Phenotypic and Functional Characterisation of Innate and Adaptive Immune Responses after Mucosal Vaccination.

By

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Abstract

The successful development of mucosal vaccines is still impeded by our lack of understanding of how the mucosal immune system regulates antigen-specific responses. As most pathogens interact with or invade their host through a mucosal surface we may learn more about mucosal immunity by investigating the interaction of pathogens and their products with host factors and cells at mucosal surfaces. Here I examine the properties of a live *Salmonella*-based vaccine and a mucosal adjuvant based on a bacterial protein. Initially I examined the immunogenicity of the *M. tuberculosis* fusion antigen Ag85B-ESAT6 using a number of different mucosal vaccination strategies. These strategies included (i) intranasal immunisation with Ag85B-ESAT6 protein with and without Heat Labile toxin as an adjuvant (ii) oral immunisation with *Salmonella enterica* Typhimurium expressing Ag85B-ESAT6 from *in vivo* inducible or constitutive promoters (with and without intranasal boosts). Mice immunised with the various vaccine candidates were found to have significant anti-Ag85B-ESAT6 serum and mucosal antibody titres as well as strong T_H1 type cytokine responses, with IFN-γ levels particularly high. Intranasal boosting served to further enhance these immune responses. Following vaccination with the constitutive *Salmonella* vector, mice challenged with *M. tuberculosis* were found to have significantly reduced CFU in the liver when compared to non-vaccinated animals. Mice primed with *Salmonella* and then boosted intranasally with Ag85B-ESAT6/LTK63 led to a significant increase in protection, equivalent to that observed in mice vaccinated with BCG.

The nasal route for vaccination offers some important opportunities for the prophylaxis of many diseases, however the description of immune responses involved early after intranasal administration of antigen have not been clearly established. In a separate study, flow cytometry and confocal microscopy were used to examine the frequencies and localisation of innate immune cells, their activation status, as well as the expression of cell adhesion molecules following intranasal immunisation. I found striking differences between the cell surface phenotype of leukocytes and their pattern of distribution in the tissues examined at all time points tested after immunisation. Following on from these results one particular cell type was examined in more depth to determine its role in adaptive immune responses.

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Finally I would like to dedicate this PhD thesis to my granddad Dan, he always thought I could do it, and I'd like to think he would be very proud.

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

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All other immunisations, tissue collections, tissue processing (including cutting of frozen sections and immunostaining) were performed by the author.

Dr Jes Dietrich (Statens Serum Instiut, Denmark) performed the aerosol *M. tuberculosis* challenge experiments following vaccination with the recombinant constitutive *Salmonella* vaccine strain in their category 3 animal facilities.

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Abbreviations

Ag85B	Antigen 85B (30kDa mycolyl transferase)
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cells
bp	Base pair
BCG	Bacille Calmette-Guérin
BSA	Bovine serum albumin
BALT	Bronchial-associated lymphoid tissue
CAMs	Cell adhesion molecules
CBA	Cytometric Bead Analysis
CD	Cluster of Differentiation
CFUs	Colony forming units
CLN	Cervical lymph nodes
CMI	Cell mediated immunity
CMIS	Common mucosal immune system
conA	Concavalin A
CT-B	Cholera toxin B-subunit
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
ELISA	Enzyme-linked immunosorbant assay
ESAT-6	Early secreted antigenic target 6kDa
FACS	Fluorescence-activated cell sorting
FDC	Follicular dendritic cells
GALT	Gut-associated lymphoid tissue
GC	Germinal Centres
GM-CSF	Granulocyte-macrophage colony stimulating factor
HEV	High Endothelial Venules
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin

i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
IL	Interleukin
kDa	Kilo Dalton
KO	Knock-out
LB	Luria Bertani
LNs	Lymph nodes
LT	Heat-labile toxin
LT-B	LT B-subunit
LTK63	LT with mutation in A subunit (ser63→lys)
mAb	Monoclonal antibody
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MCP-1	Macrophage chemoattractant protein-1
MHC	Major histocompatibility Complex
NALT	Nasal-associated lymphoid tissue
NKC	Natural Killer cells
NKT	Natural Killer T cells
NRAMP1	Natural resistance-associated macrophage protein-1
OD	Optical density
OPD	O-Phenylenediamine dihyrochloride
p	Plasmid
PBS	Phosphate Buffered Saline
PBST	PBS + 0.01% Tween 20
PCR	Polymerase chain reaction
PP	Peyer's patches
PPD	Purified protein derivative
PNA	Peanut agglutinin
PNAd	Peripheral Node Addressin
rBCG	Recombinant BCG
RD	Region of difference
rpm	Revolutions per minute
RT	Room temperature
s.c.	Subcutaneous

TBS	Tris buffered saline
TCR	T cell receptor
TetC	Tetanus toxin fragment C
T_H	T-helper
TNF	Tumour necrosis factor
TLR	Toll-like receptor
Tween	Polyoxyethylene-sorbitan monolaurate
URT	Upper respiratory tract
VCAM-1	Vascular cell adhesion molecule-1
WHO	World Health Organisation

1 Introduction

1.1 *Mycobacterium tuberculosis*

1.1.1 General features

Mycobacterium tuberculosis is a human restricted non-motile rod-shaped bacterium that is a member of the family Mycobacteriaceae within the order Actinomycetales. *M. tuberculosis* is a weakly gram positive obligate aerobe with a slow generation time of 15-20 hours. The bacterium is classified as 'acid-fast' due to its ability to retain certain dyes and stains only after being treated with an acidic solution. The cell wall structure of *M. tuberculosis* is uncommon among prokaryotes as in addition to peptidoglycan it has an unusually high lipid content (over 60%). The lipid fraction has 3 major components; mycolic acids, cord factor and wax-D, which are highly stimulatory to the host's immune system and as a result are used in Freund's adjuvant.

1.1.2 History

Tuberculosis is an ancient scourge of humankind. Skeletal remains show prehistoric humans (4000 BC) had tuberculosis, and fragments of the spinal column from Egyptian mummies, dated 2400 BC, have been shown to have significant pathological signs of tubercular decay (Herzog 1998). In the 17th and 18th centuries, *M. tuberculosis* was the cause of the "White Plague", which resulted in an almost 100% infection rate within the European population, and a 25% death rate. However, the bacillus causing tuberculosis, *M. tuberculosis*, wasn't identified and described until March 24, 1882 by Robert Koch. This pioneering tuberculosis research was facilitated by developing culture media in which to grow the organism, and then demonstrating the mode of transmission of the disease (Koch 1882).

1.1.3 Epidemiology

Currently, nearly 2 billion people, over a third of the world's population, are infected with *M. tuberculosis*, with new infections occurring at a rate of one per second. Annually, 9 million people become ill from *M. tuberculosis* and approximately 2 million die from the disease worldwide, mostly in developing countries. The World Health Organisation (WHO) estimates that the largest number of new tuberculosis cases in 2005 occurred in the South-East Asia Region, which accounted for 34% of incident cases globally. However, the estimated incidence rate in sub-Saharan Africa is nearly twice that of the South-East Asia Region, at nearly 350 cases per 100 000 population (see Figure 1.1). Tuberculosis is also the leading cause of death among Human Immunodeficiency Virus (HIV) infected people; it has been estimated that the disease accounts for up to a third of acquired immunodeficiency syndrome (AIDS) deaths worldwide and is the single most important factor determining the increased incidence of tuberculosis in Africa over the past 10 years (WHO 2007a). Along with HIV/AIDS and malaria, tuberculosis remains as one of the 3 main killers among global infectious diseases (WHO 2007b).

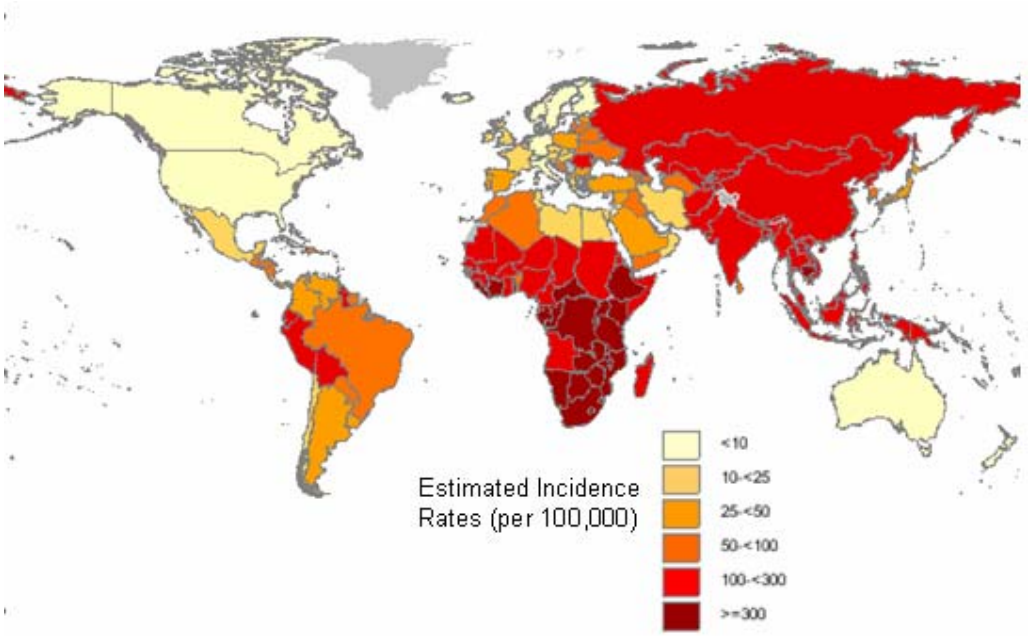


Figure 1.1: Tuberculosis incidence rates 2005 (WHO 2005).

M. tuberculosis is a human restricted pathogen spread from person to person by aerosols. Even after close contact with an infectious case, only about 10 percent of susceptible contacts acquire the infection, as determined by a delayed-type hypersensitivity response to purified protein derivative (PPD). These patients usually present with pulmonary disease; prominent symptoms are chronic, productive cough, low-grade fever, night sweats, malaise, and weight loss. *M. tuberculosis* may also spread from the lungs, causing extrapulmonary manifestations including lymphadenitis; kidney, bone, or joint involvement; meningitis; or disseminated (miliary) disease. Of those infected, only 3-4% will go onto develop active disease upon initial infection, and 5-10% within 1 year. Other factors that contribute to tuberculosis incidence is susceptibility to disease. A number of conditions that are associated with altered host cellular immunity increase the risk of developing active tuberculosis. These include HIV infection, extremes of age, immunosuppressive therapy, cancer, end stage renal disease, diabetes and severe malnutrition. Some genetic factors also predispose populations to tuberculosis including polymorphisms in the natural resistance-associated macrophage protein-1 (NRAMP1) gene, vitamin D receptors and components of the interferon gamma (IFN-γ)-signalling pathways. Reviewed by (Frieden, et al. 2003).

Tuberculosis is normally diagnosed by a skin test known as the tuberculin or Mantoux test. PPD is employed as the test antigen in the Mantoux test, where it is injected intracutaneously into the forearm and the test is read within 48-72 hours. The test is considered positive if the diameter of a resulting lesion is 10 mm or greater. Lesions are characterised by erythema (redness), swelling and induration (raised and hard).

The backbone of tuberculosis therapy is a cocktail of antibiotics that are effective primarily against Mycobacteria. A course of drug therapy usually lasts from 6-9 months. The most commonly used drugs are rifampicin, isoniazid, pyrazinamide and ethambutol or steptomycin. However, drug resistance in tuberculosis has been steadily increasing since the introduction of anti-mycobacterial medicines over 50 years ago. Multidrug-resistant tuberculosis is a form of the disease that is resistant to two or more of the primary drugs used for treatment; most commonly isoniazid and rifampicin. The WHO estimates that up to 50 million persons worldwide may be infected with drug resistant strains of *M. tuberculosis* (WHO 2007b).

1.1.4 General features of pathogenesis

Tuberculosis infection begins when the mycobacteria are inhaled and lodge in the pulmonary alveoli of the distal airways, where they then enter and replicate within alveolar macrophages. *M. tuberculosis* uses various strategies to avoid being killed by phagocytes. The bacterium can inhibit acidification of the phagosome, modify intracellular trafficking of vesicles, and cause quantities of lipoarabinomannan (LAM) to insert into glycosylphosphatidylinositol (GPI)-rich domains in the cell membrane (Sturgill-Koszycki, et al. 1994; Xu, et al. 1994). LAM is itself a GPI of unusual glycan structure that has the ability to modify numerous macrophage functions, including the response to IFN-γ and the ability to present antigen (Xu, et al. 1994). Accumulation of bacteria within the lungs leads to an influx of leukocytes, predominantly IFN-γ secreting natural killer cells (NKC) and then later $CD4^+$ T cells (T_H1-type cells) along with cytotoxic $CD8^+$ T lymphocytes (CTL) (Iho, et al. 1999; Junqueira-Kipnis, et al. 2003; Orme, et al. 1993; Serbina and Flynn 1999). IFN-γ is the central mediator of macrophage activation, which can lead to the containment and elimination of bacteria (Flynn, et al. 1993; Fritsche, et al. 2003; Ottenhoff, et al. 1998). IFN-γ also synergies with tumour necrosis factor alpha (TNF-α) in activating macrophages (Chan, et al. 2001). Macrophage control of mycobacteria occurs via a variety of mechanisms including acidification of their phagosomes, production of toxic effector molecules and apoptosis (Fayyazi, et al. 2000; Flynn and Chan 2001). Activated macrophages generate nitric oxide (NO) and related reactive nitrogen intermediates (RNI) via inducible nitric oxide synthase (iNOS2) using L-arginine as a substrate (Jagannath, et al. 1998; MacMicking, et al. 1997; Nicholson, et al. 1996; Wang, et al. 2001; Zwilling, et al. 1999) (see Figure 1.2). This innate defence mechanism has been well documented with regards to the murine system, and plays an important role in both chronic and latent infection (Chan, et al. 2001).

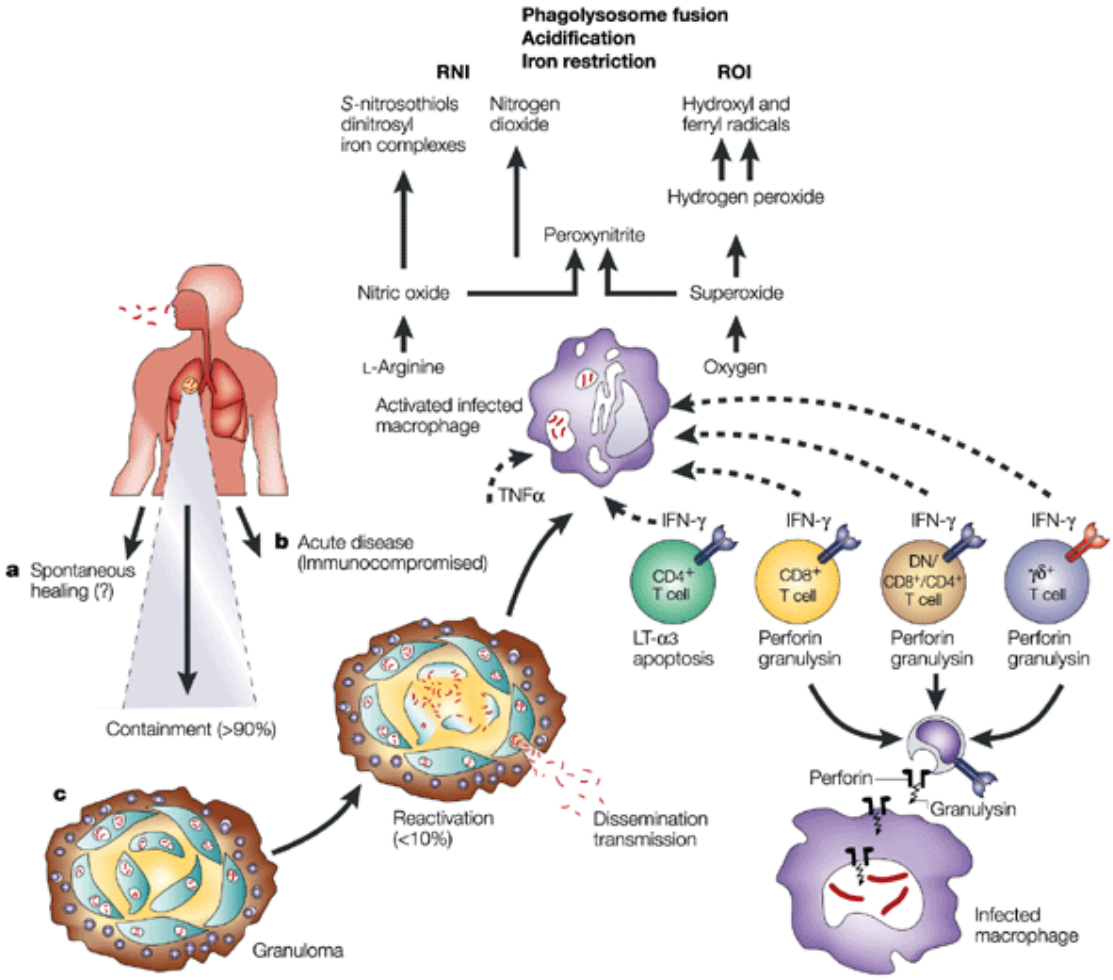


Figure 1.2: Main features of tuberculosis: from infection to host defence.

There are three potential outcomes of infection of the human host in *M. tuberculosis*. **a**. The frequency of abortive infection resulting in spontaneous healing is unknown, but is assumed to be minute. **b.** In the immunocompromised host, disease can develop directly after infection. **c.** In most cases, mycobacteria are initially contained and disease develops later as a result of reactivation. The granuloma is the site of infection, persistence, pathology and protection. Effector T cells (including conventional $CD4^+$ and $CD8^+$ T cells, and unconventional T cells, such as γδT cells, and double-negative or CD4/CD8 single-positive T cells that recognize antigen in the context of CD1) and macrophages participate in the control of tuberculosis. IFN-γ and TNF-α, produced by T cells, are important macrophage activators. Macrophage activation permits phagosomal maturation and the production of antimicrobial molecules such as reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). LT-α3, lymphotoxin-α3 (Kaufmann 2001).

The production of anti-inflammatory cytokines such as interleukin-10 (IL-10) and IL-4 in response to *M. tuberculosis* may down regulate the immune response and limit tissue injury by inhibiting excessive inflammatory responses. However, if produced in excess these cytokines may lead to a failure to control infection resulting in widely disseminated tuberculosis (Appelberg, et al. 1992; de Waal Malefyt, et al. 1991; Fulton, et al. 1998; Gong, et al. 1996; Hirsch, et al. 1999; Murray, et al. 1997; van Crevel, et al. 2002). It is therefore the balance between the inflammatory and protective immune response that determines the outcome of tuberculosis infection. $CD4^+$ T cells also produce lymphotoxin α (LTα), which participates in protection against tuberculosis (Roach, et al. 2001). At least some $CD8^+$ T cells, γδ T cells, and CD1 restricted T cells secrete perforin and granulysin which directly kill mycobacteria within macrophages (Behr-Perst, et al. 1999; Kaufmann 1999; Porcelli and Modlin 1999; Stenger 2001; Stenger, et al. 1998; Stenger, et al. 1997) (see Figure 1.2). This cell mediated immune response helps to control *M. tuberculosis* infection primarily because *M. tuberculosis* replicates within macrophages, thus T cell effector mechanisms are required for elimination of infection (see Figure 1.3 for summary of cell mediated activation). However, cell mediated immunity (CMI) is also responsible for much of the pathology associated with tuberculosis.

The humoral immune response has, for many years, been dismissed as having any defensive role with regards to *M. tuberculosis* infection. As *M. tuberculosis* is an intracellular pathogen, B cells and the antibodies that they produce may not have access to the mycobacteria and consequently may be unable to play any protective role. However some recent studies suggest that this type of immune response may in fact contribute to tuberculosis immunity (Bosio, et al. 2000; Johnson, et al. 1997; Pethe, et al. 2001; Vordermeier, et al. 1996; Williams, et al. 2004).

The next stage of infection is signalled by development of granulomatous lesions characterised by a mononuclear cell infiltrate surrounding a core of degenerating epithelioid and multinucleated giant (Langhans) cells. TNF-α has also been found to play a key role in this granuloma formation and the containment of latent infection (Kindler, et al. 1989; Mohan, et al. 2001; Senaldi, et al. 1996). This lesion (called a tubercle) may become enveloped by fibroblasts, and its centre often progresses to caseous necrosis. Liquefaction of caseous material and erosion of the tubercle into an

adjacent airway may result in cavitation and the release of massive numbers of bacilli into the sputum, greatly increasing the contagiousness of that individual. Escape of bacteria from these lesions, into the lymphatics or blood enables spread of infection to almost any anatomical location, and results in extrapulmonary tuberculosis, otherwise known as miliary tuberculosis. Within resistant hosts, the tubercle eventually becomes calcified and these lesions, known as Ghon complexes, are now readily visible upon chest X-ray. This containment of the infection within granulomas leads to latency of *M. tuberculosis* until such a point, usually impaired immunity, when reactivation can occur, years or decades later. See Figure 1.2 for the phases of infection of *M. tuberculosis*.

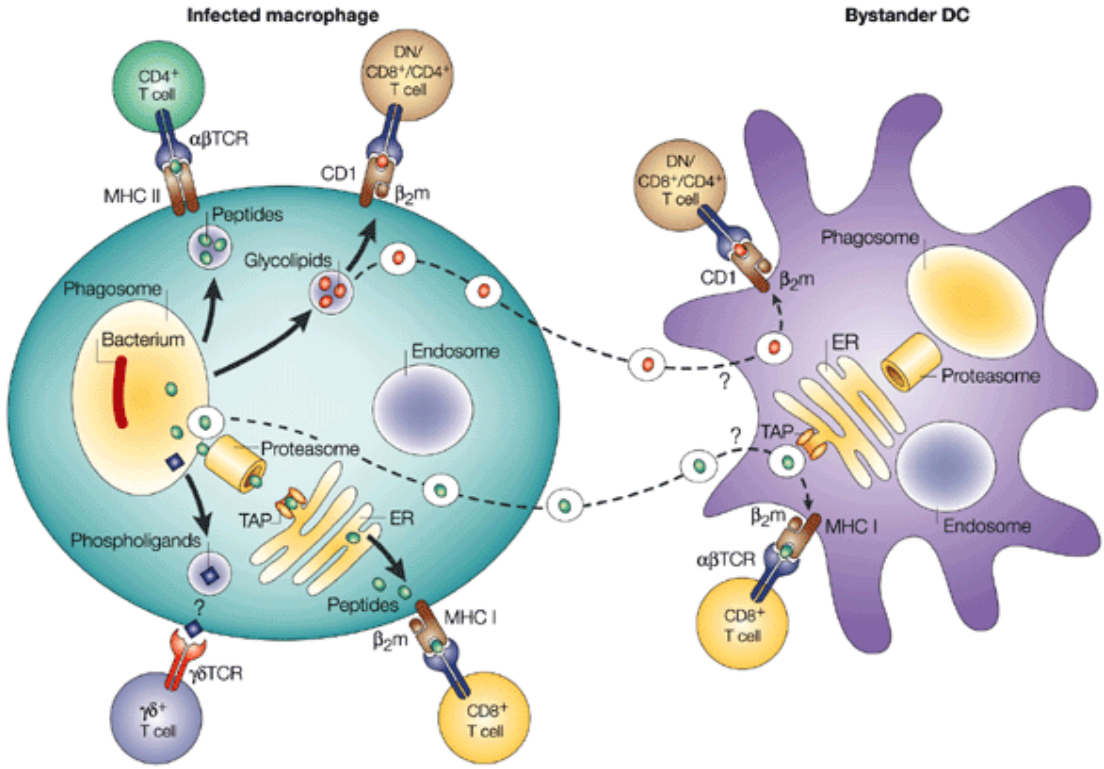


Figure 1.3: Antigen processing/presentation pathways and activation of different T-cell subsets. The different T-cell-processing pathways that result in activation of distinct T-cell populations in the immune response against *Mycobacterium tuberculosis* are shown. This pathogen preferentially resides in the phagosome of macrophages, where mycobacterial peptides have ready access to the major histocompatibility complex class II (MHC II) molecules that are shuttled to the cell surface and stimulate $CD4^+$ T cells. CD1 molecules also have access to mycobacterial glycolipids because they have contact with the phagosomal continuum at different stages of its maturation — glycolipids seem to separate from the mycobacteria and are incorporated into vesicles, which are shuttled throughout the cells and also seem to transfer antigen to bystander cells. CD1 molecules present mycobacterial glycolipids to various CD1-restricted T lymphocytes ($CD4^+$, $CD8^+$ or DN). The processing pathways for ligands for γδT cells and MHC-class-I-restricted T cells remain less understood. Mycobacterial phospholigands reach the cell surface through unknown ways, and are recognized by γδT cells in the apparent absence of specialized molecules. Although it is beyond doubt that MHC-class-I-restricted $CD8^+$ T cells are stimulated by mycobacterial peptides, the underlying mechanisms remain to be established. Some mycobacterial proteins may enter the cytosol and be introduced into MHC-class-I processing pathways, including cytosolyic proteasomes and TAP. Mycobacterial peptides and glycolipids may be transferred in vesicles along a novel pathway from infected macrophages to bystander dendritic cells. This could improve antigen presentation through MHC-class-I and CD1 pathways. Formation of these vesicles is probably stimulated during apoptosis of infected cells. β_2m, β_2-microglobulin; DN, double-negative; ER, endoplasmic reticulum; TAP, transporter of antigen processing. (Kaufmann 2001).

1.2 Current vaccines against *M. tuberculosis*

1.2.1 Bacille Calmette-Guérin (BCG) vaccine

The BCG vaccine has been used for over 80 years as the sole vaccine to protect against tuberculosis. BCG was developed by the French microbiologists Calmette and Guérin using an attenuated *Mycobacterium bovis*, obtained by serial passage in a bile enriched culture medium. Since the introduction of BCG, over 3 billion people have been vaccinated with an excellent safety record that makes it one of the safest vaccines known. In spite of a global vaccine coverage of 80%, large numbers of field trails have shown that protective efficacy of BCG may vary greatly from 0 to 80% (Fine 2001). The vaccine has consistently high protective efficacy in preventing tuberculosis in children, but unfortunately it has variable efficacy against the most common form of disease, pulmonary tuberculosis in adults (Colditz, et al. 1994). Reasons for low efficacy of the BCG vaccine may be genetic and nutritional differences in susceptibility of different human populations, strain variation in BCG preparations, along with exposure to environmental mycobacteria and chronic parasite infection, which shift the balance of the immune response. See Figure 1.4 for events that may lead to failure of BCG in developing countries.

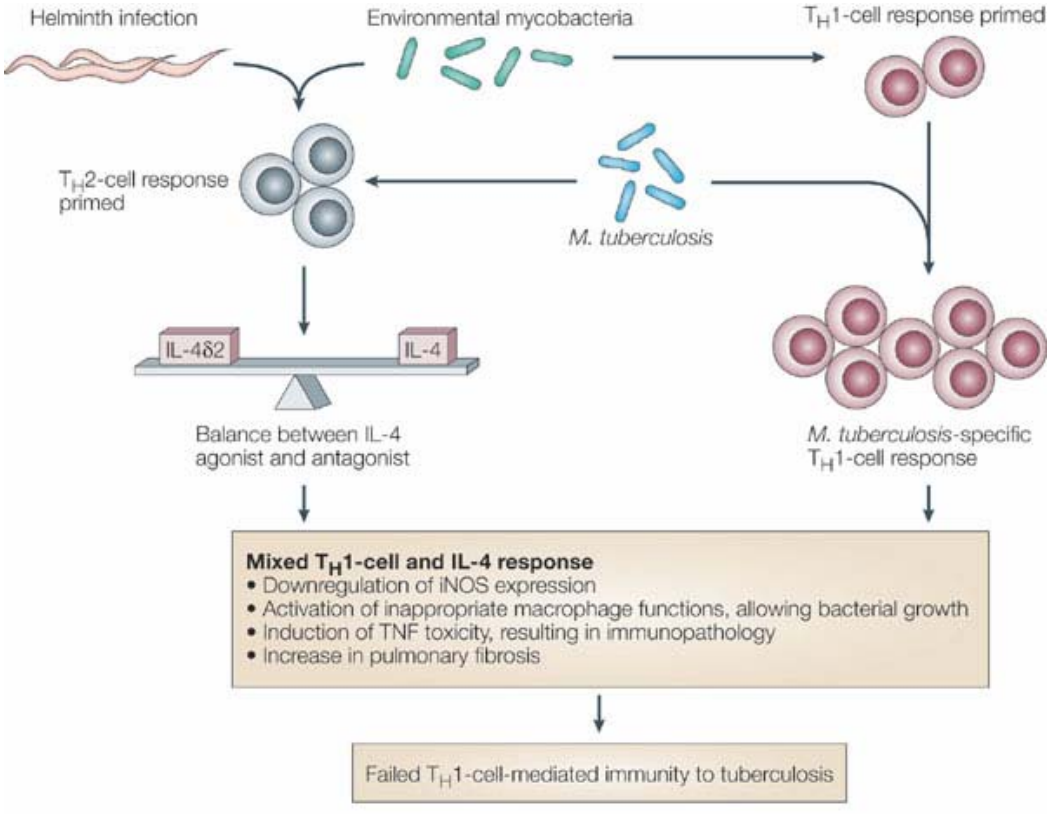


Figure 1.4: Events that might lead to failure of BCG in developing countries.

Environmental saprophytic mycobacteria prime T_H1- and T_H2-cell responses, the latter being promoted by infections with helminths in both mothers and children. Exposure to a low dose of *Mycobacterium tuberculosis* might not cause disease. Exposure to a high dose increases both the T_H1-cell and IL-4 responses. Several features of *M. tuberculosis*, particularly the Beijing genotype strains, enhance the IL-4 component of the response. IL-4 is partially counteracted by the increased expression of the splice variant IL-4δ2, which occurs in latently infected individuals, who do not develop disease. However, in many individuals, a mixed T_H1-cell and IL-4 response might compromise cell-mediated immunity to *M. tuberculosis*, because IL-4 down regulates iNOS expression and microbicidal activity, promotes TNF toxicity, and exacerbates fibrosis despite the presence of large quantities IFN-γ, which is an inhibitor of fibrosis. The result is a failure of protection and the development of disease, with unusually high levels of IL-4. An effective vaccine for citizens in developing countries might therefore need to block the IL-4 response, rather than induce a T_H1-cell response, which is already present (Rook, et al. 2005).

Another possible explanation for the inability of current live BCG to protect against pulmonary tuberculosis may be the immunisation route. BCG is delivered via the parenteral route which may not provide optimal immune responses in the lung. Delivery via mucosal routes may elicit a local respiratory mucosal immunity which may increase protection against *M. tuberculosis* infection. Reviewed in (Haile and Kallenius 2005). Oral administration was the route initially used by Calmette and Guérin, but was replaced by intradermal administration in virtually all countries after the Lubeck accident, in which 67 of 249 babies given the vaccine died due to contamination of the BCG with virulent *M. tuberculosis* (Andersen and Doherty 2005). However, the administration of oral BCG (BCG Moreau Rio de Janeiro) was maintained in Brazil and was shown capable of inducing a more substantial mucosal and systemic immune response compared to the intradermal route (Gheorghiu 1994). In fact, a recent study has shown that oral BCG vaccination induces T cells that home specifically to the lung, which provide protective local immunity in vaccinated mice (Dorer, et al. 2007). Intranasal administration of BCG is an attractive route for immunisation as intranasal vaccination may induce local immune responses in the lung, which are required for full protection of the host. In addition this local immunity may increase the speed of the overall immune responses against *M. tuberculosis* and therefore reduce initial pathology. Lyadova and colleagues showed that intranasal vaccination with BCG induced a high degree of protection against systemic challenge, and that this protection correlated with a rapid production of IFN-γ after challenge by lung T cells from vaccinated mice (Lyadova, et al. 2001). Another group reported that intranasal application of BCG-Pasteur strain was found to be highly protective against challenge infection with the pathogenic H37Rv strain given after a 4-week interval, reflected by the 100-fold reduction of CFUs in both lungs and spleens. In addition they observed that intranasal vaccination abrogated the confluent infiltration of lungs with inflammatory cells, which surrounds the granulomas in H37Rv challenged control mice (Falero-Diaz, et al. 2000).

More recent deletion analyses of the genome of different BCG strains by DNA microarray technology have shown that BCG have lost some genes now thought to be important for protective immunity. Loss of genes may have occurred during the original attenuation processes from the parental strain, or during further propagation, before the lyophilisation of seed lots was introduced in the 1960s (Hart 1967). Their analysis showed that different BCG strains lack up to 134 open reading frames (ORFs) from 16 regions of difference (RD1-RD16) compared to *M. tuberculosis* H37Rv (Behr, et al. 1999). Major antigenic proteins were found to be present in virulent mycobacteria, but either absent (early secretory antigen target 6kDa, ESAT-6; culture filtrate protein 10, CFP-10; and mycobacterial culture filtrate protein 64, MPT64) or not expressed (MPB70 and MPB83) in several BCG vaccines (Vordermeier, et al. 1999) (see Figure 1.5).

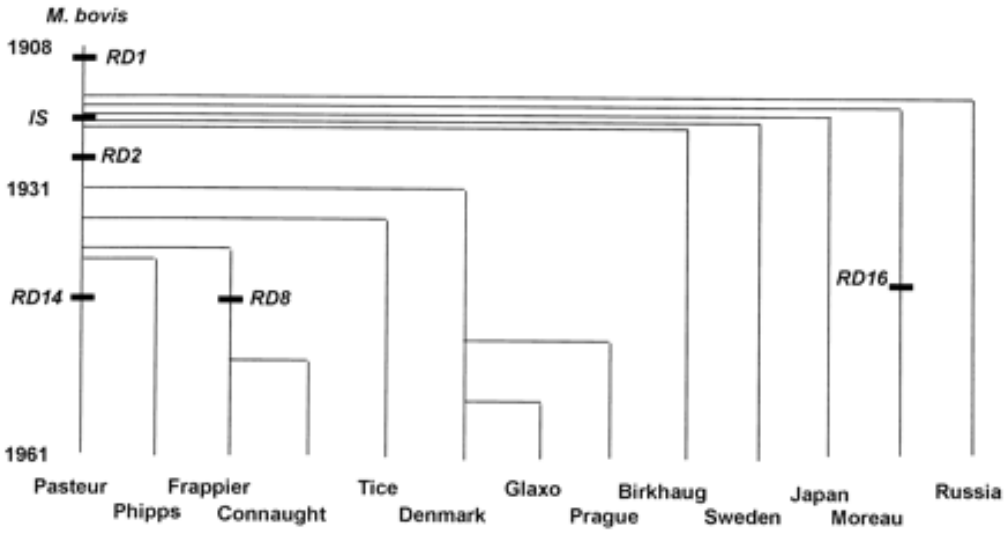


Figure 1.5: BCG historical genealogy incorporating genetic differences previously noted and newly detected genetic deletions.

Vertical axis represents time. Horizontal axis denotes different geographic locations of BCG propagation. Under this reconstruction, the *M. bovis* strain that was used to develop BCG would be missing RD3, RD4, RD5, RD6, RD7, RD9, RD10, RD11, RD12, RD13, and RD15. During serial propagation of this strain, RD1, RD2, RD8, RD14, RD16, and an IS*6110* element (IS) were deleted (Behr, et al. 1999).

1.3 New tuberculosis vaccines

Immunisation is the most effective public health tool used to control infectious disease. Moreover, it is extremely cost effective given that treatment of disease is far more expensive than disease prevention. Considering the shortcomings of the current BCG vaccine with regards to protection against adult pulmonary tuberculosis, and the overwhelming worldwide problem of tuberculosis, it is now clear that the development of a more effective vaccination strategy is urgently needed. A number of approaches can be considered in the fight to decrease global tuberculosis morbidity and mortality (see Figure 1.6 below).

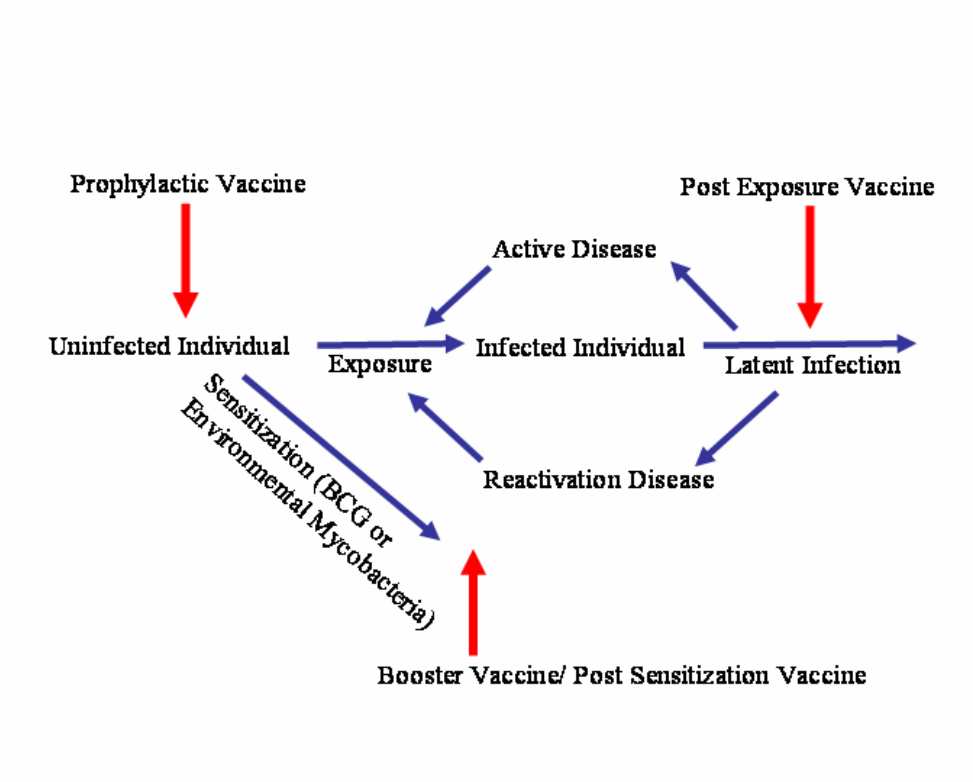


Figure 1.6: Different tuberculosis vaccination strategies.

A prophylactic vaccine to prevent primary infection and disease following exposure, a booster/post-sensitisation vaccine to boost BCG or to vaccinate on top of environmental sensitisation, and finally a post-exposure vaccine to prevent reactivation in those already infected (Olsen and Andersen 2003).

Tuberculosis research has received a major boost in funding over the past decade. This coupled with sequence data from the genomes of 2 strains of *M. tuberculosis* and 1 BCG vaccine strain as well as the introduction of many new molecular techniques has lead to nearly 200 vaccine candidates (Figure 1.7) (Brosch, et al. 2007; Cole and Barrell 1998; Philipp, et al. 1998). They include; recombinant BCG (rBCG) vaccines, live attenuated strains of *M. tuberculosis*, non-pathogenic mycobacteria, non-mycobacterial microbial vectors, DNA vaccines, and subunit vaccines.

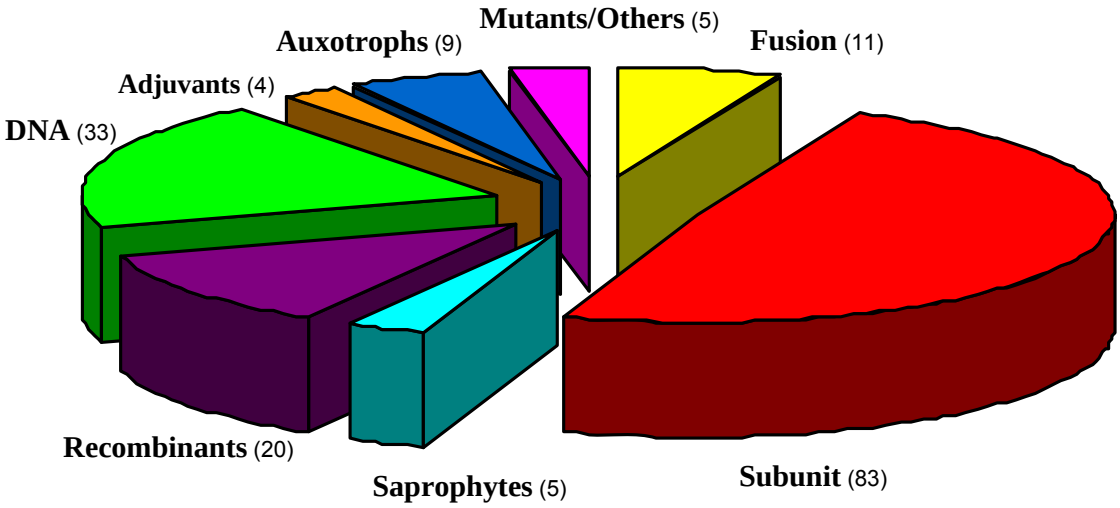


Figure 1.7: Tuberculosis vaccines currently undergoing screening.

1.3.1 Recombinant BCG vaccines

Recombinant BCG (rBCG) techniques may be useful for the development of a more effective mycobacterial vaccine than the parental BCG now in use. One rBCG vaccine that has been developed (rBCG30), over-expresses the 30kDa major secretory protein, Ag85B, of *M. tuberculosis*. This vaccine candidate has shown promising results in animal models. Animals immunised with rBCG30, when challenged by aerosol with a highly virulent strain of *M. tuberculosis*, exhibited much less lung, spleen and liver pathology, had approximately 10-fold fewer lesions, and 10-fold fewer bacilli organisms in their tissues than BCG-immunised animals. In addition, rBCG30 vaccinated animals exposed to tuberculosis infection survived significantly longer than BCG vaccinated groups (Horwitz and Harth 2003). Furthermore, this vaccine candidate was the first tuberculosis vaccine to enter clinical trials in the United States

for over 60 years, entering phase I trials in 2004 (McCarthy 2004). Pym and colleagues demonstrated that immunisation with rBCG RD1-2FP, a rBCG exporting ESAT6, which is missing in BCG, gave better protection against challenge with *M. tuberculosis* as well as decreasing severe pathology and reducing dissemination of the pathogen, compared to mice immunised with BCG alone (Pym, et al. 2003). By complementing BCG with cholera toxin B subunit (CT-B), a mucosal immunogen, an increased immune response was induced indicated by increased immunoglobulin A (IgA) and IgG production compared to mice immunised with nonrecombinant BCG (Biet, et al. 2003). Recently, a rBCG that secretes biologically active membrane-perforating listeriolysin (Hly) of *Listeria monocytogenes* has been constructed. Following aerosol challenge, the Hly-secreting rBCG (hly^+ rBCG) vaccine was shown to protect significantly better against aerosol infection with *M. tuberculosis* than the parental BCG strain. This rBCG improves MHC class I-presentation of co-phagocytosed soluble protein (Grode, et al. 2005). Other candidates shown to be effective in animal models, indicated by elevated cell-mediated immunity and significant reduction in CFUs in lungs compared to BCG vaccinated animals, are rBCGs secreting various cytokines (Luo, et al. 2004; Murray, et al. 1996).

1.3.2 Live mycobacterial vaccines

A number of groups have generated attenuated *M. tuberculosis* strains with the goal of obtaining a vaccine with superior efficacy to BCG. Perez and colleagues have used this approach to disrupt the *phoP* gene of *M. tuberculosis* which is important in regulation of several virulence genes (Perez, et al. 2001). Auxotrophic mutants of *M. tuberculosis* have also been proposed as new vaccine candidates. *M. tuberculosis* strains containing defined mutations in the genes involved in proline (*proC*) or tryptophan (*trpD*) amino acid biosynthesis were found to be significantly attenuated within Severe Combined Immunodeficient (SCID) mice indicating their potential as vaccine candidates (Smith, et al. 2001). Several non-pathogenic mycobacteria have also been proposed as potential tuberculosis vaccines, including *Mycobacterium microti*, and *Mycobacterium vaccae*. Most recently, *M. vaccae* has been evaluated as an immunotherapeutic agent in two relatively large human trials: a phase III trial in Durban, South Africa and an NIAID-supported phase I/II trial in Kampala, Uganda (Johnson, et al. 2000; Johnson, et al. 2004; Vuola, et al. 2003)

1.3.3 Non-mycobacterial vaccine vectors

Live vectors, such as recombinant viruses or bacteria expressing immunodominant *M. tuberculosis* proteins have generated some encouraging data in animal models. The recombinant vaccinia virus Ankara expressing Ag85A was the first phase I study of any recombinant vaccine against tuberculosis. McShane et al showed that recombinant modified vaccinia virus Ankara (MVA) expressing Ag85A (MVA85A) was safe and highly immunogenic in study subjects. MVA85A was found to induce high levels of antigen specific IFN-γ-secreting T cells when used alone in previously naive volunteers. Healthy individuals, who had been vaccinated 1-38 years previously with BCG, had substantially higher levels of IFN-γ-secreting T cells (McShane, et al. 2005; McShane, et al. 2004). This is the only tuberculosis vaccine currently in phase II trails and is currently being tested for efficacy in *M. tuberculosis* endemic South Africa (Ibanga, et al. 2006). Sereining and colleagues generated several subtypes of attenuated recombinant influenza A viruses expressing ESAT-6 of *M. tuberculosis* from the NS1 reading frame. Those mice vaccinated with the recombinant virus constructs were found to have strong T_H1 type immune responses. Moreover, intranasal immunisation of mice and guinea pigs with such vectors induced protection against mycobacterial challenge, similar to that induced by BCG vaccination (Sereinig, et al. 2006). Recombinant *S.* Typhimurium vaccine carrier strains expressing ESAT-6 or Ag85B have also been constructed using the haemolysin (HlyA) secretion system from uropathogenic *Escherichia coli*. Vaccination with a single dose of recombinant *S.* Typhimurium secreting ESAT-6 increased the number of IFN-γ-secreting T cells after vaccination, and reduced numbers of *M. tuberculosis* in the lungs after challenge as compared to naïve mice, but this regimen was still not as efficacious as the BCG immunised controls (Mollenkopf, et al. 2001). Hess et al have developed a recombinant *S.* Typhimurium secreting Ag85B which was shown to confer partial protection against intravenous challenge with *M. tuberculosis*. The immune response induced by recombinant *S.* Typhimurium Ag85B was accompanied by augmented IFN-γ and TNF-α levels produced by restimulated splenocytes (Hess, et al. 2000).

1.3.4 DNA vaccines

The use of a DNA vaccine offers many advantages: such vaccines can be easily manipulated, are safe, and can be easily stored and transported, hence the reason why a number of candidate DNA vaccines have emerged in recent years in the fight against tuberculosis. These candidates have been tested in various studies for their efficacy against tuberculosis in animal models and some have been shown to confer protective immunity. These plasmid vaccines code for various mycobacterial antigens including members of the mycolyl-transferase family (Ag85 complex), and heat shock proteins 60, 65, 70 (Hsp60, 65, 70). A tuberculosis DNA vaccine encoding Ag85A was found to be immunogenic with elevated IL-2 and IFN-γ levels along with antigen specific antibody and CTL responses. The DNA vaccine was also found to be protective when administered via intramuscular injection and then boosted with protein (Ag85A) and adjuvant. Prime-boosted animals exhibited reduced numbers of CFU in the lungs and spleen, compared to animals vaccinated with naked DNA (Tanghe, et al. 2001; Tanghe, et al. 2000). Recently another DNA vaccine expressing ESAT6-Ag85B (pE6/85) was shown to protect similarly to BCG in short-term vaccination studies. Furthermore, boosting with pE6/85 after an initial BCG immunisation 1 year previously, augmented protection in the lung of C57BL/6 mice (Derrick, et al. 2004). Ferraz and colleagues also used a heterologous prime-boost immunisation approach after initial immunisation with DNA plasmids encoding *M. tuberculosis* Apa (alanine-proline-rich antigen), and the immunodominant Hsp65 and Hsp70 antigens and boosting with BCG. This study showed an increase in specific anti-mycobacterial immune responses, and protection as compared to animals vaccinated with BCG alone (Ferraz, et al. 2004). These latter two studies indicate the important role that DNA vaccines may play in the prime/boosting of the current tuberculosis vaccine BCG. Recently, Parida et al used an attenuated *Salmonella aroA* strain to carry a eukaryotic expression plasmid encoding Ag85A as a DNA vaccine delivery system. Strong cellular immune responses were induced after vaccination as well as reduced *M. tuberculosis* CFU in the lungs and the spleens after challenge (Parida, et al. 2005).

1.3.5 Subunit vaccines

Key *M. tuberculosis* antigens have been identified from the plethora of mycobacterial components including proteins, lipids, and carbohydrates using a variety of biochemical, molecular, and immunologic approaches. These selected antigens have been used for the development of subunit vaccines against tuberculosis and are among some of the most prominent vaccine candidates. These include: ESAT-6, TB10.4, Ag85B, the fusion protein vaccines compromising Ag85B and ESAT-6 or TB10.4, and another consisting of Mtb32 and Mtb39 (Mtb72F). Previous work on the antigens ESAT-6 and Ag85B has shown that the fusion of Ag85B-ESAT6 was more immunogenic and gave higher levels of protection compared to the individual antigens as well as the fusion in the other orientation, i.e. ESAT6-Ag85B. The vaccination studies performed using this fusion protein (Ag85B-ESAT6) will be discussed in more detail later. Recently the Andersen group exchanged ESAT-6 with TB10.4 in an Ag85B fusion subunit vaccine. TB10.4 is a strongly immunogenic protein that belongs to a subfamily of the *esat-6* gene family and is recognised by BCG-vaccinated donors and TB patients (Skjot, et al. 2002; Skjot, et al. 2000). Subcutaneous immunisation of mice with Ag85B-TB10.4 induced superior protection against compared to the individual antigen components (Dietrich, et al. 2005). Another subunit vaccine, Mtb72F, which codes for a 72-kDa polyprotein (Mtb32(C)-Mtb39-Mtb32 (N)), has also been tested extensively in animal models. Immunisation of mice with Mtb72F protein formulated in the adjuvant AS01B generated a comprehensive and robust immune response, eliciting strong IFN-γ and antibody responses for all three components of the polyprotein vaccine, and a strong $CD8^+$ response directed against the Mtb32(C) epitope. Mtb72F immunisation resulted in the protection of C57BL/6 mice against aerosol challenge with a virulent strain of *M. tuberculosis* (Skeiky, et al. 2004). Most importantly, immunisation of guinea pigs with Mtb72F resulted in prolonged survival (> 1 yr) after aerosol challenge with virulent *M. tuberculosis* comparable to BCG immunisation (Brandt, et al. 2004). Mtb72F in the AS02A formulation (the AS02A adjuvant contains monophosphoryl lipid A (MPL), QS21 and an oil in water emulsion) is currently in phase I clinical trials, making it the first subunit tuberculosis vaccine to be tested in humans.

1.3.6 *M. tuberculosis* vaccine antigens

1.3.6.1 Antigen 85B

Antigen 85B (Ag85B) is a 30kDa mycolyl transferase and is part of the *M. tuberculosis* 85 complex, which also includes the closely related 32kDa mycolyl transferase proteins; antigen 85A and 85C (Content, et al. 1991). All three show 70-80% amino acid homology and contribute to cell wall biogenesis by catalysing transfer of the fatty acid mycolate from one trehalose monomycolate to another, resulting in trehalose dimycolate (cord factor) and free trehalose (Anderson, et al. 2001; Wiker and Harboe 1992). Ag85B is the most abundant protein exported by *M. tuberculosis*, and has been shown to bind fibronectin (Abou-Zeid, et al. 1988; Salata, et al. 1991). Ag85B induces strong T cell proliferation and IFN-γ secretion in most healthy individuals exposed to *M. tuberculosis*, and in both BCG-vaccinated mice and humans (Geluk, et al. 2000; Huygen, et al. 1994; Kariyone, et al. 2003; Launois, et al. 1994; Mustafa, et al. 2000b; Thole, et al. 1999). Arrest of the multiplication of *M. tuberculosis* caused by induction of adaptive immunity in the mouse lung, was shown to be accompanied by a 10- to 20-fold decrease in levels of mRNAs encoding the secreted Ag85 complex (Shi, et al. 2004). This data compliments another study which showed antibodies against Ag85 are most prevalent in active tuberculosis patients with decreased cellular immune responses (Van Vooren, et al. 1992). In terms of vaccine development, the most prominent characteristic of Ag85B is the fact that it has been shown to induce protection in animal models (Abou-Zeid, et al. 1988; Horwitz and Harth 2003; Horwitz, et al. 2000; Horwitz, et al. 1995).

1.3.6.2 Early Secreted Antigenic Target-6

The early secreted antigenic target-6 (ESAT6) is a small (6kDa) protein secreted by *M. tuberculosis* belonging to the ESAT-6 family. ESAT6 was isolated from short-term culture filtrate proteins (CFP) and proved to be the most potent among the isolated proteins at inducing immune responses from isolated lymphocytes in mice, cattle and later in humans (Andersen, et al. 1995; Sorensen, et al. 1995). ESAT6 was found to be a very strong T cell antigen during the early phase of infection in a number of animals, with a very high proportion of tuberculosis patients (96%) recognising ESAT-6 (Brandt, et al. 1996; Lalvani, et al. 2001; Pollock and Andersen 1997; Ravn, et al. 1999). In addition to T cell responses, strong antibody responses to ESAT6 have more recently been found in *M. tuberculosis* infected individuals, non-human primates and cattle (Brusasca, et al. 2003; Lyashchenko, et al. 1998a; Lyashchenko, et al. 1998b). The strong and frequent recognition of ESAT6 during *M. tuberculosis* infection, as well as its absence from the BCG vaccine, has stimulated strong interest in its potential to be used for both vaccine and diagnostic uses (Mahairas, et al. 1996). A number of studies have shown that ESAT-6 can confer significant protection in animal models after challenge with pathogenic *M. tuberculosis* (Brandt, et al. 2000; Huygen, et al. 1996; Kamath, et al. 1999; Olsen, et al. 2000). Several recent studies have shown that expression of ESAT-6 by *M. tuberculosis* may be associated with lower innate immune responses to infection. One study has suggested that this protein may dampen down innate immune responses though decreased production of IL-12p40, TNF-α and NO (Stanley, et al. 2003). Renshaw et al have determined the solution structure of the tight, 1:1 complex formed by ESAT-6 and CFP-10, another secreted mycobacterial protein. A striking feature of the complex is the long flexible arm formed by the C-terminus of CFP-10, which was found to be essential for binding to the surface of cells (Renshaw, et al. 2005; Renshaw, et al. 2002). Most recently, Pathak and colleagues have shown that ESAT-6 inhibited activation of transcription factor NF-κB and interferon-regulatory factors (IRFs) after TLR2 signaling. The six carboxy-terminal amino acid residues of ESAT-6 were required and sufficient for the TLR2-mediated inhibitory effect (Pathak, et al. 2007).

1.4 The mucosal immune system

Mucosal surfaces within the gastrointestinal and respiratory tracts are the main focus of both the adaptive and innate immune responses to infection (McGhee and Kiyono 1993). These surfaces enable the exchange of nutrients and gases essential for survival, and as a result the cells associated with these surfaces are vulnerable to attack by potential pathogens. The immune system associated with mucosal surfaces covers the largest area of the body (200-300 m^2) and has evolved tight regulatory mechanisms that counter infection by dangerous pathogens. However, the mucosal immune system can also discriminate commensals and harmless antigens to prevent inappropriate responses that could lead to immune pathology (Czerkinsky, et al. 1999). The mucosal system is associated with local immune responses and has specialised lymphoid tissues known as gut-associated lymphoid tissue (GALT) in the gut, bronchial-associated lymphoid tissue (BALT) in the respiratory tract and the nasal-associated lymphoid tissue (NALT) in the nose. These mucosal reactions can take place independently or synergistically with the systemic immune system. This common mucosal immune system (CMIS) connects these inductive sites (that is, the GALT and NALT) with effector sites (such as the lamina propria of the intestinal tract, and the nasal passages of the upper respiratory tract (URT)) for the generation of antigen-specific immune responses featured either by secretory IgA or cell-mediated immunity, which function as the first line of defence at mucosal surfaces (see Figure 1.8 below). Reviewed in (Kiyono, et al. 2001).

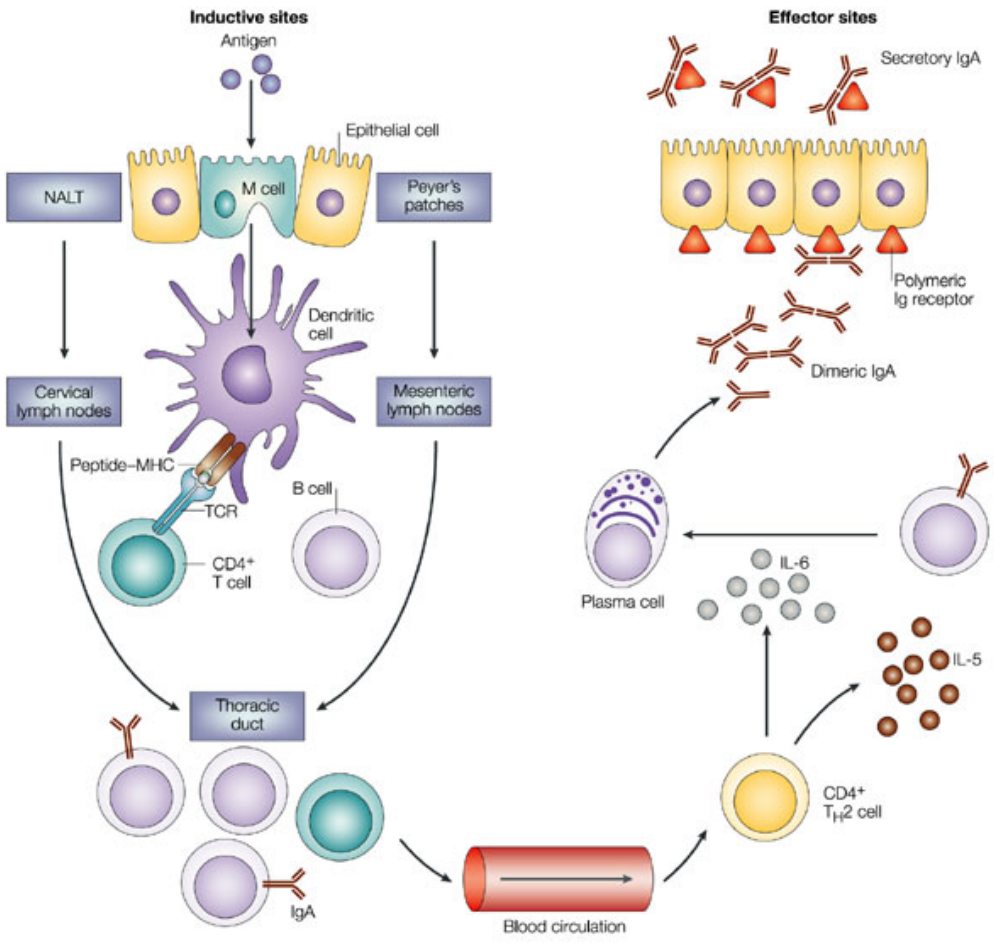


Figure 1.8: The CMIS

Luminal antigens are transported to the NALT and Peyer's patches (PP) through microfold (M) cells that are present in the epithelium overlying NALT and PP follicles. Dendritic cells (DC) process and present antigens to T cells in these lymphoid tissues. $CD4^+$ T cells that are stimulated by DC then preferentially induce IgA-committed B-cell development in the germinal centre (GC) of the lymphoid follicle. After IgA class switching and affinity maturation, B cells rapidly migrate from NALT and PP to the regional cervical lymph nodes (CLN) and mesenteric lymph nodes (MLN) respectively, through the efferent lymphatics. Finally, antigen-specific $CD4^+$ T cells and IgA^+ B cells migrate to effector sites (such as the nasal passage and intestinal lamina propria) through the thoracic duct and blood circulation. IgA^+ B cells and plasmablasts then differentiate into IgA-producing plasma cells in the presence of cytokines (such as IL-5 and IL-6) that are produced by T_H2 cells, and they subsequently produce dimeric (or polymeric) forms of IgA. These dimeric forms of IgA then become secretory IgA by binding to polymeric Ig receptors (which become the secretory component in the process of secretory IgA formation) that are displayed on the monolayer of epithelial cells lining the mucosa. Secretory IgA is then released into the nasal passage and intestinal tract. (Kiyono and Fukuyama 2004).

1.4.1 Mucosal immunisation

Most infectious agents are either restricted to the mucosal membranes or need to transit through them at some stage in the infection process. This has prompted studies aimed at the development of vaccination protocols that would lead to efficient immune responses and protection at both the mucosal and systemic level. The goal of current mucosal vaccination strategies is to prevent both initial stages of disease (colonisation and infection by pathogens) and block its development (Medina and Guzman 2000). Currently, the vast majority of vaccines available only block disease development once the pathogen has crossed the mucosal barrier into the normally sterile systemic environment (McCluskie and Davis 1999). Due to the apparent compartmentalisation of the systemic and mucosal immune systems, parental administration of vaccines is less effective in protecting against mucosal pathogens. Parenterally administered vaccines are not effective for eliciting mucosal secretory IgA (sIgA) responses and are generally ineffective against organisms that colonise mucosal surfaces and do not invade. The use of the mucosal route is associated by itself with a considerable number of advantages including low reactogenicity but high immunogenicity and reduced delivery costs. In addition, mucosal vaccines avoid the use of needles; hence the spread of diseases such as HIV via contaminated needles can be avoided (Holmgren, et al. 2003). These advantages could reduce the costs and increase the safety of vaccinations, an issue especially pertinent in the developing world. Through an understanding of the mucosal immune system as an immune communication network it is now apparent that administration of immunogens through a mucosal route not only induces immune responses at the targeted site but also has the potential to stimulate immune cells at remote mucosal sites.

1.4.2 Intranasal immunisation

The nasal mucosa is an important arm of the mucosal immune system as it is the first contact site with inhaled antigens. In fact, immunisation via the nose is an effective way to induce mucosal immune responses at remote effector sites, and a role for NALT in the induction of these responses has been recognised (Asanuma, et al. 1998; Davis 2001; Kiyono and Fukuyama 2004; Tamura, et al. 1998; Wu 1997; Zuercher, et al. 2002). As a result, intranasal immunisation has emerged as an attractive mucosal route for inducing both local and systemic immunity. The rationale behind the use of

intranasal vaccination has been considered by Partidos who has listed a number of reasons why this route is such an attractive choice for immunisation (Partidos 2000).

- Easily accessible.
- Highly vascularised.
- Presence of numerous microvilli covering the nasal epithelium generates a large absorption surface.
- After intranasal immunisation, both mucosal and systemic immune responses can be induced.
- Immune response can be induced at distant mucosal sites owing to the dissemination of effector immune cells in the CMIS.
- The nose can be used for the easy immunisation of large population groups.
- Intranasal vaccination avoids degradation of vaccine antigen caused by digestive enzymes, so requires a smaller dose of antigen than oral immunisation.
- Nasal immunisation does not require needles and syringes, which are potential sources of infection.
- Immunisation via this route does not require trained medical personnel for delivery, so reduces costs.

Intranasal immunisation is a particularly promising route of administration against respiratory infections. A number of studies have shown that following intranasal vaccination, potent antibody and cellular immune responses are generated which can subsequently lead to protection against aerogenic challenge with a number of bacterial pathogens of both the lower and upper respiratory tracts (Dietrich, et al. 2006; Jabbal-Gill, et al. 1998; Oliveira, et al. 2006; Zhu, et al. 2006; Zuercher, et al. 2006). As the nose can act as an inducer and effector site for good secretory immune responses at distal mucosal sites such as the lung and vagina via the CMIS, nasal vaccines may have an important role in the prophylaxis of diseases involving other mucosal surfaces (HIV, *Chlamydia trachomatis* and *H. pylori*) (Borsutzky, et al. 2006; Keenan, et al. 2003; Marinaro, et al. 2003; Murthy, et al. 2007). Furthermore, in 2003 the cold attenuated reassortment live flu vaccine called FluMist© was licensed for use in the United States and currently this remains the only intranasal vaccine on the market for

humans (Mossad 2003). The introduction of this vaccine now opens the way for increasing use of intranasal delivery. Other intranasal vaccines under going phase I human trials include; a vaccine against norovirus (Ligocyte 2007) and a HIV protein-based vaccine candidate which comprises of HIV gp140 protein with the V2 loop deleted, delivered along with LTK63 (IAVI 2006). A possible disadvantage for intranasal vaccination is that to induce significant immune responses you normally need to co-administer an adjuvant with the vaccine antigen (see section 1.4.3. below). In 1997, a Swiss company (BernaBiotech) received approval to market an inactivated intranasal flu vaccine with the mucosal adjuvant heat labile toxin (LT). However, a high incidence of Bell's palsy was recorded in those patients vaccinated, and subsequently it was found that LT can induce inflammatory responses in the olfactory sites and the meninges of mice when delivered intranasally (Couch 2004; Mutsch, et al. 2004).

1.4.3 The mucosal adjuvants LT and LTK63

Most traditional vaccines are still administered systemically. Mucosal vaccination offers some important advantages over systemic immunisation, such as quick action, increased compliance and decreased risk of spread of infectious diseases due to contaminated syringes. However, most vaccines are unable to induce immune responses when administered mucosally, and require the use of strong mucosal immunogens (adjuvants) for effective delivery. An immunological adjuvant is defined as a "substance used in combination with a specific antigen that produced more robust immune response than the antigen alone" (Ramon 1924). These molecules have the unique property of inducing strong immune responses after contact with mucosal surfaces and one of the most potent and well characterised of these molecules discovered to date is *E. coli* heat labile toxin (LT). LT is an extremely powerful mucosal adjuvant when co-administered with soluble antigens (Martin, et al. 2002). As a result of its strong adjuvant properties it has been used extensively in many vaccination studies against a variety of bacterial, fungal, and viral pathogens (Cheng, et al. 1999; Gluck, et al. 1999; Hathaway, et al. 1995; Katz, et al. 1997; Weltzin, et al. 1997; Wu, et al. 1997a). For a comprehensive review see (Freytag and Clements 2005).

This adjuvant/toxin has ADP-ribosylating activity and has an AB_5 structure: the A subunit has the enzymatic activity that is responsible for toxicity, whereas the B

subunit is a pentameric oligomer that binds receptor(s) located on the surface of eukaryotic cells. Due to the adjuvants toxic properties, its use in humans has been hampered (Levine, et al. 1983; Zurbriggen, et al. 2003). However, using site-directed mutagenesis a number of LT mutants have been generated that are fully non toxic or with dramatically reduced toxicity, which still retain their strong adjuvanticity at the mucosal level (Douce, et al. 1995). Among these mutants are LTK63 (serine-to-lysine substitution at position 63 in the A subunit). The X-ray structure of LTK63 shows complete identity to wild-type LT with only lysine present within the catalytic site preventing any ADP-ribosylation (Merritt, et al. 1994; Sixma, et al. 1993). A number of animal studies have shown that LTK63 is a strong mucosal adjuvant when co-delivered with many different antigens, although its activity is reduced in comparison to wild-type LT. For comprehensive reviews see (Peppoloni 2003; Pizza, et al. 2001). Currently LTK63 is undergoing phase I human trails to determine safety.

1.4.4 Nasal Associated Lymphoid Tissue

1.4.4.1 General features

Rodent NALT is a paired bell-shaped secondary lymphoid organ located at the base of the nasal cavity and is considered the equivalent of the Waldeyer's ring (tonsils and adenoids) in humans (Kuper, et al. 1990; Kuper, et al. 1992). The NALT contains 10^5-10^6 cells that form a lymphoid cell aggregate which is covered with ciliated epithelium containing microfold (M) cells and follicle-associated epithelium (FAE). The NALT, situated at the entrance to the pharyngeal duct, is the most significant well organised mucosal inductive site in the URT. As well as this organised NALT (O-NALT) there is also less well organised NALT called diffuse NALT (D-NALT) which lines the nasal passages (Asanuma, et al. 1997; Kuper, et al. 1992; Liang, et al. 2001).

1.4.4.2 Structure of the NALT

Unlike Peyer's patches (PP) and peripheral lymph nodes which are already present in the embryo, fully developed NALT does not appear in mice until 5-8 weeks after birth (time of weaning). NALT is a well organised lymphoid structure consisting of B and

T cell areas interspersed with FAE, high endothelial venules (HEV), macrophages and dendritic cells (DC). Antigen-sampling M cells are also present in the respiratory epithelium that covers the NALT and enable the efficient absorption and sampling of particulate antigens that are inhaled (Hameleers, et al. 1989; Karchev and Kabakchiev 1984; Spit 1989). It is the specialised structure of M cells that allows this transport of macromolecules and particles across the epithelium. The basolateral surface of M cells is deeply invaginated whereby large intraepithelial pockets are formed, which serve as the collect site for transcytosed material. It is within this characteristic pocket feature of M cells that interactions between antigen and lymphocytes, macrophages and antigen presenting cells (APC) can occur for induction of an immune response. Very few studies have examined M cells within the NALT directly; most have concentrated on the M cells that overlie PP. However, due to the structural and distribution similarities between NALT and PP M cells it is likely that most characteristics found in PP M cells are attributable to those in NALT (Fujimura 2000). Differences that have been studied are the lectin binding patterns. Both rat and hamster M cells within the NALT stain specifically for GSI-B4, a lectin from *Griffonia simplicifolia*, directed against α-linked galactoses, whereas M cells within PP are specific for *Ulex europaeus* agglutinin-1 which binds to α-linked fucose (Giannasca, et al. 1997; Takata, et al. 2000). In addition to M cells, which can be found in small clusters or alone, the epithelium covering the NALT also contains a few mucus goblet cells. The NALT lymphoid population contains an equal number of B and T cells with a $CD8^+$ to $CD4^+$ T cell ratio of 1:4. Unlike PP which have small developed germinal centres (GC) in naïve animals, it is only upon direct stimulation with antigen that GC develop within the NALT (Asanuma, et al. 1997; Weinstein and Cebra 1991). The NALT therefore has all the lymphoid cells required for the development of systemic and local immunity after the intranasal administration of antigen (see Figure 1.9).

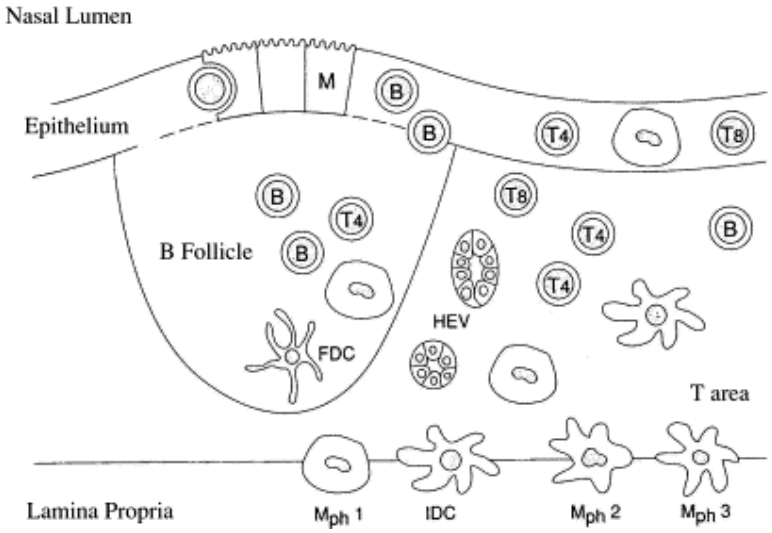


Figure 1.9: Schematic representation of compartments and cellular composition of NALT.
B, B cell; T4, CD4-positive T cell; T8, CD8-positive T cell; $M_{ph}1$, $M_{ph}2$, $M_{ph}3$, macrophages positive for ED1, ED2 or ED3 (macrophage surface antigens), respectively; IDC, interdigitating dendritic cell; FDC, follicular dendritic cell; HEV, high endothelial venule; M, microfold epithelial cell (Davis 2001).

