IMMUNE MECHANISMS OF RESPONSES TO ENVIRONMENTAL MYCOBACTERIA

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SUMMARY

Human epidemiological studies suggest that poor efficacy of the tuberculosis (TB) vaccine, *Mycobacterium bovis* bacille Calmette- Guérin (BCG), may be because of immuno-modulatory effects of exposure to environmental mycobacteria (Env). However, exactly how and why this happens remains unclear. This study examined the hypothesis that effects of Env sensitisation are related to induction of regulatory and cytotoxic T cells. *Mycobacterium chelonae* (CHE) sensitisation of Balb/c mice through various routes was used as the model system.

Heat-killed CHE intra-peritoneal sensitisation induced $CD4^+$ T cells which lysed BCG-infected macrophages *in vitro*. The cytotoxicity was dependent on IFN-γ, perforin and FasL. Sensitisation with an unrelated bacterium failed to induce cytotoxicity, therefore priming of T cells cross-reactive with BCG, and not non-specific inflammation, underlies the cytotoxicity. Sensitised mice had reduced BCG viability in the lungs upon subsequent inhalation challenge; this can explain the reduced BCG-induced protection.

Both IFN-γ and IL-10 were increased in the lungs of CHE-sensitised mice, relative to naïve mice, after BCG lung challenge. Although the frequency of systemic $CD4^+CD25^+$ cells was unremarkable after CHE sensitisation, adoptive transfer of these Tregs to naïve mice followed by BCG challenge led to reduced lung lymphocyte recruitment, reduced lung IL-2 and increased systemic IL-10

production. This suggests functional suppression of local BCG responses by $CD4^+CD25^+$ Tregs from CHE-sensitised mice.

Memory responses after transient CHE lung colonisation led to increases in Tregs weeks after no live CHE was recoverable. Different doses of inhaled CHE exposure were tested – higher doses induced stronger Treg responses and weaker BCG-specific IFN-γ responses. Subsequent experiments used repeated low dose live intra-tracheal CHE exposure to mimic natural human inhalational exposure, followed by subcutaneous BCG vaccination. Systemic IL-10, mainly produced by $CD4^+CD25^-FoxP3^+$ inducible Tregs, was increased and associated with reduced frequency of IFN-γ producing memory cells recognising a BCG-specific epitope. Thus, adaptive Tregs also have a role in suppressing BCG-specific inflammation in CHE-sensitised mice.

To explore if post-BCG CHE exposure had similar effects, BCG vaccination of weanling mice was followed by low dose CHE intra-tracheal exposures. This mainly induced natural Tregs, with minimal IL-10 induction. Suppression of inflammatory cell recruitment in the lungs to subsequent BCG lung challenge was noted, associated with reduced lung chemokines, in spite of elevated systemic IFN-γ responses. The rate of inflammatory cell recruitment to the lung early in TB infection is increasingly recognised as the critical determinant of effective immunity, more than systemic IFN-γ responses. Thus, CHE exposure even after BCG vaccination can suppress *Mycobacterium*-specific immunity.

These two mechanisms proposed for effects of CHE exposure on BCG-induced immunity are novel. This work is also the first to provide a mechanistic explanation for how Env exposure modulates an existing BCG vaccine response. This accounts for observations of lack of BCG-induced protection in humans living in Env-prevalent areas, and suggests how prospective candidate TB vaccines could be screened to avoid problems of BCG vaccine. It also explains why even early neonatal BCG vaccination fails to provide long-lasting effects against adult pulmonary TB, with implications for its continued use.

Publications arising from this thesis

Journal papers:

1) Peiying, Ho, Lin Zhang, Xing Wei and Geok Teng Seah (2009). *Mycobacterium chelonae* sensitisation induces $CD4^+$-mediated cytotoxicity against BCG. Eur J Immunol 39(7): 1841-9
2) Peiying, Ho, Xing Wei and Geok Teng Seah (2010). Regulatory T cells induced by *Mycobacterium chelonae* sensitization influence murine responses to bacille Calmette-Guérin. J Leukoc Biol 88: 1073-80.
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Conference presentations:

1) Peiying, Ho, Megan McBee, Geok Teng Seah and David Schauer. *M. chelonae* exposure post-BCG vaccination suppresses BCG-specific responses. Poster presentation. International Congress of Mucosal Immunology, July 2009, Boston, USA.

2) Effects of environmental mycobacteria post-BCG vaccination. Oral Presentation. Singapore MIT Alliance for Research and Technology (SMART) Infectious Diseases Inter-disciplinary Research Group (ID-IRG) Workshop, January 2010, Singapore. *Won best oral presentation award*.
3) Peiying, Ho, Carmen, Low, Joanne, Kang, Tse Mien Tan, Nicola Leung and Geok Teng Seah. *Mycobacterium chelonae* sensitisation prior to BCG vaccination induces regulatory T cells that suppress IFN-γ responses. Poster presentation. SMART ID-IRG Annual Workshop July 2010, Singapore.

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ABBREVIATIONS

Ab	Antibody
BAL	Bronchoalveolar lavage
BCG	*bacille* Calmette-Guérin
BSA	Bovine serum albumin
CHE	*Mycobacterium chelonae*
CTL	Cytolytic T lymphocyte
DC	Dendritic cell
DDAO-SE	dodecyldimethylamine oxide-succinimidyl ester
DOTS	Directly observed treatment, short-course
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot assay
Env	Environmental mycobacteria
FAC	Ferric ammonium citrate supplement
FBS	Foetal bovine serum
FoxP3	Forkhead-box P3
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
IFN- γ	Interferon gamma
IL	Interleukin
i.p.	Intra-peritoneal
i.n.	Intra-nasal

i.t.	Intra-tracheal
iTreg	Inducible (or adaptive) regulatory T cell
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
Mtb	*Mycobacterium tuberculosis*
NK	Natural killer cell
nTreg	Natural regulatory T cell
OADC	Oleic acid-albumin-dextrose-catalase enrichment
PBS	Phosphate-buffered saline
PMA	Phorbol myristate acetate
PPD	Purified protein derivative (of *M. tuberculosis*)
RAG	Recombination activating gene
SD	Standard deviation
SEM	Standard error of the mean
TB	Tuberculosis
TGF- β	Transforming growth factor beta
Th1	T helper type 1
Th2	T helper type 2
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cell
Tr1	Type 1 regulatory T cell
WHO	World Health Organisation

CHAPTER 1 – INTRODUCTION

1.1 The global tuberculosis situation

Tuberculosis (TB) has been declared a global emergency by the World Health Organisation (WHO) (WHO, 2003). It is estimated that one-third of the world's population is infected annually, and in 2009 there was an estimated 9.4 million cases of TB globally, with an approximate 1.3 million people dying from TB (WHO, 2010). Although there is a high cure rate with appropriate anti-TB drugs, the failure to stem the tide with drugs is related to numerous social, public health and pharmacological problems, and therefore, effective vaccination remains the holy grail in TB eradication.

Members of the *Mycobacterium tuberculosis* (Mtb) complex, which comprises of Mtb, *M. bovis*, *M. microti*, *M. africanum* and *M. canettii*, cause human TB (Cosma, 2003). A highly genetically-related species, *M. bovis* bacille Calmette-Guérin (BCG), has been used as a live attenuated vaccine for almost a century, and there is no clinically available alternative at present. However, the exact immune mechanisms through which BCG protection is conferred remain unclear. More importantly, the protective efficacy of BCG against adult pulmonary TB, which is the most prevalent form of TB, ranges from 0-80% across different parts of the world (Fine and Vynnycky, 1998). Strain differences, geographical factors and reinfection pathways (Smith, 2000; Fine, 2001a) have been suggested as causes, but the most widely supported hypothesis for the failure of BCG is that prior exposure to environmental mycobacteria (Env) affects how the host responds to

the vaccine (Black, 2001a; Brandt, 2002; Buddle, 2002; de Lisle, 2005; Lalor, 2009). However, details on how this happens have yet to be fully elucidated.

1.2 Immune responses of environmental mycobacteria exposure and effects on BCG vaccination

Prior exposure to certain Env species blocks the replication of BCG (Brandt, 2002; Demangel, 2005) or modifies the nature of immunity induced by BCG (Young, 2007). The persistence of the Env species in the host and the relatedness between the priming Env strain and BCG have been suggested as factors influencing these effects. Env exposure following BCG vaccination can also modulate protective immunity generated by BCG in mice (Flaherty, 2006). Infants with delayed BCG vaccination, allowing time for Env exposure, have poorer IFN-γ responses to BCG antigens after vaccination (Burl, 2010) and yet early neonatal vaccination of those living in areas with high prevalence of Env sensitisation still result in poorer subsequent IFN-γ responses to Mtb antigens (Lalor, 2009). These studies provide human epidemiological evidence that Env-induced immunomodulation could occur both before and after BCG vaccination.

Preliminary studies in our laboratory on human peripheral blood cells found that when lymphocytes were stimulated with each of the ten commonest Env species isolated in Singapore, there was strongest cytolytic activity against autologous BCG-infected monocytes with *Mycobacterium chelonae* (CHE) stimulation, implying best cross-reactive cytotoxicity (Zhang Lin, MSc thesis). This suggests that CHE priming had strongest potential to influence BCG responses. This

outcome was subsequently replicated in mice after *in vivo* intra-peritoneal sensitisation with various Env species, and again CHE exposure led to strongest cytotoxic responses against BCG-infected autologous macrophages (Ho, 2009). This led to our choice of using CHE sensitisation as a model for Env exposure in our murine studies on Env-induced effects against BCG but in this project, additional routes and doses of CHE were used.

1.3 Objectives and scope of project

This project evaluated the immune mechanisms underlying how exposure to Env affects host responses to BCG vaccine. The rationale was that understanding why BCG fails will allow development of vaccines that do not have similar pitfalls. This is especially pertinent given that several vaccines in the pipeline are actually modified strains of BCG (Kaufmann, 2010b).

This work investigated the hypotheses that Env sensitisation induced both regulatory T cells and cytotoxicity mechanisms, which ultimately reduced immunity induced by BCG vaccination. Sensitisation of Balb/c mice with CHE via various routes was the model for Env exposure in this project. The broad aims of the project were:

1) In CHE-sensitised mice, to analyse the role of different cell types, cytokine factors and mediators of cytotoxicity on the host's responses to BCG, and their subsequent impact on BCG survival.
2) To investigate the role of $CD4^+CD25^+$ regulatory T cells (Tregs) from CHE-sensitised mice in suppression of the BCG response, through

studying the immunomodulatory effects of such cells *in vitro* and *in vivo* upon BCG challenge after adoptive transfer into naïve mice.

3) To investigate how variations on multiple sensitisation parameters (dose, timing, *in vivo* persistence of CHE and viability of CHE) affect Treg phenotypes and systemic Th1 responses induced by CHE sensitisation, before and after subsequent BCG vaccination.
4) In a model of BCG vaccination of weanling mice that mimics human neonatal BCG vaccination, to characterise the influence of post-vaccination CHE exposure on local and systemic cytokines, Tregs and cellular recruitment, before and after *in vivo* BCG challenge.

CHAPTER 2 – LITERATURE REVIEW

2.1 Epidemiology of tuberculosis (TB)

Tuberculosis (TB) in humans is caused by Gram-negative bacteria collectively known as *Mycobacterium tuberculosis* complex, amongst which the most prevalent species is *Mycobacterium tuberculosis* (Mtb) (Dye, 2006). This disease has been declared a global emergency by the World Health Organisation (WHO) since 1993. TB causes an estimated 2 million deaths annually, and one-third of the world's population is latently infected, with the potential for reactivation (WHO, 2010). Majority of the cases of TB occur in Asia and Africa, with the highest TB incidence in India, China, South Africa, Nigeria and Indonesia (WHO, 2010). In Singapore, TB is of moderate endemicity, with near 1500 new cases each year, more than half being elderly males. A vast majority of these cases are pulmonary TB and less than 0.5% involve multiple drug resistant (MDR) TB (Ministry of Health, 2010).

2.1.1 Clinical tuberculosis

Clinically, TB disease exists in two main forms – pulmonary and extrapulmonary TB, the former being more important epidemiologically as a source of infectious Mtb (Dye, 2006). Patients diagnosed with pulmonary TB typically have a chronic cough, fever and weight loss. Extrapulmonary TB can involve any organ, but lymphadenitis and pleuritis with effusion are most common, while miliary disease and meningitis are the most fatal forms. The spread of TB infection occurs through the inhalation of airborne droplets of respiratory secretions

carrying Mtb bacteria, which are generated when infected individuals cough, sneeze, talk, or spit (Dye, 2006). Less than five percent of infected individuals develop progressive disease within five years of Mtb infection, after which the risk of reactivation of latent TB is even lower (Dye and Floyd, 2006).

TB can be treated with a cocktail of antibiotics, primarily isoniazid, rifampicin, pyrazinamide and ethambutol. WHO recommends directly observed treatment short course (DOTS), a strategy for administering treatment under direct observation. Issues contributing to continued TB transmission include inaccessibility of drugs, poor compliance to the prolonged course of chemotherapy (six months minimum), overcrowded living conditions associated with poverty in certain communities, the rise of human immunodeficiency virus (HIV) infections that compromises cellular immunity and emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of Mtb (Lawn, 2006; Cox, 2008; Gandhi, 2010).

2.1.2 Bacterium-host immune interactions

Mtb is an intracellular pathogen that survives within lung macrophages. Alveolar macrophages, believed to be the primary host cells of Mtb, function both as a first line of cellular defence, as well as a niche for bacterial survival and replication. Mtb can interfere with membrane trafficking, neutralise phagosomal pH and avoid phagolysosomal fusion, allowing them to escape the host immune response (Kusner, 2005; Vergne, 2005; Russell, 2007a). Nevertheless, phagocytes, including dendritic cells (DCs) and macrophages, that are recruited

to the lungs in infected individuals take up the bacteria and may transport Mtb to other sites during early TB (Davis and Ramakrishnan, 2009). These phagocytes can also migrate to the draining lymph nodes, where they then present Mtb antigens to T cells and initiate T helper type 1 (Th1) responses. Granulomas eventually form in response to persistent intracellular Mtb. Granulomas comprise of macrophages, DCs, T cells and B cells that surround single infected macrophages (Cosma, 2003). Mtb that remain in the host can persist in a latent state and can lead to active disease upon reactivation of such bacteria. There is some evidence that latent mycobacteria survive within granulomas, where conditions are hypoxic and generally nutrient-deprived, by reducing their metabolic activity and persisting in a slowly dividing or non-dividing state (Wayne and Sohaskey, 2001; Betts, 2002; Voskuil, 2003).

2.2 Immune responses to TB: cell types and their functions

The intracellular lifestyle of Mtb places cell-mediated immunity in greater prominence than antibodies in anti-TB immunity. The major cell types involved are described below, with a focus on their various effector functions in TB control.

2.2.1 CD4 cells

The central role of $CD4^+$ T helper cells in controlling *Mycobacterium* infections is seen in the marked susceptibility of HIV-positive patients, who have reduced $CD4^+$ T cell counts (Flynn and Chan, 2001; Elkins, 2003). Effector mechanisms that $CD4^+$ T cells engage to fight Mtb are still being elucidated. $CD4^+$ Th1 memory

cells are crucial in mediating long-term protection against TB (Sutherland, 2010). They produce a variety of pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ that are critical in the process (Flynn, 1993; Flynn, 1995; Cooper, 2009). IFN-γ production in response to infected macrophages presenting *Mycobacterium* antigens is a key effector mechanism of $CD4^+$ cells, which then activate macrophages to kill the bacteria harboured within by producing reactive nitrogen and oxygen species and promoting phagolysosome fusion (Flynn and Chan, 2001; MacMicking, 2003). The magnitude of polyfunctional *Mycobacterium*-specific Th1 cells, secreting multiple cytokines, in the lungs after murine Mtb challenge is apparently correlated with protection against disease (Forbes, 2008). However, TB patients have higher frequencies of such polyfunctional $CD4^+$ T cells relative to tuberculin-positive healthy controls (Sutherland, 2009), so the protective significance of these cells is unclear. Murine and human studies also suggest a protective role for Th17 cells, producing IL-23 and IL-17, in TB infection (Khader, 2007; Chen, 2010).

$CD4^+$ T cells are largely known to function as helper T cells, but they can also exhibit cytotoxic activity. After *in vitro* stimulation with Mtb, upregulation of mRNA for perforin, granulysin, and granzymes A and B is observed in $CD4^+$ T cells from healthy tuberculin skin-test positive individuals (Canaday, 2001). This indicates a cytolytic role of these cells against TB. In addition, $CD4^+$ cells from peripheral blood of patients with active TB display cytolytic responses against autologous Mtb-pulsed macrophages, and such activity diminishes with severity of TB (De La Barrera, 2003). However, it is unclear whether the opposite, that patients with less severe TB have better cytotoxic responses, holds true. The

same study shows that the CD4-mediated cytotoxicity observed is dependent on the Fas/ Fas-ligand mechanism. However, other studies on $CD4^+$ T cell clones have reported perforin-dependent mechanisms for cytotoxicity (Susskind, 1996; Kaneko, 2000).

2.2.2 CD8 cells

$CD8^+$ T cells with cytotoxic functions have been reported in TB patients and are important in immunity against TB (Lewinsohn, 1998; Sousa, 2000; van Pinxteren, 2000). The essentiality of $CD8^+$ T cells in murine TB immunity has been proven in some studies (Flynn, 1992; Rolph, 2001), but disputed by others (Mogues, 2001). Reduced numbers of antigen-specific effector memory CD8 cells have been linked to the poorer antimicrobial activity of patients on anti-TNF therapy, with associated increased TB incidence (Bruns, 2009). Although the major effector function of $CD8^+$ T cells is cytotoxicity against infected cells, additional roles of $CD8^+$ cells in cytokine production (Soares, 2008) and as regulatory T cells (Joosten, 2007) in TB are just emerging.

The mechanism behind $CD8^+$ cytotoxicity in TB is through exocytosis of granule contents. In humans, $CD8^+$ 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). In mice, one study claims that the perforin/ granzyme pathway is more important than the Fas/ Fas-ligand pathway in lysis of Mtb-infected macrophages by $CD8^+$ CTLs (Silva and Lowrie, 2000). Nonetheless, another study

shows that perforin inhibition does not affect restriction of Mtb growth, although granule exocytosis is required for the cytolytic activity of human $CD8^+$ T cells (Canaday, 2001). $CD8^+$ responses are elicited via the major histocompatibility complex (MHC) Class I pathway, which requires antigen presentation in the cytosol. As Mtb resides in the phagosome, its antigens are not usually available in the cytosol. However, Mtb can egress into the cytosol of infected DCs (van der Wel, 2007), otherwise, apoptosis of infected macrophages can also lead to cross-priming when DCs take up vesicles with Mtb antigens that arise from the apoptosis (Winau, 2006), thus allowing MHC Class I presentation.

2.2.3 γδ T cells

Mtb readily activates γδ T cells and induces γδ T cell–mediated production of antigen-specific IFN-γ (Ladel, 1995). γδ T cells are also a primary source of IL-17 (Lockhart, 2006). Mice with T cell receptor (TCR) δ gene deletions succumb to Mtb infection, while immunocompetent control mice survive, demonstrating the protective role of γδ T cells in TB (Ladel, 1995). In addition, γδ T cell-mediated lytic activity is observed in *ex vivo* effector cells from TB patients, suggesting a cytolytic role for these cells in TB (De La Barrera, 2003). The expansion of γδ T cells with BCG, from peripheral blood lymphocytes of tuberculin-positive donors, results in good Mtb-killing, but not if the cells are expanded with phosphoantigen, suggesting that this particular subset of cells may be involved in protective immunity (Spencer, 2008).

2.2.4 Natural killer (NK) cells

Natural killer (NK) cells are cytolytic effector cells of innate immunity. Human studies have demonstrated that NK cells respond to live Mtb *in vitro* and increased NK activity is observed in active pulmonary TB patients (Yoneda, 1983; Esin, 1996). In murine studies with *Mycobacterium bovis* bacille Calmette-Guérin (BCG) or Mtb infection, expansion of NK cells is observed (Falcone, 1993; Junqueira-Kipnis, 2003). The direct role of NK cells in mycobacteria infections, however, remains not well understood.

2.2.5 Other cells

2.2.5.1 CD1-restricted T cells

CD1-restricted T cells recognise lipid antigens and can be double negative for CD4 and CD8 (Porcelli, 1992), or expressing CD4 or CD8 (Rosat, 1999; Sieling, 2000). CD1-restricted T cells are involved in immune responses against Mtb – they recognise lipid and glycolipid antigens of Mtb and kill infected macrophages (Stenger, 1997). Mycobacteria-specific CD1-restricted T cell clones mostly have Th1 functions, producing high levels of IFN-γ and TNF-α (Rosat, 1999; Sieling, 2000). CD1-restricted T cells that are highly proliferative and IFN-γ secreting, predominantly of the CD4 subset, are also found in patients with active TB (Ulrichs, 2003). CD1-restricted T cells also contain perforin and granulysin and can exhibit cytotoxic and microbicidal functions in both the $CD4^+$ and $CD8^+$ subsets (Stenger, 1997; Stenger, 1998; Rosat, 1999).

2.2.5.2 B cells

B cells have a less apparent role in TB, but studies have shown that monoclonal antibodies against certain mycobacteria-derived products can protect TB in murine models (Teitelbaum, 1998; Pethe, 2001; Hamasur, 2004), either by reducing bacterial burden or limiting inflammatory progression (Glatman-Freedman, 2006). B cells also function as antigen-presenting cells and can influence host immunity by engaging Fcγ receptors by antibodies during Mtb infections, leading to an impact on Th1 activation (Maglione and Chan, 2009). B cells are also responsible for granuloma formation by surrounding Mtb bacteria. This is required for effective immunity against Mtb during acute infection, as well as promoting local host immune responses and prevent reactivation during chronic infection (Maglione and Chan, 2009).

2.2.5.3 Antigen presenting cells

The major antigen presenting cells in Mtb infections are macrophages and DCs, and these cells can influence the type of T cell responses mounted during infection (Dorhoi and Kaufmann, 2009). The cytokines secreted by antigen presenting cells can polarise TB responses, such as the production of IL-23 by DCs that stimulates polarisation of Th17 responses (Dorhoi and Kaufmann, 2009). The engagement of different receptors on antigen presenting cells results in different responses primed, an example being the promotion of Treg differentiation in the presence of Toll-like receptor (TLR) ligation, but in the absence of TLR ligation, Th17 responses are favoured (Torchinsky, 2009).

2.3 Relevance to vaccine development

Currently, there is insufficient understanding of the critical cell types required to contain Mtb, yet this knowledge is crucial for designing an optimal TB vaccine. Whether effector cells or memory cells are more important for protection still remains a question, as the former protects during early Mtb infection and the latter in long-term immunity (Kaufmann, 2010a). Additionally, amongst effector T cells, it is still unclear whether only some or all of effector T cells, which include $CD4^+$ Th1 cells, $CD8^+$ cells, the cytolytic cells and Th17 cells, are required during early infection (Kaufmann, 2010a). For long-term immunity, questions remain about whether effector memory cells that reside at the active Mtb infected site or central memory cells that reside further away but generate effector memory cells are more important (Kaufmann, 2010a).

2.4 BCG as a vaccine

The only currently available human vaccine against TB is live *M. bovis* bacille Calmette-Guérin (BCG), originating in Institut Pasteur from repeated *in vitro* passage of a virulent *M. bovis* strain to achieve attenuation. The WHO recommends BCG vaccination at birth, especially in TB endemic areas, but alternative vaccination policies exist (Fine, 1999). In the United Kingdom (UK) and some nations in Europe, BCG is administered once during childhood or adolescence (12-13 year olds), although some health authorities in these regions have currently moved to vaccinating only high-risk populations (Fine, 1999). Repeated or boosting BCG vaccination regimen is used in some countries, such as

Switzerland, while no routine BCG vaccination protocols exist in the USA and Netherlands (Fine, 1999). In Singapore, the national policy of BCG vaccination is to perform mass vaccination at birth, with a re-vaccination at 12-16 years of age for tuberculin non-reactors, since the 1950s (Chee, 2001). However, since 2001 following WHO recommendations, no further booster is given after BCG at birth (Ministry of Health, 2001).

BCG was previously distributed in liquid form, with ~10^6 CFU per dose, but most BCG vaccines are now distributed in lyophilised form, ranging from approximately 0.35 x 10^6 to 10^6 culturable particles per dose (Fine, 1999). The original Pasteur strain was distributed to various production centres globally, and loss of genes during passage at these centres have led to several genetically varied BCG strains being used today (Fine, 1999).

2.4.1 Measuring BCG responses

The tuberculin skin test is the classical method of assessing the BCG vaccination response by measuring the delayed type hypersensitivity response to intra-dermal PPD (Fine, 1999). Purified protein derivative of Mtb (PPD) contains Mtb protein antigens, many of which are conserved within the *Mycobacterium* genus. The Mantoux method of skin-testing requires reading the diameter of skin induration 48 h after PPD administration, and BCG-induced reactivity to tuberculin could yield a 0 – 19 mm induration (Karalliedde, 1987). Some factors that contribute to the varying size of the tuberculin skin reaction post-BCG vaccination include the dose (Ashley and Siebenmann, 1967), method of

vaccination (Landi, 1967), geographical location, which is also linked to environmental mycobacteria (Env) exposure (Floyd, 2002), and the manufacturer of the vaccine (Horwitz and Bunch-Christensen, 1972). Prior asymptomatic Mtb exposure or latent TB would also increase the PPD response, as evidenced from the use of the Mantoux test to detect latent TB (Schluger and Burzynski, 2010). These factors complicate the interpretation of Mantoux test results.

An alternative method of testing BCG-induced responses, popular in research studies in the last decade, is to measure the IFN-γ responses in peripheral blood of vaccinees, after *in vitro* stimulation of lymphocytes with PPD. The same issues of non-specificity and cross-reactivity with other *Mycobacterium* exposures exist. Such IFN-γ responses are expected to increase in vaccinated individuals following BCG vaccination (Black, 2002), but these responses start waning by 9 months after neonatal vaccination (Burl, 2010). This method of testing BCG-induced T cell immunity is also commonly used in research with murine models of vaccination.

Whether BCG protects against TB can only be observed after years of following-up vaccinees in endemic areas for their TB incidence. This can take at least 5 – 10 years even in areas where TB is prevalent (Group, 1996). The observations on BCG efficacy can be confounded if the vaccinees already had undiagnosed latent TB when vaccinated, since BCG cannot protect against reactivation of latent TB infections (Kaufmann, 2010a).

2.4.2 BCG protective efficacy

2.4.2.1 Human trials

Although BCG prevents severe childhood manifestations of TB disease, including miliary and the often fatal TB meningitis (Lanckriet, 1995; Sterne, 1998; Zodpey, 1998), little or no protection conferred by BCG against TB infections is observed in clinical trials conducted in tropical areas such as Karonga, Africa (Group, 1996), and Chingleput, India (Tuberculosis Research Centre (ICMR), 1999), while an estimated 75% protection in young adults is attributable to BCG vaccination in the UK (Sutherland and Springett, 1987). Some meta-analyses of BCG vaccine clinical trials concluded there is an average protective efficacy of 50% afforded by BCG (Colditz, 1994; Colditz, 1995), while others suggest that, depending on the geographical location, the protective efficacy of BCG can vary between 0 and 80% (Fine, 1989; Fine and Vynnycky, 1998). Reasons that have been suggested for the variability include differences in the BCG strains used (Lagranderie, 1996; Gorak-Stolinska, 2006), BCG dose (Davids, 2007), host genetic variations, vaccination route (Davids, 2006) and interference with BCG-mediated responses through exposure to Env (Fine, 1999; Black, 2001a; Weir, 2006) Protection attributable to BCG is particularly low in developing countries with high TB incidence, such as parts of Asia and Africa. This coincides with tropical regions where Env is prevalent (Chilima, 2006) and human exposure to Env is high (Black, 2001a).

2.4.2.2 Experimental models

The effects of BCG depend on its viability, as murine studies show that dead BCG does not generate protective immunity (Orme, 1988; Daugelat, 1995). BCG manufacturers have traditionally performed batch-testing for efficacy based on tuberculin conversion in guinea pigs. In research, BCG efficacy is usually determined by challenging BCG-vaccinated mice with low dose Mtb via the aerosol route 30 days after vaccination, then enumerating the lung bacterial burden one month post-infection (Orme, 2005). BCG reduces Mtb counts in the lungs of vaccinated mice or guinea pigs by 1 – 1.3 log or 2 – 3 log respectively, about a month after aerosol challenge with low-dose virulent Mtb (Huygen, 1996; Skeiky, 2004; Castanon-Arreola, 2005; Orme, 2005). Guinea pigs develop granulomas histologically similar to those in active TB patients, and can be used for both short-term protection studies and chronic disease models (McMurray, 1996; Baldwin, 1998; McMurray, 2001). Mice are naturally more resistant to TB and prime stronger immune responses. BCG usually takes a chronic course in mice, with development of long-lasting immunity (Blanden, 1969). A subcutaneous dose of BCG is still protective 30 weeks after vaccination (Aldwell, 2006). However, BCG-vaccinated mice do not achieve sterile eradication of Mtb, only a relatively small reduction in Mtb burden is observed, so there are some concerns that this model does not reproduce the ideal goal of vaccination against TB (Kaufmann, 2010a).

2.4.3 Routes of BCG administration

BCG vaccination was initially administered to humans orally. Oral vaccination requires a larger dose (higher costs), and oropharyngeal infection or intussusception in children due to enlarged Peyer's patches are potential complications. Therefore, BCG is now most commonly administered via the intra-dermal route, which is also more efficient at inducing tuberculin conversion (Fine, 1999).

Different routes of BCG administration prime different immune responses. Higher levels of Th1 cytokines in both $CD4^+$ and $CD8^+$ cells are observed with percutaneous compared to intra-dermal administrations of BCG in humans (Davids, 2006), although this may not necessarily have an impact on protective efficacy of BCG against TB (Hawkridge, 2008).

Protection afforded by BCG against murine TB infection also appears independent of the administration route, be it the subcutaneous, intravenous, rectal or aerosol route (Abolhassani, 2000; Lagranderie, 2000; Palendira, 2002), although immune changes induced may differ widely. The intravenous route yields higher frequencies of IFN-γ producing $CD4^+$ and $CD8^+$ cells than the oral route (Mittrucker, 2007). Recruitment of activated memory $CD44^{hi}CD62L^{lo}$ $CD4^+$ memory T cells to the lungs is fastest and highest in mice given inhaled BCG via aerosol, followed by the intravenous then the subcutaneous route (Palendira, 2002). In these studies, however, the differential routes and immune responses to BCG did not lead to different protective effects against Mtb challenge (Palendira, 2002; Mittrucker, 2007). In murine models, BCG vaccination is most

commonly administered via the subcutaneous or intra-dermal route, as this most closely mimics what happens in humans, even though the inhaled route of administration is likely to better engage local responses in the lungs and protects mice against inhaled TB infection (Falero-Diaz, 2000). The disadvantage of inhaled BCG is the risk of immunopathological reactions in the lungs (Nuermberger, 2004).

2.5 Cell-mediated immune responses with BCG vaccination and immune correlates of protection

Vaccination-induced protection against TB has been thought to depend on the generation of antigen-specific $CD8^+$ (Wang, 2004; Begum, 2009) cytotoxic T cells as well as $CD4^+$ Th1 T cell subsets together with the induction of IFN-γ (Pedrazzini, 1987; Yang and Mitsuyama, 1997). However, the understanding of immune correlates of vaccine protection is incomplete, and new findings are challenging several traditional concepts.

