represents a valid approach due to the robustness of the results and the large set of data obtained. Despite the importance of such studies, only a few have been published describing the transcriptome analysis in the context of BCG vaccination.

Mollenkopf et al. [25] compared the modulation of gene expression of mice immunized with BCG to those experimentally infected with *M. tuberculosis*. This strategy was useful to identify genes specifically responsive to BCG immunization. A major concern of this evaluation was the use of intravenous inoculation of the vaccine rather than subcutaneous injection, which is used in the classical vaccination protocol of BCG; nevertheless, this study is of great importance because it illustrated a rapid lung transcriptome response after BCG immunization.

Aranday Cortes and coworkers [26] studied the lung and spleen transcriptome response of vaccinated and unvaccinated mice. The data analysis allowed identifying expression profiles that could be associated with vaccination efficacy. The mice from this study were intradermally vaccinated and infected with *M. bovis*, and the results confirmed and extended the data from the previous Mollenkopf study [25]. Curiously, so far there have not been any published studies of human samples showing high-throughput transcriptome profiling following BCG immunization.

Data obtained using a more focused method, such as qRT-PCR, to evaluate 16 immune system genes, showed that groups of children vaccinated with different strains of BCG feature Th1 responses and Treg cells associated genes. Moreover, the results showed that BCG vaccines used in Brazil and Denmark induce a response that is related to the Th1 response in the PBMCs from neonates, while the response to Japan BCG vaccines is related to an inflammatory profile [27]. This study indicates the feasibility of gene expression studies in human samples following BCG vaccination. Moreover, this approach could be useful to better understand the nonspecific, beneficial effects of BCG vaccination in children, a question that remains widely neglected but represents a potential public health problem if BCG is replaced by the new sub-unit vaccines against TB [28, 29].

4. Development of Novel Therapeutic Strategies against TB

The emergence of XDR-TB represents a challenge for the development of new, effective drugs with shorter regimens of treatment [1]. The classical antibiotic research and development includes investigation of novel susceptibility pathways of the mycobacterium metabolism that could be used as targets for these compounds. Once again, transcriptome profiling could be useful to identify the differentially expressed genes in mycobacterium strains susceptible or resistant to antibiotics [30].

In parallel with these pharmacological studies, immunostimulation of TB patients has been proposed as an important element that helps improve the outcome of the disease [31, 32]. Candidate approaches are under intensive evaluation as immunotherapeutic options such as *M. vaccae* and a detoxified cellular fragment compost of *M. tuberculosis* named "RUTI", both tested in clinical trials [33–35]. However, the data indicate the necessity of a more detailed characterization of their mechanisms of action to improve the results observed, in which transcriptomal evaluations could be useful.

DNA-based vaccines constructed with an *M. leprae* 65 kDa heat-shock protein (DNA-hsp65) and developed by the team of one of us (CLS) represent a new strategy for immune intervention against TB. DNA-hsp65 immunization has been applied successfully in different experimental models of TB. Initially tested as a new vaccine candidate, the data collected since 1992 show that this vaccine stimulates a strong Th1-specific immune response towards the hsp65 immune-dominant antigen [36, 37]. Stimulation with four doses of DNA-hsp65 vaccine can be maintained by using different formulations of microspheres or liposomes or prime-boost strategy reducing up to 16 times the amounts

of DNA needed to maintain the protective efficacy [38–40]. Moreover, because mycobacterial Hsp65 is one of the most extensively studied antigens among the several components of the HSP family with regard to their antitumoral properties [41–43], we evaluated the effects of treatment with hsp65 in a phase I clinical trial. Hsp65 intratumoral vaccination resulted in no toxic effects in head and neck squamous cell carcinoma patients [44, 45].

In 1999, Lowrie and co-workers [46] showed that the DNA-hsp65 vaccine could be applied effectively to treat active TB, thus becoming the first DNA vaccination protocol to display such properties against an intracellular pathogen. Subsequent studies that demonstrated the advantages of DNA-hsp65 to treat latent TB infection and MDR-TB [47, 48] indicated their importance as one of the most promising molecules against TB [31, 49].

Although significant progress has been made towards a better understanding of the prophylactic mechanism of DNA-based vaccines in driving the immune response, systematic characterization of DNA immunotherapy was not undertaken before the work developed by our research group [50]. We reasoned that the elucidation of the molecular events underlying cellular states that occur during DNA-hsp65 immunotherapy in response to *M. tuberculosis* infection could be evaluated by quantifying differential gene expression using microarray hybridizations and qRT-PCR.

The results showed that the effects of DNA-hsp65 immunotherapy in mice with active TB could be characterized by gene expression analysis. A high number of transcripts that code for proteins associated with the immune system made it possible to distinguish treated from nontreated animals [50]. Functional analysis of this group of genes suggested that DNA-hsp65 therapy inhibits Th2 cytokines and regulates the intensity of inflammation through fine tuning of the expression of various genes, including those that code for IFN-γ, IL-17, lymphotoxin-α, TNF-α, IL-6, TGF-β, iNOS, and Foxp3. In addition, the expression levels of a large number of genes and expressed sequence tags previously unrelated to DNA therapy were modulated.

A correlation between gene expression and histopathological lesions of the lungs validated the data [50]. Because the effects of DNA therapy were reflected in the modulation of gene expression, the genes identified as differentially expressed could be considered transcriptional biomarkers of DNA-hsp65 immunotherapy against TB.

Thus, we combined the methods that involve characterization of the transcriptional signatures of lungs from mice infected with *M. tuberculosis* and treated with Hsp65 as a genetic vaccine along with microarray, qRT-PCR analysis and finally correlation of the gene expression data with the histopathological analysis of lungs. This was instrumental to obtain a better understanding of the mechanism of DNA-based immunomodulation [50]. Furthermore, we proposed the term "transcriptype" to characterize transcriptome signatures that differ among healthy individuals, infected individuals, or infected individuals treated with DNA immunotherapy [50].

5. Systems Biology Tackles the Limitations on the Interpretation of Transcriptional Data

High-throughput transcriptome analysis using microarrays is helping to identify biomarkers for TB, understand BCG efficacy/failure, and develop alternative treatments such as DNA-based immunotherapy. Nevertheless, the relatively few studies in this field reflect the striking difficulty in integrating the large amount of data generated and effectively translating it to clinical use. One of the main reasons for this problem is the lack of integrative tools that provide investigators a more complete and understandable picture of the transcriptome modulation; however, some integrated efforts are being conducted in this area. The Tuberculosis Database (TBDB) at Stanford University and the Broad Institute [51, 52] provide a platform with information regarding the *M. tuberculosis* genome, protein, and gene expression data. They also contain information regarding other mycobacterium-related species, as well as gene expression data from mouse and human samples. Moreover, future expansion of this platform is planned to generate tools that will be able to combine gene expression from the immune system with other data.

Systems biology is a relatively recent discipline that aims to combine, and therefore better understand, the high-throughput sets of genomic, transcriptomic, and proteomic data using bioinformatic tools. Recently, systems biology studies analyzed integrative data to evaluate the effectiveness of the yellow fever vaccine YF-17D [53, 54]. This vaccine represents the most successful prophylactic immunization against an infectious disease due to its high efficacy, safety, and long-lasting protection that can reach 35 years [55]. Even though more than 540 million doses have been administered to humans since its development in 1937, the mechanisms underlying its efficacy remained poorly defined until recently. Seminal studies combined data from human gene expression signatures after YF-17D vaccination using multiparameter flow cytometry analysis for cell type or cytokine evaluation and computational analysis [53, 54].

This strategy led to the identification of key elements of the innate and adaptive immune system that predict the immunogenicity and efficacy of YF-17D vaccination, thus providing a new perspective to vaccinology [56] that could be applied to the combat of other infections as TB.

6. Concluding Remarks

The application of microarray technology has been demonstrated in different settings. Currently, with the evolution of novel computational analysis tools, the integration and extraction of the vast information generated by this technology are supporting new and exciting discoveries. This systems biology approach is now being applied to the development of novel diagnostics, vaccines and, drugs against TB, as well as to other areas of TB research [57, 58].

In our opinion, some key issues from the "host point of view" that could benefit from these integrative tools are the investigation of BCG vaccine efficacy/failure, the determination of the importance of BCG nonspecific beneficial effects,

and the discovery of TB biomarkers and the evaluation of novel therapeutic strategies, such as immunotherapy and chemotherapy.

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Review Article

A Spotlight on Liquefaction: Evidence from Clinical Settings and Experimental Models in Tuberculosis

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Liquefaction is one of the most intriguing aspects of human tuberculosis. It is a major cause of the transition from the infection to active disease (tuberculosis, TB) as well as the transmission of *M. tuberculosis* to other persons. This paper reviews the natural history of liquefaction in humans from a pathological and radiological point of view and discusses how the experimental models available can be used to address the topic of liquefaction and cavity formation. Different concepts that have been related to liquefaction, from the influence of immune response to mechanical factors, are reviewed. Synchronic necrosis or apoptosis of infected macrophages in a close area, together with an ineffective fibrosis, appears to be clue in this process, in which macrophages, the immune response, and bacillary load interact usually in a particular scenario: the upper lobes of the lung. The summary would be that even if being a stochastic effect, liquefaction would result if the organization of the intragranulomatous necrosis (by means of fibrosis) would be disturbed.

1. Liquefaction of Necrotic Tissue Takes Place in the Upper Lobes in Humans

1.1. The Primary Infection Is Usually Not Seen. Primary infection is sometimes associated with Ghon's Complex, that is, the presence of a small lesion in the parenchyma, together with enlarged hilar lymph nodes. The primary infection, (about 0.5 mm of diameter in average) is not detected by the radiologist in around 85% of cases [1]. These lesions have been described as nodular-acinar due to their size and location in the bronchial tree, and from a histological point of view, they are granulomas and characterized by the induction of well-encapsulated central necrosis (or caseum) [2]. Such lesions being usually found in necropsies of subjects without any evidence of active TB. They are commonly associated with a "benign evolution" of the infection [3] in which the fibrosis plays a paramount role. Two patterns of fibrosis have been described in these lesions: a central one, based on the production of a collagen matrix to organize a small inert caseum, and a peripheral one, which is the origin of the encapsulation and in which the fibroblasts can be easily identified, and related with the external cellular ring, mostly composed by lymphocytes [3]. Usually, the necrotic tissues of these primary lesions calcify, a fact that has been used in studies aiming to detect infected subjects and/or to evaluate the effectiveness of BCG vaccination in preventing the infection [4]. Interestingly, very few if any calcified lesions carry viable bacilli [5].

1.2. The Upper Lobes: the Scenario for Cavitation. Although cavitation in the upper lobes has traditionally been associated with reactivation of old lesions, this image has been found in chest X-ray assay of both recently infected adults or in those with a latent infection (LTBI) that suffered a reactivation [6], thus highlighting the importance of this site in the development of cavitation.

The clinical features observed in immunocompetent adults are the combined result of mycobacterial replication and a destructive host immune response [1]. Thus, the classical findings in chest radiography are upper lobe infiltrates (60%) or cavitary lesions in the lung apex or upper zones of the lower lobes (30–66%) [7], with most patients with

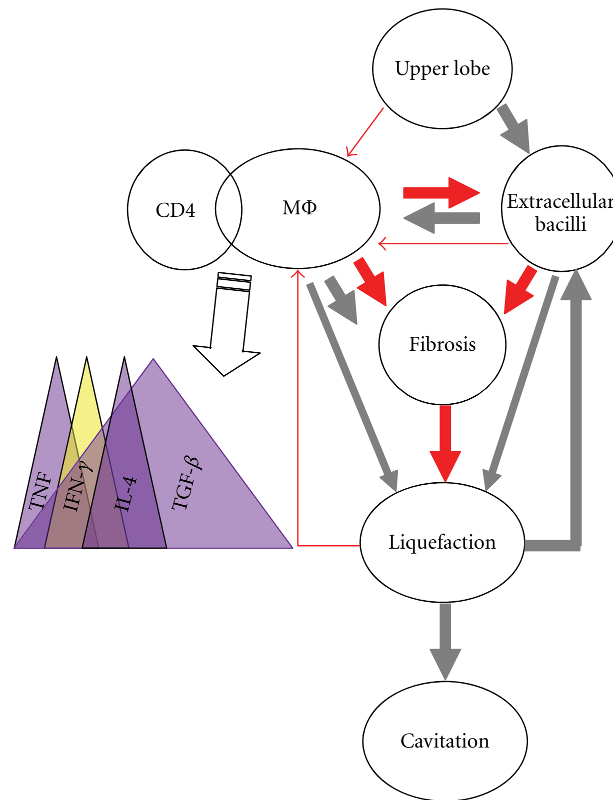

Figure 1: Interactions between the factors involved in the liquefaction process. The colour of the arrows shows the ability to induce a process (in gray) or inhibit it (in red), and the thickness of the arrow is proportional to the intensity of this induction. The upper lobe appears to be the *sine qua non* condition for the process to take place. Macrophage (MΦ) activation and the presence of CD4 is linked to the appearance of different cytokines with time: TNF initially, followed by IFN-γ and IL-4, and TGF-β from the onset and peaking at the chronic phase. All those cytokines are profibrotic (in violet) except for IFN-γ (in yellow). This site mainly undergoes a profibrotic process although there is also a nonspecific antifibrotic effect arising from the macrophages and their enzymatic activity. Extracellular bacilli also have antifibrotic activity and promote macrophage activation although they are also thought to inhibit such activation to some extent. Fibrosis prevents liquefaction whereas liquefaction is promoted by macrophages, the immune response, by promoting the apoptosis of infected macrophages, and extracellular bacilli. Liquefaction induces cavitation, inhibits macrophage activation (indeed, it appears to destroy them), and promotes extracellular bacillary growth. Overall, liquefaction comes first, and then the extracellular multiplication of bacilli occurs. Fibrosis, and thus resume of the liquefaction, would occur only after the number of extracellular bacilli is reduced sufficiently to allow attempts at healing to take place. Finally, a large number of extracellular bacilli results in tissue destruction, cavity formation, and the death of the macrophages that attempt to inhibit such bacillary growth.

pulmonary TB being observed to have multiple cavities [8] with sizes ranging from between 1 cm to more than 5 cm [9]. In contrast, severely immunosuppressed patients, such as HIV positive patients with CD4 < 200 mm^3, the upper lobe infiltrates and cavitation are reduced to 20% and 10%, respectively [7].

1.3. Does the High Oxygen Pressure Favor Liquefaction? As regards the tropism of cavity formation in the upper lobes, it has been accepted since the 1940s that relative ischemia is likely to affect the apical localization of phthisis in humans. Thus, the very low incidence of progressive apical lesions in patients with mitral stenosis can be explained by the fact that they have a higher pulmonary arterial pressure whereas the very high incidence in patients with pulmonary stenosis is due to the fact that they suffer a global ischemia in the lungs [10].

Some years later, West [11] demonstrated that the blood flow in the lowermost pulmonary regions was up to 10 times higher than in the uppermost regions whereas ventilation was only 1.5-times higher, thus generating a progressive fall in the ventilation-perfusion ratio of 3.3 : 0.63 from the apex to the base. This generates large regional variations in the alveolar partial pressures of oxygen, carbon dioxide, and nitrogen, with a difference of 43, 15, and 29 mm of Hg, respectively, and therefore an increase of 41% and a decrease of 39% and 5%, respectively, when compared with the average values.

On the other hand, the differences as regards blood gas content are much lower because of the shape of the oxygen dissociation curve, the saturation (oxygen content) falling by a 4%. In contrast, the slope of the carbon dioxide dissociation curve varies by 7%, increasing the pH to 7.5, a value outside the normal range for arterial blood.

All these factors suggest that the bacilli phagocytosed by alveolar macrophages in the upper lobes will have a much higher oxygen tension than those in the lower lobes, thereby favoring their growth [12]. In contrast, the much lower blood flow will reduce both the number of cells to come at the infectious foci and the quantity of bacilli drained by the lymphatic system, thus reducing the immunological surveillance. In addition, once a granuloma is set and local inflammatory response is induced during the course of the infection, the relative alkalosis will reduce the maturation capacity of the dendritic cells, which is stimulated by acidic pH (5.5–7.0), thereby also repressing the development of Th1 immunity [13] (Table 1).

The impact of a high oxygen pressure in promoting TB has been recently epidemiologically demonstrated by the description of a lower TB incidence in a population living at altitude (3000 m) compared to another one with similar socioeconomic status living at sea level [14].

On the other hand, the effect of the ventilation gradient is controversial: the weight of the lung itself can cause some "sagging" within the thorax, the nondependent alveoli being more expanded than the dependent alveoli, much like a "slinky", or coiled spring, hanging under its own weight, where the upper coils are further apart than the lower coils [15]. In contrast, some authors have reported that gravity has only a minor effect on pulmonary blood flow distribution and that two-thirds of the variability in perfusion distribution is determined genetically [16]; this fact could be used as a genetically driven susceptibility to acquire TB.

Moreover, mechanical stress also needs to be considered, as upper lobes have the highest values. Thus, in disease states where the structural integrity of the lung is weakened, mechanical failure occurs at this site. In practice, destruction of parenchyma in centrilobular emphysema is most marked near the apex, with a gradation as we move down the lung. Rupture of a bleb on the upper lobe is almost always the cause of spontaneous pneumothorax in an otherwise healthy young patient [17]. As occurring in the upper lobes, mechanical stress must have a relation with the induction of cavitation (Table 1).

1.4. Are Tuberculomas the Consequence of a Failed Liquefaction Process? A tuberculoma typically appears as a fairly discrete nodule or mass in which repeated infection sites have created a core of caseous necrosis surrounded by a mantle of epithelioid cells and collagen with peripheral round-cell infiltration. The majority of tuberculomas are less than 3 cm in diameter although lesions up to 5 cm have been reported and tend to be mainly found in the upper lobes [18]. Interestingly, both the tuberculomas and the cavitated lesions shown a marked inflammatory response and a high bacillary concentration, but the former having much stronger vascularization and higher proliferative activity, thus indicating a continuous local immune activity [19].

Moreover, tuberculomas and cavitated lesions are similar in size [9, 18]. It has been suggested that the former could be an intermediate phase between a controlled or "benign" nodule and a cavitated lesion, with the only difference between them being the liquefaction of the necrotic tissue. Another hypothesis proposes that tuberculomas are actually closed cavities [9].

2. The Liquefaction Process Has Only Been Studied in an Experimental Rabbit Model

Tuberculosis in rabbits has often been used as surrogate model for the study of this disease in humans. Unfortunately, the lack of appropriate reagents has led this model to be considered obsolete by immunologists, and logistical problems (i.e., housing and lack of inbred rabbits) have resulted in it being poorly used and underexploited.

The systematic work undertaken by Dannenberg [20], following in the steps of Lurie [21] and working with relatively unsophisticated tools, has provided a great deal of interesting information. The main breakthrough arose as a result of breeding susceptible and resistant rabbit strains (today commercially available rabbits resemble Lurie's resistant strains). Susceptible subjects experienced an earlier increase in bacillary growth and also required a higher bacillary load to trigger an adaptive immune response than the resistant ones, developing in the lesions larger necrosis but (contrary to what would be expected) without liquefaction. This could be due to the fact that they died before these events occurred, or because their macrophages were less activated.

The authors hypothesized on the dichotomy between delayed type hypersensitivity (DTH) and cell-mediated immunity (CMI) to explain these differences in necrosis induction. They considered the susceptible rabbits developed a weak CMI, thus continuing using the tissue-damaging immune response (necrotizing DTH) to stop the intracellular bacillary multiplication. Then, the caseous center enlarges, and local lung tissue is destroyed [20]. Eventually, following infection with virulent bacilli, the susceptible rabbits showed high DTH, because the virulent bacilli reached higher titers in them and therefore provided a larger antigenic stimulus. Nowadays, we know that the DTH response is linked to CMI, being a local manifestation of the arrival of specific lymphocytes attracted by the infected macrophages or, in the case of the tuberculin skin test, those that phagocyte tuberculin [22]. It has been argued that the concentration of the antigens that elicit each is different: Protein Purified Derivative (PPD) elicits DTH in very low concentrations whereas antigens eliciting CMI probably need higher concentrations [20, 23]. On the other hand, the role of specific immunity was clearly linked to the liquefaction process when Yamamura et al. demonstrated that desensitizing with tuberculin-active peptides protected against induction of cavitations in rabbits [24]. This model was also used to demonstrate the more intense the TST the higher the number of cavitations, which was probably due to the larger number of tubercle bacilli in these rabbits [25]. This fact correlates with clinical studies, which showed that patients with larger TSTs were more apt to develop active TB [26].

This observation led to the concept that liquefaction required the action of activated or strongly active macrophages, a fact which can nowadays be compared to the situation found in humans suffering from AIDS (see above), who usually do not develop liquefaction and cavitation of the lesions. These findings, therefore, link the action of macrophages to the presence of a specific immune response.

Macrophage hydrolytic enzymes are thought to be responsible for the liquefaction of tuberculous lesions [27]. However, although a number of such enzymes have been identified to date, there is still no direct evidence to confirm that their activity is key to the development of liquefaction as a natural progression of infection.

Finally, experiments showed that keeping *M. tuberculosis*-infected rabbits erect in a harness for eleven hours each day produced cavities in the upper lobes, resembling the situation observed in humans [28].

3. The Mouse Model and Its Focus on Necrosis Formation

Typically considered as a resistant host as it is able to survive infection for a long time, nevertheless, the mouse model does not usually develop liquefaction. In fact, we have postulated that these animals are tolerant instead of resistant, as they can harbor a high bacillary concentration without hampering their health status [29]. This can be related with their low capacity to elicit a DTH against PPD, and thus its low induction of intragranulomatous necrosis in this host [30]. Furthermore, the induction of intragranulomatous necrosis in mice has been linked to the genetic background of the animal although this not precluding a better or worse control of the bacillary load, after low-dose aerosol infection. This proposal was based on a comparison between mice with the same H-2 background that develop necrosis but are either more resistant (C57BL/6) or more susceptible (129/Sv) and those that do not develop necrosis but are more resistant (BALB/c) or more susceptible (DBA/2) to the infection [31]. Results showed that 129/Sv mice developed larger necrotic lesions than C57BL/6 and that this was found to be linked to a higher bacillary concentration in the lungs. Using the same infection route, knocking out crucial cytokines or cell types implicated in protection against infection did not prevent the induction of necrosis. Rather, it increased the size of the lesions if the knocking out was related to an increase in the bacillary load [32]. Consolidation of the necrosis was obtained by the chronic phase of the infection (i.e., week 6) in all cases, a fact that could be linked to the accumulation of foamy macrophages (FM), which have been postulated to be the source of the intragranulomatous necrosis [33, 34].

FM have been also related to the drainage of nonreplicating bacilli out from the granuloma through the alveolar spaces, as demonstrated by Sköld and Behar [35], therefore supposed to be a source of constant endogenous reinfection, a factor that has been suggested to be a way of maintaining the Latent Tuberculosis Infection (LTBI) [36]. In this regard, it can be claimed that the mice model is not a good one to reproduce LTBI as it is seen in humans, because it is thought that the main population of latent bacilli resides in the intragranulomatous necrosis, and this phenomenon is weak in mice. This is a controversial issue; as looking at the necrosed tissue in humans [3] or big mammals [37], the presence of bacilli is really very low if present. On the other hand, chronic phase in murine TB has clearly demonstrated the presence of a major population of latent bacilli [38, 39].

Indeed, data obtained in the mouse model and mathematically modulated [40] has shown how, at the onset of infection, bacillary growth occurs in individual alveolar macrophages, and that formation of granulomas is an stochastic process that takes place once a critical number of infected macrophages coincides in a time and spatial manner, and is able to persist after becoming a local spot where enough quantity of chemokines is produced and is stable through time. Then, the granuloma is able to attract specific or innate lymphocytes that would activate the infected macrophages, thus being an important defensive structure against *M. tuberculosis* infection, but that requires a constant chemokine production to persist [41]. These particularities in the onset of the infection provide an important window for constant endogenous reinfection even in the presence of protective immunity [40].

There is one mouse strain (C3HeB/FeJ) able to induce liquefaction of the necrotic tissue, with different outcomes related to the control of the bacillary load [42]. Indeed, if the infection is induced by a low-dose aerosol, this liquefaction can be resumed, and the bacillary load is controlled (Vilaplana et al., in preparation), thus showing an important fact, which is that liquefaction is a reversible process, and thus requires a maintenance whereas it persists, together with a high bacillary load, if the infection is induced by a large dose administered intravenously, causing the death of the animal [42]. Liquefaction has also been observed in SCID mice under certain conditions. Thus, if the animals are treated for a period of time with chemotherapy after infection by aerosol and the infection has been allowed to progress, the bacillary load resumes its growth and leads to liquefaction after suspension of the chemotherapy [43]. This phenomenon can be explained as a result of the sudden and massive resumption of growth in already infected macrophages, which leads to the simultaneous necrosis of them and thus to liquefaction due to an inability to fibrose the necrosis caused. This phenomenon, which we call the "synchronic effect" (Table 1), can help to explain the situation in resistant rabbits and immunocompetent humans, where the sudden onset of the immune response can induce a massive apoptosis and/or necrosis of infected macrophages, thus disturbing the balance of tissue organization and causing its liquefaction. The reason why this does not usually happen in immunocompetent mice could be due to its tolerant response against *M. tuberculosis* infection, thus maybe even when they accumulate a large bacillary concentration, it does not accumulate locally, so the "synchronic effect" does not take place. However, it can explain why some severely immunosuppressed patients develop liquefaction: they are probably suffering from a large necrosis and thus more chances for the synchronic of infected macrophages to take place.

Table 1: The factors involved in liquefaction.

(A) The upper lobes are privileged sites
- (1) because of their low perfusion/ventilation ratio, which results in:
  - (a) an increase in bacillary growth inside individual alveolar macrophages due to the high oxygen pressure in the alveolar space,
  - (b) local alkalosis, and thus inhibition of dendritic cell maturation,
  - (c) decreased local perfusion, thus delaying the presentation of antigens at the local lymph nodes,
- (2) the mechanical stress of ventilation makes stabilization of a fibrotic lesion more difficult.

(B) The fibrinolytic ability of the macrophages.

(C) Immune response
- (1) as a result of the synchronic effect, which provokes a massive apoptosis/necrosis of infected macrophages in a short period of time,
- (2) induction of high levels of IFN-γ, which promotes fibrinolysis,
- (3) promotion of a massive entry of macrophages into the lesions.

(D) Extracellular bacillary accumulation
- (1) the accumulation of plasminogen and its activation to plasmin induces fibrinolysis and allows the maintenance of the liquefaction through time,
- (2) generation of a mantle of infected macrophages which maintains the inflammatory response.

Interestingly, the modeling of a Toxoplasma infection in mice, which induces a strong Th1 response, has also provided a further clue by highlighting the delicate balance between TNF and IFN-γ in the fibrin deposits in the lesions, with IFN-γ clearly favoring fibrinolysis [44, 45], thus again favouring the liquefaction process. This information must be framed in time. It is demonstrated that at the beginning of the *M. tuberculosis* infection, IL-1 and TNF are primary cytokines that favor CMI, and thus the arrival of specific lymphocytes which are able to produce IFN-γ, that activates macrophages, so that they can inhibit the growth of tubercle bacilli that they ingest. At the same time, it must be taken into account that TGF-β is present from the very onset of the infection, once macrophages phagocyte apoptotic bodies [46], thus downregulating the CMI and favoring the fibrosis, an activity that increases its intensity as the infection progresses, together with the presence of IL-4, which has also been shown to be profibrotic, which also appears at latest time points [47]. This process is shown in the Figure 1 and shows that liquefaction process, only favored by the presence of IFN-γ is a process that has a very small window to take place. It is also interesting to note the downregulation induced by TNF to the IFN-γ activity which might be a crucial factor to avoid liquefaction [48]. It can be hypothesized that maybe this can be also behind the massive induction of active TB in patients treated with anti-TNF antibodies [49] not only because of the disorganization of the granulomas but for the local increase of IFN-γ (without the counterbalance of the TNF) that induces liquefaction.

4. Nonhuman Primates

The infection of nonhuman primates has also been shown to produce cavities [23], but in a very different manner when compared to the rabbit model, even if probably reflecting more accurately what happens in humans. Cavitation has been detected as soon as seven weeks after the challenge or later, at 28 weeks after infection although this phenomenon has not been studied exhaustively to date [50], and it can be effectively avoided by BCG vaccination in *Cynomolgus* [51]. Furthermore, it has recently been reported that reactivation of the infection occurs after the decrease in CD4 number induced by the SIV virus, without causing cavitation in the animals, thus resembling what happens in AIDS patients.

5. Minipigs Highlights the Role of Fibrosis in Infection Control

Our search for a similarly sized animal to humans with the same degree of intolerance to the bacilli [30] in which an experimental infection could be studied, led to the development of the minipig experimental model. The key point of interest for this model was to reproduce the classical nonvisible lesions of LTBI in humans, qualified as “benign” by Canetti [3]. The results showed two different fibrosis patterns: an inner one, which is well organized due to the influence of myofibroblasts, and an outer one, which was significantly related to the contact with intralobular septae, a structure not present in small mammals (including rabbits and nonhuman primates) and that structure of the lung of big mammals like minipigs (and also humans) [37]. These fibrotic patterns confirmed their similarity with human lesions according to Canetti’s descriptions in necropsies. In this model, mature lesions with calcification appeared very rapidly during progress of the infection (week 6 after challenge). Cavitation has also been described in this host [52], thus we do believe the model highlighting the role of fibrosis in the control of the infection, as with no proper fibrosis the liquefaction process occurs readily.

6. Do the Bacilli Themselves Interfere in the Fibrotic Process?

It is well known that some components of the cell wall of *M. tuberculosis* are able to bind to plasminogen and even to transform it into plasmin, thereby inhibiting the induction of fibrosis [53]. Likewise, a study in *Streptoccocus*

pyogenes showed that the ability to bind to fibrin depends on the specific mammal species concerned, thus it could explain why *M. bovis* is less active against humans than *M. tuberculosis* and vice versa for cows or goats [54]. However, the role of the bacillary bulk is likely to be limited and only relevant when a high extracellular concentration is available in the necrotic and/or the liquefied centre, in this specific way of action, that is, by directly interfering to the fibrin production process. But this process might be paramount for maintaining the liquefaction process and avoiding its reversibility. On the other hand, it has been demonstrated that *M. tuberculosis* specifically upregulates matrix metalloproteinase-1 in human macrophages (whereas BCG vaccine does not) [55]. Excess of MMP activity in the lung, therefore, results in collagen destruction, thus favouring the liquefaction process (Table 1).

7. The Role of Architecture

The alveolar spaces in humans and rabbits are much larger than in mice; therefore, this factor could also plays a role in the healing process of the granulomatous lesion. Indeed, it can be argued that the lung parenchyma, being denser in mice, could exert a protective effect by requiring less intense fibrosis to stabilize the granuloma than in humans or rabbits. Effectively, the volume of the alveolar space in humans and rabbits is 57 and 8 times higher, respectively, than in mice [56]. These data clearly show that rabbits and humans must initiate a much faster and stronger fibrotic response than mice to stabilize the necrotic tissue generated inside of the granuloma and also because the necrotic process is much intense. This finding can also be linked to the higher mechanical stress that suffers the upper lobes [17], which interferes in building a fibrotic structure to support the lesion and also may favor liquefaction processes to take place specifically in this site.

8. Towards a Holistic View of Liquefaction Induction

Although the role of genetics has been accepted for many years, research in the area of TB induction has mainly focused on the deficiencies induced in the immunological response, especially the Th1-Th2 balance, or nutritional elements that favor mycobacterial growth, such as the availability of iron or the lack of macrophage activation due to reduced levels of vitamin D [57, 58]. Somewhat surprisingly, given the fact that males suffer four times more incidence of active disease than females, the influence of sex was not definitively analyzed until recently [59]. This study suggested that this difference could arise due to the influence of estrogens on the production of IFN-γ, or in other words, on immunological factors, as suggested years before [60]. However, the influence of high ferritin concentrations in males, which favors oxidative stress, thereby increasing macrophage activity and possibly inducing fibrinolysis, and thus reducing collagen formation, or the influence of age, which results in increased collagen breakdown and thus a decreased fibrinogenic ability [61], has received little attention.

9. What Can Be Done to Further Prevent Liquefaction

Overall, the actual strategies to enhance the specific immune response against *M. tuberculosis* appear to be still good. If considering the dynamic hypothesis of LTBI, a strong cellular immune response must help to control the constant endogenous reinfection of the host. On the other hand, the nature of this immune response (cellular type) leaves a window that makes possible the constant reinfection process [40]. This gives reasons for trying to build a better response to avoid this window by building an effectively protective humoral response, which unfortunately appears to be very difficult although some results have been already obtained in this field [43, 62].

On the other hand, there is the process of induction of tolerance to *M. tuberculosis* antigens, a strategy used for a long time with tuberculins [63] that has proven to be effective in avoiding the liquefaction process, demonstrated by Yamamura et al. experimentally [24]. In this regard, it would be better to have more information about how regulatory T cells behave and how they can be specifically induced to have a more balanced immune response.

