MYCOBACTERIAL STRAIN DIVERSITY: IMPACT ON THE HOST IMMUNE RESPONSE

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Page

Table of contents		iii
Abstract		iv
Acknowledgements		v
List of Figures and Tables		vii
Publications arising from this work		ix
Abbreviations		x
Chapter 1	General Introduction *Discussion of the literature*	1
Chapter 2	General Methods *Materials and experimental techniques used*	35
Chapter 3	Tumor necrosis factor-alpha mediated growth restriction of *Mycobacterium tuberculosis* H37Ra is observed in mouse macrophages but not in human monocytes *in vitro*	57
Chapter 4	The 19-kilodalton lipoprotein of *Mycobacterium tuberculosis* inhibits *Mycobacterium smegmatis*-induced cytokine production by human macrophages *in vitro*	77
Chapter 5	IL-12 and TNF-α production in tuberculosis patients with different extents of pulmonary disease	97
Chapter 6	Growth of *Mycobacterium tuberculosis* at the surface of the cavity reflects a microenvironment with failed immunity	113
Chapter 7	Genetic polymorphism in *M. tuberculosis* isolates from patients with chronic multi-drug resistant tuberculosis	141
Chapter 8	Concluding remarks *Summary of the results and findings in this thesis*	157
References		169

University of Cape Town

ABSTRACT

This thesis examined the impact of mycobacterial strain diversity on the interaction between host phagocyte and mycobacterium *in vitro*, and the extent to which strain polymorphism is observed in selected patients with pulmonary tuberculosis (TB). Human monocytes and mouse macrophages were infected with previously characterized mycobacterial strains. Infection of human monocytes with *M. tuberculosis* (MTB) HN878, which causes more severe disease in mice, or *M. smegmatis* 19kDa, which failed to protect mice when administered as a vaccine, resulted in reduced production of tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12) and IL-6 compared to MTB strain CDC1551 and *M. smegmatis* respectively. Monocytes from patients with more severe pulmonary TB produced less TNF-α, IL-12 and IL-6 in response to infection, and these cells were more permissive for MTB replication. Whereas similar intracellular growth of H37Ra and H37Rv was observed in human monocytes, replication of H37Ra but not H37Rv was restricted by a TNF-α dependent, inducible nitric oxide synthase (iNOS) and interferon-gamma (IFN-γ) independent mechanism in mouse macrophages.

Mycobacterial strain diversity *in vivo* was examined in serial sputum samples and lung tissue from patients with chronic, multi-drug resistant (MDR) TB. Although TB in each patient was caused by a single MTB strain, the drug-resistance conferring loci were frequently polymorphic. Acquisition of additional drug resistance mutations occurred preferentially at the cavity surface where mycobacteria were most numerous. Absence of $CD4^+$ and $CD8^+$ T-lymphocytes from this area may have contributed to a reduced ability of the macrophages to control bacillary growth. In areas of the lung where $CD4^+$ and $CD8^+$ T-cells were co-localized with macrophages, IL-2, IL-12, IFN-γ and iNOS was readily detected and few if any bacilli were observed.

These results support the concept that mycobacterial isolates that elicit a robust pro-inflammatory cytokine response may enable the host to mount a more protective immune response as judged by enhanced vaccine efficacy, reduced permissiveness of phagocytes for mycobacterial replication, or less severe pulmonary disease. Even in areas with intense MTB transmission, human TB is caused by a single MTB strain that may undergo genetic changes during treatment. This genetic polymorphism may result in the presence of drug susceptible and drug resistant bacilli in the lungs of TB patients.

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University of Cape Town

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University of Cape Town

LIST OF FIGURES AND TABLES

CHAPTER 3: TNF-α mediated growth restriction of *M. tuberculosis* H37Ra

Figure 3-1 RFLP fingerprints of the *M. tuberculosis* strains H37Ra and H37Rv.

Figure 3-2 Growth of H37Ra and H37Rv in human monocytes.

Figure 3-3 Growth of H37Ra and H37Rv in mouse peritoneal macrophages.

Figure 3-4 Growth rates of H37Ra and H37Rv in peritoneal macrophages from selected gene-deficient and wild type mice.

Figure 3-5 TNF-α production by MTB infected peritoneal murine macrophages and human monocytes.

University of Cape Town

CHAPTER 4: The 19-kilodalton lipoprotein of *M. tuberculosis*

Figure 4-1 Number of intracellular mycobacteria following infection of monocyte derived macrophages with *M. smegmatis* or *M. smegmatis* 19kDa.

Figure 4-2 Electron micrographs of MDM 5h post infection with *M. smegmatis* or *M. smegmatis* 19kDa.

Figure 4-3 Cytokine production by MDM infected with *M. smegmatis* or *M. smegmatis* 19kDa.

Figure 4-4 Effect of post-translational modification of the 19kDa antigen on cytokine production.

Figure 4-5 Expression of cell surface markers on uninfected MDM or MDM infected with *M. smegmatis* or *M. smegmatis* 19kDa.

Figure 4-6 Effect of exogenously added 19kDa lipoprotein on TNF-α and IL-12 production by MDM.

CHAPTER 5: IL-12 and TNF-α production in tuberculosis patients

Table 5-1 Demographic and radiographic characteristics of the subjects

Figure 5-1 Examples of pre-treatment chest radiographs of patients classified as having limited or extensive tuberculosis disease

Figure 5-2 Plasma cytokine levels in patients with a limited pulmonary TB or extensive pulmonary disease on chest radiograph.

Figure 5-3 IL-12, TNF-α and IL-6 production by freshly isolated monocytes from patients with limited or extensive TB, infected with CDC1551 or HN878 *in vitro*.

Figure 5-4 Intracellular growth of MTB in monocytes from patients with limited or extensive TB.

CHAPTER 6: Growth of *M. tuberculosis* at the surface of the cavity

Table 6-1 Characteristics of the patients and classification of their TB disease.

Table 6-2 Histopathological, microbiological and host mRNA analysis of the sampled lesions.

Figure 6-1 Pre-operative chest radiographs and resected lungs.

Figure 6-2 IS*6110* patterns of *M. tuberculosis* isolates.

Figure 6-3 Histo-pathological section of an open granuloma

Figure 6-4 Histo-pathological section of a closed, non-cavitary granuloma

Figure 6-5 Localization of macrophages and AFB in the lung lesions.

Figure 6-6 Localization of lymphoid cells within the lung lesions.

Figure 6-5 Induction of *M. tuberculosis* stress-response mRNAs.

University of Cape Town

CHAPTER 7: Genetic polymorphism in *M. tuberculosis* isolates

Table 7-1 Characteristics of the patients with MDR TB

Table 7-2 TB treatment prior to and during the study

Table 7-3 Phenotypic and genotypic drug resistance in baseline MTB cultures

Table 7-4 Genotypic polymorphism of selected *M.tuberculosis* genes

Figure 7-1 IS*6110* fingerprints of MTB isolated from sputum of 3 patients at baseline, and after 6-56 weeks of additional treatment

PUBLICATIONS THAT HAVE ARISEN FROM THIS WORK

Post FA, Manca C, Neyrolles O, Ryffel B, Young DB, Kaplan G. *Mycobacterium tuberculosis* 19-kilodalton lipoprotein inhibits *Mycobacterium smegmatis*-induced cytokine production by human macrophages in vitro. Infect Immun. 69:1433-1439, 2001.

Post FA, Steyn LM, Kaplan G. IL-12 and TNF-α production in tuberculosis patients with limited or extensive pulmonary disease. Submitted.

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Timm J, Post FA, Bekker LG, Walther G, Wainwright H, Manganelli R, Chan W-T, Tsenova L, Gold B, Smith I, Kaplan G, McKinney JD. Differential expression of *Mycobacterium tuberculosis* stress response genes in the lungs of mouse and humans. Submitted.

Kaplan G, Post FA, Moreira AL, Wainwright H, Kreiswirth BN, Tanverdi M, Mathema B, Ramaswamy SV, Walther G, Steyn LM, Barry 3rd CE, Bekker LG. *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. Submitted

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ABSTRACTS OF CONFERENCE PRESENTATIONS

The 19kDa antigen of *M. tuberculosis* is a potent down-regulator of inflammatory cytokines induced by *M. smegmatis* in human macrophages in vitro. (Fourth International Conference on the Pathogenesis of Mycobacterial Infections – Stockholm, Sweden, July 1999)

Post-translational modification of the *Mycobacterium tuberculosis* 19kDa antigen is essential for inhibition of cytokine production by human macrophages in vitro. (Karolinska Institutet Nobel Symposium No 114: Prevention and Treatment of Tuberculosis in the coming century – Stockholm, Sweden, August 2000)

Inhibition of *Mycobacterium smegmatis*-induced cytokine production by human macrophages in vitro requires intracellular localization of glycosylated and acylated 19kDa lipoprotein. (Molecular and Cellular Aspects of Tuberculosis Research in the Post Genome Era – Taos, NM, USA, January 2001)

Impaired IL-12 production in HIV negative patients with extensive pulmonary TB. (Fourth World Congress on Tuberculosis – Washington DC, USA, June 2002)

Drug-resistance mutations in patients with chronic TB. (Tuberculosis: Integrating Host and Pathogen Biology – Taos, NM, USA, January 2003)

ABBREVIATIONS

ADC	:	Albumin, dextrose, catalase
AFB	:	Acid-fast bacilli
Ag	:	Antigen
BCG	:	*Mycobacterium bovis Bacillus Calmette-Guerin*
bp	:	Basepair
BSL-3	:	Biosafety laboratory level 3
CCR	:	C-C chemokine receptor
CD	:	Cluster of differentiation
CDC	:	Centers for Diseases Control
CF	:	Culture filtrate
CFP	:	Culture filtrate protein
CFU	:	Colony forming unit
CTAB	:	Hexadecyl trimethyl ammonium bromide
CTL	:	Cytotoxic T-lymphocyte
CTLA	:	CTL antigen
DC	:	Dendritic cell
DC-SIGN	:	DC-specific ICAM-3 grabbing nonintegrin
DMEM	:	Dulbecco's minimum essential medium
D10	:	DMEM 10% FCS
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxy nucleoside tri phosphate
DOTS	:	Directly observed treatment-short course
E	:	Ethambutol
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbent assay
FACS	:	Fluorescence-activated cell sorter
FAS I/II	:	Fatty acid synthase I/II
FasL	:	Fas ligand
FCS	:	Fetal calf serum
FITC	:	Fluorescein isothiocyanate
H	:	Isoniazid
HAART	:	Highly active anti-retroviral therapy
hARP	:	Human acidic ribosomal protein
HRP	:	Horseradish peroxidase
HIV	:	Human immunodeficiency virus
HSP	:	Heat-shock protein
ICAM	:	Intra cellular adhesion molecule
ICL	:	Isocitrate lyase-1
IFN-γ	:	Interferon-gamma
IFN-γR	:	IFN-γ receptor
IL	:	Interleukin
INH	:	Isoniazid
iNOS	:	Inducible nitric oxide synthase
IP	:	Interferon-inducing protein
IR	:	Induction ratio
kDa	:	Kilo-dalton
KO	:	Knock-out (gene-deficient)
LAM	:	Lipoarabinomannan

University of Cape Town

LJ	:	Loewenstein-Jensen
LPS	:	Lipopolysaccharide
M	:	Molar
ManLAM	:	Mannosylated LAM
MCP	:	Monocyte chemotactic protein
MDM	:	Monocyte-derived macrophages
MDR	:	Multi-drug resistant (at least isoniazid and rifampin)
MGIT	:	Mycobacteria growth indicator tube
MIP	:	Macrophage inflammatory protein
MHC	:	Major histo-compatibility complex
mRNA	:	Messenger RNA
MOI	:	Multiplicity of infection
MTB	:	*Mycobacterium tuberculosis*
NA	:	Non-acylated
NF-κB	:	Nuclear factor κB
NK	:	Natural Killer
NO	:	Nitric oxide
NOG	:	Non-O-glycosylated
NRAMP	:	Natural resistance-associated macrophage protein-1
NS	:	Non-secreted
OADC	:	Oleic acid, albumin, dextrose, catalase
OD	:	Optical density
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PE	:	Phycoerythrin or Proline-Glutamine
Phox	:	Phagocyte oxydase
PPE	:	Proline-Proline-Glutamine
PPD	:	Purified protein derivative
r	:	Recombinant
R	:	Rifampin
RANTES	:	Regulated on activation, normal, T-cell expressed and secreted
RIF	:	Rifampicin (Rifampin)
R10/R20	:	RPMI 10%/20% human AB serum
RFLP	:	Restriction fragment length polymorphysm
RNA	:	Ribonucleic acid
RNI	:	Reactive nitrogen intermediates
ROI	:	Reactive oxygen intermediates
RPMI	:	Roswell Park Memorial Institute
RT	:	Reverse transcription
SCC	:	Short-course chemotherapy
SCID	:	Severe combined immune deficiency
SDS	:	Sodium dodecyl sulfate
SPF	:	Specific pathogen free
TACO	:	Tryptophan aspartate-containing coat
Taq	:	*Thermus aquaticus*
TB	:	Tuberculosis
TGF-β	:	Transforming growth factor-beta
T_H	:	T-helper
TLR	:	Toll-like receptor

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TNF	:	Tumor necrosis factor
TNF-α	:	TNF-alpha
TNF-αR	:	TNF-α receptor
TRIS	:	Tris (hydroxy) amino methane
WCL	:	Whole cell lysate
Z	:	Pyrazinamide

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Chapter 1

GENERAL INTRODUCTION

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1.1 INTRODUCTION AND HISTORICAL PERSPECTIVE

Mycobacterium tuberculosis (MTB) is a fascinating and extremely successful pathogen. Following infection, the host immune response prevents active tuberculosis (TB) in the majority of exposed persons. However, the host response fails to eradicate the infecting bacilli, leading to a state of latent infection (Sutherland, 1976). As many as 2 billion people may be latently infected with MTB, and up to 10% of these individuals will develop "reactivation" TB, often years or even decades after the initial infection. Reactivation TB is characterized by mycobacterial replication, immune activation, granuloma and cavity formation, and bacillary transmission. The introduction of effective chemotherapy did not eliminate this "scourge of all diseases" from modern society. Rather, incidence rates of tuberculosis have risen dramatically over the past 20 years, prompting the World Health Organization to declare tuberculosis a global emergency in 1993. In 1997, MTB caused eight million incident and sixteen million prevalent TB cases, resulting in two million deaths (Dye, Scheele, Dolin, *et al.*, 1999).

Historically, tuberculosis is likely to have affected humans for at least 5-10,000 years. Lesions interpreted as spinal TB have been documented in mummies, artwork and medical texts dating as far back as 5000 BC. Based on molecular analyses, it has been estimated that MTB developed as a separate pathogen a mere 15-20,000 years ago. Although it was originally postulated that MTB and *M. bovis* might have originated from a common ancestral cattle pathogen, more recent phylogenetic studies suggest that the ancestral strain of MTB was probably already a human pathogen. (Brosch, Gordon, Marmiesse, *et al.*, 2002; Sreevatsan, Pan, Stockbauer, *et al.*, 1997; Zink, Sola, Reischl, *et al.*, 2003).

The etiologic agent of TB was identified by Robert Koch in 1882. Although the infectious nature of TB had been appreciated for some time, a modified bacterial stain (alcoholic methylene-blue/potassium-hydrate stain followed by aqueous vesuvin de-stain)

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allowed him to visualize "beautifully blue" bacilli within tuberculous lesions of patients with consumption. He could passage the bacilli several times on blood-serum agar after which they were still capable of causing disease in animals, and he showed that consumption and tubercle formation were part of the same disease process. Differential host susceptibility to developing TB had already been appreciated since "excessive numbers of tubercle bacilli were expectorated by consumptives and scattered everywhere", therefore "peculiar factors had to favor the implantation of bacilli and make infection possible" (Pinner and Pinner, 1932). More than a century on, the mechanisms that determine why some people but not others develop TB after exposure to MTB remain poorly understood.

1.2 EPIDEMIOLOGY OF TB

MTB is transmitted from individual to individual via the respiratory route. Following exposure, the majority (90%) of immune competent individuals controls the infection and never develops clinical disease. These persons may be identified by a positive skin test reaction to MTB antigens (purified protein derivative, PPD), defined as an induration of >10mm. Approximately 5% of exposed persons develop active disease within 2 years of exposure, and another 5% present with "reactivated" tuberculosis later in life (Bloom and Murray, 1992; Comstock, Livesay and Woolpert, 1974).

Tuberculosis in the late 1800's, accounted for 15 to 25 percent of deaths in Berlin and Paris (Pinner and Pinner, 1932). Studies performed in Europe in the first half of the twentieth century showed that clinical TB developed proportionate to MTB exposure, with annual TB incidence rates of 1,100-4,900/100,000 in tuberculin negative and 430-760 per 100,000 in tuberculin positive individuals when exposure to TB had been heavy, and 157 and 29/100,000 respectively in persons with minimal exposure (Stead, 1967). A gradual decline in number of TB deaths had been observed since the 1830's. Following the introduction of

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multi-drug chemotherapy, TB incidence rates declined more sharply, in the United States at 5-7% per annum between 1955 and 1984. However, an 18% rise, accounting for 39,000 excess TB cases, was observed between 1985 and 1991. Undoubtedly, human immunodeficiency virus (HIV) infection played a major role in this increase. HIV infected persons develop TB at rates of 3-13% per annum. Renewed efforts to limit the spread of TB, such as isolation of infectious cases, supervised treatment and preventing TB through administration of prophylaxis to PPD positive contacts, and recognition of the atypical presentations of HIV related TB and the availability of specific immune reconstituting therapy for HIV infected persons have resulted in a 31% decrease in TB incidence between 1992 and 1998 (1992; Girardi, Raviglione, Antonucci, *et al.*, 2000).

The socio-economic status of the underprivileged majority in sub-Saharan Africa has shown minimal improvement between 1955 and the 1980's. Even in countries such as South Africa that used chemotherapeutic regimens similar to those used in the USA, no decline of the tuberculosis incidence rate was observed during the 1960's, 1970's and 1980's, suggesting that chemotherapy on its own is insufficient to reduce the burden of tuberculosis in a society. Moreover, between 1990 and 1997, tuberculosis incidence rates in sub-Saharan Africa have increased by a further 35% as a result of HIV infection. In the worst affected countries such as Zimbabwe where HIV antenatal prevalence rates have reached 30%, TB case notification increased 9 fold between 1990 and 2000 (Corbett, Steketee, ter Kuile, *et al.*, 2002). Approximately half of all excess tuberculosis cases were HIV negative, reflecting increased MTB transmission in societies heavily affected by HIV and TB. With less than 50% of sputum smear positive cases detected, TB cure rates below 70%, and a reservoir of more than 10,000,000 people dually infected with HIV and MTB, it is more than likely that TB incidence rates in Africa will continue to increase (Girardi, Raviglione, Antonucci, *et al.*, 2000). HIV interventions, including prevention of vertical transmission, prophylaxis against

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opportunistic infections, and anti-retroviral treatment, together with active TB case finding and TB preventive therapy will have to be utilized to reduce the impact of MTB on morbidity and mortality in sub-Saharan Africa (Corbett, Steketee, ter Kuile, *et al.*, 2002).

1.3 CLINICAL FEATURES OF TUBERCULOSIS

Cough, fever, night sweats and cachexia are the cardinal clinical features of pulmonary TB. The cough generates droplet nuclei containing infectious *M. tuberculosis*, thereby allowing transmission of the pathogen. Upon inhalation of bacilli by an uninfected person, infection may be established in the lungs. Initial replication in macrophages is followed by dissemination of bacilli to the local lymph nodes, the "vulnerable" parts of the lungs (apico-posterior segments of the upper lobes or apical segments of the lower lobes) and occasionally other parts of the body. Cell mediated immunity ensues and while usually sufficient to control bacillary replication, the host response is unable to eradicate MTB. Primary MTB infection may be asymptomatic, or result in mild disease (simple primary TB or TB pleural effusion), progressive pulmonary disease, or miliary TB (Stead, Kerby, Schlueter, *et al.*, 1968).

Post-primary (reactivation) tuberculosis occurs usually in adolescents and adults and often presents as a chronic respiratory illness localized in the vulnerable parts of the lung. On occasion, the disease may involve or be limited to the meninges, skin, joints, kidneys, lymph nodes, serosa of pleural, pericardial or peritoneal cavity, bone, liver or spleen. The typical presentation is a wasting illness associated with fever and night sweats, and a non-productive cough that may be associated with haemoptysis. In HIV sero-negative patients, the chest radiograph may reveal minimal changes, fibro-cavitation or extensive fibrosis and destruction of the lungs. Features suggesting endobronchial or hematogenous spread may be present. Lung pathology, as well as many of the symptoms of TB, may result from the host immune

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response rather than from direct toxicity of the mycobacteria (Bekker, Maartens, Steyn, *et al.*, 1998; Bekker, Moreira, Bergtold, *et al.*, 2000; Moreira, Tsenova-Berkova, Wang, *et al.*, 1997).

Active TB may be confirmed by the presence of acid-fast bacilli (needle-shaped bacilli that retain carbolfuchsin dye and do not de-stain on exposure to acidic-alcohol) on microscopic examination of sputum smears. Sputum culture may be performed to confirm that the mycobacteria observed in the smear are indeed *M. tuberculosis*, to evaluate drug susceptibility profile, or to confirm the presence of MTB in sputum of "smear negative" cases.

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Patients infected with human immunodeficiency virus (HIV) infection are at increased risk of developing active TB (8% annual risk compared to the 10% lifetime risk of HIV negative persons), particularly when $CD4^+$ T-lymphocytopenia is present and exposure to MTB is high (Wood, Maartens and Lombard, 2000). Primary infection may progress to active disease in as little as 3 weeks (Daley, Small, Schecter, *et al.*, 1992; Di Perri, Cruciani, Danzi, *et al.*, 1989; Selwyn, Hartel, Lewis, *et al.*, 1989). Although pulmonary disease remains the commonest presentation (Small, Schecter, Goodman, *et al.*, 1991), extra-pulmonary disease and disseminated TB occur more frequently when HIV-induced immune suppression is advanced (Ackah, Coulibaly, Digbeu, *et al.*, 1995). Sputum smears are less often diagnostic, and chest radiographic features may be atypical (Post, Wood and Pillay, 1995). Whereas short course chemotherapy (SCC: 2 months of RHZE followed by 4 months of RH) is as effective as in HIV negative TB cases (Kassim, Sassan-Morokro, Ackah, *et al.*, 1995; Perriens, St Louis, Mukadi, *et al.*, 1995), thiacetazone based treatment is significantly more toxic in HIV infected patients and less efficacious than SCC (Okwera, Whalen, Byekwaso, *et al.*, 1994). Highly active anti-retroviral therapy (HAART) has been shown to reduce the incidence rate of TB in HIV infected persons (Badri, Wilson and Wood, 2002).

1.4 PATHOLOGICAL AND PATHOPHYSIOLOGICAL ASPECTS OF TUBERCULOSIS

The initial manifestation of MTB infection in humans (primary or Ghon complex) consists of a focal pneumonia and an inflammatory reaction in the regional lymph nodes. Ghon foci are encountered with equal frequency throughout the lungs, and when not located in the "vulnerable" parts of the lung (apico-posterior segments of the upper lobes or apical segments of the lower lobes), seldom result in progressive disease (Medlar, 1955). They commonly undergo necrosis yet heal with minimal scarring, fibrosis or calcification. Non-replicating "dormant" bacilli may persist in these lesions (or in the fibrotic scar tissue or even in normal lung tissue) for many years and cause "reactivation" TB when cellular immunity wanes (Lillebaek, Dirksen, Baess, *et al.*, 2002; Stead, 1967). Indeed, normal lung tissue from patients with latent TB was shown to contain *M. tuberculosis* DNA (Hernandez-Pando, Jeyanathan, Mengistu, *et al.*, 2000) or bacilli that gave rise to TB when injected into guinea pigs (Opie and Aronson, 1927).

Post-primary TB results from liquefaction and sloughing of a necrotic primary lesion. This occurs with increased frequency when the primary lesion is located in the "vulnerable" parts of the lung: 84% of post-primary lesions against 12% of the primary complexes are found in these areas. The early mycobacterial pneumonia, characterized by a mononuclear cell infiltrate, may progress to epithelioid and caseous granulomatous inflammation ("tubercles"). Epithelioid granulomas consist of well-organized accumulations of epithelioid macrophages and multi-nucleated giant cells surrounded by a rim of sensitized T-lymphocytes. These lesions reveal no central necrosis, contain few visible bacilli, and are considered the more favorable host response (Medlar, 1955). Caseous necrotic granulomas contain a center of distinctive granular necrosis (designated caseous because of its macroscopic appearance) and may harbor large numbers of organisms. Neutrophilic

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infiltration of the infected areas appears to favor the development of caseous necrosis. These lesions are at risk of further liquefaction and sloughing, resulting in the formation of cavities, endobronchial dissemination of the infection, and progressive lung tissue destruction. Interestingly, epitheloid and necrotic tubercles are frequently present in the same patient (Medlar, 1955).

Cavities appear to offer the ideal growth conditions for MTB with an abundance of nutrients and oxygen, enabling the bacilli to maintain a relatively high state of metabolic activity (Medlar, Bernstein and Steward, 1952). Mycobacteria obtained from cavities can usually be cultured within the normal incubation period of 8 weeks (Hobby, Auerbach, Lenert, *et al.*, 1954; Vandiviere, Loring, Melvin, *et al.*, 1956). In contrast, only 10-25% of closed granulomas (lesions that do not have an open communication with a bronchus) yield viable MTB (Beck and Yegian, 1952; Medlar, Bernstein and Steward, 1952), and spontaneous or treatment-induced closure of an open granuloma is associated with a 100 to 1000-fold reduction in the number of culturable bacilli (Wayne and Salkin, 1956), suggesting that the closed lesion is likely to be an unfavorable physical and chemical environment for the bacteria (Yegian, 1952). Nonetheless, a small subset of organisms appears to adapt to the environmental changes, assuming a lowered metabolic state, and these bacilli can only be cultured after prolonged incubation (Medlar, Bernstein and Steward, 1952). It is possible that reactivation TB may arise from these "dormant" bacilli (Hobby, Auerbach, Lenert, *et al.*, 1954). These non-replicating bacilli may not be affected by the actions of present anti-tuberculous chemotherapy (Fenhalls, Stevens, Moses, *et al.*, 2002; Hobby, Auerbach, Lenert, *et al.*, 1954; Vandiviere, Loring, Melvin, *et al.*, 1956).

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1.5 THE HOST RESPONSE AGAINST *M. TUBERCULOSIS*

Studies of mouse TB have provided detailed insight into many aspects of the host response to MTB. Aerosol infection is followed by logarithmic multiplication of MTB in the lungs and dissemination of bacilli to the regional lymph nodes and other organs. Cell mediated immunity results in the formation of granulomas consisting of sensitized T-cells and activated macrophages and inhibits mycobacterial replication. However, the bacilli are not eradicated, and progressive lung consolidation results in death of the animals between 200 and 300 days (Manca, Tsenova, Barry, *et al.*, 1999; Mogues, Goodrich, Ryan, *et al.*, 2001).

Phagocytosis of MTB. Phagocytosis of MTB by resident alveolar macrophages or dendritic cells (DC) initiates the host immune response. MTB may utilize several macrophage receptors, including the mannose, complement and scavenger receptors, to gain access to its intra-cellular niche, the phagosome (Ernst, 1998). Within macrophages, MTB resides within tightly apposed, individual vacuoles (Moreira, Wang, Tsenova-Berkova, *et al.*, 1997) where through the actions of mannose capped lipoarabinomannan (ManLAM) it resists the toxic effects of macrophage defense mechanisms by inhibiting acidification and maturation of the vacuole (Clemens and Horwitz, 1995; Fratti, Chua, Vergne, *et al.*, 2003; Sturgill-Koszycki, Schlesinger, Chakraborty, *et al.*, 1994; Xu, Cooper, Sturgill-Koszycki, *et al.*, 1994), and by inhibiting phagosome-lysosome fusion through retention of a tryptophan aspartate-containing coat (TACO) (Ferrari, Langen, Naito, *et al.*, 1999).

Cytokine production by phagocytes. Infected macrophages utilize Toll-like receptors (TLR) to sample phagosomal contents, and to activate appropriate cytokine and anti-microbial responses (Ozinsky, Underhill, Fontenot, *et al.*, 2000; Underhill, Ozinsky, Hajjar, *et al.*, 1999). Stimulation of TLR-2 (and to a lesser extent TLR-4) with mycobacterial cell wall components results in MyD88-dependent nuclear factor-kappa B (NF-κB) activation and transcription of the immune regulatory cytokine genes encoding tumor necrosis factor-

University of Cape Town

alpha (TNF-α) and interleukin-12 (IL-12) (Brightbill, Libraty, Krutzik, *et al.*, 1999; Means, Jones, Schromm, *et al.*, 2001; Means, Wang, Lien, *et al.*, 1999; Underhill, Ozinsky, Smith, *et al.*, 1999). MTB infection of human monocytes, which express high levels of TLR2 and TLR4 (Kadowaki, Ho, Antonenko, *et al.*, 2001), results in TNF-α and IL-12 production (Manca, Tsenova, Barry, *et al.*, 1999). Resistance of mice deficient in TLR2 or TLR4 to low dose aerosol MTB infection, however, suggests substantial redundancy for the Toll-like receptors with regards to the production of immune regulatory cytokines (Abel, Thieblemont, Quesniaux, *et al.*, 2002; Reiling, Holscher, Fehrenbach, *et al.*, 2002).

IL-12 is produced at the site of MTB infection, and contributes to the protective immune response by stimulating T-helper cell type 1 (T_H1) development, T-cell proliferation, cytolytic activity and the production of IFN-γ by lymphocytes and natural-killer (NK) cells (Chan, Perussia, Gupta, *et al.*, 1991; Chehimi, Starr, Frank, *et al.*, 1992; Hsieh, Macatonia, Tripp, *et al.*, 1993; Manetti, Parronchi, Giudizi, *et al.*, 1993; Murphy, Terres, Macatonia, *et al.*, 1994). Mice that are incapable of producing IL-12 (IL-12 "knock-out" (KO) mice), and mice treated with IL-12 neutralizing antibodies display an increased mortality when infected with MTB, an increased mycobacterial load, impaired granuloma formation and reduced IFN-γ production compared to wild-type mice. Treatment of MTB-infected mice with IL-12, on the other hand, results in enhanced mycobacterial immunity (Cooper, Magram, Ferrante, *et al.*, 1997; Cooper, Roberts, Rhoades, *et al.*, 1995; Flynn, Goldstein, Triebold, *et al.*, 1995; Lowrie, Tascon, Bonato, *et al.*, 1999). In humans, mutations in the genes encoding IL-12p40 or the IL-12 receptor result in decreased IFN-γ production by stimulated T-cells and increased susceptibility to (predominantly atypical) mycobacterial infections (Altare, Durandy, Lammas, *et al.*, 1998; Altare, Ensser, Breiman, *et al.*, 2001; Altare, Lammas, Revy, *et al.*, 1998; de Jong, Altare, Haagen, *et al.*, 1998; Fieschi, Dupuis, Catherinot, *et al.*, 2003; Frucht and Holland, 1996). IL-12 has been demonstrated in TB granulomas in human lung,

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tuberculous pleural fluid, and supernatants of human monocytes infected with MTB *in vitro* (Bergeron, Bonay, Kambouchner, *et al.*, 1997; Manca, Tsenova, Barry, *et al.*, 1999; Zhang, Gately, Wang, *et al.*, 1994). These observations confirm an important role for IL-12 in the induction of MTB specific immunity in man and mice. Consequently, inhibition of IL-12 production by infected macrophages may be one of the mechanisms utilized by MTB to reduce the impact of the host immune response (Nau, Richmond, Schlesinger, *et al.*, 2002).

TNF-α is another important regulator of cell-mediated immunity and required for activation of macrophages, co-localization of lymphocytes and macrophages within granulomas, and maintenance of granuloma structure (Bean, Roach, Briscoe, *et al.*, 1999; Flynn, Goldstein, Chan, *et al.*, 1995). Granuloma formation is impaired in MTB infected mice that are deficient in TNF-α or that are treated with TNF-α neutralizing antibodies, and survival is markedly shortened compared to wild-type mice. Secretion of TNF-α by recombinant *M. bovis Bacillus Calmette Guerin* (BCG) restores the ability of TNF-α deficient mice to form granulomas and control infection (Bekker, Freeman, Murray, *et al.*, 2001). TNF-α has been demonstrated in human tuberculous granulomas, and is readily detected in the supernatants of monocytes infected with MTB *in vitro* (Bergeron, Bonay, Kambouchner, *et al.*, 1997; Fenhalls, Wong, Bezuidenhout, *et al.*, 2000; Manca, Tsenova, Barry, *et al.*, 1999). Treatment of humans with TNF-α neutralizing antibodies is associated with an increased risk of developing TB (Keane, Gershon, Wise, *et al.*, 2001). Although TNF-α has an essential role in the protective immune response against TB, this cytokine is also largely responsible for the cachexia and immunopathology associated with TB (Bekker, Maartens, Steyn, *et al.*, 1998; Bekker, Moreira, Bergtold, *et al.*, 2000; Moreira, Tsenova-Berkova, Wang, *et al.*, 1997).

Several other pro-inflammatory cytokines, including IL-1β, IL-6 and IL-18, are produced at the site of disease in patients with TB (Bergeron, Bonay, Kambouchner, *et al.*,

University of Cape Town

1997; Hoheisel, Izbicki, Roth, *et al.*, 1998; Law, Weiden, Harkin, *et al.*, 1996; Vankayalapati, Wizel, Weis, *et al.*, 2000). Mice that are deficient for any of these cytokines or their receptors display an increased susceptibility to TB (Juffermans, Florquin, Camoglio, *et al.*, 2000; Ladel, Blum, Dreher, *et al.*, 1997; Sugawara, Yamada, Kaneko, *et al.*, 1999; Yamada, Mizumo, Horai, *et al.*, 2000). On the other hand, local production of IL-10 (Barnes, Lu, Abrams, *et al.*, 1993; Gerosa, Nisii, Righetti, *et al.*, 1999; Shaw, Thomas and Friedland, 2000) or transforming growth factor-beta (TGF-β) (Condos, Rom, Liu, *et al.*, 1998; Toossi, Gogate, Shiratsuchi, *et al.*, 1995) may down-regulate the host response (Fulton, Cross, Toossi, *et al.*, 1998; Gong, Zhang, Modlin, *et al.*, 1996; Hirsch, Toossi, Othieno, *et al.*, 1999; Toossi and Ellner, 1998). It is likely that a fine balance between inflammatory and inhibitory cytokines is required to regulate cell-mediated immunity and prevent excessive tissue damage.

Chemokine production by infected macrophages and dendritic cells. The successful host response requires early migration of infected macrophages and/or DC to the regional lymph nodes to allow sensitization of T-lymphocytes (Chackerian, Alt, Perera, *et al.*, 2002). Monocytes and sensitized T-cells are recruited to the site of infection, and result in the formation of granulomas. This TNF-α dependent process requires upregulation of adhesion molecules on vascular endothelium, and production of chemokines by and expression of chemokine receptors on activated macrophages and lymphocytes (Roach, Bean, Demangel, *et al.*, 2002). MTB infection of mouse macrophages results in the production of monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-2 and interferon-inducible protein-10 (IP-10), chemokines involved in the recruitment of monocytes, T-cells, NK-cells and granulocytes. These chemokines are also upregulated in the lungs of MTB infected mice (Rhoades, Cooper and Orme, 1995). Delayed chemokine expression and cellular recruitment, and impaired granuloma formation, were observed in

University of Cape Town

MTB infected TNF-α deficient mice when compared to wild-type mice (Roach, Bean, Demangel, *et al.*, 2002). Mice that are unable to produce MCP-1 show no enhanced susceptibility to MTB, suggesting a degree of redundancy within the chemokine network (Lu, Rutledge, Gu, *et al.*, 1998). In contrast, recruitment of macrophage, dendritic cells and primed lymphocytes to the lungs was markedly impaired in MTB infected C-C chemokine receptor-2 (CCR-2) deficient mice, resulting in uncontrolled mycobacterial replication and early lethality (Peters, Scott, Chambers, *et al.*, 2001). Increased levels of the chemokines MCP-1, MIP-1α, IL-8 and regulated on activation, normal T-cell expressed and secreted (RANTES) in bronchoalveolar lavage fluid from TB patients (Kurashima, Mukaida, Fujimura, *et al.*, 1997; Sadek, Sada, Toossi, *et al.*, 1998; Zhang, Broser, Cohen, *et al.*, 1995), and expression of IL-8, MCP-1, IP-10 and RANTES in granulomas from patients with pulmonary and lymphnode TB (Devergne, Marfaing-Koka, Schall, *et al.*, 1994; Ferrero, Biswas, Vettoretto, *et al.*, 2003; Lin, Gong, Zhang, *et al.*, 1998), underscore their role in human host defense against MTB.

Antigen presentation by dendritic cells. Sensitization of T-lymphocytes requires DC to present mycobacterial antigens on major histocompatibility (MHC) or MHC-like molecules, in the context of co-stimulatory molecules and pro-inflammatory cytokines (Pulendran, Palucka and Banchereau, 2001). MTB may gain access to the intracellular compartments of immature DC through ligation of DC-SIGN (Geijtenbeek, Van Vliet, Koppel, *et al.*, 2003; Tailleux, Schwartz, Herrmann, *et al.*, 2003). Internalization of MTB results in DC maturation (increased CD83 expression), upregulation of the co-stimulatory molecules CD40 (CTLA-4), CD80 (B7.1) and CD54 (ICAM-1) and MHC class I, and production of IL-12, TNF-α and IL-1 (Henderson, Watkins and Flynn, 1997). Surface expression of MHC class II on DC allows the presentation of mycobacterial antigens derived from the phagolysosome compartment to $CD4^+$ T-cells, expression of MHC class I the

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presentation of antigens from the cytosolic compartment to $CD8^+$ T-cells, and expression of CD1 the presentation of glycolipids or lipoproteins to CD1-restricted $CD4^-$ $CD8^-$ T-cells or $CD8^+$ T cells. Priming of naïve T-cells in the presence of IL-12 favors T_H1 development of lymphocytes capable of producing IFN-γ (Pulendran, Palucka and Banchereau, 2001; Trinchieri, 1997), a cytokine of principal importance in host defense against TB in mice and in humans (Cooper, Dalton, Stewart, *et al.*, 1993; Dalton, Pitts-Meek, Keshav, *et al.*, 1993; Flynn, Chan, Triebold, *et al.*, 1993; Jouanguy, Altare, Lamhamedi, *et al.*, 1996; Newport, Huxley, Huston, *et al.*, 1996).

Pathogen specific T-cell responses. The cellular immune response that controls mycobacterial replication in infected humans and mice is mediated by pathogen specific T lymphocytes that are capable of producing IFN-γ. In mice, the appearance of these cells at the site of infection is temporarily associated with control of mycobacterial replication (Chackerian, Perera and Behar, 2001; Feng, Bean, Hooi, *et al.*, 1999; Serbina and Flynn, 1999). Of the different T-lymphocyte subpopulations, MHC class II expressing, $CD4^+$ T-cells appear to be the most important mediators of host resistance to MTB (Caruso, Serbina, Klein, *et al.*, 1999; Mogues, Goodrich, Ryan, *et al.*, 2001; Muller, Cobbold, Waldmann, *et al.*, 1987; Orme, 1987). $CD4^+$ T-cells are the principal source of IFN-γ, a cytokine required for activation of mycobacteriostatic pathways within infected macrophages. In humans, $CD4^+$ T-lymphocytopenia, as observed in patients with advanced HIV infection, is associated with an increased risk of TB, and may result in severe, disseminated disease (Barnes, Bloch, Davidson, *et al.*, 1991). $CD4^+$ T-cells are present in TB granulomas, enriched at the site of disease in pleural TB (Lorgat, Keraan, Lukey, *et al.*, 1992; Tan, Canaday, Boom, *et al.*, 1997), and capable of perforin/granzyme or FAS (CD95)-FASL mediated lysis of MTB-infected macrophages (Lewinsohn, Bement, Xu, *et al.*, 1998; Oddo, Renno, Attinger, *et al.*, 1998; Oftung, Borka and Mustafa, 1998; Ottenhoff and Mutis, 1990; Tsukaguchi, Balaji and

University of Cape Town

Boom, 1995). FAS-mediated apoptosis of MTB infected macrophages by $CD4^+$ T-cells is associated with reduced mycobacterial viability (Oddo, Renno, Attinger, *et al.*, 1998).

MHC class I restricted $CD8^+$ T-lymphocytes also contribute to control of experimental MTB infection (Behar, Dascher, Grusby, *et al.*, 1999; Mogues, Goodrich, Ryan, *et al.*, 2001; Serbina and Flynn, 1999), and vaccine induced protective immunity (Bonato, Lima, Tascon, *et al.*, 1998; Tascon, Colston, Ragno, *et al.*, 1996; Tascon, Ragno, Lowrie, *et al.*, 2000). In humans, $CD8^+$ T-lymphocytes are present at the site of disease (Tan, Canaday, Boom, *et al.*, 1997) and in peripheral blood of patients with active TB or latent MTB infection (Cho, Mehra, Thoma-Uszynski, *et al.*, 2000; Gong, Stenger, Zack, *et al.*, 1998; Lalvani, Brookes, Wilkinson, *et al.*, 1998; Lewinsohn, Alderson, Briden, *et al.*, 1998; Mohagheghpour, Gammon, Kawamura, *et al.*, 1998; Rosat, Grant, Beckman, *et al.*, 1999; Stenger, Mazzaccaro, Uyemura, *et al.*, 1997). MHC class I or CD1 restricted $CD8^+$ T-cells may recognize mycobacterial antigens, produce IFN-γ, and lyse MTB infected target cells through a granule-dependent mechanism, resulting in killing of the mycobacteria (Stenger, Mazzaccaro, Uyemura, *et al.*, 1997) through the effects of granulysin, a substance with broad anti-microbial activity (Stenger, Hanson, Teitelbaum, *et al.*, 1998).

Two additional subsets of T-lymphocytes have been implicated in the host response against MTB. MTB-specific, CD1-restricted, $\alpha\beta$ T-cell receptor expressing $CD4^-CD8^-$ T-cells capable of FAS-FASL mediated target cell lysis (Beckman, Porcelli, Morita, *et al.*, 1994; Porcelli, Morita and Brenner, 1992; Sieling, Chatterjee, Porcelli, *et al.*, 1995). However, this does not affect the viability of intracellular MTB (Stenger, Mazzaccaro, Uyemura, *et al.*, 1997). Another subset of T-cells, $\gamma\delta$ T-cell receptor expressing lymphocytes, accumulates in the lungs of MTB infected mice (Griffin, Harshan, Born, *et al.*, 1991). Protective immunity in MTB infected macaques is temporarily associated with the appearance of $\gamma\delta$ T-cells in the lungs (Shen, Zhou, Qiu, *et al.*, 2002). Human $\gamma\delta$ T-cells are

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capable of granule dependent lysis of MTB infected macrophages and results in granulysin-mediated reduction of bacillary viability (Dieli, Troye-Blomberg, Ivanyi, *et al.*, 2000; Dieli, Troye-Blomberg, Ivanyi, *et al.*, 2001). The relative contribution of $CD8^+$ T-cells, double negative T-cells and γδ T-cells to mycobacterial host defense mechanisms remains to be defined, since mice deficient in γδ T-cells are not impaired in their ability to control mycobacterial replication (D'Souza, Cooper, Frank, *et al.*, 1997; Mogues, Goodrich, Ryan, *et al.*, 2001).

