Cigarette smoke and human pulmonary immune responses to mycobacteria

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Declaration

I, Richard Nellis van Zyl-Smit hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Dedication

I dedicate this thesis to my God and my family
with whom all of life has worth.

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Abbreviations

7AAD	7-aminoactinomycin D
AFB	acid fast bacilli
Ag	antigen
APC	allophycocyanin
BAL	bronchoalveolar lavage
BCG	*Bacillus Calmette-Guérin*
Ca^{2+}	calcium cation
CSE	Cigarette smoke extract
CDC	Centre for Disease Control (USA)
cDNA	copy deoxyribonucleic acid
CFP-10	culture filtrate protein 10
CFU	colony forming units
CMI	cell mediated immunity
COPD	Chronic obstructive pulmonary disease
cpm	counts per minute
CT	Cycle threshold
CTL	cytotoxic lymphocyte/s
DC	dendritic cell
DOTS	Directly observed therapy short course
EBA	Early bactericidal activity
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
ESAT-6	early secreted antigenic target 6 kDa
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FDA	Federal Drug Administration (USA)
FITC	fluorescein
gfp	green fluorescent protein
HBHA	heparin-binding haemagglutinin
HCW	Health care worker
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
HuPO	Human acidic ribosomal protein
IFN-γ	interferon gamma
Ig	immunoglobulin
IGRA	interferon gamma release assay
IL-	interleukin
IU	international units
kDa	kilo Dalton
LTBI	latent tuberculosis infection
M.avium	Mycobacterium avium
M.bovis	Mycobacterium bovis
M.tb	Mycobacterium tuberculosis
MAPK	Mitogen activated protein kinase
MDR-TB	multi-drug resistant tuberculosis
MGIT	mycobacterial growth indicator tube
MMR	macrophage mannose receptor
MOI	multiplicity of infection

mRNA	messenger ribonucleic acid
NAAT	Nucleic acid amplification test
NICE	National Institute for Clinical Excellence (UK)
NK	natural killer
NKT	natural killer T-cell
NO	nitric oxide
OADC	Oleate-albumin-dextrose-catalase
PAR	Population attributable risk
PBMC	peripheral blood mononuclear cell/s
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridin chlorophyll protein
PI3K	phosphotidyl inositol-3-kinase
PPD	purified protein derivative
QFT GIT	QuantiFERON®-TB Gold In-Tube
qPCR	Quantitative polymerase chain reaction
RD-1	Region of differentiation 1
RIN	RNA integrity number
RLU	relative light units
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RR	Relative risk
RT- PCR	Reverse transcription polymerase chain reaction
SFC	Spot forming cell
STAT	signal transducer and activator of transcription
T-reg	regulatory T-cell/s
TB	tuberculosis
TCR	T-cell receptor
Th	T-helper
TLR	toll like receptor
TNF-α	tumour necrosis factor-alpha
TSPOTTB	T-SPOT.®TB commercial interferon gamma release assay
TST	tuberculin skin test
TTP	time to positivity
TU	tuberculin unit
UCT	University of Cape Town
UK	United Kingdom
US	United States
USD	United States dollar
WHO	World Health Organisation
XDR-TB	extensively drug-resistant tuberculosis
ZAR	South African Rand

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Abstract

Introduction:

Recent epidemiological evidence suggests that up to 15% of worldwide tuberculosis (TB) cases may be attributable to tobacco smoking. The aim of the studies reported here was to gain insights into the effects of exposure to cigarette smoke on human cells that form part of the innate immune system of host defence in the lung.

Methods:

First, a reliable method for exposing human cells *in vitro* to cigarette smoke was developed. In order to reliably establish the tuberculous infection status of volunteers, a series of experiments were performed to evaluate current and new methods of testing for latent tuberculosis infection. These included comparing the performance of the tuberculin skin test with interferon gamma release assays (IGRA). Quantitation of host defences *in vitro* involves an assessment of bacterial load, the performance and utility of several methods used for this purpose were evaluated and compared. Finally, I examined the effects of cigarette smoke extract and nicotine exposure on the function of human macrophage cell lines, monocyte-derived and alveolar macrophages (from healthy adults). Functional tests included the uptake of and cytokines responses to mycobacterial infection and the killing or stasis of BCG organisms.

Results:

The studies of IGRA tests confirmed significant short-term variability of the test results. An analysis of cut-points identified a zone in which results should be considered of uncertain significance. A boosting effect of tuberculin skin testing on IGRA responses was confirmed. The study of methods for determining mycobacterial load established that colony forming units (CFU) on solid media was the most labour-intensive but cheapest. BACTEC-MGIT-960 liquid culture had a lower detection threshold (<10 organisms) than Xpert-MTB/RIF (~100 organisms) but significantly longer turn around time. Cigarette smoke extract (CSE) did not reduce mycobacterial uptake by either monocyte-derived or alveolar macrophages, but containment of intracellular organisms was reduced in 5 day cultures. In addition, production of IFN-γ, TNF-α and IL-10 by monocyte-derived macrophages was significantly reduced.

Nicotine alone reduced IL-10 production. The partial suppression of TNF-α production did not appear to be mediated via nicotine receptors.

Conclusions:

The studies evaluating the interferon gamma release assays have improved understanding of the variability of test results in individual patients, the interpretation of equivocal test results and the impact of prior exposure to tuberculin testing, each of which will have an impact on the use of these tests in research and clinical practice. The formal comparison of mycobacterial load assays, under standardised conditions, provides a useful guide to the selection of the optimal test for each indication. Demonstration of the performance, advantages and disadvantages of each, serves as a guide for clinicians and researchers.

The experiments on the pulmonary effects of cigarette smoke confirm that exposure has a significant effect upon innate host defences. Significant reductions in the production of key cytokines implicated in defences against mycobacteria were observed, not attributable to impairment of mycobacterial uptake by cigarette smoke extract exposure. Furthermore control of intracellular mycobacterial growth was impaired by cigarette smoke extract exposure.

These data have important implications for individuals and for public health strategies to address the tuberculosis epidemic. Tobacco cessation is imperative, but given the deleterious effects of nicotine, inhaled forms of nicotine used in nicotine-replacement therapies, may not reduce the risk of TB. Although there are likely to be several mechanisms responsible for susceptibility of smokers to tuberculosis, these data may be used to motivate for tobacco control and smoking cessation strategies as a priority, particularly in high prevalence regions like South Africa.

1 Introduction and literature review

1.1 Objectives and rationale

Tobacco smoking is the single most preventable cause of death in the world today with an estimated 1.3 billion smokers worldwide.[1] Eight percent of all adult deaths (> 5million people) per year are attributable to tobacco with more than 80% of those deaths occurring in the developing world.[1] Current estimates of tobacco smoking rates are 49% for men and 8% for women in low- and middle-income countries and 37% for men and 21% for women in high-income countries.[2]

One-third of the world's population are thought to be latently-infected with *M. tuberculosis* (*M.tb*).[3] Each year approximately 9.4 million new cases of active tuberculosis (TB) are diagnosed with a resultant 1.7 million deaths.[3] In Africa and particularly in South Africa, the TB epidemic is out of control with an incidence rate of approximately 971 per 100,000 people per year.[3] Although an association between tobacco smoke and TB has been debated for over a century,[4] clear epidemiological evidence has only recently emerged.

There are three comprehensive independent systematic reviews and meta-analyses that have synthesized evidence for the association between TB and tobacco smoking.[2, 5, 6] Smoking is associated with an increased risk of *M.tb* infection (relative risk~1.8), progression to active TB disease (relative risk~2.2) and increased mortality (relative risk ~2.0).[2, 5, 6] Consequently, up to 15% of worldwide TB cases each year may be attributable to tobacco exposure.[7] This estimated population-attributable fraction of ~ 15% for smoking is higher than that for HIV ~11%.[8]

There are currently very few data examining the direct effects of tobacco smoke or nicotine on defence mechanisms in human tuberculosis infection. I therefore set out to examine the effects of tobacco smoke on human immune responses to mycobacterial infection, in support of the hypothesis that exposure to tobacco smoke increases the risk of tuberculosis infection and its progression to disease.

The effects of tobacco smoke on the immune system have been extensively studied and several reviews on the subject have been published.[9-14] Interactions between smoke exposure and susceptibility to several infective agents have been described and studied, but there is almost no data on susceptibility to infection with *M.tb* or BCG. Several approaches, most involving animal models, have been used to examine the effects of smoking on immune function. These models have generally involved exposing the animals to cigarette for days or weeks and examining their response to an infective challenge. Animal models however, may have little relevance to human disease and direct evidence from the study of human tissues *in vivo* or *in vitro* are likely to be more informative.

Demonstrating mechanisms in human cells that may explain the link between smoking and TB infection is the primary aim of the research reported in this thesis. Five questions are addressed: 1) What is the effect of tobacco smoke on mycobacterial uptake by human macrophages? 2) What is the effect of tobacco smoke on cytokine production in response to infection? 3) What mechanism or mechanisms are responsible for these abnormal cytokine responses? 4) Are the abnormalities in cellular function observed attributable to the nicotine fraction of tobacco smoke? 5) What is the effect of tobacco smoke on mycobacterial stasis or killing and on macrophage survival?

A human macrophage model of *in vitro* infection with mycobacteria and *in vitro* exposure to cigarette smoke extract and nicotine was developed. Three human macrophages systems were used: human myelomonocytic cell line (THP-1) macrophages, human monocyte-derived macrophages and human alveolar macrophages from healthy non-smokers. BCG, a well accepted representative mycobacterial strain used in research on tuberculous immunity was selected for these experiments in order to establish and develop the methods and obtain data on cigarette smoke extract exposure and mycobacterial human cell interactions. The findings of these experiments with BCG pave the way for future confirmatory work involving pathogenic strains of *M.tb.*

Methods for *in vitro* exposure of cells to cigarette smoke have not been standardised and several techniques have been described. A common approach has been the preparation of a cigarette smoke extract by drawing air through a cigarette and bubbling it through a liquid medium. The current project demanded a standardised and reproducible cigarette smoke extract (CSE) to which to expose cell preparations. The first task was therefore to develop a reliable "smoke extract generation device" and a technique for exposing human cells to CSE *in vitro*.

The cell preparations used to examine the effect of *in vitro* mycobacterial infection status on immunological responses were monocyte-derived and alveolar macrophages obtained from healthy HIV negative volunteers without clinical evidence of tuberculous disease. In a high burden setting like South Africa, many such volunteers are likely to have been infected with tuberculosis, that is, may have latent infection. Since this was likely to influence the *in vitro* responses of their cells in culture, it was important to establish the latent infection status of all subjects who donated blood mononuclear cells or alveolar macrophages for experiments. Currently, there is no gold standard definition for latent TB infection (LTBI) nor is there clarity of what LBTI signifies. On the one hand, individuals with LTBI may be considered to represent those in whom sterilising immunity has failed - a susceptible immunological phenotype. On the other hand, since fewer than 10% of latently infected individuals develop active disease, LTBI may also be considered a protective phenotype. This lack of clarity on the significance of LTBI, in terms of immunological and host defence competency, prompted the investigation and review of current understanding of this state and of methods for diagnosing it.

Understanding of the significance of LTBI and its diagnosis has been revolutionized by the recent development of tests of antigen-specific T-cell responses to mycobacterial antigens. Historically, the tuberculin skin test (TST) has been the standard test for identifying patients with LTBI. However, limitations of this test, in terms of accuracy and reliability are well known.[15] IFN-γ release assays (IGRAs) that utilise peripheral blood-derived T-cell responses to relatively TB-specific antigens, early secreted antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 (CFP-10), have been shown to be sensitive and specific laboratory markers of LTBI.[16]

However, there have to date been widely divergent views on how the TST and IGRAs should be employed in screening for LTBI. Some guidelines have suggested that TST should be replaced by IGRAs,[17, 18] while others suggest that TST should be used as the initial screen for LTBI and that IGRA be used to confirm the TST results.[19, 20] A weakness of these recommendations is that most are based on clinical research performed in low TB prevalence settings. Their appropriateness in high prevalence areas needs to be assessed.

In investigating screening for LTBI in our high prevalence area, I initially adopted a dual approach, using both the TST and IGRA. However, two technical questions arose on which there appeared to be little or no published data:[21, 22] First, was the uncertainty on what constitutes a significant change in IFN-γ response, given that IFN-γ responses in individual subjects appear to vary over time. The second was the influence of a prior TST on subsequent IGRA test results. I therefore conducted studies on the within-subject variability of the IFN-γ responses over time, examined the reliability of cut-points used to define presumed LTBI and studied the effect of a TST on subsequent IGRA test responses. Using this information I was able to more accurately characterise the infection status of the participants enrolled in the studies of cellular responses to BCG infection and cigarette smoke.

A further developmental component of this body of work, necessary for assessing mycobacterial stasis or killing, was to develop a reliable method for quantifying mycobacteria in test specimens. Such methods are also relevant for research on new drugs and vaccines in tuberculosis, and have many other uses.[23-26] I evaluated and compared five methods for quantifying mycobacteria, some established and others experimental.

At present, the lack of mechanistic data on the relationship between smoking and poor TB outcomes is fuelling uncertainty about the importance of this association. Should the epidemiological link between smoking and TB be backed up by a mechanistic explanation, it would add credibility to and enhance advocacy to include tobacco cessation and prevention programs as part of National and global TB control efforts. Furthermore, it might also provide insights into known or novel mechanisms

whereby host immunity to tuberculosis is subverted *in vivo*. If specific constituents of cigarette smoke such as nicotine were shown to impair immune responses to mycobacterial infection, this would have implications for the selection of tobacco cessation strategies; using nicotine replacement therapy and the therapeutic use of nicotine-containing products such as electronic cigarettes.

Findings of this research will be of particular relevance in developing countries like South Africa, India and China, where the prevalence of both tuberculosis and tobacco smoking is high.

1.2 The epidemics of tobacco smoking and tuberculosis

At the beginning of the 21st century we are facing the convergence of several epidemics with considerable consequences for global respiratory health. At the end of the 19th century a similar pattern was seen as tobacco smoking was widespread and increasing after the invention of the cigarette-rolling machine in 1881.[27] Furthermore, the discovery of *Mycobacterium tuberculosis* by Robert Koch one year later in 1882 occurred during a time that tuberculosis (TB) was rampant in many parts of the world.

Nearly 100 years later, smoking is at an all time high with over 6,319 billion cigarettes consumed per year.[27] Tuberculosis remains uncontrolled in the developing world with increasing rates of multi-, extensively- and pan-drug resistant tuberculosis.[3] Tobacco smoking, TB, in addition to HIV infection and chronic obstructive pulmonary disease (COPD), are colliding on a global scale with the epicentre in sub-Saharan Africa.

1.2.1 Tobacco smoking

Approximately one-third of the world's population are active smokers (~3 billion people).[1] Tobacco smoke is known to be carcinogenic, the primary etiological factor for chronic obstructive airways disease (COPD) and a key component in promoting cardiovascular disease. It is considered to be the single most preventable cause of death in the world today. Eight percent of all adult deaths (> 5 million people) each year are attributable to tobacco smoking and of the 8 leading causes of death, all except diarrhoeal disease have a tobacco attributable component.[1] (Figure 1-1) Although no tobacco attributable fraction for HIV is included in this figure, there is now increasing evidence that smoking is also a risk factor for HIV disease and its co morbidities.[28-30]

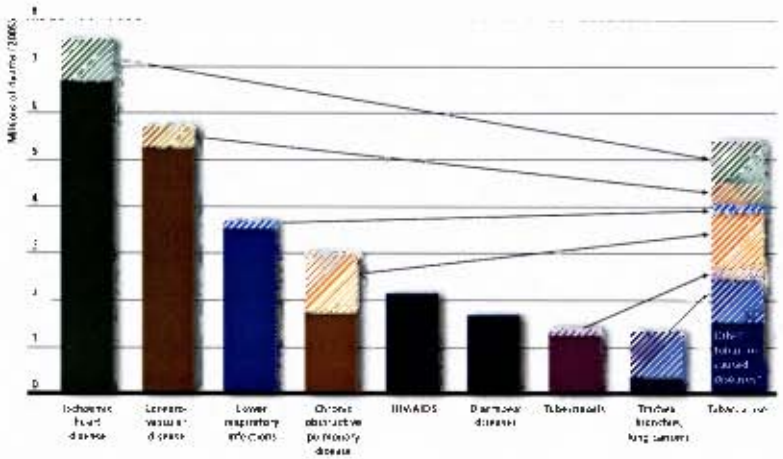


Figure 1-1 The tobacco attributable fraction for the eight leading causes of death

Each bar represents the number of deaths for each of the eight leading causes of death as reported by the WHO in 2008. The hatched fraction represents the estimated tobacco attributable fraction for each condition.[2]

Estimates of tobacco smoking rates globally are 49% for men and 8% for women in low- and middle-income countries and 37% for men and 21% for women in high-income countries.[2] (Figure 1-2) In South Africa the rates of smoking have reduced over the past 20 years: in 1992 it was estimated that 52% of men and 17% of women smoked.[31] By 2003 smoking rates had reduced for both men and women to 35% and 25% respectively.[32] The latest estimates for South Africa are 25% for men and 7.8% for women.[33] South Africa has stringent anti-tobacco legislation, which was amended in 1999 and 2008[34, 35] to be inline with the Framework Convention on Tobacco Control, which South Africa ratified in 2005.[1] This legislation includes a complete ban on smoking advertising and smoking in public spaces, which may account for some of the reduction in smoking rates. However, a study from Cape Town in 2010 found that 56% of TB patients smoked.[36]

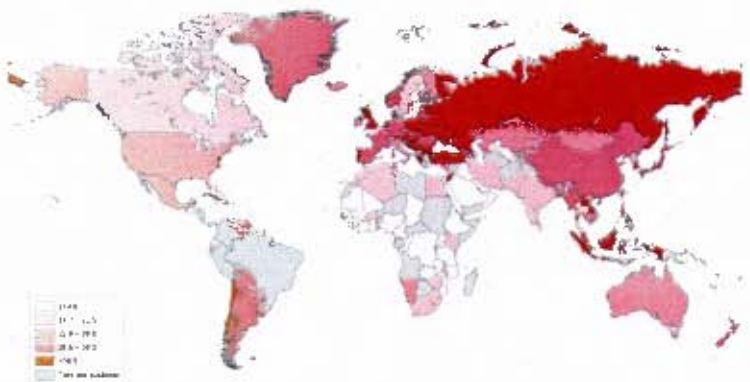


Figure 1-2 Global map of smoking rates for individual countries

Increasing intensity of pink depicts higher rates of smoking. For countries with grey shading no estimates are available. The rates depicted are for 2005.[2]

1.2.2 Tuberculosis

Over one-third of the world's population are thought to be latently infected with *M. tuberculosis*. The latest figures released by the WHO estimate that in 2009 there were 9.4 (range estimate: 8.9-9.9) million new cases of tuberculosis globally.[3] Approximately 1.7 million deaths occurred from TB: 1.3 million in HIV uninfected and 400 000 in HIV co-infected people. The countries with the highest TB burden are India (1.6–2.4 million), China (1.1–1.5 million) and South Africa (0.40–0.59 million). South Africa however has a significantly higher incidence rate of 971 per 100 000 people compared to 96 per 100 000 people for India and 168 per 100 000 people for China.[3] (Figure 1-3)

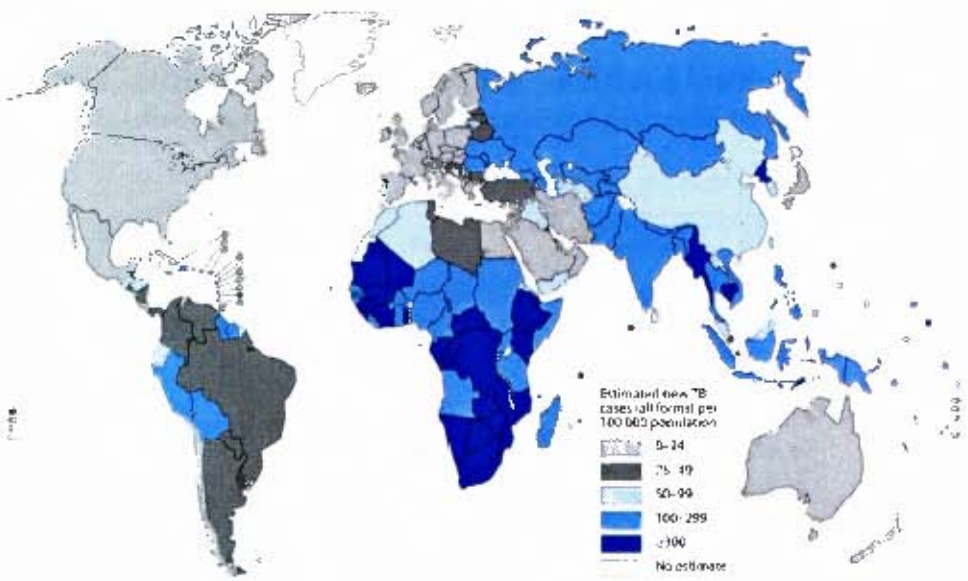


Figure 1-3 Global map of tuberculosis incidence rates

Incidence rates are depicted by increasing intensity of blue for each country. Grey shading indicates very low rates. The rates depicted are for 2009. [3]

1.2.3 The association between smoking and tuberculosis

The association between tobacco smoke and tuberculosis has been debated for almost one hundred years. In an article published in the American Review of Tuberculosis in 1918, Major Gerald Webb "listened to the chests of over 3000 soldiers" for the presence of "ronchi" and additionally documented "their preference in regard to tobacco".[4] Over 80% of the cohort were active smokers. In those who were "discharged on account of tuberculosis", 30% were stated to "not inhale cigarette smoke" or have "ronchi" and were either non-smokers or pipe/cigar smokers. Unfortunately, his final conclusion was that: "these studies would at least

suggest that the inhalation of the smoke of cigarettes does not aid in the outbreak of pulmonary tuberculosis".[4] Since then many more studies have examined this association and have been confounded by the strong association of both tuberculosis and smoking with poverty, overcrowding, malnutrition and alcohol use; all of which are risk factors for tuberculosis. However, there are now three comprehensive systematic reviews and meta-analyses that have synthesised the evidence for the association between TB and tobacco smoking.[2, 5, 6] These have examined the association of smoking and TB for three outcomes: TB infection (defined as a positive tuberculin skin test), active TB disease and death due to TB. Table 1-1 provides the outcome-specific pooled relative risk estimates from the 3 meta-analyses.

Table 1-1 The association between smoking and the relative risk of latent tuberculosis infection, progression to active disease and mortality from active TB disease.

Meta-analysis [Reference]	Pooled relative risk (95% C.I.)		
	TB infection [n= ~6 studies]	**TB disease** [n= ~15 studies]	**TB mortality** [n= ~5 studies]
Slama et al. 2007[2]	~1.8 (1.5-2.1)	~2.3 (1.8-3.0)	~2.2 (1.3-3.7)
Lin et al. 2007[5]	~1.7 – 2.2 (1.5-2.8)	~2.0 (1.6-2.6)	~2.0 (1.1-3.5)
Bates et al. 2007[6]	~1.7 (1.5-2.0)	~2.3 (2.0-2.8)	~2.1 (1.4-3.4)

n= number of studies in the meta-analysis

It is evident from these meta-analyses that smoking approximately doubles the relative risk (RR) of each outcome, namely TB infection (RR ~1.5), active TB disease (RR ~2.0) and TB mortality (RR ~2.0). The evidence is strong for TB disease, but relatively weaker for latent TB infection. Due to the widespread nature of tobacco smoking the population attributable risk (PAR %) is high. For example, if the relative risk for TB disease is estimated at 1.5 and population exposure to tobacco smoke is 30%, the PAR% is approximately 15%. In other words, 15% of the TB cases in the world each year may be attributable to tobacco exposure.[37] The estimated population attributable fraction for smoking (~ 15%) is indeed higher than that for HIV (~11%)[8] Table 1-2 summarises the global population attributable fractions.

Table 1-2 Population attributable fraction of several risk factors for TB

Risk factor	Population attributable fraction
Tobacco smoking	15.8%
HIV	11%
Malnutrition	26.9%
Air pollution	22.2%
Alcohol abuse	9.8%
Diabetes	7.5%

Data reproduced from Lönnroth et al. [8]

Since the publication of these meta-analyses, newer studies have been published and confirm the association between smoking and TB. In a large case-control study from India, Jha et al. reported excess TB deaths among smokers, as compared with non-smokers among both women (RR, 3.0; 99% CI, 2.4 to 3.9) and men (RR, 2.3; 99% CI, 2.1 to 2.6).[38] A subsequent large case-control study from India reported that those who both smoked cigarettes and drank alcohol had considerably higher active TB incidence rates than those who did neither (TB incidence RR 3.5).[39]

Similar results have been reported from Taiwan and China. In a prospective cohort study from Taiwan, Lin et al. reported that current smoking was associated with an increased risk of active TB (adjusted OR, 1.94; 95% confidence interval, 1.01-3.73).[40] In a case-control study from two rural areas of China, Wang et al. reported an adjusted OR of 1.93 (95% CI: 1.51–2.48) for smoking and TB disease.[41] A cohort study in Brazil looked at the association between smoking and a fourth outcome, TB relapse. They reported that smoking was independently associated with relapse of TB, as defined by the requirement for re-treatment within 3-5 years after successful completion of TB treatment. After adjusting for socioeconomic variables and alcohol, the OR for relapse was 2.53 (95% CI, 1.23, 5.21).[42]

A mathematical modelling study by Lin and colleagues further supports the association between smoking and TB.[43] Taking into account the accumulation of hazardous effects of risk factors on COPD and lung cancer over time, and dependency of the risk of TB infection on the prevalence of disease, their model suggested that,

provided directly observed treatment short-course (DOTS) coverage remains at 80%, smoking cessation would reduce the projected annual TB incidence in 2033 by 14-52%. This is in addition to the potential prevention of 26 million deaths from COPD and 6.3 million deaths from lung cancer if both smoking and solid fuel usage ceased.[43]

An interaction between TB and HIV infection became evident soon after the HIV epidemic started with new associations continuing to be recognised, including treatment-related complications and drug interactions.[44] The effect of the HIV epidemic on TB has been well documented with incident rates of TB particularly in Africa, rising rapidly as a result of HIV.[45] The risk of active TB doubles in the first year of HIV co-infection[46] and the risk of developing active disease in those who have latent tuberculosis infection (LTBI) is approximately 10% per year.[47] HIV-TB co-infected individuals have reduced survival[48] and are at higher risk for subsequent opportunistic infections.[49, 50] In overcrowded and poor living conditions, the combined effect of the two epidemics is magnified as evidenced by greater than 2000/100 000 population prevalence rates in certain South African communities.[51] Added complications in co-infected individuals include drug-resistant TB and immune reconstitution inflammatory syndrome (IRIS).[52]

Causality between tobacco smoke and COPD is well established and it remains the primary risk factor for COPD.[53] In addition to the documented dose dependent risk of COPD from tobacco smoke,[54] the importance of the "total burden of inhaled particles", including occupational, household and environmental exposures, is increasingly being recognised.[55-58] Post-tuberculous 'obstructive airways disease' was described in the 1950s-1960s by several authors.[59-62] Hallet and Martin reported that: "It is increasingly evident that a diffuse obstructive pulmonary syndrome is often associated with tuberculosis."[61] They observed that in the 1800's Laennec had reported the association of emphysema with TB.[60] Although this association continues to be widely recognised by clinicians in developing countries,[63-65] it has been poorly studied.[65, 66] In addition to the burden of tobacco smoking, air pollution (indoor and environmental) and poverty, HIV is prevalent in many developing countries, particularly in sub-Saharan Africa. HIV infection alone is now being studied as a potential risk factor for the development of COPD.[28, 67-71]

It is thus increasingly evident that the epidemics of tuberculosis and tobacco smoking are colliding with HIV and COPD. (Figure 1-4) These epidemics, although occurring globally are having a particular impact on respiratory health in sub Saharan Africa.

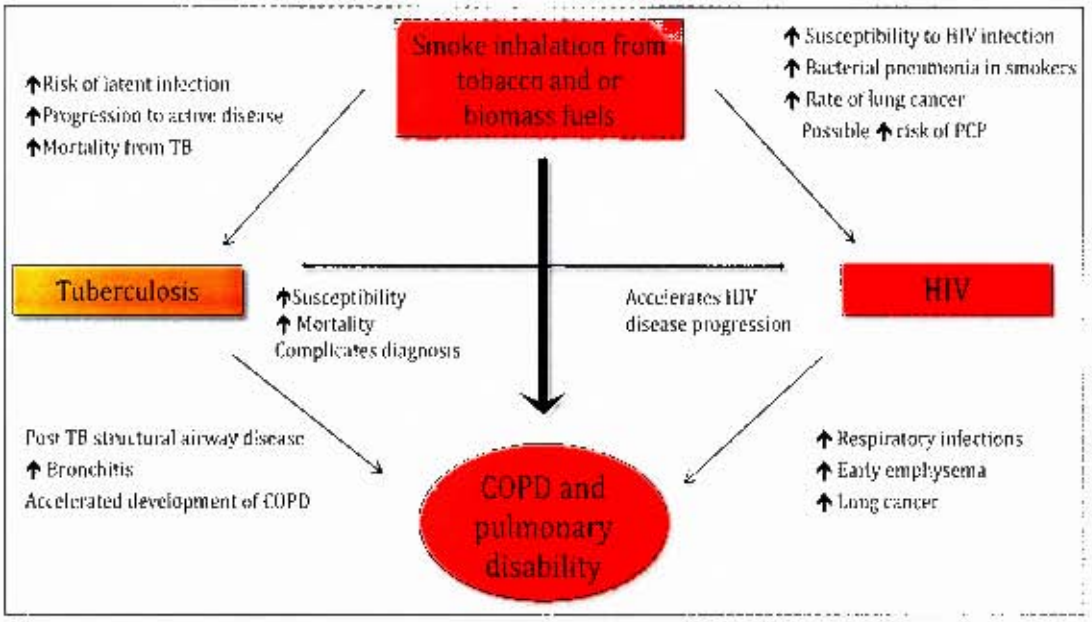


Figure 1-4 The colliding epidemics of tobacco smoking, tuberculosis, HIV and COPD

The interactions and associations between HIV, TB, smoking and COPD are depicted by the arrows connecting the individual diseases. The majority of the associates apart from smoking and COPD are based on epidemiological rather than basic science evidence.[7]

1.3 Smoking and immune responses to infection and tuberculosis

1.3.1 Tobacco smoke and immunity

The effect of tobacco/cigarette smoke on the immune system has been extensively studied with many in-depth reviews published.[9-14] Tobacco smoke is made up of both particulate matter and a gaseous phase and contains over 8000 chemicals at last report.[72] The effects on the immune system can broadly be classified into peripheral blood effects, airway effects and alveolar/cellular effects. These effects are depicted and summarised in Figure 1-5.

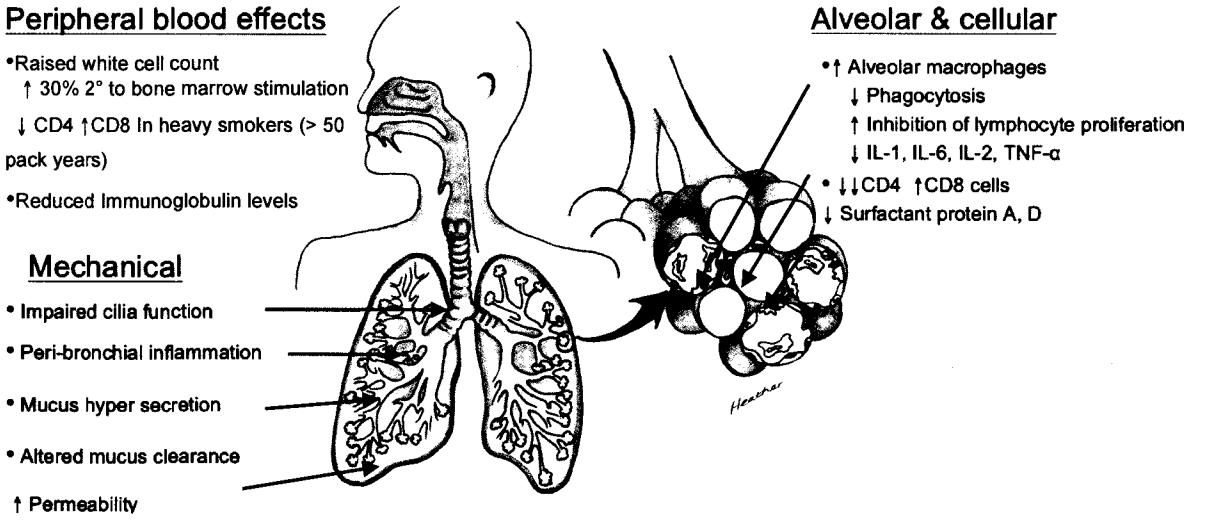


Figure 1-5 The effects of tobacco smoke on host immunity

The effects of tobacco smoke on components of the immune system with particular emphasis on the pulmonary defences are depicted.[73]

1.3.1.1 Tobacco smoke and the airways

Tobacco smoke has several effects on the structure and function of the respiratory tract. Mucus hyper secretion occurs as a result of gland hyperplasia[74] and direct secretogogue effects of nicotine.[75] In addition, it has effects on ciliary function[76-78] resulting in impaired airway clearance.[79, 80] The effect on ciliary function is mediated in part by acrolein as well as acetaldehyde (found in both tobacco smoke and alcohol).[78] At a cellular level, there is increased peribronchial inflammation[81, 82] and increased permeability.[83]

1.3.1.2 Peripheral blood

Smokers have raised peripheral blood white cell counts[84-86] and in heavy smokers (>50 pack years) alterations in the CD4:CD8 ration is evident.[87] Immunoglobulin levels are also reduced by 10 to 20% in smokers.[88-90] Smoking also raises CRP and fibrinogen levels important additional markers of cardiovascular disease.[91, 92]

1.3.1.3 Alveolar and cellular level

Smokers have increased alveolar macrophages in bronchoalveolar lavage fluid,[93-95] reduced surfactant A and D[96] and altered immunoglobulin levels.[97, 98] Alveolar macrophage phagocytic function is impaired in smokers (human and mice-exposed). Several studies have shown reduced phagocytosis of organisms such as *Listeria,*[99]

haemophilus,[100, 101] *Staphylococcus*,[93, 102] *Streptococcus*,[103] *Cryptococcus*[104] and *Candida*.[105] A reduced phagocytic index has also been demonstrated in short-term cigarette smoke (<24 hour) exposure models.[102, 103, 105, 106]

The impact of both tobacco smoke and individual components of tobacco smoke (nicotine and acrolein) on cytokine responses have been widely investigated. Cigarette smoke exposure reduced TNF alpha in response to non-typeable *Haemophilus influenzae* infection,[107] and nicotine has been shown in models of *Legionella* infection to reduce production of IL-6, IL-12 and TNF-α, but not IL-10.[108] In peripheral blood mononuclear cell models, smoke exposure reduced TNF-α, IL-1β and IFN-γ whereas acrolein reduced TNF-α and IL-2 production by T-cells following PMA stimulation.[109, 110] Two bronchoalveolar lavage cell models have shown reduction in TNF-α and IL-1β from BAL cells stimulated by LPS[94] and IFN-γ and IL-2 from BAL cells stimulated by PMA.[111] In a contrasting inflammatory model of ulcerative colitis, nicotine reduced IL-10, but not IL-2 and TNF-α production.[112]

1.3.2 Mycobacterial immune responses

The immunological mechanisms that underpin the mechanistic link between smoking and TB are unclear, though several have been proposed.[113, 114] There is a paucity of data in both humans and animal models directly investigating the effect of tobacco smoke on immune responses to mycobacterial infection/exposure.[115, 116] In a mouse model of tuberculosis and smoke exposure, Shang and colleagues showed an impaired immune response to infection in mice exposed to cigarette smoke over a 14 week period with resultant higher loads of bacilli in the lungs and spleen of exposed mice.[116] Several of the altered immune responses shown in non-tuberculosis cigarette smoke exposure models, may be relevant to the smoking and tuberculosis association.

