The Role of Tumour Necrosis Factor, Lymphotoxin Alpha and Interleukin-10 in the Host's Protective Immune Response Against Mycobacterial Infection.

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Thesis submitted to the University of Cape Town in fulfillment of the degree
Doctor of Philosophy
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Muazzam Jacobs

March 2002
Publications and Presentations

The following publications and presentations were based on the studies that are described in this thesis:

Publications.

Jacobs M, Brown N, Allie N, Guler R, Ryffel B.
Increased resistance to mycobacterial infection in the absence of interleukin-10.

Jacobs M, Marino MW, Brown N, Abel B, Bekker LG, Quesniaux VJ, Fick L, Ryffel B.
Correction of defective host response to Mycobacterium bovis BCG infection in TNF-deficient mice by bone marrow transplantation.

Jacobs M, Brown N, Allie N, Chetty K, Ryffel B.
Tumor necrosis factor receptor 2 plays a minor role for mycobacterial immunity.

Jacobs M, Brown N, Allie N, Ryffel B.
Fatal Mycobacterium bovis BCG infection in TNF-LT-alpha-deficient mice.

Manuscript in preparation.

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Lymphotoxin alpha deficient mice are susceptible to Mycobacterium tuberculosis infection.

Poster presentations.

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Jacobs M, Abel B, Brown N, Ryffel B.
Mycobacterium tuberculosis infection in LT-/- mice
Keystone Sympoias, Taos, New Mexico, USA.

2000
Jacobs M, Allie N, Brown N, Ryffel B.
Fatal Mycobacterium bovis BCG infection in TNF/LT-/- mice.
Nobel Symposium No 14, Karolinka Institute, Stockholm, Sweden.
In memory of my parents.

"I do not have money to give you, but I can give you a chance."

-My mother
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<tr>
<td>AFB</td>
<td>acid-fast bacilli</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>APES</td>
<td>3-aminopropyltriethoxysilane</td>
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<tr>
<td>BALF</td>
<td>bronchial alveolar lavage fluid</td>
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<tr>
<td>BCG</td>
<td>Bacille Calmette Guerin</td>
</tr>
<tr>
<td>CAB</td>
<td>chromotrope aniline blue</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>Cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td><em>Chlamydia trachomatis</em></td>
</tr>
<tr>
<td>DAB</td>
<td>3,3 diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
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<tr>
<td>FACS</td>
<td>fluorescent activated cell sorting</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>Fig</td>
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<tr>
<td>H/E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>ICAM</td>
<td>intercellular cell adhesion molecule</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>INH</td>
<td>isoniazid</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LAM</td>
<td>lipoarabinomannan</td>
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<tr>
<td><em>L. monocytogenes</em></td>
<td><em>Listeria monocytogenes</em></td>
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<td><em>L. major</em></td>
<td><em>Leishmania major</em></td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LT</td>
<td>lymphotoxin alpha</td>
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LTβR  lymphotxin beta receptor
M. avium  *Mycobacterium avium*
M. bovis  *Mycobacterium bovis*
MCP  monocyte chemoattractant protein
MHC  major histocompatibility complex
MIP  macrophage inflammatory protein
*M. tuberculosis*  *Mycobacterium tuberculosis*
n  sample number
ND  not detectable
NO  nitric oxide
OADC  oleic acid-albumin-dextrose-catalase
PBS  phosphate buffered saline
PDIM  phthioceroldimycocerate
PMN  polymorphonucleocytes
PPD  purified protein derivative
RANTES  regulated upon activation, normal T cell expressed and
         secreted.
RNI  reactive nitrogen intermediates
SD  standard deviation
sTNFR  soluble tumour necrosis factor receptor
TACE  TNF converting enzyme
*T. Cruzi*  *trypanasoma cruzi*
TGF  transforming growth factor
Th  T-helper
TNF  tumour necrosis factor
TNF/LT  tumour necrosis factor and lymphotoxin alpha
TNFR  tumour necrosis factor receptor
TRAF  tumour necrosis factor receptor associated factor
TRADD  tumour necrosis factor receptor associated death domain
WT  wild type
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Abstract

The aim of this study was to address the importance of tumour necrosis factor (TNF), lymphotxin alpha (LT) and interleukin-10 (IL-10) in an in vivo mouse model after challenge with either Mycobacterium bovis BCG or Mycobacterium tuberculosis H37Rv.

These studies conclude that

1. TNF"/" mice were highly susceptible to Mycobacterium bovis BCG infection. The disease was characterised by delayed and structurally disorganized granuloma formation with unrestricted pulmonary bacilli growth. Early recruitment of CD3^CD4^ and CD3^CD8^ T-lymphocytes were impaired and granuloma associated expression of the macrophage marker F4/80, adhesion molecules ICAM-1, CD11b and CD11c, and macrophage activation markers MHC Class II and iNOS were reduced. Enhanced production of the Th1 cytokines, IFNγ and IL-12, and the chemokines, MCP-1 and MIP-1α, were detectable during chronic infection and mice could induce a memory recall response in the absence of TNF. Resistance to Mycobacterium bovis BCG infection could be restored in TNF"/" mice by transplantation with WT haemopoietic precursor cells.

2. TNF/LT" mice were highly susceptible to Mycobacterium bovis BCG infection. Granuloma formation was characterised by small cellular clusters that failed to mature. Failure to control mycobacterial growth resulted in enhanced organ bacilli levels and the development of necrotic lesions. Hepatic cell clusters contained reduced expression of the lymphocytic marker CD3, macrophage marker F4/80, adhesion molecule ICAM-1 and the macrophage activation marker iNOS. Mycobacterium bovis BCG infected TNF/LT" mice could induce a memory recall response and were resistant to LPS challenge. Further it was shown that LT" mice do not succumb to Mycobacterium bovis BCG infection. Granuloma formation was structurally similar to that of WT mice. However, comparative bacilli burdens showed that LT" mice had higher bacilli levels than WT mice suggesting that the bactericidal efficacy of the granulomas to be impaired.
3. IL-10<sup>−/−</sup> mice were resistant to *Mycobacterium bovis* BCG and manifested an elevated protective immune response against infection. This was characterised by enhancements in clearance of bacilli which was accompanied by a vigorous granulomatous response. Significantly higher numbers of granulomas of increased size were established. Enhanced macrophage activation was represented by an increase in granuloma-associated expression of MHC Class II, acid phosphatase and iNOS. In the absence of IL-10, down modulation of the inflammatory response was inhibited which resulted in the persistence of enlarged granulomas subsequent to mycobacterial clearance.

4. LT<sup>−/−</sup> mice were highly susceptible to aerosol challenge with *Mycobacterium tuberculosis* H37Rv despite the ability to induce a Th1 response. Progression of infection was associated with a failure to establish defined pulmonary granulomas which resulted in elevated bacilli levels. The susceptibility of LT<sup>−/−</sup> mice was underlined by its failure to induce iNOS during infection and also displayed a lack of memory recall. Although TNF deficient and LT<sup>−/−</sup> mice displayed similar mortality rates at a standard dose (100 cfu/lung of *Mycobacterium tuberculosis* H37Rv) of infection, TNF<sup>−/−</sup> mice had a higher rate of mortality at a low infection dose (10 cfu/lung of *Mycobacterium tuberculosis* H37Rv) in comparison to LT<sup>−/−</sup> mice which suggested a more prominent role for TNF than LT in protective immunity. Irradiated WT chimeric mice, which were reconstituted with LT deficient bone marrow, had similar mortality rates as control LT<sup>−/−</sup> mice, which argued against the involvement of the developmental lymphoid deficiency in LT<sup>−/−</sup> mice to mycobacterial susceptibility.
Literature Review
Literature Review

LR. 1. Tuberculosis – A Historical Perspective.

Tuberculosis is an infectious disease that is induced by the intra-cellular pathogen *Mycobacterium tuberculosis*. Tuberculosis is primarily transmitted through the inhalation of infected aerosolised droplets. These droplets, normally ≤ 5-10 μm in diameter and containing ~1-10 bacilli and would lodge into the alveoli where they are phagocytosed by resident alveolar macrophages (McKinney et al., 1998, Smith and Moss, 1994). Other means of infection, though rare, include ingestion and inoculation. *Mycobacterium tuberculosis* is a member of the “tuberculosis complex”, a group of closely related mycobacterial pathogens, which include *Mycobacterium bovis*, *Mycobacterium microti* and *Mycobacterium africanum*. Due to its high sequence homology, common immunological properties and historical evidence it has been suggested that *Mycobacterium tuberculosis* originated from *Mycobacterium bovis* in its human host after the domestication of cattle. Recent evidence from comparative genomic studies of the different mycobacterial species that constitutes the tuberculosis complex have however argued against this possibility (Mahairas et al., 1996). Although skeletal data supporting the presence of tuberculosis in humans at ~5000 BC was available, conclusive diagnosis to the cause of disease was impossible (Haas, 1996). The first confirmed instance of tuberculosis infection in humans was noted in the deformities of the skeletal and muscular remains of Egyptian mummies at ~2400 BC (WHO website - History of TB, Haas, 1996). However, it could not be determined whether the disease was due to *M. bovis* or *M. tuberculosis*. By the year 200 BC tuberculosis was a documented global disease and was present in Europe, Middle East Asia and Far East Asia. During its emergence as a global threat several eminent scholars were confronted with the disease and made positive contributions towards understanding it. Aristotle recognised the infectious nature of the disease (Haas, 1996), Hippocrates described it as a pestilential disease (WHO website - History of TB; NJMS National Tuberculosis Center website; Haas, 1996) and Galen observed the presence of tubercles and that the disease could be obtained from an infected patient (Haas, 1996; Wits University website- The History of Human Tuberculosis). The introduction of the “germ theory” and the notion that “animalcula” or “small animals” could be the cause of tuberculosis were
revolutionary concepts that opened new avenues for exploration (WHO website - History of TB; Haas, 1996; Thomas et al., 1994). Further insight was provided through the work of Bayle and Laennec who put the tubercle as the unifying factor for the different types of pulmonary disease that was thought to be prevalent at the time (Thomas et al., 1994; Haas, 1996). Their work was extended by Villemin who showed that tuberculosis could be transferred across species by inoculating sample material from human tubercles into healthy rabbits (Thomas et al., 1994; WHO website - History of TB; Haas, 1996). Although significantly more information was available regarding tuberculosis at the time, the cause of the disease was still unknown to the scientific and medical community. It was in a lecture to the Physiological Society of Berlin in March 1882 that Robert Koch first gave notice of the isolation of the tubercle bacillus as the infecting vector in tuberculosis (Maulitz, 1982). His introduction of "tuberculin" as a cure for tuberculosis, although being a failure, was recognized as a valuable diagnostic tool for the disease. The early part of the twentieth century was dominated by the production of the first vaccine against tuberculosis. Calmette and Guerin derived an attenuated strain of the bacillus from M. bovis after 230 passages over 13 years, which offered protection against M. tuberculosis infection, but did not itself cause disease (Mckinney et al., 1998). In 1924 the first successful vaccination trial was done on 664 children in whom more than 90% protection was reported (Mckinney et al., 1998). The era of chemotherapy was ushered in with the successful use of the antibiotic streptomycin in a female patient with advance pulmonary tuberculosis in 1944. Dual therapy with para-salicylic acid and streptomycin proved more effective than the use of the drugs on their own in subsequent trials. The emergence of drug resistant strains gave impetus to the search for better and more effective drugs. This was achieved with the discovery that isoniazide and pyrazinamide were found to be more effective in clinical trials (Mckinney et al., 1998; Thomas et al., 1994). The synthesis of rifampicin and the introduction of ethambutol as chemotherapeutic agents soon followed these drugs. Underlying the advancement in the understanding of pathogen-host interaction has been the rapid development of molecular technology. This has allowed investigators to specifically alter the genetic composition of either the pathogen or the host and have resulted in the establishment of specific mutant strains that are widely used in research today. These are attested to by the large volume of articles, which involve mutant mycobacterial strains or gene targeted mutant mouse strains that were
published in the past 15 years. The most pivotal event of the last decade has certainly been the identification of the complete nucleotide sequence of the laboratory strain of *M. tuberculosis*, H37Rv (Cole et al., 1998).

**L.R. 2 Tuberculosis- A Current Perspective.**

In a seminal paper published in 1991 by Arata Kochi (Kochi, 1991) the global tuberculosis epidemic was clearly summarised and highlighted the factors, which contributed to the continuity of the disease. It was instrumental in emphasising the importance of proper management structures and served as a reference on which the implementation of the DOTS program was based. In this study the importance of BCG vaccination for the prevention of childhood tuberculosis was noted but just as important, it noted that it had a limited impact on adult tuberculosis. It identified HIV as the highest risk factor in Sub-Saharan Africa and predicted some of the dire social consequences that are currently associated with the HIV and *M. tuberculosis* in this region. What was also significant was that tuberculosis could effectively be divided into an “industrialised disease”, where most of the infections occurred in the elderly and the disease attributed to reactivation, and an “impoverished disease” where the risk of infection was high for all age groups but was most prevalent in the economically active populations. In the study new control strategies and objectives for the WHO were outlined which included ways to improve the cure rate and to expand tuberculosis services.

Tuberculosis however still remains a global health problem (WHO Report, 2001). A third of the world’s population or 1.8 billion people are reported to be infected with the disease. It is estimated that 8 million people develop active tuberculosis and that 2 million people succumb annually. The global distribution pattern indicates that the majority of cases are confined to regions with developing economies rather than industrialized regions. In South East Asia more than 3 million active cases are reported every year and in Sub-Saharan Africa the risk of infection can be as high as 2.5%. In the Balkan States of Eastern Europe the incidence of tuberculosis has reached epidemic proportions since the disintegration of the former Soviet Union. The decline in the world’s health status with respect to tuberculosis has been attributed to several factors. Although more than an 80% cure rate has been found in certain countries, which have adopted the DOTS, program as encouraged by the
Figure A
The map represents the global incidence rates of tuberculosis. Note that the darker shades of grey which represents the highest reported incidence is primarily confined to Sub-Saharan Africa. Industrialised countries show the lowest incident rates.
(Adapted from the WHO Report, 2001)
WHO, a major concern is the emergence of multi-drug resistant (MDR) strains that arise due to inadequately managed chemotherapeutic programs and incomplete drug treatment. Strains resistant to isoniazide and rifampicin have been widely reported (WHO Report, 1997). The AIDS epidemic has contributed significantly to the maintenance of the tuberculosis epidemic with the highest incidence rates of HIV-TB positive cases in sub-Saharan Africa. The effects of HIV on tuberculosis includes an increase in the rate of reactivation (7-10%/annum vs. 5-8%/lifetime), a faster rate of progression to active tuberculosis (37%/6 months vs. 5%/2 years) and higher rates of progression to extra pulmonary tuberculosis (Sepkowitz et al., 1995). Movement of people due to global travel, emigration, political or economic instability has also contributed to the status quo of tuberculosis. Although the number of countries that have implemented the DOTS strategy have increased, progress in the control of the disease has been slow (WHO Report 2001).

Mycobacterial pathogenicity, virulence and fitness can be ascribed to the physical and metabolic characteristics of M. tuberculosis to sustain and promote its presence within the host. Due to the hostility of its environment it has evolved specific biological mechanisms and physical features to adapt and survive. Thus the identification of such factors has become attractive targets for intervention therapy.

Structural analysis of M. tuberculosis has been a research subject for more than 50 years. A great deal of emphasis has been placed on the chemical identification and structural modeling of the surrounding envelope, a feature that is unique to mycobacteria. It has been postulated that the survival properties of M. tuberculosis within phagocytic cells could be attributed to this distinct structural characteristic. Examination of the cell wall by electron microscopy revealed that it consists of 4 "layers" namely the plasma membrane, an electron-dense layer, an electron transparent layer and an outer layer (Brennan and Draper, 1994). The electron dense layer is thought to contain peptidoglycan, the electron transparent layer appears to consist of arabinogalactan mycolate and the outer layer is thought to contain polysaccharide and protein. The cell envelope can therefore be described as consisting of a cell membrane, a peptidoglycan layer and a hydrophobic layer in which mycolic acids are covalently bound to the arabinogalactan skeleton. In addition
Figure C

Cell wall structure of M. tuberculosis.

The figure summarizes the constituents and the interrelated complexity that is associated with the M. tuberculosis cell wall structure. Lipoarabinomannan is depicted as anchored within the plasma membrane and proteins are shown as being distributed throughout the whole structure. (From McNeil et al, 1996)
lipoarabinomannan is anchored into the cell membrane via fatty acyl residues and transverse the cell wall. Several unique lipid moieties are associated with the cell wall in a non-covalent manner, which include trehalose dimycolate and phthioceroldimyocerate (PDIM). A schematic diagram is given in Figure B.

More difficult was to assign functional relevance to the different constituents of the outer envelope. Nevertheless, several studies have provided evidence supporting the importance of the cell wall for survival within the host. Lipoarabinomannan can inhibit IFNγ synthesis (Sibley et al., 1988, 1990) and act as a scavenger of free oxygen radicals (Chan et al., 1991) while phthiocerol dimycocerosate lipids was shown to inhibit the lymphocytic response. In addition it was shown by signature tagged mutagenesis that the absence of PDIM or failure of transport and correct localization into the cell wall leads to attenuation of pulmonary mycobacterial growth (Cox et al., 1999). Isoniazid was found to specifically inhibit a long chain enoyl-acyl carrier protein reductase, an enzyme that is essential in mycolic acid biosynthesis (Roswarski et al., 1998; Mdluli et al., 1998). It has also been demonstrated that the inhibition of mycolic acid synthesis occurs concomitantly with loss of M. tuberculosis viability (Dubnau et al., 2000). Antigen 85 is known to mediate the transfer of a mycoloyl residue from an α,α’ trehalose monomycolate to another resulting in the formation of α,α’ trehalose dimycolate. A mutant strain for Ag85C had a reduced mycolate content and had altered permeability properties (Belisle et al., 1997). Taken together, these studies illustrate that disruption of the cell wall structure alters its inherent permeable characteristics and thus influence its pathogenicity.

Macrophages act as a primary defense mechanism against foreign antigens. Uptake into early endosomes and delivery into the lysosomal pathway leads to degradation of the phagosomal contents (Pieters, 2001). Although it has been found that the route of entry could determine the subsequent fate of specific pathogens (Ishibashi and Arai, 1990, Da Silva et al., 1989), limited data is available which addresses this aspect in relation to mycobacteria. Selective blockage of the receptors CR1, CR3, CR4, mannose receptor and class A scavenger receptor did not alter the survival properties of M. tuberculosis (Zimmerli et al., 1996). An invasion mechanism that was used by pathogenic mycobacteria, and not by non-pathogenic strains, to gain entry into the
macrophages was shown to be dependent on its association with the complement cleavage product C2a. C2a would process C3 to C3b with subsequent opsonisation of the bacillus and uptake by macrophages (Schorey et al., 1997). This would suggest that the route of entry confer an advantage on the pathogenic strains, which can circumvent the bactericidal mechanism of the macrophage. A method which *M. tuberculosis* employs to prevent lysis, is to avoid fusion with lysozomes. It is known that pathogenic live bacilli are associated with early endosomes and the failure of these vacuoles to fuse with lysozomes is thought to be actively induced by the bacilli. Phagosomes, which contain live bacilli, fail to acquire Rab 7, a member of the GTPases, which regulate membrane trafficking between early and late endosomes (Via et al., 1997). In addition it shows a lack of acidification due to the exclusion of the vesicular proton ATPase (Sturgill-Koszycki et al., 1994). Recent studies have implicated the protein TACO (tryptophan aspartate-containing coat) in promoting the active inhibition of early endosomal compartment fusion with the lysosomal pathway (Ferrari et al., 1999). TACO was found in association with the membranes of phagosomes which harboured viable bacilli for prolonged periods of time while their association with phagosomes containing killed bacilli was limited thus supporting the active retention of the molecule. By retaining TACO, fusion of the bacillus-containing compartment with lysosomes is prevented. An interesting corollary was that Kupffer cells do not contain TACO and it is speculated that its absence could account for the resistance of this organ to mycobacterial infection. TACO is not an intergrated membrane protein but is attached to the phagosome via cholestrol. It has recently been shown that binding of *M. tuberculosis* to phagocytes occurs in cholesterol rich membrane areas and that depletion of cholesterol inhibits binding (Gattofield et al., 2000). Being internalised surrounded by plasma membrane rich in cholesterol assists in the retention of TACO and hence eludes fusion with the lysosome. It could therefore be erroneously interpreted that the bacilli-containing phagosome is an inert, static structure. Evidence to the contrary was shown in studies that such vacuoles could preferentially acquire constituents such as lysosomal associated membrane protein-1 (LAMP-1) yet could exclude the vesicular ATPase (Hart et al., 1991; Crowle et al., 1991). It was also shown that lipoarabinomannan could be detected in vesicles that were separate from the bacilli-containing phagosome which is suggestive of membrane trafficking into and out of the phagosome (Xu et al., 1994).
It has previously been reported that human alveolar macrophages undergo apoptosis after *M. tuberculosis* infection (Gao *et al.*, 2000). In a comparative study in which virulent or attenuated mycobacterial strains were used, significantly higher levels of apoptosis were observed in alveolar macrophages that were infected with attenuated strains (Keane *et al.*, 2000). This observation suggested that virulent mycobacterial strains could produce factors, which could actively modulate the apoptotic response. It is well established that TNF can mediate apoptosis. Such a mechanism was proposed in a study in which both virulent H37Rv and H37Ra, although inducing similar amounts of TNF from alveolar macrophages *in vitro*, caused differential release of soluble TNFR2 (Balcerwicz-Sablinska *et al.*, 1998). The increased production of soluble TNFR2 by H37Rv resulted in the formation of inactive TNF-TNFR2 complexes and hence a reduction in TNF bioactivity.

Further strategies, which mycobacteria employ to circumvent the immune response, are by down regulating or inhibiting macrophage and T-cell activation. It was found that the induction of MHC Class II and B7 molecules were significantly lower in infected *M. tuberculosis* macrophages with a consequent reduced ability to activate CD4 T-cells (Mishra *et al.*, 1994). It has also been postulated that *M. tuberculosis* induces the production of TGFβ (Hernandez-Pando *et al.*, 1997). TGFβ has previously been implicated in the inhibition of the generation of reactive oxygen and nitrogen intermediates and the secretion of Th1 cytokines (Oswald *et al.*, 1992). This has been supported in studies in which TGFβ production was detected by monocytes from infected patients (Toossi *et al.*, 1995). In addition both LAM (Dahl *et al.*, 1996) and PPD (Hirsch *et al.*, 1999) were shown to stimulate TGFβ from human monocytes. It was also found that *M. tuberculosis* infected human monocyte derived macrophages secreted the immunosuppressive cytokine IL-10 which inhibited the production of IL-12 and, together with IL-4 inhibited the production of IL-8, a chemokine which is important for mediating leukocyte recruitment to sites of infection. The generation of reactive nitrogen intermediates as a mechanism for killing mycobacteria has been well documented for mice (MacMicking *et al.*, 1997b) and more evidence is emerging supporting a role in humans (Nicholson *et al.*, 1996). Evasion of such reactive substances by the bacillus would therefore contribute to its survival. It has been
shown that NO production is inhibited by cytokines such as IL-10 and TGFβ (Gazzinelli et al., 1992a,b). The regulatory functions of these cytokines have been exploited by several different pathogens to either gain entry to the host or to down regulate its immune responses.

One of the major events that have infused urgency in several research areas of tuberculosis has been the publication of the complete genomic sequence of *M. tuberculosis* strain H37Rv (Cole et al., 1998). It has allowed researchers to compare genomes within the *M. tuberculosis* complex and to assign functional relevance of transcribed genes and synthesized proteins. *M. tuberculosis* H37Rv has a genomic sequence which consists of 4,411,529 base pairs that code for ~4000 proteins and 50 genes are transcribed as stable RNA (Cole et al., 1998). A major finding of the study was that ~9% of the genome coded for two new glycine-rich protein families namely the PE and PPE families in which the N-termini of each family are conserved. Functional data on the genes is limited but it has been implicated in survival of *M. tuberculosis* in vivo (Camacho et al., 1999) and was implicated in the growth of *M. marinum* in frogs (Ramakrishnan et al., 2000). A second surprising feature of the genome was the high number of genes that was associated with fatty acid metabolism. Several systems are in place, which can catalyse the β-oxidation of fatty acids and suggests that the bacilli can utilize different lipids as sources of carbon. In view of the identification of the polyketide, mycolactone, as a virulence factor during *M. ulcerans* infection (George et al., 1999) it was interesting to note that more than 39 genes were involved in polyketide metabolism. Comparative genomic studies of *M. bovis* and *M. bovis* BCG (Pasteur) with H37Rv indicated that there were seven deleted regions in the bovis strains (Domenech et al., 2001). Although not conclusively proven it was suggested that the differences relating to the deleted regions could be involved in host specificity or virulence. A comparison of different BCG strains revealed that a total of 16 deleted regions were detected in different combinations amongst the BCG strains. 9 of the deleted regions were absent in all BCG strains but present in H37Rv and only one region was present in all the members of the *M. tuberculosis* complex but absent in all BCG strains. It is speculated that the loss of this region, which encodes the ESAT-6 system, and some of the PE and PPE genes, could have accounted for the generation of the first attenuated BCG strain. Given the differences
in genetic composition of the different BCG strains it is now possible to speculate on
the possible reasons for the variability in the efficacy of BCG in several vaccine trials.

More relevant than the identification of genomic differences between attenuated and
virulent strains would be to investigate how these differences are converted into
functional significance. It is therefore important to identify differences in
transcription and protein synthesis between different strains under conditions of stress
and growth. In studies in which *M. tuberculosis* was exposed to INH it was found
that genes that were involved in fatty acid metabolism were transcribed (Wilson *et al*.,
1999). These included genes that were involved in fatty acid synthesis, β-oxidation of
fatty acids and cell wall synthesis. On uptake of *M. tuberculosis* in macrophages it
was discovered that transcription of sigma factors, which were implicated in stress
survival, was initiated (Fernandes *et al*., 1999). In a comparative proteomic study it
was found that ~2600 proteins were translated in members of the *M. tuberculosis*
complex which included proteins components of the cell wall and proteins that confer
drug resistance (Jungblut, 1999). Comparisons between the virulent strains H37Rv
and Erdman revealed that certain proteins, which were synthesized in one strain, were
absent in the other and that the degree of synthesis differed between the two strains
for specific proteins. Interestingly, a comparison between the avirulent BCG Chicago
strain and H37Rv revealed that although 13 genes of H37Rv were absent from BCG
Chicago, the inverse was also true. Eight proteins of BCG Chicago were absent from
H37Rv.

Understanding the growth of mycobacteria within the host has been more
complicated. *M. tuberculosis* is an obligate aerobe but can shift into an anaerobic
respiratory state depending on oxygen availability (Wayne *et al*., 1996). It is thought
that different rates of replication in different organs are at least partly influenced by
the oxygen tension within that organ. It is still a matter of conjecture as to how the
bacillus survives and replicates within granuloma, which is thought to have limited
oxygen availability. However, several findings have suggested that *M. tuberculosis* is
remarkably well adapted to persist under low oxygen tension or anaerobic conditions.
It is now believed that *M. tuberculosis* employ the glyoxylate shunt under anaerobic
conditions. Central to this pathway, which makes use of fatty acids or acetate as
sources of carbon to produce glucose, is the enzyme isocitrate lyase. It was elegantly illustrated that an isocitrate lyase mutant mycobacterial strain fails to persist during chronic infection (Mckinney et al., 2000). In addition, it was reported that bacilli, which had been isolated from mouse tissues, preferentially utilised fatty acids as a carbon source. Now that it is known that a considerable part of the \textit{M. tuberculosis} genome incorporates genes for lipid biosynthesis, does the combined evidence suggest that the organism may preferentially be using an anaerobic metabolic pathway during chronic infection. Further evidence substantiating this assertion has been the isolation of enzymes such as lactate dehydrogenase, which are required for anaerobic growth from bacillary extracts (McKinney et al., 1998). From the genomic sequence of H37Rv two hemoglobins that can bind oxygen with high affinity under low oxygen tension has been identified as well as a gene locus coding for a nitrate reductase, an enzyme that is essential for anaerobic respiration (Cole et al., 1998). In view of the identification of new genes that suggest that \textit{M. tuberculosis} is exquisitely adapted for anaerobic growth, clinical and experimental of yesteryear, which addressed mycobacterial growth under these conditions, need to be revisited. This is particularly important with respect to its relevance to latent tuberculosis infection. The resilience of this organism was elegantly illustrated in a study, which demonstrated that even after 12 years' of exposure to anaerobic conditions, the bacillus could still adapt to growing under aerobic conditions (Corper et al., 1933). The metabolic complexity of \textit{M. tuberculosis} was further illustrated in which two different populations of organisms were isolated from the same lungs; one population was drug resistant and metabolically active, while the other was drug sensitive but metabolically inert (Vandiviere et al., 1956)

\textbf{LR. 4. Animal models in experimental tuberculosis.}

One of the pioneers to use animals as models for experimental tuberculosis has been Robert Koch. Its use today is certainly as valid as it was more than a century ago. The criteria for selecting an animal model for experimental tuberculosis should be based on how faithfully the model approximates the condition in man. Practically this is not always possible, as several other criteria need to be taken into account. There are several different animal models that are currently being used. These include cattle, non-human primates, possums, armadillos, rabbits, guinea pigs and frogs. However the animals that are most widely used in research today are mice.
Mice, guinea pigs and rabbits exhibit many of the clinical features that are associated with tuberculosis in humans (McMurray, 2001; McMurray, 1994; Dannenberg, 1994; Orme and Collins, 1994; Thoen, 1994; Orme and McMurray, 1996). These animals can be infected with virulent tuberculosis strains by aerosol inhalation, which will progress to establishment of disease. The recruitment of mononuclear cells to sites of infection and the formation of granulomas characterize the immune response. Protective immunity is mediated by a Th1 response which is dependent on CD4 and CD8 positive T-Cells. Extra pulmonary dissemination occurs and can be detected in organs such as the liver, spleen and lymphnodes. A delayed type hypersensitivity response can be induced and all three animals can be protected by BCG vaccination. While caseating necrosis is present in both the rabbit and guinea pig, this process is absent in mice. It is however only in the rabbit that necrotic lesions progress to cavitation. All three animals do eventually succumb to infection but the degree of susceptibility differs with the guinea pig more susceptible than the rabbit or mouse. One of the major disadvantages of these models is that although all three animals are capable of controlling infection none of these models induce a state of latency. Infection is characterized by an acute phase of growth followed by a stationary chronic phase in which the animal continues to survive but with high bacillary titers.

The mouse has evolved as the choice animal of researchers for the investigation of several diseases. Besides the relative low maintenance cost compared to other animals, the mouse is genetically similar to man as most human genes have a murine counterpart (Malakoff, 2000). There are several well-characterized inbred strains of mice that are commercially available and rapid advances in molecular technology have assisted in specific genetic manipulation of these mouse strains. Currently, targeting specific genes for alteration is confined to mice only. Inactivation of genes has normally been generated through the insertion of a selectable neo-cassette into the gene/locus of choice. Phenotypes that are obtained in genetically altered mice require careful analysis as long-range disruption of gene expression through insertion of selectable cassettes has been reported (Pham et al., 1996). The impact of gene<sup>-/-</sup> mice in particular has contributed significantly to understanding the dual relationship of <i>M. tuberculosis</i> pathogenesis and host response. In fact, the effects of the absence of interferon during mycobacterial infections in humans (Dorman et al., 1998; Newport
et al., 1996; Jouanguy, 1996) were preceded by infection studies that were performed in mice, which were deficient for interferon signaling (Kamijo et al., 1993; Flynn et al., 1993; Cooper et al., 1993). Today the use of standard gene-" knockout mice is common. Emphasis has shifted more to the use of conditional gene-targeted mice, which can be defined as a genetic modification that is restricted to specific cell types, or developmental stages of the mouse. The use of the Cre-loxP recombination system has been extremely useful for the introduction of point mutations into genes, deletion of specific genes or the replacement of a specific gene (Barinaga, 1994). Basically, the Cre-enzyme is a recombinase that acts at a loxP sequence site and when two loxP sites are in tandem it removes the DNA sequence between them but leaves one loxP site. The number of different types of Cre transgenic mice is increasing. Two of the most popular types of strains are those in which the Cre-recombinase is under the control of a cell/tissue specific promoter or the Cre recombinase is under the control of an inducer molecule. Thus to generate e.g. a tissue/cell type specific gene deficient mouse, requires the modification of the ES cell genome by the introduction of loxP sequences. Mice that contain the loxP sequences are subsequently crossed with a Cre transgenic strain after which the correct mice are selected.