2.5.1 T helper type 1 $CD4^+$ response and IFN-γ responses

BCG induces the activation and proliferation of $CD4^+$ cells, with IL-2 expressing cells predominantly of the central memory phenotype, and IFN-γ expressing cells mainly of the effector phenotype (Soares, 2008). The magnitude of IFN-γ production following BCG vaccination has been, for a long time, considered a key immune correlate of protection (Huygen, 1992). Previously BCG-vaccinated HIV patients with higher baseline IFN-γ responses to Mtb antigens have a significantly lower risk for TB infection when followed up prospectively (Lahey,

2010). However, there is no correlation between BCG-induced IFN-γ responses and protection in mice (Mittrucker, 2007). Moreover, some candidate TB vaccines that induce higher levels of IFN-γ production than BCG, are nonetheless less protective than BCG in terms of reducing TB bacterial burden in animals (Skinner, 2003). The failure to show a consistent correlation between IFN-γ induction and protection may be partly related to effects of Th17 cells, which can mediate partial protection against TB challenge in BCG-vaccinated mice that lack IFN-γ (Wozniak, 2010).

In BCG-vaccinated humans and mice, majority of the cells produce single cytokines, i.e. different cells secreting just IFN-γ, TNF-α or IL-2 (Li, 2010), while other cells are polyfunctional and can produce these multiple cytokines simultaneously (Beveridge, 2007; Soares, 2008). *Mycobacterium*-specific polyfunctional T cells at the infection site have been associated with protection against TB challenge (Forbes, 2008). However, the latest large-scale neonatal BCG vaccination study involving multi-cytokine comparisons of those who did and did not succumb to TB within two years suggests that frequency and cytokine profile of *Mycobacterium*-specific T cells post-vaccination does not correlate with protection (Kagina, 2010).

2.5.2 $CD8^+$ T cells

BCG has traditionally been thought to be a poor inducer of $CD8^+$ T cell responses in humans (Kaufmann and McMichael, 2005). However, BCG-vaccinated macaques, if depleted of $CD8^+$ cells, have diminished immunity to subsequent

Mtb challenge (Chen, 2009). Moreover, BCG-specific proliferating $CD8^+$ T cells with functional cytotoxic potential are present in peripheral blood of vaccinated infants, ten weeks post-vaccination (Hanekom, 2005). The presence of $CD8^+$ T cells, in the absence of $CD4^+$ T cells in CD4 knock-out mice, is sufficient to mediate protection, albeit delayed, post-BCG vaccination in mice, and this effect is independent of the viability of BCG (Wang, 2004). The rapid accumulation of $CD8^+$ T cells after TB challenge in the infected tissues of BCG-vaccinated mice correlates better with protection than IFN-γ secretion (Mittrucker, 2007). $CD8^+$ T cells induced by BCG have cytolytic function (Turner and Dockrell, 1996), are antigen-specific, produce IFN-γ and can be involved in protection against TB (Begum, 2009).

2.5.3 Regulatory T cell (Treg) responses

Suppressor responses have long been known to occur with BCG vaccination (Bennett, 1978) but induction of naturally occurring and inducible regulatory T cells (Tregs) in *Mycobacterium* infections has been fairly recently described. Expansion of Tregs, both at disease sites and in peripheral blood, are observed in human TB studies, and has been linked to depressed immunity (Guyot-Revol, 2006; Hougardy, 2007a; Li, 2007). An increase in $CD4^+CD25^+$ Treg and FoxP3 expression, together with an increase in the level of anti-inflammatory cytokines IL-10 and TGF-β, is associated with BCG treatment, demonstrating that BCG induces Tregs (Li and Shen, 2009). Even in human neonates, BCG vaccination induces $CD4^+CD25^+FoxP3^+$ natural Tregs (Burl, 2010). Some suggest that $CD4^+CD25^+$ cells contribute to the IL-10 production after BCG vaccination

(Akkoc, 2010) but others have described lung cells (Mendez-Samperio, 2008) and inducible Tregs (Soares, 2008) as being responsible for increased IL-10 production in response to BCG vaccination. A murine study by Quinn demonstrates that antibody inactivation of natural Tregs – $CD25^+$ cells – three days before BCG vaccination increases the proportion and frequency of antigen-specific IFN-γ producing $CD4^+$ and $CD8^+$ T lymphocytes post-vaccination. Secondary responses to a subsequent BCG challenge occur earlier than in non-Treg depleted mice, without adverse impact on immune protection against TB (Quinn, 2006). This suggests that natural Tregs do not have a direct role to play in BCG induced protection. A separate study by Jaron, however, contradicts this. Jaron finds that murine BCG vaccination causes Treg recruitment to draining lymph nodes, and they suppress anti-mycobacteria $CD4^+$, but not $CD8^+$, T cell responses (Jaron, 2008). Moreover, $CD25^+$ Treg depletion led to a significant but moderate reduction in lung Mtb load after BCG vaccination and Mtb challenge, indicating that such Tregs have an impact on the protective capability of BCG against TB (Jaron, 2008).

2.6 Problems with BCG and novel strategies to replace or improve BCG as a TB vaccine

The failure of BCG to protect against adult pulmonary TB and latent TB has prompted efforts to develop more efficacious vaccine strategies. Many candidate vaccines for TB often fail to surpass BCG in its protection against TB in mice (Olsen, 2000; Skeiky, 2000; Orme, 2001; Doherty, 2004).

Given the reliability of BCG in protecting against disseminated TB in children, it may be unethical not to include BCG in any vaccine strategies (Andersen and Doherty, 2005). Hence, to retain the protective effects observed in childhood, a vaccination strategy that improves rather than replaces BCG would seem ideal (McShane, 2004). Several novel vaccines undergoing clinical trials are primarily based on either improving BCG antigenicity by genetic modification or boosting BCG-primed memory with subunit vaccines (Kaufmann, 2010a).

Although protection attributable to BCG is shown to be life-long in Alaskan natives and American Indians (Comstock, 1994; Aronson, 2004), most other studies demonstrate that protection only lasts for 10-20 years (Comstock, 1976; Hart and Sutherland, 1977; Sterne, 1998). To improve memory responses, new vaccines aim at improving antigenicity and immunogenicity. Some vaccines are engineered to over-express immunodominant Mtb proteins. An example is the r-BCG30, which simply over-expresses the immunodominant secretory antigen Ag85B (Horwitz, 2000). Improvement of antigen presentation has been achieved by rBCGΔure-Hly, a modified BCG strain expressing listeriolysin that causes perforation in phagosomes, allowing BCG to egress into the cytosol, and induces apoptosis of infected macrophages, thereby inducing cross-priming and increasing BCG immunogenicity (Grode, 2005). Another strategy is to design vaccines that target novel antigens such as the heparin binding haemagglutinin (HBHA) (Rouanet, 2009).

Another way to address the problem of BCG's waning responses is to boost the responses primed by an initial BCG vaccination (Andersen and Doherty, 2005).

Hence, there are scientists investigating subunit vaccine boosting after BCG priming (Schneider, 1998; McShane, 2001; McConkey, 2003). Viral vectors have been used in such vaccines, and one example is the modified vaccinia virus Ankara (MVA85A), expressing Ag85A (McShane, 2004; Sander, 2009). Given that Ag85A is shared between BCG and Mtb, it is an appropriate antigen to evoke a true booster response (Kaufmann, 2010a). Improvements of subunit vaccine antigenicity can also be achieved using novel adjuvant systems, such as monophosphoryl lipid A and QS21. This has been tested for *M. tuberculosis* 72F (M72), a fusion protein with Rv0125 and Rv1196 antigens, leading to strong T-cell immune responses and acceptable tolerability in humans (Skeiky, 2004; Von Eschen, 2009).

Being a live vaccine, BCG cannot be given to HIV and other immunocompromised patients, as this can lead to disease in such individuals (Kaufmann, 2010a). New vaccines therefore try to achieve further attenuation of BCG, such as through utilisation of BCG auxotrophs (Bange, 1996) or to improve vaccine safety with mutant Mtb strains, such as the double deletion Mtb mutants (Sambandamurthy, 2006). Another problem with BCG is that when given in neonates, a mixed Th1/ T helper type 2 (Th2)/ Treg/ T helper type 17 (Th17) response is primed, with the Th2 response persisting while the Th1 response wanes, leading to Th2 bias with BCG priming in early life (Siegrist, 2001; Burl, 2010). There is currently no obvious solution to this complex problem.

The aforementioned vaccines in research are designed as pre-exposure prophylactic vaccines, and therefore neither protect against development of

latent TB nor prevent reactivation of latent TB. Hence, scientists believe that the next generation of novel TB vaccines should target dormant Mtb and control reactivation for the lifetime of an infected individual (Kaufmann, 2006; Kaufmann). This requires a pre-exposure vaccination strategy that will induce immune mechanisms that are able to contain Mtb and sustain lifelong containment, as well as a post-exposure vaccine that will restimulate memory T cells and promote strong effector T cell responses specific for Mtb upon reactivation (Kaufmann, 2010a). There is interest in developing vaccines targeting antigens upregulated in latency, such as the DosR-regulated proteins (Lin and Ottenhoff, 2008). A multi-stage vaccine combining early antigens with latency-associated antigens has successfully controlled reactivation and reduced bacterial loads in mouse models of latent TB (Aagaard, 2011).

2.7 Environmental mycobacteria (Env)

M. tuberculosis and *M. leprae* are the main pathogens within the *Mycobacterium* genus causing severe human disease, and these are obligate intracellular bacteria. However, there are numerous species of mycobacteria that are free-living and ubiquitously found in soil, open waters and even urban water supplies (Chakrabarti, 1990; Falkinham, 2001), hence they are called environmental mycobacteria (Env) or non-tuberculous mycobacteria. Many of these are potentially pathogenic but opportunistic. Env were thought to rarely cause human disease, except upon direct inoculation or in immunocompromised individuals (Primm, 2004), where infection most frequently occurs in the lungs, skin and cervical lymph nodes (Wayne and Sramek, 1992; Tortoli, 2003).

However, there is increasing recognition that immunocompetent adults can also suffer from self-limited cutaneous to widespread Env infections such as lymphadenitis and disseminated disease (Jarzembowski and Young, 2008). Asymptomatic colonisation can occur in immunocompetent individuals. Many Env species are genetically closely related to BCG (average >95% similarity) and are likely to share many antigens with BCG (Stahl and Urbance, 1990; Tortoli, 2003).

2.7.1 Classification of Env

Non-tuberculous or environmental mycobacteria are categorised into four broad groups (Runyon Classification) according to their growth rate and pigment production (Timpe and Runyon, 1954). Slow-growers (Groups I, II, III) are distinguished from fast-growers (Group IV) based on whether the bacteria grow on agar after seven days. These are further divided into groups depending on their ability to produce pigment. Slow growers include pathogens such as *M. kansasii, M. marinum, M. scrofulaceum, M. szulgai, M. avium-intracellulare* (also known as *M. avium complex*) and the non-pathogenic *M. gordonae* and *M. flavescens.* Fast-growing Env include pathogens such as *M. fortuitum* and *M. chelonae* (CHE) and non-pathogenic *M. smegmatis* and *M. vaccae*. Slow growing Env within the *Mycobacterium* genus are phylogenetically distant from the fast growing ones. Antigens in common between species and the consequent cross-reactivity in the host reflect this phylogenetic distance (Black, 2001a).

2.7.2 *M. chelonae* (CHE)

CHE is a rapidly growing Env and, like many other Env species, CHE is normally found in soil and open waters. CHE has also been isolated from drinking water systems and urban water supply (Covert, 1999; von Baum, 2010). CHE is known to cause infections in animals, especially in aquatic species, such as disseminated granulomatous lesions in fish, tuberculosis-like lesions in turtle lungs, as well as abscesses and ulcerative lesions in manatees and some other mammalian species (Thoen, 1981; Howard and Byrd, 2000). CHE can also cause human disease, of which chronic pulmonary disease and skin or soft-tissue infections from local trauma are the most common disorders associated with CHE, along with a few other rapid growers (Falkinham, 1996; Chan, 2010). No naturally-acquired CHE murine infections have been described in the literature.

2.8 Immune responses to Env

T cell, IFN-γ or TNF-α knock-out mice have higher organ loads of Env when infected, compared to wild-type mice, indicating the importance of these factors in defending against Env infections (Byrd and Lyons, 1999; Rottman, 2007). Env also induce pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α in the host (Bradbury and Moreno, 1993; Denis and Ghadirian, 1994).

Individuals are sensitised upon repeated exposure to Env, and as with BCG, this exposure stimulates cell-mediated immune responses, which can be measured both *in vitro* (Black, 2001a) and as a skin test (Fine, 2001b; Weir, 2003). In Malawi, a strong correlation was observed between IFN-γ responses for the

closely related *M. avium, M. intracellulare, M. scrofulaceum* and *M. marinum* but not for more distantly related Env species such as fast-growing *M. fortuitum* and *M. vaccae*, indicating a role of the relatedness of the Env species in determining the strength of responses elicited (Black, 2001a). These findings suggest that different species of Env elicit different immune responses, usually as a result of their phylogenetic distance from one another. In murine studies, the slow-growing Env species *M. avium* and *M. scrofulaceum* elicit stronger responses than the fast-growing *M. vaccae* (Demangel, 2005), possibly related to their persistence in hosts. When six different isolates of Env from soil and sputum samples from Malawi were administered intravenously in mice, the fast-growing CHE and *M. fortuitum* were rapidly cleared, while strains of the slow-growing *M. avium* complex multiplied to three logs above the other strains in the spleen, liver and lungs (Brandt, 2002).

Env can also induce immunosuppressive responses, as evidenced by studies where *M. vaccae* administration reduced airway inflammation and hyper-responsiveness in asthmatic models in mice (Hopfenspirger and Agrawal, 2002) and guinea pigs (Zhao, 2003). The *M. vaccae* effect is not due to a switch to a Th1 dominated response as earlier thought (Zuany-Amorim, 2002a; Adams, 2004). An IL-10 and TGF-β dependent $CD4^{+}CD45RB^{lo}$ T cell population was found to harbour Treg activity as the adoptive transfer of these cells, taken from *M. vaccae* sensitised mice, into allergic recipient mice, blocked the response to airway challenge with antigen in these mice (Zuany-Amorim, 2002a), and this was found to be allergen specific (Zuany-Amorim, 2002b). Certain strains of *M. avium* are known to suppress co-stimulation and pro-inflammatory cytokine secretion by

human DCs (Buchan, 2009).

2.9 Effects of environmental mycobacteria exposure on BCG vaccination

It has been proposed that exposure to Env through environmental contact results in immune modulation of the response to BCG, which adversely affects the protection conferred. Numerous animal studies have long supported this notion (Edwards, 1982; Orme and Collins, 1986; Howard, 2002). Human epidemiological studies also show that BCG is more efficacious where neonates are vaccinated very early (before Env exposure) (Colditz, 1995) and there is poor BCG efficacy in areas with high Env exposure (Weir, 2006).

Two main hypotheses have been mooted for immune modulation by prior Env exposure on BCG vaccination. The first is the 'masking' hypothesis – prior Env exposure confers a level of immune protection against TB, which masks the effects of a subsequent BCG vaccination. Guinea pig studies by Palmer in 1966 (Palmer and Long, 1966) first noted that Env immunisation offers some protection against TB challenge, but reduces the apparent protection conferred by BCG. Circumstantial evidence from human epidemiological studies also supports this hypothesis, because BCG vaccine efficacy is reduced in populations highly sensitised to Env (Black, 2001a; Black, 2002). Much of such knowledge arises from studies comparing Malawi and United Kingdom, as these populations differ markedly in BCG efficacy in clinical trials (Sutherland and Springett, 1987; Group, 1996). Prevalence and magnitude of skin-test sensitivity to protein preparations derived from Env species are higher in Malawi than in the UK,

affirming that Malawians have greater Env exposure (Black, 2001b; Black, 2002; Weir, 2003). In Malawians, BCG vaccination confers no additional protection relative to unvaccinated controls, and their post-BCG cellular responses (IFN-γ and DTH) to *Mycobacterium* antigens are minimally increased (Group, 1996; Black, 2002). The opposite outcomes are noted in UK residents, where post-BCG increases in IFN-γ responses to proteins of different Env species are correlated with the relatedness of each Env species to BCG (Black, 2002). This suggests that memory T cells that respond to BCG potentially cross-react with Env antigens (Weir, 2006), and indicate that prior Env exposure potentially confers a level of protective immunity to TB that is not surpassed by subsequent BCG vaccination (Andersen and Doherty, 2005).

A second hypothesis, the 'blocking' hypothesis', based on animal studies, suggests that prior Env sensitisation modifies the host's BCG response, which limits BCG replication, thereby diminishing BCG-induced immunity (Buddle, 2002; de Lisle, 2005; Demangel, 2005). A reduction in BCG counts in host mice pre-exposed to live *M. avium* (but not to CHE or *M. fortuitum*) has been described by Brandt, correlating with the abrogation of BCG-induced protection against TB, relative to non-sensitised mice (Brandt, 2002). This occurs even though the *M. avium* is cleared with antibiotics before BCG administration. Demangel's study concurs that Env diminishes BCG replication, but differs in not observing any reduction in protective effects of BCG against TB (Demangel, 2005). Cross-reactive memory responses to shared antigens between Env and BCG could have led to the host clearing BCG more efficiently (Andersen and Doherty, 2005).

However, the precise immune mechanisms underlying the cellular response to Env and how these led to reduced BCG replication is largely unknown. A few mechanistic studies have emerged in recent years. One study has proposed that induction of Th2 responses may underlie how Env modifies the host response. Oral exposure to *M. avium* reduces IFN-γ responses and upregulates antibody responses in BCG vaccinated mice, suggesting a switch to Th2 responses upon Env exposure (Young, 2007). Another study claims that DCs exposed to *M. avium* do not express co-stimulatory molecules but upregulate programmed death-1 ligand-2 (PD-L2), thereby potentially inducing tolerance to BCG (Mendoza-Coronel, 2010).

In humans, more complex immunological explanations may account for the Env effect. A Gambian neonatal study shows that delaying BCG vaccination for 4 ½ months (allowing time for Env exposure) leads to reduced IFN-γ, IL-6, and IL-17 responses (all of which have anti-mycobacterial properties), relative to vaccination at birth, but both groups have similar anti-mycobacterium responses at nine months (Burl, 2010). The IFN-γ reduction is not directly correlated with pre-vaccine natural Treg frequencies or IL-10 levels in the delayed vaccine group. The authors suggest that low levels of IL-6 may contribute to poorer IL-17 induction, and increased sensitivity of *Mycobacterium*-specific T cells to Treg suppression of IFN-γ production (Burl, 2010).

Where BCG is administered in infancy, it could be argued that prior Env exposure is minimal. Does Env exposure *after* BCG vaccination have any effect on BCG efficacy? There is limited data, but a murine study on oral *M. avium* exposure

following BCG vaccination shows this lowers the magnitude of Mtb load reduction in the lungs conferred by BCG vaccination, unrelated to any obvious changes in T cell immunity (Flaherty, 2006). Whether the effects are because of live *M. avium* colonising the lungs is unclear. In a human study focusing on delivering BCG vaccines within the first month of life, there is still a substantially lower proportion of Malawian infants (53%) with IFN-γ responses to Mtb PPD post-vaccination, as compared to UK infants (100%) (Lalor, 2009). Although the reasons for the difference were not studied, exposure to Env after vaccination remains one of the possible explanations.

There are also papers that disagree that Env exposure is negative for BCG vaccination responses. One study states that cattle exposure of cattle to *M. avium* before BCG vaccination does not reduce BCG-specific immune responses and actually primes better responses to *M. bovis* challenge resulting in lower TB pathology (Thom, 2008).

2.10 Regulatory T cells (Tregs)

Tregs are crucial for maintaining homeostatic balance in the immune system, as evidenced by devastating consequences in hosts lacking or with disrupted Tregs (Brunkow, 2001; Fontenot, 2003; Sakaguchi and Sakaguchi, 2005). In addition, Tregs are important in limiting harmful inflammatory responses, as observed in allergic disorders (Zuany-Amorim, 2002b), inflammatory bowel disease (Powrie, 1994) and bacteria-triggered colitis (Kullberg, 2002). Tregs exert suppressive effects on immune responses, and are known to influence $CD4^+$ (Thornton and

Shevach, 2000), $CD8^+$ T cells (Piccirillo and Shevach, 2001; Green, 2003) as well as cells of innate immunity (Maloy, 2003). Tregs are hence an important consideration when evaluating efficacy of immune responses against infectious pathogens. Thymectomy studies in mice indicate that Tregs originate from the thymus (Fontenot, 2005), but peripheral T cells have also been demonstrated to acquire Treg ability (Chen, 2003), suggesting that different groups of Tregs exist.

Two main populations of Tregs have been described. They are naturally occurring thymus-derived natural Treg cells (nTregs) (Sakaguchi, 2004; Shevach, 2006) and adaptive or inducible Treg cells (iTregs) that arise in peripheral lymphoid organs (Bluestone and Abbas, 2003; O'Garra, 2004). Based on murine models of inflammatory diseases such as cancer and autoimmune disorders, various phenotypes of iTregs have been described – IL-10 secreting type 1 regulatory T (Tr1) cells, TGF-β secreting T helper type 3 (Th3) cells and converted $FoxP3^+$ cells. Both types of Tregs can control effector responses and play an important role in infections (Belkaid, 2007).

2.10.1 Natural Tregs (nTregs)

nTregs are a subset of $CD4^+$ T cells that typically express high levels of CD25 (also known as IL-2 receptor α–chain), and in some cases also the co-stimulatory molecule cytotoxic T lymphocyte antigen 4 (CTLA-4), tumour necrosis factor (TNF)-superfamily member glucocorticoid-induced TNF receptor family related (GITR) protein (Bluestone and Abbas, 2003), OX40 (Shevach, 2006), and high levels of folate receptor 4 (FR4) (Yamaguchi, 2007). However, these markers can

also be expressed on activated cells, and hence cannot be used as specific markers to identify nTregs. nTregs are able to suppress the effector functions of $CD4^+$ and $CD8^+$ T cells (Thornton and Shevach, 1998; Murakami, 2002), and the transcription factor FoxP3 is the most definitive molecular marker for these cells, particularly in mice (Fontenot, 2003; Fontenot, 2005; Roncador, 2005), though FoxP3 is also transiently upregulated on activated human T cells (Gavin, 2006).

The effects of nTregs during infections are double-edged – they can function to protect the pathogen or the host. This is shown in murine models of *Leishmania major* infection, where accumulation of nTregs regulates the function of effector cells at the site of infection, preventing eradication of the parasite (Belkaid, 2002). However, at the same time, the persistence of *L. major* that results from immune suppression by nTregs is also necessary for maintaining host immune responses against the parasite in the same model, demonstrating the fine balance between the immune responses to the host and pathogen that is influenced by nTregs (Belkaid, 2002). The immunosuppression exerted by nTregs can be excessive in some instances, thereby allowing the pathogen to replicate aggressively and compromise the survival of the host. This is observed in mice infected with *Plasmodium yoelii*, which causes lethal malaria in rodents. nTreg depletion restores effector immune responses that are otherwise limited by nTregs, eventually leading to pathogen elimination and host survival (Hisaeda, 2004). Self antigens released during tissue damage in acute diseases are probably recognised by nTregs, priming their activity, but studies of chronic infections suggest that nTregs can also recognise microbial antigens (Belkaid, 2002; Cabrera, 2004; Weiss, 2004; Hisaeda, 2005). Furthermore, instead of being anergic, nTregs

proliferate at the site of infection when they encounter microbial antigens, and may depend on these antigens for their survival in *L. major* infections (Suffia, 2006).

2.10.2 Natural Tregs in TB

In TB patients, an increase in frequency of Tregs of the $CD4^+CD25^{high}$ phenotype is observed, together with elevated mRNA expression of FoxP3, in the peripheral blood of these patients (Guyot-Revol, 2006). The authors suggest that this expansion of Tregs may contribute to suppression of immune responses against TB. Other human and murine studies have also repeatedly demonstrated that $CD4^+CD25^+$ Treg can be expanded and suppress T cell mediated protective responses during the course of TB disease, thus facilitating pathogen persistence (Dieckmann, 2005; Hougardy, 2007a; Mason, 2007). A study on effects of reconstituting recombination activating gene (RAG) deficient mice, which lack functional T and B cells, with $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ cells, shows that the Tregs directly restrict T helper cell proliferation, IFN-γ production, and abrogate the protection against Mtb challenge conferred by T helper cells (Kursar, 2007). In support of this, human *ex vivo* peripheral blood experiments suggest that $CD4^+CD25^+$ Tregs in TB patients suppress Th1 but not Th17 cells (Marin, 2010). Both these studies suggest that the effects of these $CD4^+CD25^+$ Tregs are not due to IL-10. The suppression of immunity against TB mediated by $CD4^+CD25^+FoxP3^+$ Tregs is also independent of IL-10 (Chen, 2007), suggesting that nTregs do not usually act via IL-10 production. Two studies disagree on whether depletion of $CD25^+$ cells prior to murine pulmonary infection with Mtb

affects bacterial burden or pathology (Quinn, 2006; Jaron, 2008), but it has been commented that the role for nTregs in suppressing Mtb infections may be more marked in animals not bred in sterile conditions and have more nTregs expanded through repeated Env exposure (Shafiani, 2010).

2.10.3 Adaptive or inducible Tregs (iTregs)

iTregs are less well defined, but are known to be extrathymic $CD4^+$ T cells that develop after encountering antigen presenting cells modulated by microbial antigens or upon contact with regulatory cytokines (O'Garra, 2004). Activity of antigen-driven iTregs may not require FoxP3 (Vieira, 2004), and instead requires the immunoregulatory cytokines IL-10 and TGF-β (Groux, 1997). The ability of IL-10 to inhibit both Th1 and Th2 responses has been shown in experimental models of various chronic diseases such as malaria and schistosomiasis (Li, 1999; Hoffmann, 2000), as well as in human infectious diseases including TB (Boussiotis, 2000).

Functional regulatory T cells can be expanded *in vitro* from peripheral blood of individuals latently infected with TB, but not non-infected individuals in the presence of TGF- β and BCG (Hougardy, 2007b), indicating that Tregs can be induced in TB infected patients. This is important in controlling effector immune responses beneficial to the host, as observed in murine models of *Toxoplasma gondii*, where IL-10 deficient mice, although able to control parasite numbers, die as a result of lethal immunopathology caused by uncontrolled effector immune responses (Gazzinelli, 1996). The conversion of $CD4^+CD25^-$ T cells into

FoxP3$^+$ Tregs has been documented in both mice and humans. This is achieved in the presence of different stimuli, including TGF-β upon T cell receptor ligation (Chen, 2003; Fantini, 2004; Zheng, 2007), IFN-γ (Wang, 2006) and prolonged exposure to low dose antigens (Kretschmer, 2005). Peripheral conversion of Tregs is potentially important in long-term chronic infectious diseases, such as TB, as the output of nTregs is expected to diminish with ageing, due to a reduced production of T cells over time in the face of chronic antigenic stimulation (Belkaid, 2007). Without properly defined markers to distinguish nTregs from converted Tregs, however, such issues will remain difficult to address and study.

2.10.4 IL-10 in TB disease

Monocytes and macrophages are rapidly induced to produce IL-10 during Mtb infections (Song, 2003), and T cells can also produce IL-10 upon stimulation (Boussiotis, 2000). IL-10 mediates suppression of immune responses during active TB, which includes suppressing the production of proinflammatory cytokines like IFN-γ and TNF-α (Fiorentino, 1991; Gong, 1996; Hirsch, 1999), impairing Th1 responses (Akbari, 2001) and directing responses towards the Th2 type in the lungs of infected hosts (Almeida, 2009), regulating macrophage apoptosis (Patel, 2009), inhibiting the differentiation of monocytes to DCs (Remoli, 2010) and preventing the maturation of phagosomes in Mtb infected macrophages (O'Leary, 2011). The exact role of IL-10 producing Tregs in active TB is unclear, but the involvement of IL-10 secreting Tregs in escaping immune surveillance by Mtb has been proposed in anergic TB patients (Boussiotis, 2000).

CHAPTER 3 – *Mycobacterium chelonae* sensitisation induces CD4$^+$-mediated cytotoxicity against BCG

3.1 INTRODUCTION

Palmer first observed in guinea pigs (Palmer and Long, 1966) that Env exposure may confer some immune protection against TB, masking the effects of subsequent BCG vaccination. This was later also supported by circumstantial evidence from human studies in Malawi. The population had high levels of immune recognition of Env antigens before vaccination (Black, 2001a), and subsequent BCG vaccination conferred little additional protection against TB relative to non-vaccinated controls (Ponnighaus, 1992). The above observations suggest that although Env-induced symptomatic disease is uncommon in humans, exposure to Env may nonetheless prime T cell memory responses that influence the subsequent response to BCG. However, effector mechanisms of Env-specific T cells have not been fully elucidated. In murine studies, prior Env exposure limits the survival of live BCG given subsequently (Brandt, 2002; Demangel, 2005). This may be due to cross-reactive memory responses to shared antigens, but the nature of Env-induced effector cells and how they interact subsequently with BCG-infected cells, were not described in those studies.

There is evidence for human T cells with *Mycobacterium*-specific cytotoxic activity (Tan, 1997) and that mycobacteria can be killed via cytotoxicity mechanisms (Stenger, 1997; Stenger, 1998). Cytotoxicity against *Mycobacterium*-infected cells may be influenced by FasL and IFN-γ (Boselli, 2007). Therefore, we

hypothesised that the curtailment of BCG survival after Env exposure could be due to cytotoxicity against live BCG and/or BCG-infected cells, induced by Env-specific memory cells that recognise shared (common) antigens between BCG and Env. This work also sought to identify some cells types and factors that may play a role in this Env-induced cytotoxicity. The data showed that $CD4^+$ T cells from CHE-sensitised mice, but not naïve mice, kill BCG-infected autologous macrophages and that IFN-γ, perforin and FasL may have a role in the cytotoxicity. The sensitisation also resulted in increased cellular responses to BCG, associated with decreased *in vivo* BCG survival. This work thus provides one cellular mechanism by which Env may modulate immune responses to BCG.

3.2 MATERIALS AND METHODS

3.2.1 Mice

Murine experiments were approved by the NUS Institutional Animal Care and Use Committee. BALB/c mice that were 5 – 6 weeks old were purchased from the Centre for Animal Resources (CARE) and Biological Resources Centre (BRC) and housed in filtered isolators.

3.2.2 Bacteria

M. chelonae (CHE) derived from clinical samples, was a kind 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 appendix), supplemented with oleic acid-albumin-dextrose-catalase (OADC; Difco), and single colonies picked for growing in Middlebrook 7H9 broth (Difco) + 0.1 % Tween 80 (refer to appendix). *Bacillus subtilis* (ATCC) was cultivated in either LB broth or LB agar (Difco). Some cultures were frozen in 50 % glycerol aliquots at -80 °C before use.

3.2.3 Preparation of heat-killed and live bacterial cultures

Mycobacterium bovis BCG, CHE and *B. subtilis* broth cultures were grown to mid-log phase. Required volumes of culture were then 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 syringed through a 27 G needle to reduce clumping, before absorbance was measured at 600 nm to estimate bacterial numbers (1 A_{600} ~ 1.25 x 10^8 bacteria). Bacterial suspensions were diluted to obtain 1 x 10^6 cells/ 100 µl PBS or 1 x 10^7 cells/ 50 µl PBS for murine immunisation and lymphocyte restimulation respectively. For heat-killed preparations, these aliquots were treated at 95°C for 15 min, all in one batch to ensure uniformity of treatment, then snap-frozen until use. For intra-nasal (i.n.) infection of mice, 1 x 10^5 live BCG were re-suspended in a final volume of 10 µl PBS. Such preparations were kept at 37 °C prior to infection. All bacterial preparations were subjected to purity check and counting of the actual colony-forming units (CFU) by serial dilutions and culture on solid media. BCG, CHE and *B. subtilis* colonies were counted after incubation at 37 °C for three weeks, four days and two days respectively.

3.2.4 Murine immunisation and live BCG challenge

Mice were immunised once a week via the intra-peritoneal (i.p.) route, for three weeks, with PBS or 10^6 heat-killed CHE in 100 µl sterile PBS, prepared as described above. For BCG infection, 1 x 10^5 live BCG in 10 µl sterile PBS was given via the i.n. route one week after the last immunisation. The infected mice were sacrificed at one or three weeks post-infection.

