Diagnostic biomarkers and improved vaccination against mycobacterial infection

Muhammad Jubayer Rahman

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"It is a good morning exercise for a research scientist to discard a pet hypothesis every day before breakfast - it keeps him/her young."

Konrad Lorenz (1903 - 1989)
1973 Nobel Laureate in Medicine
Summary

Tuberculosis (TB) remains one of the world’s most serious infectious diseases. It is estimated that a third of the world’s population is latently infected and 8 million new cases are recorded each year. Although BCG vaccination triggers protective immune responses in the neonates, it confers protection against only certain forms of childhood TB. Protection mediated by BCG, against pulmonary TB, is controversial as reported with variable efficacy ranging from 0-80%. In addition to the problems associated with the BCG vaccine, diagnosis of TB cannot be performed readily with the available tools. At present, an effective control of TB is highly dependent on the development of a new TB-vaccine as well as proper identification and treatment of individuals with active disease. Therefore, we particularly focused on identification of biomarker(s) of infection and the development of better vaccines, with special emphasis on the immune responses in the respiratory tract.

In the first study, we aimed to identify immune biomarker(s) of infection for better diagnosis of TB. Mice were infected with BCG administered i.n. or i.v., and the bacterial burden in the lungs, spleen and liver was examined. We measured IL-12, IFN-γ, TNF, soluble TNF receptors (sTNFR) and mycobacteria-specific antibodies in the broncho-alveolar lavage (BAL) and in serum in order to find immune correlates of infection. Results showed that sTNFR and mycobacteria-specific antibodies in BAL, but not in serum, might be useful in distinguishing active from latent infection or exposure to mycobacterial antigens.

In the second study, we investigated whether we could improve the currently used BCG vaccine. For this purpose, we tested a combination of neonatal vaccination protocol using BCG and posterior boosting with the protein heparin-binding hemagglutinin adhesion (HBHA). It has been described that immunization with native (n) HBHA but not recombinant (r) HBHA conferred protection against M. tuberculosis challenge in mice.
This protection was comparable to that afforded by the BCG vaccine. In order to improve the protective efficacy of the nHBHA vaccine we followed heterologous prime-boost strategy, comprising BCG vaccination at the neonatal age, followed by nHBHA boosting at the infant and adult ages. We also examined whether the rHBHA protein could boost BCG-mediated protective immunity. Cellular immune responses and protection as measured by control of bacterial growth in the lungs of the treated animals were followed. Our results showed an improved effect of BCG-priming on HBHA-immunization. The BCG/HBHA immunization protocol was more effective in induction of HBHA-specific immune responses, as well as in protection than when the animals received only BCG or HBHA alone. Importantly, our study revealed that nHBHA does not require co-administration with adjuvant provided that mice were primed with live BCG before boosting.

Finally, we hypothesized that in utero sensitization of the fetal immune system with nHBHA may improve nHBHA-specific immune responses after birth. The pregnant mother was immunized with nHBHA 1 week before delivery. After birth, the offspring received two doses (week 1 and week 4) of nHBHA formulated with cholera toxin. We examined HBHA-specific recall responses and protection after challenge with a high dose of BCG. We found that immune responses were improved by priming the pregnant mother, and that this also provided better protection than when the offspring received only BCG or HBHA neonatal vaccinations.
LIST OF PAPERS

This thesis is based on the following original papers (manuscripts), which will be referred to by their Roman numerals:

I. Arko-Mensah J$^*$, Rahman MJ$^{*}$, M. Singh$^2$, Fernández C$^1$. Immunodiagnosis of mycobacterial infection: Increased levels of immunological markers in the respiratory tract but not in serum correlate with active pulmonary infection in mice (Submitted).

$^*$Authors contributed equally to this work

II. Rahman MJ$^1$, Locht C$^2$ and Fernández C$^1$. Improvement of HBHA vaccination by BCG priming in a neonatal mouse model (Manuscript).
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFB</td>
<td>Acid fast bacilli</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BAL</td>
<td>Broncho-alveolar lavage</td>
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<tr>
<td>BALT</td>
<td>Bronchus-associated lymphoid tissue</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<td>BCG-BMM</td>
<td>BCG infected bone-marrow derived macrophages</td>
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<tr>
<td>CFP-10</td>
<td>10-kDa culture filtrate protein</td>
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<tr>
<td>CR</td>
<td>Complement receptor</td>
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<tr>
<td>CT</td>
<td>Cholera toxin</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ESAT-6</td>
<td>6-kDa early secretory antigenic target</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>i.n.</td>
<td>Intranasal</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>IL-12</td>
<td>Interleukin 12</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>LALT</td>
<td>Larynx-associated lymphoid tissue</td>
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<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
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<tr>
<td>MALT</td>
<td>Mucosa associated lymphoid tissue</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>NALT</td>
<td>Nose associated lymphoid tissue</td>
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<tr>
<td>nHBHA</td>
<td>Heparin-binding hemagglutinin native</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PknG</td>
<td>protein kinase G</td>
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<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
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<tr>
<td>rBCG</td>
<td>Recombinant Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>rHBHA</td>
<td>Heparin-binding hemagglutinin recombinant</td>
</tr>
<tr>
<td>sTNFR</td>
<td>Soluble tumor necrosis factor receptor</td>
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<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TLRs</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>XDR</td>
<td>Extensively drug resistant</td>
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INTRODUCTION

TUBERCULOSIS (TB) IS A GLOBAL BURDEN

‘TB anywhere is TB everywhere’-remains a serious global burden due to the contagious nature of the infection. The World Health Organization (1) recorded 1.6 million deaths resulted from TB in 2005. The highest number of deaths and mortality per capita are in Africa, where estimated incidences are nearly 350 cases per 100 000 individuals. The most important weapon to keep the disease under control is a vaccine. The BCG (live attenuated *Mycobacterium bovis* BCG) was discovered in early 1900s. Unfortunately, very poor efficacy of the BCG vaccine has been reviewed over time. BCG has been shown to protect against disseminated TB in children but not against pulmonary TB. With the current context of human immunodeficiency virus (HIV) prevalence and the continued increased number of virulent strains, the TB epidemic is exacerbated.

TUBERCULOSIS IS AN INFECTIOUS DISEASE

*Mycobacterium tuberculosis* causes TB primarily in the lungs. The bacteria are transmitted from person to person through the air by droplet nuclei, usually 1-5 µm in diameter, having only 1-5 tubercle bacilli. Organisms in the droplet nuclei survive for long periods and remain infective. Bacilli reach the alveoli within the lungs and multiply. Air droplets are formed by sneezing, coughing or speaking, as well as in the hospital or laboratory during sputum collection, bronchoscopy or tissue processing.

*M. tuberculosis*

*M. tuberculosis* was first described on March 24, 1882 by Robert Koch, who subsequently received the Nobel Prize in 1905. The *M. tuberculosis* belongs to the genus
*Mycobacterium*, which comprises a number of gram-positive, acid-fast, rod-shaped aerobic bacteria and is the only member of the family *Mycobacteriaceae* within the order *Actinomycetales*. Mycobacteria are classified into two categories, the fast-growing and the slow-growing strains. The special characteristics of the cell wall of mycobacteria are the peptidoglycan layer, free lipids and waxes that provide hydrophobic characters, acid fast properties and intracellular survival (2). Most mycobacteria live in habitats such as water or soil. A few are intracellular pathogens in animals and humans causing serious diseases include TB and leprosy. *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* are members of the tuberculosis species complex, and all cause a disease known as TB. Usually *M. tuberculosis* is pathogenic for humans while *M. bovis* is pathogenic for animals.

**Infection and outcomes**

Infection is characterized by the presence of bacilli in the host, without any clinical symptoms. As shown in Fig. 1, after inhalation, mycobacteria are taken up by the alveolar macrophages and may be killed immediately. Alternatively, bacteria may start replication in the macrophages, and afterwards may localize to the other parts of lungs or regional lymph nodes (3-5). Interactions between the pathogen and the host terminate by killing of the bacilli or result in lesions in the lungs. The lesions can be detected by X-ray but in some cases lesions can not be seen in chest radiographs. The clinical manifestations of TB after infection in the lungs are known as pulmonary TB. Bacilli continue their replication and form tubercle which can undergo so-called caseation necrosis showing semi-solid or "cheesy" consistency. A significant proportion of the patients at this caseation stage can be diagnosed by chest radiograph. At this point the patients are highly
contagious. However, failure in early detection of infection and therefore lack of treatment in a minority of cases lead disease progression or hematogenous dissemination to other parts of the body such as, bones and joints (6), kidneys and genital tract (7) or the central nervous system resulting in TB meningitis (8).

**Fig. 1: Chronological events after inhalation of *M. tuberculosis*.**


**M. tuberculosis survival program**

Under natural circumstances, alveolar macrophages take up mycobacteria from the alveolar mucosa and enclose them into the phagosomes. Subsequently, activation of
macrophages leads to the fusion of the phagosomes with lysosomes, which results in killing of bacteria. *M. tuberculosis* escapes this destruction by altering the maturation of phagolysosome fusion in some way, and extends its length of survival in the host, which is called a state of clinical latency. Bacteria set up their dormant life by the rapid upregulation of ‘dormancy regulon’ -- contains at least 48 genes. At the dormant state, bacteria survive under low oxygen level and become drug-resistant. Under such a situation bacteria shut off protein synthesis and stop replication in the host.

Three possible mechanisms have been suggested to be involved for the intracellular survival of bacteria (9-12). First, Voskuil et al demonstrated that hypoxia and low nontoxic levels of nitric oxide (NO) induced the expression of a 48-gene regulon, which limits the *M. tuberculosis* replication by inhibiting their metabolic process. Second, Walburger et al proposed that the phagosome-lysosome fusion can be interrupted by the active production of protein kinase G (PknG), which is one of several eukaryotic-like serine/threonine protein kinases found in *M. tuberculosis* and related organisms. PknG blocks the lysosomal delivery and ensures a friendly environment to the bacterium (Fig. 2).

**Fig. 2: PknG affects the intracellular traffic of *M. tuberculosis* in macrophages.**

*Nat Med.* 2007;13:282-4
Third, Rengarajan et al proposed that phosphate transport proteins may be crucial for the survival of bacteria. Probably, lung macrophages have limited levels of phosphates therefore, transport of inorganic phosphate by phosphate transport proteins is critical for the bacterial growth in macrophages.

**IMMUNE RESPONSES TO M. tuberculosis**

As other microorganisms, an infection with *M. tuberculosis* stimulates both arms of the immune system, innate and adaptive. After intracellular infection, the protective immune response to *M. tuberculosis* is mainly cell mediated. Collaborations between lymphocytes and macrophages are basically important for the successful eradication of *M. tuberculosis*.

**Innate immunity**

It is generally believed that alveolar macrophages are the primary cells that initially take up *M. tuberculosis*. Ingestion of *M. tuberculosis* by macrophages signals NF-κB mediated expression of several genes that code for cytokines and chemokines. Chemokines signal leukocytes to travel to the site of infection and this process further activates macrophages.

Many receptors have been identified for endocytosis of *M. tuberculosis* by macrophages and dendritic cells (DCs) (reviewed in ref. 13). Complement receptor 1 (CR1), CR3, CR4 (14-16) recognize and bind to the cleavage products of complement component C3 deposited on the surface of *M. tuberculosis* (opsonization) following complement activation. Mannose receptors bind to the nonopsonized *M. tuberculosis* (16). Outcomes of the interactions depend on the type of receptors. Fc receptors increase the production
of reactive oxygen intermediates that favours phagosome-lysosome fusion (17) and CR3 prevents the respiratory burst and inhibits the maturation of phagosomes (18).

Toll-like receptors (TLRs) are pattern recognition receptors that play an important role in the recognition of mycobacterial components. Thirteen TLRs (TLR1 to TLR13) have been identified in humans and mice. TRL2 recognizes the 19-kDa lipoprotein (19) and the major mycobacterial cell wall component lipoarabinomann (LAM) (20). In animal models, defective functions of TLR2 and TRL4 have been associated with susceptibility, at an early stage of infection (21). TLR-9 has also been proposed to be involved in the recognition of CpG-motifs in mycobacteria (22). Usually, stimulation of TLRs by mycobacterial components triggers a proinflammatory response by the production of IL-12 and inducible nitric oxide synthase (iNOS) (23), which can promote mycobacterial killing (24). TLR signalling is also important in the generation of cellular populations such as differentiation of monocytes into macrophages and DCs (25).

**Humoral immunity**

Mycobacterial infection is basically intracellular and therefore, the humoral immune response is considered to be non-protective. However, the benefits from antibodies have been explored in the control of intracellular infections especially chlamydial respiratory infection, and chronic infections induced by actinomycetes (26, 27). While the role of antibody in protection against TB is uncertain, several mechanisms of action mediated by antibody are considered, which could modify the outcome of infection. Antibody may interfere with the adhesion of mycobacteria (28) or bind and neutralize the function of mycobacterial products (29). Recent studies have also demonstrated the protective function of antibody in experimental mouse models of TB (30-33). Recently,
mycobacterium-specific antibodies have been found to be capable of enhancing cell-mediated immune responses to mycobacteria (34). Antimycobacterial antibodies increased the proliferation of CD4$^+$ and CD8$^+$ T cells after in vitro stimulation with BCG in the presence of antigen presenting cells (APC) (34). Disregarding the role of antibody in protection, specific antibodies to mycobacterial components can be valuable in diagnosis of TB (35).

**Cellular immunity**

*CD4$^+$ T cells*

CD4$^+$ T cells are central in driving cellular immunity to TB. Cytokines produced by CD4$^+$ T cells are of critical importance for the outcome of immune responses, for example Th1 type or Th2 type. Th1 cells secreting interleukin-2 (IL-2) and interferon-γ (IFN-γ) are associated with cell-mediated immunity, whereas Th2 cells that produce typically IL-4, IL-5, IL-10 and IL-13, are responsible for humoral immunity. After phagocytosis of mycobacteria, antigens are processed and presented to the CD4$^+$ T cells by macrophages or DCs in association with class II MHC molecules, encoded by the major histocompatibility gene complex (MHC). Activated CD4$^+$ T cells express CD40L on the surface that interacts with its receptor, CD40, on antigen presenting cells. The activated CD4$^+$ T cells secrete cytokines such as IFN-γ and tumor necrosis factor (TNF), which in turn activate macrophages to kill the ingested bacteria (36). The importance of CD4$^+$ T cells in the control of TB has been demonstrated in mice and humans. Studies with CD4$^+$ T cell-deficient mice showed a defect in IFN-γ production, which led to the increase of bacterial numbers in various organs (37). In a murine model of chronic persistent *M. tuberculosis* infection, depletion of CD4$^+$ T cells resulted in a steady
increase of bacterial growth in all organs (38). In humans, the importance of CD4+ T cells is clear because humans infected with the HIV are more susceptible to develop TB (39).