Lymphocyte migration and the binding properties of HEV within NALT are unique among lymphoid tissues. All HEV situated within the NALT express peripheral node addressin (PNAd) alone, or PNAd associated with mucosal addressin cell adhesion molecule-1 (MAdCAM-1), with initial naïve lymphocyte binding being primarily mediated via PNAd-L-selectin interactions. In addition, HEV are also found in the B cell compartments of the NALT along with T cell areas (Csencsits, et al. 1999). The greatest addressin expression profile of follicular DC (FDC) found within the B-cell areas of the NALT is MAdCAM-1, with some vascular cell adhesion molecule-1 (VCAM-1) expression, and their location may play an important role in lymphocyte recruitment and retention (Csencsits, et al. 2002). It is through these HEV that lymphocyte migration takes place, with the efferent lymphatics draining into the CLN of the upper thorax region.

1.4.4.3 Function of the NALT

Previous studies have proven that the NALT is a major mucosal inductive site (Kiyono and Fukuyama 2004; Wu 1997). Therefore, new vaccination strategies based on nasal application have been designed to prevent diseases caused by infectious agents with a mucosal portal of entry. Tracking studies, using microspheres and viruses in animals have shown that these antigens are rapidly deposited in NALT after nasal administration and that cellular activation can take place (Carr, et al. 1996; Eyles, et al. 2001a; Eyles, et al. 2001b; Kuper, et al. 1992; Velin, et al. 1997). Recently, a study examined the uptake of fluorescent microparticles into the epithelium of human adenoid tissues. Transmission microscopy revealed that microparticles were taken up by the M cells of human nasopharyngeal tissues with the smallest particles (0.2μm) showing greater uptake than those larger than 0.5μm. In addition it was also noted that surface coatings with poly-$_L$-lysin or chitosan resulted in efficient uptake into the NALT (Fujimura, et al. 2006). However, the introduction of soluble antigens into the nasal mucosa can lead to immunological non-responsiveness or tolerance (Hall, et al. 2003; Unger, et al. 2003). Hence, there are extremely complex mechanisms operating at the level of the nasal immune system to regulate highly specialised processes, such as immune reactivity and mucosal tolerance.

Intranasal delivery of particulate and soluble antigen results in uptake through M cells of the FAE, yet soluble antigens can also pass directly through the nasal epithelium of D-NALT. After antigens are sampled and transported to the underlying lymphoid cells in the submucosa, antigen processing and presentation occurs. This results in activation of T cells, which in turn provide help to B cells to develop into IgA plasma cells to induce both humoral and cellular immune responses. Recently, a strain of *Streptococcus gordonii* expressing on its surface a model vaccine antigen fused to the ovalbumin (OVA) peptide was used to study *in vivo* adoptive transfer of ovalbumin-specific transgenic T cells. The recombinant strain activated the OVA-specific $CD4^+$ T-cell population in the NALT just 3 days following intranasal immunisation. In the CLN, the percentage of proliferating cells was initially low, but it reached the peak of activation at day 5 (Medaglini, et al. 2006). This work indicates that the NALT is the site of antigen-specific T-cell priming. Several studies have indicated that intranasally

administered antigens stimulate $CD3^+CD4^+$ T cells within the NALT to become either T_H1 or T_H2 cells (Hiroi, et al. 2001; Hiroi, et al. 1998; Yanagita, et al. 1999). Hiroi and colleagues used reverse transcription-PCR (RT-PCR) to analyse the cytokine profiles of mucosal T cell within O-NALT and D-NALT, and revealed that the predominant cytokine profile expressed by $CD4^+$ T cells in O-NALT was that normally associated with naïve and resting T_H0 T cells. When these T cells were stimulated with anti-αβ TCR, anti-CD3 or mitogens, they expressed an array of both T_H1 and T_H2 cytokines, indicating that these T cells are programmed to become T_H1 or T_H2 cells immediately following antigen exposure via the nasal tract. Conversely the $CD4^+$ T cells within D-NALT, which lines the nasal passages, showed high expression levels of T_H2 cytokines including IL-4 and IL-10, along with a lower frequency of T_H1 cytokine including IL-2 and IFN-γ expressing $CD4^+$ T cells (Rodriguez-Monroy, et al. 2007). This group also found that the O-NALT contained a lower number of these spontaneously cytokine expressing T cells in comparison to D-NALT of the nasal passages (Rodriguez-Monroy, et al. 2007). These studies suggest that O-NALT may be a mucosal inductive site, whereas the D-NALT may function as an effector site. A number of studies have used nasal delivery of protein antigens (such as bacterial cell-wall components or virus-associated antigens) or live vectors together with the mucosal adjuvant CT to show the induction of T_H1 and/or T_H2 antigen-specific responses in NALT T cells. These immunisations also led to the induction of immune responses at distinct mucosal effector sites including the genito-urinary, respiratory and intestinal tracts (Hiroi, et al. 2001; Imaoka, et al. 1998; Kurono, et al. 1999; Yanagita, et al. 1999). Within the NALT, the majority of T cells express the αβ T-cell receptor (αβTCR) with few γδ TCR^+ T cells (Asanuma, et al. 1995). However, Hiroi and colleagues have shown that the D-NALT within the nasal passages contains a higher frequency of these γδ T cells than the O-NALT. This group has postulated that the nasal passages may represent an area of extrathymic differentiation of T cells, since this area contains $CD4^-CD8^+$ and $CD4^-CD8^-$ γδ T cells, similar to those of the γδ T cells in the intestinal intraepithelial lymphocytes (i-IEL) (Hiroi, et al. 1998).

Accumulating evidence suggests that NALT is also capable of generating CTL (Porgador, et al. 1998; Wiley, et al. 2001; Zuercher, et al. 2002). Zuercher et al demonstrated that virus-specific CTLs could be detected in the NALT after nasal

infection with reovirus at 5-fold higher frequency than those detected in the CLN (Zuercher, et al. 2002). Further work by Wiley and colleagues showed that influenza-specific CTLs accumulated and persisted in both the O-NALT and D-NALT following primary intranasal infection. However in comparison to D-NALT, antigen-specific CTLs within the O-NALT accumulated and diminished over a longer time course during secondary influenza infection (Wiley, et al. 2001).

Due to the large numbers of inhaled antigens that pass through the NALT, this mucosal tissue has to have high tolerogenicity. However, no, or very few, classical regulatory T cell such as those expressing $CD4^{+}CD25^{+}$ and $CD4^{+}CD45RB^{low}$ have been found within nasal tissues (Asseman, et al. 1999; Powrie, et al. 1996; Powrie and Maloy 2003). Recently, Rharbaoui and colleagues characterised a $B220^{+}$ lymphoid subpopulation that had immune modulatory functions within the NALT. They demonstrated that $B220^{low}CD4^{-}CD8^{-}CD19^{-}$ cells constituted a large subpopulation of cells within NALT and their main mechanism of cell death was Fas-independent apoptosis. These cells exhibited a capacity to down-regulate mature T cell activation via secretion of soluble factors in cooperation with $CD4^{+}$ T cells. However, the innate immunity receptor TLR2 was also highly expressed on this cell subpopulation, and when the TLR2/6 signalling cascade was stimulated *in vivo* it lead to an activated phenotype of these cells (Rharbaoui, et al. 2005).

IgA is the major isotype of antibody produced by NALT (Heritage, et al. 1997; Wu, et al. 1997b). Several in depth studies have shown that specific IgA producing B cells are induced in both the O-NALT and D-NALT after intranasal administration of antigen (Asanuma, et al. 1998; Tamura, et al. 1998; Zuercher, et al. 2002). Liang and colleagues showed that both the O-NALT and the D-NALT are capable of producing virus-specific antibody in response to influenza virus infection, although the frequency of specific antibody-forming cells in the D-NALT was much greater than the frequency observed in the O-NALT and CLN (Liang, et al. 2001). In the same study, an 18 month analysis was performed and showed that the D-NALT, but not the O-NALT, was the site of long-term virus-specific humoral immunity which lasted for the life of the animal. These data indicate that the NALT is not only a major inductive site of IgA switching, but also the site that IgA blasts can migrate from O-NALT to the effector site of D-NALT, which lines the nasal passages, to provide local long-

term specific antibody production. Zuercher and colleagues demonstrated that after intranasal infection with reovirus, production of antigen-specific IgA antibodies was preceded by the induction of GC and the expansion of IgA^+ B cells in the NALT (Zuercher, et al. 2002). Significant proportions of IgG antibodies are also produced in the NALT; however it is only IgA producing cells that show affinity maturation (Shimoda, et al. 2001; Zuercher, et al. 2002).

NALT and the nasal mucosa drain directly to the superficial and posterior CLN. These lymph nodes (LNs) are designed to optimise interaction between APC and T and B lymphocytes. Lymph fluid enters the node via the afferent lymphatics, and passes through the sinuses, which are lined with macrophages, before finally leaving via the efferent lymphatic that ultimately drains into the portal vein. Lymphocytes actually enter the node primarily from the blood, via specialised endothelia, which are located within T areas. DC migrating from the tissues also enter the node, and move into these T cell regions. B cells entering nodes from the blood must cross the T cell rich area in transit to the B cell rich areas, thus optimising the chance of T-B cell co-operation. The B cell rich areas contain mature, resting B cells organised into structures around FDC (primary follicles) (see Figure 1.10).

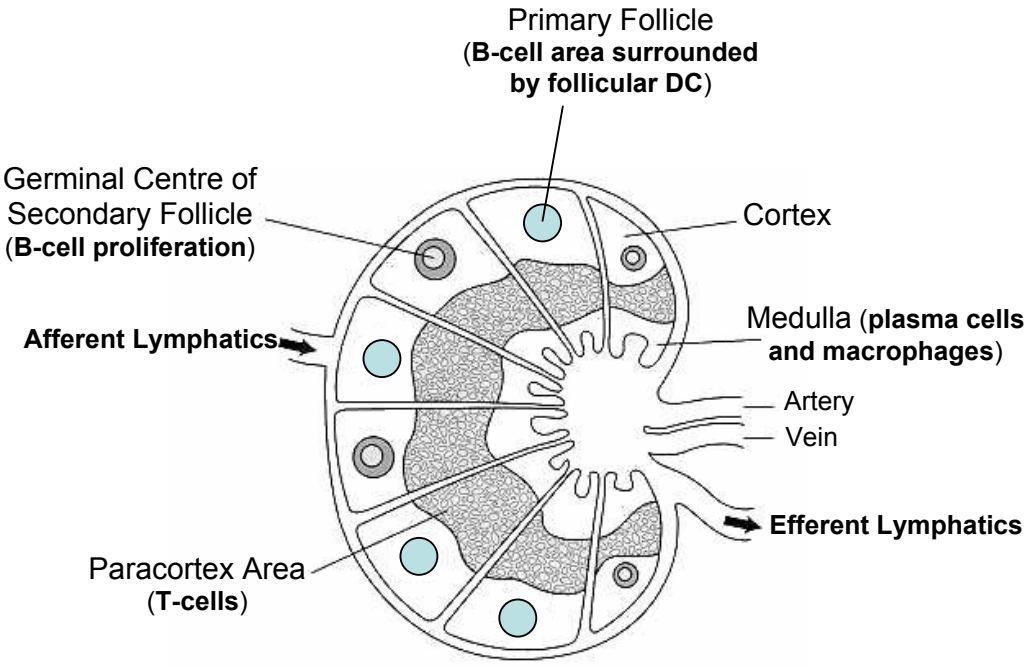


Figure 1.10: Diagram of a lymph node

Although an inhaled particulate impacting on the mucus layer of the nasal mucosa can be rapidly cleared by ciliary motion, it could also be selectively delivered to the organised NALT structures, thereby triggering immune responses (Wu, et al. 1997b). After initial induction in NALT, antigen-specific B and T lymphocytes are amplified in the draining LNs, and proceed via the lymph into the circulation, from which they 'home' to distant mucosal effector sites. These draining LNs also produce large amounts of antigen-specific IgG antibodies, which can also migrate to distant sites, leading to systemic (serum IgG) immunity. In general, NALT-targeted immunisation induces antigen-specific immunity in the respiratory and reproductive tissues, demonstrated by NALT IgA committed B cells efficiently trafficking to the respiratory and genitor-urinary tracts through complementary ligand patterns (Kunkel, et al. 2003; Lazarus, et al. 2003). A recent study by Wiley et al, where both the NALT and CLN were removed in mice, demonstrated that though both tissues participate in the generation of local immunity to influenza infections, neither was essential for the development of protective immunity and viral clearance in the URT (Wiley, et al. 2005).

1.4.4.4 Waldeyer's ring

The presence of lymphoid tissue in the human pharynx was first recognised in 1884 by Waldeyer. He described what is now know as the Waldeyer's ring, which consists of the palatine, pharyngeal (adenoid), lingual and tubal tonsils along with the lateral pharyngeal lymphoid bands (see Figure 1.11 below) (Waldeyer-Hartz 1884). The tonsils are responsible for the sampling of respiratory antigens by trapping foreign material in the crypts of the stratified squamous epithelium that covers their surface. The adenoids are covered by ciliated columnar epithelium with cleft like invaginations. This specialised architecture enables the tonsils and adenoids to initiate immunological responses in the URT (Perry and Whyte 1998).

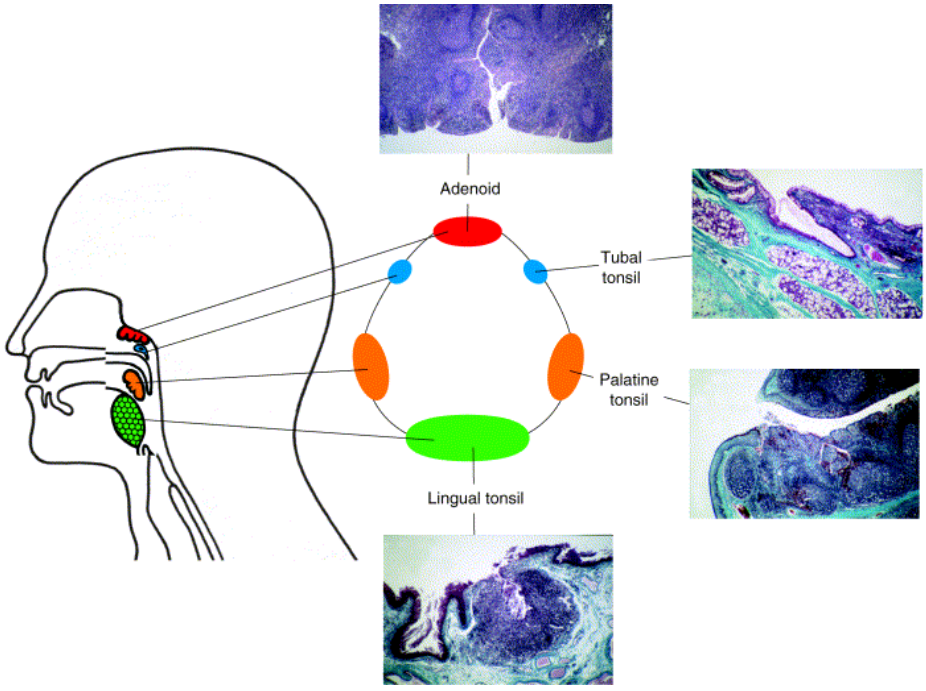


Figure 1.11: Pharyngeal lymphoid tissue of Waldeyer's ring.

Pharyngeal lymphoid tissue of Waldeyer's ring comprises the nasopharyngeal tonsil or adenoid, the paired tubal tonsils, the paired palatine tonsils and the lingual tonsil. All four micrographs show that the surface of the tonsils is in each case covered with pharyngeal epithelium (Perry and Whyte 1998).

Like the NALT, the lymphoid structures of the Waldeyer's ring have an epithelial barrier that has specialised M-cells that facilitate transport of antigens to the B and T cell zones of the tissue (Claeys, et al. 1996a; Claeys, et al. 1996b; Karchev 1996). The distribution of T and B cell subsets, cytokine patterns, and antibody isotype profiles are similar for both adenoids and tonsils. Both tissues contain predominantly B cells (~ 65%), approximately 5% macrophages, and 30% $CD3^+$ T cells. The T cells are primarily of the $CD3^+CD4^+$ subset (~80%). Tonsillar intraepithelial lymphocytes are also enriched in B cells. Analysis of dispersed cells revealed a higher frequency of cells secreting IgG than IgA and the predominant Ig subclass profiles were IgG1 > IgG3, and IgA1 > IgA2, respectively. As described in the NALT, mitogen-triggered T cells from tonsils and adenoids produce both T_H1- and T_H2-type cytokines (both IFN-γ and IL-5), exhibiting their pluripotentiality for support of cell-mediated and antibody responses (Boyaka, et al. 2000; McGhee and Kiyono 1992). Also, similar to the NALT, tonsils and adenoids do not start development until after birth, when postnatal immunological challenges initiate the tonsillar follicles and plasma cells to develop

(Zenner and Brunner 1988). It is also during childhood that the Waldeyer's ring is at its most immunologically active, although with age the follicular mantle zones and reticular crypt epithelium are reduced (Brandtzaeg 1988). Debertin and colleagues have shown that 38% of young children in a post-mortem study had a structure like the NALT that is morphologically distinct from the Waldeyer's ring. In contrast to rodent NALT, this human NALT is maybe more representative of D-NALT, as it was found disseminated in the nasal mucosa mainly in the middle concha (Debertin, et al. 2003). These anatomic differences between rodents and humans may reflect differences in breathing patterns. Animals with predominant or exclusive nose breathing, such as rodents, have well developed NALT and bronchus-associated lymphoid tissue (BALT), whereas in species with mixed breathing through nose and mouth, tonsillar structures in the pharynx can be seen, i.e. the Waldeyer's ring.

The question of whether removal of tonsils may compromise protection of the upper respiratory tract and result in humoral immunodeficiency has been the subject of debate (Dolen, et al. 1990; Donovan and Soothill 1973). Combined adenoidectomy and tonsillectomy were reported to reduce IgA titres in nasopharyngeal secretions to poliovirus and to delay or abrogate the local mucosal immune response to the live polio vaccine (Ogra 1971). Another study reported that tonsillectomy leads to a decreased serum and salivary IgA for a period of 3 years or longer (D'Amelio, et al. 1982).

1.4.5 Gut Associated Lymphoid Tissue

The gut associated lymphoid tissue (GALT) is the largest collection of lymphoid tissues in the body, consisting of both organised lymphoid tissues, such as mesenteric lymph nodes (MLN) and Peyer's patches (PP), and more diffusely scattered lymphocytes in the intestinal lamina propria (LP) and epithelium including large numbers of IgA^+ plasmablasts. Initial colonisation of the GALT with intestinal flora starts at birth and this colonisation with bacteria is critical for the normal structural and functional development and optimal function of the GALT (Macpherson, et al. 2000; Moreau and Corthier 1988; Talham, et al. 1999). The GALT is covered with a single layer of cells, the intestinal epithelium which is interspersed, in some regions, by specialised M cells. M cells use transepithelial vesicular transport to carry

microbes to APCs in the underlying GALT (Kraehenbuhl and Neutra 2000). The GALT is divided into discrete inductive and effector sites. M cells are contained within inductive sites in the GALT, known as the PP. PP are aggregations of lymphoid follicles found primarily in the distal ileum of the small intestine, located within the dome-like structure of the FAE. Like LNs, PP have B cell follicles and germinal centres that are surrounded by areas that contain predominantly T cells. Both $CD4^+$ and $CD8^+$ T cells are present within PP with the predominance of IFN-γ secreting cells and few IL-4, IL-5 or IL-10 secreting cells. A subpopulation of $CD4^+$ T cells that co-express CD25 (the α-chain of IL-2R) with regulatory function has been identified in the mucosa, and has been purported to be generated by the exposure of intestinal immune system to luminal antigens. These cells appear to play an important role in the induction and maintenance of tolerance in the normal intestine through the production of TGF-β and IL-10 (Shevach 2000). The GALT also contains loosely organised effector sites, primarily within the lamina propria of the intestinal villi. The lymphocytes found in the lamina propria are largely IgA-secreting plasma cells and memory T-effector cells. Polymeric IgA, one of the hallmarks of the intestinal humoral immune system, is produced to defend mucosal surfaces from environmental microbes (Farstad, et al. 2000). See Figure 1.12 for an overview of the GALT. For a comprehensive review see (Forchielli and Walker 2005).

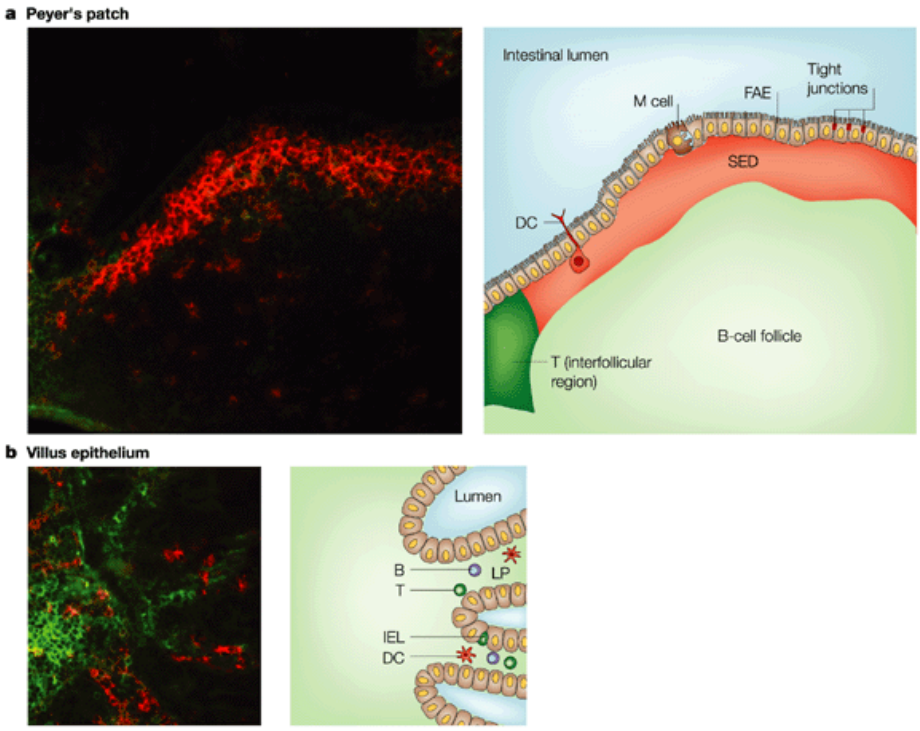


Figure 1.12: Gut associated lymphoid tissue

The gut-associated lymphoid tissue (GALT) is divided into inductive (Peyer's patch) and effector (lamina propria) sites. In the immunofluorescence images shown, T cells are green and dendritic cells (DCs) are red. a | Similar to lymph nodes, the Peyer's patch contains B-cell follicles. The follicle-associated epithelium (FAE) covers the dome of the Peyer's patch. Transport across the epithelium occurs through both specialised M cells and by DCs that extend their processes through epithelial tight junctions. DCs are present in both the subepithelial dome (SED) and the interfollicular T-cell areas and are visible as stellate (red) cells in these sites. b | The intestinal villus epithelium contains an unusual population of intraepithelial lymphocytes (IELs) which reside above the epithelial basement membrane. Scattered lamina propria (LP) effector cells — T cells (T), IgA-secreting B cells (B) and DCs — are located within the villi (Nagler-Anderson 2001).

1.5 Live recombinant bacterial vaccines

Live recombinant bacteria represent an attractive means to induce both systemic and mucosal immune responses against pathogens. Vaccines based on attenuated bacterial carriers are cost-efficient in terms of production and delivery, and frequently stimulate more potent immune responses then non-replicating formulations. These attenuated bacterial antigen vectors can be used to induce immunity to their corresponding pathogenic strain or they can be modified to deliver protective heterologous (foreign) antigens, plasmid DNA or other macromolecules such as immune modulators, and can therefore provide the possibility of conferring protection against a variety of infectious diseases or as therapy for other illnesses (Roland, et al. 2005).

1.5.1 Live, attenuated *Salmonella* vaccines and vectors

1.5.1.1 *Salmonella* pathogenesis

Salmonella are gram negative bacteria that cause gastroenteritis and typhoid fever. Pathogenic salmonellae that are ingested and survive the gastric acid barrier traverse the intestinal mucus layer before adhering and invading the intestinal epithelium. A small number of bacteria transcytose to the basolateral membrane, where *Salmonella* serotypes clinically associated with enteritis induce fluid and electrolyte secretion, and initiate recruitment and transmigration of neutrophils into the intestinal lumen. After penetration of the lamina propria and the submucosa, they are phagocytosed by macrophages. *Salmonella* serotypes that cause systemic infection enter macrophages, survive and persist in the intracellular environment by circumventing the microbiocidal functions of the phagocyte. Migration of infected phagocytes to other organs of the reticuloendothelial system, via the lymphatics and blood, may then facilitate dissemination of bacteria in the host. For a comprehensive review see (Ohl and Miller 2001).

1.5.1.2 Immune response to infection with *Salmonella*

During the initial stages, macrophages, neutrophils and DC are all recruited to the site of infection, and process antigen for presentation to T and B cells. Cell wall components of *Salmonella*, such as LPS, induce a massive inflammatory response in the surrounding tissue, resulting in the expression of an array of cytokines and chemokines. Although the innate mechanisms of the immune system are highly effective in restricting initial growth of *Salmonella*, an adaptive response is required for eventual effective elimination of the pathogen. *Salmonella* infection induces the generation of specific $CD4^+$ and $CD8^+$ T cells, as well as γδ T cells. Cytokines are also produced by T cells, as well as NKC early in infection and contribute to various steps of the host inflammatory and immune response. Primarily IFN-γ, but also TNF-α, are required for macrophage activation and stimulation of antimicrobial defence mechanisms. IFN-γ, as well as its inducing factors IL-12 and IL-18, is critical in directing the ensuing T-cell response towards the T_H1 pole. Infection with *Salmonella* also results in a profound antibody response against both non-protein antigens (e.g. LPS), and protein antigens. Serum antibodies, as well as mucosal IgA and IgM, are produced, which may help in the control of infection by prevention of bacteria binding to cell surfaces, complement activation, and opsonisation. Reviewed in (Mittrucker and Kaufmann 2000).

1.5.1.3 *Salmonella* as vaccine vectors

Salmonellae are the most widely exploited and well characterised of the bacterial vaccine carrier system, perhaps in part due to the ease in which they can be genetically manipulated (Sirard, et al. 1999). Attenuated *Salmonella* colonise on, or invade through mucosal surfaces and/or MALT, and can be used to deliver antigen or DNA to elicit mucosal, systemic and cellular immune responses to bacterial, viral, parasitic and fungal pathogens. Two *Salmonella enterica* serovars, *S.* Typhimurium and *S.* Typhi, have been the most widely used as vaccine vectors. The introduction of defined, non-reverting mutations affecting genes in metabolic pathways (*aro, pur*), cAMP regulation (*cya, crp*), or in virulence (*phoP-phoQ*) has resulted in several avirulent strains that preserve various degrees of invasiveness and immunogenicity, and are suitable for vaccination purposes (Clare and Dougan 2004).

1.5.1.3.1 *S.* Typhi vaccine vectors

Most work on developing a live recombinant vaccine against *S. enterica* serovars has concentrated on *S.* Typhi. Few attempts have been made to develop human vaccines against other *S. enterica* serovars as most normally only cause localised gastroenteritis, while *S.* Typhi causes typhoid fever in humans. In fact the strain Ty21a, which contains a *galE* mutation, was the first and only live oral typhoid vaccine to be licensed for use in humans. However, Ty21a is weakly immunogenic and genetically undefined, making it not a particular attractive for use as a live vaccine vector. Subsequently, a number of new *S.* Typhi vaccine strains have been developed that contain defined mutations allowing for more effective attenuation including *S.* Typhi CVD-908*htrA*, Ty800 and ZH9, which have all demonstrated good reactogenicity and immunogenicity in humans (Hindle, et al. 2002; Hohmann, et al. 1996; Khan, et al. 2003; Tacket, et al. 2000). Salmonellae were among the first bacteria used as recombinant vectors for antigen delivery with Formal and colleagues performing pioneer studies in the 1980s, using Ty21a to express a *Shigella* surface antigen (Curtiss 2002; Formal, et al. 1981). Animals and humans were immunised with the construct Ty21a expressing this heterologous *Shigella* antigen, which was shown to induce protection in animals after *S.* Typhi and *Shigella sonnei* challenges, and induce significant IgA titres in humans (Black, et al. 1983; Van de Verg, et al. 1990). Recently, the newly developed *S.* Typhi vaccine strains have also been modified to deliver one or more heterologous antigen(s) (Khan, et al. 2007; Microscience 2004a; Microscience 2004b).

1.5.1.3.2 *S.* Typhimurium vaccine vectors

The development of *S.* Typhimurium vectors is based on the notion that the prolonged intestinal phase of the organism may induce immune responses in the GI-tract that is qualitatively and/or quantitatively different than those elicited by *S.* Typhi. Attenuated *S.* Typhimurium SL3261 expressing *P. aeruginosa* serogroup O11 O antigen, was used to vaccinate animals to protect against *P. aeruginosa* infection in an acute fatal pneumonia model. Immunised animals showed increased survival and significantly fewer bacteria in the lungs compared to controls (DiGiandomenico, et al. 2004). Evans and colleagues used attenuated strains of *S.* Typhimurium expressing fragments of the simian immunodeficiency virus (SIV) Gag protein fused to the type III-secreted

SopE protein to induce priming of virus-specific CTL responses in rhesus macaques. Good CTL responses were detected only after three oral doses of recombinant *Salmonella*, followed by a peripheral boost with modified vaccinia virus Ankara expressing SIV Gag (MVA Gag). However, *Salmonella*-primed/MVA-boosted animals did not exhibit improved control of virus replication following a rectal challenge with SIVmac239 (Evans, et al. 2003). This vaccine is currently in phase I trials. The first *S.* Typhimurium vector expressing heterologous antigens tested in humans was performed by Angelakopoulos and colleagues. Attenuated strains of *S.* Typhimurium LH1160 expressing the *Helicobacter pylori ureAB* genes were used to orally immunise volunteers. A single oral dose was generally well tolerated, and over half of the volunteers developed antibody responses to UreAB (Angelakopoulos and Hohmann 2000). Most recently, Kotton and colleagues administered a recombinant *S.* Typhimurium vaccine vector expressing HIV Gag. Eighteen healthy adults were given single escalating oral doses of the vaccine with adverse events being mild except at the highest dose. Over 80% of the subjects developed anti-*Salmonella* immune responses. However, only a small number of subjects had any detectable anti-gag IL-2 and IFN-γ responses (Kotton, et al. 2006).

1.5.1.3.3 Immune responses generated after immunisation with *Salmonella* vectors

Oral vaccination with live attenuated *Salmonella* vectors results in the generation of both *Salmonella* and heterologous antigen specific humoral and cellular immune responses, normally biased towards the T_H1 phenotype. Studies undertaken with a *S.* Typhimurium vaccine strain expressing Tetanus toxin fragment C (TetC) resulted in TetC-specific IgG2a and mucosal IgA antibody responses. TetC stimulated $CD4^+$ T cells, isolated from both mucosal and systemic tissues, produced IFN-γ, IL-2 and IL-6 as well as IL-10, but not IL-4 or IL-5 (VanCott, et al. 1996). Immunisation with the a recombinant *S.* Typhi Ty21a vaccine vector expressing urease A and B from *H. pylori* were found to have anti-urease antibodies, as well as antigen specific IFN-γ responses in most volunteers tested (Bumann, et al. 2001).

1.6 Hypothesis

Given that *M. tuberculosis* is a pathogen that infects its host via the mucosal surface of the lung, I hypothesise that mucosal administration of the tuberculosis fusion antigen Ag85B-ESAT6 will be appropriate for stimulating both local and systemic immune responses strongly biased towards the T_H1 phenotype. Mucosal immunisation of this antigen will induce both innate and adaptive immune responses, both at the site of administration as well as distal effector sites, with these responses being potent enough to protect vaccinated animals after aerosol challenge with pathogenic *M. tuberculosis*.

1.7 Aims of this thesis

The aims of this project were to employ the *M. tuberculosis* fusion antigen Ag85B-ESAT6 in a number of different vaccination regimens. Techniques such as ELISAs and cytometric bead analysis (CBA) were used to establish levels of immunogenicity, and a challenge study was performed to determine levels of protection in animal models compared to the current BCG vaccine, with the final goal of identifying the most promising vaccine candidate. A further aim was to investigate both phenotypic and functional aspects of innate immune responses shortly following intranasal immunisation with model antigens and mucosal adjuvants. By utilising techniques such as immunofluorescence, flow cytometry and antibody depletion I hope to gain a better understanding of the underlying mechanisms, the cell populations involved, localisation of these antigens within cell populations, and the trafficking patterns of these immune cells early after immunisation in both the NALT and the CLN. From these studies I observed large increases in the percentage of NKC in both lymphoid tissues and wished to explore how these cells might influence the induction of antigen specific adaptive immune responses after mucosal immunisation.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

All chemicals were obtained from Sigma (Poole, Dorset, UK) or BDH Ltd (Poole, Dorset, UK) unless otherwise stated. Reagents were prepared and stored according to (Sambrook 1989) or manufacture's guidelines. Water was deionised using a purite water system.

2.1.2 Bacterial strains

Table 2.1: Bacterial strains used during study

Bacterial strain	Characteristics	Reference/Source
S. Typhimurium SL3261	*aroA* deletion	(Hoiseth and Stocker 1981) supplied by Derek Pickard
E. coli JM109	F´ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17*	Promega
E. coli TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
S. Typhimurium LB5010	*metA22 metE551 ilv-452 leu-3121 trpΔ2 xyl-404 galE856 hsdLT6 hsdSA29 hsdSB121 rpsL120*	(Bullas and Ryu 1983) supplied by Derek Pickard
S. Typhimurium TML	Wild-type (human gastroenteritic origin)	(Giannella, et al. 1973) supplied by Derek Pickard
M. tuberculosis Erdman	Wild-type	Statens Serum Institute, Copenhagen, Denmark
M. bovis BCG Danish 1331	Freeze-dried vaccine strain	Statens Serum Institute, Copenhagen, Denmark

2.1.3 Plasmid vectors

Table 2.2: Plasmid vectors used during study

Plasmid	Size (bp)	Comments	Source
pMCT6 (Hyb1)	5810	Contains *lacZ* promoter plus *lacIQ* repressor and *Ag85B-ESAT6*	Statens Serum Institute, Denmark
pQF50	6800	Used for cloning *ssaG* promoter driving *Ag85B-ESAT6* clone	Derek Pickard
pmyconirB	4827	Includes *nirB* promoter plus *Ag85B-ESAT6*	This study
pTOPO	3500	T-overhang vectors for cloning PCR fragments	Invitrogen
pGEM-Teasy vector	3010	T-overhang vectors for cloning PCR fragments	Promega
pMQ8	7900	Contains *Ag85B-ESAT6* in pQF50 vector	This study
pBRD940	3727	Contains *nirB* promoter	Derek Pickard
pmycossaG	8400	Includes *ssaG* promoter plus *Ag85B-ESAT6* (vector backbone is pQF50)	This study
p2795	4443	Hensel Vector for red recombinase system	(Husseiny and Hensel 2005)
pmyco2795	~ 5700	Hensel Vector plus *lacZ* Ag85B-ESAT6	This study

2.1.4 Oligonucleotide primers

Table 2.3: Details of primers used during study

Target DNA	Primer Name	Primer Sequence (5'- 3')
ssaG promoter from TML strain (primers designed to include SphI and BglII sites)	ssaGF	CCTGGCAGGGATTGGGCATGCTATTGCCATCGCGGA
	ssaGR	ACTAATTGTGCAGATCTCATAATGCTTTTCCTTAAA
Ag85B-ESAT6 from pMCT60 (primers designed to include NcoI and BamHI)	Myco Nco1F	CCATCACATCGACCATGGGAAGATCTTTCTCCCGGCCGGG GCTGCCGGTCGAG
	Myco Nco1R	TAGCTAGCTAGGATCCCGTGTTTCGCTATTC
ssaG Ag85B-ESAT6 (sequencing primers for pRA263)	QF50F	GCGACTCCTGCATTAGGAAGCAG
	QF50R	TTCCCAGTCACGACGTTGTA
	DESTM1F	TGCCATCGCGGATGTCGCCT
	DESTM4R	CGGTATATACCTGAAAACGA
nirB Ag85B-ESAT6 (sequencing primers for pmyco940)	940for	GATGTACATCAAATGGTACCCCTTGC
	940Rev2	CAGCCTAGCCGGGTCCTCAAC
	940AnjamFor	CGACCCGAACCGTGACATC
	MycoF1	CACAGGAAACAGGATCACTAAGGA
	MycoF2	AATCGGCTTGTCGATGGCCGGCTG
	MycoF3	CAGGATGCGTACAACGCCGCGGGCG
	MycoR2	CGAGCCGGCCATCGACAAGCCGATT
	MycoR3	ACATTTCCCTGGATTGCGCTTGCCGC
	MycoR4	CCGTGTTTCGCTATTCTACGCGAAC
lacZ Ag85B-EAT6 from myco-p2795 (primers designed to include *phoN* knock-in regions (Hensel Vector))	phoNhensF	GCTGTGGCCAGTTTGCGGGAAGACTTTCACCTTCAGTAATT AAGATACGACTCACTCACTATAGGGCG
	phoNhensR	CTGTTTATTATTGCCTGATCCGGAGTGAGTCTTTATGAAAAG TTGACCATGATTACGCCAAGC
lacZ Ag85B-ESAT6 (sequencing primers for chromosomal *phoN lacZ* Ag85B-ESAT6 insert)	phoNseqF	GGTATGGACAGACGATAATGCCAGGGCA
	phoNseqR	GAATTCATGAGAATCGGGGAAACCAAAG
	mycoseqR	CGTGTTTCGCTATTCTACGCGAACTCGGCG
	M13 Reverse	CAGGAAACAGCTATGAC
	M13 Forward	GTAAAACGACGGCCAG

2.1.5 Proteins for immunisations

Table 2.4: Proteins used in immunisations

Protein	Concentration (µg/mL)	Source
Ag85B-ESAT6	1, 10 or 25	Statens Serum Institute, Denmark
Wild-type LT	1	Chiron, Italy
LTK63	10 or 20	Chiron, Italy

2.1.6 ELISA antibodies

Table 2.5: Antibodies used for ELISAs

Target molecule	Host	Isotype	Conjugate	Source
IgG	rabbit	Ig	HRP	Sigma
IgA	rat	IgG1	biotin	Pharmingen
IgA	rat	IgG1	none	Pharmingen
IgG1	rat	IgG1	HRP	Pharmingen
IgG2a	rat	IgG2a	HRP	Pharmingen
IgG2b	rat	IgG2a	none	Pharmingen
IgG3	rat	IgG2a	none	Pharmingen
IgM	rat	IgG2a	none	Pharmingen
IgE	rat	IgG1	none	Pharmingen
Ig	rabbit	Ig	HRP	Dako
Streptavidin	rat	Ig	HRP	Dako
Rat Ig	rabbit	Ig	HRP	Dako

2.1.7 Immunofluorescence antibodies

Table 2.6: Antibodies for Immunofluorescence staining

Target Molecule	Host	Isotype	Conjugate	Source
F4/80	rat	IgG2b	FITC	Serotec
Ly6G	rat	IgG2b	PE	Pharmingen
VCAM-1	rat	IgG2a	unconjugated	Pharmingen
PNAd	rat	IgM	unconjugated	Pharmingen
MAdCAM-1	rat	IgG2a	unconjugated	Pharmingen
CD11c	hamster	IgG	PE	Pharmingen
ICAM-1	hamster	IgG	unconjugated	Pharmingen
PNA	*Arachis hypogaea*	N/A	rhodamine	Vector Labs
B220	rat	IgG2a	FITC	Pharmingen
Rat IgG and IgM	goat	IgG	AlexaFluor 488, 568 and 633	Invitrogen
Hamster IgG	goat	IgG	AlexaFluor 488, 568 and 633	Invitrogen
Rabbit IgG	goat	IgG	AlexaFluor 488, 568 and 633	Invitrogen

2.1.8 Antibodies for FACS

Table 2.7: Antibodies for FACS analysis

Target Molecule	Host	Isotype	Conjugate	Source
CD69	hamster	IgG	PE-Cy7	Pharmingen
CD25	rat	IgG1	APC-Cy7 or APC	Pharmingen
MHCII	rat	IgG2b	FITC or PE	Pharmingen
CD11c	hamster	IgG	PE-Cy7	Pharmingen
F4/80	rat	IgG2b	PerCP	Serotec
VCAM-1	rat	IgG2a	APC	Serotec
Ly6G	rat	IgG2b	PE	Pharmingen
DX5	rat	IgM	FITC or PE	Pharmingen
Vβ8.1, 8.2-TCR	rat	IgG1	PE-Cy5	eBioscience
Vβ8.1, 8.2-TCR	mouse	IgG2a	PE	Pharmingen

2.1.9 Mice

Female Balb/c and C57BL/6 mice (5-6 weeks) from Charles River, UK were used in all animal experiments. All animals were given food and water *ad libitum*. Mice were sacrificed by cervical dislocation or exsanguination. Animal husbandry and experimental procedures were conducted according to the United Kingdom Animals (Scientific Procedures) Act 1986.

2.2 Methods

2.2.1 Bacterial growth conditions

All stains were routinely grown at 37^{o}C on Luria Bertani (LB) agar plates or in LB broth containing the appropriate antibiotic. For growth of aromatic-dependent *Salmonella* strains, cultures and plates were supplemented with Aro mix (containing 40µg/mL each of L-phenylalanine, L-tryptophan and L-tyrosine, and 10µg/mL each of 4-para-amino benzoic acid and 2, 3-dihydroxybenzoic acid). After electroporation, strains were grown in electroporation recovery media (SOC) and incubated with agitation at 220rpm for 1.5 hours at 37°C before being transferred to LB containing appropriate antibiotic. *S.* Typhimurium were grown statically in broth cultures. *E. coli* cultures were incubated with shaking (220 rpm).

1.2.1.1 Growth conditions for overexpression of Ag85B-ESAT6 from *in vivo* inducible promoter

For the overexpression of Ag85B-ESAT6 via the *nirB* promoter, *Salmonella* were grown anaerobically in LB broth containing suitable antibiotic. Cultures were left at 37 ^{o}C for 24 hours in anaerobic jars in the presence of a palladium catalyst to remove any oxygen. For the overexpression of Ag85B-ESAT6 via the *ssaG* promoter, *Salmonella* were grown in a minimal medium based on the MM5.8 medium described by Hautefort et al. (Hautefort, et al. 2003). Specifically, the SPI II inducing medium contained; 5mM KCl, 7.5mM $(NH_4)_2SO_4$, 0.5mM K_2SO_4, 1mM KH_2PO_4, 0.1% casamino acids, 38mM glycerol, 100mM Bis-Tris, 0.2% glucose and 10μM MgCl at pH 5.8. Cultures were left at 37^{o}C for 24 hours.

1.2.1.2 Growth conditions for *M. tuberculosis*

Before aerosol challenge, *M. tuberculosis* Erdman was grown at 37°C in suspension in Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose (Andersen, et al. 1991). After challenge, whole-organ homogenates were grown on 7H11 medium. Organs from the BCG-vaccinated animals were grown on medium supplemented with 2 μg/ml 2-thiophene-carboxylic acid hydrazide to selectively inhibit the growth of the residual BCG bacteria in the test organs. Colonies were counted after 2–3 wk of incubation at 37°C.

2.2.2 Animal methods

2.2.2.1 Preparation for inoculation/immunisation

2.2.2.1.1 Oral

Cultures were grown overnight as described above (section 2.2.1). Bacteria were then centrifuged at 10000 rpm for 10 minutes at 4^{o}C and resuspended in PBS (10% original volume). Mice received 200 μL (approximately 5 x 10^9 CFU of *Salmonella*) by oral gavage.

2.2.2.1.2 Intranasal

Proteins for intranasal immunisation were diluted in PBS to obtain the appropriate concentrations. Each mouse was lightly anesthetised with isofluorane before receiving 30 µL of immunisation solution into her nostrils (15µL/nare).

2.2.2.1.3 Intraperitoneal

Proteins for i.p. immunisation were diluted in PBS to obtain appropriate concentrations. Mice were lightly anesthetised with isofluorane before being injected with 300-500 µL of immunisation solution into their peritoneal cavity.

2.2.2.1.4 Intravenous

Proteins for i.v. injection were diluted in PBS to obtain the appropriate concentration. Mice were injected with 200 µL of solution into their tail vein.

2.2.2.1.5 BCG immunisation

The freeze-dried vaccine was rehydrated in PBS before vaccination. Mice received a single dose of BCG Danish 1331 (5×10^6 CFU) injected subcutaneously at the base of the tail.

2.2.2.2 Experimental *M. tuberculosis* infections

C57BL/6 mice were challenged by the aerosol route with ~500 CFU of *M. tuberculosis* Erdman/mouse. The mice were sacrificed 5 weeks after challenge. Bacteria were enumerated in the liver, spleen, and lungs. CFUs were determined by serial 3-fold dilutions of individual whole-organ homogenates in duplicate as described below.

2.2.2.3 Determination of pathogen burden

All organs were homogenised in 5mL of sterile water using a Seward Stomacher 80 (Seward, London UK) for 2 minutes at high speed. Serial dilutions of each organ were plated onto 7H11 medium plates (described above in section 1.2.1.2) in duplicate. Colonies were counted after 2–3 wk of incubation at 37°C.

2.2.2.4 Natural Killer Cells (NKC) depletion

NKC were depleted from Balb/c mice by administration of 50µg of functional grade rabbit anti-asialo GM1 antibody (Wako Chemicals, Germany) in 0.2mL PBS by intravenous or 0.3mL intraperitoneal injection (Harshan and Gangadharam 1991; Stitz, et al. 1986). In addition, for depletion of NKC from both the NALT and CLN, mice were depleted intranasally with 5µg anti-asialo GM1 in 10µL PBS. Control animals received 50µg or 5µg of appropriate functional grade rabbit isotype control antibody (rabbit IgG, R+D Systems, UK). Injections were performed seven days prior to antigen immunisation and repeated every 3-4 days to maintain depletion. Intranasal depletions were performed five days prior to immunisation in between intravenous injections. Upon sacrifice, FACS analysis using PE or FITC-conjugated anti-DX5 antibody was performed on spleens, NALT and CLN of depleted and control mice to quantify the level of depletion.

2.2.3 Molecular Methods

2.2.3.1 Plasmid DNA extraction

Plasmid DNA was isolated from 10 mL overnight cultures of *E. coli* or *S.* Typhimurium strains harbouring plasmids using the Qiagen miniprep kit (Qiagen) as per the manufacturer's instructions.

2.2.3.2 Agarose gel electrophoresis

Plasmid DNA and PCR products and restriction enzyme digestion samples were routinely subjected to electrophoresis on 0.5 % to 1.5 % agarose gels (depending on estimated sizes of fragments). Agarose gels were prepared by boiling agarose (Sigma ultra pure grade) in TAE. Once cooled, ethidium bromide was added (final

concentration 0.5 –1.0 μg/mL). DNA samples were mixed with one-sixth volume of tracking dye (0.25 % bromophenol blue, 0.25 % xylene cyanol 30 % glycerol, 6 mM EDTA, filter sterilised - 0.22 μm filter) and loaded onto the gel (5 μL per lane for PCR products, 25 μL for restriction enzyme digests). On all agarose gels presented the size of the product is compared to 1 Kb Plus DNA Ladder™ (Invitrogen) which has 12 regularly spaced bands ranging from 1000 bp to 12,000 bp. Electrophoresis was performed at a constant voltage (80–100 V). The DNA was visualised by illumination on UV transilluminator and photographed using UVtech DNA documentation system.

2.2.3.3 DNA digestion

All restriction endonucleases used were supplied by New England Biolabs. Selection of an appropriate endonuclease was performed *in silico* by using WEBCUTTER (http://www.webcutter.com). Restriction enzyme digestions of DNA were carried out as recommended by the manufacturer (New England Biolabs). In general, 5 to 20 units of enzyme were used per μg of genomic DNA along with 5 mL of appropriate 10x buffer, made up to 50 μL with water. Digestion of plasmid DNA was normally for 1.5 - 2 hours at the recommended temperature (normally 37 ^{o}C) in a water bath, while for chromosomal DNA digestion was allowed to proceed overnight. When complete, 1 μL of 6x loading dye was added to each digest and the reaction was stored at –20 ^{o}C until required. The digests were then examined on a 1 % agarose gel.

2.2.3.4 DNA ligation

Ligations of DNA fragments and vectors were carried out using T4 DNA ligase and buffer as per the manufacture's instructions (Roche). The reactions were carried out in a total volume of 10-20 μL in the presence of ligation buffer and ATP (1 mM). Ligations were incubated overnight at 4 ^{o}C. Controls of (a) no DNA ligase or (b) no insert were also set-up at the same time as ligation of vector and insert reaction. Following ligation, samples were transformed into competent host bacteria (Section 2.2.3.10). To determine the success of the ligations, plasmid DNA was isolated from the transformants (as described in Section 2.2.3.1) and digested with the appropriate restriction enzyme. The digests were then examined on a 1 % agarose gel.

2.2.3.5 Oligonucleotides

The oligonucleotides used in this study are outlined in Table 2.1.4. Custom primers were designed for this study using several criteria; all primers designed were between 18-25 bases, all primers (apart from those designed to include restriction sites) were selected with a G-C content of approximately 50 %; the annealing temperature of the primers was calculated to be between 55 ^{o}C and 65 ^{o}C; primer pairs were designed with approximately the same length, G-C content and Tm; all primers were designed to avoid repetitive sequence and to end in either a G or C. All primers were purchased from Sigma-Genosys Ltd and were dissolved in 10 mM TE at a concentration of 40pmoles/μL.

2.2.3.6 Polymerase chain reaction (PCR)

The PCR method utilised a standard *Taq* polymerase PCR reaction using Platinum PCR SuperMix (Invitrogen). All the PCR reactions were executed on a DNA engine DYAD thermal cycler (MJ research). In those experiments where a negative control was required template was omitted from the reaction mixture (for primer details see Table 2.1.4). PCR amplifications using the Platinum PCR SuperMix were carried out as per manufacturers instructions in a total volume of 100 μL. PCRs were performed in 0.2 mL thin wall PCR tubes. The amount of template used depended on the source. For plasmid DNA this was approximately 10 ng, whilst for genomic DNA approximately 100 ng was used. When using intact bacteria, a small piece of each colony was removed and mixed in 50μL dH_2O and then boiled for 10 minutes. 2.5μL to 5μL of this mixture was added to the final PCR mix. The dNTP mixture and magnesium was supplied in the Platinum SuperMix along with the recombinant *Taq* enzyme. The quantities used in the PCR reaction mixture are outlined in Table 2.8.
DNA amplifications were performed using the following programme:

a. 1 cycle of 95°C for 5 minutes.
b. 30 cycles of 95°C for 1 minute, 55°C* for 1 minute and 72°C$^{@}$ for 2 minutes.
c. 1 cycle of 72°C for 7 minutes.
d. Held at 10 °C.

*Annealing temperature dependant on Tm of primers; 55 °C is an estimated average. Each primer set was optimised for PCR by testing at a range of temperatures between 55°C-65°C; all other parameters were kept constant throughout.

@ Elongation time was dependant on expected size of product; 1 minute per kb as a rule of thumb.

Table 2.8: PCR Mixture for Platinum SuperMix (Invitrogen)

Reagent	Volume	Final Concentration
Template DNA	2µL for purified DNA, 2.5 - 5µL lysed bacteria	2ng genomic DNA
Forward Primer	2µL	0.8pmol
Reverse Primer	2µL	0.8pmol
Platinum PCR SuperMix	94µL for DNA reaction, 91.5 - 93.5µL for intact bacteria PCR	22 U/ml complexed recombinant *Taq* DNA polymerase with Platinum® *Taq* Antibody, 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 µM dGTP, 220 µM dATP, 220 µM dTTP, 220 µM dCTP, and stabilizers.
	100µL	

The reagents, volumes and final concentrations of a standard 100 µl Platinum PCR SuperMix reaction.

2.2.3.6.1 Characterisation of PCR products

PCR amplicons were visualised and sized on a 0.5-1.5 % agarose gel (dependent on size of product) as previously described (see section 2.2.3.2). PCR products used for cloning were purified to remove excess nucleotides using a PCR purification kit (Qiagen), as per manufacturers' instructions. Any products that were excised from agarose gels were purified using the DNA gel purification kit (Qiagen) as per manufacturer's instructions.

2.2.3.7 Precipitation of DNA

In some instances DNA was concentrated before further use by ethanol precipitation. The DNA sample was adjusted to a volume of 400µL with EB buffer. The DNA was precipitated by the addition of 1mL 100% ethanol, 40uL of 3M sodium acetate (pH 7.0) and 1µL glycogen (Roche). Samples were placed at -20°C for 20 minutes, centrifuged for 30 minutes at 14,000 rpm, washed in 1.5mL of 70% ethanol and then

centrifuged for 2 minutes at 14,000 rpm. The pellet was vacuum-dried for 5 minutes at 45°C, re-suspended in 10µL dH_2O and stored at 4°C.

2.2.3.8 DNA sequencing

PCR products were cloned into pCR-XL-TOPO (Invitrogen) and then electroporated into OneShot Electrocomp Cells (Invitrogen) according to the manufactures instructions. Purified plasmids containing the insert were then sent to the Wellcome Trust Sanger Institute, Cambridge for sequencing.

2.2.3.9 Preparation of electrocompetent cells

Electroporation was used for the transformation of *Salmonella* and *E. coli* species with stable plasmids (10-100 ng). A 1:100 dilution of an overnight bacterial culture was prepared using fresh LB broth and incubated at 37 °C with aeration (200 rpm). Once the cells had reached mid-log phase (approx. 4 hours) the bacteria were harvested by centrifugation at 4000 rpm for 10 minutes (sorvall legend RT). The bacterial pellet was washed with 30 mL of ice-cold 10 % glycerol then centrifuged as above. The bacteria were washed a second time, and the pellet was re-suspended in 400 µL of ice-cold 10 % glycerol in water. This gave an approximate cell density of 10^{10} CFU/mL.

2.2.3.10 Electroporation

Aliquots (50 µL) of the competent cells were added to chilled electroporation cuvettes (Equibio) (0.1 cm for *E. coli*, 0.2 cm for *Salmonella*) and kept on ice. Plasmid DNA (10 ng-1 µg) was added to each cuvette. Competent cells with no plasmid DNA served as negative controls. Electro-competent bacteria were electroporated using a Bio-Rad Gene Pulser™ using the following conditions; *E. coli* 1.25 kV, 25 µF, 600 Ωhms. *Salmonella* 2.0 Kv, 25 µF, 600 Ωhms.

Immediately following electroporation the contents of the cuvette were transferred a microfuge tube containing 400 µl of electroporation recovery media (SOC) and incubated with agitation for 1.5 hours at 37°C. Bacterial cells were plated onto selective antibiotic plates and incubated overnight at 37°C. The following day, colony numbers were calculated and cell morphology examined. The presence of the plasmid was confirmed by visualisation on 0.7 ~ 1 % agarose gel following extraction, and in some cases by PCR.

2.2.3.11 Identification of bacteria

The identity of *Salmonella* was confirmed using standard laboratory slide agglutinations. Agglutination tests were performed with *Salmonella* antisera (ProLab) as per the manufacturer's instructions. Briefly, 15 µL of anti-sera were placed upon a glass slide and then mixed with a small amount of bacterial culture and the slide was rocked side to side. Agglutination of the bacteria within one minute was considered a positive result. Irrelevant anti-sera acted as a negative control.

2.2.3.12 Red Recombinase "one step" mutagenesis

The mutagenesis used in this study was based upon the red recombinase method described by Husseiny and Hensel (Husseiny and Hensel 2005). The recombinase genes are carried on the pKD46 plasmid; this plasmid confers Ampicillin resistance and is temperature sensitive i.e. it is stable at 30^{o}C. *S.* Typhimurium SL3261 containing pKD46 (provided by Dr R Kingsley, Wellcome Trust Sanger Institute) was grown overnight in LB broth containing aromatic mix and 100 µg/ml Ampicillin. This culture was then used to inoculate fresh LB broth containing 1mM arabinose and 100 µg/ml Ampicillin and electrocompetent cells were prepared. The total volume of the re-suspended PCR product was then electroporated into *S.* Typhimurium SL3261 containing pKD46. The cells were placed in SOC at 37^{o}C for 1.5 hours. 100 µL of culture was then removed from the SOC, plated on LB plates containing aromatic mix and kanamycin (50 µg/ml) and grown at 37^{o}C. The remainder of the SOC was left overnight at ambient temperature, and if no growth was observed from the overnight plates, this was plated as before. Any resulting colonies were sub-cultured onto

selective media and multiple colonies were selected and screened by colony blots for protein (Ag85B-ESAT6) production.

2.2.3.13 Transduction of *Salmonella* with P22 phage

Before mutants were thoroughly screened the red recombinase transformants were transduced onto a 'clean' *S*. Typhimurium SL3261 background using P22 phage. P22 stock (provided by Dr. D. Pickard, Wellcome Trust Sanger Institute) was diluted to obtain; neat, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} in λ buffer/ T2 buffer with 10µL of each phage dilution plus controls i.e. cells only, being added to 200µL of the donor *Salmonella* strain. This was left to adsorb at 37°C for 20-30 minutes statically before 3mLs of melted top agar was added, mixed and then immediately poured over LB agar plates containing appropriate antibiotic. The agar was allowed to set for 5 minutes and then incubated at 37°C for 4-6 hours until plaques grew to confluence. Plates were then covered in 3mLs λ buffer/ T2 buffer and left overnight at 4°C. The top agar layer and λ buffer/ T2 buffer was then harvested and put into tubes with 50µL chloroform which was then mixed well for ~ 1 minute. The tubes were left to shake for 30 minutes at 37°C before being centrifuged for 10 minutes at 4000rpm until a clear supernatant was produced. The supernatant was transferred into a glass bijou and 50µL chloroform was added to kill remaining cells. The solution was then filtered with a 0.45µm filter to produce the new phage lysate. The new phage lysate stock was diluted to obtain; neat, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in λ or T2 buffer and 200µL of overnight recipient *Salmonella* cells were added to 10µL of each phage dilution. This was then allowed to adsorb at 37°C for 20-30 minutes before 1mL LB broth and 5mM EGTA was added to each phage aliquot and incubated at 37°C for 1 hour 30 minutes stationary. Bacteria (100µL aliquots) were then spread onto LB agar plates containing 5mM EGTA plus appropriate antibiotic and incubated overnight at 37°C. Transduced colonies were picked and re-streaked onto fresh 5mM EGTA plates plus appropriate antibiotic and incubated again overnight at 37°C to 'cure' any remaining phage. Colonies were then tested for recombinant protein expression and any giving strong Ag85B-ESAT6 production were further examined using SDS PAGE and then western blot analysis before being stored in microbanks at -80°C.

2.2.4 ELISA methods

2.2.4.1 ELISA for total Ig, IgG and IgG subclasses in mouse sera (General ELISA protocol)

Flat-bottomed Nunc Maxisorp plates were coated overnight at 4 ^{o}C with 50 µL of a 2 µg/mL solution of relevant antigen in coating buffer (0.1 M Na_2HPO_4 at pH 9). Following one wash with PBS containing 0.01 % Tween-20 (PBST) plates were blocked with 100 µL of 3 % BSA in PBS at 25 ^{o}C for 1 hour. Plates were then washed once with PBST, and sera from experimental animal groups, was added as follows. 3 µL of sera was added to 27 µL of PBS + 0.01 % BSA (PBS-BSA), 12.5 µL of this was added to 112.5 µL of PBS-BSA in the top well, which is a 1:100 dilution of serum and then diluted (PBS-BSA) 5 fold down the plate. Each plate contained control wells with pre-immune sera, PBS alone, and known positive immune sera. The plates were then left to incubate for 1 hour at 37 ^{o}C. After 3 washes with PBST, antibodies conjugated to HRP diluted 1:1000 in PBS-BSA were added at 100 µL per well. Conjugate antibodies were either anti-mouse total Ig (Dako), anti-mouse IgG (Sigma), anti-mouse IgG1 and anti-mouse IgG2a (Pharmingen). Plates were incubated for 1 hour at room temperature, washed 3 times with PBST and developed using Sigma fast OPD tablet set (50 µL per well). Development was left to proceed for 5 minutes and stopped with 25 µL 3 M sulphuric acid. Absorbances were read at 490 nm and titres calculated based on the reciprocal dilution giving an absorbance of 0.3, using a computer based program called Endpoint.

2.2.4.2 ELISA for IgG2b, IgG3, IgE, IgA and IgM in mouse sera

The general ELISA protocol was modified as followed to determine IgG2b, IgG3, IgE, IgA an IgM titres in mouse sera. Purified rat mAb against the different antibody classes (Pharmingen) were used as secondary antibodies. All secondaries were used at 1:1000 dilutions. To detect the purified rat mAb, the plates were washed 3 times in PBST, then incubated with 100 µL per well of rabbit anti-rat Ig-HRP diluted 1:1000 in PBS-BSA for 1 hour at RT. Plates were developed as described above.

2.2.4.3 ELISA for IgA in sera and lung and nasal washes

Again the general ELISA protocol was followed with some modifications. The lung and nasal washes were collected from animals using 1 mL PBS containing a cocktail of protease inhibitors (Roche) and stored at -20 ^{o}C prior to use. The washes and serum were added to the plate as follows. All wells contained 112.5 μL PBS-BSA and 12.5 μL of the washes and serum was added to the top wells (1:10 dilution) and further diluted 1:5 down the plate. The secondary antibody used was anti-IgA, conjugated to biotin, at a dilution of 1:1000. Dilutions of the antibodies were determined using purified isotype antibodies and the calibration was carried out by Dr. C. Hale (Wellcome Trust Sanger Institute). The ELISA plates were incubated for 1 hour at 37 ^{o}C and washed x3 with PBST and then the tertiary streptavidin conjugated to HRP antibody was added at 1:1000 in PBS-BSA 100 μL per well. The plates were left for 1 hr at room temperature, washed x3 with PBST and developed as described above.

2.2.5 Measurement of cellular responses

2.2.5.1 Isolation of lymphocytes

Immunised and naive mice were sacrificed by cervical dislocation and their CLN, NALT and spleens aseptically removed into RPMI supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (RPMI^{+}). For NALT removal see separate protocol (Section 2.3.1.1). Single cell suspensions were made by passing homogenised organs through a 100 μm cell strainer (Becton Dickinson). Cells were centrifuged at 1500 rpm for 5 minutes followed by 5 minutes incubation with 0.5 % tris-ammonium chloride (pH 7.2) solution to remove erythrocytes. Cells were washed twice with RPMI, centrifuged at 1500 rpm for 5 minutes and resuspened in 1mL RPMI^{+}. Viable cells were counted, by diluting 1 to 1 with Trypan blue, using a Neubauer haemocytometer.

2.2.5.2 T-cell cytokine assay using Cytokine Bead Analysis (CBA)

Single cell suspensions were prepared as stated previously. Cells were seeded, in duplicate, into a round-bottomed 96-well tissue culture plate to a concentration of 5 x 10^{6} cells in a volume of 200 μL. The cells were allowed to settle for 1 hour at 37 ^{o}C (5

% CO_2) before being stimulated with the appropriate antigens and controls. The antigens (Ag85B-ESAT6, ESAT-6 and Ag85B peptide) were added at a concentration of 5 μg/mL with the positive controls (concavalinA) also added at a final concentration 5 μg/mL. The negative control used was $RPMI^+$. The plates were incubated, at 37 ^oC and 5 % CO_2, for 24-48 hours when 100 μL of supernatants were removed to a fresh plate and stored at -80 ^oC for subsequent CBA. The mouse cytokine flexi CBA kit (Becton Dickinson Pharmingen, USA) was used as per the manufactures instructions and set-up on the FACSAria (Becton Dickinson, USA). The cytokine levels of TNF-α, IL-12p70, IL-6, MCP-1, IL-10, IL-4, IL-5, IL-2 and IFN-γ were measured.

2.2.5.3 Lymphocyte preparation for FACS

Single cell suspensions were prepared as above and diluted as necessary with $RPMI^+$ to obtain a final concentration 5 x 10^6 cells/mL for spleens and CLN and for the NALT a concentration of 5 x 10^5 cells/mL. Cells (200μL of each cell suspension) were added to a 96-well plate corresponding to the number of FACS antibody-dye mixtures (see Table 2.1.8 for details) and centrifuged at 1500rpm for 5 minutes. The cells were then resuspended in 50μL PBS + 1% BSA and 0.05% sodium azide (azide buffer) and to this; 50μL of each dye mix was added with incubation in the dark at 4^{o}C for 30 minutes. The plate was then spun down at 1500rpm for 5 minutes and the cells washed with 100μL of azide buffer. Another spin was preformed and the cells finally resuspened in 100μL 1% paraformaldehyde and stored in the dark at 4^{o}C overnight before analysis on the FACSAria (Becton Dickinson, USA).

2.2.6 Protein overexpression

2.2.6.1 Protein gel separation

Samples were mixed with an equal volume of 2X SDS loading dye (0.125 M Tris-HCl, 4% SDS, 2% sucrose, 0.2 M Mercaptoethanol, 0.02% bromophenol blue, pH 6.8) and boiled for 5 minutes. The protein solutions, along with 5 μL SeeBlue® ladder (Promega), were separated in a 15% Precast Ready Gel (Bio-Rad) in MOPS buffer

(Bio-Rad) and run at 150 V. The gel was placed in either coomassie blue stain or transferred to nitrocellulose for western blot analysis.

2.2.6.2 Western blot analysis

Proteins were separated as described above. The gel, filter paper and nitrocellulose membrane were then allowed to soak in transfer buffer (0.19 M glycine, 25mM Tris-HCl pH 7.5, 20 % methanol) for approximately 30 minutes. The protein samples were then transferred onto the nitrocellulose membrane using semi-dry Bio-Rad transfer system at 15 V for 45 minutes. After completion of the transfer, the membrane was blocked overnight with 10 % milk in TBST (500 mM NaCl, 20 mM Tris-HCl and 0.01 % Tween-20) at 4 ^{o}C with gentle shaking. The membrane was washed twice for 5 minutes with TBST. The primary antibody, mouse anti-ESAT6 (Statens Serum Institute, Denmark), was added at 1:25 dilution in TBST (+1 % milk) and the membrane left to incubate for 1-2 hours at room temperature. The membrane was rinsed with TBST twice for 5 minutes and the secondary antibody, anti-mouse Ig (Dako) was added at 1:1000 dilution and for 1 hour at room temperature. The membrane was washed x3 with TBST for 5 minutes each time and then developed with Opti-4CN substrate (see section 2.2.6.5) for approximately 20 minutes. The reaction was stopped by washing with distilled water.

2.2.6.3 Coomassie blue stain

Gels were fixed in a solution of 0.01 % coomassie blue, 40 % ethanol and 10 % glacial acetic acid for 1 hour at room temperature. Gels were destained in 40 % ethanol and 10 % glacial acetic acid for 2-3 hours, also at room temperature.

2.2.6.4 Colony blot analysis

Dry, numbered nitrocellulose filters were placed onto LB-agar plates containing appropriate antibiotics (grids of 20 colonies/plate). Transformed colonies were picked from previous transformation reactions with sterile toothpicks and pressed onto nitrocellulose before incubation overnight along with positive and negative controls. The colonies picked with the toothpicks were also streaked onto normal LB-agar

plates containing the appropriate antibiotics and left to incubate overnight to obtain single colonies. After overnight incubation, the nitrocellulose filters were removed and placed into a dish of chloroform for 15-20 minutes to lyse the bacteria. Membranes were then washed in TBS for 5 minutes with rocking before being covered in blocking solution (3% gelatin in TBS) for 30 minutes with gentle agitation. Filters were again washed twice in TBST for 5 minutes and the immersed in appropriately diluted primary antibody (dilute in 1% gelatin TBST) and left for 2 hours at room temperature with gentle agitation. After another TBST wash membranes were covered in appropriately diluted secondary antibody HRP conjugate (dilute in 1% gelatine TBST) and again left to incubate for 2 hours at room temperature with rocking. The filters were again washed 3 times with TBST and were finally developed with Opti-4CN substrate kit (Bio-Rad).

2.2.6.5 Opti-4CN stain (Bio-Rad)

Filters were covered with one part Opti-4CN diluent concentrate with nine parts dH_20. Membranes were incubated with gentle agitation in the substrate for up to 30 minutes or until desired level sensitivity was attained. Membranes were then washed in dH_20 for 15 minutes before being documented and stored.

2.3 Tissue staining methods

2.3.1 Sectioning

2.3.1.1 NALT removal

Mice were sacrificed by cervical dislocation and surface sterilized with 70% ethanol. They were laid on their backs and the lower jaw completely removed. The NALT is located just below the soft palate at the roof of the mouth. At the third palatial ridge the NALT begins and using a very sharp scalpel, the outside of the palate was cut next to the teeth and peeled back gently, with some brief teasing with the scalpel blade point staring from the nose end. The NALT, which adheres to the soft palate, was then either prepared for frozen sectioning (see Section 2.3.1.2) or placed in $RPMI^+$ for FACS (see Section 2.2.5.3).

2.3.1.2 Frozen sectioning

Mice were sacrificed by cervical dislocation, surface sterilized with 70% ethanol and the CLN and NALT removed aseptically – refer to separate protocol (section 2.3.1.1). The CLN and NALT were covered in OTC and frozen using tetrafluroethane (Cool-Jet), before being transferred to a cryo-vial and snap-frozen in liquid nitrogen. Before sectioning, the vials were again snap frozen in liquid nitrogen and 6μm sections cut using the Shanndon cryostat. Sections were transferred to poly-lysine-L coated slides and allowed to air-dry overnight at room temperature before use.

2.3.2 Immunofluorescent staining

2.3.2.1 Single immunofluorescent staining

Sections were fixed in a 1:3 ratio of 100% acetone and 100% ethanol for 5 minutes. Following 3 times 5 minute washes in PBS, sections were blocked with normal goat serum (10% goat serum, 5% fish gelatin, 0.01% sodium azide, and 0.1% BSA, and 0.01% Tween-20) for 45 minutes. Serum was then tapped off, and primary antibody added (see Table 2.6 for primary antibodies used). Control sections for each group did not have primary antibody added; only PBS was added to these sections. Slides were incubated for 1 hour at 37^{o}C in darkness and then washed twice in PBS for 5 minutes. The sections were then covered in appropriate secondary antibody (see Table 2.6) and again incubated for 1 hour at 37^{o}C in darkness and then washed x3 with PBS. Sections were counterstained with Hoechst for 15 minutes at room temperature in the dark before again being washed x3 in PBS. Finally slides were mounted with coverslips and ProLong Gold (Invitrogen) and left to air-dry in the dark for 4-5 hours before visualisation using a Confocal Microscope.

2.3.2.2 Double or triple immunofluorescent staining

The single immunofluorescent protocol was followed with some modifications. When using directly conjugated flurochrome primaries, all antibodies were diluted in goat serum at appropriate dilutions and incubated for 1 hour at 37^{o}C in the dark. Sections were then counterstained with Hoechst before being mounted in ProLong Gold. When using a mixture of directly conjugated and non-flurochrome conjugated primaries,

antibodies not directly conjugated were diluted in goat serum and incubated on sections for 1 hour at 37^{o}C in the dark. Slides were then washed 4 times in PBS for 5 minutes each time before the appropriate secondary flurochrome conjugated antibodies and primary directly conjugated antibodies were added and allowed to incubate for another hour at 37^{o}C again in the dark. Slides were washed in PBS and then counterstained with Hoechst for 15 minutes before being mounted with coverslips and ProLong Gold and left to air-dry for 4-5 hours at room temperature in darkness.

2.3.3 Histology

The frozen sections were also used for haematoxylin and eosin staining. Sections were fixed in a 1:3 ratio of 100% acetone and 100% ethanol for 5 minutes before being rinsed in PBS for 5 minutes Slides were submerged in Mayer's haematoxylin for 2 minutes and washed in tap water for 2 minutes to remove excess stain and "blue". The sections were immersed in eosin Y for 1 minute and again washed in tap water for 3 minutes. The tissue sections were then dehydrated through ethanol, beginning with 70% ethanol (2min), followed by 90% (2min), 100% (4min) and 2 minutes in histoclear. Finally, slides were mounted in DPX, allowed to dry overnight and viewed using a light microscope.

2.4 Statistical analysis

Antibody titres were analysed using a non-parametric two-tailed Mann-Whitney U-Test statistic. Experiments with more than two groups were analysed with the non-parametric Kruskal-Wallis H one-way analysis of variance with Dunn's multiple comparison post-hoc test. Total percentages of cell populations and mean fluorescence intensity (MFI) were analysed using one-way ANOVA with Dunnett's multiple comparison post-hoc correction. A p value of < 0.05 was taken to be significant in all cases. All tests were performed using the graphing and statistical software GraphPad Prism 4 (GraphPad Software, Inc, USA).