3.2.5 Isolation of murine peritoneal macrophages

Mice were sacrificed by CO_2 asphyxiation at appropriate time points. Peritoneal macrophages were harvested by injecting 10 ml of ice-cold RPMI 1640 supplemented with 2 mM L-glutamine (RPMI, Sigma) + 10 % fetal bovine serum (FBS, BioGen) into the peritoneal cavity via a 21 G needle and the peritoneum gently massaged before withdrawal of the peritoneal fluid through a 18 G needle. All peritoneal fluid was subsequently kept on ice, until use. Cell suspensions were centrifuged at 400 x *g* at 4°C, for 10 min, and cells re-suspended in 2 ml of RPMI + 10 % FBS. Cells were then seeded into tissue culture wells to obtain adherent cells after overnight culture.

3.2.6 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 mm 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 appendix) 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.

3.2.7 Trypan Blue exclusion assay

To count viable murine cells, 10 µl of 0.04 % trypan blue dye (Sigma) was mixed with 10 µl of cell suspension. Cells were then counted under the microscope upon loading of 10 µl of the mixture into a haemocytometer. Non-viable cells stain blue because of their inability to limit the entry of the blue dye, while viable cells remain clear.

3.2.8 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.

3.2.9 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 CHE, supernatants harvested from cell cultures were assayed by Enzyme-Linked Immunosorbent Assay (ELISA) for the presence of IFN-γ and IL-10 (R&D Systems), using the respective kits according to manufacturer's instructions. All assays were based on the sandwich ELISA. In brief, 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 for detection, and streptavidin- or avidin- horse radish peroxidase (HRP) were used in conjunction with TMB substrate to produce a colorimetric 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 31.2 pg/ ml for IFN-γ and IL-10.

3.2.10 Cytotoxicity 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 a stable cytosolic enzyme – lactate dehydrogenase (LDH), which is released upon cell lysis. A red formazan product, which is formed through conversion of a tetrazolium salt (INT) by LDH released in culture supernatants, was measured by a 30 min coupled enzymatic assay. The amount of the formazan was measured at 490 nm, with the reference wavelength at 650 nm. The percentage cytotoxicity was calculated as follows:

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

3.2.11 Cytotoxicity assay experimental set-up

Freshly isolated murine peritoneal cells were seeded in triplicates in 96-well round-bottom tissue culture plates at 2 x 10^4 macrophages per 200 ml of RPMI + 10 % FBS. The adherent cells, after overnight culture, were used as target cells. Separately, to generate effector cells, $CD4^+$, $CD4^-$ 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 CHE, BCG (at a bacteria to cell ratio of 10:1) or PBS, non-adherent effector cells were harvested for viability

count, and the culture media replaced with fresh RPMI without phenol red (Invitrogen) + 2 % FBS + ferric ammonium citrate (FAC) at a working concentration of 50 μg/ml (refer to appendix). Target (adherent peritoneal) cells were infected with live *M. bovis* BCG at 10 bacteria per cell, 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. In some experiments, endotoxin-free Ab – anti-IFN-γ (10 μg/ ml, XMG1.2; BioLegend), anti-IL-10 (10 μg/ ml, JES5-16E3; Biolegend); anti-FasL (2 μg/ ml, MFL3; BioLegend), their respective isotype controls, or 3 nM concanamycin A (Sigma) were added during stimulation and just before co-culture. 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.

3.2.12 Flow cytometry

Lung cells for intracellular IFN-γ staining were stimulated with phorbol 12-mistrate 13-acetate (PMA; 50 ng/ ml; Sigma) and ionomycin (1 μM; Sigma), and treated with monensin (5 μM) for 6 h. Cells were then harvested, washed with staining buffer (PBS + 0.5 % bovine serum albumin (BSA)), and surface stained with relevant mAb or isotype controls in staining buffer for 30 min on ice, washed with cold PBS, then fixed with 4% paraformaldehyde. For intracellular perforin and IFN-γ staining, the cells were permeabilised by washing with 0.1% saponin in PBS (PBS-S), followed by incubation in 50 ml of PBS with 0.1% saponin and 0.1% BSA (PBS-S/BSA) for 30 min on ice.

Cells were subsequently stained with relevant mAb in PBS-S/ BSA, washed with PBS-S and re-suspended in PBS/BSA. Flow cytometry was performed using the Cytomics FC500 (Beckman Coulter) within 24 h. The following anti-mouse mAb were used, along with their respective isotype controls: CD3 (145-2C11, BD Biosciences), CD4 (RM4-5, BD Biosciences), CD8 (53-6.7, BioLegend), perforin (eBioOMAK-D, eBioscience) and IFN-γ (XMG1.2, BD Biosciences).

3.2.13 Statistical analysis

Means of triplicate well assays were compared using a two-tailed Student's *t* test.

Differences between groups were considered statistically significant when $p < 0.05$.

3.3 RESULTS

Splenocytes from naïve mice sensitised by intra-peritoneal (i.p.) immunisation with heat-killed *M. avium*, CHE*, M. smegmatis*, BCG or PBS, exert a range of cytotoxic responses against BCG-infected autologous macrophages upon stimulation with different heat-killed mycobacterial preparations (Ho, 2009). CHE sensitisation *in vivo*, followed by *in vitro* splenocyte restimulation with *Mycobacterium* antigens, resulted in the strongest cytotoxicity compared to other Env-sensitised mice. This is why CHE sensitisation was chosen in the present study to further investigate cytotoxicity against BCG induced by Env exposure. Fig. 3.1 shows that in mice given repeated CHE sensitisation, followed by *in vitro* heat-killed BCG restimulation, there was higher cytotoxicity against BCG-infected macrophages than in unstimulated cells or in BCG-stimulated cells from PBS mice. The most likely explanation is that the enhanced cytotoxicity is due to memory lymphocytes from CHE-sensitised mice being expanded upon BCG stimulation, due to cross-reactivity.

3.3.1 Cell subsets involved in cytotoxicity

Earlier studies in the laboratory have shown that if $CD4^+$ cells were depleted from lymphocytes of CHE-sensitised mice before co-culture with BCG-infected cells, cytotoxicity is almost completely inhibited relative to untouched lymphocyte co-cultures. There was no decrease in cytotoxicity with $CD8^+$ or $CD56^+$ cell depletion. This suggests that CHE-responsive $CD4^+$ T cells had a major role in mediating cytotoxicity against BCG-infected cells. (Ho, 2009).

To verify that $CD4^+$ cells are an important cell subset involved in CHE-mediated cytotoxicity against BCG, the cytotoxicity assay was repeated with either pure $CD4^+$ cells or CD4-depleted T cells ($CD4^-$) (Fig. 3.2). Antigen-stimulated $CD4^+$ cells in co-cultures with autologous BCG-infected macrophages showed higher cytotoxicity than co-cultures with $CD4^-$ cells ($p = 0.07$), supporting the previous results and providing direct evidence for the role of $CD4^+$ cells in CHE-induced cytotoxicity. The cytotoxicity induced by cells of CHE-sensitised mice was higher than that by cells from PBS control mice, regardless of which T cell type was used.

To confirm that cytotoxicity of infected macrophages is linked to killing of the intracellular bacteria, BCG in the wells were cultured at the end of the cytotoxicity assay. There was a significant negative correlation between % cytotoxicity and viable BCG counts, suggesting that the lysis of infected macrophages is directly correlated with and negatively affects BCG survival. (Data not shown, this work was performed by laboratory colleagues.)

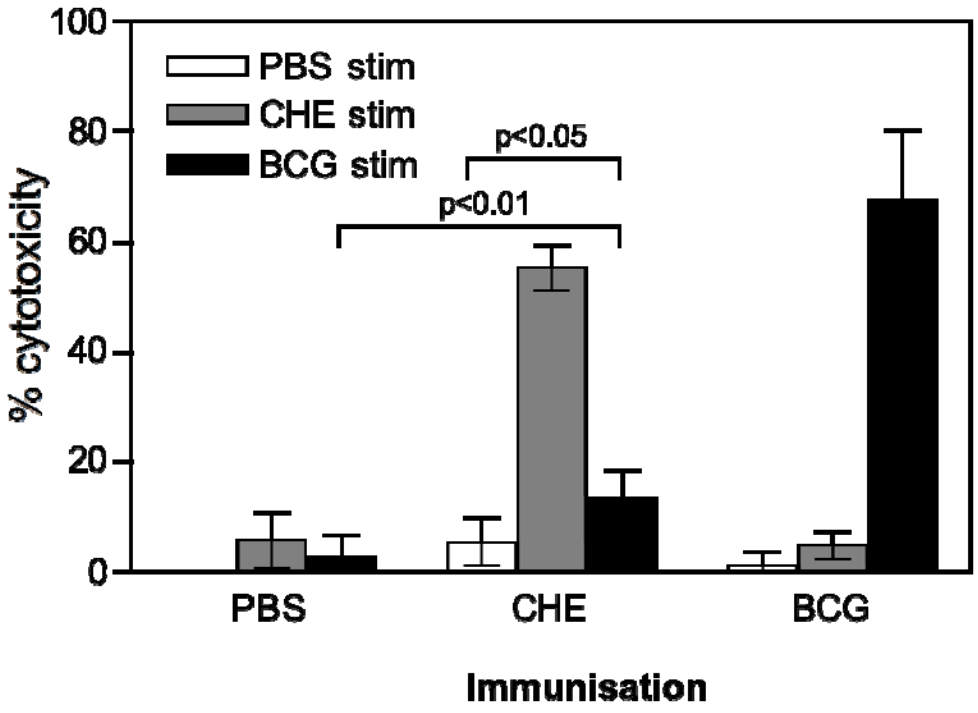

Fig. 3.1: Splenocytes of CHE-sensitised mice are cytotoxic to BCG-infected cells upon restimulation with CHE or BCG
Mice immunised three times, once a week, with i.p. heat-killed CHE, BCG or PBS, were sacrificed 1 week after the last exposure. Splenocytes were stimulated *in vitro* for 48 h with heat-killed preparations of CHE, BCG, or PBS before co-culture with autologous BCG-infected macrophages. Mean % cytotoxicity ± 1 SD of 3–5 mice per group are shown. Statistics by Student's *t* test.

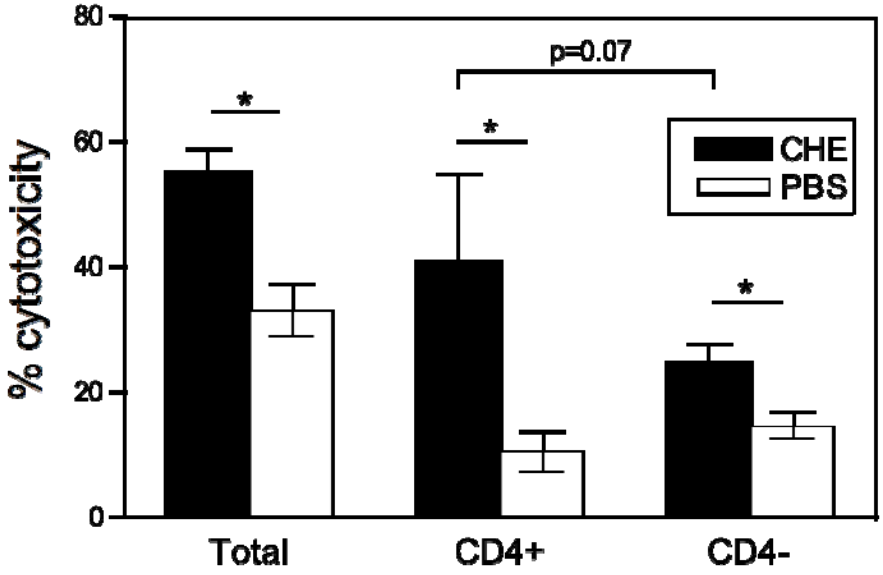

Fig. 3.2: Effect of cell subset enrichment on cytotoxic activity.
Mice were i.p. immunised with heat-killed CHE once a week for three weeks and their splenocytes harvested. $CD4^+$ positively selected cells (by autoMACS) and $CD4^-$ cells (negative fraction after CD4 selection) from the splenocytes were stimulated with heat-killed CHE or PBS and then co-cultured with BCG-infected macrophages. Mean % cytotoxicity ± 1 SD shown for 3 – 6 mice per group. *$p<0.01$ using Student's *t* test.

3.3.2 Mediators of cytotoxicity

Various factors were neutralised using inhibitors prior to performing the cytotoxicity assay in CHE-sensitised mice, to investigate some of the potential pathways and cytokine mediators involved in CHE-induced cytotoxicity. Blocking either FasL or perforin pathways led to reduced cytotoxicity, with a relatively greater reduction seen when perforin was blocked. When both pathways were concurrently inhibited, the mean cytotoxicity was further reduced but not significantly more than either pathway alone (Fig. 3.3A). Inhibition of IFN-γ significantly reduced cytotoxicity (Fig. 3.3B), but there was no effect upon blocking IL-10 (Fig. 3.3B). Thus Fas, perforin and IFN-γ, play a role in CHE-induced cytotoxicity, but IL-10 may not be an important suppressor of this activity.

3.3.3 Specificity of cytotoxic responses

In order to ascertain that the cytotoxic responses observed were induced by CHE and not a result of non-specific inflammatory responses, naïve mice were sensitised with an unrelated bacterium *Bacillus subtilis*, and cytotoxicity of splenocytes from these mice were compared against that of cells from CHE-sensitised mice. Regardless of the *in vitro* stimulation, splenocytes from *B. subtilis* sensitised mice exerted cytotoxic effects that were similar to negative control mice given PBS – significantly less than the cytotoxicity observed in co-cultures of cells from CHE-sensitised mice (Fig. 3.4) This implies that *Mycobacterium*-specific sensitisation is required for significant cytotoxicity

against BCG-infected macrophages, therefore the effects of CHE sensitisation are not likely to be attributable to a non-specific inflammatory response (Fig. 3.4).

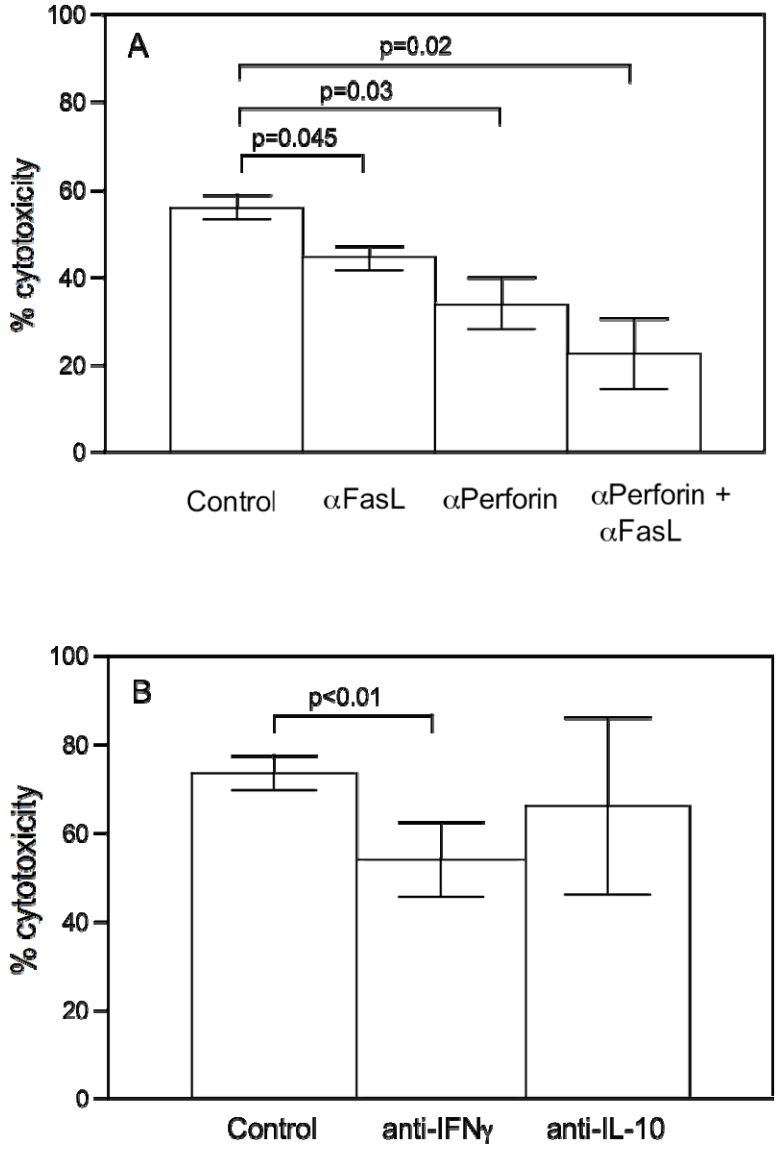

Fig. 3.3: Role of FasL, perforin, IFN-γ and IL-10 in CHE-mediated cytotoxicity
Mice were i.p. immunised with heat-killed CHE three times, once a week, and splenic T cells were re-stimulated *in vitro* with heat-killed CHE before co-culture with BCG-infected macrophages to test for cytotoxicity. Prior to and during co-culture, (A) anti-FasL Ab and/ or concanamycin A were used to block FasL (αFasL) and/ or perforin (αPerforin) respectively, while in (B) anti-IFN-γ Ab or anti-IL-10 Ab were used. Isotype control Ab was added to control wells. Mean % cytotoxicity ± 1 SD is shown for 3–6 mice per group. Statistics by Student's *t* test.

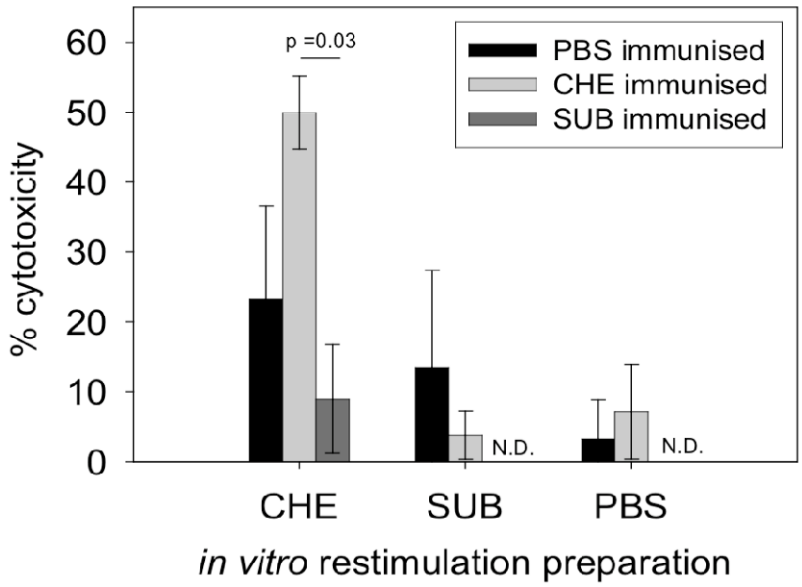

Fig. 3.4: Cytotoxicity is specific to CHE and not non-specific
Mice were immunised thrice with heat-killed CHE, *Bacillus subtilis* (SUB) or PBS, once a week. Splenocytes were stimulated for 48 h with heat-killed CHE, SUB or PBS before co-culture with BCG-infected macrophages. Means of 3 mice per group shown ± 1 SD. N.D. = Not detectable. Data represent one of two independent experiments with similar results.

3.3.4 Effect of Env sensitisation on live BCG infection

Perforin expression is a marker of cells with cytotoxic potential. After BCG infection, $CD4^+$ cells accounted for almost 70% of the perforin-positive cells in both CHE- and PBS-sensitised mice, indicating the predominance of $CD4^+$ cells among perforin-expressing cells (Fig. 3.5A). A mean of 18.6% of $CD4^+$ cells expressed perforin, while only a mean of 3.5% of $CD8^+$ cells were perforin-positive, demonstrating that a higher proportion of $CD4^+$ cells, compared to $CD8^+$ cells, harboured the cytotoxic perforin (Fig. 3.5B). However, there was no differential perforin expression between sensitised and control mice.

Of all the IFN-γ producing cells in the lungs, $CD4^+$ cells were the predominant subset, significantly more than $CD8^+$ cells (Fig. 3.5C). Within both $CD4^+$ and $CD8^+$ populations, a higher percentage of cells producing IFN-γ was observed in sensitised compared to non-sensitised mice (Fig. 3.5D). Post-BCG infection, splenic IFN-γ production after restimulation with heat-killed BCG was also higher than control mice (Fig. 3.6A) but this difference did not reach statistical significance.

To determine the effects of CHE on BCG survival *in vivo*, mice sensitised with heat-killed CHE or PBS were challenged with live BCG intra-nasally one week after the last immunisation, and BCG load in the lung examined either one or three weeks after BCG infection. Bacterial load was assayed in the lungs by measuring colony-forming units (CFU). Lung BCG counts in sensitised mice were 2- and 6-fold lower than PBS mice at one and three weeks post-infection

respectively (Fig. 3.6B). Thus, immunity induced by CHE sensitisation was functional in suppressing BCG growth.

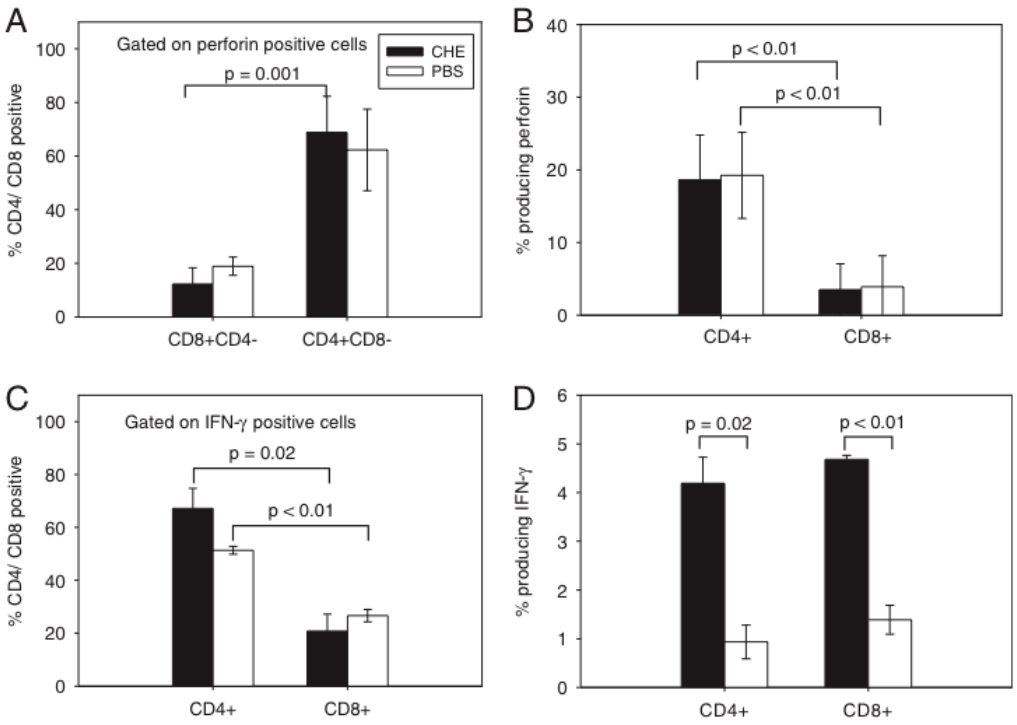

Fig. 3.5 Cells expressing perforin or IFN-γ in infected mouse lungs
Lung single-cell suspensions from CHE-immunised and PBS-control mice, one week after BCG infection *in vivo*, were analysed by flow cytometry. (A) Perforin-positive cells were gated and percentages expressing CD4 or CD8 assessed. (B) Cells were gated on either $CD4^+$ or $CD8^+$ cells, % perforin-positive cells shown. (C) $CD3^+$ IFN-γ positive cells were gated, and percentages expressing CD4 or CD8 assessed. (D) $CD3^+CD4^+$ or $CD3^+CD8^+$ cells were gated, percentage of IFN-γ positive cells shown. Means of 4 mice per group shown, ± 1 SD. Statistics by Student's *t* test.

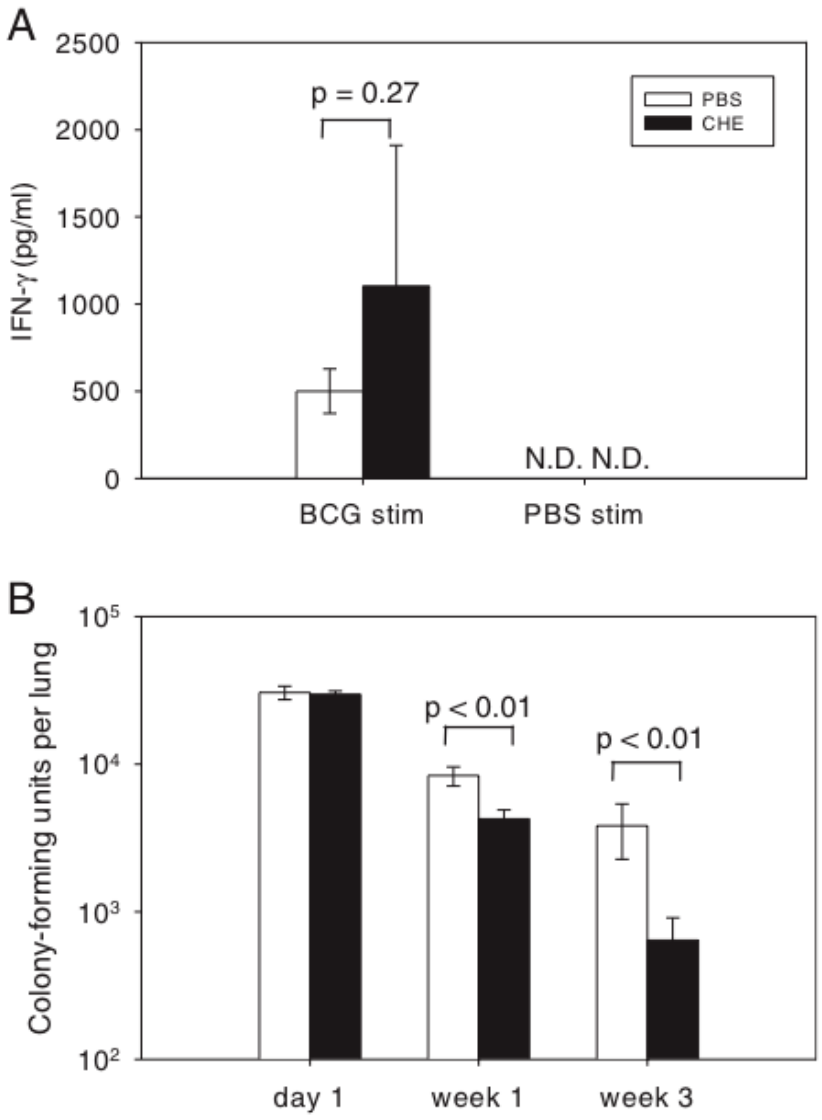

Fig. 3.6: Cytokine production and BCG counts after BCG infection *in vivo*
(A) IFN-γ production in splenocytes isolated from CHE-immunised or control mice after restimulation with heat-killed BCG or PBS one week after *in vivo* BCG infection of all mice. N.D. = not detectable. (B) BCG CFU in lungs at different time-points after *in vivo* BCG infection of CHE-immunsed and control mice. Mean data from 3 - 5 mice per group shown, ± 1 SD. Statistics by Student's *t* test.

3.4 DISCUSSION

Why BCG fails to protect against TB in certain human populations is relevant to current efforts to design an improved vaccine. This present study was founded on prior evidence that Env exposure before BCG vaccination adversely affects the vaccine's protective efficacy (Black, 2001a; Black, 2001b; Brandt, 2002; de Lisle, 2005; Demangel, 2005; Young, 2007). Those observations were extended here to describe a particular role of Env sensitisation in inducing cytotoxicity against BCG-infected cells, providing a cellular mechanism for the Env effect on BCG efficacy.

3.4.1 $CD4^+$ T cells involved in CHE-mediated cytotoxicity

$CD4^+$ T cells are known to have cytotoxic activity against *Mycobacterium*-infected cells (Mustafa and Godal, 1987; Hancock, 1989; Boom, 1991; Lorgat, 1992; Pithie, 1992; Silva and Lowrie, 2000; Boselli, 2007). Peripheral blood $CD4^+$ T cells of active TB patients display cytotoxicity against Mtb-infected macrophages, which decreases with severity of TB (De La Barrera, 2003). $CD8^+$ T cells, however, remain the most widely reported cell type exerting cytotoxic effects in TB (Sousa, 2000; Sada-Ovalle, 2006; Billeskov, 2007) and are known to harbour cytotoxic precursors such as granulysin and perforin (Stenger, 1998; Stegelmann, 2005). $CD8^+$ CTL activity is reduced in TB patients compared to healthy controls (Smith, 2000), suggesting that the lack of such activity may be related to disease. In murine studies, experiments with β2 microglobulin or MHC Class Iα gene-deleted mice suggest that $CD8^+$ T cells are required for Mtb

resistance (Flynn, 1992). However, there are also studies showing that mice lacking $CD8^+$ T cells are no different from normal mice in controlling BCG or Mtb infection, but depletion of $CD4^+$ cells in mice significantly reduced mean survival days (Xing, 1998; Mogues, 2001). This study found a major role for $CD4^+$ T cells in CHE- induced cytotoxicity, and previous studies in our laboratory noted that in CHE-sensitised mice, depletion of $CD8^+$ cells does not negatively affect killing of BCG-infected macrophages (Ho, 2009). There is precedence for such an outcome. In *M. avium* infection of MHC-Class I deficient mice, the lack of functional $CD8^+$ T cells has no effect on bacteria numbers (Bermudez and Petrofsky, 1999), whereas in wild-type mice, infection is worse and lung cytotoxicity is reduced upon depletion of $CD4^+$, but not $CD8^+$ cells (Orme, 1992; Saunders and Cheers, 1995). This supports the present finding that CHE exposure induces functional $CD4^+$ T cells with cytotoxic activity. Lazarevic noted that $CD8^+$ T cells are cytotoxic mainly in acute TB infection and cytotoxicity diminishes after exposure to high doses of mycobacterial antigens for prolonged periods (Lazarevic, 2005). This may account for the apparent lack of contribution of $CD8^+$ T cells to cytotoxicity against BCG-infected cells after repeated exposure to Env antigens.

3.4.2 CHE-induced cytotoxicity dependent on FasL and perforin

Human *Mycobacterium*-specific $CD4^+$ clones cytotoxic to *Mycobacterium*-infected cells can be FasL-dependent or FasL-independent (Lewinsohn, 1998; Oddo, 1998; Boselli, 2007). Cytotoxic $CD4^+$ T cell clones derived from Mtb-infected mice lyse target cells via the Fas-FasL pathway (Silva and Lowrie, 2000). Human studies suggest that cytotoxicity of $CD4^+$ and $CD8^+$ T cells against Mtb-infected

macrophages from healthy purified protein derivative-positive (PPD^+) individuals is reduced if perforin is blocked (Canaday, 2001). This study found a role for both FasL and perforin in CHE-induced cytotoxicity, although synergism between these two pathways was not demonstrated (Fig. 3.3A). Studies on gene-deleted mice with respect to perforin, granzyme B or FasL have demonstrated no difference in the course of Mtb infection (Cooper, 1997; Laochumroonvorapong, 1997) – however, those studies did not specifically show whether cytotoxicity was impaired in the mutant mice; possibly some redundancy in cytotoxic mechanisms exists. Perforin-negative $CD4^+$ CTLs have been known to exert cytotoxicity against Fas-sensitive target cells (Yanai, 2003).