10. A New Model for Testing Inhibitors of Liquefaction in the Rabbit Skin

Recently, a new experimental model has been validated to test future drugs to inhibit liquefaction [27]. This test is based in the induction of liquefaction in rabbit skin caused by the inoculation of high bacillary load intradermally. The only criticism that can be done is the fact that this phenomenon takes place with another cellular type (i.e., the adypocytes), and it might be difficult to validate its correlation to what it happens in the lungs, but on the other hand, it is a reproducible and reliable test that will sure impulse the knowledge in the field and the research in new tools to avoid the progression towards TB.

11. Summary: How Does the Progression to Liquefaction Occur?

Cavity formation has traditionally been considered to occur from solid caseum, and a lot of controversies were raised to understand who is the responsible of inducing liquefaction: the reactivation of the bacilli trapped in the caseum of old lesions or the macrophage through the extracellular release of hydrolytic enzymes.

We understand liquefaction as one of the three possible outcomes (the other two being control and dissemination) of the constant endogenous reinfection process which would maintain LTBI. This idea is supported by human-like experimental models (i.e., the minipig model) suggesting new lesions are constantly generated. The induction of a higher number of new lesions would increase the probability

of one of them occurring in the appropriate location to induce liquefaction as upper lobes. These lobes favor higher bacillary load before the immune response appears by directly promoting bacillary growth and delaying the local onset of the immune response. Once this response appears, however, the synchronized induction of apoptosis/necrosis of infected macrophages together with a high IFN-γ concentration and the release of metalloproteinases by new incoming macrophages would be critical factors to promote the inhibition of localized fibrosis of the lesion and thus liquefaction. A high ability to generate a nonspecific inflammatory response, which is structurally present in males (i.e., high levels of ferritin), or a lower ability to produce collagen with age, could hypothetically promote this liquefaction.

Although this process can be redirected with time, with fibrosis finally taking place, another, albeit slow factor, namely extracellular bacillary growth, should be taken into account. Such growth might be essential to allow the irreversibility of the liquefaction process already triggered due to the so-called bacillus factor, that is, fibrinolytic properties of proteins from the bacillary cell wall, or by infecting the macrophages that surrounds the liquefaction, and thus maintaining the Th1 response that favors liquefaction to persist, whereby the presence of a large volume of liquefaction product leads to the destruction of new incoming macrophages (due to the high concentration of free fatty acids [20]) and fibroblasts, thereby preventing the structuration of the site.

As conclusion, it could be said that liquefaction appears to be a stochastic effect due to disturbance in the organization of the intragranulomatous necrosis. The immune response and its magnitude, the bacillary load, the speed of the bacillary growth and the amount of extracellular bacilli, as well as mechanic and chemical factors (due to the distribution of the blood flow) are involved in it. Animal models have provided evidences to infer some of these factors, but more efforts on developing new models should be done in order to better mimic the human disease.

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Research Article

Recombinant HBHA Boosting Effect on BCG-Induced Immunity against *Mycobacterium tuberculosis* Infection

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Heterologous prime-boost regimens are effective strategies to promote long-term memory and strong cellular Th1 responses to *Mycobacterium tuberculosis*, when BCG is used in the priming step. Subcutaneous or intranasal boosting of BCG-vaccinated newborn mice with native heparin-binding haemagglutinin (nHBHA) significantly enhances protection against *M. tuberculosis.* However, nHBHA is characterized by a complex methylation pattern in its C-terminal domain, which is important for protective immunogenicity in primary vaccination. In this study we addressed the question whether boosting with recombinant, non-methylated HBHA (rHBHA) produced in *Escherichia coli* may enhance protection of BCG-primed newborn mice. We found that while subcutaneous rHBHA boosting enhanced protection of BCG-primed mice against intranasal *M. tuberculosis* infection both in spleen and lungs, enhanced protection against aerosol infection was only seen in the spleen (0.72 logs; $P < 0.05$) but not in the lungs. Thus, in BCG-primed mice the methylation of the C-terminal domain of HBHA is dispensable for the induction of enhanced protection in the lungs against intranasal but not aerosol infection, whereas it enhances protection in the spleen in both challenge models. This report thus provides evidence that rHBHA may be considered as a booster vaccine against disseminated tuberculosis.

1. Introduction

Tuberculosis (TB) remains one of the main causes of mortality in the world [1, 2]. *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) [3], a live, attenuated mycobacterial strain is still the only vaccine available against TB. While it has clear beneficial effects against pulmonary and disseminated TB in children for a limited number of years, it provides insufficient protection against pulmonary tuberculosis in adults, with highly variable protective efficacy [1, 4]. Therefore, more effective vaccines to increase or boost BCG-induced immune protection against TB are urgently needed. Recent advances have shown that non-living vaccines based on secreted proteins and prime-boost strategies [5, 6] can effectively protect against subsequent *Mycobacterium tuberculosis* infection in animal models and increase immune responses in humans [7–11].

The most desirable immunological consequence of the vaccination with BCG is the priming of a Th1-oriented $CD4^+$ T cell response that maximizes, through the release of IFN-γ, the antimicrobial properties of macrophages [12–14]. In this sense, it has been demonstrated that the mycobacterial adhesin heparin-binding haemagglutinin (HBHA) [15, 16] is a promising antigen against TB [17, 18]. HBHA is a methylated protein, and the methylation pattern in its C-terminal domain is important for the production of HBHA-specific IFN-γ by peripheral blood mononuclear cells (PBMCs) from *M. tuberculosis*-infected healthy individuals [17]. In mouse models, methylated HBHA, but not recombinant, non-methylated HBHA (rHBHA), co-administered

with the adjuvant dimethyl dioctadecylammonium bromide monophosphoryl lipid A (DDA+MPL) induced protection against intravenous (i.v.) or aerosol challenge with *M. tuberculosis* at levels similar to those induced by BCG [17–19], indicating the importance of the methylated C-terminal domain of HBHA for protection. However, the N-terminal domain is predicted to contain helix-coiled coil motifs, which are usually rich in T and B cell epitopes [20, 21]. Consistent with this, we have previously reported that rHBHA without adjuvant, administered to adult BALB/c mice by the intranasal (i.n.) or subcutaneous (s.c.) route, induces significant humoral and T cell immune responses [22], although protective immunity was not observed. On the other hand, we have also shown that native HBHA administered by the s.c. or i.n. route without adjuvant significantly enhances BCG-primed immunoprotection against *M. tuberculosis* infection [23]. In this study, we investigated whether; in a similar heterologous prime-boost regimen [23], rHBHA boosting confers enhanced protection against aerosol or i.n. *M. tuberculosis* infection in BCG-primed mice. We found that the methylation of the C-terminal domain of HBHA can be dispensable for the boosting effect of BCG-induced protective immunity in the lungs against i.n., but not aerosol *M. tuberculosis* infection, whereas it enhances protection in the spleen in both infection models. We therefore conclude that rHBHA may be considered as a booster vaccine to improve the efficacy of BCG for the prevention of disseminated TB to non-pulmonary locations.

2. Material and Methods

2.1. Animals. Pathogen-free newborn (three to four days old) BALB/c mice were obtained from Harlan, Co, France and were maintained in a specific pathogen-free environment at the Institut Pasteur of Lille throughout the entire experiment. All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee and mainly under barrier conditions in a level II and III biosafety animal facility.

2.2. Microorganisms. The BCG Pasteur strain (isolate 1173P2, World Health Organization, Stockholm, Sweden) was grown in dispersed cultures in Sauton medium for 14 days as previously described [15]. The BCG suspension was then stored at –80°C until further use. *M. tuberculosis* H37Rv used to challenge mice was grown at 37°C in Middlebrook 7H9 medium (DIFCO), supplemented with albumin-dextrose-catalase enrichment and Tween 80, collected at the end of the stationary phase and stored in Middlebrook 7H9 medium supplemented with 20% (v/v) glycerol at –80°C until they were used.

2.3. Immunizations. Recombinant HBHA was purified from *Escherichia coli* BL21(DE3)(pET-HBHA) [16] by using heparin-sepharose chromatography, followed by reverse phase high-pressure liquid chromatography, as previously described [15, 24]. Groups of 3-4-day-old neonate BALB/c mice were immunized s.c. [23] with 5×10^5 colony-forming units (CFU) BCG in 50 μL sterile phosphate-buffered saline (PBS) or mock-immunized with 50 μL of sterile PBS. At intervals of one month, for a total of three months, each group of mice was boosted s.c. with 5 μg rHBHA, dissolved in 150 μL of PBS or as control received only PBS [23]. Mice were rested one month before *M. tuberculosis* challenge infection.

2.4. M. tuberculosis Challenge. For the i.n. challenge experiments, mice were first anesthetized with an intra-peritoneal (i.p.) injection of sodium pentobarbital (1/10 of the weight mouse) and then infected i.n. with 5×10^5 CFUs of *M. tuberculosis* in 20 μL of PBS. Alternatively, mice were aerosol-challenged by using a homemade nebulizer. 3 mL of a suspension containing 2×10^7 CFUs was aerosolized to deliver an inhaled dose of 100–150 CFUs per mouse. Numbers of live bacteria in organs were measured eight weeks after infection by plating serial dilutions of whole organ homogenates on Middlebrook 7H11 solid medium supplemented with 2 mg/mL of THF. Petri dishes were kept in sealed plastic bags at 37°C for 3-4 weeks. Colonies were counted and total CFU calculated.

2.5. Cytokine Analyses. For cytokine measurements single cell suspensions of spleen or lung cells were prepared as described elsewhere [22]. Briefly, spleen tissues were smashed in a Falcon sieve using the top end of a 1 mL syringe piston. The excised lung tissue was minced and incubated for 1 h at 37°C in 500 μL of PBS containing 2% fetal bovine serum (FBS) (Buffer I), 125 U/mL collagenase I (Sigma-Aldrich), and 60 U/mL DNase I (Sigma-Aldrich). Single cell suspensions were prepared by passing the tissue through a 30-mm stainless steel mesh, washed, resuspended in Buffer I, applied carefully onto a gradient of percoll (Sigma-Aldrich), and centrifuged at 2200 rpm for 30 min at 20°C. Live cells at the interface were collected and used for *in vitro* culture. 1×10^6 viable cells were cultured in medium consisting of RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U of penicillin G per mL, 100 μg of streptomycin per mL, and 0.05 mM 2-mercaptoethanol. Cells were incubated at 37°C in an atmosphere of 5% CO_2 for 72 h in 96-well ELISA plates in the presence or absence of ConA (2.5 μg/mL) or rHBHA (2.5 μg/mL). The culture supernatants were then collected and stored at −20°C until analysis. The amounts of IL-6, IFN-γ, IL-12p70, and IL-10 in the supernatants were measured by using a specific sandwich ELISA (OptEIA; BD Biosciences-Pharmingen) according to the manufacturer's instructions. Assay sensitivities were 2.5 pg/mL for IFN-γ and 3 pg/mL for IL-6, IL12p70, and IL10. The data are expressed as the mean ± SEM for each mouse group.

2.6. Statistical Analysis. Differences between the different groups (three) were assessed by one-way analysis of variance (ANOVA) and by its nonparametric equivalent Kruskal-Wallis test of the $\log_{10}$ CFU followed by Tukey and/or Dunn's post-test, respectively. A value of $P < 0.05$ was considered statistically significant. Analysis was performed using Prism

5.0 software (Graph Pad Software, Inc., Sand Diego, Calif, USA).

3. Results

3.1. The Methylation Pattern in the C-Terminal Domain of HBHA Is Dispensable to Boost Protection of BCG-Vaccinated Newborn Mice against M. tuberculosis i.n. Challenge. The natural methylation pattern in the C-terminal domain of HBHA is important for T cell antigenicity and protective immunity [17]. No protective immunity was induced when rHBHA was co-administered with the adjuvant DDA+MPL to adult BALB/c or C57BL/6 mice challenged i.v. or through the aerosol route with *M. tuberculosis* [17, 18]. However, it has been shown that heterologous prime-boost regimens are efficient to boost BCG-induced immunity against *M. tuberculosis* [5, 9]. We have recently reported that boosting (i.n. or s.c.) with nHBHA in the absence of adjuvant was an efficient way to significantly enhance BCG-induced immunoprotection against *M. tuberculosis* infection [23]. Taking into account that rHBHA is easily purified from recombinant *E. coli*, we investigated whether this form of HBHA may also boost BCG-induced protective immunity. Therefore, newborn mice were vaccinated with BCG and then boosted with rHBHA by the s.c. route. One month after the last immunization, the mice were infected with *M. tuberculosis* and sacrificed 8 weeks after challenge to count the CFUs in spleen and lungs, as a measure of protection. BCG-primed/rHBHA-boosted mice had a significantly reduced bacterial load in the spleen (by 1.32 logs, $P < 0.05$) in comparison with PBS control mice (3.72 ± 0.12 versus 5.04 ± 0.29) (Table 1), which was significantly lower than in BCG-vaccinated, non-boosted animals (by 0.89 logs, $P < 0.05$; 3.72 ± 0.12 versus 4.15 ± 0.55) (Table 1). Furthermore, in the lungs BCG-vaccinated/rHBHA-boosted mice had also significantly reduced bacterial loads compared to the PBS controls (by 2.26 logs, $P < 0.05$; 3.98 ± 0.11 versus 6.24 ± 0.25) and compared to BCG-vaccinated non-boosted mice (by 1.26 logs, $P < 0.05$; 3.98 ± 0.11 versus 5.24 ± 0.40) (Table 1). These data indicate that the methylation pattern in the C-terminal domain of HBHA is dispensable for the booster effect of BCG-induced protective immunity after i.n. *M. tuberculosis* infection.

3.2. The Methylation Pattern in the C-Terminal Domain of HBHA Is Important to Boost Lung Protection of BCG-Vaccinated Newborn Mice against M. tuberculosis Aerosol Challenge. Next, we evaluated the rHBHA boosting effect of the BCG-induced protective immunity against *M. tuberculosis* aerosol infection. BCG-vaccinated newborn mice boosted with rHBHA were challenged by aerosol with a low dose of *M. tuberculosis* (~100–150 CFUs). Eight weeks post-challenge, BCG-vaccinated/rHBHA-boosted mice showed a significant reduction of bacteria in the spleen (by 1.35 logs, $P < 0.05$) in comparison with PBS control mice (3.15 ± 0.71 versus 4.50 ± 0.46) (Table 1), as well as in comparison with BCG-vaccinated, non-boosted mice (by 0.72 logs, $P < 0.05$; 3.15 ± 0.71 versus 3.87 ± 0.34) (Table 1). In contrast, in the lungs, both BCG-vaccinated non-boosted and BCG-vaccinated/rHBHA-boosted mice showed reduced bacterial load at a similar magnitude in comparison to the PBS control mice (by 0.59 logs) (Table 1). Thus, in contrast to the i.n. *M. tuberculosis* infection, upon aerosol challenge rHBHA boosting did not enhance BCG-induced protective immunity in the lung, while it boosted protection in the spleen. These data indicate therefore that natural methylation present in the C-terminal of nHBHA and absent in rHBHA is important to enhance BCG-induced protective immunity in the lungs, but not in the spleen against *M. tuberculosis* aerosol-infection.

3.3. rHBHA Boosting of BCG-Induced Protective Immunity against i.n. Challenge Correlates with High IL-12 Production. We have previously reported that in the absence of DDA+MPL, nHBHA boosting of the BCG-induced protective immunity against i.n. or aerosol *M. tuberculosis* infection correlates with high IL-12 and TGF-β production [23], two cytokines that are important for the maintenance of the memory T cell population [25–27], especially when boosting occurs late after priming. Therefore, we determined whether the rHBHA-mediated booster effect of the BCG-induced protective immunity after *M. tuberculosis* infection (either i.n./or aerosol) also correlates with these two cytokines induced after challenge in a long-term prime boost protocol and *in vitro* recall with rHBHA.

Splenocytes from i.n. infected mice after *in vitro* recall with rHBHA produced significant amounts of IL12p70 (3325 ± 403 pg/mL) (Figure 1A, gray bar), in comparison with PBS-immunized mice (850 ± 508 pg/mL) (Figure 1A, white bar) and BCG-vaccinated non-boosted mice (1540 ± 239 pg/mL) (Figure 1A, black bar). In contrast, the spleen cell TGF-β production in BCG-vaccinated/rHBHA-boosted mice after infection was similar (2174 ± 270 pg/mL) to that of spleen cells from the BCG-vaccinated i.n. infected mice without boost (2291 ± 203 pg/mL) (Figure 1A). BCG-vaccinated/rHBHA-boosted mice also produced slightly higher amounts of IFN-γ (505 ± 150 pg/mL, $P < 0.05$) (Figure 1A) after infection, in comparison with those induced by the BCG-vaccinated non-boosted mice (252 ± 14 pg/mL) (Figure 1A) and PBS control mice (236 ± 32) (Figure 1A). We also determined regulatory (IL-10) and Th17 type cytokines (IL-6, IL-17) and found that splenocytes from BCG-vaccinated/rHBHA-boosted i.n. infected mice induced a significant IL-10 production (1640 ± 100 pg/mL) in comparison with infected PBS control mice (76 ± 20 pg/mL) and infected BCG-vaccinated mice without boost (304 ± 14 pg/mL) (Figure 1A). Interestingly, the IL-6 spleen cell production from BCG-vaccinated/rHBHA-boosted i.n. infected mice (427 ± 89 pg/mL) was similar to that of the non-boosted group (419 ± 20 pg/mL), but significantly higher than that of the PBS control mice (96 ± 4 pg/mL) (Figure 1A). Spleen cell IL-17 production significantly changed between the three groups after i.n. infection (Figure 1A).

After aerosol infection, splenocytes from BCG-vaccinated/rHBHA-boosted mice produced similar amounts of IL-12p70 (3020 ± 42 pg/mL) as those from BCG-vaccinated

Table 1: Differential recombinant HBHA boosting effect of BCG-induced protective immunity in BALB/c newborn mice against *M. tuberculosis* H37Rv infection as measured by reduced bacterial load in spleen and lungs.

Vaccine	Lung CFUs[a]	Lung protection Versus		Spleen CFUs[a]	Spleen protection Versus	
		Control	BCG		Control	BCG
Control[b]	6.24 ± 0.25			5.04 ± 0.29		
BCG[b]	5.24 ± 0.40	1.0*		4.15 ± 0.55	0.89*	
BCG-rHBHA[b]	3.98 ± 0.11	2.26*	1.26**	3.72 ± 0.12	1.32*	0.43**
Control[c]	4.50 ± 0.71			4.50 ± 0.46		
BCG[c]	3.91 ± 0.28	0.59*		3.87 ± 0.34	0.63*	
BCG-rHBHA[c]	3.91 ± 0.47	0.59*		3.15 ± 0.71	1.35*	0.72**

[a]Data are represented as mean ± standard deviations of the $\log_{10}$-transformed bacterial (CFUs) of *M. tuberculosis* per organ; [b]intranasal challenge; [c]aerosol challenge. Protection is calculated with respect to control of vaccination (BCG) and with respect to PBS-immunized newborn mice (control). The data reported are *significant at $P < 0.05$ with respect to control mice and **significant at $P < 0.05$ with respect to BCG control of vaccination with no boost. One representative of a total of two independent experiments is shown.

non-boosted mice (3020 ± 313 pg/mL) (Figure 1B), but higher than those from the PBS control mice (2720 ± 170 pg/mL) (Figure 1B). A similar pattern was observed for the spleen cell TGF-β production (BCG: 1540 ± 367 pg/mL; BCG/rHBHA: 1755 ± 450 pg/mL; controls: 970 ± 403 pg/mL). Interestingly, after aerosol infection, spleen cells from BCG-vaccinated/rHBHA-boosted mice produced also a modest but significant amount of IFN-γ (760 ± 312 pg/mL) (Figure 1B, gray bar), higher than spleen cells from BCG-vaccinated mice without boost (300 ± 68 pg/mL) (Figure 1B, black bar) and PBS control mice (159 ± 10 pg/mL) (Figure 1B, blank bars) ($P < 0.05$). The spleen cell production of IL-10, IL-17, and IL-6 was not significantly different between the different groups of aerosol-infected mice (Figure 1B).

Thus it appears that for some cytokines the booster effect of rHBHA upon BCG priming depends on the route of infection (i.n. for IL-12, IL-10; aerosol and i.n. for IFN-γ).

3.4. Th1-Type Cytokine Induction in the Lungs from BCG-Vaccinated/rHBHA-Boosted Mice after Infection. To determine whether the enhanced lung protective effect of BCG-vaccinated/rHBHA-boosted mice against i.n. infection compared to BCG-vaccinated, non-boosted mice also correlates with high IL-12 and/or TGF-β production, eight weeks after challenge (either i.n. or aerosol), the cytokine production was determined in the supernatant of the lung cell cultures after *in vitro* recall with rHBHA.

Lung cell IL-12 production of BCG-vaccinated/rHBHA-boosted mice infected i.n. was significantly higher (3800 ± 560 pg/mL) (Figure 2A) than that of non-boosted mice (2280 ± 335 pg/mL) or PBS control mice (433 ± 222 pg/mL) (Figure 2A). Interestingly, the lung cells from BCG-vaccinated/rHBHA-boosted mice also produced higher amounts of TGF-β (2240 ± 290 pg/mL) after i.n. infection, in comparison with non-boosted mice (1707 ± 122 pg/mL) and the controls (489 ± 277). The lung cell IL-10 production was significantly higher in BCG-vaccinated/rHBHA-boosted mice (1955 ± 275 pg/mL) than in non-boosted (252 ± 42 pg/mL) and in control mice (68 ± 7 pg/mL) after i.n. infection (Figure 2A). Very low levels of IFN-γ were produced by lung cells in all three groups (Figure 2A). The lung cell IL-6 and IL-17 production was higher in the BCG-vaccinated non-boosted mice (451 ± 33 pg/mL) than in the BCG-vaccinated/rHBHA-boosted (271 ± 37) and in the PBS control mice (133 ± 7 pg/mL) after i.n. infection. No difference in lung cell IL-17 production between boosted and non-boosted mice was seen (Figure 2A).

Although the lung cells from BCG-vaccinated/rHBHA-boosted mice infected by the aerosol route with *M. tuberculosis* produced a similar cytokine pattern (IL-12, IFN-γ, IL-6, and IL-17) as those from mice infected by i.n. route, there were no statistically significant differences between rHBHA-boosted and non-boosted animals (Figure 2B). Together these results suggest that the rHBHA boosting effect of BCG-induced protective immunity after i.n. *M. tuberculosis* infection correlates essentially with high IL-12 production (Figure 2A), since, after aerosol infection, lung cells from BCG-vaccinated/rHBHA-boosted mice produced a lower magnitude of IL-12 (Figure 2B). Thus, the absence of the methylation pattern in the C-terminal domain does not affect the induction of cytokines important for the T cell memory response but is important for the magnitude, specifically of IL-10 and IL-12.

4. Discussion

The natural methylation in the C-terminal domain of mycobacterial HBHA [15, 16] plays an important role in T cell antigenicity and protective immunity [17–19]. In this work, we report that the methylation pattern in the C-terminal domain of HBHA is dispensable for the booster effect of the BCG-induced protective immunity against i.n. *M. tuberculosis* infection but is important to enhance protection in lungs after aerosol challenge.

rHBHA, a protein without the C-terminal methylation, can be easily purified from recombinant *E. coli* and is able to induce strong humoral and cellular immune responses when is administered without the adjuvant DDA+MPL by the s.c. or i.n. route to BALB/c mice [22], suggesting that the α-helical regions in the N-terminal domain can be target of B and T cell receptors [20, 21]. However, co-administration

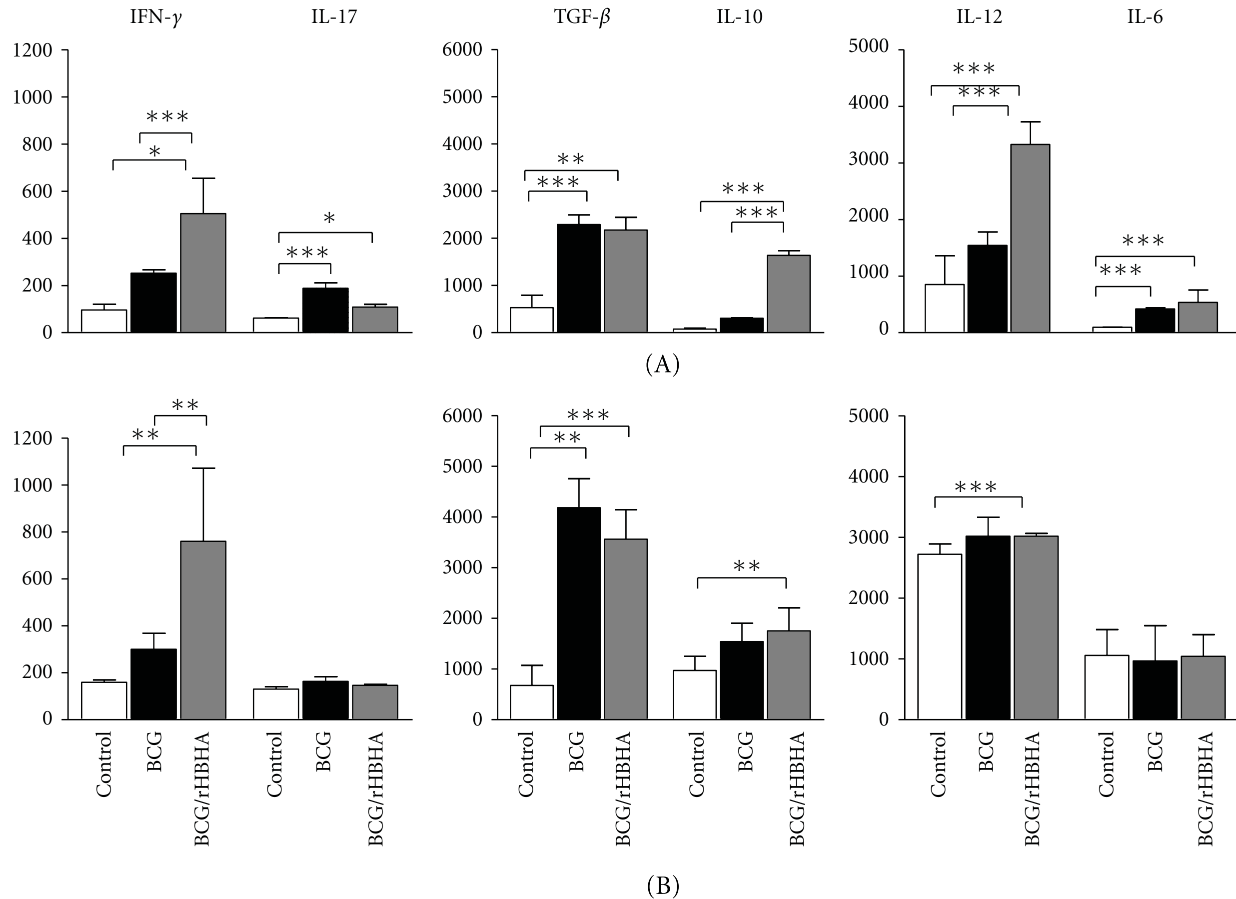

Figure 1: Spleen cell cytokine profile of BCG-vaccinated mice with or without rHBHA boost after *M. tuberculosis* infection. Eight weeks after i.n. (A) or aerosol (B) challenge spleen cells from control mice (white bars), BCG-vaccinated mice (black bars), or BCG-vaccinated/rHBHA-boosted mice (gray bars) were cultured in the presence of medium only, ConA (2.5 μg/mL), or rHBHA (5 μg/mL). Levels of cytokines were measured after 72 h culture in the supernatants by using the OptEIA kit (BD Biosciences). Values are expressed in pg/mL and represent media ± SEM of samples tested in duplicates from each group of mice. The data was considered statistically significant when $P < 0.05$ (*), $P < 0.001$ (**), and $P < 0.0001$ (***).

of rHBHA plus the DDA+MPL was unable to protect adult mice from i.v. or aerosol infection with *M. tuberculosis* [17, 18]. Heterologous prime boost regimens are an efficient way to induce long-term memory cellular immune responses leading to enhanced protection against *M. tuberculosis* [5, 9, 23]. We have recently shown that both i.n. and s.c. boosting with nHBHA in the absence of the strong Th1-adjuvant (DDA+MPL) were able to significantly enhance protection against an i.n. or aerosol *M. tuberculosis* challenge in BCG-primed, newborn mice [23]. Keeping with these observations, it was conceivable to think that, under a similar prime-boost regimen, rHBHA without methylation in the C-terminal domain and without adjuvant (DDA+MPL) could enhance BCG-induced protective immunity against *M. tuberculosis* infection. The findings of this work indicate that, while the methylation pattern in the C-terminal domain of HBHA is dispensable for the rHBHA boosting effect of the BCG-induced protective immunity against i.n. *M. tuberculosis* challenge, it is essential to induce lung protection after aerosol infection of mice with *M. tuberculosis*.

Although the most desirable effect of the vaccination with live BCG is the induction of a strong pro-Th1-type $CD4^+$ T cell response through the release of IFN-γ [12, 13, 27], several studies have shown that enhanced protection does not necessarily correlate with the presence of IFN-γ-producing circulating cells. As mentioned above, heterologous prime/boost strategies are a very effective strategy to enhance protection against *M. tuberculosis* because of the induction of cytokines (IL-12, IL-17, and TGF-β) [25, 26] that promote the development and maintenance of long-term memory T cells [28, 29]. In agreement with this, we recently reported that enhanced BCG-mediated protective immunity against *M. tuberculosis* infection correlated precisely with these cytokines in the systemic and mucosal compartments. Interestingly, in this work we have found that BCG-vaccinated/rHBHA-boosted mice were protected against i.n. *M. tuberculosis* infection, and this enhanced protection correlated with high amount of spleen and lung IL-12 production, as well as lung TGF-β production. In addition, IL-10 production was detected in spleen and lungs

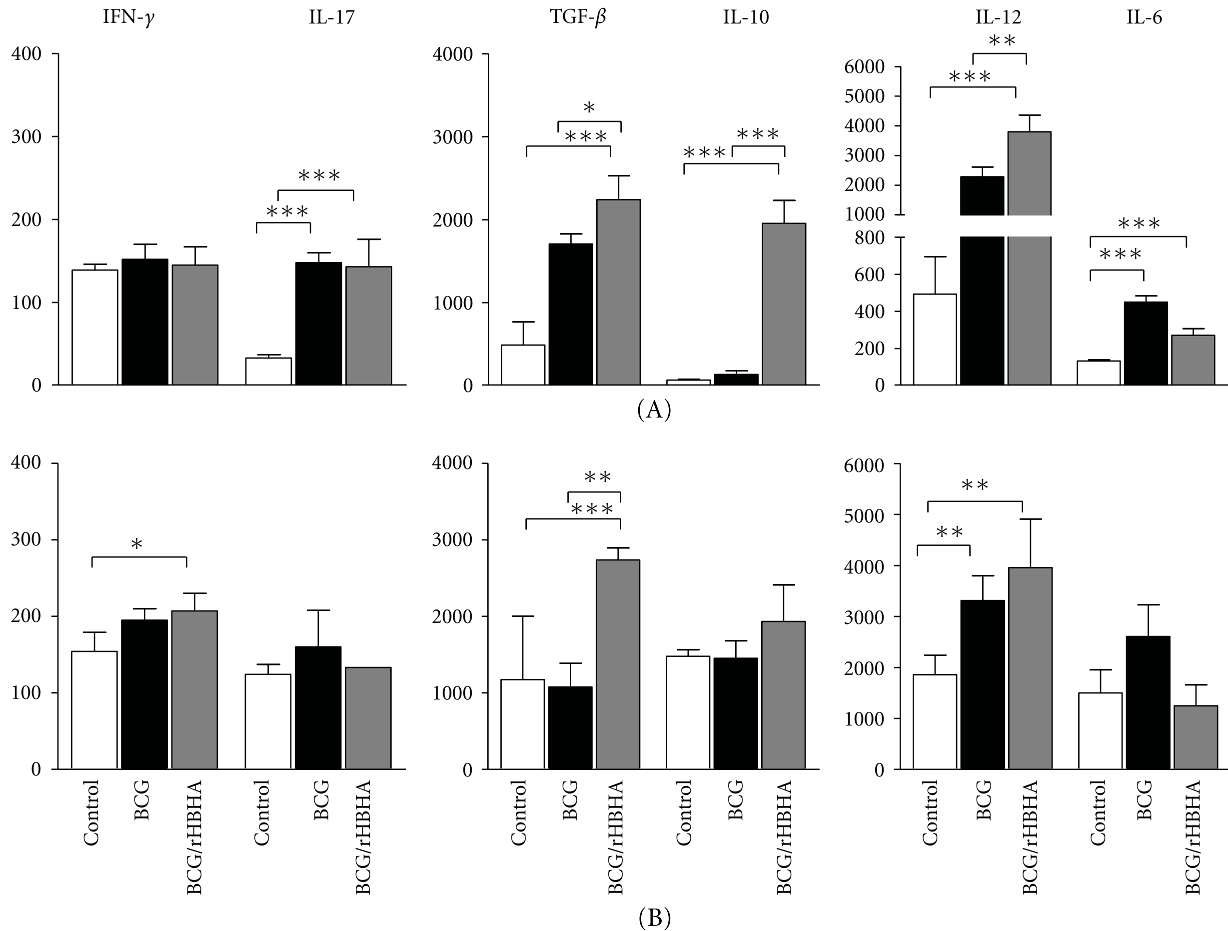

Figure 2: Lung cell cytokine profile of BCG-vaccinated mice with or without rHBHA boost after *M. tuberculosis* infection. Eight weeks after i.n. (A) or aerosol (B) challenge lung cells from control mice (white bars), BCG-vaccinated mice (black bars), or BCG-vaccinated/rHBHA-boosted mice (gray bars) were cultured in the presence of medium only, ConA (2.5 μg/mL), or rHBHA (5 μg/mL). Levels of cytokines were measured after 72 h culture in the supernatants by using the OptEIA kit (BD Biosciences). Values are expressed in pg/mL and represent media ± SEM of samples tested in duplicates from each group of mice. Statistically significant data: $P < 0.05$ (*), $P < 0.001$ (**), and $P < 0.0001$ (***).

after i.n. infection, suggesting that the lack of methylation in the C-terminal domain of HBHA could influence a more complex interplay between Th1 and regulatory type cytokines, in agreement with what has been reported elsewhere concerning the effect of IL-10 on the Th1-type cellular immune response upon vaccination [30, 31]. It is possible that, in our model, the high IL-10 production after i.n. *M. tuberculosis* infection of BCG-vaccinated/rHBHA-boosted mice promotes protection specifically at the long term, an issue that deserves future investigation. Interestingly, it has been reported recently that in early *M. tuberculosis* infection regulatory T cells could be delaying the onset of effector T cells in the lung [32].

