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The *ΔfbpA* attenuated candidate vaccine from *Mycobacterium tuberculosis*, H37Rv primes for a stronger T-bet dependent Th1 immunity in mice

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Summary

The *ΔfbpA* candidate vaccine derived from *Mycobacterium tuberculosis* (H37Rv) protects mice better than BCG against tuberculosis, and we investigated the hypothesis that *ΔfbpA* may induce a stronger Th1 immunity. Since T-bet transcription factor regulates Th1 immunity, mice infected with *ΔfbpA*, BCG vaccine and related mycobacteria were analyzed for T-bet positive T cells. Mouse dendritic cells (DCs) or macrophages were also pulsed with excretory-secreted antigens (ES; Antigen-85B, ESAT-6 and CFP10) and cocultured with T cells from immunized or naïve mice and tested for *in vitro* induction of T-bet and IFN- γ . In both models, *ΔfbpA* mutant induced a stronger response of T-bet⁺CD4 T cells, which correlated with an increased expansion of IFN- γ ⁺CD4 T cells *in vivo* and *in vitro*. When DCs pulsed with ES antigens were allowed to stimulate T cells, ESAT-6 and CFP-10 failed to induce a recall expansion of T-bet⁺IFN- γ ⁺CD4 T cells from BCG vaccinated mice. Thus, deletion of RD1 in BCG seems to reduce its ability to induce T-bet and induce stronger Th1 immunity. Finally, mice were vaccinated with *ΔfbpA* and BCG and challenged with virulent Mtb for evaluation of protection and T cell expansion. *ΔfbpA* vaccinated mice showed a rapid and stronger expansion of CD4⁺CXCR3⁺ IFN- γ ⁺ T cells in the lungs of Mtb challenged mice, compared to those which had BCG vaccine. *ΔfbpA* immunized mice also showed a better decline of the Mtb bacterial counts of the lungs. Mtb derived *ΔfbpA* candidate vaccine therefore induces qualitatively better T-bet dependent Th1 immunity than BCG vaccine.

1. Introduction

Mycobacterium tuberculosis (Mtb) causes tuberculosis which is the leading cause of death due to infections in man today, with at least 2 million deaths reported worldwide. *M. bovis* BCG is the most frequently used vaccine with over a billion doses administered to date. Although it is effective against childhood tuberculosis, it has a variable efficacy and

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geographic variation [1]. Evidence suggests that BCG lacks some immunodominant antigens (e.g., ESAT-6 and CFP-10) encoded by the RD1 region, which is intact in the wild type MTB. Thus, efforts to improve vaccination have included, re-engineered BCG that can express these antigens, besides, DNA vaccines, sub unit vaccines and gene deletion attenuated mutants of Mtb [2] [3]. Vaccines more effective than BCG in animal models are available [4]. However, the parameters that determine protection against tuberculosis still remain unclear because existing vaccines have proved less than ideal to protect non-human primate animal models against tuberculosis [5].

The search for better vaccines for tuberculosis has necessitated an understanding of the immune mechanisms operative in protection. Extensive studies show that immunity to tuberculosis is dependent both on CD4 and CD8 T cells while a non-traditional CD1d-dependent mechanism is also evident [6]. The efficacy of T cell mediated immunity is in turn, dependent upon the ability of antigen presenting cells (APCs) to process and present antigens of pathogens [7,8]. After the initial aerosol implantation of Mtb, infected alveolar macrophages (Mφs) and dendritic cells (DCs) of the respiratory tract secrete cytokines and chemokines that recruit immune cells into the lungs[9]. The initial containment of infection or the granuloma consists of infiltrating elements including T cells, neutrophils and eosinophils [10]. Emerging evidence indicates that, during the primary immune response, DCs that contain Mtb or BCG vaccine migrate to lymph nodes where they prime naïve T cells. Mφs restrict infection at the lungs and can prime locally available immune T cells. Thus, Mφs and DCs play a key role in the development and maintenance of an anti-tuberculosis immune response [11] [12] [9] [3].

HIV-1 induced depletion of CD4 T cells in humans leads to enhanced susceptibility to tuberculosis strengthening the concept that, CD4 T cells are major mediators of protection in humans and relevant to vaccine development. Consequently, T cell-regulatory factors that orchestrate Th1 immunity appear to be important. During the initial induction of Th1 immunity, DCs not only present antigen to naïve T cells but also induce T-bet, a transcription factor. T-bet drives IFN- γ synthesis and surface expression of CXCR3 among CD4 T cells, thereby affecting the ability of Th1 T cells to migrate to sites of inflammation and infection [13] [14] [15]. Likewise, antigen primed DCs can also induce either GATA or FoxP3 transcription factors, to respectively induce Th2 and T-regulatory T cells [8]. Mills and others have proposed that the nature of pathogen being phagocytosed by the DCs, secretion of distinct cytokines and stimulation of Toll-like receptors on DCs, together determine the ultimate lineage of naïve T cell differentiation [16] [8] [17,18]. In our earlier study, we found that naïve DCs infected with mycobacteria could drive Th1 differentiation and lead to the expansion of IFN- γ secreting T cells [19,20]. Using the mouse models, we also identified that newer vaccine strains derived from wild type Mtb can protect against tuberculosis. In this study, using *in vitro* and *in vivo models*, we have investigated the hypothesis that mycobacterial strains may induce different levels of T-bet transcription factor in T cells and thereby determine the efficacy of Th1 pathway. We propose an additional parameter to evaluate anti-tuberculosis vaccines.