Cytokine activation of MTB infected macrophages. Control of mycobacterial infection is the result of activation of infected macrophages by pro-inflammatory cytokines including IFN-γ and TNFα, produced in an auto- and paracrine manner by macrophages and lymphocytes in the granulomas (Fenhalls, Stevens, Bezuidenhout, *et al.*, 2002). Apoptosis (Molloy, Laochumroonvorapong and Kaplan, 1994; Oddo, Renno, Attinger, *et al.*, 1998) and lysis of infected macrophages, with exposure of MTB to granulysin (Dieli, Troye-Blomberg, Ivanyi, *et al.*, 2001; Stenger, Hanson, Teitelbaum, *et al.*, 1998), are likely to contribute to mycobacteriostasis. Since perforin, granzyme B and FAS-receptor deficient mice have normal resistance against MTB (Cooper, D'Souza, Frank, *et al.*, 1997; Laochumroonvorapong, Wang, Liu, *et al.*, 1997), cytokine production may be the predominant contribution of recruited lymphocytes to control of MTB replication in mice.

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Activation of mouse macrophages by IFN-γ and TNF-α is associated with mycobacteriostatic activity against *M. tuberculosis* (Chan, Xing, Magliozzo, *et al.*, 1992; Denis, 1991; Flesch and Kaufmann, 1987; Rook, Steele, Ainsworth, *et al.*, 1986; Sato, Akaki and Tomioka, 1998). IFN-γ stimulation of mouse macrophages results in iNOS induction and the generation of reactive nitrogen intermediates (RNI) such as nitric oxide (NO), NO_2^-, and HNO_2 (Chan, Xing, Magliozzo, *et al.*, 1992). The important contribution of RNI to the control of mycobacterial replication was confirmed *in vivo* (Chan, Tanaka, Carroll, *et al.*,

1995; MacMicking, North, LaCourse, *et al.*, 1997; Mogues, Goodrich, Ryan, *et al.*, 2001). In addition to their toxic effects on phagocytosed microbes, RNI and reactive oxygen intermediates (ROI) may also have an important regulatory role in cell signaling and gene transcription (Ehrt, Schnappinger, Bekiranov, *et al.*, 2001).

In contrast to mouse macrophages, stimulation of human phagocytes with IFN-γ does not result in mycobacteriostasis (Denis, Gregg and Ghandirian, 1990; Douvas, Looker, Vatter, *et al.*, 1985; Rook, Steele, Ainsworth, *et al.*, 1986). Although human macrophages are capable of expressing iNOS (Nicholson, Bonecini-Almeida Mda, Lapa e Silva, *et al.*, 1996; Wang, Liu, Lin, *et al.*, 1998), it remains unclear whether human phagocytes posses NO-dependent mycobacteriostatic pathways (Nozaki, Hasegawa, Ichiyama, *et al.*, 1997; Thoma-Uszynski, Stenger, Takeuchi, *et al.*, 2001). Inhibition of mycobacterial replication has been observed following activation of human monocytes by TNF-α (Bermudez and Young, 1988; Denis and Gregg, 1990; Denis, Gregg and Ghandirian, 1990), vitamin D metabolites (Rook, Steele, Fraher, *et al.*, 1986) and/or iron (Byrd, 1997).

Immune mechanisms involved in controlling latent or chronic MTB infection. Drug treatment of MTB infected mice renders the organs sterile by conventional culture techniques. However, small numbers of organisms may persist for long periods of time (McCune, Tompsett and McDermott, 1956). Recrudescence of TB may occur spontaneously, and at increased frequency when glucocorticosteroids are administered (McCune, Feldmann, Lambert, *et al.*, 1966; McCune, Tompsett and McDermott, 1956). Spontaneous reactivation of TB at increased rates is also observed in drug-"sterilized" IFN-γ and TNF-α deficient mice (J.D. McKinney and B. Ryffel, unpublished observations). Exacerbation of chronic stable TB in mice is observed following treatment with TNF-α neutralizing mAbs (Mohan, Scanga, Yu, *et al.*, 2001), NOS2 inhibitors (MacMicking, North, LaCourse, *et al.*, 1997), IFN-γ neutralizing mAbs (Scanga, Mohan, Joseph, *et al.*, 1999), or anti-CD4 antibodies (Scanga,

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Mohan, Yu, *et al.*, 2000), underscoring the importance of persistent cytokine activation of infected macrophages, RNI mediated mycobacteriostasis, and T-cell mediated immunity in the control of latent or chronic MTB infection.

Tubercle bacilli have also been shown to survive prolonged chemotherapy in humans (Hobby, Auerbach, Lenert, *et al.*, 1954; van Rie, Warren, Richardson, *et al.*, 1999; Wayne and Salkin, 1956). However, the striking feature of MTB is its ability to persist for many years without causing disease in untreated individuals (Lillebaek, Dirksen, Baess, *et al.*, 2002). The immune mechanisms that prevent active TB in the majority of infected individuals are unknown. MTB-specific $CD4^{+}$T-cells (Pathan, Wilkinson, Klenerman, *et al.*, 2001; Ulrichs, Moody, Grant, *et al.*, 2003), $CD8^{+}$T-cells (Pathan, Wilkinson, Wilkinson, *et al.*, 2000) and $CD4^{-}CD8^{-}$ T-cells (Stenger, Mazzaccaro, Uyemura, *et al.*, 1997) have been documented in peripheral blood of PPD-positive persons and household contacts of TB patients, and are likely to contribute to protection against TB. As in mice, immune suppression as a result of glucocorticosteroids administration, TNF-α neutralizing mAbs (Keane, Gershon, Wise, *et al.*, 2001), or HIV-induced $CD4^{+}$T-cell depletion (Daley, Small, Schecter, *et al.*, 1992; Di Perri, Cruciani, Danzi, *et al.*, 1989) is associated with increased rates of reactivation of latent MTB infection.

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1.6 TB – OUTCOME OF THE HOST-PATHOGEN INTERACTION

Whether an infected person contains the infection, or develops progressive primary disease or reactivation TB will depend on many host, pathogen and environmental factors. Host genetic polymorphism may confer susceptibility or resistance to TB, and factors such as nutritional status, age-related immune dysfunction and co-morbidity may impair the ability to contain the bacilli. Virulence determinants of the MTB strain and size of the inhaled inoculum size may further influence the risk of developing TB.

Host susceptibility to TB. Apart from the major genetic defects in the IL-12 – IFN-γ pathway that result in severe susceptibility to overwhelming (chiefly atypical) mycobacterial infections, several polymorphisms have been identified that confer susceptibility to or protection against TB. *NRAMP1* (*SLC11A1*) polymorphism has been associated with susceptibility to TB in West Africans (Bellamy, Ruwende, Corrah, *et al.*, 1998; Cervino, Lakiss, Sow, *et al.*, 2000). Macrophage IL-10 production was enhanced in donors with susceptible *NRAMP1* genotypes (Awomoyi, Marchant, Howson, *et al.*, 2002) and in TB patients with persistent skin anergy. These patients appeared to have impaired resistance against MTB, as suggested by an increased number of deaths and episodes of recurrent TB, as well as reduced T-cell proliferative responses and impaired antigen-specific IL-2 and IFN-γ production to MTB antigens (Delgado, Tsai, Thim, *et al.*, 2002).

Associations between genetic polymorphisms and susceptibility to TB may be population specific. For instance, *NRAMP1* alleles that were associated with susceptibility to TB in Gambians were associated with resistance against TB in Cambodians and not associated with TB in Taiwanese (Delgado, Baena, Thim, *et al.*, 2002). *NRAMP1* polymorphisms in Danish TB patients were not associated with susceptibility to TB per se, but with sputum smear positive disease. Since the *NRAMP1*-encoded protein is present on the membranes of phagolysosomes within macrophages, *NRAMP1* polymorphisms might co-determine the ability of these cells to inhibit mycobacterial replication and the extent of clinical disease (Soborg, Andersen, Madsen, *et al.*, 2002).

In accordance with the observation that vitamin D inhibits the growth of MTB in human macrophages (Crowle and Elkins, 1990; Rook, Steele, Fraher, *et al.*, 1986), low serum vitamin D levels were associated with enhanced susceptibility to TB (Wilkinson, Llewelyn, Toossi, *et al.*, 2000), and specific vitamin D receptor polymorphisms with protection against TB (Bellamy, Ruwende, Corrah, *et al.*, 1999). An association between class II HLA

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genotypes and susceptibility to or protection against TB has been observed in some populations (Bothamley, Beck, Schreuder, *et al.*, 1989; Goldfeld, Delgado, Thim, *et al.*, 1998; Ravikumar, Dheenadhayalan, Rajaram, *et al.*, 1999).

Despite the importance of cytokines in determining host resistance against MTB, few consistent associations between polymorphisms of genes encoding these proteins or their receptors and active TB have been reported (Bellamy, Ruwende, Corrah, *et al.*, 1998; Delgado, Baena, Thim, *et al.*, 2002; Goldfeld, Delgado, Thim, *et al.*, 1998; Wilkinson, Patel, Llewelyn, *et al.*, 1999). Nonetheless, persons latently infected with MTB, in whom the immune response contains MTB infection, have a significantly higher number of IFN-γ secreting $CD4^+$ T cells, and respond with increased IFN-γ production in response to MTB specific antigens (Pathan, Wilkinson, Klenerman, *et al.*, 2001; Ulrichs, Moody, Grant, *et al.*, 2003; Vankayalapati, Wizel, Weis, *et al.*, 2003; Vekemans, Lienhardt, Sillah, *et al.*, 2001). Most recently, a polymorphism in the gene for IFN-γ was found to be associated with TB in South Africans (Rossouw, Nel, Cooke, *et al.*, 2003).

Virulence factors and adaptive responses of MTB. The success of MTB as a pathogen relates to its ability to replicate within host macrophages, to resist elimination by the host immune response, and to persist in a relatively inactive state while retaining the potential for reactivation years after infection (Glickman and Jacobs, 2001). MTB has developed "virulence" mechanisms that enable the bacilli to interfere with the establishment of an efficient T_H1 response, including the ability to inhibit macrophage IL-12, IL-15 and IL-18 production (Nau, Richmond, Schlesinger, *et al.*, 2002; Nigou, Zelle-Rieser, Gilleron, *et al.*, 2001; Vankayalapati, Wizel, Weis, *et al.*, 2003), IFN-γ induced transcriptional responses (Ting, Kim, Cattamanchi, *et al.*, 1999), CD1 and MHC class II expression (Giuliani, Prete, Graziani, *et al.*, 2001; Noss, Pai, Sellati, *et al.*, 2001) and TLR mediated signaling (Means, Lien, Yoshimura, *et al.*, 1999). In addition, MTB infection may result in impaired DC

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maturation (Geijtenbeek, Van Vliet, Koppel, *et al.*, 2003) or production of immune inhibitory cytokines (IL-10 and TGFβ) (Geijtenbeek, Van Vliet, Koppel, *et al.*, 2003; Hirsch, Toossi, Othieno, *et al.*, 1999). Susceptibility to MTB may thus be a reflection of a reduced ability of the host to overcome these pathogen specific impairments of T_H1 immunity.

The MTB cell wall contains a large number of lipids, glycolipids and polysaccharides that are likely to be involved in pathogenesis (Cole, Brosch, Parkhill, *et al.*, 1998; Dubnau, Chan, Raynaud, *et al.*, 2000; Glickman, Cox and Jacobs, 2000). Some of these lipids and proteins, including the 19kDa lipoprotein, are exported from the phagolysosome to the cytoplasm (Beatty, Rhoades, Ullrich, *et al.*, 2000; Berthet, Lagranderie, Gounon, *et al.*, 1998; Neyrolles, Gould, Gares, *et al.*, 2001), where they may affect host cell functions. Others, such as the cell wall associated lipid phthiocerol dimycocerosate or the 52 kDa cell surface protein encoded by the *mce1* gene appear to be required for organ specific replication or invasion of non-phagocytic cells and survival within human macrophages (Arruda, Bomfim, Knights, *et al.*, 1993; Cox, Chen, McNeil, *et al.*, 1999; Tekaia, Gordon, Garnier, *et al.*, 1999), while antigens such as the Ag 85B may be dispensable for replication yet important for eliciting immunopathology, a requirement for bacillary transmission in man (Armitige, Jagannath, Wanger, *et al.*, 2000; Baldwin, D'Souza, Roberts, *et al.*, 1998; Wilkinson, DesJardin, Islam, *et al.*, 2001).

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Another set of potential MTB virulence factors, including the exported repetitive protein (Berthet, Lagranderie, Gounon, *et al.*, 1998), the 16 kDa alpha crystalline protein (Yuan, Crane, Simpson, *et al.*, 1998), a cluster of genes involved in lipid metabolism, phthiocerol, phenolphthiocerol and phtiocerol dimycocerosate biosynthesis (Cox, Chen, McNeil, *et al.*, 1999) and siderophore production (De Voss, Rutter, Schroeder, *et al.*, 2000), a putative polyketide synthase, a PPE family protein (Camacho, Ensergueix, Perez, *et al.*, 1999), and the enzyme glutamine synthase (Tullius, Harth and Horwitz, 2003) are required

for growth in macrophages or mouse lungs, and expression of a heparin binding haemagglutinin by MTB may facilitate early dissemination from the airways to other organs (Pethe, Alonso, Biet, *et al.*, 2001).

Approximately 10% of the MTB genome encodes 2 families of unrelated, glycine rich (PE and PPE) proteins. These could potentially represent a source of antigenic variation, interfere with immune responses by inhibiting antigen processing, or serve as an asparagine storage reservoir for the bacilli (Cole, Brosch, Parkhill, *et al.*, 1998; Tekaia, Gordon, Garnier, *et al.*, 1999). In addition, granuloma-specific expression of members of the PE/PE-PGRS family by *M. marinum* suggests a possible role for these proteins in mycobacterial persistence (Ramakrishnan, Federspiel and Falkow, 2000).

Mycobacteria in stationary phase broth culture and murine lungs use fatty acids as a carbon source (Primm, Andersen, Mizrahi, *et al.*, 2000; Segal and Bloch, 1956). Fatty acid catabolism requires the use of two metabolic pathways: the β-oxidation cycle and the glyoxylate shunt (Honer Zu Bentrup, Miczak, Swenson, *et al.*, 1999). MTB mutants deficient for isocitrate lyase (ICL) were impaired in their ability to resist elimination by the murine host immune response, suggesting an important role for fatty acid metabolism im mycobacterial persistence (McKinney, Honer zu Bentrup, Munoz-Elias, *et al.*, 2000). The expression of ICL by MTB within granulomas of patients with TB suggests that similar metabolic adaptation of MTB may be required for persistence in the human lung (Fenhalls, Stevens, Moses, *et al.*, 2002). The importance of fatty acid metabolism to MTB is underscored by the identification of some 250 regulatory enzymes (Cole, Brosch, Parkhill, *et al.*, 1998), several of which are upregulated following phagocytosis by macrophages (Dubnau, Fontan, Manganelli, *et al.*, 2002).

Adaptive responses to changes in the environment, including exposure to the host immune response, require extensive gene regulation (Shi, Jung, Tyagi, *et al.*, 2003). An

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important family of gene transcriptional regulators is the alternate RNA polymerase sigma factors (Cole, Brosch, Parkhill, *et al.*, 1998; Collins, Kawakami, de Lisle, *et al.*, 1995; DeMaio, Zhang, Ko, *et al.*, 1996; Graham and Clark-Curtiss, 1999; Kaushal, Schroeder, Tyagi, *et al.*, 2002). The large numbers of genes required for the various adaptative responses could all be considered "virulence factors" (Barry, 2001). More constructively, the interaction between *M. tuberculosis* and the mammalian host could be seen as a dynamic interplay between bacterial adaptive responses and host defense mechanisms. In man, this interaction may result in the following outcomes: progressive primary infection, latency or reactivation disease.

1.7 MOLECULAR EPIDEMIOLOGY – THE IDENTIFICATION OF CLINICAL OUTBREAK STRAINS

DNA fingerprinting (also known as restriction fragment length polymorphism or RFLP) of MTB strains has offered revolutionary insights into the epidemiology of tuberculosis, and allowed the identification of MTB strains or families of related strains. Studies from San Francisco and New York City that employed this technique demonstrated that reactivation of latent disease was less common than hitherto assumed (Alland, Kalkut, Moss, *et al.*, 1994; Small, Hopewell, Singh, *et al.*, 1994). In these cities, the isolates from 30-40% of patients were clustered, suggesting that TB in these patients had arisen from recently transmitted MTB infection. Importantly, conventional contact tracing had identified connections in only 10% of clustered cases linked by RFLP analysis (Small, Hopewell, Singh, *et al.*, 1994). In New York City, drug resistant TB, until then considered to be the result of poor compliance with therapy, was shown to be due to (recent) transmission of drug resistant strains in two-thirds of cases (Alland, Kalkut, Moss, *et al.*, 1994). Other patients developed MDR TB from a new, drug resistant strain while undergoing treatment for drug susceptible TB (Small,

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Shafer, Hopewell, *et al.*, 1993). These findings had major implications for tuberculosis control strategies, with a shift of emphasis from preventive therapy in exposed individuals to interruption of transmission by early case identification and limitation of institutional spread (Alland, Kalkut, Moss, *et al.*, 1994; Daley, Small, Schecter, *et al.*, 1992; Small, Hopewell, Singh, *et al.*, 1994; Small, Shafer, Hopewell, *et al.*, 1993). Consequently, the rates of drug-resistant TB have dropped and MDR TB is no longer associated with recent transmission (Geng, Kreiswirth, Driver, *et al.*, 2002).

RFLP has been used to document outbreaks of TB (Bifani, Plikaytis, Kapur, *et al.*, 1996; Frieden, Sherman, Maw, *et al.*, 1996; van Rie, Warren, Beyers, *et al.*, 1999), and to estimate the risk of MTB transmission. Of interest, in San Francisco, 17% of all TB cases had arisen from smear-negative source cases. The relative risk of MTB transmission from smear-negative cases was 0.22 compared to transmission from smear-positive cases. In TB endemic, resource-poor countries however, MTB transmission from smear-negative cases may be considerably higher since these patients are missed by smear-based diagnosis and therefore may remain infectious for longer periods (Behr, Warren, Salamon, *et al.*, 1999).

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In many parts of the developing world, TB case rates have reached "epidemic" levels. In such circumstances, it is to be expected that TB is predominantly the result of recently transmitted MTB infection. Surprisingly, an RFLP study from South Africa, performed in a largely HIV negative community with TB incidence rates of over 1% (2% in young adults and 3% in subsets of the community) revealed that reactivation of latent disease was the principal mode of developing TB in adults (Warren, Hauman, Beyers, *et al.*, 1996). However, when a wider definition of genotypic clustering was used, over 85% of TB was considered to have arisen from recent MTB transmission (van der Spuy, Warren, Richardson, *et al.*, 2001). In addition, two thirds of the MDR-TB cases had arisen from recently transmitted infection (Van Rie, Warren, Richardson, *et al.*, 2000; Warren, Hauman, Beyers, *et al.*, 1996), and most

(12 of 16) episodes of recurrent TB in HIV negative individuals after curative treatment were the result of exogenous re-infection (van Rie, Warren, Richardson, *et al.*, 1999).

Apart from providing an opportunity to identify outbreaks and transmission patterns of TB, RFLP has also allowed classification of MTB isolates into families of genotypically related strains. Specific isolates have been studied for their immunologic characteristics or genetic properties. For instance, a strain (CDC1551) that caused an outbreak of TB in the Mid-West of the USA in the 1990's was noted to have given rise to unusually strong delayed-type hypersensitivity skin reactions in infected persons (Valway, Sanchez, Shinnick, *et al.*, 1998). The CDC1551 strain was also shown to have reduced virulence in rabbits (Bishai, Dannenberg, Parrish, *et al.*, 1999) and in mice (Manca, Tsenova, Barry, *et al.*, 1999). Compared to H37Rv, CDC1551 induced increased levels of IL-12 and TNF-α, which resulted in earlier control of mycobacterial growth. Increased pro-inflammatory cytokine production was also observed in human monocytes infected with CDC1551 *in vitro*, and together with the enhanced skin test reactions, suggest that this strain may be highly immunogenic (Manca, Tsenova, Barry, *et al.*, 1999).

Another clinical outbreak strain, HN878 (Sreevatsan, Pan, Stockbauer, *et al.*, 1997), was shown to be hypervirulent in mice. Infection with this strain resulted in reduced IL-12 and TNF-α production, increased bacterial counts in the lungs, and markedly impaired survival of infected animals (Manca, Tsenova, Bergtold, *et al.*, 2001). It is interesting to note that HN878 belongs to the W-Beijing family of MTB isolates, related strains with a worldwide distribution and particularly high prevalence in Asia. Faster replication in human macrophages has been reported for members of the W-Beijing family (Li, Whalen, Albert, *et al.*, 2002; Zhang, Gong, Yang, *et al.*, 1999). These observations underscore the notion that these strains may have an as yet unidentified genetic advantage to cause disease in humans (Bifani, Mathema, Kurepina, *et al.*, 2002).

University of Cape Town

1.8 TB TREATMENT, ACTION OF TB DRUGS AND MECHANISMS OF DRUG RESISTANCE

Prior to the availability of chemotherapeutic agents, bed rest in sanatoria and lung collaps were readily administered with "good" success (patients not infrequently became sputum smear negative as long as they refrained from physical activity). Not infrequently, diseased lung was surgically removed in an attempt to remove residual tubercle bacilli. Despite these attempts at cure, TB in 1947 was associated with 21% one-year mortality, whereas 54% of patients remained sputum positive and 25% became sputum negative. Following introduction of streptomycin and para-amino salicylic acid (PAS), mortality was reduced to 3%, 32% remained sputum positive and 65% became non-infectious (Ferebee and Palmer, 1965).

With the availability of more potent bactericidal agents including pyrazinamide (PZA, Z), isoniazid (INH, H) and rifampicin (rifampin, RIF, R), trials were commenced to evaluate the efficacy of short course (6 months) treatment. Regimens that contained RHZ were well tolerated, highly efficacious, and bacteriological relapses were infrequent (1972; 1973; 1974; 1974). RHZ achieved rapid bactericidal activity *in vivo*, with an approximate 25-fold fall in sputum colony counts over the first 2 days, a further 10-fold decrease over the next 7 days, and another 1 log reduction over the next 20 days (Jindani, Aber, Edwards, *et al.*, 1980). A six months regimen consisting of RHZ combined with ethambutol (EMB) for the first 2 months, followed by 4 months of RH, became the standard "short course chemotherapy" for tuberculosis (1981; 1981; 1982; 1982). To facilitate direct supervision of therapy, daily RH therapy may be substituted with twice weekly RH, or, in the absence of cavitation, with weekly rifapentine/isoniazid in the continuation phase (Benator, Bhattacharya, Bozeman, *et al.*, 2002; Perriens, St Louis, Mukadi, *et al.*, 1995). Addition of drugs with superior activity against non-replicating bacilli, such as a recently described nitroimidazopyran, to current treatment regimens could result in more rapid sterilization of necrotic granulomas and

University of Cape Town

allowing a further reduction of the treatment duration (Stover, Warrener, VanDevanter, *et al.*, 2000). Adjuvant immunotherapy with *M. vaccae* did not result in improved cure or relapse rates in TB patients treated with short-course chemotherapy (1999; Johnson, Kamya, Okwera, *et al.*, 2000; Mwinga, Nunn, Ngwira, *et al.*, 2002).

As discussed earlier, fatty acid metabolism is extremely important to the mycobacterium. Fatty acid synthesis requires the action of 2 key enzymes, fatty acid synthase-1 (FAS-I), which catalyzes the conversion of Acetyl-CoA to 16-24-carbon fatty acids, and FAS-II, which adds additional acetyl-CoA units to form meromycolates (fatty acids containing more than 50 carbon atoms) that can be modified into mycolic acids. Several tuberculostatics inhibit fatty acid synthesis by targeting these enzymes. PZA, a nicotinamide analogue and a pro-drug that is converted by mycobacterial pyrazinamidase to pyrazinoic acid, inhibits FAS-I (Zimhony, Cox, Welch, *et al.*, 2000). Isoniazid and ethionamide both target the enoyl-ACP reductase (*inhA*) (Banerjee, Dubnau, Quemard, *et al.*, 1994) component of FAS-II (Draper, 2000), and β-ketoacyl ACP synthase (*kasA*) (Mdluli, Slayden, Zhu, *et al.*, 1998). Resistance to PZA may be caused by mutations in *pncA*, the gene encoding pyrazinamidase, and resistance to INH may result from reduced oxidation of this pro-drug by mycobacterial catalase peroxidase due to *katG* mutations (Zhang, Heym, Allen, *et al.*, 1992). INH resistance may also be caused by overexpression of *inhA* (Larsen, Vilcheze, Kremer, *et al.*, 2002). Ethionamide does not require intracellular oxidation; resistance to this drug as well as to isoniazid may result from mutations in enoyl-ACP reductase and/or β-ketoacyl ACP synthase.

Rifampin and streptomycin are translational inhibitors. Rifampin disrupts mRNA synthesis by binding to the RNA polymerase β subunit (*rpoB*) (Telenti, Imboden, Marchesi, *et al.*, 1993), and streptomycin inhibits bacterial protein synthesis through binding to the 16S rRNA (*rrs*) and S12 ribosomal protein (*rpsL*) (Finken, Kirschner, Meier, *et al.*, 1993).

University of Cape Town

Meier, *et al.*, 1993). Fluoroquinolones inhibit mycobacterial DNA synthesis by binding to DNA gyrase (*gyrAB*) (Drlica, Xu, Wang, *et al.*, 1996). Resistance to each of these drugs is conferred by single nucleotide substitutions in the target genes.

Ethambutol, a first line tuberculostatic, inhibits cell wall arabinogalactam synthesis. Resistance to EMB is caused by overexpression of its target arabinosyltransferase, or by mutations in the *embAB* gene encoding this enzyme (Belanger, Besra, Ford, *et al.*, 1996). Cycloserine targets cell wall peptidoglycan synthesis by inhibition of the enzymes d-alanyl, d-alanine ligase (*ddlA*) (David, Takayama and Goldman, 1969) and alanine racemase (*alr*) (Caceres, Harris, Wellehan, *et al.*, 1997), whereas PAS is a weak inhibitor of mycobacterial folic acid synthesis (Nopponpunth, Sirawaraporn, Greene, *et al.*, 1999).

In most bacterial species, drug resistance mutations result in reduced fitness of the strain. Drug pressure results in selection of drug resistant mutants by inhibiting the growth of susceptible bacilli. Following withdrawal of drug pressure, 'wild' type bacilli are able to outgrow resistant bacilli and become predominant. In contrast, cessation of drug pressure in patients with drug resistant TB does not result in the disappearance of resistant isolates, raising the possibility that resistant MTB isolates may be no less fit than pan-susceptible isolates. Indeed, a recent study revealed that MTB isolates harboring the *katG* S315T mutation grew similarly in mice as isogenic bacilli that lacked this mutation (Pym, Saint-Joanis and Cole, 2002). In addition, the *rpoB* S531L and *rpsL* mutations were not associated with reduced fitness (Cohen, Sommers and Murray, 2003). Whereas cluster studies in several populations have suggested that MDR strains may be less, equally or more transmissible than drug susceptible isolates, household studies have shown that MDR strains gave rise to similar rates of skin test conversion and clinical TB as drug susceptible strains (Snider, Kelly, Cauthen, *et al.*, 1985; Teixeira, Perkins, Johnson, *et al.*, 2001).

University of Cape Town

Interestingly, a recent study suggested that MTB may posess mechanisms to promote mutagenesis. The *dnaE2* gene that encodes an error-prone DNA polymerase was shown to be upregulated when cellular replication was arrested as a result of unrepaired DNA damage. Upregulation of *dnaE2* was also observed in bacilli obtained from the organs of infected mice following adaptive immunity. Bacilli in which *dnaE2* had been deleted were less virulent, and developed fewer *rpoB* resistance mutations following exposure to rifampin *in vivo*. Induction of error-prone DNA polymerases *in vivo* may thus contribute to the observed increased rate of drug resistance mutants in humans with TB relative to their predicted frequency based on stochastic mutation rates *in vitro*. Generation of mutants may be a strategy that MTB has developed to adapt to the stresses of host immunity and TB therapy (Boshoff, Reed, Barry, *et al.*, 2003).

1.9 TB CONTROL AND PREVENTION

As of 1995, the DOTS strategy has been proposed by the World Health Organization as one of the ways to reduce the burden of TB. DOTS "direct observation of therapy – short course" indicates that patients worldwide should be treated with optimal – rifampin containing – short course chemotherapy. The supervised aspect attempts to address the issue that a substantial number of patients interrupt treatment, largely because they feel physically much improved after the first few weeks of therapy. However, the concept of DOTS stretches well beyond this and includes government commitment, diagnosis by smear microscopy, reliable drug supply, and standardized recording and reporting of treatment outcomes (Nunn, 2001).

The potential to identify individuals who are latently infected with MTB, and who are thus at risk of reactivation TB later in life, affords the opportunity to administer prophylactic TB treatment (Jasmer, Nahid and Hopewell, 2002). In low prevalence countries, treatment of PPD-positive persons with isoniazid results in at least 50% reduction of the risk of

University of Cape Town

developing active TB. Consequently, this intervention is frequently used to limit the burden of active disease (1992; Ferebee, 1970).

Many of the countries in sub-Saharan Africa with a high TB burden also have high rates of HIV infection. HIV infected persons who are latently infected with MTB are at increased risk of developing TB and may thus benefit from chemoprophylaxis. In non-anergic, tuberculin skin test positive (>5 mm) HIV infected persons, 6-12 months of isoniazid, or three months of isoniazid-rifampin, reduces the risk of active TB by 60-83% (Pape, Jean, Ho, *et al.*, 1993; Whalen, Johnson, Okwera, *et al.*, 1997). The logistics associated with voluntary counseling and HIV-testing, and the difficulty of excluding active TB in patients with HIV infection in rural settings, however, make it unlikely that chemoprophylaxis will have a significant impact on the burden of tuberculosis in sub-Saharan Africa (Aisu, Raviglione, van Praag, *et al.*, 1995). More prolonged (12 months) treatment of patients with active TB, and secondary INH prophylaxis following treatment, are effective ways to reduce the number of relapses but fail to improve survival (Fitzgerald, Desvarieux, Severe, *et al.*, 2000; Perriens, St Louis, Mukadi, *et al.*, 1995).

The current vaccine used against TB, BCG, was obtained by repeated passaging of the bovine tubercle bacillus in the presence of bile. By 1921, after thirteen years and 230 passages, the *M. bovis* strain had become highly attenuated and could be safely administered to a variety of animal species as well as humans. Further passaging in laboratories around the world yielded a number of genetically distinct BCG strains, most of which have been used as vaccines given to billions of people in an attempt to prevent TB (Behr, 2002; Behr, Wilson, Gill, *et al.*, 1999). BCG vaccination induces a T_H1 response characterized by antigen-specific, IFN-γ producing, memory T cells (Black, Weir, Floyd, *et al.*, 2002; Marchant, Goetghebuer, Ota, *et al.*, 1999; Ravn, Boesen, Pedersen, *et al.*, 1997). When mice are vaccinated with BCG and then infected with MTB by aerosol, an accelerated recruitment of activated, IFN-γ

University of Cape Town

producing lymphocytes to the lungs is observed. This is associated with reduced tissue mycobacterial loads compared to non-vaccinated animals, but the bacilli are not eliminated (Silva, Bonato, Lima, *et al.*, 1999).

In man, BCG vaccination results in an approximate 50% reduced risk of developing tuberculosis, particularly childhood disease at extra pulmonary sites. Unfortunately, there appears to be minimal, if any, or even negative benefit with regards to the prevention of adult pulmonary tuberculosis (Fine, 2001). The efficacy of BCG is least in the tropical developing world, where sensitization due to environmental mycobacteria may be more common (Black, Weir, Floyd, *et al.*, 2002; Colditz, Brewer, Berkey, *et al.*, 1994; Rodrigues, Diwan and Wheeler, 1993). Despite almost universal BCG vaccination at birth, annual notification rates in 0-4 year olds have risen sharply between 1970 and 1996 in areas where TB is endemic (Enarson, Beyers and Zhang, 2001). Inadequate protective efficacy and a small risk of disseminated BCG infection when the vaccine is given to immune compromised neonates (Altare, Lammas, Revy, *et al.*, 1998; Jouanguy, Altare, Lamhamedi, *et al.*, 1996) has resulted in a search for new vaccine candidates.

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MTB vaccine development has rendered several candidates, including subunit vaccines, whole bacterial vaccines, and combination vaccine strategies. Among the subunit vaccines, both DNA encoding MTB antigen 85A (Ag 85A) and DNA encoding heat shock protein 65 (HSP 65) have afforded mouse protection against MTB challenge comparable to BCG (Huygen, Content, Denis, *et al.*, 1996; Tascon, Colston, Ragno, *et al.*, 1996). The HSP 65 DNA vaccine was also effective in reducing bacterial loads and improving survival of mice when administered several weeks post infection (Lowrie, Tascon, Bonato, *et al.*, 1999). Another subunit vaccine candidate is the early secreted antigenic target of 6 kDa (ESAT-6). This antigen is recognized by T-cells from patients with TB and experimentally infected guineapigs (Elhay, Oettinger and Andersen, 1998; Ravn, Demissie, Eguale, *et al.*, 1999), and

is capable of inducing high levels of protective immunity (Brandt, Elhay, Rosenkrands, *et al.*, 2000). The use of subunit vaccines has resulted in severe lung damage in mice and guinea pigs challenged with MTB by aerosol (Baldwin, D'Souza, Roberts, *et al.*, 1998; Taylor, Turner, Basaraba, *et al.*, 2003; Turner, Roberts, Frank, *et al.*, 2000), implying that the safety of these vaccines will have to be carefully monitored.

Among the whole cell vaccines, auxotroph mutants have been developed as a safe alternative to BCG. These highly attenuated BCG or MTB strains are unable to replicate in host tissue (Camacho, Ensergueix, Perez, *et al.*, 1999; Cox, Chen, McNeil, *et al.*, 1999; Jackson, Phalen, Lagranderie, *et al.*, 1999), and may induce protective responses against MTB (Guleria, Teitelbaum, McAdam, *et al.*, 1996; Sambandamurthy, Wang, Chen, *et al.*, 2002). Enhanced protective efficacy of BCG was observed when it was co-administered with IL-12, or when BCG was made to express the MTB 30 kDa major secretory protein (Ag 85B) or secrete the ESAT-6 and the 10kDa culture filtrate protein (CFP-10). MTB challenge mice had up to 0.5-1 log reduced organ bacterial loads compared to BCG vaccinated mice (Freidag, Melton, Collins, *et al.*, 2000; Horwitz, Harth, Dillon, *et al.*, 2000; Pym, Brodin, Majlessi, *et al.*, 2003).

Combination vaccines utilize a prime-boost strategy. A recently developed candidate uses BCG to prime immunity and Ag 85A (expressed in modified vaccinia Ankara or recombinant pox virus) to boost this immune response. This strategy has resulted in induction of IFN-γ secreting, MTB specific T-cells in healthy volunteers, and protective efficacy in populations at risk of TB is currently under evaluation.

1.10 STUDIES OF THE HOST-PATHOGEN INTERACTION

This thesis contains five studies. In chapters 3-5, the interaction between related yet distinct mycobacterial strains and human or mouse mononuclear phagocytes *in vitro* is examined.

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Monocytes and MDM from healthy subjects and patients with TB, and peritoneal macrophages from immune competent and gene deficient mice, are infected in parallel with H37Ra and H37Rv, *M. smegmatis* and *M. smegmatis* 19kDa, or CDC1551 and HN878. The ability of these cells to control the growth of these mycobacterial isolates, as well as the production of immune regulatory cytokines in response to the infection is examined. The extent to which mycobacterial strain diversity and bacillary heterogeneity are encountered in human TB is examined in chapters 6-7. Mycobacteria obtained from different tuberculous lung lesions and serial sputum cultures of patients with chronic, therapy-refractory TB are characterized by RFLP fingerprint and sequence analysis of the genetic targets of anti-tuberculous chemotherapy. In addition, a detailed histological, immunohistochemical and molecular analysis of the host-pathogen interaction at the site of disease is presented.

University of Cape Town

Chapter 2

GENERAL METHODS

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The studies presented in this thesis examine the interaction between *M. tuberculosis* (MTB) and human host, both *in vivo* and *in vitro*, and between MTB and mouse macrophages *in vitro*. A description of the TB patients enrolled, as well as the cell biologic, immunologic and microbiologic methods employed in these studies, is presented below. The methodology is divided in human, mouse, and mycobacterial studies.

2.1 STUDIES OF HUMAN SUBJECTS AND HUMAN PHAGOCYTES

2.1.1 Human subjects

Patients. Seventeen HIV-negative patients with active pulmonary tuberculosis (TB) were enrolled for the study presented in chapter 5. These patients were recruited from the Chapel Street TB clinic in Woodstock or Brooklyn Chest Hospital, a 350-bed inpatient TB treatment facility. All patients were South African of Coloured or Black ethnicity and tended to come from under-privileged backgrounds where joblessness and alcohol abuse are common. All patients were sputum smear and culture positive for MTB. None of the patients had specific risk factors for TB such as drug-induced immune suppression or diabetes mellitus, and none of the female patients was pregnant. Patients with prior TB treatment were excluded from this study. At the time of sampling, none of the patients had received more than 4 weeks of rifampin-based short course chemotherapy.

Six patients who underwent pneumonectomy for the complications of TB at Groote Schuur Hospital between January 2000 and December 2001 were studied in chapter 6. All patients had unilateral lung disease with (almost) complete destruction of the affected lung. Three patients (two of whom had MDR-TB) underwent surgery for chronic, sputum positive TB despite 18-24 months of supervised multi-drug therapy. The three patients who were culture negative at the time of surgery all had a history of pulmonary tuberculosis. These patients had presented with hemoptysis and other symptoms suggestive of relapse, and had

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received empirical treatment for TB for 7-15 months. Sputum cultures from these three patients at the time of presentation, however, failed to yield MTB. Lung resection in these patients was performed to relieve ongoing hemoptysis thought to have arisen from a focus in the destroyed lung. All patients received TB therapy up until the time of surgery, and none of the patients was co-infected with HIV.

Thirteen HIV-negative patients with chronic, active MDR-TB despite at least 12 (mean of 36, range 12-77) months of optimized chemotherapy were studied in chapter 7. These patients comprised the entire South African subset of a multinational, randomized, placebo controlled study to evaluate the role of aerosolized recombinant human interferon-gamma (rhuIFN-γ, InterMune, Brisbane, CA, USA) as an adjunct to chemotherapy in patients with MDR-TB (Condos, Rom and Schluger, 1997). All 13 patients had initially received treatment for drug susceptible TB; this was substituted for individualized drug regimens based on the results of phenotypic drug susceptibility tests once MDR-TB was diagnosed. Drug susceptibility had been evaluated every 2-3 months, and treatment regimens adjusted accordingly at approximately 6 monthly intervals. All patients were sputum smear and culture positive at the time of enrollment into the study, and continued to excrete bacilli in their sputum throughout the study.

Control subjects. Healthy volunteers were used as blood donors in Cape Town and New York (chapters 3 and 4). In addition, buffy coats were obtained from the New York Blood Transfusion Service and used as a source of peripheral blood mononuclear cells (PBMC). Only blood from HIV negative persons was used.

Recruitment and consent procedures. All patient studies were performed in Cape Town, South Africa. TB Patients were asked to participate in the studies, and informed both in word and in writing about the study aims and procedures. Participation was voluntary and all patients signed informed consent. A small monetary incentive was offered to compensate

University of Cape Town

for the discomfort of venapuncture to the patients in chapter 5. Healthy blood donors gave written informed consent and received a small payment for each blood donation.

Ethical approval. The Ethics Committee of the University of Cape Town and the Institutional Review Boards of The Rockefeller University and the University of Medicine and Dentistry of New Jersey approved all studies involving human subjects.

2.1.2 Clinical specimen obtained from TB patients

Blood. Ten ml of venous EDTA-blood was obtained from patients with active TB for plasma cytokine analysis and centrifuged at 800x*g* for 4 minutes. Plasma was removed and stored in aliquots at -70°C for cytokine analysis. PBMC were isolated from 60 ml of venous blood collected in heparinized syringes (approximately 3 hours after the administration of the morning dose of TB chemotherapy). Blood was layered on lymphocyte separation medium (Ficoll Hypaque, Pharmacia, Uppsala, Sweden) and centrifuged at 400x*g* for 25 minutes. PBMC were harvested from the gradient, washed twice in cold RPMI 1640 (Gibco BRL, Gaithersburg, MD) enriched with 1% human AB serum (Gemini Bio Products, Calabasas, CA) (R1), and kept on ice until use. Flowcytometry (FACScan, Becton Dickinson) was used to calculate the monocyte fraction of PBMC.

Lung tissue. Surgically removed lungs were taken immediately to the BSL-3 laboratory for dissection in a laminar flow hood. Samples from various lesions and macroscopically normal tissue were obtained for histology (immersed in formalin), for mRNA analysis (snap frozen in liquid nitrogen), and for mycobacterial culture (in a sterile container). Dissection instruments and work surfaces were frequently changed or cleaned to minimize the risk of cross contamination of specimen with mycobacteria from different sites within the lung.

University of Cape Town

Sputum. Sputum samples were obtained at baseline and at multiple time points thereafter. The bacillary load in sputum was determined by counting the number of acid-fast bacilli, using a standardized concentration procedure (incubation with 5% bleach followed by high speed centrifugation) to prepare smears of at least 2 cm^2 that were auramine stained and examined by direct fluorescent-microscopy using 400x magnification. Smears were recorded as having <4, 4-40, >40 bacilli per high power field, and scored 1, 2 or 3 respectively. Each sputum specimen was also cultured in an automated mycobacterial culture system (MGIT, Becton Dickinson, Sparks, MD). Positive cultures were examined by fluorescent microscopy for acid-fast bacilli and by PCR to confirm the presence of MTB (De Wit, Steyn, Shoemaker, *et al.*, 1990).

Phenotypic resistance analysis was performed on the baseline sputum MTB isolates of all 13 patients in chapter 7. Resistance to rifampin (RIF) and isoniazid (INH) was confirmed by growth of the isolates in liquid culture medium containing 2 μg/ml of RIF or 0.1 μg/ml of INH using the radiometric Bactec 460 system (Becton Dickinson). Susceptibility of MTB to other anti-tuberculous agents was determined by the indirect proportion method, using OADC enriched solid 7H10 medium containing critical concentrations of 7.5 μg/ml of ethambutol, 10 μg of streptomycin, 5 μg/ml of kanamycin, 2 μg/ml of ofloxacin, 10 μg/ml of ethionamide or 2 μg/ml of thiacetazone. Phenotypic susceptibility testing for pyrazinamide was not performed, since the results of this test can be difficult to reproduce and may not correlate well with drug susceptibility *in vivo* (Cutler, Wilson, Villarroel, *et al.*, 1997; Hewlett, Horn and Alfalla, 1995). Spacer oligonucleotide genotyping (spoligotyping) was carried out by amplification of the direct repeat region of each isolate. Isolates were assigned a number according to the Centers for Diseases Control (CDC) nomenclature (Mathema, Bifani, Driscoll, *et al.*, 2002). Direct DNA sequencing of the following drug resistance target

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genes was carried out: *rpoB, katG, inhA, pncA, embB, rpsL, rrs* and *gyrA* (Ramaswamy and Musser, 1998).

