Reactivation of persistent tuberculosis

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A thesis submitted for the
Degree of Doctor of Philosophy
at the University of Cape Town

Supervisor: Professor Bernhard Ryffel

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DECLARATION

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

TANIA BOTHA

DATE
Certification of supervisor

In terms of paragraph 9 of "General regulations for the degree of PhD", I as supervisor of the candidate, Tania Botha, certify that I approve of the incorporation in this thesis of material that has already been published.

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Department of Immunology
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University of Cape Town

25/07/03
Publications arising from this work


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Vir Pappa en Mamma
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<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>APES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>BCP</td>
<td>1-bromo-3-chloropropane</td>
</tr>
<tr>
<td>BCG</td>
<td><em>Mycobacterium bovis</em> Bacille Calmette-Guerin</td>
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<tr>
<td>BMDC</td>
<td>bone marrow-derived macrophages</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSL-3</td>
<td>Biosafety Level 3</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CCR2</td>
<td>chemokine receptor 2</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>cTNF-/-</td>
<td>conditional TNF gene deficient</td>
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<tr>
<td>DAB</td>
<td>3, 3-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DABCYL</td>
<td>4-(4′-dimethylaminophenylazo)benzoic acid</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<td>DMEM</td>
<td>Dulbecco's modification of Eagle's medium</td>
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<td>DMSO</td>
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<td>DTT</td>
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<td>ethylene diamine tetra acetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ES</td>
<td>embryonic stem</td>
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<tr>
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<td>fluorescence-activated cell sorter</td>
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<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>gram</td>
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<td>G</td>
<td>gauge</td>
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<td>human immunodeficiency virus</td>
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<td>IFN-γ</td>
<td>interferon-gamma</td>
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<td>IL</td>
<td>interleukin</td>
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<td>INH</td>
<td>isoniazid</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<td>interferon-gamma-inducible protein-10</td>
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<td>kanamycin resistance</td>
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<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<td>kilogram</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<td>lipopolysaccharide</td>
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<td>M</td>
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<td>mg</td>
<td>milligram</td>
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<tr>
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<tr>
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<td>messenger RNA</td>
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<td>μl</td>
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<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
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<tr>
<td>( n )</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>( neo^R )</td>
<td>neomycin resistance</td>
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<td>NO</td>
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<td>nitrate</td>
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<tr>
<td>NOS2</td>
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<td>OADC</td>
<td>oleic acid, dextrose and catalase</td>
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<td>OD</td>
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<td>p</td>
<td>probability</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>(^{32})P-dCTP</td>
<td>(^{32})P-radiolabelled deoxyctydine triphosphate</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>poly(I:C)</td>
<td>polynucleotide-polycytidylic acid</td>
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<tr>
<td>PZA</td>
<td>pyrazinamide</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated upon activation, normal T cell</td>
</tr>
<tr>
<td>RANTES</td>
<td>expressed and secreted</td>
</tr>
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<td>RE</td>
<td>restriction endonuclease</td>
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<td>RMP</td>
<td>rifampicin</td>
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<td>ribonuclease</td>
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<td>reactive nitrogen intermediates</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<td>reverse transcriptase</td>
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<td>RT-PCR</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>sigA</td>
<td>sigma factor A</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>TAE</td>
<td>Tris, acetic acid, EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris, boric acid, EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris, EDTA</td>
</tr>
<tr>
<td>$T_m$</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNF$^{-/-}$</td>
<td>tumour necrosis factor deficient</td>
</tr>
<tr>
<td>TNF-RI</td>
<td>tumour necrosis factor receptor I</td>
</tr>
<tr>
<td>TNF-RI$^{-/-}$</td>
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</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
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<tr>
<td>vol</td>
<td>volume</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>wt</td>
<td>weight</td>
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<td>ZN</td>
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ABSTRACT

Exposure to *Mycobacterium tuberculosis* results in clinical tuberculosis only in a small percentage of immunocompetent individuals. In most instances mycobacteria are controlled by the host immune system and survive in a dormant state within granuloma. Immunosuppression, however, may result in reactivation of active tuberculosis resulting in clinical disease.

Using low dose aerosol infection of *M. tuberculosis* in mice, a short-duration model of rifampicin-isoniazid (RMP-INH)-induced persistent tuberculosis is described. This persistent infection is characterised by undetectable levels of colony-forming units (CFU) in mouse organs and mice being clinically asymptomatic for prolonged periods. Reactivation of persistent tuberculosis can occur spontaneously following short-course chemotherapy or can be achieved by immunosuppression, specifically inhibition of macrophage-specific nitric oxide synthase (NOS2) by a chemical inhibitor, aminoguanidine. This model can therefore be used to characterise spontaneous or drug-induced reactivation of murine tuberculosis, as this is not feasible to study in human subjects. Additionally, this model may serve as a valuable tool for testing novel vaccines and antituberculous drugs, especially those designed to combat persistent infection.

Mycobacterial genome copy enumeration and assessment of 16S ribosomal RNA (16S rRNA) and sigma factor A (sigA) gene expression revealed that large numbers of dormant bacilli are present in lung tissue during the persistent phase of infection in this model. This finding opens up the possibility that additional gene expression profiles can be analysed with current technology, unravelling the exact metabolic status of these dormant mycobacteria.

Moreover, this model facilitates characterisation of another poorly understood aspect, namely reinfection. Preliminary aerosol reinfection
ABSTRACT

during the persistent phase of tuberculosis revealed that the primary-infected dormant \textit{M. tuberculosis} strain may be reactivated and may outgrow the primary strain during reinfection.

Tumour necrosis factor (TNF) deficient mice are known to be highly susceptible to \textit{M. tuberculosis} infection. In this study it was asked whether TNF is required for post-infectious immunity in aerosol-infected mice. This model was applied and mice were treated with RMP-INH for 4 weeks to reduce the CFU to undetectable levels. While wild-type control mice spontaneously reactivated but controlled the infection upon cessation of chemotherapy, TNF deficient mice developed fatal reactivation of infection. The increased susceptibility of TNF deficient mice was accompanied by diminished recruitment and activation of T cells and macrophages into the lung with defective granuloma formation and reduced inducible nitric oxide synthase expression. Reduced chemokine production in the lung might explain sub-optimal recruitment and activation of T cells and uncontrolled infection. Therefore, despite a massive reduction of the mycobacterial load by chemotherapy, TNF deficient mice were unable to compensate and mount a protective immune response. In conclusion, endogenous TNF is critical to maintain latent tuberculosis infection and in its absence no specific immunity is generated.
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Chapter 1: INTRODUCTION

1.1 Tuberculosis – a global problem

Tuberculosis kills at least 3 million people in the world every year, more than any other single infectious micro-organism (Dolin et al., 1994). However, this is a small number considering that one-third of the world population is infected with M. tuberculosis in a clinically asymptomatic latent state (Bloom & Murray, 1992; Kochi, 1991; Sudre et al., 1992). Reactivation of this vast reservoir of tuberculosis accounts for a substantial proportion of reactivated tuberculosis cases (Stead, 1967). In recent years, co-infection with human immunodeficiency virus (HIV) is probably the most important cause and biggest risk factor of reactivated tuberculosis (Antonucci et al., 1995; Narain et al., 1992; Raviglione et al., 1995). In view of the high incidence of HIV infection in southern Africa, tuberculosis has reached epidemic proportions and a high percentage of AIDS patients die as direct result of tuberculosis infection. It is therefore of utmost importance that factors contributing to the uncontrolled spread of this airborne disease be identified and acted upon.

1.2 Tuberculosis vaccines and chemotherapy

Despite the high mortality rate, tuberculosis is a curable disease. Existing vaccination offers only limited protection and current drug therapy involves a 6-month multi-drug regimen with non-compliance being a major concern. Linked to this is the emergence of multi-drug resistant tuberculosis bacilli, which are not curable. Therefore, more effective vaccines and shorter drug regimens with more efficient drugs need to be developed. To facilitate better understanding of the underlying failing immune mechanisms of the host and immune evasion strategies of M. tuberculosis, the mouse serves as a practical in vivo model.
1.3 **Mouse models of tuberculosis**

Little is understood of the underlying immune interaction between the host and the tubercle bacillus in its evasion of host immunity. Moreover, the mechanism of reactivation of persistent tuberculosis is difficult to investigate in humans. Animal models present opportunities to elucidate factors of the immune system involved in persistent and reactivated tuberculosis infection and better understanding of these mechanisms will facilitate improved treatment and better prevention of tuberculosis.

Several models have been developed to study persistence and reactivation of tuberculosis, among which the Cornell model was the first reported model (McCune & Tompsett, 1957; McCune et al., 1957; McCune et al., 1966a; McCune et al., 1966b). Upon intravenous administration of *M. tuberculosis* H37Rv and treatment with pyrazinamide (PZA) and isoniazid (INH) in their food for 12 weeks, mice seemed to have cleared all bacilli from organs, but after several months a substantial proportion spontaneously reactivated with acute disease (McCune & Tompsett, 1957; McCune et al., 1957). Since the Cornell model has been published, a few variations on this model have been reported (Botha & Ryffel, 2002; Flynn et al., 1998; Grosset, 1978; Scanga et al., 1999). The low bacterial burden during the persistent phase of infection makes it a realistic mouse model to study latency, however, the effect of antibiotics on the mycobacteria is not clear and may very well affect development of a protective immunity.

The alternative model that has been described, also known as the low-dose model (Flynn et al., 1998), involves low dose infection with tubercle bacilli in the absence of chemotherapy with the ensuing infection exclusively controlled by the host (Brown et al., 1995; Flynn et al., 1998; Mustafa et al., 1999; Orme, 1988; Phyu et al., 1998; Scanga et al., 1999). Although considered to reflect the human host response better, bacterial numbers in the organs of these mice remain high during the chronic persistent phase of infection, which is a disadvantage as it is not the case in humans. However,
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Immunosuppression in these mice leads to reactivation of infection, therefore it can be used to model persistence and reactivation of tuberculosis infection.

Modelling latent tuberculosis infection in the mouse remains difficult, as we do not have a clear picture of latent infection in humans. It is clear that both types of models described have advantages and disadvantages and should be chosen depending on the type of research question asked.

Reactivation of latent tuberculosis in the Cornell model has been induced with cortisone, a broad-spectrum immunosuppressant (McCune et al., 1966a; McCune et al., 1966b). Several more methods of reactivation have since been employed, including restraining mice resulting in activation of the hypothalamic-pituitary-adrenal axis (Brown et al., 1995), corticosterone administration (Phyu et al., 1998), aminoguanidine administration (Flynn et al., 1998), dexamethasone and glucocorticoid administration, and interferon-\(\gamma\) (IFN-\(\gamma\)) and TNF neutralisation (Scanga et al., 1999).

To date, both of these types of models have aided in identification of immune effectors participating in latent or chronic persistent and reactivated tuberculosis. Key immune factors that have been implicated in the control of persistent tuberculosis infection and reactivation of disease, are T cells (Flory et al., 1992; Flynn et al., 1992; Orme & Collins, 1984; Orme et al., 1992), reviewed in (Chan & Kaufmann, 1994), IFN-\(\gamma\) (Cooper et al., 1993; Flynn et al., 1993), TNF (Flynn et al., 1995), interleukin-12 (IL-12) (Cooper et al., 1997), and reactive nitrogen intermediates (RNI) (Chan et al., 1992; Chan et al., 1995; Denis & Ghadirian, 1991; Flesch & Kaufmann, 1991; MacMicking et al., 1997). A schematic representation of the cellular and cytokine components involved in cross-talk during tuberculosis infection is displayed in Figure 1.1.
Figure 1.1. Cellular immune response to tuberculosis. The response of the host upon tuberculosis infection involves a complex network of cytokine and cellular interactions. Black arrows and names indicate activating pathways, whereas dashed arrows and grey names represent inhibitory pathways.

Modified from: Trucks, 2000

1.4 Pathogenesis of tuberculosis infection

The lung is the point of entry for airborne infectious droplets, containing as little as one to three bacilli (Dannenberg & Rook, 1994). Primary infection usually occurs at the terminal alveoli, where bacilli are taken up by alveolar macrophages through various receptors (reviewed by Ehlers & Daffe, 1998). The outcome of the infection may have to do with the choice of receptor for entry into the macrophage, but it is as yet unclear. Once inside the macrophage, some mycobacteria are killed as macrophages are activated, while others survive and multiply while escaping the normal defence mechanisms, reviewed by (Flynn & Chan, 2003). When activated by IFN-γ and TNF, mouse macrophages produce bactericidal toxins, such as nitric
oxide (NO), one of two known direct killing agents against *M. tuberculosis* (Chan *et al.*, 1995; Flynn *et al.*, 1998; MacMicking *et al.*, 1997), the other being granulysin (Ernst *et al.*, 2000). Infected dendritic cells and possibly macrophages pass through lymph nodes and may disseminate mycobacteria throughout the body. Whether this results in development of active disease or latent infection depends on various factors and rests on the delicate balance between tissue-damaging and macrophage-activating immune responses (Dannenberg & Rook, 1994).

### 1.5 Persistence of tuberculosis infection

Infected individuals who develop clinically latent or persistent tuberculosis, can reactivate later in their life due to immune senescence, immunosuppression or immunosuppressive treatment. This allows dormant but viable mycobacteria to escape immune surveillance and reactivate into active tuberculosis, reviewed by (Flynn & Chan, 2001). The Wayne model has been developed to study the metabolic status of mycobacteria during dormancy *in vitro* (Wayne, 1976), reviewed in (Wayne & Sohaskey, 2001). This, however, does not necessarily reflect the status of tubercle bacilli *in vivo*, which is likely far more complex and still not understood.

De Wit and colleagues showed with conventional polymerase chain reaction (PCR) and with dot blot hybridisation that between $10^5$ and $10^6$ mycobacterial DNA copies were present during the persistent state in the Cornell model during the drug-free period when no bacilli were culturable (De Wit *et al.*, 1995). The disadvantage of this study was the uncertainty of the status of the detected mycobacteria – whether they were alive or dead could not be determined.
1.6 Reactivation of persistent tuberculosis infection

The struggle to control tuberculosis is further complicated by the ability of mycobacteria to exist in a persistent, latent, or dormant state. The mechanisms utilised by *M. tuberculosis* to establish such a persistent state and the signals that allow it to re-establish tuberculosis infection, remain unclear. Additionally, we lack in-depth understanding why and how this pathogen evades the host immune surveillance mechanisms.

Frequently, a persistent state can last for a person’s lifetime, but in 5% of cases, immunocompetent persons develop active tuberculosis within the first five years of infection. Another 5% will develop reactivated tuberculosis later on in their lives. Figure 1.2 shows the possible outcomes following exposure to *M. tuberculosis*. The specific outcome largely depends on factors influencing the integrity of the granuloma, which is responsible for isolating and containing the tubercle bacilli. The state of mycobacteria within the granuloma is not absolutely clear, but whether they slowly replicate or are dormant, they remain viable for a prolonged period. However, if the granuloma liquefies as result of tissue-damaging host immune responses, bacilli can multiply and disseminate to other parts of the lung and to the outside environment, where another person may be infected (Trucksis, 2000).
Figure 1.2. Possible outcomes following exposure to *M. tuberculosis*. After close contact, 30% of individuals become infected, with approximately 40% of them developing primary active tuberculosis and 60% harbouring a clinically asymptomatic persistent infection. Around 2 - 23% of immunocompetent individuals persistently infected will reactivate at a later stage, whereas HIV-infected patients may develop reactivated tuberculosis at a rate of 5 - 10% per year (Parrish *et al.*, 1998).

Adapted from Parrish *et al.*, 1998.

1.7 *M. tuberculosis* gene expression

In more recent advances, groups have begun to unravel gene expression profiles of *M. tuberculosis*. It was noted, using electron transmission microscopy of *M. tuberculosis*, that when bacilli are cultured under microaerobic or anaerobic conditions, the bacilli take on a spore-like form with a prominently thickened cell wall, which is not the case in aerobic growth conditions (Cunningham & Spreadbury, 1998). Additionally, they showed that mycobacteria increased expression of a 16-kDa heat shock protein, *hspX*, an α-crystallin homologue, during this state. It was hypothesised that the reduced oxygen tension of the growth conditions may trigger the mycobacteria to enter a dormant state and that this environment may be very similar to the conditions inside of a granuloma, an acidic milieu with low oxygen and high carbon dioxide levels (Cunningham & Spreadbury, 1998). In an *in vivo* study, a gene expression profile of selected genes involved in maintenance of a nonreplicating persistent state in the Wayne
model (Wayne & Sohaskey, 2001), showed a similar expression profile in the mouse lung during transition to the stationary phase of chronic infection (Shi et al., 2003).

It has been demonstrated that mycobacteria shift from an active metabolism to a low-metabolic non-multiplying dormant state during the transition from an oxygen-rich to an anaerobic milieu in vitro, when they switch to the glyoxylate cycle (Wayne & Lin, 1982). This change in metabolism permits them to utilise lipid substrates for survival in the granuloma. Thus, icl1, the gene for isocitrate lyase, which enables mycobacteria to utilise fatty acids as carbon source, is upregulated in the stationary phase of infection (McKinney et al., 2000) and is likely an important role-player in the maintenance of persistent tuberculosis.

Additionally, a sigma factor gene, sigF, has also been identified in M. tuberculosis. This sigma factor is related to sporulation sigma factors present in Streptomyces coelicolor and Bacillus subtilis. This fits in with the picture of thickening of the cell wall and other measures taken by mycobacteria to adapt and survive in a hostile environment during persistent infection (DeMaio et al., 1997). Therefore, understanding of the expression profiles of these and other candidate genes in M. tuberculosis will facilitate better understanding of the strategies employed by this pathogen in order to survive and evade host immunity and may lead to identification of novel drug targets.

1.8 The role of tumour necrosis factor in tuberculosis control

TNF plays a central role in granuloma formation, macrophage activation and bacterial killing. The protective immune response of TNF to mycobacterial infection has been assessed in vivo using two different approaches – gene knockout and antibody neutralisation technologies. Infected mice deficient in the 55 kDa TNF receptor (Flynn et al., 1995; Kindler et al., 1989), and mice in which the TNF gene has been disrupted (Bean et al., 1999; Jacobs
et al., 2000) have both shown defective granuloma formation and inability to control mycobacterial replication. Clear synergy has been demonstrated between IFN-γ and TNF for the stimulation of macrophage intracellular killing through the induction of RNI in vitro (Flesch et al., 1994) as well as in vivo by activation of inducible nitric oxide synthase (NOS2) in macrophages (Ding et al., 1988). The role of RNI has been demonstrated in NOS2 deficient mice (Garcia et al., 2000), which are highly susceptible to *M. tuberculosis* infection (MacMicking et al., 1997). Additionally, inhibition of NOS2 activity by a chemical inhibitor, aminoguanidine, results in reactivation of persistent tuberculosis in mice, which implicates that toxic nitrogen oxides are essential to control persistent infection (Botha & Ryffel, 2002; Flynn et al., 1998).

Apart from its protective effects in the immune response to *M. tuberculosis*, TNF also contributes to pathology in vivo, including caseous necrosis and cachexia, which are correlated with elevated TNF levels (Beutler & Cerami, 1988; Jacobs et al., 2000; Rook, 1990; Rook et al., 1987; Tracey & Cerami, 1992).

### 1.9 Aims of this study

Since the development of the Cornell model as drug-induced latency model (McCune & Tompsett, 1957; McCune et al., 1957; McCune et al., 1966a), it has not been extensively used due to its limitations: the intravenous route of infection, early initiation of chemotherapy, delivery of drugs in food pellets and corticosteroids by intraperitoneal (i.p.) injections, long duration of the model, large percentage of spontaneous reactivation and poor reactivation efficiency. This study aimed to provide a short-duration aerosol murine model of persistent infection and reactivation of tuberculosis and to overcome the above-mentioned limitations so that this type of model can be readily used. *M. tuberculosis* H37Rv was chosen as primary infectious agent and an antituberculous drug regimen consisting of rifampicin (RMP)
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and INH was used to attain dormancy of bacilli. Reactivation of this persistent state, defined as undetectable levels of bacilli in mouse organs for a prolonged period of time, was achieved by administration of aminoguanidine, an inhibitor of NOS2, as was first demonstrated by Flynn and colleagues (Flynn et al., 1998). The model in this study is therefore based on the original Cornell model (McCune & Tompsett, 1957; McCune et al., 1957; McCune et al., 1966a), but shows various improvements, greater reproducibility and is easier to perform.

Further characterisation of this model enabled enumeration of mycobacterial genome copy numbers by using quantitative molecular beacon PCR, which provided information on the actual numbers of unculturable organisms present in the mouse lung during the persistent phase of infection. Additionally, putative candidate genes likely to be important during persistent tuberculosis in vivo have been selected and attempts were made to quantify expression levels during the different phases of infection.