**CD8**\(^+\) T cells

CD8\(^+\) T cells are also important in protection against TB although to a lesser extent than CD4\(^+\) T cells. Antigens are processed and presented to CD8\(^+\) T cells in association with class I MHC molecules. Mice, lacking CD8\(^+\) T cells or MHC class I molecules are more susceptible to infection (40). Although CD4\(^+\) T cells are the major source for IFN-\(\gamma\) production, CD8\(^+\) T cells are also able to produce IFN-\(\gamma\) (41). CD8\(^+\) T cells are efficient in lysing infected cells and reducing bacterial load (42). The effector mechanisms mediated by CD8\(^+\) T cells are largely dependent on the production of perforin/granulysin (43).

Despite the fact that CD8\(^+\) T cells are important in protection, it is still unclear how the presentation of processed antigens to CD8\(^+\) T cells is done. As shown in Fig. 4, three
Possible mechanisms are proposed to be involved: first, the classical pathway of antigen processing and presentation by macrophages; second, *M. tuberculosis* infected cells may produce exosomes containing mycobacterial antigens, which can be presented by bystander APC for MHC class I cross processing; third, apoptosis of *M. tuberculosis* infected cells releases apoptotic vesicles containing mycobacterial antigens, which are taken up by bystander APC. The mechanisms are likely to be operated in the presence of the specific type of APC.

![Diagram](image)

**Fig. 4: Cross processing of *M. tuberculosis* (MTB) antigens for CD8+ T cells.** J. Clin. Invest. 2007;117:2092–2094

### Gamma/delta (γδ) T cells

γδ T cells are large granular lymphocytes, which comprise 10% of circulating T-lymphocytes in healthy individuals (44). γδ T cells display T cell receptor 1 (TCR-1) receptors whereas αβ T cells (CD4+ and CD8+ T cells) display TCR-2 receptors. The γδ TCRs and αβ TCRs are similar in both sequence and structure. However, γδ T cells may
directly recognize in the form of intact proteins (45) or non-protein phosphoantigens (44, 46), and αβ T cells recognize small peptides together with MHC molecules. The exact role of γδ T cells in protection against TB remains to be elucidated. It has been demonstrated that γδ T cells may produce IFN-γ during early infection, and that they can function as APC and give proliferation signals to αβ T cells (47). It has also been suggested that γδ T cells probably limit the migration of inflammatory cells to the site of infection that may cause tissue damage (48, 49). Mice infected with M. tuberculosis/BCG intranasally showed a quick localization of γδ T cells in the lungs (50, 51). It has been shown that upon in vitro stimulation with BCG-infected macrophages, γδ T cells showed cytotoxic activity (52), and blocking of γδ T cells’ functions reduced cytotoxic activity and IFN-γ production by lung CD8+ T cells (52). Thus γδ T cells have been proposed to be important for the control of infection in the period between the innate and adaptive immune responses (52). γδ T cells can also produce IL-17 during early in the infection that might help in the movement of immune cells to the site of the infection (53).

**Host’s protective kits**

Experiments in animal models showed that immune responses against TB are Th1 dominated (54-56). Macrophages ingest bacteria and produce the IL-12 cytokine, which is important for the induction and generation of Th1 immunity. Fig. 3 displays the sequence of events driven by the host after phagocytosis of bacilli. Activated CD4+ and CD8+ T-cells play a crucial role by producing IFN-γ and TNF, which together drive the immune system towards a Th1-type of immune response. In humans, defective in the receptors for IFN-γ and IL-12 reiterate the importance of these cytokines in protection (57, 58). Mice deficient in the IFN-γ-gene expression are very susceptible to fatal TB (59,
TNF is also an important cytokine in the control of TB. In mice, it was demonstrated that in absence of TNF, effective granuloma formation is impeded and bacterial growth is rapidly increased, which reduced the survival time of the mice (61, 62). However, high levels of TNF are detrimental for the host (63) and may cause severe pathology. Therefore, the persistence and the levels of TNF determine whether TNF activity is helpful or harmful. TNF functions by interacting with one of two receptors: TNFR1 (55 kDa) or TNFR2 (75 kDa) expressed on almost all nucleated cells. The extracellular domain of both receptors can be cleaved by metalloproteases and then the soluble form (sTNFR) can bind to TNF and neutralize TNF-mediated activities. It has been described that complete neutralization of TNF activity by sTNFR prevents the induction of cell-mediated immunity in mice and therefore succumb to BCG infection (64).

Both TNF and IFN-γ-signaling activate macrophages and activation leads to the synthesis of iNOS, which regulates the production of NO. NO is an important mediator in the killing of intracellular \textit{M. tuberculosis}.

\textbf{Mucosal immunity in the lung}

The immune response in the respiratory tract has been studied during the last decade, with the intention to induce high level of protection against TB. The hypothesis is that, since TB is primarily a lung disease, the immune responses in the lungs might play a significant role in protection. Mucous membrane lines up the respiratory tract and initially prevents invasion of the pathogens. The defensive mechanism of the mucosal lining predominantly involves mucosa associated lymphoid tissue (MALT), where pathogens are sampled, processed and presented to the immune cells. Lymphoid tissues in the respiratory tract can be divided into three groups (65): nose associated lymphoid
tissue (NALT), larynx-associated lymphoid tissue (LALT), and bronchus-associated lymphoid tissue (BALT). Luminal antigens are taken up by a specific type of cells called microfold (M cells), that are present in the epithelium overlying NALT. As shown in Fig. 5, DCs process and present antigens to the T lymphocytes. Activated T cells help B cells to produce antigen-specific antibodies especially of the IgA isotype. Antigen-specific T cells and IgA-producing B cells migrate to the effector sites via thoracic duct and blood circulation. After proteolysis of the polymeric Ig receptor, the dimeric IgA binds with a parts of the polymeric Ig receptor and form secretory-IgA. Secretory-IgA antibodies are then released into the mucosal secretions. Secretory IgA binds to the pathogens and block their replication and colonization (66, 67).

Fig-5: The mucosal immune system. Nature Rev. Imm 2004;4:699-710
VACCINES FOR TB

The BCG vaccine

The BCG (bacille Calmette-Guérin) vaccine was developed in the early 1900s by Albert Calmette and Camille Guérin. BCG is made of a live, attenuated strain of *M. bovis*. BCG was first given to a newborn in 1921, whose mother had died of TB and who was looked after by a grandmother, was also suffering from the disease. This vaccinated individual remained free of TB throughout his life (68). In 1928 the League of Nations suggested widespread vaccination with BCG. In the first half of the 20th Century, BCG vaccines were prepared and preserved under varying conditions by different laboratories and therefore both genotypical and phenotypical changes occurred. As shown in Fig. 6, Japanese and Danish strains were evolved from the original Pasteur BCG in 1921 and Glaxo strain from the Danish in 1331.

Fig. 6: Documented evolution of the four *M. bovis* BCG strains. DR indicates the deleted regions and the number in the arrows is the in vitro passages of bacteria which were occurred during the evolution of BCG Pasteur and daughter strains since 1921 to the freeze dried of BCG in 60th. Ann Clin Microbiol Antimicrob. 2004,3:10
The BCG vaccine is effective against disseminated and meningeal TB in infants and young children. However, the protective efficacy of BCG vaccination against pulmonary TB is highly questioned. Results from field studies showed unsatisfactory efficacy varying between 0% and 80% (69). There could be many reasons for this such as methodological differences among the clinical trials, genetic differences within and among host populations, endogenous reactivation of persistent infection, exogenous re-infection, and effects of environmental mycobacteria on the host immune response to BCG. Furthermore, there have been reports about the influence of helminth infections that divert Th1 immunity towards Th2, resulting in reduced protection (70). Today, the BCG vaccine is not considered as a secured vaccine against pulmonary TB.

**New TB vaccines**

*Live attenuated vaccines:* Several strategies have been taken into consideration in order to improve the BCG vaccine. Recent studies with comparative genomic analysis identified that some genes were missing in the BCG (71, 72). Therefore it was presumed that BCG was not a complete vaccine against *M. tuberculosis*. Accordingly, it was hypothesized that the introduction of the deleted genes to the BCG vaccine could improve the vaccination. Subsequently, vaccination of mice with a recombinant BCG (rBCG) reconstituted with the missing genes, 6-kiloDalton early secretory antigenic target (kDa ESAT-6) and CFP-10 (10-kDa culture filtrate protein) showed better protection than in the control animals immunized with BCG alone (73). rBCG over-expressing mycobacterial antigen, Ag85B, was also reported to possess a better efficacy than wild-type BCG, in guinea pigs (74), as well as, in macaques (75). Although the modified live BCG vaccines have been found to be attractive, the major concern for the
clinical use of this kind of vaccine is safety. Introduction of missing genes to the BCG vaccine might include the possibility of reactivation of virulence.

**Subunit vaccines (single or fusion proteins):** Subunit vaccines contain purified antigens rather than whole organisms. The development of subunit vaccines has been accelerated due to the sequencing of the *M. tuberculosis* genome (76). This led to the identification of many protective antigens including antigen 85 complex proteins (Ag85, 30-32 kDa), ESAT-6, CFP-10 and TB 10.4 (belong to the *esat-6* gene family), 19 and 38 kDa lipoproteins, heparin-binding hemagglutinin adhesion (HBHA) etc. However, almost all of the single subunit proteins with adjuvants used for vaccination showed a certain level of protection that is even lower than the BCG vaccination. To our knowledge, only the HBHA has been proposed to be a potent vaccine candidate against TB, based on the observation that vaccination with HBHA, in mice, could reduce bacterial counts as efficiently as vaccination with wild-type BCG (77). Another important characteristic of the HBHA protein is that lymphocytes isolated from the infected healthy subjects produce more IFN-γ in response to HBHA than patients with active disease (78). Recently, it has been described that vaccination with fusion proteins is highly protective against *M. tuberculosis* infection in animals. The fusion protein Ag85B-ESAT-6 has been tested in mice (79) and found a level of protection that was better than that with either Ag85B or ESAT-6. Exchange of ESAT-6 with TB10.4 in the Ag85B-ESAT-6 construct has been found to induce a level of protection comparable to that provided by BCG vaccination (80).

However, a major challenge in developing a subunit vaccine is the requirement of a safe and effective adjuvant.
**DNA vaccines:** DNA vaccine is injected into muscle cells usually with a "gene gun" that uses compressed gas to blow the DNA. Some muscle cells express the inoculated DNA and are able to stimulate the immune system. Over the last few years, DNA vaccinations have become a strategic way to deliver mycobacterial antigens that stimulate the cellular immune system. In mice, DNA-vaccines expressing several immunodominant antigens have been found to be protective against *M. tuberculosis* (81-85). DNA vaccines stimulate both CD4⁺ and CD8⁺ T cells (reviewed in ref. 88) and induce mixed Th1/Th2 type of immune responses (87). The efficacy of DNA vaccines can be further improved by co-delivery of multiple DNA plasmids or chimeric DNA vaccines (88, 89). Although DNA vaccines are probably a good alternative, they have been disappointing so far when tested in larger animals like guinea pigs and non-human primates (90).

With the knowledge from different types of vaccines and the context of current global BCG vaccination, it has increasingly been realized that a combination of BCG or rBCG, DNA vaccines, and subunit proteins may provide greater levels of protection. Heterologous prime-boost strategies involving BCG as a priming vaccine and either DNA-based vaccine or subunit protein as a booster have been found very attractive (91, 92).

**DIAGNOSIS OF TB**

A timely and accurate diagnosis of TB is essential for better treatment, prevention and control of the disease. Currently there are a number of methods used to diagnose mycobacterial infection but none of them are optimal. Identification of *M. tuberculosis* in conventional laboratory tests is difficult.
Conventional methods

**Microscopy and culture**

Diagnosis of active pulmonary TB by microscopic or culture techniques is based on the detection of bacilli in specimens from the respiratory tract -- the most common type is sputum. Acid fast bacilli (AFB) smear microscopy and culture on Löwenstein-Jensen medium are the “gold standard” methods for the diagnosis of active TB. AFB smear microscopy is most commonly used to identify highly contagious patients. AFB smear microscopy is a quick and inexpensive technique. However, an estimation of 5000-10,000 organisms per ml of sputum is needed to visualize the bacilli under microscope. This situation makes mycobacteriologic identification by smear difficult, especially in children who rarely produce adequate sputum. Culture based methods are more sensitive and can detect as few as 10 bacteria/ml of specimen. Culture techniques are also used to monitor drug sensitivity and resistance pattern. A real disadvantage of the culture method is that it takes 4-6 weeks for confirmation. However, a positive culture for *M. tuberculosis* confirms the diagnosis of active disease.

**Tuberculin skin test**

“Koch’s Old Tuberculin” is a heat-inactivated filtrate from cultures of *M. tuberculosis* that was first proposed for the treatment of TB. However, TB patients who received tuberculin suffered from systemic reactions, including fever, muscle aches, and abdominal discomfort with nausea and vomiting. These observations were the basis of using tuberculin, also known as purified protein derivative (PPD), for the diagnosis of TB. Tuberculin skin test was described by Mantoux, and his method became widespread. A positive Mantoux tuberculin skin test appears between 24 to 72 hours after application.
of tuberculin, is used to detect infection. But PPD skin test is not reliable to detect infection, since people exposed or vaccinated with BCG also respond. In addition, in a positive skin test, major confounding factors are exposure with mycobacteria other than *M. tuberculosis*.

**Radiology**

Radiographic screening is another way of monitoring the progression of TB disease. Although radiography is often useful to detect advanced stages of disease, it is also an initial screening method used together with PPD skin test. In active pulmonary TB, chest radiograph views cavities often in the upper lung. Chest radiographs may be suggestive of, but are never specific to TB.

**Newer methods**

*Nucleic acid amplification methods*

Polymerase Chain Reaction (PCR) is an efficient technique that allows rapid amplification of mycobacterial species. Among many genomic targets for diagnostic PCR there are, the insertion element IS6110 (present as multiple copies, 4-20, in *M. tuberculosis*), the 65 kDa heat-shock protein gene, the 126 kDa fusion protein gene, and the gene encoding the β-subunit of RNA polymerase. All of them have been found to provide accurate determination of the presence of *M. tuberculosis*. However, implementation of the PCR technique remains technically challenging because it requires strong laboratory capacities and good quality control procedures that are frequently not available in affected countries.
**Serological diagnosis**

In the case of patients who do not produce adequate sputum or sputum smears are negative or patients with extrapulmonary TB, serological diagnosis is an attractive option. Serological diagnosis is based on the detection of TB-specific antibodies in the sera of patients. There have been many studies showing TB-specific antibody responses in patients suffering from active disease (93, 94). However, the general scenario of serological diagnosis is very poor in terms of sensitivity and specificity (95, 96). Cross-reactions also occur in healthy individuals exposed to environmental mycobacteria or BCG vaccination, and therefore difficulties arise in the interpretation of the results.

A possible explanation for the low success of serological studies may be that TB is primarily a disease in the lungs and therefore measurements of specific antibodies locally in the respiratory tract would be more accurate as markers of lung infection than serological studies that would reflect a systemic response.

