

Evaluation of the Immunological Mechanisms Induced by Mycobacteria and the Potential Effect this may have on Immunity Induced by Tuberculosis Vaccines

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EVALUATION OF THE IMMUNOLOGICAL MECHANISMS INDUCED BY MYCOBACTERIA AND THE POTENTIAL EFFECT THIS MAY HAVE ON IMMUNITY INDUCED BY TUBERCULOSIS VACCINES

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The efficacy of Bacille-Calmette Guerin (BCG) vaccination in protection against pulmonary tuberculosis (TB) is highly variable between populations. One possible explanation is increased exposure of certain populations to non-tuberculous mycobacteria (NTM). Given the variable efficacy of BCG an improved vaccine against TB is required. The novel TB vaccine MVA85A has shown promising results, however, the immunogenicity of the vaccine is reduced when it is administered in the Expanded Programme on Immunisation (EPI) schedule. This thesis aims to explore: (A) the effect of exposure to NTM on the level of protection afforded by BCG vaccination against *Mycobacterium tuberculosis* (*M. tb*) and (B) the immunological mechanisms behind EPI interference with MVA85A.

The effect of *M. avium* (MA) exposure via systemic and oral routes on the efficacy of BCG was tested using *M. tb* aerosol infection in a mouse model. The adaptive immune response was profiled in BCG vaccinated mice with and without exposure to MA pre- and post- *M. tb* infection. The results showed BCG efficacy could be enhanced by exposure to dead MA by a systemic route; T helper 1 and T helper 17 responses were associated with increased protection. In contrast, BCG efficacy may have been reduced by exposure to live MA by the oral route; T helper 2 and regulatory T cells were associated with reduced protection.

To answer the second aim MVA85A was co-administered to mice with aluminium adjuvants or aluminium-containing vaccines to replicate the effect of co-administration in the EPI schedule; the adaptive immune response was profiled. T helper 2 and regulatory T cell responses induced by aluminium-containing vaccines were associated with a reduction in the immunogenicity of MVA85A.

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Abbreviations

Ag 85A - Antigen 85A

APC - Antigen presenting cell

BCG - Bacille-Calmette Geurin

CFU - Colony forming units

cLN - Cervical lymph node

DC - Dendritic cell

EPI - Expanded Programme on Immunisation

ID - Intradermal

IFNγ - Interferon gamma

iLN - Inguinal lymph node

IM - Intramuscular

IN - Intranasal

IP - Intraperitoneal

KO - Knockout mice

LAM - Lipoarabinomannan

M. tb - *Mycobacterium tuberculosis*

MA - *M. avium*

ManLAM - Mannose capped LAM

MDR - Multidrug resistant

mLN - Mesenteric lymph node

mLN/pLN Mesenteric/pulmonary lymph node

NO - Nitric oxide

NTM - Non-tuberculous mycobacteria

PFU - Plaque forming units

PPD - Purified protein derivative

SC - Subcutaneous

TB - Tuberculosis

TCM - Central memory T cell

TEff - Terminally differentiated effector

TEM - Effector memory T cell

TGFβ - Transforming growth factor beta

TNFα - Tumour necrosis factor alpha

UAC - Uric acid crystals

1 Introduction

1.1 TB disease

1.1.1 Overview

Tuberculosis disease (TB) is caused by the bacterium *Mycobacterium tuberculosis* (*M. tb*). TB has affected the human population for thousands of years and remains a major global health problem today. In 2010 it was estimated that 12 million people worldwide were suffering with TB disease and a further 8.8 million more are infected each year[3]. The number of people living with latent *M. tb* may be nearer 2 billion[4]. The incidence is greatest in Asia (59% of TB cases in 2010) and Africa (26%) but is also a concern for the rest of the world, especially with the emergence of multi-drug resistant (MDR) strains of *M. tb*.

In high incidence areas disease affects mainly young adults in the form of pulmonary TB[5]. In low incidence areas disease mainly affects the elderly, immune-compromised and immigrant populations. The probability of developing progressive disease on primary infection is 10% and this form of disease mainly affects children and the immune-compromised. Ninety per cent of those who do not develop progressive disease on initial infection mount an immune response that contains the bacteria within a granuloma. This immune response does not clear the bacteria, instead bacteria exist in the granuloma in a state of latency; the chance of latent disease reactivation in the lifetime of a healthy patient is between 5 and 10%. HIV co-infected individuals have a higher risk of active disease; the probability of developing disease is 5 – 15% for every year of infection or 50% over a lifetime. TB is the leading cause of HIV/AIDS related deaths (26%), 99% of these deaths occur in developing countries which puts a massive strain on already stretched medical infrastructures[6].

Effective diagnosis and provision of standardised treatment are two of the key components in the Stop TB strategy; a list of actions to achieve the World Health Organisation's (WHO) global target for reduction of TB disease burden by 2015[3]. When a patient is diagnosed with active TB the strategy recommends they should be entered on to the national TB control program for antibiotic therapy. Methods of detection and treatment programs vary widely dependent on the country; this is reviewed more extensively later.

1.1.2 Pathogenesis of *M. tb* Infection

TB is an infectious disease which is spread by inhalation of sputum droplets from an infected person formed when they cough, sneeze or speak. On inhalation of infected droplets the bacteria are delivered to the alveolar space of the lung and are most commonly phagocytosed by alveolar macrophages[7]. They may also interact with dendritic cells (DC) and epithelial cells. Infection results in stimulation of the innate and adaptive immune system. The protective response is centred on the activation of infected macrophages and DCs by CD4+ T cells via interferon gamma (IFNγ) signalling which leads to activation of the infected cell and killing of the bacteria[4]. Many other immune mechanisms play an important role in controlling infection which is discussed later.

Some people are capable of mounting an innate response that clears infection; evidenced by studies in house-hold TB contacts who do not develop disease and have no reaction to the tuberculin skin test (TST)[4]. Others may clear infection with an adaptive response but it is not possible to determine if the infection is cleared or lying in latency state as their TST test is positive in both circumstances.

If bacteria are not cleared the adaptive immune response acts to contain the infection by formation of a granuloma[4]. The granuloma structure prevents spread of bacteria by

surrounding them with giant multi-nuclear macrophages, T cells and B cells. This structure also benefits the bacteria by protecting them from immune attack. Bacteria enter a state of latency and remain dormant until a time when immune control is compromised, for example by HIV co-infection.

If bacteria are not controlled on primary infection or they re-activate from latent state and the immune system cannot control them they can replicate extracellularly and cause pathology in the pulmonary tissue, this is termed as active pulmonary TB disease. Inflammation in the lung damages tissue structure and leads to bacteria entering the airways where they can be transmitted in aerosolized droplets[8].

M. tb can also cause miliary disease if endothelial and lymphatic vessels within the lung are disrupted. *M. tb* can infect nearly any organ including spleen, heart and liver and can result in organ malfunction and ultimately death if untreated. These disseminated forms of disease mostly affect children and immune-compromised patients. Treatment of TB meningitis with standard antibiotic regimens is effective for drug susceptible strains.

1.1.3 Contributing Factors to Disease Outcome

Co-infection with *M.tb* and HIV exacerbates the outcome of both diseases. Depletion of CD4+ T cells is the main characteristic of HIV/AIDS and leaves the patient more susceptible to progressive TB disease after primary *M. tb* infection and to reactivation of latent infection as this cell type is essential for the control of TB[9].

Other host and environmental factors can increase the risk of infection or progression of TB disease. For example smoking is significantly associated with *M. tb* infection and development of pulmonary disease as well as increased mortality from disease and increased risk of re-

infection[10]. Diabetes mellitus is linked to increased susceptibility to active TB disease which is a worrying co-epidemic given the high prevalence of diabetes mellitus currently and forecasted increases in global prevalence[11].

Socio-economic determinants are some of the most highly associated risk factors for developing TB disease. Low economic areas are often places which are densely populated with a high burden of disease within the community; this leads to increased exposure and higher transmission rates[12]. Malnutrition compromises the cellular immune response and can increase the chance of developing active TB disease[13].

1.1.4 Global Strategy for Disease Control

TB disease in the industrialised world was controlled at a remarkable rate from the beginning of the 20th Century until the 1980's; for example mortality rates in the Netherlands dropped from 2,000 in every 100,000 in 1901 to less than 10 in every 100,000 in 1983 (Figure 1-1 A)[14].

The initial increase in disease control was due to the identification of *M. tb* bacilli as the causative agent of TB by Koch in 1882. This led to the isolation of infected patients and reduced transmission rates[14]. Improved living conditions and nutrition in the early part of the 20th century also played a large part in initial control. In 1921 the Bacillus Calmette-Guerin (BCG) vaccine was developed and subsequently grew in usage across the world; in 1974 it was included in the Expanded Program on Immunisation (EPI) schedule. The first efficacious antibiotic therapies for TB were used in 1948. The combination short course-therapy that is widely used today was developed in the 1980's. Mass vaccination programs and antibiotic treatment were successful in industrialised nations but there was not sufficient infrastructure to implement these methods in developing countries. Also the efficacy of BCG is highly

variable and is at its lowest in many developing countries[15]. These factors led to a large disparity in disease burden across the industrialised and developing world.

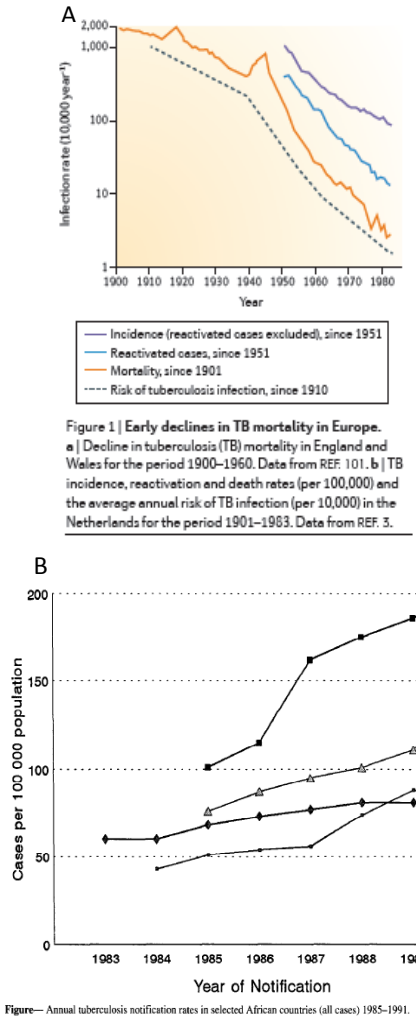


Figure 1-1: (A) Taken from a review by Lienhardt et al 2012. Graph shows incidence, reactivation, mortality and annual risk of TB in the Netherlands from 1901 to 1983. (B) From a review by Narain et al 1992[1]. Graph shows the number of cases in selected African countries from 1983 to 1990.

In 1991 the WHO set up the directly observed therapy, short-course (DOTS) strategy with 5 key elements which were hoped would lead to the global control of TB disease; government commitment to TB control, case detection by sputum smear testing, short-course antibiotic treatment for smear-positive patients, ensuring a regular supply of anti-tuberculosis drugs and reliable monitoring and recording of TB disease[14].

During the 1990's it became apparent that the HIV/ AIDs epidemic was associated with an increase in the number of tuberculosis cases in co-infected individuals (Figure 1-1 B)[1].

Between 1997 and 2001 the global incidence rate of tuberculosis increased solely due to the burden of HIV - *M. tb* co-infection in Africa[3]. Around the same time MDR strains of *M. tb* began to emerge.

On a global scale the DOTS strategy was failing to deliver the level of control expected due to these emerging risk factors. The Stop TB Strategy was devised in 2006; it was based on expansion and enhancement of the DOTS strategy and deals with HIV co-infection and MDR-TB[3]. The WHO's Global Tuberculosis Control report published in 2011 included data from 198 countries; this covered an estimated 99% of the world's TB cases. The data showed that incidence, prevalence and mortality rates are falling; however at present the goal of reducing prevalence by 50% on the value in 1990 will not be achieved by the target date of 2015. The trend of prevalence rates estimates this goal could be achieved in all regions apart from Africa.

1.1.5 Diagnosis

Accurate and fast diagnosis of active TB is key to the control of disease transmission. The tests used in industrialised nations include radiography, rapid liquid culture detection and gene amplification in accompaniment to traditional microscopy of patient samples[16]. In low-income countries there is neither the funding nor laboratory infrastructure to extend these advanced diagnostic tests to the entire population. The majority of suspected TB cases in endemic countries are diagnosed using light microscopy of sputum samples. This is an insensitive test and cannot diagnose drug-resistance.

A better diagnostic test is required for all patients in all countries. There have been recent advances in diagnostic test development including the IFNγ release assay (IGRA) which can distinguish *M. tb* infection. It is not confounded by BCG vaccination or exposure to the

majority of non-tuberculous mycobacteria as it detects IFNγ production in response to early secretory antigenic target – 6 (ESAT-6) and culture filtrate protein-10 (CFP-10)[16].

One of the most successful areas of development has been in gene amplification tests. The GeneXpert MTB/Rif assay from Cepheid detects *M. tb* and the most common determinant of Rifampicin resistance[17]. It is highly sensitive and also efficacious in immune-compromised patients and children, however it only detects one mechanism of resistance. Both of these tests require laboratory handling, are not straight-forward to perform or affordable so the implementation in resource-poor settings is still limited.

1.1.6 Antibiotic Therapy

The standard treatment regime for TB disease is the combined short-course therapy which is prescribed for pulmonary and disseminated TB. It is a combination of ethambutol, isoniazid, pyrazinamide and rifampicin administered for 2 months then a continuation phase of isoniazid and rifampicin for a minimum of 4 further months[18]. All patients are recommended to be directly observed taking the drugs to ensure compliance.

The obvious downfall of antibiotic therapy is the emergence of drug resistance. In 2010 it was estimated that of the 12 million new cases of TB disease, 650 000 of them were caused by MDR strains of *M.tb*[3]. MDR are resistant to at least isoniazid and rifampicin[19]. Second line drugs are available, such as kanamycin and fluoroquinolones, but these drugs have their problems as they may need to be injected, administered for long periods of time, have a high level of toxicity or are extremely expensive[20]. There are now reports of extensively drug resistant (XDR) and totally drug resistant strains[19]. There is a re-generated interest in research into new anti-tuberculosis drugs.

The emergence of drug resistant strains of *M. tb* has impeded the control of disease through drug regimens. It is more than likely that resistance will occur against new-line drugs. The combination of diagnostics, successful therapy and reducing transmission are effective at controlling disease but this could be achieved much faster with vaccination against primary disease and reactivation disease.

1.1.7 Vaccination

Vaccination has been practiced since the 1600s but was scientifically proven by Edward Jenner in the late 1700s[21]. Jenner performed the first vaccination with cowpox, a virus related to the human pathogen smallpox and demonstrated this process could prevent contracting the lethal disease. The success of his vaccine and the process of vaccination was proven in 1981 when smallpox was eradicated from the globe. Since the advent of vaccination, the development of vaccines against major diseases has provided the world with protection from diphtheria, tetanus, rabies, influenza, measles, mumps, rubella and many more diseases.

The only licensed vaccine against TB, BCG, is recommended for use in endemic populations and high-risk individuals[22]. The successes and limitations of BCG vaccination and the development of novel vaccine candidates are discussed later in this chapter.

1.2 Mycobacteria

1.2.1 Physical Characteristics of Mycobacteria

The mycobacterial cell wall is a complex structure which is distinct to the species (Figure 1-2). The cytoplasmic membrane of the bacteria is surrounded by a layer of peptidoglycan which is attached to arabinogalactan and forms the main backbone of the cell wall[23]. Mycolic acids, which are a defining constituent of the wall, exist as tetramers bound to the arabinogalactin

and as free molecules which are called cord factor. It is these acids that give the mycobacteria their hydrophobicity and acid-fast staining phenotype. Inserted into the wall by a phosphatidylinositol anchor is lipoarabinomannan (LAM) which is one of the immunodominant molecules of the mycobacteria. In pathogenic bacteria the LAM is capped by mannose (Man-LAM); in fast growing species, which are not pathogenic, the LAM is capped by inositol phosphate. The wall is further surrounded by a capsule of glycolipids which are inserted in the mycolic acid. These molecules differ with the species of the mycobacteria.

Due to this thick membrane nutrient acquisition is restricted which is what gives mycobacteria their small size in relation to other bacteria and their phenotypically slow growth. It is also what makes them resistant to many antibiotics; further knowledge of the structure and synthesis of the cell wall will provide new drug targets.

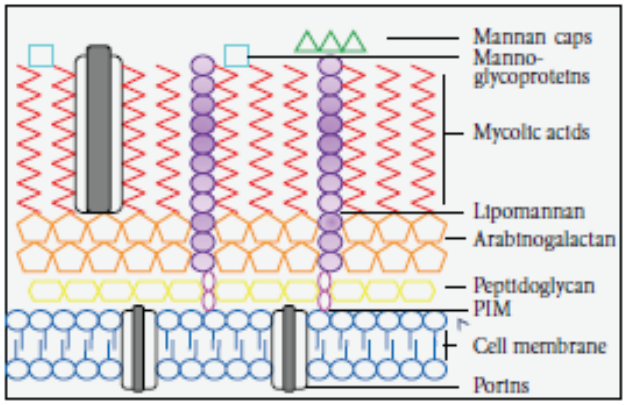


Figure 1-2: Diagram depicting the key components of the mycobacterial cell wall, taken from Kleinnijenhuis J et al[2].

1.2.2 Phylogeny of Mycobacteria

The species of the genus mycobacteria were historically defined by physical observations including fast or slow growth and pigmentation of colonies[24]. From the 1990s molecular tools were implicated in taxonomy; the most commonly used is the evaluation of the nucleotide sequence encoding 16S ribosomal RNA. The 65kDa heat-shock protein and the nucleotide

sequence between the 16S rDNA and 23S rDNA sequence may also be used in species determination.

1.2.3 The *Mycobacterium tuberculosis* Complex and Non-Tuberculous Mycobacteria

The *M.tb* complex (MTBC) is a group of mycobacterial species which are responsible for the cause of TB in humans and animals; they share a 99.9% identical genome sequence and identical 16S rRNA sequence[25]. They are *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canetti*. *M. tb* is the main cause of human TB and can also infect other animals. *M. africanum* and *M. canetti* can also cause TB in humans. *M. bovis* mainly causes disease in domestic and wild animals but can cause TB in humans. *M. microti* mainly infects small rodents.

The use of the term tuberculous does not describe the ability to cause tubercles but instead relates to the way the bacteria exist and certain phenotypes[24]. *M. tb* is not found free in the environment; it is contagious and is passed from host to host. Non-tuberculous mycobacteria (NTM) exist free in the environment and transmission from host to host has very rarely been reported. Many are not pathogenic at all and those which are, are opportunistic.

1.2.4 Pathogenic Strains of MTBC

Evolution of the MTBC species originated from a common ancestor which was most likely a human pathogen[25]. Despite the genomes of the MTBC being highly conserved, there are differences between the species and between strains of a species. *M. tb* strain differences can be identified in areas of the genome with high DNA sequence repetition; an example is insertion element IS6110 which is widely used to distinguish clinical isolates[26]. Recent reports have shown that genetic background dictates the pathogenicity and virulence of certain *M. tb* strains[27].

1.2.5 Drug-Resistant Strains

Drug resistance mutations are spontaneous mutations which occur at a predictable rate in patients who are on antibiotic therapy[19]. Mutations give the strain an advantage under selection-pressure of the drug and so they become the dominant strain in infection. These strains are then passed on to further human hosts. Acquisition of resistance is increased by sub-optimal levels of drug concentration and not completing the recommended length of treatment course. Resistance to further drugs can occur on top of the initial resistance; correct management of chemotherapy can reduce this risk[19]. Some drug resistant strains induce localised epidemics; it is suggested that some strain backgrounds, for example the Beijing strain, are better adapted to handle resistance mutations and do not incur fitness costs with the mutations[28]. Compensatory mutations may also occur to circumvent the loss of fitness which occurred with the initial mutation.

1.3 Immunology

1.3.1 Innate Immune Defences against *M. tb*

After entry to the airways, the first encounter a mycobacterium has with the host is usually an alveolar macrophage. The initial host-pathogen interactions occur between the host's pathogen recognition receptors (PRR) and the bacterial pathogen associated molecular patterns (PAMP). The bacterium is ultimately phagocytosed; the mechanism of phagocytosis and fate of the bacterium is determined by which receptors are triggered during this process. Receptor activation leads to initiation of cytokine secretion which is a key determinant of the profile of immunity induced against the pathogen.

1.3.2 Pattern Recognition Receptors

Toll-like receptors (TLRs) are expressed on the cell surface or on endocytic vesicle membranes of immune cells. There are currently 12 known receptors; each receptor

distinguishes between different types of pathogen and induces the most effective type of immune response to eliminate them. Mycobacteria have been reported to activate TLR 2, 4, 9 and possibly 8[2]. LAM, a major constituent of the bacterial cell wall, binds TLR2. Activation of these receptors induces pro-inflammatory responses including tumour necrosis factor alpha (TNFα), nitric oxide (NO), IL-1β and IL-12 secretion.

NOD-like receptors are highly conserved; the family consists of around 20 molecules. NOD2 recognises bacterial peptidoglycans and activation by *M. tb* results in a pro-inflammatory cytokine response and NO activity.

The mannose receptor is a cell surface bound C-type lectin which recognises differences between the patterns of sugar residues on the surface of pathogen cells and host cells. Interaction with this receptor gives an immune-modulatory response; many pathogenic species of mycobacteria have a modified LAM with a mannose cap (Man-LAM) to enter cells via this receptor. Another C type lectin involved in mycobacterial recognition is Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) which is expressed on the cell surface of DCs. Mycobacteria bind DC-SIGN through Man-LAM and induce an immunomodulatory response by impairing DC maturation and inducing secretion of IL-10[2].

Complement opsonisation of mycobacteria can result in phagocytosis through complement receptor 3 which is expressed on phagocytic cells. This is probably a rare event in *M. tb* infection though as complement factors are not common in the lung tissue.

1.3.3 Cytokines

1.3.3.1 Interferon gamma

IFNγ is secreted by CD4+ T cells, CD8+ T cells, neutrophils, γ:δ t cells and natural killer (NK) cells[29]. The IFNγ receptor is expressed on all cell types; among the cytokine's effects are macrophage activation, up-regulation of MHC Class I and Class II molecules, activation of NK cells, induction of T helper 1 (Th1) cell differentiation, inhibition of T helper 2 (Th2) responses and inducing differentiation of IgG antibody subclasses toward IgG2a. IFNγ is necessary for the host to mount an effective defence against *M. tb* infection.

1.3.3.2 Tumour Necrosis Factor α

Tumour necrosis factor α (TNFα) is a member of the TNF family of cytokines which also includes lymphotoxin, Fas ligand and CD40 ligand[30]. Many cell types including macrophages, CD4+ T cells, CD8+ T cells, NK cells, endothelial cells, and fibroblasts produce TNFα. TNFα can induce macrophage activation, neutrophil migration, secretion of IL-1 and IL-6 and is a key determinant of granuloma formation in *M. tb* infection[29]. Membrane bound TNFα can induce apoptosis of *M. tb* infected cells[31]. TNFα neutralization studies have demonstrated the importance of this cytokine in controlling replication, formation of granulomas and prevention of disease reactivation in latency. Reactivation of latent *M.tb* infection has been seen in arthritis patients taking anti-TNFα antibody treatment[31].

1.3.3.3 Lymphotoxin

Lymphotoxin is also a member of the TNF family of cytokines and is either membrane bound or secreted[30]. It's role in *M. tb* infection is less well defined than the role of TNFα but there are reports it plays a role in granuloma organisation[29].

1.3.3.4 IL-1α and IL-1β

IL-1α and IL-1β are part of the IL-1 family of cytokines. Il-1β is produced by macrophages, neutrophils and DCs and requires cleavage to its active form. IL-1α is produced by epithelial

cells as well as immune cells and does not require cleavage for it to be active. IL-1α and IL-1β actions include induction of IL-2 activity, leukocyte chemotaxis and generation of fever. IL-1R1 knockout (KO) mice still have functional TNFα, inducible NO production and IFNγ responses however these mice have gross lung pathology and increased mortality[29]. *IL-1* or *IL-1R1* polymorphisms in the human genome are linked to increased susceptibility to TB disease[32]. Despite these deficiencies showing susceptibility to disease the mechanism of action of IL-1α and IL-1β in the control of *M. tb* is unknown.

1.3.3.5 IL-12

The IL-12p40 subunit can form a homodimer (IL-12p80) or a heterodimer with IL-12p35 subunit (IL-12 p70)[29]. IL-12p40 is produced early in infection by the macrophage or DC by stimulation of TLR9. IL-12p80 induces DC differentiation and migration to the draining lymph nodes. It can also activate naïve T cells but cannot fully induce IFNγ so the Th1 response is not fully functional. IL-12p35 is produced in the presence of an IFNγ signal and activation of TLR7/8. IL-12p70 appears to be produced in the local tissue and in the draining lymph node as it can induce an optimal Th1 response; it is also required for maintenance of Th1 responses over the course of infection. IL-12 stimulates secretion of IFNγ and TNFα from Th1 cells, CD8+ and NK cells.

1.3.3.6 IL-23

IL-23 is formed from the IL-12p40 subunit and IL-23p19 subunit[29]. Production is stimulated by activation of NOD2 and TLR2 and it is secreted early in the *M. tb* response by alveolar macrophages. IL-23 acts to induce IL-17 production from innate cells e.g. γ:δ T cells and from the Th17 subset.

1.3.3.7 IL-2

IL-2 is produced when the T cell receptor (TCR) recognises antigen and is activated. IL-2 receptors are expressed on the surface of T cells and when they are stimulated they promote

cell differentiation, activation and proliferation[30]. IL-2 is essential for generating and maintaining an effective T cell response. IL-2 also increases expression of IL-12R on NK cells.

1.3.3.8 IL-17

IL-17 is produced from innate cells, e.g. γ:δ T cells, in early responses to *M. tb* infection; its production is stimulated by IL-23[33]. IL-17 is also produced by the Th17 subset later in infection. IL-17 induces production of granulocyte colony-stimulating factor (G-CSF) and chemokines CXCL-1, CXCL-2 CXCL-5 and CXCL-8 which recruit neutrophils. IL-17 may not be essential for the control of *M. tb* but can provide some protection if IL-12/IFNγ responses are compromised[34]. It also plays a role in granuloma formation. Continued mycobacterial antigen exposure leads to a large IL-23/IL-17 responses resulting in exacerbated infiltration of neutrophils[35].

1.3.3.9 IL-6

IL-6 is required for the differentiation of Th1 cell responses to *M. tb* and is especially crucial in high burden infections[29]. IL-6 can also act with transforming growth factor-β (TGFβ) to induce Th17 or Treg cells depending on strength of each cytokine.

1.3.3.10 IL-10

Monocytes, macrophages, CD4+ T cells and CD8+ T cells can produce IL-10[36]. IL-10 has an immune modulatory function and has been shown to block phagosome maturation in alveolar macrophages. It may play an important role in long-term infection by reducing the levels of inflammation; however it has also been associated with reactivation of disease. IL-10 has been shown to be produced by DCs when DC-SIGN is bound by mycobacteria[2].

1.3.3.11 TGFβ

TGFβ is secreted in an inactivated state and can be activated by conformational change or cleavage[37]. The TGFβ receptor is expressed by haemotopoitec cells and non-haematopoietic cells. It is produced by monocytes, macrophages, dendritic cells and CD4+ T cells[36]. Its

function is generally immunomodulatory but it can be involved in pro-inflammatory functions such as induction of Th17 cells. TGFβ can suppress antigen presenting cell (APC) co-stimulation and production of NO.

1.3.3.12 Eicosanoids

Eicosanoids include prostaglandins, lipoxins and leukotrienes[29]. Prostaglandin E2 can promote apoptosis of *M. tb* infected macrophages which limits mycobacterial replication and spread.

1.3.4 Innate Cellular Defences

1.3.4.1 Macrophages

Macrophages are stimulated to phagocytose mycobacteria by ligation of PRRs and contain them within a phagosome which is made from the plasma membrane. To destroy the bacteria the cell mounts a range of responses. One of the key antimicrobial activities is maturation of the phagosome through early, intermediate and late stages, ending with fusion with lysosomes to form phagolysosomes[38]. Throughout this process the phagosome becomes increasingly acidic due to acquisition of proton pumps in the phagosomal membrane; this environment prevents mycobacterial growth and favours the activity of specific phagolysosomal enzymes. The protein NRAMP is also acquired to the phagosomal membrane and exports iron which restricts bacterial function. TLR2 activation stimulates macrophages to produce bioactive vitamin D that results in production of the antibacterial protein cathelicidin[39]. TLR ligation also stimulates secretion of cytokines such as TNFα, IL-1β and IL-12.

Reactive Nitrogen

NO and reactive nitrogen species (RNS) are an important defence mechanism in macrophages and are highly toxic to the engulfed bacteria. NO is produced by nitric oxide

synthase (NOS2) from metabolism of L-arginine[38]. NO interacts with reactive oxygen species (ROS) within the phagosome to produce RNS.

Reactive Oxygen Species

Reactive oxygen species are another key mechanism in control of intracellular pathogens. The NOX2 NADPH oxidase generates O_2 - which is transferred into the phagosome[38]. O_2 - can produce H_2O_2 and other ROS; these molecules are highly toxic to the mycobacteria.

Mycobacterial Evasion Strategies

Pathogenic mycobacteria can inhibit phagosome maturation in the early stages which allows the mycobacteria to grow exponentially within the macrophage. The mechanism of action is still not fully understood, a number of laboratories have reported involvement of various mycobacterial factors including Man-LAM which is an analogue of the host membrane phospholipid phosphatidylinositol-3 phosphate[40].

As well as preventing phagolysosome formation, strains of the MTBC of mycobacteria can escape the phagosome and exist within the host cell cytosol. This mechanism of escape has been related to the ESAT-6 secretion system 1 (ESX-1) and the secreted proteins CFP-10 and ESAT-6 which are all encoded on the region of difference 1 (RD1) [41]. Phagosomal escape is a key characteristic of pathogenic species of mycobacteria; the RD1 locus has been lost in BCG[42]. ESAT-6 produces pores in phagosomal membranes which probably happens early in infection and allows mycobacterial PAMPs to enter the cytosol. In later stages of infection it has been shown that mycobacteria can exit the phagosome and exist in the cytosol. High levels of toxicity exist in the cell after phagosomal rupture which leads to cell death.

Lipoxin A4 induced by the mycobacterium inhibits prostaglandin E2; the inflammatory mediator produced by macrophages to stimulate apoptosis through caspases[29]. This results in necrotic cell death rather than apoptotic cell death which favours the mycobacteria.

Apoptosis leads to uptake of *M. tb* containing vesicles by DCs and priming of CD4 and CD8 T cell responses whereas necrosis does not lead to such efficient priming of the adaptive response.

1.3.4.2 Dendritic cells

Dendritic cells (DC) can phagocytose bacteria through binding of complement receptor 3, mannose receptor and DC-SIGN but unlike macrophages, mycobacteria cannot grow in DCs[31]. Large amounts of IL-12 are secreted from DCs through TLR9 activation by binding of CpG motif DNA. DCs also produce chemokines that attract monocytes and immature DCs to the inflamed lung. The defining feature of DCs in mycobacterial infection is their ability to prime the adaptive immune response. Activated infected DCs up regulate CCR7 expression and migrate to the pulmonary lymph nodes. They also up regulate CD40, CD80 and CD86 co-stimulatory molecules together with the presentation of mycobacterial antigens on MHC Class I and II molecules.

Mycobacteria can modulate the DC response by binding DC-SIGN and inducing IL-10 production which inhibits maturation[43]. IL-10 prevents signalling through CCR7 which prevents migration to the draining lymph nodes[44].

1.3.4.3 Neutrophils

Neutrophils are phagocytic cells that circulate in the blood but can enter inflamed tissue through chemotaxis. Neutrophils, like macrophages, phagocytose bacteria into a phagosome and then form a phagolysosome to kill the bacteria. Neutrophils also have specialised primary granules which contain degradive enzymes, cathepsins, antimicrobial peptides and secondary granules that contain molecules such as collagenase, heparanase and lysozyme[38]. Neutrophils also produce NO and ROS.

Neutrophil responses in mycobacterial infections are stimulated by IL-17 production and can be both protective and detrimental. Early in infection neutrophil infiltration helps to control initial infection and help build the granuloma[45]. In chronic infection or continued antigen exposure large IL-23/IL-17 responses can be induced resulting in pathological infiltration of neutrophils[35].

1.3.4.4 Gamma Delta T cells

γ:δ T cells are found in mice and humans although their development and function differs slightly between species. The γ:δ TCR does not recognise as broad a repertoire of antigens as the αβ TCR, nor does it recognise MHC Class I or II molecules. Ligands which have been identified to be recognised by γ:δ T cells include lipid-type molecules presented by CD1d molecules but still little is known about this[30,46]. Mouse γ:δ T cell are thought to be imprinted in the thymus to either IFNγ or IL-17 phenotype whereas human γ:δ T cells can be polarized in the periphery.

γ:δ T cells react early in mycobacterial infection responding to the chemokines and cytokines produced by infected macrophages and DCs, e. g. IL-12, CXCL2 and CXCL10[31]. IFNγ produced by γ:δ T cells activates macrophages and increases NOS2 activity along with other anti-mycobacterial pathways, IL-17 from γ:δ T cells has been linked to early initiation of granuloma formation and neutrophil infiltration. There are reports that IL-17 from γ:δ T cells plays a role in later stages of infection and show that the predominant IL-17A producing cells in *M. tb* infection were γ:δ T cells rather than αβ T cells[47].

1.3.4.5 Natural Killer Cells

NK cells are lymphoid cells without antigen specific receptors. NK cells can induce apoptosis through various mechanisms. Resident NK cells in the lung can be activated and mature to produce IFNγ, perforin and express cytotoxicity receptors. Production of IFNγ by NK cells in the early stages of infection activates macrophages and balances the neutrophil influx. NK

cells were shown to produce IFNγ and perforin as early as 3 days after infection[48]. Depletion studies show they are not an essential subset to the control of *M. tb* probably due to redundancy to other subsets of the innate response and the generation of adaptive immunity[49].

1.3.4.6 CD1 Restricted T cells

CD1 restricted T cells recognise CD1 molecules associated with lipids or glycolipids. There are five isoforms of CD1 (a-e); CD1 a, b and c are found in humans but CD1d is the only molecule of the family to be found in mice[50]. In humans CD1 T cells recognise a large variety of mycobacterial antigens. In mice the only known ligand for CD1d is phosphotidylinositol mannoside (PIM) and recognition leads to IFNγ production and cellular cytotoxicity. In the mouse model of infection described by Junqueira – Kipnis et al CD1d restricted T cells (also known as NKT cells) had no effect but this may be due to restriction to just CD1d in the mouse and may be different in human infection[48].

1.3.4.7 Mucosally Associated Invariant T cells

Mucosally Associated invariant T cells express an invariant TCR α chain and recognize MHC-related protein (MR-1) bound to a ligand on APCs and infected epithelial cells[51]. It is still uncertain what ligand binds to MR-1 but as signalling is conserved across different bacterial infections it is thought likely to be a host product. Antimicrobial efficacy of these cells was demonstrated by *M. abscessus* infection of MR-1 KO mice which resulted in higher colony forming units (CFU) burden in the spleen[51].

1.3.5 Adaptive Immunity

The adaptive immune response is conducted by lymphocytes; T cells elicit the cell-mediated arm of the response and B cells elicit the antibody-mediated arm. Each lymphocyte is specific for a particular antigen, when the cell sees this antigen it activates to mature and differentiate into an armed effecter that plays a specific role in the response against the

invading pathogen and any future attack that may occur by the same pathogen. Antigen is presented to naïve lymphocytes in the periphery whose antigen specificity was already decided; in the thymus for T cells and in the bone marrow for B cells. B cell and T cell responses will be described separately in further detail below. The role these lymphocytes have in *M. tb* infection will also be discussed.

1.3.5.1 B Cells

B cells are activated to secrete antibodies which circulate the body and bind specifically to antigen. Binding may inactivate the pathogenic compound directly, e.g. toxins, or it may mark the molecule for destruction via other processes, such as phagocytosis of opsonised bacteria.

Each B cell produces antibodies with one unique antigen specificity. This specificity is determined during development in the bone marrow through a process called combinatorial diversification; in brief it is the assembly of a combination of the variable (V), joining (J) and diversity (D) gene segments which encode the antibody's light and heavy chains[52]. Naive B cells circulate from the bone marrow with IgD and IgM antibodies inserted into the plasma membrane which act as antigen receptors.

Antigen binding to the antigen receptor is the first signal required for activation of B cells, they also require a second signal which is often provided by T cell help. The T cell produces cytokines such as IL-4 and IL-5 which stimulate differentiation, proliferation and antibody class switching.

The role of B cells in *M. tb* infection is under debate. The classical opinion is that B cells and antibodies play a minor role in immunity to mycobacterial infection as shown in a standard aerosol *M. tb* infection of B cell knockout (KO) mice[53]. However, a protective role was suggested in a high dose *M. tb* challenge of B cell KO mice[54]. B cell KO mice had a significantly

higher burden of *M. tb* than wild type (WT) although it must be noted the infection was not lethal as it would be in IFNγ KO mice. It was suggested in this study that B cells play a role as antigen presenting cells and it is possible secreted antibodies may act to neutralise mycobacterial products such as cord factor. Assessments of human B cell deficiencies have shown no susceptibility to mycobacterial infection[32]. Despite a role, it appears that B cells and antibodies are not a key contributor to immunity against *M. tb* infection.

1.3.5.2 T Cells

There are two arms of the T cell response; CD8+ cytotoxic T cells and CD4+ helper T cells. All naïve T cells need to recognise antigen displayed by a professional antigen presenting cell (APC) to be activated and differentiate to a mature T cell. This occurs in the peripheral lymphoid tissue. T cells display a T cell receptor (TCR) which is formed of an α and a β chain[52]. Each chain contains a variable and a constant region. The TCR development is similar to that of the B cell antigen receptor in that V, J and D segments are selected by genetic recombination from pools of genes in the TCR domain[52]. Like B cells, each T cell has an individual specificity.

TCR recognise peptide-MHC molecule complexes. There are two types of MHC; Class I which present to CD8+ T cells and class II which present to CD4+ T cells. The peptides which bind with MHC are generated from different compartments of an infected cell which in turn activates a particular arm of the T cell response against the pathogen. Peptides to be loaded on to MHC Class I molecules usually originate from pathogens which exist in the cytosol of host cells. Proteins produced by the pathogen are degraded by the proteasome and transported into the endoplasmic reticulum (ER) lumen via TAP protein where they associate with MHC Class I molecules and are transported to the cell surface[52]. Another mechanism of MHC Class I loading is under investigation as it has been realised that certain DCs are able to display peptides which are not derived from cytosolic pathogens on MHC Class I molecules; a

process termed cross-presentation[55]. The mechanism of this is unknown and each theory has research to support it but argument against. Theories include; exportation of peptides from endosomes to the cytosol where they can be processed by the proteasome, fusion of endosomes with membrane from the ER that contains MHC Class I molecules and loading complexes, loading of recycled MHC Class I molecules from the plasma membrane within the endosome with phagocytosed peptides. Peptides which associate with MHC Class II molecules are usually generated from phagocytosed particles. Endosomes containing foreign particles that enter the endosomal pathway. When lysosome fusion occurs proteolysis and enzymatic degradation produce peptides which, if of a high affinity, can bind to the MHC Class II molecules present in the phagolysosomal membrane. The peptide-MHC complex is then transported to the cell surface.

To activate naïve T cells APCs display MHC-peptide complexes to TCRs. If the TCR recognises the MHC-peptide complex it binds, the T cell also binds MHC class II or MHC Class I molecules through CD4 or CD8 respectively[52]. The mature APC will express co-stimulatory molecules as a second signal for T cell activation. CD80 or CD86 are positive co-stimulatory molecules expressed by the APC and they interact with CD28 on the T cell surface. Co-localisation of these molecules in the T cell membrane allow for TCR activation with the help of CD3 molecules. Signal transduction stimulates expression of IL-2 and the IL-2 receptor, signalling of the IL-2 receptor in the T cell membrane further activates the T cell. LFA-1 is expressed by the T cell and binds ICAM-1 on the APC to hold the T cell in firm contact with the APC whilst differentiation occurs. T helper cell subclass differentiation is determined by cytokines produced by APCs during T cell priming. The profile of cytokines produced is decided by the PRRs stimulated by the pathogen.

1.3.5.3 T helper 1

T helper subclasses were first described by Mosmann et al in 1986; they observed T helper 1 (Th1) and T helper 2 (Th2) subsets produced distinct cytokine profiles and had different effector functions[56,57]. At present up to 7 T helper subsets have been described; Th1, Th2, Th17, Treg, Th9, Th22 and T follicular helpers[58]. T cell lineage is determined by transcription factors and differentiation is driven by cytokine but the plasticity of these subsets is under much investigation.

Th1 differentiation is driven by IFNγ and IL-12 and expression of the transcription factor T-bet[59]. The characteristic cytokine profile of these cells is IFNγ, TNFα and IL-2. Mechanisms of action include macrophage activation to destroy intracellular pathogens and activation of cytotoxic CD8+ T cells. The role of Th1 in defence against *M. tb* infection is crucial to the survival of the host.

Th1 cells are primed in the mediastinal lymph nodes after aerosol infection with *M. tb*; Reiley et al showed this response was delayed until up to day 10 after infection[60]. This delay in priming of the adaptive response appears to be an *M. tb* evasion strategy that allows time for the bacteria to grow within the macrophages of the lung and disseminate to other organs without adaptive immune interference. On induction of the adaptive response *M. tb* proliferation can be controlled and a sustained population of CD4+ T cells are maintained in the lung; this occurs around day 20 after infection in aerosol mouse infections[61].

Activated Th1 track to the lung and other infected organs and secrete IFNγ and TNFα when presented peptide-MHC II complexes on infected phagocytes. IFNγ activates infected phagocytes leading to the generation of NO, RNS and ROS. IFNγ has also been related to the stimulation of macroautophagy; a process of endosome maturation that has been suggested to overcome the blockade induced by the mycobacteria[62]. TNFα is needed to control

granuloma formation in chronic stages of *M. tb*, the granuloma disintegrates in *M. avium* infection of TNFα deficient mice[63].

In 1993 Orme et al demonstrated in the mouse model of *M. tb* infection that IFNγ production from CD4+ T cells coincided with the time in *M. tb* infection when protective immunity was induced[64]. In 2001 Mogues et al used MHC Class II KO mice and CD4 depletions to show the Th1 subset was required for the control of *M. tb* infection and survival of infected mice[65]. CD4+ T cell KO mice had an 86% reduced survival time compared to WT mice. Results from mouse models showing Th1 subsets are essential in the control of *M. tb* is further supported by the destructive effect observed in patients co-infected with HIV and *M. tb* and also in human genetic studies, for example IL-12Rβ1 deficiency is related to greater risk of disseminated mycobacterial infections [32].

1.3.5.4 T helper 17

TGFβ and IL-6 are required to stimulate differentiation of Th17 cells and the activation of transcription factor Rorγt[59]. Cells produce IL-17A, IL-17F and IL-22. IL-23 and IL-21 appear to be required for Th17 activity. The effect of Th17 cells in acute *M. tb* infection is minimal; determined by bacterial load and survival being the same in WT and IL-23 KO mice[34]. Deficiency of this subset affects the cellular environment as infection progresses, most significantly by altering the neutrophil content of the granuloma. Repeated BCG vaccination after *M. tb* infection models high level antigen exposure in mycobacterial infection, the result of which is a severe pathology[35]. Cruz et al found pathology was orchestrated by the induction of high levels of IL-17 producing cells as well as IL-23 production in the lung which sustained the IL-17 response; these cytokines stimulated the infiltration of neutrophils which resulted in pathology. Interestingly the Th17 subset can mediate enhanced protection against *M. tb* in vaccinated animals[45].

1.3.5.5 Regulatory T cells

Natural T regs develop in the thymus and express FoxP3 constitutively[59]. Inducible Tregs can develop in the periphery from circulating naïve T cells and acquire expression of FoxP3 in the presence of TGFβ[66]. Regulatory T cells can also be induced by the presence of IL-10 and antigen and do not express FoxP3 these are termed IL-10-Tregs[67]. There are many reported mechanisms of regulation by Tregs including production of TGFβ, IL-10, IL-35 and contact-dependant mechanisms[68,69]. One example is the mechanism of CD39 which is expressed by active Tregs[70]. ATP can be immunostimulatory; in ATP rich environments DC express CD86, ATP also stimulates IL-1β release from epithelial cells therefore a more modulatory environment can be induced by depletion of ATP.

Regulation of the inflammatory response to *M. tb* is a delicate balance. Without control the inflammation in the lung could lead to organ failure whereas too much regulation could lead to loss of immune control of *M. tb* and unrestricted growth. As described above, ligation of DC-SIGN induces maturation of DCs to a tolerogenic phenotype and secretion IL-10[2]. Scott-Browne et al reported that Tregs accumulated in the pulmonary lymph nodes (pLN) and lungs of *M. tb* infected mice at the same rate as effector CD4+ T cells[71]. The depletion of Tregs in this model gave a reduction of the bacterial burden by 1 log colony forming units (CFU) and was associated with increased T cell activation. Shafiani et al later demonstrated that administration of transgenic Tregs specific for ESAT-6 increased bacterial burdens[72]. The action of Tregs was to delay Th1 priming in the pLN and contribute to higher bacterial burdens by delaying the effector T cells infiltrating the lung.

1.3.5.6 T helper 2 cells

IL-4 stimulates expression of transcription factor GATA-3 in the differentiation of Th2 cells[59]. Th2 produce IL-4, IL-5 and IL-13 when activated. Their effector functions include activation of B cells and they are key to protection against helminth worm infections.

Both Orme et al and Mogues et al saw IL-4 production during *M. tb* infection[64,65]. Orme found that IL-4 was produced in a second wave CD4+ T cell response at day 20 to day 60 after infection, Mogues also reported IL-4 was produced from day 20 post infection. Neither of them concluded that IL-4 played a part in the initial control of infection but Orme suggested IL-4 could play a part in long-term control and induction of antibody responses. There are reports that patients with increased IL-4 production in response to *M. tb* have an impaired Th1 response[73]. IL-4 can inhibit the differentiation of Th1 responses by suppressing IL-12Rβ expression[59].

Jung et al infected IL-4 and IL-13 knockout mice with *M. tb,* KO mice had increased Th1 responses at day 50 post infection although this did not result in a decrease in the burden of *M. tb*[63]. This study showed that Th2 responses did not play a key role in the control of infection but also that Th2 responses induced in murine infection were not detrimental to the control of *M. tb*. It would appear that, as with regulatory T cells, a balance of these responses is required.

1.3.5.7 CD8 Cytotoxic T Cells

Differentiated cytotoxic T cells migrate from the pulmonary lymph node to the lungs where infected cells displaying protein-MHC Class I activate them. All cells have the ability to express MHC Class I therefore any epithelial cells which may have been infected as well as phagocytic cells can be recognised by CD8+ T cells[30]. The primary function of CD8+ T cells is to kill infected cells by inducing apoptosis; this is mediated by the release of lytic granules that contain perforin and granzyme. CD8+ T cells also secrete IFNγ, TNFα and IL-2.

CD8+ T cells play a protective role in the early stages of *M. tb* infection. In the aerosol mouse infection study conducted by Mogues et al MHC Class I KO mice were able to control *M. tb* infection but only at day 30 after infection, 10 days later than WT mice, which resulted in a 1

log higher CFU burden[65]. Cytotoxicity is a key mechanism of CD8+ T cell mediated protection and so is IFNγ but it may play less of a role in the acute stages of infection once the CD4+ T cell response is initiated. This is possibly due to the fact the CD4+ T cell subset secretes greater amounts of IFNγ in relation to CD8 + T cells in response to mycobacteria[21]. *M. tb* specific CD8+ T cells capable of killing *M. tb* infected macrophages are found in sputum samples of patients with active TB[74].

The importance of secretion of IFNγ from CD8+ T cells during latent *M. tb* infection was shown by van Pinxteren et al using a mouse model of latent infection; depletion of the CD8+ T cell subset induced a log increase in *M. tb* burden in the lung[75].

1.3.6 Granuloma Formation and Structure

The adaptive immune responses lead to formation of the granuloma structure that is distinctive of mycobacterial infections. Infection induces the infiltration of monocytes from the blood that differentiate to become macrophages and may too become infected[76]. T cells surround macrophages that are incapable of killing the residing *M. tb*, some cannot eliminate the infection even with T cell help[77]. Sustained antigen exposure leads to chronic cytokine stimulation of the macrophage and with secretion of GM-CSF and IL-4, macrophages can be induced to differentiate to epithelioid cells[76]. Epithelioid cells can fuse with one-another to become giant multinuclear cells. A dense ring of Th1 cells and to a lesser extent CD8+ T cells and B cells surrounds the macrophages. Caseous necrosis occurs at the centre of the granuloma where there is intense cell-to-cell signalling and multinucleation. T cell secreted perforin and granulysin can induce apoptosis and secretion of RNS, and ROS from activated macrophages can induce necrosis of the giant cells. The central region becomes hypoxic as blood vessels are eliminated. Mycobacteria exist in macrophages at the edge of the caseous region but at a distance from the T cell response.

The host can control the mycobacterial infection within this structure as long as the contributing parts of the immune response are maintained. IFNγ is required for granuloma formation; mice deficient in IFNγ have lungs infiltrated with neutrophils and large areas of necrosis[76]. TNFα is key to the maintenance of the structure by providing a cytokine gradient keeping interactions between macrophages and T cells. The need for this cytokine in granuloma maintenance is evidenced by reactivation of latent disease in patients on anti-TNFα treatment[31]. If the levels of Th1 or either of these cytokines is altered IL-23/IL-17 signalling recruits infiltration of neutrophils leading to necrotic regions and reactivation to active TB.

1.3.7 Relevance of Animal models

Much of what we know about the immune response to TB has been discovered using animal models of *M. tb* infection and of these models mice are the most widely implemented. Guinea pigs, rabbits and cows are also used but the range of reagents, ease of genetic manipulation and ease of challenge are not as great as with mice. Non-human primates are also used but generally to test the efficacy of drug or vaccine candidates before they enter human trials. All these models demonstrate that the key features of the immune response against *M. tb*, namely IFNγ, TNFα and IL-2 production from CD4+ and CD8+ T cells, are comparable to the human response.

The mouse strains most frequently used to model infection are BALB/C and C57BL/6, both of these strains differ from humans in the generation of granuloma structure and the absence of caseous necrosis[78]. BALB/C mice induce a more Th2 biased response than C57BL/6 mice and are more susceptible to *M. tb* infection. Other mouse strains including CBA and DBA/2 are more susceptible to *M. tb* than both BALB/C and C57BL/6. The I/St strain induces necrotic granulomas and hypoxic areas in the lung on *M. tb* infection that is more representative of human pathology. The differences in the ability of strains to control *M. tb* infection outlines

the role genetic variation has on outcome of disease. This is replicative of the variation of disease manifestations seen in the human population. The use of BALB/C and C57BL/6 mice to study the key features of the immune response is valid although their use as a model for long-term and latent infection is perhaps not as good given the differences in pathology induced. Genetic manipulation of mice has allowed the observation of roles that are played by different arms of the immune response. This has provided an invaluable tool for elucidating the mechanisms of the immune response to *M. tb*.

Guinea pigs are highly susceptible to infection with *M. tb* and induce similar responses to humans[79]. Guinea pigs are a good animal model of TB and have been extensively used in the past however the range of reagents available for immunological assays in this species has limited their use for elucidating immune responses. A similarly representative form of human disease is induced in rabbits with many of the different stages of responses seen in humans being replicated. Caseous necrosis of lung granulomas is seen in guinea pigs and rabbits[80].

Challenge of cattle with *M. bovis* represents the exposure of an animal to its natural pathogen and can also be used to model transmission. Many of the immunological responses induced by cattle are similar to humans however they do not induce cavitating disease[81]. Macaques induce an immune response replicative of humans although they are more susceptible to progressive disease in high dose challenge[79]. The expense and regulatory issues as well as physical logistics in using these latter two models limit their use. Zebra fish infected with *M. marinum* have recently been used to model innate immune responses important in the control of mycobacteria; especially granuloma formation[80].

1.4 BCG

1.4.1 Immunity Induced by BCG

Most currently licensed vaccines mediate protection via antibody-mediated activity. Plasma cells are long –lived and responses can be boosted if protection wanes[21]. Conversely some of the world's most devastating diseases are controlled by T cell-mediated immunity; malaria, TB, HIV and hepatitis C. Presently BCG is the only licensed vaccine that mediates protection through inducing T cell immunity.

BCG was created by the serial passage on an *M. bovis* isolate over a period of 13 years[22]. There are a variety of strains that all descend from the original strain but have been altered by growth in different laboratory conditions.

Much of what we know about the protective immunological mechanisms induced by BCG has been discovered by using animal models[82]. The most common route of infection used for challenge in vaccine models is the intravenous route as it induces the adaptive response quickly, however the pulmonary route is the most favourable as it replicates human exposure. The most common read-out for vaccine-mediated protection is the comparison of the ability of vaccinated groups and naïve controls to limit the replication of *M. tb* in the lungs and spleens over 4-6 weeks. This is usually shown as around 1 log lower CFU in lungs of BCG vaccinated groups in aerosol challenge studies.

BCG induces Th1 and CD8+ T cell mediated immunity. Much of the research on the immune response to BCG vaccination has been performed in mice owing to the availability of reagents for this species and the ease of performing the experiments. Kramnick et al reported the induction of Th1 immunity by BCG vaccination in mice in 1994[83]. Work by Kaveh et al further characterised the memory properties of CD4+ T cells induced by BCG and reported that the

vaccine induced effector memory populations were present for at least 18 months after vaccination[74]. The cells were present systemically and locally in the lung and differentiated on challenge to produce IFNγ, TNFα and IL-2. As we know from the assay of immune responses in *M. tb* infection these cells are key mediators of protective immunity against infection.

CD8+ T cell responses were assayed by Begum et al, they reported that BCG induced responses appeared in the lungs of *M. tb* infected mice 7 days before those in naïve mice giving a head-start to the control of *M. tb*[77]

In the guinea pig model of *M. tb* infection BCG protects animals and is associated with the induction of CD4+ and CD8+ T cells expressing CD45 in the vaccinated animals[84]. In cattle the immunity induced by BCG against *M. bovis* is also mediated by CD4+ and CD8+ T cells, these cells produce IFNγ in response to purified protein derivative (PPD) of *M. bovis* (-B) and are highly proliferative[85].

IL-17 is important in the generation of Th1 responses by BCG vaccination. BCG stimulates the production of IL-10 from APCs which in turn prevents IL-12 production and so priming of the Th1 response is compromised[86]. Prostaglandin E2 is involved in this modulation as it enhances IL-10 responses but it also induces the production of IL-23 which drives Th17 responses. The host immune system can override the modulatory effect of IL-10 with the production of IL-23 instead; this leads to optimal induction of Th1 immune responses by BCG. BCG induced Th17 responses play a role in protection against *M. tb* infection and mechanism involves inducing early Th1 infiltration to the lungs by production of inflammatory mediators and chemokines[45].

1.4.2 Correlates of Protection

As IFNγ is an essential mediator of the protective response against *M. tb* it seems plausible that measuring the magnitude of this cytokine response would give an indication of the protection afforded by the immune response. However this is not the case and a correlate of protection against *M. tb* remains to be found.

Kagina et al retrospectively assayed BCG induced immune responses in BCG-vaccinated infants who went on to develop childhood TB at a trial site in South Africa[87]. They found frequencies of CD4+ and CD8+ T cells and production of IFNγ, TNFα and IL-2 10 weeks post BCG was not associated to protection against subsequent disease. It was surprising that no association between cellular immunity and protection was seen. It is possible that the response induced by BCG in the neonatal immune system is different to that mounted in adults. As childhood vaccination protects against disseminated forms of disease the mechanism involved in protection would be an interesting insight as this form of disease may not be so dependent on the classical Th1 response.

Despite this the importance of IFNγ in protection is clear as demonstrated by knockout mice and human genetic deficiencies and so it remains the primary readout in assessing vaccine 'take' until a better correlate is found.

1.4.3 BCG Vaccine Efficacy

BCG efficacy in preventing pulmonary TB has been a matter of debate since its use began in 1921. Much of Europe adopted the vaccine after World War 2 but many countries in the rest of the world were not as quick to adopt the policy, either through financial and infrastructure limitations or because efficacy had not been proven[22].

Rodrigues et al and Colditz et al performed a meta-analysis of BCG randomised control trials (RCT) and case-control studies in the early 1990s[15,88]. Both studies displayed a large variation in the efficacy of BCG vaccination in control of pulmonary TB between studies. Colditz et al summarised overall protective efficacy of BCG at 50% but the variation between studies ranges from 80% to 0%. Colditz also calculated that 41% of the variation between RCTs could be related to the latitude where the trial was conducted.

The MRC undertook a large RCT of adolescents in the UK and concluded BCG vaccination was 80% effective in preventing pulmonary TB[89]. Another RCT conducted by the Indian MRC and the WHO in Chingleput, India reported 0% efficacy against pulmonary TB; 0% efficacy was also found in a case control study in Malawi[90,91]. Large RCT studies in the US, where mass BCG vaccination has never been implemented showed efficacy of less than 30% in Georgia, Alabama and Puerto Rico[92].

The length of time for which protective efficacy is sustained is also variable dependant on the population studied. Follow-up of the MRC trial conducted in the UK showed that 10-15 years after BCG vaccination the protection afforded against pulmonary TB was reduced to 59% from the 80% afforded 0-5 years after vaccination[89]. A similar result was published from a study in Saudi Arabia where neonatal vaccination afforded protection to 82% of children in the 5 to 14 age group but only 20% of adults in the 25 to 34 years age group[93]. Contradictory to these results was the outcome of extended follow-up of a placebo-controlled trial of BCG conducted in American Indians and Alaskan Natives in the 1930s. At the first follow-up 0-9 years after vaccination 54% efficacy was afforded by BCG in this population[94]. Extended follow-ups showed the efficacy of BCG vaccination was 48% 40-49 years after vaccination demonstrating a more sustained level of vaccine efficacy in this population.

Despite variation in the efficacy of BCG against pulmonary disease, the protection conferred against miliary and meningeal tuberculosis are far less variable; a meta-analysis shows BCG efficacy is 81% against these forms of the disease[88]. A decrease in BCG immunity over time has been a suggested reason for this disparity; miliary and meningeal TB tend to affect infants and children whereas pulmonary TB affects adults however the evidence that efficacy of BCG is maintained past childhood is contradictory to this hypothesis. Another possible explanation would be the mechanisms involved in the immune control of the two forms of disease may be different and that BCG induced immunity is more able to control extrapulmonary forms of the disease, as BCG is administered systemically.

Given the vaccine is efficacious in preventing disseminated forms of TB disease in children and that it affords a level of protection against leprosy it is recommended by the WHO that infants born in TB endemic areas are vaccinated with BCG as soon after birth as possible[22,88]. It is important that the infant is known to be HIV negative as the risk of disseminated BCG disease is increased several hundredfold in HIV-infected infants[95]. BCG vaccination programs in other areas are dependent on the countries policy for vaccination and schedules differ widely.

1.4.4 Explanations for the Variation of BCG Efficacy

1.4.4.1 BCG Strain

The WHO has kept seed stocks of the vaccine since 1956; new batches are grown from these seeds to prevent genetic deviation[22]. The number of viable bacilli in a vial varies considerably between preparation methods, production lots and also within a lot. There are a few strains of BCG available but 90% of the world's vaccinations are with French Pasteur strain 1173 P2, Danish strain 1331, Glaxo strain 1077 and Tokyo strain 172.

It was argued early on that differences in the vaccine strain were accountable for the variation in efficacy. Differences in the immunogenicity of 5 strains of BCG were compared in BALB/C mice: BCG Pasteur 1173 P, Glaxo 1077, Japanese 172, Russian strain and Prague strain[96]. Vaccination with Pasteur, Glaxo and Russian strains gave protection against a subsequent BCG challenge demonstrating greater immunogenicity than the Japanese and Prague strains. A study in guinea pigs compared the protective efficacy of BCG Danish, Glaxo, Japanese, Connaught, Pasteur and Tice strains against pulmonary *M. tb* infection[97]. In this study BCG Glaxo was found to be less protective than the other strains tested. This was contradictory to the results in the previous study where BCG Glaxo had been efficacious but BCG Japanese had not. The difference in the efficacy of the strains in these two studies may be due to the route of administration which was intravenous in the first and intradermal in the second, the model species used which was mice in the first and guinea pigs in the second and the challenge bacteria which was BCG in the first and *M. tb* in the second.

The hypothesis that the difference in responses between trial sites was due to the strain of BCG used was challenged by results from a number of clinical studies. For example the same GSK freeze dried preparation induced a different level of efficacy in Togo and Malawi, Africa[91,98] and the Danish strain that was used in the MRC's UK trial and the Chingleput trial gave vastly different efficacy results[89,90]. Also, the immunogenicity of Glaxo 1077 and Danish 1331 was compared directly in the same population of UK school children and no effect on immunogenicity was observed as detected by IFNγ production in response to PPD and TST tests after 1 year[99]. There is evidence that strains do perform differently but overwhelmingly in population comparisons these differences cannot show significant alterations in efficacy. In addition the fact that the same strain induces significantly different efficacy in distinct populations shows that strain difference is probably not a sufficient explanation of BCG's variable efficacy.

1.4.4.2 Human Genetics

There are a number of human genetic deficiencies that pre-dispose to severe mycobacterial infections. Some examples are; CD40L deficiency, defects in the phagocyte NADPH oxidase enzyme, IFNγR1 or R2 deficiency, IL-12p40 deficiency, STAT-1 defect and NFκB defects[32]. Also HIV infection and anti-TNFα therapy pre-disposes to TB disease. These deficiencies give a pathological pre-disposition to those who are affected but do not occur at a high enough frequency within the populations tested at the time of the trials to explain the variable efficacy of BCG. There is no evidence for role for host genetics in vaccine induced protection.

1.4.4.3 Pathogenic Strains of *M. tb*

Introduction of DNA sequence analysis in the 1990s showed TB was not caused by one strain of *M. tb* but in fact there were a whole range of strains that differed in virulence[100]. This was not known at the time of the BCG efficacy trials reported in the meta-analysis reviews. TB epidemics usually consist of a number of genetically different strains of *M. tb*, however there has been recent scrutiny of the pathogenicity of the Beijing strain due to emergence of epidemics consisting of this one strain.

The Beijing strain was first characterized in 1995 in the region of Beijing and Mongolia. It is now the most common strain in Asia contributing to 50% of infections and its emergence has spread worldwide contributing at least 13% of total *M. tb* infections[101]. What is unusual about the Beijing strain compared to other strains is the ability it has to cause large epidemics but with minimal genetic divergence. This would suggest the strain has a genetic advantage compared to other strains of *M. tb*. In 1992-3 a study was conducted in Vietnam to type the strains of TB in the population; at this time there was a high incidence of TB cases in the country. It was found that the Beijing strain accounted for 53% of infections and was strongly associated with infections in younger people, demonstrating these were new cases and showed it was this strain which had the ability to cause an epidemic[102]. A study of TB patients

in Cape Town, South Africa, where the burden of TB is very high, showed that over 12 years the incidence of TB caused by the Beijing strain increased exponentially[103]. The incidence of original clades in the population, Haarlem, LAM, Quebec and Low-Copy Clades, remained constant over the same period. The Beijing strain was reported to be more likely to cause active disease on initial infection and patients had higher levels of bacilli in their sputum.

In a mouse model of *M. tb* infection Lopez et al demonstrated that the Beijing strain is more pathogenic than the laboratory strain H37Rv and isolates of the Somali and Haarlem strains[27]. Pathogenicity was measured by bacterial load, pneumonia and death; the outcome of all was greater in the Beijing strain infected mice compared to H37Rv. The other isolates had intermediate pathogenicity. Mice responded to Beijing strain infection with initially high levels of TNFα and NOS but these were transient and an IFNγ response was not developed to the same magnitude as in H37Rv infected mice. These results suggest that initial responses were good but sustained immune control, especially induction of adaptive responses, was modulated by the bacteria which enhanced its pathogenicity. BCG vaccine efficacy was impaired against challenge with the Beijing strain compared to H37Rv.

These results show how strains in certain regions can be more virulent and transmissible as well as less susceptible to BCG induced vaccine protection. There is a strong possibility this could be a contributory factor to explain the variable results of BCG efficacy trials. It also highlights the importance of testing new vaccines against virulent clinical isolates.

1.4.4.4 Co-infection with Regional Pathogens

Helminths

In regions where BCG efficacy is lowest there is often a high burden of other infections. Chronic helminth infection is known to induce a Th2 type immune response and Treg production of TGFβ and IL-10. In patients with active TB, helminth infection was related to

reduced Th1 and increased IL-10 responses[104]. Malhotra et al reported Kenyan infants born to mothers who suffered from filariasis or schistosomiasis infections during pregnancy had a reduced BCG induced IFNγ response compared to children of un-infected mothers[105]. Elias et al administered BCG to volunteers who had been treated for worm infection and control volunteers who had not[106]. The infected group had reduced PPD specific IFNγ and IL-12 responses after BCG vaccination compared to un-infected controls; infected patients had greater levels of TGFβ production but not Th2 associated IL-4 or IL-5.

Potian et al conducted a series of experiments to determine the mechanisms involved in co-infection with *Nippostrongylus brasiliensis* and *M. tb* after aerosol infection[107]. This worm resides in the lungs from day 11 and up to day 50 after subcutaneous vaccination. In the lung and pulmonary LN the worm induces high levels of IL-4 and alternative macrophage differentiation. *M. tb* burden was significantly greater in co-infected mice 4 weeks after infection. Interestingly IFNγ production remained comparable with WT mice indicating Th1 priming was not affected. The authors demonstrated that the differentiation of alternative macrophages was the determining mechanism of increased *M. tb* load. How alternative macrophages lead to increased burden is still unknown; the authors discuss the activity of arginase (ARG)-1 a molecule that is expressed on alternative macrophages and competes with NOS2 for their substrate L-arginine to convert it to urea and L-orthinine[108]. The autophagy pathway in alternative macrophages is blocked, this may be another mechanism by which *M. tb* killing is avoided. These are both pathways which the protective adaptive response controls infection. This would also delay vaccine induced Th1 and CD8+ T cell immunity from having an effect until the worm had passed from the lung; which may well be too late to provide the host with any protection.

Elias et al tested this theory in *Schistosoma mansoni* infection of BCG vaccinated mice that were then challenged with *M. tb* intravenously[109]. They reported schistosome infection

reduced the efficacy of BCG to control the *M. tb* infection. CFU burden and inflammation were significantly greater in lungs of co-infected animals compared to controls. This was related to an increased systemic Th2 environment and decreased PPD specific IFNγ and NO production.

Seasonal Variation in Efficacy

Lalor et al conducted a trial of BCG vaccinated infants in the UK and Malawi. Malawian children had received BCG in the first 198 days of life, most in the first week and UK children in the first 3 – 13 weeks after birth[110]. 100% of UK infants produced a PPD specific IFNγ response compared to only 53% of Malawian infants and those which did had a lower magnitude of IFNγ production. The magnitude of response of both cohorts was associated with the season they were born in; Malawian infants born in the hot-dry season had greater responses than those born in the cool or rainy seasons. UK infants born in summer had the greatest magnitude of IFNγ although the variation over the population was not as great as that in Malawi. The authors related these differences to changes in seasonal infections; malaria and respiratory infections are greater in the wet months in Malawi and respiratory infections greater in the UK during the winter. Maternal diseases were also suggested to play a role. Parra et al showed in a mouse model of *M. tb* aerosol infection that malaria infection had no effect on the immunogenicity of BCG[73].

There is a lot of evidence supporting the theory that co-infection can result in diminishing the immunogenicity of BCG and this may well contribute to the variance seen in BCG efficacy trials. If this is a cause of reduced BCG efficacy the battle to rid populations of co-infecting pathogens is a formidable challenge.

1.4.4.5 Exposure to Non-Tuberculous Mycobacteria

The question of NTM and their effect on BCG vaccine efficacy was first raised by Palmer and Long in 1966 and later resurfaced in 1981 in a publication by Stanford et al[98]. In this report Stanford suggests two types of immune response can be mounted to mycobacteria and individuals may be directed towards one path before BCG vaccination by exposure to NTM, upon vaccination, BCG will enhance whichever type of response pre-existed[98]. He linked the type of response to the size of the TST and concluded that those who had a small TST before BCG mounted a protective response compared to those with a large TST who had reduced vaccine efficacy. This raised the idea that some species of NTM or some level of exposure to NTM can induce protective responses with subsequent BCG vaccination whereas some NTM may induce an ineffective response which is boosted by BCG or high levels of exposure is detrimental.

1.4.5 NTM species

Presently 55 NTM species have been identified[22]. They are divided into fast and slow growers and further differentiated by genotyping or morphological distinctions (Figure 1-3). Around half of the species may cause infection in humans but generally NTM are considered to be non-pathogenic and are only opportunistic where infection does occur. Healthy immune-competent people are rarely susceptible to infection with NTM. NTM species are ubiquitous in the environment, more prevalent in hot climates compared to cold climates and are often associated with soil and water supplies. In an investigation of composting soil 89% of samples were found to contain NTM which included the species *M. avium* (27%) and *M . intracellulare* (28%)[111]. *M. gordonae* and *M. kansasii* are also frequently isolated species. NTM are capable of forming biofilms and are resistant to chlorination, which leads to them disseminating into local water supplies[112]. An analysis of water supplies in various towns in South Africa detected *M. gordonae, M. fortuitum, M. abscessus, M. gilvum* and *M. spepticum.*

1.4.5.1 Evidence for Sub-Clinical exposure to NTM

Black et al examined the PPD reactivity of adolescents and young adults in the Karonga District of Malawi[113]. They found that 60% of population produced an IFNγ response and the magnitude of response ranged from 62pg/ml up to 4000 pg/ml. The same study was performed in UK adolescents; 21% of the population produced a PPD specific IFNγ response with a lower magnitude than the Malawian group. The frequency of responders increased to 70% of the Malawian population over a year but remained unchanged in the UK. All these responses were attributable to NTM and clearly show the exposure is greater in Malawi and exposure is onging, as demonstrated by increase in responders in a year.

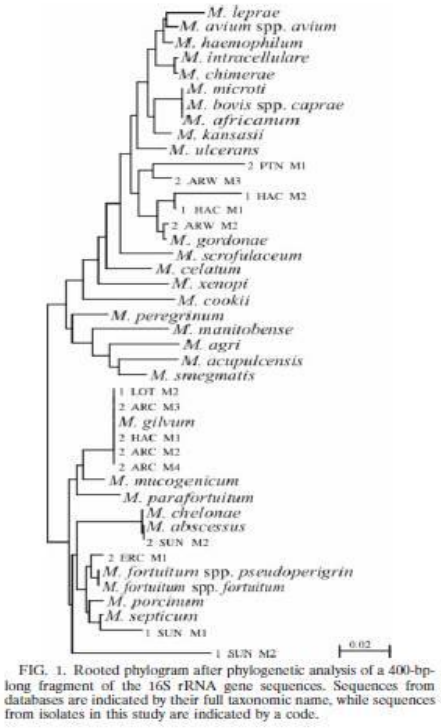


FIG. 1. Rooted phylogram after phylogenetic analysis of a 400-bp-long fragment of the 16S rRNA gene sequences. Sequences from databases are indicated by their full taxonomic name, while sequences from isolates in this study are indicated by a code.

Figure 1-3: Phylogenetic tree based on the 16s rRNA sequence of NTM taken from September et al. Figure displays the NTM species taken from databases and the isoltes found in water supplies in South Africa.

Chilima et al conducted an analysis of the NTM species present in soil and water supplies of the Karonga district and found NTM were prevalent in both samples[114]. Interestingly there were more recoverable bacteria in the dry season rather than the wet season but this may be due to difficulties in sampling during the wet season. The frequency of exposure to certain

species in the Karonga district was analysed by comparing responses to PPD preparations of common NTM; *M. intracellulare, M. avium and M. scrofulaceum* were the most frequently recognised[115]. The same study was performed in UK schoolchildren and the same species were the most frequently recognised however the number of responders in the UK population was far lower than in the Malawian population and the magnitude of response was less[116].

1.4.5.2 Evidence for NTM Interference on BCG Efficacy: Human Trials

The level of PPD responsiveness prior to vaccination gives evidence of exposure to NTM. Baseline PPD responses in the Malawian population were found to be higher than in the UK and comparing the efficacy of BCG in the two sites shows 80% efficacy in the UK and 0% in Malawi[91].

In South India where the efficacy of BCG was also 0%, the population is highly reactive to PPD prior to BCG vaccination[117]. Another result to consider is the 80% efficacy observed in the original UK MRC trial; in this trial volunteers were screened for PPD reactivity prior to vaccination and excluded from the study if any level of reactivity was seen. This would select for evaluating BCG efficacy in a population which had no previous exposure to NTM[89]. The theory of NTM interaction was not supported in Puerto Rico though where BCG efficacy was 31% in both high pre-vaccination PPD responders and low pre-vaccination PPD responders.

Another contradiction of the theory that exposure to NTM may have a detrimental effect on BCG conferred immunity came from a trial conducted with *M. vaccae* used as a booster for prior BCG vaccination[118]. In this phase III efficacy trial conducted in HIV infected patients in Tanzania, an increased protection against laboratory confirmed TB was found in comparison with the BCG vaccinated control group. However the study did not meet the primary endpoint, and further studies are needed to see if this finding can be reproduced.