Chest radiographs. Pretreatment chest radiographs were classified and scored according to the number of lung zones affected by tuberculosis, the degree of involvement of each zone, and the size of the cavities. The lungs were divided into 6 zones: upper (apex to second rib), middle (second to fourth rib) and lower zones (fourth rib to diaphragm), and scored 0-2 points each for the degree of pulmonary involvement: 0 (uninvolved), 1 (<50% of the lung diseased) or 2 (>50% of the lung involved in the disease process). Cavities were scored 0.25, 0.5 or 1 points if single and measuring <2 cm, 2-4 cm, or >4 cm respectively. Multiple cavities scored 0.5, 1 or 2 points if <2 cm, 2-4 cm, or >4 cm in diameter. The chest radiograph severity score used in chapter 7 thus ranged from 0 to 24 points. In chapter 5, patients were classified as having limited disease if the pulmonary infiltrates confined to the upper lobes and/or apical segments of the lower lobes. Disease was classified as extensive when, in addition to upper zone infiltrates, the radiographs revealed infiltrates in the lower and/or mid-zones.

University of Cape Town

2.1.3 Culture, infection and stimulation of human monocytes *in vitro*

Culture of monocytes. PBMC were plated at a density of $3x10^6$ cells/well in 24 well Falcon tissue culture plates (Becton Dickenson Labware, Lincoln Park, NJ). After 1-2h of incubation at 37°C and under 5% CO_2, non-adherent cells were removed by gently washing the monolayers with warm culture medium. The adherent monocytes were infected immediately, or cultured for up to 7 days to generate monocyte-derived macrophages (MDM). In other experiments, non-adherent monocytes (cultured in Teflon beakers; Savillix, Minnetonka, MN) were obtained from PBMC initially plated on 100 mm Falcon tissue culture dishes (Becton Dickenson Labware, Lincoln Park, NJ). Following removal of non-adherent

lymphocytes, the monocytes were re-incubated for a further 24 h, after which the culture medium was replaced with ice-cold phosphate buffered saline (PBS) (Gibco) containing 0.2% EDTA. The detached monocytes were harvested after 15 minutes, and the remaining adherent cells removed with cell scrapers (Costar, Cambridge, MA). The harvested monocytes were washed, suspended in R20, counted and plated in 24 well plastic plates or in Teflon wells at a density of $3x10^5$ monocytes per well, and then infected (day 1).

***In vitro* infection of human monocytes/MDM with mycobacteria.** Monocytes (approximately $3x10^5$ cells/well) were infected on the day of isolation with MTB strains H37Ra or H37Rv (chapter 3), or with MTB strains CDC1551 or HN878 (chapter 5), or incubated in R10 for 6 days (recombinant *M. smegmatis* experiments, chapter 4) or incubated in R20 for 7 days (H37Ra and H37Rv experiments, chapter 3) prior to infection. The cells were infected by replacing the culture medium with fresh medium containing the mycobacteria at the desired multiplicity of infection (MOI) of 1:1 for MTB and 3:1 for *M. smegmatis*. In the experiments in which adherent and non-adherent monocytes were used, infection was carried out by adding MTB containing medium to the cell culture medium, rather than replacing it. Since MTB was fully phagocytosed by 6h (Laochumroonvorapong, Paul, Manca, *et al.*, 1997), monocyte cultures infected with MTB were not washed after the addition of mycobacteria. The number of viable intracellular bacilli in MTB infected monocytes and MDM was determined on days 1, 2, (and 4) post infection.

M. smegmatis infected cells were pulsed with gentamycin (Gibco) 3h post infection, at a final concentration 200 µg/ml, to inhibit the growth of extra-cellular organisms. At 5h post infection, the culture medium was replaced with fresh R10 medium, without antibiotics. The infection was followed over a 96h time course. Monocyte viability was monitored by trypan blue exclusion in all experiments.

University of Cape Town

***In vitro* stimulation of MDM with purified 19kDa lipoprotein.** The effect of the addition of exogenous recombinant 19kDa lipoprotein on cytokine production by infected monocytes was explored by adding 100-400 ng/mL of mycobacterium-derived 19kDa antigen to the MDM, prior to infection with *M. smegmatis*. The effects of native 19kDa antigen purified from H37Rv (Dr. J. Belisle, Colorado State University, Fort Collins, CO), from recombinant *M. vaccae* 19kDa culture filtrates (rCF) or from recombinant *M.vaccae* 19kDa whole-cell lysate (rWCL; purified from the sonicated bacterial pellet) (Dr. D. B. Young, Imperial College, London, UK)(Abou-Zeid, Gares, Inwald, *et al.*, 1997) were compared. Since only mycobacterium-derived 19kDa protein has undergone post-translational acylation and glycosylation (Abou-Zeid, Gares, Inwald, *et al.*, 1997; Prestidge, Grandison, Chuk, *et al.*, 1995), recombinant 19kDa antigen from mycobacterial and not from an *E. coli* source was used.

2.1.4 Analysis of the host response to TB *in vitro*

Morphology of infected monocytes. Monocytes were cultured on Thermanox cover slips (Nunc, Naperville, IL) for 6 days, and then infected with *M. smegmatis* or *M. smegmatis* 19kDa at an MOI of 5:1. Five hours post infection, the cells were harvested and fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate and 0.1 M dextrose (pH 7.4), post-fixed with 1% osmium tetroxide, stained with 2% uranyl acetate, dehydrated in graded ethanol solutions, and embedded in Epon 812. Thin sections were post-stained with lead citrate and examined with a Jeol JEM 100cx transmission electron microscope.

Enumeration of intracellular mycobacteria. The number of viable organisms in mycobacterium-infected monocytes and MDM was determined. An aliquot of the culture supernatant was removed for cytokine analysis, and the contents of each well expanded with PBS containing 0.25% Tween 80 (Sigma, St Louis, MO) to a volume of 1 ml, probe

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sonicated for 4 x 5s (3W), and harvested for colony counts. Ten fold serial dilutions of the mycobacterial suspension were plated on 7H11 agar containing glucose (2%) and hygromycin B (50μg/ml), incubated at 37°C, and the number of colony-forming units (CFU) read after 48-72h (*M. smegmatis*), or plated on 7H10 agar containing 10% OADC and read after 2-3 weeks (MTB).

Cytokine analysis. TNF-α, IL-6, IL-12 (p70 and p40) and IL-10 concentrations were measured in the supernatants of infected (and uninfected) macrophages by ELISA (Endogen, Woburn, MA, USA). Briefly, 96 well plates coated with monoclonal antibodies against the relevant cytokine were incubated with 50 μl of culture supernatant and primary antibody for 2h. Following a thorough wash, the wells were incubated with a secondary, fluorochrome labeled monoclonal antibody for 30 minutes. Excess antibody was removed and TMB substrate added to convert the fluorochrome into a blue dye. Another chemical (stop solution) halted this reaction, and rendered the solution yellow proportionate to the amount of cytokine that was originally present in the supernatant. The intensity of the yellow discoloration was measured as an optical density (OD) in an ELISA reader. Cytokine concentrations were calculated from the OD readings plotted against a series of standards. Samples and standards were run in duplicates or triplicates.

Flow cytometric analysis (FACS). Uninfected, *M. smegmatis* and *M. smegmatis* 19kDa infected MDM were harvested 24, 48 and 72h post-infection, using ice cold PBS with 0.02% EDTA (pH 7.2), and washed in FACS buffer (PBS containing 3% fetal calf serum (FCS) (Gemini Bio Products, Calabasas, CA) and 0.1% sodium azide). Macrophages were labeled on ice for 30 minutes with one of the following monoclonal antibodies: phycoerythrin (PE)-anti-CD14, PE-anti-CD80 (B7.1), PE-anti-HLA-DR (Becton Dickinson, San Jose, CA), PE-anti-CD86 (B7.2) or fluorescein isothiocyanate (FITC)-anti-CD40 (Pharmingen, San Diego, CA), washed in FACS buffer, and fixed with 1% paraformaldehyde in PBS.

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T-Lymphocyte proliferation assays. T-lymphocytes ($1x10^5$) from PPD positive donors, enriched by E-rosetting and depleted of monocytes by passage through a nylon wool fiber column (Polysciences Inc., Warrington, PA), were added to *M. smegmatis* or *M. smegmatis* 19kDa infected MDM. Proliferative responses were assayed by [^{3}H]-thymidine (NEN Research Products, Boston, MA) incorporation during the last 6 or 18h of 72-120h cultures. DNA was harvested onto fiber mats with an automatic cell harvester (Skatron Instruments Inc., Sterling, VA), and [^{3}H]-thymidine incorporation measured with a β plate liquid scintillation counter (model LKB 1205; Wallac, Gaithersburg, MD).

2.1.5 Analysis of the human immune response to MTB *in vivo*

Plasma. Cytokine levels were measured in undiluted plasma from TB patients by ELISA, using commercially available cytokine kits (Endogen, Woburn, MA, USA for IL-12, IL-6 and IL-10 and Biosource, Nivelles, Belgium for TNF-α).

Processing of lung tissues. The resected lungs were immediately transferred to the BSL-3 facility for pathological dissection. TB lesions were identified macroscopically; approximately 0.5g of tissue from each lesion were snap frozen in liquid nitrogen for mRNA analysis; another 0.5g from each lesion were homogenized and subjected to prolonged culture (up to 1 year) in mycobacterial growth medium (MGIT, Becton Dickinson, Sparks, MD) and on Lowenstein-Jensen (LJ) slants. The remainder of the lung was immersed in formalin and prepared for microscopic analysis of the selected lesions.

Histology and immune histology of lung tissue. Tissue sections (2μm) were stained with haematoxylin and eosin (H&E) or with carbolfuchsin (Ziehl-Neelsen). The number of mycobacteria in each area of the section was quantified using a 40x objective, as none, scant (individual bacilli found in each granuloma), moderate (1-10 bacilli in each granuloma), or numerous (clumps of more than 10 bacilli found in each granuloma). For

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immunohistology, additional sections were collected on charged glass slides (Superfrost/Plus, Fisher Scientific, Pittsburgh, PA), de-paraffinized, rehydrated in alcohol and submitted to antigen retrieval by boiling in 0.1 M citrate buffer pH 7.0 (CD3, CD8, CD68 or TIA-1) or in 0.1 M EDTA buffer pH 7.0 (CD4) for 20 min using a microwave oven. The phenotype of the cellular infiltrate in the tissue sections was determined using monoclonal antibodies against a pan T-cell marker CD3 (Ventana Tucson, AZ) at a dilution of 1:100, against the T-cell subsets CD4 (Nova Castra, New Castle upon Tyne, UK) at a dilution of 1:20 and CD8 (Dako, Carpinteria, CA) at a dilution of 1:20, and against the cytotoxic cell marker TIA-1 (Coulter Inc, Hialeah, FL) at a dilution of 1:300. KP1 (CD68) antibody (Dako) was used as a marker of histiocytes/macrophages (dilution 1:500). Reactions were carried out in an automated immunostainer (Ventana) using an immunoperoxidase-diaminobenzidene kit (Ventana) (Siddiqui, Moreira, Negesse, *et al.*, 2002).

Real-time quantitative polymerase chain reaction (PCR) (TaqMan). To evaluate the expression level of the IL-2, IL-12, IFN-γ and iNOS genes in the lung lesions, quantitative RT-PCR was performed with real-time TaqMan technology (Sequence Detection System model 7700, Perkin Elmer Wellesly, MA). Gene specific primers and 6-carboxy-fluorescein (6-FAM) probes were designed using Primer Express software and synthesized by Perkin Elmer. RT-PCR was carried out with the TaqMan RT-PCR core Reagents kit (PE Applied Biosystems, Foster City, CA). Briefly, 5ng of RNA extracted from the lung tissues was reverse transcribed and amplified in TaqMan EZ buffer containing 300 μmol/L dATP, dCTP, and dGTP, 600 μmol/L dUTP, 3 mmol/L manganese acetate, 0.1U/μL DNA polymerase, 0.01 U/μL AmpErase uracil N-glycosylase (UNG), 200 nmol/L of each primer and 100 nmol/L of each detection probe. Thermal cycling conditions were as follows: 2' at 50°C (initial step), 30' at 60°C (reverse transcription), 5' at 95°C (deactivation of UNG), 40 cycles of 15" at 95°C for (denaturation), and 1' at 60°C (annealing and extension).

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Sequence-specific amplification was detected as 6-FAM fluorescence exceeding the threshold limit (10 times the standard deviation of the baseline) during the amplification cycle. Gene-specific mRNA was quantified using standard curves established from PCR amplifications of serial dilutions of known mRNA levels (serial 10 fold dilutions with 10 to 3.2 ng per reaction well). Amplification of the gene for human acidic ribosomal protein (hARP) was performed on all samples tested to control for variability in the amount of RNA. The quantity of cDNA for each experimental gene was normalized to the amount of hARP in each sample. Levels of gene-specific messages were graphed as normalized message units as determined from the standard curve. A no-template control was included in each amplification reaction to control for contaminating templates. For valid sample analysis the fluorescent intensity in the no-template control was required to be zero.

University of Cape Town

2.1.6 Statistical analysis

The two-tailed Student's *T*-test was used to compare CFU counts, cytokine production and replication times (chapter 3); CFU counts, cytokine concentrations and co-stimulatory molecule expression (chapter 4); number of zones on chest radiograph affected by TB, cytokine concentrations in plasma and culture supernatants, and replication times (chapter 5); and durations of treatment (Chapter 7). The two-tailed Mann-Whitney test was used to compare sputum bacterial loads and chest radiographic scores in chapter 7. *P* values of less than 0.05 were considered statistically significant.

MS Excel (MS Office 2000) was used to draw linear trend lines through logarithmically plotted CFU. Replication times over the 72h or 24h time periods were calculated as follows: [72xlog(2)]/[log(CFUday4)-log(CFUday1)] or [24xlog(2)]/[log(CFUday2)-log(CFUday1)].

2.2 STUDIES OF MTB INFECTED MICE AND MOUSE MACROPHAGES

2.2.1 Mouse strains used for the experiments

Eight to twelve week old C57BL/6 and 129 (wild type) specific pathogen free (SPF) mice were obtained from the University of Cape Town and the University of Medicine and Dentistry of New Jersey animal facilities, and The Jackson Laboratories, Bar Harbor, ME. In addition, 8-12 week old homozygous TNF-α-deficient (TNF-α-KO), interferon-γ (IFN-γ-KO), inducible nitric oxide synthase (iNOS-KO), TNF-α receptor 1 (p55; TNF-αR1-KO) and TNF-α receptor 2 gene disrupted mice (p75; TNF-αR2-KO) on a C57BL/6 genetic background, and IFN-γ receptor (IFN-γR-KO) deficient mice on a 129 genetic background were obtained from the above institutions and used as a source of peritoneal macrophages. The animal use and care committees at the Rockefeller University, New York, and the University of Cape Town, South Africa approved all protocols involving mice.

2.2.2 Mouse macrophages

Mice were sacrificed by CO_2 narcosis. Resting peritoneal macrophages were obtained by lavage from the peritoneal cavity (Cohn and Benson, 1965). The peritoneum was exposed, and the peritoneal cavity flushed with 4 ml of ice-cold DMEM using a heparinized syringe and a 26-gauge needle. Lavage fluid containing the macrophages was removed with a sterile glass pipette. Peritoneal cells were washed twice with DMEM, and plated in 48 well plates at a density of 10^6 macrophages per well. Monolayers were washed with warm DMEM after 3h to remove non-adherent cells, and incubated in DMEM supplemented with 10% FCS (D10) at 37°C and under 5% CO_2 for an additional 3 days prior to infection. No antibiotics were added to the culture medium at any time during the experiments.

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2.2.3 Experimental procedures

Genotyping of mice. Mouse tail specimens were digested overnight at 55°C with proteinase K (0.5mg/ml) in TRIS (50mM), EDTA (100mM), NaCl (100mM) and 1% SDS. Protein was precipitated from the digest with supersaturated (6M) NaCl and pelleted by ultra centrifugation (14000 rpm in a bench top Eppendorf centrifuge); DNA in the upper phase was precipitated with isopropanol, pelleted by ultra centrifugation, washed in absolute ethanol, and dissolved in 500 μl of water.

Three primers (Ransom Hill Bioscience, Ramona, CA, USA) were used in each PCR: oIMR449 and oIMR450 to amplify a 300 base pair (bp) product from the TNF-α1R-KO mutant allele, and oIMR449 and oIMR448 to amplify a 470 bp product from the wild type allele. Sequences of the primers used were as follows: TGT GAA AAG GGC ACC TTT ACG GC (oIMR448); GGC TGC AGT CCA CGC ACT GG (oIMR449); and ATT CGC CAA TGA CAA GAC GCT GG (oIMR450). The primers were used at a concentration of 6.25 μM. Each PCR reaction was carried out with 2μl of DNA, 5μl of 10x PCR buffer, 4μl of dNTP (2.5 mM), 2μl of each primer, 0.1μl of *Taq* DNA polymerase and 33μl H_2O. The following thermocycling conditions were used: 94°C for 3', 12 cycles of [94°C x 20", 64°C x 30" (-0.5°C per cycle), 72°C x 35"], 25 cycles of [94°C x 20", 58°C x 30", 72°C x 35"], and 72°C for 2'. The PCR products were separated on a 1.6% agarose gel stained with ethidium bromide using 150 V current for 45 minutes, and a 1000 bp ladder was used to estimate the band sizes.

***In vivo* infection of mice.** To examine the effect of the host immune response on mycobacterial gene expression *in vivo*, female C57Bl/6 wild type and IFN-γ-KO mice 6-8 weeks of age were infected with H37Rv using a Lovelace nebulizer (InTox Products, Moriarty, NM) to deliver 500-1,000 bacterial CFU to the lungs (Moreira, Wang, Tsenova-Berkova, *et al.*, 1997). The bacterial load at selected timepoints was evaluated by

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homogenizing the left lung in PBS containing 0.05% Tween 80 and plating 10-fold serial dilutions of lung homogenate on 7H10 agar supplemented with 10% OADC and 0.5% glycerol, while the lung was used mycobacterial mRNA analysis.

***In vitro* infection of murine peritoneal macrophages cultures.** H37Ra and H37Rv were suspended in D10 medium. On day 3, mouse macrophages were washed and infected with H37Ra and H37Rv suspended in 500μl of D10, at an MOI of 3:1 (cytokine analysis: 12 h time point) or 1:3 (growth curves: days 1, 2 and 4). Phagocytosis of mycobacteria was complete with no extracellular organisms remaining by 6h post infection. Cultures were therefore not washed after the addition of mycobacteria. Macrophage viability was checked throughout the experiments by trypan blue exclusion.

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2.2.4 Analysis of mouse macrophages infected with MTB *in vitro*

Mycobacterial replication. The number of viable organisms in infected macrophages was determined after removal of an aliquot of the culture supernatants for cytokine analysis. The contents of each well was expanded with PBS containing 0.25% Tween 80 to a volume of 1 ml, probe sonicated for 4 x 5s (3W), and harvested for CFU counts. Ten fold serial dilutions of the mycobacterial suspension were plated on 7H10 agar containing 10% OADC and read after 2-3 weeks.

TNF-α production. Production of TNF-α by mouse macrophages in response to infection with H37Ra or H37Rv, as well as TNF-α production by uninfected macrophages, was measured in the culture supernatants by ELISA (Endogen, Woburn, MA, USA).

2.2.5 Statistical analysis

Replication times for H37Ra and H37Rv in the macrophages from different mouse strains were calculated from day 1 to day 2 and from day 2 to day 4, using the formulae as used for

the calculation of mycobacterial replication times in human monocytes. Replication times were compared using the two-tailed Student's *T*-test. *P* values <0.05 were considered statistically significant.

2.3 CULTURE AND ANALYSIS OF MYCOBACTERIA

2.3.1 Mycobacterial strains used for the experiments

The following MTB strains were used: H37Ra (Trudeau Institute, Saranac Lake, NY) and H37Rv (Pasteur Institute, Paris), CDC1551 (Dr. T. M. Shinnick, CDC, Atlanta, GA) and HN878 (Dr. J. Musser, NIH, Hamilton, MT). H37Ra and H37Rv were derived after serial passage of H37, a clinical MTB isolate dating back from 1905. These strains have been noted for their differential ability to cause disease in mice and guinea pigs (North and Izzo, 1993; Steenken and Gardner, 1946). The clinical isolates CDC1551 and HN878 were responsible for outbreaks of tuberculosis in the Mid-West and Houston, USA, respectively during the 1990's (Sreevatsan, Pan, Stockbauer, *et al.*, 1997; Valway, Sanchez, Shinnick, *et al.*, 1998), and have since been noted for their differential ability to induce pro-inflammatory cytokines (Manca, Tsenova, Barry, *et al.*, 1999; Manca, Tsenova, Bergtold, *et al.*, 2001).

We also used recombinant *M. smegmatis* $mc^2/1$-2c strains (Dr D. B. Young, Imperial College, London, UK) containing hygromycin-resistance determining pSMT3-based plasmids encoding the 19kDa lipoprotein of MTB. In addition, *M. smegmatis* carrying plasmids in which the 19kDa gene had been modified by site-directed mutagenesis (pSMT3-19NOG encoding non-O-glycosylated 19kDa; pSMT3-19NS encoding non-secreted 19kDa; and pSMT3-19NA encoding a non-acylated form of the 19kDa) were used (Garbe, Barathi, Barnini, *et al.*, 1994; Herrmann, O'Gaora, Gallagher, *et al.*, 1996; Neyrolles, Gould, Gares, *et al.*, 2001). Mutant proteins were expressed at levels comparable to those of the wild-type

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protein, and *M. smegmatis* (vector), containing the plasmid vector only, was used as a control in all infection experiments.

2.3.2 Culture of mycobacteria

MTB was grown in lipopolysaccharide (LPS)-free 7H9 broth (Difco, Detroit, MI) enriched with albumin, dextrose, catalase (ADC) and glycerol to mid-log phase, in the presence of 0.25% Tween 80, and recombinant *M. smegmatis* was grown in 7H9 broth enriched with glycerol and glucose (2g/100ml), in the presence of hygromycin B (50μg/ml) (Boehringer Mannheim, Indianapolis, IN). All cultures were sonicated at 3W for 4x5s (Fisher Scientific dismembrator 60, Pittsburgh, PA), enumerated and stored at -70°C until use.

Sputum mycobacterial culture, as well as culture of lung tissue homogenate, was performed in an automated mycobacterial culture system (MGIT). LJ slants were inoculated with 500 μl of mycobacterial suspension from the MGIT. Mycobacteria were harvested for DNA fingerprinting approximately 4-6 weeks later.

University of Cape Town

2.3.3 Restriction fragment length polymorphism (RFLP)

Isolation of DNA from MTB isolates. MTB was harvested from LJ slopes and heat-killed for 30' at 80°C. Bacteria were digested with proteinase K (10 mg/ml) and 10% SDS on a 60°C thermo-shaker. Preheated 5M NaCl and 10% hexadecyl trimethyl ammonium bromide (CTAB) were added to the digest for the last 15'. The suspension was briefly frozen at -70°C before adding chloroform/isoamyl alcohol (24:1). Tubes were inverted by hand and centrifuged at 14,000 rpm for 10 minutes. DNA was extracted from the aqueous phase with isopropanol, washed in 80% ethanol, dried in the speed vacuum for 5-10', and dissolved in 50μl H_20. Five μl of DNA was mixed with 1μl of dye and run on a 1% agarose gel (100 V, 1h) for visual estimation of DNA quality and concentration.

Restriction endonuclease digestion and Southern transfer of DNA. DNA (2-22μl) was cut with restriction endonuclease *PvuII* for 3h at 37°C, and the fragments separated on a 1% agarose gel (36V, overnight). The DNA fragments were Southern transferred onto a Hybond-N+ membrane (Amersham Pharmacia biotech, Piscataway, NJ) by flooding the membrane with different solutions (10% SSC (0.15 M Na_3Citrate + 1.5 M NaCl, pH=7); 1/100 HCl; 0.5 M NaOH+1.5 M NaCl; 0.5 M TRIS+1.5 M NaCl (pH=7); 10x SSC) while 50 cm of H_2O vacuum was applied. Membranes onto which the DNA had been transferred were air-dried for 10 minutes and twice UV irradiated (Optimal cross-link).

Hybridization and detection assay. The IS*6110* probe was denatured in boiling water, labeled with horseradish peroxidase (HRP) and cross-linked with glutaraldehyde. Labeled IS*6110* probe was hybridized to the DNA on the membrane in a roller bottle at 42°C overnight. Excess hybridization solution was removed with primary (360 g urea, 4 g SDS, and 25 ml 20x SSC in 1000 ml dH_2O) and secondary (2x SSC) wash buffers. Detection solutions (enhanced chemiluminescence, Amersham Pharmacia biotech) converted peroxidase-generated H_2O_2 (at the IS*6110* sites) to light by oxidation of luminol. The IS*6110* insertion patterns thus visualized were analyzed with Whole Band Analyzer software, version 3.4 (BioImage). MTB isolates that belonged to the W-Beijing family (Bifani, Mathema, Kurepina, *et al.*, 2002) were identified on the basis of high IS*6110* copy numbers, the presence of characteristic IS*6110* insertions in the origin of replication region, and spoligotype S00034 (Bifani, Mathema, Kurepina, *et al.*, 2002; Kurepina, Sreevatsan, Plikaytis, *et al.*, 1998).

2.3.4 Mycobacterial gene expression

Human lung tissue and entire right lungs of infected mice were flash frozen in liquid nitrogen and stored at -80°C until use. Lung tissue was homogenized in TRI reagent (Molecular

University of Cape Town

Research Center, Cincinnati, OH) with a Polytron homogenizer (Pro Scientific, Oxford, CT). Homogenates were centrifuged at 3000x*g* for 5' at 4°C to collect bacteria. Bacterial pellets were resuspended in 0.5 ml TRI containing 1% polyacryl carrier (Molecular Research Center), transferred to srewcap tubes containing 0.25 ml of zirconia/silica beads (Biospec Products, Bartlesville, OK), and broken using a bead-beater homogenizer (Biopsec Products). RNA was isolated using TRI Reagent and treated with DNase I (DNA-free kit, Ambion, Austin, TX) to eliminate contaminating genomic DNA.

Design of primers and molecular beacons. PCR primers (Genosys, The Woodlands, TX) were designed to amplify internal gene fragments of 50-150 bp. Reverse transcription (RT) primers were located as close as possible to the reverse PCR primers. Primers with comparable melting temperatures (60-66°C) were designed using PRIMER 3 software. Molecular beacons (Eurogentec, Philadelphia, PA) were designed and tested in PCR reactions using 10-fold serial dilutions of H37Rv chromosomal DNA as template.

Reverse transcription and real-time PCR with molecular beacons. RT reactions, typically containing 7 gene-specific primers, were carried out using the Klenow fragment of DNA polymerase from *Carboxythermus hydrogenoformans* (*C. therm* kit, Roche, Indianapolis, IN), as follows: annealing step 95°C (1'), 65°C (3'), 57°C (3'); polymerisation step 60°C (30'); RT inactivation step 95°C (5'). Real-time PCR reactions typically contained 2 μl of the RT mixes and were run on an ABI7900 real-time PCR machine and analyzed according to the manufacturer's instructions. The PCR conditions used were as follows: Amplitaq Gold activation step 95°C (10'); 15 cycles of 95°C (30"), 65°C (-0.5°C step down each cycle, 30"), 72°C (30"); 25 cycles of 95°C (30"), 57°C (30"), 72°C (30").

Calculation of induction ratios (IR). RNA samples were prepared from bacteria grown *in vitro* (to rule out potential strain specific quantitative differences with respect to the ability to express the genes studied) and *in vivo*. For each sample, *sigA* mRNA and target

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mRNAs were reversed-transcribed together in a single reaction, and the resulting cDNAs were quantified in parallel by real-time PCR with molecular beacons. The target cDNA was internally calibrated to the *sigA* cDNA in the same sample, and expressed as the induction ratio (IR): [Target mRNA]/[*sigA* mRNA]. IR>1 suggests that expression of the target gene was induced *in vivo*; an IR<1 reflects repression *in vivo*.

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Chapter 3

Tumor necrosis factor-alpha mediated growth restriction of *Mycobacterium tuberculosis* H37Ra is observed in mouse macrophages but not in human monocytes *in vitro*

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3.1 ABSTRACT

M. tuberculosis strains H37Ra and H37Rv are distinguished by their differential ability to cause progressive disease in mice and guinea pigs. To understand the difference in virulence of the two strains, we evaluated their growth and tumor necrosis factor-alpha (TNF-α) producing capacity in cultures of human monocytes and peritoneal macrophages from wild type and gene-disrupted mice. Similar growth of H37Ra and H37Rv was observed in human monocytes. Monocyte maturation, but not cyto-adherence, resulted in growth restriction of both strains. In wild type mouse macrophages, H37Rv grew logarithmically while growth of H37Ra slowed down significantly by 48h post infection. Similar differences in growth of H37Rv versus H37Ra were observed in macrophages obtained from mice with disruptions in the genes encoding inducible nitric oxide synthase (iNOS), interferon-gamma (IFN-γ) or the IFN-γ receptor. In contrast, mouse macrophages obtained from mice deficient in TNF-α were unable to control the growth of H37Ra. In both mouse and human macrophages, infection with H37Rv induced higher levels of TNF-α production than infection with H37Ra. Our observations suggest that growth restriction of H37Ra in mouse macrophages is mediated through a TNF-α dependent, iNOS and IFN-γ independent pathway that may be absent in human monocyte-derived macrophages.

3.2 INTRODUCTION

Mycobacterium tuberculosis H37Ra and H37Rv could be seen as the paradigm strains to study the effects of related mycobacterial strains with diverse biological properties on the interaction between host cell and pathogen. The parental strain of H37Ra and H37Rv, H37, was isolated from sputum of a 19 years old male with chronic pulmonary TB in 1905. Multiple passages on artificial media did not appear to affect the virulence of H37 until 1922, when it was noted that some H37 strains had lost their ability to cause progressive disease in

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guinea pigs. Continued cultivation of these variant strains on potato-bile and fresh egg medium yielded an almost avirulent strain (Steenken and Gardner, 1946). The strains derived from H37 that had remained virulent became known as H37Rv, and the attenuated variants as H37Ra. To date, the genetic mutations or deletions that resulted in attenuation of H37Ra remain unknown (Brosch, Philipp, Stavropoulos, *et al.*, 1999; Pascopella, Collins, Martin, *et al.*, 1994).

The differential ability of H37Ra and H37Rv to cause disease in guinea pigs and mice has been confirmed in several studies (Middlebrook, Dubos and Pierce, 1947; North and Izzo, 1993; Pierce, Dubos and Middlebrook, 1947; Sever and Youmans, 1957). These strains have been used extensively since to study mycobacterial growth, survival and virulence. In immunocompetent mice, H37Rv was shown to cause progressive disease in the lungs, while the growth of H37Ra was controlled. In T-lymphocyte deficient mice (SCID mice), the growth of H37Ra was, at least in part, controlled by a glucocorticosteroid-sensitive mycobacteriostatic pathway within macrophages (North and Izzo, 1993). Growth of H37Ra was also controlled by aerosol infected mice deficient in iNOS and/or phagocyte oxidase (Phox); mycobacteriostasis of H37Ra *in vivo* therefore appears to result from an iNOS independent mechanism (Jung, LaCourse, Ryan, *et al.*, 2002).

Infection of macrophages *in vitro* is a useful model to study the growth and control of growth of *M. tuberculosis* in host cells. The relative virulence of H37Ra and H37Rv is retained when these strains are used to infect murine or guinea pig peritoneal macrophages. In these cells, H37Rv grows logarithmically until the host cell monolayer is destroyed by the heavy bacterial burden. In contrast, H37Ra fails to grow in these cells (Falcone, Bassey, Toniolo, *et al.*, 1994; Paul, Laochumroonvorapong and Kaplan, 1996; Suter, 1952). If the murine macrophages are activated by treatment with the cytokines IFN-γ and TNF-α, or by

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exposure to LPS, then the growth of H37Rv is also controlled (Chan, Xing, Magliozzo, *et al.*, 1992; Denis, 1991; Flesch and Kaufmann, 1987; Rook, Steele, Ainsworth, *et al.*, 1986).

A number of recent studies have shown that human monocyte-derived macrophages (MDM) are permissive for the growth of H37Rv and other mycobacterial strains. For example, we have reported that H37Ra and H37Rv grow similarly in human macrophages *in vitro* (Laochumroonvorapong, Paul, Manca, *et al.*, 1997; Paul, Laochumroonvorapong and Kaplan, 1996). Growth of H37Ra was also demonstrated in human monocytes and alveolar macrophages (Hirsch, Ellner, Russell, *et al.*, 1994). However, other investigators have found that while H37Rv grows in human macrophages, H37Ra does not (Engele, Stobetael, Castiglione, *et al.*, 2002; Silver, Li and Ellner, 1998), or that H37Ra grows significantly slower than H37Rv (Zhang, Gong, Lin, *et al.*, 1998). The mechanism underlying the differences in the ability of murine and human macrophages to regulate the growth of *M. tuberculosis* strains is not understood.

Apoptosis has been put forward as one of the host defense mechanisms that contributes to mycobacteriostasis (Molloy, Laochumroonvorapong and Kaplan, 1994; Oddo, Renno, Attinger, *et al.*, 1998). At high MOI's, MTB induces apoptosis of infected macrophages by a TNF-α mediated mechanism (Keane, Balcewicz-Sablinska, Remold, *et al.*, 1997). FASL or TNF-α mediated apoptosis of infected human MDM results in reduced viability of intracellular H37Ra and H37Rv (Oddo, Renno, Attinger, *et al.*, 1998). Infection of human macrophages with H37Ra has been shown to result in more host cell apoptosis than infection with H37Rv (Keane, Remold and Kornfeld, 2000). Apoptosis of H37Rv infected macrophages may be prevented by upregulation of Mcl-1 (an anti-apoptotic member of the Bcl-2 family) by infected cells (Sly, Hingley-Wilson, Reiner, *et al.*, 2003), or by increased IL-10 production and shedding of soluble TNF-α receptor II, resulting in TNF-α neutralization (Balcewicz-Sablinska, Keane, Kornfeld, *et al.*, 1998). Differential rates of

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induction of host cell apoptosis may be one of the mechanisms to explain the different growth rates of H37Ra and H37Rv in human macrophages. However, no apoptosis was observed in macrophages infected with H37Ra or H37Rv at low inoculum, yet these cells were perfectly capable of restricting the growth of H37Ra but not H37Rv (Engele, Stobetael, Castiglione, *et al.*, 2002). It is therefore likely that other mycobacteriostatic mechanisms exist.

H37Ra and H37Rv are easily distinguished by IS*6110* restriction fragment length polymorphism (RFLP). Propagation of H37Ra and H37Rv since the 1920's has resulted in polymorphic variants of these strains that have circulated widely among laboratories (Bifani, Moghazeh, Shopsin, *et al.*, 2000). Few studies have confirmed or provided the genotypic identity of the H37Ra and H37Rv strains used. It is possible that subtle genotypic polymorphism between strains has given rise to biological differences among different isolates of H37Ra and H37Rv used in these studies.

In this chapter, we have investigated potential mechanism that could account for the different growth characteristics of avirulent and virulent *M. tuberculosis* in mouse and human macrophages. We performed RFLP analysis to confirm the identity of the H37Ra and H37Rv strains used in the experiments. We investigated the effect of cell adherence and maturation on the ability of human peripheral blood monocytes to restrict intracellular growth of H37Ra and H37Rv in a low-dose *in vitro* infection model. In addition, we used peritoneal macrophages from mice with disruptions in immune regulatory genes involved in macrophage activation and anti-microbial activity to study the contribution of these gene products to mycobacterial growth restriction. Finally, the ability of H37Ra and H37Rv to induce TNF-α production by human and mouse macrophages was studied by measuring the concentration of this cytokine in the culture supernatants.

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3.3 RESULTS

Confirmation of strains H37Ra and H37Rv. *M. tuberculosis* H37Ra (Trudeau Institute, Saranac Lake, NY) and H37Rv (Pasteur Institute, Paris) were used for the experiments. To first confirm that the *M. tuberculosis* strains were indeed H37Ra and H37Rv, RFLP analysis was carried out. The RFLP pattern for our laboratory strain of H37Rv was identical to a polymorphic variant of H37Rv previously characterized by Bifani *et al.* (Bifani, Moghazeh, Shopsin, *et al.*, 2000) (Figure 3-1). Our strain of H37Ra was clearly distinguishable from H37Rv, and its IS*6110* RFLP pattern indistinguishable from the H37Ra polymorphism recently described (Bifani, Moghazeh, Shopsin, *et al.*, 2000). The results presented in Figure 3-1 confirm that the two isolates used were the appropriate strains. These strains were used in experiments carried out both in New York and in Cape Town.

Growth of H37Ra and H37Rv in human monocytes and monocyte-derived macrophages *in vitro*. Human monocytes were isolated from PBMC obtained from healthy persons. Monocytes, enriched by cytoadherence, were infected with H37Ra and H37Rv at an MOI of 1:1. The ability of the *M. tuberculosis* strains to replicate within these cells was examined. No significant difference in the replication times of H37Ra and H37Rv in freshly isolated monocytes was observed (Figure 3-2A).

In monocytes infected immediately after introduction into culture, H37Ra and H37Rv showed a mean replication rate of 27.6 h and 31.9 h respectively over a 3-day period of infection. If monocytes were first cultured for 7 days and then infected, both strains grew more slowly with a generation time of 62.3 h and 56.9 h respectively (Figure 3-2A). Viability of either fresh monocytes or cultured monocytes was >90% throughout the experiments. To evaluate whether culture conditions, such as adherence to plastic, affected the growth rate of these two strains, human monocytes were cultured and infected in plastic wells (adherent) or teflon wells (non-adherent). The growth rates of H37Ra and H37Rv were similar in adherent

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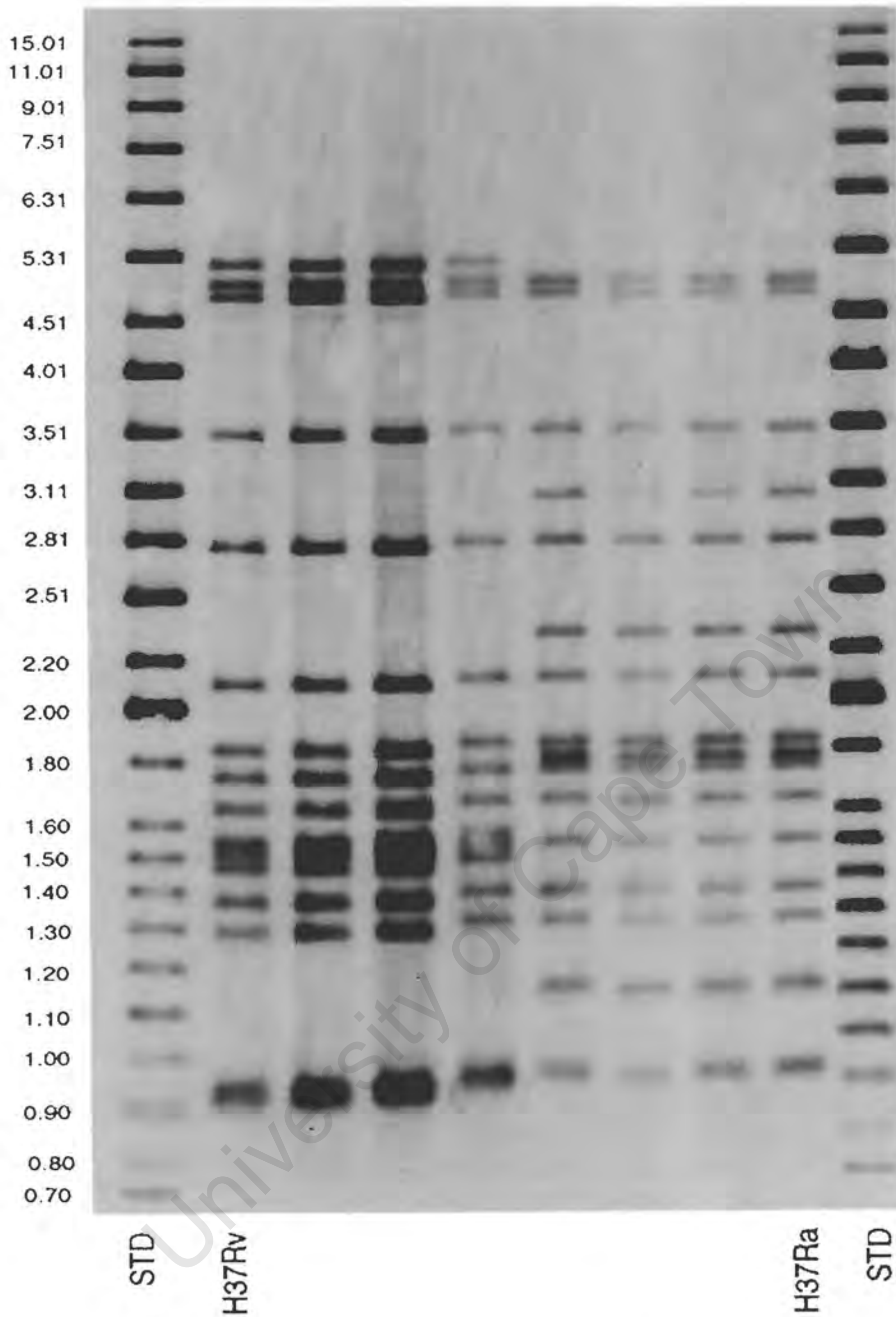

Figure 3-1 RFLP fingerprints of the *M. tuberculosis* strains H37Ra and H37Rv used for these experiments. DNA was isolated from three cultures of each strain, digested with restriction endonuclease *PvuII*, Southern hybridized with labeled IS*6110* probe, and visualized by enhanced chemiluminescence. H37Rv (lanes 3-5) and H37Ra (lanes 6-8) were identical to their respective reference strains (lanes 2 and 9 respectively).