2. Results

Mycobacterial strains induce a variable level of T-bet transcription factor in CD4 T cells of mice

T-bet determines multiple parameters of Th1 immunity including the expansion of IFN- γ ⁺ CD4 and CD8 T cells, and surface expression of chemokine receptors that enable migration of T cells to sites of infection and inflammation [13] [21] [22]. Thus, we initially sought to determine if virulent and attenuated strains of mycobacteria induced T-bet in Th1 T cells that could affect their infection profile. Fig. 1a shows that after i.p. infection, mycobacterial

strains induced variable levels of T-bet, with virulent *M.tuberculosis* inducing the strongest response followed by *M.avium*, BCG and *M. smegmatis*. Interestingly, all mycobacteria grew to detectable levels in the spleens by day 7 and then, either increased up to week 4 for Mtb-H37Rv and *M.avium* or plateaued for BCG and *M.smegmatis*. On day 7, when bacterial counts were comparable, virulent Mtb-H37Rv had the strongest T-bet induction suggesting that, induction of T-bet responses was not related to bacterial growth, at least over the initial 7 days of infection (Fig. 1b). Splenic T cells were also typed for IFN- γ secreting CD4 T cells. On day 14, Mtb-H37Rv induced elevated levels of CD4 IFN- γ ⁺ T cells, compared to other mycobacteria (Fig. 1c).

$\Delta fbpA$ candidate vaccine induces stronger Th1 responses in mice compared to BCG vaccine

In our previous studies, we demonstrated that an attenuated candidate vaccine derived from Mtb-H37Rv was able to protect mice against tuberculosis better than BCG vaccine [23]. We hypothesized that $\Delta fbpA$ induced a stronger Th1 response. Since T-bet is known to affect IFN- γ and CXCR3 expression of Th1 T cells, mice were infected i.p or s.c. with $\Delta fbpA$ and BCG followed by weekly T cell analysis. T cell profiles of mice following i.p. or s.c infections were comparable and thus, only i.p. profiles are presented. Flow cytometric analysis indicated that $\Delta fbpA$ induced an increased expansion of T-bet⁺(Fig. 2A), CXCR3⁺(Fig. 2B) and IFN- γ ⁺CD4 T cells (Fig. 2C) in the spleens of mice, during the 4 weeks of infection. Interestingly, both $\Delta fbpA$ mutant and BCG vaccine grew similarly within spleens (Fig. 2D).

Dendritic cells infected with $\Delta fbpA$ mutant drive a better recall T-bet response in T cells *in vitro* compared to BCG vaccine

During an ongoing infection, DCs present antigens to T cells under the regulatory influence of cytokines to recall Th1 expansion. To determine whether APCs infected with $\Delta fbpA$ and BCG vaccine strains prime T cells differently, DCs were infected *in vitro* with $\Delta fbpA$ or various sub-strains of BCG and overlaid with splenic T cells from mice infected with $\Delta fbpA$ or BCG. T cells were analyzed for T-bet, GATA-3 and FoxP3 and culture supernatants measured for soluble IFN- γ by ELISA. These experiments revealed that $\Delta fbpA$ infected DCs drive a stronger recall T-bet response compared to BCG (Fig. 3a), which correlated with a stronger expression of IFN- γ (Fig. 3b). Interestingly, none of the DC cocultures induced a significant GATA-3 or FoxP3 recall response among immune T cells *in vitro* (Fig. 3c).

The ES antigens of $\Delta fbpA$ mutant and BCG vaccine induce T-bet response in T cells

Since mycobacteria induced different levels of T-bet in mice (Fig. 1 & 2) and T cells activated by DCs *in vitro* (Fig. 3), we sought to determine, if ES antigens of mycobacteria could account for differences in the ability to prime for T-bet. ES antigens are a cluster of secreted antigens capable of modulating Th1 responses to tuberculosis. DCs treated with two major ES antigens were therefore overlaid with T cells from mice immunized with different mycobacteria (H37Rv, H37Ra, BCG and $\Delta fbpA$) and T-bet and IFN- γ expression evaluated using flow cytometry. Fig. 4A. illustrates the recall expansion of IFN- γ ⁺CD4 T cells by DCs treated with ES antigens. Fig. 4B summarizes data from three separate experiments of T cell analysis. First, Ag85B treated DCs uniformly expanded IFN- γ secreting CD4 T cells from mycobacteria. This was anticipated, since it is present in all the mycobacteria tested. T cells from naïve mice overlaid on antigen pulsed DCs yielded <5% of IFN- γ ⁺ T cells, indicating that there was no non-specific expansion. In contrast, DCs with ESAT-6 and CFP-10 failed to drive Th1 expansion among T cells from BCG infected mice, which in turn, correlated with a decreased T-bet induction. It is well established that Mtb-H37Rv, H37Ra and $\Delta fbpA$ have intact ESAT-6 and CFP10 while BCG lacks them. Reduced Th1 expansion by BCG infected DCs is therefore likely due to the absence of ESAT6 and CFP10 antigens in the

BCG vaccine. We conclude that ES antigens can induce T-bet and differences in the content or expression of ES antigens can alter the ability of mycobacteria to induce Th1 response.