Cytokines crucial to the immune response to tuberculosis such as IFN-γ, IL-12 and TNF-α are reduced by smoke exposure. In addition, TNF-α production is reduced by nicotine through an interaction with α7 nicotinic acetylcholine receptors.[108] Apoptosis, an important mechanism to control intracellular growth of bacilli[117, 118]

has been shown in some non-TB macrophage models to be reduced by smoke exposure,[119, 120] but increased in others.[121-123] Furthermore, cigarette smoke prevents pathogen-specific expansion and activation of CD_4 T cells[124] and reduces IFN-γ producing adenoid-specific CD_4 and CD_8 T cell numbers.[119] Other potential mechanisms by which smoking may attenuate pulmonary immunity include, increased oxidative stress on the lung, mechanical disruption of ciliary function and other clearance mechanisms in the tracheo-bronchial tree. [125]

Given the large body of evidence of the negative effects of tobacco smoke on immunity, it is likely that smoking will additionally impair TB specific human pulmonary immune responses, although this is as yet unproven.

1.4 Determining LTBI status in healthy subjects

In many countries with a low incidence of tuberculosis (TB), serial (repeated) testing for latent TB infection (LTBI) is done for individuals at high risk of TB exposure.[21] There is however no gold standard for defining LTBI. Asymptomatic infection by *M.tb* organisms is detected by the demonstration of a cutaneous delayed hypersensitivity reaction to the intra-dermal injection of purified protein derivative (PPD) – the tuberculin skin test (TST). The diagnosis is 'presumed' and until the development of the interferon gamma release assay, this was the only test available. Although widely used, the TST has limitations in accuracy and reliability.[15] Furthermore, interpretation of serial TST results is complicated by the problems of non-specificity, boosting, conversions, and reversions.[126-128]

Recently, the development of more specific *in vitro* assays for LTBI – interferon-gamma (IFN-γ) release assays (IGRAs), has offered an alternative approach to LTBI diagnosis.[129] IGRAs are blood tests based on IFN-γ release after stimulation by relatively TB specific antigens such as early secreted antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7. These antigens are more specific to *M. tuberculosis* than purified protein derivative (PPD) used in the TST.[129] IGRAs are

highly specific, also in BCG vaccinated populations.[16, 130] IGRAs additionally have features that make them ideal for serial testing: they are more specific than TST, they are *ex-vivo* assays and can be repeated any number of times without sensitisation and boosting. The testing protocol does not require a second visit to read the TST response, and unlike the TST there is no need for a baseline two-step testing protocol.

While some guidelines have recommended the use of IGRAs for serial testing,[17, 18] others have been more cautious.[19, 20] Some guidelines have suggested that TST may be replaced by IGRAs,[17, 18] while others have suggested initial testing with TST, with IGRA as a follow-up option to confirm TST results.[19, 20] Regardless of the approach, widespread use of IGRAs in serial testing is hampered by lack of evidence on several key questions:[21, 22] 1) What is the within-person reproducibility of T cell responses over time (in other words, what amount of variation is expected when IGRAs are repeated)? 2) Given a certain degree of "inherent variability", how does one interpret a single test result close to the assay cut point? 3) Will a TST boost or affect the results of subsequent IGRA testing and what is the optimum time gap between the two tests? 4) What is an IGRA "reversion" and what threshold should be used to define reversion? 5) What is the clinical significance and prognosis of an IGRA reversion? 6) What is an IGRA "conversion" and what threshold (cut-off) should be used to define conversion? 7) What is the prognosis (i.e. predictive value) of an IGRA conversion and will treatment of individuals with IGRA conversions reduce their risk of progression to active disease?

To achieve an accurate and consistent definition of LTBI in our research subjects, I set out to answer some of the questions outlined above. Further details and rationale for the study are provided in Chapter 3.

1.5 Quantifying mycobacterial load in research studies

In recent years, tuberculosis research has increased dramatically as new vaccines, drugs and diagnostics are sought to curb the expanding epidemic of both sensitive

and drug resistant tuberculosis.[131-133] Accurate determination of mycobacterial load is a basic requirement of many laboratory and clinical studies. Although several techniques exist to determine bacillary load in clinical and research samples, each is associated with limitations including, their cost, methodological complexity, delay in obtaining results (turn-around-time) and the detection limits of the test.

Culture on solid media using colony forming units (CFU), is widely considered to be the gold standard for determining the number of viable organisms in clinical or laboratory samples, but is labour intensive and has a long turn-around-time.[134, 135] Several alternative techniques are however available to determine mycobacterial burden. These include the incorporation of tritiated uracil into mycobacterial DNA, bioluminescence assays that use a reporter construct, quantitative real-time polymerase chain reactions (PCR) and time to positivity (TTP) in automated liquid culture systems (BACTEC MGIT 960].

Bioluminescence and uracil incorporation assays both provide rapid turn around time and are extensively used in laboratory studies.[25, 136, 137] They however have no application in clinical studies due to the requirement for specific *M.tb*-luciferase constructs and susceptibility to bacterial contamination respectively. Quantitative and conventional PCR that amplify *M.tb*-specific DNA to determine mycobacterial burden has been limited by the inability to distinguish viable from degraded organisms and detecting *M.tb*-specific mRNA from viable organisms by RT-PCR is technically challenging.[138] More recently, newer technologies such as the Xpert® MTB/RIF system (Cepheid, Sunnyvale, USA) have been developed for the rapid detection of TB using clinical samples. It may represent an accurate quantitative tool as through an intermediary wash step, contaminating DNA is discarded. Only DNA from intact organisms trapped in a mesh is amplified in the PCR step.[139, 140]

Liquid culture using the automated BACTEC 960 MGIT system is also a very attractive technology for both clinical and laboratory studies. Already incorporated into EBA studies,[134, 135] time to positivity has been well equated with bacterial load.[135, 141, 142] Although the semi-automation, high discriminative ability and low detection threshold makes it very suitable for laboratory studies, the slow turn around time

and cost exceed those of solid culture.[143] To establish the optimal methods for quantifying mycobacterial load in this research project, a comparative study of available techniques was performed. Further details and rationale for the study are contained in Chapter 4.

In summary, the review of the literature on the association between tobacco smoke and tuberculosis confirms that although substantiated epidemiologically, further work was required to identify mechanisms underlying this association. To date most work on this subject has been performed in animals, but studies in humans are lacking. To study this association in humans, the infection status of subjects had to be accurately established. The tuberculin skin test, the standard test for this purpose has significant limitations, but newer interferon gamma release assays show promise for improving this assessment, but require further study to improve their application and interpretation of results. Several techniques to quantify mycobacterial load in this study were available, but the most suitable required clarification.

The questions identified as requiring study were as follows:

1. How could a consistent definition of latent TB infection in the research subjects be achieved?
2. What mycobacterial quantification assay would be most suitable?
3. What is the effect of tobacco smoke on mycobacterial uptake by human macrophages?
4. What is the effect of tobacco smoke on cytokine production in response to infection?
5. What mechanism or mechanisms are responsible for these abnormal cytokine responses?
6. Are the abnormalities in cellular function observed attributable to the nicotine fraction of tobacco smoke?
7. What is the effect of tobacco smoke on mycobacterial stasis or killing and on macrophage survival?

These needs and the methods used to address them are described in the chapters that follow.

2 General methods and validation

2.1 Subject recruitment

2.1.1 Latent TB infection and monocyte-derived macrophage (MDM) studies

Volunteers for the latent TB infection and monocyte-derived macrophage (MDM) studies included health care workers, medical students and laboratory personnel. Participants were in good health and informed consent was obtained prior to participation in LTBI screening and phlebotomy. (Details about subject recruitment for the LTBI study are provided in Chapter 3.

2.1.2 Alveolar macrophage studies

Ethical approval from the University of Cape Town Health Sciences Research Ethics Committee was obtained to undertake bronchoscopy and lavage of healthy volunteers in addition to venesection. Participants were adult staff-members from within the Groote Schuur Hospital complex and asymptomatic tuberculosis-exposed family members of participants enrolled in on going local TB diagnostics studies.

All subjects provided informed consent prior to participation in the study and were compensated according to local ethical policy. Only physically healthy subjects were enrolled for bronchoscopy. Exclusion criteria included asthma, uncontrolled hypertension, ischaemic heart disease, diabetes, pregnancy and immunosuppression. A CXR was performed to exclude any active respiratory disease and a full blood count, and coagulation profile was done to screen for possible bleeding tendencies. Details of bronchoscopy preparation and lavage techniques are detailed in section 2.2.4.

2.2 Acquisition and preparation of human cells

2.2.1 THP-1 cells

Frozen stocks of the human myelomonocytic cell line THP-1 (American Type Culture Col- lection) in a volume of 0.5ml were thawed in a 37°C water bath and transferred into a 15ml conical tube without delay. The cells were reconstituted by adding very

small volumes of RPMI (Roswell Park memorial Institute)-1640 with 25mM Hepes and L-glutamine (Biowhitiker; Lonza, Wakersville, MD, USA) containing 5% foetal bovine serum (FBS)(Gibco®; Invitrogen, Grand Island, NY, USA) to a volume of 1ml. Further diluting of the cells was performed slowly to preserve the integrity of the cells until a total volume of 15ml was obtained.

The cells were subsequently washed 3 times at 1100rpm for 10min at 4°C. The final cell pellet was resuspended in 10ml of complete medium (10% FBS in RPMI). The cells were transferred to 25cm^2 cell culture flasks (.2μm vent cap, #430639, Corning; Corning, NY, USA) and incubated at 37^{0}C in a 5% CO_2 humidified incubator for 48 hours. Culture flasks were placed upright until significant cell growth had occurred then placed horizontally and maintained at 1x10^5 cells per ml concentration by twice weekly replacement of fresh media.

In order to differentiate THP-1 cells into adherent cells, 2x10^5 cells/ml per well were seeded in a 24 well plate (Falcon; BD Biosciences, San Jose, CA, USA) in complete medium with the addition of PMA (phorbol 12-myristate 13-acetate, Sigma, St Louis, MO) at a final concentration of 5pg/ml for 48 hours. Fresh complete medium was added to the resultant adherent macrophages after 48 hours.

2.2.2 Peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected into sodium heparin VACUTAINER® tubes and diluted with an equal volume of Ca^+ and Mg^+ free phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMC) were isolated by density sedimentation using Ficoll-Hypaque (Sigma-Aldrich, Steinham, Germany) using standardised techniques. Briefly, diluted blood in PBS was layered onto Ficoll and centrifuged at 400g for 25 minutes at room temperature. The resultant interface was removed and washed twice with PBS and centrifuged at 300g for 10 minutes. After the final wash the PBMC were adjusted to 1x10^6/ml in complete medium.

2.2.3 Preparation of monocyte-derived macrophages

PBMCs (obtained by density sedimentation as described in 2.2.2) were seeded at a concentration of $1x10^6$/ml into either 24 well plates (Corning) or 96 well plates (Corning) for mycobacterial uptake studies or cytokine and mycobacterial stasis assays, respectively. The cells were incubated at 37°C with 5% CO_2 for 6 days to ensure that the monocytes had acquired a macrophage phenotype (MDM). The adherent macrophages were presumed to be at $1x10^5$/ml, as mononuclear cells comprise approximately 10% of monocytes. Non-adherent cells were removed by washing with warmed RPMI prior to any further experiments.

2.2.4 Harvesting and preparation of alveolar macrophages

2.2.4.1 Bronchoscopy and lavage

2.2.4.1.1 Participant safety

To determine whether the subjects were presumed latently infected with TB, interferon gamma release assays (IGRA) including QuantiFERON®TB Gold-in-Tube and T-SPOT.®TB, in addition to a tuberculin skin test (TST) were performed. HIV testing was performed following appropriate counselling. All subjects undergoing bronchoscopy were evaluated by a certified pulmonologist (respiratory physician) and the CXR, safety bloods and medical history were reviewed prior to the procedure. All participants undergoing bronchoscopy signed a separate informed consent form. An experienced bronchoscopist performed all lavages. Additional safeguards undertaken to minimise risk included the following:

- Neither brushings nor transbronchial biopsies were taken.
- Participants with asthma, ischaemic heart disease, uncontrolled hypertension, any arrhythmia or significant medical disorders were excluded.
- Venous access was maintained during the procedure and pulse and oxygen saturation monitored continuously.
- Supplemental oxygen was provided during the procedure to minimise the risk of hypoxia and arrhythmias.

- Participants were nil per mouth for at least 6 hours prior to the procedure and remained starved for at least 3 hours after the procedure to minimise the risk of aspiration.
- Full resuscitation facilities were available and intensive care facilities, if required, were accessible.
- Sedation with midazolam was allowed in 1mg increments until conscious sedation was achieved. Using this approach it was unlikely that doses greater than 0.05mg/kg would be required. At this dose, respiration was not adversely affected.
- The total amount of lignocaine used as topical anaesthesia was maintained below the 6-8mg/kg maximal recommend dose[144, 145] thus providing a margin of safety.
- All bronchoscopes were sterilised prior to bronchoscopy so that there was no risk of transmission of infection to study subjects.
- Subjects were monitored during recovery and were not allowed to drive a vehicle once discharged (following sedative drug administration). Subjects were provided with an emergency number to contact the research team at all times, and a telephonic follow-up was performed one-week post procedure.

2.2.4.1.2 Bronchoscopy and lavage technique

Alveolar lavage cells were obtained by bronchoscopy as follows. Following the administration of local anaesthetic gel and spray to the nose and pharynx, a flexible video-bronchoscope was passed through a nostril to the level of the vocal cords. Further lignocaine was sprayed onto the cords. No fluid was aspirated prior to passing the cords to minimize the risk of contamination of the bronchoscope suction channel with nasopharyngeal organisms.

Following further administration of lignocaine to the lower airways, the tip of the bronchoscope was wedged into the right middle lobe bronchus. A 300ml lavage using sterile saline, in 60 ml aliquots with a dwell time of 10 seconds was performed with low suction (<20cm H_20). The lavage fluid was aspirated into a sterile glass container and a maximal return attempted (patient tolerance and physical return). The fluid was then transported on ice directly to the laboratory for processing.

2.2.4.2 Preparation of alveolar macrophages

The volume of the BAL fluid obtained was documented. The BAL was passed through 2-ply gauze to remove any mucus and particulate debris then transferred into sterile 50ml conical tubes. Following centrifuging at 300g for 10 min at room temperature, the resultant pellets were combined and reconstituted with 50 ml PBS. The cells were washed and centrifuged at 300g twice more then resuspended in 2ml of RPMI containing 10% human AB serum and 0.1%Fungin™ (Invivogen, France) and 100u/ml penicillin (Sigma Aldrich). A diff quick cell count and viability count was determined by counting on a haemocytometer with trypan blue exclusion dye (Sigma Aldrich). Appropriate cell concentrations were prepared for each of the various experiments performed. Non-adherent cells were washed off after three hours.

2.2.5 Assessing macrophage viability

Several techniques were used to determine macrophage viability dependent on the specific experimental question. To determine viability of macrophages exposed to cigarette smoke, macrophages were stained with trypan blue exclusion dye and counted using a haemocytometer. For flow cytometry experiments, macrophage viability was determined by 7AAD viability dye (eBiosciences) staining. Immediately prior to acquisition of the cells 10µl of 7AAD was added to the cells. Once acquired the cells were analysed on a FACSCalibur flow cytometer using *Cell Quest* software to determine the proportion of viable cells. For fluorescent microscopy, 7AAD was added to the cell suspension (or culture medium of adherent cells) immediately before viewing under a fluorescent microscope.

2.3 Preparation of mycobacterial stock

Mycobacterium Bovis Bacillus Calmette Guérin expressing green fluorescent protein (BCG-gfp); a kind gift from Prof. B Ryffel, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, South Africa was grown in Middlebrook 7H9 broth (Difco, Detroit, Michigan) supplemented with 10% OADC (oleate-albumin-dextrose-catalase) enrichment media (Becton Dickenson) and 0.02% glycerol (Merck, Darmstadt, Germany). The media was supplemented with

Hygromycin B (50ug/ml; Boehringer Mannheim, Germany) for selection of recombinant mycobacteria and cultures were grown at 37°C in a 5% CO_2 humidified chamber and shaken and assessed daily for growth. At mid-log phase the cultures where snap frozen in 10% glycerol and stored at -80°C.

To determine the colony forming units (CFU), 3 tubes of the frozen BCG were thawed. The bacilli were subjected to sonication (Microson Ultrasonic Cell disruptor, Misonix Inc., Farmingdale, NY, USA) in order to disperse clumps of organisms and obtain single bacilli in suspension. BCG were then plated onto Middlebrook 7H10 agar plates (Falcon, BD Microbiology Systems, Maryland, USA, as shown below) for determination of CFU by counting the colonies using an Olympus S751 microscope (Olympus Corporation, Hamburg, Germany). Cultures were set up in triplicate and plated onto a plate with 6 grids, so that the average of 6 replicates was determined in each of the triplicate cultures. (Figure 2-1)The CFUs were enumerated between day 10 and day 14. CFU/ml were determined by multiplying the mean colony count (of 6 squares) by 100 (as 10µl was plated) and then multiplied by the dilution factor.

Figure 2-1 Micro-grid 7H10 agar plate

Cultures were set up in triplicates using plates with grids as shown. Each grid comprised of 6x6 squares so for each triplicate there were 6 squares each giving a total of 18 squares for each result.

2.4 Infection of macrophages

Monocyte-derived macrophages were infected with BCG-gfp at a multiplicity of infection (MOI) of 2:1 and for alveolar macrophages at an MOI of 2.5:1. These MOI were chosen based on preliminary optimisation experiments demonstrating that lower MOI resulted in poor BCG uptake after 18 hours. For infection of adherent

macrophages, aliquots were thawed and mycobacterial clumps disrupted by passing the bacteria through a 27g insulin syringe several times. After 18 hours the macrophages were washed with warm PBS to remove any bacteria that did not gain access into the cells.

2.4.1 Determination of mycobacterial uptake by flow cytometry

Flow cytometric analysis was performed to determine the number of macrophages containing intracellular BCG-gfp. Cold PBS with 20mM EDTA (Sigma-Aldrich) was added to all wells for 10 minutes to facilitate detachment of adherent macrophages. Macrophages were washed in PBS containing 1% human serum and 0.1% sodium azide (Sigma-Aldrich) and resuspended in the same buffer (FACS buffer). Immediately prior to acquisition of the cells, 10µl of 7AAD (eBiosciences) was added in order to establish cell viability. Once acquired, the cells were analysed on a FACSCalibur using *Cell Quest* software. The BCG-gfp, read in the Fl1 channel, was plotted against the 7ADD that was read in the Fl3 channel, thereby determining mycobacterial uptake and macrophage viability.

2.5 Cytokine assays

Cytokine concentrations were determined using commercially available ELISA kits. Supernatants were removed from the culture wells containing infected and uninfected macrophages and spun down to remove cellular debris. Where possible, supernatants from multiple wells for each experimental condition were pooled. Triplicates of each experimental condition were prepared whenever sufficient cells were available. Supernatants were stored at -80°C to enable batching of the ELISAs. ELISAs were performed according to the manufacturers instructions following thawing of supernatants (methods described below for each cytokine). All individual samples were run in triplicate wells for each ELISA performed. Mean cytokine concentration was calculated from the three replicates for the given experimental condition.

2.5.1 Interferon gamma (IFN-γ)

IFN-γ concentration was determined using the QuantiFERON®-TB Gold (In-tube) whole blood IFN-gamma ELISA kit (Cellestis, Carnegie Victoria, Australia). Briefly, supernatants were thawed and brought to room temperature with the specified kit reagents. Replicate kit standards were prepared ranging from 0-4IU/ml (160pg/ml). 50µl of supernatant was combined with 50µl of reconstituted conjugate and incubated for 120 min after which the plates were manually washed 6 times with wash buffer. After a 30 minute incubation with enzyme substrate the reaction was stopped and the optical density (OD) values were obtained within 5 minutes with a 450nm filter and a 620nm reference filter. OD values were manually inputted into the QuantiFERON®-TB Gold IT Analysis Software (Cellestis). Automated software quality control confirmed validity of the data and generated IFN-γ concentrations for each well.

2.5.2 Tumour necrosis factor alpha (TNF-α)

TNF-α concentration was determined using the Human TNF-α Ready-SET-Go! ELISA Kit (eBiosciences, #88-7347). Briefly, supernatants were thawed and brought to room temperature with the specified kit reagents. Standards were prepared to generate a standard curve range of 4 - 500 pg/ml. 100µl of supernatant was combined with 100µl of conjugate and incubated overnight at 4°C. After 5 manual washes with diluted wash buffer, 100µl of detection antibody was added to each well and incubated for 1 hour. A further 7 wash steps were followed by the addition of 100µl of substrate. The subsequent 15-minute incubation was halted by 50µl of stop solution. The plate was read within 5 minutes using a wavelength of 450nm. OD values were manually entered into an Excel spreadsheet and a standard curve was generated using the serial standard dilutions. TNF-α concentrations were then calculated by reading the OD of the test samples off the standard curve.

2.5.3 Interleukin 10 (IL-10)

IL-10 concentration was determined using the Human IL-10 Ready-SET-Go! ELISA Kit (eBiosciences, #88-7906). Standard preparation was the same as for TNF-α except that the standards were prepared to generate a standard curve range of 2- 300 pg/ml.

2.6 Preparation of cigarette smoke extract

2.6.1 Cigarette brand

Cigarette smoke extract (CSE) was obtained from the combustion of commercially available medium tar (10 mg tar & 0.8 mg nicotine) Marlboro Red® (Phillip Morris, USA) cigarettes. A single carton containing 10 individually wrapped cigarette cartons was purchased and the individual boxes were stored in sealed plastic bags at -20°C until required for use.[14b] Individual boxes were allowed to equilibrate to ambient room temperature and humidity over 48 hours prior to use in experiments.

2.6.2 Cigarette smoking apparatus

A standardized cigarette-smoking device was constructed based on the apparatus used in several studies published by Freed and co-workers.[109, 110] (Figure 2-2) A single filtered cigarette was connected via 5mm high flow PVC tubing (Gilson) to a peristaltic pump (Minipulse® evolution, Gilson). A sterile 50cc conical tube (Corning, Corning NY, USA) was inserted inline with the cigarette and the pump using a rubber stopper with two glass tubes as connection ports. For each extract preparation, new sterilised tubing and connections were used. The sterile 50ml conical flask was filled with 10ml warmed (37°C) RPMI and the rubber stopper and connections inserted into the tube under sterile conditions. The tube and connectors where then transferred to the fume hood for preparation of the extract.

Figure 2-2 Cigarette smoking apparatus

The cigarette smoking apparatus consists of the peristaltic pump (left panel), PVC tubing and 50ml conical flask containing culture medium (middle panel). Smoke exhausting after passage through the culture medium is shown in the right panel.

2.6.3 Optimisation of cigarette smoke extract preparation

2.6.3.1 Smoking time

All smoke extract preparation was performed in a fume hood. The hood cover was closed to the same height for all experiments and the extraction fan only switched on after completion of the smoking time. A fixed pump rate of 45 RPM with 5mm PVC tubing achieved a flow rate of 125cc/min equivalent to published methods.[109, 110] Several cigarette brands were tested to establish the time taken to smoke the cigarette to within 1 cm of the filter. (Figure 2-3)

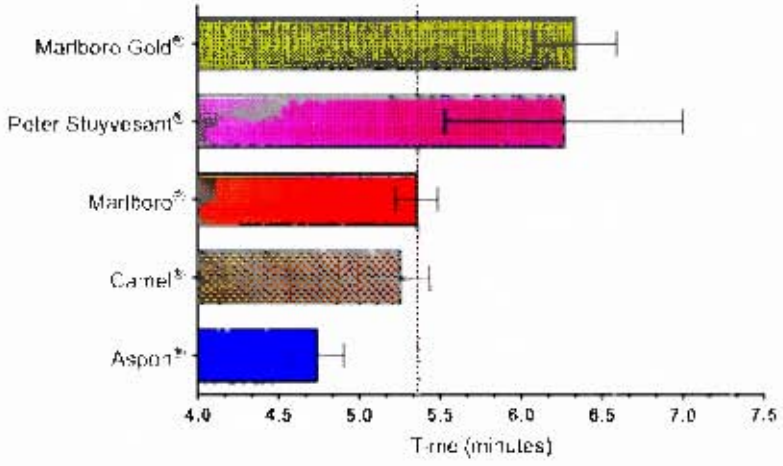


Figure 2-3 Smoking time for various cigarette brands

The time taken for 5 cigarettes of each brand to burn to 1 cm from the filter was recorded using a fixed pump rate. The individual bars represent the mean 'smoking time' with error bars depicting the standard deviation. The dotted vertical line represents the 5min 21 sec 'smoking time' for the brand of cigarette used in all experiments

Using the Marlboro Red® cigarettes (Phillip Morris, USA) at a fixed flow rate of 125cc/min, a smoking time of 5 minutes and 21 seconds after an initial 5 second ignition period was established. (Table 2-1) This time reliably smoked the cigarette to 10mm (= 0.8mm) from the filter. Smoke remaining within the 50ml conical tube after completion of the smoking time, was allowed to dissolve in the medium by gentle shaking for 30 seconds.

Table 2-1 Variability of 'smoking time' between cigarette brands and products within brand products

Cigarette brand	Mean time to burn to 1cm of filter	Standard deviation	Coefficient of variance
Aspen®	4min 44 sec	10 sec	3.5%
Peter Stuyvesant®	6 min 16 sec	44 sec	11.7%
Marlboro Red®	5 min 21 sec	8 sec	2.5%
Marlboro Gold®	6 min 20 sec	15 sec	4.1%
Camel®	5 min 15 sec	11 sec	3.5%

The concentration of CSE produced by this method was defined (by convention) as a 100% solution. Dependent on the experimental protocol, appropriate dilutions were prepared to produce a final concentration ranging between 0.1% - 10% in cell culture medium. Cigarette smoke extract was produced freshly for each experimental intervention and was used within 20 minutes of preparation. Two aliquots of 100% cigarette smoke extract were immediately frozen and stored at -80°C for mass spectrometry determination of nicotine content.

2.6.3.2 Determination of nicotine concentration

2.6.3.2.1 Mass spectrometry

The University of Cape Town Division of Pharmacology analytical & research laboratory performed the mass spectrometry for the determination of nicotine concentrations.

Briefly, aliquots of cigarette smoke extract were analysed using a ABSciex 3200 Qtrap mass spectrometer connected to an Aglient 1200 Series HPLC (High Performance Liquid Chromatography). A series of nicotine standards (Sigma) and quality controls were prepared in HPLC grade water (Merck, Germany). Standards, controls and unknowns were diluted 1000 fold, in 50% Acetonitrile: 0.1% Formic acid (Merck, Germany), with Reserpine (Sigma) as an internal standard. 5µl of the samples were injected onto a Phenomenex Luna Hilic column (50 x 2mm x 3 micron), using Acetonitrile and 0.1% formic acid as mobile phase.

2.6.3.3 Reproducibility of the cigarette smoke extract

2.6.3.3.1 Cigarette smoke extract reproducibility between brands

5 Marlboro Red® cigarettes (10 mg tar and 0.8 mg nicotine) were sequentially smoked using the standardized protocol. Individual nicotine concentrations were calculated for each of the five prepared extracts. The mean (SD) concentration of nicotine obtained from the 5 sequentially smoked cigarettes was 12.5(3.4) µg/ml and the coefficient of variance was 28.17%. Nicotine concentrations are known to vary within brands (Marlboro Gold® vs. Marlboro Red®) and within brand products (Marlboro Red® bought in Kenya vs. Marlboro Red® bought in America).[146]

The nicotine concentrations of one low tar and two medium tar brands (used in the smoking time experiments shown in (Figure 2-3) were compared. Camel® (medium tar: 10 mg tar, 0.8 mg nicotine), Marlboro Red® (medium tar: 10 mg tar, 0.8 mg nicotine) and Marlboro Gold® (low tar: 0.8 mg tar, 0.5 mg nicotine) were compared. Nicotine concentrations in CSE differed across all three brands. CSE prepared from Camel® cigarettes had the highest mean (SD) concentration of nicotine: 13.9(2.4) µg/ml compared to Marlboro Red® 9.1(1.9) µg/ml and Marlboro Gold® 4.9(0.9) µg/ml; p<0.001. (Figure 2-4) The coefficient of variance was similar across all three brands: 17.6%, 18.3% and 21.1% respectively.

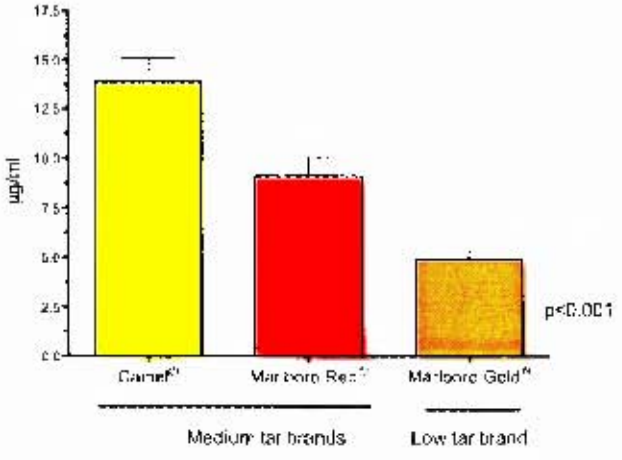


Figure 2-4 Nicotine concentrations in cigarette smoke extract from different tobacco brands

Each bar represents the mean nicotine concentration in prepared cigarette smoke extract from two medium and one low tar brand. Five cigarettes were smoked for each brand with error bars representing the SEM. Each brand produced statistically different nicotine concentrations (P<0.001; ANOVA).

2.6.3.3.2 Cigarette smoke extract reproducibility over time

Two aliquots of all prepared cigarette smoke extract were immediately frozen and stored at -80°C for later batched nicotine determination. Using nicotine standards ranging from 30–200 µg/ml the coefficient of variance of the mass spectrometer determined standard concentrations was 14.9%.

Reproducibility of the cigarette extract was best when multiple extracts were produced on the same day (set up phase): mean (SD) nicotine concentration of 12.5(3.4) µg/ml coefficient of variance (CV%) 28.2%. During the first 6-month optimisation phase (total of 16 separate CSE prepared), the mean (SD) concentration of nicotine was 10.7(5.2) µg/ml (CV%= 48.4%). For the 9 month experimental phase (total of 26 prepared extracts) the mean (SD) nicotine concentration was 6.4(2.6) µg/ml (p=0.006 compared to optimisation phase); CV%=40.3%. (Figure 2-5) The level of reproducibility is similar to that published by Vassalo et al who had a coefficient of variance of 32% in nicotine concentration using a similar technique of liquid chromatography-tandem mass spectrometry.[147]

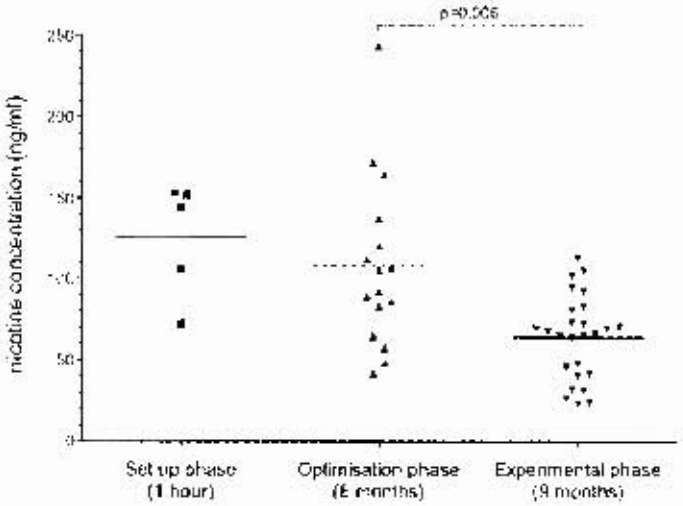


Figure 2-5 Reproducibility of nicotine concentrations in cigarette smoke extract over time.

Nicotine concentrations for each cigarette smoke extract (CSE) prepared are depicted for three separate time periods. Setup-phase: 5 extracts prepared over 1 hour, optimization-phase: 16 cigarette extracts prepared over 6 months, experimental-phase: 26 extracts prepared over 9 months. The horizontal bars represent mean concentrations for each phase.

2.6.4 Statistical methods

Data in the text are expressed as mean with standard deviation (SD) and in the figures as mean with standard error of the mean (SEM). Where data was not normally distributed, the data is reported as median with inter-quartile range. Statistical comparisons were made with the appropriate parametric and non-parametric tests. For unpaired normally distributed data a t-test was used (with Welch's correction if unequal variance) and the Mann Whitney U test for non-parametric data. Where paired data were analysed, the paired t-test or Wilcoxon matched-pairs signed rank test were used. For data involving more than two categories, an analysis of variance (ANOVA) was used: One-way ANOVA or repeated measures ANOVA as appropriate. To correct for multiple comparisons, the Tukey test was used. A p value of 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism version 5.00 for Mac, GraphPad Software, San Diego California USA, www.graphpad.com, OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version 2.3.1. www.OpenEpi.com, updated 2010/19/09 and Excel®, Microsoft corporation, USA. In Chapter 3 specific statistical methods were used and are described in section 3.2.3.

3 Clarifying the latent tuberculosis infection status of participants and evaluating the reproducibility, cut-point reliability and TST-mediated boosting of IGRAs

3.1 Introduction

Latent TB infection (LTBI) is an asymptomatic state with no clinical or radiological evidence of active TB but with viable *M. tuberculosis* (*M.tb*) organisms within body tissues. It is likely that not all individuals exposed to aerosolised *M.tb* droplet nuclei become infected. Some individuals may possess sterilizing immunity and may thus 'clear' the organisms without mounting an immunological response. In others, organisms are phagocytosed by alveolar macrophages[148-151] and dendritic cells[152, 153] and are transported to regional lymph nodes where T cells are primed and clonally expanded.[36] In such individuals, a detectable immune response to TB antigens is likely.

There is no gold standard for the diagnosis of LTBI. The tuberculin skin test (TST) was the favoured method for diagnosing presumed LTBI until the development of IFN-γ release assays (IGRAs). IGRAs, which include QuantiFERON®TB Gold In-Tube (QFT) and T-SPOT.®TB (TSPOT), detect peripheral blood-derived T-cell responses to relatively *M.tb*-specific RD-1 (region of diffentiation-1) antigens including early secreted antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 (CFP-10). IGRAs have been shown to be sensitive and specific laboratory markers of presumed LTBI.[16] However, it is well documented that there is not 100% agreement between IGRA and TST results.[16] This is due, in part, to the TST being a composite 48 to 72 hour readout of the pro-inflammatory effects of antigen presenting cells, chemokines and lymphocytes in response to purified protein derivative (PPD), a culture filtrate of *M. tuberculosis*. In contrast, IGRA's represent a single cytokine response (IFN-γ) after overnight stimulation of T-cells by relatively *M.tb* specific antigens (ESAT-6, CFP-10 and also TB 7.7).[154]

Identification and treatment of individuals with latent TB infection (LTBI) is an important public health control measure, which substantially reduces the risk of reactivation TB.[155, 156] Detection of LTBI is particularly important in persons at high

risk of infection such as contacts, health care workers, immunocompromised individuals and children who may benefit from chemoprophylaxis.[157] In vaccine and therapeutic studies, accurate detection of latent TB infection is crucial in the assessment of efficacy of the interventions being used.[158] Furthermore, determination of TB infection status forms the basis of epidemiological studies, following the course of the epidemic and for assessing risk factors for TB infection such as smoking, HIV and malnutrition. Individuals who have no evidence of presumed LTBI infection (TST and IGRA negative) but strong evidence of exposure to infectious individuals may presumably have 'sterilising immunity' though this is unproven. They display no evidence of immunological memory to TB. These individuals may serve as negative controls in experimental conditions examining the immune responses to either antigen or live organism exposure.

There are widely divergent views on how the TST and IGRAs should be use to screen for LTBI. The US Centres for Disease Control (CDC) guidelines recommend that a single IGRA can replace the TST.[17, 18, 159] The UK-based National Institute for Clinical Excellence (NICE)[20] and revised 2008 Canadian guidelines[19] however recommend a two-step strategy namely a TST followed by an IGRA (up to 6 weeks later in the UK guidelines) for the detection of LTBI.

As persons with LTBI (versus no LTBI) may have different cytokine and antigen-specific host immune responses and profiles, and to delineate this from the effect of CSE, it was necessary to determine and standardise the LTBI status of the participants. To confirm the nature of the LTBI status in this research project, a dual approach was undertaken using both the TST and IGRA. This however raised several issues for which there was little published data:[21, 22] 1) What magnitude of variation could be expected in IGRA responses when subjects had a confirmatory IGRA 1 to 2 weeks after the first. 2) If significant variability is demonstrated, what is the appropriate cut point that should be used to define LTBI status (a positive test or conversion/ reversion if serial testing is used)? 3) Would the TST administered at the time of the first IGRA potentially affect the second test by 'boosting' the interferon gamma responses? Given these questions, it was important to examine the within-

subject variability of the IFN-γ responses in our local population when serially testing individuals as well as the effect of an antecedent TST on subsequent IGRA responses.