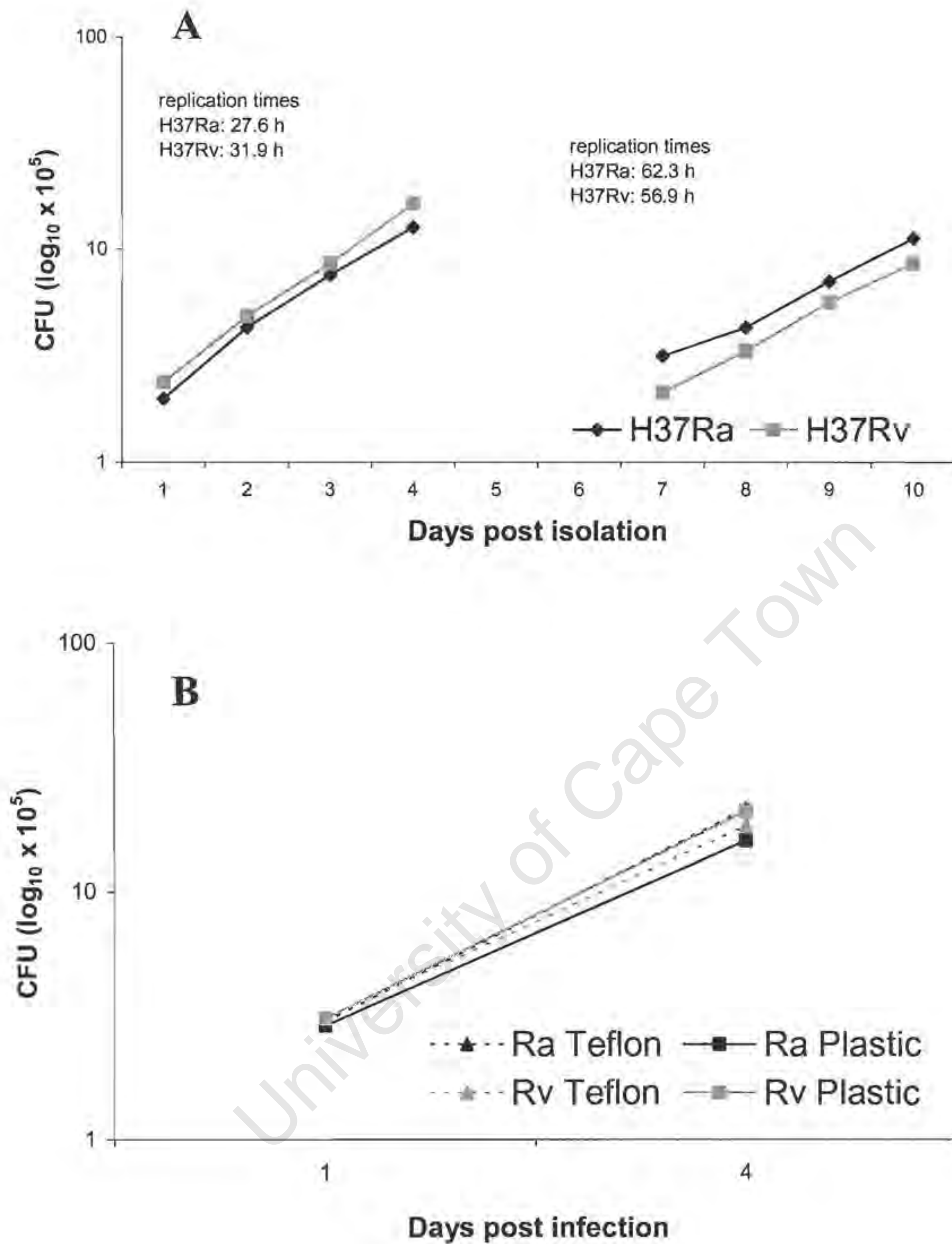

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Figure 3-2 Growth of H37Ra and H37Rv in human monocytes maintained in culture for 0 or 7 days prior to infection (A), and in human monocytes cultured in teflon (non-adherent) or plastic (adherent) culture wells (B). Representative experiments of 2-4 experiments each are shown; standard deviations between replicate culture wells were less than 10%.

and non-adherent cells. Replication times for H37Ra and H37Rv were 35 h and 34.3 h respectively in non-adherent macrophages, and 34.1 h and 36.7 h in adherent macrophages (Figure 3-2 B). During the 3 day experimental period following infection, the viability of monocytes in either culture system was >90%. Thus, whether the monocytes were adherent or non-adherent, freshly isolated and introduced into culture or cultured for 7 days prior to infection, the growth rates of H37Ra and H37Rv were similar during 3 days of infection.

Growth of H37Ra and H37Rv in mouse macrophages *in vitro*. Unstimulated mouse peritoneal macrophages were obtained by lavage from C57Bl/6 and 129 (wild-type) mice. After 3 days of culture, the cells were infected with H37Ra or H37Rv at an MOI of 1 bacillus per 3 cells, and bacillary growth was evaluated. A clear difference in the growth of the two strains was noted (Figure 3-3A, $p=0.006$ and Figure 3-3B, $p=0.04$). In macrophages from 129 mice, H37Rv demonstrated fast logarithmic growth, with a mean replication time of 21.6 h from day 1-2 and 32.1 h from day 2-4 of infection. In comparison, H37Ra initially multiplied with a mean replication time of 22.1 h from day 1-2. However, from day 2-4, the growth rate of H37Ra was significantly reduced (replication time 102.5 h). Thus, in macrophages obtained from 129 mice, from days 2-4 the numbers of H37Ra increased by only 35%, while the numbers of H37Rv increased about 300%. In macrophages from C57Bl/6 mice, H37Rv replicated somewhat faster, with a mean replication time of 17.6 h from day 1-2, and 23.8 h from days 2-4 (Figure 3-3). H37Ra showed a replication time of 23.3 h from day 1-2 and 216 h from day 2-4. Thus, in macrophages obtained from C57Bl/6 mice, the numbers of H37Ra increased from days 2-4 by 18% while H37Rv increased by 400%, a slightly greater rate of increase than that observed in macrophages obtained from 129 mice. Throughout the experimental period, the viability of the macrophage cultures remained >90%.

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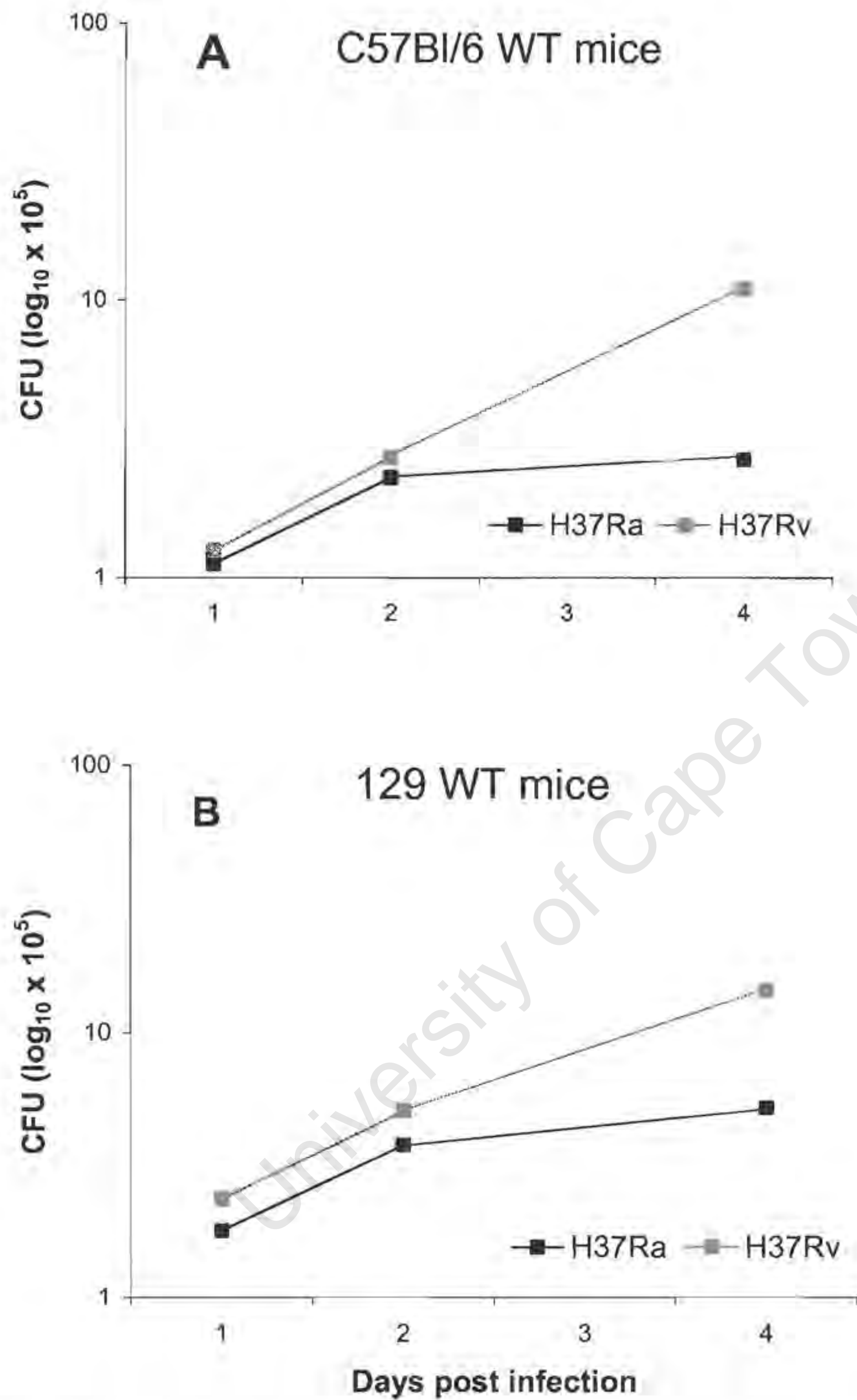

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Figure 3-3 Growth of H37Ra and H37Rv in peritoneal macrophages from C57Bl/6 (A) and 129 (B) wild-type mice. Representative experiments of 2-4 experiments each are shown; standard deviations between replicate culture wells were below 10%.

Figure 3-4 Growth rates of H37Ra (black bars) and H37Rv (gray bars) in peritoneal macrophages from selected gene-deficient and wild type mice. Growth rates from day 1 to 2 are displayed as hatched bars, from day 2 to 4 as solid bars. The ability of mouse macrophages to restrict the growth of H37Ra does not require signaling trough the IFN-γ receptor or iNOS (A). In contrast, cells deficient in TNF-α or its receptors are significantly more permissive for replication of H37Ra. Results are the mean of 3 experiments (TNF-α-KO, iNOS-KO and C57BL/6), 2 experiments (TNF-αR1-KO), or a single experiment (TNF-αR2-KO, IFN-γ-KO, and IFN-γR-KO). Error bars indicate standard deviations.

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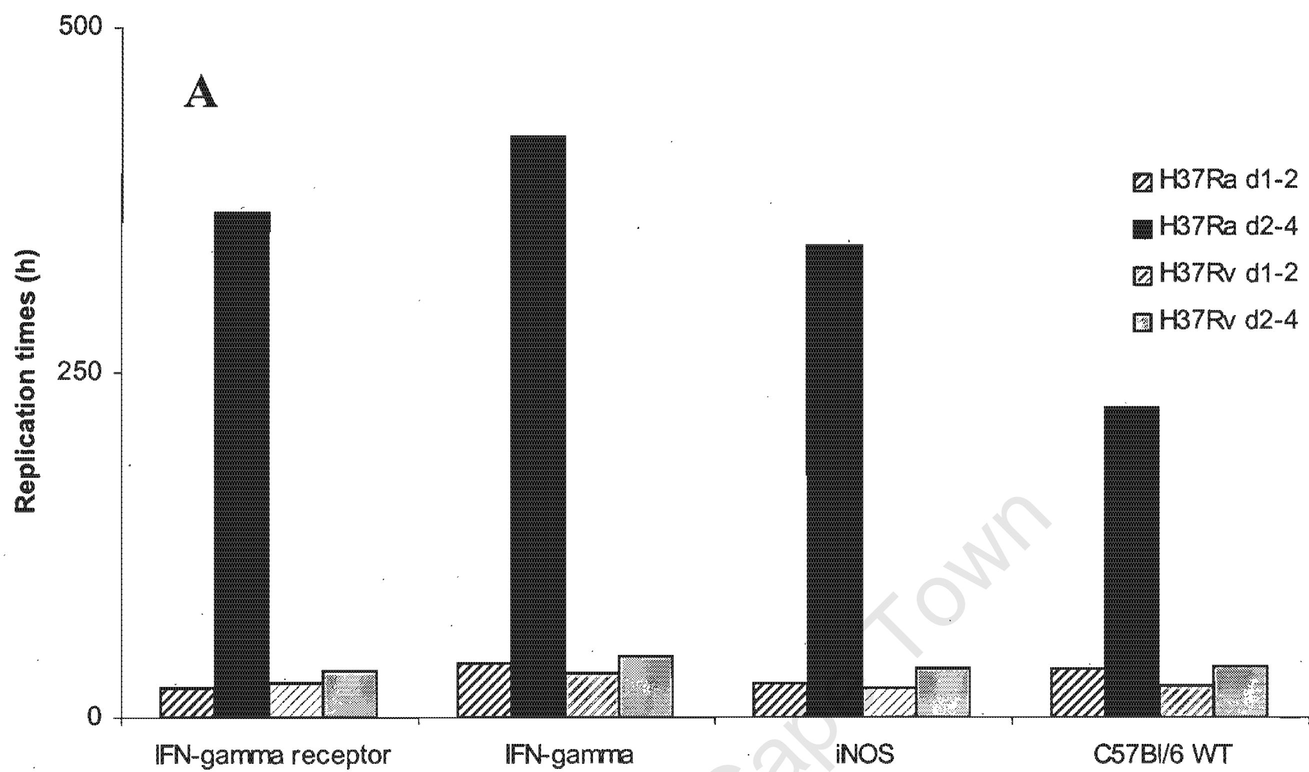

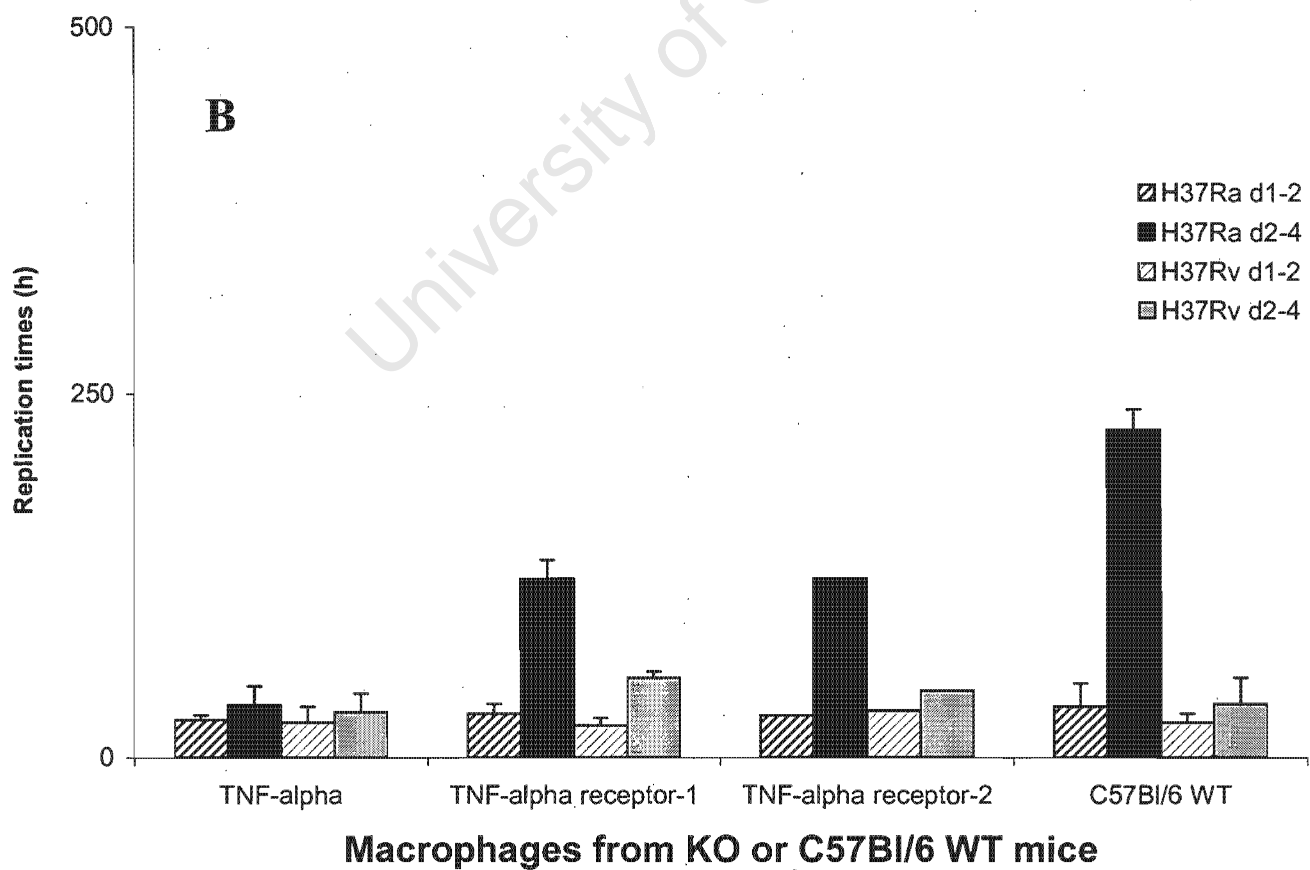

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Growth of H37Ra and H37Rv in macrophages from gene-disrupted mice. To establish whether specific immune regulatory pathways were involved in the control of growth of H37Ra, macrophages prepared from iNOS-KO, IFN-γ-KO, IFN-γR-KO, TNF-αR1-KO, TNF-αR2-KO and TNF-α-KO mice were infected with either H37Ra or H37Rv. Growth of the bacilli was evaluated over 3 days. H37Rv grew rapidly in all macrophages, both on days 1-2 and on days 2-4. While H37Ra replicated as well as H37Rv on days 1-2 in all the macrophages tested, by days 2-4, a difference in the growth of H37Ra was apparent (Figures 3-4A and 3-4B). In wild-type, iNOS-KO, IFN-γ-KO, and IFN-γR-KO macrophages, growth of H37Ra was controlled from days 2-4 (generation times of 224 h, 343 h, 422 h, 366 h, respectively). In contrast, in TNF-α-KO macrophages, control of growth of H37Ra from days 2-4 of the infection was not seen (generation time of 36 h). When macrophages obtained from TNF-αR1-KO or TNF-αR2-KO mice were infected with H37Ra, the growth rate from days 2-4 was intermediate (132 and 114 h respectively) between that seen in wild-type macrophages and that seen in TNF-α-KO macrophages (Figure 3-4B). These results suggested that TNF-α or a TNF-α induced pathway might be involved in the control of growth of H37Ra in murine macrophages.

TNF-α production in response to infection of murine and human macrophages with H37Ra or H37Rv. To determine the effect of infection with H37Ra or H37Rv on TNF-α production, wild-type murine macrophages were infected at an MOI of 3:1. At 12 h post infection, H37Rv induced significantly higher levels of TNF-α than H37Ra ($p<0.0001$). H37Rv also induced higher levels of TNF-α production in macrophages from IFN-γ-KO and TNF-αR1-KO mice, compared to H37Ra (Figure 3-5A). In general, levels of TNF-α induced by infection of TNF-αR1-KO and IFN-γ-KO macrophages with either *M. tuberculosis* strain were much lower than those induced in wild-type macrophages. Of course, no TNF-α was

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produced in TNF-α-KO macrophages following infection with either strain. Infection of human monocyte-derived macrophages with either strain induced efficient TNF-α production (Figure 3-5B). Again, H37Rv infection was associated with significantly higher levels of TNF-α (p=0.01) at all time points. In human and mouse macrophages therefore, growth rates of H37Ra and H37Rv were unrelated to the absolute concentration of TNF-α induced by the infection.

3.4 DISCUSSION

We have shown that growth of *M. tuberculosis* H37Ra in mouse macrophages is restricted by a TNF-α dependent, iNOS and IFN-γ independent mechanism. We observed that incomplete ablation of TNF-α signaling, as occurred in mouse macrophages deficient for either the p55 or p75 TNF-α receptors, resulted in partial growth suppression of H37Ra. Several lines of evidence support the presence in mouse macrophages of a mycobacteriostatic pathway mediated through TNF-α. Recent studies demonstrated that growth of *M. bovis BCG* in mouse macrophages is controlled through both iNOS dependent and iNOS independent mechanisms (Bekker, Freeman, Murray, *et al.*, 2001). TNF-α was shown to stimulate macrophages to control the growth of intracellular *M. bovis BCG* in macrophages from iNOS-KO and TNF-α-KO mice, and in macrophages from wild-type mice when iNOS activity was chemically blocked. Similarly, reduced growth of *M. avium* was observed when TNF-α was added to infected mouse macrophage cultures (Appelberg and Orme, 1993; Furney, Skinner, Roberts, *et al.*, 1992), whereas neutralization of TNF-α resulted in enhancement of growth of the bacilli (Sarmento and Appelberg, 1995). Like H37Ra in our experiments, the TNF-α mediated growth restriction of *M. avium* was independent of iNOS or the production of reactive nitrogen intermediates (RNI) (Appelberg and Orme, 1993;

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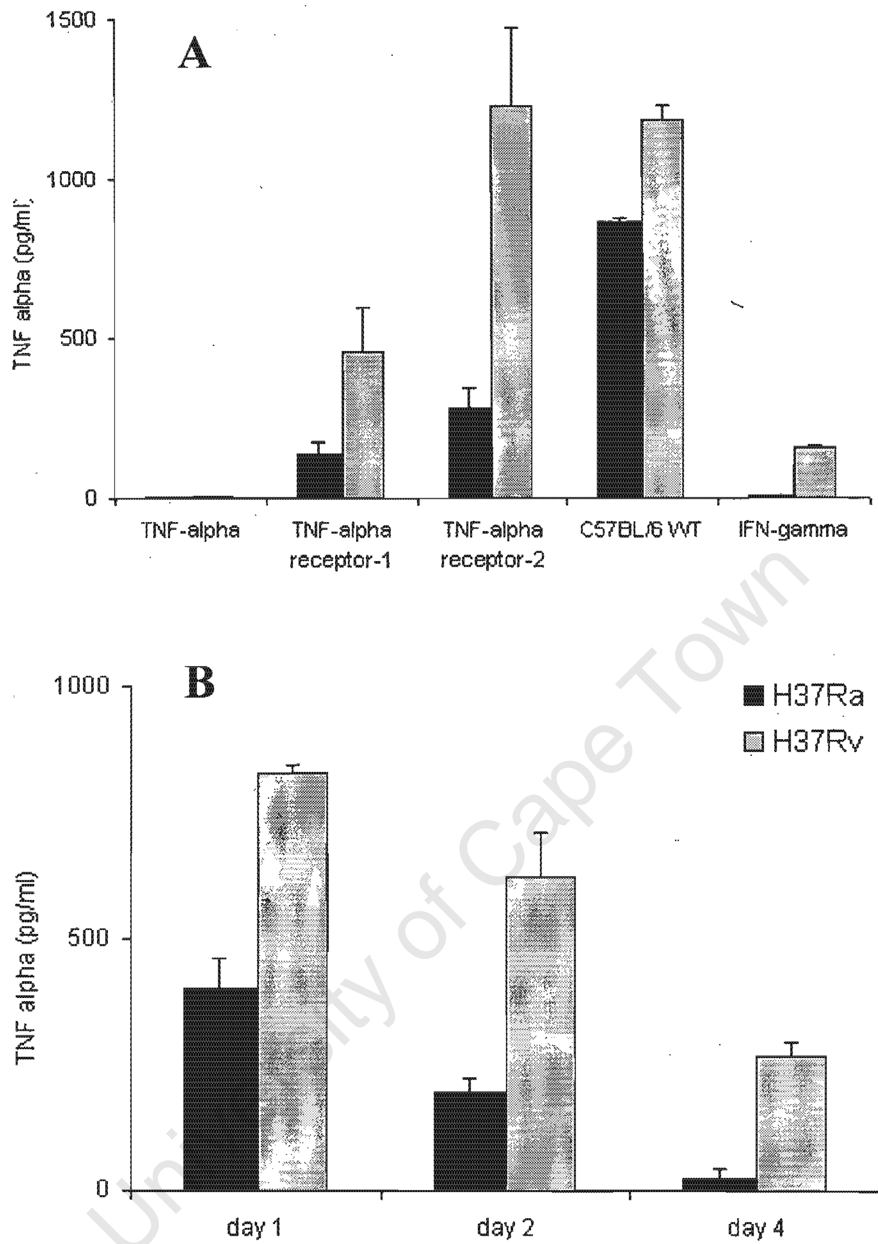

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Figure 3-5 TNF-α production by infected peritoneal murine macrophages (A) and human monocytes (B). Supernatants were collected 12 hours after infection of murine macrophages with H37Ra (black bars) or H37Rv (open bars), or 1 to 3 days after infection of human monocytes. At each time point, the number of viable organisms recovered from infected macrophages was similar for H37Ra and H37Rv. TNF-α levels were measured by ELISA. No TNF-α was detected in uninfected macrophage cultures.

Gomes, Florido, Pais, *et al.*, 1999). In addition, neutralization of TNF-α has been reported to enhance growth of H37Ra in mouse macrophages *in vitro* (Appelberg, Sarmento and Castro, 1995). Taken together these observations show that mouse macrophages are capable of restricting mycobacterial growth through a TNF-α dependent, iNOS independent pathway.

In the mouse, TNF-α is the ligand for at least 2 distinct receptors on the surface of the macrophage (p55 or TNF-α receptor 1 and p75 or TNF-α receptor 2) (Erickson, de Sauvage, Kikly, *et al.*, 1994; Pfeffer, Matsuyama, Kundig, *et al.*, 1993). TNF-αR1 deficient mice are highly susceptible to intracellular pathogens including *M. tuberculosis*, whereas TNF-αR2 deficient mice display an increased susceptibility to *Listeria monocytogenes*, but their resistance to *M. tuberculosis* is unknown (Erickson, de Sauvage, Kikly, *et al.*, 1994; Flynn, Goldstein, Chan, *et al.*, 1995; Pfeffer, Matsuyama, Kundig, *et al.*, 1993). We observed a similar degree of growth inhibition of H37Ra in macrophages from TNF-αR1-KO and TNF-αR2-KO mice. Incomplete abrogation of TNF-α signaling may explain the partial growth inhibition of H37Ra in these cells. Similar growth rates in macrophages deficient in p55 or p75 TNF-α receptor might imply that the pathway of mycobacterial growth restriction is not dependent on NF-κB activation, since only TNF-αR1 is reported to activate this transcription factor (Kalb, Bluethmann, Moore, *et al.*, 1996; Pfeffer, Matsuyama, Kundig, *et al.*, 1993).

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In contrast to the differential growth of H37Ra and H37Rv in murine macrophages, similar rates of replication were observed in human monocytes. Several differences between murine and human macrophages with respect to restricting mycobacterial replication have been reported. *M. bovis BCG* is unable to replicate in unstimulated murine peritoneal macrophages (Bekker, Freeman, Murray, *et al.*, 2001; Falcone, Bassey, Toniolo, *et al.*, 1994), whereas human monocytes were found to be permissive for the growth of this organism (Laochumroonvorapong, Paul, Manca, *et al.*, 1997; Molloy, Meyn, Smith, *et al.*, 1993; Silver, Li and Ellner, 1998). Also, murine and human macrophages differ in their response to

activation by IFN-γ. While IFN-γ treated murine macrophages display enhanced mycobacteriostatic activity against *M. tuberculosis* (Chan, Xing, Magliozzo, *et al.*, 1992; Denis, 1991; Flesch and Kaufmann, 1987; Sato, Akaki and Tomioka, 1998), IFN-γ stimulation of human monocytes does not result in increased mycobacteriostasis (Denis, Gregg and Ghandirian, 1990; Douvas, Looker, Vatter, *et al.*, 1985; Rook, Steele, Ainsworth, *et al.*, 1986; Thoma-Uszynski, Stenger, Takeuchi, *et al.*, 2001). In addition, opposite effects of TNF-α neutralizing antibodies have been reported, with enhanced replication of H37Ra in murine macrophages (Appelberg, Sarmento and Castro, 1995) and reduced growth in human phagocytes (Engele, Stobetael, Castiglione, *et al.*, 2002). Finally, it was recently demonstrated that murine and human macrophages utilize different mycobacteriostatic pathways following ligation of Toll-like receptor-2 (Thoma-Uszynski, Stenger, Takeuchi, *et al.*, 2001). It is therefore plausible that biological differences between murine and human macrophages may account for the observed difference in growth patterns of H37Ra in these cells.

The lack of growth control of H37Ra in human monocyte-derived macrophages observed in our experiments despite ample TNF-α production, suggests that the TNF-α dependent pathway of mycobacterial growth restriction in mouse macrophages may not be operational in human phagocytes. In both human and mouse macrophages, increased TNF-α production was observed after infection with H37Rv relative to H37Ra. Relatively increased TNF-α production by H37Rv infected human monocytes and alveolar macrophages has been reported previously (Engele, Stobetael, Castiglione, *et al.*, 2002; Silver, Li and Ellner, 1998), and has led to the suggestion that TNF-α may support the growth of virulent mycobacteria (Byrd, 1997; Engele, Stobetael, Castiglione, *et al.*, 2002).

Our observation that H37Ra and H37Rv display similar growth in human monocytes is in keeping with our earlier observations (Laochumroonvorapong, Paul, Manca, *et al.*, 1997;

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Paul, Laochumroonvorapong and Kaplan, 1996). Similar growth of H37Ra and H37Rv has also been observed in human type II alveolar cells (Bermudez and Goodman, 1996), and exponential growth of H37Ra was reported in human monocytes, and to a lesser extent human alveolar macrophages (Hirsch, Ellner, Russell, *et al.*, 1994). However, others have reported reduced growth (Zhang, Gong, Lin, *et al.*, 1998) or absent growth (Engele, Stobetael, Castiglione, *et al.*, 2002; Keane, Remold and Kornfeld, 2000; Silver, Li and Ellner, 1998) of H37Ra in human monocytes or alveolar macrophages. The controversy surrounding the ability of the avirulent strain of *M. tuberculosis* to grow in human macrophages may relate to the culture conditions of the cells, or the multiplicity of infection or to the particular strain of H37Ra used for the experiments. Here we evaluated the effect of host cell maturation and adherence on their ability to inhibit mycobacterial replication. Whereas adherence of monocytes had no effect on mycobacterial growth, maturation of cells in tissue culture was associated with reduced growth of *M. tuberculosis*. However, growth of H37Ra and H37Rv were inhibited to the same extent in mature cells. We also performed RFLP analysis of the strains used for these experiments. This confirmed the identity of both H37Ra and H37Rv. It has become apparent that although H37Rv has retained its virulence in experimental murine infection, very little is known about the effect of serial passage of H37Ra on the degree of attenuation of this strain. A recent study demonstrated several polymorphisms among reference H37Ra strains (Bifani, Moghazeh, Shopsin, *et al.*, 2000). The genetic diversity of H37Ra may be one of the variables that could explain the different results obtained in the studies published to date.

In conclusion, here we show that the reduced ability of *M. tuberculosis* H37Ra to grow in murine macrophages may be due to the action of a TNF-α dependent pathway. This mycobacteriostatic pathway does not affect the ability of H37Rv to replicate in mouse macrophages. In addition, several lines of evidence suggest a difference in biologic behavior

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between mouse and human macrophages following mycobacterial infection. These differences should be taken into consideration when extrapolations are made from experimental murine tuberculosis to the human disease.

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Chapter 4

The 19-kilodalton lipoprotein of *Mycobacterium tuberculosis* inhibits *Mycobacterium smegmatis*-induced cytokine production by human macrophages *in vitro*

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4.1 ABSTRACT

Vaccination of mice with *M. vaccae* or *M. smegmatis* induces some protection against *M. tuberculosis* (MTB) challenge. The 19kDa lipoprotein of MTB, expressed in *M. vaccae* or *M. smegmatis* (*M. smegmatis* 19kDa), abrogates this protective immunity. To investigate the mechanism of this suppression of immunity, human monocyte-derived macrophages (MDM) were infected with *M. smegmatis* 19kDa. Infection resulted in reduced production of tumor necrosis factor-alpha (TNF-α) ($P < 0.01$), interleukin-12 (IL-12) ($P < 0.05$), IL-6 ($P < 0.05$) and IL-10 ($P < 0.05$), compared to infection with *M. smegmatis* (vector). The 19kDa antigen had no differential effect on expression of co-stimulatory molecules on the surface of *M. smegmatis* infected MDM, nor did it affect the proliferation of pre-sensitized T-cells co-cultured with infected MDM. The immunosuppressive effect was dependent on the presence of glycosylated and acylated 19kDa lipoprotein in the mycobacteria-containing phagosomes. These results suggest that the diminished protection against challenge with *M. tuberculosis* seen in mice vaccinated with *M. smegmatis* expressing the 19kDa lipoprotein might have been the result of reduced TNF-α and IL-12 production, resulting in reduced T-cell activation.

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4.2 INTRODUCTION

Recent vaccination studies have suggested that the 19kDa antigen of *M. tuberculosis* may possess immune-inhibitory effects. In this chapter, two recombinant mycobacterial strains expressing the 19kDa lipoprotein were used to infect human monocyte-derived macrophages (MDM), to study the effect of this antigen on host cell cytokine production and expression of antigen-presenting and co-stimulatory molecules. Expression of the 19kDa antigen, a glycosylated lipoprotein, by recombinant mycobacteria allowed us to study the immunological properties of this antigen in the context of other mycobacterial cell wall

antigens. The 19kDa lipoprotein has been extensively studied for its unusual structure, and as immunodiagnostic, vaccine candidate, and ligand for the Toll-like receptor 2 (TLR-2).

The 19kDa lipoprotein of *M. tuberculosis.* The 19kDa lipoprotein is a secreted, cell wall associated protein (Young and Garbe, 1991), and is restricted to the MTB complex (which includes *M. tuberculosis*, *M. bovis BCG*, *M. microti* and *M. africanum*) (Nair, Rouse and Morris, 1992; Oftung, Mustafa, Husson, *et al.*, 1987). The 19kDa antigens of MTB and *M. bovis BCG* are identical, bear no structural homology to other proteins, and have no known function ascribed to them (Ashbridge, Booth, Watson, *et al.*, 1989; Collins, Patki, Wall, *et al.*, 1990). The MTB 19kDa antigen exists as a glycosylated lipoprotein (Fifis, Costopoulos, Radford, *et al.*, 1991; Garbe, Harris, Vordermeier, *et al.*, 1993; Young and Garbe, 1991). The unusual lipid and carbohydrate moieties may serve to influence antigen presentation or binding to major histocompatibility complex (MHC) molecules (Garbe, Harris, Vordermeier, *et al.*, 1993) or prevent proteolysis (Herrmann, O'Gaora, Gallagher, *et al.*, 1996). The lipid tail is essential for intracellular trafficking of the 19kDa antigen, which occurs after phagocytosis of the mycobacterium. Its subsequent entry into the class I antigen presentation pathway may serve immune recognition of MTB infected phagocytes by $CD8^{+}$T-lymphocytes (Neyrolles, Gould, Gares, *et al.*, 2001).

Innate immunity and the 19kDa antigen. The innate immune response serves to protect the host during the earliest stages of infection, and provides the appropriate co-stimulatory and cytokine signals required for the induction of adaptive (cell-mediated) immunity. Toll-like receptors have recently been described as a novel class of receptors on mononuclear phagocytes that mediate innate immune responses to microbial ligands including lipoproteins (Aderem and Ulevitch, 2000). Toll-like receptors enable the macrophages to sample the contents of their vacuoles, and elicit a microbe-specific inflammatory response (Underhill, Ozinsky, Hajjar, *et al.*, 1999). Ligation of TLR results in

NF-κB mediated cytokine production and co-stimulatory molecule expression (Medzhitov, Preston-Hurlburt and Janeway, 1997).

MTB and MTB cell wall components, including the 19kDa lipoprotein, lipoarabinomannan (LAM), and cell wall lipids can activate macrophages through TLR-2 dependent signaling, which results in the production of TNF-α and IL-12, two key cytokines involved in regulating the adaptive immune response (Brightbill, Libraty, Krutzik, *et al.*, 1999; Faure, Equils, Sieling, *et al.*, 2000; Means, Wang, Lien, *et al.*, 1999; Underhill, Ozinsky, Smith, *et al.*, 1999). Ligation of TLR-2 by the 19kDa has been shown to result in iNOS expression and nitric oxide (NO) production (Brightbill, Libraty, Krutzik, *et al.*, 1999), apoptosis (Aliprantis, Yang, Mark, *et al.*, 1999; Lopez, Sly, Luu, *et al.*, 2003), dendritic cell maturation (Hertz, Kiertscher, Godowski, *et al.*, 2001), and inhibition of MHC class II expression (Noss, Pai, Sellati, *et al.*, 2001). Ligation of TLR-2 by the 19kDa results in activation of NO-dependent mycobacteriostatic pathways in mouse macrophages and NO-independent pathways in human monocyte-derived macrophages (Thoma-Uszynski, Stenger, Takeuchi, *et al.*, 2001).

Acquired immunity and the 19kDa antigen. The MTB 19kDa antigen elicits both humeral and cellular immune responses. Antibodies against the 19kDa are present in mice infected with MTB or *M. bovis BCG* (Ashbridge, Prestidge, Booth, *et al.*, 1990; Verbon, Kuijper, Jansen, *et al.*, 1992), and T-cell clones reactive against the 19kDa or 19kDa-derived peptides may be generated from their lymph nodes (Ashbridge, Backstrom, Liu, *et al.*, 1992; Boom, Husson, Young, *et al.*, 1987; Harris, Vordermeier, Friscia, *et al.*, 1993; Harris, Vordermeier, Roman, *et al.*, 1991). Antibodies against the 19kDa, and 19kDa-specific, IFN-γ secreting T-cells are frequently encountered in patients with tuberculosis and healthy, PPD-positive persons (Harris, Vordermeier, Friscia, *et al.*, 1993; Jackett, Bothamley, Batra, *et al.*, 1988; Oftung, Mustafa, Husson, *et al.*, 1987; Surcel, Troye-Blomberg, Paulie, *et al.*, 1994).

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Cytotoxic T-lymphocytes (CTL) specific for a 19kDa derived peptide can recognize and lyse MTB infected monocytes (Mohagheghpour, Gammon, Kawamura, *et al.*, 1998). These observations suggest that the 19kDa is available for immune recognition, and since secreted MTB antigens tend to be potent inducers of memory T-cell responses (Andersen, Askgaard, Ljungqvist, *et al.*, 1991), a number of 19kDa-based vaccination strategies have been devised.

Vaccines based on the 19kDa antigen. The current vaccine used to protect humans against TB, *M. bovis BCG*, elicits a type-1 response mediated by IFN-γ producing T-cells (Marchant, Goetghebuer, Ota, *et al.*, 1999; Ravn, Boesen, Pedersen, *et al.*, 1997). In the mouse model, exposure to MTB is followed by recruitment of IFN-γ producing lymphocytes to the site of infection (Flynn, Chan, Triebold, *et al.*, 1993). When mice are vaccinated with BCG and then challenged with MTB, accelerated recruitment of activated, IFN-γ producing T-lymphocytes to the site of infection is observed (Silva, Bonato, Lima, *et al.*, 1999). This is associated with a reduction in the tissue bacillary load but not with elimination of the infection.

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Vaccination of mice with DNA encoding the 19kDa, or peptides derived from the 19kDa antigen, resulted in the induction of a type-1 response and generation of CTL (Erb, Kirman, Woodfield, *et al.*, 1998; Fonseca, Joosten, Snippe, *et al.*, 2000; Yeremeev, Lyadova, Nikonenko, *et al.*, 2000). This immune response, however, has failed to protect mice against MTB challenge (Erb, Kirman, Woodfield, *et al.*, 1998; Yeremeev, Lyadova, Nikonenko, *et al.*, 2000).

The saprophytic mycobacteria *M. vaccae* and *M. smegmatis*, when administered to mice as a vaccine, induce a short-lived type-1 T-cell response, as demonstrated by the ability of splenocytes to proliferate, produce IFN-γ, and mediate CTL activity in response to mycobacterial antigens (Skinner, Yuan, Prestidge, *et al.*, 1997). This immune response affords the animals some protection against MTB challenge (Abou-Zeid, Gares, Inwald, *et*

al., 1997; Yeremeev, Lyadova, Nikonenko, *et al.*, 2000). Since *M. vaccae* and *M. smegmatis* do not express the 19kDa antigen, it was anticipated that recombinant expression of the 19kDa lipoprotein on the surface of these mycobacteria might enhance the protective efficacy of these vaccine strains. However, the limited protection afforded by immunization with *M. vaccae* or *M. smegmatis* was abrogated when the 19kDa antigen was expressed by these strains (Abou-Zeid, Gares, Inwald, *et al.*, 1997; Yeremeev, Lyadova, Nikonenko, *et al.*, 2000). In addition, immunization of mice with the 19kDa-expressing strains failed to induce the accelerated recruitment of IFN-γ producing T-cells to the site of infection, and these mice were noted to have impaired delayed-type hypersensitivity (DTH) responses to intradermal PPD (Yeremeev, Lyadova, Nikonenko, *et al.*, 2000). Thus, the 19kDa antigen appeared to have an inhibitory effect on the generation of protective immunity.

We hypothesized that the immune inhibitory effect of the 19kDa antigen might be the result of impaired pro-inflammatory cytokine production or co-stimulatory molecule expression, or enhanced IL-10 production by infected antigen-presenting cells. In the studies presented in this chapter, we infected human monocyte-derived macrophages with *M. smegmatis* vector (*M. smegmatis*) or *M. smegmatis* that expressed the 19kDa antigen (*M. smegmatis* 19kDa), and measured TNF-α, IL-12, IL-6 and IL-10 production. The effect of post-translational modification of the 19kDa protein on cytokine production was also studied by infecting the cells with recombinant *M. smegmatis* that expressed non-O-glycosylated, non-acylated, or non-secreted 19kDa mutants. Surface expression of CD14, HLA-DR, and the co-stimulatory molecules CD40, CD80, and CD86 on uninfected, and *M. smegmatis* or *M. smegmatis* 19kDa infected MDM was also compared.

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4.3 RESULTS

Survival of *M. smegmatis* in cultures of human MDM. Peripheral blood monocytes from healthy volunteers were maintained in tissue culture for 6 days prior to infection with *M. smegmatis* (vector) or *M. smegmatis* 19kDa at an MOI of 3:1. Three hours post infection, cell cultures were pulsed with gentamycin which resulted in killing of the extra-cellular bacilli. At 5 h post infection, 22 to 56% (mean 36%) of the organisms had been phagocytosed, and culture medium was replaced by fresh medium without antibiotics. A progressive decline in the number of viable intracellular organisms was observed over the next 4 days. However, bacterial counts were similar for both strains throughout the experiments (Figure 4-1). MDM viability, as determined by trypan blue exclusion, was similar for uninfected, *M. smegmatis*-infected, and *M. smegmatis* 19kDa-infected cells (range, 79 to 97%). The morphological aspect of macrophages and phagocytosed bacilli examined by electron microscopy was also similar, with both *M. smegmatis* and *M. smegmatis* 19kDa residing in individual vacuoles surrounded by a double membrane (Figure 4-2).