1.4.5.3 Evidence for NTM Interference on BCG Efficacy: Animal Models

Paper	Model species	Species Strain	Route	Dose	Live/ Dead	Dose repeats	BCG	Challenge	Protection
Brandt 2002	Mice	Mix of MA MI MV	SC	$2x10^6$	Live	3	After sens.	Erdmann	No
Demangel 2005	Mice	MA MI MV	SC	$2x10^6$	Live	3	After sens.	H37Rv	Yes
Collins 1971	Mice	MK MI MA MF MS	IV	$1x10^6$	Live	1	After sens.	Erdmann	Yes
Orme 1984	Mice	MK MS MA M Sim	Aer	$1x10^4$	Live	1	After sens.	H37Rv	Yes
Orme 1986	Mice	MA MK	SC	$1x10^4$	Live	1	After sens.	H37Rv	Yes
Flaherty 2006	Mice	MA	Oral	$1x10^2$	Live	8	Before sens.	Erdmann	No
Buddle 2002	Cow	?	?	?	?	?	After sens.	*M. bovis*	No
Thom 2008	Cow	MA	SC	$1x10^6$	Live	1	After sens.	*M. bovis*	Yes
Edwards 1982	Guinea Pig	MI	ID	$1x10^6$	Live	1	After sens.	H37Rv Clinical Isolate	Yes/ No
de Lisle 2005	Guinea Pig	MA 206 207	Oral	$1x10^8$	Live	3	After sens.	*M. bovis*	No Yes

Figure 1-4: Table summarising the published animal models of NTM interference in BCG vaccine efficacy. Abbreviations: MA = *M. avium*, MI = *M. intracellulare*, MK = *M. kansasaii*, MS = *M. scrofulaceum,* MV = *M. vaccae*, M Sim = *M. simiae*. MF = *M.* fortuitum. SC = subcutaneous, IV = intravenous, Aer = aerosol, ID = intradermal. Sens. = sensitisation.

The model of NTM exposure in mice published by Brandt et al demonstrates that memory responses induced by NTM are capable of inhibiting BCG efficacy[119]. Mice were administered a mix of *M. avium, M. scrofulaceum* and *M. vaccae* 3 times at 2 week intervals, this infection was left for 3 weeks then treated with antibiotics. IFNγ levels were initially induced but began to drop off which correlated with the time BCG CFU decreased. After subsequent *M. tb* challenge, there was no protection in mice sensitised and then BCG vaccinated. Interestingly this group had less protection than mice which were sensitised to NTM with no BCG vaccination. Brandt et al also tested NTM isolates from the Karonga District in Malawi; two of

the isolates tested, both *M. avium*, induced persistent infections in C57BL/6 mice, whereas other isolates were cleared by the immune response. Infection was cleared with antibiotics and mice were then vaccinated with BCG; BCG replication was inhibited and IFNγ was reduced in the mice which received *M. avium* but not the other isolates.

This study led to the conclusion that prior NTM exposure restricted BCG replication and this was the cause of reduced efficacy of the vaccine. In this study the effect appeared only to be induced by certain isolates which could cause persistent infections or possibly by giving a mix of species.

Demangel et al used this model of NTM exposure in a later study and found that *M. avium*, *M. vaccae* and *M. scrofulaceum* alone did not result in reduced efficacy of BCG against *M. tb* challenge[120]. In fact *M. scrofulaceum* sensitisation gave significantly more protection than BCG alone. *M. avium* alone did reduce the replication of BCG after vaccination and IFNγ levels were lower than in BCG only controls, although the effect was not enough to decrease efficacy of the vaccine. The disparity in this assay may come from the strains of bacteria used, that Brandt et al used a mix or even the strain of BCG used for vaccination.

Collins and Orme tested exposure of many species of NTM in 3 separate studies which are reviewed in Chapter 3, these same studies also observed the effect of subsequent BCG vaccination after NTM infection[121-123]. In Collin's studies, mice were infected with NTM intravenously (IV) then vaccinated with BCG Tice IV. None of the strains, even those which produced a persistent infection, reduced the efficacy of BCG against *M. tb* challenge. Orme et al infected mice via aerosol route then vaccinated with BCG IV, similarly efficacy of BCG was not compromised by prior sensitisation. Orme et al also studied subcutaneous infection with NTM followed by subcutaneous vaccination with BCG and again there was no decrease in the efficacy of BCG. These studies showed that despite some of these NTM species being able to

induce a persistent infection and many of them inducing a level of immunity against *M. tb* they did not reduce the efficacy of BCG. This contradicts Brandt's observations although the effect of multiple bacteria or multiple doses was not tested.

The induction of regulatory responses by NTM was suggested as a mechanism to reduce BCG efficacy in a recent study by Ho et al[124]. They reported that *M. chelonae* sensitisation with 3 doses of dead bacteria induces CD4+ CD25+ T cells which are functionally suppressive in BCG infection. Sensitisation induced production of IL-10 which may have led to a priming of regulatory type responses in the initial exposure; IFNγ responses on subsequent infection with BCG were reduced. Whether or not *M. chelonae* exposure affected the efficacy of BCG vaccination was not tested

Sensitising guinea pigs with multiple doses of a particular strain of *M. avium* (WAg206) reduced the efficacy of subsequent BCG vaccination against *M. bovis* challenge[125]. Another strain of *M. avium* (WAg207) was tested in parallel and produced no effect of BCG efficacy. A subsequent report on infections with these strains in mice showed that Wag 206 was more pathogenic, did not induce activation of macrophages and upon BCG vaccination induced a large antibody mediated response skewed toward the Th2 IgG1 subclass[126]. This study demonstrates how different strains of NTM can induce a different type of response to BCG vaccination ultimately affecting the outcome of the vaccination. Another interesting point to consider with this model is that *M. avium* was given orally which may induce different responses to the infection than would be induced by systemic administration.

In a study by Thom et al exposure of cattle to *M. avium* prior to BCG did not compromise BCG efficacy against *M. bovis* challenge, in fact there was lower TB pathology in these animals than in BCG vaccinated only animals[127]. High levels of purified protein derivative of *M. avium* (PPD-A) specific IFNγ was induced by sensitisation but an equivalent PPD of *M. bovis* (PPD-B)

specific response was induced on challenge compared to BCG vaccinated animals. *M. avium* and BCG were given subcutaneously in this study and only one dose of *M. avium* was given.

The most representative model of human exposure was performed as an accident by Buddle et al[128]. Calves were entered into a BCG vaccine study and were found to have higher levels of PPD-A responses than had been recorded in two previous studies; these calves had been naturally exposed to NTM prior to entering the trial. On challenge with *M. bovis,* BCG was not effective at preventing disease.

1.4.5.4 Evidence for NTM Interference on BCG Efficacy: In Vitro Assays

Cheng et al conducted a killing assay using cells isolated from volunteers in South India and the UK[129]. The capability of the volunteer's cells to kill *M. microti* was assayed as a read-out of the capability to control *M. tb* infection, the assay was performed pre and post BCG vaccination. Indian volunteers were more capable of controlling *M. microti* pre-vaccination than UK volunteers. BCG vaccination enhanced *M. microti* growth restriction in UK volunteers but there was no enhancement of the response in Indian volunteers. The outcome was explained with the theory that BCG vaccination could not improve on the magnitude of response already generated by pre-exposure to NTM.

Weir et al who conducted analysis of IFNγ responses pre- and post-vaccination in UK and Malawian adolescents observed a similar outcome[130]. They analysed the response induced to PPDs from a range of NTM as well as PPD from *M. tb* and *M. bovis*. Malawian volunteers had responses to a diverse range of species prior to vaccination as previously discussed. After vaccination the UK volunteers induced the greatest magnitude of responses against PPDs of mycobacteria most closely related to BCG which included *M. tb*. The response to those PPDs had been of a high magnitude in Malawian volunteers before vaccination and BCG vaccination did not improve on the magnitude of the IFNγ responses to these species' PPDs. Interestingly

the percentage of responders to PPDs of species less closely related to BCG was increased by BCG despite them being less genetically related.

1.4.5.5 Innate Responses

Innate responses in UK and Malawian volunteers were analysed in a further study conducted by Weir et al[131]. IL-1β, TNFα and IL-10 production were all greater in Malawian volunteers compared to UK volunteers before vaccination. After vaccination the concentration of IL-10 was far greater in Malawian volunteers than UK volunteers; IL-1β and TNFα were similar between the groups. The authors suggested the increased regulatory cytokines production by innate cells in Malawian volunteers may be due to different levels of monocytes or activation of PRRs on those monocytes by regular exposure to PAMPs.

1.5 Novel TB Vaccines

1.5.1 Design of Vaccines

There are two main groups of vaccines; the live attenuated vaccines which are attenuated forms of the pathogen that induce a similar immune response but are not pathogenic, eg BCG[132]. The second group include subunit vaccines (e.g. Hepatitis B), toxoid vaccines (e.g. diphtheria and tetanus), carbohydrate vaccines (e.g. pneumococcal) and conjugate vaccines (e.g. *Haemophilus influenzae* type B) and are often administered with adjuvants to increase immunogenicity.

1.5.2 Vaccine Adjuvants

Adjuvants are formulations of chemical compounds which can induce a greater immune response to vaccination. They can also skew the type of immune response induced toward humoral or cellular mediated immunity

1.5.2.1 T Cell Inducing Adjuvant

GSK's adjuvants AS01 and AS02 contain 2 immunostimulants combined with liposomes (AS01) or in an oil-in-water emulsion (AS02). One of the immunostimulants is monophosphoryl lipid A (MPL), the other molecule *Quillaja saponaria* fraction 1 (QS21) has been shown to enhance cell-mediated responses induced by MPL[133]. These adjuvants in combination with Mtb72, a fusion protein of serine proteases Mtb32A and Mtb39A from *M. tb* which was developed as a novel TB vaccine, have been shown to induce antigen specific CD4+ T cells in human volunteers.

1.5.2.2 Aluminium Adjuvants

Aluminium salts are the most widely used adjuvant and have been used in vaccination for decades despite not knowing how they work. Aluminium hydroxide and aluminium phosphate are the most commonly used aluminium salts in vaccination. Originally it was thought these adjuvants acted through the induction of a depot effect[134]. Contradictory to this idea is a recent report that showed removal of the site of vaccination did not decrease the efficacy of vaccination.

Several studies have showed aluminium adjuvants can induce the NACHT, LRR and PYD domains-containing protein 3 (Nalp3) inflammasome leading to production of IL-1β, IL-18 and IL-33[135]. This may be activated directly by the aluminium salt or indirectly possibly by induction of cytotoxicity signals such as uric acid or by inducing membrane disruption and potassium release[136].

Aluminium adjuvants recruit inflammatory monocytes, DCs, neutrophils and eosinophils to the site of vaccination[135]. A recent study with aluminium hydroxide showed cellular infiltration predominantly consisted of neutrophils and eosinophils[134]. Eosinophil infiltration and production of IL-4 and IL-5 has been associated with induction of Th2 skewed immunity.

Aluminium adjuvants are classically associated with inducing a Th2 and antibody mediated responses.

1.5.3 Novel TB Vaccine Candidates

The efficacy of BCG in preventing childhood forms of systemic disease mean it would be unethical to stop vaccination with BCG. This has led to development of either 'improved' BCG vaccines or agents which 'boost' the immune responses induced by BCG vaccination. Twelve novel TB vaccines are currently in clinical trials[137]. They range in their design and administration routine and include recombinant BCGs, attenuated *M. tb* strains, adjuvanted-proteins as a boost on prior BCG vaccination and recombinant viral vectors as a boost on prior BCG vaccination. Vaccines may be designed to prevent infection, prevent primary disease, latent disease, reactivation of latent disease or shorten antibiotic therapy duration.

1.5.4 Novel TB Vaccine Candidate MVA85A

1.5.4.1 Modified vaccinia Ankara Vector

Modified vaccinia vaccine Ankara (MVA) is an attenuated poxvirus[138]. It was developed as a safer alternative for smallpox vaccination than the parental vaccinia virus and was administered to over 100 000 people in the smallpox eradication campaign. MVA is replication deficient but can produce similar levels of recombinant proteins as other replication competent vectors making it equally as immunogenic and remaining extremely safe. Insertion of the recombinant protein Antigen 85A (Ag 85A) into MVA has provided a promising vaccine for efficacy against *M. tb*.

1.5.4.2 Antigen 85A

Ag 85A is one of three members of a family of secreted proteins which are highly expressed by all mycobacterial species. The protein is highly immunogenic. Murine epitopes for both CD4+ and CD8+ T cells have been found in the 85A sequence and specific cells from both arms

of the T cell response have been identified in TB patients[139]. There is 90% homology between the Ag 85A protein produced by BCG and *M. tb* which makes it an ideal candidate for use in prime-boost strategies[140].

1.5.4.3 Clinical Trials

MVA85A administered to BCG naïve volunteers in the UK induces high levels of Ag 85A specific IFNγ which is predominantly produced by CD4+ T cells[141]. Using MVA85A as a boost after BCG vaccination induced greater magnitude of T cell responses and a longer-lived immune response than either vaccine alone. These preliminary phase I studies proved the MVA85A vaccine was safe and immunogenic. Further studies in the UK showed that the same magnitude of response was induced by MVA85A whether it was administered 4 weeks after BCG or up to 10 years after BCG[142].

The safety and immunogenicity of MVA85A has been proven in The Gambia and South Africa[143,144]. MVA85A was capable of boosting frequencies of polyfunctional Th1 cells, Th1 which can produce a combination of IFNγ, TNFα and IL-2, which were theorised to be a better predictor of vaccine efficacy. Induction of CD8+ T cells was recorded in the Gambian study but not in the UK or South Africa. Safety of the vaccine has also been proven in *M. tb* infected patients, children and adolescents and HIV infected patients[145-147]. MVA85A is currently in a phase IIb efficacy trial to determine prevention of *M. tb* infection in infants.

1.5.4.4 Further Information from Pre-Clinical Models

It has also been shown in mice that administering BCG intranasally induces strong responses in the pulmonary lymph nodes which can be further boosted by MVA85A administered intranasally[148]. These mice receiving intranasal boosting with MVA85A are significantly better protected in *M. tb* challenge than mice vaccinated intradermally with BCG or those vaccinated intranasally with BCG.

In cattle BCG followed by intradermal MVA85A vaccination boosted protection against *M. bovis* challenge compared to BCG alone[149]. Immunological analysis showed boosting with MVA 85A induced stronger IFNγ responses, recognition of a wider range of T cell epitopes and stronger memory T cell responses. IL-17 responses were also induced by MVA85A and were correlated with lower pathology scores

A regimen of subcutaneous BCG followed by intradermal MVA85A and subsequent fowlpox 85A protected guinea pigs from *M. bovis* infection at a greater level than BCG[141,144,150]. Interestingly when this regimen was tested in humans, Fowlpox did not enhance the immunogenicity induced by BCG and MVA85A. The improved efficacy of an MVA85A boost after BCG vaccination, compared to BCG vaccination alone, was also demonstrated in rhesus macaques[151].

1.5.5 Evidence of Interference with Immunogenicity Induced by MVA85A

There may be an effect of NTM exposure on the immunogenicity induced by MVA85A, a publication comparing the immunogenicity of MVA85A in different trial sites is in preparation (Checkley et al). Median IFNγ responses induced 7 days after vaccination are of lower magnitude in South African and Gambian volunteers compared to UK volunteers; this is an interesting result and resonates with what is seen in BCG vaccination [141,143,144] . Despite the lower peak magnitude the long-term frequencies of Th1 responses are comparable if not higher than in UK vaccinees and may demonstrate a beneficial effect of NTM exposure.

There are other factors which may interfere with immunogenicity of vaccines including existing vaccine regimens. A novel vaccine would ideally be incorporated into the EPI schedule to ensure maximum coverage of a population. The immunogenicity of novel malaria vaccine RTS,S/AS02 was evaluated when combined with the existing EPI vaccine against Diphtheria, tetanus, whole cell pertussis and *Haemophilus influenzae* B (DTwP-Hib)[152]. Inclusion of the

malaria vaccine in the EPI schedule reduced antibody responses induced by the existing EPI vaccine as well as antibody responses against the malarial sporozoite antigens in RTS,S. A similar deleterious effect on antibody titres against tetanus toxoid was recorded when a new pneumococcal vaccine was introduced with DTwP-Hib[153]. Although conversely, combining the vaccines enhanced the Hib and pneumococcal antibody titres.

Co-administering MVA85A with vaccines in the EPI schedule in the Gambia was assessed for safety and immunogenicity. No effect on the immunogenicity of the EPI vaccines was observed but there was a significant reduction in the Ag85A specific IFNγ response when vaccines were co-administered. This result was assumed to be due to the presence of alum in the EPI vaccines which is known to induce a Th2 skewed response.

1.6 Study Aims

1.6.1 The Effect of NTM Exposure on BCG Efficacy against *M. tb*

A leading theory as to why BCG efficacy against TB is reduced in tropical climates is that exposure to NTM in the environment interferes with vaccine induced immune responses. No conclusive evidence of this effect has been provided from publications of animal models that assay the effect of NTM exposure on BCG conferred protection. A lack of evidence is due to highly variable outcomes on the level of BCG efficacy across the models and a limited analysis of the immune response mechanisms involved. The aim of this study was to perform a model of NTM exposure after BCG vaccination which would reduce the level of protection afforded by BCG against *M. tb* challenge. Profiling the adaptive immune response would hopefully show the mechanism by which BCG efficacy is reduced. The C57BL/6 mouse model was used for this study and *M. avium* was chosen as the NTM for exposures. Exposures were performed after BCG vaccination to model what happens in human populations. Two models of exposure were performed; dead *M. avium* systemically and live *M. avium* orally. The effect of both

sensitisation regimens on the efficacy of BCG was tested by *M. tb* aerosol challenge. Extensive profiling of the adaptive immune response was performed pre- and post-challenge to determine what immunological mechanisms are involved. Flow cytometry and Flow Cytomix were used to evaluate the adaptive immune response profiles.

1.6.2 Interference between EPI Vaccines and MVA85A

Given the variability in the protection afforded by BCG there is a need for development of a new TB vaccine. Inclusion of a novel TB vaccine in the EPI schedule would ensure maximum coverage of the population, however, there is evidence of interference from pre-existing vaccines in the schedule on the immunogenicity induced by the novel TB vaccine MVA85A. To investigate this effect further a study was designed to model the interaction between MVA85A and aluminium adjuvants or aluminium-containing vaccines in BALB/C mice. Adaptive immune responses were profiled using flow cytometry to elucidate the mechanisms by which MVA85A immunogenicity may be reduced. The effect of co-administering aluminium adjuvant at the same time as MVA85A was analysed, as was the effect of this regimen in BCG vaccinated mice. A replication of the infant study performed in The Gambia by Ota et al was performed in BALB/C mice by administering 2 EPI vaccines in a 4 month schedule and vaccinating with MVA85A at the final dose time point. In each experiment it was also tested whether interference could be avoided by vaccinating with MVA85A intranasally rather than intradermally.

2 Materials and Methods

2.1 Materials

Product	Supplier	Catalogue Number
85A 66p	Peptide Protein Research Ltd	N/A
ADC	BD bioscience	212352
Alhydrogel (3% aluminium hydroxide)	Brenntag Biosector, Denmark via Lausane GADI lab	N/A
Adju-Phos (2% aluminium phosphate)	Brenntag Biosector, Denmark	N/A
Collagenase	Sigma	C7657-500MG
DMSO	Sigma	D2650
DNAse	Sigma	D5025-150KU
ENGERIX-B®	GlaxoSmithKline, UK	
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, UK	E7889
FBS GOLD EU Approved	PAA Laboratories Ltd	A15-151
Flowcytomix basic kit	eBioscience	BMS8440FF
Flowcytomix IFNY	eBioscience	BMS8606/2FF
Flowcytomix IL-10	eBioscience	BMS8614/2FF
Flowcytomix IL-4	eBioscience	BMS8613FF
Flowcytomix IL-5	eBioscience	BMS8610FF

Flowcytomix TGFβ	eBioscience	BMS8608FF
Glycerol	Sigma	49767
Iso-Flo Isofluorane	Oxford University Veterinary Services	N/A
L-glutamine solution 200mM	Sigma	G7513
Low Phosphate PBS	Gibco-Invitrogen	10010
Middlebrook 7H10 agar	Sigma	M0303
Middlebrook 7H9 media	BD bioscience	271310
OADC enrichment	BD bioscience	212240
PEDIACEL®	Sanofi Pasteur MSD Ltd, UK	
Penicillin-streptomycin	Sigma	P0781
PHA	Sigma	L4144
Phosphate Buffered Saline (PBS)	Sigma	D8537
PPD-A (*M. avium* PPD)	Veterinary Laboratories Agency, Surrey	N/A
PPD-T (Tuberculin PPD)	Staten Serum Institute	2391
RPMI + L-glutamine + HEPES	Sigma	R0883
RPMI-1640 Medium	Sigma	R0883
TAPI	Merck Biosciences Ltd	579052
Tween 80	Difco, BD	231181

2.2 Equipment and Software

Equipment/ Software	Supplier	Product Number/ Version
15 ml Falcon Tube	Corning	430791

150ml Universal Container	Sterilin	
50 ml Falcon Tube	VWR	89039-658
5ml Syringe	Plastipak	302187
6 well tissue culture plate	Corning	3516
7 ml Bijou	Sterilin	129A
70µm Sieve	Fisher Brand	22363548
96 well U-bottom Tissue Culture Plate	Falcon	3077
Casy Counter	Innovatis	Model TT
Category Level 3 Containment Mouse Cage	Techniplast, Italy	N/A
Centrifuge – for cryovials	Eppendorf	5430
Centrifuge – large	Sorvall	Legend RT
Collision Nebuliser	BGI INC, USA	N/A
Cryotube Vials	Thermo Scientific	377267
Dosing Needle	Harvard Apparatus	34-0322
Facs Diva	BD	Version 6.0
Flow Jo	Tree Star Inc	Version 8.8.7 and 9
LSR II Flow Cytometer	BD	N/A
Manifold for Pump	Whatman	N/A
Microlance 3 26G Needle	BD Medical	304300
Nose-Only Mouse Restrainers	Biaera Technologies, USA	N/A
Plate Shaker	Stovall Life Science	BDHWB220
Polypropylene Cluster Tubes	Costar	4401

Polystyrene Facs tubes	Falcon	2058
Prism	GraphPad Software	Version 5.0
Rotating incubater	New Brunswick Scientific, USA	Innova 40
Sonicator	VWR	Ultrasonic Cleaner
Spectrophotometer	Beckman Coulter	DV 730
Spice	National Institute of Allergy and Infectious Disease	Version 5.2
U-100 29G Syringe	BD Medical	324891
Vacuum Pump	Charles Austen Pumps	DA7C

2.3 Mycobacteria

2.3.1 Species, Strains and Sources

BCG Vaccine Danish strain 1331: Staten Serum Institute, Copenhagen supplied as lyophilized BCG with a separate solvent. Average colony forming units (CFU) of batch used was 7.82 x 10^5 per vial. BCG Pasteur: a kind donation from Ann Rawkins, Health Protection Agency, Porton Down, UK. *M. avium* 2-151: a kind donation from Professor Ian Orme, University of Colorado, USA. *M. avium* 724: a kind donation from Ian Orme.

M. avium 104: a kind donation from Professor Andrea Cooper, Trudeau Institute, USA. *M.tb* Erdmann KO1: supplied by the US Food and Drug Administration (FDA) or BEI Resources (Manassas Virginia, USA).

2.3.2 Culturing *M. avium* and BCG in Liquid Broth

A 15ml Universal container containing 10ml of Middlebrook 7H9 media (made up as directed on container with 10% ADC supplement, 0.02% Glycerol and 0.05% Tween 80) was inoculated

with a seed stock of bacteria and incubated in a rotating incubator at 30°C for *M. avium* and 37°C for BCG. After around 5 days the optical density (OD) was checked using a spectrophotometer at 600nm. When *M. avium* cultures reached an OD between 0.6 and 1 the bacteria was prepared for freezing. BCG was prepared for freezing at 0.4 OD. In experiments in Chapter 3 and experiment 2-151 in Chapter 4 the liquid culture method for *M. avium* was used

2.3.3 Culturing *M. avium* on Solid Agar

In experiment 724 in Chapter 4 and experiments in Chapter 5 and Chapter 6 when the liquid culture reached an OD between 0.6 and 1 it was diluted 1 in 100,000 and plated onto Middlebrook 7H10 plates with 10% OADC enrichment and 0.05% glycerol, a sterile spreader was used to distribute bacteria over the plate then incubated at 30°C until individual colonies were visible (2 weeks- 3 weeks). Smooth transparent, smooth opaque and rough morphotypes can be distinguished on the agar, only the smooth transparent and rough colonies were picked and suspended in 7H9 media then incubated in rotating incubator at 30°C overnight to reduce clumping.

2.3.4 Freezing BCG and *M. avium* cultures

Broth cultures were transferred to 50 ml Falcon Tubes and centrifuged at 3000rpm for 30 minutes. The supernatant was discarded and the pellet resuspended in 30ml sterile PBS. The culture was centrifuged again at the same setting. For seed stocks mycobacteria was resuspended in a solution of 50% 7H9 Broth and 50% glycerol, aliquoted to cryovials and stored at -80°C. Bacteria used for vaccinations were frozen in sterile PBS at -80°C in 1ml aliquots.

2.3.5 Quantifying CFU of BCG, *M. avium* and *M. tb*

Frozen aliquots of bacteria were thawed and sonicated three times for 1 minute with a 1 minute rest on ice between each sonication. Bacteria were then serially diluted in sterile PBS

before plating on Middlebrook 7H10 plates. Colonies could be counted after 2 – 4 weeks incubation at 30°C for *M. avium* and 37°C for BCG and *M. tb.*

2.3.6 Preparing BCG, *M. avium* and *M. tb* for Administration to Mice

Frozen aliquots of bacteria were thawed and sonicated three times for 1 minute with a 1 minute rest on ice between each sonication. Where *M. avium* was to be given heat-killed the aliquots was placed in a water bath at 80°C for 25 minutes. Bacteria were then diluted to the required concentration in sterile PBS before administration.

2.4 Vaccines and Adjuvants

2.4.1 MVA85A

Modified vaccinia Ankara (MVA) expressing amino acids 1-323 of antigen 85A under the control of vaccinia virus P7.5 early/late promotor. Aliquots of virus were stored at -80°C and thawed before use. Vaccine was diluted in sterile PBS to required plaque forming unit (pfu) concentration and kept on ice until vaccination.

2.4.2 Aluminium Adjuvants

A volume of 8.5µl of Alhydrogel containing 85µg aluminium (Al^{3+}) was given in 100µl volume diluted in low phosphate PBS. 17µl of Adju-Phos, which contains 85µg of Al^{3+}, was given in a 100µl volume of low phosphate PBS.

2.4.3 ENGERIX-B®

A volume of 100µl of ENGERIX-B® was administered to each mouse, this contains; 2µg of hepatitis B surface antigen and 0.05 mg Al^{3+}.

2.4.4 PEDIACEL®

Each vial of 500µl contains; Diphtheria toxoid < 30 international units (IU), Tetanus toxoid <40 IU, Acellular Pertussis antigens (Pertussis toxoid 20µg, filamentous haemagglutinin 20µg, pertactin 3µg, fimbriae types 2 and 3 5µg), Inactivated Poliomyelitis (type 1 40D antigen units, type 2 8D antigen units, type 3 32D antigen units), *Haemophilus influenzae* type b polysaccharide (polyribosylribitol phosphate 10µg) conjugated to 20µg Tetanus toxoid and 1.5mg aluminium phosphate (0.33mg Al^{3+}). Mice received 100µl of the vaccine.

2.5 Animals

2.5.1 Strains

Female C57BL/6 J mice from Harlan Laboratories, UK were used in the environmental mycobacteria exposure models. Female Balb/C mice from Harlan Laboratories, UK or Charles Rivers UK Ltd were used in MVA85A experiments. Animals were generally ordered at six weeks of age. Ex-breeders and 9 week old mice were sometimes used as Naïve controls in aerosol challenge experiments.

2.5.2 Welfare

All procedures were performed under project license 30.2414 until the 13th February 2012, then under PPL 30.2414 after this date, by me (personal license number 30/8539) and sometimes with assistance of a fully trained and licensed colleague. Groups of animals were kept in a specific pathogen free facility within individually ventilated cages. All housing and procedures were in compliance with the 'Code of Practice for the Housing and Care of Animals used in Scientific Procedures' and followed the 'Guidance on the Operation of the Animals (Scientific Procedures) Act 1986'.

2.5.3 Anaesthetic

For intramuscular, intradermal and intranasal vaccinations mice were anaesthetised by inhalation of Isofluorane (2-4%) and oxygen (2L/min) in air. After the procedure mice were monitored for full recovery from anesthetic.

2.5.4 Oral Gavage

Mice received 100µl of *M. avium* suspended in PBS via a dosing cannula inserted directly in to the stomach. Mice were restrained but not anaesthetised for the procedure.

2.5.5 Intraperitoneal Injection

A volume of 100µl of *M. avium* suspended in PBS was administered with a U-100 29G syringe in the right or left side of the peritoneal cavity. Mice were restrained but not anaesthetised for the procedure.

2.5.6 Subcutaneous Injection

A volume of 100µl of BCG in PBS was injected at the base of the tail using a U-100 29G syringe. Mice were restrained but not anaesthetised for the procedure.

2.5.7 Intradermal Injection

MVA85A or BCG was administered as two 50µl volumes in PBS over two ears with a U-100 29G syringe. Mice were anaesthetised for the procedure.

2.5.8 Intranasal Administration

A volume of 100µl volume of MVA85A suspended in PBS was gradually pipetted on to one nostril of the mouse. Mice were anaesthetised for the procedure.

2.5.9 Intramuscular Injection

Aluminium adjuvants were suspended in PBS and 50µl administered in each hind limb with a BD Microlance 3 26G needle. Mice were anaesthetised for the procedure.

2.5.10 *M. tb* Aerosol Challenge

Mice were transferred to the category level III (CL3) suite and loaded into nose-only restrainers. Restrainers were inserted into an exposure chamber inside a CL3 isolator. *M.tb* was diluted in sterile PBS and put in to a collision nebuliser. Dose escalation studies were performed to determine the concentration of *M.tb* needed to deliver 100 CFU to each mouse in a 10 minute period of nebuliser activity followed by a 5 minute purge cycle with clean air. The Biaera aeroMP controller monitored the airflow and pressure in the apparatus. After exposure mice are removed from the restrainers and housed in CL3 containment cages.

2.5.11 Collection and Preparation of Spleen and Lymph Nodes

Mice were culled by cervical dislocation and spleens sterilely dissected and placed in PBS. Spleens were mashed through a 70µm sieve with the end of a 5ml syringe in wells of a 6 well culture plate then resuspended in PBS and transferred to a 50ml Falcon tube. Cells were then spun at 1500 rpm for 5 minutes. Lymph nodes were resuspended in RPMI supplemented with 10% FBS, 2mM L-glutamine and 1% pen-strep (R10). Red blood cells in the spleen samples were lysed with ACK lysis buffer (0.15M NH_4Cl, 1mM KCO_3, 0.1mM Na_2EDTA) over 5 minutes then washed in PBS before resuspending in R10. At this stage a clump of debris from the cell lysis step was removed with a Pasteur pipette. A Casy Counter (settings: 5.50-10.80µm, X-axis 0-20µm) was used if cell suspensions were counted. Generally spleens were resuspended in 5ml of R10 and a 1 in 2000 dilution of cells made for counting. Lymph nodes were reuspended in the volume of R10 required for the number of wells to be plated and the remainder counted at 1 in 2000.

2.5.12 Collection and Preparation of Lungs

Mice were culled by cervical dislocation. Lungs were perfused before dissection by injecting roughly 2 ml of sterile PBS into the heart. Lungs were then dissected into PBS, diced into 1mm pieces and suspended in 5ml of RPMI with 700µg/ml collagenase and 30µg/ml DNAse (Sigma) for 30minutes at 37°C. The reaction was stopped by adding R10 then the suspension was poured through a 70µm seive and mashed with a 5ml syringe. Samples were spun at 1500rpm for 10 minutes then treated with ACK lysis buffer, spun again and resuspended in R10. Lung samples were not counted.

2.5.13 Collection and Preparation of Blood

Mice were anaesthetised and maintained on isofluorane whilst a U-100 syringe was inserted into the heart. Blood was extracted into the syringe and transferred to a bijou containing 4ml of 10mM EDTA in PBS. The mouse was terminated by cervical dislocation immediately after the blood was withdrawn. Blood was transferred to a 15 ml falcon tube and 5 ml ACK lysis buffer added for 5 minutes then the sample spun at 1500 rpm for 5 minutes. Samples were resuspended in R10 to the volume needed to plate the number of wells in the assay. Samples were counted at 1 in 2000 at the same settings as for spleen and lymph nodes (see above).

2.5.14 Cell stimulation for ICS and Flow Cytomix

A volume of 150µl of cells suspended in R10 was transferred to a 96 well cell culture plate. Generally 3 x 10^6 splenocytes would be plated per well for flow cytometry and 0.25 x 10^6 for flow cytomix. Blood, lung, lymph node and all CL3 samples were not counted prior to plating so the number of cells in the wells varied although the volume was always 150µl. Stimulations were added to the cells in 50µl volumes; peptide and protein stocks were kept in PBS at -20°C, thawed and diluted in R10 before use. Purified protein derivative from *M. tb* (PPD)-T and PPD from *M. avium* (PPD-A) were used at a final concentration of 10µg/ml in the well. A pool of 66 peptides which span the length of antigen 85A (85A 66p) were used at a final concentration of

2µg/ml. Lectin from *Phaseolus vulgaris* (PHA) was used at 10µg/ml final concentration as a positive control in Flow Cytomix. All samples had an un-stimulated control well (50µl of R10) which provided a background activity level for subtraction in analysis of stimulation specific responses. Plates were incubated at 37°C with 5% CO2.

An adaptation of this protocol was required when staining stimulated cells for CD62L. TNFα proteolysis inhibitor (TAPI)-2 prevents cleavage of CD62L when cells are stimulated[154]. 1 mg of TAPI-2 was diluted in 960µl of sterile PBS, 6µl of this solution was added to each well of the cell culture plate before cells were plated. Cells and TAPI-2 were then incubated at 37°C for one hour before stimulation. 2µl per well of TAPI-2 is added into the 50µl stimulation mix.

Cells for flow cytometry were stimulated for 6 hours, if cytokines were to be analysed 0.22µl of GolgiPlug in a 20µl volume of R10 was added to the cells 2 hours after stimulation was started. One exception was the assays performed 13 and 16 days after *M. tb* challenge where the cells were stimulated overnight rather than for 6 hours. Cells for flow cytomix were stimulated for 3 days and for RNA extractions cells were stimulated overnight.

2.5.15 Staining Cells for Flow Cytometry

The Flow cytometry antibodies and reagents used throughout this project are listed in Table 2-1. After stimulation, cells for flow analysis were stored at 4°C before staining started. Plates of cells were spun at 1500 rpm for 3 minutes then resuspended in PBS and transferred to a V bottom well plate. Cells were spun again as above, all spins in this protocol are the same, then stained with 10µl of live/dead fixable stain diluted in PBS at the required concentration for 10 minutes. All incubations in this protocol were on ice and in the dark. Cell surface specific antibodies were diluted in PBS with 2% FBS (PBS-FBS) at a volume of 40µl per well. The cocktail of surface antibodies was added on top of the live/dead stain and incubated for 30 minutes. After incubation 150µl of PBS-FBS was added and cells spun.

If cells were to be stained for intracellular markers they were incubated for 10 minutes with BD fixation/ permeabilisation solution, unless they were to be stained for FoxP3 when they were incubated for 30 minutes with eBioscience fixation/ permeabilisation solution. After fixing and permeabilising the cells were spun and resuspended in perm/wash solution twice; BD or eBioscience depending on whether FoxP3 was to be used. After this 50µl of intracellular staining cocktail, diluted in the perm/wash previously used, was added to the cells for 30 minutes. Cells were spun and resuspended twice more in perm/wash and finally resuspended in PBS-FBS.

2.5.16 Fixing *M. tb* Infected Samples for Flow Cytometry

The protocols for cell processing, stimulations and staining were followed as above but at CL3 containment. After the final stage of staining the plate of cells in PBS-FBS was spun again then the cells resuspended in 100µl of PBS-FBS with 4% paraformaldehyde and incubated on ice in the dark for 30 minutes. The plate was spun again and the samples resuspended in PBS-FBS then transferred to a sterile 96 well U-bottom plate and removed from category level 3.

2.5.17 Compensation Beads

To compensate for fluorescence overlap in multicolour flow cytometry I used compensation beads stained with each of the fluorochrome labeled antibodies used in the assay. In polystyrene facs tubes 100µl of PBS-FBS, 1µl of one of the fluorochrome labelled antibodies, a drop of vortexed positive and of negative compensation beads was mixed. This was repeated for each of the fluorchromes used in the assay then all were incubated for 15-30 minutes at room temperature. After incubation 2ml of PBS-FBS was added to the tubes and spun at 1500 rpm for 3 minutes. The supernatant was poured off, the tube vortexed and beads resuspended in 300µl of PBS-FBS then stored in the fridge until ready to be run on the flow cytometer.

2.5.18 Flow Cytometer and Data Analysis

Samples were transferred from the 96 well plates to polypropelene cluster tubes. All data was collected with a BD LSRII cytometer linked to Facs Diva software. Generally 250,000 total events were acquired from spleen samples and lymph node samples. Occasionally lymph node samples would not contain this number of cells and in lung preparations this was often the case. At these times the entire contents of the sample would be collected. In mice un-infected with aerosolised *M. tb* between 30, 000 and 75, 000 events was a normal collection range for the lung. In mice infected with *M. tb* events from the lung could range from 30, 000 up to the 250, 000 cut-off.

Files were then analysed with FlowJo; an example of a typical gating strategy is displayed in Figure 2-1. Results from FlowJo were exported to Excel. For gating of cytokines the media control sample was used to determine where the negative population fell and so where positive cytokine gates should be drawn. A low level of cytokine producing cells was detected in media control samples which represents a background level of cytokine production. This value is deducted from stimulated cultures to give the stimulation specific response. As a representation of the values usually detected in these models the following values and graphical representation (Figure 2-2) are taken from the model of *M. avium* exposure with live *M. avium* given orally: pre-challenge data (presented in Chapter 5). In the lymph nodes IFNγ+ CD4+ T cells in the media control samples ranged between 0.0038% and 0.036%, IL-2+ CD4+ T cells ranged between 0% and 0.019%, IL-4+ CD4+ T cells ranged between 0.015% and 0.095% and TNFα+ CD8+ T cells ranged between 0.002% and 0.02%. In the spleen IFNγ+ CD4+ T cells in the media control samples ranged between 0.0084% and 0.064%, IL-2+ CD4+ T cells ranged between 0.055% and 0.26%, IL-4+ CD4+ T cells ranged between 0.0048% and 0.075% and TNFα+ CD8+ T cells ranged between 0% and 0.048%.

Experiments in Chapter 3 included a naïve control group which gave an indication of the background level of cytokine producing cells which were stimulated non-specifically from incubation with PPD-T, PPD-A and 85A 66p(Figure 3-2a). Using this data a cut-off of 0.01% was determined for the background level of response once the media control well values had been subtracted; any cytokine level in a vaccinated animal's sample falling at or below this frequency was considered to be background noise.

In the case of the cell surface markers CD62L, CD127 and CD44 a preliminary experiment was performed using fluorochrome-minus-one (FMO) for each of these markers to determine the level of expression and whether a distinct positive population could be gated. The expression of CD127 was not easily distinguishable as it occurred as a blurred signal from the negative population to positive. In all experiments where this marker was used an FMO for CD127 was included. The CD62L and CD44 positive populations were easily distinguishable.

Prism was used to graph the data and apply statistical tests. The median value is used as average and significance between groups tested with the Mann-Whitney U test unless stated otherwise. Statistically significant differences were determined if $p< 0.05$. For some experiments SPICE was used to display complicated data comparisons.

In the memory analysis presented in Chapter 6 a fluctuation in the frequencies of the memory subsets in the naïve mice was seen over the 56 day time course. This made it difficult to see the change of the memory subset frequencies over time which was induced by infection in the infected mice. In order to show the 'infection induced fluctuation' of the memory subsets the natural deviation in frequency that occurred in the naïve group was corrected for in the data of the infected mice. This was done by calculating how much the central memory, effecter memory and terminally differentiated effecter subset frequencies in the naïve group

at day 28, 42 or 56 had differed from day 14. This value was then added to the raw data from all mice in the infected groups at day 28, 42 or 56.

Eg Calculation of central memory frequency:

1: **Y** @ d28 or 42 or 56 = Median of of naïve group @ d14 – median of central memory frequency of naïve group at d28 or 42 or 56

2: Corrected infected group data = Infected mouse central memory frequency @ d28 or 42 or 56 + **Y** @ d28 or 42 or 56

Table 2-1 Flow Cytometry Reagents

Anti-mouse antibody	Company	Catalogue number	Clone	Concentration	Dilution
CD16/32 Fc block	eBioscience	14-0161-86	93	0.5 mg/ml	1:50
Live/Dead Pacific Blue	Invitrogen	L34955	N/A	Unknown	1:300
Live/Dead PE-Texas Red	Invitrogen	L23102	N/A	Unknown	1:200
B220 AF700	eBioscience	56-0452-82	RA3-6B2	0.2 mg/ml	1:300
B220 PE-Texas red	BD Pharmingen	551489	RA3-6B2	0.2 mg/ml	1:300
CD3 PerCP-Cy5.5	eBioscience	45-0031-82	145-2c11	0.2 mg/ml	1:200
CD3 AF700	Biolegend	100216	17A2	0.5 mg/ml	1:400
α/β TCR Pacific Blue	Invitrogen	HM3628	H57-597	0.2 mg/ml	1:100
Y/δ TCR eFluor710	eBioscience	46-5711-82	eBioGL3	0.2 mg/ml	1:500

CD8a APC-AF750	Invitrogen	MCD0827	5H10	0.1 mg/ml	1:30
CD8a APC-ef780	eBioscience	45-0081-80	53-6.7	0.2 mg/ml	1:50
CD4 Pacific Orange	Invitrogen	MCD0430	Monoclonal	0.1 mg/ml	1:300
CD4 eFluor 650NC	eBioscience	95-0041-42	GK1.5	Unknown	1:100
IFNY APC	eBioscience	17-7311-81	XMG1.2	0.2 mg/ml	1:200
IFNY Brilliant Violet 421	Biolegend	505829	XMG1.2	0.025 mg/ml	1:100
IL-2 PE-Cy7	eBioscience	25-7021-82	JES6-5H4	0.2 mg/ml	1:100
IL-2 PE	eBioscience	12-7021-82	JES6-5H4	0.2 mg/ml	1:100
IL-17a PE	eBioscience	12-7177-81	eBio17B7	0.2 mg/ml	1:200
IL-17a PerCP-Cy5.5	eBioscience	45-7177-82	eBio17B7	0.2 mg/ml	1:200
TNFα FITC	eBioscience	11-7321-82	MP6-XT22	0.5 mg/ml	1:200
IL-4 PE	eBioscience	12-7041-81	11B11	0.2 mg/ml	1:100
IL-10 APC	eBioscience	505010	JES5-16E3	0.2 mg/ml	1:50
CD25 APC	eBioscience	17-0251-82	PC61.5	0.2 mg/ml	1:400

FoxP3 PE	eBioscience	12-5773-82	FJK-16S	0.2 mg/ml	1:100
CD39 PE-Cy7	eBioscience	25-0391-82	24DMS1	0.2 mg/ml	1:100
CD127 ef450	eBioscience	48-1271-82	A7R34	0.2 mg/ml	1:50
CD44 AF700	eBioscience	56-0441-82	IM7	0.2 mg/ml	1:200
CD62L PE-Cy7	eBioscience	25-0621-81	MEL-14	0.2 mg/ml	1:100
Anti-Rat/Hampster Compensation beads	BD biosciences	51-90-9000949	N/A	Unknown	N/A
Cytofix/cytoperm kit	BD biosciences	555028	N/A	N/A	N/A
Fixperm concentrate	eBioscience	00-5123-43	N/A	N/A	N/A
Fixperm diluent	eBioscience	00-5223-56	N/A	N/A	N/A
Golgi Stop transport inhibitor	BD biosciences	554742	N/A	N/A	N/A
Golgi Plug transport inhibitor	BD biosciences	51-2301K2	N/A	N/A	N/A

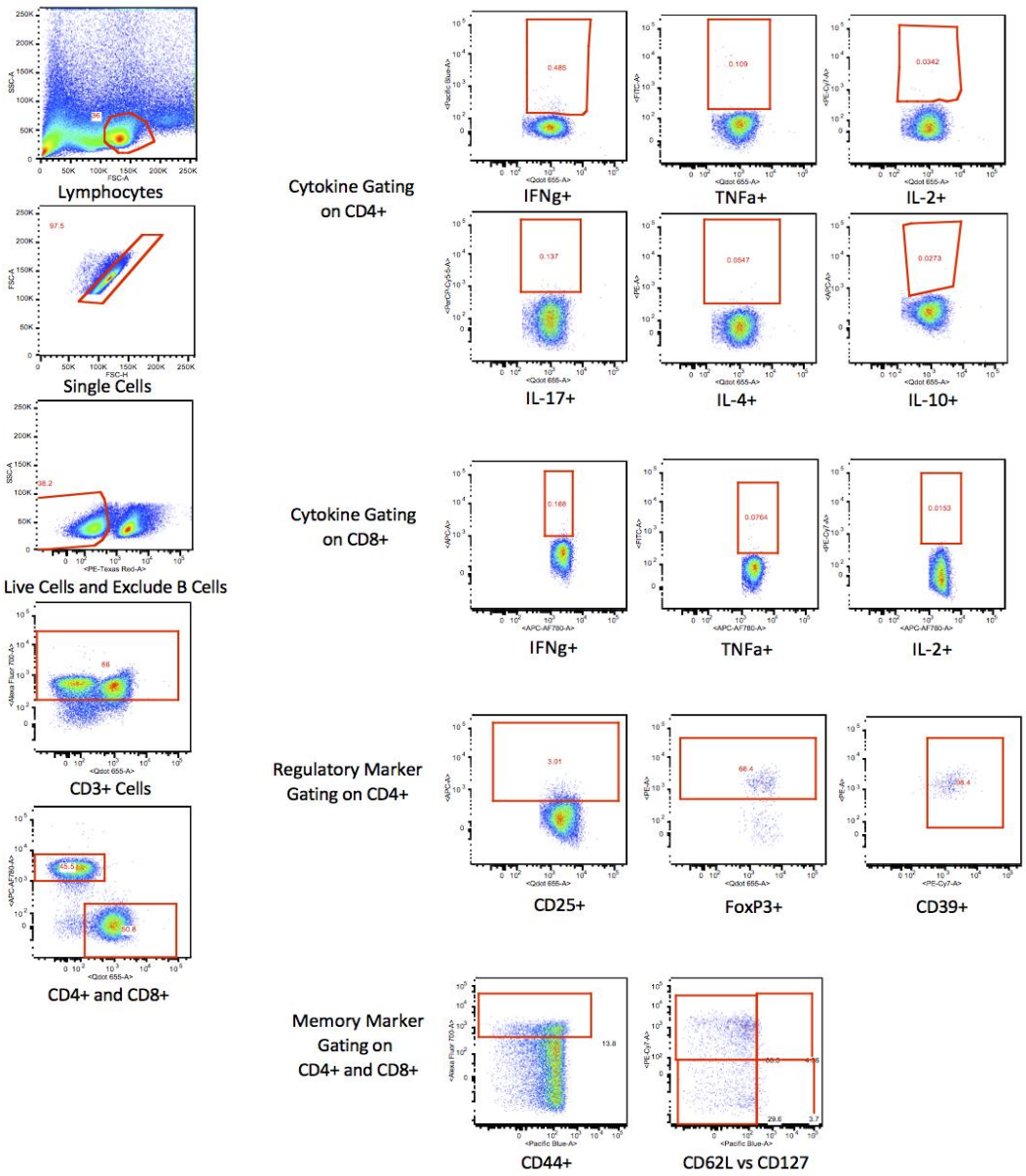


Figure 2-1: Flow cytometry data gating strategy. Gating was performed in the order; lymphocytes, single cells, dead and B cells excluded, CD3+ cells, CD4+ and CD8+ cells. From the CD4+ and CD8 + T cell subsets cytokine, regulatory marker and memory marker gating was performed. All plots representing lymphocyte gating through to cytokine and regulatory marker gating are from one mouse which was BCG vaccinated and exposed to dead *M. avium* 724 IP (results presented in Chapter 4). This was a spleen sample which had been stimulated with PPD-T. Representation of memory marker gating was from a PPD-T stimulated lymph node sample taken from a mouse which had been BCG vaccinated and exposed to dead *M. avium* 724 IP (results presented in Chapter 6). All gating on this lymph node sample prior to memory markers was comparable to the spleen plots shown.

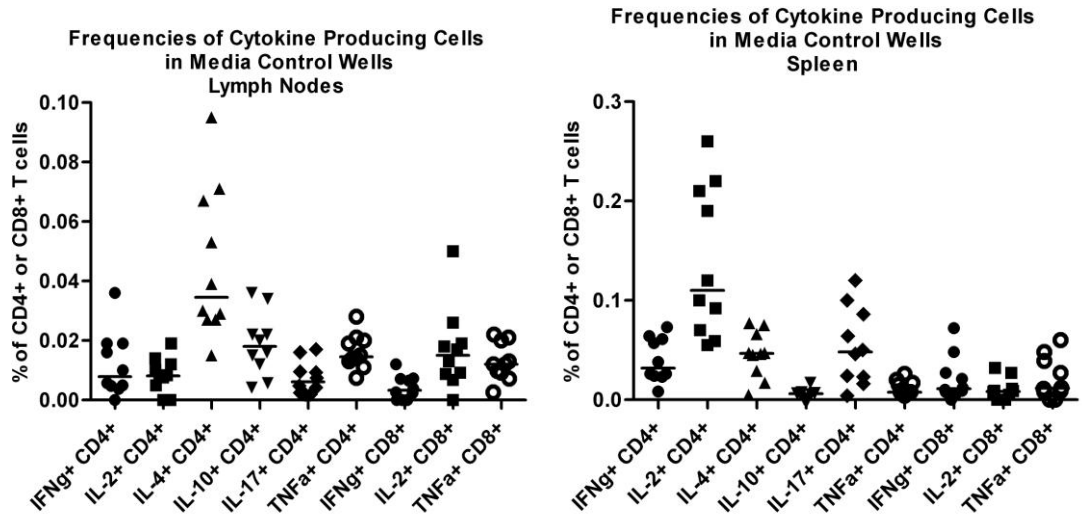


Figure 2-2: Production of cytokine from media control wells. This data is taken from BCG only vaccinated mice and mice vaccinated with BCG which were then exposed to *M. avium* in the model of *M. avium* exposure with live *M. avium* given orally: pre-challenge data (presented in Chapter 5). Data in the left graph is from the mesenteric lymph node and in the right graph from the spleen. Data shows the frequency of cells which secrete cytokine when the sample is not stimulated with antigen.

2.5.19 FlowCytomix

The eBioscience Basic Kit for mouse/rat samples and simplex kits for specific cytokines came with all reagents needed and a protocol that is briefly described below. The assay was performed in the 96 well filter plate supplied with the kit. Liquid had to be pulled through the filter in all wash steps of the assay; which required the use of a vacuum pump and manifold. Supernatants were thawed and the assay buffer supplied in the kit was diluted 1 in 10 with deionized water. Cytokine standard vials were spun in a microfuge briefly then the specified volume of distilled water added and left for 30 minutes. 10µl of each standard to be used in the assay was added to a cryovial and the volume made up to 200µl with assay buffer. A series of serial dilutions at 1 in 3 was then made in 6 further cryovials. 25µl of each of the analyte specific Cytomix beads were needed for each assay well; beads were pooled in a 15ml falcon and washed in assay buffer then resuspended in assay buffer at a volume of 25µl per well. Biotin conjugate specific for each analyte was diluted 1 in 20 in assay buffer to give a volume of 50µl per well. 50µl of assay buffer was added to all wells of the filter plate then removed through the filter with the vacuum pump. 25µl of cytokine standard mixture was

added to standard curve wells, 25µl of assay buffer to blanks and 25µl of supernatant to sample wells. 25µl of the bead mix was then added to all wells followed by 50µl of biotin conjugate. The plate was covered with an adhesive plate cover and foil and left on a shaker at 500rpm for 2 hours. After incubation wells were emptied through the filter and washed twice with 100µl of assay buffer. 100µl of assay buffer was put in each well followed by 1.6µl of streptavidin conjugate diluted in assay buffer to a volume of 50µl per well. The plate was covered by a film and foil and left on the shaker for 1 hour. The plate was washed twice with 100µl of assay buffer. Finally 200µl of assay buffer was added to all wells and mixed with a pipette before transferring to cluster tubes and run on the LSR II flow cytometer. Instructions for cytometer set-up and sample acquisition were included with the kit.

FlowCytomix Pro software can be downloaded from eBioscience.com to analyse the data acquired on the cytometer. The software provides a PDF and data file of results that includes the concentration of cytokine for each sample tested in picograms/ml. I analysed the results in Excel. R10 blank well values were subtracted from all sample well values then media control supernatant samples subtracted from PPD or 66p 85A stimulated supernatant samples to give values for antigen specific cytokine production. These results were then transferred to Prism for graphical presentation and statistical analysis. Median was used as average and data spreads compared with the Mann-Whitney U test. Statistically significant differences were determined if $p < 0.05$.

3 Profiling the Immune Response to *Mycobacterium avium* and BCG

3.1 Introduction

3.1.1 Infections Caused by NTM

The incidence of human disease caused by NTM has increased in recent years. The most accountable reason for this is the rise in the number of people living with HIV and other forms of immune suppression; which are mainly the result of medical interventions such as immune suppression after transplantation[155]. Also the advances in isolation and increased sensitivity of genetic sequencing techniques to identify mycobacteria from clinical samples have undoubtedly led to increases in reported cases. Despite the increases in reported cases, NTM remain opportunistic pathogens in healthy humans. The effect of exposure in the general population is a sub-clinical infection with the induction of a level of immunity against mycobacterial antigens as determined by reactivity to PPDs[130].

Infection can lead to five main forms of disease; pulmonary disease, lymphadenopathy, skin lesions, localised non-pulmonary lesions and disseminated disease[155]. *M. avium* was the most regularly identified species in clinical samples in Europe followed by *M. gordonae*[156]. *M. kansasii* was the most frequently isolated species from pulmonary infections in a study of South African miners[157]. A study of Taiwanese patients presenting with skin lesions defined rapidly growing NTM species as the cause of 43% of infections, of these *M. abscessus* was the most frequently isolated[158]. *M. marinum* was another frequently isolated species from skin lesions and was associated with patients keeping aquatic pets. Lymphadenitis is associated with elderly patients and the immune-suppressed as well as young children with T cell deficiencies[155]. Disseminated disease is most commonly identified in patients with HIV; the *M.*

avium complex (*M. avium*, *M. scrofulaceum* and *M. intracellulare*) are the most common species isolated in these infections. Treatment with highly active anti-retroviral therapy (HAART) has led to a decrease in the incidence of disseminated disease in HIV infected patients[155]. A study in Italy showed that the incidence was reduced by 74% in patients on HAART[159].

Johne's disease caused by *M. avium* subspecies paratuberculosis infects ruminants such as cattle, sheep, goats and deer[160]. Infection causes a wasting disease via granulomatous degenerative enteritis. There is a great economic impact of this disease in agricultural animals as infected herds have reduced milk production and may need to be culled to prevent spreading the infection. This is one example of pathogenic infection however there is much more evidence of sub-clinical exposure in animals; PPD responsiveness has been tested in domestic animals without pathogenic infection and *M. avium* can be cultured from minor lesions in the intestine of cattle and deer[125]. This latter report indicates these animals are likely to be infected by the oral route.

Like BCG, other species of mycobacteria can induce protective immunity against *M. tb* infection. In the MRC BCG efficacy trial conducted in the UK the efficacy of vaccination with *M. microti* was also tested and proven to confer the same level of protection as BCG over a 20 year follow-up[161].

3.1.2 The Efficacy of NTM in protection Against *M. tb*; Analysis of Animal Models

3.1.2.1 Cows

Experimental infection of calves with *M. avium* strain D4ER given subcutaneously induced PPD of *M. avium* (-A) and PPD-B specific IFNγ responses[162]. Calves did not suffer from pathogenic infection with the strain. Buddle et al vaccinated calves with *M. avium* D4ER and this provided protection against challenge with *M. bovis*.

3.1.2.2 Guinea Pigs

Guinea pigs are also resistant to pathogenic infections from NTM. A study of *M. avium* infection via oral or subcutaneous route by de Lisle et al showed no pathological outcome although there was a subclinical infection present 8 weeks after administration[125]. High levels of PPD responsiveness was recorded after *M. avium* infection; the subcutaneous route induced a greater response than the oral route. In a further study guinea pigs were infected orally with two strains of *M. avium* (WAg206 and WAg207) and subsequently challenged with *M. bovis*; both strains afforded significant protection.

Edwards performed exposures in guinea pigs with the *M. avium-intracellulare* complex[163]. The report showed that PPD sensitivity was only induced by intradermal vaccination of CFU doses greater than 1×10^6 and not induced by the oral route or respiratory route of exposure. Intradermal vaccination significantly protected the animals from challenge with a highly virulent strain of *M. tb* and H37Rv although significantly less protection was afforded compared to BCG.

3.1.2.3 Mice

Mice have been the most extensively used species to model NTM infection owing to their ease of handling and access to a wide range of immunological reagents. This model may not

be a true representation of the responses induced in humans as the majority of NTM species cause a pathological or persistent infection in mice.

Early studies did not assay immune responses induced by NTM infection but often challenged the mice to see what protective efficacy was provided by the infection. These studies provide interesting insights into the variation which exists between species of NTM. In 1971 Collins presented a systematic comparison of the ability of different strains of mycobacteria to persist in CD-1 mice after intravenous infection[121]. Intravenous challenge of these animals with *M. tb* Erdmann demonstrated a protective effect of prior infection with some of the species; there was a direct association between the ability of the NTM to persist in the host and the level of protection conferred against *M. tb*

A similar outcome was seen in a study by Orme and Collins where mice were infected by the aerosol route; a more comparable route to human exposure than intravenous[122]. Bacteria which persisted in the lungs for 30 days provided protection against *M. tb* H37Rv aerosol challenge whereas *M. scrofulaceum* that did not persist provided no protection. It should be noted that protective efficacy against *M. tb* challenge was less than that conferred by BCG.

In 1986 Orme et al found that intravenous administration of *M. avium* and *M. kansasii* was protective against aerosol *M. tb* infection whereas subcutaneous administration was not[123]. The induction of adaptive responses would be faster in the intravenous model which may provide the increased efficacy. Both NTM persisted in the lymph node draining the site of subcutaneous vaccination but did not induce the same level of protection against *M. tb* as intravenous infection did.

Brandt et al infected mice with a mix of *M. avium, M. vaccae and M. intracellulare* then cleared the infection with antibiotics after 3 weeks of infection[119]. Mice induced a low level of

protection against *M. tb* challenge; despite being a low magnitude of efficacy the results demonstrate that immunological memory can be induced by NTM.

The effect of killed *M. vaccae* sensitisation was examined by Hernandez-Pando et al[164]. The report provided an interesting result; protection was afforded by dosing with 1×10^7 CFU but was not with 1×10^9 CFU. The low dose group had greater level of IFNγ response than the high dose group; the high dose group had a sharp rise in the level of IL-4 transcription where the low dose group did not. The authors concluded that the high dose of antigen leads to induction of detrimental Th1/Th2 balance.

Despite there being a range of results in the experiments summarised above, some patterns do emerge; an ability of NTM to induce a persistent infection *in vivo* is important for generation of protective immunity. The route of administration also appears to be important with intravenous and intradermal routes of exposure inducing some consistent levels of protective efficacy, whilst oral, aerosol and subcutaneous routes are more variable in outcome. These studies are summarised in Table 2.

Author	NTM	Route	Dose	Live/ dead	Challenged	Protected	Notes
Cattle							
Howard 2002	*M. avium* D4ER	SC	1×10^5	Live	-	-	IFNγ response primed
Hope 2005	*M. avium* D4ER	SC	1×10^6	Live	*M. bovis*	Yes	IFNγ response
Guinea Pig							
Edwards	*M. avium* – *intracellulare* complex	ID	1×10^6	Live	H37Rv and high virulence strain	Yes	
de Lisle 2004	*M. avium* WAG 206	oral	1×10^8	Live	*M. bovis*	Yes	Infection detected at 8 weeks
Mice							
Collins 1971	*M. kansasii* 1201, 1203	IV	1×10^6	Live	Erdmann	Yes	Persistent infection
	M. kansasii 1204	IV	1×10^6	Live	Erdmann	No	Did not persist
	M. avium 705	IV	1×10^6	Live	Erdmann	Yes	Persistent infection

	M. intracellulare Dungar 647	IV	1 x 10^6	Live	Erdmann	Yes	Persistent infection
	M. kansasii 1204	IV	1 x 10^6	Live	Erdmann	No	Did not persist
	M. intracellulare 1403, 1406, 1467	IV	1 x 10^6	Live	Erdmann	No	Did not persist
	M. fortuitum 1530	IV	1 x 10^6	Live	Erdmann	No	Did not persist
	M. scrofulaceum 1515	IV	1 x 10^6	Live	Erdmann	No	Did not persist
Orme 1984	*M. kansasii* 120e	Aer	1 x 10^4	Live	H37Rv	Yes	30 day infection
	M. simiae 1226	Aer	1 x 10^4	Live	H37Rv	Yes	30 day infection
	M. avium 724	Aer	1 x 10^4	Live	H37Rv	Yes	persistent infection
	M. scrofulaceum 1306	Aer	1 x 10^4	Live	H37Rv	No	Did not persist
Orme 1986	*M. avium* 724	IV	1 x 10^4	Live	H37Rv	Yes	Persistent infection

	M. kansasii 1023	IV	1 x 10^4	Live	H37Rv	Yes	Persistent infection
	M. avium 724	SC	1 x 10^6	Live	H37Rv	No	Persistent infection, SC vaccinated
	M. kansasii 1023	SC	1 x 10^6	Live	H37Rv	No	Persistent infection, SC vaccinated
Brandt	*M. avium* + *M. vaccae* + *M. scrofulaceum*	SC	2 x 10^6 3 times	Live	Erdmann	Yes	Protection without on-going infection
Hernandez-Pando	*M. vaccae* NCTC 11659	SC	1 x 10^7	Dead	H37Rv	Yes	Low dose protect
	M. vaccae NCTC 11659	SC	1 x 10^9	Dead	H37Rv	No	High dose no protection

Table 2: Summary of the published models of NTM exposure and challenge

3.1.3 Immune Response Studies

The fact that exposure to mycobacteria induces IFNγ responses has been extensively discussed. Lozes et al showed that the magnitude of the IFNγ response does not correlate with the pathogenicity of the mycobacteria[140]. The magnitude of IFNγ and IL-2 production was in fact correlated with the ability of the host to clear this infection.

The presence of IS901 insertion element is a possible virulence factor for *M. avium* species[126]. Strain WAg 206 is more virulent than WAg 207 and is able to disseminate from the gastrointestinal tract. WAg 207 replicates rapidly within DCs and stimulates activation and subsequent IL-1β, IL-6 and TNFα production. The induction of these cytokines by WAg 206 is far less. WAg 206 induces stronger IgG titres than WAg207 which may indicate a switch in the dominance of Th1 to Th2 mediated immunity in this infection. This may be of relevance to the disparity seen in the efficacy of *M. kansasii* strains in the 1971 study by Collins given one strain persisted and one did not.

M. avium 101 oral infection in the mouse model has been shown to induce immunomodulatory cytokines. In vitro infection of monocytes and macrophages leads to production of TGFβ, IL-6 and IL-10[165].

3.1.4 Aims of this Experiment

The overview of published works shows it is clear there is much disparity in the results from NTM infection studies. These differences come from variations in the NTM species used as well as the strains of those species. The animal species in which the work was performed may also provide a different result to another species. Considering just the mouse model, there is further variation in the ability of certain strains of mice to control mycobacterial infection; for example C57BL/6 mice are more resistant to infection with mycobacteria compared to BALB/C as they induce a stronger Th1 response[166]. On top of this researchers use a variety of

doses administered via different routes. It is impossible to predict what response an NTM isolate will induce based on the existing published data therefore it was necessary to assay the immune response profile of the NTM isolate selected for use in this study.

The *M. avium* strain 2-151 was selected for use in this study. This experiment was designed to assess the adaptive immune response profile induced by this strain of *M. avium*. The effect of administering live or dead bacteria would also be compared. BCG vaccinated mice were assayed in parallel as a control of a known level of immunogenicity; as immune responses induced by BCG are known to be protective against *M. tb*. *M. avium* would be administered by the intraperitoneal route as this is a model of systemic exposure which is also a reproducible method of vaccination so variation in the data could be limited. C57BL/6 mice were chosen as the experimental model based on the epitopes this strain recognises in the Ag 85A sequence.

3.1.5 Experiment Plan

Mice were infected with BCG, $1x10^5$ CFU subcutaneously (SC), live MA 2-151, $1x10^5$ CFU intraperitoneally (IP) or heat-killed MA 2-151, $1x10^8$ CFU IP. At 1, 2 and 3 months post-infection, 5 mice from each group were culled and the spleens and the inguinal lymph nodes, which drain from both the IP and SC vaccination site, were stimulated ex vivo with PPD-T, PPD-A and Ag 85A. T helper 1, T helper 17, CD8, T helper 2 and regulatory T cell responses were assayed by cell surface marker expression and production of subset specific cytokines.



3.2 Results

3.2.1 T helper 1 Responses Induced by *M. avium* and BCG

Th1 responses induced by live or dead *M. avium* (MA) and BCG were quantified by the frequency of CD4+ T cells producing IFNγ, TNFα and IL-2 in stimulated lymph node and spleen cultures. Ag 85A stimulated cultures were only performed if there were enough cells; therefore there are not always 5 data points in each group for this stimulation.

3.2.1.1 Lymph Node

One Month

All three infections induced an IFNγ, TNFα and IL-2 response to all antigens tested. Total frequencies of IFNγ and TNFα producing cells were greater than IL-2 producing cells. 1 month after infection, dead MA and BCG induced greater frequencies of PPD-T specific IFNγ+ and TNFα+ CD4+ T cells compared to live MA (Figure 3-1 A). Production of IL-2 was similar across the groups.

PPD-A specific IFNγ and TNFα responses were similar to PPD-T specific responses; IL-2 responses were greater in the dead MA group compared to live MA and BCG group (Figure 3-1 D).

Dead MA and BCG induced comparable frequencies of all cytokines in response to Ag 85A (Figure 3-1 G).

Two Months

The magnitude of the live MA and BCG IFNγ response to PPD-T at 2 months was greater than the response at 1 month, the dead MA response was maintained at a similar level to the 1 month response (Figure 3-1 B). Live MA induced greater frequencies of PPD-T specific IFNγ+ CD4+ T cells than dead MA and BCG. Dead MA induced greater frequencies of TNFα+ CD4+ T cells than live MA and BCG. Production of IL-2 was similar across the groups but at a lower magnitude than at 1 month after infection.

Live MA and dead MA induced a similar magnitude of PPD-A specific IFNγ+ and TNFα+ CD4+ T cells; this was greater than the BCG induced response (Figure 3-1 E). IL-2 responses were similar across the groups.

There were too few data points in the Ag 85A specific analysis to interpret the results (Figure 3-1 H).

Three months

The magnitude of PPD-T specific responses induced by all infections was lower at 3 months than at 2 months (Figure 3-1 C). Live MA and BCG induced PPD-T specific IFNγ+ CD4+ T cells; no IFNγ response was induced by dead MA. All three infections induced TNFα+ CD4+ T cells; BCG induced more than live and dead MA. A low level IL-2 response was induced by all infections.

PPD-A specific responses were different to PPD-T specific responses. All groups induced an IFNγ response (Figure 3-1 F). Live MA induced significantly more IFNγ+ and TNFα+ CD4+ T cells than dead MA and more than BCG; the magnitude of these responses was greater than at 2 months. Live MA also induced more IL-2 than the other two groups.

There were too few data points in the Ag 85A specific analysis to interpret the results (Figure 3-1 I).

3.2.1.2 Spleen

One Month

One month after infection, dead MA and BCG induced a Th1 response to all stimulations, live MA only induced a PPD-A specific response. No IL-2 was produced from splenocytes 1 month after infection. Dead MA and BCG had comparable frequencies of PPD-T specific IFNγ+ and TNFα+ CD4+ T cells (Figure 3-2 A).

BCG induced greater frequencies of PPD-A specific IFNγ+ CD4+ T cells than dead MA (Figure 3-2 D). Live MA did not induce IFNγ+ CD4+ T cells but did induce TNFα+ CD4+ T cells, although at a lower frequency than dead MA and BCG.

Dead MA induced a greater frequency of Ag 85A specific IFNγ+ and TNFα+ CD4+ T cells than BCG (Figure 3-2 G).

Two Months

Two months after infection the peak magnitude of response to all stimulations was lower than at 1 month. PPD-T specific responses were induced by all infections (Figure 3-2 B). Dead MA induced a greater frequency of PPD-T specific IFNγ+ CD4+ T cells than live MA and BCG. Live MA induced a greater frequency of TNFα+ CD4+ T cells than dead MA and BCG. Levels of IL-2+ CD4+ T cells were similar over the groups.

PPD-A specific responses were induced by all infections and were of a greater magnitude than PPD-T and Ag 85A specific responses. Frequencies of IFNγ+ CD4+ T cells were similar over the groups (Figure 3-2 E). Live MA induced a significantly greater frequency of TNFα+ CD4+ T cells than dead MA and BCG.

Ag 85A responses were comparable between the three groups (Figure 3-2 H).

Three Months

At 3 months the magnitude of PPD-T responses remained similar to the level they were at 2 months (Figure 3-2 C). A similar frequency of IFNγ+ CD4+ T cells was induced by all infections. Dead MA and BCG induced a greater frequency of TNFα+ CD4+ T cells than live MA. Levels of IL-2+ CD4+ T cells were similar over the groups.

The magnitude of the PPD-A specific response was greater than the response to the other stimulations and had increased from the level seen at 2 months (Figure 3-2 F). Live MA and BCG induced a similar frequency of IFNγ+ and TNFα+ CD4+ T cells, dead MA did not induce a response.

Low levels of Ag 85A responses were induced by all three infections (Figure 3-2 I).

Responses in the lymph nodes and spleen were a similar magnitude. In all stimulations live MA did not induce a response until 2 and 3 months after infection whereas BCG and dead MA induced responses at 1 month. All infection regimes induced a similar magnitude of PPD-T

specific responses. Live MA induced the greatest PPD-A specific response; the magnitude of which was greater than any PPD-T or Ag 85A response recorded in this experiment. Dead MA induced the greatest Ag 85A specific response; the magnitude was lower than PPD-T and PPD-A responses.

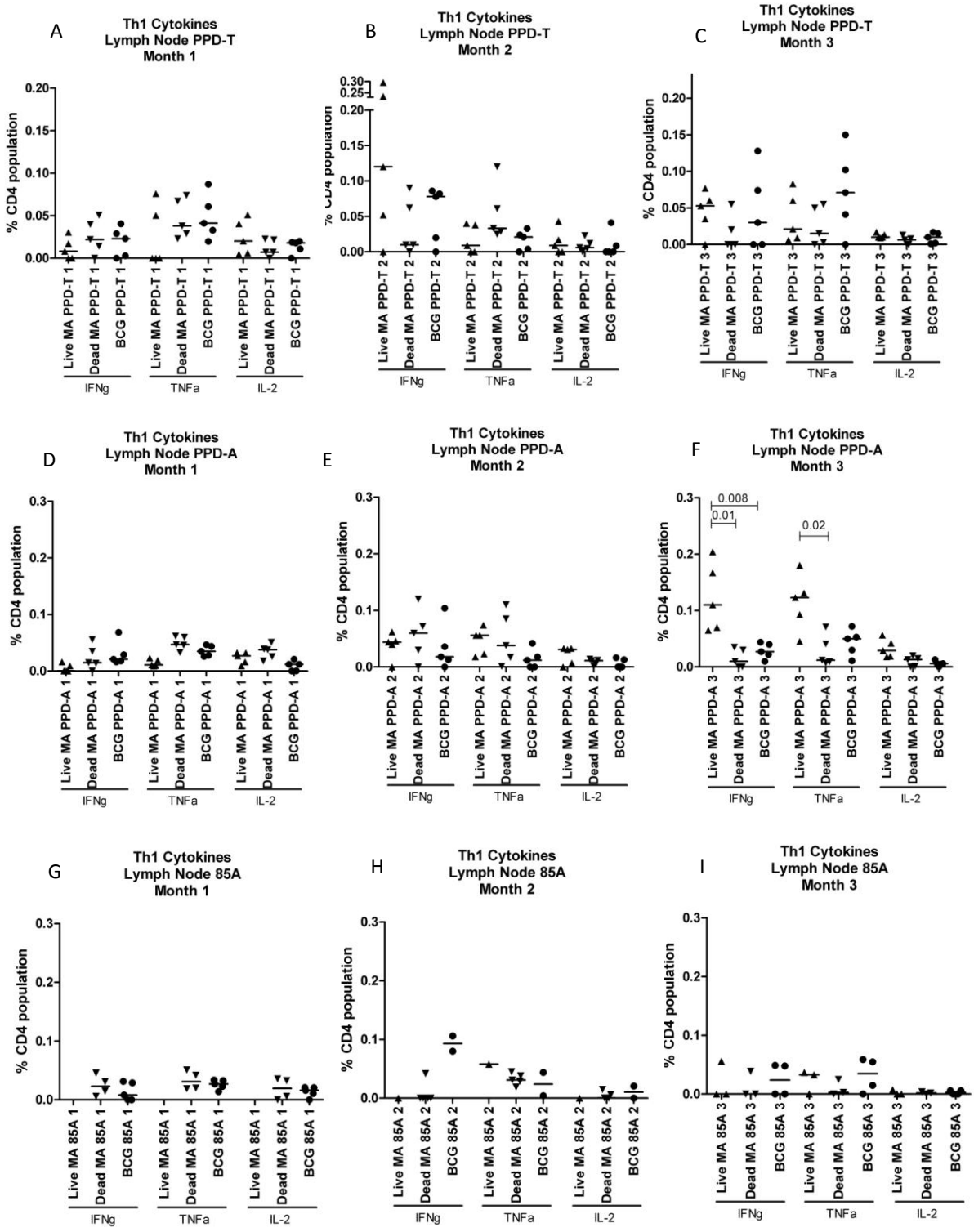


Figure 3-1: IFNγ, TNFα and IL-2 production by CD4+ T cells in the lymph nodes over 3 months of infection. Each graph shows all 3 cytokines at one time point. (A-C) are PPDT specific responses, (D-F) are PPD-A specific responses and (G – I) are Ag 85A specific responses. Data is antigen specific cytokine production; media-only control well values have been subtracted. Data points for all mice are displayed, n=5 apart from Ag 85A stimulated results where the number of cultures was dependent on cell numbers available. The median of the group is shown, statistical significance was determined by the Mann Whitney U test and p values are displayed where there was a significant difference.