Modeling latency in animals has proven to be difficult and selecting a model that best mimics the situation in humans is controversial. Nonetheless, there are currently two basic mouse models, which carry favour with investigators. The Cornell-model was first introduced in the 1950's after which several variations were established (McCune et al., 1957a-b). It basically involves the establishment of infection after which the animal is treated with a regimen of antimycobacterial drugs until bacilli are undetectable by culture. Immediately after the removal of the drugs, a period occurs in which further mycobacterial is inhibited, the so-called latency period. The reinitiation of mycobacterial growth can either occur spontaneously or can be induced by an immunosuppressant. However questions have been raised regarding the use of chemotherapy and its possible consequences on the protective immune response (Flynn et al., 2001). The second model involves the establishment and progression of infection without the intervention of chemotherapy. In this model infection is allowed to develop until equilibrium is established whereby mycobacterial growth, although being at high titer, remains static (Flynn et al., 1998). Mice in this state can continue to control infection for up to a year. It has been argued that this equilibrium situation
emulates the latent state in humans better than one that is induced by chemotherapy. During immunosuppression of the immune response the high mycobacterial titres increase even further and lead to death of the animal. Controversy surrounds the high titers that are present in this mouse model. Comparatively it does not resemble the infection in humans and hence doubt has been cast on its representation as an applicable model.

**LR. 5. The immune response against *M. tuberculosis.***

The immune response against *M. tuberculosis* can be divided into two components i.e. innate immunity and adaptive immunity. Innate immunity can be described as a nonspecific cellular and humoral response that includes cells such as macrophages, NK cells, neutrophils and proteins molecules such as those of the compliment system, lysozymes, specific cellular receptors (e.g. LPS receptor, mannose receptor) and acute phase proteins. Adaptive immunity is a learned memory-recall response of which T- and B-lymphocytes are the central effector cells. Emerging evidence suggests that these two systems are not mutually exclusive but that the innate immune response has an instructive role in adaptive immunity (Fearon et al., 1996; Song et al., 2000; Metzhitov et al., 1997). The involvement of the complement system has been illustrated in studies, which demonstrated that *M. tuberculosis* could invade macrophage through opsinization with C3 (Schorey et al., 1997) with subsequent binding to CR1, CR3 and CR4 (Ernst, 1998). Other receptors that have been implicated in *M. tuberculosis* uptake are the mannose and CD14 receptors, putatively using LAM as the binding ligand, surfactant protein-A, class A scavenger receptors and Fcy receptors which bind immunoglobulin G coated mycobacteria (Ehlers et al., 1998; Ernst, 1998). One of the most significant recent findings that lend support to a direct association between the innate and adaptive response has been the identification of the Toll-like receptor family of proteins. These proteins, which were first discovered in Drosophila, were postulated to be involved in immunity through the similarities that existed with the IL-1 receptor/I-k B/NF-κ B cytokine pathway (Medzhitov et al., 2000). Initial evidence for its involvement in recognising *M. tuberculosis* was provided by studies which showed that the 19Kd *M. tuberculosis* lipoprotein could induce NF-κ B with subsequent production of IL-12 as well as iNOS promoter activity and that these activities were dependent on TLR2 (Brightbill
Further evidence was provided in studies, which showed that ara-LAM, mycolylarabinogalactan-peptidoglycan and M. tuberculosis lipids could activate murine macrophages by signaling through TLR2 (Means et al., 1999). These observations were confirmed in in vitro studies, which demonstrated that murine macrophages could be activated by M. tuberculosis through either TLR2 or TLR4 in a CD14 independent manner (Means et al., 1999).

As an intracellular pathogen, M. tuberculosis requires an adaptive immune response i.e., cell mediated immunity rather than a humoral response. Cell mediated immunity can be classified into two major subsets namely a Th1 response or a Th2 response (Mosmann et al., 1996). Th1 cells are characterised by the production of IFNγ, LT and IL-2 (Mosmann et al., 1996) whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Rogge et al., 1997; Mosmann et al., 1996). Both helper cell lines originate from a common IL-2 secreting progenitor and although it has been attempted to define distinctive cell markers for each subset (Xu et al., 1998), the subject remains controversial. The differentiation of a naïve T helper cell to either a Th1 or Th2 cell type is critical to the outcome of disease and thus the factors determining differentiation becomes crucial. One of the major factors, which are believed to determine this outcome, is the cytokine environment. The presence of IL-12 and IFNγ are the primary inducers of a Th1 response whereas IL-4 is important for a Th2 response. Because of the low number of antigen specific T-cells that are present during the primary response it is thought that cells which form part of the innate immune response provide the cytokine environment. IL-12 production through signaling of the Toll-like receptors has been discussed and it has been shown that natural killer cells can produce IFNγ in response to IL-12 (Scott, 1993). Mast cells (Plaut et al., 1989), basophils (Seder et al., 1991) and CD3+ CD4+ NK1.1 cells (Scott et al., 1990) are all capable of synthesizing IL-4. Other factors that influence naïve T cell differentiation includes antigen concentration with low concentrations of antigen inducing a Th1 response and high antigen concentrations inducing a Th2 response (Constant et al., 1997). APC have a direct effect on T-cell differentiation through regulation of its co-stimulatory molecules B7.1 and B7.2. It has been shown that antibodies against B7.1 and B7.2 inhibit the development of a Th1 and Th2 response respectively (Constant et al., 1997). Thus, it is evident from the literature that the
prerequisites on which a critical decision such as T-cell differentiation is based, is multi-factorial and tightly regulated.

For the effective control of *M. tuberculosis* infection the host requires to develop a cell mediated Th1 response (Cooper *et al.*, 1995b). The importance of IFNγ signaling in the Th1 response and hence in the control of mycobacteria was illustrated in studies which identified dissemination of bacilli in Maltese children with a truncated form of the IFNγ receptor (Levin *et al.*, 1995) as well as in a child with a deficiency for IFNγ-R who received *M. bovis* BCG vaccination (Dorman *et al.*, 1998). These findings were supported by studies in which IFNγ - or IFNγ-R⁺⁻ mice were challenged with *M. tuberculosis* (Flynn *et al.*, 1993; Cooper *et al.*, 1993) or *M. bovis* BCG (Kamijo *et al.*, 1993). These mutant strains lacked protective immunity, had high bacilli burdens and eventually succumbed to the infection.

IL-12 is a potent inducer of IFNγ production and central to the development of a Th1 response (Cooper *et al.*, 1995a). However IL-12 independent production of IFNγ by CD8⁺ T-cells has also been reported (Kaplan *et al.*, 1998; Carter *et al.*, 1999). Individuals who are defective for the IL-12R have dissemination of bacilli after *M. bovis* BCG vaccination and are susceptible to salmonella infection (de Jong *et al.*, 1998). Concomitantly they had reduced levels of IFNγ. The importance of IL-12 in the protective immune response against mycobacterial infection was demonstrated in several murine studies (Flynn *et al.*, 1995b; Cooper *et al.*, 1997b; Castro *et al.*, 1995; Wakeham *et al.*, 1998). *M. tuberculosis* was shown to induce IL-12 production both *in vivo* and *in vitro* (Cooper *et al.*, 1995b). IL-12 treatment afforded better protection to the relatively susceptible Balb/c mouse strain, which doubled their survival times (Flynn *et al.*, 1995b). The inability of IL-12p40⁻⁻ mice to control *M. tuberculosis* infection conclusively proved its importance in protective immunity (Cooper *et al.*, 1997b). These mice developed high bacilli titers and had reduced levels of IFNγ production. It was demonstrated that IL-18 acted synergistically with IL-12 to induce IFNγ production (Munder *et al.*, 1998; Micalef *et al.*, 1996; Robinson *et al.*, 1997) but that induction of transcription was differentially regulated (Barbulescu *et al.*, 1998). Its importance in the Th1 response was implied in studies, which showed that the IL-18R was expressed only in Th1 T-cell clones (Hoshino *et al.*, 1999). A direct
role in the elimination of mycobacteria was confirmed in studies in which IL-18-/- mice were infected with either *M. tuberculosis* or *M. bovis* (Sugawara et al. 1999, Takeda et al., 1998). These mice developed enlarged granulomatous lesions and had impaired IFNγ production.

A major consequence of the cooperative action of IFNγ and TNF is the generation of reactive nitrogen intermediates by activated macrophages. Central to this process are the nitric oxide synthases. By employing nitric oxide synthases inhibitors it was shown that NO is effective against a range of infectious agents such as viruses, parasites, helminthes and intracellular bacteria (MacMicking et al., 1997). Encouraging evidence reporting the detection of iNOS in patients with active tuberculosis lends credence to the use of NO as an active component against *M. tuberculosis* infection in humans (Nicholson et al., 1996). In contrast, its inhibitory role during mycobacterial infection in mice is well described. Mice, which have a homozygous disruption in their NOS2 allele, are susceptible to *M. tuberculosis* infection (Scanga et al., 2001; MacMicking et al., 1997) and *M. bovis* BCG infection (Garcia et al., 2000) and rapidly succumb. NO has also been implicated in the control of mycobacterial growth during latency and chronic infection in mice (Scanga et al., 1999; Flynn et al., 1998). Administration of aminoguanidine, an iNOS specific inhibitor, to mice with chronic infection resulted in excessive mycobacterial growth and an increase in mortality (Flynn et al., 1998). Mice, in which a latent infection was induced by treatment with isoniazid and pyrazinamide, however did not reactivate after treatment with the iNOS inhibitor, L-Nil possibly due to mycobacterial sterility of the mice (Scanga et al., 1999). It is interesting to note that inactivating genes which are involved in IL-12 or IFNγ signaling result in defective iNOS activity and that this consequential effect is, at least partially, responsible for the susceptibility of these strains.

**LR. 6. T-cells in tuberculosis.**

The intracellular location of the infecting pathogen is important for its detection and elimination and determines the presentation pathway of the MHC. Generally, antigenic determinants from pathogens that reside within the intracellular vacuoles are presented to CD4+ T-cells in the context of MHC class II presentation (Cresswell and
Howard, 1997; Pieters, 1997) whereas those antigens which are derived from pathogens residing in the cytosol are presented in the context of MHC class I molecules to CD8+ T-cells (Cresswell and Howard, 1997; Koopmann et al., 1997). It is thought that although M. tuberculosis and M. bovis BCG resides within phagosomes of cells both CD4+ and CD8+ T-cells are involved protective immunity (Cooper and Flynn, 1995). The importance of lymphocytes for the control of mycobacterial infection was aptly illustrated by infecting SCID mice (North and Izzo, 1993) and RAG-/- mice (Ladel et al., 1995), which are essentially devoid of lymphocytes, with M. bovis BCG. These mice, unlike their WT counterparts, were unable to resolve the infection.

The requirement for CD8+ T-cells in protective immunity against M. tuberculosis infection was first established through adoptive transfer experiments (Orme and Collins, 1983; Orme and Collins, 1984) and through the depletion of specific subsets (Muller et al., 1987). It was shown that CD8+ T-cells are able to recognise mycobacterial antigens which are presented either by MHC class I (Mohagheghpour et al., 1998; Serbina et al., 2000) or CD1 molecules (Jullien et al., 1997; Stenger et al., 1997). M. tuberculosis antigens, which were immunogenic and capable of eliciting a CTL response, include ESAT-6 (Lalvani et al., 1998), 19Kd lipoprotein (Mohagheghpour et al., 1998) and the 38 Kd protein (Zhu et al., 1997). During M. tuberculosis infection, CD8+ T-cells are recruited to the lungs where they are capable of secreting IFNγ (Serbina and Flynn, 1999; Feng et al., 1999). In the absence of CD4 T-cells, IFNγ secretion can be augmented by CD8+ T-cells but is not enough to fully rescue infected animals, which eventually succumb from infection (Caruso et al., 1999). Several studies have implicated CD8+ T-cells, which are potent producers of IFNγ, in protective immunity against mycobacterial infection. It is thought that CD8+ T-cells can mediate its effects in the following ways: (a) activation of macrophages through secretion of IFNγ and TNF to produce NO which result in killing of intracellular bacilli, (b) secretion of the cytolysis molecule granulysin in association with perforin (Stenger et al., 1998). Perforin is a granule molecule, which can form pores in cell membranes and facilitate access of granulysin to intracellular bacteria. It was shown that granulysin can kill mycobacteria directly, (c) inducing apoptosis through FAS–FASL interactions of inert infected macrophages that are incapable of
being activated. Released mycobacteria are than available for uptake by newly recruited phagocytic cells (Kaufmann, 1988; Kaufmann, 1993). A role for perforin in the control of mycobacterial infection was supported by studies in which CD8+ T-cells, which express perforin, were isolated from *M. tuberculosis* infected murine lungs (Serbina *et al.*, 2000). Data contesting a role for perforin, granzyme B and FAS in immunity against *M. tuberculosis* have been published (Cooper *et al.*, 1997; Laochumroonvorapong *et al.*, 1997). Mice deficient for these genes did not show marked differences in comparison to the WT strain in controlling infection. Controversy still surrounds the relative importance of CD8+ T-cells in *M. tuberculosis* infected mice. Studies, which indicated that the absence of CD8+ T-cell mediated immunity resulted in high susceptibility (Flynn *et al.*, 1992) and low susceptibility (Mogues *et al.*, 2001), have been published. Further evidence has been forwarded for a role of CD8+ T-cells in latent infection (van Pinxteren *et al.*, 2000). The acute phase of infection correlated with an increase in IFNγ producing CD4+ T-cells in *M. tuberculosis* infected mice. However the establishment of latency through chemotherapy coincided with an increase in IFNγ producing CD8+ T-cells.

The observation of the increased risk of HIV/AIDS positive patients in contracting tuberculosis is probably the most poignant statistic to emphasise the importance of CD4+ T-cells in combating *M. tuberculosis* infection. These individuals have an annual risk of 10% in contracting tuberculosis in comparison to a lifetime risk of 10%. Early studies have shown that CD4+ T cells can transfer immunity (Orme and Collins, 1983; Orme and Collins, 1984). CD4+ T cells specific for mycobacterial antigens have been identified (Geluk *et al.*, 1998). After infection with *M. tuberculosis* activated CD4+ T-cells can be isolated from the lungs and draining lymphnodes of mice (Feng *et al.*, 1999). The significance of CD4+ T-cells was confirmed in *M. tuberculosis* and *M. bovis* BCG infection studies using MHC class II+ mice and CD4+ mice (Caruso *et al.*, 1999). These mice have a profound deficiency in CD4+ T-cells and were unable to control the infection. They had reduced granulomatous lesion sizes that were histologically different from their WT counterparts, reduced iNOS expression and eventually succumbed to infection. The survival rates of infected MHC class II+ mice were mirrored by CD4+ T-cell depletion experiments in WT mice, which were infected with *M. tuberculosis* (Flory *et al.*, 1992; Leveton *et al.*, 1989). Several
studies have concentrated on the ability of CD4+ T-cells to activate macrophages through the production and action of IFNγ. MHC class II+ mice produce significantly reduced levels of IFNγ on challenge with M. tuberculosis (Caruso et al., 1999) and M. bovis BCG (Xing et al., 1998) during the acute phase of infection, which confirm CD4+ T-cells as a major source of IFNγ. IFNγ however requires the synergistic signaling of TNF to fully activate macrophages. It has previously been shown that T cells are capable of producing TNF (Barnes et al., 1993) and CD4+ T cells could therefore activate macrophages independent of any other source of cytokines. Although it is universally accepted that CD4+ T-cells are crucial for controlling mycobacterial infection during the acute phase of infection, its importance during the latent phase of infection still requires further investigation. In two studies, one in which a chronic infection mouse model was used and the other in which a latent state was obtained in mice after chemotherapy reported different findings. Depletion of CD4+ T cells after M. tuberculosis infection in the chronic infection mouse model resulted in uncontrolled growth despite the continued presence of IFNγ and NOS2 activity (Scanga et al., 2000). In contrast, the absence of CD4+ T cells in the latter infection model did not result in reactivation of the latent infection (van Pinxteren et al., 2000). CD4+ T cells are important in mediating immunological memory. Mice, which have previously been rendered immune by primary infection and antibiotic treatment, displayed rapid recruitment of activated CD4+ T-cells proficient for IFNγ secretion after reinfection with M. tuberculosis (Orme, 1988).

Other cell types have been implicated in protective immunity to a lesser degree. It was shown that CD4+CD8+ T lymphocytes could effectively lyse M. tuberculosis infected macrophages and that this process was mediated through FAS-FASL interaction but that this had no effect on the viability of mycobacteria (Stenger et al., 1997). A role for γδ T cells have initially been implied in studies which showed elevated levels of this subset in the lungs and draining lymphnodes of M. tuberculosis infected mice (Janis et al., 1989). Whether it influences protective immunity is still unresolved. Data, which support the involvement of γδ T cells at high doses of infection, have been published (Ladel et al., 1995) whereas a recent study showed that the absence of γδ T cells has no influence on mycobacterial growth (Mogues et al., 2001). CD4+NK1+ T-cells of the liver are normally potent producers of IL-4 and
promote a Th2 response. However, after *M. bovis* BCG infection, CD4^+^NK1^+^ T-cells, which secrete IFNγ were isolated (Emoto *et al.*, 1999). The generation of this population was inhibited in the presence of IL-12 antibodies and was accompanied by an increase in mycobacterial growth.

**LR.7. Establishment of granulomas during mycobacterial infection.**

Granuloma formation is the first pathological manifestation in the host immune response to mycobacterial infection. Simplicistically, it can be regarded as a process that facilitates the isolation of bacilli to prevent further spreading. However, it is a metabolically active structure, which promotes the coordinated action of phenotypically different cell subsets and cell types and is formed as a consequence of cytokine and chemokine stimuli that regulates the expression of adhesion molecules and induce recruitment of cells. Maintaining the integrity of the granuloma structure is necessary for effective control of infection. Granulomas are characterised by a central core of macrophages, which is surrounded by a lymphocytic mantle. In humans, granulomas often progress to caseous necrosis and liquefy which can lead to dissemination of bacilli. Granuloma formation in the rabbit and guinea pig resemble those in human closely whereas mice, although having a similar cellular composition, display different structural properties (McMurray, 2001). A central core of macrophages can often be observed but these are usually interpersed and surrounded with infiltrating lymphocytes. Several factors drive the formation and stability of granulomas, one of which is the cytokine environment. IFNγ, IL-12 and TNF are all essential for granuloma formation as mice which are deficient for any of these cytokines, have an abnormal granulomatous response on challenge with *M. tuberculosis*, display uncontrolled mycobacterial growth and eventually succumb (Cooper *et al.*, 1993; Cooper *et al.*, 1997^2^; Bean *et al.*, 1999). The significance of CD4^+^ T-cells in granuloma formation was illustrated in CD4 deficient and MHC Class II^+^ mice after *M. tuberculosis* infection (Caruso *et al.*, 1999). Both mouse strains have a lack of CD4^+^ T-cells and do not form proper granulomas. These observations were confirmed in CD4 depletion studies using *M. avium*, which demonstrated that such mice lack the ability to initiate granuloma formation during early stages of infection (Hansch *et al.*, 1996).
Granuloma formation requires coordinated recruitment of cells to sites of infection, which is regulated by chemokine production. Chemokines are small chemotactic proteins that can be distinguished as belonging either to the CXC or CC subset (Schluger and Rom, 1997). Most chemokine receptors are promiscuous and allow for more than one binding ligand (Premack and Schall, 1996). With respect to granuloma formation, the most important chemokines are those that are involved in lymphocyte and monocyte trafficking. These include RANTES, MIP-1α, MIP-1β, MCP 1-5 and IL-8 (Adams and Loyd, 1997). Several studies have associated chemokine expression or chemokine receptor expression with tuberculosis infection. IP-10, which is secreted by bronchial epithelial cells and is selective for the recruitment of IL-2 activated T-lymphocytes was detected in elevated concentrations in airways of tuberculosis patients (Sauty et al., 1999). MCP-1 secretion was induced in a human alveolar epithelial after M. tuberculosis infection (Lin et al., 1998b) and isolated monocytes from tuberculosis patients spontaneously expressed higher levels of MCP-1 mRNA (Lin et al., 1998b). Mice, which over express MCP-1, were more susceptible to infection (Rutledge et al., 1995) whereas MCP-1−/− mice were unable to mount a Th2 response and synthesised significantly lower levels of IL-4, IL-5 and IL-10 (Lu et al., 1998). It is therefore interesting to speculate whether the induction of MCP-1 during infection is to indirectly inhibit a Th1 response through induction of Th2 cytokines. MCP-1−/− mice however are not susceptible to M. tuberculosis infection, an observation which supports its non-involvement in mounting a Th1 response (Lu et al., 1998). CCR2−/− mice are thought to have a role in protective immunity against M. tuberculosis as mice, which are deficient for this gene, are highly susceptible to infection (Peters et al., 2001). The expression of CCR5, a receptor for MIP1α, MIP-1β and RANTES was upregulated in monocyte derived macrophages and alveolar macrophages from tuberculosis patients only (Fraziano et al., 1999). Recruitment of activated T cells is important in inflammatory diseases. TNF and IFNγ acted synergistically to induce the expression of the IP-10, Mig and I-TAC, CXC chemokines which specifically attracts activated T- cells (Sauty et al., 1999). In addition expression of IP-10 were associated with bronchial epithelium and brochoalveolar lavage cells of tuberculosis patients. A direct relationship was established between TNF and the expression of the CXC chemokines MIP-2 and CIN and the CC chemokines MIP1α, MIP-1β and MCP-1 in a rat lung injury model.
(Czermak et al., 1999). Neutralization of TNF by anti-TNF treatment inhibited cytokine expression and underscores the role of TNF as promoting secretion of both CXC and CC chemokines. In view of the importance of TNF in granuloma formation the data provides and indirect relationship between granuloma formation and chemokines. A direct relationship was established between RANTES and M. bovis derived PPD induced granuloma formation (Chensue et al., 1999). Depletion of RANTES significantly reduced granuloma lesion area, which suggested that RANTES promotes granuloma formation. IL-8 is a chemotactant for neutrophils, T cells and monocytes. IL-8 has isolated from the brochoalveolar lavage fluids from TB infected patients, which correlated with leukocyte numbers and were higher in plasma of patients that died from TB than in patients who survived. It was shown to assist in granuloma formation because anti-IL8 treatment inhibited reactivity during tuberculin skin tests.

**L.R. 8. Tumour Necrosis Factor and Lymphotoxin**  
TNF, LTα and LTβ, and its receptors are members of extended super gene families (Locksley et al., 2001). Although several different cell types can synthesize TNF under in vitro conditions (Vasalli, 1992) its production is mainly attributed to macrophages and T-cells whereas LTα and LTβ are primarily synthesized by activated T, B and NK cells. TNF, LTα and LTβ are structurally homologous proteins that are encoded by genes, which are clustered on human chromosome 6 and mouse chromosome 17 within the MHC locus (Locksley et al., 2001). TNF can be expressed in two biologically active forms namely as a precursor membrane bound protein (26 kD) which can be cleaved to a soluble mature protein (17 kD) by TACE (Perez et al., 1990). LTα exists either as a soluble homotrimer or mediates its effects as a heterotrimer in association with the membrane constrained LTβ protein. Soluble TNF, membrane bound TNF and soluble LTα can bind to TNFR1 and TNFR2 while the LTα1LTβ2 heterotrimer bind to the LTβR (Orlinick and Chao, 1998). Production of the heterorimer LTα2LTβ1 have been demonstrated under in vitro conditions but its role under in vivo conditions are still to be clarified. TNFR1 and TNFR2 are
Membrane bound and soluble homotrimeric TNF are able to bind to both TNFR1 and TNFR2. Similarly, homotrimeric LTα can also signal through both TNFR1 and TNFR2. However, membrane bound LTαβ2 can associate only with LTβR. The cartoon also illustrates that membrane bound TNF can be cleaved by TACE and that TNFR1 and TNFR2 can be cleaved to soluble forms. (Adapted from Schluter and Deckert, 2000)
expressed on a broad spectrum of cell types and can exist in both a soluble form or can be attached to the membrane. It displays sequence homology with respect to its extra cellular cysteine-rich domains but lacks distinctive similarities in its intracellular cytoplasmic tail (Orlinick and Chao, 1998; Locksley et al., 2001). One of the major differences is the presence of the "death domain" in TNFR1, an ~80 amino acid sequence which is common to some members of the TNFR super family such as DR3, DR4, DR5, FAS and CAR-1 but is absent in others, including TNFR2. Therefore, although both receptors bind its ligands with similar affinities, subsequent intracellular signaling events are different. Both receptors can induce NFκB and JNK/AP-1 activation, and apoptosis through recruitment of intermediate signaling proteins such as TRAF2 (Van Antwerp et al., 1998). In contrast to TNFR2, which can bind TRAF2 directly, TNFR1 interacts with TRAF2 through the intermediary molecule, TRADD to induce NFκB and JNK/AP-1 activation (Wajant et al., 2001). TRADD can also associate with FADD instead of TRAF2 to induce apoptosis and it is thought that the balance between the formation of the complexes TNF-TNFR1-TRADD-TRAF2 and TNF-TNFR1-TRADD-FADD contributes to determining the fate of the signal (Wajant et al., 2001). The apoptotic signal involves the initiation of the caspase cascade and leads to proteolysis of enzymes that are responsible for maintenance of structural and metabolic integrity, which eventually ends in cell death. In contrast, the activation of NFκB and JNK/AP-1 is a survival signal that activate several pro-inflammatory and immunomodulatory genes and has recently been implicated in inhibiting apoptosis (Mayo et al., 1997; Van Antwerp et al., 1998).

A role for TNF in human tuberculosis has been identified (Barnes et al., 1990). After infection with M. tuberculosis, human macrophages secrete TNF (Engele et al., 2002). Addition of exogenous TNF to M. tuberculosis infected monocytes in culture results in attenuation of intracellular bacilli viability whereas neutralization of TNF, by the administration of anti-TNF antibodies, in H37Ra infected human alveolar macrophage cultures promotes mycobacterial growth (Appelberg et al., 1995). TNF and/or LTα have been associated with protective immunity against bacterial, viral, fungal and protozoal infections in studies in which TNFR1Δ mice were found to be susceptible to the respective pathogens (Schluter and Deckert, 2000). Perhaps the most compelling findings providing direct evidence of the importance of TNF in controlling M. tuberculosis in humans was published by Keane et al., (2001). In this
the presence of TGFβ (Maeda et al., 1995). In PBMC cultures the combined inhibitory effects on PPD induced IFNγ production was significantly higher than the inhibitory effects of either cytokine alone (Othieno et al., 1999).

LR. 10. The validity of using a *M. bovis* BCG infected mouse model for tuberculosis.

Several studies have used *M. bovis* BCG as the infecting agent to gain insight into the host immune response. Reasons for using *M. bovis* BCG in animal models could be statutory or financial e.g. prohibitive regulations regarding the use of virulent strains such as H37Rv or clinical isolates and would therefore necessitate the researcher to search for an avirulent alternative. The use of virulent organisms also requires the presence of a biohazard level–3 facility and the maintenance cost that is associated with such a facility could be restrictive. *M. bovis* BCG is however genetically different from *M. tuberculosis* (Domenech et al., 2001). An additional complication is that several different strains of *M. bovis* BCG are available and it has been shown that these strains can vary with respect to protein expression and lipid composition. Relative to *M. tuberculosis* it was found that the lost of specific genetically deletion regions were variable between *M. bovis* BCG strains (Domenech et al., 2001). The combined effect of these differences were probably reflected in the disparate growth rates that were observed between different *M. bovis* BCG strains after infection in mice (Lagrannderie et al., 1996).

To add legitimacy to the use of *M. bovis* BCG as a relevant substitute for *M. tuberculosis*, it is necessary to identify similarities in the host response that is invoked by both organisms. It is known that after uptake of both organisms, the host initiates a cell-mediated immune response that is characterised by recruitment of leukocytes, CD4⁺ and CD8⁺ T-cells which are intricately involved in granuloma formation. Associated with this process is the activation of lymphocytes and macrophages, which is characterised by the upregulation of specific markers such as CD44 or MHC Class II, respectively. Both organisms elicit the production of a range of similar cytokines that is involved in the control of the immune response, which, in mice, leads to production of reactive nitrogen intermediates and induction of apoptosis. Its relevance was more aptly illustrated in studies in which *M. bovis* BCG was employed as the infecting organism in gene−/− mice to identify the importance of cytokines such
as IFNγ, IL-12 and TNF or the role of CD4 and CD8 cells during the host response. Although there was distinct differences in the rate at which disease progressed, comparatively the phenotypes of the mice, whether infected with *M. bovis* BCG or *M. tuberculosis*, were the same.

The use of intravenous infection during mycobacterial studies has been widely used as the method of necessity for infecting mice. Although it can be used to seed the lungs with an initial source of bacilli it does not resemble the natural manner of respiratory infection. It has been argued that extra-pulmonary dissemination of the bacilli occurs after the establishment of cell-mediated immunity and that hemotogenous spread of bacilli by intravenous infection disregards this aspect of the disease. Nonetheless, several studies in which this route of infection was utilised contributed immensely to understanding the pathogenesis of tuberculosis. It is however with caution and in proper perspective that data from such studies are to be interpreted. Often it is non-scientific criteria, which determine parameters such as the route of infection. For proper simulation of respiratory infection an aerosol inhalation chamber, which is capable of generating a specific size of droplet that contains the bacillus, is required and should be capable of housing a sufficient number of animals that are required for experimental purposes. Such specialised equipment, which is housed within a biohazard level-3 facility, is not always readily accessible and together with restrictive legislation could leave the researcher with a limited choice.

A major consideration when using animal models for experimental tuberculosis is the safety of the researcher. All experimental work requires biohazard level three facilities and the area available for research could dictate the number of animals that can be housed and therefore the type of animal that will be used. It is e.g. possible to house more mice in the same area as rabbits. Commercial availability of a wide selection of genetically modified mouse strains enables the researcher to investigate several different aspects not only of the specific gene but also in areas that are related to it. Currently the number of reagents that are specific for mice far exceeds that of any other animal. The ease of commercial availability of the majority of these reagents allows for the investigation of numerous parameters within a mouse.
Materials and Methods
Materials and Methods

MM. 1. Mice.

Mice were bred and housed under specific pathogen free conditions in the animal facility of the University of Cape Town. Both male and female mice between 8-12 weeks were used in experiments. Mice were housed in filtertop cages for the duration of experiments and were given food and water *ad libitum*. Genotypes of mice were confirmed by PCR analysis of tail biopsies. The Animal Ethics Committee of the University of Cape Town approved all experiments.

Genetically modified mice were obtained from the following sources:

- TNF<sup>-/-</sup> mice were obtained from Dr Mike Marino (Marino et al., 1997).
- TNFR1<sup>-/-</sup> mice were obtained from Dr Horst Bluethmann (Rothe et al., 1993)
- TNFR2<sup>-/-</sup> mice were obtained from Dr Horst Bluethmann (Erickson et al., 1994)
- TNF/LT<sup>-/-</sup> mice were obtained from Prof. Rylliel (Eugster et al., 1996).
- LT<sup>-/-</sup> mice were obtained from Dr Theresa Banks (Banks et al., 1995)
- IL-10<sup>-/-</sup> mice were obtained from Prof. Frank Brombacher (Khun et al., 1993)

All strains were generated on a C57Bl/6 x 129Sv/Ev background. C57Bl/6 x 129Sv/Ev mice were used as controls in all experimental studies.

MM. 2. Mycobacteria.

*Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* strain H37Rv were obtained from the Trudeau Mycobacterial Culture Collection. Mycobacterial strains were grown in Difco Middlesbrook 7H9 medium containing 0.5% glycerol and enriched with 10% OADC. Cultures were incubated at 37°C and grown until log phase. Sample aliquots (0.5ml) were frozen and stored at -70°C in screw cap vials (Nalge Nunc International, Naperville, IL, USA). To determine the mycobacterial concentrations (defined as cfu/ml) of frozen stocks, an aliquot was thawed and passed 30x through a 29.5G needle (B.Braun, Melsungen, Germany) to disperse clumps and 100μl of sample was plated in duplicate on Difco Middlesbrook 7H10 agar plates in 10 fold serial dilutions. Plates were semi-sealed in plastic bags, incubated for 17-21 days at 37°C after which the number of mycobacterial colonies were counted and the batch
concentration was calculated. The batch concentration was verified independently by at least two individuals.