Cytokine production by MDM following infection with *M. smegmatis* strains. Pro-inflammatory cytokine production by MDM following infection with *M. smegmatis* or *M. smegmatis* 19kDa was measured in the culture supernatants (Figure 4-3). In response to infection, the kinetics of production of the various cytokines differed, and substantial donor to donor variability was observed in the absolute concentrations of cytokines (up to 8-fold differences in the concentrations of TNF-α and IL-10; and up to 100-fold differences for IL-6, and IL-12). The concentrations of TNF-α, IL-12, IL-6, and IL-10 were sufficiently elevated to be detectable at 24 h in all donors. However, to allow meaningful comparison of cytokine production by MDM in response to infection with either *M. smegmatis* strain, the results for each cytokine were normalized to the 24 h time point. When thus analyzed, infection with *M. smegmatis* 19kDa resulted, in each donor, on average in 2.1-fold (range 1.6

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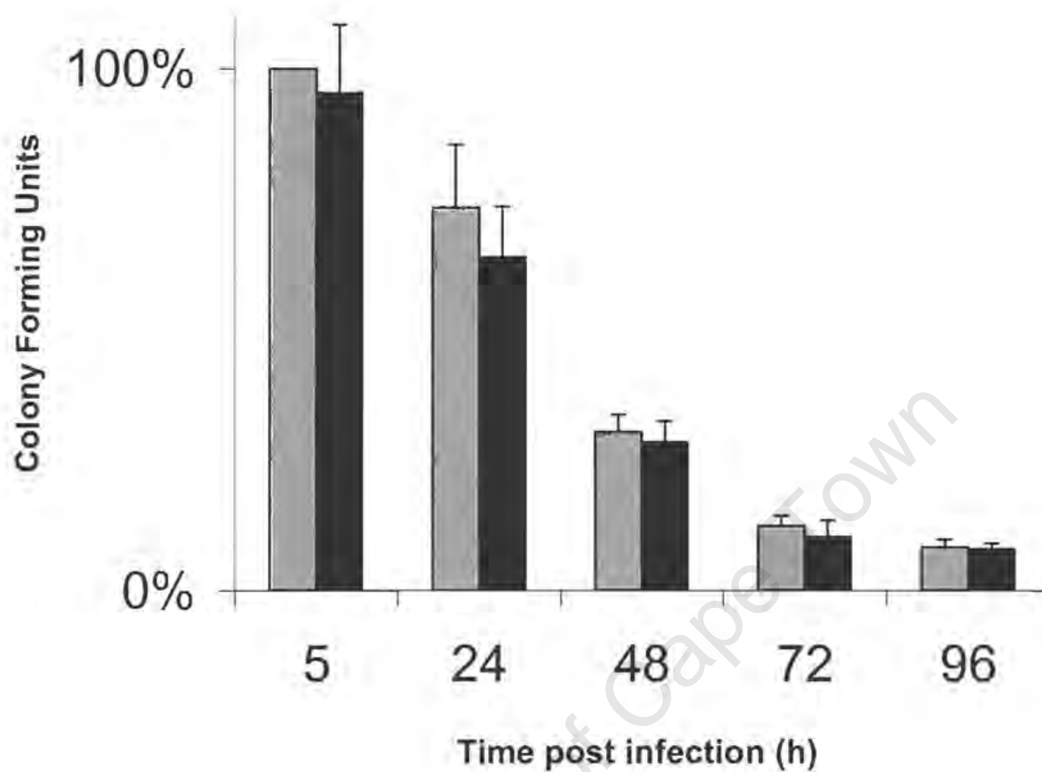

Figure 4-1 Number of intracellular viable mycobacteria following infection of MDM with *M. smegmatis* (vector) (gray bars) or *M. smegmatis* 19kDa (black bars). Cells were infected at an MOI of 3:1 for 3 h and pulsed with gentamycin for 2 h. The results are expressed as the percentage of CFU normalized to the number of CFU of *M. smegmatis* at 5 h. Results are means of six experiments done in duplicate or triplicate, carried out on monocytes of unrelated donors. Error bars indicate standard error of the mean.

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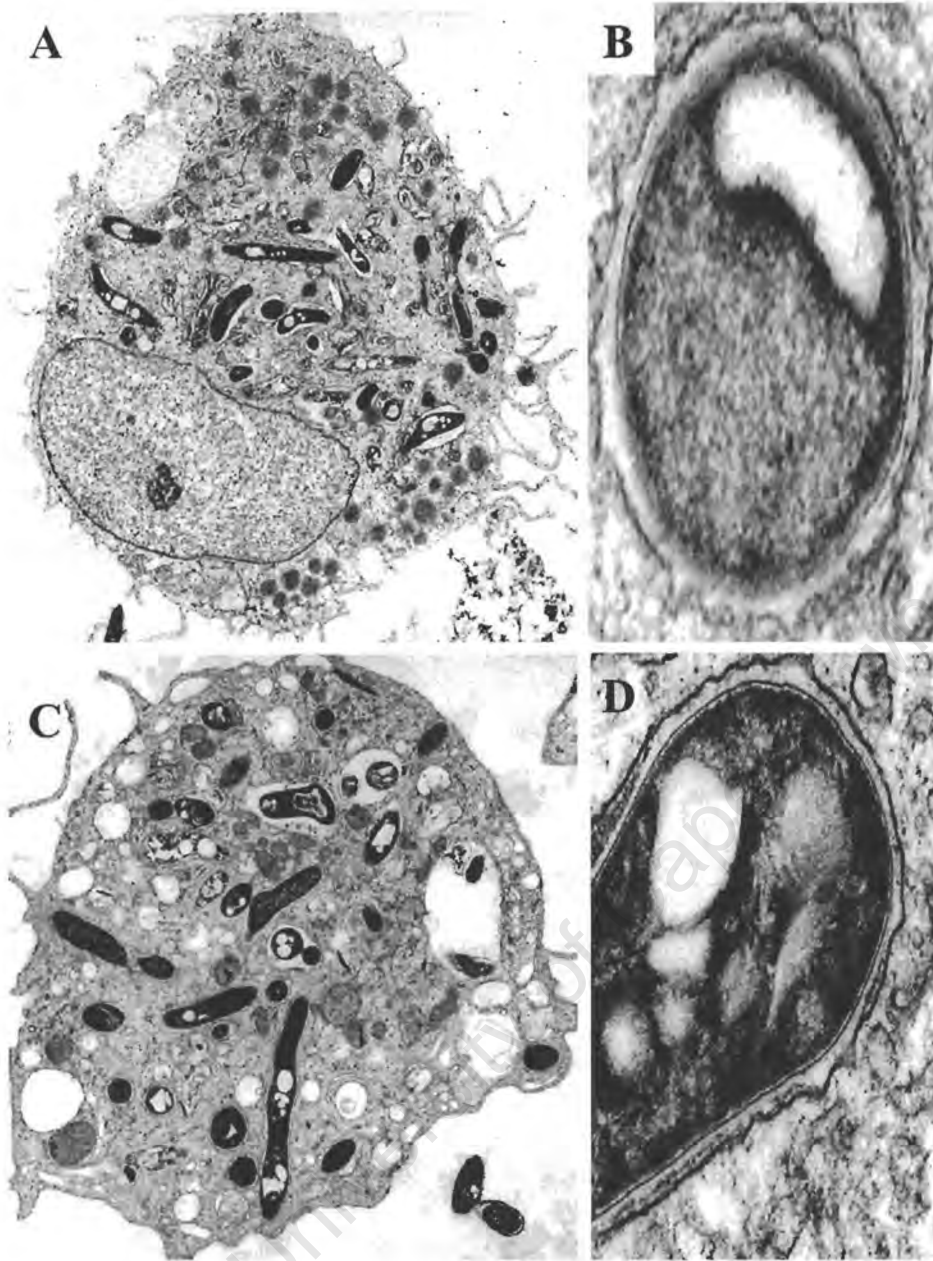

Figure 4-2 Electron micrographs of monocyte-derived macrophages 5h post infection with *M. smegmatis* (a, b) or *M. smegmatis* 19kDa (c, d). Both recombinant mycobacterial strains occupy individual vacuoles surrounded by tightly apposed, double membranes. Original magnification: 3300x (A), 13000x (B), 5000x (C), 26000x (D).

-2.5) reduced production of TNF-α ($P < 0.01$), IL-12 ($P < 0.05$), IL-6 ($P < 0.05$), and IL-10 ($P < 0.05$) compared to infection with *M. smegmatis* (Figure 4-3). Reduced production of TNF-α, IL-6, and IL-12, therefore, did not result from increased production of IL-10.

Effect of post-translational modification of the 19kDa antigen on cytokine production by MDM infected with *M. smegmatis* strains. To study the effect of acylation and glycosylation of the 19kDa antigen on cytokine production, MDM were infected with *M. smegmatis* expressing the complete glycosylated and acylated 19kDa molecule (*M. smegmatis* 19kDa), the non-O-glycosylated (*M. smegmatis* 19NOG), non-secreted (*M. smegmatis* 19NS), or non-acylated (*M. smegmatis* 19NA) forms of the 19kDa antigen, or *M. smegmatis* (vector). The inhibitory effect of the 19kDa on TNF-α and IL-12 production was observed only when the 19kDa antigen was both acylated and O-glycosylated (Figure 4-4).

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Effect of the 19kDa antigen on co-stimulatory molecule expression and T-cell proliferation. Adherent MDM infected with *M. smegmatis* or *M. smegmatis* 19kDa were detached at 24, 48, and 72 h post infection and analyzed for cell surface markers by flow cytometry. Comparative analysis of the mean fluorescent intensity for uninfected, *M. smegmatis*-infected, and *M. smegmatis* 19kDa-infected cells revealed an infection-induced down-regulation of CD14 and increased expression of CD40 and CD80, which reached a maximum at 48 h post infection (Figure 4-5). Down-regulation of CD14 and increased CD40 and CD80 expression were similar on *M. smegmatis* and *M. smegmatis* 19kDa infected cells. No significant difference in CD86 or HLA-DR expression was observed between uninfected MDM and MDM infected with *M. smegmatis* or *M. smegmatis* 19kDa (Figure 4-5). When T-cells from PPD^+ individuals were exposed to autologous infected MDM, the recall proliferative responses were slightly higher (22% ± 16% higher) in the presence of *M. smegmatis* 19kDa than in the presence of *M. smegmatis*. These results suggest that the 19

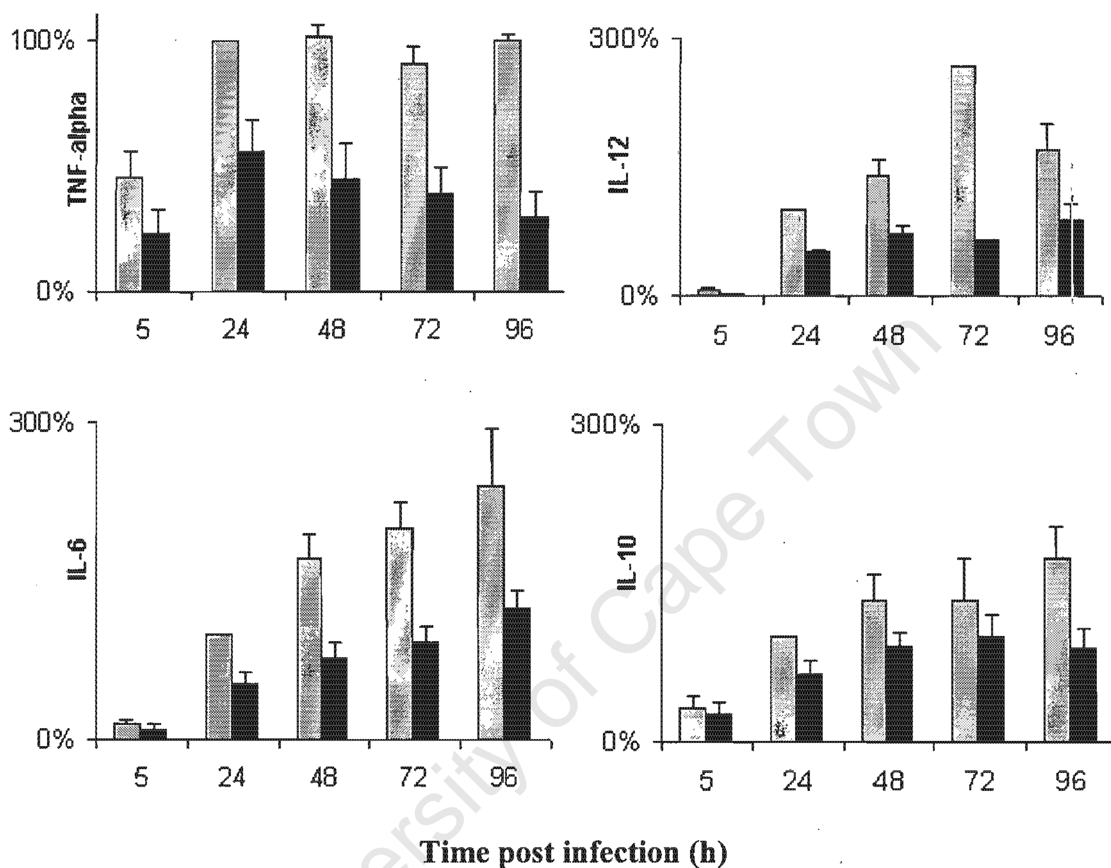

Figure 4-3 Cytokine production by MDM infected with *M. smegmatis* (grey bars) or *M. smegmatis* 19kDa (black bars). Cytokine production was normalized to the level of the relevant cytokine measured in the supernatants of *M. smegmatis* infected MDM at 24 h post infection. Results are presented as percent activity and represent the means of four to six experiments; error bars reflect the standard error of the mean.

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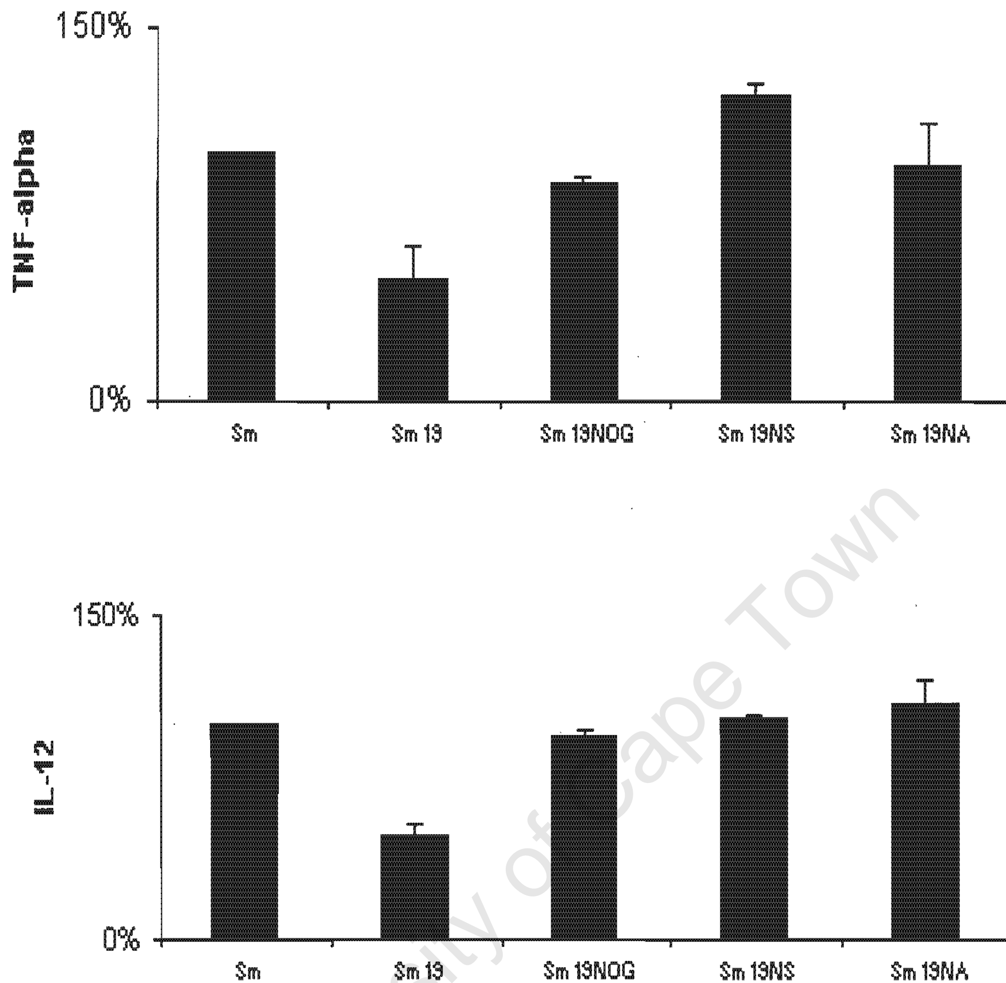

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Figure 4-4 Effect of post-translational modification of the 19kDa antigen on cytokine production. TNF-α (5 h post infection) and IL-12 (24 h post infection) production by MDM infected with *M. smegmatis* (Sm), *M. smegmatis* 19kDa (Sm 19), or *M. smegmatis* expressing non-O-glycosylated (Sm 19NOG), non-secreted (Sm 19NS) or non-acylated (Sm 19NA) forms of the 19kDa lipoprotein is shown. The results are shown as the means and standard error of the mean of three or four experiments; cytokine values are normalized to TNF-α or IL-12 production by *M. smegmatis* infected cells.

kDa antigen did not directly affect the expression of co-stimulatory molecules on MDM. The slight increases in *in vitro* T-cell proliferation may have been due to recognition of the 19kDa lipoprotein by T-cells previously sensitized during (asymptomatic) MTB infection or BCG vaccination.

Effect of exogenously added 19kDa antigen on cytokine production by MDM infected with *M. smegmatis* strains. We next investigated whether cytokine production by MDM could be modified by addition of purified recombinant or purified native 19kDa lipoprotein to the culture medium during infection with *M. smegmatis*. When different concentrations of recombinant 19kDa lipoprotein purified from whole-cell lysates (rWCL) were added to uninfected MDM, low levels of TNF-α were produced in a dose-dependent manner. When added to MDM infected with either *M. smegmatis* or *M. smegmatis* 19kDa, rWCL at 100 ng/ml had no effect on TNF-α production (Figure 4-6). The extent of inhibition (~50%) of TNF-α production in the *M. smegmatis* 19kDa-infected cells compared to that in the *M. smegmatis*-infected MDM remained the same. Addition of recombinant 19-kDa antigen purified from culture filtrate (rCF) to uninfected MDM had no stimulatory effect on TNF-α production (Figure 4-6). When rCF at 100 ng/ml was added to MDM infected with *M. smegmatis* or *M. smegmatis* 19kDa, the inhibitory effect on TNF-α production was minimal. When the purified native 19kDa antigen (100 ng/ml) was added to uninfected MDM, very low levels of TNF-α and no IL-12 were produced (Figure 4-6). Native lipoprotein added to MDM infected with *M. smegmatis* or *M. smegmatis* 19kDa had little or no effect on TNF-α or IL-12 production. Taken together, these results suggest that exogenously added 19kDa antigen has only limited effects on cytokine production. It would appear that the 19kDa lipoprotein has to be intracellular and probably in the same cellular compartment as *M. smegmatis* 19kDa to exert its maximal inhibitory effect on MDM cytokine production.

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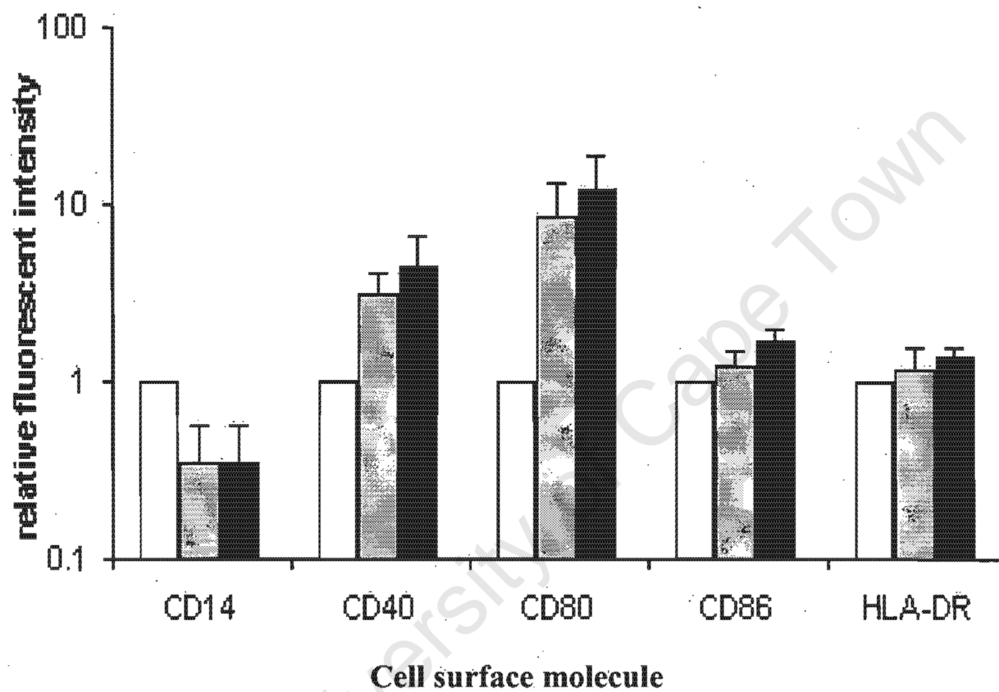

Figure 4-5 Expression of cell surface markers on uninfected MDM (open bars) or MDM infected for 48 h with *M. smegmatis* (gray bars) or *M. smegmatis* 19kDa (black bars). The results are normalized to the values for uninfected cells (set at 1) and are means and standard error of the mean of three experiments.

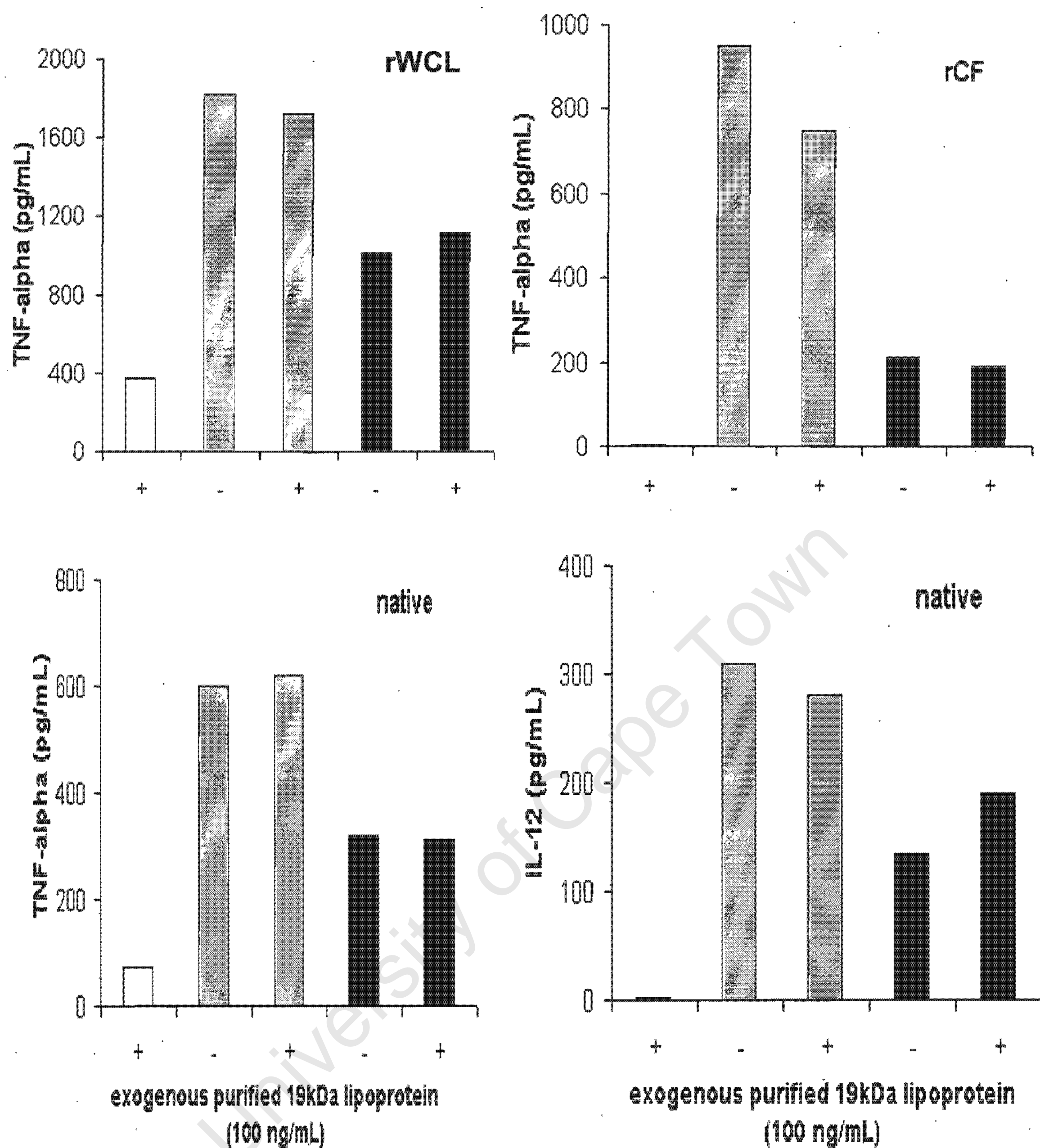

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Figure 4-6 Effect of exogenously added 19kDa lipoprotein on TNF-α and IL-12 production by MDM. MDM were uninfected (open bars) or infected with *M. smegmatis* (gray bars) or *M. smegmatis* 19kDa (black bars) in the presence (+) or absence (-) of 100 ng of purified recombinant 19kDa protein per ml isolated from whole recombinant *M. smegmatis* 19kDa cell lysate (rWCL) or *M. smegmatis* 19kDa culture filtrate (rCF), or 19kDa protein purified from *M. tuberculosis* (native).

4.4 DISCUSSION

Protective immunity against MTB depends on IL-12 and TNF-α production to regulate T-cell activation and stimulation of mycobacteriostatic pathways within infected macrophages (Kaplan and Freedman, 1996). Following aerosol MTB infection, mice deficient in IL-12 have decreased and delayed expression of mRNA for IFN-γ, TNF-α, and inducible nitric oxide synthase in infected tissues and reduced lymphocyte recruitment to the site of infection, resulting in overwhelming tissue bacillary loads and early mortality (Cooper, Magram, Ferrante, *et al.*, 1997). When mice are treated with exogenous IL-12, enhanced, IFN-γ-dependent resistance to infection with MTB is observed (Cooper, Roberts, Rhoades, *et al.*, 1995; Flynn, Goldstein, Triebold, *et al.*, 1995). As a vaccine adjuvant, IL-12 enhances the development of a protective Th1 response (Afonso, Scharton, Vieira, *et al.*, 1994; Freidag, Melton, Collins, *et al.*, 2000), and when IL-12 DNA therapy is administered to MTB-infected mice, a phenotypic switch from Th2-type cytokine production to IFN-γ-secreting Th1-type cells is observed (Lowrie, Tascon, Bonato, *et al.*, 1999). Therefore, IL-12 is a key cytokine involved in establishing protective immunity against MTB.

A critical role in protection against murine tuberculosis has also been demonstrated for the pro-inflammatory cytokine TNF-α. Mice lacking TNF-α succumb to overwhelming MTB infection. In the absence TNF-α, delayed macrophage activation and chemokine expression result in impaired T-cell migration into granulomas despite the presence of adequate numbers of both $CD4^+$ and $CD8^+$ T cells in the liver and lungs (Bean, Roach, Briscoe, *et al.*, 1999; Flynn, Goldstein, Chan, *et al.*, 1995; Roach, Bean, Demangel, *et al.*, 2002). TNF-α has also been shown to enhance IFN-γ-induced production of reactive nitrogen intermediates *in vitro* (Flesch, Hess, Oswald, *et al.*, 1994). Recent studies have also suggested an important role for IL-6 in stimulating early IFN-γ production during MTB infection (Saunders, Frank, Orme, *et al.*, 2000) and in priming of T cells after vaccination with MTB

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proteins (Leal, Smedegard, Andersen, *et al.*, 1999). We have shown here that following *in vitro* infection of human MDM with *M. smegmatis* expressing the MTB 19kDa lipoprotein, there is reduced production of the pro-inflammatory cytokines IL-12, TNF-α and IL-6. The inhibition of pro-inflammatory cytokine production by the 19kDa lipoprotein may be an explanation why *M. smegmatis* strains expressing the 19kDa antigen were ineffective as vaccines (Abou-Zeid, Gares, Inwald, *et al.*, 1997; Yeremeev, Lyadova, Nikonenko, *et al.*, 2000).

Experiments in which the 19kDa lipoprotein was used to elicit immune responses have yielded contrasting results. Some studies have reported antigen-specific IFN-γ production by splenocytes and a slightly increased survival time after MTB challenge in the case of a 19kDa DNA vaccine (Abou-Zeid, Gares, Inwald, *et al.*, 1997; Yeremeev, Lyadova, Nikonenko, *et al.*, 2000). A degree of protection against MTB challenge was also observed in mice immunized with a 19kDa recombinant vaccinia virus construct (Zhu, Venkataprasad, Ivanyi, *et al.*, 1997). Although Erb *et al.* could readily detect antibodies against the 19kDa lipoprotein following DNA vaccination, no proliferative or CTL activity was induced and protection against *M. bovis* BCG challenge was not achieved (Erb, Kirman, Woodfield, *et al.*, 1998), and T cells obtained from mice immunized with 19kDa-antigen-pulsed dendritic cells showed no antigen-specific IFN-γ production (Baird, Hart, Abernethy, *et al.*, 1995). Nonetheless, others have documented that a peptide fragment of the 19kDa antigen, presented to T cells by human dendritic cells *in vitro*, could induce HLA class I-restricted CTL activity (Mohagheghpour, Gammon, Kawamura, *et al.*, 1998).

A recent study showed the 19kDa antigen to be a ligand for the TLR-2 receptor, and stimulation of TLR-2 expressing THP-1 cells with purified 19kDa resulted in high IL-12 production (Brightbill, Libraty, Krutzik, *et al.*, 1999). In some of our experiments, we stimulated human MDM with purified 19kDa antigen. Although monocytes express high

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levels of TLR2 (Kadowaki, Ho, Antonenko, *et al.*, 2001), we observed no IL-12 and only modest TNF-α production in response to purified 19kDa antigen, in contrast to the high levels of TNF-α and IL-12 produced in response to infection with recombinant *M. smegmatis* expressing the 19kDa antigen. The lack of IL-12 production in response to the purified antigen in our study might reflect a difference in biologic behavior between monocytes and macrophages, as suggested by a recent study (Stewart *et al.*, Tuberculosis: Integrating Host and Pathogen Biology, Taos, Jan 25-30, 2003. Abstract 240).

MTB has developed a number of mechanisms to impair effective generation of cell-mediated immunity by the infected host, including inhibition of pro-inflammatory cytokine production by macrophages (Nau, Richmond, Schlesinger, *et al.*, 2002; Nigou, Zelle-Rieser, Gilleron, *et al.*, 2001; Vankayalapati, Wizel, Weis, *et al.*, 2003), expression of antigen-presenting molecules (Giuliani, Prete, Graziani, *et al.*, 2001; Noss, Pai, Sellati, *et al.*, 2001), TLR mediated signaling (Means, Lien, Yoshimura, *et al.*, 1999) and dendritic cell maturation (Geijtenbeek, Van Vliet, Koppel, *et al.*, 2003). It is possible that the inhibitory effects of MTB on IL-12 production, as observed by Nau and colleagues, may have been mediated by the 19kDa antigen (Nau, Richmond, Schlesinger, *et al.*, 2002). We observed that intracellular *M. smegmatis* 19kDa had stronger immune-modulating effects than exogenously added 19kDa antigen, and that the intact 19kDa molecule was required for the immune-modulatory activity. Interestingly, a previous study showed that within 1 h of macrophage phagocytosis of *M. bovis* BCG, *M. tuberculosis*, or *M. smegmatis* 19kDa, the 19kDa lipoprotein traffics out from the phagosome by insertion into plasma membranes. This trafficking did not occur with mutant 19kDa lipoproteins that were non-acylated and non-glycosylated (Neyrolles, Gould, Gares, *et al.*, 2001). It is possible that the inhibition of cytokine production by *M. smegmatis* 19kDa seen here may require the 19kDa lipoprotein to be initially part of the same cellular compartment as the mycobacterium. This would allow the 19kDa lipoprotein to leave the

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phagosome by insertion into the phagosome membrane, thereby gaining access to the regulatory pathways for cytokine production. The idea that an MTB component may affect host cell immune function by insertion into the cell membrane has been suggested in another system. Ting *et al.* have observed that MTB blocks human macrophage responses to IFN-γ by preventing the interaction of STAT1 with the transcriptional machinery in the nucleus (Ting, Kim, Cattamanchi, *et al.*, 1999). This may occur through sequestration of transcriptional co-activators at extra-nuclear sites adjacent to the phagosome membrane, and be mediated by a component of the mycobacterial cell wall. It is possible that the 19kDa lipoprotein may be such a component.

Any factor that contributes to the success of the pathogen by interfering with the host protective response can be considered a virulence factor for *M. tuberculosis*. Our observation that the 19kDa antigen influences host cytokine responses is compatible with a role for this protein in mycobacterial virulence. Additional data supporting this view were reported in a study in which a naturally occurring mutant of *M. tuberculosis* H37Rv (I 2646), which does not express the 19kDa antigen, showed reduced growth in B10 mice (Lathigra, Zhang, Hill, *et al.*, 1996). When this strain was transformed with the 19kDa antigen, increased bacterial loads were observed compared to those in the vector control. However, deletion or overexpression of the 19kDa antigen in BCG did not affect its protective efficacy against MTB challenge in mice (Yeremeev, Stewart, Neyrolles, *et al.*, 2000).

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Chapter 5

IL-12 and TNF-α production in tuberculosis patients with different extents of pulmonary disease

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5.1 ABSTRACT

In this chapter, we examined whether production of the pro-inflammatory cytokines interleukin-12 (IL-12) and tumor necrosis factor-alpha (TNF-α) is associated with the severity of pulmonary disease in patients with tuberculosis (TB). Plasma cytokine levels were measured in patients who had limited or extensive disease on chest radiograph. Plasma IL-12 levels were higher in patients with limited pulmonary disease compared to patients with extensive disease ($p=0.02$). In addition, peripheral blood monocytes from patients with limited disease produced more IL-12, TNF-α and IL-6 in response to *in vitro* infection with *M. tuberculosis* (MTB) than monocytes from patients with extensive disease ($p<0.05$), and MTB grew faster in cultured monocytes from patients with extensive disease ($p<0.05$). Pro-inflammatory cytokine production and intracellular growth rates were inversely related among different MTB clinical isolates. Thus, enhanced pro-inflammatory cytokine production in response to MTB infection may reduce bacillary growth and restrict the extent of pulmonary disease in TB patients.

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5.2 INTRODUCTION

An estimated 90% of humans infected with MTB do not develop clinical TB (Bloom and Murray, 1992). It is assumed that these individuals mount a cellular immune response to the bacilli that is essentially protective. Monocyte cytokines have been shown to be involved in the protective cellular immune response to MTB (Kaplan and Freedman, 1996). When mice are infected via the aerosol route with MTB, expression of pro-inflammatory cytokines including IL-12 and TNF-α mRNA at the site of disease in the lungs is observed (Moreira, Tsenova-Berkova, Wang, *et al.*, 1997). In addition, infection of mice with MTB CDC1551 (Valway, Sanchez, Shinnick, *et al.*, 1998), a clinical strain that induces high levels of IL-12 and TNF-α, results in early control of mycobacterial growth and prolonged survival of the

infected animals (Manca, Tsenova, Barry, *et al.*, 1999). In contrast, infection of mice with MTB clinical strain HN878 (Sreevatsan, Pan, Stockbauer, *et al.*, 1997) results in delayed and reduced IL-12 and TNF-α production, a 10 fold higher bacterial load in the lungs, and markedly impaired survival of the infected animals (Manca, Tsenova, Bergtold, *et al.*, 2001). When mice are genetically disabled in their ability to produce IL-12 or TNF-α, complete failure to control the growth of MTB in the lungs is observed, and these mice rapidly succumb to overwhelming mycobacterial infection (Bean, Roach, Briscoe, *et al.*, 1999; Cooper, Magram, Ferrante, *et al.*, 1997; Flynn, Goldstein, Chan, *et al.*, 1995). Thus, in MTB infected mice, a rapid and strong pro-inflammatory cytokine response in the lungs is associated with less severe disease.

Direct involvement of IL-12 and TNF-α in the host defense against TB in humans is suggested by the presence of mRNA for these cytokines within tuberculosis granulomas in infected lungs and lymph nodes (Bergeron, Bonay, Kambouchner, *et al.*, 1997; Fenhalls, Stevens, Bezuidenhout, *et al.*, 2002; Fenhalls, Wong, Bezuidenhout, *et al.*, 2000), and elevated concentrations of these proteins in TB pleural effusions, a disease manifestation which is often self limiting (Barnes, Fong, Brennan, *et al.*, 1990; Zhang, Gately, Wang, *et al.*, 1994). *In vitro,* IL-12 and TNF-α are readily produced by human monocytes in response to infection with MTB or following exposure to mycobacterial cell wall components (Brightbill, Libraty, Krutzik, *et al.*, 1999; Manca, Tsenova, Barry, *et al.*, 1999; Means, Jones, Schromm, *et al.*, 2001; Underhill, Ozinsky, Smith, *et al.*, 1999). Similar to what is noted in animal experiments, persons with genetic defects in the IL-12 signaling pathway, and patients who have received treatment with TNF-α neutralizing antibodies have been shown to be at increased risk of mycobacterial infections including TB (Altare, Durandy, Lammas, *et al.*, 1998; Altare, Ensser, Breiman, *et al.*, 2001; Altare, Lammas, Revy, *et al.*, 1998; de Jong,

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Altare, Haagen, *et al.*, 1998; Keane, Gershon, Wise, *et al.*, 2001; Picard, Fieschi, Altare, *et al.*, 2002).

We hypothesized that impaired IL-12 and TNF-α production in response to MTB infection may be associated with more severe tuberculous lung disease in humans. The extent of lung disease was determined on pre-treatment chest radiographs, and plasma cytokine levels were measured. In addition, we evaluated cytokine production by the monocytes obtained from patients with limited or extensive pulmonary disease infected *in vitro* with MTB strains CDC1551 and HN878. The ability of these monocytes to control the growth of MTB *in vitro* was also analyzed.

5.3 RESULTS

Seventeen human immunodeficiency virus (HIV) seronegative patients with active pulmonary tuberculosis from Cape Town, South Africa, were randomly selected and recruited into the study. Patients included South African Black and Coloured (mixed ethnicity) males and females 18-62 years old (Table 5-1). Patients with prior TB, and those who had known risk factors for TB including drug-induced immune suppression, diabetes mellitus and pregnancy in females, were excluded. All patients had MTB isolated from sputum and none had received more than 4 weeks of short course chemotherapy (rifampin, isoniazid, pyrazinamide and ethambutol) at the time of sampling.

On the basis of the extent of disease on pretreatment chest radiographs, patients were classified as having limited or extensive disease. Patients in whom the pulmonary TB infiltrates were confined to the upper zones and/or apical segments of the lower lobes (typical post-primary infiltrates) were classified as having limited disease (n=8) as shown in Figures 5-1A and 5-1B. Patients were classified as having extensive disease when, in addition to upper zone infiltrates, radiographs revealed infiltrates in the lower and/or mid-zones (n=9)

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Classification of Patients	No	Ethnicity	Age	Gender	Rx (d)	Pretreatment Chest Radiograph	
						No. of affected zones	cavitation
Limited Disease:	1	Coloured	37	M	12	3	yes
	2	Coloured	62	M	15	3	no
	3	Coloured	18	F	13	3	yes
	4	Coloured	37	M	20	2	yes
	5	Coloured	52	M	10	2	yes
	6	Black	52	M	13	1	no
	7	Coloured	61	M	19	2	yes
	8	Black	19	F	26	1	yes
		Mean ± SD	42 ± 17	--	16 ± 5	2.1 ± 0.8	--
Extensive Disease:	9	Coloured	28	M	0	6	yes
	10	Coloured	43	F	14	4	yes
	11	Black	55	M	7	6	yes
	12	Black	51	F	21	5	yes
	13	Coloured	36	M	15	5	yes
	14	Coloured	35	F	0	5	yes
	15	Black	30	M	7	4	yes
	16	Black	56	F	17	6	yes
	17	Coloured	37	F	9	4	yes
		Mean ± SD	41 ± 11	--	10 ± 7	5.0 ± 0.9	--

Table 5-1 Demographic and radiographic characteristics of the study subjects.

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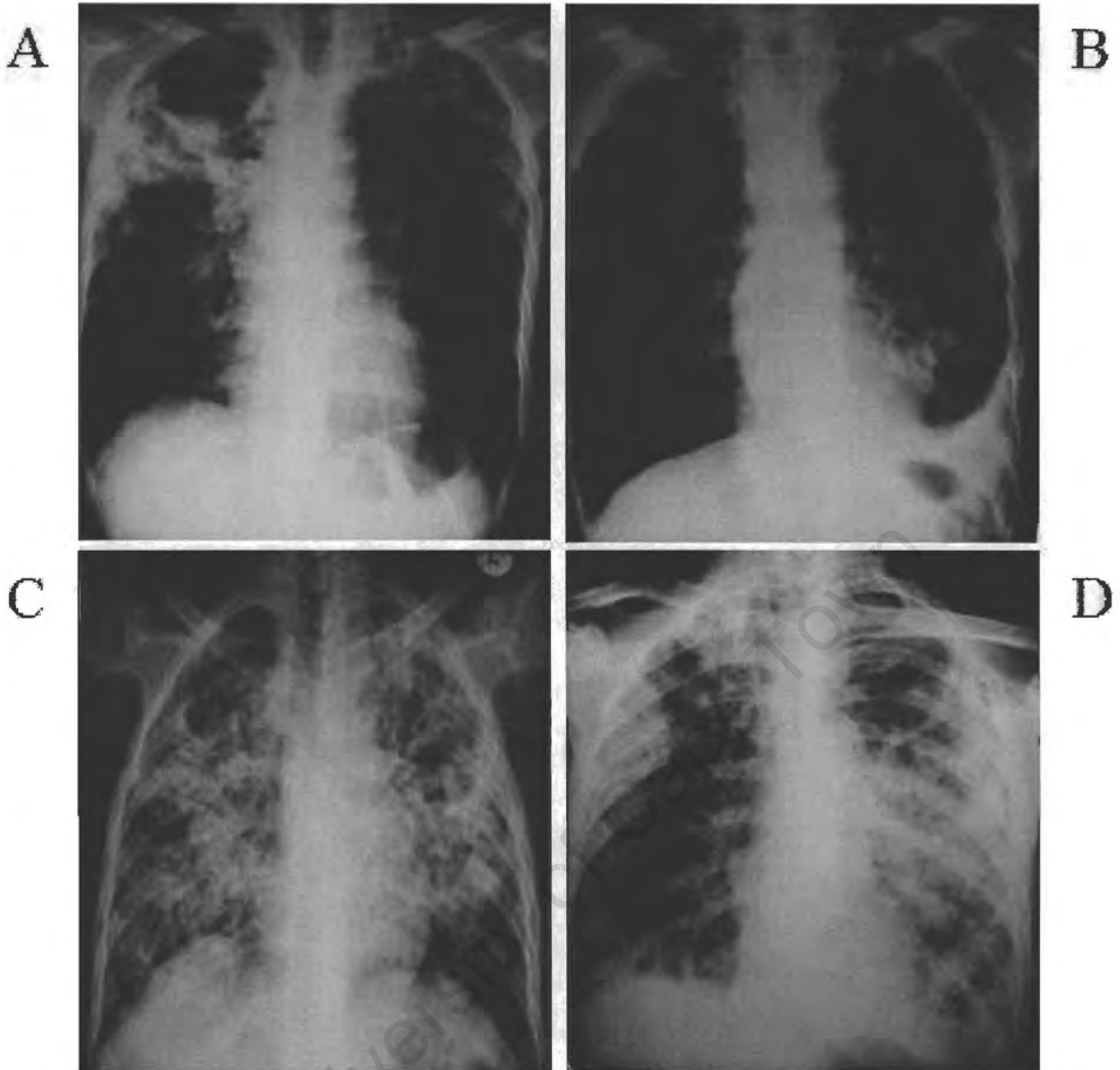

Figure 5-1 Examples of pre-treatment chest radiographs of patients classified as having limited (A, B) or extensive (C, D) tuberculosis disease.