**Cell mediated immunity and diagnosis**

The only widely used method based on cell-mediated immune response is the PPD. However, a major drawback of PPD is that it gives false positive results in people exposed to the environmental mycobacteria or vaccinated with BCG. Thus, efforts have been made to identify antigens that are not cross-reactive with BCG. Antigens, such as ESAT-6 and CFP-10 have been proposed for TB diagnosis (97, 98). This diagnosis method is based on the assumption that T cells from sensitized individuals produce IFN-γ when they re-encounter antigens of *M. tuberculosis* (99). One of the recently developed assays is QuantiFERON-TB Gold, which is the advanced version of QuantiFERON-TB. A positive test indicates that the *M. tuberculosis* infection is likely and a negative test
indicates infection is unlikely. However, one of the shortcomings of using ESAT-6 and CFP-10 is that orthologues of these antigens in other mycobacteria (M. smegmatis) might influence the test results (100). Also, QuantiFERON-TB Gold test alone is not sufficient to differentiate between active and latent infection because QuantiFERON-TB Gold test can be positive for both active and latent cases. Moreover, some patients with advanced TB have been found to be not responsive to the antigenic stimulation, which also limits the use of QuantiFERON-TB Gold test.

**Immune biomarker(s) of infection**

Biomarkers are biological features or substances that can be used as indicators of infection. Recently, it has been described that cytokine levels in broncho-alveolar lavage (BAL) could be good markers of infection. TNF, IFN-γ and IL-2 levels in BAL of patients with smear-negative pulmonary TB are increased significantly compared to the other pulmonary disease (101). Moreover, other candidate biomarkers for TB infection such as lactoferrin, CD64, and the Rab33A (member of the Ras-associated GTPase) have recently been suggested (102). Expression of CD64 on monocytes has been found higher in TB patients than M. tuberculosis-infected healthy subjects. Similarly, a higher gene expression of lactoferrin and Rab33A was reported in TB patients. However, these molecules whether or not specific for TB are yet to be defined.
PRESENT STUDY

Aims

TB is primarily a lung disease and the respiratory tract is the natural route of \textit{M. tuberculosis} infection. In order to improve diagnostic methods and protection against TB, in the present study we have aimed at investigating the immune responses at the entry port of mycobacteria and downstream along the respiratory tract.

Our specific objectives were:

- To investigate the induction of immune responses in mice to mycobacterial infection and to identify immunological parameters or biomarkers associated with infection (Paper I, Submitted)
- To increase the protective efficacy of the HBHA vaccine based on the induction of HBHA-specific immune responses in the lungs (Paper II, Manuscript).
- To examine the effect of maternal immunization with HBHA on postnatal immunity in the offspring (Preliminary results)

Materials and Methods

The materials and methods for these studies are described in the separate papers.
Results and discussion

*Identification of immune biomarker(s) of mycobacterial infection for better diagnosis of TB (Paper I)*

Diagnosis of TB based on the immune responses to mycobacterial antigens (103-107) has been extensively studied over the past years. However, methods based on the detection of marker(s) in serum are limited by low sensitivity and specificity. Diagnosis based on the local but not peripheral immune responses might be highly sensitive and specific. In this regard, we modeled natural infection in mice by using BCG administered intranasally (i.n.). We examined levels of several immune parameters both in the lungs and in serum and analysed immune correlates of active infection.

The major findings of this study are:

a) Active mycobacterial infection, but not exposure to non-replicating mycobacteria resulted in elevation of IL-12, IFN-γ and sTNFR in BAL independently of the route of infection. Serum levels were not equally conclusive.

b) Reactivation of controlled BCG infection resulted in increased bacteria growth in the lungs and in increased levels of sTNFR in BAL.

c) Active infection, but not exposure to non-replicating mycobacteria resulted in production of antigen-specific IgG or IgA in BAL.

In recent times, attention has been focused on the detection of immune parameters locally in the lungs which might be of good correlates of infection. Expression of IL-12 and IFN-γ mRNA in the BAL (108) and elevated levels of cytokines such as IFN-γ, IL-12, IL-1β, IL-8 and TNF produced by broncho-alveolar cells were described to be associated with
active pulmonary TB (109). In addition to the cytokine levels, cytokine receptors, such as sTNFR, have been reported to be sensitive marker of infection. Increased shedding of sTNFR1 into serum is predominantly associated with TB rather than HIV infection. The levels of sTNFR1 are reduced even after anti-tuberculosis treatment (110). Transgenic mice expressing high serum levels of sTNFR1 exhibited reduced bactericidal activity, undifferentiated granulomas and succumbed to BCG infection (64). In the present study, we, however, measured cytokines TNF, IL-12 and IFN-γ or sTNFR levels in both BAL and in serum after active infection as well as treatment with heat-killed BCG or BCG-lysate and compared with the bacterial burden in the lungs. In contrast to the previous reports, we found that increased serum levels of sTNFR were not restricted to active infection but induced after treatment of mice with killed-BCG. However, increased levels of sTNFR, IL-12 and IFN-γ in BAL correlated better with the bacteria counts. This suggests that levels of cytokines or soluble TNF receptors in BAL, but not in serum, are more proper to detect infection in the lungs. We also confirmed our findings in the mouse model of active infection induced by the systemic route. After intravenous route of infection with live BCG, we observed a similar correlation between the bacterial burden and the sTNFR levels in the lungs. The undetectable TNF might indicate that TNF and sTNFR levels are inversely correlated.

sTNFR levels are not only increased in mycobacterial infections, but also in many other inflammatory diseases (111-113). Therefore, we reasoned that detection of BCG-specific antibodies, together with cytokines or soluble cytokine receptors, could be used as biomarkers for distinguishing an active infection from exposure to killed bacteria. We detected higher levels of antibodies in both BAL and serum at week-9 after infection,
compared to the early time points. We detected antigen-specific IgA only in the BAL but not in the serum. The reason for this could be that our maximum observation time was week-9 after infection, which might limit the detection of systemic IgA. In contrast to the results with live BCG, treatment with killed-BCG or BCG-lysate induced only detectable levels of antibodies in serum. Live BCG is a very strong stimulant and therefore, probably, continuous stimulation of immune cells by the secreted antigens resulted in significant production of antibodies over time. Specific antibodies in BAL but not in serum probably reflect better with the results of sTNFR in BAL.

In conclusion, our observation suggested a new strategy of detecting mycobacterial infection based on the determination of more than one immune marker in the lung microenvironment.

**Improved immunogenicity and protective efficacy of HBHA vaccine (Paper II)**

BCG vaccine is the most widely used immunization in the world (144). The failure of BCG vaccine in life-long protection against TB is one of the important factors for the current threat of TB. This suggests that a new vaccine against TB is urgently needed in the control of the disease. In recent times, there have been significant advances in the development of a new TB vaccine particularly in improving BCG vaccination. The best model for the improvement of BCG vaccination is probably the heterologous prime-boost strategy (115). In this protocol, priming with BCG and boosting with either DNA-based vaccine or fusion protein vaccine have been reported to be very efficient (92, 116). In this study, we evaluated a single protein, HBHA as a BCG boost vaccine. HBHA is one of the most potent mycobacterial antigens that has been shown to induce protection against *M. tuberculosis* challenge in mice similar to the protection provided by the BCG (117). We
tested immunogenicity and protective efficacy of HBHA in a neonatal BCG-vaccinated mouse model.

The major findings of this study are:

a) Neonatal BCG vaccination primed the immune system for native (n) HBHA and therefore upon nHBHA boosting, recall immune responses to HBHA were improved.

b) BCG/nHBHA combination significantly improved protection against BCG infection independently of the route of nHBHA immunization and the coadministration of adjuvant.

c) Boosting with rHBHA induced protection in the BCG-primed mice.

The neonatal immune system is not fully matured and biased to Th2 type response. The challenge of neonatal vaccination arises due to the poor response of neonates to most vaccines. Neonatal BCG vaccination is one of the few examples that can shift the immune system from a Th2 to Th1 type response (118). Although the BCG-promoted immunity wanes with time, BCG vaccination can induce even longer protective immunity than subunit vaccines (119).

In this study, following vaccination with BCG/HBHA combination we observed that BCG vaccination not only primed the immune system but also BCG itself acted as an immune adjuvant. We found that introduction of cholera toxin (CT) in nHBHA vaccine did not show a better protection than the nHBHA formulated without CT. Interestingly, priming with BCG improved protective immune responses even for recombinant (r) HBHA, which was previously reported to be non-protective (117). We observed that lung lymphocytes from the BCG-primed and rHBHA-boosted mice produced large amounts of
IFN-γ upon stimulation with BCG-infected bone-marrow derived macrophages (BCG-BMM), a methodological approach suggested to correlate with the level of protection. IFN-γ levels upon stimulation with HBHA were also induced but were unable to correlate with the level of protection. It is possible that upon BCG infection, BMM present mostly the protective epitope to the lymphocytes from HBHA-immunized mice, which is not the case with HBHA stimulation. In this regard, it has been reported that some mycobacterial antigens may induce higher levels of IFN-γ but do not offer protection (120). Presently, we cannot explain the mechanism of protection induced after rHBHA boosting. Most probably rHBHA acted as a carrier molecule for the protective epitopes generated after BCG vaccination.

We examined the effect of route of nHBHA administration on the immune responses and protection under the BCG/nHBHA vaccination protocol. We found that, provided the animals were primed with BCG, the level of protection was comparable between the i.n. and subcutaneous (s.c.) routes of immunization. In vitro stimulation of lung cells with BCG-BMM showed comparable levels of IFN-γ between the i.n. and s.c. groups, which correlated with the levels of protection. Neonatal BCG vaccination predominantly induced IFN-γ^+CD4^+ T cells as measured by the expression of CD40L on the CD4^+ T cells. Consistent with this, in human neonates, it has also been found that BCG vaccination induced higher frequency of IFN-γ^+CD4^+ T cells than IFN-γ^+CD8^+ T cells. Thus, the general consensus is that BCG vaccination is highly important for the induction of Th1 dominated immune response in the Th2 biased neonates.
Collectively, our study showed an improvement of HBHA vaccination, when combined with the neonatal BCG priming, which would have a significant impact on future TB-vaccine development.

**Effect of maternal priming with HBHA during the gestation period on postnatal cellular immunity in the offspring (Preliminary results)**

Previously, it was described that vaccination of adult mice with nHBHA could induce a level of protection similar to that provided by the BCG vaccination (117). However, in our neonatal model, we have experienced that the protection, offered by the nHBHA vaccination alone, was lower than that offered by the BCG vaccination. In order to improve the nHBHA vaccination in neonates, we hypothesized that *in utero* sensitization of the fetal immune system with nHBHA may improve nHBHA-specific immune responses after birth.

It has been reported previously that not only antibodies but also antigens can be transferred from the mother to the fetus through the placenta, and to the neonates via milk (121-123). Recently, increasing numbers of evidence suggest that transplacental sensitization of the fetal immune system could improve postnatal immune responses (123, 124). In this context, we were especially interested to improve postnatal T-cell immunity by immunizing the pregnant mother at 2 weeks of gestation. Immunization of pregnant mice at 1-week ahead of delivery might exclude the possibility of HBHA-specific antibody transportation through the placenta from the mother to the fetus. Within this short period, IgM, but not IgG can be produced. However, IgG is the only class of immunoglobulin that can cross the placental barrier. Therefore, our hypothesis was that immunization of pregnant mother at 2\textsuperscript{nd} week of gestation will prime the fetal cellular
immune system and will exclude HBHA-specific antibody-mediated effect on the offspring.

In this study, we vaccinated the pregnant mice with n- or rHBHA at 2 weeks of gestation. Following delivery, mice were vaccinated with either BCG s.c. or n- or rHBHA i.n. at 1-week and later boosted with n- or rHBHA i.n. at 4 weeks of age. Mice vaccinated with only BCG, at 1-week, were used as a control. One week after the last immunization, lung lymphocytes were isolated and restimulated in vitro with rHBHA in order to assess the recall immune responses. As shown in Fig. 7, offspring from the immunized mother had significantly higher recall immune responses to rHBHA than the offspring from the unimmunized mother. Maternal priming also improved recall immune responses in the BCG-primed-rHBHA-boosted neonates although the effect was not statistically significant.

We next examined whether the improved recall immune responses, due to maternal immunization, could impact on better protection. To examine this, we used nHBHA instead of rHBHA and followed the similar immunization protocol as mentioned above. Four weeks after the last immunization, mice were challenged with a high dose of BCG i.n., and 3 weeks postchallenge the bacterial burden in the lungs was determined. As shown in Table 1, the offspring from the immunized mother controlled infection better than the offspring from the unimmunized mother, which was even higher than the BCG control group. The effect of maternal priming was less clear in the group that received BCG instead of nHBHA at 1-week and boosted later with nHBHA.
Results were analyzed from two independent experiments. p value (s) was calculated by comparing two groups using t test. * significant when p<0.05.

Fig. 7: Maternal immunization primes for improved postnatal cellular immunity. Pregnant mice at 2\textsuperscript{nd} week of gestation were primed with rHBHA s.c. Following delivery, neonates were immunized with BCG s.c or rHBHA+CT i.n. at one week of age. Second booster dose of rHBHA+CT was applied i.n. at 4-week of age. One week after the last immunization, lung cells from the rHBHA-immunized animals were re-stimulated \textit{in vitro} with rHBHA to measure recall IFN-\(\gamma\) responses. Data for IFN-\(\gamma\) shows mean stimulation index with s.d. calculated as a ratio of HBHA-stimulated IFN-\(\gamma\) levels to Con A-stimulated IFN-\(\gamma\) levels multiplied by a factor 10. Data for IFN-\(\gamma\) represents mean±sd of triplicate wells. Cells were pooled from four animals per group. Results were analyzed from two independent experiments. p value (s) was calculated by comparing two groups using \(t\) test. * significant when p<0.05.

Taken together, these results suggest that maternal immunization with HBHA could prime the fetal immune system, and therefore subsequent immunization of the offspring with HBHA induces better protection.

The results from this study were encouraging because when compared with the BCG vaccinated control group, the HBHA vaccinated group (maternal and postnatal immunization) had higher levels of recall responses and protection. This indicates that a combination of maternal and postnatal immunization with only nHBHA might be beneficial for the group of children who are at risk of developing BCGosis due to the BCG vaccination. The effect of maternal priming in the BCG vaccinated group was not so prominent and this could be due the fact that BCG itself is a very strong immunostimulant and therefore BCG/HBHA vaccination of the naive offspring was sufficient to induce protection. Under the natural circumstances, heterologous prime-boost strategy with BCG/HBHA vaccination is a better approach as we have seen in our previous study.
Vaccination was done with nHBHA in the pregnant mother at 2\textsuperscript{th} week of gestation which followed offspring immunizations with BCG or nHBHA+CT at 1 week and nHBHA+CT at 4 weeks of age.