3 Immunogenicity of the tuberculosis fusion antigen, Ag85B-ESAT6, after an intranasal prime-boost regimen

3.1 Introduction

Prevention and control of tuberculosis involves two main approaches; the first step is to identify those infected with *M. tuberculosis*, as well as their contacts. Infected patients are isolated and treated with the available antibiotic cocktail for up to 12 months to completely eliminate the bacterium. The other approach is to vaccinate young children to protect against tuberculosis. Currently the only licensed vaccine in the fight against tuberculosis is the BCG vaccine. This vaccine has been used for over 80 years and is 80% effective against serious forms of the disease, e.g. meningitis, in children. However, its protective efficacy in adults against the most common form of the disease, pulmonary tuberculosis, ranges from 0 to 80% (Colditz, et al. 1994).

The development of several new vaccines to prevent tuberculosis is currently ongoing. They include; rBCG vaccines, live attenuated strains of *M. tuberculosis*, non-pathogenic Mycobacteria, non-mycobacterial microbial vectors, DNA vaccines, and subunit vaccines. However, as previously discussed in Chapter 1, there are only a very small number of vaccines currently in human trials. A new more effective vaccine would be expected to improve tuberculosis control substantially, and therefore vaccine development is one of the highest priorities in tuberculosis research.

3.1.1 The tuberculosis fusion antigen, Ag85B-ESAT6

Two major antigens produced by *M. tuberculosis* during infection are Ag85B (a 30kDa mycolyl transferase) and ESAT6 (a small 6kDa protein secreted by *M. tuberculosis* belonging to the ESAT-6 family). As already discussed in the introduction, these individual antigens have been shown to induce strong immune responses in a number of animal models (Brandt, et al. 2000; Geluk, et al. 2000; Huygen, et al. 1996; Huygen, et al. 1994; Kamath, et al. 1999; Kariyone, et al. 2003; Launois, et al. 1994; Mustafa, et al. 2000b; Olsen, et al. 2000; Thole, et al. 1999). In addition, previous work on the antigens ESAT-6 and Ag85B have shown that the fusion of Ag85B-ESAT6 is more immunogenic, and gives higher levels of protection

compared to the individual antigens, as well as the fusion in the other orientation, i.e. ESAT6-Ag85B. The initial study by Peter Andersen's group showed that administration of the fusion protein with the adjuvant dimethyl dioctadeclammonium bromide-monophosphoryl lipid A (DDA) stimulated a strong dose-dependent immune response, and also induced high levels of protection comparable to those induced by BCG. Their definition of protection was the reduction in *M. tuberculosis* CFUs in the lungs, spleen and liver when compared to BCG vaccinated animals. Their vaccine also induced efficient immunological memory, which remained stable 30 weeks post vaccination (Olsen, et al. 2001). In the second study, the same fusion vaccine was administered to mice and guinea pigs, either orally with the adjuvants MPL, CT-B or LT, or subcutaneously in MPL. A separate group of mice received the subcutaneous vaccine followed by oral boosting, the aim being to stimulate efficient immunity in the lung by targeting the gut mucosa. Oral priming alone resulted in increased IFN-γ production by splenocytes; however this was not enough to prevent infection by *M. tuberculosis*. In the heterologous priming and boosting group, there was a significant increase in systemic type 1 responses along with reduced CFU counts in both the lungs and liver after aerosol challenge with virulent *M. tuberculosis*, which were comparable to the subcutaneous immunisation protocols (Doherty, et al. 2002). Another recent study by this group with the fusion vaccine demonstrated that when Ag85B-ESAT6 was aerosol administered to guinea pigs in a liposomal adjuvant, it conferred a significant level of protection compared to the individual or a cocktail of the antigens (Olsen, et al. 2004). Langermans et al investigated the efficacy of the Ag85B-ESAT6 fusion protein vaccine in a non-human primate model for tuberculosis. In this study, vaccination of cynomolgus monkeys with the fusion in two different adjuvants (DDA/MPL and AS02A) resulted in a reduction in bacterial numbers and/or lung pathology in animals challenged with *M. tuberculosis* (Langermans, et al. 2005).

3.1.2 Intranasal immunisation and *M. tuberculosis*

M. tuberculosis is an airborne pathogen that usually enters the host via the mucosal surface of the lung after inhalation of infectious droplets from an infected individual. Since the respiratory tract is the natural route of *M. tuberculosis* infection, mucosal, and in particular, intranasal immunisation is an attractive route for vaccination, and a number of studies have demonstrated that this route may provide strong protection against *M. tuberculosis* challenge (Chen, et al. 2004; Falero-Diaz, et al. 2000; Giri, et al. 2005; Wang, et al. 2004). One explanation for the efficiency of mucosal administration against *M. tuberculosis* infection could be the engaging of local immune responses in the lung, particularly antigen specific memory T cells which may preferentially home back to the site of vaccination (Kamath, et al. 2004). Further, the location of T cells in the airway at the time of infection is of importance (Santosuosso, et al. 2006; Santosuosso, et al. 2005). As already stated with nasal delivery, no needles are employed and given the high rate of co-infection with *M. tuberculosis* and HIV, especially in developing countries, this certainly suggests that this route of vaccine delivery deserves further attention.

Considering the information described above, I hypothesise that intranasal immunisation with the *M. tuberculosis* fusion antigen, Ag85B-ESAT6 with the mucosal adjuvants LT and LTK63 will be effective at stimulating strong T_H1 immune responses both locally, i.e. within the NALT, CLN and lungs, as well as systemically. Consequently, I decided to determine the validity of this system as a vaccination regimen against *M. tuberculosis*.

3.2 Results

3.2.1 An intranasal prime-boost immunisation regimen with Ag85B-ESAT6 induces serum Ig

To investigate immune responses following Ag85B-ESAT6 immunisations over time, mice were immunised as outlined in Table 3.1. Serum collected on day 21, 35 and 42 was analysed for specific anti-Ag85B-ESAT6 Ig antibodies by ELISA to determine if a number of different prime-boost regimens were sufficient to trigger a measurable antibody response. As already discussed LTK63 is a mutant form of LT and consequently does not have such a strong adjuvant activity as the wild-type form. Previous studies have shown that ten times more LTK63 is needed to induce similar immune responses to those observed with LT, hence the choice of adjuvant doses in this vaccine study (i.e. 1µg LT and 10µg LTK63) (Peppoloni 2003).

Table 3.1: Ag85B-ESAT6 prime-boost immunisation regimen (5-10 mice/group)

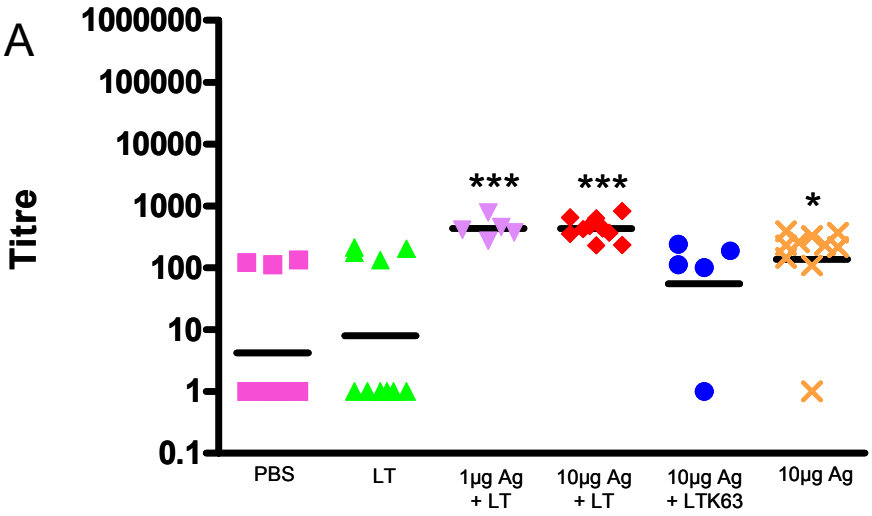
Day	Group	Immunised antigen	Procedure	Route
0	1	naïve	immunisation	i.n.
	2	1µg LT		i.n.
	3	1µg Ag85B-ESAT6 + 1µg LT		i.n.
	4	10µg Ag85B-ESAT6 + 1µg LT		i.n.
	5	10µg Ag85B-ESAT6 + 10µg LTK63		i.n.
	6	10µg Ag85B-ESAT6		i.n.
21	All groups		sample bleed	
28	1	naïve	boost	i.n.
	2	1µg LT		i.n.
	3	1µg Ag85B-ESAT6 + 1µg LT		i.n.
	4	10µg Ag85B-ESAT6 + 1µg LT		i.n.
	5	10µg Ag85B-ESAT6 + 10µg LTK63		i.n.
	6	10µg Ag85B-ESAT6		i.n.
35	All groups		sample bleed	
36	1	naïve	boost	i.p.
	2	1µg LT		i.p.
	3	1µg Ag85B-ESAT6 + 1µg LT		i.p.
	4	10µg Ag85B-ESAT6 + 1µg LT		i.p.
	5	10µg Ag85B-ESAT6 + 10µg LTK63		i.p.
	6	10µg Ag85B-ESAT6		i.p.
42	All groups	sample bleed, lung and nasal washes and spleens for CBAs	END	

As shown in Figure 3.1, mice immunised with Ag85B-ESAT6, with or without LT or LTK63, developed a significant antibody response by day 21 (Figure 3.1A) after initial intranasal priming when compared to naïve animals, which lasted throughout the experimental study. Following an intranasal boost, groups receiving Ag85B-ESAT6 and the adjuvant LT displayed a further significant increase in serum Ig titres by day 35 (Figure 3.1B). Notably, mice receiving LT alone also showed an increase in antibody levels, but this increase was significantly less than groups receiving Ag85B-ESAT6 as well ($p < 0.01$) (Fig 3.1B). Following a further intraperitoneal boost, mice receiving 10µg Ag85B-ESAT6 plus adjuvant (LT or LTK63) showed a further significant increase in antibody titres compared to both naïve and mice receiving LT alone on completion of the experiment (day 42) (Figure 3.1C). Animals receiving purified Ag85B-ESAT6 alone showed no obvious increases in antibody titres after the initial intranasal priming (Figures 3.1A, B and C). Mice immunised with 10µg Ag85B-ESAT6 and 1µg LT gave the highest total Ig antibody titres following two intranasal and one intraperitoneal immunisation.

Figure 3.1: Serum total Ig responses to Ag85B-ESAT6 after i.n. immunisation.

Balb/c mice were primed intranasally with different concentrations of Ag85B-ESAT6 protein with or without wild-type LT and LTK63. Mice were then boosted via intranasal immunisation at day 28, and then again via intraperitoneal injection at day 35, to determine a dose response to the fusion antigen. Mice were left for 21, 35 and 42 days and then sample bled to determine anti-Ag85B-ESAT6 Ig antibodies. Naïve (Balb/c mice immunised with PBS) and wild-type LT immunised mice were also sample bled. Table 3.1 shows the immunisation regimen and groups involved in this study. Ag85B-ESAT6 specific antibody titres were determined by ELISA. ■ represents naïve animals, ▲ shows mice immunised with 1µg LT alone, ▼ and ◆ indicates those animals vaccinated with 1µg LT and 1µg or 10µg Ag85B-ESAT6 respectively, ● indicates mice immunised with 10µg fusion and 10µg LTK63 with ✕ showing those animals vaccinated with 10 µg Ag85B-ESAT6 alone. Total Ig titres (A day 21, B day 35 and C day 42) from naïve and immunised animals are expressed as antibody titre plotted on a logarithmic scale using a cut off of OD 0.3. The black bar shows the geometric mean titre from the group and the * indicates significant values of $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ as determined Kruskal-Wallis test, followed by Dunn's Multiple Comparison test compared to PBS immunised controls. Data is representative of two independent experiments.

3.1A


Titre
1000000
100000
10000
1000
100
10
1
0.1
***
***
*
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LT
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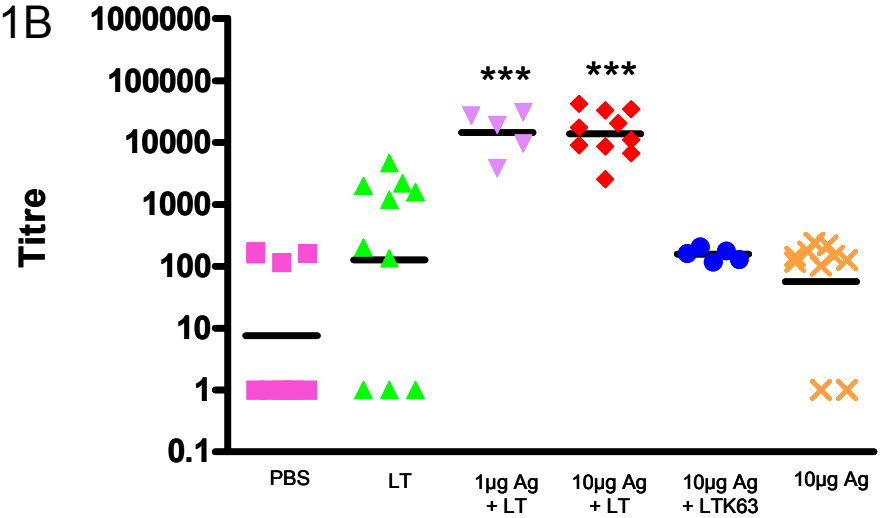
10µg Ag + LT
10µg Ag + LTK63
10µg Ag

3.1B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100000
10000
1000
100
10
1
0.1
***
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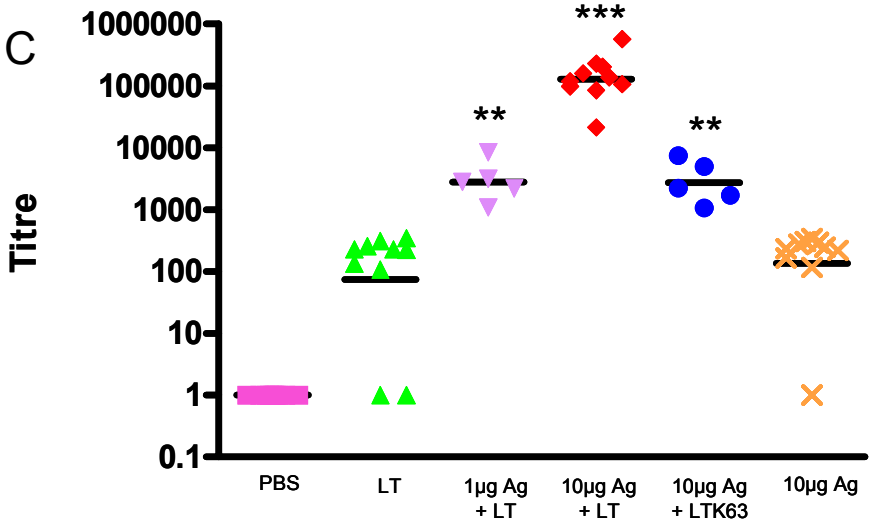
PBS
LT
1µg Ag + LT
10µg Ag + LT
10µg Ag + LTK63
10µg Ag

3.1C

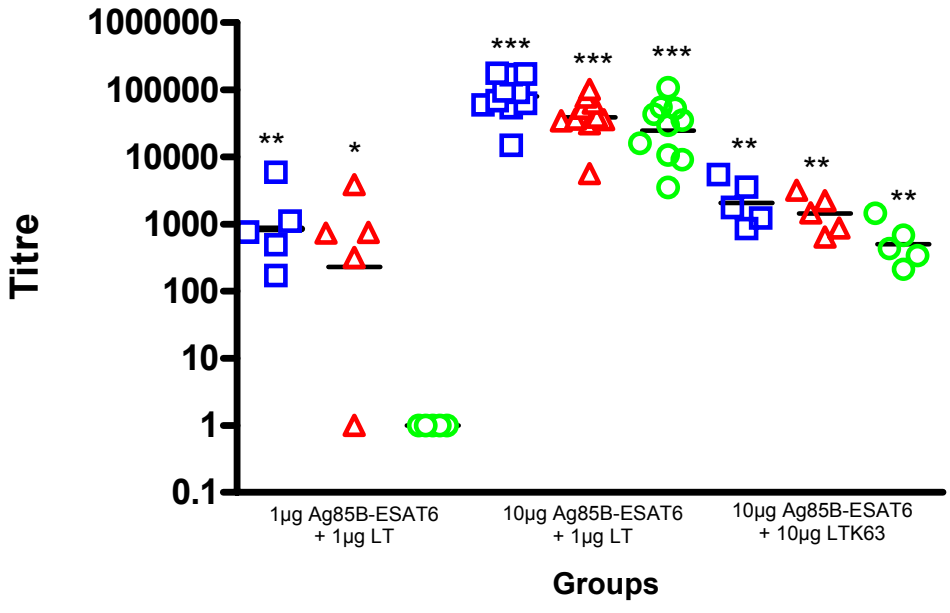

Titre
1000000
100000
10000
1000
100
10
1
0.1
***
**
**
PBS
LT
1µg Ag + LT
10µg Ag + LT
10µg Ag + LTK63
10µg Ag

3.2.2 IgG1:IgG2a ratios after intranasal administration of Ag85B-ESAT6 and mucosal adjuvants

Total IgG, IgG1 and IgG2a antibody responses in sera from vaccinated animals (as summarised in Table 3.1) were analysed for specificity to Ag85B-ESAT6 by ELISA. IgG subtypes were analysed to determine if any of the vaccination regimens were sufficient to trigger a specific measurable antibody response. The pattern of immunogenicity achieved by the various immunisation strategies was monitored by the IgG1/IgG2a ratio as a surrogate marker for the T_H1-T_H2 balance (Figure 3.2). Ag85B-ESAT6 without adjuvant failed to induce statistically significant levels of serum IgG when compared to negative control groups (data not shown). Vaccination with 1µg Ag85B-ESAT6 plus 1µg LT resulted in an exclusive IgG1 response ($p < 0.001$), while mice receiving 10µg Ag85B-ESAT6 and 1µg LT or 10µg LTK63 yielded both strong IgG1 and IgG2a antibody responses that were not statistically different ($p > 0.05$).

Figure 3.2: IgG1:IgG2a ratios after i.n. immunisation with Ag85B-ESAT6.

Balb/c mice were immunised as outlined in Table 3.1. Ag85B-ESAT6 specific antibody titres were determined by ELISA. Total IgG, IgG1 and IgG2a titres (day 42) from immunised animals are expressed as Log10 antibody titre using a cut off of OD 0.3. PBS, LT and fusion alone immunised mice had undetectable IgG sub-type titres and are not shown in figure. □ represents total IgG, red △ shows IgG1 and ○ indicates IgG2a antibody subtypes. The black bar shows the geometric mean titre from the group and the * indicates significant values of $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ as determined Kruskal-Wallis test, followed by Dunn's Multiple Comparison test compared to PBS immunised controls. Data is representative of two independent experiments.



3.2.3 The fusion protein plus LT induces significant serum IgA, and mucosal IgA in lung and nasal washes following immunisation

To determine serum and mucosal IgA antibody responses after prime-boosting with Ag85B-ESAT6, mice were immunised as outlined in Table 3.1. Levels of antigen specific IgA in serum and lung or nasal washes were determined using ELISA at day 42. Mice receiving 10µg Ag85B-ESAT6 plus 1µg LT were the only group to have any detectable serum or mucosal IgA antibody production when compared to naïve animals (Table 3.2). Levels of specific anti-Ag85B-ESAT6 IgA antibodies were highest in the serum of immunised animals, with lung washings containing comparable IgA titres. Nasal washings also contained antigen specific IgA antibodies, but at lower levels, with two animals not showing any detectable titres (data not shown)

Table 3.2: Ag85B-ESAT6 specific mucosal IgA responses after i.n. immunisation.

Serum and lung or nasal washes were collected from Balb/c mice immunised as outlined in Table 3.1. Mucosal samples were obtained by washing with 1mL of protease inhibitor cocktail solution (Roche) through both the nasal passages and lungs of each mouse. Specific anti-Ag85B-ESAT6 IgA antibodies were determined via ELISA. Average IgA titres are shown using a cut off of OD 0.3. Titres shown are the mean ± standard deviation of 5 animals for groups 3 and 5 and 10 animals for the remaining groups. † indicates less than 0.3OD detected with *** indicating significant values of $p < 0.001$ and ** indicating $p < 0.01$ as determined Kruskal-Wallis test, followed by Dunn's Multiple Comparison test compared to PBS immunised controls. Data is representative of two independent experiments.

		average IgA titres		
Group	**Immunisation**	**serum**	**BAL (lung washings)**	**nasal washings**
1	naïve	0†	0†	0†
2	LT	0†	0†	0†
3	1µg Ag85B-ESAT6 + 1µg LT	0†	0†	0†
4	10µg Ag85B-ESAT6 + 1 µg LT	28 ± 9***	22 ± 7***	7 ± 6**
5	10µg Ag85B-ESAT6 + 10µg LTK63	0†	0†	0†
6	10µg Ag85B-ESAT6	0†	0†	0†

3.2.4 Strong pro-inflammatory cytokine responses are induced following immunisation

Cytokines are the major mediator of immune responses towards pathogens. Production of cytokines by leukocytes in response to *M. tuberculosis* plays a crucial role in the inflammatory response, and the balance between pro-inflammatory and anti-inflammatory cytokines is crucial in the determination of immune activation and disease clearance (Flynn and Chan 2001). To compare cytokine profiles from splenocytes of vaccinated and naïve mice, I evaluated splenocytes supernatants stimulated with the single antigenic components, Ag85B and ESAT6, as well as the fusion protein Ag85B-ESAT6, plus appropriate controls after 24-48 hours. The levels of a number of cytokines were measured using the Mouse Cytokine Flexi Kit (Becton Dickinson, USA) and included; TNF-α, IL-12p70, IL-6, MCP-1, IL-10, IL-4, IL-5, IL-2 and IFN-γ.

Mice immunised with Ag85B-ESAT6 plus either the adjuvant LT or LTK63 induced significant increases in antigen stimulated splenocytes for T_H1 (IL-2, IFN-γ, TNF-α, IL-12) and T_H2 (IL-4, IL-5, IL-6, IL-10) type cytokines ($p < 0.05$) including the chemokine MCP-1 when compared to naïve animals (Table 3.3 and graphic representation of data in Figure 3.3). Notably, the greatest increases in cytokine levels were observed in with the T_H1 cytokine profile, with the exception of IL-6 and IL-5 production, which were also high. Interestingly, even though those mice immunised with fusion antigen in the adjuvant LTK63 showed increases in both T_H1 and T_H2 cytokines, levels of IFN-γ, IL-2, IL-5 and IL-6 were still significantly lower when compared to those mice receiving the wild-type adjuvant LT ($p < 0.05$). In contrast, mice vaccinated with Ag85B-ESAT6 alone only showed significant increases in TNF-α ($p < 0.01$) and MCP-1 ($p < 0.05$) when compared to negative control animals. Control mice immunised with either LT or LTK63 showed no significant increases ($p > 0.05$) in cytokine responses in antigen stimulated cultures during the course of the study.

Table 3.3: Ag85B-ESAT6 cytokine responses in i.n. immunised mice.

Supernatants from stimulated splenocytes of immunised Balb/c mice (see Table 3.1 for details) were analysed for cytokine levels using CBA after 24-48 hours. Stimulants used in the T-cell assay included Ag85B-ESAT6, ESAT-6, Ag85B peptide, and the positive control as concavalinA (all at 5µg/mL) and, the negative control, $RPMI^{+}$. Results are expressed in pg/mL (mean ± standard deviation) with the * indicating significant values of $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$ (Kruskal-Wallis test followed by Dunn's Multiple Comparison test compared to PBS immunised controls). † indicates $p < 0.05$ when animals immunised with Ag85B-ESAT6 + LT are compared to those receiving Ag85B-ESAT6 + LTK63. There are 5-10 animals per groups, from two independent experiments.

		Immunisation				
Cytokine	**Stimulant**	**1 µg LT**	**10 µgLTK63**	**10µg Ag85B-ESAT6 + 1µg LT**	**10µg Ag85B-ESAT6 + 10µg LTK63**	**10µg Ag85B-ESAT6**
IL-4	Ag85 B-ESAT6	<1	<1	**24 ± 15***	**20 ± 7***	<1
	ESAT-6	<1	<1	<1	<1	<1
	Ag85B peptide	<1	<1	<1	<1	<1
	conA	35 ± 13	42 ± 18	41 ± 13	39 ± 8	32 ± 11
	unstimulated	<1	<1	<1	<1	<1
IFN-γ	Ag85 B-ESAT6	5 ± 2	3 ± 1	**1845 ± 82 ***†**	**278 ± 37*****	<1
	ESAT-6	7 ± 3	16 ± 4	**157 ± 90 **†**	19 ± 7	12 ± 10
	Ag85B peptide	3 ± 1	3 ± 1	3 ± 1	7 ± 2	<1
	conA	546 ± 45	789 ± 102	1030 ± 5	967 ± 267	613 ± 15
	unstimulated	4 ± 2	3 ± 1	4 ± 2	<1	<1
IL-2	Ag85 B-ESAT6	5 ± 2	6 ± 1	**265 ± 56***†**	**58 ± 10****	<1
	ESAT-6	4 ± 2	5 ± 1	**46 ± 6****	**26 ± 9***	<1
	Ag85B peptide	4 ± 1	5 ± 1	6 ± 3	<1	<1
	conA	203 ± 67	274 ± 38	218 ± 111	367 ± 89	233 ± 88
	unstimulated	3 ± 2	5 ± 2	8 ± 5	<1	<1
IL-5	Ag85 B-ESAT6	26 ± 24	40 ± 16	**825 ± 63 ***†**	**121 ± 20****	26 ± 20
	ESAT-6	2 ± 1	9 ± 4	**158 ± 39 **†**	23 ± 8	<1
	Ag85B peptide	<1	8 ± 3	18 ± 4	<1	<1
	conA	46 ± 19	51 ± 12	38 ± 8	77 ± 34	42 ± 11
	unstimulated	<1	6 ± 3	3 ± 1	<1	<1
IL-6	Ag85 B-ESAT6	196 ± 70	198 ± 60	**2291 ± 295 ***†**	**466 ± 63****	355 ± 141
	ESAT-6	44 ± 21	101 ± 23	**1955 ± 445 **†**	**386 ± 67***	316 ± 68
	Ag85B peptide	21 ± 9	48 ± 8	103 ± 39	65 ± 10	90 ± 34
	conA	289 ± 56	390 ± 79	209 ± 38	456 ± 134	333 ± 42
	unstimulated	32 ± 11	50 ± 7	99 ± 23	55 ± 27	98 ± 38

IL-10	Ag85 B-ESAT6			**209 ± 46*** **		32 ± 17
	ESAT-6			**190 ± 33** **		71 ± 45
	Ag85B peptide			86 ± 14		20 ± 8
	conA			79 ± 32		24 ± 11
	unstimulated			43 ± 8		29 ± 12
IL-12p70	Ag85 B-ESAT6			**34 ± 6 ** **		9 ± 4
	ESAT-6			7 ± 3		4 ± 3
	Ag85B peptide			5 ± 2		5 ± 2
	conA			9 ± 2		7 ± 1
	unstimulated			<1		<1
TNF-α	Ag85 B-ESAT6			**209 ± 23 ***		**234 ± 34****
	ESAT-6			**158 ± 39 ***		**165 ± 39***
	Ag85B peptide			<1		<1
	conA			83 ± 12		47 ± 15
	unstimulated			<1		<1
MCP-1	Ag85 B-ESAT6			**450 ± 45 *** **		**50 ± 14***
	ESAT-6			**210 ± 5 ***		<1
	Ag85B peptide			29 ± 17		<1
	conA			155 ± 37		70 ± 4
	unstimulated			<1		<1

Figure 3.3: Graphic representation of Ag85B-ESAT6-specific cytokine responses.

Graphic representation of Table 3.3 of Ag85B-ESAT6-specific cytokine responses in Balb/c mice primed and boosted with purified fusion antigen plus LT or LTK63. For details see legend of Table 3.1. The data shown is for Ag85B-ESAT6 *in vitro* stimulation of splenocytes after 24-48 hours. (1) Naïve animals are represented by pink, (2) those mice receiving 1µg LT by green, (3) the 10µg LTK63 alone immunisation is shown by purple, (4) animals vaccinated with 10µg Ag85B-ESAT6 plus 1µg LT is indicated by red, (5) those receiving the fusion and LTK63 represented by blue, (6) finally those mice receiving 10µg Ag85B-ESAT6 alone are indicated by the orange columns.

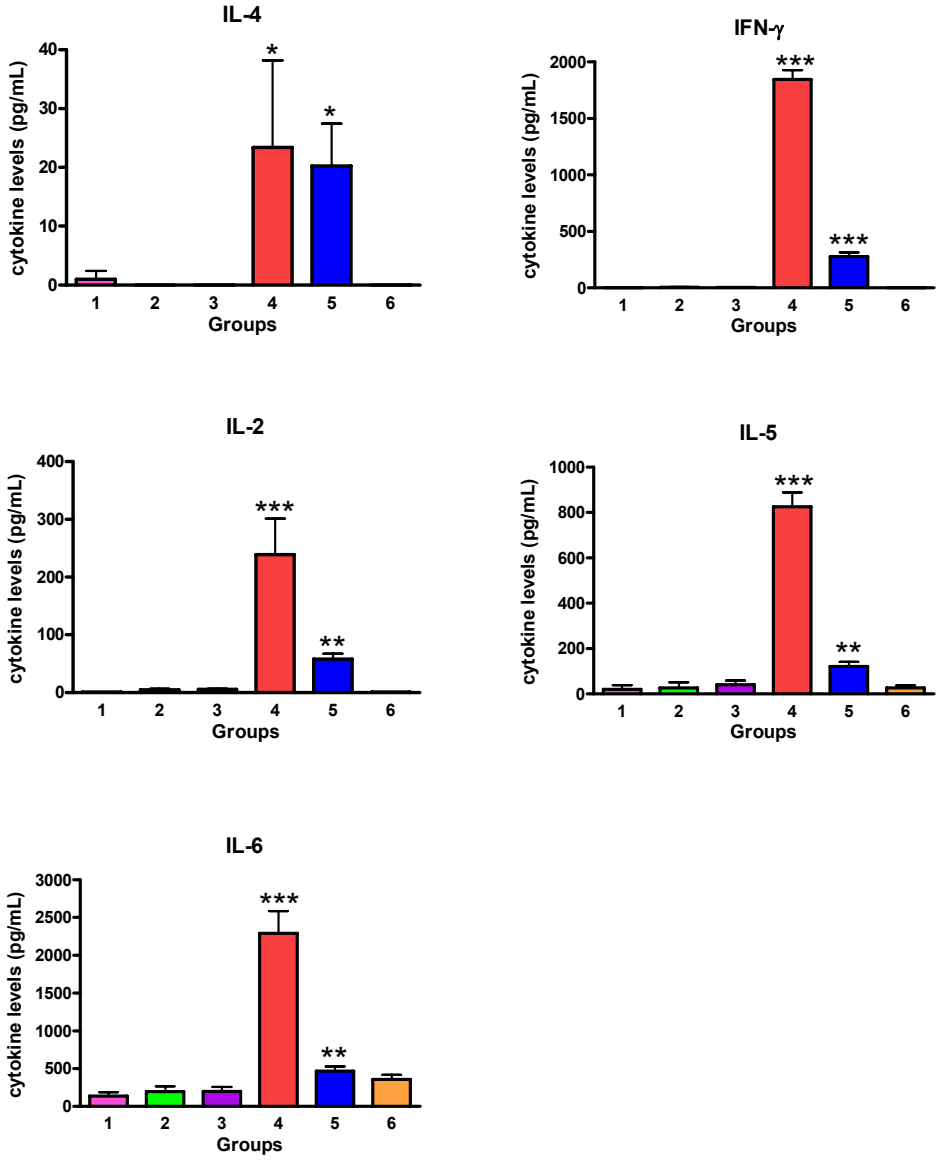
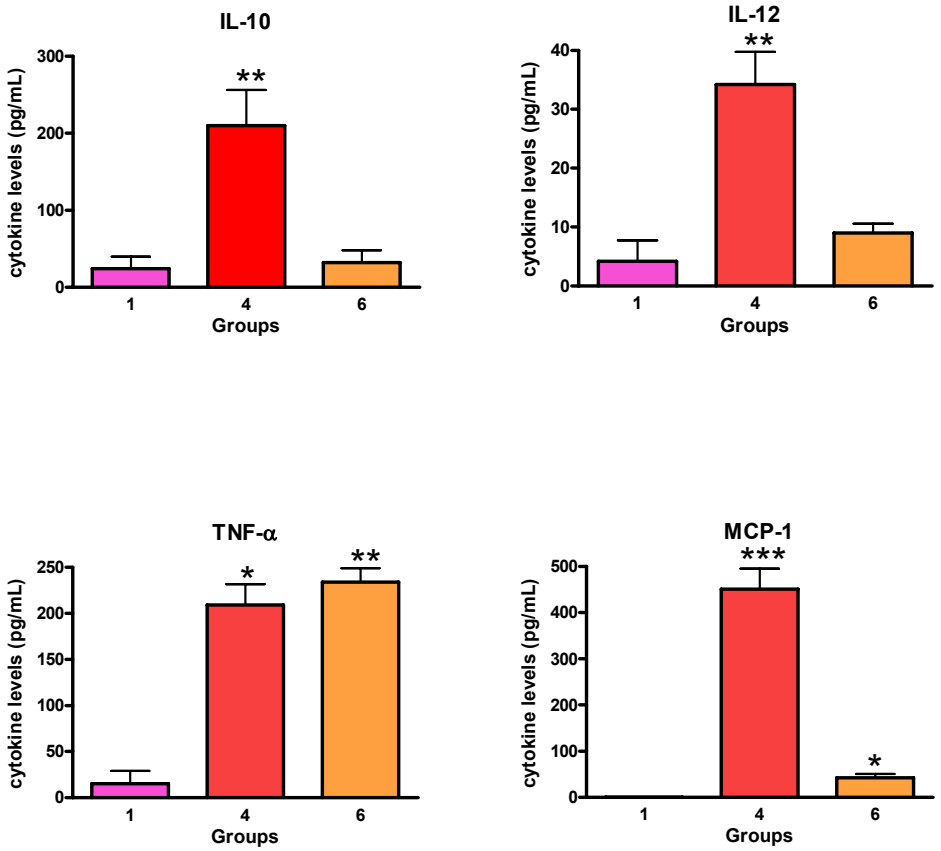


Figure 3.4: Graphic representation of Ag85B-ESAT6-specific cytokine responses.

Graphic representation of Table 3.3 of Ag85B-ESAT6-specific cytokine responses in Balb/c mice primed and boosted with purified fusion antigen plus LT or LTK63. For details see legend of Table 3.1. The data shown is for Ag85B-ESAT6 *in vitro* stimulation of splenocytes after 24-48 hours. (1) Naïve animals are represented by pink, (2) those mice receiving 1µg LT by green, (3) the 10µg LTK63 alone immunisation is shown by purple, (4) animals vaccinated with 10µg Ag85B-ESAT6 plus 1µg LT is indicated by red, (5) those receiving the fusion and LTK63 represented by blue, (6) finally those mice receiving 10µg Ag85B-ESAT6 alone are indicated by the orange columns.



3.3 Discussion

In this study I explored the immunogenicity of the subunit vaccine candidate, Ag85B-ESAT6 after an intranasal prime-boost regimen. The *M. tuberculosis* fusion antigen Ag85B-ESAT6 used in these vaccination studies has been shown to previously induce protection in a number of animal models when administered parenterally (Doherty, et al. 2002; Langermans, et al. 2005; Olsen, et al. 2001; Olsen, et al. 2004). At the time of this study little was known about the efficacy of this antigen with regards to mucosal delivery. However, some recent studies have examined the administration of the fusion molecule intranasally along with a number of different mucosal adjuvants. In the first study, three immunisation doses of Ag85B-ESAT6 plus LTK63 were given at two week intervals, which induced strong IFN-γ responses from T cells as well as providing significant reduction in *M. tuberculosis* CFUs in both the lungs and the spleen, comparable to that seen in the BCG controls (Dietrich, et al. 2006). Most recently another intranasal vaccine, comprising the antigenic fusion protein Ag85B-ESAT6 and the mucosal combined adjuvant vector CTA1-DD/ISCOMs (composed of immune stimulating complexes (ISCOMs) and the cholera toxin-derived fusion protein CTA1-DD), was demonstrated to strongly promote a T_H1-specific immune response, dominated by IFN-γ-secreting $CD4^+$ T cells. Mucosal administration of Ag85B-ESAT6 mixed with CTA1-DD/ISCOMs also strongly boosted prior BCG immunity, leading to a highly increased recruitment of antigen-specific cells to the site of infection, with a reduced bacterial burden in the lung compared to non-boosted control animals (Andersen, et al. 2007).

The nasal route for vaccination offers some important opportunities, especially for the prophylaxis against respiratory diseases. The highly vascularised and immunogenic nasal mucosa offers potential advantages in terms of quick action, improved bio-availability and patient compliance, as well as improved immune response for vaccines (Davis 2001). LT is a well characterised adjuvant which has been widely used to induce mucosal immunity against viral and bacterial pathogens (Freytag and Clements 2005). This, along with a mutant form of LT, LTK63, were employed as agents to boost the immunogenicity of the mucosally delivered antigen.

Our results show that mice immunised twice intranasally and boosted once intraperitoneally with the fusion antigen and the adjuvant LT produced high titres of anti-Ag85B-ESAT6 serum antibodies and modest specific mucosal IgA antibodies. Notably, after only two intranasal doses of the fusion antigen plus LT, high antibody titres were induced. LTK63 only induced high serum anti-Ag85B-ESAT6 antibodies after the third boost. LTK63 has a mutation in the A subunit (Ser63→Lys), which reduces the toxicity of the adjuvant, while still retaining its strong mucosal adjuvanticity (Pizza, et al. 2001). However, a number of studies have shown that while LTK63 is a strong mucosal adjuvant, it does not confer the same high levels of boosting observed when compared to the wild-type toxin (Salmond, et al. 2002).

Antibody titres were examined to confirm that the immune system was recognising Ag85B-ESAT6. However, the humoral immune response has, for many years, been dismissed as not having any protective role with regards to *M. tuberculosis* infection. The concept about the effectiveness of antibodies against intracellular bacterial infections, including tuberculosis, is contentious partly due to the notion that antibodies cannot easily reach the intracellular pathogens. This reservation has been applied particularly to organisms, which are confined to phagosomes (i.e. *M. tuberculosis*), and thus further segregated from the cytoplasm. However, this view is under reconsideration in the light of increasing evidence that antibodies interfering with some extracellular stages of the infection can influence the intracellular fate of the pathogen. Recently, Williams and co-workers reported a 10-fold reduction in lung bacterial burdens after intranasal inoculations of mice with an IgA monoclonal antibody (mAb) against the α-crystallin antigen of *M. tuberculosis* (Williams, et al. 2004). Another study indicated the possible role antibodies may play in preventing the dissemination of *M. tuberculosis* from the lungs to the distal organs such as the spleen and liver (Pethe, et al. 2001). Pethe and co-workers showed that coating wild-type Mycobacteria with monoclonal anti-HBHA (heparin-binding haemagglutinin adhesion) antibody impaired dissemination after intranasal infection indicated by reduced CFU in the spleen but not in the lung. A number of clinical studies have also suggested the possible protective role of antibodies against the natural course of tuberculosis infection. They reported that antibody titres to lipoarabinomannan or Ag85 antigens were higher in patients with milder forms of active tuberculosis (Costello, et al. 1992; Sanchez-Rodriguez, et al. 2002). The Dietrich LTK63 mucosal

vaccination paper (Dietrich, et al. 2006), like many other *M. tuberculosis* immunisation studies, failed to account for humoral immune responses, despite the potential importance of antibodies in preventing infection or modifying the course of experimental tuberculosis. This present study reported mucosal anti-Ag85B-ESAT6 IgA antibodies in both lung and nasal washes, as well as high serum titres of antigen specific IgG, therefore this antibody production may play a role in protection against a future *M. tuberculosis* challenge. Retrospectively, statistical analysis would be performed comparing all negative control groups (i.e. PBS and LT immunised) with all vaccination cohorts to obtain a clearer picture of vaccination efficacy.

In mice, the T_H1 phenotype is normally associated with production of IgG2a antibodies, while the IgG1 subtype is normally indicative of a T_H2 type immune response (Finkelman, et al. 1988; Snapper and Paul 1987). In the present study, a balanced T_H1-T_H2 type immune response pattern was observed for both groups receiving adjuvant and 10μg Ag85B-ESAT6, while those mice receiving only 1μg of the fusion plus LT gave antibody responses which were more obviously biased toward T_H2 type response as only IgG1 titres were observed. These results suggest that the dose of Ag85B-ESAT6 affects the type of immune response initiated after intranasal immunisation, i.e. the higher the dose of fusion antigen the more balanced the immune response. In fact, studies carried out on Ag85B and ESAT6 have shown that they are both strong inducers of a T_H1-type immune responses (Mustafa, et al. 2000a; Mustafa, et al. 2003; Takatsu and Kariyone 2003). It could be postulated that an even higher dose of Ag85B-ESAT6 may in fact push the immune response to be more biased towards T_H1, which may be an advantage for the vaccination against tuberculosis as a strong T_H1-type immune response is needed to clear the disease (Flynn and Chan 2001). Both the recently published intranasal papers employing the fusion protein used a dose of 25μg. From their results it is apparent that this higher dose of Ag85B-ESAT6 induces significant IFN-γ responses with only very low IL-5 levels, indicating a strong biased T_H1-type immune response.

M. tuberculosis is a classic example of a pathogen for which the protective response relies on CMI. This is primarily because *M. tuberculosis* replicates within macrophages, thus T cell effector mechanisms are required for elimination of infection. T cells contribute to the defence against infection by producing specific

patterns of cytokines. The inflammatory response to this pathogen is crucial to the control of the infection but may also contribute to the chronic infection and associated pathology (Flynn and Chan 2001). This is why cytokine profiles from the splenocytes of immunised mice were examined including; IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, MCP-1 and TNF-α. The serum IgG1-IgG2a ratios obtained in this study indicated that immunisation with 10μg Ag85B-ESAT6 plus 1μg LT gave balanced T_H1-T_H2 type immune responses as discussed previously. CBA data has shown the predominance of pro-inflammatory cytokine secretion of IFN-γ, TNF-α, IL-2, IL-5 and IL-6 and chemokine MCP-1 by antigen stimulated splenocytes in immunised animals. Immunologic control of *M. tuberculosis* infection is based on a type 1 T cell response and this study shows that type 1 cytokines i.e. IL-2, IFN-γ and TNF-α, were significantly increased in comparison to negative control groups. In contrast, the production of anti-inflammatory cytokines such as IL-4 and IL-10 in mice immunised with the subunit antigen and LT were low, or not significant when compared to naïve animals. IL-10 and IL-4 antagonise the proinflammatory response by down-regulation of production of cytokines IFN-γ, TNF-α and IL-12 in response to *M. tuberculosis* infection. This leads to suppression of an effective T_H1 type immune response (Fulton, et al. 1998; Gong, et al. 1996; Hirsch, et al. 1999). However, the production of these anti-inflammatory cytokines in response to *M. tuberculosis* may limit tissue injury by inhibiting excessive inflammatory response (Murray, et al. 1997). It is therefore the balance between the inflammatory and protective immune response that determines the outcome of tuberculosis infection. The classic cytokine produced during *M. tuberculosis* infection that is indicative of protection is IFN-γ (Flynn, et al. 1993). IFN-γ contributes to protective immunity against *M. tuberculosis* by activating macrophages to more effectively eliminate this pathogen. Individuals defective in genes for IFN-γ and IFN-γ receptors are prone to serious *M. tuberculosis* infections (Ottenhoff, et al. 1998). CBA data from this study shows high levels of antigen specific IFN-γ production in those mice immunised with 10μg Ag85B-ESAT6 and LT (approx. 2000pg/mL). Docherty et al showed similar levels of IFN-γ in mice immunised with the subunit vaccine, plus the adjuvant monophosphoryl lipid A (MPL). This study also showed a substantial reduction in both lung and spleen *M. tuberculosis* CFUs after aerosol infection with virulent *M. tuberculosis* (Doherty, et al. 2002), which was comparable to BCG vaccinated animals. Our data therefore suggests that this intranasal prime-boost immunisation regimen, using LT and Ag85B-ESAT6, would possibly provide protection against a future pathogenic *M. tuberculosis* challenge. Both this study, and the Dietrich study employed intranasal

delivery of the adjuvant LTK63 in combination with the fusion protein Ag85B-ESAT6 (Dietrich, et al. 2006). The Dietrich study reported high levels of IFNγ (9000pg/mL) in animal intranasally immunised three times, whereas this study only shows modest levels of IFN-γ in mice immunised twice intranasally and once intraperitoneally with the adjuvant and protein (300pg/mL). As already discussed, the differences observed in the IFN-γ levels between this study and the Dietrich study may be due to the levels of Ag85B-ESAT6 used (i.e. 10μg compared to 25μg), and may also be due in part to the differences in the route of the third immunisation boost (i.p. compared to i.n.). The published study gave in total 45μg more fusion protein than this study, which may be a major factor in inducing significant IFN-γ immune responses. In fact, a dose response study done by Jes Dietrich indicated significant increases in IFN-γ levels when using the larger (i.e. 25μg) Ag85B-ESAT6 dose when compared to just a 10μg dose (personal communication). A number of studies have reported that the route of immunisation with either homologous or heterologous boosting does influence the magnitude of the antibody response as well as the IgG1/IgG2a ratio (Glynn, et al. 2005a) (Vajdy, et al. 2004). Mucosal priming followed by systemic boosting can be more effective in inducing serum antibody responses, as well as strong cellular immune responses, than a homologous vaccination regimen (Glynn, et al. 2005a; Vajdy, et al. 2004). However, more often than not the differences observed did not affect the outcome of further a challenge study (Glynn, et al. 2005b). Therefore, for this present study it can be postulated that the reason for the lower IFN-γ levels when compared to the Dietrich paper is possibly due to the differences in the doses of antigen, rather than due to the variation in the third immunisation route. The final boosting of animals via intraperitoneal route was used in this study as it has previously been shown to significantly increase humoral responses in a number of vaccination regimens PAPER. However, as the main focus of this thesis is to examine immune responses induced after mucosal immunisation it would be interesting to repeat the experiment using a final intranasal boost.

In conclusion, an intranasal/intraperitoneal heterologous vaccination regimen, utilising the *M. tuberculosis* fusion antigen Ag85B-ESAT6 and the mucosal adjuvant LT is successful at inducing strong and significant serum and mucosal antibody responses along with a strong T_H1 cytokine profile.

4 Characterisation of innate immune responses shortly after intranasal immunisation of model antigens.

4.1 General introduction

The nasal route for vaccination offers some important opportunities for the prophylaxis of many diseases. It is a particularly attractive approach due to the functionality of the CMIS, i.e. induction of immune responses at local sites can lead to effector responses at distant mucosal sites. Consequently, the rational design of nasal vaccines for clinical use depends on the availability of information about the mechanisms that lead to a mucosal immune response after intranasal vaccination (Ogra, et al. 2001).

The role of lymphoid tissues in respiratory tract defences includes antigen uptake and processing and presentation for the induction of mucosal immune responses. This has been found to occur in the secondary organised lymphoid aggregates, called the BALT, situated in the bronchial wall, and the NALT, which is situated at the nasal entrance to the pharyngeal duct. Other important parts of the respiratory tract that play roles in immune development include the draining LNs. In fact, it is the NALT that is the first point of contact for many inhaled antigens, and consequently plays a major role in both induction and effector immune responses, which are then further amplified in the draining CLN. Reviewed in (Bienenstock and McDermott 2005). In humans, the nasopharyngeal region also contains a high density of immune competent cells similar to the NALT, most notable in the Waldeyer's ring which consists of the tonsils and adenoids (Perry and Whyte 1998). As described in Chapter 1, NALT is a well organised structure, consisting of B and T-cell areas with surrounding APC, which are covered by an epithelial layer containing M-cells (Asanuma, et al. 1997; Karchev and Kabakchiev 1984; Kuper, et al. 1990). See Figure 4.1 for location and structure of the NALT.

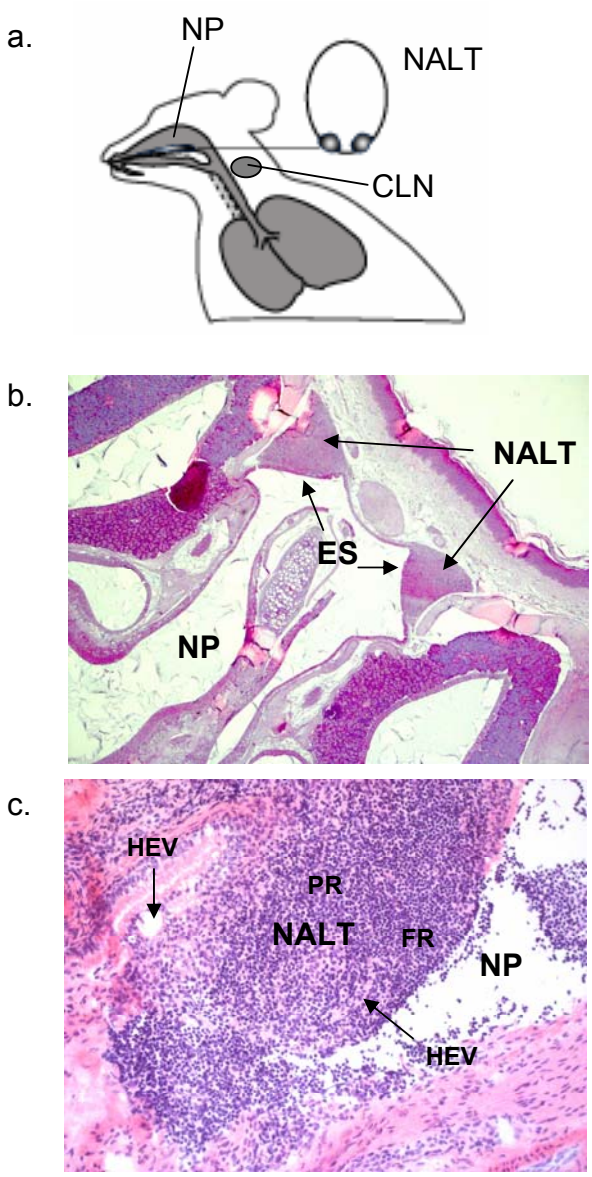


Figure 4.1: Location and structure of the NALT

a. Schematic diagram showing the anatomical location of murine NALT, NP (nasal passages) and CLN. b. Anatomic locations of NALT in naive Balb/c mice after hematoxylin-eosin (H+E) staining. Magnification = 5. c. Frozen NALT section from naive Balb/c mice after H+E staining. FR indicates follicular regions; PR, parafollicular regions; HEV, high endothelial venules and; ES, epithelial sides. Magnification = 20

Intranasal administration of antigens to the mucosal surface can lead to different outcomes depending on the nature of the antigen and interactions at the inductive site. Factors such as dose, the ability to bind, the use of adjuvant, and frequency of administration are all contributing factors. After intranasal immunisation, both humoral and cellular immune responses can occur. Small soluble antigens can penetrate the nasal lumen directly and interact with underlying APC, such as DC and macrophages, whereas the specialised M-cells can allow selective transport of both soluble and particulate antigen across the epithelium for further interaction with a wide variety of leukocytes. Once APCs in the M-cell pocket take up antigen, they process the antigen and migrate into the interfollicular areas, where they present antigenic epitopes in the context of MHC molecules (Fujimura 2000). However, the detailed pathway for the antigen uptake and their migration within the NALT is still unclear. This antigen processing and presentation by DC and macrophages leads to T-cell activation of both T-helper and CTL. A number of studies have revealed a dominant T_H0 cytokine profile, indicating that these T cells are capable of becoming T_H1 or T_H2 cells immediately after antigen exposure through the nasal tract. This in turn leads to the release of cytokines and chemokines as well as B-cell proliferation, differentiation, and maturation into IgA and IgG producing plasma cells (Hiroi, et al. 2001; Hiroi, et al. 1998; Yanagita, et al. 1999). In fact, after intranasal immunisation, the antibody response induced in the NALT and the associated draining LNs can be strong and may retain long term memory (Liang, et al. 2001; Tamura, et al. 1998; Zuercher, et al. 2002).

The first point of call for draining antigen activated immune cells is the superficial CLN, which then drains into the posterior CLN. These LNs are organised bean-shaped organs consisting of an outer B-cell cortex region, the paracortex, which is particularly rich in T cells, and the medulla which contains both B lymphocytes, plasma cells and macrophages (see Section 1.4.4.3 for more details). After initial induction in the NALT, immune responses are further amplified in these draining LNs following an onset delay. The CLN show a more pronounced expansion of lymphocytes when compared to the NALT, particularly after viral infection (11-fold vs. 2-fold). Even more strongly than in the NALT, B cells within the draining LNs show high production of specific IgG antibodies, which after entering the blood leads

to the generation of systemic IgG after intranasal vaccination. Reviewed in (Zuercher 2003). From the CLN, specific effector cells are distributed via lymph fluid and blood to both mucosal and systemic sites around the body. In fact, numerous studies have reported antigen specific immune responses from NALT primed lymphoid cells at local and distant mucosal effector sites (e.g. the D-NALT, the genitourinary tract and the gut) operated by the CMIS (Kunkel, et al. 2003; Lazarus, et al. 2003). However, the mechanisms of homing to these sites are largely unknown to date.

The role played by the GALT, such as PPs and ILFs, in antigen sampling, innate immune responses and in the subsequent generation of antigen-specific immune responses is well-characterised. For a comprehensive review see (Nochi and Kiyono 2006). Despite a central role in mucosal immunity, little is known about the nasal immune system. The phenotype and functions of the different cellular subpopulations are not fully characterised. The majority of studies looking at the NALT and CLN have focussed on the induction of adaptive immunity, and have therefore tended to examine later time-points. However, the characterisation of innate immune responses early after intranasal administration of antigens/pathogens in the upper respiratory tract is not fully understood. Gueirard et al examined immune responses after intranasal administration of the respiratory pathogen *Bordetella bronchiseptica*. $CD11c^+$ leukocytes were present in the NALT subepithelium of naive mice, but were not found to be homogeneously distributed. At 5 and 24 hours after *B. bronchiseptica* delivery, an increased number of cells expressing CD11c were detected and located exclusively along the epithelial side of each NALT aggregate without specific localisation to the T cell area. However, by 48 hours post challenge $CD11c^+$ cells had migrated to these areas (Gueirard, et al. 2003). Another study examined the NALT and CLN after influenza virus infection, and found that three days post administration slightly higher numbers of $CD4^+$ and $CD8^+$ T cells and lower $B220^+$ cell numbers were detectable in the NALT. At 4 days post infection, IgA producing plasma cells were found in the NALT, whereas another two days was needed before antibody responses were detected in the CLN (day 6) (Liang, et al. 2001). Recently, Park and colleagues constructed a group A streptococcus (GAS) strain that expressed the model OVA epitope on the bacterial cell surface, and used this strain in adoptive transfer experiments to study CD4 T cell response to bacterial infection in NALT. After

intranasal infection of mice with the recombinant strain, OVA-specific CD4 T cells were detected in the NALT at three days. The level of CD69, an early activation marker, on OVA-specific T cells in NALT was also assessed at an earlier time point. 36 hours after infection, a great number of T cells in NALT and CLN expressed CD69. Both levels of $CD69^+$ expression on T cells and their frequency were higher in NALT than in CLN (Park, et al. 2004).

Given the rapid nature of the innate immune system, I hypothesise that intranasal immunisation of antigen and adjuvant will lead to the induction of significant early immune responses in a number of different innate cell populations in both local URT lymphoid tissues, i.e. the NALT and CLN. In addition intranasal immunisation will result in significant up regulation of CAMs and the generation of GC.

The aim of this work is to contribute to the phenotypic and functional description of NALT and CLN early after intranasal administration of antigen. Balb/c mice were intranasally immunised with; PBS (naïve), the adjuvant LT or LT plus the tuberculosis fusion antigen Ag85B-ESAT6. I isolated cells from both compartments in mice 5, 24 and 72 hours after immunisation, and using flow cytometry and confocal microscopy, determined the frequencies, localisation and activation status of innate immune cell populations including; DC, macrophages, neutrophils, and NKC. I also examined the expression of cell adhesion molecules (MAdCAM-1, PNAd, ICAM-1 and VCAM-1) and germinal centre formation.

I found striking differences between the cell surface phenotype of leukocytes and their pattern of distribution from the NALT and CLN at all time points tested after immunisation. Altogether, these data provide evidence indicating the distinctive and complex phenotypic and functional features that exist in the immune cell population at early time points after intranasal administration of antigen.

4.1.1 Technical Note

I had to develop an approach for removal and analysis of the NALT (see Section 2.3.1.1 for removal details). Unfortunately, decalcification and formalin fixing of mouse heads destroys many antigen markers and therefore I could not use this process for examining sections of the NALT. At first I tried immunohistochemical staining methods on our frozen NALT sections; however I found that the background staining was very high and therefore not suitable for our study. I consequently decided to utilise mAb staining with flurochromes and found this to be a far more superior method for staining of the NALT.

4.2 Identification of cell populations early after intranasal immunisation

4.2.1 Introduction

The innate immune system has the important function of identifying and eradicating microbes (i.e. heterologous antigens) and alerting the adaptive immune system to their presence. The faster-acting innate immune responses provide a necessary first line of defence because of the relatively slow nature of adaptive immunity. For comprehensive reviews see (Medzhitov and Janeway 2000; Medzhitov and Janeway 1997). Innate immune responses, among their many effects, leads to a rapid burst of inflammatory mediators which results in the infiltration of various cell types to the site of stimulation. Innate immune processes are conducted by cells relatively unrestricted in antigen specificity, including NKC, DC, neutrophils and macrophages. See Figure 4.2 for origin and differentiation of immune cells.

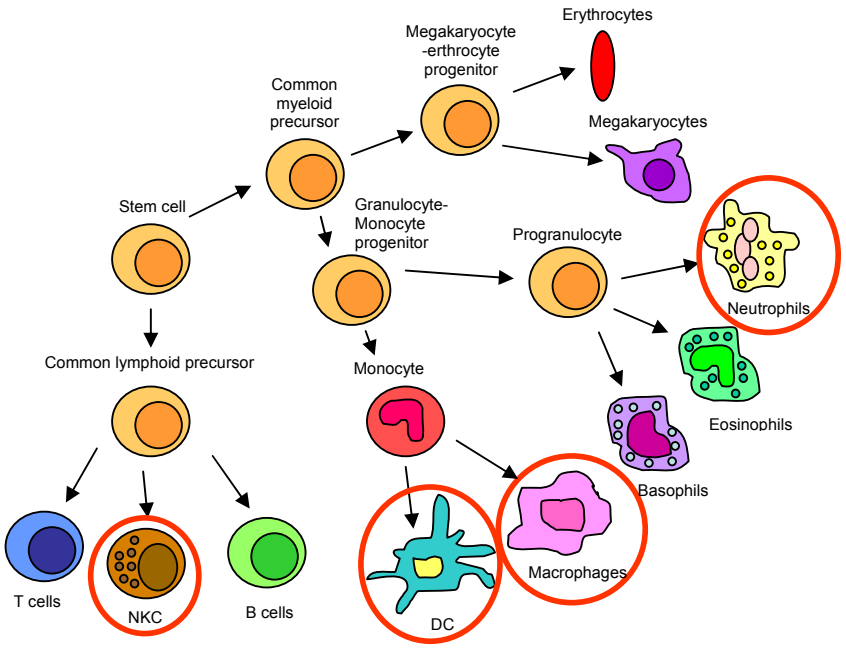


Figure 4.2: Origin and haematopoietic differentiation of immune cells

All hematopoietic cells are derived from pluripotent stem cells which give rise to two main lineages: one for lymphoid cells and one for myeloid cells. The common lymphoid progenitor has the capacity to differentiate into T cells, B cells or NKC depending on the microenvironment to which it homes. The myeloid cells differentiate into all other cells mentioned. Cells circled in red indicate the cell populations examined in this study.

Innate stimulation of DC triggers their differentiation into immunogenic APC, which consequently allows presentation of antigen material to T cells as well as providing the necessary inflammatory signals to modulate T cell differentiation (Reis e Sousa 2004; Steinman and Hemmi 2006). In bacterial infections, a newly discovered DC subpopulation termed iNOS/TNFα-producing "Tip"-DC has been reported to be critical, as demonstrated in a model of infection with *L. monocytogenes*, indicating that DC may also have a role to play in innate immune defences (Serbina, et al. 2003). Macrophages are essential effector cells of innate immunity that play a pivotal role in the recognition and elimination of invasive micro-organisms. Mediators released by activated macrophages orchestrate innate and adaptive immune host responses. As with DC, they also have a role to play in antigen presentation via MHC II, in addition to their other main phagocyctic role (Gordon and Read 2002). Neutrophils are an integral part of the innate immune response and are normally one of the first cell types

to arrive at an inflammatory site due to their high motility. As with macrophages, they are active phagocytes and ingest and destroy foreign antigens during a "respiratory burst" (Kobayashi, et al. 2005). NKC are a form of cytotoxic lymphocyte which constitute a major component of the innate immune system. NKC play a major role in the host-rejection of both tumors and pathogen infected cells and activated NKC also exhibit rapid secretion of several cytokines, notably IFN-γ (Young and Ortaldo 2006). Considering the above points I therefore speculated that intranasal immunisation may affect migration and activation of these cell populations within both the NALT and CLN at early time-points. As far as I know, this present study is the first to characterise the innate immune responses taking place in the upper respiratory tract lymphoid tissues. It will hopefully provide a better understanding of the inductive and effector factors involved in the early regulation of mucosal immunity which will help in the design of safer mucosal vaccines to elicit the appropriate protective immune response to a given pathogen.

4.2.2 Results

4.2.2.1 Percentage, distribution and activation status of NALT and CLN innate cell populations after intranasal immunisation

In order to identify the cell populations involved in early immune responses after intranasal administration of antigen, cells were isolated 5, 24 and 72 hours from both the NALT and CLN of immunised mice. The number of cells recovered from isolated NALT of naïve Balb/c mice was 1.2 (± 0.8) x 10^5 cells for a single mouse. In contrast, the number of cells recovered from the CLN of naïve mice was over 20-fold higher at 2.8 (± 1.1) x 10^6 cells per mouse. The phenotype of NALT and CLN innate immune cells were analysed by flow cytometry as shown in Table 5.1. In the NALT of naïve mice, DC ($CD11c^+$), macrophages ($F4/80^+$), NKC ($DX5^+$) and neutrophils ($Ly6G^+$) were more abundant in terms to total percentage than in the CLN, however in terms of total cell numbers these cell types were more profuse in the CLN. The percentages/numbers of innate leukocytes observed in this present study, for both the NALT and CLN, are similar to those reported previously in Balb/c mice (Gueirard, et al. 2003; Liang, et al. 2001; Rodriguez-Monroy, et al. 2007; Tanigawa, et al. 2000; van Helvoort, et al. 2004; Woolard, et al. 2004; Wu, et al. 1997b; Yeo, et al. 2006).

Kinetic analysis of intranasal immunisation revealed that administration of LT plus Ag85B-ESAT6 differentially influenced several cell populations early after intranasal immunisation of antigen in both the NALT and CLN, and dramatic changes in cell number percentage were evident as early as 5 hours post-immunisation (Table 4.1 and Figure 8.1).

In order to confirm the flow cytometry data as well as examine the distribution of innate immune cell populations early after intranasal immunisation, serial frozen NALT and CLN sections of control (naïve) and immunised (LT + Ag85B-ESAT6) mice were stained for $CD11c^+$, $F4/80^+$ and $Ly6G^+$ cells. Unfortunately examination of $DX5^+$ (NKC) cells was not possible as no antibodies are currently commercially available for immunohistochemistry. Immunofluorescence analysis of $CD11c^+$, $F4/80^+$ and $Ly6G^+$ cells in the NALT and CLN indicated the same kinetics as the flow cytometric analysis (Figures 4.3A, 4.4A, 4.5A, 4.6A, 4.7).

To evaluate the effects of intranasal immunisation on the activation status of innate immune cells ($CD11c^+$, $F4/80^+$, $Ly6G^+$ and $DX5^+$) at early time-points, I examined both the number of cells expressing CD69, CD25, MHCII and VCAM-,1 and the Mean Fluorescence Intensity (MFI) of these markers by flow cytometry. The CD69 (Leu-23) antigen is a cell surface glycoprotein which is expressed on activated T and B lymphocytes, as well as NKC and neutrophils (Lanier, et al. 1988). CD69 expression is induced 4-6 hours after activation, reaches a peak at 18-24 hours and is still detectable on the cell surface 24 hours after stimulus is removed (Testi, et al. 1994; Testi, et al. 1989). Another marker that is expressed early after NKC activation is the IL-2 receptor (CD25). This antigen plays a role in IL-2 induced NKC proliferation and cytotoxic activity (Kehri, et al. 1988; Lanier, et al. 1985; Lanier, et al. 1988; Siegel, et al. 1987; Tsudo, et al. 1987). Class II MHC molecules are integral membrane proteins expressed at the cell surface, which bind peptide fragments of antigen and present them to T cells that express a complimentary T cell receptor for antigen. MHC class II molecules are only expressed on so-called “professional” APC, such as DC, macrophages and B lymphocytes. MHC II expression is upregulated after antigen stimulation and is therefore associated with APC activation (Santambrogio and Strominger 2006; Santana and Esquivel-Guadarrama 2006; Wetzel and Parker

2006). The MHC II expressed in Balb/c mice is known as I-A^d. VCAM-1 is also expressed at low levels on macrophages and DC, but can be induced after antigenic stimulation. This adhesion molecule can therefore be used an indicator for activation of these cell types (Balogh, et al. 2002; Rice, et al. 1991). From this analysis, it was determined that differences in expression of both MHC II and VCAM-1 expression on $CD11c^+$ and $F4/80^+$ cells were maximal in the NALT at early time-points with the CLN showing the greatest differences at later time-points (Figures 4.3B, 4.4B, 4.5B, 4.6B and Table 8.2).

The flow cytometry results are shown as percentages from 10,000 recorded events. I did not tie these percentages back to total cell numbers obtained after processing of both the NALT and CLN as it is very unlikely that the whole tissues could be dissected out. Therefore any data obtained from these calculations (i.e. percentages multiplied back to total cell numbers) would not be an accurate representation of the responses induced.

Table 4.1: Surface phenotype of isolated cells from NALT and CLN 5, 24 and 72 hours after intranasal immunisation.

Cells were isolated from NALT and CLN of Balb/c mice 5, 24 and 72hours after intranasal immunisation with PBS (naïve mice), 1µg LT or 1µg LT + 10µg Ag85B-ESAT6, and stained with flurochrome-labelled mAbs and analysed by flow cytometry in which 10,000 events were recorded. Data represents the mean percentage of expression ± standard deviation from groups of ten individual mice, from two independent experiments. The * indicates significant values of $p<0.05$ and **, $p<0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Black represents those percentage values not significantly different to those seen in naïve mice; red indicates values significantly increased, and blue shows percentages significantly decreased in comparison to negative control mice.

Cell Marker	Immunisation	NALT %				CLN %			
		0hrs	5hrs	24hrs	72hrs	0hrs	5hrs	24hrs	72hrs
CD11c$^+$	LT	6.6 ± 1.5	5.2 ± 0.8*	5.3 ± 0.7*	5.9 ± 0.5	4.2 ± 0.3	4.4 ± 0.4	2.9 ± 0.8**	5.3 ± 0.7*
	LT + Ag	6.5 ± 1.1	5.3 ± 1.1*	7.9 ± 1.2*	6.8 ± 1.0	4.3 ± 0.6	4.3 ± 0.2	2.4 ± 0.3**	5.3 ± 0.9*
F4/80$^+$	LT	7.7 ± 1.2	6.2 ± 0.9*	6.0 ± 1.0*	7.8 ± 1.0	3.4 ± 0.8	3.3 ± 0.5	4.0 ± 0.3	5.7 ± 0.6**
	LT + Ag	7.6 ± 1.3	4.9 ± 1.1**	7.6 ± 1.2	7.9 ± 1.2	3.6 ± 0.7	3.2 ± 0.2	3.9 ± 0.6	6.7 ± 0.9**
DX5$^+$	LT	5.4 ± 1.6	5.6 ± 0.5	7.8 ± 2.1*	3.2 ± 0.8*	4.3 ± 0.9	5.8 ± 0.7*	8.2 ± 3.1**	4.7 ± 1.9
	LT + Ag	5.3 ± 1.2	5.0 ± 0.6	10.3 ± 3.1**	7.6 ± 2.1*	3.9 ± 1.4	10.4 ± 3.4**	8.9 ± 2.2**	3.6 ± 0.7
Ly6G$^+$	LT	7.6 ± 1.8	6.9 ± 1.7	9.0 ± 1.1*	7.0 ± 1.4	3.4 ± 0.7	3.0 ± 0.6	5.9 ± 1.2**	5.6 ± 1.2**
	LT + Ag	7.4 ± 1.6	4.8 ± 1.1**	5.8 ± 1.1*	7.8 ± 1.2	3.5 ± 0.7	5.5 ± 1.2**	3.2 ± 1.9	2.1 ± 0.3**

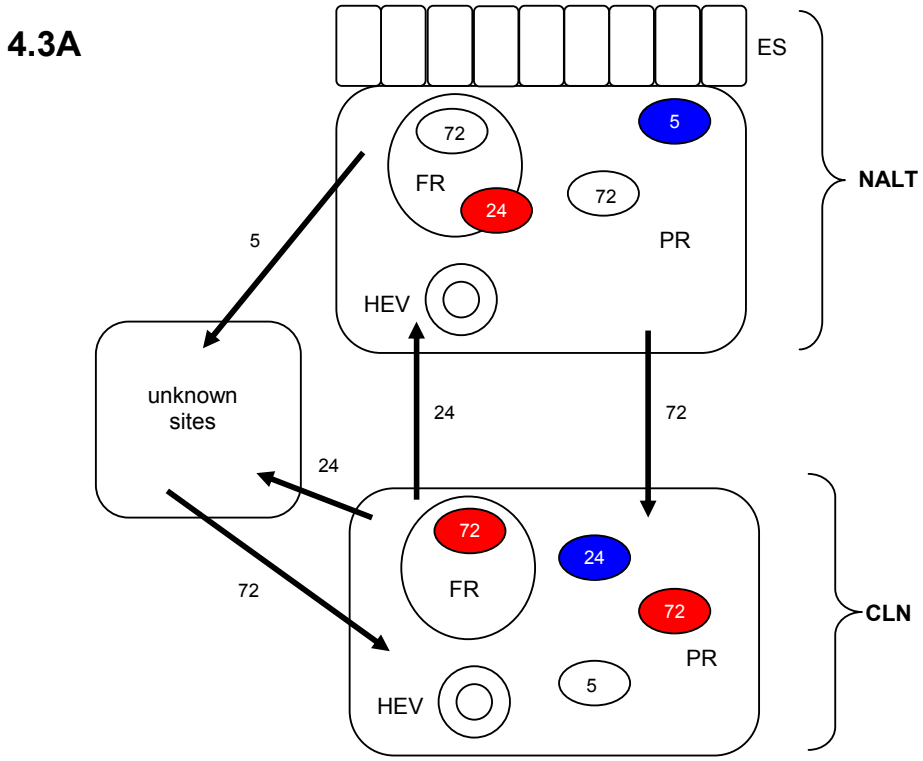
4.2.2.1.1 Dendritic Cells

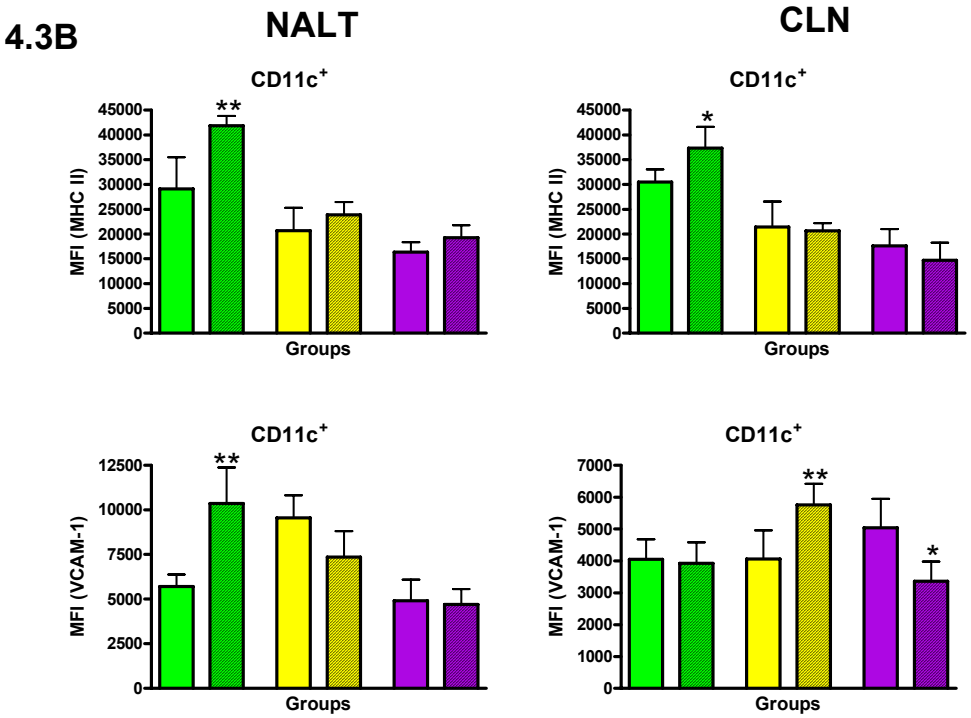
Figure 4.3: Schematic diagram showing an overview of DC movement and activation within the NALT and CLN 5, 24, and 72 hours after intranasal immunisation

A. Ovals represent cells and numbers within the ovals indicate individual time-points. White ovals represent that the percentage change is not statistically different (i.e. $p > 0.05$) from naïve animals, blue shows a significant decrease (i.e. $p < 0.05$) and red indicates a significant increase (i.e. $p > 0.05$) in the cell population when compared to control mice. The arrows indicate direction of movement at particular time-points. FR stands for follicular regions (i.e. B cell areas), PR is for parafollicular regions (i.e. T cell areas), ES is for epithelial sides, and HEV stands for high endothelial venules.