A very moderate increase in BCG-induced spleen IFN-γ production (independent of the route of infection, i.n. versus aerosol) of infected mice by the s.c. rHBHA boosting was found in comparison with those reported from BCG-vaccinated nHBHA/boosted [23]. In the previous report, we proposed that the nHBHA boosting effect of BCG-induced protective immunity could be through a mechanism independent of IFN-γ [23]. In this study, we do not know the impact of the moderate systemic IFN-γ production on the control of *M. tuberculosis* infection. However, from the data it is also possible that, although the reduction of bacterial load in the spleen is significant ($P < 0.05$) upon aerosol infection (Table 1), the methylated pattern in the C-terminal domain of HBHA is required to promote the development of a more sustained cellular response in the lung compartment, as it was observed for the i.n. infected mice. However, more experiments are necessary to address this issue.

Thus, in agreement with our previous findings, an environment of IL-12 and TGF-β production in long-term prime-boost regimens could promote enhanced BCG-induced protective immunity to contain *M. tuberculosis* locally and at peripheral locations, suggesting that effector and memory T cells could be involved [25, 26, 33–35]. In

agreement with this, it has been reported that protection against *M. tuberculosis* induced by the prime-boost either parenterally, i.n., or through other routes did not necessarily correlate with the presence of IFN-γ-producing circulating T cells [36, 37].

In summary, several points arise from this study: first, the data are consistent with the fact that heterologous prime-boost protocols are a very effective way to enhance BCG-induced protective immunity and, second, the dispensability of the methylation pattern in the C-terminal domain of HBHA to enhance this protection after i.n. but not after aerosol *M. tuberculosis* infection. It is the IL-12 production and not IFN-γ that is very important not only to promote Th1 cellular immune responses but also for the development and maintenance of long-term memory T cells of infected mice. In addition, this report provides evidence that rHBHA may be used to improve current BCG vaccination schemes in heterologous prime/boost regimens, particularly relevant for reducing the incidence of disseminated tuberculosis.

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Research Article

Immunogenicity and Protective Efficacy against Murine Tuberculosis of a Prime-Boost Regimen with BCG and a DNA Vaccine Expressing ESAT-6 and Ag85A Fusion Protein

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Heterologous prime-boost regimens utilizing BCG as a prime vaccine probably represent the best hope for the development of novel tuberculosis (TB) vaccines. In this study, we examined the immunogenicity and protective efficacy of DNA vaccine (pcD685A) expressing the fusion protein of Ag85A and ESAT-6 (r685A) and its booster effects in BCG-immunized mice. The recombinant r685A fusion protein stimulated higher level of antigen-specific IFN-γ release in tuberculin skin test- (TST-) positive healthy household contacts of active pulmonary TB patients than that in TST-negative population. Vaccination of C57BL/6 mice with pcD685A resulted in significant protection against challenge with virulent *Mycobacterium tuberculosis* H37Rv when compared with the control group. Most importantly, pcD685A could act as a BCG booster and amplify Th1-type cell-mediated immunity in the lung of BCG-vaccinated mice as shown the increased expression of IFN-γ. The most significant reduction in bacterial load of both spleen and lung was obtained in mice vaccinated with BCG prime and pcD685A DNA booster when compared with BCG or pcD685A alone. Thus, our study indicates that pcD685A may be an efficient booster vaccine against TB with a strong ability to enhance prior BCG immunity.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a leading infectious killer worldwide [1]. The emergence and spread of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) and coinfection with TB/HIV pose serious challenges to effective TB control [2]. *M. bovis* BCG is currently the only available vaccine against TB, with over 120 million doses administered annually [3]. BCG immunization provides protection against severe forms of TB in children, including tuberculous meningitis and miliary TB [4]. However, BCG induced-protection lasts less than 15 years because antituberculous protective immunity wanes gradually after the initial immunization [5]. Consequently, developing new, more effective vaccines and immunization strategies aimed to boost waning BCG-induced protective responses, is urgently needed.

DNA vaccines can stimulate both humoral and cell-mediated immunity in different animal models of TB and is thought to be a promising strategy in the development of new vaccines against TB [6]. DNA vaccine candidates expressing several antigens of *M. tuberculosis* have been shown to provide protective immune responses against TB [6] and to boost BCG efficacy using prime/boost strategies [7]. In our previous study, we constructed two DNA vaccine candidates separately encoding antigen Ag85A and ESAT-6 from *M. tuberculosis*, and both DNA vaccines could induce strong humoral and cell-mediated immunity in vaccinated mice, which resulted in some degree of protection in mice challenged with virulent *M. tuberculosis* [8]. DNA vaccine

expressing ESAT-6 protein could enhance the protective efficacy of BCG vaccination in mice vaccinated with a combination strategy of BCG and DNA vaccine [9]. In the present study, we evaluated the immune responses generated against DNA vaccine expressing the fusion protein of ESAT-6 and Ag85A (r685A) and the immunogenicity of r685A fusion protein in tuberculin skin test- (TST-) positive healthy populations. In addition, we evaluated the use of a BCG prime plus DNA vaccine in a prime/boost strategy to induce protection against virulent *M. tuberculosis* challenge in mice.

2. Materials and Methods

2.1. Bacterial Strain and Culture Media. Escherichia coli DH5α and BL21 (DE3) strains were used for cloning and overexpression, respectively. Both bacteria were cultured in Luria-Bertani (LB) medium with or without agar. When required, ampicillin was added to a final concentration of 100 μg/mL. *M. tuberculosis* H37Rv and *M. bovis* BCG China were cultivated in Middlebrook 7H9 medium or enumerated on 7H11 agar (BD, Sparks, USA), supplemented with 10% ADC, 0.5% glycerol, and 0.05% Tween 80.

2.2. Construction of Recombinant Plasmids. Genes coding ESAT-6 (*esxA, Rv3875*) and Ag85A (*fbpA, Rv3804c*) were amplified, respectively, by PCR with primers (listed in Table 1) and the genomic DNA of *M. tuberculosis* H37Rv as the template. The gene encoding the fusion protein of ESAT-6 and Ag85A was generated by a second PCR according to the gene splicing with the overlap extension (GeneSOEing) method [10]. The PCR products were first digested with *Bam*HI and *Eco*RI and then cloned into the corresponding sites of prokaryotic expression vector pProEXHTb (Invitrogen, Carlsbad, CA, USA) and eukaryotic expression vector pcDNA3.1(+), resulting in recombinant plasmids named pPro685A and pcD685A, respectively. The correctness of recombinant plasmids was confirmed by DNA sequencing and enzyme digestion. Plasmids pcDNA3.1(+) and pcD685A for DNA immunization were transformed into competent *Escherichia coli* DH5α, and endotoxin-free plasmid DNA was purified using the Qiagen Plasmid Giga kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

2.3. Overexpression of r685A Protein in E. coli. E. coli BL21 (DE3) strain harboring the plasmid pPro685A was cultured overnight. Overnight cultures were inoculated into fresh LB medium (1 : 100) containing ampicillin and incubated at 37°C with shaking, until OD_{600} nm reached 0.6. The expression of the fusion protein r685A was induced with isopropyl thio-β-D-galactoside (IPTG) at a final concentration of 0.1 mM for 0, 2, 4, or 6 hours. Bacterial pellets were collected by centrifugation, prepared in sample buffer, and subjected to SDS-PAGE analysis. Proteins were stained with a Coomassie blue dye.

Purification of r685A protein was performed using the Ni-NTA Purification System according to the manual instructions (Invitrogen, USA). The purified r685A protein was lyophilized and diluted in normal saline, using pyrogen-free reagents, and tested to exclude endotoxin contamination. The protein concentration was determined by using the Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China) and stored at −20°C.

To verify the specificity of purified r685A fusion protein, the protein samples were first separated by SDS-PAGE and then transferred onto nitrocellulose membranes and probed with a rabbit polyclonal antibody to ESAT-6 (Abcam 45073, Cambridge Science Park, Cambridge, UK) and a chicken polyclonal antibody to Ag85A (Abcam 36731, Cambridge Science Park, Cambridge, UK) as the primary antibody. Alkaline phosphatase-conjugated goat antirabbit IgG antibody and rabbit antichicken IgG antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) were as the secondary antibody, respectively.

2.4. Overexpression of r685A Protein In Vitro. In vitro overexpression of r685A fusion protein was verified by Western blot analysis of transfected HeLa cells. Cells were cultured in wells of a 6-well plate and transfected with 1 μg of pcD685A or empty vector (pcDNA3.1). G418-resistant colonies (250 mg/L) were selected and harvested for the Western blotting analysis with mouse polyclonal antibody to r685A.

2.5. Immunogenicity of r685A Protein. The study protocol was approved by the Ethics Committee of Tongji Medical College. Written informed consents were obtained from all subjects involved in this study. Seventeen household contacts with recent sputum-positive TB patients (mean age: 37 ± 19 years; male/female ratio: 7/10) were enrolled. Active TB was excluded for all household contacts based on radiologic and clinical examinations, sputum microscopy, and bacteriologic culture. After heparinized whole blood sample from each participants was collected, TST and whole blood IFN-γ assay (WBIA) based on the r685A protein were performed as previously described, respectively [11]. Reactions of <5 mm and ≥5 mm were considered TST negative and positive, respectively. Whole blood from each donor (1 mL) was seeded in 24-well plates and incubated with 20 μL r685A protein at final concentrations of 0 or 10 μg/mL for 24 h at 37°C. After stimulation, 200 μL of plasma was then taken from each well and stored at −20°C until use. The concentrations of IFN-γ in collected samples were determined in duplicate, using a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Dakewei Biotech, Shenzhen, China).

2.6. Animal Immunization. Specific pathogen-free, 6- to 8-week-old, female C57BL/6 mice (Vital River Lab Animal, Beijing, China) were bred in cages on the animal feeding cabinet (VentiRack, Chester, CA, USA) in a biosafety level 3 laboratory. Mice received free access to food and water throughout the study. The research protocol was reviewed and approved by Tongji Medical School Committees on Biosaftey and Animal Care and Use Committee of China.

Table 1: Primers and thermal cycle parameters for cloning of *M. tuberculosis* antigens.

Protein Names	Primer names and sequences (5′-3′)	Cycle parameters
ESAT-6	ESAT6F: GGATCCATGACAGAGCAGCAGTG ESAT6R: GCTGCCGCCACCGCCGCTTCCGCCACCGCCGCTTCCACCGCC-ACCTGCGAACATCCCAGTGACGTTGCCTTC	95°C 5 min; 94°C 45 s, 60°C 45 s, 72°C 50 s, 30 cycles; 72°C 10 min
Ag85A	Ag85AF: GGTGGCGGTGGAAGCGGCGGTGGCGGAAGCGGCGGTGGCGGC-AGCGCATTTTCCCGGCCGGGCTTG Ag85AR: GGAATTCTGTTCGGAGCTAGGCGCCCTGGG	95°C 5 min; 94°C 45 s, 60°C 45 s, 72°C 50 s, 30 cycles; 72°C 10 min
r685A	ESAT6F Ag85AR	95°C 5 min; 94°C 1 min, 60°C 1 min, 72°C 90 s, 30 cycles; 72°C 10 min

Mice were randomly divided into (12 mice in each group): nonvaccinated control, vector control, pcD685A, BCG, BCG prime plus vector booster, and BCG prime plus pcD685A booster. Mice were injected with 30 μL of 0.25% Bupivacaine in the quadriceps muscle of each hind leg three days before DNA immunization. Plasmid DNA (50 μg) was injected intramuscularly in the same area, and immunization was repeated thrice with 2-week intervals. The latter two groups were firstly immunized with 10^6 CFU BCG once subcutaneously on the first day, then boosted with the plasmids following the DNA vaccination process. Two weeks after the completion of gene immunization, five mice of each group were used for immunological analysis, while the others were challenged with virulent *M. tuberculosis* H37Rv.

2.7. Antibody Response. Sera were collected from each mouse two weeks after immunization. Antigen-specific antibody responses were measured in an ELISA using microtiter plates, precoated overnight at 4°C with 100 μL r685A protein (5 μg/mL) in carbonate/bicarbonate buffer (pH 9.6). After blocking with 1% BSA in PBS, serum samples were diluted to appropriate concentrations and were incubated for 2 h at 37°C. After washing, the plates were incubated for 2 h at 37°C with HRP-conjugated goat antimouse IgG antibody. Orthophenylenediamine (OPD) was used for color development as an indicator. Sera from naive mice were used as negative controls. Data are presented as mean of optical density value at 490 nm per group.

2.8. qRT-PCR Analysis of IFN-γ and IL-10 Expression in Lungs of Vaccinated Mice. About 100 mg of lung tissue was crushed with a syringe plunger and the DNA-free RNA samples were extracted with TRIzol reagent (Invitrogen). For qRT-PCR, 2 μg of RNA was reverse transcribed with M-MLV Reverse Transcriptase (Promega, USA) to obtain cDNA samples. For the real-time reaction, each primer (250 nM) and 7.5 μL of template reaction (1 : 20 dilution) in 25 μL volume with SYBR Green kit (Roche, USA) were used. Triplicate samples were run on an Applied Rotor-Gene 3000 Real-Time system (Gene, USA). Tested cDNAs were normalized to the endogenous RNA levels of the internal control GAPDH. Gene expression was determined using the relative quantification $\Delta\Delta CT = (CT_{Test} - CT_{GAPDH})_{sample} - (CT_{Test} - CT_{GAPDH})_{Control}$. CT is the fractional cycle number that reaches a fixed threshold, CT_{Test} is the test of each vaccinated group, and $CT_{Control}$ is the reference control from the control group. $\Delta\Delta$CT is the difference between gene expression in each vaccinated group and the control group. The increase fold was calculated using $2^{-\Delta\Delta CT}$. Primer sequences and cycle parameters used for qRT-PCR are listed in Supplementary Table 1 (see Supplementary Table 1 in Supplementary Material available online at doi:10:1155/2011/61789g).

2.9. M. tuberculosis Challenge. C57BL/6 mice were infected intravenously through the lateral tail vein at the dose of 10^6 CFU with exponentially growing *M. tuberculosis* suspended in 100 μL PBS. Four weeks later, seven mice per group were killed, and bacterial burden was determined by plating serial dilutions of lung and spleen homogenates onto Middlebrook 7H11 agar plates supplemented with 10% (v/v) ADC enrichment and 0.5% (v/v) glycerol, containing 2-thiophenecarboxylic acid hydrazide (10 μg/mL), which inhibits residual BCG but not *M. tuberculosis*. Mouse protocols were approved by the Biosafety Committee of Tongji Medical College.

Right lung lobes from different vaccine groups were fixed in 10% PBS-buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) followed with analyzing by a pathologist with no prior knowledge for the treatment group and recording the results under a light microscope.

2.10. Statistical Analysis. Student's *t* test was used to compare the mean organ burdens of each group of mice, and a *P* value was less than .05 was considered statistically significant.

3. Results

3.1. Construction and Overexpression of Recombinant r685A Protein in E. coli. The genes of ESAT-6 and Ag85A were first amplified by PCR and *M. tuberculosis* H37Rv genomic DNA as the template (Figure 1). The fusion gene of *esat-6* and *fbpA* was then amplified using a mixture of PCR products of *esat-6* and *fbpA* as template with the upstream primer of ESAT-6 and the downstream primer of Ag85A (Figure 1). The purified PCR productions were digested with *Bam*HI and *Eco*RI and separately cloned into the plasmid

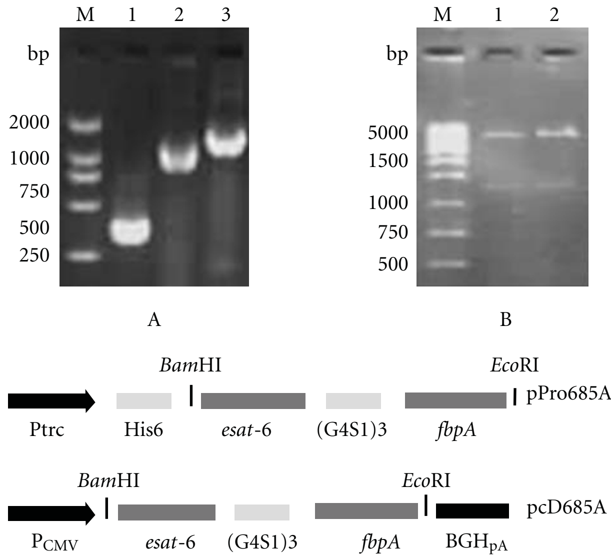

Figure 1: Construction of recombinant plasmids. Genes encoding ESAT-6, Ag85A, the fusion protein of ESAT-6, and Ag85A with a 45 bp linker, were cloned from *M. tuberculosis* H37Rv genomic DNA, respectively (A). The PCR products of the fusion genes were inserted into the *Bam*HI and *Eco*RI sites of pProExHTb or pcDNA3.1, resulting in the recombinant plasmids pPro685A and pcD685A, respectively. The recombinant plasmids were identified by enzyme digestion (B). Lane M: DNA molecular marker; lane A1: PCR product of *esat-6*; lane A2: PCR product of *fbpA*; lane A3: PCR product of *esat-6-fbpA*; lane B1: products of pPro685A digested with enzymes; lane B2: products of pcD685A digested with enzymes.

pProEXHTb and pcDNA3.1(+) predigested with the same restriction enzymes, in order to construct prokaryotic expression plasmid pPro685A and eukaryotic expression plasmid pcD685A (Figure 1). DNA sequencing and enzyme digestion confirmed the successful constructions (Figure 1).

E. coli BL21 (DE3) strain containing plasmid pPro685A was induced with IPTG, and recombinant fusion protein r685A was identified by 12% SDS-PAGE with a molecular mass of about 38 kDa as expected (Figure 2). r685A protein was expressed in *E. coli* in the inclusion body, and purification was performed using the Ni-NTA purification system. The process of purification was monitored by SDS-PAGE (Figure 2). The specificity of the purified r685A protein was verified by Western blotting with anti-ESAT-6 and anti-Ag85A antibodies, respectively, (Figure 2).

3.2. Expression of pcD685A DNA Construct. After plasmid pcD685A was transfected into HeLa cells by electroporation, cell lines of stable resistance to G418 were obtained by G418 selection. The expression of protein r685A was confirmed by Western blotting (Figure 3). HeLa cells transfected with vector pcDNA3.1(+) were also detected as control and showed the negative result as expected (Figure 3).

3.3. Immunogenicity of r685A Protein. Whole blood samples were collected from 17 household contacts with active TB patients and subjected to r685A-WBIA (Figure 4). The levels of IFN-γ in all samples without antigen stimuli were below 30 pg/mL (10.6 ± 5.9 pg/mL) but significantly increased to 1401.1 ± 1084.3 pg/mL after r685A protein stimulation. Analysis of the distribution of IFN-γ levels in TST-positive and TST-negative samples showed a significant correlation between TST positivity and IFN-γ level (Figure 4). The mean IFN-γ levels in samples from TST-positive group detected by r685A-WBIA were significantly higher than those for TST-negative group ($P < .05$).

3.4. Antibody Response. Sera IgG antibodies against r685A protein were determined by ELISA. As shown in Figure 5, strong IgG response to r685A protein was elicited in BCG, pcD685A, BCG plus vector, and BCG plus pcD685A-treated mice groups. As expected, empty DNA vector and control groups stimulated little or very weak IgG response.

3.5. Expression of IFN-γ and IL-10 in Lungs. To determine cell-mediated immune responses in the lung, lung tissues from immunized mice were prepared 2 weeks after the last DNA vaccination. The expression levels of IFN-γ and IL-10 in lungs of vaccinated mice were determined by qRT-PCR. As shown in Figure 6, IFN-γ response increased in all groups except the vector control group. Mice vaccinated with BCG plus pcD685A induced the highest levels of IFN-γ responses in the lungs. Combination with BCG increased significantly the expression of IFN-γ when compared with BCG or pcD685A alone. In addition, BCG plus vector group showed the most significant IL10 response in the lung, and high expression of IFN-γ was also produced. The expression of both IFN-γ and IL-10 decreased in vector group when compared with control mice.

3.6. BCG Prime Plus pcD685A Booster Produced Better Protective Effect against Virulent Challenge of M. tuberculosis in Mice than BCG Alone. In order to determine the protection effect, mice were challenged with *M. tuberculosis* H37Rv. The results of bacterial load in different organs were shown in Figure 7. Most importantly, mice vaccinated with BCG plus pcD685A produced the strongest protective effect and significantly inhibited the growth of *M. tuberculosis* in the lung (-1.922 log) and spleen (-1.609 log) when compared with BCG or pcD685A alone ($P < .05$). In addition, pcD685A also evoked a significant protection (-0.663 log) in the lung. Highest bacterial load was seen in the lung and spleen in both control and empty vector groups.

3.7. Decreased Lung Pathology in Mice Vaccinated with BCG Plus pcD685A Compared with BCG. Consistent with varying bacterial loads in the lung, HE-stained sections from different groups showed clear differences (Figure 8). The lung sections from both control and vector control mice showed severe interstitial pneumonia and intense inflammation throughout the lung with the appearance of early granuloma formation (Figures 8(a) and 8(b)). Slight damage in alveolar tissues with aggregated, relatively large number of lymphocytes was also observed in the lung from single pcD685A-vaccinated group (Figure 8(c)). BCG- and

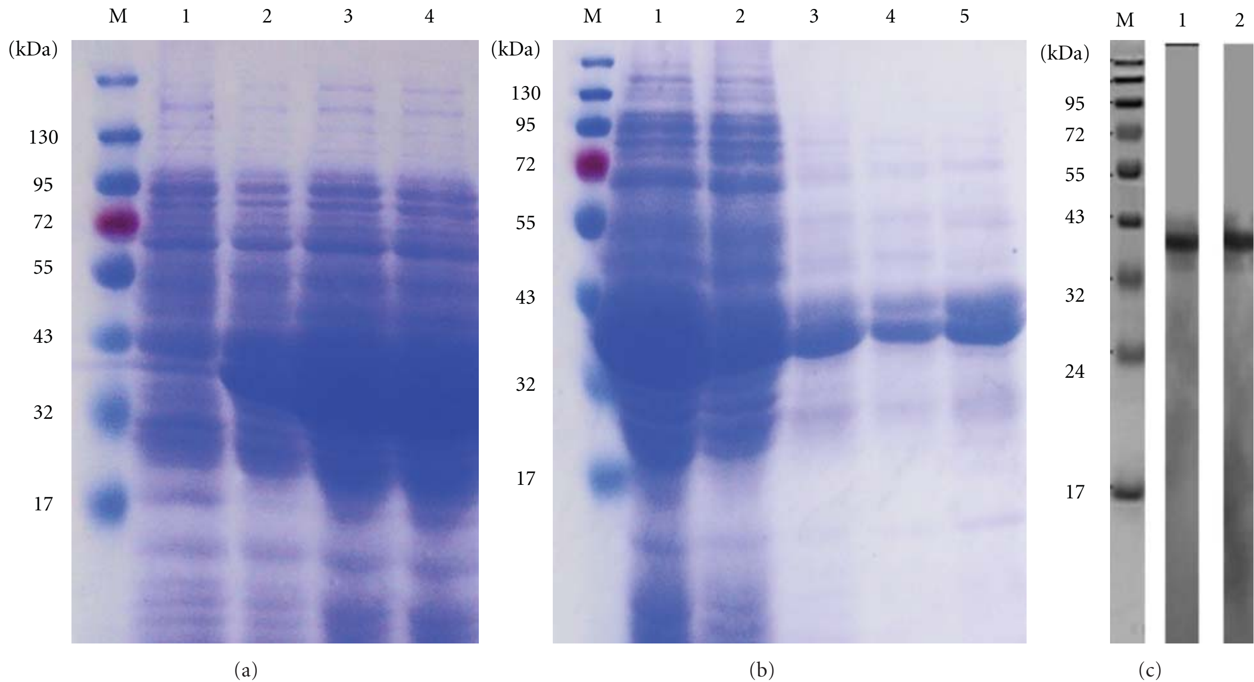

Figure 2: Expression, purification, and identification of recombinant fusion protein. *E. coli* BL21 (DE3) harboring pPro685A was cultured with IPTG. The expression (a) and the purification procession of r685A fusion protein (b) were confirmed by SDS-PAGE and Western blotting (c). Lane M: protein molecular size marker (kDa); lane a1: *E. coli* strain without IPTG; lane a2: *E. coli* strain 2 h after IPTG induction; lane a3: *E. coli* strain 4 h after IPTG induction; lane a4: *E. coli* strain 6 h after IPTG induction; lane b1: cell lysis; lane b2: fraction from Ni-NTA column after wash with denaturing binding buffer; lanes b3 and b4: fraction after wash with wash buffer; lane b5: r685A protein eluted with native elution buffer; lane c1: anti-ESAT-6 antibody; lane c2: anti-Ag85A antibody.

Figure 3: Western blot analysis of cell lysates from Hela cells transfected with the pcD685A construct (lane 2) or empty vector (lane 1). The primary antibody used was mouse antisera after pcD685A DNA vaccination.

BCG plus vector-vaccinated mice showed slight interstitial pneumonia (Figures 8(d) and 8(e)). Alveolar tissue from mice vaccination with BCG plus pcD685A combination appeared to be intact with very limited lung inflammation (Figure 8(f)).

4. Discussion

Developing more effective vaccines than BCG remains a high priority in global TB control. The replacement of currently used BCG with different platforms might not be a practical goal because it is the most widely used vaccine in humans and has over 80% efficacy against severe forms of childhood TB, and because no other vaccine candidates (based on different platforms except BCG) are superior to BCG so far. Therefore, a vaccine that can work as an effective booster of BCG vaccine may be ideal because it will be more effective regardless of current vaccination strategy. In this study, our data clearly demonstrated that mice vaccinated with the recombinant plasmid pcD685A expressing the

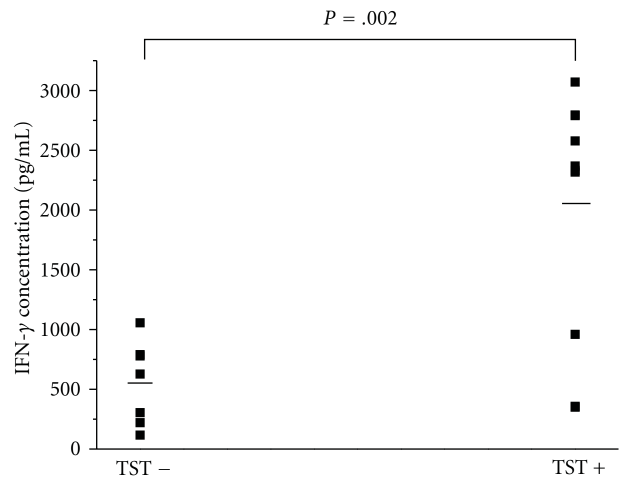

Figure 4: Immunogenicity of r685A fusion protein in healthy population. A total of 17 household contacts with active TB patients were tested by TST and r685A-WBIA, respectively. Each square represents the IFN-γ concentration in a sample, and median values for TST− ($n = 7$) and TST+ ($n = 10$) groups are indicated by horizontal lines.

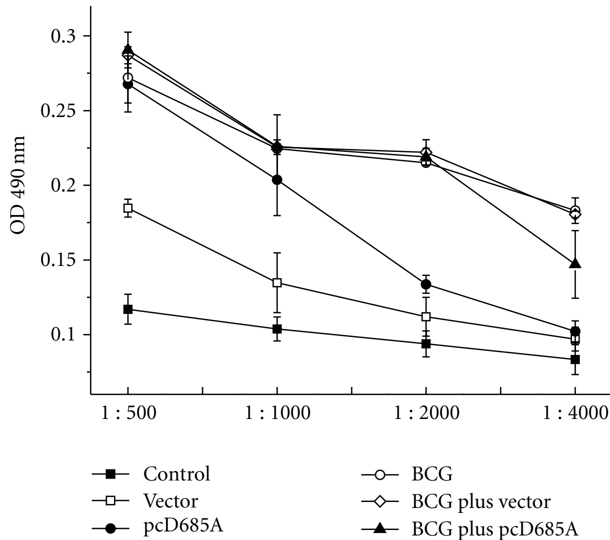

Figure 5: r685A-specific IgG antibody-induced in immunized mice. Mice were immunized and bled at day 14 following the last immunization; serum antibody levels were assessed by ELISA. Sera from five animals in each group were evaluated individually at the dilutions indicated. Results shown are the mean and standard error.

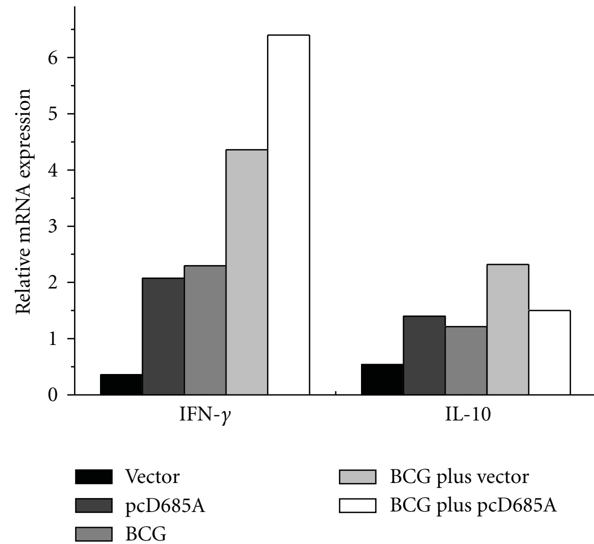

Figure 6: Differential expression of IFN-γ and IL-10 in the lungs of vaccinated mice ($n = 3$). Two weeks after DNA vaccination, RNA extracted from lung of each mouse in different groups was used for the detection of cytokines mRNA concentrations by qRT-PCR analysis. The levels of the cytokine mRNAs for each group were normalized based on the levels of GAPDH. Cytokine mRNA levels were expressed as values relative to the control group.

fusion protein of ESAT6 and Ag85A markedly reduced the bacterial load in the spleen and lung of vaccinated mice when compared with control group, but did not increase efficacy over BCG. More importantly, BCG prime followed by pcD685A DNA booster vaccination produced a greater protection as shown by reduced bacterial load and more pronounced antituberculous protective immune responses than BCG or pcD685A alone. The results of IFN-γ assay in a TST-positive population, cytokines assay of the lungs, postchallenge lung and spleen bacterial load, and pathological examination indicate that BCG prime plus pcD685A booster is an effective vaccine strategy against murine TB.

The antigen Ag85 complex was shared in by *M. tuberculosis*, BCG, and other mycobacteria, consisting of a family of three proteins (Ag85A, Ag85B, and Ag85C) with a MW range of 30–32000 daltons [12, 13]. These proteins possess mycolyl-transferase activity, which are responsible for the transfer of mycolic acids to a-a′-trehalose to form the cord factor and play an important role in the biogenesis of cell wall of mycobacteria [14]. The gene for ESAT-6 is absent in all BCG strains distributed worldwide, but is present in *M. tuberculosis* complex [15]. ESAT-6 is considered a dominant antigen for cell-mediated immunity [16] and is a major target for memory T cells in mice infected with *M. tuberculosis* [17]. Recently, different gene transferring systems such as modified vaccinia virus Ankara [18], alphavirus [19], vaccinia [20], or adenovirus [21, 22], BCG [23], and plasmid DNA [24–27] have been used to express Ag85A. The results of these studies showed that Ag85A can induce humoral and cell-mediated immunity and provide significant protection against TB in different animal models. Subunit vaccine based on antigen ESAT-6 also could enhance

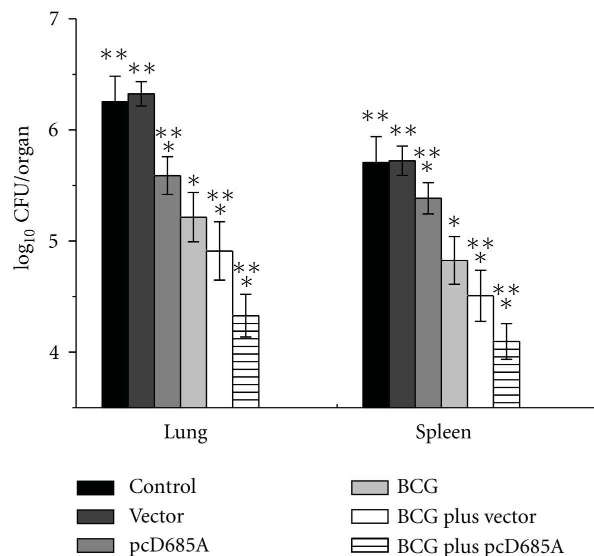

Figure 7: Bacterial load per lung and spleen in C57BL/6 mice at 4 weeks after challenge ($n = 7$). Vaccinated C57BL/6 mice ($n = 7$) were challenged i.v. with 10^6 CFU virulent *M. tuberculosis* H37Rv strain. Four weeks after challenge, lungs and spleens were harvested aseptically and numbers of bacterial CFU per organ were enumerated. Results are shown as the mean (±SEM) $\log_{10}$ CFU/organ. $^*P < .05$ versus control; $^{**}P < .05$ versus BCG. This experiment was repeated twice with similar results.