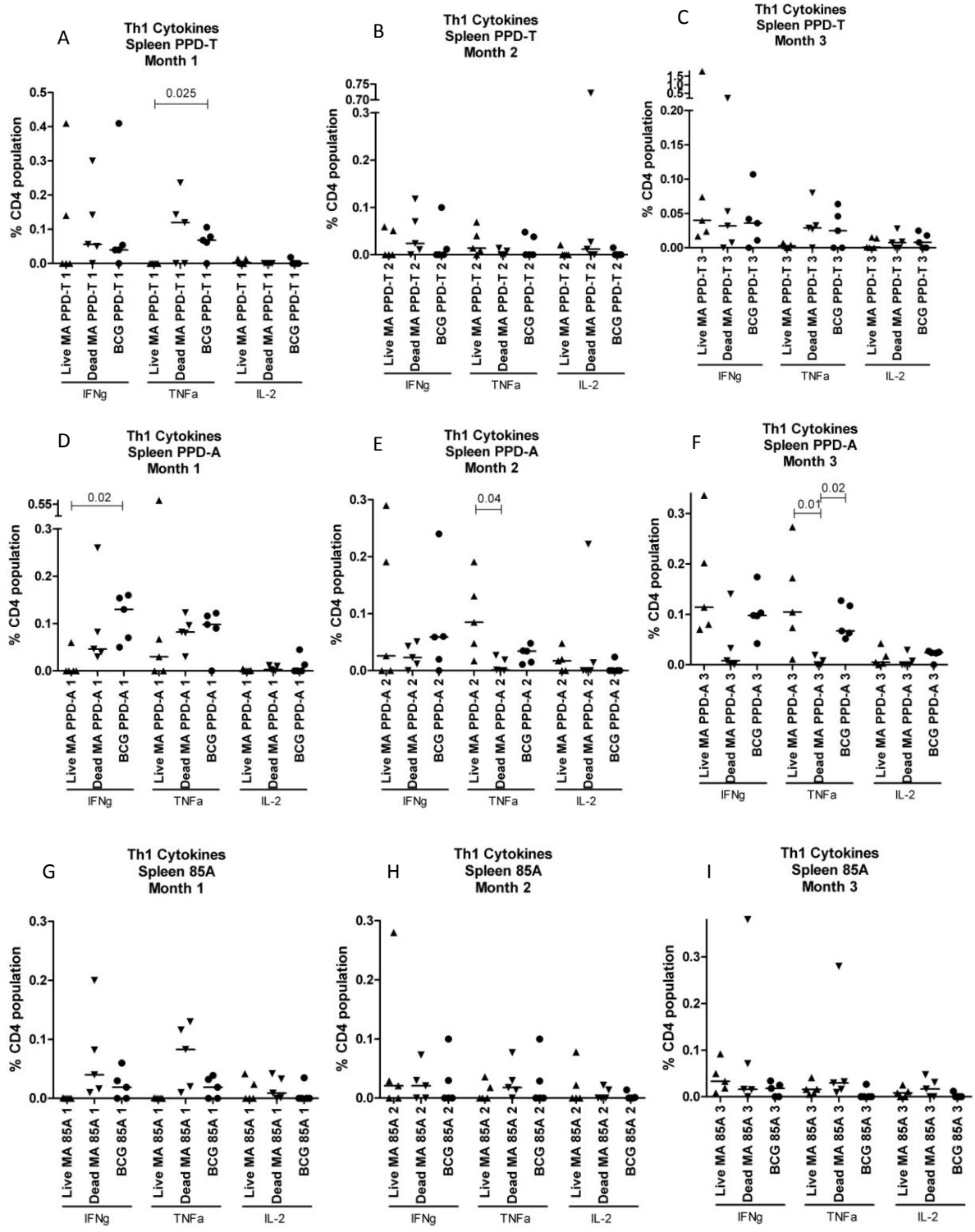


Figure 3-2: IFNγ, TNFα and IL-2 production by CD4+ T cells in the spleen over 3 months of infection. Each graph shows all 3 cytokines at one time point. (A-C) are PPDT specific responses, (D-F) are PPD-A specific responses and (G – I) are Ag 85A specific responses. Data is antigen specific cytokine production; media-only control well values have been subtracted. Data points for all mice are displayed, n=5 apart from Ag 85A stimulated results where the number of cultures was dependent on cell numbers available. The median of the group is shown, statistical significance was determined by the Mann Whitney U test and p values are displayed where there was a significant difference.

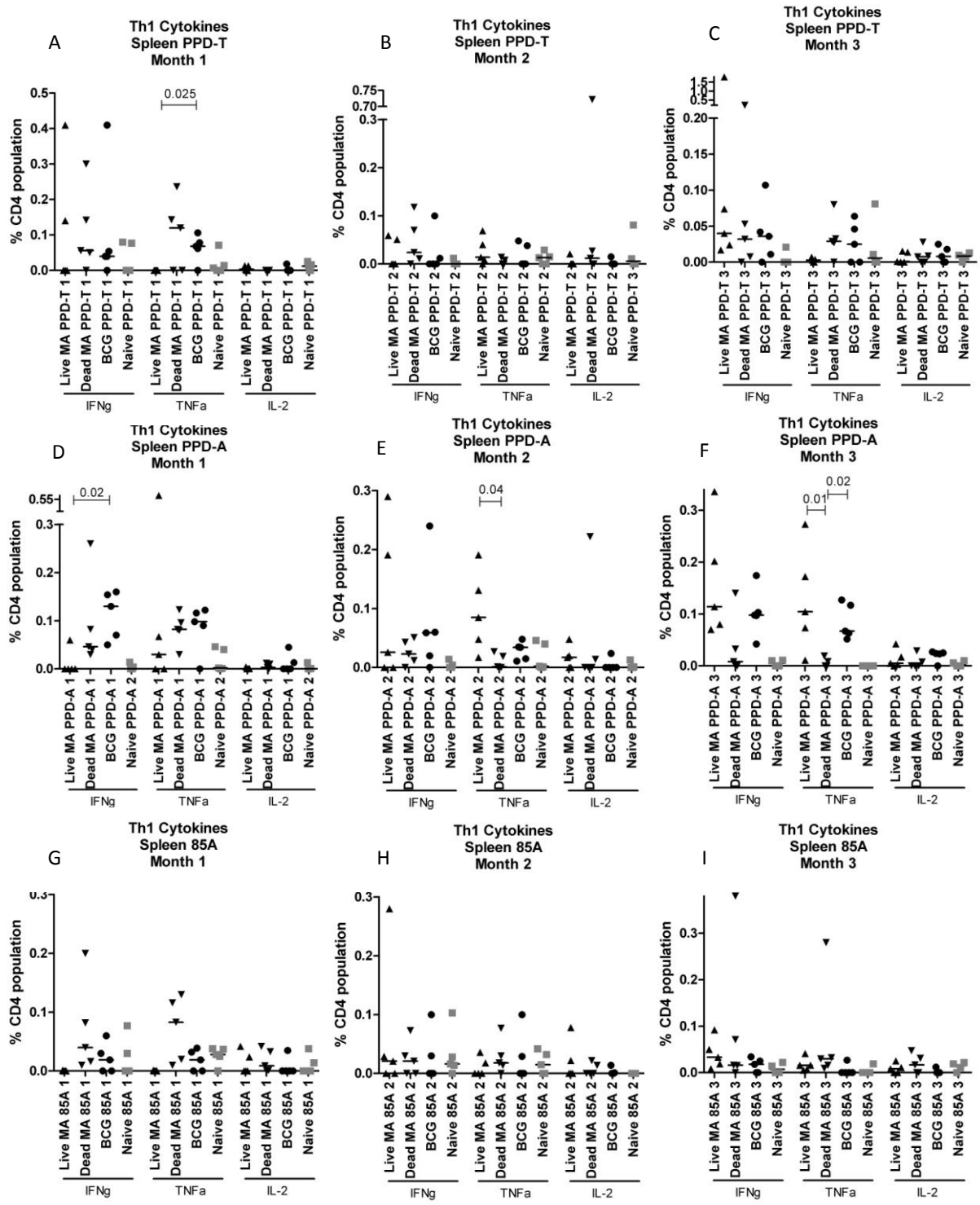


Figure 3-2a: IFNγ, TNFα and IL-2 production by CD4+ T cells in the spleen over 3 months of infection with values from naïve control mice included to show background level of cytokine producing cells which were stimulated non-specifically from incubation with PPD-T, PPD-A and 85A 66p. Each graph shows all 3 cytokines at one time point. (A-C) are PPDT specific responses, (D-F) are PPD-A specific responses and (G – I) are Ag 85A specific responses. Data is antigen specific cytokine production; media-only control well values have been subtracted. Data points for all mice are displayed, n=5 apart from Ag 85A stimulated results where the number of cultures was dependent on cell numbers available. The median of the group is shown, statistical significance was determined by the Mann Whitney U test and p values are displayed where there was a significant difference.

3.2.2 Polyfunctional Th1 Responses

Polyfunctional analysis of Th1 responses in this experiment are shown below. PPD-T specific results at the 3 month time point are shown as these are representative of results from PPD-A and Ag 85A stimulated CD4+ T cells at 1 and 2 months. One exception to this is the results of PPD-A stimulated lymph nodes at 3 months which are also shown.

Live MA Infection

The dominant cytokine producing CD4+ T cells specific for PPD-T were IFNγ+ (γ+) (Figure 3-3 A). TNFα+ (α+) and γ+α+ were also induced and at a lower frequency γ+α+2+ CD4+ T cells were also detected. The response to PPD-A induced by live MA was dominated by γ+α+ CD4+ T cells (Figure 3-3 B). High frequencies of γ+α+2+, γ+ and α+ CD4+ T cells were also induced. In the spleen only γ+ CD4+ T cells were detected (Figure 3-3 C).

Dead MA Infection

The greatest frequencies of cytokine producing cells were γ+α+ and α+ CD4+ T cells γ+α+2+ and γ+ CD4+ T cells were also induced (Figure 3-3 A). This did not differ between PPD-T and PPDA specific responses. In the spleen the greatest frequency of cytokine producing cells were γ+α+ (Figure 3-3 C).

BCG Infection

The greatest frequency of cytokine producing cells were γ+α+ (Figure 3-3 A). A high frequency of α+ CD4+ T cells and a lower frequency of γ+α+2+ CD4+ T cells were also induced; the response to PPD-T and PPD-A stimulation was the same. In the spleen the frequency of γ+α+ CD4+ T cells was slightly higher than other cytokine producing cells (Figure 3-3 C).

The response induced by live MA to PPD-T was dominated by monofunctional cells whereas the response to PPD-A was more polyfunctional. Dead MA and BCG induced polyfunctional responses in lymph nodes and spleen to PPD-T. The dominant functional phenotype induced by BCG and dead MA were γ+α+ and α+ CD4+ T cells.

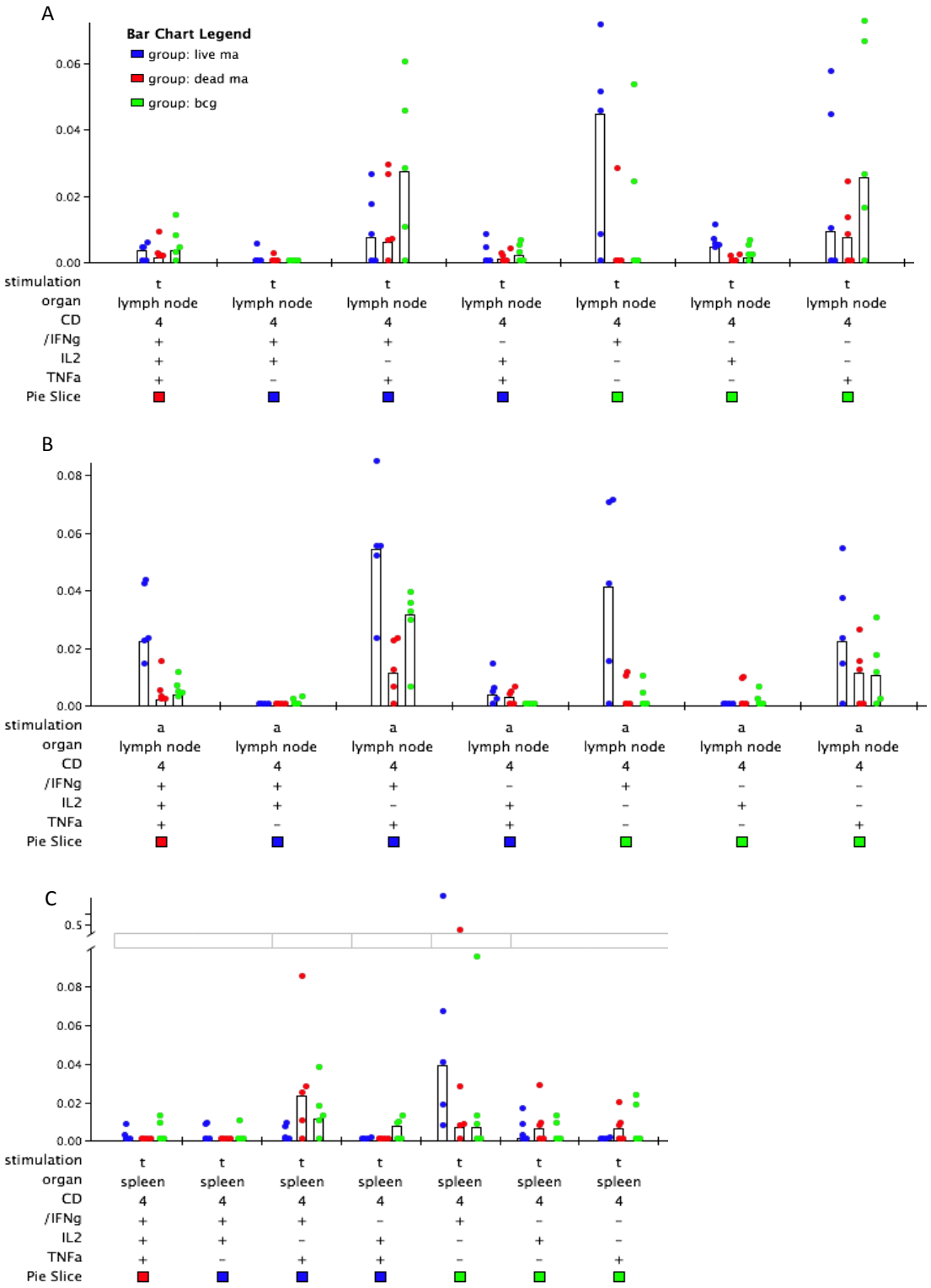


Figure 3-3: Graph showing the secretion of one or more of IFNγ, TNFα and IL-2 from CD4+ T cells 3 months after infection. Data is expressed as the % of CD4+ T cells and is antigen specific; media only control wells are subtracted. A is lymph node cells stimulated with PPD-T, B is lymph node cells stimulated with PPD-A and C is spleen cells stimulated with PPD-T. All mice are shown as a separate dot, n = 5 and the median is displayed.

3.2.3 Cytokine Production from CD8+ Cells

3.2.3.1 Lymph Nodes

One Month

All infections induced a CD8+ T cell response to at least one of the stimulations tested. When looking at the cytokines produced by these antigen specific CD8+ T cells, no IL-2 was induced in the lymph node by any of the infections at any time point assayed. None of the infections induced an IFNγ response to PPD-T 1 month after infection (Figure 3-4 A). Live MA induced a PPD-T specific TNFα response. BCG was the only infection to induce a PPD-A specific IFNγ response (Figure 3-4 D). BCG and dead MA induced a similar Ag 85A specific TNFα response; BCG also induced an Ag 85A specific IFNγ response (Figure 3-4 G).

Two Months

No TNFα was detected at 2 months. Live MA induced PPD-T specific IFNγ+ CD8+ T cells; dead MA and BCG did not induce a response to PPD-T (Figure 3-4 B). Live MA and dead MA induced PPD-A specific IFNγ+ CD8+ T cells; BCG did not (Figure 3-4 E). There were too few data points in Ag 85A specific results to determine if one group had a greater response than another (Figure 3-4 H).

Three Months

Live MA induced a PPD-T specific TNFα response; dead MA and BCG did not (Figure 3-4 C). None of the groups had an IFNγ response against PPD-T. Live MA and dead MA induced a PPD-A specific IFNγ response; the magnitude was lower than the response at 2 months (Figure 3-4 F). None of the groups had a PPD-A specific TNFα response. There were too few data points in Ag 85A data to conclude a difference between the groups (Figure 3-4 I).

3.2.3.2 Spleen

One Month

Spleen responses were a greater magnitude than lymph node responses and IFNγ, TNFα and IL-2 were all induced by infections. One month after infection all groups had a PPD-T specific CD8+ T cell response; dead MA induced a greater frequency of IFNγ+ CD8s than live MA or BCG (Figure 3-5 A). The same was seen for TNFα but at lower magnitude than IFNγ. Low frequencies of IL-2+ CD8+ T cell were induced in all groups. Dead MA also induced a PPD-A specific IFNγ and IL-2 response but live MA and BCG did not; none of the groups induced TNFα (Figure 3-5 D). Dead MA induced an Ag 85A specific IFNγ response, all infections induced TNFα and IL-2 responses of similar magnitude (Figure 3-5 G).

Two Months

All three infections induced PPD-T specific responses; dead MA induced a greater IFNγ response than live MA and BCG and was the only infection to induce a TNFα and IL-2 CD8+ T cell to this stimulation (Figure 3-5 B). Live MA was the only infection to induce PPD-A specific IFNγ production; no other cytokine was detected against this stimulation (Figure 3-5 E). Live MA and dead MA induced IFNγ in response to stimulation with Ag 85A: dead MA also induced TNFα and IL-2 in response to Ag 85A stimulation (Figure 3-5 H).

Three Months

At 3 months BCG was the only infection to induce a response to PPD-T; only IFNγ was detected (Figure 3-5 C). Dead MA was the only infection to induce a PPD-A response; again only IFNγ was detected (Figure 3-5 F). All groups induced a TNFα response to Ag 85A but only dead MA induced IFNγ and IL-2 as well (Figure 3-5 I).

Spleen responses were of a greater magnitude than lymph node responses. Lymph node responses were mainly IFNγ+ whereas all three cytokines tested were produced by CD8+ T cell in the spleen. Dead MA induced the greatest magnitude of response to all 3 stimulations in the spleen.

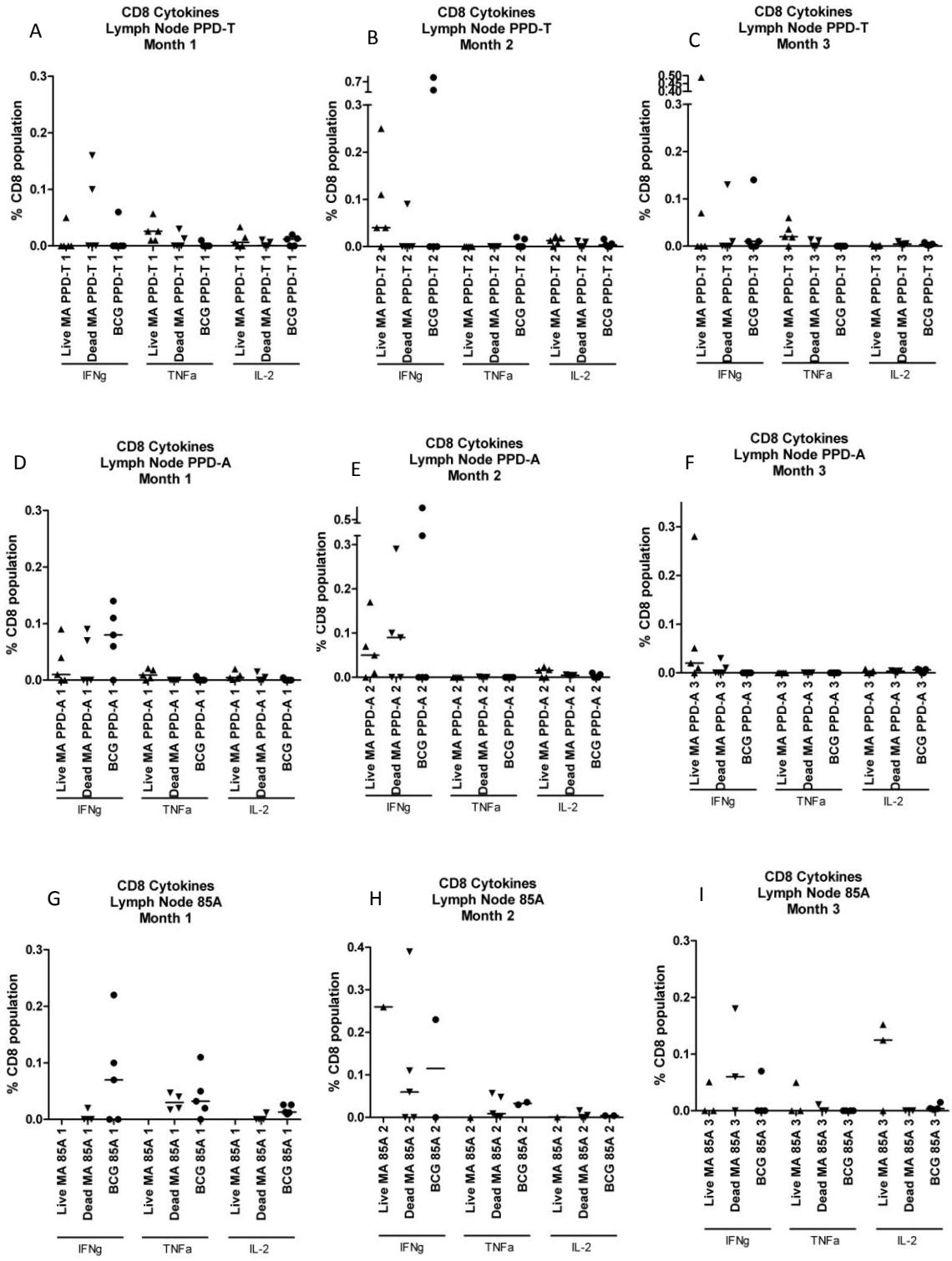


Figure 3-4: IFNγ, TNFα and IL-2 production by CD8+ T cells in the lymph nodes over 3 months of infection. Each graph shows all 3 cytokines at one time point. (A-C) are PPD-T specific responses, (D-F) are PPD-A specific responses and (G – I) are Ag 85A specific responses. Data is antigen specific cytokine production; media-only control well values have been subtracted. Data points for all mice are displayed, n=5 apart from Ag 85A stimulated results where the number of cultures was dependent on cell numbers available. The median of the group is shown; statistical significance was determined by the Mann Whitney U test and p values are displayed where there was a significant difference.

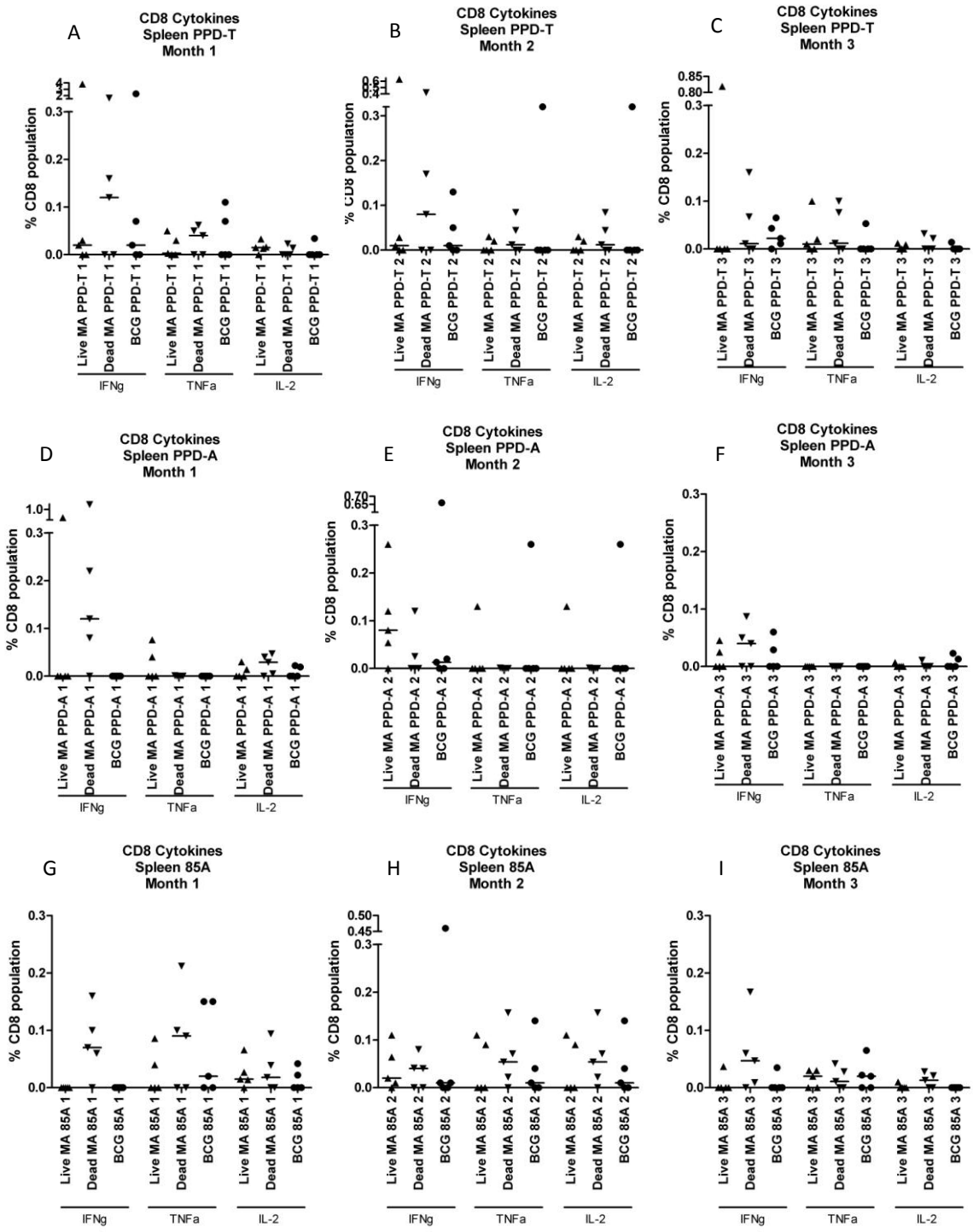


Figure 3-5: IFNγ, TNFα and IL-2 production by CD8+ cells in the spleen over 3 months of infection. Each graph shows all 3 cytokines at one time point. (A-C) are PPDT specific responses, (D-F) are PPD-A specific responses and (G – I) are Ag 85A specific responses. Data is antigen specific cytokine production; media-only control well values have been subtracted. Data points for all mice are displayed, n=5 apart from Ag 85A stimulated results where the number of cultures was dependent on cell numbers available. The median of the group is shown; statistical significance was determined by the Mann Whitney U test and p values are displayed where there was a significant difference.

3.2.4 IL-17 Secretion from CD4+ T Cells

Frequencies of IL-17+ CD4+ T cells were very low in magnitude which meant the results obtained were highly variable.

3.2.4.1 Lymph Node

One Month

Only BCG infection induced a PPD-T specific IL-17 response 1 month after infection (Figure 3-6 A). Dead MA and BCG infection induced an IL-17 response to PPD-A, live MA did not (Figure 3-6 B). Dead MA and BCG induced a similar response to Ag 85A (Figure 3-6 C).

Two Months

Two months after infection, all groups had very low or no IL-17+ CD4+ T cells specific for PPD-T and PPD-A (Figure 3-6 A & B)). There were too few data points in the Ag 85A specific data to determine if one group has a greater response than another (Figure 3-6 C).

Three Months

Three months after infection all groups had very low levels of IL-17+ CD4+ T cells specific for PPD-T (Figure 3-6 A). Live MA and dead MA induced a similar PPD-A specific IL-17 response, BCG did not (Figure 3-6 B). There were too few data points in the Ag 85A specific data to make any definitive statements (Figure 3-6 C).

3.2.4.2 Spleen

One Month and Two Months

In the spleen, 1 and 2 months after infection, all groups had very low or no frequencies of IL-17+ CD4+ T cells specific for PPD-T and PPD-A (Figure 3-6 D and E). BCG induced an Ag 85A specific response 1 month after infection, live MA and dead MA did not.

Three Months

Three months after infection, live MA induced an IL-17 response to PPD-T, PPD-A and Ag 85A (Figure 3-6 D, E & F). Dead MA induced a similar magnitude response against PPD-A as live

MA but no response any other stimulation. BCG induced no IL-17 response to any antigenic stimulation.

3.2.5 Th2 Responses

Th2 responses were measured by production of IL-4 and IL-5 from splenocytes in response to stimulation.

3.2.5.1 IL-5

No PPD-T, PPD-A or Ag 85A specific IL-5 was induced by any of the infections (Figure 3-6 G-H).

3.2.5.2 IL-4

One month and 3 months after infection, BCG induced a PPD-T specific IL-4 response; no IL-4 was induced by live MA or dead MA (Figure 3-6 J). IL-4 was detected in some cultures stimulated with PPD-A and Ag 85A but not enough mice responded per group to conclude whether or not the infection induced a consistent response (Figure 3-6 K&L).

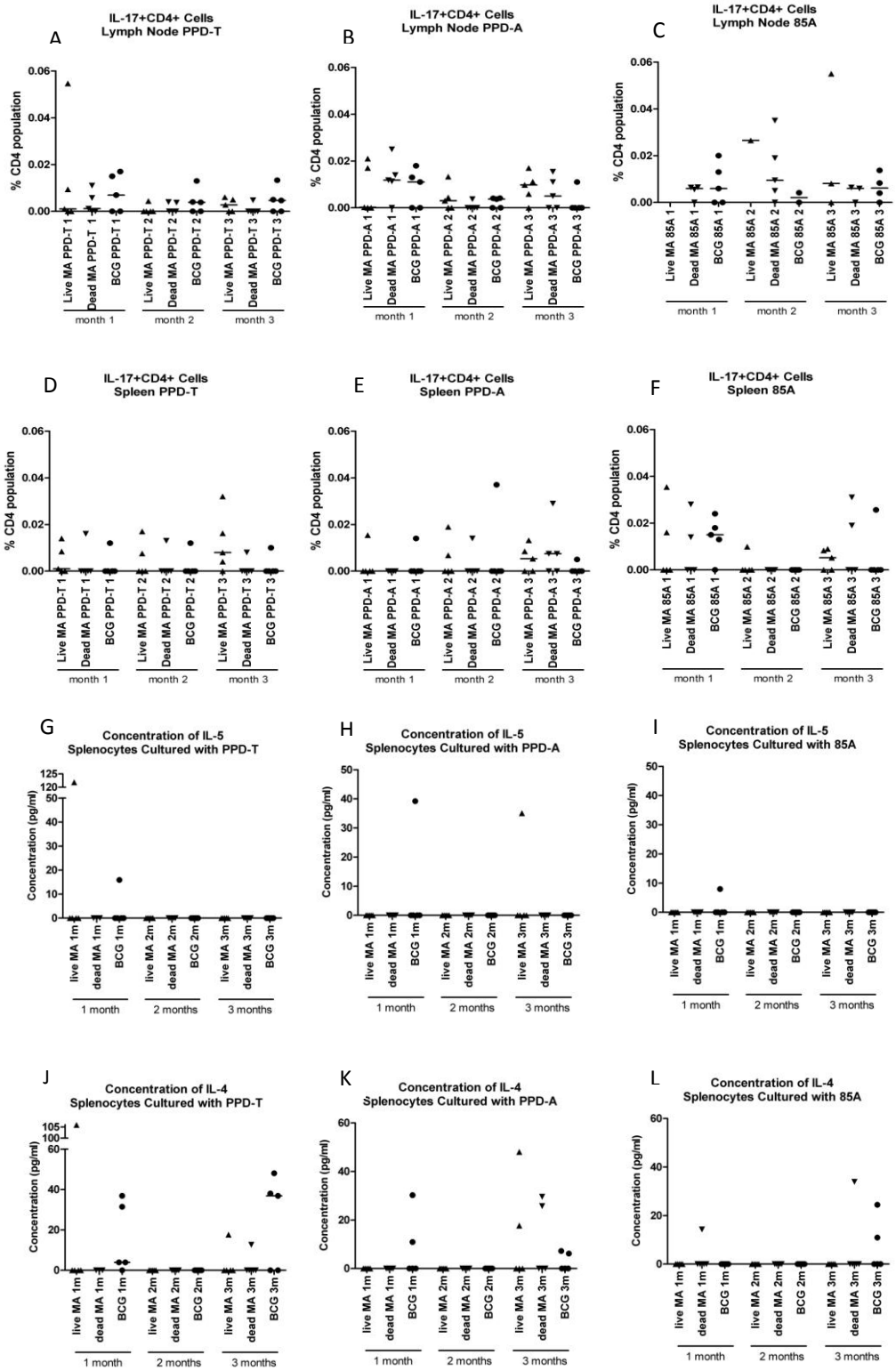


Figure 3-6: IL-17, IL-5 and IL-4 production. Graphs show all 3 time points assayed and each graph shows specific responses to one stimulant. (A-C) are IL-17+ CD4+ T cells in the lymph nodes, (D-F) are IL-17+ CD4+ T cells in the spleen, (G – H) are IL-5 concentration in spleen cultures, (J – L) are IL-4 concentration in spleen cultures. Data is antigen specific production; media-only control well values have been subtracted. Each data point represents one mouse and the median is displayed. Lymph node group sizes in Ag 85A cultures vary due to availability of cells, all other cultures n=5). Statistical significance was determined using the Mann-Whitney U test, none was calculated.

3.2.6 Regulatory T Cell Responses

To investigate regulatory responses the frequency of Tregs, defined as CD4+CD25+FoxP3+CD39+ T cells, and the concentration IL-10 and TGFβ were assayed.

3.2.6.1 Frequencies of Tregs

Lymph Node

All groups had between 5 and 10% Tregs in the lymph nodes at all 3 time points. One month after infection the live MA group had significantly greater frequencies of Tregs than the BCG group and greater frequencies than the dead MA group (Figure 3-7 A). 2 months after infection the dead MA group had a slightly greater frequency of Tregs than the live MA and BCG groups. 3 months after infection the live MA group had a slightly greater frequency of Tregs than the dead MA and BCG group.

Spleen

Frequencies of Tregs were lower in the spleen than the lymph nodes. 1 month after infection the median frequency of Tregs was between 4 and 6%, at 2 and 3 months after infection this dropped to between 1 and 3%. 1 month after infection dead MA and BCG groups had greater frequencies of Tregs in the spleen than the live MA group (Figure 3-7 B). 2 months after infection Treg frequencies were similar across the groups. At 3 months the live MA group had slightly greater frequencies of Tregs than the other groups, but these differences were not significant.

3.2.6.2 IL-10

No PPD-T specific IL-10 was detected 1 or 2 months after infection (Figure 3-7 C). At 1 month dead MA induced a low level PPD-A specific response (Figure 3-7 D). Live MA induced a PPD-A specific response at 2 months and an Ag 85A specific response at 1 and 2 months (Figure 3-7 E). At 3 months the concentration of IL-10 in PPD-T and PPD-A stimulated cultures was very high in all groups. This recorded increase in cytokine level was a technical issue and not a real result as the assay detected equivalent concentrations in cultures of splenocytes from naïve

mice. Splenocytes from naïve mice should not respond to antigen stimulation and the equivalent levels of cytokine detected in these cultures indicates a technical issue.

3.2.6.3 TGFβ

One month after infection live MA and BCG induced a PPD-T specific TGFβ response, dead MA did not (Figure 3-7 F). There was no response to Ag 85A at 1 month. No TGFβ was produced in response to PPD-A at any time point. At 2 months dead MA induced a TGFβ response against PPD-T and Ag 85A, the other infections did not. At 3 months BCG induced TGFβ in response to PPD-T; dead MA induced TGFβ against Ag 85A.

BCG and dead MA induced the greatest level of TGFβ; both peak responses were at 3 months after infection in response to PPD-T and Ag 85A respectively. All infections induced a low level of IL-10 production. Peak magnitudes of TGFβ and IL-10 did not coincide with peak frequencies of Tregs.

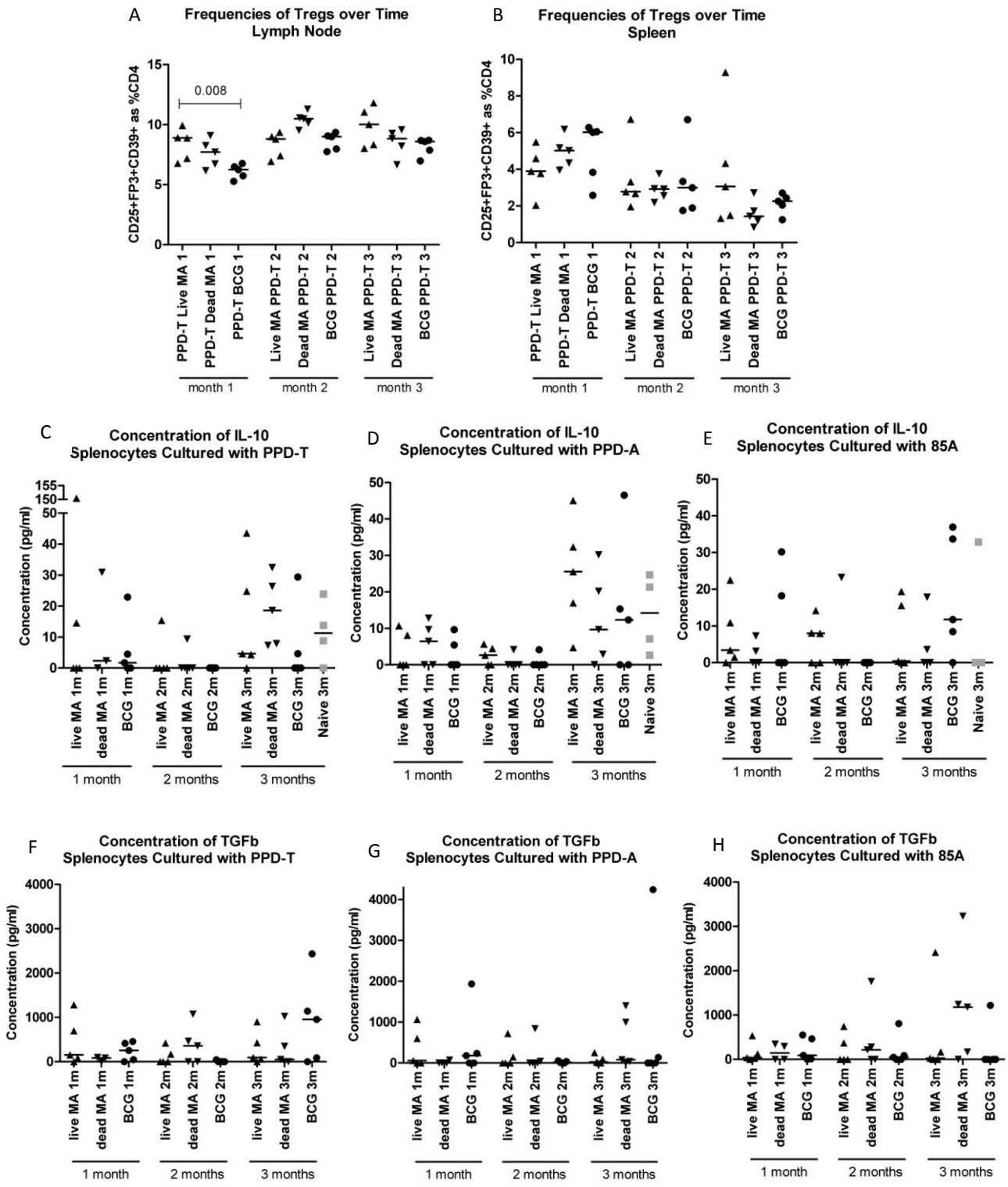


Figure 3-7: Regulatory responses. Graphs A and B show the frequency of CD4+ T cells which have a regulatory phenotype defined as CD25+ FoxP3+ CD39+ cells, measured by flow cytometry. Lymph node and spleen data for all 3 time points in PPD-T stimulated cultures. Responses are not antigen specific. (C-E) show the concentration of IL-10 in PPD-T, PPD-A and Ag 85A stimulated splenocyte cultures respectively, measured by FlowCytomix. Graphs F, G and H show concentration of TGFβ in PPD-T, PPD-A and Ag 85A stimulated splenocyte cultures respectively, measured by FlowCytomix. Cytokine concentrations are antigen specific as media only control well concentrations have been subtracted. N=5 apart from naïve groups which n=4. Statistical significance was determined by the Mann-Witney U test and is shown where achieved.

3.3 Discussion

This experiment showed that the 2-151 strain of MA induces Th1, Th17, CD8 and regulatory T cell responses specific for PPD-T, PPD-A and Ag 85A. It also allowed the comparison of adaptive immune responses induced by MA to those induced by BCG which are known to be immunogenic and protect against *M. tb*.

The induction of IFNγ by MA has been reported in all studies where this immune response was tested so it was not surprising IFNγ responses were detected in this study. Results show that the source of this cytokine is both CD4+ T cells and CD8+ T cells although the magnitude of the response from CD4+ T cells is far greater than the CD8+ T cell response, similar to what is seen after BCG.

Production of TNFα and IL-2 was assayed in combination with IFNγ. CD8+ T cell responses predominantly produced only IFNγ; when TNFα+ or IL-2+ CD8+ T cells were detected they were at very low frequencies. TNFα production was shown to be strongly correlated with IFNγ production in CD4+ T cells; often similar frequencies of the two cytokine producing cells were detected. IL-2 was not detected as frequently as the other two cytokines. The production of IFNγ and TNFα in MA infection was studied by Appelberg et al[167]. They showed the two cytokines peaked at the same time in infection and that depletion of CD4+ T cells abrogated the response of both cytokines which is in agreement with my data.

The kinetic of the immune response induced by live MA differed to that induced by dead MA. Dead MA responses were induced more rapidly, after infection, and the maximum magnitude of response was detected at 1 or 2 months after infection and then dropped at 3 months. Live MA infection induced Th1 responses which were detected 2 months after infection and increased in magnitude at 3 months.

Differences in the responses of live and dead BCG vaccination was studied by Chambers et al[168]. The IFNγ response at the site of administration was followed to day 42. At 14 and 28 days the group which received dead BCG had significantly greater levels of IFNγ than the group which received live BCG which is similar to what I saw at 1 month. The response was not followed further than day 28 but I suspect the live BCG response would begin to increase after this time point whereas the dead BCG response would decrease.

The authors investigated persistence of the bacteria at the site of infection and found that dead BCG persisted at the site whereas live BCG CFU declined; this was in correlation with the levels of IFNγ detected. The ability of pathogenic mycobacteria to exist within the host cell after phagocytosis is an active process requiring the secretion of proteins; this process delays immune activation. Dead bacteria do not secrete the proteins necessary for this response and so would assumedly be phagocytosed and destroyed directly after initial infection leading to large amounts of inflammatory cytokines. Live MA may delay the onset of the immune response by existing in an intracellular stage; this may explain the later appearance of adaptive immune responses in this group. Interestingly though, responses induced by BCG were detected at 1 month in the lymph nodes and spleen which may show a difference in the ability of these bacteria to locate to the lymph nodes and prime adaptive immunity.

Despite the difference in the kinetic of the response both live and dead infections induced a similar magnitude of PPD-T specific Th1 response in the 3 months assayed. BCG induced a PPD-A response, this demonstrates the high level cross-recognition of antigens in the two stimulants. Live MA induced a significantly greater PPD-A response than dead MA; the magnitude of this response was greater than the PPD-T specific response. It is not surprising that PPD-A specific responses would be greater than PPD-T specific responses. Despite the high level of cross-recognition of antigens between PPDs mycobacteria generally stimulate a greater response to their species specific PPD. This result was shown by Howard et al who

administered MA to calves and measured a greater PPD-A specific IFNγ response compared to PPD-B specific response[162]. What was surprising was that dead MA did not induce a greater magnitude of response against PPD-A than it did against PPD-T.

It was also surprising that dead MA induced a response to secreted Ag 85A as this protein is only produced in live infection; in fact the greatest magnitude of an Ag 85A specific response was recorded 1 month after infection with dead bacteria. The bacteria were killed by heating the entire bacterial preparation so it is plausible some Ag 85A protein was present in the preparation which was administered. The preparation was certified to have been successfully killed by plating a sample on agar so I am certain it was not the presence of live bacteria which was inducing this response.

Th1 responses are key in the vaccine mediated protection afforded by BCG[74]. The response directed toward PPD-T is the best indicator in this study of how cells will respond to *M. tb*. Dead and live MA both induced magnitudes of Th1 responses comparable to those in BCG vaccinated mice.

A polyfunctional Th1 profile has been linked to improved vaccine efficacy against *M. tb* infection in mice[169]. BCG vaccination induced polyfunctional responses which were detected from the first month and were maintained throughout the experiment. The same was seen in mice infected with dead MA. Live MA infection induced a monofunctional Th1 response to PPD-T but a polyfunctional response to PPD-A. This is an interesting result as clearly live MA is capable of inducing polyfunctional cells but not specific to PPD-T. Given that dead MA is able to induce polyfunctional cells against PPD-T it is not a restriction of MA to prime PPD-T specific polyfunctional CD4+ T cells. A possible explanation is that polyfunctional responses are only induced against the most highly immunogenic antigens, in live MA infection these immunogenic antigens were specific to PPD-A which is why they were not detected in PPD-T

stimulated cultures. Dead MA infection must not induce a response to these same antigens, they are perhaps only produced by live infection, which allowed polyfunctional responses to be induced to antigens in PPD-T. The lack of polyfunctional cells specific to PPD-T may lead to a reduced ability of live MA to control *M. tb* infection in comparison to BCG; which was seen in some of the NTM exposure models discussed in the introduction to this chapter[122].

As with the Th1 responses CD8+ T cell responses were detected at 1 month after infection with dead MA and BCG but not until 2 and 3 months after infection with live MA. Dead MA induced the greatest responses of all the infections; specificity was to PPD-T and Ag 85A.

Th17 cells have been reported to play a role in the generation of protective immunity in BCG vaccination and in vaccine mediated protection in *M. tb* infection[86]. The IL-23/IL-17 response overcomes IL-10 induced by BCG infection to help generate Th1 responses. Frequencies of Th17 cells were low in this study. PPD-T, PPD-A and Ag 85A specific Th17 were induced by BCG and detected in the lymph nodes. Dead and live MA induced Th17 but of a lower magnitude than BCG. There appears to be no effect of lower Th17 responses on the induction of Th1 responses in MA infected mice.

It has been suggested by some laboratories that NTM induce Th2 skewed immunity. As discussed in the introduction, Young et al found a particular strain of MA (WAg206) did not induce activation of macrophages or production of pro-inflammatory cytokines. Exposure to this strain also induced high titres of IgG subclass 1 antibodies after BCG vaccination which is indicative of Th2 skewed immunity. The effect of IFNγ and IL-4 on BALB/C infected macrophages was examined by Denis and Gregg[170]. It was found that addition of either cytokine alone had no effect on the capability of the macrophage to kill MA but administered together they increased the microbicidal effect. Conversely a similar study conducted by Appelberg et al showed IFNγ could arrest MA growth and that addition of IL-4 with IFNγ did

not increase this effect[171]. What may explain this disparity is that the macrophages used in the latter study were from C57BL/6 mice rather than BALB/C mice and the strain of MA used was different in both studies. Despite the latter two studies being *in vitro* they demonstrate how different outcomes can be induced by study design. In this current study no IL-4 was induced by infection with MA 2-151 given live or dead which suggests that Th2 skewed immunity was not induced by this strain.

Ag 85A specific IL-10 was induced by live MA infection and dead MA induced PPD-A specific IL-10. Azouaou et al showed IL-10 is produced in the later stages of infection in their IV model of MA infection[172]. The increase in IL-10 they observed was associated with a decrease in IFNγ production. IL-10 inhibits IL-12 production by DCs and so has an inhibitory effect on the priming of IFNγ responses[86]. IL-10 production was detected from the first time point in mice infected with dead and live MA in the current study; the decrease in IFNγ observed at 3 months in the dead MA infected mice was not associated with an increase in regulatory cytokines at 3 months so I do not think this was the mechanism of reduction of IFNγ in these mice. Azouaou et al note that the strain of MA which they used induces less IFNγ than other strains of MA. If this strain induces greater regulatory responses and less cytotoxic responses this may be why there is a discrepancy in the IFNγ /IL-10 response in their study and mine.

Levels of TGFβ production from human macrophages infected with different strains of MA showed that more pathogenic strains induced greater levels of TGFβ[173]. The induction of TGFβ by MA 2-151 was minimal, some cytokine was detected at 3 months in dead MA infected mice but it was not a consistent effect observed in other stimulations or at other time points.

In the reports discussed here, IL-10 production and TGFβ production are linked to the pathogenicity of the strain of MA. The magnitude of the IFNγ response is inversely correlated to the pathogenicity of the strain of *M. tb* in reports by Lozes et al. MA 2-151 given live or

dead induces good levels of Th1 and CD8+ T cell immune responses but little TGFβ or IL-10 which may indicate a low pathogenicity of this strain in C57BL/6 mice.

Frequencies of regulatory cells in the lymph nodes of live MA infected mice were significantly greater than frequencies in BCG vaccinated mice. This may account for the difference in the kinetic of the responses seen at 1 month after infection. Tregs induced by *M. tb* have been shown to slow priming of the adaptive response; this may be what is occurring in mice infected with live MA[72]. Some IL-10 was induced in live MA mice at 1 month but at low levels and only in Ag 85A stimulated cultures; it does not seem enough to account this cytokine as the regulatory mechanism.

In summary MA 2-151 administered systemically either live or dead induces strong Th1 immune responses. Adaptive immune responses induced by live MA infection take up to 2 months to be induced to a detectable level which is slower than seen in BCG vaccination and infection with dead MA. Live MA induces a strongly PPD-A specific immune response whereas dead MA does not. Th2 skewed immunity was not induced by MA 2-151 and neither was IL-10 or TGFβ. Regulatory T cells may play a role in the delay of adaptive immunity in live MA infection.

4 Model of *M. avium* Exposure after BCG Vaccination: Heat-Killed *M. avium* Administered Intraperitoneally

4.1 Introduction

4.1.1 Exposure to NTM after BCG Vaccination

The effect of prior exposure to NTM on the vaccine efficacy of BCG is an area of intense debate. There is no doubt that in populations where BCG is less effective, there is a high level of exposure to NTM but the use of animal models to show a reduction in BCG-induced efficacy does not provide consistent results.

The aim of this study was to model what happens in human populations to better understand the effect NTM has in populations where BCG fails to protect against pulmonary TB. The assessment of how NTM exposure may affect pre-existing BCG immunity was of interest given the change in the WHO's recommendations for BCG vaccination and for it now to be given at birth in endemic populations[22].

Burl et al compared the immune responses in Gambian infants receiving BCG vaccine at birth and those receiving it at 4.5 months old[174]. PPD specific IFNγ+ cells, as well as PPD specific Tregs and IL-10 production was detected in infants in the delayed vaccine group prior to BCG. No ESAT-6 response was detected eliminating *M. tb* infection as the cause of this response so it was concluded it was induced by exposure to NTM. Infants vaccinated with BCG at birth generated high levels of PPD specific Th1, Th2, IL-6, IL-17 and regulatory T cell responses 4.5 months after vaccination. Infants in the delayed vaccine group were assayed 4.5 months after vaccination as well and it was found the PPD specific IFNγ, IL-6 and IL-17 responses were of

lower magnitude compared to the group vaccinated at birth. These results show that withholding BCG by 4.5 months results in a reduction in the levels of immunity induced by BCG.

Of further note, the group vaccinated at birth were assayed again 9 months after vaccination and it was found the Th1 and Th17 responses were comparable to the frequencies measured in the delayed group at 4.5 months after vaccination[174]. This shows a lack of durability of the BCG induced response which may be due to a deleterious effect of post-vaccination NTM exposure on BCG immunogenicity.

Contradictory to the these results was a study in Cape Town, South Africa where neonatal BCG vaccination was delayed by 10 weeks in infants; this resulted in higher frequencies of Th1 producing CD4+ T cells and a greater polyfunctional capability of these cells compared to infants vaccinated at birth[175]. The obvious disparity between these studies is the time by which BCG is delayed and the effect this has on exposure to NTM; in fact infants in the delayed vaccine group in Cape Town did not have mycobacterial immune responses prior to vaccination.

The study by Lalor et al described in the Introduction showed that BCG induced strong PPD specific IFNγ responses, as measured by whole blood ELISA, in 53% of Malawian infants vaccinated in the first 28 weeks after birth. 100% of UK infants vaccinated in the first 13 weeks after birth induced strong PPD specific responses. The authors discount NTM exposure as the cause of reduced responses in the Malawian population based 62% of the samples being taken in the first week after birth, but as PPD responses pre-vaccination were not measured NTM exposure cannot be eliminated as a cause of the reduced immune response generated in these infants.

Follow-up 12 months after vaccination showed that the median magnitude of PPD specific IFNγ had fallen in UK infants but had remained at a constant level in Malawian infants. This result may indicate a positive effect rather than a negative effect of NTM exposure after vaccination in keeping levels of mycobacterial responses primed. It may, however, be due to infection with *M. tb*. The incidence of TB is higher in Malawi compared to the UK and a diagnosis was not performed in the subjects to eliminate this confounding factor.

At present there is only one published animal model which investigates exposure to NTM after BCG vaccination. Flaherty et al reported that oral administration of *M. avium* after BCG reduced BCG efficacy against *M. tb* challenge[176]. The mechanism of this effect was not discovered in the study but it was suggested that exhaustion of T cell mediated immunity may have been the cause as the protective capability of the immune response decreased over long term *M. tb* infection.

4.1.2 Hypothesis

The aim of this experiment was to model exposure to NTM after BCG vaccination, to model the real life situation that BCG is administered at birth. To evaluate whether this was detrimental to BCG efficacy an *M. tb* challenge was performed.

The hypothesis was that exposure to *M. avium* would reduce the efficacy of BCG. Immunogenicity would be monitored throughout the experiment to identify possible mechanisms of immunological interaction between NTM and BCG.

4.1.3 Experiment Plan

Intraperitoneal sensitisation with dead mycobacteria was tested with two strains of *M. avium* (MA). A BCG only and naïve control group was included in both experiments.

4.1.3.1 Experiment 2-151

All mice were vaccinated with BCG. Three months after BCG, half the mice were exposed to dead MA strain 2-151 monthly for 7 months (Figure 4-1). Cellular immune responses of exposed mice and BCG only controls were compared 4 weeks after the first dose of MA (4 months) and 4 weeks after the 7th dose of MA (10 months). Cells from inguinal lymph nodes and spleen were stimulated with PPD-T, PPD-A and Ag 85A to assess the specificity of the response to these mycobacterial antigens.

At 10 months mice were exposed to *M. tb* by aerosol delivery. 28 days after *M. tb* infection PPD-T specific immune responses were assayed and *M. tb* load in the lungs and spleens were determined.

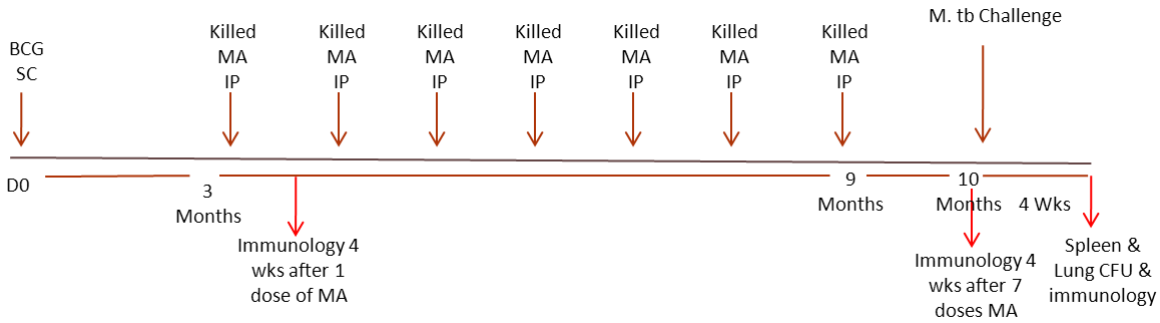


Figure 4-1: Experiment 2-151 plan. Administration time points shown on top of the line; immunology and CFU time points shown on the bottom of the line. 1x10^{5} CFU of BCG was given subcutaneously (SC). 1x10^{8} CFU of heat-killed MA 2-151 was given intraperitoneally (IP). *M.tb* Erdmann was given by aerosol delivery.

4.1.3.2 Experiment 724

All mice were vaccinated with BCG. 6 months after BCG, half the mice were exposed to dead MA strain 724 monthly for 6 months (Figure 4-2). 4 weeks after the 6th dose of MA (13 months) cells from the inguinal lymph nodes, spleen and lungs were stimulated with PPD-T, PPD-A or Ag 85A to assess the specificity of the response to these mycobacterial antigens.

At 13 months mice were exposed to *M. tb* by aerosol delivery. 13 and 16 days after *M. tb* infection cellular immune responses specific for PPD-T were assayed in lungs, pulmonary lymph nodes and spleens. 5 weeks after *M. tb* infection bacterial load in lungs and spleens was determined.

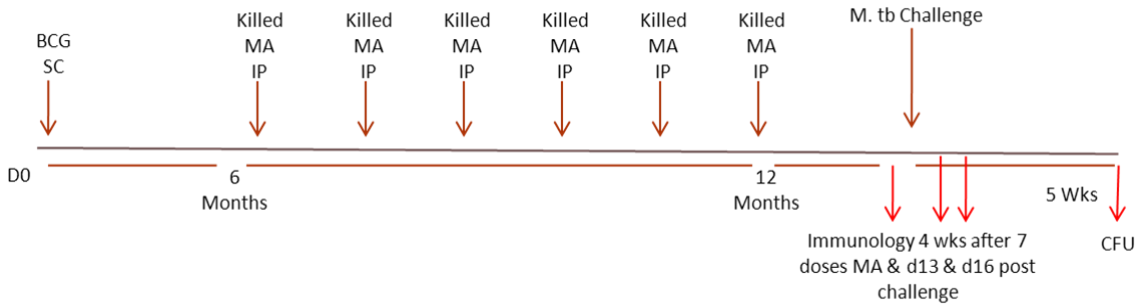


Figure 4-2: Experiment 724 plan. Administration time points shown on top of the line; immunology and CFU time points shown on the bottom of the line. $1x10^5$ CFU of BCG was given subcutaneously. $1x10^8$ CFU of heat-killed MA 724 was given IP. 100 CFU of *M.tb* Erdmann was given by aerosol delivery

4.2 Results: Pre-Challenge

4.2.1 Th1 Responses: Experiment 2-151

4.2.1.1 Lymph Node

4 Months

Neither the BCG only group nor the BCG + MA sensitisation (B + MA) group had induced IFNγ+ CD4+ T cells to any stimulation (Figure 4-3 A, C, E). B + MA induced a TNFα+ CD4+ T cells to all 3 stimulations and an IL-2+ CD4+ T cells to PPD-A; TNFα and IL-2 responses were not detected in the BCG only group.

10 Months

At 10 months PPD-T specific TNFα+ CD4+ T cells and PPD-A specific IL-2+ CD4+ T cells were induced in the BCG only group (Figure 4-3 B, D, F). B + MA induced IFNγ+, TNFα+ and IL-2+ CD4+ T cells to all three stimulations. The B+MA group had significantly greater frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells specific for PPD-T, significantly greater frequencies of TNFα+ CD4+ T cells to PPD-A and greater frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells

specific to Ag 85A compared to BCG alone. The BCG only group had significantly greater frequencies of IL-2+ CD4+ T cells specific for PPD-A.

4.2.1.2 Spleen

4 Months

At 4 months BCG only and B + MA induced a similar TNFα response to PPD-A and TNFα and IL-2 responses to Ag 85A (Figure 4-4 C & E). B + MA induced IFNγ+ CD4+ T cells in response to PPD-T (Figure 4-4 A). The BCG only group induced IFNγ+ CD4+ T cells in response to PPD-A.

10 Months

BCG only and B + MA induced IFNγ+, TNFα+ and IL-2+ CD4+ T cells specific for all 3 stimulations (Figure 4-4 B, D & E). Frequencies of cells specific for PPD-T and PPD-A were greater than Ag 85A specific responses. Compared to BCG alone B + MA had significantly greater frequencies of IFNγ+ CD4+ T cells specific for PPD-T and TNFα+ CD4+ cells specific for PPD-A and Ag 85A.

The B + MA group had greater frequencies of IFNγ+, TNFα+ and IL-2+ CD4s in lymph nodes and spleen compared to the BCG only group. The effect was enhanced with repeated doses of MA and in most cases became significantly different to BCG only controls. The magnitude of response was greater in the lymph node than the spleen. PPD-T and PPD-A specific responses were a greater magnitude than Ag 85A specific responses.

4.2.2 Th1 Responses: Experiment 724

4.2.2.1 Lymph Node

BCG alone induced IFNγ+ and TNFα+ CD4+ T cells specific to PPD-T and TNFα+ CD4+ T cells specific to Ag 85A (Figure 4-5 A & C). B + MA induced IFNγ+, TNFα+ and IL-2 + CD4+ T cells to all 3 stimulations; frequencies were greater than those induced by BCG alone (Figure 4-5 A, B & C). Response to PPD-T was of greater magnitude than to PPD-A and Ag 85A.

4.2.2.2 Spleen

BCG alone induced an IFNγ+ and TNFα+ response to PPD-T but no response to PPD-A or Ag 85A (Figure 4-5 D, E and F). B + MA induced IFNγ+, TNFα+ and IL-2 + CD4+ T cells to all 3 stimulations. Frequencies induced by B + MA were greater than those induced by BCG alone; this was significant for Ag 85A specific TNFα+ CD4+ T cells. PPD-T and Ag 85A responses were of greater magnitude than PPD-A responses.

4.2.2.3 Lung

BCG alone induced a strong PPD-A specific IFNγ response (Figure 4-5 H). B + MA did not induce any PPD-A specific response but did induce IFNγ+ CD4+ T cells specific to PPD-T (Figure 4-5 G). BCG only and B + MA induced a similar TNFα response to PPD-T. No Ag 85A specific responses were detected (Figure 4-5 I).

The greatest frequencies of Th1 were induced in the lungs and lymph node responses were greater than spleen responses. In lymph nodes and spleen the B + MA group had greater frequencies of Th1 against all cytokines compared to BCG. In the lung a PPD-T specific response was induced by both groups. PPD-A specific IFNγ responses were greater in the BCG only group compared to the B + MA group.

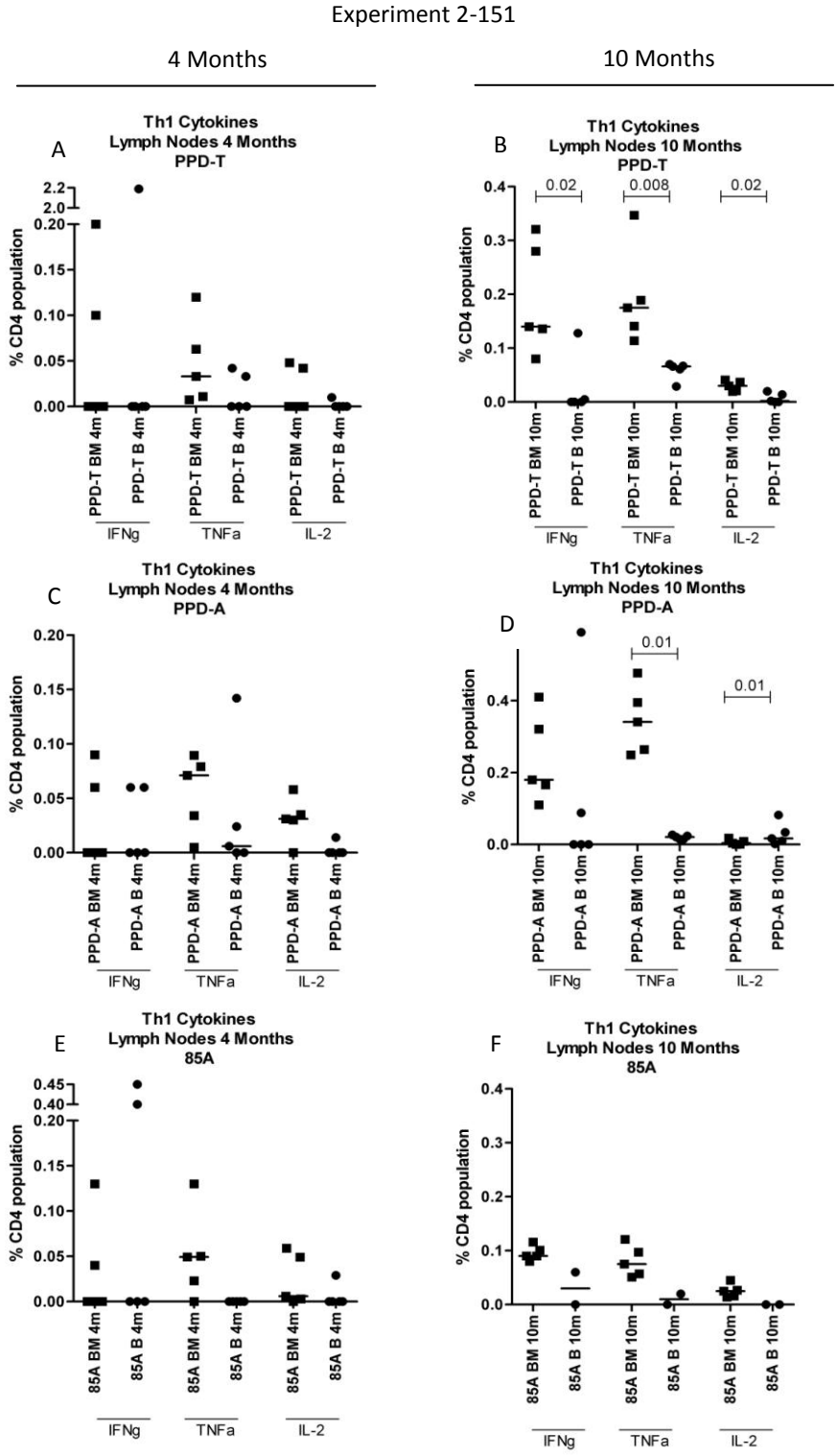


Figure 4-3: Th1 responses in BCG vaccinated mice and mice which were sensitised with MA 2-151 after BCG vaccination. Graphs on the left are results from the 4 month time point; graphs on the right are from the 10 month time point. Cells from inguinal lymph nodes were stimulated with PPD-T, PPD-A or 85A. IFNγ, TNFα and IL-2 production from CD4+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to determine statistical significance and these are displayed on the graph.

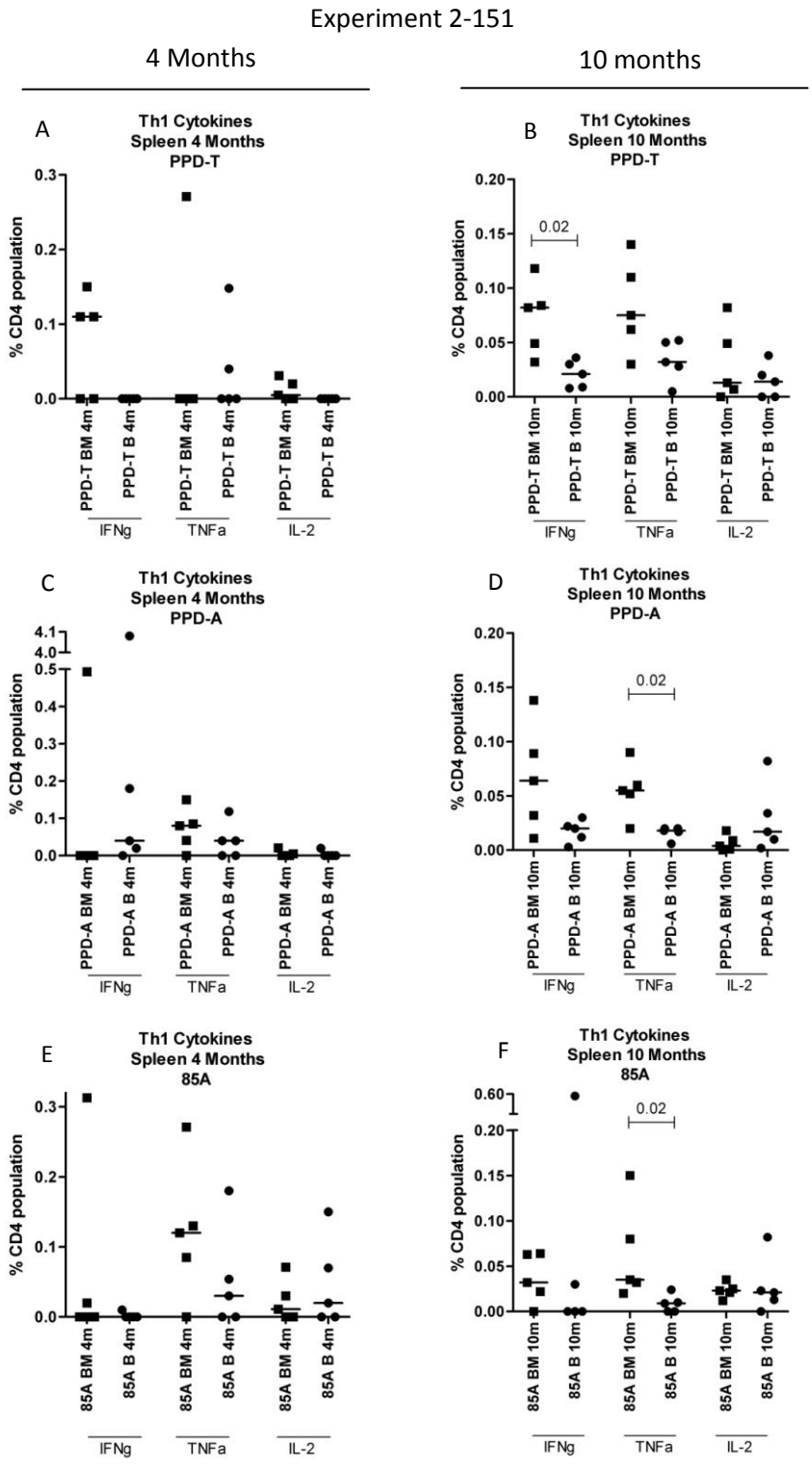


Figure 4-4: Th1 responses in BCG vaccinated mice and mice which were sensitised with MA 2-151 after BCG vaccination. Graphs on the left are results from the 4 month time point; graphs on the right are from the 10 month time point. Cells from the spleen were stimulated with PPD-T, PPD-A or 85A. IFNγ, TNFα and IL-2 production from CD4+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to determine statistical significance and these are displayed on the graph.

Experiment 724

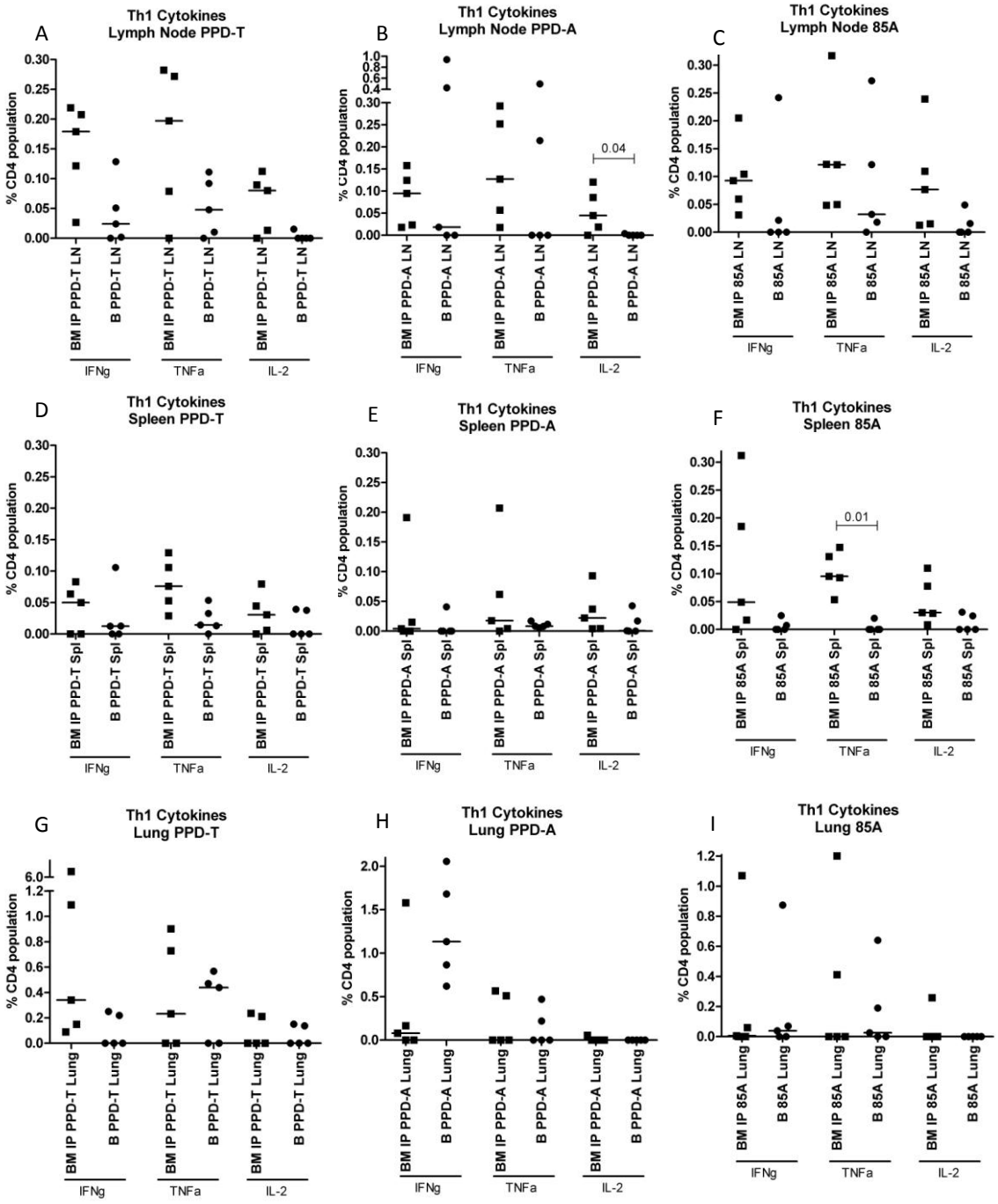


Figure 4-5: Th1 responses in BCG vaccinated mice and mice which were sensitised with MA 724 after BCG vaccination. Cells from inguinal lymph nodes, spleens and lungs were stimulated with PPD-T, PPD-A or 85A. A – C is the response in the lymph nodes, D – F is the response in the spleen and G – I is the response in the lung. IFNγ, TNFα and IL-2 production from CD4+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to determine statistical significance and these are displayed on the graph.