B. Cells were isolated from the NALT and CLN of control (PBS immunised) and immunised animals, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $CD11c^+$ (Figure 4.3B) expression, as well as MHC II and VCAM-1 expression. For analysis, gates were set on the innate subset marker positive ($CD11c^+$) cells and the MFI of MHC II and VCAM-1 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, yellow at 24 hours and purple at 72 hours. Block colours represent naïve (PBS immunised) animals and, diagonal lines show animals intranasally immunised with adjuvant plus Ag85B-ESAT6. Scales are representational of individual activation marker MFI for each tissue – always refer to y-axis scale for each graph.

4.2.2.1.1 Dendritic Cells





4.2.2.1.1.1 5 hour time-point

Cells from the NALT of immunised mice at the 5 hour time-point were found to have a significantly lower ($p < 0.05$) percentage of $CD11c^+$ cells, as well as $CD11c^+$/VCAM-1^+ cells, when compared to PBS immunised animals (Tables 4.1 and 8.2A and Figure 8.1A). I also observed that both MHC II and VCAM-1 fluorescence increased ($p < 0.01$) significantly in all immunised mice on DC at this time-point (Figure 5.3B). However, the distribution of this cell type remained similar to that seen in naïve mice (Figure 4.7A). Within the CLN, as early as 5 hours post immunisation, DC were more widely distributed, this was in comparison to the DC pattern in naïve animals which appeared more clustered (Figure 4.7A). I also observed an increase in MHC II fluorescence ($p < 0.05$) on $CD11c^+$ cells from immunised animals compared to that of naïve mice (Figure 4.3B).

4.2.2.1.1.2 24 hour time-point

24 hours post intranasal immunisation, $CD11c^+$ and $CD11c^+$/$MHCII^+$ cells were significantly increased ($p < 0.05$) in the NALT (Tables 4.1 and 8.2A and Figure 8.1B). In addition I also observed a more wide-spread pattern of $CD11c^+$ cells in immunised mice compared to that seen in the naive mice (Figure 4.7A). Within the CLN, immunised mice were found to have a significantly lower percentage ($p < 0.01$) of $CD11c^+$ cells and the subpopulations of $CD11c^+$ cells positive for MHC II and VCAM-1 when compared to naïve animals (Tables 4.1 and 8.2A and Figure 8.1C). Conversely, I observed a significant increase in VCAM-1 expression on DC at 24 hours within the CLN of immunised mice (Figure 4.3B).

4.2.2.1.1.3 72 hour time-point

Even though the percentage of $CD11c^+$ cells were similar 72 hours after immunisation in all animals tested, I did also observe a more widespread distribution of DC within the NALT of immunised animals, particularly the appearance of DC within follicular regions (Table 4.1 and Figures 4.7A). I also noted that $CD11c^+$ cells expressing VCAM-1 significantly increased ($p < 0.01$) in those animals receiving adjuvant and antigen (Figure 4.3B). The CLN DC population as well as the $CD11c^+/MHCII^+$ and $CD11c^+/VCAM\text{-}1^+$ subpopulations were significantly increased ($p < 0.05$) in all immunised mice compared to PBS immunised animals at this final time-point (Tables 4.1 and 8.2A and Figure 8.1D). As observed in the NALT, I also saw DC within follicular and parafollicular regions (Figure 4.7A). In addition, VCAM-1 expression was observed to significantly decrease ($p < 0.05$) 72 hours after immunisation (Figure 4.3B). For overview see Figure 4.3.

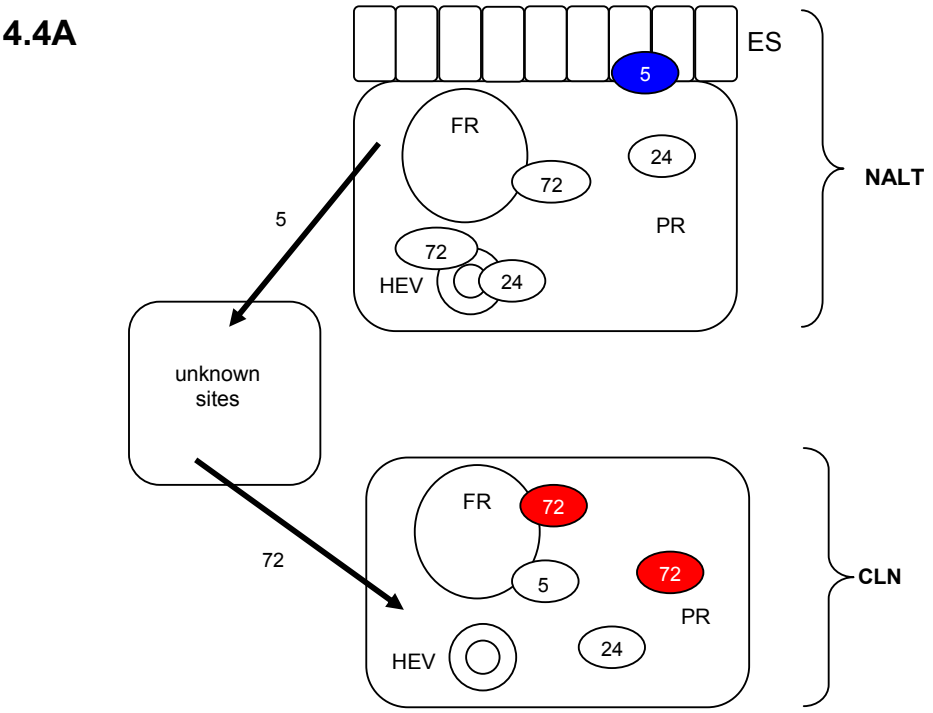
4.2.2.1.2 Macrophages

Figure 4.4: Schematic diagram showing an overview of macrophage movement and activation within the NALT and CLN 5, 24, and 72 hours after intranasal immunisation

A. Ovals represent cells and numbers within the ovals indicate individual time-points. White ovals represent that the percentage change is not statistically different (i.e. $p > 0.05$) from naïve animals, blue shows a significant decrease (i.e. $p < 0.05$) and red indicates a significant increase (i.e. $p > 0.05$) in the cell population when compared to control mice. The arrows indicate direction of movement at particular time-points. FR stands for follicular regions (i.e. B cell areas), PR is for parafollicular regions (i.e. T cell areas), ES is for epithelial sides, and HEV stands for high endothelial venules.

B. Cells were isolated from the NALT and CLN of control (PBS immunised) and immunised animals, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $F4/80^+$ (Figure 4.4B) expression, as well as MHC II and VCAM-1 expression. For analysis, gates were set on the innate subset marker positive ($F4/80^+$) cells and the MFI of MHC II and VCAM-1 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, yellow at 24 hours and purple at 72 hours. Block colours represent naïve (PBS immunised) animals and, diagonal lines show animals intranasally immunised with adjuvant plus Ag85B-ESAT6. Scales are representational of individual activation marker MFI for each tissue – always refer to y-axis scale for each graph.

4.2.2.1.2. Macrophages



4.2.2.1.2.1 5 hour time-point

The percentage of F4/80$^+$ cells (macrophages) and those cells also positive for VCAM-1 were observed to significantly decrease ($p < 0.05$) within the NALT in intranasally immunised mice as early as 5 hours (Tables 4.1 and 8.2A and Figure 8.1A). The F4/80$^+$ cells from both naïve (PBS immunised) and immunised animals at this time-point were mostly located around the epithelial sides of the tissue; however a small number of macrophages in the NALT from immunised animals were also located in parafollicular T cell areas (Figure 4.7B). In addition, F4/80$^+$ cells also had a significant increase ($p < 0.01$) in MHC II expression as early as 5 hours after immunisation (Figure 4.4B). The F4/80$^+$ population in the CLN of immunised mice was similar to that seen in naive mice, however I did observe a significant increase in F4/80$^+$/VCAM-1$^+$ cells and MHC II expression at this early 5 hour time-point (Tables 4.1 and 8.2A and Figure 4.4B).

4.2.2.1.2.2 24 hour time-point

At 24 hours post administration of antigen, F4/80$^+$ cells had started to return to similar levels observed in the NALT of naïve mice (Table 4.1). In addition, the macrophages present were located even more centrally, as well as surrounding HEV in immunised mice (Figure 4.7B). Again as described for the F4/80$^+$ population at 5 hours within the CLN, the percentages of these cells were comparable to control mice (Table 4.1). However, I did observe that macrophages positive for MHC II and VCAM-1 were actually decreased ($p < 0.01$) in all immunised mice 24 hours post administration of antigen (Table 8.2A). I also noted that the fluorescence intensity of VCAM-1 significantly increased ($p < 0.01$) on CLN macrophages (Figure 4.4B). In terms of their distribution, the macrophages within CLN of immunised were mice were also observed in more central areas compared to the more peripheral pattern seen in naive animals 24 hours after immunisation (Figure 4.7B).

4.2.2.1.2.3 72 hour time-point

By 72 hours all immunised mice appeared to have similar numbers of NALT macrophages as naive animals, with most surrounding HEV (Table 4.1 and Figure 4.7B). Within the CLN I observed the same diffuse pattern as at 24 hours and in addition there was a significant increase ($p < 0.05$) in $F4/80^+$ cells as well as the subpopulations, $F4/80^+/MHCII^+$ and $F4/80^+/VCAM\text{-}1^+$ at this final time-point (Tables 4.1 and 8.2A and Figures 8.1D and 4.7B). I also observed a significant decrease ($p < 0.05$) in VCAM-1 expression 72 hours after immunisation on $F4/80^+$ cells within the CLN (Figure 4.4B). For overview see Figure 4.4.

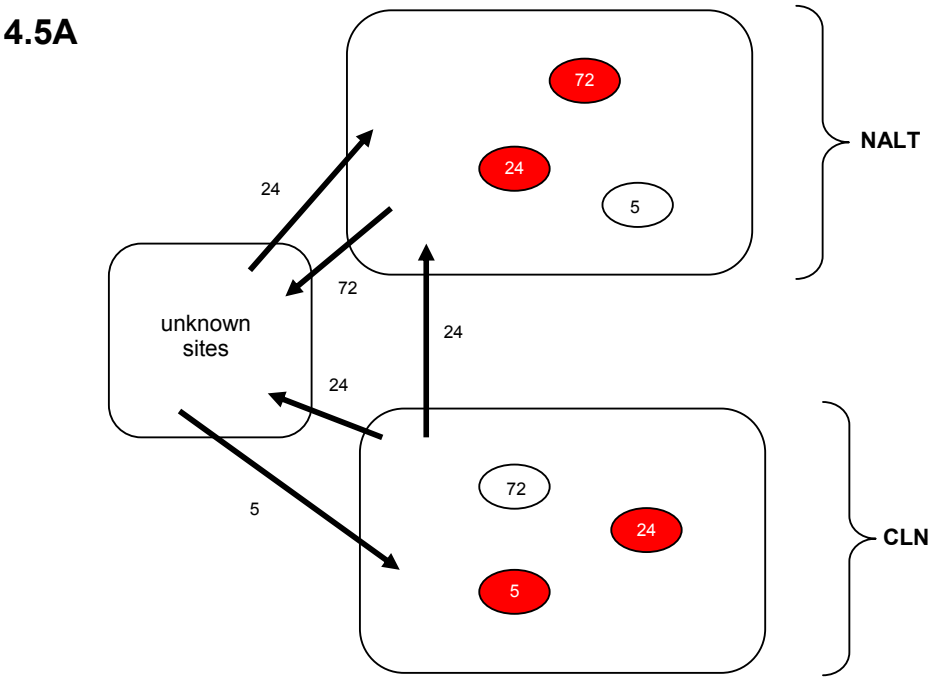
4.2.2.1.3 Natural Killer Cells

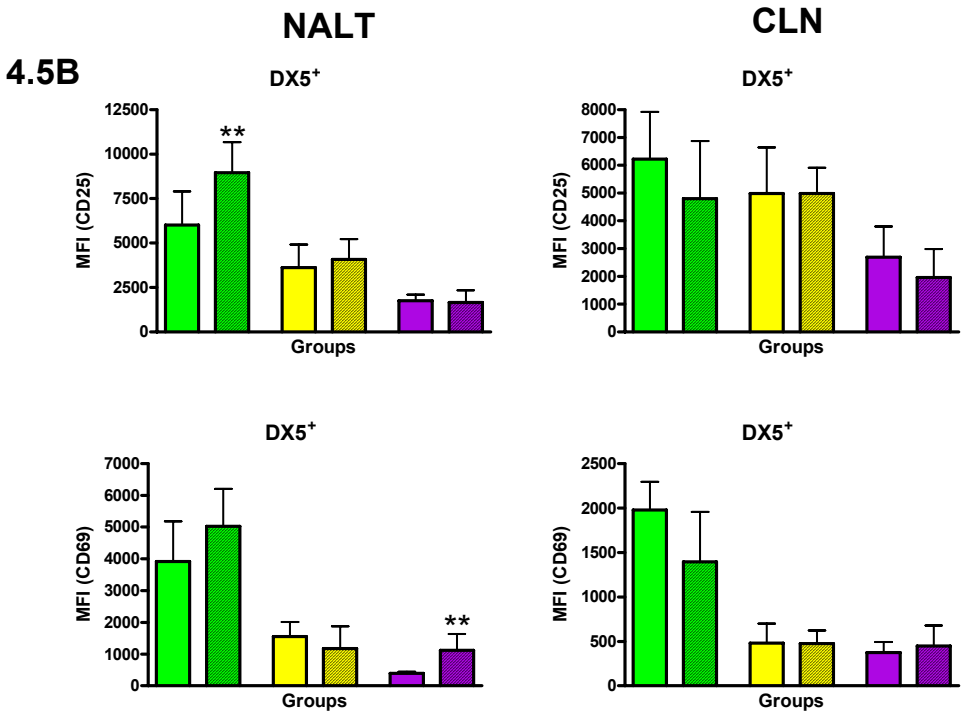
Figure 4.5: Schematic diagram showing an overview of NKC movement and activation within the NALT and CLN 5, 24, and 72 hours after intranasal immunisation

A. Ovals represent cells and numbers within the ovals indicate individual time-points. White ovals represent that the percentage change is not statistically different (i.e. $p > 0.05$) from naïve animals, blue shows a significant decrease (i.e. $p < 0.05$) and red indicates a significant increase (i.e. $p > 0.05$) in the cell population when compared to control mice. The arrows indicate direction of movement at particular time-points. FR stands for follicular regions (i.e. B cell areas), PR is for parafollicular regions (i.e. T cell areas), ES is for epithelial sides, and HEV stands for high endothelial venules.

B. Cells were isolated from the NALT and CLN of control (PBS immunised) and immunised animals, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $DX5^+$ expression as well as CD25 and CD69 expression (Figure 4.5B). For analysis, gates were set on the innate subset marker positive ($DX5^+$) cells and the MFI of CD25 and CD69 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, yellow at 24 hours and purple at 72 hours. Block colours represent naïve (PBS immunised) animals and, diagonal lines show animals intranasally immunised with adjuvant plus Ag85B-ESAT6. Scales are representational of individual activation marker MFI for each tissue – always refer to y-axis scale for each graph.

4.2.2.1.3 Natural Killer Cells





4.2.2.1.3.1 5 hour time-point

The percentage of $DX5^+$ cells within the NALT was found not to be significantly different ($p > 0.05$) from those seen in naïve mice 5 hours after immunisation (Table 4.1). I also observed increased ($p < 0.05$) CD25 expression on $DX5^+$ cells, but significantly lower ($p < 0.01$) number of $DX5^+/CD69^+$ cells at 5 hours within the NALT (Table 8.2B and Figure 4.5B). The CLN percentage of NKC and $DX5^+/CD25^+$ cells in immunised mice were increased ($p < 0.01$) by more than 2x compared to naive mice (Tables 4.1 and 8.2B and Figure 8.1H). Conversely to what was observed in the NALT, all animals immunised with adjuvant and antigen showed an increase ($p < 0.01$) in $DX5^+/CD69^+$ cells 5 hours after immunisation within the CLN (Table 8.2B).

4.2.2.1.3.2 24 hour time-point

By 24 hours the percentage of $DX5^+$ and $DX5^+/CD25^+$ cells found within the NALT was significantly increased ($p < 0.01$) in immunised mice, with an approximately 2x higher percentage of NKC than naïve animals (Tables 4.1 and 8.2B and Figure 8.1F). Within the CLN, the percentages of $DX5^+$ and $DX5^+/CD25^+$ cells in immunised mice were still found to be significantly higher ($p < 0.01$) than naïve animals 24 hours after intranasal immunisation (Tables 4.1 and 8.2B and Figures 8.1I).

4.2.2.1.3.3 72 hour time-point

Within the NALT, 72 hours post immunisation, there continued to be a greater percentage ($p < 0.05$) of NKC, $DX5^+/CD25^+$ and $DX5^+/CD69^+$ cells compared to naïve mice but the percentage was lower than that seen at 24 hours (Tables 4.1 and 8.2B and Figure 8.1G). In addition, I observed a significant increase in surface CD69 expression on these cells (Figure 4.5B). For the CLN $DX5^+$ population I observed that NKC percentages in immunised mice returned to those seen for naïve animals ($p > 0.05$) (Table 4.1). However, there was still a significant increase ($p < 0.01$) in the $DX5^+/CD25^+$ subpopulation in immunised mice at this final time-point (Table 8.2B). For overview see Figure 4.5.

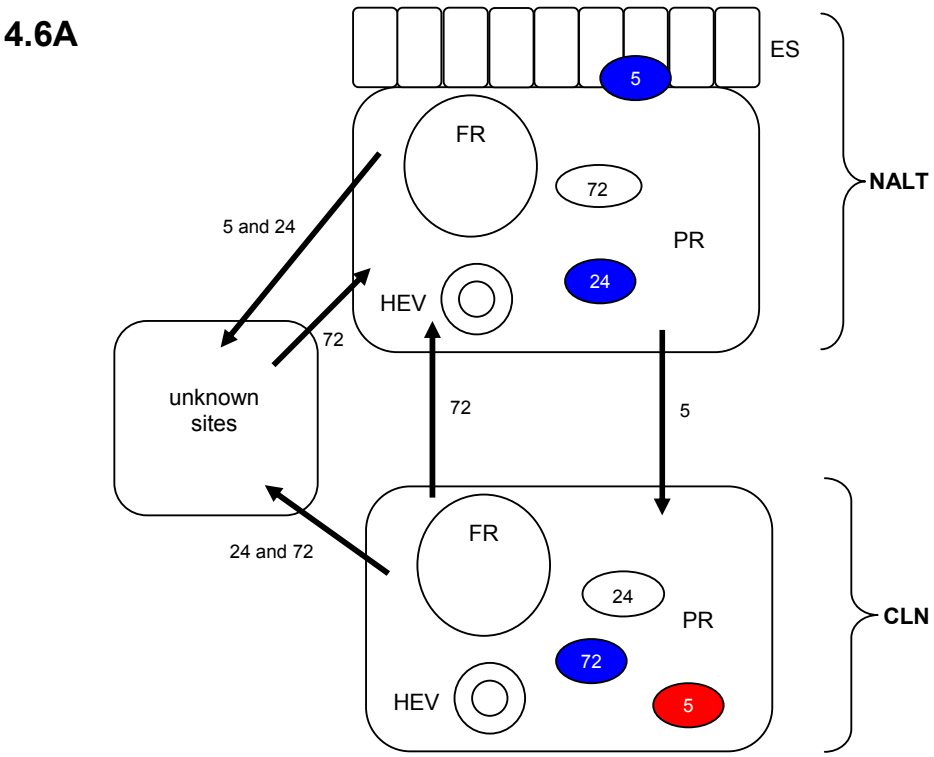
4.2.2.1.4 Neutrophils

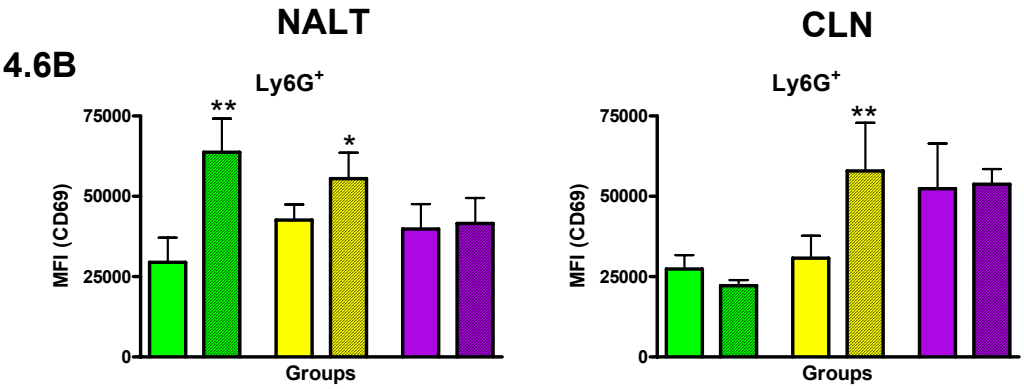
Figure 4.6: Schematic diagram showing an overview of neutrophil movement and activation within the NALT and CLN 5, 24, and 72 hours after intranasal immunisation

A. Ovals represent cells and numbers within the ovals indicate individual time-points. White ovals represent that the percentage change is not statistically different (i.e. $p > 0.05$) from naïve animals, blue shows a significant decrease (i.e. $p < 0.05$) and red indicates a significant increase (i.e. $p > 0.05$) in the cell population when compared to control mice. The arrows indicate direction of movement at particular time-points. FR stands for follicular regions (i.e. B cell areas), PR is for parafollicular regions (i.e. T cell areas), ES is for epithelial sides, and HEV stands for high endothelial venules.

B. Cells were isolated from the NALT and CLN of control (PBS immunised) and immunised animals, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $Ly6G^+$ (Figure 4.6B) expression, as well as CD69 expression. For analysis, gates were set on the innate subset marker positive ($Ly6G^+$) cells and the MFI of CD69 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, yellow at 24 hours and purple at 72 hours. Block colours represent naïve (PBS immunised) animals and, diagonal lines show animals intranasally immunised with adjuvant plus Ag85B-ESAT6. Scales are representational of individual activation marker MFI for each tissue – always refer to y-axis scale for each graph.

4.2.2.1.4 Neutrophils





4.2.2.1.4.1 5 hour time-point

Neutrophil numbers were observed to significantly decrease ($p < 0.05$), but the MFI of CD69 significantly increased ($p < 0.01$) on these cells in immunised animals within the NALT, compared to control mice at the 5 hour time-point (Table 4.1 and Figures 8.1E and 4.6B). The distribution of remaining cells was still similar to control animals (Figure 4.7C). I also observed that neutrophils ($Ly6G^+$) positive for CD69 decreased ($p < 0.01$) in all immunised mice after 5 hours within the NALT (Table 8.2B). Contrary to what I observed within the NALT, immunised animals had increased percentages ($p < 0.01$) of $Ly6G^+$ cells within their CLN at this early 5 hour time-point (Table 4.1 and Figure 8.1H).

4.2.2.1.4.2 24 hour time-point

By 24 hours, mice immunised with LT + Ag85B-ESAT6 had an increase in the percentage of $Ly6G^+$ cells compared to the percentage seen at 5 hours, however this was still significantly lower ($p < 0.05$) than that seen for naïve animals (Table 4.1 and Figure 8.1F). I also observed that CD69 expression was still higher ($p < 0.05$) in these animals when compared to controls (Figure 4.6B). I also observed movement of $Ly6G^+$ cells from the periphery into more central locations after immunisation (Figures 4.7C). Within the CLN mice receiving adjuvant and antigen showed an increase in CD69 expression on their $Ly6G^+$ cells when compared to naïve animals (Figure 4.6B). However, in terms of the percentage of $Ly6G^+$ cells I did not observe a significant difference ($p > 0.05$) between immunised and naïve animals (Table 4.1).

4.2.2.1.4.3 72 hour time-point

By 72 hours the $Ly6G^+$ NALT population was similar in all animals tested, but neutrophils of immunised mice still showed a more central wide-spread distribution than observed in control animals (Table 4.1 and Figure 4.7C). Within the CLN, I observed a depletion of the $Ly6G^+$ population in immunised mice (Table 4.1 and Figure 8.1J). In terms of distribution, I observed a similar pattern of $Ly6G^+$ cells in all immunised and naïve animals at all time-points tested within the CLN, i.e. within the parafollicular region (Figure 4.7C). For overview see Figure 4.6.

Figure 4.7: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT + Ag85B-ESAT6) Balb/c mice were compared. Figure 4.7A represents staining of frozen sections for CD11c in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and CD11c (red). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES). For H + E image reference see Figure 4.1.

Figure 4.7A:

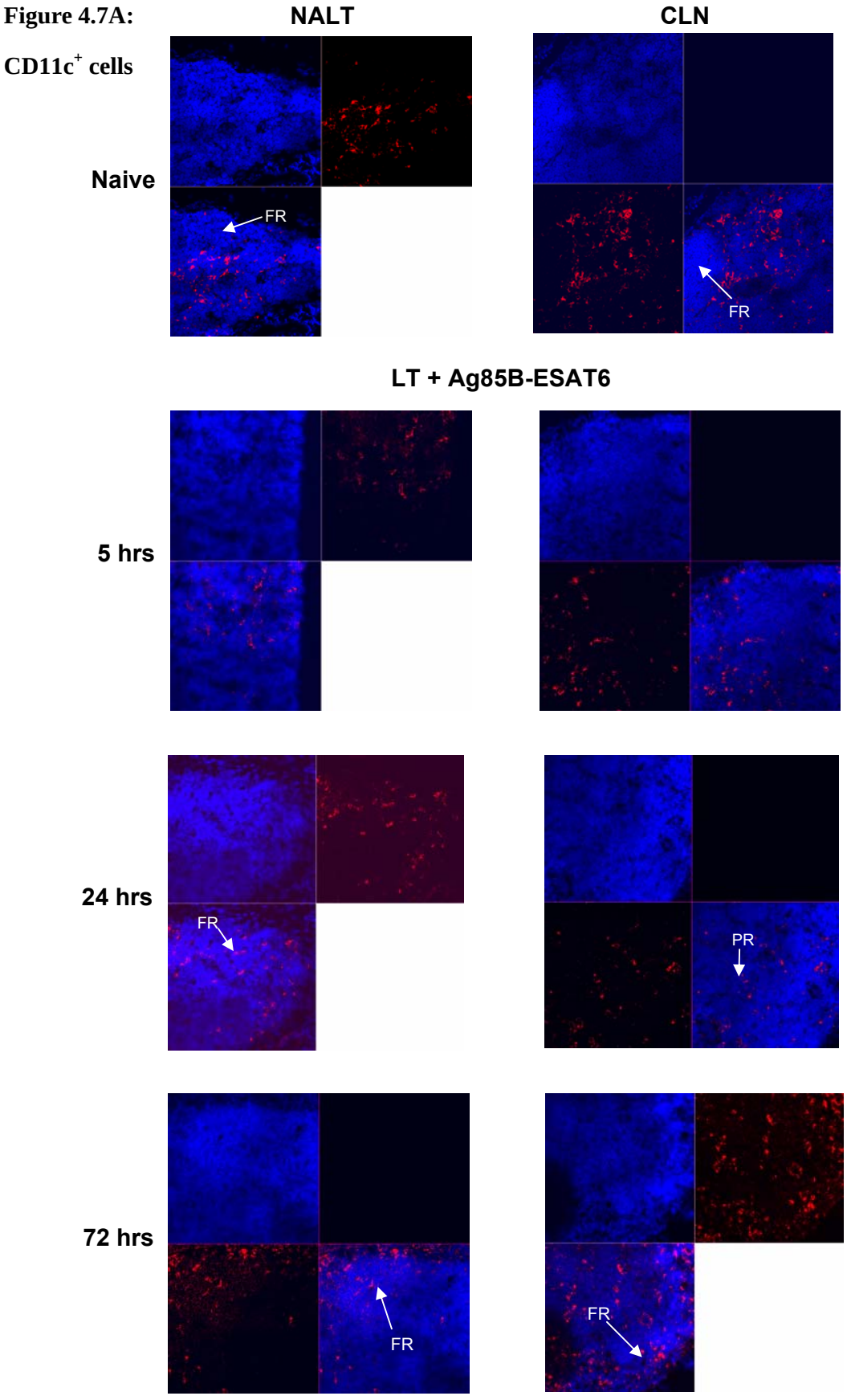

NALT
CLN
CD11c+ cells
Naive
FR
FR
LT + Ag85B-ESAT6
5 hrs
24 hrs
FR
PR
72 hrs
FR
FR

Figure 4.7: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT + Ag85B-ESAT6) Balb/c mice were compared. Figure 4.7B represents staining of frozen sections for F4/80 in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and F4/80 (green). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES). For H + E image reference see Figure 4.1.

Figure 4.7B:

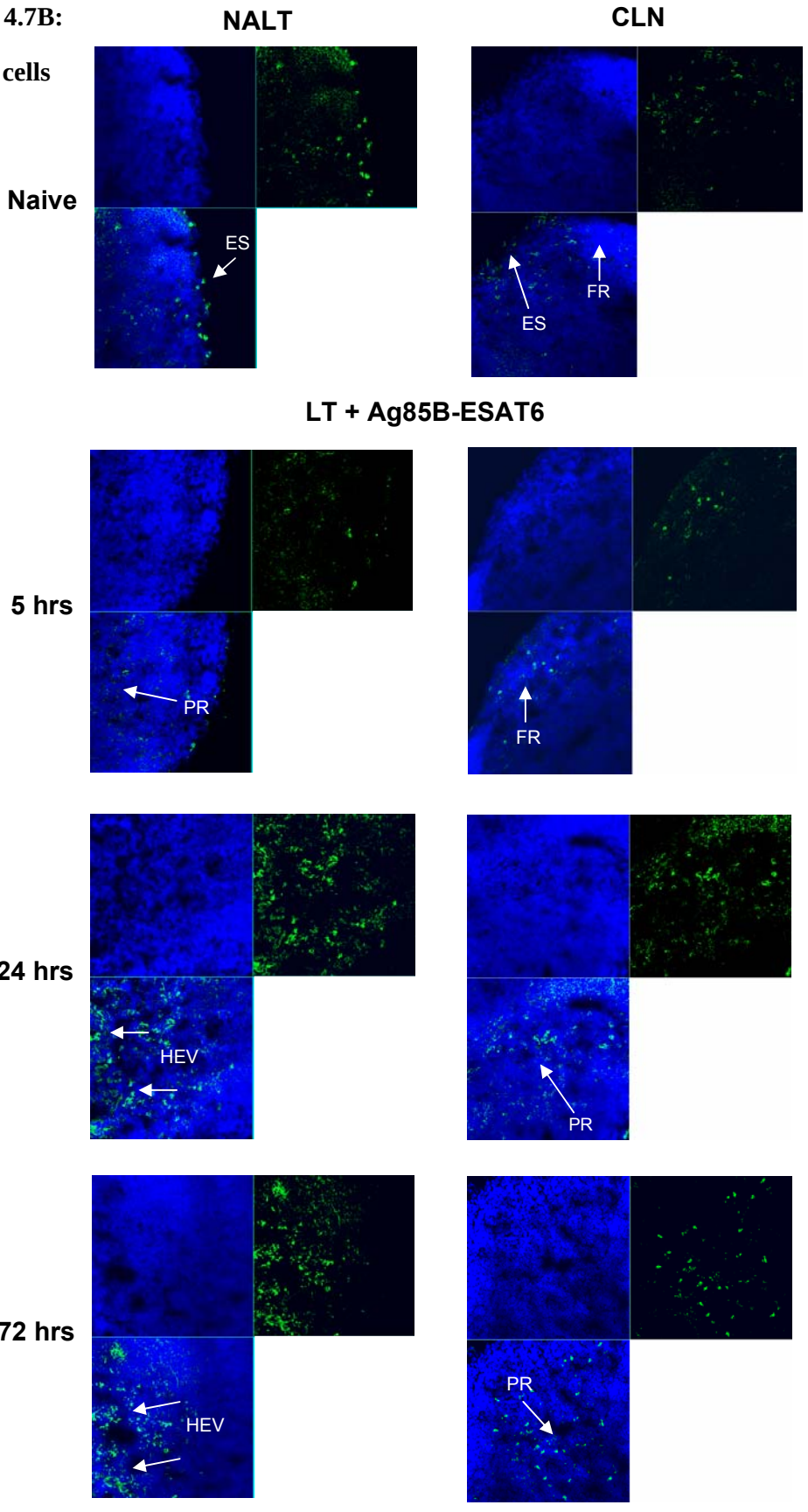
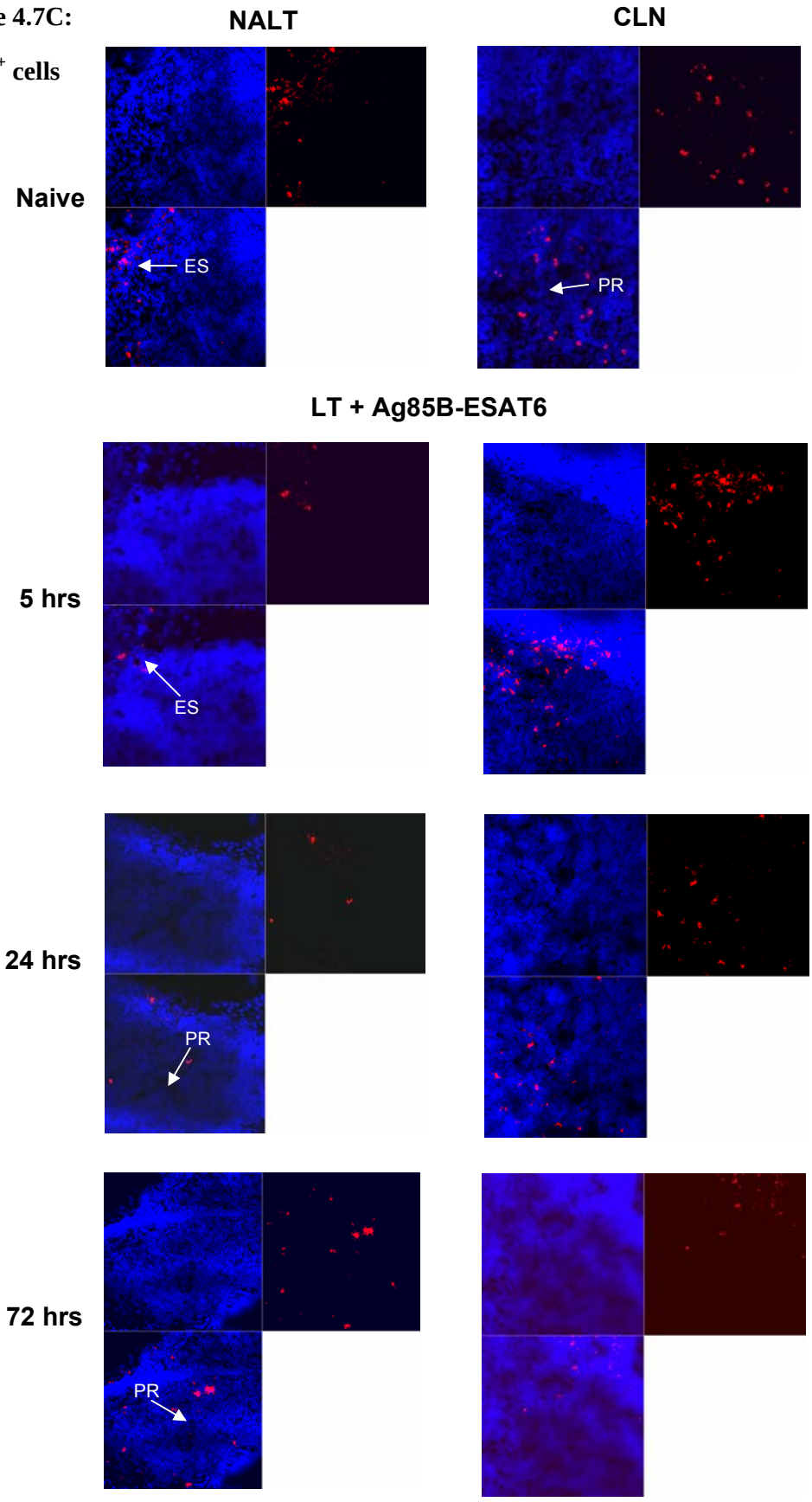

NALT
CLN
F4/80+ cells
Naive
ES
ES
FR
LT + Ag85B-ESAT6
5 hrs
PR
FR
24 hrs
HEV
PR
72 hrs
HEV
PR

Figure 4.7: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT + Ag85B-ESAT6) Balb/c mice were compared. Figure 4.7C represents staining of frozen sections for Ly6G in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and Ly6G (red). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES). For H + E image reference see Figure 4.1.

Figure 4.7C:


NALT
CLN
Ly6G+ cells
Naive
ES
PR
LT + Ag85B-ESAT6
5 hrs
ES
24 hrs
PR
72 hrs
PR

4.2.3 Discussion

The innate immune response reacts rapidly to foreign antigens with activation and migration of its cellular components (Janeway and Medzhitov 2002; Medzhitov and Janeway 2000). The relevance of these individual components to host survival against pathogen infections is beyond doubt, with the protective contribution of DC, macrophages, NKC and neutrophils, as well as various cytokines being demonstrated in a number of infections (Bodnar, et al. 2001; Harshan and Gangadharam 1991; Murphy, et al. 2004; Pedrosa, et al. 2000; Rogers and Unanue 1993; Samsom, et al. 1997; Schafer and Eisenstein 1992; Weissman and Fauci 1997). This has lead to an appreciation in recent years that innate immunity is central to protection against infectious diseases and the induction of adaptive immune responses. However, an integrated picture of innate responses after mucosal vaccination has not been established. The current study addresses this by characterising changes in defined cell populations, their activation status, cell adhesion molecule expression and GC formation in the first few hours and days following intranasal immunisation with a mucosal adjuvant and model antigen. Hopefully this study will accentuate the importance of understanding the nature of innate immune responses at the mucosae for the design of improved vaccines.

4.2.3.1 5 hour time-point

The earliest changes in NALT cell populations detected were a rapid and significant reduction in the total percentages of DC, macrophages and neutrophils and a concomitant increase in CLN neutrophils along with an increase in NKC. The 2-fold increase in NKC percentages that occurred 5 hours after intranasal immunisation transformed this minor CLN population to the most abundant innate cell type in the CLN. NKC contribute to innate immunity by the production of cytokines, particularly IFN-γ, along with other immunomodulatory signals early after their activation. In addition to the increase of $DX5^+$ cells seen within the CLN, I also observed a significant increase of $DX5^+CD69^+$ and $DX5^+/CD25^+$ cells. As already discussed, the CD69 and CD25 antigens are some of the earliest markers expressed on activated NKC following stimulation by a variety of mitogenic agents. Our *in vivo* observations in immunised mice show that even though the percentage of these cells increases,

their MFI does not, possibly indicating that this influx of NKC cells in the CLN precedes their activation. Contrary to the activation status observed in the CLN NKC, I did observe a significant increase in CD25 expression on NALT NKC, suggesting that the cells already present within this lymphoid tissue may be activated. The earlier activation status of these cells within the NALT highlights the fact that the NALT is the first area that uptake of soluble antigen occurs before it can gain access to nose-draining LNs such as the superficial CLN. Although I did observe migration of NKC cells into the CLN, I did not see a concurrent reduction in percentages within the NALT, suggesting that influx of NKC within the CLN is due to migration of this innate cell population from another site, as yet unidentified. In this present study I removed the O-NALT; however there is also another, less organised, lymphoid site situated within the nasal passages called the D-NALT (Kuper, et al. 1992). Studies to date suggest that the O-NALT is rich in unswitched, naive B cells and naive T cells, suggesting that it is a mucosal inductive site, whereas the D-NALT may function as an effector site (Hiroi, et al. 1998; Wu, et al. 1996). With these points in mind it may be that the other cell populations undergoing changes during this early 5 hour time-point are in fact trafficking to this area to 'pick-up' and process antigen before moving back to the inductive O-NALT or other lymphoid tissues. Whereas this may be true for the macrophage and DC populations, with regards to the $Ly6G^{+}$ population, it appears that the disappearance of neutrophils from the NALT probably results in the migration and influx of this cell population into the CLN 5 hours after immunisation. As described for the up regulation of CD25 on NKC, I also observed a significant increase of CD69 on neutrophils suggesting that these cells were activated within the NALT, but not the CLN. Neutrophils are usually thought of as the leukocyte population involved in acute inflammatory responses, acting as a first line of defense against invading microorganisms. They are also able to synthesise cytokines in response to a variety of inflammatory stimuli. For a comprehensive review see (Nathan 2006). Therefore, activation of this innate cell population may induce further cellular and vascular innate immune mechanisms at very early time-points after immunisation. In addition to the decrease in percentages of APC within the NALT, I also observed an increase in activation marker expression (i.e. MHC II and VCAM-1), suggesting that the NALT cells present may be activated and ready to process antigen. In lymphoid tissues, mature FDC play key roles in organising the

formation of GC. FDC interact avidly with B cells by virtue of the adhesion molecules ICAM-1 and VCAM-1 on the FDC, which interact with $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrins on the B cell. These physical interactions are crucial for the survival of B cells proliferating in the GC (Koopman, et al. 1994; Tew, et al. 2001). Therefore, the presence of DC expressing VCAM-1 to high levels may indicate why I observe GC formation as early as 5 hours post immunisation within these lymphoid tissues (see Section 5.4). I also noted that distribution of APC in immunised NALT was very similar to that seen in naïve animals, i.e. surrounding the edge of the tissue. This localisation of these DC is comparable to another study which examined $CD11c^+$ cell distribution early after *B. bronchiseptica* challenge (Gueirard, et al. 2003). Even though cell percentages of the APC, DC and macrophages did not change in the CLN of immunised mice 5 hours post immunisation, I did observe an increase in the MFI of MHCII on both $CD11c^+$ and $F4/80^+$ cells present, indicating that these cell types may in fact be activated. These activated cells may have already processed antigen or become activated via immunomodulatory signals secreted from the NKC and neutrophils in the O-NALT or D-NALT, and have consequently migrated down to the CLN. In addition, I also observed the movement of these cells to more central areas within the CLN, possibly indicating the involvement of these APC in presenting antigen via MHC II to lymphocytes. Several studies show that LT toxin exerted detectable regulatory effects on DC and macrophages, altering both their surface phenotype and their ability to process and present protein antigens (Martin, et al. 2002; Petrovska, et al. 2003).These observations may explain why I see increased activation of these cell types as early as 5 hours post immunisation. The FACS plots (Figure 8.1) had some diagonal smearing which may be due to the presence of dead cells. In order to confirm and exclude this data a live/dead marker could be added next time to allow this differentiation.

4.2.3.2 24 hour time-point

By 24 hours post immunisation I observed a dramatic increase in the percentages of NKC within the NALT when compared to naïve animals. I also again observed an increase in this cell population with the CLN; however the increase was not as great as that seen 5 hours after immunisation. Therefore, it is tempting to speculate that the

influx of NKC observed within the NALT may be due to migration of these $DX5^+$ cells from the CLN. Neutrophils within the NALT immunised animals were again decreased in comparison to the percentage seen in control (PBS immunised) animals however, percentages seen in the CLN were back to those observed in naïve animals. The reduction in the $Ly6G^+$ population within the NALT was not as significant as that seen at the 5 hour time-point, and this may account for the percentage observed within the CLN at 24 hours. It was also noted that the neutrophils present in these immunised animals appeared to be activated due to up regulation of the CD69 antigen. Within the NALT, $F4/80^+$ cells from immunised mice had returned to those seen in naïve mice and DC percentages were significantly increased. The increase in these cell populations may be due to macrophage and DC returning from the D-NALT ready to present antigen, in fact the APC present in the NALT of these mice were located within both B and T cell areas supporting this hypothesise. As already discussed, NKC and major producers of IFN-γ and the influx of NKC within the CLN at both 5 and 24 hours post immunisation may indicate why I do not see any movement of macrophages out of this tissue. IFN-γ can induce production of macrophage migration inhibitory factor which in turn can prevent macrophages from leaving the tissue. Even though I did not see any differences in percentages of $F4/80^+$ cells within the CLN at the 24 hour time-point, I again observed a more diffuse staining pattern, again highlighting that those macrophages present may in fact be processing and presenting antigens to lymphocytes. I also observed that the macrophages and DC within the CLN had up regulated expression of VCAM-1. APC expressing this cell adhesion molecule are known to increase presentation of antigen to GC B cells and this subset of cells may be involved in the induction of antigen-specific humoral immune responses shortly after intranasal immunisation. DC populations within the CLN decreased within the CLN of immunised animals, including the $CD11c^+/MHCII^+$ and $CD11c^+/VCAM\text{-}1^+$ subset. This migration of DC may be to the spleen for further induction of immune responses, however as I did not examine splenocytes populations I can not speculate further.

4.2.3.3 72 hour time-point

At the final time-point examined (i.e. 72 hours), there was a decrease in the NKC population within the NALT in those animals immunised, when compared to that seen at 24 hours after immunisation. The reduction in percentages of NKC may signal their movement from the CLN to other secondary lymphoid organs within the mucosal immune systems, as well as movement systemically. The $DX5^+$ cells present also had significantly more expression of the CD69 antigen when compared to control animals. As already discussed, CD69 is an early marker of NKC activation, however high expression is still seen up to 48-72 hours after stimulus is removed. Therefore, this high CD69 expression on $DX5^+$ cells may show the population of NALT NKC cells that were activated very shortly after intranasal immunisation, or the cells may in fact be a newly activated population that has migrated in from another site. In addition, previous work on IL-12 stimulation of NKC cells suggests that CD69 expression also identifies cells in a state of anergy post function, not cells that are preactivated and ready to function (Craston, et al. 1997). Consequently this may be another explanation as to why there are $DX5^+/CD69^+$ cells 72 hours after immunisation. The CLN $DX5^+$ cell population were back to those percentages seen in control mice by 72 hours, with those cells present having no significant increase in their CD69 or CD25 expression. This may indicate that this cell population has returned to basal levels by 72 hours, as other populations are now beginning to modulate immune responses, e.g. lymphocytes. I also observed a return to those percentages consistent with naïve animals in the $Ly6G^+$ NALT population. As mentioned above, neutrophils are one of the first cell types to migrate to sites of inflammation and consequently are also one of the first to disappear after stimulus has been removed. This cell type only has a very short life-span, 6-48 hours, after their activation and this may in part explain why I no longer observe changes in the percentage of neutrophils within the NALT at this later time-point. Conversely, I did observe changes in the percentages of $Ly6G^+$ cells with the CLN 72 hours after immunisation. This may simply be due to the onset delay, through trafficking of local NALT immune responses, to the CLN after initial induction within the NALT. As described for neutrophils responses within the NALT at 72 hours, I also no longer observed any changes in both the DC and macrophage populations when compared to naïve animals. Examining tissue sections of the NALT I did see that both populations were still in more central areas rather than surrounding

the periphery, which is the case in PBS immunised animals, suggesting that these APC are still processing and presenting antigen to lymphocytes. I also observed macrophages surrounding HEV, indicating the migration of this cell type from the NALT through the lymphatics to other lymphoid tissues for induction of further immune responses. The increase in number of $CD11c^+$ DC, and $F4/80^+$ macrophage cells in the CLN by 72 hours is impressive, and their position in the T and B cell areas might reflect their involvement in immunologic reactions taking place in this lymphoid tissue.

4.2.3.4 Summary

In summary, I demonstrate for the first time that intranasal immunisation with adjuvant and antigen results in the dramatic changes in innate cell populations within both the NALT and CLN. APC and neutrophil percentages decrease within the NALT as early as 5 hours post immunisation, and it isn't until day 3 that levels return to those seen in control animals. A reduction in DC CLN percentages is not observed until 24 hours after immunisation, but a significant APC influx is observed by 72 hours. Neutrophils accumulate within 5 hours in the CLN and remain present until day 3. Both macrophages and DC start to migrate into more central areas of these lymphoid tissues by 24 hours indicating their involvement in the linking of adaptive and innate immune responses. The innate cell population showing the most drastic changes in percentage are NKC. These cells migrate into the CLN first, before moving up to the NALT. Immunisation also results in a rapid and transient activation of all cells first in the NALT, and then in the CLN, highlighting the fact that the NALT is the first point of call for inhaled antigens. Overall the innate immune response after intranasal immunisation is a complicated one, with cells rapidly moving to and fro between both the NALT and the CLN, as well as yet undefined tissues. Further studies are needed to understand the functional implications of all this leukocyte migration early after intranasal immunisation.

4.3 Cell adhesion molecule expression early after intranasal immunisation

4.3.1 Introduction

4.3.1.1 Leukocyte trafficking and cell adhesion molecules

The trafficking of immune cells in blood and lymph into secondary lymphoid tissues is an important part of the immune response that is required for an efficient and targeted response to antigenic challenge (Butcher and Picker 1996). During innate immune responses, inflammatory mediator such as cytokines; including IL-1β, TNF-α and IFN-α/β, are released in response to foreign antigens. Along with these cytokines, various chemokines (such as IL-8, monocytes chemoattractant proteins and macrophage inflammatory proteins) are released and their receptors are expressed on the surface of activated cells. As a result, vascular endothelial cells may alter their surface expression of selectins and intracellular adhesion molecules, leading to the extravasation and selective retention of some leukocytes at the inflamed site (Fabbri, et al. 1999). Leukocytes exit the blood at specialised HEV, and are regulated in part by multistep cascades, involving sequential leukocyte/endothelial adhesion and activation events (Gowans and Knight 1964). At sites of chronic inflammation, HEV-like vessels are often induced and allow large numbers of leukocytes to emigrate from blood and accumulate in extra lymphoid tissues (Freemont 1987). The first of these interactions causes the leukocyte to roll along the vessel wall (Bargatze, et al. 1994). Within seconds, the rolling leukocyte becomes firmly adherent, and the leukocyte transmigrates from the blood vessel to tissue (Warnock, et al. 1998; Warnock, et al. 2000). These cascades can be tissue-specific, with much of the specificity determined by selective expression of adhesion molecules by endothelia in various tissues, and of their ligands by circulating leukocytes (Butcher, et al. 1999). There are four major families of cell adhesion molecules; the Ig superfamily cell adhesion molecules (CAMs), integrins, cadherins, and selectins. A selection of cell adhesion molecules and the receptors involved in each step of leukocyte extravasation to lymphoid tissues are listed in Table 4.2.

Table 4.2: Cell Adhesion Molecules and Their Receptors

Molecule	Expression/Distribution	Ligand
MAdCAM-1	HEV and DC	$\alpha_4\beta_7$ integrin
PNAd	HEV	L-Selectin
ICAM-1 (CD54)	Monocytes; Endothelial cells; DC; Fibroblasts; Epithelium; Synovial cells and T lymphocytes	$\alpha_L\beta_2$ integrin (LFA-1, CD11/CD18)
VCAM-1 (CD106)	Monocytes; DC; Synovial cells and Activated Endothelial cells	$\alpha_4\beta_1$ integrin (VLA-4)

4.3.1.2 Cell adhesion molecule expression in the NALT and CLN

As already discussed, the murine NALT and CLN are involved in the generation of local immune responses within the upper respiratory tract. Previous intranasal immunisation studies have shown that the CLN drain the NALT, and therefore for this present study cell adhesion molecule expression will be examined in the both these upper respiratory tract lymphoid tissues to determine expression profiles. All NALT HEV express PNAd, either associated with MAdCAM-1 or alone, while NALT FDC express both MAdCAM-1 and VCAM-1. Interestingly, these expression profiles are distinct from those of the gut mucosal inductive site PP, where predominately MAdCAM-1-$\alpha_4\beta_7$ interactions mediate leukocyte homing (Csencsits, et al. 1999; Csencsits, et al. 2002). Intracellular adhesion molecule-1 (ICAM-1) expression within the NALT, and the epithelium overlying the NALT, has been shown to be constitutively low in naïve mice (Hussain, et al. 2001). Within the CLN, HEV also utilise PNAd-L-selectin interactions and MAdCAM-1-$\alpha_4\beta_7$ interactions for leukocyte binding, although not all HEV express MAdCAM-1 (Csencsits, et al. 2002; Wolvers, et al. 1999). ICAM-1 is also constitutively expressed at low to moderate levels on HEV of LNs, and all other vascular endothelium. In the CLN, staining for diffuse MAdCAM-1 is found to correlate with the expression of MAdCAM-1 on its HEV, and is located within the follicular stromal elements. The CLN also displays diffuse staining of VCAM-1 and ICAM-1 within its follicles. Evidence from cell ligand blocking experiments and the Stamper-Woodruff *ex vivo* binding assays, indicates that PNAd-L-selectin interactions mediate the majority of naive-leukocyte binding in both the NALT and CLN, with lesser roles for MAdCAM-1-$\alpha_4\beta_7$ and VCAM-1-$\alpha_4\beta_1$ interactions (Csencsits, et al. 2002).

4.3.2 Results

4.3.2.1 Intranasal immunisation increases both expression and distribution of cell adhesion molecules at early time-points in the NALT and CLN.

In order to identify the cell adhesion molecule expression profile early after intranasal immunisation, serial frozen NALT and CLN sections of control (naïve) and immunised (LT + Ag85B-ESAT6) mice were investigated for the distribution and expression of MAdCAM-1, PNAd, ICAM-1 and VCAM-1 at 5, 24 and 72 hours. As described in previous studies for the NALT and CLN, the HEV of naïve (PBS immunised) mice were found to express both MAdCAM and PNAd (Figures 4.8A and 4.8B). However, no diffuse MAdCAM-1 expression was observed in either the NALT or CLN (Figure 4.8A). ICAM-1 was observed mainly in the vascular endothelium, and weak expression of ICAM-1 was also shown around micro-vessels as well as on the surface of some cells in the NALT (Figure 4.8C), whereas ICAM-1 expression was found to be restricted to HEV and microvessels in the CLN (Figure 4.8C). Weak diffuse VCAM-1 expression was observed in the NALT and was localised on the surface of cells as well as around blood vessels, but not on HEV within the NALT of naïve animals (Figure 4.8D). Within CLN, VCAM-1 was observed on venular endothelium, vessels, and in addition on cells within this tissue (Figures 4.8D). After intranasal immunisation, I visually observed a greater intensity of MAdCAM-1 expression, which was mainly located on the vascular endothelium of HEV, and was higher than that seen in naïve animals in both the NALT and CLN at all time-points (Figure 4.8A). In addition to venular endothelium, MAdCAM-1 was also shown on the surface of infiltrating cells in the inflamed tissues after only 5 hours in the NALT (Figure 4.8A). All immunised mice were found to have some degree of diffuse MAdCAM-1 staining at all time-points tested within the CLN (Figure 4.8A). An increase in both PNAd expression and the number of HEV expressing this cell adhesion molecule was seen in the NALT of mice as early as 5 hours after intranasal immunisation (Figure 4.8B). This expression profile was still present at 24 hours, although by day 3 (72 hours) expression levels of PNAd were back down to that seen in naïve animals (Figure 4.8B). Expression of this addressin in the CLN was also found to have a similar profile to that seen in the NALT (Figure 4.8B). In intranasally immunised mice, higher expression of ICAM-1 was observed on HEV, micro-vessels,

and on the surface of cells within the NALT (Figure 4.8C). The pattern of diffuse ICAM-1 staining was also more widespread and of greater intensity than that seen in naïve animals at both 24 and 72 hours in this tissue (Figure 4.8C). Expression of ICAM-1 was also up-regulated in the CLN following immunisation, on both vascular endothelium, and with extension to many cell surfaces within CLN at all time-points examined (Figure 4.8C). As with ICAM-1 expression, VCAM-1 was also observed to be more intense and widespread on HEV, blood vessels and cells in the NALT of immunised mice (Figure 4.8D). Intranasal immunisation also increased VCAM-1 expression and distribution in CLN, especially at 24 hours (Figure 4.8D). Expression of this cell adhesion molecule was also more widely distributed at both 5 hours and 72 hours within the CLN, however levels of expression were only moderately increased at the 72 hour time-point when compared to naïve animals (Figure 4.8D).

Figure 4.8: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 4.8A represents staining of frozen sections for MAdCAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT + Ag85B-ESAT6 in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and MAdCAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.8A:

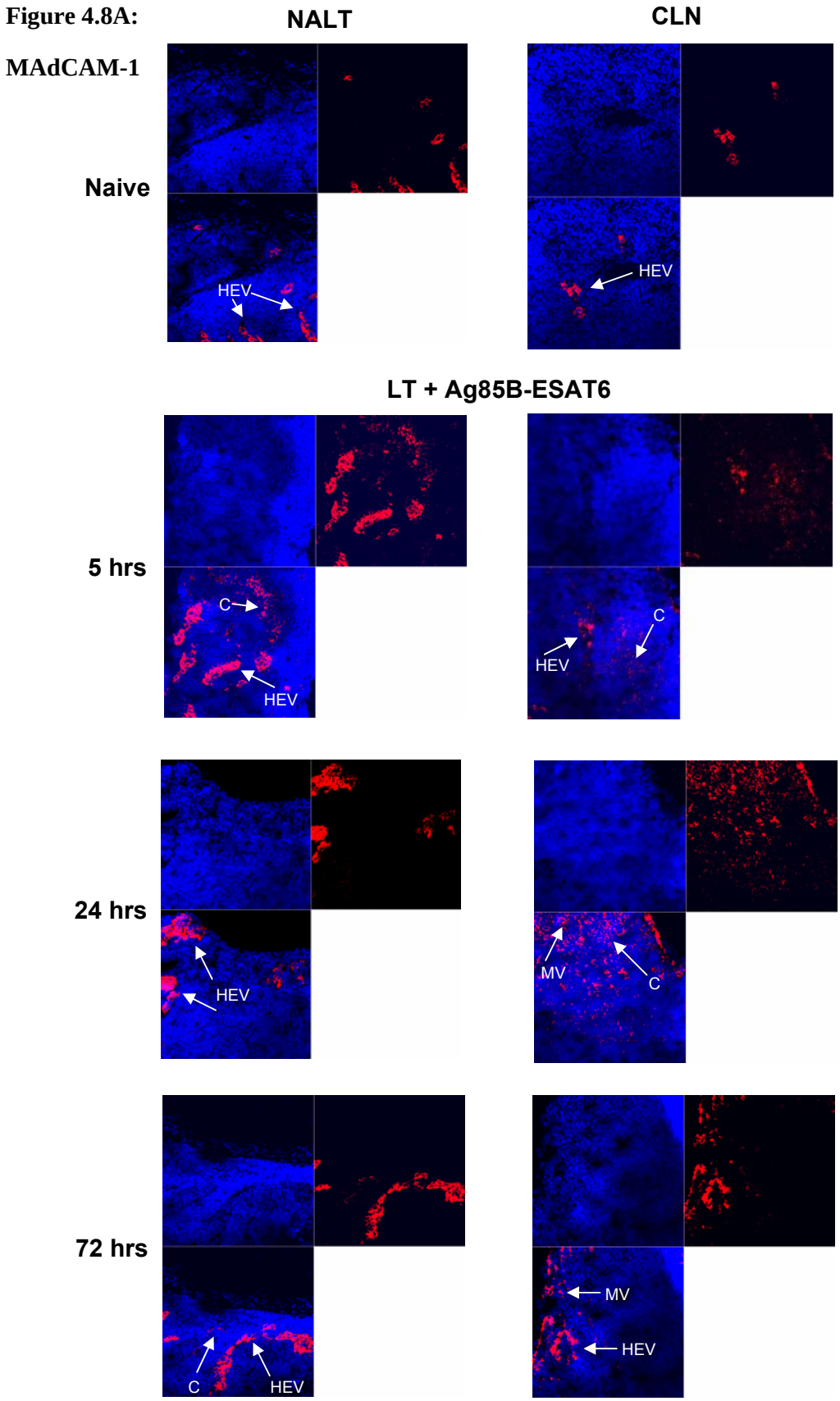

NALT
CLN
MAdCAM-1
Naive
HEV
HEV
LT + Ag85B-ESAT6
5 hrs
C
HEV
HEV
C
24 hrs
HEV
MV
C
72 hrs
C
HEV
MV
HEV

Figure 4.8: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 4.8B represents staining of frozen sections for PNAd in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT + Ag85B-ESAT6 in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and PNAd (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.8B:

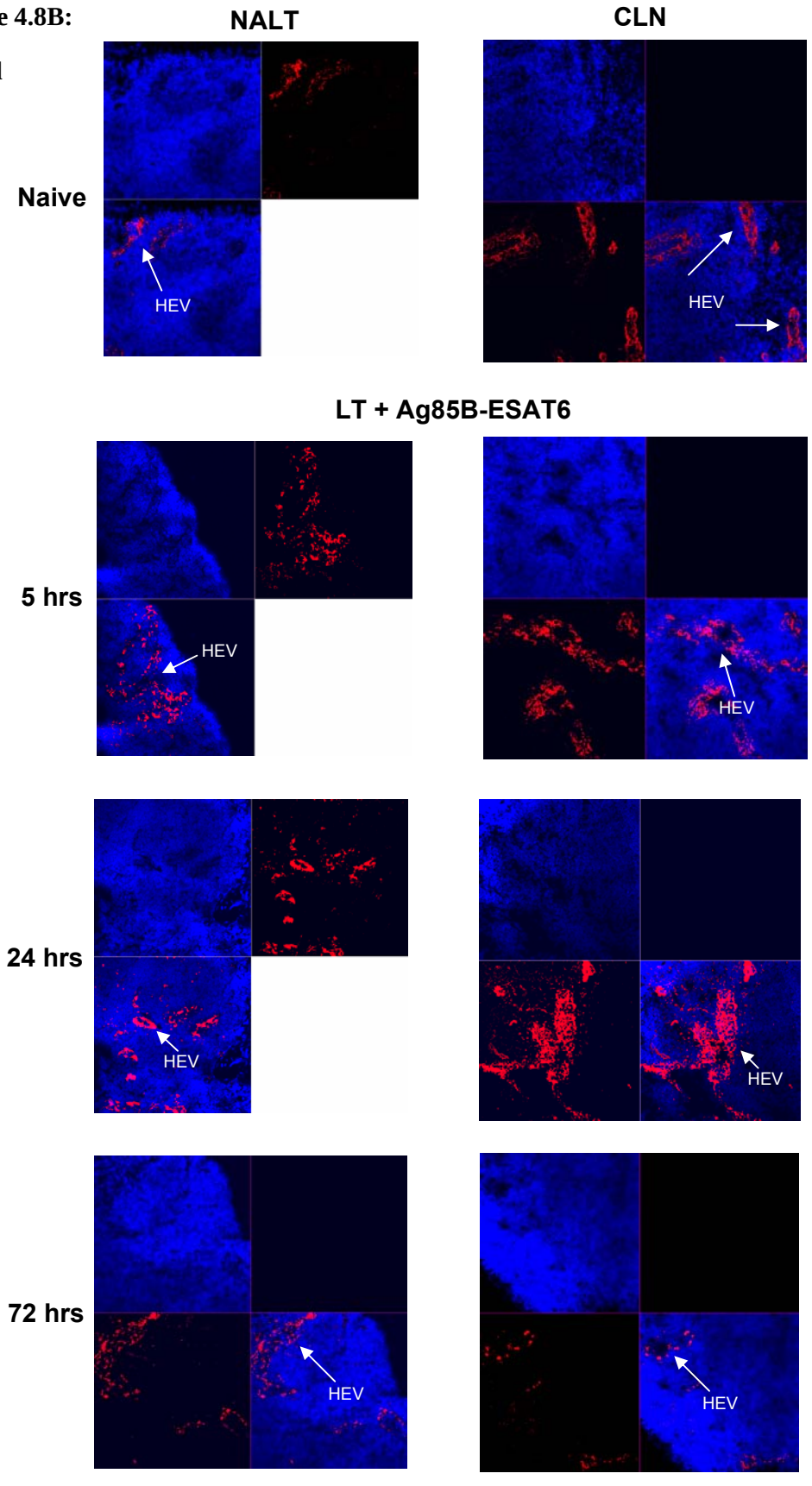

NALT
CLN
PNAd
Naive
HEV
HEV
LT + Ag85B-ESAT6
5 hrs
HEV
HEV
24 hrs
HEV
HEV
72 hrs
HEV
HEV

Figure 4.8: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 4.8C represents staining of frozen sections for ICAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT + Ag85B-ESAT6 in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and ICAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.8C:

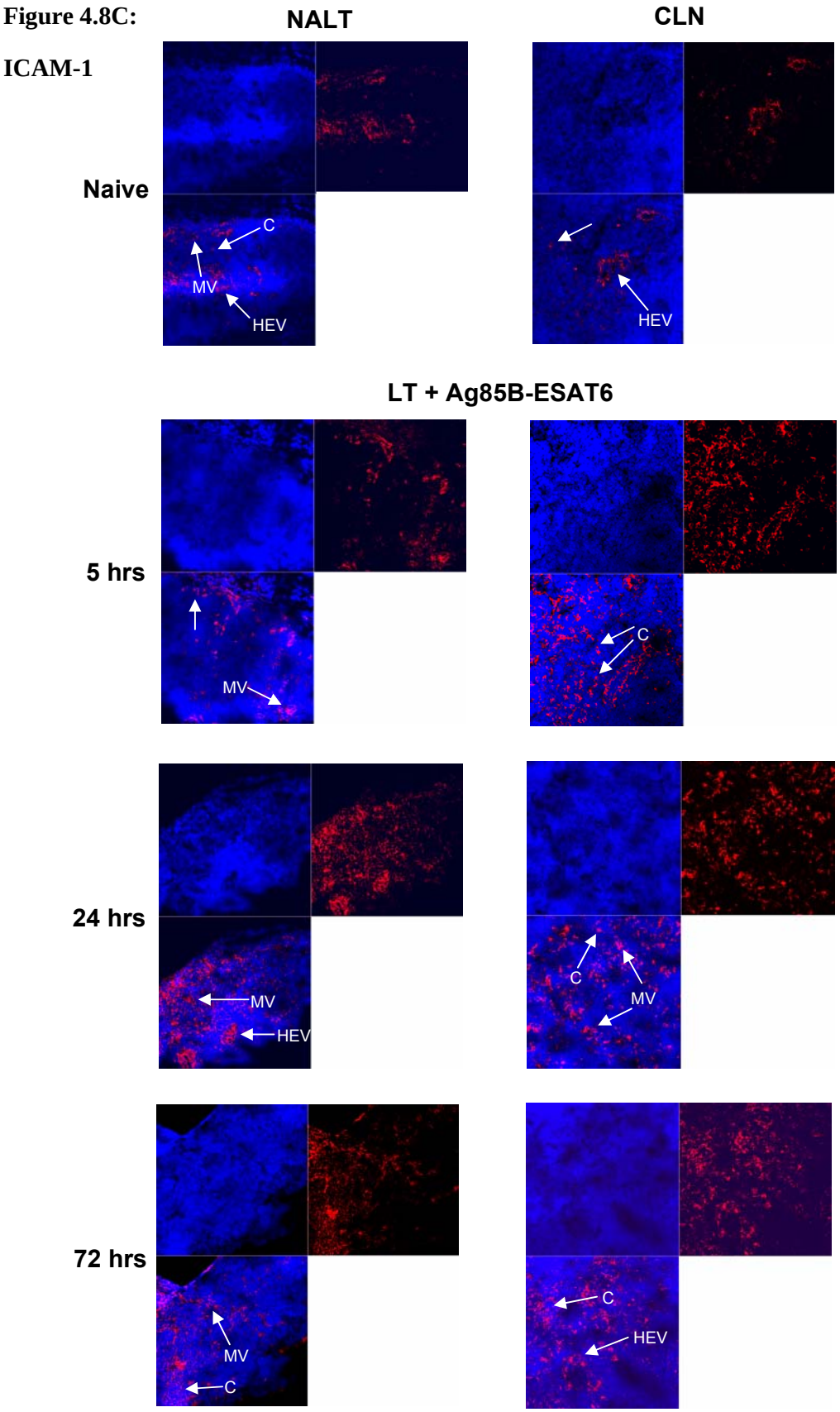
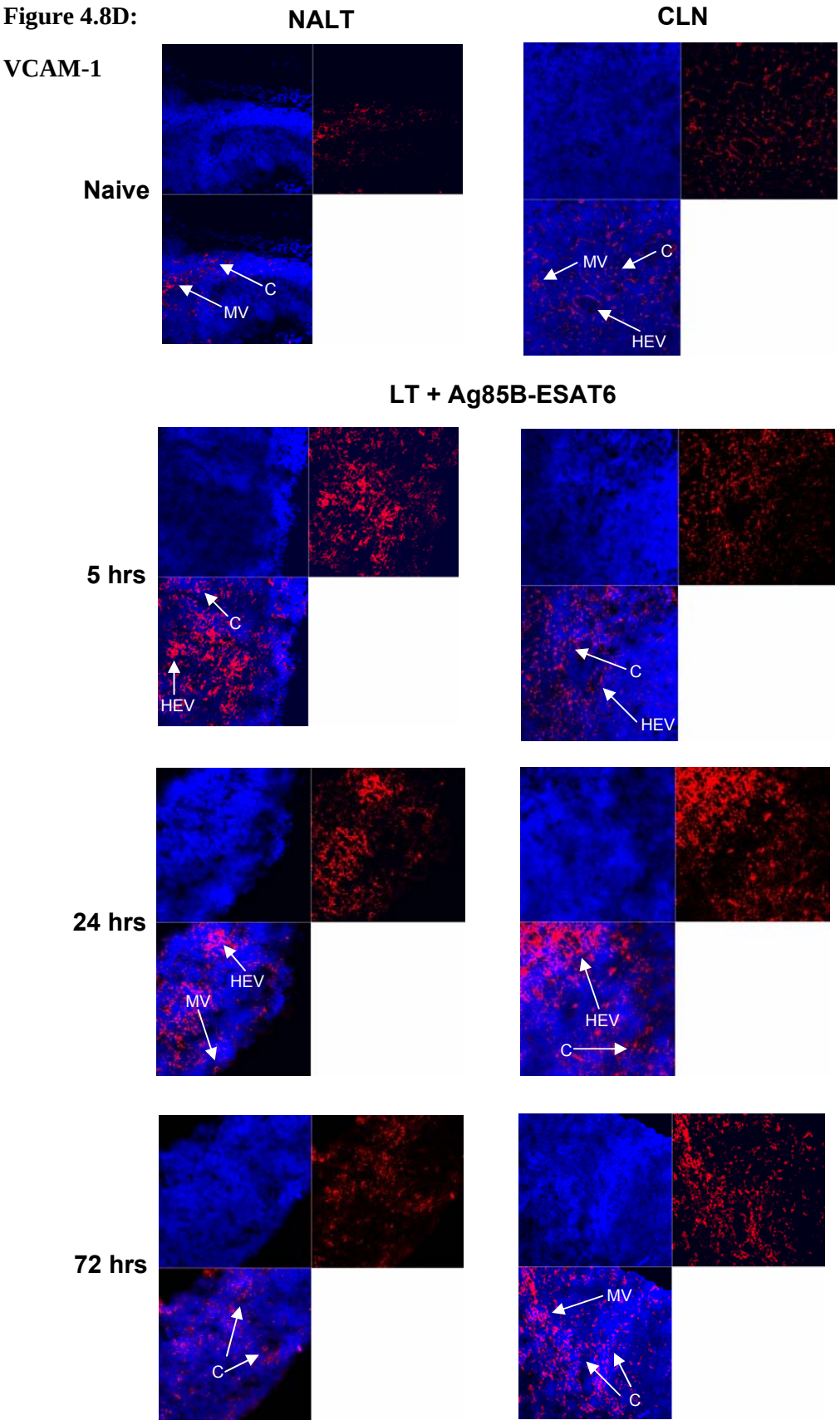

NALT
CLN
ICAM-1
Naive
C
MV
HEV
HEV
LT + Ag85B-ESAT6
5 hrs
MV
C
24 hrs
MV
HEV
C
MV
72 hrs
MV
C
C
HEV

Figure 4.8: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 4.8D represents staining of frozen sections for VCAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT + Ag85B-ESAT6 in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and VCAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.8D:


NALT
CLN
VCAM-1
Naive
C
MV
MV
C
HEV
LT + Ag85B-ESAT6
5 hrs
C
HEV
C
HEV
24 hrs
HEV
MV
HEV
C
72 hrs
C
MV
C

4.3.3 Discussion

The impact of immunisation on the expression of mucosal homing receptors on circulating immune cells, as well as MAdCAM-1 expression on endothelium, has been rather well studied, particularly with regards to the gut (Kantele, et al. 2005; Kantele, et al. 1999; Lindholm, et al. 2004; Quiding-Jarbrink, et al. 1997; Rydstrom and Wick 2007). The migration of antigen-sensitised cells is preferentially determined by the concurrent expression of homing-specific adhesion molecules in the tissue endothelium, and the specific receptors (integrins) expressed on the activated lymphoid cells. Oral (intestinal) mucosal exposure to antigen seems to stimulate expression of $\alpha_4\beta_7$ integrins, and intranasal immunisation has been shown to induce expression of L-selectin as well as $\alpha_4\beta_7$ integrins (Quiding-Jarbrink, et al. 1997). However, it is still unknown whether this homing of specific cells is mediated by altered cell adhesion molecule expression after intranasal vaccination in the upper respiratory tract lymphoid tissues (i.e. NALT and CLN). In this present study I show that cell adhesion molecule expression of MAdCAM-1, PNAd, ICAM-1 and VCAM-1 is both increased, and more widely distributed, early after intranasal immunisation in both the NALT and CLN.