The role of TNF in the development of post-infectious immunity was elucidated using TNF deficient mice in this aerosol infection model where rapid and lethal reactivation of tuberculosis in TNF deficient mice was demonstrated despite complete clearance of culturable bacilli following chemotherapy. Reactivation correlated with reduced T cell recruitment and activation, as well as diminished chemokine production, preventing the formation of granulomas. Finally, newly generated conditional TNF-targeted mutant mice were characterised for their suitability in this model and future tuberculosis studies.
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2.1 Mice

2.1.1 Wild-type mice

C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA; University of Cape Town breeding stock, Cape Town, South Africa) of 8 to 12 weeks old were used as wild-type mice in this study. Mice were kept under specific pathogen-free conditions in autoclaved cages with microfilter lids, autoclaved bedding, food and water. All experiments performed were in accordance with the guidelines of the Animal Research Ethics Board of the University of Cape Town (Cape Town, South Africa), National Cancer Institute (Frederick, MD, USA) and The Rockefeller University (New York, NY, USA).

2.1.2 Gene-deficient mice

Homozygous TNF (Marino et al., 1997) gene-deficient mice (Taconic, Germantown, NY, USA) were kept under specific pathogen-free conditions as described above (section 2.1.1). The genotype of TNF−/− mice was confirmed by PCR analysis of DNA obtained from tail biopsies of the breeding colony maintained at the University of Cape Town.

2.1.2.1 DNA extraction from mouse tail biopsies

A 1-cm length mouse tail biopsy was suspended in 1.2 ml digestion buffer, consisting of 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% SDS (Biofluids, New England Biolabs Incorporated, Beverly, MA, USA), and 0.5 mg/ml Proteinase K (GIBCO-BRL, Carlsbad, CA, USA). It was incubated at 55°C with shaking for 12 – 16 hours. The digested tissue was centrifuged for 5 min at 13,000 rpm in a microcentrifuge, after which 700 µl
solution, excluding the top phase and the loose pellet, was transferred to a tube containing an equal volume equilibrated phenol/chloroform/isoamyl alcohol 25:24:1 (Sigma-Aldrich, St. Louis, MI, USA). Five hundred microlitres of the top organic phase was transferred to a fresh tube containing 0.7 volumes isopropanol (Sigma-Aldrich) after it was mixed and centrifuged at 13,000 rpm for 10 min. It was mixed and the DNA recovered by centrifugation at 13,000 rpm for 5 min. The DNA pellet was washed with 1 ml 70% ethanol (AAPER Alcohol and Chemical, Shelbyville, KY, USA) by centrifuging it for 5 min at 13,000 rpm. The ethanol was aspirated and the pellet was allowed to air dry for 5 min, after which the DNA was resuspended in 100 µl Tris-EDTA (TE) buffer (0.01 M Tris, 0.001 M EDTA, pH 8.0; New England Biolabs).

2.1.2.2 Determination of DNA concentration
In order to determine the DNA concentration spectrophotometrically, an aliquot of DNA was diluted 1:200 in sterile water and the absorbance determined at 260 nm (A$_{260}$) in an Ultrospec® 2000 (Pharmacia Biotech, Piscataway, NJ, USA). An absorbance value A$_{260}$ = 1 corresponds to 50 µg/ml double stranded DNA. The A$_{260}$ : A$_{280}$ ratio was determined to assess the purity of the DNA.

2.1.2.3 PCR genotyping of TNF$^-^>$ mice
TNF$^-^>$ mice were bred at the Animal Facility of the University of Cape Town and were distinguished from wild-type mice by yielding a 1200 bp product after PCR amplification with the JT428-JT427 primer set (Table I$^1$). Wild-type mice yielded a 750 bp product after PCR amplification with the JT429-JT427 primer set. Each PCR reaction was performed with 0.5 µg DNA in a final volume of 25 µl, with the addition of 2.5 µl 10x PCR buffer, 0.25 µl 10 mM dNTPs, 2.5 µl of each primer at a working concentration of 10 µM, and 0.25 µl 5 U/µl Taq polymerase (Promega, Southampton, UK).

$^1$ Primer sequences kindly provided by Dr. M. Marino, Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, NY, USA.
The PCR programme proceeded as follows:
Initial denaturation at 94°C for 5 min, followed by 30 cycles of:

- **Denaturing**: 94°C for 1 min;
- **Annealing**: 55°C for 1 min;
- **Extension**: 72°C for 2 min;

The final extension step was continued for 10 min and the samples were held at 4°C.

### Table I. PCR primers for genotyping TNF$^\alpha$ mice$^2$

<table>
<thead>
<tr>
<th>Primer function</th>
<th>Name</th>
<th>Orientation</th>
<th>Primer sequence</th>
<th>$T_m$</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF$^\alpha$ amplification</td>
<td>JT428</td>
<td>Forward</td>
<td>5'-CCT TCT ATC GCC TTC TTG ACG-3'</td>
<td>59.8°C</td>
<td>1200 bp</td>
</tr>
<tr>
<td></td>
<td>JT427</td>
<td>Reverse</td>
<td>5'-AGA TAG CAA ATC GGC TGA CGG-3'</td>
<td>59.8°C</td>
<td></td>
</tr>
<tr>
<td>WT amplification</td>
<td>JT429</td>
<td>Forward</td>
<td>5'-ATC AGT TCT ATG GCC CAG ACC-3'</td>
<td>59.8°C</td>
<td>750 bp</td>
</tr>
<tr>
<td></td>
<td>JT427</td>
<td>Reverse</td>
<td>5'-AGA TAG CAA ATC GGC TGA CGG-3'</td>
<td>59.8°C</td>
<td></td>
</tr>
</tbody>
</table>

PCR products were separated and visualised on a 1.6% agarose gel (Seakem, Cambrex Bio Science, Rockland, ME, USA), made with TBE buffer (0.89 M Tris, 0.89 M boric acid, 20 mM Na$_2$EDTA, pH 8.3; Merck Ltd., Poole, Dorset, UK).

#### 2.1.3 Conditional gene targeted mice

Conditional TNF gene targeted mice were obtained from Drs. S. Nedospasov$^3$ and D. Kuprash$^4$ and were generated as follows. Homologous recombination in embryonic stem (ES) cells was employed to generate mice bearing the floxed TNF allele. LoxP sites were inserted on both sides of the TNF locus in a targeting vector and a neomycin resistance cassette was inserted upstream of the TNF locus. The vector was linearised by digestion with BamHI and electroporated into mouse ES cells. Transfected cells were

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$^2$ Primers were obtained from the Department of Biochemistry, University of Cape Town, Cape Town, South Africa.

$^3$ Laboratory of Molecular Immunoregulation, National Cancer Institute, Frederick, MD, USA; Engelhardt Institute of Molecular Biology, Russian Academy of Science, Moscow State University, Moscow, Russia.

$^4$ Engelhardt Institute of Molecular Biology, Russian Academy of Science, Moscow State University, Moscow, Russia.
cultured in the presence of geneticin (G418; Sigma-Aldrich) and G418-resistant clones were further expanded. DNA was prepared and clones with homologous recombination were identified by Southern blot analysis using a probe, designated mER4 (Figure 2.1). A homologous recombinant ES clone was injected into a blastocyst of a C57BL/6 mouse and chimeras were born. Germ line transmission of the floxed TNF allele was obtained by crossing chimeras with C57BL/6 mice and confirmed by Southern blot analysis of tail biopsy DNA using the mER4 probe. Mice homozygous for floxed TNF alleles were crossed with Mx-Cre transgenic mice (cre gene under the control of the Mx promoter) to obtain TNF$^{fl}$ x Cre mice.

Figure 2.1. Conditional gene targeting of TNF. (A) Schematic representation of the wild-type TNF allele in wild-type mice indicating the position of the mER4 probe, the adjacent genes, Lymphotoxin-β (LT-β) and Lymphotoxin-α (LT-α), as well as respective orientations. (B) The targeting vector, with loxP sites and neomycin resistance (neo') cassette introduced. (C) The floxed TNF allele as result of homologous recombination, which will yield a 11 kb fragment when digested with BamHI and probed with mER4. (D) The deleted TNF allele as result of Cre-mediated recombination at the loxP sites, which will yield a 8.6 kb fragment when digested with BamHI and probed with mER4.

2.1.3.1 Genotyping of TNF$^{fl}$ x Cre mice
During the breeding of TNF$^{fl}$ and TNF$^{fl}$-mice with Cre mice, mice were genotyped for the presence of the cre transgene and for homozygous floxed TNF alleles. Each PCR reaction was performed separately with 0.5 μg DNA in a final volume of 20 μl, with the addition of 2 μl 10x MgCl₂-free PCR II buffer, 2 μl 100 mM dNTPs, 1.2 μl 25 mM MgCl₂ (Perkin-Elmer, Norwalk,
CT, USA), 0.2 µl of each primer at a working concentration of 1 µM, and 0.2 µl 5 U/µl AmpliTaq™ DNA polymerase and 0.2 µl AntiTaq™ antibody (Perkin-Elmer). The AmpliTaq™ polymerase and AntiTaq™ antibody were mixed together and left to incubate for 5 min at room temperature before they were added to the rest of the PCR mixture.

The interleukin-2 (IL-2) gene was amplified as internal control in a separate reaction as amplification of the cre transgene and the floxed TNF alleles. Primer sequences are provided in Table II.

### Table II. PCR primers for genotyping TNF<sup>fl</sup> × Cre mice

<table>
<thead>
<tr>
<th>Primer function</th>
<th>Name</th>
<th>Orientation</th>
<th>Primer sequence</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Product size</th>
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<tr>
<td>IL-2 amplification</td>
<td>IMR0042</td>
<td>Forward</td>
<td>5'-CTA GGC CAC AGA ATT GAA AGA TCT AGC ATC ATC-3'</td>
<td>65.2°C</td>
<td>323 bp</td>
</tr>
<tr>
<td></td>
<td>IMR0043</td>
<td>Reverse</td>
<td>3'-GTA GGT GGA AAT TCT AGC ATC ATC-5'</td>
<td>65.2°C</td>
<td></td>
</tr>
<tr>
<td>Cre amplification</td>
<td>IMR1084</td>
<td>Forward</td>
<td>5'-GCG GTC TGG CAG TAA AM CTA TC-3'</td>
<td>66.6°C</td>
<td>101 bp</td>
</tr>
<tr>
<td></td>
<td>IMR1085</td>
<td>Reverse</td>
<td>3'-GTG AAA CAG CAT TGC TGT CAC TT-5'</td>
<td>65.1°C</td>
<td></td>
</tr>
<tr>
<td>TNF&lt;sup&gt;fl&lt;/sup&gt; amplification</td>
<td>K041</td>
<td>Forward</td>
<td>5'-TGA GTC TGT CTT AAC TAA CC-3'</td>
<td>61.4°C</td>
<td>WT 350 bp</td>
</tr>
<tr>
<td></td>
<td>K042</td>
<td>Reverse</td>
<td>3'-CCC TTC ATT CTC AAG GCA CA-5'</td>
<td>64.8°C</td>
<td>TNF&lt;sup&gt;fl&lt;/sup&gt; 400 bp</td>
</tr>
</tbody>
</table>

All primer sets were used in the following PCR programme:

Initial denaturation at 94°C for 2 min, followed by 35 cycles of:
- Denaturing: 94°C for 1 min;
- Annealing: 62°C for 1 min;
- Extension: 72°C for 1 min 30 sec;

After the last cycle samples were held at 4°C.

PCR products were separated and visualised on a 1.8% agarose gel made up with TBE buffer.
2.2 Mycobacteria

Mice were infected with *M. tuberculosis* H37Rv\textsuperscript{5} and *M. tuberculosis* Erdman, respectively, to ascertain whether genetic diversity between these two strains would influence the outcome of infection. In the reinfection study, mice were primary infected with *M. tuberculosis* H37Rv, then reininfected with *M. tuberculosis* H37Rv-5\textsuperscript{6}, a kanamycin resistant (*\text{kan}^R*) variant of the parent strain, in order to quantify the strains of primary infection and reinfection differentially. Briefly, this strain was generated using a pUC19 backbone plasmid, containing the complete L5 integrase gene (*int*), a phage attachment site (*\text{attP}*), and an insert conferring *\text{kan}^R* (*aph*).

2.2.1 Liquid culturing of *M. tuberculosis* H37Rv, H37Rv-pIVT and Erdman

*M. tuberculosis* H37Rv and Erdman, and the *\text{kan}^R* strain H37Rv-5 were cultured in 50 – 100 ml volumes Middlebrook 7H9 medium (Difco Laboratories, Sparks, MD, USA), supplemented with 10% Middlebrook oleic acid, dextrose, and catalase (OADC) enrichment medium (Becton Dickinson Microbiology Systems, Sparks, MD, USA), 0.5% glycerol (Fisher Scientific, Pittsburgh, PA, USA), and 0.05% Tween 80 (Sigma-Aldrich). Cultures were incubated at 37°C and growth was monitored by measuring optical densitites at OD\text{600}. Cultures were harvested during mid-log phase, typically at an OD\text{600} value between 0.4 and 0.8. Aliquots were either freshly prepared for infection or frozen at -80°C in phosphate-buffered saline (PBS) containing 15% glycerol and 0.05% Tween 80. Titres were determined by plating 10-fold serial dilutions onto 7H10 agar, supplemented with 10% OADC and 0.5% glycerol and enumerating mycobacterial colonies after incubation for 21 days at 37°C.

\textsuperscript{5} Kindly provided by Dr. G. Kaplan, originally from The Rockefeller University, New York, USA; currently at the Laboratory of Mycobacterial Immunity and Pathogenesis, Public Health Research Institute, Newark, New Jersey, USA.

\textsuperscript{6} Kindly provided by Dr. J. Gomez, Department of Infection Biology, The Rockefeller University, New York, USA.
2.2.2 Preparation of fresh bacterial cultures for aerosol infection

An aliquot, approximately 30 ml mycobacterial culture, was harvested during mid-log phase, pelleted for 5 min at 3000 rpm in a benchtop centrifuge, and resuspended in 10 ml sterile PBS containing 0.05% Tween 80. This suspension was then diluted to $10^6 - 10^8$ bacteria/ml ($\text{OD}_{600} = 0.01 - 0.1$) in a final volume of 40 ml for aerosol infection, as determined by the $\text{OD}_{600}$, subsequently confirmed by plating.

2.2.3 Preparation of bacteria from frozen stocks for aerosol infection

Prior to use, an aliquot of frozen bacteria was thawed, vortexed and diluted in sterile PBS containing 0.05% Tween 80 and clumping disrupted by aspirating through a 29-G needle (Omnican®, Braun, Kronberg, Germany) 20 times.

2.3 Aerosol infection of mice

Mice were exposed to an aerosol of mycobacteria produced by nebulising 5 ml of a suspension of $10^6 - 10^8$ bacilli/ml in PBS containing 0.05% Tween 80, using an inhalation exposure system (Glas-Col, Terre Haute, Indiana, USA). Alternatively, mice were exposed to an infective aerosol in a custom-built aerosol exposure chamber (Mechanical Engineering Shops, University of Wisconsin, WI, USA) by nebulising 30 ml of a $10^6 - 10^8$ bacilli/ml suspension in PBS containing 0.05% Tween 80 for exactly 20 minutes.

2.4 Quantitation of viable mycobacteria in mouse organs: Colony forming units

The initial infective dose of *M. tuberculosis* was verified by sacrificing 5 to 10 mice 24 hours after aerosol exposure. Mice were euthanased by carbon
dioxide narcosis and lungs were aseptically removed, weighed, homogenised in PBS containing 0.05% Tween 80. Ten-fold serial dilutions were plated onto 7H10 agar, supplemented with 10% OADC and 0.5% glycerol, in duplicate. During the reinfection study, *M. tuberculosis* H37Rv-5 was cultured on plates containing 12.5 μg/ml kanamycin (Sigma-Aldrich) in order to differentiate it from the primary H37Rv strain. Plates were incubated at 37°C and CFU enumerated after 21 days, except when culturing mycobacteria during and following exposure to antibiotics, when plates were incubated for 6 weeks before enumeration. At specific time points, mice were sacrificed and their lungs, livers and spleens aseptically removed and weighed. Each organ was homogenised in PBS containing 0.05% Tween 80 for CFU enumeration as described above.

2.5 Antibiotic treatment of mice

Two weeks post-infection, groups of mice were treated with 0.1g/L RMP and 0.1g/L INH (Sigma-Aldrich) delivered *ad libitum* in drinking water (changed weekly) for 4, 8 or 12 weeks, respectively. The experimental design, indicating variations in treatment and reactivation of models A, B and C, is depicted in Figure 2.2. *M. tuberculosis*-infected control mice received plain drinking water *ad libitum*. 

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2.6 Immunosuppression of mice

In models B and C (Figure 2.2) mice were given 2.5% (wt/vol) aminoguanidine (Sigma-Aldrich) ad libitum in drinking water, which was changed weekly, in order to inhibit NOS2 and induce reactivation of tuberculosis (Flynn et al., 1998). Volumes of drinking water containing drugs were monitored weekly to verify that mice consumed adequate dosages. M. tuberculosis-infected control mice received plain drinking water ad libitum.

2.7 Induction of gene deletion in TNF\textsuperscript{ff} x Cre mice

In order to induce Cre-mediated deletion of the floxed TNF gene, 6- to 10-week-old TNF\textsuperscript{ff} x Cre mice were injected i.p. with a series of 250 μg polyinosine-polycytidylic acid (poly(I:C)) (Sigma-Aldrich) in 250 μl saline (0.83% NaCl) on days 0, 2, and 5, adapted from Kuhn and colleagues (Kuhn et al., 1995). Forty-eight hours after the last injection, on day 7, mice were
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sacrificed and various organs obtained for further analysis. Poly(I:C) is a synthetic analogue of double stranded RNA which induces interferon-α/β (IFN-α/β) expression, which in turn activates the Mx-promoter, which is responsible for Cre recombinase expression and subsequent excision of the LoxP-marked TNF alleles. The action of poly(I:C) on Cre-mediated deletion is depicted in Figure 2.3.

Figure 2.3. Action of poly(I:C) on Cre-mediated TNF deletion. Poly(I:C), double-stranded RNA, induces IFN-α/β expression, which in turn activates the Mx-promoter, which controls Cre recombinase expression. The action of Cre recombinase on floxed TNF alleles will result in recombination at the loxP sites, therefore the deletion of TNF.

2.8 Analysis of gene deletion in TNF<sup>fl/fl</sup> × Cre mice

2.8.1 Collection of mouse organs

After euthanasing mice, the following organs were obtained: peripheral blood through cardiac puncture (300 – 500 µl), inguinal lymph nodes (both nodes), mesenteric lymph nodes (all), liver (10%), spleen (25%), pancreas (total), kidney (20%), duodenum (total), bone marrow (from one femur), muscle (2 g), lung (25% of left lobe), heart (25%), thymus (25%), brain (25%), and tail (1 cm length). Tissues were collected in microfuge tubes.
and flash-frozen in liquid nitrogen, except for the blood, bone marrow and tail. Blood was obtained through cardiac puncture into a heparinised syringe with a 20G needle (BD Biosciences, Franklin Lakes, NJ, USA) and transferred to a microfuge tube containing 10 μl heparin (Sigma-Aldrich). Blood was kept at 4°C until further processing, at which time red blood cells were lysed by addition of 2 ml ACK lysing buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA; New England Biolabs) and incubation on ice for 5 min. The suspension was centrifuged in a microfuge at 1,200 rpm for 5 min at 4°C and the supernatant discarded. The pellet was resuspended in 700 μl digestion buffer containing Proteinase K and the sample was incubated at 55°C with shaking for 12 – 16 hours. Each mouse femur was collected into a microfuge tube and kept on ice until bone marrow was extracted. Both sides of the femur were cut off and the bone marrow flushed out with 1.2 ml digestion buffer containing Proteinase K. Mouse tails were digested in 1.2 ml digestion buffer containing Proteinase K without prior processing. Flash-frozen tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle. The ground organs were each suspended in 1.2 ml digestion buffer, containing Proteinase K, and were digested by incubation at 55°C for 12 – 16 hours with shaking.

### 2.8.2 Genomic DNA extraction from mouse tissue

After tissue digestion was complete, 700 μl of each tissue suspension was mixed with an equal volume equilibrated phenol/chloroform/isoamyl alcohol and centrifuged in a microcentrifuge at 13,000 rpm for 10 min. The supernatant from each was transferred to a fresh tube and residual RNA removed by incubating for 1 hour at 37°C with the addition of 100 μg RNase (Promega). The DNA was re-extracted by an equal volume of phenol/chloroform/isoamyl alcohol and centrifuged for 10 min at 13,000 rpm. The supernatant from each tube was transferred to a fresh tube containing 2 volumes absolute ethanol and 0.5 volumes ammonium acetate (New England Biolabs). The solutions were mixed and centrifuged for 5 min at 13,000 rpm. The supernatants were aspirated and the pellets washed with 1 ml 70% ethanol for 5 min at 13,000 rpm. After aspirating the ethanol, the
pellets were briefly air-dried and the DNA resuspended in 300 – 500 µl TE buffer. Mouse-tail DNA was isolated as described in before (Section 2.1.2.1).

DNA concentration was assessed spectrophotometrically as described before (Section 2.1.2.2) and 100 ng DNA of each sample was electrophoresed on 0.6% agarose for visual assessment.