3.4.3 CHE-induced cytotoxicity associated with mycobactericidal activity

There are reports that infected macrophage lysis can be associated with *Mycobacterium* killing (Stenger, 1998; Cho, 2000; Thoma-Uszynski, 2000), but others suggest that human *Mycobacterium*-specific $CD4^+$ T cell clones, although able to lyse BCG-infected cells, may not reduce BCG viability (Fazal, 1995; Canaday, 2001). Our laboratory has demonstrated *in vitro* that % cytotoxicity in our research model is inversely correlated with the viable BCG count (Ho, 2009), thereby supporting the probability that Env-mediated cytotoxicity kills the intracellular BCG. Activated $CD8^+$ CTLs can certainly lyse Mtb directly using cytotoxic granules such as pore-forming perforin and antibacterial granulysin (Stenger, 1998). However, evidence on whether FasL or perforin mediated cytotoxicity is relevant to killing of mycobacteria in the literature is conflicting. Some studies suggest that FasL and perforin may not play a major role in limiting

early *Mycobacterium* growth (Laochumroonvorapong, 1997; Turner, 2001), but the latter study by Turner provides *in vivo* evidence that in chronic murine Mtb infection, FasL but not perforin is important in limiting *Mycobacterium* load. A study on cytotoxic effects of human Vgamma9/Vdelta2 T lymphocytes (Dieli, 2000) shows that perforin plays an important role in killing of both infected macrophages and intracellular mycobacteria. It is probable that the enhanced cytotoxicity against BCG-infected macrophages in sensitised mice does enhance functional bactericidal activity, because there were fewer BCG in the lungs post-challenge (Fig. 3.6B).

3.4.4 IFN-γ dependent CHE-mediated cytotoxicity

In TB patients, IL-10 and IFN-γ modulate the cytotoxicity induced by both $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes on Mtb-pulsed macrophages (de la Barrera, 2004). In the same study, Ab neutralisation of endogenous IL-10, as well as the addition of exogenous IFN-γ to $CD4^+$ or $CD8^+$ cells, increased cytotoxicity. FasL expression on human $CD4^+$ T cells is regulated by IFN-γ, and lowered expression of FasL results in reduced $CD4^+$ CTL activity against mycobacteria (Boselli, 2007). IFN-γ depletion reduces cytotoxicity in *M. avium*-infected mice (Saunders and Cheers, 1995). Similarly, this study found that CHE-induced cytotoxicity was dependent on IFN-γ, but could not establish any effect for IL-10 (Fig. 3.3B). In wells treated with anti-IL-10, IFN-γ concentration was higher (data not shown), but this was not associated with improved cytotoxicity.

$CD4^+$ cells constituted the significant majority of IFN-γ producing cells in the lungs post-BCG (Fig. 3.5C). After intra-tracheal BCG infection, $CD4^{-/-}$ mice have reduced IFN-γ production (Xing, 1998), suggesting that $CD8^+$ cells cannot replace $CD4^+$ cells in IFN-γ production, although $CD8^+$ cells can produce IFN-γ (Lazarevic, 2005), as this study has also noted (Fig. 3.5D). CHE sensitisation induced more $CD4^+$ T cell recruitment (Ho, 2009) and IFN-γ production (Fig. 3.5D) in the BCG-infected lungs than PBS sensitisation. Taken together with the *in vitro* findings that CHE-induced cytotoxicity against BCG-infected macrophages was dependent on $CD4^+$ cells and IFN-γ, this would explain the reduced BCG survival in the lungs of sensitised mice (Fig. 3.6B).

3.4.5 Potential reasons for differential responses to Env sensitisation

Prior papers by Brandt (Brandt, 2002) and Demangel (Demangel, 2005) agree with our observations that different Env species have different effects on the subsequent BCG response. They suggest that cross recognition of antigens shared between Env and BCG underlies these effects. Brandt finds that pre-sensitisation with live *M. avium* and *M. fortuitum*, but not CHE, reduces BCG replication in mice. This disparity with the present study with regards to effects of CHE on BCG could be due to differences in the mouse strain used, method of sensitisation and notably, their use of live Env for sensitisation, since killed Env antigens were used for sensitisation in this study. We recognise that live Env sensitisation would prime the primary response more effectively than heat-killed bacteria. However, it is also probable that natural exposure to Env in the environment consists of a mixture of live and non-viable Env. A standard, batch-

processed, non-viable CHE preparation was chosen for all assays in this study due to the need for optimal consistency in reproducing the Env antigen load in all sensitised mice, because antigen load critically determines the CTL response (Lazarevic, 2005). In accordance with Brown (Brown, 1985), this project hypothesised that the dose, route and timing of Env administration, the species/strain used, and usage of live or dead Env, all significantly influence the nature of the primary response and consequently, the extent to which Env exposure enhances, masks or interferes with effects of BCG. Therefore, in further studies, the differential effects of viable and non-viable Env administered through different routes and at different doses will be addressed (Chapters 5 and 6).

The ability of the Env species and BCG to colonise the host is a critical determinant of their combined immune effects (Brandt, 2002; Young, 2007). In Demangel's study, pre-sensitisation with *M. avium* decreases BCG specific IFN-γ responses and inhibits BCG growth; but under conditions of Env sensitisation that still allow a minimum persistence of BCG, the BCG response can boost the Env-induced primary response and improve protection against TB (Demangel, 2005). This is consistent with observations in this study that after BCG challenge, IFN-γ responses to BCG were increased in CHE-sensitised mice, relative to non-sensitised mice (Fig. 3.5D, 3.6A), suggesting that CHE sensitisation promoted a stronger recall response to BCG antigens upon BCG challenge.

3.4.6 Conclusion

The present findings on the cytotoxicity against BCG induced by Env sensitisation provide a novel explanation for the limited BCG efficacy following Env exposure. This study accepts that there may be other explanations; for example, some believe that the Env effect consists of Th2-mediated suppression of BCG-induced responses, associated with reduced IFN-γ responses to BCG after BCG challenge (Young, 2007). Another important area for research is on mechanisms underlying the potential effect of Env exposure after BCG vaccination, since the latter is given to neonates in many countries and there is murine evidence that *M. avium* exposure post-BCG vaccination also impairs the protection against TB (Flaherty, 2006). Reasons for the failure of BCG have major implications for the next generation of TB vaccines (Andersen and Doherty, 2005), in particular those involving live BCG, if they are to be used in areas where Env exposure is prevalent. Identification of vaccines which are not affected by prior Env sensitisation (Brandt, 2002; Demangel, 2005), or could even boost responses to primary Env exposure, should be a priority.

CHAPTER 4 – Evidence for regulatory T cell activity in *Mycobacterium chelonae* sensitised mice and functional impact of $CD4^+CD25^+$ cells on BCG responses

4.1 INTRODUCTION

The BCG vaccine has a consistently low efficacy in many tropical regions in Africa and India where the vaccine is most needed (Colditz, 1995; Fine, 1995). One possible reason is that prior exposure to other non-tuberculous species of Env, which is common in the tropics, has a role in this phenomenon (Colditz, 1995; Fine, 1995). Consistent with this theory, individuals living in a rural district of Malawi, where BCG provides little protection, have considerable exposure to Env, whereas BCG efficacy is high in the UK where such sensitisation is less evident (Black, 2001a). Moreover, BCG is consistently efficient in 'clean' animal models of TB (Smith, 1985), as well as in protecting newborns (without prior exposure to Env) against childhood manifestations of tuberculosis (Colditz, 1995). However, the precise immunological mechanism(s), particularly cell-specific responses, in the regulation of BCG responses by Env are not known.

Killed *Mycobacterium vaccae*, one of many Env species found in the environment, induce regulatory T cells (Tregs), which, when adoptively transferred into naïve mice, suppress airway inflammatory responses upon allergen challenge (Zuany-Amorim, 2002b). It was hypothesised that sensitisation to some Env species could, likewise, exert an immunomodulatory effect by inducing *Mycobacterium*-

specific Tregs that suppress the response to live BCG vaccine given later. Murine evidence from this study showed that CHE sensitisation led to enhanced IL-10 and reduced inflammatory responses to BCG *in vitro*. $CD4^+CD25^+$ Tregs have been described to suppress human and murine responses to BCG (Jaron, 2008; Wammes, 2010). This study found that $CD4^+CD25^+$ Tregs induced by CHE sensitisation, when adoptively transferred *in vivo*, suppressed the inflammatory response to BCG challenge in naïve mice. This provides an immunological mechanism to explain Env effects on BCG host responses.

4.2 MATERIALS AND METHODS

4.2.1 Mice & Immunisation

Female specific pathogen-free BALB/c mice (6-10 weeks old) were used under protocols approved by the NUS Institutional Animal Care and Use Committee (IACUC). Bacteria strains and preparation have been previously described (Chapter 3, section 3.2.2 and 3.2.3). For murine sensitisation, i.p. administration of one million cells of heat-killed CHE or PBS in 100 µl PBS was performed once a week for three consecutive weeks. In some experiments, mice were intra-nasally administered with one million live BCG cells suspended in 10 µl PBS. After sacrificing the mice, single cell suspensions of lungs and splenocytes were obtained as previously described (Chapter 3, section 3.2.6).

4.2.2 Bronchoalveolar lavage (BAL)

BAL fluid was collected by flushing the trachea twice with 600 µl PBS, and aspirating the washings. Cell-free BAL fluid was then kept at -20 °C until use for cytokine analysis by ELISA. Cells were concentrated on slides using Cytospin 3 (Shandon) and stained with Giemsa stain for microscopic analysis.

4.2.3 Cell sorting and adoptive transfer

Regulatory T cells were isolated from splenocytes using the $CD4^+CD25^+$ isolation kit (Miltenyi Biotec). Briefly, non-$CD4^+$ cells were first depleted using antibodies targeting $CD8^+$ T cells, B cells, macrophages, NK cells and erythrocytes. $CD25^+$

cells were then positively selected using PE-labelled anti-CD25 (7D4) and anti-PE microbeads. $CD25^-$ cells were also collected as the unlabelled fraction. One million of either $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from PBS or CHE-sensitised mice were intravenously transferred into naïve mice.

4.2.4 Co-culture proliferation suppression assay

$CD4^+CD25^-$ cells from PBS-immunised mice were stained with CFSE (Invitrogen). Separately, four different types of cells – $CD4^+CD25^+$ or $CD4^+CD25^-$ cells of PBS or CHE-sensitised mice – were stained with CellTrace Far Red DDAO-SE (Invitrogen). The CFSE-stained cells were co-cultured with the DDAO-stained cells at a 1:5 ratio in 96-well plates. To perform the cell staining, two to five million cells were incubated with either 10 μM CFSE or 20 μM CellTrace Far Red DDAO-SE in RPMI containing 5 % FBS for 15 min at 37 ˚C in the dark. Cells were then washed with RPMI containing 20 % FBS twice, before re-suspension in RPMI containing 10 % FBS for seeding.

The co-cultures were then stimulated with either endotoxin-free anti-CD3 (1 μg/ ml) and anti-CD28 (2 μg/ ml; Biolegend) for 5 days, or with heat-killed BCG (10 BCG per cell) for 48 h before analysis of CFSE fluorescence by flow cytometry and assay of IFN-γ production in the supernatants, respectively. By flow cytometry, in the 5 day experiment, the proliferative response of the $CFSE^+DDAO^-$ cells (i.e. $CD4^+CD25^-$ cells of naïve mice) was the subject of analysis. In the 48 h experiments, the $CFSE^+$ and $CFSE^-$ phenotype was used to distinguish

between the effector cells ($CD4^+CD25^-$ cells of naïve mice) and the other cells used in the co-culture.

4.2.5 ELISA

Lung cells and T cell enriched splenocytes were seeded at 0.25 x 10^6 cells/ well in 96-well U bottom tissue culture plates and stimulated with heat-killed CHE, BCG (10 bacteria: 1 cell) or PBS for 48 h before supernatants were harvested for cytokine analysis and kept at -20 °C until use. Otherwise, supernatants from co-cultures (described above in section 4.2.4) were kept at -20 °C until use. Mouse IL-2 ELISA MAX kits (BioLegend), IL-10 DuoSet (R&D Systems) and IFN-γ OptEIA ELISA Set (BD Pharmingen) were used to evaluate the cytokine levels in supernatants of cell cultures and BAL fluid. Cytokine levels were measured based on a sandwich ELISA method following the manufacturer's instructions, as previously described (Chapter 3, section 3.2.9).

4.2.6 Flow cytometry

Cells to be analysed via flow cytometry were washed with PBS containing 0.5% BSA, then stained with relevant fluorochrome-conjugated antibodies at 4 ˚C for 30 min in the dark and fixed with 4 % paraformaldehyde before use. For intracellular staining, cells were permeabilised with PBS containing 0.1 % saponin (Sigma) after fixing, then blocked with PBS containing 0.5% BSA and 0.1 % saponin for 30 min at 4 ˚C, before staining with relevant antibodies. The antibodies used were anti-mouse CD3 (BD Bioscience, 145-2C11), CD4 (BioLegend, GK1.5), CD25 (BD Bioscience, PC61), CD103 (BD Bioscience, M290),

FoxP3 (BioLegend, 150D), and IL-2 (BioLegend, JES6-5H4), along with relevant isotype control antibodies staining negative control samples.

4.2.7 Statistics

Differences between group means were tested pairwise using the two-tailed Student's *t*-test. Results were expressed as group means ± SD, with 3 – 8 mice per group. A p value < 0.05 was considered significant.

4.3 RESULTS

4.3.1 CHE sensitisation reduces IFN-γ and increases IL-10 production with associated reduced lymphocyte activity

To investigate whether CHE sensitisation modulates systemic cytokine production, levels of the pro-inflammatory cytokine, IFN-γ, and anti-inflammatory cytokine, IL-10, were measured from heat-killed CHE-stimulated splenocyte cultures. One week after the last CHE exposure, total splenocyte cultures from CHE-sensitised mice contained significantly less IFN-γ and more IL-10, relative to non-sensitised PBS mice, after *in vitro* heat-killed CHE-restimulation (Fig 4.1A, B). Interestingly, both IFN-γ and IL-10 levels in CHE-sensitised mice were significantly higher in CD4-depleted (i.e. $CD4^-$ fraction) splenocytes, relative to total splenocyte cultures (Fig. 4.1A, B), suggesting an ability of $CD4^+$ cells in these mice to suppress expression of both these cytokines, and hence may be tolerogenic or suppressive in nature.

The majority of the IFN-γ and IL-10 producing cells in the above experiments were T cells, but approximately 30% was derived from DCs and macrophages. Cytokine production by lymphocytes alone was also investigated, using flow cytometry (Fig 4.1C). In CHE-sensitised mice, there was a significantly lower frequency of IFN-γ positive lymphocytes and but a similar percentage of IL-10 positive lymphocytes, relative to non-sensitised mice. This complements the ELISA data and confirms that CHE-sensitised lymphocytes have lower inflammatory responses. However, it is difficult to conclude that there was substantial induction of Tr1 due to CHE sensitisation, as the percentage of IL-10

expressing T cells specific for CHE was not higher relative to non-sensitised mice. In response to non-specific stimulation, IL-2 production by $CD4^+$ cells of CHE-sensitised mice was also significantly lower than in non-sensitised mice (Fig 4.1D). This suggested that, compared to non-sensitised mice, there was reduced lymphocyte activity in CHE-sensitised mice, possibly related to soluble factors induced by CHE sensitisation.

4.3.2 Reduced lung inflammatory cells and increased lung cytokines upon BCG lung infection after CHE sensitisation

CHE-sensitised and non-sensitised mice were given live i.n. BCG and examined one or three weeks later. The frequency of IFN-γ and IL-10 producing lymphocytes in the lungs of CHE-sensitised mice at one week post-BCG was significantly higher (Fig 4.2A), suggesting possible cross-reactive T cells recognising common antigens between CHE and BCG. In Chapter 3, however, post-BCG splenic IFN-γ production by ELISA was similar between sensitised and non-sensitised mice (Fig. 3.6A). Splenic IL-10 concentrations were lower three weeks after BCG administration (Fig 4.2B) than before (Fig 4.1A), probably due to inflammation induced by BCG driving a predominantly type 1 cytokine response. In spite of this, three-week post-BCG splenic IL-10 production was significantly higher in CHE-sensitised mice than non-sensitised mice (Fig 4.2B). Moreover, the BAL of sensitised mice contained fewer inflammatory cells, including macrophages, lymphocytes and neutrophils (Fig 4.2C). Thus, systemic CHE priming resulted in a reduced inflammatory response to BCG at the site of infection, and heightened systemic IL-10 responses, suggesting the presence of factors dampening the *in vivo* adaptive response.

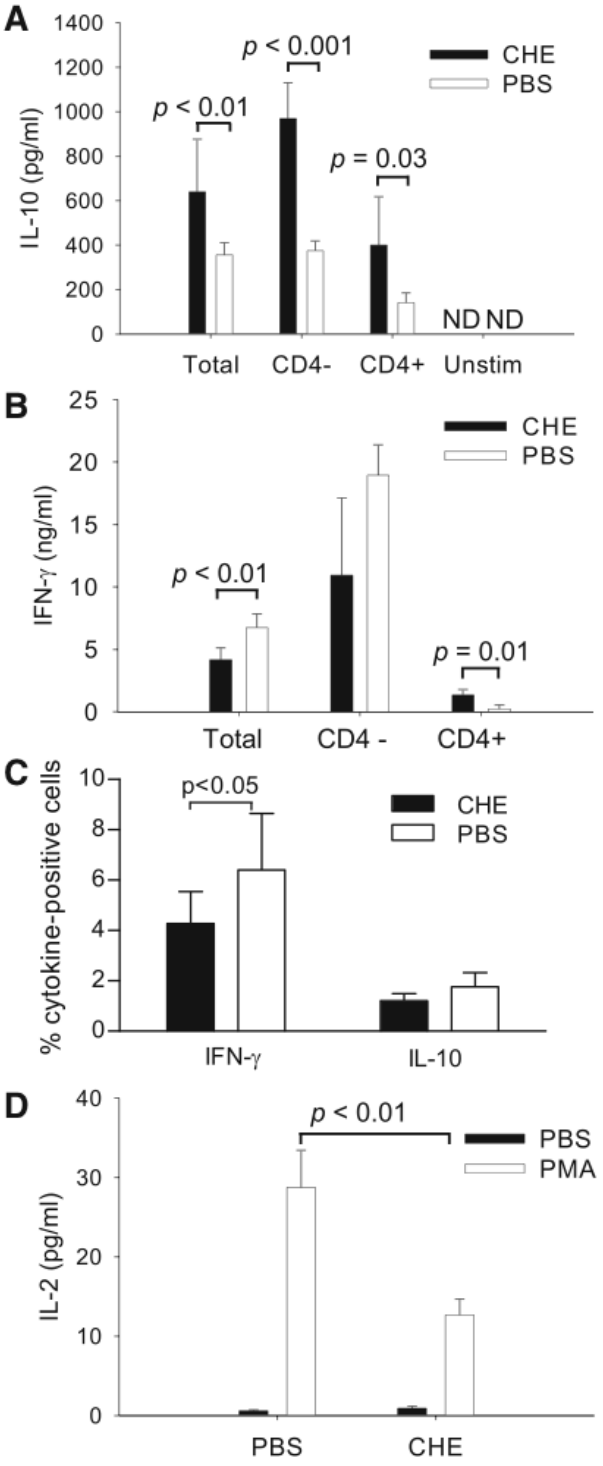

Fig. 4.1: Cytokine production by splenocytes from CHE-sensitised mice
Mice were either given i.p. heat-killed CHE (dark bars) or PBS (control, white bars) once every week for three weeks, then sacrificed a week after the last exposure. The splenocytes were used directly ("total"), or magnetically sorted into $CD4^+$ and $CD4^-$ (CD4-depleted) fractions before seeding. Cells were stimulated with heat-killed CHE or PBS ("unstim") for 48 h. Supernatants were

analysed by ELISA for (A) IL-10 and (B) IFN-γ. ND = not detectable. (C) The percentages of stimulated lymphocytes expressing IFN-γ or IL-10 were analysed by flow cytometry after intracellular staining and lymphocyte gating. (D) In a separate experiment, $CD4^+$ cells were obtained from CHE- or PBS-immunised mice and IL-2 production after 48 h stimulation with PMA and ionomycin was measured.

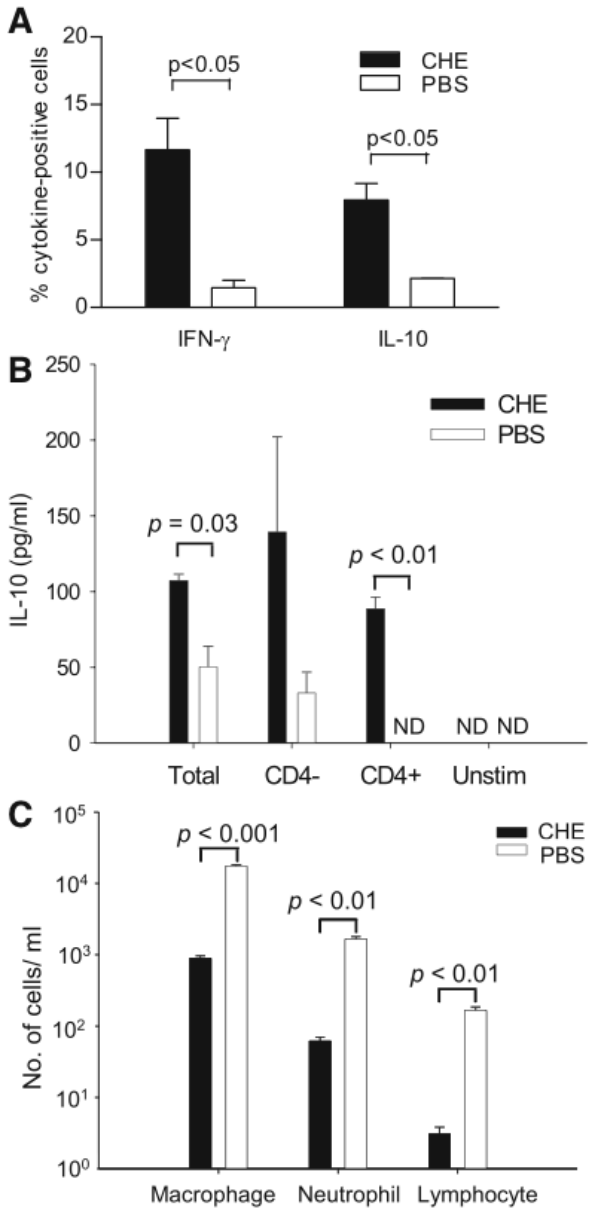

Fig. 4.2: Response to live BCG after CHE sensitisation

Mice were sensitised with i.p. heat-killed CHE (black bars) or PBS (white bars), once every week for three weeks. This was followed one week later by i.n. administration of 1 x 10^6 live BCG, and the mice were sacrificed one or three weeks post-BCG administration. (A) One week post-BCG, lung cells were stimulated with PMA and ionomycin for 6 h, and percentages of T cells producing IFN-γ or IL-10 were analysed by flow cytometry after gating on $CD3^+$ cells. (B) Three weeks post-BCG, splenocytes (Total, $CD4^-$, or $CD4^+$, as described in Fig. 4.1) were stimulated *in vitro* with heat-killed BCG for 48 h and IL-10 production in supernatants analysed by ELISA. (C) Cells from a known volume of aspirated BAL fluid were concentrated and counted. There were 3–5 mice/group.

4.3.3 Unremarkable frequency of $CD4^+CD25^+$ cells with CHE sensitisation

Some Treg phenotypes were investigated in CHE-sensitised mice, prior to BCG administration, by flow cytometry. The frequency of $CD4^+CD25^+$ cells was only marginally elevated in CHE-sensitised mice, compared to non-sensitised mice (Fig 4.3A). The difference in proportion of $CD4^+CD25^+$ cells expressing FoxP3, in CHE-sensitised mice, was unremarkable relative to non-sensitised mice (Fig 4.3B). However, as other authors have suggested that there could be differences in functional $CD4^+CD25^+$ activity despite no marked difference in frequency upon microbial sensitisation (Wammes, 2010), this study investigated qualitative differences in $CD4^+CD25^+$ cells in sensitised and non-sensitised mice.

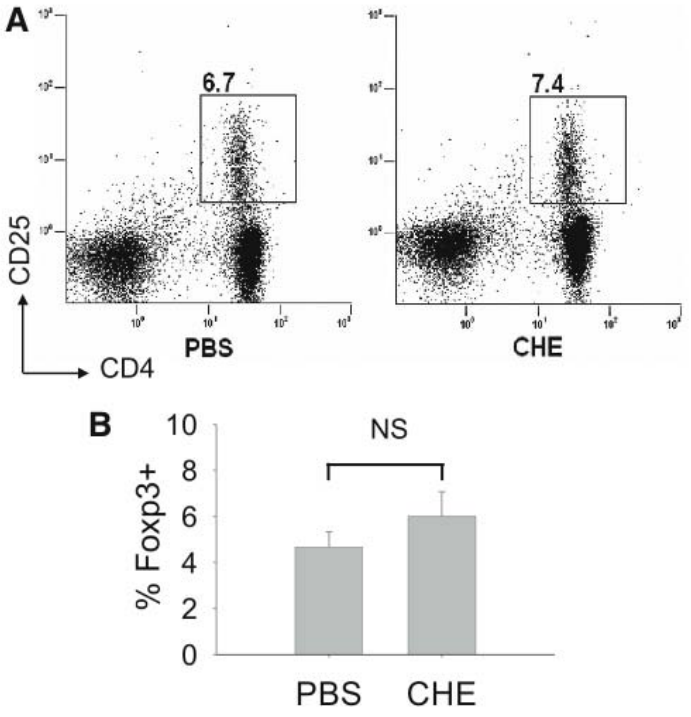

Fig. 4.3: Frequency of $CD4^+CD25^+$ and $FoxP3^+$ cells in CHE-sensitised mice
Mice were given i.p. heat-killed CHE or PBS weekly for three weeks, and sacrificed one week later. Their splenocytes were analysed by flow cytometry. (A) Percentage of $CD4^+CD25^+$ cells in lymphocyte gate. (B) Percentage of $FoxP3^+$ cells within the $CD4^+CD25^+$ population. NS = not significant. Statistics by Student's *t* test.

4.3.4 $CD4^+CD25^+$ cells from sensitised mice reduce IFN-γ production and proliferation of co-cultured effector cells

Therefore, the functional ability of splenic $CD4^+CD25^+$ cells of CHE-sensitised mice to suppress BCG-induced, as well as non-specific, lymphocyte responses in $CD4^+CD25^-$ effector cells from PBS (naïve) mice was next investigated. This effector cell fraction was co-cultured with either $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from CHE or PBS mice. Upon BCG antigen stimulation, there was lower total IFN-γ production in the co-cultures with CHE-derived $CD4^+CD25^+$ cells, than the $CD4^+CD25^-$ fraction (Fig 4.4A). This implies that the former fraction caused greater suppression of IFN-γ production by the effector cells than the latter, as CHE-derived $CD4^+CD25^+$ and $CD25^-$ cells, on their own, produced similar levels of IFN-γ (C+ vs C-: 609.7 ± 246 vs 507.7 ± 150.8 pg/ ml). It was noted, however, that both the CHE-derived fractions produced higher levels of IFN-γ than the equivalent fractions from PBS mice (P+ vs C+: 24.2 ± 14.1 vs 609.7 ± 246 pg/ ml, p=0.078; P- vs C-: 60.6 ± 1.6 vs 507.7 ± 150.8 pg/ ml, p=0.052), probably because of presence of *Mycobacterium*-specific effector cells in CHE-derived fractions. Therefore, direct comparison of the magnitude of suppression induced by $CD4^+CD25^+$ cells from CHE versus PBS mice, on the effector cell population, could not be made, in this context of *Mycobacterium* antigen-specific stimulation.

This co-culture experiment was then modified to investigate modulation by different cell fractions on non-specific proliferation of $CD4^+CD25^-$ cells from naïve mice, in response to anti-CD3 and anti-CD28 (Fig 4.4B, C). Significant proliferation was detected in the co-culture with $CD4^+CD25^-$ cells from PBS mice

(i.e. P- co-cultures, Fig. 4.4C), showing that the effector cells from naïve mice responded sufficiently to the non-specific stimulation for suppression to be observed in other co-cultures. The $CD4^+CD25^+$ cells from both PBS and CHE-sensitised mice were, as expected, more suppressive than the respective $CD4^+CD25^-$ cells from the same sources. More notably, significantly lower lymphocyte proliferative activity was observed in co-cultures with $CD4^+CD25^+$ cells from CHE-sensitised mice than PBS mice (Fig. 4.4B, C), although the absolute difference was not large.

After BCG antigen-stimulation, the co-cultures containing CHE-derived $CD4^+CD25^+$ cells expressed the highest levels of CD103 in the proliferating cells ($CFSE^-$ population, Fig 4.4D) and FoxP3 in the lymphocytes (Fig 4.4E). This supports the probability that CHE-derived $CD4^+CD25^+$ cells were most enriched for Treg cells, comparing the four co-cultured cell types. There was also evidence (Fig 4.4E) that this cell type led to *in vitro* conversion of a fraction of the naïve $CD4^+CD25^-$ cells to Treg cells. After BCG antigen-stimulated culture with $CD4^+CD25^+$ cells from CHE-immunised mice, the $CFSE^+$ cells, which were the naïve T cells originally derived from PBS-immunised mice, showed the largest increase in FoxP3 expression (Fig 4.4E).

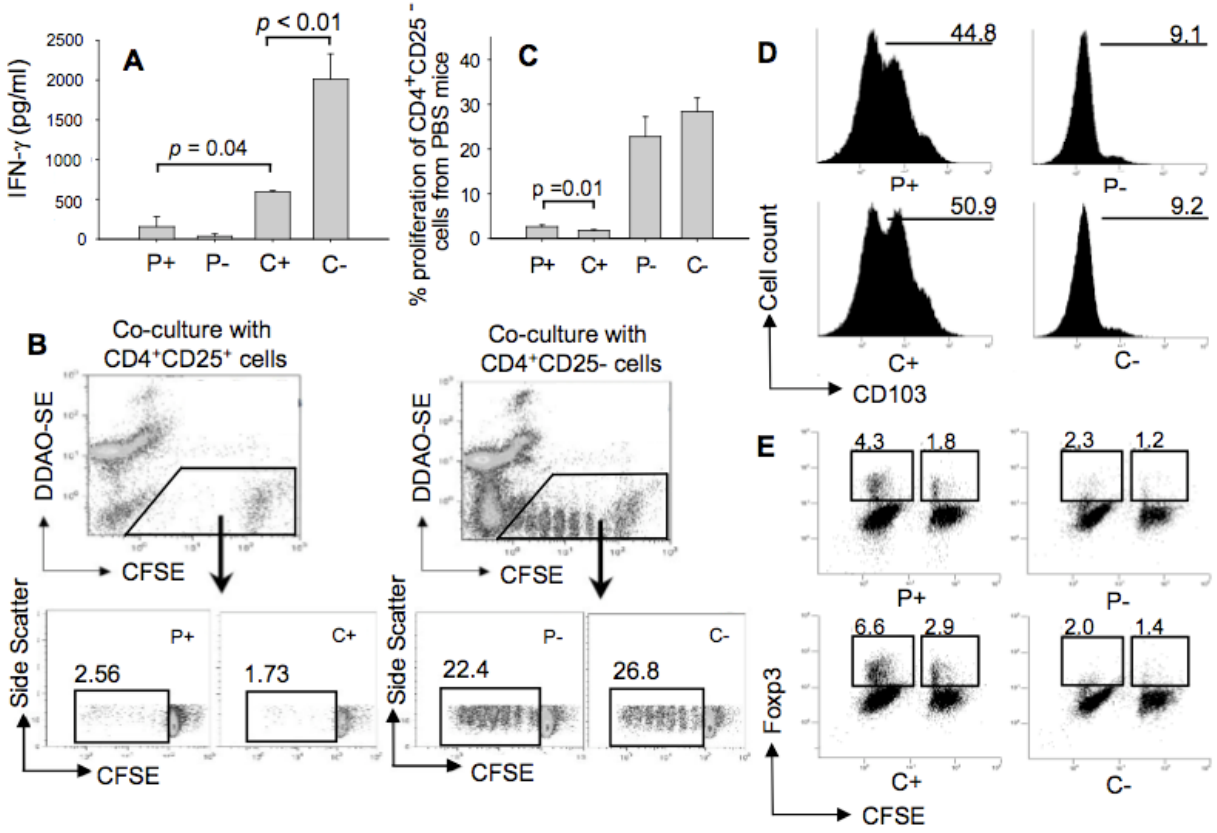

Fig. 4.4: Presence and functional activity of CD4⁺CD25⁺ regulatory T cells from CHE-sensitised mice

CD4⁺CD25⁺ and CD4⁺CD25⁻ cells from splenocytes of CHE-sensitised mice are denoted C+ and C- respectively. The corresponding subsets from PBS-immunised mice are denoted P+ and P- respectively. This experiment aimed to study effects of the four cell types on 'effector'

$CD4^+CD25^-$ cells from naïve mice. The co-cultures with P- cells represent the negative control, as they are identical to the effector cells. CFSE-labelled $CD4^+CD25^-$ splenocytes (2.5×10^4 per well) from PBS-immunised mice were seeded into wells, and co-cultured with DDAO-labelled P+, P-, C+ or C- cells, in the presence of heat-killed BCG at 10 BCG per cell for 48 h (A, D and E) or anti-CD3/anti-CD28 for 5 days (B and C). (A) Supernatants from the co-cultures were assayed for IFN-γ by ELISA. (B) Proliferation of $DDAO^-CFSE^+$ cells was analysed via flow cytometry gated on this population (boxed). In the lower panel, proliferating cells undergoing cell divisions ($CFSE^{lo}$) are shown as a percentage of the gated population, and in (C) the mean % proliferation of 4 mice per group is shown ± SD (this is a graphical representation of panel B). Data are from one of two representative experiments with similar results. The co-cultured cells were then stained for (D) CD103, gated on $CFSE^-$ population, and (E) FoxP3, gated on lymphocytes. In (E), as the cells were stimulated only for 48 h, there were minimal cell divisions by CFSE-labelled $CD4^+CD25^-$ cells from the naïve mice, thus they are largely observed as $CFSE^+$ cells. The $CFSE^-$ population represents the DDAO-labelled P+, P-, C+ and C- cells used in the co-cultures.