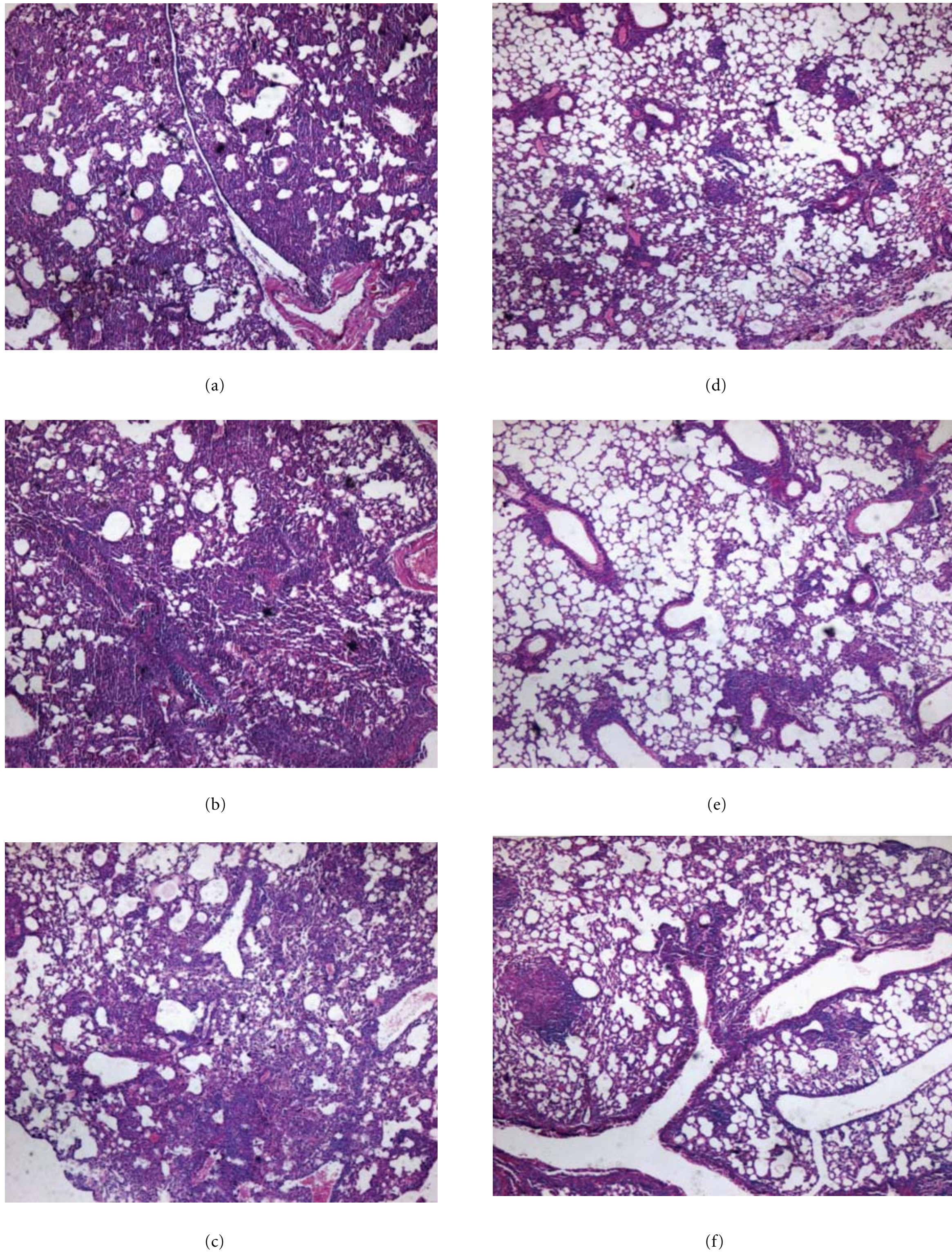

FIGURE 8: Representative lung pathology of C57BL/6 mice after challenge. Vaccinated C57BL/6 mice were challenged i.v with 10^6 CFU virulent *Mycobacterium tuberculosis* H37Rv strain. Four weeks after infection, lung tissue sections from different vaccine groups were prepared for HE staining (amplification 10×4), (a) control; (b) vector control; (c) pcD685A; (d) BCG; (e) BCG plus vector; (f) BCG plus pcD685A.

the efficacy of BCG [9], and the vaccine of the fusion protein of ESAT-6 with Ag85B which promotes strong and long-lived *M. tuberculosis*-specific T cell responses in naïve human volunteers has been evaluated in clinical study [28]. Recombinant BCG expressing ESAT-6 also conferred enhanced immunogenicity and protection against TB when compared with the parent BCG vaccine [29, 30]. Other vaccine platforms such as DNA vaccine [31–33], *Influenza* virus [34], and *Salmonella typhimurium* [35] also demonstrated that ESAT-6 is a potential immunodominant antigen for the development of TB vaccines. In addition, a chimeric protein of Ag85A and ESAT-6 with strong immunogenicity showed a treatment effect on MDR-TB in mice [36, 37]. In order to combine the immunological advantage of both antigens, we designed the fusion protein of Ag85A and ESAT-6 with a linker of 15 amino acid peptides, aimed to keep the naive configuration of these proteins. With the stimulation of purified r685A fusion protein, IFN-γ level in the peripheral blood from the TST-positive household contacts was much higher than that of TST negative group. Without the stimulation of r685A protein, IFN-γ levels in both groups are very low or none. These results indicate that the production of IFN-γ in the peripheral blood is r685A antigen specific and that r685A could stimulate the release of IFN-γ from sensitized T lymphocytes in the peripheral blood of household contacts. IFN-γ is an important indicator for Th1-type cellular immune response and is an essential mediator of the protective immune response to TB [38]. Therefore, we assume that the expression of r685A protein in vivo might be a promising stimulator of Th1-type cellular immune response against TB and might amplify the protective efficacy of BCG. In this study, pcD685A expressing r685A protein can protect mice against a primary *M. tuberculosis* infection with comparable pathological changes in lung with BCG, although bacterial load of lung and spleen in pcD685A vaccinated mice was higher than that of BCG.

Previous studies have shown that repeat vaccination with BCG may be deleterious to protection against TB [39], and heterologous boost vaccines are likely to be used to enhance specific immunity primed by BCG [40] because BCG is not an effective booster vaccine itself. Several studies have demonstrated that recombinant modified vaccinia virus or adenovirus expressing Ag85A could enhance the waning BCG immunity and both have been under clinical evaluation [41, 42]. DNA vaccine expressing ESAT-6 [43] or the fusion protein of Ag85B with ESAT-6 also could amplify the protective immune responses of BCG prime [44, 45]. In our study, BCG prime and pcD685A booster showed the greatest protection against *M. tuberculosis* infection in mice. pcD685A could amplify antituberculous protective immune response in mice previously vaccinated with BCG, and markedly enhanced protection was correlated with increased IFN-γ level in the lungs of mice models prior to challenge. Although the gene encoding ESAT-6 is deleted in BCG, ESAT-6 may amplify overall anti-TB immunity in BCG immune animals by activating ESAT-6-specific T cells and play an important role in producing the booster effect of r685A protein.

5. Conclusion

In this study, our results clearly demonstrated the vaccination of C57BL/6 mice with DNA vaccine (pcD685A) expressing the fusion protein of Ag85A and ESAT-6 (r685A). pcD685A resulted in significant protection against challenge with virulent *M. tuberculosis* H37Rv when compared with the control group. Most importantly, BCG prime and pcD685A booster resulted in the most significant reduction in bacterial load of both spleen and lung when compared with BCG or pcD685A alone. Thus, our study indicates that pcD685A may be an efficient booster vaccine against TB with a strong ability to enhance prior BCG immunity.

Acknowledgments

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Research Article

Novel Prophylactic Vaccine Using a Prime-Boost Method and Hemagglutinating Virus of Japan-Envelope against Tuberculosis

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Objective. *Mycobacterium tuberculosis* infection is a major global threat to human health. The only tuberculosis (TB) vaccine currently available is bacillus Calmette-Guérin (BCG), although it has no efficacy in adults. Therefore, the development of a novel vaccine against TB for adults is desired. *Method*. A novel TB vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan- (HVJ)- envelope was evaluated against TB infection in mice. Bacterial load reductions and histopathological assessments were used to determine efficacy. *Results*. Vaccination by BCG prime with IgHSP65+murine IL-12/HVJ-envelope boost resulted in significant protective efficacy (>10, 000-fold versus BCG alone) against TB infection in the lungs of mice. In addition to bacterial loads, significant protective efficacy was demonstrated by histopathological analysis of the lungs. Furthermore, the vaccine increased the number of T cells secreting IFN-γ. *Conclusion*. This vaccine showed extremely significant protection against TB in a mouse model, consistent with results from a similar paper on cynomolgus monkeys. The results suggest that further development of the vaccine for eventual testing in clinical trials may be warranted.

1. Introduction

Tuberculosis (TB) is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* infection. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guerin (BCG), although its efficacy against adult TB disease is unclear. Furthermore, multidrug-resistant TB (MDR-TB) and extremely drug-resistant TB (XDR-TB) are becoming big problems worldwide. For these reasons, a prophylactic and therapeutic vaccine against TB is sought. TB vaccines are classified into 4 main groups: (1) DNA vaccines, (2) recombinant BCG vaccines, (3) subunit vaccines, and (4) attenuated vaccines.

It is well established that protective immunity to *M. tuberculosis* depends on both CD4$^+$ and CD8$^+$ T cells [1–6]. DNA vaccines induce cellular immune responses, including the Th-1-type cellular immune response, and they prevent infections in animal models [7, 8]. In fact, several human clinical trials have recently been initiated to test the efficacy of DNA vaccines against emerging and re-emerging infectious diseases including hepatitis B [9], malaria [10–12], and HIV infections [13]. DNA vaccines have also shown their potential as TB vaccines in mouse

models [14–17]. However, in a guinea pig model, which is one of the most biologically relevant systems available for studying human pulmonary TB, DNA vaccines have not been proven more efficacious than BCG [18]. The efficacy of any experimental TB vaccine must be evaluated in human clinical trials, and a vaccine against TB is still anxiously awaited.

We have been developing a novel TB vaccine that is a DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12), delivered by the hemagglutinating virus of Japan- (HVJ)- liposome or -envelope (HVJ-E) (HSP65 + IL-12/HVJ) [19–22]. The former vaccine was 100-fold more efficacious than BCG in a murine model on the basis of the elimination of *M. tuberculosis* [19]. In the present study, we demonstrated that the combination of BCG prime with HSP65 + IL-12/HVJ-E vaccine-boost was 10,000-fold more efficacious than BCG alone in a murine TB prophylactic model.

2. Materials and Methods

2.1. Bacteria. *M. tuberculosis* strains H37Rv and *M. bovis* BCG Tokyo, were kindly provided by Dr. I. Sugawara (JATA, Tokyo, Japan). *M. bovis* BCG Tokyo was maintained in synthetic Sauton's medium (Wako Chemicals, Osaka, Japan). For the mouse infection studies, a single colony of *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 medium (DIFCO Laboratories, Detroit, MI; lot 137971 XA MD) supplemented with albumin-dextrose complex and grown at 37°C until approximately midlog phase. Aliquots were stored at −80°C and thawed 10 days before use. Each bacterium was grown to midlog phase in 7H9 medium.

2.2. Animals. Inbred and specific pathogen-free female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained in isolator cages, manipulated in laminar flow hoods, and used between 8 and 10 weeks of age. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. All vaccinations and experiments on isolated tissues were performed on anesthetized animals with sevoflurane. Infected animals were housed in individual microisolator cages in Biosafety Level (BL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center.

2.3. Plasmid Construction. The *HSP65* gene was amplified from *M. tuberculosis* H37Rv genomic DNA, and cloned into pcDNA3.1 (+) (Invitrogen, San Diego, CA) to generate pcDNA-hsp65 (designated as HSP65 DNA) as described previously [19]. The *hsp65* gene was fused with mouse Igκ secretion signal sequence, and pcDNA-Ighsp65 (designated as IgHSP65 DNA) was generated. For construction of the mouse IL-12 (mIL-12) *p40* and *p35* single-chain genes, *mIL12p35* and *mIL12p40* genes were cloned from pcDNA-p40p35 [21], fused and cloned into pcDNA3.1 (+) to generate pcDNA-mIL12p40p35-F (designated as mIL-12 DNA).

2.4. HVJ-E Vaccination. HVJ-E was prepared as described previously (Figure 1) [19–23]. The HVJ-E complex was aliquoted and stored at −70°C until use. Groups of BALB/c mice were vaccinated 3 times at 3-week intervals with 100 μL of HVJ-E solution containing 50 μg of pcDNA-IgHSP65 and 50 μg of mIL12 DNA. These DNA vaccines were injected into both anterior muscles in the tibia. Mice were vaccinated using 1×10^6 colony-forming units (CFU) *M. bovis* BCG Tokyo by subcutaneous injection at 4 different sites (left-upper, right-upper, left-lower, and right-lower back). HVJ-E DNA vaccine containing pcDNA-IgHSP65 and -mIL12 DNA was designated as IgHSP65 + mIL-12/HVJ-E in this text.

2.5. Challenge Infection of Vaccinated Animals and Bacterial Load Determination. Mice were challenged by the intravenous route with 5×10^5 CFU of *M. tuberculosis* H37Rv 4 weeks after the third vaccination as described previously (Figure 2) [19, 24]. 0.2 mL of saline containing 5×10^5 CFU of H37Rv *Mycobacterium tuberculosis* were injected into tail vein of mice. At 5 and 10 weeks after *M. tuberculosis* H37Rv challenge, lungs, spleens, and livers were aseptically homogenized by using a homogenizer in saline, and serial dilutions of the organ homogenates were plated on 7H11 Middlebrook agar (Kyokuto. Tokyo, Japan). Plates were sealed and incubated at 37°C, and the number of colonies was counted 2 weeks later. Results were converted to $\log_{10}$ values. The $\log_{10}$ [mean ± standard deviation (S.D.)] values for CFU/organs/animals were calculated for each experimental group. Weight of lungs, liver, or spleen was measured by a balance (Sartorius Co. LP620S).

2.6. Histological Analysis. The lungs obtained from the mice were fixed with 10% buffered formalin and embedded in paraffin. Each block was sliced into 4-μm-thick sections and stained using hematoxylin and eosin. Semiquantitative morphometric analysis of pathological slides was performed by a method modified over that of Dascher et al. (2003) using a micrometer-attached microscope (Microphot-FXA, Nikon, Japan) [19, 25, 26]. The longer axis and minor axis of each granuloma in the field (×40 magnification) were measured and then multiplied and summed. Three random fields from each tissue section of mice were evaluated. The average score of the fields was designated as the granuloma index ($\times 10^{-2}$ mm^2). This method for the evaluation of granuloma area was significantly correlated with the granuloma area determined by a hematoxylin and eosin section scanning method.

2.7. ELISPOT Assay. The spleens were removed aseptically from vaccinated mice 3 weeks after the third vaccination. Antigen-specific IFN-γ-producing cells were determined by enzyme-linked immunosorbent spot (ELISPOT) as described previously [19]. Briefly, ELISPOT plates (MultiScreen IP Filtration plate MAIPS45; Millipore, Bedford, MA) were coated with antimouse IFN-γ MAb R4-6A2 (BD Biosciences Pharmingen. San Diego, CA). Spleen cells from vaccinated mice were suspended at 1×10^7 cells/mL (1×10^6 cells/well). The cells were placed into 6 antibody-coated wells, and rHSP65 protein (10 μg/mL) or PPD

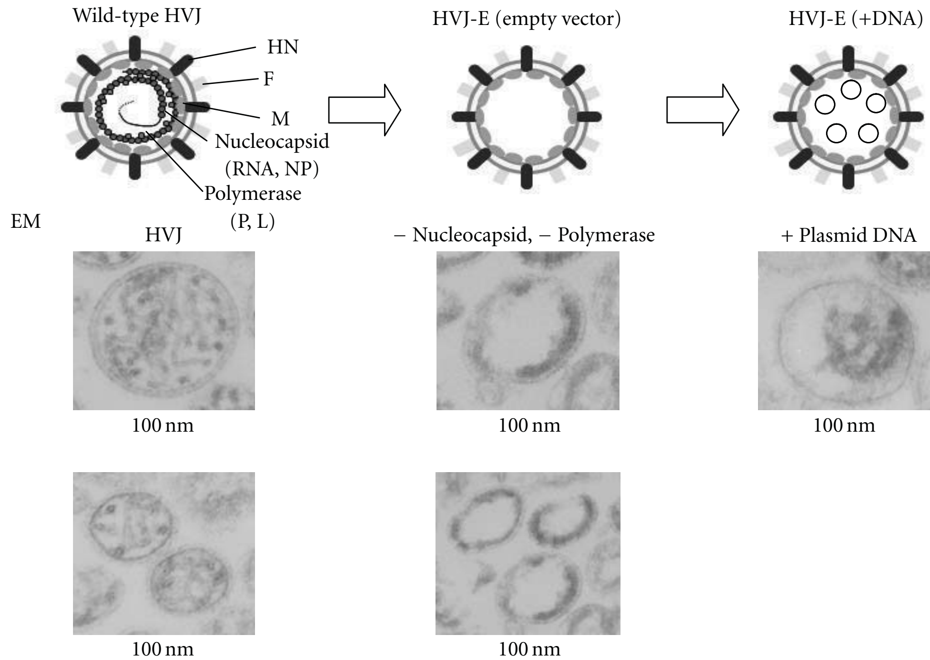

FIGURE 1: Hemagglutinating virus of Japan- (HVJ)- envelope vaccination: pcDNA3.1/HSP65DNA + IL-12DNA were incorporated into an HVJ-envelope empty vector (nonviral vector). Graphical representations of the HVJ-envelope empty vector in the presence or absence of DNA are shown. Electronic microscopy (EM) photographs of the HVJ-envelope empty vector are also shown.

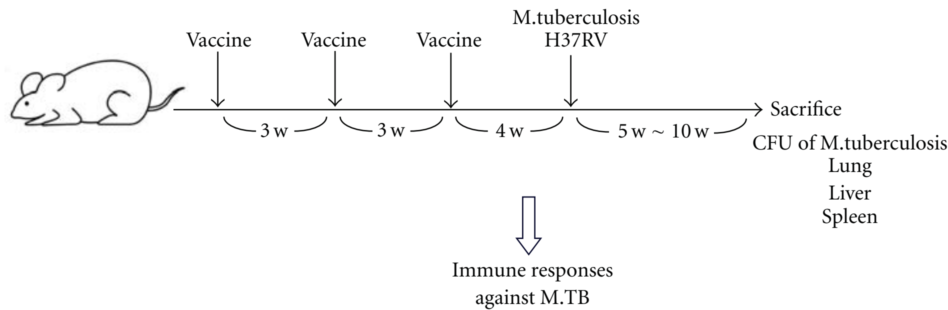

FIGURE 2: Groups of mice were vaccinated 3 times every 3 weeks using the prime-boost method and challenged intravenously with *M. tuberculosis* H37Rv as described in the Materials and Methods section. Five or 10 weeks after challenge with TB, protection was measured by enumerating bacterial loads (CFU) in the lungs, liver, and spleen of the vaccinated mice.

(10 μg/mL) was added to each well. After 20 h of incubation at 37°C, cells were removed by washing the plates, and the site of cytokine secretions was detected using biotinylated antimouse IFN-γ MAb XMG1.2 (BD Biosciences Pharmingen) and streptavidin-alkaline phosphatase conjugate (BD Biosciences Pharmingen). The enzyme reaction was developed with BCIP-NBT substrate (Vector Laboratories Inc., Burlingame, CA). Spot-forming cells (SFCs) were enumerated using the KS ELISPOT system (Carl Zeiss, Hallbergmoos, Germany).

2.8. Statistical Analysis. Dunnett's tests (multiple comparisons) were used to compare $\log_{10}$ values of CFUs between groups following challenge and used to compare T-cell responses between groups in the ELISPOT assay. A *P*-value of < .05 was considered significant.

3. Results and Discussion

3.1. Results

3.1.1. Prophylactic Efficacy Using Prime-Boost Method. The IgHSP65 + mIL-12/HVJ-E and BCG vaccines were administered using the prime-boost method as shown in Table 1.

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H37Rv, the number of CFUs in the lungs, spleen, and liver were determined. Figure 3(a) shows the result of bacterial loads 5 weeks after challenge.

Table 1: BCG-HVJ-E/HSP65 DNA + IL-12 DNA Prime/Boost Experiment. Groups of mice were vaccinated 2 or 3 times with IgHSP65 + mIL-12/HVJ-E vaccine and/or BCG by using the prime-boost method. IgHSP65 + mIL-12/HVJ-E vaccine was injected intramuscularly, and BCG was injected subcutaneously. 4 weeks after the last immunization, *M. tuberculosis* H37Rv was challenged intravenously. 5 weeks and 10 weeks after TB challenge, protection was measured by enumerating bacterial loads (CFU) in the lungs, liver, and spleen from vaccinated mice. One week before the TB challenge, the immune responses of cytotoxic T cells, proliferation of T cells, and cytokines (IFN-γ, IL-2, IL-6) production were assayed.

Group	First immunization	Second immunization	Third immunization
1	—	—	—
2	—	—	BCG
3	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E
4	BCG	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E
5	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E	BCG

13 mice per group.
3 mice for the *in vitro* assay prior to challenge (IFN-γ ELISPOT, etc.).
10 mice for the protection study (half of the mice were used for necropsy at 5 weeks after challenge and half at 10 weeks).

Vaccination by BCG prime + IgHSP65 + mIL-12/HVJ-E boost showed significant protective effects on the bacterial loads in the lungs as compared to BCG alone ($P < .01$). The prime-boost method using DNA and BCG vaccines showed extremely strong protective efficacy (>10,000-fold versus BCG alone) regardless of the order of administration (Figure 3(a)). Vaccination with BCG vaccine alone decreased TB CFUs in the lungs by 1 log unit as compared to nonvaccinated mice.

Vaccination with IgHSP65 + mIL-12/HVJ-E and BCG by the prime-boost method also showed significant protective efficacy on the bacterial loads in the liver as compared to BCG (>100-fold, $P < .05$; Figure 3(b)). The combination of 2 vaccines and administration by the prime-boost method also exerted a significant protective effect on the bacterial load in the spleen as compared to naive control group (10-fold higher, $P < .05$; Figure 3(c)).

Body weight of vaccinated mice was similar in all vaccinated groups. Tissue weights of spleens and livers in the prime-boost groups were lower than those of naive group (Figures 4 and 5).

We also confirmed the greater enhancement of protective effects in the BCG-DNA vaccine combination groups than those in the naive control group or BCG-alone group 10 weeks after challenge (data not shown). These results indicate that treatment using 2 vaccines by the prime-boost method was more effective than BCG alone.

3.1.2. Histological Analysis. In addition to the reduction of bacterial loads, the efficacies of each vaccine were assessed by histological analysis. The number and size of granulomatous lesions in the lungs were significantly lower and smaller, respectively, in the mice vaccinated by the BCG prime-DNA boost group than in the naive control mice and BCG control mice groups (Figure 6). Quantitative evaluation of the granulomatous lesions clearly showed that the BCG prime with IgHSP65 + mIL-12/HVJ-E boost significantly reduced the granuloma index in the lungs as compared to naive and BCG groups ($P < .05$; Figure 7). Thus, vaccination by the prime-boost method has the capability to reduce pulmonary lesions caused by *M. tuberculosis* infection.

3.1.3. Immunological Analysis. Furthermore, BCG prime with IgHSP65 + mIL-12/HVJ-E boost augmented the proliferation and IFN-γ production of HSP65 antigen-specific T cells in the K-S ELISPOT Assay. The efficacy of BCG prime with IgHSP65 + mIL-12/HVJ-E boost was higher compared with BCG Tokyo alone or IgHSP65 + mIL-12/HVJ-E prime with BCG boost (Figure 8).

These data indicate that the protective efficacies of BCG prime with IgHSP65 + mIL-12/HVJ-E boost are strongly associated with the number and activity of IFN-γ-secreting and HSP65-specific T cells. Taken together, combinational vaccination with BCG and IgHSP65 + mIL-12/HVJ-E by the prime-boost method is capable of augmenting T-cell activation. In addition, increase of IFN-γ-secreting cells is involved in the reduction of bacterial burden and lesions in the lungs. The efficacies of the prime-boost method are greater than those achieved by vaccination with BCG alone.

3.2. Discussion. In this study, we evaluated the protective efficacy of IgHSP65 + mIL-12/HVJ-E vaccine, using the prime-boost method. One of the significant findings was that the combination of IgHSP65 + mIL-12/HVJ-E and BCG led to a remarkably high degree of protection against intravenous challenge infection with virulent *M. tuberculosis*; bacterial numbers exponentially declined in 3 organs, especially in the lungs (10,000-fold lower than that of mice vaccinated with BCG alone; Figure 3(a)).

The pathological parameters of protection included reductions in the mean lung granulomatous lesion score in our study. The protective efficacies of BCG with IgHSP65 + mIL-12/HVJ-E administered by the prime-boost method were indicated on the basis of histopathological methods as well as bacterial loads. Histopathological analysis showed that mice vaccinated with BCG prime with IgHSP65 + mIL-12/HVJ-E boost had fewer and smaller lesions in the lungs and significantly less lung granuloma than naive mice and mice treated with BCG alone. These results suggest that severe toxicities (Koch phenomenon) were suppressed by the combination of two kinds of vaccines.

The data in the present study also show that the protective efficacy of BCG prime with IgHSP65 + mIL-12/HVJ-E boost is strongly associated with the emergence of IFN-γ-secreting T cells upon stimulation with HSP65. In the previous study, we demonstrated that *in vivo* function of CD8-positive T cells as well as CD4-positive T cells is involved in prophylactic efficacy of the IgHSP65 + mIL-12/HVJ-E in mice [22].

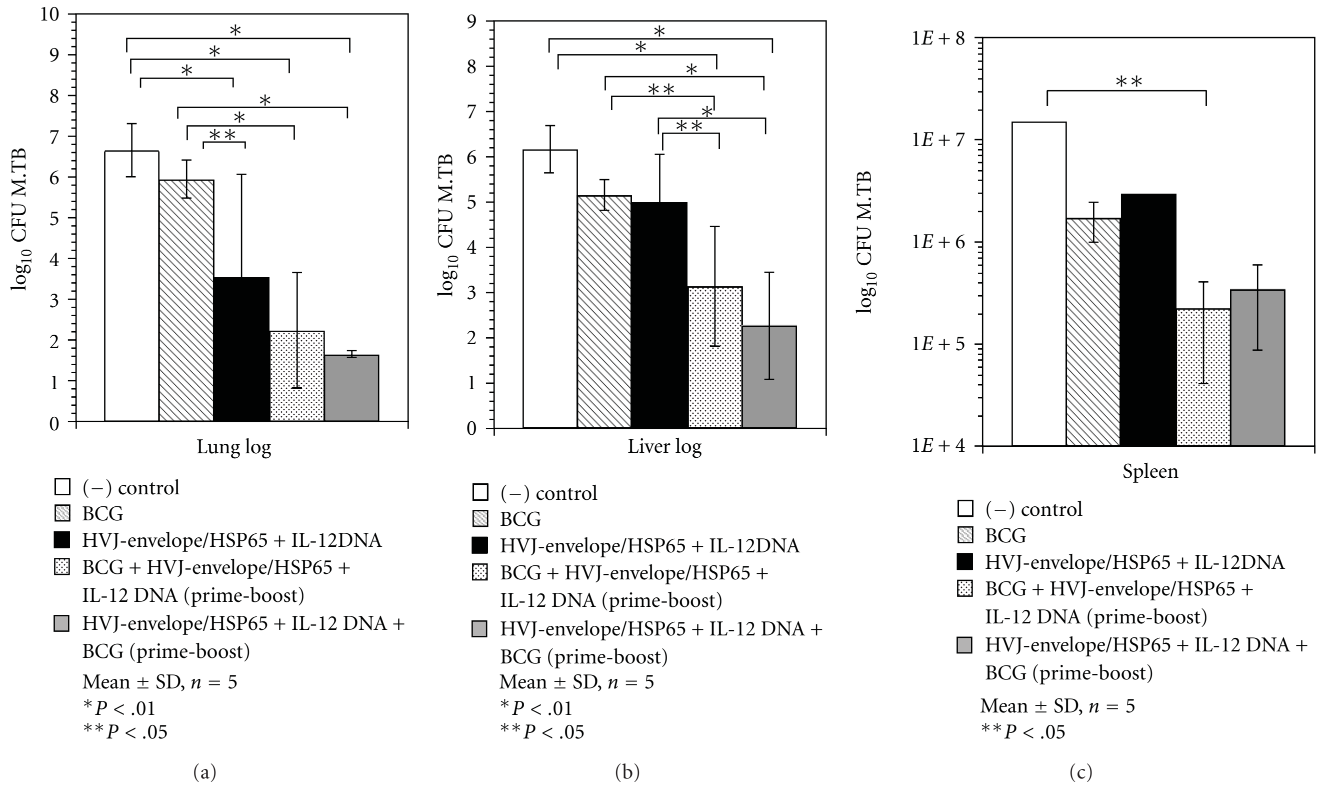

Figure 3: (a) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-envelope (HVJ-E) DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the lungs. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the CFU number was determined by Dunnett test ($n = 5$); $^*P < .01$ and $^{**}P < .05$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). The statistical significance of differences ($^{**}P < .05$) of the G2 group (BCG-alone group) compared to the G3 group (DNA/DNA/DNA), that of differences ($P < .01$) of the G2 group compared to the G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). (b) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the liver. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the CFU number was determined by Dunnett test ($n = 5$), $^*P < .01$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). The statistical significance of differences ($P < .05$) of the G2 group (BCG-alone group) compared to the G4 group (BCG/DNA/DNA). The statistical significance of differences($P < .01$) of the G2 group compared to the G5 group (DNA/DNA/BCG). The statistical significance of differences ($P < .05$) of the G3 group (DNA/DNA/DNA) compared to G4 (BCG/DNA/DNA). That of differences ($P < .01$) of the G3 group compared to the G5 group. (c) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the spleen. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the number of CFU was determined by Dunnett test ($n = 5$); $^{**}P < .05$; the statistical significance of differences ($P < .05$) of the G1 (naive) group compared to the G4 group (BCG/DNA/DNA).

In this study, we used the murine model of TB, which may not reflect the pathologic status of human TB. As to the difference of the infection route, our previous results in a guinea pig model used in a collaborative study with Dr. D. McMurray (Texas A&M University) showed that vaccination with HSP65 + guinea pig IL-12/HVJ resulted in better protection against pulmonary pathology caused by aerosol challenge with *M. tuberculosis* than BCG vaccination (data not shown).

In addition, we have recently confirmed that the prime-boost method was also effective in a cynomolgus monkey model [20–22]. We evaluated our HSP65 + human IL-12/HVJ (HSP65 + hIL-12/HVJ) in the monkey model infected by an intratracheal instillation (aerogenic route), which is currently the best animal model of human TB. Vaccination with HSP65 + hIL-12/HVJ resulted in better protective efficacy than that with BCG alone on the basis of the erythrocyte sedimentation rate test, chest X-ray findings, and immune responses. In addition, vaccination with HSP65 + hIL-12/HVJ resulted in increased survival for over a year. This was the first report of successful DNA vaccination against M. tuberculosis in a monkey model [21].