**MM. 3. Infections.**

**MM. 3.1 Intravenous Infection.**

A frozen aliquot of *Mycobacterium bovis* BCG was rapidly thawed at 37°C, passed 30× through a 29.5G needle (B.Braun, Melsungen, Germany) to remove clumps and diluted in sterile saline to a concentration of 10⁷ cfu/ml. Prior to injection, mice were placed under an infrared lamp and subjected to gentle heating for ~3-5 minutes to dilate their tail veins. Mice were immediately placed in a restrainer and a standard dose (1×10⁶ cfu), or a dose as indicated, was injected into the lateral tail vein using a 29.5G needle (B.Braun, Melsungen, Germany). Applying pressure at the site of injection stopped bleeding. All intravenous infection procedures were done under sterile conditions in a laminar flow cabinet. To confirm the required concentration, 100µl of the inoculum was plated on Difco Middlebrook 7H10 agar plates in 10 fold serial dilutions. Plates were semi-sealed in plastic bags, incubated for 17-21 days at 37°C after which the number of mycobacterial colonies were counted and the concentration was calculated.

**MM. 3.2 Aerosol Inhalation Infection.**

The aerosol inhalation infection procedures were performed in an Animal Biosafety Level-3 Laboratory in the Heart Research Centre at the University of Cape Town. Mice were infected by aerosol inhalation using a Glas-Col Inhalation Exposure System Model A4224. Prior to the infection procedure the exposure chamber was sterilised by washing 1x with 3% Virkon (Antec Africa, South Africa) and 2x with 70% ethanol. The drum cage unit and deflecting plate was autoclaved before use. A frozen aliquot of *Mycobacterium tuberculosis* H37Rv was rapidly thawed at 37°C, passed 30× through a 29.5G needle (B.Braun, Melsungen, Germany) to remove clumps and diluted in sterile saline to the required inoculum concentration. 5 ml of the inoculum was transferred to the Nebuliser-Venturi unit using a 15G needle. The operating program and instrument settings were as follows:

- **Preheat time:** 900 seconds
- **Nebulising time:** 2400 seconds
• Cloud decay time: 2400 seconds
• UV decontamination time: 900 seconds
• Main air flow: 60 cubic feet per hour
• Compressed air flow: 10 cubic feet per hour

To confirm the required concentration, 100μl of the inoculum was plated on Difco Middlebrook 7H10 agar plates in 10 fold serial dilutions. Plates were semi-sealed in plastic bags, incubated for 17-21 days at 37°C after which the number of mycobacterial colonies were counted and the concentration was calculated. Typically a concentration of 2x10⁶ cfu/ml would result in a pulmonary infection dose of ~100 cfu and a concentration of 2x10⁵ cfu/ml would result in a pulmonary infection dose of ~10 cfu as for the above program and instrument settings. The pulmonary infection dose was confirmed by sacrificing 10 mice 1 day after infection and plating the homogenised lung tissue on Difco Middlesbrook 7H10 agar plates in 10 fold serial dilutions. Plates were semi-sealed in plastic bags, incubated for 17-21 days at 37°C after which the number of mycobacterial colonies were counted and the infection dose was calculated.

**MM. 4  Delayed Type Hypersensitivity.**

DTH was determined at 4 weeks post infection. Mice were anaesthetised by intraperitoneal injection with 300μl of general anaesthetic after which 50μl of PPD (0.1mg/ml) (Central Veterinary Laboratory, Surrey, England) was injected subcutaneously into the right hind footpad using a 29.5G needle (B.Braun, Melsungen, Germany). As a control, 50μl of saline was injected into the left hind footpad. Footpad swelling was measured 42 hours later using a dial-gauge callipers (Mitutoyo, Japan).

**MM. 5  Reconstitution of lethally irradiated mice with bone marrow from donor mice.**

Mice were reconstituted with bone marrow according to modified procedure as described (Muller et al., 1996). The radiation procedure was performed in the Department of Radiology at the University of Cape Town in adherence to the safety regulations of the institute. Briefly, designated recipient mice received an optimised
lethal total body irradiation dose of 1000 Rad using a $^{60}$Cobalt irradiation source. Mice were rested for 24 hours to recover from irradiation trauma. Bone marrow was removed from the femurs and tibias of donor mice by aspiration with PBS using a 29.5G needle (B.Braun, Melsungen, Germany) and kept on ice. Cells from different donor mice were pooled and viewed under phase contrast using a Nikon, model TMS microscope. Cells were counted using a haemocytometer (Neubauer Superior - Marienfeld, Germany) and viability was determined by trypan blue exclusion. Irradiated mice were reconstituted with $2 \times 10^6$ fresh unseparated bone marrow cells by intravenous injection in the lateral tail vein using a 29.5G needle (B.Braun, Melsungen, Germany). Mice were left for three months to fully reconstitute and recover prior to infection.

**MM.6  LPS challenge.**

25μg LPS from *Esterichia Coli* 0127:B8 (Sigma Chemical Co, St Louis, USA) were administered intravenously via the lateral tail vein to *M. bovis* BCG infected mice using a 29.5G needle (B.Braun, Melsungen, Germany). Mortality of mice was monitored for 72 hours.

**MM.7  Bronchial Alveolar Lavage.**

Mice were euthanased by intra peritoneal injection with a lethal dose of anaesthetic. A 20-gauge Intracan catheter (B.Braun, Germany) was inserted into the exposed trachea. The lungs were lavaged 10x with two volumes of 300μl PBS. 500μl of fluid was routinely recovered. Lavage fluid were centrifuged at 1000rpm for 5 minutes, the supernatant was removed, aliquoted and stored at $-80^\circ$C for cytokine and chemokine analysis. Lungs were subsequently lavaged 8x with 4 volumes of 800μl PBS. To remove traces of red blood cells, pooled samples were incubated in 1ml red cell lysis buffer for 5 minutes, washed 2x with 5ml PBS. Samples were centrifuged at 1000rpm for 5 minutes and the cells were used for flow cytometric analysis. Procedures were performed under sterile conditions and samples kept on ice for the duration of the experiment.
MM.8 Flow Cytometry.
1x10^6 cells were incubated with 25μl FACS blocking solution for 30 minutes after which they were washed with 1 ml FACS buffer and centrifuged at 1000rpm for 5 minutes. Cells were incubated with 2μg/ml anti-CD3 PE (clone 145-2C11) and either 2μg/ml anti-CD4 FITC (clone H129-19) or 2μg/ml anti-CD8 FITC (clone 53-6-7) in a total volume of 50μl for 30 minutes. Cells were washed with 1ml FACS buffer, centrifuged at 1000rpm for 5 minutes and resuspended in FACS fixation buffer. Samples were kept on ice for the duration of the labelling procedure. Fluorescently labelled antibodies were purchased from BD Pharmingen. Samples were analysed on a FACS Calibur (Beckton Dickinson) flow cytometer using Cell Quest software (Beckton Dickinson).

MM.9 Enzyme linked immunosorbant assay.
96-well Maxisorb microtiter plates (Nalge Nunc International, Naperville, IL, USA) were incubated overnight with “capturing antibody” (10μg/ml) at 4°C. Plates were washed 4x with Washing Buffer and incubated overnight with 200μl Blocking Buffer at 4°C. The Blocking Buffer was removed and recombinant mouse chemokine or cytokine standards were added in three fold dilutions at concentrations ranging from 100ng/ml - 0.6pg/ml. Samples were added in three fold dilutions (undiluted – 1/9) and the plates incubated overnight at 4°C. Plates were washed 4x and incubated overnight with a biotinilated “detecting antibody” (2μg/ml) at 4°C. Plates were washed 4x and incubated with streptavidin-alkaline phosphatase (BD Pharmingen) (1:1000 dilution) for 2 hours at room temperature. The plates were subsequently washed, incubated with p-nitrophenyl phosphate (1mg/ml) (Boehringer Mannheim, Germany) and the enzymatic reaction were read at 405nm using a microplate spectrophotometer (Molecular Devices, spectra MAXGemini).

MM.10 Paraffin wax embedding.
All tissue sections were immersed in formalin at least 10x the volume of the tissue. For paraffin wax embedding tissues were dehydrated in an automated tissue processor (Shandon Elliot) according to the following program:
70% alcohol 30 minutes
96% alcohol (2x) 45 minutes
100% alcohol (4x)  45 minutes
xylol (2x)  60 minutes
Wax [55°C - 60°C] (2x)  45 minutes with vacuum

Tissues were sectioned at 2μm using a microtome (Leica, model RM-2125) and fixed onto a glass slide by overnight incubation at 37°C. Prior to staining tissue sections were incubated at 60°C for 2 hours – 18 hours to remove wax.

**MM.11  Haemotoxylin and Eosin staining**

Tissues were rehydrated according to the following procedure:

xylol  3 minutes
xylol  1 minute (2x)
Absolute Alcohol  1 minute (2x)
96% Alcohol  1 minute (2x)
70% Alcohol  1 minute
Water  1 minute

Sections were stained with Haemotoxylin for 8 minutes and rinsed in water. Sections were immersed in 1% acid alcohol for 10 seconds and rinsed in running water for 30 minutes. Sections were counterstained with 1% eosin for 2 minutes and rinsed in water. Sections were dehydrated by immersion for 10 seconds in 70% alcohol, 96% alcohol and xylol. Sections were mounted using Canada Balsam.

**MM.12  Ziehl-Neelson Staining**

Tissue sections were immersed in a filtered Cabol Fuchsine solution, flamed and cooled for 5 minutes (2x). After rinsing in water, sections were immersed in 1% acid alcohol for 30 seconds to remove excess stain and rinsed again in water. Tissue sections were submerged in 25% H₂SO₄ for 20 minutes and washed in running water for 10 minutes. Sections were stained with Loefflers’ Methylene Blue for 1 minute and rapidly rinsed in water. Tissue sections were dehydrated by immersion for 10 seconds in 70% alcohol, 96% alcohol and xylol. Sections were mounted using Entellan (Merck).
MM.13  CAB staining

Tissues were rehydrated by passing it through xylol for 3 minutes, xylol for 1 minute (2x), absolute alcohol for 1 minute (2x), 96% alcohol for 1 minute (2x), 70% alcohol for 1 minute (1x) and water for 1 minute (1x). Tissue sections were immersed in Weigert's haematoxylin solution for 10 minutes and rinsed with distilled water. Section were immersed in phosphomolybdic acid for 3 minutes and rinsed with distilled water. Sections were stained with CAB solution for 8 minutes, rinsed in distilled water and dried. Tissue sections were dehydrated by immersion for 10 seconds in 70% alcohol, 96% alcohol and xylol.

MM.14  Acid Phosphatase (Gomori-lead method)

Tissue sections were immersed in acid phosphatase incubating medium for 2 hours at 37°C and rinsed in distilled water. Sections were then immersed in freshly prepared 1% ammonium sulphide for 2 minutes, rinse in distilled water and counterstained with 2% Methyl Green. After rinsing in distilled water, sections were mounted in glycerine jelly and sealed with coverslips.

MM.15  Immunohistochemistry on frozen sections

Tissues were removed and immediately frozen on solid CO₂ blocks in Tissue-Tek cryomoulds (Miles Inc, Elkhart, IN, USA) containing Tissue-Tek OCT compound (Sakura Finetechanical Co, Torrence, CA, USA) and stored at -80°C until further processing. Frozen tissues were sectioned at 5µm, mounted on APES coated slides and air-dried for 2 hours. Tissue sections were fixed in acetone for 10 minutes and air dried for 15 minutes. Endogenous peroxidase activity was inhibited by incubation in 1% H₂O₂/methanol for 30 minutes and washed with 1x PBS. For liver sections, endogenous biotin was blocked by incubation in 0.1% avidin/PBS for 30 minutes, followed by incubation in 0.01% biotin/PBS for 20 minutes. Non-specific binding sites were saturated by incubation with 1.5% serum/PBS (serum was used from animal in which the secondary antibody was raised) for 30 minutes. 100µl of primary antibody (at an optimised concentration) was added to sections, incubated overnight at 4°C and washed with PBS. Sections were incubated in secondary antibody (1.5% normal mouse serum, 0.5% biotinilated IgG) for 30 minutes and washed with PBS. Sections were incubated in ABC Vector kit (Vector Laboratories, Berlingame, CA, USA) for 30 minutes and washed with PBS. Freshly prepared DAB substrate was
added to sections, incubated for 10 minutes and washed in running water. Staining was enhanced by immersing sections in 1% CuSO₄ for 2 minutes which were washed in running tap water for 2 minutes. Sections were counterstained in Mayer’s haematoxylin for 4 minutes and washed in running water for 5 minutes. Sections were dehydrated by immersion 2x in 70% alcohol, 1x 96% alcohol, 2x 100% alcohol and 2x xylol. Sections were mounted in Entellan (Merck). All incubations were done at room temperature in a humidified chamber unless otherwise stated.

**MM.16 Immunohistochemistry on paraffin embedded sections.**

Paraffin sectioned slides were incubated at 65°C overnight. Tissue sections were rehydrated by passing it through xylol (2x), 100% ethanol (2x), 96% ethanol (1x), 70% ethanol (2x) and water. To demask epitopes, sections were immersed in citrate buffer (pH 6), microwave heated for 5 minutes (2x) on a medium heat setting and allowed to cool for 20 minutes. Sections were fixed for 10 minutes in acetone and air dried for 15 minutes. Endogenous peroxidase activity was blocked by incubating tissue sections in 1% H₂O₂/methanol for 30 minutes after which it was washed once in PBS (pH 7.4). None specific binding sites were blocked by incubating tissue sections in 1.5% serum/PBS (use serum in which secondary antibody as raised) for 30 minutes. After removing the blocking solution, tissue sections were incubated in 50ul of the primary detecting antibody (at an optimised concentration) and incubated at 4°C overnight. Tissue sections were washed 1x in PBS (pH 7.4) and incubated with secondary antibody (1.5% normal mouse serum, 0.5% biotinilated IgG) for 30 minutes at room temperature. After washing 1x with PBS (pH 7.4) tissue sections were incubated with ABC Vector kit (Vector Laboratories, Berlingame, CA, USA) for 30 minutes. Tissue sections were washed 1x in PBS (pH 7.4), incubated in freshly prepared DAB substrate for 10 minutes and washed again in running water. Tissue sections were incubated in 1% CuSO₄ for 2 minutes, washed in running water for 2 minutes and counter stained in Mayers haematoxylin for 4 minutes. After washing in running water for 5 minutes, tissue sections were rehydrated by passing it through 70% ethanol (2x), 96% ethanol (1x), 100% ethanol (2x) and xylol (2x), and mounted in Entellan (Merck).
MM.17 Imaging and Software
Images of stained tissue sections were captured using a Nikon DXM 1200 digital still camera attached to a Nikon Eclipse E400 microscope and the ACT-1 software application program. Images of whole organs were captured using a Panasonic WV-CP410/G digital camera and the Matrox Inteliicam 2.0 software application program.

MM.18 Statistical Analysis
The data is expressed as the mean ± SD. Statistical analysis was performed using the Student's t test. For all tests, a p value of < 0.05 was considered significant.

Reagents

All chemical reagents were of analytical grade and were purchased from the following companies unless otherwise stated:
Merck Laboratory Supplies- South Africa
Sigma-Aldrich South Africa
BDH Chemicals Ltd, Poole, England

General Reagents
Agar
Dissolve 19g Difco Middlebrook 7H10 agar in 900ml distilled H₂O. Add 5 ml glycerol. Autoclave at 121°C for 10 minutes. Cool to 55°C and add 100ml OADC (State Vaccine Institute, South Africa). Pour 7ml in each of a two-compartment 90mm petri dish (Bibby Sterilin, Staffordshire, England). Allow to set and store at 4°C for up to 2 weeks.

Phosphate Buffered Saline
Dissolve 8g NaCl, 0.2g KCl, 1.44g Na₂HPO₄ and 0.24g KH₂PO₄ in 900ml distilled H₂O. Adjust to pH 7.4 with HCl. Adjust volume to 1000ml. Sterilise by autoclaving for 30 minutes at 121°C.
Saline
Dissolve 9g NaCl in 900ml distilled H₂O. Adjust volume to 1000ml. Sterilise at 121°C for 30 minutes. Store at room temperature.

LPS
Dissolve 1mg of LPS from *Esterichia Coli* 0127:B8 (Sigma Chemical Co, St Louis, USA) in 2ml of PBS. Sterilise through a 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4°C.

Homogenising buffer for organs.
Dissolve 9g NaCl in 900ml distilled H₂O. Add 0.4ml Tween 80. Sterilise at 121°C for 30 minutes. Store at room temperature.

Lethal Anaesthetic
Add 0.5 ml Rompun (Bayer Pty Ltd, Germany) and 2ml Anaket-V (Centaur Labs, Premier Pharmaceutical Company, Bryanston, South Africa) to 9ml saline. Pass through 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4°C.

General Anaesthetic
Add 0.25 ml Rompun (Bayer Pty Ltd, Germany) and 2ml Anaket-V (Centaur Labs, Premier Pharmaceutical Company, Bryanston, South Africa) to 11ml saline. Pass through 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4°C.

Buffered formalin
Add 100 ml 40% formaldehyde to 900 ml PBS. Store in the dark at room temperature.

FACS Reagents
FACS buffer
Dissolve 1g Bovine Serum Albumin (Boehringer Mannheim, Germany) and 0.1g NaN₃ in 900ml PBS (pH 7.4). Adjust volume to 1000 ml. Sterilise by filtering through a 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4°C.
Fixation Buffer
Dissolve 4g NaOH in 100ml PBS. Add 20g paraformaldehyde and dissolve. Adjust pH to 7.2. Adjust volume to 1000 ml and sterilise through 0.45µm filter (Millipore Corporation, Bedford, USA). Store in the dark at 4°C.

FACS Blocking Solution
Make up 2.5% normal rat serum, 2.5% normal mouse serum and 6.25 µg/ml anti-CD32/CD16c (anti-FCγRIII/II) (Clone: 2.4G2, Pharmingen) in FACS buffer.

ELISA reagents

Table A: Antibodies used in ELISAs for cytokine detection.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Capturing Antibody</th>
<th>Detecting Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clone Number</td>
<td>Clone Number</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(biotinilated)</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>BVD4 - 1D11</td>
<td>BVD6 - 24G2</td>
<td>Pharmingen</td>
</tr>
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<td>TRFK4</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>IL-12</td>
<td>C15.6</td>
<td>C17.8</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>IFNγ</td>
<td>R4 - 6A2</td>
<td>XMG1.2</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>TNF</td>
<td>MP6 - XT22</td>
<td>MP6 – XT3</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2H5</td>
<td>4E2MCP</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>39624</td>
<td>polyclonal</td>
<td>R&amp;D Systems</td>
</tr>
</tbody>
</table>

Coating Buffer
Dissolve 0.2g NaN₃ in 800ml PBS (pH7.2). Adjust the volume to 1000ml. Sterilise by passing the solution through a 0.45µm filter (Millipore Corporation, Bedford, USA) and store at 4°C.
Dilution Buffer
Dissolve 10g Bovine Serum Albumin (Boehringer Mannheim, Germany) and 0.2g NaN₃ in 800ml PBS (pH7.4). Adjust volume to 1000ml. Filter through a 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4°C.

Blocking Buffer
Dissolve 40g Bovine Serum Albumin (Boehringer Mannheim, Germany) and 0.2g NaN₃ in 800ml PBS (pH7.4) and store at 4°C.

20x Washing Buffer
Dissolve 20g KCl, 20g KH₂PO₄, 144g NaH₂PO₄ and 800g NaCl in 4.5 liters distilled H₂O. Add 50ml Tween 20 and 100ml 10% NaN₃ solution. Adjust volume to 5 liters. Sterilise through a 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4°C.

Substrate Buffer
Dissolve 0.2g NaN₃, and 0.8g MgCl₂ in 700ml distilled H₂O. Add 97 ml liquefied diethanolamine. Adjust to pH 9.8. Adjust volume to 1000ml distilled H₂O. Sterilise through a 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4°C.

Reagents and Material for Tissue Staining

Table B: Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Name</th>
<th>Clone Number</th>
<th>Source</th>
</tr>
</thead>
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<td>anti-CD3</td>
<td>KT3</td>
<td>BMA Biomedicals AG</td>
</tr>
<tr>
<td>anti-CD4</td>
<td>GK 1.5</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>anti-CD8a</td>
<td>53-6.7</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>anti-F4/80</td>
<td>CI:A3-1</td>
<td>BMA Biomedicals AG</td>
</tr>
<tr>
<td>anti-ICAM-1</td>
<td>K181</td>
<td>BMA Biomedicals AG</td>
</tr>
<tr>
<td>anti-CD11b</td>
<td>M1/70</td>
<td>BMA Biomedicals AG</td>
</tr>
<tr>
<td>anti-CD11c</td>
<td>HL3</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>anti-iNOS</td>
<td>polyclonal</td>
<td>Gift from Dr Pfeilshiffer</td>
</tr>
</tbody>
</table>
Mayer's haematoxylin
Dissolve 1g haematoxylin in 800ml distilled H₂O. Add 50g aluminium ammonium sulphate and dissolve. Add 0.2 g sodium iodate, 1g citric acid and 50g chloral hydrate ensuring to dissolve each compound before the addition of the other. Make up to 1000ml, filter through Whatmann filter paper no 1 and store in the dark at room temperature.

Wegert's haematoxylin
Solution A.
Dissolve 1 g of haematoxylin in 100ml absolute alcohol.

Solution B
Add 4ml 30% Aqueous Ferric Chloride (anhydrate) and 1ml concentrated HCl to 95ml of distilled H₂O.
Mix equal volumes before use.

CAB solution
Dissolve 6g Chromotrope 2R in 150ml distilled H₂O. Dissolve 1.5g Aniline Blue in 2.5 ml concentrated HCL. Add to chromotrope solution. Adjust volume to 200ml.

Eosin
Add 150ml 1% Eosin solution to 75ml 1% Phloxine solution. Adjust volume to 450ml with distilled H₂O. Filter through Whatmann filter paper no. 1 and store at room temperature.

Carbol Fuchsin
Add 10ml 6% Basic fuchsins (in absolute alcohol) to 90ml 5% carbolic acid solution. Filter solution through Whatmann paper no 1 and store at room temperature.

Loeffers’ Methylene Blue
Add 1ml 1% KOH to 99 ml distilled H₂O. Add 30ml 0.8% Methylene Blue (in absolute alcohol). Filter through Whatmann paper no 1 and store at room temperature.
**Veronal acetate buffer**

_Solution A:_
Dissolve 1.94g sodium acetate and 2.94g sodium barbitol in 80ml distilled H₂O. Adjust volume to 100ml.

_Solution B_
0.1N HCl
Add 5ml solution A, 9ml solution B and 9ml distilled H₂O. Mix thoroughly.

**Citrate buffer (pH 6)**
Solution A – Dissolve 1.05g citric acid in 500ml distilled H₂O.
Solution B – Dissolve 2.94 sodium citrate in 100ml distilled H₂O.
Add 9ml Solution A to 41ml Solution B and adjust volume to 500ml with distilled H₂O.

**Acid phosphatase incubating medium**
Dissolve 0.02g lead nitrate in 10ml veronal acetate buffer with stirring. Add 0.032g sodium β-glycerophosphate with heat. Check pH to be ~5.0. Filter through Whatmann paper no 1 and store at room temperature.

**APES coated slides**
Wash slides in 1% Teepol for 10 minutes at room temperature. Wash in running tap water for 1hour and dry slides overnight at 37°C. Immerse slides in 3.3% APES for 10-20 seconds, 10 seconds in acetone and rinse with distilled H₂O. Dry slides overnight at 37°C and store at room temperature.
Experimental Studies
*Mycobacterium bovis* BCG infection

in the absence of TNF signaling.
Summary

This study illustrates the importance of tumour necrosis factor (TNF) during the induction of protective immunity against *Mycobacterial bovis* BCG infection. Mice, which are deficient for TNF, are highly susceptible and succumb to infection. TNFR2 is less important than TNFR1 for the control of *Mycobacterial bovis* BCG infection as demonstrated by the higher susceptibility of TNFR1+/− mice in comparison to TNFR2+/− mice. TNF−/− mice display impeded locomotor activity and emaciation with progressive infection. Disease is characterized by unrestricted pulmonary mycobacterial growth but partial clearance in both the liver and spleen was observed. Underlying this difference was the delayed formation of granulomatous lesions, which was observed in the liver whereas pulmonary granulomas were absent. In contrast to well-defined hepatic granulomas, which developed in response to *Mycobacterial bovis* BCG infection in WT mice, granulomatous structures in TNF−/− mice were uncohesive. Reduced granuloma-associated expression of the lymphocytic marker CD3, the macrophage marker F4/80 and adhesion molecules ICAM, CD11b and CD11c in confirmed the qualitative differences in the immune response between WT mice and TNF−/− mice. TNF−/− mice also exhibited impaired recruitment of the subsets CD3+CD4+ T-cells and CD3+CD8+ T-cells. In the absence of TNF, persistent cellular infiltration, severe lesion formation and necrosis characterized *Mycobacterial bovis* BCG induced pulmonary pathology. Susceptibility to infection was observed despite the ability of TNF−/− mice to induce a Th1 response with elevated levels of IFNγ and IL-12 being detected during the chronic stage of infection. In addition, enhanced production of the proinflammatory chemokines, MIP-1α and MCP-1, was observed. *Mycobacterial bovis* BCG infected TNF−/− mice could elicit a specific memory recall response. On challenge with PPD, increased footpad swelling was observed in comparison to challenge with saline. Resistance to *Mycobacterial bovis* BCG in TNF−/− mice could be restored completely by transplantation of haemopoetic precursor cells.
Results

1.1 TNF signalling is required for protective immunity against *M. bovis* BCG infection.

To determine whether TNF is involved in protective immunity, WT and TNF<sup>−/−</sup> mice were challenged with *M. bovis* BCG. Groups of 10 mice per strain were intravenously infected with 10<sup>6</sup> cfu and their rate of mortality was recorded. TNF<sup>−/−</sup> mice were highly susceptible and succumbed to *M. bovis* BCG infection in contrast to their WT littermates that survived (Fig. 1.1A). It has been established that TNF can induce its biological effects through both TNFR1 and TNFR2. To determine the relative importance of these receptors in mediating protective immunity, TNFR1<sup>−/−</sup> and TNFR2<sup>−/−</sup> mice were intravenously infected with 10<sup>6</sup> cfu of *M. bovis* BCG. TNFR1<sup>−/−</sup> mice were highly susceptible to the infection and succumbed by day 43 post infection whereas ~70% of TNFR2<sup>−/−</sup> mice survived the experiment (Fig. 1.1B). Mortality was not observed in the WT control group. A significant observation was that TNFR1<sup>−/−</sup> mice had a notably higher rate of mortality in comparison to TNF<sup>−/−</sup> mice on challenge with *M. bovis* BCG.

1.2 Progressive loss of bodyweight in *M. bovis* BCG infected TNF<sup>−/−</sup> mice.

Rapid weight loss is a well-documented symptom of patients with chronic tuberculosis infection. The effect of *M. bovis* BCG infection on bodyweight was therefore investigated in TNF<sup>−/−</sup> mice (Fig. 1.2). A gain in average bodyweight of ~10% of the initial bodyweight was observed for both WT and TNF<sup>−/−</sup> mice at 28 days after infection. The average bodyweight of WT mice stabilised and at 56 days post infection was comparable to that measured at 28 days post infection. In contrast rapid weight loss was observed in TNF<sup>−/−</sup> mice with an average weight loss of ~40% at 56 days compared to 28 days post infection.

1.3 Inflammation in *M. bovis* BCG infected TNF<sup>−/−</sup> mice is delayed.

The organ weights and organ-bodyweight ratios of *M. bovis* BCG infected WT and TNF<sup>−/−</sup> mice were determined as an indication of inflammation in response to *M. bovis*
Figure 1.1
Susceptibility of TNF−/− mice to M. bovis BCG.

WT mice and TNF−/− mice (panel A) or TNFR1−/− and TNFR2−/− (panel B) mice were intravenously infected with 10^6 cfu of M. bovis BCG and monitored for mortality. The data indicate the percentage of surviving mice during the course of infection. The results are representative of 5 similar experiments. (n = 10 mice/strain)

Figure 1.2
Body weight Ratio.

WT and TNF−/− mice were intravenously infected with 10^6 cfu of M. Bovis BCG and their body weights were measured at 1, 14, 28 and 56 days after infection. Data points represent the average weight ratios relative to day 1. Each time point represents the average body weight ratios of 4 mice. The results are representative of 4 similar experiments.
BCG infection. The data in Figure 1.3 is represented either as the absolute organ weight (panels A-C) or is expressed as the organ-body weight mass ratio (panels D-F). The organ weights of M. bovis BCG infected WT and TNF\(^{−/−}\) mice were similar and not significantly different after 1 day of infection. However significant weight gain of the spleen and liver (\(p < 0.005\)) was measured in WT mice but not in TNF\(^{−/−}\) mice after 14 days. Fourteen days post infection represented the maximum inflammatory time point for both the liver and spleen in WT mice, as a significant increase of organ weights was not further observed. However, WT mice displayed significant weight loss of the spleen and liver after 56 days of infection (\(p < 0.01\)). In contrast a delayed, but dramatic weight increase was observed for both organs in TNF\(^{−/−}\) mice after 28 days of infection. The increase in weight in the spleen and liver was maintained at 56 days post infection and not significantly different from organ weights at 28 days post infection. Pulmonary inflammation in WT mice was reflected by a significant increase in lung weights after 28 days of infection (\(p < 0.05\)) and displayed significant reduction after 56 days of infection (\(p < 0.05\)). TNF\(^{−/−}\) mice manifested a delayed inflammatory response as evident by a significant gain in lung weight only after 56 days of infection (\(p < 0.005\)).

There were no significant differences in the ratios in all the organs between the two strains at day 1 post infection. The liver-bodyweight ratio and the spleen-bodyweight ratio of WT mice were significantly higher (\(p < 0.005\)) compared to TNF\(^{−/−}\) mice at 14 days post infection whereas a significant difference was not observed in the lung-body weight ratios. However, the reverse state was observed at 28 days post infection. The liver-bodyweight ratio and the spleen-bodyweight ratio of TNF\(^{−/−}\) mice were significantly higher (\(p < 0.001\)) than that of WT mice whereas the lung-body weight ratios of both strains remained unchanged. WT mice had a further reduction in the averages of the liver-bodyweight ratio, the spleen-bodyweight ratio and the lung-bodyweight ratio at 56 days post infection. TNF however displayed an increase in the liver-bodyweight ratio and the spleen-bodyweight ratio, which were significantly higher (\(p < 0.01\)) than the values that were observed in WT mice. In addition, the lung-bodyweight ratio of TNF\(^{−/−}\) mice increased notably and was significantly higher (\(p < 0.005\)) than that of WT mice.
Figure 1.3

Organ weights and organ-bodyweight ratios of *M. bovis* BCG infected WT and TNF−/− mice.

WT and TNF−/− mice were intravenously infected with 10^6 cfu of *M. bovis* BCG and their organ weights and body weights were measured at 1, 14, 28 and 56 days after infection. Panels A-C represent the absolute organ weights of the lungs (panel A), spleen (panel B) and liver (panel C). Panels D-F represent the organ-bodyweight ratios of the lungs (panel D), spleen (panel E) and liver (panel F). Each data point represents the average ± SD of 4 mice. The results represent one of three similar experiments.
1.4 Macroscopic pulmonary pathology is characterised by severe lesion formation in \textit{M. bovis} BCG infected TNF$^{-/-}$ mice.

The effects of \textit{M. bovis} BCG on the pathology of the lungs in TNF$^{-/-}$ mice were investigated due to the strain’s susceptibility to infection. Whole lungs of WT and TNF$^{-/-}$ mice were examined and compared for lesion formation at the indicated time points after infection (Fig. 1.4). WT and TNF$^{-/-}$ mice had normal pathology at 14 days post infection (Fig. 1.4A and 1.4D). Both strains developed small, visible lesions which were distributed uniformly throughout the lungs at 28 days post infection with no noticeable difference in pathology (Fig. 1.4B and 1.4E). WT mice had reduced visible lesions at 56 days after infection (Fig. 1.4C) with pathology resembling, that which was observed at day 14 post infection. In contrast to WT mice, enlarged lungs with severe lesions of increased size at 56 days post infection (Fig.1.4F) distinguished pulmonary pathology of TNF$^{-/-}$ mice.