***ΔfbpA* mutant offers better protection than BCG vaccine in mice against virulent challenge with Mtb**

Following aerosol infection of mice with Mtb, Th1 T cells infiltrate into lungs and help to contain infection through the secretion of IFN- γ and other cytokines. The ability to infiltrate into lungs is dependent upon the expression of chemokine receptors such as CXCR3 for Th1 T cells. We sought to determine if *ΔfbpA* or BCG which induce different types of Th1 differ in their ability to protect against tuberculosis. Thus, mice were immunized s.c. with *ΔfbpA* or BCG and challenged four weeks later with low dose aerosol (<100 CFU per mouse) using Mtb-Erdman strain. Fig. 5A illustrates that on day 28 after challenge, there were more IFN- γ secreting CD4 T cells in the lungs of mice vaccinated with *ΔfbpA* mutant than those with BCG vaccine or control groups. Such mice also showed a better protection against tuberculosis indicated by a decline in the bacterial counts of lungs (Fig. 5B). Fig. 5C summarizes the flow cytometric data of mouse lungs analyzed weeks after challenge. *ΔfbpA* vaccinated mice showed uniform increase in the number of CXCR3⁺IFN- γ ⁺CD4 and CD8 T cells.

3. Discussion

Th1 immunity is known to be protective against tuberculosis and anti-tuberculosis vaccines induce Th1 signatures in animal models. Use of gene knock out strains of mice has shown that the Th1 response mediated by IFN- γ is a critical determinant of anti-tuberculosis immunity, while other cytokines like TNF α and IL-2 also play a major role [24]. It is generally held that Th1 immunity is initiated by immature DCs that upon engulfment of microbes migrate to lymphoid organs and within the cytokine rich milieu, process antigens from microbes and prime naïve T cells. The latter develop into IFN- γ secreting CD4 and CD8 T cells that also express appropriate homing receptors to enable to migrate into sites of infection and inflammation. While T-bet induction was found necessary for IFN- γ production, it also determines the efficacy with which T cells express CXCR3. Thus, efficacy of Th1 immunity seems to depend upon T-bet.

We demonstrated that mycobacteria differ in their ability induce T-bet in T cells of mice after infection. Curiously, virulent Mtb induced stronger T-bet and significant IFN- γ responses in mice. This observation can be explained as follows. First, virulent Mtb appears to have unique survival mechanisms. The ability of Mtb to remain sequestered in semi-permeable phagosomes of M ϕ s is well known. Mtb also has the ability to down-regulate the antigen processing mechanisms including, expression of MHC-II, CD1d, costimulatory molecules and alter the sensitivity of M ϕ s to IFN- γ [25] [26] [27] [28] [29]. In addition, Mtb derived lipids and lipoproteins have been reported to alter the phenotype of DCs, affect maturation and suppress or alter T cell priming function [30] [31] [32]. Since Mtb infects predominantly through aerosol route, we suggest that the minimal numbers of Mtb in respiratory tract may parasitize the M ϕ s and DCs and prevent efficient generation of Th1 immunity through evasion mechanisms. The significant peak of T-bet response in mice to Mtb we observed therefore seems to be due to size of the inoculum given into the peritoneal cavity, that enables rapid access of Mtb to APCs and T cells (Fig. 1). It should be noted that the growth of Mtb and *M.avium* continued despite Th1 response after day 7, while, BCG and *M.smegmatis* declined. It therefore appears that, the pathogens Mtb and *M.avium* have virulence factors that enable their growth while attenuated BCG and *M.smegmatis* are controlled by Th1 mechanisms. These observations indicate that, homologous Th1 responses fail to protect against tuberculosis while such responses effective against the infection with BCG vaccine strain. Paradoxically, BCG vaccine is only moderately effective against

tuberculosis in C57Bl/6 mice, and prior vaccination yields about 1-log₁₀ decrease in the growth of Mtb within lungs of challenged mice[5,33]. We therefore hypothesized that a better vaccine to prevent tuberculosis perhaps needs to prime for a stronger and longer lasting, Th1 immunity.