2.8.3 Restriction endonuclease digestion and agarose gel-electrophoresis of genomic DNA

Ten microgram genomic DNA from each sample was digested with 100 U BamHI restriction endonuclease in 1 x NE Buffer (150 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9; New England Biolabs), supplemented with 100 µg/ml bovine serum albumin (BSA) in a final volume of 300 µl. Digestion proceeded at 37°C for 12 – 16 hours, after which DNA was extracted with an equal volume phenol/chloroform/isoamyl alcohol and centrifugation at 13,000 rpm for 10 min. The upper phase was transferred to a tube containing 2 volumes absolute ethanol and 0.5 volumes ammonium acetate. The solutions were mixed and centrifuged for 5 min at 13,000 rpm. The supernatants were aspirated and the pellets washed with 1 ml 70% ethanol for 5 min at 13,000 rpm. After aspirating the ethanol, the pellets were briefly air-dried and the DNA resuspended in 13 µl TE buffer. Two microlitres DNA loading buffer (50% glycerol, 0.4% bromophenol blue, 0.4% xylene cyanol; Sigma-Aldrich) was added and the DNA electrophoresed in tracks on a 0.7% agarose gel in TAE buffer (0.89 M Tris, 0.89 M acetic acid, 20 mM Na₂EDTA, pH 8.3; New England Biolabs). Four micrograms of a 1 Kb DNA ladder molecular weight marker (Gibco-BRL) was run alongside and electrophoresis proceeded in a sealed chamber at 15 V for 24 hrs.
2.8.4 Preparation of the mER4 probe

The mER4 probe, homologous to part of the lymphotoxin-β gene (Figure 2.1), was cloned and propagated in a plasmid vector. The plasmid was transformed into competent *Escherichia coli* HB101 cells (Promega), using the heat-shock method. Ten nanograms plasmid DNA in a volume of 1 – 5 µl was added to 25 µl chilled competent HB101 cells, gently mixed by swirling and incubated on ice for 30 min. This mixture was heat-shocked for 45 sec at 42°C. Cells were incubated on ice for 2 min, resuspended in 975 µl SOC medium (New England Biolabs) and incubated at 37°C for 1 hour. Cells were plated on Luria-Bertani (LB) agar plates (1% NaCl, 1% tryptone, 0.5% yeast extract, 1.5% agar; Difco Laboratories) containing 50 µg/ml ampicillin (Sigma-Aldrich) and incubated at 37°C for 16 hours. Transformants were grown in LB medium at 37°C for 16 hours and DNA isolated with a Qiagen® plasmid midi kit (Qiagen, Valencia, CA, USA), using the manufacturer's protocol. DNA was analysed on a 1% agarose gel and the mER4 fragment excised from the plasmid by restriction endonuclease digestion with *Bam*HI and *Eco*RI (New England Biolabs) for 1.5 hours at 37°C. The linearised plasmid and mER4 insert DNA were separated on a 0.6% agarose gel, the mER4 DNA excised and purified using a QIAquick™ gel extraction kit (Qiagen), then quantified by spectroscopy and agarose gel visualisation.

2.8.5 Southern blotting and radio-labelling of the mER4 probe

Following electrophoresis of genomic DNA samples, the agarose gel was photographed alongside a ruler for subsequent orientation of bands on the Southern blot. The gel was rinsed by gently shaking it for 5 min in distilled water. The DNA in the gel was denatured by gently shaking the gel in 500 ml denaturing solution (New England Biolabs) twice for 20 min, followed by neutralisation in 500 ml neutralisation solution (New England Biolabs) twice for 20 min. The gel was rinsed in water before setting it up for Southern blotting as follows. Two long wicks adsorbent paper, prewetted with 10 x SSC buffer (New England Biolabs), were set up in a Southern blot
chamber, extending into both buffer reservoirs of the chamber. The gel was placed on the SSC-soaked wicks and a nylon membrane (Hybond™-N\textsuperscript{+}; Amersham Pharmacia Biotech, Piscataway, NJ, USA), prewetted in distilled water, placed on top of the gel. Air bubbles were rolled out carefully and an 8-cm stack blotting paper was placed on top of the membrane. Finally, a glass plate was placed on top with a 300 g weight. The buffer reservoirs were filled with 10 x SSC buffer and Southern transfer was allowed to proceed for 16 hours. After the Southern blot chamber was dismantled, the membrane was rinsed for 5 min in 2 x SSC buffer while rotating on a platform shaker. The blot was allowed to air-dry for 10 min, after which the DNA was cross-linked under UV light in a UV Stratalinker™ 2400 (Stratagene, La Jolla, CA, USA). The membrane was stored at room temperature between 2 sheets of blotting paper until hybridisation.

The mER4 probe was labelled with $^{32}$P-dCTP (Amersham Pharmacia) prior to hybridisation with the digested mouse genomic DNA. Fifty nanograms probe DNA was combined with water to a final volume of 50 µl and was denatured by boiling for 3 min and flash-cooling on ice. The denatured DNA was transferred to a single reaction mixture bead using a Ready-To-Go™ labelling beads (-dCTP) kit (Amersham Pharmacia) and mixed with 5 µl (50 µCi) $^{32}$P-dCTP. Radioactive incorporation proceeded during incubation for 30 min at 37°C, after which the reaction was stopped by addition of 5 µl EDTA (pH 8.0). A ProbeQuant™ micro G-50 column (Amersham Pharmacia) was prepared according to the manufacturer's recommendations and the labelled probe mixture was centrifuged through the column for 2 min at 3,000 rpm in order to remove unincorporated nucleotides. A 2-µl aliquot was removed prior to centrifugation and another 2-µl aliquot after purification to assess the efficiency of radioactive incorporation before proceeding with hybridisation of the membrane.

2.8.6 Hybridisation of mouse genomic DNA to mER4

Prior to hybridisation, the membrane containing BamHI-digested genomic DNA was moistened in 6 x SSC buffer, then prehybridised for 2 hours in 10
ml prewarmed ExpressHyb™ (BD Biosciences Clontech, Palo Alto, CA, USA) hybridisation solution at 65°C with gentle rolling. The purified radio-labelled probe was denatured by boiling for 3 min and flash-cooling on ice. It was mixed with 10 ml fresh prewarmed hybridisation solution and the membrane was hybridised for 16 hours at 65°C with gentle rolling.

The hybridisation solution was decanted and the membrane rinsed in a prewarmed (65°C) wash solution of 2x SSC/0.1% SDS. The membrane was then washed twice at 65°C for 20 min in 2x SSC/0.1% SDS, then twice at 65°C for 20 min in 0.2x SSC/0.1% SDS, followed by two more washes at 65°C for 20 min in 0.1x SSC/0.1% SDS. The membrane was finally rinsed in 2x SSC buffer, sealed in Saran wrap and exposed to a phosphorimager for 24 – 72 hours at room temperature.

2.9 Assessment of TNF levels in TNF$^{fr}$ x Cre mice ex vivo

TNF levels were determined in different cellular compartments in TNF$^{fr}$ x Cre mice before and after Cre-mediated deletion of the TNF locus. Wild-type and TNF$^{fr}$ mice served as controls.

2.9.1 Lipopolysaccharide stimulation in vivo

Wild-type C57BL/6 control, TNF$^{fr}$ control, and TNF$^{fr}$ x Cre mice before or after poly(I:C)-induced gene deletion were stimulated lipopolysaccharide (LPS, *E. coli* serotype 0111:B4; Sigma-Aldrich) by injecting them i.p with 100 µg LPS in 500 µl PBS, using a 1 ml-syringe with a 29-G needle. Mice were euthanased 90 min later and blood, spleen and bone marrow obtained and processed as follows.

2.9.2 Preparation of whole blood

Whole blood (300 – 500 µl) was obtained from cardiac puncture immediately after euthanasing each mouse using with a heparinised syringe with a
29-G needle. Blood was diluted 1:2 with PBS-Heparin at 4 U heparin per millilitre PBS. This mixture was split in 2 equal aliquots into 2 wells of a flat bottom 24-well plate (Corning Incorporated, Corning, NY, USA), and incubated at 37°C with 5% CO₂.

2.9.3 Preparation of splenocytes

The whole spleen was aseptically removed from each mouse 90 min following LPS stimulation and placed into 5 ml cold PBS buffer and kept on ice. The spleen was washed through a 100 µm FALCON® nylon mesh cell strainer (BD Biosciences) with 10 ml cold PBS buffer, after which 3 ml ACK Lysing buffer was added and the mixture incubated on ice for 3 min. Splenocytes were collected by centrifugation at 1,200 rpm for 5 min at 4°C in a benchtop centrifuge. The supernatant was discarded and the cell pellet was resuspended in 5 ml cold PBS buffer. The splenocytes were washed by centrifuging for 5 min at 1,200 rpm at 4°C and were resuspended in 5 ml cold complete RPMI-1640 medium (BD Biosciences), containing 10% foetal calf serum (FCS) (BD Biosciences). The cell suspension was passed through a 70-µm nylon mesh strainer and washed through with an additional 5 ml complete RPMI-1640 medium. Splenocytes were pelleted by centrifuging at 1,200 rpm for 5 min at 4°C and resuspended in 5 ml cold complete RPMI-1640 medium and kept on ice while a 5 µl aliquot of cells were counted in a haemacytometer after staining with Trypan blue (Sigma-Aldrich). Splenocytes were then plated at 2 x 10⁶ cells/ml (2 ml total volume) in 2 wells of a flat bottom 12-well plate (Corning) and incubated at 37°C with 5% CO₂.

2.9.4 Preparation and culturing of bone marrow-derived macrophages

Both femurs and tibia were aseptically removed from each mouse 90 min following LPS administration. Tips were cut off the ends of bones and bone marrow washed through a 100-µm nylon mesh strainer with cold PBS buffer. After each bone was rinsed twice, the strainer was rinsed with additional
cold PBS buffer. Cells were washed essentially as described before (Section 2.9.3). After cells were collected by centrifugation at 1,200 rpm for 5 min at 4°C, the supernatant was discarded and the cell pellet resuspended in 5 ml cold PBS buffer. The cells were washed again and resuspended in cold complete DMEM medium (BD Biosciences), supplemented with 20% horse serum (BD Biosciences), 30% leukocyte conditioned medium (LCM; BD Biosciences), as source of cytokines, 1% Glutamine/Penicillin/Streptomycin (Sigma-Aldrich) and 1% HEPES™ (BD Biosciences). The cell suspension was plated in a microbiological petri dish (Fisher Scientific) and incubated at at 37°C with 5% CO₂ for 6 days to allow the BMDM to expand. On Day 6, the supernatant was transferred to a prechilled 50 ml FALCON™ screw-cap tube (BD Biosciences) and the petri dish rinsed with 10 ml cold PBS buffer after the PBS was left in the plate on ice for 10 min for the adherent macrophages to detach. BMDM were pelleted at 1,200 rpm for 5 min at 4°C in a benchtop centrifuge and resuspended in 5 ml complete DMEM medium, after which a 5-μl aliquot of cells was counted in a haemacytometer. Cells were plated at 2 x 10⁶ cells/ml (in a 2 ml total volume) in duplicate in wells of a flat bottom 12-well plate and incubated at 37°C with 5% CO₂ for a further 4 days to expand before LPS stimulation.

2.9.5 Lipopolysaccharide re-stimulation of cells

Whole blood and splenocyte samples were restimulated with LPS immediately after preparation, whereas the BMDM culture was restimulated on Day 10, after sufficient expansion and maturation of cells. In each case, one well was used for restimulation with LPS, while the other served as an unstimulated control. LPS was added to each test well at 10 ng/ml and incubated for 4 hours at 37°C with 5% CO₂, after which the contents of each well was transferred to a microfuge tube and centrifuged at 3,000 rpm for 3 min. The supernatants were transferred to clean tubes and stored at -20°C until ELISA analysis.
2.9.6 **TNF measurement**

TNF ELISA was performed with each of the whole blood, splenocyte and BMDM samples and their controls in duplicate, using a Quantakine® Mouse TNF-\(\alpha\) Immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The assay employed the quantitative sandwich enzyme immunoassay technique with a pre-coated affinity-purified polyclonal antibody specific for mouse TNF.

2.10 **Assessment of the effect of poly(I:C) on *M. tuberculosis* infection in C57BL/6 mice**

C57BL/6 mice were infected with 30 CFU *M. tuberculosis* H37Rv via aerosol, as described before (Section 2.3). To assess if poly(I:C) administration to mice during *M. tuberculosis* infection would have any effect on disease progression, a group of infected mice received a series of poly(I:C) i.p. injections, as previously described (Section 2.7), on days 0, 2 and 5, starting 2 weeks post-infection. The clinical health of mice and mouse body weights were monitored during this period. Groups of mice were sacrificed 2 days following the last injection (1 week since the first injection), as well as 1 week later, in order to assess mouse organ weights, organ histopathology, and mycobacterial replication in the lungs, livers and spleens.

2.11 **Quantitation of mycobacterial genes in mouse lung tissue**

2.11.1 **M. tuberculosis DNA isolation from lung tissue**

The right lobes of each mouse lung were homogenised in 3 ml PBS containing 0.05% Tween and used for plating to enumerate CFU (Section 2.4). One-and-a-half millilitres of lung homogenates were frozen at -80°C until DNA extraction. Each homogenate was thawed and centrifuged at
3000 rpm for 5 min at 4°C to pellet the bacteria. The pellet was resuspended in 1 ml TE buffer and two equal aliquots transferred to two 2 ml o-ring tubes containing warm phenol (70°C) (Fisher Scientific) and 250 μl 0.1 mm sterile zirconia/silica beads (Biospec Products, Bartlesville, OK, USA). These suspensions were bead-beated in a bead-beater (Biospec Products) 3 times for 1 min at a time, with 1 min intervals, after which they were centrifuged at 12,000 g for 10 min and supernatants transferred to clean microfuge tubes. Tubes were decontaminated at this point and transferred from the Biosafety Level 3 laboratory. The phenol extraction step was repeated and followed by a chloroform (Fisher Scientific) extraction. DNA was finally precipitated at 4°C by addition of 0.1 volumes 5M NaCl and 0.7 volumes isopropanol (Fisher Scientific). DNA was recovered by centrifugation at 12,000 g for 10 min, washed with 70% ethanol and resuspended in 100 μl sterile water. This mixture of prokaryotic and eukaryotic DNA was then used to amplify M. tuberculosis DNA using the molecular beacon technology to enhance sensitivity.

2.11.2 M. tuberculosis RNA isolation from lung tissue

The left lobe of each mouse lung was snap-frozen in liquid nitrogen in a 2 ml screw-cap microfuge tube with an o-ring at time points at which mycobacterial DNA was to be quantitated. These organs were stored at -80°C until RNA was extracted. The lung tissue was then homogenised in 3 – 4 ml TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) using a tissue homogeniser. The sample was incubated on ice for 10 min and transferred to a pre-chilled 15 ml conical tube (BD Biosciences). It was centrifuged at 3000 rpm for 10 min at 4°C to collect the mycobacteria, after which the pellet was resuspended in 1 ml TRI Reagent containing 1% Polyacryl Carrier (Molecular Research Center) and aliquoted into two o-ring tubes containing 250 μl zirconia/silica beads each. The suspensions were bead-beated twice for 1 min at a time with 1 min intervals, after which they were centrifuged for 10 min at 12,000 g to pellet the beads. The supernatants were transferred to clean tubes and 50 μl 1-bromo-3-chloropropane (BCP) added and shaken vigorously for 15 sec. After
incubation at room temperature for 10 min, samples were centrifuged at 12,000 g for 10 min at 4°C and the upper phase of each transferred to a tube containing 250 μl isopropanol, mixed and incubated at room temperature for 10 min. The isopropanol-precipitated RNA was centrifuged at 12,000 g for 10 min at 4°C, supernatants were discarded, and pellets were washed with 300 μl 75% ethanol. The pellets were briefly air dried then resuspended in 50 μl DEPC-water. The TRI Reagent extraction step was repeated with 500 μl, and the RNA finally resuspended in 50 μl DEPC-water.

2.11.3 Primer and molecular beacon design

Molecular beacons are novel hybridisation probes that are extremely specific and allow the user to detect low-level gene activity, such as mycobacterial gene expression amidst extensive host gene expression. These probes emit fluorescence specifically upon interaction with their target sequence, which is the amplified mycobacterial gene of interest. They are hairpin-shaped oligonucleotides with their central part complementary to the target, flanked by two 5 – 6 bp inverted repeats that can form a stable stem. A fluorescent tag, usually TET or FAM phosphoramidite, is covalently linked to the 5’ end of the probe and a quenching moiety, 4-(4’-dimethylaminophenylazo)benzoic acid (DABCYL), covalently linked to the 3’ end. The stem keeps the fluorophore and quencher in close proximity to each other, which quenches the fluorescent signal, but when the molecular beacon specifically binds to its target, its conformation changes and this results in a fluorescent signal as illustrated in Figure 2.4 (Tyagi & Kramer, 1996).
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**Figure 2.4. Mechanism of action of molecular beacons.** When the complementary bases in the probe form a stem structure, the quencher (●) keeps the fluorophore (○) from emitting fluorescence, but when the probe hybridises to its complementary target sequence, the new conformation separates the quencher from the fluorophore, thereby emitting a fluorescent signal.

When molecular beacons are used in conjunction with PCR where their target is the amplification product, they can form stable hybrids with the amplicon during the annealing step and can be quantitated. The intensity of fluorescence during the annealing step of each amplification cycle is therefore a measure of amplicon concentration (Manganelli et al., 1999; Tyagi et al., 1998), as visually explained in **Figure 2.5**.

**Figure 2.5. Real-time measurement of amplicon synthesis during PCR using molecular beacons.** Four reactions, each initiated with a different number of template molecules (indicated), were incubated simultaneously in the spectrofluoro- metric thermal cycler (A). Primers and molecular beacon specific for the *M. tuberculosis* sigA gene were used in the amplification reactions. The target was represented by *M. tuberculosis* H37Rv chromosomal DNA. The amount of chromosome equivalents per µl was calculated considering the length of the *M. tuberculosis* chromosome, 4.4 megabases (Cole & Barrell, 1998). (B) Inverse relationship between the threshold cycle (the cycle at which the fluorescent signal becomes detectable above the background) and the logarithm of the initial number of template molecules. The concentration of amplicons that were present after each cycle of amplification was determined by measuring fluorescence during the last few seconds of the annealing step (Tyagi et al., 1998).

Figure reprinted from Manganelli et al., 1999.
PCR primers (Genosys) were designed to amplify gene fragments of 50 to 150 bp. Reverse transcriptase (RT) primers (Genosys) were located close to the reverse PCR primers and all primers had comparable melting temperatures of 60 – 66°C. Primers were designed using PRIMER 3 software (Rozen & Skaletsky, 2000) and were tested with conventional PCR reaction conditions with 100 *M. tuberculosis* H24Rv genome equivalents as template and the resulting amplicons evaluated by agarose gel electrophoresis. Molecular beacons (Eurogentec) were designed as described (http://www.molecular-beacons.org) and tested in PCR reactions using 10-fold serial dilutions of *M. tuberculosis* H37Rv DNA as template. The sequences of primers and beacons used to quantitate *M. tuberculosis* DNA are listed in Table III and the primer and beacon sequences used to quantitate *M. tuberculosis* mRNA expression are listed in Table IV.

**Table III. PCR primers for quantification of *M. tuberculosis* DNA**

<table>
<thead>
<tr>
<th>Primer function</th>
<th>Name</th>
<th>Orientation</th>
<th>Primer sequence</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid metabolism</td>
<td>FadE15</td>
<td>Forward</td>
<td>5'-GAT TGC GCA GAT GTT CAC GG-3'</td>
<td>65.0°C</td>
<td>121 bp</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-GCC CTG CAC CCG CTC CTT GG-3'</td>
<td>73.5°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beacon</td>
<td>5'-GCA GCC CGC ATG ACC ATC GGA GTT AAG TGG CTG C-3'</td>
<td>63.7°C</td>
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</table>
Table IV. PCR primers for quantification of *M. tuberculosis* mRNA

<table>
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<tr>
<th>Function</th>
<th>Name</th>
<th>Orientation</th>
<th>Primer sequence</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA synthesis</td>
<td>16SrRNA</td>
<td>RT</td>
<td>5'-GCC CGC ACA CTC ACA G TT AAG-3'</td>
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<tr>
<td></td>
<td></td>
<td>Forward</td>
<td>5'-ATG ACG GCC TTC GGG TGG TAA-3'</td>
<td>69.3°C</td>
<td>109 bp</td>
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<td></td>
<td></td>
<td>Reverse</td>
<td>5'-CGG CTC CTG GCA CTC AGT TG-3'</td>
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<tr>
<td></td>
<td></td>
<td>Beacon</td>
<td>5'-CCA CGC GGA CGA AAG TGG GCTG TGG TG CCG GGG-3'</td>
<td>66.3°C</td>
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<td>RNA synthesis</td>
<td>sigA</td>
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<td>5'-CTG ACA TGG GGG CCC GCT ACG-3'</td>
<td>78.8°C</td>
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### 2.11.4 Real-time PCR of *M. tuberculosis* DNA

A real-time PCR reaction typically contained 2 μl of DNA (Section 2.11.1), 1x PCR Gold® buffer containing 0.1% Rox reference dye (Invitrogen), 0.25 mM dNTPs, 4 mM MgCl₂, 0.5 μM of each FadE15 forward and reverse primers, 2.5 ng FadE15 molecular beacon, and 0.5 μl 5 U/μl AmpliTaq® Gold DNA polymerase made up to a final volume of 50 μl with DEPC-water. Standards of *M. tuberculosis* H37Rv at 10<sup>6</sup> to 10<sup>2</sup> copy numbers were
included with each run. Samples were run in duplicate in 20 μl volumes in 384-well optical plates (Applied Biosystems, Foster City, CA, USA) in an ABI 7900 real-time PCR machine (Applied Biosystems) and analysed according to the manufacturer’s instructions. PCR conditions were as follows:

AmpliTaq® Gold activation step at 95°C for 10 min;
40 cycles at 95°C for 30 sec;
57°C for 30 sec;
72°C for 30 sec;
Samples were held at 4°C.