4.3.5 Adoptive transfer of $CD4^+CD25^+$ Tregs from CHE-sensitised mice suppresses BCG responses

To confirm that $CD4^+CD25^+$ cells from CHE-sensitised mice could mediate suppressive effects *in vivo* during BCG infection, adoptive transfer of different $CD4^+$ cell fractions from CHE or PBS mice to naïve mice was performed, just before administering live BCG to the recipients. $CD4^+CD25^+$ cells from CHE- or PBS-sensitised mice (denoted as C+ and P+ cells, respectively) were intravenously transferred into naïve recipient mice (denoted as C+ and P+ mice respectively). As a control, the same number of $CD4^+CD25^-$ cells from CHE or PBS-sensitised mice (denoted as C- and P- cells respectively) were separately transferred to naïve recipient mice (denoted C- and P- mice) respectively. To investigate the modulation of initial responses to BCG in recipient naïve mice that was due to the transferred cells, the mice were sacrificed 48 hours after live i.n. BCG was administered to all recipient mice. Local responses in the lung, which is the first site for BCG infection, were examined. Published reports have shown that within two days post-BCG infection by either the intra-tracheal (Umemura, 2007) or intra-thoracic route (Souza, 2008), there is up-regulation of inflammatory cells and cytokine signals in the lungs of infected mice, indicating that responses to BCG are detectable soon after infection.

BCG-specific IL-10 production in the lungs was higher in C+ mice than C- mice, and much elevated over P+ and P- mice, which showed minimal IL-10 responses (Fig 4.5A). Dendritic cells (DCs) formed the majority of IL-10 expressing leucocytes (53.8 % ± 4.3 %), followed by T lymphocytes (39.2 % ± 16 %) with

only a minor (2 % ± 0.7 %) macrophage contribution. The BAL fluid of C+ recipient mice had the lowest lymphocyte counts (Fig 4.5B), but there was no difference in other BAL leucocytes (not shown). The frequency of IL-2 producing cells in the lungs was lower in C+ mice compared to P+ mice (Fig 4.5C). The differential early lung immune responses to BCG in the various recipient mice are likely to be related to immune modulation by the respective transferred cells. Overall, the data from the adoptive transfer experiments suggests that the small number of transferred $CD4^{+}CD25^{+}$ Tregs from CHE-sensitised mice can significantly limit the host's lung responses to live BCG soon after infection.

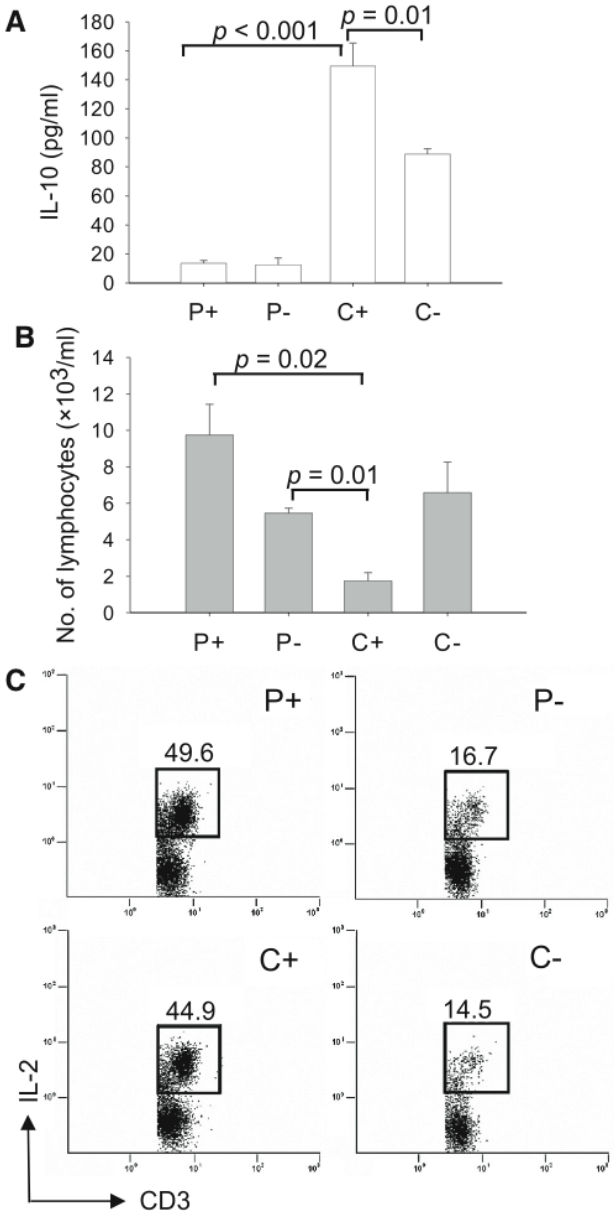

Fig. 4.5: Immune response to BCG in adoptive transfer recipient mice
Donor mice were given, weekly, either CHE or PBS, thrice, and their splenocytes harvested one week after the final exposure. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were obtained from each mouse by magnetic sorting. These cells (denoted as P+, P-, C+ or C- as in Fig 4.4 legend) were separately transferred intravenously into different naïve recipient mice, which were given one million live BCG cells i.n. on the same day, and sacrificed after 48 h. (A) Lung cells of recipient mice were cultured *in vitro* with heat-killed BCG for 48 h and supernatants assayed for IL-10 production by ELISA. Data represent three experiments with similar results. (B) BAL fluid was concentrated for microscopic examination of lymphocytes according to Fig 4.2C, and results expressed as cell count per ml of BAL fluid. (C) Frequency of IL-2 producing cells in lungs of recipient mice, gated on the $CD3^+$ population. Data represent three experiments with similar results.

4.4 DISCUSSION

4.4.1 Natural and induced Tregs with CHE sensitisation

Both naturally-occurring and adaptive Tregs have been described in infections (Belkaid, 2007). Natural Tregs are thymus-derived cells that serve to limit peripheral self-reactive T lymphocytes, and are most commonly characterised as $CD4^+CD25^+$ and $FoxP3^+$ (Sakaguchi, 2004; Horwitz, 2008). Infection-induced adaptive Tregs are less well defined phenotypically. They have been described as extrathymic $CD4^+$ T cells that develop after encountering antigen presenting cells modulated by microbial antigens or upon contact with regulatory cytokines (O'Garra, 2004). Adaptive Tregs may also have the phenotype $CD4^+CD25^+$ after conversion from $CD25^-$ cells in peripheral lymphoid organs (Horwitz, 2008). Both IL-10 and TGF-β have been described as key mediators of Treg-induced effects (Sakaguchi, 2004; Horwitz, 2008). Some IL-10 secreting Tregs, usually known as Tr1 cells, have comparable regulatory properties with naturally-occurring Tregs but do not express CD25 or FoxP3 (Vieira, 2004). $CD4^+CD25^+$ cells in the CHE-sensitised mice model in this study may potentially include both natural and adaptive Tregs. Although this study failed to find a significant elevation of cells fulfilling the Tr1 phenotype in the CHE-sensitised mice, this does not exclude a role for adaptive Tregs in suppression of the BCG response.

4.4.2 Usage of dead CHE for sensitisation

In this study, mice were sensitised with heat-killed CHE. Although a more effective primary response could potentially be primed with live Env

sensitisation instead, natural human exposure to Env probably involves both live and dead Env – there is no evidence that only live Env exposure can result in human sensitisation. In parallel studies (described in Chapter 5), responses to three weekly doses of intra-nasally administered 10^6 CFU live versus heat-killed CHE were compared, and no differences in IFN-γ or IL-10 production from their splenocyte cultures were observed. Thus, both live and dead CHE may have similar effects in this context. As immune responses are highly dependent on antigen loads (Dhar, 2003; Lazarevic, 2005; Russell, 2007b), it is important to be mindful of the need for maximal consistency in reproducing the antigen load in all sensitised mice. Therefore, in this study, a standard, batch-processed, non-viable preparation of CHE was chosen and it was introduced through the most reproducible route (i.e. intra-peritoneally).

4.4.3 Qualitative suppressive activity of $CD4^+CD25^+$ Tregs without quantitative changes

$CD4^+CD25^+$ cells were the focus of this study because of the many published reports on such cells found in *Mycobacterium* infected hosts. $CD4^+CD25^+$ Tregs can be expanded during the course of human and murine tuberculosis infection and suppress T cell mediated protective responses (Guyot-Revol, 2006; Hougardy, 2007a; Mason, 2007), thus facilitating pathogen persistence. $CD4^+CD25^+$ cells are known to suppress human immune responses to BCG (Wammes, 2010), and impair Mtb control by $CD4^+$ effector cells in mice (Kursar, 2007). Inactivation of such cells improves protection conferred by BCG against Mtb challenge (Jaron, 2008). Findings from this study, that the $CD4^+CD25^+$

fraction in CHE-sensitised mice was a Treg population with functional suppressive activity, with a role in modifying subsequent responses to BCG, is consistent with the above reports.

Some $CD4^+CD25^+$ cells may be activated effector T cells, although the adoptively transferred population in this study was predominantly $CD25^{hi}$. That adoptive transfer of such cells from CHE-sensitised mice led to overall suppression of BCG responses (Fig 4.5) implies that the suppressive effects of the Tregs within this fraction overshadow the direct *Mycobacterium*-specific effector functions of the small fraction of activated cells. Since this occurred upon adoptive transfer to wild-type recipient mice (with their existing full complement of $CD4^+$ T cells), it suggests a potent effect of very small numbers of CHE-derived $CD4^+CD25^+$ Tregs in mediating systemic suppressive effects. This study is also the first to demonstrate that functional activity of such Tregs from mice with environmental mycobacteria priming is qualitatively different (more suppressive) than $CD4^+CD25^+$ cells from naïve mice, with respect to modulating a subsequent BCG response. A qualitative (but not quantitative) difference is also shown in $CD4^+CD25^+$ cells from geohelminth-infected people, which substantially impair immune responses to BCG, relative to the same cells from uninfected people (Wammes, 2010). The unremarkable frequency of $CD4^+CD25^+$ cells in CHE-sensitised mice (Fig 4.3) may be because of concurrent expansion of both Tregs and the total T cell population (including effector cells) in *Mycobacterium* exposed mice. This makes the prominent reduction in BAL immune cell counts (relative to non-sensitised mice), in response to live BCG, more remarkable (Fig 4.2), and likely to be due to Treg activity.

4.4.4 Tregs may affect non-T cell types

Since $CD4^+CD25^+$ cells may induce not just T cells, but also DCs (Veldhoen, 2006) and macrophages (Liu, 2010) to produce more IL-10, this explains why the observed increase in systemic IL-10 production in CHE-sensitised mice is not entirely arising from IL-10 secreting Tregs. In fact, IL-10 producing leucocytes in the BCG-challenged lungs comprised of 54% DCs and 40% T cells, consistent with the fact that $CD4^+CD25^+$ Tregs can skew pathogen-stimulated DCs towards production of more IL-10, whereas memory $CD4^+$ T cells do not (Veldhoen, 2006). Such an effect of the adoptively transferred $CD4^+CD25^+$ cells would account for the enhanced IL-10 production in the lungs (Fig 4.5A), which in turn is likely to have contributed to the suppression of local lymphocyte recruitment post-BCG infection.

4.4.5 Potential mechanisms for CHE-induced Treg activity

More than one cell type in CHE-sensitised mice could have Treg activity, for example BCG-immunised mice and humans have $CD8^+$ Tregs with suppressive activity (Joosten, 2007). Whether Tregs in CHE-sensitised mice could operate via other cytokines or non-cytokine mechanisms was also not investigated. For example, TGF-β from $CD4^+$ Treg cells, has a role in limiting airway inflammatory responses after *M. vaccae* sensitisation (Zuany-Amorim, 2002b). Studies in both mice and humans have suggested that $CD4^+CD25^-FoxP3^-$ cells could undergo 'peripheral conversion' to $FoxP3^+$ Treg cells under different stimuli, including TGF-β (Chen, 2003; Fantini, 2004; Zheng, 2007), IFN-γ (Wang, 2006), IL-4 and

IL-13 (Skapenko, 2005), or prolonged exposure to low-dose antigen (Kretschmer, 2005). In this study, some $CD4^+CD25^-$ T cells from naïve mice acquired the FoxP3 marker *in vitro* when co-cultured with $CD4^+CD25^+$ Treg cells from CHE-sensitised mice together with BCG stimulation (Fig 4.4E), suggesting that the Treg cells from CHE-sensitised mice may, likewise, be able to induce non-Treg cells from the recipient mice to become Tregs by peripheral conversion, thereby providing a possible additional mechanism for the *in vivo* effects upon adoptive transfer. The higher level of expression of CD103 observed in $CD4^+CD25^+$ cells from CHE-sensitised mice than PBS mice after co-culture (Fig 4.4D) may also be relevant to their *in vivo* functions, as studies of Treg cells in murine *L. major* infection have suggested that CD103 is required for the retention of Tregs at the infection site (Suffia, 2005).

4.4.6 Conclusion

Tregs could be acting to prevent exhaustion of *Mycobacterium*-specific T cells in the face of constant antigenic stimulus during chronic persistent infections. The equilibrium established between effector and regulatory T cells has been elegantly demonstrated in the chronic *L. major* murine infection model (Belkaid, 2002). Repeated human Env exposure through environmental sources could likewise result in a constant stimulus for both such cell types, and the effects of either, or both, cell type could modulate the host response to BCG. The findings in this study demonstrate, at the cellular level, one potential explanation for the reduced protective efficacy of BCG vaccination in areas where the population is highly sensitised with repeated Env exposure (Black, 2001a). Tregs can inhibit

memory T cell responses (Murakami, 2002; Suvas, 2003) and it is possible that this, rather than an insufficient initial Th1 response to BCG, explains the lack of BCG efficacy in some areas and the waning of BCG-induced immunity over time in vaccinated populations (Hart and Sutherland, 1977; Doherty and Rook, 2006). A recent paper has suggested that in humans, prior exposure to *Mycobacterium* antigens induces $CD4^{+}CD25^{hi}CD39^{+}$ regulatory cells associated with a poorer Th17 response to a new tuberculosis vaccine currently in clinical trials (de Cassan, 2010). As Env exposure is inevitable with certain geographical localities and lifestyles (Weir, 2006), the findings in this study contribute to the growing need to understand mechanisms by which Env exposure affects efficacy of present and future TB vaccines.

CHAPTER 5 – Differential effects of varying *Mycobacterium chelonae* exposure parameters and effects of increased IL-10 producing regulatory T cells with low dose inhaled exposure

5.1 INTRODUCTION

Cross-reactivity to common antigens shared between Env and BCG may be one reason for the influence of Env priming on subsequent BCG responses in mice (Brandt, 2002; Demangel, 2005). In mice sensitised intra-peritoneally with different heat-killed Env species, it was previously noted (Ho, 2009) that sensitisation with CHE primed higher cross-reactive cytotoxic responses against autologous BCG-infected macrophages, compared to *M. avium* or *M. smegmatis*. Lower lung BCG counts after i.n. BCG infection of CHE-sensitised mice, relative to unexposed mice were also noted (Chapter 3). In contrast, Brandt worked with mice pre-sensitised subcutaneously with live CHE and did not detect differences from naïve mice in their splenic BCG counts after intravenous BCG (Brandt, 2002). The dose, route, viability and timing of CHE sensitisation were different from our study; therefore at least some of these factors may have caused the differences in BCG responses. The present work investigates how bacterial dose and viability in CHE sensitisation affect host immunity.

There is evidence that the *Mycobacterium* dose critically affects the nature of priming of cellular immunity (Hernandez-Pando, 1997). The differential effects of priming with live versus dead Env are less clear. To explore the relative effects

of these factors, outcomes of murine CHE priming experiments with high or low doses of live CHE, and to live and dead CHE were compared. Additionally, this study investigated whether immune responses primed by live CHE depended on the persistence of live organisms in the murine host. This study found that higher doses of live CHE given intra-nasally resulted in a stronger induction of Tregs, but this was, surprisingly, not dependent on the viability of CHE. It was also observed that dead CHE led to sustained strong levels of BCG-specific IFN-γ over time, and the effects of live CHE on immune cell recruitment to the site of sensitisation persisted even after no remaining live organisms were recoverable.

To uncover how CHE sensitisation influences *in vivo* BCG responses, subsequent studies compared BCG vaccination responses in mice with and without prior intra-tracheal (i.t.) low dose CHE sensitisation. This route was chosen as an enhancement over the i.n. route, to allow more accurate delivery of small numbers of CHE into the lungs. The reduction in BCG-specific memory Th1 responses, associated with an increase in frequency of IL-10 producing $CD4^+$ lymphocytes in CHE-sensitised mice, was consistent with suppression of BCG vaccination responses by prior CHE exposure.

5.2 MATERIALS AND METHODS

5.2.1 Bacteria strains, mice and immunisation protocols

Female specific pathogen-free BALB/c mice between 6 and 10 weeks old were purchased from A*Star Biological Resource Centre (BRC) and used under protocols approved by the NUS Institutional Animal Care and Use Committee (IACUC). All mice were housed in filtered isolators to limit exposure to environmental organisms. To determine the persistence of CHE in the lungs, mice were initially given 10^3 or 10^6 CFU live CHE via the i.n. route, once a week for three weeks, then sacrificed at weekly intervals for lung colony enumeration. In some experiments, mice were given either live or killed CHE intra-nasally (prepared as in Chapter 3, section 3.2.2 and 3.2.3), then sacrificed at either two or six weeks after the first dose. In later experiments, mice were sensitised with 500 CFU live CHE administered with the same dosing schedule as above, via the i.t. route, subsequently vaccinated subcutaneously at the hock with 10^6 CFU live BCG four weeks after the last dose of CHE and sacrificed eight weeks after BCG vaccination. Lung and spleen cells were isolated according to previously described methods (Chapter 3, section 3.2.6).

5.2.2 ELISpot

To detect IFN-γ and IL-10 secretion by individual cells after antigen stimulation, BD ELISpot mouse IFN-γ and IL-10 ELISpot sets (BD Biosciences) were utilised according to manufacturer's instructions. Briefly, ELISpot plates (Millipore) were

coated with capture antibodies (anti-IFN-γ or anti-IL-10) overnight at 4 °C, and blocked with RPMI + 10% FBS for 2 h at room temperature prior to use. T cell enriched splenocytes were seeded at 0.5 x 10^6 cells/ well in ELISpot plates and stimulated with heat-killed BCG (10 bacteria: 1 cell) or PBS in triplicate wells for 24 h or 48 h, for IFN-γ and IL-10 respectively, at 37 °C to detect IFN-γ secretion. After incubation, cells were removed and plates washed with deionised water and PBS + Tween 20. Secondary biotinylated antibodies were used for detection, and streptavidin-alkaline phosphatase was added before using the 3-substrate 3-amino-9-ethylcarbazole substrate reagent kit to produce a color change. The reaction was stopped, after spots had developed, by running water over the wells. Spots were then enumerated on the Bioreader 4000 (BIO-SYS).

5.2.3 ELISA

T cell enriched splenocytes were seeded at 0.25 x 10^6 cells/ well in 96-well U-bottom tissue culture plates and stimulated with heat-killed BCG (10 bacteria: 1 cell) for 24 h or 48 h to detect IFN-γ and IL-10 respectively. Mouse IFN-γ OptEIA ELISA Set (BD Pharmingen) and IL-10 DuoSet (R&D Systems) were used, and cytokine levels in the supernatants were measured based on a sandwich ELISA method following the manufacturer's instructions, as previously described. (Chapter 3, section 3.2.9).

5.2.4 Flow cytometry

Cells to be analysed via flow cytometry were washed with PBS containing 0.5% BSA, then stained with relevant fluorochrome-conjugated antibodies at 4 ˚C for

30 min in the dark and fixed with 4 % paraformaldehyde before use. For intracellular staining, cells were permeabilised with PBS containing 0.1 % saponin (Sigma) or with the intracellular (IC) staining or permeabilisation buffer set (eBioscience) after fixing, then blocked with PBS containing 0.5% BSA and 0.1 % saponin for 30 min at 4 ˚C, before staining with relevant antibodies. The following anti-mouse antibodies were used: CD3 (145-2C11, BD Biosciences), Gr-1 (RB6-8C5, BioLegend), CD11c (N418, BioLegend), F4/80 (BM-8, BioLegend), CD4 (RM4-5, BD Biosciences), CD8 (53-6.7, BioLegend), CD25 (PC61, BD Biosciences), CD62L (MEL-14, BioLegend), CD44 (IM7, BD Biosciences), CD127 (SB/199, BioLegend), IFN-γ (XMG1.2, BD Biosciences), GITR (YGITR 765, BioLegend), FoxP3 (150D, BioLegend), along with relevant isotype control antibodies in negative control samples.

In some experiments, the absolute cell numbers of each cell type in the lungs was determined by spiking the samples with fixed volumes of known concentrations of Flow-Count Fluorosphere® (Beckman Coulter) which provided the reference for cell numbers. Cells were then analysed on the Cytomics FC500 (Beckman Coulter) cytometer or the LSR-II (BD Biosciences) within 24 h.

5.2.5 Cytokine Secretion Assay

Based on the published sequence of TB10.4 (Skjot, 2000), a 15-mer peptide was synthesised from with the following sequence: Thr-His-Glu-Ala-Asn-Thr-Met-Ala-Met-Met-Ala-Arg-Asp-Thr-Ala (GL Biochem (Shanghai)). The TB10.4 (74-88): TA-15 peptide was supplied in the synthesised form and diluted in water containing

0.5 % demethyl sulfoxide to a concentration of 5 mg/ ml and kept in aliquots at -80 ˚C until use. To determine the proportion of splenocytes actively secreting IFN-γ after TB10.4 (5 μg/ ml) antigen or PBS stimulation, the mouse IFN-γ secretion assay – cell enrichment and detection kit (Miltenyi Biotec) was utilised according to manufacturer's instructions. Briefly, splenocytes were seeded in tissue culture plates and harvested after 24 h of antigen stimulation. Cells were then labelled with an IFN-γ specific 'catch reagent' at 4 °C for 15 min, then topped up with warm RPMI and incubated at 37 °C with constant mixing for 4 h. Cells were then washed with cold PBS + 0.5 % BSA and stained with detection antibody (conjugated to PE) together with antibodies specific for other cell surface markers for 30 min, washed and fixed with 4 % paraformaldehyde. Samples were then analysed on the flow cytometer within 24 h.

5.2.6 Statistics

Differences in group means of two different treatment groups were tested using the two-tailed Student's *t*-test. One-way ANOVA was used to test differences between multiple treatment groups. Results were expressed as group means ± SD, with 3 – 8 mice per group. A p value < 0.05 was considered significant.

5.3 RESULTS

5.3.1 Differential persistence of CHE in murine lungs at different intra-nasal doses

Lung CHE sensitisation with 10^3 CFU CHE thrice one week apart sufficed to suppress *in vivo* BCG responses – this is described more fully in Fig 5.5A. With this preliminary knowledge on minimal dosage for a suppressive effect, the differences in immune responses primed by a low (10^3 CFU) or high (10^6 CFU) dose of live CHE given thrice once a week via the i.n. route were investigated, to mimic inhalation of aerosolised bacteria from environmental sources. Lung viable bacterial counts were assayed at different time points after the first CHE dose (Fig. 5.1A). Two weeks after initiating low dose CHE, live bacteria were no longer recoverable from the lungs. This suggests that the immunity built-up from the earlier doses was sufficient to eliminate the low dose of CHE preventing even transient colonisation at week 2. As expected, the higher dose resulted in slightly more prolonged lung colonisation, but all bacteria were nonetheless eliminated by six weeks post-infection (Fig. 5.1A). Based on these results, immune responses two- and six-weeks post-CHE were subsequently studied to determine if viable CHE in the lungs were required to maintain responses.

5.3.2 Dose and timing of CHE exposure affects systemic IFN-γ but not IL-10 responses

To determine the effects of CHE viability on pro- and anti-inflammatory responses, in mice given high or low dose i.n. CHE sensitisation, levels of splenic IFN-γ and IL-10 production in response to *in vitro* heat-killed BCG stimulation at two and six weeks post-infection were first assessed. Such responses are likely to be due to cross-reactivity to BCG antigens primed by common antigens in CHE. At the early time point, IFN-γ response levels were markedly higher in the mice given high dose than low dose CHE, consistent with the higher antigen load. In low dose CHE-sensitised mice, IFN-γ production was higher at week 6 than week 2, although CHE was cleared by week 2. This suggests a build-up of memory responses to the common antigens even without presence of viable CHE. The reverse trend (i.e. reduced IFN-γ response at week 6 relative to week 2) was surprisingly observed in mice given 10^6 CFU (Fig. 5.1B, left). In fact, at week 6 the recipients of low dose CHE had stronger IFN-γ responses than those given high dose CHE ($p = 0.09$). This study speculated that the higher dose of CHE induced Tregs early, and these Tregs subsequently dampened the initial IFN-γ response, as has been noted with *M. vaccae* sensitisation (Hernandez-Pando, 1997). However, IL-10 was unlikely to be the mediator of these suppressive effects, as splenic IL-10 levels were similar at weeks 2 and 6, regardless of the CHE dose (Fig. 5.1B, right).

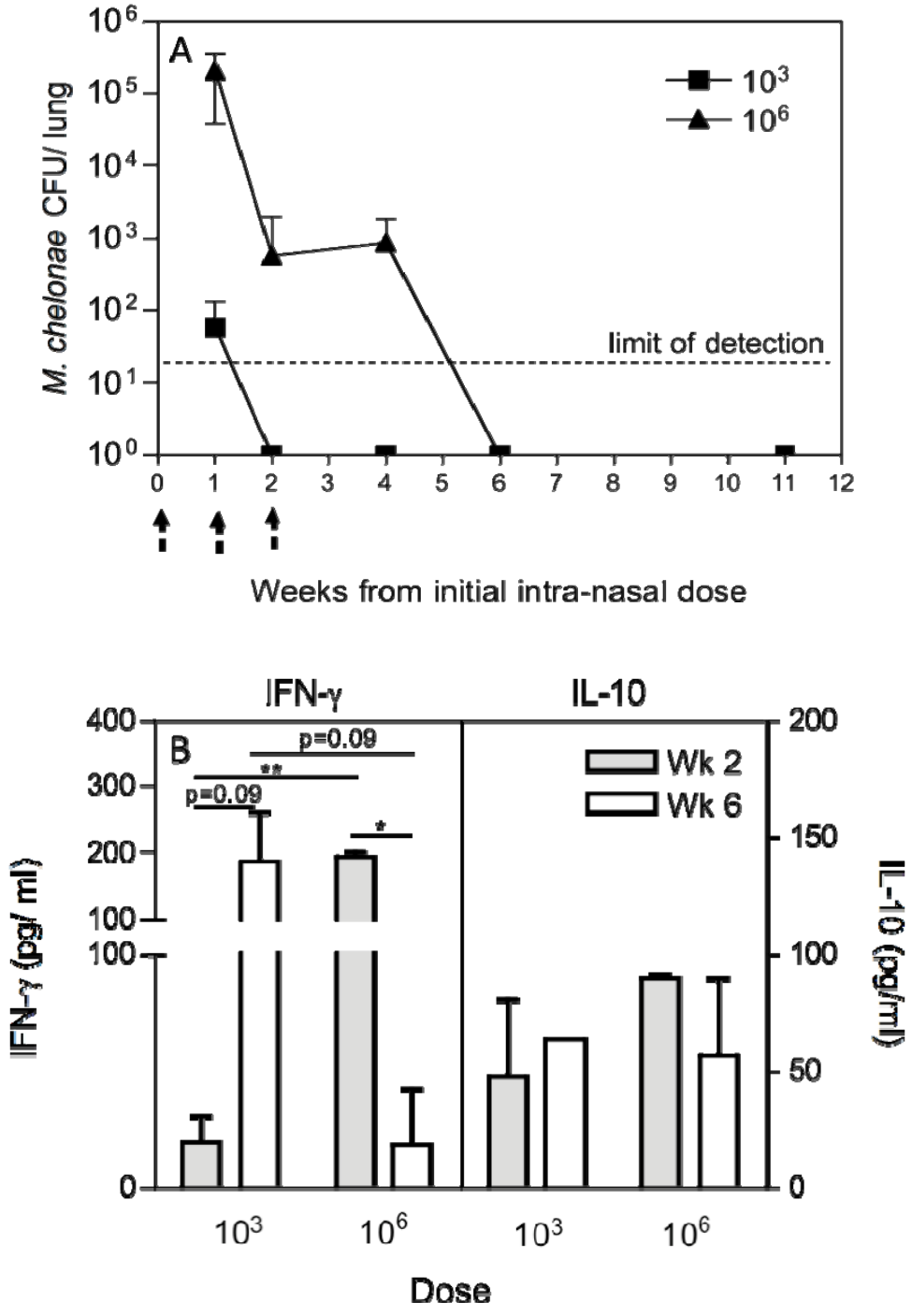

Fig. 5.1: Persistence of CHE in the lungs and dose dependent splenic IFN-γ but not IL-10 responses

Mice were given a low (10^3 CFU) or high (10^6 CFU) dose of live CHE three times, once a week, via the i.n. route. (A) Viable bacterial counts in the lungs were assayed for 11 weeks at various time points after the first CHE dose. (B) Splenocytes were stimulated *in vitro* with heat-killed BCG for 48 h, and IFN-γ (left) and IL-10 (right) production in supernatants were analysed by ELISA. *p<0.05, **p<0.01 by Student's *t* test.

5.3.3 High dose CHE induced stronger Treg responses and weaker IFN-γ responses

To verify if high dose CHE induced stronger regulatory T cell responses, the frequency of lung and splenic Tregs was studied by flow cytometry. The higher CHE dose led to significantly stronger induction of $CD4^+CD25^+$ cells and $CD4^+$ cells of $CD25^+GITR^+$ phenotype, respectively in the lungs at the early (Fig. 5.2A) and spleens at the late time-point (Fig. 5.2B) compared to lower dose CHE sensitisation. Thus, the dose of CHE affects the extent of Treg induction, with local (lung) effects being observable earlier than systemic (splenic) effects. However, the absolute magnitude of difference was small. Treg responses to low dose CHE were significantly stronger at week 6 than week 2**,** despite the clearance of CHE from the lungs (Fig. 5.2A). This could be due to persistence of inflammatory processes triggered by CHE exposures maintaining Treg expansion. In summary, higher levels of Treg responses and weaker IFN-γ responses were associated with the higher dose CHE sensitisation.