3.1.1 Hypothesis

In a TB-endemic setting the within-person variability of IGRAs is high, variability about the cut-point is significant and TST administration will boost downstream RD-1 antigen-specific host immune responses.

3.1.2 Specific aims

1) To determine the magnitude of within subject variability in a cohort of individuals from a high burden TB country and its impact on determining assay cut-points.
2) To determine the effect of TST administration on subsequent IGRA (QFT & TSPOT) responses.
3) To define thresholds for conversion (previously negative IGRA or TST becomes positive on retesting) and reversion (previous positive TST or IGRA becomes negative upon repeat testing) of antigen responses.

3.2 Methods

3.2.1 Subject recruitment & testing schedule

Healthy health care workers, medical students and volunteers were recruited to undergo repeated IGRA testing prior to, and following a TST as shown in **Figure 3-1**. Information regarding previous TB, HIV status, smoking status, alcohol consumption, current TB symptoms and estimated level of domestic and occupational exposure to TB during the previous 3 months was recorded from each participant. In addition, exposures were recorded during the first 3 months of the study.

Symptoms of active TB were sought at each visit and all subjects were followed up for 6 months following the final blood sampling to document the development of symptoms or illness. Chest x-rays were taken at the time of recruitment to exclude

active disease and to record changes suggestive of healed disease. A specialist pulmonologist and radiologist independently reported the Chest x-rays.

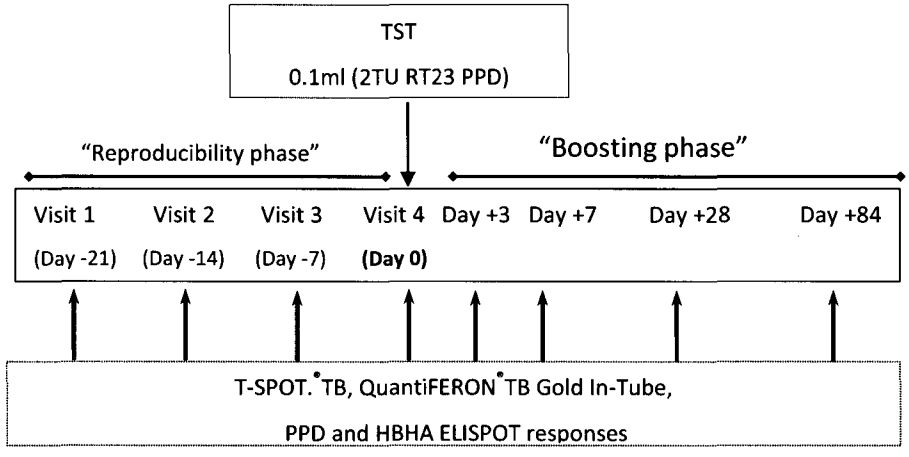


Figure 3-1 Subject testing schedule

Commercially available interferon-γ release assays (IGRA), were performed over a 4-month period, divided by a pre and post TST phase. QuantiFERON®TB Gold-IT as well as T-SPOT.®TB including additional PPD and HBHA (heparin binding haemagglutinin) responses were measured at each time point. The TST (tuberculin skin test) was administered on day 0 following venesection and read on day 3 (~ 72 hours).

Subjects were allocated to three risk groups based on TB exposure.

- **High risk:** exposure to TB patients on the majority of working days (respiratory clinic and thoracic surgery wards).
- **Medium risk:** hospital workers without recognised exposure to TB patients (gastroenterology outpatients, rheumatology clinics).
- **Low risk:** non-hospital workers with no known TB exposure (preclinical medical students and healthy volunteers from low TB risk urban areas). However, as South Africa is a high TB incidence country, it was not possible to rule out exposures in even the low risk group.

High and medium exposure risk subjects were recruited from Groote Schuur Hospital. In Cape Town, South Africa there is currently no policy for latent tuberculosis screening of health care workers.

3.2.2 Latent TB infection testing

All subjects underwent testing as described in Chapter 2.

3.2.2.1 Tuberculin skin test

A tuberculin skin test was performed at the fourth visit defined as study day zero. 0.1ml (2TU) of RT23 PPD (Staten Serum Institute, Denmark) was injected intradermally on the volar aspect of the non-dominant forearm. The extent of induration was read on day three (approximately 72 hours) using the ballpoint pen method i.e. the edges of induration being detected by increased resistance to movement of a pen across the skin. The two widest distances were recorded and the average diameter calculated to determine the extent of the induration. As all subjects were HIV un-infected, and BCG vaccination occurs at birth in South Africa, a cut-point of $\geq$10 mm was used to define latent TB infection as per the South African guidelines.

3.2.2.2 Interferon gamma release assays

Both commercially available interferon gamma release assays were used: QuantiFERON®TB Gold In-Tube (QFT-GIT) (Cellestis, Victoria Australia) and T-SPOT.®TB (Oxford Immunotec, Oxford, England). Both assays were performed at each predefined time point. (Figure 3-1)

3.2.2.3 QuantiFERON®TB Gold-In-Tube

The QuantiFERON®TB Gold In-Tube assay, was performed according to the manufactures' guidelines contained in the assay kit.[160] In summary, 1ml of whole blood was collected directly into the provided nil, TB antigen and mitogen tubes and transported directly to the laboratory. After 16- 24 hours incubation, the tubes were spun down and the supernatants stored at 4°C.

The QuantiFERON®TB GOLD (In-Tube Method) ELISA was carried out according to manufacturer's instructions. The ELISA was performed manually without automated washing or pipetting. After incubation of the ELISA plate, the optical density was read using an Organon Keknika ELISA plate reader (Anthos Labtec Instruments GmbH) with a 450nm filter and a 620 to 650nm reference filter. The OD values were then entered into the QFT Gold analysis software v2.5 to calculate the IFN-γ values and test results.

3.2.2.4 T-SPOT.®TB

The T-SPOT.®TB assay was performed according to the manufacturer's instructions.[161] Preparation of peripheral blood mononuclear cells (PBMCs) for use in the T-SPOT.®TB assay are described in Chapter 2.

In summary, PBMCs were purified from whole blood drawn into a heparin containing blood tube. After washing and counting, PBMCs were plated at a final concentration of 250,000 PBMCs per 100μl medium/well. The standard 4 wells containing ESAT-6, CFP-10, positive and negative controls were prepared with four additional duplicate wells containing PPD 20μg/ml (Staten Serum Institute, Denmark), and HBHA 100μg/ml[162, 163] a novel antigen that may distinguish latent from active TB to evaluate within-subject variability for this antigen. After 24 hours incubation in a humidified $C0_2$ incubator, the plate was washed and incubated with the provided conjugate and substrate. Plates were allowed to dry overnight before spot counting was performed.

Automated spot counting was performed using the automated AID reader (AID Autoimmun Diagnostika GmbH) employing standard reading software. Spot count settings were provided by Oxford Immunotec: (intensity: minimum = 35, maximum =250; size: minimum=70, maximum =20000; gradient: minimum =10, maximum =90)

3.2.2.4.1 Assessing ELISPOT automated reader variability

It has been documented that IGRA responses are not constant over time. To establish the variability in a high burden setting, it was important to first establish the contribution of technical factors to variability. To determine the technical machine/apparatus variability for both assays, repeat analysis of the same assay plates was undertaken.

3.2.2.4.1.1 Automated ELISPOT count variability

For the assessment of apparatus induced variability in the ELISPOT assay, three separate plates were read daily for three days using an AID EliSpot reader (Autoimmun Diagnostika, Strasberg, Germany). Spot counting settings/thresholds

supplied by Oxford Immunotec were used throughout the study without changing the settings on the apparatus. Manual addition of spots (by marking spots thought by the operator to have been missed by the automated camera reading) was not performed and "spot removal (unmarking spots that were thought by the operator to have been over read) was only done if artefacts were present. A spot count of 250 spots was set as the limit for the maximum readable spot count. Higher counts were not analysed in the automated reader reproducibility tests. A total of 216 wells were read with the highest count of 129 spots per well.

3.2.2.4.1.2 QuantiFERON®TB GIT ELISA reader variability

For technical reasons it was not possible to assess the intra-test variability of the QuantiFERON®TB ELISA, in the same manner as performed for the T-SPOT.TB test. Instead, 'repeat counting' of the same ELISA plate was performed over two hours to determine the within-test variability. The ELISA plate was kept in the dark for the entire 2-hour period.

3.2.3 Statistical methods used for the analysis of variability and boosting

The data collected represented multiple tests, from multiple individuals, at multiple time points. Repeatability was assessed by the method of Bland.[164] Wilcoxon-matched pairs test, and where necessary, Mann-Whitney tests were used to compare subject time points following the TST. A p value of <0.05 was considered significant. Specific statistical methodology was required to perform the analysis of within subject variability over time, given the number of subjects, tests and time points. This analysis was undertaken with assistance from the faculty of statistical sciences at the University of Cape Town. (Section 3.2.3.1 and 3.2.3.2)

3.2.3.1 Linear mixed effects model

Linear mixed effect models were fitted to the logged QFT values assuming they were approximately normally distributed. Residual plots from the fitted models showed this assumption was valid.

To assess reproducibility of the data, linear mixed effects models were fitted by residual maximum likelihood (REML) to the T-SPOT.®TB spot counts (TB antigen wells minus nil well) and logged (to normalize the distribution) QuantiFERON®TB GIT IFN-γ responses (TB antigen minus nil). These models allowed the difference in both the mean change (i.e. expected values) at each time and the correlation structure and variance to be estimated (i.e. their covariance structure) and assessed. Akaike Information Criterion (AIC) was used to assess covariance structure (model fit). This criterion adjusts the log-likelihood for the number of parameters fitted assuming the same pattern for the mean value, so that models with different covariance can be compared. Residual plots from the fitted models showed that this assumption was valid. Assessment of variability was made from the REML plots as well as a coefficient of variance analysis (see3.2.3.2).

3.2.3.2 Coefficient of variance analysis

Mean IGRA responses were calculated for each subject over the four visits during the reproducibility phase. At QFT IFN-γ values below 0.1, small numerical changes conferred very high variance in the QFT responses. As values below 0.1 are clinically meaningless, the final analysis was performed after excluding these values. Within-subject variability was calculated by determining the mean standard deviation (SD) for all subjects over the reproducibility phase. Expressing this SD value as a percentage of the IFN-γ response enabled calculation of the 95% confidence intervals (2SD) and hence test variability. For the T-SPOT.®TB responses, mean and standard deviations were calculated as above. Within-subject variability was reported as a spot count, as percentage changes were not appropriate to report.

3.3 Results

3.3.1 Assessment of within-test variability of interferon gamma release assays

3.3.1.1 Technical automated ELISPOT reader and ELISA within-test variability

The AID ELiSpot automated reader was assessed by recounting the same three plates over three days as described previously. When repeat counting of the same wells over three days was performed, an increase in the variability (as denoted by the 95% confidence intervals) with increasing spot counts was noted. At spot counts of 0-2 a change of only one spot was accounted for by the 95% confidence intervals around the mean. This increased to 3 spots at values of greater than three. Table 3-1

Table 3-1 Repeat counting of 216 ELISPOT wells using the automated reader

Spot count range	Mean count	SD	Variance
Spot counts 0-2	0.7	0.4	0.2
Spot counts 3-8	5.7	1.4	3.7
Spot counts 9-15	11.4	1.4	4.5
Spot counts 16-30	21.1	2.2	10.8
Spot counts >30	42.1	2.3	18.9

3.3.1.2 QuantiFERON®TB GIT ELISA reader variability

It was not possible for technical reasons to assess the ELISA machine variability in the same manner as the ELISPOT reader. A single QuantiFERON®TB GOLD IFN-γ ELISA plate was thus read repeatedly over two hours. (It is recommended by the manufacturer that the plate be read within 5 minutes of stopping the reaction.) No significant change in IFN-γ values was noted within this 5-minute period when three readings were performed or over the full 2-hour period (p=0.69). (Table 3-2)

Table 3-2 Variability in ELISA plate IFN-γ values when optical density (OD) readings were repeatedly performed over 2 hours

	Repeated readings at different time points									
	0 min	2.5min	5 min	30min	60min	90min	120min			
Subject	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ	IFN-γ		Mean	SD
ID 1	0.01	0.01	0.01	0.00	0.01	0.01	0.01		0.01	0.004
ID 2	0.02	0.00	0.01	0.01	0.02	0.01	-0.01		0.01	0.011
ID 3	0.55	0.55	0.55	0.55	0.55	0.54	0.52		0.54	0.011
ID 4	0.51	0.53	0.53	0.50	0.52	0.50	0.50		0.51	0.014
ID 5	0.03	0.06	0.06	0.02	0.06	0.05	0.04		0.05	0.016
ID 6	0.19	0.18	0.17	0.18	0.17	0.16	0.16		0.17	0.011
ID 7	2.39	2.36	2.37	2.38	2.40	2.39	2.39		2.38	0.014
ID 8	1.04	1.05	1.06	1.05	1.06	1.06	1.05		1.05	0.008
ID 9	0.05	0.05	0.05	0.05	0.02	0.05	0.02		0.04	0.015
ID 10	0.06	0.06	0.06	0.06	0.06	0.07	0.07		0.06	0.005
ID 11	0.14	0.14	0.14	0.14	0.14	0.13	0.13		0.14	0.005
ID 12	0.63	0.62	0.63	0.62	0.62	0.61	0.59		0.62	0.014
ID 13	-0.05	-0.06	-0.05	-0.06	-0.08	-0.10	-0.10		-0.07	0.022
ID 14	0.00	0.00	0.00	0.02	0.03	0.05	0.07		0.02	0.028
ID 15	0.01	0.01	0.02	0.01	0.01	0.02	0.01		0.01	0.005
ID 16	0.01	0.01	0.00	0.01	0.01	0.01	0.01		0.01	0.004
ID 17	0.01	0.00	0.00	0.01	0.01	0.01	0.00		0.01	0.005
ID 18	-0.36	-0.35	-0.35	-0.35	-0.36	-0.35	-0.35		-0.35	0.005
ID 19	-0.19	-0.19	-0.20	-0.19	-0.20	-0.19	-0.18		-0.19	0.007

3.3.2 Demographic characteristics of study participants

Twenty-eight healthy volunteers were recruited. Four participants failed to complete the study (one participant withdrew after the first visit, one did not receive a TST, one did not return for blood testing after the TST, and one subject withdrew consent during the study period). Thus, a total of 26 subjects were available for the reproducibility analysis and 24 for boosting analysis. Of the 26 subjects, 11 were classified as high risk, 10 as medium risk, and 5 as low risk for exposure.

All subjects were HIV negative and reported receiving BCG vaccination at birth. A single subject recorded having TB disease 40 years previously. The exposure risk groups were similarly matched except that the low risk group was younger and exclusively of European ancestry. (Table 3-3) No episodes of unprotected TB exposure were reported during the study.

Table 3-3 Demographic details of study participants

	Total	High risk	Medium risk	Low risk
Number of subject	26	11	10	5
Age mean (SD)	39 (10)	43 (7)[+]	41 (10)*	29 (11)[+]*
Racial group				
Black African	4 (15%)	3 (27%)	1 (10%)	0
Mixed ancestry	14 (54%)	7 (63%)	7 (70%)	0
European ancestry	8 (31%)	1 (10%)	2 (20%)	5 (100%)
Current smoker	6 (23%)	2 (18%)	3 (30%)	1 (20%)
HIV status	All HIV negative			
BCG vaccinated	All vaccinated			
Previous TB (n)	1	1	0	0

[+] p=0.05 High vs. Low * p< 0.01 Medium vs. Low

3.3.3 IGRA and TST agreement/discordance

A total of 88 paired IGRA (T-SPOT.®TB and QFT-GIT) results were available for comparison prior to the TST administration. Multiple conversions or reversions, over the three week testing period, resulted in rates of discordant T-SPOT.®TB and QFT-GIT results varying from 4% (1/25) to 11.5% (3/26). Overall, the rate of IGRA discordant results was 10% (9/88). TST positive IGRA negative discordance varied between 20% (5/25) to 24% (6/25) for QFT-GIT and 24% (6/25) to 32% (8/25) for T-SPOT.®TB. All discordant results (except one paired test at visit 4) were TST positive / IGRA negative. Rates of positive IGRA and TST results are shown in Table 3-4.

Table 3-4 Rates of QuantiFERON®TB GIT and T-SPOT.®TB discordance during the reproducibility (pre-TST) phase

	IFN-γ release assay results				TST results
Visits prior to TST	No. of evaluable paired results	QFT GIT positive n (%)	T-SPOT.® TB positive n (%)	Discordant IGRA results n (%)	TST positive n (%)*
Visit 1	13*	7 (54%)	5 (38.5%)	2 (15.4%)	Not applicable
Visit 2	25	9 (36%)	8 (32%)	1 (4%)	Not applicable
Visit 3	24	9 (37.5%)	8 (33.3%)	2 (8%)	Not applicable
Visit 4 (day of TST)	26	10 (38.5%)	9 (34.5%)	3 (11.5%)	15 (60%)

IGRA discordance was calculated for each visit using the available results. * No low risk subjects were recruited at visit 1. [+] The tuberculin skin test (TST; cut-point of 10mm) was performed once at visit 4 after venesection.

3.3.4 Within-subject variability

All 5 low exposure risk participants were IGRA negative and remained IGRA negative through out the study. Seven of the 21 medium or high-risk subjects had a conversion or a reversion around the manufacturer predefined cut-point (defined as ≥ 6 spot forming cells per well (SFC/well) for T-SPOT.®TB and IFN-gamma ≥ 0.35 IU/ml for QFT-GIT), at least once during the 3 week (4 test) reproducibility phase. (Table 3-5) Conversions or reversions were only seen in the high and medium exposure risk groups and occurred more commonly for the T-SPOT.®TB (6 episodes) than for QFT-GIT (1 episode) p =0.049. (Table 3-5) If the FDA recommended T-SPOT.®TB borderline zone of 5, 6 and 7 spots was applied to the results, 1 less conversion and 1 less reversion would have been defined.

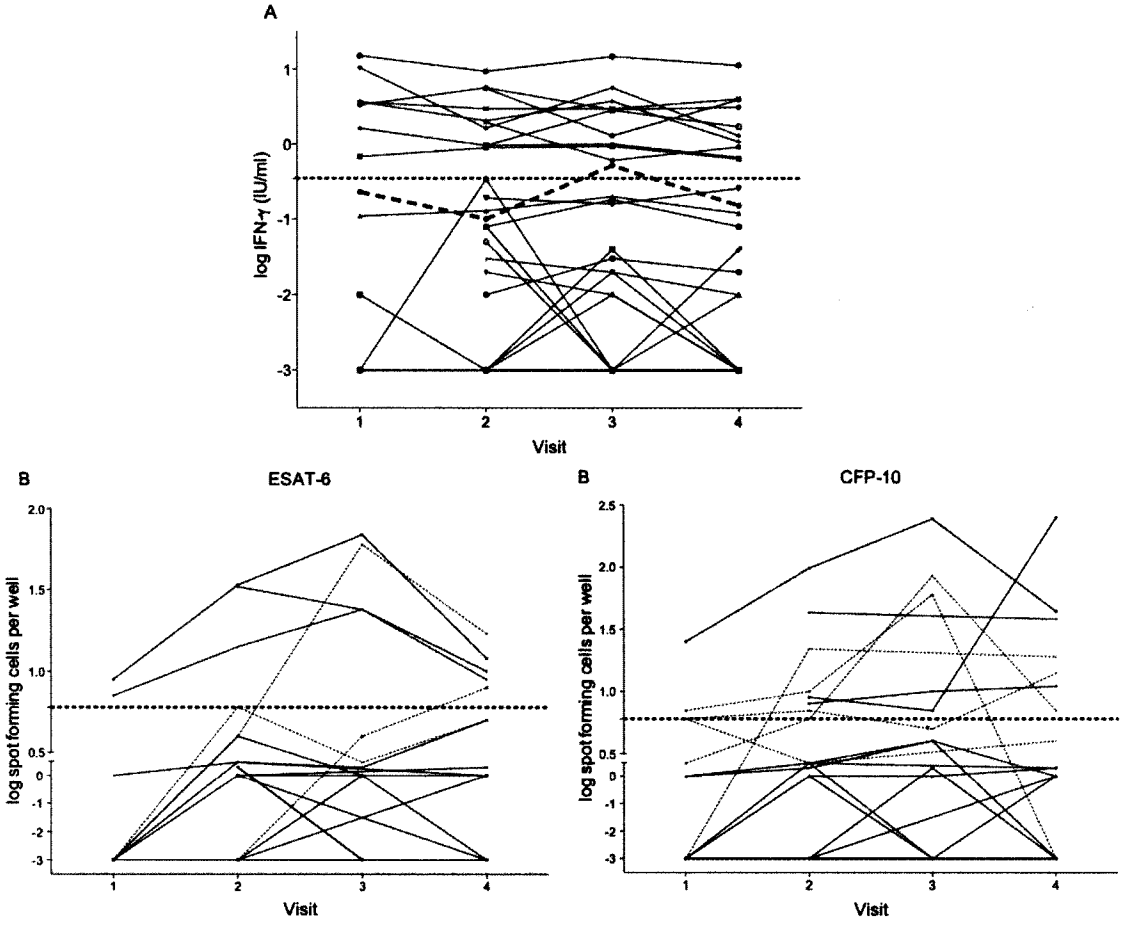
Table 3-5 Subjects demonstrating a change in IGRA status during the reproducibility (pre-TST) phase of the study

Subject	IGRA assay	Reproducibility phase visits (3 weeks)				TST
		Visit 1 (Day-21)	Visit 2 (Day-14)	Visit 3 (Day -7)	Visit 4 (Day 0)	
1	QFT- GIT+	Positive (3.29)	Positive (3.29)	Positive (2.89)	Positive (3.09)	15.5mm
	TSPOT.®TB*	Positive (0/6)	Positive (6/7)	**NEGATIVE** (3/5)	Positive (5/14)	
5	QFT- GIT	Positive (10.56)	Positive (1.61)	Positive (5.67)	Positive (1.30)	19mm
	T-SPOT.®TB	**NEGATIVE** (0/0)	Positive (0/22)	Not done	Positive (0/19)	
12	QFT- GIT	Positive (1.64)	Positive (0.95)	Positive (0.99)	Positive (0.66)	19mm
	T-SPOT.®TB	**POSITIVE** (1/6)	Negative (3/3)	Not done	Negative (1/4)	
13	QFT- GIT	Positive (3.61)	Positive (2.02)	Positive (3.70)	Positive (1.07)	No test
	T-SPOT.®TB	**NEGATIVE** (1/3)	Positive (4/6)	Positive (60/85)	Positive (17/7)	
14	QFT- GIT	Positive (3.66)	Positive (2.97)	Positive (2.97)	Positive (3.97)	18.5mm
	T-SPOT.®TB	Positive (0/7)	Positive (0/10)	Positive (1/60)	**NEGATIVE** (0/0)	
15	QFT- GIT	Negative (0.00)	Negative (0.34)	Negative (0.00)	Negative (0.00)	0mm
	T-SPOT.®TB	Negative (0/0)	Negative (0/0)	Negative (4/2)	**POSITIVE** (8/0)	
19	QFT- GIT	Negative (0.23)	Negative (0.10)	**POSITIVE** (0.51)	Negative (0.15)	22mm
	T-SPOT.®TB	Negative (0/0)	Negative (3/1)	Negative (2/1)	Negative (5/2)	

The details of subjects who converted or reverted (defined as crossing the predefined manufacturer suggested cut-point of 0.35 IU/ml for QFT-GIT and ≥ 6 spots for T-SPOT.®TB) are shown. Subjects 1 and 12 had T-SPOT.®TB values within the FDA recommended borderline zone and would not be considered a conversion or reversion. All other subjects remained either positive or negative over the three-week reproducibility phase prior to tuberculin skin testing (TST) administration at visit 4 (day 0). The categorical results as well as the IFN-γ responses (TB antigen minus nil) are detailed for each individual. Conversions and reversions are indicated by capital letters at the specific time-points. + QFT-GIT values are IU/ml. *T-SPOT.®TB values are depicted as (ESAT-6/CFP-10) spot counts.

For the QFT-GIT assay, a linear mixed effects model, as well as coefficient of variance analysis was used to determine within-subject variability. Ninety-five percent of the variability of IFN-γ responses (TB antigen minus nil control) occurred within 80% of the mean IFN-γ response. Therefore an 80% increase or decrease in QuantiFERON®TB GIT IFN-γ responses over the short-term can be regarded as within normal limits.

For the T-SPOT.®TB assay, variability in IFN-γ responses (TB antigen minus negative control SFC/well) increased with increasing spot counts. At a response of between 3 and 8 spots, using a coefficient of variance analysis, a change of 3 spots in either direction accounted for 95% of the within-subject variability in either the ESAT-6 or CFP-10 well. The linear mixed effects model confirmed this. PPD, in contrast to HBHA and the standardised IGRAs, showed greater variability over time. (Figure 3-2)



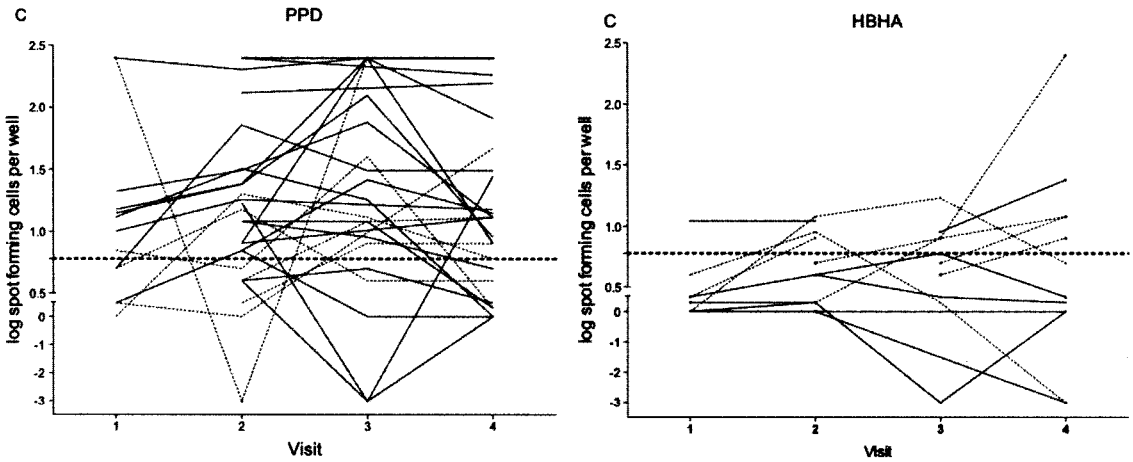
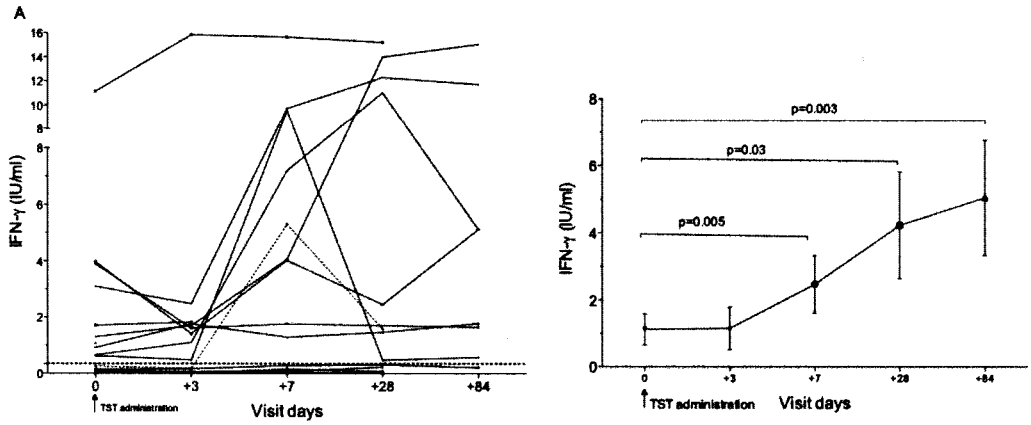


Figure 3-2 Within subject variability for QuantiFERON®TB GIT and T-SPOT.®TB including PPD and HBHA responses

Reproducibility for the Interferon gamma release assays was measured over four visits: (A) QuantiFERON®TB-Gold-IT, (B) T-SPOT.®TB using ESAT-6 and CFP-10, and (C) PPD and HBHA ELISPOT assay Log transformed data is presented. The manufacturer's predefined test cut-point is indicated by a stippled horizontal line, except PPD and HBHA where a laboratory derived cut-point was used. A dashed line depicts individual subjects who crossed the cut-point.

3.3.5 Tuberculin skin test mediates IGRA boosting

A significant increase in the mean QFT-GIT IFN-γ levels was noted by day seven post-TST, in comparison to the IFN-γ levels observed during the pre-TST period (p=0.005). (Figure 3-3A) T-SPOT.®TB ESAT-6 (p= 0.029) and CFP-10 (p=0.006) spot counts were also significantly raised by day 7, as compared to the spot counts observed during the pre-TST period. (Figure 3-3B) By contrast for PPD (p= 0.02) and HBHA (p=0.03), elevated spots counts were evident as early as day three post-TST. (Figure 3-3C) In several subjects, elevated levels of IGRA responses were detectable for up to three months after the TST.



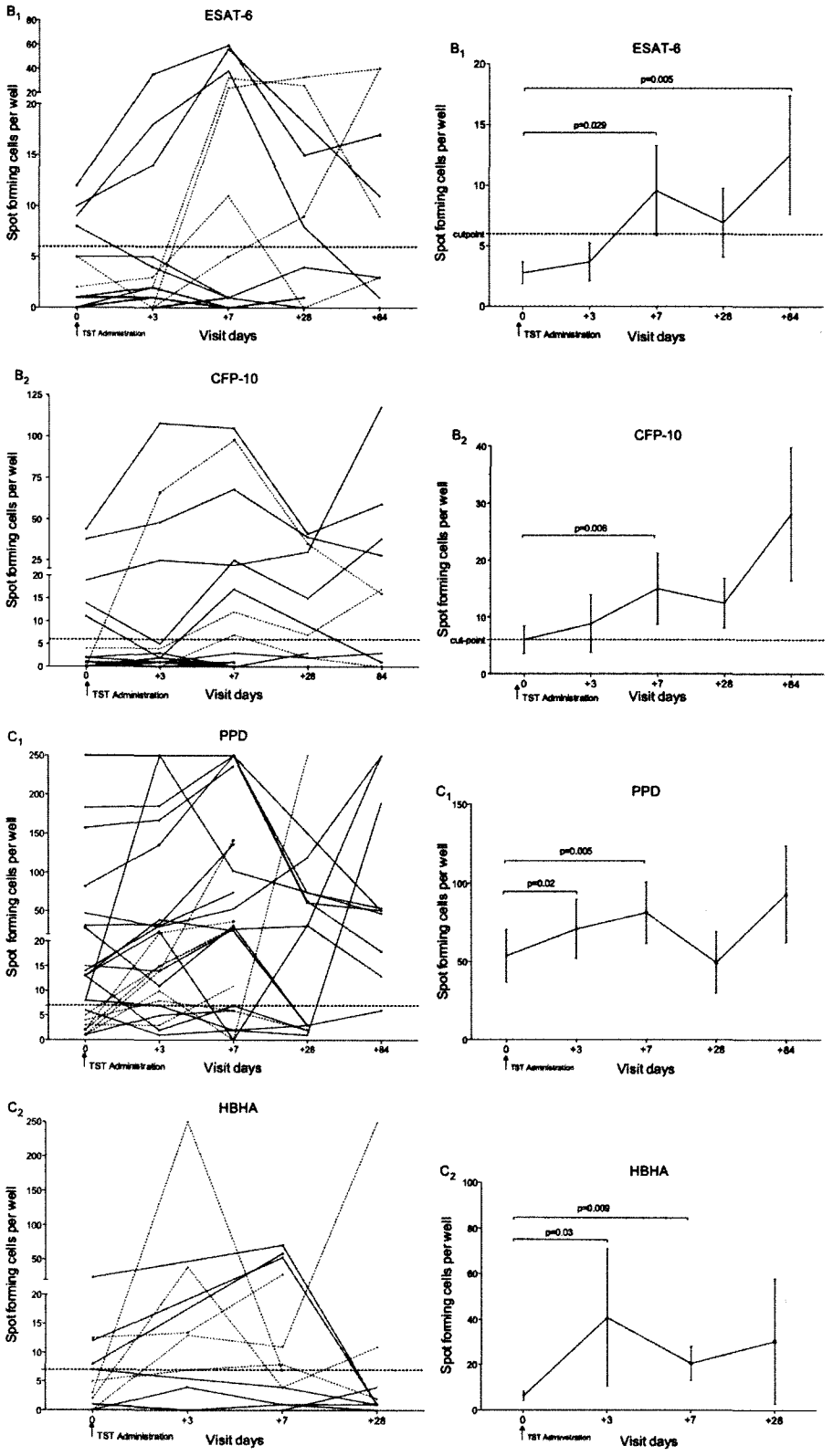


Figure 3-3 Boosting of interferon gamma responses following TST administration

Post TST boosting of QuantiFERON®TB GOLD-IT (A), T-SPOT.®TB using ESAT-6 (B_1) and CFP-10(B_2), and PPD(C_1) and HBHA(C_2) ELISPOT assay. The summation graph (right panel) shows mean IFN-γ responses (IU/ml or spot count). Error bars designate the standard error of the mean (SEM) at each time point.

Because an ±80% variation in QFT-GIT IFN-γ levels and ±3 spots in T-SPOT.®TB counts was observed during the reproducibility phase, boosting of IFN-γ responses was defined as a >80% increase in IFN-γ or a 3 spot increase from the pre-TST IGRA response. Boosting was demonstrated in 75% (6/8) of pre-TST IGRA positive subjects and in 12.5% (3/16) of pre-TST IGRA negative subjects (who also converted to an IGRA positive status. (Table 3-6) All the IGRA negative subjects who boosted were TST positive. Details of IGRA negative subjects who boosted are outlined in Table 3-6. A detailed comparison of both IGRA assays results is contained in Table 3-7.

Table 3-6 Characteristics of individuals who were QuantiFERON®TB GIT or T-SPOT.®TB negative, or with borderline results, who displayed boosting of their responses after TST administration

			Visit							
			Pre TST "reproducibility phase"				***Post TST "boosting phase"***			
Subject	Demographics	Test format	Visit 1	Visit 2	Visit 3	Visit 4 Day 0	*Day +3*	*Day +7*	*Day +28*	*Day +84*
1	Female, 48 years, non smoker, TST=15.5 mm High risk	ESAT-6 (SFU/well)	0	6	3	5	0	**24**	**33**	**40**
		CFP-10 (SFU/well)	6	7	5	14	5	**25**	**15**	**38**
		IFN-γ (IU/ml)	3.29	5.59	2.89	3.09	2.48	9.72	12.29	11.72
9	Female, 45 years, non smoker, TST=19 mm Medium risk	ESAT-6 (SFU/well)	No test result availabl e	1	No test result available	2	3	**11**	0	3
		CFP-10 (SFU/well)		3		2	3	0	0	0
		IFN-γ (IU/ml)		0.19	0.16	0.26	0.18	**5.30**	**1.59**	**4.20**
12	Female, 31 years, smoker, TST =19mm Medium risk	ESAT-6 (SFU/well)	1	3	No test result available	1	3	**32**	**26**	**9**
		CFP-10 (SFU/well)	6	3		4	5	**12**	**7**	**17**
		IFN-γ (IU/ml)	1.64	0.95	0.99	0.66	1.10	**7.20**	**11.04**	**5.12**

For each subject the individual IGRA results (where available) are tabulated across the table. The TST was administered at visit 4 (Day 0).SFU= Spot forming units. Results in bold indicate where a significantly boosted result occurred.