Mice were challenged with BCG i.n. at a dose of 10 million CFU/animal four weeks after the last immunization. Three weeks postchallenge bacterial burden in the lungs was determined.

Percent of reduction was calculated with respect to the unimmunized group. Results were analyzed from two independent experiments. \( p \) value(s) was calculated by comparing two groups using \( t \) test.

\( **p<0.001 \) and \( *p <0.05 \), when compared with BCG group.

### Table 1. Bacterial burden in the lungs of offspring mice born from the mother primed with or without nHBHA

<table>
<thead>
<tr>
<th>Vaccination(^1)</th>
<th>Neonates</th>
<th>Protection, nHBHA(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant Mother</td>
<td></td>
<td>CFU in the lungs ( \text{mean} \pm \text{sd} \times 10^4 )</td>
</tr>
<tr>
<td></td>
<td>W1</td>
<td>W4</td>
</tr>
<tr>
<td>nHBHA+CT</td>
<td>BCG</td>
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\( ^1 \) Vaccination was done with nHBHA in the pregnant mother at 2\textsuperscript{th} week of gestation which followed offspring immunizations with BCG or nHBHA+CT at 1 week and nHBHA+CT at 4 weeks of age.

\( ^2 \) Mice were challenged with BCG i.n. at a dose of 10 million CFU/animal four weeks after the last immunization. Three weeks postchallenge bacterial burden in the lungs was determined.

\( ^3 \) Percent of reduction was calculated with respect to the unimmunized group. Results were analyzed from two independent experiments. \( p \) value(s) was calculated by comparing two groups using \( t \) test.

\( **p<0.001 \) and \( *p <0.05 \), when compared with BCG group.
Concluding remarks

An effective control of TB is highly depended on the development of a new TB-vaccine, as well as proper identification and treatment of individuals with active disease. From our study with the identification of immune markers for better diagnosis of mycobacterial infection, we can conclude that sTNFR and mycobacteria-specific antibodies in BAL, but not in serum might be useful in distinguishing active from latent infection or exposure to mycobacterial antigens. Cytokines such as IL-12 and IFN-γ can also be used as markers of infection. Although we could not detect TNF in our particular model of infection, BAL TNF has been reported to be a poor marker of detecting smear-negative pulmonary TB (101).

The experience with the BCG and HBHA subunit vaccines in a neonatal mouse model indicates that BCG vaccination primes the immune system for nHBHA. Priming with BCG and boosting with nHBHA could substantially improve BCG-derived immune responses and induce improved protection. Boosting with a non-protective antigens, rHBHA, could also induce protection, however, only when the mice were primed with BCG. Finally, upon BCG priming, HBHA immunization induces higher levels of protection irrespective of the route of administration and the co-administration of adjuvant.

An alternative strategy of improving neonatal nHBHA vaccination is the priming of the fetal immune system by vaccinating the pregnant mother at 2\textsuperscript{th} week of gestation. This strategy might only be useful for the children who are at risk of developing BCGosis. Otherwise, BCG/HBHA combinations seem to be the best effective vaccine in our model against mycobacterial infection.
Future studies

1. How long-lasting is the memory induced after BCG/HBHA vaccination?
In the previous study we have assessed the recall immune responses and protection 1-week and 4-week, respectively, after the last immunization with HBHA. Therefore, we will investigate the presence of protective memory, at 2 months and 4 months, after the last immunization in a longitudinal experiment.

2. How long does the effect of BCG priming last?
In the previous study we considered only 3 weeks interval between prime and boost vaccination. We will therefore examine the kinetics of BCG-primed immunity in order to choose the best time point for boosting BCG-mediated immune response with HBHA.

3. Does BCG vaccination prime for only BCG-related antigens or has it a more general adjuvant effect?
We have seen that BCG primes the immune system for HBHA, which is a BCG antigen. In the future study we will ask whether the effect of BCG priming is specific to BCG-related or unrelated antigens. A comparative study between HBHA and other immunodominant antigens (BCG-related or unrelated) will be done by following heterologous prime-boost approach.

4. Do mycobacteria other than BCG improve HBHA vaccination?
*M. vaccae* is a non-pathogenic mycobacterium, has been reported to exert adjuvant effects (125). We will examine whether vaccination with *M. vaccae* prior to the HBHA immunization could induce HBHA-specific immune responses and protection.

5. Is the effect of maternal priming with HBHA antibody mediated or cell mediated or both?
The preliminary experiment with the maternal immunization focused exclusively on the cellular immunity. Transferable maternal antibodies are crucial during the period when the fetal T-cell immunity is not fully developed. In the future experiments we will
examine whether the milk of HBHA-primed mother has any influence on protection in
the offspring. We will also investigate the effect of maternal antibody in protection by
using IFN-γ-gene knockout mice, since IFN-γ is critical for the cell-mediated immune
response and protection against mycobacterial infection.
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Immunodiagnosis of mycobacterial infection: Increased levels of immunological markers in the respiratory tract but not in serum correlate with active pulmonary infection in mice.

Arko-Mensah J\textsuperscript{1*}, Rahman MJ\textsuperscript{1*}, M. Singh\textsuperscript{2}, Fernández C\textsuperscript{1}

\textsuperscript{1}Department of Immunology, Wenner-Gren Institute, Stockholm University, S-10691, Stockholm, Sweden

\textsuperscript{2}Lionex Diagnostics and Therapeutics GmbH, Braunschweig, Germany

*Both authors contributed equally to the manuscript

Corresponding author: John Arko-Mensah

Department of Immunology, The Wenner-Gren Institute, Stockholm University, S-10691 Stockholm, Sweden

Tel: 00468164174; Fax: 004686129542

Email: john.arko-mensah@imun.su.se
Abstract

In developing countries where the vast majority of *Mycobacterium tuberculosis* (*M. tuberculosis*) infections occur, diagnosis relies mainly on identification of acid-fast bacilli in unprocessed sputum smears, which fails to identify approximately 50% of active pulmonary disease cases, or mycobacteria culture which is usually slow and could take several weeks. On the other hand, immunological tests for the diagnosis of TB have relied mostly on detection of immune markers in serum or release of cytokines by mononuclear cells *in vitro*. Here, we have modelled the natural route of mycobacterial infection by infecting mice intranasally (i.n), but also systemically with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and assessed the immune response generated at the site of infection and in serum. We demonstrate that active infection of mice with BCG, but not exposure to non-replicating mycobacteria, induced production of IL-12, IFN-γ and sTNFR locally in the lungs as detected in the broncho-alveolar lavage (BAL). There was a positive relationship between bacteria growth in the lungs and levels of sTNFR or IL-12, and to some extent IFN-γ in BAL. Moreover, sTNFR levels increased significantly in BAL after reactivation of controlled infection with dexamethasone, which resulted in increased bacteria growth in the lungs. Finally, infection but not exposure to non-replicating mycobacteria induced specific IgG and IgA antibodies in BAL. Taken together, the detection of sTNFR and mycobacteria specific antibodies, especially IgA locally in the lungs to elected antigens expressed at different stages of disease manifestation may be used as immunological markers for the diagnosis of TB.
1. Introduction

The global epidemic of tuberculosis (TB) results in eight to ten million new cases of disease per year (1), with an annual projected increased rate of 3%. It is estimated that between 5 and 10% of immunocompetent individuals are susceptible to TB, and of these, 85% develop pulmonary disease (2) in the first 2 years after exposure. The chronicity or latency of *Mycobacterium tuberculosis* infection has made eradication of TB a very difficult goal.

Immunity against TB depends on several factors, including cytokines, chemokines, antibodies, macrophages, neutrophils, several T-cell subsets and specific pattern of T-cell migration (3, 4). The infected host generates a T helper (Th)1 type of immune response in which mycobacterial antigen-specific T lymphocytes are recruited to the lungs, and play a significant role in protection against *M. tuberculosis* infection (5, 6). Interferon gamma (IFN-γ) is the central mediator in protection against *M. tuberculosis* infection and synergises with tumour necrosis factor (TNF) in activating macrophages (7). Interleukin (IL)-12, together with TNF and IFN-γ, plays a critical role in the development of protective granulomas, which contain activated macrophages producing specific enzymes, such as inducible nitric oxide synthase (iNOS) or NOS2, responsible for the elimination of bacteria. These mechanisms form the basis for protection against mycobacterial spreading (8, 9). Even if less clear, specific antibodies particularly IgA in mucosal secretions have been related to protective immunity against pulmonary infections (10-12).
To effectively control the global TB epidemic, an improvement in the currently available diagnostic tools is of utmost importance. In the vast majority of low- and middle-income countries, where TB is prevalent, diagnosis primarily relies on identification of acid-fast bacilli in unprocessed sputum smears using microscopy. Although acid-fast staining is relatively quick, sensitivity is variable, ranging from 20-80% (13), because greater than \(10^4\) bacilli/ml of sputum are required for reliable detection. Subsequently, approximately 50% of active pulmonary TB is sputum smear negative, the failure rate being greater for paediatric and HIV-associated TB (14, 15). While mycobacteria culture is the ultimate proof of *M. tuberculosis* infection and often used as a reference method for diagnosis due to its high sensitivity and specificity (16, 17), it involves direct handling of live bacteria and also takes 4-6 weeks to grow on solid culture medium. The Mantoux or Tuberculin skin test is used widely in several countries as the standard method for the diagnosis of latent TB. The interpretation of this test, which is based on the detection of delayed type hypersensitivity to purified protein derivative (PPD) after intradermal injection, could however be affected by factors such as age, exposure to environmental mycobacteria or BCG vaccination (18).

In recent times, the diagnostic potential of immune-based tests for the identification of *M. tuberculosis* infection has been assessed and thought to offer the potential to improve case detection in a general population. In this regard, a number of alternative testing strategies, some based on IFN-\(\gamma\) release have been developed to address some of the problems presented by the Tuberculin skin test in the detection of latent *M. tuberculosis* infection. IFN-\(\gamma\) release assays involve stimulation of blood lymphocytes with the early secreted antigenic target 6 (ESAT-6) or culture filtrate protein (CFP-10), followed by the
Measurement of IFN-\(\gamma\) by enzyme-linked immunosorbent assay (ELISA) or the detection of IFN-\(\gamma\)-producing cells by ELISPOT (19, 20). However, the infrastructure required to collect specimen, deliver them to the proper laboratory and perform the test make it difficult to establish in resource-limited countries (21). The development of serodiagnostic tests for the detection of antibodies, antigens and immune complexes has been attempted for decades. Serological methods are usually simple, rapid, inexpensive and relatively non-invasive. To this end, different mycobacterial antigens have been evaluated, culture filtrate proteins (22), mycobacterial sonicates (23), and purified proteins (24, 25). Until recently, researchers have looked mainly in serum for identification of specific mycobacterial antibodies as markers of infection. With regard to the use of cytokines or cytokine receptors as surrogate markers of mycobacterial infection, levels have been assessed mainly in serum of TB patients (26-30). Serological methods have been used with limited success. One possible explanation is that serum may not be the best biologic material to be analyzed.

Since TB is a disease of the lungs, antibodies and cytokines detected locally in the lungs and associated fluids will more likely reflect infection status compared to serum. In this study, we aimed to identify both cellular markers and specific mycobacterial antibodies locally in broncho-alveolar lavage (BAL) and serum which in combination or separately may be indicative of active mycobacterial infection. We demonstrate that active infection of mice but not exposure to non-replicating mycobacteria resulted in increased levels of IL-12, IFN-\(\gamma\) and soluble TNF receptors (sTNFR) in the BAL. Moreover, infection but not exposure to non-replicating mycobacteria induced production of specific IgA antibodies in BAL, which were undetectable in serum.
2. Materials and Methods

2.1. Mice

The studies were performed using 8-12 weeks old female BALB/c mice purchased from Taconic Europe, Denmark and housed in pathogen free conditions. All animals were kept at the Animal Department of the Arrhenius Laboratories, Stockholm University, Sweden. All experiments were done in accordance with the guideline of the animal research ethics board at Stockholm University. Mice were supervised daily and sentinel mice were used to assess and ensure pathogen free conditions in the facility.

2.2. Bacteria cultivation

*M. bovis* BCG (Pasteur strain) obtained from Dr. Ann Williams, HPA, Salisbury, UK, was grown in Middlebrook 7H9 broth with glycerol supplemented with albumin-dextrose-catalase (ADC) at 37°C, and aliquots frozen in PBS at -70°C. Three vials picked randomly from the stock were thawed, serially diluted in plating buffer (PBS with 0.05% Tween-80 [vol/vol]) and colony forming units (CFU) counted at 2-3 weeks after plating on Middlebrook 7H11 agar (Difco, Sparks, MD, USA), with glycerol and oleic-acid-albumin-dextrose-catalase (OADC) enrichment.

2.3. Preparation of heat killed (hk)- and soluble BCG lysate

Bacteria were grown until they reached approximately 5-10 × 10^7 CFU/ml. To prepare hk-BCG, 10^7 CFU/ml of BCG were autoclaved at 121°C for 15 min. Killed bacteria were washed once and re-suspended in sterile PBS before use. For the preparation of BCG lysate, 10^7 CFU/ml bacteria were pelleted by spinning at 8,000 × g, resuspended in 0.05% Tween 80 in PBS and washed two more times in this solution. The bacteria were then
resuspended in 5 ml of ice-cold PBS and sonicated on ice for 14 cycles of 1 minute each as described by Power CA et al (31). The sonicated suspension was spun at 20,000 rpm for 30 minutes at 4°C to remove particulate matter and the supernatant containing soluble antigens (referred to as BCG lysate) was collected. Protein concentration of the lysate was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, CA, USA) according to the manufacturers instructions. Soluble Bovine serum albumin fraction V (BSA) was used as protein standard, and the supernatant was stored at –20°C.

2.4. Single mycobacteria antigens

The antigen 85 complex (Ag85c) was obtained from the Colorado State University. Ag85c is a major secreted protein belonging to the mycolyl transferase family of the M. tuberculosis complex (32). Purified 38-kDa, 19-kDa and 16-kDa antigens were all obtained from LIONEX Diagnostics & Therapeutics GmbH, Germany. These antigens, also secreted, have been commonly used in the serodiagnosis of TB (33-35).

2.5. Experimental infection or treatment with non-replicating BCG, determination of CFU and reactivation of controlled infection

Mice were infected i.n. with $10^7$ CFU of live BCG or treated with $10^7$ CFU of non-replicating BCG (BCG lysate or hk-BCG) or PBS as control in 30 µl PBS. Before infection or treatment, mice were anaesthetised with isofluorane (Baxter Medical AB, Kista, Sweden). I.n. administration was carried out by inoculation of live or non-replicating BCG to the nostrils (15 µl per nostril given in two doses) and mice allowed to breathe the suspension into the lung naturally (36). At day 3, weeks 1, 3, 5, and 9 after infection, mice were sacrificed by cervical dislocation. Briefly, lungs, spleen and liver
were removed aseptically and placed in 2 ml PBS with 0.05% Tween-80 and homogenized in glass homogenizers. Serial dilutions of the lung homogenates were plated on Middlebrook 7H11 agar plates with OADC enrichment and incubated at 37°C. The number of CFU was determined 2-3 weeks after plating. Reactivation of controlled infection was done as described by Lowrie et al. (37) with some modifications. Briefly, each mouse was injected intraperitoneally (i.p.) at week 10 after infection (day 0) with 180 µg dexamethasone in 100µl PBS. I.p. injection of dexamethasone was repeated each other day (days 0, 2, 4, and 6) for a total of 4 doses.