BCG vaccine is an attenuated derivative of *M.bovis* and known to induce significant CD4 T cell responses, with moderate CD8 T cell responses in mice. Since CD4 T cells seem to protect against acute tuberculosis infection and CD8 T cells against chronic or latent stages of tuberculosis, BCG has been modified to stimulate better CD4 and CD8 responses. Early research showed that BCG induced CD8 T cell response in mice can be enhanced through addition of listeriolysin, that seems increase release BCG antigens from phagosomes into the cytosol [34] [35,36]. Others have cloned several proteins of wild type Mtb into BCG, which led to an increase in the efficacy of the vaccine. Recombinant BCG (rBCG) expressing ESAT-6, Ag85B and LLO is a major, improved candidate vaccine under human trials. Even with these improvements, however, the immunological basis for protecting against tuberculosis remains far from clear. For example, many vaccines that protect better against mouse tuberculosis do not protect non-human primates against tuberculosis. We sought an alternate to BCG vaccine by evaluating an attenuated candidate vaccine from Mtb-H37Rv. The rationale was that the mutant would have nearly all antigens of wild type Mtb and those missing in BCG such as RD1 encoded antigens may help to confer better protection. Our previous studies showed that the *ΔfbpA* mutant protects mice better than BCG [23].

In this study, we observed that *ΔfbpA* mutant induced better Th1 responses in mice compared to BCG vaccine. First, infection studies in mice demonstrated that *ΔfbpA* induced enhanced levels of T-bet positive T cells that also secreted IFN- γ and expressed CXCR3 (Fig. 2 & 3). A striking observation was that the ES antigens of *ΔfbpA* were capable for priming for recall T-bet expression in T cells and amplify IFN- γ responses (Fig. 4). Finally, *ΔfbpA* mutant offered a better protection against tuberculosis in mice that correlated with a better accumulation of CXCR3⁺IFN- γ ⁺CD4 and CD8 T cells in the lungs of vaccinated and Mtb challenged mice (Fig. 5).

It is apparent that the presence of ESAT-6 and CFP-10 (ES) antigens in *ΔfbpA*, is likely responsible for the better immunogenicity of *ΔfbpA* mutant vaccine. These antigens are deleted in BCG and are known to carry major T cell epitopes and capable of boosting anti-tuberculosis immunity in mice. A second possibility for the increased efficacy of *ΔfbpA* candidate vaccine centers on its ability to undergo limited phagosome maturation. Using an *in vitro* antigen-85B presentation system to T cells, we found that APCs with BCG vaccine had a reduced efficacy in presenting Ag85B to T cells *in vitro* compared to those infected with *ΔfbpA* mutant[37]. Since DCs process and present peptide antigens to prime the Th1 immunity [38] [7] [39] [8], it seems reasonable to propose that the ability of vaccines to undergo efficient processing in APCs can affect their immunogenicity. In summary, we have demonstrated that novel attenuated strains of Mtb can induce qualitatively and quantitatively different Th1 responses in mice that translate into better protection against tuberculosis. If safety can be ensured, attenuated Mtb-derived vaccines can perhaps replace BCG vaccine.

4. Methods

DCs

C57Bl/6 mice derived bone marrow DCs were prepared by culturing bone marrow cells in IDM with 10 ng/mL mouse GM-CSF or 10% culture supernatant from NIH-3T3 fibroblasts secreting IL-4 (Kind gift of Kammertoens T, Institute of Immunology, Charite Campus, Benjamin Franklin, Berlin, Germany). After 7 days, the non-adherent cells were gently flushed and further purified using CD11c magnetic beads (Miltenyi Inc, USA). Resulting

cells were more than 98% pure as analyzed by flow cytometry. The DCs were immunophenotyped with fluorophore labeled antibodies and largely found to contain CD8+ with lesser numbers of double negative DCs, all of which were CD11c positive but F4/80 negative, thus excluding contamination with macrophages.

Mycobacteria

Stock cultures from ATCC were grown in Dubos' broth and used fresh after three washes in PBS. They were routinely > 90% viable as evaluated by fluorescein diacetate stains. Wild type Mtb-H37Rv (# 27294), and BCG (# 35734) were prepared from ATCC stock. Mtb-Erdman with acriflavine resistance gene was a kind gift of Dr. D. Kernodle from Vanderbilt University. *AfbpA* mutant (cultured in 25 µg/ml kanamycin medium) has been described by us before and were cultured in Dubos' broth with 25 µg/mL kanamycin [40]. BCG Pasteur and other sub strains were obtained from ATCC (MD). All mycobacteria were grown in 7H9 broth to log phase and frozen stored as > 90% viable suspensions until use.

Recombinant antigens

Early secreted antigenic target (ESAT-6) and culture filtrate protein (CFP-10) were expressed and purified as described earlier [41]. They were ultra-purified to enable biophysical studies on complex formation between ESAT-6 and CFP-10 (kind gift of Dr. Arora CDRI India).

Immune and naïve T cells

Mycobacteria primed mice—Live Mtb-H37Rv, Mtb-H37Ra, BCG or *AfbpA* were given i.p. (or s.c) at 10^6 CFU as one dose on day 0 to C57Bl/6 mice of 4–6 weeks age and mice were sacrificed for T cells 2 or 4 weeks later. For each immunization, 6 mice were used and CD3⁺ T cells from mouse spleens were cocultured with DCs. The entire experiment was repeated 3 times. Splenic T cells from immune and naïve non immunized mice were negatively isolated using a Pan-T cell kit (Miltenyi Inc, USA) and used for coculture experiments with DCs.

