Immune evasion and identification of biomarkers associated with mycobacterial infection

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SUMMARY

Over 90% of the two billion people infected with *M. tuberculosis* are able to contain the infection without developing disease although the pathogen is not completely eliminated. Whether or not infection will lead to development of disease depends on the outcome of a complex interaction between the pathogen and the host’s immune response. Therefore, deepening our understanding of the pathogen-host interactions, especially in the lungs and its microenvironment will facilitate the design of superior vaccines or drugs against mycobacterial infections. Moreover, understanding the immune response generated could facilitate in distinguishing acute from latent infection or immunization and serve as a non invasive tool for diagnosis of tuberculosis (TB).

The aims of these studies were, first to investigate TLR signalling as an evasive mechanism for mycobacteria survival in macrophages. Second we aimed to study the immune response generated in mice after mycobacterial infection and to identify immunological parameters (biomarkers) which could be used for a non invasive, immune based diagnosis of infection. In the first paper, we demonstrate that prolonged TLR2 but not TLR4 signalling interferes with IFN-γ mediated killing of ingested mycobacteria by murine macrophages. TLR2 signalling did not affect the proliferation of macrophages or induce antimycobacterial activity. In terms of mechanisms, neither TNF production nor NO secretion was significantly affected after TLR2 ligation. Finally, we show that the refractoriness induced after TLR2 signalling could be reversed with increasing concentrations of IFN-γ.

In the second manuscript, we show that there is a positive relationship between the bacteria load in the lungs and secretion of soluble TNF receptors (sTNFR) in the broncho-alveolar lavage (BAL). We found that unlike the systemic, the immune
response in the lungs was very much dependent on the presence of live bacteria. Moreover, mycobacteria infection induced IgA antibody production in BAL but not serum. Finally, we show that the pattern of the immune response in C57BL/6 mice, known to have a lower susceptibility to mycobacterial infections was similar to that of BALB/c mice.
LIST OF PAPERS

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


*Authors contributed equally to this work
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<tbody>
<tr>
<td>BAL</td>
<td>Broncho-alveolar lavage</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<td>BMM</td>
<td>Bone marrow macrophages</td>
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<td>CDC</td>
<td>Centers for Disease Control</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>CW&lt;sub&gt;BCG&lt;/sub&gt;</td>
<td>BCG cell wall</td>
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<tr>
<td>CW&lt;sub&gt;M.vaccae&lt;/sub&gt;</td>
<td>M. vaccae cell wall</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DC-SIGN</td>
<td>DC-specific intercellular adhesion molecule-3-grabbing nonintegrin</td>
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<tr>
<td>DOTS</td>
<td>Directly observed treatment-short course</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>hk-BCG</td>
<td>Heat killed BCG</td>
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<tr>
<td>i.n.</td>
<td>Intranasal</td>
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<tr>
<td>i.m.</td>
<td>Intramuscular</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>IL-12</td>
<td>Interleukin 12</td>
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<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
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<tr>
<td>NK-T</td>
<td>Natural killer T cells</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
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<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PPD</td>
<td>Purified protein derivative</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediate</td>
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<td>ROI</td>
<td>Reactive oxygen intermediates</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
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<td>sTNF</td>
<td>Soluble tumor necrosis factor</td>
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<tr>
<td>TACE</td>
<td>Tumor necrosis factor converting enzyme</td>
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<tr>
<td>TACO</td>
<td>Tryptophan aspartate rich coat protein</td>
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<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TmTNF</td>
<td>Transmembrane tumor necrosis factor receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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INTRODUCTION

Tuberculosis

Tuberculosis (TB), also known as the 'white plague' and human immunodeficiency virus (HIV) are the major infectious killers of adults in the developing world, and about 13 million people are infected with these two pathogens. The global epidemic of TB results in 8-10 million new cases every year, with an annual projected increase rate of 3%. It is estimated that between 5 and 10% of immunocompetent individuals are susceptible to TB, of which, 85% develop pulmonary disease. In 1993 and also 2002, the World Health Organization (WHO) declared TB a global public health emergency. In 2002, the number of new cases of TB was projected to reach 12 million annually by the year 2006 if existing control efforts were not strengthened. The resurgence in the incidence of TB in the last two decades has been attributed to the emergence of multi-drug resistant strains of Mycobacterium tuberculosis, the causative organism of TB, co-infection with the HIV and immigration of infected persons from TB prevalent to less prevalent areas.

Mycobacterium tuberculosis complex

The M. tuberculosis complex is the cause of TB and is comprised of M. tuberculosis, M. bovis, M. africanum, M. canettii and M. microti. The mycobacteria grouped in the complex are characterised by 99.9% similarity at the nucleotide level and identical 16S rRNA sequences, but differ widely in terms of their host tropisms, phenotypes, and pathogenicity. Some are exclusively human pathogens (M. tuberculosis, M. africanum, M. canettii) or rodent M. microti whereas M. bovis have a
wide host spectrum \cite{10}. All members of the complex are slow-growing, with generation time ranging from 12 to 24 hrs depending on environmental and microbial variables.

**Mycobacterial infections**

*M. tuberculosis* is an obligate, aerobic, intracellular pathogen, which has a predilection for the lung tissue rich in oxygen. TB occurs almost exclusively from inhalation of droplet nuclei containing *M. tuberculosis*, which disperse primarily through coughing, singing and other forced respiratory maneuvers by a person with active pulmonary TB. Normally, repeated exposure to a TB patient is necessary for infection to take place. Inhaled droplets are deposited in the alveolar spaces, where the bacteria are taken up by phagocytic cells, mainly alveolar macrophages, event of which induces a rapid inflammatory response and accumulation of cells. A number of studies addressing the macrophage surface receptors involved in *M. tuberculosis* uptake have shown that complement receptors and complement opsonization of mycobacteria make up the major route of entry \cite{11, 12}. Other receptors have been shown to interact with mycobacteria: mannose receptors \cite{11}, surfactant protein A (Sp-A) and its receptors, scavenger receptor class A and CD14 (reviewed in \cite{12}). The mode of entry into macrophages is considered as predetermining the subsequent intracellular fate of mycobacteria. However, experiments have shown that blocking individual receptors does not significantly alter *M. tuberculosis* intracellular trafficking \cite{12}.
After *M. tuberculosis* has entered the lung, one of four potential fates might occur (Dannenberg, Jr., 1994):

i. The initial response can be effective in the killing and elimination of the bacilli, and these individuals do not develop TB at any time point in the future.

ii. The bacilli can grow and multiply immediately after infection, causing clinical disease (primary TB).

iii. The bacilli may become dormant and never cause disease at all, resulting in a latent infection that is manifested only as positive tuberculin skin test (latent TB)

iv. The dormant bacilli can eventually begin to grow with resultant disease (reactivation TB).

**Pathogenesis of tuberculosis**

There are two major patterns of TB:

Primary tuberculosis: seen as an initial infection, usually in children. The initial focus of infection is a small subpleural granuloma accompanied by granulomatous lymph node infection, together known as the “Ghon complex”. In nearly all cases, these granulomas resolve and there is no further spread of the infection.

Secondary tuberculosis: seen mostly in adults as a reactivation of previous infection (latent TB) or reinfection, particularly when health status declines. The granulomatous inflammation is much more florid and widespread. Typically, the upper lung lobes are most affected, and cavitation can occur.

Dissemination of tuberculosis outside the lungs (extrapulmonary TB) is more common in children and HIV infected individuals leading to the appearance of a number of uncommon findings with characteristic patterns: skeletal TB, involves
mainly the thoracic and lumbar vertebrae also known as Pott's disease, genital tract TB involves the fallopian tube, prostate and epididymis. Others are: urinary tract TB, TB of the central nervous system, cardiac TB and scrofula (lymphadenitis TB) \(^{15}\).

**Receptor signalling by *M. tuberculosis***

One of the earliest indications that the body has been infected with an invading microbe is the activation of signaling pathways upon recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) \(^{16}\) (Figure 1).

\[\text{Adapted from Nature immunology 2001}\]

**Figure 1:** TLRs and their ligands. TLR4 signals through MyD88-dependent and independent pathways.
Although T cells provide the crucial element of specificity, the immune response is regulated by the level of danger posed by the infection, which is sensed primarily by innate immune mechanisms. TLRs are expressed on many cells, including phagocytes, and mediate the activation of cells of the innate immune system, resulting in destruction of the invading microorganism through activation of several signalling cascades. TLRs signal either in a MyD88-dependent or -independent manner, leading to the nuclear translocation of nuclear factor-κB.

In vitro analyses of the responses of murine and human macrophages to *M. tuberculosis* infection indicate that these cells produce a robust proinflammatory response through the activity of TLR agonists (stimulators of the host's TLRs) that are abundant on the surface of the bacteria. These components can activate cells through heterodimers of TLR1 and TLR2, as well as through TLR4 and TLR6. The exact role of these TLRs in vivo remain to be established and might be dependent on the actual dose, administration route or the animal model in which it is tested.

Stimulation of TLR2 by lipoproteins triggers a proinflammatory response, which can promote mycobacterial killing, but also reduce antigen presentation through interference with IFN-γ signalling or promote apoptosis of infected cells. Other signals also contribute to the proinflammatory response; TLR-1/TLR6 and TLR4 have been implicated in responses to *M. tuberculosis*. It was recently demonstrated that nucleotide-binding oligomerization domain 2 (NOD2) is a nonredundant PRR of *M. tuberculosis*, which synergizes with TLRs in stimulation of cytokine production by phagocytic cells. Furthermore, mannose-capped lipoarabinomannan (LAM), a component of *M. Tuberculosis* cell wall, can deliver anti-inflammatory signals through dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), a C-type lectin receptor on DCs, thereby reducing antimycobacterial
activity and stimulating the release of IL-10. TLR signalling also triggers differentiation of monocytes into macrophages and DCs, generating the cellular populations necessary for a potent innate and adaptive immune response.

**Immune responses to *M. tuberculosis* infection**

*Macrophages*

Macrophages play a central effector role in the immune response to *M. tuberculosis* infection. Once infected by the bacterium, macrophages present antigens on both class I and II major histocompatibility complex (MHC) to T cells, which in turn secrete IFN-γ resulting in activation of the macrophages to kill the bacteria. Tumor necrosis factor (TNF), is an important proinflammatory cytokine secreted by activated monocytes/macrophages, which synergizes with IFN-γ to induce antimycobacterial effects of murine macrophages *in vitro*. Furthermore, the proinflammatory cytokines IL-1 and IL-6 secreted during inflammation play an important role in recruitment of cells to the site of infection. A major effector mechanism responsible for the antimycobacterial activity of IFN-γ and TNF is the induction of nitric oxide (NO) and related reactive nitrogen intermediates (RNIs) by macrophages via the action of the inducible form of nitric oxide synthase (NOS). Whereas the antimycobacterial property of RNI is well documented both *in vitro* and *in vivo* in the murine model, there has been conflicting data on the role of RNI in human TB. However, recent data support a protective role for these reactive intermediates in humans TB as well. Other antimycobacterial mechanisms of macrophages are; phagolysosome fusion, a process which exposes ingested bacteria in the phagosome to
lytic enzymes in the lysosome \(^{34}\); apoptosis \(^{24}\) of infected macrophages which removes the niche for growth and therefore restricts multiplication of bacteria.

**Dendritic cells**

It is now established that DCs are also involved in an effector role against *M. tuberculosis* infection \(^{35, 36}\), and are central to the generation of acquired immunity after carriage of antigens to draining lymph nodes, where recognition by T cells can be maximized \(^{36, 37}\). The interaction of structural components of mycobacteria with DC-SIGN has been reported as one of the major examples of how this receptor can influence DC function \(^{26}\). The immune response limiting and switching off infection during primary TB is presumably initiated when upon exposure to *M. tuberculosis*, the efficient antigen-capturing immature DCs \(^{38}\) are transformed into mature T cell stimulating DCs, which migrate with high efficiency into draining lymph nodes. In these compartments, the stimulatory capacity of mature DCs ultimately leads to effector T cell differentiation and memory T cell expansion, which in turn, confer protection against *M. tuberculosis* in the lungs \(^{39, 40}\).