2.11.5 Reverse transcription and real-time PCR of M. tuberculosis mRNA

RT and PCR reactions were performed as described (Manganelli et al., 2001), with modifications noted. Different primers were used for RT and PCR reactions to increase specificity of the assay. Control reactions without RT were performed on each RNA sample to rule out DNA contamination. RT reactions, typically containing seven gene-specific primers, were carried out using the Klenow fragment of DNA polymerase from Carboxydothermus hydrogenoformans (C. therm kit, Roche Applied Science, Indianapolis, IN, USA) as follows:

Annealing step at 95°C for 1 min, 65°C for 3 min, 57°C for 3 min;
Polymerisation step at 60°C for 30 min;
RT inactivation step at 95°C for 5 min.

Each PCR reaction contained 2 μl of the RT reaction mix, 1x PCR Gold® buffer containing 0.1% Rox reference dye (Invitrogen), 0.25 mM dNTPs, 4 mM MgCl2, 0.5 μM of each forward and reverse primers for the different genes analysed, 2.5 ng matching molecular beacon, and 0.5 μl 5 U/μl AmpliTaq® Gold DNA polymerase made up to a final volume of 50 μl with DEPC-water. Samples were run in duplicate in 20 μl volumes in 384-well
optical plates (Applied Biosystems) as described above. PCR conditions were as follows.

For 16SrRNA (rrs):

- AmpliTaq® Gold activation step at 95°C for 10 min;
- 40 cycles at 95°C for 30 sec;
  - 57°C for 30 sec;
  - 72°C for 30 sec;
- Samples were held at 4°C.

For sigA, icl1, hspX, pckA, mbtB and sigF:

- AmpliTaq® Gold activation step at 95°C for 10 min;
- 15 cycles at 95°C for 30 sec;
  - 65°C for 30 sec (and a -0.5°C step down each cycle);
  - 72°C for 30 sec;
- 25 cycles at 95°C for 30 sec;
  - 57°C for 30 sec;
  - 72°C for 30 sec;
- Samples were held at 4°C.

2.12 Histopathological analysis

One third of lungs, livers and spleens of mice were prepared by fixing in 10% buffered formalin (BDH, Laboratory Supplies, Poole, UK) for 24 hours before embedment in paraffin wax (Histosec Pastilles, Merck, Germany). Sections were cut at 2 – 3 μm thickness on a rotary microtome (Leica, model RM-2125).

2.12.1 Haematoxylin and eosin staining

Lung, liver and spleen sections were stained with haematoxylin and eosin according to standard procedures for evaluation of pathologic changes during the various stages of disease.
2.12.2 Ziehl-Neelsen staining for acid-fast bacilli
Lung, liver and spleen sections were stained with the Ziehl-Neelsen acid-fast stain and counterstained with haematoxylin to detect mycobacteria located within tissue.

2.13 Immunohistochemistry

2.13.1 NOS2 immunostaining
Formalin-fixed, paraffin-embedded sections were deparaffinised and rehydrated through a series of graded alcohols. Sections were mounted on 3-aminopropyltriethoxysilane (APES) coated slides and were incubated with a rabbit anti-mouse antibody\(^7\), specific for NOS2, at 1:2000 dilution for 16 hours at 4°C, followed by rinses in PBS. Sections were then incubated with a rat anti-rabbit secondary antibody for 30 minutes at room temperature, rinsed in PBS and incubated with ABC Vector (Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature. Subsequently, sections were rinsed in PBS, incubated with 3,3-diaminobenzidene tetrahydrochloride (DAB) substrate (Vector Laboratories) for 10 minutes at room temperature, washed in running water, counterstained in haematoxylin and mounted in entellen (Merck).

2.14 FACS analysis of cell surface markers
Lung cells were obtained from mice at 10 weeks as described before. Briefly, lungs were perfused, chopped into small pieces and incubated with RPMI-1640 (Sigma-Aldrich) containing optimal concentrations of collagenase (Sigma-Aldrich) and DNAase (Sigma-Aldrich) for 60 min at 37°C. The cell suspension was passed through a 100-µm pore size mesh

\(^7\) Kindly provided by Dr. J. Pfeilschifter, University of Frankfurt, Germany.
and cells were collected by centrifugation. Cells were stained with the following antibodies (BD Biosciences Pharmingen, San Diego, CA, USA): CD3 (anti-CD3 PE, clone 145.2C11), CD4 (anti-CD4 FITC, clone H129.19), CD8 (anti-CD8 FITC, clone 53-6.7), CD11a (anti-CD11a PE, clone M17/4), CD44 (anti-CD44 PE, clone IM7), I-A/I-E (anti-I-A/I-E PE, clone M5/114.15.2), and CD16/32 (clone 2.4G2). All staining procedures were performed in PBS containing 0.1% BSA and 0.1% sodium azide (FACS buffer) for 20 minutes at 4°C. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for at least 1 hour and analysed by flow cytometry using CellQuest® software (BD Immunocytometry Systems, San Jose, CA). Cells were gated on the lymphocyte or monocyte population by forward and side scatter.

2.15 Chemokine assays in the lung

Mice infected with *M. tuberculosis* H37Rv and treated with RMP and INH from weeks 2 to 6, were sacrificed at 10 weeks post-infection. Whole lungs were removed from infected mice and were homogenized in 1 ml saline containing 0.04% Tween 80. Supernatants were collected after low-speed centrifugation, aliquoted and frozen at -80°C. Supernatants were then assayed for chemokine content using commercially available ELISA reagents for macrophage inflammatory protein-1 alpha (MIP-1α), monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T cell expressed and secreted (RANTES) (R&D Systems, Abingdon, UK and BD PharMingen, San Diego, CA).

2.16 Statistical analysis

Data are presented as means and SD indicated by error bars. Statistical significance was determined using Student’s t test. P values equal to or less than 0.05 were considered significant.
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Chapter 3. RESULTS

3.1 Liquid culturing of *M. tuberculosis* H37Rv, H37Rv-plVT and Erdman strains

Different strains of *M. tuberculosis* – H37Rv, kanamycin-resistant H37Rv-plVT and Erdman were used in mouse aerosol infection experiments, and it first had to be established that these cultures showed comparable growth kinetics *in vitro* before use *in vivo*. Moreover, in order to harvest bacteria during mid-log phase for aerosol infection, growth curves for these strains had to be established *in vitro*, as depicted in Figure 3.1. All strains behaved similar during *in vitro* culturing conditions, with the exception of H37Rv-plVT, which showed a slightly extended lag phase (Figure 3.1 B). During reinfection studies, *M. tuberculosis* H37Rv and H37Rv-plVT were used to simultaneously infect mice, therefore, co-culturing of these strains *in vitro* with and without the presence of kanamycin was observed. *M. tuberculosis* H37Rv-plVT was kanamycin-sensitive, as expected, and when both *M. tuberculosis* H37Rv and H37Rv-plVT were co-cultured in the absence of kanamycin, they multiplied at similar rates.

3.2 Optimising antituberculous therapy in wild-type mice in the drug model

In an attempt to establish a relatively short-duration model to study spontaneous and drug-induced reactivation of latent infection, C57BL/6 mice were infected with a low aerosol exposure *M. tuberculosis* H37Rv (30 CFU), and RMP-INH antituberculous therapy was commenced two weeks post-infection. The duration of antituberculous therapy was varied between 4 and 12 weeks, as displayed in Figure 3.2.
Figure 3.1. *In vitro* growth curves of *M. tuberculosis* H37Rv, H37Rv-pIVT and Erdman. An aliquot of approximately $10^{10}$ bacteria from *M. tuberculosis* H37Rv (solid triangles), H37Rv-pIVT (solid squares) and Erdman (open circles) frozen stock was each inoculated into 50 ml 7H9 culture medium, containing 0.5% glycerol, 0.05% Tween 80, enriched with 10% OADC, and incubated at 37°C with gentle rolling. (A) Growth was monitored by assessment of culture optical density at 600 nm (OD$_{600}$) during a 2-week period and plotting these values against time. (B) The logarithmic representation of culture growth indicates more subtle differences, especially during the lag phase.
Figure 3.2. Experimental design of antituberculous treatment regimes. Schematic diagram of the experimental design for establishing the optimal length of RMP-INH treatment in mice to achieve latency of tuberculosis. In the 3 different models, mice were treated for varying times – Model A (4 weeks), Model B (8 weeks), and Model C (12 weeks), starting 2 weeks post-infection. Clear blocked areas represent periods of RMP-INH therapy, while solid blocked areas indicate periods of aminoguanidine administration.

Firstly, aerosol infected mice were treated for four weeks. The combined administration of RMP and INH for four weeks eliminated the bacilli completely, and no viable colonies could be detected in the lungs (sensitivity of detection 5 CFU), spleen or liver (sensitivity of detection 10 CFU) (Figure 3.3). This finding is consistent with effective therapy. In contrast to this, the bacterial load in the lungs of untreated mice was approximately $10^5$ CFU, and in the spleen and liver $10^4$ CFU and $10^3$ CFU per organ, respectively. However, 8 weeks upon cessation of drug treatment, mycobacteria were found in the lungs, spleen and liver ($10^4$, $10^3$ and $10^2$ CFU) in 100% of treated mice and persisted over the 24-week experimental period. Therefore, all mice reactivated with tuberculosis infection spontaneously. The mycobacterial load, however, remained lower as compared to the untreated control mice and all mice survived the infection during the experimental period.
Figure 3.3. Model A: Bacterial burden of mice treated with RMP-INH for 4 weeks. C57BL/6 mice were infected with 30 CFU *M. tuberculosis* H37Rv via aerosol. Two weeks post-infection, one group of mice (open circles) received 0.1 g/L RMP-INH, delivered in drinking water *ad libitum* (indicated by arrows) for a period of 4 weeks, whereas infected control mice (closed circles) received plain drinking water *ad libitum*. At specific time intervals up to 24 weeks post-infection, viable bacilli in the lung, spleen and liver were determined by plating organ homogenates and enumerating CFU. No viable bacilli were detected in organs of mice upon cessation of antituberculous therapy, however, mice spontaneously reactivated and bacilli could be cultured 8 weeks later (open circles). Both infected controls and reactivated mice controlled the infection (100% survival). Results are expressed as means ± SD and are representative of three independent experiments with 3–5 mice per group per time point (p > 0.05).
In conclusion, the duration of 4 weeks of chemotherapy was insufficient to eliminate the mycobacteria following an initial infective dose of 30 CFU. Although no viable bacilli could be detected with conventional plating methods following drug treatment, spontaneous reactivation of infection occurred in all mice.

Secondly, in order to have a long-lasting effect on the infection RMP-INH chemotherapy was given for 8 weeks (Figure 3.4). The bacilli, as assessed by CFU, were completely eliminated from the lungs, and remained undetectable in spleen and liver (sensitivity of detection 2 CFU in all organs) after an 8-week course of antituberculous therapy, while untreated controls had $10^5$ CFU in the lungs and spread of infection to the spleen and liver at $10^4$ and $10^3$ CFU, respectively. Interestingly, no spontaneous reactivation was detected over the duration of the experiment over 55 weeks, suggesting effective elimination of bacilli from the host. However, administration of aminoguanidine, an inhibitor of NOS2, at any time 6 to 20 weeks after RMP-INH chemotherapy, resulted in rapid reactivation of *M. tuberculosis* infection. As early as two weeks following reactivation, the mycobacterial load had increased to $10^5$ CFU in the lungs, with lower levels in spleen and liver ($10^4$ and $10^3$ CFU, respectively), which was comparable to untreated controls. Therefore, treatment for 8 weeks resulted in long-term absence of infection. Since aminoguanidine-induced inhibition of NOS2 allowed rapid reactivation, viable mycobacteria were still present in the host during the persistent phase, although not detectable by conventional culture plating methods.
Figure 3.4. Model B: Bacterial burden of mice treated with RMP-INH for 8 weeks. C57BL/6 mice were infected with 30 CFU *M. tuberculosis* H37Rv via aerosol. Two weeks post-infection, two groups of mice (open circles and open squares) received 0.1 g/L RMP-INH, delivered in drinking water *ad libitum* (open arrows) for a period of 8 weeks, whereas the control group (closed circles) received plain drinking water *ad libitum*. At specific time intervals up to 55 weeks post-infection, viable bacilli in the lung, spleen and liver were determined by plating organ homogenates and enumerating CFU. No viable bacilli were detected in the organs of mice upon completion of chemotherapy. After administration of 2.5% aminoguanidine containing 10% glucose in drinking water (solid arrows) from week 30 – 55, reactivated infection was observed in 67% of mice (open squares – representative of reactivated mice only), although they survived (100% survival) and seemed to control the infection. The group of mice with latent infection (open circles) remained culture negative, with no viable CFU detected in organs for the duration of the experiment (55 weeks). Infected control mice (solid circles) showed a rapid initial increase in CFU in lung, liver and spleen, although these mice contained the reactivated infection successfully for the duration of the experiment (100% survival). Results are expressed as means ± SD and are representative of three independent experiments with 3 – 5 mice per group per time point.
Thirdly, we extended the RMP-INH antituberculous therapy for 12 weeks to achieve a cure in infected mice (Figure 3.5). The bacilli were eliminated from the lung tissue after therapy. Furthermore, no colonies were detectable from any organ during the 44 weeks duration of the experiment, while the untreated controls developed chronic tuberculosis. In order to ascertain whether mycobacteria have indeed been eradicated, aminoguanidine was administered at week 40, 26 weeks after RMP-INH treatment. No reactivation was observed within the subsequent 4 weeks or at time points beyond that. In conclusion, RMP-INH drug therapy over 12 weeks under these experimental conditions cured *M. tuberculosis* infection, while 8 weeks of therapy induced a persistent form of infection.

TNF gene-deficient mice, which are unable to control and survive mycobacterial infection, were included as controls and to corroborate the notion that the immune system plays an important part in reactivation of tuberculosis. Although no bacilli were detected after 4 weeks RMP-INH chemotherapy, TNF deficient mice reactivated faster than C57BL/6 wild-type control mice and succumbed to infection within 5 weeks after cessation of treatment. After 8 weeks of chemotherapy, TNF deficient mice reactivated spontaneously and succumbed to infection unlike wild-type control mice.

By contrast, no reactivation was observed in TNF deficient mice treated with RMP-INH for 12 weeks following cessation of treatment. Therefore, this extensive treatment apparently had eliminated all viable bacilli.
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Figure 3.5. Model C: Bacterial burden of mice treated with RMP-INH for 12 weeks. C57BL/6 mice were infected with 30 CFU *M. tuberculosis* H37Rv via aerosol. Two weeks post-infection, one group of mice (open circles) received 0.1 g/L RMP-INH, delivered in drinking water *ad libitum* (open arrows) for a period of 12 weeks, whereas the control group (closed circles) received plain drinking water *ad libitum*. At specific time intervals up to 44 weeks post-infection, viable bacilli in the lung, spleen and liver were determined by plating organ homogenates and enumerating CFU. No viable bacilli were detected in the organs of mice after completion of chemotherapy. Mice treated with RMP-INH for 12 weeks and subsequently with aminoguanidine for 4 weeks (solid arrows) failed to reactivate. Results are expressed as means ± SD and are representative of three independent experiments with 3 – 5 mice per group per time point.
3.3 Kinetics of mycobacterial reduction during rifampicin-isoniazid chemotherapy

The kinetics of killing of mycobacteria during RMP-INH therapy, which was monitored during the 8-week drug treatment period, is depicted in Figure 3.6. At the start of therapy, 2 weeks post-infection, average CFU in the lungs, spleen and liver were $1.83 \times 10^5$, $3.0 \times 10^2$ and $2.5 \times 10^2$ CFU per organ, respectively. In the lungs, during the first week of treatment with RMP-INH, the number of culturable CFU varied significantly between $10^4$ and $10^1$ in mice ($n = 5$) (Figure 3.6 A). Two weeks after initiation of therapy CFU in the lungs of mice were clustered closer together, around $10^4$ to $10^3$ CFU per mouse. At three weeks after treatment, 3 out of 5 mice had undetectable CFU in their lungs, plated to a sensitivity of 2 CFU per organ, while the other two mice had $10^3$ and $10^2$ CFU per lung, respectively. Halfway through therapy, one mouse had $10^2$ viable *M. tuberculosis* in the lung, while another one had 50 CFU. Two more mice had 4 and 3 CFU per lung, respectively, and one had undetectable CFU plated to a sensitivity of 2 CFU per organ. At the end of the 8-week RMP-INH therapy, no bacilli could be cultured in the lungs by plating at a sensitivity of 2 CFU per organ ($n = 5$).

During RMP-INH treatment in the spleen (Figure 3.6 B), mycobacteria were also reduced immediately from the first week of therapy. At the start of therapy, mice had an average of 300 CFU per spleen, which increased to $1.4 \times 10^4$ CFU in the untreated control mice within the same week. In the treated group, however, all mice had undetectable levels of bacilli at a plating sensitivity of 50 CFU per organ ($n = 5$). At both 3 and 4 weeks after drug treatment, all spleens were culture negative at a reduced plating sensitivity of 10 CFU per organ. At the end of the 8-week therapy, spleens were plated to a sensitivity of 2 CFU per organ, and no bacilli were detected ($n = 5$).
Figure 3.6. Kinetics of mycobacterial killing during RMP-INH therapy. Mouse (A) lung, (B) spleen and (C) liver homogenates were plated to a sensitivity of up to 2 CFU each to assess the killing kinetics of mycobacteria during the 8-week course of chemotherapeutic treatment. CFU of individual drug-treated mice are indicated by horizontal bars, whereas the diamonds represent the average CFU of chronic infected untreated control mice at the corresponding time points (n = 5 mice per group per time point).
Mycobacterial numbers in the liver (Figure 3.6 C) averaged at 250 CFU per organ at the onset of RMP-INH therapy and showed a similar profile as in the spleen at the time points studied, with some exceptions. Although mouse liver CFU after the first 2 weeks of therapy were below the limit of detection (50 CFU per organ), one mouse had 150 CFU in its liver at 2 weeks after the initiation of therapy. Sensitivity of plating was decreased from 50 CFU to 10 CFU and finally to 2 CFU per organ, as it was done for the spleen. At the final time point, which marked the end of chemotherapy, four mice had undetectable CFU, but one mouse had 44 CFU in the liver. As mentioned before, no mycobacteria were detected in the lungs or spleen of this mouse.

All plates harbouring organ homogenates plated from drug-treated mice were incubated for 6 weeks, twice as long as usual, before final scoring.

3.4 Reactivation of tuberculosis in wild-type mice during immuno-suppression with aminoguanidine

Although no mycobacteria were detectable by plate culturing following 4 weeks of RMP-INH drug treatment, viable bacilli were still persistent in the host. This explains the spontaneous reactivation of infection with a dramatic increase of bacilli ($10^4 - 10^5$ CFU) in the lung after discontinuing drug administration, which stabilised over the following 10 weeks (Figure 3.3).

An extended RMP-INH treatment over 8 weeks was able to control infection and no spontaneous reactivation was observed. However, interfering with the host defence by inhibiting NOS2 activity with aminoguanidine resulted in reactivation of infection, with a similar rapid increase in mycobacterial loads (Figure 3.4).

The percentage of mice that reactivated upon aminoguanidine administration was 67% on average and this percentage is represented in
Figure 3.4. Two weeks following reactivation, 100% mice had positive cultures in lung, liver and spleen. However, 4 weeks after reactivation, CFU decreased by 50% in all organs. Therefore, it would be optimal to further characterise the reactivated state 2 weeks after aminoguanidine administration. This time point coincides with a peak in CFU in all organs following reactivation.

A 12-week RMP-INH antituberculous course, however, eradicated the infection, and immunosuppression with aminoguanidine did not cause reactivation (Figure 3.5). These results suggest that a 4-week course of RMP-INH treatment is insufficient to mount protective immunity, while extended treatment regimens allow for immunological control by the host or the longer course of 8 weeks kills more bacteria.