Table 3-7 Comparison of QuantiFERON®TB GIT and T-SPOT.®TB variability, borderline zones and proposed threshold for conversion

	QuantiFERON®TB GIT	T-SPOT.®TB
Manufacturer defined assay cut-point	> 0.35 IU/ml	≥ 6 Spots *
Within- subject short term variability	± 80% of IFN-γ response	± 3 Spots
Borderline or uncertainty	0.2-0.7 IU/ml	4 – 8 spots (inclusive)
Proposed conversion threshold	Increase from below 0.35 to above 0.7 IU/ml	Increase from below 6 to above 9 spots (inclusive)

*The FDA borderline zone for result interpretation includes values of 5, 6 & 7 spots.

3.4 Discussion

The results of this study confirm that short-term variability in IGRA responses is common. During a three-week observation period 7/26 (27%) subjects spontaneously converted and reverted around the cut-point at least once. It is thus necessary, when interpreting IGRA results, to consider within-subject variability and assay reproducibility. Secondly there was clear evidence of boosting of the IGRA responses in IGRA positive ("sensitized") subjects by day seven post-TST administration, which in several subjects persisted for at least three months. Significantly, boosting was not evident before day 3. Moreover, in 12.5% (3/16) of IGRA negative subjects boosting resulted in conversion to IGRA positivity (using manufacturer-recommended cut-points). The increases in IFN-γ levels and spot counts were significantly greater than the random within-subject variability observed in these subjects.

Data about within-subject variability and TST-induced boosting of IGRA responses are limited.[165, 166] These data are summarised in Table 3-8 and Table 3-9. A recent study evaluated 3-month post-TST IGRA responses and found a 6% conversion rate (post-TST values were all close to the cut-point).[165, 167] It is not clear from the reported results however, how much of this represented TST-mediated boosting rather than random within-subject variation. Pai and colleagues, in a study of 14 subjects tested 4 times over 12 days, found that most within-subject variability could be explained within 15% of the specified IFN-γ ELISA-derived value.[127] My study,

performed in high burden setting, evaluated a broader range of commercially available IGRAs, additional novel antigens, and studied variability over a longer period. Our results confirm that 95% of the variability over 3 weeks can be explained by a change, in either direction, of 80% of the average ELISA-derived IFN-γ value (QFT-GIT) or a 3 spot change in the mean T-SPOT.®TB spot count.

Instrument-specific variability contributed little to variability around the manufacturer-recommended, cut-points. At higher levels, there is greater within-person variability. This may be related to a hitherto unexplained biological effect in addition to technical factors, as both the ELISPOT and ELISA plate reader have technical limitations reducing the accuracy of results at high levels. As the documented variability in both assays increase at higher levels, these variability estimates should not be applied to high responses.

Table 3-8 Studies on within-person variability of interferon gamma release assays in high and low burden countries

Paper	Country (TB Burden)	Subjects	TB Exposure during study	BCG status	IGRA	Time points (days)	Internal quality control	Study results summary (within subject variability)	Comment
Veerapathran et al [127] '08	India (High)	14 HCWs (clinical and laboratory workers)*	Likely but all tests were done within a 2-week period	All vaccinated	QFT-GIT	0, 3, 9, 12	Yes	Over a 2 weeks period, 2 of 14 persons had a QFT reversion. With quantitative results, an increase in 16% of IFN-γ response was considered within the 'normal' expected within subject variability.	Subjects who had conversions or reversions had initial values close to the cut point
Van Zyl-Smit et al [126] '09	South Africa (High)	26 HCWs and low risk volunteers+	Likely but all tests were done within a 3 week period	All vaccinated	QFT-GIT T-SPOT.TB	0, 7, 14, 21	Yes	Over a 3-week period, 7 of 26 persons had a conversion or reversion (1 QFT and 6 TSPOT.TB). With quantitative results, a change of ±80% of any given IFN-γ response (QFT-GIT) or ±3 spots (T-SPOT.TB) was considered to fall within the 'normal' expected within subject variability.	Subjects who had conversions or reversions had initial values close to the cut point
Detjen et al [168] '09	South Africa (High)	27 HCW's (Clinical and laboratory workers)*	Likely but all tests were done within a 3-day period	All vaccinated	QFT-GIT	0, 3	Yes	**Over a 3-day period, no changes in qualitative results were noted for 15 persons. With quantitative results, considerable intra-individual variability occurred in the magnitude of IFN-γ responses; intra-class correlation was 0.80.**	
Belknap et al [169] '09 (abstract)#	USA (Low)	117 HCWs	Unlikely and all tests were done within a 3 week period	Unknown	QFT-GIT T-SPOT.TB	0, 7-21	Yes	Over a 3 week period, 7 of 117 (6%) persons had a conversion or reversion with QFT-GIT and 8 of 105 (7.6%) with T-SPOT.TB	Quantitative results not yet available
Ringshausen et al [170] '10	Germany (Low)	182 HCWs	Unlikely	52% vaccinated	QFT-GIT	0, 126	No	Over 18 weeks 6/18 (33%) had a reversion and 3/ 162 a conversion	Some subjects had TST 3 months prior to first IGRA

* India and South Africa are high burden TB countries with high risk of exposure to health care workers (HCW). HCW's were divided into two groups – medical doctors or laboratory workers. + South Africa is a high burden TB country with high risk of exposure to health care workers (HCW). HCW's were stratified into: High risk (daily potential TB exposure) Medium risk (HCW, but no daily expected TB exposure) Low risk group (pre-clinical medical students and non-clinical volunteers) # updated preliminary data presented at the Second international IGRA symposium. Daley C. Evaluation of interferon-g release assays in the diagnosis of latent TB infection in US healthcare workers: preliminary results of Task Order #18. 31 May 2009; Dubrovnik, Croatia2009.

Table 3-9 Studies on boosting effect of TST on IGRA results

Paper	Country/ Population recruited (TB burden)	Subjects	TST	IGRA	Time points (days after TST administration)	Study results summary	Comment	Study conclusion on boosting
van Zyl-Smit et al[126] '09	South Africa HCW's and healthy volunteers (High)	24	2TU RT23	QFT-GIT TSPOT.TB	0,3,7,28,84	Day 3: no categorical changes Day 7: Significant increase in mean IFN-γ, QFT-GIT 1/12 (8%) negative to positive, 5/8 (62.5%) positive ↑ in INF-γ responses Day 7: T-SPOT.TB 2/16 (12.5%) negative to positive, 6/8 (75%) positive ↑ in INF-γ responses	IGRA negative subjects who boosted were TST positive.	Yes
Baker et al [171] '09	USA Immigrants/ refugees in US less than 6 months (mainly high burden countries)	114	5TU PPD-S	QFT-GIT	0, 14-112	<35 days: 2nd IGRA 87%↑ in INF-γ responses, 35 -112 days: 69%2nd IGRA ↑ in INF-γ responses IGRA positive 86% showed boosting* IGRA negative 68% showed boosting	*TST positive boosted by 82% whereas TST negative by 62% (p=0.06)	Yes
Belknap et al [172] '09 [abstract] #	USA HCWs (equal number of TST +/TST -) (Low)	125	5TU Tubersol	QFT-GIT TSPOT.TB	7-21+	QFT-GIT: 12 (10%) negative to positive, T-SPOT.TB: 12 (10%) negative to positive	Exact testing days not specified Only IGRA negative recruited TST status did not predict boosting	Yes
Vilaplana et al [173] '08	Spain TB researchers (Low)	9	2TU RT23	ELISPOT & WBA IFN-γ*	0,7, 14, 28	IGRA neg / TST neg 5-60 x ↑@ day 7* (4 subjects) IGRA pos / TST pos 20-400 x ↑ @ day 7* (3 subjects) IGRA pos /TST neg 5-80 x ↑ @ day7 * (2 subjects)	* Depending on antigen used + Cellestis Ltd.	Yes
Choi et al[174] '08	South Korea HCWs in pulmonary medicine working >1 year (Medium)	59	2TU RT23	QFT G	0, 14-28	Median IFN-γ responses ↑ @ visit post TST 0.05 to 0.19IU/ml increase in TST positive group (p=0.01) IGRA neg/ TST pos 3/18 (16.7%) became IGRA positive IGRA neg/TST neg zero became positive (p=0.11)		Yes
Perry et al [165] '08	Infectious disease cohort (Low)	63	5TU Tubersol	QFT-GIT	0, 84 (3 mo)	Day 84: 3/48 (6%) QFT negative became positive Day 84: Mean IFN-γ responses ↑ in initially QFT positive subjects	Non significant trend for inconsistent QFT results to be discordant by TST at baseline	Yes
Richeldi et al[175] '08 *	Italy, paediatric TB contacts (Low)	70 & 81	5TU PPD S	QFT-G /QFT-GIT	0, 56-77	QFT-G: 1/51(2%) negative became positive (no change in mean QFT levels in negative subjects) QFT-GIT 1/63 (1.5%) negative became positive		No

Paper	Country/ Population recruited (TB burden)	Subjects	TST	IGRA	Time points (days after TST administration)	Study results summary	Comment	Study conclusion on boosting
Leyten et al [176] '07	The Netherlands known TST 0mm (n=15) and known TST ≥10mm (n=51) (Low)	66	2TU RT23	QFT GIT	0, 3, (10,11)*	Day 3: no categorical changes Day10: 1 negative to positive Day11: 1 positive ↑ in INF-γ response	*Boosting shown only in two with delayed processing, and this was not statistically significant	No @ 3days Yes @ 10 days
Igariet al[177] '07	Japan, University Medical students, negative baseline QFT and TST <15mm (Low)	33	3TU PPD	QFT-G	0, 42	Day 42: IGRA neg/ TST neg; 5(15%) became positive	Only concordant baseline negatives had second IGRA	Yes
Naseer et al. [178] '07	UK Subjects not specified No Hx of TB contact or disease (Low)	10	Not reported	QFT-G TSPOT.TB	0, 2, 42	Day 42: 3/9 (33%) QFT negative became positive Day 42: 0 T-SPOT negative became positive	No qualitative results reported No boosting if blood drawn at TST administration	Yes
Cellestis Int, Australia - QFT USA Package insert[179] '07	USA (Low)	530	Not reported	QFT-GIT	0, 28-35	IGRA negative 3 became positive (total number of negatives not reported), 5 initially positive reverted	Industry study not published	No
Richeldi et al [180] '06 *	Italy TB contacts (Low)	44	5TU PPD S	TSPOT.TB	0, 9, 15 24 months (Post TB exposure)	Month 24: all subjects remained IGRA negative, although 3 converted by TST.	All subject TST and IGRA negative at first visit	No
Nguyen et al [181] '05	USA Infectious disease cohort (Low)	48	5TU Tubersol	QFT- TB	0, 84 (3 mo)	Day 84: 1/27 (4%) negative became positive (p=0.10)	This study primarily investigated TST-TST boosting (PPD) responses) and discordance.	No

Updated preliminary data presented at the Second international IGRA symposium. Daley C. Evaluation of interferon-g release assays in the diagnosis of latent TB infection in US healthcare workers: preliminary results of Task Order #18. 31 May 2009; Dubrovnik, Croatia2009. * Retrospective studies [**Table 3-8** and **Table 3-9** updated from [166]]

In this high burden setting different exposure categories were used to control for chance *M.tb* exposure being an explanation for the observed boosting of RD-1 responses. Thus, given similar boosting in the high and medium HCW exposure categories, and the temporal relationship of boosted responses to the TST, from a stable baseline, the effects seen are unlikely to be attributed to chance *M.tb* exposure. Secondly, for the purposes of generalising within-person variability to clinical practice, a diverse group of participants was needed. The finding that variability occurred in all groups increases the robustness of the findings.

These data have several important implications. Firstly, they suggests that variation around the assay cut-points of 0.35IU/ml for QFT-GIT and 6 spots for TSPOT TB is present and assay results near these cut points should be interpreted with caution and within the clinical context. This borderline zone: (0.2 to 0.7 IU/ml for QFT-GIT and 4 to 8 spots (inclusive) for T-SPOT.®TB) is in keeping with the "uncertainty zone" proposed for interpretation of serial IGRA results.[182] Also, clinicians should be made aware of this and should not view minor elevations in IGRA test results as confirming new infection when serial testing is performed in healthcare worker monitoring. This is important as IGRA results are often dichotomised when reported to the referring clinician. Laboratories should provide absolute IFN-γ or spot count results and avoid simply reporting results as positive or negative.

Secondly, it provides clinically appropriate thresholds for conversions and reversions. The data supports the recommendation by Pai et al that a higher threshold (e.g. 0.70 IU/ml) for QFT-GIT should be used to define conversions.[183] In order to avoid minor changes around the cut-off values being misinterpreted as real conversions. In addition these data provide evidence for the extent of an uncertainty zone for the TSPOT.TB assay of 4-8 spots, which has not previously been reported. Furthermore, a 9 or greater spot count threshold for a T-SPOT.TB assay conversion would be comparable to that suggested (0.7IU/ml) for QFT-GIT. Thirdly, by taking into account within-subject variability, boosting of IFN-γ responses by the TST can clearly be defined (i.e. distinguishing boosting from random day-to-day variability). This concept is depicted in Figure 3-4.

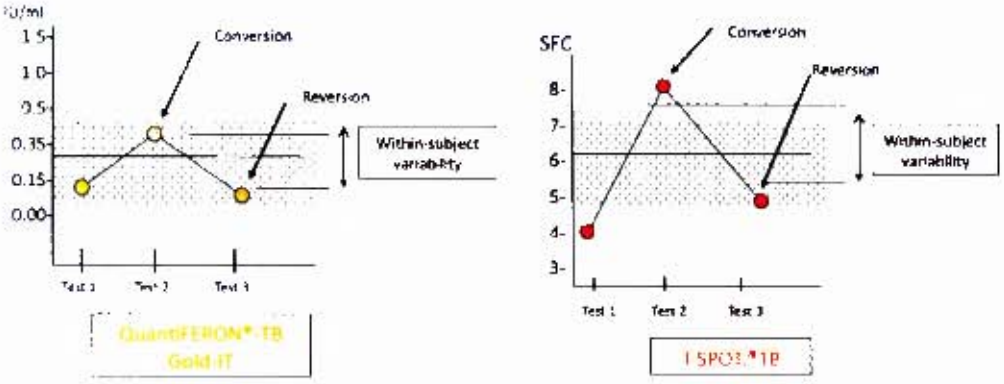


Figure 3-4 Graphic depiction of the concept of "conversion and reversion" and "within-subject variability"

The conversion and reversion points depicted are based on the manufacture's definitions with a hypothetical within-subject variability or border line/grey zone indicated. The shaded area for the T-SPOT.TB diagram is the FDA defined grey zone. [108]

The observed variability of IGRA responses may be due to several technical and biological factors including random variability, laboratory and operator variability, diurnal variation in T cell levels,[104, 105] possible cyclical fluctuations in ESAT-6 antigen levels,[106, 107] exposure to RD-1 homologue-producing non tuberculous mycobacterium e.g. *M. marinum* and degeneracy of the four-point ELISA dilution for values less than 1IU/ml.[165] There may also be inherent test-retest variability ('measurement noise').

The optimal method for confirming LTBI is under debate; whether to use a single IGRA or to use the 2-step approach, a TST followed by IGRA. The results of previous studies investing TST mediated IGRA boosting, have varied with some studies showing boosting[173, 176, 177, 178] and others not.[176, 180, 181] Possible reasons for these conflicting results are the timing of the IGRA after TST. This has varied from 3 days to 9 months in different reports.[176, 180] Secondly several studies used older versions of the commercially available IGRAs,[174, 177, 181] or in-house assays,[173] rather than standardised ones. In many cases analysis was retrospective and boosting was not a pre-specified end-point. Finally, these studies have been performed in different exposure settings[173, 176, 177] and only some evaluating IGRA/TST negative subjects.[180]

This study prospectively evaluated both commercially available IGRA formats (T-SPOT.®TB and QFT-GIT) using pre-specified end-points, after first defining within-subject variability. These data indicate that the QFT-GIT or T-SPOT.®TB test result may be misleading if performed more that 3 days after TST in patients being evaluated by the 2 step strategy. Fortuitously this is a convenient time-point as it generally coincides with the date on which the TST should be read. A recent study from the Netherlands found no evidence of QFT-GIT boosting at the 3 day post-TST time-point but two subjects whose repeat testing was delayed to day 10 showed evidence of boosting.[176]

A further significant observation in this study is that boosting by TST was more common in IGRA positive (i.e. sensitized) subjects. This finding is in keeping with previously published data that suggest that only IGRA or TST positive subjects show post-TST boosting. [174] It is therefore reasonable to speculate that pre-dominantly those with pre-existing effectors memory T-cell responses, and hence LTBI, will boost their subsequent responses. However, the small sample size precludes any definite conclusions and several IGRA-negative/TST-positive subjects did boost their responses (it is possible that these subjects were sensitized to TB antigens, but not detected by the IGRA). It is interesting that a single TST, which contains only nanogram amounts of ESAT-6 and CFP-10, is sufficient to elicit a systemic T cell response. This supports the concept that the IGRAs detect recent exposure to TB antigen and hence LTBI. It is not clear what number of latent organisms (burden of infection) is required for a positive test result.

The inability of RD-1 antigen responses to distinguish latent from active TB has prompted the search for alternative *M.tb*-specific antigens. One such novel candidate is HBHA.[188, 189] It is therefore useful to have data on within-subject variability for this antigen and the effect of TST on the results of this test. Interestingly, like PPD but in contrast to RD-1 antigens, HBHA-boosted responses were detectable by day 3. The reasons for this are unclear but may be related to differential modulation of antigen-specific responses by regulatory T cells.[190, 191]

There are several limitations of this study. Firstly, the study population is small. However, as each subject had multiple tests performed over several weeks the number of tests evaluated was large (greater than 300). The conclusions are thus both clinically and statistically valid. On the other hand, as not all subjects demonstrated boosting in T cell responses, the study may not have been adequately powered to exclude the possibility that some subjects might demonstrate RD-1 boosting before day three, post-TST. However, Leyten and colleagues observed no QFT-GIT boosting at day 3 post-TST.[176]

Due to the high tuberculosis exposure in the health care workers, transient chance exposure as the cause of boosting cannot be ruled out. However, this is unlikely given that similar boosting patterns were seen in the different exposure risk categories and that the same boosting pattern was observed over several months of recruitment. It remains unclear how long the booster effect of tuberculin skin testing persists.

There are three conclusions:

Firstly, I have shown that there is significant within-subject variability of IFN-γ responses over time. This variability can be used to define an 'uncertainty zone' around the cut-point where results are unreliable and necessitates repetition of the test.

Secondly, based on these results, a threshold for a 'true conversion' in a high exposure environment has been proposed: An increase in IFN-γ response from below 0.35 IU/ml to above 0.70 IU/ml for QuantiFERON®TB Gold-IT, and from below 6 spots to above 9 spots for the T-SPOT.®TB assay. This may explain much of what previously were regarded as conversions and reversions because of 'movement' across the cut-point. Larger long-term studies in different populations are necessary to establish the relevance of these findings to other parts of the world.

Thirdly I have confirmed that IGRA results, in both IGRA positive and to a lesser extent in IGRA negative subjects, are influenced by recent TST administration. Boosting is evident by day seven but not day three post-TST administration. Thus, a

three-day cut-off appears to be a safe window within which to perform a standardised IGRA after TST. Results of IGRAs repeated one week after an initial IGRA test and TST may be falsely elevated.

A gold standard definition for latent TB infection remains elusive and given the variability over time and discordant IGRA-TST results, LTBI remains a presumptive diagnosis. Individuals, who are both TST negative and have very low IFN-γ response, are likely to be uninfected and test results are likely to remain negative over time. By contrast, individuals who have a strong TST response (>10mm) and IFN-γ responses above the defined manufacturer's cut point for positivity are likely to be latently infected. Individuals who have either discordant results or results within the 'uncertainty zone' should not be viewed as having LTBI. For researchers, stratifying subjects as double negative (IGRA and TST) or double positive provides greater confidence about the absence or presence of T cell memory. It remains unclear on how to interpret the data where low positive or discordant results occur.

4 Determination of mycobacterial burden

4.1 Introduction/Background of assays

Determination of mycobacterial load is an important measure in many laboratory and clinical studies including those assessing disease severity and the efficacy of new therapies and tuberculosis (TB) interventions,[131-133] all of which have now become urgent with the emerging public health threat of multidrug and extensively drug resistant TB.[192, 193] The usual method used for clinical purposes and in some studies is the grade of smear positivity on microscopy (scanty, 1+ 2+, and 3+). It is used to evaluate the infectiousness of cases in the context of public health contact tracing and screening,[141, 194] but at best is semi quantitative. More accurate determination of mycobacterial numbers was required for the work described in this thesis, examining the effects of cigarette smoke on pulmonary immune responses and the mycobactericidal-modulating effect of cigarette smoke extract (CSE).

Such methods are also necessary in translational research in research on drug and vaccines for tuberculosis, whether in humans or murine models of disease.[23-26] Although several techniques for determining mycobacterial burden exist, each is associated with significant limitations such as inaccuracy, turn-around-time, limited reproducibility, cost, methodological complexity, relative discriminative ability and detection threshold.

Culture on solid media using colony-forming units (CFU) is widely considered to be the gold standard for determining the number of viable organisms in a specimen or experimental condition, but is labour-intensive and has a long turn-around-time.[134, 135] Alternative techniques include the incorporation of tritiated uracil into mycobacterial DNA, bioluminescence assays that use a reporter construct, quantitative real-time polymerase chain reactions (PCR) and time to positivity (TTP) in automated liquid culture systems (BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960). Each of the later has its own set of performance characteristics that determine its suitability for different applications.

More recently, newer technologies such as the Xpert MTB/RIF system (Cepheid, Sunnyvale, USA) have been developed for the rapid detection of TB using clinical samples. However PCR methods have been limited by their inability to distinguish viable from degraded organisms. Whilst detecting *M.tb*-specific mRNA from viable organisms is a potential solution, like PCR[195, 196] real time PCR, is technically demanding.[138] Xpert MTB/RIF has the potential to circumvent this problem as contaminating extracellular debris is removed in an intermediary step by washing. DNA from intact organisms trapped in a mesh is subsequently amplified by PCR.[139, 140] Its quantitative accuracy however, has not yet been compared to that of automated culture, uracil incorporation and bioluminescence techniques.

There are limited reports comparing the performance of different mycobacterial load determination techniques in laboratory-based experiments. This study therefore compared the performance of five quantitative load determination techniques.

4.1.1 Hypothesis

Newer automated techniques for quantifying mycobacteria in human and experimental specimens may perform as well as traditional measures such as CFU determination of solid media and could have advantages, making them preferable for a variety of clinical and experimental uses.

4.1.2 Specific aims

1. To determine the performance of five assays to determine mycobacterial burden in serial dilutions of *Mycobacterium bovis* and *Mycobacterium tuberculosis* H37RV. The assays studied were:
    - Automated liquid culture (BACTEC-MGIT-960)
    - [^{3}H]-uracil incorporation assays
    - Luciferase-reporter construct bioluminescence
    - Quantitative PCR using the Xpert-MTB/RIF system
    - Mycobacterial colony-forming units (CFU) using 7H10-Middlebrook solid media (reference standard)

2. To determine the optimal assay for use in clinical and laboratory research by comparing:
    - Turn-around-time
    - Detection threshold
    - Dynamic range
    - Labour intensiveness/complexity
    - Reproducibility
    - Quantitative discriminative ability
    - Cost

4.2 Methods for determining mycobacterial burden

4.2.1 Preparation of mycobacteria for assays

Detailed methods for the growth, storage and preparation of mycobacteria are outlined in Chapter 2.

Briefly, both BCG and H37RV luciferase reporter constructs (pSMT1 luciferase)[197] were used for all assays (gift of Muazaam Jacobs from the Institute for Infectious Diseases and Molecular Medicine University of Cape Town). Triplicate serial dilutions were prepared in sterile phosphate buffer solution (PBS) from the frozen stock for each strain in aliquots ranging from 1 to 1 x 10^6 CFU per ml. The five predetermined assays were each undertaken using one of the prepared aliquots. In addition, all dilutions were inoculated onto solid media to confirm the number of CFUs at each dilution.

4.2.2 Solid culture determination of colony forming units (reference standard)

Aliquots of 1, 10 and 100 CFU were plated in 6 replicates of 10µl on 7h10 Middlebrook enriched with OADC (oleate-albumin-dextrose-catalase). Plates were sealed in airtight bags and incubated at 37°C. Colonies were counted daily between days 7 and 14 using an inverted microscope. The colony counts were done over two days by two readers.

4.2.3 Uracil incorporation assays

200µl of each dilution was pipetted into 6 wells in 4 separate U-bottomed 96 well plates and 50ul of ^{3}H uracil (final concentration of 1µCi/well) was added to each well. The plates were incubated for 24 hours at 37°C in a 5% CO_2 humidified chamber and harvested onto fibreglass filter mats. The discs were placed into scintillation bottles containing 1ml of Quicksafe (Zinnser Analytic, Frankfurt, Germany) and the amount of tritiated uridine incorporated determined using a liquid scintillation counter and reported as counts per minute (CPM).

4.2.4 Bioluminescence assay

Luminescence was measured using a Modulus™ Microplate Multimode Luminometer (Turner). 25µl of 1% (v/v) n-decyl aldehyde (Sigma), was injected into each well containing serial dilutions (1:10 with 0.25% Tween/PBS) to a final volume of 125µl, using a 0.5s delay and 0.5s integration time. The final results were expressed as relative light units (RLU).

4.2.5 Liquid culture

A 500µl aliquot of PBS containing the specified number of organisms was injected into pre-prepared mycobacterial growth indicator tubes (MGITs) (Becton Dickinson, Sparks, Maryland) and the MGITs then incubated using the BACTEC-MGIT-960 automated culture system, which monitors the tubes for the presence of growth on a constant basis. Each dilution was prepared in triplicate and blinded to the MGIT operator. Time to positivity was recorded by instrument as the time between start of incubation and detection of growth.

4.2.6 Polymerase chain reaction (PCR) using Xpert® MTB/RIF assay

A 1ml serial dilution of H37RV and BCG was treated with sample buffer supplied by the manufacturer as recommended and 2 ml of the resultant mixture was later transferred into an Xpert MTB/RIF cartridge. The cartridge was loaded into the Gene Xpert IV instrument and the automated procedure started. Results and C_T values of probes were obtained using the software (Xpert MTB/RIF version 2.0).

4.3 Determination of performance characteristics

4.3.1 Turn-around-time

The turn-around-time was defined as the time taken from the start of each assay after preparation of the aliquots, until a bacterial load determination was possible (either automated output or colony counting).

4.3.2 Detection threshold

The detection threshold was defined as the lowest colony number detected by the assay: The results from 2 out of 3 MGIT bottles, 2 out of 3 Xpert MTB/RIF cartridges or >50% of CFU/RLU/CPM replicates for a particular dilution were used as the reliable lower limit of detection for comparative purposes.

4.3.3 Discriminative ability

The ability of the assay to detect a difference between serial dilutions was assessed using a 1 way ANOVA with correction for multiple comparisons (Tukey).

4.3.4 Reproducibility

Reproducibility of each assay was determined by calculating the coefficient of variance (SD/mean) across all dilutions. To compare the reproducibility between assays (across the various range and readout units such as time, colony counts, cycle threshold, etc.) the mean coefficient of variance for BCG dilutions was calculated for each assay.

4.3.5 Determination of assay costs

The cost of each technique was assessed by taking into account consumables used. Quotes were requested from local suppliers for both major equipment as well as specific consumables required for each individual experiment. Costing was calculated in South African Rand (ZAR) and converted to United States dollars (USD). Personnel costs, general laboratory consumables and bio-safety equipment, were considered to be similar for all assays and thus not included in the final simple

costing. The capital cost of major laboratory equipment (incubator, beta-counter, automated MGIT system etc.) was calculated for comparative purposes but not included in the basic cost per experiment.

A hypothetical single experiment, containing 2 conditions (control and intervention) performed in triplicate, was used to standardise cost between assays. Where appropriate, 'pooling of wells' was allowed to reduce costs e.g. a single Xpert MTB/RIF cartridge for each condition (total of 2) as opposed to a single cartridge for each replicate (total of 6). Details of the costing sheet and costs for each assay are contained in Table 4-2.

4.3.6 Labour intensiveness /complexity

The complexity and labour intensiveness of each experiment was assessed on a qualitative scale. Factors included were: number of technical steps, degree of automation and use of specialized reagents such as radioactive isotopes or luminescence substrates, which require specialised handling.

4.4 Results for the performance of each assay including costs

A comparative overview of all the mycobacterial load assays is summarised in Table 4-1 with the breakdown of the cost of each assay contained in Table 4-2. The performance characteristics of each assay are detailed separately.

Table 4-1 Performance characteristics of assays used to determine mycobacterial burden

Assay	Turn-around time	Detection threshold (CFU)	Dynamic range *	Reproducibility (Coefficient of Variance)[+]	Cost per experiment[#]	Labour intensiveness/ complexity
Culture on 7H10 solid media	Days to weeks	1 CFU	Wide provided appropriate dilutions are made.	Fair (22%)	Low ≈ ZAR 49 / USD 7	Labour intensive, learning curve for counting colonies –
Liquid culture using MGIT 960 system	Days to weeks	1-10 CFU	Excellent at range used from 1 to 1x 10^6 CFU	Good (2%)	Low/medium ≈ZAR 84/ USD 12	Automated system once bottles inoculated – requires BACTEC 960 MGIT system
Tritiated Uracil assay	24 hours	1000 CFU	Poor below 1x 10^3 CFU able to detect dilution of up to 1x 10^6 CFU	Poor (38%)	Low/medium ≈ZAR 98/ USD 14	Requires radioactive isotope handling skills and regulatory approval
Luminescence assay using reporter construct	< 2hours	100 CFU	Poor below 1x10^3 CFU- but able to detect dilutions of up to 1x 10^6 CFU	Fair (19%)	Low ≈ZAR 70/ USD 10	Extensive optimization required for each strain and batch – requires organism with construct
PCR using Xpert MTB/RIF	2 hours	100 CFU	Good at ranges between 1x10^2 and 1x 10^6 CFU	Good (3%)	High ≈ZAR 252/ USD 36	Limited skills required prior to automated processing – requires Gene Xpert machine and supplies

*All experiments apart from CFU, require a standard curve to allow for actual CFU calculation from assay readout

Cost was calculated for the specific consumables for a hypothetical experiment containing 2 conditions performed in triplicate. For MGIT and PCR triplicate wells were pooled into a single bottle/cartridge to reduce costs. Costing does not include major equipment: Gene Xpert IV machine ≈ZAR 126000/USD 18000, Scintillation counter ≈ZAR 595000/USD 85000, Luminometer ≈ZAR 59500/USD 8500, BACTEC 960 MGIT machine ≈ZAR 49000/USD 70000

+ Reproducibility was determined by the mean coefficient of variance for the specific readout (CFU, RLU etc.) across all dilutions using the only the BCG experiments.

4.4.1 Costing for all assays

The detailed costing for each assay is contained in Table 4-2. The assay with the lowest associated costs was CFU by solid media: ZAR 50.31 / USD 7.19. The most expensive assay was the automated gene Xpert MTB/RIF PCR at ZAR 255.70/ USD 36.53. Although the uracil incorporation and automated liquid culture assays were less expensive than the Xpert MTB/RIF, the capital cost of the equipment was approximately 5 times higher. Further details of the comparative costing are described below for each assay as part of the overall performance.

Table 4-2 Costing overview of mycobacterial load quantification assays

Experiment	Equipment	Basic cost/volume	Unit cost	No. per experiment	Cost per experiment	Total cost ZAR /USD
PCR using Xpert MTB/RIF	Gene Xpert IV machine	R127 500,00				
	XPERT MTB/Rif Cartridge	R126,00	R126,00	2	R252,00	R255,70 / $36,53
	Micro centrifuge tubes	R400/1000	R0,40	3	R1,20	
	Pipette tips	R245/1000	R0,25	10	R2,50	
Tritiated Uracil assay	Beta counter	R524 000				R99,89/ $14,27
	Harvester	R100 000				
	[^{3}H]- Uracil	R2692/ml	R2,70	6 µl	R16,20	
	Opaque 96 well plate	R858/50	R17,00	1	R17,00	
	Fibre filter paper	R5000/100sheets	R50,00	1	R50,00	
	Pipette tips	R245/1000	R0,25	36	R9,00	
	15ml conical tubes	R865/500	R1,73	3	R5,19	
	RPMI	R124/500ml	R0,25	10 ml	R2,50	
	Scintillation fluid	R3110/ 5 l	R0,62	6 ml	R3,72	
	Scintillation tubes	3623/2500	R1,45	6 ml	R8,70	
Culture on 7H10 solid media	Incubator	R63 000				R50,31 / $7,19
	Autoclave	R22 000				
	Microscope	R10 000				
	7h10 Agar	R493/500g	R0,99	1,18 g	R1,18	
	OADC	R536/200ml	R2,68	7 ml	R18,76	
	Micro centrifuge tubes	R400/1000	R0,40	16	R6,40	
	Lysate (saponin)	R742/100g	R0,01	6 mg	R0,06	
	Autoclave tape	R211 for 20m roll	R11,00	0,01 m	R0,11	
	Pipette tips	R245/1000	R0,25	66	R16,50	
	Agar plate	R2195/300	R7,31	1	R7,31	
Luminescence assay using reporter construct	Luminometer	R63 000				R71,07 / $10.15
	Opaque 96 well plates	R5700/100	R57,00	1	R57,00	
	Pipette tips	R245/1000	R0,25	20	R5,00	
	Lysate (saponin)	R742/100g	R0,01	6 mg	R0,06	
	Decanal	R344/250ml	R1,37	0,5ml	R0,69	
	70% Ethanol	R120/L 70%	R1,20	0,5ml	R0,60	
	Sterile water	R83/l	R0,80	1ml	R0,80	
	15ml conical tubes	R865/500	R1,73	4	R6,92	
Liquid culture using MGIT 960 system	BACTEC 960 MGIT machine	R490 000				R81,06 / $11,58
	MGIT bottles	R2596/100	R25,96	2	R51,92	
	Culture supplement	R837/100 tests	R8,37	2	R16,74	
	Micro centrifuge tubes	R400/1000	R0,40	16	R6,40	
	Pipette tips	R245/1000	R0,25	24	R6,00	

Conditions in triplicate = 6 wells pooled for analysis either 6 or 2 tests
USD:ZAR exchange rate = 1:7

4.4.2 Solid media culture

Although technically simple to perform, plating for CFU is time consuming and pipetting skills as well as visual counting of colonies affect accuracy and reproducibility. Counting of CFUs and determination of mycobacterial load was possible approximately 10 – 14 days after plating. (Figure 4-1) Determination of the CFU count was limited by the visual ability to accurately count organisms and thus only dilutions of 1, $1x10^1$ and $1x10^2$ CFU were used for plating on 7H10 agar. The coefficient of variance across the three dilutions ranges was 22%. The assay is relatively cheap to perform at approximately ZAR 50.31/ USD $7.19 and requires only standard laboratory equipment such as an incubator, autoclave and microscope

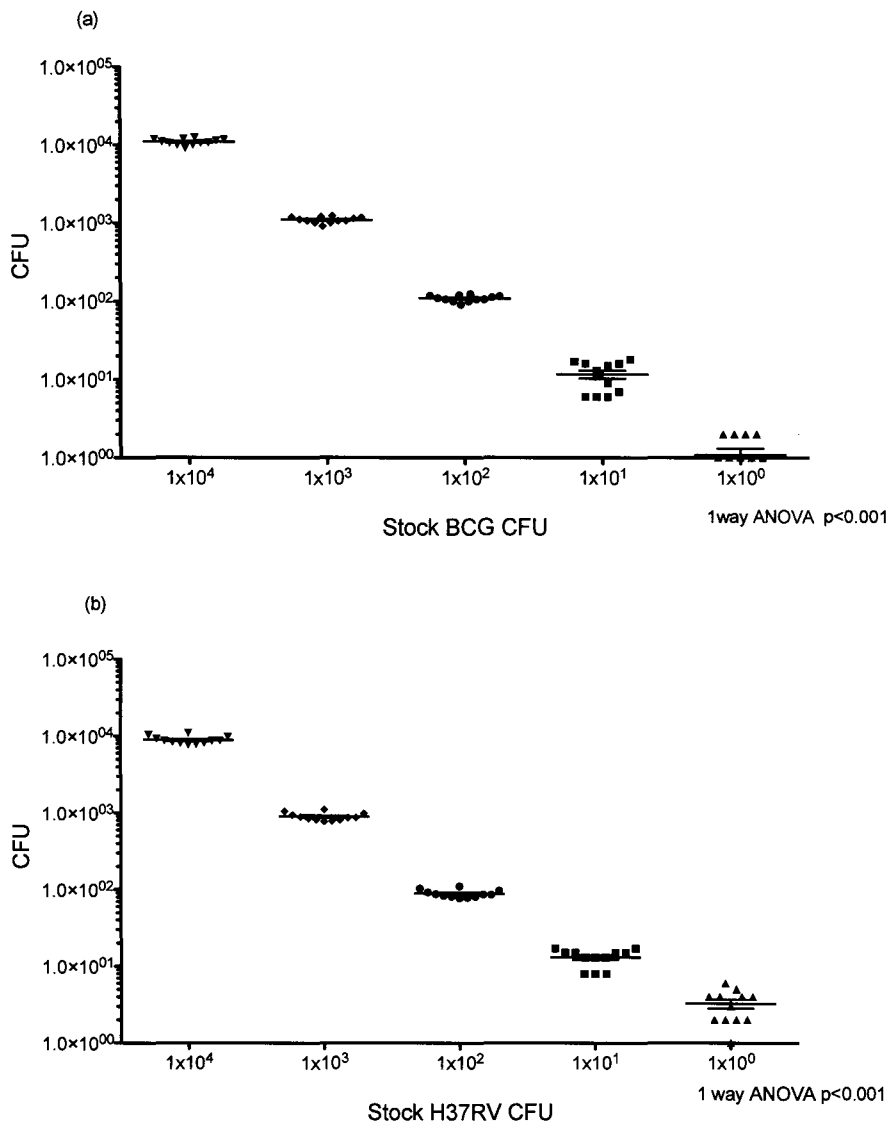


Figure 4-1 Mycobacterial load determination using solid media (Middlebrook 7H10)
Stock dilutions are presented on the x-axis for BCG (a) and H37RV (b) with the calculated CFU on the y-axis (log scale) # values for dilutions $1x10^4$ and $1x10^3$ extrapolated from serial dilutions to $1x10^2$.