**Formation of granuloma**

The granulomatous response is the hallmark of chronic *M. tuberculosis* infection, which is a desperate attempt by the host immune system to contain multiplication and further dissemination of bacteria to other organs. It is postulated that stimulated alveolar macrophages in the airways invade the lung epithelium following internalization of inhaled bacteria \(^{41-43}\). Production of TNF and inflammatory chemokines from infected macrophages drive the recruitment of successive waves of neutrophils, natural killer (NK) T cells, CD4\(^+\) T and CD8\(^+\) T
cells, DCs and B cells, each of which produce their own complement of cytokines that amplify cellular recruitment and remodelling of the infection site 41-43.

This inflammatory cascade is regulated and superceded by a specific, cellular immune response that is linked to the production of IFN-γ. At this stage, formation of the 'stable' granuloma responsible for immune containment during latent or subclinical infection becomes recognizable and stratification of the structure emerge 44, 45. The granuloma subsequently develops central areas of necrosis (called caseum, from the word ‘cheese’), resulting in the death of the majority of bacteria and destruction of the surrounding host tissue. The surviving bacilli exist in a latent state and can become reactivated leading to development of active disease. The granuloma serves 3 major purposes; it is a local environment in which immune cells can interact to kill bacteria, a focus of inflammatory cells that prevent inflammation from occurring throughout the lungs, and a barrier to dissemination of bacteria throughout the lungs and other organs 43. Disruption of the granuloma structure or function appears to be detrimental to the control of bacterial replication and the control of immunopathology in the lung.

**CD4⁺ T cells**

Although various cells contribute to immunity against *M. tuberculosis*, T cells, notably effector CD4⁺ T cells play a dominant role 69. *M. tuberculosis* resides primarily in a vacuole within the macrophage resulting in MHC II presentation of mycobacterial antigens to CD4⁺ T cells. Upon activation, CD4⁺ T cells secrete IFN-γ and TNF, which in turn induce antimycobacterial mechanisms in macrophages 28. Studies in mouse models deficient in CD4⁺ T cells demonstrated clearly that the CD4⁺ T cell subset is required for the control of infection 70. Other roles played by CD4⁺ T
cells include induction of apoptosis suggested to be important in controlling *M. tuberculosis* infection, conditioning of antigen-presenting cells, help for B cells and CD8\(^+\) T cells, and production of other cytokines. CD4\(^+\) T cells can also contribute to the control of acute mycobacterial infections through IFN-\(\gamma\) independent mechanisms, which have been demonstrated in experimental models using antibody depletion or mouse strains deficient in either CD4 or MHC class II molecules.

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

It has been demonstrated that mycobacterial antigens derived from infected cells can be presented by MHC I to CD8\(^+\) T cells in humans and in mice, and antigens recognized by these cells have been identified. CD8\(^+\) T cells also recognize various antigens from *M. tuberculosis* that are not presented by classical MHC I molecules, but by a closely related group of molecules, the Class Ib molecules. These are non-polymorphic, and include the CD1 molecules (reviewed in) as well as H2-M3. CD1 molecules primarily present lipid antigens from *M. tuberculosis* to CD8\(^+\) T cells, thereby increasing the possible antigen source greatly. In humans, CD8\(^+\) T cells can kill intracellular mycobacteria via the release of the antimicrobial peptide granulysin; however, this molecule is not present in the mouse. The fact that no mouse analog of granulysin exists may in part explain why CD8\(^+\) T cells are not as important in the control of infection in mouse models of TB.

The cytotoxic potential of CD8\(^+\) T cells to kill infected cells (Cytotoxic T cell; CTL activity) in vivo has been shown to be dependent on CD4\(^+\) T cells in the mouse model, suggesting that the susceptibility of CD4\(^+\) T cells knockout mice to *M. tuberculosis* infection might be due in part to impaired CTL activity. CD8\(^+\) T cells
also produce cytokines (IFN-γ and TNF) during *M. tuberculosis* infection, which probably participates in activation of macrophages.

*B cells and antibodies in *M. tuberculosis* infection*

Historically, the view that protective immunity against TB is mediated exclusively by T cells, involving cytokines, mainly IFN-γ-mediated activation of infected macrophages, rather than antibodies had determined all strategies of TB vaccine research. This view has been sustained by the knowledge that antibodies cannot reach the bacilli within the phagosomes of infected macrophages ⁷⁸. However, the fact that TB develops despite the presence of abundant T helper immunity ⁷⁹, coupled with the observation that T-cell targeted vaccination does not always induce optimal protection either in humans or in experimental animals have made it necessary to investigate alternative immune mechanisms of protection ⁸⁰. To this end, the protective role of antibodies in TB has been elucidated recently using modern approaches and tools (reviewed in ⁸¹, ⁸²). Role for B cells in protection against *M. tuberculosis* infection was suggested on grounds of raised bacterial load in the organs of mice genetically depleted of B cells (µ chain knockout) or defective for IgA production ⁸³-⁸⁵. The possible role of antibodies in humans to natural course of *M. tuberculosis* infection was indicated in clinical studies, which reported higher antibody titres to lipoarabinomannan (LAM) or Ag85 in patients with milder forms of active tuberculosis ⁸⁶.
Cytokines and *M. tuberculosis* infection

**IL-12**

*M. tuberculosis* infection results in the induction of a large number of cytokines, and a subset of these have been demonstrated to be essential for control of the infection. Immunologic control of *M. tuberculosis* infection is based on a type 1 T-cell response. Production of interleukin 12 (IL-12) by *M. tuberculosis*-infected DCs is essential for the priming of potent Th1 responses characterized by IFN-γ production by CD4⁺ and CD8⁺ T cells. Mycobacteria are such strong IL-12 inducers that mycobacterial infection can skew the response to a secondary antigen towards a Th1 phenotype. IL-12 is a crucial cytokine in controlling *M. tuberculosis* infection. For example, exogenous administration of IL-12 to BALB/c mice can prolong survival, and IL-12 deficient mice are susceptible to *M. tuberculosis* infection. Humans with mutations in IL-12p40 or the IL-12 receptor genes present with reduced but not absent IFN-γ production, and are more susceptible to mycobacterial infections (reviewed in). It has been shown that the administration of IL-12 DNA could substantially reduce bacterial numbers in mice with a chronic *M. tuberculosis* infection, suggesting that the induction of this cytokine is an important factor in the design of tuberculosis vaccines.

**IFN-γ**

IFN-γ is central to the control of *M. tuberculosis* infection. This cytokine is produced by CD4⁺, CD8⁺ T cells and NK(T) during *M. tuberculosis* infection, and is important in macrophage activation and perhaps other functions. Individuals defective in genes for IFN-γ or IFN-γ receptors are susceptible to serious
mycobacterial infections, including *M. tuberculosis* ⁵¹. In a large study, it was reported that patients with IFN-γ receptor deficiency presented disseminated infection with *M. bovis* BCG or environmental mycobacteria, which in some cases resulted in death of about half of the patients and required continuous antimycobacterial treatment in the survivors (reviewed in ⁵⁴). In mice, IFN-γ knockout strains are the most susceptible to virulent *M. tuberculosis* infection ⁵⁵; with defective macrophage activation and low NOS2 expression ⁵⁵, ⁵⁶.

*M. tuberculosis* has developed mechanisms to limit the activation of macrophages by IFN-γ ¹⁹-²³, suggesting that the amount of IFN-γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine. In this regard, it has been shown that the level of IFN-γ produced by a mouse in response to a candidate vaccine does not always correlate with the effectiveness of the vaccine during *M. tuberculosis* challenge ⁵⁷. Similarly, evaluation of the efficacy of human BCG vaccination using several assays demonstrated that mycobacterial growth inhibition did not correlate with IFN-γ response ⁵⁸. Thus, although IFN-γ is essential for the development of an immune response that prolongs the life span of an infected animal, it is not sufficient to eliminate an *M. tuberculosis* infection.

**TNF and soluble TNF Receptors**

The importance of TNF in the generation and maintenance of a protective immune response against *M. tuberculosis* and a host of other bacterial and viral pathogens has been clearly demonstrated ⁵⁹-⁶¹. Although TNF is not required for the generation of an antigen-specific T cell response, it is essential for controlling the recruitment of inflammatory cells to sites of infection and the development of a
protective granulomatous response, resulting in containment of bacilli growth and survival of infected animals \textsuperscript{43,61-63}. During \textit{M. tuberculosis} infection, TNF is involved in almost every stage of the inflammatory response, from the initial macrophage response, to the attachment, migration, and trafficking of leukocytes through blood vessels, and retention at the site of infection \textsuperscript{64}. TNF is produced primarily by activated monocytes/macrophages in response to pathogens \textsuperscript{65}, but can also be expressed by activated T cells, B lymphocytes, NK cells, and some tumour cells.

TNF is first synthesized as a transmembrane (TmTNF) precursor and cleaved by membrane-bound metalloprotease disintegrin, including tumor necrosis factor converting enzyme (TACE), generating a soluble TNF molecule \textsuperscript{66}. Both forms of TNF function physiologically by interacting with one of two receptors; TNFR1 (55 kDa) and TNFR2 (75 kDa) expressed on a diverse range of cell types \textsuperscript{65}. Upon stimulation, these receptors could be cleaved from the cell surface, or directly expressed as soluble isoforms lacking the transmembrane domain. TNF mainly binds to TNFR1 while the TmTNF binds to TNFR2 \textsuperscript{67,68}. Mice deficient in TNF or sTNFR1 succumbed quickly to \textit{M. tuberculosis} infection, with substantially higher bacterial burdens compared to their wild type (WT) counterparts \textsuperscript{59}. TNFR1 signalling is required for the modulation of T-cell response because in TNFR1-deficient mice, T-cell dependant granuloma decomposition is observed \textsuperscript{63}, while TNFR2 seems to have a lesser role in granuloma formation and mycobacterial immunity. sTNFR neutralization of TNF is important for homeostasis, since excessive production could lead to exaggerated inflammation resulting in immunopathologies.
Mucosal immunity

Mucosal immunization has received increasing attention because the respiratory tract is the natural route of *M. tuberculosis* infection, and it is believed that mucosal vaccination provides the best protection from mucosal infectious diseases. Emerging evidence suggest that respiratory mucosal vaccination provides better immune protection against pulmonary TB than parenteral vaccination. For example, respiratory mucosal immunization uniquely elicited higher numbers of antigen-specific CD4+ and CD8+ T cells in the airways capable of IFN-γ production, cytotoxic lysis of target cells, and immune protection against *M. tuberculosis* infection. In comparison, parenteral intramuscular (i.m.) immunization led to activation of T cells, particularly CD8+ T cells, in the peripheral lymphoid organs, but failed to elicit airway luminal T cells or protect the lung from *M. tuberculosis* infection. The immunoprotective role of mucosally induced IgA or passively administered IgA against *M. tuberculosis* infection has been demonstrated.

Immune evasion

Hosts infected with *M. tuberculosis* mount a strong immune response, eliciting CD4+ and CD8+ T cells as well as antibodies specific for mycobacterial antigens. Although this response is usually sufficient to prevent progression to active disease, the microorganism persists in the host. Thus, the strong immune response can control, but not eliminate the infection, indicating that *M. tuberculosis* has evolved mechanisms to modulate or avoid detection by the host. *M. tuberculosis* persist within macrophages through a variety of immune evasion strategies:
Entry into macrophages via multiple receptors

Entry of mycobacteria into phagocytic cells can occur through binding to multiple receptors, all leading to the delivery of the bacilli into macrophage phagosomes. Although the precise receptor that mediates mycobacterial uptake \textit{in vivo} is yet to be established, multiple molecules have been shown to trigger phagocytosis \textit{in vitro} (Reviewed in \textsuperscript{12}).