(Figures 5-1C and 5-1D). The mean number of lung zones involved in the group of patients with limited disease was 2.1 and in patients with extensive TB it was 5.0 ($p<0.0001$).

Plasma cytokine levels in patients with TB. Cytokine levels in plasma from patients with limited or extensive TB were measured by ELISA. IL-12 levels were 2-fold higher in patients with limited pulmonary disease compared to patients who had extensive TB ($p=0.02$) (Figure 5-2A). IL-6 and IL-10 levels were similar in the plasma of patients with limited and extensive disease. However, levels of TNF-α were somewhat higher ($p=0.12$) in plasma of patients with limited TB (Figure 2B). These results suggest that IL-12, and possibly TNF-α production may be increased in TB patients who have less extensive pulmonary disease.

Cytokine production by monocytes from patients with limited or extensive TB. Peripheral blood monocytes from some of the patients were infected with the MTB clinical isolates CDC1551 or HN878 *in vitro*, and release of IL-12 and TNF-α into the culture supernatants was measured. Up to 5 fold higher IL-12 production was observed when monocytes from patients with limited disease were infected with CDC1551 or HN878 (Figure 5-3A) compared to monocytes from patients with extensive disease ($p<0.05$). Monocytes from patients with limited disease also produced higher amounts of TNF-α ($p<0.05$) and IL-6 ($p<0.005$) (Figure 5-3B and 5-3C). When monocytes of the same patients were compared, infection with CDC1551 resulted in more IL-12 ($p=0.00003$), TNF-α ($p=0.009$), and IL-6 ($p=0.0005$) production than infection with HN878.

Replication of *M. tuberculosis* in monocytes from TB patients. We next examined the intracellular growth of MTB *in vitro* in monocytes from patients with limited or extensive disease. Growth rates were calculated from 24 h to 48 h post infection. Substantially faster growth of MTB was observed in monocytes obtained from patients with extensive disease. Mean replication times of CDC1551 within the monocytes from patients with limited disease

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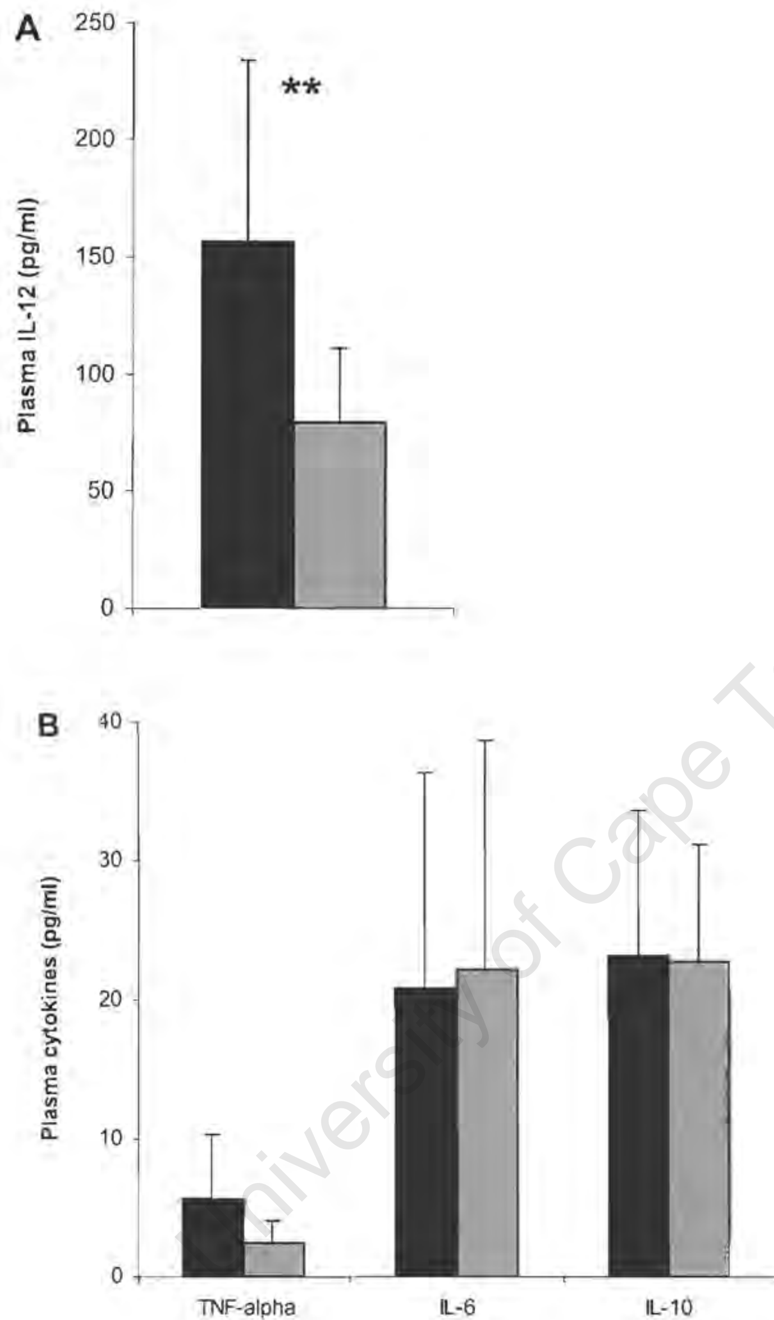

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Figure 5-2 Plasma cytokine levels in patients with a pulmonary TB who have limited disease (n=8, black bars) or extensive disease (n=9, gray bars) on chest radiograph. Error bars indicate standard deviations. IL-12 levels in patients with limited disease were increased compared to patients with extensive TB (p=0.02).

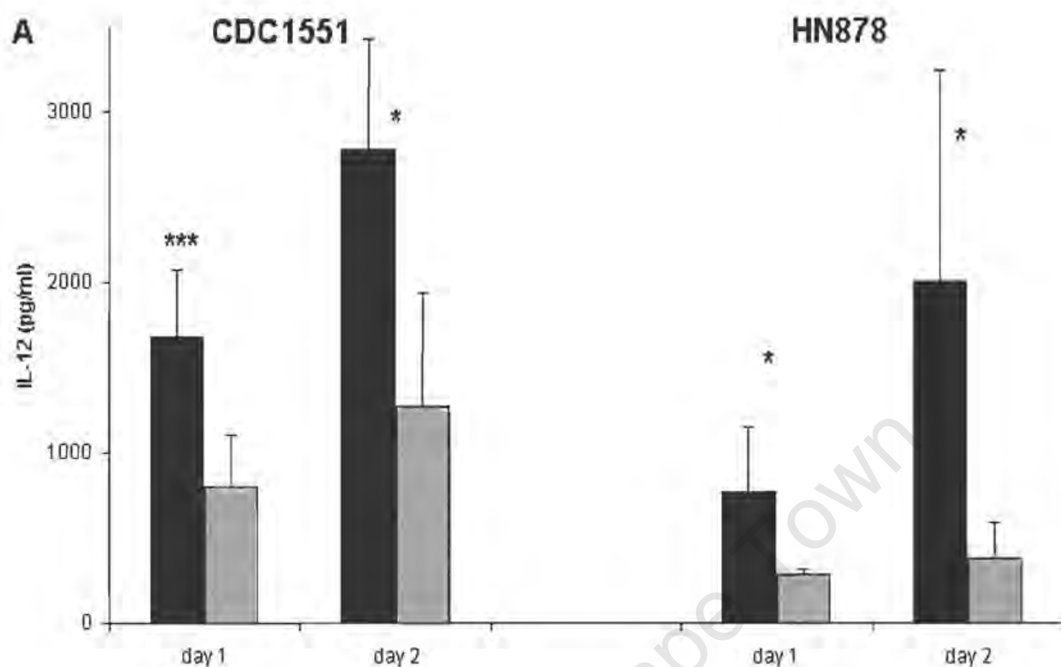

Figure 5-3 IL-12 (A), TNF-α (B) and IL-6 (C) production by freshly isolated monocytes from patients with limited disease (black bars) or from patients with extensive disease (gray bars), infected with MTB strains CDC1551 or HN878 *in vitro*. Supernatants from triplicate cultures were harvested on Day 1 and Day 2 post infection. The graphs display mean cytokine production by infected monocytes in a single representative experiment, using 2 patients with limited TB and 2 patients with extensive TB as a source of monocytes. Error bars indicate standard deviations.

Significance levels: * $p<0.05$, ** $p<0.01$ and *** $p<0.005$.

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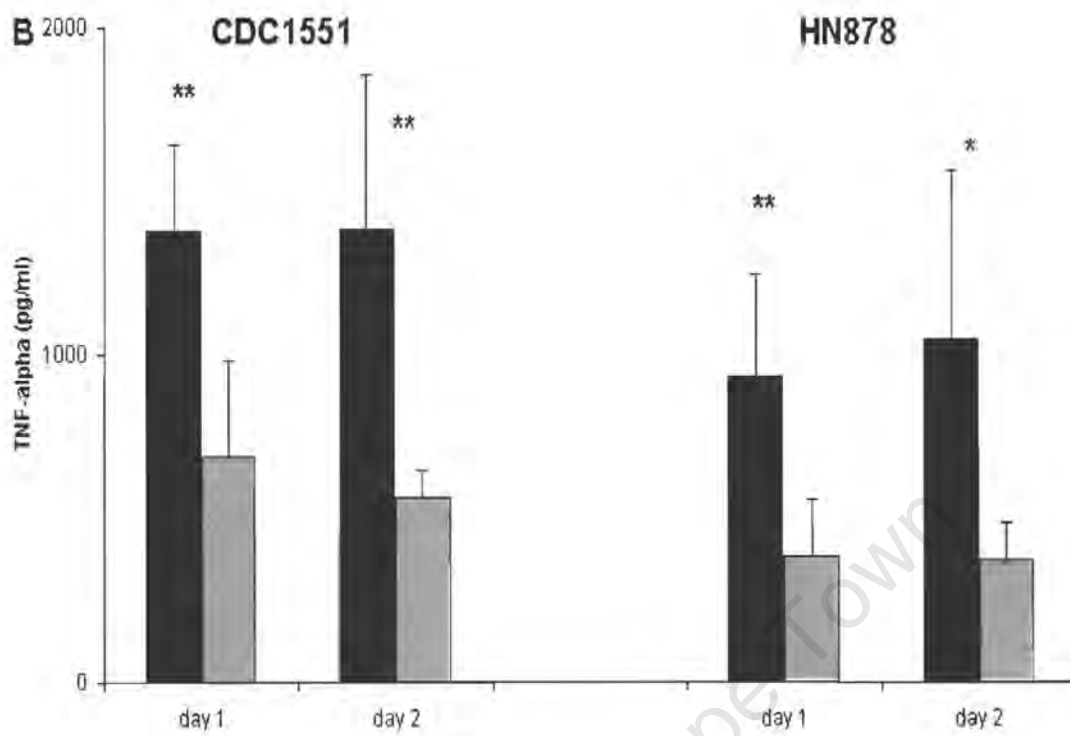

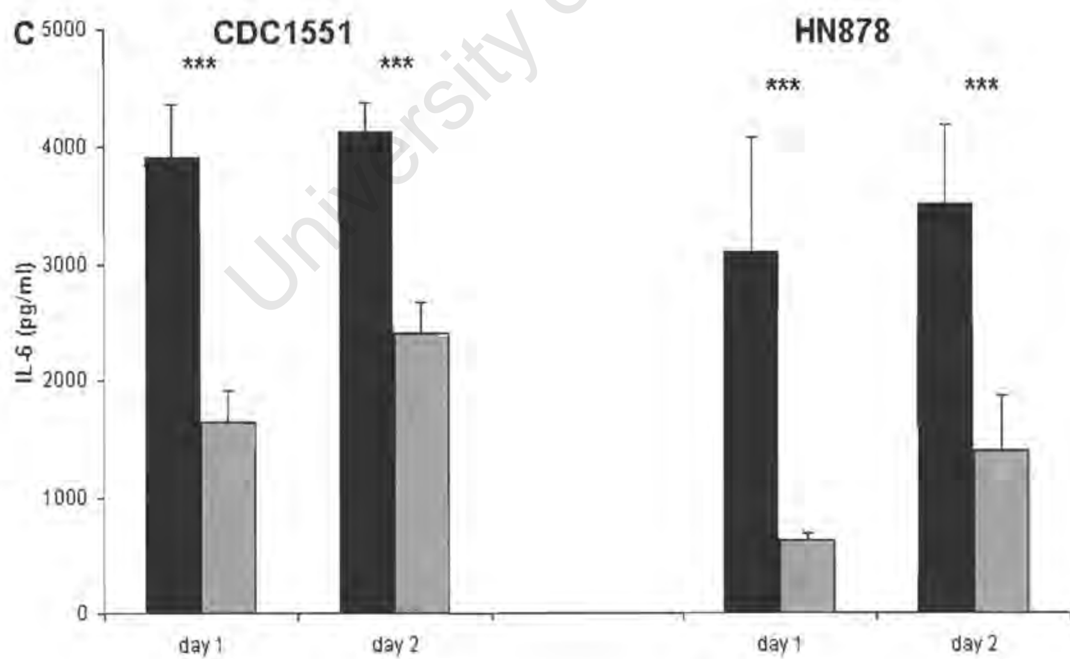

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or extensive TB were 32.1 h and 20.3 h respectively (p=0.04); mean replication times of HN878 were 21.1 h and 16.6 h respectively (p=0.01) (Figure 5-4). When the intracellular growth of CDC1551 and HN878 was directly compared within monocytes of the same donor, HN878 was noted to replicate significantly faster (p=0.007).

5.4 DISCUSSION

In this study, we observed that more extensive pulmonary TB disease (more extensive lung involvement on chest radiograph) was associated with lower pro-inflammatory cytokine (IL-12, TNF-α and IL-6) production by monocytes in response to MTB. In addition, more extensive disease was associated with a reduced ability of patient monocytes to control mycobacterial replication. Faster replication within patient monocytes was also observed for the MTB isolate that induced lower cytokine production by these cells. Thus, an impaired ability of the host to mount a pro-inflammatory cytokine response and restrict mycobacterial growth *in vitro*, or infection with an MTB strain that induces low levels of these cytokines, may constitute risk factors for developing more severe disease.

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The observation that a less robust cytokine response may be detrimental to the host is supported by a number of prior studies. Mice display more severe disease following infection with MTB strains that induce low levels of IL-12 and TNF-α (Manca, Tsenova, Barry, *et al.*, 1999; Manca, Tsenova, Bergtold, *et al.*, 2001). Patients with smear positive TB have been shown to have lower serum IL-12 levels than patients who have paucibacillary, smear negative disease (Verbon, Juffermans, Van Deventer, *et al.*, 1999). Also, patients with advanced pulmonary TB have been shown to have lower serum interferon-gamma (IFN-γ) levels than patients with mild or moderate disease as determined from chest radiograph (Dlugovitzky, Torres-Morales, Rateni, *et al.*, 1997). Lymphocytes from patients with more advanced disease also produced less IFN-γ in response to heat killed MTB (Sodhi, Gong,

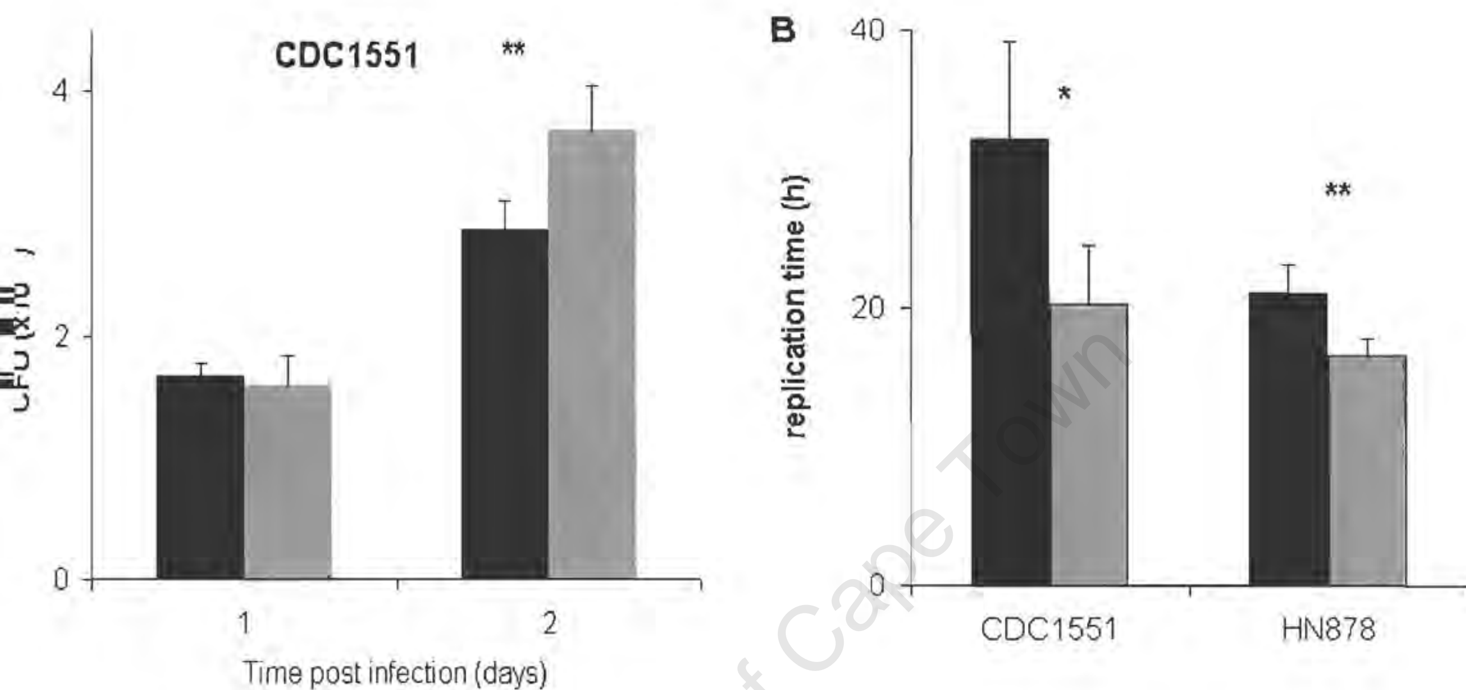

Figure 5-4 Intracellular growth of MTB in monocytes from patients with limited (n=5, black bars) or extensive (n=3, gray bars) TB. Faster growth of MTB CDC1551 and HN878 was observed in monocytes from patients with extensive TB. This resulted in higher numbers of colony forming units on Day 2 (A) and shortened replication times (B). Error bars indicate standard deviations; p values indicate significance levels: * $p<0.05$, ** $p<0.01$.

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Silva, *et al.*, 1997). Thus, in mice and humans, activation of IL-12 production and signaling through the IL-12 receptor pathway may result in enhanced cell-mediated immunity and reduced TB disease. Indeed, administration of IL-12 to MTB infected mice reduces the bacterial load in infected tissues and improves survival (Flynn, Goldstein, Triebold, *et al.*, 1995). Recently, IL-12 administration was also shown to be beneficial in a patient with disseminated TB who was refractory to chemotherapy (Greinert, Ernst, Schlaak, *et al.*, 2001).

Several pathogens, including measles virus, *Histoplasma capsulatum*, *Leishmania major*, and human immunodeficiency virus have developed mechanisms to impair the host IL-12 response (Mosser and Karp, 1999; Nau, Richmond, Schlesinger, *et al.*, 2002). Two such mechanisms have been described for MTB. Mannosylated lipoarabinomannan (ManLAM), a compound of the mycobacterial cell wall, was shown to inhibit IL-12 and TNF-α induction in LPS-stimulated human mononuclear phagocytes through increased SHP-1 tyrosine phosphatase activity (Knutson, Hmama, Herrera-Velit, *et al.*, 1998). In addition, the 19-kDa lipoprotein, an antigen secreted by MTB, was shown to inhibit IL-12 and TNF-α production by recombinant *M. smegmatis* (Post, Manca, Neyrolles, *et al.*, 2001). Of interest, both the 19kDa antigen and ManLAM required acyl moieties to inhibit cytokine production (Nigou, Zelle-Rieser, Gilleron, *et al.*, 2001; Post, Manca, Neyrolles, *et al.*, 2001). It is possible that monocytes from patients with limited and extensive TB display differential susceptibility to the inhibitory effects of these MTB molecules on cytokine production. Alternatively, impaired TNF-α and IL-6 production by monocytes from patients with extensive disease may have resulted in decreased cellular activation and impaired innate resistance to intracellular mycobacterial growth (Flesch and Kaufmann, 1990; Hirsch, Ellner, Russell, *et al.*, 1994).

One previous study reported higher IL-12 and TNF-α concentrations following *in vitro* stimulation of PBMC isolated from patients with advanced TB compared to cells from

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patients with moderately advanced disease (Dlugovitzky, Bay, Rateni, *et al.*, 2000). Plasma cytokine levels were not measured in that study nor were patients with early or limited disease included. In the present study, the inclusion of patients across a wider age spectrum, the use of a different clinical and radiographic classification and alternative methods for *in vitro* cytokine induction, may have contributed to an apparent difference between our results and the observations made in the study by Dlugovitzky *et al.* (Dlugovitzky, Bay, Rateni, *et al.*, 2000). Earlier observations by these authors, however, suggested that patients with advanced TB have elevated serum IL-10 levels (Dlugovitzky, Torres-Morales, Rateni, *et al.*, 1997), an inhibitory cytokine that may have contributed to the impaired IL-12 production observed in our patients with advanced disease (Trinchieri, 1997).

The HN878 isolate is a member of the MTB W-Beijing family of strains (Bifani, Mathema, Kurepina, *et al.*, 2002). Epidemiological surveys suggest that isolates belonging to the W-Beijing family may be more transmissible. It is interesting to observe that *in vitro* infection of patient monocytes with the HN878 strain resulted in impaired cytokine production and faster intracellular replication. Our study raises the possibility that patients infected with specific W-Beijing isolates may have more extensive lung disease and an increased bacillary burden. Increased numbers of bacilli in sputum would favor more efficient transmission of these MTB isolates.

The optimal cytokine response that follows infection with MTB and results in protective immunity in most individuals remains to be defined. Studies comparing innate immune responses to MTB in latently infected persons and in patients with reactivation disease may elucidate whether impaired IL-12 and TNF-α responses are also associated with progression from latent TB infection to clinically overt tuberculosis.

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Chapter 6

Growth of *Mycobacterium tuberculosis* at the surface of the cavity reflects a microenvironment with failed immunity

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6.1 ABSTRACT

Protective immunity against pulmonary tuberculosis (TB) is characterized by the formation of granulomas consisting of macrophages and activated T-cells producing tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), both of which are required for the activation of the phagocytes. In 90% of immune competent humans, this response controls the infection. To understand why immunity fails in the other 10% we studied the lungs of six patients who underwent surgery for incurable TB. Histologic examination of different lesions of the affected lungs revealed numerous acid-fast bacilli only at the luminal surface of the cavities. The mutation profile of these isolates suggested that a single founder strain of *M. tuberculosis* (MTB) may undergo genetic changes during treatment, leading to the acquisition of drug resistance independently in discrete physical locales. Additional drug resistance was preferentially observed at the cavity surface. An analysis of cytokine gene expression revealed that failure to control the bacilli was not associated with a generalized suppression of cellular immunity since cytokine mRNA was up regulated in all lesions tested. Rather, a selective absence of $CD4^+$ and $CD8^+$ T-cells was noted at the luminal surface of the cavity, thereby preventing direct T-cell-macrophage interactions allowing phagocytes to remain permissive for bacillary growth. In contrast, at the other side of the necrotic zone of the granulomas, the two cell types were co-localized, and the paucity of bacilli in these macrophages suggests that in this microenvironment an efficient bacteriostatic/bacteriocidal phagocyte population was generated. The observed blunting of MTB stress responses in the bacilli at the surface of the cavity further supports the concept that this area represents a microenvironment with failed anti-mycobacterial immunity.

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6.2 INTRODUCTION

Mycobacterium tuberculosis is an extremely successful pathogen spreading from individual to individual via the aerosolization of infectious nuclei droplets. The infectious particles are released from the lungs of patients with cavitary pulmonary disease while coughing. Once inhaled and phagocytosed by resident alveolar macrophages, the tubercle bacilli elicit the production of soluble effector molecules including the cytokines TNF-α and interleukin 12 (IL-12) as well as a large number of chemokines (Henderson, Watkins and Flynn, 1997; Manca, Tsenova, Barry, *et al.*, 1999; Roach, Bean, Demangel, *et al.*, 2002). These molecules regulate the development of the host cellular immune response that presumably controls the infection in the majority (90%) of immunocompetent individuals (Bloom and Murray, 1992).

Protective immunity is characterized by the formation of granulomas at the site of infection. These tumor-like aggregates consist primarily of activated MTB-infected macrophages and T-cells. In the mouse model of MTB infection, the maturation and maintenance of granulomas, and the control of bacillary replication within macrophages is dependent upon the continued production of TNF-α and IFN-γ by macrophages and activated T-cells respectively (Bean, Roach, Briscoe, *et al.*, 1999; Cooper, Dalton, Stewart, *et al.*, 1993; Flynn, Chan, Triebold, *et al.*, 1993; Flynn, Goldstein, Chan, *et al.*, 1995), and the expression of inducible nitric oxide synthase (iNOS) in infected macrophages (Chan, Xing, Magliozzo, *et al.*, 1992; MacMicking, North, LaCourse, *et al.*, 1997). In mice, $CD4^+$ T-lymphocytes are the principal mediators of resistance to TB, and $CD8^+$ T-lymphocytes have been shown to contribute to this resistance (Mogues, Goodrich, Ryan, *et al.*, 2001). An important role for $CD4^+$ T-cells in protecting the human host from TB is underscored by the marked susceptibility to TB of patients with advanced HIV-induced $CD4^+$ T-cell depletion (Daley, Small, Schecter, *et al.*, 1992; Di Perri, Cruciani, Danzi, *et al.*, 1989; Selwyn, Hartel, Lewis, *et al.*, 1989). In addition, human $CD4^+$ T-cells and $CD8^+$ T-cells may induce death of

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MTB infected macrophages, resulting in reduced viability of the bacilli (Dieli, Troye-Blomberg, Ivanyi, *et al.*, 2000; Oddo, Renno, Attinger, *et al.*, 1998; Stenger, Mazzaccaro, Uyemura, *et al.*, 1997).

In 10% of immunocompetent persons, the infection is not contained by the host immune response. Why protective immunity fails in these individuals is not understood. Progressive bacillary replication results in disease manifestations, tissue necrosis and cavity formation (Medlar, 1955). The host immune response directed at the infecting bacilli is believed to be the main cause of tissue necrosis, which may be the result of cytokine-mediated toxicity as well as the release of activated proteolytic enzymes by macrophages (Chang, Wysocki, Tchou-Wong, *et al.*, 1996; Condos, Rom, Liu, *et al.*, 1998; Schluger and Rom, 1998). Most TB patients, however, respond to antibiotic treatment by clearance of the bacilli from the sputum, partial reversal of the granulomatous inflammatory process, and successful clinical cure. In patients that fail to respond to chemotherapy, including patients with multi-drug resistant (MDR) TB, chronic progressive disease may be observed. Pneumonectomy is sometimes employed in these patients in an attempt to reduce the bacillary load in the lungs, to achieve sputum conversion, and/or to reduce spread of the infection to the remaining healthy lung. In others, surgery may be performed to reduce the life threatening complications of TB such as severe hemoptysis (Blumberg, Burman, Chaisson, *et al.*, 2003; van Leuven, De Groot, Shean, *et al.*, 1997).

To better understand the etiology of progressive chronic TB, we studied the lungs of six patients who underwent surgery for incurable TB and/or complications related to their TB. The excised lung tissue from these patients provided a unique opportunity to study the pathogenic process that occurs during long-term antibiotic therapy and disease progression. By studying the histology, immunohistology, host cellular immune response, and by

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characterizing the bacterial populations present as well as the gene expression of MTB in situ, we hoped to gain insight into the dynamics of the infectious process.

6.3 RESULTS

Patients. Six patients who underwent pulmonary resection for complications arising from TB were studied. All six patients had unilateral lung disease with (almost) complete destruction of the affected lung (Figure 6-1). Three patients (two of whom had MDR-TB) underwent surgery for chronic, sputum positive TB despite 18-24 months of supervised multi-drug therapy (Table 6-1). The other three patients who were culture negative at the time of surgery all had a history of pulmonary tuberculosis. These patients had presented with hemoptysis and other symptoms suggestive of relapse, and had received empirical treatment for TB for 7-15 months. Lung resection in these patients was performed to relieve ongoing hemoptysis thought to have arisen from a focus in the destroyed lung. Sputum cultures from these three patients at the time of presentation, however, failed to yield *M. tuberculosis* (Table 6-1). All patients received TB therapy up until the time of surgery, and none of the patients was co-infected with HIV.

Presence of mycobacteria in lesions obtained from the resected lungs. The lungs of all six patients revealed evidence of tuberculosis, with cavitation, fibrosis, nodules, granulomas and bronchiectasis. Macroscopic caseation (Figure 6-1) was only observed in patients with active tuberculosis (Patients #1-3, Table 6-1). Dissection of the lungs allowed us to identify and sample a number of macroscopically distinct lesions (2-6 per patient) and, in some cases, lung tissue which appeared uninvolved (Table 6-2). Each lesion was examined for the presence of acid-fast bacilli (AFB), and categorized according to its continuity with the airways. AFB were almost exclusively observed in the lesions of patients with sputum-

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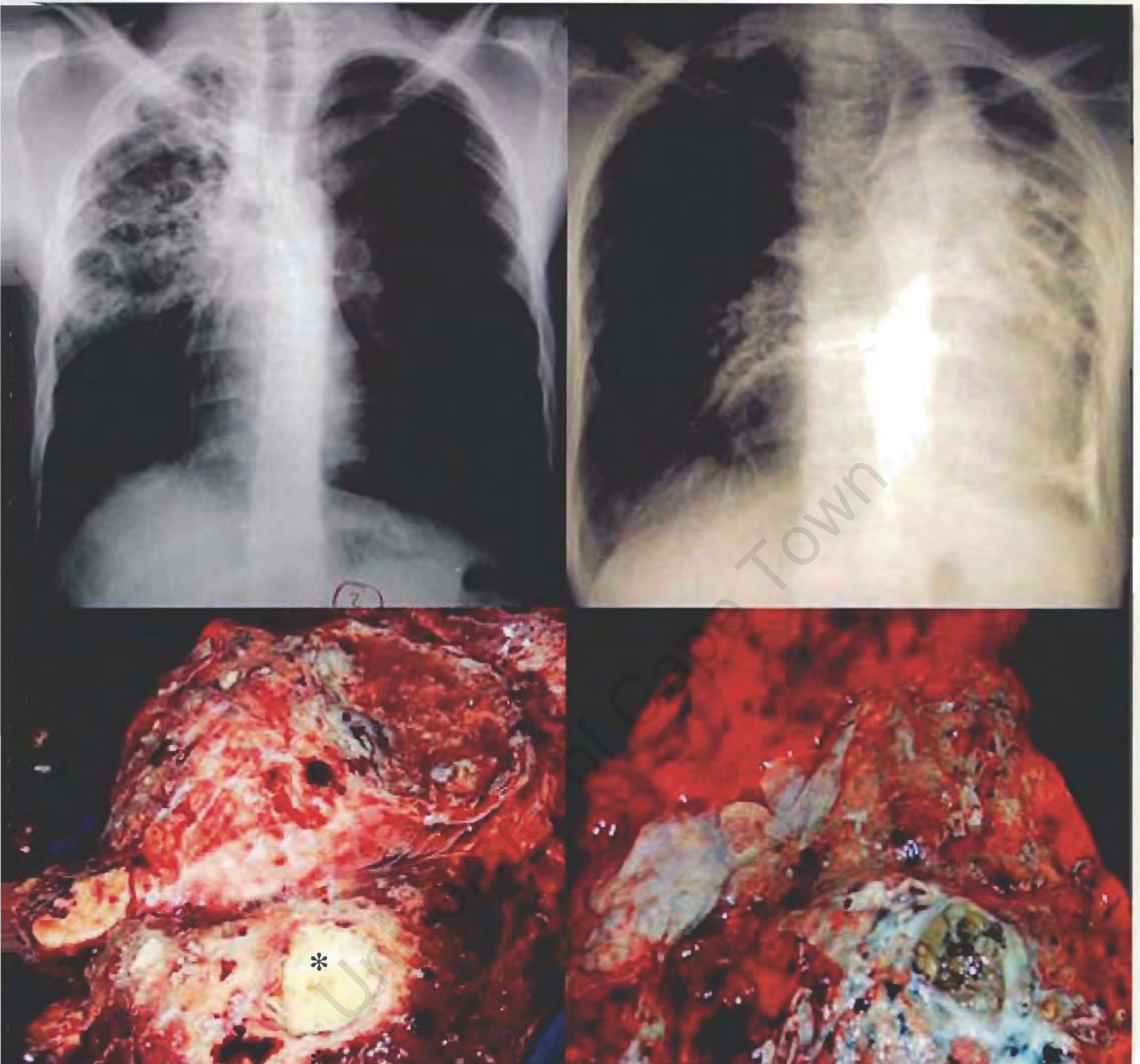
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Figure 6-1 Pre-operative chest radiographs and resected lungs of patients #2 (left) and #5 (right). The resected lungs were almost entirely destroyed. Both lungs contained areas of cavitation, fibrosis, nodules and granulomas with central necrosis, but only the lungs of patients with active disease contained caseous necrosis (*).

Patient #	Age	Gender	Anti-TB Treatment at Surgery		Sputum**		Indication	Macroscopic			
			Drugs	Months	Smear	Culture	for Surgery	Description	Caseation	Cavitation	Fibrosis
1	19	F	R H Z E S	18	pos	pos	persistent TB	destroyed L lung	Y	Y	Y
2*	17	M	R H Z E S Et Th Km Of	18	pos	pos	persistent TB	destroyed R lung	Y	Y	Y
3*	35	M	R H Z E Et Th Km Of Cl	24	pos	pos	persistent TB	destroyed L lung	Y	Y	Y
4	54	M	R H Z E	7	neg	neg	hemoptysis	destroyed L lung	N	Y	Y
5	59	F	R H Z E	9	neg	neg	hemoptysis	destroyed L lung	N	Y	Y
6	45	M	R H Z E	15	neg	neg	hemoptysis	destroyed L lung	N	Y	Y

*MDR-TB; **at the time of surgery

R=rifampin; H=isoniazid; Z=pyrazinamide; E=ethambutol; S=streptomycin; Et=ethionamide; Th=thiacetazone; Km=kanamycin; Of=ofoxacin; Cl=clofazamine

Table 6-1 Characteristics of the patients and classification of their TB disease.

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positive disease. *M. tuberculosis* was cultured from all lesions of sputum-positive patients, and from none of the lesions of sputum negative subjects.

Genetic analysis of *M. tuberculosis* cultures obtained from the lesions of the resected lungs. Genetic analysis was carried out on all isolates cultured from pre-operative sputum samples and lung lesions. IS*6110*-based RFLP analysis revealed a homogenous bacterial population and no evidence of mixed infection in each of the three patients with active disease (Figure 6-2). In contrast, sequence analysis of several genes implicated in drug resistance revealed heterogeneity in the resistance-associated alleles among the isolates recovered from different lung lesions of the same patient (Table 6-2). For example, the isolate cultured from the upper lobe cavity of patient #1 contained the K43R resistance mutation in the genetic target of streptomycin (*rpsL*), whereas the wild type gene was present in the isolate obtained from sputum as well as a closed, lower lobe granuloma (Table 6-2). Moreover, in the lung from patient #2, two mutations in the fluoroquinolone resistance associated gene *gyrA* (D89N and D94G) were found in all 3 isolates obtained from the open lesions, whereas the bacilli from the sputum and the 2 closed lesions bore wild type *gyrA* alleles. Most strikingly, patient #3 had three discrete bacillary populations identified with different alleles of the resistance-associated genes. Apparently normal lung tissue from this patient contained few *M. tuberculosis* isolates and these bore the wild type alleles in the *katG* (a target for INH), *embB* (a target for EMB) and *rrs* (a target for amino glycosides) genes. Bacteria isolated from the sputum of patient #3 and from four other pathologic sites of the lung had identical *katG* (S315T) and *embB* (M306V) mutations but were wild type for *rrs*. A sixth site contained bacilli that, in addition to the *katG* and *embB* mutations, had acquired a resistance mutation (G1484T) in the *rrs* gene. The mutation profiles of these isolates suggest that the acquisition of drug resistance is a dynamic process whereby an initial infecting strain may spread from one pulmonary site to another becoming the founder for acquisition of

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Patient #	Site	Histopathological examination		Bacteriology		Genotypic analysis of selected drug targets								mRNA expression			
		Lesion	Airway	AFB	Culture	*rpoB*	*katG*	*inhA*	*pncA*	*embB*	*rpsL*	*rrs*	*gyrA*	IL-2	IL-12	IFN-γ	iNOS
1	sputum	-	-	Numerous	*M.tuberculosis*	WT	WT	WT	WT	WT	WT	WT	WT	-	-	-	-
	LUL	Cavity wall (caseous necrosis)	Open	Numerous	*M.tuberculosis*	WT	WT	WT	WT	WT	**K43R**	WT	WT	120	190	5400	nd
	LLL	Cavity wall (caseous necrosis)	Open	Numerous	nd	-	-	-	-	-	-	-	-	100	220	6300	nd
	LLL	Granuloma (fibro-caseous)	Closed	Scanty	*M.tuberculosis*	WT	WT	WT	WT	WT	WT	WT	WT	85	415	5000	nd
	LN	Granuloma (non-necrotic)	Closed	None	nd	-	-	-	-	-	-	-	-	80	340	8700	nd
2	sputum	-	-	Numerous	*M.tuberculosis*	S531L	WT	C15T	WT	M306V	K88R	WT	WT	-	-	-	-
	RUL	Cavity wall (caseous necrosis)	Open	Moderate	*M.tuberculosis*	S531L	WT	C15T	WT	M306V	K88R	WT	**D89N**	107	244	3149	46
	RLL	Cavity wall (caseous necrosis)	Open	Numerous	*M.tuberculosis*	S531L	WT	C15T	WT	M306V	K88R	WT	**D94G**	89	345	1630	621
	RLL	Cavity wall (caseous necrosis)	Open	Scanty	*M.tuberculosis*	S531L	WT	C15T	WT	M306V	K88R	WT	**D89N**	28	136	1465	21
	RML	Small nodule (caseous)	Closed	Scanty	*M.tuberculosis*	S531L	WT	C15T	WT	M306V	K88R	WT	WT	32	240	1196	90
	RLL	Infarction (coagulative necrosis)	Closed	Scanty	*M.tuberculosis*	S531L	WT	C15T	WT	M306V	K88R	WT	WT	13	89	7243	135
3	sputum	-	-	Numerous	*M.tuberculosis*	S531L	S315T	WT	173 (T del)	M306V	K43R	WT	D94G	-	-	-	-
	LUL	Cavity wall (caseous necrosis)	Open	Moderate	*M.tuberculosis*	S531L	S315T	WT	173 (T del)	M306V	K43R	WT	D94G	80	199	7536	207
	LLL	Cavity wall (caseous necrosis)	Open	Scanty	*M.tuberculosis*	S531L	S315T	WT	173 (T del)	M306V	K43R	WT	D94G	92	290	715	119
	LLL	Fibrotic nodule (liquefaction)	Open	Moderate	*M.tuberculosis*	S531L	S315T	WT	173 (T del)	M306V	K43R	**G1484T**	D94G	80	388	1142	129
	LUL	Granuloma (caseous necrosis)	Closed	Moderate	*M.tuberculosis*	S531L	S315T	WT	173 (T del)	M306V	K43R	WT	D94G	175	421	1861	38
	LLL	Miliary nodules (caseous)	Closed	Scanty	*M.tuberculosis*	S531L	S315T	WT	173 (T del)	M306V	K43R	WT	D94G	64	78	202	181
	LLL	"Normal" lung tissue	-	Scanty	*M.tuberculosis*	S531L	**WT**	WT	173 (T del)	**WT**	K43R	WT	nd	64	138	605	274
4	LUL	Granuloma (non-necrotic)	Closed	None	Negative	-	-		-	-	-	-	-	101	197	857	96
	LUL	Granuloma (non-caseous)	Closed	Scanty	Negative	-	-		-	-	-	-	-	313	289	1201	103
	LLL	"Normal" lung tissue	-	None	Negative	-	-		-	-	-	-	-	78	107	362	373
5	LUL	Cavity wall (no necrosis)	Open	None	Negative	-	-		-	-	-	-	-	168	409	2407	102
	LLL	Granuloma (fibrotic)	Closed	None	Negative	-	-		-	-	-	-	-	76	325	2066	14
6	LUL	Cavity wall (no necrosis)	Open	None	Negative	-	-		-	-	-	-	-	87	234	1666	145
	LUL	Granuloma (caseous)	Closed	None	Negative	-	-		-	-	-	-	-	159	385	2541	115
	LLL	Miliary nodules (caseous)	Closed	Scanty	Negative	-	-		-	-	-	-	-	90	152	1321	270

LUL/RUL=Left/Right upper lobe; LN=lymphnode; nd = not done

Table 6-2 Results of the histopathological, microbiological and host mRNA analysis carried out on the different lung lesions.