4.2.3 Polyfunctional Th1 Responses

The Th1 data was analysed to show whether cells were producing more than one of IFNγ, TNFα and IL-2. Results from experiment 724 are shown as responses were similar in both experiments. PPD-T stimulated results are shown as the response to all stimulations was similar.

4.2.3.1 BCG + MA 724

Lymph Node

The Th1 response was dominated by IFNγ+TNFα+IL-2+ (γ+α+2+) CD4+ T cells, γ+α+ CD4s and α+ CD4+ T cells (Figure 4-6 A). α+2+, γ+ and 2+ CD4+ T cells were also detected.

Spleen

The dominant cytokine producing cells were γ+α+ CD4+ T cells followed by γ+α+2+ CD4+ T cells (Figure 4-6 B). α+2+ and α+ CD4+ T cells were also detected.

Lung

The cytokine response in the lung was produced by γ+α+ CD4+ T cells and γ+ CD4+ T cells (Figure 4-6 C). Other combinations of cytokine were not detected.

4.2.3.2 BCG Only

Lymph Node

The greatest frequency of cytokine producing cells were α+ CD4+ T cells, γ+ CD4s were also detected (Figure 4-6 A).

Spleen

The greatest frequency of cytokine producing cells in the spleen were α+ CD4+ T cells (Figure 4-6 B). γ+α+2+, γ+α+ and γ+ CD4+ T cells were also detected.

Lung

The response in the lung was dominated by α+ CD4+ T cells, γ+ CD4s were also detected (Figure 4-6 C).

The B + MA group had a more polyfunctional Th1 response than the BCG only group; the BCG only group's response in the lymph nodes and lungs was purely monofunctional γ+ and α+ CD4s.

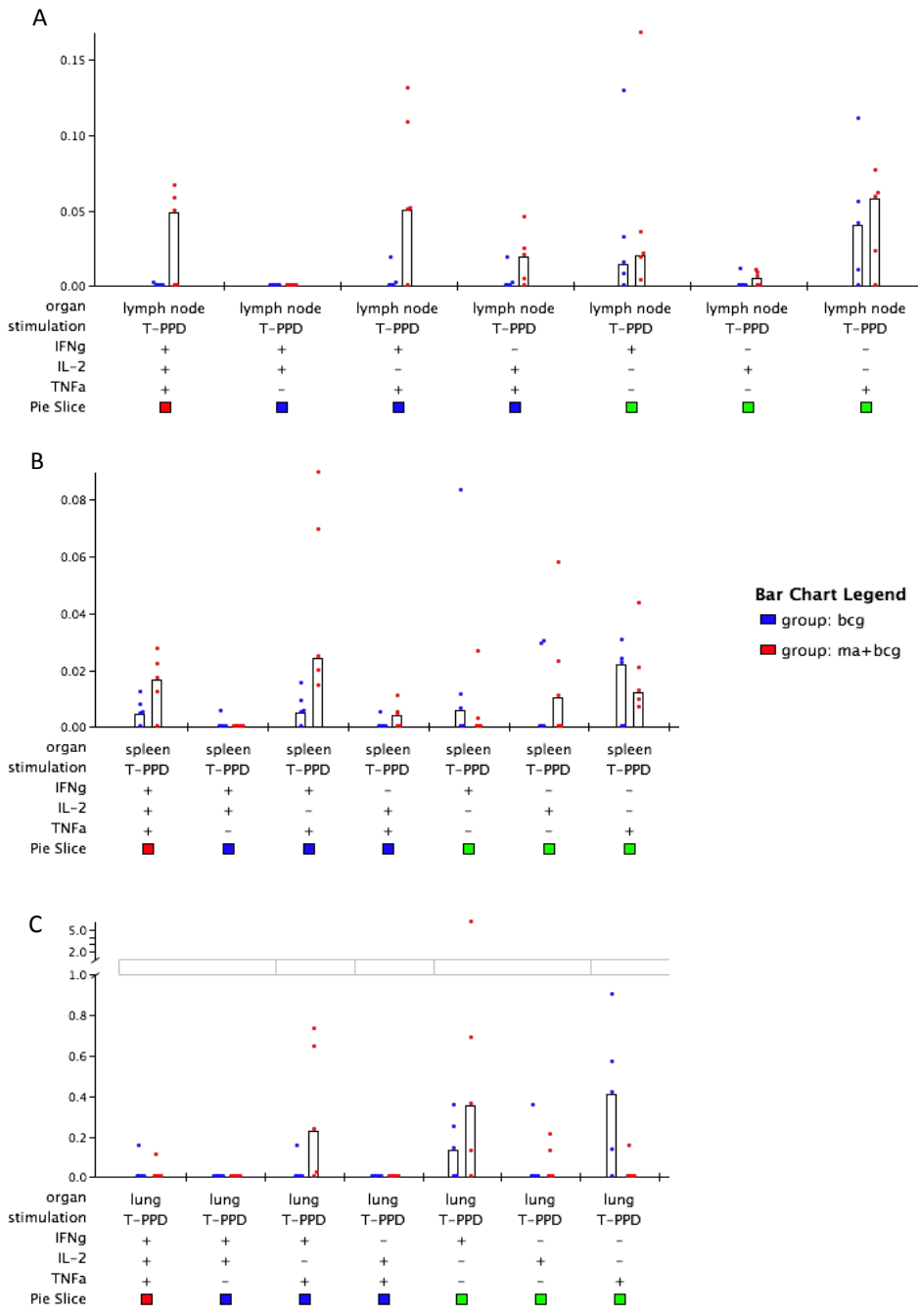


Figure 4-6: Polyfunctional Th1 response in mice vaccinated with BCG and mice sensitised with MA 724 after BCG. Graphs show the secretion of one or more of IFNγ, TNFα and IL-2 from CD4+ cells stimulated with PPD-T. Data is expressed as the % of CD4+ cells and is antigen specific; media only control wells are subtracted. A is lymph node and B is spleen, C is lung. Each data point represents one mouse and the median is displayed, n=5.

4.2.4 Th17 Responses: Experiment 2-151

4.2.4.1 Lymph Nodes

At 4 months IL-17 was produced by both groups in response to PPD-A but not to PPD-T or Ag 85A (Figure 4-7 A). At 10 months BCG only and B + MA induced IL-17 in response to PPD-T, PPD-A and Ag 85A; frequencies were of greater magnitude than recorded at 4 months (Figure 4-7 B). B + MA induced greater frequencies of PPD-T, PPD-A and Ag 85A specific IL-17+ CD4+ T cells than BCG alone.

4.2.4.2 Spleen

At 4 months B + M induced IL-17+ CD4+ T cells specific for PPD-T; BCG alone induced IL-17+ CD4+ T cells specific for Ag 85A (Figure 4-7 C). There was no PPD-A specific response. At 10 months BCG alone and B + MA induced a similar response to PPD-T (Figure 4-7 D). BCG alone induced a PPD-A specific response; B + MA induced an Ag 85A specific response.

4.2.5 Th17 Responses: Experiment 724

4.2.5.1 Lymph Node

BCG alone and B + MA induced an Ag 85A response; BCG alone induced a PPD-T response (Figure 4-7 E). Neither group induced a PPD-A response.

4.2.5.2 Spleen

B + MA induced significantly more PPD-A and Ag 85A specific and more PPD-T specific IL-17+ CD4+ T cells than BCG alone (Figure 4-7 F).

4.2.5.3 Lung

The BCG only group induced a significantly greater PPD-T specific IL-17 response and a greater Ag 85A response compared to the B + MA group (Figure 4-7 G).

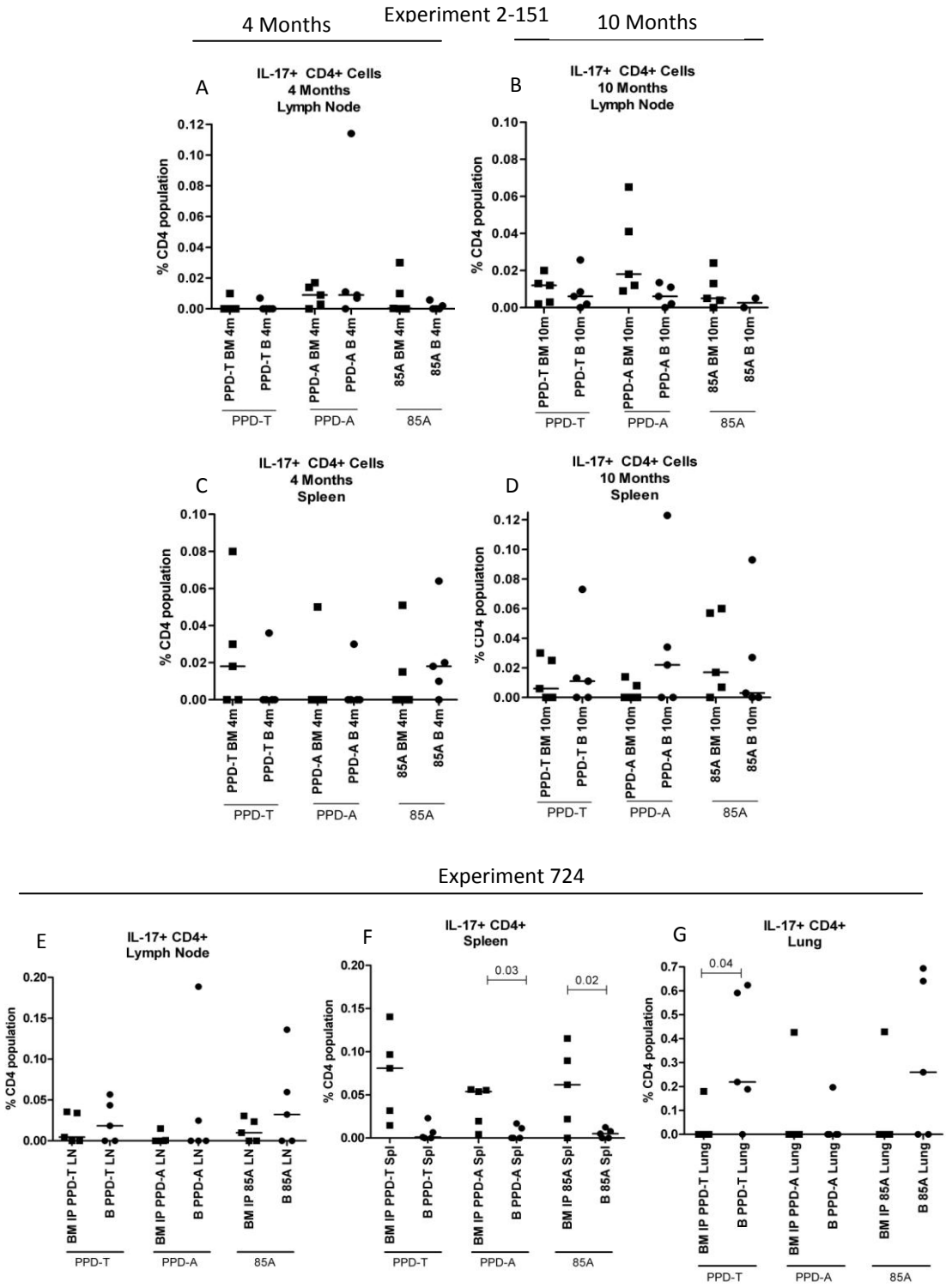


Figure 4-7: Th17 responses in BCG vaccinated mice and mice which were sensitised with MA 2-151 or 724 after BCG vaccination. Cells from the inguinal lymph nodes, spleens and lungs were stimulated with PPD-T, PPD-A or 85A. (A) LN at 4 months in 2-151, (B) LN at 10 month in 2-151, (C) spleen at 4 months in 2-151, (D) spleen at 10 months in 2-151, (E) LN in 724, (F) spleen in 724 and (G) lungs in 724. Il-17 production from CD4+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to determine statistical significance; none were calculated.

4.2.6 CD8+ T Cell Responses: Experiment 2-151

4.2.6.1 Lymph Nodes

4 Months

No IFNγ was detected in either group at 4 months (Figure 4-8 A, C & E). B + MA induced a TNFα response to all 3 stimulations. A low level IL-2 response was detected in both groups to all stimulations.

10 Months

BCG alone and B + MA induced a PPD-A specific IFNγ response (Figure 4-8 D). There were no PPD-T or Ag 85A responses detected in either group (Figure 4-8 B & F).

4.2.6.2 Spleen

4 Months

BCG alone induced PPD-T specific IL-2+ CD8+ T cells and PPD-A specific IFNγ+ CD8+ T cells (Figure 4-9 A & C). B + MA induced PPD-T specific IFNγ+ and TNFα+ CD8+ T cells and a PPD-A specific IFNγ+ response, which was greater than that induced by BCG. B + MA also induced IFNγ+, TNFα+ and IL-2+ Ag85A specific CD8+ T cells (Figure 4-9 E).

10 Months

At 10 months PPD-A specific IL-2+ CD8+ T cells were detected in both groups (Figure 4-9 D). BCG alone also induced PPD-T and Ag85A specific IL-2+ CD8+ cells (Figure 4-9 B & F).

CD8 responses were low in both groups. The strongest response was detected in the spleen of the B + MA group at 4 months in response to PPD-A and Ag 85A stimulation.

4.2.7 CD8+ T Cell Responses: Experiment 724

4.2.7.1 Lymph Nodes

B + MA induced PPD-T, PPD-A and Ag85A specific IFNγ+ CD8+ T cells; BCG alone induced no responses in lymph nodes (Figure 4-10 A, B & C).

4.2.7.2 Spleen

BCG alone induced PPD-A and Ag 85A specific IFNγ+ CD8+ T cells (Figure 4-10 E & F). B + MA induced IFNγ+ CD8+ T cells specific for all 3 stimulants; PPD-A specific responses were similar and Ag 85A specific responses were greater compared to BCG alone (Figure 4-10 D, E & F).

4.2.7.3 Lung

B + MA induced PPD-T, PPD-A and Ag 85A specific IFNγ+ CD8+ T cells ; TNFα and IL-2 were not produced (Figure 4-10 G, H & I). BCG alone did not induce a response.

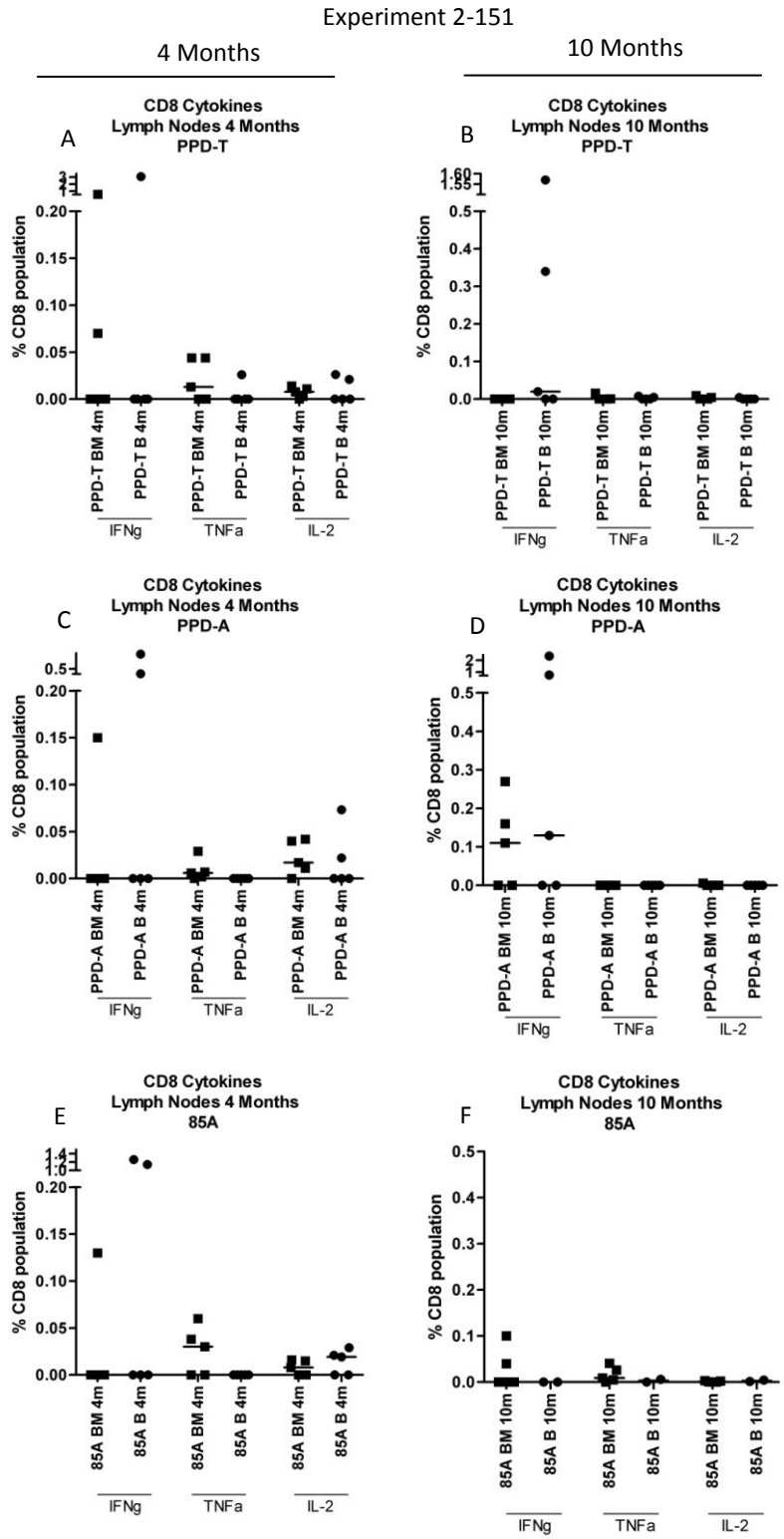


Figure 4-8: CD8+ T cell cytokine responses in BCG vaccinated mice and mice which were sensitised with MA 2-151 after BCG vaccination. Graphs on the left are results from the 4 month time point; graphs on the right are from the 10 month time point. Cells from inguinal lymph nodes were stimulated with PPD-T, PPD-A or 85A. IFNγ, TNFα and IL-2 production from CD8+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD8+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; none were calculated.

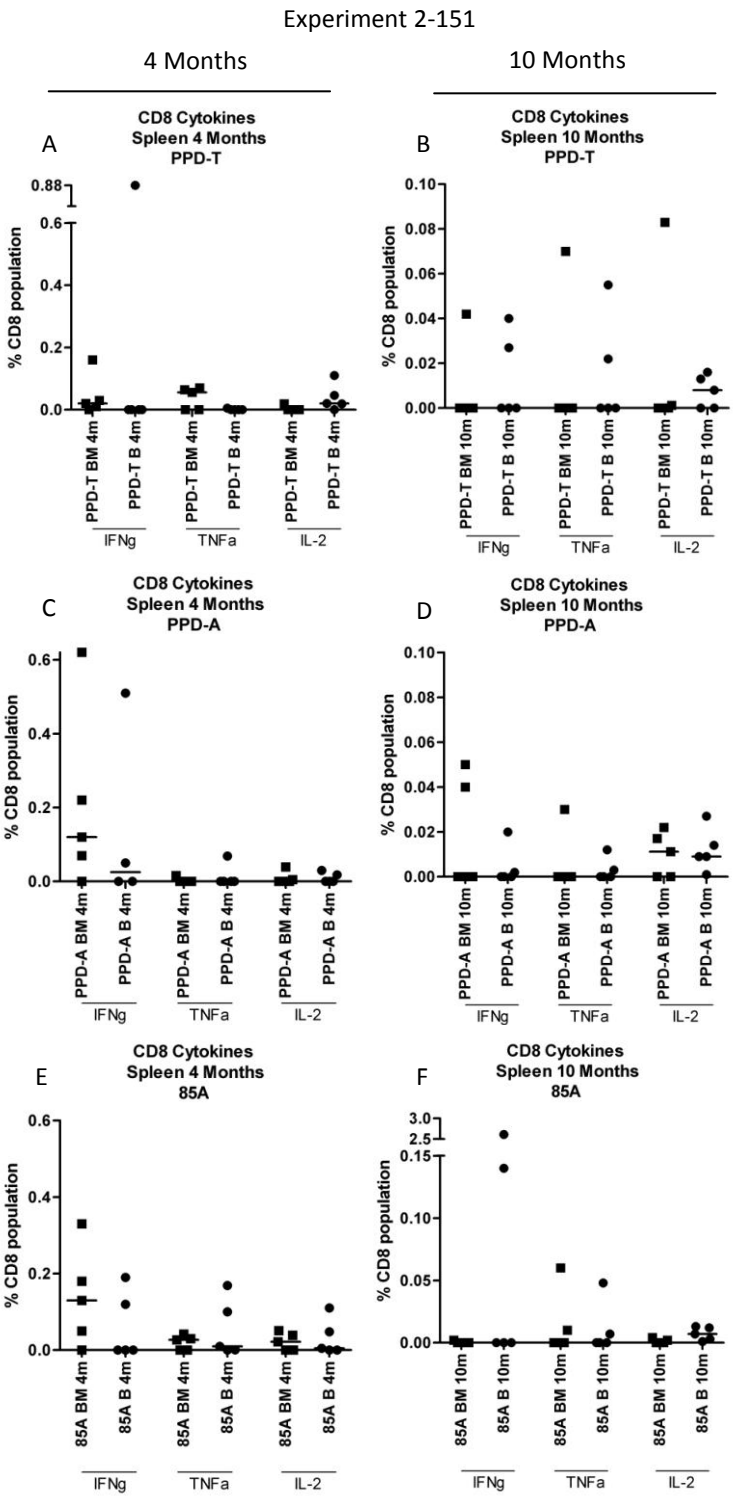


Figure 4-9: CD8+ T cell cytokine responses in BCG vaccinated mice and mice which were sensitised with MA 2-151 after BCG vaccination. Graphs on the left are results from the 4 month time point; graphs on the right are from the 10 month time point. Cells from the spleen were stimulated with PPD-T, PPD-A or 85A. IFNγ, TNFα and IL-2 production from CD8+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD8+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; none was calculated.

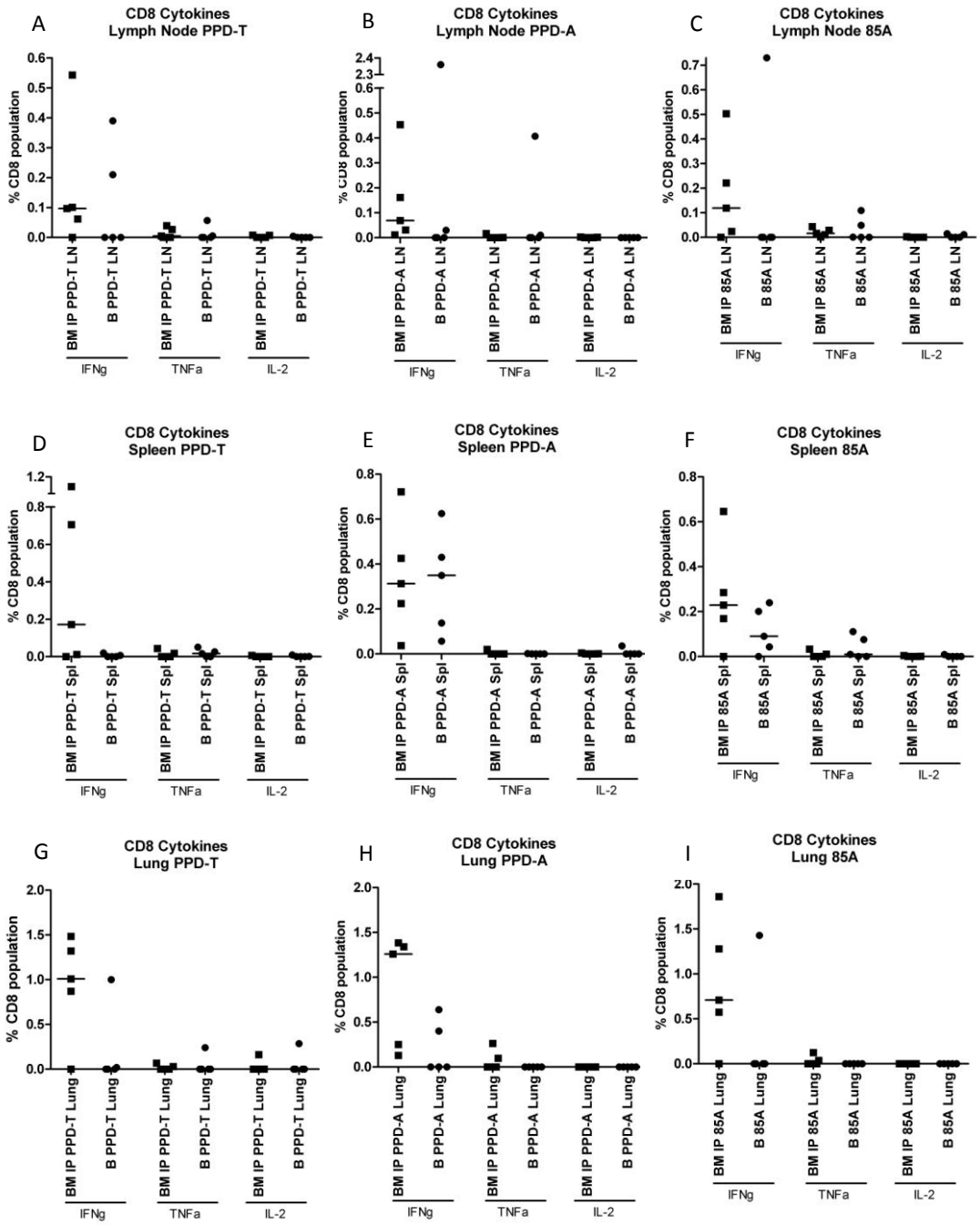


Figure 4-10: CD8+ T cell cytokine responses in BCG vaccinated mice and mice which were sensitised with MA 724 after BCG vaccination. Cells from the inguinal lymph node (A-C), spleen (D-F) and lungs (G-H) were stimulated with PPD-T, PPD-A or 85A. IFNγ, TNFα and IL-2 production from CD8+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD8+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; none was calculated.

4.2.8 Th2 Responses

The Th2 response was measured by production of IL-4. In experiment 2-151 the concentration of IL-4 in 3-day culture supernatants was measured by the FlowCytomix assay. In experiment 724 the frequency of Il-4+ CD4+ T cells was assayed by flow cytometry.

4.2.8.1 Experiment 2-151

No IL-4 was detected in lymph node cultures at 4 months (data not shown). IL-4 was detected in the spleens of both groups at 4 months; BCG alone induced PPD-A specific response and B + MA induced a PPD-T and PPD-A specific response (Figure 4-11 A). At 10 months IL-4 was induced to a similar level in both groups in response to stimulation with both PPD-T and PPD-A (Figure 4-11 B). The concentration of cytokine in PPD-T cultures was greater at 10 months than at 4 months.

4.2.8.2 Experiment 724

In the lymph node BCG and B + MA induced IL-4+ CD4+ T cells; there were significantly more induced by B + MA compared to BCG alone (Figure 4-11 C)). In the spleen B + MA induced IL-4+ CD4+ T cells specific for all 3 stimulants (Figure 4-11 D). BCG alone did not. BCG alone induced a PPD-T specific response in the lung (Figure 4-11 E).

B + MA induced more IL-4 than BCG alone at 4 months in experiment 2-151. In experiment 724 B + MA induced significantly more IL-4+ CD4+ T cells in lymph nodes and in spleen compared to BCG alone.

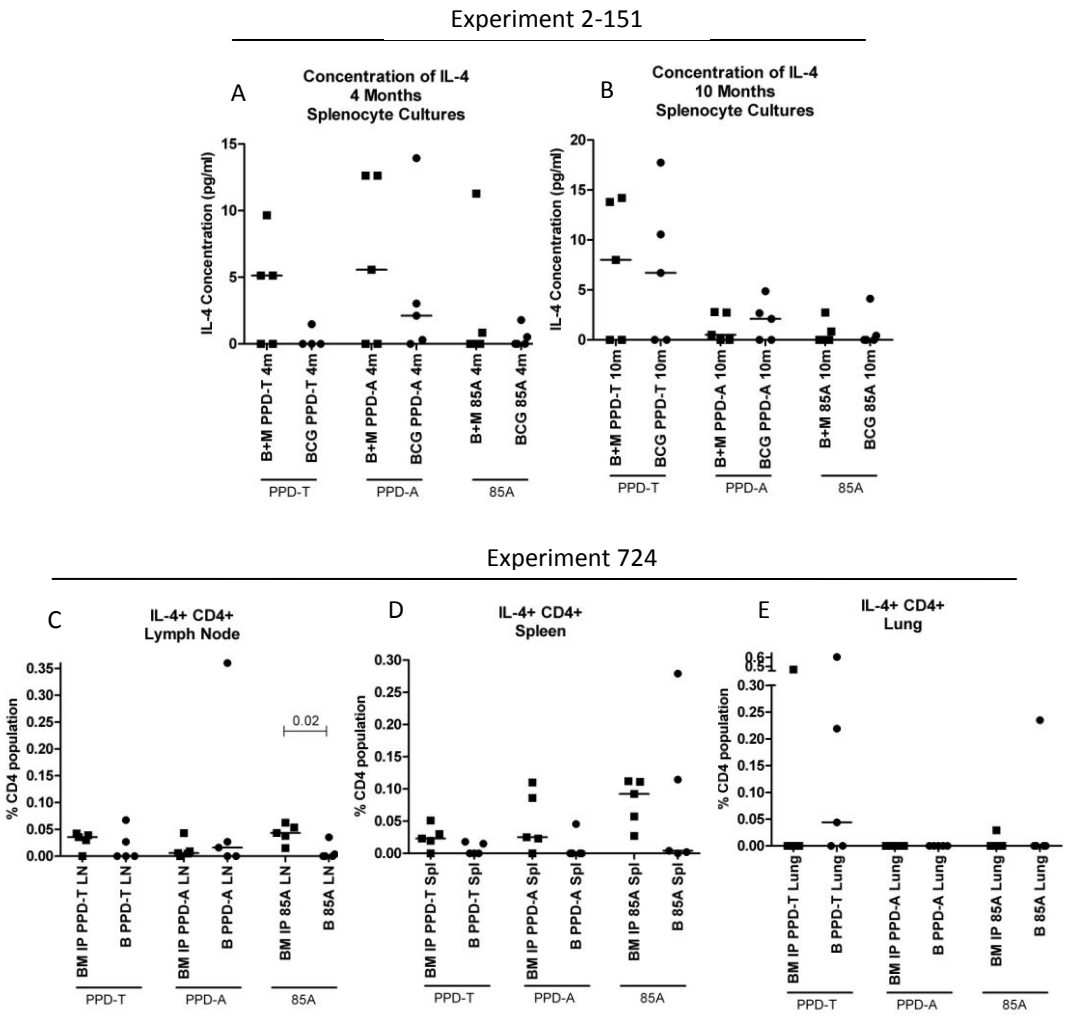


Figure 4-11: Th2 responses in BCG vaccinated mice and mice sensitised with MA 2-151 or 724 after BCG vaccination. (A & B) Splenocytes were cultured with PPD-T, PPD-A or 85A for 3 days. The concentration of IL-4 in the culture supernatant was determined with the FlowCytomix assay. (C-E) Cells from lymph nodes, spleen, or lungs were stimulated with PPD-T, PPD-A or 85A and the production of IL-4 detected by flow cytometry. All results are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance differences; these are shown where calculated.

4.2.9 Regulatory T Cell Responses: Experiment 2-151

Tregs were defined as CD4+ T cells expressing CD25, FoxP3 and CD39. Flow cytometry was used to assay cell frequencies in experiment 2-151 and 724. In experiment 2-151 the concentration of IL-10 and TGFβ in 3-day culture supernatants was measured by the FlowCytomix assay. In experiment 724 production of IL-10 from CD4+ T cells was detected by flow cytometry.

4.2.9.1 Treg frequency

Lymph Node

Around 5% of CD4+ T cells were Tregs in BCG alone and B + MA mice at 4 months; this increased to around 12 to 15% at 10 months. There were significantly higher frequencies of Tregs in the lymph nodes of the B + MA group compared to the BCG only group at 10 months (Figure 4-12 A).

Spleen

The frequency of Tregs at 4 months was between 0.5 and 2.5% (Figure 4-12 B). At 10 months it was between 1 and 3%. The frequencies of the 2 groups were similar.

4.2.9.2 IL-10 and TGFβ

IL-10

Very few mice produced IL-10 (Figure 4-12 C - E). 3 of 5 mice from the BCG only group responded to PPD-T at 10 months.

TGFβ

TGFβ was produced in lymph node cultures from BCG only mice 4 months after vaccination (Figure 4-12 F). It was not detected in any other culture.

4.2.10 Regulatory T Cell Responses: Experiment 724

4.2.10.1 Treg frequency

Lymph node

Treg frequencies were around 10 to 18%; median frequency was greatest in the BCG only group compared to B + MA group (Figure 4-13 A).

Spleen

Frequencies in the spleen were lower compared to the lymph nodes at around 3%; the groups had similar levels of Treg cells.

Lung

Frequencies in the lungs were around 3-5%, similar to the spleen and no difference between the groups was detected.

4.2.10.2 IL-10

Lymph Node

BCG alone induced IL-10+ CD4+ T cells in the lymph node specific to PPD-T and PPD-A; significantly more than in B + MA cultures (Figure 4-13 C).

Spleen

BCG alone induced IL-10+ CD4+ T cells specific for PPD-T and Ag 85A (Figure 4-13 D). There were lower frequencies of IL-10 + CD4+ T cells in the spleen than the lymph nodes

Lung

BCG alone induced IL-10+ CD4+ T cells specific to Ag 85A; B + MA induced a PPD-T and PPD-A specific response (Figure 4-13 B).

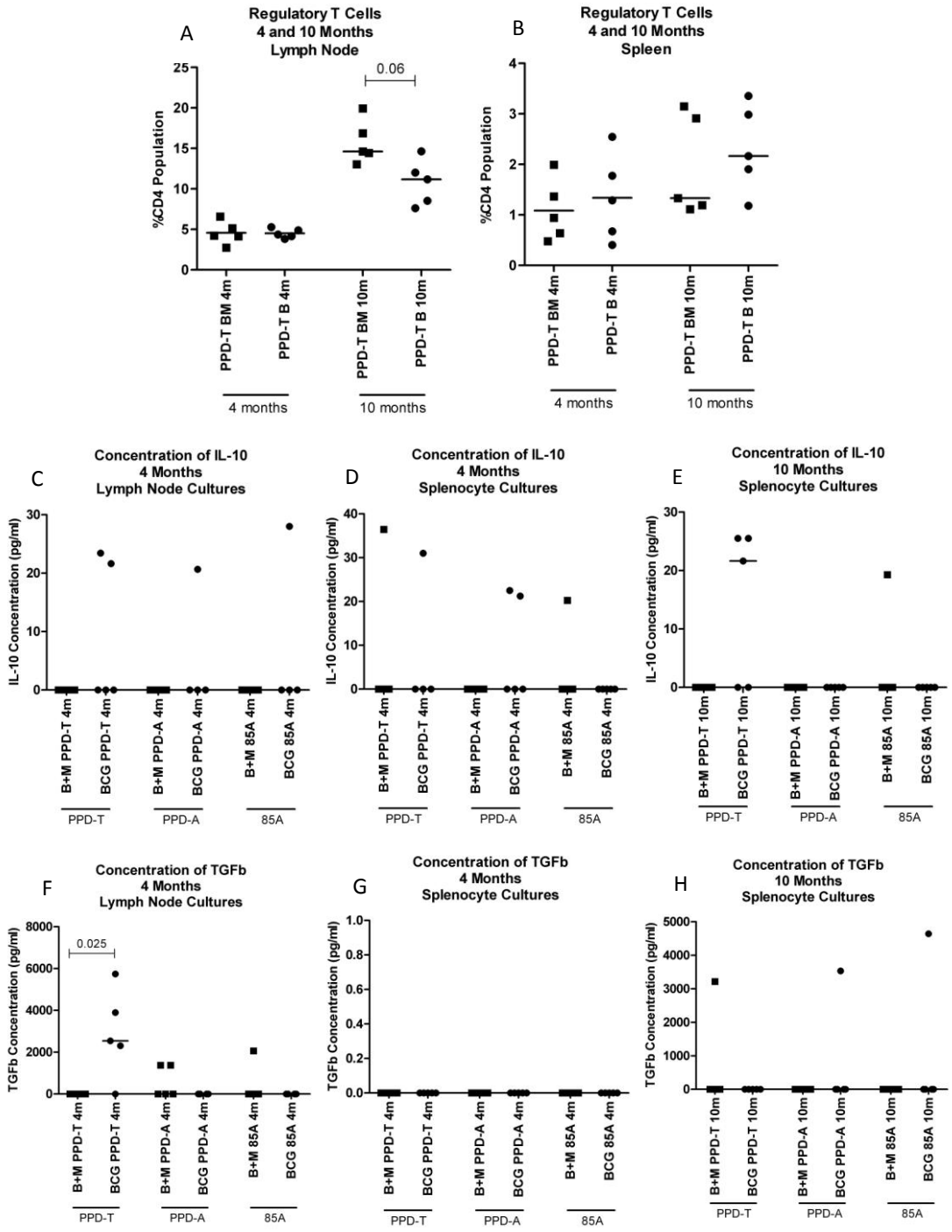


Figure 4-12: Treg responses in BCG vaccinated mice and mice which were sensitised with MA 2-151 after BCG vaccination. (A & B) Treg frequencies in lymph node and spleen were assayed by flow cytometry. (C-E) IL-10 concentration in lymph node cultures at 4 months and spleen cultures at 4 and 10 months. (F-H) TGFβ concentration in lymph node cultures at 4 months and spleen cultures at 4 and 10 months. Cells were cultured with PPD-T, PPD-A or 85A for 3 days and the concentration of cytokines in the culture supernatant was determined with the FlowCytomix assay. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance and is shown where calculated.

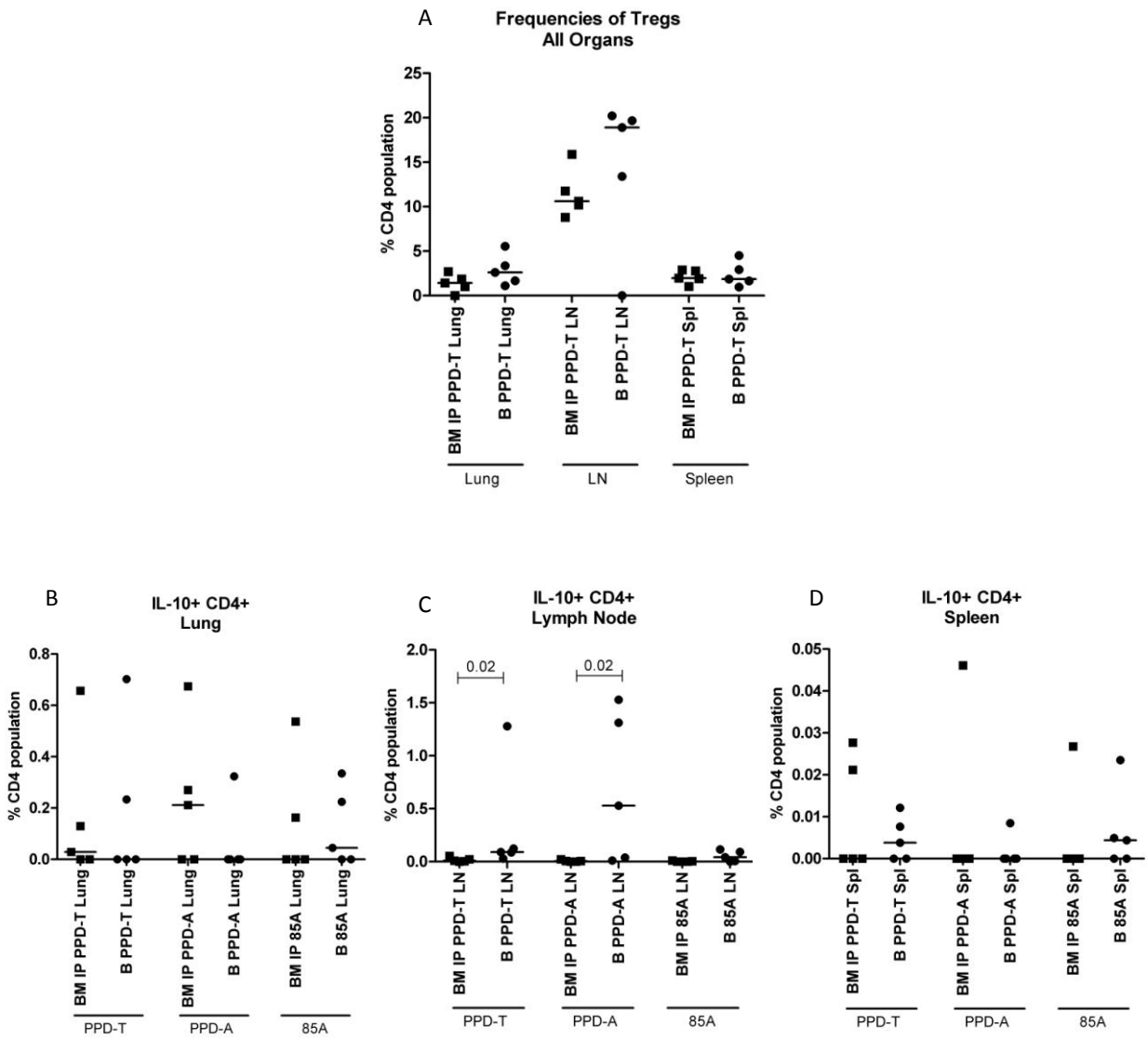


Figure 4-13: Treg responses in BCG vaccinated mice and mice which were sensitised with MA 724 after BCG vaccination. (A) Flow cytometry was used to assay the frequency of cells expressing regulatory phenotype CD4+CD25+FoxP3+CD39+ in inguinal lymph nodes and spleens. (B – D) IL-10 production from CD4+ cells was assayed by flow cytometry. Cells were stimulated with PPD-T, PPD-A or 85A; the media control well value is not subtracted for Treg frequency but is for IL-10 production to show antigen specific response. Results are expressed as a % of the CD4 population. Each data point represents one mouse and the median is shown, n=5. The Mann-Whitney U test was applied to test statistically significant results and these are shown on the graph.

4.3 Results: Post-Challenge

4.3.1 Experiment 2-151

Lung

Naïve mice had a median CFU of 2.9 (log 10), mice vaccinated with BCG 6 weeks prior to challenge had a median of 2.6 (log 10) (Figure 4-14 A). Mice vaccinated with BCG 10 months prior to challenge had median CFU 3.24 (log 10), those mice which received BCG 10 months prior to challenge and then exposed to MA had a median CFU of 3.15 (log 10). Although the median CFU of mice vaccinated with BCG 6 weeks before challenge was lower than all the other groups, this was not statistically significant. The CFU counts in the lungs of mice vaccinated with BCG 10 months before challenge and those exposed to MA were not different to naïve mice.

Spleen

The median CFU in the naïve group was 3.32 (log 10), the median CFU of mice vaccinated 6 weeks before challenge was 0 (Figure 4-14 B). Mice vaccinated with BCG 10 months before challenge had a median CFU of 1.45 (log 10) and those which were exposed to MA after BCG vaccination had a median CFU of 1.602 (log 10).Many of the plated spleen samples had no *M. tb* growth. There were no differences in CFU counts between any groups.

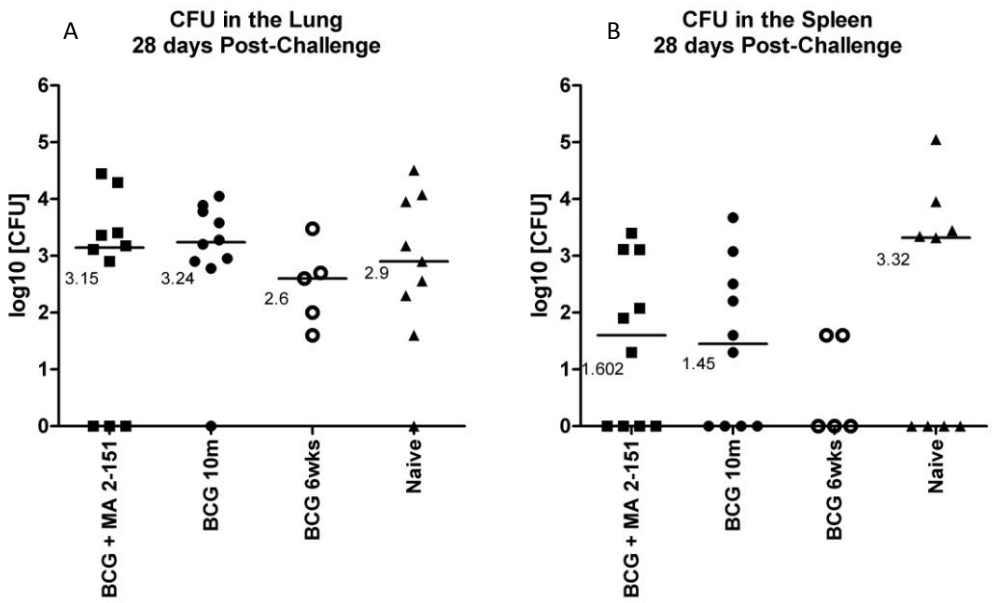


Figure 4-14: *M. tb* burden in the lungs and spleens after aerosol challenge. Log 10 of the CFU count of the whole organ is displayed. Each point represents one mouse; the median is displayed as a line and the value in text next to the line. N=5. The Mann-Whitney U test was used to test for statistical significance; none was calculated.

4.3.1.1 Th1 Responses: Post-Challenge

Lung

Four weeks after *M. tb* challenge the B + MA group had a PPD-T specific IFNγ and IL-2 response in the lung, no response was detected in the BCG only and naïve group (Figure 4-15 A).

Spleen

A TNFα response to PPD-T was detected in the spleens of all 3 groups, only the naïve group produced IFNγ and IL-2 (Figure 4-15 B). The naïve group had significantly greater frequencies of IFNγ, TNFα and IL-2 producing cells compared to the other two groups.

4.3.1.2 CD8+ T Cell Responses

Lung

An IFNγ+ CD8+ T cell response was detected in the lungs of all groups; greater frequencies were recorded in the naïve and BCG only groups compared to the B + MA group (Figure 4-15 C).

Spleen

A TNFα response was detected in the spleens of naïve mice; an IL-2 response was detected in the spleens of B + MA mice (Figure 4-15 D). Cytokine producing CD8+ T cells were at a lower frequency in the spleen compared to the lung.

4.3.1.3 Th17 Responses

A PPD-T specific IL-17 response was detected in the lungs of BCG alone and B + MA mice, the response was greater in the B + MA group but the difference was not significant (Figure 4-15 E). No IL-17 was detected in the spleens.

4.3.1.4 Treg Responses

Frequencies of Tregs in the lungs were highly variable (Figure 4-15 F). The median frequency of the BCG alone group was greater than the B + MA group and the naïve group. Frequencies in the spleen were less variable, between 2 and 6%, and comparable across the groups.

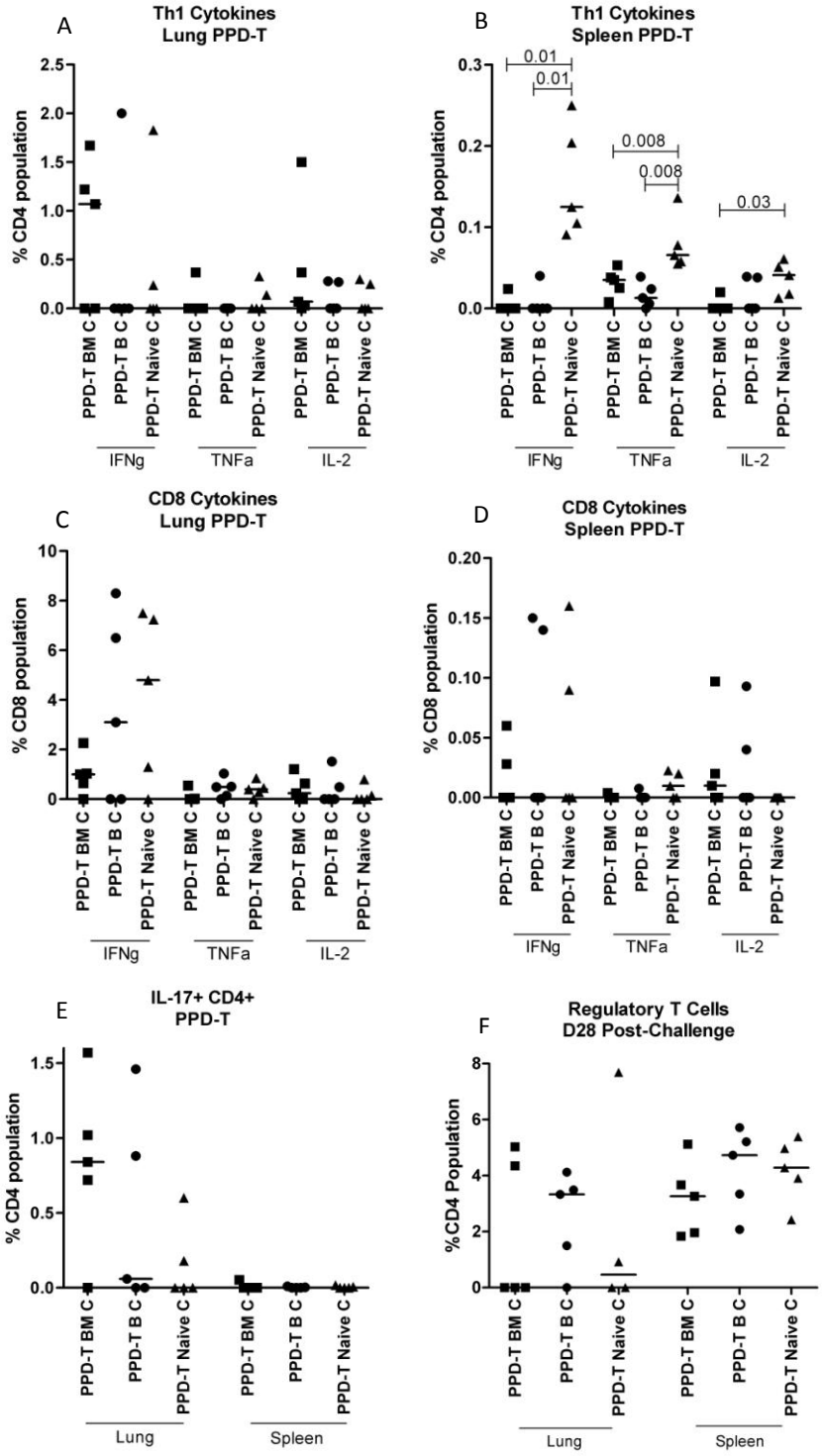


Figure 4-15: Th1, CD8+ T cell, Th17 cell and Treg cell responses in *M. tb* infected lung and spleens 4 weeks after challenge. Cells isolated from lungs and spleens were stimulated with PPD-T. (A & B) IFNγ, TNFα and IL-2 production from CD4+ T cells, (C & D) IFNγ, TNFα and IL-2 production from CD8+ T cells, (E) IL-17 production from CD4+ cells. Results for Th1, CD8+ T cells and Th17 cells are expressed as a percentage of the CD8+ T cell or CD4+ T cell population and are antigen specific; media only control well values are subtracted. (F) Expression of CD25, FoxP3 and CD39 on CD4+ T cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ T cell population; the media control well value is not subtracted for Treg frequency. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; none were calculated.

4.3.2 Experiment 724

4.3.2.1 CFU

Lung

The median CFU in the lungs of naïve mice was 6.4 (log 10) (Figure 4-16 A). The median CFU of mice vaccinated 3 months before infection was 5.6 (log 10); median CFU of mice vaccinated 13 months before infection was 5.9 (log 10). Mice vaccinated 13 months before infection then exposed to MA 724 had a median CFU of 5.7 (log 10).

Groups of mice vaccinated with BCG 3 months and 13 months prior to challenge and mice vaccinated then exposed to MA 724 had significantly lower CFU counts in the lungs compared to naïve mice. There were no other differences between groups.

Spleen

Spleens of naïve mice had a median CFU of 5.2 (log 10) (Figure 4-16 B). Median CFU of mice vaccinated 3 months before challenge was 4.3 (log 10); median CFU of mice vaccinated 13 months before challenge was 4.4 (log 10). Mice vaccinated then exposed to MA had a median CFU of 4.1 (log 10).

Mice vaccinated 3 months and 13 months before challenge and mice vaccinated then exposed to MA had significantly lower CFU counts in the spleens compared to naïve mice. Mice vaccinated 13 months before challenge had significantly more CFU than mice vaccinated then exposed to MA 724.

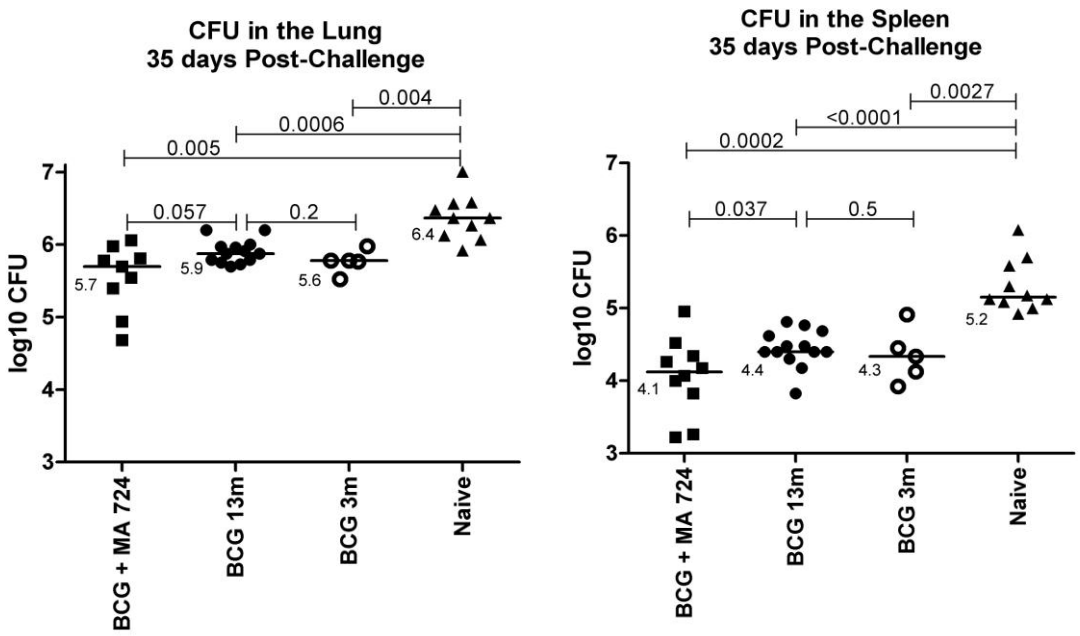


Figure 4-16: *M. tb* burden in the lungs and spleens 35 days after challenge. Homogenised lung (A) and spleen (B) tissue taken 35 days after challenge was plated on 7H10 agar to quantify M. tb burden. Log 10 of the CFU count of the whole organ is displayed. Each point represents one mouse; the median is displayed as a line and the value in text next to the line. The Mann-Whitney U test was used to determine statistical significance; the p value for each comparison is displayed.

4.3.2.2 CD44+ CD4+ T Cells: Post-Challenge

Lung

In naïve mice around 10% of lymphocytes in the lung were activated CD4+ T cells at day 13 after infection (Figure 4-17 A). BCG vaccinated and B + MA mice had around 30% CD44+ CD4+ T cells which was significantly more than naïve mice.

At day 16 after infection naïve mice still had around 10% CD44+ CD4+ T cells (Figure 4-17 B). BCG and B + MA mice had around 12 – 15% CD44+ CD4+ T cells; this was less than at day 13 but still significantly more than naïve mice.

Lymph Node

Thirteen days after infection around 5% of lymphocytes in naïve mice were CD44+ CD4+ (Figure 4-17 A). Around 20% of lymphocytes in BCG vaccinated and B + MA mice were CD44+ CD4+, this was significantly more than in naïve mice

At day 16 frequencies of CD44+ CD4+ T cells in naïve mice remained around 5%; frequencies in BCG and B + MA mice had dropped to around 15% but remained significantly more than naïve mice (Figure 4-17 B).

Spleen

The frequency of CD44+ CD4+ T cells in the spleens of naïve mice 13 days after infection was around 10% (Figure 4-17 A). The frequency of CD44+ CD4+ T cells in BCG mice and B + MA mice was around 35% which was significantly more than naïve mice.

At day 16 after infection the frequency of CD44+ CD4+ T cells in naïve mice remained around 10% (Figure 4-17 B). The frequency of CD44+ CD4+ cells in B + MA mice was around 30% and in BCG only mice around 22%; these frequencies were significantly greater than in naïve mice.

4.3.2.3 CD44+ CD8+ T Cells: Post-Challenge

Lung

At day 13 the frequency of CD44+ CD8+ T cells in the lymphocyte population was 12% in naïve mice and around 22% in BCG and B + MA mice (Figure 4-17 C). BCG alone mice had significantly more CD44+ CD8+ T cells than naïve mice.

The frequency of CD44+ CD8+ cells 16 days after infection was between 0 and 3% in all mice (Figure 4-17 D).

Lymph Node

The frequency of CD44+ CD8+ T cells in naïve mice at day 13 was around 5% (Figure 4-17 C). The frequency of CD44+ CD8+ T cells in BCG and B + MA mice was around 12% which was significantly more than naïve mice.

At day 16 the frequency of CD44+ CD8+ T cells had dropped in naïve mice to around 1% and in BCG and B + MA mice to around 4% (Figure 4-17 D). BCG and B + MA mice had significantly more CD44+ CD8+ T cells compared to naïve mice.

Spleen

The frequency of CD44+ CD8+ T cells in naïve mice at day 13 was around 5% (Figure 4-17 C). The frequency of CD44+ CD8+ T cells in BCG mice and B + MA mice was around 12%; which was significantly greater than naïve mice.

At day 16 the frequency of CD44+ CD8+ T cells in all mice was around 1 – 2% (Figure 4-17 D).

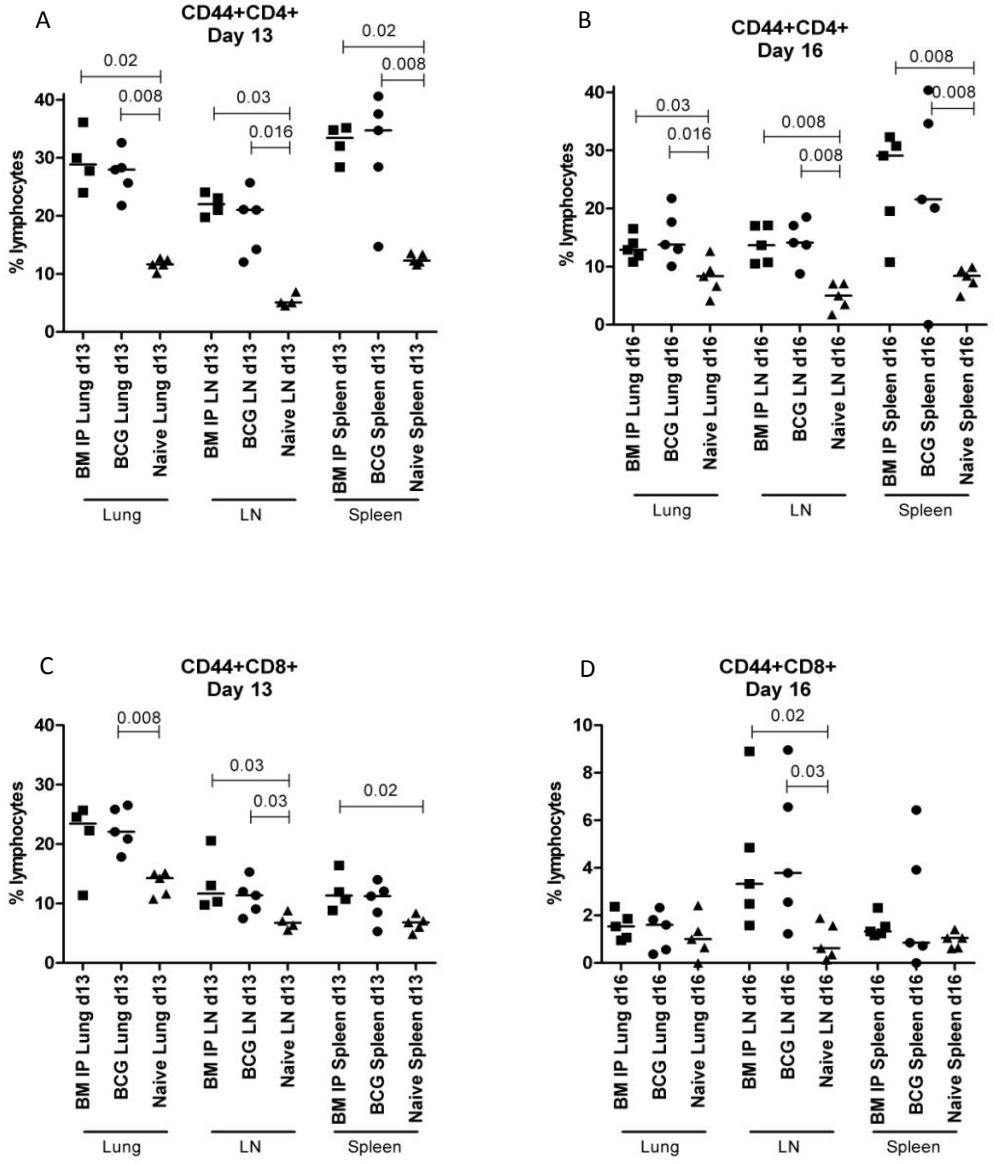


Figure 4-17: Percentage of activated CD4 and CD8 T cells 13 and 16 days after M. tb aerosol challenge. CD44 expression on CD4+ T cells (A and B) and CD8+ T cells (C and D) was assayed by flow cytometry. Cells from lungs, pulmonary lymph nodes and spleens of M. tb infected mice were assayed 13 and 16 days after M. tb infection. Results are expressed as the % of CD44+ CD4+ or CD8+ T cells in the lymphocyte population. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to determine significance and the p value is displayed where significance was found.

4.3.2.4 Th1 Responses: Post-Challenge

Lymph Node

At day 13 all groups had a PPD-T specific IFNγ response; the BCG and the naïve group also had a TNFα and IL-2 response (Figure 4-18 A). The BCG group had greater PPD-T specific responses in all 3 cytokines than the B + MA and naïve groups.

At day 16 all groups had a PPD-T specific IFNγ and TNFα response; BCG and B + MA groups also had an IL-2 response (Figure 4-18 B). The frequency of IFNγ+ CD4+ T cells in B + MA mice was greater than in BCG and naïve mice. Frequencies of TNFα+ and IL-2+ CD4+ T cells were similar between BCG and B + MA mice and greater compared to naïve mice but the difference was not significant.

Spleen

BCG and B + MA groups had IFNγ and TNFα responses in the spleen at day 13 after infection; naïve mice did not have an IFNγ or TNFα response but did have an IL-2 response (Figure 4-18 C). Day 16 data was excluded as there was a technical issue with the assay.

Lung

The BCG and B + MA groups both had IFNγ and TNFα responses in the lungs at day 13 after infection (Figure 4-18 D). The B + MA group also had an IL-2 response. No response was detected in naïve mice. Frequencies of IFNγ+ and TNFα+ CD4+ T cells were greater in the B + MA group compared to the BCG alone group but the difference was not significant.

At day 16 all groups had an IFNγ response but only the BCG and B + MA groups had a TNFα response (Figure 4-18 E). None of the groups had an IL-2 response. Frequencies of IFNγ+ and TNFα+ CD4+ T cells were greater in the B + MA group compared to BCG alone and naïve groups but the difference was not significant.

4.3.2.5 CD8+ T Cell Responses: Post-Challenge

Lymph Node

At day 13 the BCG and B + MA groups had an IFNγ response but naïve mice did not (Figure 4-19 A). Naïve mice had a small IL-2 response. None of the groups had a TNFα response. The B + MA group had significantly more IFNγ+ CD8+ T cells compared to the BCG alone group.

At day 16 there were no CD8 responses detected in any of the groups (Figure 4-19 B).

Spleen

A small TNFα response was detected in naïve mice at day 13 (Figure 4-19 C). No other responses were detected. Day 16 data was excluded as there was a technical issue with the assay.

Lung

All three groups had an IFNγ response in the lung 13 days after infection (Figure 4-19 D). The BCG alone and B + MA groups also had a small TNFα response.

This was similar at day 16 (Figure 4-19 E). The frequency of IFNγ+ CD8+ T cells in the B + MA group had increased compared to day 13; the response was greater compared to BCG alone and naïve mice, though not significant.

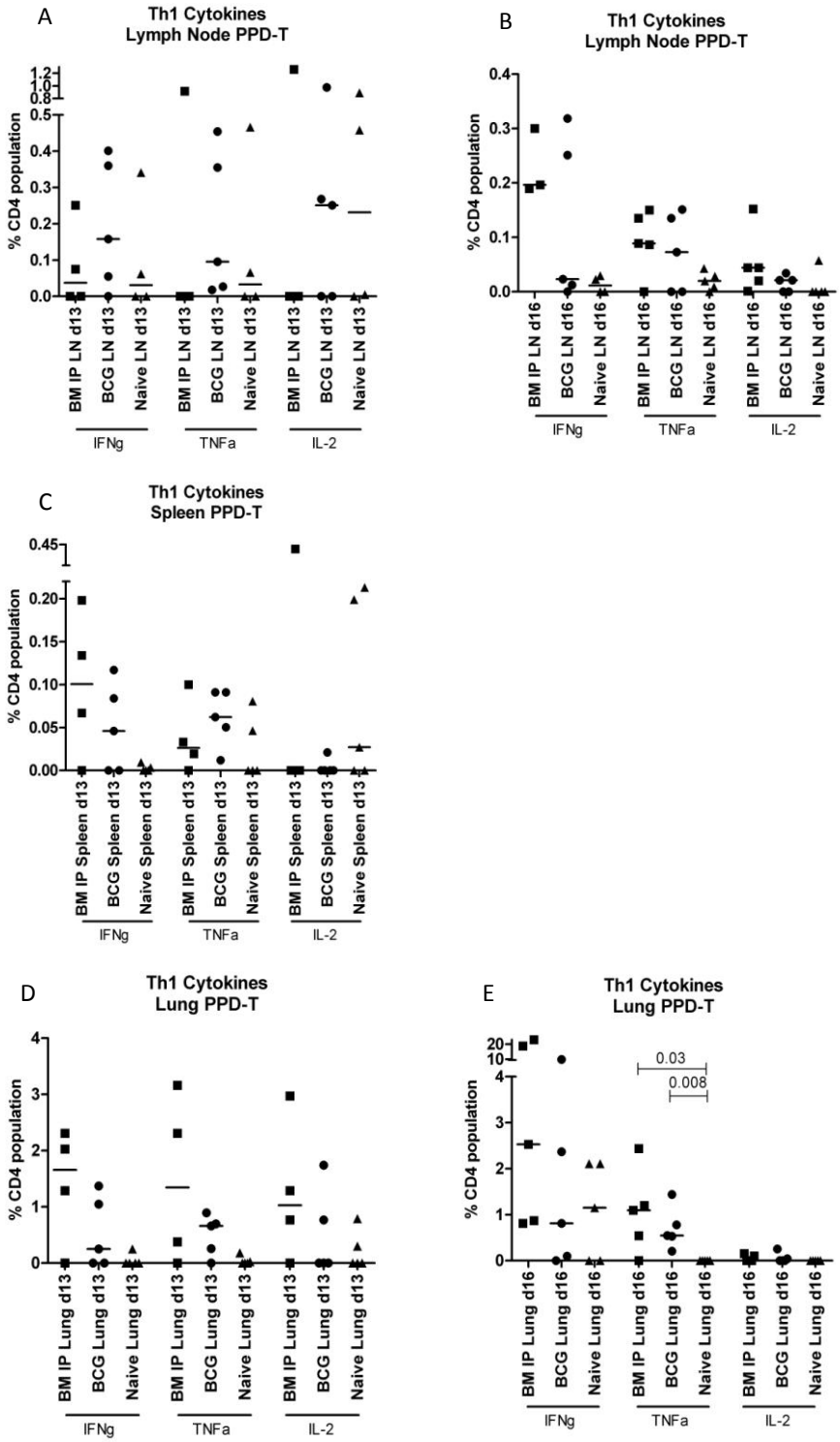


Figure 4-18: Th1 immune responses 13 and 16 days after *M. tb* challenge. Cells isolated from pulmonary lymph nodes (A and B), spleens (C) and lungs (D and E) were stimulated with PPD-T. IFNγ, TNFα and IL-2 production from CD4+ T cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ T cell population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=4 or 5. The Mann-Whitney U test was used to determine significance; these are displayed where significance was calculated.

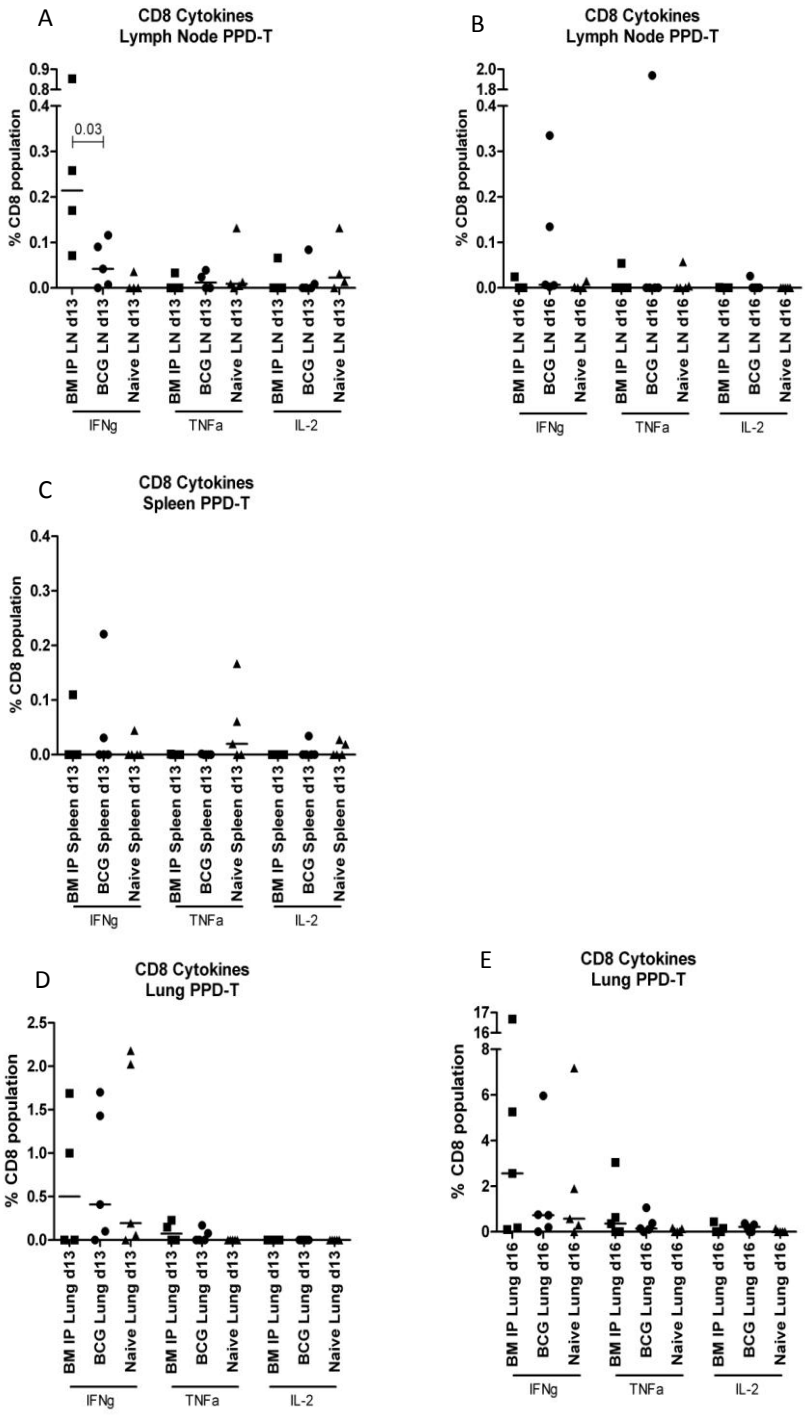


Figure 4-19: CD8+ T cell immune responses 13 and 16 days after *M. tb* challenge. Cells isolated from pulmonary lymph nodes (A and B), spleens (C) and lungs (D and E) were stimulated with PPD-T. IFNγ, TNFα and IL-2 production from CD8+ T cells was assayed by flow cytometry. Results are expressed as a percentage of the CD8+ T cell population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=4 or 5. The Mann-Whitney U test was used to determine significance; no significant differences were calculated.

