EFFECTS OF EXPOSURE TO ENVIRONMENTAL MYCOBACTERIA ON IMMUNITY CONFERRED BY BACILLE CALMETTE-GUÉRIN (BCG) VACCINE

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Summary

Epidemiological evidence suggests that the efficacy of *Mycobacterium bovis* bacille Calmette-Guérin (BCG), as a tuberculosis (TB) vaccine in human populations, is influenced by prior sensitisation to environmental mycobacteria (Env). After priming with certain Env species and subsequent vaccination with BCG, murine hosts show reduced proliferation of BCG *in vivo*. This may be because memory responses to Env antigens are cross-reactive with antigens of other mycobacterium species. However, the immunological mechanisms underlying these effects remain unknown. This project aimed to uncover these mechanisms using a murine model of *Mycobacterium chelonae* sensitisation followed by intranasal BCG infection. Cytotoxic responses of splenocytes against autologous BCG-infected macrophages of mice sensitised with *M. chelonae* (a representation of Env), with or without subsequent intranasal BCG infection, were measured by a non-radioactive cytotoxicity assay. Splenocytes were sorted into CD4 and non-CD4 subsets to investigate the T cell subsets involved in these cytotoxic responses. The levels of relevant cytokines produced by splenic $CD4^+$ and $CD4^-$ T cells were determined by ELISA. Env sensitisation increased cytotoxicity of splenic T cells against autologous BCG-infected macrophages, both before and after BCG challenge. This was especially noted at 3 weeks post-infection in the $CD4^+$ fraction, which also contributed largely to the perforin production in those mice. However, the cytotoxicity was not directly correlated with IFN-γ production. Cytokine production and inflammatory cell count, at the site of infection (i.e. lung) was also determined, by flow cytometry. Reduced percentages of all inflammatory cells in the lungs of sensitised mice in response to

intranasal BCG, and a higher proportion of IL-10 producing cells in the lung tissue, relative to control mice, suggest induction of regulatory T cells following Env sensitisation. Thus, $CD4^+$ mediated cytotoxicity in Env-primed mice against BCG-infected cells is a mechanism behind the effect of Env exposure on subsequent BCG vaccination. The results of this work have an impact on the use of BCG as a vaccine as well as development of future vaccines against TB, given that many candidate TB vaccines on clinical trials currently involve BCG in prime-boost strategies or genetically-modified BCG as a vector to carry novel antigens.

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ABBREVIATIONS

APC	Allophycocyanin
autoMACS	Automated magnetic cell sorting
BALF	Bronchoalveolar lavage fluid
BCG	Bacillus Calmette-Guérin
BSA	Bovine serum albumin
CMV	Cytomegalovirus
CTL	Cytolytic T lymphocyte
DC	Dendritic cell
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
Env	Environmental mycobacteria
FITC	Fluorescein isothiocyanate
FAC	Ferric ammonium citrate supplement
FBS	Foetal bovine serum
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitrogen oxide synthase
i.p.	Intraperitoneal
i.n.	Intranasal
KO	Knockout

LDH	Lactate dehydrogenase
MHC	Major histocompatibility complex
mAb	Monoclonal antibody
MOI	Multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
NK	Natural killer cell
OADC	Oleic acid-albumin-dextrose-catalase enrichment
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanate 7
PMA	Phorbol myristate acetate
PPD	Purified protein derivative
SD	Standard deviation
TB	Tuberculosis
TLR	Toll-like receptor
Treg	Regulatory T cell
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2

CHAPTER 1 LITERATURE REVIEW

1.1 Tuberculosis situation in the world

Tuberculosis (TB) is amongst the global leading causes of death by a single infectious pathogen. Human disease is mainly caused by members of the *Mycobacterium tuberculosis* (Mtb) complex, comprising of Mtb*, M. bovis, M. africanum M. canettii* and *M. microti* (Cosma, 2003). The World Health Organization (WHO) has declared TB a 'global emergency', and estimates that two million people die from this curable disease annually. TB can be treated with a cocktail of antibiotics but this requires at least six months, with potential toxicity and cost issues. Due to poor availability or compliance to drug treatment, especially in poor developing areas, direct observed therapy (DOTS) is advocated but is difficult to administer. With the rising trend in HIV (human immunodeficiency virus) infections as well as the appearance of multiple-drug resistant (MDR) strains of Mtb, the TB situation worldwide is worsening, with almost nine million new cases in 2004 (WHO, 2006).

1.2 *Mycobacterim tuberculosis* – an intracellular pathogen

Tubercle bacilli are intracellular pathogens, surviving within lung macrophages after the human host inhales airborne droplets containing the bacteria. Alveolar macrophages, which are believed to be the principal host cells of the bacteria, play dual roles in the lifestyle of Mtb – as a first line of cellular defence, as well as a site for bacterial survival and replication. The bacteria can escape the host immune system by interfering with

membrane trafficking and avoiding phagolysosomal fusion. Nonetheless, in infected individuals, dendritic cells (DCs) and macrophages recruited to the lung take up the bacteria, migrate to the draining lymph nodes and initiate T-helper 1 (Th1) responses by presenting Mtb antigens to T cells. Eventually, granulomas form in response to persistent intracellular Mtb. In these structures, macrophages, DCs, T cells and B cells surround single infected macrophages (Cosma, 2003). Any remaining Mtb can persist in a latent state in the host and reactivation of such bacteria leads to active disease. There is some evidence that latent mycobacteria survive under conditions of nutrient deprivation and hypoxia within granulomas by reducing their metabolic activity and persisting in a non-dividing or slowly dividing state (Raja 2004).

1.3 Immune responses to TB

Protective immune responses against all mycobacteria depends on cell-mediated immunity provided by T cells. The intracellular lifestyle of Mtb makes T cell effector functions more important than antibodies in controlling or eliminating Mtb infections. Two major effector functions are the T helper and cytotoxic activities, which shall be further described below.

1.3.1 T helper cells

$CD4^+$ T cells are the most important subset of T cells for controlling Mtb infections. This is clearly seen in numerous murine studies as well as in HIV-infected individuals, who have a significantly lowered $CD4^+$ T cell count and are markedly more susceptible to TB (Flynn and Chan 2001; Elkins, 2003). The full range of effector mechanisms utilised by

$CD4^+$ T cells in combating TB remains to be elucidated. However, the production of IFN-γ in activating macrophages to release reactive oxygen and nitrogen intermediates is generally recognised as a key effector mechanism of $CD4^+$ cells in murine models of TB (Flynn and Chan 2001).

1.3.2 Cytotoxicity in response to *M. tuberculosis*

Cytotoxic T lymphocytes (CTLs) have increasingly been reported in TB patients, and are likely to have major roles in anti-TB immunity (Lewinsohn, 1998). Potential cytolytic cell subsets involved in lysis of Mtb-infected macrophages are $CD4^+$, $CD8^+$ and γδ T cells, as well as natural killer (NK) cells.

1.3.2.1 Natural killer (NK) cells

NK cells are cytolytic effector cells of innate immunity, and have been shown to be involved in immune responses against TB. Human NK cells have been demonstrated to respond to live Mtb *in vitro* and increased NK activity is observed in active pulmonary TB patients (Yoneda, 1983; Esin, 1996). The expansion of NK cells after *Mycobacterium bovis* bacille Calmette- Guérin (BCG), or Mtb infection in mice has also been reported, suggesting a role for NK cells in immune responses against TB (Falcone, 1993; Junqueira-Kipnis, 2003). The direct role of NK cells in mycobacteria infections, however, is not well understood.

1.3.2.2 $CD4^+$ cytolytic T cells

Apart from being involved in T helper responses, $CD4^+$ T cells can also exhibit cytotoxicity. Upregulation of mRNA for granulysin, perforin and granzymes A and B, is observed in human $CD4^+$ T cells after *in vitro* stimulation with Mtb, indicating a cytolytic role of these cells against TB (Canaday, 2001). Furthermore, $CD4^+$ cells from peripheral blood of patients with active TB have been reported to display cytotoxic responses against autologous Mtb-pulsed macrophages, and this cytotoxicity diminishes with severity of TB. However, it is unclear whether the opposite, where patients with less severe TB have better cytotoxic responses, holds true (De La Barrera, 2003). The same study shows that the CD4-mediated cytotoxicity occurs via the Fas/ Fas-ligand mechanism. However, other studies on $CD4^+$ T cell clones have reported perforin-dependent mechanisms for their cytolytic activity (Susskind, 1996; Kaneko 2000).

1.3.2.3 $CD8^+$ T cells

The most widely reported cell type exhibiting cytotoxicity in TB studies is the $CD8^+$ cell (Sousa, 2000; van Pinxteren, 2000). There is evidence for exocytosis of granule contents as the mechanism behind $CD8^+$ CTLs in TB. Human $CD8^+$ T cells exert cytotoxicity on Mtb-infected macrophages via a granule (perforin/ granzyme or granulysin)-dependent mechanism that is independent of Fas/ Fas-ligand interaction (Stenger, 1997; Stenger, 1998). The perforin/ granzmye pathway is also suggested to be more important than the Fas/ Fas-ligand pathway in lysis of Mtb-infected macrophages by $CD8^+$ CTLs in mice (Silva and Lowrie 2000). Another study showed that although granule exocytosis is

required for the cytotoxic activity of human $CD8^+$ T cells, perforin inhibition did not affect restriction of Mtb growth (Canaday, 2001).

1.3.2.4 γδ T cells

γδ T cells are readily activated by Mtb and secrete antigen-specific IFN-γ (Ladel, 1995a). Murine studies with T cell receptor (TCR) δ gene deletion mutants show that γδ T cells play a major role in protective responses against TB, as these mice died after Mtb infection, while immunocompetent control mice survived (Ladel, 1995b). Futhermore, γδ T cell-mediated lytic activity is observed in *ex vivo* effector cells from TB patients (De La Barrera, 2003).

1.4 Regulatory T cells (Treg)

Regulatory T cells (Treg) exert suppressive effects on immune responses, and therefore are an important consideration when evaluating efficacy of immunity against infectious pathogens. Two Treg populations have been described, but not in infectious disease models – IL-10 secreting and naturally occurring Treg cells (O'Garra, 2004). Naturally occurring Tregs are a subset of $CD4^+$ T cells that are able to suppress the effector functions of $CD4^+$ and $CD8^+$ T cells (Thornton and Shevach 1998; Murakami, 2002). These are of the $CD4^+CD25^+$ phenotype, and the transcription factor FoxP3 is known as a specific molecular marker for such cells (Fontenot, 2003; Fontenot and Rudensky 2005; Roncador, 2005). Activity of antigen-driven IL-10 secreting Treg cells does not seem to need FoxP3 (Vieira, 2004), but requires IL-10 and TGF-β (Groux, 1997). Treg cells of

the $CD4^+CD25^{high}$ phenotype have been recently reported in TB patients, and an increase in frequency of these cells, together with elevated mRNA expression of FoxP3, is observed in the peripheral blood of these patients (Guyot-Revol, 2006). The authors suggest that Tregs expanded in patients with TB may contribute to suppression of immune responses against TB. In a murine study, however, antibody-mediated depletion of $CD25^+$ cells prior to pulmonary infection with Mtb and BCG does not affect bacterial burden or pathology. The authors interpret this as implying a minor role for Tregs in the pathogenesis of Mtb infections in mice (Quinn, 2006).

1.5 Roles of cytokines in *M. tuberculosis* infection

Cytokines are produced by activated immune cells, often in response to an infection in general, or specifically to an antigen. Given the chronicity of Mtb infection, the role of cytokines in polarising the immune response at the inflammation site is significant as demonstrated by cytokine gene-deficient mice. The cytokines of relevance to this study will be described here.

1.5.1 Interferon γ (IFN-γ)

IFN-γ is a key cytokine required for protection in Mtb infections. It is produced by NK cells early, and later by activated $CD4^+$, $CD8^+$ and γδ T cells, in Mtb infections. Although insufficient in limiting Mtb infections by itself, IFN-γ plays an important role of activating macrophages by inducing phagosome maturation and upregulating their antimicrobial molecules, such as iNOS (inducible nitrogen oxide synthase), reactive nitrogen intermediates and reactive oxygen species, against intracellular Mtb. Humans

who have genes defective for IFN-γ are susceptible to serious mycobacterial infections (Cooper, 1993; Jouanguy, 1996). In addition, IFN-γ gene disruption murine experiments proved a high susceptibility to Mtb in these mice (Cooper, 1993; Dalton, 1993; Flynn, 1993). However, IFN-γ is weakly produced in patients with active pulmonary TB (Onwubalili, 1985; Vilcek, 1986), and some authors have suggested that this may be, in part, a cause for their susceptibility.

Human studies in Malawi have demonstrated that among BCG vacinees, increases in IFN-γ responses to Mtb antigens were highest among those with low initial responsiveness to environmental mycobacterial (Env) antigens (Black, 2001a). Later studies done by the same group showed that prior to BCG vaccination, Malawi residents already have a higher IFN-γ response to Mtb purified protein derivative *(*PPD) and some Env species than UK individuals, likely due to Env sensitisation (Black, 2002; Weir, 2006). An increased frequency of IFN-γ responses to Env was also observed in Malawi, but not in the UK, over time in non-vaccinated controls, reflecting the higher level of natural exposure to Env in Malawi than the UK (Weir, 2006). Different levels of natural exposure to Env have an impact on subsequent BCG vaccination, which will be discussed later.

1.5.2 Interleukin 4 (IL-4)

There have been studies showing increased expression of the Th2 cytokine IL-4 in human TB patients as well as murine TB models (Hernandez-Pando, 1996; Seah, 2000; van Crevel, 2000; Lienhardt, 2002). Some roles that IL-4 may play in immunity against TB as

well as in immunopathology have been suggested. Findings include activation of an inappropriate type of macrophages, a decrease in Toll-like receptor 2 (TLR2) expression and signalling, in addition to a downregulation of inducible nitric oxide synthase (iNOS) by IL-4 (Bogdan, 1994; Krutzik, 2003; Kahnert, 2006). IL-4 knockout (KO) studies in Balb/c mice have demonstrated that IL-4 KO mice were better able to control bacterial replication and produce Th1 cytokines like IFN-γ to combat the disease progression of TB than control mice (Hernandez-Pando, 2004). These findings point towards IL-4 as a cause for decreased immunity and increased immunopathology in TB.

1.5.3 Transforming growth factor β (TGF-β)

It has been shown that *Mycobacterium vaccae*–induced Treg cells priming anti-inflammatory responses to ovalbumin produce IL-10 and transforming growth factor-β (TGF-β) (Zuany-Amorim, 2002). These cytokines have been described to have immunosuppressive roles and are produced by Treg cells. Treg cells have been shown to be expanded in TB patients and likely have roles in suppression of Th1-type immune responses in TB disease (Guyot-Revol, 2006). IL-10 and TGF-β have been suggested to down-regulate host immune responses against TB in lungs of human patients, which then lead to overt disease (Bonecini-Almeida, 2004). TGF-β has also been indicated, *in vitro*, to play a part in suppressing T cell responses to mycobacterial antigens in peripheral blood mononuclear cells (PBMCs) (Hirsch, 1996; Ellner 1997; Hirsch, 1997; Toossi and Ellner 1998). Some mechanisms behind the suppressive role of TGF-β include inhibition of lymphocyte proliferation and function, suppression of IL-2 production and blocking of IFN-γ –induced macrophage activation (Allen, 2004; Hernandez-Pando, 2006). A recent

study by Hernández-Pando *et al* (2006) demonstrated that the administration of TGF-β antagonist and cyclooxygenase inhibitor in mice controlled pulmonary TB to a similar extent as anti-microbial treatment alone. These experiments suggest that TGF-β is an important player in the defective cell mediated immunity (CMI) that leads to TB progression.