DC-T cell cocultures and Th1 recall expansion

The purified DCs were washed and plated into 6-well plates. DCs were either treated with antigens as indicated or were infected for 4 hrs with mycobacteria an MOI of 1:1 and washed before coculture with T cells. The splenic CD3⁺ T cells from immunized mice or naïve mice were cocultured with the DCs (5×10^6 T cells per 1×10^6 DCs, triplicate cultures per mouse, 3 mice per strain) [42] [19]. At the end of 72 hr coculture, dead cells were removed on Ficoll-Hypaque columns and viable cells were stained for flow cytometry using antibodies as needed. IFN- γ levels were determined using a sandwich ELISA kit from R & D.

Vaccine model

C57Bl/6 mice were immunized with one dose of either BCG or *AfbpA* mutant or left unvaccinated (naïve). They were challenged after 4 weeks with virulent acriflavine resistant Mtb-Erdman strain implanting about 100 CFU per lungs. Mice were sacrificed weekly for colony counts of Mtb-Erdman on acriflavine containing 7H11 agar plates over the next 4 weeks. Mice were sacrificed separately for CFU counts and T cells. The lung derived cells were immunotyped for the Th1-transcription markers T-bet, CXCR3 and intracellular IFN- γ as previously described [19]. Total lung cells were counted before staining and the numbers of T cells positive for markers were enumerated using flow cytometry [19] [43] [44].

Flow Cytometry

DCs were typed for purity using CD11c (Integrin α_x chain), while T cells were typed for both surface markers (CD4, CD8) as well as intracellular cytokines as follows. DCs were gated based on forward scatter and isotype control of Rat IgG2a (Caltag Laboratories, R2a04) was used. DCs were dispensed at 10^5 cells per pellet and added with Fc Receptor Blocker (CD16/32, Caltag Laboratories, MFCR00) and incubated for 15 min on ice. The appropriate antibody (CD11c) was added to each sample and incubated for 30 min in the dark. The cells were washed and then fixed in 2% paraformaldehyde for 30 min before acquisition using a BD FACSCAN. DCs were also stained with F4/80 to rule out contamination with M ϕ s. The intracellular staining for T cells was performed as follows. DC-T cell cocultures were treated on the day of harvest with Golgi stop and Golgi plug (GolgiStop 554715, BD Biosciences) for 6 h. T cells harvested on ficoll columns from cocultures were added with Fc Receptor Blocker (CD16/32; Caltag Laboratories, MFCR00) and incubated on ice for 15 min. The appropriate antibodies were added to each sample (CD4, L3T4; Caltag Laboratories MCD0401 or MCD0404), CD8 α (Ly-2; Caltag Laboratories RM2201-3 or MCD0804-3) and incubated for 30 min in the dark. The cells were washed and then fixed with 2.7% paraformaldehyde for 30 min, followed by staining with anti-IFN- γ (Caltag Laboratories RM9001-3) diluted in a perm buffer containing 0.1% Triton-X 100, 0.1% Saponin, 0.001% Digitonin, and PBS for 2 h or overnight at 4°C. The samples were washed in PBS and acquired using BD FACSCAN and Cellquest software.

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Key words

Mtb-H37Rv	Strain of <i>Mycobacterium tuberculosis</i>
<i>AfbpA</i>	fibronectin binding protein A deletion mutant
BCG	<i>M.bovis</i> BCG Bacille Calmette-Guérin vaccine
Ag85B	Antigen 85 complex B protein
ESAT-6	Early secreted antigenic target-6
CFP-10	Culture filtrate protein-10

References

- McMurray DN. Recent advances in improved tuberculosis vaccines. Indian J Pediatr. 2000; 67:S58–62. [PubMed: 11129910]
- Andersen P, Doherty TM. The success and failure of BCG - implications for a novel tuberculosis vaccine. Nat Rev Microbiol. 2005; 3:656–662. [PubMed: 16012514]
- Majlessi L, Brodin P, Brosch R, Rojas MJ, Khun H, Huerre M, Cole ST, Leclerc C. Influence of ESAT-6 secretion system 1 (RD1) of *Mycobacterium tuberculosis* on the interaction between mycobacteria and the host immune system. J Immunol. 2005; 174:3570–3579. [PubMed: 15749894]
- Doherty TM, Andersen P. Vaccines for tuberculosis: novel concepts and recent progress. Clin Microbiol Rev. 2005; 18:687–702. [PubMed: 16223953]
- Orme IM. The use of animal models to guide rational vaccine design. Microbes Infect. 2005; 7:905–910. [PubMed: 15878834]
- Flynn JL. Immunology of tuberculosis and implications in vaccine development. Tuberculosis (Edinb). 2004; 84:93–101. [PubMed: 14670350]