4.3.2.6 Th17 Responses: Post-Challenge

Lung

The B + MA group had an IL-17 response at 13 days after infection (Figure 4-20 A). No response was detected at day 16 (Figure 4-20 B).

Lymph Node

Low frequencies of IL-17+ CD4+ T cells were detected in all groups at day 13 (Figure 4-20 A). At day 16 the B + MA group and the naïve group had greater IL-17 responses compared to day 13; frequencies in the BCG group remained the same (Figure 4-20 B). The difference in frequency between B + MA and BCG was significant.

Spleen

No IL-17+ CD4+ T cells were detected in the spleen at day 13 (Figure 4-20 A). At day 16 a high IL-17 response was seen in all groups (Figure 4-20 B). Frequencies of IL-17+ CD4+ cells were greater in the spleen than the pulmonary lymph nodes.

4.3.2.7 Th2 Responses: Post-Challenge

Lung

At day 13 a large frequency of PPD-T specific IL-4+ CD4+ T cells was detected in the lungs of B + MA mice (Figure 4-20 C). At day 16 this was not seen; a small frequency of IL-4+ CD4+ T cells were detected in BCG only mice (Figure 4-20 D).

Lymph Nodes

A small frequency of IL-4+ CD4+ T cells were detected in the BCG only and B + MA groups at day 13 (Figure 4-20 C). No IL-4 was detected at day 16 (Figure 4-20 D).

Spleen

There was no IL-4 detected in the spleen at day 13 or day 16 (Figure 4-20 C & D).

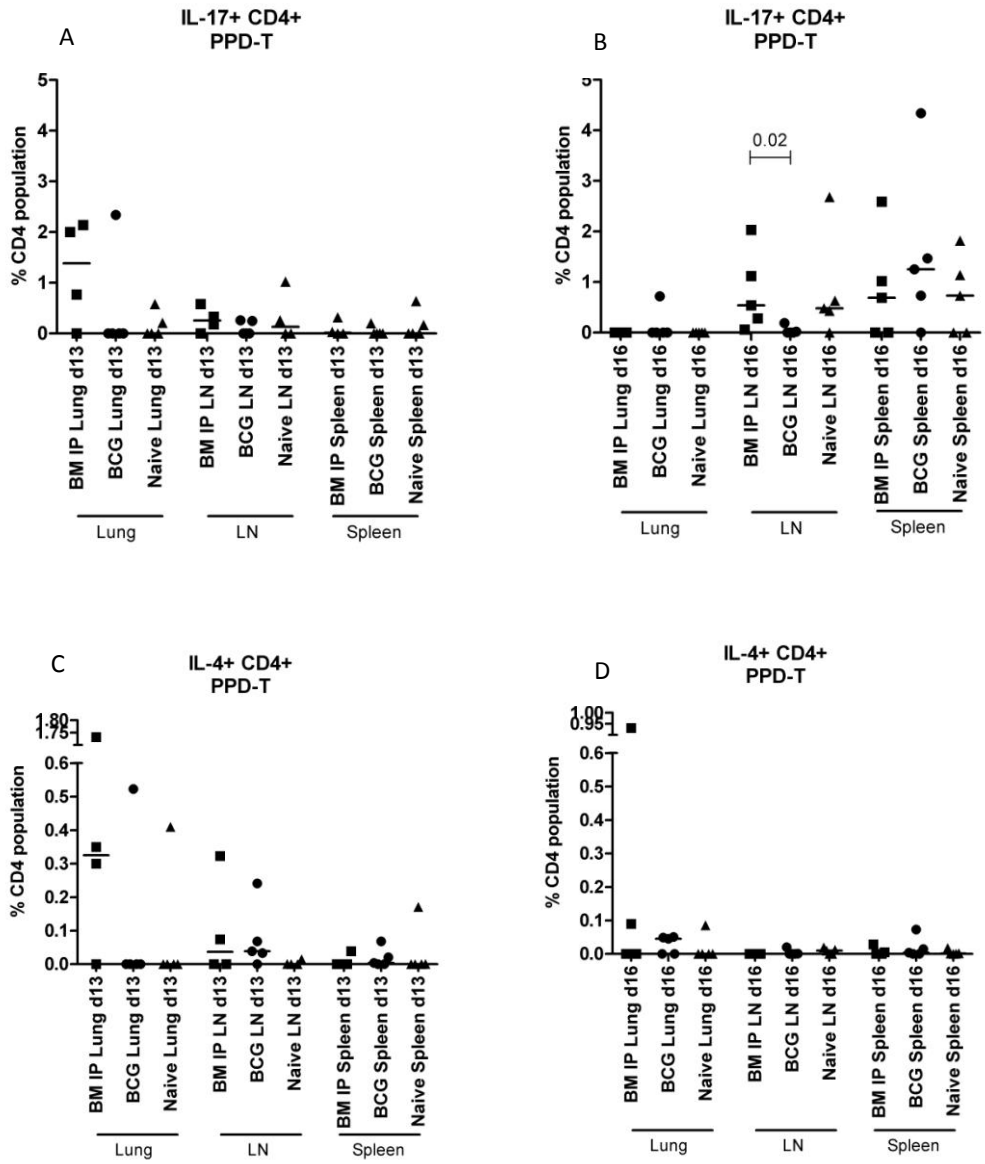


Figure 4-20: Th17 and Th2 responses 13 and 16 days after *M. tb* infection. Cells from the pulmonary lymph nodes, spleens and lungs were stimulated with PPD-T. IL-17 (A and B) or IL-4 production (C and D) from CD4+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ T cell population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=4 or 5. The Mann-Whitney U test was applied to test for statistically significant differences and these are displayed on the graph.

4.3.2.8 Regulatory T Cell Responses: Post-Challenge

Lungs

The frequency of Tregs at day 13 was around 1% in all groups (Figure 4-21 A). At day 16 this had risen to between 1 and 5% and was similar across the groups (Figure 4-21 B).

Lymph Nodes

The frequency of Tregs in naïve mice at day 13 was around 3% (Figure 4-21 A). In the BCG only group the frequency was around 5% and in the B + MA group it was around 6-7%. BCG only mice had a significantly higher frequency of Tregs compared to naïve mice. At day 16 the frequencies had increased; in naïve mice and the BCG only mice frequencies were around 7%, in B + MA mice frequencies were around 9% (Figure 4-21 B). The difference in frequency between the B + MA group and naïve group was significant.

Spleen

At day 13 the frequency of Tregs in all 3 groups was around 1% (Figure 4-21 A). At day 16 the frequencies had increased; in the naïve group frequencies were around 7%, in the BCG only group and the B + MA group frequencies were around 10% (Figure 4-21 B).

4.3.2.9 IL-10+ CD4+ T Cells: Post-Challenge

Lung

At day 13 some mice produced IL-10 but not enough to conclude a definite response was induced (Figure 4-21 C). No IL-10 was detected at day 16 (Figure 4-21 D).

Lymph Node

At day 13 some IL-10 was detected but not enough mice per group responded to conclude a result (Figure 4-21 C). A small frequency of IL-10+ CD4+ T cells was detected in the BCG only group at day 16 (Figure 4-21 D).

Spleen

At day 13 IL-10+ CD4+ T cells were detected in all groups. The magnitude of response was similar across the groups (Figure 4-21 C). IL-10+ CD4+ T cells were detected in the BCG only group and the naïve group at day 16 (Figure 4-21 D).

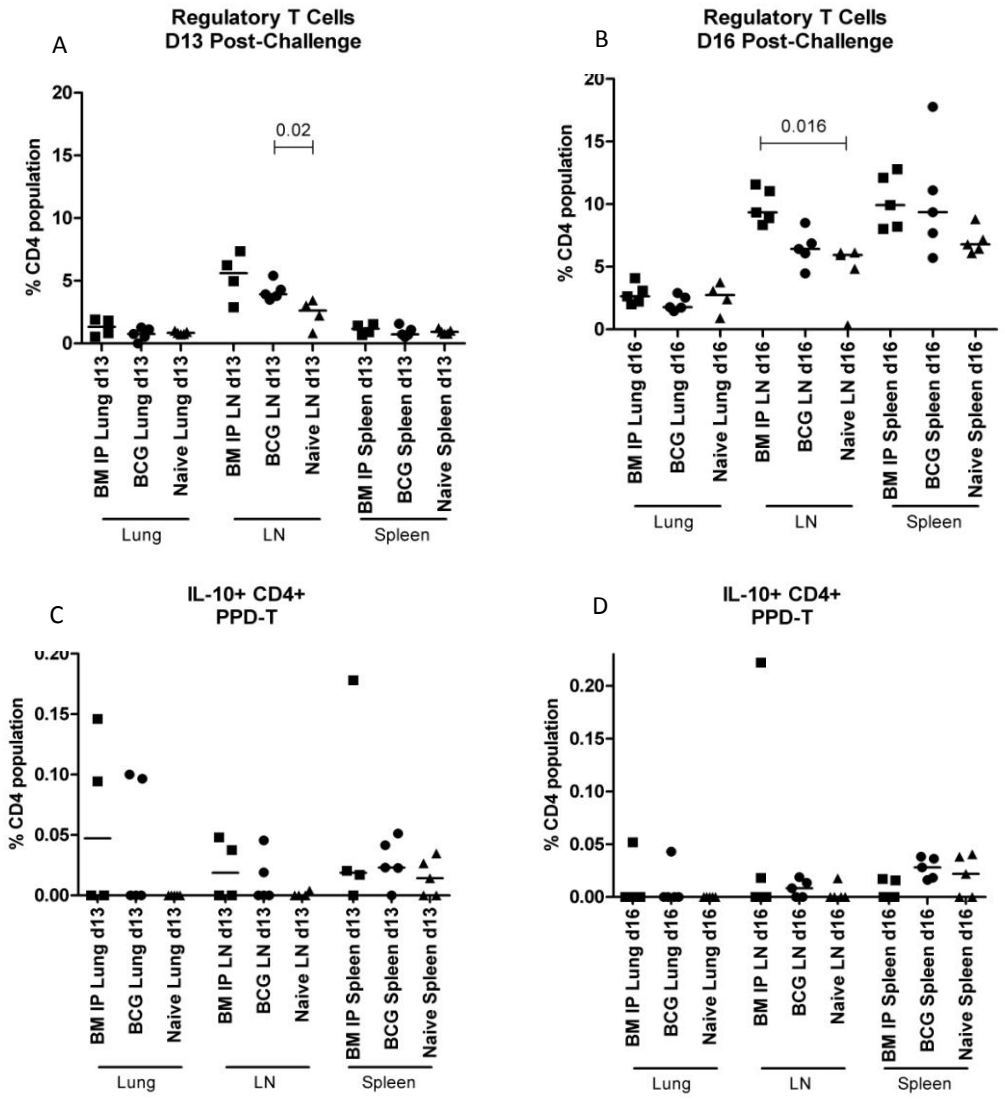


Figure 4-21: Regulatory T cell frequencies and IL-10 production 13 and 16 days after M. tb challenge. (A and B) Expression of CD25, FoxP3 and CD39 on CD4+ T cells was assayed by flow cytometry. (C and D) IL-10 production from CD4+ T cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ T cell population; the media control well value is not subtracted from Treg frequency or IL-10 production. Each data point represents one mouse and the median is displayed, n=4 or 5. The Mann-Whitney U test was applied to test for statistically significant differences and p values are shown where calculated.

4.4 Discussion

4.4.1 Hypothesis

The experiment was designed to model exposure to NTM after BCG vaccination. The hypothesis was that exposure would reduce the efficacy of BCG in protecting against *M. tb* challenge. *M. avium* was the species of NTM chosen to be studied as this species is the most well characterised in animal models and in vitro studies. It is also a common isolate from the environment and responses to this species are detected in populations where BCG efficacy is at its lowest[115].

To mimic natural exposure in areas of high NTM prevalence *M. avium* was administered repeatedly over an extended period of time. Because of these repeated doses it was decided that dead *M. avium* would be used so as not to induce a high level of infection in the mice as this would not represent what occurs in human exposure to NTM. The intraperitoneal route of administration was selected as this would best mimic systemic exposure, it is also a highly replicable route which was favourable to keep variability as low as possible.

4.4.2 Experiment 2-151 Challenge

Experiment 2-151 was the first of this model to be conducted. It was also the first challenge experiment to be done using new aerosol delivery equipment. There had not been time to optimise the procedure using this new apparatus so we had to estimate what concentration of *M. tb* to put in the collision nebuliser. To test vaccine efficacy, a dose of 100 CFU of *M. tb* is usually delivered per mouse; it was estimated that around 5 CFU was delivered to mice in experiment 2-151. This resulted in low CFU burdens at 4 weeks which does not give enough power to show a difference in the ability of the groups to control *M. tb* growth.

Immunology data from this experiment showed that during *M. tb* infection greater magnitudes of Th1 and Th17 responses appeared in the lungs of MA exposed mice; both these subsets are known to be protective in *M. tb* infection. These results suggested that MA exposure may enhance protective responses which was contradictory to the hypothesis, but without successful challenge data no conclusion could be made. Pre-challenge data had shown Th1 and Th17 responses had been enhanced by exposure and were of greater magnitude than BCG only mice just before challenge.

The experiment had to be repeated, as the challenge had failed, but rather than directly repeat experiment 2-151, some changes were made to the model based on data which had been collected and advice from collaborators. The immunology results from experiment 2-151 implied the hypothesis had been disproved in this model. It was considered that the strain 2-151 was possibly not inducing the right type of immune responses to interfere with BCG induced immunity. In the report by Brandt et al, only the most persistent isolate of NTM from the Karonga District induced a debilitating effect on induction of BCG immunity; and it is possible that 2-151 is not a pathogenic enough strain to see a debilitating effect[119].

Another mechanism to consider is the induction of immune modulation by this isolate. As discussed in the introduction, the pathogenicity of *M.tb* is in part due to the mannose capping of LAM[23]. Uncapped LAM, which is present in the fast growing non-pathogenic species of NTM, can bind directly with CD14 and TLR2 to induce phagocyte activation and induction of pro-inflammatory IL-6 and IL-1β[131]. Mannose capped LAM (ManLAM) avoids binding to TLR2 and is one of the mechanisms of immune evasion employed by pathogenic strains. Binding of ManLAM to DC-SIGN induces production of modulatory IL-10 from the DCs[2]. Species of MA, *M. intracellulare* and *M. scrofulaceum* have some degree of mannose capping[115]. If induction of a regulatory environment is a mechanism by which NTM might reduce BCG induced immunity, the degree of mannose capping of LAM expressed by the strain would be a

determining factor of the regulatory responses. Ho et al reported the ability of *M. chelonae* to induce regulatory T cells and IL-10 that reduced BCG induced immune responses[124]. Treg frequencies were increased by exposure to 2-151 however no IL-10 was induced in cultures; strain 2-151 may not have enough mannose capping to induce a strong enough regulatory environment.

This exposure model had been devised in collaboration with Professor Ian Orme, Colorardo State University, based on work conducted in his laboratory. His group reported that administration of MA 724 after BCG vaccination reduced the efficacy of BCG in guinea pigs (Orme, personal communication). MA 724 was used in the repeat experiment to try and replicate this result. MA 724 has been defined in aerosol, intravenous and subcutaneous infection models in mice and is capable of inducing a persistent infection[122,123]. It was also advised by Professor Andrea Cooper, Trudeau Institute, to adopt an alternative growth method to ensure pathogenic morphotypes of the strain were being administered to the mice[177].

In addition to changes in the MA strain used, the time point assayed post challenge was also modified. It was thought that BCG vaccinated mice would mount a greater response against *M. tb* than naïve mice, given that memory immune responses can protect against *M. tb*. Results post challenge in experiment 2-151 showed naïve mice had significantly higher Th1 immunity in the spleens compared to BCG vaccinated and BCG vaccinated-MA exposed mice. This was contrary to what was expected.

Jung et al showed memory responses against *M. tb*, primed by a prior *M. tb* infection which had been cured by antibiotics, were induced more quickly on aerosol *M. tb* challenge than the primary response of naïve mice. This secondary recall response controls *M. tb* growth earlier and so a lower CFU burden is achieved in the lung and spleen[61]. After growth of *M. tb* has

been arrested the magnitude of the immune response correlates to the load of *M. tb* in the organ. These results are in agreement with what I saw at 4 weeks; naïve mice had a greater immune response in the spleen compared to the vaccinated mice as the load of *M. tb* in the organ was greater.

Adaptive immune responses in *M. tb* infection are induced in the pulmonary lymph nodes. Reiley et al showed activation of CD4+ T cells occurs from day 12 and peaks at day 15 in naïve mice, cell infiltration to the lungs occurred at day 15[60]. Results from Jung et al showed that in immune mice the CD4+ T cell response was seen 3 days earlier than in naïve mice[61]. Begum et al reported that CD8+ T cell responses in BCG vaccinated mice are induced 7 days earlier than in naïve mice[77]. I therefore decided to measure immunogenicity at the onset of the adaptive immune response, in order to compare the levels of the vaccine induced response and the naïve response to *M. tb* and determine any differences in the profile of protective immunity induced at this critical time point. Based on work by Reiley and Jung, day 13 and 16 after challenge were chosen as the time points to assay as they should cover the vaccine induced immune response and the primary response to *M. tb* infection[60 61]. It was also reported by Shafianai et al that Tregs induced in *M. tb* infection were able to delay the priming of the effecter response to *M. tb*; this interaction would take place over the same time period in the pulmonary lymph nodes[72].

4.4.3 Experiment 724 Challenge

The *M. tb* aerosol challenge of experiment 724 was successful; both BCG control groups had significantly less *M. tb* in the lungs and spleens 35 days after challenge compared to naïve controls. The median CFU of *M. tb* in the lungs of mice vaccinated with BCG 10 months before challenge was greater than the CFU in lungs of mice vaccinated then exposed to MA. This difference was not significant; however there was a significant difference between these groups in the spleen. This result disproved my hypothesis that MA 724 exposure would

reduce the efficacy of BCG. This experiment suggests that protective efficacy may have been enhanced by exposure.

The frequencies of activated CD4+ T cells in the pulmonary lymph nodes, lungs and spleens were significantly greater in the two vaccinated groups compared to the naïve group at day 13 and day 16 after infection. This was in agreement with what I had expected to see in vaccinated mice. CD8+ T cells were also of a higher magnitude in vaccinated mice in all 3 organs. There was no difference in the frequencies of activated cells between mice exposed to MA after BCG vaccination and those which were not.

At day 13 Th1 responses in the lymph nodes appeared greater in BCG only mice compared to those exposed to MA but by day 16 there were comparable frequencies of this cell type between the two groups. Interestingly there was a higher frequency of Th1 in the lungs of BCG + MA mice at day 13 compared to BCG only mice which may imply these cells translocated to the lung earlier than cells in the BCG only group. This may be why there were lower frequencies of Th1 in the lymph nodes of this group at this time point. Without having an earlier time point this cannot be proven.

Systemically the two vaccinated groups were very similar with comparable Th1 frequencies being detected in the spleen at day 13. It would appear the responses of naïve mice had not begun to be primed in the lymph node at the two time points tested as there was no change in frequencies of activated CD4+ T cells or production of cytokine. A Th1 response was detected in the lungs at day 16 but this is contradictory to Reiley et als report which shows Th1 responses are primed in the lymph nodes and not directly in the lungs[60].

There is a positive role of CD8+ T cells in early responses to *M. tb* and their early accumulation is correlated to vaccine mediated protection[178]. CD8+T cell responses were

detected in the lymph nodes of B + MA mice and in the lungs of both vaccinated groups at day 13 after infection. At day 16 this response persisted in lungs of vaccinated groups and a response appeared in naïve mice.

Early Th17 responses in the lung help the generation of the Th1 responses by down regulating the induction of IL-10 in *M. tb* infection. They also induce the secretion of chemokines which induce chemotaxis of Th1 responses to this site. Sensitised mice had a greater Th17 response than BCG only mice; measured in the lungs at day 13 and in the lymph nodes at day 16.

4.4.4 Protective Responses were Enhanced by Sensitisation Pre-Challenge

Adaptive immune mediators were quantified in experiment MA 2-151 and MA 724 prior to challenge. Th1 responses were the most dramatically affected by MA exposure; the magnitude of IFNγ, TNFα and IL-2 producing CD4+ T cells were all greater in the MA exposed mice, than in BCG alone mice. Experiment 2-151 demonstrated that 7 doses compared to just 1 dose produced a highly significant enhancement of the Th1 response. The response was specific for PPD-T, PPD-A and Ag 85A demonstrating that despite dosing with MA the specificity of the response was not skewed toward MA specific antigens. Also worthy of note was that the Th1 response had greater polyfunctionality in MA exposed mice.

Th17 responses were significantly increased by MA 724 but the effect MA 2-151 had on this subset was less dramatic as there was only a trend to increased frequencies.

The same was seen in CD8+ T cell responses; MA 724 had a stronger effect than MA 2-151. CD8+ T cell responses were greatly enhanced by sensitisation, prior to challenge subsets were detected in the lungs and lymph nodes of exposed mice but not BCG only mice. The BCG only

response appeared to exist in the spleen but exposure to MA had somehow promoted the translocation of this subset to the lung.

IL-4 responses were analysed to test for any skew MA may have induced toward Th2 sided immunity. There was a trend toward the induction of these responses by exposure to MA; seen after one dose of MA-2151 and in the lymph nodes and spleens of mice exposed to MA 724. This response was not detected post-challenge and had no deleterious effect on the generation of sufficient Th1 immune responses against *M. tb*.

The two strains of MA used induced different regulatory T cell responses. A significantly greater frequency of Tregs was induced by exposure to MA 2-151 whereas there was no difference between BCG only mice and those which received MA 724. Interestingly BCG only mice in experiment 724 had greater frequencies of IL-10+ CD4+ T cells than mice exposed to MA. The difference in the ability of the two strains to induce Tregs may be due to the difference in the level of mannose capping of LAM. Post-challenge a greater frequency of Tregs was recorded in both vaccinated groups in experiment 724 compared to naïve controls; this was most likely a compensatory expansion of regulatory cells in correlation with the increase in effecter cells and it had no deleterious effect on the generation of effecter responses. Neither strain induced large frequencies of Tregs which may possibly be explained by heat-killing of MA degrading the mannose capping of LAM. Kang et al reported this effect with heat-killing of *M. tb*[40].

4.4.5 Sensitisation after BCG Vaccination may Increase Protection

The enhancement of protective Th1, Th17 and CD8+ T cell responses by exposure to MA after BCG was correlated with a significantly lower CFU burden in the spleens of *M. tb* challenged mice.

Enhanced protection was induced by MAI exposure after BCG vaccination in a study by Edwards et al using the guinea pig model of *M. tb* aerosol infection. Interestingly there were fewer CFU in the organs of guinea pigs which were exposed to MAI after BCG vaccination compared to those which were exposed to MAI prior to BCG vaccination. Both of these groups had lower CFUs than animals vaccinated with BCG only. TST response size was positively correlated with control of *M. tb*.

Howard et al also report enhanced immunogenicity with MA sensitisation and BCG vaccination; proliferative responses and IFNγ production were greater than in BCG only vaccinated calves. This study was performed in calves with MA given prior to vaccination but efficacy of BCG was not tested by challenge. Despite this the report provides further evidence that exposure to NTM can induce enhanced immunogenicity in the setting of BCG vaccination and not a deleterious effect.

The immunological responses post-challenge give some evidence of which effectors may mediate increased protection. The infiltration of Th1 responses to the lung at day 13 was seen in the lungs of MA exposed mice but not BCG only mice. This may be associated with the presence of Th17 cells in this location at the same time. Both of these subsets were enhanced by exposure to NTM pre-challenge therefore it is a plausible explanation that the enhanced frequency of these subsets offered increased protection against challenge.

The *M. tb* aerosol challenge model is a well-tested, robust assay for vaccine efficacy. The frequencies of Th1 and Th17 were so much higher in the BCG + MA mice pre-challenge that it is interesting not more protection was seen in this group compared to the BCG only group. A limitation with the aerosol infection model is the extremely high level of protection which is afforded by BCG vaccination. This makes it difficult to see any improvement on the level of protection BCG alone confers, the fact that significant reduction of CFU in the spleen was

seen in the BCG + MA group, however small the difference was, is actually a very significant result.

A further limitation in this model is the time which it takes to complete. Ideally I would have wanted to test other species of NTM to ascertain whether the same result would be reproduced. Despite the change to a different strain of MA there was still no induction of IL-10. It would be interesting to see whether an NTM isolate known to induce high levels of regulatory responses could induce the same result or whether BCG conferred protection would be compromised.

Other limitations include the variability of the results; there was a large range in the CFU counts in the BCG + MA group. This is most likely due to the difference in the mice's response to the exposures and the repetitive dosing would only enhance this variability. However results from experiment 2-151 show that repeated dosing induces a greater response than just one dose. With more time it would be interesting to see how many doses are needed to induce the level of response measured after 7 doses. This may also lead to the shortening of the exposure frame allowing for more repetitions of the model to be performed.

A further limitation with this model is whether it is actually a true representation of human exposure. It is not known how people are exposed to NTM but most likely it is a continuous event, to a variety of NTM species, by a variety of routes and to a mixture of live and dead bacteria and not the exposure to a high bolus of antigen once every month.

The prevalence of NTM in water supplies leads to the suspicion that many people are exposed via the gastric route. Subclinical infections have been described in the intestinal tracts of wild and domesticated animals[125]. This route of exposure was deemed an important area of study and was investigated, the results of which are presented in Chapter 5.

5 Model of *M. avium* Exposure after BCG Vaccination: Live *M. avium* Administered Orally

5.1 Introduction

5.1.1 Replication of a Published Model of Oral *M. avium* Exposure after BCG Vaccination

It is very likely that in humans, the oral route of NTM exposure accounts for a significant degree of exposure. A model of this route of exposure to NTM after BCG vaccination had been published by Flaherty et al and reported to reduce the efficacy of BCG against *M. tb* challenge[176]. I decided to try and replicate this model and evaluate the adaptive immune response to determine how *M. avium* induces this effect. The model was performed in mice. Flaherty et al chose the oral route as it is likely to model the majority of exposure to NTM which occurs in the field[176]. Repeated doses of live *M. avium* 104 (MA 104) was administered to mice; a low dose of 100 CFU was given to induce a sub-clinical infection but not a pathological infection. One strain of *M. avium* was chosen to give more control of the model and to reduce variability. MA 104 was chosen as it is a well characterised strain; it is a clinical isolate, proven to cause a persistent infection in mice but not mortality and it can invade the gut epithelium and disseminate when given orally.

The virulence, colony morphotypes and glycopeptidolipid expression of MA 104 was investigated by Torelles et al to complement the genetic sequence of the strain[177]. Virulence was assessed by aerosol infection and it was found that replication was controlled in a chronic infection after 2 months. The strain exhibits 3 morphotypes with differing pathogenicity. This was taken in to consideration in this experiment and a culture method to select for the virulent rough and smooth-transparent morphotypes was adopted.

5.1.2 Gut Associated Lymphoid Tissue

The intestinal immune system is highly specialised and designed to deal with constant antigen stimulation from food antigens and the gut bacterial flora[37]. Gut associated lymphoid tissue (GALT) is comprised of; peyers patches which are lymphoid structures designed to sample antigens in the gut transported through M cells, isolated lymphoid follicles, the lamina propria which is a loose connective tissue that supports intestinal epithelia and harbors many of the gut specific lymphocytes and the mesenteric lymph nodes to which the other structures drain to (Figure 5-1). The identity of macrophages and DCs in the GALT is not clear cut[179]. Mucosal APCs are derived from unique monocyte precursors and their functions are distinct from those of their systemic counterparts[180]. Intestinal macrophages induce a tolerising environment which is essential to prevent inflammation against harmless antigens in the intestine. These macrophages are unresponsive to TLR stimulation, constitutively express IL-10 and induce the differentiation of T cells to a regulatory phenotype[181]. In the absence of inflammatory stimulus dendritic cells in the system promote differentiation of T cells to secrete IL-10, IL-4 and TGFβ and to express a mucosal homing phenotype through expression of α4β1 integrins[180]. The tolerising macrophages can prevent DCs from priming IL-17+ T cell responses in inflammatory conditions.

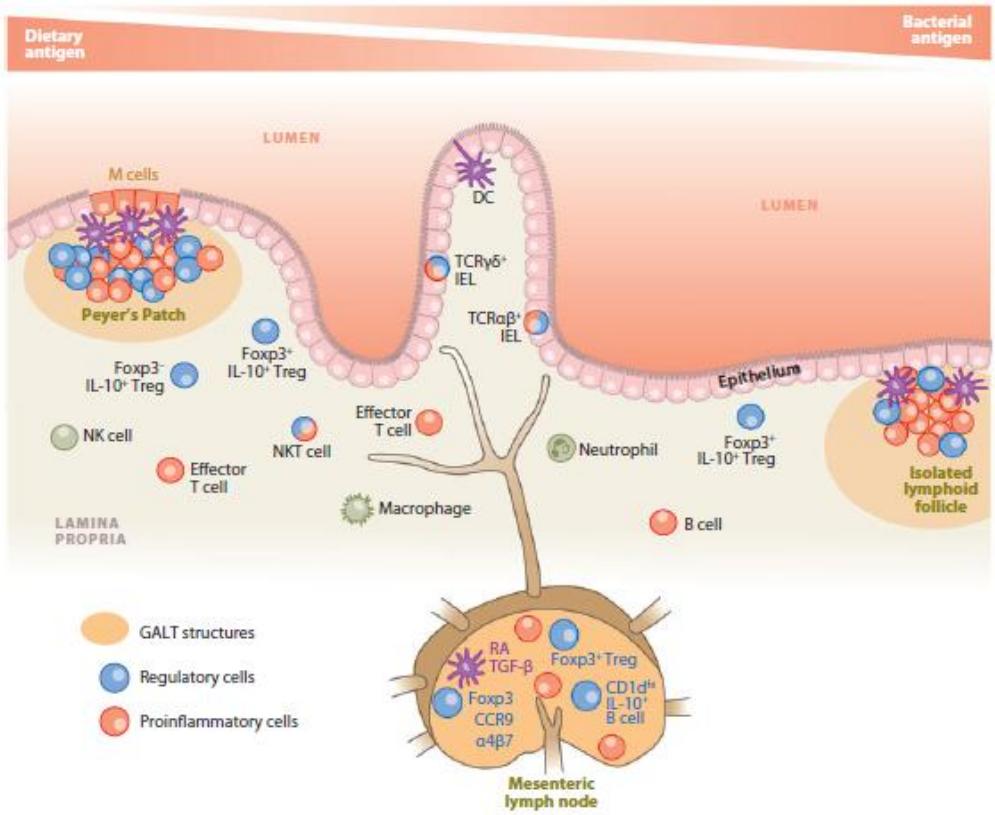


Figure 1

Intestinal immune environment. Intestinal immune responses take place in the epithelium, lamina propria, and gut-associated lymphoid tissue (GALT). The gut harbors cell populations specialized in antigen sampling and presentation, antimicrobial immunity, and maintenance of tolerance toward harmless exogenous antigens. (Abbreviations: IEL, intraepithelial lymphocytes; NKT, natural killer T cell; DC, dendritic cell; Treg, regulatory T cell.)

Figure 5-1: Representation of the GALT immune system. Taken from Izcue et al[37].

5.1.3 Oral Infections with *M. avium*

Sangari et al reported that MA 104 invades the intestinal mucosa through enterocytes rather than M cells[182]. This gives the bacteria a route for dissemination as entering through an M cell would most likely target it for destruction. Petrofsky et al demonstrated oral infection with MA 101 induced inflammatory CD4+ and CD8+ T cells in the mesenteric lymph nodes which produce IFNγ, TNFα and regulatory IL-10 responses. MA existed in macrophages within the mesenteric lymph nodes and dissemination did not occur until 4 months after infection. They noted that the macrophages in the lymph nodes produced high concentrations of IL-10, which suggests MA infection did not stimulate a high level of inflammatory mediators. Bermudez et al administered a high dose of MA 101 orally which resulted in systemic IL-10 being induced from day 7 after infection[165].

Hsu et al found intestinal macrophages react differently to MA compared to splenic and peritoneal macrophages[183]. Lower levels of TNFα were secreted by intestinal macrophages compared to those from other areas of the immune system. Blocking TGFβ and IL-10 allowed these macrophages to kill the intracellular mycobacteria.

It appears MA may have adapted to infect via the oral route as it can hi-jack the tolerising environment in this location and survive almost undetected in its macrophage niche. The generation of T cell responses in this location are skewed toward regulatory and Th2 phenotypes which are not protective in mycobacterial infections[180]. Induction of mycobacteria-specific immune responses of these phenotypes may well interfere with the protective immune responses induced by BCG.

5.1.4 Hypothesis

The hypothesis of this experiment was that sensitising mice orally with MA after BCG would induce the production of MA specific tolerising immune responses which may interact detrimentally with the BCG induced protective Th1 responses. A thorough analysis of the adaptive immune responses was performed pre- and post-challenge to test this hypothesis.

5.1.5 Experiment plan

The protocol published by Flaherty et al was replicated[176]. All mice were vaccinated with BCG; 6 weeks after BCG, half the mice began an exposure regime where MA 104 was given by oral gavage every 2 weeks for 16 weeks. 6 weeks after the final gavage the mice were challenged with aerosolised *M. tb*. Cellular immune responses of BCG vaccinated-exposed mice and BCG only controls were compared just before *M. tb* challenge. Cells from mesenteric lymph nodes, lungs and spleens were stimulated with PPD-T, PPD-A and Ag 85A to assess the specificity of the adaptive immune response to these mycobacterial antigens.

Thirteen and sixteen days after *M. tb* infection cellular immune responses specific for PPD-T were assayed in lungs, pulmonary lymph nodes and spleens. 5 weeks after *M. tb* infection bacterial load in lungs and spleens was determined.

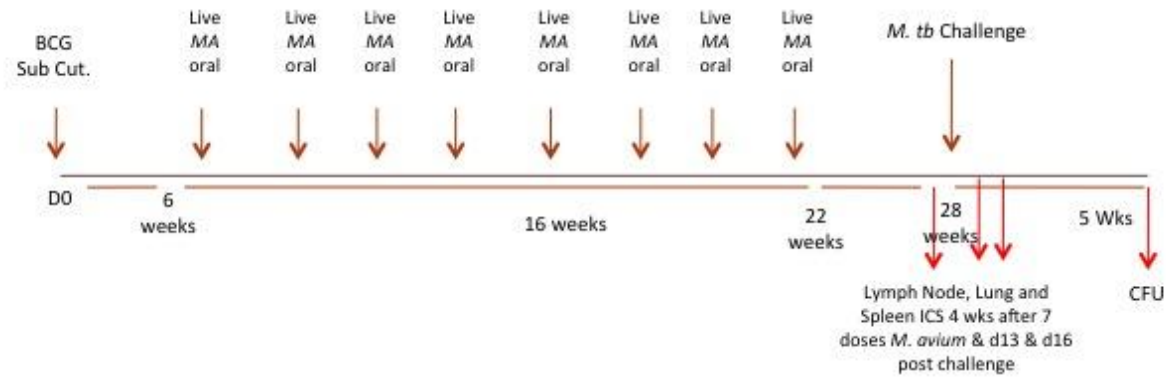


Figure 5-2: Experiment plan for oral MA 104 exposure. Administration time points shown on top of the line; immunology and CFU time points shown on the bottom of the line. $1x10^5$ CFU of BCG was given subcutaneously (SC). 100 CFU of live MA 104 was given by oral gavage. Around 35 CFU of *M.tb* Erdmann was given by aerosol delivery.

5.2 Results: Pre-Challenge

5.2.1 Th1 Responses

5.2.1.1 Mesenteric Lymph Node

PPD-T-specific IFNγ+ CD4+ T cells and TNFα+ CD4+ T cells were not detected in either BCG vaccinated only mice or BCG vaccinated and MA exposed (B + MA) mice (Figure 5-3 A). IL-2+ CD4+ T cells were detected in B + MA mice.

IFNγ+ CD4+ T cells specific to PPD-A were detected in the BCG only group. IL-2+ CD4+ T cells were detected in BCG + MA mice. No TNFα+ CD4+ cells were detected in either group (Figure 5-3 B).

Ag 85A specific IFNγ+ and TNFα+ CD4+ T cells were detected in both groups (Figure 5-3 C). IL-2+ CD4+ T cells were detected in the B + MA mice. The frequencies of IFNγ+ and TNFα CD4+ T cells were comparable between the groups.

5.2.1.2 Spleen

Both BCG only mice and B + MA mice had populations of PPD-T, PPD-A and Ag 85A specific IFNγ, TNFα and IL-2+ CD4+ T cells (Figure 5-3 D, E & F).

The frequencies of PPD-T specific IFNγ and TNFα+ CD4+ T cells were similar between the groups. B + MA mice had higher frequencies of IL-2+ CD4+ T cells compared to BCG only mice.

Frequencies of PPD-A specific IFNγ, TNFα and IL-2+ CD4+ T cells were greater in the B + MA mice compared to the BCG only mice.

Frequencies of Ag 85A specific IFNγ, TNFα and IL-2+ CD4+ T cells were greater in the BCG only mice compared to the B + MA only mice, these differences were not statistically significant.

5.2.1.3 Lung

PPD-T specific IFNγ+ CD4+ T cells were detected in the B + MA group but not the BCG only group (Figure 5-3 G). No PPD-T specific TNFα or IL-2+ CD4+ T cells were detected in either group.

PPD-A specific IFNγ+ CD4+ T cells were detected in both groups (Figure 5-3 H). BCG only mice had a small frequency of PPD-A specific TNFα+ CD4+ T cells. No IL-2+ CD4+ T cells were detected against this stimulation in either group.

The B + MA group had Ag 85A specific IFNγ+ and TNFα+ CD4+ T cells (Figure 5-3 I). IFNγ+ CD4+ T cells were detected in the BCG only group but at a lower frequency than in B + MA mice.

The highest frequencies of Th1 responses were recorded in the lungs and the lowest frequencies in the mesenteric lymph nodes. The groups had similar responses in the lymph nodes. In the spleen B + MA mice had a higher frequency of PPD-A specific responses than BCG only mice whereas BCG only mice had greater Ag 85A specific responses compared to B + MA mice. In the lungs B + MA had greater PPD-T and Ag 85A responses but PPD-A specific responses were similar between the groups.

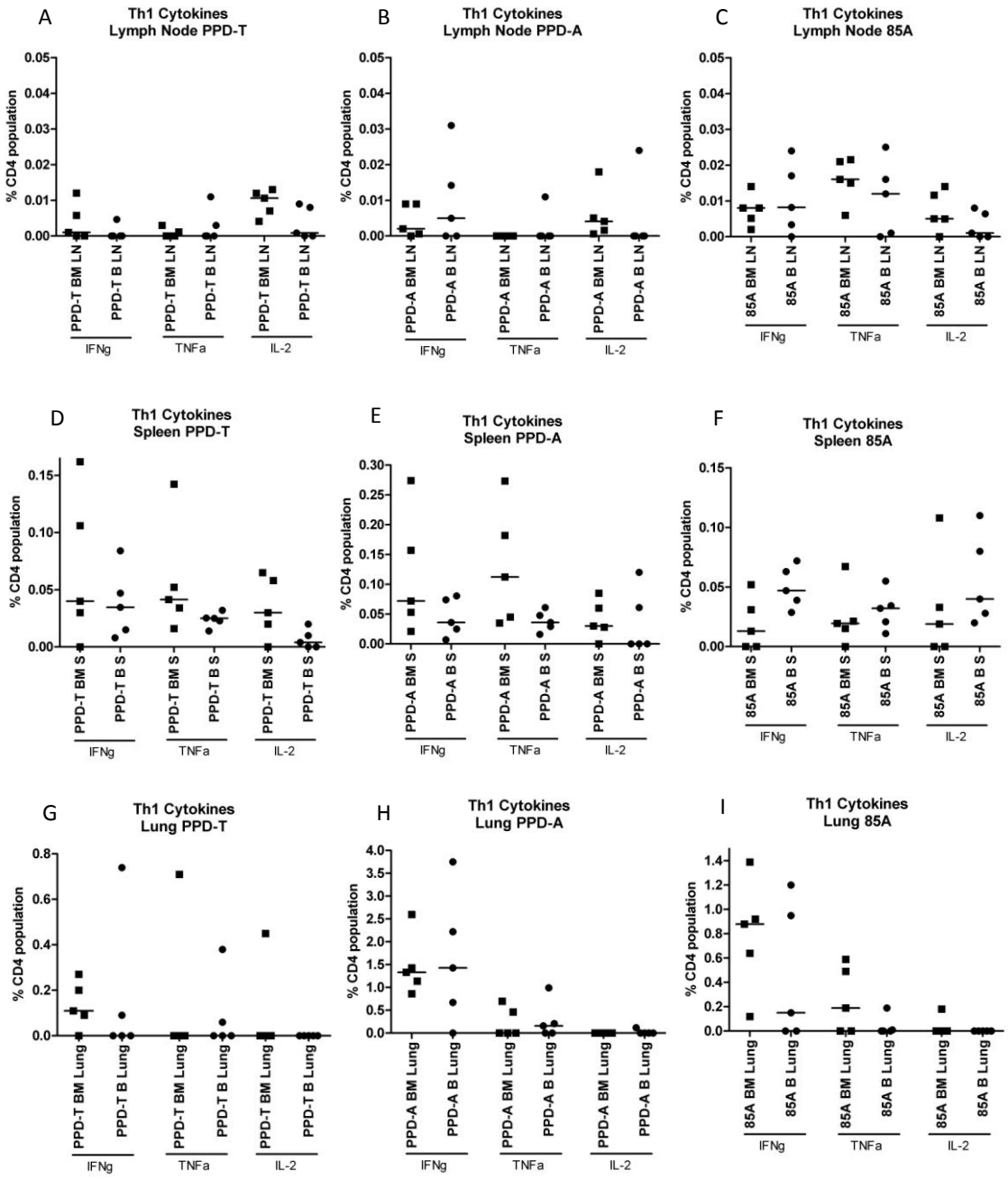


Figure 5-3: Th1 responses in BCG vaccinated mice and mice sensitised with MA 104 after BCG vaccination. Cells from mesenteric lymph nodes, spleens and lungs were stimulated with PPD-T, PPD-A or Ag 85A. (A – C) is the response in the lymph nodes, (D – F) is the response in the spleen and (G – I) is the response in the lung. IFNγ, TNFα and IL-2 production from CD4+ T cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to determine statistical significance; none was calculated.

5.2.2 CD8+ T Cell Responses

5.2.2.1 Mesenteric Lymph Node

A low frequency of PPD-T specific IFNγ+ CD8+ T cells were detected in the B + MA mice but not the BCG only mice. No PPD-T specific TNFα or IL-2 was detected (Figure 5-4A). PPD-A specific responses were the same as PPD-T (Figure 5-4 B).

Ag 85A specific IFNγ+ and TNFα+ CD8+ T cells were detected in both groups, no IL-2+ CD8+ T cells were detected (Figure 5-4 C). The frequencies of IFNγ+ and TNFα+ CD8+ T cells were greater in the BCG only mice compared to B + MA mice. Ag 85A responses were greater than PPD-T and PPD-A specific responses.

5.2.2.2 Spleen

PPD-T specific IFNγ+, TNFα+ and IL-2+ CD8+ T cells were detected in the BCG only mice, IFNγ+ and IL-2+ CD8+ T cells were detected in the B + MA mice (Figure 5-4 D). Frequencies of IFNγ+ and IL-2+ CD8+ T cells were similar between the groups.

PPD-A specific IFNγ+ CD8+ T cells were detected in B + MA mice but not in BCG only mice (Figure 5-4 E). IL-2+ CD8+ T cells were detected in BCG only mice but not B + MA mice.

Ag 85A specific IFNγ+, TNFα+ and IL-2+ CD8+ T cells were detected in the BCG only mice, TNFα+ and IL-2+ CD8+ T cells were detected in B + MA mice (Figure 5-4 F). Frequencies of TNFα+ and IL-2+ CD8+ T cells were similar between the groups. PPD-T specific and Ag 85A specific responses were greater than PPD-A specific responses.

5.2.2.3 Lung

PPD-T specific IFNγ+ CD8+ T cells were detected in B + MA mice, IL-2+ CD8+ T cells were detected in BCG only mice (Figure 5-4 G). PPD-A specific IFNγ+ CD8+ T cells were detected in the B + MA and the BCG only group. Il-2 was induced by the BCG only group. The frequency of IFNγ+ CD8+ T cells in B + MA mice was greater than in BCG only mice (Figure 5-4 H). Ag 85A specific IL-2+ CD8+ T cells were detected in both groups at a similar frequency (Figure 5-4 I).

The lowest frequencies of CD8 responses were seen in the mesenteric lymph nodes and the highest frequencies were seen in the lungs. There were more PPD-T and Ag 85A specific responses than PPD-A specific responses recorded in both groups. The CD8 response was similar between the groups.

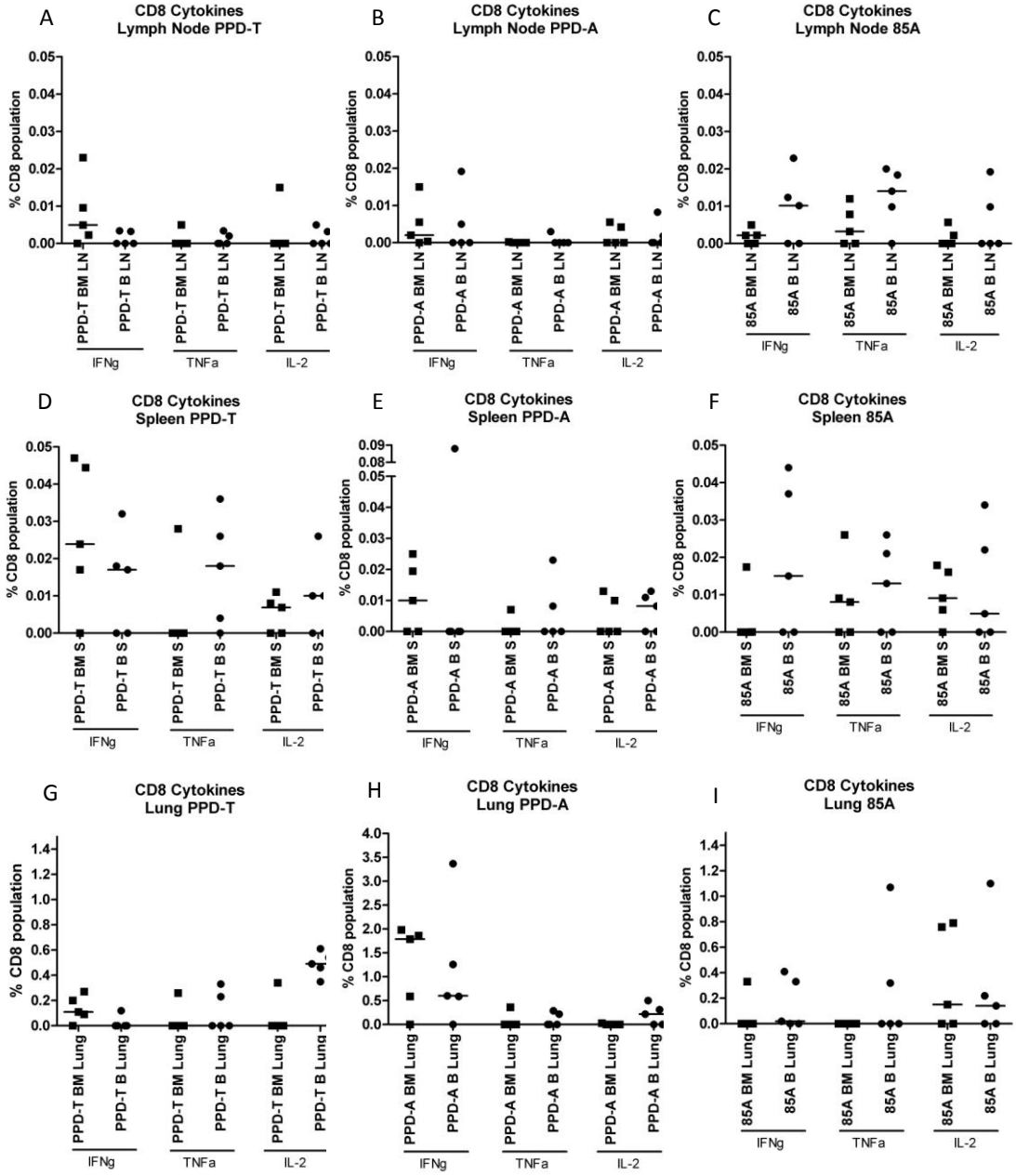


Figure 5-4: CD8+ T cell responses in BCG vaccinated mice and BCG-MA exposed mice. Cells from mesenteric lymph nodes, spleens and lungs were stimulated with PPD-T, PPD-A or Ag 85A. (A – C) is the response in the lymph nodes, (D – F) is the response in the spleen and (G – I) is the response in the lung. IFNγ, TNFα and IL-2 production from CD8+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD8+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; none was calculated.

5.2.3 Th17 Responses

5.2.3.1 Mesenteric Lymph Nodes

Antigen 85A specific IL-17+ CD4+ T cells were detected in B + MA mice (Figure 5-5 A). No PPD-T or PPD-A specific IL-17+CD4+T cells were detected in either group.

5.2.3.2 Spleen

PPD-T, PPD-A and Ag 85A specific IL-17+CD4+ T cells were detected in both groups (Figure 5-5 B). There were greater frequencies of Ag 85A specific cells than PPD-T or PPD-A specific cells. Both groups had a similar magnitude of IL-17+ CD4+ T cells.

5.2.3.3 Lung

No IL-17+ CD4+ T cells specific to any of the stimulations were detected in either group (Figure 5-5 C).

5.2.4 Th2 Responses

5.2.4.1 Mesenteric Lymph Nodes

PPD-T, PPD-A and Ag 85A specific Il-4+ CD4+ T cells were detected in the B + MA group (Figure 5-5 D). No IL-4+ CD4+ T cells were detected in the BCG only group.

5.2.4.2 Spleen

PPD-T, PPD-A and Ag 85A specific Il-4+ CD4+ T cells were detected in both groups (Figure 5-5 E). Frequencies were greater in the BCG only mice compared to B + MA mice.

5.2.4.3 Lung

Ag 85A specific IL-4+ CD4+ T cells were detected in B + MA mice but not BCG only mice (Figure 5-5 F). No PPD-T or PPD-A specific response was detected in either group.

In the mesenteric lymph node the B + MA group had an IL-4 response where the BCG only group did not, however in the spleen where a response was detected in both groups the frequencies were greater in the BCG only group compared to the B + MA group.

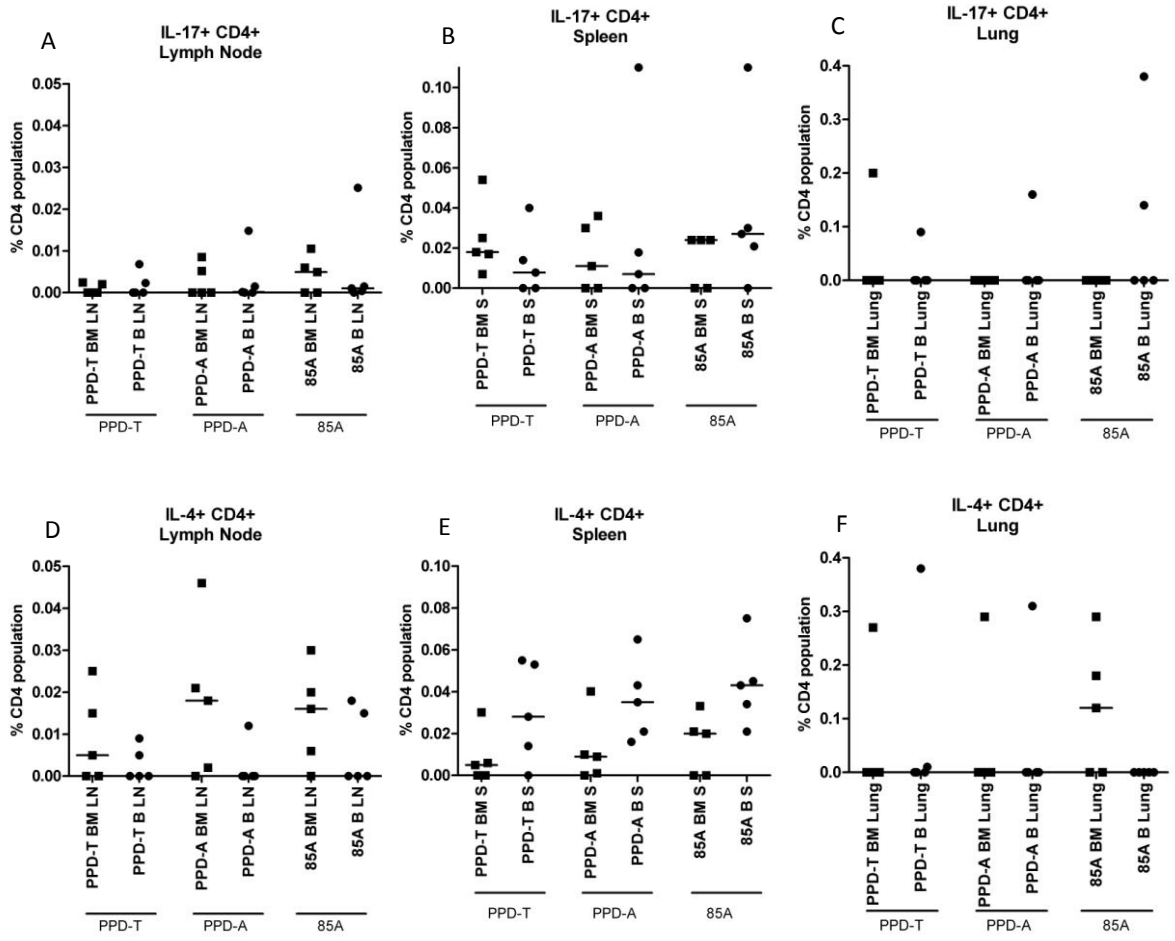


Figure 5-5: Th17 and Th2 responses from BCG vaccinated mice and BCG vaccinated -MA exposed mice. IL-17+ CD4+ T Cells from mesenteric lymph nodes (A), spleen (B) and lungs (C) IL-4+ CD4+ T cells in mesenteric lymph nodes (D), spleen (E) and lungs (F). Cells were stimulated with PPD-T, PPD-A or Ag 85A and cytokines detected using flow cytometry. Results are expressed as a percentage of the CD4+ T cell population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance, none was calculated.

5.2.5 Regulatory T Cell Responses

5.2.5.1 Treg Frequency

In the lung the frequencies of Tregs were between 0 and 7%; frequencies were greater in the B + MA mice compared to BCG only mice (Figure 5-6 A).

In the mesenteric lymph nodes frequencies were between around 7 and 15%. There were significantly more Tregs in the lymph nodes of B + MA mice compared to BCG only mice.

Frequencies in the spleen were between 1 and 6% and similar between the groups.

5.2.5.2 IL-10+ CD4+ T cells

Mesenteric lymph nodes

PPD-T, PPD-A and Ag 85A specific IL-10+ CD4+ T cells were detected in lymph nodes of BCG only mice (Figure 5-6 B). PPD-T specific IL-10+ CD4+ T cells were a similar frequency in B + MA mice, no PPD-A specific IL-10+ CD4+ T cells were detected in this group. The greatest magnitude of response was against Ag 85A, both groups had similar responses.

Spleen

IL-10+ CD4+ T cells specific for all stimulations were detected in both groups, the frequencies were similar between the groups (Figure 5-6 C).

Lungs

IL-10+ CD4+ T cells were detected in PPD-A stimulated cultures from B + MA mice but not BCG only mice. No PPD-T or Ag 85A specific IL-10 was detected (Figure 5-6 D).

Treg frequencies and IL-10 production was highest in the mesenteric lymph nodes. The B + MA group had significantly higher frequencies of Tregs compared to the BCG only group but IL-10 production was similar between the groups.

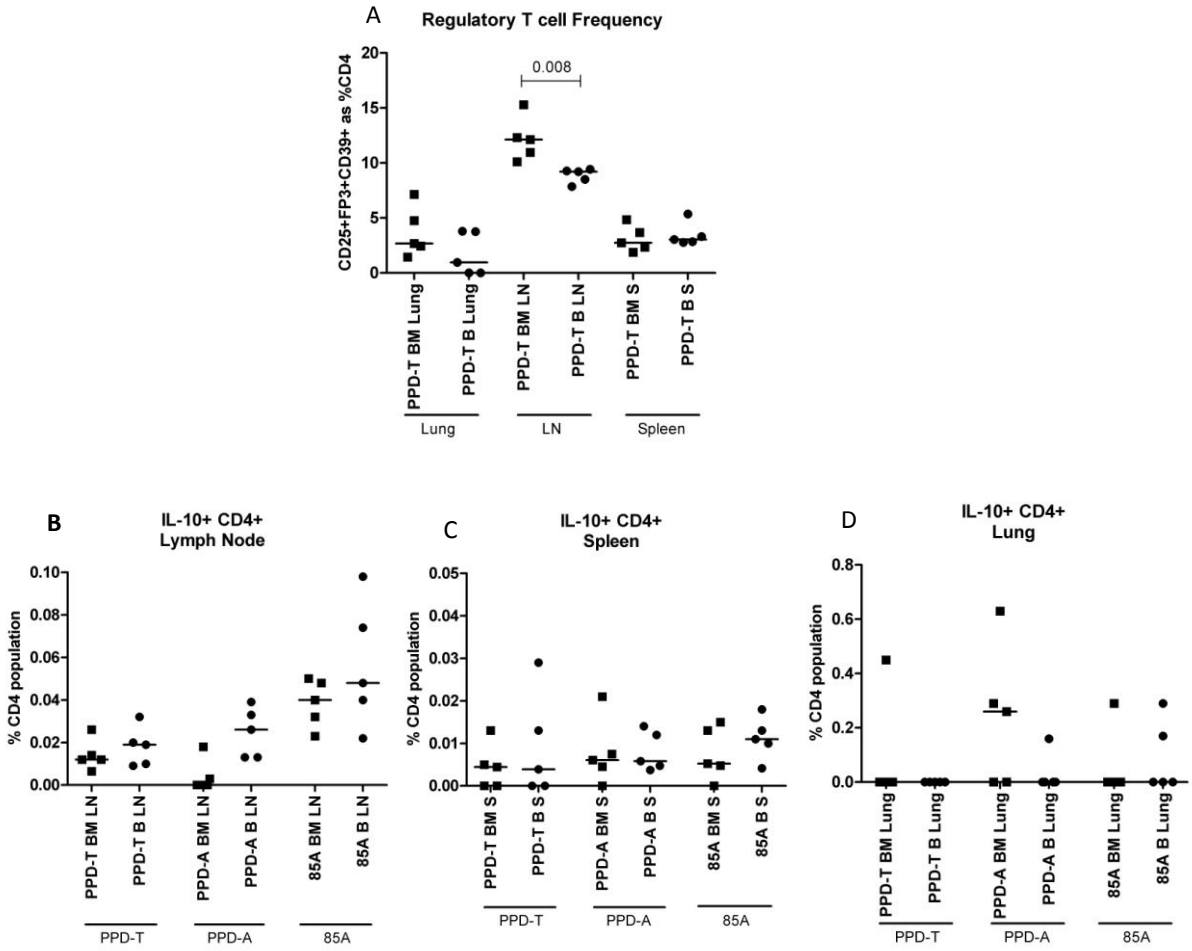


Figure 5-6: Regulatory responses in BCG vaccinated mice and mice exposed to MA. (A) Expression of CD25, FoxP3 and CD39 on CD4+ T cells was assayed by flow cytometry. (B - D) IL-10+ CD4+ T cells in mesenteric lymph nodes, spleen and lungs stimulated with PPD-T, PPD-A and Ag 85A, measured by flow cytometry. Results are expressed as a percentage of the CD4+ T cell population; the media control well value is not subtracted from Treg frequency but is from IL-10 results. Each data point represents one mouse and the median is displayed, n= 5. The Mann-Whitney U test was used to test for statistical significance and p values are shown where calculated.

5.3 Results: Post-Challenge

Around 35 CFU of *M. tb* Erdmann was administered by aerosol to 4 groups of mice; naïve controls, mice vaccinated with BCG 3 months before *M. tb* challenge, mice vaccinated with BCG 6 months before challenge and mice vaccinated 6 months before challenge then exposed to repeated doses of live MA 104. Lungs and spleens were taken 35 days after *M. tb* challenge, homogenised and plated on 7H10 agar to determine the burden of *M. tb* in the organs. Cellular immune responses 13 and 16 days after infection were assayed in lungs, pulmonary lymph nodes and spleens of naïve mice, mice vaccinated 6 months before challenge and vaccinated and MA exposed mice. Cytokine responses were not detectable at day 13 so these results are not shown.

5.3.1 CFU

5.3.1.1 Lungs

The median CFU in the lungs of naïve mice was 5.6 (log 10), in mice vaccinated 3 months before challenge 4.9 (log 10), in mice vaccinated 6 months before challenge 4.96 (log 10) and in mice vaccinated then exposed to MA 5 (log 10) (Figure 5-7 A). Both BCG vaccinated groups and the B + MA group had significantly lower CFU burden compared to naïve mice. There was no statistically significant difference in CFU between mice vaccinated with BCG 3 months before challenge or 6 months before challenge. There was no statistically significant difference between mice exposed to MA after vaccination and those which were not.

5.3.1.2 Spleen

The median CFU in the spleens of naïve mice was 4.2 (log 10), in mice vaccinated 3 months before challenge 3.6 (log 10), in mice vaccinated 6 months before challenge 3.1 (log 10) and in mice vaccinated then exposed to MA 3.7 (log 10) (Figure 5-7 B). Both BCG vaccinated groups and the B + MA group had significantly lower CFU in the spleens compared to the naïve group. There was no statistically significant difference in the CFU between mice vaccinated 3 months

before challenge or 6 months before challenge. There was no statistically significant difference in the CFU between mice exposed to MA after vaccination and those which were not.

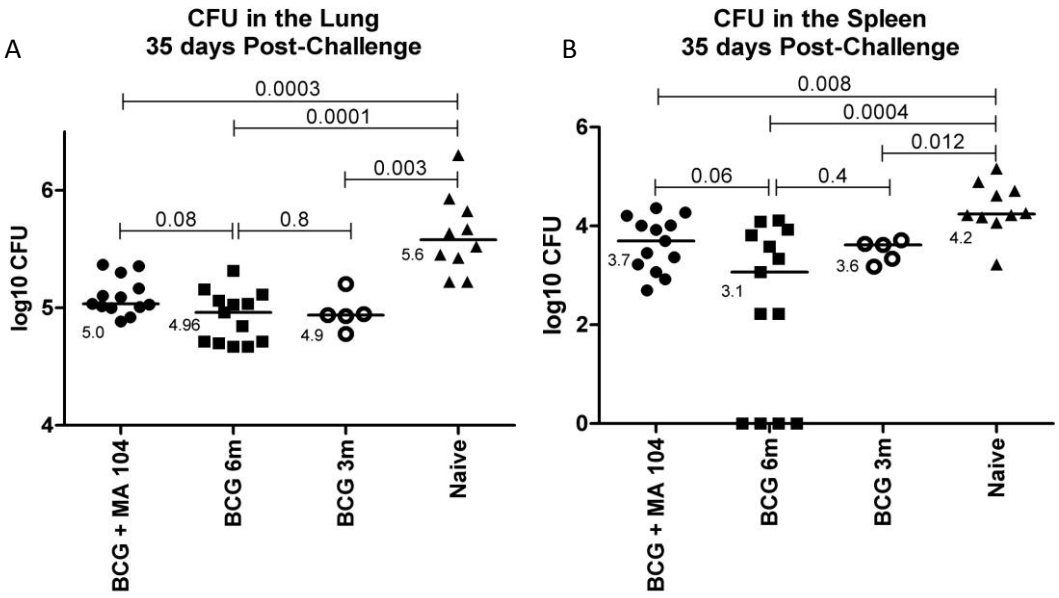


Figure 5-7: *M. tb* burden in the lungs and spleens 35 days after aerosol challenge with *M. tb*. Homogenised lung (A) and spleen (B) tissue was plated on 7H10 agar to quantify the CFU in the organ. Log 10 of the CFU count for the whole organ is displayed. Each point represents one mouse; the median is represented as a line and the value in text next to the line. The Mann-Whitney U test was applied to test for statistically significant differences; the p value for each comparison is displayed.

5.3.2 CD44+ CD4+ T Cells: Post-Challenge

5.3.2.1 Lung

In naïve mice around 5% of lymphocytes in the lung were activated CD4+ T cells at day 13 after challenge (Figure 5-8 A). The frequency of CD44+ CD4+ T cells in B + MA mice was around 8% and in BCG only mice the frequency was around 12%; significantly greater than B + MA mice. At day 16 after challenge the frequency of CD44+ CD4+ T cells in naïve mice was around 15% and in BCG only mice and B + MA mice the frequency was around 30% (Figure 5-8 B).

5.3.2.2 Pulmonary Lymph Nodes

Thirteen days after challenge, the frequency of CD44+ CD4+ T cells in the lymph nodes of all mice was around 17% (Figure 5-8 A). At day 16 after challenge the frequency was the same in BCG only mice and B + MA mice but was around 10% in naïve mice (Figure 5-8 B).

5.3.2.3 Spleen

The frequency of CD44+ CD4+ T cells in the spleens of naïve mice at day 13 was around 22%, the frequency in BCG only mice was around 30% and in B + MA mice much less at around 5% (Figure 5-8 A). At day 16 after challenge the frequency of CD44+ CD4+ T cells in naïve mice was around 12%, in BCG only mice the frequency remained around 30% and in B + MA mice the frequency had risen to 30% (Figure 5-8 B).

5.3.3 CD44+ CD8+ T Cells

5.3.3.1 Lungs

Thirteen days after challenge the frequency of CD44+ CD8+ T cells in all groups was 0 to 2% (Figure 5-8 C). At day 16 the frequency in naïve mice was 7%, in BCG only mice the frequency was around 8% and in B + MA mice the frequency was greater at around 13% (Figure 5-8 D).

5.3.3.2 Pulmonary lymph Nodes

The frequency of CD44+ CD8+ T cells 13 days after challenge was around 3% in all groups (Figure 5-8 C). 16 days after challenge the frequency remained the same in naïve mice but had risen in BCG only and B + MA mice to around 8% (Figure 5-8 D).

5.3.3.3 Spleen

At day 13 the frequency of CD44+ CD8+ T cells in the spleens from BCG only and B + MA mice was 0 to 2% (Figure 5-8 C). There were no CD44+ CD8+ T cells in the spleens of naïve mice. At day 16 after challenge the frequency in naïve mice was around 5% and around 8% in BCG only and B + MA mice (Figure 5-8 D).

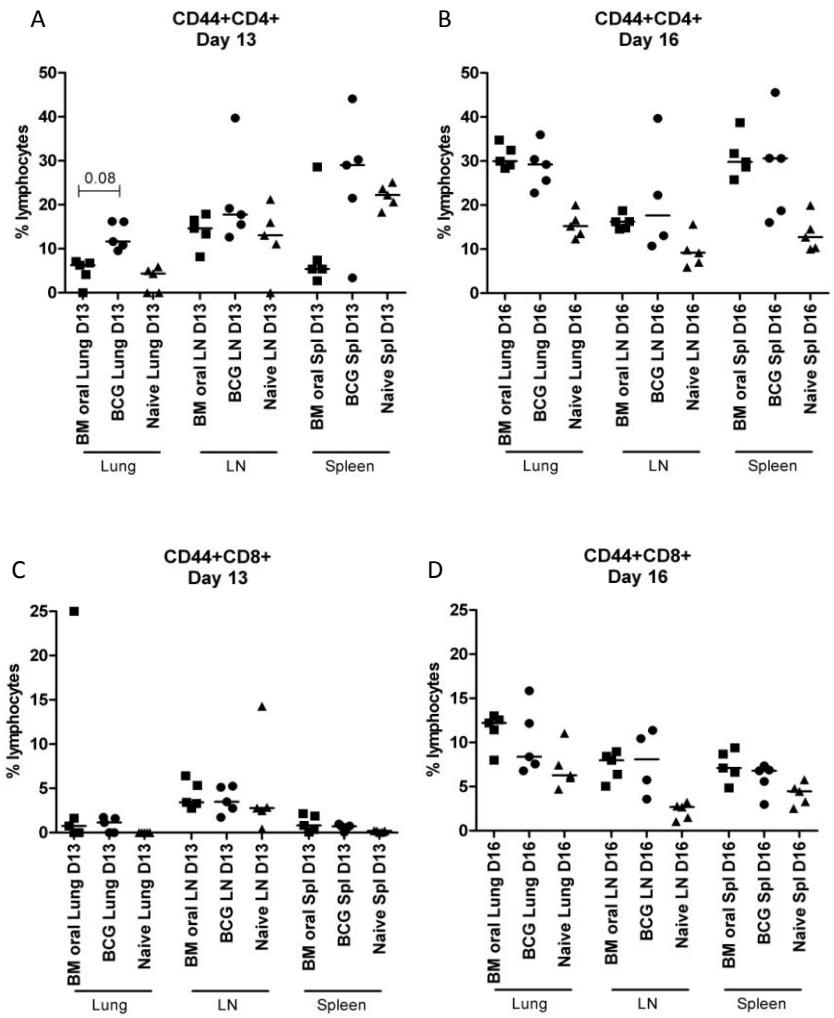


Figure 5-8: Percentage of activated CD4 and CD8 T cells 13 and 16 days after *M. tb* aerosol challenge. Cells from lungs, pulmonary lymph nodes and spleens were assayed 13 and 16 days after *M. tb* infection. CD44 expression on CD4+ T cells (A and B) and CD8+ T cells (C and D) was assayed by flow cytometry. Results are expressed as the % of CD44+ CD4+ or CD8+ T cells in the lymphocyte population. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance and the p value is displayed where significance was found.

5.3.4 Th1 Responses: Post-Challenge

5.3.4.1 Pulmonary Lymph Nodes

Sixteen days after challenge PPD-T specific IFNγ+ and TNFα+ CD4+ T cells were detected in all groups (Figure 5-9A). There were greater frequencies of IFNγ+ CD4+ T cells in naïve mice and B + MA mice compared to BCG only mice. Frequencies of TNFα+ CD4+ T cells were slightly greater in B + MA compared to the other groups. No IL-2+ CD4+ T cells were detected.

5.3.4.2 Spleen

IFNγ+ CD4+ T cells were detected in the B + MA group and the naïve group but not the BCG only group (Figure 5-9 B). TNFα+ CD4+ T cells were detected in all groups but at low frequency. No IL-2+ CD4+ T cells were detected.

5.3.4.3 Lung

IFNγ+ and TNFα+ CD4+ T cells were detected in the lungs of BCG only mice and B + MA mice but not in naïve mice (Figure 5-9 C). No IL-2+ CD4+ T cells were detected.

All groups had a PPD-T specific Th1 response 16 days after challenge. In the lymph nodes and spleen the B + MA group and the naïve group had a similar magnitude of response and this was greater than that in the BCG only group. The responses in the lungs were different; the naïve group had no PPD-T specific Th1 cells whereas the BCG only and B + MA groups had a high frequency of responding cells.

5.3.5 CD8+ T Cell Responses: Post-Challenge

5.3.5.1 Pulmonary Lymph Nodes

All groups had an IFNγ+ CD8+ T cell response 16 days after challenge; frequencies were similar over the groups (Figure 5-9 D). TNFα+ CD8+ T cells were detected in the B + MA group.

5.3.5.2 Spleen

Low frequencies of IFNγ+ CD8+ T cells were detected in the spleens of all groups (Figure 5-9 E). TNFα+ CD8+ T cells were detected in the B + MA group.

5.3.5.3 Lung

IFNγ+ CD8+ T cells were detected in the BCG only group but not in the B + MA group or the naïve group (Figure 5-9 F). TNFα+ CD8+ T cells were detected in the B + MA group and the naïve group but not the BCG only group.

CD8 responses were of greatest magnitude in the pulmonary lymph nodes. IFNγ responses were similar across the groups in the lymph nodes and spleen but in the lung only the BCG group had an IFNγ response. B + MA mice had a higher TNFα+ CD8+ response than the BCG only mice and the naïve mice.