1.5.4 Interleukin 10 (IL-10)

There is evidence to show that IL-10 antagonises anti-microbial effector functions of macrophages and reduces the presentation of major histocompatibility complex (MHC) class II-peptide complexes at monocyte plasma membranes (Koppelman, 1997; Redpath, 2001; de la Barrera, 2004). A recent study found that IL-10 in BCG-infected cells inhibits cathepsin S-dependent processing of the MHC class II invariant chain in human macrophages, therefore escaping immune surveillance by inhibiting the export of mature MHC class II molecules to the cell surface and reducing the presentation of mycobacterial peptides to $CD4^+$ T cells (Sendide, 2005). Elevated levels of IL-10 are also seen in mice made susceptible to Mtb due to the absence of the transcription factor T-bet, implying that IL-10 has a part to play in TB progression as well (Sullivan, 2005).

1.6 BCG as a vaccine

Currently, BCG is the only available human vaccine against TB, and has seen almost a century of human usage. BCG is an attenuated strain of *M. bovis*, and was obtained after many years of continuous *in vitro* passage of a virulent *M. bovis* strain. In spite of the long history, it is not yet clear what are the exact immune mechanisms underlying

protection conferred by this vaccine. More importantly, scientists are now intensively investigating reasons why BCG has poor efficacy against adult forms of TB. The protective efficacy of BCG varies dramatically across different parts of the world – a geographical variation in BCG efficacy is observed, with between 0 – 80% efficacy noted in different areas. BCG-attributable protection is especially low in developing countries, such as parts of Asia and Africa, which are also the areas of high TB incidence.

BCG has consistent 'efficacy' as a vaccine in murine models of TB – in this field, this is defined with respect to the ability to diminish Mtb bacterial burden upon subsequent TB infectious challenge – but even in mice, BCG vaccination never results in host elimination of subsequent TB infection. Other candidate TB vaccines have not even been able to outshine this 'protection' provided by BCG in mice (Olsen, 2000; Skeiky, 2000; Orme, 2001; Doherty, 2004). In mice, BCG does induce high levels of IFN-γ production, and it has been argued that the magnitude of this response may be an immune correlate of protection (Al-Attiyah, 2004; Castanon-Arreola, 2005; Hovav, 2005). However, it is also evident that some candidate TB vaccines which elicit stronger IFN-γ responses than BCG are nonetheless less protective than BCG in terms of reducing TB bacterial burden. (Skinner, 2003).

1.7 Environmental mycobacteria (Env)

There are numerous species of mycobacteria that are free-living and ubiquitous in soil and open waters, termed Env, which are also known as non-tuberculous mycobacterium. Many of these are opportunistic pathogens. They rarely cause human disease, except

upon direct inoculation, but are common pathogens to people with immunocompromising conditions (Primm, 2004).

1.8 Effect of environmental mycobacteria (Env) exposure on subsequent BCG vaccination

Recent studies have proposed that immune modulation through exposure to Env affects the efficacy of BCG. These non-pathogenic mycobacteria belong to the same genus as Mtb and BCG, and many are genetically closely related to BCG. Human epidemiological studies have shown circumstantial evidence that efficacy of BCG vaccination is reduced in populations with high levels of exposure to Env (Black, 2001a; Black, 2002). BCG-vaccinated individuals in the United Kingdom (UK) have post-vaccination increases in IFN-γ responses to PPDs from different species of Env, and the degree of change is correlated to the relatedness of the Env species to BCG, thereby providing evidence that memory T cells responding to BCG cross-react with Env antigens (Weir, 2006). The efficacy of BCG has been demonstrated to be better in the UK compared to rural African areas such as Malawi, where exposure to Env is believed to be higher. The prevalence and magnitude of sensitivity to PPDs from Env before BCG vaccination have been shown to be higher in Malawi individuals than those in the UK, affirming that Env exposure is indeed higher in Malawi than in the UK (Black, 2001b, 2002, Weir, 2003). Malawi adults, upon BCG vaccination, have only moderate increases in IFN-γ and delayed type hypersensitivity (DTH) responses, while greater increases are seen in the UK individuals. The difference in BCG-attributable increases in IFN-γ and DTH responses together with the difference in Env exposure between these two populations

indicate a possible role of Env in interfering with the protective efficacy of BCG. The authors suggest that Env could possibly confer a level of immune protection to TB which subsequent BCG vaccination does not surpass. As a result, there may be little additional protection observed post-BCG in these populations, but the overall level of protection is still inadequate to completely prevent adult forms of TB. This 'masking hypothesis' thus suggests that Env-generated immunity masks the effects of BCG (Andersen and Doherty, 2005).

A second hypothesis – the 'blocking' hypothesis' – is based on murine studies showing that prior sensitisation with certain species of Env reduces the replication of live BCG in the host, possibly through immune responses to antigens that are cross-reactive with BCG antigens (Buddle, 2002; de Lisle, 2005; Demangel, 2005). Brandt *et al* (2001) show that in mice exposed to live Env, subsequent BCG vaccinations result in transient immune responses that limit BCG multiplication, thereby reducing its numbers, and are unable to protect against TB. Another study also demonstrated that exposure to live Env, which are cleared with antibiotic treatment, followed by immunisation with BCG results in limitation in the replication of BCG in these mice as well as reduced protective effects of BCG against TB (Demangel, 2004). These studies support the 'blocking' hypothesis, which attributes the lack of BCG activity to the possibility that with prior Env exposure, memory responses cross-reactive with BCG antigens result in limitation of BCG multiplication thereby attenuating the desired effects of the live vaccine in continuously stimulating T cell responses. However, the specific nature of immunity invoked by Env

and therefore the reasons why the BCG showed reduced replication in Env-sensitised hosts were not addressed in those studies.

CHAPTER 2 AIMS AND OBJECTIVES

Epidemiological evidence suggests that the efficacy of *Mycobacterium bovis* bacille Calmette- Guérin (BCG) as a tuberculosis vaccine may be influenced by prior host sensitisation to environmental mycobacteria (Env). Recent work in our lab showed that mice sensitised with *M. chelonae* had cytotoxicity responses against autologous macrophages infected with BCG. Such cross-protective cytotoxic responses were most significant with *M. chelonae* amongst many Env species tested, and this formed the basis for the use of *M. chelonae* in our current project. This prior work of our lab thus suggests that it is cytotoxicity against BCG-infected macrophages that could be responsible for the observed reduction in BCG replication in Env-sensitised mice. However, another hypothesis may also be possible to explain the lack of BCG efficacy after Env sensitisation. A study by Zuany-Amorim *et al* (2002), showed that sensitisation with heat-killed *M. vaccae* (an Env species) gave rise to ovalbumin-specific regulatory T cells (Treg) that reduced the airway inflammation in mice with ovalbumin-induced eosinophilic airway inflammation. In our lab, after *M. chelonae* sensitisation followed by intranasal BCG administration, both lung BCG load as well as recruitment of inflammatory cells in these mice were markedly decreased. We showed that the adoptive transfer of a subset of T cells from Env-sensitised mice was responsible for this effect (Zhang *et al*, manuscript in preparation). This demonstrated that Env species, such as *M. chelonae*, have immunomodulatory effects that reduce the immune response to BCG.

In this project, murine *M. chelonae* sensitisation followed by intranasal BCG infection was used as the model to understand the phenomenon in humans of diminished vaccine efficacy of BCG after exposure to Env. We wished to test the hypotheses that cytotoxicity plays a role in immune responses induced by *M. chelonae* (as a representative of Env) against BCG, and that there was also a regulatory T cell response induced by Env sensitisation.

The objectives of this study are:

1) To determine if Env sensitisation in a murine model primes for cytotoxicity against BCG-infected cells, and the cell subsets and cytokines involved.
2) To examine evidence for a regulatory T cell response invoked by Env sensitisation, and the functional consequences on subsequent live BCG exposure.

CHAPTER 3 MATERIALS AND METHODS

3.1 Mice

BALB/c mice between 5 – 6 weeks old were purchased from the Centre for Animal Resources (CARE) and Biological Resources Centre (BRC). Mice were maintained in the departmental animal facility, housed in individual isolator cages (Alternative Design, US) with filter tops. Food and water were supplied *ad lib*, and autoclaved beddings were changed twice a week. All experiments were carried out with the approval of the institutional animal care and use committee.

3.2 Bacteria

Mycobacterium chelonae derived from clinical samples cultured on Lowenstein-Jensen media, was a generous gift from Dr Pam Nye, University College London Hospitals (UK). *Mycobacterium bovis* BCG (Pasteur) vaccine strain was donated by Dr William Jacobs, Jr (Albert Einstein College of Medicine, USA). All species were subsequently cultivated on Middlebrook 7H10 agar (refer to appendices), supplemented with oleic acid-albumin-dextrose-catalase (OADC; Difco), and single colonies picked for growing in Middlebrook 7H9 broth (Difco) + 20 % Tween 80 (refer to appendices). Some cultures were stored in 50 % glycerol aliquots at -80 °C before use.

3.3 Preparation of heat-killed and live mycobacterial cultures

Mycobacterium bovis Bacille Calmette-Guérin (BCG) and *Mycobacterium chelonae* broth cultures were grown to mid-log phase. Required volumes of culture were subsequently centrifuged at 2500 x *g* for 10 min and washed twice with sterile phosphate buffered saline (PBS, prepared with nanopure water) before re-suspension in PBS. This bacterial suspension was passed via a syringe through a 27 G needle to reduce clumping, before absorbance was measured at 600 nm to estimate bacterial numbers (1 A_{600} ~ $2x10^8$ bacteria). Bacterial suspensions were diluted to obtain 1 x 10^6 cells/ 10 µl PBS or 1 x 10^7 cells/ 50 µl PBS for murine immunisation and lymphocyte restimulation respectively. These preparations were subsequently heat-killed at 95 °C for 10 min, and stored at -20 ºC until use. For intranasal infection of mice, 0.15 - 1 x 10^6 live BCG were re-suspended in a final volume of 10 µl PBS. Such preparations were kept at 37 ºC prior to infection. All BCG preparations were subjected to purity check and counting of the actual colony-forming units (CFU) – bacteria were re-suspended in Middlebrook 7H9 and serial dilutions plated on Middlebrook 7H10 agar. Bacterial colonies were counted 3 weeks after incubation at 37 °C.

3.4 Murine immunisation and live BCG challenge

Mice were immunised thrice at weekly intervals with 10^6 heat-killed *M. chelonae* in 100 µl sterile PBS, prepared as described above, via the intraperitoneal (i.p.) route. If live

BCG was given, it was prepared with sterile PBS. The infected mice were sacrificed at one week or three weeks post-infection.

3.5 Trypan Blue exclusion assay

To count viable murine cells, 10 µl of cell suspension was added to 10 µl of 0.04 % trypan blue dye (Merck, Germany) at room temperature and mixed by pipetting. Subsequently, 10 µl of the mixture was loaded into a single chamber of a haemocytometer for cell counting. Non-viable cells were stained blue because of their inability to limit the entry of the blue dye, while viable cells remain clear. Only unstained cells were enumerated.

3.6 Isolation of murine peritoneal macrophages

Mice were sacrificed by CO_2 asphyxiation at appropriate time points. To harvest peritoneal macrophages, 5 ml of ice-cold RPMI 1640 supplemented with 2 mM L-glutamine (RPMI) + 10 % fetal bovine serum (FBS) was injected into the peritoneal cavity via an 18G needle and the peritoneum gently massaged before withdrawal of the peritoneal fluid. This process was repeated with a new needle, and all peritoneal fluid was subsequently kept on ice, until use. Cell suspensions were centrifuged at 400 x *g* for 10 min at 4°C, and cell pellet re-suspended in 2 ml of RPMI + 10 % FBS. Cell numbers were obtained and cells seeded into tissue culture wells to obtain adherent cells after overnight culture.

3.7 Isolation of murine splenocytes and lung tissue

Lung tissue was subjected to enzymatic digestion in 1 ml of 0.34 PZ-U/ ml collagenase (NB 4 standard grade; Serva, Germany) at 37 °C for 1 h, but splenocytes were not treated. Both organs were homogenised through sterile 40 μm nylon cell strainers (BD Falcon). The cells were suspended in 5 ml of RPMI + 5 % FBS, centrifuged at 350 x *g* for 10 min, and the pellet re-suspended in 1 ml of 0.17 M NH_4Cl (refer to appendices) for 90 sec to lyse the red blood cells. The cells were immediately diluted in an additional 5 ml of RPMI + 5 % FBS, centrifuged at 350 x *g* for 10 min, and cells re-suspended in RPMI + 5 % FBS before cell numbers were counted.

B cells were depleted from splenocytes using Dynabeads Mouse pan B (B220; Dynal Biotech ASA, Oslo, Norway) at 1 bead: 1 splenocyte ratio. According to the manufacturer's instructions, briefly, splenocytes were labelled with anti-CD19 linked to magnetic beads, in RPMI + 5 % FBS for 30 min at 4 °C. After negative magnetic selection, a portion of the B cell-depleted splenocytes were seeded at 2 x 10^6 cells/ ml of RPMI + 5 % FBS in a 24-well plate (Greiner) for antigen restimulation while the remaining B cell-depleted splenocytes underwent further CD4-sorting (see below). All experiments utilising murine splenocytes were derived following this treatment.

3.8 Positive cell selection using magnetic beads

$CD4^+$ and $CD4^-$ T lymphocytes from B cell-depleted splenocytes were derived using the

$CD4^+$ positive selection MACS kit (Miltenyi Biotec). Splenocytes were incubated in staining buffer, with CD4-specific antibodies coupled to magnetic beads at 4 °C for 15 min in the dark, according to manufacturer's instructions. Following processing through the AutoMACS (automated Magnetic Cell Sorting; Miltenyi Biotec) column, magnetically-labelled cells were separated from non-labelled cells using the 'positive selection' mode. The labelled and non-labelled cells were collected from the positive and negative ports respectively, in 5 ml of RPMI + 5 % FBS. Cells were subsequently counted, centrifuged at 350 x *g* for 10 min before re-suspension in appropriate volumes of RPMI + 5% FBS for experiments.

3.9 Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) was performed immediately after sacrificing the mice subjected to BCG challenge. Sterile PBS (600 µl) was instilled via the trachea into the lungs twice, and the BAL fluid withdrawn and centrifuged at 600 x *g* for 5 min. The supernatant was stored at -20 °C prior to cytokine analysis whereas the cells were re-suspended in 160 µl of PBS, counted and diluted, if required, to a maximum concentration of 2 x 10^5 cells/ 150 µl PBS. The 150 µl cell suspension was loaded onto the Cytospin 3 (Thermo Shandon Fisher Scientific) centrifuge and cells concentrated onto a single spot on glass slides after spinning at 550 rpm for 5 min. The slides were heat-treated, fixed in methanol for 15 min and stained with 10% Giemsa (Applichem, Germany) for 20 min. The number of macrophages, neutrophils, lymphocytes and eosinophils were obtained by visually counting the cells under the microscope, and their adjusted numbers in 1 ml of BAL fluid was calculated.