2.6. Sample collection

Serum and BAL were collected from each group of mice at the different time points indicated above. To obtain sera, mice were bled from the tail vein and serum collected after centrifugation. BAL was obtained by flushing 1.5 ml of PBS into the lungs of sacrificed mice. All samples were kept at -20°C until use.

2.7. Cytokine and cytokine receptor detection assays

TNF and sTNFR enzyme-linked immunosorbent assay (ELISA) was performed using the commercially available DuoSet ELISA Development Systems (R&D Systems Europe, Abingdon, UK) according to the manufacturer’s recommendations, with slight modifications. Streptavidin conjugated to alkaline phosphatase (MABTECH, Nacka, Sweden) was used instead of horseradish peroxidase at 1:1000 dilution. IL-12 and IFN-γ ELISA was performed using commercially available kits (MABTECH, Nacka, Sweden). The enzyme-substrate reaction was developed using p-nitrophenyl phosphate (SIGMA, St. Louis, MO, USA). Optical density was read in a multiscan plate reader (Anthos
Labtech Instruments, Salzburg, Austria) at 405 nm and concentrations were obtained by comparison with calibration curves established with recombinant TNF, TNFR1 & 2, IL-12 or IFN-γ standards.

2.8. Detection of antibodies in serum and BAL

Antibodies in serum and BAL were analyzed by ELISA. ELISA plates (Costar, high binding, NY, USA) were coated with either BCG lysate (20 µg/ml) or Ag85c, 38-kDa, 19-kDa or 16-kDa (2 µg/ml) in carbonate-bicarbonate buffer pH 9.6, overnight (ON) at room temperature (RT). Plates were washed four times with washing buffer (0.9% NaCl-0.05% Tween-20 [vol/vol]). After washing, pools of samples (4 mice per treatment) serially diluted were incubated in the antigen coated plates, starting as follows 1:100 (sera) and 1:5 (BAL) and plates were then incubated ON at RT. Following sample incubation, plates were washed and incubated for 2 h at RT with alkaline-phosphatase (ALP) labelled goat anti-mouse IgG or IgA (Southern Biotech, Birmingham, USA) and the enzyme substrate reaction developed using p-nitrophenyl phosphate as substrate. Absorbance was measured in a multiscan reader at 405 nm. Non-specific antibodies were determined in parallel using BSA as unrelated antigens, and these values were deducted from those obtained for specific anti-BCG antibodies.

2.9. Statistical analysis

Data are presented as the mean value ± SEM. Student’s $t$ test was used to determine statistical significance between treatment points. Differences were considered significant when (*, $p<0.05$, (**, $p<0.01$).
3. Results

3.1. Intranasal infection but not treatment of mice with non-replicating BCG induces elevated levels of IL-12, IFN-γ and sTNFR in the lungs

TNF, IL-12 and IFN-γ are essential components of the protective immune response against mycobacterial infection, and elevated levels of these cytokines in the lungs, the principal organ involved during *M. tuberculosis* infection, could be indicative of disease activity. To assess this, BAL from mice infected i.n. with BCG or treated with non-replicating BCG (lysate or hk-BCG) was collected at different time points and analyzed for TNF, IL-12 and IFN-γ amounts. To quantify bacterial load in lungs, spleen and liver, serial dilutions of organ homogenates were plated on Middlebrook 7H11 agar, and CFU counted 2-3 weeks after plating.

Live, but not non-replicating BCG induced levels of IL-12 and IFN-γ in the lungs (Figure 1a-b). The amount of IL-12 detected in BAL progressively increased during infection to week 3, and declined significantly (*P< 0.05*) by week 9. There was a direct relationship between IL-12 levels in BAL and bacteria growth in the lungs (Figure 1c). The highest level of IFN-γ in BAL, unlike IL-12, did not exactly coincide with the peak bacteria growth in the lungs, but was indicative of active infection. In contrast to IL-12 or IFN-γ, minimal amounts of TNF were detected at all time points (data not shown). Since TNF was undetectable in BAL we reasoned that elevated levels of sTNFR could result in TNF neutralization (38). To assess this, we measured the levels of sTNFR1 and 2. Similarly to IL-12 and IFN-γ, live, but not non-replicating BCG induced levels of sTNFR in the lungs as detected in BAL (Figure 1d-e). The concentrations of sTNFRs, like IL-12 and to some
extent IFN-\(\gamma\), seem to have a direct relationship with bacteria growth in the lungs. Compared to the lungs, there was significantly lower infectivity of the spleen or liver after i.n. infection of mice with BCG.

### 3.2. Elevated levels of sTNFR and IL-12 in serum are more indicative of exposure to mycobacterial antigens than to active infection

Generally, peripheral blood is used for assessing the state of infection in several diseases. To determine whether serum levels of sTNFR or IL-12 could be used similarly as BAL levels to predict an ongoing mycobacterial infection, sera collected from the tail-vein at the time points mentioned above were analyzed for presence of cytokines. Increased levels of sTNFR and IL-12 were measured in serum not only after infection, but also after treatment of mice with non-replicating BCG (Figure 2a-c). There was no direct relationship between sTNFR or IL-12 levels and bacterial growth (Figure 1c) in the lungs, spleen or liver, indicating that presence of sTNFR or IL-12 in serum may be more indicative of antigen exposure rather than active infection. In these experiments, TNF and IFN-\(\gamma\) were undetectable in serum. These results suggest that the detection of cytokines or soluble receptors in serum may not be reflective of bacteria growth in the lungs, and therefore not reliable for prediction of an ongoing mycobacterial infection.

### 3.3. Intravenous infection of mice with BCG results in elevated levels of sTNFR in BAL which correlate with bacterial growth in the lungs

Since i.n. infection did not result in significant bacterial growth in the spleen and liver, we administered BCG systemically via the i.v. route, as infection via the i.v. route results in generalized infection of the lungs, spleen and liver. From the results obtained with i.n.
infection, levels of sTNFR were measured since concentrations seemed to have the strongest correlation with bacteria growth. Intravenous administration of BCG resulted in a generalized infection, with bacteria growth in all three organs (Figure 3a). Similarly to the observation in BAL after i.n. infection, elevated levels of sTNFR (Figure 3b-c), followed increased bacteria growth in the lungs. Overall, higher amounts of sTNFR were detected in the serum of i.v., compared to i.n. infected mice. There was however no direct relationship between levels of sTNFR in serum and bacterial load in the lungs, spleen or liver, perhaps because many organs are infected and their contributions are unclear. These results corroborate the observation that the local immune response in the lung microenvironment but not the generalized or systemic may reflect an ongoing bacterial growth in the lungs.

3.4. Reactivation of mycobacterial infection results in elevated levels of sTNFR in BAL

In a lifetime, 5-10% of people latently infected with M. tuberculosis reactivate their infection as a consequence of immunosuppression, which results in increased bacterial growth in the lungs and other organs. In animal experiments, reactivation of controlled mycobacterial infection has been achieved by the administration of dexamethasone (34, 35), a corticosteroid that suppresses the effector functions of T cells central to the control of infection. To determine whether increased bacteria growth in the lungs will result in elevated levels of sTNFR in BAL, mice infected, but with controlled infection (week 10 after i.n. infection) were treated with dexamethasone as described above and BAL and sera analyzed for presence of sTNFR.
Administration of dexamethasone resulted in increased bacterial growth in the lungs (Figure 4a) by week 2 after treatment. There was also a slight but not significant increase in bacteria load in the spleen and liver (data not shown). sTNFR levels increased significantly in the BAL 2 weeks after dexamethasone treatment (Figure 4b-c), which correlated with the bacteria growth in the lungs. In contrast, sTNFR levels in serum after dexamethasone treatment were comparable to that of untreated mice (data not shown). These results confirm our observation that sTNFR levels in BAL correlate with bacteria growth in the lungs, and higher sTNFR amounts may be indicative of an ongoing but not controlled chronic mycobacterial infection.

3.5. Infection but not treatment of mice with non-replicating BCG results in production of BCG or antigen specific antibodies in BAL and serum

Thus far, our results have demonstrated a correlation between bacterial load in the lungs and sTNFR levels in the lung microenvironment. However, several infections, especially upper respiratory tract infections may induce inflammation and increase cytokine production in the respiratory tract including the lungs. To confirm that infection was caused by mycobacteria, we analyzed BAL and sera for the production of specific IgG and IgA antibodies using BCG lysate as antigen. Only infected mice produced detectable levels of antibodies in BAL and serum (Figure 5a-b), which increased over time. BCG specific IgA was detected in BAL, but not serum, and had a similar kinetics as IgG (Figure 5a). Next, we determined whether detection of antibodies to single mycobacterial antigens in BAL and serum could be used to predict an ongoing infection compared to total mycobacterial antigens. Similarly, to the results obtained with BCG lysate, only active infection resulted in production of antigen specific IgG or IgA in BAL (Fig. 5c, d).
In contrast to BCG lysate, moderate to high amounts of IgG to single antigens were detectable in serum (Fig. 5e). Overall, these data suggest that detection of antibodies to multiple or single mycobacterial antigens in BAL, especially IgA may be predictive of an ongoing infection in the lungs.
4. Discussion

A critical element of TB control is early diagnosis of infection and treatment. The obtention of a microbiologically confirmed diagnosis of TB by the traditional methods are disadvantaged by low sensitivity in sputum smear microscopy or slow growth in the case of mycobacteria culture. These shortcomings, which could lead to diagnostic delay, underscore the need for alternative diagnostic tests that are simple, rapid and sensitive. In recent years, there has been renewed interest in the development of immune-based assays for the diagnosis of *M. tuberculosis* infection. In this regard, IFN-γ release (19, 20) or antibody based diagnostic assays (reviewed in ref. 39) have been developed. Notwithstanding, the biological complexity of *M. tuberculosis* infection means that election of a single immunological marker as predictive of infection or disease activity would probably have limited diagnostic value, and analysis of several biomarkers would offer the possibility of enhanced diagnosis. The importance of our study therefore lies in the fact that we have assessed elements of the Th1 immune profile, known to be important for the control of *M. tuberculosis* infection (5, 6), and specific mycobacterial antibodies in serum and at the site of infection.

In the present study, we have demonstrated that: 1) active mycobacterial infection, but not exposure to non-replicating mycobacteria resulted in concomitant elevation of IL-12, IFN-γ and sTNFR in BAL; 2) levels of IL-12, sTNFR and IFN-γ in BAL depend on bacteria growth in the lungs; 3) serum levels of IL-12 and sTNFR are probably more related to exposure to mycobacterial antigens since live and non-replicating BGC induced similar levels of IL-12 and sTNFR; 4) independently of the route of infection, sTNFR levels in BAL correlate with bacteria growth in the lungs, but bacteria growth in the
spleen or liver do not seem to directly influence sTNFR levels in BAL; 5) reactivation of controlled BCG infection with dexamethasone results in increased bacteria growth in the lungs, which correlated with sTNFR levels in BAL; 6) active infection, but not exposure to non-replicating mycobacteria resulted in production of antigen specific IgG or IgA in BAL.

Over the years, the majority of studies that have investigated into cytokines or their soluble receptors as markers of active disease in TB patients have looked mainly in the serum (26-30). However, *M. tuberculosis* infection occurs via the respiratory tract, and currently, there is the belief that in TB, as well as in other lung diseases, the markers of disease activity need to be measured locally in the lungs, and not in peripheral blood. In the present study, elevated levels of IL-12 or sTNFR in serum were associated with exposure to mycobacterial antigens in general and not exclusively to active infection. On the other hand, elevated levels of IL-12, IFN-γ in BAL, or the shedding of sTNFR depended on the infection status. In TB, several studies have directly demonstrated a relationship between cytokine production in the lungs and active tuberculosis based on cytokine release by mononuclear cells at the site of infection (41-42). In these studies, there was significant increase in release of IFN-γ, IL-12, IL-1β, IL-8 and TNF by broncho-alveolar cells lavaged from patients with pulmonary TB.

In contrast, few studies have systematically assessed cytokines or their soluble receptors locally in the lungs of TB patients. For example, Condos and co-workers (42) demonstrated elevated levels of TNF and IL-1β in BAL from patients with active pulmonary TB compared with that from healthy subjects. However, in their study, a TB
patient was defined as any patient with chest radiograph suggestive of TB regardless of HIV status. In this regard, a possible contribution by some other co-infections cannot be entirely excluded. In their work, Kupeli and co workers (43) demonstrated elevated levels of TNF in BAL of smear-negative pulmonary TB patients compared to healthy controls. However, BAL IL-2 and IFN-γ levels were not significantly different from that obtained from patients with other pulmonary diseases. In our study, IFN-γ level in BAL was lower, Compared to IL-12, and peaked later at week 5. IL-12 produced by activated antigen presenting cells acts as a pro-inflammatory cytokine that bridges the innate and adaptive immune responses and skews T-cell reactivity toward a Th1 cytokine pattern (44).

The two forms of TNF, soluble TNF and transmembrane TNF function physiologically by interacting with TNFR1 or TNFR2 expressed on a diverse range of cell types (45). Elevated levels of sTNFR were measured in BAL after i.n. or i.v. infection of mice with BCG. It is notable that mycobacteria have a predilection for the lungs even when inoculated systemically. The bioavailability of TNF is modulated by binding to TNFR. Although TNF neutralization is an effective therapy in some debilitating conditions like rheumatoid arthritis, it could increase the risk of reactivation of latent TB (46). For example, transgenic mice expressing high serum levels of TNFR1 exhibited reduced bactericidal activity, had undifferentiated granulomas and succumbed to BCG infection (47). TNF neutralization may explain our inability to detect TNF and account for the relatively lower levels of TNFR1, known to account mainly for binding to TNF. While elevated levels of TNF were found in the BAL of TB patients (42, 48), its been suggested that low TNF levels relative to sTNFR are found in chronic infection like TB, compared
to high TNF levels in acute infections which could overcome the potential neutralizing activity of sTNFR in the circulation (49). Overall, our findings strengthen the notion that TNF, IFN-γ, IL-12 and sTNFR are important components of the anti-mycobacterial protection systems in humans, even if it is still unclear which levels could be considered adequate, and elevated levels may be used as surrogate markers of disease activity.