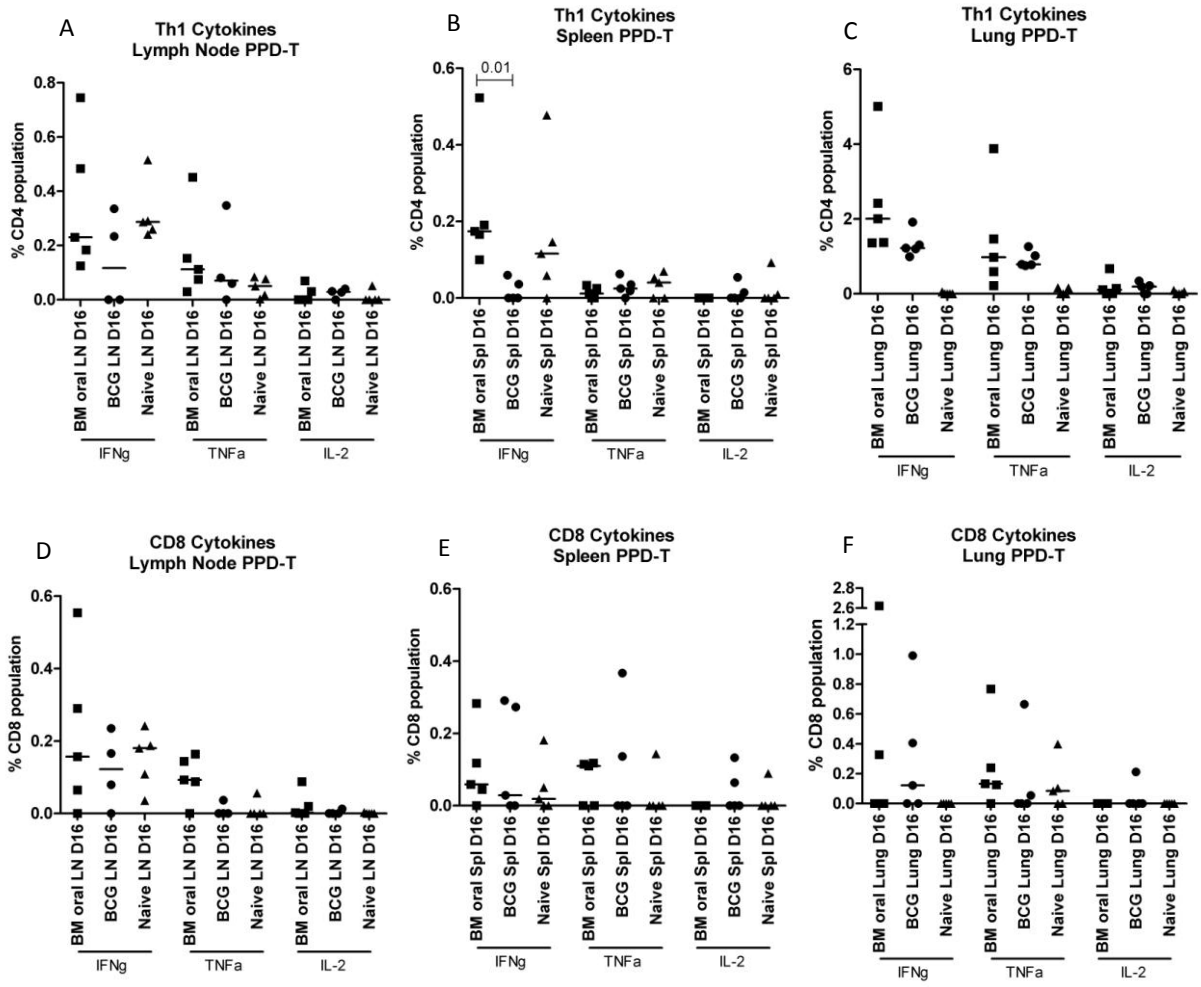


Figure 5-9: Th1 and CD8+ T cell responses 16 days after *M. tb* challenge. Cells isolated from pulmonary lymph nodes, spleens and lungs were stimulated with PPD-T. (A – C) are Th1 responses. (D – F) are CD8+ T cell responses. IFNγ, TNFα and IL-2 production from CD4+ and CD8+ T cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ or CD8+ T cell population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n= 5. The Mann-Whitney U test was used to test for statistical significance and are shown where significance was calculated.

5.3.6 Th17 Responses: Post-Challenge

5.3.6.1 Lung

The BCG only group and the B + MA group had a high frequency of PPD-T specific IL-17+ CD4+ T cells in the lungs; significantly more were detected in the B + MA group compared to the BCG only group (Figure 5-10 A).

5.3.6.2 Pulmonary Lymph Nodes

IL-17+ CD4+ T cells were detected in the BCG only group and B + MA group (Figure 5-10 A). B + MA mice had higher frequency compared to BCG only mice but the difference was not significant. Frequencies were lower than in the lungs.

5.3.6.3 Spleen

Low frequencies of IL-17+ CD4+ T cells were detected in the spleens of B + MA mice but not BCG only or naïve mice (Figure 5-10 A).

5.3.7 Th2 Responses: Post-Challenge

5.3.7.1 Lung

PPD-T specific Il-4+ CD4+ T cells were detected in the lungs of B + MA mice and BCG only mice (Figure 5-10 B). There were significantly more IL-4+ CD4+ T cells in B + MA mice compared to BCG only mice.

5.3.7.2 Pulmonary Lymph Nodes and Spleen

No IL-4 was detected in the lymph nodes or spleens of any group (Figure 5-10 B).

5.3.8 Regulatory T Cell Responses: Post-Challenge

5.3.8.1 Treg frequencies

All mice had a Treg frequency of between 1 and 2% in the lungs 16 days after *M. tb* challenge (Figure 5-10 C).

The naïve group had a median frequency of 4% Tregs in the pulmonary lymph nodes; the BCG only group had a slightly higher frequency of around 5.5% and the B + MA group slightly more at around 6%.

The naïve group had the highest frequency of Tregs in the spleen with a median of 4%. The BCG only group and the B + MA group had a median frequency of 3%.

5.3.8.2 IL-10+ CD4+ T Cells

IL-10+ CD4+ T cells were detected in the lungs of all mice; frequencies in B + MA mice and naïve mice were greater than in BCG only mice (Figure 5-10 D).

Low frequencies of IL-10+ CD4+ T cells were detected in the lymph nodes of all groups. The BCG only group had slightly greater frequencies than the B + MA and naïve groups.

Similar frequencies of IL-10+ CD4+ T cells were detected in the spleens of all mice. The frequencies in the spleen were greater than what was detected in the lymph nodes but less than in the lungs.

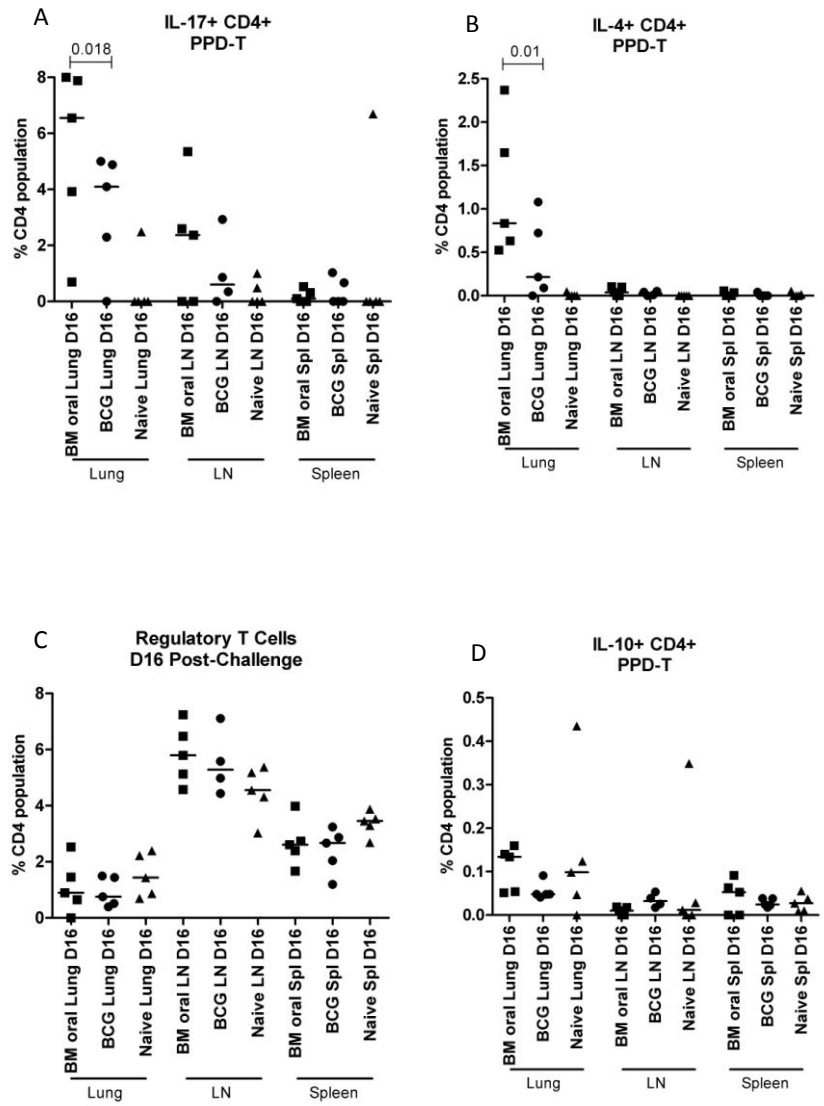


Figure 5-10: Th17, Th2, Treg frequencies and IL-10 production 16 days after *M. tb* challenge. (A) IL-17+ CD4+ T cells, (B) IL-4+ CD4+ T cells, (C) expression of CD25, FoxP3 and CD39 on CD4+ T cells, (D) IL-10 production from CD4+ T cells. Cells from lungs, pulmonary lymph nodes and spleens were stimulated with PPD-T and cell surface markers and cytokine production assayed by flow cytometry. Results are expressed as a percentage of the CD4+ T cell population; the media control well value is not subtracted from Treg frequency but is for cytokine production. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance and is shown where calculated.

5.4 Discussion

In this study a published model of oral exposure to MA after BCG vaccination was replicated. In the publication the efficacy of BCG had been reduced by sensitisation. I hypothesised that a reduction in the efficacy of BCG would be replicated and that the mechanism by which this occurred may be attributable to a regulatory T cell response.

5.4.1 Adaptive Immunity Induced Pre-Challenge

In the previous chapter, Th1 and Th17 subsets had been enhanced by repeated doses of MA after BCG in a systemic model of MA exposure. By contrast, in this model of oral exposure, there was no difference in the Th17 responses and in the Th1 responses only a trend of enhancement was observed in the spleen and the lung of B + MA mice; not the same magnitude of difference which had been seen in the systemic model. This is partly due to the time since BCG vaccination in the BCG only group; responses were assayed 6 months after BCG in this model whereas in the systemic model it was 10 months after BCG. The BCG only group's response is of greater magnitude in this model which is why only a small difference in Th1 responses between the groups is seen. The enhancement of Th17 responses was of lower magnitude compared to Th1 in the systemic model, this probably explains why no difference in this subset is detectable in this model.

The greatest difference in Th1 magnitudes between the groups was seen in PPD-A specific responses in the spleen. The frequency induced by B + MA was greater against this stimulation than PPD-T or Ag 85A. An enhanced ability of live MA compared to BCG to induce PPD-A responses was seen in the initial experiments presented in Chapter 3. The greatest magnitude of response BCG + MA mice induced against PPD-A was detected in the lungs. Contradictory to this, were responses recorded in the spleen where the BCG only group induced the same frequency of PPD-A specific Th1 in this organ as the B + MA group.

The Th1 responses in the mesenteric lymph nodes are a particularly interesting result. They are of much lower magnitude than those detected in the spleen which is opposite to what was seen in the inguinal lymph nodes in the systemic model. This result is in accordance with publications showing inflammatory responses are not primed to the same degree in the intestinal immune system compared to the peripheral[183]. The Ag 85A responses are greater compared to PPD-T and PPD-A responses in this location. This is another interesting observation and may imply that somehow this antigen is preferentially recognised, maybe as a result of it being particularly immunogenic and so can induce differentiation toward Th1 responses in this environment. The fact that both groups induce this response in the lymph node indicates BCG vaccination is capable of priming Ag 85A specific responses in the gut mucosal tissue.

IL-4+ CD4+ T cells were induced by MA exposure in the mesenteric lymph nodes. This Th2 response is in accordance with reports that DCs in the mesenteric lymph nodes can preferentially skew lymphocyte differentiation toward this lineage[180] . Conversely MA induced Th2 responses were not detected in the spleen; in fact the frequency of Th2 responses were lower here than in the BCG only mice. In support of the observation that oral MA induced Th2 responses was Young et al's report of the induction of mycobacterial specific IgG1 antibody responses after BCG vaccination in mice previously exposed to MA orally[126]. It is possible that prior oral MA infection imprinted mycobacterial specific Th2 responses which were boosted on BCG vaccination; this could be measured by antibody production. Results from the current study show MA can induce mycobacterial specific Th2 responses in the gut which would probably lead to the activation of B cells however antibodies were not measured in this model so the results cannot be directly compared to Young et als data.

As hypothesised, oral MA exposure increased the frequency of regulatory T cells in the mesenteric lymph nodes. A greater frequency of Tregs was also seen in the lungs of these

mice however there was no difference in the frequencies in the spleen compared to BCG only mice. Surprisingly the increased frequency of Tregs did not correlate with an increase in IL-10+ CD4+ T cells, which suggests that either these Tregs did not perform regulatory function through secretion of IL-10 or that they were not activate suppressors.

5.4.2 Results of *M. tb* Challenge

The *M. tb* challenge was successful in that it showed BCG vaccinated mice were significantly more capable of controlling the growth of *M. tb* compared to naïve controls. The dose administered to the mice was around 35 CFU; lower than the intended 100 CFU. This resulted in lower numbers of *M. tb* in the lungs and spleens which gives less power to see a difference in the ability of the groups to control infection.

BCG + MA mice had a 0.04 (log 10) CFU more in the lungs compared to BCG only mice, this difference was not significantly different. In the spleen, BCG + MA mice had 0.6 (log 10) CFU more compared to BCG only mice which was very close to a significant increase. In Flaherty's report they measured a 0.25 (log 10) increase in CFU in the lungs of MA exposed mice which is a greater difference than I recorded. This difference could be explained by the lower challenge dose given in my experiment but this is not conclusive. Without a significant difference in the burden of CFU between the BCG-exposed and un-exposed groups I cannot confidently say that I was able to replicate Flaherty et als result and reduce the efficacy of BCG by exposure to MA.

The immunology recorded post challenge in the BCG + MA group compared to the BCG alone group will be discussed with the thought of how exposure after BCG may provide less protection against *M. tb* but keeping in mind that no conclusive evidence of interference has been found.

CD44+ CD8+ T cells were at the same frequency in all groups at day 13 which indicates this response had not been activated by *M. tb* infection at this time point. At day 16 frequencies of activated CD8+ T cells were increased in the two vaccinated groups showing the earlier activation of this response by vaccination.

Activated CD4+ T cells had infiltrated the lungs of BCG only mice at day 13 after challenge measured by the significant increase in the frequency of these cells compared to naïve mice. This infiltration was delayed in MA exposed mice. At day 16 both vaccinated groups had greater frequencies of activated CD4+ T cells in all organs compared to naïve mice which is in agreement with the results published by Flaherty et al 21 days after infection. They did not assay earlier time points than this so I cannot compare with their model the earlier infiltration of cells in the BCG only group I recorded. The lower dose of *M. tb* in this model slowed the kinetic of infection which lead to later activation of the immune response; cytokine responses were not detected at the day 13 time point so this data was not presented. This means the population of CD44+ CD4+ T cells in the lungs of the BCG only group at day 13 was not producing cytokine and so the subclass of these CD4+ T cells is unknown. Despite not knowing the subclass I suspect this earlier infiltration of CD4+ T cells would be a likely explanation of an increased ability to control infection in this group. Why the response was delayed in the MA exposed mice is an important question to be answered.

It could be explained by slightly higher frequencies of Tregs in the pulmonary lymph nodes compared to BCG only mice but this difference was not substantial so it is unlikely to be the only mechanism. There was no IL-10 production so this was not the mechanism. Frequencies of both these cell subsets were the same at day 13 so were not shown. It is possible that activation of a mycobacterial specific response in the lungs was later because of the MA infection in the intestine.

In the systemic model of infection I had seen the appearance of Th1 and Th17 populations earlier in the lungs of BCG + MA mice compared to BCG only mice. This Th1/Th17 response is reported by Khader et al to be a key component of vaccine mediated protection. Th17 infiltrate the lungs before Th1; attracting Th1 infiltration by chemokine and cytokine signalling[45]. As I had seen significantly lower CFU in the spleens of exposed mice compared to BCG alone mice in the systemic model I considered this response to be protective. It is possible that the BCG only group had an earlier infiltration of Th17 and Th1 however, no cytokine was detected so even if this was the case the cells were not actively producing this effect. An extensive time course after *M. tb* infection would give more detailed information on the kinetics of adaptive immunity, be certain to assay the peak of each response and the interplay with regulatory responses.

The BCG only group actually had lower frequencies of Th1 cells in the lymph nodes and spleen at day 16 compared to BCG + MA group and naïve mice. The Th17 responses were also lower in the BCG only mice compared to the BCG + MA mice. Th1 frequencies in the lungs were comparable between BCG only and B + MA mice and greater than naïve mice. If the protective response is reduced by exposure to MA it appears reducing the production of cytokine by these subsets is not the mechanism.

A significant result was the appearance of Th2 immune responses in the lungs of MA exposed mice during *M. tb* infection. These cells were likely the ones primed in the gut seen in the pre-challenge immunology. It may be the production of IL-4 by these cells in the lungs during *M. tb* infection that has a negative impact on control. IL-4 can inhibit the activity of iNOS which is a key mechanism in the control of CFU burdens[184]. The negative role of IL-4 induction in helminth and *M. tb* co-infection was attributed to the activation of macrohpages toward the alternative pathway[107]. There are also reports that frequencies of IL-4+ CD4+ T cells are increased in TB patients however a study by Zhang et al found that the frequency of this

population actually remained the same in TB patients, and it was a decrease in Th1 responses which was associated with disease[185]. This result confirms the importance of Th1 in protection but implies that Th2 are not responsible for the reduction of the Th1 subset in human disease.

IL-4 KO mice are not better at controlling *M. tb* infection than C57BL/6 WT controls showing normal levels of Th2 responses are not detrimental in *M. tb* infection[63]. BALB/C mice mount a Th2 skewed response and are more susceptible to *M. tb* than C57BL/6; 80-90% of BALB/C mice die before 4 months post-infection[186]. IL-4 is produced in late-stages of infection and is linked to pathology in BALB/C mice[64,186]. These reports imply that a higher ratio of Th2/Th1 in long-term infection is pathological. It is plausible the Th2 response may have begun to expand at day 35 in the oral model of MA exposure which is why there was some degree of decrease in BCG efficacy at this time point. Following the *M. tb* infection of the oral model to day 120 as Flaherty et al did may well have shown a far more increased Th2 response which was the cause of the further decrease in BCG efficacy seen at this later time point[176].

The innate immune response was not assayed in this study and may act to reduce BCG efficacy in this oral model of exposure. As previously discussed mycobacteria employ several immune evasion strategies in macrophages and dendritic cells that affect the priming of protective immune responses[2,165]. Innate immune responses may be directly modified by persistent infection with NTM that may in turn affect the ability of innate cells to control *M. tb* infection. This theory is contradicted by the number of reports showing NTM infection can provide a level of protection against *M. tb* and does not render the host more susceptible[121,122]. Whether MA was directly suppressing the immune response was discounted by Flaherty et al as an MA alone control group had been challenged and they showed no more susceptibility to *M. tb* than naïve mice. Also, altered innate immune responses would likely be evidenced by changes in the adaptive response e.g. induction of IL-10 producing cells or Th2 skewed responses; which has already been studied.

It was also considered that MA could have deleted antigen specific T cells by apoptosis which has been reported to occur in MA infection[187,188]. Flaherty et al reported an increased expression of CD95, which binds to FAS and can induce apoptosis, was detected on the surface of CD4+ and CD8+ T cells in the BCG + MA group, however, was not associated to a decrease in the frequencies of activated T cells.

There is a significant difference between my replication of the model and what Flaherty et al reported which may be another contributing factor to why I did not measure a significant reduction in the efficacy of BCG. Mice in Flaherty et als study had a high burden of MA infection in the lungs; up to 3 (log 10) CFU was detected up to 35 days after *M. tb* infection. I detected no MA infection in the lungs of my mice. The reason for this difference is not clear as I had followed their protocol exactly; even to the point of importing the same MA 104 isolate from their source in the US. The only reason I can think of for this disparity is that it occurred during the action of dosing the mice. It is a possibility that their method of gastric gavage led to administration of the MA into the respiratory tract therefore implanting the infection directly to the lungs, however without directly plating lungs after this process this cannot be proven. I also cannot rule out the possibility that I did not administer the same dose or that my cultures became attenuated in some way therefore leading to reduced dissemination of the bacteria in the model.

A pathological response is induced by repeatedly administering BCG to mice infected with *M. tb*. Cruz et al report this response is mediated by IL-17 and IL-23 production which stimulates the infiltration of neutrophils[35]. This model of high antigen exposure during infection is similar to the dual *M. tb* and MA infection in Flaherty et als mice and this is supported by their observation of neutrophil infiltration to the granulomas. The effect of dual infection in the lungs of their mice would presumably put the immune response under much more strain than the single *M. tb* infection in my mice.

Models of high antigen exposure in viral infections such as lymphocytic choriomeningitis virus (LCMV) have shown that induction of memory cell subsets can be limited due to a sustained high level of antigen during infection[189]. In TB the T cell pool is maintained but where these new effectors come from is still not known[50]. Memory subsets may well provide replenishment of the effecter T cell pool; high antigen levels and inflammatory cytokine can induce the differentiation of memory cells to terminally differentiated effectors[190]. The issue with this is that sustained antigen exposure may well deplete the memory pool and prevent the generation of further effecter T cell responses[191].

In my model there was not as high an antigen load as in Flaherty et als as my MA infection was not present in the lungs. Continued antigen load may be the cause of reduced BCG efficacy as it prevents the generation of T cell memory or exhausts what memory exists. This concept was tested in another model of NTM exposure which is presented in Chapter 6.

6 Model of Continuous *M. avium* Exposure after BCG Vaccination: Dead *M. avium* Administered Intraperitoneally

6.1 Introduction

6.1.1 T Cell Memory Subsets

Upon infection the naïve immune response becomes activated and expands rapidly. In infections where the pathogen is cleared by this effector response there is a contraction phase where more than 90% of the effector cells are depleted through apoptosis[190]. Cells which remain are directed toward a memory phenotype and exist in the host's memory repertoire ready to respond if reinfection occurs.

It is now widely accepted that there are two distinct types of memory T cells; effector memory T cells which patrol the tissues and central memory T cells which preferentially home to lymph nodes[190]. Both sets of memory cells express the IL-7 receptor (CD127); IL-7 and IL-15 are essential in the long-term survival of the memory cell[190]. The expression of CD62L allows memory cells to locate to the lymph nodes and so expression of this cell surface marker is limited to central memory cells and not effector memory cells. Central memory cells have a limited effector function which may be attributed to the fact that they are less differentiated. On re-infection this means CD4+ central memory T cells may be able to give rise to different CD4+ T cell lineages. Central memory cells have a low activation threshold, express high levels of IL-2 and have a high proliferative potential. Effector memory cells reside in tissues and can be activated here directly to produce Th1 or Th2 mediated activity or CTL function; CD4+ effector memory T cells are generally thought to be already committed to one subset profile.

The generation of these memory subsets and terminally differentiated effector cells has been suggested to follow a linear differentiation model with the observation that antigenic stimulation of central memory cells leads to differentiation towards effector memory phenotype[190]. This model relies on the theory of antigenic strength to determine the extent of differentiation; with high antigen loads ultimately resulting in differentiation to terminally differentiated effector T cells. As central memory cells are highly proliferative they are more effective at replenishing T cell responses during infection than effector memory cells[192,193]. Effector memory cells can proliferate but owing to a higher degree of apoptosis in this subset they cannot replenish a T cell pool during infection[190].

6.1.2 Induction of Memory in Mycobacterial Infection

The chronic nature of mycobacterial infections lends itself to continued antigen exposure which can impede the generation of central memory T cells. BCG persists in the host[194]. Henao- Tamayo et al reported that BCG vaccination leads to a T cell response dominated by terminally differentiated effector cells and effector memory cells. The central memory population was 50 fold lower than these effector populations. The authors correlated long-term antigen exposure with the lack of central memory populations. Kaveh et al also reported that BCG vaccination induces an effector memory phenotype and found these cells resided in the lungs as well as the spleen[74].

The study by Henao- Tamayo also assessed the induction of memory subsets during chronic *M. tb* infection and found the same dominance of effector memory and terminally differentiated effector cells with 70 fold fewer central memory cells[194]. A greater number of terminally differentiated effector cells were seen in chronic *M. tb* infection compared to BCG vaccination assumedly due to the higher antigen burden and immune stimulation.

Obar et al showed IL-12 is responsible for the generation of more terminally differentiated cells compared to central memory cells in a mouse model of *L. monocytogenes* infection[195]. Given the high level of IL-12 produced in mycobacterial infection this cytokine may well influence the skew away from central memory priming, the production of IL-12 is correlated to mycobacterial antigen load.

Millington et al observed that when TB patients are treated with antibiotics, there is an increase in the frequency of CD4+ T cells secreting IL-2 and cells secreting both IFNγ and IL-2 over the course of treatment[196]. The secretion of IL-2 is associated with a central memory phenotype and supports the theory of a relationship between decreased antigen load and generation of central memory populations.

The inability of BCG to prime a central memory population may be an explanation to the reduction in efficacy over time; as observed in the UK MRC trial and a Saudi Arabian trial (discussed in full in the Introduction) [89,93]. Contradictory to these results was the observation of sustained efficacy of BCG for up to 49 years in North American Indians and Alaskan Natives[94].

6.1.3 Hypothesis

NTM exposure may compromise BCG induced memory responses in 2 ways. Firstly; high antigen loads which are sustained for a period of time prevent the generation of central memory and effector memory, therefore high level exposure to NTM from the time of vaccination may prevent a strong memory T cell response from being formed. Secondly; if BCG induces a memory response which is mainly comprised of effector memory T cells, these cells are more prone to apoptosis on antigen activation than the central memory population. Depletion of the memory pool which was induced by BCG may be accelerated if the host is under constant antigen exposure from exposure to NTM.

To test this hypothesis, a model of NTM exposure directly after BCG was designed to test whether the induction of memory T cell responses by BCG was prevented by exposure to NTM. The ability of *M. avium* to induce memory responses was also assayed.

6.1.4 Experiment plan

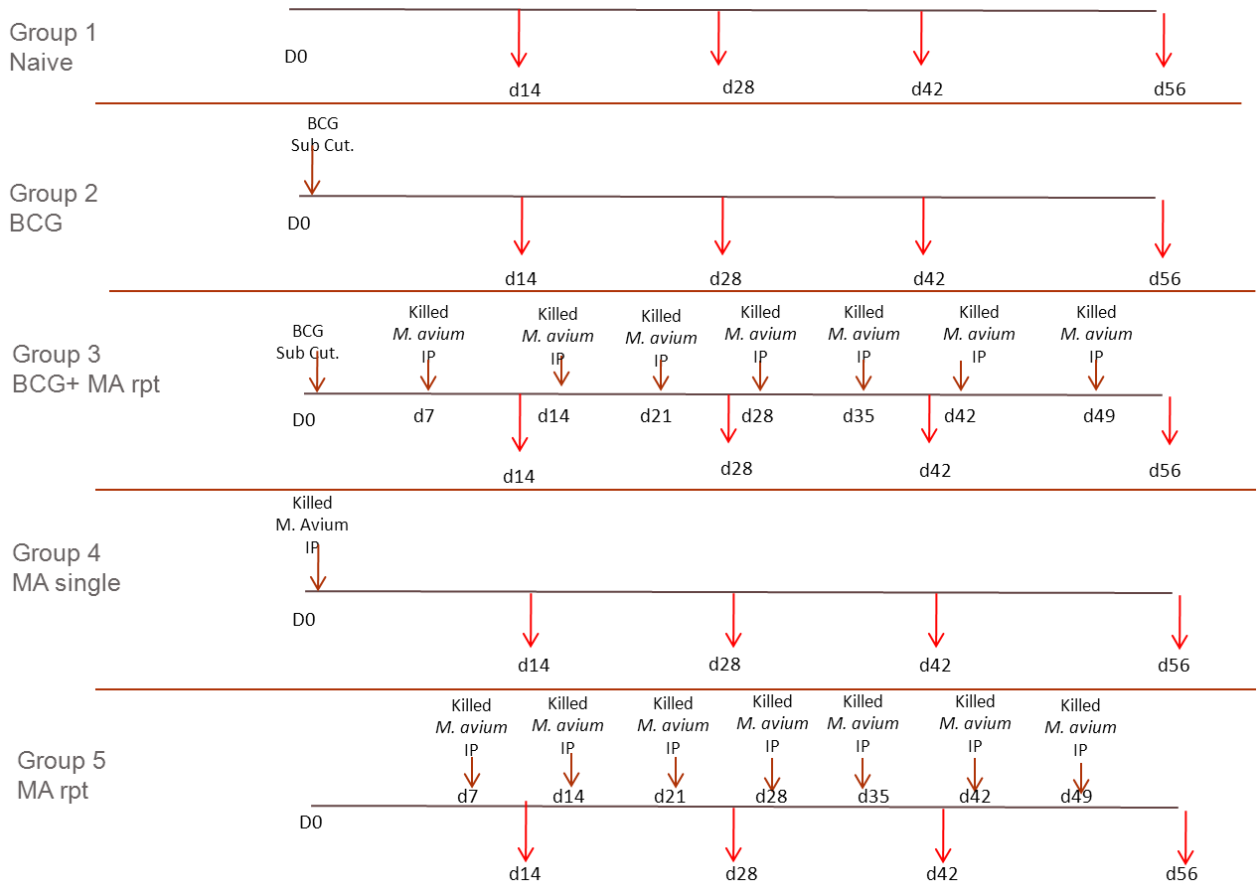


Figure 6-1: Experiment plan to assay induction of CD4+ and CD8+ T cell memory subsets and cytokine expression. Administration time points are shown on the top of the line and immunology time points are shown on the bottom of the line for each group. $1x10^5$ CFU of BCG was given subcutaneously (SC). $1x10^6$ CFU of heat-killed MA 724 was given intraperitoneally.

The administrations of mycobacteria to each group were performed as shown in Figure 6-1.

On day 0 groups 2 and 3 were vaccinated with BCG; group 4 was given dead MA IP.

On day 7, groups 3 and 5 began an MA exposure regime; dead MA IP every 7 days.

Mice in group 1 were naïve controls and left un-manipulated.

On days 14, 28, 42 and 56 mice were taken from each group and lungs, inguinal lymph nodes and spleens assayed for CD4+ and CD8+ T cell memory populations and PPD-T specific Th1 and CD8+ T cell immune responses.

6.2 Results: BCG Compared with BCG + Repeated MA Exposure

6.2.1 CD4+ T Cell Memory Populations

6.2.1.1 Lymph Node

Central Memory (TCM)

Central memory cells were defined as CD44+ CD4+ T cells expressing CD127 and CD62L (TCM). At day 13 after infection the frequency of TCM in both BCG vaccinated and BCG + MA mice was similar to naïve mice: around 3 – 5% of the CD44+ population (Figure 6-2 A). The frequency of TCM in both groups increased from day 14 to day 27 where it peaked at 12 – 14%. At day 56 the frequency had dropped slightly to 10%. There was a similar kinetic of TCM frequency and no difference in the magnitudes of the population between the BCG only and B + MA groups.

Effector Memory (TEM)

Effector memory T cells were defined as CD44+ CD4+ T cells expressing CD127 but not CD62L. At day 14 7% and 5% of CD44+ cells were TEM in the BCG only group and the B + MA group respectively, this was greater than the frequency in naïve mice (Figure 6-2 D). The frequency was slightly less at day 28 then increased at day 41 to 6 and 8% in the BCG only and B + MA group respectively. Frequencies remained at this level at day 56.

Terminally Differentiated Effectors (TEff)

Terminally differentiated effector cells were defined as CD44+CD4+ T cells which did not express CD127 or CD62L. At day 14 and day 28 the frequency of Teff in BCG only mice and B + MA mice were less than naïve mice; around 37 and 33% respectively (Figure 6-2 G). The

frequency rose at day 42 to 41% in B + MA mice and 45% in BCG only mice. Both groups had around 43% Teff at day 56.

6.2.1.2 Spleen

Central Memory

TCM responses in the spleen of both the BCG only and B + MA groups were a similar frequency to naïve mice throughout the study; remaining between 3 and 5% (Figure 6-2 B).

Effector Memory

At day 14 the frequency of TEM was around 14% in the B + MA group and 11% in the BCG only group (Figure 6-2 E). From day 28 to 56 frequencies were similar to naïve mice at around 10% of CD44+ CD4+ T cells.

Terminally Differentiated Effectors

At day 14 TEff frequencies were slightly lower than naïve mice in the B + MA group; frequencies in the BCG only group were the same as naïve mice at 60% (Figure 6-2 H). From day 28 to 56 the frequency of TEff remained the same as naïve mice.

6.2.1.3 Lung

Central Memory

At day 14 the frequency of TCM in the lungs of BCG only mice was 4%; the frequency in BCG + MA mice was the same as naïve mice at around 6% (Figure 6-2 C). At day 28 the frequency in BCG only and BCG + MA mice rose to 10% but then dropped at day 42 and further at day 56 to around 4% which was less than in naïve mice.

Effector Memory

At day 14 BCG + MA mice had a similar frequency oe TEM as naïve mice at around 19% (Figure 6-2 F). BCG only mice had slightly less at 16%. The frequency of TEM in B + MA mice rose at day 28 to 22% then dropped at day 42 and further at day 56 to 9%. In BCG only mice the response peaked at day 42 at 19% which was similar to naïve mice then dropped at day 56 to 14%. The pattern of the response was similar between BCG only and B + MA mice

although the kinetic appeared faster in the B + MA mice as their peak frequency was earlier than BCG only mice.

Terminally Differentiated Effectors

At day 14 the frequency of TEff was comparable in all groups at around 59% (Figure 6-2 I). At day 28 the frequency increased in both BCG only and B + MA mice to 79% then decreased at day 42 before rising again at day 56 to 65% in BCG only mice and around 77% in B +MA mice. This difference was not significant.

Frequencies of CD4+ TCM were greatest in the lymph nodes and lowest in the spleen. The TCM response peaked at day 42 in the lymph node and day 28 in the lung, it did not change in the spleen. The greatest frequency of TEff was in the lungs, the lowest was in the lymph nodes. The profile of memory subsets was comparable between the BCG only and the B + MA group; peak frequencies were similar as was the kinetic.

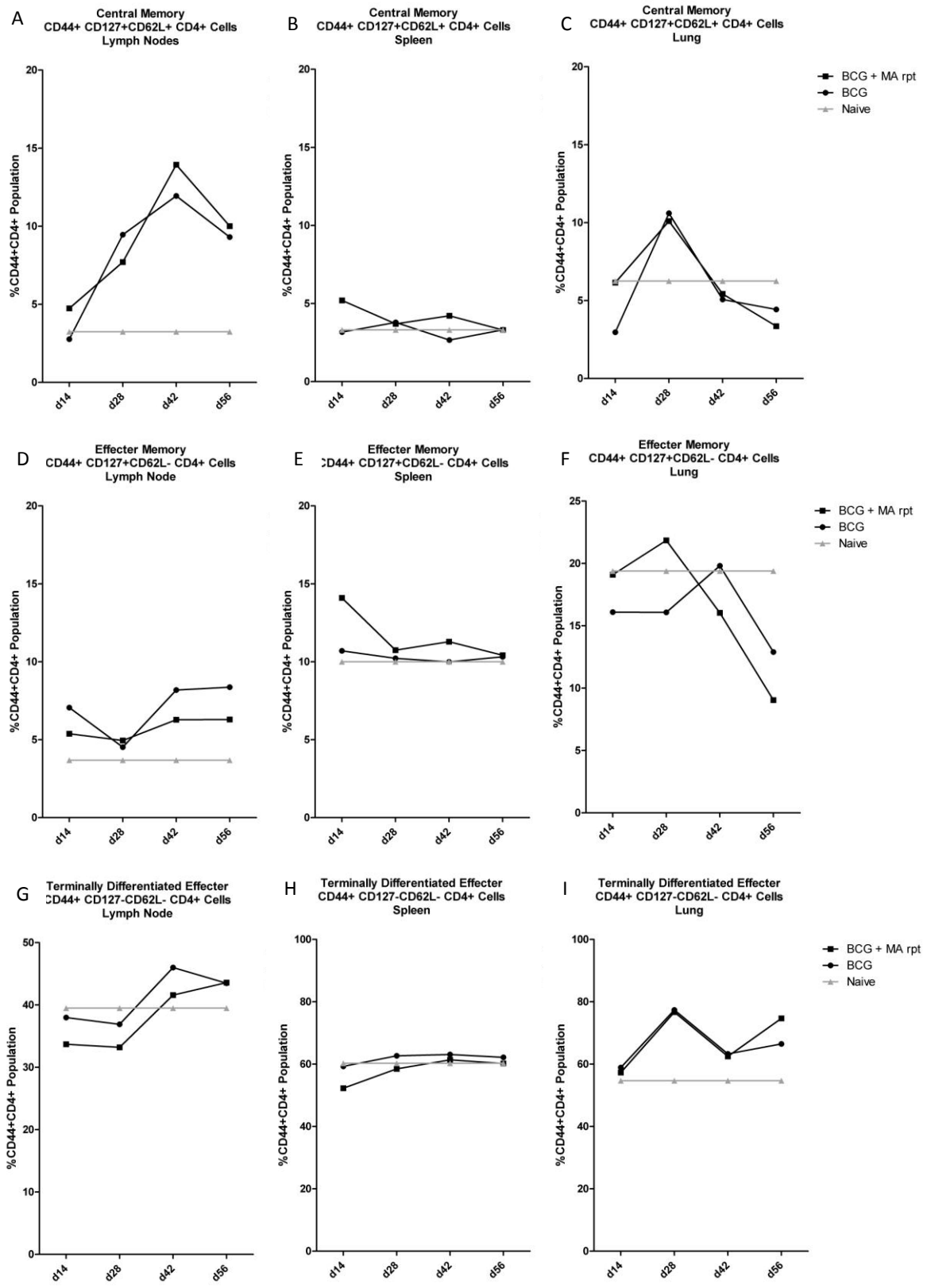


Figure 6-2: CD4+ T cell memory populations in BCG vaccinated and BCG - MA exposed mice. Flow cytometry was used to characterize central memory, effecter memory and terminally differentiated effecter populations by measuring expression of CD44, CD127 and CD62L. Cells were isolated from lymph nodes (A, D, G), spleens (B, E, H) and lungs (C, F, I). Results are expressed as a frequency of the CD44+ CD4+ population. The median of the group is displayed. Results from naïve animals were used to adjust to the frequency measured at day 14 to correct for natural variation in populations over time. Statistical difference between BCG only and B + MA mice at day 56 was measured by the Man-Whitney U test, no significant differences were detected.

6.2.2 CD8+ T Cell Memory Populations

6.2.2.1 Lymph Node

Central Memory

At day 14 the frequency of TCM in the BCG and B + MA group was similar to naïve mice at around 30% (Figure 6-3 A). This increased at day 28 to 42 – 44% then dropped at day 42 to around 40%. Frequency remained at the same level at day 56.

Effector Memory

The frequencies of TEM in BCG only and B + MA mice was similar to naïve mice throughout the experiment at around 6 – 7 % of CD44+ CD8+ T cells (Figure 6-3 D).

Terminally Differentiated Effector cells

Frequencies of TEff in BCG only and B + MA mice remained similar to naïve mice throughout the experiment at around 15 – 19% (Figure 6-3 G).

6.2.2.2 Spleen

Central Memory

The frequency of TCM in the spleen of B + MA mice was 32% at day 14 (Figure 6-3 B). The frequency in BCG only mice was similar to naïve mice at 29%. At day 28 TCM frequency peaked in BCG + MA mice and BCG only mice; 45% and 31% respectively. On day 42 and 56 frequencies returned to the same level as naïve mice at 29%. Although the kinetic of the response was similar the frequencies were greater in B + MA mice than BCG only mice, this difference was not significant.

Effector Memory

At day 14 frequencies of TEM in BCG only and B + MA mice were lower than naïve mice; around 13% compared to 17% (Figure 6-3 E). Frequencies increased at day 28; the BCG only group to a percentage of 16% and the B + MA group at 20%. Frequencies at day 42 and 56 frequencies fell to the same level as naïve mice.

Terminally Differentiated Effector cells

Frequencies of TEff in BCG only and B + MA mice were comparable to naïve mice and remained at around 20 to 30% throughout the experiment (Figure 6-3 H).

6.2.2.3 Lung

Central Memory

At day 14 the frequency of TCM in BCG only mice was around 21%; in BCG + MA mice the frequency was 31% which was more comparable to naïve mice (Figure 6-3 C). Frequencies peaked at day 42 to 38% in both BCG only and B + MA mice. Frequencies then fell at day 42 and further at day 56 to 18-20%. The kinetic of response and frequencies were similar between BCG only and B + MA mice from day 28.

Effector Memory

At day 14 the frequency of TEM in the BCG only group was around 18% which was lower than naïve mice; in the BCG + MA group the frequency was around 30% which was higher than naïve mice (Figure 6-3 F). Frequencies peaked at day 28; 28% in BCG only mice and 36% in BCG + MA mice. At day 42 TEff frequencies were comparable to naïve mice and at day 56 frequencies in BCG and B + MA mce were lower than naïve mice at 18 – 20%.

Terminallly Differentiated Effector cells

At day 14 BCG only mice had a frequency of TEff of 41% which was greater than naïve mice; B + MA mice had a frequency of around 25% which was similar to naïve mice (Figure 6-3 I). BCG only mice had slightly reduced frequencies at day 28 which then increased to 58% at day 42 and was maintained at this frequency on day 56. B + MA mice had a sustained frequency of TEff at 48% at day 28 and 42, this dropped at day 56 to around 40%. The kinetic of the TEff response differed between BCG only and B + MA mice and BCG only mice had a greater frequency of this subset.

The greatest frequencies of TCM were induced in the lymph nodes; B + MA mice also had a high frequency of this subset in the spleen. TCM frequencies peaked at day 28. Frequencies of effector memory cells were greatest in the lungs; B + MA mice had a greater frequency than BCG only mice. TEff cells were only induced in the lungs, responses did not differ to naïve mice in the lymph nodes or spleens. Frequencies of TEff rose over the course of the experiment in the lungs and were greater in BCG only mice although this difference was not significant.

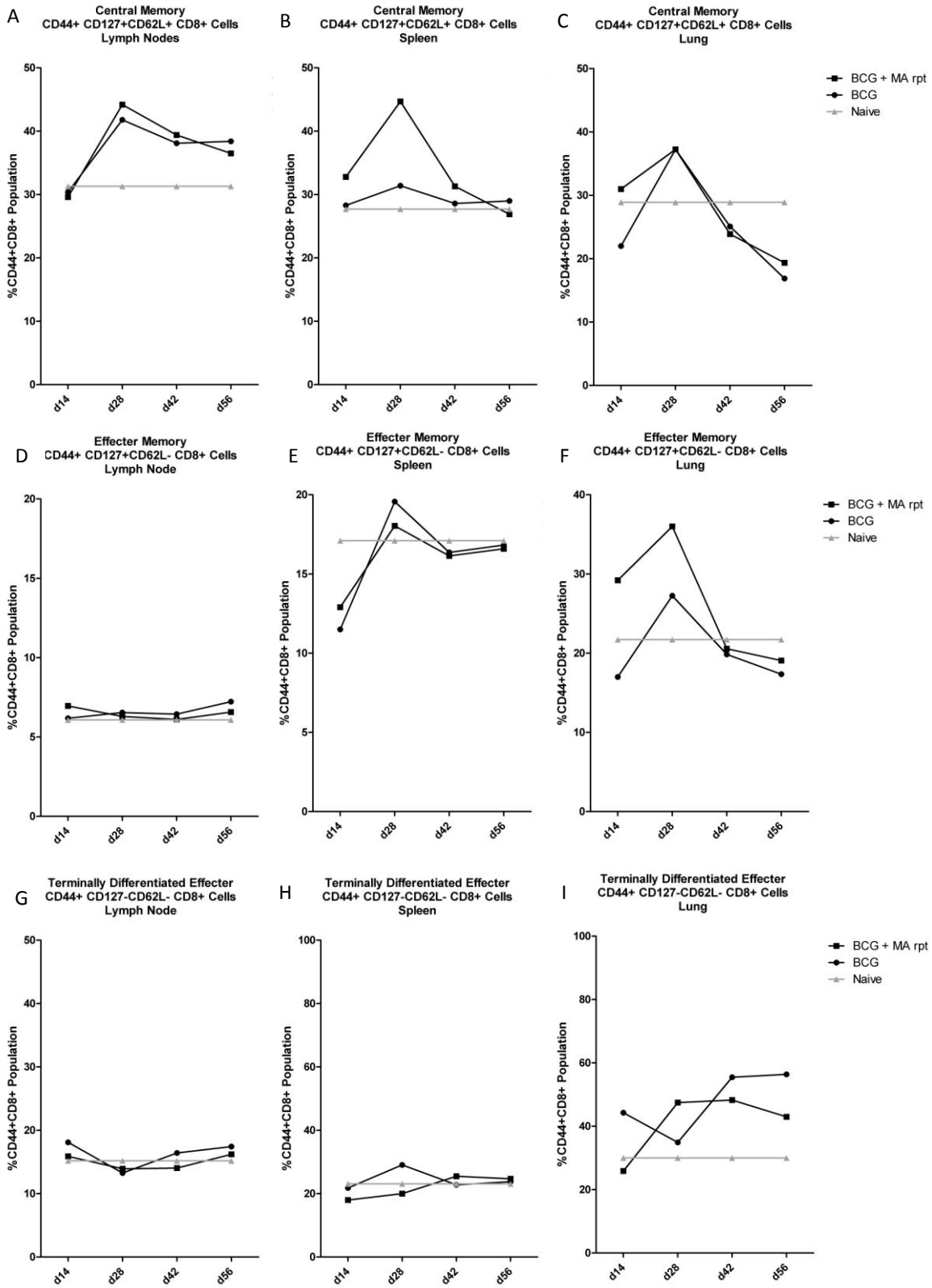


Figure 6-3: CD8+ T cell memory populations in BCG vaccinated and BCG - MA exposed mice. Flow cytometry was used to characterise central memory, effecter memory and terminally differentiated effecter populations by measuring expression of CD44, CD127 and CD62L. Cells were isolated from lymph nodes (A, D, G), spleens (B, E, H) and lungs (C, F, I). Results are expressed as a frequency of the CD44+ CD8+ population. Results from naïve animals were used to adjust to the frequency measured at day 14 to correct for natural variation in populations over time. Statistical difference between BCG only and B + MA mice at day 56 was measured by the Man-Whitney U test, no significant differences were detected.

6.2.3 Cytokine Production from Th1 Cells

6.2.3.1 Lymph Node

At day 14 low levels of PPD-T specific IFNγ+ and TNFα+ CD4+ T cells were detected in both groups, no IL-2+ CD4+ T cells were detected (Figure 6-4 A-D)). At day 28 frequencies remained low in the BCG only group and still no IL-2 was detected. IFNγ+, TNFα+ and IL-2+ responses increased in BCG + MA mice at day 28 and increased again at day 42. On day 42 frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells increased in BCG only mice but decreased again at day 56 whereas in BCG + MA mice they remained the same.

The BCG + MA group had greater frequencies of IFNγ+ , TNFα+ and IL-2+ CD4+ T cells compared to BCG at day 28, 42 and 56. This was significant for TNFα at day 56.

6.2.3.2 Spleen

PPD-T specific IFNγ+ and TNFα+ CD4+ T cells were detected in both groups at all time points (Figure 6-4 E-H). Frequencies in the BCG only mice were lower at day 28 than day 14. In the B + MA group IFNγ+, TNFα+ and IL-2+ CD4+ T cell frequencies rose from day 14 to day 28. In B + MA mice IFNγ+ and TNFα+ CD4+ T cell frequencies remained similar for day 42 and 56 but the IL-2 response decreased. In BCG only mice frequencies of TNFα+ CD4+ T cells increased at day 42 and a population of IL-2+ CD4+ T cells was detected. TNFα+ CD4+ T cells remained at this level at day 56 and frequencies of IFNγ+ CD4+ T cells increased at this time point.

On day 14 greater frequencies of IFNγ+ and TNFα+ CD4+ T cells were detected in the BCG only group but from day 28 to day 56 there were greater frequencies in the BCG + MA group. This was significant in TNFα responses at day 23 and day 56. IL-2 responses followed a different pattern, the response was greater in BCG + MA mice at day 28 but at day 42 it was greater in BCG only mice.

6.2.3.3 Lung

A TNFα response was detected in BCG only mice at day 14 but no other cytokines were produced (Figure 6-4 I-L). There were no detectable responses in either group at day 28. On day 42 IFNγ+,TNFα+ and IL-2+ CD4+ T cells were detected in both groups and were significantly greater in the BCG + MA mice compared to BCG only group. At day 56 only a low frequency of TNFα+ CD4+ T cells was detected.

Th1 responses in BCG only mice peaked at day 42 in the lymph nodes and lungs, response was similar at day 42 and 56 in the spleens. The B + MA mice had a similar kinetic of response in the lungs and spleen as the BCG only mice but in the lymph nodes the frequencies of cytokine positive cells peaked at day 56. In all organs the BCG + MA mice had a greater Th1 response than the BCG only mice.

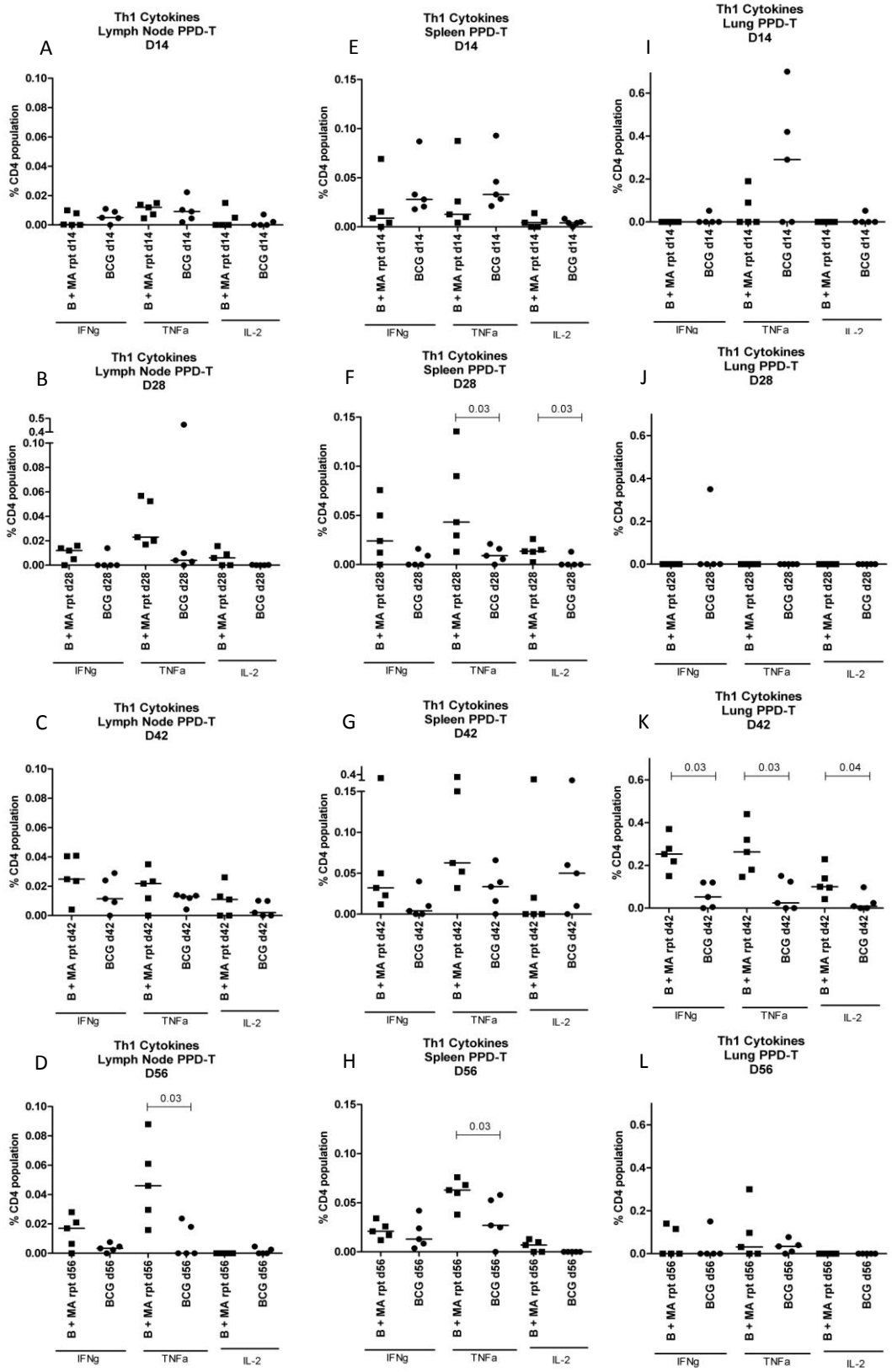


Figure 6-4: Th1 responses in BCG vaccinated and BCG and MA exposed mice. Immune responses were assayed on day 14, 28, 42 and 56 of the experiment. Lymph node (A-D), spleen (E-H)and lung (I-L) cells were stimulated with PPD-T. IFNγ, TNFα and IL-2 production from CD4+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; shown where significance was found.

6.2.4 Polyfuntional Th1 Responses

The Th1 data was analysed for polyfunctional capability. Day 42 data was chosen as representative data as this was the peak time point of response in the majority of organs.

6.2.4.1 BCG only

The Th1 response in lymph nodes was dominated by CD4+ T cells making only TNFα (T+) and cells making TNFα and IL-2 (T+2+) (Figure 6-5 A).Responses in the spleen were mainly produced by T+ CD4+ T cells. T+2+ CD4+ T cells, I+T+ and IFNγ+, TNFα+ and IL-2+ (I+T+2+) CD4+ T cells were also detected (Figure 6-5 B). T+ CD4+ T cells were the dominant cytokine producing cells in the lung. I+T+2+ and I+2+ CD4+ T cells also produced the Th1 response (Figure 6-5 C).

6.2.4.2 BCG + MA

T+ CD4+ T cells were the dominant cytokine producing cell. 2+T+ and I+T+ CD4+ T cells were also detected (Figure 6-5 A). T+ CD4+ T cells were the dominant cytokine producing cells in the spleen. T+2+ CD4+ T cells and I+T+2+ CD4+ T cells were also detected (Figure 6-5 B). T+ CD4+ T cells were the dominant cytokine producing cells in the lungs (Figure 6-5 C). A high number of I+T+2+ CD4+ T cells and a lower number of I+T+ CD4+ T cells was also detected.

A similar pattern of polyfunctionality was seen in BCG only and B + MA mice. The most frequently observed cytokine profile was T+ CD4+ T cells. In the lung the BCG + MA mice had a high frequency of I+T+2+ CD4+ T cells where BCG only mice did not.

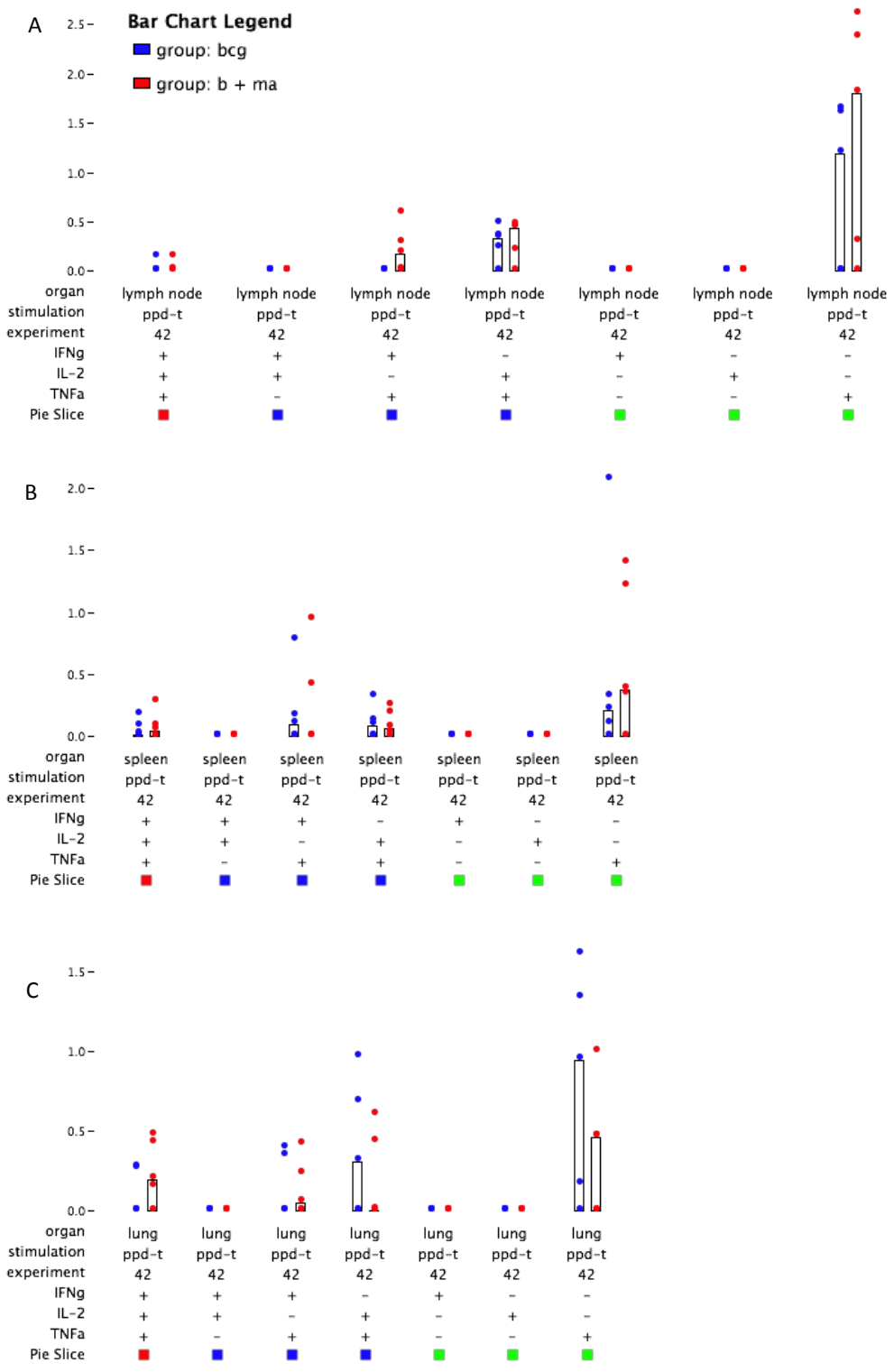


Figure 6-5: Polyfunctional Th1 response of BCG vaccinated and BCG-MA exposed mice on day 42 of the experiment. Secretion of one or more of IFNγ, TNFα and IL-2 from CD4+ T cells stimulated with PPD-T. Data is expressed as the % of CD4+ T cells and is antigen specific; media only control wells are subtracted. Cells from lymph nodes (A), spleen (B) and lungs (C). All mice are shown as a separate dot, n = 5 and the median is displayed.

6.2.5 CD8+ T Cell Cytokine Responses

6.2.5.1 Lymph Node

At day 14, an IFNγ response was detected in BCG only mice but not BCG + MA mice (Figure 6-6 A-D). No CD8 response was detected in either group at day 28. A low level of IFNγ+ CD8+ T cells were detected in both groups at day 42. No response was detected in either group at day 56.

6.2.5.2 Spleen

IFNγ+ and TNFα+ CD8+ T cells were detected in BCG only mice at day 14; no response was detected in B + MA mice (Figure 6-6 E-H). At day 28 high frequencies of IFNγ+ CD8+ T cells were detected in both groups at a similar magnitude. IFNγ+ CD8+ T cells were detected in both groups again at day 42 but at a lower frequency than at day 28. IL-2+ CD8+ T cells were also detected in BCG only mice at this time point. At day 56 the IFNγ response remained similar to day 42, TNFα+ CD4+ T cells were detected in the BCG only group.

6.2.5.3 Lung

TNFα+ CD8+ T cells were detected in the lungs of both groups at day 14 (Figure 6-6 I-L). IFNγ+ CD8+ T cells were detected in the B + MA group at day 28. No CD8 response was detected in the lungs of either group at day 42 or 56.

The greatest CD8 response was detected in the spleen at day 28, the magnitude was similar in both groups. BCG induced a large IFNγ response in the lymph node at day 14 which was not detected in B + MA mice. BCG also induced an IL-2 response in the spleen at day 42, IL-2 from CD8+ T cells was not detected anywhere else. Lung responses were detected in both groups at day 42 but only B + MA mice at day 28.

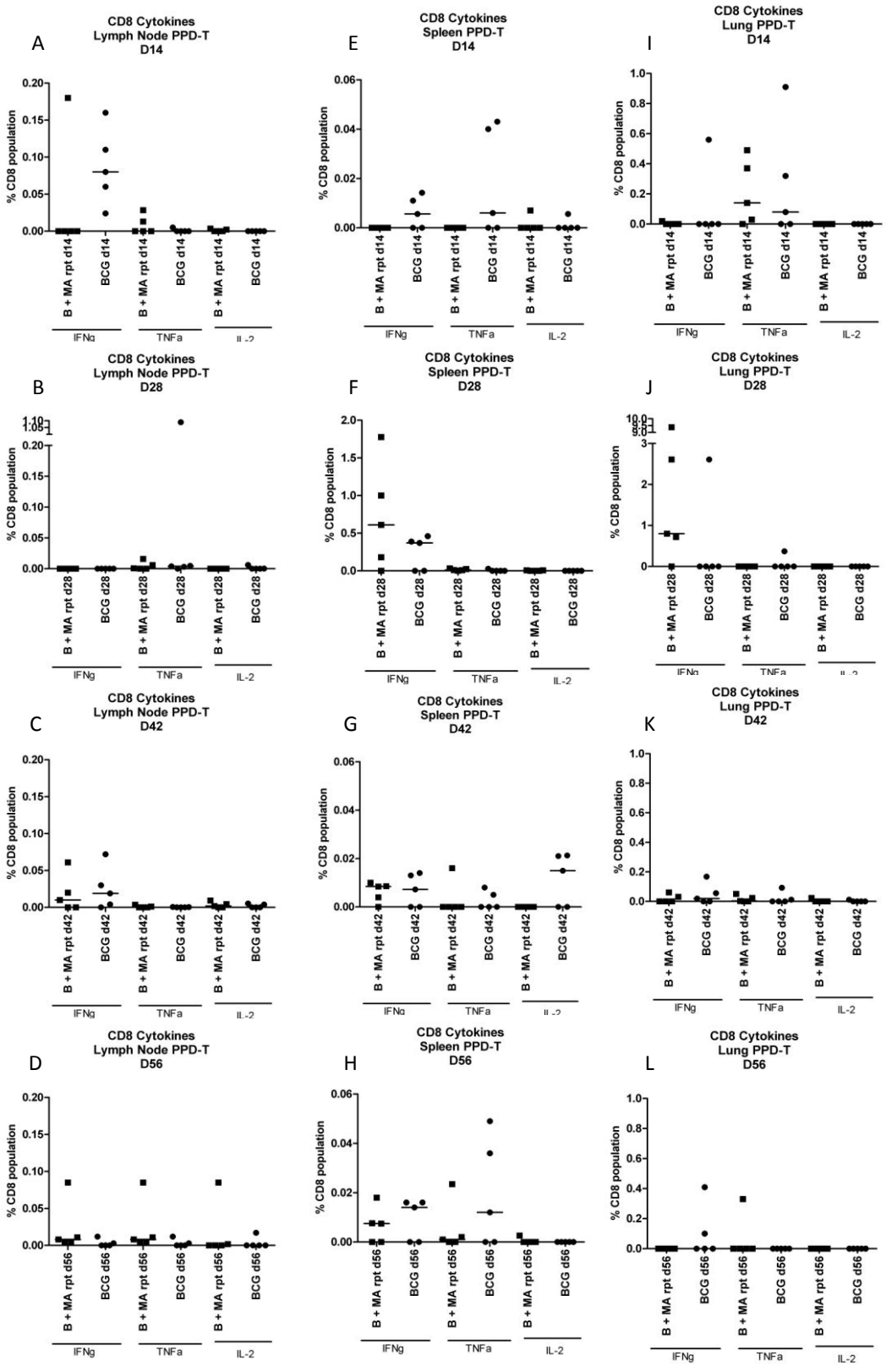


Figure 6-6: CD8+ T cell responses in BCG vaccinated and BCG and MA exposed mice. Immune responses were assayed on day 14, 28, 42 and 56 of the experiment. Lymph node (A-D), Spleen (E-H) and lung (I-L) cells were stimulated with PPD-T. IFNγ, TNFα and IL-2 production from CD8+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD8+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; shown where significance was found.

6.3 Results: BCG compared to MA

6.3.1 CD4+ T Cell Memory Populations

6.3.1.1 Lymph Node

Central Memory (TCM)

At day 14 BCG vaccinated mice and mice receiving MA repeatedly (MA rpt) mice had a frequency of TCM around 3% which was similar to naive mice (Figure 6-7 A). Mice receiving MA once (MA x1) had a slightly lower frequency of TCM at around 2%. In all groups the frequencies of TCM increased to a peak at day 42; BCG 12%, MA rpt 8% and MA x1 6%. Frequencies in all groups dropped at day 56; 10% in the BCG group and around 4% both MA groups. The frequency of TCM was significantly greater in BCG mice compared to both MA groups at day 56.

Effector Memory (TEM)

MA x 1 and MA rpt mice had similar frequencies of TEM as naïve mice at around 4% throughout the experiment (Figure 6-7 D). BCG vaccinated mice had a frequency of 7% at day 14 which dropped at day 28 to the frequency similar to the other groups then increased at day 42 to 8% and was maintained at this level at day 56. The frequency of TEM in BCG mice at day 56 was significantly greater than the frequency in MA x 1 mice.

Terminally Differentiated Effector cells (TEff)

The frequency of TEff measured in BCG vaccinated mice was around 40% and was maintained at this frequency throughout the study (Figure 6-7 G). The MA x 1 group and MA rpt group had a lower frequency of TEff at around 28-30% at day 14. The frequency of TEff in the MA x 1 group increased to a similar level as BCG mice at day 42 whereas in MA rpt mice this frequency increased further to around 45% at day 42 before dropping slightly at day 56.

6.3.1.2 Spleen

Central Memory (TCM)

At day 14 the frequency of TCM in BCG vaccinated mice was recorded as 3% which was similar to naïve mice and was maintained around this frequency throughout the study (Figure 6-7 B). In MA x 1 mice and MA rpt mice the frequency of TCM was around 3% and 5% respectively. Frequencies of TCM in the MA groups increased at day 28 and again at day 42 with a frequency of 7% in MA rpt mice and 6% in MA x 1 mice but decreased at day 56 so that the frequencies of all groups were similar to naïve mice.

Effector Memory (TEM)

Frequencies of TEM in the spleens of BCG mice were recorded at around 10%, the same as naïve mice, throughout the study. At day 14 MA x 1 mice had a similar frequency but this increased at day 28 to around 12% before decreasing at day 42 and again at day 56 to a frequency of around 8% (Figure 6-7 E). The frequency of TEM recorded in MA rpt mice at day 14 was around 12%, this was maintained at day 27 but dropped at day 42 and 56 to a similar level as MA x 1 mice.

Terminally Differentiated Effector cells (TEff)

Frequencies of TEff in BCG vaccinated mice remained similar to the level in naïve mice throughout the study; around 60%. MA x 1 and MA rpt mice maintained a lower frequency of around 50 – 55% throughout the study which was significantly lower than BCG vaccinated mice (Figure 6-7 H).

6.3.1.3 Lung

Central Memory (TCM)

At day 14 frequencies of TCM in the lungs of the MA x 1 and the MA rpt group was 9%; greater than naïve mice (Figure 6-7 C). The frequency recorded in BCG vaccinated mice at this time point was 4%; less than naïve mice. Frequencies in all groups rose at day 28 but dropped at day 42 and again at day 56 to between 4 and 5% in all groups.

Effector Memory (TEM)

TEM frequencies measured in MA rpt and MA x 1 mice were 32 and 28% respectively which were both greater than frequencies in naïve mice (Figure 6-7 F). BCG vaccinated mice had a lower frequency of TEM than naïve mice at 18%. The frequencies in both MA groups dropped at day 28 and day 42 whereas frequencies increased in BCG mice so that at day 42 all groups had a similar frequency of TEM as naïve mice. Frequencies in all 3 groups then dropped below the level of naïve mice at day 56 to between 12 and 17%.

Terminally Differentiated Effector cells (TEff)

At day 14 a TEff frequency of 60% was recorded in BCG vaccinated mice; greater than naïve mice. Both MA groups had a frequency of 40% at day 14; lower than naïve mice (Figure 6-7 I). Frequencies of TEM in all groups increased at day 28 to nearly 80% in the BCG group and around 65% in both MA groups. Frequencies dropped at day 42 but increased slightly again at day 56 to a similar frequency in all groups at around 63%.

Frequencies and the kinetic of memory subsets in mice receiving MA once or repeatedly were similar in all organs. BCG vaccination induced greater frequencies of central memory and effector memory CD4+ T cells in the lymph nodes compared to the two groups receiving MA. Frequencies of memory subsets in the spleen were not as high in magnitude as the lymph nodes or the lungs. BCG vaccinated mice had similar responses to naïve mice in the spleen. Both groups of MA exposed mice had greater frequencies of central memory cells and effector memory CD4+ T cells up to day 56 when frequencies dropped.

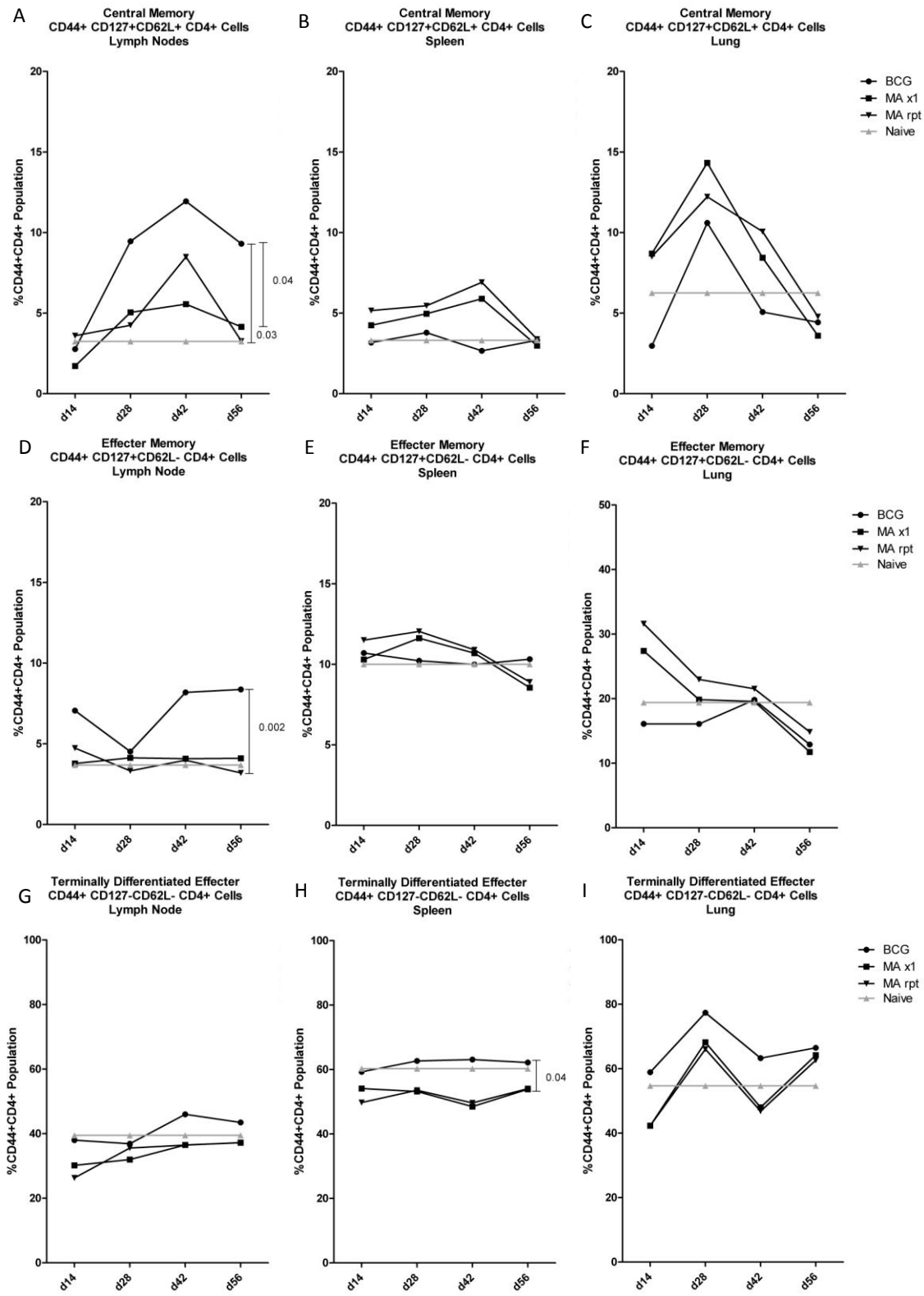


Figure 6-7: CD4+ T cell memory populations in BCG vaccinated mice, mice exposed to MA once and MA repeatedly. Flow cytometry was used to characterize central memory, effecter memory and terminally differentiated effecter populations by measuring expression of CD44, CD127 and CD62L. Cells were isolated from lymph nodes (A, D, G), spleens (B, E, H) and lungs (C, F, I). Results are expressed as a frequency of the CD44+ CD4+ population. Results from naïve animals were used to adjust to the frequency measured at day 14 to correct for natural variation in populations over time. Statistical difference between the groups at day 56 was measured by the Man-Whitney U test; where significance was calculated it is displayed on the graph.

6.3.2 CD8+ T Cell Memory Frequencies

6.3.2.1 Lymph Nodes

Central Memory (TCM)

At day 14 frequencies of TCM in the MA rpt group and the MA x 1 group were around 55% and 48% respectively (Figure 6-8 A). These levels decreased gradually over the course of the experiment to a frequency of around 38%; this frequency was greater than naïve mice. BCG vaccinated mice had the same frequency of TCM as naïve mice at day 14 which was 30%. This increased at day 28 and was maintained around 38% for the rest of the time points so at day 56 all groups had a similar frequency.