3.10 Cytokine analysis by ELISA

Total splenocytes, $CD4^+$ and non-$CD4^+$ (CD4-) splenocytes were seeded at 2 x 10^6 cells/ ml in each well of a 24–well plate for re-stimulation. After 48 h of stimulation with heat-killed *M. chelonae*, supernatants harvested from cell cultures were assayed by Enzyme-Linked Immunosorbent Assay (ELISA) for the presence of IFN-γ, TGF-β (BD Pharmingen), IL-10 (R&D Systems) and IL-2, IL-4 (BioLegend), using the respective kits according to manufacturer's instructions. All assays were based on the sandwich ELISA. The ELISA plate (Co-star or BD Falcon) wells were coated with diluted cytokine-specific capture antibody overnight, blocked using assay diluent, and subsequently incubated with culture supernatant or diluted recombinant cytokine standards. Biotinylated antibodies specific for the respective cytokines were used as the detecting antibody, and streptavidin- or avidin- horse radish peroxidase (HRP) were used in conjunction with TMB substrate to produce a colormetric change. The absorbance was read at 450 nm with a correction wavelength of 570 nm using the Magellan ELISA reader (Tecan, Switzerland) and the amount of cytokine in the samples was derived by interpolation from the standard curve. The detection limit for the ELISA assay used was 1 pg/ ml for IL-4 and IL-2, 31.2 pg/ ml for IFN-γ and 62.5 pg/ ml for TGF-β.

3.11 BCG killing assay by peritoneal macrophages

Freshly isolated peritoneal cells were seeded at 2 x 10^5 cells/ 200 µl RPMI + 5% FBS in 96-well round-bottom tissue culture plates and incubated overnight at 37 °C in

humidified air containing 5% CO_2. The non-adherent cells were removed the next day, and fresh medium added with ferric ammonium citrate (FAC) at a working concentration of 50 μg/ml (refer to appendices). Wells were included for counting macrophages seeded after trypsinisation of the adherent cells.

The macrophages in each of the test wells were infected with live BCG in 10 μl of Middlebrook 7H9 medium, at a MOI of 10:1, for 4 hours at 37 °C with 5 % CO_2. Middlebrook 7H9 medium alone was added to negative control wells. At the end of the 4 hour incubation, extracellular BCG was removed by washing the wells gently with 100 μl of RPMI + 5 % FBS. Macrophages were lysed by adding 200 μl of freshly prepared 0.1% saponin (Sigma-Aldrich). The supernatants from each well were centrifuged at 2000 x *g* for 10 min, and re-suspended in 100 μl of 7H9 medium. Serial dilutions of the resultant bacteria suspension were cultured in triplicates and colonies counted after 3 weeks of incubation.

3.12 Flow Cytometry

3.12.1 Cell surface markers

Lung cells designated for flow cytometry were PBS-washed and 0.5 – 1 x 10^6 cells/tube re-suspended in 50 μl of staining buffer (PBS + 0.5 % bovine serum albumin (BSA; Sigma). The cells were stained for various cell surface markers by adding 2 μl of relevant antibodies per tube and incubated for 30 min on ice in the dark. Unbound antibodies were removed by washing with 1 ml of ice-cold PBS, and cells were fixed in 4 %

paraformaldehyde. Samples were kept at 4 °C in the dark before analysis by the flow cytometer (Cytomics FC500, Beckman Coulter). Fluorescence was analysed by measuring fluorescent intensity of the fluorochromes used, i.e. fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or phycoerythrin-cyanate 7 (PE-Cy7).

Mouse specific mAbs CD3-FITC (hamster IgG1, κ), CD4-APC (rat IgG2a, κ), CD3-PE (hamster IgG1 κ), CD11b-FITC (rat IgG2b κ), CD11c-APC (hamster IgG1) were purchased from BD Bioscience, while biotin-CD8 (rat IgG2a, κ), streptavidin PE-Cy7, and CD49b/Dx5 (pan-NK) –FITC (rat IgM, κ) were purchased from BioLegend, and F4/80-biotin (rat IgG2b, κ) from Serotec. These mAbs were used with relevant isotype controls. CD49b (Dx5) is mainly expressed on NK cells and NKT cells, and can be used for the identification and isolation of NK cells. CD3, CD11b, F4/80 and CD11c are cellular markers for determining lymphocytes, neutrophils, macrophages and dendritic cells respectively. To determine absolute cell numbers of each cell type, the samples were spiked with fixed volumes of known concentrations of Flow-Count Fluorosphere® (Beckman Coulter) which provided the reference for cell numbers.

3.12.2 Intracellular cytokine and perforin staining

Lung cells for perforin staining were used directly, while those for cytokine staining were seeded at 1 x 10^6 cells in 1 ml of RPMI + 10 % FBS per well in 24 well flat-bottom tissue culture plates. In each well, 1 μl of 10 mM ionomycin and 200 μg/ml of phorbol

myristate acetate (PMA), in the presence of 3 μM monensin (Sigma), were added. After 6 h of culture, the cells were centrifuged at 300 x g, 4 °C for 10 min, washed once in 1 ml of ice-cold staining buffer, surface stained and fixed for 5 min as described above. The cells were then permeabilised in 1 ml of PBS + 0.1 % saponin + 1 % FBS (PBS-S), and incubated in 50 μl of PBS + 0.1 % saponin + 0.1 % BSA (PBS-S/BSA) for 30 min on ice. Cells were subsequently stained in the dark with IL-10 PE, IFN-γ-PE or perforin-PE mAbs in PBS-S/BSA for 30 min at 4 °C, washed with PBS-S and finally re-suspended in 200 μl of PBS/BSA. The anti-mouse mAbs used were IL-10-PE (rat IgG2b, κ, BioLegend) or IFN-γ-PE (rat IgG1 κ, BD Bioscience) or perforin-PE (eBiosciences), with relevant isotype controls. Samples were then analysed on the flow cytometer within 24 h.

3.13 Cytotoxicity assay

3.13.1 Principle of assay

A non-radioactive CytoTox 96® Assay kit (Promega) was used to measure cell-mediated cytotoxic responses following antigen stimulation. The CytoTox 96® Non-radioactive Assay is a colorimetric assay that quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. The conversion of a tetrazolium salt (INT) into a red formazan product by LDH released in culture supernatants was measured by a 30 min coupled enzymatic assay. The amount of red formazan formed is proportional to the amount of LDH released, which is also proportional to the number of lysed cells when the appropriate controls were subtracted. The amount of the formazan

was measured at 490 nm, with the reference wavelength at 650 nm. The percentage cytotoxicity was calculated as:

$$\% \text{ Cytotoxicity} = \frac{(\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}) \times 100)}{\text{Target Maximum} - \text{Target Spontaneous}}$$

3.13.2 Cytotoxicity assay experimental set-up

Freshly isolated murine peritoneal cells were seeded in triplicates at 1 x 10^5 and 2.5 x 10^5 in 200 μl of RPMI + 5 % FBS for PBS- and *M. chelonae*- immunised mice respectively, in 96-well round-bottom tissue culture plates. The adherent cells after overnight culture were used as target cells. Separately, to generate effector cells, total B cell-depleted splenocytes were seeded at 1.5 x 10^6 cells/ ml in tissue culture flasks, while $CD4^+$, $CD4^-$ and total B-cell depleted splenocytes were seeded at 2 x 10^6 cells/ ml in 24 well tissue culture plates for antigen stimulation.

After 48 h of antigen stimulation using heat-killed *M. chelonae* at a bacteria to cell ratio of 10:1, non-adherent effector cells were harvested for viability count, and the culture media replaced with fresh RPMI without phenol red (Invitrogen) + 2 % FBS + FAC at a working concentration of 50 μg/ml. To evaluate adherent cell numbers, in certain wells, these cells were trypsinised and counted by trypan blue exclusion. Target (adherent) cells were infected with live *M. bovis* BCG at an infection ratio (MOI) of 10:1, with added FAC at a working concentration of 50 μg/ml to enhance intracellular mycobacteria growth. Extracellular bacteria were removed after 4 hours by gently aspirating the supernatant and washing the wells once with fresh complete media. The effector and

target cells were then co-cultured at an effector to target cell ratio of 10:1 and the entire plate was centrifuged at 250 x *g* for 4 min to allow for maximum contact between the effector cells and target macrophages. The plate was then incubated for 12 h at 37 °C in a humidified chamber with 5 % CO_2. At the end of the co-culture, the plate was again centrifuged at 250 x g for 4 min to obtain the cell-free supernatant. Certain control wells were also set up – effector cells added to wells without target cells ('Effector Spontaneous'), target cells without effector cells ('Target Spontaneous'), and target cells vigorously scraped off the plate, subjected to freeze-thawing for 10 sec to lyse cells completely ('Target Maximum').

Fifty microlitres of supernatant from each well were transferred into 96-well flat-bottom non-sterile plates and 50 µl of reconstituted substrate mixture from the assay kit was added to each well for 30 min at room temperature in the dark. Thereafter, 50 µl of stop solution was added. Intensity of colour change in individual wells was measured using the Magellan ELISA Reader (Tecan) at 490 nm with reference wavelength at 650 nm and the percentage cytotoxicity was calculated according to the formula given above.

3.14 Statistical analysis

Means of triplicate well assays were compared using a two-tailed Student *t* test. Where the distribution of data (especially from replicate mice) did not conform to a normal distribution, the medians of the experimental groups were compared using the non-parametric Mann-Whitney *U* test, and the 25^{th} and 75^{th} percentiles were described for the distribution. Differences between groups were considered statistically significant when $p < 0.05$.

CHAPTER 4 RESULTS

4.1 Distribution of inflammatory cells in lungs of BCG-infected mice

The distribution of inflammatory cells in the lungs of PBS- and *M. chelonae* -immunised mice following BCG infection was examined. Three weeks after intranasal BCG instillation, cells in the bronchoalveolar lavage fluid (BALF) of infected mice were concentrated on slides and the number of macrophages, eosinophils, lymphocytes and neutrophils counted, based on their morphology (Fig. 1). Absolute cell counts of total lung cells, dendritic cells (DCs), neutrophils, T cells and macrophages extracted from the inflamed whole lung tissue 1 week post-challenge were measured by flow cytometry (Fig. 2).

In the BALF, overall there was a lower number of inflammatory cells induced by BCG infection in *M. chelonae*-sensitised mice compared to control mice (Fig. 1B), although the differences were not statistically significant. The absolute number of total cells, DCs, neutrophils, T cells and macrophages in the inflamed lung tissue 1 week after BCG infection was also lower in *M. chelonae*-immunised mice compared to control mice (Fig. 2). This was especially evident in total cell count and macrophage count, where there was an approximately 2-fold difference between *M. chelonae*-immunised and control mice ($p<0.05$, Fig. 2).

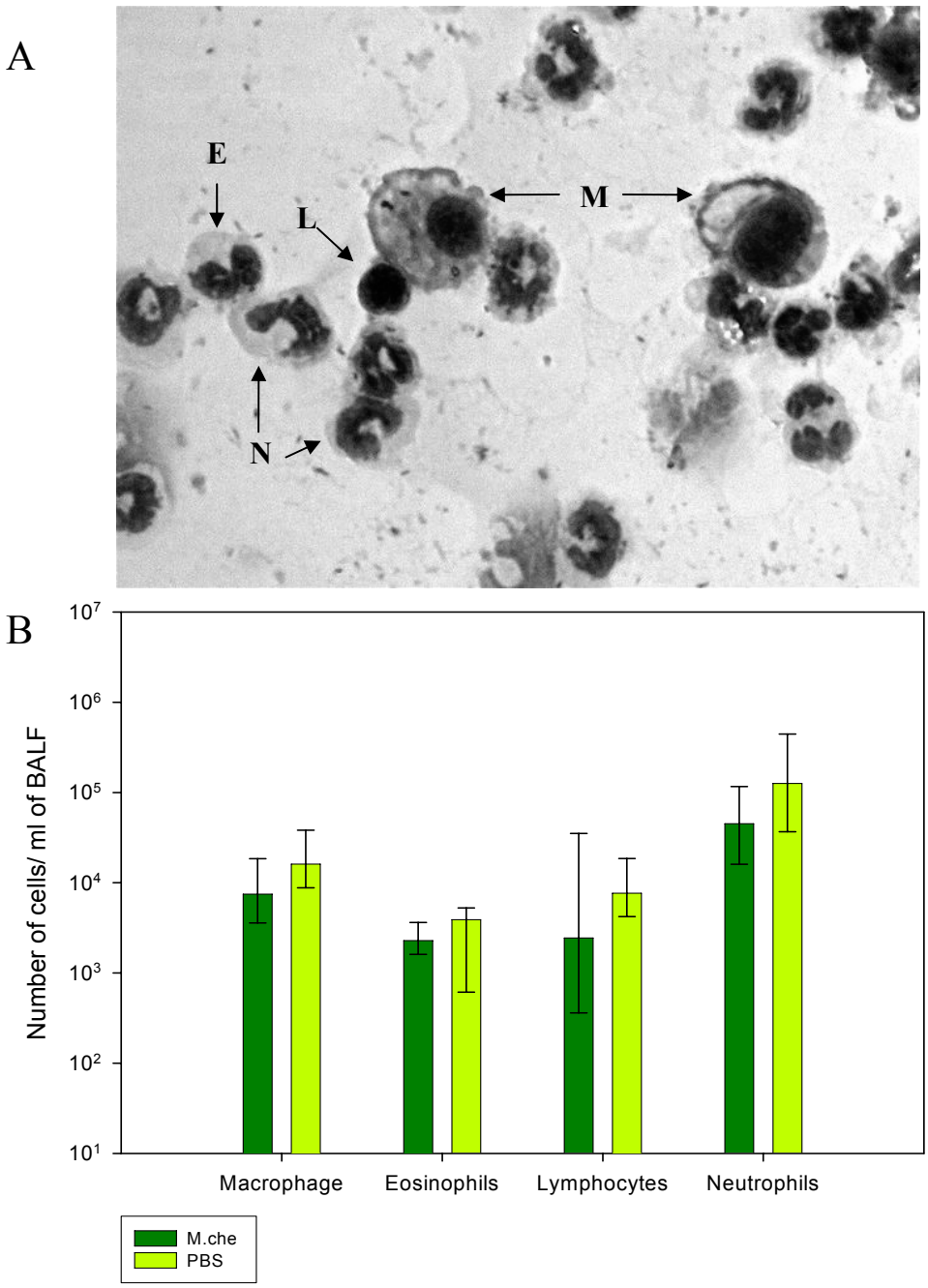


Fig. 1: Cell counts of immune cells in the bronchoalveolar lavage fluid (BALF) of M. chelonae (M.che) sensitised and control (PBS) mice after BCG infection. BALF of immunised and control mice 3 weeks after BCG infection was obtained by flushing the lungs with PBS. Cells from the BALF were concentrated on glass slides, Giemsa stained, and cell numbers enumerated under the microscope. **A:** Immune cells were differentiated according to their morphology. M: macrophage, N: neutrophil, L: lymphocyte, E: eosinophil. **B**: Number of different immune cell types per ml of BALF. Data represents an average of four individual experiments comprising four mice per experimental group. Results are expressed as medians, 75th and 25th percentiles.