In the first part of our study, we identified cellular immune markers associated with mycobacterial infection. Later, we analyzed mycobacteria-specific antibodies in BAL and serum after active infection of mice or treatment with non-replicating antigens by using BCG lysate or selected mycobacterial antigens commonly used in the serodiagnosis of TB. The use of crude antigens like whole-culture filtrate (22) or lysate (23) composed of a mixture of several antigens has had the limitation of lack of sensitivity and/or specificity. In the last decade however, studies of new assays that use various purified and well-characterised proteins (24, 25) and lipid antigens (50, 51) for measurement of serum antibodies in TB patients have been reported. While antibody production during a primary immune response is usually low, the chronicity of mycobacterial infection means that there is continuous stimulation of immune cells by surface exposed as well as secreted antigens, resulting in significant production of antibodies over time. The higher levels of antibodies measured in both BAL and serum at week 9, compared to the early time points is therefore expected. In contrast, non-replicating antigens would need continuous priming as well as help from relevant adjuvants to be optimally immunogenic. This may explain the inability of the non-replicating antigens administered as single doses to induce detectable antibodies in BAL. Nevertheless, detectable levels of specific IgG were measured in serum but still not in BAL of mice treated with non-replicating
antigens. One explanation could be that the single antigens used, which were purified antigens, had better affinity and higher specificity compared to BCG lysate antigens. In this regard, it has been suggested that antibody-based diagnostics that utilize purified or multiple antigens would achieve high levels of sensitivity and specificity (52).

In this study, IgA was detectable only in BAL. IgA is the major immunoglobulin in mucosal secretions and our group and others have demonstrated the induction of IgA in mucosal secretions after i.n. immunization with mycobacterial antigens (10-12) or infection with mycobacteria (53). Nevertheless, mycobacteria specific IgA was detected in the serum of adult TB patients (54, 55). This is not entirely surprising, considering the protracted nature of TB in humans, which could result in constant release of secretory mycobacterial antigens into the general circulation resulting in continuous priming of IgA secreting B-cells. The four antigens selected have been commonly used for the serodiagnosis of TB (32-35). In contrast to Ag85c, 38-kDa or 19-kDa, lower amounts of anti-16-kDa antibodies were detectable in BAL and serum. The 16-kDa antigen is a cytosolic regulatory protein specific to the *M. tuberculosis* complex and expressed during latency (56). A systematic analysis of humoral immune responses of TB patients has shown that the profile of antigenic proteins of *M. tuberculosis* recognized by antibodies differs at different stages of infection and disease progression (57). This suggests that an accurate diagnostic test for TB will need to be based on a combination of antigens. At this point, it is conceivable to propose the detection of mycobacterial antigens in BAL, saliva or serum as an alternative to soluble immune markers. However, serological tests based on detection of mycobacterial antigens, like the skin test, could be confounded by cross-reactivity with non-pathogenic mycobacteria or immunization.
It's noteworthy to mention that measurement of immune markers directly in saliva would be rapid, simple, inexpensive and non-invasive compared to obtention of BAL. Moreover, the use of BAL may be technically demanding in resource-limited settings. So far, we have been unsuccessful in detecting cytokines or antibodies to single mycobacterial antigens in saliva from infected mice. Notwithstanding, we are working to improve our detection assays in saliva. In conclusion, detection of a tailored combination of mycobacteria specific antibodies and cellular markers in the respiratory tract would be useful for the immunodiagnosis of TB, and may help prediction of the clinical course of disease.
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Figure Legends

Figure 1
Intranasal infection but not treatment of mice with non-replicating BCG induces elevated levels of sTNFR, IL-12 and IFN-γ in the lungs. Mice were infected i.n. with $10^7$ CFU of BCG or treated with corresponding amounts of BCG lysate or hk-BCG. Control mice were treated with PBS. BAL was collected at day 3, weeks 1, 3, 5 and 9 after infection or treatment. Levels of IL-12 (a), IFN-γ (b) and sTNFR (d and e) were measured with standard ELISA kits and mean concentrations expressed as pg/ml. Serial dilutions of lungs, spleen and liver were plated on Middlebrook 7H11 agar and bacterial loads were determined 2-3 weeks after plating (c). Results are expressed as mean concentration (a,b), (d,e) or CFU $\times 10^3$ (c) ± SEM from 4 mice per group. A representative of three different experiments is shown. * $p<0.05$, ** $p<0.01$ versus D3.

Figure 2
Elevated levels of sTNFR and IL-12 in serum are more indicative of exposure to mycobacterial antigens than to active infection. Sera from mice infected with live or non-replicating BCG as described in Figure 1 were analyzed for presence of sTNFR (a-b) and IL-12 (c) with standard ELISA kits and mean concentrations expressed as pg/ml. Results are expressed as mean concentration (a-c) ± SEM from 4 mice per group. A representative of three different experiments is shown.
Figure 3

Intravenous infection of mice with BCG results in elevated levels of sTNFR in BAL, which correlate with bacterial growth in the lungs. Mice were infected i.v. with $10^7$ CFU of BCG and BAL and sera collected at day 3, weeks 1, 3, 5 and 9 after infection. Levels of sTNFR in BAL (b-c) and sera (d-e) were assayed as described above and mean concentrations expressed as pg/ml. Serial dilutions of lungs, spleen and liver were plated on Middlebrook 7H11 agar and bacterial loads determined 2-3 weeks after plating (a). Results are expressed as mean concentration (b-e) or CFU $\times 10^3$ (a) ± SEM from 4 mice per group. A representative of three different experiments is shown. * $p<0.05$, ** $p<0.01$ versus D3.

Figure 4

Reactivation of mycobacterial infection results in elevated levels of sTNFR in BAL. Mice perceived to have controlled BCG growth in the lungs at week 10 after i.n. infection with $10^7$ CFU of BCG were injected intraperitoneally with 180ug dexamethasone per mouse every other day for a total of 4 injections. BAL collected at weeks 1, 2 and 3 after dexamethasone treatment was assayed as described above for the presence of sTNFR, and mean concentrations expressed as pg/ml (b-c). Serial dilutions of lungs were plated on Middlebrook 7H11 agar and bacterial loads determined 2-3 weeks after plating (a). Results are expressed as mean concentration (b-c) or CFU $\times 10^3$ (a) ± SEM from 4 mice per group. A representative of three different experiments is shown. * $p<0.05$, ** $p<0.01$ versus D3.
Figure 5

Infection but not treatment of mice with non-replicating BCG results in production of BCG or antigen specific antibodies in BAL and serum. Mice were infected i.n. with $10^7$ CFU of BCG or treated with $10^7$ CFU non-replicating BCG, and BAL and sera collected as above. Micro-titre plates were coated with 25 µg/ml of BCG lysate as antigen overnight in bicarbonate buffer, pH 9.6, and the amounts of BCG-specific antibodies assayed in BAL (a) and serum (b) serially diluted in PBS. 2 µg/ml of purified Ag85c, 38-kDa, 19-kDa or 16-kDa was coated as described and antigen specific antibodies assayed in BAL (c, d) and in serum (e). OD was read at 405nm and was in the linearity phase. Results are expressed as mean OD from 4 mice per group, and a representative of three different experiments is shown.
Fig. 1
Fig. 2
Fig. 3

a) Bacteria load

- Lung
- Spleen
- Liver

CFU × 10^3

b) BAL

sTNFR1

(pg/ml)

c) sTNFR2

(pg/ml)

d) SERUM

sTNFR1

(pg/ml)

e) sTNFR2

(pg/ml)
a) Bacteria load

- CFU x 10^3
- w9, w11, w12, w13
- DXM treatment

b) BAL

- sTNFR1
- (pg/ml)
- w9, w11, w12, w13
- DXM treatment

- BCG(-DXM)
- BCG(+DXM)

c) sTNFR2

- (pg/ml)
- w9, w11, w12, w13
- DXM treatment

Fig. 4
Fig. 5
Improvement of HBHA vaccination by BCG priming in a neonatal mouse model

Rahman MJ\textsuperscript{1}, Locht C\textsuperscript{2} and Fernández C\textsuperscript{1}

\textsuperscript{1}Department of Immunology, Wenner-Gren Institute, Stockholm University, S-10691, Stockholm, Sweden
\textsuperscript{2}INSERM U629, Institut Pasteur de Lille, 1, rue du Prof. Calmette, F-59019 Lille Cedex, France.

Corresponding author: Muhammad Jubayer Rahman
Department of Immunology, The Wenner-Gren Institute, S-10691, Stockholm University, Stockholm, Sweden
Tel: 00468164174; Fax: 004686129542
Email: jubayer.rahman@imun.su.se
Abstract
The neonatal immune system is not fully developed which results in suboptimal responses to many vaccines. The Mycobacterium bovis BCG vaccine is one of the few examples that can actively induce Th1 cellular immunity in the neonates and confers protection against severe forms of disseminated but not pulmonary tuberculosis in children. Moreover, protection mediated by the BCG vaccine wanes over time. There is a need to develop an effective booster vaccine, which can improve the BCG-stimulated protective immunity. Since tuberculosis (TB) is primarily a pulmonary disease we addressed whether the route of administration and the election of a good antigen could be of importance in the improvement of neonatal BCG vaccination. For this purpose, we have tested the mycobacterial antigen heparin-binding hemagglutinin (HBHA), a potent new vaccine candidate. Our results showed that priming with BCG at the neonatal period followed by boosting with native (n) HBHA, improved HBHA-specific immune responses in the lungs, including the frequency of IFN-γ producing CD4+ and CD8+ T cells. Interestingly, priming with BCG biased the immune response towards a Th1 type since the vast majority of CD4/CD40L expressing cells was also found to be IFN-γ positive. A comparison of intranasal (i.n.) and subcutaneous (s.c) immunization with nHBHA showed that i.n. immunization induced higher levels of IFN-γ as observed upon in vitro stimulation of lung cells with HBHA. However, IFN-γ levels upon stimulation with BCG-infected macrophages and protection against high-dose of BCG infection were comparable between the i.n. and s.c. groups. Also, the use of adjuvant in the boosting with nHBHA was not necessary provided that the animals were previously primed with BCG. Immunological responses correlated with the levels of protection measured by control of bacterial growth in the lungs upon i.n. infection. Finally, priming with BCG improved the immunogenicity and protection promoted by the recombinant form of HBHA regarded previously as non-protective. We discuss the improvement of HBHA vaccination for better protection against TB.
1. Introduction

Tuberculosis (TB) is a disease of global concern caused by the pathogen *Mycobacterium tuberculosis*. The World Health Organization (WHO) reported that there were 8.8 million new cases of TB in 2005 and a total of 1.6 million people died of TB, including patients infected with HIV (1). Nearly 80% of the world’s population is vaccinated with *Mycobacterium bovis* bacille Calmette-Guérin (BCG) (2), which has been the only licensed vaccine against TB. Despite this large coverage, TB remains one of the leading causes of human death (3). In a number of field studies, it has been reported that the protective efficacy of BCG vaccination against pulmonary TB varies from 0-80% (4-5). Only well-nourished children vaccinated with BCG at the neonatal stage have protection against TB with an average of 50% (5-6), which has been reported as a highly cost effective intervention against severe childhood TB such as meningitis or miliary TB (7). Therefore, vaccination with BCG is still recommended in many countries as the standard for TB prevention in infants and young children. Unfortunately, BCG-mediated protection lasts only during the first years (5) of life and revaccination with BCG does not provide additional protection (8). With the increasing appearance of multi-drug resistant TB and the co-infection with human immune deficiency virus (HIV), incidence of TB is now escalating. All together, there is an urgent need to develop an effective vaccine.

BCG is a live attenuated strain of *M. bovis*, which was altered with many undefined genetic changes during the process of attenuation. The reasons for the variable efficacy of BCG in protection against *M. tuberculosis* are not well understood. Possible explanations have been suggested (reviewed in refs 9, 10) including influence of environmental
mycobacteria on the immune response to BCG, deletion of protective antigens, route of administration, and genetic differences among vaccines. In order to improve the BCG vaccine or to develop a new TB vaccine, several approaches have already been addressed in different experimental models with recombinant BCG (rBCG), DNA vaccines or subunit proteins. It has been shown that rBCG over-expressing Ag85B provided better protection than the BCG vaccine (11, 12). Another strategy is the use of subunit vaccines but the majority of them failed to impart better protection than the BCG vaccine (13, 14). DNA vaccines expressing mycobacterial antigens have shown protection in mice but they have been disappointing in the non-human primate model of TB (15). Heterologous prime-boost strategies using DNA and subunit proteins have been demonstrated promising in mice, although protection was not significantly better than BCG when tested in the larger animals like guinea pigs (16). Subsequently, it was found that BCG prime-DNA boost and BCG prime-fusion protein boost regimes were efficient for enhancing protective immunity in mouse models of TB (17, 18).

The mycobacterial heparin-binding hemagglutinin adhesion (HBHA) has been reported to be a potent mycobacterial antigen that displayed a level of protection similar to the BCG vaccine in a mouse model of TB (19, 20). HBHA is a surface associated protein involved in adherence to epithelial cells (21), and has also been shown to be important for extrapulmonary dissemination of *M. tuberculosis* (22). In mice, protection mediated by HBHA was found to be dependent on the methylation of the c-terminal site of native (n) HBHA but not to the recombinant (r) HBHA without methylation (20). In humans, methylation-dependent T-cell immunity to nHBHA was proposed to be protective against TB, since T lymphocytes from healthy subjects infected with *M. tuberculosis* produce
significant amounts of HBHA-specific interferon-γ (IFN-γ), but T cells from patients with active disease do not (23).

One of the requirements for an effective vaccine against pulmonary TB is that it should be able to induce long-lasting protective immunity in the lungs, where the initial infection takes place. Many investigations have focused on the examination of the mucosal immune responses at the entry port of the mycobacteria and downstream along the respiratory tract, which have profiled the significant strength of vaccine-induced lung immunity against TB (24, 25). Generally, the mucosal route of immunization enhances immune responses on the mucosal sites, provided that antigen is delivered with a strong adjuvant (26). In this context, cholera toxin (CT) and \textit{E. coli} heat-labile enterotoxin (LT) have been described as successful mucosal adjuvants (reviewed in ref. 27), although both are toxic for human use. However, progress has been made to modify CT and LT in a way that could reduce the toxicity but retain the adjuvanticity (28, 29).

Protection against pulmonary TB concerns the development of a new vaccine with improved immunogenicity and protection, which is especially difficult to achieve by vaccinating children after birth. Studies in newborn mice demonstrated that they are biased to a Th2-type of immune response and basically unable to induce a Th1-type of immune response (30, 31). Importantly, BCG is an example of one of the few vaccines that can induce Th1-type immune responses at birth (32), and this might be one of the reasons why BCG is effective in reducing the incidence of childhood TB. Since BCG-mediated protection wanes over time and repeated vaccination with BCG is not effective, strategy for future vaccines could be to focus on the boosting of BCG-induced protective
immune responses and this may be best accomplished by a subunit booster vaccine. Due to the protective capacity of HBHA, we evaluated a heterologous prime-boost protocol by priming with BCG at the neonatal stage and boosting with n- or rHBHA at the systemic or mucosal routes during the infant and adult ages.