Aminoguanidine-induced reactivation of latent tuberculosis infection following 8 weeks of RMP-INH treatment results in a rapid bacillary increase in the lungs of mice from undetectable levels to around $10^5$ CFU within 2 weeks, comparable to levels found at a similar time point following primary infection (Figure 3.7). More detailed analysis of reactivation kinetics revealed a rapid increase of mycobacteria in the lungs from undetectable levels (at a plating sensitivity of 2 CFU per organ) following 8 weeks of RMP-INH treatment to $10^3$ CFU per organ within the first week of aminoguanidine administration ($n = 8$) (Figure 3.8 A). After a second week of aminoguanidine administration, the CFU in the lungs increased to between $10^4$ and $10^5$ per mouse. In contrast to the closely clustered CFU levels within the first two weeks of induced reactivation, CFU levels varied significantly between individual mice at later time points and showed a tendency to decrease on average, similar to previous observations in Model B.
Figure 3.7. Comparison of bacterial burden in lung, spleen and liver of mice following reactivation versus primary infection. C57BL/6 mice were infected with 30 CFU *M. tuberculosis* H37Rv and CFU determined 2 weeks post-infection (solid bars). Mice were treated with RMP-INH for 8 weeks to achieve latency (with no viable bacilli detected in organs). After 20 weeks in this latent state, a group of mice received 2.5% aminoguanidine containing 10% glucose in drinking water *ad libitum* to induce reactivation. Bacterial increases following reactivated infection are shown 2 weeks after aminoguanidine administration (open bars). Results are expressed as means ± SD with 3 – 5 mice per group per time point.
Figure 3.8. Reactivation of tuberculosis during immunosuppression with aminoguanidine. Mouse (A) lung, (B) spleen and (C) liver CFU during 4 weeks of aminoguanidine administration. CFU were undetectable in all organs at the start of immunosuppression, plated to a sensitivity of 2 CFU per organ. CFU of individual AG-treated mice are indicated by horizontal bars, whereas diamonds represent the average CFU of chronic infected untreated control mice at the corresponding time points (n = 8 mice per group per time point).
Reactivation kinetics in the spleen (Figure 3.8 B) showed an increase from undetectable levels to $10^2 - 10^3$ CFU per organ (at a plating sensitivity of 2 CFU per spleen) at 2 weeks following aminoguanidine administration. One week following induced reactivation no culturable bacilli were detected at a plating sensitivity of 50 CFU per organ ($n = 8$). Interestingly, at later time points, half of the mice had undetectable mycobacterial levels in the spleen at a plating sensitivity of 50 CFU per organ while others showed an increase in CFU.

Similarly, in the liver, levels of mycobacteria at a plating sensitivity of 50 CFU per organ were only detectable after 2 weeks of aminoguanidine administration, in the order of $10^2$ CFU per mouse (Figure 3.8 C). Five out of eight mice at both 3 and 4 weeks following induced reactivation had undetectable CFU in the liver, while the remaining mice showed an increase in CFU levels. Chronically infected untreated control mice maintained relatively stable mycobacterial counts in all organs studied over this period.

3.5 Spontaneous reactivation of tuberculosis in wild-type mice

The question was asked whether the early initiation of chemotherapy at 2 weeks post-infection would contribute to the relatively easy relapse of tuberculosis infection when mice are immunosuppressed with aminoguanidine during the latent phase of infection. To assess this, an experiment was performed in which one group of mice was treated with RMP-INH for 4 weeks, starting 2 weeks post-infection (similar to Model A, see Section 3.2), designated Group 1. Whereas treatment started before host immunity had sufficient time to develop in Group 1, another group of mice (Group 2) was treated for the same length of time, but chemotherapy started 8 weeks post-infection, long after the infection reached steady CFU that implicated adequate immune control by the host. This variation of Model A (Figure 3.3) would allow comparison of the killing kinetics and the kinetics of spontaneous reactivation in both groups in order to assess whether full development of the host immune system would protect against
relapse of infection. Additionally, *M. tuberculosis* Erdman was used to determine whether this strain behaved in a similar manner as *M. tuberculosis* H37Rv in the drug model. CFU in the lungs (Figure 3.9 A) and spleens (Figure 3.9 B) of both Group 1 and Group 2 were monitored during RMP-INH chemotherapy and for 10 weeks following treatment during spontaneous reactivation.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 3.9.** Mycobacterial burden in the lungs and spleens of mice following early and late RMP-INH chemotherapy. Mice were infected with 900 CFU *M. tuberculosis* Erdman via aerosol, after which a 4-week course of RMP-INH chemotherapy was started 2 weeks post-infection in one group (open symbols; open arrow), while it was started 8 weeks post-infection in another group (shaded symbols; shaded arrow). Control infected untreated mouse CFU are indicated (solid diamonds). Mouse (A) lung and (B) spleen homogenates were plated and CFU enumerated during chemotherapy and 10 weeks thereafter during spontaneous reactivation. Results are expressed as means ± SD and are representative of 5 mice per group per time point. The dashed line indicates the sensitivity of detection at 50 CFU in the spleen.
A graphic representation of the mycobacterial reduction during RMP-INH chemotherapy for both the early-treated Group 1 and late-treated Group 2 is given in Figure 3.10. Following an initial aerosol infection with 900 CFU *M. tuberculosis* Erdman, there was a 4.5 log expansion in the lungs before the 4-week RMP-INH chemotherapy was initiated in Group 1. The average mycobacterial load was \(3.4 \times 10^7\) CFU in the lungs for Group 1, with a similar load in Group 2 \((1.0 \times 10^7\) CFU) at the start of therapy. Total reduction of the bacillary load in the lungs for Group 1 after 4 weeks of chemotherapy was 3.6 log, whereas it was a comparable 3.0 log for Group 2 (Figure 3.10 A). There were some differences in the kinetics of killing in the lungs between the two groups. The most striking difference being the reduction of CFU in the lungs and spleens between the third and fourth weeks of chemotherapy. In the lungs there was a 17-fold reduction between the third and the fourth week of chemotherapy in Group 1, whereas there was only a 1.5-fold reduction of CFU in Group 2. A similar pattern was evident in the spleen in the last week of chemotherapy, with a dramatic 34-fold reduction in CFU in Group 1 and a smaller 7-fold reduction in Group 2 CFU (Figure 3.10 B). However, the overall reduction in CFU throughout the total period of chemotherapy was similar in the two groups – a total reduction of 3.4 log and 3.7 log for Group 1 and 2, respectively. The average spleen CFU at the time of initiation of chemotherapy was \(1.2 \times 10^5\) for Group 1 and \(5.9 \times 10^5\) CFU per organ for Group 2.

Soon after cessation of the 4-week, RMP-INH treatment, mice spontaneously reactivated with infection. The kinetics of reactivation in the lungs and spleens for Group 1 and 2 is shown in Figure 3.11. CFU at the end of chemotherapy in the lungs in both groups were similar at \(9 \times 10^3\) CFU per organ, despite the altered initiation of therapy (Figure 3.11 A). A delayed effect of RMP was evident in Group 1 at 1 week post-treatment, with a further albeit marginal decrease in CFU, after which there was a fairly steady increase up to 10 weeks after therapy. The most pronounced increase in the CFU of the lungs in Group 1 was between 8 and 10 weeks, with almost 1 log increase in CFU. In Group 2 there was an immediate increase observed in CFU in the lungs up to 4 weeks post-therapy, after
Figure 3.10. Kinetics of mycobacterial reduction in the lungs and spleens of mice following early and late RMP-INH chemotherapy. Mice were infected with 900 CFU *M. tuberculosis* Erdman via aerosol, after which a 4-week course of RMP-INH chemotherapy was started 2 weeks post-infection in one group (open symbols), while it was started 8 weeks post-infection in another group (shaded symbols). Mouse (A) lung and (B) spleen homogenates were plated and CFU enumerated weekly during chemotherapy. Results are expressed as means ± SD and are representative of 5 mice per group per time point. The dashed line indicates the sensitivity of detection at 50 CFU in the spleen.
Figure 3.11. Kinetics of spontaneous reactivation in the lungs and spleens of mice following early and late RMP-INH chemotherapy. Mice were infected with 900 CFU *M. tuberculosis* Erdman via aerosol, after which a 4-week course of RMP-INH chemotherapy was started 2 weeks post-infection in one group (open symbols), while it was started 8 weeks post-infection in another group (shaded symbols). Mouse (A) lung and (B) spleen homogenates were plated and CFU enumerated at 2-weekly intervals following chemotherapy in each group. Results are expressed as means ± SD and are representative of 5 mice per group per time point. The dashed line indicates the sensitivity of detection at 50 CFU in the spleen.
which it temporarily stabilised before further increasing until the end of the experimental period. The total increase in lung CFU since the termination of chemotherapy was 1.6 log for Group 1 and 2.1 log for Group 2. In the spleen CFU levels at the end of chemotherapy was undetectable at a sensitivity of 50 CFU per organ for Group 1, whereas it was $10^2$ in Group 2 (Figure 3.11 B). The subsequent increases in CFU in the spleen of Group 1 was rapid initially, with an increase of 1.8 log in the first 2 weeks post-chemotherapy, after which a marginal reduction followed by stabilisation was seen until the sixth week since cessation of RMP-INH treatment. Between the sixth and eighth week, another increase of 1.1 log was observed and a final smaller increase of 0.3 log until the final time point. The total CFU increase in the spleen in Group 1 was 3.0 log, 2-fold higher than the total increase in the lungs of this group. The profile of spontaneous reactivation in Group 2 in the spleen also showed a rapid initial increase of 1.2 log for each of the two 2-weekly intervals since the cessation of chemotherapy. Thereafter there was a much-reduced rate of increase until the end of the experimental period, with a total increase of 2.8 log in the 10 weeks since the end of chemotherapy, 1.3-fold higher than the increase in the lungs for this group.

3.6 Pathology of latent and chronic tuberculosis infection

Examination of the H&E-stained tissue sections of the lungs at 2 weeks post-infection revealed recruitment of lymphocytes and macrophages with an incipient granuloma formation (Figure 3.12 A). At 10 weeks post-infection, differences between antituberculous treated and non-treated mice were evident. Untreated controls had a well-developed granulomatous response with lymphoid aggregates, while the treated mice had only small granulomata (Figure 3.12 B, C). At 55 weeks untreated mice had displayed chronic tuberculosis with abundant granulomata containing lipid loaded macrophages and lymphoid cell aggregates (Figure 3.12 D). Mice immunosuppressed with aminoguanidine revealed increased recruitment of mononuclear cells and loss of granuloma structures (Figure 3.12 E).
Figure 3.12. Morphology of the lungs of mice during active, persistent and reactivated tuberculosis infection. Lungs were examined at 2 weeks (A), 10 weeks (B, C), and 55 weeks (D – F) post-infection. Panel A represents a C57BL/6 mouse 2 weeks post-infection, prior to chemotherapy, panels B and C 10 weeks post-infection – an infected control (B) and a latently infected mouse after 8 weeks of RMP-INH chemotherapy (C). Panels D, E and F represent mice 55 weeks post-infection – an infected control (D, F) and a mouse reactivated after aminoguanidine administration (E). Sections were stained with haematoxylin and eosin (A – E) or with Ziehl-Neelsen (F). Magnification, panels A to E, x100; panel F, x400.

The lung sections stained with Ziehl-Neelsen were analysed for the presence of bacilli during latent infection. No bacilli were detected in RMP-INH treated mice, while acid-fast bacilli were observed in untreated control lungs (Figure 3.12 F).
Figure 3.13. NOS2 expression in lung tissue in mice during active, persistent and reactivated tuberculosis infection. Mice were aerogenically infected with 30 CFU \textit{M. tuberculosis} H37Rv, after which a group was treated with RMP-INH for 8 weeks to induce latency of infection. Lung tissue of C57BL/6 mice was examined at 32 weeks post-infection. No NOS2 staining was detected in mice during latent infection (A), whereas intense NOS2 expression was observed in infected control mice (B). An augmented NOS2 staining pattern was observed in mice 2 weeks after aminoguanidine-induced reactivation (C). Panel D represents a magnification of a granuloma in the liver of an infected control mouse to illustrate the origin of NOS2 protein from activated macrophages. Magnification, panels A to C, x100; panel D, x400.

We next examined the expression of NOS2 on lung sections from mice with latent infection. While chronically infected untreated lungs strongly expressed NOS2 (Figure 3.13 B), it was non-detectable during latent infection in RMP-INH treated mice (Figure 3.13 A). Aminoguanidine-induced reactivation of infection augmented the NOS2 expression in the lungs (Figure 3.13 C).
3.7 Mycobacterial resistance to rifampicin and isoniazid

There was some concern that the reactivated mycobacteria following RMP-INH chemotherapy could be drug resistant, therefore, lung homogenates containing reactivated mycobacteria of least $10^5$ CFU per organ of seven mice were plated to determine drug sensitivity. Separate 7H10 plates containing the minimum inhibitory concentration (MIC) of RMP at 0.1 μg/ml and INH at 0.05 μg/ml were used to plate total organ homogenates and results were scored at 6 weeks of incubation at 37°C and re-examined until 12 weeks of incubation. No colonies were detected, therefore all reactivated mycobacteria were still sensitive to both RMP and INH.

3.8 Genome copy numbers during rifampicin-isoniazid treatment, persistence and reactivation

Quantitative real-time PCR with molecular beacons was used to determine mycobacterial genome copy numbers during the different phases of the drug model. Primers for a housekeeping gene, fadE15, which encodes acyl-coenzyme A dehydrogenase in the lipid metabolism of M. tuberculosis, was used to quantitatively amplify each M. tuberculosis genome copy in the mouse lung. This technique provided valuable information on mycobacterial clearance following RMP-INH chemotherapy, reactivation kinetics, as well as reinfection kinetics.
Figure 3.14. *M. tuberculosis* genome copy numbers during chemotherapy and aminoguanidine-induced reactivation in the mouse lung. C57BL/6 mice were infected with 10 CFU *M. tuberculosis* H37Rv and a group (small open triangles) was treated with RMP-INH for 8 weeks (open arrow), starting 2 weeks post-infection. After a 6-week rest period, aminoguanidine was administered (solid arrow) to a group of mice (small solid triangles) for 4 weeks to induce reactivation of tuberculosis. Lung CFU of untreated control infected mice are indicated (small solid diamonds). Quantitative molecular beacon PCR was used to determine mycobacterial genome copy numbers of untreated control mice at 2, 16 and 20 weeks post-infection (large solid diamonds) and of chemotherapeutically treated mice half-way through therapy, at the end of the 8-week course of RMP-INH, and just before aminoguanidine-induced reactivation (large open triangles). Additionally, genome copy numbers were determined in reactivated mice at 1, 2 and 4 weeks following aminoguanidine administration (large solid triangles). Results are expressed as means ± SD and are representative of 4 – 8 mice per group per time point and the dashed line indicates the sensitivity of detection at 2 CFU per lung. (Connected small symbols indicate CFU, whereas free large symbols indicate genome copies.)

From Figure 3.14 it is evident that CFU of viable mycobacteria in untreated control mice correlate remarkably well with the actual genome copy numbers at the time points assessed over the 20-week experimental period. Interestingly, the mycobacterial genome numbers halfway through the 8-week RMP-INH chemotherapy in the treated group remained similar to the initial genome copy number at the start of therapy. At the end of chemotherapy, there was a 1.1 log reduction in mycobacterial genome copy numbers compared to 4 weeks before, whereas the CFU over the same period decreased by 1.5 log. However, the difference between genome
copy number and viable CFU was around 4 log at both time points. While the CFU remained stable and undetectable at a sensitivity of 2 CFU at the end of chemotherapy until the start of aminoguanidine administration 6 weeks later, the genome copy number also stabilised, albeit at higher numbers, around $10^4$. One week following aminoguanidine-induced reactivation of tuberculosis, the number of viable mycobacteria increased from undetectable levels to $2.9 \times 10^3$ CFU per lung, whereas the genome copy numbers increased by 0.4 log to $1.4 \times 10^5$ genome copies per lung. The subsequent increase in CFU after another week of aminoguanidine administration by 0.9 log was more than the 0.4 log increase in genome copies at that point, but both seemed to stabilise at 4 weeks of reactivation.

3.9 Mycobacterial gene expression during reactivation

Attempts were made to quantitate certain mycobacterial candidate genes involved in persistence of tuberculosis infection in the mouse lung during the different phases of infection in the drug model. The left lobe of every mouse was flash-frozen in liquid nitrogen and kept at -80°C until mycobacterial mRNA was extracted. Specific reverse transcription anchoring primers were used to reverse transcribe each mRNA analysed after which quantitative real-time PCR with different sets of gene-specific primers and molecular beacons was used to amplify mycobacterial genes of interest. Additionally, unique molecular beacon probes were used to further enhance specificity and sensitivity of each gene-specific PCR.

The sensitivity of this methodology regrettably was not low enough to obtain meaningful quantitative data. During most of the experimental phases in the drug model, notably during persistence of infection, mycobacterial numbers in the frozen lung portions were too low to detect mycobacterial gene expression reproducibly in some persistence gene candidates, such as icl1, hspX, pckA, mbtB and sigF. However, 16S ribosomal RNA (16S rRNA) and sigA gene expression during the different phases of RMP-INH chemotherapy, dormancy, aminoguanidine-induced reactivation and
reinfection is graphically displayed in Figure 3.15. 16S rRNA expression was almost 1 log higher than CFU at 2 weeks post-infection, during optimal growth of *M. tuberculosis* H37Rv (Figure 3.15 A), after which it gradually decreases during RMP-INH chemotherapy, albeit slightly less than the CFU reduction. Remarkably, six weeks after the end of therapy, during the persistent phase of infection, the 16S rRNA gene expression increased by 3.5 log and increased a little more during the first week of aminoguanidine-induced reactivation of tuberculosis. Interestingly, this level of gene expression was similar to the level in chronically infected control mice. 16S rRNA gene expression during reinfection of persistently infected mice increased to levels slightly in excess of the levels reached during reactivation of disease. A similar profile was evident with *sigA* gene expression (Figure 3.15 B), but there was only a 1.2 log increase in the six weeks since the end of therapy and start of reactivation or reinfection in different groups of mice. During subsequent reactivation and reinfection of tuberculosis, *sigA* gene expression levels much more resembled CFU levels of the chronically infected mice during those time points, and gene expression of reactivated and reinfected mice were similar.
Figure 3.15. Lung CFU versus 16S rRNA and sigA gene expression at different time points during reactivation and reinfection of tuberculosis. C57BL/6 mice were primary infected with 10 CFU M. tuberculosis H37Rv, treated with RMP-INH for 8 weeks to induce a persistent state (connected open triangles), rested for 6 weeks, then reactivated with aminoguanidine (connected solid triangles) or reinjected with 60 CFU M. tuberculosis H37Rv-pIVT via aerosol. Genome copy numbers were determined at different stages in RMP-INH treated and aminoguanidine-reactivated mice (free solid triangles), reinjected mice (free X-symbols), and untreated control infected mice (free open diamonds). Results are represented as means ± SD of 4–8 mice per group per time point.
3.10 Reinfection with *M. tuberculosis* H37Rv-PlVT during latent tuberculosis infection

Another aspect of this study was to investigate the dynamics of reinfection of *M. tuberculosis* in C57BL/6 mice during the persistent stage of disease. A kanamycin-resistant strain of *M. tuberculosis* H37Rv, designated H37Rv-PlVT, was used for this purpose. Mice that were initially infected with 10 CFU *M. tuberculosis* H37Rv were chemotherapeutically treated with RMP-INH for 8 weeks to achieve a latent state, characterised by no detectable CFU, plated to a sensitivity of 2 CFU. Six weeks after the end of treatment, after reconfirmation of undetectable CFU at this point, a group of mice were reinfected via aerosol with 60 CFU *M. tuberculosis* H37Rv-PlVT. A naïve uninfected group of mice was primary infected with the same strain to serve as controls. Still another group of mice were administrered aminoguanidine at the same time to achieve reactivation of tuberculosis infection. All groups of mice were monitored for the subsequent 8 weeks and organs harvested at various time points.

Firstly, mouse body weights were monitored as crude indicator of disease (Figure 3.16). For the first 3 weeks following reinfection, there was little deviation in mouse body weights compared to primary infected mice. However, from week 4 to 8 post-reinfection the primary infected mice steadily gained body weight, whereas the co-infected group lost a small proportion during the fourth and fifth week before they started to gain some weight. At the eighth week post-reinfection, the body weight deviation of almost 10% between the 2 groups varied significantly (p < 0.001). It should be noted, however, that naïve-infected mice were much younger (12 weeks of age) than reinfected mice at the time of reinfection, which might have been a contributing factor to the weight differences.
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Figure 3.16. Mouse body weight deviation during reinfection with *M. tuberculosis* H37Rv-pIVT. C57BL/6 mice were primary infected with 10 CFU *M. tuberculosis* H37Rv, treated with RMP-INH for 8 weeks to induce a latent state, rested for 6 weeks, then reinfectec with 60 CFU *M. tuberculosis* H37Rv-pIVT via aerosol (open squares). A control group of naïve C57BL/6 mice were primary infected with 60 CFU *M. tuberculosis* H37Rv-pIVT (solid circles). Body weights of reinfected mice decreased between the third and fourth week post-reinfection, whereas newly infected gained weight consistently during the experimental period. Mouse body weights were monitored weekly and are presented as means ± SD of the percentage body weight deviation compared to the start of *M. tuberculosis* H37Rv-pIVT infection of 5 mice per group per time point.
Combined *M. tuberculosis* H37Rv and H37Rv-pLVT CFU and individual H37Rv-pLVT CFU in lungs and spleens of mice were assessed in reinfected mice (Figure 3.17). Results show a steady but similar increase in both organs up to 6 weeks post-reinfection, however, at 8 weeks post-reinfection there were significant differences noted in both the lung (p < 0.001) (Figure 3.17 A) and spleen mycobacterial load (p = 0.01) (Figure 3.17 B). The 1.7 log and 1.1 log difference in CFU at that point in the lungs and spleens, respectively, appeared to be attributable to the increase in the previously dormant *M. tuberculosis* H37Rv strain in excess of the reinfected H37Rv-pLVT strain. This is indeed a very interesting observation.