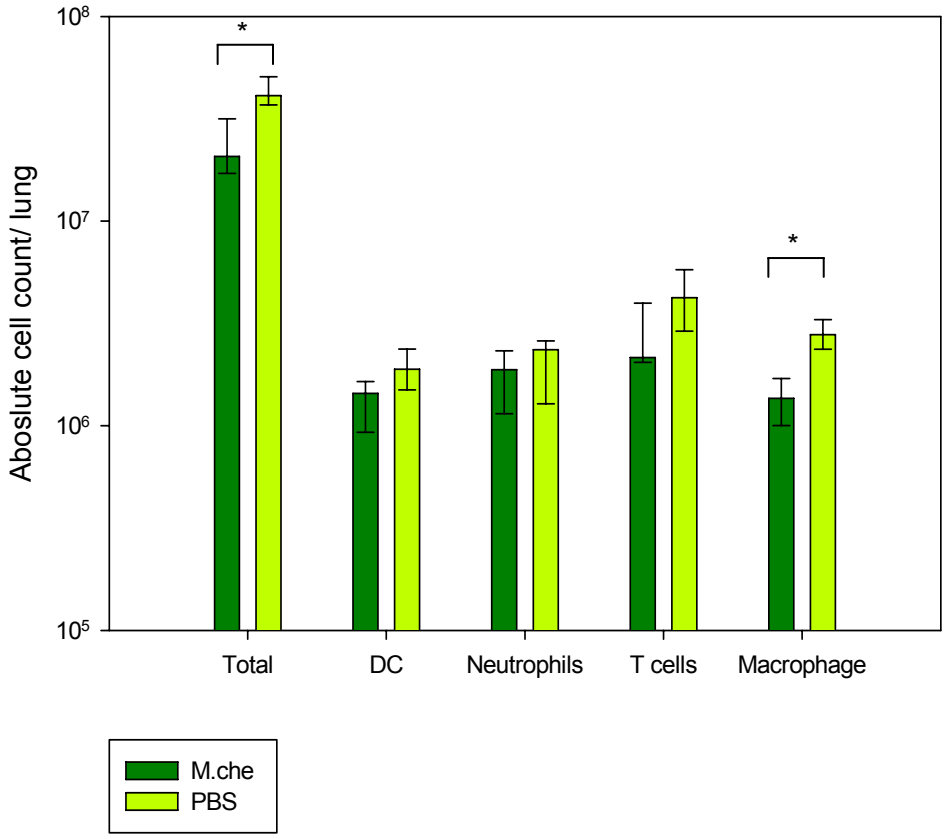


Fig. 2: Absolute cell count of immune cells in the lungs of M. chelonae sensitised and control mice after BCG infection. Lung single-cell suspensions from immunised (M.che) and control (PBS) mice 1 week post BCG infection were stained with monoclonal antibodies against CD11c (dendritic cells, DC), CD3 (T cells), CD11b (neutrophils) and F4/80 (macrophages), and analyzed by flow cytometry. Fluorospheres of known concentration were used concurrently to obtain an absolute cell count. The figure represents an average of two separate experiments comprising four mice per experimental group. Results are expressed as medians, 75^{th} and 25^{th} percentiles. Statistical significance was determined by non-parametric Mann Whitney-U test. *$p<0.05$ comparing immunised with control mice.

In order to further evaluate the distribution of possible effector immune cells in the inflamed lung, the distribution of cells with particular markers of interest, namely CD3 (T cells), CD4, CD8 (T cell subsets), and CD49b (NK cell) were observed by flow cytometry (Tables 1, 2, Fig. 3). The percentage of $CD49b^+$, $CD3^+$ and $CD8^+CD4^-$ cells in the total lung cell population cell populations in the lung remained relatively similar for sensitised and control mice (Table 1). The proportion of $CD4^+CD8^-$ cells in the T cell population was significantly higher in *M. chelonae*-immunised mice compared to control mice ($p<0.01$, *M. chelonae*- vs PBS-immunised mice; Table 2, Fig. 3). This suggests that *M. chelonae* sensitisation prior to BCG infection induces the expansion of $CD4^+$ cells to a greater extent than without pre-sensitisation.

	Cell marker	**PBS**	***M. che***
% of total lung cells	**CD49b+**	0.5 ± 0.2	0.2 ± 0.2
	CD3+	21.0 ± 6.2	22.4 ± 3.9
	CD8+CD4-	6.6 ± 1.2	6.3 ± 1.8
	CD4+CD8-*	7.6 ± 1.4	12.8 ± 1.6

Table 1: Percentage of different subsets of cells out of total lung cells. Lung single-cell suspensions from immunised and control mice, 1 week post BCG infection, were stained with CD3, CD4, CD8 and/ or CD49b (natural killer cell, NK)-specific mAbs, followed by flow cytometry. Results are expressed as mean ± standard deviation (SD) of two individual experiments. Each experimental group comprises four mice. Statistical significance was determined by two-tailed Student's t test. *$p<0.01$ comparing immunised with control mice.

	Cell marker	PBS	*M. che*
% of CD3+ cells (lymphocyte gate)	CD4+CD8-*	38.9 ± 9.2	51.4 ± 6.6
	CD8+CD4-	21.8 ± 6.1	17.4 ± 4.7
	CD4+CD8+	6.6 ± 3.9	8.5 ± 5.3
	CD8-CD4-	33.0 ± 17.7	23.3 ± 14.1

Table 2: Percentage of different subsets of cells out of lymphocyte gate. Lung single-cell suspensions from immunised and control mice, 1 week post BCG infection, were stained with CD4, CD8 and CD3 (T cell)-specific mAbs, and analysed by flow cytometry. Data shown are gated on CD3 cells.The mean ± 1 SD for the whole experimental group is shown (4 mice per group) and incorporates data from two independent experiments. Statistical significance was determined by two-tailed Student's t test. *p<0.01 comparing immunised with control mice.

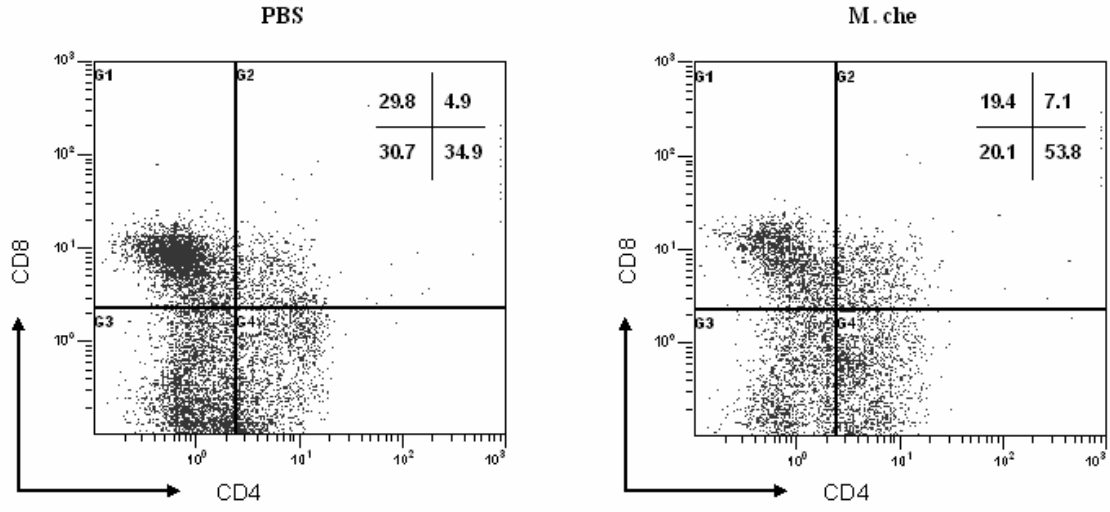


Fig. 3: Distribution of different subsets of cells in the infected lung (gated on $CD3^+$ T cells). Data shown are gated on CD3 cells. For the same experiment illustrated above, a single representative experiment is shown here, mean data for the experimental groups is presented in the table above.

4.2 Cytokine expression in different cell subsets in BCG-infected lungs

It was postulated that differential cytokine expression may account for the observed differences in inflammatory responses between *M. chelonae*-sensitised and control mice one week after BCG infection. Thus, the percentage of lung T cells producing IFN-γ or IL-10 and their surface markers ($CD4^+$ or $CD8^+$) in the infected mice was analysed by flow cytometry (Figs. 4-6, Tables 3, 4).

A predominant percentage of IFN-γ producing T cells were $CD4^+$ in both control and sensitised mice ($CD4^+$: $CD8^+$ ratio 1.6 and 2.2 respectively; Fig. 4A). *M. chelonae*-sensitised mice had a higher proportion of $CD4^+$ cells among T cells producing IFN-γ (55.5 ± 5.9 %) compared to control mice (48.1 ± 7.3 %). In particular, among the IFN-γ producing T cells, cells of the $CD4^+CD8^-$ phenotype were of a significantly higher proportion in *M. chelonae*-sensitised mice (45.8 ± 4.8 %) compared to control mice (35.75 ± 2.5 %) ($p<0.05$).

Within each of the $CD4^+$ and $CD8^+$ T cell populations, a higher percentage of IFN-γ^+ cells was observed in the lungs of sensitised mice compared to control mice (Table 3, Fig. 5). This difference was more obvious in the $CD4^+$ T cell population, where 13.3 ± 0.81 % of $CD4^+$ cells was producing IFN-γ in sensitised mice, as compared to 8.8 ± 2.5 % in control mice ($p<0.05$, Fig. 5, Table 3).

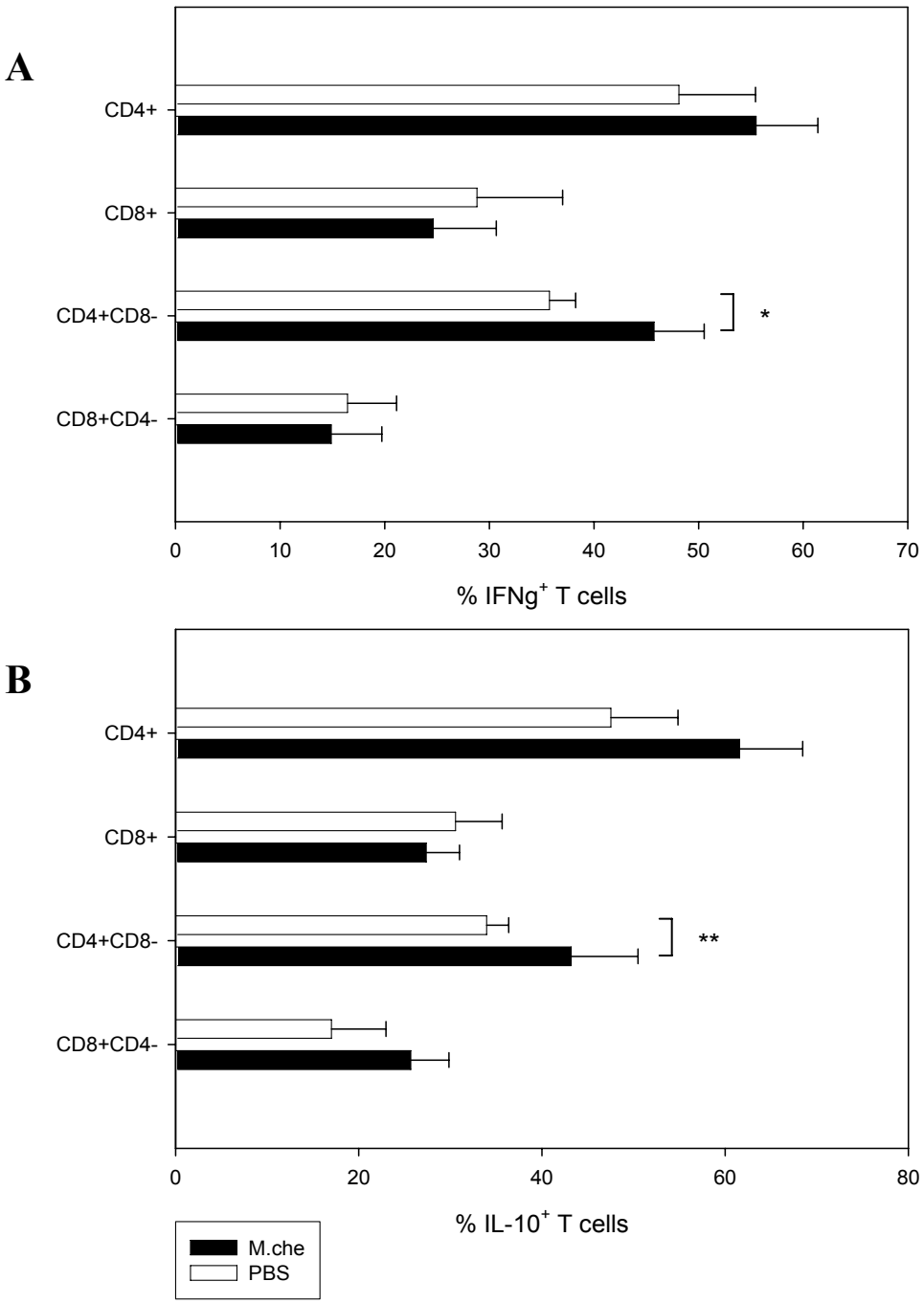


Fig. 4: Distribution of various cell types among CD3$^+$ cells producing IFN-γ or IL-10. Lung single-cell suspensions from immunised and control mice, 1 week post *BCG* infection, were stimulated with PMA and ionomycin, and treated with monensin for 6 h. Cells were stained for expression of CD4, CD8, CD3, and either IFN-γ or IL-10 before analysis by flow cytometry. The analysis was focused only on cytokine-positive cells within the CD3 (T cell) gate. Results for the whole experimental group is shown (4 mice per group) and are expressed as mean % of each cell subset out of all IFN-γ^+ **(A)** or IL-10^+ **(B)** cells ± 1 SD. Results incorporate data from two independent experiments. Statistical significance was determined by two-tailed Student's t test. *$p<0.05$, **$p<0.001$ comparing immunised with control mice.

A similar trend for $CD4^+$ T cells was observed for IL-10 production. The $CD4^+$ cells were predominant over $CD8^+$ cells in the IL-10^+ T cell population of both experimental groups of mice ($CD4^+$: $CD8^+$ ratio 1.6 and 2.2 respectively in control and *M. chelonae*-sensitised mice; Fig. 4B). Considering only the T cells producing IL-10, *M. chelonae*-sensitised mice had a higher percentage in the $CD4^+$ population (61.6 ± 6.9 %) compared to control mice (47.5 ± 7.3 %). This was particularly evident in the $CD4^+CD8^-$ population (47.1 ± 0.6 % and 34.0 ± 2.4 % for sensitised and control mice respectively, $p < 0.01$, Fig. 4B).

Considering the $CD4^+$ and $CD8^+$ T cell populations separately, a higher percentage of cells producing IL-10 was observed in sensitised mice compared to control mice (Fig, Table 4, Fig. 6). Within the $CD4^+$ T cell population, 14.4 ± 1.9 % in sensitised mice were producing IL-10, as compared to 10.2 ± 2.2 % in control mice, while in the $CD8^+$ population, 10.6 ± 1.8 % and 6.7 ± 1.3 % was producing IL-10 for sensitised and control mice respectively (Fig. 6, Table 4).

In summary, with respect to cytokine production in BCG-infected lungs, $CD4^+$ cells comprised the predominant population of cells producing IFN-γ and IL-10. In addition, it was generally observed that a higher level of cytokine, in *M. chelonae*-sensitised than control mice, was secreted within the $CD4^+$ and $CD8^+$ populations.