Manipulation of the phagosome

Phagocytosis of pathogenic microorganisms by “professional” phagocytes such as macrophages and neutrophils is the first step in their eventual degradation, as the phagosome eventually matures into a phagolysosome rich in acid hydrolases with degradative and microbicidal capacity. When normal phagolysosome fusion occurs, the bacteria could encounter a hostile environment that include acid pH, reactive oxygen intermediates (ROI), lysosomal enzymes and toxic peptides. To persist in the host, \textit{M. tuberculosis} arrests the maturation of bacilli-containing phagosomes into phagolysosomes \textsuperscript{91, 92}. Another mechanism by which mycobacteria could interfere with phagolysosomal fusion is by retention of host protein TACO (tryptophan aspartate rich coat protein, also known as coronin 1) on the phagosome \textsuperscript{34}, thereby behaving as self antigens. For example, J774 macrophages containing live, but not dead BCG were associated with the TACO protein.

Avoidance of the toxic effects of reactive nitrogen intermediates

The most comprehensively studied antimycobacterial mechanism of activated macrophages is the nitric oxide synthase 2 (NOS2)-dependent pathway, which generates NO and other RNI toxic to mycobacteria \textsuperscript{93}. Mice deficient in RNI
displayed markedly enhanced susceptibility to *M. tuberculosis* infection. Although NOS2 dependent NO and RNI are essential for containment of *M. tuberculosis*, infection persists in both mice and humans. This feature suggests that *M. tuberculosis* expresses genes that counteract the bactericidal or bacteriostatic effects of RNI.

*Modulation of antigen presentation and interference with IFN-γ signalling*

The recognition of infected macrophages by CD^4^ T cells depends on constitutively expressed MHC II on professional antigen-presenting cells, level of which is upregulated upon activation with IFN-γ. One mechanism by which *M. tuberculosis* avoids elimination by the immune system after infection is through the inhibition of antigen processing or presentation by macrophages. Further, it is well established that prolonged signalling through TLR2 by the 19-kDa lipoprotein of *M. tuberculosis* interferes with IFN-γ signalling in both murine and human macrophages. It was recently demonstrated that *M. tuberculosis* uses at least two mechanisms to block responses to IFN-γ; one initiated by lipoproteins acting through TLR2/MyD88, whereas the other is initiated by mycobacterial peptidoglycans, acting in a TLR2-, MyD88-independent manner.

**Diagnosis of TB**

Tests for diagnosis of TB varies in sensitivity, specificity, speed and cost

*Microscopy*

The use of stained-sputum microscopy (Ziehl-Neelsen, Kinyoun, or fluorochrome) for acid-fast bacilli still remains the most available, easy to perform, inexpensive, and rapid diagnostic test for tuberculosis. This is especially true for
laboratories in developing countries, where limited resources often do not allow culture isolation as a diagnostic option. The greatest difficulty in diagnosing tuberculosis and other mycobacterial infections by sputum microscopy is the test’s lack of sensitivity and specificity \(^{97}\). Further, diagnosis of TB by microscopy is difficult especially in children who rarely produce adequate sputum. Currently, the sensitivity of this test has improved considerably with improved techniques and standardization of sputum preparation, and the use of auramine-rhodamine/fluorochrome method instead of the classic Ziehl-Neelsen stain which uses carbol-fuchsin \(^{98}\). Identification of smear positive patients is of major importance because only smear positive pulmonary TB patients are regarded as highly infectious to others \(^{99}\).

**Bacteria cultivation**

Mycobacteria culture is the ultimate proof of mycobacterial infection and is often used as a reference method due to its high sensitivity and specificity \(^{100, 101}\). However, it takes 4-6 weeks for *M. tuberculosis* to grow on solid culture medium (e.g. agar based Middlebrook 7H10 or 7H11 or the egg-based Lowenstein-Jensson medium), and 3 weeks to grow in liquid 7H9 medium \(^{102}\). Notwithstanding the long culture, it is still a requirement for definitive diagnosis of tuberculosis and in drug-susceptibility testing \(^{103}\).

**Tuberculin skin test**

The Mantoux test (Tuberculin Sensitivity Test, Pirquet test, or Purified Protein Derivative (PPD) test) is a diagnostic tool for tuberculosis. The TB skin test is based upon the type 4 hypersensitivity reaction, in which a standard dose of 5 Tuberculin
units is injected intradermally into the forearm and read 48 to 72 hours later \(^{104}\). Sensitized lymphocytes as a result of previous exposure react with the bacterial proteins in the skin. The reaction is read by measuring the diameter of induration across the forearm, perpendicular to the long axis in millimeters. No induration is recorded as "0 mm", whereas reactions over 10 mm in size are considered positive in non-immunocompromised persons. However, several factors may contribute to false-negative results such as age, poor nutrition, acute illness or immunosuppression induced by medication or HIV infection \(^{99}\). On the other hand, false-positive results can occur in individuals exposed to other mycobacteria or immunized with BCG.

**QuantiFERON-TB Gold test**

As a replacement for the Mantoux test, several other tests are being developed. QuantiFERON-TB Gold test is an indirect test for *M. tuberculosis*-complex. The readout of this test is the measurement of IFN-\(\gamma\) production in whole blood upon stimulation with PPD. The QuantiFERON-TB Gold test addresses the operational problems with the tuberculin skin test, but, as the test is based on PPD, it still has a low specificity in populations vaccinated with the BCG vaccine \(^{105}\). The test is used in conjunction with risk assessment, radiography and other medical and diagnostic assays. Guidelines for the use of QuantiFERON-TB Gold were released by the Centers for Disease Control (CDC) in December 2005. QuantiFERON-TB Gold has been approved by the Food and Drugs Administration in the United States, as well as in Europe and Japan.
Molecular methods

Nucleic acid amplification tests, such as polymerase chain reaction (PCR) have contributed to a more rapid and reliable diagnosis of pulmonary tuberculosis: These technologies allow for the amplification of specific target sequences of nucleic acids that can be detected through the use of nucleic acid probes; both RNA and DNA amplification systems are commercially available. Amplification methods for \textit{M. tuberculosis} however have low sensitivity, and the absence of specific internal controls for the detection of inhibitors of the reaction means it cannot completely replace the classical diagnostic techniques.

Treatment

The WHO has been tackling the global problem of inadequate tuberculosis control for some years and launched a new programme of integrated care in 1994, called directly observed treatment, short course (DOTS). A combination of drugs referred to as first line drugs (Isoniazid, rifampicin, pyrazinamide and ethambutol) are used together in initial treatment for 6 months under close supervision. Other antibiotics are active against TB and are used primarily for multi-drug resistant (MDR) TB. The 2 most important classes are the aminoglycosides (streptomycin, kanamycin, amikacin) and fluoroquinolones (levofloxacin, moxifloxacin).

BCG vaccine

Robert Koch (1843-1910) elucidated the aetiology of TB, and Calmette (1863-1933) together with Guérin (1872-1961) developed the BCG vaccine in the 20th century, which is still the only vaccine available against TB. The first clinical studies took place from 1921 to 1927 in France and Belgium, and showed that BCG was
highly efficient in protecting against TB in children\textsuperscript{109}. Unfortunately, despite the early success, the BCG vaccine has had a limited effect against the TB epidemic in developing countries\textsuperscript{110}. Although BCG protects children efficiently against the early manifestations of TB, estimates of protection against adult pulmonary TB range from 0–80\%, based on large, well-controlled field trials\textsuperscript{111}.

Among the hypotheses for low protective efficacy of BCG is improper storage of vaccine, loss of capacity to stimulate a durable immune response and continuous exposure to environmental mycobacteria, suggested to block or mask BCG vaccination-induced immune responses\textsuperscript{112}. The current route of vaccination, the subcutaneous (s.c.) route is thought of as not inducing an optimal immune response. Consequently, mucosal vaccination via the i.n. route has been found to be effective in conferring protection against several diseases of the respiratory tract\textsuperscript{113}. Further, it has been demonstrated that i.n. BCG vaccination is superior to the s.c. route for protection against pulmonary TB in mice\textsuperscript{114}.

\textit{Novel vaccine candidates}

It is now clear that a new vaccine is needed to either replace or boost BCG. In this direction, two types of vaccines are under development. The first group of vaccines called subunit vaccines are made up of one or a few mycobacteria antigens, and are generally considered as vaccines to be used on top of BCG as a booster vaccine following a conventional BCG prime vaccination. The second group or recombinant viable vaccines are anticipated to be superior alternatives to BCG, hence are intended to replace conventional BCG vaccination in the newborn. Whereas a vaccine intended to replace BCG needs to demonstrate superior efficacy to BCG and be safe to be seriously considered, booster vaccines are often no more effective than
BCG at generating primary immune response \cite{110}. Further, they have the additional requirement to be effective in sensitized as well as naive recipients, a test which BCG significantly fails. The most advanced TB vaccine candidates were recently reviewed\cite{110,115,116}.

**The mouse model in tuberculosis**

Undoubtedly, the mouse is the most sophisticated and cost-efficient animal model in biomedical research. The immune response of the mouse is very well understood, and reagents such as monoclonal antibodies against surface markers and cytokines are available. Furthermore, the genetic manipulation of mice is highly advanced. For example, transgene expression, gene knockout, gene knock-in, both constitutive and conditional, have all become standard technologies and also a large variety of mouse mutants with defined immunodeficiencies are available to researchers studying the role of distinct cells and surface molecules in the in vivo setting of tuberculosis. Moreover, the mouse genome has been completely sequenced, making the blueprint for future experiments available \cite{117}. Notwithstanding, there are differences in the host defense mechanisms between mice and humans, and evaluation of data in murine experiments should be done cautiously.
THE PRESENT STUDY

Aims

With the declaration that tuberculosis is a major public health problem worldwide, the overall aim of this study was to increase our understanding of the interaction between *M. tuberculosis* and the host, a prerequisite for accurate diagnosis, design of better vaccines and effective treatment: Our specific objectives were:

- To investigate the role of TLR signalling as an evasive mechanism for mycobacteria survival and persistence in macrophages (paper I)
- To investigate the induction of immune response in mice to i.n. mycobacterial infection and to identify immunological parameters or biomarkers associated with infection (paper II)

Materials and Methods

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

Inhibition of IFN-γ induced killing of mycobacteria by murine macrophages (Paper I)

*M. tuberculosis* is a highly successful pathogen that can infect, persist, and cause progressive disease in humans and experimental animals with apparently normal immune responses. Individuals infected with *M. tuberculosis* develop appropriate cellular immune responses with priming, expansion, differentiation and trafficking of antigen-specific CD4⁺ and CD8⁺ T cells resulting in IFN-γ and TNF production required for protective immunity at the site of infection ¹¹⁸,¹¹⁹. This suggests that *M. tuberculosis* has evolved mechanisms to avoid elimination by normal mechanisms of immunity. It was previously observed that continuous exposure of macrophages to *M. tuberculosis* or its components inhibited IFN-γ mediated MHC II expression ¹²⁰. Subsequently, several studies have demonstrated that prolonged signaling of TLR2 by the 19-kDa lipoprotein of *M. tuberculosis* result in downregulation of some IFN-γ inducible genes.

In this study, we evaluated the functional implications of prolonged TLR2 signalling, with regard to the ability of IFN-γ activated macrophages to kill ingested mycobacteria. To this end, we have used zymosan, a TLR2 ligand but of yeast origin, lipopolysaccharide (LPS), a well described TLR4 ligand as well as the cell wall of BCG (CW<sub>BCG</sub>) or *M. vaccae* (CW<sub>M.vaccae</sub>) in addition to 19-kDa. Whereas BCG expresses the 19-kDa in the cell wall, *M. vaccae* does not express this antigen. We found that prior exposure of the macrophage cell line, J774 cells to 19-kDa or zymosan but not LPS impaired their ability to kill ingested mycobacteria after IFN-γ activation. Similarly, pretreatment with CW<sub>BCG</sub>, but not CW<sub>M.vaccae</sub> inhibited killing of ingested mycobacteria. These findings were confirmed using bone marrow
macrophages (BMM) from TLR2, TLR4 deficient or wild type (WT) mice. In support of our observation with zymosan, it has been demonstrated that inhibition of macrophage responses to IFN-γ by live virulent *M. tuberculosis* is independent of lipoproteins, but dependent on TLR2 signaling.