It has been demonstrated that both NALT and CLN express MAdCAM-1 and PNAd on their HEV (Csencsits, et al. 1999; Csencsits, et al. 2002). I observed expression of both these addressins on HEV in naïve mice confirming these previous studies. In addition, I found that as early as 5 hours post intranasal immunisation, expression of MAdCAM-1 and PNAd was further upregulated on HEV in both tissues. The changes in CAM expression between naïve and immunised mice was by visual assessment only. This increased MAdCAM-1 expression is similar to that observed in other studies after either oral immunisation or challenge in the gut mucosa (Lindholm, et al. 2004; Quiding-Jarbrink, et al. 1997; Rydstrom and Wick 2007). Significant induction of PNAd also takes place during gut inflammation, allowing the entrance of leukocytes to inflamed mucosal sites (Salmi, et al. 1994; Salmi, et al. 1993). It is likely that the increased MAdCAM-1 and PNAd expression in the respiratory mucosa after immunisation is one of the mechanisms controlling the recruitment of leukocytes to the site of inflammation. This is supported by studies that show expression of the homing receptors $\alpha_4\beta_7$ and L-selectin on leukocytes that are found in both the NALT

and CLN (Csencsits, et al. 2002). The presence of MAdCAM-1 staining within the NALT and CLN may suggest a mechanism for the entrance of intestinal leukocytes, which have been shown to preferentially express $\alpha_4\beta_7$ and enter lymphoid tissues primarily through MADCAM-1-$\alpha_4\beta_7$ interactions (Bargatze, et al. 1995; Streeter, et al. 1988). Though the HEV of the NALT and CLN do express MAdCAM-1, most cell binding, as well as short- and long-term leukocyte homing, appears to be mediated through PNAd-L-selectin interactions (Csencsits, et al. 1999). In this present study I also observed that expression of PNAd is more abundant than MAdCAM-1 on HEV in both the NALT and CLN (particularly in CLN) of both naïve and immunised mice, again highlighting the importance of peripheral addressins in leukocyte trafficking to and from the nasal passages and LNs. As well as MAdCAM-1 expression on HEV, diffuse staining was also observed in both the NALT and CLN after intranasal immunisation. This expression appears to be on the surface cells and may be on infiltrating immune cells, or on existing cell populations within these lymphoid tissues. This diffuse MAdCAM-1 staining has been observed previously in both the NALT and CLN within B cell areas, as well as on FDC in the NALT. The fact that this diffuse MAdCAM-1 staining was only observed after intranasal immunisation supports other studies that have shown that addressin expression by interfollicular DC and macrophages is important in leukocyte recruitment and retention in LNs (Szabo, et al. 1997).

In addition to MAdCAM-1 and PNAd, I observed that both ICAM-1 and VCAM-1 were constitutively expressed in the normal NALT and CLN, supporting the findings of previous studies (Csencsits, et al. 1999; Csencsits, et al. 2002; Hussain, et al. 2001). I also found that expression was dramatically induced, following intranasal immunisation, on both vascular endothelium and cell surfaces. Other studies have also shown that upon inflammatory stimuli, both ICAM-1 and VCAM-1 become strongly up-regulated on endothelial cell lines (up to 40-fold), and are found in increased levels in a variety of inflamed tissues (Dustin, et al. 1986; Henninger, et al. 1997; Meager 1999). Leukocytes migrating into sites of both mucosal and systemic inflammation are also found to express the $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrins that direct adherence to endothelial cells expressing ICAM-1 and VCAM-1, respectively (Engelhardt, et al. 1994; Steffen, et al. 1996). It therefore appears that leukocyte trafficking, after

intranasal immunisation, to both the NALT and CLN may also depend on the interactions of $\alpha_L\beta_2$ and $\alpha_4\beta_1$ cells with vascular ICAM-1 and/or VCAM-1 in addition to MAdCAM-1 and PNAd. It is interesting to note that the same molecules, i.e. ICAM-1 and VCAM-1, are involved in trafficking in the genitor-urinary tract, which may explain why high levels of antigen-specific immune responses are induced in the genital tract after nasal immunisation (Perry, et al. 1998). In addition to ICAM-1/VCAM-1 staining on vascular endothelium, I also observed diffuse staining on the surface of cells, particularly after intranasal immunisation in both the NALT and CLN. The adhesion molecules ICAM-1 and VCAM-1 are expressed basally at low levels on the surface of macrophages and DC, and can be induced to high levels after antigenic stimulation (Colic and Drabek 1991; Perretti, et al. 1996; Rice, et al. 1991; Yasukawa, et al. 1997). It has been suggested that leukocytes migrate, within tonsils in humans and NALT in rodents, via B cells binding directly to FDC through $\alpha_4\beta_1$/VCAM-1 and $\alpha_L\beta_2$/ICAM-1 interactions. This prevents apoptosis of B cells and is thus crucial for their fate (Csencsits, et al. 1999; Koopman, et al. 1994; Koopman, et al. 1991). A similar adhesion mechanism using $\alpha_4\beta_1$/VCAM-1 interactions has been demonstrated to be involved in the migration of T cells into GC, and this may play a role in B cell retention within GC (Schriever, et al. 1997). In fact, increased expression of VCAM-1 on FDC has been found to coincide with GC formation in both the spleen and peripheral LNs (Balogh, et al. 2002). However, VCAM-1, as a counter-receptor for both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ heterodimers, suggests that the $\alpha_4\beta_7$ leukocytes might also bind in GC through this route as well (Ruegg, et al. 1992). Interaction of $\alpha_L\beta_2$/ICAM-1 also facilitates membrane antigen recognition by B cells (Carrasco, et al. 2004). In addition, ICAM-1 binding to $\alpha_L\beta_2$ on T cells also forms part of the stable T cell-APC immunological synapse, which is essential for antigen presentation (Dustin and Shaw 1999; Monks, et al. 1998). This increased expression of VCAM-1 and ICAM-1 on the surface of leukocytes indicates a possible role in early recruitment and retention of immune cells, enabling increased antigen presentation, and the consequent induction of antigen specific immune responses in the NALT and CLN after intranasal immunisation.

Finally, our results indicate that there are differences in the kinetics of cell adhesion molecule expression early after intranasal immunisation. This data presents interesting implications for the complex nature of immune cell trafficking in both the NALT and CLN and the induction of immune responses. Based on what is known about the role of these ligands and leukocyte receptors in leukocyte extravasation, I expect that induction of the addressins precedes cell entry into both the NALT and CLN. However, as early as 5 hours post intranasal immunisation I could not separate the onset of cell influx with the induction of cell adhesion molecules, suggesting that these two events are very closely linked. In fact, it has been shown that soluble factors released by activated macrophages or lymphocytes may regulate PNAd and MAdCAM-1 expression on HEV in LNs (Hendriks, et al. 1987; Hendriks and Eestermans 1983; Mebius, et al. 1993; Panes and Granger 1998). Similarly, VCAM-1 expression on endothelial cells is induced by several inflammatory factors (Aplin, et al. 1998; Aplin, et al. 1999; Hanada and Yoshimura 2002). With these points in mind, it is notable that I observed increased expression of MAdCAM-1 and VCAM-1 as early as 5 hours in the NALT, but this was not seen in the CLN until 24 hours post immunisation. This may be due, in part, to the earlier activation of leukocytes in the NALT, the site of antigen administration, before these cells drain into the CLN, therefore inducing immune responses slightly later. In addition, the low frequency of L-selectin ligand (PNAd)-expressing vessels 72 hours after intranasal immunisation suggests the possibility that other adhesion molecules, including MAdCAM-1, ICAM-1 and VCAM-1, could mediate later stages of the inflammation. These results are similar to studies performed using the radiolabeled mAb technique, which have revealed that inflammatory stimuli (e.g., cytokines, endotoxin) elicit a time-dependent increase in the expression of all endothelial CAMs in different tissues of the digestive system (Eppihimer, et al. 1996; Henninger, et al. 1997; Panes and Granger 1998).

The work presented here suggests that the recruitment and retention of immune cells early after intranasal immunisation relies in part on modulation of expression of the cell adhesion molecules; PNAd, MAdCAM-1, ICAM-1 and VCAM-1. The expression profile observed indicates the complex nature of leukocyte trafficking after intranasal administration of antigen, and may provide insights into strategies to regulate effector cell populations after mucosal vaccination.

4.4 Germinal centre formation early after intranasal immunisation

4.4.1 Introduction

After antigen challenge, GC are formed within the secondary lymphoid organs (LNs, PP, spleen or tonsils/NALT). GC are the site where B cells proliferate and differentiate into Ig-producing plasma cells, generate high-affinity antigen-specific B-cell receptors by affinity maturation, and differentiate into memory cells (Kelsoe 1996; Kosco-Vilbois, et al. 1997a; Kosco-Vilbois, et al. 1997b; Thorbecke, et al. 1994). The major cell populations encountered in GC are centroblasts and centrocytes, B cells that can be labelled with peanut agglutinin (PNA) (Rose, et al. 1980). Within GC, PNA^+ centrocytes are associated with FDC and T cells (Kosco-Vilbois, et al. 1997b). These FDC retain native antigen and present it to centrocytes, resulting in selection and activation of antigen-specific B cells with high-affinity Ig receptors. Outside the GC, B cells with receptors of high affinity differentiate to plasma cells or memory cells (Kelsoe 1996).

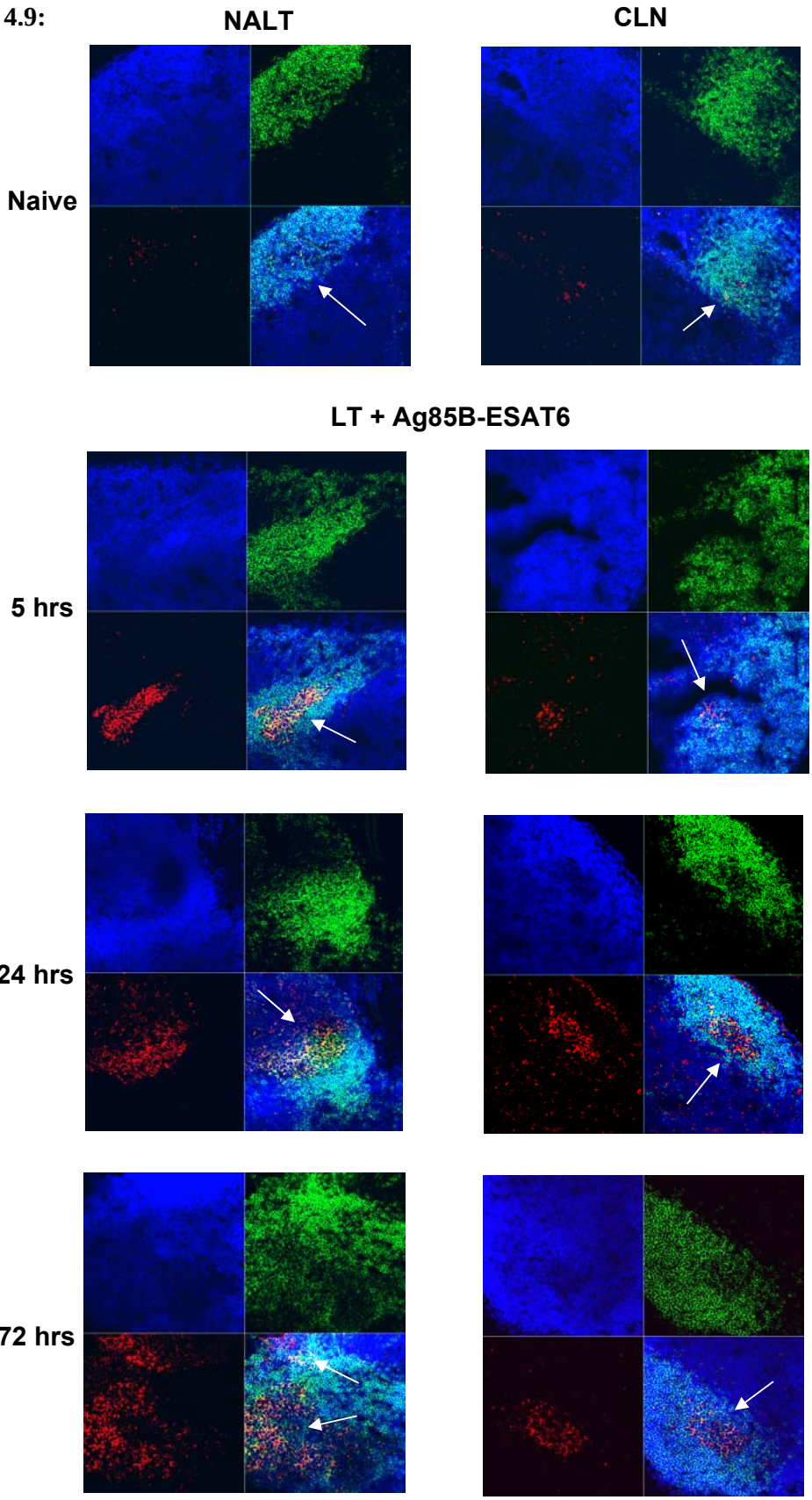
4.4.2 Results

To assess the formation of GC occurring in situ early after intranasal immunisation, cryosections were prepared from both the NALT and CLN, 5, 24 and 72 hours post immunisation. As early as 5 hours post intranasal immunisation, GC were seen in both the NALT and CLN of immunised mice and were still present at completion of the experiment on day 3, as indicated by PNA-binding cells (in red), which are representative of GC B cells (Figure 4.9). At the 5 hour time-point, at least 1 GC was seen per CLN/NALT, whereas those from unvaccinated animals displayed minimally reactive or no GC (Figures 4.9). By 24 hours, vaccinated animals contained a larger number of expanded GC (approximately 2 per tissue) in both lymphoid tissues (Figures 4.9). Finally, 3 days after intranasal immunisation GC were still found to be present, both in larger size and number (3 per NALT and 3-4 per CLN) (Figures 4.9).

Figure 4.9: Immunofluorescence evaluation of GC in both CLN and NALT from immunised Balb/c mice.

Balb/c mice were immunised with either PBS (naïve) or 1µg LT + 25µg Ag85B-ESAT6. Figure 4.9 reveals the presence of GC in the NALT and the CLN of immunised mice at 5, 24 and 72 hours post intranasal immunisation. Triple immunolabeling of B220-positive cells (green), PNA binding cells (red) and cell nuclei by Hoechst (blue) is shown for all images. White arrows indicate GC, or lack of in naïve animals. There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.9: GC



4.4.3 Discussion

Antigenic stimulation induces the formation of GC in lymphoid tissue. GC represent critical sites within organised lymphoid tissues, in which B cell responses to antigen are amplified and refined in specificity (Guzman-Rojas, et al. 2002). Mucosal but not peripheral, B cells are driven into GC through interaction of innate immune receptors with microbial antigens independent of B cell receptor specificity. This process requires T cells, and recruitment depends on innate immune mechanisms (Casola, et al. 2004). In this present study, I observed the formation of $PNA^{+}B220^{+}$ cells within B cell areas as early as 5 hours post intranasal immunisation. These GC continued to grow and expand in size and number up until the end of the experiment (72 hours) in both the NALT and CLN. This provides direct evidence for the induction of a regional response as GC reactions were induced in both these lymphoid tissues following immunisation. Previous studies have also shown GC formation in the NALT and CLN, but not until 3 days or later after intranasal immunisation or challenge (Asakura, et al. 1998; Eriksson, et al. 2004; Shimoda, et al. 2001; Zuercher, et al. 2002). However, Savransky et al have shown GC formation as early as 6 hours post intranasal immunisation with the Staphylococcal Enterotoxin B in the both the lungs and the spleen (Savransky, et al. 2003). In addition, 72 hours post immunisation, the PNA^{+} cells appeared to be moving away from the B cell areas, particularly within the NALT. Previous studies have shown that more B cells expressing switched transcripts are actually found outside GC, before seeding to other mucosal areas (Kelsoe 1996). This suggests that maturing and differentiating plasma cells are already moving away from B cell follicles at this time-point. The B7 molecules present on APC and B cells play a critical role in both isotype switching and the formation of GC (Han, et al. 1995). Previous studies have shown that *in vivo* adjuvant properties of LT appeared to be mediated by both B7-1 and B7-2, possibly explaining why I see development of GC at these early time-points (Martin, et al. 2002; Petrovska, et al. 2003). Formation of these GC in both the NALT and the CLN highlights the possible mechanisms responsible for induction of humoral immunity following intranasal immunisation.

4.5 Summary discussion

The work presented in this chapter provides insights into the complex nature of innate immune responses induced following intranasal immunisation within the upper respiratory tract. I show that a significant influx and efflux of cells occurs in both the NALT and CLN as early as 5 hour post immunisation, which correlates with the increased expression of cell adhesion molecules also at this time. Examination of a number of activation markers indicates that the innate cell types examined (i.e. DC, macrophages, NKC and neutrophils) are stimulated. These activated cells may be releasing pro-inflammatory cytokines that are enabling vascular endothelial cells to increase their expression of adhesion molecules such as PNAd, MAdCAM-1, ICAM-1 and VCAM-1. In addition, antigen-derived stimuli combined with host cell factors, such as cytokines and chemokines, may recruit and activate additional cells. Macrophages, DC, neutrophils and NKC are probably part of the first wave of direct responders, since I observed increases and decreases of these cell populations as early as 5 hours within the NALT and CLN. Leukocyte homing to both normal tissues and sites of inflammation is, in part, regulated by differential expression of cell surface homing receptors and their selective interactions with tissue selective vascular adhesion molecules at sites of leukocyte recruitment. The differential expression of these cell adhesion molecules shortly after intranasal immunisation may be consistent with the homing of leukocytes into and from both the NALT and CLN from various sites in a time-dependent manner. In order to characterise this migration more fully I can examine the specific integrins on the cells present in these tissues to determine their origin. Leukocytes present in both the NALT and CLN were found to move from peripheral areas into B and T cell areas after intranasal immunisation, suggesting their involvement in the initiation of adaptive immune cell responses; this was especially true for both DC and macrophages. Increased expression of MHC II and movement of these cell types into parafollicular regions within the NALT and CLN suggests their interaction with $CD4^+$ T_H cells. In addition, I observed increased expression of VCAM-1 and ICAM-1 on APC cells, which may correlate with the generation of GC within both lymphoid tissues and subsequent antigen-specific antibody responses. Antigen administered intranasally would not be expected to persist for very long as uptake by APC would result in its rapid breakdown ready for presentation to T cells. In fact, when I probed both the NALT and CLN with mAb against ESAT-6 I did not

observe any protein within the tissues as early as 5 hours, therefore supporting this hypothesis.

As already postulated in the Chapters 3, the differences observed in immune responses between animals immunised with adjuvant alone or adjuvant plus antigen may be down to the differences in the amount of antigen the mouse is receiving rather than a modulating effect by the model antigen (i.e. 1µg LT compared to 1µg LT plus 25µg Ag85B-ESAT6). These data suggest that the variation in innate responses we observe in mice immunised with adjuvant alone compared to those animals receiving adjuvant plus antigen may, to some extent, be influenced by the differential amplification of early innate factors through antigen dose. As already discussed the adjuvant LT is known to modulate innate responses, particular through its interaction and activation of professional APC. However, we cannot exclude the possibility that the fusion antigen is also modulating these early immune responses. Recently, Latchumanan and colleagues observed that when bone marrow cells are incubated with either Ag85B or ESAT-6 they differentiate into DC-like APC, with high expression of surface MHC and co-stimulatory molecules (Latchumanan, et al. 2005; Latchumanan, et al. 2002). In addition, Olsen et al have shown that ESAT-6 can induce the production of IFN-γ from bovine NKC with the response mediated through stimulation of APC (Olsen, et al. 2005). This indicates that both proteins may actually have adjuvant properties similar to those seen in LT. Ouhara *et al*, have reported that the outer membrane protein 100 of *Actinobacillus actinomycetemcomitans* binds to fibronectin inducing antimicrobial peptides production via the MAP kinase pathway (Ouhara, et al. 2006). As already discussed, Ag85B has previously been shown to bind selectively to fibronectin. It is tempting to speculate that Ag85B may also induce antimicrobial peptide production through fibronectin binding interactions. Antimicrobial peptides are important components of innate immune defence. They exhibit large cationic patches on their molecular surface which enables them to depolarise and/or pierce bacterial cell membranes. In addition to their innate antibacterial role, they have been demonstrated to have a number of immunomodulatory functions, including the ability to alter host gene expression, act as chemokines and/or induce chemokine production, promote wound healing, and modulate the responses of DC and lymphocytes (reviewed in (Hancock and Sahl 2006). This work suggests that if Ag85B could promote antimicrobial peptide

production through fibronectin binding and both Ag85B and ESAT6 can activate DC, then it is possible that the fusion protein is also modulating innate immune responses along with LT. However, as already discussed ESAT-6 may also attenuate the innate immune response by dampening production of IL-12p40, TNF-α and NO (Stanley, et al. 2003). In addition some very recent work has shown that ESAT-6 inhibits activation of transcription factor NF-κB and interferon regulatory factors (IRFs) after TLR signaling (Pathak, et al. 2007). Hence, it may be the differences we are seeing in early immune responses in those animals receiving adjuvant and the fusion protein is actually a direct result of the dampening down of TLR induced innate immune responses. These data therefore provide some other possible elucidations as to why we see variations in early immune responses between adjuvant and adjuvant plus antigen immunised mice.

It is hoped that this current research will provide new insights into the function of upper respiratory tract mucosal tissues and the interplay of innate and adaptive immune responses that results in immune protection at mucosal surfaces. Characterisation of these innate immune response mechanisms involved early after intranasal immunisation may help clarify the concepts and provide the tools that are needed to exploit the full potential of mucosal vaccines

5 Natural killer cell depletion influences antigen specific cytokine and antibody responses after intranasal immunisation

5.1 Introduction

NKC, a component of the innate immune system, mediate cellular cytotoxicity and produce chemokines and inflammatory cytokines, such as IFN-γ and TNF-α (Anegon, et al. 1988; Arase, et al. 1995; Arase, et al. 1996; Kim, et al. 2000; Kim and Yokoyama 1998; Ortaldo and Young 2003; Trinchieri 1989). They are an important constituent of innate resistance to viruses, bacteria and certain parasites as well as providing immune surveillance against the development of tumours (Biron, et al. 1989; French and Yokoyama 2003; Karre, et al. 1986; Lieke, et al. 2006; Lodoen and Lanier 2006; Roetynck, et al. 2006; Schafer and Eisenstein 1992; Storkus, et al. 1989; Yang, et al. 2006). NKC also interface with adaptive immunity by stimulating DC and by promoting T cell responses. NKC are bone marrow-derived granular lymphocytes, distinct from T and B cells that are widespread throughout the body being present in both lymphoid organs and non-lymphoid peripheral tissues. For a comprehensive review see (Hamerman, et al. 2005). NKC express a range of distinct receptors on their surface that are involved in recognising and responding to pathogens. There are two general types of NKC receptors, i.e., inhibitory and activation receptors (Lanier 1998). NKC express inhibitory receptors that monitor for normal expression of host proteins, and their function underpins the 'missing self' hypothesis. The best characterised are those receptors that bind classical and non-classical MHC class I, the expression of which is often targeted by pathogens or malignancies to subvert immune responses by T cells (Lanier 2005). The term 'NKC activating receptor' generally refers to those receptors that trigger release of cytolytic granules and typically induce cytokine production. Cellular ligands for NKC activating receptors are normally absent on healthy cells, but can be induced in response to cellular stress, such as infection. In addition, NKC express receptors that are a form of pathogen-associated pattern recognition system. The mouse Ly49H NKC receptor has been found to bind to a product *m157* of mouse cytomegalovirus (MCMV), and activation through these receptor ligand interactions enable NKC cells to limit early-stages of

MCMV infection and to undergo considerable proliferation (Arase, et al. 2002; Smith, et al. 2002). NKC have also been reported to express most of the known Toll-like receptors (TLR), including TLR3, TLR5 and TLR9. However, the role and the expression pattern of TLR on NKC has not been well characterised (Yokoyama and Scalzo 2002). Activated NKC exhibit rapid secretion of several cytokines, including IFN- γ, as well as other immunoregulatory mediators, including TGF-β, TNF-α, TNF-β, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1α, IL-1, IL-2, IL-3, IL-5, IL-8 and IL-10 (Biron 1997; Scharton-Kersten and Sher 1997; Warren, et al. 1995). Additionally, infected or activated macrophages and DC produce cytokines and chemokines such as IFN-α/β, IL-12, IL-15 and IL-18 that stimulate NKC to rapidly produce other cytokines (Andrews, et al. 2005; Biron, et al. 1999; Walzer, et al. 2005; Zitvogel, et al. 2006). Recent work suggests that crosstalk between DC and NKC may influence the adaptive immune response (Ferlazzo, et al. 2003; Kalinski, et al. 2005; Walzer, et al. 2005; Zitvogel, et al. 2006). Activation of NKC *in vivo* may be in part due to receptor-ligand interactions with DC, as well as DC-derived cytokines. Communication between these two cell types is not just unidirectional, as NKC can also stimulate maturation of DC by up-regulation of MHC complexes and co-stimulatory molecules, and therefore may represent a key mechanism to bridge NKC response to the stimulation of T cell responses (see Figure 5.1) (Raulet 2004).

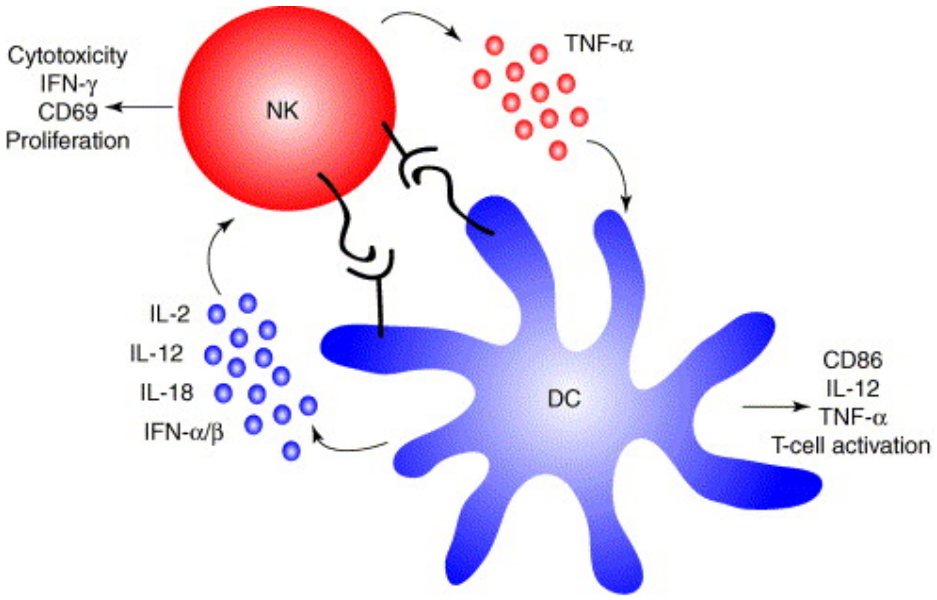


Figure 5.1: Crosstalk between NKC and DC.

NKC and DC have the ability to reciprocally activate one-another, both *in vitro* and *in vivo*. This crosstalk includes cell contact involving unknown receptor-ligand pairs and soluble mediators produced by the two cells. The cytokines, TNF-α, IL-2, IL-12, IL-18 and IFNs, have all been implicated in this process. The end result of these interactions is NKC activated for cytotoxicity, IFNγ production, and proliferation, and DC that have matured and are capable of cytokine production and T cell activation (Hamerman, et al. 2005).

Evidence from a number of studies have also suggested that rapid IFN-γ production by NKC may affect the characteristics of antigen-specific immune response, particularly by promoting T_H1 polarisation (Fearon and Locksley 1996; Korsgren, et al. 1999; Martin-Fontecha, et al. 2004; McKnight, et al. 1994; O'Leary, et al. 2006; Romagnani 1992; Scharton-Kersten and Scott 1995; Scharton and Scott 1993; Wang, et al. 1998). Collectively these data have led to a broadly based consensus that early NKC responses may have a profound effect on later adaptive immune responses. However, little is know about the role of NKC in induction of immune responses after mucosal administration of antigen. Previously I have shown that NKC are the most abundant innate cell type recruited to both the NALT and CLN early after intranasal immunisation. These $DX5^+$ cells were observed to express high levels of the markers CD69 and CD25, suggesting that these NKC were also activated. Given this influx of NKC post intranasal immunisation, I hypothesised that these innate immune cells may

be involved in processes that lead to antigen-specific immune responses, i.e. adaptive immunity. Specifically, this study examines whether NKC depleted mice have differences in both antigen-specific B and T cells responses through examination of antibody subtypes, their titres and cytokine production, when compared to control animals.

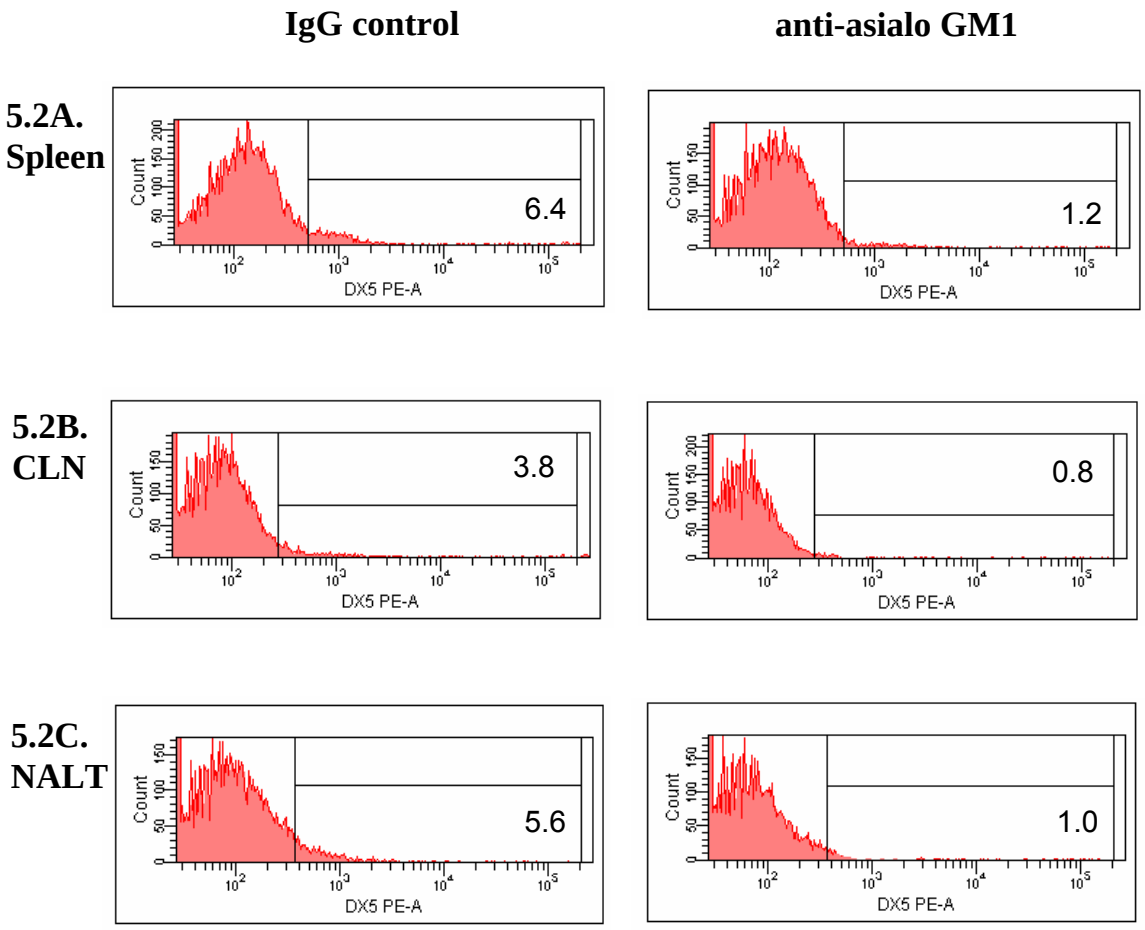
5.2 Results

5.2.1 Anti-Asialo GM1 treatment successfully depletes Balb/c mice of NKC

In order to study the effects of NKC on the induction of antigen-specific immune responses I employed chronic administration of polyclonal anti-asialo GM1 antibody to Balb/c mice. Virtually all murine NKC express this marker and, as widely demonstrated, this approach effectively depletes functional and phenotypic NKC activity *in vivo* (Yu, et al. 1992). In addition, this antibody does not activate NKC when binding. Controls were treated with an equivalent concentration of normal rabbit IgG. NKC depletion was effective as shown by histogram plots of $DX5^+$ cells within the spleens, CLN and NALT of antibody depleted and control mice (Figure 5.2A, B and C). This marker is also expressed at low levels on murine macrophages and NK T cells, so in order to make sure these cell types were not being reduced I examined the percentages of $F4/80^+$ cells and $\alpha\beta TCR^+/DX5^+$ cells within spleens of naive mice. I did not observe any significant reduction ($p > 0.05$) in these cells types in depleted mice when compared to control animals (Figure 5.3). To ensure NKC depletion, antibody treatment was given at 3-4 day intervals, beginning 7 days before intranasal immunisation and continuing until sacrifice. I also selected mice from within each cohort at the end of the study (day 21) to confirm that NKC remained at undetectable levels (Figures 5.2D, E and F).

Figure 5.2: Impact of *in vivo* administration of anti-asialo GM1 on NKC percentages in Balb/c mice.

Balb/c mice were treated with 50µg anti-asialo GM1 antibody or 50µg rabbit IgG antibody and spleens, CLN and NALT were assessed for $DX5^+$ cells (NKC) after 2 doses of antibody (day 0) and after 8 doses of antibody (day 21) by flow cytometry. Figure 5.2A represent naïve spleens from IgG control and anti-asialo GM1 depleted mice at beginning of experiment (day 0). Figure 5.2B shows the CLN and C represents the NALT also at beginning of experiment (day 0). Figures 5.2D-F represents naive spleen, CLN and NALT, but at the end of the experiment (day 21). Histogram plots represent the $DX5^+$ population within each tissue and the number on the right hand side indicates the total percentages present. Plots shown are from five individual representative mice, and the mean values are indicated.



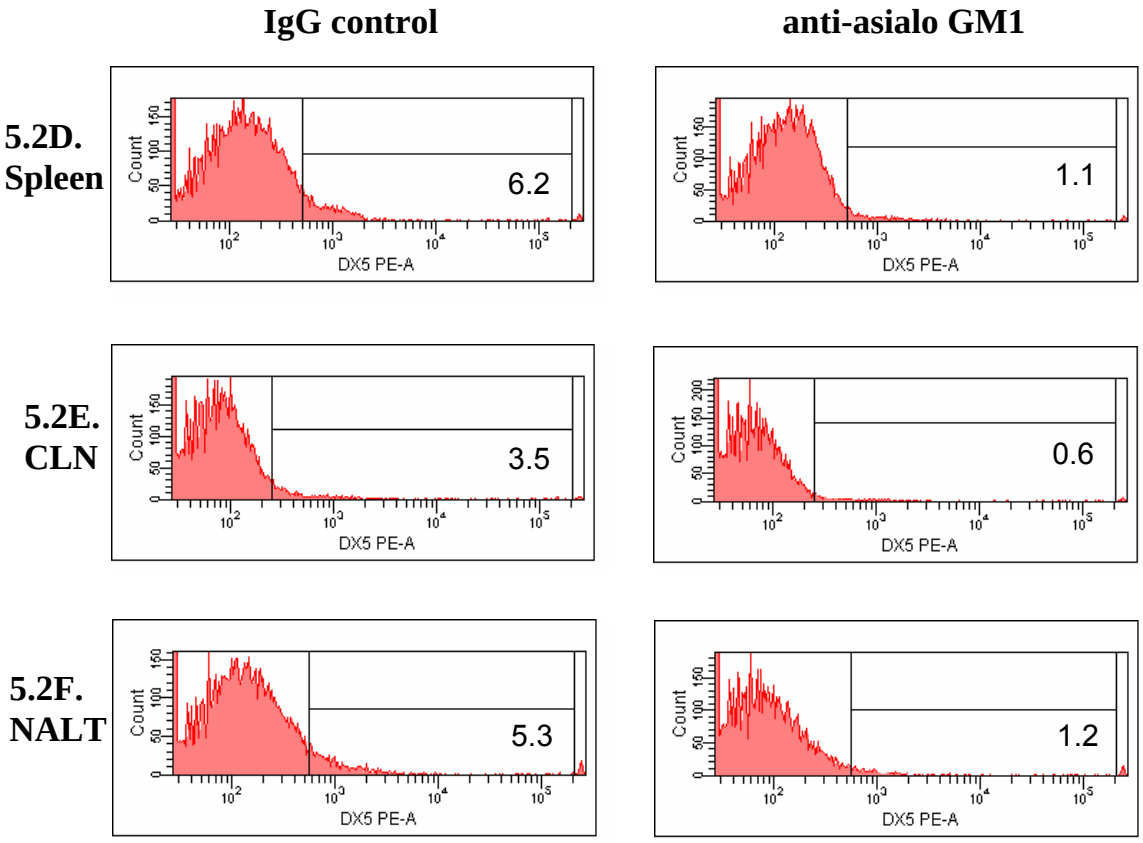
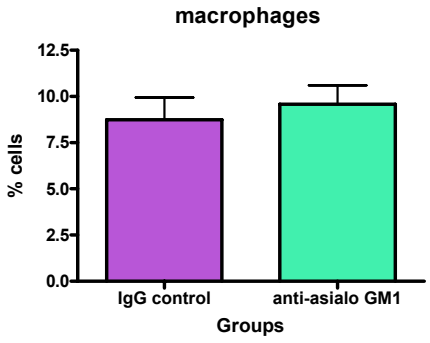

IgG control
anti-asialo GM1
5.2D. Spleen
5.2E. CLN
5.2F. NALT
Count
DX5 PE-A
6.2
1.1
3.5
0.6
5.3
1.2

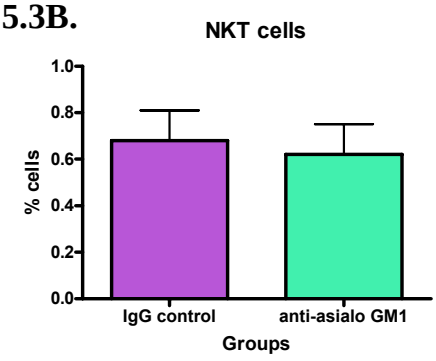
Figure 5.3: Percentages of macrophages and NKT cells in naïve normal IgG and anti-asialo GM1 treated mice.

Balb/c mice were treated with 50µg anti-asialo GM1 antibody or 50µg rabbit IgG antibody and spleens were assessed for $F4/80^+$ macrophages cells and $\alpha\beta TCR^+/DX5^+$ cells after 2 doses of antibody by flow cytometry. Figure 5.3A represent percentages of $F4/80^+$ cells from naïve spleens of IgG control and anti-asialo GM1 depleted mice. Figure 5.3B shows $\alpha\beta TCR^+/DX5^+$ cells from naïve spleens of IgG control and anti-asialo GM1 treated mice. Columns represent the mean (± SD) percentages of cells from five animals per group.

5.3A.



5.3B.



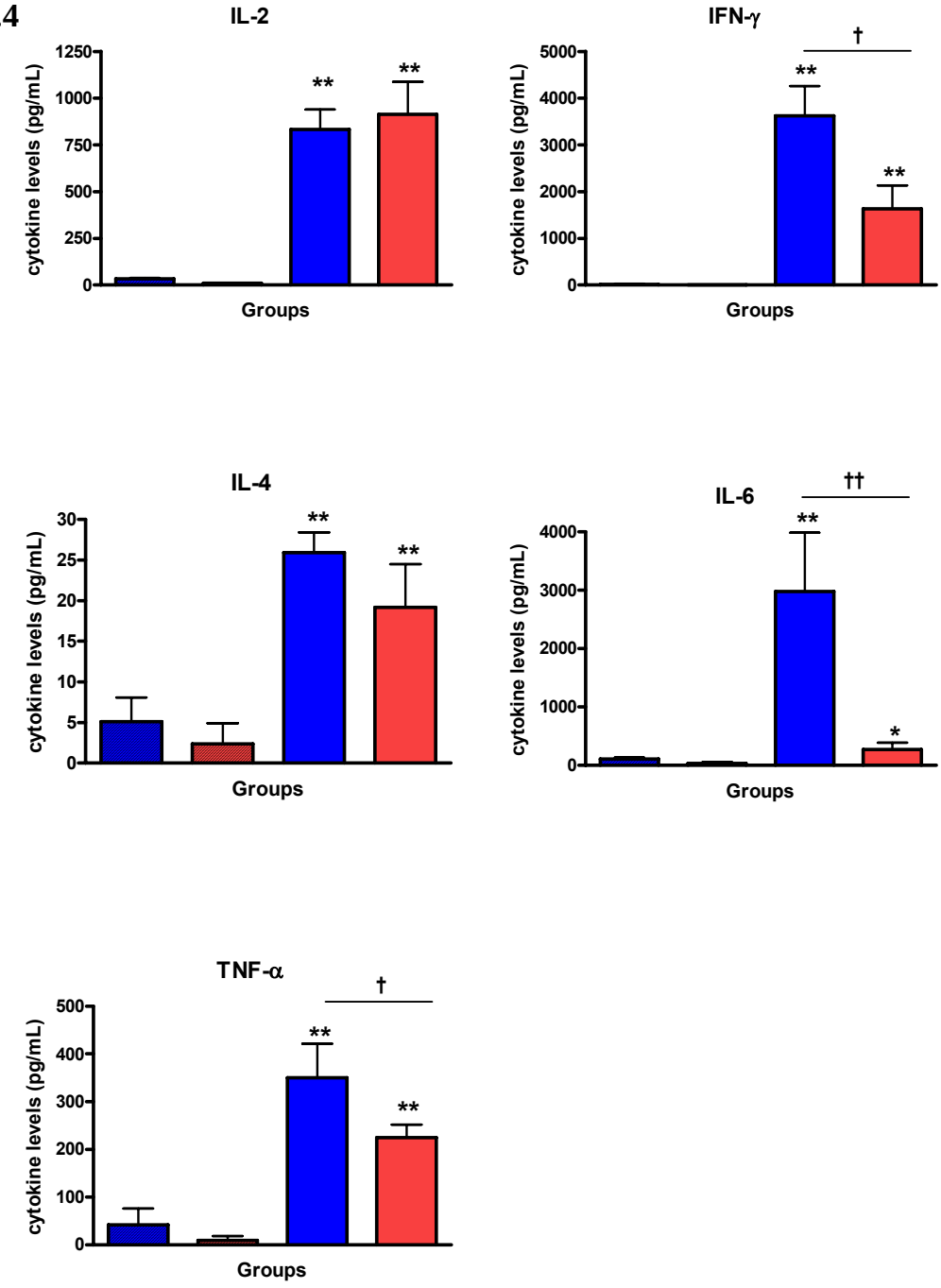
5.2.2 *In vivo* depletion of NKC affects induction of antigen-specific cytokine responses

Balb/c mice treated with anti-asialo GM1 and controls treated with an equivalent concentration of normal rabbit IgG were immunised with adjuvant and Ag85B-ESAT6 to assess the intensity of T_H1 and T_H2 associated cytokine synthesis (IL-2, IFN-γ, TNF-α, IL-4 and IL-6) As shown in Figure 5.4 all immunised mice had significantly higher levels ($p < 0.05$) of each cytokine when compared to their controls, i.e. naïve IgG control mice compared to immunised IgG control mice and naïve (PBS immunised) NKC depleted mice compared to adjuvant and antigen immunised NKC depleted mice. When IgG control immunised mice were compared to anti-asialo GM1 treated immunised mice, both IL-2 and IL-4 levels were found not to be significantly different ($p > 0.05$) between these cohorts. However, IFN-γ, TNF-α and IL-6 were all found to be significantly lower in immunised NKC depleted mice when compared to IgG control immunised animals ($p < 0.05$), with the reduction in IL-6 levels being the most significant ($p < 0.01$ vs. $p < 0.05$).

Figure 5.4: Anti-asialo GM1 antibody treatment alters the induction of antigen-specific cytokines.

Balb/c mice were treated with anti-asialo GM1 or normal rabbit IgG on days -7, -4, -2 then intranasally immunised with 1µg LT and 25µg Ag85B-ESAT6 or PBS on day 0. Mice were further treated ever 3-4 days, before sacrifice on day 21 when spleens were removed for T-cell assays. Cytokine responses were measured upon *in vitro* stimulation with Ag85B-ESAT6 (5µg/mL) for 36-42 hours. Cells were also stimulated with conA (positive control) and $RPMI^{+}$ media (negative control) (data not shown). Columns represent the mean (± SD) stimulation indices of splenocytes from five animals per group. The differences in cytokine levels between either control, or NKC depleted naïve and immunised animals is indicated by *, $p < 0.05$; and **, $p < 0.01$ with differences in immunised IgG control or anti-asialo GM1 treated animals shown by †, $p < 0.05$ and ††, $p < 0.01$ using a Kruskal-Wallis test followed by Dunn's Multiple Comparison test. The sensitivities of the CBA was > 1pg/mL for each cytokine. Blue indicates rabbit IgG control treated mice and red shows anti-asialo GM1 treated animals with diagonal lines and block colours representing naïve and immunised groups, respectively.

5.4


IL-2
cytokine levels (pg/mL)
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1000
750
500
250
0
**
**
Groups
IFN-γ
cytokine levels (pg/mL)
5000
4000
3000
2000
1000
0
†
**
**
Groups
IL-4
cytokine levels (pg/mL)
30
25
20
15
10
5
0
**
**
Groups
IL-6
cytokine levels (pg/mL)
4000
3000
2000
1000
0
††
**
*
Groups
TNF-α
cytokine levels (pg/mL)
500
400
300
200
100
0
†
**
**
Groups

5.2.3 Mice depleted of NKC have reduced antigen-specific antibody responses

To assess antigen-specific antibody production in both control (i.e. rabbit IgG treated mice) and anti-asialo GM1 treated immunised mice, I measured both LT and Ag85B-ESAT6 specific total Ig, IgG, IgG1, IgG2a, IgG2b, IgG3, IgA, IgE and IgM levels in plasma using ELISA at day 14 and day 21 after intranasal immunisation. Figure 5.5A shows that immunised anti-asialo GM1 treated animals have a modest decrease in total both LT and Ag85B-ESAT6-specific Ig titres when compared to immunised rabbit IgG treated control animals on day 14. However, this decrease was not found to be significant ($p > 0.05$). By 21 days post immunisation, it is clear that all mice have made a vigorous total Ig anti-LT and anti-Ag85B-ESAT6 response, regardless of whether they were treated with anti-asialo GM1 or normal rabbit IgG, suggesting that depletion of NKC does not affect total Ig responses at this later time-point (Figure 5.5B).

I also did not observe a difference ($p > 0.05$) in Ag85B-ESAT6-specific IgG responses at either time-point between immunised rabbit IgG treated control mice or anti-asialo GM1 treated mice (Figure 5.5A and B). Examination of the IgG subtype titres, IgG1 and IgG2a, showed that NKC depleted mice were able to mount an apparently normal IgG1 response to Ag85B-ESAT6, whereas I could not detect serum IgG2a anti-Ag85B-ESAT6 ($p = 0.008$) above pre-immune levels throughout the experiment in any of the anti-asialo GM1 treated mice (Figures 5.5A and B). Interestingly, mice treated with anti-asialo GM1 were not able to mount a detectable LT-specific IgG response, including IgG1 and IgG2a ($p = 0.008$), 14 days after intranasal immunisation (Figure 5.5A). However by day 21, I did observe anti-LT IgG and IgG1 responses in anti-asialo GM1 treated animals, but as observed for anti-Ag85B-ESAT6 response I did not observe any detectable IgG2a titres ($p = 0.008$) (Figure 5.5B). On day 21 after immunisation I had more serum as it was the end of the experiment, compared to the tail bleeds performed on day 14, therefore I decided to examine both antigen-specific IgG2b, IgG3, IgE, IgA and IgM responses in control and NKC depleted mice to further characterise the *in vivo* antibody responses. As with IgG2a responses I did not detect any anti-LT or anti-Ag85B-ESAT6 IgG2b responses ($p = 0.008$) in all of the animals treated with anti-asialo GM1 (Figure 5.5B). I

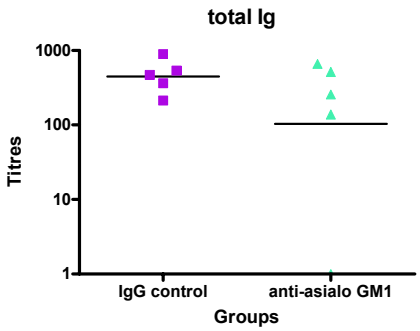
observed that control mice mounted both a modest IgE and IgM antigen-specific response after intranasal immunisation with LT and Ag85B-ESAT6 (Figure 5.5B). Conversely, I could not detect any anti-LT IgE and IgM responses in mice treated with anti-asialo GM1 ($p = 0.008$). Immunised control animals also had significantly higher ($p = 0.008$) IgE anti-Ag85B-ESAT6 titres compared to anti-asialo treated animals, though one of the five mice depleted of NKC did have detectable anti-Ag85B-ESAT6 IgE titres (Figure 5.5B). Notably, anti-Ag85B-ESAT6 IgM responses did not appear to be affected by anti-asialo GM1 treatment (Figure 5.5B). I could not detect any serum antigen-specific IgG3 or IgA in either rabbit IgG treated, or anti-asialo GM1 treated immunised animals (data not shown). Collectively, these data argue that anti-asialo GM1-mediated NKC depletion impacts the development of antigen specific immune response to antigens and adjuvant after intranasal immunisation.

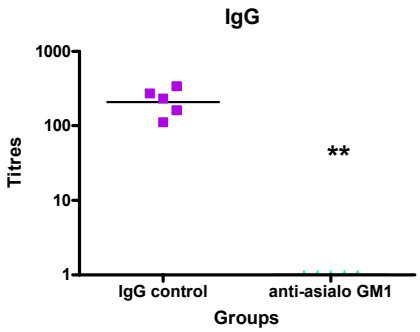
Figure 5.5: Anti-asialo GM1 treatment influences the development of antigen-specific antibody responses.

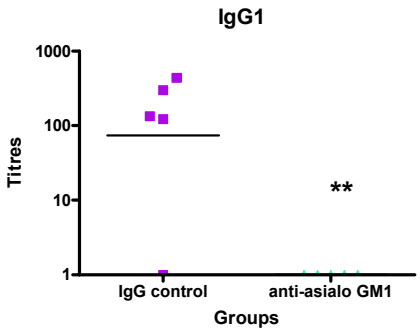
Balb/c mice were treated as described in Material and Methods (purple squares represent normal rabbit IgG treatment and green triangles indicate anti-asialo GM1 treatment) and intranasally immunised with Ag85B-ESAT6 and LT, then bled on days 14 and 21 for evaluation of primary antigen-specific total Ig, IgG, IgG1, IgG2a, IgE and IgM synthesis by ELISA. Figures 5.5A and B show anti-LT and anti-Ag85B-ESAT6 titres 14 and 21 days after immunisation, respectively. Data reflect five individual mice per group (minus background naïve animal titres) expressed as total antibody titre using a cut off of OD 0.3., with the black bar showing the geometric mean. Significant differences as calculated with the Mann-Whitney U-Test are marked with **; $p = 0.008$.

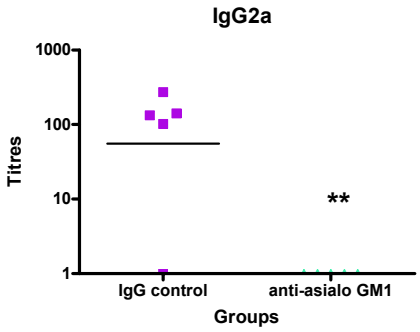
Day 14

5.5A. LT-specific titres

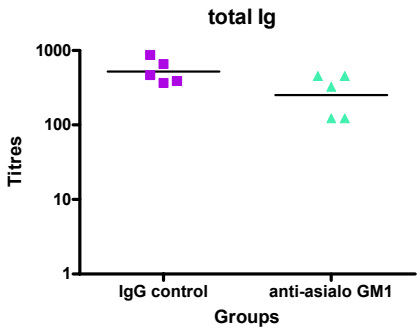


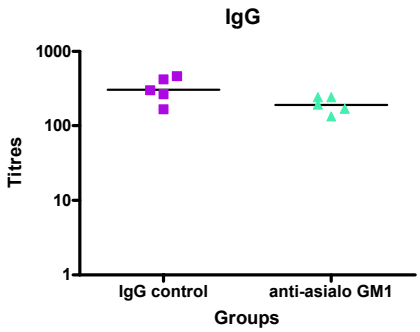


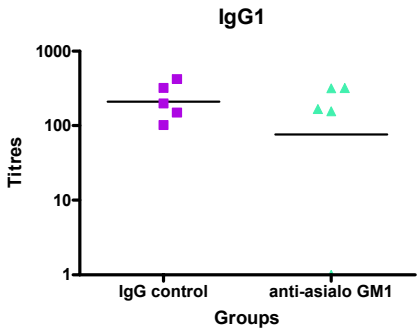


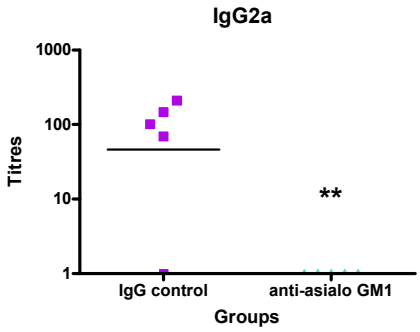


Ag85B-ESAT6-specific titres



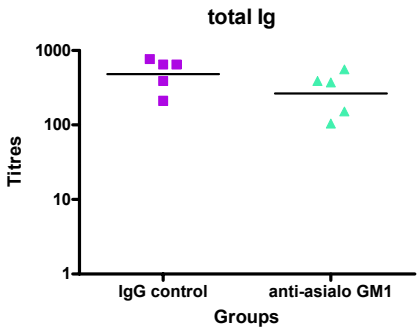


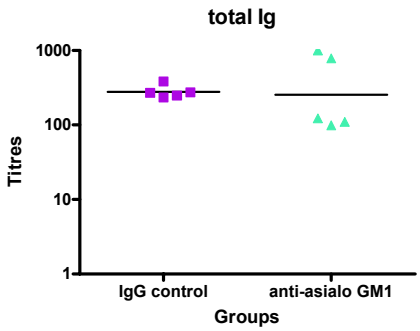


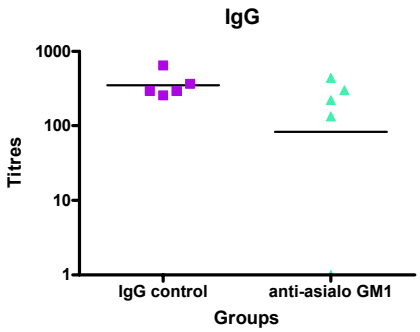


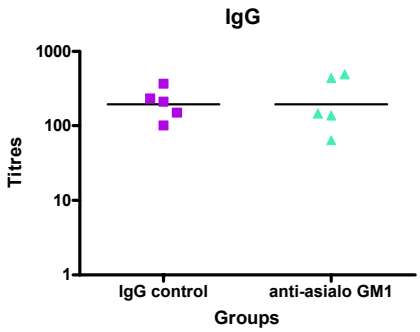
Day 21

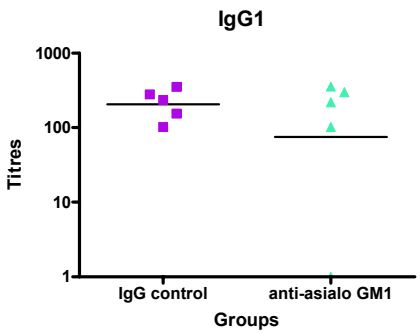
5.5B. LT-specific titres **Ag85B-ESAT6-specific titres**

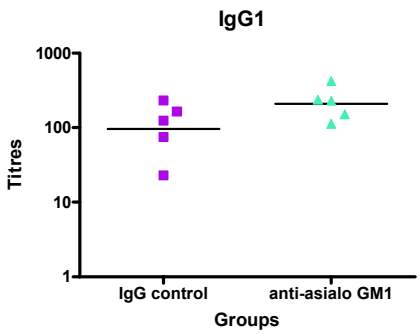


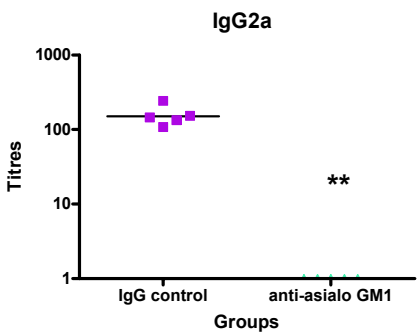


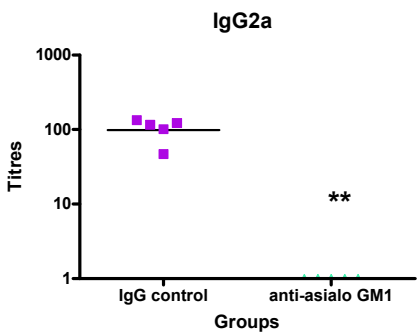






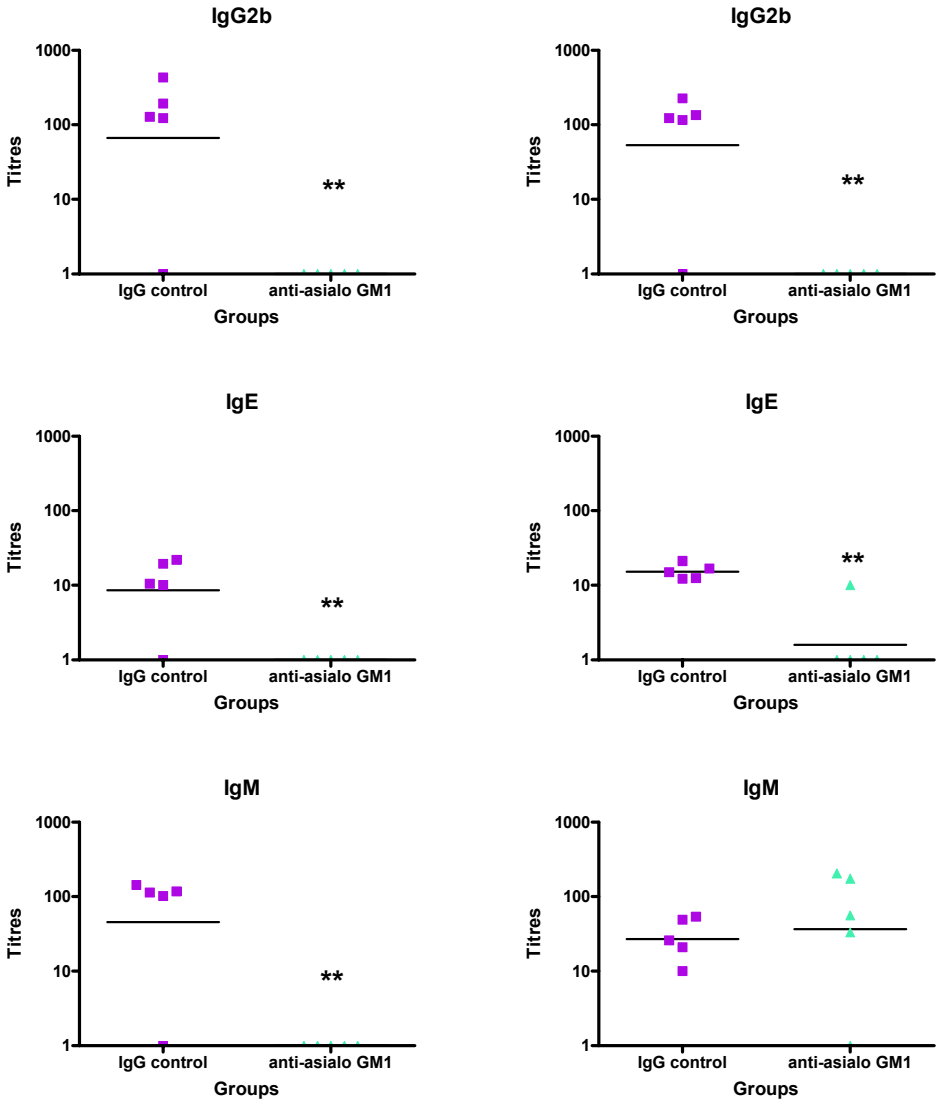






Day 21

5.5B. LT-specific titres Ag85B-ESAT6-specific titres



5.3 Discussion

In this study I provide evidence that NKC, a major arm of innate immunity, participate in the development of antigen-specific immune responses after mucosal immunisation. The requirement of NKC in the induction of adaptive immunity was reflected by the significantly decreased production of IFN-γ, TNF-α and IL-6 in anti-asialo GM1 treated mice when compared to IgG control animals. NKC were also involved in antigen–specific antibody responses, as mice depleted of NKC failed to mount both IgG2a and IgG2b responses. Interestingly, examination of specific adjuvant and antigen antibody responses gave diverse results, i.e. early (day 14) anti-LT antibody responses lacked any specific IgG titres. However, this was not the case for anti-Ag85B-ESAT6 responses as I observed both IgG and IgG1 at this time-point. Additionally, adjuvant antibody responses at day 21 failed to elicit any detectable IgM, whereas this was not found for antigen antibody responses.

Antibody depletion was utilised in this study for the removal of NKC as there are, as yet, no animal models in which NKC activities are genetically and selectively deficient. Most mutations inserted into mice to study the role of NKC affect the granules of many other cell types (e.g. CTL) and do not ablate other NKC activities such as cytokine production (Kim, et al. 2000). Most recently, some transgenic mice have been constructed that express the diphtheria toxin receptor under the control of the NKp46 promoter. Addition of diphtheria toxin to these mice results in death of only the cells expressing the toxin receptor. However, this data is still unpublished and the mice are not currently available. For *in vivo* depletion of NKC in Balb/c mice I utilised the anti-asialo GM1 antibody. This antibody has been employed in many previous NKC depletion experiments with effective results. However, asialo GM1 is also expressed at low levels on the surface of other cell types. NKC and NKT cells show different susceptibilities to *in vivo* treatment with anti-asialo GM1, possibly due to the difference in their expression of asialo GM1 (Sonoda, et al. 1999). By taking advantage of this difference, I could selectively deplete NKC without affecting NKT cells. Asialo GM1 is also expressed at low levels on the surface of macrophages, however this present study and previous ones have shown that treatment with anti-asialo GM1 does not affect macrophage numbers or their activity (Keller, et al. 1983). These data therefore suggest that only NKC are being depleted in mice and that the

observations noted throughout the study are a direct cause by the absence of NKC and not any other cell type.

The mechanisms by which NKC modulate adaptive immune responses are not fully understood. Factors that affect APC and determine the differentiation of naïve T cells into either T_H1 (IL-2, IFN-γ, TNF-α), or T_H2 (IL-4, IL-10) phenotypes and B cells into antibody producing cells would be expected to have an impact on the development of antigen specific immune responses after intranasal immunisation. NKC contribute to the innate immune defence through the early release of IFN-γ and TNF-α. Our results show that levels of IFN-γ and TNF-α from stimulated splenocytes were significantly lower in NKC depleted mice than in IgG control animals. These data indicate that NKC may be a major source of these cytokines after mucosal immunisation, and consequently may drive T_H1 adaptive immune responses. These data are also consistent with other findings that show that NKC depletion reduces IFN-γ production after systemic administration of antigen/pathogen (Korsgren, et al. 1999; Martin-Fontecha, et al. 2004; Sayeh, et al. 2004; Scharton and Scott 1993). As already discussed, NKC are also involved in crosstalk and activation of DC. TNF-α is also produced by activated DC, and this may also explain why I observed a significant decrease in production of this cytokine in anti-asialo GM1 treated animals. Studies have failed to detect IL-4 production from NKC, however it has been hypothesised that NKT cells may be an important source of IL-4 and, therefore in the development of T_H2 type immune response. In this study I showed that NKT cells were not depleted in anti-asialo GM1 treated animals, possibly explaining why I did not observe any significant decrease in antigen-specific IL-4 production from NKC depleted mice. No significant reduction in IL-4 levels was also observed by Satoskar and colleagues in NK^-T^+ mice. However, they also did not detect a significant reduction in IFN-γ levels (Satoskar, et al. 1999). IL-6 is an important mediator of the immune response during acute phases of stimulation. Levels of IL-6 were significantly higher in non-depleted controls than in anti-asialo GM1 treated animals after stimulation of splenocytes by antigen. As IL-6 is a cytokine produced by activated macrophages and DC, these data corroborate the involvement of NKC in promoting APC activation (Akira and Kishimoto 1996; Banchereau, et al. 2000; Grohmann, et al. 2001). IL-2 is produced by T cells and enhances NKC cytotoxicity and cytokine production as well as inducing proliferation of activated T and B cells.

In this present study I did not observe any differences in IL-2 production in anti-asialo GM1 treated mice compared to IgG control immunised mice. Consequently it appears that NKC do not affect T cell proliferation, whereas they do appear to modulate antigen-specific cytokine production.

NKC have also been shown to regulate B cell differentiation and antibody production. However, their role in regulating antibody secretion, both *in vitro* and *in vivo*, is uncertain as many studies contradict one another, some suggesting that NKC down-regulate antibody production, others proposing that they actually enhance it (Arai, et al. 1983; Jensen, et al. 2004; Khater, et al. 1986; Korsgren, et al. 1999; Robles and Pollack 1989; Satoskar, et al. 1999; Snapper, et al. 1994; Wang, et al. 1998; Wilder, et al. 1996; Yuan, et al. 2004). Many different methods were utilised in these studies to deplete NKC and this may, in part, explain these inconsistent findings. These problems may be overcome if genetically modified mice with a specific deletion of NKC can be constructed, however this has yet to be achieved. The fact that the IgG2a subclass of antibody was the one most affected by anti-asialo GM1 treatment is not surprising, given the fact that its production is sensitive to levels of IFN-γ in the milieu (Finkelman, et al. 1988; Snapper and Paul 1987; Stevens, et al. 1988). IFN-γ is one of the cytokines secreted by NKC, and IL-2 activated NKC have been shown *in vitro* to enhance IgG2a secretion by LPS-stimulated B cells, in a manner dependent upon IFN-γ secretion (Amigorena, et al. 1990; Michael, et al. 1991). In accordance with these observations I also noted a significant decrease in IFN-γ production from antigen stimulated splenocytes, and this may account for the lack of any detectable IgG2a titres. These results are consistent with other studies that have also shown that changes in IgG2a levels are NKC-dependent (Jensen, et al. 2004; Korsgren, et al. 1999; Satoskar, et al. 1999; Wilder, et al. 1996). In relation to the effects observed on IgG2b concentrations in NKC-depleted mice, it has been proposed that IFN-γ also regulates production of IgG2b to T cell dependent antigens (Snapper and Mond 1993). Again the significant reduction of IFN-γ observed in this present study may account for this disparity in IgG2b titres. I did not observe any significant differences in anti-Ag85B-ESAT6 IgG1 levels in anti-asialo GM1 treated animals when compared to control mice. This may correlate with our cytokine data, as IFN-γ has been shown to inhibit IgG1, whereas production of these antibody subtypes is supported by IL-4 (Finkelman, et al. 1988; Yoshimoto and Paul 1994). A number of

studies have also shown that NKC depletion does not affect antigen-specific IgG1 responses after challenge/immunisation (Satoskar, et al. 1999; Wilder, et al. 1996). Animals treated with anti-asialo GM1 were not able to mount either an anti-LT or anti-Ag85B-ESAT6 IgE response. As already described, I also observed a significant reduction in IL-6 production from antigen stimulated splenocytes. In addition to IL-4, IL-6 is also known to modulate IgE class-switching, and a reduction in IL-6 levels may explain why I do not see any significant IgE titres in NKC depleted mice (Maggi, et al. 1989). Korsgren et al show a reduction in antigen specific IgE responses in NK1.1 treated mice when using a murine model of allergic asthma (Korsgren, et al. 1999). In the immune response to a foreign protein, the first antibodies to appear are of the IgM class. Shaping of the secondary antibody repertoire is generated by means of class-switch recombination, which replaces IgM with other isotypes such as IgA, IgE and IgG. One reason I may not be detecting any LT-specific IgG responses at day 14 may be that it is too early for LT specific antibody class-switching, and therefore the main subtype present would be IgM. Unfortunately, I did not have enough serum at day 14 to perform these IgM ELISAs and therefore I cannot confirm this hypothesis. I did perform IgM ELISAs at day 21 and found that mice treated with anti-asialo GM1 were found to have similar levels of anti-Ag85B-ESAT6 IgM compared to control animals, but undetectable anti-LT IgM levels on day 21. The failure of mice depleted of NKC to mount an efficient anti-LT IgM response at this time-point may explain why I see no anti-LT IgG responses at day 14, i.e. most IgM has now been class-switched to other isotypes.