4.4.3 Automated liquid culture

The turn around time for the BACTEC-MGIT-960 system was defined as time to positivity (TTP) ranged from 117 hours (~ 5 days) to 467 hours (~ 19 days) for BCG and 123 hours (~ 5 days) to 528 hours (~22 days) for H37RV (dilution range $1x10^6$ to $1x10^0$ CFU). (Figure 4-2)

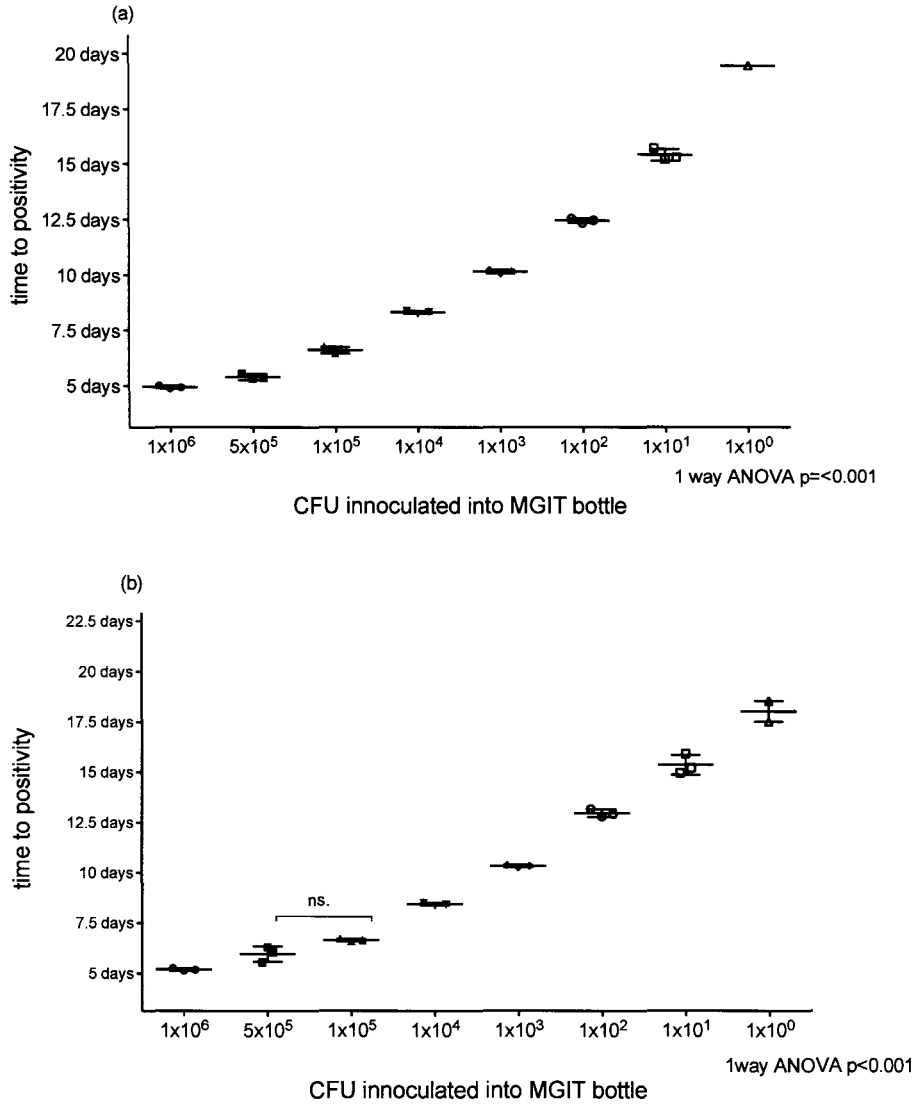


Figure 4-2 Automated liquid culture (using BACTEC MGIT 960) time to positivity

Time to positivity was calculated as the automated time from culture initiation to bacterial detection (on the y-axis) for serial dilutions of BCG (a) and H37RV (b) on the x-axis

The lower limit of detection was less than 10 CFU (1 CFU detected in 3/3 BCG bottles and 2/3 H37RV bottles). Reproducibility of the BACTEC-MGIT-960 system was excellent as most replicates become positive within a few hours of each other at all dilutions (coefficient of variance = 2%). The range of detection was from 1 to $1x10^6$ CFU. BACTEC-MGIT-960 was able to detect differences in mycobacterial load as small as $1x10^2$ organisms at low concentrations and $1x10^5$ at higher concentrations. The

major cost for the experiment was the culture bottles, and estimated experimental costs were ZAR 81.56/ USD 11.08. The BACTEC MGIT machine costs approximately ZAR 490 000/ USD 70 000 (developing country pricing).

4.4.4 Luminescence assay

The lower limit of detection was 1x 10^2 CFU as no discrimination was possible below 100 organisms. Discrimination between mycobacterial loads was possible at ranges of 1x10^2 to 1x10^5 CFU. (Figure 4-3)

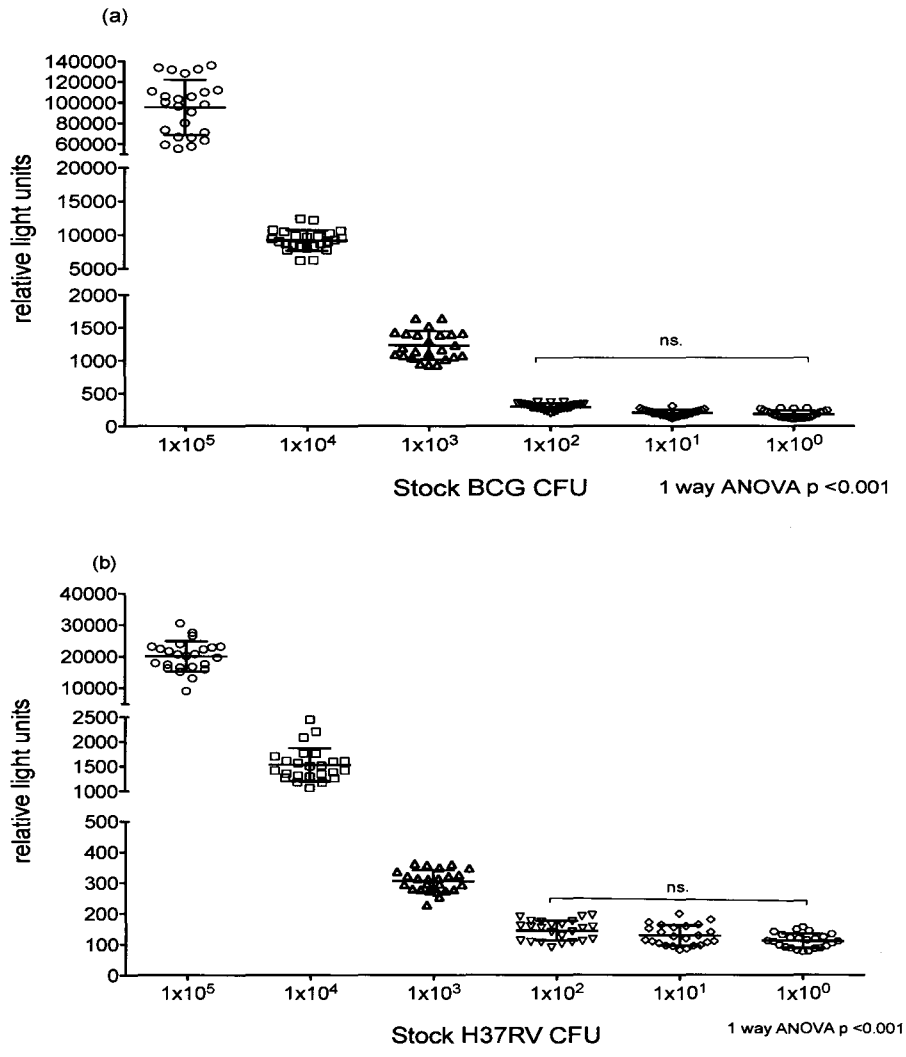


Figure 4-3 Luminescence assay (using luminescent reporter construct) relative light units

Relative light units measure for each serial dilution are represented on the y-axis with serial dilutions of BCG (a) and H37RV (b) on the x-axis

Reproducibility was comparable to CFU with a coefficient of variance of 19%. Mycobacterial load determination was potentially available within minutes of RLU determination using a pre-prepared standard curve of RLU vs. CFU. The cost of the

assay was approximately ZAR 71.07 / USD 10.15 but requires a luminometer (≈ZAR 63 000/ USD 8,500), to be permanently situated in the bio safety laboratory.

4.4.5 Uracil incorporation

Uracil incorporation assays were complex and time consuming to perform although the time to acquiring a result was approximately 24 hours. Specific training and equipment was required to use radioisotopes as well as provision for isotope storage and waste disposal. The effective lower limit of detection was 1000 CFU as the tritiated uracil assay could detect, but not discriminate between loads at ranges below 1x 10^3 CFU. However, it was effective at 1x10^3 to 1x10^5 CFU. (Figure 4-4) The cost per experiment was approximately ZAR 99.89/ USD 14.27 but requires a harvester and scintillation counter (≈ ZAR 524 000/ USD 85,000).

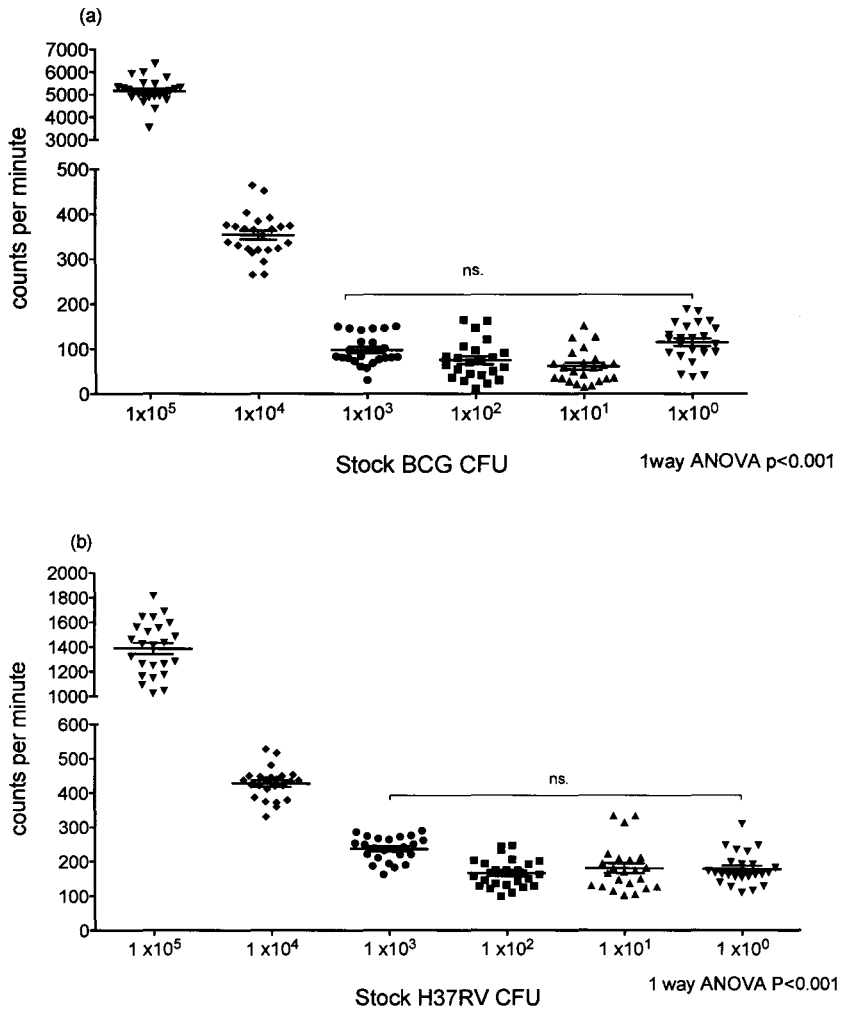


Figure 4-4 Uracil incorporation assay counts per minutes (CPM)

Counts per minute are depicted on the y-axis with serial dilutions of BCG (a) and H37RV (b) on the x-axis

4.4.6 PCR using the Xpert MTB/RIF system

The automated real time MTB/RIF cartridge in the Gene Xpert IV system was user friendly with minimal technical steps required prior to inserting the cartridge into the machine. Xpert MTB/RIF results were available within 2 hours of the experiment using the Gene Xpert IV machine with 4 bays (only 4 samples could be run at once). BCG reproducibility was excellent (coefficient of variance 3%) and the automated PCR reliably detected 100 organisms. (Figure 4-5)

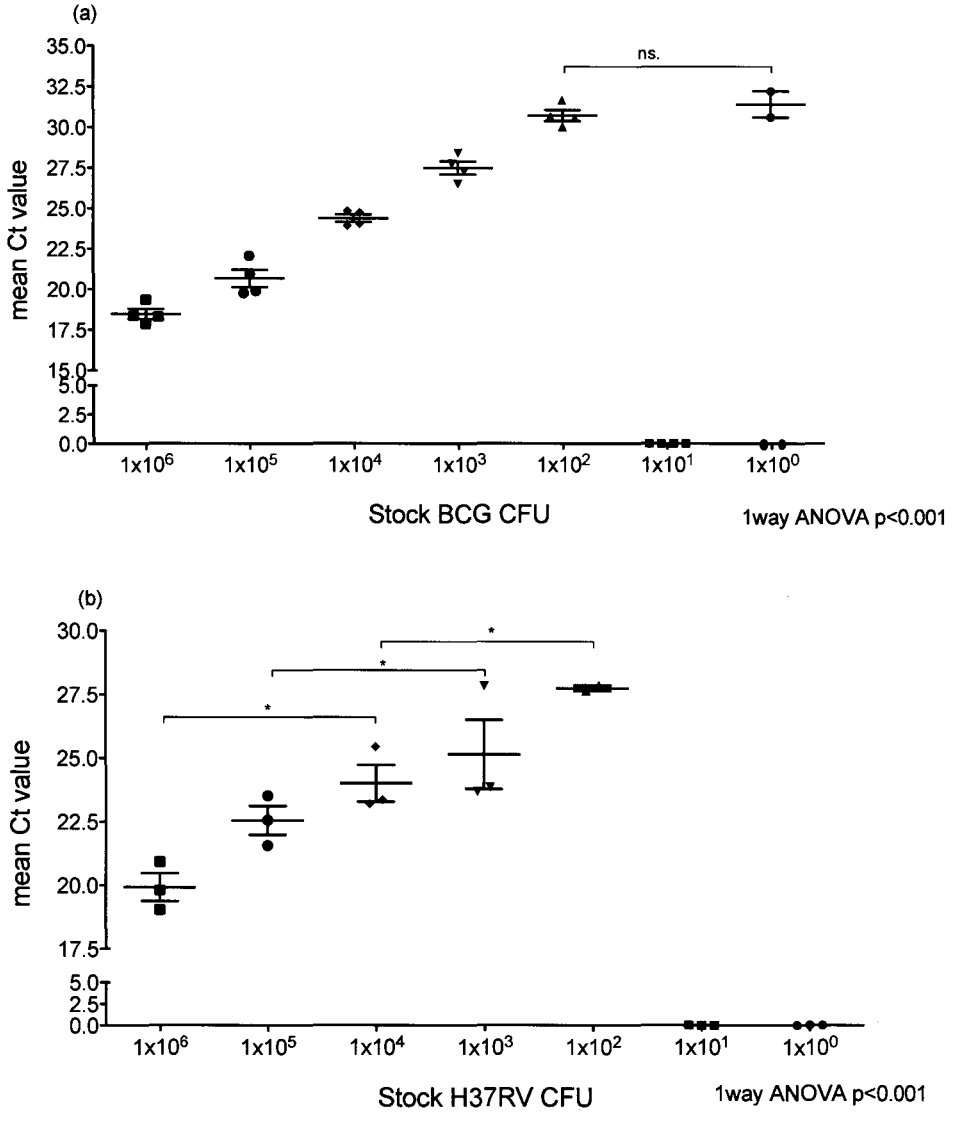


Figure 4-5 Automated PCR (using Xpert MTB/RIF) changes in cycle threshold (C_T)

Cycle threshold (C_T) is depicted on the y-axis with serial dilutions of BCG(a) and H37RV(b) on the x-axis. For BCG, C_T values were significantly different between serial dilutions (following correcting for multiple comparisons) except where indicated. For H37Rv, significant differences between C_T values were only present for 2 log changes in CFU when correcting for multiple comparison (*) C_T values of zero indicate failure of Xpert MTB/RIF to detect mycobacteria.

At the lowest dilution of 1 CFU, only 2 out of 3 cartridges were positive. The H37RV had greater variability in cycle threshold (C_T) values as compared to BCG (coefficient of variance 6%) and did not detect less than 100 CFU. For the BCG experiments mean C_T values were statistically significant across all ranges thus providing excellent discriminative ability. Although the mean C_T values across the range of loads tested were statistically different for the H37RV experiments (1way ANOVA $p < 0.001$), when correcting for multiple comparison (Tukey), statistical significance was only present at a 2 log change in CFU i.e. $1x10^4$ vs. $1x10^6$.

For the costing analysis the 3 replicates were pooled so that only 2 cartridges were used. The Xpert MTB/RIF PCR was the most expensive costing a total of ZAR 255.70/ USD 36.53 per experiment. The cost of the Gene Xpert IV machine and software is approximately ZAR 127 500/ USD 18,000 (2011 pricing).

4.5 Discussion

Accurate determination of mycobacterial burden is an essential requirement in basic and translational research and for effective implementation of TB control programmes. Although determination of mycobacterial burden by CFU is considered the gold standard, newer techniques offer a variety of advantages but also limitations. These have been examined in the series of experiments described here.

The key findings of these studies are that (i) Xpert MTB/RIF is a rapid and accurate measure of mycobacterial load above a threshold of 100 organisms/sample; (ii) TTP using MGIT 960, whilst also an accurate measure of burden with better discriminative ability and a detection threshold down to 1 CFU, has a substantially longer turn-around-time, and (iii) bioluminescence and uracil incorporation assays are limited by lack of discrimination below 1000 organisms.

Thus, no single assay has 'ideal' performance characteristics and the selection of an assay will be determined by the intended use or requirement of the experiment. These included study design, the anticipated load of organisms, the importance of

obtaining rapid results, desired reproducibility and financial and laboratory resources available. In resource-limited settings CFU using solid media is likely to remain the method of choice as it remains the cheapest alternative.

The Xpert MTB/RIF assay may in theory overcome several drawbacks of real time PCR. DNA from degraded organisms is removed in a pre-PCR wash step with only intact organisms retained in the cartridge mesh for the PCR step.[140] This allows for quantification of organism load with cycle threshold[198] not possible with traditional PCR. The data presented here supports this notion as Xpert-related results correlated with those obtained from solid culture CFU counts. However, Xpert MTB/RIF assay detects intact organisms and cannot distinguish viable from non-viable organisms and thus suffers from similar drawbacks to conventional NAATs and smear microscopy. Whilst C_T correlated with mycobacterial burden as has been shown in other studies,[198] to what extent it will correlate with risk of infection in contacts of index cases remains to be determined. It is possible that Xpert MTB/RIF C_T might correlate poorly with infectiousness, as discrimination between viable and non-viable organisms is likely to be poor. Given its rapid turn-around-time and detection of smear negative patients, clinical studies will be required to evaluate the utility of Xpert for monitoring treatment response, disease prognosis and for evaluating risk of disease transmission.

Rapid turn-around-time is an attractive advantage of the Xpert assay particularly in drug development-related EBA studies providing 'real time' serial quantification of bacterial burden. By contrast liquid culture results although accurate, may take up to 6 weeks. The clinical value of rapid turn around will need to be weighed up against the greater cost ≈ ZAR1750/USD250 (ZAR126/USD18 per cartridge) compared to ZAR686/USD98 (ZAR49/USD7 per MGIT bottle) per subject over a 14 day EBA study]. Consequently, in basic science laboratory study settings the rapid turn around of results is highly attractive but comes at a significantly higher cost compared to solid culture. Another key drawback in this setting is the detection threshold of 100 organisms, which may be inadequate for experimental models where low numbers of organism are used or need to be detected.

Liquid culture using the automated BACTEC 960 MGIT system is an attractive technology for both clinical and laboratory studies. Already incorporated into EBA studies[134, 135] time to positivity serves as a surrogate for bacterial load.[135, 141, 142] Its user-friendly format, automation, high discriminative ability and low detection threshold (less than 10 organisms) makes it well suited for laboratory studies. The key disadvantages compared to Xpert MTB/RIF and solid culture, are slow turn around time (days to weeks) and cost respectively.[143]

Bioluminescence and uracil incorporation assays both provide rapid turn around time and have been widely used in laboratory studies.[25, 136, 137] They have limited application to clinical and public health studies given the requirement for specific *M.tb*-reporter construct cultures and the high risk of bacterial contamination respectively. In addition both methods have limited discriminative ability below 1000 organisms/ sample. Uracil incorporation methods offer little advantage over bioluminescence yet require significant additional infrastructure to accommodate the storage and disposal of radioactive waste. Establishing this technique in a new laboratory is unlikely to be cost effective especially in resource-poor settings. As with all the other "indirect" techniques of quantitative load determination, a standard curve is required for calculation of CFUs.

The studies described here have several limitations. Firstly, they were performed in a research rather than a service laboratory. Secondly, assays were performed with only a single strain of virulent mycobacteria, and were prepared in a standard medium. The findings might therefore not apply to other clinical mycobacterial strains and samples in which contaminating biological materials might dilute or inhibit mycobacteria. The former will apply most to the findings of the MGIT method. Thirdly, in costing each method we assumed that the time spent by a technologist would be the same for each method. Nevertheless, economy of scale will apply in high throughput laboratories. However in this setting, the savings in labour costs (time spent plating and counting CFU on solid media) may be offset by the cost of the more expensive automated assays.

In summary, no single mycobacterial quantitative technique has ideal performance characteristics. Thus, the choice of assay will largely depend on the research context, study question, and the relative tradeoffs such as cost versus turn around time. Automated systems like MGIT are sensitive and discriminatory but have a long turn around time. Xpert MTB/RIF is a good quantitative tool with rapid turn-around-time, but its detection threshold was not as good as automated liquid culture. Although solid culture is the most labour-intensive, it remains the cheapest option for a highly discriminative quantification of mycobacterial load over a wide dynamic range.

5 Mycobacteria-induced monocyte-derived macrophage responses to nicotine and cigarette smoke extract

5.1 Introduction

The immune response to mycobacterial infection is a complex interaction between innate and adaptive immune responses.[199] The immune response is initiated after ingestion of aerosolised *M.tb* organisms by alveolar macrophages.[148, 151] In active disease there is an exuberant local pulmonary immune response characterized by inflammation in which recently recruited immature macrophages play a central role.[200] This reaction is strongly enhanced by *M.tb* antigen-specific Th1 responses[201, 202] with large amounts of locally secreted IFN-γ. *M.tb*-induced immune evasion mechanisms[203] with production of suppressive cytokines and effector molecules[204, 205] may counteract protective immune responses and abrogate bactericidal immune mechanisms.[206]

The important role of IFN-γ and TNF-α has been demonstrated by susceptibility to active TB in patients receiving therapeutic TNF-alpha blockade[207, 208] and with hereditary IFN-γ and IL-12 receptor abnormalities.[209, 210] TNF-α is likely to play an important role in killing intracellular *M.tb* through reactive nitrogen intermediates and IFN-γ is important in granuloma formation.[211] TNF-α is furthermore important in the control of latent TB infection since the anti-TNF antibody Infliximab (Remicade®) increases the risk of activating latent TB.[207]

Although the effects of tobacco smoke on the immune system in general have been well described,[12, 14] there are little data on the impact on mycobacteria-specific immune responses and the mechanisms involved.[12, 113, 114] Cigarette smoke turns off production of TNF-α by macrophages in the lungs[212, 213] and alveolar macrophages from smokers have reduced phagocytic ability.[214] Cigarette smoke prevents pathogen-specific expansion and activation of CD4 T cells[124] and reduces IFN-γ-producing adenoid-specific CD4 and CD8 T cell numbers.[119] Nicotine enhances the replication of intracellular organisms such as *Legionella* and can down regulate TNF-α through alpha7 (α7) receptors.[212] Furthermore nicotine may reduce macrophage

apoptosis,[64] an important component of mycobacterial control[117, 118] which promotes the killing of virulent mycobacteria.[215]

Although there is epidemiological evidence of a harmful smoking-TB association, several factors including; poverty, alcohol and socio-economic status are confounders and the lack of convincing mechanistic proof has led to uncertainty about the importance of cigarette smoke as opposed to other social or environmental factors in this apparent association. Proof of association will assist advocacy for smoking cessation programmes and elevate their status as part of global TB control efforts, and inform policy on appropriate smoking cessation strategies. These considerations provided the basis for the experiments described in this thesis to examine the effects of cigarette smoke constituents on host defence against mycobacterial infection. The focus on their effects on important defence mechanisms against mycobacteria in human monocyte-derived macrophages in Chapter 5 and *in vitro* infected bronchoalveolar lavage harvested alveolar macrophages in Chapter 6.

5.1.1 Hypothesis

Constituents of tobacco smoke (nicotine vs. whole CSE) attenuate protective mycobactericidal host immune responses (effector cytokines and bacterial stasis).

5.1.2 Specific aims

1. To determine whether tobacco smoke constituents modulate mycobacterial ingestion by macrophages.
2. To determine whether cytokine production in response to mycobacterial infection, is impaired by exposure to cigarette smoke extract and nicotine in particular.
3. To determine if nicotine impairs cytokine production, and if this is mediated via the nicotinic α-7 receptor.
4. To determine whether tobacco smoke-exposed human monocyte-derived macrophages have attenuated capacity for stasis and/or killing of mycobacteria.

5.2 Overview of methods

5.2.1 Preparation of monocyte-derived macrophages

Monocyte-derived macrophages (MDM) were prepared from whole blood obtained from healthy HIV negative volunteers. As detailed in Chapter 2 peripheral blood mononuclear cells were prepared by density centrifugation and plated in flat bottom tissue culture plates. Following a 6-day culture, non-adherent cells were washed off and fresh media added prior to infection or exposure. THP-1 cells were prepared as described in Chapter 2.

5.2.2 Preparation of cigarette smoke and nicotine extract

Fresh cigarette smoke extract was prepared for each experiment using the standardised protocol described in Chapter 2. CSE was prepared from Marlboro Red® cigarettes and used at a final concentration of 10%. Nicotine was diluted in PBS to achieve a final concentration of 1μg/ml that was employed for all nicotine experiments. CSE was added to the cells immediately prior to BCG infection.

5.2.3 Infection of macrophages

Macrophages were infected with BCG at a multiplicity of infection (MOI) of 2:1. Aliquots of frozen stock were thawed immediately prior to infection. Following 18-hour exposure, non-ingested bacteria were washed off prior to further experimental steps. A 4-hour infection period was used for certain preliminary experiments. Infection of macrophages by green fluorescent protein (gfp) labelled BCG was confirmed by light and fluorescent microscopy following washing of the cells to remove surface adherent cells. (Figure 5-1)

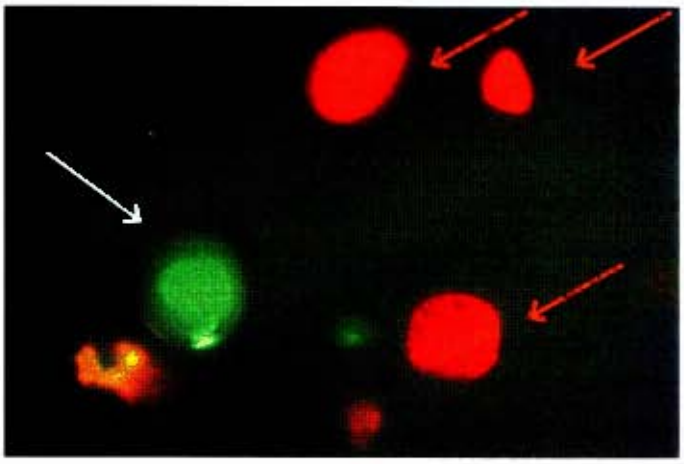


Figure 5-1 Monocyte-derived macrophages infected with green fluorescent protein labelled BCG organisms

The green arrow indicates a macrophage with an ingested BCG-gfp organism. Three red (7AAD positive) non viable macrophages (red arrows) are shown.

5.2.4 Determination of cytokine production following mycobacterial infection

Cytokine production was measured at 18hours post infection using commercially available ELISA kits as described in Chapter 2.

5.2.4.1 Defining the cellular subtypes producing interferon gamma

To define the cell type producing interferon gamma (IFN-γ), monocyte derived macrophages cells were surface and intracellularly stained to identify macrophages containing intracellular IFN-γ. Adherent macrophages were harvested following BCG infection and cigarette smoke exposure. Harvested cells were washed with FACS buffer prior to staining with surface markers for CD3 (Alexa700), CD4 (APC-H7), CD8 (PE), CD14 (PECy7), and CD33 (APC). Following surface staining, cells were permeabilised with FACS™ Permeabilising Solution (BD Biosciences) according to manufacturer's instructions and then stained for IFN-γ (PercP). Cells were washed and resuspended in FACS buffer prior to acquisition on the BD LSRII (BD Biosciences) and analysed using the BD FACSDiva™ software V6.1.3.

To further characterize the cell types producing IFN-γ following infection and exposure, harvested cells were incubated with CD14 magnetic Dynabeads (Invitrogen) to separate the CD14 positive cells (CD14+ fraction) from non-CD14 cells (non-CD14+ fraction). CD14 magnetic beads (500µl) were washed in a PBS-FBA

solution prior to adding 25µl of magnetic beads to the cell suspensions. Cells were then incubated on a rotator/mixer at 4°C with the magnetic beads. CD14-bead-bound cells were adhered using magnetic attraction and the remaining cells aspirated and stored as the "non–CD14 fraction". CD14-bead-bound cells were washed a further 3 times to remove possible non-bound cells. Aliquots of cells to be used for PCR analysis were spun down and preserved in RLT buffer and stored at -80 °C.

To assess the purity of the extraction method, an aliquot of cells was retained prior to RLT preservation for flow cytometric analysis. Cells were stained with CD33 (APC) to identify macrophages as the CD14 receptor was bound to the magnetic beads. CD3 (PE), CD4 (PerCP) were used to indentify any contaminating lymphocytes. Following surface staining, cells were resuspended in 4% paraformaldehyde (PFA) prior to acquisition using the BD LSRII (BD Biosciences) flow cytometer and analysed using the BD FACSDiva™ software V6.1.3.

5.2.4.2 Determining IFN-γ production by qRT-PCR

PCR was performed with assistance from the Centre for Proteomics and Genomics and the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town.

IFN-γ mRNA production was determined using two-step RT-qPCR. RNA was isolated from harvested CD14+ cells using the RNeasy® Mini Plus kit (Qiagen) according to the manufacturer's instructions and all precautions for handling and storage of RNA were considered. The purified RNA was quantitatively and qualitatively assessed using Agilent 2100 Bioanalyzer with the RNA 6000 Pico Labchip kit (Agilent Technologies, Palo Alto, CA, USA). The amount of total RNA extracted ranged from 30 – 300ng and RIN values obtained were > 7.0. Reverse transcription reactions were performed using the Sensiscript® Reverse Transcriptase (Qiagen) kit according to the manufacturer's instructions.

All PCR reactions were run on the Rotor-Gene 6000 (Qiagen). The PCR reactions were set up using the Rotor-Gene SYBR Green PCR kit (Qiagen) according to the

manufacturer's instructions. IFN-γ mRNA values were normalized using a previously validated reference gene, HuPO.[195] Primer sequences for IFN-γ and HuPO were used from a previously published study[196] and primers were added at a concentration of 800nM. cDNA copy numbers were quantified by absolute quantification. Standard curves were generated using serially dilutions of linearised plasmids containing the appropriate gene targets. All reactions were run in duplicate. The PCR cycling conditions were: initial step of 95°C for 10 min, followed by 40 cycles of 90°C for 10 s and 60°C for 30 s, in a 12.5-μL reaction volume). Reaction efficiencies were within acceptable limits and R^2 values were >0.99. IFN-γ copy numbers were expressed per 10^6 copies of HuPO.

5.2.5 Determination of monocyte-derived macrophage stasis/killing of ingested mycobacteria

Macrophages were infected as described in 5.2.3. 18 hours following infection non-ingested organisms were removed by washing adherent cells three times with warm RPMI. Fresh culture medium supplemented with 10% FBS was added to all experimental wells. CSE was added to triplicate wells post infection to a final concentration of 10%. No further CSE was added on subsequent days.

At each time point (days 1, 2, 3, 5) visual inspection of control and CSE exposed wells was performed to identify excess cell loss in either condition. At day five, triplicate wells of CSE exposed and unexposed infected macrophages were harvested with cold PBS and the number of viable cells in both conditions was determined using trypan blue exclusion dye. Additional adherent cells prepared on glass chamber slides and exposed to the same conditions, were stained with 7AAD for fluorescent microscopy.

On days 1, 2, 3 and 5 post infection, supernatants were removed and adherent cells lysed with 0.3% saponin (Sigma Aldrich) to release intracellular organisms for plating for CFU. Following lysis of the macrophages, serial dilutions were made and plated on 7H10 Middlebrook solid media agar to establish the colony count of BCG organisms at each time point. (Described in chapter 4) Three dilutions for each condition were plated in 6 replicates. CFU counting was performed using an inverted microscope and was performed between days 10 and 14 following plating.

5.3 RESULTS

5.3.1 The effect of cigarette smoke extract on macrophage survival

Adherent THP-1 cells and monocyte-derived macrophages were prepared as described previously. Macrophages were exposed to freshly prepared cigarette smoke extract in increasing concentrations for 24 hours and then harvested using cold PBS with 20mM EDTA.