We did not find any direct relationship between TLR2 signalling and cell proliferation or induction of antimycobacterial activity in macrophages. Mechanistically, neither TNF nor NO production by IFN-γ activated macrophages was significantly affected by exposure to TLR2 ligands. It is well established that NO plays a significant role in the induction of antimycobacterial properties, at least in murine macrophages. However, it was shown recently that 19-kDa could inhibit IFN-γ signalling through mechanism(s) other than the production of NO. We finally demonstrated that the refractoriness induced in macrophages after prolonged TLR2 ligation could be reversed with increasing amounts of IFN-γ.

The general consensus is that exposure to mycobacteria or to 19-kDa neither affect the expression of IFN-γ receptors on the cell surface, nor the IFN-γ proximal signalling steps. Presently, we cannot explain the mechanism(s) underlying this observation. It is possible that certain IFN-γ responsive genes are upregulated with increasing amounts of IFN-γ. While the 19-kDa-TLR2 signalling paradigm is well accepted as an important evasive mechanism employed by mycobacteria to persist in the host, it has been demonstrated that mycobacterial peptidoglycans acting in a TLR2-and MyD88-independent pathways can also inhibit macrophage responses to IFN-γ. Since peptidoglycan, a component of bacterial cell wall signal through the intracellular PRR, NOD proteins, it is possible that these NOD receptors are involved in inhibition to IFN-γ responses. At this point, it is important to emphasise the predominant role of TLR2 in immune recognition of *M. tuberculosis*, as well as in
the activation of sentinel cells like macrophages and dendritic cells. Taken together, it is evident that although IFN-γ is essential for the development of an immune response that prolongs the life span of an infected animal, it is not sufficient to eliminate an *M. tuberculosis* infection. It is therefore important to define other correlates of protection or pathology, factors important for the design of better vaccines and accurate diagnosis.

*Induction of immune response and identification of biomarkers associated with mycobacterial infection in mice (paper II)*

Infection with *M. tuberculosis* generates a complex immune response not only in the lungs, but also in the periphery resulting in secretion of several immune mediators. The outcome of infection depends, at least in part on the early immunological events, involving mainly innate mechanisms. Recognition of mycobacteria by PRR including TLR on phagocytic cells result in receptor mediated phagocytosis and activation of innate cells, mainly macrophages and DCs. On the other hand, chronic TLR2 signalling of macrophages could induce a state of refractoriness to IFN-γ activation, resulting in mycobacteria persistence. Elucidating the immune response generated to mycobacterial infections especially in the lung microenvironment is a prerequisite for vaccine design, and could provide the basis for a and non invasive, immune based diagnosis of TB.

In paper II, we first investigated the induction of TNF or sTNFR secretion in the lung microenvironment (BAL) or in the blood (serum) after i.n. infection of mice with BCG, or treatment with hk-BCG or BCG lysate. Our results indicated that infection with BCG induced sTNFR secretion in BAL, which had a positive relationship with the bacteria load in the lungs. In contrast, sTNFR secretion in serum was independent
of live BCG, as i.n. treatment of mice with either hk-BCG or BCG lysate resulted in induction of sTNFR secretion. These findings suggest that the nature of immune response mounted to mycobacterial infection in the lung microenvironment is probably dependent on successful colonization and growth of bacteria in the lungs.

TNF is important in controlling mycobacterial infections, and the importance of TNF in phagocytosis and killing of mycobacteria has been demonstrated in vitro. In vivo, TNF production is a requirement for granuloma formation, important for restriction of mycobacteria dissemination to other organs, and regulation of other cytokines. We found that the highest sTNFR secretion coincided with the peak of infection. This observation is in agreement with published data which showed that transgenic mice expressing high serum sTNFR exhibited reduced bactericidal activity and succumbed to BCG infection. In this light, sTNFR neutralization of TNF may explain our inability to measure this cytokine in our experiments.

In order to identify other immunological markers associated with mycobacterial infections, we evaluated antibody production in BAL, saliva and serum. I.n. infection of BALB/c mice with BCG resulted in antibody production in BAL. Moreover, IgA was detected in BAL but not serum. Until recently, the prevailing opinion has been that antibodies have no role in protection against TB. However, several studies have provided data on the protective role of antibodies (Reviewed in 80-82). In this study, our particular interest was to find the relationship between antibody production and infection. In this regard, detection of mucosal IgA is likely to indicate the presence of mycobacteria in the lungs, rather than exposure to mycobacterial antigens. This assertion is based on the fact that i.n. immunization with single mycobacterial antigens is able to induce antibody production when formulated with potent mucosal adjuvants, and on their own induce little or no antibodies.
We reasoned that detection of mycobacterial antibodies in saliva would be useful for TB diagnosis, since it is relatively easy and would be cost-effective in the field. Unfortunately, anti-mycobacterial antibodies in saliva turned out to be highly crossreactive compared to BAL. Since mycobacteria infection induces production of antibodies to several antigens, it is possible that some antibodies are redundant and therefore masked the detection of the antibodies of interest. However, it is most likely that the picture will be different in humans who naturally produce saliva. It is noteworthy to mention that unlike sTNFR secretion, antibody production did not follow bacteria growth in the lungs.

It is established that different mouse strains respond differently to intracellular pathogens. In this regard, BALB/c and C57BL/6 have been used extensively in susceptibility studies\textsuperscript{125}. In this light, we evaluated TNF and sTNFR secretion as well as antibody production after BCG infection. Overall, C57BL/6 generated a similar pattern, but lower immune response to mycobacterial. Both C57BL/6 and BALB/c are susceptible to infection with mycobacteria (\textit{bcg}\textsuperscript{3}) and therefore should not display differences in their \textit{bcg}-controlled innate responses\textsuperscript{126}. However, several factors have been suggested to account for the differences in immune response to intracellular pathogens, including the H-2 and other non-H-2 genes. In addition, higher type 1 immune response in C57BL/6\textsuperscript{127}, as opposed to type 2 response in BALB/c\textsuperscript{128}, have been suggested to account for the differences in response to mycobacteria and other infections. Taken together, correlating sTNFR induction or antibody production to acute mycobacterial infections may provide a basis for a non invasive, immune based diagnosis of infection.
Concluding remarks

The BCG vaccine has been in existence for eight decades, and currently, a vast majority of the world’s population have been vaccinated with BCG. Despite this, TB remains the second leading cause of death by an infectious disease worldwide²,⁴, and is also the major complication in HIV infections⁶,⁷. Whether or not exposure to M. tuberculosis infection will result in disease development is dependent on the outcome of the host-pathogen interactions, which generates a complex immune response locally in the lungs as well as the periphery. We have shown that TLR signalling of macrophages by mycobacteria is an important evasive mechanism for survival. In addition, we have shown that specific immunological markers like sTNFR or IgA associated with the mucosal immune response generated after mycobacteria infections could probably be used to distinguish acute from latent infection or immunization.

Future plans

Our study (paper II) showed a positive relationship between sTNFR secretion locally, and bacteria load in the lungs. Even though mice naturally control BCG infection, this infection could be reactivated with immunosuppressive chemicals like corticosteroids. We hypothesize that sTNFR secretion will increase with an increased bacteria load in the lungs after reactivation of controlled infection. We are currently conducting experiments in order to confirm this. We will investigate further differences in immunological parameters between C57BL/6 and BALB/c mice, resulting in differences in response to mycobacterial infections. In addition, we will investigate the role of antibodies in innate immune mechanisms with regards to phagocytosis and killing of mycobacteria in vitro. For the specific role of IgA in protection against mycobacterial infections, IgA deficient or WT mice will be used.
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Introduction

Tuberculosis, one of the oldest recorded human afflictions, is still one of the biggest killers among the infectious diseases. Estimates from the World Health Organization (WHO) suggests that worldwide there are approximately eight million new cases and two million deaths caused by this disease each year [1]. Macrophages are critical to the control of Mycobacterium tuberculosis infection, because they harbour the bacteria in intracellular compartments and present M. tuberculosis antigens via major histocompatibility complex class II (MHC II) to CD4+ T cells [2]. During the early stages of M. tuberculosis infection, the extent of bacterial survival and proliferation is mainly determined by the efficacy of the immune response [3]. Gamma-interferon (IFN-γ) plays a critical role in host responses to a wide variety of viral and microbial pathogens [4, 5], including M. tuberculosis, and synergises with tumour necrosis factor (TNF) in activating macrophages [6].

In mice, IFN-γ activation of macrophages stimulates nitric oxide (NO) production, which is considered to contribute to the killing of M. tuberculosis bacilli [7, 8]. By contrast, direct activation of human macrophages by IFN-γ does not result in increased killing of intracellular bacilli [9–11]. However, children defective in the genes for IFN-γ or the IFN-γ receptor 1 show a predisposition to infection even with poorly virulent mycobacteria strains as well as to severe and recurrent tuberculosis [12]. Mycobacterium tuberculosis is a highly successful intracellular pathogen that is able to survive and cause disease despite the induction of innate and adaptive immune responses.

Abstract

Gamma-interferon (IFN-γ) plays a determinant role in activating macrophages that are critical to control Mycobacterium tuberculosis infection. However, M. tuberculosis can escape killing by attenuating the response of macrophages to IFN-γ by blocking the transcription of a subset of IFN-γ inducible genes. This inhibition occurs after signalling through Toll-like receptor 2 (TLR2). While most studies have investigated the inhibition of IFN-γ responsive genes after TLR2 signalling, the present study focuses on the functional implications of inhibition of IFN-γ signalling in macrophages with regard to mycobacteria killing. Here, we provide evidence that exposure of the murine macrophage cell line J774 to the TLR2 ligands; 19-kDa or zymosan, 19-kDa or zymosan, results in an impairment of IFN-γ-mediated killing. We demonstrate that 19-kDa and zymosan inhibit the ability of IFN-γ to activate murine macrophages to kill BCG without inhibiting nitric oxide (NO) or tumour necrosis factor (TNF) production. Finally, we demonstrate that the inhibitory effect of 19-kDa on IFN-γ signalling is overcome with increasing amounts of IFN-γ indicating that the refractoriness could be reversed at optimal IFN-γ concentrations. The critical role of TLR2 but not TLR4 signalling in the inhibition of IFN-γ promoted killing of mycobacteria is discussed.
While IFN-γ is clearly required for the prevention of rapidly progressive M. tuberculosis infection [13], even the development of an immune response with local production of IFN-γ is still unable to reliably eradicate M. tuberculosis infection in mice or humans. Studies of patients with tuberculosis have demonstrated the presence of IFN-γ in the pleural fluid [14, 15], lung fluid [16], lymph nodes [17] and within the granuloma [14, 18], suggesting that a defect in response to IFN-γ rather than the absence of its production allows tuberculosis to progress.

Several mechanisms have been described that may be used by M. tuberculosis to evade host immune responses (reviewed in Ref. [19]), potentially contributing to its persistence in the infected host. For example, M. tuberculosis attenuates the response of infected macrophages to IFN-γ [2, 20–26], in part by blocking the transcription of a subset of IFN-γ-induced genes including the type I receptor for the Fc domain of IgG (FcγRI or CD64) [20, 23–25] and the MHC class II transactivator [2, 24–26], which regulates MHC class II expression [27, 28]. The inhibition occurs by blocking IFN-γ signalling through a Toll-like receptor 2 (TLR2)-dependent mechanism [2, 22–24, 26, 29].

To further understand the role of TLR signalling in the inhibition of IFN-γ-mediated killing of intracellular mycobacteria, we included the ligands zymosan and lipopolysaccharide (LPS) from Escherichia coli in this study in addition to the M. tuberculosis 19-kDa lipoprotein. Zymosan is a constituent of yeast cell wall and a well-described TLR2 agonist, while LPS signals through TLR4. We present here evidence that, similar to 19-kDa, zymosan but not LPS is able to inhibit IFN-γ-mediated killing reinforcing the importance of TLR2 signalling for this type of inhibition.

Materials and methods

Mice. The studies were performed using 7- to 12-week-old C57BL/6 (Taconic, Eby, Denmark), and TLR2 [30] and TLR4-deficient mice [31] with a C57BL/6 background. Breeding pairs of the TLR-knockout mice were obtained from the Karolinska Institute, Sweden, with the permission of S. Akira (Osaka University, Japan), and kept at the Animal Department of the Arhenius Laboratories, Stockholm University, Sweden. All procedures were performed with the permission of the Stockholm Norstedts Ethical Board (Stockholm, Sweden). The use of animals was in accordance with the Swedish law on animal experiments.