### 3.11 Chemotherapy in mice deficient in TNF signalling

C57BL/6 and TNF deficient mice were infected with 30 CFU *M. tuberculosis* H37Rv via the aerosol route and treated with RMP-INH for a period of 4 weeks starting 2 weeks post-infection. While C57BL/6 mice survived for the duration of the experiment (44 weeks), all untreated TNF deficient mice succumbed to infection within 5 to 6 weeks, whereas TNF deficient mice succumbed to infection between 7 and 12 weeks after cessation of chemotherapy (Figure 3.18). Untreated wild-type mice, also infected with 30 CFU *M. tuberculosis* H37Rv, seemed to control the infection during the experimental period (100% survival). While all TNF deficient mice reactivated spontaneously with tuberculosis following cessation of RMP-INH treatment and succumbed to infection, reactivation in wild-type mice was sub-clinical and could only be assessed by the determination of bacterial counts.
Figure 3.17. Lung and spleen CFU during reinfection with *M. tuberculosis* H37Rv-pIVT. C57BL/6 mice were primary infected with 10 CFU *M. tuberculosis* H37Rv, treated with RMP-INH for 8 weeks to induce a latent state, rested for 6 weeks, then reinfected with 60 CFU *M. tuberculosis* H37Rv-pIVT via aerosol (open squares). A control group of naïve C57BL/6 mice were primary infected with 60 CFU *M. tuberculosis* H37Rv-pIVT (solid circles). Progression of infection was similar until 8 weeks post-reinfection, when lung (p < 0.001) and spleen (p = 0.01) CFU differed significantly between the naïve-infected and reinfected groups. Lung and spleen total CFU were determined and are presented as means ± SD of 5 mice per group per time point.
Figure 3.18. Survival of TNF deficient mice following primary infection and reactivated tuberculosis. C57BL/6 and TNF deficient mice were infected with 30 CFU *M. tuberculosis* H37Rv and treated with RMP-INH starting 2 weeks post-infection for a period of 4 weeks (indicated by arrow). Both groups of mice spontaneously reactivated with tuberculosis infection (100% for both groups) following cessation of RMP-INH treatment. Reactivated C57BL/6 mice (open circles) survived (100% survival) whereas TNF deficient mice (open squares) succumbed to infection 7 to 12 weeks after cessation of chemotherapy. Control untreated TNF deficient mice succumbed to infection between 5 and 6 weeks post-infection (solid squares), whereas untreated C57BL/6 mice (solid circles) survived for the duration of the experiment (*n = 9 - 15*).

Upon reactivation in both wild-type and TNF deficient mice, only TNF deficient mice lost body weight significantly (19.75 g ± 4.37) as compared to reactivated wild-type mice (27.71 g ± 2.46) (*p < 0.001*) (Figure 3.19). Furthermore, lung weights, as indicator of an inflammatory process, dramatically increased at week 14 post-infection in TNF deficient, but not in wild-type mice (Figure 3.20 A). Mean lung weights of reactivated TNF deficient mice (5.40% ± 1.33%), expressed as percentages of body weights, were significantly higher (*p < 0.001*) than their reactivated wild-type counterparts (0.647% ± 0.10) due to extensive inflammation, as confirmed by histopathology. Gross lung pathology of reactivated C57BL/6 and TNF gene deficient mice at this time point is shown in Figure 3.20 B. Whereas wild-type reactivated mice presented with many small granulomas with a grey appearance, TNF deficient mice had fewer, but large yellow caseous lesions. There were no significant differences in the lung weights between untreated wild-type infected controls (0.765% ± 0.01) and reactivated wild-
type mice at this time point ($p < 0.01$). In contrast, infected but untreated TNF deficient mice lost 24% of their body weight within four weeks of infection, preceding death. At post-mortem their lung weights were increased almost two-fold and microscopically the lungs displayed abundant recruitment of mononuclear cells in the absence of granuloma formation. The data are in accordance with previously published data (Bean et al., 1999; Roach et al., 2001). Gross splenomegaly was observed in reactivated TNF deficient mice (1.480% ± 0.43) as compared to reactivated wild-type mice (0.333% ± 0.08) ($p = 0.001$), which indicates systemic inflammation. By contrast, no differences were observed in spleen weights between reactivated wild-type and chronically infected wild-type mice (0.415% ± 0.01) ($p = 0.124$). Lastly, gross hepatomegaly was found in TNF deficient mice following reactivation of infection (8.917% ± 1.31) as compared to wild-type reactivated mice ($p = 0.016$).

![Figure 3.19. Effect of reactivated tuberculosis on TNF deficient mouse body weights. C57BL/6 and TNF deficient mice were aerogenically infected with 30 CFU *M. tuberculosis* H37Rv and chemotherapeutically treated with RMP-INH for a period of 4 weeks starting 2 weeks post-infection (arrow). Treated C57BL/6 mice (open circles) reactivated after cessation of treatment (100% reactivation) but controlled the infection (100% survival), whereas TNF deficient mice (open squares) reactivated but rapidly succumbed to infection (100% reactivation, 0% survival). The body weights of C57BL/6 infected control mice are represented by solid circles. Following spontaneous reactivation in both C57BL/6 and TNF deficient mice (100% reactivation for both groups), body weights (* $p < 0.005$) were significantly different between these two groups. Body weights are expressed as means ± SD with 3 – 5 mice per group per time point.](image-url)
Figure 3.20. **Effect of reactivated tuberculosis on TNF deficient mouse lung weights.** (A) C57BL/6 and TNF deficient mice were aerogenically infected with 30 CFU *M. tuberculosis* H37Rv and chemotherapeutically treated with RMP-INH for a period of 4 weeks starting 2 weeks post-infection (arrow). Treated C57BL/6 mice (open circles) spontaneously reactivated with tuberculosis after cessation of treatment (100% reactivation) but controlled the infection (100% survival), whereas TNF deficient mice (open squares) reactivated but rapidly succumbed to infection (100% reactivation, 0% survival). The lung weights of C57BL/6 infected control mice are represented by solid circles. Following spontaneous reactivation in both C57BL/6 and TNF deficient mice (100% reactivation for both groups), lung weights (** p < 0.005) were significantly different between these two groups. Lung weights are expressed as means of percentages of body weights ± SD with 3 – 5 mice per group per time point. (B) Macroscopic appearance of lungs of C57BL/6 (left) and TNF deficient mice (right) 14 weeks post-infection during reactivation of tuberculosis.
3.12 Course of infection in TNF gene-deficient mice following short-course antituberculous chemotherapy

In view of the rapid deterioration of the health status and weight changes observed in mice, we asked whether those were correlated with increased bacillary growth in the lungs, spleen and liver following aerosol infection with 30 CFU *M. tuberculosis* H37Rv. A rapid increase of bacilli in lungs, spleens and livers was observed upon cessation of chemotherapy, with a plateau in bacterial load representing a stable and controlled infection in untreated wild-type mice (Figure 3.21).

RMP-INH treatment for 4 weeks controlled infection in both TNF deficient and C57BL/6 mice with undetectable CFU in lungs, spleen and liver in both groups of mice at the end of chemotherapy (week 6). However, the 4-week chemotherapy course did not prevent spontaneous reactivation in both groups of mice. In wild-type mice, the bacterial load increased from undetectable levels to approximately $10^4$, $10^3$ and $10^2$ CFU in lungs, spleen and liver, respectively. By contrast, in TNF deficient mice the reappearance of bacilli following reactivation was much more rapid and uncontrolled. The bacillary burden in the lungs of TNF deficient mice sharply rose to approximately $10^9$ CFU within 8 weeks after therapy, significantly higher than the $10^4$ CFU in the lungs of reactivated wild-type mice at the same time point ($p < 0.01$). A similar pattern was observed in reactivation in the spleen and liver, with an increase from undetectable levels to $10^6$ and $10^7$ CFU, respectively, in TNF deficient mice, which is significantly higher than in wild-type mice (liver $p = 0.05$ and spleen $p < 0.01$). By contrast, infected but untreated TNF deficient mice are unable to control infection and show a dramatic increase of CFU in the lungs ($1.2 \times 10^9$ CFU), spleen ($9.0 \times 10^6$ CFU) and liver ($1.4 \times 10^7$ CFU) at 4 weeks post-infection, prior to death.
Figure 3.21. Uncontrolled tuberculosis in TNF deficient mice following aerosol challenge and RMP-INH chemotherapy. C57BL/6 and TNF deficient mice were infected with 30 CFU *M. tuberculosis* H37Rv and treated for 4 weeks with RMP-INH starting 2 weeks post-infection (indicated by arrows). Viable mycobacteria were enumerated in lungs, spleens and livers at specific time intervals in C57BL/6 infected control mice (solid circles), RMP-INH treated C57BL/6 mice (open circles) and RMP-INH treated TNF deficient mice (open squares). Data are expressed as log_{10} CFU per organ of means ± SD of 3 – 5 mice per group per time point. (* p < 0.005; ** p = 0.05; *** p = 0.001).
3.13 Pathology of TNF gene-deficient mice following short-course chemotherapy and reactivation

Two weeks following aerosol infection a slightly increased cellularity was observed in the alveolar septae of both experimental groups (Figure 3.22 A, D, G). Untreated wild-type mice developed substantial peribronchial inflammation with granuloma formation at 6 weeks post-infection (Figure 3.22 B), which gradually progressed to chronic pneumonia at 14 weeks (Figure 3.22 C). By contrast, chemotherapy treated wild-type and TNF deficient mice presented largely normal lungs at 6 weeks post-infection (Figure 3.22 E, H). Upon cessation of therapy, reactivation of infection was visible at 14 weeks with mild chronic pneumonia and granuloma formation with abundant macrophages in wild-type mice (Figure 3.22 F), while TNF deficient mice developed severe pneumonia with focal necrosis and abundant neutrophil infiltration and absence of proper granulomas (Figure 3.22 I). Abundant acid-fast bacilli were detected in reactivated TNF deficient lung tissues at 14 weeks post-infection (Figure 3.23 A), which were difficult to identify in wild-type mice (Figure 3.23 B). Therefore, TNF deficient mice, which had an undetectable bacterial load at 6 weeks, were unable to control infection upon cessation of chemotherapy and succumbed to overwhelming infection.
Figure 3.22. Lung morphology of RMP-INH treated C57BL/6 and TNF deficient mice during primary infection, persistent infection and reactivation. Lung morphology appears largely normal in RMP-INH treated C57BL/6 and TNF deficient mice, but severe focal necrosis is observed in TNF deficient mice during reactivation of tuberculosis infection. Mice were aerogenically infected with 30 CFU *M. tuberculosis* H37Rv and treated with short-course RMP-INH for 4 weeks, starting 2 weeks post-infection. Panels A, B and C represent lung morphology of infected control wild-type mice during primary infection (A), 6 weeks post-infection (B), corresponding to the period after chemotherapy of the other groups, and at 14 weeks post-infection (C), corresponding to the time point of reactivated disease in the treated C57BL/6 (D, E and F) and TNF gene-deficient mice (G, H and I). Magnification x40.
Figure 3.23. ZN stain showing acid fast bacilli in the lung tissue of TNF deficient and C57BL/6 mice during reactivation of tuberculosis. (A) Abundant mycobacteria can be seen in TNF deficient mice versus low numbers of bacilli in (B) C57BL/6 mice 14 weeks post-infection during reactivation in both groups. Mice were aerogenically infected with 30 CFU \textit{M. tuberculosis} H37Rv and treated with short-course RMP-INH for 4 weeks, starting 2 weeks post-infection, shortly after which mice spontaneously reactivated with tuberculosis. Tissue was fixed in buffered formalin, sectioned and ZN stained to detect acid-fast mycobacteria. Magnification A, x40; B, x100.
3.14 Granulomatous response in TNF gene-deficient mice following short-course chemotherapy and reactivation

As no proper granulomas could be identified in the lungs of TNF deficient mice, we analysed granuloma formation in the liver. While untreated wild-type mice presented with distinct hepatic granulomas at 6 weeks post-infection, no inflammatory reaction was observed in chemotherapeutically treated wild-type and TNF deficient mice. By 14 weeks post-infection, untreated wild-type mice had small hepatic granulomas, which were comparable to that of wild-type mice after chemotherapy and reactivation. By contrast, TNF deficient mice developed large and in part necrotic inflammatory foci in the liver at 14 weeks (Figure 3.24).

We further tested the expression of NOS2 as a marker of macrophage activation in liver (data not shown) and lungs. While wild-type mice with distinct granulomas expressed abundant NOS2 immunoreactivity in the lungs, NOS2 expression in TNF deficient mice was distinctly reduced (Figure 3.25).

Concomitant with reduced NOS2 expression nitric oxide levels in the lung homogenate at 4 weeks after cessation of drug treatment were significantly lower in TNF deficient mice as compared to wild-type controls ($p < 0.005$). Untreated wild-type mice had slightly lower levels than treated wild-type controls (Figure 3.26). Therefore, the results demonstrate that TNF is required for NOS2 expression and nitric oxide production in addition to its pivotal role in granuloma formation and corroborates the finding of other ups (Flynn et al., 1995; Scanga et al., 1999; Flynn & Chan, 2001).
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Figure 3.24. Defective granulomatous response in TNF deficient mice during reactivation of tuberculosis. Mice were aerogenically infected with 30 CFU *M. tuberculosis* H37Rv and treated with short-course RMP-INH for 4 weeks, starting 2 weeks post-infection. Liver tissue was obtained during the reactivation of infection at 14 weeks post-infection, fixed in buffered formalin, sectioned and stained with H&E. Magnification x40.
Figure 3.25. Reduced NOS2 expression in the lung tissue of TNF deficient mice during reactivated tuberculosis. Mice were aerogenically infected with 30 CFU *M. tuberculosis* H37Rv and treated with short-course RMP-INH for 4 weeks, starting 2 weeks post-infection. Lung tissue was obtained during reactivation of infection, 14 weeks post-infection, fixed in buffered formalin, sectioned and stained with a NOS2 antibody to indicate NOS2 expression (brown staining). Magnification x40.
3.15 Lymphocyte recruitment and activation in TNF deficient mice with reactivated *M. tuberculosis* infection

Recruitment of T cells to the site of infection has been shown to be critical to form granulomas and control *M. tuberculosis* infection. In accordance with the microscopic findings, the total mononuclear cell counts obtained from infected lungs at 4 weeks after cessation of RMP-INH chemotherapy was reduced in TNF deficient mice. Flow cytometric analysis revealed $4.8 \times 10^6$ T cells from the lungs of TNF deficient mice and $7.3 \times 10^6$ for wild-type mice. We further quantified T cell subpopulations and assessed activation status. Recruitment of CD4$^+$ T cells was significantly decreased in TNF deficient mice at 10 weeks post-infection, while CD8$^+$ T cell counts were similar in both groups (Figure 3.27). Using CD11a and CD44 as activation markers of CD4$^+$ cells, TNF deficient mice revealed significantly lower numbers of activated T cells at 10 weeks post-infection ($p < 0.001$). Furthermore, CD11b$^+$ positive macrophages expressing MHC class II were also significantly reduced in TNF deficient mice ($p < 0.001$). Thus, both lymphocyte and macrophage recruitment and activation were decreased in the absence of TNF. Since chemokines orchestrate the recruitment of
mononuclear cells, we asked whether the production of chemokines was altered in TNF deficient mice.

Figure 3.27. TNF deficient mice show reduced lymphocyte and macrophage recruitment and activation in the lung in response to reactivated tuberculosis. Lung cells were obtained from wild-type (solid bars) and TNF deficient mice (open bars) 4 weeks after cessation of RMP-INH chemotherapy and analysed by flow cytometry. Results are pooled from two experiments and expressed as absolute cell numbers of the cell populations indicated (mean ± SD for four mice per group, * p < 0.001).

3.16 Chemokine production in the absence of TNF

TNF dependence of chemokine expression has been reported in vitro (Hornung et al., 2000), and decreased CXC and CC chemokine expression upon tuberculosis infection has been shown in TNF deficient mice (Roach et al., 2002). We therefore assessed the production of selected chemokines in the lungs upon reactivation of tuberculosis infection. We determined the pulmonary levels of MCP-1, MIP-1α and RANTES after reactivation of infection (Figure 3.28). MCP-1 levels in the lung were significantly lower in TNF deficient mice at 10 weeks following M. tuberculosis infection as compared to wild-type controls (p < 0.001). Similarly, pulmonary production of MIP-1α and RANTES were significantly decreased in TNF deficient mice (p < 0.001). These data suggest that reduced chemokine production might explain in part reduced cell recruitment and granuloma formation.
3.17 Characterisation of conditional gene-targeted TNF deficient mice

Conditional TNF knockout mice were generated using the Cre-loxP-mediated site-specific recombination strategy, as described by Kuhn et al. (1995). To establish mice with conditional gene-targeted TNF, loxP sites were inserted upstream and downstream of the coding region of TNF in the mouse genome, together with a neomycin phosphotransferase gene-expressing cassette (Figure 2.1). ES cells were electroporated with this construct and neomycin-resistant clones were screened by Southern blot analysis, using the mER4 radiolabelled probe, which detects part of the coding region of lymphotoxin-β, upstream of the TNF locus. A few homologous recombinant ES clones were injected into C57BL/6 mouse blastocysts in order to generate chimeric mice. Mouse-tail DNA was analysed by Southern blot to assess transmission of the targeted allele. Mice with either of the homozygous (TNF^{fl/fl}) or heterozygous (TNF^{fl/+}) floxed TNF alleles showed no visible developmental change without Cre
recombinase expression. Deletional inactivation of the TNF locus by Cre expression was achieved by breeding mice with the floxed TNF allele with Mx-Cre transgenic mice (mice in which the Cre recombinase is under the control of the Mx promoter) (Kuhn et al., 1995). Mice containing the floxed TNF allele were then genotyped for the presence of the Cre recombinase transgene, using the cre-specific primers IMR1084 and IMR1085 (Figure 3.29). Mice containing both the floxed TNF alleles and the Cre recombinase transgene were used in experiments.

![Figure 3.29. PCR genotyping of TNF<sup>+/−</sup> x Cre mice. A 1.6% agarose gel with a DNA ladder molecular weight marker in lane 1 and PCR-amplified genomic mouse DNA samples in lanes 3 – 9. Samples of previously genotyped TNF deficient mice were amplified with cre-specific primers IMR1084 and IMR1085 to establish which mice had insertions of the cre transgene. The presence of a 123 bp amplified product in lanes 3 and 5 – 9 indicates mice positive for the cre recombinase gene. The mouse DNA in lane 4 is negative for the presence cre.](image-url)
Conditional inactivation of TNF was induced upon injection of a series of three injections of poly(I:C), an inducer of IFN-α/β, and analysed by Southern blot hybridisation using the mER4 probe. BamHI-digested genomic DNA probed with mER4 gave rise to 11 kb and 8.6 kb bands for wild-type and deleted TNF alleles, respectively (Figure 3.30 A). Efficiency of deletion in various mouse tissues were assessed, and banding patterns were analysed by densitometry and percentage deletion calculated using ImageQuant® software (Figure 3.30 B). In some tissues, for example bone marrow, peripheral blood and liver, injection of poly(I:C) induced complete deletion of the floxed TNF gene fragment, whereas deletion efficiency in other tissues varied and ranged from 87% for spleen to 14% for tail (Figure 3.31 A). Furthermore, a number of mice were analysed for spontaneous deletion of floxed TNF (Figure 3.31 A), which could have resulted from mice secreting small amounts of IFN-α/β as result of a viral pathogen or non-sterile animal housing conditions. This type of spontaneous gene deletion occurred only in some mice and only in some organs of those mice. The spleen seemed to be the most sensitive organ concerning spontaneous gene deletion, as in some mice this was the only organ that showed spontaneous deletion. Additionally, the extent of spontaneous deletion was most varied in the spleen. Some mice had spontaneous deletion at 19% whereas others had a very high percentage (67%) of gene deletion, with the average gene deletion in the spleen at 46% (n = 7). Furthermore, the spleen seems to be the most sensitive of the organs studied, as in some mice only the spleen showed spontaneous gene deletion, with no spontaneous deletion detected in any other organ. In other organs, such as the liver, no spontaneous deletion was ever observed, whereas deletion was consistently 100% following poly(I:C)-induced gene deletion (Figure 3.31 A).