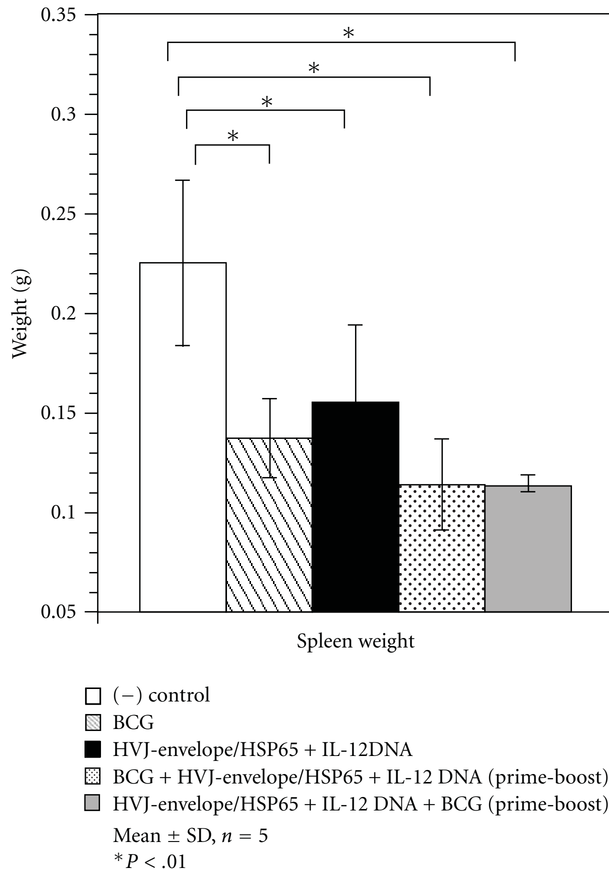

Figure 4: Tissue weight in mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, spleen weight was measured. Results are expressed as the mean ± S.D. in grams (g). The statistical significance of differences between individual groups in the weight was determined by Dunnett test ($n = 5$), $^*P \leq .01$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G2 group (BCG-alone group), G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or G5 group (DNA/DNA/BCG).

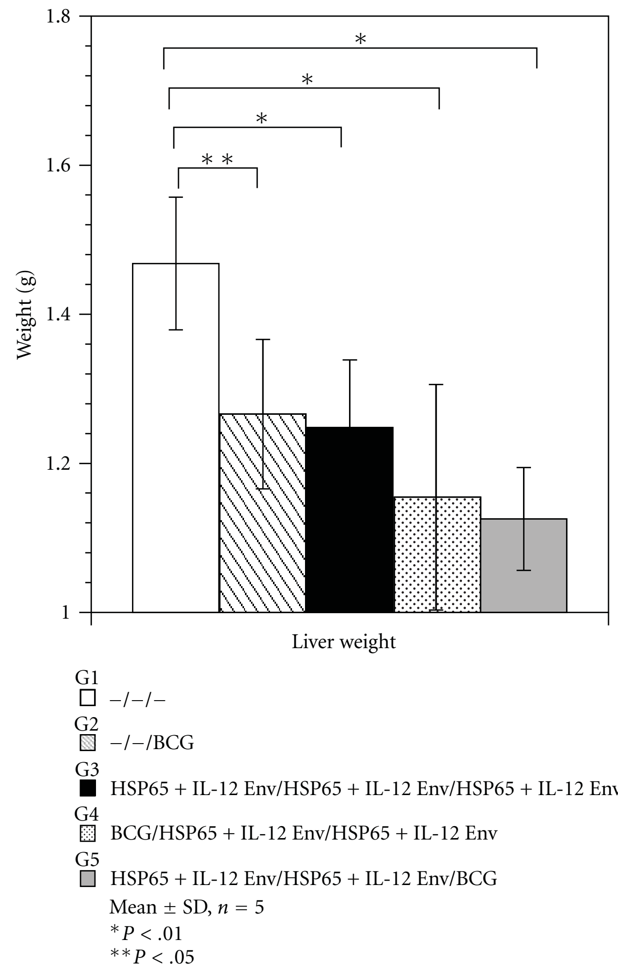

Figure 5: Tissue weight in mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, liver weight was measured. Results are expressed as the mean ± S.D. in grams (g). The statistical significance of differences between individual groups in the weight was determined by Dunnett test ($n = 5$), $^*P < .01$; the statistical significance of differences ($P \leq .01$) of the G1 (naive) group compared to the G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or G5 group (DNA/DNA/BCG). $^{**}P < .05$; that of differences ($P < .05$) of the G1 group compared to the G2 group (BCG alone group).

Most importantly, protective efficacy was augmented when BCG and HSP65 + hIL-12/HVJ were administered by the prime-boost method. Survival rates of BCG alone, saline control, HSP65 + hIL-12/HVJ-prime with BCG-boost, and BCG-prime with HSP65 + hIL-12/HVJ-boost groups were 33%(2/6), 50%(3/6), 50%(2/4), and 100%(4/4) at 12 months after the infection (aerogenic route), respectively [21]. We also evaluated immune responses in the monkey model of TB. Antigen-specific IFN-γ-production and proliferation of peripheral blood lymphocyte (PBL) were enhanced by the vaccination using the prime-boost method.

We also demonstrated efficacies in the monkey model when the boost was performed after a long-term period (4 months) from the prime. The prolongation of the survival was observed in the BCG-prime and HSP65 + IL-12/HVJ-booster group [27]. Improvement of ESR, increase of the body weight and augmentation of IFN-γ production, and proliferation of PBL were also observed in the BCG-prime and HSP65 + IL-12/HVJ-booster group.

Taken together, these results clearly demonstrated that BCG-prime with HSP65 + hIL-12/HVJ-boost could provide extremely strong protective efficacy against M. tuberculosis in a cynomolgus monkey model (intratracheal infection route), which is currently the best animal model of human TB [21].

The prime-boost method was reported in a study of the MVA85A vaccine, which is a modified vaccinia virus Ankara (MVA) strain expressing antigen 85A. In phase I studies in humans, this vaccine has induced high immune responses in previously BCG-vaccinated individuals [28].

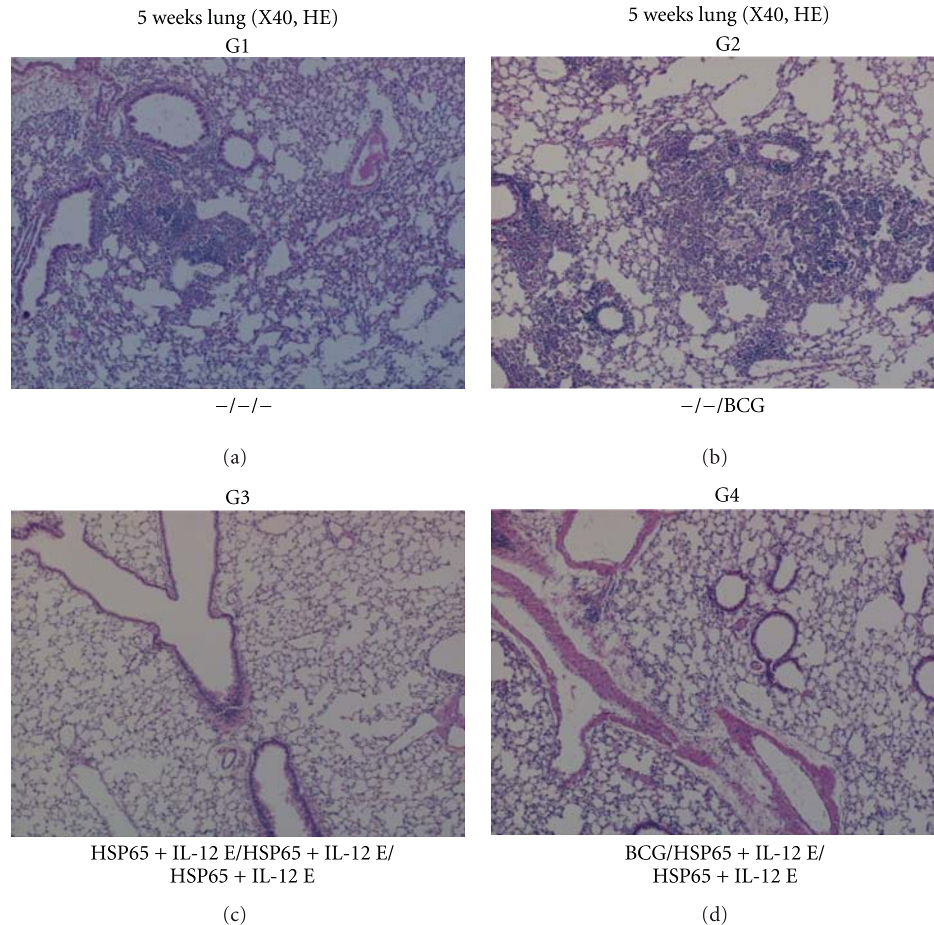

Figure 6: Histopathological analysis of vaccinated mice 5 weeks after *M. tuberculosis* challenge. Representative photomicrographs of lung tissue sections harvested from the G1 naive control group, G2 (BCG alone) group, G3 group (DNA/DNA/DNA), and G4 group (BCG/DNA/DNA) are shown (5 weeks after *M. tuberculosis* challenge, hematoxylin and eosin staining, × 4 objective). There was much infiltration of mononuclear cells and extensive parenchymal destruction by large, poorly demarcated granuloma in the lungs from the G1 (naive control) group and G2 (BCG alone) group. In the G3 (DNA/DNA/DNA) group and G4 (BCG/DNA/DNA) group, there was less inflammation, and only a few granulomas were observed.

Boosting BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGF-β1 [29]. Aeras-402 DNA (DNA that expressed 85A, 85B, and TB10.4) vaccine using adenovirus vector is intended for use as a boosting vaccine in BCG-primed individuals [30]. Several vaccines have been used with a prime-boost strategy to complement immune responses [31].

DNA vaccines are a relatively new approach to induce immunities for the protection of infectious diseases [14, 19, 22, 32–34]. Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as HVJ-liposome, HVJ-E, adenovirus vector, adenoassociated virus vector, and lentivirus vector [19–22, 35, 36]. In order to explore the preclinical use of a tuberculosis DNA vaccine combination of *IL-12* DNA with *hsp65* DNA, we chose the HVJ-based delivery system (HVJ-liposome and HVJ-E). These systems have high transfection efficiency and are available for repeated *in vivo* gene transfection without reduction of gene transfer efficiency or apparent toxicity. These characters of HVJ-liposomes support the feasibility of its clinical application not only for cancer gene therapy but also for DNA vaccinations. In a recent study, highly efficient gene expression in muscle cells was observed for several weeks when pcDNA3 plasmid containing the human tumor antigen genes, *MAGE-1* and *MAGE-3*, were encapsulated in HVJ-liposomes and injected intramuscularly in mice [37]. Effective induction of $CD4^+$ T-cell responses by a hepatitis B core particle-based HIV vaccine was achieved by subcutaneous administration of HVJ-liposomes in mice [38]. HVJ-liposomes were also very effective as a mucosal vaccine against HIV infection [39]. Thus, it is likely that HVJ proteins may be responsible for the induction of a robust immune response. No side effects were observed when repetitive injections of HVJ-liposomes were performed in mice, rats, or monkeys. We have previously developed an HVJ-E using inactivated Sendai virus, as a nonviral vector for drug delivery [40–42]. It can be used for efficient delivery of DNAs, siRNAs, proteins, and anticancer drugs into cells

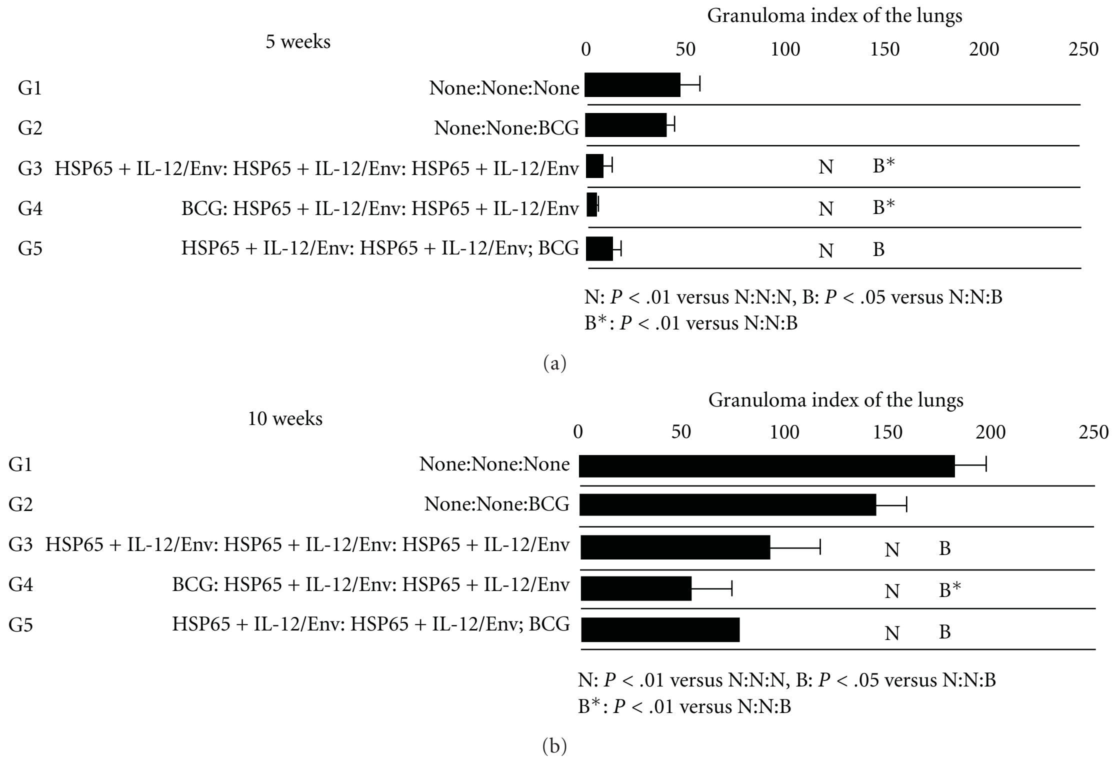

Figure 7: Granuloma index of the G1, G2, G3, G4, and G5 (DNA/DNA/BCG) groups in the lungs 5 weeks and 10 weeks after *M. tuberculosis* challenge. Results are expressed as the mean ± S.D. of triplicates of 5 mice per group. The statistical significance of differences between the groups was determined by Dunnett test, $P < .01$ as compared with the naive (N) group or the BCG alone (B) group. $P < .05$ as compared with the BCG alone (B*) group. The statistical significance of differences ($P < .05$) of granuloma index of 5 weeks G3 group compared to the G4 group.

both *in vitro* and *in vivo* [40, 43, 44]. Therefore, HVJ-E was used as an efficient and safe vector for DNA vaccine against TB in the present study.

Mycobacterial heat shock protein 65 (HSP65) is a potential target for protective immunity and has been studied extensively [19]. Several studies have reported that *hsp65* DNA vaccines can strongly induce protective immune responses in mice against virulent *M. tuberculosis* infections [20–22]. Protection is attributed to the establishment of a cellular immune response dominated by HSP65-specific T cells which produce IFN-γ and are cytotoxic towards infected cells. Furthermore, Lowrie and colleagues have reported that this vaccine reduces bacterial loads in mice infected with *M. tuberculosis* when given therapeutically after infection [32].

One of the major roles of IL-12 is the induction of IFN-γ-mediated immune responses to microbial pathogens. Cooper and colleagues have demonstrated the importance of IL-12 in generation of the protective response to tuberculosis [45]. Coadministration of the *IL-12* gene, which induces an IFN-γ-mediated immune response to microbial pathogens, with various tuberculosis DNA vaccines including *hsp65* DNA [46], and 35 K MW DNA [47], may boost the efficacy of these DNA vaccines to the levels achieved with BCG in the mouse model, although an inhibitory effect rather than a synergistic effect on immunotherapy was observed in mice coadministered *hsp65* DNA vaccine plus the *IL-12* gene [32].

In conclusion, we have shown efficacy of a novel HVJ-E DNA vaccine encapsulating HSP65 DNA with IL-12 DNA in the mouse model of TB. These results suggest that HSP65 + IL-12/HVJ could be a promising candidate for a new tuberculosis vaccine superior to BCG. To this aim, protective efficacy and immune responses were further studied in nonhuman primates before proceeding to human clinical trials.

In Japan and other countries, BCG is inoculated into human infants up to 6 months after birth. Therefore, BCG prime in infants and HSP65 + hIL-12/HVJ boost in adults (including junior high school students, high school students, and the elderly) may be required for significant improvement of clinical protective efficacy against TB. Thus, our results with the HSP65 + hIL-12/HVJ vaccine in a murine prophylactic model and cynomolgus monkey prophylactic model provide a significant rationale for moving this vaccine into clinical trials. Indeed, multiple animal models are available to accumulate essential data on the HVJ-E DNA vaccine in anticipation of a phase I clinical trial.

4. Conclusions

Vaccination by BCG prime with a novel vaccine (IgHSP65 + mIL-12/HVJ-E) boost resulted in significant protective efficacy (10,000-fold greater than BCG alone) against TB

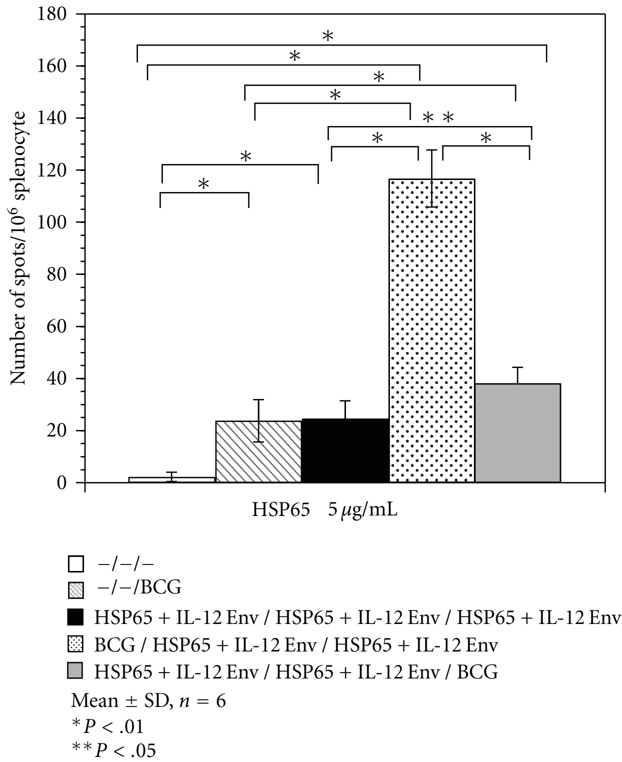

Figure 8: ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with rHSP65 protein. Spleen cell cultures were stimulated with rHSP65 protein for 20 h. The numbers of IFN-γ-secreting cells specific for rHSP65 protein per million cells were determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of 6 wells of 3 mice per group. The statistical significance of differences between individual groups in the number of IFN-γ-secreting cells was determined by Dunnett test. The statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G2 (BCG alone group), G3 (DNA/DNA/DNA), G4 (BCG/DNA/DNA), or G5 (DNA/DNA/BCG). The statistical significance of the G2 group difference ($P < .01$) compared to the G4 or the G5. The statistical significance of the G3 group differences ($P < .01$) compared to the G4. $P < .01$; the G4 group compared to the G5. The statistical significance of the G3 group differences ($P < .05$) compared to the G5.

infection in the lungs of mice. In addition to bacterial loads, significant protective immunity was demonstrated by histopathological analysis of the lungs. This vaccine showed extremely significant protection against TB, suggesting that further development for eventual testing in clinical trials may be warranted.

Acknowledgments

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Research Article

The *M. tuberculosis* Phosphate-Binding Lipoproteins PstS1 and PstS3 Induce Th1 and Th17 Responses That Are Not Associated with Protection against *M. tuberculosis* Infection

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The *M. tuberculosis* phosphate-binding transporter lipoproteins PstS1 and PstS3 were good immunogens inducing $CD8^+$ T-cell activation and both Th1 and Th17 immunity in mice. However, this antigen-specific immunity, even when amplified by administration of the protein with the adjuvant LTK63 or by the DNA priming/protein boosting regimen, was not able to contain *M. tuberculosis* replication in the lungs of infected mice. The lack of protection might be ascribed with the scarce/absent capacity of PstS1/PstS3 antigens to modulate the IFN-γ response elicited by *M. tuberculosis* infection during which, however, PstS1-specific IL-17 secreting cells were generated in both unvaccinated and BCG-vaccinated mice. In spite of a lack of protection by PstS1/PstS3 immunizations, our results do show that PstS1 is able to induce IL-17 response upon *M. tuberculosis* infection which is of interest in the study of anti-*M. tuberculosis* immunity and as potential immunomodulator in combined vaccines.

1. Introduction

Tuberculosis (TB) remains a leading human infectious disease and a major public health problem in low-income countries [1]. Despite the availability of the Bacillus Calmette-Guérin (BCG) vaccine for more than 80 years, until now an effective tuberculosis vaccine is still unavailable and still unknown are the correlates of protection against this disease [2]. Several protective antigens in particular those belonging to the immunodominant complex Ag85 [3–5] have been studied but immune responses against most of them are still not precisely defined. Hence, the search for new protective *Mycobacterium tuberculosis (M. tuberculosis)* antigens remain a vital field, particularly for proteins exposed on the bacterial cell surface exerting important physiological functions and/or acting as virulence factors.

We recently focused our attention on phosphate-specific transporter (pst) lipoproteins of *M. tuberculosis*. Pst is a membrane-associated complex that belongs to the ABC transporter superfamily [6]. In *M. tuberculosis*, three putative pst operons have been identified pstS1, pstS2, and pstS3 [7], which probably constitute a subtle biochemical adaptation of this microorganism for its growth and survival under different phosphate-limiting conditions during its infectious cycle [8]. The three genes coding these proteins are very similar (about 75% similarity between *pstS1* and *pstS2* or *pstS3* and 94% similarity between PstS2 and PstS3), and all proteins have a lipoprotein consensus signal [8]. These phosphate transport receptors are exposed not only on the cell surface of *M. tuberculosis* but also on the surface of *M. bovis* BCG [8].

Disruption of genes encoding *pstS1* and *pstS2* reduced *in vivo* multiplication of *M. tuberculosis* suggesting that the high-affinity phosphate-specific transporters are also virulence factors of *M. tuberculosis* and *M. bovis* [9]. Specific immunity against PstS lipoprotein has been detected in TB patients, and in particular antibodies (Abs) against PstS1 have been reported to be a valuable tool in the serodiagnosis

of active TB [10–12]. Moreover, mice vaccinated with DNA coding for *pstS3* demonstrated significant and sustained reduction in bacterial load in spleen and lungs for 3 months after *M. tuberculosis* challenge, as compared with CFU counts in control unvaccinated mice [13]. Conversely, immunizations with DNA coding for *pstS1* resulted in contradictory evidence regarding protection [13, 14] possibly due to different plasmid vector backbone and different experimental protocol of immunization and infection.

In this paper, we have reassessed the role of PstS3 and PstS1 antigens on B and T cell-mediated immunity and protection against *M. tuberculosis* infection. To expand and diversify the immune responses induced by immunizations with DNA only, we used a combination of DNA and protein in a prime-boosting regimen and also the protein administered with the adjuvant LTK63. In fact, our previous studies on Ag85B, an abundant secreted protein of replicating MTB which is currently evaluated in various TB vaccine formulations [5], revealed that antigen-specific immunity can be enhanced by priming/boosting regimen [3]. In addition, the potent mucosal as well as systemic adjuvant LTK63, a nontoxic derivative of heat-labile enterotoxin of *Escherichia coli* [15], can exert positive and negative control on Ag85B-specific Th1 polarization [4, 5]. The attention was focused especially on the Th1 and Th17 responses, in view of their relevant role in TB immunity. The importance of IFN-γ-producing $CD4^+$ T cells in primary resistance to *M. tuberculosis* has been established both in humans and mice [16–18]. IL-17, a proinflammatory cytokine mostly produced by Th17 cells, can provide IFN-γ-dependent or IFN-γ-independent protection to M. tuberculosis infection [19, 20]. On the other hand, both the IFN-γ- and/or IL-17-induced inflammation needs to be tightly controlled during *M. tuberculosis* infection otherwise they can have important pathological consequences [20–24].

Therefore the aim of the work was to dissect the PstS1- and PstS3-specific immunity, through the use of different protocols of immunizations, suitable to the identification of protective responses. Some of the immune responses induced by PstS1 and PstS3 have been compared with those induced by Ag85B, taken as protective antigen.

2. Materials and Methods

2.1. Ethics Statement. The handling of mice was conducted in accordance with the regulations set forward by the institutional animal care committee of the Italian Ministry of Health, and in compliance with European Community Directive 86/609 and the US Association for Laboratory Animal Care recommendations for the care and use of laboratory animals.

2.2. Microorganisms. M. tuberculosis H37Rv (ATCC 27294) and *M. bovis* BCG strain Pasteur (ATCC 27291) were grown at 37°C in Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase enrichment under agitation (120 rpm) up to mid-exponential phase. Aliquoted stocks were stored at −70°C until use; the titers of stocks were verified on a regular basis by counting the numbers of colony forming unit (CFU) on Middlebrook 7H10 agar plates. For the manipulation of plasmids, *Escherichia coli (E. coli)* (strains: MC1061, K12 CAG629, and Rosetta) was grown on Luria-Bertani (LB) broth or agar supplemented with ampicillin (100 μg/mL), as required.

2.3. Production of DNA-PstS1, DNA-PstS3, or DNA-Ag85B Vaccines and Recombinant PstS1, PstS3, or Ag85B Proteins. Plasmid DNA coding for PstS1 or PstS3 was prepared as previously described [13, 14, 25]. Briefly, plasmid DNA was isolated from *E. coli* host strains by standard plasmid preparation technique using the QIAGEN Plasmid Maxi Kit according to the manufacturers recommendations. Bacteria were cultured over night in LB Broth containing the appropriate antibiotics. Following plasmid isolation, the quality of DNA was determined using agarose gel electrophoresis. All plasmid DNA contained their own signal sequence and the human tpa signal sequence. The plasmid sequences were verified by DNA sequencing. Recombinant PstS1 protein was prepared in *E. coli* strain K12 CAG629 containing the expression plasmid for recombinant PstS1 [25]. After cell disruption by sonication, the inclusion body bound protein was solubilized in buffer containing 8 M urea and refolded by gel filtration on Sephadex G25 fine medium (GE Healthcare). Protein contaminants were separated from PstS1 by performing anion exchange chromatography using Q Sepharose High Performance (GE Healthcare). The protein solution was concentrated by crossflow filtration with a module of 100 kDa molecular weight cutoff to a concentration of approximately 1 mg/mL. Finally a buffer exchange step into volatile buffer was performed by gel filtration on Sephadex G25 fine medium (GE Healthcare), and the protein was freeze dried. Recombinant PstS3 protein was prepared in *E. coli* strain Rosetta (DE3) (Novagen) containing the expression plasmid for recombinant PstS3. Cells were grown in LB medium in shaking flasks at 37°C and induced with 1 mM IPTG. 4 h after induction cells were harvested. After cell disruption by sonication the inclusion body bound protein was solubilized in buffer containing 8 M urea. A metal chelate affinity chromatography under denturating conditions was performed (Ni-NTA resin, QIAGEN), and bound protein was eluted in an increasing imidazole gradient. PstS3 containing fractions were refolded by gel filtration on Sephadex G25 fine medium (GE Healthcare) in potassium phosphate buffer. The refolded protein was concentrated using VivaCell concentrators (10 kDa molecular weight cutoff; Sartorius stedim) to a concentration of approximately 150 μg/mL and stored at 4–8°C. The LPS content of all protein preparations was measured by a *Lymulus* amebocyte lysate test and shown to be below 4.3 EU/μg of protein.

Recombinant Ag85B protein and plasmid DNA coding for Ag85B were prepared as previously described in [3, 4].

2.4. Immunization and Mycobacterial Infection. C57BL/6 female mice were supplied as specific pathogen-free mice by Harlan (Udine, Italy) and were maintained in specific pathogen-free conditions. Food and water were available ad libitum. Seven- to 8-week-old mice were immunized. Fifty μg of plasmid DNA-*pstS1*, DNA-*pstS3*, or DNA-*Ag85B*

was injected i.m. in 50 μl PBS into the hind leg. On the dorsum of the mice, 10 μg of recombinant PstS1, PstS3, or Ag85B protein was administered s.c with or without 10 μg LTK63 (Novartis Vaccine and Diagnostics Srl, Siena, Italy). Mice were immunized, at 2-week intervals, 2 or 4 times with DNA, and 2 times with recombinant protein alone or together with LTK63 adjuvant. Some mice were boosted twice with recombinant Ag85B protein coadministered with LTK63 adjuvant after priming with DNA. As a positive control, a single dose of BCG (10^5 CFU) was injected s.c. (Figure 1).

Four weeks after the last immunization, mice were challenged i.v. in a lateral tail vein with 10^5 CFU of *M. tuberculosis* H37Rv. Infection studies were performed in a biosafety level 3 facility; mice were housed in microisolator cages and fed with autoclaved food and water at libitum. After 4 weeks, the mice were killed by cervical dislocation, and the number of bacteria in lungs was enumerated by homogenizing the tissue and plating 10-fold dilutions, prepared in distilled water, on Middlebrook 7H10 agar. The colonies were counted visually after 21 days of incubation.

2.5. Antibody Measurement. Sera from immunized mice were collected by retro-orbital bleeding 4 weeks after the last immunization. The levels of total anti-PstS1 and anti-PstS3 IgG antibodies (Abs) were determined by ELISA. Briefly, polyvinyl microtiter plates (Nunc) were coated overnight at 4°C with 10 μg/mL each recombinant antigen (PstS-1, PstS-3) in PBS. Several dilutions of mouse serum were incubated for 45 min at 37°C prior to the addition of antimouse immunoglobulin G-peroxidase conjugate and then tetramethylbenzidine substrate. The mean absorbance of naive mouse sera, diluted 1 : 100, plus 3 standard deviations was adopted as the cutoff absorbance for determining Ab titers.

2.6. Splenocyte Preparation and Cell Culture. Four weeks after the last immunization, single cell suspensions were prepared from the pooled spleens (3 mice/group), passed through Falcon 2360 cell strainers (BD Discovery Labware), centrifuged, aliquoted, and then frozen in liquid nitrogen. Some spleen cells were prepared from unvaccinated mice or BCG-vaccinated mice infected with *M. tuberculosis* as described above. Spleens of infected mice were recovered after 4 or 11 weeks from the challenge and processed in a biosafety level 3 facility. From single spleen cell suspensions, depletion of $CD4^+$ T-cell population was obtained by the magnetically labeled fractions isolated from the specific mouse $CD4^+$T-cell isolation kits (Miltenyi Biotec Inc., Auburn, CA), in accordance with the manufacturer's instructions. By FACS analysis, negligible fluorescence was observed in depleted fractions during the labeling of cells with the monoclonal Ab CD4-PE corresponding to the depleted cell population. Cells isolated from spleens of naïve, vaccinated, or MTB-infected mice were cultured at $4 \times 10^5/200\,\mu$l in 96-well flat plates in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES buffer, 50 μM 2-β-Mercaptoethanol, 50 U/mL penicillin, and 50 μg/mL streptomycin (complete RPMI, cRPMI) and stimulated with Ag85B, PstS1, or PstS3 proteins (5 μg/mL each).

2.7. Cytokine Detection. Culture supernatants after 4 days of culture were assayed for IFN-γ and IL-17, by specific quantitative sandwich ELISA Kits (mouse Quantikine, R&D System, Inc., Minneapolis, MN), in accordance with the manufacture's instructions. Quantitation was made against a standard curve obtained for individual cytokine standards provided by the manufacturer.

2.8. Cell Proliferation by CFSE Staining. Spleen cells (10^7 cells/mL) were stained with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Invitrogen Life Technologies) at 1 μM in PBS 1% FBS for 10 min at 37°C in the dark. Cells were washed and cultured in 96-well plates, as previously described, for 4 days. After the incubation time, cells were washed with FACS buffer and stained for 20 min at 4°C with PE antimouse CD4 and PerCP antimouse CD8, (BD Biosciences Pharmingen) or the isotype controls. Cells were washed and analyzed on a FACScan flow cytometer with the Cell Quest software programs.

2.9. Cell Proliferation by H^3-Thymidine Incorporation. After 4 days of culture, the cells were pulsed with [H^3]thymidine (1 μCi/well) (Perkin Elmer Life and Analytical Sciences, Boston, MA) for additional 18 h. Incorporation of [H^3]thymidine was measured by β-scintillation counting (Microβ counter, Perkin Elmer). Values were expressed as mean counts per minute (cpm) in the cultures.

3. Results

3.1. Immunization with Protein Was Required to Induce Specific Anti-PstS1 or Anti-PstS3 Ab Responses, with PstS1 Being a Better Inducer. In a first series of experiments, mice were immunized with the antigens PstS1 and PstS3 by using several protocols of immunizations, as reported in Figure 1. In particular, DNA was given two or four times, protein, alone or together with LTK63 adjuvant, two times, and priming with two DNA injections and boosting with the protein in LTK63 adjuvant twice. These protocols were selected based on previous work made with the immunodominant *M. tuberculosis* antigen Ag85B, which generated differential humoral and cell-mediated responses associated or not with protection against *M. tuberculosis* challenge (Table 1).