Media and cell culture. J774A1 (referred as J774 cells in the text) macrophage cell line was obtained from the European Type Tissue Culture Collection (CAMR, Salisbury, UK). The cells were grown at 37 °C and 5% CO2 in DMEM containing glucose, t-glutamine and 20 mM HEPES (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin in 50 cm2 tissue culture flasks (Costar, Corning, NY, USA). BMM were obtained as previously described [32, 33]. Briefly, mice were killed, and the femur and tibia of the hind legs flushed with sterile PBS. The bone marrow cells were washed and resuspended in DMEM containing glucose and supplemented with 10% FCS, 20% L929 cell-conditioned medium (as a source of M-CSF), 100 μg/ml streptomycin and 100 U/ml penicillin. Bone marrow cells were plated in 12-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.

Bacteria cultivation. Mycobacterium bovis Bacillus Calmette–Guerin (BCG) (Pasteur strain) has been genetically tagged with the green fluorescent protein from the jellyfish Aequorea victoria [34], were obtained from R. Reljic, Guy’s Hospital Campus of Kings College, London. BCG was grown in Middlebrook 7H9 broth with glycerol supplemented with albumin–dextrose–catalase (ADC) at 37 °C. Aliquots were frozen in PBS at −70 °C. Representative vials from stock were thawed, serially diluted in plating buffer [0.15 M NaCl/PBS/0.05% Tween-80 (vol/vol)] and colony-forming units (CFU) counted after plating on Middlebrook 7H11 agar (Difco, Sparks, MD, USA), with glycerol and oleic acid–albumin–dextrose–catalase (OADC) enrichment.

Mycobacterial antigens and other stimuli. Mycobacterium tuberculosis 19-kDa lipoprotein was provided by LIONEX Diagnostics & Therapeutics GmbH, Braunschweig, Germany. Zymosan from Saccharomyces cerevisiae and LPS from Escherichia coli 055:B5 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). To prepare BCG cell wall, lyophilized BCG (SSI, Copenhagen, Denmark) was grown on Middlebrook 7H11 at 37 °C for 14 days. Colonies were scraped from agar into glass tubes, and non-covalently attached molecules were extracted first with chloroform–methanol (1:2 vol/vol) and then with chloroform–methanol (2:1 vol/vol), with continuous stirring for 2 days in each extraction. Pooled organic extracts were partitioned with chloroform–methanol–water (8:4:2 vol/vol). The organic phase was separated, evaporated to dryness, diluted in PBS [35] and aliquoted (cell wall fraction: CWBCG). For the extraction of M. vaccae cell wall (CW_M. vaccae), bacteria was grown on Tryptone Soy peptone Agar (TSA) media for 5 days, and prepared as described above. All preparations were kept at −70 °C until use.

Macrophage infections and IFN-γ stimulation. Before each experiment, J774 cells were harvested by gentle scraping with a cell scraper (Costar), and cultured at a density of 105 per ml in 12-well flat-bottom tissue culture plates (Corning, NY, USA) at 37 °C overnight to allow adherence of cells. Pretreatment of macrophages with all stimuli was done for 24 h before BCG infection. Before use, aliquots of BCG were thawed and vortexed vigorously, then resuspended in antibiotic-free culture medium supplemented with 5% heat-inactivated FCS. To disrupt bac-
teria clumps, bacteria was vortexed vigorously with sterile glass beads, and the final suspension used to infect cells at MOI of 5:1–10:1. Four hours after infection, monolayers were washed three times with medium to remove extracellular bacteria, and then stimulated with recombinant mouse IFN-γ (MABTECH, Stockholm, Sweden) in complete medium and incubated at 37°C and 5% CO2 for 72 h. At 72 h, culture supernatant was first collected and centrifuged at 600 g to pellet down detached cells and any extracellular bacteria, and stored at −80°C. Infected cells were lysed with 0.2% Triton X-100 for 15 min, and cell lysates serially diluted in plating buffer and plated onto 7H11 agar plates, and CFU counted in 21 days.

**Proliferation assay.** J774 cells were seeded at 2 × 10^4 per well in 96-well culture plates (Costar) and stimulated or left untreated. Thymidine incorporation was done by adding 1 μCi of [3H]thymidine per well to cultures at 24, 48 and 72 h. Arithmetic mean of incorporation counts of radioactivity from triplicate wells were determined by standard procedures.

**TNF detection and NO measurement.** Cytokine enzyme-linked immunosorbent assay (ELISA) was performed using the commercially available DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s recommendations, with slight modifications. Streptavidin conjugated to alkaline phosphatase (MABTECH, Sweden) was used instead of hors eradish peroxidase at 1:1000 dilution. The enzyme-substrate reaction was developed using P-nitrophenyl phosphate (Sigma). Optical density was read in a multi-scan plate reader at 405 nm and concentrations were obtained by comparison with calibration curves established with an rmTNF standard. NO production was assessed by measuring nitrite concentrations using the Griess reaction (Sigma).

**Statistical analysis.** Data are presented as mean ± SD. Student’s t-test was used to determine statistical significance between two differentially treated cultures. Differences were considered significant when P < 0.05.

**Results**

**Exposure of J774 cells to zymosan or 19-kDa impairs the ability of IFN-γ to induce mycobacteria killing**

Previous studies have evidenced that prolonged exposure of macrophages to M. tuberculosis 19-kDa lipoprotein interferes with IFN-γ signalling by blocking the transcription of a subset of IFN-γ responsive genes. To assess if signalling through TLR2 is inhibitory to IFN-γ-mediated bacterial killing, we tested the ability of the TLR2 ligand zymosan to inhibit macrophage responses to IFN-γ compared with 19-kDa. For this purpose, J774 cells were incubated for 24 h with either zymosan (20 μg/ml) or 19-kDa (200 ng/ml) or left untreated and infected with M. bovis BCG. Maximal inhibition of HLA-DR Ag processing was measured at concentrations of 200 ng of 19-kDa lipoprotein per ml and above [23]. After 4 h of infection, infected J774 monolayers were extensively washed with medium and subsequently stimulated with IFN-γ (20 ng/ml). We found that macrophages incubated with either 19-kDa or zymosan before IFN-γ activation were less able to control intracellular replication of mycobacteria compared with non-treated but IFN-γ-activated macrophages (*P < 0.05) (Fig. 1). Non-activated macrophages were not able to restrict mycobacterial growth, resulting at 72 h post-infection, in a roughly 4.0-fold increase in CFU relative to the infecting inoculum at time t = 0.

To further confirm the involvement of TLR2 signalling in the refractoriness of macrophages to IFN-γ-dependent killing of mycobacteria, we compared the effect of 19-kDa and zymosan with the effect of LPS, which signals through TLR4. As many biological features of LPS are concentration-dependent, we tested the ability of LPS to interfere with IFN-γ-mediated killing of mycobacteria with varying concentrations (50, 300 ng/ml and 5 μg/ml). We also used the cell wall fraction of BCG (CW_{BCG}), which contains the 19-kDa lipoprotein, and the cell wall from M. vaccae (CW_{M. vaccae}), which lacks 19-kDa [36]. As shown in Fig. 1, pretreatment of J774 cells with low (50 ng/ml) to high (5 μg/ml) concentrations of LPS failed to interfere with IFN-γ-mediated killing of mycobacteria. As expected, CW_{BCG} interfered with IFN-γ-induced killing of mycobacteria, even if to a lesser extent than the pure protein (*P < 0.05), whereas CW_{M. vaccae} did not.

To determine if the inhibitors could compete with IFN-γ when added simultaneously, we treated infected cells with either 19-kDa or zymosan together with IFN-γ, and compared bacteria killing with cells pretreated 24 h before infection and IFN-γ activation. Simultaneous treatment of J774 cells with 19-kDa and IFN-γ or zymosan and IFN-γ resulted in less inhibition of IFN-γ-activated killing compared with pretreatment before IFN-γ stimulation (results not shown).

**Inhibition of IFN-γ-induced killing of BCG by 19-kDa and zymosan involves TLR2**

To further analyse the role of TLR2 signalling on IFN-γ stimulation of macrophages, BMM from TLR2- and TLR4-deficient and also WT C57BL/6 mice were pretreated with 19-kDa, zymosan or LPS. Pre-incubation of BMM derived from TLR4-deficient and WT mice with 19-kDa or zymosan resulted in a statistically significant (*P < 0.05) inhibition of IFN-γ-activated killing of mycobacteria compared with IFN-γ-activated cells
By contrast, both 19-kDa and zymosan failed to interfere with IFN-γ-mediated killing of mycobacteria in BMM from TLR2-deficient mice, and bacteria killing was comparable with that observed in IFN-γ-activated cells. Taken together, these results suggest that inhibition of IFN-γ-activated killing of mycobacteria in murine macrophages critically involves TLR2 and not TLR4 signalling.

TLR2 signalling does not inhibit intracellular mycobacterial growth

To investigate the possibility that either 19-kDa or zymosan could directly stimulate macrophages to kill mycobacteria, we treated J774 cells with the 19-kDa, zymosan, LPS or CWBCG. Contrary to previous findings [37], we did not observe any direct involvement of the 19-kDa or zymosan with mycobacteria killing. At 72 h, bacterial growth in macrophages pre-treated with 19-kDa or zymosan was comparable with that observed in unstimulated cells (Fig. 3). A possible explanation could be the lower concentration of 19-kDa used in our studies. LPS stimulation, however, resulted in approximately 20% reduction in the number of viable bacilli recovered after plating compared with the amount recovered from unstimulated cells. As expected, there was a statistically significant reduction of CFU in IFN-γ-activated cells at 72 h (*P < 0.05).

TLR2 signalling has no inhibitory effect on macrophage proliferation

Continuous stimulation of J774 cells with either LPS or IFN-γ resulted in suppression of cell proliferation [38]. Next, we assessed the effect of TLR2 signalling on J774-cell proliferation. We seeded 2 × 10⁴ J774 cells in 96-well tissue culture plates and treated with 19-kDa, zymosan or CWBCG and compared cell proliferation using [³H] thymidine incorporation at 24, 48 and 72 h to cells treated with LPS or IFN-γ, both known to suppress
J774 cells treated with either 19-kDa, zymosan or CWBCG proliferated to a level comparable with unstimulated cells (Fig. 4). By contrast, IFN-\(\gamma\) and LPS treatment suppressed J774-cell growth. Together, these data imply that neither the 19-kDa nor zymosan directly inhibit either bacterial growth or J774-cell proliferation.

Inhibition of IFN-\(\gamma\)-induced bacterial killing is not dependent on TNF production

Tumour necrosis factor is a pro-inflammatory cytokine known to play an important role in the control of mycobacterial replication both in mice [39, 40] and in humans [41, 42]. Therefore, we considered of interest to investigate if pretreatment of macrophages with 19-kDa or zymosan affected TNF production. J774 and BMM from WT mice were pretreated with LPS, 19-kDa, zymosan or left untreated, and culture supernatants were assayed for the presence of TNF 72 h after infection and IFN-\(\gamma\) stimulation (Fig. 5A). Infection of macrophages with BCG resulted in TNF production that was increased upon IFN-\(\gamma\) stimulation. Pretreatment of J774 cells with TLR

Figure 4 Toll-like receptor 2 signalling has no direct inhibitory effect on macrophage proliferation. J774 cells were seeded at \(10^5\) per ml in 12-well tissue culture plates overnight and treated with 19-kDa (200 ng/ml), zymosan (20 \(\mu\)g/ml), LPS (500 ng/ml), CWBCG (20 \(\mu\)g/ml) or IFN-\(\gamma\) (20 ng/ml) or left untreated for 24 h. After 24 h, cell monolayers were infected with BCG in DMEM containing 5% FCS for 4 h, washed three times with medium, and incubated with complete medium for 72 h. Data are expressed as mean CFU, and error bars represent the SD between one of four independent experiments. *\(P < 0.05\) versus non-treated cells.