5.3.1.1 THP-1 cells

Viability of harvested adherent THP-1 cells as assessed using trypan blue exclusion dye, showed a mean (SD) viability of 96.6%(3.0) in unexposed THP-1 cells, 94.6%(2.3) in 10% CSE exposed and 89.33%(7.6) in 25% CSE exposed THP-1 cells respectively; p=0.71(ANOVA). (Figure 5-2)

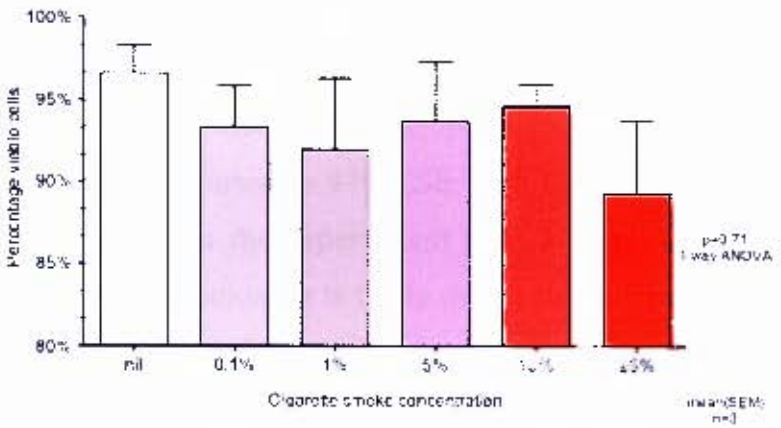


Figure 5-2 Adherent THP-1 cell viability after 24-hour exposure to increasing concentrations of cigarette smoke extract

5.3.1.2 Monocyte-derived macrophages

Viability of monocyte-derived macrophages assessed at 24 hours showed a similar pattern to THP-1 cells: no significant difference in cell viability between unexposed and 10% CSE exposed: 86.5%(5.3) and 75.7%(5.6) respectively (p=0.13). A marked reduction in cell viability at concentrations of 20% and greater was noted: 20% CSE exposure 54.8%(0.3); 50% CSE exposure: 62.5%(1.6); and 90% CSE exposure: 9.1%(2.3); p= <0.001 (ANOVA). (Figure 5-3)

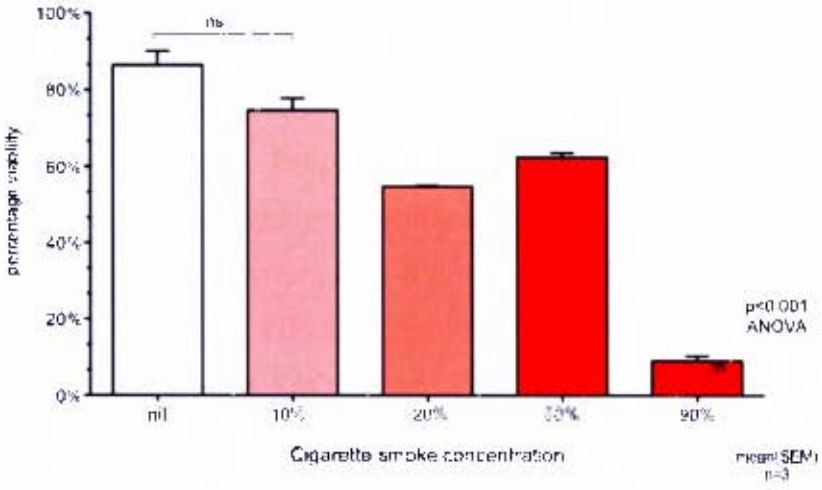


Figure 5-3 Adherent monocyte-derived macrophage viability after 24-hour exposure to increasing concentrations of cigarette smoke extract

The experiment was repeated to assess the effect of increasing concentrations of CSE on cell detachment. After 24 hours the viability of both spontaneously detached cells (in the culture supernatant) and harvested (adherent) cells were compared. The detached fraction (6%) and viability (100%) of detached macrophages was equivalent in unexposed macrophages compared to 10% CSE exposed macrophages. At 20% and 50% exposure, a significantly higher number of macrophages had detached during the 24 hours of exposure although 83% and 79% remained viable respectively. When exposed to 90% CSE, only 15% of the original number of plated cells were recovered in the supernatant with 29% remaining viable. The low cell recovery at 90% CSE exposure is likely due toxicity with resultant cell lysis. (Figure 5-4)

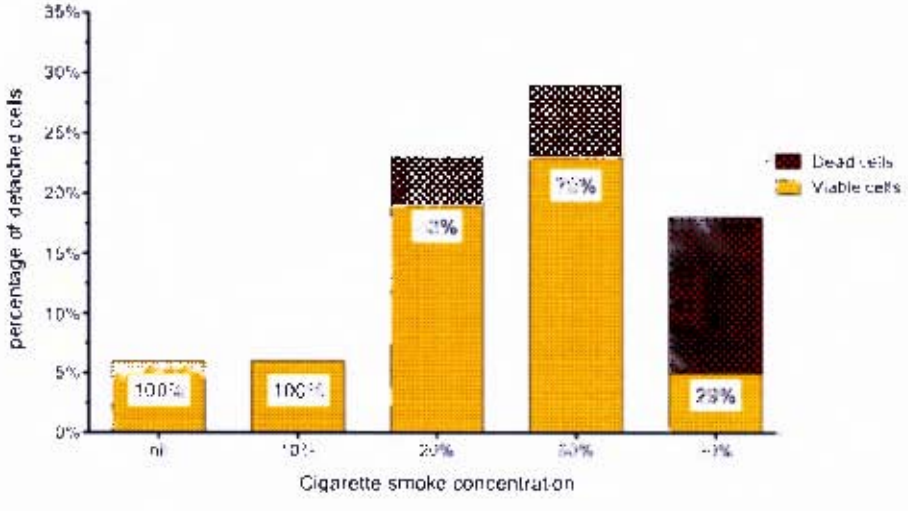


Figure 5-4 The effect of cigarette smoke on macrophage detachment and viability

Each bar represents the percentage of cells recovered in the supernatant after 24 hours. The yellow shaded fraction and number indicates the proportion of the recovered cells that were viable, the red fraction the proportion that were non viable (positive for trypan blue). (n=3)

5.3.2 The effect of tobacco smoke extract on mycobacterial uptake by monocyte-derived macrophages

Mycobacterial uptake was assessed using gfp-labelled BCG organisms and confirmed by fluorescent microscopy. Prepared macrophages were infected at an MOI of 2:1 in triplicate wells. Macrophages were either co-exposed to 10% CSE during infection or unexposed. Following infection, non-adherent cells were removed and adherent cells were harvested using cold PBS and 20mM EDTA. Cells were washed in FACs buffer prior to acquisition on the FACSCalibur (BD, BD Biosciences). Immediately before acquiring the cells on the flow cytometer 10µl of 7AAD was added to assess the viability of the cells.

Macrophage viability was not affected by BCG infection after 18 hours or by co-exposure to either exposure to nicotine or cigarette smoke. After 18 hours, uninfected unexposed macrophages had a mean (SD) viability of 63.3%(5.5) as determined by 7AAD staining. Viability was unchanged by exposure to 10% cigarette smoke: 67.7%(4.05) or 1µg/ml nicotine 64.1%(3.8); p=0.59. Following the 18 hour BCG infection, viability was 67.3%(5.8) which was not significantly different from the uninfected macrophages (exposed or unexposed) nor the infected/10% cigarette exposed macrophages: 68.1%(2.7) or infected/1µg/ml nicotine exposed macrophages: 60.9%(9.7); p= 0.63. (Figure 5-5)

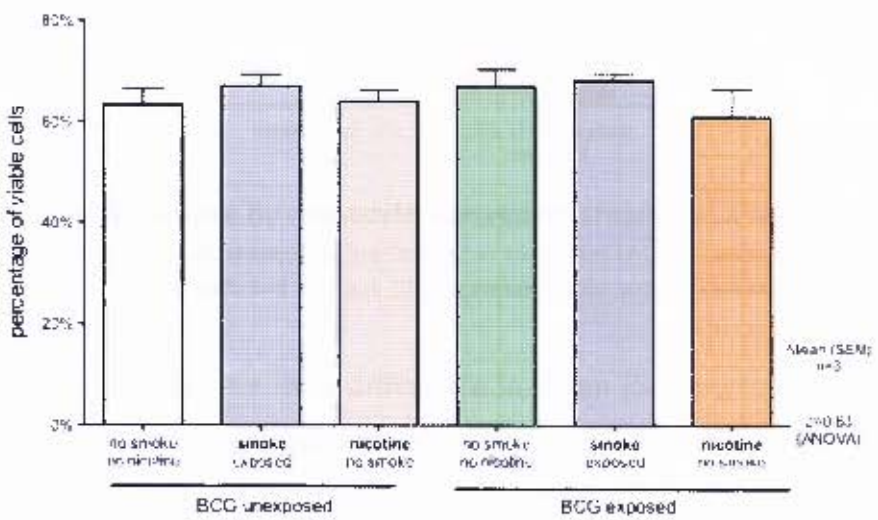


Figure 5-5 Viability of monocyte-derived macrophages after 18 hour BCG infection

Each bar represents the mean viability (7AAD negative) of MDMs unexposed or exposed to 10% cigarette smoke or 1µg/ml nicotine with or without infection by BCG for 18hours.

Mycobacterial uptake was assessed at 4 and 18 hours. Variability in uptake was seen between individuals at both times points. (Figure 5-6A, Figure 5-7A) At 4 hours, the percentage of macrophages that were BCG-gfp positive in the unexposed wells compared to the cigarette smoke extract exposed wells was: mean (SD) 4.5%(2.6) and 3.15%(1.6) respectively (p=0.028). (Figure 5-6B)

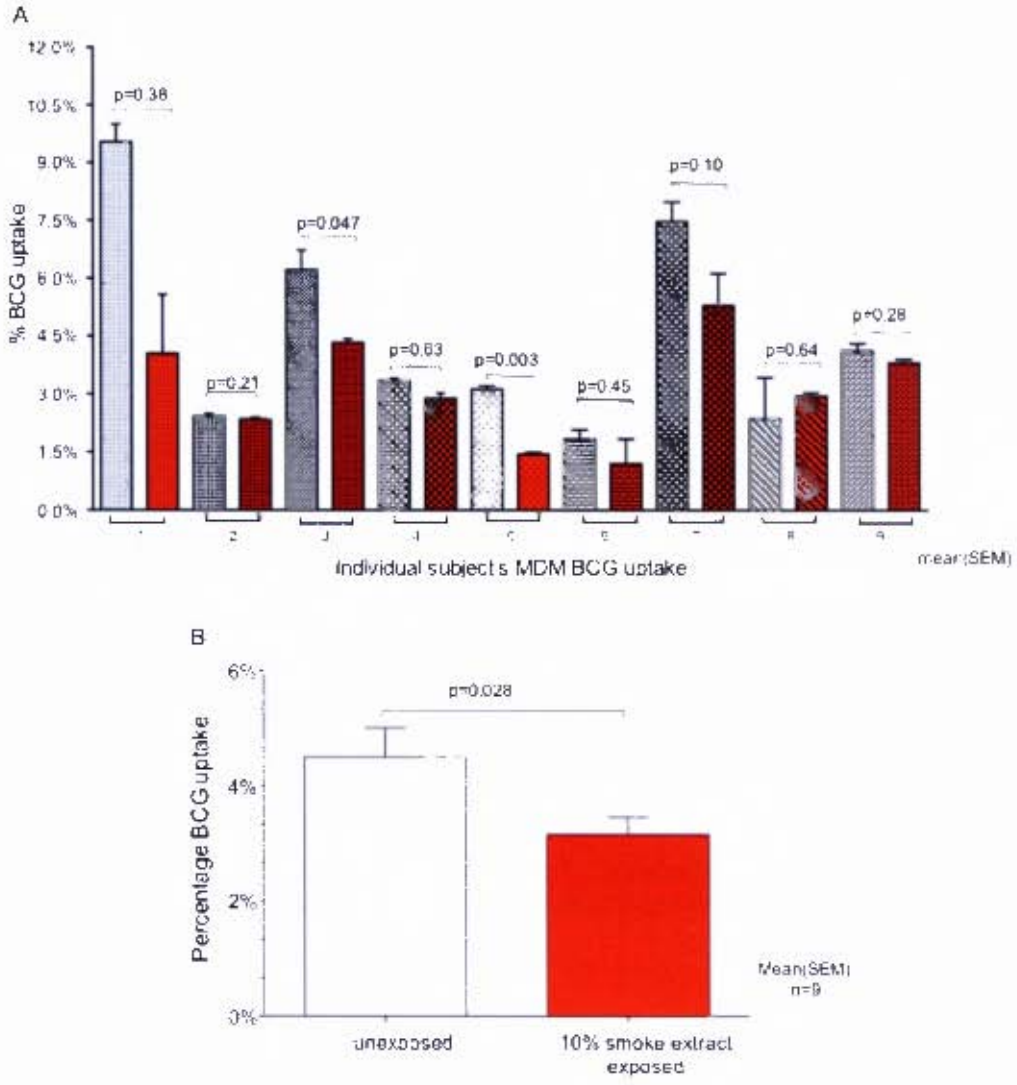


Figure 5-6 BCG- gfp uptake by monocyte-derived macrophages after *4 hours*

Uptake is depicted for individual subjects (performed in triplicate) (A) and summated results of all subjects (B) with exposure (red bars) with and without 10% cigarette smoke extract (white/shaded).

However, at 18 hours very little difference in mean (SD) mycobacterial uptake was seen: 23.2%(7.9) in unexposed control wells compared to 20.12%(7.0) in cigarette smoke exposed wells (p=0.16). (Figure 5-7B)

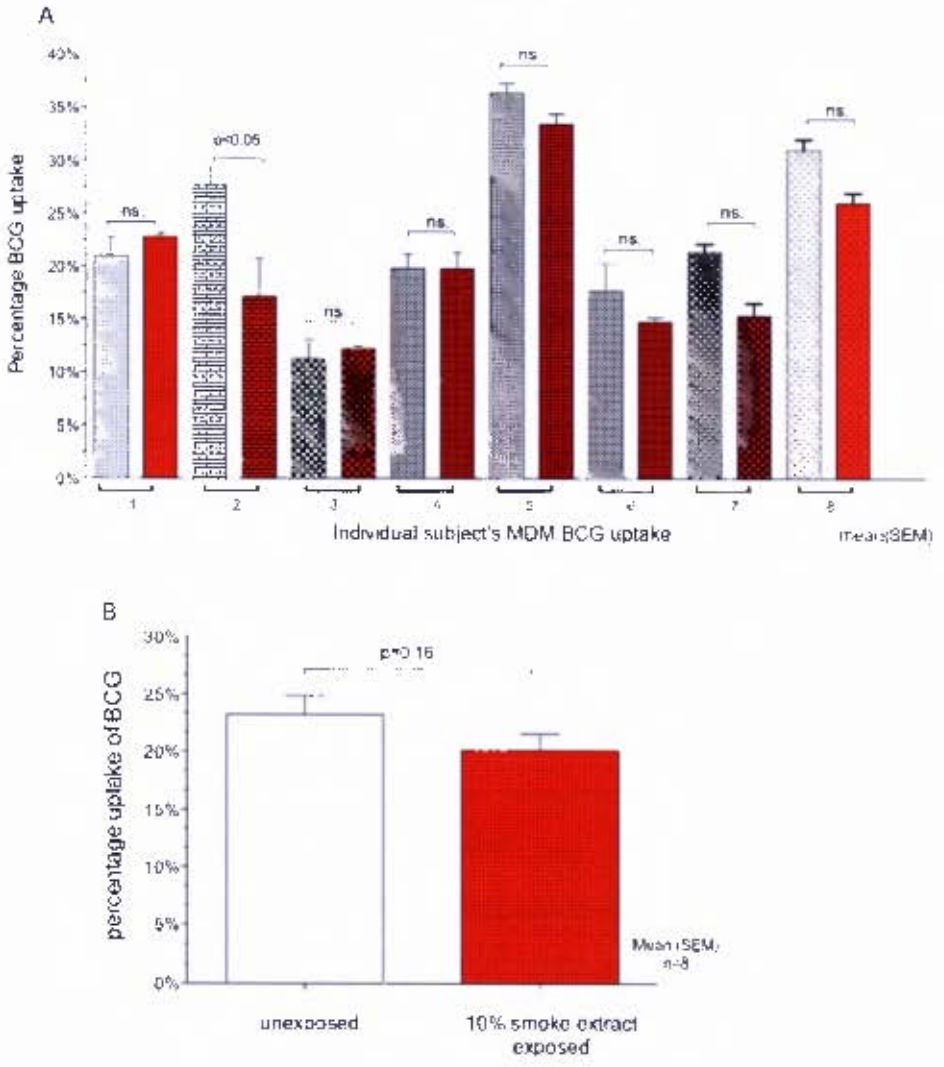


Figure 5-7 BCG-gfp uptake by monocyte-derived macrophages after 18 hours

Uptake is depicted for individual subjects performed in triplicate (A) and summated results of all subjects (B) with exposure (red bars) with and without 10% cigarette smoke extract (white/shaded).

When subjects were stratified by LTBI status (LTBI negative: TST and IGRA negative vs. LTBI positive: TST or IGRA positive), significantly higher BCG-gfp uptake was noted in LTBI positive subjects 28.1%(7.6) compared to LTBI negative 18.3%(4.84); p=0.001. Uptake was not affected by cigarette smoke co-exposure and did not differ by LTBI status. (Figure 5-8)

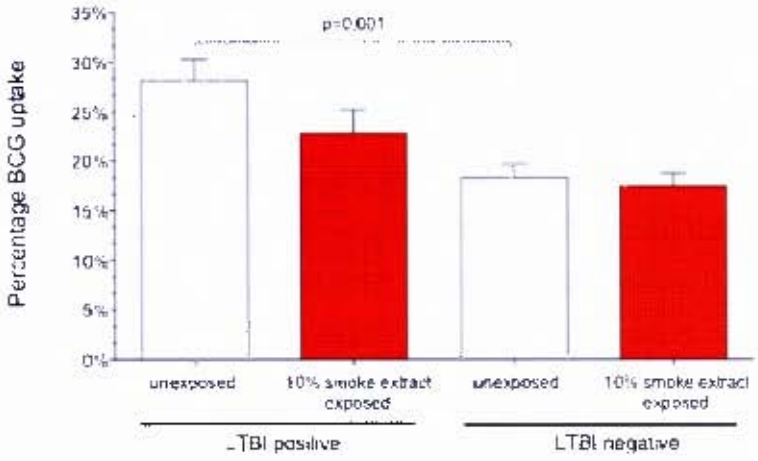


Figure 5-8 Monocyte-derived macrophages BCG-gfp uptake stratified by presumed LTBI status

Uptake is depicted for 10% cigarette smoke exposed (red bars) and unexposed (clear bars) and stratified by latent TB infection status. (n= 4 for LTBI positive and 3 for LTBI negative)

5.3.3 Cytokine production following mycobacterial infection

Given attenuation of secretion of all the cytokines studied, to exclude a possible effect of cigarette smoke extract on the reagents used in the commercial ELISA, IFN-γ standards provided in the QuantiFERON®TB Gold-In-Tube ELISA Kit were made up in both RPMI and RPMI containing 10% CSE. Using three separate standards, no difference in detected IFN-γ was demonstrated in the standards made up in 10% CSE/RPMI compared to RPMI alone. (Figure 5-9)

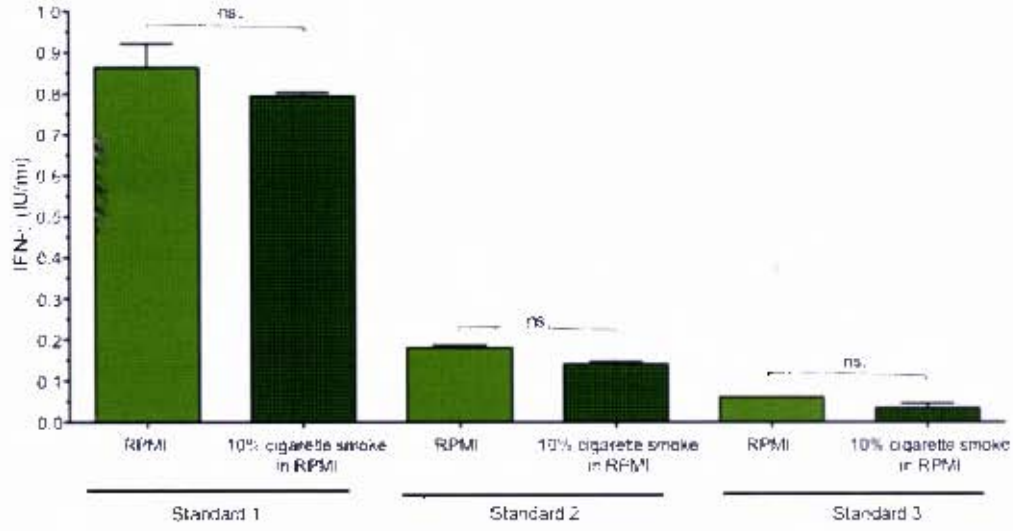


Figure 5-9 The effect of 10% CSE on IFN-γ concentrations detected by the commercial QFT-GIT ELISA kit

Each bar represents the mean (SEM) concentration of IFN-γ as determined by the ELISA kit using the three suggested standards either prepared in RPMI or PRMI containing 10% cigarette smoke extract. (n=3)

5.3.3.1 Interferon gamma (IFN-γ)

IFN-γ production by monocyte-derived macrophages was measured at both 4 and 18 hours post infection. At four hours, the mean (SD) IFN-γ production was minimal at 0.05(0.01) IU/ml and not affected by co-exposure to 10% CSE 0.04(0.18) IU/ml (p=0.43). At 18 hours, IFN-γ production by un-stimulated macrophages as well as macrophages exposed to 10% CSE, remained negligible: 0.06(0.03) IU/ml and 0.06(0.03) IU/ml respectively (p=1.0). Following BCG infection, a significant rise in mean (SD) IFN-γ was detected 0.28(0.18) IU/ml. However, macrophages co-exposed with 10% CSE during infection, demonstrated significantly less IFN-γ production: mean (SD) 0.10(0.04) IU/ml; p=0.001. (Figure 5-10) The production of IFN-γ and the effect of tobacco smoke extract was not significantly different when stratified by LTBI status (data contained in appendix A).

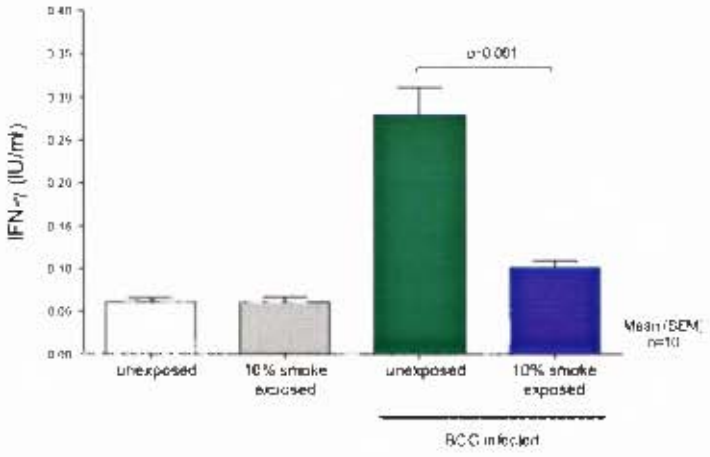


Figure 5-10 Interferon gamma production at 18 hours by monocyte derived macrophages with and without exposure to cigarette smoke extract

5.3.3.1.1 Confirming the production of IFN-γ by monocyte-derived macrophages

Human and murine macrophages have been shown to produce IFN-γ, although T cells predominantly secrete it in response to infection.[117, 216, 217] Therefore to confirm that the macrophages in this MDM model were producing IFN-γ, further experiments were conducted. Three additional subjects were recruited and following the same experimental methods as previously described, harvested cells were prepared for flow cytometric analysis.

Cells were stained with surface markers CD3, CD4, CD33, CD14 and for intracellular IFN-γ. The cells were identified by a general gate from a dot-plot depicting forward and side scatter characteristics. The gating strategy is depicted below: Briefly, CD3+ cells were gated to identify T- lymphocytes and NK T-cells and this gate was excluded from further analysis. Cells negative for the myeloid marker CD33 were also excluded. The macrophage population was subsequently identified by positive staining with anti CD14, a LPS receptor found on macrophages. (Figure 5-11)

Using this gating strategy 40% of the cells were CD33 positive and 70% of CD33+ cells were CD14 positive. In the CD33+CD14+ population, 60% were positive for IFN-γ. (Figure 5-11) In the excluded CD3+ve population (not shown) 0.2% were IFN-γ positive.

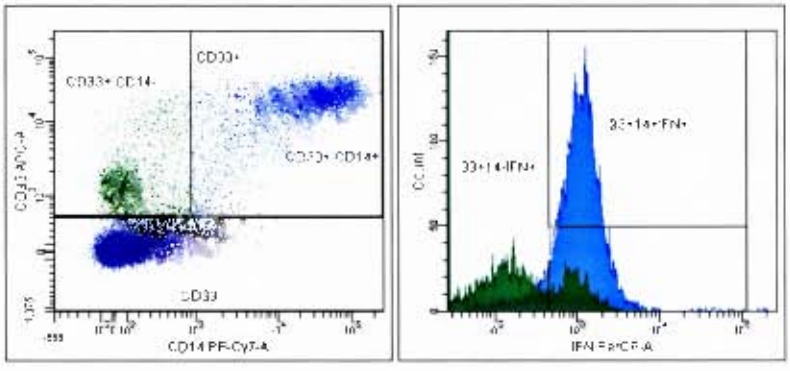


Figure 5-11 Flow cytometry gating strategy for the definition of cell types producing IFN-γ in response to BCG infection

Macrophages have IFN-γ receptors and consequently positive intracellular staining does not prove production of IFN-γ by the macrophages but may represent internalization of bound IFN-γ. To determine the source of IFN-γ production, further experiments were conducted in order to isolate the CD14 positive cells and to determine the production of IFN-γ in these cells using PCR.

CD14 positive cells were isolated as described in 5.2.4.1. To confirm the purity of the CD14 positive fraction prior to PCR, dual staining of anti CD3-/CD33+ was shown to be greater than 99% (duplicate experiments) in the CD14 fraction. (Table 5-1) A small percentage of CD33+ cells remained in the non-CD14 fraction, which was in keeping with the previous experiments demonstrating the presence of CD33 positive but CD14 negative cells.

Table 5-1 Purity of CD14 magnetic bead extracted cell fraction in unexposed and BCG infected conditions

		% CD3 -ve	% CD33 +ve	$CD3^-CD33^+$ purity
Unexposed cells	CD14 fraction	99.8 %	99.4 %	**99.2 %**
	Non CD14 fraction	67%	42.5%	29%
BCG-infected	CD14 fraction	100%	99.6%	**99.6 %**
	Non CD14 fraction	88.4%	22.8%	68.4%

5.3.3.1.2 PCR for IFN-γ mRNA

PCR for IFN-γ was performed on the CD14 +ve cell fraction as described above. Minimal IFN-γ mRNA copies, mean (SD), were present in the control (unexposed and uninfected) macrophages: 94.5(106.8) mRNA copies per 10^6 copies of HuPO. Following BCG infection, IFN-γ mRNA expression increased significantly to a mean (SD) 1016(16.97) copies per 10^6 copies of HuPO; p= 0.003. In BCG infected and CSE exposed macrophages the level of IFN-γ expression, mean(SD), was numerically lower: 320 (390) but did not reach statistical significance (p=0.06). These data, although only from two individuals, demonstrate the production of IFN-γ mRNA by the CD14 positive cells shown in the prior experiments to be IFN-γ positive by intracellular staining. The trend towards a reduced IFN-γ by co-exposure to cigarette smoke supports the ELISA findings.

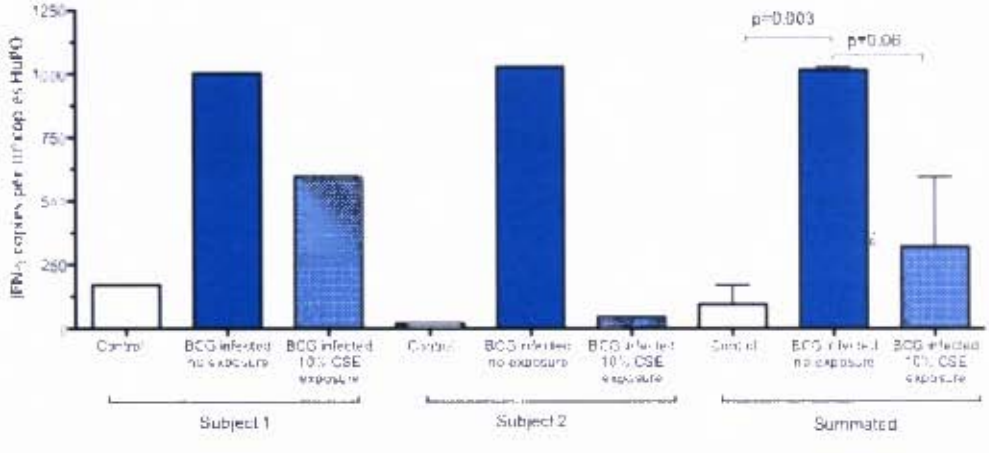


Figure 5-12 Interferon gamma (IFN-γ) mRNA transcription following BCG infection in CD14 positive cells

IFN-γ mRNA was transcribed from CD14 cells (macrophages) purified by magnetic bead separation. Following conversion of RNA to cDNA, real time PCR was performed and quantitative amounts of IFN γ mRNA calculated relative to the house keeping gene HPO. (For the summated graph mean and SEM are depicted)

5.3.3.2 Tumour necrosis factor alpha (TNF-α)

TNF-α production was measured at 18 hours post infection with BCG. As seen with IFN-γ negligible amounts of TNF were secreted by un-stimulated and CSE exposed macrophages: mean (SD) 0.73(1.8) pg/ml and 1.7(5.3) pg/ml respectively (p=0.3). Following infection, TNF-α production increased significantly to a mean (SD) of 137.5(111.7) pg/ml. TNF-α production was significantly reduced with co-exposure to 10% CSE: 21.63(45.97) pg/ml (p<0.001). (Figure 5-13) The production of TNF-α and the effect of tobacco smoke extract was not significantly different when stratified by LTBI status (data contained in appendix A).

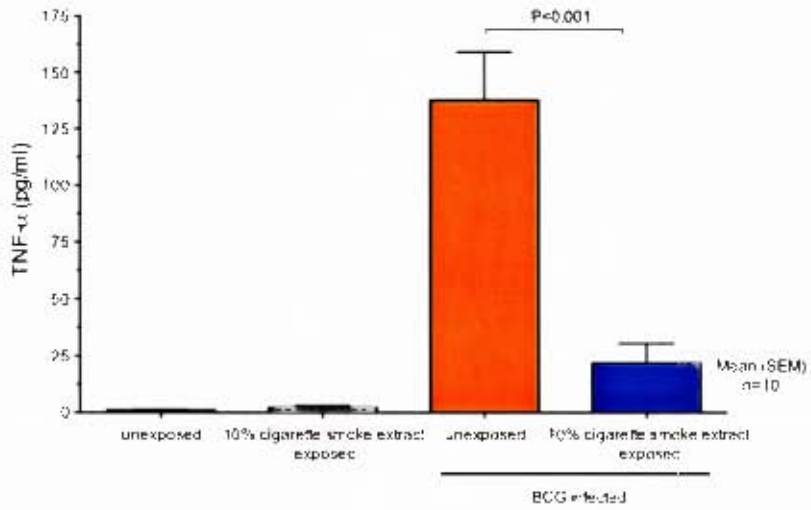


Figure 5-13 TNF-α production by monocyte-derived macrophages

Each bar represents either unexposed or CSE exposed MDM production of TNF-α in uninfected conditions (left) or following an 18-hour BCG infection (right).

5.3.3.2.1 The effect of nicotine (vs. whole CSE) on TNF alpha production

It has been suggested that nicotine, as depicted in Figure 5-14, acting through nicotine alpha 7 receptors, can turn off TNF-α production. As TNF-α is involved the maintenance of the latent state within macrophages, reactivation may occur.[125] To explore this hypothesis, further experiments were conducted using the commercially available α7-receptor blocker, α-bungarotoxin (Sigma Aldrich). Macrophages were pre-incubated with α-bungarotoxin-FITC (1.5μg/ml) for 15 minutes prior to the addition of nicotine and infection with BCG.

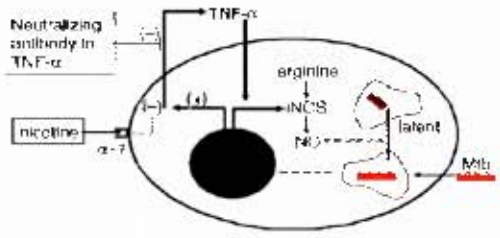


Figure 5-14 Hypothetical effect of nicotine acting through the α-7 receptor promoting *M.tb* infection

Figure reproduced from Davies et al. with permission. *M.tb* taken up into a phagosome increases the background synthesis and release of TNF-α. The latent state is maintained until the equilibrium is disturbed. This can be due neutralization of TNF-α by specific therapeutic TNF-α blockers or to down-regulation of TNF-α by nicotine binding to the α7 subunit of the nicotinic acetylcholine receptors.[145]

The level of TNF-α produced by macrophages infected with BCG and co-exposed to nicotine, although numerically lower, did not reach statistical significance: mean (SD) TNF-α in unexposed 181.6(186.1) pg/ml compared to nicotine co-exposed 78.7(52.48) pg/ml (p=0.08). Addition of the nicotine α-7 receptor blocker for the 18-hour infection period did not restore TNF-α production. (Figure 5-15) Because of the negative results using the α7-receptor blocker no further experiments were conducted with α-bungarotoxin.

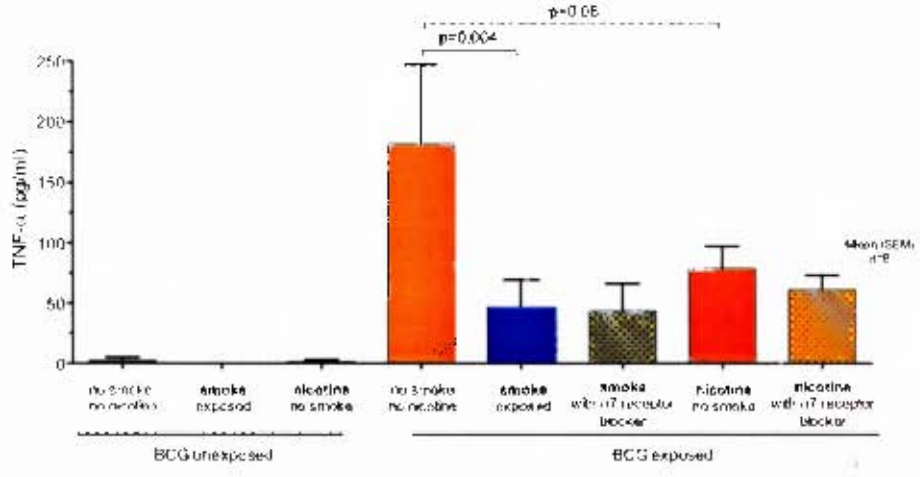


Figure 5-15 The effect of nicotine on TNF-α production by monocyte-derived macrophages

Each bar represent the production of TNF-α by monocyte-derived macrophages either unexposed or exposed to 10% CSF or 1μg/ml nicotine. BCG infected macrophages (18hours) were exposed during infection to the CSE/nicotine and a α7 nicotine receptor blocker (α-bungarotoxin).

5.3.3.3 Interleukin 10 (IL-10)

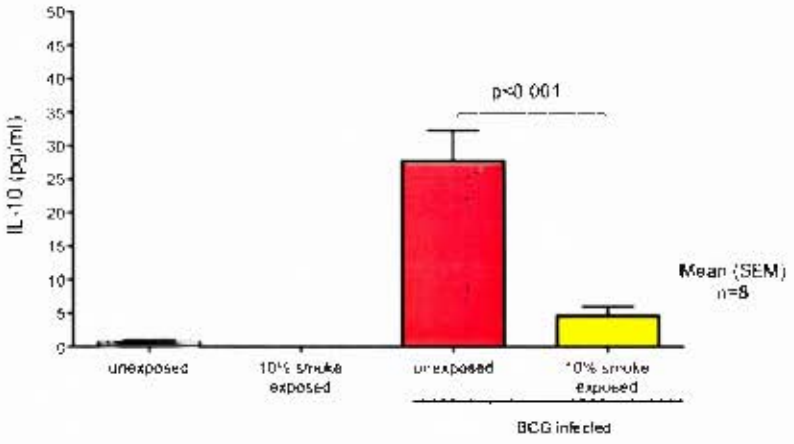


Figure 5-16 The effect of 10% CSE on monocyte-derived macrophage IL-10 production

Each bar represents either unexposed or CSE exposed MDM production of IL-10 in uninfected conditions (left) or following an 18-hour BCG infection (right).