5.3.4 Increasing cellular recruitment to lung over time after CHE inhalation

In mice given 10^6 CFU CHE, the number of leucocytes at the site of CHE exposure (lungs) was generally higher at week 6 than week 2, especially $CD3^+$ T cells and $CD11c^+$ DCs (Fig. 5.3). Thus, at week 6 after high dose i.n. CHE, there was increased local lung lymphocyte activity relative to week 2, although the systemic (splenic) IFN-γ response had declined (Fig. 5.1B). The expansion of lung lymphocytes could comprise of both effectors and Tregs.

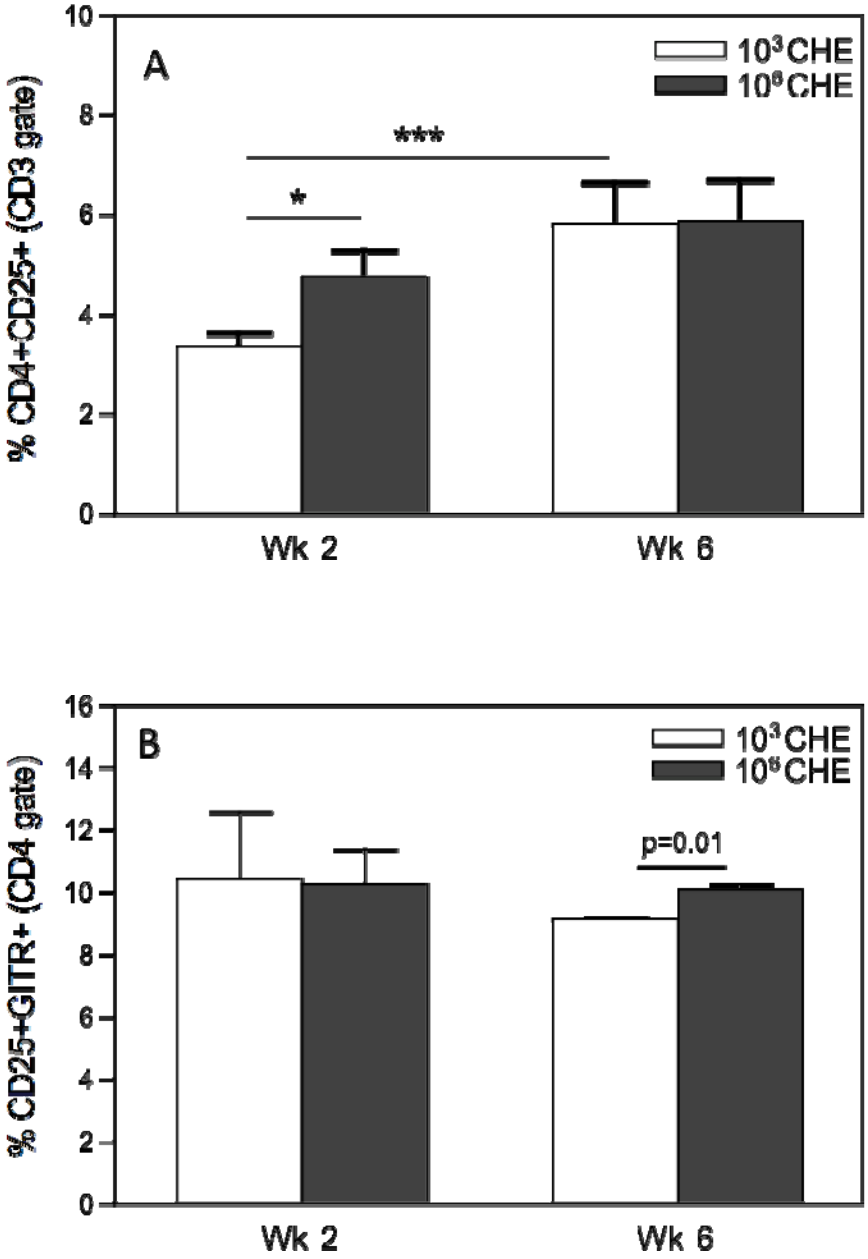

Fig. 5.2: Frequency of $CD4^+CD25^+$ $CD3^+$ T cells in the lungs and $CD25^+GITR^+$ $CD4^+$ cells in the spleens of CHE-sensitised mice

Mice were given i.t. 10^3 or 10^6 CFU CHE or PBS as described in Fig. 5.1A, and sacrificed two or six weeks after initial administration of CHE. The frequency of (A) $CD4^+CD25^+$ cells gated on $CD3^+$ cells in the lungs and (B) $CD25^+GITR^+$ cells, gated on $CD4^+$ cells, in the spleen were determined by flow cytometry. *$p<0.05$, ***$p<0.001$

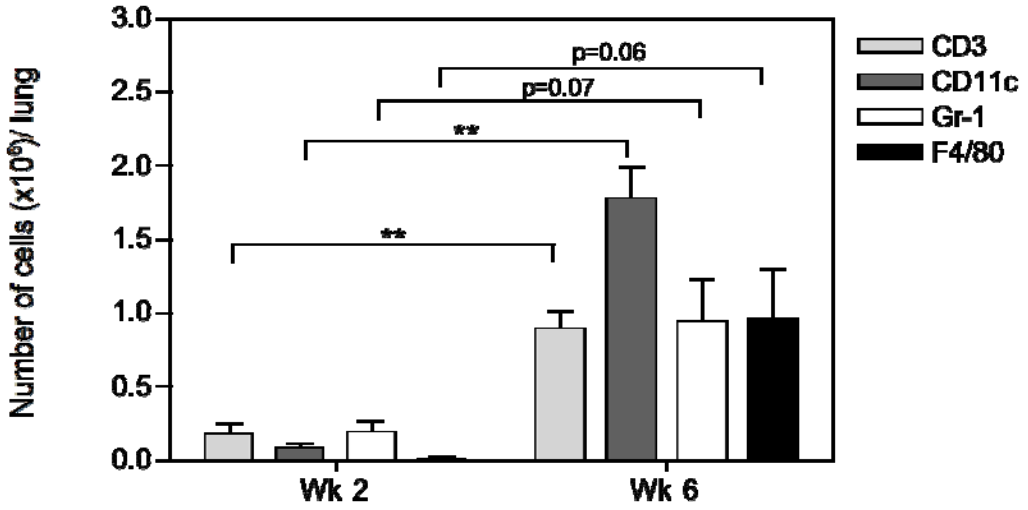

Fig. 5.3: Recruitment of inflammatory cells to the lungs of CHE-sensitised mice

Inflammatory cells, namely T cells (CD3), DCs (CD11c), neutrophils (Gr-1) and macrophages (F4/80), from lung tissue of mice sensitised with 10^6 CFU live CHE at two or six weeks after initial CHE sensitisation were analysed by flow cytometry. Fluorospheres of known concentration were used concurrently to obtain an absolute cell count. Results are expressed as means ± S.D. **$p<0.01$ by Student's *t* test.

5.3.5 Live and dead CHE induce similar levels of nTregs and IL-10

This study next investigated the responses to live versus dead 10^6 CFU CHE to directly compare their effects on Treg induction and IFN-γ production. The frequency of $CD25^+GITR^+$ cells out of all splenic $CD4^+$ lymphocytes was similar between mice given live and dead CHE (Fig. 5.4A) at both weeks 2 and 6. This Treg frequency persisted even after CHE was cleared at week 6. This indicated that the viability of CHE used for sensitisation did not significantly affect induction of $CD4^+$ Tregs with the $CD25^+GITR^+$ phenotype. Although the difference was not statistically significant, there was a 3-fold higher IFN-γ response in mice given dead CHE than those given live CHE at week 6 (Fig. 5.4B). This difference was not observed at week 2. IL-10 levels were the same in live and dead CHE sensitised mice at both time-points (Fig. 5.4B).

5.3.6 Suppression of BCG-specific memory IFN-γ producing cells by CHE exposure before BCG vaccination

Subsequent experiments were carried out with low dose CHE, as this dosage was more likely to mimic natural CHE exposure in the environment. The next stage of investigations focused on determining aspects of BCG vaccination responses that are suppressed by prior CHE sensitisation. The $CD62L^{lo}CD127^{hi}$ subset of memory cells is expanded in the organs of Mtb-infected mice and they are believed to be important for protection against TB (Henao-Tamayo, 2010). TB10.4 is highly immunogenic in BCG-vaccinated individuals and TB patients, inducing strong IFN-γ responses in such persons (Skjot, 2000; Skjot, 2002). An increase in TB10.4-specific IFN-γ responses in Treg-neutralised mice is

associated with reduced Mtb load upon challenge (Jaron, 2008). High frequencies of TB10.4 specific $CD4^+$ T cells correlate with protection against Mtb infection (Hervas-Stubbs, 2006), therefore memory responses to this antigen were studied as a surrogate for protective BCG responses.

After vaccination, mice pre-sensitised with repeated i.t. CHE exposures showed a significant reduction in frequency of BCG-specific IFN-γ producing cells, upon stimulation with heat-killed BCG (Fig. 5.5A) and TB10.4 epitope (Fig. 5.5B), compared to non-sensitised mice. Memory responses to BCG vaccination were reduced in CHE sensitised mice, evidenced by a smaller proportion of TB10.4-specific IFN-γ^+ memory cells of the $CD127^+CD62L^{lo}$ phenotypes (Fig. 5.6). Thus, CHE sensitisation suppressed BCG-specific memory IFN-γ responses. As the TB10.4 epitope is not a CHE antigen, this suppression is unlikely to be due to cross-reactive Tregs. However, *Mycobacterium*-specific nTregs can restrict expansion of effector T cells whose epitope specificity differs from the epitope recognised by the Tregs (Shafiani, 2010). It is also possible that the suppression in this case is non-specific, mediated by cytokines such as IL-10 produced by adaptive Tregs (Roncarolo, 2006), so this was investigated next.

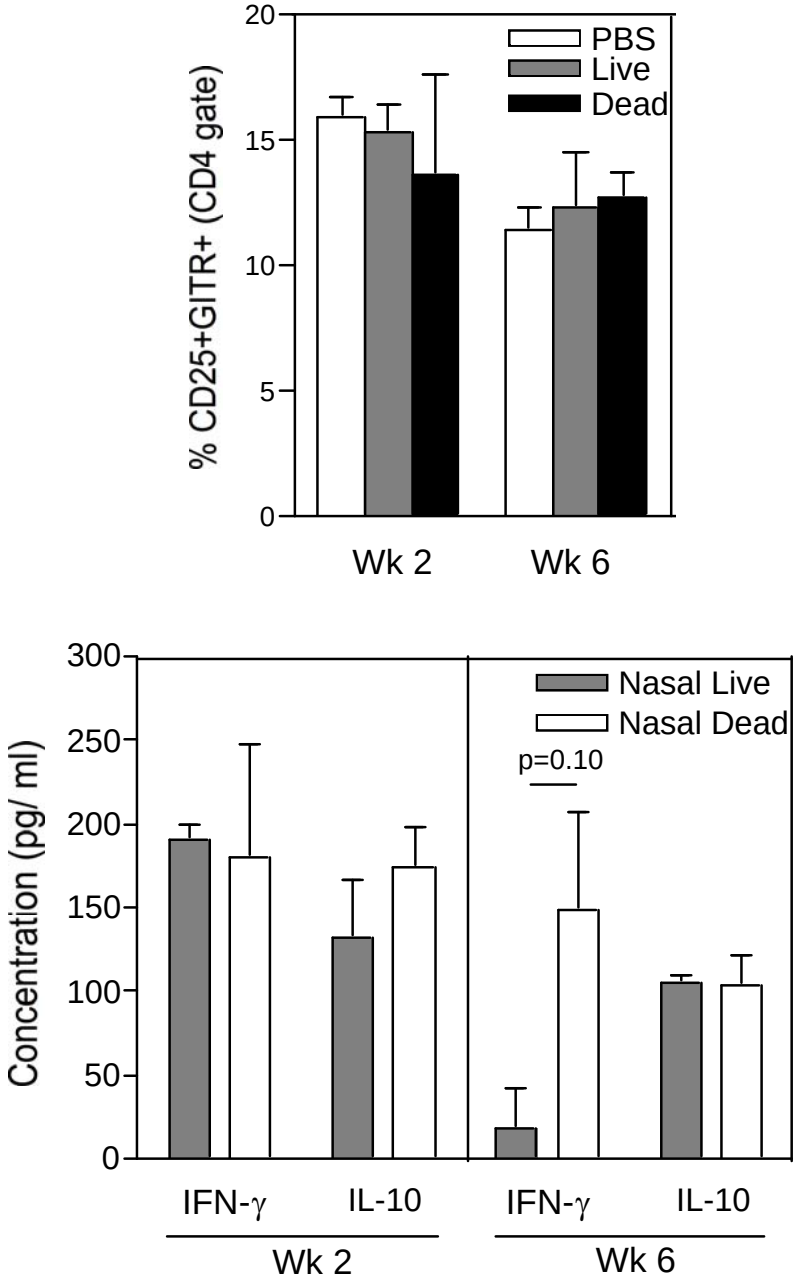

Fig. 5.4: Effects of CHE viability on Treg and cytokine responses
(A) The proportion of $CD25^{+}GITR^{+}$ cells, gated on $CD4^{+}$ cells, in the spleens of mice given i.t. live (10^{6}), dead (heat-killed) CHE or PBS was determined by flow cytometry. (B) Splenocytes were stimulated *in vitro* with heat-killed BCG for 48 h, and IFN-γ and IL-10 levels in supernatants were determined by ELISA. Results are means ± S.D.

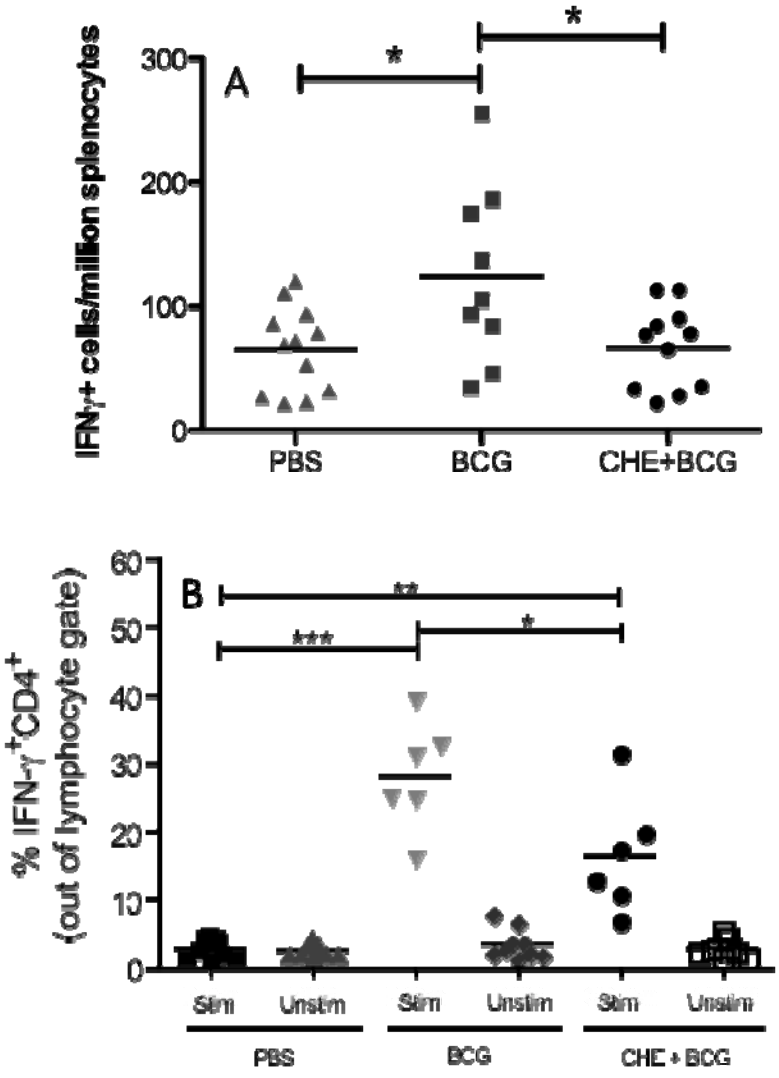

Fig. 5.5: Reduced frequency of BCG specific IFN-γ producing cells in vaccinated mice with prior CHE sensitisation

Splenocytes from vaccinated mice (BCG), CHE sensitised and vaccinated (CHE+BCG) or control (PBS) mice were stimulated with (A) heat-killed BCG or (B) a TB10.4 peptide (a BCG epitope not found in CHE; "stim") or PBS ("unstim") for 24h and frequency of IFN-γ^+ cells determined by (A) ELISpot or (B) cytokine capture followed by flow cytometry. Analysis was gated on lymphocytes (B) and the proportion of IFN-γ producing memory cells was determined. Data shown are individual mice (data points) and group means (short bars). *p<0.05, **p<0.01, ***p<0.001 by Student's *t* test.

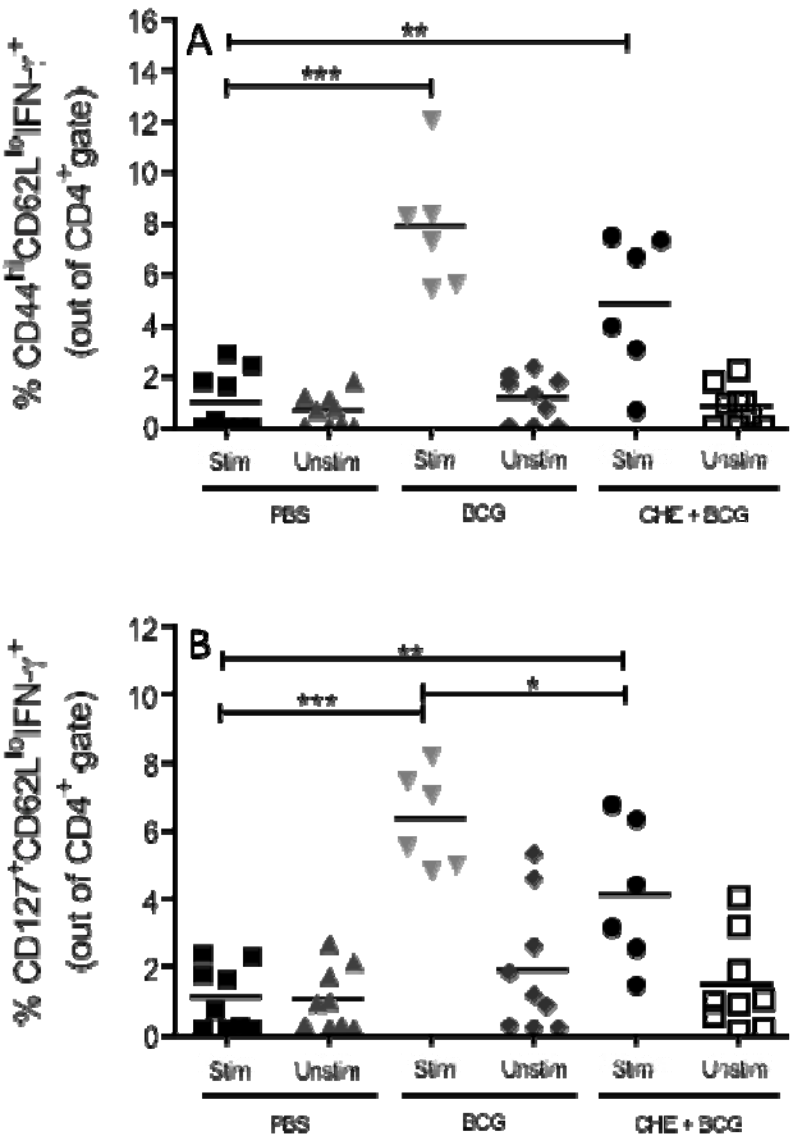

Fig. 5.6: Reduced frequency of IFN-γ producing epitope specific memory cells in vaccinated mice with prior CHE sensitisation

Splenocytes from vaccinated mice (BCG), CHE sensitised and vaccinated (CHE+BCG) or control (PBS) mice were stimulated with TB10.4 peptide ("stim") or PBS ("unstim") for 24h and the frequency of IFN-γ^+ cells was determined by cytokine capture and flow cytometry. Analysis was gated on $CD4^+$ lymphocytes and the proportion of (A) $CD44^{hi}CD62L^{lo}$ and (B) $CD127^+CD62L^{lo}$ memory cell subsets producing IFN-γ. gated on $CD4^+$ cells, was determined. Data shown are individual mice (data points) and group means (short bars). *p<0.05, **p<0.01, ***p<0.001 by Student's *t* test.

5.3.7 Systemic increase in IL-10 producing cells with CHE sensitisation before BCG vaccination

After BCG vaccination, significantly higher frequencies of splenic IL-10 producing cells (by ELISpot) and $CD4^+$ IL-10-secreting lymphocytes (by flow cytometry) were observed in CHE-sensitised mice (Fig. 5.7), compared to non-sensitised mice, in response to heat-killed BCG stimulation. This suggests the presence of adaptive Tregs induced upon prior CHE exposure followed by BCG vaccination. This increase in systemic IL-10 may be one possible explanation for the suppression of splenic IFN-γ responses to BCG antigens in CHE-sensitised mice post-vaccination. Other laboratory colleagues subsequently showed that neutralisation of IL-10 led to restoration of the IFN-γ response in this experimental system (not shown).

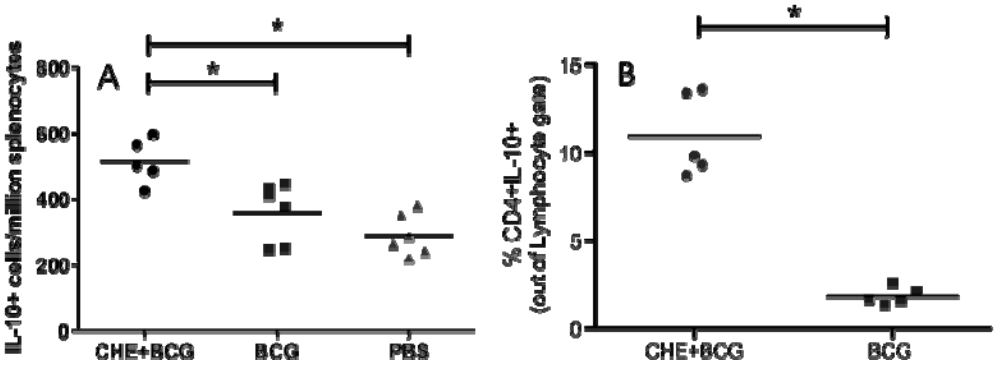

Fig. 5.7: Higher frequency of IL-10 producing adaptive Tregs in BCG-vaccinated mice with prior CHE exposure
Splenocytes from vaccinated mice (BCG) or vaccinated mice with prior CHE sensitisation (CHE+BCG) were stimulated with heat-killed BCG for 72 h. (A) $IL\text{-}10^+$ cells were then detected via ELISpot or (B) stained by cytokine capture for IL-10, then analysed by flow cytometry. Data represent individual mice and group means. *$p<0.05$ by Student's *t* test.

5.3.8 Differential phenotype of IL-10 producing cells in BCG vaccinated mice with and without CHE sensitisation

The surface phenotype of IL-10 producing cells was investigated. About 80% of IL-10 producing $CD4^+$ cells in BCG-vaccinated mice were of the conventionally described Tr1 cell phenotype (i.e. $CD25^-FoxP3^-$). However, in mice with CHE sensitisation before BCG vaccination, $CD4^+IL\text{-}10^+$ cells were predominantly $CD25^-FoxP3^+$ (~70%), suggesting that CHE sensitisation induced a different type of IL-10 secreting adaptive Tregs, relative to BCG vaccination alone. These $FoxP3^+$ cells could have arisen by peripheral conversion of $CD4^+CD25^-$ effector cells by $CD4^+CD25^+$ Tregs in CHE-sensitised mice, as described in Chapter 4 (Fig. 4.4E).

5.3.9 Expansion of nTregs with CHE sensitisation before BCG vaccination

The frequencies of nTregs in spleens of CHE-sensitised, BCG-vaccinated mice were also investigated. Most (~80%) of $CD4^+CD25^+$ cells were $GITR^+$. There were no differences in proportions of $CD4^+CD25^+$ cells between BCG-vaccinated mice with and without prior CHE sensitisation. However, the CHE-sensitised mice had a significantly higher frequency of $FoxP3^+$ lymphocytes and cells with the $CD25^+FoxP3^+$ phenotype within the $CD4^+$ lymphocyte population (Fig. 5.8). This is a phenotype commonly associated with nTregs. It suggests that with repeated CHE exposure before BCG vaccination, a higher frequency of nTregs can be expanded upon *in vitro* BCG restimulation, than with single BCG vaccination alone.

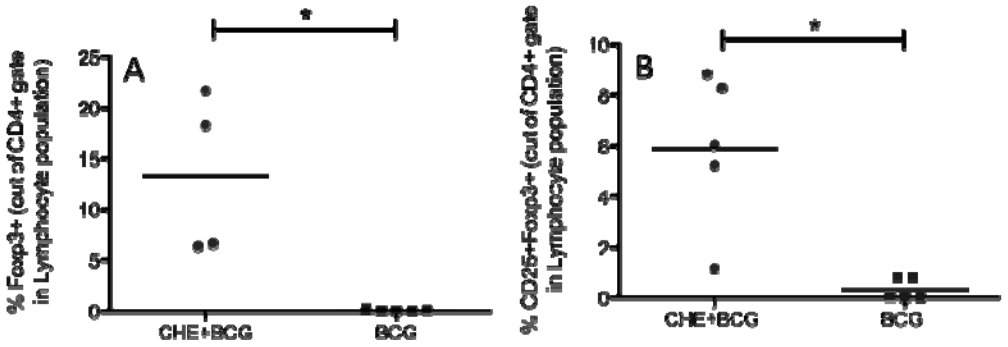

Fig. 5.8: Increased frequency of FoxP3$^+$ and CD25$^+$FoxP3$^+$ CD4$^+$ Treg in vaccinated mice with prior CHE exposure upon BCG stimulation.
Splenocytes either from vaccinated mice (BCG) or vaccinated and CHE sensitised (CHE+BCG) mice were stimulated with heat-killed BCG for 72 h. The proportion of (A) FoxP3$^+$ and (B) CD25$^+$FoxP3$^+$ was determined, gated on CD4$^+$ lymphocytes. Data points represent individual mice, and group means are indicated as short bars. *$p<0.05$ by Student's *t* test.

5.4 DISCUSSION

Before evaluating how Env exposure can potentially affect *in vivo* BCG responses in the host, it was important to investigate how different exposure parameters affect the initial and subsequent host responses to Env. The present findings suggest that higher bacterial loads lead to more Treg induction, associated with subsequently weaker M*ycobacterium*-specific IFN-γ responses. However, persistence of live CHE in the host was not required for build-up of Tregs or IFN-γ responses over time, and sensitisation with dead CHE surprisingly gave similar responses to live CHE.

5.4.1 Higher CHE doses induce more pro- and anti-inflammatory responses

M. tuberculosis at low doses induces antigen-specific Th1 cellular responses in the lungs more slowly than high doses (Yahagi, 2010). Likewise, with inhaled CHE at low doses, the induction of systemic *Mycobacterium*-specific IFN-γ response was slower than with high dose exposures. However, high doses of Env are known to induce both pro- and anti-inflammatory responses (Power, 1998), and this was in agreement with observations in this study. A higher frequency of $CD25^{+}GITR^{+}$ type of $CD4^{+}$ cells, which are known to have regulatory functions (McHugh, 2002), was observed within a week of first exposure in the lungs of mice given high dose CHE, even though at that same time point (week 2), there were strong systemic IFN-γ responses. Given the temporal link of higher frequency of splenic Tregs at week 6 (relative to low dose group) with lower systemic IFN-γ responses in the high dose group, it is tempting to suspect that

Mycobacterium-specific nTregs may have contributed to this. GITR is highly expressed on Tregs (Belkaid, 2007). GITR signalling triggers the expansion of functional Tregs (Ji, 2004; Nishioka, 2008), and GITR expression on Treg cells is higher in TB patients who have reduced BCG-specific IFN-γ response in $CD4^+$ cells (Li, 2007). This implies that $GITR^+$ cells may have a role in mediating suppression of *Mycobacterium*-specific responses. Induction of IL-10, typically a product of adaptive Tregs, appears unrelated to suppression of BCG-specific IFN-γ, as its levels were unaltered with dose and time post-infection.

5.4.2 CHE induced responses not dependent on viability of CHE sensitisation

Whether live and dead mycobacteria prime different immune responses, and the nature of their interaction with the host is controversial. There is evidence that in murine studies, live actively replicating BCG, but not dead BCG, are effective in protecting against TB challenge (Orme, 1988), possibly due to the importance of activating T cell subsets specific for proteins secreted by live mycobacteria. (Daugelat, 1995). In contrast, other studies suggest that live and dead BCG induce similar local responses (Chambers, 1997). Findings in Chapter 3 showed that heat-killed CHE sensitisation induced cytotoxicity against BCG-infected macrophages, and reduced BCG replication, therefore immune priming against BCG clearly occurs even with dead CHE. A study on oral administration of different live strains of *M. avium* (Young, 2007) suggests that the strain that persists longer in the host is less likely to trigger pro-inflammatory cytokine secretion, and also tends to induce greater suppression of IFN-γ responses and

antibody responses biased towards a Th2 phenotype. This suggests that Env species with longer persistence may limit the BCG response more significantly. However, elimination of live *M. avium* with antibiotics given to the host before BCG vaccination still led to suppression of BCG counts and IFN-γ responses (Brandt, 2002), so the nature of memory responses imprinted in the host may not be entirely determined by the persistence of the species during BCG vaccination. Indeed, heat-killing or antibiotic mediated lysis of mycobacteria may release intracellular antigens, contributing to greater immunogenicity than live mycobacteria, which usually have means of evading host immunity if they live intracellularly. In this present study, splenic Treg or BCG-specific IFN-γ responses primed by i.n. CHE exposure were not substantially different with live or dead (heat-killed) CHE sensitisation (Fig. 5.4). Moreover, most responses were undiminished or even elevated when tested beyond the point of *in vivo* live persistence of CHE (Figs 5.2 and 5.3). This implies that the memory and regulatory responses induced by CHE are similar whether the host was sensitised by live or dead CHE.

5.4.3 Implications of dose-dependent immune induction with CHE exposure

The multiple Env species often identified in urban water sources (Vaerewijck, 2005) could be live or dead at the point of human contact. The fact that even dead Env could have similar impact as live Env in terms of immune priming and response to BCG antigens obviously enlarges the potential influence of such Env priming in the urban human population. This study showed that different doses

of CHE induced different immune-modulatory effects, thus caution is needed in comparing studies investigating the impact of Env on BCG vaccine efficacy. As higher doses of CHE led to higher levels of Treg responses and weaker BCG-specific IFN-γ responses pre-vaccination, it is tempting to speculate that people who are highly exposed to Env may be primed to have poorer responses to BCG. The converse point is also interesting – that low dose repeated inhaled CHE priming could lead to enhanced memory to BCG antigens over time through cross-reactivity, as this method of sensitisation has less immediate impact on systemic Treg induction. Indeed, this led us to directly examine whether low dose priming would boost BCG responses post-vaccination and maintain low Treg responses.