Figure 5 Toll-like receptor signalling does not inhibit TNF production induced by IFN-\(\gamma\). (A) J774 cells were treated with 19-kDa, zymosan, LPS, CWBCG and CW\(\text{M. vaccae}\) or left untreated for 24 h, infected as described and then stimulated with IFN-\(\gamma\). (B) BMM derived from TLR2- and TLR4-deficient and WT C57BL/6 mice were treated with 19-kDa, zymosan and LPS or left untreated for 24 h, infected as described previously and stimulated with IFN-\(\gamma\). Culture supernatants were collected 72 h after infection with BCG. TNF production was assayed with a standard ELISA kit and mean concentration expressed as pg/ml. Three replicates were performed for each condition. Error bars represent the SD between replicates from one of three (A) and two (B) independent experiments. *\(P < 0.05\) versus non-treated but IFN-\(\gamma\)-stimulated cells.
ligands before IFN-γ stimulation did not modify significantly the levels of TNF (P < 0.05) compared with the levels induced by IFN-γ alone (Fig. 5A). Similar results were observed in the culture supernatants of BMM from WT mice (Fig. 5B). No differences were either observed in the TNF levels from cultures of BMM derived from TLR2 or TLR4 knockout mice (data not shown).

19-kDa and zymosan do not interfere with IFN-γ-induced NO production in macrophages

A key role of IFN-γ in the killing of intracellular *M. tuberculosis* is the induction of NO production in murine macrophages, which has direct anti-microbial properties [7, 8]. Thus, we next assessed if impairment of IFN-γ-mediated killing was correlated to a decrease in NO production. Culture supernatants collected were assayed for nitrite production. Nitrite (NO$_2^-$) is a stable catabolite of NO, and levels of nitrite in culture supernatants are a good indirect indicator of NO production by macrophages. As expected, IFN-γ-induced high levels of NO production whereas BCG infection induced only marginal levels in both J774 cells and BMM (Fig. 6A and B). We found that none of the stimuli tested inhibited NO production induced by IFN-γ. Similar to TNF, NO production in IFN-γ-activated BMM from WT mice was similar to that of TLR-deficient BMM (data not shown).

The inhibitory effect of 19-kDa on IFN-γ signalling is overcome with increasing amounts of IFN-γ

Finally, we asked whether the inhibitory effect of 19-kDa on IFN-γ-mediated killing could be overcome with increasing amounts of IFN-γ. We addressed this question by pretreating J774 cells as before with the 19-kDa lipoprotein, infected with BCG for 4 h, and stimulated with IFN-γ as before (20 ng/ml), or with lower (2 ng/ml) as well as higher (80, 200 ng/ml) concentrations. As shown in Fig. 7, increased amounts of IFN-γ reversed the impairment of IFN-γ signalling induced by 19-kDa, and resulted in increased killing of BCG.

Discussion

The mechanism(s) used by *M. tuberculosis* to persist in macrophages in the face of highly developed and active acquired T-cell responses is poorly understood [23]. Previously published data have demonstrated that infection of macrophages with mycobacteria or exposure to mycobacterial constituents can inhibit their subsequent responsiveness to IFN-γ [2, 20–26], critically important for the induction of microbicidal function. Defective recognition of infected macrophages by T cells and/or defective responses of infected macrophages to effectors of adaptive immunity may contribute to the ability of *M. tuberculosis* to persist and progress. The 19-kDa lipoprotein of *M. tuberculosis*, a constituent of the cell wall, and usually secreted in culture medium, was identified to be responsible for interference with IFN-γ signalling in both murine and human macrophages [2, 22–24, 26]. It has also been shown that inhibition was dependent on TLR2 signalling, and required prolonged exposure. This may be one of the mechanisms used by *M. tuberculosis* to survive inside macrophages, consequently contributing to dissemination of bacteria and persistence of infection. Although substantial attention has been focused on the ability of the 19-kDa lipoprotein to inhibit up-regulation of IFN-γ inducible genes in macrophages, the present work focuses on the functional implications of interfer-
To conclude, the inhibition of IFN-γ-induced killing by the 19-kDa glycan (mAGP) complex, a TLR2 ligand, is critical for the control of infection. Although the evidence available favours the conclusion that TLR2 signalling is a crucial requirement for interference with IFN-γ signalling, it is apparent that other types of signalling may be required and consequently, a number of questions remain unresolved. For example, the cell wall mycolylarabinogalactan peptidoglycan (mAGP) complex, a TLR2 ligand, has been shown to inhibit IFN-γ signalling by down-regulating IFNγR expression at the cell surface. By contrast, most of the evidence available now seems to suggest that neither the expression of IFNγRs on the cell surface, nor the IFN-γ proximal signalling steps are modified by exposure to TLR2 ligands. Even though NO produced by IFN-γ-activated macrophages was significantly affected by exposure to TLR2 ligands, it has been shown by others that Mycobacterium tuberculosis 19-kDa could inhibit IFN-γ signalling through mechanism(s) other than the production of NO (24).

As possible mechanisms to explain the inhibition of IFN-γ function, we studied the direct effect of TLR2 ligation in mycobacterial killing, cell proliferation or production of active mediators, such as TNF and NO. We did not find any antimicrobial activity promoted by 19-kDAs, in contrast to the findings of Thoma-Uszynski et al. [37]. A possible explanation could be the lower concentration of 19-kDAs in our studies or the use of different mycobacterial strains. Furthermore, neither TNF nor NO production by IFN-γ-activated macrophages was significantly affected by exposure to TLR2 ligands. Even though NO is critical for the control of M. tuberculosis [7, 8, 44, 45], it has been shown by others that M. tuberculosis 19-kDa could inhibit IFN-γ signalling through mechanism(s) other than the production of NO (24).

Finally, we found that increasing amounts of IFN-γ could overcome the unresponsiveness induced by 19-kDa (Fig. 7). IFN-γ signalling is initiated when the cytokine binds to its receptor composed of two subunits, IFNGR1 and IFNGR2. Central to the response of IFN-γ is the activation of the non-receptor tyrosine kinases Janus kinase 1 (JAK1) and JAK2, and the subsequent phosphorylation of the signal transducer and activator of transcription 1 (STAT1) [25]. Several distinct mechanisms of inhibition of IFN-γ signalling have been described. For example, Mycobacterium avium [46] and Leishmania donovani [47] have both been shown to inhibit IFN-γ signalling by down-regulating IFNγR expression at the cell surface. We speculate that if the number of receptors is not changed, then their binding capacity may be altered after persistent TLR2 ligation. We do not have at present any formal evidence to support this speculation and other mechanisms may be implicated.

Although the evidence available favours the conclusion that TLR2 signalling is a crucial requirement for interference with IFN-γ signalling, it is apparent that other types of signalling may be required and consequently, a number of questions remain unresolved. For example, the cell wall mycolylarabinogalactan peptidoglycan (mAGP) complex, a TLR2 ligand, inhibits macrophage responses to IFN-γ signalling independently of TLR2, TLR4 and myeloid differentiation factor 88 [24]. Also intriguing is our observation that LPS, which uses the myeloid differentiation factor 88-dependent pathways for activation, and inhibits MHC-II antigen processing by macrophages similar to the 19-kDa antigen [49], failed to interfere with IFN-γ activation.

Table 1

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*P < 0.05 versus non-treated but IFN-γ-stimulated (20 ng/ml) cells.
In contrast to the inhibitory effects of TLR2 signalling discussed here, it is important to remember the predominant role of TLR2 in immune recognition of *M. tuberculosis*, as well as in the activation of macrophages and dendritic cells. A higher susceptibility to BCG infection has been demonstrated in TLR2-deficient mice compared with wild-type mice [50, 51]. TLR2-deficient mice aerogenically infected with *M. tuberculosis* were found to lack functional granulomas during the chronic stages of infection, and developed fatal chronic pneumonia [52]. In their work, Underhill et al. found TLR2 as the principal mediator of the proinflammatory signal induced by *M. tuberculosis* [53]. Recently, Blander and Medzhitov demonstrated defective phagosome maturation into late endosomal and lysosomal stages in TLR2-deficient macrophages [54]. TLR activation is also known to promote the formation of both reactive oxygen and nitrogen species, both critical for effective anti-microbial mechanism against intracellular pathogens [55, 56]. Furthermore, it has been shown that TLR2 activation leads to killing of intracellular tubercle bacilli in both human and mouse macrophages and also to the induction of proinflammatory responses [37, 57]. In their work, Pecora et al. [58] characterized a new *M. tuberculosis* lipoprotein, LprA, also a TLR2 agonist which was found to induce dendritic cell maturation, cytokine production and also regulated the antigen-presenting function.

Thus, putting together the available information on TLR2 signalling in macrophages, it is obvious that, similar to other immunological mediators, TLR2 has a double face. Short exposure to TLR ligands may be beneficial because it mediates phagosome maturation in macrophages. On the contrary, persistent exposure could make macrophages refractory to IFN-γ and decrease their ability to process and present antigens. This decrease in response to IFN-γ may be required to avoid the deleterious effects of a chronic inflammatory response but, in addition, it can be used by mycobacterium to escape the immune system and survive inside these refractory macrophages. Clearly, there is the need for careful analysis of the molecular mechanisms and understanding of the complex signalling events associated in subversion of host immune responses by mycobacterial products to provide better insights into the mechanism of *M. tuberculosis* pathogenesis. In conclusion, efforts to develop new vaccines for tuberculosis should not solely rely on T-lymphocyte production of IFN-γ as the *in vitro* correlate of potential efficacy of candidate vaccines.

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Induction of immune responses and identification of biomarkers associated with mycobacterial infection in mice

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Abstract

Tuberculosis is primarily a disease of the lung, and therefore a better understanding of the immune mechanisms especially in the lungs and its microenvironment would be useful not only for development of new vaccines, but also for optimal diagnosis of disease. In this study, we demonstrated that significant levels of soluble tumor necrosis factor receptor (sTNFR) 1 and 2 were induced in the lungs BALB/c or C57BL/6 mice after intranasal (i.n.) infection with *M. bovis* bacillus Calmette-Guérin (BCG). There was a positive relationship between sTNFR secretion and bacteria growth in the lungs, with the highest sTNFR secretion coinciding with the peak bacterial load. At the time points studied, lower bacterial growth was observed in the more resistant strain C57BL/6, compared to BALB/c mice. Neither heat killed BCG (hk-BCG) nor BCG lysate induced sTNFR in the broncho-alveolar lavage (BAL). There was no clear relationship between sTNFR levels in serum, and bacteria load in the lungs. Overall, the magnitude of sTNFR2 was much higher than sTNFR1 in either BAL or serum. Further, we assessed the humoral immune responses in both BALB/c and C57BL/6 after i.n. infection with BCG. Overall, BALB/c mice produced higher antibody levels in either BAL or serum, compared to C57BL/6. Moreover, BCG infection induced IgA antibodies in BAL, but not serum. Infection of mice, but not treatment with hk-BCG or BCG lysate resulted in sTNFR secretion or antibody production in BAL. Taken together, the presence of sTNFR or mycobacteria specific antibodies, especially IgA in the lung microenvironment could indicate active bacterial infection, and may be used for pulmonary TB diagnosis.
1. Introduction

The global epidemic of tuberculosis (TB) results in eight to ten million new TB cases 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). The chronicity or latency of *Mycobacterium tuberculosis* infection has made eradication of TB a very difficult goal. The attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) has been widely used for over 80 years as the only TB vaccine, but has proven unreliable to fully protect against pulmonary TB in adults (3-5). BCG has been administered intradermally or percutaneously to humans (6), and among several factors that are suspected to cause the failures of BCG is the route of vaccination. Vaccination at the mucosal site is believed to be superior to vaccination at other sites for eliciting protective immune responses against mucosal infectious diseases including *M. tuberculosis* (7-9). Although the mechanisms remain to be fully understood, mucosal vaccination via intranasal (i.n.), intragastric, and intrarectal routes was found to be effective in conferring protection against several nontuberculous mucosal infectious diseases (10).