1.5 Unrestricted pulmonary mycobacterial growth in TNF$^{-/-}$ mice.

The importance of TNF for the control of mycobacterial growth in the liver, lungs and spleen were investigated due to the susceptibility of TNF$^{-/-}$ mice to \textit{M. bovis} BCG infection. Mice were infected intravenously with $10^6$ cfu \textit{M. bovis} BCG and were sacrificed at 1 day, 14 days, 28 days and 56 days post infection. The mycobacterial burdens in the liver, lung and spleen were determined (Fig. 1.5). The mycobacterial distribution in the different organs, as determined on day 1 post infection, was on average as follows; $\sim$90% of the mycobacteria were found in the liver, $\sim$9% of the mycobacteria were found in the spleen and $\sim$1% of the mycobacteria were found in the lungs. Both WT and TNF$^{-/-}$ mice had analogous but significant increases (p < 0.01) in mycobacterial burdens in all organs that were examined at 14 days post infection in comparison with 1day post infection. Subsequent elimination of bacilli occurred in both the liver and spleen of WT mice as indicated by the significantly lower burden at 28 days post infection (p < 0.01) and reached values equivalent to 1 day post infection. Even further clearance of bacilli was noticed at 56 days post infection with burdens significantly lower (p < 0.05) than at 28 days post infection. Control but not elimination of mycobacterial burdens was evident in the lungs of WT mice at 28 days post infection as similar bacilli levels were measured at both day 14 and day 28. However, elimination of mycobacteria was noted at 56 days post infection with significantly lower (p < 0.001) bacilli levels present.
Figure 1.4

Excessive lesion formation in *M. bovis* BCG infected TNF−/− mice.

WT and TNF−/− mice were infected with 10^6 cfu of *M. bovis* BCG. Mice were sacrificed at 14, 28 and 56 days after infection and the progressive macroscopic pulmonary pathology of the lungs were examined and recorded. The lungs are representative of 4 mice/strain at each time point and the results denotes 1 of 4 experiments with similar findings. (Magnification = 2.5x)
Figure 1.5

Mycobacterial burden in the liver, lungs and spleens of WT and TNF<sup>-/-</sup> mice after *M. bovis* BCG infection.

Mice were intravenously infected with 10<sup>6</sup> cfu of *M. bovis* BCG and the bacilli burdens in the liver, spleen and lungs were assessed at the indicated time points as described in Materials and Methods. The liver (panel A) and spleen (panel B) display partial control of mycobacterial growth whereas uncontrolled bacilli growth occurred in the lungs (panel C). The panels represent 4 mice per strain at each time point. The data is representative of 4 similar experiments.
In contrast TNF$^{-}$ mice had a significantly higher (p < 0.01) mycobacterial burden in the liver (Fig. 1.5A) and spleen (Fig. 1.5B) at 28 days post infection when compared to WT mice. Although the mycobacterial burdens in the liver and spleen of TNF$^{-}$ mice were significantly reduced (p < 0.05) at 56 days post infection in comparison to 28 days post infection, the levels were still significantly higher (p < 0.05) than those of WT mice. Equivalent mycobacterial burdens were found in the lungs (Fig 1.5C) of both strains at 28 days post infection. The pulmonary mycobacterial load of TNF$^{-}$ mice showed a significant increase (p < 0.001) at 56 days post infection in comparison to the mycobacterial burden at day 28 and was two orders of magnitude higher than the pulmonary mycobacterial load of WT mice at 56 days post infection.

1.6 Delayed hepatic granuloma formation in TNF$^{-}$ mice.

It has previously been established that a protective immune response against M. bovis BCG infection is dependent on the host's ability to form granulomas that are well defined (Kindler et al., 1989). The effect of TNF on the kinetic response of granuloma formation in the liver and lungs was investigated due to the inability of TNF$^{-}$ mice to effectively clear the mycobacterial infection. Hepatic granulomatous lesions were used for quantitative assessment because it was better demarcated than splenic or pulmonary granulomatous lesions.

The livers of WT mice displayed typical granulomas at 14 days post infection. These structures were characterised by a central core of macrophages surrounded by lymphoid cells (Fig 1.6A). Although the number of granulomas was similar at 28 days post infection the average granuloma size was increased (Fig. 1.6B). At 56 days post infection the number and size of the granulomas were reduced when compared to that which were observed at 28 days post infection (Fig. 1.6C).

In contrast, TNF$^{-}$ mice did not develop typical granulomas at 14 days post infection although sparsely distributed, small clusters of mononuclear cells were observed (Fig. 1.6D). These clusters developed into predominantly immature, uncohesive granulomatous structures at 28 days post infection (Fig. 1.6E). The granulomatous lesions were significantly fewer (p < 0.005) in TNF$^{-}$ mice when compared to WT mice (Fig 1.7). Hepatic pathology at 56 days post infection was characterised by further lymphohcytic infiltration and an increase in the number of granulomatous
WT and TNF−/− mice were infected with $10^6$ cfu of *M. bovis* BCG and were sacrificed at 14 days, 28 days and 56 days post infection. Livers were removed and fixed in PBS buffered formalin. Paraffin embedded tissue were sectioned at 5μm and stained with haematoxylin and eosin. Panels A-C represents granuloma formation in WT mice and panels D-F represents TNF−/− mice. Sections are representative of 4 mice/strain at each time point. (Magnification = 400x)

*Figure 1.6*  
Irregular and delayed hepatic granuloma formation in *M. bovis* BCG infected TNF−/− mice.
lesions. The number of granulomatous lesions was significantly higher (p < 0.05) in TNF−/− mice than in WT mice at 56 days post infection (Fig. 1.7). However these granulomatous lesions remained poorly defined and failed to develop to maturity (Fig. 1.6F).

Typical granulomatous lesions of WT mice had few (1-3) AFB present in the epitheloid macrophage core (Fig. 1.8A) as opposed to TNF−/− mice which had, on average, 5-10 times more AFB in their granulomatous lesions (Fig. 1.8B). This observation supported the comparative hepatic mycobacterial burdens that were observed in the two strains at 56 days post infection.

1.7 Absence of pulmonary granuloma formation in TNF−/− mice.

Pulmonary pathology in both mouse strains at 14 days post infection was characterised by areas that had normal septae as well as thickened septae and clear alveolar spaces (Fig. 1.9A and 1.9D). The pathology in WT mice was dominated by the formation of well-defined granulomatous lesions at 28 days post infection (Fig. 1.9B). These were characterised by foamy epitheloid like macrophages with surrounded and interspersed lymphocytes. Lymphocytic infiltration was prominent in and around the vasculature. In contrast, TNF−/− mice showed further thickening of septae but did not develop granulomatous lesions (Fig 1.9E). Lymphocytic infiltration around the vesculature was limited. WT mice showed evidence of resolving granulomatous lesions at 56 days post infection (Fig. 1.9C). Granulomatous lesions were smaller and the lymphocytic infiltration around the vesculature was reduced when compared to 28 days post infection. Necrotic regions devoid of tissue structure primarily defined the contrasting pulmonary pathology of TNF−/− mice with nuclear debris being prominent (Fig. 1.9F). Increase lymphocytic infiltration resulted in peri-vescular and peri-bronchial cuffing. There was however no evidence of further structural re-organisation of infiltrating lymphocytes and macrophages to form well structured granulomatous lesions. An increase in the PMN was observed in TNF−/− mice and was found in regions of necrosis.

Staining for AFB at 56 days post infection confirmed the pulmonary mycobacterial burden data. Few bacilli were associated with granulomas, residing within epitheloid macrophages in WT mice (Fig. 1.8C). In contrast, tissue sections of TNF−/− mice had
Figure 1.7
Granuloma formation on challenge with *M. bovis* BCG.

The number of hepatic granulomas of WT (n = 5) and TNF⁻/⁻ (n = 5) mice was counted under 100x magnification after intravenous challenge with *M. bovis* BCG. Mononuclear cell clusters of ≥15 cells were defined as granulomas. The data is expressed as the average and standard deviation of 15 microscopic fields and is representative of 4 similar experiments.

Figure 1.8
AFB in the livers and lungs of *M. bovis* BCG infected WT and TNF⁻/⁻ mice.

WT and TNF⁻/⁻ mice were infected with 10⁶ cfu of *M. bovis* BCG and were sacrificed at 56 days post infection. Livers were removed and fixed in PBS buffered formalin. Paraffin embedded tissue were sectioned at 5μm and stained according to the Ziehl-Neelson staining method. In WT mice, AFB in the liver (panel A) and lungs are intracellular, confined to well-demarcated granulomas and significantly fewer (black arrows) in comparison TNF⁻/⁻ mice (panels B and D, respectively). Sections are representative of 4 mice/strain at each time point. (Magnification = 1000x)
WT and TNF$^{-/-}$ mice were infected with $10^6$ cfu of M. bovis BCG and were sacrificed at 14 days, 28 days and 56 days post infection. Lungs were removed and fixed in PBS buffered formalin. Paraffin embedded tissue were sectioned at $5\mu$m and stained with haematoxylin and eosin. Panels A-C represents pulmonary granuloma formation in WT mice and panels D-F represents TNF$^{-/-}$ mice. Sections are representative of 4 mice/strain at each time point. (Magnification = 100x)
significantly higher number of AFB under the same magnification (Fig. 1.8D). AFB was predominantly extra-cellular as a result of degenerating epitheloid macrophages and was generally detected within areas of necrosis.

1.8 Hepatic granulomatous lesion formation in TNF−/− mice is qualitatively different from WT mice.

The cellular composition of hepatic granulomas was compared after challenge with *M. bovis* BCG to determine whether the inflammatory responses of WT and TNF−/− mice were qualitatively comparable. Liver sections were stained with the indicated cellular markers at 28 days post infection and compared. Typical hepatic granulomas in WT mice consisted of CD3+ T-cells on the periphery (Fig. 1.10A) and F4/80+ macrophages constituting the core (Fig. 1.11A). Granulomas were also found to have significant expression of the adhesion molecules ICAM-1 (Fig. 1.12A), CD11b (Fig. 1.12C) and CD11c (Fig. 1.12E). In contrast, the hepatic inflammatory response in TNF−/− mice was predominated by a diffuse F4/80+ cellular infiltration (Fig. 1.11B) with reduced expression of the marker associated with the granulomatous lesion. ICAM-1 (Fig. 1.12B), CD11b (Fig. 1.12D) and CD11c (Fig. 1.12F) expression were also diminished.

The bactericidal activity of the granulomas in WT and TNF−/− mice were compared by staining hepatic tissue sections for the expression of MHC Class II and iNOS. High expression of both markers were localised to the granulomas of WT mice (Fig. 1.11C and 1.11E, respectively) whereas activation of macrophages in TNF−/− mice were arrested as indicated by the reduced immunoreactivity of MHC Class II and iNOS (Fig. 1.11D and 1.11F, respectively).

1.9 Pulmonary inflammation of CD3+CD4+ T-cells and CD3+CD8+ T-cells is reduced in *M. bovis* BCG infected TNF−/− mice.

It is well established that T-lymphocytes are essential for the formation of well-demarcated granulomatous lesions. Due to the inability of TNF−/− mice to mount an effective granulomatous response to control pulmonary *M. bovis* BCG infection the pulmonary lymphocytic response was investigated 28 days after infection. Cells, which were obtained after bronchial alveolar lavage, were stained with fluorecently labeled antibodies for the expression of the typical T-cell markers CD3, CD4 and
Reduced T-lymphocytes in hepatic granulomatous lesions in *M. bovis* BCG infected TNF−/− mice.

WT and TNF−/− mice were infected with 10⁶ cfu of *M. bovis* BCG and sacrificed at 28 days post infection. Frozen liver tissue sections were prepared as described in Materials and Methods and stained with antibodies specific for CD3, CD4 or CD8 respectively. Panels A, C and E represent WT control mice and panels B, D and F represent TNF−/− mice. The sections are representative of 4 mice/strain. (Magnification = 400x)
Figure 1.11
Granuloma-associated macrophage activation marker expression is reduced in the liver of
*M. bovis* BCG infected TNF<sup>-/-</sup> mice.

WT and TNF<sup>-/-</sup> mice were infected with 10<sup>6</sup> cfu of *M. bovis* BCG and sacrificed at 28 days post
infection. Frozen liver tissue sections were prepared as described in Materials and Methods and
stained with antibodies specific for F4/80, MHC class II or iNOS, respectively. Panels A, C and E
represent WT control mice and panels B, D and F represent TNF<sup>-/-</sup> mice. The sections are
representative of 4 mice/strain. (Magnification = 400x)
Granuloma-associated adhesion molecule expression is reduced in the liver of *M. bovis* BCG infected TNF-/- mice.

WT and TNF-/- mice were infected with $10^6$ cfu of *M. bovis* BCG and sacrificed at 28 days post infection. Frozen liver tissue sections were prepared as described in Materials and Methods and stained with antibodies specific for ICAM-1, CD11b, or CD11c respectively. Panels A, C and E represent WT control mice and panels B, D and F represent TNF-/- mice. The sections are representative of 4 mice/strain. (Magnification = 400x)
CD8, and were analysed by flowcytometry. A 7-fold reduction in the recruitment of both CD3+CD4+ T-cells (Fig. 1.13B vs Fig. 1.13E) and CD3+CD8+ T-cells (Fig. 1.13C vs Fig. 1.13F) in TNF−/− mice quantitatively confirmed the microscopic observation of limited pulmonary lymphocytic infiltration in comparison to WT mice.

Recruitment of the T-lymphocyte subsets was further investigated in context of its distribution within hepatic granulomas. In WT mice, granulomas contained both CD4+ T-cells (Fig. 1.10C) and CD8+ T-cells (Fig. 1.10E), which stained positive within the surrounding lymphocytic border. Similarly CD4+ T-cells (Fig. 1.10D) and CD8+ T-cells (Fig. 1.10F) were detected within the granulomatous lesions of TNF−/− mice. However the number of cells, which were associated with these lesions, appeared to be fewer as evident by reduced staining. More importantly, T-cells were arranged randomly within the lesion and occupied areas within the core as well as the periphery thus reflecting its aberrant structure.

1.10 TNF−/− mice can mount a Th1 response against *M. bovis* BCG infection.

The kinetic response of the cytokines IL-12, IFNγ, IL-4 and IL-5 were determined in *M. bovis* BCG infected TNF−/− mice to examine whether these mice have a bias towards a Th1 or Th2 response. IL-12 (Fig. 1.14B) was detected in similar concentrations in the BALF of both WT and TNF−/− mice at 14 days post infection. WT mice had a significant increase (p < 0.01) in IL-12 production at 28 days post infection compared to 14 days post infection. There was however not a significant difference in the levels of IL-12 BALF concentrations at day 28 when compared to 14 days post infection in TNF−/− mice. Comparative IL-12 levels between the two strains was not significantly different at 28 days post infection. WT mice maintained IL-12 production at 56 days post infection at levels similar to that which had been observed at 28 days post infection. In contrast, TNF−/− mice had a 10 fold increase in IL-12 production at 56 days post infection in comparison to the measured concentration at 28 days post infection and was significantly higher (p < 0.05) than that of WT mice.

TNF−/− mice had significantly higher IFNγ concentrations (Fig. 1.14A) in the BALF in comparison to WT mice at 28 days post infection. In contrast to WT mice that did not show any detectable IFNγ at 42 days post infection, TNF−/− mice showed a continual
WT and TNF−/− mice were intravenously infected with 10⁶ cfu of *M. bovis* BCG WT and TNF−/− mice. Lungs were lavaged, cells collected for double staining with anti-CD3 PE and anti-CD4 FITC (panels B and E) or anti-CD8 FITC (panels C and F) and analysis by flow cytometry as described in Materials and Methods. Cells labeled with isotypic control antibody are shown in panels A and D. The data represents 1 of 2 experiments with similar findings.

**Figure 1.13**
Pulmonary recruitment of CD3⁺CD4⁺ and CD3⁺CD8⁺ T-lymphocytes in *M. bovis* BCG WT and TNF−/− mice.
increase in IFNγ production which was significantly higher (p < 0.05) than the
concentrations which were measured at 28 days post infection. (IFNγ in the BALF of
either strain was undetectable at 14 days post infection – assay sensitivity = 5pg/ml).
IL-4 and IL-5 was not detectable in the BALF of either WT or TNF mice at any of the
time points that was investigated after *M. bovis* BCG infection (assay sensitivity =
5pg/ml).

### 1.11 MCP-1 and MIP-1α is induced in *M. bovis* BCG infected TNF<sup>−/−</sup> mice.

MCP-1 and MIP-1α have previously been shown to mediate cellular recruitment
(Baggiolini and Loetscher, 2000). In view of the poor recruitment of inflammatory
cells to sites of infection in *M. bovis* BCG infected TNF<sup>−/−</sup> mice, MCP-1 (Fig. 1.15A)
and MIP-1α (Fig. 1.15B) concentrations were measured in BALF at 28 days and 56
days post-infection. There was not a significant difference in MCP-1 levels between
WT and TNF<sup>−/−</sup> mice at 28 days post infection. However, 56 days after infection
MCP-1 levels were significantly higher (p<0.05) in TNF<sup>−/−</sup> mice when compared to
WT mice. It was possible to measure MIP-1α production in BALF of TNF<sup>−/−</sup> mice at
28 days and 56 days post infection but not in WT mice under the assay conditions that
were used (assay sensitivity = 5pg/ml).

### 1.12 TNF<sup>−/−</sup> mice can generate an antigen specific delayed type hypersensitivity
response.

The ability of TNF<sup>−/−</sup> mice to mount a specific antigenic response against *M. bovis*
BCG infection was investigated to determine whether it was a contributory factor to
susceptibility of the strain. Footpad swelling was measured in *M. bovis* BCG infected
WT and TNF<sup>−/−</sup> mice after PPD challenge at 28 days post infection. Both mouse
strains elicited a DTH response and no significant difference was observed in the
degree of footpad swelling (Fig. 1.16).

### 1.13 Determination of the minimum lethal irradiation dose for mice using a
60Cobalt source.

WT mice were exposed to different radiation doses and were monitored for 30 days to
determine the minimum lethal radiation dose using a 60Cobalt source (Table 1). Mice,
which received radiation doses of ≤ 900 Rad, all survived the experiment. Mice,
Figure 1.14
IFNγ and IL-12 concentrations in BALF of M. bovis BCG infected WT and TNF−/− mice.
Mice were infected with 10^6 cfu of M. bovis BCG and BALF was obtained at 14, 28 and 56 days post infection as described in Materials and Methods. IFNγ and IL-12 concentrations of the BALF were measured by ELISA. Each data point denotes the average ± SD of 3-4 mice. The results are representative of 3 similar experiments.

Figure 1.15
MCP-1 and MIP-1α concentrations in BALF of M. bovis BCG infected WT and TNF−/− mice.
Mice were infected with 10^6 cfu of M. bovis BCG and BALF was obtained at 14, 28 and 56 days post infection as described in Materials and Methods. MCP-1 and MIP-1α concentrations of the BALF were measured by ELISA. Each data point denotes the average ± SD of 3-4 mice. The results are representative of 3 similar experiments.
Figure 1.16
Delayed hypersensitivity in *M. bovis* BCG infected WT and TNF^-/- mice.

WT and TNF^-/- mice were infected with 10^6 cfu of *M. bovis* BCG. Mice were subcutaneously challenged with 5μg of PPD in the right hind footpad and saline in the left hind footpad. Swelling was measured after 42 hours. The data indicates the average ± SD of 5 mice per strain. The results are representative of 3 similar experiments.
which received a radiation dose of 1000 Rad, all succumbed within 12 days and mice, which received radiation doses of 1100 Rad and 1200 Rad succumbed within 11 days. All subsequent radiation procedures were therefore performed at 1000 Rad.

1.14 Bone marrow reconstitution of irradiated mice.
Mice, which were lethally irradiated with a dose of 1000 Rad and received 2x10^6 cells of unseparated bone marrow from donor mice, were compared to lethally irradiated control mice that received saline, for mortality. WT (Fig. 1.17 A) or TNF^{−/−} mice (Fig. 1.17B) could be successfully reconstituted with bone marrow from either WT mice or TNF^{−/−} mice. In contrast both WT and TNF^{−/−} control mice, which received saline, succumbed after irradiation.

1.15 Hematopoietic precursor cells are sufficient to restore resistance to M. bovis BCG infection in TNF^{−/−} mice.
The impaired immune response of TNF^{−/−} mice was investigated to determine whether it could be rectified by only hematopoietically derived TNF. It was previously established that TNF release was restored in lethally irradiated TNF/LT^{−/−} mice that were reconstituted with WT bone marrow after LPS injection (Muller et al., 1996). Lethally irradiated TNF^{−/−} mice were reconstituted with 2x10^6 unseparated bone marrow cells that were obtained from WT donor mice (TNF-WTBM). For the control experiment lethally irradiated WT and TNF^{−/−} mice received autologous bone marrow (WT-WTBM and TNF-TNFBM). Four months after reconstitution, mice were infected intravenously with 10^6 cfu of M. bovis BCG and the granulomatous response in chimeric mice was assessed after 28 days as an indication of a proper immune response.

Liver sections of TNF-WTBM mice (Fig. 1.18B) revealed the presence of well-demarcated granulomas, typical and comparable to structures that were observed in the control WT-WTBM mice (Fig 1.18A). In contrast, TNF-TNFBM mice (Fig. 1.18C) displayed an increase in lymphocytic infiltration with granulomatous lesions of less defined structure, similar to cellular arrangements, which were observed in TNF^{−/−} mice.
Table 1

Determination of the minimum radiation dose.

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<th>Radiation Dose (Rad)</th>
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To determine the minimum lethal irradiation dose, WT mice were exposed to different radiation doses and mortality was recorded. The data represents the number of mice that succumbed to the indicated radiation doses. The results represent one of two experiments.

Figure 1.17

Survival of chimeric mice

WT mice (panel A) or TNF⁻/⁻ (panel B) mice received an irradiation dose of 1000 Rad. After 24 hours each mouse received 2x10⁶ unseparated bone marrow cells from either WT donor mice or TNF⁻/⁻ donor mice by intravenous injection whereas control mice received saline. Mice were monitored for mortality and the data represented as the percentage of surviving mice. The results are representative of 2 experiments with similar results.
The pulmonary pathology in chimeric mice after *M. bovis* BCG infection were investigated due to the differential response of the liver and lungs in the control of mycobacterial growth in TNF−/− mice. Restoration of the granulomatous response extended to the lungs in TNF-WTBM as indicated by the well-defined granulomas that were present (Fig. 1.18E). These structures appeared similar to that of WT-WTBM control mice (Fig. 1.18D). In contrast continual pulmonary inflammation was observed in TNF-TNFBM mice without proper definition of granuloma structures (Fig. 1.18F).

The mycobacterial burden in the liver, spleen and lungs were determined after 28 days of infection to assess if the structural reconstituted granulomatous response in TNF-WTBM mice was also translated into functionally efficiency. The results in Figure 1.19 indicate that WT-WTBM mice had significantly lower (p < 0.05) mycobacterial burdens than TNF-TNFBM mice in all organs that were examined. More importantly the results show that TNF-WTBM mice displayed effective control of the infection because it had significantly lower mycobacterial burdens in the liver (p < 0.01), spleen (p < 0.01) and lungs (p < 0.05) than TNF-TNFBM mice. The bacilli load in TNF-WTBM mice in comparison to TNF-TNFBM mice was ~10 fold lower in all organs. The mycobacterial burdens of WT-WTBM mice and TNF-WTBM mice were comparable and significant differences were not found in any of the organs that were examined.
Figure 1.18
Restoration of hepatic and pulmonary granuloma formation in M. bovis BCG infected TNF-WTBM chimeric mice.

Chimeric mice were generated as described in Materials and Methods. Mice were intravenously infected with $10^6$ cfu of M. bovis BCG. After 28 days mice were sacrificed and the livers were fixed in PBS buffered formalin. Tissue sections were prepared and stained with haematoxylin and eosin. Black arrows indicate the formation of well-demarcated granulomas in the liver (panel A) or lungs (panel D) of WT-WTBM control chimeric mice. Irregular granuloma formation is observed in the livers (panel C) and lungs (panel F) of TNF-TNFBM control chimeric mice. Yellow arrows indicate restoration of granulomas in the livers (panel B) and lungs (panel E) of TNF-WTBM chimeric mice. Sections are representative of 4 mice/strain. (Liver sections- Magnification = 400x, Lung sections- Magnification = 100x)
Figure 1.19
Correction of host resistance to *M. bovis* BCG infection in TNF<sup>−/−</sup> mice.

Chimeric mice were generated as described in Materials and Methods. Mice were intravenously infected with 10<sup>6</sup> cfu of *M. bovis* BCG. After 28 days mice were sacrificed and the bacilli burden in the liver, lung and spleen were determined. The data shows that hemopoetically derived TNF is sufficient to generate protective immunity against *M. bovis* BCG infection. The data represent the average ± SD of 4 mice per strain.
Discussion

This study addressed the importance of TNF in protective immunity against *M. bovis* BCG infection. It demonstrated that there is an absolute dependence on TNF in mediating an effective immune response and that the absence of TNF is fatal. The high susceptibility of TNFR1−/− mice in comparison to TNFR2−/− mice suggests that TNFR1 is the predominant receptor that mediates TNF signaling. This study supported the findings of (Flynn *et al.*, 1995) who showed that the neutralization of TNF through the administration of anti-TNF antibodies is fatal in *M. tuberculosis* infected mice and that TNFR1 signaling is a requirement for the control of *M. tuberculosis* infection. It was also in accordance with the reports of Bean *et al.* (1999) and Kaneko *et al.* who showed that TNF, as the binding ligand, is crucial during *M. tuberculosis* infection. However, the findings in this study differ from the results which was obtained by Kaneko *et al.* (1999) in *M. bovis* BCG infection studies of TNF−/− mice which showed that this avirulent strain did not induce mortality as infected mice could survive for 6 months. The reason for the discrepancy is not known.

Systemic production of TNF is thought to be the cause of the clinical manifestations of disease in many instances. Some of the major systemic effects of disease in patients with active tuberculosis are fever, malaise and weight loss; effects which have been associated with especially TNF production (Smith and Moss, 1994). This study shows progressive weight loss and impeded movement of mice in the absence of TNF and suggests that factors other than TNF are important in mediating tuberculosis associated systemic effects.

Mycobacterial growth occurred rapidly within the first 2 weeks in WT mice in all the organs that were tested after which the infection was effectively controlled to levels similar (lung) or lower (liver, spleen) than the original infection doses. Two important observations were made regarding the control of mycobacterial infection in the absence of TNF namely (1) that the bacilli burden was reduced in the liver and spleen but not in the lung and that (2) although mycobacterial clearance occurred in both the spleen and the liver, the rate of clearance was reduced and hence clearance
was delayed. This suggested that although TNF was involved, it was not an absolute requirement for the control of infection in the liver and spleen and that bactericidal mechanisms independent of TNF were present. In contrast to hepatic and splenic infections, the presence of TNF was an absolute requirement for the effective control of pulmonary mycobacterial infection. The findings in this study pertaining to the control of mycobacterial infection were confirmed by the results of Bean et al. (1999). In their findings TNF$^{-/-}$ mice were unable to control the pulmonary infection but, like WT mice, displayed an acute phase of infection that was followed by a chronic phase in the liver and spleen. This chronic phase of infection is thought to reflect an equilibrium state between the ability of the host to control infection and mycobacterial growth. It is interesting to note that in M. bovis BCG infected SCID mice, the growth rate of bacilli was higher in the lungs in comparison to the liver and the spleen (North and Izzo, 1993). This suggested that, despite the inability of these mice to generate T cell mediated immunity as a result of the absence of functional T and B-lymphocytes; inherent protective differences existed between the liver and spleen, and the lungs. It has been suggested that oxygen deprivation could contribute and account, at least in part, for these observations. Further it is thought that the absence of TACO, which promotes the inhibition of endosome-lysosome fusion (Ferrari et al., 1999), in Kupffer cells contributes to hepatic resistance against mycobacterial infection. In addition, the observations from this study suggest that differences with respect to the granulomatous response between the liver and spleen, and the lungs of TNF$^{-/-}$ mice have to be considered to account for the contrasting organ resistance.

The differences in organ weights and organ/bodyweight ratios were compared between WT and TNF$^{-/-}$ mice as an indication of inflammation and immune responses. The significantly higher weights of the spleen and the liver at 14 days in comparison to day 1 post infection in WT mice probably reflects an early inflammatory response which was absent in TNF$^{-/-}$ mice. This rapid recruitment of cells, which coincided with the initial acute phase of the infection, is TNF dependent and appears to be important for the expeditious elimination of bacilli from the liver and spleen. The eventual increase of spleen and liver weights in TNF$^{-/-}$ mice is indicative of a TNF independent mediated inflammatory response, which corresponded with elimination of bacilli from these organs.
It has previously been established that granuloma formation is a critical component in protective immunity. In the absence of a Th1 cell mediated immune response either due to failure of specific cytokine signalling such as is found in IFNγ- (Cooper et al., 1993), IFNγR- (Kamijo et al., 1993) and IL-12 (Cooper et al., 1997b) or due to the absence of specific cell subsets as is found in TCR Aβ- (Mogues et al., 2001), MHC Class II- (Caruso et al., 1999) and TCRβ-/- mice (Ladel et al., 1995) as well as SCID mice (North and Izzo, 1993), granuloma formation is defective. In all of these mutant strains this defect is associated with uncontrolled mycobacterial growth and eventual death. In this study two pertinent observations were made regarding the granulomatous response on challenge with M. bovis BCG in the absence of TNF namely (1) the complete absence of organised pulmonary granuloma formation and (2) the delayed formation of hepatic granulomatous lesions that were predominantly, although not exclusively, immature and uncohesive. A reduction in mycobacterial growth in the liver was only observed subsequent to the establishment of the granulomatous lesions, which indicated that the formation of lesions in TNF+/- mice offered at least partial protective immunity. These observations supported the findings of Kindler et al. (1989) who demonstrated that TNF was important for the early establishment of granuloma formation and that its presence was required for maintaining the integrity of the granuloma structure. Delayed granuloma formation in the absence of TNF signalling was reported in a M. tuberculosis infection study (Flynn et al., 1995a). However, the delay in the establishment of granulomatous lesions in response to the M. tuberculosis infection in comparison to M. bovis BCG was shorter (~14 days vs. ~28 days) which suggested that mycobacterial virulence might have had an influence on the rate at which these lesions were formed. In contrast to partial protective hepatic immunity, which was associated with a delayed granulomatous response, the absence of well-defined pulmonary granulomas was paralleled by uncontrolled mycobacterial growth in TNF+/- mice. This suggested that pulmonary immunity against M. bovis BCG infection is completely dependent on TNF. The description of the pulmonary granulomatous response in this study is consistent with the findings of Bean et al. (1999), Flynn et al. (1995a) and Kaneko et al. (1999), which highlighted the lack of normal granuloma formation, and the presence excessive necrosis, which is found in the absence of TNF.
TNF can contribute to Th1 cell mediated immunity at different levels and have been implicated in cytokine and chemokine stimulation, cellular recruitment and inducing cellular activation. All of these parameters are important for the successful establishment of bactericidal granulomas and have been addressed in the context of TNF deficiency. A defective pulmonary granulomatous response and delayed hepatic granuloma formation was clearly indicative of a failure to rapidly recruit mononuclear cells to sites of infection. This was reflected in the reduced number of CD3⁺CD4⁺ lymphocytes and CD3⁺CD8⁺ lymphocytes that was associated with hepatic granulomas and which was recruited to the lungs. Previously it has been established that both CD4⁺ T-cells and CD8⁺ T-cells capable of IFNγ secretion are recruited to the lung following *M. tuberculosis* infection (Feng et al., 1999). CD4⁺ T cell derived IFNγ has been identified as the primary cytokine source, which drives Th1 differentiation. *M. tuberculosis* and *M. bovis* BCG infection studies, in which mice with a deficiency for either IFNγ or the IFNγ-R succumb, have unequivocally defined its importance in Th1 cell mediated immunity against mycobacterial invasion. It is therefore reasonable to deduce that the lack of early recruitment of CD4⁺ and CD8⁺ T-cells, in the absence of TNF would lead to reduce IFNγ levels and thus an impaired Th1 cell mediated immune response. This is supported by the significantly lower IFNγ mRNA levels that were detected during early infection of *M. tuberculosis* in CD4⁻/⁻ and MHC Class II⁻/⁻ mice, two mutant strains that are severely depleted of CD4⁺ T-cells (Caruso et al., 1999). However these mice displayed IFNγ levels that were equivalent to that of WT mice during late stages of infection. In this study IFNγ levels in the BAL fluid were undetectable during the early stages of infection. Nevertheless, these findings confirmed the published data of Bean et al. (1999), which illustrated that TNF⁻/⁻ mice were able to produce IFNγ in response to mycobacterial infection. In fact, IFNγ secretion increased significantly over the course of infection in TNF⁻/⁻ mice, probably in response to the continual increasing bacilli numbers. In a *M. bovis* BCG infection model using IFN-R⁻/⁻ mice Erb et al. (1999) concluded that IFNγ inhibited the development of a Th2 response. In this study elevated IFNγ concentrations were present in TNF⁻/⁻ mice, in contrast to the Th2 defining cytokines, IL-4 and IL-5, which were not detected during infection. These findings therefore supported an inhibitory effect of IFNγ on Th2 development.
Other than acting as a source of IFNγ CD4+ T cells and CD8+ T cells were shown to exert lytic activity. A role for CD8+ T cells in anti-mycobacterial activity have been suggested by the isolation of CD8+ T cell clones able to recognise mycobacterial antigens (Stenger et al., 1997). In addition M. tuberculosis specific CTL were isolated from lungs and lymphnodes of infected mice, which expressed perforin and could lyse infected macrophages (Serbina et al., 2000). Although emerging evidence have strengthened support for a cytolysis role of CD8+ T-cells during mycobacterial infection contrasting reports citing that granzyme or perforin mediated cytolyis are not required during M. tuberculosis infection have been published (Cooper et al., 1997b).