Four weeks after the last immunization, the sera were analyzed for specific anti-PstS1 or anti-PstS3 immunoglobulins (Ig) (Figures 2(a) and 2(b)). Specific IgG were detected exclusively in mice receiving protein with or without adjuvant. PstS1 antigen was a good inducer of antigen-specific Ab response while the Ab-specific production in mice immunized with PstS3 was very weak. In addition, in mice immunized with PstS1 the adjuvant LTK63 greatly enhanced the anti-PstS1 Ab production, and a cross-reactivity with PstS3 antigen was also observed. On the other hand, in mice immunized with PstS3 antigen the antigen-specific IgG titer was minimally enhanced by the adjuvant LTK63 and no cross-reactivity with PstS1 antigen was found. These data suggested a superior capacity of PstS1 to act as cross-stimulating Ab inducer.

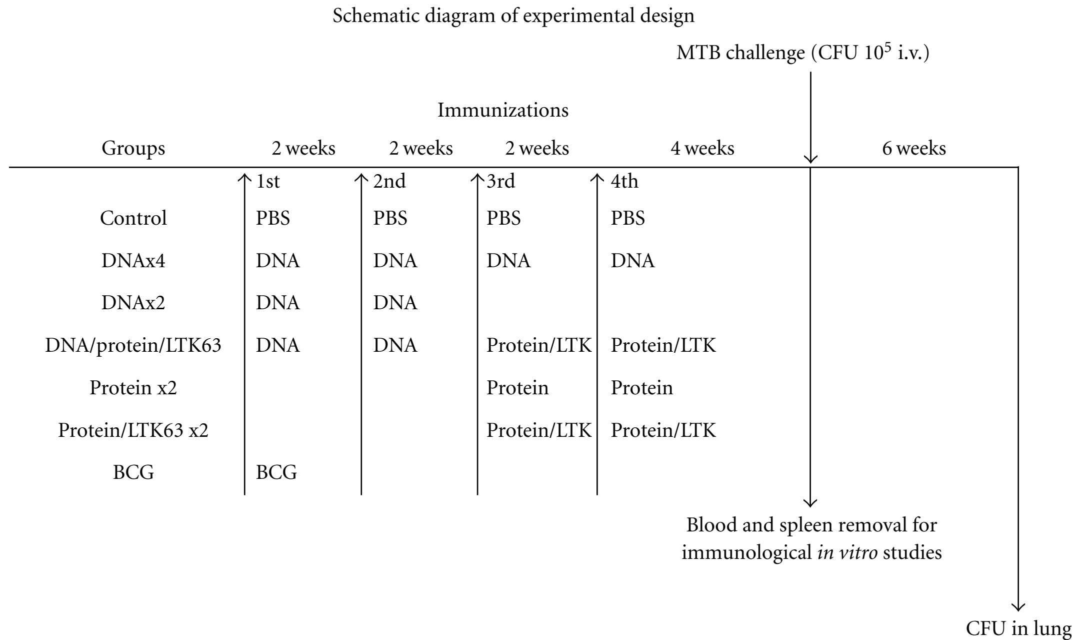

Figure 1: Schematic diagram of experimental design. C57BL/6 female mice were immunized twice or four times at 2-week intervals, with plasmid DNA (coding for *pstS1*, *pstS3,* or *ag85B*) or recombinant proteins (PstS1, PstS3, or Ag85B) in the presence or absence of LTK63 adjuvant. As a positive control, a single dose of BCG was injected s.c. Four weeks after the last immunization, mice were challenged i.v. with 10^5 CFU of *M. tuberculosis* H37Rv or killed to recover blood and spleen. Control: naive C57BL/6 female mice receiving PBS; DNA: plasmid DNA coding *pstS1*, *pstS3,* or *ag85B* 50 μg/injection i.m.; protein: PstS1, PstS3, or Ag85B proteins 10 μg/injection s.c.; protein/LTK63: PstS1, PstS3, or Ag85B protein together with the adjuvant LTK63 10 μg each/injection s.c.; BCG: BCG Pasteur 10^5 CFU s.c.

Table 1: Effects of different immunizations with the mycobacterial antigen Ag85B on protection against *Mycobacterium tuberculosis* challenge and antigen-specific immunity in mice.

Immunizations[a]	IgG titer[b]	Cell proliferation (cpm)[c]	IFN-γ (pg/mL)[d]	IL-17 (pg/mL)[e]	$-\Delta$ CFU(log)/protection[f]	Reference[g]
DNA x2	800 ± 200	3500 ± 300	450 ± 30	0 ± 0	−0.7/yes	[3]
DNA x4	2000 ± 350	3937 ± 1064	559 ± 60	0 ± 0	−0.74/yes	[3]
DNA/protein-LTK63	43053 ± 4746	22037 ± 2695	6214 ± 168	320 ± 52	−0.32/yes	[4]
protein-LTK63	64507 ± 12388	16714 ± 326	3839 ± 150	420 ± 90	−0.31/yes	
protein	2800 ± 542	7579 ± 645	333 ± 100	10 ± 5	−0.02/no	[3]
BCG					−1.1/yes	[3, 4]

[a] mice (5 mice/group) were immunized with Ag85B according to Figure 1, and some mice challenged also with *M. tuberculosis* H37Rv.
[b] sera of immunized mice recovered 4 weeks from the last immunizations were analyzed by ELISA for the presence of anti-Ag85B Ab using a conjugated secondary Abs specific total IgG. Data are plotted as geometric mean ELISA titer ± SEM of 3 independent experiments.
[c] spleen cells of Ag85B-immunized mice were cultured at 4×10^5 cells/well and re-stimulated *ex vivo* with Ag85B protein (5 μg/mL) for 5 days before measuring thymidine incorporation in proliferating cells. Cell proliferation data are represented as mean cpm (subtracted from the cpm of unstimulated spleen cells) ± SEM of 3 independent experiments.
[d] spleen cells of Ag85B-immunized mice were cultured at 4×10^5 cells/well and re-stimulated *ex vivo* with Ag85B protein (5 μg/mL) for 4 days before measuring in cell culture supernatants IFN-γ by a specific ELISA kit. IFN-γ data are represented as mean (pg/mL) ± SEM of 3 independent experiments.
[e] spleen cells of Ag85B-immunized mice were cultured at 4×10^5 cells/well and re-stimulated *ex vivo* with Ag85B protein (5 μg/mL) for 4 days before measuring in cell culture supernatants IL-17 by a specific ELISA kit. IL-17 data are represented as mean (pg/mL) ± SEM of 3 independent experiments.
[f] After 4 weeks from *M. tuberculosis* challenge, the bacteria in the lung of infected mice were enumerated as described in Materials and Methods. Data are expressed as the Δ in CFU/lung (log) between vaccinated and unvaccinated control mice and it is indicated whether or not the vaccination was protective against *M. tuberculosis* challenge.
[g] Reference reporting some of the data.

3.2. Antigen-Specific Cell Proliferation Was Mainly due to CD8$^+$ T Cells and Was Higher in PstS1- than in PstS3-Immunized Mice: PstS1 Fully Activated Cells of Mice Immunized with PstS3 Antigen while the Effect of PstS3 Re-Stimulation Was Only Partial on Cells of PstS1-Immunized Mice. Spleen cells of immunized mice were re-stimulated *ex vivo* with PstS1 or PstS3 antigens and cell proliferation was measured by thymidine incorporation in total spleen cells at 5 days of culture or by CFSE dilution in CD4$^+$ or CD8$^+$ T cell populations after 4 days of culture (Figure 3).

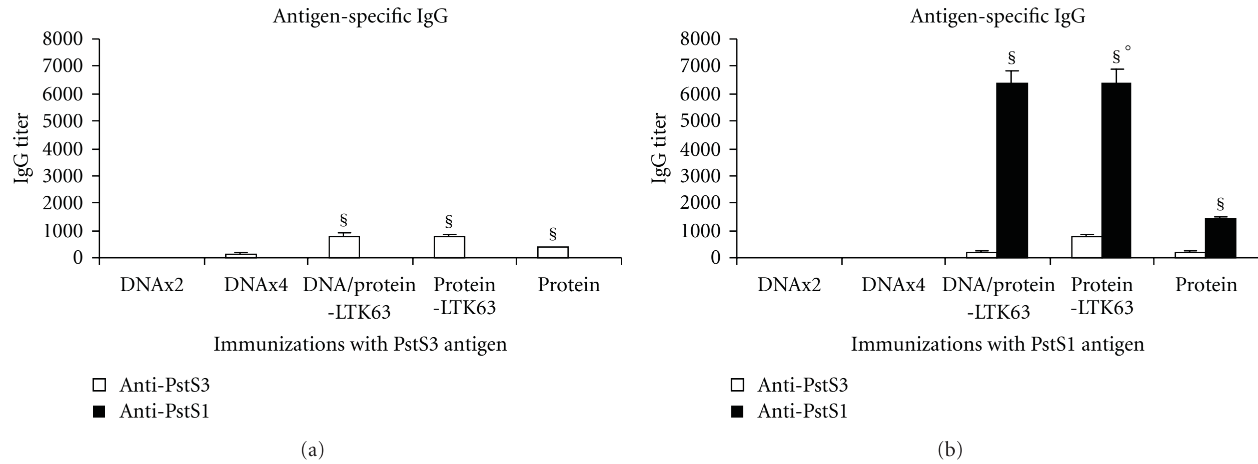

Figure 2: PstS1 and PstS3-specific Ab production in immunized mice. Pooled serum samples (3 mice/group) of mice immunized with antigen PstS3 (a) or PstS1 (b) were analyzed by ELISA for the presence of anti-PstS1 or anti-PstS3 Abs using a conjugated secondary Abs specific total IgG. Data, combined from 3 independent experiments, are plotted as geometric mean ELISA titer ± SEM. §Statistical significant difference among anti-PstS1 Ab titer and anti-PstS3 Ab titer in each group of immunized mice ($P < .05$ or $P < .01$ determined by a two-tailed Student's t-test); °statistical significant difference in anti-PstS1 Ab titer between mice immunized with PstS1 protein alone and mice immunized with protein together with LTK63 adjuvant, $P < .01$ determined by a two-tailed Student's t-test.

Spleen cells of both PstS1 or PstS3 immunized mice proliferated in response to antigens with a greater contribution of CD8$^+$ T cells rather than CD4$^+$ T cells (Figures 3(c), 3(e), 3(d), and 3(f)). Proliferation was significantly higher by cells of mice immunized with protein rather than in cells of mice immunized with DNA especially in mice immunized with pstS1 antigen (Figures 3(a), 3(b), and 3(f)). Moreover in mice immunized with DNA only, CD8$^+$ T cells were the cells mainly involved in proliferation (Figures 3(e) and 3(f)). While scarce or absent was the contribution of CD4$^+$ T cells (Figures 3(c) and 3(d)). Administration of either PstS1 or PstS3 protein with LTK63 adjuvant did not significantly modify the antigen-specific memory response. Here again, some important differences between the two antigens were found. Mice immunized with PstS1 showed a greater cell proliferation compared to mice immunized with PstS3, while an anti-PstS1-specific CD4$^+$ T cell proliferation, although weak, was found also in mice immunized with DNA only (Figure 3(d)).

Cells of mice immunized with PstS1 proliferated also in response to PstS3 antigen stimulation but the effect was restricted to CD8$^+$ T cell population, and the proliferation was significantly lower than that induced by PstS1 re-stimulation (Figure 3(f)). On the other hand, in cells of mice immunized with PstS3 antigen the magnitude of proliferation in response to both PstS1 and PstS3 antigen re-stimulation was similar and PstS1 was even better in re-activating CD8$^+$ T cell proliferation in mice immunized four times with DNA or in the group immunized with protein and LTK63 (Figure 3(e)). Moreover, CD4$^+$ T cells of some PstS3 immunizations (DNA/proteinLTK63 and protein LTK63-immunized mice) were able to proliferate in response to antigenic recall with PstS1 protein (Figure 3(c)).

These data suggested that PstS1 and PstS3 also differed in the capacity to induce and/or recall antigen-specific memory T cell proliferation.

3.3. PstS3 Antigen Was More Potent than PstS1 Antigen in Generating Memory IFN-γ-Producing Cells but Only PstS1 Protein Induced IL-17. Spleen cells of immunized mice were re-stimulated in vitro with PstS1 or PstS3 antigen and after 4 days the supernatants of culture were analyzed to detect IFN-γor IL-17 by ELISA. All kind of immunizations generated antigen-specific memory IFN-γ-secreting cells although the secretion was very low in mice immunized with protein alone or DNA only except in those animals receiving four administrations of DNA coding for *pstS3* (Figures 4(a) and 4(c)). Administration of protein with LTK63 greatly enhanced the IFN-γ response in the immunization with PstS3 antigen (Figure 4(a)) while the increase was modest, although still statistically significant, in PstS1 immunizations (Figure 4(c)). CD4$^+$ T cells were mandatory for IFN-γ production as indicated by CD4$^+$ T cell-depletion studies (Figures 4(e) and 4(f)). A weak cross-reactivity was observed with the related protein in all immunizations. In general, PstS3 antigen was a better inducer than PstS1 of memory IFN-γ-producing cells.

The production of IL-17 was associated with the administration of PstS3 protein with adjuvant LTK63 (Figure 4(b)), while immunization with PstS1 protein even in the absence of adjuvant induced a specific Th17 response (Figure 4(d)). The production of IL-17 was higher in PstS1 immunizations compared to PstS3 immunizations. In PstS1-immunized mice the ratio of IL-17/IFN-γ was higher or around 1 indicating that the amount of the two cytokines released was similar (Figures 4(c) and 4(d)), while in PstS3 immunizations the ratio was much lower than 1 since the production of IFN-γ

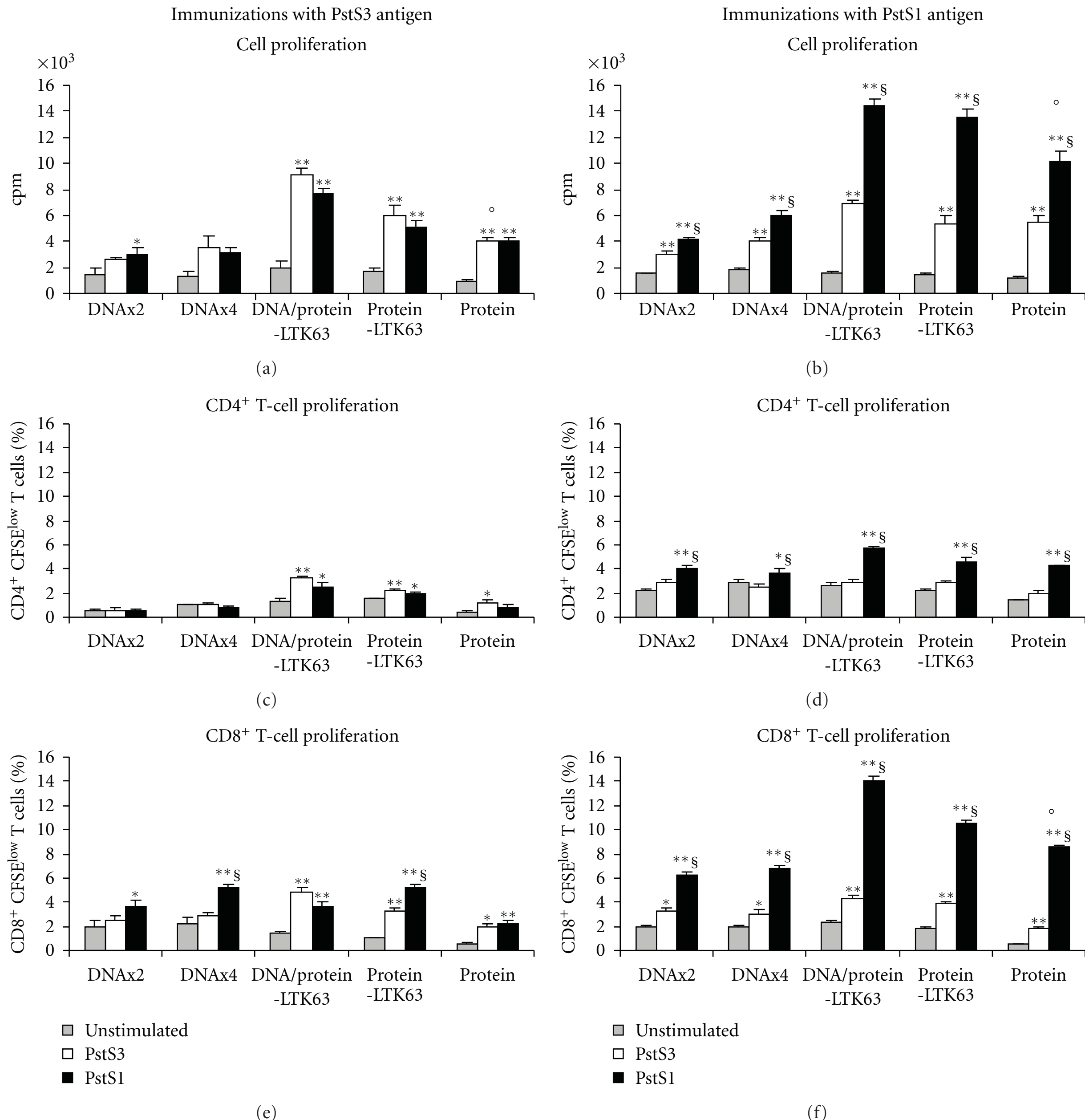

FIGURE 3: Cell proliferation in response to PstS1 or PstS3 proteins in spleen cells of immunized mice. Pooled spleen cells (3 mice/group) of mice vaccinated with PstS3 antigen (panels (a), (c), and (e)) or with PstS1 antigen (panels (b), (d), and (f)) were cultured at 4×10^5 cells/well and re-stimulated *ex vivo* with PstS1 or PstS3 proteins (5 μg/mL each) for 5 days before measuring thymidine incorporation in proliferating cells ((a) and (b)) or for 4 days before measuring CFSE dilution in replicating $CD4^+$((c) and (d)) or $CD8^+$ T cell populations ((e) and (f)). Data were combined from 3 independent experiments and are presented as mean. Error bars indicate SEM. The level of statistical significance for differences in each group of immunized mice was determined by a two-tailed Student's t-test ($^*P < .05$; $^{**}P < .01$ PstS1 or PstS3 stimulation versus unstimulated cells; §between PstS1- and PstS3-induced responses in each group; $^\circ$between antigen-specific responses observed in mice immunized with DNA or protein alone).

was always greater than IL-17 secretion (Figures 4(a) and 4(b)).

3.4. Neither PstS3 nor PstS1 Immunizations Reduced the Mycobacterial Load in the Lungs of Mice Challenged with M. tuberculosis. Four weeks after the last immunization, and concomitantly with the assessment of antigen-specific memory immune responses, groups of mice were challenged with virulent *M. tuberculosis* and the bacterial load in the lungs was measured 4 weeks after the challenge. In parallel experiments, protection induced by immunization with Ag85B under identical schedule was assessed (Table 1 [3, 4]). As shown in Figure 5, none of the vaccinations with PstS1 or PstS3 antigens was able to reduce the bacterial

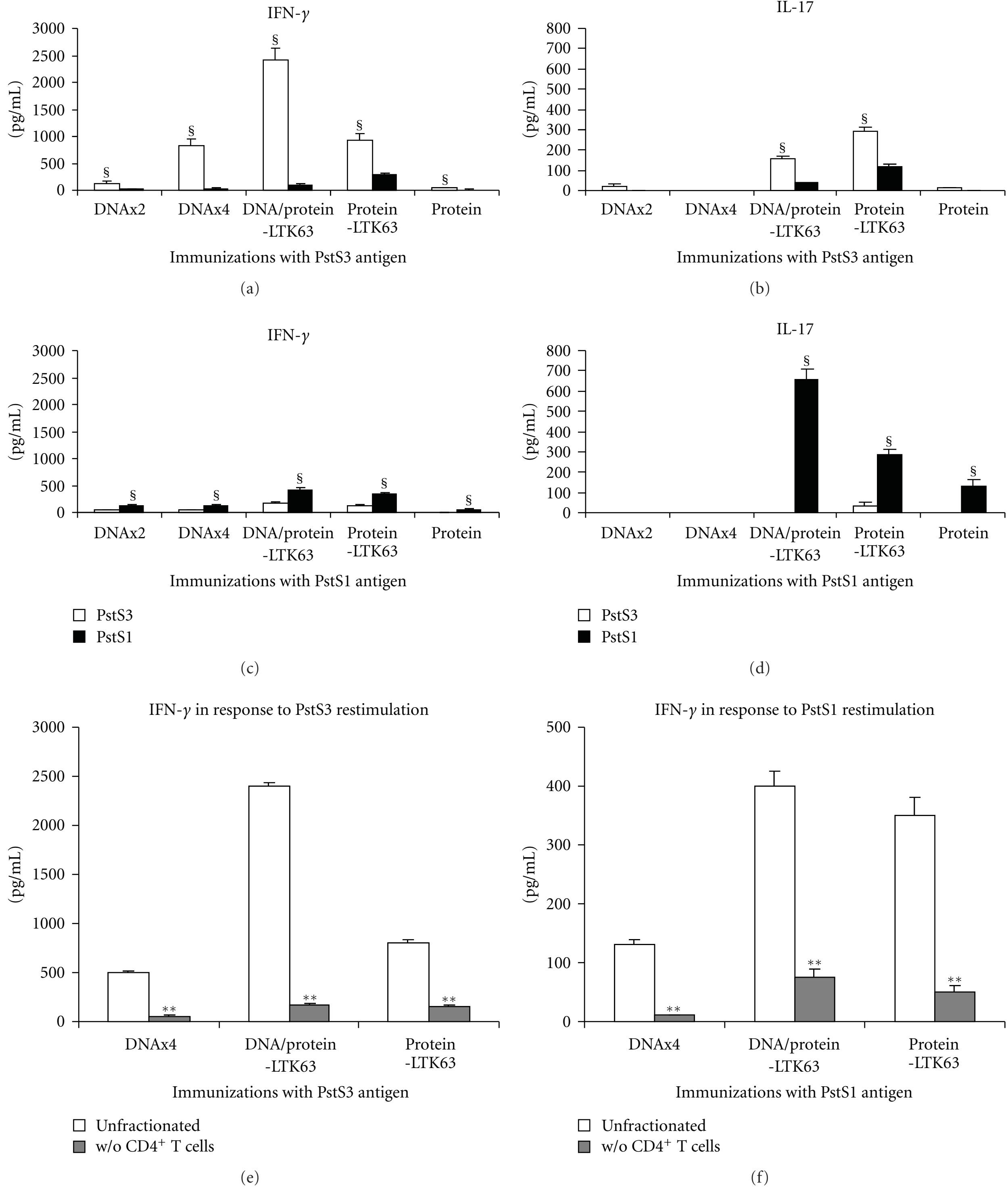

Figure 4: Cytokine production in response to PstS1 or PstS3 proteins in spleen cells of immunized mice. Pooled spleen cells (3 mice/group) of mice vaccinated with PstS3 antigen (panels (a) and (b)) or with PstS1 antigen (panels (c) and (d)) were cultured at 4×10^5 cells/well and re-stimulated *ex vivo* with PstS1 or PstS3 proteins (5 μg/mL each) for 4 days before measuring IFN-γ ((a) and (c)) or IL-17 ((b) and (d)) in the supernatant of culture by specific ELISA kit. Data were combined from 3 independent experiments and are presented as mean. Error bars indicate SEM. §Statistical significant difference between PstS1- and PstS3-induced responses in each group of immunized mice ($P < .05$ or $P < .01$ determined by a two-tailed Student's t-test). In some experiments spleen cells of DNA x4-, DNA/protein-LTK63-, or protein/LTK63-immunized mice with both PstS3 (e) or PstS1 (f) antigens were depleted of $CD4^+$ T cells by using magnetic beads as described in Materials and Methods. Undepleted cells and $CD4^+$ T cell-depleted subset were re-stimulated with PstS1 or PstS3 protein (5 μg/mL) for 4 days before testing the IFN-γ by a specific ELISA kit ((e) and (f)). Error bars indicate SEM. The level of statistical significance for differences among undepleted cells and $CD4^+$ T cell-depleted subset in each group was determined by Student's t-test ($^*P < .05$; $^{**}P < .01$).

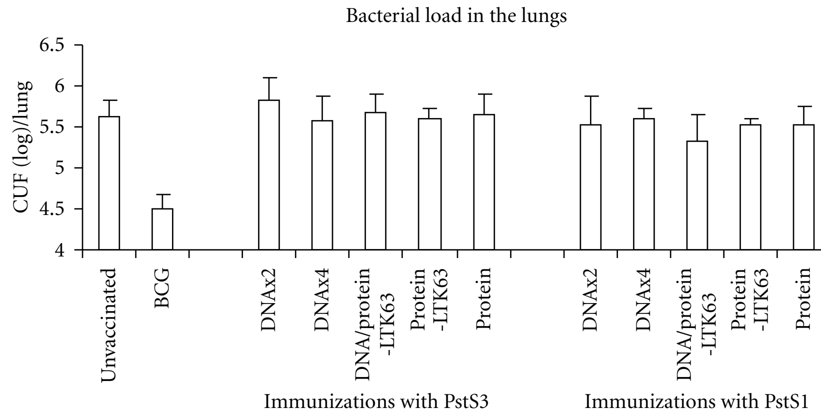

Figure 5: Effects of PstS1- or PstS3-immunizations on protection against *M. tuberculosis* challenge in mice. Mice (5 mice/group) were immunized with PstS1 or PstS3 antigens according to Figure 1, and challenged with *M. tuberculosis* H37Rv. Four weeks after infection, the bacteria in the lung were enumerated as described in Materials and Methods. Data are expressed as mean of the five individual mice ± SE. The level of statistical significance for differences between test groups and the control unvaccinated mice were determined by ANOVA test (*significant).

load in the lungs of infected mice. On the contrary, BCG vaccination (Figure 5 and Table 1) and all the several protocols of vaccination with the antigen Ag85, except only for immunization with Ag85B protein in absence of adjuvant, induced significant protection in the lungs of *M. tuberculosis*-infected mice (Table 1).

3.5. PstS1 and PstS3 Antigens in Contrast to Ag85B Were Not Recognized by IFN-γ-Secreting Spleen Cells of M. tuberculosis-Infected Mice: However PstS1 Stimulated IL-17 Production. To explain why the phosphate-binding protein immunizations were unsuccessful in protection against *M. tuberculosis* infection we investigated whether PstS1, or PstS3 specific immune reactions were raised during *M. tuberculosis* infection in unvaccinated or BCG-vaccinated mice. Therefore spleen cells of naïve unvaccinated or BCG-vaccinated mice recovered before *M. tuberculosis* infection or after 4 or 11 weeks from *M. tuberculosis* challenge were re-stimulated *ex vivo* with PstS1 or PstS3 proteins. Ag85B protein was also assayed as a protective comparator. The IFN-γ-secreting spleen cells of *M. tuberculosis*-infected mice responded to Ag85B protein, and the production of IFN-γ was greatly enhanced in mice vaccinated with BCG and infected for 4 weeks with *M. tuberculosis* (Figure 6). On the contrary, neither PstS3 nor PstS1 antigens were able to induce IFN-γ release by spleen cells of unvaccinated or BCG-vaccinated mice infected with *M. tuberculosis* for 4 or 11 weeks. Therefore, despite that PstS1 and especially PstS3 were good immunogens for activation of Th1 responses, the IFN-γ responses generated during early or late time points of *M. tuberculosis* infection (both in unvaccinated and BCG-vaccinated mice) were insensitive to these antigens.

Completely different results were obtained for IL-17-secreting cells present in spleen cells of *M. tuberculosis*-infected mice. In fact, PstS1, but not PstS3, antigen stimulated Th17 response in *M. tuberculosis*-infected mice. An important production of IL-17 was found in spleen cells of unvaccinated mice infected with *M. tuberculosis* for 4 weeks and the PstS1-responding IL-17-secreting cells decreased with progression of *M. tuberculosis* infection. In mice vaccinated with BCG and infected with *M. tuberculosis* the PstS1-activated IL-17 response was similar at the two time points of infections. Moreover, PstS1 was a better inducer than Ag85B of Th17 response in *M. tuberculosis*-infected mice.

4. Discussion

The medical need of identifying new candidate antigens for vaccine development against TB led us to investigate the phosphate-binding transporter proteins of *M. tuberculosis*. In fact, these membrane lipoproteins have been reported to be virulence factors for *M. tuberculosis* [9], are expressed also on surface of BCG [8], and can induce specific immune response in patients with active TB [10, 12, 26]. Importantly, protection against *M. tuberculosis* challenge was reported in mice immunized with plasmid DNA coding for *pstS1* [14] or *pstS3* [13]. The aim of this paper was to reassess immunogenicity and protective capacity of the above proteins by using different protocols of vaccinations with PstS1 and PstS3 to diversify the antigen-specific immune response. Moreover, the data were compared with a well-known immunogenic and protective antigen, the Ag85B [3–5]. We noticed that both PstS1 and PstS3 are capable of inducing, to a various degree and magnitude, a number of immune responses which are usually considered to be relevant for immune protection, including activation of T cell-mediated immunity as exemplified by $CD4^+$ and/or $CD8^+$ T-cell proliferation and production of different amount of IFN-γ and/or IL-17. Although considered of scarce importance in protection against *M. tuberculosis* infection, antigen-specific humoral responses, especially in PstS1-immunized mice, were also induced. Nonetheless, neither PstS1- nor PstS3-immunized mice were protected from challenge with *M. tuberculosis,* at variance with BCG- or Ag85B-immunized mice. Lack of protection was observed regardless antigen

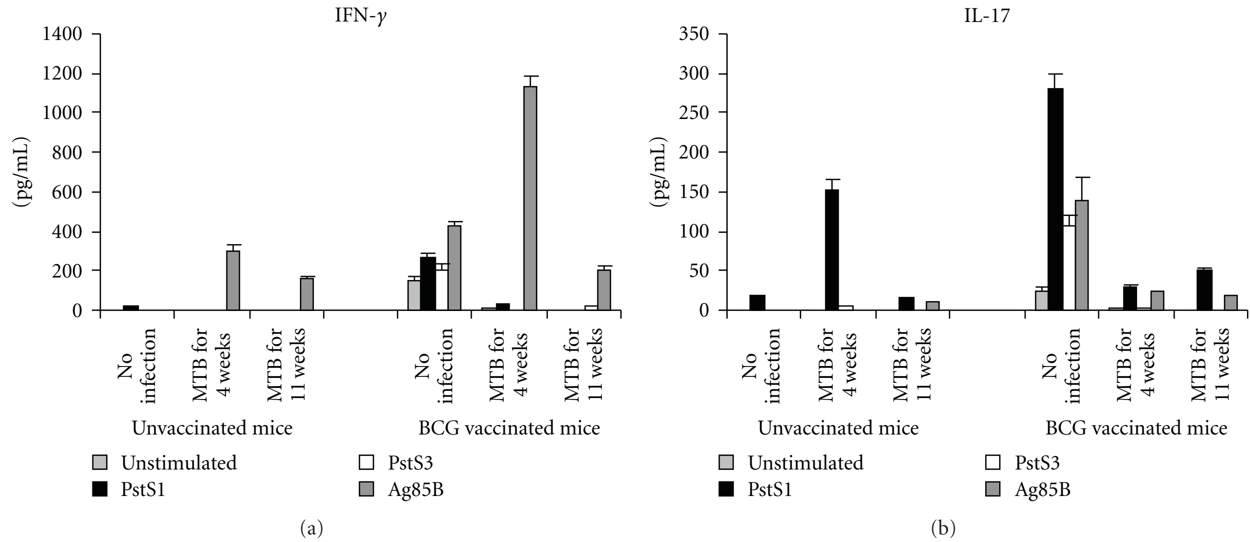

Figure 6: PstS1-specific IL-17—but not IFN-γ—secreting cells were generated during *M. tuberculosis* infection. Naïve unvaccinated or BCG-vaccinated mice (5 mice/group) were infected or not with *M. tuberculosis* for 4 or 11 weeks. Spleen cells recovered from mice were stimulated *ex vivo* (4×10^5 cells/well) with PstS1, PstS3, or Ag85B proteins (5 μg/mL each) for 4 days and culture cell supernatants assayed for IFN-γ (a) or IL-17 (b) by specific ELISA kits. Data were combined from 3 independent experiments and are presented as mean. Error bars indicate SEM.

formulation and expression, as protein or DNA or prime-boost immunization. Variations in methodology may explain why other research groups have found DNA-*pstS1* [14] and DNA–*pstS3* [13] protective: Zhu et al. [14] challenged DNA-*pstS1*-immunized mice i.p. (not i.v.) with *M. tuberculosis* just 2 weeks (not 4 weeks) after the last booster. Tanghe et al. [13] challenged DNA-*pstS3*-immunized mice with a higher (10^6 rather than 10^5 mycobacterial cells) *M. tuberculosis* burden and this burden in the lungs was evaluated at 8 weeks (not 4 weeks).

The scarce/absent involvement of PstS3 and PstS1 antigen in the IFN-γ response, the essential arm of protective TB immunity [16–19], mounted in response to *M. tuberculosis* during natural infection may be the cause of inefficacy of vaccinations with these antigens. In fact, IFN-γ-secreting cells generated following *M. tuberculosis* infection, both in unvaccinated or BCG-vaccinated mice, did not respond to antigenic stimulation with PstS1 or PstS3 proteins while they were specific for the protective antigen Ag85B. The irrelevance of PstS3-specific IFN-γ immunity during *M. tuberculosis* infection was further confirmed by the fact that amplification of this response by priming/boosting regimen did not affect the replication of *M. tuberculosis* compared to unvaccinated or PstS3-vaccinated mice inducing low-IFN-γ response.