5.4.4 Role of IL-10 in CHE-mediated suppression of BCG vaccine response

With BCG vaccination after CHE exposure, a different pattern of Treg induction was observed from inhaled CHE exposure alone. In fact, induction of both systemic nTregs and adaptive (infection-induced) Tregs was noted. IL-10 is an anti-inflammatory cytokine that is particularly important in suppressive effects induced by adaptive Tr1 cells (Roncarolo, 2006). Env such as *M. avium* can induce the production of IL-10 (Bermudez and Champsi, 1993) and neutralisation of IL-10 improves resistance to systemic murine *M. avium* infection, associated with enhanced macrophage activity (Denis and Ghadirian, 1993). Excessive IL-10 production in IL-10 transgenic mice increases *M. avium* infection and decreases macrophage effector function (Feng, 2002). Anergic TB patients who do not respond to intra-dermal purified protein derivative (PPD)

have high peripheral blood levels of IL-10, which is additionally linked to suppression of IFN-γ production and T cell proliferation (Boussiotis, 2000). These studies clearly show that IL-10 impairs *Mycobacterium* pro-inflammatory responses. CHE-sensitised mice had more IL-10 producing cells post vaccination (Fig. 5.7), which are more likely to be CHE-induced adaptive Tregs, although their phenotype was not consistent with Tr1 cells. As discussed earlier, these cells may potentially be due to peripheral conversion of $CD4^+CD25^-$ cells in the presence of bacteria (Littman and Rudensky, 2010). The IL-10 in CHE-sensitised mice post-vaccination appears to be the main cause of suppressed *Mycobacterium*-specific IFN-γ. This is supported by the temporal association with the latter, and by restoration of systemic BCG-specific IFN-γ levels with IL-10 neutralisation (shown experimentally by others in our laboratory using the same model). This contrasts with inhaled CHE alone, for which even high dose CHE did not alter IL-10 production (Fig 5.1), but did elevate nTreg frequencies over low dose CHE (Fig. 5.2). Therefore, nTregs were the more likely reason for the systemic decline in BCG-specific IFN-γ responses six weeks after high dose CHE exposure alone.

5.4.5 Conclusion

It is notable that with BCG vaccination alone, restimulation of splenic lymphocytes *in vitro* with BCG antigens gave very minimal expansion of nTregs ($CD4^+CD25^+FoxP3^+$), whereas repeated inhaled CHE exposure before BCG vaccination increased systemic frequency of such cells (Fig 5.8). This phenomenon is consistent with Shafiani's observations in the context of Mtb

infection (Shafiani, 2010); thus, expansion of *Mycobacterium*-specific nTregs with repeated exposure to *Mycobacterium* antigens in common between CHE and BCG is the likely explanation.

In CHE-primed mice, the post-vaccination response involved both nTregs and IL-10-producing adaptive Tregs, with associated suppression of IFN-γ memory responses specific for BCG antigens. These factors are known to be correlated with poor protection against TB, and are therefore likely to result in negative effects of CHE priming on BCG vaccine-induced protection. Importantly, marked differences were observed in nature of systemic Treg responses before and after BCG vaccination in CHE-primed mice. These differences may explain why in a study of human infants with delayed BCG vaccination, the frequency of peripheral blood nTregs before vaccination did not correlate with the levels of *Mycobacterium*-specific IFN-γ after vaccination (Burl, 2010), although it was noted that vaccination delay (allowing time for natural Env exposure) led to poorer IFN-γ responses compared to vaccination at birth. In the above study, it is possible that the induction of adaptive IL-10 producing Tregs and expansion of nTregs post-BCG vaccination in Env-primed subjects, rather than nTreg frequency *before* vaccination, may be more correlated with the post-BCG IFN-γ suppression. The implication is that post-BCG vaccination Treg assays may be more pertinent for assessing the real effect of Env priming. The findings in this study lend support to the theoretical proposition (Shafiani, 2010) that neutralisation of Tregs before BCG vaccination may lead to better vaccination outcomes.

CHAPTER 6 – *Mycobacterium chelonae* exposure after BCG vaccination reduces local inflammatory cell recruitment despite increasing systemic BCG-specific responses

6.1 INTRODUCTION

A large body of evidence suggests that exposure to non-tuberculous Env interferes with BCG-attributable protection, but most studies were conducted in the context of pre-BCG Env exposure (Collins, 1971; Orme and Collins, 1984; Black, 2001a; Brandt, 2002; Demangel, 2005). Given that the WHO recommends that BCG vaccination be administered at birth (WHO, 2005), there should theoretically be little Env exposure before vaccination, unless vaccination is delayed. However, post-vaccination exposure to *M. avium* also compromises subsequent BCG-induced protection in mice (Flaherty, 2006) and human studies find that in areas with prevalent Env exposure, there are weaker responses to BCG three months post-vaccination, even when vaccination takes place within a month of birth (Lalor, 2009). This gives rise to the possibility that post-BCG Env exposure also affects longer term BCG responses.

This study focused on discovering how post-BCG vaccination exposure to Env alters BCG responses in mice. Effects of the rapid grower – CHE, were studied, following earlier findings (Chapters 4 and 5) that pre-BCG exposure to this Env species negatively affected subsequent BCG recall responses. Oral and inhaled routes of CHE exposure were studied because these are the more likely routes for natural human exposure to Env (Primm, 2004). After BCG vaccination of

weanling mice, the impact of giving CHE early post-vaccination during initial development of memory responses to BCG, was compared against late CHE exposure. The findings suggest that CHE exposure late after BCG vaccination induces both pro- and anti-inflammatory responses, and influences recruitment of immune cells to the lungs upon subsequent lung *Mycobacterium* challenge.

6.2 MATERIALS AND METHODS

6.2.1 Mice

Specific pathogen-free BALB/c mice (AnN from Taconic) were bred within the animal facility approved by Association for Assessment and Accreditation of Laboratory Animal Care International, under protocols approved by Massachusetts Institute of Technology (MIT) Committee on Animal Care. Breeder mice were housed in individual isolator cages with filter tops. Newly weaned mice were then sexed and removed to new cages with sterile synthetic bedding, to minimise exposure to mycobacterial antigens. Sterile rodent diet and water were supplied *ad lib*, and beddings were changed twice a week.

6.2.2 Immunisation

Bacteria strains and preparation were as previously described in Chapter 3 (Section 3.2.2 and 3.2.3). Newly weaned mice were vaccinated with live BCG subcutaneously with 10^6 CFU live BCG, and were subsequently immunised with 500 CFU live CHE via the intra-tracheal (i.t.) route at either two or twelve weeks post-vaccination, once a week for three weeks, or given continuous live CHE exposure in drinking water at 50 CFU/ ml. Mice were subsequently sacrificed two weeks after the last CHE exposure. In some experiments, mice were challenged with 10^6 live BCG via the i.t. route, two weeks after the last CHE exposure, and sacrificed three weeks post-BCG infection. Cells were isolated from the spleens and lungs as previously described in Chapter 3 (section 3.2.6).

In certain experiments, the lungs were weighed immediately upon resection for normalisation purposes, and a portion snap-frozen and kept for total protein content analysis at a later time.

6.2.3 ELISpot

T cell enriched splenocytes were seeded at 0.5 x 10^6 cells/ well in 96-well ELISpot plates and stimulated with heat-killed BCG (10 bacteria: 1 cell) or PBS for 24 h to detect IFN-γ secretion via the ELISpot, according to previously described methods (Chapter 5, section 5.2.2).

6.2.4 Cytokine Multiplex Array

T cell enriched splenocytes were seeded at 0.25 x 10^6 cells/ well in 96-well U-bottom tissue culture plates and stimulated with heat-killed BCG (10 bacteria: 1 cell) or PBS for 48 h to detect cytokine secretion. Supernatants were harvested and kept at -20 °C until use. Analysis of cytokines and chemokines in the lungs was conducted after protein isolation. Protein from lung samples was isolated directly from frozen tissue by homogenisation in cell lysis buffer containing phosphatase and protease inhibitors according to manufacturer's protocol (Bio-Rad). Cytokine and chemokine levels were measured in supernatants and lung lysates using Bio-Plex Pro Mouse cytokine/ chemokine multiplex assay according to manufacturer's instructions (Bio-Rad). Briefly, relevant wells on plates were pre-wet with buffer and beads conjugated to antibodies specific for various cytokines were added. Wells were washed with washing buffer and fluid removed by vacuum, before standards and samples were added and incubated for 30 min.

Detection antibody and streptavidin-PE were next added with 30 min and 10 min incubation periods respectively, with washing in between. A final wash step is performed before the beads were resuspended in diluent buffer. Plates were then processed on the Luminex 200 suspension array system using the low RP1 target setting (High PMT) for maximum sensitivity. Data was analysed on the Bio-Plex Manager™ 5.0 software and cytokine concentrations calculated using a standard curve generated by either 4PL or 5PL curve fitting. Total protein in each lung sample was measured using the micro BCA assay kit (Thermo Fischer Scientific Inc.). Lung cytokines and chemokines were normalised to total protein concentration of each sample.

6.2.5 Flow cytometry

Cells to be analysed via flow cytometry were washed with PBS containing 0.5% BSA, then stained with relevant fluorochrome-conjugated antibodies at 4 ˚C for 30 min and fixed with 4 % paraformaldehyde before use. For intracellular staining, cells were first surface stained as described above, then fixed and permeabilised using the FoxP3 staining buffer set (eBioscience) according to manufacturer's instructions. Cells were next blocked with purified anti-mouse CD16/CD32 antibodies (BD Pharmingen) in permeabilisation buffer for 30 min at 4 ˚C, before staining with relevant intracellular antibodies, with relevant isotype controls. In some experiments, splenocytes were seeded at 0.25 x 10^6 cells/ well in 96-well U-bottom tissue culture plates and stimulated with PMA (12.5ng/ ml) and ionomycin (500 ng/ ml) for 18-19 h before harvesting for surface staining and flow cytometry. The following anti-mouse antibodies were used: CD3e (145-2C11, eBioscience), Gr1 (RB6-8C5, BD Pharmingen), CD11b

(M1/70, eBioscience), F4/80 (BM8, eBioscience), CD4 (GK1.5, eBioscience), CD8a (53-6.7, eBioscience), CD25 (PC61.5, eBioscience), CD62L (MEL-14, eBioscience), CD44 (IM7, eBioscience), GITR (DTA-1, eBioscience), and FoxP3 (FJK-16s, eBioscience), along with relevant isotype controls in negative control samples. Samples were then analysed on the LSR-II (BD Biosciences) or the C6 flow cytometer (Accuri Cytometers) within 24 h.

In some experiments, the absolute number of lung cells per piece of lung tissue was obtained by determining the concentration of cells within the flow cytometry sample via the C6 flow cytometer (Accuri Cytometers). The absolute number of lung cells was then calculated by multiplying that by the total volume in the sample, and normalised to total number of cells per mg of lung tissue obtained.

6.2.6 Statistics

Multiple group comparisons were tested using one-way ANOVA, while smaller group comparisons were tested using two-tailed Student's *t*-tests. Results were expressed as mean ± SEM. A p value < 0.05 was considered significant.

6.3 RESULTS

To simulate BCG vaccination at birth, newly weaned Balb/c mice were BCG-vaccinated and rested for two weeks (early) or 12 weeks (late) before commencing live CHE administration either through three doses of once weekly intra-tracheal (i.t.) exposures or four weeks of continuous oral exposure via the drinking water supply, as illustrated in the schematic diagram (Fig. 6.1A).

6.3.1 Early, but not late, lung CHE exposure increased systemic BCG-specific IFN-γ responses

The effect of CHE exposure on systemic BCG-specific responses was evaluated by enumerating frequencies of splenic IFN-γ secreting cells after heat-killed BCG stimulation. After BCG vaccination, early CHE exposure led to an increase in BCG-specific IFN-γ secreting cells upon lung, but not oral exposure (Fig. 6.1B). At late exposure, neither route of CHE exposure altered IFN-γ responses to BCG. It is possible that BCG responses that could be boosted by CHE were relatively short-lived, since responses were no longer enhanced when BCG vaccination and CHE exposure were further apart in time. Alternatively, CHE may have induced stronger Treg responses when exposure occurred late post-vaccination. The latter is supported by the significant reduction in BCG-specific IFN-γ responses in mice given late compared to early CHE exposure post-BCG ($p<0.001$, Fig. 6.1B vs 6.1C).

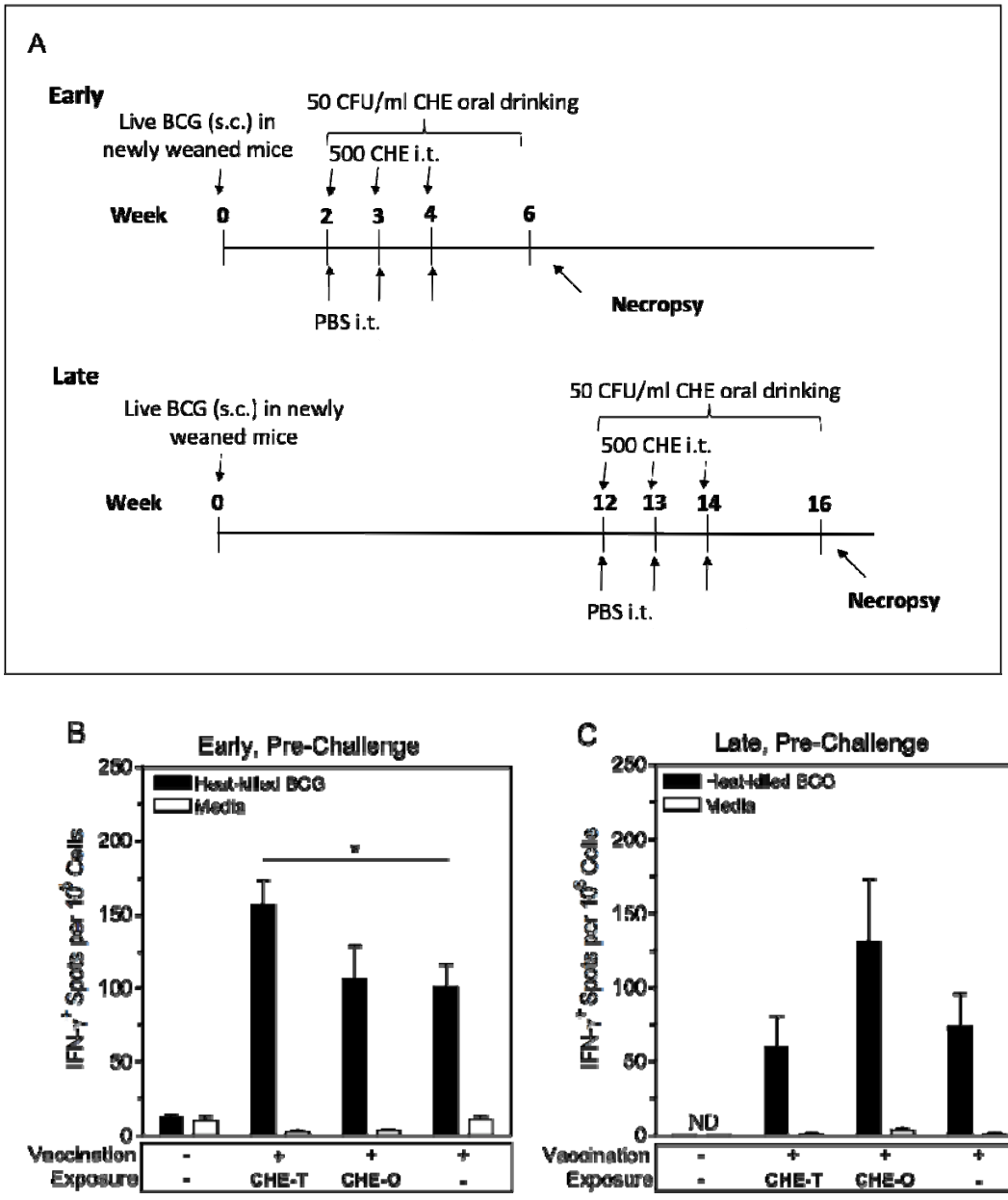

Fig. 6.1: Intra-tracheal CHE exposure reduces BCG-specific IFN-γ secreting cells in the spleen

(A) Pre-challenge time course for BCG vaccination, exposure to CHE intratracheally (CHE-T) or orally through the water supply (CHE-O), and necropsy. (B, C) The frequency of IFN-γ producing splenocytes was determined by ELISpot. Splenocytes from vaccinated and CHE exposed mice at (B) six weeks (early) or (C) 16 weeks (late) post-BCG vaccination were restimulated with heat-killed BCG or media, then assayed for IFN-γ producing cells via ELISpot. Statistics were done via 2-way ANOVA, then a Bonferroni post-test. Data shows mean spots per million cells (of 6-16 mice from multiple necropsies) ± SEM. ND = not detectable. *$p<0.05$. All experimental groups were significantly different from mice with neither BCG vaccination nor CHE exposure.

6.3.2 Both IFN-γ and IL-10 production reduced at late, relative to early CHE exposure

Subsequent studies focused on lung exposure to CHE, since this route had a greater influence on BCG-specific IFN-γ responses than oral CHE exposure. After splenocyte stimulation with heat-killed BCG for 48 h, no differences in IFN-γ production by ELISA were observed in vaccinated mice with and without lung CHE exposure, at either the early or late time points. Nonetheless, IFN-γ production was still reduced with late CHE exposure compared to early exposure (1.90 ± 2.00 vs 9.28 ± 3.56 pg/ ml, $p<0.01$), complementing the ELISpot observations above. Relative to vaccinated mice without CHE exposure, mice with early CHE lung exposure had comparable IL-10 secretion, but mice with late CHE lung exposure had reduced IL-10 levels (1.99 ±1.56 vs 0.47 ± 0.31 pg/ ml, $p<0.05$). The absolute differences were small, but the reduced IFN-γ levels at late CHE exposure are unlikely to be due to suppressive effects of IL-10, because late CHE exposure reduced IL-10 secretion relative to early exposure (0.47 ± 0.31 vs 2.14 ± 1.02 pg/ ml, $p<0.01$).

6.3.3 Lung CHE exposure did not alter proportions of $CD4^+$ Tregs

We hypothesised that the reduced BCG-induced IFN-γ responses with late CHE exposure may be related to enhanced induction of Tregs, relative to early CHE exposure. Thus, the proportions of $FoxP3^+$, $CD25^+FoxP3^+$, $CD25^+GITR^{hi}FoxP3^+$ and $CD25^+FR4^{hi}FoxP3^+$ $CD4^+$ cells in spleens of vaccinated mice after CHE lung exposure were determined by flow cytometry. However, no significant

differences in any of these Treg populations were found between early and late CHE exposed mice. There were significantly higher $CD25^{+}GITR^{hi}FoxP3^{+}$ and $CD25^{+}FR4^{hi}FoxP3^{+}$ $CD4^{+}$ frequencies in late exposed relative to CHE unexposed mice, but the absolute differences were minimal (7.32 ± 1.01 % vs 6.27 ± 0.65 %, $p<0.05$, and 7.92 ± 1.14 % vs 6.89 ± 0.77 %, $p<0.05$, respectively).

6.3.4 Post-vaccination late CHE exposure increased systemic BCG-specific IFN-γ responses upon secondary BCG challenge

Whether CHE altered recall responses to BCG *in vivo* was explored next, together with a study of local immunological changes at the site of infection. Mice, BCG-vaccinated at weaning, were challenged with live BCG via the i.t. route, either at six weeks (early) or 16 weeks (late) post-vaccination, following respective early and late CHE exposures similar to earlier experiments (Fig. 6.2A). As expected, a higher splenic IFN-γ response was observed in previously BCG-vaccinated than in unvaccinated mice (Fig. 6.2B). Early CHE exposure did not significantly alter this response, relative to unexposed mice (Fig. 6.2B). However, late CHE exposure led to significantly higher frequencies of IFN-γ producing cells, relative to unexposed mice (Fig 6.2C). Thus, the early BCG priming can be boosted by late, but not early, CHE exposure post-vaccination, leading to an enhanced secondary BCG recall response. Notably, the naïve mice, and indeed, almost all treatment groups, showed stronger IFN-γ responses to BCG challenge at the later time point (comparing same treatment groups pairwise $p<0.05$ for all groups except BCG-vaccinated and challenged mice ($p=0.14$), Fig 6.2B vs 6.2C). This may

be because the older mice with more mature immune systems mounted more robust responses to both CHE and BCG challenge.

6.3.5 Late CHE exposure increased systemic $CD4^+$ regulatory T cells in vaccinated mice after BCG challenge

To determine whether CHE exposure altered systemic Treg populations during BCG challenge, splenocytes from mouse groups treated as described in Fig. 6.2A were analysed for Treg surface markers and FoxP3 expression. Vaccination had no impact on proportions of $CD4^+$ cells expressing $CD25^+FoxP3^+$ (Fig. 6.3A), but increased the proportions of $CD25^+GITR^{hi}$ $CD4^+$ Treg in BCG challenged mice at the early time point, relative to unvaccinated but challenged mice (Fig. 6.3C). Therefore, BCG vaccination increased the Treg response upon subsequent BCG challenge. Early CHE exposure did not significantly alter proportions of Tregs (Fig. 6.3A, 6.3C). In contrast, in mice exposed late to CHE, relative to mice without CHE exposure, systemic frequency of $CD4^+$ cells expressing both CD25 and $GITR^{hi}$ were elevated post-BCG challenge (Fig. 6.3D). There was a smaller increase in $CD4^+CD25^+FoxP3^+$ cells (Fig 6.3B, significant by *t*-test comparison with non-exposed mice, but not by ANOVA). This could mean that only later CHE exposure post-vaccination results in Treg induction, or perhaps murine age and maturity of the immune system have an influence on Treg induction. The increased Treg frequency in late CHE exposure group did not negatively affect their systemic IFN-γ response, as this group saw an increase in IFN-γ responses (Fig. 6.2C).

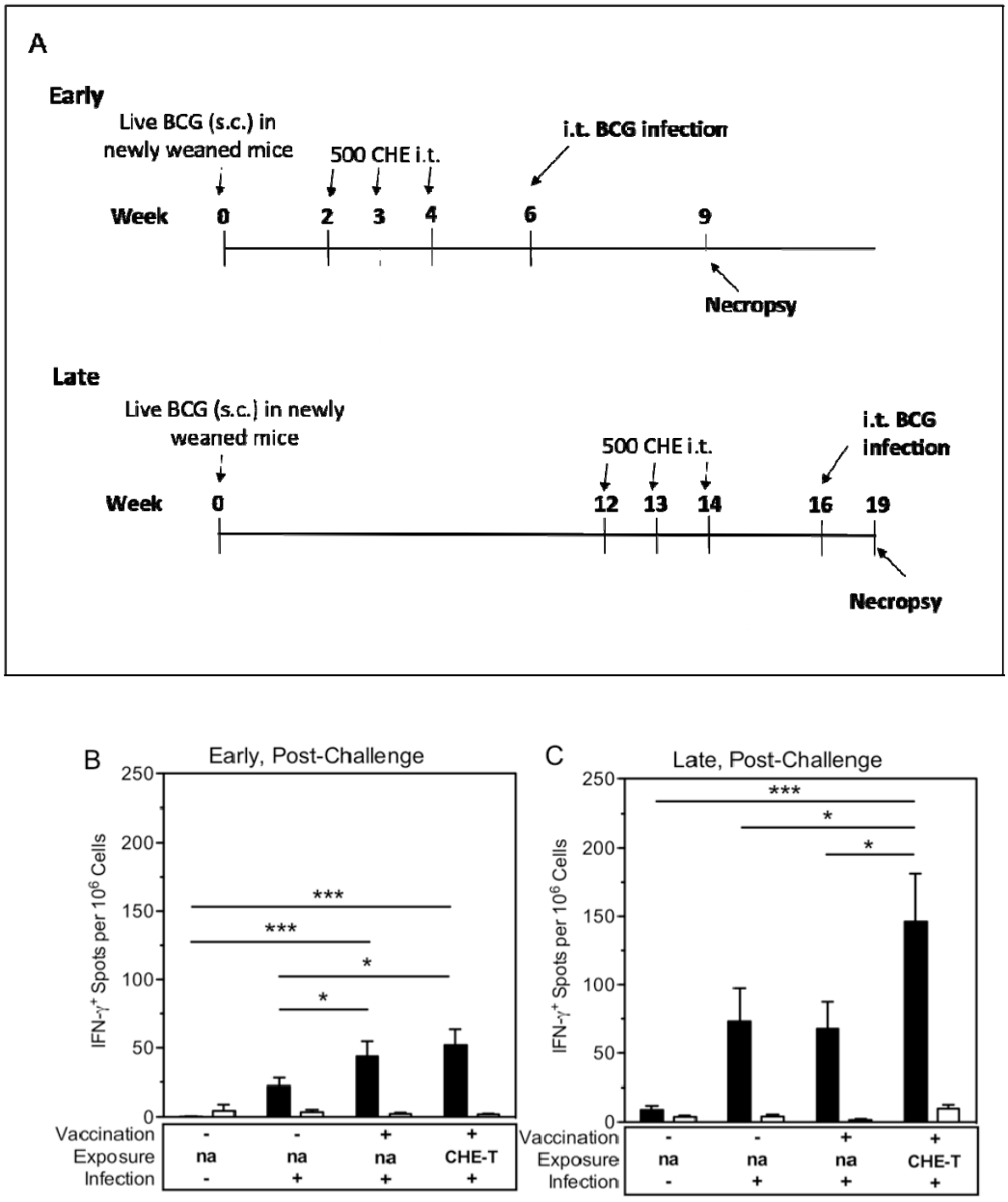

Fig. 6.2: Late exposure to CHE increases BCG-specific IFN-γ secreting cells after BCG lung challenge.

(A) Post-BCG challenge scheme for vaccination and CHE exposure, at early and late time points. Frequency of BCG-specific IFN-γ producing splenocytes three weeks post-BCG challenge from (B) early or (C) late CHE i.t. exposure as shown in A. Splenocytes were restimulated with heat-killed BCG or media with IFN-γ producing cells determined via ELISpot. Data shows mean spots per million cells from 5-8 individual mice per experimental group. The experiment was repeated with similar results. Statistics were done via 2-way ANOVA followed by Bonferroni post-tests. *$p<0.05$, ***$p<0.001$.

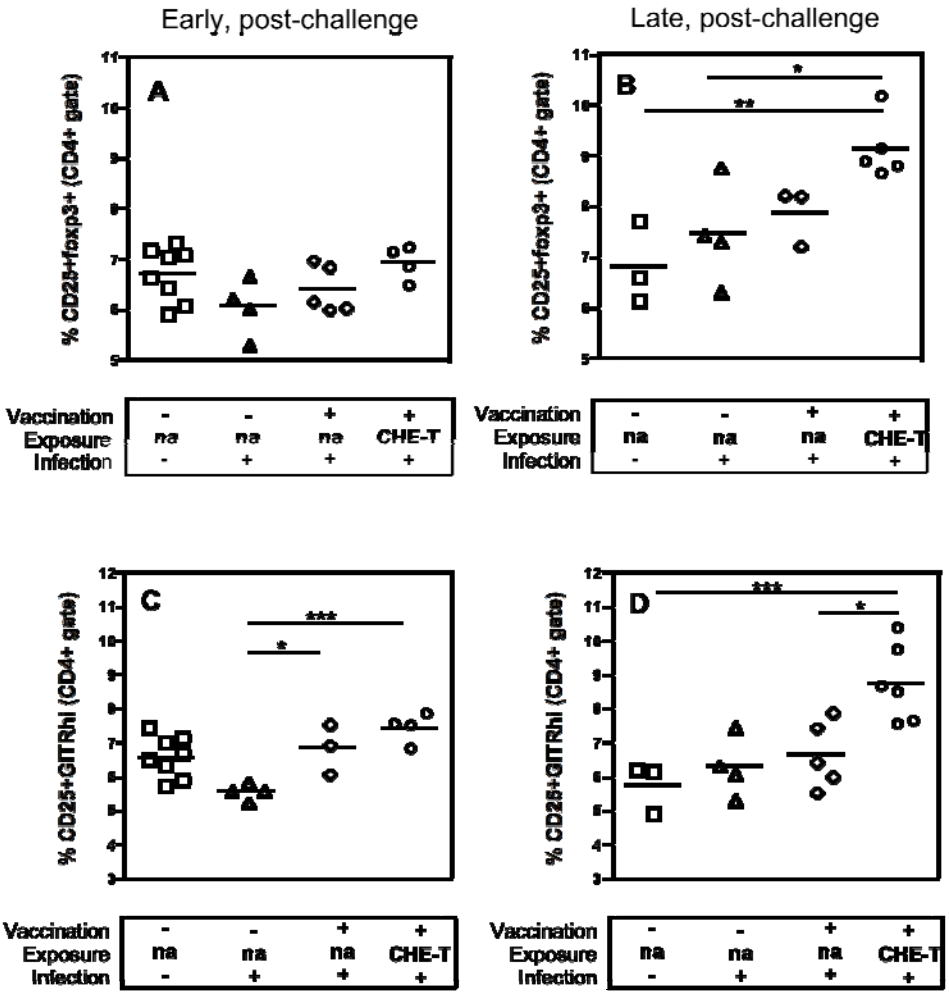

Fig. 6.3: Late CHE exposure increases proportion of systemic CD4⁺ Tregs post-BCG challenge

Proportion of splenic (A, B) $CD25^{+}FoxP3^{+}$ and (C, D) $CD25^{+}GITR^{hi}$ of $CD4^{+}$ cells in post-challenge mice treated according to Fig. 6.2A, with (A, C) early and (B, D) late exposure to CHE *p< 0.05, **p< 0.01, ***p< 0.001 by 1-way ANOVA with Bonferroni post-test. Data shows individual and mean % cells from 3-8 mice per experimental group. The experiment was repeated with similar results.

6.3.6 CHE exposure had no effect on $CD44^{hi}CD62L^{lo}$ memory cell populations

Next, the effect of CHE exposure on the splenic memory cell population, defined based on surface phenotype, was evaluated. These cells were not defined based on CHE- or BCG-specificity. Post-BCG challenge, $CD44^{hi}CD62L^{lo}$ cells in both $CD4^{+}$ and $CD8^{+}$ populations were similar between unexposed and early CHE exposed mice (Fig. 6.4A, 6.4C). Late CHE exposure also resulted in no significant difference in the same two groups (Fig. 6.4B, 6.4D). Frequencies of these memory cells were therefore unaffected by CHE exposure, despite the increased frequency of Tregs with late exposure. This implies that the Tregs do not suppress overall memory cell frequencies, but whether they suppress BCG-specific memory cell numbers was not studied.

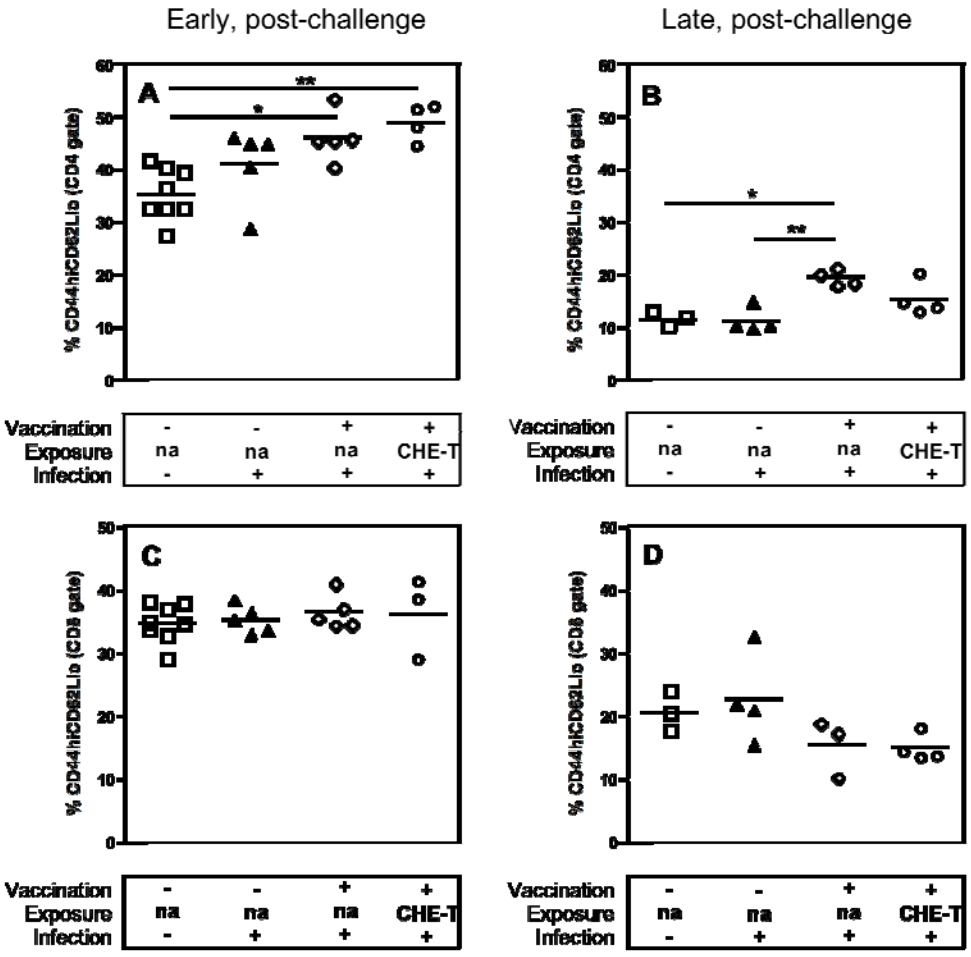

Fig. 6.4: CHE exposure had little effect on memory cell populations
Proportion memory cells of (A,B) $CD4^+$ or (C,D) $CD8^+$ splenocytes post-stimulation was determined by flow cytometry. Splenocytes from unvaccinated mice or mice at nine weeks (early, A,C) or 19 weeks (late, B,D) post-BCG vaccination (with or without CHE i.t. infection as shown in Fig. 6.1A), with or without subsequent BCG infection i.t. (as in Fig. 6.2A), were stimulated with PMA/ionomycin for 16-18h then harvested and analysed via flow cytometry. Statistics were done via 1-way ANOVA, then a Bonferroni post-test. *$p<0.05$, **$p<0.01$. Data shows individual and mean % cells from 3-8 mice per experimental group.