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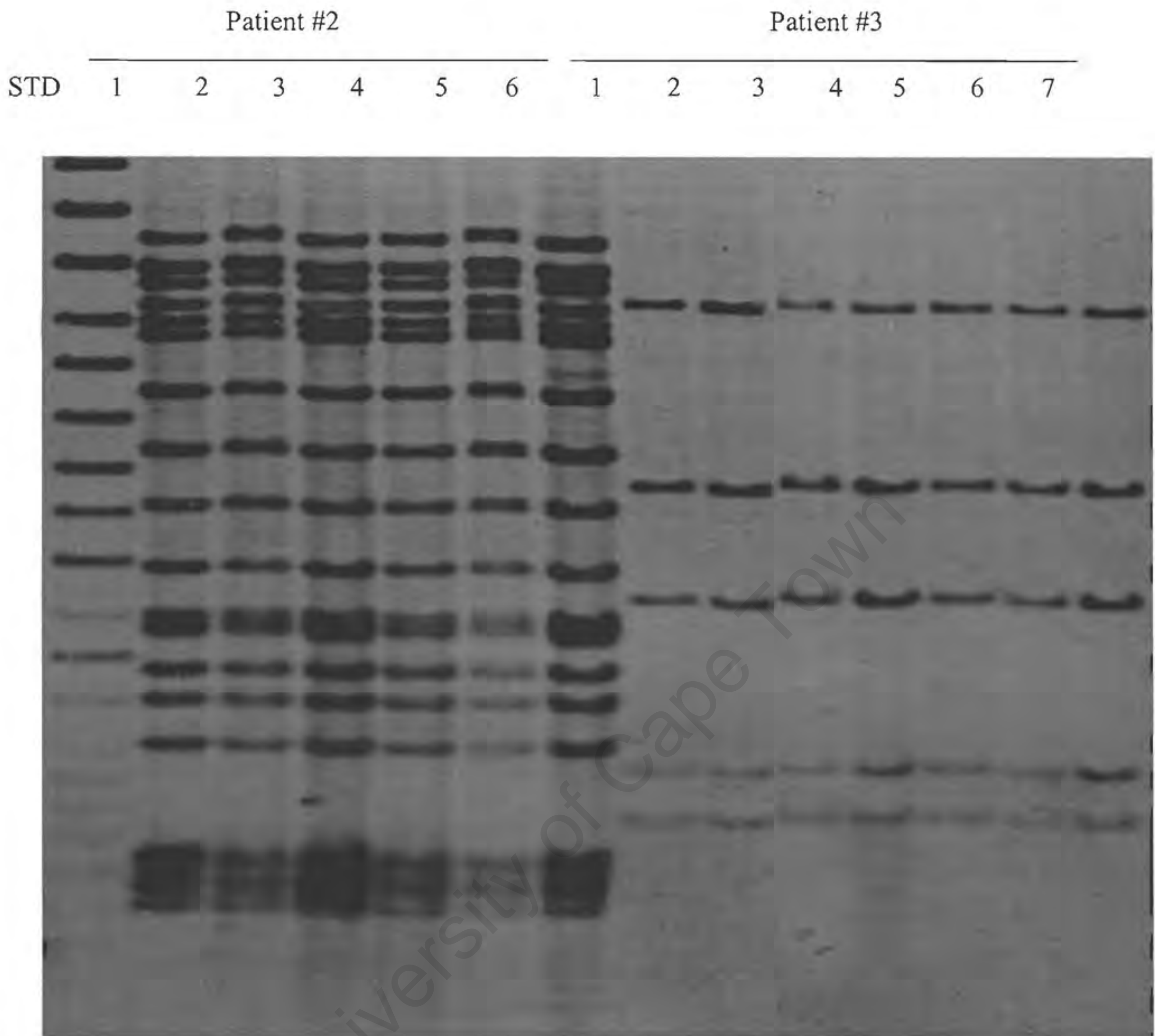

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Figure 6-2 IS*6110* Southern blot hybridization patterns of *M. tuberculosis* isolates recovered from multiple anatomical sites of the lungs of Patients #2 and #3. The six isolates from Patient #2 were recovered from (1) sputum, (2) right upper lobe open, (3) right lower lobe open, (4) right lower lobe open, (5) right middle lobe closed, and (6) right lower lobe closed lesions. The seven isolates from Patient #3 were recovered from (1) sputum, (2) left upper lobe open, (3) left lower lobe open, (4) left lower lobe open, (5) left upper lobe closed, (6) left lower lobe closed lesions, and (7) left lower lobe "normal" (see Table 6-2). STD refers to the molecular weight standard.

additional antibiotic resistance at secondary sites. Interestingly, in all three patients, the additionally acquired drug resistance mutations were seen in *M. tuberculosis* cultured from open lesions *i.e.* lesions connected to an airway (Table 6-2).

Cytokine and iNOS gene expression in the lesions of the resected lungs. The different lung samples obtained from all six patients were evaluated for expression of mRNA for IL-2, IL-12, IFN-γ, and iNOS. Cytokine and iNOS gene expression was observed in all lesions from patients with active TB as well as from those who had post tuberculous, culture negative lung disease (Table 6-2). No apparent correlation of the level of expression of the different genes and the type of lesion or the presence of AFB was observed. Rather, our results showed variable levels of immune activation in the tissue samples obtained from the different types of lesions of all subjects studied. This heterogeneity of cytokine expression suggests that the relatively large tissue fragments collected for study (about 0.5g each) contained a mixture of microenvironments rather than a single specific histologic microenvironment (see below). Our results also suggested that failure to control the growth of the bacilli was not associated with a global suppression of cellular immunity in the lungs of the three patients with chronic sputum positive disease.

Histopathologic analysis of lesions of the resected lungs. Examination of histopathology sections of the various lesions revealed that the luminal surface of the cavities in lungs from patients with sputum positive disease consisted of a superficial layer containing numerous mononuclear cells overlying a layer of caseous necrotic material. Subtending the necrotic layer there was granulomatous/fibrotic tissue with a mixed mononuclear cell infiltrate consisting of multi-nuclear (Langhans-type) giant cells, sheets of epithelioid macrophages and many scattered lymphocytes (Figure 6-3). In the closed (non-cavitary) necrotizing granulomas, the central area of caseous necrotic material was surrounded by fibrotic tissue and a multi-nuclear giant cells containing mononuclear cell infiltrate (Figure

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6-4). The lung tissue surrounding these granulomas frequently contained an increased number of mononuclear cells.

In the open granulomas, large numbers of apparently cell-associated bacilli were detected at the surface of the cavity (Figure 6-5D). The area of necrotic material had few, if any, bacilli, and the granulomatous/fibrotic layer with abundant macrophages and giant cells was essentially devoid of bacilli (Figure 6-5F). In the closed granulomas, occasionally, small to moderate numbers of AFB were observed in macrophages infiltrating the necrotic area, usually in areas with breakdown (Figure 6-5H). Thus, in the three patients with sputum-positive disease, AFB were most numerous at the luminal surface of the cavities *i.e.* in granulomas with a patent connection to the airways.

In comparison, in the patients that were sputum negative the surface of the cavities appeared inactive with re-epithelization over fibrotic tissue (not shown). Multiple mononuclear cells including multi-nucleated giant cells, epithelioid macrophages and lymphocytes were seen in the granulomatous/fibrotic tissue despite the absence of any visible AFB and the failure to grow bacilli from this tissue. The extensive cellular immune response may explain the high levels of expression of iNOS and cytokine genes in these lesions, suggesting the persistence of antigen in the absence of intact visible and/or culturable bacilli (Table 6-2).

Immunohistologic localization of macrophages and T-lymphocytes. Immunohistologic examination of lung sections revealed that $CD68^+$ macrophages were most abundant in the granulomatous/fibrotic area immediately below the necrotic layer, and at the cavity surface (Figure 6-5). In addition, scattered $CD68^+$ macrophages were seen among the fibroblasts of fibrotic areas and large numbers of alveolar macrophages were seen within the airspaces (not shown). The macrophages within the granulomatous/fibrotic layer appeared free of visible bacilli, while the macrophages at the cavity surface appeared to be infected

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Figure 6-3. Cross-section of an open granuloma (a TB lesion connected to an airway) of patient #1. The luminal surface of the cavity (*) consists of a superficial layer of mononuclear cells (E) which overlies a layer of caseous necrotic material (D,E). Subtending the necrotic layer there was granulomatous/fibrotic tissue with a mixed mononuclear cell infiltrate consisting of multi-nuclear giant cells (C), sheets of epithelioid macrophages (B) and many scattered lymphocytes (A).

(Original magnification: right hand panel x10; A-E x40)

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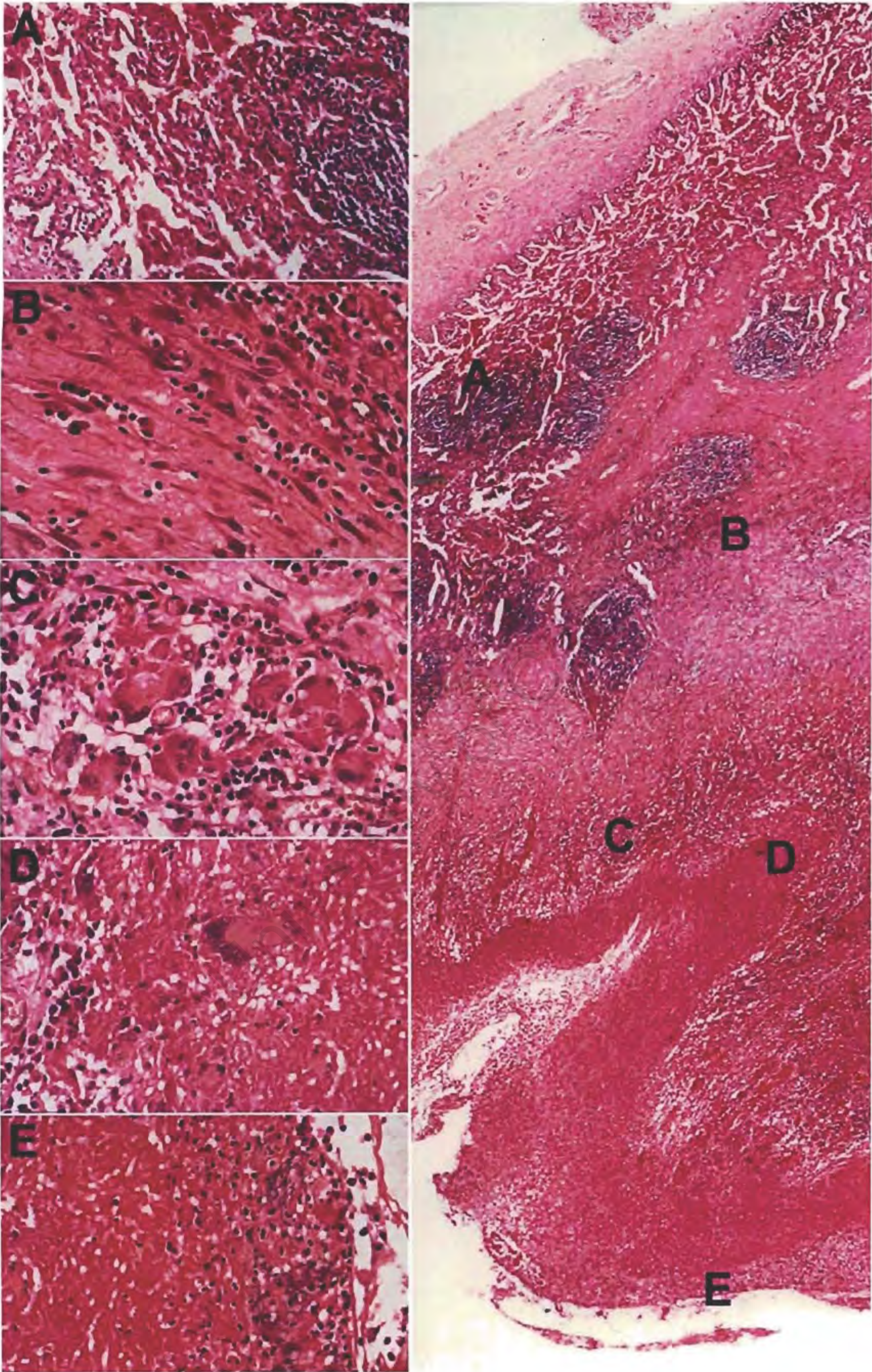
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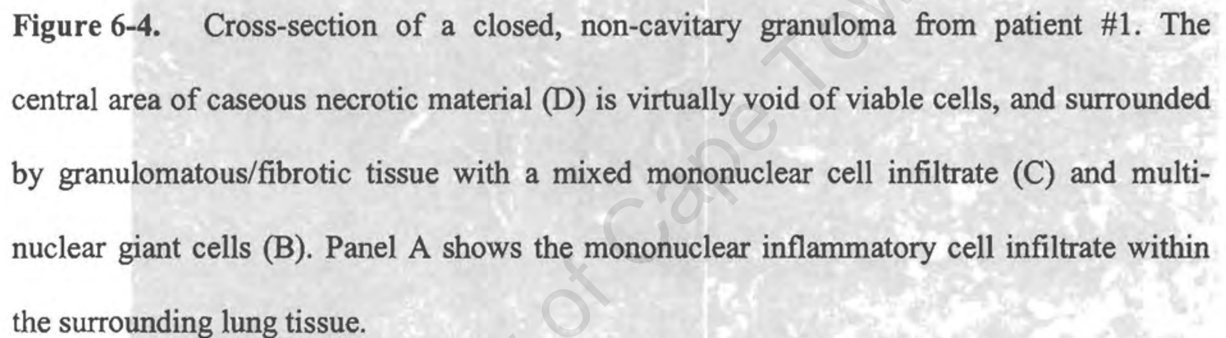

Figure 6-4. Cross-section of a closed, non-cavitary granuloma from patient #1. The central area of caseous necrotic material (D) is virtually void of viable cells, and surrounded by granulomatous/fibrotic tissue with a mixed mononuclear cell infiltrate (C) and multi-nuclear giant cells (B). Panel A shows the mononuclear inflammatory cell infiltrate within the surrounding lung tissue.

(Original magnification: right hand panel x10; A-D x40)

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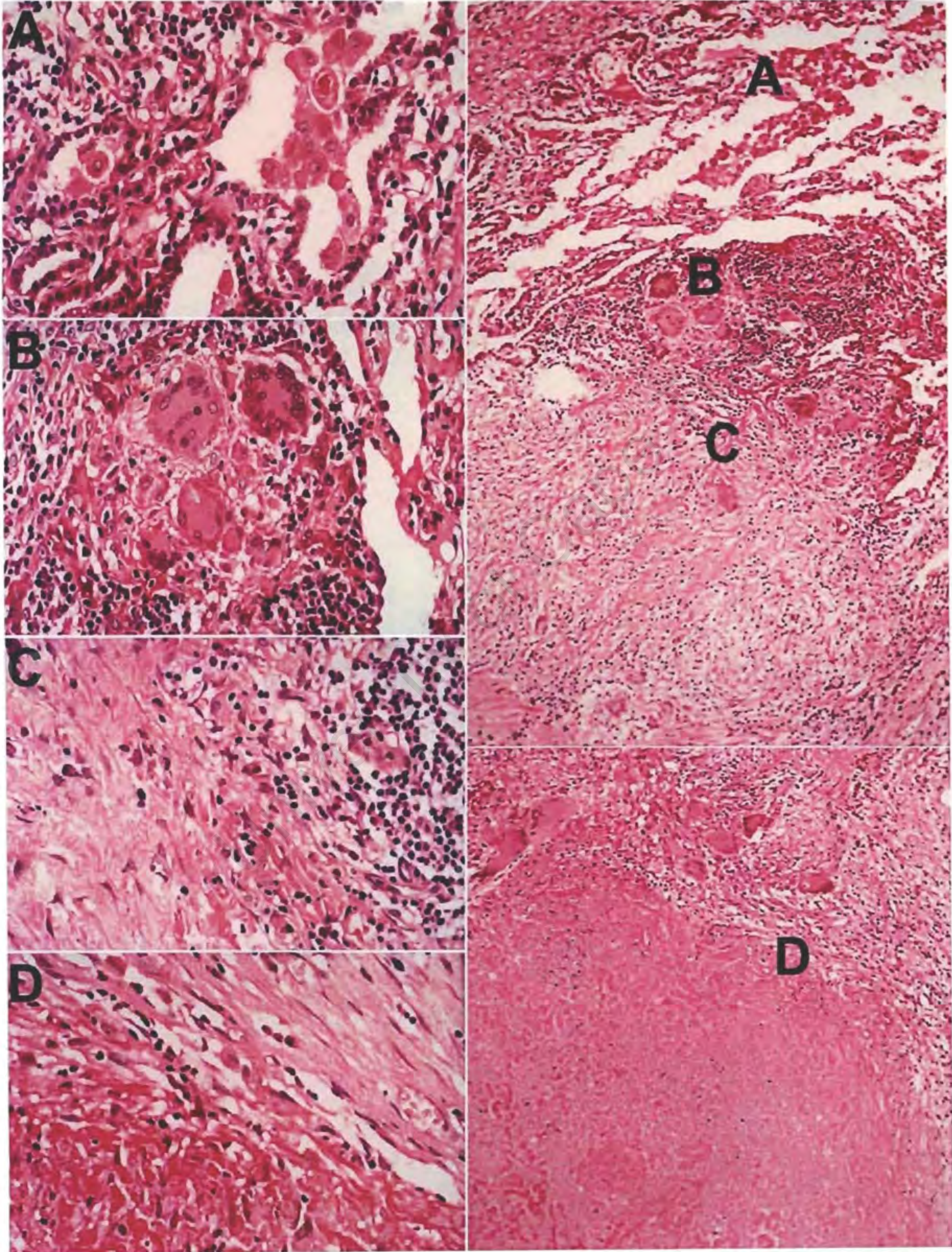
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C
D

Figure 6-5 The localization of macrophages and AFB in the resected lung lesions. Macrophages, stained with CD68 (A, C, E) and AFB stained with carbolfuchsin (B, D, F) in the LUL cavity wall of the open lesion of patient #1 are shown. Staining for macrophages is observed at the cavity (cav) surface (A and C), within the necrotic area (nec) (A and C), and in the granulomatous area below the necrotic area (E). AFB are seen predominantly within macrophages at the cavity surface (D) and not in the Langhans cells or macrophages of the granulomtous tissue (F). The insert in D shows AFB in cells at the luminal surface of the RLL cavity of patient #2. Few AFB are also seen in macrophages within the liquefied (liq) caseous necrotic (nec) center of the LLL fibrotic nodule of patient #3 (H).

(Original magnification: x10 in A and B; x40 in C, E, F and G; x200 in D and H)

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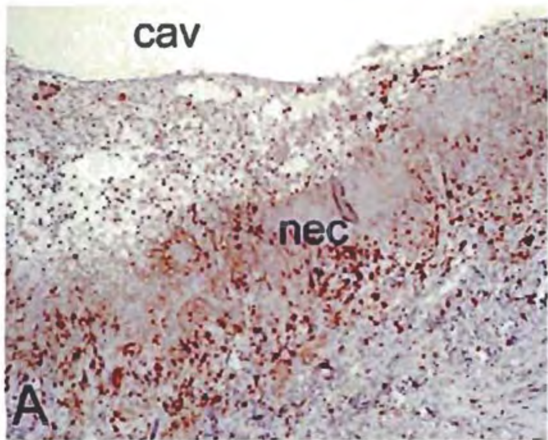
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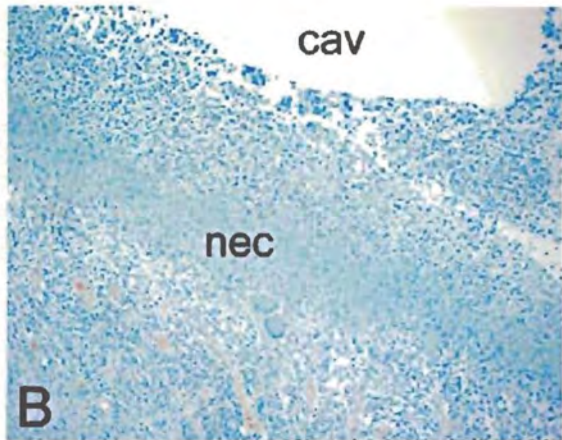
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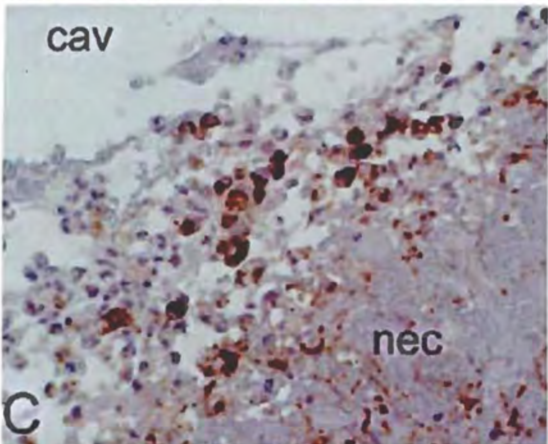
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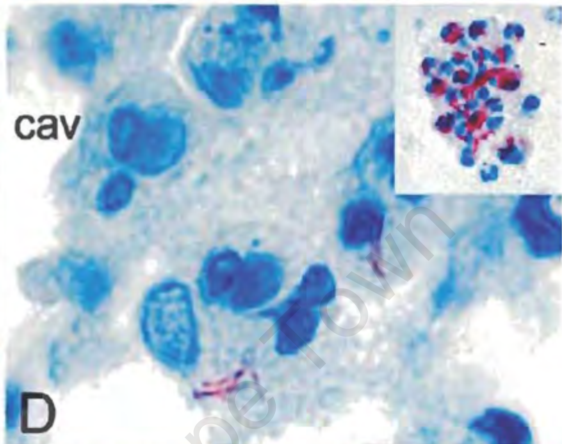
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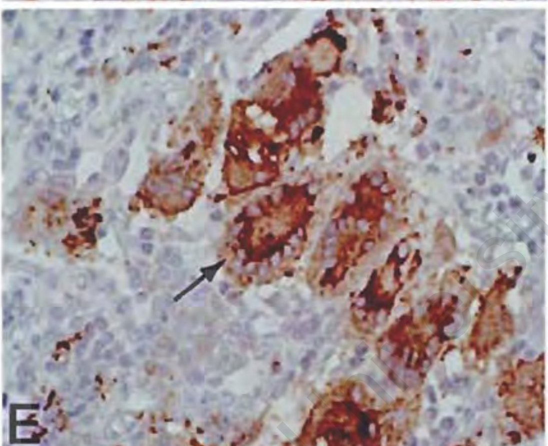
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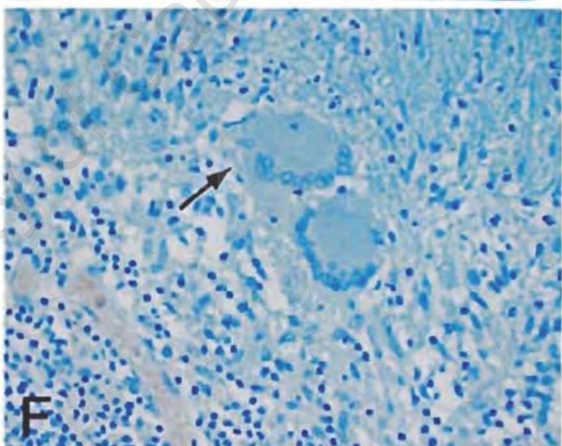
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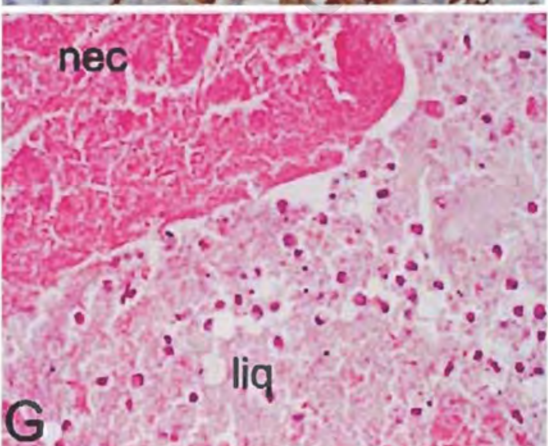
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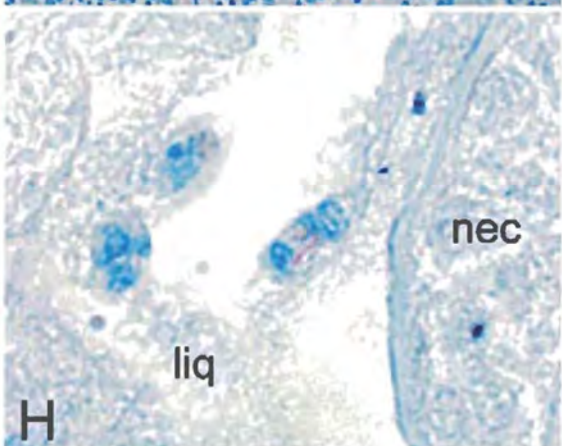
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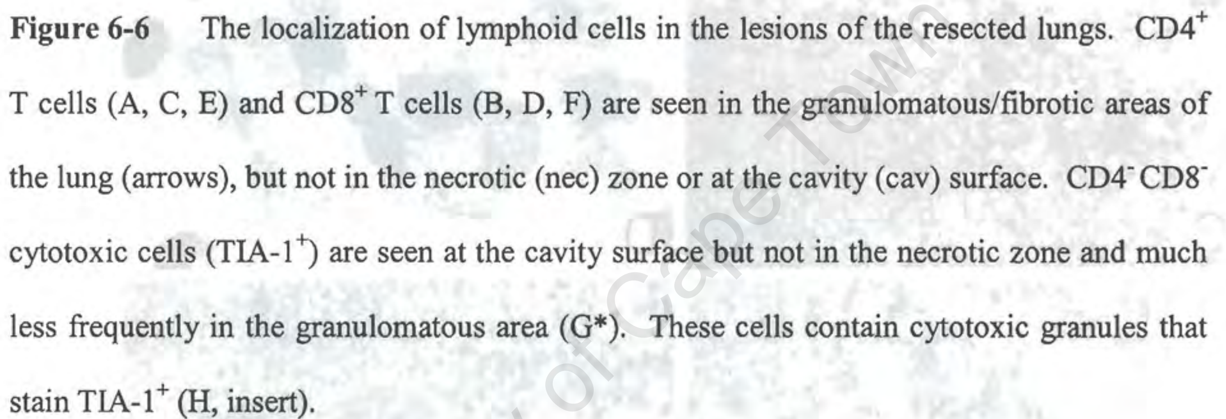

Figure 6-6 The localization of lymphoid cells in the lesions of the resected lungs. $CD4^+$ T cells (A, C, E) and $CD8^+$ T cells (B, D, F) are seen in the granulomatous/fibrotic areas of the lung (arrows), but not in the necrotic (nec) zone or at the cavity (cav) surface. $CD4^-CD8^-$ cytotoxic cells ($TIA\text{-}1^+$) are seen at the cavity surface but not in the necrotic zone and much less frequently in the granulomatous area (G*). These cells contain cytotoxic granules that stain $TIA\text{-}1^+$ (H, insert).

(Original magnificaiton: x4 in A, B and G; x40 in C, D, E, F and H; x100 in H, insert)

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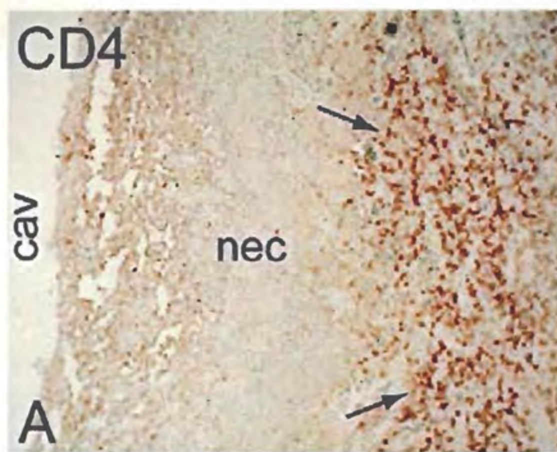
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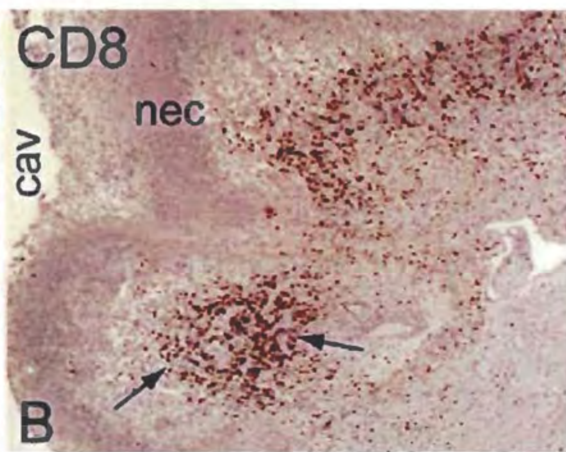
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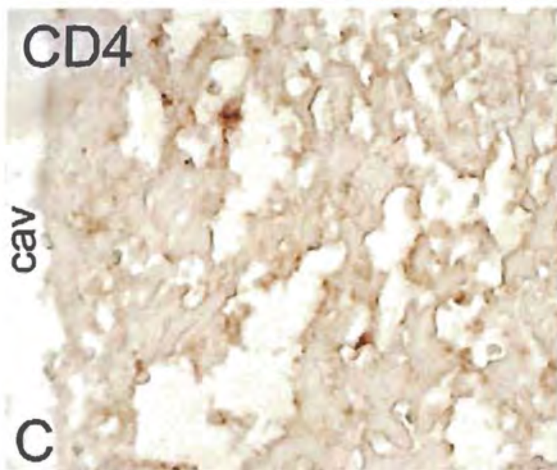
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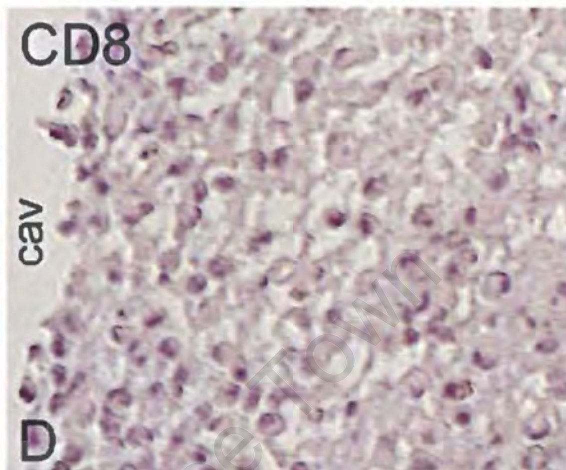
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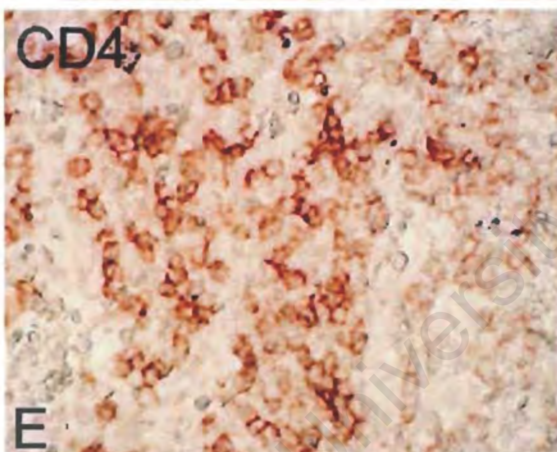
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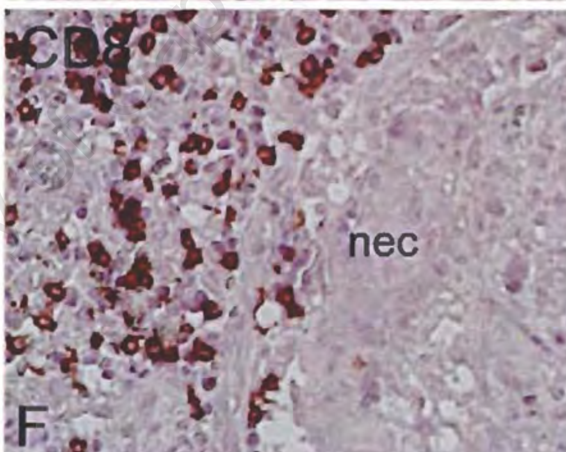
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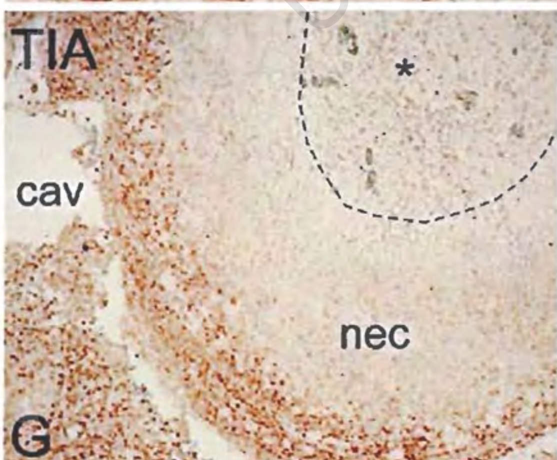
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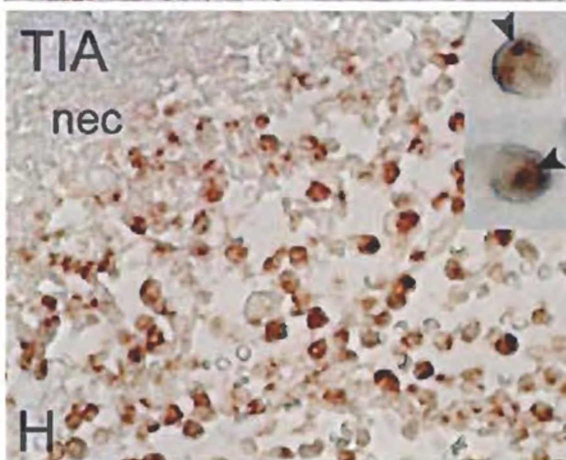
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with multiple bacilli (Figure 6-5). Staining for the presence of $CD3^+ CD4^+$ and $CD3^+CD8^+$ T-lymphocytes revealed an abundance of these cells within the granulomatous/fibrotic layer and in lymphoid aggregates (Figure 6-6). Scattered T-lymphocytes were seen within the fibrotic areas and in the airspaces (not shown). In contrast, a striking absence of $CD4^+$ and $CD8^+$ T-cells was noted at the luminal surface of the cavity. This area, however, contained large numbers of $CD4^-$ $CD8^-$ mononuclear cells with lymphoid morphology that stained for the presence of cytotoxic granules (TIA^+ cells) (Figure 6-6). Taken together, these results suggest that the luminal surface of the cavity represents a microenvironment within the lung in which macrophages and T-cells are not co-localized, thereby preventing direct T-cell-macrophage interactions at those sites. In contrast, only mm away, at the other side of the necrotic zone, another microenvironment exists in which the two cell types are co-localized and free to interact directly, resulting in an efficient immune response capable of inhibiting mycobacterial replication.

Mycobacterial gene expression in the human lung. To gain insight in the adaptive responses of *M. tuberculosis* to the effects of the host immune response, we studied mycobacterial gene expression in bacilli from the open (cavitary) lung lesions of the 3 patients with active TB. Levels of bacillary mRNA expression in the human lung were compared to the level of gene expression by mycobacteria in the lungs of immune competent mice and IFN-γ deficient mice. Studies in mice of MTB gene expression had revealed that four genes (*mbtB*, *pckA*, *icl* and *hspX*) were strongly induced following bacterial growth restriction by the adaptive immune response. These genes encode enzymes involved in iron scavenging, gluconeogenesis, fatty acid metabolism and counteracting the effects of nitric oxide and hypoxia respectively. When MTB gene expression in human and mouse lungs was compared, marked differences were noted. Whereas the host immune response in mice was associated with marked upregulation of *mbtB*, *pckA*, and *icl* expression, minimal induction of

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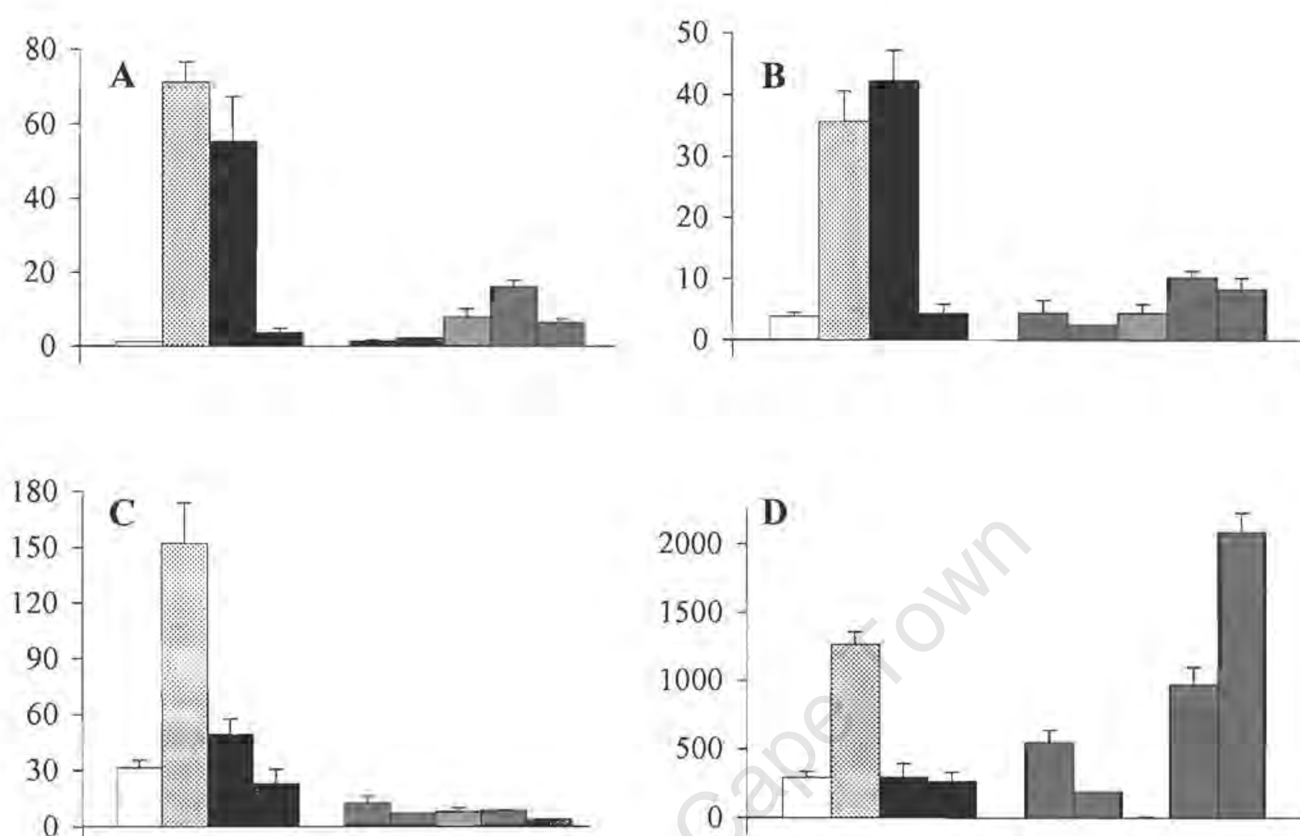

Figure 6-7 Induction of *M. tuberculosis* stress-response mRNAs in mouse and human lungs. Mice were infected by aerosol with ~1000 CFU of H37Rv, and lung samples were obtained from C57Bl/6 mice at 2 w (lane 1), 4 w (lane 2) and 9 w (lane 3), and from IFN-γ deficient mice (lane 4) at 4 w post-infection. Human lung biopsies (lanes 5-9) were obtained from the cavities of 3 HIV-negative patients with chronic, active TB: patient 1 (lanes 5,6), patient 2 (lane 7), patient 3 (lanes 8,9). Target mRNAs were *mbtB* (A), *pckA* (B), *icl* (C) and *hspX* (D), which are markers of the bacterial responses to iron limitation (A), glucose starvation (B), fatty acid catabolism (C), and nitric oxide exposure (D). Target mRNAs were quantified by real-time RT-PCR, internally calibrated to *sigA* mRNA, and are expressed as induction ratios (IR). Values represent averages for at least four independent RT-PCR reactions; error bars indicate standard deviations.

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these genes by MTB obtained from the human cavities was observed (Figure 6-7A, 6-7B and 6-7C). These results suggest that, in contrast to the granulomas in the lungs of immune competent mice, the availability of iron and carbon sources at the surface of the human cavity may be less restricted. The mRNA expression profile for *mbtB*, *pckA*, and *icl* in the bacilli located at the human cavity was remarkably similar to the gene expression profile of MTB in the lungs of IFN-γ deficient mice (Figure 6-7). However, in contrast to the blunted expression of *mbtB*, *pckA*, and *icl* in human lung tissue, potent induction of *hspX* expression, the gene encoding the chaperonin alpha-crystallin, was noted (Figure 6-7D).

6.4 DISCUSSION

In this chapter, we present data supporting the idea that in the lungs of patients affected by TB, a single founder strain of *M. tuberculosis* may undergo mutagenesis during treatment leading to the acquisition of drug resistance independently in discrete physical locales, resulting in heterogeneous subpopulations of drug resistant bacilli. We also show that the lung of a chronic TB patient contains a diversity of micro anatomical niches created by the different immunologic processes occurring at these sites. Such anatomical/immunologic variability appears to be associated with discrete subpopulations of bacilli. We observe that clonal expansion of isolates harboring new drug resistance mutations is preferentially localized to the microenvironment where bacillary growth appears to be most active, the luminal surface of the cavities. At these sites, the macrophages appear to remain non-activated, and thus permissive for bacillary growth.

Lack of macrophage activation to a bacteriostatic state may be due to the selective exclusion of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T-cells from the lumen of the cavity (Figure 6-6). The underlying mechanism for the exclusion of $CD3^+$ T-cells from the cavity surface is unknown. Interestingly, this exclusion appears to be selective as there is a relative

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enrichment at this site for $CD68^+$ macrophages as well as a population of as yet undefined $TIA\text{-}1^+$ cytotoxic lymphoid cells. TIA-1 is a monoclonal antibody that recognizes the 17kD granule membrane protein (GMP-17) expressed predominantly in the granules of $CD8^+$ $\alpha\beta$ T cell receptor (TCR), natural killer (NK) cells, as well as $\gamma\delta$ TCR^+ cells and some $CD4^+$ $\alpha\beta$ TCR^+ cells (Anderson, Nagler-Anderson, O'Brien, *et al.*, 1990; Matutes, Coelho, Aguado, *et al.*, 1996; Medley, Kedersha, O'Brien, *et al.*, 1996). Although the role of GMP-17 remains obscure, the protein is known to translocate to the cytotoxic T-cell surface after fusion of the granules with the cell membrane, and to have sequence homology with calcium channel proteins (Medley, Kedersha, O'Brien, *et al.*, 1996). $TIA\text{-}1^+$ cells have been shown to kill target cells by two distinct mechanisms, Fas receptor-mediated apoptosis and granule exocytosis (Kagi, Vignaux, Ledermann, *et al.*, 1994; Meehan, McCluskey, Pascual, *et al.*, 1997; Nagata and Golstein, 1995). However, in the present study there is no direct evidence of any cytotoxic activity of these cells nor do the cells appear to activate the adjacent macrophages to a bacteriostatic/bactericidal phenotype. In contrast, where macrophages and T-cells are co-localized and potentially in close contact with each other, as observed in the granulomatous/fibrotic areas, the macrophages are morphologically activated (multinucleated giant cells or epithelioid) and few if any bacilli are seen. The phenotypic identification of the $TIA\text{-}1^+$ leukocytes seen at the surface of the cavities in patients with active TB must await the immunohistologic probing of unfixed frozen tissue that can only be performed in the BSL-3 facility.