The differences in anti-adjuvant and anti-antigen antibody responses suggest that there is a mechanism of NKC involvement associated with activation events initiated by adjuvant. The actions of LT are multifactorial, involving both direct activation of various APC, as well as secondary activation via cytokine secretion (Martin, et al. 2002; Petrovska, et al. 2003). Our findings here indicate that NKC may be implicated in these processes. Part of the action of the adjuvant may be to activate the NKC directly, or may be the stimulation of NKC through other cell types e.g. DC. In fact, one study has shown that LT can bind directly to asialo GM1 therefore substantiating the theory that these adjuvants may be inducing direct effects on NKC (Fukuta, et al. 1988). However, further studies will have to be performed in order to substantiate these theories.

In conclusion, the present data indicate that NKC have a critical role in mucosal immunisation and subsequent development of adaptive immune responses. These new data suggest the possibility of a central role for the increased numbers of NKC observed in the NALT and CLN shortly after intranasal immunisation, and suggest that NKC activity may be one of the factors governing the development of both antigen-specific cytokine and antibody responses, particularly the T_H1 response, after mucosal vaccination.

6 Live recombinant *Salmonella* vaccine candidates expressing the tuberculosis fusion antigen, Ag85B-ESAT6

6.1 General introduction

6.1.1 Live vector vaccines

Effective mucosal immunity is efficiently induced by mucosal delivery of vaccines, due to the specialised and interlinked nature of the mucosal lymphoid tissues. Different strategies can be used to deliver vaccine antigens via the mucosal route, with living attenuated bacterial vaccines being among the most promising candidates. Broadly, live bacterial vaccines can be classified as a self-limiting asymptomatic infection stimulating an immune response to one or more expressed antigens. Attenuation can be accomplished through deletion of different genes, including virulence, regulatory, and metabolic genes. Live bacterial vaccines avoid some of the downsides of parenterally administered vaccines, as they mimic the route of entry of many pathogens, and are therefore able to elicit effective humoral and cellular immune responses, at the level of both systemic and mucosal compartments. The vaccines can also be administered orally or nasally, which offers advantages of simplicity and safety compared to needle-based delivery, and reduces the need for a professional healthcare infra structure. Other advantages associated with using live attenuated bacteria as vaccine vectors include low batch preparation costs with increased shelf-life, and stability in the field, i.e. formulations can be lyophilised. Furthermore, attenuated bacterial vaccines can not only be used to induce immunity to their corresponding pathogenic strain, but they can also be modified to produce one or more heterologous antigens. For a comprehensive review see (Roland, et al. 2005). These bacterial vectors can be constructed to produce protein or DNA from any number of pathogens, as well as allergens or therapeutic agents, with the antigen encoding gene being located on a plasmid or integrated within the chromosome. However, only very few of the promising candidates have survived the licensing process and become registered for use in humans, indicating the difficulty in developing a commercial live vaccine (Table 6.1). Currently, no recombinant bacterial vector vaccines expressing heterologous antigens are available for general use, but there are a number both in pre-clinical and human trials (Table 6.2).

Table 6.1: Current live attenuated bacterial licensed vaccines

Vaccine Name	Route	Disease Protection
BCG	i.d. or s.c.	Tuberculosis
S. Typhi Ty21a	oral	Typhoid Fever
CVD 103-HgR	oral	Cholera

Table 6.2: Recombinant bacterial vector vaccine candidates

Attenuated Bacterial Strain	Foreign Antigen	Route	Test Species	Comments	Reference(s)
L. monocytogenes	HIV gp-120	oral	mice	*in vitro* delivery to CD4+ T cells via MHC II	(Guzman, et al. 1998)
E. coli	CFA/I	oral	humans	No adverse vaccination effects and strong antibody responses	(Turner, et al. 2006)
Streptococcus gordonii	none	i.n. + oral	humans	Strong colonisation and well tolerated	(Kotloff, et al. 2005)
S. Typhi	*S. sonnei* surface antigen	oral	mice/humans	protection in animal models and good IgA titres in volunteers	(Formal, et al. 1981; Van de Verg, et al. 1990)
	H. pylori urease	oral	humans	no immune responses detected, despite boosting with LT + urease	(DiPetrillo, et al. 1999)
	hepatitis B core antigen	oral	humans	vaccine safe and well tolerated and induced cellular immune responses in volunteers	(Microscience 2004b)
	LT-B and HBV-core	i.n. + s.c.	mice	Immunisation of mice stimulated potent antigen-specific serum IgG responses to the heterologous antigens	(Stratford, et al. 2005)
	Yersinia pestis F1-V	i.n.	mice	2 intranasal doses induced good immune responses and protected over half the mice from challenge with Y. pestis	(Morton, et al. 2004)
S. Typhimurium	*H. pylori* UreAB	oral	humans	well tolerated and induced anti-urease immune responses in volunteers	(Angelakopoulos and Hohmann 2000)
	LT-B	oral	mice	expression of LT-B seen after intra-macrophage survival and immunised mice mounted good anti-LT-B antibody responses	(McKelvie, et al. 2004)
	SopE-Gag	oral	primates	good CTL responses detected after immunisation + MVA boosting, however viral replication was not reduced after challenge	(Evans, et al. 2003)
	ESAT6	i.v.	mice	high levels of antigen-specific IFN-γ, but protection lower than BCG vaccinated animals	(Mollenkopf, et al. 2001)
	Ag85B	i.v.	mice	partial protection against challenge	(Hess, et al. 2000)
	HIV-1 Gag	oral	humans	Modest anti-gag immune responses (IL-2 and IFN-γ)	(Kotton, et al. 2006)
S. flexneri 2a	measles virus hemagglutinin	i.n.	rats	MV titres were significantly reduced in lungs of infected animals	(Pasetti, et al. 2003)
	S. dysenteriae O-antigens	i.p.	mice	good protection against challenge	(Klee, et al. 1997; Tzschaschel, et al. 1996)
M. bovis	Ag85B	i.d.	guinea pigs/humans	vaccinated animals exposed to tuberculosis infection survived significantly longer than BCG vaccinated groups/ also in phase I human trails	(Horwitz and Harth 2003; Horwitz, et al. 2000).
	ESAT6	s.c. or i.p.	mice/guinea pigs	immunised animals showed better protection after challenge compared to BCG immunised animals and less lung pathology	(Pym, et al. 2003).

6.1.2 *Salmonella* as vaccine vectors

Attenuated derivatives of *S. enterica* have been proposed as vehicles for the delivery of heterologous antigens to the mammalian immune system, and as a basis for multivalent vaccines. In fact, strains of *S*. Typhi and *S*. Typhimurium were among the first bacterial recombinant vaccine vectors used to deliver heterologous antigens (Curtiss 2002). *S. enterica* is attractive as a delivery vehicle because strains have the ability to invade host tissues and persist, while continuing to produce a heterologous antigen(s), and vaccines can potentially be delivered via mucosal routes of immunisation. Immunisation with attenuated strains of *Salmonella* primes the host to produce antigen-specific immune responses, including the production of $CD8^+$ and $CD4^+$ T lymphocytes. *Salmonella* vaccine vectors also induce the production of multiple cytokines including, TNF-α, IFN-γ and IL-12, as well as pro-inflammatory mediators such as NO, which enhance early innate immune responses and create a local environment favourable to antigen presentation. All these points are considered to be significant advantages for the development of a mucosal vaccine.

Over the years live *Salmonella* vector vaccines have been evaluated in numerous animal and human studies. Results with recombinant attenuated *S.* Typhimurium vaccines in animals suggest that recombinant attenuated *S.* Typhi vaccines could be developed to protect against viral infections caused by Hepatitis B virus (HBV), papillomaviruses, herpes simplex, influenza and HIV; as well as many bacterial pathogens including, *Streptococcus pneumoniae*, *H. pylori*, *Y. pestis*, human enterotoxigenic and *E. coli* strains, *M. tuberculosis*, *L. monocytogenes*, *Bacillus anthracis*, *Clostridium tetani*, *Clostridium difficile* and *Bordetella pertussis* (Bowen, et al. 1995; Corthesy-Theulaz, et al. 1998; Dalla Pozza, et al. 1998; Hess, et al. 1997; Hess, et al. 2000; Lasaro, et al. 2005; Nardelli-Haefliger, et al. 1997; Nayak, et al. 1998; Stokes, et al. 2006; Stratford, et al. 2005; Tite, et al. 1990; Tsunetsugu-Yokota, et al. 2007; Vindurampulle, et al. 2004; Ward, et al. 1999; Yang, et al. 2007). In addition, some progress is being made in developing vaccines to protect against parasitic infections due to *Plasmodium falciparium*, *Leishmania major*, and *Schistosoma mansoni* (Gomez-Duarte, et al. 2001; Pacheco, et al. 2005; Xu, et al. 1995). Recently, Yang and colleagues cloned the *Y. pestis caf*1 operon, encoding the F1-Ag and virulence Ag (V-Ag) into attenuated *Salmonella* vaccine vectors.

Immunised mice gave strong antigen specific serum IgG antibody titres as well as elevated mucosal IgA responses. When vaccinated mice were challenged with bubonic and pneumonic plague, significant protection was detected showing that a single *Salmonella* vaccine can deliver both F1- and V-Ags to effect both systemic and mucosal immune protection against *Y. pestis* (Yang, et al. 2007). Hess et al, used an *aroA S*. Typhimurium strain as a vector to express and secrete the somatic protein of *L. monocytogenes,* superoxide dismutase. Vaccination of mice with the construct induced protection against a lethal challenge with this intracellular pathogen (Hess, et al. 1997). Another study used *Salmonella* to express codon-optimised HIV type 1 Gag protein. Mice intranasally primed with purified Gag and CT adjuvant, and boosted with the recombinant *Salmonella* strain induced significant mucosal IgA as well as $CD8^+$ T-cell responses (Tsunetsugu-Yokota, et al. 2007).

The development of new *S*. Typhi-based vectors has been limited by the narrow host range of *S*. Typhi and paucity of appropriate animal models. Since humans are the only known natural host for *S*. Typhi, many strategies for producing attenuated vaccine vectors were initially identified as immunogenic in *S*. Typhimurium as mentioned above. Currently the only licensed live attenuated vaccine against typhoid fever is the live *S*. Typhi strain, Ty21a. This vaccine was derived by chemical mutagenesis of the wild-type virulent bacterial isolate, and is associated with a defect in the *galE* gene, as well as other undefined mutations (Germanier and Fuer 1975); this technology is now not considered appropriate for development of new live attenuated bacterial strains. Even though Ty21a is well tolerated in humans it is weakly immunogenic with three or four oral doses being required for significant, but incomplete, protection against typhoid fever (Cryz, et al. 1988). It was this avirulent *Salmonella* strain that was first used to successfully express foreign antigens and induce antigen-specific immune responses (Formal, et al. 1981; Herrington, et al. 1990). Subsequent studies in humans reported limited application of this strain as a vaccine vector. This may have been due to low antigen expression *in vivo* or weak immunogenicity of the vector. With the improvements in bacterial genetics, a number of new live vaccine vector candidates have been constructed by introducing defined non-reverting mutations in both virulence and house-keeping genes, resulting in rational attenuation. Often more than one defined mutation is introduced to minimise the chance of reversion to wild-type virulent phenotype (see Table 6.3).

Table 6.3: Characteristics of live vaccines developed against typhoid fever

Strains	Mutation	Safety	Comments	References
Ty21a	*galE* + undefined	safe	Weakly immunogenic, 3-4 oral doses required	Commercially available
CVD906-htrA CVD908-hrtA	*aroC aroD htrA*	mild diarrhoea and fever in small number of volunteers	Antibody and T-cell responses after single dose	(Tacket, et al. 1997; Tacket, et al. 2000)
ZH9	*aroC ssaV*	safe	Antibody and T-cell responses after single dose, currently undergoing phase III trials	(Kirkpatrick, et al. 2006)
541Ty 543Ty	*aroA purA Vi*	safe	Weakly immunogenic, lower than Ty21a	(Edwards and Stocker 1988)
Ty800	*phoP phoQ*	safe	Antibody responses	(Hohmann, et al. 1996)

The potential application of newly developed vaccine strains as vectors for delivering one or heterologous antigen(s) has been recently demonstrated in clinical studies. Microscience Ltd have carried out phase I clinical studies on volunteers with recombinant *S.* Typhi ZH9 expressing the LT B-subunit (LT-B) of *E. coli* (ZH9/LT-B). The ZH9/LT-B vaccine was well tolerated and immunogenic following 2 oral doses (Khan, et al. 2007; Microscience 2004a). ZH9 has also been manipulated to express hepatitis B virus core antigen (HBcAg), which when given orally to volunteers elicited production of proliferative T cell responses to HBcAg (Microscience 2004b). Considering all the above points, attenuated *Salmonella* strains constitute an attractive carrier system for the delivery of the *M. tuberculosis* protein Ag85B-ESAT6.

6.2 Mucosally delivered live *Salmonella, in vivo* inducible, vector vaccines elicit significant immune responses against the tuberculosis fusion antigen, Ag85B-ESAT6

6.2.1 Introduction

Oral live *Salmonella* vaccine vectors expressing recombinant heterologous antigens help stimulate systemic, mucosal, humoral, and cell-mediated immune responses against *Salmonella* and recombinant antigens (Garmory, et al. 2003). Therefore, it may be possible to protect against *M. tuberculosis* by using *Salmonella* as a vector to express the immunodominant tuberculosis fusion antigen, Ag85B-ESAT6. In general, there are two common methods of expressing foreign antigen in salmonellae: from plasmid vectors, or from the bacterial chromosome. The main advantage for using a plasmid based expression system is the ability to produce high levels of protein from large or high-copy number plasmids. However, overexpression of many proteins can be toxic to *Salmonella*, probably due to the increased metabolic burden on the cell. One way to overcome these deleterious effects associated with constitutive expression of foreign proteins is to use inducible promoters that are regulated and only expressed in specific environments that the *Salmonella* encounters within the host. Thus, the use of an *in vivo* inducible promoter such as *nirB* or *ssaG* is an applicable approach to obtaining the stable *in vivo* expression of heterologous antigens in *Salmonella* vaccine strains.

The *ssaG* gene and its promoter are located in the *S. enterica* chromosome within *Salmonella* Pathogenicity Island-2 (SPI-2), which encodes a type III secretion system involved in adapting the pathogen to its intravacuole environment within mammalian cells (Shea, et al. 1996). The macrophage-inducible virulence factor (*ssaG*) is part of an operon that includes other essential components of the type III secretion apparatus, such as *ssaJ* (Hensel, et al. 1997). Strains deleted in *ssaG* have a strong defect in intracellular replication, and are unable to translocate effector proteins within macrophages as well as having an absence of appendage formation (i.e. functional type III secretion apparatus) (Chakravortty, et al. 2005). The *ssaG* promoter has been studied extensively as a means for controlled expression of heterologous antigens in *Salmonella*-based vaccines (McKelvie, et al. 2004; Stratford, et al. 2005). This

promoter has also previously been shown to be up-regulated at least 400-fold in macrophages (Valdivia and Falkow 1997). McKelvie and colleagues have used the *ssaG* promoter to express the *E. coli* LT-B within *S.* Typhimurium vaccine strains. They reported that expression of LT-B by the *Salmonella* constructs was detectable at significant levels after intra-macrophage survival, and mice immunised with these derivatives mounted marked anti-LT-B humoral antibody responses (McKelvie, et al. 2004). In addition Stratford et al used the *ssaG* promoter to effectively control expression of the HepB core antigen and LT-B after integration of these antigens into the chromosome of *S.* Typhi ZH9 (Stratford, et al. 2005).

The *nirB* gene, which encodes the *E. coli* NADH-dependent nitrite reductase, is the first gene in an operon that also includes *nirD*, *nirC* and *cycG*. The *nirB* promoter is tightly regulated by nitrite and by changes in oxygen tension of the environment, and becomes active under anaerobic conditions (Peakman, et al. 1990). Mutants of *E. coli* K12 defective in the *nirB* gene lack NADH-dependent nitrite reductase activity and reduce nitrite slowly during anaerobic growth (Cole, et al. 1980). Chatfield et al previously used the anaerobically inducible *nirB* promoter to drive significant expression of the non-toxic immunogenic fragment C of tetanus toxin in the *Salmonella* vector BRD847. Oral immunisation with this vaccine construct induced high levels of circulating anti-TetC antibodies, and mice were solidly protected against tetanus toxin challenge (Chatfield, et al. 1992). Recently, Salam et al used a *Salmonella* vector expressing the cloned saliva-binding region (SBR) of *Streptococcus mutans* or SBR linked to the A2 and B subunits of cholera toxin (CTA2/B) under the control of both the *T7* and *nirB* promoters (*T7-nirB* dual promoter). *Salmonella* clones expressing SBR or SBR-CAT2/B under the control of either *T7-nirB* promoter induced a high and persistent mucosal and systemic anti-SBR antibody response (Salam, et al. 2006).

As already discussed *Salmonella* can be modified to carry and express heterologous antigens. Numerous studies have observed that immunisation of animals with various recombinant *Salmonella* constructs induces potent T_H1 immune responses to both the vector as well as the heterologous antigen. I therefore hypothesise that mice orally immunised with the various *Salmonella* constructs producing Ag85B-ESAT6, via *in*

vivo inducible plasmid based expression systems, will have significant immune responses that are appropriate for protection after challenge with *M. tuberculosis*.

The aim of this study was therefore to evaluate the expression and immunogenicity of Ag85B-ESAT6 expressed under the control of the *ssaG* and *nirB* promoters as multiple copies in *Salmonella*. The immunogenicity of the recombinant *Salmonella* constructs was determined by examining both antigen-specific antibody and cytokine production.

6.2.2 Results

6.2.2.1 Construction of recombinant *Salmonella* vaccine strains and *in vitro* expression of Ag85B-ESAT6.

Two plasmids were constructed which directed the expression of the *Ag85b-esat6* gene from the *nirB* and *ssaG* promoters. The plasmids pmycossaG (*ssaG* plus fusion) and pmyconirB (*nirB* plus fusion) were cloned using the strategy outlined in Figure 6.1 and 6.2 respectively with help from Dr D Pickard and Dr M Abd E L Ghany (Wellcome Trust Sanger Institute). A set of primers were designed to confirm the cloned sequence expected including the promoter and fusion regions. Based on the sequence supplied by Dr J Dietrich (Statens Serum Institute, Denmark), sequencing results indicated no errors and no frame shift within the cloned regions. The two recombinant plasmids were isolated from *E. coli* and transferred into *S.* Typhimurium SL3261 (*aroA*) by electroporation after passage through *S.* Typhimurium LB5010 to ensure methylation of the DNA in the absence of restriction.

Figure 6.1: Schematic diagram showing the construction of the plasmid used to express Ag85B-ESAT6 via the *ssaG* promoter.

(A) The *ag85B-esat6* gene (pink symbol) was removed from pMCT6 using the restriction enzyme sites BglII/BamHI. (B) The fragment was then inserted into BglII (black line) and BamHI (grey line) sites within pMQ8, which also contained the ampicillin resistance gene (green symbol). (C) The *ssaG* promoter was obtained from amplification of *S*. Typhimurium TML strain DNA by primers containing SphI (red line) and BglII sequences. (D) This was subsequently cloned into t-overhang sites within the pGEM-Teasy vector. (E) The promoter fragment was then removed and inserted into the SphI/BglII sites within the pMQ8 vector in front of the Ag85B-ESAT6 fragment to give the *ssaG* Ag8B-ESAT6 expression plasmid (pmycossaG).

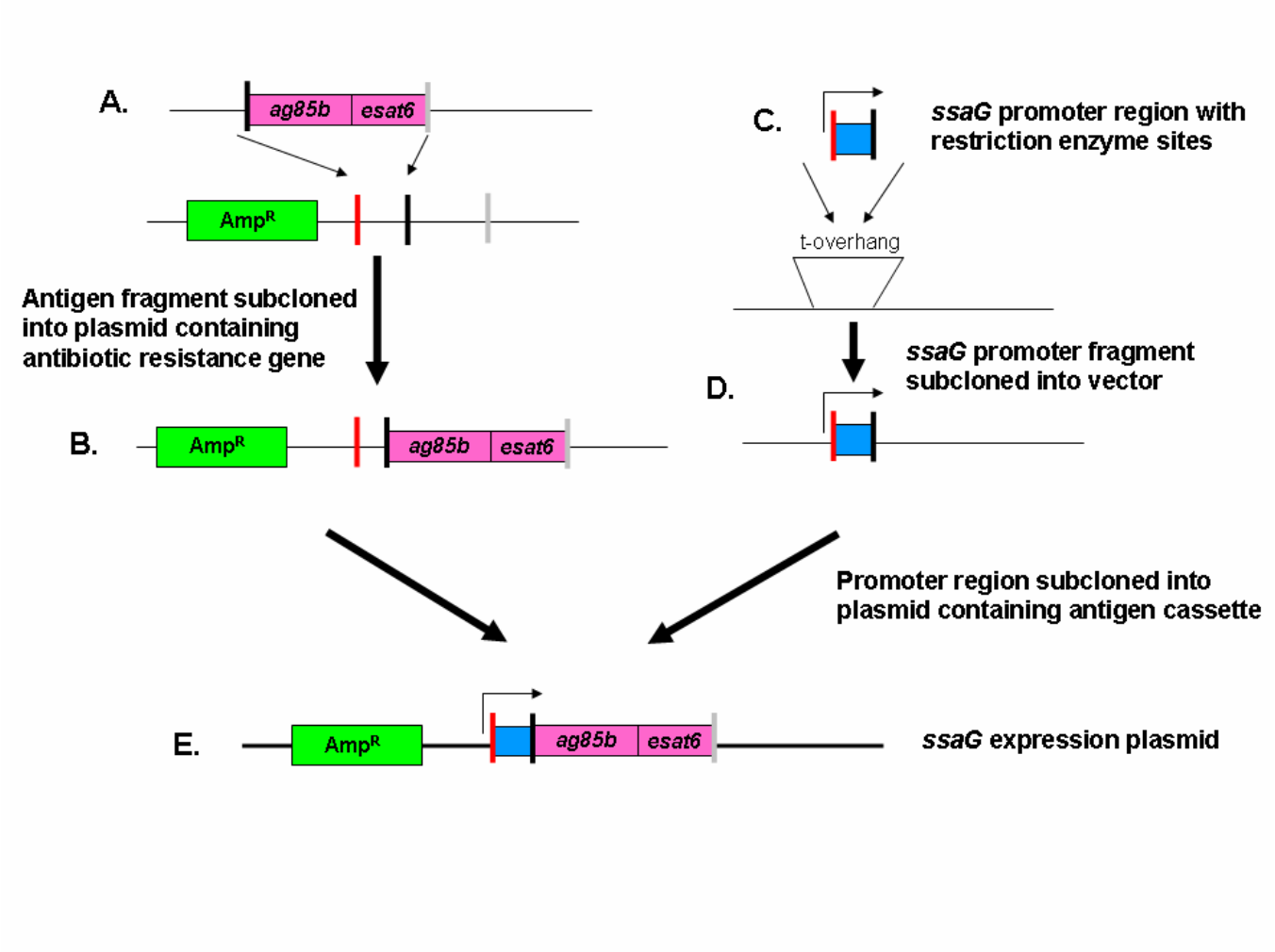
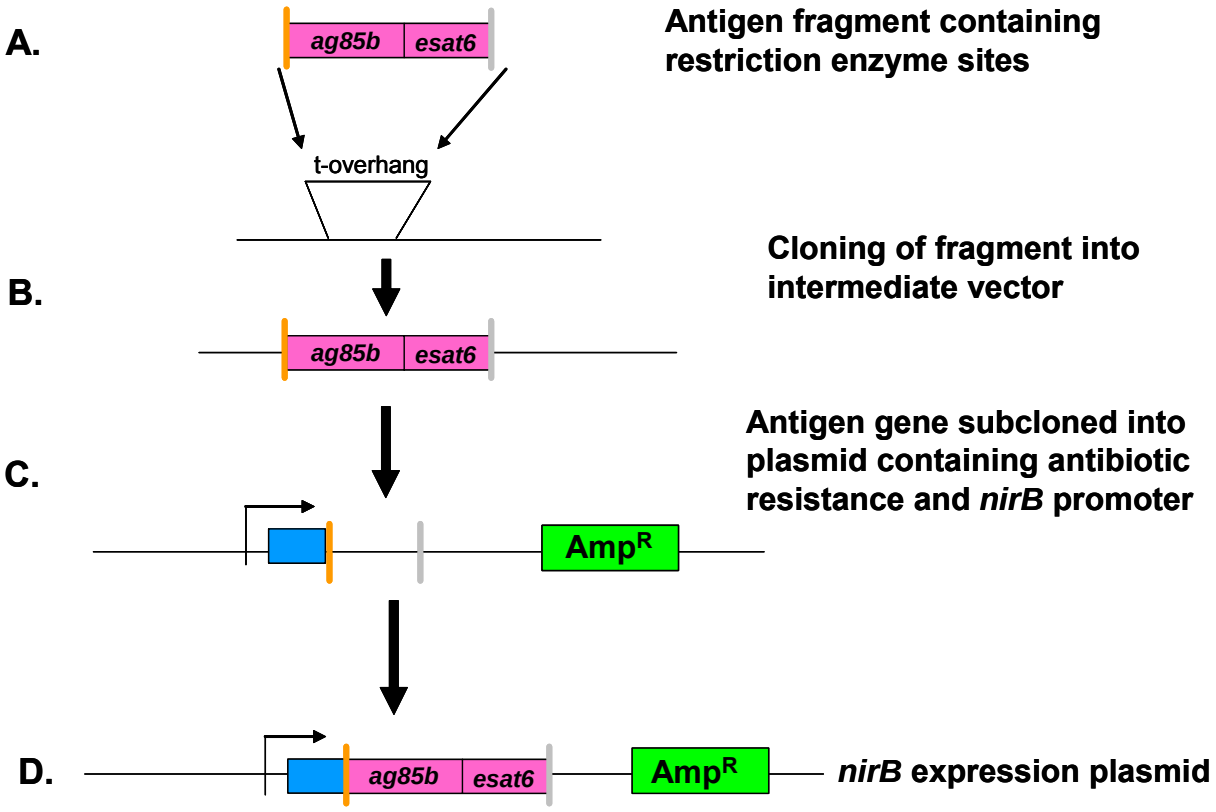


Figure 6.2: Schematic diagram showing the construction of the plasmid used to express Ag85B-ESAT6 via the *nirB* promoter.

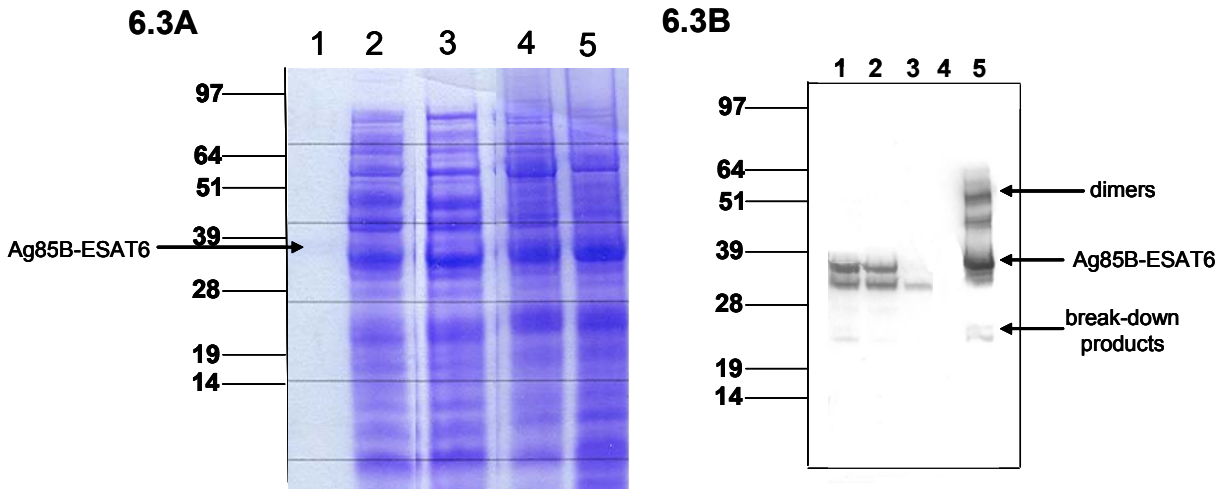
(A) The *ag85B-esat6* gene (pink symbol) was amplified from pMCT6 using primers containing NcoI (orange line) and BamHI (grey line) sequences. (B) The fragment was then cloned into t-overhang sites within the pTOPO vector. (C) The NcoI/BamHI antigen fragment was removed from the intermediate vector and subcloned into the NcoI and BamHI sites within pBRD940 in frame with the *nirB* promoter (blue symbol) which also contained an ampicillin gene (green symbol). (D) Leading to the *nirB* Ag85B-ESAT6 expression plasmid (pmyconirB).



The expression of Ag85B-ESAT6 was analysed in the recombinant SL3261 strains containing plasmids pmyconirB and pmycossaG by SDS-PAGE and Western blotting. The promoter *nirB* is inducible under anaerobic conditions, so strains were grown in an anaerobic jar in the presence of a palladium catalyst to remove any oxygen. The expression of Ag85B-ESAT6 protein was confirmed by Western blotting using anti-ESAT6 serum. Figure 6.3 shows that control purified Ag85B-ESAT6 protein and Ag85B-ESAT6 expressed in *Salmonella* containing pmyconirB reacted with the anti-ESAT6 antisera, and that no reaction was detected with *Salmonella* SL3261 parental strain. The electrophoretic mobility of these bands corresponded to that expected for the Ag85B-ESAT6 fusion protein, 36kDa. The expression levels of the other construct, pmycossaG, was also analysed by a Western blotting experiment. *In vivo*-inducible promoters, such as the *ssaG* promoter, are poorly induced when *Salmonella* are grown on normal laboratory medium and to facilitate detection of expression of heterologous antigens, the use of a modified growth medium was investigated. Minimal medium was designed to mimic some of the environmental conditions experienced by *Salmonella* inside the host macrophage. Minimal medium used in this study is based on MM5.8 medium described by Hautefort et al. (Hautefort, et al. 2003). MM5.8 medium has a pH 5.8, which mimics the acidic pH of the vacuole inside macrophages in which *Salmonella* resides. Figure 6.3 shows that Ag85B-ESAT6 was expressed strongly from the *ssaG* promoter when *Salmonella* strains harbouring pmycossaG were grown under minimal media conditions. In this gel it is evident that the antiserum cross reacts with putative break-down products and dimers, both above and below the position of the 36 kDa Ag85B-ESAT6 protein, but a clear reaction with the Ag85B-ESAT6 protein is nevertheless observed in lanes 1, 2, 3, and 5.

Figure 6.3: Coomassie gel and western blot analysis of recombinant *Salmonella* cultures.

Figure 6.3A shows a coomassie stained gel of 1µg Ag85B-ESAT6 (lane 1), and approximately 1.0 OD log phase cultures of SL3261 parent strain (lane 2), SL3261(pmyconirB) (lane3) and SL3261(pmycossaG) (lanes 4 and 5). Figure 6.3B shows the western blot analysis of the previous protein gel. Lane 3 shows SL3261(pmyconirB), lanes 1 and 2 are SL3261(pmycossaG), lane 4 shows the SL3261 parent strain and 1µg Ag85B-ESAT6 protein is located in lane 5. Nitrocellulose membranes were probed with 1:25 dilution of monoclonal anti-ESAT6 antibodies (Statens Serum Institute, Denmark).



6.2.2.2 Intragastric priming with recombinant SL3216 strains plus an intranasal Ag85B-ESAT6 boost and Ig responses

To investigate the ability of SL3261 recombinant strains expressing Ag85B-ESAT6 to elicit specific antibody responses against Ag85B-ESAT6, mice were immunised as summarised in Table 6.4. Serum collected at day 21, day 42 and day 56 was analysed for specific anti-Ag85B-ESAT6 Ig antibodies to determine if the SL3261 vaccine strains were expressing sufficient Ag85B-ESAT6 to trigger a measurable antibody response.

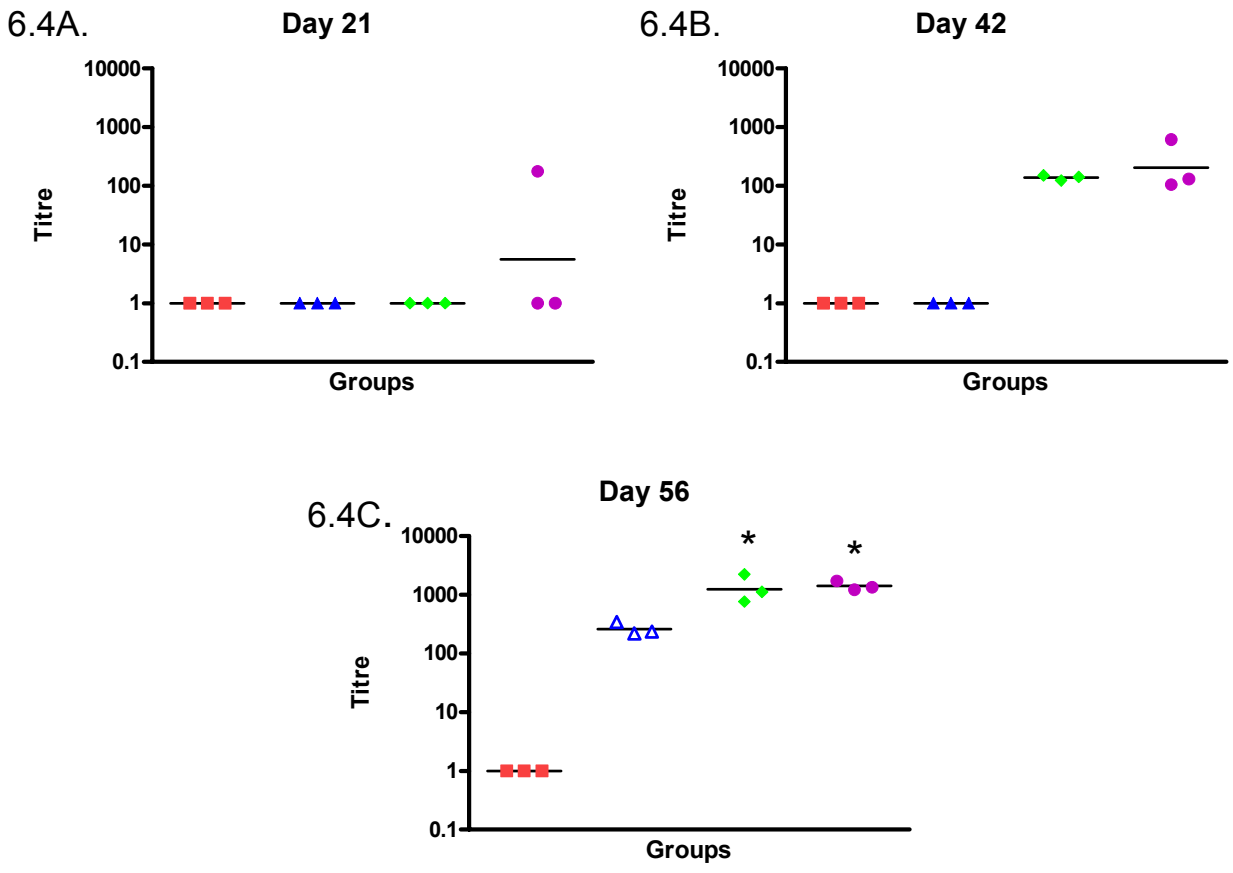
Table 6.4: Recombinant SL3261 immunisation regimen (3 mice/group)

Day	Group	Construct/Antigen	Procedure	Route
0	1	naïve (PBS)	immunisation	oral gavage
	2	SL3261		oral gavage
	3	SL3261(pmycossaG)		oral gavage
	4	SL3261(pmyconirB)		oral gavage
21	All groups		sample bleed	
35	1	naïve (PBS)	boost	oral gavage
	2	SL3261		oral gavage
	3	SL3261(pmycossaG)		oral gavage
	4	SL3261(pmyconirB)		oral gavage
42	All groups		sample bleed	
49	1	naïve (PBS)	boost	intranasal
	2	10µg Ag85B-ESAT6 + 1µg LT		intranasal
	3	10µg Ag85B-ESAT6 + 1µg LT		intranasal
	4	10µg Ag85B-ESAT6 + 1µg LT		intranasal
56	All groups	sample bleed, lung and nasal washes and spleens for CBAs	END	

Following the first dose of oral vaccination with SL3261 recombinant strains, only SL3261(pmyconirB) had any detectable serum Ig antibody titres at day 21 (1 out of 3 animals), when compared to the negative control groups (Figure 6.4A). After a second oral immunisation (day 35), all mice that received recombinant *Salmonella* strains expressing Ag85B-ESAT6 had modest titres of total anti-Ag85B-ESAT6 Ig, when compared to PBS and SL3261 immunised animals (Figure 6.4B). Following a third intranasal boost with 10µg Ag85B-ESAT6 plus 1µg LT all mice immunised with recombinant *Salmonella* strains expressing fusion protein showed a significant ($p < 0.05$) increase in Ig titres when compared to naïve animals (Figure 6.4C). On day 56, mice primed with parental SL3261 and boosted with protein and adjuvant also had anti-Ag85B-ESAT6 titres, but these were not significant when compared to negative control animals ($p > 0.05$).

Figure 6.4: Ag85B-ESAT6-specific total Ig titres in mice immunised with recombinant *in vivo* inducible *Salmonella* strains.

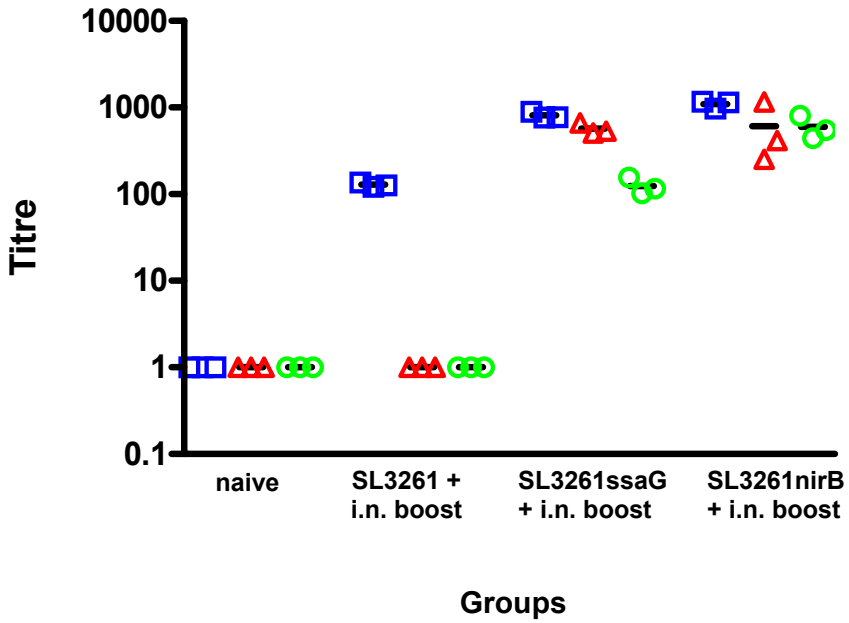
As outlined in Table 6.4, intragastric administration of recombinant SL3261 strains; SL3261*ssaG* and SL3261*nirB*, into Balb/c mice was performed at day 0 (immunisation) and day 35 (boost) with a final intranasal boost of 10µg Ag85B-ESAT6 + 1µg LT (day 49). Mice were inoculated with approximately 5 x 10^9 CFU of *Salmonella*. Mice were left for 21, 42 and 56 days and then sample bled to determine anti-Ag85B-ESAT6 Ig antibodies. Ag85B-ESAT6 specific antibody titres were determined by ELISA. Total serum Ig titres (A day 21, B day 42, and C day 56) from naïve and immunised animals are expressed as total antibody titre using a cut off of OD 0.3. ■ indicates naïve animals; ▲ represent the SL3261 parental strain with △ showing the SL3261 parental strain plus the intranasal boost of LT and Ag85B-ESAT6. ◆ shows animals immunised with SL3261 containing pmycossaG with ● indicating mice vaccinated with *Salmonella* strain harbouring the pmyconirB. The black bar shows the geometric mean from the group and the * indicates significant values of $p < 0.05$ as determined using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test compared to PBS and SL3261 immunised controls.



6.2.2.3 IgG1/IgG2a profile after recombinant *Salmonella* immunisation

To determine the subclass distribution of serum IgG antibody responses, samples from mice orally immunised with the recombinant SL3261(pmycossaG) and SL3261(pmyconirB) were analysed for levels of IgG1 and IgG2a antibody responses, which are indicative of a T_H2 or T_H1 responses, respectively (Figure 6.5). Serum IgG antibodies to Ag85B-ESAT6 from mice immunised with SL3261(pmycossaG) belonged predominantly to the IgG1 subclass (IgG1/IgG2a ratio = 4.6), but a mixed IgG2a and IgG1 response pattern (IgG2a/IgG1 = 1.0) was observed in those animals vaccinated with SL3261(pmyconirB). However, the shift in terms of IgG1/IgG2a ratios was not statistically significant ($p > 0.05$) in any of the vaccinated animals. No detectable IgG1 or IgG2a subtype titres were observed in animals immunised with SL3261 and boosted intranasally with LT and Ag85B-ESAT6 along with naive (PBS immunised) mice.

Figure 6.5: IgG1/IgG2a profile after recombinant *Salmonella* immunisation.
Balb/c mice were immunised as depicted in Table 6.4 and sample bled on day 56 to determine IgG and subtypes IgG1 and IgG2a titres. Ag85B-ESAT6 specific antibody titres were determined by ELISA. Total serum IgG, IgG1 and IgG2a titres from naïve and immunised animals are expressed as total antibody titre using a cut off of OD 0.3. □ shows IgG, △ IgG1, ○ indicates IgG2a and the black line gives the geometric mean.



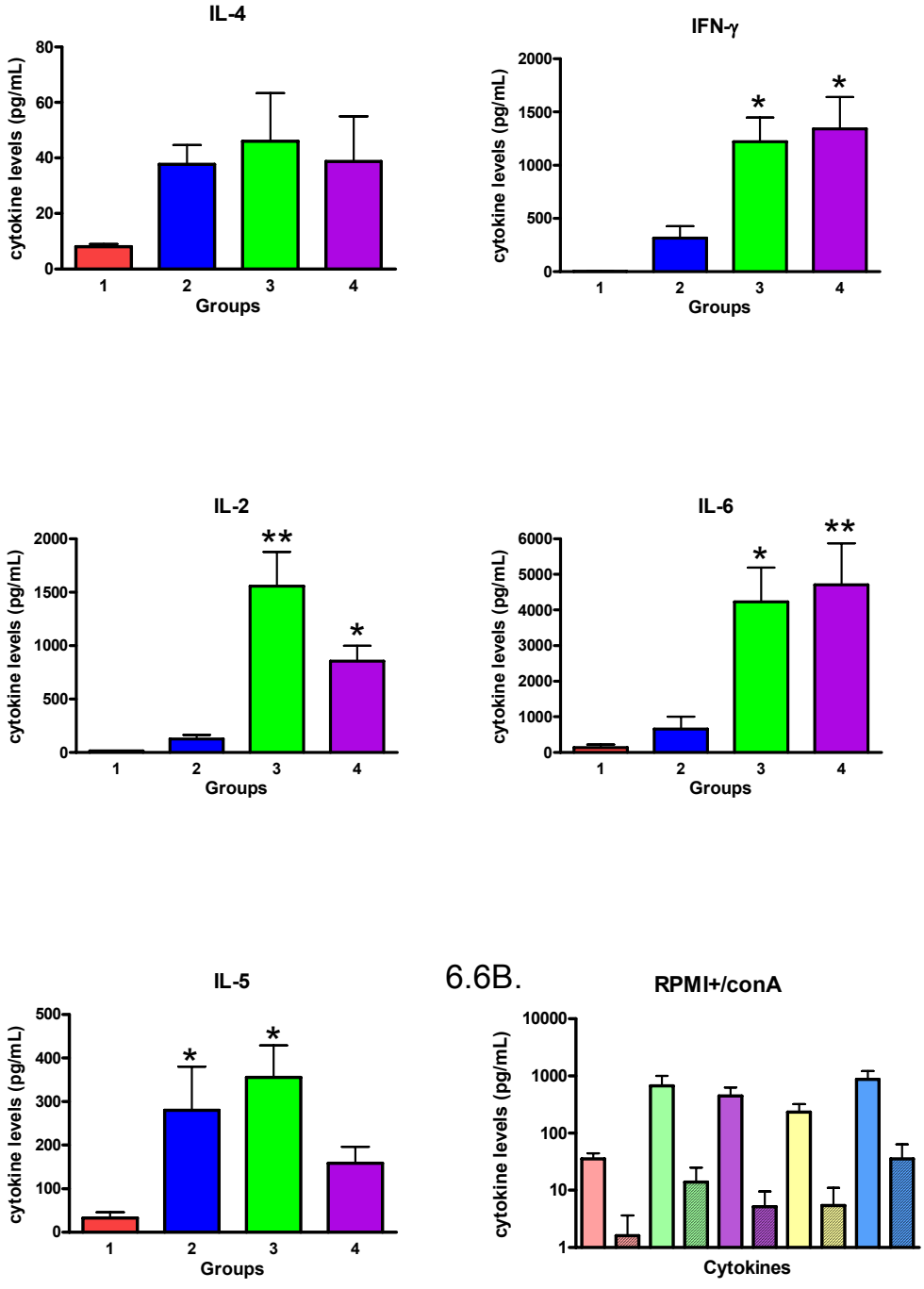
6.2.2.4 Intragastric priming with recombinant SL3216 strains plus an intranasal Ag85B-ESAT6 boost and cytokine responses

In order to examine the cellular immune responses induced after *Salmonella* vaccination T-cell assays were performed using splenocytes from immunised and naive animals. Cells were stimulated with 5 μg/ml Ag85B-ESAT6 and the supernatants were tested for IFN-γ, IL-4, IL-2, IL-5 and IL-6 production (Figure 6.6). The recombinant *Salmonella* vaccinated mice showed high and significant IFN-γ, IL-2, IL-6 responses at day 56 after priming and boosting. In contrast, those mice receiving *S.* Typhimurium SL3261 parental strain, and boosted intranasally with purified Ag85B-ESAT6 and the adjuvant LT, only produced significant levels of IL-5 when compared to naïve animals. Low and not significant IL-4 production ($p > 0.05$) was detected in the supernatants of Ag85B-ESAT6-stimulated splenocytes from either the vaccinated or control mice. For the SL3261(pmycossaG) vaccinated group, IL-5 production was significant when compared to negative control mice, whereas splenocytes from the group immunised with SL3261(pmyconirB) produced only modest levels of IL-5 that were not significant ($p > 0.05$). Mice vaccinated with SL3261(pmycossaG) gave significantly higher IL-2 levels when compared to those immunised with SL3261(pmyconirB) ($p < 0.01$ compared to $p < 0.05$). For IL-6 the reverse was true i.e. SL3261(pmyconirB) gave significantly higher levels of IL-6 than SL3261(pmycossaG) ($p < 0.01$ compared to $p < 0.05$).

Figure 6.6: Ag85B-ESAT6-specififc cytokine production from splenocytes of recombinant *Salmonella* immunised animals.

Supernatants were removed from stimulated splenocytes obtained from Balb/c mice vaccinated with PBS, SL3261 and *Salmonella* strains harbouring plasmids expressing Ag85B-ESAT6 from either the *ssaG* or *nirB* promoters, 56 days after initial priming and boosting and were assessed for different cytokines by CBA (Figure 6.6A). For full immunisation schedule see Table 6.4. Cytokine responses were measured upon *in vitro* stimulation with Ag85B-ESAT6 for 36-42 hours. Cells were also stimulated with conA (positive control) and media (negative control) (Figure 4.6B). Columns represent the mean (± SD) stimulation indices of splenocytes from three animals per group. The sensitivities of the CBA was >1pg/mL for each cytokine. (1) The red columns indicate naïve animals; (2) the blue represents mice immunised with the SL3261 parental strain and then intranasally boosted with LT and Ag85B-ESAT6. (3) The green shows animals immunised with SL3261 containing pmycossaG and, (4) the purple indicating mice vaccinated with *Salmonella* strain harbouring the pmyconirB. The * indicates significant values of $p < 0.05$ and ** indicates $p < 0.01$ as determined using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test compared to PBS immunised controls. For Figure 6.6B, block colours represent conA stimulated splenocytes with diagonal lines indicating $RPMI^+$ stimulated splenocytes. Pink shows IFN-γ, green IL-4, purple IL-2, yellow IL-6 and blue IL-5.

6.6A.


IL-4
cytokine levels (pg/mL)
80
60
40
20
0
1
2
3
4
Groups
IFN-γ
cytokine levels (pg/mL)
2000
1500
1000
500
0
*
*
1
2
3
4
Groups
IL-2
cytokine levels (pg/mL)
2000
1500
1000
500
0
**
*
1
2
3
4
Groups
IL-6
cytokine levels (pg/mL)
6000
5000
4000
3000
2000
1000
0
*
**
1
2
3
4
Groups
IL-5
cytokine levels (pg/mL)
500
400
300
200
100
0
*
*
1
2
3
4
Groups

6.6B.

RPMI+/conA
cytokine levels (pg/mL)
10000
1000
100
10
1
Cytokines

6.2.3 Discussion

The use of live attenuated bacteria as vectors offers many technical and clinical advantages. They are easy and relatively inexpensive to produce, are able to carry large or multiple antigens or adjuvants, and can be eradicated with antibiotics should the need arise. These attributes may make attenuated bacterial vaccinations more attractive for the developing world. Compared to injectable vaccines, oral delivery should result in increased compliance, safety, and ease of administration. In particular bacterial vectors may mimic natural infection and therefore interact with the mucosal, humoral, and cellular compartments of the immune system inducing both a systemic and mucosal immune response to the delivered heterologous antigen (Kotton and Hohmann 2004). Here I describe the construction of two recombinant vaccine candidates, SL3261(pmycossaG) and SL3261(pmyconirB), which express the tuberculosis fusion antigen Ag85B-ESAT6. As already discussed, the promoters chosen for this study have previously been shown to drive expression and specific immune responses against heterologous antigens delivered by *Salmonella* vaccine constructs, hence our decision to utilise these particular promoters (Chatfield, et al. 1992; Valdivia and Falkow 1997). Expression of the immunodominant tuberculosis antigen, Ag85B-ESAT6, via the *ssaG* and *nirB* was achieved by integrating the heterologous gene and appropriate promoter onto plasmids (pmycossaG and pmyconirB). These plasmids were then transformed into *S.* Typhimurium SL3261. The unregulated expression of foreign genes within *S.* Typhimurium can lead to plasmid instability, and yet stable expression of the guest antigen *in vivo* is necessary for presentation of the antigen to immune effector cells and the induction of a protective response. One approach to promote the stable expression of guest antigens involves the use of promoters which are induced within the host so that immune effector cells are presented with the guest antigen. As the gene is only expressed after certain environmental cues have been recognised, this approach might reduce the selective pressure towards deleting the heterologous gene (Curtiss 2002).

Previous studies by Hess et al reported the expression of the single components of the fusion antigen, Ag85B and ESAT6 by the *Salmonella* SL7207 strain endowed with the haemolysin secretion system of *E. coli* (Hess, et al. 2000; Mollenkopf, et al. 2001). Immunisation with both recombinant *Salmonella* strains demonstrated reduced numbers of tubercle bacilli in the lungs and spleens throughout the course of infection comparable to BCG vaccinated control animals. To our knowledge this study is the first to use a recombinant *Salmonella* vector to express the heterologous immunodominant fusion antigen, Ag85B-ESAT6.

I compared the immune responses to Ag85B-ESAT6 in mice immunised with a live *Salmonella* vector expressing Ag85B-ESAT6 from two different regulated promoters, *ssaG* and *nirB*. The purpose was to determine if either or both of the *in vivo* inducible promoters has the utility for expressing the foreign fusion antigen in *Salmonella*, and also if there were advantages of using a particular expression system. The *nirB* promoter is regulated by changes in oxygen tension in the local microenvironment. This regulation is effective since Ag85B-ESAT6 was readily detectable under reduced oxygen tension *in vitro* (Figure 6.3). The *ssaG* promoter is regulated under low pH conditions which are similar to those found inside macrophage vacuoles. The strong production of Ag85B-ESAT6 obtained after *Salmonella* were grown under *in vitro* minimal media conditions also confirms that this regulation is efficient (Figure 6.3).

I found that both *Salmonella* strains were very similar in terms of anti-Ag85B-ESAT6 Ig responses through the later stages of the study. However, only one out of the three mice immunised with SL3261(pmyconirB), and none of the animals immunised with SL3261(pmycossaG), had detectable Ig antibody titres after one oral dose (day 21). One possible reason for lack of detectable antibody titres after one oral immunisation may be due to loss of the plasmids. I did not check plasmid stability *in vivo*, and this may be a factor in the poor antibody responses observed at day 21. I do not know the reason why the anti-Ag85B-ESAT6 immune responses were stronger and more rapid in SL3261(pmyconirB) immunised mice. The only differences between the two plasmid systems are the promoters controlling Ag85B-ESAT6 expression. It is possible that Ag85B-ESAT6 expression is switched on earlier and for longer *in vivo* in SL3261(pmyconirB) than in SL3261(pmycossaG), or that *in vivo* expression of Ag85B-ESAT6 is higher using pmyconirB than pmycossaG. Notably, both strains

stimulated modest anti-Ag85B-ESAT6 titres after a second oral dose of the recombinant *Salmonella* strains in all mice immunised (day 42), therefore demonstrating the utility of the *in vivo* inducible promoters in driving immunologically relevant levels of antigen. To further increase the immune response to Ag85B-ESAT6, mice were intranasally boosted with the subunit protein plus the adjuvant LT. This third boost significantly increased the antibody titres in those mice primed with the recombinant *Salmonella* strains. I also observed that those mice receiving the SL3261 parental strain plus the Ag85B-ESAT6 and adjuvant boost also showed modest anti-Ag85B-ESAT6 responses when compared to PBS immunised controls. However, these responses were not found to be significant. When I examined the difference in antibody titres between mice immunised with the parental strain of SL3261 and boosted, compared to animals primed with recombinant *Salmonella* strains and subsequently boosted, I observed that titres were lower, but this difference was not found to be statistically significant. This may just be due to the very low number of animals (i.e. three) used in each group, making it difficult to statistically differentiate between each cohort. Nonetheless, these data suggests that priming with recombinant *Salmonella* and boosting is superior to intranasal vaccination alone at inducing antigen-specific antibody responses.

Salmonella (as well as other intracellular micro-organisms) generally induce a T_H1-type response characterised by high levels of IFN-γ and IgG2a antibodies (Klimpel, et al. 1995; Ramarathinam, et al. 1991; VanCott, et al. 1996). Significant cellular responses were only detected in those mice immunised with two oral doses of both the SL3261(pmyconirB) and SL3261(pmycossaG), and boosted intranasally with the adjuvant LT and Ag85B-ESAT6. Splenocytes from recombinant *Salmonella* immunised mice proliferated strongly against purified Ag85B-ESAT6 and secreted significant levels of IFN-γ, IL-2, IL-5 and IL-6, but not IL-4 when stimulated *in vitro*. These data indicate that both T_H1- and T_H2-type responses were elicited against Ag85B-ESAT6. The analysis of the subclasses of serum Ag85B-ESAT6-specific IgG also supported the presence of a mixed T_H1- and T_H2-type response. Similar observations were reported for mice immunised with recombinant *Salmonella* expressing TetC (VanCott, et al. 1996). Interestingly, from the cytokine profiles it appears that SL3261(pmyconirB) may induce a stronger T_H1 response, as represented by the significantly lower IL-5 levels and IgG1 antibody titres compared to

SL3261(pmycossaG). This suggests that the *nirB* promoter may be more appropriate than the *ssaG* promoter for expressing the Ag85B-ESAT6 antigen as a polarised T_H1 response is required for immunity to *M. tuberculosis* (Boom 1996; Flynn and Chan 2001). However, the underlying mechanism for the induction of different types of immune response by the use of specific promoters is unclear (Medina, et al. 2000). Notably, the immunisation regimens incorporating the recombinant *Salmonella* strains produced significant levels of IFN-γ (i.e. over 1250pg/mL). As already discussed this cytokine is a key indicator to levels of protection possible after challenge with pathogenic *M. tuberculosis* and previous studies have shown similar levels of IFN-γ to correlate with a reduction in both lung and spleen *M. tuberculosis* CFUs (Doherty, et al. 2002). Our data therefore suggests that priming with these recombinant *Salmonella* strains, and intranasally boosting with adjuvant and protein, would possibly provide protection against a future pathogenic *M. tuberculosis* challenge.

This study outlines the value of combining *in vivo* inducible promoter driven antigen expression in *S.* Typhimurium SL3261 background plus an intranasal boost as a prime-boost strategy for developing clinically acceptable, immunogenic recombinant *Salmonella*-based vaccine against *M. tuberculosis*.

6.3 A recombinant attenuated *S.* Typhimurium vaccine encoding the *M. tuberculosis* antigen, Ag85B-ESAT6, confers potent specific immune responses and induces significant reduction of bacterial colonisation of mice.

6.3.1 Introduction

Attenuated live oral vaccines based on attenuated *S.* Typhi or *S.* Typhimurium have been successfully used as vehicles to deliver heterologous antigens and induce protective immune responses in a variety of animal models (Bowe, et al. 2003; Kotton and Hohmann 2004). A few clinical trials have also shown their safety and immunogenicity in humans. As already discussed, there are two frequently used methods for expressing heterologous antigens, from both plasmids and from the chromosome. In the previous study, the *ssaG* and *nirB* promoters were used to efficiently drive *Ag85b-esat6* gene expression from multicopy plasmids. However, the use of multicopy plasmids has several disadvantages when it comes to the generation of vaccines. For example, the metabolic burden resulting from the expression of foreign protein creates a selective pressure for plasmid loss during *in vitro* growth, or early in the *in vivo* colonisation process following administration. Furthermore, the toxic effects of overexpression of heterologous proteins on bacterial vectors may lead to poor immunogenicity despite high levels of antigen expression. One method that could be used to overcome this stability issue would be to insert the heterologous sequence into the bacterial chromosome.

The recombinant *Salmonella* strains constructed in Section 6.2 were used as a proof of principle i.e. to show that *Salmonella* vaccine vectors can be engineered to express the *M. tuberculosis* fusion antigen Ag85B-ESAT6. As discussed above plasmid based expression systems have a number of disadvantages and therefore I decided to utilise chromosomal integration of Ag85B-ESAT6 as this system is more appropriate for use in human trials. I hypothesise that mice immunised with attenuated *S.* Typhimurium SL3261 expressing the Ag85B-ESAT6 antigen from the chromosome would induce specific immune responses and provide protection against subsequent challenge with virulent *M. tuberculosis*.

6.3.2 Results

6.3.2.1 Construction of recombinant *Salmonella* vaccine strain and *in vitro* expression of Ag85B-ESAT6.

Several systems have been developed that facilitate chromosomal expression and one such system, based upon *phoN* as an integration site was selected for this study (Husseiny and Hensel 2005). This approach relies on a single copy of the heterologous gene to drive immunologically relevant levels of antigen expression. As only one copy of the foreign antigen is produced from the recombinant *Salmonella*, the promoter *lacZ* was chosen, as deletion of the *lacIQ* repressor allows constitutive expression of the protein of interest. The red recombinase approach was utilised for integration of expression cassettes into the chromosome of *S.* Typhimurium. Briefly, an expression cassette was constructed that contained the constitutive promoter *lacZ* to control expression of the heterologous fusion antigen *ag85B-esat6* with help from Dr D Pickard and Dr M Abd E L Ghany (Wellcome Trust Sanger Institute). The *lacZ* promoter was derived from the original expression plasmid (pMCT6) with a region of the repressor (*lacIQ*) deleted to allow constitutive expression of the protein. The expression cassette was inserted into a plasmid (p2795) that contained a kanamycin resistance gene (Figure 6.7A). The resulting targeting construct was amplified by PCR with primers complementary to the flanks of the targeting construct and the chromosomal gene selected for integration i.e. *phoN* (Fig 6.7B). *phoN* is not required for normal growth of *S.* Typhimurium and mutations in the *phoN* gene do not result in any further attenuation of virulence (Lodge, et al. 1995). By homologous recombination mediated by red recombinase plasmid (pKD46), the *phoN* gene was replaced by the targeting construct to give SL3261mycolacZ (Fig 6.7C and D). A set of primers were designed to confirm the expected cloned sequence including the promoter and antigen regions. Based on the sequence supplied by Dr J Dietrich (Statens Serum Institute, Denmark), sequencing results indicated no errors and no frame shifts within the cloned regions. Whole-cell lysates of recombinant strain SL3261mycolacZ, or of the parental control strain, were grown statically at 37°C to allow Ag85B-ESAT6 expression and were then probed with mAb against ESAT-6. A positive control of purified Ag85B-ESAT6 protein was also probed. The blots revealed one major immunoreactive band in SL3261mycolacZ lysates and Ag85B-ESAT6 protein that were absent from wild-type SL3261. The electrophoretic mobility of these bands corresponded to that expected for the Ag85B-ESAT6 fusion protein, 36kDa (Fig 6.8).

Figure 6.7: Schematic diagram for the construction of targeting construct and chromosomal integration of expression cassette.

(A) Expression cassette consists of a constitutive promoter (*lacZ*), a gene fragment encoding the model vaccine fusion antigen Ag85B-ESAT6 (pink symbol). Expression cassette for expression of Ag85B-ESAT6 was inserted into the multiple cloning site of p2795. p2795 contains the kanamycin resistance gene (green symbol) and binding sites for primers (blue symbol). (B) The targeting construct consisting of expression cassette and resistance gene was amplified by knock-in primers containing sequences complementary to the chromosomal target gene, *phoN* (orange symbol). (C) *S.* Typhimurium strain SL3261 harbouring pKD46 for expression of Red recombinase was transformed with linear DNA of targeting construct. (D) Recombinant colonies with replacements of a chromosomal target gene, *phoN* (purple symbol) by the targeting construct were selected.

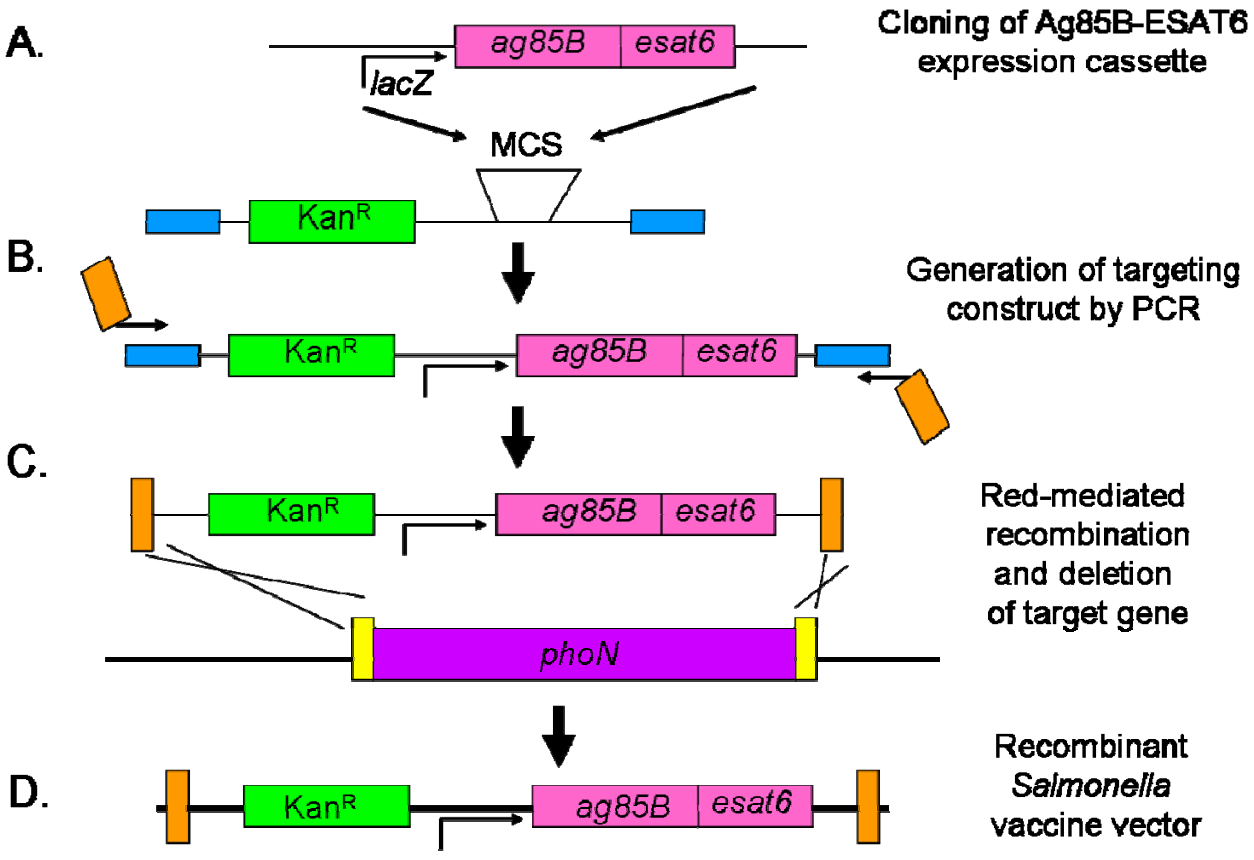
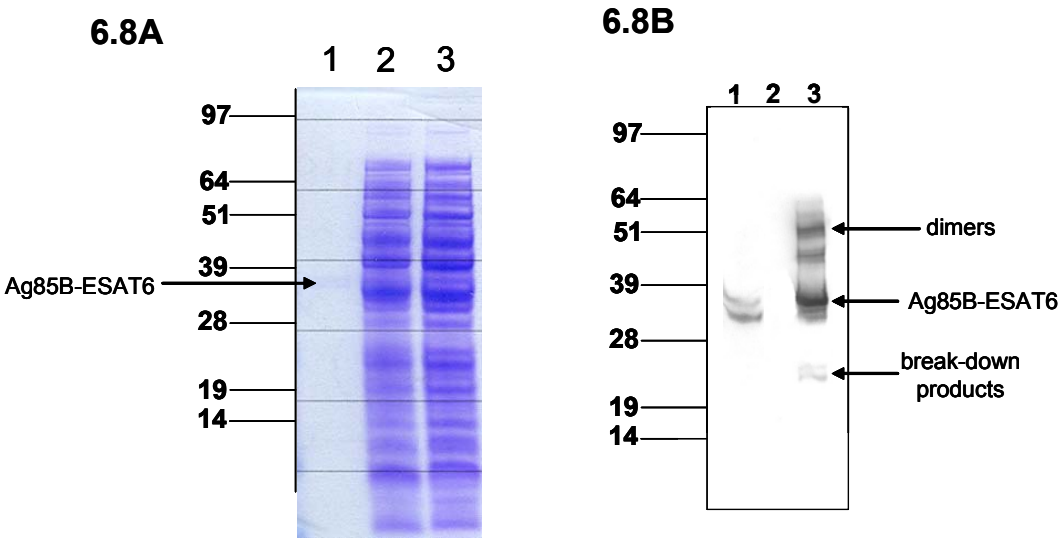


Figure 6.8: Coomassie gel and western blot analysis of recombinant chromosomal *Salmonella* construct.

Figure 6.8A shows a coomassie stained gel of 1µg Ag85B-ESAT6 (lane 1), and approximately 1.0 OD log phase cultures of SL3261 parent strain (lane 2), SL3261mycolacZ (lane3). Figure 6.8B shows the western blot analysis of the previous protein gel. Lane 3 shows 1µg Ag85B-ESAT6 protein, log phase static cultures of the SL3261 parent strain are shown in lane 2 and SL3261mycolacZ cultures are in lane 1. Nitrocellulose membranes were probed with 1:25 dilution of monoclonal anti-ESAT6 antibodies (Statens Serum Institute, Denmark).



6.3.2.2 Humoral immunogenicity of the oral recombinant *Salmonella* priming-intranasal protein boost vaccine regimen.

The concept about the effectiveness of antibodies against intracellular bacterial infections, including tuberculosis, is contentious partly due to the notion that antibodies cannot easily reach intracellular pathogens. This reservation has been applied particularly to organisms, which are confined to phagosomes (i.e. *M. tuberculosis*). However, this view is under reconsideration in the light of increasing evidence that antibodies interfering with some extracellular stages of the infection can influence the intracellular fate of the pathogen (Casadevall 2003; Edelson and Unanue 2001; Li, et al. 2001; Mukherjee, et al. 1995a; Mukherjee, et al. 1995b). In order to determine if SL3261mycolacZ was effective in stimulating humoral immune responses, sera collected at various time points were tested for the kinetics of Ig antibody responses by ELISA. Seventy-five C57BL/6 mice were allocated to eleven groups (5-10 animals per group) and vaccinated according to the regimens shown in Table 6.5. The *Salmonella* vaccine strain SL3261 used in these studies has been shown to persist for 3-4 weeks within immunised mice (Dunstan, et al. 1998). To ensure no non-specific activation of macrophages and therefore of immune responses it is important to ensure that all *Salmonella* have been cleared before boosting, this is why mice were orally immunised on day 0 before being intranasally boosted on day 50. Group 2 shows the antibody response that follows a single immunisation with SL3261mycolacZ administered orally; mice were found to have modest but significant titres 21 days post priming when compared to PBS immunised and SL3261 parental strain vaccinated mice (Group 1 and Group 3, respectively) (Figure 6.9A). These anti-Ag85B-ESAT6 titres were observed to increase over ten times by day 50, where 100% of the animals showed high titres (Figure 6.9B). Boosting intranasally with 20µg LTK63 and 25µg Ag85B-ESAT6 in SL3261mycolacZ primed mice (Group 2c) appeared to somewhat enhance serum Ig anti-Ag85B-ESAT6 responses at both 7 days and 70 days post boost (Figure 6.9C and D respectively). Although not significant, the difference between the anti-Ag85B-ESAT6 responses of Groups 2 and 2c is consistent with the elevated Ag85B-ESAT6-specific cytokine levels observed in the latter group (see Section 6.3.2.3). Control mice vaccinated with SL3261mycolacZ, and then boosted with either PBS (Group 2a) or LTK63 (Group 2b), did not show any increase in Ag85B-ESAT6 specific titres 7 days after boosting (Figure 6.9C). Group 4

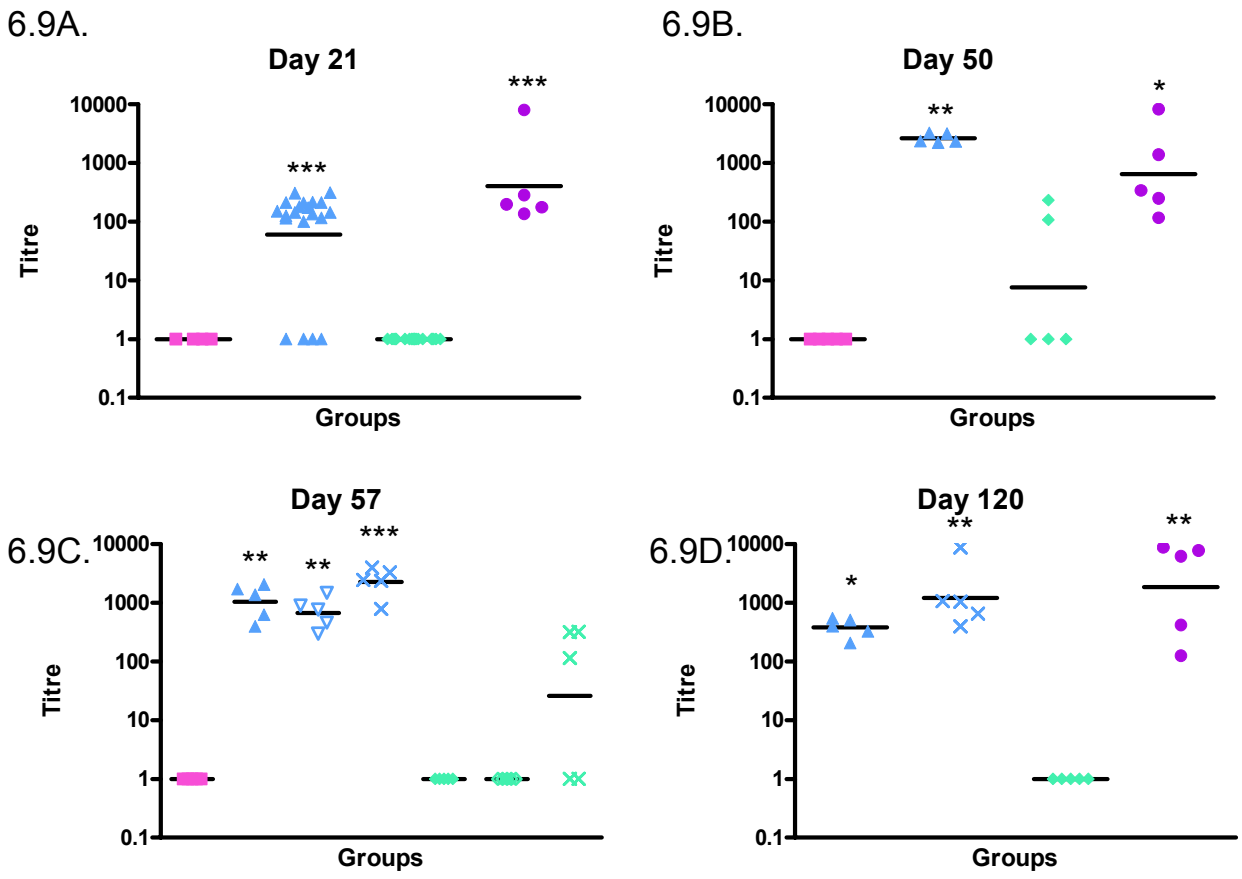
shows the serum anti-Ag85B-ESAT6 responses achieved when one dose of Ag85B-ESAT6 plus LT was administered intranasally and was considered a positive control group as this regimen has already been shown to induce significant anti-Ag85B-ESAT6 titres (see Chapter 3). As with Groups 2, 2a, 2b and 2c the animals in Group 4 attained high titres after just a single dose of protein and adjuvant (day 50 and 120) (Figures 6.9B and D). As expected, mice primed with the parental *Salmonella* strain and boosted with either PBS (Group 3a) or LTK63 (Group 3b) did not show any detectable anti-Ag85B-ESAT6 titres (Figure 6.9C). Group 3c demonstrates the serological response that follows oral priming with the parental SL3261 strain and intranasal boosting with purified Ag85B-ESAT6 and LTK63. This group did have modest anti-Ag85B-ESAT6 titres 7 days after boosting, however only 60% of the animals seroconverted (Figure 6.9C).