The initial recruitment of macrophages was arrested during early infection as evident by the small number of epitheloid macrophages that was present in the hepatic granulomas and the absence of well-organised granulomas in the lungs of TNFα+ mice. As the primary cell responsible for pathogenic uptake, its absence held severe consequences, not only in terms of phagocytosis but also in the provision of cytokines and chemokines that are essential to drive a cell mediated immune response. Macrophage also provides the cellular platform in which NO is generated as the bactericidal mechanism against invading bacilli. In this study it was shown that IL-12 production can occur in the absence of TNF but although significant elevation of IL-12 production in WT mice was shown during early infection, significance could not be demonstrated in the absence of TNF. This inhibition of IL-12 production during early infection was transient as significantly higher levels were detected during late infection. The similar secretion pattern of IFNγ and IL-12 was not surprising given the metabolic interdependence of these two cytokines. The regulatory effects of TNF on IL-12 are controversial, as inhibitory and stimulating effects have been reported in different experimental set-ups (Ma, 2001). Notwithstanding, the importance of IL-12 in protective immunity have been illustrated in mice (Cooper et al., 1997b; Holscher et al., 2001) and humans (de Jong et al., 1998); however despite the high levels of both IL-12 and IFNγ in the late phase of infection, it could not compensate for the absence of TNF in generating full protective immunity.

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This study has further addressed the activation status of hepatic and pulmonary granulomas by immunohistochemistry on challenge with \textit{M. bovis} BCG. The data shows that F4/80, MHC Class II, acid phosphatase and iNOS staining of granulomas in TNF$^{-/-}$ mice are reduced. In addition, specific staining for the adhesion molecule ICAM-1 was diminished and for the first time, a reduction in expression of granuloma associated CD11b and lack of CD11c staining was demonstrated in the absence of TNF. Previously CD11b and CD11c upregulation was only reported on peripheral blood leukocytes of patients that were infected with tuberculosis (Yassin and Hamblin, 1994). The reduction in positive staining for the respective macrophage activation markers that was associated with granulomas in TNF$^{-/-}$ mice could be the consequence of either reduced cellular recruitment or reduced expression of the proteins or a combination of both processes. Nevertheless it was interesting to note that granuloma associated macrophage activation did occur, albeit at a lower level in the absence of TNF. It has previously been shown that iNOS expression was induced in the absence of TNF signalling (Bean \textit{et al.}, 1999). The importance of MHC class II (Caruso \textit{et al.}, 1999) and iNOS (MacMicking \textit{et al.}, 1997; Garcia \textit{et al.}, 2000) have been illustrated in gene deletion studies which showed that the absence of these genes results in fatality on challenge with \textit{M. tuberculosis} or \textit{M. bovis} BCG. Interestingly, studies by Cooper \textit{et al.} (2000) have reported that iNOS expression is not essential for early control of \textit{M. tuberculosis} during pulmonary infection and suggested that other mechanisms independent of NO generation were involved in limiting bacilli growth.

The reduction in adhesion molecule expression could imply functional relevance with respect to cellular migration and leukocyte interaction particularly within the confines of the granuloma structure. In the absence of ICAM-1, mice displayed defective inflammatory and immune responses with ICAM-1 deficient cells providing negligible stimulation in a mixed lymphocytic reaction (Sligh \textit{et al.}, 1993). Johnson \textit{et al.} (1998) reported an adequate expression of protective immunity against \textit{M. tuberculosis} infection in ICAM-1$^{-/-}$ mice up to 100 days post infection. Interestingly these mice had impaired macrophage recruitment and lacked the formation of proper granulomas. In a mouse model for reactivation Turner \textit{et al.} (2001) associated a degenerative granulomatous response and consequent increase in bacilli growth with a reduction in CD11a and ICAM-1 lymphocytic expression. CR3 [CD11b/CD18] is an important receptor on macrophages that facilitates uptake of mycobacteria (Ernst,
1998; Ehlers and Daffe, 1998) and was increased in patients with active tuberculosis (Kuo et al., 1996). In addition Juffermans et al. (2001) showed that CD11b on monocytes could be upregulated by LAM. Both VCAM-1 and ICAM induction in a mouse model for experimental allergic encaphalomyelitis (EAE) were demonstrated to be TNF dependent (Barten and Ruddle, 1994; Shrikant et al., 1994). Furthermore Feng et al. (2000) demonstrated that VCAM-1 expression was upregulated during M. 

Chemokines and its corresponding receptors have been implicated in several bacterial infections (Schluger and Rom, 1997), including mycobacterial infections (Rhoades et al., 1995). They are important in leukocyte trafficking (Baggiolini and Loetscher, 2000) and essential for the proper establishment of intact granulomas (Boring et al., 1997). TNF dependent chemokine secretion was shown to be necessary for the homing of T and B cells in the spleen (Sedgewick et al., 2000) but more relevant was the report of Czermak et al. (1999) who demonstrated a requirement for TNF to produce MIP-1α and MCP-1 in a lung injury model. In this study the role of TNF dependent MIP-1α and MCP-1 dependent secretion was investigated during M. bovis BCG infection and showed that TNF was not required for the production of MCP-1 or MIP-1α and therefore excluded these chemokines as essential for contributing to the granulomatous response and protective immunity. The findings regarding MCP-1 were not surprising because it had been implicated in promoting a Th2 response (Gu et al., 2000; Chensue et al., 1996). The association of elevated MCP-1 concentrations and susceptibility to mycobacterial infection was in accordance with the findings of Rutledge et al. (1995) who found that high levels of MCP-1 expression in transgenic mice increased their susceptibility to M. tuberculosis infection. Previously Lin et al. (1998a) showed that MCP-1 could be induced independently of TNF in a M. tuberculosis infected human alveolar cell line. It was also found that spontaneous MCP-1 secretion by monocytes from tuberculosis-infected patients was higher than healthy individuals (Lin et al., 1998b). Taken together this data suggests that the induction of MCP-1 is primarily required for a Th2 type response and that its presence
in tuberculosis patients, rather than being characteristic of a protective immunity, is indicative of a poor prognosis.

In this study the contributory source of TNF during *M. bovis* BCG infection was also addressed. It has previously been reported that TNF could be produced from hematopoetic as well as non-hematopoetic cells (Vasalli, 1992). In bone marrow chimeric studies it was shown that TNF of hematopoetic origin is sufficient to reconstitute protective immunity in TNF−/− mice against *M. bovis* BCG infection. Similarly in a mouse model of experimental allergic encapholomyelitis it was revealed that the replacement of the haemopoetic compartment in TNF deficient with WT cells induces early onset of disease and visa versa (Sedgwick et al., 2000). In both instances haematopoetically derived TNF was sufficient to induce either EAE or protection against *M. bovis* BCG infection.
*Mycobacterium bovis* BCG infection

in TNF/LT$^{-/-}$ and LT$^{-/-}$ mice.
Summary

In this study the effects of *Mycobacterium bovis* BCG infection in the absence of TNF and LT was investigated. In contrast to WT mice, TNF/LT<sup>−/−</sup> mice were highly susceptible and succumbed after intravenous infection. Determination of mycobacterial burdens in the liver, spleen and lungs indicated an inability of TNF/LT<sup>−/−</sup> mice to contain the infection. Characterisation of liver pathology showed that the granulomatous response was severely compromised. The induction of granuloma formation was delayed and its reorganisation and development to mature bactericidal structures did not occur. Granulomas in TNF/LT<sup>−/−</sup> mice, therefore, consisted primarily of small cellular aggregates. Similarly well-demarcated pulmonary granulomas failed to be established and the inability to eliminate the infection resulted in persistent inflammation with the eventual development of tissue necrosis. Staining for AFB revealed high numbers of bacilli within pulmonary epitheloid macrophages during early infection that progressed to being essentially extra-cellular and resident within necrotic tissue and nuclear debris. Cellular aggregates in the liver in TNF/LT<sup>−/−</sup> mice also contained an increased number of AFB in contrast to few bacilli in granulomas of WT mice. Characterisation of the cellular aggregates revealed a diminished presence of lymphocytes and macrophages. In addition, reduction of ICAM-1 expression was noted and the lower expression of iNOS confirmed the limitation of TNF/LT<sup>−/−</sup> mice to clear infection.

In a comparative study, the contribution of LT to generate protective immunity against an avirulent infection, was investigated. LT<sup>−/−</sup> mice, although displaying partial susceptibility to infection, did not succumb after challenge with *Mycobacterium bovis* BCG. Mycobacterial burden studies revealed that LT<sup>−/−</sup> mice could control bacilli growth in the liver and spleen similarly to TNF<sup>−/−</sup> mice but not as well as WT mice. Unlike TNF<sup>−/−</sup> mice, which could not control pulmonary mycobacterial growth, partial elimination of infection was observed in LT<sup>−/−</sup> mice. Early induction of granulomas in LT<sup>−/−</sup> mice was supported by early reduction of bacilli load in the liver. However the bactericidal activity of granulomas appeared to be compromised due to the absence of LT as the rate of mycobacterial elimination could not be sustained.
In conclusion, the results of this study argue for the importance of both TNF and LT in generating a complete and effective protective immune response against *Mycobacterium bovis* BCG infection and that both molecules occupy a unique niche in cell mediated immunity.
Results

2.1 TNF/LT−/− mice succumb to Mycobacterium bovis BCG infection.
WT mice and TNF/LT−/− mice were intravenously challenged with 10⁶ cfu of M. bovis BCG and their respective mortality rates were monitored (Fig. 2.1). In contrast to WT mice, which survived the experiment, TNF/LT−/− mice was highly susceptible and succumbed to the infection between 8-11 weeks.

2.2 Ineffective control of mycobacterial replication in TNF/LT−/− mice.
The mycobacterial burden in different organs of TNF/LT−/− mice was determined due to its susceptibility to M. bovis BCG infection (Fig 2.2). The peak of hepatic mycobacterial growth (Fig. 2.2A) was observed at 14 days post infection in WT mice and at 56 days post infection in TNF/LT−/− mice. In contrast to WT mice that significantly cleared (p < 0.005) the infection after 28 days, TNF/LT−/− mice maintained the same mycobacterial load which resulted in significantly higher (p < 0.01) hepatic bacilli counts in TNF/LT−/− mice. A further significant reduction (p < 0.01) was observed in the hepatic bacilli load of WT mice at 56 days after infection. Although the bacilli load in TNF/LT was not significantly different after 56 days of infection when compared to 28 days post infection, it was significantly higher (p < 0.05) than the mycobacterial burden at 14 days post infection. The 48-fold difference that existed between the two mouse strains at 56 days post infection was highly significant (p < 0.01).

Mycobacterial clearance in the spleen (Fig. 2.2B) followed a similar pattern to hepatic mycobacterial clearance in WT mice. Significant clearance (p < 0.001) was observed at 28 days post infection compared to 14 days post infection and was further significantly reduced (p < 0.05) after 56 days of infection. In contrast, the splenic bacilli burden of TNF/LT−/− mice increased significantly (p < 0.005) at 28 days post infection in comparison to 14 days post infection. Continuous uncontrolled splenic mycobacterial growth resulted in significantly higher (p < 0.01) bacilli burdens at 56 days post infection compared to 28 days post infection.
Figure 2.1.
Survival of TNF/LT<sup>+</sup> and LT<sup>-</sup> mice on challenge with M. bovis BCG.

WT, TNF/LT<sup>+</sup> and LT<sup>-</sup> mice were intravenously infected with 10<sup>6</sup> cfu of M.bovis BCG and were monitored for mortality. The data is expressed as the percentage of surviving mice during the course of infection. The results are representative of 3 experiments with similar findings.
Figure 2.2
Mycobacterial burden in the liver, spleen and lungs of M. bovis BCG infected WT and TNF/LT-/- mice.

WT and TNF/LT+ mice were intravenously infected with $10^6$ cfu of M. bovis BCG. Mice were sacrificed at 14 days, 28 days and 56 days post infection and the bacilli burdens in the liver (panel A), spleen (panel B) and lungs (panel C) were assessed as described in Materials and Methods. Each time point represents the average ± SD of 4 mice per strain. The results are representative of 2 experiments with similar findings.
The pulmonary mycobacterial burden (Fig. 2.2C) in WT mice was equivalent at 14 days and 28 days post infection. A significant reduction (p < 0.005) was only observed after 56 days of infection. An inability of TNF/LT^- mice to control pulmonary bacilli growth was reflected in the significantly higher (p < 0.05) bacilli number at day 28 post infection in comparison to day 14 post infection. At day 56 post infection mycobacterial growth was further enhanced and significantly higher (p < 0.05) than at day 28 post infection. Comparative bacilli loads between the two strains revealed significantly higher levels in TNF/LT^- mice at 28 days (p < 0.001) and 56 days (p < 0.01) post infection.

Specific staining for AFB confirmed that uncontrolled mycobacterial replication occurred in TNF/LT^- mice. Fig 2.3 represents lung sections of WT mice and TNF/LT^- mice after Ziehl-Neelson staining. AFB resided within epitheloid macrophages in both mouse strains at 28 days post infection. However the number of AFB that were detected within these cells were clearly higher in TNF/LT^- mice (Fig. 2.3B) than in WT mice (Fig. 2.3A). Few bacilli were still present within epitheloid macrophages of WT mice at 56 days post infection (Fig. 2.3C). In contrast, TNF/LT^- mice had excessive numbers of bacilli that were predominantly extra-cellular and epitheloid macrophages, which contained bacilli, were fully compacted (Fig. 2.3D).

Staining for the presence of AFB in liver granulomas of WT mice or cellular aggregates in the livers TNF/LT^- mice at 56 days post infection likewise validated the observation of uncontrolled mycobacterial growth in the absence of TNF and LT. The number of AFB that was associated with granulomas in WT mice were few (1-3 bacilli) in contrast to higher number (>10 bacilli) of AFB that was normally found allied to cellular aggregates in TNF/LT^- mice (Fig. 2.4).

A semi-quantitative assessment of the bacilli burden in liver tissue was performed by counting the number of AFB that was visible under high magnification (x1000) at 56 days post infection. The results of Fig 2.5A indicate that the average number of bacilli present in a single microscopic field was significantly higher (p < 0.01) in TNF/LT^- mice than in WT mice.
WT and TNF/LT−/− mice were infected intravenously with $10^6$ cfu of *M. bovis* BCG. Mice were sacrificed at 28 days and 56 days post infection. Lungs were fixed in formalin, imbedded in paraffin wax, sectioned and stained with Ziehl-Neelson stain. Significantly fewer intracellular bacilli (black arrows) were detected in WT mice (panel A) than TNF/LT−/− mice (panel B) at 28 days post infection. Mycobacteria remained intracellular in WT mice (panel C) but were predominantly extracellular in TNF/LT−/− mice (panel D) at 56 days post infection. Tissue sections represent 4 mice per strain. The results are representative of 2 similar experiments. (Magnification = 1000x)
Figure 2.4
Acid fast bacilli in the livers of M. bovis BCG infected WT and TNF/LT−/− mice.

WT and TNF/LT−/− mice were infected intravenously with 10⁶ cfu of M. bovis BCG. Mice were sacrificed at 56 days post infection and lungs were fixed in formalin, imbedded in paraffin wax, sectioned and stained with Ziehl-Neelson stain. Resolving granulomas in WT mice (left panel) have few (1-3) bacilli confined to epitheloid cells whereas TNF/LT−/− mice (right panel) have significantly more (>10) bacilli associated with newly establish small cellular aggregates. Tissue sections represent 4 mice per strain. The results are representative of 2 similar experiments. (Magnification = 1000x)

Figure 2.5
Granuloma formation and granuloma-associated AFB in M. bovis BCG infected WT and TNF/LT−/− mice.

WT and TNF/LT−/− mice (n = 8 mice/strain) were intravenously infected with 10⁶ cfu of M. bovis BCG. Mice were sacrificed at 56 days post infection; livers were fixed in phosphate buffered formalin, sectioned and stained with haematoxylin and eosin. The number of AFB (left panel) was counted under 1000x magnification and the number of granulomas was assessed under 400x magnification. The results denote the average ± SD of 80 microscopic fields and represent 4 mice per strain. The data depicts 1 of 2 experiments with similar findings.
2.3 Defective granuloma formation in *M. bovis* infected TNF/LT<sup>−/−</sup> mice.

Effective control of mycobacterial infection is associated with the early establishment of well-demarcated granulomas. Hepatic and pulmonary granuloma formation was therefore examined in TNF/LT<sup>−/−</sup> mice due to the inability of the strain to clear the infection. Fig. 2.6 compares the kinetic hepatic granulomatous response of WT mice and TNF/LT<sup>−/−</sup> mice after intravenous infection with 1x10<sup>6</sup> cfu of *M. bovis* BCG. Histological examination of liver sections of WT mice revealed the presence of typical mature granulomas which comprised of macrophages, epitheloid cells and surrounding lymphocytes at 14 days post infection (Fig. 2.6A). These granulomas persisted at 28 days post infection (Fig. 2.6B) but were diminished in size at 56 days post infection (Fig. 2.6C) and resembled end stage resolving structures. In contrast, granuloma formation in TNF/LT deficient mouse was absent at 14 days post infection (Fig. 2.6D). Cellular aggregates dictated hepatic pathology and developed into small clusters of lymphocytes and epitheloid cells at 28 days post infection (Fig. 2.6E). These cellular clusters failed to structurally mature and were present in larger numbers at day 56 post infection (Fig. 2.6F). Necrosis was not evident in the liver sections of WT or TNF/LT<sup>−/−</sup> mice.

Pulmonary granuloma formation was examined in WT mice and TNF/LT<sup>−/−</sup> mice subsequent to intravenous infection with *M. bovis* BCG (Fig. 2.7). Inspection of lung tissue sections at 14 days post infection indicated no specific differences in pathology, both sections being characterised by limited cellular infiltration and enlarged septae in certain areas (Fig 2.7A and 2.7D). WT mice developed distinct granulomas at day 28 post infection, which comprised of mainly epitheloid cells and interspersed lymphocytes although PMN were visible occasionally (Fig. 2.7B). Consolidation of infiltrating cells into well-demarcated granulomas was completely absent in TNF/LT<sup>−/−</sup> mice (Fig. 2.7E). Lung pathology was characterised by cellular infiltration that lacked order and appeared random. In Fig. 2.7C and Fig 2.7F lung tissue sections of WT mice and TNF/LT<sup>−/−</sup> mice are compared at 56 days post infection. WT mice had clearly defined, compact granulomas with unobstructed alveolar spaces. In contrast, TNF/LT<sup>−/−</sup> mice had large necrotic lesions with no alveolar structure. Extensive lymphocyte infiltration was evident, surrounding areas of necrosis, and being found in peri-vascular and peri-bronchial areas.

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WT, TNF/LT− and LT− mice were infected intravenously with 10^6 cfu of M. bovis BCG. Mice were sacrificed at 14 days, 28 days and 56 days post infection. Livers were fixed in formalin, imbedded in paraffin wax, sectioned and stained with hematoxylin and eosin. Defined granulomas were established in WT mice (panel A) and LT− mice (panel G) but were absent in TNF/LT− mice (panel D) at 14 days post infection. Granulomas developed to maturity in both WT mice (panel B) and (panel H) but not in TNF/LT− mice (panel E) at 28 days post infection. Smaller resolving granulomas were present in both WT (panel C) and LT− (panel I) mice but pathology in TNF/LT− mice (panel F) were characterised by small newly established cellular aggregates. The sections represent 4 mice per strain. The results are representative of two similar experiments. (Magnification = 100x)
WT, TNF/LT\textsuperscript{-/-} and LT\textsuperscript{-/-} mice were infected intravenously with 10\textsuperscript{6} cfu of \textit{M. bovis BCG}. Mice were sacrificed at 14 days, 28 days and 56 days post infection. Lungs were fixed in formalin, imbedded in paraffin wax, sectioned and stained with hematoxylin and eosin. Similar pathology, which was characterised by limited cellular infiltration, was observed in WT (panel A), TNF/LT\textsuperscript{-/-} (panel D) and LT\textsuperscript{-/-} (panel G) mice at 14 days post infection. Granulomas (black arrows) were established in WT (panel B) and LT\textsuperscript{-/-} (panel H) mice but were absent in TNF/LT\textsuperscript{-/-} (panel E) mice at 28 days post infection. Defined granulomas were still present in WT (panel C) and LT\textsuperscript{-/-} (panel I) mice but tissue of TNF/LT\textsuperscript{-/-} mice (panel F) contained advanced necrotic lesions. Notice the difference in airspaces between LT\textsuperscript{-/-} and TNF/LT\textsuperscript{-/-} mice at 56 days post infection. The sections represent 4 mice per strain. The results are representative of two similar experiments. (Magnification = 40x)
The number of hepatic lesions was quantified in WT mice and TNF/LT$^{-/-}$ mice as an additional indication of the efficacy of the granulomatous response. Lesions were defined as an aggregation of 10 or more cells. The significantly higher ($p < 0.01$) number of granulomas in Fig. 2.5B shows a rapid response in WT mice at 14 days post infection. The number of granulomas was maintained at day 28 post infection and were significantly higher ($p < 0.01$) than in TNF/LT$^{-/-}$ mice. A significantly lower ($p < 0.05$) number of lesions were observed in WT mice at day 56 compared to day 28 post infection. In contrast, small, sparse cell clusters were present at 14 days post infection, which increased significantly ($p < 0.05$) in quantity but not quality at day 28 post infection in TNF/LT$^{-/-}$ mice. Although a further significant increase in the number of granulomatous lesions/cell clusters were observed at day 56, equivalent to that found in WT mice, the quality of the lesions did not improve and failed to mature into the well-demarcated and defined structures of WT mice.

2.4 Cellular composition of granulomas in M. bovis BCG infected TNF/LT$^{-/-}$ mice is altered.

The inability of TNF/LT$^{-/-}$ mice to contain bacilli growth was analysed further by immunohistochemical staining of granulomas using specific cellular and activation markers (Fig. 2.8)). Staining liver sections with anti-CD3 revealed that lymphocytes are organised around the periphery of granulomas in WT mice (Fig. 2.8A). In TNF/LT$^{-/-}$ mice CD3 positive cells appeared in small, tight clusters or encircled small numbers of epitheloid cells (Fig. 2.8B). The central core of granulomas in WT mice stained strongly for the macrophage markers F4/80 (Fig. 2.8C) and ICAM-1 (Fig. 2.8E) whereas these markers were either diminished and less intense or absent in TNF/LT$^{-/-}$ mice (Fig. 2.8D and Fig. 2.8F). The ability to eliminate mycobacterial infection in WT mice was reflected in the strong staining for the enzyme, iNOS (Fig. 2.8G). Granulomas that stained positive for iNOS were few and sparsely-distributed in TNF/LT$^{-/-}$ mice (Fig. 2.8H).

2.5 Cutaneous delayed hypersensitivity to PPD is unaffected in M. bovis BCG infected TNF/LT$^{-/-}$ mice.

The ability of TNF/LT$^{-/-}$ mice to elicit a cell-mediated immune response was tested due to its importance in controlling mycobacterial infection. Differences in footpad swelling were measured in M. bovis BCG infected mice after challenge with PPD at
Figure 2.8
Cellular characterisation of hepatic granulomas in M. bovis BCG infected WT and TNF/LT−/− mice.

WT and TNF/LT−/− mice were infected intravenously with 2x 10^6 cfu of M. bovis BCG. Mice were sacrificed at 28 days post infection and frozen tissue sections were prepared as described in Materials and Methods. Tissue sections were stained with antibodies specific for CD3 (panels A and B), F4/80 (panels C and D), ICAM-1 (panels E and F) and iNOS (panels G and H). Reduced "granuloma"-associated expression of all markers that tested was found in TNF/LT−/− mice. Tissue sections represent 4 mice per strain. The results are representative of 2 similar experiments. (Magnification = 100x)
28 days of infection. The results in Fig. 2.9 indicate that the absence of TNF and LT did not impair the DTH response and there was no significant difference in footpad swelling between the two strains of mice.

2.6 *M. bovis* BCG infected TNF/LT<sup>−/−</sup> mice is resistant to LPS induced endotoxic shock.

*M. bovis* BCG infection renders mice highly sensitive to the effects of endotoxic shock. Endotoxic shock is associated with elevated levels of TNF and was shown to be dependent on IFNγ signalling (Kamijo *et al.*, 1993). To determine whether *M. bovis* BCG infected TNF/LT<sup>−/−</sup> mice develop hyper reactivity, mice were intravenously challenged with 25μg LPS and mortality was followed for 36 hours. The results in Fig. 2.10 shows that *M. bovis* BCG infected WT mice were highly susceptible to LPS and succumbed within 36 hours. In contrast, TNF/LT<sup>−/−</sup> mice remained resistant to LPS despite being sensitised with *M. bovis* BCG.

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*M. bovis* BCG infection in TNF/LT<sup>−/−</sup> and LT<sup>−/−</sup> mice – a comparative study.

It was previously shown that TNF and signalling through TNFR1 is imperative for generating a protective cell mediated immune response against *M. bovis* BCG infection (Jacobs *et al.*, 2000; Flynn *et al.*, 1995; Senaldi *et al.*, 1996). Although it is known that both TNF and LT can bind to TNFR1, the contribution of LT to protective immunity was undetermined. In this study the ability TNF/LT<sup>−/−</sup> and LT<sup>−/−</sup> mice to control *M. bovis* BCG infection mice were compared to investigate whether LT is associated with protective immunity.

2.7 LT<sup>−/−</sup> mice are resistant to *M. bovis* BCG infection.

Comparative mortality rates between LT<sup>+</sup> and TNF/LT<sup>−/−</sup> mice after intravenous challenge with 10<sup>6</sup> cfu of *M. bovis* BCG are represented in Fig 2.1. Mice were observed for 120 days and, similar to WT mice, LT<sup>−/−</sup> mice were resistant to infection whereas TNF/LT<sup>−/−</sup> was highly susceptible. This observation was interesting in view
Figure 2.9

Cutaneous delayed hypersensitivity in M. bovis BCG infected WT and TNF/LT<sup>−/−</sup> mice.

WT and TNF/LT<sup>−/−</sup> mice were intravenously infected with 10⁶ cfu of M. bovis BCG. On day 28 post infection mice were challenged with 5μg of PPD in the right hind footpad while the saline was injected into the left hind footpad. Footpad swelling was measured after 42 hours. The data indicate the difference in swelling measurements between the PPD and saline challenged hind footpads. The results represent the average and standard deviation of 5 mice and depicts 1 of 2 experiments with similar findings.

Figure 2.10

Survival of M. bovis BCG infected WT and TNF/LT<sup>−/−</sup> mice after challenge with LPS.

WT and TNF/LT<sup>−/−</sup> mice (n = 8 mice/strain) were intravenously infected with 10⁶ cfu of M. bovis BCG. On day 14 post infection, each mice received 25μg of LPS intravenously. Mortality was monitored for 72 hours. The results represent 1 of 2 similar findings.
of the well-characterised defective phenotype that is associated with the secondary peripheral lymphoid organs in both LT$^{-/-}$ and TNF/LT$^{-/-}$ mice (Eugster et al., 1996; Banks et al., 1995).

2.8 Pathology in *M. bovis* BCG infected LT$^{-/-}$ and TNF/LT$^{-/-}$ mice.

Pathology of the lungs and spleens between LT$^{-/-}$ and TNF/LT$^{-/-}$ mice were compared due to the different susceptibilities that were exhibited by the two mutant strains to *M. bovis* BCG infection. Macroscopic pathology of *M. bovis* BCG infected WT, LT$^{-/-}$ and TNF/LT$^{-/-}$ mice at 56 days post infection is represented in Fig. 2.11. Lungs of WT and LT$^{-/-}$ mice were similar in appearance (Fig. 2.11A and 2.11B), having small lesions notwithstanding the higher significant bacilli load that was observed between the two strains (Fig. 2.12C). In contrast, the lungs of TNF/LT$^{-/-}$ mice were enlarged and its appearance were characterised by large multiple lesions that were spread throughout the lungs (Fig. 2.11C). Similarly, spleens of WT and LT$^{-/-}$ mice were of similar size (Fig. 2.11D and 2.11E) whereas spleens of TNF/LT$^{-/-}$ mice were enlarged (Fig. 2.11F).

Microscopic analysis of hepatic granuloma formation in LT$^{-/-}$ and TNF/LT$^{-/-}$ mice was compared (Fig. 2.6). Analogous to pathology in WT mice, established granulomas were found in LT$^{-/-}$ mice at 14 days post infection (Fig. 2.6G) but were absent in TNF/LT$^{-/-}$ mice (Fig. 2.6D). In LT$^{-/-}$ mice mature granulomas remained visible at 28 days post infection (Fig. 2.6H) and resolved at 56 days post infection (Fig. 2.6I). In contrast, maturation of granulomas was completely absent in TNF/LT$^{-/-}$ mice (Fig. 2.6E and 2.6F). Structurally, hepatic granulomas of LT$^{-/-}$ mice differed from that of the WT strain. The lymphocytic rim of the granulomas in LT$^{-/-}$ mice appeared enlarged containing a higher cellular content at 14 days and 28 days post infection.

2.9 TNF and LT are both important for the control of *M. bovis* BCG infection.

In this study the relative importance of TNF and LT in protective immunity was addressed by comparing the ability and rate of clearance of bacilli in infected mice. WT, LT$^{-/-}$, TNF$^{-/-}$ and TNF/LT$^{-/-}$ mice were infected intravenously with a dose of $10^6$ cfu of *M. bovis* BCG and the mycobacterial burden was determined in the lungs, liver and spleen over time (Fig. 2.12).
Figure 2.11
Macroscopic pathology of the liver and spleen of *M. bovis* BCG infected WT, TNF/LT<sup>−/−</sup> and LT<sup>−/−</sup> mice.