Despite the fact that an s.c. immunization with PstS1 protein proved to be a nonprotective, it was recognized by IL-17-secreting cells generated during *M. tuberculosis* infection. The role of IL-17 in protection against *M. tuberculosis* infection is not fully elucidated. Infection of IL17$^{-/-}$ mice with *M. tuberculosis* revealed that IL-17 was not essential to control the growth of *M. tuberculosis* during acute infection [19] suggesting that IFN-γ-secreting CD4$^+$ and effector CD8$^+$ T cells were sufficient to inhibit mycobacterial replication in the absence of IL-17. Recently, it has been reported that *M. bovis* BCG-specific Th17 cells confer partial protection against *M. tuberculosis* infection in the absence of IFN-γ [20], indicating that also Th17 cells per se, independently from IFN-γ response, may contribute to the early control of *M. tuberculosis* infection. However, the short-term protective effect provided by the IL-17-secreting cells occurred at the cost of increased tissue damage characterized by a marked neutrophil infiltrate [20]. In this context, one aspect of Th17 response is particular attracting in TB vaccination. IL-17 accelerates antigen-specific Th1 memory response in the lungs of vaccinated mice infected with *M. tuberculosis* [19]. One of the central improvements required in the development of effective TB vaccines is to shorten the delay in recruitment of antigen-specific Th1 cells in the lungs. Therefore, the immunogenic feature of PstS1 antigen to induce Th17 responses recognized during *M. tuberculosis* infection should be further investigated in TB vaccine development to study whether PstS1 given in combination with antigens inducing protective Th1 immunity could accelerate the expression of protective Th1 immunity in the lungs upon infection.

Another aspect of immunogenic features of PstS1 deserves to be further investigated. PstS1, even in the immunization schedules with protein alone, activated CD8$^+$ T-cell proliferation even better than the CD4$^+$ T-cell counterpart. Although the role of these PstS1-specific CD8$^+$ T cells appears not uncoupled with a direct effect on protection, these cells might participate in the network of cellular regulation during infection. In fact, in addition to a recognized, direct role in protection against TB through their cytotoxic activity on *M. tuberculosis*-infected cells [27],

$CD8^+$ T cells specific for mycobacterial antigens can suppress IFN-γ production and proliferation by $CD4^+$ T cells [28, 29].

LTK63 is a good adjuvant improving protection when used in vaccination with protective TB antigens [4, 5]. In vaccinations with antigen Ag85B, LTK63 improved protection against *M. tuberculosis* challenge by modulating memory IFN-γ-secreting $CD4^+$ T cells with opposite effects. The INF-γ response was enhanced in unprimed mice vaccinated with Ag85B protein and adjuvant (Table 1) but reduced in mice primed with DNA and boosted with protein associated with the adjuvant [4]. In the latter case, coadministration of Ag85B protein with the adjuvant LTK63 reduced the generation of nonprotective Ag85B-specific IFN-γ-secreting cells, that led to a partial recovery in protection [4]. In fact, in DNA-primed mice boosted with adjuvant-free Ag85B protein, the expansion of an Ag85B-specific $CD4^+$ T-cell subset secreting elevated IFN-γ amounts was associated with the loss of that protection conferred by immunization with DNA only [3]. Now we also report that LTK63 drives the commitment of memory antigen-specific T cells towards Th17 response, as observed not only in Ag85B but also in PstS1 and PstS3 immunizations. Considering that IFN-γ and IL-17 responses are negatively regulated by each other [30–32], the induction at the same time of both responses may help to prevent that one response, at the expense of the other, can expand without control causing inflammation-mediated lung damage during *M. tuberculosis* infection. In fact, the balance between protection and pathological consequences is the crux of TB pathogenesis. The ability to activate several mechanisms to contain uncontrolled expansion of Th1/Th17-mediated inflammatory process may be the reason of the efficacy of LTK63 adjuvant in TB vaccination.

5. Conclusion

We demonstrated that the *M. tuberculosis* phosphate-binding transporter lipoproteins PstS1 and PstS3 are excellent immunogens inducing $CD8^+$ T-cell activation and both Th1 and Th17 immunity. These antigen-specific responses were not able, however, to contain *M. tuberculosis* replication in the lungs of infected mice even when amplified by administration of the protein with the adjuvant LTK63 or by the DNA priming/protein boosting regimen. The lack of protection might be ascribed to the scarce/absent immunogenicity/presentation of these antigens during natural infection. In fact, neither IFN-γ- nor IL-17-secreting cells specific for PstS3 were generated in spleen of unvaccinated or BCG-vaccinated mice infected with *M. tuberculosis*. Also PstS1 antigen, a weak inducer of Th1, did not react with IFN-γ-secreting cells generated during early or late infection with *M. tuberculosis*. However, although not determinant for protection, PstS1-specific IL-17-secreting cells were generated during *M. tuberculosis* infection both in unvaccinated and BCG-vaccinated mice. Although PstS1 is a nonprotective antigen "per se," it could be utilized in TB vaccination, as modulator, in association with antigens making protective Th1 immunity. In fact, the PstS1 ability to drive Th17 immunity recognized upon *M. tuberculosis* infection could accelerate the recruitment of protective IFN-γ cells in the lungs of infected animals. To shorten the delay in recruitment of antigen-specific Th1 cells in the lungs is one of the central improvements required in the development of effective TB vaccines.

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Review Article

Tuberculosis Immunity: Opportunities from Studies with Cattle

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Mycobacterium tuberculosis and *M. bovis* share >99% genetic identity and induce similar host responses and disease profiles upon infection. There is a rich history of codiscovery in the development of control measures applicable to both human and bovine tuberculosis (TB) including skin-testing procedures, *M. bovis* BCG vaccination, and interferon-γ release assays. The calf TB infection model offers several opportunities to further our understanding of TB immunopathogenesis. Recent observations include correlation of central memory immune responses with TB vaccine efficacy, association of SIRPα^+ cells in ESAT-6:CFP10-elicited multinucleate giant cell formation, early $\gamma\delta$ T cell responses to TB, antimycobacterial activity of memory CD4$^+$ T cells via granulysin production, association of specific antibody with antigen burden, and suppression of innate immune gene expression in infected animals. Partnerships teaming researchers with veterinary and medical perspectives will continue to provide mutual benefit to TB research in man and animals.

1. Introduction: History of Codiscovery

Three essential tools developed in cattle and used for the control of human tuberculosis (TB) include (1) vaccination with an attenuated *Mycobacterium bovis* Bacillus Calmette Guerin (BCG, [1]), (2) use of tuberculin as an *in vivo* diagnostic reagent, and (3) antigen-induced interferon- (IFN-) γ as an *in vitro* biomarker of TB infection. In 1913 at the Pasteur Institute (Lille, France), Albert Calmette and Camille Guerin vaccinated 9 cows with *M. bovis* (Nocard strain) attenuated by serial passage on glycerol-soaked potato slices in ox bile (i.e., BCG) [1]. All 9 animals were protected from challenge with virulent *M. bovis,* thereby, demonstrating the potential use of BCG vaccination against *M. tuberculosis* infection of humans. In 1921, BCG was administered to a newborn child (6 mg orally) and has since been used widely for the control of human TB. Within a few years of the discovery of tuberculin by Robert Koch, veterinary investigators in Russia (Professor Gutman), the UK (John McFadyean), Denmark (Bernhard Bang), and the US (Leonard Pearson and Maz'yck Ravenel) were administering tuberculin to cattle as an *in vivo* diagnostic reagent (infection indicated by a rise in temperature within 24 hours) [2]. Clemens von Pirquet and Charles Mantoux later (circa 1907/1908) adapted and improved (e.g., subcutaneous to intradermal) this technology for application in the diagnosis of TB in humans, coincidently defining the principles of allergy and delayed type hypersensitivity. During the 1980s, an *in vitro* IFN-γ release assay was developed for the diagnosis of TB in cattle [3]; a modified version of this assay is now

widely used in the diagnosis of both human and bovine TB. Together, these findings demonstrate the mutual benefit for cooperative veterinary and medical research. As stated by Emil von Behring in his Nobel Prize acceptance speech [4], "I need hardly add that the fight against cattle tuberculosis only marks a stage on the road which leads finally to the effective protection of human beings against the disease." The current review highlights recent observations on immunity to bovine TB of relevance for understanding the disease, both in cattle and humans.

2. The Neonatal Calf as a Model for the Study of TB

2.1. Mycobacterium bovis. Mycobacterium bovis, a member of the *M. tuberculosis* complex, has a wide host range as compared to other species in this disease complex, is infectious to humans, and is the species most often isolated from tuberculous cattle. Prior to implementation of widescale pasteurization, it is estimated that 20–40% of TB cases in humans resulted from infection with *M. bovis* [5–7]. An explanation, not apparent at the time, suggests a difference in the capacity of *M. tuberculosis* and *M. bovis* to infect and cause disease in cattle. Genome comparisons show that *M. bovis* and *M. tuberculosis* evolved into two clades from a common prototypic ancestor some 40,000 years ago: clades defined by presence or absence of *M. tuberculosis* deletion 1 (TbD1) [8]. The data suggest that both clades arose in humans, with the $TbD1^{-}$ clade 1 coevolving mainly in humans and the $TbD1^{+}$ clade 2 coevolving in humans, ruminants, and other species. The difference in host range shows that evolution of *M. bovis* and *M. tuberculosis* has included development of a difference in virulence and the capacity to cause disease in different species. This difference may prove useful in comparative studies designed to elucidate the mechanisms of immunopathogenesis and development of vaccines. Approximately 90% of humans exposed to *M. tuberculosis* develop an immune response that controls but does not eliminate the pathogen. Immune control of this persistent (latent) stage of infection may persist for a lifetime or become dysregulated, allowing for disease progression. It is not clear whether a comparable proportion of humans infected with *M. bovis* develop an immune response that controls infection. Recent direct comparison of *M. bovis* and *M. tuberculosis* infection in cattle has demonstrated that *M. tuberculosis* is less virulent for cattle; however, the *M. tuberculosis* strain used for these studies was a laboratory-adapted strain ($H_{37}R_v$) [9]. However, experimental transmission studies (conducted in the late 1800s by Theobald Smith (physician scientist) and veterinarians Austin Peters and Langdon Frothingham using calves experimentally inoculated with sputum from humans with tuberculosis), demonstrated that human bacilli possess a low virulence for cattle [10]. Other studies clearly demonstrated that nonlaboratory-adapted strains of *M. tuberculosis* were less virulent in cattle than those of *M. bovis* (reviewed by Whelan et al., 2010 [9]). Analysis of the difference in the immune response to the two pathogens may provide insight into the mechanisms used by both bacteria to circumvent protective immunity [11].

2.2. Aerosol Infection Model. Aerosol inoculation of *M. bovis* to calves results in a respiratory tract infection (i.e., lungs and lung-associated lymph nodes), severity is dose-dependent, and the disease closely mimics natural infection of cattle [12]. As related to human disease, studies with neonatal calves are particularly relevant as this is the primary target population for human vaccination and exposure to TB often occurs at a very young age. For calf vaccine studies, parameters to demonstrate efficacy include quantitative and qualitative mycobacterial culture, gross and microscopic disease scoring, radiographic morphometry of lung lesions, and disease-associated immune parameters. Opportunities afforded by use of the calf model include (1) large numbers of affordable age-, gender-, and breed-matched animals available throughout the year (nonhuman primates (NHPs) are seasonal breeders and are costly), (2) cattle are out-bred species, thus, experimental variance is similar to what is expected for NHP and humans, (3) size allows dose titration studies and full immunologic assessment via frequent collection of large volumes of blood which facilitates studies on immune response kinetics, (4) the nutritional status (e.g., vitamin deficiencies and protein malnutrition) can be manipulated to achieve similar levels of deficiency as may occur in target human populations in the developing world, (5) serves as an additional safety screen for evaluation of vaccines, adjuvants, or other administered biologics, and (6) feasibility of duration of immunity studies.

2.3. Parallel Testing of Vaccine Candidates in the Primate and Calf Model-mc²6030. A double deletion mutant of *M. tuberculosis* $H_{37}R_v$ (i.e., ΔRD1-the primary attenuating mutation of BCG and ΔpanCD-deletion of pantothenate synthesis genes) was evaluated in parallel with BCG (Copenhagen strain) in both the calf model and adolescent Cynomologus monkeys (*Macaca fascicularis*). The *M. tuberculosis* ΔRD1 X panCD mutant ($mc^2$6030) undergoes limited replication in mice and is safer than BCG in immunocompromised mice (i.e., SCID, IFN-γ receptor-deficient and CD4-deficient mice) [13]. Immunization with this mutant strain of $H_{37}R_v$ induces prolonged protective immune responses that promote survival of both wild type and CD4-deficient mice against an aerosol challenge with virulent *M. tuberculosis*. Antibody depletion and adoptive transfer studies revealed that protection in CD4-deficient mice was mediated in the absence of $CD4^{+}$, $CD8^{+}$, $\gamma\delta^{+}$, and NK1.1^{+} T cells, thus, indicating a surprising capacity for protection to be elicited by $CD4^{-}CD8^{-}$ TCR$-\alpha\beta^{+}$ cells [14]. For second tier testing, $mc^2$6030 was evaluated for efficacy in the newborn calf model and adolescent Cynomologus monkeys. In both calves and monkeys, the vaccine was ineffective [15, 16]; thus, in this instance, responses in mice were not predictive of efficacy in models using natural hosts of infection. For cattle, attenuated *M. tuberculosis* mutants may be less immunogenic as compared to those produced on a virulent *M. bovis* or BCG strain; thus, cattle may not be as useful as other

models (e.g., monkeys) for the study of vaccine efficacy using *M. tuberculosis* mutants. Further studies are required to directly compare immunogenicity and virulence of *M. tuberculosis* versus *M. bovis* background mutants in cattle.

3. Bovine DCs and Macrophages

The role of signal regulatory protein alpha-expressing (SIRPα^+) *cells in the adaptive response to tuberculous mycobacteria via interactions with ESAT-6/CFP-10.* Multiple functions are proposed for the RD1 proteins ESAT-6 and CFP-10 [17, 18]. For instance, ESAT-6 interacts with biomembranes after dissociation from its putative CFP-10 chaperone within the acidic phagolysosome [19], thereby affording a "phagolysosome escape" mechanism for the pathogen. ESAT-6 deletion mutants of *M. tuberculosis* have reduced tissue invasiveness, likely due to loss of cytolytic activity [20]. In addition, use of the *M. marinum*/zebrafish granuloma model demonstrates that RD1 components are required for efficient recruitment of macrophages to granulomas "creating new bacterial growth niches" [21, 22]. RD-1 proteins, including ESAT-6/CFP-10, likely elicit more rapid granuloma formation offering a distinct growth advantage for the pathogen [22]. In addition to enhancing recruitment of cells susceptible to infection, the stable ESAT-6/CFP-10 complex binds to host cells [23]; thereby, modulating the host response favourably for the pathogen via down-regulation of host cell killing mechanisms and immune cell activation [24].

A specific receptor (TLR2) for ESAT-6 has been identified using mouse monocyte/macrophage cell lines [25]. Studies with leukocytes obtained from cattle have also demonstrated a specific interaction of the ESAT-6/CFP-10 complex with CD172a (SIRPα)-expressing cells [26]. Stimulation of peripheral blood mononuclear cell cultures from *M. bovis*-infected calves with ESAT-6/CFP-10 results in the specific expansion of SIRPα^+ cells, with binding of the fusion protein bound to the surface of SIRPα^+ cells [26]. SIRPα-CD47 interactions are essential for efficient migration of DCs to skin [27] and secondary lymphoid organs [28]. Thus, ESAT-6/CFP-10-induced expansion of SIRPα-expressing cells may favour migration of DC's/macrophages to infection sites, thereby, promoting efficient granuloma formation and early dissemination of *M. tuberculosis* complex mycobacteria [22]. With the bovine TB model, intradermal injection of rESAT-6:CFP-10 elicits granuloma formation with infiltration of numerous T cells, SIRPα^+ cells, and CD14$^+$ cells in *M. bovis*-infected calves, further supporting a role for ESAT-6/CFP-10 in the recruitment of naïve cells to sites of granuloma formation [26]. A unique aspect of the cellular infiltrates at rESAT-6:CFP-10 injection sites in cattle is the presence of numerous multinucleated giant cells. Multinucleated giant cell formation is mediated, in part, by SIRPα (also termed macrophage fusion receptor). Cell surface expression of SIRPα is strongly and transiently induced upon giant cell formation. As opposed to phagocytosis, SIRPα-CD47 interactions provide "self recognition" signals that prevent killing of internalized (i.e., fused) cells. Thus, cattle may serve as a useful model for the molecular characterization of multinucleate giant cell formation within tuberculous granulomas. Additionally, comparative studies examining ESAT-6/CFP-10 interactions with SIRPα and TLR2 in mouse, human, and bovine tissues are needed to determine if host factors affect ESAT-6/CFP-10 specificity.

4. T Cell Subsets and Effector Mechanisms

4.1. Cell-Mediated Immunity (CMI). Ongoing studies have shown the adaptive immune system of cattle is quite similar to the human system [29]. Importantly, comparisons at the genomic level indicate that genes encoding cytokines known to play a role in regulating immune responses in humans are present in cattle, including cytokines not found in mice (e.g., IL-26). Cell-mediated immunity is essential for protection against bovine and human TB and there appears to be significant similarity in the primary mechanisms of antimycobacterial CMI between humans (as reviewed in [30]) and cattle (as reviewed in [31]). Similarities include roles of T cell subsets (e.g., CD4$^+$, CD8$^+$, and $\gamma\delta$ TCR$^+$), protective function and cellular sources of IFNγ and cytotoxic granule proteins; the reduction of mycobacterial numbers within macrophages by cytotoxic T cells and NK cells, the relative levels of antigen specific Th1 and Th2 cytokines, and expression of memory markers by antigen specific T cells [32–42]. Widely utilized for TB diagnosis, antigen-specific release of IFN-γ is clearly an important function of the CMI response to TB in cattle and humans [43, 44]. In contrast to rodents, human and bovine immunity to TB appears to be less reliant on antigen-specific IFN-γ activation of macrophages [45, 46] and may employ cytotoxic immune cells in a more active role.

Protective immunity to TB in cattle, as in human and other animal models of TB, correlates to the induction of Type 1 T cell cytokines following antigen specific stimulation [45, 47]. The balance of cytokines (IFN-γ, IL-4) elicited by mycobacterial antigens in bovine T cells, however, is more similar to cytokine profiles observed following immunization of humans than of mice. Relative levels of IFN-γ and IL-4 expressed by lymph node T cells correlate to tissue pathology and bacterial load in vaccination/challenge studies of bovine TB [15, 48]. In BCG-vaccinated neonatal calves, antigen-specific expression of IL-2 and IFN-γ by peripheral blood leukocytes correlated with clinical protection following challenge with virulent *M. bovis* [48]. A potential role for the Th2/Th17 cytokine IL-21 in protection against mycobacterial disease was recently identified for the first time in a calf model of TB [35]. IL-21 is a member of the common gamma chain family of cytokines (IL-2, IL-7, IL-15) and is secreted primarily by CD4$^+$ T cells [49]. Following vaccination with BCG, CD4$^+$ T cells from immunized cattle expressed IL-21 upon *in vitro* stimulation with PPD [35]. Expression of IL-21 in these studies correlated with cytotoxic activity and effector molecule expression by antigen-stimulated CD4$^+$ T cells and occurred late in the antigen-specific response, similar to perforin. The role of IL-21 and other key regulatory factors

for maintenance and induction of IFN-γ (IL-12, IL-18, IL-23, and IL-27) and NK cell function in protective immunity to TB is an important avenue of investigation in the efforts to develop a vaccine for humans and cattle.

4.2. $\gamma\delta$ T Cells in Bovine Tuberculosis. $\gamma\delta$ T cells may form a key component linking innate and adaptive responses to *M. bovis* infection in cattle given their active release of IFN-γ and relatively high prevalence in the blood of young calves as compared to adults [50]. There are two phenotypically distinct subsets of $\gamma\delta$ T cells in cattle, workshop cluster 1 (WC1)$^+$ CD2$^-$ and WC1$^-$ CD2$^+\gamma\delta$ T cells. Unique to *Artiodactyla*, WC1 is a member of the scavenger cysteine rich gene family that includes CD5 and CD6 [51]. Differences in abundance, tissue distribution, patterns of circulation, and TCR gene usage suggest that the two major $\gamma\delta$ T cell subsets (WC1$^+$ and WC1$^-$) play different roles in host defense [52–55]. Orthologues of WC1 have only been identified in pigs and camelids [51, 56–59] but there is no known orthologue of WC1 in primates. Isoforms of WC1 are encoded by a cluster of thirteen genes distributed between two loci in cattle [51, 56–58] and studies have demonstrated that multiple gene products from the two loci are coexpressed forming two essentially mutually exclusive populations with an apparent dichotomy in function [60, 61]. These two subsets—referred to as WC1.1$^+$ and WC1.2$^+$—are defined by differential staining with specific monoclonal antibodies. Functional studies have demonstrated that the subset expressing WC1.1 is a major source of IFN-γ following antigenic stimulation [55] and is likely an early source of perforin and granulysin. The relative proportions of WC1.1$^+$and WC1.2$^+$cells change with age, with a predominance of WC1.1$^+\gamma\delta$ T cells in young cattle [62]. In addition, we showed that WC1$^+\gamma\delta$ T cells from young cattle had a greater capacity for IFNγ secretion, compared to WC1$^+\gamma\delta$ T cells from adult cattle, and that this was due to a higher number of WC1.1$^+$ cells in the young calves [63].

$\gamma\delta$ T cells respond to mycobacterial antigens including crude and defined antigens, heat shock proteins, and other nonproteinaceous components which may be expressed relatively early in infection with *M. bovis* or other mycobacterial species [64–66]. The effector contribution of the WC1$^+\gamma\delta$ T cells to *M. bovis* immunity in cattle is not fully elucidated but early roles postinfection and postvaccination are indicated from a number of studies in cattle and in mice. Vaccination of calves with BCG induced an early increase in circulating WC1$^+\gamma\delta$ T cells which was associated with an increase in the secretion of antigen-specific IFN-γ [67]. We also showed that intranasal administration of BCG induced significant increases in WC1.1$^+$ T cells in the lungs of immunised calves and that these cells clustered with DC in tissues [50]. In *M. bovis*-infected cattle, WC1$^+\gamma\delta$ T cells were detected within lesions early in the course of infection [68, 69]. Concurrently, numbers of circulating WC1$^+\gamma\delta$ T cells decrease shortly after infection, followed by a rapid increase [66]. *In vivo* depletion of WC1$^+\gamma\delta$ T cells from cattle prior to infection did not alter the course of disease [70] but, rather, significantly influenced the immune bias of the antigen-specific response. These data suggest that WC1$^+$ cells may be involved in directing immune bias through IFN-γ secretion, as the WC1$^+\gamma\delta$ T cell-depleted cattle had increased IL-4 expression and altered immunoglobulin isotype profile as compared to nondepleted, *M. bovis*-infected controls [70]. Studies in mice have also revealed roles for $\gamma\delta$ T cells in immune responses to *Mycobacteria spp.*. The WC1-bearing $\gamma\delta$ T-cell population was shown to have an essential role in regulating inflammation in both the liver and the spleen of *M. bovis*-infected SCID-bovine heterochimeric (mouse-bovine) chimeras [37]. A similar regulatory effect of $\gamma\delta$ T lymphocytes in inflammatory responses induced by *M. tuberculosis* was also reported to influence bacterial survival within tissues [71]. The early presence of $\gamma\delta$ T cells in tuberculous lesions likely promotes containment of *M. bovis* via cytokine (e.g., IL-12 and IFN-γ), granulysin, and chemokine release stimulating macrophage activation and T cell recruitment [70, 72, 73]. However, recent studies in the SCID-bovine heterochimeric mouse model have shown that $\gamma\delta$ T cells are not a primary source of chemokines in response to various agonists but may influence other cells types (including macrophages) in the production of chemokines and granuloma formation [74]. Treatment of mice with an anti-WC1 monoclonal antibody resulted in an apparent loss of control of the inflammatory response confirming important roles for WC1$^+\gamma\delta$ T cells in vivo in regulation of the immune response. Similar findings were obtained with mycobacterial infection of $\gamma\delta$ TCR knockout mice [75, 76]. Interestingly other studies have also shown that it is the bovine WC1.1$^+$ and WC1.2$^+\gamma\delta$ T cells which act as T regulatory cells, not CD4$^+$CD25$^+$FoxP3$^+$ cells as has been observed in other species [77]. The available data suggest that bovine WC1$^+\gamma\delta$ T cells have multiple roles and can be both regulatory and stimulatory through the expression of cytokines. Their exact roles in *M. bovis* immunity remain to be fully revealed.

4.3. Cytotoxic Lymphocytes (CTLs) and Biomarkers for Effector Mechanisms in Bovine TB. Human and murine CD4$^+$, CD8$^+$, and $\gamma\delta$ T cells exhibit CTL activity against mycobacterial-infected targets, indicating a role in immunity to TB [78–81]. Production of granulysin by CTL reduces intracellular mycobacteria and appears to require perforin to gain access to the interior of the infected cell. Bovine T cells express a homologue of human granulysin, a potent antimicrobial protein stored with perforin in cytotoxic granules [34]. Memory CD4$^+$ T cells (CD45R0$^+$) from BCG-vaccinated animals are efficient at reducing colony forming units of BCG in infected macrophages following antigen specific stimulation [35]. This antimycobacterial activity correlates with expression of perforin, granulysin, and IFN-γ by the same memory subset. Expression of the bovine granulysin gene can be induced in CD4$^+$, CD8$^+$, and $\gamma\delta$ T cells resulting in antimycobacterial activity similar to human granulysin [34, 35]. Using laser capture microdissection, granulysin mRNA was detected in the lymphocytic cuff of a forming granuloma, simultaneous with *M. bovis* DNA, in an experimentally challenged calf [34]. Granulysin and perforin gene expression are upregulated in peripheral blood CD4$^+$ and CD8$^+$ T cells in both BCG- and *M. bovis*

ΔRD1-vaccinated calves (protected) as compared with nonvaccinated (not protected) calves [82], demonstrating the potential of these biomarkers as correlates of protection for prioritizing vaccine candidates. To date, a murine and guinea pig orthologue of granulysin has not been identified, precluding studies of the full repertoire of lytic granule proteins in rodent models of TB. Further studies of protective biomarkers in peripheral blood and granulomas of vaccinated and experimentally infected cattle have significant potential for testing strategies for augmenting immunity by vaccination.

Bovine NK cells have been identified with a mAb specific for CD335 [83, 84] and numbers of NK cells in peripheral blood were found to be highest in young calves [83, 85]. The population is comprised of $CD2^+$ and $CD2^-$ subsets expressing combinations of killer immunoglobulin-like receptors (KIRs), leukocyte-receptor complex (LRC) CD94/NKG2C (inhibitory) and CD94/NKG2A (activating), NKG2D, and lectin-like receptors Ly49, CD69, NKP-R1, and KLRJ (reviewed in [86]). Initial studies suggest that NK cells play a significant role in the innate response to mycobacterial pathogens [33, 87–89]. They are an initial source of IFN-γ, IL-17, and IL-22 that play a role in the inflammatory response to intracellular pathogens, including *M. tuberculosis*, and a source of perforin and granulysin. Bovine neonatal NK cells were shown to be a major source of IFNγ through interactions with BCG-infected DCs and this may be a pivotal early influence *in vivo* [88].

NKT cells have not been identified in cattle. Analysis of the CD1 family of proteins involved in antigen presentation to NK and NKT cells suggests NKT cells may not be present in cattle or that receptor usage differs markedly from that in humans and mice. The CD1 family in cattle is comprised of genes encoding CD1a, multiple CD1b molecules, and CD1e. An orthologue of the human CD1c gene is not present in the bovine genome. Both identified CD1d genes are pseudogenes supporting the possibility that NKT cells may be absent [90].

5. Immunological Parameters as Correlates of Protection versus Indicators of Disease

5.1. T Cell Central Memory (TcM) Immune Responses. Costly and protracted efficacy studies using various and often multiple animal models are currently used to evaluate human TB vaccine candidates [91]. Vaccine-elicited immune parameters (i.e., correlates of protection) are very much needed to prioritize the multitude of candidates. Several vaccine studies with cattle have demonstrated that TcM responses [92] negatively correlate with mycobacterial burden [93] and TB-associated pathology [94]; thus, TcM responses are positive correlates of protection. With the TcM assay, short-term T cell lines are generated via stimulation of peripheral blood mononuclear cells with specific antigens including Ag85A/TB10.4 and *M. bovis* PPD. Early effector T cell responses wane over time and memory cells are maintained via addition of IL-2 and fresh medium. On the final day of culture (∼13d), cells are moved to plates containing autologous antigen presenting cells and antigen for elicitation of TcM responses as measured by IFN-γ ELISPOT. With two independent vaccine efficacy trials [93, 94], protected calves had greater TcM responses as compared to nonprotected calves. As with TcM responses, IL-17 responses (as measured by real time RT-PCR) also correlated with protection [94]. Recent findings indicate that IL-21 and IL-22 may also be good candidates for further evaluation (*Davis and Waters, unpublished observations*). These data demonstrate the potential for defining a protective signature elicited by vaccination to prioritize candidates for efficacy testing within calves.

5.2. Immune Activation as a Positive Indicator of Disease and Negative Indicator of Vaccine Efficacy. Positive prognostic indicators (i.e., as measured after challenge) for vaccine efficacy include reduced antigen-specific IFN-γ, iNOS, IL-4, and MIP1-α (CCL3) responses, reduced expansion of $CD4^+$ cells in culture, and a diminished activation profile (i.e, ↓ expression of CD25 and CD44 and ↑ expression of CD62L) on T cells with antigen-stimulated cultures [15]. In particular, reduced responses to ESAT-6/CFP-10 upon experimental challenge are positive prognostic indicators for vaccine efficacy [44, 93, 95] whereas robust or increasing cellular immune responses (with the exception of IL-17 [94]) to ESAT-6/CFP-10 are generally a negative prognostic indicator of vaccine efficacy. These findings are consistent with the diagnostic capacity for ESAT-6/CFP-10 as antigens in cellular immune assays. Prognostic indicators offer antemortem monitoring techniques for vaccine efficacy studies.

5.3. Association of Antibody Responses to Antigen Burden. Antibody responses generally correlate to mycobacterial-elicited pathology [96] in accordance with the belief that *Mycobacteria spp.* induce antibody primarily late in the course of infection. To further evaluate the correlation of antibody responses to disease expression, calves were inoculated with *M. bovis*, *M. kansasii*, or *M. tuberculosis* and immune responses evaluated [9, 11]. Disease expression ranged from mycobacterial colonization with associated pathology (*M. bovis*), colonization without pathology (*M. tuberculosis*), to no colonization or pathology (*M. kansasii*). Antibody responses were associated with antigen burden; cellular responses (i.e., to PPD) correlated with infection but not necessarily with pathology or bacterial burden; exposure to mycobacterial antigens (in this case, injection of PPD for skin test) boosted antibody responses in presensitized animals. Thus, evaluation of antibody response to mycobacterial infections may be useful for correlation to antigen burden and prior mycobacterial sensitization. Further studies are warranted.

6. Global Strategies for Discovery: Gene Expression Profiling

One argument for use of rodent models in human tuberculosis research was the widespread availability of reagents to detect cytokines, chemokines, and for differentiation of immune cells. The advent of functional

genomics, proteomics, and completion of the bovine genome sequence have dramatically improved our ability to study immunopathogenesis of TB in cattle. In addition, there has been a steady increase in numbers of antibody reagents either prepared directly against bovine proteins or validated for cross-reactivity against bovine orthologues. To date, functional genomics studies of *M. bovis* infection in cattle have been concentrated in two main areas: (1) *in vitro* studies aimed at defining changes in macrophage gene expression profiles following infection with various *M. bovis* isolates and (2) *ex-vivo* studies to define a "gene expression signature" that could be used to detect *M. bovis*-infected cattle. Many of these studies have relied upon early renditions of cDNA microarrays focused on bovine genes encoding proteins known to be important in immunity [97–100].

Wedlock et al. compared gene expression patterns in primary bovine alveolar macrophages infected with a virulent *M. bovis* strain and its attenuated isogenic counterpart [101]. This study employed a cDNA microarray containing over 20,000 bovine-expressed sequence tags (ESTs) as well as amplicons representing various bovine and cervine cytokines [102]. While virulent and attenuated *M. bovis* isolates grew at comparable rates in the alveolar macrophages, initial analyses suggested that as many as 45% of the ESTs were differentially expressed between the two infection groups [101]. Of the 20 most differentially expressed genes, IL-8, monocyte chemotactant protein (MCP)-1 (CCL2), epithelial cell inflammatory protein-1, Groα (CXCL1), CDC-like kinase 3, and fibrinogen-like protein-2 were prominent. Wedlock et al. concluded that alveolar macrophages infected with virulent *M. bovis* adopted a much more proinflammatory gene expression profile than similar cells infected with the attenuated isogenic strain [101]. Another study using microarray technology found that lower levels of chemokines were expressed by *M. bovis*-infected alveolar macrophages than *M. tuberculosis*-infected cells, highlighted by the authors as a potential mechanism by which *M. bovis* can circumvent activation of the host chemotactic response and evade killing [103]. Of note, there appears to be species differences in the response of macrophages to *M. bovis* as human monocytes and monocyte-derived macrophages (MDM cells) infected with *M. bovis* do not produce significant IL-8 [104]. In addition, human cells infected with virulent *M. tuberculosis* produce large amounts of IL-10 [104], which was not observed in *M. bovis*-infected alveolar macrophages. However, as acknowledged by the authors, it remains a possibility that these discrepancies are due to differences between MDM cells and primary alveolar macrophages rather than actual species differences. Experiments to refine these observations have not been reported to date.