Table 6.5: Recombinant *Salmonella* prime-boost immunisation schedule

Day	Group	Number mice/group	Construct/Antigen	Procedure	Route
0	1	5	Naïve (PBS)	Immunisation	Oral gavage
	1a	5	Naïve (PBS)		Oral gavage
	2	10	SL3261mycolacZ		Oral gavage
	2a	5	SL3261mycolacZ		Oral gavage
	2b	5	SL3261mycolacZ		Oral gavage
	2c	10	SL3261mycolacZ		Oral gavage
	3	10	SL3261		Oral gavage
	3a	5	SL3261		Oral gavage
	3b	5	SL3261		Oral gavage
	3c	5	SL3261		Oral gavage
	4	10	25µg Ag85B-ESAT6 + 1µg LT		Intranasal
21	All groups			Sample bleed	
50	1, 2, 3 and 4	5	Sample bleed, lung and nasal washes and spleens	END	
50	1a	5	PBS	Boost	Intranasal
	2a	5	PBS		Intranasal
	2b	5	20µg LTK63		Intranasal
	2c	10	25µg Ag85B-ESAT6 + 20µg LTK63		Intranasal
	3a	5	PBS		Intranasal
	3b	5	20µg LTK63		Intranasal
	3c	5	25µg Ag85B-ESAT6 + 20µg LTK63		Intranasal
57	1a, 2a, 2b, 2c, 3a, 3b and 3c	5	Sample bleed, lung and nasal washes and spleens	END	
120	2, 2c, 3 and 4	5	Sample bleed, lung and nasal washes and spleens	END	

Figure 6.9: Time-course of the serum Ig anti-Ag85B-ESAT6 antibody response in mice following oral *Salmonella* prime and intranasal protein boost.

As outlined in Table 6.5 C57BL/6 were orally vaccinated with approximately 5 x 10^9 CFU of SL3261mycolacZ on day 0 and then boosted with 25µg Ag85B-ESAT6 + 20µg LTK63 or appropriate antigen controls on day 50. Negative control mice were immunised with wild-type SL3261 or PBS, and then also boosted on day 50 with the suitable antigens. Positive controls received 1µg LT plus 25µg Ag85B-ESAT6 intranasally at day 0. Mice were left for 21, 50, 57 and 120 days and then sample bled to determine anti-Ag85B-ESAT6 specific Ig antibodies, which were determined by ELISA. Total serum Ig titres (A day 21, B day 50, C day 57 and D day 120) from naïve and immunised animals are expressed in total antibody titres using a cut off of OD 0.3. The black bar shows the geometric mean from the group with the * indicates significant values of $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ as determined using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test compared to negative controls. ■ represents naïve (PBS immunised) animals, ▲ indicates animals immunised SL3261mycolacZ ◆ represents mice vaccinated with the SL3261 parental control strain and ● shows the positive control mice, i.e. those immunised with 1µg LT plus 25µg Ag85B-ESAT6. After priming with SL3261mycolacZ ▽ indicates a further boost with 20µg LTK63 and × shows mice intranasally boosted with 20µg LTK63 plus 25µg Ag85B-ESAT6. Those animals primed with the negative SL3261 control strain also received a 20µg LTK63 boost shown by ◇ and × shows mice receiving the 20µg LTK63 plus 25µg Ag85B-ESAT6 intranasal boost.

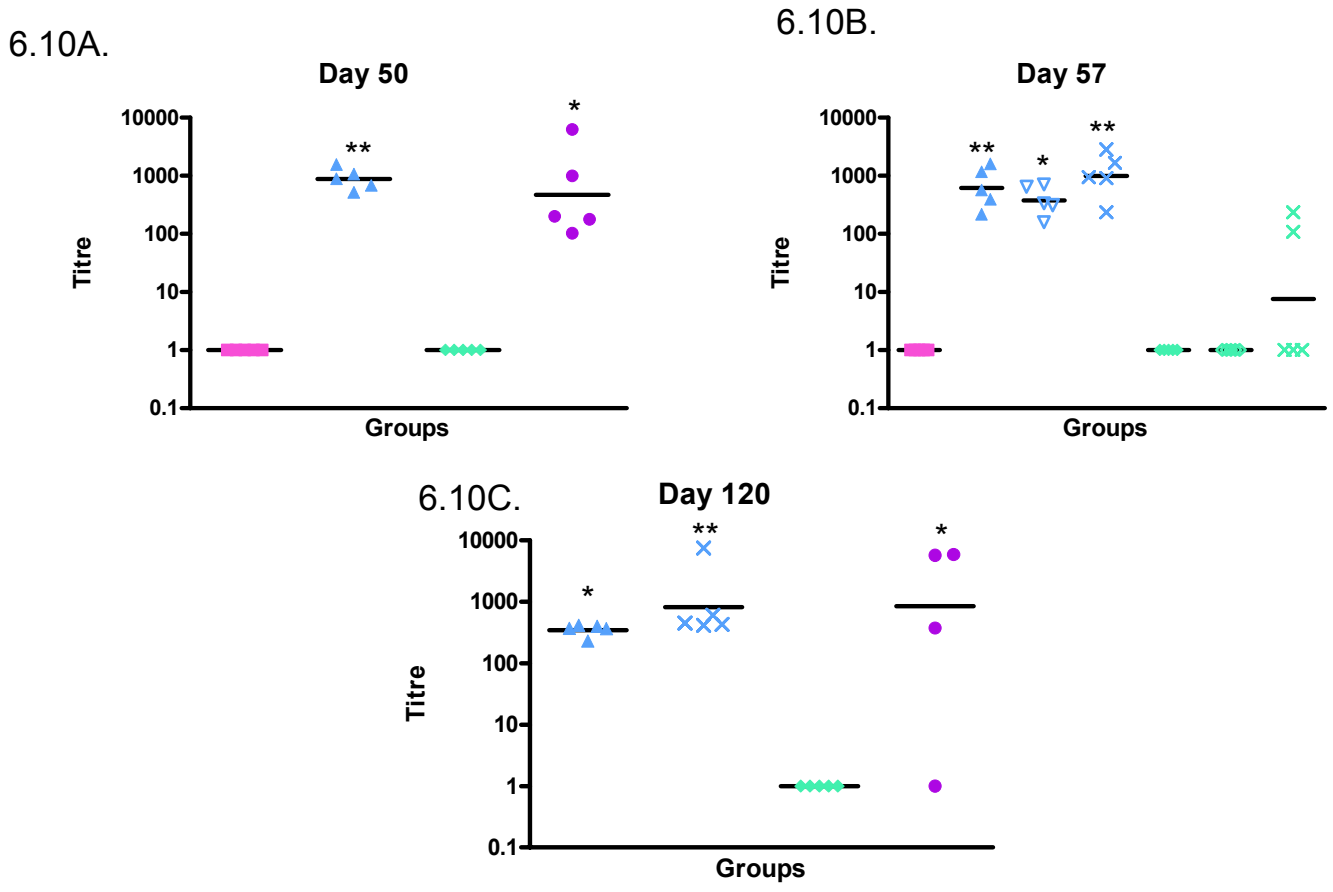


6.3.2.2.1 Serum IgG antibody responses

In order to determine if vaccination with SL3261mycolacZ was sufficient to induce humoral IgG antibodies, mice were primed with SL3261mycolacZ and boosted intranasally as depicted in Table 6.5. Responses to Ag85B-ESAT6 were seen in serum following immunisation and boosting intranasally with Ag85B-ESAT6 and adjuvant LTK63. All mice receiving SL3261mycolacZ, plus or minus an intranasal boost (Group 2, 2a, 2b and 2c), seroconverted to high and significant IgG anti-Ag85B-ESAT6 titres at day 50 and throughout the experimental period including the positive control group 4 when compared to negative control animals (Groups 1, 1a, 3, 3a, 3b, 3c). Again, IgG responses in serum tended only to be slightly higher, but not significantly so, following immunisation and boosting (Group 2c), compared to those mice receiving SL3261mycolacZ only (Group 2) (Figure 6.10).

Figure 6.10: Time-course of the serum IgG anti-Ag85B-ESAT6 antibody responses.

As outlined in Table 6.5 C57BL/6 were orally vaccinated with approximately 5 x 10^9 CFU of SL3261mycolacZ on day 0 and then boosted with 25µg Ag85B-ESAT6 + 20µg LTK63 or appropriate antigen controls on day 50. Negative control mice were immunised with wild-type SL3261 or PBS, and then also boosted on day 50 with the suitable antigens. Positive controls received 1µg LT plus 25µg Ag85B-ESAT6 intranasally at day 0. Mice were left for 50, 57 and 120 days and then sample bled to determine anti-Ag85B-ESAT6 specific Ig antibodies, which were determined by ELISA. Serum IgG titres (A day 50, B day 57, and C day 120) from naïve and immunised animals are expressed in total antibody titres using a cut off of OD 0.3. The black bar shows the geometric mean from the group with the * indicates significant values of $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ as determined using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test compared to negative controls. ■ represents naïve (PBS immunised) animals, ▲ indicates animals immunised SL3261mycolacZ ◆ represents mice vaccinated with the SL3261 parental control strain and ● shows the positive control mice, i.e. those immunised with 1µg LT plus 25µg Ag85B-ESAT6. After priming with SL3261mycolacZ ▽ indicates a further boost with 20µg LTK63 and × shows mice intranasally boosted with 20µg LTK63 plus 25µg Ag85B-ESAT6. Those animals primed with the negative SL3261 control strain also received a 20µg LTK63 boost shown by ◇ and × shows mice receiving the 20µg LTK63 plus 25µg Ag85B-ESAT6 intranasal boost.

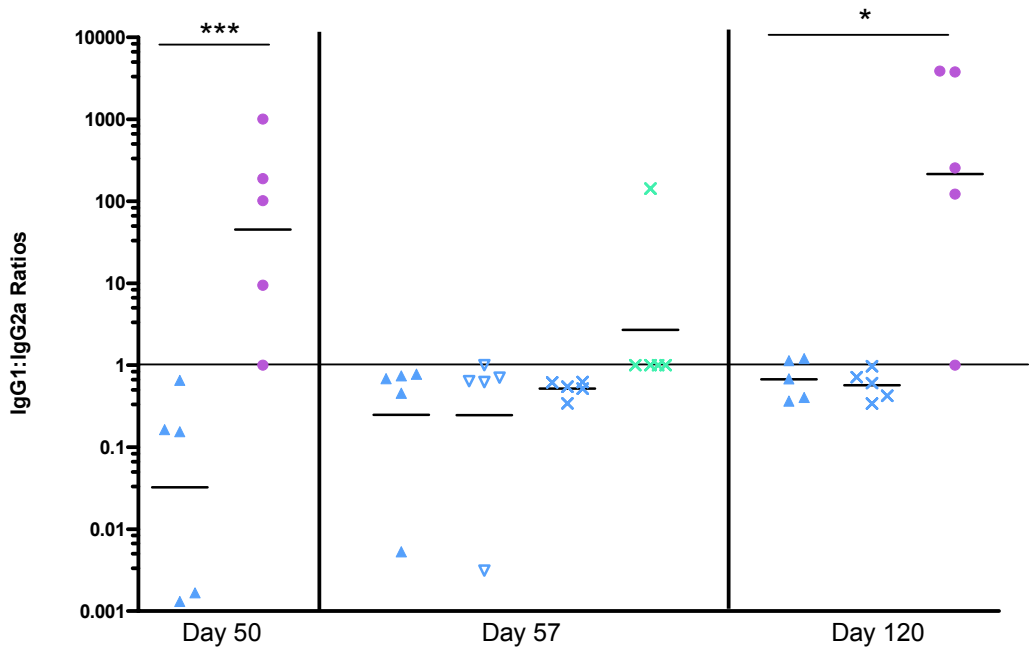


6.3.2.2.2 IgG sub-class responses

Sera prepared on days 50, 57 and 120 from primed and/or boosted mice were further analysed to ascertain Ag85B-ESAT6-specific IgG_1:IgG_{2a} sub-class ratios as an indirect assessment of the T-helper cell response bias (Figure 6.11). On all days, mice primed with SL3261mycolacZ (Groups 2) and boosted with PBS, LTK63 or Ag85B-ESAT6 plus LTK63 (Groups 2a, 2b and 2c) were found to be more IgG2a-biased, as depicted by the low IgG1:IgG2a ratios. However, between these groups there was no development of IgG_1:IgG_{2a} profiles that were statistically different. Mice vaccinated with the SL3261 and then boosted with adjuvant and Ag85B-ESAT6 (Group 3c) had an IgG1 biased response, however it was not statistically different from groups 2, 2a, 2b and 2c. Notably, mice dosed once with the adjuvant LT and Ag85B-ESAT6 (Group 4) engendered a significantly greater proportion of IgG_1-specific anti-Ag85B-ESAT6 antibodies than those primed with SL3261mycolacZ (Groups 2 and 2c) on both days 50 and 120 ($p < 0.001$ on day 50 and $p < 0.05$ on day 120).

Figure 6.11: Day 50, 57 and 120 serum anti-Ag85B-ESAT6 IgG1:IgG2a responses.

IgG1:IgG2a anti-Ag85B-ESAT6 responses were estimated by ELISA with sera prepared on days 50, 57 and 120 from C57BL/6 mice primed with SL3261mycolacZ (Group 2 ( ), mice primed and boosted with LTK63 (Group 2b ( ), mice primed and boosted with LTK63 plus Ag85B-ESAT6 (Group 2c ( ), mice primed with parental *Salmonella* strain and boosted with LTK63 and Ag85B-ESAT6 (Group 3c ( ) and mice intranasally immunised with adjuvant LT and Ag85B-ESAT6 (Group 4 ( ). Negative control mice i.e. PBS and SL3261 immunised had no detectable IgG subtype titres (data not shown). Statistical significance was determined by using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test (* $p < 0.05$ and *** $p < 0.001$).

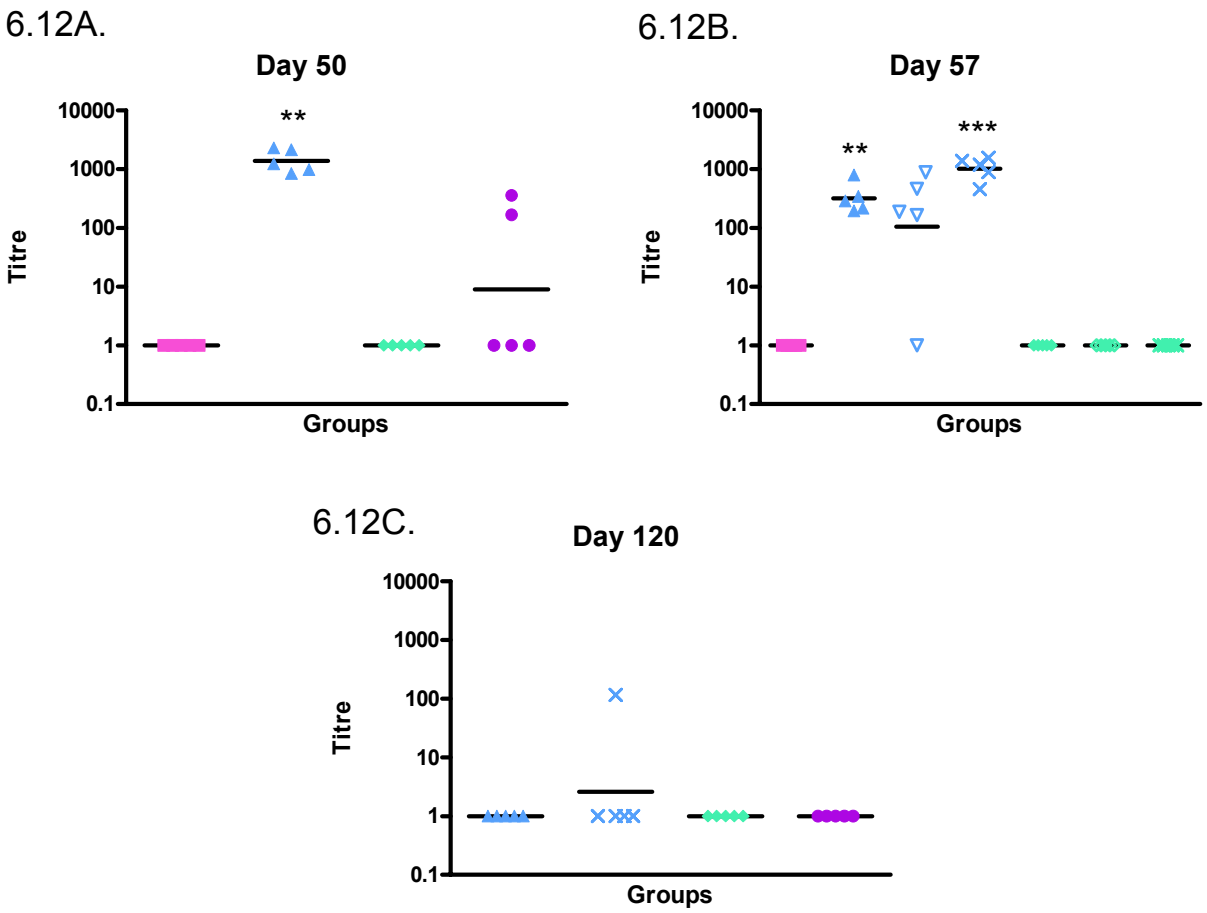


6.3.2.2.3 Serum IgA responses.

For a number of years the humoral response was considered non-protective against a number of intracellular pathogens (Andersen 1997; Collins 1991; Dunlap and Briles 1993). Nevertheless, antibodies can protect against many viruses, all of which are obligate intracellular pathogens, or even against more complex intracellular micro-organisms such as *Toxoplasma gondii*. Reviewed in (Burton 2002; Mineo, et al. 1994). Although the role of antibody mediated immunity in protection against *M. tuberculosis* remains uncertain, exposure to *M. tuberculosis* does elicit the production of antibodies to several antigens (Laal, et al. 1997; Samanich, et al. 2001). In fact a number of studies have shown that IgA deficient mice are more susceptible to *M. tuberculosis* infection (Rodriguez, et al. 2005; Tjarnlund, et al. 2006). With this research in mind, systemic IgA response following administration of the SL3261mycolacZ inoculum was also analysed (see Table 6.5 for immunisation regimen). The levels of anti-Ag85B-ESAT6 IgA in the serum of the eleven groups of mice is shown in Figure 6.12. As already described for the systemic total Ig and IgG response, the anti-Ag85B-ESAT6 IgA was significantly higher in mice immunised with SL3261mycolacZ (groups 2, 2a, and 2c) when compared to negative control animals (groups 1, 1a, 3, 3a, 3b and 3c). However, only 80% of the mice that received SL3261mycolacZ plus an LTK63 boost (group 2b) seroconverted and were found not to be significantly different from the negative control mice. The anti-Ag85B-ESAT6 IgA response in positive control mice (group 4) was 100-fold lower than that elicited by the other groups immunised with the candidate vaccine and only 40% of animals seroconverted (Figure 6.12A). Nasal boosting with antigen and adjuvant (group 2c) appeared to further enhance induction of anti-Ag85B-ESAT6 IgA in serum at day 57 (Figure 6.12B). At the final time point (day 120), the titres of anti-Ag85B-ESAT6 IgA induced by immunisation with SL3261mycolacZ reached levels similar to those obtained from the negative control mice (i.e. undetectable). Notably, only one mouse in group 2c still had detectable IgA levels at this time point (Figure 6.12C).

Figure 6.12: Time-course of the serum IgA anti-Ag85B-ESAT6 antibody responses.

As outlined in Table 6.5 C57BL/6 were orally vaccinated with approximately 5 x 10^9 CFU of SL3261mycolacZ on day 0 and then boosted with 25µg Ag85B-ESAT6 + 20µg LTK63 or appropriate antigen controls on day 50. Negative control mice were immunised with wild-type SL3261 or PBS, and then also boosted on day 50 with the suitable antigens. Positive controls received 1µg LT plus 25µg Ag85B-ESAT6 intranasally at day 0. Mice were left for 50, 57 and 120 days and then sample bled to determine anti-Ag85B-ESAT6 specific Ig antibodies, which were determined by ELISA. Serum IgA titres (A day 50, B day 57, and C day 120) from naïve and immunised animals are expressed in total antibody titres using a cut off of OD 0.3. The black bar shows the geometric mean from the group with the * indicates significant values of $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ as determined using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test compared to negative controls. ■ represents naïve (PBS immunised) animals, ▲ indicates animals immunised SL3261mycolacZ ◆ represents mice vaccinated with the SL3261 parental control strain and ● shows the positive control mice, i.e. those immunised with 1µg LT plus 25µg Ag85B-ESAT6. After priming with SL3261mycolacZ ▽ indicates a further boost with 20µg LTK63 and × shows mice intranasally boosted with 20µg LTK63 plus 25µg Ag85B-ESAT6. Those animals primed with the negative SL3261 control strain also received a 20µg LTK63 boost shown by ◇ and × shows mice receiving the 20µg LTK63 plus 25µg Ag85B-ESAT6 intranasal boost.

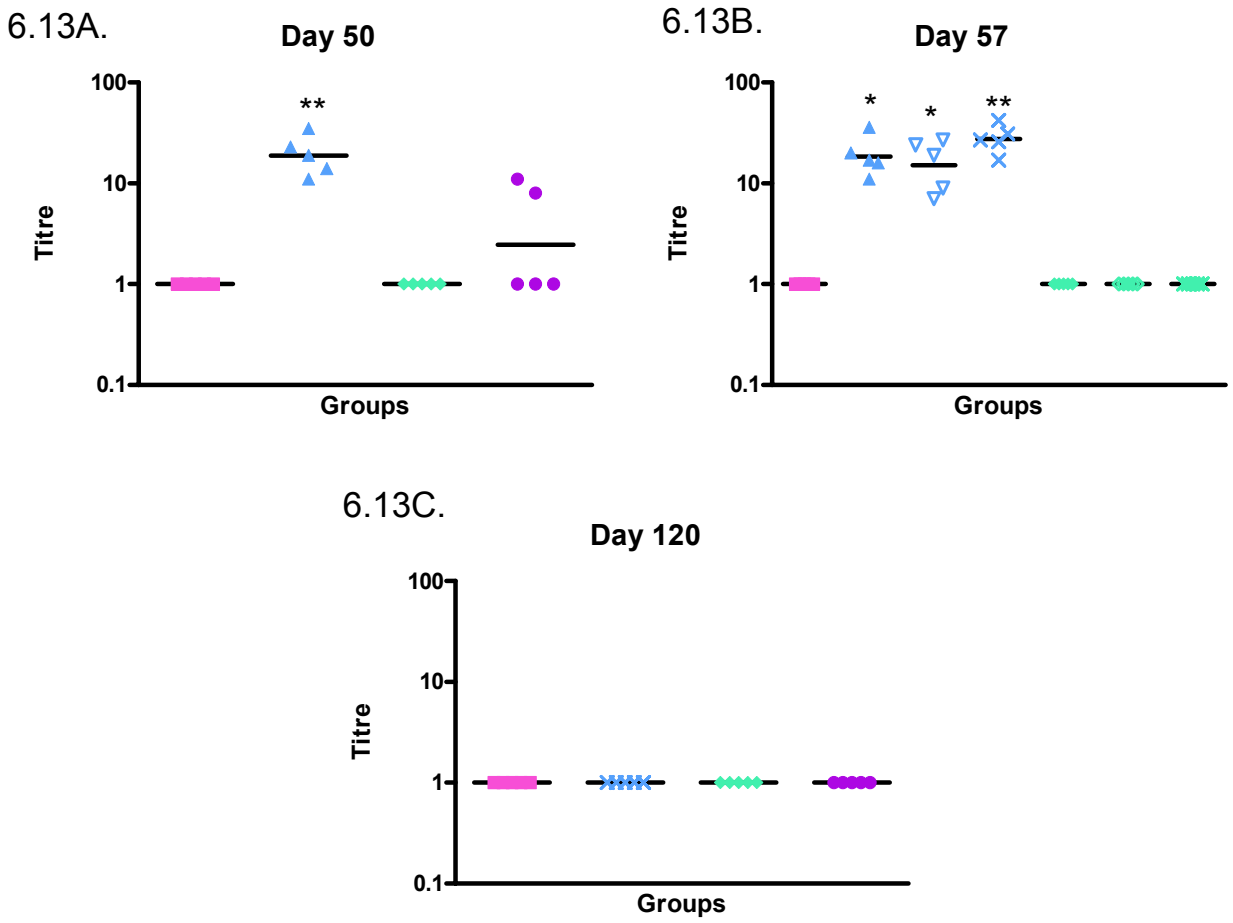


6.3.2.2.4 Mucosal immune responses.

Secretory IgA (sIgA), being prominent at mucosal surfaces, plays an important role in the early defensive mechanisms against invading pathogens in the gastrointestinal, respiratory and urogenital tracts. sIgA can bind and intercept invading pathogens in the mucosal fluids, leading to their neutralisation or 'exclusion' of the infection by a number of mechanisms including; the agglutination of microbes, inhibition of their motility, blocking of their attachment to the mucosal epithelium by targeting bacterial adhesins, clearance of microbial products and activation of phagocytic cells. Reviewed in (Lamm 1997; Monteiro and Van De Winkel 2003; van Egmond, et al. 2001). A recent study reported that an intranasally administered monoclonal IgA antibody significantly reduced the *M. tuberculosis* load in the infected lungs, and that this protective effect of IgA could be further extended by co-inoculation with IFN-γ (Reljic, et al. 2006). Since vaccination through the oral and nasal route has the potential to induce mucosal protective responses, I investigated the induction of IgA in nasal and lung washes of mice immunised with SL3261mycolacZ vaccine. I also compared the levels of mucosal IgA after the intranasal boosting. Table 6.5 summaries the immunisation schedule. Mucosal Ag85B-ESAT6 specific IgA titres were demonstrated in both nasal and lung washes following immunisation with SL3261mycolacZ (group 2); responses were readily detected 50 days after the first dose (day 0) and were still present 7 days after boosting (day 57) (Figures 6.13A and B respectively). However, no mucosal IgA was observed in any groups after completion of the experiment at day 120 (Figure 6.13C). A slightly higher titre of IgA was observed in lung and nasal washes from mice immunised with SL3261mycolacZ and boosted intranasally with antigen plus adjuvant (group 2c) than in mucosal washes from those receiving SL3261mycolacZ with or without the negative control boosts (groups 2, 2a and 2b). No IgA antibodies were found in mice immunised with SL3261 (group 3) or PBS (group 1) (negative controls) including those mice receiving wild-type SL3261 plus LTK63 and Ag85B-ESAT6 boost (group 3c). An important observation was that systemic IgA (Figure 6.12) was 10- 100-fold higher in serum compared to those titres detected in mucosal washes (Figure 6.13).

Figure 6.13: Mucosal IgA induced by immunisation with *Salmonella* live vector vaccine.

As outlined in Table 6.5 C57BL/6 were orally vaccinated with approximately 5 x 10^9 CFU of SL3261mycolacZ on day 0 and then boosted with 25µg Ag85B-ESAT6 + 20µg LTK63 or appropriate antigen controls on day 50. Negative control mice were immunised with wild-type SL3261 or PBS, and then also boosted on day 50 with the suitable antigens. Positive controls received 1µg LT plus 25µg Ag85B-ESAT6 intranasally at day 0. Lung and nasal washes were obtained on day 50 (A), day 57 (B) and on day 120 (C). Ag85B-ESAT6-specific IgA responses were measured by ELISA. Washes isolated from mice immunised with SL3261 parental strain or PBS served as negative controls. The black bar shows the geometric mean from the group with the * indicates significant values of $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ as determined using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test compared to negative controls. ■ represents naïve (PBS immunised) animals, ▲ indicates animals immunised SL3261mycolacZ ◆ represents mice vaccinated with the SL3261 parental control strain and ● shows the positive control mice, i.e. those immunised with 1µg LT plus 25µg Ag85B-ESAT6. After priming with SL3261mycolacZ ▽ indicates a further boost with 20µg LTK63 and × shows mice intranasally boosted with 20µg LTK63 plus 25µg Ag85B-ESAT6. Those animals primed with the negative SL3261 control strain also received a 20µg LTK63 boost shown by ◇ and × shows mice receiving the 20µg LTK63 plus 25µg Ag85B-ESAT6 intranasal boost.



6.3.2.3 Evaluation of cytokine responses after recombinant *Salmonella* vaccination

Cytokines are key mediator molecules in the expression of acquired immunity in response to *M. tuberculosis* infection, with a range of cytokine needed for protection both during the active and latent stages of disease. T cells are the mediators of immunity, with macrophages being the effector cells. It is the production of cytokines by T cells, in response to infection, that activates resting macrophages harbouring mycobacteria within their phagosomes to induce antibacterial mechanisms, including reactive oxygen and nitrogen intermediates which can kill or arrest *M. tuberculosis* growth. Reviewed in (Flesch and Kaufmann 1993). The cytokine IFN-γ is the central mediator of macrophage activation (Raupach and Kaufmann 2001). However, the role of cytokines to control mycobacterial growth is complex, with some having activating and some deactivating properties. During acute infection IL-2, IL-12 and IFN-γ are indicative of a protective T_H1 type immune response with IL-6 also being implicated. Whereas T_H2 type immune responses characterised by IL-4, IL-5 and IL-10 have been described as cross regulatory as they promote antibody production and can inhibit macrophage activation. However, a number of studies have shown that after the initial T_H1 protective immune response, a later T_H2 type response may serve to limit the inflammatory response and minimise tissue damage at the site of infection Reviewed in (Kaufmann 2001; Rook and Hernandez-Pando 1996; Rook, et al. 2001). It is therefore the balance between both these anti-inflammatory and pro-inflammatory cytokines throughout the course of infection that determines the outcome of the disease.

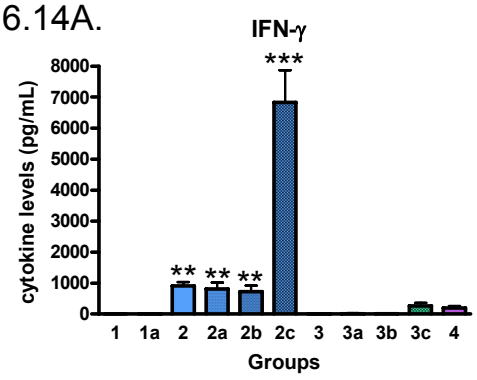
To analyse the T-helper type of immune response induced in the different groups of immunised mice, I evaluated the cytokines secreted in cell culture supernatants derived from splenocytes after *in vitro* restimulation with the specific antigen (purified Ag85B-ESAT6 protein). CBA was used to determine the secreted levels of the cytokines; IFN-γ, IL-4, IL-6, and IL-2 and IL-5. Figure 6.14 illustrates the mean concentrations (pg/mL) of the various cytokines secreted from spleen cells for each group. As seen in Figure 6.14A, high and significant levels of IFN-γ secretion were observed from those groups of mice receiving SL3261mycolacZ plus or minus a boost (groups 2, 2a, 2b and 2c) when compared to both the negative control mice (groups 1,

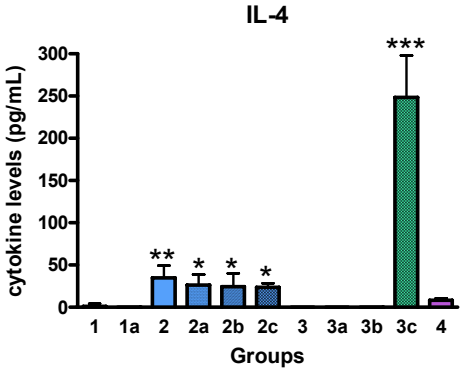
1a, 3, 3a, 3b and 3c) as well as the positive control group (group 4) on days 50 and 57. In fact, the increase in IFN-γ secretion by mice primed with SL3261mycolacZ and boosted with Ag85B-ESAT6 plus LTK63 (group 2c) was significantly higher when compared to those just immunised with SL3261mycolacZ alone (group 2) ($p < 0.001$ compared to $p < 0.01$ respectively). Moreover, IFN-γ levels persisted throughout the experimental period up until day 120, with those mice vaccinated and boosted showing significantly higher levels than any of the negative control mice (Figure 6.14C). In contrast, spleen cells from groups 2, 2a, 2b and 2c secreted low, but significant, levels of IL-4 ($p < 0.05$) when compared to groups all other groups (except group 3c) at day 50 and 57. IL-4 production from mice receiving SL3261 plus Ag85B-ESAT6 and LTK63 intranasal boost (group 3c) was significantly higher than any other experimental group ($p < 0.001$) also at this time-point (Figure 6.14A). At day 120, mice in groups 2 still had modest but significant IL-4 levels, along with groups 2c and 4. Levels of IL-2 secreted in splenocyte supernatants were significantly increased 50 and then 120 days after oral immunisation with the SL3261mycolacZ and 7 and 70 days after the intranasal boosting (day 57 and day 120) when compared to groups 1, 1a, 3, 3a and 3b (Figure 6.14A and C). Notably, IL-2 levels as seen for IFN-γ levels were observed to be significantly higher in group 2c, compared to group 2 at these time-points. High levels of IL-6 were also observed in mice immunised with SL3261mycolacZ alone at day 50, with those animals primed and boosted found to have a further significant increase of IL-6 (day 57) over the vector alone immunised animals ($p < 0.01$ compared to $p < 0.05$) (Figure 6.14A). As was observed for IL-4 cytokine levels, IL-6 production from mice in group 3c was found to be significantly higher than any of the experimental groups ($p < 0.001$) (Figure 6.14A). IL-6 levels continued to remain significantly higher throughout the study in groups 2c and 4 up until day 120 (Figure 6.14C). Lastly, levels of IL-5 secreted from those experimental groups receiving Ag85B-ESAT6 either through SL3261mycolacZ (groups 2, 2a, 2b and 2c) or purified protein (group 3c) were also significantly higher when compared to the negative control group's at all experimental time-points, with group 4 only showing significant levels at day 120 (Figure 6.14A and C).

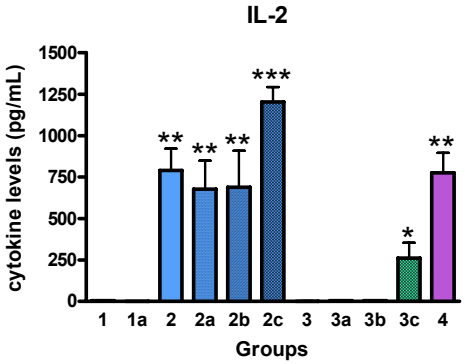
Figure 6.14: Ag85B-ESAT6-specific cytokine responses in mice immunised with *Salmonella* live vector vaccines.

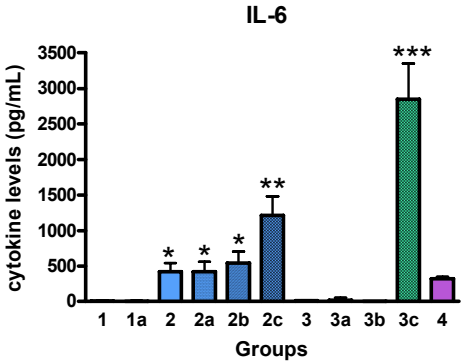
(A) Mice were immunised as described in Table 6.5 with spleens harvested on day 50 and 57, (C) and 120. Cytokine responses were measured upon *in vitro* stimulation with Ag85B-ESAT6 for 36-42 hours. (B) Cells were also stimulated with conA (positive control) and $RPMI^+$ media (negative control). Columns represent the mean (± SD) stimulation indices of splenocytes from five animals per group. The increase in cytokine levels between SL3261mycolacZ-primed, intranasally-boosted mice, compared to negative control mice, is indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) as determined using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test. The sensitivities of the CBA was >1pg/mL for each cytokine. For Figures 6.14A and C, (1 and 1a) pink represents naïve (PBS immunised) animals, (2) blue indicates animals immunised with SL3261mycolacZ vaccine, (3) green represents mice vaccinated with the SL3261 parental control strain and, (4) purple shows the positive control mice, i.e. those immunised with 1µg LT plus 25µg Ag85B-ESAT6. After priming with SL3261mycolacZ, (2a) dotted blue shows a PBS boost, (2b) blue lines indicates a further boost with 20µg LTK63 and, (2c) blue hatched shows mice intranasally boosted with 20µg LTK63 plus 25µg Ag85B-ESAT6. (3a) those animals primed with the negative SL3261 control strain also received a PBS boost indicated by the dotted green, (3b) 20µg LTK63 boost shown by green lines and, (3c) green hatched shows mice receiving the 20µg LTK63 plus 25µg Ag85B-ESAT6 intranasal boost. For Figure 4.14B, block colours represent conA stimulated splenocytes with diagonal lines indicating $RPMI^+$ stimulated splenocytes. Red shows IFN-γ, blue IL-4, pink IL-2, green IL-6 and purple IL-5.

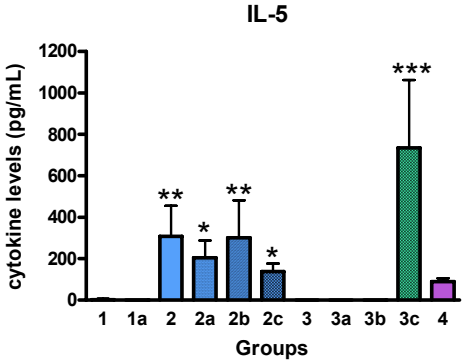
6.14A.


IFN-γ
cytokine levels (pg/mL)
8000
7000
6000
5000
4000
3000
2000
1000
0
1 1a 2 2a 2b 2c 3 3a 3b 3c 4
Groups

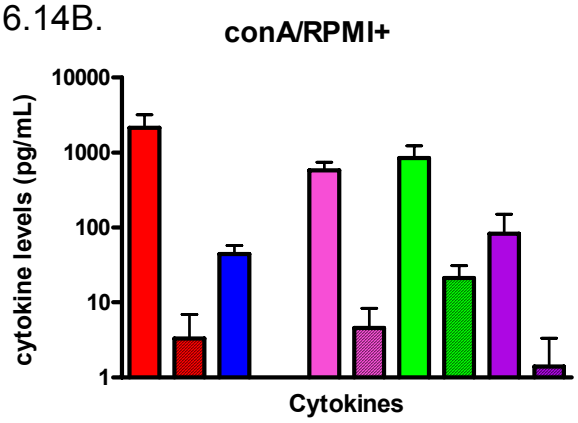

IL-4
cytokine levels (pg/mL)
300
250
200
150
100
50
0
1 1a 2 2a 2b 2c 3 3a 3b 3c 4
Groups


IL-2
cytokine levels (pg/mL)
1500
1250
1000
750
500
250
0
1 1a 2 2a 2b 2c 3 3a 3b 3c 4
Groups

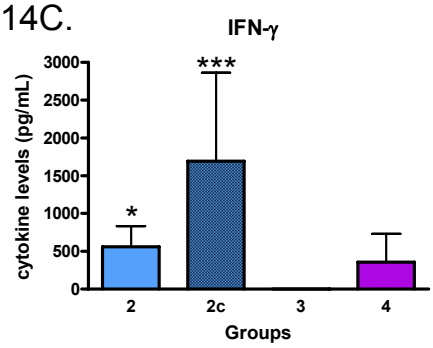

IL-6
cytokine levels (pg/mL)
3500
3000
2500
2000
1500
1000
500
0
1 1a 2 2a 2b 2c 3 3a 3b 3c 4
Groups

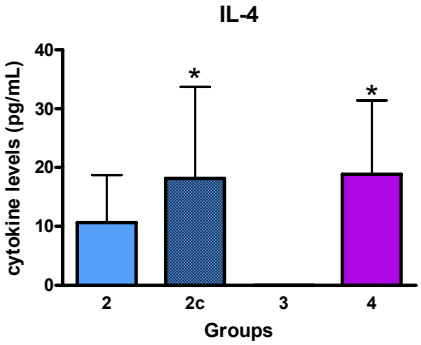

IL-5
cytokine levels (pg/mL)
1200
1000
800
600
400
200
0
1 1a 2 2a 2b 2c 3 3a 3b 3c 4
Groups

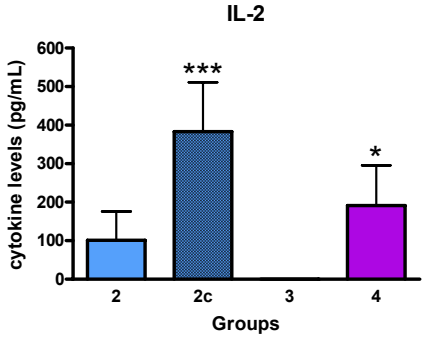
6.14B.

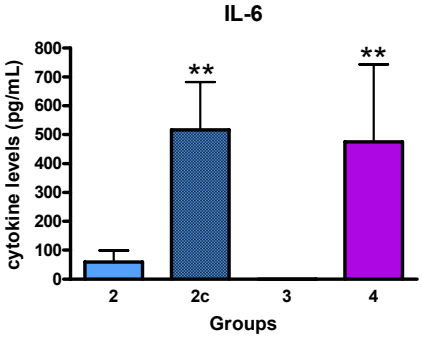

conA/RPMI+
cytokine levels (pg/mL)
10000
1000
100
10
1
Cytokines

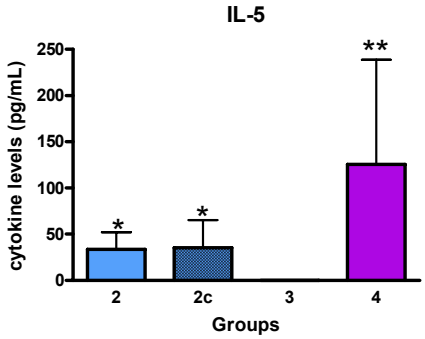
6.14C.


IFN-γ
cytokine levels (pg/mL)
3000
2500
2000
1500
1000
500
0
*
***
2
2c
3
4
Groups


IL-4
cytokine levels (pg/mL)
40
30
20
10
0
*
*
2
2c
3
4
Groups


IL-2
cytokine levels (pg/mL)
600
500
400
300
200
100
0
***
*
2
2c
3
4
Groups


IL-6
cytokine levels (pg/mL)
800
700
600
500
400
300
200
100
0
**
**
2
2c
3
4
Groups

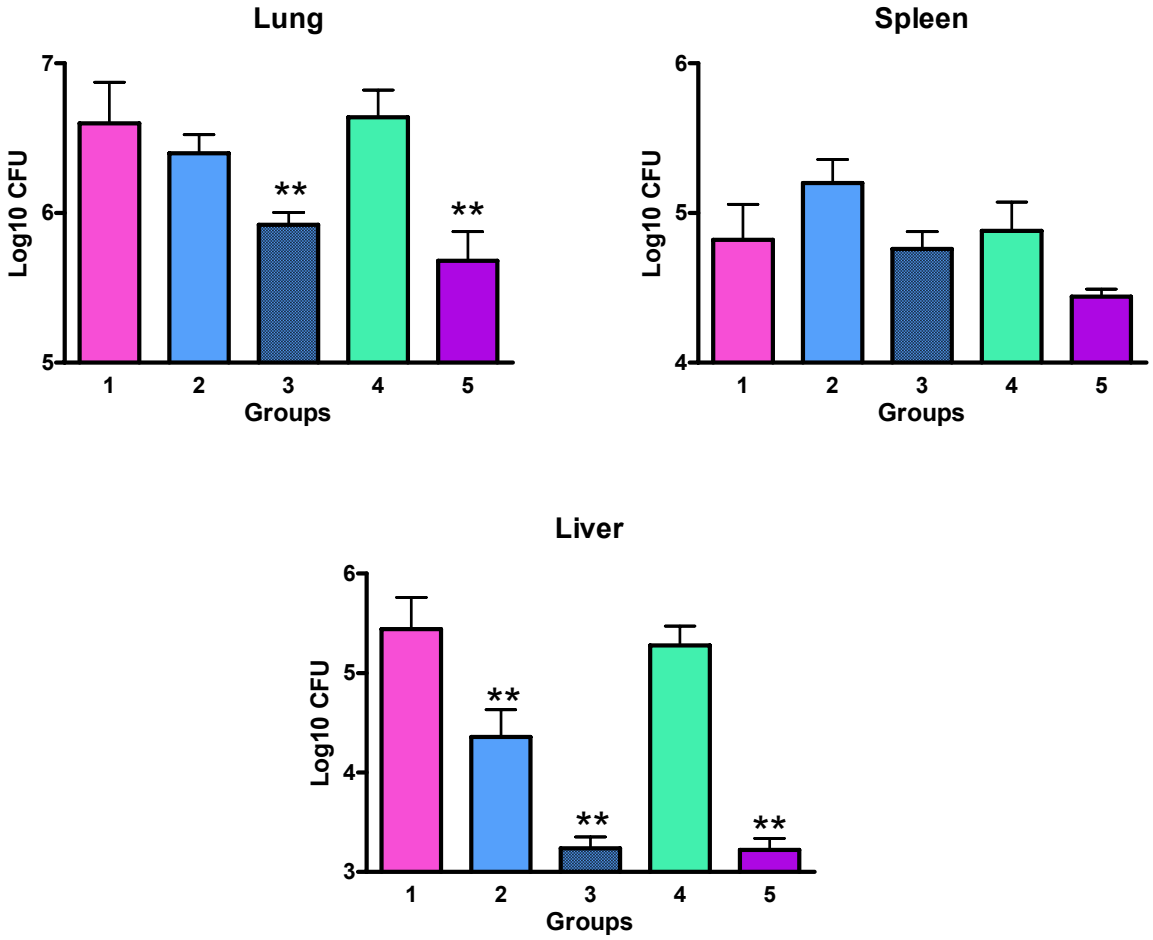

IL-5
cytokine levels (pg/mL)
250
200
150
100
50
0
*
*
**
2
2c
3
4
Groups

6.3.2.4 Protective efficacy of the recombinant *Salmonella* vaccine candidate

Challenge experiments were performed by Dr Jes Dietrich (Statens Serum Institute, Denmark) to evaluate whether the enhanced immune responses to Ag85B-ESAT6 observed following mucosal priming with SL3261mycolacZ, and boosting intranasally with Ag85B-ESAT6 in combination with LTK63, conferred protection against *M. tuberculosis* challenge. One group of C57BL/6 mice were immunised with SL3261mycolacZ alone with another receiving SL3261mycolacZ plus an intranasal boost with purified Ag85B-ESAT6 in LTK63 twenty days after the initial oral vaccination. Mice in the negative control groups received either the parental strain of SL3261 or were left unvaccinated. As the vaccine BCG has consistently demonstrated good efficacy against *M. tuberculosis* infection in animal models, BCG was included in the experiment as a "gold standard" against which efficacy can be assessed. Four weeks after the last vaccination, the mice were subjected to an aerosol challenge with virulent *M. tuberculosis*. Another five weeks after challenge, the mice were killed and the bacterial numbers were determined in the lungs, spleen and liver. The results (Figure 6.15) showed that vaccination with SL3261mycolacZ lead to a bacterial (*M. tuberculosis*) burden of 6.4 ± 0.12 $\log_{10}$ CFU in the lungs, which wasn't significantly different than the bacterial burden observed in naïve and SL3261 vaccinated mice. Mice receiving SL3261mycolacZ plus the intranasal boost showed a bacterial burden of 5.9 ± 0.09 $\log_{10}$ CFU, significantly lower than those mice in the negative control groups ($p < 0.01$) but not significantly different from BCG-vaccinated mice. In the spleen none of the immunised mice showed a significance difference in *M. tuberculosis* burden including those vaccinated with BCG when compared to those mice in the negative control groups. All mice vaccinated with SL3261mycolacZ with or without the intranasal boost were significantly different from both the naïve group and those mice receiving the SL3261 control strain in the liver ($p < 0.01$). Notably, the bacterial burden in those mice immunised with SL3261mycolacZ alone (4.35 ± 0.2 $\log_{10}$ CFU) was still significantly higher ($p < 0.01$) when compared to the BCG vaccinated animals (3.25 ± 0.25 $\log_{10}$ CFU) and those mice primed and intranasally boosted (3.2 ± 0.11 $\log_{10}$ CFU).

Figure 6.15: Bacterial burden in vaccinated mice challenged with *M. tuberculosis*.

Bacterial burden in vaccinated C57BL/6 mice (expressed as $\log_{10}$ CFU ± SD) compared with unvaccinated (naïve) and SL3261 parental immunised controls challenged by the aerosol route with pathogenic *M. tuberculosis* 8 weeks after the first vaccination. Five weeks post challenge, the mice were killed and the bacterial burden (CFU) was measured in the lungs, spleen and liver. The reduction in bacterial numbers in BCG vaccinated and SL3261mycolacZ-primed, intranasally-boosted mice, compared to negative control mice, is indicated (*, $p < 0.05$; **, $p < 0.01$) as determined by the Kruskal-Wallis test followed by Dunn's Multiple Comparison test). (1) pink columns represent naïve (PBS immunised mice), (2) blue indicates mice vaccinated with SL3261mycolacZ, (3) blue hatched columns indicate SL3261mycolacZ and an intranasal boost with LTK63 and Ag85B-ESAT6, (4) green shows mice vaccinated with the SL3261 parental strain and, (5) finally purple represents BCG immunised animals.



6.3.3 Discussion

In the present study I have shown that a heterologous prime-boost strategy utilising oral priming with a live attenuated recombinant *S.* Typhimurium SL3261 strain (SL3261mycolacZ), plus intranasal boosting with Ag85B-ESAT6 and the adjuvant LTK63, reduced numbers of tubercle bacilli in the lungs and livers throughout the course of *M. tuberculosis* infection comparable to BCG vaccinated control animals.

Vaccination remains one of the most effective methods for managing infectious diseases and diminishing their negative economic impact. Ideally a vaccine should elicit innate and adaptive immune responses that result in protection of vaccinated animals against a challenge by the targeted pathogen. In addition, a vaccine should be easily administered and its use economically feasible (WHO 2007b).

Attenuated *Salmonella* are an attractive means of delivering recombinant antigens for a number of reasons; they are inexpensive to manufacture, they are practical for large scale distribution, mucosal delivery of antigen is possible (i.e. oral), and the ability of *Salmonella* to colonise GALT and invade via the intestinal mucosa means they can induce both mucosal and systemic immune responses. As bacterial vectors, both *S.* Typhi and *S.* Typhimurium have been extensively studied using many bacterial, viral and protozoan antigens in both animal models and humans. Reviewed in (Kraehenbuhl JP 1998; Sirard, et al. 1999). As already discussed, immunisation at one mucosal inductive site (e.g., intestinal PP) can lead to an immune response at another, anatomically remote, mucosal effector site (e.g., lungs) due to the sub-networks that exist within the mucosal immune system, in particular the GALT-lung sub-network (Allen, et al. 2000; Challacombe, et al. 1997; Forrest, et al. 1991; Ruedl, et al. 1994). I reasoned, therefore, that a *Salmonella*-based live vector vaccine could be an excellent candidate to prime immune responses by delivering the foreign fusion antigen, Ag85B-ESAT6, directly into APC, while providing a strong T_H1 cytokine milieu and other immunomodulatory signals that are important for clearance and protection.

Heterologous antigen delivery by *Salmonella* is largely dependent upon antibiotic selectable genes or by alternative methods, including no antibiotic-based vectors, balanced lethal stabilisation systems, or by chromosomal insertions. Owing to the apparent drawbacks of stability and antibiotic gene leakage, the antibiotic/plasmid-based vector delivery systems means that further development for human use is doubtful (Bowe, et al. 2003). Chromosomal-dependent delivery systems do have promise as they are more stable, and constitutive expression of the protein of interest may allow enough antigen to induce potent immune responses. In fact, phase I human trails using the *S.* Typhi vaccine candidate ZH9 expressing LT-B and HepB core antigens were found to be safe and immunogenic, again highlighting the advantages of utilising this antigen delivery system (Khan, et al. 2007; Microscience 2004a; Microscience 2004b).

I have successfully cloned the tuberculosis fusion antigen Ag85B-ESAT6 into the chromosomal *phoN* gene of the attenuated *Salmonella* vaccine strain SL3261. The *ag85B-esat6* gene is under the constitutive control of the *lacZ* promoter with strong Ag85B-ESAT6 production readily detectable under *in vitro* growth conditions. Previously, Hess and colleagues used *S.* Typhimurium to express and secrete the *M. tuberculosis* protein Ag85B. They transformed SL7027 with a plasmid encoding the HlyB/HlyD/TolC export machinery (from *E. coli*) and the *ag85b* gene. Those mice vaccinated with the recombinant *Salmonella* strain were offered partial protection after intravenous challenge with *M. tuberculosis* (Hess, et al. 2000). A further study also used *S.* Typhimurium as a vector to export another *M. tuberculosis* antigen, ESAT-6, via the haemolysin (HlyA) secretion system. Immunised mice were found to have reduced numbers of tubercle bacilli in the lungs throughout the course of *M. tuberculosis* infection (Mollenkopf, et al. 2001). These previous studies have utilised plasmid based expression systems. However as already discussed, the use of multicopy plasmids has several physical and regulatory implications in the generation of vaccines intended for use in humans. In this present study I have overcome this stability issue by inserting the heterologous sequence into the bacterial chromosome. Therefore this expression system would hopefully be a more realistic basis for an efficient *Salmonella* delivery system of Ag85B-ESAT6 for use in humans based upon a *S.* Typhi construct.

Salmonella as well as *M. tuberculosis* are confined to the phagosome of macrophages and therefore have access to MHC II and presentation to $CD4^+$ T cells. Lysis of macrophages enables cross priming of dendritic cells via vesicles that are carrying pathogen antigens and allows the subsequent generation of CTL via MHC I presentation. I observed generation of antigen specific immune responses through both antibodies as well as cytokine production from splenocytes. High titres of anti-Ag85B-ESAT6 IgG were detected in the serum of mice primed orally with SL3261mycolacZ and in animals primed and intranasally boosted with purified Ag85B-ESAT6 and the adjuvant LTK63, with prominence of the IgG2a subtype. The differential production of cytokines has important effects on the Ig isotype, and the high ratio of IgG2a to IgG1 antibodies specific for Ag85B-ESAT6 is consistent with a heightened T_H1 response, since IFN-γ is required to stimulate IgG2a secretion and is associated with inhibition of IgG1 production (Mosmann and Coffman 1989). Indeed, the immunisation using attenuated recombinant *Salmonella* has already been reported as inducing a T_H1-type response preferentially (Klimpel, et al. 1995; VanCott, et al. 1996). Mice primed and then boosted with or without Ag85B-ESAT6 plus LTK63 also exhibited significantly high IgA titres in both serum and lung and nasal washes. Importantly, the presence of antibodies in mucosal secretions indicates the induction of a specific mucosal immune response after *Salmonella* colonisation and expression of the foreign antigen in the GALT and trafficking of these responses to remote effector sites via the CMIS.

The mouse strain used in this particular vaccine study was C57BL/6. Previous studies have shown that while vaccinated Balb/c mice tend to induce stronger T_H2 type immune responses, C57BL/6 mice show a more robust T_H1 phenotype. This mouse model is consequently used in many tuberculosis vaccination studies as T_H1 immune responses are needed for subsequent protection after *M. tuberculosis* challenge.

Determination of the cytokine profiles revealed that splenocytes from mice vaccinated with SL3261mycolacZ elicited significant inflammatory responses. As shown in Figure 6.14, priming mice with SL3261mycolacZ was sufficient to induce significant and long-lasting levels of all cytokines tested i.e. IL-4, IL-2, IL-5, IL-6 and IFN-γ. For a number of cytokines, their production was further elevated after intranasally boosting with Ag85B-ESAT6 and LTK63 (i.e. IL-6, IL-2 and IFN-γ). Notably, the T

cell response was dominated by IFN-γ, the importance of which for the protection against *M. tuberculosis* has been shown in several studies (Kaufmann 2000). Interestingly, those animals primed with the parental SL3261 strain, and boosted with purified protein and adjuvant, exhibited strong production of the T_H2-associated cytokines IL-4 and IL-5, however I only observed a minor T_H2 response in SL3261mycolacZ immunised animals. A number of studies have shown that these cytokines, if produced in excess, may result in failure to control infection resulting in widely disseminated tuberculosis (Murray, et al. 1997). The differences in cytokine profiles between mice primed with the vaccine strain compared to those immunised with the negative control strain after boosting indicates the significant T_H1 environment the recombinant SL3261mycolacZ strain induces after administration.

Finally, after a robust aerosol challenge with pathogenic *M. tuberculosis* mice immunised with SL3261mycolacZ were found to have a protective effect of 1.1 $\log_{10}$ CFU reduction in the liver. Notably, priming mice with SL3261mycolacZ served to further enhance between a 0.6 and 3.2 $\log_{10}$ CFU reduction in the lung and liver respectively following boosting intranasally with Ag85B-ESAT6 and LTK63. Importantly I found that this protective effect was comparable to BCG vaccinated controls. As already discussed the *Salmonella* vaccine strain SL3261 can persist in mice for 3-4 weeks after administration. For the challenge study it was therefore important that all the recombinant *Salmonella* had been cleared before mice were infected with *M. tuberculosis* to ensure that immune responses being generated were only against *M. tuberculosis* and not any *Salmonella* present. This is why mice were not challenged with *M. tuberculosis* until 8 weeks after oral immunisation of SL3261mycolacZ. Due to experimental constraints the challenge study was carried out by collaborators at the Statens Serum Institute, Denmark. Unfortunately, important control groups, i.e. SL3261mycolacZ plus adjuvant alone and LTK63 plus Ag85B-ESAT6 intranasal vaccination alone, were not included in these challenge studies and should be included when the vaccine candidate is tested again. However, from the Dietrich study it appears that one dose is not effective at stimulating potent enough immune responses to protect animals after *M. tuberculosis* challenge. They reported that three intranasal doses of Ag85B-ESAT6 plus LTK63 was optimum for significant reduction in *M. tuberculosis* CFU counts in both the spleen and the liver (Dietrich, et al. 2006). In addition, mice are normally challenged with a low dose (~

50 CFU) of *M. tuberculosis* for vaccination studies. The large dose used in this study (~ 500 CFU) may possibly have overwhelmed the immune responses generated after vaccination with the recombinant *Salmonella* vaccine SL3261mycolacZ and therefore may not be the most appropriate for obtaining a clear picture of the immunogenicity of the vaccine candidate. In addition, this large dose of pathogenic *M. tuberculosis* may have increased the chance of dissemination of the mycobacteria throughout the host and does not therefore represent pulmonary infection. Consequently, this experiment should be repeated, but next time using a low dose challenge to determine the potential of this novel tuberculosis vaccine candidate.

In conclusion, I have demonstrated for the first time that the mice vaccination with attenuated *S.* Typhimurium expressing the Ag85B-ESAT6 protein via the chromosome is able to induce significant humoral and cellular immune responses and subsequently confer protection against experimental *M. tuberculosis*.

7 Final discussion

Vaccination remains one of the most important strategies in the fight against infectious diseases, as it is based on the age-old adage “Prevention is better than cure”. The pathogens responsible for the greatest burden of human disease make initial contact at mucosal surfaces such as the respiratory tract, gastrointestinal tract or genitourinary tract. Administration of vaccines via this route is often required to induce protective immune responses, and there is consequently a great interest in developing mucosal vaccines against a variety of microbial pathogens. Mucosal vaccination has several advantages over parenteral vaccination, including the possibility of inducing immune responses at the site of administration, as well as induction of specific responses at distant sites, providing protective mucosal immunity. In addition to local responses against mucosally-acquired pathogens, mucosal vaccines have the potential to induce systemic immunity, including humoral and cell-mediated responses. The administration of mucosal vaccines also does not require the use of needles, potentially increasing vaccine compliance, reducing logistical burden, and minimising the risks of blood transmissible infections (e.g. HIV).

Both intranasal vaccination and oral delivery of live attenuated recombinant vectors are promising mucosal immunisation strategies. It has been nearly 20 years since the first Phase I clinical trial of a live recombinant bacterial vaccine, and in that time there have been many more animal studies, as well as human trials, that have indicated the important role these biologicals may play in health-care programs in both developed and developing countries. Much of the progress in developing bacterial vectors has concentrated on the construction of attenuated recombinant strains of *Salmonella*, *E. coli*, *Shigella*, *Listeria* and BCG. As live vectors in general mimic natural infection, they can interact with the mucosal, humoral and cellular compartments of the immune system. In addition, recombinant bacteria are also able to stimulate innate immunity with APC, neutrophils and NKC playing central roles.

In this present body of work I have utilised *Salmonella* as a delivery system for the *M. tuberculosis* fusion antigen Ag85B-ESAT6. As already discussed, Ag85B-ESAT6 has been proven to induce significant antigen-specific immune responses and protection in a number of different vaccination regimens. However, as far as I know, this is the first study to express this immunodominant antigen in a live *Salmonella* vector. I employed both the *in vivo* inducible *nirB* and *ssaG* promoters in a plasmid based expression system as well as constitutive expression of the gene from the *lacZ* promoter, which was incorporated onto the *Salmonella* chromosome. These vaccine candidates were then tested for their ability to induce both systemic and mucosal antigen-specific immune responses. Expression of heterologous antigens in vaccine strains is currently largely plasmid-based, one reason being that plasmid copy number facilitates expression of sufficient antigen to engender an immune response. Both recombinant *Salmonella* vectors driving expression of Ag85B-ESAT6 from the *in vivo* inducible promoters were found to have significant immunogenicity, but only after a second oral dose and an intranasal protein boost. Chromosomal expression, with a single-copy nature and inherent stability, is preferable in clinical studies and consequently I developed the recombinant SL3261mycolacZ vector. I found that only one oral dose of this vaccine candidate was required to induce significant titres of both systemic and mucosal antibodies, as well as potent inflammatory cytokine responses. Furthermore, incorporating this recombinant *Salmonella* vaccine into a heterologous oral priming-intranasal boosting regimen increased these antigen specific immune responses further. As this vaccine candidate induced such significant immune responses after immunisation, I subsequently tested its ability to provide protection against aerosol *M. tuberculosis* challenge. I observed a significant reduction in *M. tuberculosis* CFUs in vaccinated mice that were comparable to the mice vaccinated with the current BCG vaccine. Regarding BCG, several studies have shown that the protective efficacy of BCG wanes significantly over a period of 10–15 years which emphasises the urgent need for a BCG booster vaccine that efficiently boosts immunity in BCG-vaccinated individuals. If possible I would also use our recombinant *Salmonella* vaccine to boost pre-BCG immune animals and determine if the vaccine would be suitable as either as a replacement for BCG, and/or as an additional boost for BCG vaccinated individuals.

The *S.* Typhi ZH9 strain (Ty2 Δ*aroC* Δ*ssaV*) is an oral vaccine candidate against the disease typhoid fever, and is currently about to enter a 50,000 cohort Phase III efficacy study in India. As already discussed, this particular attenuated *Salmonella* vector has also been modified to deliver a number of heterologous antigens, and been shown to stimulate antigen-specific antibody responses in mice immunised with these *S.* Typhi ZH9 derivatives (Khan, et al. 2007; Microscience 2004a; Microscience 2004b; Stratford, et al. 2005). Due to the success of manipulating this particular vaccine candidate as a vector to deliver foreign antigens in both human subjects and animal models, it may be possible to construct a recombinant strain expressing the tuberculosis fusion Ag85B-ESAT6 antigen via the *ssaG* or *lacZ* promoter within the *Salmonella* chromosome at the site of the attenuating *aroC* deletion. Animal studies would be performed to determine immunogenicity. As this particular *Salmonella* vaccine strain has already been tested in humans and was shown to be well tolerated, and induce specific immunity to the delivered foreign antigens, there may be the possibility of setting up a phase I clinical trial to look at reactogenicity/safety and immunogenicity of this oral vaccine candidate in human volunteers.

Many also consider that intranasal immunisation is potentially a very effective route for inducing both mucosal and systemic immunity to an infectious agent, and believe that the exploitation of the advantages of this route of administration will be the basis for the next generation of vaccines (see Section 1.4.2 for more details). In this present study I found that when mice were vaccinated (with LT and Ag85B-ESAT6) via this route, the vaccine induced significant mucosal and systemic antigen-specific antibodies as well as high levels of a number of cytokines. The work in Chapter 3 therefore highlights the possibilities of using this administration route as a viable vaccination strategy.

In addition to the generation of adaptive immune responses, the induction of innate immunity is also crucial for vaccines to elicit potent antigen specific immune responses and a greater understanding of innate immunity at mucosal surfaces is therefore required. The work presented in Chapter 5 comprises a comprehensive study into innate immune responses generated early after intranasal immunisation. I observed dynamic and diverse responses as early as 5 hours post intranasal administration of adjuvant and antigen, including significant increases and decreases of innate immune cell populations, their increased activation, and movement of cells within both the NALT and CLN into different compartments of these lymphoid tissues, i.e. follicular and parafollicular regions. The changes observed in the DC, macrophage, NKC and neutrophil populations suggests the movement of these cell types to and from the NALT and CLN, as well as trafficking back and forth from as yet undefined locations. However, it may be that the decrease I observe in APC at the early 5 hour time-point within the NALT and the 24 hour time-point in the CLN is actually apoptosis of resident cell populations, rather than their movement out of these tissues. A number of studies have shown that LT can actually induce apoptosis of $CD8^+$ T cells, and it may be that one of the local effects of LT is to modulate regulatory cells populations which in turn may lead to apoptosis of these APC (Fraser, et al. 2003). Nevertheless, as I have not followed these cell populations I cannot speculate further and this may be something that can be performed so that I can obtain a more complete trafficking picture. Examination of CAMs did reveal a complex pattern of expression in the two tissues at all time-points. It appears that the NALT is a “cross-roads” for the movement of cells from several different mucosal compartments, due to the wide variety of CAMs expressed. This may help explain why intranasal vaccination is able to induce immune responses in several distal mucosal sites, including the gut and the genitourinary tract. As already discussed these molecules play key roles in inflammation and subsequent trafficking of cell populations, and our data may help with the design of preferential targeting/homing of vaccines to specific mucosal areas most appropriate for protection. The adjuvant LT can also affect antigen processing and presentation by macrophages and dendritic cells, and these may be why I see activation of these cell populations at these early time-points. Activation of these cells types may in turn induce activation of other cells types, i.e. NKC and neutrophils, through release of soluble immunomodulatory factors or direct interactions. Another explanation is that LT may be directing some

unknown effects on regulatory cell populations, which is turn leads to the unregulated activation of other cell populations. Shortly after intranasal immunisation I also observed that APC were located in areas not observed in naïve mice, and their position in the T and B cell areas might reflect their involvement in immunologic reactions taking place in these lymphoid tissues. I also observed the induction of GC within both the NALT and the CLN, as well as increased expression of VCAM-1 on APC, which may be involved in stabilising this GC formation. These data therefore highlight the importance of innate immune responses in the induction of adaptive immunity.

Data obtained from the above study indicated that NKC were an important cell population involved in early immune responses after intranasal immunisation. Consequently I went on to examine their possible role in adaptive immunity after mucosal vaccination. I found that NKC depletion reduced T_H1 type immune responses as indicated by undetectable IgG2a and IgG2b antibody titres, and significantly reduced IFN-γ and TNF-α production from stimulated splenocytes after intranasal immunisation. Previous work has shown us that NKC produce a variety of cytokines, including IFN-γ and TNF-α, after their activation, and this may explain why I see a reduction in antigen-specific cytokine production in addition to an absence of the above antibody isotypes, as these cytokines are known to be involved in both IgG2a and IgG2b class-switching. In addition, I also observed that IL-6 production was significantly reduced. As already discussed, IL-6 is not detectably produced by NKC, but is by activated macrophages and dendritic cells. Therefore, the reduction in levels of this particular cytokine emphasises the role that NKC have in the ‘cross-talk’ and ensuing activation of APC. Previous studies have shown that the adjuvant LT is known to directly modulate APC. It is temping to speculate that it may also be directly interacting with NKC, possibly through their activation or inhibition receptors. It would therefore be of interest to analyse the expression of a selection of NKC receptors after contact with LT, and to possibly further characterise the mechanisms of LT, which may help in the rational design of new and improved mucosal adjuvants.

The work in this thesis has led to the development of a number of vaccines utilising the *M. tuberculosis* fusion antigen, Ag85B-ESAT6. I have demonstrated potent immunogenicity in all vaccine candidates, and in addition I observed that vaccination with the chromosomal recombinant *Salmonella* vaccine candidate, plus an intranasal boost, inferred levels of protection similar to those seen in BCG vaccinated mice. I have also outlined the complexity of innate immune responses induced early after intranasal immunisation. I have demonstrated that a number of innate immune cell populations are activated as early as 5 hours after administration of antigen. These cells possibly move between different mucosal compartments as well as traffic into areas within both the NALT and the CLN that are involved in the induction of adaptive immune responses (i.e. T and B cell areas), as shown by the formation of GC. I have also demonstrated the diversity of CAMs expression on both vascular endothelium and cells after vaccination. It is hoped that the characterisation of these innate immune responses may help with the more rational design of mucosal vaccines. Finally, I have also demonstrated that NKC are important for the induction of antigen-specific adaptive immune responses after mucosal vaccination, and that the mechanism of induction is possibly through cytokine production as well as 'cross-talk' and activation of APC.

References

Abou-Zeid, C., Ratliff, T. L., Wiker, H. G., Harboe, M., Bennedsen, J. and Rook, G. A. 1988 'Characterization of fibronectin-binding antigens released by Mycobacterium tuberculosis and Mycobacterium bovis BCG', *Infect Immun* 56(12): 3046-51.

Akira, S. and Kishimoto, T. 1996 'Role of interleukin-6 in macrophage function', *Curr Opin Hematol* 3(1): 87-93.

Allen, J. S., Dougan, G. and Strugnell, R. A. 2000 'Kinetics of the mucosal antibody secreting cell response and evidence of specific lymphocyte migration to the lung after oral immunisation with attenuated S. enterica var. typhimurium', *FEMS Immunol Med Microbiol* 27(4): 275-81.

Amigorena, S., Bonnerot, C., Fridman, W. H. and Teillaud, J. L. 1990 'Recombinant interleukin 2-activated natural killer cells regulate IgG2a production', *Eur J Immunol* 20(8): 1781-7.