WT, TNF/LT<sup>−/−</sup> and LT<sup>−/−</sup> mice were intravenously infected with $10^6$ cfu of *M. bovis* BCG. Mice were sacrificed 56 days post infection and the liver and spleen were fixed in phosphate buffered formalin. Captured images represent 4 mice per strain. (Magnification = 2.5x)
The pulmonary bacilli burden (Fig 2.12C) was not significantly different in WT, LT<sup>−/−</sup> and TNF<sup>−/−</sup> mice at 14 days post infection. The bacilli burden in TNF/LT<sup>−/−</sup> mice however was significantly higher (p < 0.05) (5.7 fold) in comparison to the other three strains. WT mice had a small (<2 fold), but significant increase (p < 0.05) in bacilli burden at 28 days post infection in comparison to 14 days post infection. Significant increases (p < 0.05) of 5.8 fold and 4 fold were also observed in TNF<sup>−/−</sup> mice and TNF/LT<sup>−/−</sup> mice respectively. A comparison of mycobacterial burdens between mouse strains after 28 days of infection did not show a significant difference between WT and LT<sup>−/−</sup> mice. However, the bacilli burden in TNF<sup>−/−</sup> mice and TNF/LT<sup>−/−</sup> mice relative to WT mice were significantly higher (p < 0.05 and p < 0.001 respectively) having a 3.3 fold and 14 fold difference respectively. The bacilli burden in TNF<sup>−/−</sup> mice and TNF/LT<sup>−/−</sup> mice were also significantly higher (p < 0.05 and p < 0.01 respectively) in comparison to LT<sup>−/−</sup> mice, the difference being 4.2 fold and 18 fold respectively. In turn, the results showed that the pulmonary bacilli burden of TNF/LT<sup>−/−</sup> mice were significantly higher (p < 0.01) than TNF<sup>−/−</sup> mice by a 4.3 fold difference at 28 days post infection. TNF<sup>−/−</sup> mice and TNF/LT<sup>−/−</sup> mice had a 3.3 fold and 12 fold significant increase (p < 0.01) in their respective bacilli loads at 56 days post infection relative to 28 days post infection. The bacilli burden of LT<sup>−/−</sup> mice remained undifferentiated whereas a 6 fold significant decrease (p < 0.0001) was observed in WT mice. Hence, all three mouse strains had significantly higher (TNF<sup>−/−</sup> mice: p < 0.0005, LT<sup>−/−</sup> mice: p < 0.01, TNF/LT<sup>−/−</sup> mice: p < 0.0005) mycobacterial loads relative to WT mice. Differences in bacilli burden were 9 fold, 63 fold and 1000 fold for LT<sup>−/−</sup> mice, TNF<sup>−/−</sup> mice and TNF/LT<sup>−/−</sup> mice respectively.

Significant clearance was observed in the hepatic mycobacterial burden (Fig. 2.12A) of WT mice (p < 0.005) and LT<sup>−/−</sup> mice (p ≤ 0.001) at 28 days post infection when compared to 14 days post infection. In contrast, the bacilli burden of TNF<sup>−/−</sup> mice and TNF/LT<sup>−/−</sup> mice at 28 days post infection did not deviate significantly from levels obtained at 14 days post infection. Thus, a comparison of bacilli burden at 28 days post infection showed that relative to WT mice, significantly higher levels (p < 0.01) were found in TNF<sup>−/−</sup> mice (4 fold) and in TNF/LT<sup>−/−</sup> mice (11 fold). These significant differences (p < 0.01) were also found in relation to LT<sup>−/−</sup> mice with TNF<sup>−/−</sup> mice and TNF/LT<sup>−/−</sup> mice having a 3 fold and 8 fold higher bacilli burden, respectively. The
Figure 2.12
Comparative mycobacterial burden in the liver, spleen and lungs of WT, TNF\(^\text{--}\), TNF/LT\(^\text{--}\) and LT\(^\text{--}\) mice after M. bovis BCG infection.

WT, TNF\(^\text{--}\), TNF/LT\(^\text{--}\) and LT\(^\text{--}\) mice were intravenously infected with 10\(^6\) cfu of M. bovis BCG. Mice were sacrificed at 14 days, 28 days and 56 days post infection and the bacilli burdens in the liver (panel A), spleen (panel B) and lungs (panel C) were assessed as described in Materials and Methods. Each time point represents the average ± SD of 4 mice per strain.
bacilli burden in WT mice and LT\(^{+/}\) mice were comparable and the differences not significant. However, TNF/LT\(^{+/}\) mice had a 2.7 fold significantly higher bacilli load than TNF\(^{+/}\) mice. The bacilli load in LT\(^{+/}\) mice and in TNF/LT\(^{+/}\) mice remained unchanged at 56 days post infection. In contrast, significant clearance was observed in WT mice (p < 0.01) (2.7 fold) and in TNF\(^{+/}\) mice (p < 0.05) (2.7 fold). Comparatively the hepatic bacilli loads of all three gene deficient mouse strains were significantly higher than the WT strain (TNF\(^{+/}\) mice: p < 0.05 (4 fold), LT\(^{+/}\) mice: p < 0.05 (4 fold), TNF/LT\(^{+/}\) mice: p < 0.01(48 fold)). LT\(^{+/}\) mice and TNF\(^{+/}\) mice had similar bacilli burdens that were not significantly different. However, the mycobacterial burden in TNF/LT\(^{+/}\) mice, relative to the other two mutant strains, was 12 fold higher with a significance of p < 0.05.

Protective immunity in the spleens (Fig. 2.12B) of WT mice and LT\(^{+/}\) mice was reflected in the significantly lower (p < 0.001) mycobacterial burdens that was observed at 28 days post infection in comparison to 14 days post infection. In contrast, ineffective control was evident in the significantly higher (p < 0.005) bacilli burdens of TNF\(^{+/}\) mice and TNF/LT\(^{+/}\) mice. The splenic bacilli burdens of the three gene deficient strains however, were still significantly higher than that of WT mice (LT\(^{+/}\) mice: p < 0.005, TNF\(^{+/}\) mice: p < 0.005, TNF/LT\(^{+/}\) mice: p < 0.01). In relation to LT\(^{+/}\) mice, both TNF\(^{+/}\) mice and TNF/LT\(^{+/}\) mice had significantly higher (p < 0.05 and p < 0.01 respectively) bacilli levels at day 28 post infection. In turn the bacilli burden of TNF/LT\(^{+/}\) mice were significantly higher (p < 0.05) than TNF\(^{+/}\) mice. Effective elimination of bacilli occurred in WT mice and TNF\(^{+/}\) mice at 56 days post infection as witnessed by the significantly lower (p < 0.01) bacilli loads in both strains. Although the bacilli burden of LT\(^{+/}\) mice did not alter significantly, the bacilli burden in TNF/LT\(^{+/}\) mice increased significantly (p < 0.01). TNF\(^{+/}\) mice and LT\(^{+/}\) mice had similar mycobacterial burdens that were 5.4 fold higher (p < 0.05) than that of WT mice. The difference in bacilli load between TNF/LT deficient and WT mice were 71 fold with a significance of p < 0.0005. The bacilli load of TNF/LT deficient was also significantly higher (p < 0.001) than both LT\(^{+/}\) mice and TNF mice.

Similarly, pulmonary pathology in LT\(^{+/}\) mice mirrored that of WT mice (Fig. 2.7). Granulomas were visible at 28 days (Fig. 2.71) and 56 days post infection (Fig. 2.71) in LT\(^{+/}\) mice. Unlike the extensive degree of alveolitis and tissue necrosis that was
observed in TNF/LT$^{-/-}$ mice (Fig. 2.7F), the lung composition of WT (Fig. 2.7C) and LT$^{-/-}$ mice (Fig. 2.7I) constituted areas of large alveolar spaces.
Discussion

In this study the combined effects of TNF and LT in the host immune response against *M. bovis* BCG were evaluated. The findings conclude that the absence of TNF and LT was fatal on challenge with *M. bovis* BCG. The susceptibility of TNF/LT<sup>−/−</sup> mice was associated with severely delayed hepatic granuloma formation and the absence of an organized pulmonary granulomatous response, which results in uncontrolled mycobacterial growth. In addition, immunohistochemical staining of tissues indicated that the quality of granulomas in TNF/LT<sup>−/−</sup> mice was inferior to that of their WT counterparts.

TNF/LT<sup>−/−</sup> mice display a complete disruption of the TNF/LT system, hence cannot mediate signaling through TNFR1 and TNFR2 nor mediate LT-dependent LTβR signaling. Previous studies in which soluble TNFR1 (Senaldi et al., 1996) or TNFR1<sup>−/−</sup> mice (Senaldi et al., 1996; Flynn et al., 1995<sup>a</sup>) were used, have indicated a role for either TNF or LT, or both molecules in protective immunity against mycobacterial infection since both these cytokines can act as binding ligands for TNFR1. Controversy exists regarding the mutual redundancy of these two molecules due to its binding affinities for the same receptors. TNF and LT share common biological properties e.g. both cytokines induce proinflammatory effects and can regulate leukocyte trafficking (Sedgwick et al., 2000, Bazzoni et al., 1995; Borgstrom et al., 1997; Pober et al., 1987). Conclusive evidence in favour of distinct biological activities for each cytokine was reported in an autoimmune inflammation mouse model (Sean-Riminton et al., 1998). Further support for separate functional roles were found in two infection studies, which employed mutant mice defective for each of these cytokines. In both studies a virulent strain of *M. tuberculosis* i.e. H37Rv was used as the infecting agent. Bean et al. (1999) reported that TNF<sup>−/−</sup> mice were highly susceptible and succumbed to infection. In their studies LT could not compensate for the absence of TNF. Likewise, Roach et al. (2001) reported that virulent H37Rv was fatal in the absence of LT and that TNF could not compensate for it absence. The finding in this study of a higher susceptibility exhibited by TNF/LT<sup>−/−</sup> mice in comparison to TNF<sup>−/−</sup> or LT<sup>−/−</sup> mice to mycobacterial infection indicated that the absence of both cytokines results in an additive effect. It therefore is in agreement
with the postulate that TNF and LT have exclusive properties in the generation of protective immunity against *M. bovis* BCG.

The observation that LT<sup>−/−</sup> mice could control mycobacterial growth sufficiently to survive the experimental period was novel. In view of the findings of Roach *et al.* (2001) who showed that mice, which were deficient for LT, were highly susceptible to *M. tuberculosis* H37Rv infection, the ability of LT<sup>−/−</sup> mice to resist *M. bovis* BCG infection suggested that the influence of LT in generating protective immunity is dependent on strain virulence. The data may also reflect a partial redundant functional role for TNF in the absence of LT during an avirulent mycobacterial infection, which would suggest that although mutually exclusive functions could be attributed to each of these cytokines, their functions were not absolutely exclusive. More recently Bopst *et al.* (2001) have demonstrated that the reintroduction of LT as a transgene into TNF/LT<sup>−/−</sup> mice significantly increased survival after *M. bovis* BCG infection. The observation that the introduction of LT could not fully substitute for the absence of TNF further supports the theory of distinctive functions for each cytokine in *M. bovis* BCG infection.

The capacity to control *M. bovis* BCG infection was compared between different mutant strains. Interestingly both TNF deficient and LT<sup>−/−</sup> mice displayed partial cytokine dependent protection in the liver and the spleen to a similar extent. However the difference in susceptibility between the TNF<sup>−/−</sup> mice and LT<sup>−/−</sup> mice was reflected in their ability to control pulmonary mycobacterial growth. In contrast to LT<sup>−/−</sup> mice, TNF<sup>−/−</sup> mice failed to initiate an early pulmonary protective immune response and succumbed to infection (Jacobs *et al.*, 2000<sup>6</sup>). LT<sup>−/−</sup> mice displayed partial control of pulmonary mycobacterial growth, sufficient to survive the infection. Conversely, TNF/LT<sup>−/−</sup> mice failed to control bacilli growth in any of the organs that were investigated. This may imply that the TNF independent control of mycobacterial growth that is observed in the spleens and livers of TNF<sup>−/−</sup> mice could be attributed to LT dependent immune responses and *visa versa* for LT<sup>−/−</sup> mice. Nevertheless, the higher bacilli burdens TNF/LT<sup>−/−</sup> mice in comparison to either TNF deficient or LT<sup>−/−</sup> mice again confirm that both cytokines are involved in protective immunity. However, a comparison of the bacilli burdens amongst all three strains suggested that
in pulmonary immunity, although both TNF and LT are required for protective immunity, TNF is more important than LT.

The differences in susceptibility between TNF/LT$^{-/-}$ and LT$^{-/-}$ mice also addressed the importance of the peripheral lymphoid developmental abnormalities that are associated with these two mutant strains. Mice, which are defective for the expression of LT, lack lymphnodes, Peyers patches and do not form proper germinal centres. The importance of the lymphoid deficiencies of LT$^{-/-}$ mice in generating proper immune responses is still unresolved. Previous viral infection studies have suggested that an intact lymphoid system is required for inducing effective immunity (Eugster et al., 1996; Berger et al., 1999). In contrast, Kumaraguru et al. (2001) who showed that LT$^{-/-}$ mice induced normal recruitment of CD8$^+$ T-cells on challenge with HSV demonstrated that a competent immune response could be generated in the absence of LT. Further support was provided by the investigations of Gajewska et al. (2001). In their study the authors showed that a properly constituted Th2 response could be induced in an experimental allergic airways model in the absence of lymphnodes. Differences in susceptibilities between TNF/LT$^{-/-}$ and LT$^{-/-}$ mice, at least against M. bovis BCG infection, suggested that the impaired development of secondary lymphoid organs did not have a determining influence.

The underlying mechanism of the susceptibility of TNF/LT$^{-/-}$ mice to M. bovis BCG infection is its inability to establish defined granulomas during early infection. In this study the quality of granulomas was severely compromised. In comparison to TNF$^{-/-}$ mice (Jacobs et al., 2000) the granulomatous response in TNF/LT$^{-/-}$ mice was inhibited to a greater extent. Interestingly, the absence of LT had a limited impact on the structure of granuloma formation with the development of granulomas occurring with kinetics comparable to that in WT mice. The enhanced lymphocytic rim of granulomas in LT$^{-/-}$ mice could have been due to the larger perivascular lymphocytic infiltrate, which is normally resident in tissues. Nevertheless, differences in the kinetics of granuloma formation between TNF$^{-/-}$ and LT$^{-/-}$ mice indicated that TNF assumed a more prominent role in the induction of granuloma formation. The differences in the granulomatous response between TNF$^{-/-}$ and TNF/LT$^{-/-}$ mice would suggest that both TNF and LT contribute to the establishment of well-demarcated granulomas. It is therefore interesting to speculate on possible cooperative roles for
TNF and LT in the generation and maintenance of granulomas. It is plausible that TNF is required for the rapid initiation of granulomas and that although this could partially be substituted for by LT, possibly through its shared signaling ability through TNFR1, the process is delayed. However, despite the early establishment of granulomas in LT<sup>−/−</sup> mice, only partial clearance of the hepatic bacilli load was noted. This indicated that the bactericidal capabilities of these granulomas were impaired even though the granulomas were structurally similar in appearance to that of WT mice.

TNF and LT are both considered to be potent proinflammatory cytokines and can induce the expression of molecules which are involved in leukocyte trafficking (Sacca et al., 1998). VCAM, ICAM, E-selectin and MadCAM were upregulated by LT and TNF in culture (Cuff et al., 1998; Cuff et al., 1999; Sikorski et al., 1993). Further Borgstrom et al. (1997) demonstrated that LT dependent regulation of E selectin, P selectin and β2 Integrin stimulate rolling, adhesion and transmigration of leukocytes in angiogenic vessels. Similarly, Hickey et al. (1997) showed that TNF treatment significantly increased rolling and adhesion in feline mesenteric venules. It would therefore be reasonable to conclude that the absence of both these cytokines would restrict the expression of these molecules considerably and thereby inhibiting proper recruitment of cells to sites of infection. Indeed, Eugster et al. (1996) have shown that TNF/LT<sup>−/−</sup> mice are defective for the expression of VCAM, ICAM and Mac-1 expression. In accord with this finding, the results of this study indicated that TNF/LT<sup>−/−</sup> mice had reduced granulomatous associated ICAM expression on challenge with <i>M. bovis</i> BCG. It can therefore be concluded that the severely limited granulomatous response was, at least in part, the consequence of an inadequate inflammatory response which was caused by the failure to induce adhesion molecules during acute mycobacterial infection in the absence of TNF and LT. Several other cytokines have been positively associated with tuberculous granulomas (Bergeron et al., 1997) of which IL-1β, IFNγ, GM-CSF and IL-12 showed increase levels of expression. These findings are supported by <i>M. tuberculosis</i> infection studies in IFNγ deficient mice (Flynn et al., 1993; Kamijo et al., 1993) and IL-12<sup>−/−</sup> mice (Cooper et al., 1997), which showed that these cytokines were required for a proper granulomatous response. Notwithstanding the importance of these cytokines in Th1
type granuloma formation, none could substitute for the combined absence of TNF and LT. The severely impaired granulomatous response was accentuated by limited cellular recruitment, which included both lymphocytes and macrophages and was reflected in the reduced presence of CD3 and the macrophage maturation marker F4/80. In addition was the bactericidal activity of the rudimentary granulomatous lesions profoundly inhibited as represented by its low iNOS content.

Previously septic shock was described in patients with active tuberculosis and was contributed to elevated levels of systemic TNF (George et al., 1996). Similarly, in this study, mice were sensitized with \textit{M. bovis} BCG and the observation that TNF/LT \textsuperscript{-/-} mice were resistant to LPS challenge supports established data that TNF induces endotoxic shock. Whether the absence of LT contributes to resistance in this mouse model is not known.

In conclusion, this study has unequivocally demonstrated a role for both TNF and LT in \textit{M. bovis} BCG infection. However research into its kinetic expression and metabolic regulation will greatly enhance the understanding of the relative contribution of each cytokine during mycobacterial pathogenesis.
Mycobacterium bovis BCG infection in IL-10^{-/-} mice.
Summary

In this study the importance of the modulatory cytokine, Interleukin 10 (IL-10), during *Mycobacterium bovis* BCG infection was addressed. Similar to WT mice, IL-10^-/-_ mice were resistant to intravenous challenge with *Mycobacterium bovis* BCG. However, IL-10^-/-_ mice manifested an elevated protective immune response. Determination of bacilli burden in IL-10^-/-_ mice showed accelerated clearance in the lungs, spleen and the liver in comparison to WT mice. This was confirmed by significantly lower numbers of AFB that was detected under high magnification in the livers of IL-10^-/-_ mice. Accelerated mycobacterial clearance was accompanied by a vigorous granulomatous response. IL-10^-/-_ mice induced significantly higher numbers of granulomas which were enlarged and had an increased cellular content. Further characterisation revealed that granulomas of IL-10^-/-_ mice had an increased lymphocytic content and displayed enhanced expression of the macrophage activation markers F4/80, MHC class II, acid phosphatase and iNOS. Upregulation of granuloma associated adhesion molecules was also detected. Down modulation of the inflammatory cellular response was inhibited in the absence of IL-10. Both WT and IL-10^-/-_ mice showed significant increases in lung and spleen weights during the acute stage of infection. However, in contrast to WT mice, which showed significant reduction in lung and spleen weights during the later stage of infection, significant reduction of organ weights could not be measured in IL-10^-/-_ mice. Further it was found that resolution of hepatic granulomas was delayed in IL-10^-/-_ mice subsequent to mycobacterial clearance. Elevated plasma cytokine levels of IL-12 and TNF distinguished the enhanced protective immune response in IL-10^-/-_ mice. On challenge with PPD it was found that the absence of IL-10 did not influence memory recall. In conclusion, this study has shown that cell mediated immunity is enhanced in the absence of IL-10 which is associated with a vigorous granulomatous response that induces accelerated clearance of infection. The results therefore support an attenuated role for IL-10 during *Mycobacterium bovis* BCG infection.
Results

3.1 IL-10<sup>−/−</sup> mice are resistant to M. bovis BCG infection.
The susceptibility of WT and IL-10<sup>−/−</sup> mice was investigated by intravenous challenge with 1x10<sup>6</sup> cfu M. bovis BCG (Fig. 3.1). Groups of 12 mice were observed for 120 days after infection. IL-10<sup>−/−</sup> mice were completely resistant to M. bovis BCG infection. A 10-fold increase in the infection dose (1x10<sup>7</sup> cfu M. bovis BCG) did not induce mortality. Mice did not show classical symptoms associated with progressive infection such as weight loss, impeded movement, hunched posture or roughed fur. Similarly, WT control mice survived the experiment.

3.2 Enhanced clearance of M. bovis BCG infection in IL-10<sup>−/−</sup> mice.
WT and IL-10<sup>−/−</sup> mice were intravenously infected with 1x10<sup>6</sup> cfu of M. bovis BCG and the course of infection was followed for 56 days (Fig. 3.2). There was not a significant difference in the mycobacterial uptake between WT and IL-10<sup>−/−</sup> mice in any of the organs that were examined as assessed by the bacilli burdens on day 1 after infection.

The mycobacterial burden in the livers (Fig. 3.2A) of IL-10<sup>−/−</sup> mice after 14 days was similar to the initial uptake as determined on day 1. In contrast, WT mice had an increased hepatic mycobacterial burden at 14 days post infection, which was significantly higher (p < 0.01) than that of IL-10<sup>−/−</sup> mice. Both mouse strains showed a significant reduction (p < 0.0001) in hepatic mycobacterial burdens at 28 days compared to 14 days post infection. There was however no significant difference in the hepatic mycobacterial burden between the two strains. The kinetic rates of clearance in both strains were decreased between 28 days and 56 days after infection. Nevertheless, there was a further significant decrease (p < 0.01) in the hepatic mycobacterial burdens in both strains at 56 days when compared to 28 days post infection. In addition, the hepatic mycobacterial burden in IL-10<sup>−/−</sup> mice was significantly less (p < 0.05) in comparison to WT mice at 56 days post infection.

The splenic mycobacterial burden (Fig. 3.2B) in WT and IL-10<sup>−/−</sup> mice increased significantly (p < 0.01) after 14 days of infection. IL-10<sup>−/−</sup> mice however, had a
Figure 3.1
Survival of *M. bovis* BCG infected WT and IL-10−/− mice.

WT mice and IL-10−/− mice were intravenously infected with 10⁸ cfu of *M. bovis* BCG and were monitored for mortality. The data represents the percentage of surviving mice during the course of infection. Each group consisted of 12 mice per strain. The results are representative of 3 similar experiments.
Figure 3.2
*Mycobacterial burden in M. bovis BCG infected WT and IL-10-/- mice.*

WT mice and IL-10-/- mice were intravenously infected with $10^6$ cfu of *M. bovis* BCG and were sacrificed at the indicated time points. Bacilli burdens in the (panel A) liver, spleen (panel B) and lungs (panel C) were determined as described in Materials and Methods. The data shows an enhanced rate of bacilli clearance in IL-10-/- mice. Each time point indicates the average ± SD of 4 mice. The results are representative of 2 similar experiments.
significantly lower (p < 0.05) splenic mycobacterial load at 14 days post infection when compared to WT mice. Effective clearance in both strains resulted in significantly lower (WT = p < 0.01; IL-10^{-/-} = p < 0.001) mycobacterial loads in the spleen at 28 days post infection with no significant difference between the two strains. The mycobacterial loads in both strains remained static after 28 days with no significant differences being detected at 56 days post infection.

The pulmonary mycobacterial burden (Fig. 3.2C) in WT and IL-10^{-/-} mice increased significantly (p < 0.01) after 14 days of infection. The mycobacterial burden in IL-10^{-/-} mice was however, significantly lower (p < 0.05) in IL-10^{-/-} mice. The mycobacterial burden in both strains were equivalent at 14 days and 28 days post infection and maintained the statistical difference (p < 0.01). There was however a significant (p < 0.01) reduction in the pulmonary mycobacterial burden in both strains at 56 days when compared 14 days post infection. A comparison of WT and IL-10^{-/-} mice revealed that IL-10 mice had significantly fewer (p < 0.05) mycobacteria in the lungs than WT mice at 56 days post infection.

3.3 *M. bovis* BCG infected IL-10^{-/-} mice have an enhanced immune response.

In previous infection studies with *L. monocytogenes* and *T. cruzi* the absence of IL-10 were associated with an increase in the inflammatory cytokines IL-12, TNF, IFNγ and IL-1α (Dai et al., 1997; Holscher et al., 2000). To determine if the absence of IL-10 enhances the Th1 response against *M. bovis* BCG, plasma IL-12, TNF and IFNγ levels were measured at the indicated time points during infection (Fig. 3.3). The plasma concentrations of IL-12 (Fig. 3.3A) were comparable after 1 day of infection in the two mouse strains but increased significantly in WT mice (p < 0.01) and in IL-10^{-/-} mice (p < 0.05) after 14 days of infection. There was however not a significant difference between the mouse strains. A further significant increase (p < 0.05) in IL-12 plasma concentrations were observed in WT mice and IL-10^{-/-} mice at 28 days post infection in comparison to 14 days post infection. A comparison of IL-12 plasma concentrations revealed significantly higher (p < 0.05) IL-12 levels in IL-10^{-/-} mice. The high IL-12 plasma concentrations were maintained at 56 days post infection with a significantly higher level (p < 0.05) being confirmed in IL-10^{-/-} mice. Plasma TNF concentrations (Fig. 3.3B) of IL-10^{-/-} mice were significantly higher (p < 0.05) than
Figure 3.3
Cytokine production in *M. bovis* BCG infected WT and IL-10<sup>−/−</sup> mice.

WT mice and IL-10<sup>−/−</sup> mice were intravenously infected with 10<sup>6</sup> cfu of *M. bovis* BCG and sera were collected at the indicated time points. Cytokine concentrations were determined by ELISA as described in Materials and Methods. The results represent the average ± SD of 4 mice per strain and denote 1 of 2 experiments with similar findings.
that of WT mice at 28 days post infection. There was however not a significant difference in plasma TNF concentrations between WT and IL-10<sup>−/−</sup> mice at 56 days post infection. Although IFNγ could be measured in the sera of both WT and IL-10<sup>−/−</sup> mice, cytokine concentrations were not significantly different (Fig. 3.3C).

### 3.4 Persistent inflammation subsequent to mycobacterial clearance in <i>M. bovis</i> BCG infected IL-10<sup>−/−</sup> mice.

It has been established that IL-10 has potent anti-inflammatory properties (Tan <em>et al.</em>, 1995; Ameixa and Friedland, 2001). To determine the effect of infection in the absence of IL-10, organ weights were measured as an indication of inflammation. WT and IL-10<sup>−/−</sup> mice were matched for age, gender and weight and intravenously infected with 1x10<sup>6</sup> cfu <i>M. bovis</i> BCG. Mice were sacrificed at the indicated time points and the weights of the lungs and spleens were compared (Fig 3.4).

Organ weights of WT mice and IL-10 were not significantly different after 1 day of infection. Spleen weights (Fig 3.4A) of both mouse strains increased significantly after 14 days of infection (WT = p < 0.05, IL-10<sup>−/−</sup> = p < 0.01) but there was no significant difference between the two strains. In WT mice, spleen weights remained constant at 28 days after infection and were not significantly different to the weights measured after 14 days of infection. IL-10<sup>−/−</sup> mice however, had significantly higher (p < 0.05) spleen weights at 28 days post infection in comparison to 14 days post infection. The comparable spleen weights of IL-10<sup>−/−</sup> mice were thus significantly higher (p < 0.0001) than that of their WT littermates at 28 days post infection. WT mice displayed significantly (p < 0.01) reduced spleen weights at 56 days post infection in comparison to 28 days after infection. Contrary to the findings in WT mice, IL-10<sup>−/−</sup> mice maintained their spleen weights to that of 28 days post infection. This resulted in significantly higher (p < 0.01) spleen weights in IL-10<sup>−/−</sup> mice in comparison to WT mice.

The lung weights (Fig. 3.4B) in both strains were unaffected at 14 days post infection. However, the lung weights in WT and IL-10<sup>−/−</sup> mice increased significantly (p < 0.01) at 28 days post infection compared to 1 day after infection but no significant difference between the two strains was observed. A significant decrease (p < 0.05) in
Figure 3.4
Organ weights in *M. bovis* BCG infected WT and IL-10−/− mice.

WT mice and IL-10−/− mice were intravenously infected with 10⁶ cfu of *M. bovis* BCG and were sacrificed at the indicated time points. The spleen (panel A) and lungs (panel B) were removed and the weights recorded. Each data point represents the average ± SD of 4 mice. The results are representative of 2 similar experiments.
lung weights could be demonstrated in WT mice but a similar decrease was absent in IL-10−/− mice at 56 days post infection.

3.5 Enhanced granuloma formation in *M. bovis* BCG infected IL-10−/− mice.

Hepatic granuloma formation was investigated in WT and IL-10−/− mice due to the enhanced rate of mycobacterial clearance in IL-10−/− mice (Fig. 3.5). Microscopic tissue sections were compared and analysed at 14 days, 28 days and 56 days subsequent to *M. bovis* BCG infection. Typical granulomas were already evident in both WT and IL-10−/− mice at 14 days post infection with no discernible difference in pathology between WT and IL-10−/− mice (Figs. 3.5A and 3.5D). Granulomas were characterised by a central core of activated epithelioid macrophages surrounded by lymphocytes. Hepatic morphology in IL-10−/− mice (Fig. 3.5E) differed from WT mice (Fig. 3.5B) with respect to the following at 28 days post infection: (a) IL-10−/− mice had an increase in size of granulomas (b) granulomas in IL-10−/− mice had a higher cellularity and (c) a higher degree of cellular infiltrate was present in IL-10−/− mice as evident by the predominance of peri-vascular cuffing. Hepatic pathology in WT mice were distinguished by the presence of smaller, resolving granulomas at 56 days post infection (Fig 3.5C). In contrast, IL-10−/− mice maintained morphology similar to that which was observed at 28 days post infection. Granulomas in IL-10−/− mice were larger in size with evidence of continual inflammation (Fig. 3.5F).

Semi-quantitative assessment of hepatic granuloma formation was done at 14 days post infection. This indicated that the number of granulomas were significantly higher (p < 0.001) in the absence of IL-10 (Fig. 3.6A). In addition, the number of AFB under a 1000x magnification was determined in liver sections. AFB were located within the central epithelioid macrophage core of the granulomas (Fig 3.7) and the number of bacilli per microscopic field was significantly lower (p < 0.05) in IL-10−/− mice in comparison to WT mice (Fig. 3.6B).

In contrast to WT mice, IL-10−/− mice did not manifest resolution of hepatic granulomatous lesions subsequent to mycobacterial clearance. Rather, mature granulomas persisted, being structurally similar at both 28 days and 56 days post infection. To quantitatively verify the differences that was observed in granulomas between WT and IL-10−/− mice, the area size of 100 randomly chosen granulomas
WT and IL-10⁻/⁻ mice were infected with 10⁶ cfu of *M. bovis* BCG and sacrificed at 14 days, 28 days and 56 days post infection. Livers were fixed in PBS buffered formalin and embedded in paraffin wax. Tissue sections were stained with hematoxylin and eosin. Well-defined granulomatous structures were formed at 14 days post infection in both WT and IL-10⁻/⁻ mice (panels A and D, respectively). Mature granulomas in IL-10⁻/⁻ mice were enlarged (panel E) in comparison to WT mice (panel B). In contrast to WT mice which showed vestiges of well-defined granulomas (panel C) at 56 days post infection, granulomas of IL-10⁻/⁻ mice remained enlarged (panel F). Tissue sections are representative of 4 mice/strain. (Magnification = 100x)
Granuloma formation in *M. bovis* BCG infected WT and IL-10⁻/⁻ mice.

WT mice and IL-10⁻/⁻ mice were intravenously infected with 10⁶ cfu of *M. bovis* BCG and were sacrificed at 14 days post infection. Livers were transferred to phosphate-buffered formalin, sectioned and stained with hematoxylin and eosin or with Ziehl-Neelson stain. The data indicates the number of granulomas (panel A) and AFB (panel B) that were counted per microscopic field at 400x and 1000x magnification, respectively. 20 microscopic fields were counted for each mouse and the data represents the average ± SD of 4 mice per strain. The results are representative of 2 similar experiments.

AFB in the livers of *M. bovis* BCG infected WT and IL-10⁻/⁻ mice.

WT and IL-10⁻/⁻ mice were infected with 10⁶ cfu of *M. bovis* BCG and sacrificed at 14 days and 56 days post infection. Liver sections were stained with Ziehl-Neelson stain to detect AFB. The number of AFB present within granulomas was similar in both WT and IL-10⁻/⁻ mice panels (panels A and C, respectively). However the number of granulomas, which contained AFB, was significantly fewer in IL-10⁻/⁻ mice in comparison to WT mice. Despite displaying delayed resolution, granulomas of IL-10⁻/⁻ mice at 56 days post infection (panel D), similarly to WT mice (panel B), were bactericidal as indicated by the absence of AFB. Tissue sections represent 4 mice per strain. (Magnification = 400x)
representing 4 mice/strain were measured at 56 days post infection. Fig 3.8 shows that the average granuloma size of IL-10⁻/⁻ mice was significantly larger (p < 0.0001) than the granulomas which were measured in WT mice.