We show here that priming with BCG at the neonatal age and boosting with nHBHA later improved protection, which was significantly better than the protection provided by BCG alone. Protection correlated with the enhanced levels of IFN-γ in the respiratory tract.
2. Materials and Methods

2.1. Animals
The studies were performed in neonatal (1-week-old), infant (4 to 6-week-old) and adult (more than 8-week-old) BALB/c mice. Adult mice were purchased from Taconic Europe, Denmark and housed in pathogen free conditions. Neonates were obtained from laboratory breeding facilities followed by timed (hand) mating protocol using adult males and females manually placing them together over night. Three weeks after birth mice were weaned and separated from their mothers. All animals were kept at the Animal Department of the Arrhenius Laboratories, Stockholm University, Sweden. All experiments were done in accordance with the relevant guideline of the animal research ethics board at Stockholm University. Mice were supervised daily and sentinel mice were used to assess and ensure pathogen free conditions in the facility.

2.2. Antigens and adjuvants
Both n- and rHBHA were kindly provided by Dr. Camille Locht, Pasteur de Lille, France. Briefly, nHBHA was extracted from *M. bovis* or *M. tuberculosis* H37Ra and purified by heparin-sepharose chromatography (21), followed by high-performance liquid chromatography (HPLC) as described previously by Masungi et al. (23). rHBHA was expressed in *E. coli* and purified by nickel chromatography as previously described (33). Cholera toxin was obtained from Quadratech Ltd, Surrey, UK.

2.3. Mycobacteria
*M. bovis* BCG (Pasteur strain) obtained from Dr. Ann Williams, UK was grown in Middlebrook 7H9 (DIFCO, Sparks, MD, USA) broth supplemented with albumin-
dextrose-catalase (ADC) enrichment, 0.5% glycerol and 0.05% Tween 80 (vol/vol) for 10-15 days at 37°C. Aliquots frozen in phosphate buffer saline (PBS) with 10% glycerol were kept at -70°C. Three vials picked randomly from the stock were thawed, serially diluted in plating buffer (PBS with 0.05% Tween-80 [vol/vol]) and colony forming units (CFU) counted 2-3 weeks after plating on Middlebrook 7H11 agar (Difco, Sparks, MD, USA), with glycerol and oleic acid-albumin-dextrose-catalase (OADC) enrichment.

2.4. Immunization and challenge

As shown in Fig. 1, neonates at 1-week of age were primed with either BCG or nHBHA formulated with CT administered subcutaneously (s.c.). A total of $10^5$ CFU of BCG or nHBHA (1 µg) formulated with CT (0.2 µg) in 50 µl of phosphate buffer saline (PBS) was administered s.c. at the dorsal neck region. For boosting studies, mice were given n- or rHBHA intranasally (i.n.) or s.c. three times at 2-week interval from the age of 4 weeks. For i.n.-boosting, mice were anesthetized with isofluorane (Baxter Medical AB, Kista, Sweden) and given 5 µg of HBHA together with 1 µg of CT in 20 µl total volume (5 µl per nostril given in two doses). Mice were allowed to breathe the suspension into the lung naturally. For s.c.-boosting, 5 µg of nHBHA was formulated with 1 µg of CT in 100 µl of PBS and administered into the dorsal neck region. Adult mice were vaccinated similarly as mentioned above with five times the neonatal doses. Age-matched control mice received PBS (i.n. or s.c.) or BCG s.c. Some mice were sacrificed one week after the last immunization for in vitro cellular responses. The rest of the mice were infected with BCG i.n. ($10^7$ CFU per animal) four weeks after the last immunization. Three weeks after the challenge, mice were sacrificed for the observation of bacterial burden in the lungs as described previously (34).
2.5. Mononuclear cell isolation

One week after the last immunization with HBHA, lungs and spleens were removed aseptically and placed in sterile PBS. Lungs were cut into small pieces (2-3mm) and incubated for one hour at 37°C with collagenase type I (1 mg/ml, Roche, Mannheim, Germany) and DNase (30 U/ml, Sigma, St. Louis, MO, USA). Lung pieces were then homogenised using a glass homogenizer and the cell suspension was filtered through a 70 µm nylon membrane (BD, Franklin Lakes, NJ USA) and subjected to gradient separation using Lympholyte M (CEDARLANE laboratories, Ontario, Canada). After centrifugation at 1500 g for 20 minutes, the interface was collected and washed twice with PBS. Viable cells were enumerated using trypan blue. Thereafter, cells were cultured in complete DMEM medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM sodium pyruvate (all from Invitrogen, Paisley, UK).

2.6. Generation of bone marrow derived macrophages

Bone marrow derived macrophages (BMM) were generated as previously described (35). Briefly, after sacrifice, the femur and tibia of the hind legs were taken. Bone marrow cavities were flushed with cold, sterile PBS followed by washings and resuspension of the cells in complete DMEM supplemented with 20% L929 cell conditioned medium (as a source of M-CSF). Bone marrow cells were plated in 6-well plates and incubated for 7 days at 37°C, 5% CO2, with replacement of medium every second day. Before use, BMM were washed vigorously to remove non-adherent cells.
2.7. In vitro stimulation of lymphocytes

**Ag stimulation:** Mononuclear cells isolated from the lungs were plated at a concentration of 10^6 cells/ml in 96-well flat bottom plates (Costar, NY, USA) and stimulated with HBHA (5 µg/ml) or Con A (2 µg/ml) for 72 h at 37°C, 5% CO2. Cell culture supernatant was collected and stored at -20°C until tested for cytokines by Enzyme-linked immunosorbent assay (ELISA) assay.

**Stimulation with BCG-infected BMM (BCG-BMM):** It has been described that IFN-γ levels upon in vitro stimulation of lymphocytes from HBHA-immunized animals with BCG- or *M. tuberculosis* pulsed macrophages correlate better to protection against *M. tuberculosis* (19, 20). Macrophages were infected with BCG for 4 h at a multiplicity of infection of 5:1. Extracellular bacteria were removed by vigorous washings and cultures incubated again for 24 h at 37°C. Lung cells were cultured with BCG-BMM for 72 h at a ratio of 10:1. Culture supernatants were collected and analyzed for IFN-γ by captured ELISA.

2.8. IFN-γ ELISA

A commercially available kit for IFN-γ (MABTECH, Stockholm, Sweden) was used to determine the cytokine levels in the culture supernatants according to the manufacturer’s recommendations, with slight modifications. Streptavidin conjugated to alkaline phosphatase (MABTECH, Stockholm, Sweden) was used instead of horseradish peroxidase at 1:1000 dilution. The enzyme-substrate reaction was developed using p-nitrophenyl phosphate (Sigma). Optical density was measured in a multiscan ELISA reader at 405 nm and concentrations were calculated from the standard curves established
with corresponding purified recombinant mouse IFN-\(\gamma\). Data were represented as a stimulation index calculated as a ratio of HBHA or BCG-BMM-stimulated IFN-\(\gamma\) levels to Con A-stimulated IFN-\(\gamma\) levels multiplied by a factor 10.

### 2.9. Intracellular IFN-\(\gamma\) staining

Lymphocytes were isolated from the lungs as described above. Lymphocytes at 10\(^6\) cells/ml were stimulated with HBHA (5 \(\mu\)g/ml), BCG-BMM (10\(^5\) cells/ml) or Con A as a positive control (2 \(\mu\)g/ml, Sigma) for 6 h at 37\(^\circ\)C. GolgiStop (BD), containing monensin was added for the last 4 h of stimulation. Thereafter, cells were washed and incubated with mouse FcBlock (Fc\(\gamma\)II/III, BD). Following washings, cells were stained for surface markers: allophycocyanin conjugated anti-CD3 (eBioscience, San Diego, USA), fluorescein isothiocyanate (FITC) conjugated anti-CD4 (BD) and phycoerythrin (PE) conjugated anti-CD8 (BD). At this stage, cells were permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), and stained for intracellular R-PE-coupled cyanine dye (PE-cy7) conjugated anti-IFN-\(\gamma\) antibody (BD). After washing, cells were resuspended in PBS containing 0.1% sodium azide and analyzed by FACS Calibur.

Detection of CD40L on the cell surface was done by stimulation of cells according to the protocol described previously (36). Briefly, cells were cocultured with PE-conjugated anti-CD40L antibody (BD) in the presence of CD28-specific antibody (BD) during the stimulation with HBHA or BCG-BMM as described above. After stimulation, cells were washed and blocked with Fc receptors (Fc\(\gamma\)II/III, BD) and stained for the surface markers CD3 and CD4 and subsequently permeabilized for intracellular IFN-\(\gamma\) staining as described above. Following washings, cells were resuspended in PBS containing 0.1% sodium azide and analyzed by FACS Calibur.
2.10. Statistics

Comparison between two experimental groups was done by Student’s $t$ test. A p value of <0.05 was considered significant.
3. Results

3.1. Improvement of nHBHA-immunization by neonatal BCG priming

3.1.1. BCG priming improves immune responses to nHBHA boostings

To examine whether BCG vaccination could prime the immune system for the induction of HBHA-specific immune responses, we vaccinated mice at 1-week of age with BCG s.c. and later boosted them s.c. with nHBHA three times at 2-week intervals (Fig. 1). One week after the last immunization, lung lymphocytes were prepared and stimulated in vitro with n- or rHBHA. IFN-γ levels were measured in the culture supernatants as a sign of cell activation. Both the native and recombinant proteins were equally efficient in the in vitro restimulation of lung cells (data not shown). We here present only data obtained with recombinant form of HBHA. Since rHBHA is easier to obtain, and in the following restimulation experiments only rHBHA was used. As shown in Fig. 2A, significantly higher levels of IFN-γ were produced by cells from BCG-primed-nHBHA-immunized animals (BCG/nHBHA) (Gr. 5) compared to the control groups receiving only with PBS (Gr. 1), BCG (Gr. 2) or nHBHA (Gr. 3) alone. Similar results were observed when lung cells were cultured with BCG-BMM (Fig. 2B: Gr. 5). Collectively, these results showed an improved effect of BCG priming on recall immune responses to HBHA.

3.1.2. Effect of BCG as adjuvant

It has been shown in adult mice that nHBHA, contrary to other mycobacterial antigens, induced a degree of protection similar to that provided by BCG. In these experiments HBHA was administered with adjuvant (19, 20). Generally, adjuvants make proteins immunogenic but unfortunately, there are not good adjuvants for human use. The current
ones are either too toxic or not so efficient. Therefore, it was of interest to test the need of adjuvant in our immunization protocol. nHBHA was administered with or without CT to mice primed neonatally with BCG. Lung lymphocytes from the immunized mice were isolated and restimulated in vitro as described above. IFN-γ levels in culture supernatants were determined. Data showed that in the BCG-primed mice, vaccination with nHBHA given together with CT did not result in a significantly higher IFN-γ response compared to nHBHA administered without CT (Fig. 2A, B: Gr. 4 and 5). Our observations suggested that adjuvants might not be necessary provided that mice were primed with live BCG before boosting.

3.1.3. The effect of route of immunization with nHBHA

The i.n. route of immunization preferentially induces better immune responses in the lungs compared to the parenteral immunization (37). In order to examine this issue in our BCG/nHBHA model, we compared the recall immune responses induced after immunization following i.n.- or s.c.-routes. One week after the last immunization with nHBHA, cells were isolated from the lungs and restimulated with rHBHA or BCG-BMM and IFN-γ levels were measured in the culture supernatants. Restimulation with rHBHA induced significantly higher levels of IFN-γ in the i.n. group compared to the s.c. group (Fig. 2A: Gr. 5 and 6). In contrast, IFN-γ responses after BCG-BMM stimulation were comparable in both groups (Fig. 2B: Gr. 5 and 6). Thus, both i.n. and s.c. immunizations may be similar in the stimulation of lung cells at least in the model described here using BCG as a priming agent.
3.1.4. Boosting with nHBHA significantly enhances protection promoted by BCG vaccination

We next examined whether the higher levels of IFN-γ observed in the animals primed with live BCG and boosted with nHBHA under the various conditions mentioned above correlated with higher protection against BCG infection. Four weeks after the last immunization, mice were challenged with a high dose of BCG given i.n. and three weeks later, bacterial numbers in the lungs were enumerated. Our results showed that in all groups, independently of the route of immunization or the co-administration with adjuvant, there was a better protection (Fig. 2C: Gr.4, 5, 6) when the mice were neonatally primed with BCG.

3.2. Identification of cells producing IFN-γ following prime-boost immunization

Priming with BCG followed by boostings with nHBHA i.n. or s.c. significantly improved IFN-γ responses and protection. We next examined the frequency of IFN-γ producing CD4+ and CD8+ T cells. Mice were immunized as described above, and 1-week after the last boost, lymphocytes were isolated from the lungs and stimulated with rHBHA or BCG-BMM for 6 h. Cells were permeabilized and stained for intracellular IFN-γ. As shown in Table 1, upregulation of CD4+IFN-γ+ and CD8+IFN-γ+ cells was observed in both the i.n.- and s.c.-boosted groups. Restimulation with BCG-BMM induced higher percentages IFN-γ producing cells than restimulation with rHBHA. Compared with the BCG-vaccinated control group, i.n. and s.c. groups had higher percentages of CD4+IFN-γ+ and CD8+IFN-γ+ cells. Compared to each other, higher percentages of IFN-γ+ cells were observed in the i.n. immunized mice.
CD40L/CD154 is an activation marker that is expressed transiently mostly by activated CD4\(^+\) T cells (36). To closely assess the proportion of antigen-specific CD4\(^+\) T cells and IFN-\(\gamma\) production in our experimental conditions, we further investigated the proportion of activated CD4\(^+\) T cells in presence of HBHA or BCG-BMM by cell surface staining with anti-CD40L antibody. The proportion of CD4\(^+\) T cells expressing CD40L (33% and 38% respectively upon stimulation with HBHA or BCG-BMM) was higher in the i.n. group compared to the s.c. and BCG-vaccinated groups (Table 2). Intracellular staining for IFN-\(\gamma\) showed that the majority of the CD40L\(^+\)CD4\(^+\) T cells were IFN-\(\gamma\) expressing cells (74% and 80% respectively upon stimulation with HBHA or BCG-BMM, respectively) suggesting that after BCG priming, T cells had a tendency to produce a Th1 type of immune response.

Using a cell depletion assay we next examined the contribution of CD4\(^+\) T cells on IFN-\(\gamma\) production. Lung cells were depleted of CD4\(^+\) T cells using anti-CD4 antibody tagged magnetic beads and cultured with BCG-BMM for 72 hours. Undepleted cells as well as isolated CD4\(^+\) T cells were cultured with BCG-BMM. Levels of IFN-\(\gamma\) in the culture supernatants were detected by ELISA. We observed more than 70% reduction following depletion of CD4\(^+\) T cells from the lung cell suspensions (Fig. 3). Reconstitution of lung cell suspensions by adding CD4\(^+\) T back to the culture resumed IFN-\(\gamma\) levels.