7. de Jong EC, Smits HH, Kapsenberg ML. Dendritic cell-mediated T cell polarization. Springer Semin Immunopathol. 2005; 26:289–307. [PubMed: 15609003]
8. Mills KH, McGuirk P. Antigen-specific regulatory T cells--their induction and role in infection. Semin Immunol. 2004; 16:107–117. [PubMed: 15036234]
9. Humphreys IR, Stewart GR, Turner DJ, Patel J, Karamanou D, Snelgrove RJ, Young DB. A role for dendritic cells in the dissemination of mycobacterial infection. Microbes Infect. 2006; 8:1339–1346. [PubMed: 16697232]
10. Kaufmann SH. Protection against tuberculosis: cytokines, T cells, and macrophages. Ann Rheum Dis. 2002; 61(Suppl 2):ii54–58. [PubMed: 12379623]
11. Russell DG. Mycobacterium tuberculosis: here today, and here tomorrow. Nat Rev Mol Cell Biol. 2001; 2:569–577. [PubMed: 11483990]
12. Herrmann JL, Lagrange PH. Dendritic cells and Mycobacterium tuberculosis: which is the Trojan horse? Pathol Biol (Paris). 2005; 53:35–40. [PubMed: 15620608]
13. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000; 100:655–669. [PubMed: 10761931]
14. Lametschwandtner G, Biedermann T, Schwarzler C, Gunther C, Kund J, Fassl S, Hinteregger S, Carballido-Perrig N, Szabo SJ, Glimcher LH, Carballido JM. Sustained T-bet expression confers polarized human TH2 cells with TH1-like cytokine production and migratory capacities. J Allergy Clin Immunol. 2004; 113:987–994. [PubMed: 15131585]
15. Dorfman DM, van den Elzen P, Weng AP, Shahsafaei A, Glimcher LH. Differential expression of T-bet, a T-box transcription factor required for Th1 T-cell development, in peripheral T-cell lymphomas. Am J Clin Pathol. 2003; 120:866–873. [PubMed: 14671975]
16. Zola H. Markers of cell lineage, differentiation and activation. J Biol Regul Homeost Agents. 2000; 14:218–219. [PubMed: 11037056]
17. Mazzoni A, Segal DM. Controlling the Toll road to dendritic cell polarization. J Leukoc Biol. 2004; 75:721–730. [PubMed: 14726500]
18. Steinman RM. Some interfaces of dendritic cell biology. Apmis. 2003; 111:675–697. [PubMed: 12974772]
19. Connelly MA, Moulton RA, Smith AK, Lindsey DR, Sinha M, Wetsel RA, Jagannath C. Mycobacteria-primed macrophages and dendritic cells induce an up-regulation of complement C5a anaphylatoxin receptor (CD88) in CD3+ murine T cells. J Leukoc Biol. 2007; 81:212–220. [PubMed: 16997854]
20. Moulton RA, Mashruwala MA, Smith AK, Lindsey DR, Wetsel RA, Haviland DL, Hunter RL, Jagannath C. Complement C5a anaphylatoxin is an innate determinant of dendritic cell-induced Th1 immunity to Mycobacterium bovis BCG infection in mice. J Leukoc Biol. 2007; 82:956–967. [PubMed: 17675563]
21. Hwang ES, Szabo SJ, Schwartzberg PL, Glimcher LH. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science. 2005; 307:430–433. [PubMed: 15662016]
22. Lord GM, Rao RM, Choe H, Sullivan BM, Lichtman AH, Luscinskas FW, Glimcher LH. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. Blood. 2005; 106:3432–3439. [PubMed: 16014561]
23. Copenhaver RH, Sepulveda E, Armitage LY, Actor JK, Wanger A, Norris SJ, Hunter RL, Jagannath C. A mutant of Mycobacterium tuberculosis H37Rv that lacks expression of antigen 85A is attenuated in mice but retains vaccinating potential. Infect Immun. 2004; 72:7084–7095. [PubMed: 15557632]
24. Bennekov T, Dietrich J, Rosenkrands I, Stryhn A, Doherty TM, Andersen P. Alteration of epitope recognition pattern in Ag85B and ESAT-6 has a profound influence on vaccine-induced protection against Mycobacterium tuberculosis. Eur J Immunol. 2006; 36:3346–3355. [PubMed: 17109467]
25. Fratti RA, Vergne I, Chua J, Skidmore J, Deretic V. Regulators of membrane trafficking and Mycobacterium tuberculosis phagosome maturation block. Electrophoresis. 2000; 21:3378–3385. [PubMed: 11079558]
26. Russell DG. Mycobacterium and Leishmania: stowaways in the endosomal network. Trends Cell Biol. 1995; 5:125–128. [PubMed: 14732168]