Meade et al. conducted a series of studies aimed at defining the gene expression profiles of bovine peripheral blood mononuclear cells (PBMCs) from *M. bovis*-infected cattle following stimulation with antigens of *M. bovis* (PPD-B) in vitro [99]. Samples were collected throughout a 24 hour time course of stimulation. Comparisons were made primarily between PPD-B-stimulated and -unstimulated PBMC mRNA profiles. This study utilized a bovine-specific cDNA microarray (BOTL-4) designed primarily for immune studies in cattle and containing over 1300 ESTs and amplicons spotted in triplicate [105, 106]. Statistical analysis revealed that of the >1300 genes present on the BOTL-4 microarray, 224 (~17%) were differentially expressed in PPD-B-stimulated PBMCs when compared to unstimulated PBMCs from the same chronically infected animals [99]. Major ontological classes of genes that showed significant differential expression included those encoding proteins involved in metabolism, cell communication, response to biotic stimulus, death, and development. Molecular functions most affected by stimulation were catalytic activity, protein binding, and nucleic acid binding. During the 24-hour stimulation time course, the authors observed a cyclical gene expression pattern with larger numbers of transcripts differentially expressed at 3-hour and 12-hour post stimulation relative to 6-hour and 24-hour time points. Although this could be related to cyclical receptor signalling, the fact that very few transcripts were commonly affected throughout the time course suggests that it is due to activation of early response transcripts followed by a lag (possibly due to transcription/translation of early response transcripts) followed by a secondary wave of gene expression. Alternatively, this pattern could be due to immediate response to mycobacterial antigens through, for example, Toll-like receptor signalling followed by a secondary stimulation after uptake, processing, and antigen presentation. The authors suggested that this early response pattern might represent a signature of *M. bovis* infection [99].

In a series of subsequent studies, Meade et al. pursued the idea that a unique and rapid gene expression pattern in PBMCs could be used to reliably detect *M. bovis*-infected cattle [107, 108]. Biomarker discovery using genomics and proteomics has been applied to infections with other pathogens in several host species, including humans and cattle [109–112]. Meade et al. demonstrated that total leukocytes from late-stage *M. bovis*-infected cattle contained more lymphocytes (72%) than similar samples from control healthy cattle (43%) and that healthy controls contained more neutrophils (40%) than cells from late-stage *M. bovis*-infected cattle (14%) [113]. Given such differences, one would expect that total leukocyte gene expression profiles from late-stage *M. bovis*-infected cattle would be different than those from healthy controls. Indeed, of the >1,300 genes represented on the BOTL-5 microarray, 378 (27%) showed significant differential expression ($P < .05$) between total leukocytes from healthy and infected cattle. Importantly, the suppression of innate immune gene expression detected in chronically infected animals [113] could be one mechanism by which *M. bovis* infection persists [31], and current diagnostics fail to identify all infected animals. Of importance, application of a hierarchical clustering algorithm identified a subset of 15 genes whose combined expression pattern appeared to be indicative of infection status [99]. It may also be the case that the early and transient profile of differential gene expression detected in these studies, could shed light on the mechanisms and kinetics of the shift in the immune system toward a nonprotective, antibody-mediated response associated with progression to chronic disease [31, 47]. It is also possible that expression patterns of this gene subset

could be used to diagnose *M. bovis* infection in cattle. Unfortunately, this was not rigorously tested using a blinded set of samples in the present study. However a recent review from this group suggests that work in this area is on-going [114]. It waits to be seen if changes in expression of such a gene subset are specific to *M. bovis* infection or are a common response to bacterial invasion.

In order to address the issue of an *M. bovis* specific gene expression pattern, Meade et al. performed another time course study using *M. bovis* antigen stimulation to elicit changes in gene expression that would not necessarily be common with any other bacterial infection [107, 108]. This study revealed a subset of 18 genes that showed opposite expression changes (up- or downregulated) in cells from healthy control cattle and *M. bovis*-infected cattle. In keeping with previous results and relevant literature, the nature of the differentially expressed genes suggested a significant role for Toll-like receptor signalling in response to *M. bovis* antigens [107, 108, 113, 114]. This is postulated to have significant consequences for the development of disease in humans [115], and the same may hold true in cattle.

While there may be some species differences between the response of humans and cattle to mycobacterial infections, as discussed elsewhere in this article, there are fewer significant differences between cattle and humans than between mice and humans. This can have significant advantages for translational understanding of the mechanisms of pathogenesis [116]. Detailed pan-genomic transcriptomic analyses will also aid the understanding and potential therapeutic targeting of *M. bovis* infections in other natural infection models, including wildlife species that pose threats as reservoirs of infection [117–119]. Significant progress has been made in defining a gene expression signature that may be diagnostic for *M. bovis* infection in cattle. In many cases, eradication of *M. bovis* is difficult because currently used diagnostic tests do not detect all infected cattle. Development of an improved test, based on genomic or proteomic biomarkers, would be a great improvement. Particular focus also needs to be paid to the development of early stage indicators of infection in order to minimize the losses and risks associated with advanced or chronic infection. Evidence for differential cytokine gene expression, found to be associated with pathology in *M. bovis* infected cattle [120], and a pre-existing gene expression profile in infected animals holds promise for such a resolution [108, 113]. In fact a recent study has made considerable progress in this area using comparative proteomic analysis to identify biomarkers of subclinical (latent) *M. bovis* infection [121]. Serum levels from experimentally infected animals showed marked increases of alpha-1-microglobulin/bikunin precursor (AMBP) protein, alpha-1-acid glycoprotein, alpha-1-microglobulin and fetulin proteins in *M. bovis* infected animals, which were absent from animals challenged with other closely related mycobacteria. As yet, genomic and proteomic tests have not been subjected to rigorous field testing, presumably because funding sources for a large blinded study have not been available. In addition, transfer of genomics information from *M. bovis* infected cattle to *M. tuberculosis* (and *M. bovis*) infections in humans has not been forthcoming. However, functional genomics has added new dimensions to our understanding of the bovine immune response to *M. bovis* infection, and the advent of new technologies including next-generation sequencing holds significant potential for the development of novel intervention strategies against this recalcitrant disease.

7. Summary

The advent of genomic resources for cattle has dramatically improved our ability to use this species in studies of immunity to a variety of diseases, including zoonotic infections such as *M. bovis*. Although the mouse model has proven useful in comparative studies of tuberculosis, recent advances in the characterization of the immune system of cattle now afford an opportunity to gain further insight into the mechanisms of immunopathogenesis utilizing a natural host/pathogen relationship. It is clear from recent investigations that the Th1/Th2 paradigm must be expanded to include the newly identified CD4 and CD8 T cells subsets that comprise the effector and regulatory subsets responding to mycobacterial antigens during different stages of infection. Further studies are needed to determine the relative contribution of Treg and Th17 subsets in the response of cattle to *M. bovis* infection/vaccination. Efforts to elucidate differences between the immune response profile of individuals with latent infection and patients with progressive disease suggest that latency is associated with maintenance of subsets of CD4 T cells [117]. The calf TB model affords a unique opportunity for evaluation of neonatal immune responses to vaccination/infection. Promising TB vaccines may also be evaluated in the calf (safety and efficacy) prior to testing in costly NHPs (thereby prioritizing candidates), and as presented in this review, important mechanisms of immunity may be uncovered by the use of the calf for the study of TB.

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Review Article

Biosensing Technologies for *Mycobacterium tuberculosis* Detection: Status and New Developments

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Biosensing technologies promise to improve *Mycobacterium tuberculosis* (*M. tuberculosis*) detection and management in clinical diagnosis, food analysis, bioprocess, and environmental monitoring. A variety of portable, rapid, and sensitive biosensors with immediate "on-the-spot" interpretation have been developed for *M. tuberculosis* detection based on different biological elements recognition systems and basic signal transducer principles. Here, we present a synopsis of current developments of biosensing technologies for *M. tuberculosis* detection, which are classified on the basis of basic signal transducer principles, including piezoelectric quartz crystal biosensors, electrochemical biosensors, and magnetoelastic biosensors. Special attention is paid to the methods for improving the framework and analytical parameters of the biosensors, including sensitivity and analysis time as well as automation of analysis procedures. Challenges and perspectives of biosensing technologies development for *M. tuberculosis* detection are also discussed in the final part of this paper.

1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is a much dangerous pathogenic bacterium that causes tuberculosis (TB)—one of the leading causes of death from infectious diseases [1]. Currently, about one-third of the human population is infected with TB worldwide [2]. The infection of TB is a serious public health concern because it may emerge as a complication of acquired immune deficiency syndrome infection. The rapid diagnosis and treatment of infectors is considered crucial for the effective control of TB because one patient is known to transmit the disease to 12–15 people/year on average through respiratory tract infection [3]. Areas including clinical diagnosis, water and environmental analysis, food safety, and biodefense are quite critical for sensitive detection of *M. tuberculosis*. Therefore, it is very important and crucial for global public health protection to detect, identify, and quantify *M. tuberculosis*. Traditional microbial culture-based tests are the most common methodologies currently used [4, 5]. Usually these methods involve cell culture, cell counts, and cell enrichment, but this process is time consuming and laborious, especially for the slow-growing bacteria like *M. tuberculosis*. The heavy global public health burden of TB worldwide demands for the development of more rapid and sensitive detection methods. To date, many methods and techniques have been developed for rapid detection of *M. tuberculosis*, such as polymerase chain reaction (PCR) [6–9], latex agglutination [10], enzyme-linked immunosorbent assay (ELISA) [11–13], radiometric detection [14], gen-probe amplified *M. Tuberculosis* direct test (AMTDT) [15], TB rapid cultivation detection technique, such as MB/Bact system, BactecMGIT 960 system [16, 17] and flow cytometry [18]. These methods are more sensitive and rapid than the traditional microbial culture-based methods, as summarized in Table 1. However, they cannot provide the detection results in real-time and most of these methods are centralized in large stationary laboratories because complex instrumentation and highly qualified technical staff are required. As a result, the development of portable, real-time, sensitive, rapid, and accurate methods for *M. tuberculosis* detection is essential to effectively prevent TB infection [19, 20].

Table 1: The various mentioned non-biosensing techniques for bacteria detection.

Method and technique type	Samples analyzed	Detection limit	References
PCR	*M. tuberculosis*	with the true positivity of 95.5%	Thomson et al. [7]
Latex agglutination	*M. tuberculosis*	with the true positivity of 73.6%	Krambovitis et al. [10]
ELISA	*M. tuberculosis*	with the true positivity of 68%	Delacourt et al. [13]
The AMTDT	*M. tuberculosis*	with the sensitivity of 94.3%	Gamboa et al. [15]
Radiometric detection	*M. tuberculosis*	—	Middlebrook et al. [14]
Flow cytometry	*M. tuberculosis*	3.5×10^3 cells/mL	Qin et al. [18]
MB/Bact system	*M. tuberculosis*	—	Horvath et al. 2004
MB/Bact system	*Mycobacteria*	—	Cambau et al. [17]

In recent years, with the improvement of sensing technology research, biosensing technologies are well suited for the purpose. The areas where biosensors show particular importance are clinical assay, disease diagnostics, food security, bioprocess, and environmental monitoring. The importance of biosensors results from their high specificity and sensitivity, which allows the detection of a broad spectrum of analytes in complex sample matrices (saliva, serum, and urine) with minimum samples pretreatment [21–32]. This short review mainly focuses on the discussion of piezoelectric quartz crystal biosensors, electrochemical biosensors, and magnetoelastic biosensors as examples to summarize the development of different biosensors for the use for *M. tuberculosis* detection. Lastly we discuss the future perspectives of biosensors for bacteria, viruses, and other microorganisms' detection.

2. Development of Biosensors for *M. tuberculosis* Detection

The biosensor for *M. tuberculosis* detection is generally defined as a compact analytical device incorporating a biological sensing element with a physicochemical transducer. Depending on the biological element employed, nucleic acid and antibody-based biosensors have been developed. According to the methods of signal transduction, it can be divided into piezoelectric, electrochemical, and optical biosensors for *M. tuberculosis* detection [33–35]. By comparison with culturing system detection and nucleic acid amplification systems detection, there are many advantages associated with the use of biosensing technologies as a rapid and sensitive detection method for *M. tuberculosis* detection, which allow the detection of *M. tuberculosis* in complex sample matrices (serum, urine, or saliva) [36]. For example, (1) it is high specificity by using the biological sensing elements, which can distinguish the targets from other microorganisms; (2) the response time is rapid; (3) it has the capability to provide continuous data with minimal quantity of the samples; (4) it can detect the analytes on-line due to the sensor and the signal transducer in series; (5) some of the biological elements, which are used in detection process, can be reused for other samples [37].

2.1. Piezoelectric Quartz Crystal Biosensors. The piezoelectric quartz crystal (PQC) sensor is one of the new bioelectrochemical devices used for direct detection of *M. tuberculosis*. By combining the high sensitivity with mass and surface characteristics of quartz crystal, such as viscosity, density, dielectric constant, and conductance with the high specificity of biological molecules, the PQC sensor has attracted many analysts because of its high sensitivity, low cost, small size, online detection, and easy operation [38, 39]. On the basis of different parameter responses, the piezoelectric quartz crystal sensors can be classified into two different types: quartz crystal microbalance and series piezoelectric quartz crystal [40–44]. The results acquired from the quartz crystal microbalance are usually by measuring the change in frequency of a quartz crystal resonator. The resonance is changed with the addition or removal of a small mass due to oxide growth/decay or film deposition at the surface of the acoustic resonator [45]. The quartz crystal microbalance can be used under vacuum, in gas phase and recently in liquid environments. It is very useful for monitoring the rate of deposition in thin film deposition systems under vacuum. In liquid, it is highly effective at determining the affinity of molecules (proteins, in particular) to surfaces functionalized as recognition sites. Larger entities such as *M. tuberculosis* and polymers are investigated, as well. Frequency measurements are easily made to high precision. Hence, it can be easy to respond to changes from mass loading on electrode surface down to a nanogram level [45]. Piezo-immunological sensor, which is recently developed on the basis of the quartz crystal microbalance technology, has been used for *M. tuberculosis* detection [46]. For example, He and zhang reported a novel piezo-immunological sensor for *M. tuberculosis* detection. In this method, the quartz crystal was first coated by the styrene-butadiene-styrene copolymer; the antibody was then successfully immobilized onto the membrane surface. After incubation with *M. tuberculosis*, the results were acquired based on the resonant frequency change. With this method, it can detect 10^5 cells/mL *M. tuberculosis* [47]. Though this method is rapid, simple, and unlabeled, the results of this kind of sensors are easily affected by external factors, such as density, viscosity, dielectric constant, and conductivity of the solution [48]. The series piezoelectric crystal quartz sensor (SPQC), which is constructed by combining in series a pair of

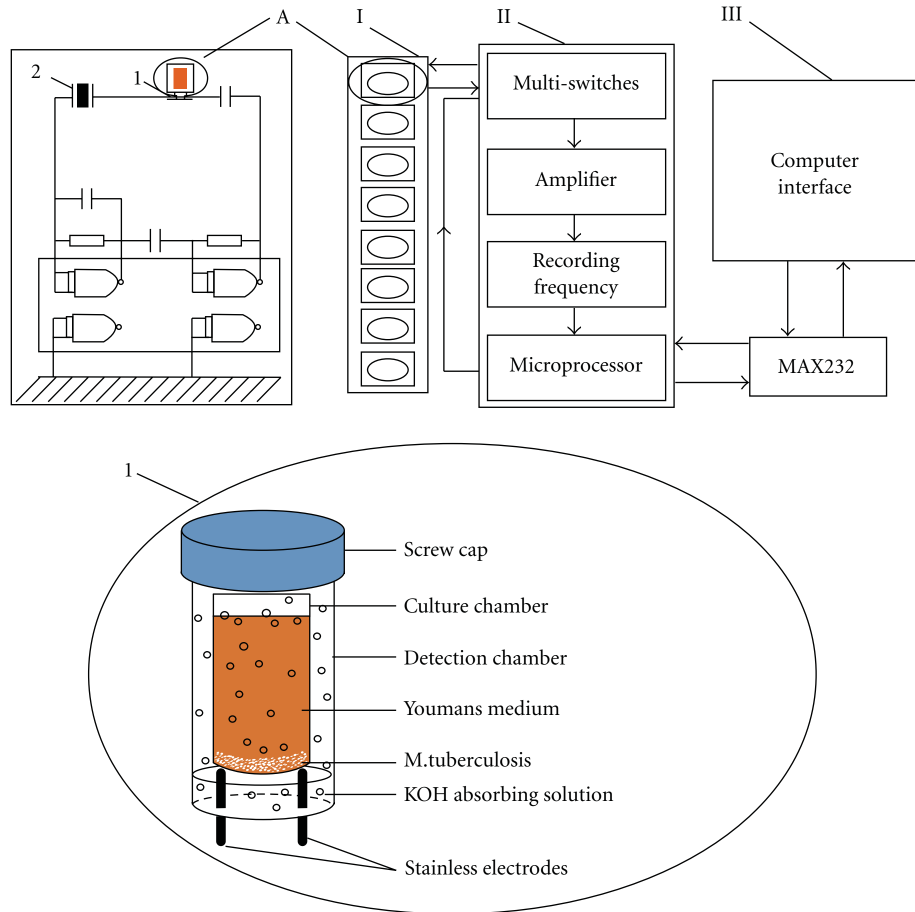

Figure 1: Block diagram drawing of the multichannel series piezoelectric quartz crystal sensor system. The system consists of 3 major components (I) eight samples detection system (A) the circuit of the single oscillator (1) detection cell; (2) 9 MHz AT-cut piezoelectric quartz crystal), (II) microprocessor system, (III) data output system; Reprinted with permission from [48]. Copyright 2008, Biosensors. Bioelectronics.

electrodes immersing in a liquid with a piezoelectric quartz crystal in oscillating circuit, is a very unique device in all of PQC sensors. It has a sensitive frequency response to electric parameters of the solution. SPQC sensor can respond to the changes of liquid conductivity with excellent frequency stability. Compared with conventional conductive methods, this method can detect a smaller conductivity change in the presence of electrolyte [49, 50]. Based on this superiority, the method has been extensively used as a highly sensitive biological and chemical sensor in various fields, such as the analysis of biochemical oxygen demand in environmental monitoring [38], the assay and detection of bacteria in food safety, clinic diagnosis, and so on [51]. He et al. also used it to detect and quantify *M. tuberculosis* H37Ra [39]. With the growth of *M. tuberculosis*, the conductivity of the culture medium was monitored using the sensor through the frequency response curve, where x-axis was the culture time and y-axis was the frequency shift. This method is rapid, sensitive, cheap, and the detection limit is as low as 2×10^3 cells/mL.

Recently, the potential use of volatile production patterns of *M. tuberculosis* and associated cells for early disease diagnosis including TB, urinary tract infections, and breast cancer based on electronic nose has been recognized [52–54]. Ren et al. has reported that the combination of the typical volatile production pattern produced by *M. tuberculosis* with the sensitive conductive response of the series piezoelectric quartz crystal sensor was a new automated continuous multichannel series piezoelectric quartz crystal (MSPQC) sensor system [48]. This system included a detection system for eight samples, a microprocessor system and data output system (Figure 1). In the detection system, it contained two chambers. One of the chambers was full of KOH absorbing solution with a pair of conductive electrodes at its bottom. The other chamber, which was inserted inside the detection chamber, was called the culture chamber with a special growth medium for *M. tuberculosis*. With the growth of *M. tuberculosis*, the component of the medium was decomposed into volatile NH_3 and CO_2. Then, the volatile NH_3 and CO_2 were absorbed by the KOH absorbing solution. The impedance changed in KOH solution was detected by the pair of conductive electrodes. It was connected to the piezoelectric quartz crystal coated with silver disc at two sides in series, which can change the oscillating frequency.

This new automated, continuous and multichannel method was proposed for rapid separation and sensitive detection of *M. tuberculosis*. The detection limit was as low as 10 cells/mL. Compared with other methods, this system was more rapid, much cheaper and the detection limit is much lower. It can detect eight samples at the same time. Therefore, this method was promising to detect *M. tuberculosis* cheaply and quickly for clinic microbiological laboratories and other fields.

2.2. Electrochemical Biosensors. The field of electrochemical biosensors has significant growth in recent years due to their potential application for assay and detection of enzyme, nucleic acid, and microorganism [55, 56]. Usually in the detection process, the solid electrodes are used as the basic electrode. After the biological sensitive molecules are fixed on the electrode surface, the target molecules can be identified and captured onto the electrode surface through the specific recognition of biological molecules. The basic electrode, which works as a signal transmitter, can switch the signal produced by the specific recognition between biological molecules into the electrical signal including current, potential, impedance, and coulometry. Therefore, electrochemical biosensors can sensitively detect and quantify the analysis targets. The choice of the basis electrode is a crucial step for the development of biosensors. There is a wide variety of electrodes for choice to fabricate sensor devices, such as carbon paste electrodes [57], gold electrodes [58], and glassy carbon electrodes [59]. The immobilization of a biomolecule (e.g., DNA, and antibody) [60–63] onto a desired electrode surface is another important step. According to the immobilization of different biomolecules for *M. tuberculosis* detection, electrochemical biosensors can be divided into electrochemical immunosensors [61] and electrochemical DNA biosensors [60, 63].

Electrochemical immunosensors, which combine the high specificity of conventional immunochemical methods with electrochemical system, provides a potential opportunity to gain new insights to create sensitive and simple immunoassay devices for *M. tuberculosis* detection. Díaz-González et al. has developed an enzymatic voltammetric immunosensor for the determination of *M. tuberculosis* antigen [61]. A screen-printed carbon electrode, modified with the streptavidin, was used in this method as a signal transduction element. The biotinylated rabbit anti-*M. tuberculosis* antibodies were immobilized onto the electrode surface through the specific streptavidin-biotin reaction. In the presence of *M. tuberculosis* antigens and monoclonal antibodies against *M. tuberculosis*, a sandwich immune complexes of rabbit anti-*M. tuberculosis*/*M. tuberculosis* antigens/monoclonal antibodies against *M. tuberculosis* could be formed. The alkaline phosphatase (AP) labeled rabbit IgG anti-mouse immunoglobulin G, which was used as detector antibodies, was further adsorbed onto the monoclonal antibodies. When the 3-indoxyl phosphate was used as an electrochemical substrate, the resulting enzymatic product could cause the change of electrochemical behaviors on the electrode surface. Using this technique it was possible to detect *M. tuberculosis* antigens with detection limit of 1.0 ng/mL.

Electrochemical DNA biosensors, which are based on nucleic acid hybridization, have attracted considerable attention due to their potential application for assay and diagnosis of TB and other diseases. Depending on the probes employed, the PNA (peptide nucleic acid) probes electrochemical biosensor [55] and DNA probe electrochemical biosensor have been developed [60, 63]. Nirmal Arora et al. has reported a method for *M. tuberculosis* detection using PNA probes [55]. In this method, the 21-mer PNA probe specific to 16 s–23 s rRNA spacer region of *M. tuberculosis*, has been covalently immobilized onto the polypyrrole-polyvinylsulphonate (PPy-PVS) film. The film was then electrochemically deposited onto indium-tin-oxide (ITO) glass to form the PPy-PVS/ITO electrode. The PNA probe was used for the hybridization detection with complementary sequence of *M. tuberculosis* DNA with a detection limit of 2.5 pg/μL. The whole detection process can be finished within about 60 min. Das et al. detected *M. tuberculosis* using the Zirconia- (ZrO_2-) based nucleic acid sensor [63]. ZrO_2 is an attractive inorganic metal oxide with thermal stability, chemical inertness, nontoxicity, and affinity for groups which contain oxygen. These groups facilitate covalent immobilization without using any crosslinker which may limit the sensitivity of the fabricated sensor. So it is an ideal material to immobilize the biomolecules with oxygen groups [60, 62]. Moreover, ZrO_2 has pH stability, which plays an important role as corrosion-resistant coatings on sensor applications. Based on the superiorities of ZrO_2, the nanostructured ZrO_2 film was electrochemically deposited onto the Au electrode surface to fabricate a DNA biosensor for *M. tuberculosis* detection. As can be seen in Figure 2, the ssDNA was the 21-mer oligonucleotide specific to *M. tuberculosis*. First, nano ZrO_2 film was deposited onto the bare gold electrode [63, 64]. After the incubation of ssDNA with ZrO_2/Au surface by utilizing the affinity between oxygen atom of phosphoric group and zirconium, the DNA biosensor was successfully fabricated. With this method, the detection limit was 0.065 ng/μL and the whole detection process can be done within 60 s. Therefore, this kind of DNA-ZrO_2/Au bioelectrodes can be used for early, sensitive and rapid assay, detection and diagnosis of *M. tuberculosis*.

2.3. Magnetoelastic Biosensors. The development and application of magnetoelastic biosensor techniques have been reported in the past few years [65–68]. Typically, this kind of sensors is a free-standing, ribbon-like magnetoelastic film coupled with a chemical or biochemical sensing layer such as enzyme. In response to an externally applied magnetic field, the sensor mechanically vibrates at a characteristic resonance frequency, launching a return magnetic field which can be remotely detected by a pickup coil. Since there is no physical contact between the sensor and the detection system, various magnetoelastic sensors have been developed for remote-query monitoring of different physical and chemical parameters (e.g., microorganisms [69], flow velocity [70], temperature [71], pressure [72], elasticity [73], mass loading [74–76], density, and liquid viscosity [77–79]). Pang et al. used the magnetoelastic biosensor for the direct, real-time detection and quantification of

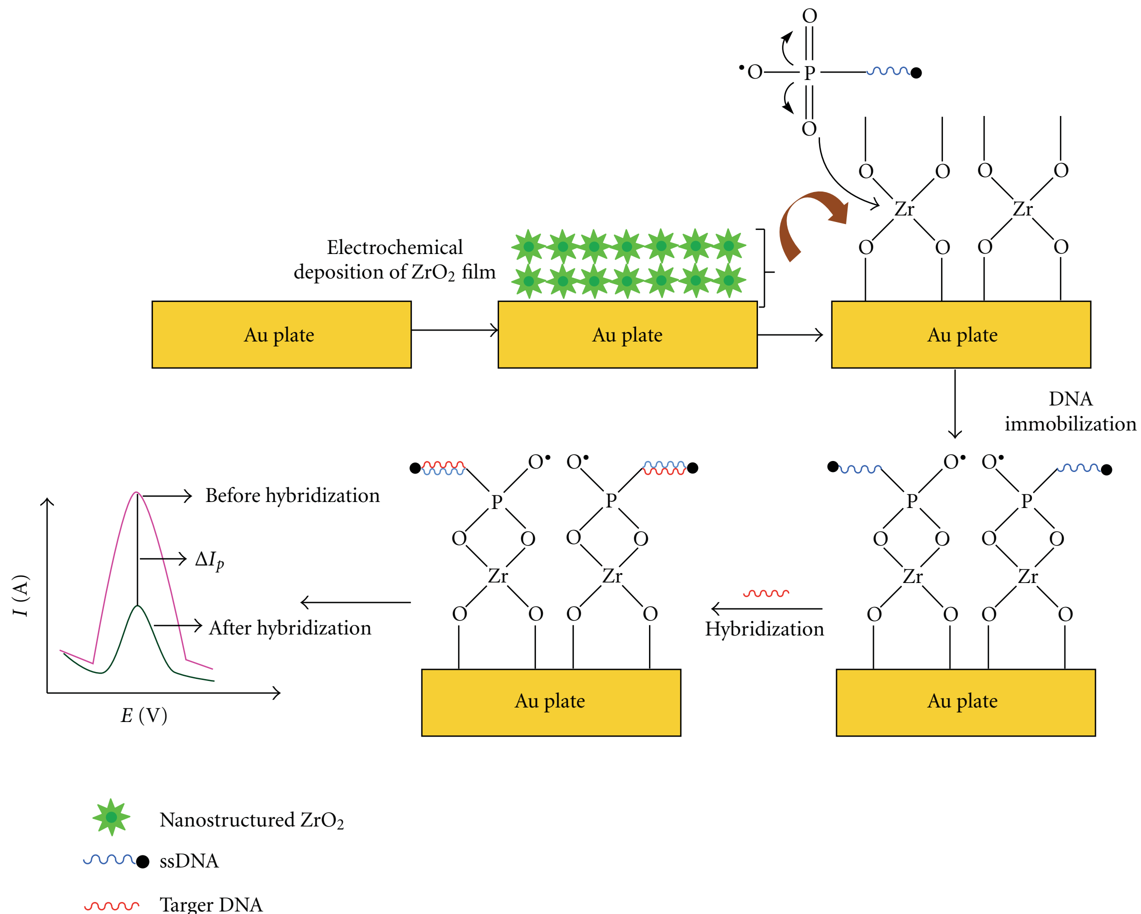

Figure 2: Proposed schematic for the fabrication of nano-ZrO_2/Au-based DNA biosensor. Reprinted with permission from [63]. Copyright 2010, Applied Physics Letters.

M. tuberculosis with a liquid medium. The sensor used in this method was fabricated by coating a magnetoelastic ribbon with a polyurethane protecting film [69]. With the growth and proliferation of *M. tuberculosis*, cells consumed the nutrients and decomposed the macromolecules into small molecules (e.g., CO_2, NH_3, organic acid and so on) within a liquid culture medium. Consequently, the medium properties, which were determined by the composition and concentration of the medium, could be changed by these small molecules. This, in turn, the resonant frequency of the magnetoelastic sensor was changed. Therefore, the sensor response sensitivity was affected by the characters of culture medium. Pang et al. discovered that the response at standard culture medium concentration was the best. On the basis of this concentration of the culture medium, they detected a linear relationship with the bacterial concentration in the range of 1×10^4 to 1×10^9 cells/mL. The detection limit was 10^4 cells/mL. In addition, this method was also used in sputum sample. All of the results indicated that the magnetoelastic biosensor can be effectively used for monitoring and detecting the growth of *M. tuberculosis* both in culture medium and real samples because of its low cost and remote query nature.

3. Challenges and Perspectives

Analysis of published literatures has shown that the rapid development of biosensor technology has opened enormous opportunities for *M. tuberculosis* detection, as listed in Table 2. However, there are still many considerable challenges and issues remained for reliable and effective use in routine applications with biosensors. The biosensor system must have the adaptability and flexibility to detect different analytes with relatively simple and inexpensive structures. Although the sensitivity for detection of *M. tuberculosis* has been highly improved and the detection limit of *M. tuberculosis* can be as low as 10 cells/mL when detected by the new automated continuous multichannel series piezoelectric quartz crystal (MSPQC) sensor system [48], a biosensor must be able to provide a detection limit as low as single coliform organism in 100 mL of potable water [36]. Thus, the sensitivity is still a very important issue that requires improvement. Another problem is that the price of most of biosensors is too high to acquire, only a few biosensors for bacterial detection are relatively cheap to acquire and the main reasons for this are both the technology and market related reasons. This problem seriously influences the online

Table 2: Different kinds of biosensors for *M. tuberculosis* detection.

Biosensor devices	Samples analyzed	Detection limit	References
Piezo-immunological sensor	*M. tuberculosis*	10^5 cells/mL	He et al. [46]
The series piezoelectric crystal quartz sensor	*M. tuberculosis* H37Ra	2×10^3 cells/mL	He et al. [39]
Multichannel series piezoelectric quartz crystal sensor	*M. tuberculosis*	10 cells/mL	Ren et al. [48]
Electrochemical immunosensor	*M. tuberculosis* antigens	1.0 ng/mL	Díaz-González et al. [61]
Electrochemical DNA biosensor	*M. tuberculosis* DNA	0.065 ng/μL	Das et al. [63]
Magnetoelastic biosensor	*M. tuberculosis*	10^4 cells/mL	Pang et al. [69]
Acoustic wave impedance biosensor	*M. tuberculosis*	2×10^3 cells/mL	He et al. [39]
Surface plasmon resonance sensor	*M. tuberculosis* complex	30 ng/μL	Duman et al. 2010

detection of infectious bacteria (e.g., *M. tuberculosis*) worldwide. Especially for developing countries, this problem is more seriously.

In recent years, with the successful development of nanotechnology, highly sensitive and accurate biosensor systems based on the combination of nanotechnology and biosensing technology have great application in the medical diagnostics, clinical medicine, environmental monitoring, food quality control, defense, and other industries. The applications of these biosensor systems have been also started to flourish in the field of *M. tuberculosis* detection. Therefore, the continued collaborations in various fields including chemistry, physics, materials science, molecular biology, and manufacture, will with no doubt speed up the translational process and eventually realize the great impact of biosensors for *M. tuberculosis* detection.

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