### 3.3. Priming with BCG improves also protection promoted by rHBHA

It has been described previously that both n- and rHBHA were immunogenic in terms of cellular and humoral immune responses but that a protective immune response was only generated by nHBHA (20). The only difference was observed when splenocytes from
immunized animals were restimulated with mycobacteria-pulsed BMM. Under these conditions, significantly more IFN-γ was secreted by the lymphocytes from nHBHA-immunized mice than by those from rHBHA-immunized mice (20). In order to see if neonatal vaccination with BCG could impact on rHBHA-boosters in the generation of protective immune responses, we tested i.n.-rHBHA-immunization in the BCG-primed neonates and compared with the BCG/nHBHA (Fig. 2: Gr. 6, i.n.) and BCG-vaccinated (Fig. 2: Gr. 2) groups. Results showed that restimulation of lung lymphocytes with rHBHA induced similar levels of IFN-γ production in both n- or rHBHA vaccinated groups (Table 3). More interesting, restimulation with BCG-BMM showed increased levels of IFN-γ production in both groups particularly in the rHBHA-boosted group (Table 3), which was previously reported to be an indication of protective immune responses. To test whether this immune response was protective, mice were challenged with BCG i.n. Results showed that there was a remarkable decrease of the bacterial load (51%) in the rHBHA-boosted animals, which was higher, even if not statistically significant than the reduction observed in the BCG-vaccinated (37%) group (Table 3). Taken together, these observations indicate a possible role of protection by rHBHA when given to the BCG-primed animals.
4. Discussion

In this study, we have aimed to improve the protection provided by neonatal vaccination with BCG by additional boosting with the mycobacterial antigen HBHA later in life (infant and adult mice). We chose HBHA because it has been found to be one of the most potent antigens able to provide a protection against *M. tuberculosis* challenge in mice similar to the protection provided by BCG (20). This type of immunization protocol has been successfully used before but with relatively less protective mycobacterial antigens (17, 18). Therefore, we expected to be able to induce even higher levels of protection by using HBHA. Also of interest was to optimise protective immunization in neonates more prone to Th2 type of responses (30, 31). BCG is an immunomodulator that can induce better Th1 type of immune responses than the conventional adjuvant mono phosphoryl lipid A (38). Upon vaccination with BCG, human neonatal immune response to BCG has been found to be Th1 dominated which is characterized by the higher frequencies IFN-γ⁺-CD4⁺ T lymphocytes (32).

Induction of protective immune responses in the respiratory compartment is probably the most effective way to prevent pulmonary TB since the respiratory tract is the natural route of *M. tuberculosis* infection (24, 25). Thus we have tested the efficacy of HBHA on the induction of protection against mycobacterial infection based on the lung immunity. Based on previous studies, our assumption was that for the induction of strong and protective immune responses in the respiratory tract, i.n. route of immunization would be better than s.c. route. We show here that in fact, vaccination of neonatal mice with BCG drives the immune system to Th1 responses, possibly more protective regarding a mycobacterial infection. We also show that the BCG/nHBHA combination, significantly
improved protection against lung infection compared to vaccination with BCG or nHBHA administered separately and that, under these conditions, adjuvant was not required. Moreover, priming with BCG improved protective immune responses even for rHBHA, which was previously reported to be non-protective (20). In contrast to our expectations we found that, provided the animals were primed with BCG, the level of protection was comparable between the i.n. and s.c. routes of immunization.

Increasing attention has recently been paid on BCG prime-boost vaccination strategies not only towards the development of vaccines against TB (17, 18) but also against other diseases (39-42). It is known that live BCG, but not killed, is a good immunostimulant (43). It has been proposed that the viability of BCG may be crucial for the induction of immune responses because live BCG but not killed could migrate to the draining lymph nodes, which results in an increase in mononuclear cells in the lymph nodes and expression of inducible nitric oxide synthase (iNOS) both in the lymph nodes and at the site of infection (43). Consistent with the notion that BCG can act as an adjuvant, we observed an immunostimulatory and/or adjuvant role of BCG in the mice vaccinated with BCG/nHBHA, where addition of CT did not show any effect on the immune response and protection. Apart from the adjuvant effect, we show here that BCG vaccination primes the immune system for nHBHA. There are two possible explanations for this: first, immunization of the BCG-primed mice with nHBHA may amplify HBHA-specific immunity because BCG itself contains nHBHA protein; second, live-BCG probably acts as an immune adjuvant, enhancing the immunogenicity of HBHA.
Since BCG priming shows immunostimulatory or adjuvant effects regarding nHBHA, we asked if this immunization protocol could also have an effect on rHBHA, not only enhancing immune responses but also providing higher levels of protection. We found that IFN-\(\gamma\) levels upon stimulation of lung cells with BCG-BMM were increased and that protection was also higher in the BCG/rHBHA compared to the BCG group. The mechanisms behind the improved immune responses and protection in the rHBHA-boosted mice are not clear to us. In this context, it has been shown in an \textit{in vitro} experiment that stimulation of lymphocytes from \textit{M. tuberculosis}-infected healthy individuals with methylated peptide together with rHBHA increased IFN-\(\gamma\) production (20). This was assumed to be a carrier protein effect on immunogenicity. In our \textit{in vivo} situation, we speculated that rHBHA probably acted as a carrier molecule for the methylated peptides generated after BCG priming and therefore it could enhance protective immune responses specific to the methylated epitope.

Generally i.n. immunization with protein antigens induces higher immune responses and better protection in the lungs than systemic immunization (25). In contrast to this general experience, we have observed in the present study that administration of BCG improved immune responses and protection regardless of the route of administration of HBHA. Recently, a comparative study between i.n. and intramuscular (i.m.) boosting of BCG-primed mice showed that both routes induced similar frequencies of IFN-\(\gamma^+\)CD4\(^+\) T cells in the lungs (44). However, the i.n. route of vaccination showed better protection than the i.m. route. In contrast, we observed that i.n. boosting with HBHA induced higher frequencies of IFN-\(\gamma^+\)CD4\(^+\)- and CD8\(^+\)-T cells than the s.c. boosting in response to BCG-BMM, as observed by the flow cytometric analysis of intracellular staining for IFN-\(\gamma\).
However, IFN-\(\gamma\) levels measured by ELISA were similar in both groups. Thus, these results might indicate that the capacity of the cells to produce IFN-\(\gamma\) is more important than the higher proportion of cells, which probably correlates with the protection.

The importance of CD4\(^+\) and CD8\(^+\) T cells in protection has been evaluated in several studies (45-47). In fact, IFN-\(\gamma\) producing CD4\(^+\) T cells were found important in the early phases of the acquired immune response (48) and in absence of CD4\(^+\) T cells, \(M.\) \textit{tuberculosis} infection caused severe pathology and reduced survival time (47). Consistent with this notion, we show in this study that after priming with BCG, the majority of the activated CD4\(^+\) T cells were driven to the IFN-\(\gamma\) production as demonstrated by the detection of CD40L expression. We speculate that the primary vaccination with BCG is critical, and favors the establishment of a strong Th1 type of immune response.

In conclusion, this study has shown the importance of BCG priming in the development of protective vaccines against mycobacterial infection. Neonatal BCG vaccination is fairly effective against severe forms of TB but not consistent with the level of protection against pulmonary TB and unable to provide sufficiently long memory. Therefore, if the HBHA boosting can improve the BCG derived protection, this would have a significant impact on the future TB vaccine development.
Acknowledgments

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Table 1. IFN-γ producing CD4/CD8 lung cells

<table>
<thead>
<tr>
<th>Prime</th>
<th>Boost</th>
<th>IFN-γ producing cells (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD4</td>
<td>CD8</td>
</tr>
<tr>
<td>BCG</td>
<td>-</td>
<td>5.12</td>
<td>3.03</td>
<td>6.11</td>
</tr>
<tr>
<td>BCG</td>
<td>nHBHA+CT (i.n.)</td>
<td>7.98 (56)</td>
<td>3.58(18)</td>
<td>9.64(58)</td>
</tr>
<tr>
<td>BCG</td>
<td>nHBHA+CT (s.c.)</td>
<td>5.66 (11)</td>
<td>3.00(0)</td>
<td>7.39(21)</td>
</tr>
</tbody>
</table>

In vitro restimulation was done for 6 h in presence of GolgiStop followed by surface staining of CD3, CD4 and CD8 markers. After the restimulation, cells were permeabilized and stained for intracellular IFN-γ expression. Data represent pooled samples from 3 animals per group. Cells producing IFN-γ were expressed as percentage of total lymphocytes. Values shown in the parentheses are increased cell population by HBHA immunization over BCG control group. (HBHA-immunized – BCG-immunized)×100/ BCG-immunized
Table 2. IFN-γ\(^{+}\)CD40L\(^{+}\) cells within CD4\(^{+}\) T cells

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>(^{1})rHBHA</th>
<th>(^{1})BCG-BMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prime</td>
<td>CD40LCD4</td>
<td>IFN-γCD40LCD4</td>
</tr>
<tr>
<td>Boost</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCG</td>
<td>22</td>
<td>60</td>
</tr>
<tr>
<td>BCG</td>
<td>nHBHA+CT (i.n.)</td>
<td>33</td>
</tr>
<tr>
<td>BCG</td>
<td>nHBHA+CT (s.c.)</td>
<td>24</td>
</tr>
</tbody>
</table>

\(^{1}\)In vitro restimulation was done for 6 h in presence CD40L antibody and GolgiStop followed by surface staining of CD3 and CD4 markers. After the restimulation, cells were permeabilized and stained for intracellular IFN-γ expression. Data represents pooled samples from 3 animals per group. Cell expresses CD40L was represented as percentage of total CD4\(^{+}\) T lymphocytes and cell expresses IFN-γ was represented as percentage of total CD40L\(^{+}\)CD4\(^{+}\) T lymphocytes.
Table 3. Immune response and protection induced by rHBHA immunization in the BCG-primed animals

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>IFN-γ index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CFU counts&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% of reduction&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prime</td>
<td>Booster</td>
<td>rHBHA</td>
<td>BCG-BMM</td>
</tr>
<tr>
<td>1&lt;sup&gt;BCG&lt;/sup&gt;</td>
<td>-</td>
<td>0.74±0.47</td>
<td>0.96±0.22</td>
</tr>
<tr>
<td>1&lt;sup&gt;BCG&lt;/sup&gt;</td>
<td>nHBHA+CT (i.n.)</td>
<td>4.71±1.41**</td>
<td>3.03±0.84**</td>
</tr>
<tr>
<td>BCG</td>
<td>rHBHA+CT (i.n.)</td>
<td>4.33±1.04**</td>
<td>1.79±0.39**</td>
</tr>
</tbody>
</table>

<sup>a</sup>PProaming with BCG and boosting with rHBHA improve protective immunity. One week after the last immunization, cells from the lungs were isolated and stimulated in vitro with rHBHA or BCG-BMM for 72 h. IFN-γ levels were determined in the culture supernatants. IFN-γ index is a ratio of HBHA-stimulated IFN-γ levels to Con A-stimulated IFN-γ levels multiplied by a factor 10.

<sup>b</sup>Four weeks after the last immunization mice were challenged with 10<sup>7</sup> CFU of BCG given i.n. Three weeks postchallenge, bacterial load in the lungs was enumerated. Data represents mean ±sd.

<sup>1</sup>Data were taken from Fig. 2 A and B

<sup>2</sup>Percent of reduction of CFU was calculated with respect to the unimmunized group in Fig. 2C.

**p<0.05 and *p=0.18, when compared with BCG group.
Figure legends

Figure 1. Schematic representation of vaccination schedule. Neonatal mice were primed with BCG or nHBHA s.c. and boosted later with n- or rHBHA (A). Adult mice were primed with BCG and boosted later with nHBHA (B). One week after booster vaccination, some of mice were sacrificed and lung-derived cells were tested for immunogenicity. The rest of the mice were challenged with BCG administered i.n. 4-week after the last immunization. Challenged mice were killed after 3-week and bacterial counts were determined in the lungs by colony forming unit (CFU) assay.

Figure 2. Strong immune response and protection by nHBHA-immunization in the BCG-primed animals. IFN-γ levels after in vitro restimulation of lymphocytes with HBHA (A) and BCG-BMM (B). Mice at 1-week were immunized with BCG s.c. nHBHA boosters were formulated with or without CT and administered via i.n./s.c route thrice at two weeks interval after weaning at 3 weeks of age. Lung lymphocytes from immunized or unimmunized animals were cultured with HBHA or BCG-BMM for 72 h and IFN-γ levels in the culture supernatants were measured. Data for IFN-γ shows mean stimulation index with s.d. calculated as a ratio of HBHA or BCG-BMM-stimulated IFN-γ levels to Con A-stimulated IFN-γ levels multiplied by a factor 10. Cells were pooled from four animals per group. Data represents mean±sd of triplicate wells. C, protection against i.n.-BCG challenge compared with unimmunized and BCG controls. Four weeks after the last immunization, mice were challenged with BCG i.n. Three weeks postchallenge, bacterial load in the lungs was determined. Graph shows bacterial counts from individual animal
with mean of ten animals per group. Results were analyzed from two independent experiments. \( p \) value(s) was calculated by comparing two groups using \( t \) test. *, \( p<0.05 \).

**Figure 3.** CD4\(^+\) T cells are the major source of IFN-\( \gamma \) production. Adult mice were primed with BCG (5\( \times \)10\(^5\) CFU) s.c. at dorsal region of the neck and were rested for 3 weeks. Afterward, mice were immunized three times i.n. at 2 weeks interval with nHBHA (5 \( \mu \)g/animal) formulated with CT (1 \( \mu \)g/animal). Animals primed with only BCG were considered as a control. One week after the last immunization, lung lymphocytes from the immunized mice were collected and allowed for magnetic depletion of >95\% CD4\(^+\) T cells. Coculture experiment of lymphocytes and BCG-BMM (10:1) showed removal of the CD4\(^+\) T cells are major IFN-\( \gamma \)-producers as detected in ELISA. Depletion of CD4\(^+\) T cells affected IFN-\( \gamma \) production. IFN-\( \gamma \) levels were resumed in the coculture of the lymphocytes reconstituted with CD4\(^+\) T cells and BCG-BMM. Data represents mean\( \pm \)s.d. of triplicate wells prepared from the pooled samples of 4 animals per group. \( p \) value(s) was calculated by comparing two groups using \( t \) test. *, \( p<0.05 \).
BCG or nHBHA (s.c.)

**W0**  **W3**  **W5**  **W7**  **W8**  **W11**  **W14**

**Immunogenicity**  **Challenge**  **Lung CFU count**

**Neonates**

n- or rHBHA (i.n/s.c.)

**Fig. 1**

**Adults**

**BCG (s.c.)**

**W0**  **W3**  **W5**  **W7**  **W8**

**Immunogenicity**

nHBHA (i.n.)
Fig. 3

BCG
BCG/nHBHA+CT

Undepleted CD4+T-depleted re-constituted with CD4+T

IFN-γ (pg/ml)