5.3.3.3.1 The effect of nicotine alone on IL-10 production

A reduction in IL-10 production has been demonstrated in non-TB models following exposure to nicotine.[112] To test the effect of nicotine in a mycobacterial model, the BCG infection experiments were repeated with the addition of nicotine alone. (Figure 5-17) Following 18 hours of BCG exposure, the mean (SD) production of IL-10 by monocyte-derived macrophages was 40.1(60.3) pg/ml. When infection occurred with co-exposure to either CSE or nicotine, exposed macrophages produced significantly less IL-10 with a mean (SD) production of 6.9(12) pg/ml (p=0.02) and 11.3(16.4) pg/ml (p=0.03) respectively. The effect of nicotine was less pronounced than that of CSE (p=0.032).

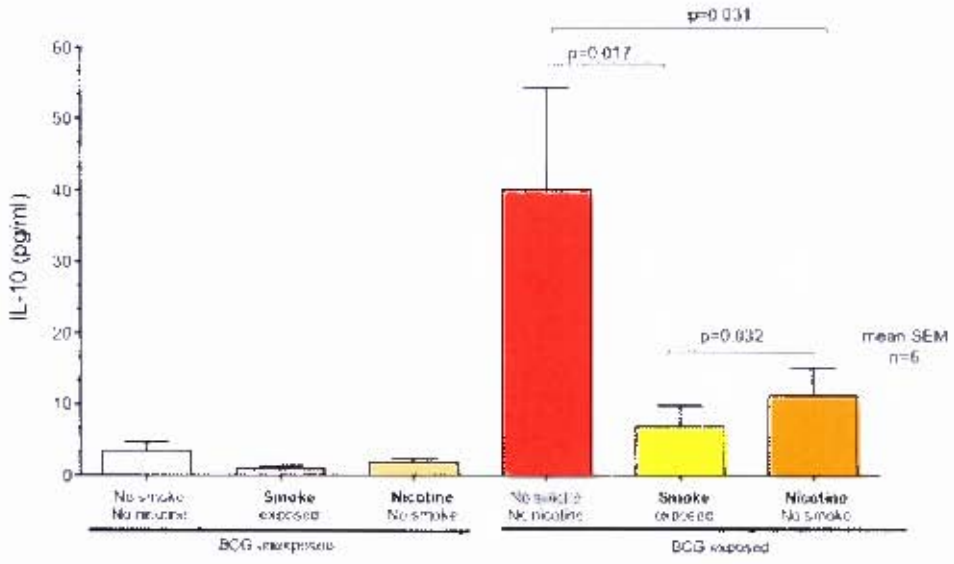


Figure 5-17 The effect of nicotine alone on monocyte-derived macrophage IL-10 production

Each bar represent the production of IL 10 by monocyte-derived macrophages either unexposed or exposed to 10% CSE or 1µg/ml nicotine. Uninfected macrophage responses are on the left with BCG responses following infection depicted on the right.

5.3.4 The effect of tobacco smoke exposure over 5 days on BCG stasis/killing by monocyte-derived macrophages.

Following overnight infection with BCG, MDMs were washed to remove non-ingested mycobacteria and fresh media added with or without 10% CSE. Macrophages were lysed on days 0, 1, 2, 3 and 5 to release the intracellular organisms, which were plated for CFUs.

To compare macrophage viability at day 5 post BCG infection between CSE and non-CSE exposed experiments, additional experiments were prepared on glass chamber slides and culture plates. Harvested cells were stained with trypan blue exclusion dye and no difference in cell viability was noted between exposed and unexposed macrophages. Adherent macrophages were washed with PBS and then stained with 7AAD viability dye in situ to avoid any trauma to cells associated with harvesting. No difference in cell numbers was seen between CSE exposed and unexposed macrophages nor in cell viability (number of non-7AAD staining cells). (Figure 5-18)

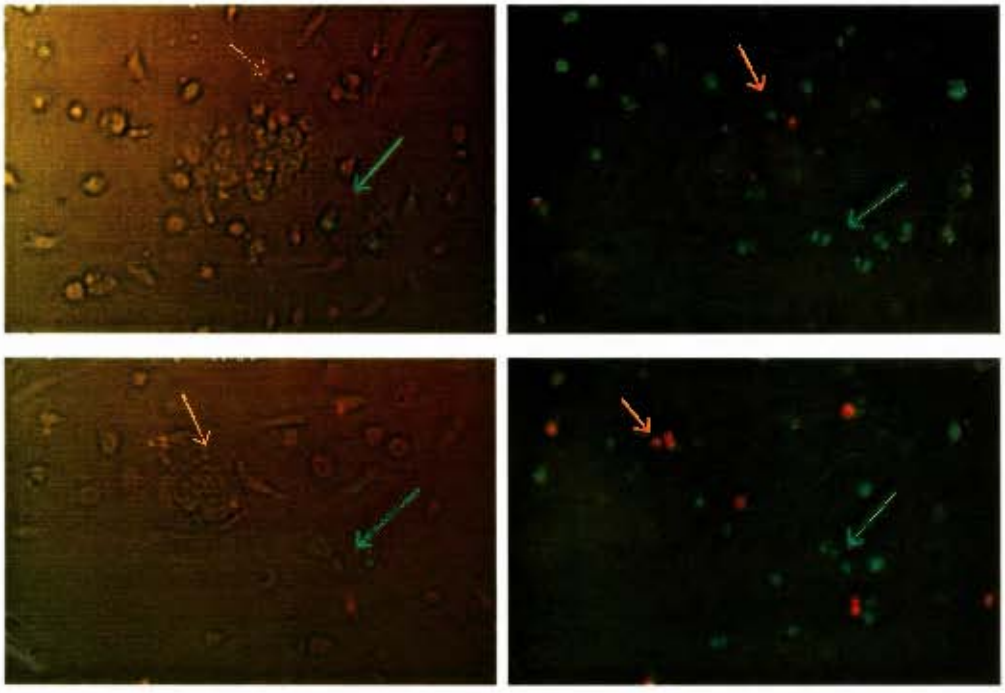


Figure 5-18 Monocyte derived macrophage viability after 5 day BCG infection

Adherent monocyte derived macrophages stained with 7AAD viability dye (red) following 5 days infection with BCG-gfp (green fluorescent protein). The upper two panels depict infected but unexposed macrophages and the lower two panels cigarette smoke exposed. Dead cells are indicated with red arrows, and viable cells with intracellular BCG with green arrows in the light microscopy/ fluorescence panels on the left and fluorescent microscopy panels on the right

The CFU trends for both CSE-exposed and unexposed macrophages are depicted in Figure 5-19. Day zero CFU counts, following infection without CSE exposure, were considered to be equal: mean (SD) $1.4x10^4$ ($5.7x10^3$) CFU. From day 1 to day 3 the CSE exposed wells had marginally higher CFU counts although not statistically significant. On day 5, CFU counts in the CSE exposed macrophage wells were significantly higher compared to the non-CSE exposed macrophages: mean (SD) CFU count of $5.6x10^4$ ($7.5x10^3$) CFU vs. $4.1x10^4$ ($4.6x10^3$) CFU respectively; p=0.008. No significant differences in CFU counts over the 5-day period were discernable when stratifying for LTBI status. (Appendix B)

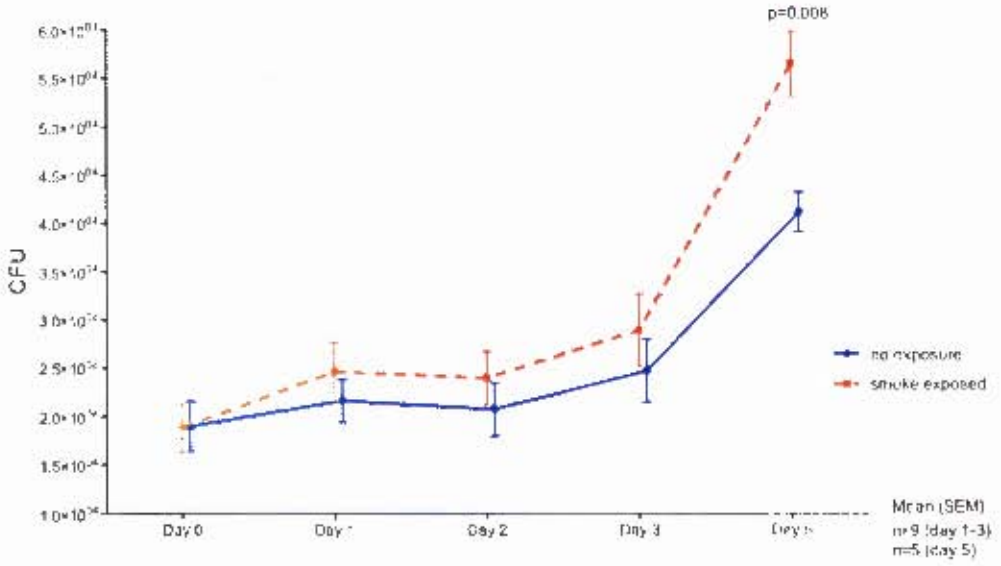


Figure 5-19 Serial BCG colony counts over 5 days in monocyte-derived macrophages

Monocyte derived macrophages infected with BCG were kept in culture for 5 days after the addition of 10% CSE to culture wells on day zero (post infection). The solid blue line represents unexposed macrophages and the stippled red line CSE exposed macrophages. Each day represents the time point post infection that macrophages were lysed and organism load calculated by colony counts on solid media.

5.4 Discussion

In this monocyte-derived macrophage model of mycobacterial (BCG) infection and acute cigarette smoke exposure (CSE), I have shown that viability of macrophages is not affected by 10% CSE exposure. Production of key cytokines in the immune response to TB infection, namely TNF-α, IFN-γ and IL-10, however, are significantly reduced by smoke exposure. Furthermore, production of IL-10 and TNF-α are also reduced by exposure to nicotine alone (TNF-α not reaching statistical significance.) Nicotine receptor blockade did not restore TNF-α production. Despite the observation that no effect of cigarette smokes extract on the uptake of organisms over 18 hours was observed, over a 5-day period, macrophages exposed to CSE had significantly higher intracellular bacillary loads.

In this acute cigarette smoke extract exposure model, although small differences in BCG uptake, as assessed by flow cytometry were noted at 4 hours after infection, no

differences were evident at 18 hours. Significant inter-subject variation in uptake was seen between individuals, which probably reflects both biological differences in macrophage phagocytic function, and experimental variability in actual MOI. When stratifying by LTBI status those with LTBI had a higher uptake of BCG although the effect of cigarette smoke remained constant.

The effect of tobacco smoke on macrophage phagocytic function has been examined using several models. In chronic cigarette exposure models in humans and mice using a variety of infecting organisms, phagocytosis of several bacteria and fungal species has been shown to be reduced by chronic cigarette smoke exposure ("smokers" vs. "non smokers"). These include bacteria such as *Listeria,*[99] *Haemophilus,*[100, 101] *Staphylococcus*[93, 102] and *Streptococci*[103] in addition to fungi such as *Cryptococcus*[104] and *Candida.*[105] A reduction in phagocytic index has also been demonstrated in acute cigarette smoke/extract (<24 hour) exposure models.[102, 103, 105, 106] Interestingly, Berenson demonstrated reduced phagocytosis of *Haemophilus* in alveolar but not blood-derived macrophages from smokers suggesting functional differences between cells of the small lineage from different sites.[100]

There are limited data for mycobacterial uptake. In a myelomonocytic cell line (THP-1) model, Shang and colleagues infected differentiated THP-1 cells with H37Rv during co – exposure to cigarette smoke extract or nicotine. The number of ingested mycobacteria was only measured at 1 hour post infection at which time no difference in uptake was noted.[116] There are no published data on the effect of cigarette smoke exposure on monocyte-derived phagocytic function for mycobacterium (*M.tb* or BCG). (The effect of cigarette smoke exposure on alveolar macrophages will be discussed in chapter 6.)

Production of IFN-γ and TNF-α and IL-10 was consistently reduced by co-exposure to tobacco smoke. A possible effect of the cigarette smoke extract on the ELISA reagents was excluded and no difference in cell viability in exposed and unexposed macrophages was shown to account for the reduced cytokine production. These data are novel as there are no published studies documenting the cytokine responses

following mycobacterial infection and co-exposure to tobacco smoke or nicotine using human cells.

The reduction in cytokine production shown in this study is consistent with several published non–TB models. Ouyang demonstrated reduced TNF-α, IL-β, and IFN-γ in a human peripheral blood mononuclear cell (PBMC) model stimulated with PHA following CSE exposure. Wewers and Hagiwara, in a bronchoalveolar lavage cell model, showed reduction in TNF-α (following LPS stimulation) and IFN-γ (following PMA stimulation) respectively.[94, 111]

The effect of cigarette smoke and nicotine on IL-10 production has not been consistent across non-TB-related experimental models with some demonstrating a reduction in IL-10 production[112] but not others.[108] The reduced IL-10 production by MDM exposure to either nicotine or cigarette smoke is in keeping with the Madretsma study, in which in a model of healthy non-smokers exposed to nicotine patches, IL-10 production by PBMCs was reduced.[112] In contrast, Matsunaga and colleagues, using a murine alveolar macrophage cell line infected with *Legionella,* showed a reduction in IL-6, IL-12, and TNF-alpha, but not IL-10 when infected macrophages were exposed to nicotine.[108]

Although a consistent effect of nicotine in reducing cytokine production was shown for IL-10, there was significant variability in TNF-α production with a non-significant reduction in TNF-α production. Blocking of the α7-receptor additionally did not show any reversal of the effect of nicotine. The hypothesis suggested by Davies et al. that the reduction in TNF-α production is potentially a nicotine effect, remains convincing although the current experiments neither support nor refute this hypothesis. A trend towards a reduction in TNF-α production was seen, but this did not reach statistical significance ($p=0.08$). Furthermore a restoration of TNF-α production should occur if the mechanism is purely mediated through the α7-receptor and the α-bungarotoxin receptor blockade over the 18-hour infection period was effective. Alternatively, the effect of nicotine on TNF-α production may be mediated through Toll like receptors (TLRs) as Means et al. have shown that *M.tb-*

induced TNF-α production is TLR dependent.[218] The expression of TLR2 on alveolar macrophages is reduced in patients with COPD[219] and furthermore, there is evidence from non-tuberculous models that macrophages from smokers have impaired signalling through TLR2/4.[220] Tobacco smoke potentially impairs three major pathways resulting in impaired cytokine production (NFκB, PI3K and MAPK), which could further facilitate escape of *M.tb* from the protective immune response.[221, 222]

It appears from these results that both pro inflammatory and anti-inflammatory cytokines responses are impaired by cigarette exposure. Stampfli and Andersen suggested that the effects of tobacco smoke may not merely suppress cytokine function but may skew the 'inflammatory mediator profile' and the nature of the skewing may be being a determinant of the disease.[14, 107] Macrophages do not act in isolation but are involved in complex interactions with several other immune cells such as T-cells, NK-cells, and neutrophils. Contamination by T-cells may account for some of the minor differences seen when correcting for LTBI status, as macrophages do not have immunological memory.

Although regulatory T cells (T-reg) have not been implicated in control of macrophage function, they appear to play a pivotal role in preventing immunopathology and limiting collateral damage to the host caused by exuberant immune responses to the pathogen. Yet, T-regs significantly inhibit protective Th1 responses especially with respect to IFN-γ production.[191, 223] T-regs are increased in active TB.[190, 191, 224] Additionally, there is evidence that regulatory T cell number may be affected by smoking in conditions like COPD.[225, 226] It remains unclear what effect smoke exposure may have on T-reg function in the context of TB or on the induction of alternatively activated macrophages.

Failure to inhibit intracellular mycobacterial replication is a key factor in disease progression and is related to both the virulence of the infecting organisms and to bacterial load.[118, 227] TNF-α is important for granuloma formation and impaired TNF-α production may result in progression of primary disease. In the 5-day infection model, day 5 intracellular CFU counts of adherent macrophages were significantly

higher in macrophages exposed to tobacco smoke. This suggests that cigarette smoke impairs pathways involved in the containment of intracellular infection. Possible mechanisms are impairment of phagosome-lysosome fusion, or modulation of TLR responses.

There are no published studies examining intracellular BCG growth in monocyte-derived macrophages exposed to cigarette smoke extract in humans. In the experiments over 5 days, excess cell death in the CSE exposed macrophages might release organisms resulting in a higher MOI. No difference in cell numbers or viability was detected between exposed and unexposed macrophages after the 5-day culture. Thus excess cell death is an unlikely explanation. This model is limited as the timing of macrophage exposure to smoke and M.tb in the human lung is highly variable – these experiments thus need to be expanded on in alveolar macrophages from smokers and non-smokers.

In a mouse model where mice were exposed to cigarette smoke for 14 weeks prior to aerosolised infection, smoke exposed mice had significantly higher bacterial burden in the lungs and spleen 30 days after infection.[116] Shang and colleagues suggested that in this mouse model, the higher CFU counts were the result of a smoke induced impairment of both innate and adaptive responses resulting in a greater number of foci of inflammatory cells but reduced influx of CD4 and CD8 effector and memory T cells.

In summary and to my knowledge, I have demonstrated for the first time a direct deleterious effect of cigarette smoke extract on cytokine production in response to mycobacterial infection using a human *in vitro* model. This study has also shown that cigarette smoke extract exposure facilitates intracellular mycobacterial (BCG) growth in macrophages. The signalling pathways affected by CSE exposure are yet to be defined, as are the intracellular mechanisms by which intracellular mycobacterial growth is facilitated.

6 The effect of tobacco smoke constituents on mycobacteria-induced alveolar macrophage responses

6.1 Introduction

The lung is the primary route of entry for most cases of tuberculosis. Inhaled infectious droplet nuclei if not trapped by the upper airway cilia and airway mucus, may settle in the lower respiratory tract. Alveolar macrophages in concert with dendritic cells[152, 153] and neutrophils[228] constitute the primary defence against TB; phagocytosing the organisms.[148 151] If organisms are not cleared, active disease develops, characterised by exuberant inflammation involving recently recruited immature macrophages[200] and *M.tb* antigen-specific Th1 responses[201, 202] with large amounts of locally secreted IFN-γ. These protective immune responses may be counteracted by *M.tb*-induced immune evasion[203] involving the production of suppressive cytokines and effector molecules.[204, 205,206] These interactions are summarized in Figure 6-1, reproduced from a recent article by Schwander and Dheda.[199]

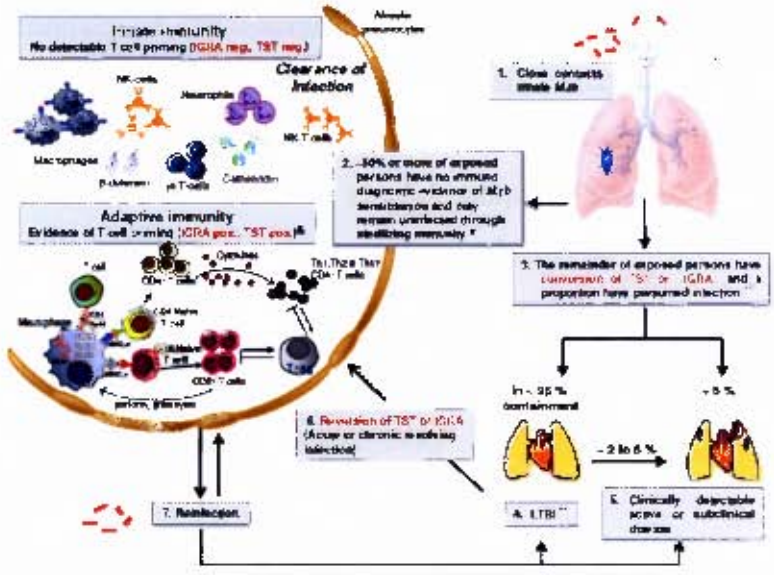


Figure 6-1 The spectrum and immunopathogenesis of *M. tuberculosis* infection

Mycobacteria are inhaled into the lung alveoli (1) and may be cleared (2) by sterilizing innate or adaptive immune mechanisms (which may determine the results of the TST and IGRA assays. In the remainder (3) infection may progress to LTBI (4), or to active TB (<5%) (5). Some individuals may revert to negative after a transient period of positivity (6). Those after clearing their infection (2) may be re-infected may clear their infection (2) progress to LTBI (4) or active disease (5), depending on the prevailing immunity. Figure and legend reproduced with permission. [199]

TNF-α, in conjunction with IFN-γ, plays an important role, not only in killing intracellular *M.tb* through reactive nitrogen intermediates, but also in granuloma

formation. Therapeutic TNF-alpha blockade[208, 229] and hereditary IFN-γ and IL-12 receptor abnormalities are associated with susceptibility to TB.[209, 210] TNF-α drives polymorph apoptosis and inhibits clearance of apoptotic cells[230] controlling mycobacterial growth by promoting the killing of virulent mycobacteria.[117, 118] Tobacco smoke is thought to increase macrophage apoptosis,[231] although not all studies support this view.[119-123]

As discussed in Chapter 5 there is little mechanistic data on the effect of tobacco smoke on TB-specific immune responses. In a mouse model examining the role of tobacco smoke in mycobacterial infections, Shang and colleagues confirmed a greater number of organisms in both the lungs and splenic tissue 14 to 30 days in mice exposed for 14 weeks to both a aerosol of *M.tuberculosis* Erdman and tobacco smoke, than those exposed to the organisms alone.[116] Furthermore they showed that in alveolar macrophages from 2 healthy non-smokers (human) infected with *M.tb* over a 4 day period, higher *M.tb* CFU were recovered from 10% CSE exposed macrophages compared to unexposed macrophages.[116] The effects of nicotine and cigarette smoke on cell viability, acute cytokine production and apoptosis were not studied. Since mouse models of mycobacterial infections do not adequately represent human disease, evidence from studies using human cells and pathology are required.[199] The experiments described below were designed to examine the short-term effects of cigarette smoke and nicotine on the responses of human alveolar macrophages infected *in vitro* with non-pathogenic mycobacteria.

6.1.1 Hypothesis and specific aims

Constituents of tobacco smoke may attenuate protective alveolar macrophage effector cytokine responses to mycobacteria.

6.1.2 Specific aims

1. To determine whether cigarette smoke constituents modulate mycobacterial uptake by alveolar macrophages.
2. To determine whether mycobacteria-infected human alveolar macrophages exposed to cigarette smoke extract, have reduced or augmented rates of apoptosis.

3. To determine if effector cytokine production by mycobacteria-infected alveolar macrophages is impaired by short-term *in vitro* cigarette smoke extract exposure.
4. To determine if impairment of alveolar macrophage TNF-α production is mediated through the nicotinic α-7 receptor pathway.

6.2 Overview of methods

6.2.1 Preparation of alveolar macrophages

Alveolar macrophages were obtained via bronchoalveolar lavage from healthy HIV negative, non-smoking controls, as described in Chapter 2. Briefly, harvested alveolar macrophages were allowed to adhere to flat bottom tissue culture plates for three hours in complete culture medium (10% AB serum in RPMI supplemented with 100U/ml Penicillin and 0.1%Fungin™). The cells were then washed to clear the culture of non-adherent cells prior to infection with mycobacteria or exposure to tobacco smoke.

6.2.2 Preparation of cigarette smoke and nicotine extract

Fresh cigarette smoke extract (CSE) was prepared for each experiment using the standardised protocol described in Chapter 2. CSE was prepared from Marlboro Red® cigarettes and used at a final concentration of 10%. Nicotine was diluted in PBS to achieve a final concentration of 1 μg/ml, which was used for all nicotine exposure experiments.

6.2.3 Infection of macrophages

Macrophages were infected with BCG at an MOI of 2.5:1 as described in Chapter 2. Aliquots of frozen stock where thawed prior to infection. Following an 18-hour exposure, non-ingested bacteria were washed off prior to further experimental steps.

6.2.4 Macrophage viability and extent of apoptosis

Flow cytometric analysis was performed to determine macrophage viability (necrotic cell death (7AAD) or evidence of apoptosis (Annexin-V)) following infection and

exposure to cigarette smoke extract and nicotine. Adherent macrophages were dislodged from the culture plate with cold PBS containing 20% EDTA (Sigma-Aldrich). Macrophages were washed with FACS buffer and Annexin-binding buffer (10 mM Hepes, 0.14 M NaCl; 2.5 mM $CaCl_2$; pH 7.4) (eBiosciences). Annexin-V APC (eBiosciences) was added to the cell pellet and incubated for 15 min in the dark at room temperature. After a final wash and immediately preceding cell acquisition, 10 µl of 7AAD (eBiosciences) was added to the cells to enumerate cell death. Macrophages were acquired using the FACSCalibur (BD biosciences) flow cytometer and analysed using *Cell Quest and Flow Jo* software v9.2. With the application of three different fluorochromes, the uptake of the bacteria (BCG-gfp) as well as the apoptotic (Annexin V) and necrotic indices (7AAD) of the cells could be assessed.

6.2.5 Cytokine production

Cytokine production was measured in the culture supernatants after 18-hour infection with or without CSE or nicotine exposure as described in Chapter 2. 10% CSE or 1µg/ml nicotine were used for exposure experiments. To examine the potential role of the nicotinic α7-receptor in TNF-α responses to infection and nicotine exposure, alpha bungarotoxin was used as described in Chapter 5.

6.3 Results

6.3.1 The effect of cigarette smoke extract and nicotine on BCG uptake by alveolar macrophages

BCG-gfp uptake was assessed at 18 hours following infection and exposure to either cigarette smoke extract or nicotine alone. Infection of macrophages by gfp labelled BCG was confirmed by light and fluorescent microscopy: (Figure 6-2)

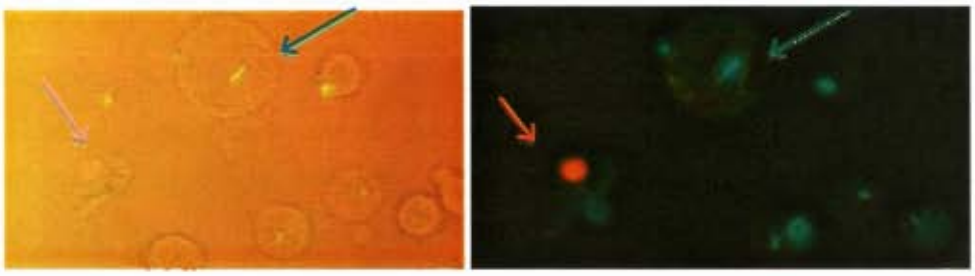


Figure 6-2 Alveolar macrophages containing ingested green fluorescent protein (gfp) labelled BCG organisms

The green arrows depict BCG-gfp within macrophages and the red arrows a single non-viable 7AAD positive macrophage in the two panels: the left panel shows a light microscopic view of non-adherent alveolar macrophages containing fluorescing BCG organisms. The right panel demonstrates the same macrophages by fluorescence microscopy, again demonstrating florescent organisms, but also some non-specific macrophage auto-fluorescence.

The mean (SD) percentage of alveolar macrophages (unexposed to smoke or nicotine) positive for gfp-labelled BCG was: 35.8%(20.3) and was not different to that in cigarette smoke and nicotine exposed alveolar macrophages 37.0%(19.7) and 32.0%(18.5) respectively; p=0.28. (Figure 6-3) No difference in uptake was seen when stratifying by LTBI status (Appendix C). Thus cigarette smoke and nicotine did not affect the uptake of BCG into alveolar macrophages *in vitro*.

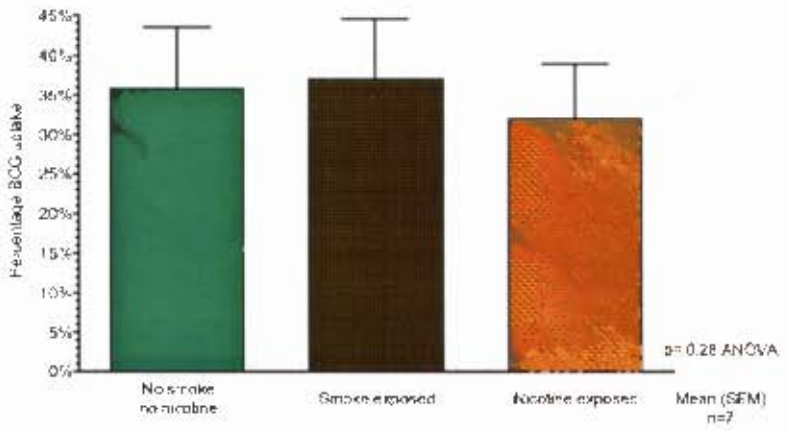


Figure 6-3 Uptake of BCG organisms by alveolar macrophages

Macrophages were either unexposed or exposed to tobacco smoke or nicotine during the 18-hour period of mycobacterial infection. From left to right the three bars represent unexposed macrophages, 10% cigarette smoke exposed and 1ug/ml nicotine exposed macrophages. Uptake was measured by flow cytometry.

6.3.2 The effect of tobacco smoke extract, nicotine and BCG-gfp uptake on alveolar macrophage death

In unexposed and uninfected alveolar macrophages, viability was greater than 70%; mean (SD) 7AAD positive cells: 28.7%(14.9). This was not significantly different in

uninfected alveolar macrophages exposed to either cigarette smoke extract or nicotine: 28.0%(17.1) and 29.0%(15.0) 7AAD positive cells respectively (p=0.14). Although numerically lower, the viability of macrophages was not significantly affected by BCG infection alone or when co-exposure to cigarette smoke extract or nicotine occurred during infection: Viability of BCG infected/unexposed alveolar macrophages was nearly 80%: mean (SD) 7AAD positive cells: 20.8%(9.4). This was comparable to the BCG infected/smoke exposed macrophages: 18.1% (5.1) and BCG infected/nicotine exposed macrophages 24.6%(17.0); p =0.11. (Figure 6-4)

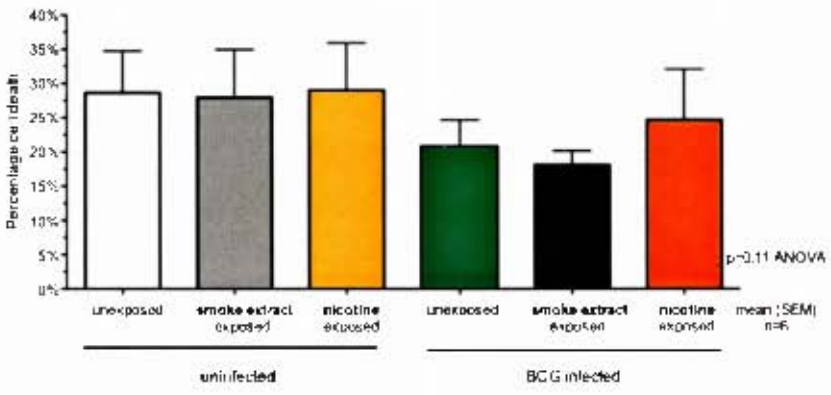


Figure 6-4 Viability of alveolar macrophages following BCG infection

Alveolar macrophage viability was determined by 7AAD staining following 18 hour infection and exposure to either tobacco smoke extract or nicotine. Percentage cell death depicted on the y-axis represents the percentage staining positive with 7AAD.

Representative dot plots of macrophages viability staining with 7AAD for BCG infected/ unexposed alveolar macrophages and BCG infected 10% cigarette smoke or 1 μg/ml nicotine exposed alveolar macrophages are shown in Figure 6-5. Viability (7AAD negative) of BCG-gfp positive macrophages was approximately 51% for exposed and unexposed alveolar macrophages. Neither cigarette smoke nor nicotine affected macrophage viability pre or post -BCG infection.

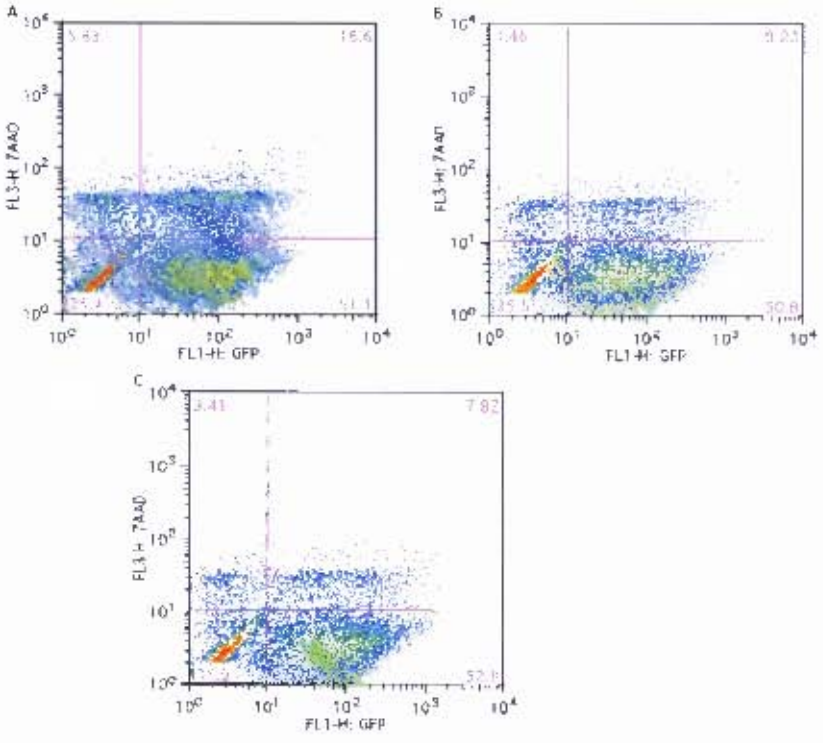


Figure 6-5 Flow cytometry dot-plots of alveolar macrophages BCG-gfp uptake and viability

The representative flow cytometry dot-plots depict BCG-gfp uptake on the x-axis and 7AAD viability staining on the y-axis. **Panel A:** BCG gfp infection without exposure. **Panel B:** Infection with co exposure to 10% CSE. **Panel C:** Infection with co exposure to 1 ug/ml nicotine. The bottom right quadrant of all panels represents alive and BCG-gfp infected macrophages.

6.3.3 Alveolar macrophage apoptosis following BCG infection and exposure to cigarette smoke extract or nicotine.

As cell death was not affected by ether cigarette smoke extract (CSE) or nicotine, the effect on apoptosis was investigated. Apoptosis was determined by flow cytometry positive Annexin-V (eBiosciences) staining using a FACSCalibur (BD biosciences) flow cytometer FACS calibur after harvesting of the adherent cells post infection. There was no difference in macrophage apoptosis (Annexin V positive cells) between uninfected control, and CSE or nicotine exposed alveolar macrophages: mean%(SD) control 7.2%(4.2), CSE exposed 8.8%(5.6) and nicotine exposed 8.9%(6.2); p=0.4. (Figure 6-6) Following infection the extent of apoptosis in infected macrophages was no different from uninfected macrophages. Although a similar pattern of slight increases in apoptosis in CSE and nicotine exposed macrophages was seen following infection, this was not statistically significant: 6.4%(4.5), 7.3%(4.3) and 7.9%(7.2) respectively (p=0.54).

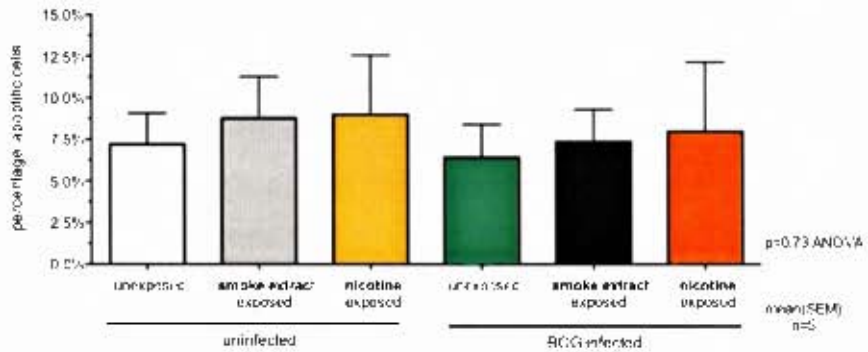


Figure 6-6 Apoptosis of alveolar macrophages following BCG infection

Apoptosis was determined by positive Annexin-V staining. Each separate bar represents an exposure condition. The left three bars being uninfected and the right three bars BCG infected macrophages respectively.

Representative flow cytometry dot plots depicting BCG-gfp uptake and Annexin-V staining in unexposed and CSE or nicotine exposed alveolar macrophages with and without BGC infection are shown in Figure 6-7. Neither necrosis nor programmed cell death of alveolar macrophages appeared to be affected by exposure to cigarette smoke extract or nicotine in this model.