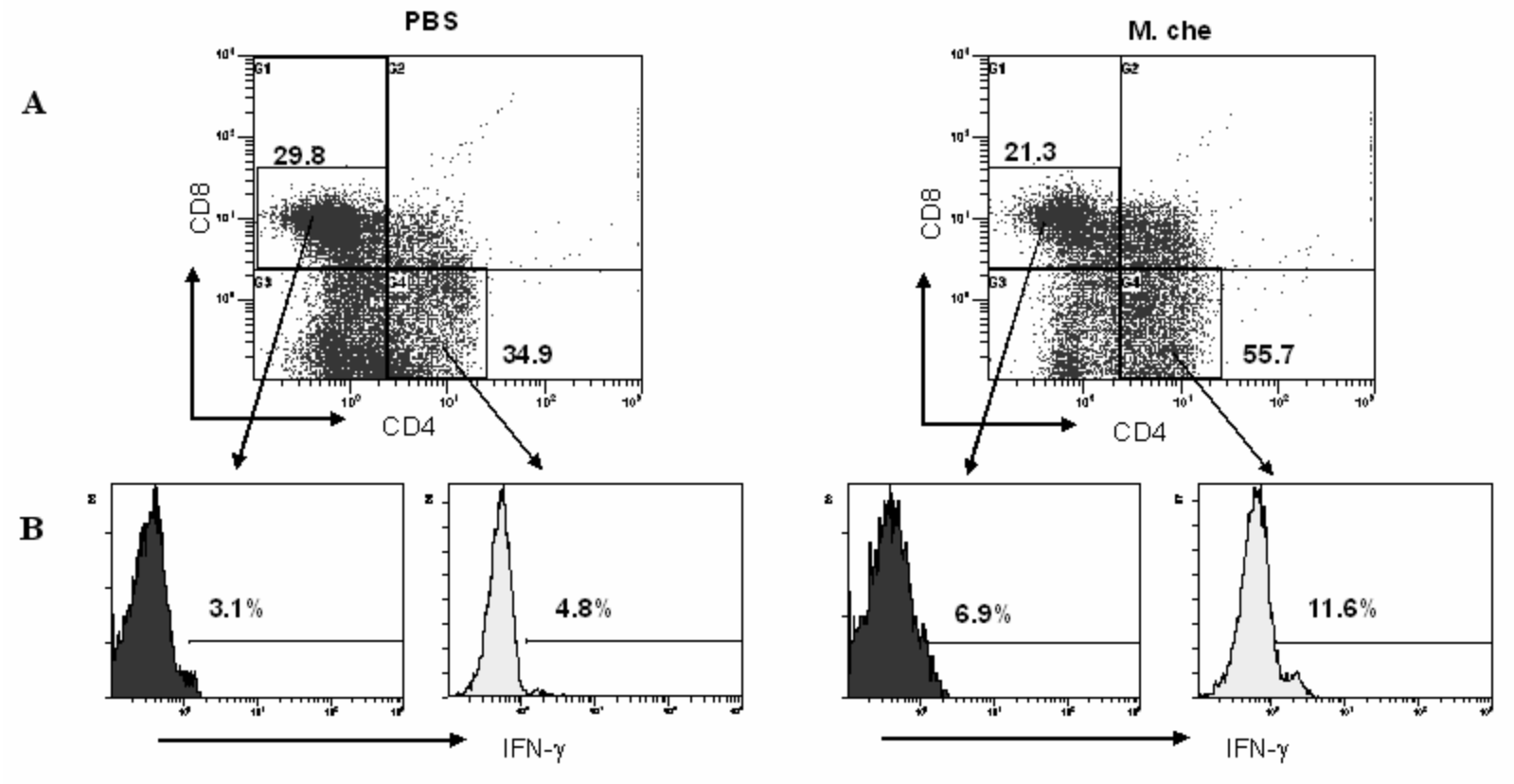


Fig. 5: IFN-γ production in lung T cell subsets. Lung single-cell suspensions from immunised and control mice, 1 week post BCG infection, were stimulated with PMA and ionomycin, and treated with monensin for 6 h. Cells were stained for expression of CD4, CD8, CD3, and IFN-γ before analysis by flow cytometry. **(A):** Distribution of CD4 and CD8 T cells, after gating on CD3 cells, in PBS and *M. chelonae*-immunised mice. (**B):** Percentage of IFN-γ^+ cells, gated on $CD4^+CD8^-$ and $CD8^+CD4^-$ cells. A single representative experiment is shown in A and B, the group mean data is presented in the table below.

	% of each cell subset expressing IFN-γ (CD3 gated cells)	
Cell types	**PBS**	***M. che***
CD4+*	8.8 ± 2.5	13.3 ± 0.81
CD8+	6.4 ± 3.1	9.2 ± 2.0
CD4+CD8-*	6.3 ± 2.8	11.2 ± 1.2
CD8+CD4-	4.7 ± 3.1	7.6 ± 2.3

Table 3: Percentage of $CD4^+$ or $CD8^+$ cells expressing IFN-γ (CD3 gated cells). Based on the same experiment described above in Fig. 5, the mean ± 1 SD for the whole experimental group is shown (4 mice per group) and incorporates data from two independent experiments. Statistical significance was determined by Student's t test. *$p<0.05$ comparing immunised with control mice.

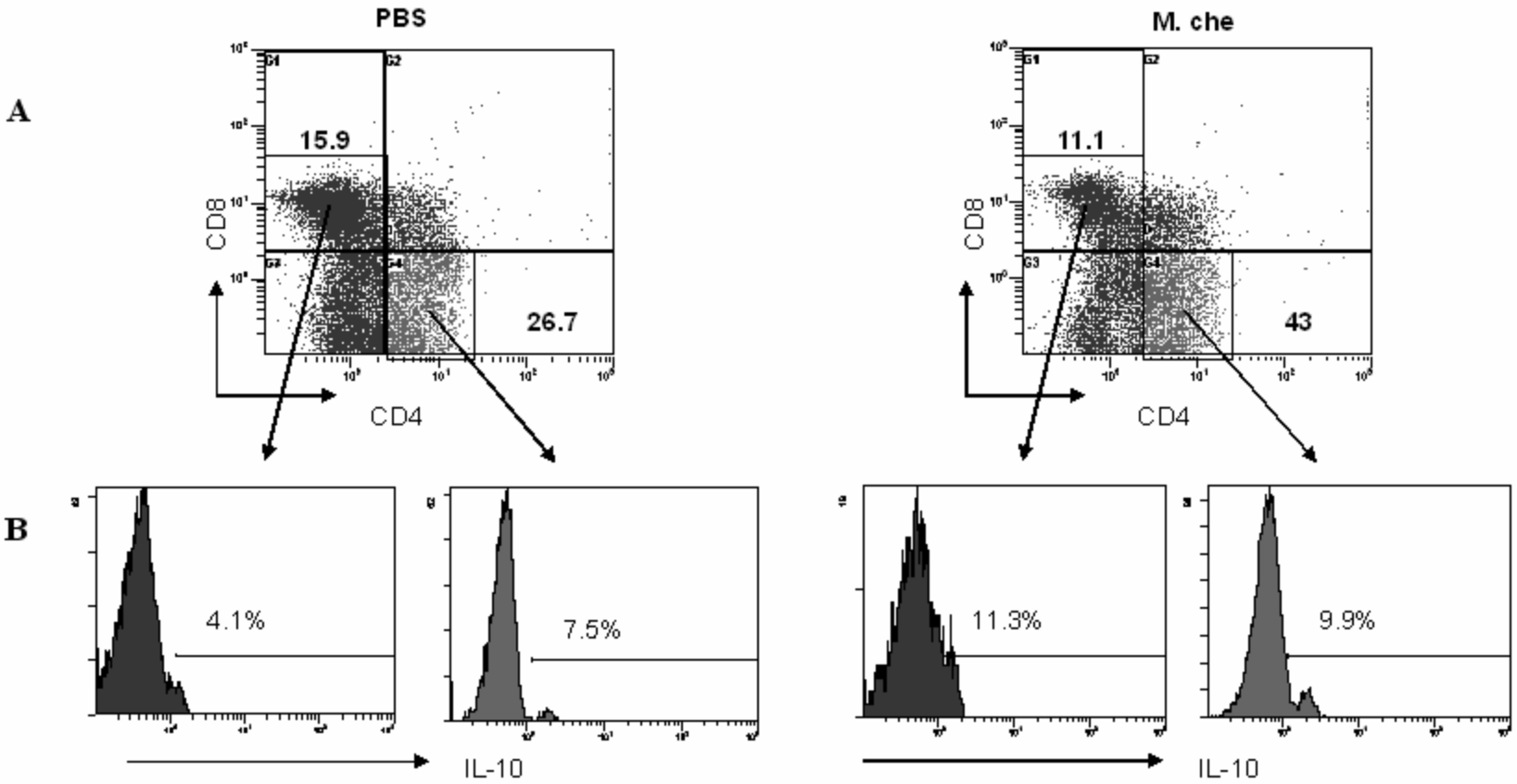


Fig. 6: IL-10 production in lung T cell subsets. Lung single-cell suspensions from immunised and control mice, 1 week post BCG infection, were stimulated with PMA and ionomycin, and treated with monensin for 6 h. Cells were stained for expression of CD4, CD8, CD3, and IL-10 before analysis by flow cytometry. **(A):** Distribution of CD4 and CD8 T cells, after gating on CD3 cells, in PBS and *M. chelonae*-immunised mice. **(B):** Percentage of IL-10^{+} cells, gated on $CD4^{+}CD8^{-}$ and $CD8^{+}CD4^{-}$ cells. A single representative experiment is shown in A and B, the group mean data is presented in the table below.

	% of each cell subset expressing IL-10 (CD3 gated cells)	
Cell types	**PBS**	***M. che***
CD4+	10.2 ± 2.2	14.4 ± 1.9
CD8+*	6.7 ± 1.3	10.6 ± 1.8
CD4+CD8-	6.9 ± 2.1	10.6 ± 3.4
CD8+CD4-	5.2 ± 1.1	8.5 ± 2.4

Table 4: Percentage of $CD4^+$ or $CD8^+$ cells expressing IL-10 (CD3 gated cells). For the same experiment illustrated above in Fig. 6, the mean ± 1 SD for the whole experimental group is shown (4 mice per group) and incorporates data from two independent experiments. Statistical significance was determined by Student's t test. *$p<0.05$ comparing immunised with control mice.

4.3 Distribution of $CD4^+$ and $CD4^-$ cells in the spleen

Splenocytes from mice were subjected to red cell and B cell depletion, then magnetically sorted into $CD4^+$ or $CD4^-$ cells. This was done to compare the cytotoxic activity of the two cell populations, in control and *M. chelonae*-sensitised mice, with and without subsequent BCG infection. The relative distribution of the two cell types was first assessed by direct cell counting after sorting. As shown in Table 5, the proportions of $CD4^+$ and $CD4^-$ cells were similar in both sensitised and control mice, and the proportions were not significantly affected by BCG infection.

	% of each cell subset in splenocytes			
	Non-infected		BCG infected	
	CD4+	CD4-	CD4+	CD4-
PBS	44 ± 9	57 ± 9	46 ± 10	54 ± 10
M. che	50 ± 5	50 ± 5	52 ± 4	48 ± 4

Table 5: Percentages of CD4 ($CD4^+$) and non-CD4 ($CD4^-$) cells in murine splenocytes in presence or absence of BCG infection. Single-cell suspensions of splenocytes from immunised and control mice were magnetically sorted into CD4 or non-CD4 cell portions. Cells were counted and numbers of each cell population calculated as a percentage of the total splenocyte cell number. Data are expressed as mean ± 1 SD. Results for the whole experimental group is shown (4 mice per group) and incorporates data from four (non-infected) and five (BCG infected) independent experiments.

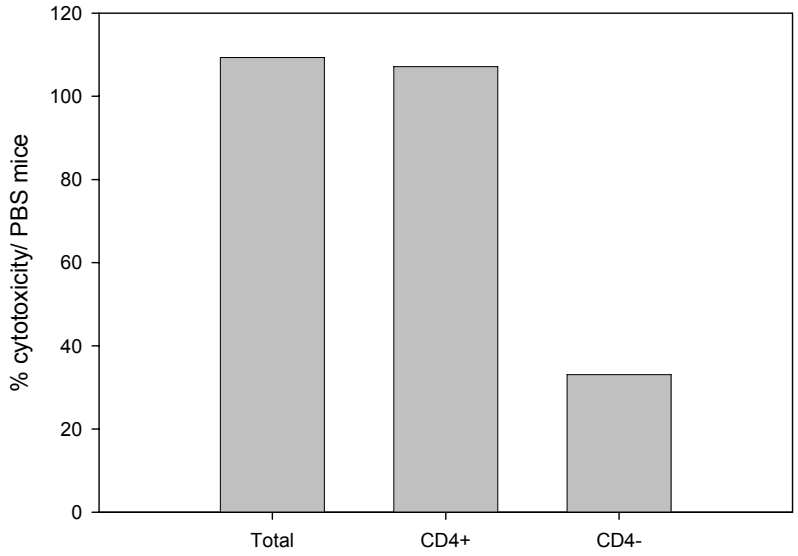
4.4 Cytotoxic activity following BCG challenge

Prior work in this lab has found that, comparing *M. chelonae*-immunised and control mice subjected to live BCG intra-nasal infection, the BCG load in the lungs of sensitised mice was 1 log lower than in control mice, at 3 weeks post-infection. It was postulated that this may be due to lymphocytes in *M. chelonae*-sensitised mice having higher cytotoxic activity than those in control mice. Thus, the cytotoxicity of different cell fractions in the infected murine spleens, against autologous BCG-infected macrophages was investigated.

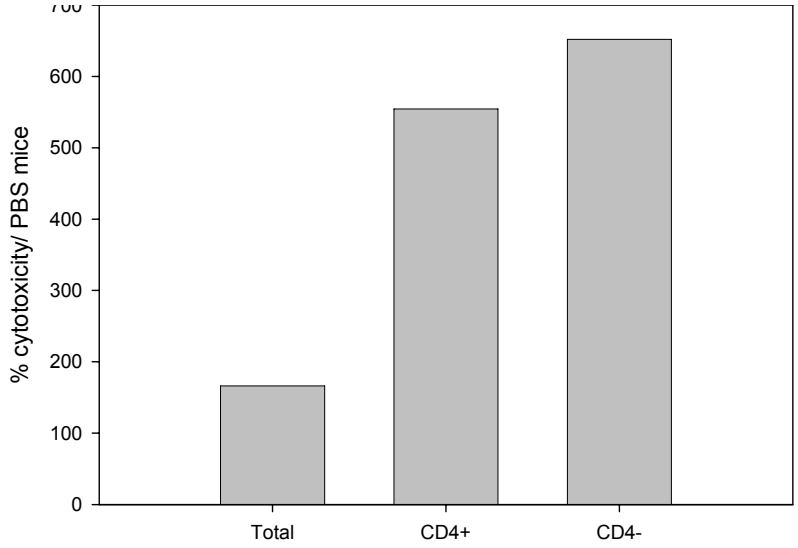
Cytotoxicity against BCG-infected autologous target cells was measured in splenocytes from *M. chelonae*-immunised and control mice 1 week and 3 weeks following intranasal BCG infection. All splenocytes were stimulated with heat-killed BCG for 48 h before carrying out the cyotoxicity assay.