3.6 Increased lymphocytic recruitment, macrophage activation and adhesion molecule expression in the granulomatous lesions of M. bovis BCG infected IL-10⁻/⁻ mice.

The lymphocytic composition, macrophage activation and expression of adhesion molecules of the granulomas in IL-10⁻/⁻ mice were examined as indicators of bactericidal efficacy. The recruitment of T-cells during the establishment of pulmonary and hepatic granulomas was enhanced as indicated by the increased staining for CD3 in the absence of IL-10 (Fig. 3.9). Staining for F4/80 positive macrophages in liver tissue sections revealed an enhanced recruitment to granulomas in IL-10⁻/⁻ mice (Fig. 3.10). The activation of epitheloid macrophages within granulomas was assessed by staining for MHC Class II expression, iNOS expression and acid phosphatase activity. Hepatic granulomas showed increased expression of MHC Class II, iNOS and acid phosphatase activity (Fig. 3.10) and pulmonary granulomas lesions had heightened expression of MHC Class II and iNos (Fig. 3.11).

The expression of the adhesion molecules ICAM-1, CD11b and CD11c were investigated due to the increase in cellular recruitment in the absence of IL-10. All three markers showed enhanced expression in hepatic granulomas of IL-10⁻/⁻ mice in comparison to WT mice (Fig. 3.12).

3.7 Delayed Type Hypersensitivity responses in IL-10⁻/⁻ mice and WT mice are similar.

To test whether the accelerated rate of mycobacterial clearance could be attributed to differences in cell-mediated responses, M. bovis BCG infected WT and IL-10⁻/⁻ mice were challenged with PPD after 28 days. Both mouse strains exhibited a DTH response as determined by differences in footpad swelling after 42 hours (Fig. 3.13). There was however no significant difference in the footpad swelling of WT and IL-10⁻/⁻ mice.
Figure 3.8
Measurement of granuloma size in M. bovis BCG infected WT and IL-10⁻/⁻ mice.

WT mice and IL-10⁻/⁻ mice were intravenously infected with 10⁶ cfu of M. bovis BCG and were sacrificed at 56 days post infection. Livers were transferred to phosphate-buffered formalin, sectioned and stained with hematoxylin and eosin. The data represents the average granuloma area size of 100 randomly chosen granulomas that were measured at 400x magnification. The data represent 4 mice per strain and denotes 1 of 2 experiments with similar findings.

Figure 3.9
T-cell recruitment in M. bovis BCG infected WT and IL-10⁻/⁻ mice.

WT and IL-10⁻/⁻ mice were infected with 10⁶ cfu of M. bovis BCG and sacrificed at 28 days post infection. Cryosections of lung and liver tissue were prepared and stained with anti-CD3 antibodies. IL-10⁻/⁻ mice (right panel) displayed an increase in recruitment of T-lymphocytes to sites of infection in both lung and liver tissue in contrast to WT mice (left panel). Tissue sections are representative of 4 mice per strain. (Magnification = 400x)
Figure 3.10

*Macrophage recruitment and activation in the livers of M. bovis BCG infected WT and IL-10^+ mice.*

WT and IL-10^+ mice were infected with 10^6 cfu of *M. bovis* BCG and sacrificed at 28 days post infection. Cryosections were prepared as described in Materials and Methods and stained with antibodies specific for F4/80, MHC class II, acid phosphatase and iNOS. Granuloma-associated expression of the macrophage maturation marker, F4/80 (panel B) was significantly higher in comparison to WT mice (panel A). Macrophage activation in IL-10^+ mice, defined by the expression of MHC class II (panel D), acid phosphatase (panel F) and iNOS (panel H) was significantly higher than in WT mice (panels C, E and G, respectively). Tissue sections are representative of 4 mice per strain. (Magnification = 400x [panels A-D], 100x [panels E-H])
WT and IL-10⁻/⁻ mice were infected with 10⁶ cfu of \textit{M. bovis} BCG and sacrificed at 28 days post infection. Lung cryosections were prepared as described in Materials and Methods and stained with antibodies specific for either iNOS (panels A and B) or MHC class II (panels C and D). Significantly higher induction of iNOS was observed in IL-10⁻/⁻ mice (panel B) in comparison to WT mice (panel A). Similarly, higher levels of MHC class II expression was observed in IL-10⁻/⁻ mice (panel D) in comparison to WT mice (panel C). Tissue sections represents 4 mice per strain. (Magnification = 100x [panels A and B], 400x [panels C and D])

Figure 3.11

Macrophage activation in the lungs of \textit{M. bovis} BCG infected WT and IL-10⁻/⁻ mice.
WT and IL-10⁻/⁻ mice were infected with 10⁶ cfu of *M. bovis* BCG and sacrificed at 28 days post infection. Cryosections were prepared as described in Materials and Methods and stained with antibodies specific for ICAM-1, CD11b and CD11c. Granuloma-associated expression in IL-10⁻/⁻ mice of ICAM-1 (panel B), CD11b (panel D) and CD11c (panel F) were higher in comparison to WT mice (panels A, C and E respectively). Tissue sections are representative of 4 mice per strain. (Magnification = 400x)
Figure 3.13
Delayed type hypersensitivity in M. bovis BCG infected WT and IL-10^- mice.

WT mice and IL-10^- mice were intravenously infected with 10^8 cfu of M. bovis BCG. On day 28 post infection mice were challenged with 5µg of PPD in the right hind footpad whilst the left hind footpad received saline. Footpad swelling was measured after 42 hours. The data represents the difference in swelling between PPD and saline challenged footpads. The data indicates the average ± SD of 5 mice per strain. The results represent 2 experiments with similar findings.
Discussion

Although mycobacterial infection is governed by a Th1 cell-mediated response it also induces a Th2 component. The Th2 response serves as biological regulatory mechanism to control the severity of Th1 mediated systemic and proinflammatory pathological effects. The role of endogenous IL-10 during mycobacterial infection as defined by studies in experimental animals though, is still controversial. Transgenic expression of IL-10 antagonized macrophage function during *M. bovis* BCG infection (Murray *et al.*, 1997) and neutralization of IL-10 enhanced resistance to *M. avium* infection (Denis and Ghaadirian, 1993). Studies that had been done with IL-10" mice were unresolved. In agreement with the above studies Murray and Young (1999) reported an increase in antimycobacterial immunity in IL-10" mice. In contrast Erb *et al.* (1998) and North (1998) reported that the absence of IL-10 had no effect on the immune response.

In this study the role of IL-10 in protective immunity on challenge with *M. bovis* BCG was investigated. IL-10" mice were completely resistant to a high dose of *M. bovis* BCG and survived the experiment. These mice did not manifest any clinically related symptoms evident of physical deterioration or endotoxic shock. This is in contrast to the critical role of IL-10 in controlling TNF production by macrophages subsequent to parasitic infections. *Trypanosoma cruzi* infected IL-10" mice, although able to reduce the parasitaemia load through an increased inflammatory response, succumbed to TNF mediated endotoxic shock (Holscher *et al.*, 2000). The absence of acute toxicity on challenge with *M. bovis* BCG is rather similar to the reported findings of Yang *et al.* (1999) in *Chlamydia trachomatis* infection studies. These authors reported control of infection in IL-10" mice without the development of toxic shock. IL-10" mice were found to be highly sensitive to LPS and succumb after challenge (Berg *et al.*, 1995). Together, these findings suggest that factors which are involved in mediating endotoxic shock are differentially regulated in *M. bovis* BCG and *C. trachomatis* infections as opposed to *T. Cruzi* infection and is influenced by the nature of the pathogen.
IL-10−/− mice were able to control the *M. bovis* BCG infection in all the organs that were investigated but in addition exhibited an enhanced rate of clearance, clearly establishing a regulatory role for IL-10 during mycobacterial infection. The data of Murray and Young (1999) who showed an increase in resistance during early *M. bovis* BCG infection in the absence of IL-10 corroborated the findings in this study. However, the report confined resistance to the liver and spleen. The results of this study extended these findings and demonstrated that increased resistance to mycobacterial infection was also observed in the lungs. The enhanced protective efficacy against *M. bovis* BCG infection in the absence of IL-10 contributes to an impressive list of pathogenic infection studies with similar findings. Kane and Mosser (2001) illustrated that *L. major* infection in IL-10−/− mice was controlled and that the reduced lesion size was associated with a thousand fold fewer parasites. Dai et al. (1997) showed that the earlier clearance of *L. monocytogenes* resulted in reduced tissue damage whereas Yang et al. (1999) illustrated accelerated chlamydial clearance with decreased morbidity in IL-10−/− mice. A contrasting approach to investigate the immunoregulatory role of IL-10 was to study infection in IL-10 transgenic mice. Murray et al. (1997) illustrated that these mice were highly susceptible and succumbed after *M. bovis* BCG infection. Likewise, Groux et al. (1999) demonstrated that *L. monocytogenes* and *L. major* infection led to mortality in IL-10 transgenic mice. From its role as a down modulating cytokine of Th1 cell mediated immunity it was reasonable to deduce that the increase in resistance manifested against *M. bovis* BCG infection in IL-10−/− mice was due to enhanced Th1 induced protective immunity. In several of these infection models the absence of IL-10 resulted in an augmented Th1 cytokine profile thus supporting the hypothesis that an elevated Th1 response underlined the enhanced protective immunity in IL-10−/− mice against *M. bovis* BCG infection. Following infection with the obligate intracellular bacterium *Chlamydia trachomatis* IL-10−/− mice displayed significantly higher IFNγ, IL-12 and TNF production (Yang et al., 1999). Similarly, significantly higher plasma levels of these cytokines were also found in IL-10−/− mice subsequent to infection with *Trypanosoma cruzi* (Holscher et al., 2000). The cytokine response in IL-10−/− mice after infection with *Listeria monocytogenes* was characterised by an enhanced proinflammatory cytokine profile with elevated levels of IL-12, IFNγ, TNF, IL-1 and IL-6 being reported (Dai et al., 1997). In addition it was found that binding
Leishmania amastigotes to macrophages induced IL-10 secretion which inhibited macrophage activation and diminished their production of IL-12 and TNF (Kane and Mosser, 2001). Current literature however fails to provide evidence for an enhanced Th1 cell mediated response on challenge with M. bovis BCG infection in IL-10<sup>-/-</sup> mice. This study, for the first time, shows that IL-12 and TNF production is enhanced in M. bovis BCG infected IL-10<sup>-/-</sup> mice. Although the TNF concentrations were low, it was not expected to rival that induced through parasitic infection in view of the absence of endotoxemia. Similarly, in C. trachomatis infected IL-10<sup>-/-</sup> mice that survived the infection, systemic expression of cytokines were undetectable (Kane and Mosser, 2001). Despite that it could be established that IL-10<sup>-/-</sup> mice secreted IFNγ, this study could not demonstrate a significant difference in plasma levels in comparison to WT mice; in agreement with the findings of Erb et al. (1998) and Murray and Young (1999). This however did not exclude the possibility that the local IFNγ concentrations at sites of infections in IL-10<sup>-/-</sup> mice were higher than their WT counterparts. The requirement of IL-12 for IFNγ secretion has been defined in M. bovis BCG (Wakeham et al., 1998) and M. tuberculosis (Cooper et al., 1997<sup>a</sup>) studies, which demonstrated the necessity for both of these cytokines to resolve mycobacterial infection. A direct correlate was established between IL-10, IFNγ and IL-12 by Gong et al. (1996) in tuberculosis patients when the authors showed that anti-IL10 treatment enhances IFNγ by upregulating IL-12 production. In the present study the higher IL-12 levels argued for an enhanced state of macrophage activation in IL-10<sup>-/-</sup> mice. The activational state of macrophages in M. bovis BCG infected mice was therefore characterized within the confines of granuloma formation. WT mice responded with the early establishment of typical granulomas during M. bovis BCG infection. The higher rate of clearance in IL-10<sup>-/-</sup> mice was associated with a more pronounced granulomatous response that was characterized by an increase in number as well as size of granulomas. The increase in resistance to intracellular pathogens and its association with a prominent granulomatous response is not automatically interrelated as the increased clearance of C. trachomatis in IL-10<sup>-/-</sup> mice occurred in the absence of granuloma formation (Yang et al., 1999). An interesting observation was the decrease in the number of bacilli that was associated with hepatic granulomas in IL-10<sup>-/-</sup> mice. This reflected the higher rate of clearance that was observed in the liver but more significantly, indicated that these granulomas displayed a higher bactericidal
activity than granulomas in the WT mice. Therefore it can be concluded that at least three factors contributed to the higher resistance of IL-10−/− mice during M. bovis BCG infection namely (1) an increase in the number of granulomas that are formed, (2) an increase in the size of the granulomas and (3) an increase in the bactericidal activity of the granulomas. The finding of persistently enlarged granulomas subsequent to mycobacterial clearance in IL-10−/− mice is novel. This observation supports a role for IL-10 in the regulation of granuloma formation during M. bovis BCG infection, probably indirectly through an inhibitory function on granuloma promoting cytokines. Kindler et al. (1989) have shown that TNF is required for the initiation and maintenance of granulomas. Further it was illustrated that IL-10 is directly involved in the inhibition of TNF synthesis (Gerard et al., 1993). It is therefore plausible to suggest that during M. bovis BCG infection the inhibitory effect on TNF secretion is reduced in IL-10−/− mice. The continual presence of cytokines such as TNF could therefore sustain the integrity of the granuloma. Chensue et al. (1995) reported that anti-IFNγ and anti-TNF treatment caused a reduction in granuloma size which were induced by M. tuberculosis PPD coated beads but found that anti-IL-10 treatment did not affect granuloma formation. The authors did however indicate that anti-IL-10 treatment was associated with an increase in IFNγ suggesting that a decrease in Th1 associated cytokines resulted in the inhibition of granuloma formation but that an increase in Th1 associated cytokines did not affect the granulomatous response. The results however have to be interpreted with caution as the absence of any effects that were found with anti-IL-10 treatment could have been due to incomplete neutralization. An interesting corollary for the importance of IL-10 in granulomatous formation was described by Ludviksson et al. (1998) in patients with Wegener granulomatosis. This disease is characterized by the presence of granulomatous inflammatory lesions similar to those associated with a variety of intracellular infections. PBMC cultures from diseased patients exhibited an unbalanced Th1-type cytokine profile with elevated levels of IFNγ and TNF while monocytes from these patients secreted increased levels of IL-12. The addition of IL-10 to PBMC cultures inhibited IFNγ production and hence has been implicated as a possible therapeutic agent in controlling the disease. The relationship between Th1 defining cytokines and IL-10 is autoregulatory. IL-10 secretion is stimulated by cytokines defining a predominantly Th1 cytokine profile which include IL-12, TNF, IL-6 and IL-1
(Daftarian et al., 1996; Wanidwonaran and Strober, 1993; Fiorentino et al., 1991) and in turn is downregulated by IL-10.

Identification of the cellular composition of the enlarged hepatic granulomas in IL-10−/− mice revealed the presence of F4/80 expressing epitheloid macrophages with a surrounding CD3+ lymphocytic rim. The influence of IL-10 on regulating the cellular migration could transpire at several junctions, one of which is chemokine-induced inflammation. Ameixa and Friedland (2001) illustrated that IL-8, a CXC chemokine that has a pivotal role in leukocyte recruitment to areas of granuloma formation in tuberculosis, is down regulated by IL-10 in M. tuberculosis infected monocytes. Following on from this observation Tan et al. (1995) found that IL-10 inhibited the chemotactic response of CD4+ T lymphocytes towards RANTES and IL-8. Increased production and degradation of the extracellular matrix, activated macrophages and the accumulation of chemokines and cytokines characterize chronic inflammation. Hyaluronan, a component of the extra cellular matrix, were shown to control macrophage effector functions such as the secretion of inflammatory chemokines. Horton et al. (1998) found that IL-10 could inhibit hyaluronan-induced expression of MIP-1α, MIP-1β and KC at the level of both transcription and translation. Chemokines, in contrast, also exerts a regulatory effect on IL-10 as illustrated by Byrnes et al. who found that MIP-3β could enhance IL-10 production by activated human peripheral blood monocytes and T lymphocytes.

The importance of specific T regulatory lymphocytes that could influence the Th1 immune response during mycobacterial infection through secretion of suppressive cytokines is unknown. However IL-10 secreting CD4+ T-cells are involved in metabolic regulation of autoimmune diseases and the induction of transplant tolerance (Hara et al., 2001) and therefore is it interesting to speculate if such specific immunosuppressive cell types are involved in the control of granuloma formation. Sebastiani et al. (2001) have described the regulation of chemokine receptor expression on such CD4+ T cells, which specifically releases high levels of IL-10. In contrast to resting cells which had a migratory capacity to most chemokine stimuli, activation caused the down regulation of most of the chemokine receptor expression and resulted in these cells retaining migratory capacity for specific stimuli only.
Inflammation is mediated through the induction of proinflammatory cytokines which regulate adhesion molecule expression. In this study IL-10<sup>−/−</sup> mice had significantly higher lung and spleen weights notwithstanding an accelerated clearance of bacilli. This increase in organ weights is consistent with the concept of a sustained inflammatory response. Similarly, *T. gondii* infected mice IL-10<sup>−/−</sup> mice controlled the infection and displayed liver pathology that was characterized by increased cellular infiltration (Gazzinelli et al., 1996). In line with these observations it was found that ICAM expression associated with granuloma formation on challenge with *M. bovis* BCG was enhanced in the absence of IL-10. This finding is novel and is supported by Shrikant et al. (1995) who found that IL-10 suppresses ICAM expression on rat astrocytes and glial cells, and initiates its effects at the translational and/or post-translational level. It is therefore possible that IL-10 downregulates adhesion molecule expression such as ICAM during *M. bovis* BCG infection. In IL-10<sup>−/−</sup> mice however this suppressive effect is abolished which could contribute to a sustained inflammatory response.

The increase in granuloma-associated expression of MHC Class II, acid phosphatase and iNOS was indicative of enhanced macrophage activation in *M. bovis* BCG infected IL-10<sup>−/−</sup> mice. Macrophage activation and effector functions are either directly or indirectly inhibited in the presence of IL-10. It therefore follows that the absence of IL-10 could lead to elevated macrophage functional activity. Supporting this hypothesis it was established that both soluble (Park and Skerret, 1996) and membrane expressed IL-10 (Fleming and Campbell, 1996) suppressed macrophage/monocyte bactericidal activity which is abolished after neutralization. Further it was found that IL-10 inhibits the expression of MHC class II (Frei et al., 1994; Flores et al., 1994) and B7 (Ding et al., 1993; Flores et al., 1994) on the cell surface of macrophages. More specifically, Gazzinelli et al. (1992<sup>2</sup>) observed that IFNγ-induced microbicidal activity of macrophages is inhibited by IL-10 and that it correlated with the suppression of nitrogen oxide metabolite generation. In this study the lymphocyte content of the granulomas in the absence of IL-10 were enhanced. T cells increase IL-10 production on stimulation with IL-12 (Daftarian et al., 1996), which can inhibit antigen specific proliferation, and cytokine production (Del Prete, et al., 1993). It is therefore highly likely that production of IL-10 by macrophages and T
cells in the context of granuloma formation has autoregulatory suppressing effects and has a profound influence on controlling the protective immune response.

In summary, this study has demonstrated that IL-10−/− mice induced an enhanced protective immune response against *M. bovis* BCG infection. In view of the findings in this study, further investigation in the role of IL-10 using virulent *M. tuberculosis* is warranted. The absence of an endotoxic shock response during infection with *M. bovis* BCG, together with an enhanced protective immune response in the absence of IL-10 presents this cytokine as an attractive target for chemotherapy.
Mycobacterium tuberculosis H37Rv

infection in LT−/− mice.
Summary

In this study the importance of lymphotoxin alpha (LT) in the host’s immune response against a virulent mycobacterial infection was investigated. Both WT and LT⁻/⁻ mice could induce the Th1 defining cytokines, IFNγ and IL-12, in response to *Mycobacterium tuberculosis* H37Rv infection. Despite the ability to induce a Th1 cell mediated immune response, LT⁻/⁻ mice were highly susceptible to infection by aerosol inhalation. Comparative mortality rates of TNF⁻/⁻ mice and LT⁻/⁻ mice, at a standard infection dose of 100cfu/lung, were similar. However, at a 10 fold lower dose, TNF⁻/⁻ mice displayed a higher mortality rate than LT⁻/⁻ mice indicating distinct functional differences for TNF and LT. Progression of infection in LT⁻/⁻ mice was associated with loss of bodyweight and impeded movement. Lungs were enlarged with significant weight increases during the latter stage of infection and macroscopic pathology was characterised by large nodule formation under the pleura. Splenomegaly was also observed during the course of infection. LT⁻/⁻ mice were unable to control mycobacterial growth as evident by the significantly higher mycobacterial burdens that were detected in the livers, spleens and lungs of infected mice. In contrast to WT mice, the formation of well-demarcated pulmonary granulomas in response *Mycobacterium tuberculosis* H37Rv were absent in LT⁻/⁻ mice. Microscopic pulmonary pathology in LT⁻/⁻ mice was characterised by persistent cellular infiltration, with the consequent development of tissue necrosis. Uncontrolled mycobacterial growth resulted in accelerated haematogenic dissemination of bacilli as evident by the early formation of hepatic granulomas in LT⁻/⁻ mice. In WT mice, AFB in the lungs were primarily confined to epitheloid macrophages within granulomas during the course of infection. In LT⁻/⁻ mice higher numbers of AFB were intracellular, residing within epitheloid macrophages, during the early stage of infection. However, concomitant with tissue necrosis in the lungs during the latter stages of infection, most AFB was found to be extra-cellular in LT⁻/⁻ mice. The inability of LT⁻/⁻ mice to control pulmonary mycobacterial growth was corroborated by the reduction of iNOS syntheses in the lungs. In addition, it was established that LT⁻/⁻ mice had a diminished specific memory recall response in response to challenge with PPD. The contribution of the inherent developmental lymphoid abnormalities of LT⁻/⁻ mice to *Mycobacterium tuberculosis* H37Rv infection was addressed in survival
studies using chimeric mice. Irradiated WT mice which were reconstituted with LT deficient bone marrow, remained susceptible to infection, but more importantly, had similar mortality rates as control chimeric LT⁻/⁻ mice, thus supporting the non-involvement of developmental lymphoid deficiency to mycobacterial susceptibility. In conclusion, the results of this study have shown that LT are necessary to establish a protective cell mediated immune response against virulent mycobacterial infection.
Results

4.1 Rapid mortality of *M. tuberculosis* H37Rv infected LT<sup>−/−</sup> mice.

To determine whether LT<sup>−/−</sup> mice are susceptible to *M. tuberculosis* H37Rv infection, mice were infected with a low dose (~10 cfu/lung) and standard dose (~100 cfu/lung) by aerosol inhalation. These doses were confirmed by sacrificing 10 mice 1 day after infection. In these experiments the susceptibility of LT<sup>−/−</sup> mice were compared to TNF<sup>−/−</sup> mice and the wild type control strain. There was a dose dependent effect on the susceptibility of LT<sup>−/−</sup> mice when compared to TNF<sup>−/−</sup> mice. A significant difference in susceptibility between the two mutant strains at an infection dose of ~100cfu/lung was not observed (Fig. 4.1A). Both mutant strains were highly susceptible with similar mortality rates and succumbed between day 35 and day 39 while wild type mice survived the experiment. At an infection dose of 10cfu/lung, however there was a significant difference in the mortality rates between the two mutant strains (Fig. 4.1B). TNF<sup>−/−</sup> mice succumbed rapidly to the infection between day 47 and day 54. In contrast, mortality for LT<sup>−/−</sup> mice was significantly delayed, occurring between day 42 and day 218 whereas >70% of the WT strain survived the experiment.

4.2 Progressive emaciation and increase in organ weights of LT<sup>−/−</sup> mice during *M. tuberculosis* H37Rv infection.

Weight loss is a well established symptom of progressive tuberculosis. To determine whether a correlation exists between weight and progressive disease in the absence of LT, mice were infected with ~100 cfu/lung and their body weights were recorded for the duration of the experiment (Fig. 4.2). There were not significant differences in body weights during the early stages of infection between wild type mice and LT<sup>−/−</sup> mice. However 32 days after infection, LT<sup>−/−</sup> mice showed rapid loss of body weight and appeared emaciated with impeded movement. In contrast, WT mice had a significantly higher body weight (p < 0.05) and appeared healthy.

The pathology of whole lungs of WT mice and LT<sup>−/−</sup> mice were examined and compared after 35 days of infection (Fig. 4.3). WT mice had a healthy appearance
Figure 4.1
Susceptibility of LT$^{-}$ and TNF$^{-}$ mice to M. tuberculosis H37Rv infection.

WT mice ($n = 14$), LT$^{-}$ mice ($n = 10$) and TNF$^{-}$ mice ($n = 10$) were infected with $\sim 100$ cfu (panel A) or $\sim 10$ cfu (panel B) of M. tuberculosis H37Rv by aerosol inhalation. The data indicates the percentage of mice surviving over the course of infection. The results are representative of 2 similar experiments.

Figure 4.2
Bodyweight determination during the course of M. tuberculosis H37Rv infection.

Body weights of M. tuberculosis H37Rv infected WT and LT$^{-}$ mice were recorded for the duration of the experiment. The data represents the average ± SD for each group. Each data point represents 13-17 mice per time point. The results are representative of 2 similar experiments.
Figure 4.3
Macroscopic pathology of lungs in \( M. \) \( \text{tuberculosis} \) H37Rv infected WT and LT\(^{-/-}\) mice.

WT and LT\(^{-/-}\) mice were infected with 100 cfu of \( M. \) \( \text{tuberculosis} \) H37Rv by aerosol inhalation and were sacrificed at 35 days post infection. Lungs of WT mice had small, discrete lesions (black arrows) in contrast to the large nodular appearance (red arrows) of LT\(^{-/-}\) mice. Results are representative of 4 mice/strain.

Figure 4.4
Organ weights in \( M. \) \( \text{tuberculosis} \) H37Rv infected WT and LT\(^{-/-}\) mice.

WT and LT\(^{-/-}\) mice were infected with ~100 cfu of \( M. \) \( \text{tuberculosis} \) H37Rv by aerosol inhalation. After sacrifice at the indicated time points, lungs (panel A) or spleens (panel B) were removed and the weights recorded. The data represents the average ± SD of 4 mice per time point. The results are representative of 2 similar experiments.
with fine lesions visible on occasions. In contrast, lungs of LT<sup>-/-</sup> were enlarged with large white nodules visible under the pleura.

Fig. 4.4 represents the recorded weights of the lungs and spleens of wild type mice and LT<sup>-/-</sup> mice at 21 days and 35 days post infection. Although a significantly lower lung weight were recorded in LT<sup>-/-</sup> mice when compared to wild type mice after 21 days of infection (p < 0.01), this trend was reversed after 35 days with LT<sup>-/-</sup> mice having a significantly higher lung weight (Fig. 4.4A). The spleen weights in the two mouse strains were not significantly different at 21 days post infection. Splenomegaly was however evident in LT<sup>-/-</sup> mice which had a significantly higher average spleen weight after 35 days of infection (Fig. 4.4B).

4.3 Enhanced mycobacterial burden in *M. tuberculosis* H37Rv infected LT<sup>-/-</sup> mice.

The mycobacterial burden in the liver, lung and spleen were determined due to the differences in mortality rates that were observed between the wild type and LT<sup>-/-</sup> mouse strains (Fig. 4.5). After an infection dose of ~100 cfu/lung, mice were sacrificed at the indicated time points.

The pulmonary mycobacterial burden (Fig. 4.5A) in both mouse strains increased significantly (p < 0.001) after 14 days of infection. The mycobacterial burden between the two strains was however not significantly different. A further significant increase in the mycobacterial burden of WT mice (p < 0.001) and LT<sup>-/-</sup> mice (p < 0.05) was observed after 21 days of infection in comparison to that at 14 days. Of interest however, was the repeated and consistent finding of a small (>3 fold) but significantly higher (p < 0.01) mycobacterial burden in WT mice. WT mice had a significantly lower mycobacterial load at 28 days post infection in comparison to day 21 days after infection (p < 0.05) whereas the mycobacterial load in LT<sup>-/-</sup> mice remained constant. There was no significant difference between the two strains at this point. A continual significant reduction (p < 0.05) of the mycobacterial burden was observed in WT mice at 35 days post infection in comparison to LT<sup>-/-</sup> mice. In contrast, the mycobacterial burden in LT<sup>-/-</sup> mice increased ~2 orders of magnitude more and was significantly higher (p < 0.01) than the levels that were observed at 28
Figure 4.5
*Mycobacterial burden in the lungs spleen and liver of WT and LT−/− mice.*

Mice were infected with ~100 cfu of *M. tuberculosis* H37Rv by aerosol inhalation and sacrificed at the indicated time points. Homogenised liver, spleen and lung tissue samples were plated on OADC enriched agar and incubated at 37°C for 18-21 days. The plates were scored for mycobacterial colonies and the bacilli load of each organ determined. The data represents the average ± SD of 4 mice per time point. The results are representative of 2 similar experiments. Lungs (panel A), Spleen (panel B), Liver (panel C).
days post infection. A comparison of the mycobacterial burden in the two strains revealed significantly higher numbers ($p < 0.01$) in the lungs of LT$^{-/}$ mice.

Dissemination of bacilli into the spleen (Fig. 4.5B) was evident in WT and LT$^{-/}$ mice at 14 days post infection but no significant difference was detected in the number of organisms between the two mouse strains. There was a significant increase in the splenic mycobacterial burden in both strains (WT mice $p < 0.01$, LT$^{-/}$ mice $p < 0.001$) at 21 day post infection in comparison to 14 days post infection. However, the number of bacilli that were recovered from LT$^{-/}$ mice was significantly higher than that of their WT littermates. Continual progression of infection in both mouse strains was characterised by further significant growth of bacilli after 28 days of infection ($p < 0.05$) with the number of organisms remaining significantly higher in LT$^{-/}$ mice ($p < 0.05$). Mycobacterial growth continued as established at 35 days post infection and a significantly higher ($p < 0.05$) number of organisms was present in both mouse strains in comparison to day 28 post infection. Despite the increase in mycobacterial burden in WT mice over the course of infection, what was significant was the increase in the difference in mycobacterial burden between LT$^{-/}$ mice and WT mice with progression of disease. This difference ranged from 12 fold on day 21 post infection to 138 fold on day 35 post infection.

Hepatic bacilli (Fig. 4.5C) were already detectable in both mouse strains in comparable burdens at 14 days post infection. A significant increase was found in bacilli load in WT ($p < 0.001$) and LT$^{-/}$ mice ($p < 0.01$) 21 days after infection compared to 14 days post infection with significantly higher ($p < 0.01$) levels being observed in LT$^{-/}$ mice. Further significant increases ($p < 0.05$) in mycobacterial loads were observed in both strains at 28 days post infection in comparison to 21 days post infection with the significantly higher ($p < 0.05$) bacilli load in LT$^{-/}$ mice being maintained. The difference in bacilli load however increased from 50 fold to 138 fold during this time period. The mycobacterial load of WT mice remained constant. In contrast, the bacilli load in LT$^{-/}$ mice was significantly higher ($p < 0.01$) at 35 days post infection in comparison to 28 days post infection and the 728 fold difference that was observed between the mouse strains, being significantly different ($p < 0.001$).
4.4 Granulomatous lesion formation in *M. tuberculosis* H37Rv infected LT<sup>−/+</sup> mice.

**Pulmonary granuloma formation**

It has previously been established that a rapid granulomatous response is essential for controlling mycobacterial infection (Kindler *et al.*, 1989). Pulmonary granulomatous lesion formation was therefore investigated due to the high bacilli burden and associated mortality of LT<sup>−/+</sup> mice.