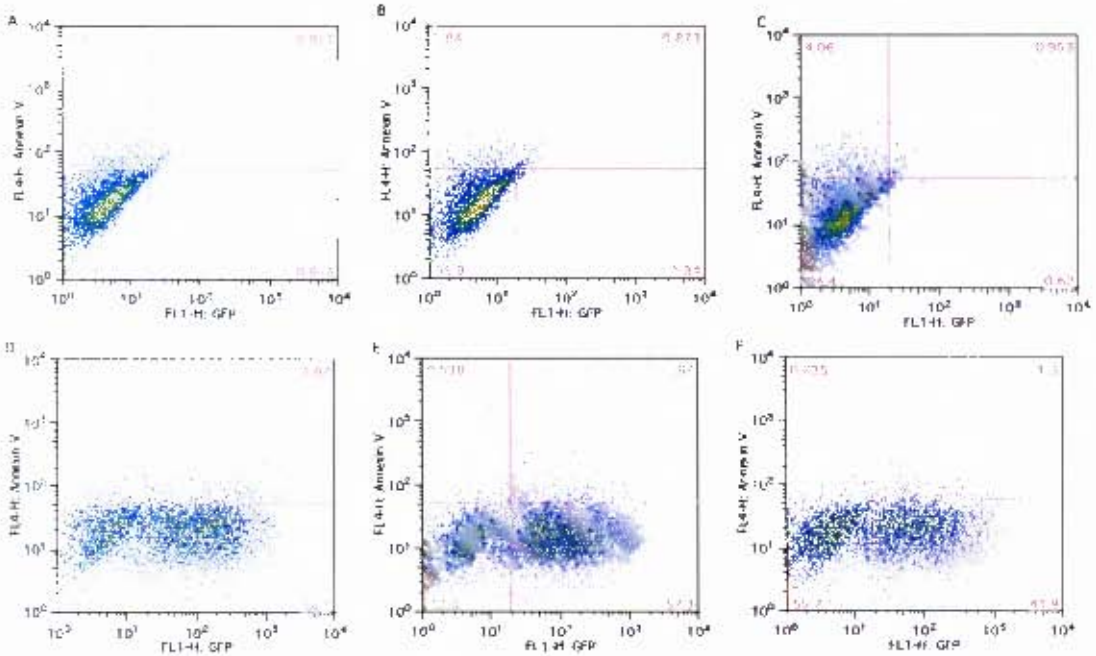


Figure 6-7 Flow cytometry dot-plots of alveolar macrophages with Annexin-V staining

The 6 panels depict representative flow cytometry dot-plots from one experiment with BCG-gfp uptake on the x-axis and Annexin-V staining on the y-axis. The top three panels are BCG uninfected conditions. **Panel A:** Uninfected/unexposed cells; **Panel B**: Uninfected/10% cigarette smoke extract exposed; **Panel C**: Uninfected/1µg/ml nicotine exposed. The lower three panels are BCG infected conditions: **Panel D:** BCG-gfp infected/unexposed; **Panel E**: BCG-gfp infected/10% cigarette smoke extract exposed; **Panel F**: BCG-gfp infected/1µg/ml nicotine exposed. The upper right-hand quadrant of each panel represents both infected and apoptotic macrophages.

6.3.4 Cytokine production following mycobacterial infection

6.3.4.1 Interferon gamma (IFN-γ)

The basal (un-stimulated) production, mean (SD) of IFN-γ after 18-hours was 0.05(0.001) IU/ml. Cigarette smoke exposure did not alter IFN-γ production mean (SD): 0.05(0.02) IU/ml (p=0.43). In contrast, post BCG infection (18 hours), a small but significant increase in IFN-γ occurred with a mean (SD) production of IFN-γ by CSE unexposed alveolar macrophage of 0.09(0.03) IU/ml (p=0.01). Another significant finding was of a small but statistically significant reduction in IFN-γ production by alveolar macrophages exposed to 10% CSE during infection: 0.07(0.01) IU/ml compared to unexposed macrophages: 0.09(0.03) IU/ml; (p=0.049). (Figure 6-8)

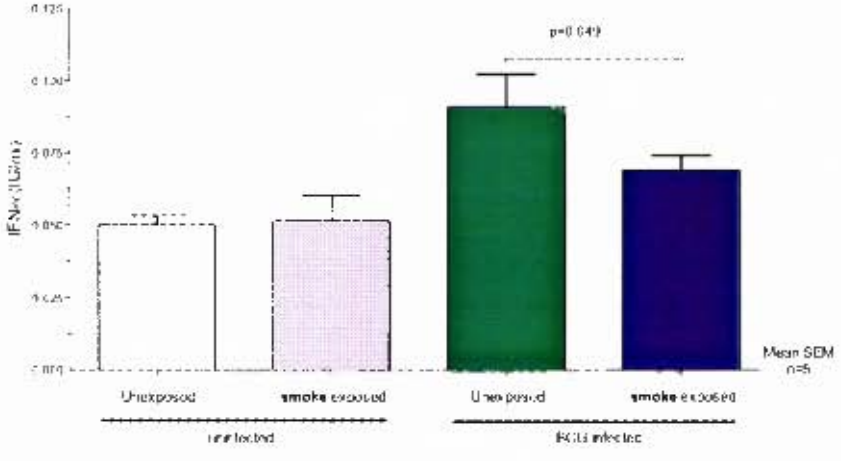


Figure 6-8 Interferon gamma production by alveolar macrophages

IFN-γ production is reported in IU/ml on the Y-axis. The left two bars represent uninfected conditions (18 hour uninfected culture) and the two right bars 18 hour BCG infection with or without 10% cigarette smoke exposure.

6.3.4.2 Tumour necrosis factor alpha (TNF-α)

The basal (un-stimulated) production of TNF-α by alveolar macrophages was high: mean (SD) 929.1(379.8) pg/ml in unexposed macrophages compared to 720.5(425.6) pg/ml and 840.1(395.6) pg/ml in smoke exposed and nicotine-exposed macrophages respectively (p=0.3). (Figure 6-9) Following 18-hour infection, the production of TNF-α was significantly lower than that from basal conditions: BCG infected macrophages 429.4(436.7) pg/ml vs. uninfected macrophages 929.1(379.8) pg/ml (p<0.001). No effect of either cigarette smoke extract or nicotine was demonstrable

on TNF-α production when BCG-infected and unexposed macrophages 411.0(457.2) pg/ml and 480.3(511.5) pg/ml vs. 429.4(436.7) pg/ml respectively were compared (p=0.2). (Figure 6-9)

To determine whether nicotine alone may impair cytokine production through the nicotine α7-receptor, the nicotine α7-receptor blocker α-bungarotoxin was added to cultures prior to nicotine exposure. This did not significantly alter TNF-α production in either cigarette smoke exposed 411.0(457.2) pg/ml to 413.2(473.8) pg/ml nor nicotine exposed macrophages 480.3(511.5) pg/ml to 477.2(535.6) pg/ml; (p = 0.21). (Figure 6-9)

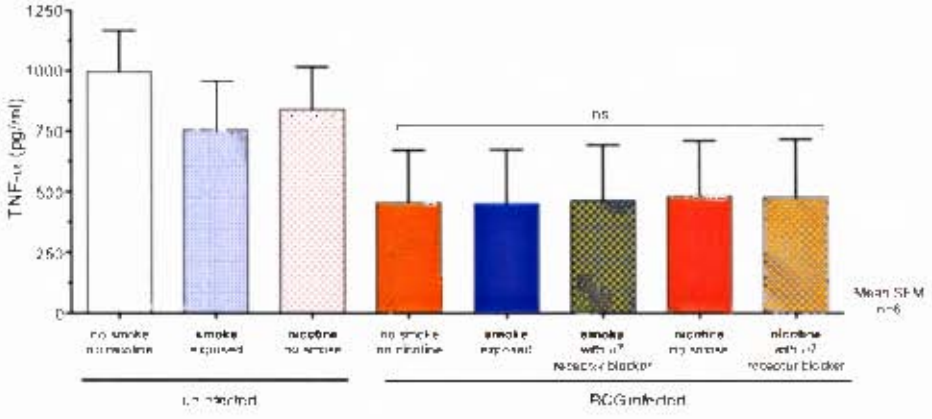


Figure 6-9 TNF-alpha production by alveolar macrophages

Replicate experiments of alveolar macrophages are depicted by each bar with the left three bars uninfected basal conditions unexposed or exposed to 10% CSE to 1μg/ml nicotine. The 5 right-sided bars represent infected conditions with or with exposure to cigarette smoke extract / nicotine in the presence or absence of a nicotine receptor blocker α-bungarotoxin.

6.3.4.3 Interleukin 10 (IL-10)

As for TNF-α, production of IL-10 by un-stimulated alveolar macrophages, was high: mean (SD) 19.36(11.67) pg/ml. There was no significant change in IL-10 production in uninfected macrophages when exposed to cigarette smoke extract or nicotine 15.5(8.6) pg/ml and 19.1(12.2) pg/ml respectively (p=0.42). (Figure 6-10) IL-10 production by alveolar macrophages after 18 hours BCG infection was numerically lower compared to uninfected macrophages 7.4(6.8) pg/ml vs. 19.36(11.7) pg/ml (p=0.06). No effect of co-exposure of BCG infected macrophages to either cigarette smoke or nicotine on IL-10 production was observed. IL-10 values were 7.4(6.7)

pg/ml vs. 5.1(7.0) pg/ml and 7.1(7.0) pg/ml in unexposed, cigarette smoke extract and nicotine-exposed cultures respectively (p=0.31, ANOVA). (Figure 6-10)

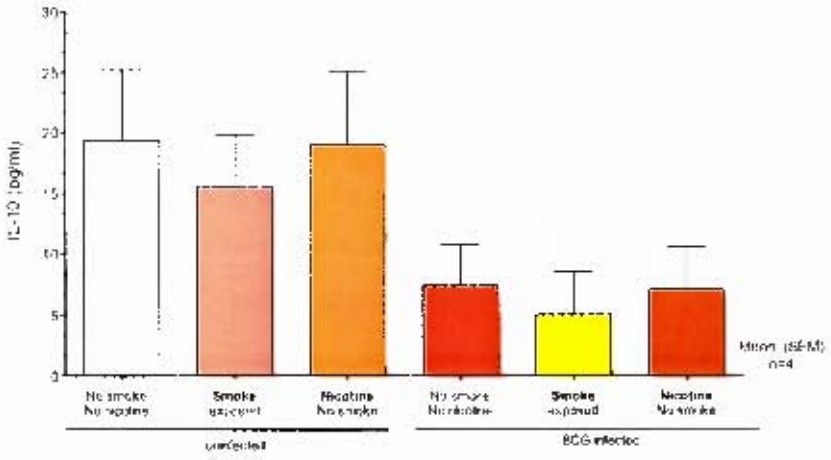


Figure 6-10 IL-10 production by alveolar macrophages

Replicate wells of alveolar macrophages are depicted by each bar with the left three bars uninfected basal conditions unexposed or exposed to 10% CSE to 1ug/ml nicotine. The right-sided three bars represent infected conditions with or with exposure to cigarette smoke extract or nicotine.

6.4 Discussion

This alveolar macrophage BCG infection model was set up to expand on the monocyte-derived macrophage (MDM) BCG-infection/exposure model reported in Chapter 5 to determine the effect of cigarette smoke and nicotine on cells from the potential site of infection, the lung. Short-term exposure (18 hours) to cigarette smoke extract (CSE) or nicotine appeared not to affect on alveolar macrophage viability or phagocytosis of BCG organisms. In addition, no consistent effect of these exposures or infection with BCG on apoptosis could be demonstrated. Cytokine production in these experiments was difficult to interpret due to the high basal production of TNF-α and IL-10 within the first 24 hours of bronchoalveolar lavage. The reduction in IFN-γ seen with CSE exposure, although small, corresponds with that seen in the MDM model.

A key finding in these experiments was that alveolar macrophage phagocytosis of BCG was not affected by short-term exposure to either cigarette smoke extract or nicotine. This extends the findings in monocyte-derived macrophage experiments.

To date, there are no reports documenting the use of cigarette smoke extract in BCG-infected alveolar macrophages. However, Ando et al. reported no difference in the phagocytosis of BCG by alveolar macrophages from individuals who smoked compared to those from non-smokers.[232] In view of these findings, it is possible that the increased epidemiological risk for TB infection and disease associated with smoking is not due to impaired alveolar macrophage phagocytosis, but to other mechanisms. In contrast to these findings for BCG, in non-TB models of short term *in vitro* alveolar macrophage exposure to cigarette smoke extract, a reduced phagocytic index for *Staphylococci*[102] and *Streptococci*[103] was observed. Smoking is a recognised risk factor for several forms of pneumonia. For pneumococcal pneumonia an odds ratio of 4.1 (95%CI: 2.4-7.3) and a population attributable fraction of 51% for active smokers has been reported.[233]

Cigarette smoke and nicotine exposure did not alter apoptosis in this model, nor could an effect of BCG infection on alveolar macrophage apoptosis be shown. This may reflect an inadequate length or intensity (dose) of exposure, or might have been confounded by the high degree of biological variability seen in the experiments. In all experimental conditions, the number of cells positive for Annexin-V staining was less than 15% at 18 hours, necrotic cells (7AAD positive) were however more common: ~30%. This could have been a consequence of technical issues such as disruption caused by harvesting of the adherent cells for flow cytometry. *In-situ* staining of adherent cells would be required to further investigate this question.

Previous studies of the effect of cigarette smoke on apoptosis in non-TB models have also yielded inconsistent results. Tomita *et al.* showed reduced apoptosis in alveolar macrophages from smokers[119] but several other authors have demonstrated increased apoptosis.[121-123] Others have suggested that attenuated strains of mycobacteria induce apoptosis while virulent strains have the opposite effect and suppress apoptosis.[118, 234, 235] In addition the burden of infection may influence the mechanism of cells death. High organism numbers per cell may trigger necrosis rather than apoptosis.[227, 236, 237] Intracellular growth of virulent organisms is facilitated by suppression of apoptosis prior to necrotic cell death when a significantly high bacterial load is established within the cell.[227, 236]

Apoptosis has been shown to be important to intracellular control of mycobacterium [117, 118] and is in part mediated via TNF-α with suppressed pro-apoptotic gene expression following virulent *M.tb* infection.[234] Resident alveolar macrophages in the lungs of smokers may thus have reduced TNF-α-induced apoptosis via two independent mechanisms viz. *M.tb* induced repression of pro-apoptotic gene expression and cigarette smoke- induced reduction in TNF-α production.

The basal/spontaneous production of TNF-α and IL-10 by alveolar macrophages was higher than expected. Hyper-stimulation may explain the cytokine reduction post infection. Significant effects of tobacco smoke, as seen with the monocyte derived macrophages, could only be confirmed for IFN-γ following BCG infection and cigarette smoke exposure. Moderate basal production (>400 pg/ml per 10^6 macrophages) of TNF-α has been shown in some studies of healthy volunteers[238-240] and is high (>2000pg/ml) in patients with interstitial fibrosis, sarcoid and rheumatoid arthritis.[241-244] All subjects recruited for lavage in this study were close contacts of TB index cases although otherwise healthy without symptoms. The high TNF-α and IL-10 production may reflect the high burden of exposure to both mycobacteria and pollutants in Cape Town or possibly activation of macrophages acutely following lavage. To my knowledge there are no other reports from high burden countries examining basal macrophage cytokine production in healthy controls.

Cells from healthy volunteers compared to smokers were used as acute *in vitro* macrophage exposure models from healthy uninfected non-smokers offer several advantages over macrophages from chronic smokers. Acute effects and responses can be studied over a wide exposure gradient. The effect of individual smoke components such as nicotine, acrolein, tetrahydrocannabiol (marijuana) in addition to exposure to either the particulate or soluble components, can also be evaluated.[102, 110, 245-248] Furthermore, smoke from tobacco, marijuana, crack cocaine etc. can be studied. In short-term exposure models however, the magnitude of the effect on a particular response has to be balanced with the toxicity of the extract to the cells as demonstrated in Chapter 2. Using alveolar macrophages from smokers represents more chronic exposure – as in most mouse models examining the effects of smoking. Smoker's macrophages are different structurally and often contain significant amounts of ingested particles such as carbon, tar, etc.[93, 249, 250] These changes may

persist for several years following smoking cessation and result in excess auto fluorescence by flow cytometry, making the interpretation of results very difficult.[250-253] In addition it is recognized that in COPD patients a persistent CD8 infiltrate is present even after the individual has stopped smoking suggesting an altered regulator phenotype that may affect cellular responses.[254]

The experimental design, as proof of concept, utilised alveolar macrophages from healthy non-smokers with *in vitro* BCG infection and short-term cigarette smoke extract exposure. To confirm and advance these data, further experiments would be required using virulent *M.tb* strains and macrophages from both smokers and non-smokers with and without *in vitro* CSE exposure. Flow cytometric analysis may however prove more difficult given the complexities of auto-fluorescence. Inactivation of virulent *M.tb* strains (usually with paraformaldehyde) prior flow cytometry will necessitate alternative techniques to identify cell death and apoptosis.

The optimal concentration of CSE that should be used in *in-vitro* models that reflects physiological concentration in smokers remains controversial. Su and colleagues likened the concentration of cigarette smoke used (10%), to a "physiological" level of smoking greater than one pack per day. They equated a 2.5% - 10% cigarette smoke extract (using 1 cigarette per 10 ml media) to smoking "slightly less than 0.5 packs per day, to slightly more than 2 packs per day".[248] The majority of cigarette smoke extract models employ a 10% solution for exposure. [115, 248, 255, 256] Acute exposure models however do not mimic the true air/liquid interface within the alveoli nor the chronic but intermittent nature of smoking.

In summary, the findings of the alveolar macrophage experiments have expanded on some aspects of the monocyte derived macrophage work, suggesting that acute exposure to cigarette smoke extract or nicotine at low concentrations does not impair phagocytosis of mycobacteria (BCG) in cells from the site of disease. Apoptosis was not significantly altered by BCG infection or cigarette smoke extract or nicotine exposure in this model. The cytokine responses are not easily interpretable due to the high baseline cytokine production, which may be a reflection of the recruits having been exposed to pollutants, mycobacteria or mechanical stimuli. Further studies will be required to clarify these data.

7 Future directions

The data presented in this dissertation and the work recently published by Shang and colleagues[116] are preliminary steps in identifying the biological and molecular pathways responsible for the association between smoking and tuberculosis. However, many questions remain unanswered, some relating to the work presented and others of a more general nature.

Firstly, in respect of the experiments reported here, it is important to establish whether the effects of cigarette smoke extract on macrophage function are influenced by the virulence of the organism. This can be addressed by repeating the experiments on cytokine production, apoptosis and mycobacterial stasis using different strains of virulent *M.tb* instead of BCG. Secondly, alternative protocols for harvesting and preparing alveolar macrophages are needed to explore the reason for the high background levels of cytokine production; whether this reflects basal function *in vivo* or is a consequence of the method of lavaging the lung or the processing the cells. The latter is unlikely, as the methods used have been widely used in other studies. A more general question is the need to identify the intracellular signalling pathways responsible for the cellular and cytokine effects demonstrated.

Evidence from non-tuberculous models of infection, suggest that macrophages from smokers have impaired signalling through TLR2/4.[220] Engaging the TLRs initiates a complex cascade of intracellular and intercellular signals that facilitate protection against pathogens. Potentially, tobacco smoke may impair three major cytokine production pathways, those involving NFκB, PI3K and MAPK. This may enable *M.tb* organisms to avoid protective immune responses.[257, 258] This issue needs to be studied in a human TB model.

M. leprae, a pathogen that evades host immunity, has been shown to down-regulate the induction of some pro-inflammatory (IL-6) cytokines and induce high levels of inhibitory cytokines and chemokines (IL-1Ra, MCP-1).[259] These effects are mediated through activation of PI3K. Phosphorylation of PI3K mediates the activation of the downstream target Akt and in turn the phosphorylation of the glycogen synthase kinase (GSK)-3β. This pathway is a potential target of tobacco smoke as described by

Zhang et al.[260] that may account for the reduced production of selected cytokines by BCG-infected macrophages. Thus examining the role of the PI3K pathway in the modulation of the profile of pro- and anti-inflammatory cytokines and chemokines in *M.tb* and BCG-infected macrophages by tobacco smoke, may yield important results.

MAPK activation leads to a wide range of cellular responses including cytokine production, inflammation and apoptosis. Tobacco smoke may inhibit or delay the activation of the MAPK pathway, thus affecting the nature and the magnitude of the cytokine and chemokine response. Tobacco smoke may also interfere with the activation of this signalling pathway independently of or via the PI3K /Akt pathway.

The role of the nicotine α-7 receptor in the inhibition of macrophage cytokine production and apoptosis remains unanswered. There are other nicotinic receptors such as the α2β4 receptor the target of the anti-smoking drugs varenicline and cytosine although these receptors and the α7 receptor are not present on cell lines nor mice. [261, 262] Closer examination of the pathways by using an inhibitor of the nicotine α-7 receptor to confirm its role in the PI3K dependent reduced cytokine production may offer clearer insights. Furthermore it is known that activation of PI3K/Akt pathway can prevent apoptosis through inhibition of Capsase-9 activation.[263] To determine if the effect of tobacco smoke on Capsase-9 is through the nicotine α-7 receptor/PI3K/Akt pathway, further alternative methods of receptor antagonism such as pre treatment of macrophages with the nicotine α-7 receptor antagonist (quinolizidine) may reveal changes in caspase-9 activation. Given the technical complexities of flow cytometry using alveolar macrophages, alternative strategies for the detection of apoptosis such as in-situ staining of adherent cells (prior to fluorescent microscopy with Annexin-V and propidium iodide) in addition to the cell Tunel staining assay may be employed.[117]

8 Conclusion and recommendations

History appears to be repeating itself. This time however, it might have a sting in its tail. A century ago a tobacco smoking was widespread and tuberculosis (TB) was rampant in many parts of the world. In 1918 the "Mother of all pandemics",[264] Spanish flu killed nearly 50 million people worldwide.[264, 265] An interaction between smoking, tuberculosis and the influenza virus was not, to my knowledge, suspected or reported, but, in the light of modern research might well have played a significant role. Interaction between organisms, host and the environment are now recognised as the rule in all infections, including those that reach epidemic proportions, and likely determine their impact.

At the beginning of the 21st century, the globe is facing the convergence of several potentially devastating infections. Faced with declining markets in the North, the tobacco industry is moving south, where smoking is on the increase, especially in countries such as China and India. Tuberculosis remains uncontrolled in the developing world with increasing rates of multi-, extensively- and pan-drug resistant tuberculosis. HIV has added a new facet to respiratory disease with associated increased rates of TB and pneumonia. In addition we are facing the looming possibility of a highly virulent H5N1 "avian" influenza pandemic, with the novel strain of H1N1 "swine" flu recently added to the mix.

This dissertation, explores one aspect of these colliding epidemics namely the interaction of tobacco smoking and immunity to tuberculosis. Before this could be addressed several questions and issues including the standardisation of methodology, accurately defining study subjects with respect to their TB infection/immune status, and identifying the most accurate method to determine mycobacterial load in the proposed studies, needed to be answered.

In basic science studies clear delineation of the TB infection status (uninfected, versus latent infection, versus active tuberculous disease) is important as it may affect immune responses. Additionally it will aid in distinguishing between TB-driven immune responses due to immunological memory rather than the intervention being investigated. Interferon gamma release assays have been proposed and accepted in

several guidelines as a replacement for the tuberculin skin test. However, as reported in this thesis, this decision is not without problems. The interpretation of results requires refinement because of within-subject variability. Based on my data, an uncertainty zone around the cut-point was defined and a threshold for defining true conversions and reversions of LTBI status has been suggested. It was also shown that an antecedent tuberculin skin test can affect subsequent IFN-γ responses. To avoid this potential boosting of IFN-γ responses, a three 3-day window has been proposed in which to perform a confirmatory IGRA. This fortuitously falls on the same day required for reading of the TST. The extent and importance of this variability and boosting of IGRAs by prior TST requires investigation in low burden settings. Furthermore, my work has demonstrated that guidelines developed for low burden settings might not be valid in high burden countries.

Having unravelled how IGRA results could optimally be defined and interpreted, the next step was to determine the best way to quantify mycobacterial load in the proposed studies. Although several techniques exist to quantify mycobacterial load, none has ideal performance characteristics. With the growth and development of TB research, newer approaches to quantifying mycobacterial load have emerged. For example, newer technologies such as automated liquid culture have been adopted in drug development in early bactericidal activity studies.[266] Novel technologies such as Xpert MTB/RIF remain untested. In this dissertation, 5 techniques were evaluated using a wide range of performance characteristics to determine the optimal techniques for use in laboratory-based studies. Liquid culture and Xpert MTB/RIF offered advantages of sensitivity and rapid turn around time respectively but had other limitations, particularly higher cost. Solid culture, despite its limitations, remains the cheapest option and is therefore likely to remain the best option in resource-limited settings. The final choice of assay however, will largely depend on the experimental design and research context with a trade-off of cost versus turnaround time. Although the data is of primary relevance to basic science researchers, it has applicability to clinical researchers in both well resourced and resource constrained environments. Furthermore it provides a structured comparison of technologies that will aid decision making in capacity development situations where new research infrastructures are being developed and technologies need to be established.

The paucity of data examining the cellular effects of tobacco smoke on mycobacterial-specific immunity is striking. In this dissertation a human *in vitro* model of mycobacterial infection and exposure to a standardised cigarette smoke extract, using both monocyte-derived and alveolar macrophages is presented. Infecting macrophages with BCG organisms with co-exposure to cigarette smoke extract, showed a clear effect of reducing cytokine production. No effect on mycobacterial uptake or cell viability was demonstrated to account for this phenomenon. Intracellular containment of BCG growth was furthermore impaired by cigarette smoke extract over a 5-day period. These data are intriguing given the lack of published data on human tissues and supports recent findings in mouse models. The preliminary evidence that nicotine alone may have similar effects supports the call to regulate nicotine-containing products[267, 268] and raises a concern about prescribing nicotine replacement therapy in tobacco cessation programmes in high TB burden settings. Randomised controlled trials are currently exploring the benefit of smoking cessation in active TB patients. It will be interesting to speculate that nicotine replacement therapy might be to blame if no beneficial outcome is seen in the tobacco cessation group. Together these data go some way in providing a mechanistic explanation for the epidemiological association between smoking and tuberculosis.

The alveolar macrophage experiments expanded on several aspects of the monocyte-derived macrophage work. The lack of effect on mycobacterial uptake was confirmed for both cigarette smoke extract and nicotine. The cytokine responses were difficult to interpret because of the high background cytokine production. Further work is required to clarify the findings. Although no conclusive effect on apoptosis could be demonstrated, apoptosis and intracellular survival of mycobacteria will need to be evaluated using virulent mycobacteria.

This dissertation has presented evidence to support the epidemiological association between smoking and TB. Much work remains to be done and the molecular pathways involved have not yet been defined. Given that epidemiological data remains confounded by socioeconomic factors, these data provide policy makers with scientific evidence to support and strengthen tobacco control measures. In South Africa, tuberculosis, HIV and smoking-related diseases such as COPD place an

enormous burden on the health care service. The final number of people who will succumb to TB, HIV and COPD will depend on the combined efforts of government, health agencies, non-governmental bodies and researchers.

Karl Marx said: "History repeats itself – first as a tragedy, second as a farce". It remains to be seen whether over the past hundred years we have learned enough to effectively combat the looming epidemics or whether we are doomed to see history repeat itself.

Appendix A

Cytokine production by LTBI status

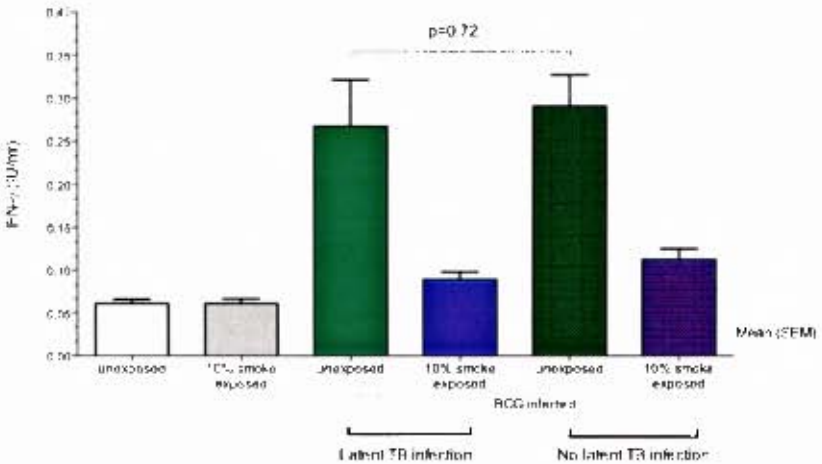


Figure A 0-1 Monocyte-derived macrophage IFN-γ production stratified by subject LTBI status

LTBI positive subjects are depicted in solid colours with LTBI–negative subjects in shaded bars. The mean (SD) IFN-γ production following infection by unexposed compared to CSE exposed monocyte-derived macrophages was: 0.27 (0.21) pg/ml vs. 0.29 (0.14) pg/ml (p=0.73).

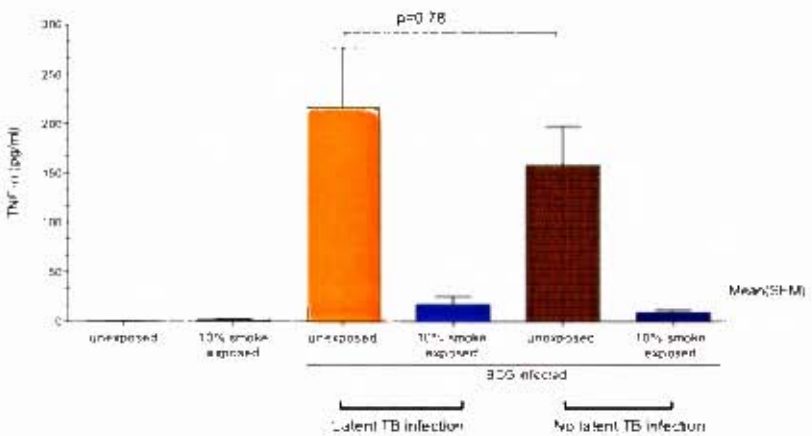


Figure A-0-2 Monocyte-derived macrophage TNF-α production stratified by subject LTBI status

LTBI positive subjects are depicted in solid colours with LTBI–negative subjects in shaded bars. The mean (SD) TNF-α production following infection by unexposed compared to CSE exposed monocyte-derived macrophages was: 216.0 (235.6) pg/ml vs. 157.4 (135.5) pg/ml (p=0.98).

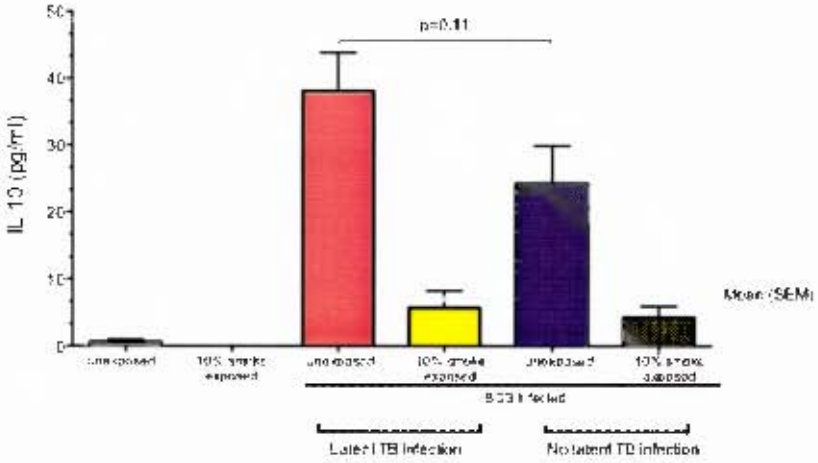


Figure A- 0-3 Monocyte-derived macrophage IL-10 production stratified by subject LTBI status

LTBI-positive subjects are depicted in solid colours with LTBI–negative subjects in shaded bars. The mean (SD) IL-10 production following infection by unexposed compared to CSE exposed monocyte-derived macrophages was: 38.1 (14.2) pg/ml vs. 24.2 (24) pg/ml (p=0.11).

Appendix B

Monocyte-derived macrophage BCG-stasis assays stratified by LTBI status

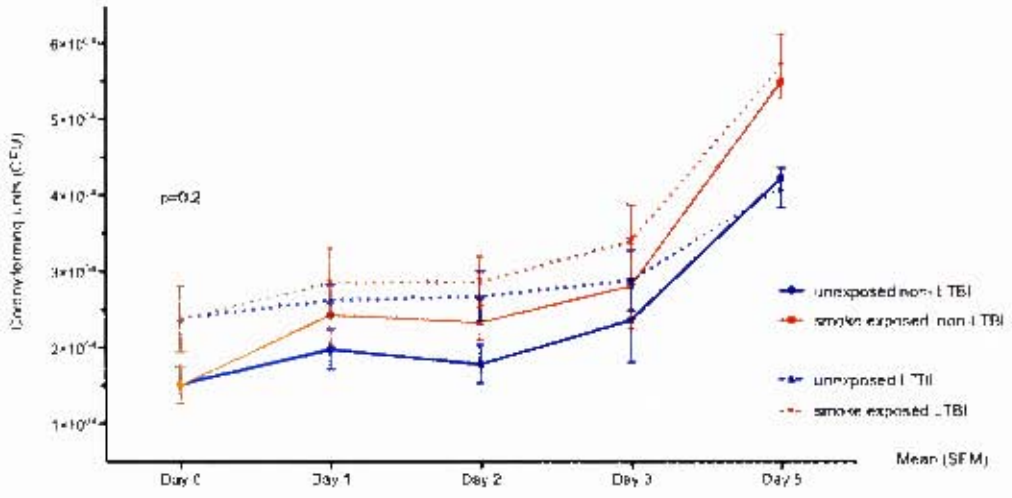


Figure B-0-1 Serial BCG colony counts over 5 days in monocyte-derived macrophages

Monocyte-derived macrophages infected with BCG were kept in culture for 5 days after the addition of 10% CSE to culture wells on day zero (post infection). The solid lines represent latent TB infected subjects and the stippled lines latent TB uninfected subjects. The blue line represents unexposed macrophages and the red line CSE exposed macrophages. Each day represents the time point post infection that macrophages were lysed and organism load calculated by colony counts on solid media. At day zero (pre CSE exposure) the colony counts were slightly higher in the LTBI positive group compared to the LTBI negative group as seen in previous experiment in chapter 5.3 but was not statistically significant; $2.4X10^4$ ($8.8x10^3$) CFU/ml vs. $1.5x10^4$ ($4.9x10^3$) CFU/ml ($p = 0.2$).

Appendix C

Alveolar macrophage BCG uptake by LTBI status

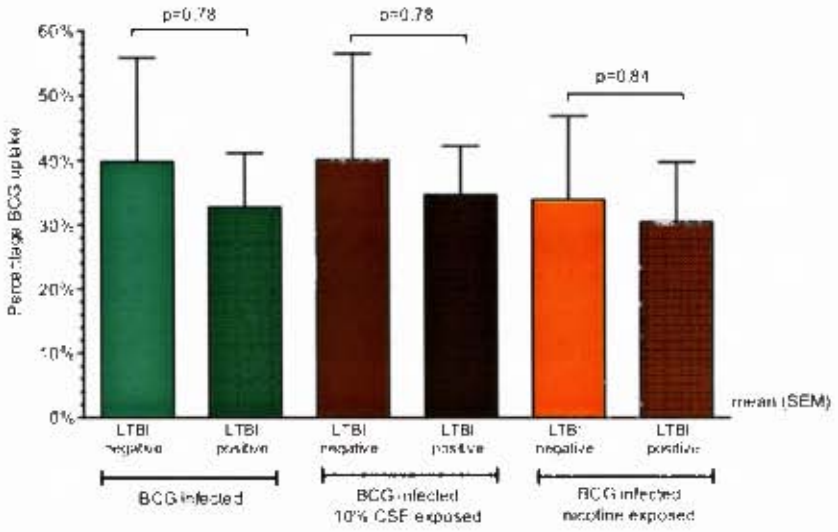


Figure C 0-1 Alveolar macrophage BCG uptake by LTBI status

LTBI-positive subjects are depicted in solid colours (n=4) with LTBI–negative subjects in shaded bars (n=3). The mean (SD) BCG uptake for unexposed alveolar macrophages was: LTBI negative 39.8% (27.8) and LTBI positive 32.8% (16.8) p = 0.78. No difference in uptake was demonstrated following exposure to 10% CSE or nicotine when stratified by LTBI status.

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