At 1 week post-infection, total splenocytes and $CD4^+$ splenocytes from immunised mice had similar cytotoxic activity as the same cell subsets from PBS mice (Fig. 7A). $CD4^-$ splenocytes, on the other hand, showed lower cytotoxicity in sensitised mice, and a 3-fold difference was seen between sensitised and control mice (Fig. 7A). In contrast, at 3 weeks post-infection, *M. chelonae*-sensitised mice showed 5- and 6-fold higher cytotoxic activity in $CD4^+$ and $CD4^-$ cells respectively, compared to control mice (Fig. 7B). The difference was less marked in the total splenocyte population – there was approximately 2-fold higher cytotoxicity in sensitised mice (Fig. 7B).

A


120
100
80
60
40
20
0
% cytotoxicity/ PBS mice
Total
CD4+
CD4-

B


600
500
400
300
200
100
0
% cytotoxicity/ PBS mice
Total
CD4+
CD4-

C

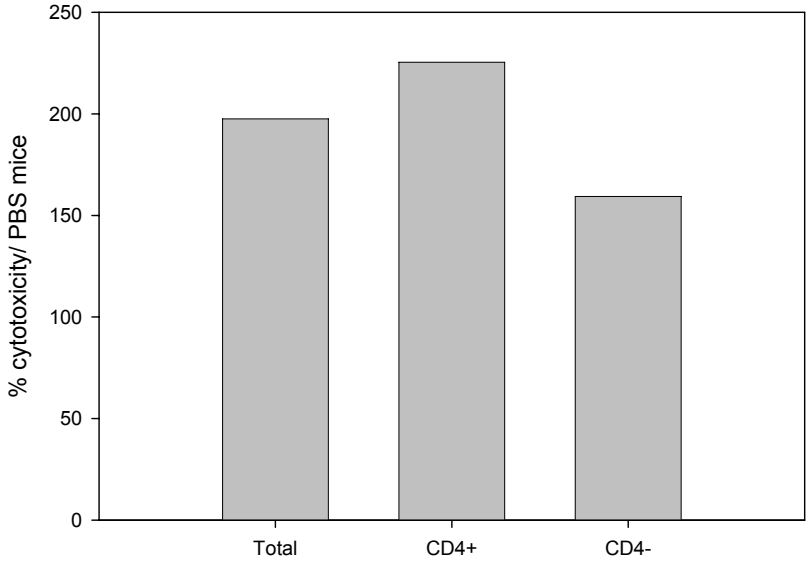

250
200
150
100
50
0
% cytotoxicity/ PBS mice
Total
CD4+
CD4-

Fig. 7: % cytotoxicity attributable to M. chelonae sensitisation. Splenocytes from immunised and control mice were sorted into different fractions ($CD4^+$ or $CD4^-$) and re-stimulated in vitro with heat-killed *M. chelonae*. Cytotoxicity of stimulated cells against autologous BCG-infected macrophages was quantified. The ratio of cytotoxicity exhibited by cells of immunised relative to control mice is shown (i.e. cytotoxicity of immunised mice/PBS mice x 100%). Data represent a mean of 3 independent experiments. The graphs show cells from mice 1 week **(A)**, 3 weeks **(B)** after intra-nasal BCG infection and without BCG infection **(C)**.

4.5 Cytotoxic activity in *M. chelonae*-sensitised mice

The same experiments, except with heat-killed *M. chelonae* stimulation for 48 h, were performed in the sensitised and control mice without BCG challenge, to see if this differential cytotoxicity already existed prior to the BCG infection. This was proven to be true, but the differences were less prominent than in the infected mice. Total splenocytes in sensitised mice had almost 2-fold higher cytotoxic activity in comparison to control mice (Fig. 7C). For $CD4^+$ and $CD4^-$ cell subsets, the *M. chelonae*-sensitised mice showed approximately 2-fold and 1.5-fold higher cytotoxicity respectively, compared to control mice (Fig. 7C).

The data on cytotoxicity suggest that *M. chelonae* sensitisation induces better cytotoxicity than without sensitisation, be it at a pre-infection or 3-weeks post-infection stage. It is notable though, that at 1 week post-BCG challenge, *M. chelonae* sensitisation did not seem to have an augmentative effect on cytotoxicity compared to control mice.

4.6 Perforin expression in BCG-infected lungs

To look at differential expression of perforin as a marker of cells with cytotoxic potential within infected murine lungs, the percentage of $CD4^+$, $CD8^+$ or $CD49b^+$ cells expressing perforin in mice with or without pre-sensitisation with *M. chelonae* was obtained by flow cytometry (Fig. 8, Table 6).

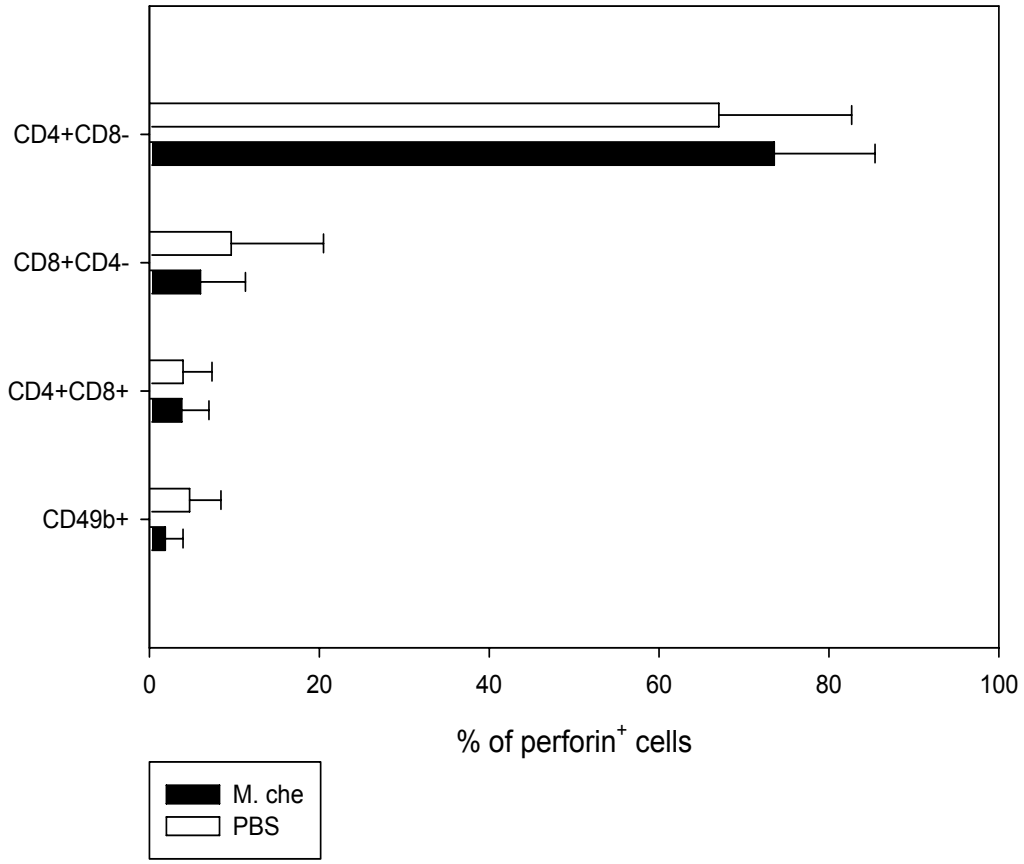


Fig. 8: Distribution of perforin producing cells in the lung. Lung single-cell suspensions from immunised and control mice, 1 week post BCG infection, were stained for expression of CD4, CD8, CD49b (NK cell), and perforin before analysis by flow cytometry. The analysis was focused only on perforin-positive cells. % of each cell subset within the perforin-expressing population is expressed as mean ± 1 SD. Results for the whole experimental group is shown (4 mice per group) and incorporates data from two independent experiments.

When only the perforin-positive cells were considered, the $CD4^+CD8^-$ were found to be the predominant perforin-producing cell type, accounting for approximately 70% of the $perforin^+$ cells, in both *M. chelonae*-sensitised and control mice (Fig. 8). Although not statistically significant, sensitised mice had a greater percentage of lung cells expressing perforin that were $CD4^+CD8^-$ than control mice (Fig. 8). However, in the control mice, the $CD49b^+$ population accounted for a higher percentage of the perforin-expressing cells than in sensitised mice (4.7% vs 1.9% of $perforin^+$ cells respectively, Fig. 8).

	% of each cell subset producing perforin (total lung cellls)	
Cell types	**PBS**	***M. che***
CD4+	19.2 ± 5.9	18.6 ± 6.2
CD8+	3.9 ± 4.3	3.5 ± 3.6
CD49b+	7.6 ± 3.4	14.7 ± 4.4

Table 6: Percentage of perforin-expressing cells within each immune cell subset in the lung. Lung single-cell suspensions from immunised and control mice 1 week post BCG infection were stained for expression of CD4, CD8, CD49b and perforin before analysis by flow cytometry. Each cell subset was gated separately, to derive the % of perforin-expressing cells within each cell subset. The mean ± 1 SD for the whole experimental group is shown (4 mice per group) and incorporates data from two independent experiments.

In both sensitised and control mice, there were 3-fold more $CD4^+$ cells producing perforin than $CD8^+$ cells (Table 6). Considering perforin expression within each individual cell population (i.e. $CD4^+$, $CD8^+$ and $CD49b^+$), the proportion of $CD4^+$ and $CD8^+$ cells producing perforin was similar in sensitised and control mice. However within the $CD49b^+$ cell population of *M. chelonae*-sensitised mice, 14.7% expressed perforin, whereas only 7.6% of the $CD49b^+$ cells in control mice were perforin-positive (Table 6).

4.7 Macrophage mycobactericidal activity

The mycobactericidal activity of macrophages has an important role in limiting BCG growth. As *M. chelonae* sensitisation is known to result in reduced BCG survival in vivo 3 weeks after infection, the reduced BCG load could be related to increased cytotoxic T cell activity and/or improved macrophage bactericidal activity. To evaluate if the macrophages of sensitised and control mice differed in their BCG killing abilities, peritoneal macrophages from each group of mice were infected *in vitro* with BCG for 4 hours and the surviving bacteria counts were subsequently enumerated by culture. There was no difference in the BCG colony-forming units in the wells derived from sensitised and control mice ($3.1 \pm 3.2\%$ vs $3.4 \pm 4.3\%$ of the original amount of BCG inoculated for PBS- vs *M. chelonae*-immunised mice), indicating that *M. chelonae* sensitisation had no direct effect on the ability of macrophages to kill BCG.

4.8 Cytokine production following *M. chelonae* sensitisation

It was postulated that the differential cytotoxicity and differential recruitment of immune cells to the BCG infected lungs attributable to *M. chelonae* sensitisation could be related to the cytokines produced by the cells post-sensitisation. Cytokines were assayed 1 week after *M. chelonae* or PBS immunisation, as well as 1 and 3 weeks after sensitisation followed by intranasal BCG infection. Only splenocytes were studied as it was not possible to obtain sufficient lung cells to perform the same assay. Total splenocytes, $CD4^+$ or $CD4^-$splenocytes were cultured for 48 hours with either heat-killed *M. chelonae* (pre-infection mice) or BCG (post-infection mice), and cytokine production measured in their supernatants by ELISA.

4.8.1 IL-10 production

IL-10 was investigated as a possible reason for the reduced inflammatory cell recruitment to the lungs of infected mice. In the pre-infection mice, in all the different cell fractions, *M. chelonae*-immunised mice showed significantly higher levels of IL-10 production than PBS-immunised mice ($p<0.01$, $p<0.001$ and $p<0.05$ for total splenocytes, $CD4^-$ and $CD4^+$ cells respectively; Fig. 9A). In cells from *M. chelonae*-sensitised mice, $CD4^-$ cells produced the highest amount of IL-10, and this was significantly higher than the levels of IL-10 produced by total splenocytes ($p<0.05$) and $CD4^+$ cells ($p<0.001$; Fig. 9A).

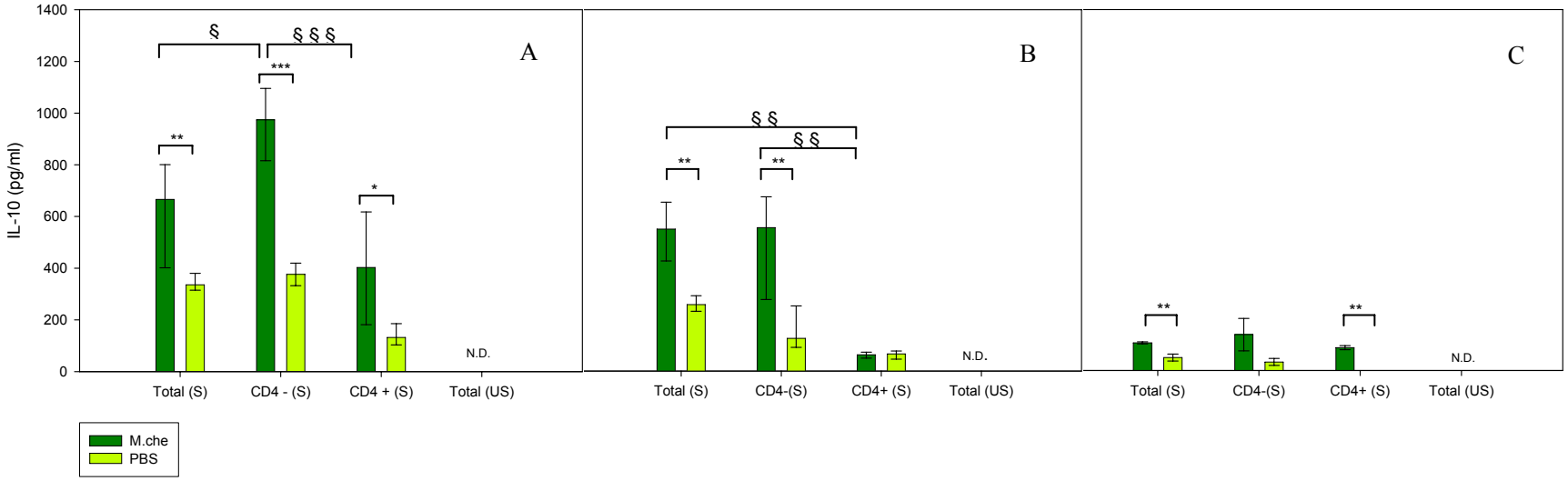


Fig. 9: IL-10 production by splenocytes from M. chelonae-immunised and control (PBS) mice pre- and post-BCG infection. Splenocytes from immunised and control mice pre-infection **(A),** 1 week post-infection **(B)** and 3 weeks post-infection **(C)**, were sorted into different fractions ($CD4^+$ or $CD4^-$) and re-stimulated in vitro with either heat-killed *M. chelonae* (S = stimulated) or PBS (US = unstimulated). The concentration of secreted IL-10 in the cell supernatants was determined by ELISA. Data shown in A and B represent one of two independent sets of experiments with similar results. Results in A and B are expressed as medians, 75th and 25th percentile of four mice per experimental group. Statistical significance was determined by non-parametric Mann-Whitney U test. Data shown in B represent a single experiment, expressed as mean ± 1 SD. Statistical significance for B was determined by Student's *t* test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ comparing immunised with control mice. §: $p<0.05$, §§: $p<0.01$, §§§: $p<0.001$ comparing between different cell types in immunised mice. N.D. = not detectable or below detection limit of the assay kit.