Effector Memory (TEM)

BCG vaccinated mice had a similar frequency of TEM as naïve mice throughout the experiment (Figure 6-8 D). At day 14 MA x1 and MA rpt mice had around 4% TEM which was fewer than BCG vaccinated mice but the frequency increased to similar level at day 28 and was maintained for the rest of the time points.

Terminally Differentiated Effectors (TEff)

BCG vaccinated mice had a similar frequency of TEff as naïve mice in the lymph nodes throughout the study (Figure 6-8 G). Both MA groups had fewer TEff at a frequency of 5% at day 14. Over the course of the study this frequency increased gradually so that at day 56 the same level as recorded in BCG vaccinated mice was reached.

6.3.2.2 Spleen

Central Memory (TCM)

Frequencies of TCM in the MA rpt and MA x 1 group were recorded at 38% and 32% respectively at day 14 (Figure 6-8 B). Frequencies rose at day 28 to 42% and 37% respectively then decreased over day 42 and 56 to around 32% and 28%. This was similar to the frequency of BCG vaccinated mice and naïve mice; levels of TCM in BCG vaccinated mice had remained around 28% for the course of the study.

Effector Memory (TEM)

At day 14 frequencies of TEM in the MA x 1 group were greater than naïve mice at 20% (Figure 6-8 E). Frequencies in the BCG vaccinated and MA rpt group were lower than naïve mice at 11 to 14%. Frequencies of TEM in the BCG and MA rpt group increased at day 28 whereas frequencies in the MA x 1 group decreased so that all groups had around 16 to 18%. This was similar at days 42 and 56 and comparable to the frequency in naive mice.

Terminally Differentiated Effector cells (TEff)

All groups had between 17 and 23% TEff throughout the study which was similar to naïve mice (Figure 6-8 H).

6.3.2.3 Lung

Central Memory (TCM)

At day 14 frequencies of TCM in the MA x 1 and MA rpt groups were around 45% which was greater than naïve mice (Figure 6-8 C). Frequencies in the BCG vaccinated group were around 22% at day 14 which was lower than naïve mice. Frequencies in BCG vaccinated mice increased to around 38% at day 28. Frequencies of TCM in all groups decreased on days 42 and day 56 to around 37, 36 and 18% in the MA rpt, MA x 1 and BCG groups respectively. BCG vaccinated mice had fewer TCM than naïve mice.

Effector Memory (TEM)

At day 14 TEM frequencies in the MA x 1 group were around 26%, in the MA rpt group around 23 % which was similar to naïve mice and in the BCG group around 18% (Figure 6-8 F). Frequencies in all groups increased at day 28 before declining to a similar level between 18 and 20% at day 42 and 56.

Terminally Differentiated Effector cells (TEff)

At day 14 TEff frequencies were recorded at around 45% in BCG vaccinated mice and 15% in both MA groups (Figure 6-8 I). Frequencies of TEff in the BCG vaccinated mice dropped at day

28 but rose again at day 42 and 56 to around 60%. Frequencies in both MA groups rose slightly and at day 56 and were recorded at 20 to 25%.

Frequencies of central memory CD8+ T cells were greatest in the lymph nodes of all groups and all groups had a similar frequency at day 56. BCG vaccinated mice had fewer central memory CD8+ T cells in the lungs compared to both MA exposed groups; in negative correlation with central memory cells terminally differentiated effecteor frequencies were greater in the lungs of BCG vaccinated mice compared to MA exposed mice.

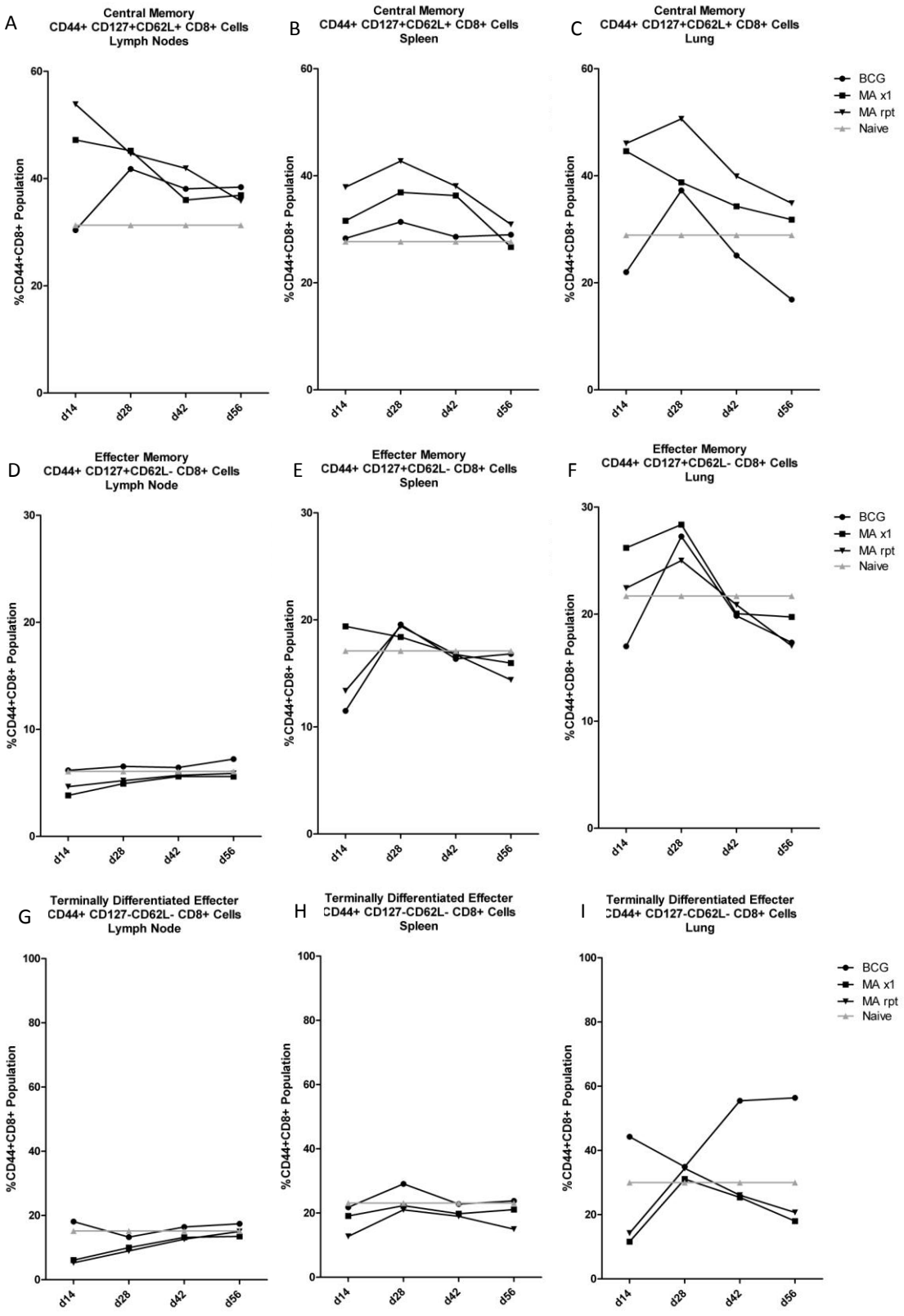


Figure 6-8: CD8+ T cell memory populations in BCG vaccinated mice, mice exposed to MA once and MA repeatedly. Flow cytometry was used to characterise central memory, effecter memory and terminally differentiated effecter populations by measuring expression of CD44, CD127 and CD62L. Cells were isolated from lymph nodes (A, D, G), spleens (B, E, H) and lungs (C, F, I). Results are expressed as a frequency of the CD44+ CD8+ T cell population. Results from naïve animals were used to adjust to the frequency measured at day 14 to correct for natural variation in populations over time. Statistical differences between the groups at day 56 was measured by the Man-Whitney U test, no significant differences were detected.

6.3.3 Th1 Cytokine Responses

6.3.3.1 Lymph Node

A similar frequency of IFNγ+ CD4+ T cells was detected in all groups at day 14 (Figure 6-9 A-D). A population of TNFα+ CD4+ T cells was also detected in the BCG vaccinated group but not the MA groups. At day 28 the only Th1 response was a low frequency of TNFα+ CD4+ T cells in the BCG vaccinated mice. BCG vaccinated and the MA rpt group induced significantly greater IFNγ+ and TNFα+ CD4+ T cell frequencies compared to the MA x 1 group at day 42 although all groups induced a high IL-2 response at this time point. Frequencies of Th1 responses were low in all groups at day 56.

6.3.3.2 Spleen

Th1 responses were detected in the spleens of all groups at all time points (Figure 6-9 E-H). BCG vaccination induced significantly greater frequencies of IFNγ+ and TNFα+ CD4+ T cells at day 14 compared to both MA groups. At day 28 and 42 the frequencies of IFNγ+ and TNFα+ CD4+ T cells in the BCG vaccinated group had decreased and a similar response was detected across the groups. IL-2 responses in BCG vaccinated mice were greater at day 42 compared to other time points. At day 56 BCG induced a significantly greater frequency of IFNγ+CD4+ T cells compared to the MA groups.

6.3.3.3 Lung

Mice given MA repeatedly were the only group to induce a response at all time points; this was a TNFα+ response at all time points other than day 42 when IFNγ was also detected (Figure 6-9 I-L). A similar response was induced by BCG vaccination apart from no cytokine was detected at day 28. A low level IL-2 response at day 42 was induced by MA x 1.

BCG vaccination induced significantly greater Th1 responses in the spleens compared to both MA regimens. BCG and MA repeat induced significantly more Th1 responses in the lymph nodes than MA x 1, this trend was also seen in the lungs.

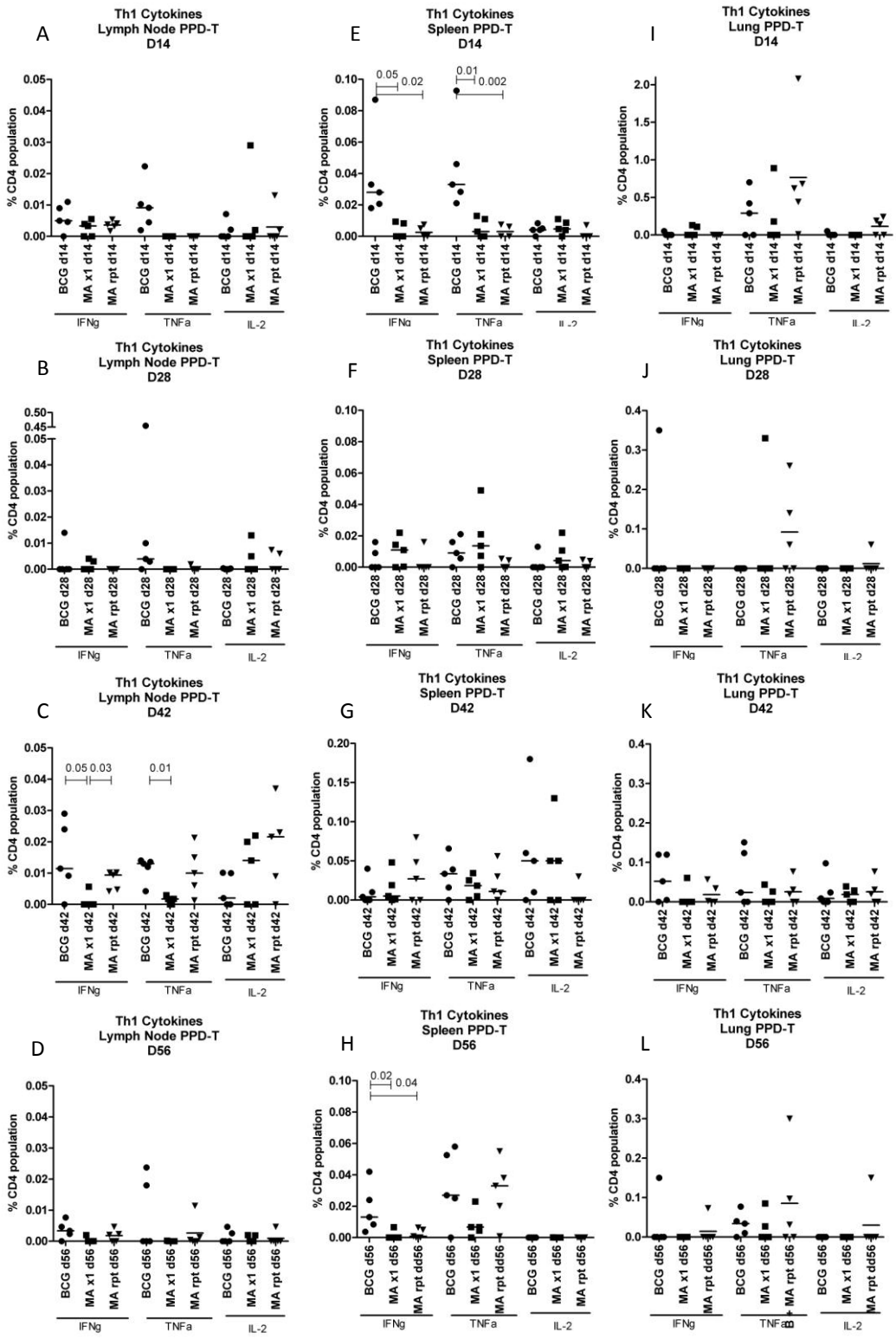


Figure 6-9: Th1 responses in BCG vaccinated mice, mice exposed to MA once and MA repeatedly. Immune responses were assayed on day 14, 28, 42 and 56 of the experiment. Lymph node (A-D), spleen (E-H) and lung (I-L) cells were stimulated with PPD-T. IFNγ, TNFα and IL-2 production from CD4+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD4+ population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; shown where significance was found.

6.3.4 Polyfunctional Th1 Responses

The Th1 data was analysed for polyfunctional capability. Day 42 data was chosen as representative data as this was the peak time point of response in the majority of organs.

6.3.4.1 BCG only

These results were presented in the first section of the results chapter. Briefly; the BCG induced Th1 response was dominated by T+ CD4+ T cells in all 3 organs tested (Figure 6-10). T+2+ cells were detected in lymph nodes and spleens. I+T+2+ CD4+ T cells were detected in spleens and lungs.

6.3.4.2 MA x 1

The Th1 response in the lymph nodes was dominated by T+ CD4+ T cells. I+T+CD4+ T cells were also detected (Figure 6-10). T+2+ CD4+ T cells were the dominant phenotype in the spleen. I+T+2+ CD4+ T cells were also detected. T+ and I+T+CD4+ CD4+ T cells produced the Th1 response in the lung.

6.3.4.3 MA repeat

T+ CD4+ T cells were the dominant phenotype and T+2+CD4+ CD4+ T cells were also detected in the lymph nodes (Figure 6-10). T+ were the dominant phenotype of CD4+ T cells in the spleen. T+2+, I+T+ and I+T+2+ CD4+ T cells were also detected. In the lung T+ were the dominant phenotype of CD4+ T cells. T+2+ CD4+ T cells were also detected.

T+ CD4+ T cells were the dominant cytokine producing cells detected in all groups. I+T+2+ CD4+ T cells were detected in the spleens of all groups and in the lymph nodes of MA x1 and BCG vaccinated mice. MA rpt induced T+2+ CD4+ T cells whereas MA x 1 and BCG induced I+T+ CD4+ T cells in the lymph nodes and lungs.

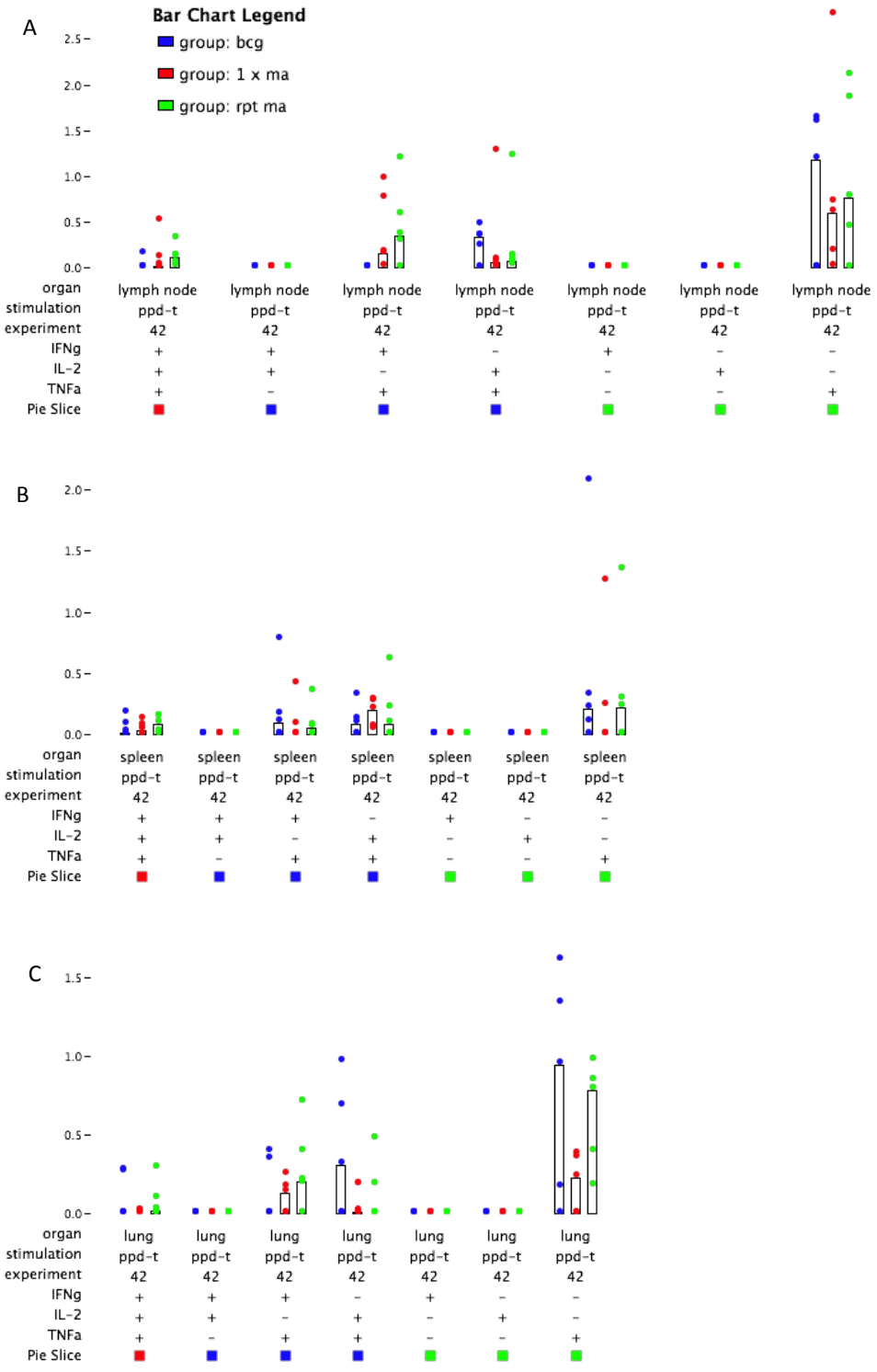


Figure 6-10: Polyfunctional Th1 response of BCG vaccinated mice, mice exposed to MA once and MA repeatedly on day 42 of the experiment. Secretion of one or more of IFNγ, TNFα and IL-2 from CD4+ T cells stimulated with PPD-T. Data is expressed as the % of CD4+ T cells and is antigen specific; media only control wells are subtracted. Cells from lymph node (A), spleen (B) and lungs (C). All mice are shown as a separate dot, n = 5 and the median is displayed.

6.3.5 CD8+ T Cell Cytokine Responses

6.3.5.1 Lymph Node

All 3 groups had a detectable IFNγ+ CD8+ T cell response at day 14 and at day 42 (Figure 6-11 A-D). Frequencies across the groups were similar at day 14 but at day 42 the frequency in the MA rpt group was greater.

6.3.5.2 Spleen

The only CD8+ T cell responses detected in the spleen was an IFNγ+ response in BCG vaccinated mice at day 28 (Figure 6-11 E-H).

6.3.5.3 Lungs

MA rpt induced IFNγ+ and TNFα+ CD8+ T cells at day 14 and 56. High frequencies of IFNγ+ CD8+ T cells were detected in the MA x 1 group at day 28 (Figure 6-11 I-L). Low levels of IFNγ+ and TNFα+ CD8+ T cells were detected in all groups at day 42.

All 3 groups induced a CD8+ T cell response. The greatest magnitude of response in the BCG vaccinated group was in the lymph nodes whereas in the two MA exposed groups it was in the lungs.

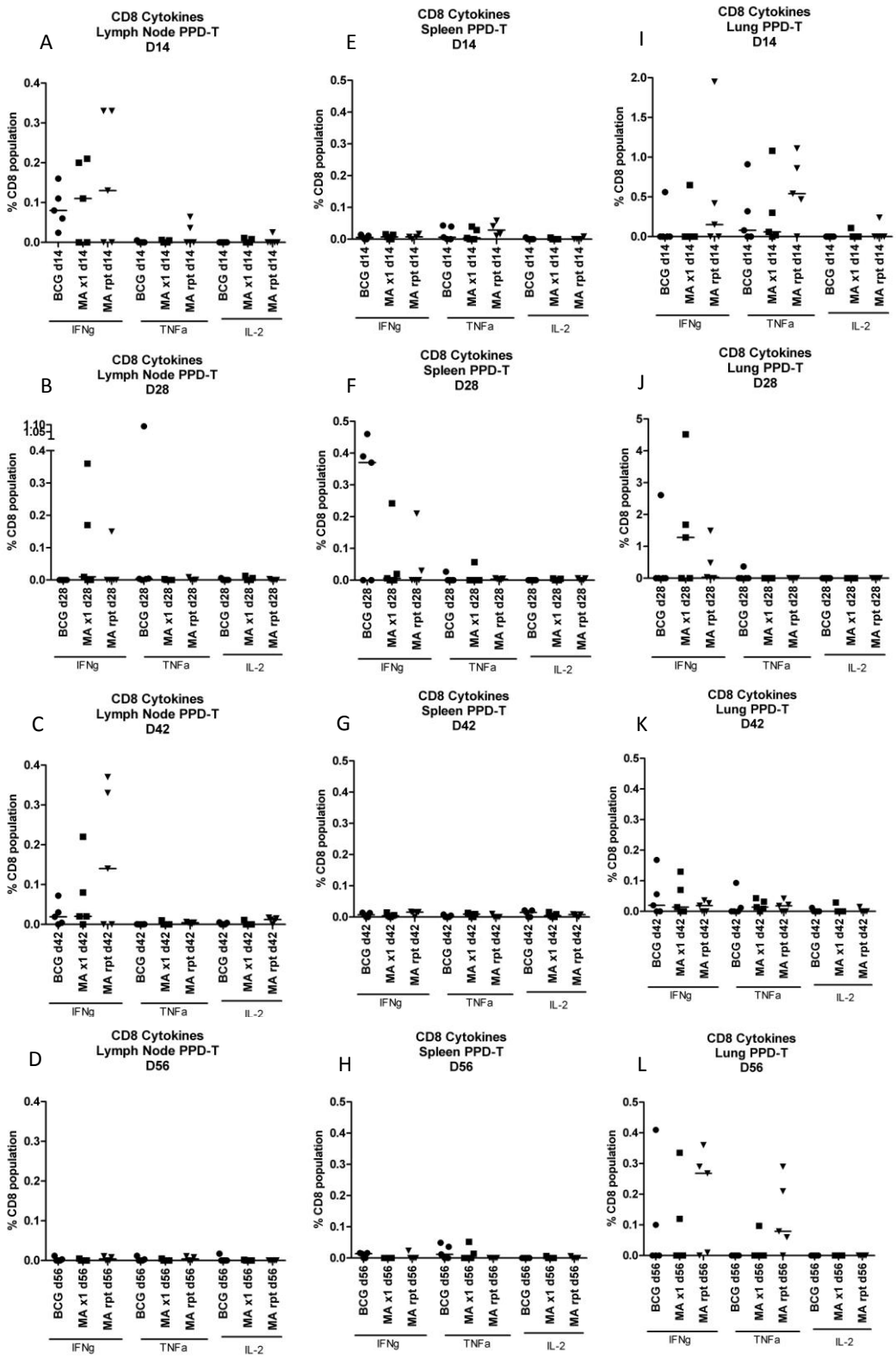


Figure 6-11: CD8+ T cell responses in BCG vaccinated mice, mice exposed to MA once and MA repeatedly. Immune responses were assayed on day 14, 28, 42 and 56 of the experiment. Lymph node (A-D), Spleen (E-H) and lung (I-L) cells were stimulated with PPD-T. IFNγ, TNFα and IL-2 production from CD8+ cells was assayed by flow cytometry. Results are expressed as a percentage of the CD8+ T cell population and are stimulation specific; media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; none was calculated.

6.4 Discussion

The hypothesis tested in this experiment was that exposure to NTM directly after BCG vaccination would interfere with the generation of protective memory T cell subsets. To model the effect in areas of high NTM exposure, MA was administered continuously directly after BCG vaccination. This differed to the previous models where MA exposure was delayed to 3 months or 6 weeks after BCG vaccination when sufficient memory to protect against *M. tb* challenge has already been generated.

Dead MA strain 724 was used because the immunogenicity of this strain in sensitisations had been well characterised in previous experiments in this study. Mice were administered $1x10^6$ CFU of dead MA every 7 days. A previous experiment had shown the immune response generated against dead MA lasted at least 1 month so dosing every 7 days would ensure constant activation of the immune system. Dead MA was used to avoid generating a high burden MA infection which would not be representative of human exposure.

BCG vaccination induced both TCM and TEM CD4+ T cell populations. The greatest frequency of both memory subsets at the final time point assayed was found in the inguinal lymph nodes which drain the site of vaccination. There was a higher percentage of TCM compared to TEM. In the spleen there was no change in the frequency of memory response; frequencies of all subsets were the same as in naïve mice. In the lung there was an initial increase in both memory subsets but this decreased with time, which was associated with an increase in TEff cells in this organ.

These data contradict what was reported by Henao- Tamayo et al, who saw a greater number of TEM compared to TCM[194]. They also reported that at day 50 the number of memory cells recorded in lungs and spleens of BCG vaccinated mice was greater compared to naïve mice.

This discrepancy with my data may be explained by the fact they did not distinguish TEff cells from TEM cells. This would explain why greater frequencies of TEM, which were actually both TEM and TEff, were seen in the lungs and spleens of BCG vaccinated compared to naïve mice in their experiment. The finding that there was no alteration in memory subsets or TEff in the spleen of the current experiment may reflect an absence of BCG dissemination to this organ. Disseminated BCG infection may have induced the memory responses in the spleen in Henao-Tamayo et al's report.

Henao- Tamayo et al also report that frequencies of TCM and TEM were greater in the lungs compared to the lymphoid organs; spleen and lymph nodes. This seems contradictory to the role of TCM and their location to lymphoid organs by expression of CD62L. Greater frequencies of TCM were detected in the lymph nodes compared to lungs and spleens in the current study which is what was expected.

The expression of the data as a total number of cells in Henao- Tamayo et al's report may also explain the discrepancies between the studies. I expressed memory populations as a percentage of the CD44+ CD4+ or CD8+ T cell population. It seems relevant to express the data as a percentage of CD44+ T cells as all of the memory subsets were gated on the expression of this molecule so their frequency is relative to each other.

TEM were the predominant memory phenotype defined in Kaveh et als report[74]. They defined memory after gating for cytokine production, more specifically polyfunctional Th1. TCM have a limited effector function and the ability to produce cytokine increases with differentiation so it is not surprising that the majority of memory cells they defined were TEM[190]. To define total levels of TCM gating for memory markers would need to be done before gating on cytokine production.

CD8+ T cell memory populations followed a similar pattern as CD4+ memory populations. This similarity between the 2 arms of the cellular response was also observed in Henao-Tamayo et al's report.

The exposure of BCG vaccinated mice to repeated doses of dead MA had no effect on the generation of memory T cell subsets. Both the BCG only and the BCG + MA groups had similar peak magnitudes of responses in all the organs and there was a similar response kinetic over the 56 day time course.

The effect of MA exposure after BCG vaccination on cytokine production was also evaluated. The BCG + MA group had greater frequencies of cytokine producing Th1 cells compared to BCG only mice. This was also seen in the other model of exposure to dead MA after BCG which was conducted in this study (Chapter 4). The memory phenotype of these cytokine producing cells was predominantly that of terminally differentiated effector cells (data not shown). The increased frequency of cytokine producing Th1 cells in the B + MA group was not correlated to an increase in any of the memory phenotypes.

In the study by Kaveh et al, polyfunctional Th1 cells induced by BCG were associated to TEM cells[74]. I found the majority of cells producing the Th1 response in the BCG group were TNFα single cytokine producers. This was in accordance with Kaveh et al, however I did not detect the triple cytokine producers in the BCG only group as they had done. I did detect triple cytokine producers in the lungs of the B + MA group but they did not correlate with an increased frequency of TEM compared to the BCG only group.

As previously discussed in this report, BCG induces a greater level of protection against *M. tb* than NTM do. The comparison of the memory populations induced by BCG and those induced

by MA allows an opportunity to consider what features of the BCG induced response may correlate with increased protection against *M. tb* challenge.

BCG vaccination induced significantly more TCM and TEM CD4+ T cells in the lymph nodes than either MA given once or MA given repeatedly. BCG vaccinated mice had significantly greater frequencies of TEff in the spleen compared to both MA groups, however, the frequency was at the same level as naïve mice indicating MA x 1 and MA rpt mice had reduced frequencies of TEff. The frequency of memory subsets in the spleen were greater in the MA groups which may explain the lower levels of TEff, however, the durability of this memory is questionable as it was not detected at day 56.

The location of memory responses may indicate a preferential site of priming; in BCG vaccinated mice it is in the draining lymph nodes where the bacteria are most probably residing and replicating[168]. In the MA x 1 and MA rpt mice, priming of a memory responses is associated with the spleen, a more systemic response, as the dead bacteria do not persist in one location and are quickly cleared.

BCG induced a significantly greater Th1 cytokine response in the spleen than either MA group. Repeated administration of MA induced comparable Th1 responses in the LN as BCG, this was of significantly greater magnitude than induced by one dose of MA.

There was not much difference in the ability of the mycobacteria to induce CD8+ T cell immune responses in the lymph nodes and the spleen. Both MA groups had a lower TEff response in the lungs than the BCG vaccinated group. This was associated with higher TCM and TEM responses in both MA x 1 and MA rpt mice, however this difference was not significant.

Antigen persistence is linked to the failure of BCG vaccination to induce an effective memory response[191]. The administration of a single bolus of antigen did not increase the priming of memory T cells in the MA x 1 mice. In fact these mice had significantly reduced cytokine production compared to BCG vaccinated mice and mice repeatedly exposed to MA. This implies a certain level of sustained antigen exposure is required to generate an effective memory response[21]. The magnitude of the initial response against an antigen dictates the number of memory CTL which are generated in the contraction phase[21]. The dose of antigen given in one dose of MA was not as high as would be produced by BCG vaccination and was not immunogenic enough to stimulate a high level of antigen specific T cells. Treatment of BCG with antibiotic therapy at a range of time points is possibly the best way to test whether sustained antigen exposure from BCG is the cause of inferior memory responses.

In summary repeated exposure to dead MA directly after BCG vaccination did not affect the generation of memory T cell responses. Comparison of memory responses induced by BCG with those induced by MA showed BCG was superior at inducing central and effector memory CD4+ T cell populations and the production of Th1 cytokines. This implies greater frequencies of memory T cells as well as increased production of cytokine are protective responses primed by BCG against *M. tb* as has been speculated[191].

7 Investigating Interference between MVA85A and Aluminium-Containing Vaccines

7.1 Introduction

The prevalence of TB in young children and adolescents means that infants are an important target population for a new TB vaccine. Each country has an Expanded Programme on Immunisation (EPI) schedule that instructs which vaccines should be administered to the population and the times at which they should be given[197]. A novel vaccine would ideally be incorporated into the EPI schedule to ensure maximum coverage of the population.

A two stage trial was conducted to assess safety and immunogenicity when MVA85A is co-administered with the EPI schedule vaccines in The Gambia[198]. The current EPI schedule for the Gambia is displayed in Figure 7-1. At the infant's 3 month EPI vaccination, volunteers were enrolled and randomised in to 3 groups to receive at their 4 month appointment either EPI alone, EPI and MVA85A simultaneously or MVA85A followed by EPI a week later. MVA85A was administered intradermally in the arm and the EPI vaccines were administered intramuscularly in the thigh. In this trial, Stage 1 assessed a low (2.5×10^7 plaque forming units (PFU)) and standard (5×10^7PFU) dose of MVA85A and found the standard dose gave significantly greater immune responses with a comparable safety profile compared to the low dose. Stage 2 continued with the standard dose of MVA85A.

Vaccine	Birth	1m	2m	3m	4m	6m	9m	1yr+
BCG	✓							
DTwP								✓
DTwP-Hib-HepB			✓	✓	✓			
Hep B	✓							
Measles							✓	
OPV	✓		✓	✓	✓		✓	✓
Pneum-conj			✓	✓	✓			
Tetanus-toxoid	✓	✓				✓		✓

Figure 7-1: Table showing the current vaccine schedule for infants in the Gambia. Adapted from information supplied by the WHO on Immunisation profiles and current as of July 2012[199]. DTwP: Diptheria and tetanus toxoid with whole cell pertussis, Hib: *Haemophilus influenzae* b, Hep B: Hepatitis B surface antigen, OPV: oral polio vaccine, Pneum-conj: pneumococcal conjugate vaccine.

At the time this trial was conducted, the EPI schedule at 4 months consisted of DTwP-Hib, Hep B and oral polio vaccine; pneumococcal conjugate vaccine was a new addition to the schedule after the trial had been completed. Specific antibody titres against *Haemophilus influenzae* b polysaccharide, diphtheria toxoid, tetanus toxoid, hepatitis B surface antigen, pertussis toxoid and pertussis filamentous haemaglutinin were not reduced by the administration of MVA85A at the same time as EPI vaccines.

Conversely the IFNγ response to Ag 85A was significantly reduced in infants who received MVA85A at the time of EPI vaccination compared to those who received MVA85A a week after EPI vaccination (Figure 7-2). This was recorded at 1, 4 and 20 weeks after vaccination. Although the responses to Ag 85A were lower in the co-administered group compared to the

group receiving MVA85A a week later they were still significantly greater than responses pre-vaccination.

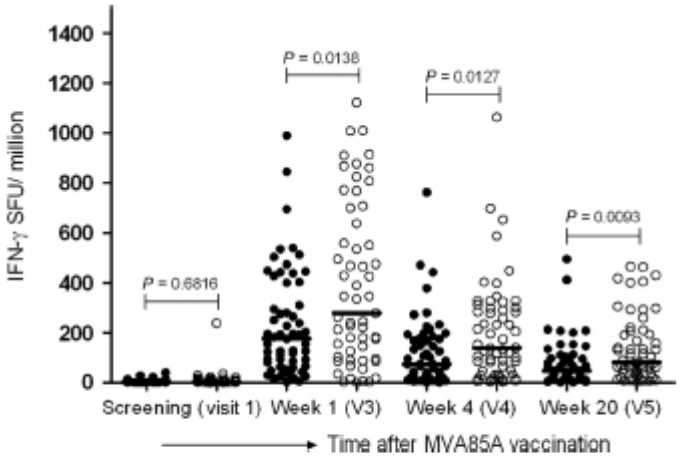


Fig. 2. Immunogenicity of MVA85A. Immunogenicity was measured by ex vivo IFN-γ ELISPOT assay to a single pool of antigen 85A peptides. The figure compares responses in group 2 (EPI + MVA85A, filled circles) and group 3 (MVA85A alone, open circles) at baseline and 1, 4, and 20 weeks after vaccination. The horizontal lines indicate median values.

Figure 7-2: Figure taken from Ota et al showing the IFNγ response to Ag 85A measured by ELISpot assay[198].

The most likely explanation for this reduction in Th1 immunogenicity in the co-administered group is that the aluminium salt-containing adjuvants in the EPI vaccines induced a Th2 skewed response. A Th2 skewed environment may inhibit the generation of Th1 responses which are known to be the predominant cell type induced by MVA85A[136].

Vaccines containing aluminium salts induce high levels of antibodies that serve to protect against infection in vaccinated individuals. The mechanism by which aluminium salts activate the immune response is still not known and is intensively researched. How aluminium salts induce Th2 skewed immunity is also not known. Recent reports have shown that aluminium salt containing vaccines induce an influx of eosinophils to the site of vaccination[136]. These cells are potent IL-4 producers and IL-4 is known to induce Th2 differentiation[200]. IL-4 also prevents the production of IL-12 which is required for the differentiation and maintenance of Th1 responses[201]. IL-4 and IL-13 which are produced by Th2 cells are connected to induction of

alternatively activated macrophages which are less pro-inflammatory than the classically activated macrophages associated with Th1 responses[202].

7.1.1 Hypothesis

The hypothesis was that aluminium salts induce a Th2 skewed immune response that would reduce Th1 responses induced by MVA85A leading to reduced levels of IFNγ being produced in response to Ag85A.

Mice were used as a model and administered alum intramuscularly (IM) and MVA85A at a separate site either intradermally (ID) or intranasally (IN). The effect of BCG priming and subsequent MVA85A boosting was also tested, as was the exposure of mice to EPI vaccines in the same schedule received by the infants in the Gambian trial. The adaptive cellular responses induced in these models were assayed.

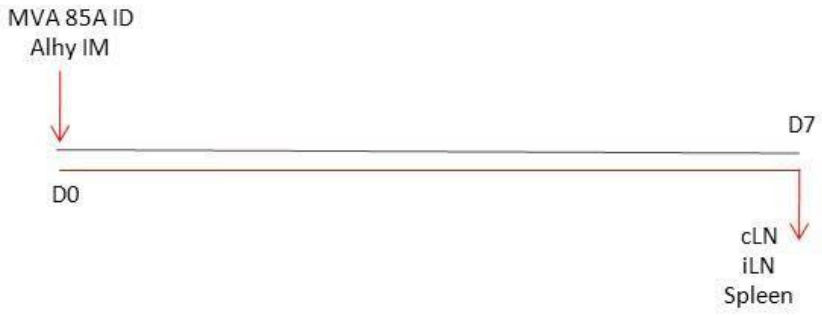
7.1.2 Experiment plan

In all experiments mice received 1 x 10^7 PFU of MVA85A ID or IN. In BCG prime experiments 1 x 10^5 CFU of BCG was given ID. Where alum adjuvant was used 85µg of Al^{3+} was administered per mouse IM, mice in the control group received a PBS placebo at the same volume as was administered to the alum group. In the EPI models 100µl of Hepatitis B surface antigen vaccine Engerix B® (GSK) and/or 100µl of DTaP-Hib-IPV vaccine Pedacil® (Sanofi Pasteur MSD) was administered to each mouse. These doses would have administered 50µg of $Al(OH)_3$ with Engerix B® vaccine and 300µg of $AlPO_4$ with Pediacil®. Dosing schedules for each experiment are described in the results section.

Cellular responses induced by MVA85A were assayed 1 week after MVA85A administration by stimulating cells from specific organs with Ag 85A and analysing cytokine production or cell surface maker expression by flow cytometry.

7.2 Results

7.2.1 MVA85A Intradermally and Alhydrogel Intramuscularly



7.2.1.1 Th1 Responses

Cervical lymph nodes (cLN)

In the cervical lymph nodes (cLN), which drain the site of MVA85A vaccination, MVA85A induced median frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells of between 0.02% and 0.04% (Figure 7-3 A). In the group which received Alhydrogel IM, median frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells were less than 0.02%.

Spleen

In the spleen MVA85A induced around 0.035% IFNγ+ CD4+ T cells and 0.002% TNFα+ and IL-2+ CD4+ T cells (Figure 7-3 B). Mice in the Alhydrogel group induced around 0.04% IFNγ+ CD4+ T cells and 0.002% TNFα+ and IL-2+ CD4+ T cells.

Inguinal lymph nodes (iLN)

In the inguinal lymph nodes (iLN), which drain the site of Alhydrogel administration, MVA85A did not induce any detectable levels of Th1 cytokines (Figure 7-3 C). A low frequency of IFNγ+ CD4+ T cells was detected in the Alhydrogel group.

Alhydrogel reduced the frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells in the cLN but did not reduce responses in the spleen or iLN; IFNγ responses were slightly increased in the Alhydrogel group in these 2 organs but the difference was not significant.

7.2.1.2 CD8+ T Cell Responses

cLN

In the cLN MVA85A induced median frequencies of IFNγ+ and TNFα+ CD8+ T cells of between 0.01 and 0.02% (Figure 7-3 D). The frequency of IFNγ+ CD8+ T cells in the Alhydrogel group was also around 0.01% but no TNFα+ CD8+ T cells were detected. No IL-2 was detected in either group.

Spleen

In the spleen a very low frequency of IFNγ+ CD8+ T cells, no TNFα+ CD8+ T cells and around 0.03% IL-2+ CD8+ T cells were induced by MVA85A (Figure 7-3 E). In the Alhydrogel group around 0.03% IFNγ+ CD8+ T cells and 0.01% of TNFα+ and IL-2+ CD8+ T cells were detected.

iLN

MVA85A induced 0.015% IFNγ+ CD8+ T cells (Figure 7-3 F). No TNFα or IL-2 was detected. In the Alhydrogel group around 0.05% of CD8+ T cells were IFNγ+, around 0.04% were TNFα+ and 0.02% were IL-2+ CD8+ T cells.

Administration of Alhydrogel reduced the TNFα response in the cLN but did not affect IFNγ production. Alhydrogel increased the frequency of CD8+ T cells in the spleen and iLN that produced cytokine in response to Ag85A; most dramatically in the iLN where frequencies of IFNγ+ CD8+ T cells were significantly greater than the group receiving MVA85A alone. Alhydrogel also increased the number of cytokines produced by CD8+ T cells in the spleen and iLN.

7.2.1.3 Polyfunctional Th1 Reponses

The ability of Th1 cells to produce a combination of IFNγ, TNFα and IL-2 was investigated (Figure 7-3 G). There was no difference in the polyfunctional capability of Th1 in the spleen and iLN between the 2 groups so this data is not shown. In the cLN of MVA85A-only vaccinated mice the greatest frequencies of cytokine producing cells were single IFNγ+ CD4+ T cells and TNFα+ IL-2+ CD4+ T cells. There were also high frequencies of IFNγ+ TNFα+ IL-2+ and IL-2+ CD4+ T cells. In the group receiving Alhydrogel the dominant cytokine producing cells were TNFα+ or IL-2+ single cytokine producers. No single IFNγ+ nor TNFα+ IL-2+ CD4+ T cells were detected in this group, and only low levels of IFNγ+ TNFα+ IL-2+ CD4+ T cells were detected.

Administration of Alhydrogel reduced the polyfunctional capability of Th1 cells in the cLN. It did not affect the polyfunctional profile of Th1 in the spleen or iLN.

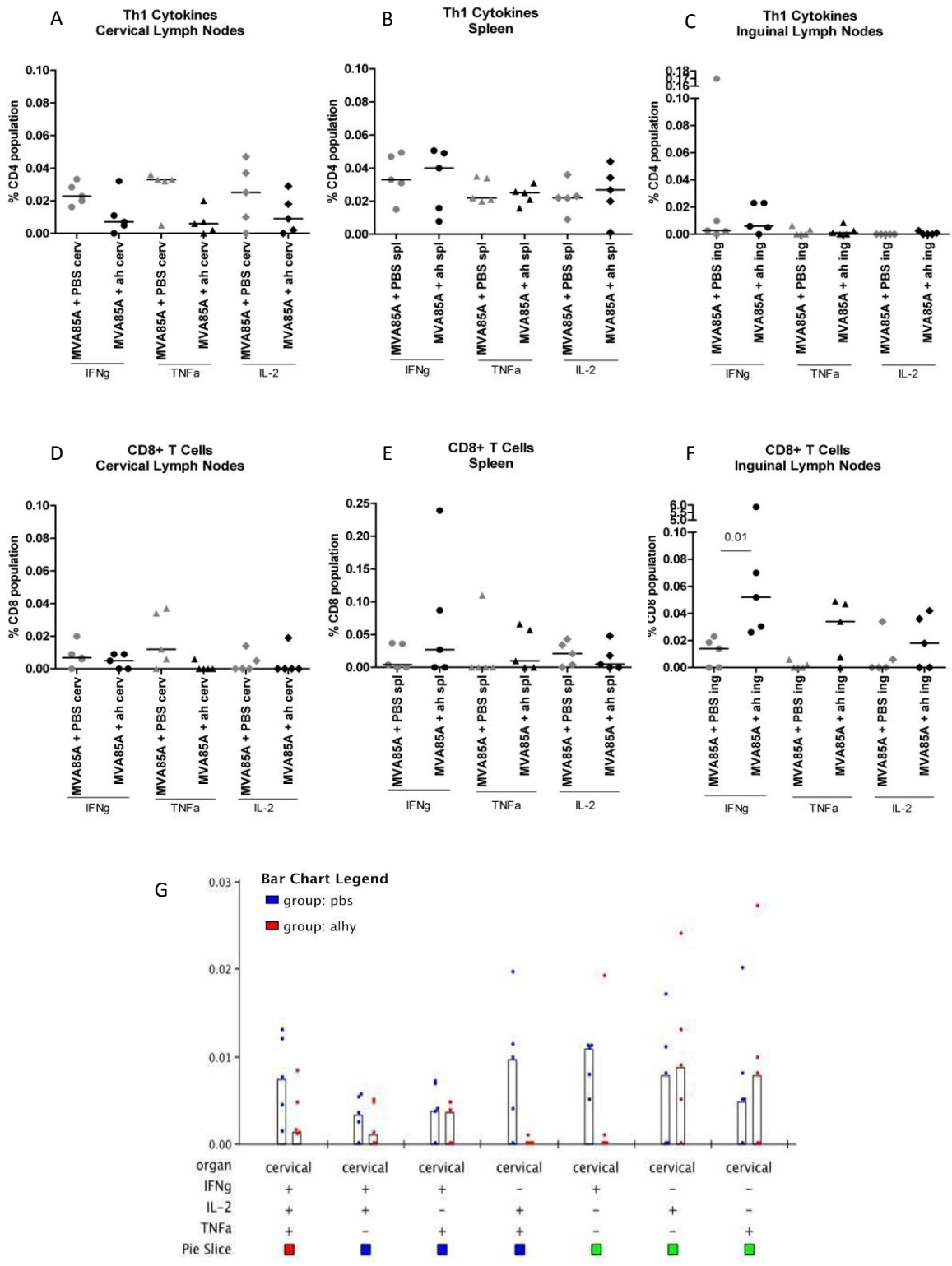


Figure 7-3: Cellular immune responses in mice vaccinated with MVA85A ID with (MVA85A + ah) or without (MVA85A + PBS) the administration of Alhydrogel IM. A-C are Th1 responses in the cLN, spleen and iLN, D-F are the CD8+ T cell responses in the cLN, spleen and iLN, G is the polyfunctional cytokine profile of Th1 cells in the cLN. Organs were taken 7 days after MVA85A and stimulated with Ag85A before staining and analysis using flow cytometry. Cytokine responses are Ag85A specific, the media only control well values are subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was applied to test for statistical significance and the value is displayed where significance was achieved.

7.2.1.4 IL-4+ CD4+ T Cells

MVA85A induced Ag 85A specific IL-4+ CD4+ T cells; frequencies in the cLN and iLN were around 0.002% (Figure 7-4 A). Alhydrogel administration induced around 0.01% IL-4+ CD4+ T cells in the cLN and 0.002% in the iLN. No IL-4 was detected in the spleens of either group.

Alhydrogel increased the frequency of IL-4+ CD4+ T cells in the cLN.

7.2.1.5 IL-17+ CD4+ T Cells

MVA85A induced around 0.002% IL-17+ T cells in the cLN (Figure 7-4 B). 0.002% IL-17+ CD4+ T cells were detected in the cLN of the Alhydrogel group as well. No IL-17 was detected in the iLN or spleens of either group.

Alhydrogel had no effect on IL-17 production from CD4+ T cells.

7.2.1.6 Regulatory T Cells

The frequencies of Tregs in the MVA85A-only mice were around 7% in the cLN, 5% in the iLN and 5% in the spleen (Figure 7-4 C). The frequencies in the Alhydrogel group were around 8% in the cLN, 6-7% in the iLN and 5% in the spleen.

Treg frequencies in the cLN and iLN of the Alhydrogel group were slightly greater than in the MVA85A-alone group.

7.2.1.7 IL-10+ CD4+ T Cells

MVA85A did not induce production of Ag 85A specific IL-10 from CD4+ T cells (Figure 7-4 D). Alhydrogel administration induced 0.002% IL-10+ CD4+ T cells in the cLN but none in the iLN or the spleen. Alhydrogel induced IL-10 production in the cLN.

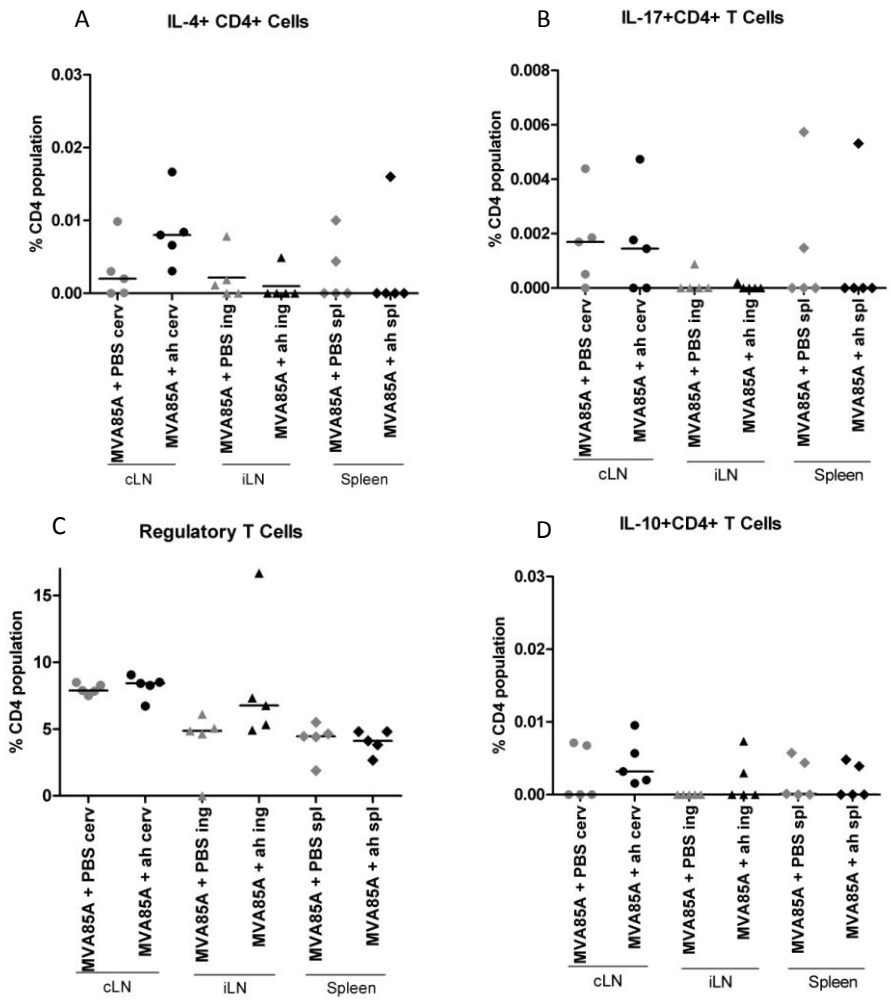


Figure 7-4: Cellular immune responses in mice vaccinated with MVA85A ID with (MVA85A + ah) or without (MVA85A + PBS) the administration of Alhydrogel IM. A is the IL-4+ CD4+ T cells response, B is the IL-17+ CD4+ T cell response, C is the frequency of Tregs and D is the IL-10+ CD4+ T cell response. cLN, iLN and spleens were taken 7 days after MVA85A and stimulated with Ag85A before staining and analysis using flow cytometry. Cytokine responses are Ag85A specific, the media only control well values are subtracted. Tregs were defined as CD25+FoxP3+CD39+ CD4+ T cells and are expressed as a frequency of CD4+ T cells, results do not have media well values subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was applied to test for statistical significance, none of the comparisons achieved this.

7.2.1.8 Alhydrogel Compared with Adjuphos

The effect of two aluminium salts on the immune responses induced by MVA85A was compared; Alhydrogel $Al(OH)_3$ and Adjuphos $AlPO_4$. The effect of the 2 adjuvants on Th1, Th2 and CD8+ T cells is shown below as these were the cell subsets that were of most interest in the effect of aluminium salt adjuvants.

Th1 Responses

Frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells in the cLN were all reduced by Alhydrogel (Figure 7-5 A). IFNγ+ and TNFα+ CD4+ T cell frequencies were reduced by Adjuphos but not to the same extent as with Alhydrogel. IL-2 production was not affected by Adjuphos.

Frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells in the spleen were slightly increased by administration of Alhydrogel (Figure 7-5 B). This was also seen with Adjuphos with a slightly more enhanced effect.

IL-4+ CD4+ T cells

Alhydrogel increased the frequency of IL-4+ CD4+ T cells in the cLN. Adjuphos also induced this effect (Figure 7-5 C).

CD8+ T cells

Alhydrogel significantly increased the frequency of IFNγ+ CD8+ T cells in the iLN and induced production of TNFα and IL-2 from CD8+ T cells (Figure 7-5 D). Adjuphos induced TNFα and IL-2 production from CD8+ T cells but had no effect of IFNγ.

Alhydrogel was selected for use in subsequent experiments.

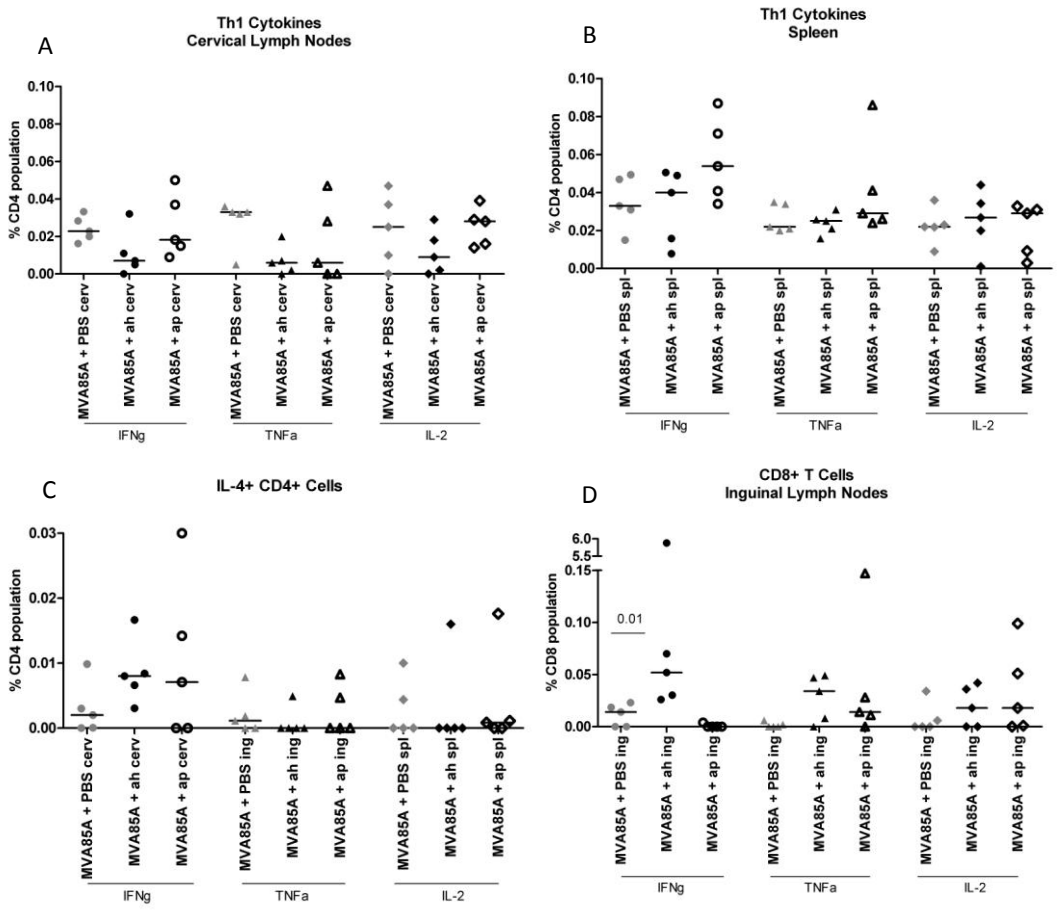
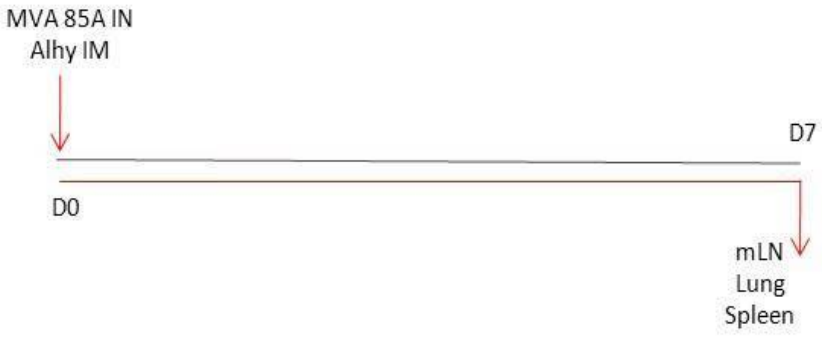


Figure 7-5: Cellular immune responses in mice vaccinated with MVA85A ID with the administration of Alhydrogel (MVA85A + ah), with the administration of Adjuphos (MVA85A + ap) or with PBS (MVA85A + PBS) IM. A is the Th1 response in the cLN, B is the Th1 response in the spleen, C is the Il-4+ CD4+ T cell response in all organs tested and D is the CD8+ T cell response in the iLN. Organs were taken 7 days after MVA85A and stimulated with Ag85A before staining and analysis using flow cytometry. Cytokine responses are Ag85A specific; the media only control well values are subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; no significant differences were achieved.

7.2.2 MVA85A Intranasally and Alhydrogel Intramuscularly



7.2.2.1 Th1 Responses

Mediastinal lymph nodes (mLN)

In the mediastinal lymph nodes (mLN), which is where IN vaccination primes responses, MVA85A induced frequencies of IFNγ+ and TNFα+CD4+ T cells of around 0.09% and IL-2+ CD4+ T cells of around 0.06% (Figure 7-6 A). Frequencies in the mLN of mice receiving Alhydrogel were IFNγ+ CD4+ T cells of around 0.05%, TNFα+CD4+ T cells of around 0.04% and IL-2+ CD4+ T cells of around 0.002%.

Lungs

In the lungs MVA85A induced IFNγ+ CD4+ T cells at 1%, TNFα+CD4+ T cells at 0.5% and IL-2+ CD4+ T cells at 0.4% (Figure 7-6 B). Mice receiving Alhydrogel induced IFNγ+ CD4+ T cells at a level of 1.3% and TNFα+ and IL-2+ CD4+ T cells at a level of 0.4%

Spleen

In the spleen MVA85A induced IFNγ+ CD4+ T cells at 0.025% and TNFα+CD4+ T cells at 0.02% (Figure 7-6 C). No IL-2 was detected. Mice receiving Alhydrogel induced IFNγ+ CD4+ T cells at 0.035%, TNFα+CD4+ T cells at 0.06% and IL-2+ CD4+ T cells at 0.02%.

Alhydrogel reduced the frequencies of IFNγ+, TNFα+ and IL-2+ CD4+ T cells in the mLN but increased the frequency of CD4+ T cells producing these cytokines in the spleen. Alhydrogel also increased the frequencies of IFNγ+ CD4+ T cells in the lungs.

7.2.2.2 CD8+ T Cell Responses

mLN

In the mLN MVA85A induced frequencies of IFNγ+ CD8+ T cells of around 0.09%, TNFα+ CD8+ T cells of around 0.06% and IL-2+ CD8+ T cells of around 0.03% (Figure 7-6 D). Mice administered Alhydrogel induced IFNγ+ CD8+ T cell frequencies of around 0.1%, TNFα+ CD8+ T cells at 0.06% and IL-2+ CD8+ T cells at 0.01%.

Lungs

In the lungs MVA85A induced IFNγ+ and TNFα+ CD8+ T cells at 1% and IL2+ CD8+ T cells at around 0.2% (Figure 7-6 E). In mice receiving Alhydrogel frequencies of IFNγ+ CD8+ T cells were 1.7%, TNFα+ CD8+ T cells were 1.3% and IL-2+ CD8+ T cells were 0.2%.

Spleen

In the spleen MVA85A induced IFNγ+ and TNFα+ CD8+ T cells at 0.08% and very low frequencies of IL-2+ CD8+ T cells (Figure 7-6 F). In mice administered Alhydrogel IFNγ+ and TNFα+ CD8+ T cells frequencies were 0.06% and IL-2+ CD8+ T cells were around 0.01%.

Alhydrogel had no effect on the frequency of cytokine producing CD8+ T cells in the mLN. Alhydrogel increased cytokine production in the lungs but in the spleen it was slightly reduced compared mice which received only MVA85A.

7.2.2.3 Polyfunctional Th1 Responses

Polyfunctional profiles of Th1 cells were similar between the groups in mLN, lungs and spleens; the mLN data is shown as an example.

The dominant cytokine producing Th1 cells induced by MVA85A in the mLN were IFNγ+ TNFα+ IL-2+ (Figure 7-6 G). The next most frequent cells were IFNγ+ TNFα+ and IFNγ+ single cytokine producers. In mice receiving Alhydrogel the greatest frequency of cells were IFNγ+ TNFα+ IL-2+ followed by IFNγ+ TNFα+ and IFNγ+ single cytokine producers.

Alhydrogel did not alter the profile of polyfunctional Th1 induced by MVA85A in the mLN, lungs or spleen. The only effect was that frequencies of the cells in the mLN were lower compared to mice receiving MVA85A alone.

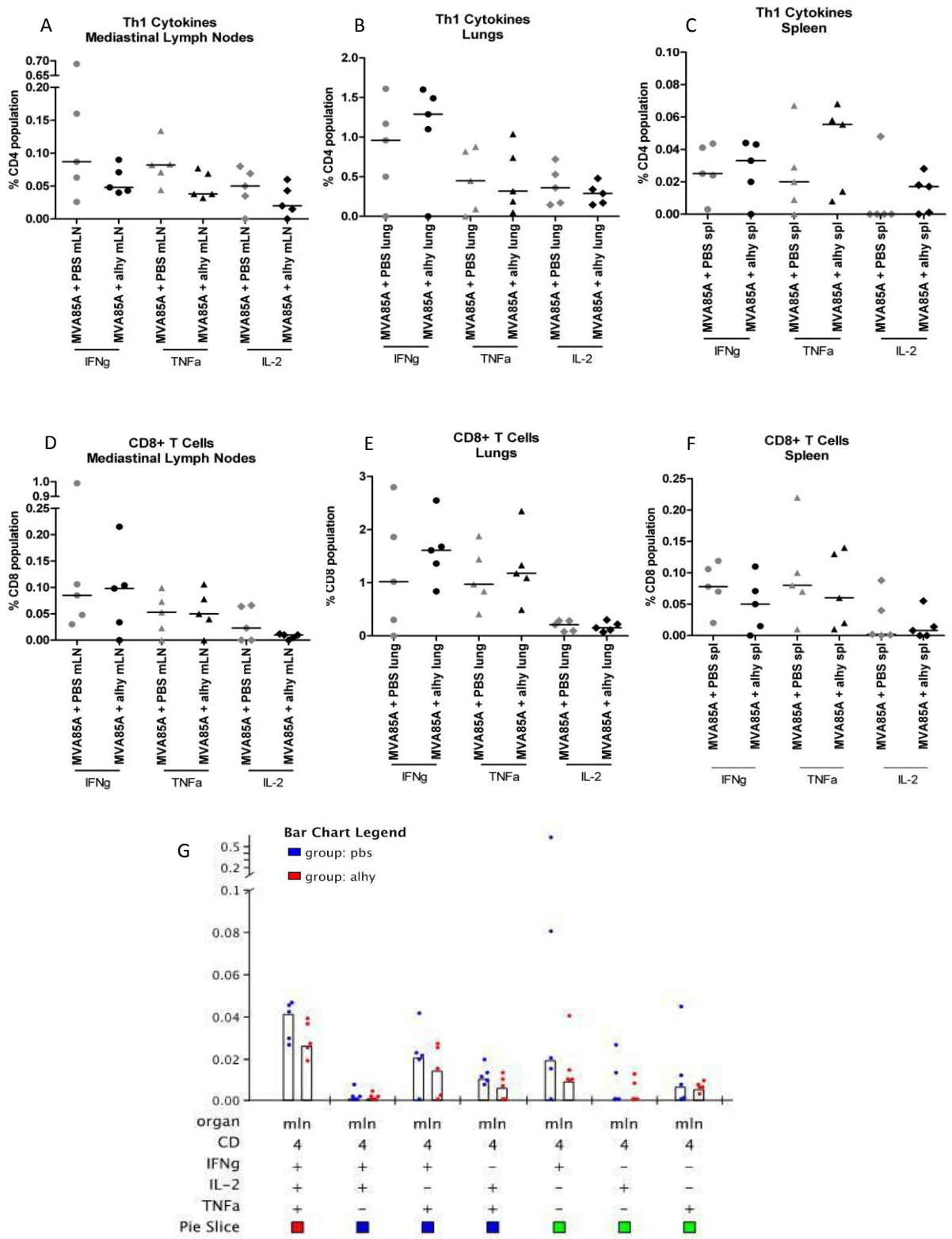


Figure 7-6: Cellular immune responses in mice vaccinated with MVA85A IN with the administration of Alhydrogel (MVA85A + alhy) or with PBS (MVA85A + PBS) IM. (A-C) is Th1 responses in the mLN, lungs and spleen, (D-F) are the CD8+ T cell responses in the mLN, lungs and spleen. (G) is the polyfunctional cytokine profile of Th1 cells in the cLN. Organs were taken 7 days after MVA85A and stimulated with Ag85A before staining and analysis using flow cytometry. Cytokine responses are Ag85A specific; the media only control well values are subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was used to test for statistical significance; no significant differences were calculated.

7.2.2.4 IL-4+ CD4+ T Cells

MVA85A induced IL-4+ CD4+ T cells in the mLN at 0.01%, the lung at 0.15% and the spleen at 0.01% (Figure 7-7 A). Mice administered Alhydrogel induced around 0.001% IL-4+ CD4+ T cells in the mLN and 0.01% in the spleen. None were detected in the lung.

Alhydrogel lowered the frequency of IL-4+ CD4+ T cells in the mLN and lungs and had no effect on the frequency in the spleen.

7.2.2.5 IL-17+ CD4+ T Cells

MVA85A induced IL-17+ CD4+ T cells in the lung at a median frequency of around 0.18% (Figure 7-7 B). No IL-17+ CD4+ T cells were detected in the mLN or the spleen of this group. In mice administered Alhydrogel the median frequency of IL-17+ CD4+ T cells in the lung was 0.03% and in the spleen 0.05%.

There was a wide variation in the production of IL-17 in the lung which makes it hard to compare responses between the groups; it does not appear Alhydrogel reduced the production of IL-17. Alhydrogel increased IL-17 production in the spleen.

7.2.2.6 IL-10+ CD4+ T Cells

IL-10+ CD4+ T cells were induced in the lungs of MVA85A vaccinated mice at a frequency of 0.26% (Figure 7-7 C). Mice receiving Alhydrogel induced a frequency of IL-10+ CD4+ T cells at 0.35%.

Alhydrogel increased the frequency of IL-10+ CD4+ T cells in the lungs of MVA85A vaccinated mice. Regulatory T cell frequencies were not different between the groups (data not shown).

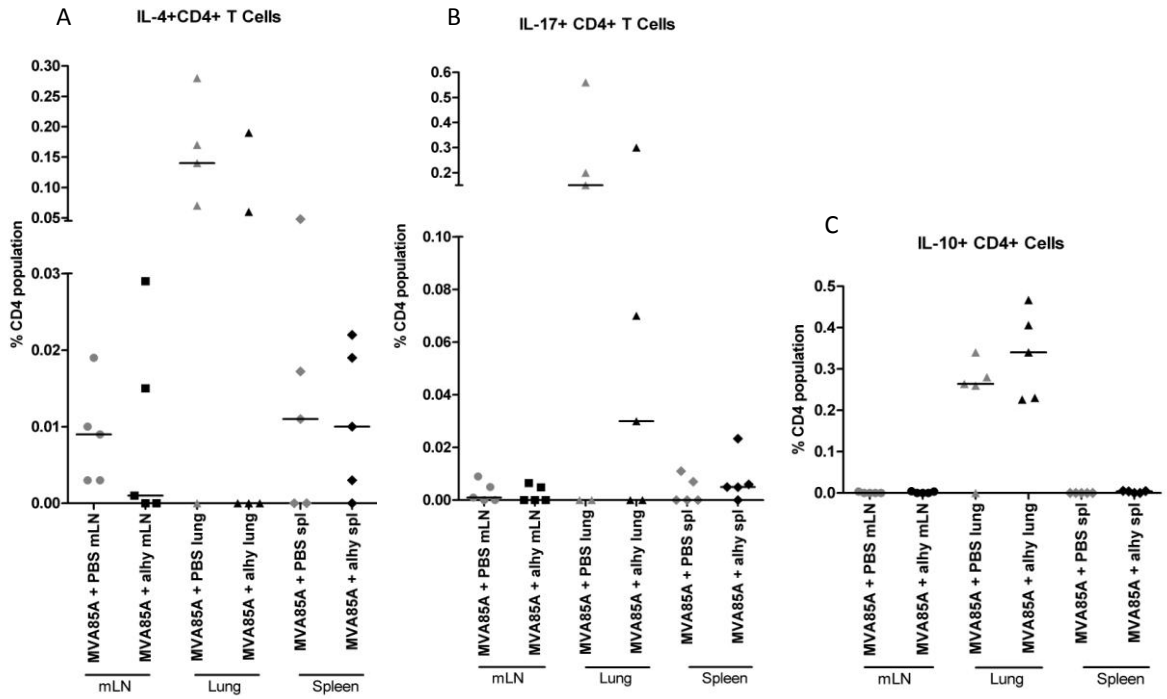
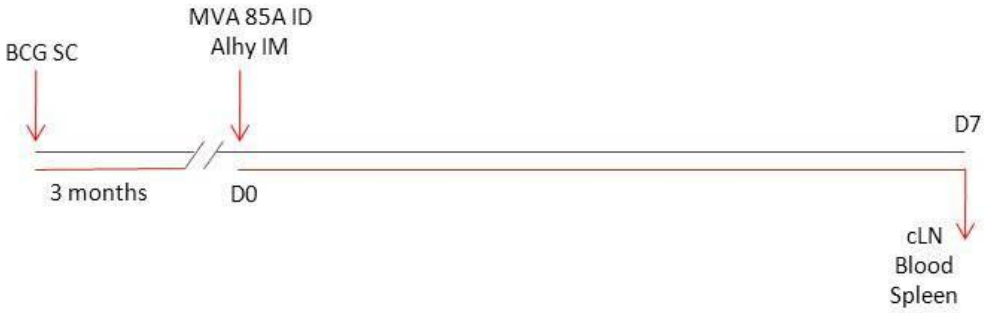


Figure 7-7: Cellular immune responses in mice vaccinated with MVA85A IN with Alhydrogel (MVA85A + alhy) or with PBS (MVA85A + PBS) IM. (A) is the IL-4+ CD4+ T cells response, (B) is the IL-17+ CD4+ T cell response, (C) is the IL-10+ CD4+ T cell response. MLN, lungs and spleens were taken 7 days after MVA85A and stimulated with Ag85A before staining and were analysed using flow cytometry. Cytokine responses are Ag85A specific; the media only control well values are subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was used to test for statistical significance, this was not achieved.

7.2.3 MVA85A Intradermally and Alhydrogel Intramuscularly in BCG Primed Mice



7.2.3.1 Th1 Responses

cLN

BCG prime followed by MVA85A boost induces 0.02% IFNγ+ CD4+ T cells in the cLN, no TNFα+ CD4+ T cells and around 0.005% IL-2+ CD4+ T cells (Figure 7-8 A). In mice receiving Alhydrogel IFNγ+ CD4+ T cell frequencies were recorded at 0.018% and TNFα+ CD4+ T cell frequencies at 0.005%. No IL-2 response was detected.

Blood

BCG prime-MVA85A boost induced no Th1 response in the blood (Figure 7-8 B). In mice receiving Alhydrogel a frequency of 0.2% was recorded for IFNγ+ CD4+ T cells, 0.04% for TNFα+ and around 0.1% for IL-2+ CD4+ T cells.

Spleen

BCG prime-MVA85A boost induced 0.1% IFNγ+ CD4+ T cells, 0.15% TNFα+ and 0.08% IL-2+ CD4+ T cells in the spleen (Figure 7-8 C). In mice administered Alhydrogel the frequency of IFNγ+ CD4+ T cells recorded was 0.18%, TNFα+ CD4+ T cells was 0.2% and IL-2+ CD4+ T cells was 0.15%.

Alhydrogel had no effect on the frequency of Th1 in the cLN but had an enhancing effect on the responses in the blood and the spleen.

7.2.3.2 CD8+ T Cell Responses

cLN

BCG prime-MVA85A boost induced IFNγ secretion from 0.04% CD8+ T cells in the cLN, TNFα from 0.02% and no IL-2 secretion (Figure 7-8 D). Mice administered Alhydrogel had 0.03% IFNγ+ and TNFα+ CD8+ T cells and 0.01% IL-2+ CD8+ T cells.

Blood

BCG prime-MVA85A boost induced IFNγ+ and TNFα+ CD8+ T cells in the blood at a frequency of 0.7% (Figure 7-8 E). A frequency of 0.1% IL-2+ CD8+ T cells was detected. In the Alhydrogel group, the frequency of IFNγ+ CD8+ T cells was 0.7%, TNFα+ CD8+ T cells was 0.8% and IL-2+ CD4+ T cells was 0.1%.