The overall efficiencies of poly(I:C)-induced deletion in this study correlates well with the findings of Kuhn and colleagues for the various tissues that were analysed in both studies, as displayed in Figure 3.31 B (Kuhn et al., 1995).
Figure 3.30. (A) Cartoon illustrating the potential banding pattern of Southern blot results and (B) a Southern blot of wild-type and TNF$^+/-$ x Cre mice. Genomic DNA was digested with BamHI and probed with mER4. In this example of wild-type C57BL/6 and TNF$^+/-$ x Cre mice, liver (LIV), spleen (SPL), thymus (THY) and mesenteric lymph node (MLN) DNA show band patterns that were scanned by a phosphor-imager and percentage deletion in each organ calculated using ImageQuant® software.
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Figure 3.31. Cre-mediated gene deletion following poly(I:C) administration. (A) Groups of TNF^x Cre mice were injected i.p. with 250 µg poly(I:C) on days 0, 2 and 5 and mice were sacrificed for analysis on day 7, 2 days after the last injection. Genomic DNA was isolated from various organs, BamHI digested, electrophoresed, Southern blotted and probed with mER4 to distinguish banding patterns before (open bars) and after poly(I:C)-induced deletion (solid bars). Results were analysed from phosphor-images and calculated with ImageQuant® software.

(B) Comparison of Cre-mediated deletion data (solid bars) with data previously published (striped bars) by Kuhn et al. (1995) on organs analysed in both studies. Results are expressed as means ± SD for 4 – 7 mice per group per time point.
Levels of TNF were measured by ELISA in the plasma of groups of TNF<sup>ff</sup> x Cre mice before and after conditional inactivation of the TNF allele (n = 4 per group). C57BL/6 and TNF gene deficient mice served as controls. Groups of TNF<sup>ff</sup> x Cre mice were injected i.p. with 250 μg poly(I:C) on days 0, 2 and 5 to allow Cre-mediated deletion of the floxed TNF alleles. Forty eight hours later, groups of mice were injected i.p. with LPS and sacrificed 90 min later. Blood was obtained through cardiac puncture, diluted and restimulated with LPS ex vivo, after which plasma TNF levels were assessed by ELISA (Figure 3.32). C57BL/6 control mice had an average of 5.83 ng/ml TNF in plasma following restimulation ex vivo, whereas TNF<sup>ff</sup> x Cre mice before induction of gene deletion had TNF levels at an average of 1.37 ng/ml, 4-fold less than wild-type controls. However, levels were 5-fold reduced to 0.26 ng/ml following poly(I:C) induction of TNF gene deletion. No TNF was detected in the plasma of TNF<sup>−/−</sup> control mice.

![Figure 3.32. Plasma TNF levels in TNF<sup>ff</sup> x Cre mice upon LPS re-stimulation.](image)

Mice were injected i.p. with 100 μg LPS in 500 μl PBS and were sacrificed for analysis 90 min later. Whole blood was obtained in a heparinised syringe through cardiac puncture and was re-stimulated ex vivo with 10 ng/ml LPS for 4 hours at 37°C, after which TNF levels in plasma were determined by ELISA. TNF levels were reduced 5-fold following Cre-mediated deletion and LPS re-stimulation in TNF<sup>ff</sup> x Cre mice. Results are expressed as means ± SD of 4 mice per group.
As described above, TNF levels were also determined by ELISA in cultured BMDM after restimulation with LPS. Groups of TNF<sup>fl</sup> x Cre, C57BL/6 and TNF deficient mice were injected with LPS 48 hours following the last poly(I:C) injection, while control groups were not injected (n = 4 per group). BMDM were obtained and cultured to allow macrophages to expand and mature, after which cells were restimulated with LPS ex vivo and TNF levels measured in culture supernatants (Figure 3.33). No significant differences were noted between wild-type mice with or without poly(I:C) administration as expected, whereas TNF levels in conditional targeted TNF mice were 10-fold decreased after poly(I:C)-induced gene deletion (p < 0.01). As before, no TNF was detected in BMDM of TNF<sup>-/-</sup> control mice. Very similar results were obtained after groups of mice were stimulated with 1 µg/ml \( M.\) \textit{tuberculosis} \( \text{H37Ra} \) (data not shown).

![Graph](image)

**Figure 3.33.** TNF levels in BMDM from TNF<sup>fl</sup> x Cre mice upon LPS re-stimulation. Mice were injected with 100 µg LPS in 500 µl PBS and were sacrificed 90 min later for analysis. Bone marrow was obtained and BMDM cultured for 10 days before re-stimulation with 10 ng/ml LPS ex vivo, after which TNF levels of unstimulated and re-stimulated BMDM were determined by ELISA. A 10-fold reduction in TNF levels was observed following Cre-mediated deletion and LPS re-stimulation in TNF<sup>fl</sup> x Cre mice. Results are expressed as means ± SD of 4 mice per group.
In order to utilise the TNF\textsuperscript{d} x Cre mice in future tuberculosis studies, certain parameters were tested. Firstly, the effect of poly(I:C) administration on tuberculosis-infected C57BL/6 mouse body weights were assessed (Figure 3.34). Wild-type mice were infected with \textit{M. tuberculosis} H37Rv at 30 CFU per lung and were injected with the standard series of poly(I:C) 2 weeks post-infection (n = 10). A control group of C57BL/6 mice received no injections (n = 10). Mice were weighed at weekly intervals for 4 weeks and no significant differences in mouse body weights were observed between the group receiving poly(I:C) and the uninjected group (p = 1.034 at week 4).

![Figure 3.34. The effect of poly(I:C) administration on mouse body weights. Mice were infected with 30 CFU \textit{M. tuberculosis} H37Rv through aerosol and injected i.p. with a series of three poly(I:C) injections 2 weeks post-infection (arrows) to determine the effect of the double-stranded RNA on mice during tuberculosis infection progression (solid squares). Control infected mice did not receive poly(I:C) injections (open squares). Results are expressed as means ± SD of 5 mice per group.]

Secondly, tuberculosis-infected mouse organ weights were monitored before and after poly(I:C) administration. Mice were infected as described above and a series of three i.p. poly(I:C) injections were given to a group of mice (n = 10) between week 2 and week 3 post-infection and lung, spleen and liver weights monitored (Figure 3.35). Each organ weight is expressed as the percentage of the mouse body weight at that time point. The organ weights at week 2 represent the point immediately prior to the poly(I:C) injection series. In the lung, at week 3, 2 days after the last poly(I:C)
Figure 3.35. The effect of poly(I:C) administration on mouse (A) lung, (B) spleen, and (C) liver weights. Mice were infected with 30 CFU *M. tuberculosis* through aerosol and injected i.p. with a series of three poly(I:C) injections between week 2 and 3 post-infection to determine the effect of the double-stranded RNA on mouse lung, spleen and liver weights in poly(I:C)-injected mice (solid bars) during tuberculosis infection. Uninjected mice (open bars) served as controls. Results are expressed as means ± SD of 5 mice per group.
injection, no significant differences were observed \((p = 0.273)\), but a delayed effect was evident with significant differences the the lung weights of uninjected control versus poly(I:C) injected mice \((p = 0.023\) at week 4) (Figure 3.35 A). Another phenomenon was observed in the liver and spleen, with a more pronounced phenotype in the liver. Hepatosplenomegaly was immediately observed at week 3, 2 days after the last poly(I:C) injection \((p < 0.001\) for both liver and spleen) (Figure 3.35 B, C). One week later, hepatosplenomegaly was still evident, but much less pronounced and approaching normalisation \((p = 0.050\) for liver; \(p = 0.017\) for spleen). Thus, the hepatosplenomegaly observed seemed to have been a transient effect immediately following the poly(I:C) injections.

Lastly, the effect of poly(I:C) injections on the replication of \(M.\) \textit{tuberculosis} was assessed and the findings presented in Figure 3.36. Following aerosol infection with 30 CFU per lung, normal expansion of \(M.\) \textit{tuberculosis} H37Rv was observed in the lung at weeks 3 and 4 post-infection, following poly(I:C) injections between weeks 2 and 3 (Figure 3.36 A). Similar expansion was observed in the spleen (Figure 3.36 B) with no significant differences observed between poly(I:C)-injected and control groups at any time point. The only significant difference in CFU was observed in the liver (Figure 3.36 C). There seemed to have been a delayed effect with a significant increase in the liver CFU of the poly(I:C)-injected group at week 4 post-infection, slightly more than 1 week after the last poly(I:C) injection \((p = 0.008)\).
Figure 3.36. The effect of poly(I:C) administration on the progression of tuberculosis infection. Mice were infected with 30 CFU *M. tuberculosis* H37Rv through aerosol and injected i.p. with a series of three poly(I:C) injections (arrows) to determine the effect of the double-stranded RNA on mycobacterial replication during tuberculosis infection. CFU were monitored in the (A) lungs, (B) spleens and (C) livers of poly(I:C)-injected mice (closed squares) and non-injected mice (open squares). Results are expressed as means ± SD of 5 mice per group.
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4.1 Optimising antituberculous therapy in wild-type mice in the drug model

One aspect of this study describes a short-duration low dose aerosol murine infection model that can be used to study aspects of reactivation of persistent tuberculosis infection. A persistent state was attained through RMP-INH drug therapy and by varying the periods of treatment, reactivation could occur either spontaneously or could be induced by aminoguanidine, an inhibitor of NOS2. Reactivation has been characterised in both wild-type and TNF deficient mice, assessing the ability of mice to mount a protective immunity. Additionally, the model was used to analyse the outcome of reinfection of *M. tuberculosis* during the persistent phase of infection.

Few other animal models have been described in which latency is induced with antituberculous drugs (Flynn *et al.*, 1998; McCune *et al.*, 1966a; McCune *et al.*, 1966b; Scanga *et al.*, 1999), however, PZA and INH were used to contain *M. tuberculosis* in these models. We opted for chemotherapeutic treatment of tuberculosis with a combination of RMP and INH because of their current use as first-line drugs in both ‘intensive phase’ and ‘continuation phase’ of chemotherapeutic patient treatment (Barry, 1997). Additionally, RMP is active against tubercle bacilli both intracellular and extracellular, whereas PZA is only active against intracellular bacteria because of its optimal functioning at acidic pH (Clini & Grassi, 1970). Moreover, the pharmacokinetics and dosage of RMP are far more similar in mouse and man than PZA (Grosset, 1978). In the mouse model described by Grosset (1978), RMP-INH combination therapy was used for 12 months following intravenous infection with *M. tuberculosis* but did not result in mycobacterial elimination. Following treatment, sixty percent of mice reactivated with tuberculosis after cortisone administration, he therefore questioned whether it was possible to eradicate tubercle bacilli using RMP-
INH combination therapy in mice (Grosset, 1978). In contrast to his findings, it has been shown in this study that it is indeed possible to eliminate bacilli using this drug regime for only 12 weeks following low dose aerosol challenge, even in TNF deficient mice. Aminoguanidine administration in both wild-type and TNF deficient mice failed to reactivate tuberculosis. In contrast, reactivation induced with aminoguanidine after 8 weeks of RMP-INH therapy resulted in faster and more efficient reactivation of tuberculosis in mice (67 – 100%) compared to 30% reactivation in the Cornell model, with no spontaneous reactivation in latently infected animals (McCune & Tompsett, 1957; McCune et al., 1957; McCune et al., 1966a; McCune et al., 1966b).

The results emphasise the fine equilibrium that exists between the initial bacterial burden and length of RMP-INH antituberculous chemotherapy, as short treatment (4 weeks) resulted in spontaneous reactivation of tuberculosis in mice, while long treatment (12 weeks) eradicated all bacilli. Eight weeks of antituberculous therapy seemed to be optimal in this model to achieve a persistent state under the experimental conditions. Treatment induced stable latency in mice and could be reactivated with aminoguanidine, which inhibits macrophage NOS2 therefore preventing production of bactericidal RNI.

The optimal model (model B in this study) initially proceeded over an experimental period of 55 weeks. This was done in order to verify sustained latency and no spontaneous reactivation during this period. It was, however, ascertained that following 8 weeks RMP-INH chemotherapy and a rest period of 4 weeks, aminoguanidine administration could be commenced in order to induce reactivation of infection. This reduces the experimental period to 18 weeks, hence it can be considered a short-duration model.
4.2 Kinetics of mycobacterial reduction during rifampicin-isoniazid chemotherapy

The kinetics of mycobacterial reduction following RMP-INH treatment show a rapid initial killing rate in the lungs, spleens and livers of mice, followed by a more gradual reduction to very low or undetectable levels at a plating sensitivity of 2 CFU per organ. This observation was especially striking in context of the continuing increase of CFU in untreated mice until a steady state was achieved between 3 and 4 weeks post-infection. A study on the numbers of culturable mycobacteria recovered from human sputum during the first 2 weeks of treatment indicated that the most rapid rate of reduction appeared within the first 2 days (Jindani et al., 1980). One hypothesis is that the majority of drug-sensitive bacilli are killed first and more rapidly than the proportion less susceptible mycobacteria that may be in the process of adapting to a persistent state (Wayne, 1994). However, exactly when and how bacteria undergo this shift in vivo is not clear.

There is, however, another consideration with regards to the killing kinetics of RMP-INH combination chemotherapy. Due to the mechanism of action of RMP against RNA synthesis (Telenti et al., 1993) and INH against cell wall mycolic acid synthesis (Banerjee et al., 1994), it is expected that mycobacteria will be most susceptible to these chemotherapeutic agents during active replication, which occurs during the first 3 weeks of growth in vivo at doubling times of 17 to 18 hours (North & Izzo, 1993). The ensuing plateau phase, when the host adaptive immune response exhibits some control over mycobacterial replication, may result in the drugs being less efficient when administered during similar time frames. This aspect was investigated in this study by comparing the killing kinetics during the first 4 weeks of chemotherapy in a group of mice treated with RMP-INH starting 2 weeks post-infection with another group of mice where chemotherapy was initiated during the plateau phase at 8 weeks post-infection. The initial number of bacteria in the lungs and spleens of these two groups of mice at the start of therapy were comparable with less than 1 log difference between the groups. Surprisingly, at the end of the respective 4-week chemotherapy
courses, mycobacterial loads were reduced comparably in both the lungs and spleens of these groups.

4.3 Aminoguanidine-induced reactivation of tuberculosis

The two types of murine tuberculosis models that have been described before are the low dose or chronic infection model (Brown et al., 1995; Flynn et al., 1998; Mustafa et al., 1999; Orme, 1988) and the drug model (Flynn et al., 1998; Grosset, 1978; McCune et al., 1966a; McCune et al., 1966b; Mustafa et al., 1999; Scanga et al., 1999). These models show different patterns of disease progression and reactivation. Aminoguanidine-induced reactivation in C57BL/6 mice chronically infected with *M. tuberculosis* resulted in a fatal infection, whereas aminoguanidine-induced reactivation in drug-treated latent mice was controlled and mice stably maintained the bacillary burden (Flynn et al., 1998). It was clear that as yet unidentified NOS2-independent mechanisms played a role in controlling reactivated tuberculosis in these mice (Flynn et al., 1998). This study showed similar observations with aminoguanidine-induced reactivation of tuberculosis in model B, in that all mice survived and managed to control reactivated infection in a similar way than they controlled chronic infection.

It was investigated whether drug-treated mice harboured RMP- or INH-resistant mycobacteria upon reactivation. The estimated frequency of drug-resistant mutants is generally low for RMP at $3.1 \times 10^{-8}$ and slightly higher for INH at $3.5 \times 10^{-6}$ (Victor et al., 2002). Extensive plating of lung homogenates revealed that recovered bacilli were still sensitive to both drugs used during chemotherapy. This finding is in line with a study on human lung biopsy specimens from patients where *M. tuberculosis* was isolated from closed lung cavities and was still found to be drug-sensitive (Vandiviere et al., 1956).
4.4 Spontaneous reactivation of tuberculosis

In contrast to aminoguanidinie-induced reactivation after 8 weeks of chemotherapy, short-course RMP-INH treatment of 4 weeks resulted in spontaneous reactivation of tuberculosis infection (Model A in this study). The question was asked whether RMP-INH treatment starting 2 weeks post-infection in a group of mice, before an adequate host immune response was established, would allow relapse of infection as result of early initiation of treatment. Likewise, whether initiation of RMP-INH treatment at 8 weeks post-infection in another group of mice would allow development of protective immunity against reactivated infection. Following 4 weeks of chemotherapy in both early- and late-treated groups, reactivation kinetics was compared between them. Subtle differences were observed in the lungs and spleens of these two groups, but overall trends were similar. In addition, the total increase in CFU over the 10-week period following chemotherapy was comparable in both organs of both groups. Therefore, it can be concluded that by allowing adequate time for a full immune response to develop before therapy commences, no protection against reactivated tuberculosis is conferred compared to the control group.

4.5 Mycobacterial genome copy numbers as assessed by real-time PCR with molecular beacons

Quantification of genome copies and comparison of these to culturable numbers of *M. tuberculosis* in mice revealed two interesting observations. Firstly, it appeared that the rate of clearance of dead tubercle bacilli from the host after RMP-INH treatment was much slower than reduction of viable mycobacteria. After an initial delay of more than four weeks, genome numbers were only 10-fold reduced and remained at these levels throughout the period of persistent infection. This is in contrast with the dramatic 100,000-fold reduction of viable colonies over the same period. The second interesting observation was that the sustained number of mycobacterial genome copies, which possibly represented mostly dormant bacilli, only
marginally increased during subsequent aminoguanidine-induced reactivation, which suggested that there might have been much larger numbers of persisters in the host organs than what could be cultured. *M. tuberculosis* DNA copy number quantification in the classic Cornell model has been performed in the past (De Wit et al., 1995). Despite the advancement of molecular beacon technology, which facilitates more sensitive and specific detection of mycobacterial genome copies, the results of this study and that of De Wit and colleagues are remarkably similar, even though the routes of infection and treatment regimes differed. They found a similar level of genome copies and viable colony counts at the onset of PZA and INH chemotherapy. The subsequent decrease in CFU during the 14-week course of therapy was more than 1,000,000-fold, whereas the reduction in genome copies was only 15-fold before it stabilised (De Wit et al., 1995). Unfortunately, they did not examine reactivated tuberculosis.

The major shortcoming in that study was the lack of discrimination between dormant persisters, dead bacilli and free mycobacterial DNA, however, the quantification of mRNA may have indicated whether the majority of detected genome copies were from viable bacilli or not. This aspect was investigated in this study and is discussed below.

4.6 Mycobacterial gene expression as assessed by real-time PCR with molecular beacons

Quantification of mycobacterial gene expression in mice during active tuberculosis, persistence and reactivation was therefore investigated in this model using molecular beacon real-time PCR. It has been demonstrated that mycobacteria behave very differently *in vitro* and *in vivo* and very little information is available of their metabolic status *in vivo* during the persistent phase of infection (Segal & Bloch, 1956). A few candidate genes were selected on the basis of their putative roles in persistent murine tuberculosis infection. For example, it has been shown that *icl1*, a gene encoding isocitrate lyase, which is essential in the catabolism of fatty acids, is
upregulated during the chronic phase of infection in mice (McKinney et al., 2000). An α-crystallin chaperone homologue, encoded by \textit{hspX} in \textit{M. tuberculosis}, is another product associated with increased expression during the persistent phase of infection (Flynn et al., 1998; Yuan et al., 1998). \textit{MbtB}, a peptide synthetase involved in siderophore synthesis, seems to be an essential element in iron uptake and metabolism in \textit{M. tuberculosis} during long-term survival (De Voss et al., 2000), whereas \textit{pckA}, encoding phosphoenolpyruvate carboxykinase, is involved in gluconeogenesis, a process thought to be important for survival of mycobacteria during glucose starvation (Liu et al., 2003). Lastly, upregulation of \textit{sigF}, which encodes a product related to sporulation sigma factors from \textit{Streptomyces coelicolor} and \textit{Bacillus subtilis}, raises an interesting possibility that \textit{M. tuberculosis} might enter a spore-like state that might enable it to survive in the hostile host environment during persistent infection (Chen et al., 2000; DeMaio et al., 1997).

Detection of the above-mentioned genes was technically difficult due to the sensitivity of the method used and limited mycobacterial numbers in the portions of lungs used in this study. The time points of interest was during the peak of primary infection, at the end of RMP-INH chemotherapy, just before aminoguanidine-induced reactivation or reinfection, and during the peaks of reactivation or reinfection with \textit{M. tuberculosis} H37Rv-pIVT. \textit{icl1}, \textit{hspX}, \textit{pckA} and \textit{mbtB} genes were all detected at most of these time points, however, not reproducibly and with enough sensitivity to make final conclusions. At least $10^4$ mRNA transcripts are usually required for detection using this technique (Dr. J. Timm, personal communication), and the fact that only the left lobe of each lung per mouse was used, mycobacterial numbers generally were too low.

Despite the lack of information from the above-mentioned genes, real-time RT-PCR with molecular beacons assessing \textit{16S} r\textit{RNA} and \textit{sigA} expression yielded some interesting results. Quantitative analysis of mycobacterial genome numbers using molecular beacon PCR and quantitative RT-PCR of
the sigA gene produced very similar results, with the only exception at the
time point at the end of the persistent phase, 16 weeks post-infection. Apart
from confirming that there are likely much higher numbers of mycobacteria
present in the host during the persistent phase of infection, these bacteria
seemed to be metabolically active during this phase, as indicated by
expression of sigA. Most likely, these bacteria represent persisters, as they
could not be detected by conventional plating methods during that time.
Whereas DNA quantification of genome copies may have included dead
bacteria as well as dormant forms, sigA expression would only be detected
in actively metabolising bacteria. This was a shortcoming in the study by De
Wit and colleagues who could not discriminate between live and dead
mycobacteria during the persistent phase of infection, using conventional
PCR DNA amplification (De Wit et al., 1995). The upward trend during the
six weeks of persistent infection following drug treatment is confirmed by the
expression of 16S rRNA.