One week following BCG infection, a similar trend was observed. Total splenocytes and CD4- cells of *M. chelonae*-sensitised mice secreted more IL-10 than the corresponding fractions in control mice ($p<0.01$; Fig. 9B). There was no difference in IL-10 production by $CD4^+$ cells between immunised and PBS mice. The secretion of IL-10 was approximately 5-fold higher in total splenocytes and CD4- cells than $CD4^+$ cells in *M. chelonae*-sensitised mice ($p<0.01$; Fig. 9B). At 3 weeks post-BCG challenge, IL-10 levels remained higher for all cell subsets in sensitised mice than control mice ($p<0.01$ for total and $CD4^+$ cells; Fig. 9C). However, it is evident that the levels of IL-10 production were decreasing in the order pre-infection > 1 week > 3 weeks post-infection.

4.8.2 IL-4 and TGF-β production

IL-4 and TGF-β are known to have immunosuppressive roles, and are cytokines produced by certain types of regulatory T cells (Mills and McGuirk 2004). In view of the reduced inflammatory cell count in lungs of *M. chelonae*-sensitised mice, these cytokines were studied in the splenocytes of these mice and the PBS-immunised mice. Both cytokines were not detectable in any of the splenocytes fractions, for both *M. chelonae*-immunised and control mice, with and without BCG infection.

4.8.3 IL-2 production

As IL-2 is produced by proliferating T cells, the IL-2 levels secreted by different subsets of splenocytes were studied. In the pre-infection mice, the levels of IL-2 were marginally higher in total and $CD4^+$ splenocytes of sensitised mice than PBS mice, but this was not statistically significant. IL-2 was not detectable in CD4- cells in both groups of mice (Fig. 10A). At both 1 and 3 weeks following BCG challenge, the total splenocytes in *M. chelonae*-sensitised mice produced higher levels of IL-2 than control mice, but the IL-2 production in $CD4^-$ splenocytes was still not detectable (Fig. 10B, 10C).

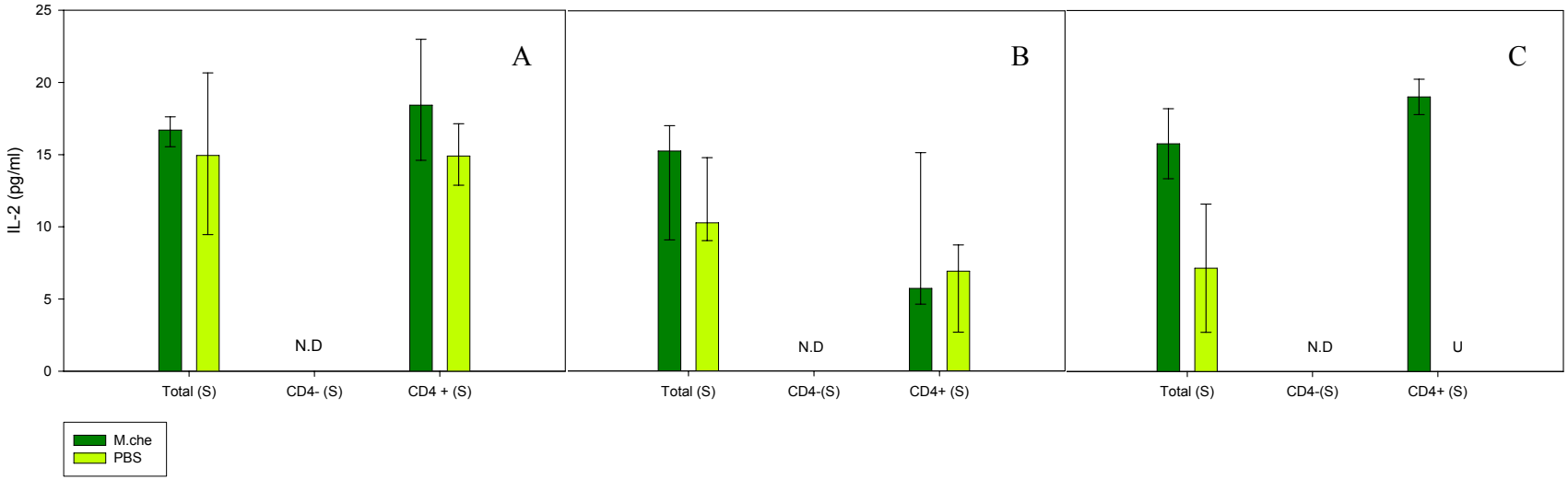


Fig. 10: IL-2 production by splenocytes from M. chelonae-immunised and control (PBS) mice pre- and post-BCG infection. Splenocytes from immunised and control mice pre-infection **(A)**, 1 week post-infection **(B)** and 3 weeks post-infection **(C)**, were sorted into different fractions ($CD4^+$ or $CD4^-$) and re-stimulated in vitro with either heat-killed *M. chelonae* (S = stimulated) or PBS (US = unstimulated). The concentration of secreted IL-2 in the cell supernatants was determined by ELISA. Data shown in A and B represent one of two independent sets of experiments with similar results. Results in A and B are expressed as medians, 75th and 25th percentile of four mice per experimental group. Data shown in B represent a single experiment, expressed as mean ± 1 SD. N.D. = not detectable or below detection limit of the assay kit.

4.8.4 IFN- γ production

IFN- γ is known to be a cytokine required for good Th1 type immunity against pathogenic mycobacteria, and it is important for activation of macrophages as well as cytotoxic T cells. It was postulated that differential IFN- γ production may account for the differences between the mice in cytotoxicity and reduction of BCG load.

Mice subjected to *M. chelonae* sensitisation showed a small deficit of IFN-γ secretion relative to control mice in total ($p<0.05$) and $CD4^-$ (not statistically significant) cells in the splenocytes (Fig. 11A). On the other hand, $CD4^+$ cells in *M. chelonae*-immunised mice secreted significantly more IFN-γ than control mice ($p<0.05$; Fig. 11A). Comparing IFN-γ levels among the different subsets of splenocytes in the mice post-sensitisation but prior to BCG infection, $CD4^-$ cells were the predominant IFN-γ producing population, secreting the highest levels of IFN-γ ($p<0.05$ $CD4^-$ vs $CD4^+$; Fig. 11A).

In contrast, after BCG infection, *M. chelonae* sensitisation clearly induced an increased secretion of IFN-γ in infected mice. All subsets of splenocytes in sensitised mice had higher levels of IFN-γ than in PBS mice at 1 week post BCG infection ($p<0.05$ and $p<0.01$ for $CD4^-$ and $CD4^+$ cells respectively; Fig. 11B). $CD4^+$ cells were the major contributor to production of IFN-γ, in the *M. chelonae*-sensitised mice. However, just as for IL-10, the levels of IFN-γ produced in all subsets generally were lower in the mice 1

week post-infection than in the immunised mice without infection. Data for mice 3 weeks post-infection were not available.

Overall, the cytokine studies suggest that significant levels of IFN-γ, IL-10 and IL-2 induction in the spleen may be attributable to *M. chelonae* sensitisation, but compared to the lung cytokine-expressing cells, the cell types involved in each organ may be different.

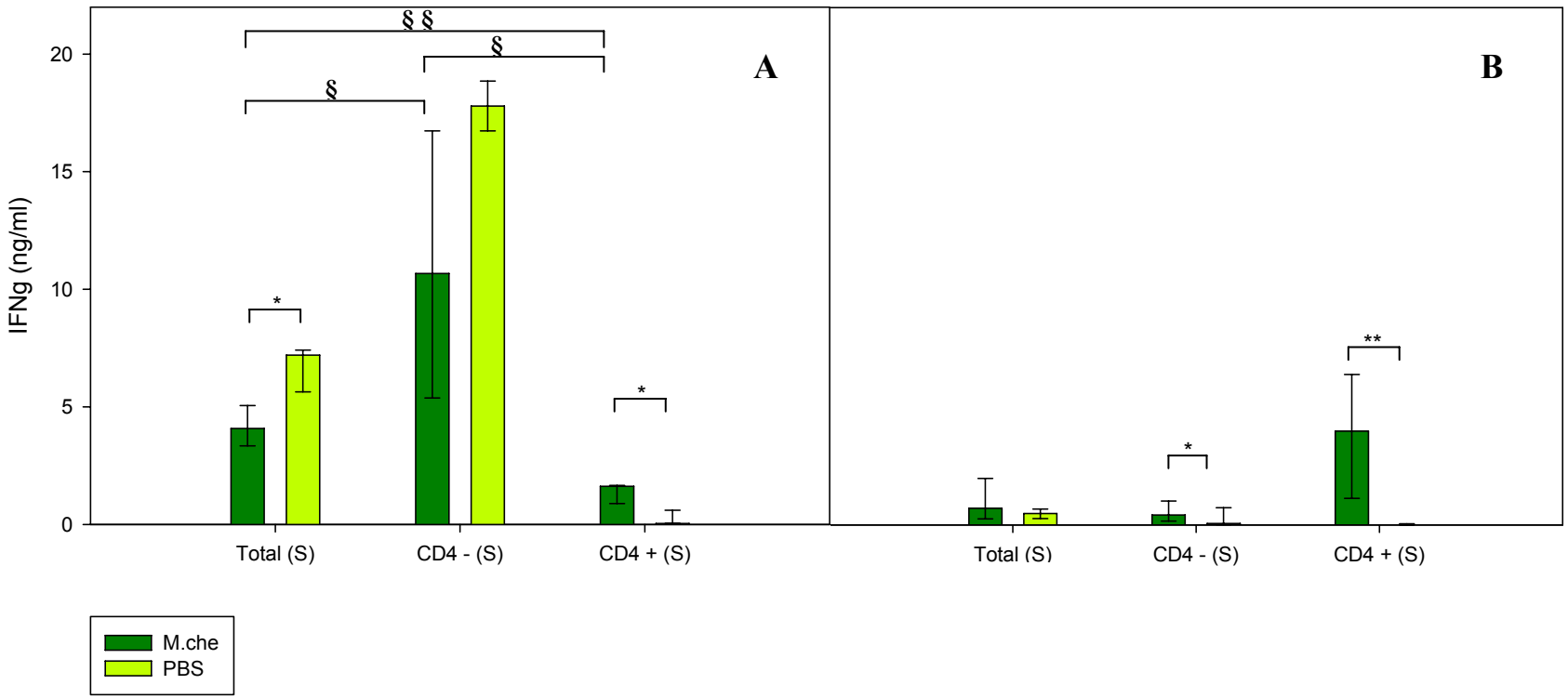


Fig. 11: IFN- γ production by splenocytes from M. chelonae-immunised and control (PBS) mice pre-infection and at 1 week post BCG infection. Splenocytes from immunised and control mice pre-infection **(A)** and 1 week post-infection **(B)**, were sorted into different fractions (CD4+ or CD4-) and re-stimulated in vitro with either heat-killed *M. chelonae* (S = stimulated) or PBS (US = unstimulated). The concentration of IFN- γ secreted in the cell supernatants was determined by ELISA. Data shown represent one of two independent sets of experiments with similar results. Results are expressed as medians, 75th and 25th percentile of four mice per experimental group. Statistical significance was determined by non-parametric Mann-Whitney U test. *$p<0.05$, **$p<0.01$, comparing immunised with control mice. §: $p<0.05$, §§: $p<0.01$ comparing between cell types in immunised mice. N.D. = not detectable or below detection limit of the assay kit.

CHAPTER 5 DISCUSSION

Why BCG fails to protect against TB in certain human populations is of much research interest as it affects current efforts to design an improved TB vaccine. Several studies have proposed that pre-exposure to Env has an adverse effect on the efficacy of subsequent BCG vaccination (Black, 2001; de Lisle, 2005; Demangel, 2005). However, the immunological mechanisms behind this phenomenon have not been explained. Prior work in this lab has shown that cytotoxicity against autologous BCG-infected macrophages in *M. chelonae*-sensitised mice was higher than in PBS-immunised control mice, and also relative to several other Env. In addition, BCG counts in the lungs 3 weeks after intra-nasal infection were 1 log lower in *M. chelonae*-immunised mice relative to control mice. This project therefore sought to further explore the role of cytotoxicity as a possible reason for the Env effect on BCG as well as the cell types and cytokines involved.

5.1 Cytolytic activity of different cell subsets

For many years, helper T cells were the main focus in numerous TB-related studies due to their ability to activate macrophage killing of intracellular mycobacteria by producing IFN-γ. In recent years, however, studies have shown that cytolytic T cells, mainly $CD8^+$ cells, also have a role to play in immune responses against Mtb (Sousa, 2000; van Pinxteren, 2000). It would seem that lysis of cells harbouring mycobacteria would be an effective protective mechanism in mycobacteria infections, hence the importance of cytolytic cells against intracellular pathogens such as Mtb. This has clearly been seen in

murine studies where CD8 T cells are necessary for limiting Mtb infection (Flynn, 1992; Sousa, 2000; van Pinxteren, 2000; Rolph, 2001), although some studies have also suggested otherwise (Mogues, 2001).

In this study, both $CD4^+$ and $CD4^-$ splenocytes in *M. chelonae*-sensitised mice induced greater cytotoxicity against autologous BCG-infected macrophages than control mice 3 weeks following BCG challenge (Fig. 7B). As the ability of macrophages alone in *M. chelonae*-immunised mice to kill BCG was the same as control mice (refer to Section 4.7), the difference seen in cytotoxicity of sensitised mice is likely to be due to the effector cells and not the macrophages.

Natural killer (NK) cells have also been involved in immune responses against TB, as shown by increased NK activity in active pulmonary TB patients, as well as an increase in NK cell number after BCG or Mtb infection in mice (Yoneda, 1983; Falcone, 1993; Junqueira-Kipnis, 2003). However, as NK cell depletion in mice infected with Mtb does not affect bacterial load in the lung, it is proposed that NK cells probably contributes to early resistance, but do not have a substantial role in TB immunity (Junqueira-Kipnis, 2003). Given the low percentage of perforin producing cells that were $CD49b^+$ (Fig. 8), as well as the low proportion of $CD49b^+$ cells present in the total lung population (Table 1), it was likely that the role of NK cells in killing BCG-infected lung cells was small. Nonetheless, the percentage of $CD49b^+$ cells producing perforin was higher in *M. chelonae*-immunised than control mice (Table 6), suggesting the possibility that *M.*

chelonae sensitisation increased the killing activity of NK cells against BCG, thereby contributing to clearing of the infection to some extent.

5.2 Cytotoxic $CD4^+$ T cells

$CD4^+$ T cells are commonly known to have 'helper' functions. They have been characterised as Th1 or Th2 cells based on the cytokines produced, and how these affect cellular and humoral immunity. There is now abundant evidence that $CD4^+$ cells can also have cytotoxic functions, with lytic capacity observed in cell lines and $CD4^+$ T cell clones of mice and humans (Lukacher, 1985; Littaua, 1992; Mahon, 1995; Norris, 2001; Aslan, 2006). In addition, recent work has reported the presence of cytolytic $CD4^+$ cells *in vivo* in a range of human pathologies such as viral infections (HIV, viral hepatitis and CMV) (Appay, 2002; Zaunders, 2004; Aslan, 2006). It is suggested that in infection immunity, especially in chronic viral infections, $CD4^+$ CTLs have a major effector role in limiting the infection, as the frequency of such CTLs increases with disease, but decreases with clearance of infection. Furthermore, $CD4^+$ CTLs have been shown to include antigen-experienced (i.e. memory) cells *in vivo*, and are shown to display a surface phenotype and functional profile characteristic of terminally differentiated effector cells (Appay, 2002; Aslan, 2006). This implies $CD4^+$ CTLs as fully functional effector cells, and not merely being present in infections.