Spleen

In the spleen, BCG prime-MVA85A boost induced IFNγ+ and TNFα+ CD8+ T cells at a frequency of 0.2% and IL-2+ CD8+ T cells at a frequency of 0.1% (Figure 7-8 F). Mice administered alhydrogel induced IFNγ+ CD8+ T cells at a frequency of 0.3%, TNFα+ CD8+ T cells at a frequency of 0.5% and IL-2+ CD8+ T cells at 0.1%.

There was no effect of Alhydrogel on CD8+ T cell responses in the cLN. Alhydrogel induced an enhanced CD8+ T cell response in the blood and spleen.

7.2.3.3 IL-4+ CD4+ T Cells

BCG prime-MVA85A boost induced 0.04% IL-4+ CD4+ T cells in the cLN, none in the blood and 0.03% in the spleen (Figure 7-8 G). In the group administered Alhydrogel no IL-4+ CD4+ T cell response was detected in the spleen, in the blood 0.12% of CD4+ T cells were IL-4+ and in the spleen 0.05% of CD4+ T cells were IL-4+.

Alhydrogel reduced the frequency of IL-4+ CD4+ T cells in the cLN but increased frequencies in the blood and spleen.

7.2.3.4 IL-10+ CD4+ T Cells

BCG prime-MVA85A boost induced 0.058% of CD4+ T cells to produce IL-10 in the blood but no IL-10 was detected in the cLN or the spleen (Figure 7-8 H). In mice administered Alhydrogel 0.01% of CD4+ T cells in the blood were IL-10+ and 0.005% in the spleen were IL-10+.

Alhydrogel reduced the frequency of IL-10 producing CD4+ T cells in the blood but increased the frequency on the spleen compared to giving MVA85A alone.

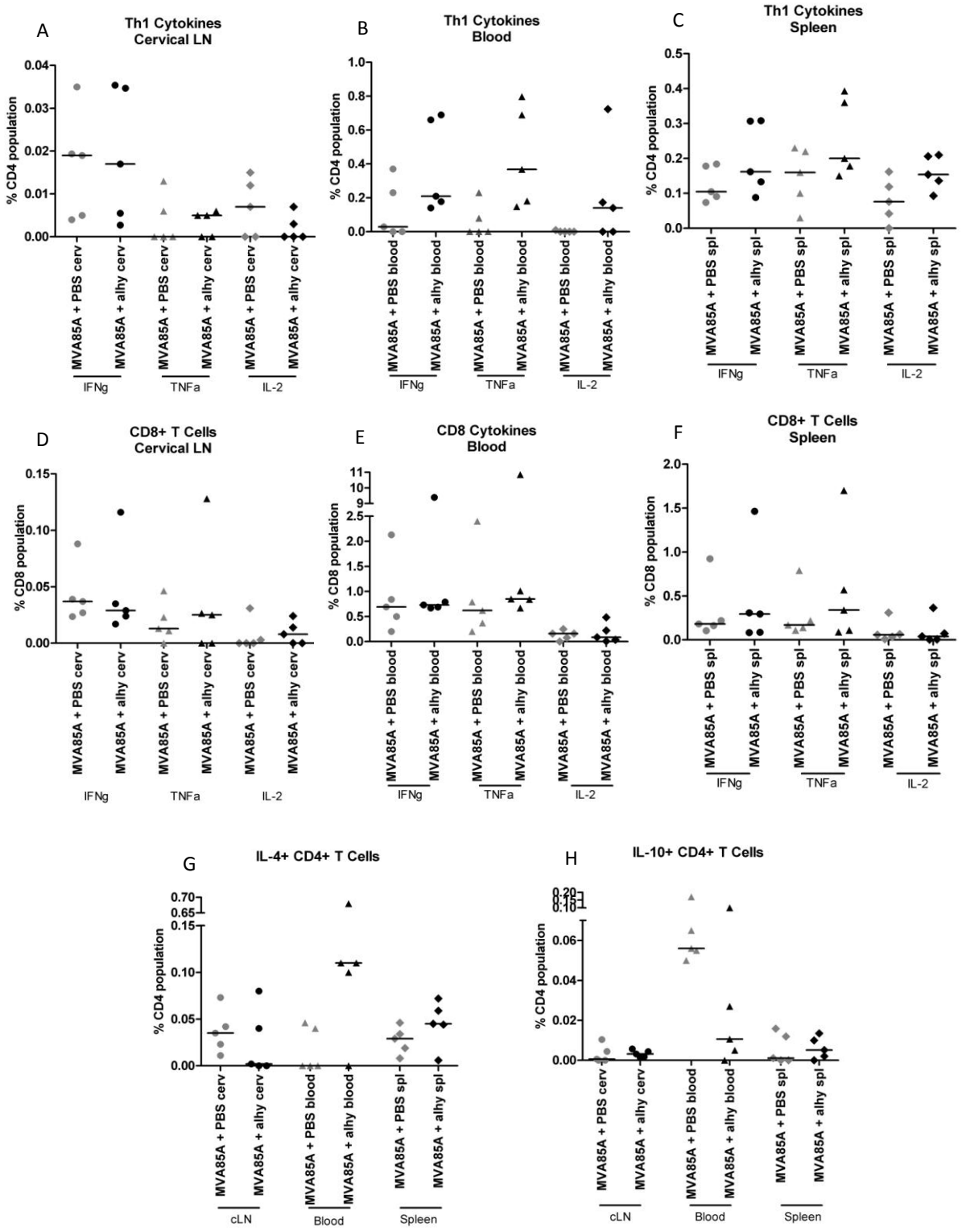
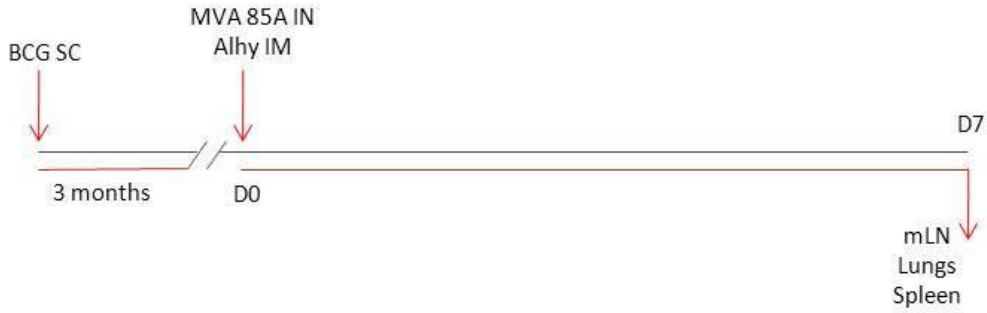


Figure 7-8: Cellular immune responses in mice vaccinated with BCG followed by MVA85A ID with the administration of Alhydrogel (MVA85A + ah) or PBS (MVA85A + PBS) IM. (A-C) is the Th1 response in cLN, blood and spleen, (D-F) is the CD8+ T cell response in cLN, blood and spleen, (G) is the IL-4+ CD4+ T cell response in cLN, blood and spleen and (H) is the IL-10+ CD4+ T cell response in cLN, blood and spleen. Cytokine responses are Ag85A specific, the media only control well values are subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was used to test for statistical significance, none were achieved.

7.2.4 MVA85A Intranasally and Alhydrogel Intramuscularly in BCG Primed Mice



7.2.4.1 Th1 Responses

mLN

BCG prime-MVA85A boost induced IFNγ+ CD4+ T cells at a frequency of 0.06%, TNFα+ CD4+ T cells at 0.04% and IL-2+ CD4+ T cells at 0.03% in the mLN (Figure 7-9 A). Mice administered Alhydrogel had a frequency of IFNγ+ CD4+ T cells of 0.06% and TNFα+ CD4+ T cells of 0.01%. No Il-2 was detected in this group.

Lungs

In the lungs BCG prime-MVA85A boost induced IFNγ+ CD4+ T cells at a frequency of 1%, TNFα+ CD4+ T cells at 0.04% and IL-2+ CD4+ T cells at 0.02% (Figure 7-9 B). In mice administered Alhydrogel, frequencies of IFNγ+ CD4+ T cells in the lungs were recorded at 1.5%, TNFα+ CD4+ T cells at 0.7% and IL-2+ CD4+ T cells at 0.03%.

Spleen

BCG prime-MVA85A boost induced IFNγ+ CD4+ T cells at a frequency of 0.03% and TNFα+ and IL-2+ CD4+ T cells at around 0.02% (Figure 7-9 C). Mice administered Alhydrogel induced IFNγ+ CD4+ T cell frequencies of 0.05%, TNFα+ CD4+ T cells at 0.03% and IL-2+ CD4+ T cells at 0.01%.

Alhydrogel reduced the production of TNFα and IL-2 in the mLN but did not affect IFNγ production. In the lungs and spleen Alhydrogel enhanced the Th1 response.

7.2.4.2 CD8+ T Cell Responses

mLN

In the mLN BCG prime-MVA85A boost induced IFNγ production from 0.02% of CD8+ T cells and TNFα from 0.04% but there was no IL-2 production (Figure 7-9 D). Mice administered Alhydrogel induced IFNγ, TNFα and IL-2 production from 0.03% of CD8+ T cells.

Lungs

In the lungs, BCG prime-MVA85A boost induced 0.5% IFNγ+ and TNFα CD8+ T cells and 0.1% IL-2+ CD8+ T cells (Figure 7-9 E). In mice administered Alhydrogel, 1.8% IFNγ+ CD8+ T cells, 1% TNFα+ and 0.2% IL-2+ CD8+ T cells were induced.

Spleen

In the spleen no IFNγ or IL-2 was induced by BCG prime-MVA85A boost and 0.08% of CD8+ T cells were TNFα+ (Figure 7-9 F). In mice administered Alhydrogel, 0.5% of CD8+ T cells produced IFNγ, 0.8% TNFα and 0.02% IL-2.

Alhydrogel had no effect on CD8+ T cell responses in the mLN. Alhydrogel significantly increased the production of IFNγ and TNFα from CD8+ T cells in the lung and an enhanced the response of all 3 cytokines in the spleen.

7.2.4.3 IL-4+ CD4+ T Cells

BCG prime-MVA85A boost induced production of IL-4 from 0.001% of CD4+ T cells in the lungs and a very small frequency were detected in the mLN (Figure 7-9 G). In mice administered Alhydrogel no IL-4 was detected.

Alhydrogel ablated the production of IL-4 from CD4+ T cells in BCG primed-MVA85A boosted mice.

7.2.4.4 IL-10+ CD4+ T Cells

BCG prime-MVA85A boost induced IL-10 production from 0.25% of CD4+ T cells in the lungs and 0.05% in the spleen (Figure 7-9 H). In mice administered Alhydrogel 0.7% of CD4+ T cells in the lung produced IL-10. No IL-10 response was detected in mLN or the spleen.

Alhydrogel increased the frequency of IL-10 producing CD4+ T cells in the lung but inhibited this response in the spleen.

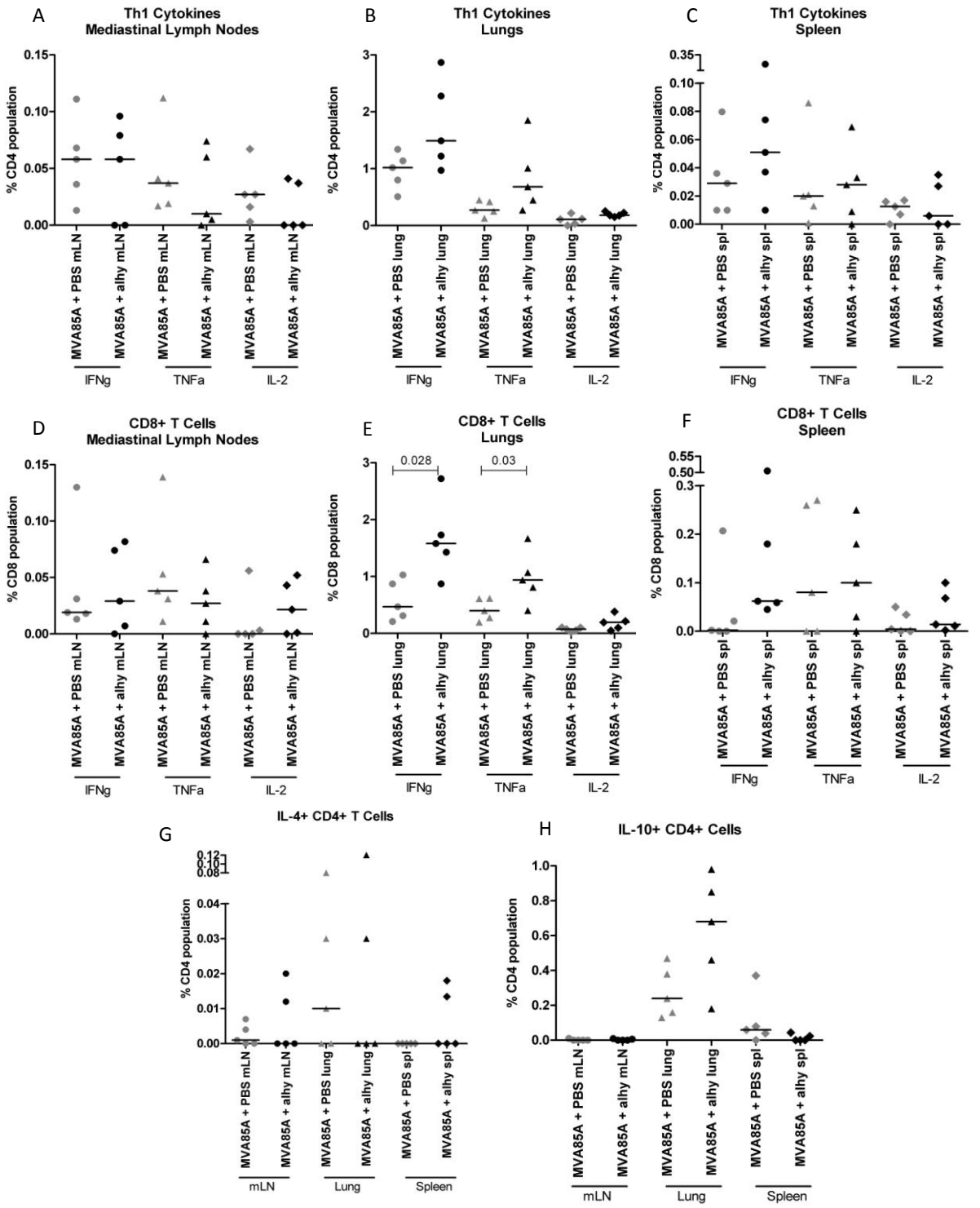
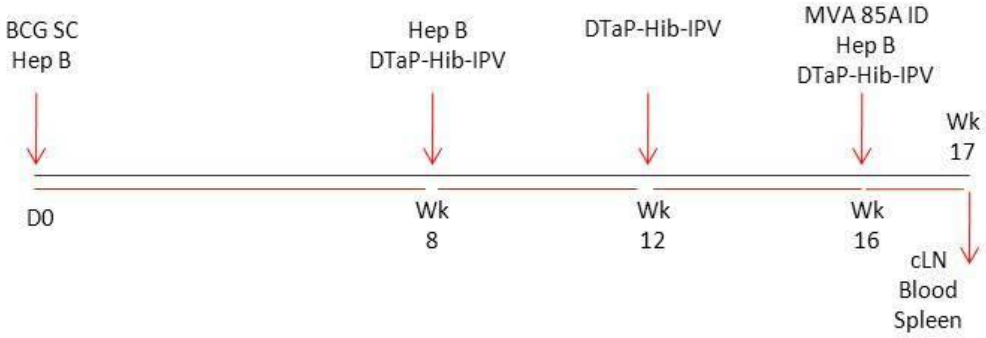


Figure 7-9: Cellular immune responses in mice vaccinated with BCG followed by MVA85A IN with the administration of Alhydrogel (MVA85A + ah) or PBS (MVA85A + PBS) IM. (A-C) is the Th1 response in mLN, lungs and spleen, (D-F) is the CD8+ T cell response in mLN, lungs and spleen, (G) is the IL-4+ CD4+ T cell response in mLN, lungs and spleen and (H) is the IL-10+ CD4+ T cell response in mLN, lungs and spleen. Cytokine responses are Ag85A specific; the media only control well values are subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was used to test for statistical significance and are shown where this was achieved.

7.2.5 MVA85A Intradermally in Mice Administered an EPI Vaccine Schedule



Group 1 (B-MVA) received BCG at day 0 then MVA85A at week 16

Group 2 (B-MVA+H+D) received BCG and Hep B at day 0, Hep B and DTaP-Hib-IPV at week 8, DTaP-Hib-IPV at week 12. At week 16 this group received Hep B and DTaP-Hib-IPV along with MVA85A.

Group 3 (B-MVA+H) received BCG and Hep B at day 0, Hep B at wk 8 and at week 16 Hep B and MVA85A.

Group 4 (B-MVA+D) received BCG at day 0 then DTaP-Hib-IPV at week 8 and week 12. At week 16 this group received DTaP-Hib-IPV and MVA85A.

7.2.5.1 Th1 Responses

cLN

In the cLN, B-MVA induced 0.065% CD4+ T cells to produce IFNγ, 0.03% were TNFα+ and 0.04% were IL-2+ (Figure 7-10 A). B-MVA+H+D induced the same level of response in all three cytokines. B-MVA+H and B-MVA+D vaccinated mice had reduced frequencies of IFNγ and IL-2. B-MVA+D vaccinated mice had increased frequencies of TNFα+ CD4+ T cells whereas B-MVA+H vaccinated mice had reduced frequencies in comparison to B-MVA mice.

Blood

In the blood, B-MVA induced 1.2% of CD4+ T cells to produce IFNγ, 1% were TNFα+ and 0.03% were IL-2+ (Figure 7-10 B). B-MVA+H+D vaccinated mice had lower frequencies of all three cytokines compared to the B-MVA group, B-MVA+H vaccinated mice had reduced frequencies of IFNγ and IL-2 but equivalent frequencies of TNFα and B-MVA+D vaccinated

mice had reduced frequencies of TNFα and IL-2 but equivalent frequencies of IFNγ to the B-MVA group.

Spleen

In the spleen B-MVA induced 0.2% of CD4+ T cells to produce IFNγ, 0.2% were TNFα+ and 0.15% were IL-2+ (Figure 7-10 C). All groups of mice receiving EPI vaccines had a lower frequency of all 3 cytokines tested. This was significantly different for IFNγ between B-MVA and B-MVA+H and B-MVA+D vaccinated mice. The difference was also significant for TNFα and IL-2 between B-MVA and B-MVA+D mice. Mice administered just one of the EPI vaccines had lower responses than mice receiving both EPI vaccines.

The administration of EPI vaccines had an overall deleterious effect on the immune response against Ag85A however this effect was variable. The greatest effect was seen in the spleen where all EPI vaccinated groups reduced Ag85A specific responses.

7.2.5.2 CD8+ T Cell Responses

cLN

In the cLN, B-MVA induced 0.1% of CD8+ T cells to produce IFNγ, 0.02% were TNFα+ and 0.01% were IL-2+ (Figure 7-10 D). B-MVA+H+D induced greater frequencies of IFNγ+ CD8+ T cells but lower frequencies of TNFα+ and IL-2+ CD8+ T cells. B-MVA+H and B-MVA+D vaccinated mice had reduced frequencies of IFNγ+ CD8+ T cells and equivalent frequencies of TNFα+ CD8+ T cells compared to B-MVA vaccinated mice. IL-2 was equivalent in the B-MVA+H mice but reduced in the B-MVA+D vaccinated mice compared to the B-MVA vaccinated group.

Blood

In the blood, B-MVA induced 1% of CD8+ T cells to secrete IFNγ and 1% were TNFα+ (Figure 7-10 E). No IL-2+ CD8+ T cells were detected in any of the groups. B-MVA+H+D vaccinated mice had lower frequencies of IFNγ+ and TNFα+ CD8+ T cells. B-MVA+H and B-MVA+D

vaccinated mice had equivalent frequencies of IFNγ+ and TNFα+ CD8+ T cells as B-MVA vaccinated mice.

Spleen

In the spleen, B-MVA induced 0.13% of CD8+ T cells to secrete IFNγ, 0.15% were TNFα+ and none were IL-2+ (Figure 7-10 F). B-MVA+H+D vaccinated mice had lower frequencies of IFNγ+ and TNFα+ CD8+ T cells. No Il-2 was detected. B-MVA+H vaccinated mice had lower frequencies of IFNγ+ and TNFα+ CD8+ T cells, 0.02% of CD8+ T cells were IL-2+. B-MVA+D vaccinated mice had reduced frequencies of IFNγ+ CD8+ T cells, equivalent TNFα+ and greater frequencies at 0.02% of IL-2+ CD8+ T cells compared to B-MVA vaccinated mice.

Administration of one of the EPI vaccines appeared to increase the CD8+ T cell response in the cLN and in the blood; administering a combination of both EPI vaccines did not induce as much of an effect. In the spleen the effect of EPI vaccination was deleterious to IFNγ production.

7.2.5.3 Polyfunctional Th1 Responses

The polyfunctionality of Th1 responses in the cLN of B-MVA and B-MVA+H+D vaccinated mice were compared. The dominant cytokine producing cells in the B-MVA vaccinated mice were IFNγ+ TNFα+ IL-2+ CD4+ T cells followed by IFNγ single cytokine producers (Figure 7-10 G). The dominant cytokine producers in B-MVA+H+D vaccinated mice were IFNγ single cytokine producers followed by IL-2 single cytokine producers and to a lesser extent IFNγ+ TNFα+ IL-2+ CD4+ T cells.

Administration of EPI vaccines reduced the polyfunctional capability of Th1 cells in the cLN. Less of an effect was seen in the blood and spleen (data not shown).

7.2.5.4 Treg Responses

The frequency of Tregs in the B-MVA group was 8% in the cLN, 0% in the blood and 3% in the spleen (Figure 7-10 H). Frequencies in the B-MVA+H+D vaccinated mice were lower in the cLN, greater in the blood and equivalent in the spleen compared to B-MVA vaccinated mice. Frequencies in the B-MVA+H group were equivalent to B-MVA vaccinated mice. Frequencies in B-MVA+D vaccinated mice were equivalent to B-MVA vaccinated mice in the cLN and blood but greater in the spleen.

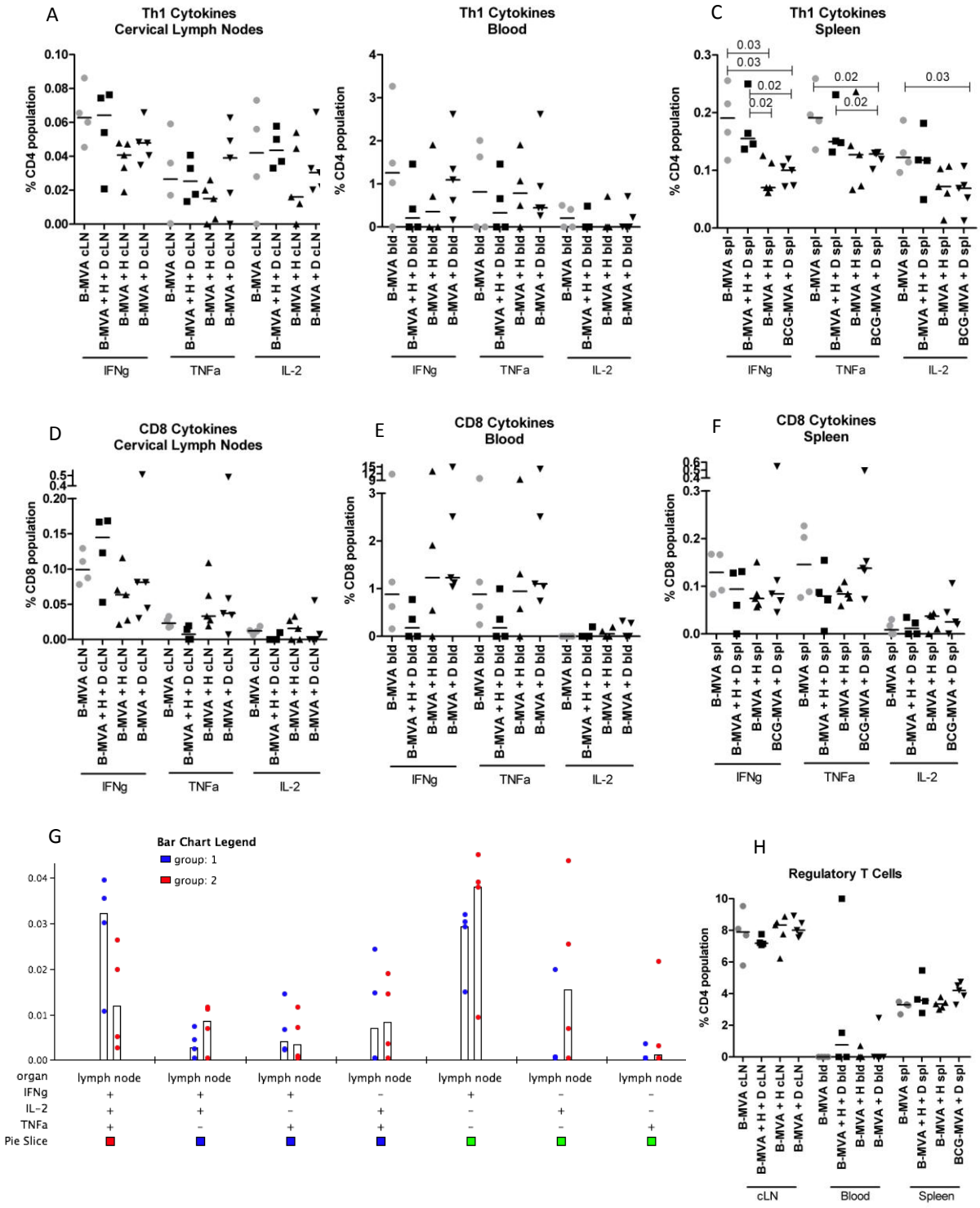


Figure 7-10: Cellular immune responses in mice administered a schedule of BCG and Hep B (H) and/or DTaP-Hib-IPV (D) with MVA85A ID at 16 weeks or mice vaccinated with BCG then MVA85A ID at 16 weeks. (A-C) is the Th1 response in cLN, blood and spleen, (D-F) is the CD8+ T cell response in cLN, blood and spleen, (G) is the polyfunctional profile of the Th1 response in cLN, group 1 is B-MVA and group 2 is B-MVA+H+D. (H) is the frequencies of Tregs in cLN, blood and spleen. Cytokine responses are Ag85A specific; the media only control well values are subtracted. Tregs were defined as CD25+FoxP3+CD39+ and expressed as a % of CD4+ T cells; media well values were not subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was used to test for statistical significance and values are displayed where significance was achieved.

7.2.5.5 IL-4+ CD4+ T Cells

cLN

In the cLN, B-MVA induced 0.005% of CD4+ T cells to secrete IL-4 (Figure 7-11 A). The frequency of IL-4+ CD4+ T cells was greater in B-MVA+H+D vaccinated mice, equivalent in B-MVA+H vaccinated mice and lower in B-MVA+D vaccinated mice in comparison to the B-MVA group.

Blood

In the blood B-MVA induced 0.7% CD4+ T cells to secrete IL-4 (Figure 7-11 B). The frequency of IL-4+ CD4+ T cells was equivalent in B-MVA+H+D vaccinated mice and lower in both B-MVA+H and B-MVA+D vaccinated mice compared to the B-MVA group.

Spleen

In the spleen B-MVA induced 0.03% of CD4+ T cells to secrete IL-4 (Figure 7-11 C). The frequency of IL-4+ CD4+ T cells in B-MVA+H+D and B-MVA+H vaccinated mice was lower and in B-MVA+D vaccinated mice the frequency was higher in comparison to the B-MVA group.

Giving both EPI vaccines simultaneously increased the frequency of Th2 in the cLN but did not have this effect in the blood or the spleen.

7.2.5.6 IL-17+ CD4+ T Cells

cLN

In the cLN, B-MVA induced 0.03% of CD4+ T cells to secrete IL-17 (Figure 7-11 D). IL-17+ CD4+ T cells were increased in the B-MVA+H+D vaccinated mice but equivalent in B-MVA+H and B-MVA+D vaccinated mice compared to the B-MVA group.

Blood

In the blood, B-MVA induced 1.5% of CD4+ T cells to secrete IL-17 (Figure 7-11 E). No IL-17+ CD4+ T cells were detected in B-MVA+H+D or B-MVA+H vaccinated mice. 0.1% of CD4+ T cells were IL-17+ in B-MVA+D vaccinated mice.

Spleen

In the spleen B-MVA did not induce an IL-17 response (Figure 7-11 F). B-MVA+H+D, B-MVA+H and B-MVA+D vaccinated mice induced between 0.05 and 0.1% IL-17+ CD4+ T cells.

Th17 responses in the blood were lowered by all EPI vaccine regimes but increased in the spleen. Administering both EPI vaccines but not just one enhanced Th17 responses in the cLN.

7.2.5.7 IL-10+ CD4+ T Cells

cLN

In the cLN, B-MVA induced 0.0025% of CD4+ T cells to produce IL-10 (Figure 7-11 G). The frequency of IL-10+ CD4+ T cells was greater in the B-MVA+H+D and B-MVA+D vaccinated mice but reduced in the B-MVA+H vaccinated mice compared to the B-MVA group.

Blood

In the blood no IL-10+ CD4+ T cells were detected in the B-MVA vaccinated group (Figure 7-11 H). No IL-10+ CD4+ T cells were detected in the B-MVA+H+D or B-MVA+D vaccinated mice. 0.2% of CD4+ T cells in the B-MVA+H group were IL-10+.

Spleen

In the spleen B-MVA induced 0.01% of CD4+ T cells to secrete IL-10 (Figure 7-11 I). The frequency of IL-10+ CD4+ T cells was greater in B-MVA+H+D and B-MVA+D vaccinated mice but equivalent in B-MVA+H vaccinated mice compared to the B-MVA group.

Administering the combined EPI or DTaP-Hib-IPV alone induced IL-10+ CD4+ T cells in the cLN and the spleen. Hep B alone induced IL-10+ CD4+ T cells in the blood.

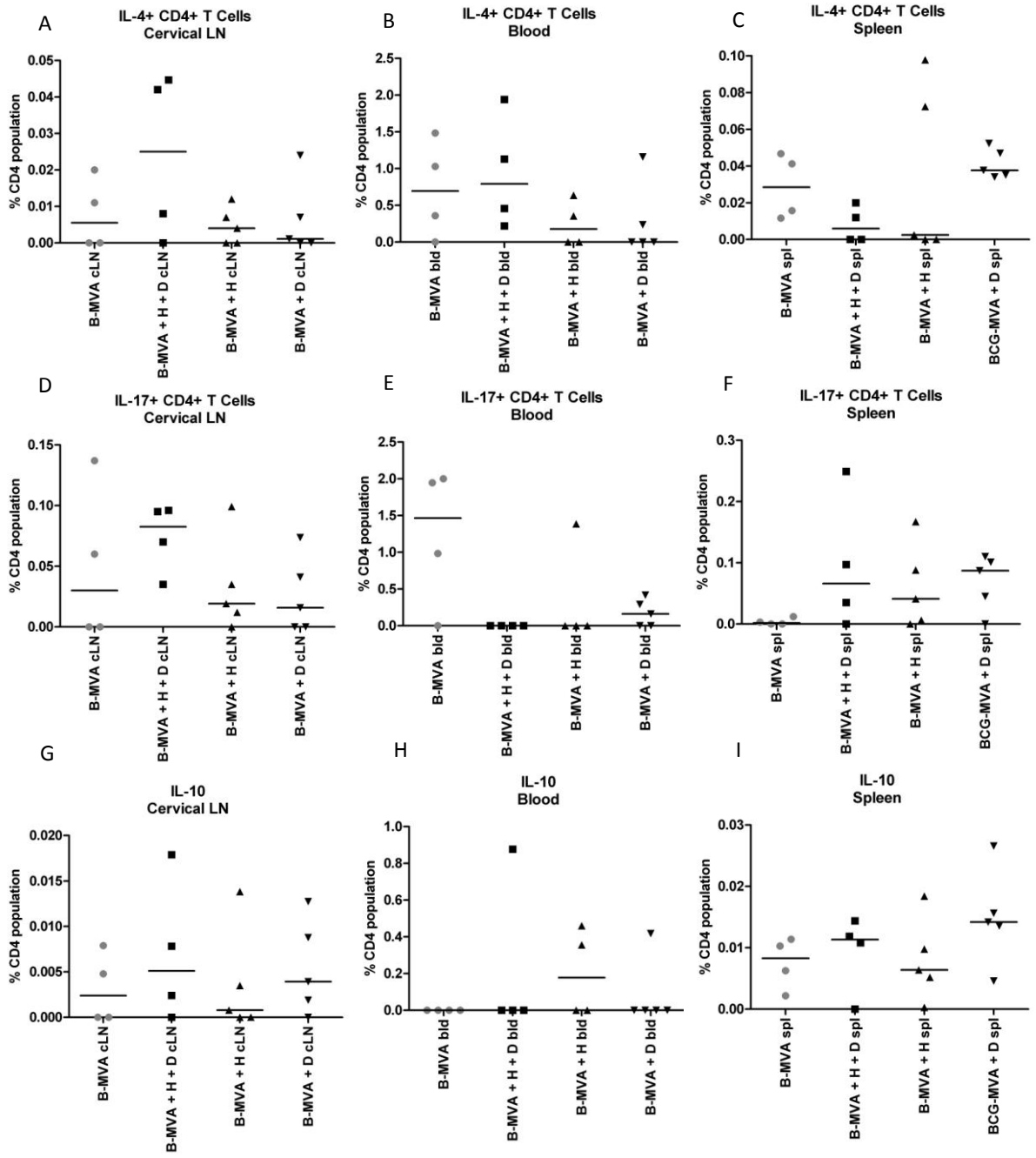
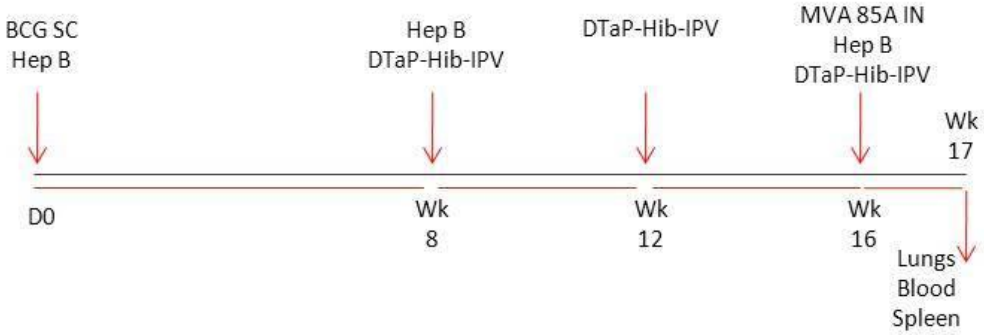


Figure 7-11: Cellular immune responses in mice administered a schedule of BCG and Hep B (H) and/or DTaP-Hib-IPV (D) with MVA85A ID at 16 weeks or mice vaccinated with BCG then MVA85A ID at 16 weeks. (A-C) is the IL-4+ CD4+ T cell response in cLN, blood and spleen, (D-F) is the IL-17+ CD4+ T cell response in cLN, blood and spleen, (G-I) is IL-10+ CD4+ T cell response in cLN, blood and spleen. Cytokine responses are Ag85A specific; the media only control well values are subtracted. Each data point represents one mouse and the median in displayed, n=4 or 5. The Mann-Whitney U test was used to test for statistical significance, none was calculated.

7.2.6 MVA85A Intranasally in Mice Administered an EPI Vaccine Schedule



Group 1 (B-MVA) received BCG at day 0 then MVA85A at week 16

Group 2 (B-MVA+H+D) received BCG and Hep B at day 0, Hep B and DTaP-Hib-IPV at week 8 and DTaP-Hib-IPV at week 12. At week 16 this group received Hep B and DTaP-Hib-IPV as well as MVA85A.

Group 3(B-MVA+H) received BCG and Hep B at day 0, Hep B at wk 8 and at week 16 Hep B and MVA85A.

Group 4 (B-MVA+D) received BCG at day 0 then DTaP-Hib-IPV at week 8 and week 12. At week 16 this group received DTaP-Hib-IPV and MVA85A.

7.2.6.1 Th1 Responses

Lungs

In the lungs B-MVA induced 4% of CD4+ T cells to produce IFNγ, 3.5% were TNFα+ and 1% were IL-2+ (Figure 7-12 A). All 3 EPI groups had reduced frequencies of all three cytokines. The greatest reduction was seen in the B-MVA+D group in which frequencies of all 3 cytokines were significantly lower than the B-MVA group.

Blood

B-MVA did not induce any Th1 response in the blood (Figure 7-12 B). Only the B-MVA+H vaccinated group induced an IFNγ response.

Spleen

In the spleen, B-MVA induced 0.07% of CD4+ T cells to produce IFNγ, 0.07% were TNFα+ and 0.02% were IL-2+ (Figure 7-12 C). All groups of mice receiving EPI vaccines had a lower

frequency of IFNγ and TNFα compared to B-MVA vaccinated mice. The effect was most pronounced in the B-MVA+H+D vaccinated mice. B-MVA+H vaccinated mice had equivalent and B-MVA+D vaccinated mice had greater frequencies of IL-2+ CD4+ T cells compared to B-MVA vaccinated mice.

The effect of EPI administration was detrimental to Th1 responses in the lungs and spleen. A greater effect was observed in mice administered both EPI vaccines compared to those receiving just one of them.

7.2.6.2 CD8+ T Cell Responses

Lungs

In the lungs, B-MVA induced 0.5% of CD8+ T cells to produce IFNγ and 0.2% were TNFα (Figure 7-12 D). No IL-2 was detected in any group. B-MVA+H+D vaccinated mice induced greater frequencies of IFNγ+ and TNFα+ CD8+ T cells. B-MVA+H and B-MVA+D vaccinated mice also had greater frequencies of IFNγ+ and TNFα+ CD8+ T cells. The magnitude of IFNγ+ and TNFα+ CD8+ T cells was greatest in the B-MVA+D vaccinated mice and this was significant when compared to B-MVA vaccinated mice.

Blood

In the blood, B-MVA did not induce any CD8+ T cell responses (Figure 7-12 E). B-MVA+H+D vaccinated mice had no detectable CD8+ T cell responses. B-MVA+H and B-MVA+D vaccinated mice induced IFNγ+ and TNFα+ CD8+ T cells, the B-MVA+D group had higher levels than the B-MVA+H group.

Spleen

In the spleen B-MVA induced 0.1% CD8+ T cells to secrete IFNγ, 0.05% were TNFα+ and 0.01% were IL-2+ (Figure 7-12 F). B-MVA+H+D vaccinated mice had lower frequencies of IFNγ+ and TNFα+ CD8+ T cells and an equivalent frequency of IL-2+ CD8+ T cells. B-MVA+H vaccinated mice had equivalent frequencies of IFNγ+ TNFα+ and IL-2+ CD8+ T cells compared to B-MVA

vaccinated mice. B-MVA+D vaccinated mice had greater frequencies of all 3 cytokines compared to B-MVA vaccinated mice.

Administration of one of the EPI vaccines strongly enhanced CD8+ T cell responses. Giving both EPI vaccines enhanced the response in the lung, did nothing in the blood and reduced the response in the spleen.

7.2.6.3 Polyfunctional Th1 Responses

The polyfunctionality of Th1 responses in the lungs of B-MVA and B-MVA+H+D vaccinated mice were compared. The dominant cytokine producing cells in the B-MVA vaccinated mice were IFNγ+ TNFα CD4+ T cells followed by IFNγ single cytokine producers and IFNγ+ TNFα+ IL-2+ CD4+ T cells (Figure 7-12 G). The dominant cytokine producers in B-MVA+H+D vaccinated mice were IFNγ+ TNFα CD4+ T cells followed by IFNγ single cytokine producers and IFNγ+ TNFα+ IL-2+ CD4+ T cells

Administration of EPI vaccines had no effect of the profile of polyfunctional responses induced by Th1 cells in the lungs. The same was seen in the blood and spleen (data not shown).

7.2.6.4 Regulatory T Cell Responses

The frequency of Tregs in the B-MVA group was 7% in the lungs, 3% in the blood and 1% in the spleen (Figure 7-12 H). Frequencies in the B-MVA+H+D vaccinated mice were greater in the lung, lower in the blood and greater in the spleen compared to B-MVA vaccinated mice. Frequencies in the B-MVA+H group were greater in the lung, lower in the blood and equivalent in the spleen. Frequencies in B-MVA+D vaccinated mice were greater compared to B-MVA vaccinated mice in the lung, blood and spleen.

All three EPI groups had greater frequencies of Tregs in the lungs. The B-MVA+H+D group and the B-MVA+D group induced the greatest frequencies of Tregs in both the lung and spleen; the B-MVA+D group also had the greatest frequency in the blood.

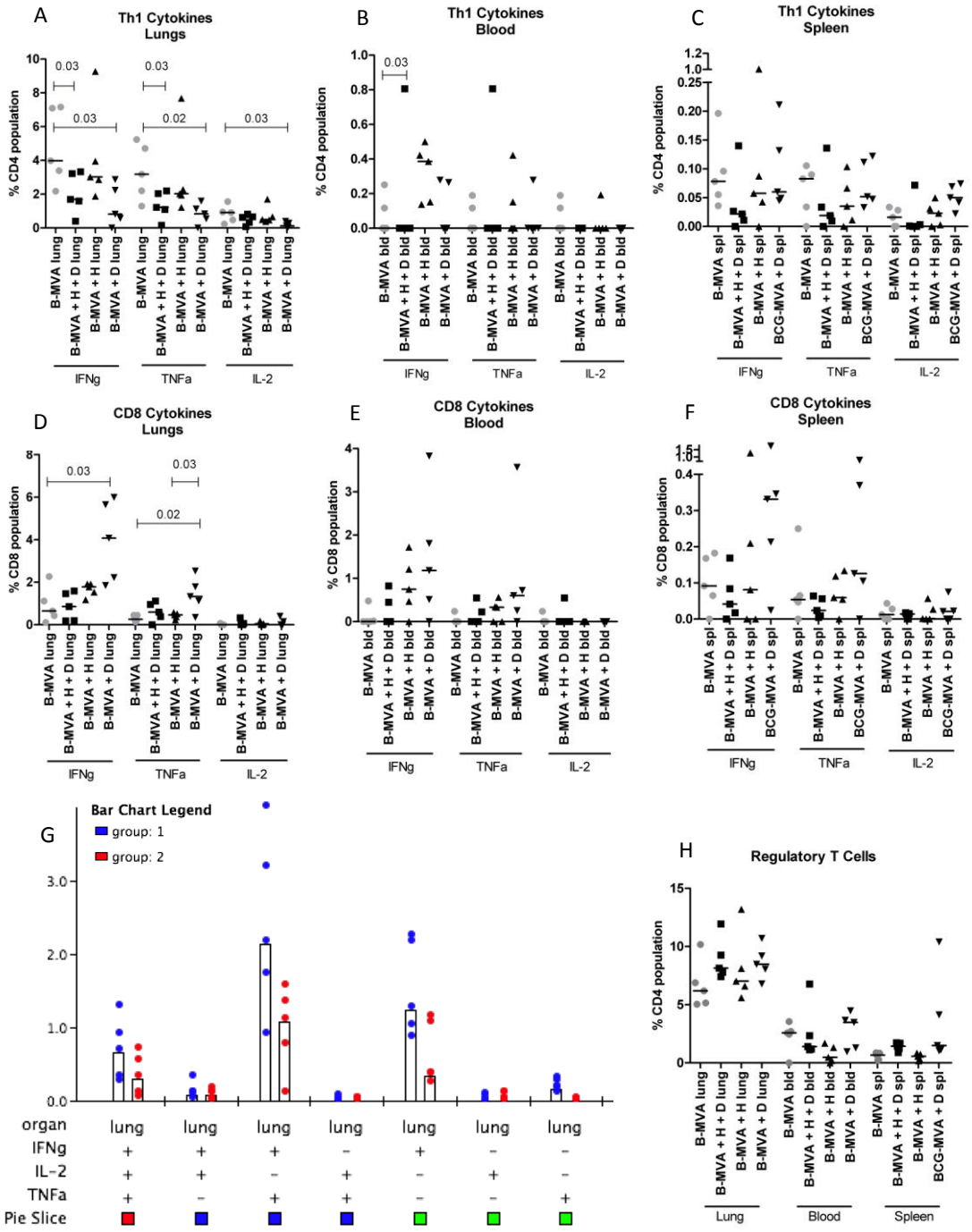


Figure 7-12: Cellular immune responses in mice administered a schedule of BCG and Hep B (H) and/or DTaP-Hib-IPV (D) with MVA85A IN at 16 weeks or mice vaccinated with BCG then MVA85A IN at 16 weeks. (A-C) is the Th1 response in lungs, blood and spleen, (D-F) is the CD8+ T cell response in lungs, blood and spleen, (G) is the polyfunctional profile of the Th1 response in lung, group 1 is B-MVA and group 2 is B-MVA+H+D. (H) is the frequencies of Tregs in lungs, blood and spleen. Cytokine responses are Ag85A specific; the media only control well values are subtracted. Tregs were defined as CD25+FoxP3+CD39+ and expressed as a % of CD4+ T cells; media well values were not subtracted. Each data point represents one mouse and the median in displayed, n=5. The Mann-Whitney U test was used to test for statistical significance and values are displayed where significance was achieved.

7.2.6.5 IL-4+ CD4+ T Cells

In the lung, B-MVA induced 0.7% of CD4+ T cells to secrete IL-4 (Figure 7-13 A). The frequency of IL-4+ CD4+ T cells was less in all 3 EPI groups at around 0.3%. No IL-4+ CD4+ T cells were detected in the blood (Figure 7-13 B). The only group to induce IL-4+ CD4+ T cells in the spleen was the B-MVA+H+D group (Figure 7-13 C).

EPI vaccines reduced Th2 responses in the lungs. The combined vaccine group induced an Ag 85A specific Th2 response in the spleen that was not seen in any other group.

7.2.6.6 IL-17+ CD4+ T Cells

Lungs

In the lungs, B-MVA induced an extremely low frequency of CD4+ T cells to secrete IL-17 (Figure 7-13 D). IL-17+ CD4+ T cells were increased in all 3 EPI groups; to the greatest extent in the B-MVA+D group

Blood

In the blood B-MVA induced 0.28% of CD4+ T cells to secrete IL-17 (Figure 7-13 E). The frequency of IL-17+ CD4+ T cells in B-MVA+H+D vaccinated mice was equivalent to the B-MVA group. No IL-17+ CD4+ T cells were detected in B-MVA+H or B-MVA+D vaccinated mice.

Spleen

In the spleen B-MVA induced 0.03% of CD4+ T cells to secrete IL-17 (Figure 7-13 F). B-MVA+H+D and B-MVA+H vaccinated mice had reduced frequencies of IL-17+ CD4+ T cells compared to the B-MVA group. No Il-17 was detected in the B-MVA+D vaccinated mice.

Th17 responses in the lung were increased by EPI vaccines but were reduced in the blood and the spleen. The greatest effect was seen in B-MVA+D vaccinated mice.

7.2.6.7 IL-10+ CD4+ T Cells

Lung

In the lung, B-MVA did not induce any CD4+ T cells to produce IL-10 (Figure 7-13 G). No IL-10+ CD4+ T cells were detected in the B-MVA+H+D group. Between 0.2 and 0.3% of CD4+ T cells secreted IL-10 in the B-MVA+H and B-MVA+D groups.

Blood

In the blood, B-MVA did not induce any CD4+ T cells to produce IL-10 (Figure 7-13 H). B-MVA+H+D vaccinated mice and B-MVA+H vaccinated mice had increased frequencies of IL-10+ CD4+ T cells. B-MVA+D vaccinated mice did not induce IL-10.

Spleen

In the spleen B-MVA induced 0.03% of CD4+ T cells to secrete IL-10 (Figure 7-13 I). No IL-10 response was detected in the B-MVA+H+D group or the B-MVA+D group. The frequency of IL-10+ CD4+ T cells was equivalent in the B-MVA+H group compared to the B-MVA group.

Administering one of the EPI vaccines increased IL-10 responses in the lung. IL-10 production was induced in the blood by giving a combination of the 2 vaccines.

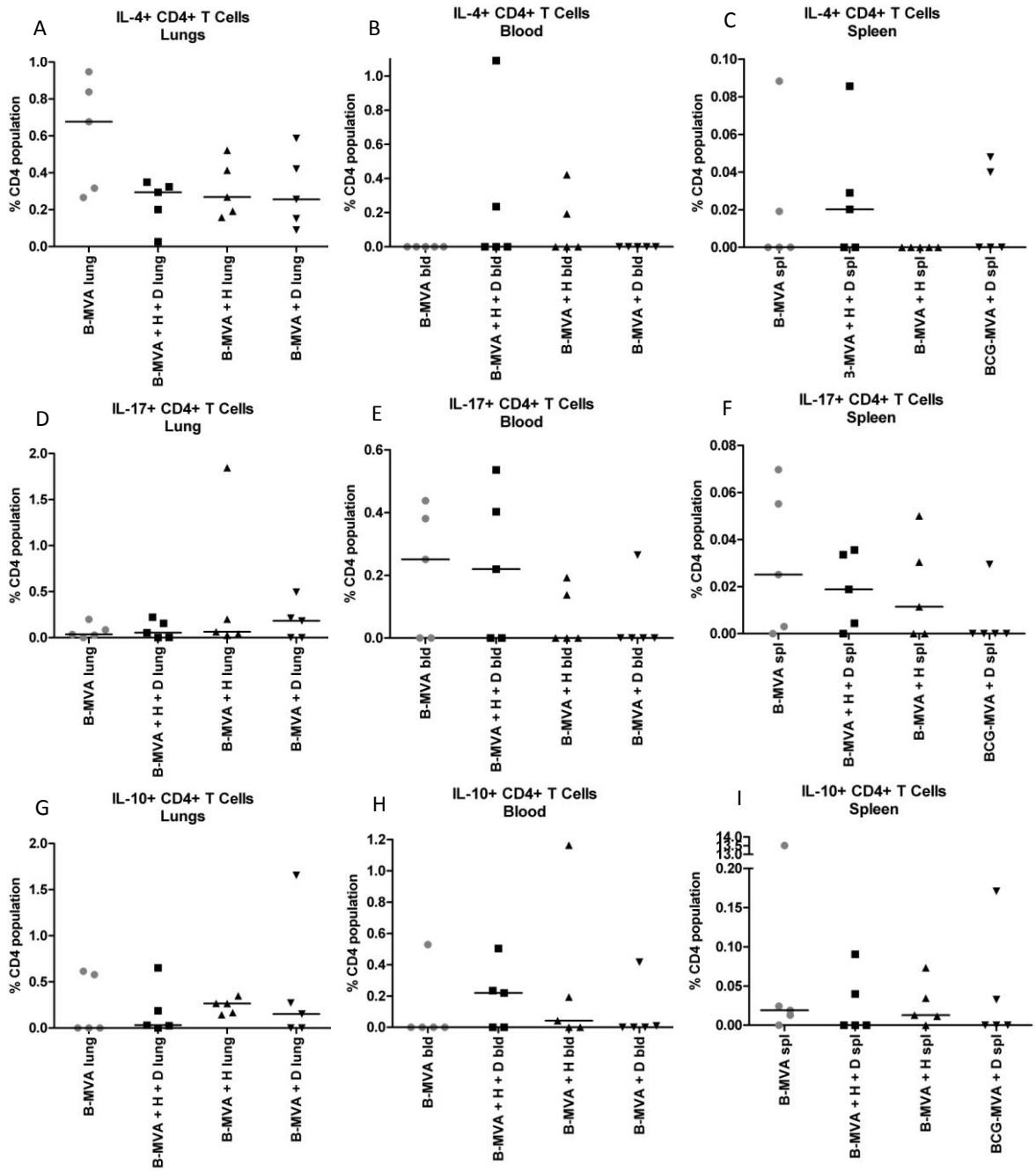


Figure 7-13: Cellular immune responses in mice administered a schedule of BCG and Hep B (H) and/or DTaP-Hib-IPV (D) with MVA85A IN at 16 weeks or mice vaccinated with BCG then MVA85A IN at 16 weeks. (A-C) is the IL-4+ CD4+ T cell response in lungs, blood and spleen, (D-F) is the IL-17+ CD4+ T cell response in lungs, blood and spleen, (G-I) is IL-10+ CD4+ T cell response in lungs, blood and spleen. Cytokine responses are Ag85A specific; the media only control well values are subtracted. Each data point represents one mouse and the median is displayed, n=4 or 5. The Mann-Whitney U test was used to test for statistical significance, none was detected.

7.3 Discussion

The hypothesis was that administering aluminium adjuvant at the same time as MVA85A would reduce Ag 85A specific Th1 responses by inducing a skew toward Th2 immunity.

7.3.1 Administering Alhydrogel IM at the same time as MVA85A ID or IN

The administration of Alhydrogel at the same time as vaccination with MVA85A ID or IN reduced the frequency of Th1 immune responses in the lymph nodes draining the site of MVA85A vaccination. The polyfunctional profile of Th1 responses were also reduced in the cLN of mice receiving Alhydrogel at the same time as MVA85A ID. In ID vaccinated mice the reduction in Th1 immunity was associated with an increase in Ag 85A specific IL-4 production which supports the hypothesis that aluminium adjuvants skew towards a Th2 response. Ag 85A specific IL-10 was also increased in the cLN of the ID vaccinated mice receiving Alhydrogel.

Aluminium vaccines are associated with the infiltration of neutrophils, eosinophils and monocytes that are induced to differentiate to dendritic cells (DCs) at the site of administration[203]. The production of IL-4 from eosinophils and neutrophils is associated with the induction of Th2 priming environment and may well explain the skew toward this arm of the CD4+ T cell response[134]. Th2 cells secrete IL-4, IL-5 and IL-13 ensuring the maintenance of this response. IL-4 and IL-13 can also stimulate differentiation of alternatively activated macrophages and these cells are capable of making high levels of IL-10 but not IL-12[202]. The presence of IL-10 producing CD4+ T cells implies that a regulatory inducing environment may have been present; priming of these responses may be due to the presence of IL-10 secreting DCs or alternatively activated macrophages[204].

The induction of IL-4+ and IL-10+ CD4+ T cells by Alhydrogel was not seen in the mediastinal lymph nodes of mice receiving MVA85A IN, in fact IL-4 production in response to Ag 85A was

reduced in the group receiving Alhydrogel. Lack of induction of IL-4 and IL-10 responses in mLN may be due to the compartmentalisation of the pulmonary immune responses from those in the periphery where Alhydrogel was administered. Responses primed in the periphery are not as likely to enter the pulmonary lymphatic system due to expression of specific homing markers, therefore whatever skews the immune response in the cLN has less effect in the mLN. For example T cells primed in peripheral lymph nodes are directed by DCs to express peripheral homing markers such as P- and E- selectin and possibly CCR4, whereas T cells which are primed in the lung and home to the lung express $\alpha_4\beta_7$ integrins[169,205]. Despite the lack of IL-4 and IL-10 in the mLN the Th1 response was reduced by administration of Alhydrogel; the mechanism of this is unclear.

In both cLN of ID vaccinated mice and mLN of IN vaccinated mice, priming of CD8+ T cell responses was unaffected which indicates there is a reduction in Th1 mediated immunity rather than a reduced priming of the total immune response.

A significant result was the finding that Alhydrogel does not reduce the Th1 responses in peripheral tissue but in fact increased IFNγ production from CD4+ T cells in the spleen and iLN in ID vaccinated mice. Alhydrogel also increased all Th1 cytokines in the spleen and IFNγ production from CD4+ T cells in the lungs of IN vaccinated mice. The enhancing effect of Alhydrogel in peripheral tissue was seen to a greater extent with CD8+ T cell responses. Mice vaccinated IN had greater frequencies of all cytokines from CD8+ T cells in the lungs and mice vaccinated ID had greater CD8+ T cell responses in the spleen and iLN when Alhydrogel was given. Alhydrogel would drain from the site of intramuscular vaccination to the iLN and this was where the greatest increase in CD8+ T cell response was recorded. This suggests it is a direct effect of the Alhydrogel on enhancing the T cell responses.

Th2 responses induced by alum are impaired if protein and adjuvant are administered separately[136]. Alum activates the Nalp3 inflammasome as does crystallised uric acid (UAC); there are several reports which imply alum acts indirectly through the induction of UAC[206]. Behrens et al showed that administering UAC with ovalbumin or dead cell lysates increased IgG1 subclass antibodies but not IgG2a which demonstrates a skew to Th2 mediated immunity[207]. Administration of ovalbumin and UAC also induced IL-5, IL-10 and IL-6 from DCs which is similar to the effects of alum[134]. Conversely Shi et al showed UAC could activate CD8+ T cell responses[208]. Uric acid is also related to the induction of inflammation in gout which is an inflammatory cell-mediated response. In Shi et als study, gp120 protein was bound to latex beads and administered with UAC to induce gp120 specific T cell responses. Binding of the protein to latex beads would have prevented the UAC associating with the protein. In Behren et als study, the protein was not bound to beads. These reports show that Nalp3 inflammasome activation can lead to activation of cellular or humoral responses and that protein may need to be associated to the adjuvant to induce Th2 skewed immunity, whereas if the protein is not associated to the adjuvant a Th1 and CD8+ T cell response is induced. In the current study the Alhydrogel was administered without associated protein which may have induced an inflammatory environment in the periphery to enhance CD8+ T cell responses.

There is a possibility Alhydrogel could have entered the cLN as it is known to disseminate from the site of administration and does not remain in a depot effect as previously thought[134]. In the cLN it may have associated with secreted Ag 85A protein from MVA85A infected cells. The association of Alhydrogel and Ag 85A may have stimulated the priming of Th2 skewed Ag 85A specific responses in the cLN. Whatever the mechanism of reducing Th1 responses in the cLN and mLN it seems the effect is not detrimental as equivalent, if not greater, peripheral Th1 responses were generated. It may be that a sufficient Th1 response is primed in the LN for it to expand further in the peripheral tissue away from the IL-4 or IL-10 secreting cells.

7.3.2 Administration of Alhydrogel at the time of MVA85A in BCG Primed Mice

BCG priming of the Ag 85A specific immune response prevents Alhydrogel reducing IFNγ+ CD4+ T cells in cLN or mLN. No IL-4 or IL-10 was detected in the cLN of BCG primed animals whereas these cytokines had in mice not primed with BCG. The hypothesis behind reduced Th1 responses was that Alhydrogel induced a skew away from Th1 priming toward Th2; the priming of Ag 85A specific T cells by BCG would have induced a dominant Th1 memory response which was boosted by MVA85A[139]. A memory cell is more likely to produce the cytokine profile from its original lineage on re-stimulation due to the expression of transcription factors GATA-3 in Th2 cells and T-bet in Th1 cells[59]. Memory T cell responses have a level of plasticity to their lineage phenotype, i.e. a Th1 memory cell can be activated in a Th2 environment to secrete IL-4[209]. The experiments conducted by Krawczyk et al showing this plasticity used very highly polarizing antigens. In the current study the BCG induced Th1 response which is boosted by MVA85A may be prevented from being skewed by Alhydrogel due to its original lineage commitment. The Th2 skewing environment was not strong enough to induce a switch of lineage.

A reduction of Ag 85A specific IFNγ was observed in the blood of the Gambian infants. This tissue was examined in the ID vaccinated mice to eliminate the possibility that a different response was induced in this organ compared to the spleen or other tissue which had been tested previously. Alhydrogel administration had no deleterious effect on effecter cell responses in any organ in BCG primed mice and without BCG priming the only deleterious effect was recorded in the mLN and cLN. In fact Alhydrogel enhanced frequencies of both Th1 and CD8+ T cells which was the opposite result of what had been recorded in the infant study. It was considered that the EPI vaccines themselves may need to be administered to reduce the effecter T cell response. The hypothesis was that a large Th2 skewed immune response would be primed by vaccination with the EPI vaccines and that this would be boosted at the time of MVA85A vaccination as a third dose of EPI vaccine was given at the same time. This

would induce a large Th2 skewed environment which would have a deleterious effect on the Ag 85A specific Th1 responses boosted by MVA85A.

7.3.3 Vaccinating with MVA85A in the EPI Vaccine Schedule

Ag 85A specific Th1 responses were reduced by the EPI vaccination regime in mice receiving MVA85A ID or IN. The greatest effect in mice vaccinated with MVA85A ID was seen in the blood and the spleen where all groups receiving either DTaP-Hib-IPV or Hep B vaccine alone or in a combination had a reduced Th1 response. Mice which received either DTaP-Hib-IPV or Hep B alone had a reduced Th1 response in the cLN, but no effect was seen in the group which received the combination of EPI vaccines. In mice vaccinated with MVA85A IN all EPI vaccine regimens reduced Th1 responses in the lungs and spleens; no response was induced by MVA85A in the blood. In these mice the combination regimen of DTaP-Hib-IPV and Hep B had a more deleterious effect on Th1 responses compared to just one of the vaccines. It is unclear why in mice administered MVA85A ID the administration of the combination of EPI vaccines had less of an effect on the Th1 responses than the single vaccine regimens. In the IN vaccination experiment, combined vaccines did induce a greater effect on the Th1 response than either of the single vaccine regimens, which was the expected result. These discrepant findings may be explained by the variability in the data. Variability was probably due to some mice inducing larger responses to the EPI vaccines than others and repeated doses of the vaccines probably enhanced this variability. Overall the results show that the administration of EPI vaccines reduced the frequency of the Th1 response induced by MVA85A. Ideally repeating this experiment with larger group sizes would achieve statistically significant differences between the EPI groups compared to BCG-MVA alone.

Vaccination with the combined regimen increased the frequency of Ag 85A specific Th2 responses in the cLN of mice vaccinated with MVA85A ID. Administration of DTaP-Hib-IPV induced Ag 85A specific Th2 responses in the spleen. In mice vaccinated with MVA85A IN only

the combined vaccine regime induced Ag 85A specific Th2 response in the spleen. Less of a Th2 response was induced in the IN vaccinated mice compared to the ID vaccinated mice, which was similar to what was seen in the mice that received Alhydrogel with MVA85A without a BCG prime. The lack of Th2 responses in the lung may again be explained by compartmentalisation of the lung mucosal system from the systemic system. Priming with BCG could overcome Alhydrogel inducing an Ag 85A specific Th2 response in the cLN of ID vaccinated mice. In the EPI vaccinated mice, the strength of the Th2 skewed environment may have been strong enough to induce a lineage switch of the memory response primed by BCG from Th1 to Th2.

Administration of the DTaP-Hib-IPV regime induced a small increase in the frequency of Ag 85A specific IL-10 from CD4+ T cells in the spleen of mice vaccinated with MVA85A ID. The frequency of Tregs was also slightly increased by this regime. In mice vaccinated with MVA85A IN the 2 single vaccine regimens induced Ag 85A specific IL-10+ CD4+ T cells in the lungs and the combined vaccine regimen increased IL-10+ CD4+ T cells in the blood. The combined vaccine regimen and the DTaP-Hib-IPV regimen induced a small increase in Treg frequencies in the lung and spleen.

The combined vaccine regimen and the DTaP-Hib-IPV regimen induced greater frequencies of Tregs in the lungs and spleen of IN vaccinated mice than the Hep B regimen. The combined vaccine regimen and the DTaP-Hib-IPV regimen also induced higher frequencies of IL-10+CD4+ T cells in the cLN and spleen of ID vaccinated mice compared to the Hep B regimen. There may be an association between the induction of regulatory T cell responses by the EPI vaccines and the reduction of Th1 responses. The fact that the combined regimen and the DTaP-Hib-IPV regimen induced greater frequencies of Tregs and Ag 85A specific IL-10+ CD4+ T cells may show that the number of antigens included in the regimen can influence the induction of greater frequencies of Tregs.

UAC was reported to increase the frequency of Tregs in the periphery by Behrens et al[207]. Trials with the novel malaria vaccine RTS,S/AS02D demonstrate that when this vaccine is administered with DTwP-Hib, reduced antibody responses against the DTwP-Hib antigens and against the malarial sporozoite antigens are found [152]. This demonstrates that inclusion of novel antibody inducing vaccines into the EPI schedule can have an inhibitory effect on induction of antibody titres; this effect would not be due to the skewing of T helper lineages and may be due to regulatory responses. IL-10 can inhibit Th2 responses and reduce IL-4 and IL-5 production that would have an effect on T cell help given to B cells[210].

The effect of the administration of EPI on CD8+ T cells was not as detrimental as the effect observed on Th1 responses. In the IN model, DTaP-Hib-IPV and Hep B increased CD8+ T cell responses in the lungs, blood and spleen. In mice vaccinated with MVA85A ID the single vaccine regimes enhanced the frequencies of CD8+ T cells in the blood however the combined regime did not; the combined regime induced a high frequency of Tregs in the blood. In MVA85A ID mice CD8+ T cell responses were decreased in the spleens of all the EPI vaccine groups. It may be possible that Tregs inhibited the CD8+ T cell response. In the uric acid model by Behrens et al depletion of Tregs lead to enhanced CD8+ T cells[207]. Why there was a deleterious effect in the ID but not the IN model is uncertain.

These experiments need to be repeated with larger group sizes to control for variability. Repetition would also test if differences seen between single EPI vaccine regimens and the combined EPI vaccine regimen can be reproduced.

The induction of a Th2 skewed environment needs to be examined further. So far only responses to Ag 85A have been tested; by the frequency of Ag 85A specific IL-4+ CD4+ T cells and also by Ag 85A specific IgG1 and IgG2a antibody subclasses (of which none were

detected, data not shown). The EPI specific Th2 response has not been examined and it may be the strength of these responses which alters Ag 85A specific Th1 priming. To test whether the environment is skewed toward a Th2 profile the ratio of IgG1 to IgG2a subclass antibodies could be measured as this would indicate whether the antibody responses were directed by Th2 or Th1 cells respectively. The production of cytokines by other cell types would also be of interest, for example production of IL-10 or IL-4 from DCs and macrophages. This could be measured by flow cytometry or from culture supernatants of cells from lymph nodes, blood or other organs.

Regulatory T cell responses were detected in both the EPI with ID-MVA85A and EPI with IN-MVA85A models. It would be important to demonstrate the suppressive capability of these Tregs if this may be the mechanism by which Th1 responses are reduced. Furthermore depletion of this subset or IL-10 blockade in vivo may restore the Th1 responses in EPI vaccinated mice. Tests to determine further mechanism of suppression employed by these T cells could also be performed.

The results from the RTS,S/AS02 trial and a pneumococcal conjugate vaccine trial both showed reduced antibody responses when vaccines were co-administered with the existing EPI schedule[152,153]. Despite the antibody responses being lower in comparison to the EPI only control groups, they were not low enough to compromise seroprotection against the diseases. It may be that the Th1 responses recorded in the Gambian infants which received MVA85A vaccination in the EPI schedule may still be of a functional level. Without a correlate of protection or information on the protective efficacy of MVA85A in humans we cannot conclude whether vaccine efficacy would be lost. Data from the MVA85A Phase IIb efficacy trial is hoped to give more information to help find a correlate of protection against *M. tb*.

In summary Alhydrogel administration at the same time, but separate site, as MVA85A increases the frequencies of Ag 85A specific Th1 and CD8+ T cell responses in the periphery. Administration of EPI vaccines DTaP-Hib-IPV and Hep B in a 3 dose schedule reduces Ag 85A specific Th1 responses induced by MVA85A. The reduction of Th1 responses was correlated with an increase in Ag 85A specific Th2 responses and regulatory T cell responses.

8 Summary

8.1 The Effect of NTM Exposure on the Efficacy of BCG against *M. tb*

The hypothesis set out to be investigated was that exposure to NTM after BCG vaccination would reduce the efficacy of pre-existing BCG mediated immunity. The results in this thesis show that exposure to NTM can enhance BCG efficacy but that it may also reduce BCG efficacy. The administration of dead MA IP enhanced Th1 and Th17 responses which were related to protection against *M. tb*. The administration of live MA orally induced Th2 skewed immune responses and regulatory T cells which were associated with decreased protection against *M. tb*. The induction of memory T cell subsets was shown not to be affected by continuous antigen exposure from NTM.

The main limitation of the NTM exposure model is the high level of variability. Administration of mycobacteria to a group of mice induces a wide range of response magnitudes. Administration of live bacteria is more variable than dead as isolates may replicate more or less easily within the mice. Moreover there is always an uncertainty as to how much dead bacteria is administered with the live bacteria and this could not be controlled for. Repetitive dosing enhanced these effects. The *M. tb* challenge is also a highly variable end-point to use in evaluation of the effect of NTM sensitisation. This is due to the variation in the ability of mice to control *M. tb* and the variable level of protection afforded by BCG. In addition to the above, the variability of the response induced by NTM sensitisation makes it extremely difficult to achieve statistically significant differences between the groups when 10 mice are used per group.

Comparing results from this thesis with results found in previous publications in this field is extremely difficult as the models that are used differ widely; variations include the model

species, the strain and species of the mycobacteria, as well as the route, dose and repetition of doses. Also the immunological assays used by different laboratories differ widely. To further the investigations into NTM exposure on BCG efficacy laboratories should work together to standardise these models. It would then be more likely for differences to be detected and conclusions to be made in this field of work.

Many of the current models of NTM exposure try to replicate what happens in human exposure but this could be improved. Multiple strains of NTM, multiple routes of administration and a mix of live and dead bacteria could be performed. Increasing the number of parameters may increase the variability of the effect on BCG efficacy, however it may provide a more consistent outcome. With a consistent outcome we could work backwards to elucidate the key mechanisms required to reduce the efficacy of BCG. Some of which may include the number of doses needed, the route of administration, the strain or whether multiple strains of NTM are needed. With each of these alterations the changes in the immunological response could be assayed. As shown in Buddle et als report the use of cows would be a more representative model as they are a natural host. Profiling of the immune response in this species has been limited by a lack of reagents, there have been recent developments in reagents for the species and with this the cow could be a valuable model for the field.[128].

Because exposure to NTM is likely to be an important factor in the reduction of BCG efficacy, a reproducible model of this effect would be extremely valuable to test whether NTM exposure would reduce the efficacy of novel TB vaccines. Brandt et al showed efficacy of a subunit vaccine was not affected by pre-exposure to NTM[119]. It may also be possible to demonstrate that a novel vaccine could reverse the detrimental effect of NTM on BCG mediated protection. For example using a subunit vaccine such as MVA85A to boost large Th1 mediated responses it may be possible to reconstitute protective efficacy. In the evaluation of

new vaccines it may also be of importance to model other factors which reduce BCG vaccine efficacy such as exposure to helminths and virulent strains of *M. tb*. These could be performed in combination with NTM exposure.

The first further experiment to be performed would be a repeat of the oral model to confirm whether or not BCG efficacy is reduced. The oral route of exposure and administration of live bacteria appears to be important in the detrimental effect on BCG efficacy as the administration of dead bacteria systemically enhanced BCG efficacy. At present it appears that the Th2 response may have more of a role than regulatory responses as this was the only adaptive response which was induced in the oral model but not the systemic model, however only an association can be made at the moment. If the repeat confirms a deleterious effect on BCG efficacy and the Th2 and regulatory responses were again associated, specific cell depletion studies could be performed to confirm that one if not both of these responses are the mechanism by which efficacy is reduced. It would also be of importance to follow the *M. tb* infection out to a later time point to ascertain whether the effect of the Th2 skewed response increases over time.

Given that the oral model used in this study gives a variable outcome there is a need for it to be developed further to become more reproducible. The first option could be the administration of a different strain of MA or species of NTM; maybe one which has been previously proven to induce Th2 skewed or regulatory responses that may enhance the effect on BCG efficacy and make the mechanism of action clearer. A higher dose of bacteria may make the model more definitive Trying to ascertain whether the model can be shortened by reducing the number of doses would also be useful as then more repetitions could be performed and would allow for faster evaluation in subsequent studies.

8.2 Interference between EPI Vaccines and MVA85A

The hypothesis of this study was that co-administering aluminium adjuvant-containing vaccines with MVA85A would have a detrimental effect on the T cell immunogenicity of MVA85A. It was found that aluminium adjuvant alone did not have a detrimental effect on Th1 responses but that administration of MVA85A at the third dose of an EPI vaccine schedule confirmed this hypothesis. These results, together with the findings from the clinical trial, are important in determining whether or not MVA85A may be co-administered with other vaccines within an EPI schedule or whether it would need to be administered by itself.

There was a modest increase in the frequency of Tregs and Ag85A specific Th2 and IL-10+ CD4+ T cells in the mice receiving EPI vaccines. However, there were not significant increases in Th2 and Treg cell subsets therefore the explanation that these responses are the mechanism for reduction of Th1 responses can only be speculated. IL-4 or regulatory T cell depletion studies could be performed or knockout mice could be used in the model to confirm the mechanism these subsets play in reducing Th1 responses induced by MVA85A.

The limitation of this model was, again, variability within the groups; a significant difference was not detected between groups which received EPI vaccines and those which did not. It is difficult to think of a way to reduce variability in the model as it is down to the variability of BCG and the variability in immune responses induced by the EPI vaccines; enhanced by repetitive dosing. With larger group sizes a significant reduction of the immunogenicity of MVA85A should be achieved, therefore this should be the first further experiment to be performed.

It would also be important to know how many doses of EPI need to be administered to induce an inhibitory effect as it may be that MVA85A could be administered earlier in the EPI schedule to circumvent the deleterious effect. To demonstrate whether protective efficacy of

BCG prime-MVA85A boost is affected by the administration of MVA85A in the EPI schedule an *M. tb* challenge could be included in the model.

A better understanding of the mechanisms of BCG mediated protection and a correlate for protection would undoubtedly help to further the field of TB vaccine development. After many years of study the reasons behind the variable efficacy of BCG is still not known. Exposure to NTM is hugely complex and has proven extremely difficult to model in animals. The effect of NTM may also have implications on the efficacy of novel TB vaccines regimens. Answering these fundamental questions on the mechanisms of BCG protection and the reasons behind its variable efficacy is of great importance. The knowledge will be invaluable for the development of an efficacious TB vaccine for all populations.

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