4.7 Reinfec tion during the persistent phase of infection

In the light of high incidence tuberculosis communities, reinfection is a facet
that should be considered critically (Caminero et al., 2001; Chaves et al.,
1999; Garcia de Viedma et al., 2002; Richardson et al., 2002; Shafer et al.,
1995; van Rie et al., 1999). To date, very little experimental data is
available on this aspect (Andersen & Smedegaard, 2000; Cardona et al.,
2002; Repique et al., 2002), mostly because of the lack of suitable models
and the comprehensive nature of such experiments.

In this study a group of wild-type mice was primary infected with
*M. tuberculosis* H37Rv via aerosol, chemotherapeutically treated with RMP-
INH for 8 weeks to achieve a latent state, then reinfected via aerosol with a
*kan*R antibiotic-resistant strain of *M. tuberculosis* H37Rv. Both primary
infected and reinfected groups of mice appeared clinically healthy
throughout the experiment, however, according to body weight assessment,
reinfected mice underwent a marginal dip in body weights 4 to 5 weeks
following reinfection. Lung and spleen CFU data were evaluated in both strains and showed that during the first 6 weeks following reinfection, both primary and reinfected strains appeared to grow at equal rates, but interestingly, 8 weeks post-reinfection, during the stationary phase of infection, there was a pronounced increase of more than 1 log in the reinfected group, whereas CFU in the primary infected group remained stable. This was most noticeable in the lung, although the same pattern was seen in the spleen. It appeared that the persistent strain went through an extended lag phase and gradually increased in CFU until it outgrew the newly infected strain. Unfortunately this reinfection experiment was only followed for 8 weeks beyond the reinfection, because of the large numbers of mice and the length of the experiment, but it would have been ideal to follow these events for longer. These results are in contrast to the results of Serbina et al. (2001), who found a “memory” response and a concomitant 10-fold reduction in CFU in a reinfected group of mice. The data presented in this study is preliminary and should be repeated before any conclusion can be reached.

4.8 Reactivation of tuberculosis in TNF deficient mice

When the drug-induced latency model was applied to TNF deficient mice, it was evident that TNF is required to control latent *M. tuberculosis* infection upon cessation of chemotherapy in aerosol-infected mice (Flynn et al., 1995). Despite elimination of bacilli to undetectable levels after short-course RMP-INH treatment, massive reactivation with necrosis and death of mice was observed in the absence of TNF. TNF deficient mice usually succumb to a primary aerosol infection with 30 CFU within 5 to 6 weeks, whereas these mice survived 7 to 12 weeks post-chemotherapy. The survival times of primary and reactivated infection were comparable and the small difference in time could be ascribed to the lower number of persistent bacilli that survived chemotherapy as compared to the infective dose. Whereas both wild-type and TNF deficient mice reactivated spontaneously following short-course RMP-INH chemotherapy, wild-type mice presented with sub-
clinical tuberculosis, unlike mice deficient in TNF, which reactivated rapidly with fatal infection. Differences between wild-type and TNF deficient mouse body weights, as well as gross differences in lung, liver and spleen weights were noted, exemplifying characteristics of clinical disease versus subclinical infection.

The kinetics of mycobacterial replication showed a rapidly progressing infection with CFU reaching fatal numbers in the organs of TNF deficient mice within 7 to 12 weeks of reactivated infection. Despite initially undetectable CFU following chemotherapy, TNF deficient mice were unable to control bacterial growth, while bacteria in wild-type mice reached a plateau following reactivation, during which time the infection was controlled. Additionally, abundant acid-fast bacilli were detected both intracellular and extracellular in TNF deficient mice shortly before they succumbed as a result of overwhelming infection. The importance of TNF in protection and pathogenesis of tuberculosis has been investigated by the use of neutralising antibodies and gene deficient mice. Both TNF and TNF receptor deficient mice showed delayed granuloma formation and lack of control of mycobacterial infection (Bean et al., 1999; Flynn et al., 1995; Jacobs et al., 2000). Furthermore, signalling through the TNF type 2 receptor was not found to be critical to control mycobacterial infection (Jacobs et al., 2000).

4.9 Pathology during reactivation in TNF deficient mice

Apart from the role of TNF in controlling multiplication of mycobacteria during infection, it also contributes to the destructive pathology typically seen in tuberculosis (Bekker et al., 2000; Rook, 1990; Rook et al., 1987). We have observed progressive pathology, most notably in lung tissue, in the absence of TNF, which might be due to compensatory production of other pro-inflammatory cytokines. TNF deficient mice developed severe pneumonia with focal necrosis, associated with an increase in bacterial
burden within a short time after reactivation of tuberculosis following primary aerosol infection.

Granulomas, a hallmark of mycobacterial infection, are formed when antigen-specific T cells are recruited and activate macrophages at the localised site of infection (Andersen, 1997; Gordon et al., 1994; Kaufmann & Ladel, 1994). Containment of \textit{M. tuberculosis} depends on this granulomatous response, and any defect that prevents efficient granuloma formation results in the failure to isolate mycobacteria from surrounding tissue (Emile et al., 1997; Kindler et al., 1989). Following RMP-INH chemotherapeutic treatment in this study, TNF deficient mice presented with minimal lung and liver pathology, but during reactivation of tuberculosis necrotic lesions formed because of the lack of orchestrated granuloma differentiation. The results from this study confirm the importance of rapid and efficient granuloma formation, as TNF deficient mice, lacking these abilities, rapidly succumbed to overwhelming infection as result of uncontrolled replication and dissemination of mycobacteria. It was found that T cells and macrophages were not efficiently recruited to the lung and no proper granulomas were formed. Additionally, NOS2 expression and nitrite levels in the lungs of TNF deficient mice were low compared to levels in reactivated wild-type mice. By contrast, NOS2 expression and serum nitrite levels in the acute infection model (MacMicking et al., 1997) and in the persistent model of tuberculosis infection (Flynn et al., 1998) is apparently TNF independent.

4.10 Nitric oxide as mycobactericidal agent

IFN-\textgamma{} and IL-12 were elevated in TNF deficient mice (unpublished observation), therefore suggesting that IFN-\textgamma{} and IL-12 alone are not sufficient to activate macrophages to produce NO in the face of an acute primary or reactivated mycobacterial infection. The exact concerted mechanism by which TNF and IFN-\textgamma{} activate macrophages to produce NO
is likely to be more complex than our current understanding. It seems that early activation and production of NO is essential to control mycobacterial replication. A delay in RNI production in splenic macrophages and in lung granulomas was observed by Flynn et al. (1995) in both TNF p55 receptor deficient mice and mice in which TNF was neutralised with a monoclonal anti-TNF antibody (Flynn et al., 1995; Mohan et al., 2001). The fine equilibrium between host immune control and mycobacterial burden seemed to be a determining component in the outcome of primary infection as well as reactivated infection. Scanga and colleagues investigated the relative importance of RNI in both aerosol and intravenous murine infection models. They found that RNI indeed played an important role in vivo, regardless whether laboratory strains or clinical isolates of *M. tuberculosis* were used, in both aerogenic and intravenous infection models (Scanga et al., 2001).

The role of RNI in host defence in human tuberculosis has been a controversial point for quite some time. However, localised expression of NOS2 in human lung lesions within granulomas, alveolar macrophages and epithelial cells in pneumonitis areas has been demonstrated recently (Choi et al., 2002). This study provides specific evidence that NOS2 is expressed at the foci of *M. tuberculosis* infection in the human lung. In addition, NO is produced by human macrophages in vitro (reviewed in (Fang & Vazquez-Torres, 2002), and is able to kill *M. tuberculosis* (Chan et al., 2001). Moreover, NOS2 expression and NO production have been demonstrated in alveolar macrophages from tuberculosis patients (Kuo et al., 2000; Nicholson et al., 1996). Therefore, the overall significance of RNI against *M. tuberculosis* in humans is likely to be an important antimycobacterial mechanism.

### 4.11 T cell response in the absence of TNF

An intact T cell response is essential for adequate host immune control and granuloma formation in tuberculosis infection, as demonstrated in T cell deficient mice (Amiri et al., 1992). After their recruitment to the site of
infection, T lymphocytes release cytokines that activate macrophages and drive epithelioid cell differentiation, eventually culminating in granuloma formation (Amiri et al., 1992; Huffnagle et al., 1995). In this study we observed reduced recruitment and activation of CD4 T cells and macrophages into the lung upon reactivation of infection. Conversely, the recruitment and activation of CD8 T cells were not altered, unlike the findings in the study using CD4 deficient mice (Serbera et al., 2001). In the acute infection model, a qualitative flow cytometric study suggested TNF being independent in T cell recruitment, while the recruitment of neutrophils and macrophages was TNF dependent (Roach et al., 2002). However, in accordance with these findings, that study demonstrated an essential role for TNF in granuloma formation.

A critical question was whether TNF is required to generate a protective immunity, or whether spontaneous reactivation of infection merely is a reflection of a defect of innate immunity. Although we have not investigated antigen-specific responses, our data using a low dose aerosol infection (3 – 10 CFU, which is fatal in TNF deficient mice between 6 and 8 weeks post-infection, unpublished observation) and the prolonged drug treatment in this study suggest that a sufficient time frame was given for the host to mount an acquired immune response. Since both approaches, however, failed and TNF deficient mice succumbed to overwhelming tuberculosis infection, the data suggest that TNF is an essential component to generate a protective immunity.

4.12 The role of chemokines in the absence of TNF

Chemokines are critical for cell recruitment and cell activation (Baggiolini, 1998; Zlotnik & Yoshie, 2000). Several chemokines including RANTES, MIP-1α, MCP-1 and IFN-γ-inducible protein-10 (IP-10) were found elevated in both in vitro and in vivo M. tuberculosis murine infections (Orme & Cooper, 1999; Rhoades et al., 1995) as well as in human pulmonary
tuberculosis (Kurashima et al., 1997; Lin et al., 1998; Sadek et al., 1998), reviewed in (Peters & Ernst, 2003). A critical role for chemokine receptor 2 (CCR2) has been indicated for the recruitment of mononuclear cells into the infected lung and to control infection (Peters et al., 2001). They found that CCR2 deficient mice rapidly succumb to a high dose intravenous infection with *M. tuberculosis* H37Rv, whereas another group showed that these mice are able to survive low dose aerosol or intravenous infection with the same strain, even though they show delayed cellular migration to the lungs (Scott et al., 2003).

TNF appears to be required for the induction of several chemokines such as MIP-1α, MIP-1β and RANTES in human lymphocytes (Hornung et al., 2000). Delayed chemokine transcription of MCP-1, MIP-1α and MIP-1β, MIP-2, eotaxin and RANTES had been reported in the liver of *M. smegmatis* and *M. tuberculosis*-infected TNF deficient mice (Kurashima et al., 1997). Chemokine transcripts in the liver in the intravenous infection model were normalised or even increased at 4 weeks of infection, but no data on protein expression are available and results may be different upon aerosol infection as in the present infection. Therefore, data in this study showing reduced MCP-1, MIP-1α and RANTES production in the lung upon reactivation in TNF deficient mice might contribute to reduced cell recruitment and activation, hence control of infection. A reduction of pulmonary RANTES, MIP-1α and MCP-1 was found in TNF deficient mice as compared to wild-type mice. The delayed and defective granuloma formation in these mice might therefore be directly as result of reduced chemokine secretion. These data are in agreement with decreased CXC and CC class chemokine expression shown in TNF deficient mice (Roach et al., 2002). Reduced CXC and CC chemokine expression was associated with reduced CD11b⁺ macrophage and CD4⁺ T cell recruitment to areas of granuloma formation. Furthermore, a critical role of CCR2 signalling had been demonstrated in the generation of mycobactericidal granulomas (Peters et al., 2001), but MCP-1, one of several CCR2 ligands, appeared not to be involved (Lu et al., 1998).
4.13 Reactivation of tuberculosis in patients treated for inflammatory disorders

Several publications address reactivation of tuberculosis in patients receiving anti-TNF therapy for a variety of inflammatory disorders, such as Crohn's disease and rheumatoid arthritis (Braun et al., 2002; Criscione & St Clair, 2002; Fleischmann et al., 2002; Keane et al., 2001; Keating & Perry, 2002; Mayordomo et al., 2002; Nunez Martinez et al., 2001; Roth et al., 2002; Shanahan & St Clair, 2002; Sieper & Braun, 2001; Van Den Bosch et al., 2002; Vonkeman et al., 2002; Weisman, 2002), of which some develop severely disseminated forms. Considering the estimates that one third of the world population is persistently infected with tuberculosis, the use of these anti-TNF agents might have devastating long-term effects. Moreover, testing and identification of persistent tuberculosis is not a practical option with the currently used Mantoux skin test in countries where people have been BCG-vaccinated. The implications of findings in this study, together with that of others, show that TNF is a critical cytokine that is essential for the early control of primary infection, maintenance of persistent infection, and safeguard against reactivated tuberculosis in mouse and man.

In summary, it has been shown here that the immune system is unable to control persistent tuberculosis infection in the absence of TNF. It is clear that TNF is required for the secretion of chemokines that enhance the recruitment and activation of T cells and macrophages to form granulomas and prevent dissemination of disease.

4.14 Characterisation of conditionally targeted TNF deficient mice

Another aspect of this study describes the characterisation of inducible TNF gene deficient mice for their utilisation in tuberculosis studies. It was considered to use the newly generated inducible TNF deficient mice in the tuberculosis reactivation model described in this study, as reactivation of latent tuberculosis can be induced in a more similar way than
aminoguanidine-induced reactivation. Therefore, these mice needed to be characterised for suitability. Firstly, efficiency of poly(I:C)-induced gene deletion was analysed. A group of mice received a series of three poly(I:C) i.p. injections at 2 weeks post-infection after an aerosol dose of 30 CFU *M. tuberculosis* H37Rv, after which genomic DNA was hybridised with the TNF gene-specific probe, mER4. Results showed that this gene was efficiently inactivated in bone marrow, peripheral blood and in the liver. Interestingly, deletion in the spleen was high on average, around 87%, but quite variable between one animal and the next. The lowest level of deletion was seen in the tail, a similar observation made by Kuhn *et al.* (1995). Of concern was the fact that some mice showed quite extensive spontaneous deletion of the TNF allele, without poly(I:C) administration, most notably in the spleen. However, this seemed to be a random event that may have been due to other factors that resulted in strong induction of IFN-α or -β, for example a viral infection, but Kuhn and colleagues did not address this occurrence in their paper (Kuhn *et al.*, 1995). Spontaneous deletion events also occurred in other immune-modulatory organs, like the bone marrow, peripheral blood, lymph nodes and thymus. The overall deletion efficiencies between this data and that of Kuhn *et al.* (1995) correlated very well in all mouse organs analysed. With the aim towards tuberculosis studies, deletion of 63% on average in the lung appeared less optimal, however, no spontaneous deletion was observed, thus with adequate controls, valuable information can be gained using these mice.

Determination of TNF levels in the blood of mice with and without *in vivo* LPS stimulation followed by *ex vivo* re-stimulation revealed a reduction of TNF levels in TNF*ff* x Cre mice compared to wild-type mice, however, plasma TNF levels before and after poly(I:C) administration varied significantly. Likewise, in BMDM cultures that were re-stimulated with LPS *ex vivo* before and after the series of poly(I:C) injections, showed a marked reduction in TNF to almost undetectable levels following induced gene deletion. Therefore, with the appropriate controls, conditional gene targeted mice will be useful in future studies in which normal functionally developed
mice can be induced to cease their TNF production at a specific point during tuberculosis infection.

The question was asked whether poly(I:C) would have any effect on mouse body weights or organ weights during tuberculosis infection. Mouse body weights and lung, spleen and liver weights were monitored during the first four weeks of infection in one group of mice following a series of poly(I:C) injections and in another group without. Mouse body weights appeared unaffected, but hepatosplenomegaly was observed immediately following the last of the series of poly(I:C) injections, although this was a transient event that normalised one week later. Interestingly, a different observation was made assessing lung weights. No significant change in lung weights was observed immediately following the injections, but a slight increase was seen a week later. Experimental error cannot be excluded for this time point, as control mouse lung weights decreased marginally between the third and fourth week of infection, which may be why poly(I:C)-treated mice appeared to have increased lung weights. Unfortunately, the unavailability of subsequent time points precluded further clarification of this matter.

Bacillary multiplication in the presence of poly(I:C) was analysed during the first four weeks of infection and followed for two weeks after poly(I:C) administration was completed. No significant differences between uninjected control mice and poly(I:C)-injected mice were observed in the lungs and spleens, but a delayed increase in CFU in the liver was apparent in poly(I:C)-injected mice, more than one week following the final injection of the series. As mentioned before, no subsequent time points were analysed, therefore it was not clear whether this phenomenon was a transient occurrence or not.

According to this preliminary data it appears that murine tuberculosis infection proceeded in the usual way following administration of a series of poly(I:C) i.p. injections in wild-type mice. Therefore, conditional TNF gene-targeted mice should be infected with *M. tuberculosis* and the progression of infection observed against appropriate controls before a final conclusion
regarding the suitability of these animals in experimental tuberculosis infections can be made.
Chapter 5. CONCLUSION

The model presented in this study entails generation of a persistent tuberculosis infection induced by RMP-INH chemotherapy and subsequent reactivation of infection by immune suppression through aminoguanidine. In contrast to previous models, this one is relatively short and is easy to perform, with all drugs delivered in the drinking water of mice. A stable persistent infection is attainable, which can be reactivated with high efficiency, and no spontaneous reactivation occurs. Infection with *M. tuberculosis* H37Rv is considered the strain of choice, as this strain maintains virulence through multiple passages in the mouse, but it has been shown by others (Flynn et al., 1998; Scanga et al., 1999) and in this study that similar results are obtainable with *M. tuberculosis* Erdman. Additionally, infection via the aerosol route is the most relevant route of infection mimicking human tuberculosis and shows a different bacillary distribution amongst the mouse organs as compared to intravenous infection. Therefore, the aerogenic infection model may be most appropriate to investigate the immune response during persistence and reactivation of tuberculosis.

Moreover, due to increasing drug resistance, poor adherence to lengthy therapy and adverse effects of current antituberculous drugs, research towards improved and shorter therapy has taken high priority. In addition to new generation antituberculous drugs, novel vaccines will have to be developed in order to combat the most prevalent form of tuberculosis, namely latent tuberculosis. This model may be a beneficial tool to validate the efficacy of novel drugs and vaccines in persistent tuberculosis infection. For the first time, these experimental studies demonstrate that it is indeed possible to control and eventually eradicate tuberculosis with efficient RMP-INH treatment, even in an immuno-compromised state, such as seen with the high numbers of HIV and tuberculosis co-infected individuals in high-burden countries.
Chapter 5: CONCLUSION

Enumeration of mycobacterial DNA and mRNA revealed that many more persistent organisms are present in lung tissue than can be cultured by conventional methods. This raises the possibility that, with careful experimental design and sufficient amounts of tissue, the persistent mycobacteria can be characterised and their exact metabolic status determined using molecular beacon real-time PCR or similar technology. Therefore, this model will enable elucidation of aspects of persistence that has in the past decades only been speculated about, even though the importance of this matter has been recognised and emphasised. Comparison of the status of persistent mycobacteria from this model, representing drug-induced latency, should however be carefully compared to persistent mycobacteria from the low dose latency model. This will reveal the whether antibiotic treatment actually affects the behaviour of these persistent bacteria. Future projects leading from this study will be used to address this question.

Another crucial question in a poorly understood field is that of reinfection with *M. tuberculosis* during a persistent infection. Exciting preliminary data indicate, against expectations, that the persistent strain reactivates and that it is not completely dominated by the secondary strain. This is another aspect that will be further characterised in future studies stemming from this project.

Further, we asked whether TNF is required for post-infectious immunity in aerosol-infected mice. TNF deficient mice were treated with RMP-INH for 4 weeks to reduce CFU to undetectable levels, but they rapidly developed fatal reactivation of tuberculosis. Reactivation was associated with diminished recruitment and activation of T cells and macrophages in the lung with poorly developed granuloma formation and reduced levels of NOS2 expression. Reduced chemokine production in the lung might be the cause of the inefficient recruitment and activation of T cells that resulted in uncontrolled infection. Therefore, the data convincingly demonstrate that
endogenous TNF is essential to control persistent tuberculosis infection and no specific immunity is generated in these mice.

Lastly, characterisation of conditional TNF gene targeted mice for their suitability in tuberculosis reactivation studies will be continued. These mice should first be infected with *M. tuberculosis* H37Rv and carefully monitored for longer time periods to ensure that the apparently minor differences will not interfere with further analysis, but their use in reactivation of tuberculosis will be novel and informative.
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