Tuberculosis is primarily a respiratory airway infectious disease, and inhaled bacilli in droplet nuclei (11) target the alveolar macrophages as its preferred habitat and avoid the host immune system through different mechanisms (12). 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 (13,14). Interferon gamma (IFN-\(\gamma\)) is the central mediator in protection against *M. tuberculosis* infection and synergises with tumour necrosis factor (TNF) in activating macrophages (15). The release of
TNF in response to mycobacterial infection has several beneficial effects. *In vitro* studies show that this cytokine increases the ability of macrophages to phagocytose and kill mycobacteria (15, 16). TNF production is a requirement for granuloma formation, important for prevention of mycobacteria dissemination to other cells and organs. TNF exists as either transmembrane TNF expressed by most cells, or soluble TNF after cleavage by metalloproteases (17). Both forms of TNF function physiologically by interacting with receptors, TNFR1 (55 kDa) and TNFR2 (75 kDa) expressed on a diverse range of cell types (18). Upon stimulation, these receptors could be cleaved from the cell surface, or directly expressed as soluble isoforms lacking the transmembrane domain.

Experiments in animals have shown that TNFR1 is important in granuloma formation during *M. tuberculosis* infection (19) and in susceptibility to intracellular pathogens (20).

Mycobacterial infections are characterised by production of antibodies, and the use of modern approaches and tools have helped to elucidate the protective role of anti-mycobacteria antibodies in immunity to TB (reviewed in 21, 22). For example, the immunoprotective potential of mycobacterial antigens through induction of mucosal IgA has been highlighted (9, 23-25). A possible protective role for B cells or antibodies against *M. tuberculosis* infection was suggested on grounds of raised bacterial load in the organs of mice genetically depleted of B cells (µ chain knockout) or defective for IgA production (24, 26).

Increasing evidence from both human and experimental animal studies suggests that host genetic heterogeneity affects the nature and/or the level of immune responses to intracellular pathogens (27). In this regard, BALB/c and C57BL/6 differ in their susceptibilities to infections with certain intracellular pathogens (28, 29), even though
they both bear the same susceptibility allele of the \textit{Bcg} gene responsible for innate resistance (30). These mouse strains have been used widely for characterisation of susceptibilities to a wide range of infections.

In this study, we aimed to identify immune parameters likely to be indicative of active \textit{M. tuberculosis} infection as well as assessed the impact of genetic heterogeneity on the host immune responses to mycobacteria infections. We demonstrate that intranasal (i.n.) instillation with live but not hk-BCG or BCG lysate induced secretion of sTNFR in the broncho-alveolar lavage (BAL), which coincided with the bacteria load. Moreover, mycobacterial IgA antibodies were detectable in BAL, but not in serum after i.n. infection of mice with BCG. Overall, though BALB/c had a higher susceptibility to BCG infection compared to C57BL/6 mice, the pattern of response was similar in the two mice strains.
2. Materials and Methods

2.1. Mice

The studies were performed using 8-12 weeks old female BALB/c and C57BL/6 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, United Kingdom 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 (0.9% NaCl and 0.05% Tween-80 [vol/vol]) and colony forming units (CFU) counted at 2-3 weeks after plating on Middlebrook 7H11 agar (Difco, USA), with glycerol and oleic-acid-albumin-dextrose-catalase (OADC) enrichment.

2.3. Preparation of *hk-BCG* and soluble *BCG* antigens (*BCG* lysate)

Bacteria were grown until they reached approximately 5-10 x 10^7/ml. To prepare hk-BCG, 10^7/ml of BCG was autoclaved at 121°C for 20 min. Killed bacteria were washed once and re-suspended in sterile PBS before use. For the preparation of BCG lysate, bacteria were pelleted by spinning at 8,000 x 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. The soluble fraction Bovine serum albumin (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 under the TB vaccine testing and research materials contract. The Ag85c, (Ag85A, Ag85B and Ag85C) is an early-secreted antigen and a major secretory constituent of mycobacterial culture filtrate. Purified 38-kDa, 19-kDa and 16-kDa antigens were all obtained from LIONEX Diagnostics & Therapeutics GmbH, Germany. The 38-kDa and 19-kDa are important mycobacterial antigens. The 16-kDa protein belongs to the α-crystallin superfamily, and a major antigen with epitopes restricted to the M. tuberculosis complex (32).

2.5. Experimental infection with BCG and determination of CFU in the lungs

Mice were infected i.n. with 10⁷ CFU of live BCG. Before infection, mice were anaesthetised with isofluorane (Baxter Medical AB, Kista, Sweden). I.n. administration was carried out by inoculation of a total volume of 30 µl of BCG suspension in PBS to the nostrils delivered in two times by using a pipette and tip, and the mouse was allowed to breathe the suspension into the lung naturally (33). Mice were sacrificed at day 3, weeks 1, 3, 5, and 9. Samples were collected and the numbers of viable bacteria in the lungs were determined. Briefly, lungs were removed
aseptically and placed in 2 ml 0.9 % NaCl 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.

2.6. Sample collection

Serum, BAL and saliva were collected from each group of mice at different time points post infection. Briefly, mice anaesthetised with 0.4% isofluorane were injected intraperitoneally (i.p.) with 100 μl of pilocarpine, 1 mg/ml (Tika Lakemedel AB, Lund, Sweden) to induce salivation, and saliva collected into eppendorf tubes and kept on ice. Mice were bled from the tail vein and serum collected after centrifugation. BAL was obtained by flushing 1 ml of PBS into the lungs of sacrificed mice. All samples were kept at -20°C until use.

2.7. Detection of antibodies in serum, BAL and saliva

Antibodies in serum, BAL and saliva were analyzed by enzyme-linked immunosorbent assay (ELISA). ELISA plates (Costar, high binding, NY, USA) were coated with either (25 μg/ml) of BCG lysate or (2 μg/ml) of Ag85c, 38-kDa, 19-kDa or 16-kDa 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) were incubated in the antigen coated plates doing serial dilutions, starting as follows 1:100 (sera), 1:5 (BAL) or 1:2 (saliva) and the plates were then incubated ON at RT. Following sample incubation, the plates were washed and incubated for 2 h at RT with alkaline-phosphatase (ALP) labelled goat anti-mouse total Ig, IgG, or IgA
(Southern Biotech, CALTAG, UK) and the enzyme substrate reaction was developed using p-nitrophenyl phosphate (Sigma Chemical Co, USA) as substrate. Absorbance was measured in a multiscan (Anthos Labtech Instruments, Salzburg, Austria) reader at 405 nm. To correct for cross-reactive antibodies, all samples were tested against a non-relevant purified protein, bovine serum albumin (BSA) and the results obtained subtracted from that obtained with either total BCG antigen lysate or single mycobacterial antigens.

2.8. Detection of TNF and sTNFR1 and 2 in BAL and serum

Cytokine 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, Sweden) was used instead of horseradish peroxidase at 1:1000 dilution. The enzyme-substrate reaction was developed using p-nitrophenyl phosphate (SIGMA, USA). Optical density was read in a multiscan plate reader at 405 nm and concentrations were obtained by comparison with calibration curves established with recombinant TNF, TNFR1 & 2 standards.

2.9. Statistical analysis

Data are presented as the mean value ± S.D. Student’s t test was used to determine statistical significance between mouse strains. Differences were considered significant when (*, p<0.05).
3. Results

3.1. Live but not hk-BCG or BCG lysate induced significant levels of sTNFR in the lungs of BALB/c mice

It is well established that TNF plays a pivotal role in host mechanism against intracellular pathogens. For example, TNF knockout mice have been found to be susceptible to mycobacteria infection (34, 35). We reasoned that the production of TNF or its soluble receptors (sTNFR1 and 2) will be induced at the site of infection and therefore be a reliable marker for the diagnosis of active mycobacterial infection. To assess this, BAL from BALB/c mice infected with $10^7$ CFU of BCG or treated i.n. with hk-BCG ($10^7$), BCG lysate or PBS as a negative control was collected at day 3, weeks 1, 3, 5, and 9 and tested for the presence of TNF or sTNFR1 and 2. To quantify bacterial load in the lungs of infected mice, serial dilutions of lung homogenates were plated on Middlebrook 7H11 agar, and CFU counted 2-3 weeks after plating.

In this experiment, minimal levels of TNF were detected at all the time points that we collected our samples (data not shown). Our results showed that at day 3 and week 1 after BCG infection or immunisation with hk-BCG or BCG lysate, sTNFR1 and 2 secretion was similar to that of mice treated with PBS (Figure 1). However, sTNFR secretion increased significantly at week 3 in mice infected with live BCG, compared to the animals immunised with hk-BCG or BCG lysate, which maintained their basal production. The highest secretion of sTNFR coincided with the highest bacterial load in the lungs of mice infected with live BCG, which occurred at week 3, and the patterns of sTNFR and CFU decline were similar by weeks 5 and 9. In contrast to the observation in BAL where sTNFR induction was dependent on the presence of live bacteria, significant levels of sTNFR were induced after treatment with hk-BCG or BCG lysate (Figure 2).
3.2. Higher levels of sTNFR1 and 2 were detected in the BAL of BALB/c mice infected i.n. with BCG compared with C57BL/6 and induction was dependent on the bacterial load in the lungs

Generally, BALB/c mice have been found to be less effective than C57BL in the control of mycobacterial growth. To assess in our experimental model the relationship between genetic heterogeneity and susceptibility to mycobacterial infection, we infected C57BL/6 with $10^7$ CFU of live BCG or administered with hk-BCG ($10^7$) or BCG lysate or treated with PBS following the same conditions as with BALB/c mice. BAL and serum were collected at the time points previously described and assessed for the presence of TNF or sTNFR. We observed a similar pattern in the secretion of sTNFR in the BAL between C57BL/6 and BALB/c, with the peak sTNFR production coinciding with the highest bacteria load in the lungs at week 3 after infection (Figure 3). However, the amount of sTNFR secreted by C57BL/6 mice at weeks 3 and 5 was significantly lower (*, $p<0.05$) than that of BALB/c, which also had a significantly higher (*, $p<0.05$) bacterial load at these time points compared to C57BL/6.

3.3. I.n. infection of BALB/c mice with BCG induced BCG-specific IgA in BAL but not serum

Although *M. tuberculosis* infection is controlled mainly by cell mediated immunity, the humoral immune response may also be of importance. Moreover, knowledge of the humoral response at various stages of infection and in different compartments of the body may help us to elucidate the complex interaction between the mycobacteria pathogen and host (36, 37). To assess the humoral immune response in the lung microenvironment as compared to serum, BAL, saliva and serum collected at weeks 1, 3, 5, and 9 from BALB/c mice infected with $10^7$ CFU of live BCG or treated i.n. with hk-BCG ($10^7$), BCG lysate or PBS as a negative control, were
analysed for anti-BCG IgG and IgA antibodies. To correct for cross-reactive antibodies, all samples were tested against BSA and the results obtained subtracted from those obtained with the BCG lysate. Overall, only live BCG induced production of detectable antibodies in either BAL or serum (Figure 4). I.n. immunisation of mice with either hk-BCG or BCG lysate did not induce detectable anti-BCG antibodies locally or in serum even at week 9 after treatment (data not shown). Anti-BCG IgA antibodies were detected only in BAL and not serum (Figure 4). Antibodies detected in saliva were highly cross reactive to BSA (data not shown).

3.4. I.n. infection of mice with BCG induced significant levels of antibodies to major mycobacterial antigens

We next considered of importance to investigate the extent to which i.n. infection of mice with BCG induces specific antibodies to some of the major secreted mycobacterial antigens. BAL and sera collected from BALB/c or C57BL/6 mice, as previously described, were tested against four single mycobacterial antigens; the Ag85c, the 38-kDa 19-kDa and 16-kDa. Similarly to the results obtained using total BCG soluble antigens, i.n. infected mice produced specific IgG in both BAL and serum whereas IgA was detected in BAL but not serum (Figure 5). Again, i.n. immunisation of mice with hk-BCG or BCG lysate did not induce detectable levels of IgG or IgA in the lung microenvironment. In contrast to the results obtained using BCG lysate, immunisation of mice with hk-BCG or BCG lysate induced detectable levels of specific IgG antibodies to all four antigens tested in serum (Figure 6). Compared to BALB/c, i.n. BCG infection of C57BL/6 mice induced lower antibody production not only to single mycobacteria antigens (Figure 7), but also to total BCG soluble antigens (results not shown).
Discussion

The control of TB depends largely on early detection of infection and proper treatment. In this regard, efforts have been made to identify suitable antigens to be used for the serodiagnosis of TB. For example, crude *M. tuberculosis* antigens such as purified protein derivative (PPD), whole-culture filtrate and sonicate have 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 (38-41) and lipid antigens (42, 43) for measurement of serum antibodies to *M. tuberculosis* in patients with tuberculosis have been reported. However, almost all of the assays are limited by low sensitivity, especially in smear-negative TB patients. The biological complexity of *M. tuberculosis* infection means that using single immunological markers or biomarkers as indicative of infection would probably have limited diagnostic value, and analysis of several biomarkers may offer the possibility of enhanced diagnosis.