Histological examination of lung tissue sections after 14 days of infection revealed limited inflammation with concomitant thickening of alveolar septae in both mouse strains (Figs. 4.6A and 4.6C). Granulomatous lesions were however absent. Discrete granulomatous foci were detected in ~20% of the lung tissue sections of WT mice after 21 days of infection (Fig. 4.6B). In contrast, the pulmonary response in LT<sup>−/+</sup> mice was characterised by continual cellular infiltration but granulomatous foci was absent (Fig. 4.6F). Investigation under high magnification revealed that PMN were more abundant in the cellular infiltrate in LT<sup>−/+</sup> mice. Granulomatous lesions in WT mice increased in size and number, and were well demarcated relative to the surrounding lung tissue at 28 days post infection (Fig. 4.6C). These were characterised by a central core of predominantly epitheloid mononuclear leukocytes, which was either partially or fully surrounded by a mantle of lymphocytes. Frequently, granulomatous lesions were coalescent. In contrast, LT<sup>−/+</sup> mice were devoid of any form of defined granulomatous lesions, although limited areas of leukocyte aggregation were observed (Fig. 4.6G). Within such areas initial stages of necrosis was evident. At 35 days post infection, granulomatous lesions in WT mice were characterised by lymphocytes that were rearranged into smaller, concentrated foci as well as being evenly dispersed throughout the epitheloid macrophages (Fig. 4.6D). In contrast, large areas of necrosis were evident in lung tissue of moribund LT<sup>−/+</sup> mice (Fig. 4.6H). These areas were surrounded by and contained cellular and nuclear debris. Major portions of lung tissue had the alveoli destroyed. Bronchi of WT mice were intact and clear of cellular debris. However tissue damage in LT<sup>−/+</sup> mice extended to the bronchi allowing infiltration of purulent debris into the air spaces (Fig. 4.7).

The ability to repair tissue was investigated in the lungs by staining for collagen deposition as an indication of fibrosis (Fig. 4.8). Lung tissue of WT mice clearly
Figure 4.6
Pulmonary granuloma formation in WT mice and LT<sup>+</sup> mice after challenge with M. tuberculosis H37Rv.

Mice were infected with 100 cfu of M. tuberculosis H37Rv by aerosol inhalation and sacrificed at 14, 21, 28 and 35 days after infection. Organs were fixed in phosphate buffered formalin, sectioned and stained with hematoxylin and eosin. Panels A-D represent progressive establishment of well-defined pulmonary granulomas in WT mice. LT<sup>+</sup> mice (panels E-H) display continual inflammation, lack of proper granulomas and present necrosis at day 35. Pathology is representative of 4 mice per strain at each time point. (Magnification = 40x)
Figure 4.7
Microscopy of bronchi in *M. tuberculosis* H37Rv infected WT and LT<sup>-/-</sup> mice.

WT and LT<sup>-/-</sup> mice were infected with 100 cfu of *M. tuberculosis* H37Rv by aerosol inhalation and were sacrificed at 35 days post infection. Lungs were fixed in phosphate buffered formalin, sectioned and stained with hematoxylin and eosin. WT mice (left panel) displayed predominantly intact bronchi free of cellular debris. In contrast, LT<sup>-/-</sup> mice (right panel) had damaged bronchi with air spaces filled with purulent debris. Tissue sections are representative of 4 mice per strain. (Magnification = 400x)

Figure 4.8
Fibrosis in lung tissue sections of *M. tuberculosis* H37Rv infected WT and LT<sup>-/-</sup> mice.

WT and LT<sup>-/-</sup> mice were infected with 100 cfu *M. tuberculosis* H37Rv by aerosol inhalation and were sacrificed after 28 days. Lungs were fixed in phosphate buffered formalin, embedded in paraffin wax, sectioned and stained for the presence collagen as described in Materials and Methods. Deposition of collagen (blue staining denoted by arrows) as an indication of fibrosis is present in WT mice but absent in LT<sup>-/-</sup> mice. Tissue sections are representative of 4 mice per strain. (Magnification = 400x)
showed collagen deposition confined to granulomas, either on the periphery or more abundantly, in the central areas of the lesions. These coincided with areas that had limited cellular structure. In contrast, collagen staining in the lungs of LT−/− mice was largely absent.

**Hepatic granuloma formation.**

Hepatic granuloma formation was investigated as an indication of systemic dissemination of the infection (Fig. 4.9). The establishment of small cellular aggregates in the liver, consistent with the initial stages of granuloma formation, at 21 days post infection accompanied early spread of bacilli from the lungs of LT−/− mice (Fig. 4.9F). These developed into larger, maturer granulomas at 28 days post infection (Fig. 4.9G). Hepatic pathology of LT−/− mice after 35 days post infection was characterised by mature granulomatous lesions interspersed with smaller, cellular foci that probably represented the initial stages of new granulomatous lesions as a consequence of continual dissemination of bacilli (Fig. 4.9H). In contrast, WT mice exhibited a delay in disease dissemination with the small, cellular foci only becoming evident at 28 days post infection (Fig. 4.9C). Hepatic pathology of WT mice at 35 days post infection displayed predominantly mature granulomas with smaller newly established granulomas, as found in LT−/− mice, being largely absent (Fig. 4.9D).

### 4.5 Detection of acid fast bacilli

Bacilli were completely confined to discrete pulmonary granulomatous lesions of WT and resided within epitheloid macrophages at 21 days post infection (Fig. 4.10A). The number of bacilli that was found within these macrophages ranged from 1-3 AFB/cell and was evenly distributed with lymphocytes throughout the granulomatous lesion. Despite the absence of granulomas AFB in LT−/− mice were contained within epitheloid macrophages (Fig. 4.10D). In WT mice, the increase in granulomatous lesion size was accompanied by the formation of pockets of epitheloid cells that were isolated and surrounded by lymphocytes within the lesion (Fig. 4.10B). The average number of bacilli/epitheloid cell within these clusters was higher in comparison to 21 days post infection. Although distinct granulomatous lesions were absent in LT−/− mice cellular infiltration was evident. Specific pockets of epitheloid cells, which harboured AFB, were absent. Instead bacilli were scattered randomly within the tissue mass either being found intra- or extra cellular (Fig 4.10E). Nuclear and
Hepatic granuloma formation in *M. tuberculosis* H37Rv infected WT and LT−/− mice as an indication of systemic dissemination of infection.

WT and LT−/− mice were infected with 100 cfu *M. tuberculosis* H37Rv by aerosol inhalation and were sacrificed at 14 days, 21 days, 28 days and 35 days post infection. Lungs were fixed in phosphate buffered formalin, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin. Granuloma formation was absent in WT and LT−/− mice at 14 days post infection (panels A and E). Early initiation of granuloma formation in LT−/− mice (panel F) developed to mature granulomas (panel G). In addition, new granulomas dominated hepatic pathology during chronic infection (panel H). In contrast, hepatic granuloma formation was delayed in WT mice (panel C) and developed to maturity (panel D). Tissue sections represent 4 mice per strain. (Magnification = 100x)
WT and LT<sup>−/−</sup> mice were infected with 100 cfu <i>M. tuberculosis</i> H37Rv by aerosol inhalation and were sacrificed at 21 days, 28 days and 35 days post infection. Lungs were fixed in phosphate buffered formalin, embedded in paraffin wax, sectioned and stained for the presence of AFB using the Ziehl-Neelson staining method. Mycobacteria in WT mice (panels A-C) were found intra-cellular in clusters of epitheloid macrophages which were surrounded by infiltrating lymphocytes. In contrast, LT<sup>−/−</sup> mice (panels D-F) displayed uncontrolled mycobacterial growth with AFB being predominantly extra-cellular (panel F). Tissue sections are representative of 4 mice per strain. (Magnification = 400x)
cellular debris was distinctly visible in areas of extra cellular AFB. Granulomatous lesions in WT mice were characterised by tight clusters of epitheloid cells that were surrounded by lymphocytes after 35 days of infection. These epitheloid clusters appeared smaller than those found at 28 days post infection but the macrophages within these clusters contained higher numbers of bacilli/cell (Fig. 4.10C). In contrast, necrotic lung tissue of LT<sup>−/−</sup> mice had an uncontrolled spread of bacilli that was predominantly extra cellular (Fig. 4.10F). Columnar epithelial layers were damaged and bronchioles were filled with tissue and nuclear debris, which contained AFB. Intact epitheloid macrophages were compacted with bacilli.

Hepatic granulomatous lesions were examined for the presence of AFB in WT and LT<sup>−/−</sup> mice at 35 days post infection (Fig. 4.11). Typical granulomatous lesions of WT mice were either clear or contained fewer than 5 bacilli. In contrast, the small cell clusters that were found in LT<sup>−/−</sup> mice contained on average more than 15 bacilli/lesion.

4.6 iNOS induction is reduced in <i>M. tuberculosis</i> H37Rv infected LT<sup>−/−</sup> mice.

Control of <i>M. tuberculosis</i> H37Rv infection is dependent on nitric oxide production. Central to this metabolic pathway is the inducible isoform of nitric oxide synthase. The ability of LT<sup>−/−</sup> mice to produce nitric oxide in the lungs was indirectly measured by staining for the presence of iNOS due to the uncontrolled mycobacterial growth that was observed in these mice (Fig. 4.12). Immunohistochemical staining of lung sections disclosed that pulmonary granulomatous lesions in WT mice stained strongly for iNOS at 28 days post infection. In contrast, very weak staining was observed in LT<sup>−/−</sup> mice in condensed areas of lung tissue resembling pseudo-granulomatous lesions.

4.7 <i>M. tuberculosis</i> H37Rv infected LT<sup>−/−</sup> mice are able to elicit a Th1 response.

The susceptibility of LT<sup>−/−</sup> mice to <i>M. tuberculosis</i> H37Rv infection raised questions regarding their ability to mount a Th1 response at the primary site of infection. To address this question, IL-12 and IFNg were measured in the supernatants of homogenised whole lungs after 28 days of infection. The results in Fig. 4.13 indicate that WT mice and LT<sup>−/−</sup> mice had detectable levels of both IL-12 and IFNg in the lung
WT and LT-/- mice were infected with 100 cfu of *M. tuberculosis* H37Rv and were sacrificed at 28 days post infection. Livers were fixed in phosphate buffered formalin, embedded in paraffin wax and the presence of AFB was detected by Ziehl-Neelson staining. Granulomas of LT-/- mice showed a significantly higher number of AFB present within epitheloid macrophages. Tissue sections are representative of 4 mice per strain. (Magnification = 1000x)

**Figure 4.11**
*AFB in hepatic granulomas in M. tuberculosis H37Rv infected WT and LT-/ mice.*

WT and LT-/- mice were infected with 100 cfu of *M. tuberculosis* H37Rv by aerosol inhalation and were sacrificed at 28 days. Lungs were fixed in phosphate buffered formalin, embedded in paraffin wax, sectioned and stained with an anti-iNOS specific antibody. Sections of WT mice showed intense expression of iNOS within well-demarcated granulomas in contrast to the weak staining that was evident in LT-/- mice. Tissue sections are representative of 4 mice per strain. (Magnification = 40x)

**Figure 4.12**
*Pulmonary iNos induction in M. tuberculosis H37Rv infected WT and LT-/ mice.*
Figure 4.13
Pulmonary cytokine concentrations in M. tuberculosis H37Rv infected WT mice and LT−/− mice.

Mice were infected with ~100 cfu of M. tuberculosis H37Rv by aerosol inhalation and sacrificed after 28 days. Whole lungs were homogenised in 1ml of saline, centrifuged and the supernatants were analysed for IFNγ (panel A), IL-12p40 (panel B) and TNF (panel C) by ELISA. Each data point represents the average ± SD of 4 mice. The results are representative of 2 similar experiments.
homogenates. There was however not a significant difference in the concentrations of these cytokines between the two mouse strains.

4.8 Pulmonary TNF production is normal in *M. tuberculosis* H37Rv infected LT^{-} mice.

The metabolic effects LT and TNF can be mediated through their common receptors, TNFR1 and TNFR2 (Orlinick and Chao, 1998). To exclude the possibility that reduced levels of TNF, and hence impaired signalling of TNF, could be responsible for the susceptibility of LT^{-} mice to *M. tuberculosis* H37Rv infection, TNF concentrations were measured in the supernatants of homogenised lung tissue of WT and LT^{-} mice after 28 days of infection. The result in Fig. 4.13 indicates that there is not a significant difference in the pulmonary TNF concentration levels between WT and LT^{-} mice.

4.9 Memory recall is diminished in *M. tuberculosis* H37Rv infected LT^{-} mice.

To assess whether LT^{-} mice can elicit a memory recall response, differences in footpad swelling against saline and PPD were measured after 28 days of infection. Distinct footpad swelling was observed against PPD in WT mice while LT^{-} mice elicited a minimal response (Fig 4.14). The DTH response of WT mice was significantly higher (p < 0.05) in comparison to LT^{-} mice.

4.10 The peripheral lymphoid developmental abnormalities of LT^{-} mice do not contribute to its susceptibility to *M. tuberculosis* H37Rv infection.

Developmental deficiencies of LT^{-} mice include the absence of lymphnodes and Peyer’s patches, the absence splenic primary B cell follicles, follicular dendritic cell networks and defined germinal centres, and an inability of splenic T and B cell zones to segregate. To address whether the susceptibility of LT^{-} mice to *M. tuberculosis* H37Rv infection could be attributed to the peripheral lymphoid developmental abnormalities of the strain, irradiated WT mice were reconstituted with LT^{-} bone marrow (WT-LTBM). For controls, irradiated wild type mice received wild type bone marrow (WT-WTBM) and irradiated LT^{-} mice were reconstituted with LT^{-} bone marrow (LT-LTBM). Mice were infected with *M. tuberculosis* H37Rv by aerosol inhalation at a low dose (~10cfu/lung) and mortality was recorded. WT-LTBM mice
Figure 4.14
Delayed type hypersensitivity of *M. tuberculosis* H37Rv infected mice after challenge with PPD.

Mice (*n* = 6/group) were infected with ~100 cfu of *M. tuberculosis* H37Rv by aerosol inhalation. On day 28 post infection mice were subcutaneously injected with 5µg PPD in the right hind footpad and saline in the left hind footpad. Footpad swellings were measured after 48 hours. The data represents the differences in swelling between PPD and saline challenge footpads. The results are representative of 2 similar experiments.

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Figure 4.15
Susceptibility of *M. tuberculosis* H37Rv infected chimeric mice.

Mice (*n* = 8/group) were infected with ~10 cfu of *M. tuberculosis* H37Rv by aerosol inhalation and mortality was monitored. WT-LTBM and LT-LTBM chimeric mice had similar mortality rates in contrast to WT-WTBM chimeric mice which survived the experiment.
and LT-LTBM mice had comparable mortality rates and succumbed to infection by
day 213 whereas WT-WTBM mice survived the experiment (Fig. 4.15).
Discussion

The structural similarities between LT and TNF and its ability to bind to the same receptors have been well documented. Although it has been thought that TNF and LT are biologically redundant molecules this concept has been challenged in a mouse model of EAE, which demonstrated that these two cytokines are functionally distinct entities in vivo (Sean-Riminton et al., 1998). Previous studies have demonstrated that TNFR1 (Flynn et al., 1995b) and TNF (Bean et al., 1999) are important in mediating protective immunity against M. tuberculosis infection yet the importance of LT in contributing to Th1 cell mediated immunity was still unknown. The findings of this study reveal an absolute dependence on LT for host immunity against M. tuberculosis infection and exclude that TNF deficiency at the site of infection is a contributory factor. The inadequacy of TNF to restore protective immunity in LT−/− mice and the failure of LT to compensate in TNF−/− mice confirms its functional distinct identities. A novel finding was the relative dose dependent susceptibility of TNF−/− and LT−/− mice to M. tuberculosis. The higher susceptibility of TNF−/− mice at the lower dose suggest that TNF is significantly more important for protective immunity than LT. It is known that both macrophages and lymphocytes can secrete TNF although the relative significance of each cell type, as a TNF source during mycobacterial infection, is unknown. However, LT is predominantly derived from T cells, B-cells and NK cells (Ware et al., 1992). The importance of TNF during acute phase of infection could therefore reflect the critical role of phagocytic macrophages as an early source of TNF to drive a Th1 cell mediated response to allow recruitment of activated lymphocytes as an additional source of TNF and LT. In the absence of early macrophage derived TNF, mycobacterial infection is fatal. The inability of TNF to substitute for LT after a standard infection dose could be due to differences in kinetic cytokine regulation. Millet et al. (1994) have found that activated murine T cell clones differentially regulate the expression of LT and TNF at both transcriptional and post-transcriptional levels. Susceptibility of LT−/− mice could also indicate a requirement of a unique receptor for LT signalling during infection. Currently, such a receptor has not yet been described.
Current literature regarding signalling through LTβR is controversial and is complicated by the presence of LIGHT, which can also act as a ligand for the receptor (Zhai et al., 1998). A further possibility could include differential signalling effects of each cytokine subsequent to binding to the same receptor.

Several of the underlying biological effects of protective immunity were investigated. In this study it was shown that LT$^{-/-}$ mice could induce a Th1 cell mediated immune response at the sight of infection equivalent to that of WT mice. IFNγ is crucial for resolving mycobacterial infection (Kamijo et al., 1993) and can act in synergy with LT (Paul and Ruddle, 1988). Importantly, IFNγ is crucial for generating NO. Similarly, LT dependent production of nitric oxide mediated by nuclear transition of NFκB was necessary to inhibit bacterial growth during in vitro infection studies (Matsushima et al., 1999). However the importance of the synergistic action of IFNγ and LT in the generation of NO for controlling mycobacterial infection is not known. The findings of this study show reduced levels of iNOS expression in the absence of LT after M. tuberculosis infection which indicated that the presence of IFNγ was not sufficient to generate NO but that cellular bactericidal concentrations of NO is dependent on the presence of both IFNγ and LT. Given the absolute requirement of NO for mycobacterial resolution (MacMicking et al., 1997; Garcia et al., 2000) it is conceivable that NO deficiency contributed significantly to the susceptibility of LT$^{-/-}$ mice.

Susceptibility of LT$^{-/-}$ mice to M. tuberculosis infection was associated with the absence of organised granulomatous structures with consequential high bacilli titres. Previously Bean et al. (1999) highlighted the importance of establishing intact granulomas during M. tuberculosis infection by illustrating that mice with a TNF deficiency are unable to form proper granulomas which becomes fatal. Granuloma formation is a multi-factorial dependent event, which include establishing the proper cytokine and chemokine milieu, regulation of adhesion molecules and initiating recruitment of required cells. It therefore follows that a disruption of any of these events could impact negatively on granuloma formation. Previously it was shown that transgenic expression of LT induced inflammation and required the interaction of LT$_3$ with TNFR1 (Sacca et al., 1998). Inflammation is dependent on the induction of
adhesion molecules and it was found that LT could regulate the expression of ICAM, VCAM, E selectin and MadCAM in cultures (Pober et al., 1987; Cavender et al., 1989; Cuff et al., 1998). ICAM deficiency leads to impaired inflammatory and immune responses (Sligh et al., 1993) and an inability to establish granulomas on challenge with *M. tuberculosis* (Johnson et al., 1998). In addition it was found that VCAM expression is crucial for the recruitment of activated lymphocytes and its neutralisation during *M. tuberculosis* infection results in the absence of organised granulomas. An additional level of control in the establishment granulomas could involve chemokine regulation. Chemokine secretion is an integral component that controls the pro-inflammatory response and has been detected after infection with different pathogens including *M. tuberculosis* infection. This includes the production of IL-8 by human alveolar macrophages (Schluger and Rom, 1997) polymorphonuclear granulocytes (Riedel and Kaufmann, 1997) and epithelial cells (Lin et al., 1998) in culture. In addition murine macrophages secreted MIP-1α, MIP-1β, IP-10 and MCP-1 during infection (Rhoades et al., 1995). Significantly, a direct correlation was established between RANTES and mycobacterial antigen induced granulomatous lesions. Providing evidence for LT dependent regulation of chemokines (Cuff et al., 1998) illustrated that LT could induce the expression of RANTES, IP-10 and MCP-1 murine endothelial cell cultures. Thus current literature strongly supports a proinflammatory role for LT during *M. tuberculosis* infection. Taken together, it can therefore be deduced that, on challenge with *M. tuberculosis*, LT deficiency results in a defective inflammatory response due inhibited chemokine secretion and the lack of adhesion molecule expression and hence an inability to establish proper granulomas.

LT<sup>−/−</sup> mice had a diminished memory recall response. Previous data (Ruddle and Waksman, 1968) showing a correlation between a positive DTH reaction with the production of LT in culture supports this finding thereby providing further evidence of the importance of LT in mediating pro-inflammatory responses. Interestingly, it was found that *M. tuberculosis* infected IL-12<sup>−/−</sup> mice were unable to generate a DTH response against PPD and fail to generate properly defined granulomas (Cooper et al., 1997), similar to the observations in this study. The lack of antigen specific recall response was thought to result from the diminished IFNγ levels. This however could
not be confirmed as DTH activity in \textit{M. tuberculosis} infected IFN$\gamma^{/-}$ mice was inconclusive. Nevertheless the accumulative data would suggest that LT together with IFN$\gamma$ is required for mediating a memory recall response.

The development of peripheral lymphoid abnormalities is inherent in the generation of LT$^{-/-}$ mice due to it role in lymphoid organogenesis. The results in this study indicate that LT deficient chimeric mice display similar susceptibilities to \textit{M. tuberculosis} irrespective of the presence or absence peripheral lymphoid abnormalities. In addition it can be argued that equivalent pulmonary concentrations of IFN$\gamma$, IL-12 and TNF is indicative of similar cellular activation in both strains. These findings provide compelling evidence that the peripheral lymphoid developmental abnormalities do not contribute to its susceptibility during \textit{M tuberculosis} infection.

Recently, in an elegant study by Roach \textit{et al}. (2001), several of these observations were verified. These authors illustrated that LT deficient chimeric mice are highly susceptible to \textit{M. tuberculosis} infection, do not form intact granulomas and develop high bacilli organ burdens. The study confirms that LT$^{-/-}$ mice are able to produce IFN$\gamma$ at levels similar to WT mice and have normal expression of TNF. The study however differs from the findings in this report with respect to NO generation and DTH response indicating that both of these events can occur independent of LT. These differences are possibly due to the contrasting experimental approaches of the two studies with specific relevance to the mouse strains that have been employed and therefore warrant further investigation.

The role of the LT$\beta$R is still controversial. Neutralisation of the LT$\beta$ through binding with LT$\beta$R-Ig fusion protein displayed enhanced mycobacterial growth in the spleens of \textit{M. bovis} BCG infected mice (Lucas \textit{et al}., 1999). This is in contrast to the findings of Roach \textit{et al}. (2001) that did not find any differences in susceptibility of LT$\beta$ deficient chimeric mice in comparison to controls.

In conclusion, this study has illustrated an absolute dependence on LT to induce protective immunity against \textit{M. tuberculosis} H37Rv infection. Notwithstanding the
common binding properties of TNF and LT, could TNF not compensate for the absence of LT.
Conclusions
Conclusions

In this study the importance of the Th1 associated cytokines, Tumour Necrosis Factor and Lymphotoxin alpha, and the immunomodulatory cytokine, Interleukin-10 in the host’s response against either *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis* H37Rv infection was addressed. These findings have unequivocally established an absolute requirement for TNF and LT in cell mediated immunity during infection and that, in the absence of these cytokines, the host’s protective immune response is compromised, irrespective of the virulence of the strain it encounters. It has confirmed that TNFR1 acts as the primary receptor responsible for TNF and LT signaling. In addition, it was shown that the combined absence of both TNF and LT exacerbates susceptibility to *Mycobacterium bovis* BCG. Comparatively, TNF/LT⁺/⁻ mice developed the highest mycobacterial burdens, followed by TNF⁻/⁻ mice, LT⁻/⁻ mice and WT mice, respectively. The relative differences in susceptibilities of TNF⁺/⁻ and LT⁺/⁻ mice to *Mycobacterium bovis* BCG challenged the concept of absolute redundancy between TNF and LT but rather indicated that TNF and LT do have unique functions. However, a more complex relationship incorporating overlapping functions during cell-mediated immunity during mycobacterial infection cannot be excluded. Although TNF and LT contain structural similarity and can signal through the same receptors, the higher susceptibility of TNF⁺/⁻ mice in comparison to LT⁻/⁻ mice indicates that TNF, rather than LT, is more important in the host’s immune response.

Central to protective immunity is the induction of an effective granulomatous response. In the absence of TNF the kinetics of hepatic granuloma formation is altered. Not only is the induction of granulomas delayed but also is its structural integrity compromised. Interestingly, the absence of LT did not alter the rate at which granulomas were established, nor did it influence its structure. The absence of both TNF and LT however, resulted in a more severe pathology on challenge with *Mycobacterium bovis* BCG. Unlike TNF⁺/⁻ mice, which developed a delayed uncohesive granulomatous structure, hepatic pathology in TNF/LT⁻/⁻ mice at best was characterised by the establishment of small cellular clusters. Therefore, whereas delayed clearance was observed in the livers and spleens of TNF⁺/⁻ mice, uncontrolled
mycobacterial growth in TNF/LT$^{-/-}$ mice was supported by the impaired severity of its granulomatous response in these organs. The early initiation of hepatic granuloma formation is thus a TNF dependent event. It is therefore possible that, in the absence of TNF, LT could partially substitute for its biological functions during the latter stages of infection. In contrast to delayed hepatic granuloma formation, defined pulmonary granulomas were completely absent in TNF$^{-/-}$ mice. Progressive infection was accompanied by enhanced inflammation, the development of tissue necrosis and eventual death of the animals. Similarly pulmonary pathology was displayed by TNF/LT$^{-/-}$ mice which failed to develop granulomas on challenge with Mycobacterium bovis BCG.

Underlying the failure to establish an effective granulomatous response is the inability of the host to induce early cellular recruitment in response to infection in the absence of TNF. Although cellular recruitment is partially rectified in the latter stages of infection in TNF$^{-/-}$ mice, the additional absence of LT in TNF/LT$^{-/-}$ mice and the failure of these mice to establish granulomatous lesions indicate that LT is necessary for cellular recruitment during the progressive stages of infection. Characterisation of granulomas in TNF$^{-/-}$ or TNF/LT$^{-/-}$ mice showed impaired macrophage activation together with reduced adhesion molecule expression contributing to an inhibited bactericidal response and the consequent higher mycobacterial burdens. TNF constitutes an irreplaceable component of the Th1 response. Despite the induction of a Th1 response, as represented by IL-12 and IFN$\gamma$ secretion, mice deficient for TNF still succumbed to infection. Interestingly, it could be established that bone marrow reconstitution could rectify the abnormalities which were associated with TNF deficiency. This would indicate that haemopoietically derived TNF, probably from macrophages, are sufficient to restore these defects at least on a short term bases.

Infection of LT$^{-/-}$ and TNF$^{-/-}$ mice with Mycobacterium tuberculosis H37Rv established the absolute requirement for these cytokines during protective immunity against virulent mycobacterial infection. In addition, it confirmed the relative importance of TNF and LT in the immune response. Although at the high dose of 100cfu/lung both mouse strains displayed similar mortality rates, susceptibility of TNF$^{-/-}$ mice was distinctly higher than LT$^{-/-}$ mice at the lower dose of 10 cfu/lung.
The inability to establish pulmonary granulomas together with inefficient macrophage activation, as indicated by reduced iNOS induction, directly contributed to the high bacilli burden in LT−/− mice. Pathology was characterised by persistent cellular infiltration in response to an increasing mycobacterial burden resulting in tissue damage and early haematopoetic dissemination of bacilli. The presence of TNF was inadequate to substitute for LT, again supporting the unique role of each cytokine in the protective immunity.

The results of this study have also unequivocally demonstrated the downmodulatory role of IL-10 during the generation of a Th1 immune response against M. bovis BCG infection. In the absence of IL-10 mice developed an improved immune response as manifested by an increase in IL-12 and TNF production, an enhanced and persistent granulomatous response, increased cellular recruitment to sites of infection and an increase in macrophage activation, which all contributed to an accelerated rate of clearance of bacilli. It was interesting to note that the immunomodulatory role of IL-10 was not replaced by any other cytokine thus supporting a unique regulatory role for IL-10.
Future Investigations
Future Investigations

The importance of TNF in the host's immune response against mycobacterial infection has been unequivocally established (Bean et al., 1999; Jacobs et al., 2000; Kaneko et al., 1999). It is an omnipotent cytokine with biological effects influencing the immune system on several levels. However, several aspects of its metabolic function still remain to be elucidated. It is known that TNF exists in a soluble and trans-membrane form with both molecular forms being biologically active. Trans-membrane TNF was demonstrated to induce cytotoxicity (Kriegler et al., 1988; Decker et al., 1987), polyclonal B cell activation (Aversa et al., 1993) and modulate IL-10 production of monocytes (Parry et al., 1997). In addition, it was shown to be a proinflammatory cell surface molecule, which transmits bipolar signals during inflammation (Harashima et al., 2001). The contributions of each of these molecular forms to protective immunity against mycobacterial infection have not been addressed. The most informative experimental approach to address this question would encompass the use of an in vivo mouse infection model in which the cleavage site for TACE has been modified allowing the mouse only to induce the membrane form of TNF. Although transgenic trans-membrane mouse strains have been generated (Georgopoulos et al., 1996), these mice display an overproduction of trans-membrane TNF and display an abnormal phenotype. Ideally, a "TNF knock-in mouse model" in which the original TNF gene is replaced by a modified version under control of its endogenous promoter and shown to be fully functional, would contribute significantly to understanding the contribution of trans-membrane TNF. A recent publication by Ruuls et al. (2001) describing the phenotypic characteristics of such a strain holds enormous promise to investigate the role of trans-membrane TNF. Similarly, a mouse model, which incorporated a modified N-terminus, thus allowing only the production of secreted TNF, could be an invaluable tool to understanding its role in pathogenesis.

Several cell types are known to express TNF (Vasalli, 1992). With the advent of tissue and cell specific gene expression mouse models it would be interesting to investigate the contribution of each of these cell types in the protective immune response against mycobacterial infections. It is known that macrophages are the
predominant cells, which secrete TNF. Findings in this report, which indicates that the TNF deficiency could be rectified by bone marrow transplantation, supports this observation. However, T-cells have been shown to be able to produce TNF (Tsukaguchi et al., 1999) and together, with macrophages, form integral components of the adaptive and innate immune responses against mycobacterial infection, respectively. Metabolic cooperativity between these cell types is important for the initiation and establishment of an effective granulomatous response. It would therefore be insightful to establish the TNF contribution of each of these cell types to cell mediated immunity against mycobacterial infection.

The relative contribution of TNFR1 and TNFR2 as mediators of TNF and/or LT signaling during mycobacterial infection is still to be resolved. Although several publications (Flynn et al., 1995a; Senaldi et al., 1996) have illustrated the importance of TNFR1 in generating protective immunity, the importance of TNFR2 has not been addressed. The results in this study indicated that TNFR2 contributes to the host’s immune response albeit with reduced significance when compared to TNFR1. However, in this study avirulent Mycobacterium bovis BCG was used to challenge TNFR2−/− mice. A complete characterisation of the influence of TNFR2 and its role in virulent mycobacterial pathogenesis requires urgent investigation.

The importance of LT as a molecule of crucial importance to inhibit virulent mycobacterial infection has been illustrated in this study and by others (Roach et al., 2000). However, several lymphocytic subsets are thought to be important in protective immunity against mycobacterial pathogenesis. It would therefore be interesting to investigate the importance of each of these subsets to the contribution of the LT production pool in order to elucidate their respective functional relevance. The technology is available to inactivate LT in each of these cellular subsets in mice and would act as valuable investigative tools, not only in mycobacterial research, but more generally in infectious disease research applications. An interesting area of research would be to investigate the relative contributions of TNF and LT, given the homologous nature of the two molecules and the common receptors that they share. It is tempting to speculate on possible cooperation between these two molecules, particularly with respect to possible kinetic interdependence during the immune response.
IL-10 is generally regarded as a negative regulatory cytokine of the Th1 immune response, yet it has also been shown to actively promote the Th1 response during dendritic cell development (Fortsch et al., 2000). This dichotomous role of IL-10 indicates that metabolic function(s) of this molecule is rather more subtle than previously thought. From the findings in this study it was concluded that the absence of IL-10 enhanced mycobacterial clearance. IL-10 therefore could therefore potentially act as a drug target to enhance the immune response against mycobacterial infection. Further areas of investigation could also include the investigation of the combined effects of the absence of IL-10 and other potential negative regulatory cytokines such as TGFβ and IL-4 in infectious disease models.
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