27. Noss EH, Harding CV, Boom WH. Mycobacterium tuberculosis inhibits MHC class II antigen processing in murine bone marrow macrophages. *Cell Immunol.* 2000; 201:63–74. [PubMed: 10805975]
28. Hmama Z, Gabathuler R, Jefferies WA, de Jong G, Reiner NE. Attenuation of HLA-DR expression by mononuclear phagocytes infected with Mycobacterium tuberculosis is related to intracellular sequestration of immature class II heterodimers. *J Immunol.* 1998; 161:4882–4893. [PubMed: 9794422]
29. Banaiee N, Kincaid EZ, Buchwald U, Jacobs WR Jr, Ernst JD. Potent inhibition of macrophage responses to IFN-gamma by live virulent Mycobacterium tuberculosis is independent of mature mycobacterial lipoproteins but dependent on TLR2. *J Immunol.* 2006; 176:3019–3027. [PubMed: 16493060]
30. Gagliardi MC, Teloni R, Giannoni F, Pardini M, Sargentini V, Brunori L, Fattorini L, Nisini R. Mycobacterium bovis Bacillus Calmette-Guerin infects DC-SIGN- dendritic cell and causes the inhibition of IL-12 and the enhancement of IL-10 production. *J Leukoc Biol.* 2005; 78:106–113. [PubMed: 15845642]
31. Geijtenbeek TB, Van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, Appelmelk B, Van Kooyk Y. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med.* 2003; 197:7–17. [PubMed: 12515809]
32. Martino A, Sacchi A, Sanarico N, Spadaro F, Ramoni C, Ciaramella A, Pucillo LP, Colizzi V, Vendetti S. Dendritic cells derived from BCG-infected precursors induce Th2-like immune response. *J Leukoc Biol.* 2004; 76:827–834. [PubMed: 15240755]
33. Orme IM. New vaccines against tuberculosis. The status of current research. *Infect Dis Clin North Am.* 1999; 13:169–185. vii–viii. [PubMed: 10198798]
34. Hess J, Miko D, Catic A, Lehmensiek V, Russell DG, Kaufmann SH. Mycobacterium bovis Bacille Calmette-Guerin strains secreting listeriolysin of Listeria monocytogenes. *Proc Natl Acad Sci U S A.* 1998; 95:5299–5304. [PubMed: 9560270]
35. Dietrich J, Lundberg CV, Andersen P. TB vaccine strategies--what is needed to solve a complex problem? *Tuberculosis (Edinb).* 2006; 86:163–168. [PubMed: 16678489]
36. Dietrich J, Weldingh K, Andersen P. Prospects for a novel vaccine against tuberculosis. *Vet Microbiol.* 2006; 112:163–169. [PubMed: 16325357]
37. Jagannath C, Lindsey DR, Dhandayuthapani S, Xu Y, Hunter RL Jr, Eissa NT. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med.* 2009; 15:267–276. [PubMed: 19252503]
38. Pulendran B. Modulating TH1/TH2 responses with microbes, dendritic cells, and pathogen recognition receptors. *Immunol Res.* 2004; 29:187–196. [PubMed: 15181281]
39. Curtis JL. Cell-mediated adaptive immune defense of the lungs. *Proc Am Thorac Soc.* 2005; 2:412–416. [PubMed: 16322591]
40. Armitige LY, Jagannath C, Wanger AR, Norris SJ. Disruption of the genes encoding antigen 85A and antigen 85B of Mycobacterium tuberculosis H37Rv: effect on growth in culture and in macrophages. *Infect Immun.* 2000; 68:767–778. [PubMed: 10639445]
41. Meher AK, Bal NC, Chary KV, Arora A. Mycobacterium tuberculosis H37Rv ESAT-6-CFP-10 complex formation confers thermodynamic and biochemical stability. *Febs J.* 2006; 273:1445–1462. [PubMed: 16689931]
42. Dodge IL, Carr MW, Cernadas M, Brenner MB. IL-6 production by pulmonary dendritic cells impedes Th1 immune responses. *J Immunol.* 2003; 170:4457–4464. [PubMed: 12707321]
43. Phyu S, Sornes S, Mustafa T, Tadesse A, Jonsson R, Bjune G. Changes in T-lymphocyte subsets in lungs and spleens of mice with slowly progressive primary mycobacterium tuberculosis infection: involvement of unconventional T-cell subsets. *Scand J Immunol.* 1999; 50:137–144. [PubMed: 10447917]
44. Hernandez-Pando R, Orozco H, Sampieri A, Pavon L, Velasquillo C, Larriva-Sahd J, Alcocer JM, Madrid MV. Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. *Immunology.* 1996; 89:26–33. [PubMed: 8911136]

Figure 1. Mycobacteria induce a variable level of Th1-regulating T-bet transcription factor in the T cells of mice after intra-peritoneal infection

Four to 6 week old C57Bl/6 mice were infected i.p. with various mycobacterial strains at 10^6 CFU per mouse and either at weekly intervals or on day 14, splenic T cells were stained with fluorescent antibodies for intracellular expression of T-bet and IFN- γ and analyzed using flow cytometry and Cellquest software, and expressed as percent positive T cells. Spleens were also cultured on 7H11 agar at weekly intervals for bacterial counts. **A:** Mtb-H37Rv induces stronger expansion of T-bet⁺CD4 T cells. (percent positive T cells $\pm$ 3 mice per time point). **B:** T-bet expression correlates with IFN- γ expressing CD4 T cells (percent positive T cells $\pm$ 3 mice on day 14) **C:** Virulent *Mycobacterium tuberculosis* (Mtb-H37Rv) and *M. avium* grow more than BCG or *M. smegmatis* in the spleens (3 mice per time point per strain; $\pm$ SD).