The growth of *M. tuberculosis* is well known to occur in proportion to oxygen tension (Wayne, 1977). Thus, another factor contributing to the florid bacterial growth seen at the luminal cavity surface could be improved access to oxygen in this microenvironment. In the open granulomas, the extent of bacterial growth appeared to follow an intuitive pattern with respect to oxygen concentration, with necrotic and deeper fibrotic regions, anticipated to be

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largely anoxic, appearing almost sterile. Nonetheless, the gene encoding alpha-crystallin, a protein thought to protect MTB against the effects of hypoxic stress (Yuan, Crane and Barry, 1996) as well as the actions of NO (Garbe, Hibler and Deretic, 1999), was strongly upregulated in the bacilli at the surface of the cavity where oxygen is most abundant. The extent to which oxygen concentration and immune pressure combine to suppress bacterial growth remains to be determined.

Our mycobacterial gene expression studies suggested a relative abundance of iron at the surface of the human cavity. Iron is an essential nutrient for most pathogens, including MTB, and iron limitation at the sites of infection is an important mechanism of host defense (De Voss, Rutter, Schroeder, *et al.*, 1999; De Voss, Rutter, Schroeder, *et al.*, 2000; Manabe, Saviola, Sun, *et al.*, 1999; Rodriguez, Gold, Gomez, *et al.*, 1999). In accordance, induction of *mbtB* as well as repression of *bfrA* (which encodes an iron-storing bacterioferritin) in the mouse lung was temporarily associated with the onset of the adaptive immune response. Persistence of MTB in the mouse lung in the face of the cell-mediated immune response requires isocitrate lyase (ICL), an enzyme involved in lipid metabolism (McKinney, Honer zu Bentrup, Munoz-Elias, *et al.*, 2000). In keeping with the essential role of ICL *in vivo*, strong induction of *icl* expression was observed in MTB obtained from the lungs of immune competent mice. In contrast, the quantitative expression of *icl* by MTB in human tissue resembled *icl* expression in immune deficient mice. The very low IR values for *icl* and *pckA* suggest that availability of carbon sources may be less restricted in patients with chronic, active TB than in the lungs of chronically infected mice. It is possible that relatively unrestricted access to iron and carbon sources in the human lung may have contributed to the ability of MTB to grow and survive at the surface of the cavity.

The presence of discrete populations of bacteria in patients presumed to have acquired drug resistance during therapy has not been carefully studied previously. Our results

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suggest that relying on drug-susceptibility tests of organisms isolated from patient sputum may not provide an accurate representation of the bacterial susceptibility in all sub-populations within the lung. Because quantitation of the bacterial subpopulations and their absolute resistance levels was not performed in this study, it is not possible to make specific therapeutic recommendations. However, our observations suggest the possibility that a careful analysis of resistance levels and bacillary population size might lead to a recommendation in some cases to continue therapy with a first line or even a second line drug in the face of resistance to a given drug in the sputum isolate.

These studies provide a preliminary analysis of the immunologic and bacterial dynamics in tissues from human TB patients. Continued study of lung tissues from patients with active TB will provide important benchmarks for validation of animal models of disease and may suggest alternative therapeutic strategies for the treatment of chronic and MDR-TB.

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Chapter 7

Genetic polymorphism in *M. tuberculosis* isolates from patients with chronic multi-drug resistant tuberculosis

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7.1 ABSTRACT

Multi-drug resistant tuberculosis (MDR-TB) is a major public health problem because treatment is complicated, cure rates are well below those of drug susceptible TB, and patients may remain infectious for months or years despite best available therapy. To gain a better understanding of MDR-TB, we characterized serial isolates from 13 HIV-negative MDR-TB patients by IS*6110* fingerprint, spoligotype, and sequence of *rpoB*, *katG*, *inhA*, *pncA*, *embB*, *rpsL*, *rrs* and *gyrA*. In all 13 patients, chronic MDR-TB was caused by a single *M. tuberculosis* (MTB) strain, 8 (62%) of which belonged to the W-Beijing family. In 4/13 (31%) patients, the patient isolate acquired additional drug resistance mutations during the study. In these 4 patients, heterogeneous populations of bacilli with different resistance mutations as well as mixtures of drug susceptible and drug resistant phenotypes were observed. This genetic heterogeneity requires treatment targeted at both resistant and susceptible phenotypes, i.e. new drugs to control the growth of resistant bacilli should be added to the drugs that are active against the residual susceptible bacilli.

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7.2 INTRODUCTION

Multi-drug resistant tuberculosis, caused by MTB isolates resistant to at least rifampin (RIF) and isoniazid (INH), is a serious public health hazard. High rates of MDR-TB are considered a reflection of poor case management and an ineffective TB control program (Espinal, Laszlo, Simonsen, *et al.*, 2001; Iseman, 2000). For example, in Estonia a country where the TB control infrastructure has recently deteriorated, MDR-TB accounts for 14% of new TB cases and up to 48% of TB in previously treated patients (Espinal, Laszlo, Simonsen, *et al.*, 2001). Treatment of MDR-TB can be very difficult because loss of the two most potent antituberculous drugs (i.e. INH and RIF) leaves only less effective "first line" therapy and "second line" drugs that are more toxic and less efficacious (Iseman, 1993). Thus, while

some patients with MDR-TB can be cured by short-course chemotherapy (1999; Espinal, Kim, Suarez, *et al.*, 2000; Mitchison and Nunn, 1986), in others bacillary growth is merely suppressed as long as treatment is continued (Goble, Iseman, Madsen, *et al.*, 1993). Furthermore, between 8 and 35% of patients have persistently active disease refractory to multi-drug therapy (Goble, Iseman, Madsen, *et al.*, 1993; Saenghirunvattana, Charoenpan, Vathesatogkit, *et al.*, 1996; Schaaf, Botha, Beyers, *et al.*, 1996; Suarez, Floyd, Portocarrero, *et al.*, 2002; Tahaoglu, Torun, Sevim, *et al.*, 2001). Consequently, in most studies, the cure rates of MDR-TB remain well below those for drug susceptible TB, and mortality, even among HIV-negative patients, may be substantial (Goble, Iseman, Madsen, *et al.*, 1993). In addition, MDR-TB patients that do not respond to treatment are a constant source of transmission of MDR-MTB (Bifani, Plikaytis, Kapur, *et al.*, 1996; Frieden, Sherman, Maw, *et al.*, 1996; Ridzon, Kent, Valway, *et al.*, 1997; Schaaf, Van Rie, Gie, *et al.*, 2000; Van Rie, Warren, Richardson, *et al.*, 2000; van Rie, Warren, Beyers, *et al.*, 1999).

Drug resistance in MTB results from mutations (nucleotide substitutions, insertions or deletions) in the genetic targets of the drugs used for antituberculous chemotherapy (Ramaswamy and Musser, 1998). Resistance conferring mutations in MTB occur spontaneously at a very low frequency and are not transmitted between bacilli. Drug selection for resistance is associated with inadequate therapy or sub-therapeutic drug levels, and leads to the resistant phenotype becoming clinically significant and ultimately predominating in persons in whom the disease was originally caused by drug susceptible isolates (Iseman, 1993). In patients with MDR-TB, additional mutations may be selected for by adding a single drug to a failing regime (Farmer, Bayona, Becerra, *et al.*, 1998). Since most drug resistance mutations appear not to affect the fitness of MTB (Cohen, Sommers and Murray, 2003), once acquired, these mutations persist even after selective drug pressure has been withdrawn (Iseman, 2000).

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In the human lung, acquisition of drug resistance mutations by MTB occurs predominantly within cavities where high bacterial loads, active mycobacterial replication, and reduced exposure to host defense mechanisms have been reported (Canetti, 1965; Vandiviere, Loring, Melvin, *et al.*, 1956). Since MTB in sputum samples of TB patients originate from lung cavities, genetic analysis of serial sputum isolates allowed us to study the genetic evolution of drug resistance in situ.

7.3 RESULTS

Patients. We studied the MTB isolates from 13 HIV-negative MDR-TB patients (8 males, 5 females; mean age 35.8 years, range 22-60) who were refractory to at least 12 months of chemotherapy (Table 7-1). These patients comprised the entire South African subset of a multinational, randomized, placebo controlled study to evaluate the role of aerosolized recombinant human interferon-gamma (rhuIFN-γ, InterMune, Brisbane, CA, USA) as an adjunct to chemotherapy in patients with MDR-TB (Condos, Rom and Schluger, 1997). All subjects were residents of the Western Cape region of South Africa, where TB is endemic (incidence rate 372/100,000) (Medical Officer of Health, 1994/1995). Five patients had had prior TB; none of the patients had extra-pulmonary TB; two patients had diabetes mellitus (patients #5 and #9) and no other patients had specific risk factors for tuberculosis. None of the patients were related or had known epidemiologic links, and none were geographically clustered. At entry into the study, all patients had sputum smear-positive, MDR-TB and had received therapy for a mean of 36 (12-77) months at entry. Most patients excreted large numbers of bacilli in sputum (median score 2.0, Table 7-1), and most patients had extensive disease on chest radiograph (median score 11.0).

Due to a low prevalence of MDR in this area (1.1% in new and 4% in previously treated patients respectively) (Weyer, Groenewald, Zwarenstein, *et al.*, 1995), at diagnosis all

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patients received treatment for drug susceptible TB (2 months of INH, RIF, PZA, and EMB followed by INH and RIF or INH, RIF and PZA for an extra 4 months). Once MDR-TB was diagnosed, drug regimens were individualized on the basis of the results of phenotypic drug susceptibility tests (Table 7-2). Drug susceptibility was evaluated every 2-3 months, and treatment regimens adjusted accordingly at approximately 6 monthly intervals in unresponsive patients. Upon entry into the study, therapy was again adjusted according to phenotypic drug susceptibility, treatment history and side effect profile. Eight of 13 patients received aerosolized rhuIFN-γ as an adjunct to treatment (Table 7-2).

All 13 patients remained sputum positive for MTB throughout the study. Eight patients died during the 56 weeks of the study, most likely as a result of cachexia and/or chronic respiratory failure. Patients who died had more extensive disease (median score 14.5) compared to those who survived (median score 9.0) ($p=0.012$) despite similar disease duration (41.7 vs 47.2 months, $p=0.64$) (Table 7-1). A mean of 7.6 (range 2-16) cultures per patient were available for analysis.

Phenotypic and genotypic resistance profile of *M. tuberculosis*. The phenotypic and genetic drug resistance for each patient is shown in Table 7-3. All isolates displayed phenotypic resistance to INH. Eight had single nucleotide substitutions in *katG*, and 4 had mutations in *inhA* C15T. In one INH resistant strain, neither *katG* nor *inhA* mutations were identified despite the presence of high level (>10 μg/ml) phenotypic isoniazid resistance. Eleven patients were continued on INH despite documented resistance to this drug. Eight of these received thiacetazone, which was only available as an INH-containing combination. Phenotypic and genotypic (*rpoB*) resistance testing confirmed that all isolates were resistant to RIF. Phenotypic resistance to EMB was not observed in any of the 13 isolates. However, sequence analysis of *embB*, the genetic target of EMB, revealed resistance mutations in 62% of isolates. The isolates of 69% of patients harbored *pncA* mutations. The presence of

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Patient #	Age (y)	Gender	Prior TB	Duration of Treatment (m)	MDR strain Spoligotype	Sputum AFB load *	Chest Radiograph Severity Score *	Number of Cultures **	Study Outcome Status **
1	60	F	Y	30	34 (W321)	2.5	11.0	16	alive
2	27	M	N	51	157	2.0	9.0	5	deceased
3	29	F	N	28	34 (MH)	1.5	17.0	2	deceased
4	32	M	Y	26	34 (W616)	3.0	13.5	4	deceased
5	47	F	N	17	2	1.0	2.0	13	alive
6	39	M	N	61	857	1.0	3.0	10	alive
7	36	M	Y	69	2	1.5	14.5	11	deceased
8	33	M	N	21	34 (W616)	3.0	15.5	6	deceased
9	32	M	N	83	34 (W451)	2.0	8.0	4	alive
10	25	F	Y	43	34 (W451)	1.5	10.0	2	alive
11	44	F	N	41	79	1.0	13.0	8	deceased
12	40	M	N	49	34 (W616)	2.5	11.0	7	alive
13	22	M	Y	56	34 (W616)	2.5	15.0	11	deceased

* See methods for quantification of sputum smears and disease severity score; ** during the study

Table 7-1 Characteristics of the patients with MDR TB .

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Patient #	Initial TB Treatment: Drugs	(m)*	Treatment since Diagnosis of MDR-TB: Drugs	(m)*	Treatment During Study: Drugs	(w)*
1**	R H Z E	12	R H Z E Et Th Sm K	18	E O Cf	56
2	R H Z E	3	H Z E Et Th Sm K O Cf Te	48	E Et O Cf	12
3**	R H Z E	4	H Z E Et Th A K O Cf Cy Cla	24	H Th A O Cf Cla	3
4**	R H Z E	8	H Z Th K O	18	H Z Et Th O	14
5	R H Z E	5	H Z E Et Th Sm O	12	H Z E Et Th O	53
6**	R H Z E	5	H Z E Et Th Sm K O Cf Te	56	H Z E Et Th O Cf Te	52
7**	R H Z E	16	H Z E Et Th Sm K O Cy	53	H Z E Sm O	56
8	R H Z E	3	H Z E Et Th Sm K O Cf	18	H Z E Th O	30
9**	R H Z E	6	R H Z E Et Th Sm K O Cf	77	H Z E Et Sm	28
10**	R H Z E	13	H Z E Et Th Sm O	30	H Z E Th O	8
11	R H Z E	7	R H Z E Et Th Sm K O Cf Cla	34	H Z E Et Th O	17
12**	R H Z E	7	H Z E K O	42	H Z E O	23
13	R H Z E	13	H Z E Et Th Sm K O Cf	43	H Z E Et Th O	22

R=RIF; H=INH; Z=PZA; E=EMB; Et=ethionamide; Th=thiacetazone; Sm=streptomycin; K=kanamycin; A=amikacin; O=ofloxacin; Cf=clofazamine; Cy=cycloserine; Cla=clarithromycin; Te=terizidone

* duration of treatment in months (m) or weeks (w) ** patients who received aerosolized rhuIFN-g

Table 7-2 TB treatment prior to and during the study.

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aminoglycoside resistance mutations in *rrs* was poorly predicted by the results of phenotypic susceptibility tests. Taken together, these results suggest that the isolates from all 13 patients were highly resistant to the most potent first and second line agents.

Genetic analysis of serial sputum isolates. Despite living in an area with high rates of MTB transmission, no evidence of co-infection with a second MTB strain was observed in any of the patients. This was shown by the fact that all MTB isolates recovered from the same patient showed genetic stability as IS*6110* fingerprint profiles and spoligotype patterns did not vary over time (Figure 7-1). It is interesting to note that 8/13 (62%) isolates from the patients with treatment refractory MDR-TB belonged to the W-Beijing family (n=7) or the W-Beijing lineage (n=1) (Bifani, Mathema, Kurepina, *et al.*, 2002). Two of the W-Beijing strains were found in clusters: W451 was cultured from patients #9 and #10 and W616 was identified in patients #4,#8,#12 and #13 (Table 7-1). No clustering of strains was observed among the 5 isolates that were not W-Beijing isolates. Patients infected with W-Beijing isolates had higher sputum bacterial loads (median bacillary load 2.5) than patients with non W-Beijing isolates (median bacillary load 1.0) (p=0.019).

Sequence analysis in serial *M. tuberculosis* sputum isolates. MTB drug resistance polymorphism was explored by sequencing the genes of serial isolates (2-3 per patient). In 9 patients, all drug targets examined remained unchanged from baseline over the 3-56 week study period (Table 7-3). However, in 4 patients (31%), additional drug resistance mutations had been acquired during treatment. In patient #1, polymorphisms were noted in *katG*, *embB* and *gyrA*. The *gyrA* gene mutation was found in some but not all bacilli at multiple time points. Patient #2 had *pncA*, *gyrA* and *rrs* polymorphisms; the *pncA* gene was mutated in some but not all the bacilli at 2 time points. Patient #6 had polymorphisms in *gyrA*, *pncA*, *rpoB* and *katG* and the *gyrA* gene was mutated in some but not all the bacilli at 1 time point. In patient #10, both *gyrA* and *pncA* had polymorphisms and the *pncA* gene was mutated in

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Patient #	RIF	*rpoB*	INH	*katG*	*inhA*	PZA	*pncA*	EMB	*embB*	Sm	*rpsL, rrs*	K	*rrs*	O	*gyrA*
1	R	D516V	**R**	**WT***	**WT**	nd	96 (CAA ins)	S	WT	S	WT	**S**	**A1401G**	S	WT
2	R	S531L	R	S315T	WT	nd	WT	**S**	**M306I**	**S**	**C492T,G217T (*rrs*)**	S	WT	R	A90V
3	R	L511R,D516V	R	S315T	G17T	nd	173 (G ins)	**S**	**M306I**	**S**	**A514C (*rrs*)**	R	A1401G	R	D94G
4	R	S531L	R	WT	C15T	nd	Y103STOP	S	WT	R	K43R (*rpsL*)	R	C1402T	R	D94G
5	R	S531L	R	S315T	WT	nd	L85R	S	WT	S	WT	S	WT	S	WT
6	R	D516V	**R**	**WT***	**WT**	nd	WT	S	WT	**S**	**C492T (*rrs*)**	S	WT	S	WT
7	R	S531W	R	S315N	WT	nd	WT	**S**	**M306V**	S	WT	R	A1401G	R	A90V
8	R	S531L	R	WT	C15T	nd	Y103STOP	**S**	**M306V**	R	K43R (*rpsL*)	**S**	**A1401G**	S	WT
9	R	S531L	R	L101P	WT	nd	18 (24 bp del)	**S**	**M306V**	R	A514C (*rrs*)	S	WT	S	WT
10	R	S531L	**R**	**WT**	**WT**	nd	L27R	**S**	**M306I**	S	WT	S	WT	S	WT
11	R	S531L	R	S315T	WT	nd	D8H	**S**	**M306I**	S	WT	S	WT	R	D94G
12	R	S531L	R	WT	C15T	nd	Y103STOP	**S**	**M306V**	R	K43R (*rpsL*)	S	WT	S	WT
13	R	H526Y	R	WT	C15T	nd	WT	S	WT	S	WT	**S**	**A1401G**	**S**	**A90V**

Discordance between phenotypic and genotypic drug susceptibility is highlighted in bold; R=resistant,S=susceptible, Sm=streptomycin,K=kanamycin,O=ofloxacin
Genotypic resistance indicates amino acid substitution (*rpoB,katG,pncA,embB,rpsL,gyrA*) or nucleotide substitution (*inhA,pncA,rrs*)
* resistance-conferring mutations were identified at later timepoints (see Table 7-4)

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Table 7-3 Phenotypic and genotypic drug resistance in baseline MTB cultures.

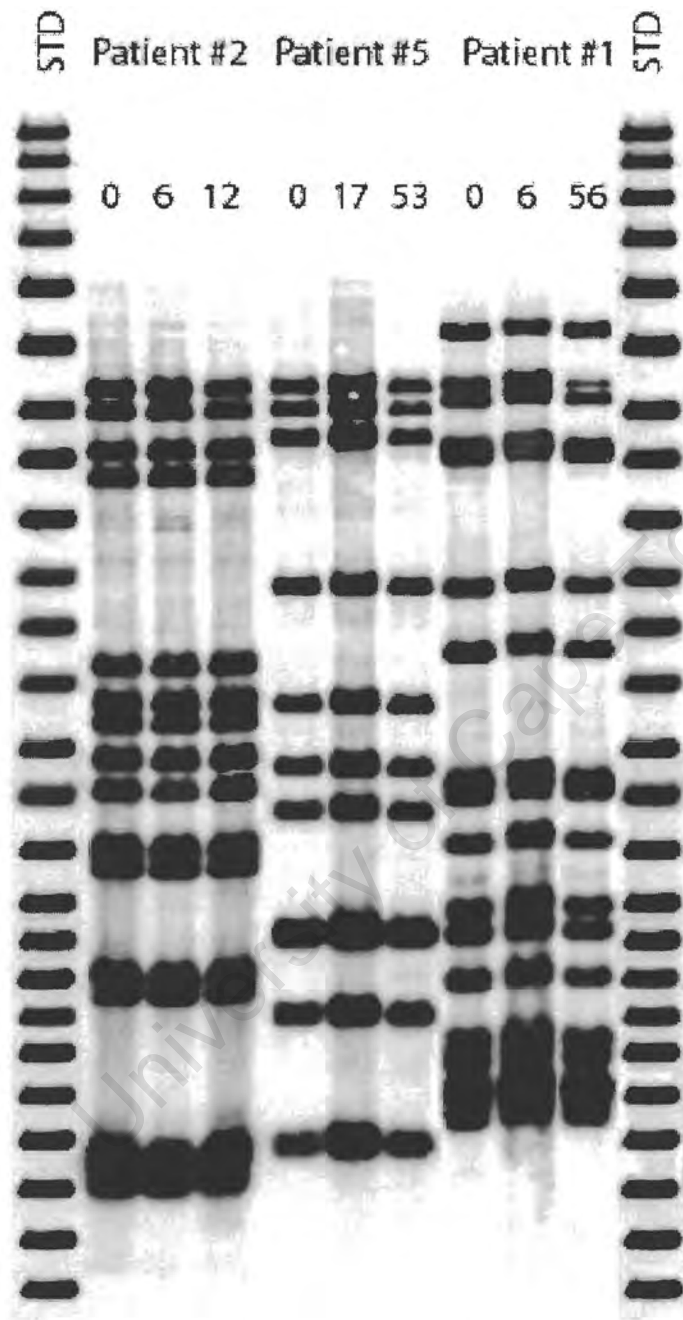

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Figure 7-1 IS*6110* fingerprints of MTB isolated from sputum of 3 patients at baseline, and after 6-56 weeks of additional treatment.

Rx	Patient #1			Patient #2			Patient #6				Patient #10	
(w)	*katG*	*embB*	*gyrA*	*pncA*	*gyrA*	*rrs*	*gyrA*	*pncA*	*rpoB*	*katG*	*gyrA*	*pncA*
0	WT	WT	WT	WT	A90V	G217T,C492T	WT	WT	D516V	WT	WT	L27R/WT
1	WT	WT	WT									
2	WT	WT	WT	**D12A/WT**	**D94Y**	**C492T**						
4	**G118A**	WT	WT									
6	G118A	**G406D**	**D94Y/WT**	**D12A**	D94Y	C492T	WT	WT	**S531W**	**E261K**		
8	**WT**	G406D	D94Y/WT	**D12A/WT**	D94Y	C492T	WT	WT	S531W	E261K	**D94G**	**WT**
12	WT	G406D	D94Y/WT	**D12A**	D94Y	C492T						
16	WT	G406D	D94Y/WT				WT	WT	S531W	E261K		
24	WT	G406D	D94Y/WT									
30	WT	G406D	**D94G**				WT	WT	S531W	**WT**		
36	WT	G406D	**D94Y**				**G88A**	**V157G**	S531W	**E261K**		
40	WT	G406D	**D94C/WT**				**WT**	**131 (GG ins)**	S531W	E261K		
44	WT	G406D	D94C/WT				**G88A/WT**	131 (GG ins)	S531W	E261K		
48	WT	G406D	D94C/WT				**WT**	131 (GG ins)	S531W	E261K		
52	WT	G406D	**D94G**				WT	131 (GG ins)	S531W	E261K		
56	WT	G406D	D94G									

Changes in genotype are highlighted in bold

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Table 7-4 Genotypic polymorphism of selected *M.tuberculosis* genes.

some but not all the bacilli isolated at 1 time point. In 3 of these patients, different nucleotide substitutions accounted for drug resistance in bacillary subpopulations (*gyrA* D94G, D94Y and G94C for fluoroquinolone resistance in patient #1, *gyrA* A90V and D94Y in patient #2 and *rpoB* D516V and S531W for rifampin resistance in patient #6; both the *pncA* V157G substitution and 131GG insertion accounted for PZA resistance in patient #6). As seen in patients #1 and #6, INH susceptible isolates were still recovered several years after the patient had developed MDR-TB (Table 7-4).

7.4 DISCUSSION

Our studies show that the acquisition of drug resistance (which is a stochastic event) during the treatment of tuberculosis results in a mixture of subpopulations of bacilli, some of which are drug susceptible while others are drug resistant (Table 7-4). This may explain why the resistance phenotype reported by the diagnostic laboratory can change over time between susceptible to resistant and back to susceptible (depending on the population that predominates at the time of sputum sampling). Phenotypic drug resistance is commonly determined by the proportion method, where an MTB isolate is considered to be resistant to a particular drug if at least 1% of colonies grow on agar containing a critical concentration of that particular drug (2000). Consequently, the particular drug is often withdrawn or withheld (Blumberg, Burman, Chaisson, *et al.*, 2003; Iseman, 1993). Our observations suggest that it may not be prudent to withdraw the drug(s) following documentation of drug resistance. Rather, the existing drug(s) should be maintained to target the bacilli that are still drug susceptible. In addition new drugs (preferably at least two) should be added to the treatment regimen to target the newly drug resistant organisms.

Despite the very high disease burden and rates of TB transmission in the Western Cape and what appears to be constant exposure to TB in the environment (van Rie, Warren,

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Richardson, *et al.*, 1999), the IS*6110* patterns of all 13 MTB isolates did not change over the study period (7.2 person-years of observation). Thus, no super-infection occurred and no change in the genetic background of the strains was noted. In most published studies, evolutionary changes in IS*6110* patterns occurred in fewer than 10% of patients, and it has been suggested that these events might be more frequent soon after infection when replication is most active (de Boer, Borgdorff, de Haas, *et al.*, 1999; Niemann, Richter and Rusch-Gerdes, 1999; Warren, Van Der Spuy, Richardson, *et al.*, 2002). The small number of patients in our study and the long periods of active disease prior to enrollment may have contributed to the lack of any IS*6110* instability in this study. In accordance with previous studies, acquisition of drug resistance mutations was not associated with changes of the IS*6110* profile (Niemann, Richter and Rusch-Gerdes, 1999; Yeh, Ponce de Leon, Agasino, *et al.*, 1998).

In our patients, 62% of disease was caused by MTB strains belonging to the W-Beijing family of strains (Bifani, Mathema, Kurepina, *et al.*, 2002). These strains comprise around 20% of all MTB isolates in the Western Cape (van Helden, Warren, Victor, *et al.*, 2002). The high frequency of W-Beijing strains seen in our study may simply reflect a sampling bias; however, it may reflect a specific predilection of this family of strains to developing drug resistance (Anh, Borgdorff, Van, *et al.*, 2000). W-Beijing isolates are considered highly successful from an epidemiologic perspective, and members of this family have caused outbreaks in institutions as well as many communities (Bifani, Mathema, Liu, *et al.*, 1999; van Rie, Warren, Beyers, *et al.*, 1999). Our study raises the possibility that certain W-Beijing strains may be more likely to acquire MDR. Alternatively, MDR W-Beijing strains may have a higher propensity to spread in the community. This is supported by higher baseline bacillary loads in sputum of patients infected with strains that are members of the W-Beijing family (Table 7-1).

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In agreement with previous studies, we observed a good correlation between phenotypic and genotypic susceptibility for INH and RIF (Escalante, Ramaswamy, Sanabria, *et al.*, 1998; Laszlo, Rahman, Espinal, *et al.*, 2002; Torres, Criado, Palomares, *et al.*, 2000). The single patient in whom no *katG* or *inhA* mutations were identified may have had mutations in *kasA*, *ahpC* (Ramaswamy and Musser, 1998) or the recently identified INH target gene *ndh* encoding the enzyme NADH dehydrogenase (Lee, Teo and Wong, 2001). In contrast, phenotypic and genotypic susceptibility to EMB correlated poorly (Mokrousov, Otten, Vyshnevskiy, *et al.*, 2002; Van Rie, Warren, Mshanga, *et al.*, 2001) and, together with non-availability of susceptibility data for PZA, may have resulted in an overestimation of the number of active drugs in most of the patients. There is good evidence that a large number of active drugs can be effective in treating highly MDR-TB patients. A seven-drug regimen comprising of RIF, INH, PZA, EMB, clarithromycin, cycloserine, and ethionamide administered to patients with chronic MDR-TB for up to 55 months resulted in a remarkable 81% cure rate (1998). In addition, improved outcome of patients with MDR-TB maintained on INH together with the other drugs has been reported (Frieden, Sherman, Maw, *et al.*, 1996). The clinical benefit of INH therapy in these patients was thought to have been due to low or intermediate levels of resistance to the drug. Our results suggest that the improved outcome observed by Frieden *et al.* may also have related to activity of INH against susceptible bacillary subpopulations within the lungs of MDR-TB patients.

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Chapter 8

CONCLUDING REMARKS

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Mycobacterium tuberculosis (MTB) has been a human pathogen for centuries. Tuberculosis (TB), a predominantly pulmonary disease that results when MTB infection is inadequately contained by the host immune response, accounted for nearly a quarter of all deaths in the major European cities around 1800. The number of TB cases has markedly declined since, presumably as a result of the improvement in the standard of living and the introduction of chemotherapy. Consequently, it was anticipated that TB would disappear as a human disease. However, MTB has proved this prediction to be premature. Fuelled by the HIV epidemic, global rates of TB have soared over the last 15 years, particularly in the developing world, prompting the World Health Organization to declare tuberculosis a global emergency in 1993. Despite the relatively straightforward clinical features of pulmonary TB and the fairly simple diagnostic tools required to confirm infectious TB, many TB cases are not identified until extensive transmission of MTB has occurred. In addition, a substantial proportion of TB patients fail to complete the six months of therapy required for cure. These persons may develop drug-resistant TB, progressive lung tissue destruction resulting in respiratory failure, or chronic respiratory tract infections that may mimic or mask TB.

Tuberculosis develops when host immunity is unable or no longer able to control bacillary replication. Commonly, the initial infection is controlled when macrophages are activated by pathogen-specific T-lymphocytes, typically resulting in the formation of granulomas. The bacilli however are not eliminated by the immune response and are able to persist for decades within these lesions. Reactivation TB may develop when liquefaction of the necrotic center of a granuloma occurs and macrophages are no longer able to contain the bacilli. The ineffective immune response is associated with caseous necrosis and formation of pulmonary cavities. Bacilli are typically numerous at the surface of the cavity and, together with the caseous contents of the cavity, expectorated over time, thereby allowing MTB to establish infection in new, susceptible hosts.

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Control of mycobacterial replication in the human lung requires the generation of T-cell mediated immunity. Efficient sensitization and expansion of T-lymphocytes that are able to recognize MTB infected macrophages depends on the production of interleukin-2 (IL-2) and IL-12 (and a number of other cytokines). These cells are recruited to the site of infection through the localized production of chemokines and expression of adhesion molecules on the vascular endothelium. Production of tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) by the mononuclear cells allows activation of mycobacteriostatic, inducible nitric oxide synthase (iNOS) mediated pathways in infected macrophages. In addition, $CD4^+$ T-cells, $CD8^+$ T-cells, and $CD4^-CD8^-$ T-cells may lyse MTB infected macrophages or induce apoptosis of the infected cells, either of which may be associated with reduced viability of the bacilli (Flynn and Chan, 2001). Since pro-inflammatory cytokines and MTB-reactive $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ T-cells are readily demonstrable in TB patients, it remains difficult to understand why the immune response fails to control MTB infection in humans who develop TB.

We performed a detailed analysis of the host immune response in the lungs of six patients with chronic TB in whom the immune response had failed to control MTB infection. Three of these patients had developed active TB, which was successfully cured by chemotherapy. The other 3 patients had chronic active disease despite prolonged administration of multi-drug treatment. Two of these patients were infected with multi-drug resistant (MDR) MTB. Whereas only the lungs of patients with active TB showed macroscopic caseation, the lungs of all 3 patients with post-tuberculous lung disease who had been cured by chemotherapy contained microscopic foci of caseous necrosis as well as epithelioid granulomas without central necrosis. The presence of epithelioid as well as caseous necrotic granulomas suggested the presence of several micro-anatomical and/or immunologic environments. In the patients with active disease, numerous bacilli at the

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luminal surface of the cavity suggested a failure of the immune response to control bacillary replication in this location. Below the necrotic zone, we noted an abundance of mononuclear cells as well as multi-nuclear giant cells. The virtual absence of acid-fast bacilli (AFB) at this site suggested that mycobacterial replication was adequately controlled. The demonstration of abundant IL-2, IL-12, IFN-γ and iNOS gene expression in the tissue samples underscored that TB in these patients did not result from a global deficiency or inability to produce these molecules, or a general lack of macrophage activation.

Immunophenotyping of the mononuclear cells in the various TB lesions revealed a striking absence of $CD4^+$ and $CD8^+$ T-lymphocytes from the surface of the cavity. It is possible that the cytokine gene expression levels reflected production of these proteins by activated $CD4^+$ and $CD8^+$ T-cells within the inflammatory cell infiltrate below the necrotic rim, where few if any bacilli were observed and macrophages showed signs of activation (epithelioid morphology and multi-nucleated giant cells). The selective exclusion of $CD4^+$ and $CD8^+$ T-lymphocytes from the surface of the cavity may explain the inability of the host to control mycobacterial replication despite the presence of appropriate cytokine signals and T-cell phenotypes in the TB lesions. Clearly, the $TIA\text{-}1^+$ cytotoxic T-cells (which may kill target cells by Fas-mediated apoptosis or granule exocytosis) had little if any effect on the replication of MTB within macrophages at the surface of the cavity. In addition, mycobacterial access to iron and carbon sources in the human lung appeared relatively unrestricted since the bacillary adaptive responses to iron limitation and carbon depletion were poorly induced. Since iron is an essential nutrient for most pathogens and iron restriction at the site of infection an important host defense mechanism, the relatively unrestricted access to iron and other nutrients presents another indication of impaired immunity at the surface of the cavity. However, since the patients from whom the lungs were

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obtained all suffered from chronic, treatment refractory TB, these observations may not be representative of all TB patients.

By performing mycobacterial cultures on different TB lesions in the lung specimens, we wished to examine the contribution of infection with different MTB strains to the differences in granulomatous response within each patient. However, when up to 6 different pulmonary sites were cultured, a single MTB isolate was recovered from each lesion. In addition, IS*6110* fingerprinting failed to show genetic evolution among the bacilli recovered from the different lesions. Similar results were obtained when genotyping of up to 16 longitudinally collected sputum isolates from 13 patients with chronic, treatment-refractory MDR TB was performed. We thus obtained no evidence that pulmonary TB in these patients was caused by infection with more than one MTB isolate, and since these patients were living in an area with extremely high rates of MTB transmission, it would appear that while the immune response failed to control replication of the founder MTB strain, it prevented progressive infection with additional isolates to which the patients were undoubtedly exposed.

The contribution of MTB strain polymorphism to chronic TB was also examined by sequence analysis of several of the genes implicated in drug resistance. In contrast to the stability of the IS*6110* fingerprint, remarkable heterogeneity of the resistance-associated alleles was observed among MTB isolates recovered from different lung lesions as well as MTB isolates obtained from serial sputum specimens. The genetic drug resistance profiles of the lung isolates suggested that an initial infecting strain may spread from one pulmonary site to another, to become the founder for acquisition of additional antibiotic resistance at secondary sites. The additional drug resistance mutations were preferentially observed in *M. tuberculosis* cultured from the open granulomas, i.e. the cavities, where bacillary growth appeared to be most active. The presence of heterogeneous subpopulations of drug resistant

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bacilli in the human lung suggest that drug-susceptibility tests performed on organisms isolated from the patient's sputum may not provide an accurate representation of the susceptibility of all bacterial sub-populations within the lung. In addition, sequence analysis of multiple sputum isolates revealed that drug-susceptible bacilli may remain present among the resistant bacilli for several months or years. Careful analysis of the level of drug-resistance as well as the size of bacillary subpopulations may therefore result in a recommendation in some patients to continue therapy with a first line or even a second line drug to which the sputum isolate has become resistant.

Genotypic analysis revealed that several of the patients with chronic TB were infected with MTB isolates that belonged to the W-Beijing family of strains (Bifani, Mathema, Kurepina, *et al.*, 2002). Whereas these isolates comprise approximately 17% of community isolates in Cape Town, we noted an increased prevalence of 35% among 200 consecutively obtained MDR isolates and 63% among the 16 patients with treatment-refractory TB. The relative enrichment of W-Beijing isolates among patients with MDR TB is in keeping with observations from South East Asia, where these isolates are highly prevalent and commonly drug-resistant (Anh, Borgdorff, Van, *et al.*, 2000). The increased sputum bacterial loads in our patients infected with W-Beijing isolates might be of relevance to their epidemiologic success. W-Beijing isolates have been associated with outbreaks in institutions as well as communities, more extensive disease in mice (Lopez, Aguilar, Orozco, *et al.*, 2003; Manca, Tsenova, Bergtold, *et al.*, 2001) and faster replication within human monocytes (Zhang, Gong, Yang, *et al.*, 1999). The propensity to cause more extensive disease may relate to their ability to induce rather weak pro-inflammatory cytokine responses and reduced T-cell activation. We noted that reduced cytokine production in patients with TB was associated with more severe lung disease. High bacillary loads may also contribute to the increased rates of drug resistance, since infrequent stochastic mutational events would be encountered more

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frequently. It would be of considerable interest to compare *dnaE2* gene expression (Boshoff, Reed, Barry, *et al.*, 2003) in human lung tissue from patients infected with W-Beijing and other isolates to evaluate if increased expression of this low-fidelity DNA polymerase may further contribute to the increased frequency of drug resistance among W-Beijing isolates.

In addition to the studies of *M. tuberculosis* polymorphism in human lung and sputum samples, we examined the effects of well characterized, different or related mycobacterial strains on the innate responses of host phagocytes. Human monocytes and mouse macrophages were infected *in vitro* with the related MTB strains H37Ra and H37Rv, with polymorphic variants of *M. smegmatis* (*M. smegmatis* and. *M. smegmatis* 19kDa), or the genetically distinct clinical MTB isolates CDC1551 and HN878. Intracellular growth rates and production of immune regulatory cytokines by the infected phagocytes were measured.

The MTB strains H37Ra and H37Rv (Steenken and Gardner, 1946) are distinguished by their differential ability to cause progressive disease in mice and guinea pigs. In an attempt to understand the difference in virulence of the two strains, we evaluated their growth and TNF-α producing capacity in cultures of human monocytes and peritoneal macrophages from wild type and gene-disrupted mice. Similar growth of H37Ra and H37Rv was observed in human monocytes, and monocyte maturation resulted in growth restriction of both strains. In wild type mouse macrophages and macrophages deficient in iNOS, IFN-γ or the IFN-γ receptor, H37Rv grew logarithmically while growth of H37Ra slowed down significantly by 48h post infection. In contrast, mouse macrophages deficient in TNF-α were unable to control the growth of H37Ra. Infection of both mouse and human macrophages with H37Rv induced higher levels of TNF-α production than infection with H37Ra. These results suggest that growth restriction of H37Ra in mouse macrophages is mediated through a TNF-α dependent, iNOS and IFN-γ independent pathway, and that this pathway may be absent in human monocyte-derived macrophages (MDM).

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The 19kDa lipoprotein of MTB (Young and Garbe, 1991), when expressed in *M. smegmatis* (*M. smegmatis* 19kDa), was shown to abrogate the limited protection against MTB challenge afforded to mice by the administration of *M. smegmatis*. To investigate the mechanism of this suppression of immunity, human MDM were infected with *M. smegmatis* or *M. smegmatis* 19kDa. Infection with *M. smegmatis* 19kDa resulted in reduced production of TNF-α, IL-12, IL-6 and IL-10 compared to infection with *M. smegmatis*. The 19kDa antigen had no differential effect on expression of co-stimulatory molecules on the surface of *M. smegmatis* infected MDM, nor did it affect the proliferation of pre-sensitized T-cells co-cultured with infected MDM. The immunosuppressive effect was dependent on the presence of glycosylated and acylated 19kDa lipoprotein in the mycobacteria-containing phagosomes. These results suggest that the diminished protection against challenge with *M. tuberculosis* seen in mice vaccinated with *M. smegmatis* expressing the 19kDa lipoprotein might have been the result of reduced pro-inflammatory cytokine production. These results contrast with the demonstrated potential of the 19kDa to stimulate IL-12 production through Toll-like receptor-2 (TLR-2) ligation, and suggest that the effect of the interaction between MTB antigens and host phagocyte receptors may depend on the cellular compartment in which immune recognition takes place, or the context in which receptor ligation occurs.

The MTB clinical isolates CDC1551 (Manca, Tsenova, Barry, *et al.*, 1999) and HN878 (Manca, Tsenova, Bergtold, *et al.*, 2001) are distinguished by their differential ability to cause pathology in mice. Infection of mice with HN878 results in delayed and reduced IL-12 and TNF-α production compared to infection with CDC1551, and this is associated with an approximately 10-fold higher bacillary load in the lungs and markedly impaired survival of the infected animals. In MTB infected mice therefore, a rapid and strong pro-inflammatory cytokine response in the lungs may be associated with less severe disease. When peripheral blood monocytes from patients with TB were infected with CDC1551 or HN878, reduced IL-

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12, TNF-α and IL-6 production by HN878 infected monocytes was observed, and HN878 displayed faster intracellular replication than CDC1551. Reduced TNF-α and IL-6 production in response to MTB infection may have resulted in decreased cellular activation and impaired innate resistance to intracellular mycobacterial growth *in vitro*.

When cytokine production by MTB infected monocytes and intracellular bacillary growth rates were compared for cells obtained from patients with limited pulmonary disease and monocytes from patients with extensive disease on chest radiograph, it was noted that monocytes from patients with more extensive disease produced lower amounts of IL-12, TNF-α and IL-6 in response to infection, and that these cells were more permissive for bacillary replication. Reduced IL-12 and TNF-α production by the monocytes from patients with extensive disease concurred with the plasma measurements of these cytokines. In addition to the direct effects of TNF-α and IL-6 on monocyte activation, reduced IL-12 and TNF-α production may also have resulted in reduced T-lymphocyte sensitization and IFN-γ induced mycobacteriostasis. Taken together, these observations support the idea that robust pro-inflammatory cytokine responses to MTB may limit the extent of pulmonary disease in humans.

The mouse model of TB has provided valuable and detailed insight in the host defense mechanisms against MTB. However, substantial differences exist in the ability of human and murine phagocytes to control the growth of *M. tuberculosis* H37Ra and *M. bovis BCG*. In addition, the histology of human and mouse pulmonary TB differs substantially, with cavitation, caseous necrosis, the presence of multi-nuclear giant cells and extensive fibrosis being the pathognomonic features of human disease. Finally, the dramatic differences in gene expression profile of *M. tuberculosis* residing in human and mouse lungs suggest that the microenvironment to which MTB is exposed may vary considerably between the two host species. These differences underscore the importance of studying TB patients as

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well as humans infected with MTB to advance our knowledge of protective immunity and the reasons for immunologic failure. This knowledge may serve to direct vaccine development and predict vaccine efficacy.

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