This project showed that in sensitised mice, $CD4^+$ cells are the most important subset of splenocytes involved in cytotoxicity against BCG-infected cells. This supports prior work

in our lab showing that in the *M. chelonae*-sensitised mice, the splenocytes that are depleted of CD4 cells showed significantly reduced cytotoxicity. The cytolysis mediated by $CD4^+$ T cells may act via the perforin-granzyme pathway, since at 1-week post-BCG infection, $CD4^+$ cells in the lung comprised the predominant cell subset among perforin-expressing cells in both *M. chelonae*- and PBS-immunised mice (Fig. 8). An increased frequency of $CD4^+$ CTLs expressing granzymes and perforin, compared to healthy controls, is observed in humans with viral infections, indicating perforin as a possible mechanism of cytolysis (Aslan, 2006). More directly, cytolytic activity of $CD4^+$ CTLs has been shown to be mediated by perforin in an *ex vivo* experiment, where cytotoxicity was markedly reduced upon inhibition of the perforin pathway (Appay, 2002).

It has been reported in murine studies that cytotoxic activity of $CD4^+$ CTLs are primarily perforin dependent rather than Fas/ Fas-ligand dependent, but only in the absence of $CD8^+$ cells (Williams and Engelhard 1996; Williams and Engelhard 1997). $CD4^+$ CTLs act via both pathways in the presence of $CD8^+$ cells, indicating a possible role of $CD8^+$ cells in regulating the perforin-mediated pathway of cytotoxicity (Williams and Engelhard 1997). However, it is observed in *gld* transgenic mice with defective Fas ligand, that cytotoxicity of $CD4^+$ cytolytic T cells is substantially reduced, suggesting that in other model systems, the Fas /Fas ligand may be the main pathway of murine $CD4^+$-mediated cytotoxicity (Hanabuchi, 1994; Ju, 1994; Stalder, 1994). This pathway was not studied in the current project, and warrants further investigation. To elucidate the mechanisms behind the cytotoxic activity of $CD4^+$ cells, it will be useful to inhibit the

different cytolytic pathways, e.g. through the use of anti-Fas antibodies or concanamycin A, and subsequently measure the cytotoxicity of $CD4^+$ cells in the future.

5.3 Cytotoxicity is higher at later time-points after BCG infection

As shown in Fig. 7C, *M. chelonae* sensitisation induced higher cytotoxicity of autologous BCG-infected macrophages than PBS-immunised mice in all splenocyte fractions. This trend was augmented at 3 weeks post-BCG infection, but was not observed at 1-week post-infection (Fig. 7A, 7B). The influence of *M. chelonae* on immunity following subsequent pathogenic mycobacteria (i.e. BCG) exposure is likely to be due to induction of memory T cells by *M. chelonae* — others in our lab have shown that the population of $CD44^+$ $CD62L^-$ cells is expanded in the sensitised mice. As such, it was likely that the higher cytotoxicity observed in sensitised mice compared to control mice pre-infection and at 3 weeks following BCG challenge was due to memory T cells cross-reactive to common mycobacterial antigens. The difference in cytotoxicity seen between 1-week and 3 weeks post-BCG challenge could be attributable to the presence of regulatory T cells (Treg). This is evidenced by the higher levels of IL-10 secreted by splenocytes at 1-week post-BCG challenge than at 3 weeks in sensitised mice (Fig. 9B, 9C). These may have contributed to the lower inflammatory cell recruitment and thus lower cytotoxicity in *M. chelonae*-sensitised mice at 1-week post-BCG infection. (Figs. 2, 9B)

5.4 Possible induction of regulatory T cells by *M. chelonae* sensitisation

M. vaccae (an Env species) induces ovalbumin-specific $CD4^+CD45RB^{low}$ Treg cells that secrete IL-10 and TGF-β. These have been shown to reduce the airway inflammation in mice with ovalbumin-induced eosinophilic airway inflammation, after sensitisation with heat-killed *M. vaccae* (Zuany-Amorim, 2002). Our present data show that recruitment of inflammatory cells to the lungs of *M. chelonae* -sensitised mice was decreased 1 week after BCG challenge, as shown by cell counts in the BALF (Fig. 1) and absolute cell counts in the lung (Fig. 2). This could be attributable to an upregulation of Treg cells in the lung after *M. chelonae* sensitisation.

Cells of the $CD4^+CD25^+$ phenotype are known to be Treg cells, which secrete IL-10, and have been shown to exist in TB patients (Dieckmann, 2005; Guyot-Revol, 2006; Shevach, 2006). Extensive flow cytometric phenotyping of cells from *M. chelonae*-sensitised mice performed previously in our lab has shown that the sensitisation markedly increases the regulatory T cell proportions – these cells are $CD4^+CD25^+CD27^+Foxp3^+CD44^+CD62L^-$. There is some debate as to whether such Treg cells actually diminish the anti-mycobacterial immunity of the host, or are simply a response to the inflammation induced by TB infection (Guyot-Revol, 2006; Quinn, 2006). In the lungs of sensitised mice at 1-week post-infection, $CD4^+$ T cells were the predominant subset among IL-10 producing cells (Fig. 4B). Moreover, comparing *M. chelonae*- and PBS-immunised mice, the percentage of $IL\text{-}10^+$ cells that were $CD4^+CD8^-$ was significantly higher in *M. chelonae*-sensitised mice (Fig. 4B). *M. chelonae* immunisation also induced a significant increase in the proportion of $CD4^+$ and $CD8^+$

cells producing IL-10 in the lung (Table 4, Fig. 6). These data provide evidence that at the site of BCG instillation, which is also the organ with maximal inflammation, there is upregulation of Treg responses that is augmented by prior *M. chelonae*-sensitisation.

It is intriguing to note that, in contrast to the lungs, the highest production of IL-10 in the spleens of these *M. chelonae*- immunised mice appears to be attributable to the $CD4^-$ cells, which produced the highest amounts of IL-10 (Fig. 9B). We also know from prior work in this lab, that splenic BCG counts were several logs lower than in the lungs. It is possible that suppressor T cells are present in the $CD4^-$ population of splenocytes. This could explain the very low levels of IL-2 in this cell fraction pre- and post- infection (Fig. 10). $CD8^+$ cells have been known to produce IL-10 in multiple sclerosis (Killestein, 2003). Additionally, recent studies have demonstrated the presence of a $CD8^+CD25^+$ subset of cells in MHC class-II deficient mice exhibiting regulatory activity, as well as a phenotypically distinct subset of $CD8^+$ cells secreting IL-10 that is a lineage of suppressor T cells (Bienvenu, 2005; Noble, 2006). Hence, it is possible that the high levels of IL-10 seen in the $CD4^-$ fraction of splenocytes were secreted by a subset of $CD8^+$ suppressor cells. Such cells warrant further characterisation in my future work.

IL-10 has been shown to reduce presentation of mycobacterial antigens to $CD4^+$ T cells by inhibiting cathepsin-S dependent processing of MHC class II invariant chain in human macrophages infected with BCG (Sendide, 2005). This suggests that IL-10 induction is an immune evasion strategy of mycobacteria to enable their persistence in the host. It is therefore also possible that the IL-10 levels observed in the culture supernatants (Fig. 9)

were attributable to BCG-infected macrophages. However, given that IL-10 was more significantly expressed in splenocyte fractions of sensitised mice that had significant cytotoxic activity than in control mice, this explanation is less likely. Instead, it is more possible that Tregs secreting IL-10 were down-regulating the murine inflammatory responses to BCG, similar to the requirement for IL-10 in suppressing excessive intestinal inflammation observed in IL-10 deficient mice (Kuhn, 1993; Berg, 1996). Hence, whereas Tregs may have an important role in reducing the potentially immuno-pathogenic inflammatory responses in TB disease, this mechanism may also likewise reduce the host's immune cell recruitment and inflammatory responses which are crucial for the response to live BCG administered as a vaccine. To further confirm that Treg cells are responsible for the IL-10 production in our model system, Treg phenotyping (looking for $FoxP3^+$ $CD25^+$ $CD127^-$ cells) in the infected lung at both 1 week and 3 weeks following BCG infection will be done in future work.

5.5 Role of IFN-γ in cytotoxic responses

The $CD4^+$ cells were the predominant population producing IFN-γ in the BCG-infected lung, and this was augmented by *M. chelonae*-sensitisation. This was in spite of the elevated expression of IL-10 in such mice. IFN-γ, a proinflammatory cytokine produced in T helper 1 (Th1) responses, activates macrophages by inducing phagosome maturation and upregulating their antimicrobial molecules, such as iNOS and reactive oxygen species, against intracellular Mtb. IFN-γ is also known to be involved in T cell cytolytic activity, and in some TB- as well as non-TB-related studies, and the extent of cytotoxicity is proportional to levels of IFN-γ production (Maraskovsky, 1989; Diamond and Gill

2000; Pathan, 2000). However, with respect to our experimental system, total and $CD4^-$ splenocytes had less IFN-γ production but higher cytotoxic activity in sensitised mice (Figs. 7C, 11A). The only exception was the splenic $CD4^+$ cell fraction, which showed more than 2-fold higher cytotoxic activity in *M. chelonae*- than PBS-immunised mice, correlating with the higher levels of IFN-γ produced by the former (Fig. 7C, 11A). At 1-week post-BCG infection, splenocytes generally produced higher levels of IFN-γ in sensitised than control mice, with $CD4^+$ cells secreting 4-fold higher IFN-γ than the other subsets of cells in immunised mice (Fig. 11B). However, cytotoxic activity of total and $CD4^+$ cells was similar in immunised and control mice, while $CD4^-$ cells in sensitised mice showed 3-fold lower cytotoxicity than control mice (Fig. 7A). The above results suggest that in our experimental system, the cytotoxicity of sensitised mice against BCG-infected cells may not be entirely linked to IFN-γ production levels.

5.6 Effects of Env sensitisation on BCG-induced immunity

Together with evidence from earlier work in this lab showing that lungs of *M. chelonae*-sensitised mice had lower BCG counts than that of control mice 3 weeks post-BCG challenge, pre-sensitisation to Env, such as *M. chelonae,* induced better cytotoxicity and would seem protective if BCG infection were considered as a surrogate model for TB infection. This is consistent with the observation that priming with Env provides, to some extent, protective immunity to other mycobacteria (Brown, 1985).

However, the dual effects of Env-sensitisation described in this study – induction of cytolytic T cells against BCG-infected macrophages and down-regulation of

inflammatory responses to BCG – are not beneficial for the prospects of BCG as a vaccine. BCG works only as a live vaccine, and immune responses to Mtb wanes if BCG is no longer viable (Olsen, 2004). As such, the replication of BCG is essential for it to be effective as a vaccine. Immunity primed by Env sensitisation blocks BCG replication, thereby reducing its protective efficacy, so such priming is disadvantageous to BCG as a vaccine (Brandt, 2002). Nonetheless, this effect may be species-specific. Demangel *et al* have shown that murine sensitisation with certain species of Env reduces BCG counts without diminishing protective efficacy against Mtb challenge (Demangel, 2005).

The data presented show that *M. chelonae* could induce Treg cells, thereby reducing the extent of inflammation at the site of infection, and yet Env-sensitisation clearly also increases the cytolytic activity of T cells against infected macrophages. Whereas Env sensitisation has a negative influence on BCG vaccine, the direct effects of immunity conferred by such sensitisation may be helpful in the response to pathogenic TB infection. A good balance of regulatory and activated pathogen-specific cells in Env-immunised mice, where inflammatory responses are not excessive, yet appropriate effector T cell responses are maintained, would be optimal. This could be the basis on which another well-studied Env species, *M. vaccae*, may work as a TB vaccine – it is currently undergoing clinical trials (Stanford and Stanford 1994; Corlan, 1997; Waddell, 2000; Mwinga, 2002; Vuola, 2003). It is important to note, though, that pre-sensitisation to naturally acquired doses of Env are unlikely to protect fully against TB, since rural populations in many African countries have been shown to have high levels of exposure to Env but still have high TB incidence.

It has also been shown, in a small scale study, that when the BCG vaccine is given early at birth, before possible exposure to Env, the longevity of the recall response to BCG, and hence the BCG-attributable immunity, is still lower in Malawi, where Env exposure is high than in the UK where Env exposure is low (Ben-Smith, communicated in 'TB Vaccines for the World 2006' conference, Vienna). It is possible that Env exposure even after BCG vaccination may limit the memory response induced by BCG. We intend to model this in young mice and extend the present study to understand how *M. chelonae* priming affects post-BCG memory responses.

Given our present understanding on the effects of Env priming, such effects would be likely to affect any vaccine approach that is based on live BCG. This raises doubts of whether development of future vaccines against TB should revolve around live BCG as the mainstay with or without genetic modifications. Unfortunately, such is the approach of at least three new TB candidates on clinical trial now (Bosch 2004; Matee, 2007). This is of concern as populations in most high TB incidence areas also have high Env exposure. Perhaps emphasis in research for new TB vaccines should therefore be focused on vaccines that do not cross-react with Env antigens – candidates in this direction would include subunit vaccines based on TB-specific proteins such as ESAT-6, which are not found in BCG.

5.7 Conclusion

This study demonstrated that murine *M. chelonae* sensitisation induced an increase in cytotoxic responses against autologous BCG-infected macrophages, both pre- and post-BCG challenge. $CD4^+$ T cells were the major T cell subset involved in cytotoxicity primed by *M. chelonae* against BCG and peforin played a role in this process. Upon *M. chelonae*- sensitisation and subsequent infection with BCG, there was a reduction in lung inflammatory responses, and increase in IL-10 production. Taken together with the phenotypic characterization of the cells involved by others in our lab, this suggests the induction of Tregs which diminish the recruitment of inflammatory cells to the site of live BCG vaccination. This is the first work that elucidates the actual immune mechanisms that govern the epidemiologically observed phenomenon of reduced BCG vaccine efficacy in populations exposed to environmental mycobacteria.

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APPENDICES

Middlebrook 7H9 broth

Volume	Distilled water	7H9 broth powder	50% Glycerol	20% Tween 80	OADC
400 ml	356.4 ml	1.88 g	1.6 ml	2 ml	40 ml

All ingredients, except OADC, were added and autoclaved at 121 °C for 15 min. OADC was added before use.

Middlebrook 7H10 broth

Volume	Distilled water	7H9 broth powder	50% Glycerol	20% Tween 80	OADC
400 ml	353.6 ml	7.6 g	4 ml	2 ml	40 ml

All ingredients, except OADC, were added and autoclaved at 121 °C for 15 min. The agar was left to cool to 55 °C before OADC was added. The liquefied agar was aliquoted into 60 mm petri dishes and allowed to set.

Complete supplement for BCG infection (10X FAC)

Volume	Distilled water	Ferric Ammonium Citrate (FAC)	Sodium glutamine	L-asparagine
50 ml	50 ml	25 mg	1 g	1g

All ingredients were added at room temperature and mixed by stirring. Medium was sterilised by filter through a 0.22 μm filter.

Red blood cell lysis solution (0.17 M NH_4Cl)

Volume	Nanopure water	Ammonim chloride (NH_4Cl)
10 ml	10 ml	90 mg

All ingredients were added at room temperature and mixed by stirring. Adjust pH to 7.3 and autoclave at 121 °C for 15 min. Solution was stored at 4 °C before use.