Figure 2. The attenuated Δ fbpA mutant derived from Mtb H37Rv induces a stronger Th1 response after intraperitoneal infection of mice compared to BCG vaccine
Mice were infected with candidate vaccine strains as in Fig. 1 and splenic T cells were immunotyped for intracellular T-bet, surface expression of CXCR3 and intracellular IFN- γ (3 mice per time point per strain $\pm$ SD). Δ fbpA vaccine induces a stronger T-bet $^{+}$ (A), CXCR3 (B), and IFN- γ^{+} (C) (* $p < 0.01$ vs. BCG, t test) CD4 T cell response compared to BCG vaccine. The vaccines showed only a moderate difference in the growth within spleens (D).

Figure 3. Dendritic cells infected with *AfbpA* vaccine drive a better recall T-bet response in T cells *in vitro* compared to BCG vaccine

Bone marrow derived dendritic cells (DCs) were infected for 4 hr *in vitro* with *ΔfbpA* or various sub-strains of BCG as indicated (MOI=1). After washing, DCs were overlaid with splenic T cells (5×10⁶ T cells per 1×10⁶ DCs per well in triplicates for per mouse; 3 mice per strain) from mice infected i.p. 2 weeks earlier with *ΔfbpA* or various strains of BCG. After 72 hr of coculture, T cells were stained for surface CD3, T-bet, GATA-3 and FoxP3 and culture supernatants measured for soluble IFN-γ by ELISA. **A–B:** *ΔfbpA* infected DCs drive a stronger recall T-bet response which correlates with a stronger production of IFN-γ (* p < 0.009 vs. BCG induced T-bet or cytokine; *t* test). **C:** None of the DC cocultures induced significant GATA-3 or Fox-P3 responses in T cells *in vitro*.

Figure 4. DCs pulsed with excretory-secreted (ES) antigens, Ag85B, ESAT-6 and CFP-10 can drive the recall T-bet and IFN- γ responses *in vitro*

DCs were pulsed for 24 hr *in vitro* with Ag85B, ESAT-6 or CFP-10 antigens (1 μ g/10⁶ DCs in triplicate wells for per mouse; 3 mice per strain), washed and were cocultured with T cells from spleens of mice infected i.p. 2 weeks earlier with Δ fbpA, H37Rv, H37Ra or BCG Pasteur strains (10⁶ CFU per mouse, 3 mice per strain). After 72 hr, the cocultured T cells were stained for intracellular T-bet, IFN- γ and supernatants tested for IFN- γ by ELISA. **A:** Histograms illustrate that DCs pulsed with Ag85B, ESAT-6 and CFP-10 induce a better recall expansion of IFN- γ ⁺ CD4⁺ T cells in Δ fbpA immunized mice compared to T cells from Mtb H37Rv, H37Ra or BCG immunized mice. **B:** Cocultured T cells were stained for IFN- γ ⁺ T-bet⁺ CD4⁺ T cells and analyzed by flow cytometry using a BD FacsCalibur and

Cellquest software. Top two panels show that ES antigens drive a strong expansion Th1 expansion reflected by an increase in IFN- γ^+ CD4 $^+$ T cells and secretion of IFN- γ into medium. T cells from naïve mice activated by DCs showed < 5% IFN- γ^+ T cells. ESAT-6 and CFP-10 fail to drive Th1 expansion among T cells from BCG infected mice, while Ag85B alone was effective to expand BCG primed T cells. ES antigens also induced an expansion of T-bet in T cells but not in BCG primed T cells.

Figure 5. *ΔfbpA* mutant offers better protection than BCG vaccine against tuberculosis in mice which correlates with accumulation of CD4⁺CXCR3⁺IFN- γ ⁺ T cells

C57Bl/6 mice (4–6 weeks) were immunized s.c. with *ΔfbpA* or BCG strains at 10^6 CFU per mouse and challenged 4 weeks later via aerosol with 100 CFU of Mtb-Erdman strain per mouse. Mice were sacrificed and lung homogenates were plated on 7H11 agar for bacterial counts or lung-derived cells analyzed for T cell phenotype. **A:** Lung harvested T cells were stained for intracellular IFN- γ on day 28 post challenge. Histograms show IFN- γ ⁺CD4⁺T cells among naïve, naïve infected with Mtb-Erdman and vaccinated mice challenged with Mtb-Erdman. *ΔfbpA* vaccine induces a stronger Th1 response (Inset numbers show percent positive IFN- γ ⁺CD4⁺T cells; 3 mice per group). **B:** *ΔfbpA* vaccine protects mice better than BCG (4 mice for CFU per time point, per group; * < 0.007 vs. BCG or naïve group on week 4; ANOVA, 2 experiments; SEM). **C:** At weekly intervals after challenge mice were sacrificed and lung cell stained for surface phenotype (CD4 or CD8 and CXCR3) in

combination with intracellular IFN- γ using three colored antibodies followed by flow cytometric analysis. Increased protection by *AfbpA* vaccine correlates with a better accumulation of IFN- γ^+ CXCR3 $^+$ T cells in the lungs after aerosol challenge (numbers per lungs; 3 mice per group per time point; SD; one experiment shown of similar 2 experiments).