In this study, we aimed to identify some biomarkers that could be indicative of an ongoing infection or pathology. First, we investigated TNF or sTNFR secretion in BALB/c mice after i.n. infection with BCG. We observed a differential induction of TNFR secretion in the lung microenvironment, compared to the systemic. There was a positive relationship between sTNFR secretion in BAL and the bacteria load in the lungs, and treatment of mice with either hk-BCG or BCG lysate did result in induction of sTNFR secretion. On the other hand, sTNFR secretion in serum was independent of BCG growth in the lungs, and treatment with either hk-BGC or BCG lysate resulted in sTNFR secretion. This observation suggests that the mucosal immune response to mycobacterial infection is more specific than the systemic.
The importance of TNF in the generation and maintenance of a protective immune response against *M. tuberculosis* and a host of other bacterial and viral pathogens has been clearly demonstrated (44, 45). It is established that receptor binding to TNF results in either biological activation or neutralisation. Although TNF neutralisation 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, had undifferentiated granulomas and succumbed to BCG infection (47). This may probably explain our inability to detect TNF in this study as well as the lower levels of TNFR1 in both BAL and serum, since this receptor is known to bind to sTNF. Neutralisation of TNF may account for the increase in bacteria load at the peak of TNFR secretion, since TNF is required for effective control of mycobacterial replication in macrophages. Ollerus and colleagues however demonstrated that although sTNF is important, TmTNF expression and function is sufficient to induce an efficient cell-mediated immunity and resistance to BCG infection (48).

Another important parameter in the response to mycobacterial infections is the production of antibodies. We reasoned that detection of antibodies in mucosal secretion could reflect the presence or absence of bacteria or pathology. I.n. infection of BALB/c mice with BCG resulted in IgG production in both BAL and serum. In contrast IgA was detected in BAL but not serum. IgA is the major immunoglobulin in mucosal secretions (49), and we (24) and others (9, 10) have demonstrated the induction of IgA in mucosal secretions after i.n. immunisation with mycobacterial antigens or infection with mycobacteria including BCG (50). Since BCG lysate contains several antigens and has the limitation of lack of sensitivity and/or specificity, we next used single mycobacterial antigens to detect antibody production
as done previously. Consistent with our previous results, IgA was detected in BAL but not serum after i.n. infection with BCG. In contrast to BCG lysate antigens however, we could detect IgG in serum to single mycobacterial antigens, again suggesting some specificity in the mucosal immune response.

Antibodies to all four single antigens were detected in both BAL and serum. An increasing number of authors have argued that secreted antigens, present in large amounts in mycobacterial culture filtrate, rather than intracellular components may be essential for the induction of protective immunity (51). Since saliva is relatively easy to collect compared to BAL, we tested for presence of mycobacterial antibodies, which turned out to be highly cross-reactive (to BSA) and unreliable. Possible reasons for this could be due to the presence of several redundant antibodies or proteins, thereby masking the presence of any mycobacteria specific antibodies present.

Different mouse strains are known to respond differently to intracellular pathogens. We next performed a similar experiment in C57BL/6 mice and compared the immune response to that of BALB/c. Overall, the pattern of either sTNFR secretion or antibody production in BAL or serum, as well as bacteria growth in the lungs was similar to BALB/c. In terms of magnitude, lower amounts of sTNFR or antibodies were measured in C57BL/6, which also had significantly lower bacteria load at the peak of infection, compared to BALB/c mice. C57BL/6 and BALB/c mice have been used extensively for the characterisation of susceptibility to intracellular infections (28, 29, 52). Even though both strains of mice bear the Nramp1 (natural resistance-associated macrophage protein 1) gene (53), known to influence the rate of replication of certain intracellular pathogens in macrophages, they differ in their susceptibilities to these pathogens, including BCG (54). Differences in the H-2 and other non-H-2 genes may account for differences in their immune response to
intracellular pathogens. Previous studies have attributed resistance in C57BL/6 to efficient production of IFN-γ and a strong Th1 response, and susceptibility in BALB/c mice to less efficient production of IFN-γ and a strong Th2 response (28, 29, 52). In their work, Shibuya and colleagues demonstrated that BALB/c mice required IL-1α and TNF as additional co-factors for IL-12-driven Th1 development. In contrast, these co-factors were not a requirement for IL-12 driven Th1 development in C57BL/6 mice (55). In this regard, exogenous administration of IL-12 to BALB/c mice prolonged survival to *M. tuberculosis* infection (56). In this study, both mouse strains were able to control bacteria growth in as many weeks, suggesting that the adaptive immune response, but not the early innate response, may be critical to host resistance to pulmonary mycobacterial infection. Taken together, correlating sTNFR induction or antibody production to acute mycobacterial infections may provide a basis for diagnosis of infection.
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Figure Legends

Figure 1
Live but not hk-BCG or BCG lysate induced significant levels of sTNFR secretion in the lungs of BALB/c mice compared to serum. BALB/c mice were infected i.n. with $10^7$ CFU of BCG or treated with $10^7$ hk-BCG or BCG lysate or PBS, and BAL collected at day 3, weeks 1, 3, 5 and 9. sTNFR1 and 2 secretion (A and B) was assayed with a standard ELISA kit and mean concentrations expressed as pg/ml. (C) Lungs were removed aseptically, homogenized and serial dilutions plated on Middlebrook 7H11 agar. Viable bacterial counts were evaluated 2-3 weeks after plating. Results are expressed as mean concentration (A and B) or CFU x $10^3$ (C) ± SD from 4 mice per group. A representative of two different experiments is shown.

Figure 2
Induction of sTNFR secretion in serum was not dependent on presence of live bacteria in the lungs. BALB/c mice were infected i.n. with $10^7$ CFU of BCG or treated with $10^7$ hk-BCG or BCG lysate or PBS, and BAL and serum collected at day 3, weeks 1, 3, 5 and 9. sTNFR1 (A) and 2 (B) secretion was assayed with a standard ELISA kit and mean concentrations expressed as pg/ml. Results are expressed as mean concentration ± SD from 4 mice per group. A representative of two different experiments is shown.

Figure 3
Higher levels of sTNFR1 and 2 were measured in the BAL of BALB/c mice infected i.n. with BCG compared with C57BL/6 and induction was dependent on the bacterial load in the lungs.
BALB/c or C57BL/6 mice were infected i.n. with $10^7$ CFU of BCG or treated with $10^7$ hk-BCG or BCG lysate or PBS, and BAL and serum collected at day 3, weeks 1, 3, 5 and 9. sTNFR1 and 2 secretion (A and B) was assayed with a standard ELISA kit and mean concentrations expressed as pg/ml. (C) Lungs were removed aseptically, homogenized and serial dilutions plated on Middlebrook 7H11 agar. Viable bacterial counts were evaluated 2-3 weeks after plating. Results are expressed as mean concentration (A and B) or CFU x $10^3$ (C) ± SD from 4 mice per group. A representative of two different experiments is shown.

* $p<0.05$ versus C57BL/6.

**Figure 4**

I.n. infection of BALB/c mice with BCG induced BCG-specific IgA in BAL but not serum. BALB/c mice were infected i.n. with $10^7$ CFU of BCG and BAL and serum collected at day 3, weeks 1, 3, 5 and 9. Pooled BAL (A) or serum (B) were analysed for BCG specific total, IgG and IgA antibodies against BCG lysate in an ELISA. Results are expressed as mean optical density (OD) values, from 4 mice per group, after subtraction of background reactivity with BSA at corresponding dilutions. PBS treated mice produced background levels of antibodies. A representative of two different experiments is shown.

**Figure 5**

I.n. infection of mice with BCG induced significant levels of antibodies to major mycobacterial antigens. BALB/c mice were infected i.n. with $10^7$ CFU of BCG or treated with $10^7$ hk-BCG or BCG lysate or PBS and BAL collected at day 3, weeks 1, 3, 5 and 9. Pooled BAL was analysed for IgG (A) and IgA (B) against Ag85c, 38-
kDa, 19-kDa or 16-kDa in an ELISA. Results are expressed as mean optical density (OD) values, from 4 mice per group, after subtraction of background reactivity with BSA at corresponding dilutions. PBS treated mice produced background levels of antibodies. A representative of two different experiments is shown.

**Figure 6**

I.n. treatment of mice with hk-BCG or BCG lysate induced antibodies to major mycobacterial antigens in serum. BALB/c mice were infected i.n. with $10^7$ CFU of BCG or treated with $10^7$ hk-BCG or BCG lysate or PBS and serum collected at day 3, weeks 1, 3, 5 and 9. Pooled BAL was analysed for IgG against Ag85c, 38-kDa, 19-kDa or 16-kDa in an ELISA. Results are expressed as mean optical density (OD) values, from 4 mice per group, after subtraction of background reactivity with BSA at corresponding dilutions. PBS treated mice produced background levels of antibodies. A representative of two different experiments is shown.

**Figure 7**

I.n. infection of BALB/c mice with BCG induced higher levels of antibodies to major mycobacterial antigens in BAL and serum compared to C57BL/6 mice. BALB/c or C57BL/6 mice were infected i.n. with $10^7$ CFU of BCG or treated with $10^7$ hk-BCG or BCG lysate or PBS and BAL collected at day 3, weeks 1, 3, 5 and 9. Pooled BAL and serum were analysed for IgG (A) and IgA (B) against Ag85c, 38-kDa, 19-kDa or 16-kDa in an ELISA. Results are expressed as mean optical density (OD) values, from 4 mice per group, after subtraction of background reactivity with BSA at corresponding dilutions. PBS treated mice produced background levels of antibodies. A representative of two different experiments is shown.
Figure 1

A

sTNFR1

- Live BCG
- BCG lysate
- hk-BCG
- PBS treated

Concentration (pg)

D3 w1 w3 w5 w9

B

sTNFRII

- Live BCG
- BCG lysate
- hk-BCG
- PBS

Concentration (pg)

D3 w1 w3 w5 w9

C

CFU

CFU x 10^3

D3 w1 w3 w5 w9

Figure 1
Figure 2

Serum

A

sTNFRI

Concentration (pg)

D3 w1 w3 w5 w9

Live BCG
BCG lysate
hk-BCG
PBS treated

B

sTNFRII

Concentration (pg)

D3 w1 w3 w5 w9

Live BCG
BCG lysate
hk-BCG
PBS
Figure 3

**A**

**sTNFR1**

Concentration (pg)

-BALB/c
- C57BL/6
- PBS treated

**B**

**sTNFRII**

Concentration (pg)

-BALB/c
- C57BL/6
- PBS

**C**

**CFU**

CFU x 10^3

-BALB/c
- C57BL/6

Figure 3
Figure 4

A  anti-BCG antibodies in BAL

B  anti-BCG antibodies in serum

Lysate
**Figure 5**

A

![Graph A](image1)

B

![Graph B](image2)

**Figure 5**
Figure 6

IgG

O.D.

Live BCG lysate hk-BCG

Serum

- Ag85c
- 38-kDa
- 19-kDa
- 16-kDa
- 14-kDa
- 12-kDa

w1 w3 w5 w9 w1 w3 w5 w1 w3 w5 w9

Live BCG lysate hk-BCG

Figure 6
Figure 7

**IgG in BAL**

![Graph A: IgG in BAL](image)

**IgA in BAL**

![Graph B: IgA in BAL](image)

**IgG in serum**

![Graph C: IgG in serum](image)