6.3.7 CHE exposure reduced lung inflammatory infiltration upon secondary BCG exposure

Lung infiltration of immune cells after BCG challenge was measured by flow cytometry to determine whether post-vaccination CHE exposure affects local responses to BCG lung challenge. At the early time-point, compared to untreated mice, BCG challenge of naïve mice did not increase any of the leucocyte populations measured (Fig. 6.5A). However, BCG-vaccinated mice had a significant increase in numbers of activated macrophages ($F4/80^+CD11b^+$) recruited to the lungs ($p<0.01$, Fig. 6.5A) following lung challenge, compared to naïve mice. With early CHE exposure, there was a much smaller increase in recruitment of activated macrophages to the lungs ($p<0.05$, Fig. 6.5A).

At the late time point, there was an even more marked post-challenge increase in lung populations of macrophages ($F4/80^+$), activated macrophages ($F4/80^+CD11b^+$) and T cells ($CD3^+$), but not neutrophils ($Gr1^+$), in BCG-vaccinated relative to naïve mice (Fig. 6.5B). This indicated that BCG priming enhanced inflammatory cell recruitment upon secondary exposure. With intervening CHE exposure, there was substantially reduced recruitment of macrophages, activated macrophages and T cells to the lungs ($p<0.01$, Fig. 6.5B), which clearly abrogated the effect of BCG vaccination on inflammatory cell recruitment. Thus, the impact of CHE exposure was more notable at the late than early time-point.

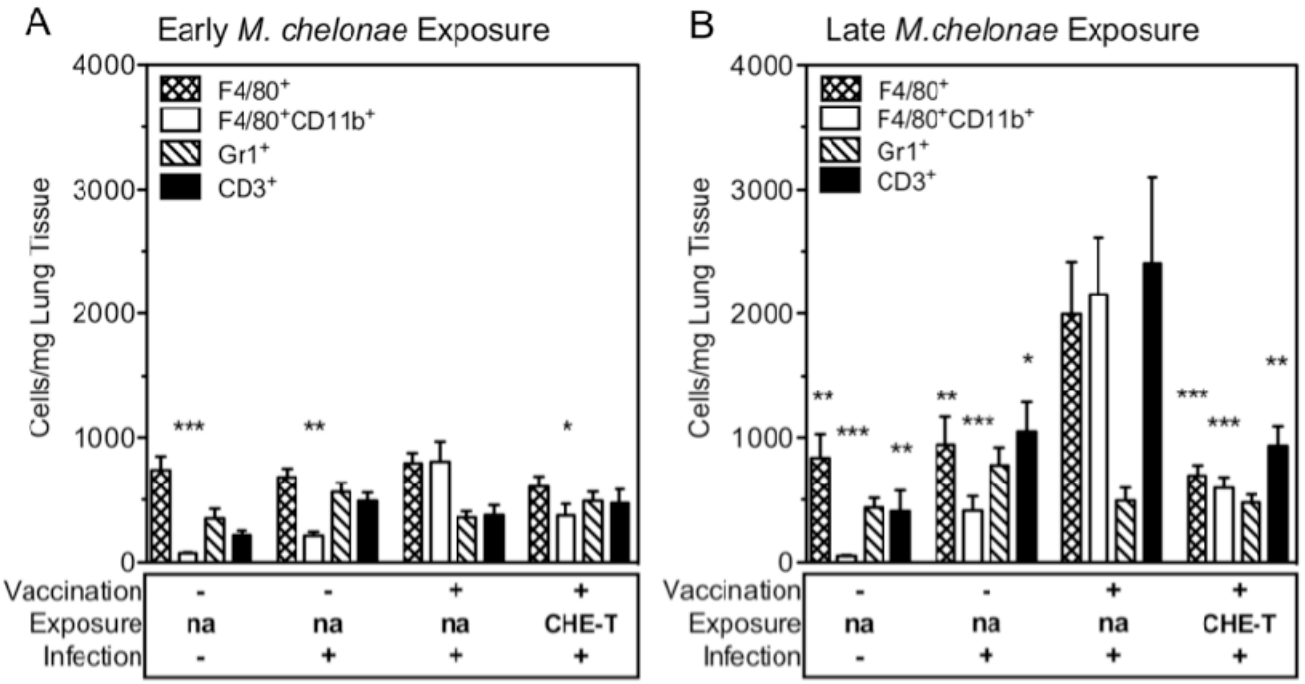

Fig. 6.5: CHE exposure decreases T cell and macrophage recruitment to the lungs upon lung challenge with BCG
Lung cells isolated three weeks post-BCG lung infection from mice with (A) early or (B) late CHE exposure according to Fig. 6.2A were analysed via flow cytometry. Absolute counts of inflammatory cells positive for Gr1 (neutrophils), CD3 (T cells), F4/80 (macrophages) and CD11b (together with F4/80 = activated macrophages) analysed by flow cytometry were normalised to volume analysed and tissue weight. Data shows mean number of cells/g tissue ± SEM from 6-12 individual mice. Statistics are indicated for all groups compared against mice given BCG-vaccination followed by BCG challenge. *p<0.05, **p< 0.01, and ***p<0.001 by one-way ANOVA with Bonferroni post-tests.

6.3.8 Reduced levels of lung cytokines and chemokines after BCG challenge in CHE exposed mice

The previous experiment demonstrated that CHE exposure late after vaccination reduced the numbers, and possibly also the activation status (proportion of $F4/80^{+}CD11b^{+}$ cells), of lung immune cells locally post-BCG challenge. To further investigate the factors responsible for this observation, various inflammatory mediators in lung tissue were measured at the late time point. Upon BCG lung challenge, BCG-vaccinated mice had significantly elevated levels of IL-4, IL-17, MIP-1α, MIP-1β, RANTES, IL-1β, and IL-12/23p40 in the lungs, compared with naive mice ($p<0.05$, Fig. 6.6). In vaccinated mice, CHE exposure between BCG priming and challenge significantly reduced the post-challenge levels of MIP-1α, MIP-1β, RANTES, IL-12/23p40 (Fig. 6.6). The other three cytokines (IL-4, IL-17, and IL-1β,) were also reduced, but did not reach statistical significance. Interestingly, post-challenge IL-10 levels were also decreased by CHE exposure ($p<0.05$, Fig. 6.6), indicating that the suppression of immune cell recruitment was not due to IL-10. Taken together, the heightened immune cell recruitment as well as lung tissue cytokine and chemokine levels upon BCG lung challenge suggest an enhanced local innate and adaptive immune response in BCG-vaccinated relative to naïve mice. CHE exposure late post-vaccination reduced post-challenge chemokine levels in the lungs, and this may underlie why CHE exposure substantially reduced local immune cell recruitment.

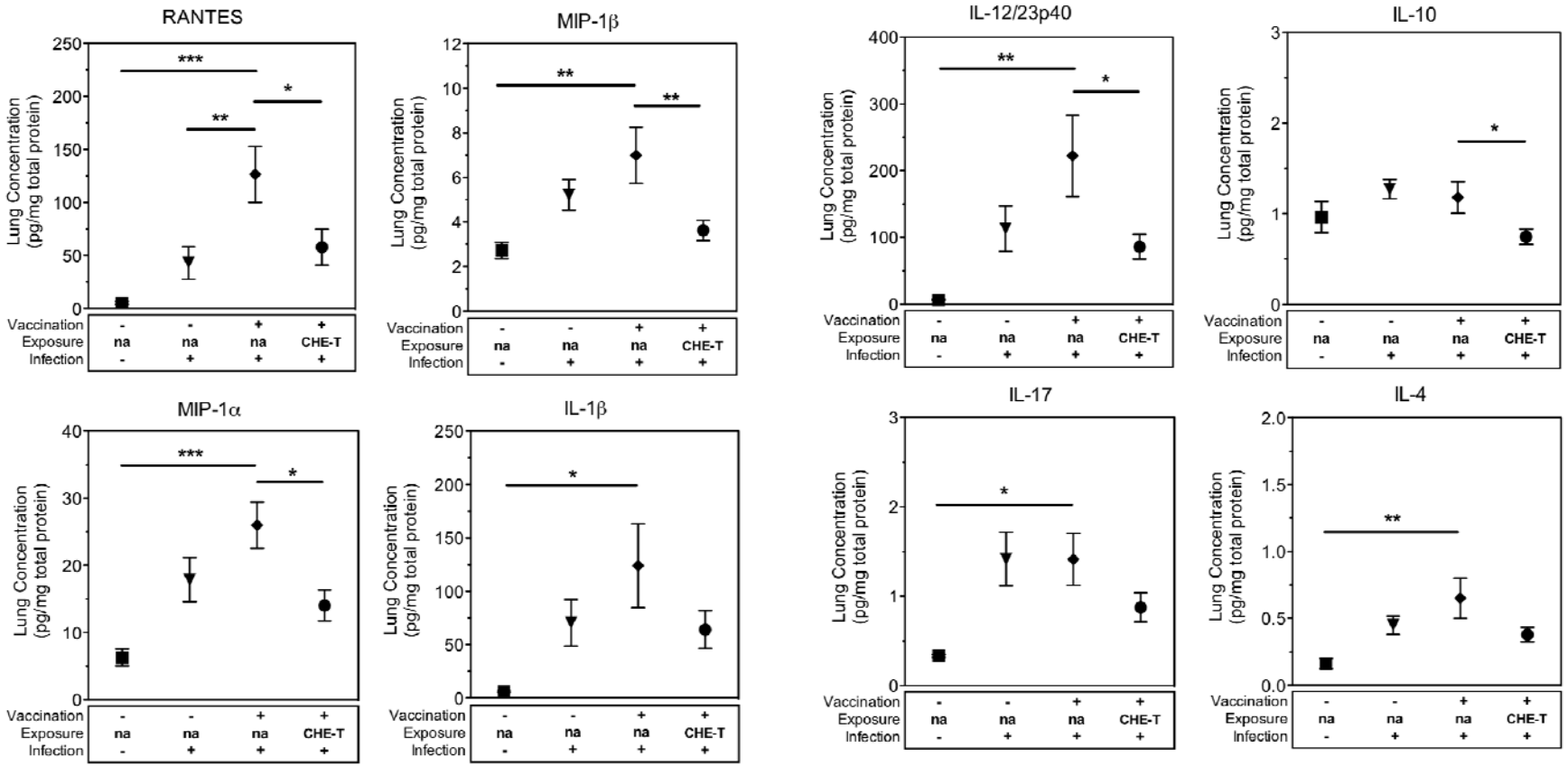

Fig. 6.6: Inflammatory mediators elevated in lung tissue upon BCG lung challenge are decreased by prior exposure to CHE
Cytokine and chemokine levels in lung tissue at three weeks post BCG lung infection, in mice given late CHE exposure according to Fig.

6.2A. Cytokine and chemokine concentrations were normalised to total protein in homogenised lung. Data shown as mean concentration per mg lung tissue ± SEM from 6-10 individual mice. * $p< 0.05$, ** $p< 0.01$, and ***$p<0.001$ by one-way ANOVA with Bonferroni post-tests.

6.4 DISCUSSION

6.4.1 Minimal effect of oral CHE

M. vaccae given by either intra-gastric or subcutaneous routes is equally able to suppress an existing inflammatory lung asthma response (Hunt, 2005) and oral *M. avium* reduces subsequent splenic IFN-γ responses to BCG (Young, 2007). The literature suggests that Treg induction should be favored in the gut (Coombes, 2007), but no systemic IFN-γ changes (Fig 6.1B, C) or upregulation of splenic nTregs (not shown) was noted when post-vaccination CHE exposure was given orally. The findings in this study concur with others showing that BCG vaccination followed by repeated oral *M. avium* by gavage does not alter Mtb antigen-specific IFN-γ upon subsequent Mtb aerosol challenge (Flaherty, 2006). Perhaps the strong systemic pro-inflammatory responses induced by BCG priming exceed a small tolerogenic effect of subsequent oral Env exposure. Different extents of Env persistence in host organs are also likely to affect extent of host immune stimulation (Brandt, 2002; Demangel, 2005). It has been suggested that sequestration of protective T cells to brachial lymph nodes or immune exhaustion due to persistent *M. avium* colonisation of the lungs (after oral gavage) may account for subsequent poor Mtb control (Flaherty, 2006). Since CHE is rapidly cleared from the lungs within two weeks even with high dose inhalation exposure (Chapter 5, Fig 5.1A), memory responses induced by CHE exposure, rather than immune exhaustion or sequestration due to on-going CHE infection, are more likely to underlie the post-CHE immune phenomena.

6.4.2 Cross-reactive boosting of systemic IFN-γ not suppressed by increased nTreg frequency in CHE exposed mice post-BCG vaccination

The *Mycobacterium* genus is quite conserved, so cross-reactive responses to the many antigens shared between CHE and BCG can be expected. Therefore, the BCG-specific IFN-γ response was predictably increased with CHE boosting of BCG-vaccination primed immunity (Fig 6.1B), and also with CHE priming just before BCG challenge (Fig 6.2C). Notably, the booster effect was diminished with elapse of time between priming and boosting (Fig 6.1C), which supports cross-reactive memory cells (rather than non-specific inflammation induced by CHE) as the basis for the enhanced BCG-specific IFN-γ response.

Less predictable is the level of Treg response induced by CHE, and whether this may specifically or non-specifically downregulate the BCG-specific IFN-γ response. The literature contains many examples of Treg activity following infection with Env (Bermudez and Champsi, 1993; Zuany-Amorim, 2002b; Hernandez-Pando, 2008; Mendoza-Coronel, 2010). There is evidence that existing inflammatory lung responses can be reduced non-specifically by Env-induced Tregs (Zuany-Amorim, 2002b). However, the role of Tregs present before BCG vaccination (presumed to be Env-induced) in suppressing BCG-induced IFN-γ levels is uncertain because $CD4^+CD25^+FoxP3^+$ Treg frequencies and IL-10 production before BCG vaccination do not correlate with IFN-γ levels post-vaccination in human infants (Burl, 2010). Likewise, post-BCG vaccination i.t. CHE exposure in this study led to increased Tregs upon subsequent BCG challenge, but this failed to suppress the BCG-induced IFN-γ response (Fig 6.2B,

C), although it was associated with suppressed BCG-induced inflammatory cell recruitment (Fig 6.5). The latter may yet be the critical outcome of CHE-induced Treg responses on Mtb challenge (discussed later).

6.4.3 Contrast with *M. vaccae* model of IL-10 mediated lung suppressive effects

Good evidence for suppression of existing inflammation by Env comes from an ovalbumin-induced murine allergy model. Oral or subcutaneous treatment with dead *M. vaccae* significantly reduces pulmonary inflammatory infiltrates, through increasing lung IL-10 production (Hunt, 2005). In this model, the *M. vaccae* induced Treg response is non-specific, affecting lung inflammation triggered by allergens distinct from *Mycobacterium* antigens. However, the suppression occurs primarily at the inflammed (lung) site – the effect on splenic IL-10 and IFN-γ is minimal. In this study, CHE clearly induces suppressive lung effects via different mechanisms from *M. vaccae*, as lung IL-10 levels were actually decreased in i.t. CHE exposed mice after BCG challenge (Fig. 6.6), so IL-10 cannot account for the suppression in inflammatory cell recruitment. The difference in the two models probably lies in that, unlike the asthma model, systemic (splenic) BCG-specific IFN-γ responses were strongly induced by cross-reactive T cells in CHE-exposed mice after BCG challenge.

6.4.4 nTreg expansion with CHE exposure after BCG vaccination explains suppressed lung inflammation

The systemic increase in $CD25^{+}GITR^{hi}$ cells (and $CD4^{+}CD25^{+}FoxP3^{+}$ cells to a lesser extent) amongst the $CD4^{+}$ lymphocytes (Fig 6.3B, D) suggests an increase of nTregs, rather than adaptive Tregs, when CHE was given post-BCG. Indeed, BCG vaccination alone induced $CD4^{+}CD25^{+}GITR^{hi}$ nTregs (Fig. 6.3C). Notably, with CHE sensitisation alone, the frequency of $CD4^{+}CD25^{+}FoxP3^{+}$ nTregs and IL-10 producing cells were unremarkable (Chapters 4 and 5). Therefore, it appears that nTregs are not primarily induced with CHE exposure, but rather, nTregs produced upon BCG priming are possibly expanded with subsequent CHE exposure.

Mtb-specific Th17 cells are crucial for inducing rapid recruitment of Th1 effector cells to the lungs of immunised mice (Khader, 2007) and Mtb-specific Tregs have the opposite effect of retarding the recruitment (Shafiani, 2010). Mtb infection does not cause non-specific proliferation of all $CD4^{+}FoxP3^{+}$ cells, but leads to Mtb-specific Tregs being preferentially expanded from a pre-existing repertoire of nTregs in the murine host (Shafiani, 2010). Such specific Tregs can restrict expansion of effector T cells whose epitope specificity differs from the epitope recognised by the Tregs. The authors suggest that this may be due to Mtb-specific Tregs recognising particular immunodominant antigens presented on DCs, leading to interactions which impair the DCs' co-stimulatory functions (Shafiani, 2010). By extension, this mechanism could also account for how BCG-primed Tregs could be re-activated and expanded by cross-reactive antigens upon subsequent CHE exposures. Higher frequencies of expanded

Mycobacterium-specific Tregs in the lungs, following repeated lung CHE exposures, would explain the poorer inflammatory cell recruitment to subsequent lung infection by BCG (or Mtb), as noted in this study (Fig 6.5). There is increasing support for the notion that the rate at which effector T cells reach the lung, rather than the level of systemic *Mycobacterium*-specific IFN-γ response, is the critical feature of protective immunity in Mtb infection (Cooper, 2009). Likewise, higher levels of systemic BCG-specific cells in CHE exposed mice may not prevent the undermining of lymphocyte recruitment to the BCG-challenged lungs caused by the CHE-expanded Tregs. This explains the abrogation of BCG vaccine-induced lymphocyte accumulation in the lungs with CHE exposure after challenge (Fig 6.5), in spite of higher systemic BCG-specific IFN-γ (Fig 6.2C) and reduced lung IL-10 levels (Fig 6.6).

6.4.5 Differential Treg responses primed with pre- versus post-BCG vaccination CHE exposure

The fact that BCG vaccination alone increases nTregs (but not IL-10) in infants after neonatal vaccination (Burl, 2010) may explain why a BCG-prime-CHE-boost model in this study led to expanded nTregs upon BCG challenge, rather than increased IL-10 production. Conversely, when CHE sensitisation occurred before BCG challenge, IL-10 was increased (Chapter 4, Fig 4.2B and Chapter 5, Fig 5.7) but the increase in frequency of $CD4^+CD25^+$ nTregs was relatively modest (Chapter 4, Fig 4.3A and Chapter 5, Fig 5.8), consistent with a predominantly adaptive Treg response. This may be because BCG vaccination provides a stronger initial priming stimulus for Th1 mediated inflammation and

concomitant expansion of *Mycobacterium*-specific nTregs, that is boosted by subsequent repeated CHE exposure. In contrast, CHE priming – being a commonly encountered Env tolerated by the immune system – leads to less inflammation and less nTreg expansion, but repeated low dose antigen exposure can induce peripheral conversion of $CD4^+CD25^-$ effector cells to $FoxP3^+$ Tregs (Kretschmer, 2005). This phenomenon was illustrated in Chapter 4, Fig 4.4E with CHE-sensitised mice, and $CD4^+CD25^-FoxP3^+$ cells were the predominant IL-10 producing adaptive Tregs after BCG vaccination of CHE-sensitised mice (Chapter 5). The impact of $CD4^+CD25^+$ nTregs appeared to be in suppressing leucocyte recruitment to the *Mycobacterium* infected lung (shown in Chapter 4, Fig 4.5B and this study, Fig 6.5B), possibly related to local induction of IL-10 production by lung DCs (Chapter 4, Fig 4.5A), whereas increased systemic IL-10 production (Chapter 5, Fig 5.7) by adaptive Tregs is associated with suppressed BCG-specific systemic IFN-γ memory cell responses (Chapter 5, Fig 5.5).

6.4.6 Conclusion

This study shows that BCG vaccine induced immunity may be compromised via several mechanisms by CHE exposure, even when the exposure occurs after vaccination. In humans, Env exposure is inevitable throughout life (Vaerewijck, 2005), and cumulative Env exposure could form part of the explanation for the waning of BCG-induced protection (Comstock, 1976; Sterne, 1998). The findings from this study also explain findings in human infants that the levels of peripheral blood cytokines post-BCG vaccination do not correlate with two-year risk of TB infection (Kagina, 2010), since the impact of Env exposure after BCG

vaccination may be primarily through reducing local lung inflammatory cell recruitment early in TB infection, rather than through suppressing systemic BCG-specific cytokine or memory responses. It could be inferred from Shafiani's work (Shafiani, 2010) that an assay of *Mycobacterium*-specific nTregs that can be expanded upon restimulation with Mtb antigens, rather than peripheral blood IFN-γ, may be a more useful predictor of TB risk in BCG-vaccinated persons.

CHAPTER 7 – CONCLUSION AND FUTURE WORK

7.1 Key findings and their implications

The failure of BCG as a vaccine for TB is a matter of global concern and intense research effort because new vaccine development is hampered by an incomplete understanding of why BCG fails. The present work is significant in that reasons discovered for the immunomodulatory effects of CHE on BCG can be tested in new vaccines to check if similar pitfalls exist. Two novel mechanisms were described here. First, CHE sensitisation induced $CD4^+$ mediated cytotoxicity against BCG infected cells that limited the replication of BCG, and was dependent on IFN-γ, perforin and FasL. Second, CHE sensitisation induced various types of Tregs upon BCG vaccination, including IL-10 producing iTregs and $CD4^+CD25^+$ nTregs, that suppressed systemic BCG-specific memory responses and local immune cell recruitment in response to BCG. This work is the first to propose the existence of these two mechanisms, and to demonstrate how they explain the effects of Env exposure on BCG responses.

An important finding was that $CD4^+CD25^+$ nTregs from CHE-sensitised mice, though unremarkable in frequency, proved to be responsible for reducing early lung lymphocyte recruitment to local *Mycobacterium* infection, which has been described as the critical factor determining outcome of Mtb infections (Cooper, 2009). CHE exposure after BCG vaccination likewise led to expansion of nTregs with similar suppression of cellular recruitment without systemic IFN-γ suppression. This study is also the first to show a mechanism that explains how

Env exposures can modulate an existing BCG vaccine response, which has relevance to neonatal vaccination programmes. Adaptive (inducible) Tregs producing IL-10 were not induced when BCG vaccination occurred before CHE exposure, but CHE sensitisation before vaccination led to increases in both systemic IL-10 and nTregs. IL-10 was a main cause of diminished BCG-specific systemic memory cell IFN-γ production in mice given CHE priming before BCG, and was mainly produced by $CD4^{+}CD25^{-}FoxP3^{+}$ iTregs, rather than Tr1 cells. This distinct activity of iTregs and nTregs, and the fact that CHE exposure occurring before or after BCG vaccination causes differential induction of such Tregs, may explain the failure of human studies to draw a link between Env-induced Tregs and suppression of BCG-induced immunity post-vaccination (Burl, 2010; Kagina, 2010). It also means that post-BCG assays of *Mycobacterium*-specific nTregs and IL-10 producing iTregs may be useful indicators of vaccine-induced protective outcomes, rather than the current tests of IFN-γ producing cells.

Both live and dead CHE gave similar responses, but memory responses after transient CHE lung colonisation led to a build-up of inflammatory and regulatory T cells for weeks. Indeed, expansion of memory cells (nTregs, cytotoxic T cells and BCG-specific IFN-γ producing cells) occurs with both CHE-prime-BCG boost and BCG-prime-CHE-boost models, but to different extents. The priming influence seemed to bias the subsequent response. For example, BCG priming mainly induced nTregs and IFN-γ with minimal IL-10 induction and this pattern of responses predominated after CHE boosting. On the other hand, repeated CHE priming alone increased IL-10 and reduced IFN-γ with relatively smaller effects

on nTregs, and correspondingly with subsequent BCG vaccination, there was an expansion of these effects.

The findings in this study contribute to the understanding of why BCG has failed as a vaccine, particularly in regions where Env exposure is prevalent. The results suggest that future vaccines that revolve around the use of recombinant BCG may likewise be affected by host Env exposure. It would therefore be prudent to include testing for effects of Env exposure during the testing of vaccine efficacy when developing such vaccines. Where cytotoxic or regulatory responses by Env negatively affect the vaccine's protective capability, strategies to block such responses in conjunction with the administration of the vaccine has to be considered. This work has additionally uncovered a novel reason for why even neonatal BCG vaccination fails to provide long-lasting effects against adult pulmonary TB, which has implications for the continued use of BCG in infants and in adolescents.

7.2 Limitations & future work

7.2.1 Exploring antigen specificity of Tregs

Although functional suppressive cells, which could inhibit BCG-induced responses, were observed in CHE-sensitised mice, this study did not ascertain the antigen specificity of these cells. It remains to be determined whether the suppressive effects observed in this model resulted from bystander suppression, whereby active Tregs exerted their effects on surrounding cells regardless of

antigen specificity, or from antigen-specific Tregs recognising antigens shared between CHE and BCG. One possible way to investigate this is to use RAG gene knock-out mice, which are devoid of functional T and B cells, a model used effectively in previous Treg studies (Kursar, 2007). These mice can be reconstituted with various phenotypes of Tregs from CHE mice and functional T helper cells from BCG-vaccinated mice, to determine if antigen specificity is an important factor in mediating the suppression.

7.2.2 Effects of IL-10 – direct role in suppression?

In CHE-exposed mice vaccinated with BCG, IL-10 secreting $CD4^+$ cells were elevated in frequency. Others in our laboratory have ascertained that removal of these cells *in vivo* reverses the suppression of BCG induced IFN-γ responses (data not shown). Both murine (Kursar, 2007) and human (Burl, 2010; Marin, 2010) studies suggest that IL-10 is not correlated with Treg-mediated suppression of BCG or TB responses. However, most of those studies were primarily looking at $CD4^+CD25^+$ Tregs, rather than iTregs that are more likely to produce IL-10. An investigation of the effects on BCG responses upon adoptive transfer of the IL-10 secreting iTregs described in CHE-sensitised mice in this study, into BCG-vaccinated mice would confirm their ability to suppress BCG induced systemic IFN-γ responses. The differential effects of CHE-induced nTregs and iTregs can also be tested in this experimental model.

7.2.3 Effects on Th17 and polyfunctional Th1 cells

Polyfunctional *Mycobacterium*-specific Th1 cells that secrete multiple cytokines (Forbes, 2008), as well as Th17 cells that produce IL-23 and IL-17 (Khader, 2007; Chen, 2010), have been suggested to be important for protection against TB infection. It would therefore be interesting to study whether Env-sensitised mice are altered in their production of such cells. Furthermore, Marin *et al* show that *in vitro* Treg depletion before antigen stimulation of cells from active and latent TB patients increases the frequency of IFN-γ secreting but not IL-17 secreting cells (Marin, 2010). Hence, future studies should investigate whether Env-induced Tregs found in this study influences BCG-mediated responses in a similar way – affecting the protective Th1 responses but not the IL-17 responses. It would also be interesting to explore whether these changes to Th1 and Th17 responses are associated with BCG-attributable protection against Mtb challenge.

7.2.4 Suppressive effects of other Env species

Since only one species of Env (CHE) was studied here, the findings may only represent a proportion of the Env interference on human immunity. The addition of other Env species may compound the influences of CHE on BCG-mediated responses, and this should be explored. Brandt demonstrated that in mice, prior to BCG vaccination, exposure to a combination of *M. avium*, *M. fortuitum* and *M. vaccae* interferes with BCG efficacy (Brandt, 2002). However, Demangel found only marginal interference by *M. avium*, whereas *M. fortuitum* and *M. vaccae* did not affect BCG protection when each species was given individually before BCG

vaccination in mice (Demangel, 2005). Demangel opined that slow growing mycobacteria (closer genetic relations to BCG) may be more likely to affect BCG responses, but only studied a limited spectrum of cellular responses to BCG. Further studies should explore whether the effects of CHE exposure on cytotoxicity and Tregs that were uncovered in the present work are similarly noted with *M. avium* sensitised mice, since some of these effects may not be related to antigen specificity. It is possible that different Env species may affect BCG responses via different mechanisms.

7.2.5 Improving the research model

A major limitation of the present work is that the CHE-induced suppressive effects documented were not directly verified through Mtb challenge. This was not possible in the time-span of this work due to unavailability of appropriate containment facilities. Now that various mechanistic leads have been established, removing/neutralising the suppressive pathways from CHE-sensitised mice and/or adoptive transfer of the relevant suppressive cells, followed by Mtb challenge would be a more rigorous model for testing the relevance of these mechanisms. For example, antibody-mediated depletion of $CD4^+CD25^+$ cells (Jaron, 2008) or IL-10 secreting cells from CHE-sensitised mice before BCG vaccination and Mtb challenge would enable confirmation if these cells suppress the protective effects of BCG. Likewise, depletion of $CD4^+$ cytotoxic cells from CHE-sensitised mice should abrogate the suppressive effects on BCG growth, and we can then confirm how this affects the response to TB.

7.2.6 Immune correlates of BCG protection

This study provided evidence for immunomodulatory effects of CHE exposure on BCG-mediated responses. However, the relevance of our observations depends on having studied the appropriate BCG-induced responses that correlate with BCG-attributable protection against TB. The concern is that Env exposure may affect many aspects of BCG-induced cellular immunity, but these may not directly affect BCG-induced protection against Mtb. For a long time, vaccine-induced IFN-γ responses were accepted as the main surrogate marker for protection in vaccine studies (Flynn, 2004; Scriba, 2010), but currently this is in some doubt (Burl, 2010; Kagina, 2010). This underscores the problem that factors clearly required for protection against TB may not be immune correlates of protection for TB vaccines. The poor understanding of immune correlates of BCG protection has been a big obstacle in developing new vaccines, and is the subject of intense global research efforts. A recent large-scale neonatal BCG study exploring immune outcomes of BCG vaccination, in relation to pre-BCG vaccination immune parameters, such as T cell frequency and cytokine expression, found that none of these were correlated with risk of TB infection (Kagina, 2010). Therefore, considerations on future work regarding what effects Env have on BCG responses must be taken together with knowing what BCG-induced parameters, if suppressed, have the greatest impact on BCG-conferred protection. This means that we must join in the global effect to uncover the elusive immune correlates of BCG protection or BCG failure. Based on the findings in this study, it would be reasonable to start with a prospective study in human infants given neonatal BCG, testing whether post-vaccination assays of

Mycobacterium-specific nTregs and IL-10 producing iTregs are correlated with two-year risk of TB infection in a high prevalence region.

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APPENDIX

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 mm 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.