Andersen, C. S., Dietrich, J., Agger, E. M., Lycke, N. Y., Lovgren, K. and Andersen, P. 2007 'The Combined CTA1-DD/ISCOMs Vector Is an Effective Intranasal Adjuvant for Boosting Prior Mycobacterium bovis BCG Immunity to Mycobacterium tuberculosis', *Infect Immun* 75(1): 408-16.

Andersen, P. 1997 'Host responses and antigens involved in protective immunity to Mycobacterium tuberculosis', *Scand J Immunol* 45(2): 115-31.

Andersen, P., Andersen, A. B., Sorensen, A. L. and Nagai, S. 1995 'Recall of long-lived immunity to Mycobacterium tuberculosis infection in mice', *J Immunol* 154(7): 3359-72.

Andersen, P., Askgaard, D., Ljungqvist, L., Bentzon, M. W. and Heron, I. 1991 'T-cell proliferative response to antigens secreted by Mycobacterium tuberculosis', *Infect Immun* 59(4): 1558-63.

Andersen, P. and Doherty, T. M. 2005 'The success and failure of BCG - implications for a novel tuberculosis vaccine', *Nat Rev Microbiol* 3(8): 656-62.

Anderson, D. H., Harth, G., Horwitz, M. A. and Eisenberg, D. 2001 'An interfacial mechanism and a class of inhibitors inferred from two crystal structures of the Mycobacterium tuberculosis 30 kDa major secretory protein (Antigen 85B), a mycolyl transferase', *J Mol Biol* 307(2): 671-81.

Andrews, D. M., Andoniou, C. E., Scalzo, A. A., van Dommelen, S. L., Wallace, M. E., Smyth, M. J. and Degli-Esposti, M. A. 2005 'Cross-talk between dendritic cells and natural killer cells in viral infection', *Mol Immunol* 42(4): 547-55.

Anegon, I., Cuturi, M. C., Trinchieri, G. and Perussia, B. 1988 'Interaction of Fc receptor (CD16) ligands induces transcription of interleukin 2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells', *J Exp Med* 167(2): 452-72.

Angelakopoulos, H. and Hohmann, E. L. 2000 'Pilot study of phoP/phoQ-deleted Salmonella enterica serovar typhimurium expressing Helicobacter pylori urease in adult volunteers', *Infect Immun* 68(4): 2135-41.

Aplin, A. E., Howe, A., Alahari, S. K. and Juliano, R. L. 1998 'Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins', *Pharmacol Rev* 50(2): 197-263.

Aplin, A. E., Howe, A. K. and Juliano, R. L. 1999 'Cell adhesion molecules, signal transduction and cell growth', *Curr Opin Cell Biol* 11(6): 737-44.

Appelberg, R., Orme, I. M., Pinto de Sousa, M. I. and Silva, M. T. 1992 'In vitro effects of interleukin-4 on interferon-gamma-induced macrophage activation', *Immunology* 76(4): 553-9.

Arai, S., Yamamoto, H., Itoh, K. and Kumagai, K. 1983 'Suppressive effect of human natural killer cells on pokeweed mitogen-induced B cell differentiation', *J Immunol* 131(2): 651-7.

Arase, H., Arase, N. and Saito, T. 1995 'Fas-mediated cytotoxicity by freshly isolated natural killer cells', *J Exp Med* 181(3): 1235-8.

— 1996 'Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking', *J Exp Med* 183(5): 2391-6.

Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B. and Lanier, L. L. 2002 'Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors', *Science* 296(5571): 1323-6.

Asakura, K., Saito, H., Hata, M. and Kataura, A. 1998 'Antigen-specific IgA response of NALT and cervical lymph node cells in antigen-primed rats', *Acta Otolaryngol* 118(6): 859-63.

Asanuma, H., Aizawa, C., Kurata, T. and Tamura, S. 1998 'IgA antibody-forming cell responses in the nasal-associated lymphoid tissue of mice vaccinated by intranasal, intravenous and/or subcutaneous administration', *Vaccine* 16(13): 1257-1262.

Asanuma, H., Inaba, Y., Aizawa, C., Kurata, T. and Tamura, S. 1995 'Characterization of mouse nasal lymphocytes isolated by enzymatic extraction with collagenase', *J Immunol Methods* 187(1): 41-51.

Asanuma, H., Thompson, A. H., Iwasaki, T., Sato, Y., Inaba, Y., Aizawa, C., Kurata, T. and Tamura, S. I. 1997 'Isolation and characterization of mouse nasal-associated lymphoid tissue', *Journal of Immunological Methods* 202(2): 123-131.

Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. and Powrie, F. 1999 'An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation', *J Exp Med* 190(7): 995-1004.

Balogh, P., Aydar, Y., Tew, J. G. and Szakal, A. K. 2002 'Appearance and phenotype of murine follicular dendritic cells expressing VCAM-1', *Anat Rec* 268(2): 160-8.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. and Palucka, K. 2000 'Immunobiology of dendritic cells', *Annu Rev Immunol* 18: 767-811.

Bargatze, R. F., Jutila, M. A. and Butcher, E. C. 1995 'Distinct roles of L-selectin and integrins alpha 4 beta 7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined', *Immunity* 3(1): 99-108.

Bargatze, R. F., Kurk, S., Butcher, E. C. and Jutila, M. A. 1994 'Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells', *J Exp Med* 180(5): 1785-92.

Behr-Perst, S. I., Munk, M. E., Schaberg, T., Ulrichs, T., Schulz, R. J. and Kaufmann, S. H. 1999 'Phenotypically activated gammadelta T lymphocytes in the peripheral blood of patients with tuberculosis', *J Infect Dis* 180(1): 141-9.

Behr, M. A., Wilson, M. A., Gill, W. P., Salamon, H., Schoolnik, G. K., Rane, S. and Small, P. M. 1999 'Comparative genomics of BCG vaccines by whole-genome DNA microarray', *Science* 284(5419): 1520-3.

Bienenstock, J. and McDermott, M. R. 2005 'Bronchus- and nasal-associated lymphoid tissues', *Immunol Rev* 206: 22-31.

Biet, F., Kremer, L., Wolowczuk, I., Delacre, M. and Locht, C. 2003 'Immune response induced by recombinant Mycobacterium bovis BCG producing the cholera toxin B subunit', *Infect Immun* 71(5): 2933-7.

Biron, C. A. 1997 'Activation and function of natural killer cell responses during viral infections', *Curr Opin Immunol* 9(1): 24-34.

Biron, C. A., Byron, K. S. and Sullivan, J. L. 1989 'Severe herpesvirus infections in an adolescent without natural killer cells', *N Engl J Med* 320(26): 1731-5.

Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. and Salazar-Mather, T. P. 1999 'Natural killer cells in antiviral defense: function and regulation by innate cytokines', *Annu Rev Immunol* 17: 189-220.

Black, R., Levine, M. M., Young, C., Rooney, J., Levine, S., Clements, M. L., O'Donnell, S., Hugues, T. and Germanier, R. 1983 'Immunogenicity of Ty21a attenuated "Salmonella typhi" given with sodium bicarbonate or in enteric-coated capsules', *Dev Biol Stand* 53: 9-14.

Bodnar, K. A., Serbina, N. V. and Flynn, J. L. 2001 'Fate of Mycobacterium tuberculosis within murine dendritic cells', *Infect Immun* 69(2): 800-9.

Boom, W. H. 1996 'The role of T-cell subsets in Mycobacterium tuberculosis infection', *Infect Agents Dis* 5(2): 73-81.

Borsutzky, S., Ebensen, T., Link, C., Becker, P. D., Fiorelli, V., Cafaro, A., Ensoli, B. and Guzman, C. A. 2006 'Efficient systemic and mucosal responses against the HIV-1 Tat protein by prime/boost vaccination using the lipopeptide MALP-2 as adjuvant', *Vaccine* 24(12): 2049-56.

Bosio, C. M., Gardner, D. and Elkins, K. L. 2000 'Infection of B cell-deficient mice with CDC 1551, a clinical isolate of Mycobacterium tuberculosis: delay in dissemination and development of lung pathology', *J Immunol* 164(12): 6417-25.

Bowe, F., Pickard, D. J., Anderson, R. J., Londono-Arcila, P. and Dougan, G. 2003 'Development of attenuated Salmonella strains that express heterologous antigens', *Methods Mol Med* 87: 83-100.

Bowen, J. C., Reddy, R. M., Alpar, H. O., Brown, M. R. and Rouse, B. T. 1995 'Expression and stability of herpes simplex virus antigens in Salmonella typhimurium', *Adv Exp Med Biol* 371B: 1543-6.

Boyaka, P. N., Wright, P. F., Marinaro, M., Kiyono, H., Johnson, J. E., Gonzales, R. A., Ikizler, M. R., Werkhaven, J. A., Jackson, R. J., Fujihashi, K., Di Fabio, S., Staats, H. F. and McGhee, J. R. 2000 'Human nasopharyngeal-associated lymphoreticular tissues. Functional analysis of subepithelial and intraepithelial B and T cells from adenoids and tonsils', *Am J Pathol* 157(6): 2023-35.

Brandt, L., Elhay, M., Rosenkrands, I., Lindblad, E. B. and Andersen, P. 2000 'ESAT-6 subunit vaccination against Mycobacterium tuberculosis', *Infection and Immunity* 68(2): 791-795.

Brandt, L., Oettinger, T., Holm, A., Andersen, A. B. and Andersen, P. 1996 'Key epitopes on the ESAT-6 antigen recognized in mice during the recall of protective immunity to Mycobacterium tuberculosis', *J Immunol* 157(8): 3527-33.

Brandt, L., Skeiky, Y. A., Alderson, M. R., Lobet, Y., Dalemans, W., Turner, O. C., Basaraba, R. J., Izzo, A. A., Lasco, T. M., Chapman, P. L., Reed, S. G. and Orme, I. M. 2004 'The protective effect of the Mycobacterium bovis BCG vaccine is increased by coadministration with the Mycobacterium tuberculosis 72-kilodalton fusion polyprotein Mtb72F in M. tuberculosis-infected guinea pigs', *Infect Immun* 72(11): 6622-32.

Brandtzaeg, P. 1988 'Immunobarriers of the mucosa of the upper respiratory and digestive pathways', *Acta Otolaryngol* 105(1-2): 172-80.

Brosch, R., Gordon, S. V., Garnier, T., Eiglmeier, K., Frigui, W., Valenti, P., Dos Santos, S., Duthoy, S., Lacroix, C., Garcia-Pelayo, C., Inwald, J. K., Golby, P., Garcia, J. N., Hewinson, R. G., Behr, M. A., Quail, M. A., Churcher, C., Barrell, B. G., Parkhill, J. and Cole, S. T. 2007 'Genome plasticity of BCG and impact on vaccine efficacy', *Proc Natl Acad Sci U S A* 104(13): 5596-601.

Brusasca, P. N., Peters, R. L., Motzel, S. L., Klein, H. J. and Gennaro, M. L. 2003 'Antigen recognition by serum antibodies in non-human primates experimentally infected with Mycobacterium tuberculosis', *Comp Med* 53(2): 165-72.

Bullas, L. R. and Ryu, J. I. 1983 'Salmonella typhimurium LT2 strains which are r- m+ for all three chromosomally located systems of DNA restriction and modification', *J Bacteriol* 156(1): 471-4.

Bumann, D., Metzger, W. G., Mansouri, E., Palme, O., Wendland, M., Hurwitz, R., Haas, G., Aebischer, T., von Specht, B. U. and Meyer, T. F. 2001 'Safety and immunogenicity of live recombinant Salmonella enterica serovar Typhi Ty21a expressing urease A and B from Helicobacter pylori in human volunteers', *Vaccine* 20(5-6): 845-52.

Burton, D. R. 2002 'Antibodies, viruses and vaccines', *Nat Rev Immunol* 2(9): 706-13.

Butcher, E. C. and Picker, L. J. 1996 'Lymphocyte homing and homeostasis', *Science* 272(5258): 60-6.

Butcher, E. C., Williams, M., Youngman, K., Rott, L. and Briskin, M. 1999 'Lymphocyte trafficking and regional immunity', *Adv Immunol* 72: 209-53.

Carr, R. M., Lolachi, C. M., Albaran, R. G., Ridley, D. M., Montgomery, P. C. and O'Sullivan, N. L. 1996 'Nasal-associated lymphoid tissue is an inductive site for rat tear IgA antibody responses', *Immunol Invest* 25(5-6): 387-96.

Carrasco, Y. R., Fleire, S. J., Cameron, T., Dustin, M. L. and Batista, F. D. 2004 'LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation', *Immunity* 20(5): 589-99.

Casadevall, A. 2003 'Antibody-mediated immunity against intracellular pathogens: Two-dimensional thinking comes full circle', *Infection and Immunity* 71(8): 4225-4228.

Casola, S., Otipoby, K. L., Alimzhanov, M., Humme, S., Uyttersprot, N., Kutok, J. L., Carroll, M. C. and Rajewsky, K. 2004 'B cell receptor signal strength determines B cell fate', *Nat Immunol* 5(3): 317-27.

Chakravortty, D., Rohde, M., Jager, L., Deiwick, J. and Hensel, M. 2005 'Formation of a novel surface structure encoded by Salmonella Pathogenicity Island 2', *Embo J* 24(11): 2043-52.

Challacombe, S. J., Rahman, D. and O'Hagan, D. T. 1997 'Salivary, gut, vaginal and nasal antibody responses after oral immunization with biodegradable microparticles', *Vaccine* 15(2): 169-75.

Chan, E. D., Chan, J. and Schluger, N. W. 2001 'What is the role of nitric oxide in murine and human host defense against tuberculosis?Current knowledge', *Am J Respir Cell Mol Biol* 25(5): 606-12.

Chatfield, S. N., Charles, I. G., Makoff, A. J., Oxer, M. D., Dougan, G., Pickard, D., Slater, D. and Fairweather, N. F. 1992 'Use of the nirB promoter to direct the stable expression of heterologous antigens in Salmonella oral vaccine strains: development of a single-dose oral tetanus vaccine', *Biotechnology (N Y)* 10(8): 888-92.

Chen, L., Wang, J., Zganiacz, A. and Xing, Z. 2004 'Single intranasal mucosal Mycobacterium bovis BCG vaccination confers improved protection compared to subcutaneous vaccination against pulmonary tuberculosis', *Infect Immun* 72(1): 238-46.

Cheng, E., Cardenas-Freytag, L. and Clements, J. D. 1999 'The role of cAMP in mucosal adjuvanticity of Escherichia coli heat-labile enterotoxin (LT)', *Vaccine* 18(1-2): 38-49.

Claeys, S., Cuvelier, C., Quatacker, J. and Van Cauwenberge, P. 1996a 'Ultrastructural investigation of M-cells and lymphoepithelial contacts in naso-pharyngeal associated lymphoid tissue (NALT)', *Acta Otolaryngol Suppl* 523: 40-2.

Claeys, S., Cuvelier, C. and Van Cauwenberge, P. 1996b 'Immunohistochemical analyses of the lymphoepithelium in human nasopharyngeal associated lymphoid tissue (NALT): preliminary results', *Acta Otolaryngol Suppl* 523: 38-9.

Clare, S. and Dougan, G. 2004 *Novel Vaccination Strategies*, Weinheim: Wiley-VCH.

Colditz, G. A., Brewer, T. F., Berkey, C. S., Wilson, M. E., Burdick, E., Fineberg, H. V. and Mosteller, F. 1994 'Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature', *Jama* 271(9): 698-702.

Cole, J. A., Newman, B. M. and White, P. 1980 'Biochemical and genetic characterization of nirB mutants of Escherichia coli K 12 pleiotropically defective in nitrite and sulphite reduction', *J Gen Microbiol* 120(2): 475-83.

Cole, S. T. and Barrell, B. G. 1998 'Analysis of the genome of Mycobacterium tuberculosis H37Rv', *Novartis Found Symp* 217: 160-72; discussion 172-7.

Colic, M. and Drabek, D. 1991 'Expression and function of intercellular adhesion molecule 1 (ICAM-1) on rat thymic macrophages in culture', *Immunol Lett* 28(3): 251-7.

Collins, F. M. 1991 'Antituberculous immunity: new solutions to an old problem', *Rev Infect Dis* 13(5): 940-50.

Content, J., de la Cuvellerie, A., De Wit, L., Vincent-Levy-Frebault, V., Ooms, J. and De Bruyn, J. 1991 'The genes coding for the antigen 85 complexes of Mycobacterium tuberculosis and Mycobacterium bovis BCG are members of a gene family: cloning, sequence determination, and genomic organization of the gene coding for antigen 85-C of M. tuberculosis', *Infect Immun* 59(9): 3205-12.

Corthesy-Theulaz, I. E., Hopkins, S., Bachmann, D., Saldinger, P. F., Porta, N., Haas, R., Zheng-Xin, Y., Meyer, T., Bouzourene, H., Blum, A. L. and Kraehenbuhl, J. P. 1998 'Mice are protected from Helicobacter pylori infection by nasal immunization with attenuated Salmonella typhimurium phoPc expressing urease A and B subunits', *Infect Immun* 66(2): 581-6.

Costello, A. M., Kumar, A., Narayan, V., Akbar, M. S., Ahmed, S., Abou-Zeid, C., Rook, G. A., Stanford, J. and Moreno, C. 1992 'Does antibody to mycobacterial antigens, including lipoarabinomannan, limit dissemination in childhood tuberculosis?' *Trans R Soc Trop Med Hyg* 86(6): 686-92.

Couch, R. B. 2004 'Nasal vaccination, Escherichia coli enterotoxin, and Bell's palsy', *N Engl J Med* 350(9): 860-1.

Craston, R., Koh, M., Mc Dermott, A., Ray, N., Prentice, H. G. and Lowdell, M. W. 1997 'Temporal dynamics of CD69 expression on lymphoid cells', *J Immunol Methods* 209(1): 37-45.

Cryz, S. J., Jr., Furer, E. and Levine, M. M. 1988 '[Effectiveness of oral, attenuated live Salmonella typhi Ty 21a vaccine in controlled field trials]', *Schweiz Med Wochenschr* 118(13): 467-70.

Csencsits, K. L., Jutila, M. A. and Pascual, D. W. 1999 'Nasal-associated lymphoid tissue: Phenotypic and functional evidence for the primary role of peripheral node addressin in naive lymphocyte adhesion to high endothelial venules in a mucosal site', *Journal of Immunology* 163(3): 1382-1389.

— 2002 'Mucosal addressin expression and binding-interactions with naive lymphocytes vary among the cranial, oral, and nasal- associated lymphoid tissues', *European Journal of Immunology* 32(11): 3029-3039.

Curtiss, R., 3rd 2002 'Bacterial infectious disease control by vaccine development', *J Clin Invest* 110(8): 1061-6.

Czerkinsky, C., Anjuere, F. and McGhee, J. R. 1999 'Mucosal immunity and tolerance: relevance to vaccine development', *Immunol. Rev.* 170: 197-222.

D'Amelio, R., Palmisano, L., Le Moli, S., Seminara, R. and Aiuti, F. 1982 'Serum and salivary IgA levels in normal subjects: comparison between tonsillectomized and non-tonsillectomized subjects', *Int Arch Allergy Appl Immunol* 68(3): 256-9.

Dalla Pozza, T., Yan, H., Meek, D., Guzman, C. A. and Walker, M. J. 1998 'Construction and characterisation of Salmonella typhimurium aroA simultaneously expressing the five pertussis toxin subunits', *Vaccine* 16(5): 522-9.

Davis, S. S. 2001 'Nasal Vaccines', *Advanced Drug Delivery Reviews* 51: 21-42.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M. G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H. and de Vries, J. E. 1991 'Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression', *J Exp Med* 174(4): 915-24.

Debertin, A. S., Tschernig, T., Tonjes, H., Kleemann, W. J., Troger, H. D. and Pabst, R. 2003 'Nasal-associated lymphoid tissue (NALT): frequency and localization in young children', *Clinical and Experimental Immunology* 134(3): 503-507.

Derrick, S. C., Yang, A. L. and Morris, S. L. 2004 'A polyvalent DNA vaccine expressing an ESAT6-Ag85B fusion protein protects mice against a primary infection with Mycobacterium tuberculosis and boosts BCG-induced protective immunity', *Vaccine* 23(6): 780-8.

Dietrich, J., Aagaard, C., Leah, R., Olsen, A. W., Stryhn, A., Doherty, T. M. and Andersen, P. 2005 'Exchanging ESAT6 with TB10.4 in an Ag85B fusion molecule-based tuberculosis subunit vaccine: efficient protection and ESAT6-based sensitive monitoring of vaccine efficacy', *J Immunol* 174(10): 6332-9.

Dietrich, J., Andersen, C., Rappuoli, R., Doherty, T. M., Jensen, C. G. and Andersen, P. 2006 'Mucosal administration of Ag85B-ESAT-6 protects against infection with Mycobacterium tuberculosis and boosts prior bacillus Calmette-Guerin immunity', *J Immunol* 177(9): 6353-60.

DiGiandomenico, A., Rao, J. and Goldberg, J. B. 2004 'Oral vaccination of BALB/c mice with Salmonella enterica serovar Typhimurium expressing Pseudomonas aeruginosa O antigen promotes increased survival in an acute fatal pneumonia model', *Infect Immun* 72(12): 7012-21.

DiPetrillo, M. D., Tibbetts, T., Kleanthous, H., Killeen, K. P. and Hohmann, E. L. 1999 'Safety and immunogenicity of phoP/phoQ-deleted Salmonella typhi expressing Helicobacter pylori urease in adult volunteers', *Vaccine* 18(5-6): 449-59.

Doherty, T. M., Olsen, A. W., van Pinxteren, L. and Andersen, P. 2002 'Oral vaccination with subunit vaccines protects animals against aerosol infection with Mycobacterium tuberculosis', *Infection and Immunity* 70(6): 3111-3121.

Dolen, W. K., Spofford, B. and Selner, J. C. 1990 'The hidden tonsils of Waldeyer's ring', *Ann Allergy* 65(4): 244-8.

Donovan, R. and Soothill, J. F. 1973 'Immunological studies in children undergoing tonsillectomy', *Clin Exp Immunol* 14(3): 347-57.

Dorer, D. E., Czepluch, W., Lambeth, M. R., Dunn, A. C., Reitinger, C., Aldwell, F. E. and McLellan, A. D. 2007 'Lymphatic tracing and T cell responses following oral vaccination with live Mycobacterium bovis (BCG)', *Cell Microbiol* 9(2): 544-53.

Douce, G., Turcotte, C., Cropley, I., Roberts, M., Pizza, M., Domenghini, M., Rappuoli, R. and Dougan, G. 1995 'Mutants of Escherichia coli heat-labile toxin lacking ADP-ribosyltransferase activity act as nontoxic, mucosal adjuvants', *Proc Natl Acad Sci U S A* 92(5): 1644-8.

Dunlap, N. E. and Briles, D. E. 1993 'Immunology of tuberculosis', *Med Clin North Am* 77(6): 1235-51.

Dunstan, S. J., Simmons, C. P. and Strugnell, R. A. 1998 'Comparison of the abilities of different attenuated Salmonella typhimurium strains to elicit humoral immune responses against a heterologous antigen', *Infect Immun* 66(2): 732-40.

Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A. and Springer, T. A. 1986 'Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1)', *J Immunol* 137(1): 245-54.

Dustin, M. L. and Shaw, A. S. 1999 'Costimulation: building an immunological synapse', *Science* 283(5402): 649-50.

Edelson, B. T. and Unanue, E. R. 2001 'Intracellular antibody neutralizes Listeria growth', *Immunity* 14(5): 503-12.

Edwards, M. F. and Stocker, B. A. 1988 'Construction of delta aroA his delta pur strains of Salmonella typhi', *J Bacteriol* 170(9): 3991-5.

Engelhardt, B., Conley, F. K. and Butcher, E. C. 1994 'Cell adhesion molecules on vessels during inflammation in the mouse central nervous system', *J Neuroimmunol* 51(2): 199-208.

Eppihimer, M. J., Wolitzky, B., Anderson, D. C., Labow, M. A. and Granger, D. N. 1996 'Heterogeneity of expression of E- and P-selectins in vivo', *Circ Res* 79(3): 560-9.

Eriksson, A. M., Schon, K. M. and Lycke, N. Y. 2004 'The cholera toxin-derived CTA1-DD vaccine adjuvant administered intranasally does not cause inflammation or accumulate in the nervous tissues', *J Immunol* 173(5): 3310-9.

Evans, D. T., Chen, L. M., Gillis, J., Lin, K. C., Harty, B., Mazzara, G. P., Donis, R. O., Mansfield, K. G., Lifson, J. D., Desrosiers, R. C., Galan, J. E. and Johnson, R. P. 2003 'Mucosal priming of simian immunodeficiency virus-specific cytotoxic T-lymphocyte responses in rhesus macaques by the Salmonella type III secretion antigen delivery system', *J Virol* 77(4): 2400-9.

Eyles, J. E., Bramwell, V. W., Williamson, E. D. and Alpar, H. O. 2001a 'Microsphere translocation and immunopotentiation in systemic tissues following intranasal administration', *Vaccine* 19(32): 4732-4742.

Eyles, J. E., Spiers, I. D., Williamson, E. D. and Alpar, H. O. 2001b 'Tissue distribution of radioactivity following intranasal administration of radioactive microspheres', *Journal of Pharmacy and Pharmacology* 53(5): 601-607.

Fabbri, M., Bianchi, E., Fumagalli, L. and Pardi, R. 1999 'Regulation of lymphocyte traffic by adhesion molecules', *Inflamm Res* 48(5): 239-46.

Falero-Diaz, G., Challacombe, S., Banerjee, D., Douce, G., Boyd, A. and Ivanyi, J. 2000 'Intranasal vaccination of mice against infection with Mycobacterium tuberculosis', *Vaccine* 18(28): 3223-9.

Farstad, I. N., Carlsen, H., Morton, H. C. and Brandtzaeg, P. 2000 'Immunoglobulin A cell distribution in the human small intestine: phenotypic and functional characteristics', *Immunology* 101(3): 354-63.

Fayyazi, A., Eichmeyer, B., Soruri, A., Schweyer, S., Herms, J., Schwarz, P. and Radzun, H. J. 2000 'Apoptosis of macrophages and T cells in tuberculosis associated caseous necrosis', *J Pathol* 191(4): 417-25.

Fearon, D. T. and Locksley, R. M. 1996 'The instructive role of innate immunity in the acquired immune response', *Science* 272(5258): 50-3.

Ferlazzo, G., Morandi, B., D'Agostino, A., Meazza, R., Melioli, G., Moretta, A. and Moretta, L. 2003 'The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells', *Eur J Immunol* 33(2): 306-13.

Ferraz, J. C., Stavropoulos, E., Yang, M., Coade, S., Espitia, C., Lowrie, D. B., Colston, M. J. and Tascon, R. E. 2004 'A heterologous DNA priming-Mycobacterium bovis BCG boosting immunization strategy using mycobacterial Hsp70, Hsp65, and Apa antigens improves protection against tuberculosis in mice', *Infect Immun* 72(12): 6945-50.

Fine, P. E. 2001 'BCG: the challenge continues', *Scand J Infect Dis* 33(4): 243-5.

Finkelman, F. D., Katona, I. M., Mosmann, T. R. and Coffman, R. L. 1988 'IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses', *J Immunol* 140(4): 1022-7.

Flesch, I. E. and Kaufmann, S. H. 1993 'Role of cytokines in tuberculosis', *Immunobiology* 189(3-4): 316-39.

Flynn, J. L. and Chan, J. 2001 'Immunology of tuberculosis', *Annu Rev Immunol* 19: 93-129.

Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A. and Bloom, B. R. 1993 'An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection', *J Exp Med* 178(6): 2249-54.

Forchielli, M. L. and Walker, W. A. 2005 'The role of gut-associated lymphoid tissues and mucosal defence', *Br J Nutr* 93 Suppl 1: S41-8.

Formal, S. B., Baron, L. S., Kopecko, D. J., Washington, O., Powell, C. and Life, C. A. 1981 'Construction of a potential bivalent vaccine strain: introduction of Shigella sonnei form I antigen genes into the galE Salmonella typhi Ty21a typhoid vaccine strain', *Infect Immun* 34(3): 746-50.

Forrest, B. D., LaBrooy, J. T., Robinson, P., Dearlove, C. E. and Shearman, D. J. 1991 'Specific immune response in the human respiratory tract following oral immunization with live typhoid vaccine', *Infect Immun* 59(3): 1206-9.

Fraser, S. A., de Haan, L., Hearn, A. R., Bone, H. K., Salmond, R. J., Rivett, A. J., Williams, N. A. and Hirst, T. R. 2003 'Mutant Escherichia coli heat-labile toxin B subunit that separates toxoid-mediated signaling and immunomodulatory action from trafficking and delivery functions', *Infect Immun* 71(3): 1527-37.

Freemont, A. J. 1987 'Molecules controlling lymphocyte-endothelial interactions in lymph nodes are produced in vessels of inflamed synovium', *Ann Rheum Dis* 46(12): 924-8.

French, A. R. and Yokoyama, W. M. 2003 'Natural killer cells and viral infections', *Curr Opin Immunol* 15(1): 45-51.

Freytag, L. C. and Clements, J. D. 2005 'Mucosal adjuvants', *Vaccine* 23(15): 1804-13.

Frieden, T. R., Sterling, T. R., Munsiff, S. S., Watt, C. J. and Dye, C. 2003 'Tuberculosis', *Lancet* 362(9387): 887-99.

Fritsche, G., Dlaska, M., Barton, H., Theurl, I., Garimorth, K. and Weiss, G. 2003 'Nramp1 functionality increases inducible nitric oxide synthase transcription via stimulation of IFN regulatory factor 1 expression', *J Immunol* 171(4): 1994-8.

Fujimura, Y. 2000 'Evidence of M cells as portals of entry for antigens in the nasopharyngeal lymphoid tissue of humans', *Virchows Arch* 436(6): 560-6.

Fujimura, Y., Akisada, T., Harada, T. and Haruma, K. 2006 'Uptake of microparticles into the epithelium of human nasopharyngeal lymphoid tissue', *Med Mol Morphol* 39(4): 181-6.

Fukuta, S., Magnani, J. L., Twiddy, E. M., Holmes, R. K. and Ginsburg, V. 1988 'Comparison of the carbohydrate-binding specificities of cholera toxin and Escherichia coli heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb', *Infect Immun* 56(7): 1748-53.

Fulton, S. A., Cross, J. V., Toossi, Z. T. and Boom, W. H. 1998 'Regulation of interleukin-12 by interleukin-10, transforming growth factor-beta, tumor necrosis factor-alpha, and interferon-gamma in human monocytes infected with Mycobacterium tuberculosis H37Ra', *J Infect Dis* 178(4): 1105-14.

Garmory, H. S., Leary, S. E., Griffin, K. F., Williamson, E. D., Brown, K. A. and Titball, R. W. 2003 'The use of live attenuated bacteria as a delivery system for heterologous antigens', *J Drug Target* 11(8-10): 471-9.

Geluk, A., van Meijgaarden, K. E., Franken, K. L., Drijfhout, J. W., D'Souza, S., Necker, A., Huygen, K. and Ottenhoff, T. H. 2000 'Identification of major epitopes of Mycobacterium tuberculosis AG85B that are recognized by HLA-A*0201-restricted CD8+ T cells in HLA-transgenic mice and humans', *J Immunol* 165(11): 6463-71.

Germanier, R. and Fuer, E. 1975 'Isolation and characterization of Gal E mutant Ty 21a of Salmonella typhi: a candidate strain for a live, oral typhoid vaccine', *J Infect Dis* 131(5): 553-8.

Gheorghiu, M. 1994 'BCG-induced mucosal immune responses', *Int J Immunopharmacol* 16(5-6): 435-44.

Giannasca, P. J., Boden, J. A. and Monath, T. P. 1997 'Targeted delivery of antigen to hamster nasal lymphoid tissue with M-cell-directed lectins', *Infect Immun* 65(10): 4288-98.

Giannella, R. A., Formal, S. B., Dammin, G. J. and Collins, H. 1973 'Pathogenesis of salmonellosis. Studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum', *J Clin Invest* 52(2): 441-53.

Giri, P. K., Sable, S. B., Verma, I. and Khuller, G. K. 2005 'Comparative evaluation of intranasal and subcutaneous route of immunization for development of mucosal vaccine against experimental tuberculosis', *FEMS Immunol Med Microbiol* 45(1): 87-93.

Gluck, U., Gebbers, J. O. and Gluck, R. 1999 'Phase 1 evaluation of intranasal virosomal influenza vaccine with and without Escherichia coli heat-labile toxin in adult volunteers', *J Virol* 73(9): 7780-6.

Glynn, A., Freytag, L. C. and Clements, J. D. 2005a 'Effect of homologous and heterologous prime-boost on the immune response to recombinant plague antigens', *Vaccine* 23(16): 1957-65.

Glynn, A., Roy, C. J., Powell, B. S., Adamovicz, J. J., Freytag, L. C. and Clements, J. D. 2005b 'Protection against aerosolized Yersinia pestis challenge following homologous and heterologous prime-boost with recombinant plague antigens', *Infect Immun* 73(8): 5256-61.

Gomez-Duarte, O. G., Pasetti, M. F., Santiago, A., Sztein, M. B., Hoffman, S. L. and Levine, M. M. 2001 'Expression, extracellular secretion, and immunogenicity of the Plasmodium falciparum sporozoite surface protein 2 in Salmonella vaccine strains', *Infect Immun* 69(2): 1192-8.

Gong, J. H., Zhang, M., Modlin, R. L., Linsley, P. S., Iyer, D., Lin, Y. and Barnes, P. F. 1996 'Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression', *Infect Immun* 64(3): 913-8.

Gordon, S. B. and Read, R. C. 2002 'Macrophage defences against respiratory tract infections', *Br Med Bull* 61: 45-61.

Gowans, J. L. and Knight, E. J. 1964 'The Route of Re-Circulation of Lymphocytes in the Rat', *Proc R Soc Lond B Biol Sci* 159: 257-82.

Grode, L., Seiler, P., Baumann, S., Hess, J., Brinkmann, V., Nasser Eddine, A., Mann, P., Goosmann, C., Bandermann, S., Smith, D., Bancroft, G. J., Reyrat, J. M., van Soolingen, D., Raupach, B. and Kaufmann, S. H. 2005 'Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin', *J Clin Invest* 115(9): 2472-9.

Grohmann, U., Fallarino, F., Bianchi, R., Belladonna, M. L., Vacca, C., Orabona, C., Uyttenhove, C., Fioretti, M. C. and Puccetti, P. 2001 'IL-6 inhibits the tolerogenic function of CD8 alpha+ dendritic cells expressing indoleamine 2,3-dioxygenase', *J Immunol* 167(2): 708-14.

Gueirard, P., Ave, P., Balazuc, A. M., Thiberge, S., Huerre, M., Milon, G. and Guiso, N. 2003 'Bordetella bronchiseptica persists in the nasal cavities of mice and triggers early delivery of dendritic cells in the lymph nodes draining the lower and upper respiratory tract', *Infect Immun* 71(7): 4137-43.

Guzman-Rojas, L., Sims-Mourtada, J. C., Rangel, R. and Martinez-Valdez, H. 2002 'Life and death within germinal centres: a double-edged sword', *Immunology* 107(2): 167-75.

Guzman, C. A., Saverino, D., Medina, E., Fenoglio, D., Gerstel, B., Merlo, A., Li Pira, G., Buffa, F., Chakraborty, T. and Manca, F. 1998 'Attenuated Listeria monocytogenes carrier strains can deliver an HIV-1 gp120 T helper epitope to MHC class II-restricted human CD4+ T cells', *Eur J Immunol* 28(6): 1807-14.

Haile, M. and Kallenius, G. 2005 'Recent developments in tuberculosis vaccines', *Curr Opin Infect Dis* 18(3): 211-5.

Hall, G., Houghton, C. G., Rahbek, J. U., Lamb, J. R. and Jarman, E. R. 2003 'Suppression of allergen reactive Th2 mediated responses and pulmonary eosinophilia by intranasal administration of an immunodominant peptide is linked to IL-10 production', *Vaccine* 21(5-6): 549-61.

Hameleers, D. M., van der Ende, M., Biewenga, J. and Sminia, T. 1989 'An immunohistochemical study on the postnatal development of rat nasal-associated lymphoid tissue (NALT)', *Cell Tissue Res* 256(2): 431-8.

Hamerman, J. A., Ogasawara, K. and Lanier, L. L. 2005 'NK cells in innate immunity', *Curr Opin Immunol* 17(1): 29-35.

Han, S., Hathcock, K., Zheng, B., Kepler, T. B., Hodes, R. and Kelsoe, G. 1995 'Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers', *J Immunol* 155(2): 556-67.

Hanada, T. and Yoshimura, A. 2002 'Regulation of cytokine signaling and inflammation', *Cytokine Growth Factor Rev* 13(4-5): 413-21.

Hancock, R. E. and Sahl, H. G. 2006 'Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies', *Nat Biotechnol* 24(12): 1551-7.

Harshan, K. V. and Gangadharam, P. R. 1991 'In vivo depletion of natural killer cell activity leads to enhanced multiplication of Mycobacterium avium complex in mice', *Infect Immun* 59(8): 2818-21.

Hart, P. D. 1967 'Efficacy and applicability of mass B. C.G. vaccination in tuberculosis control', *Br Med J* 1(540): 587-92.

Hathaway, L. J., Partidos, C. D., Vohra, P. and Steward, M. W. 1995 'Induction of systemic immune responses to measles virus synthetic peptides administered intranasally', *Vaccine* 13(16): 1495-500.

Hautefort, I., Proenca, M. J. and Hinton, J. C. 2003 'Single-copy green fluorescent protein gene fusions allow accurate measurement of Salmonella gene expression in vitro and during infection of mammalian cells', *Appl Environ Microbiol* 69(12): 7480-91.

Hendriks, H. R., Duijvestijn, A. M. and Kraal, G. 1987 'Rapid decrease in lymphocyte adherence to high endothelial venules in lymph nodes deprived of afferent lymphatic vessels', *Eur J Immunol* 17(12): 1691-5.

Hendriks, H. R. and Eestermans, I. L. 1983 'Disappearance and reappearance of high endothelial venules and immigrating lymphocytes in lymph nodes deprived of afferent lymphatic vessels: a possible regulatory role of macrophages in lymphocyte migration', *Eur J Immunol* 13(8): 663-9.

Henninger, D. D., Panes, J., Eppihimer, M., Russell, J., Gerritsen, M., Anderson, D. C. and Granger, D. N. 1997 'Cytokine-induced VCAM-1 and ICAM-1 expression in different organs of the mouse', *J Immunol* 158(4): 1825-32.

Hensel, M., Shea, J. E., Raupach, B., Monack, D., Falkow, S., Gleeson, C., Kubo, T. and Holden, D. W. 1997 'Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of Salmonella Pathogenicity Island 2', *Mol Microbiol* 24(1): 155-67.

Heritage, P. L., Underdown, B. J., Arsenault, A. L., Snider, D. P. and McDermott, M. R. 1997 'Comparison of murine nasal-associated lymphoid tissue and Peyer's patches', *American Journal of Respiratory and Critical Care Medicine* 156(4): 1256-1262.

Herrington, D. A., Van de Verg, L., Formal, S. B., Hale, T. L., Tall, B. D., Cryz, S. J., Tramont, E. C. and Levine, M. M. 1990 'Studies in volunteers to evaluate candidate Shigella vaccines: further experience with a bivalent Salmonella typhi-Shigella sonnei vaccine and protection conferred by previous Shigella sonnei disease', *Vaccine* 8(4): 353-7.

Herzog, H. 1998 'History of tuberculosis', *Respiration* 65(1): 5-15.

Hess, J., Dietrich, G., Gentschev, I., Miko, D., Goebel, W. and Kaufmann, S. H. 1997 'Protection against murine listeriosis by an attenuated recombinant Salmonella typhimurium vaccine strain that secretes the naturally somatic antigen superoxide dismutase', *Infect Immun* 65(4): 1286-92.

Hess, J., Grode, L., Hellwig, J., Conradt, P., Gentschev, I., Goebel, W., Ladel, C. and Kaufmann, S. H. 2000 'Protection against murine tuberculosis by an attenuated recombinant Salmonella typhimurium vaccine strain that secretes the 30-kDa antigen of Mycobacterium bovis BCG', *FEMS Immunol Med Microbiol* 27(4): 283-9.

Hindle, Z., Chatfield, S. N., Phillimore, J., Bentley, M., Johnson, J., Cosgrove, C. A., Ghaem-Maghami, M., Sexton, A., Khan, M., Brennan, F. R., Everest, P., Wu, T., Pickard, D., Holden, D. W., Dougan, G., Griffin, G. E., House, D., Santangelo, J. D., Khan, S. A., Shea, J. E., Feldman, R. G. and Lewis, D. J. 2002 'Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers', *Infect Immun* 70(7): 3457-67.

Hiroi, T., Goto, H., Someya, K., Yanagita, M., Honda, M., Yamanaka, N. and Kiyono, H. 2001 'HIV mucosal vaccine: nasal immunization with rBCG-V3J1 induces a long term V3J1 peptide-specific neutralizing immunity in Th1- and Th2-deficient conditions', *J Immunol* 167(10): 5862-7.

Hiroi, T., Iwatani, K., Iijima, H., Kodama, S., Yanagita, M. and Kiyono, H. 1998 'Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively', *Eur J Immunol* 28(10): 3346-53.

Hirsch, C. S., Toossi, Z., Othieno, C., Johnson, J. L., Schwander, S. K., Robertson, S., Wallis, R. S., Edmonds, K., Okwera, A., Mugerwa, R., Peters, P. and Ellner, J. J. 1999 'Depressed T-cell interferon-gamma responses in pulmonary tuberculosis: analysis of underlying mechanisms and modulation with therapy', *J Infect Dis* 180(6): 2069-73.

Hohmann, E. L., Oletta, C. A., Killeen, K. P. and Miller, S. I. 1996 'phoP/phoQ-deleted Salmonella typhi (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers', *J Infect Dis* 173(6): 1408-14.

Hoiseth, S. K. and Stocker, B. A. 1981 'Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines', *Nature* 291(5812): 238-9.

Holmgren, J., Czerkinsky, C., Eriksson, K. and Mharandi, A. 2003 'Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges', *Vaccine* 21: S89-S95.

Horwitz, M. A. and Harth, G. 2003 'A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis', *Infect Immun* 71(4): 1672-9.

Horwitz, M. A., Harth, G., Dillon, B. J. and Maslesa-Galic, S. 2000 'Recombinant bacillus calmette-guerin (BCG) vaccines expressing the Mycobacterium tuberculosis 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model', *Proc Natl Acad Sci U S A* 97(25): 13853-8.

Horwitz, M. A., Lee, B. W., Dillon, B. J. and Harth, G. 1995 'Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of Mycobacterium tuberculosis', *Proc Natl Acad Sci U S A* 92(5): 1530-4.

Hussain, I., Randolph, D., Brody, S. L., Song, S. K., Hsu, A., Kahn, A. M., Chaplin, D. D. and Hamilos, D. L. 2001 'Induction, distribution and modulation of upper airway allergic inflammation in mice', *Clin Exp Allergy* 31(7): 1048-59.

Husseiny, M. I. and Hensel, M. 2005 'Rapid method for the construction of Salmonella enterica Serovar Typhimurium vaccine carrier strains', *Infect Immun* 73(3): 1598-605.

Huygen, K., Content, J., Denis, O., Montgomery, D. L., Yawman, A. M., Deck, R. R., DeWitt, C. M., Orme, I. M., Baldwin, S., D'Souza, C., Drowart, A., Lozes, E., Vandenbussche, P., Van Vooren, J. P., Liu, M. A. and Ulmer, J. B. 1996 'Immunogenicity and protective efficacy of a tuberculosis DNA vaccine', *Nat Med* 2(8): 893-8.

Huygen, K., Lozes, E., Gilles, B., Drowart, A., Palfliet, K., Jurion, F., Roland, I., Art, M., Dufaux, M., Nyabenda, J. and et al. 1994 'Mapping of TH1 helper T-cell epitopes on major secreted mycobacterial antigen 85A in mice infected with live Mycobacterium bovis BCG', *Infect Immun* 62(2): 363-70.

IAVI 2006 'Nasal administration of AIDS vaccine candidate': IAVI.

Ibanga, H. B., Brookes, R. H., Hill, P. C., Owiafe, P. K., Fletcher, H. A., Lienhardt, C., Hill, A. V., Adegbola, R. A. and McShane, H. 2006 'Early clinical trials with a new tuberculosis vaccine, MVA85A, in tuberculosis-endemic countries: issues in study design', *Lancet Infect Dis* 6(8): 522-8.

Iho, S., Yamamoto, T., Takahashi, T. and Yamamoto, S. 1999 'Oligodeoxynucleotides containing palindrome sequences with internal 5'-CpG-3' act directly on human NK and activated T cells to induce IFN-gamma production in vitro', *J Immunol* 163(7): 3642-52.

Imaoka, K., Miller, C. J., Kubota, M., McChesney, M. B., Lohman, B., Yamamoto, M., Fujihashi, K., Someya, K., Honda, M., McGhee, J. R. and Kiyono, H. 1998 'Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues', *J Immunol* 161(11): 5952-8.

Jabbal-Gill, I., Fisher, A. N., Rappuoli, R., Davis, S. S. and Illum, L. 1998 'Stimulation of mucosal and systemic antibody responses against Bordetella pertussis filamentous haemagglutinin and recombinant pertussis toxin after nasal administration with chitosan in mice', *Vaccine* 16(20): 2039-46.

Jagannath, C., Actor, J. K. and Hunter, R. L., Jr. 1998 'Induction of nitric oxide in human monocytes and monocyte cell lines by Mycobacterium tuberculosis', *Nitric Oxide* 2(3): 174-86.

Janeway, C. A., Jr. and Medzhitov, R. 2002 'Innate immune recognition', *Annu Rev Immunol* 20: 197-216.

Jensen, M., Tawadros, S., Sedlacek, H. H., Schultze, J. L. and Berthold, F. 2004 'NK cell depletion diminish tumour-specific B cell responses', *Immunol Lett* 93(2-3): 205-10.

Johnson, C. M., Cooper, A. M., Frank, A. A., Bonorino, C. B., Wysoki, L. J. and Orme, I. M. 1997 'Mycobacterium tuberculosis aerogenic rechallenge infections in B cell-deficient mice', *Tuber Lung Dis* 78(5-6): 257-61.

Johnson, J. L., Kamya, R. M., Okwera, A., Loughlin, A. M., Nyole, S., Hom, D. L., Wallis, R. S., Hirsch, C. S., Wolski, K., Foulds, J., Mugerwa, R. D. and Ellner, J. J. 2000 'Randomized controlled trial of Mycobacterium vaccae immunotherapy in non-human immunodeficiency virus-infected ugandan adults with newly diagnosed pulmonary tuberculosis. The Uganda-Case Western Reserve University Research Collaboration', *J Infect Dis* 181(4): 1304-12.

Johnson, J. L., Nunn, A. J., Fourie, P. B., Ormerod, L. P., Mugerwa, R. D., Mwinga, A., Chintu, C., Ngwira, B., Onyebujoh, P. and Zumla, A. 2004 'Effect of Mycobacterium vaccae (SRL172) immunotherapy on radiographic healing in tuberculosis', *Int J Tuberc Lung Dis* 8(11): 1348-54.

Junqueira-Kipnis, A. P., Kipnis, A., Jamieson, A., Juarrero, M. G., Diefenbach, A., Raulet, D. H., Turner, J. and Orme, I. M. 2003 'NK cells respond to pulmonary infection with Mycobacterium tuberculosis, but play a minimal role in protection', *J Immunol* 171(11): 6039-45.

Kalinski, P., Mailliard, R. B., Giermasz, A., Zeh, H. J., Basse, P., Bartlett, D. L., Kirkwood, J. M., Lotze, M. T. and Herberman, R. B. 2005 'Natural killer-dendritic cell cross-talk in cancer immunotherapy', *Expert Opin Biol Ther* 5(10): 1303-15.

Kamath, A. B., Woodworth, J., Xiong, X., Taylor, C., Weng, Y. and Behar, S. M. 2004 'Cytolytic CD8+ T cells recognizing CFP10 are recruited to the lung after Mycobacterium tuberculosis infection', *J Exp Med* 200(11): 1479-89.

Kamath, A. T., Feng, C. G., Macdonald, M., Briscoe, H. and Britton, W. J. 1999 'Differential protective efficacy of DNA vaccines expressing secreted proteins of Mycobacterium tuberculosis', *Infect Immun* 67(4): 1702-7.

Kantele, A., Arvilommi, H., Iikkanen, K., Savilahti, E., Makela, H. P., Herzog, C., Furer, E. and Kantele, J. M. 2005 'Unique characteristics of the intestinal immune system as an inductive site after antigen reencounter', *J Infect Dis* 191(2): 312-7.

Kantele, A., Zivny, J., Hakkinen, M., Elson, C. O. and Mestecky, J. 1999 'Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans', *J Immunol* 162(9): 5173-7.

Karchev, T. 1996 'Significance of M-cells in the panautoprotection of the body', *Acta Otolaryngol Suppl* 523: 43-6.

Karchev, T. and Kabakchiev, P. 1984 'M-cells in the epithelium of the nasopharyngeal tonsil', *Rhinology* 22(3): 201-10.

Kariyone, A., Tamura, T., Kano, H., Iwakura, Y., Takeda, K., Akira, S. and Takatsu, K. 2003 'Immunogenicity of Peptide-25 of Ag85B in Th1 development: role of IFN-gamma', *Int Immunol* 15(10): 1183-94.

Karre, K., Ljunggren, H. G., Piontek, G. and Kiessling, R. 1986 'Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy', *Nature* 319(6055): 675-8.

Katz, J. M., Lu, X., Young, S. A. and Galphin, J. C. 1997 'Adjuvant activity of the heat-labile enterotoxin from enterotoxigenic Escherichia coli for oral administration of inactivated influenza virus vaccine', *J Infect Dis* 175(2): 352-63.

Kaufmann, S. H. 1999 'Killing vs suicide in antibacterial defence', *Trends Microbiol* 7(2): 59-61.

— 2000 'Is the development of a new tuberculosis vaccine possible?' *Nat Med* 6(9): 955-60.

— 2001 'How can immunology contribute to the control of tuberculosis?' *Nat Rev Immunol* 1(1): 20-30.

Keenan, J. I., Rijpkema, S. G., Durrani, Z. and Roake, J. A. 2003 'Differences in immunogenicity and protection in mice and guinea pigs following intranasal immunization with Helicobacter pylori outer membrane antigens', *FEMS Immunol Med Microbiol* 36(3): 199-205.

Kehri, J. H., Dukovich, M., Whalen, G., Katz, P., Fauci, A. S. and Greene, W. C. 1988 'Novel interleukin 2 (IL-2) receptor appears to mediate IL-2-induced activation of natural killer cells', *J Clin Invest* 81(1): 200-5.

Keller, R., Bachi, T. and Okumura, K. 1983 'Discrimination between macrophage-and NK-type tumoricidal activities via anti-asialo GM1 antibody', *Exp Cell Biol* 51(3): 158-64.

Kelsoe, G. 1996 'Life and death in germinal centers (redux)', *Immunity* 4(2): 107-11.

Khan, S., Chatfield, S., Stratford, R., Bedwell, J., Bentley, M., Sulsh, S., Giemza, R., Smith, S., Bongard, E., Cosgrove, C. A., Johnson, J., Dougan, G., Griffin, G. E., Makin, J. and Lewis, D. J. 2007 'Ability of SPI2 mutant of S. typhi to effectively induce antibody responses to the mucosal antigen enterotoxigenic E. coli heat labile toxin B subunit after oral delivery to humans', *Vaccine* 25(21): 4175-82.

Khan, S. A., Stratford, R., Wu, T., McKelvie, N., Bellaby, T., Hindle, Z., Sinha, K. A., Eltze, S., Mastroeni, P., Pickard, D., Dougan, G., Chatfield, S. N. and Brennan, F. R. 2003 'Salmonella typhi and S typhimurium derivatives harbouring deletions in aromatic biosynthesis and Salmonella Pathogenicity Island-2 (SPI-2) genes as vaccines and vectors', *Vaccine* 21(5-6): 538-48.

Khater, M., Macai, J., Genyea, C. and Kaplan, J. 1986 'Natural killer cell regulation of age-related and type-specific variations in antibody responses to pneumococcal polysaccharides', *J Exp Med* 164(5): 1505-15.

Kim, S., Iizuka, K., Aguila, H. L., Weissman, I. L. and Yokoyama, W. M. 2000 'In vivo natural killer cell activities revealed by natural killer cell-deficient mice', *Proc Natl Acad Sci U S A* 97(6): 2731-6.

Kim, S. and Yokoyama, W. M. 1998 'NK cell granule exocytosis and cytokine production inhibited by Ly-49A engagement', *Cell Immunol* 183(2): 106-12.

Kindler, V., Sappino, A. P., Grau, G. E., Piguet, P. F. and Vassalli, P. 1989 'The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection', *Cell* 56(5): 731-40.

Kirkpatrick, B. D., McKenzie, R., O'Neill, J. P., Larsson, C. J., Bourgeois, A. L., Shimko, J., Bentley, M., Makin, J., Chatfield, S., Hindle, Z., Fidler, C., Robinson, B. E., Ventrone, C. H., Bansal, N., Carpenter, C. M., Kutzko, D., Hamlet, S., LaPointe, C. and Taylor, D. N. 2006 'Evaluation of Salmonella enterica serovar Typhi (Ty2 aroC-ssaV-) M01ZH09, with a defined mutation in the Salmonella pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers', *Vaccine* 24(2): 116-23.

Kiyono, H. and Fukuyama, S. 2004 'NALT- versus Peyer's-patch-mediated mucosal immunity', *Nat Rev Immunol* 4(9): 699-710.

Kiyono, H., Kweon, M. N., Hiroi, T. and Takahashi, I. 2001 'The mucosal immune system: from specialized immune defense to inflammation and allergy', *Acta Odontol Scand* 59(3): 145-53.

Klee, S. R., Tzschaschel, B. D., Falt, I., Karnell, A., Lindberg, A. A., Timmis, K. N. and Guzman, C. A. 1997 'Construction and characterization of a live attenuated vaccine candidate against Shigella dysenteriae type 1', *Infect Immun* 65(6): 2112-8.

Klimpel, G. R., Asuncion, M., Haithcoat, J. and Niesel, D. W. 1995 'Cholera toxin and Salmonella typhimurium induce different cytokine profiles in the gastrointestinal tract', *Infect Immun* 63(3): 1134-7.

Kobayashi, S. D., Voyich, J. M., Burlak, C. and DeLeo, F. R. 2005 'Neutrophils in the innate immune response', *Arch Immunol Ther Exp (Warsz)* 53(6): 505-17.

Koch, R. 1882 'Die Aetiologie der Tuberculose', *Berliner Klinische Wochenschrift* 19: 221-230.

Koopman, G., Keehnen, R. M., Lindhout, E., Newman, W., Shimizu, Y., van Seventer, G. A., de Groot, C. and Pals, S. T. 1994 'Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells', *J Immunol* 152(8): 3760-7.

Koopman, G., Parmentier, H. K., Schuurman, H. J., Newman, W., Meijer, C. J. and Pals, S. T. 1991 'Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways', *J Exp Med* 173(6): 1297-304.

Korsgren, M., Persson, C. G., Sundler, F., Bjerke, T., Hansson, T., Chambers, B. J., Hong, S., Van Kaer, L., Ljunggren, H. G. and Korsgren, O. 1999 'Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice', *J Exp Med* 189(3): 553-62.

Kosco-Vilbois, M. H., Bonnefoy, J. Y. and Chvatchko, Y. 1997a 'The physiology of murine germinal center reactions', *Immunol Rev* 156: 127-36.

Kosco-Vilbois, M. H., Zentgraf, H., Gerdes, J. and Bonnefoy, J. Y. 1997b 'To 'B' or not to 'B' a germinal center?' *Immunol Today* 18(5): 225-30.

Kotloff, K. L., Wasserman, S. S., Jones, K. F., Livio, S., Hruby, D. E., Franke, C. A. and Fischetti, V. A. 2005 'Clinical and microbiological responses of volunteers to combined intranasal and oral inoculation with a Streptococcus gordonii carrier strain intended for future use as a group A streptococcus vaccine', *Infect Immun* 73(4): 2360-6.

Kotton, C. N. and Hohmann, E. L. 2004 'Enteric pathogens as vaccine vectors for foreign antigen delivery', *Infect Immun* 72(10): 5535-47.

Kotton, C. N., Lankowski, A. J., Scott, N., Sisul, D., Chen, L. M., Raschke, K., Borders, G., Boaz, M., Spentzou, A., Galan, J. E. and Hohmann, E. L. 2006 'Safety and immunogenicity of attenuated Salmonella enterica serovar Typhimurium delivering an HIV-1 Gag antigen via the Salmonella Type III secretion system', *Vaccine* 24(37-39): 6216-24.

Kraehenbuhl, J. P. and Neutra, M. R. 2000 'Epithelial M cells: differentiation and function', *Annu Rev Cell Dev Biol* 16: 301-32.

Kraehenbuhl JP, N. M. 1998 *Defense of mucosal surfaces: pathogenicity, immunity and vaccines*, Vol. 236, New York: Springer-Verlag.

Kunkel, E. J., Kim, C. H., Lazarus, N. H., Vierra, M. A., Soler, D., Bowman, E. P. and Butcher, E. C. 2003 'CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells', *J Clin Invest* 111(7): 1001-10.

Kuper, C. F., Hameleers, D. M., Bruijntjes, J. P., van der Ven, I., Biewenga, J. and Sminia, T. 1990 'Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study', *Cell Tissue Res* 259(2): 371-7.

Kuper, C. F., Koornstra, P. J., Hameleers, D. M. H., Biewenga, J., Spit, B. J., Duijvestijn, A. M., Vriesman, P. and Sminia, T. 1992 'The Role of Nasopharyngeal Lymphoid-Tissue', *Immunology Today* 13(6): 219-224.

Kurono, Y., Yamamoto, M., Fujihashi, K., Kodama, S., Suzuki, M., Mogi, G., McGhee, J. R. and Kiyono, H. 1999 'Nasal immunization induces Haemophilus influenzae-specific Th1 and Th2 responses with mucosal IgA and systemic IgG antibodies for protective immunity', *J Infect Dis* 180(1): 122-32.

Laal, S., Samanich, K. M., Sonnenberg, M. G., Zolla-Pazner, S., Phadtare, J. M. and Belisle, J. T. 1997 'Human humoral responses to antigens of Mycobacterium tuberculosis: immunodominance of high-molecular-mass antigens', *Clin Diagn Lab Immunol* 4(1): 49-56.

Lalvani, A., Pathan, A. A., McShane, H., Wilkinson, R. J., Latif, M., Conlon, C. P., Pasvol, G. and Hill, A. V. 2001 'Rapid detection of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells', *Am J Respir Crit Care Med* 163(4): 824-8.

Lamm, M. E. 1997 'Interaction of antigens and antibodies at mucosal surfaces', *Annu Rev Microbiol* 51: 311-40.

Langermans, J. A., Doherty, T. M., Vervenne, R. A., van der Laan, T., Lyashchenko, K., Greenwald, R., Agger, E. M., Aagaard, C., Weiler, H., van Soolingen, D., Dalemans, W., Thomas, A. W. and Andersen, P. 2005 'Protection of macaques against Mycobacterium tuberculosis infection by a subunit vaccine based on a fusion protein of antigen 85B and ESAT-6', *Vaccine* 23(21): 2740-50.

Lanier, L. L. 1998 'NK cell receptors', *Annu Rev Immunol* 16: 359-93.

— 2005 'NK cell recognition', *Annu Rev Immunol* 23: 225-74.

Lanier, L. L., Benike, C. J., Phillips, J. H. and Engleman, E. G. 1985 'Recombinant interleukin 2 enhanced natural killer cell-mediated cytotoxicity in human lymphocyte subpopulations expressing the Leu 7 and Leu 11 antigens', *J Immunol* 134(2): 794-801.

Lanier, L. L., Buck, D. W., Rhodes, L., Ding, A., Evans, E., Barney, C. and Phillips, J. H. 1988 'Interleukin 2 activation of natural killer cells rapidly induces the expression and phosphorylation of the Leu-23 activation antigen', *J Exp Med* 167(5): 1572-85.

Lasaro, M. O., Luiz, W. B., Sbrogio-Almeida, M. E. and Ferreira, L. C. 2005 'Prime-boost vaccine regimen confers protective immunity to human-derived enterotoxigenic Escherichia coli', *Vaccine* 23(19): 2430-8.

Latchumanan, V. K., Balkhi, M. Y., Sinha, A., Singh, B., Sharma, P. and Natarajan, K. 2005 'Regulation of immune responses to Mycobacterium tuberculosis secretory antigens by dendritic cells', *Tuberculosis (Edinb)* 85(5-6): 377-83.

Latchumanan, V. K., Singh, B., Sharma, P. and Natarajan, K. 2002 'Mycobacterium tuberculosis antigens induce the differentiation of dendritic cells from bone marrow', *J Immunol* 169(12): 6856-64.

Launois, P., DeLeys, R., Niang, M. N., Drowart, A., Andrien, M., Dierckx, P., Cartel, J. L., Sarthou, J. L., Van Vooren, J. P. and Huygen, K. 1994 'T-cell-epitope mapping of the major secreted mycobacterial antigen Ag85A in tuberculosis and leprosy', *Infect Immun* 62(9): 3679-87.

Lazarus, N. H., Kunkel, E. J., Johnston, B., Wilson, E., Youngman, K. R. and Butcher, E. C. 2003 'A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts', *J Immunol* 170(7): 3799-805.

Levine, M. M., Kaper, J. B., Black, R. E. and Clements, M. L. 1983 'New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development', *Microbiol Rev* 47(4): 510-50.

Li, J. S., Yager, E., Reilly, M., Freeman, C., Reddy, G. R., Reilly, A. A., Chu, F. K. and Winslow, G. M. 2001 'Outer membrane protein-specific monoclonal antibodies protect SCID mice from fatal infection by the obligate intracellular bacterial pathogen Ehrlichia chaffeensis', *J Immunol* 166(3): 1855-62.

Liang, B., Hyland, L. and Hou, S. 2001 'Nasal-associated lymphoid tissue is a site of long-term virus-specific antibody production following respiratory virus infection of mice', *J Virol* 75(11): 5416-20.

Lieke, T., Steeg, C., Graefe, S. E., Fleischer, B. and Jacobs, T. 2006 'Interaction of natural killer cells with Trypanosoma cruzi-infected fibroblasts', *Clin Exp Immunol* 145(2): 357-64.

Ligocyte 2007 'Norovirus vaccine program'.

Lindholm, C., Naylor, A., Johansson, E. L. and Quiding-Jarbrink, M. 2004 'Mucosal vaccination increases endothelial expression of mucosal addressin cell adhesion molecule 1 in the human gastrointestinal tract', *Infect Immun* 72(2): 1004-9.

Lodge, J., Douce, G. R., Amin, II, Bolton, A. J., Martin, G. D., Chatfield, S., Dougan, G., Brown, N. L. and Stephen, J. 1995 'Biological and genetic characterization of TnphoA mutants of Salmonella typhimurium TML in the context of gastroenteritis', *Infect Immun* 63(3): 762-9.

Lodoen, M. B. and Lanier, L. L. 2006 'Natural killer cells as an initial defense against pathogens', *Curr Opin Immunol* 18(4): 391-8.

Luo, Y., Yamada, H., Chen, X., Ryan, A. A., Evanoff, D. P., Triccas, J. A. and O'Donnell, M. A. 2004 'Recombinant Mycobacterium bovis bacillus Calmette-Guerin (BCG) expressing mouse IL-18 augments Th1 immunity and macrophage cytotoxicity', *Clin Exp Immunol* 137(1): 24-34.

Lyadova, I. V., Vordermeier, H. M., Eruslanov, E. B., Khaidukov, S. V., Apt, A. S. and Hewinson, R. G. 2001 'Intranasal BCG vaccination protects BALB/c mice against virulent Mycobacterium bovis and accelerates production of IFN-gamma in their lungs', *Clin Exp Immunol* 126(2): 274-9.

Lyashchenko, K., Colangeli, R., Houde, M., Al Jahdali, H., Menzies, D. and Gennaro, M. L. 1998a 'Heterogeneous antibody responses in tuberculosis', *Infect Immun* 66(8): 3936-40.

Lyashchenko, K. P., Pollock, J. M., Colangeli, R. and Gennaro, M. L. 1998b 'Diversity of antigen recognition by serum antibodies in experimental bovine tuberculosis', *Infect Immun* 66(11): 5344-9.

MacMicking, J. D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K. and Nathan, C. F. 1997 'Identification of nitric oxide synthase as a protective locus against tuberculosis', *Proc Natl Acad Sci U S A* 94(10): 5243-8.

Macpherson, A. J., Gatto, D., Sainsbury, E., Harriman, G. R., Hengartner, H. and Zinkernagel, R. M. 2000 'A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria', *Science* 288(5474): 2222-6.

Maggi, E., Del Prete, G. F., Parronchi, P., Tiri, A., Macchia, D., Biswas, P., Simonelli, C., Ricci, M. and Romagnani, S. 1989 'Role for T cells, IL-2 and IL-6 in the IL-4-dependent in vitro human IgE synthesis', *Immunology* 68(3): 300-6.

Mahairas, G. G., Sabo, P. J., Hickey, M. J., Singh, D. C. and Stover, C. K. 1996 'Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis', *J Bacteriol* 178(5): 1274-82.

Marinaro, M., Riccomi, A., Rappuoli, R., Pizza, M., Fiorelli, V., Tripiciano, A., Cafaro, A., Ensoli, B. and De Magistris, M. T. 2003 'Mucosal delivery of the human immunodeficiency virus-1 Tat protein in mice elicits systemic neutralizing antibodies, cytotoxic T lymphocytes and mucosal IgA', *Vaccine* 21(25-26): 3972-81.

Martin-Fontecha, A., Thomsen, L. L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A. and Sallusto, F. 2004 'Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming', *Nat Immunol* 5(12): 1260-5.

Martin, M., Sharpe, A., Clements, J. D. and Michalek, S. M. 2002 'Role of B7 costimulatory molecules in the adjuvant activity of the heat-labile enterotoxin of Escherichia coli', *J Immunol* 169(4): 1744-52.

McCarthy, M. 2004 'Gates grant boosts tuberculosis vaccine research. US82.9 million dollars grant will double global spending on new tuberculosis vaccines', *Lancet* 363(9408): 537.

McCluskie, M. J. and Davis, H. L. 1999 'Mucosal immunization with DNA vaccines', *Microbes Infect* 1(9): 685-98.

McGhee, J. R. and Kiyono, H. 1992 'Mucosal immunity to vaccines: current concepts for vaccine development and immune response analysis', *Adv Exp Med Biol* 327: 3-12.

— 1993 'New perspectives in vaccine development: mucosal immunity to infections.' *Infect. Agent. Dis.* 2: 55-73.

McKelvie, N. D., Stratford, R., Wu, T., Bellaby, T., Aldred, E., Hughes, N. J., Chatfield, S. N., Pickard, D., Hale, C., Dougan, G. and Khan, S. A. 2004 'Expression of heterologous antigens in Salmonella Typhimurium vaccine vectors using the in vivo-inducible, SPI-2 promoter, ssaG', *Vaccine* 22(25-26): 3243-55.

McKnight, A. J., Zimmer, G. J., Fogelman, I., Wolf, S. F. and Abbas, A. K. 1994 'Effects of IL-12 on helper T cell-dependent immune responses in vivo', *J Immunol* 152(5): 2172-9.

McShane, H., Pathan, A. A., Sander, C. R., Goonetilleke, N. P., Fletcher, H. A. and Hill, A. V. 2005 'Boosting BCG with MVA85A: the first candidate subunit vaccine for tuberculosis in clinical trials', *Tuberculosis (Edinb)* 85(1-2): 47-52.

McShane, H., Pathan, A. A., Sander, C. R., Keating, S. M., Gilbert, S. C., Huygen, K., Fletcher, H. A. and Hill, A. V. 2004 'Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans', *Nat Med* 10(11): 1240-4.

Meager, A. 1999 'Cytokine regulation of cellular adhesion molecule expression in inflammation', *Cytokine Growth Factor Rev* 10(1): 27-39.

Mebius, R. E., Dowbenko, D., Williams, A., Fennie, C., Lasky, L. A. and Watson, S. R. 1993 'Expression of GlyCAM-1, an endothelial ligand for L-selectin, is affected by afferent lymphatic flow', *J Immunol* 151(12): 6769-76.

Medaglini, D., Ciabattini, A., Cuppone, A. M., Costa, C., Ricci, S., Costalonga, M. and Pozzi, G. 2006 'In vivo activation of naive CD4+ T cells in nasal mucosa-associated lymphoid tissue following intranasal immunization with recombinant Streptococcus gordonii', *Infect Immun* 74(5): 2760-6.

Medina, E. and Guzman, C. A. 2000 'Modulation of immune responses following antigen administration by mucosal route', *FEMS Immunol Med Microbiol* 27(4): 305-11.

Medina, E., Paglia, P., Rohde, M., Colombo, M. P. and Guzman, C. A. 2000 'Modulation of host immune responses stimulated by Salmonella vaccine carrier strains by using different promoters to drive the expression of the recombinant antigen', *Eur J Immunol* 30(3): 768-77.

Medzhitov, R. and Janeway, C., Jr. 2000 'Innate immunity', *N Engl J Med* 343(5): 338-44.

Medzhitov, R. and Janeway, C. A., Jr. 1997 'Innate immunity: the virtues of a nonclonal system of recognition', *Cell* 91(3): 295-8.

Merritt, E. A., Pronk, S. E., Sixma, T. K., Kalk, K. H., van Zanten, B. A. and Hol, W. G. 1994 'Structure of partially-activated E. coli heat-labile enterotoxin (LT) at 2.6 A resolution', *FEBS Lett* 337(1): 88-92.

Michael, A., Shao, A. and Yuan, D. 1991 'Productive interactions between B and natural killer cells', *Nat Immun Cell Growth Regul* 10(2): 71-82.

Microscience 2004a 'Latest trial results move British company ahead in the race to develop new oral vaccine to protect against travellers' diarrhoea' *Press Release*.

— 2004b 'Successful phase I clinical trial of spi-VECTM oral hepatitis B vaccine paves way for phase II efficacy trail' *Press Release*.

Mineo, J. R., Khan, I. A. and Kasper, L. H. 1994 'Toxoplasma gondii: a monoclonal antibody that inhibits intracellular replication', *Exp Parasitol* 79(3): 351-61.

Mittrucker, H. W. and Kaufmann, S. H. 2000 'Immune response to infection with Salmonella typhimurium in mice', *J Leukoc Biol* 67(4): 457-63.

Mohan, V. P., Scanga, C. A., Yu, K., Scott, H. M., Tanaka, K. E., Tsang, E., Tsai, M. M., Flynn, J. L. and Chan, J. 2001 'Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology', *Infect Immun* 69(3): 1847-55.

Mollenkopf, H. J., Groine-Triebkorn, D., Andersen, P., Hess, J. and Kaufmann, S. H. E. 2001 'Protective efficacy against tuberculosis of ESAT-6 secreted by a live Salmonella typhimurium vaccine carrier strain and expressed by naked DNA', *Vaccine* 19(28-29): 4028-4035.

Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. and Kupfer, A. 1998 'Three-dimensional segregation of supramolecular activation clusters in T cells', *Nature* 395(6697): 82-6.

Monteiro, R. C. and Van De Winkel, J. G. 2003 'IgA Fc receptors', *Annu Rev Immunol* 21: 177-204.

Moreau, M. C. and Corthier, G. 1988 'Effect of the gastrointestinal microflora on induction and maintenance of oral tolerance to ovalbumin in C3H/HeJ mice', *Infect Immun* 56(10): 2766-8.

Morton, M., Garmory, H. S., Perkins, S. D., O'Dowd, A. M., Griffin, K. F., Turner, A. K., Bennett, A. M. and Titball, R. W. 2004 'A Salmonella enterica serovar Typhi vaccine expressing Yersinia pestis F1 antigen on its surface provides protection against plague in mice', *Vaccine* 22(20): 2524-32.

Mosmann, T. R. and Coffman, R. L. 1989 'TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties', *Annu Rev Immunol* 7: 145-73.

Mossad, S. B. 2003 'Demystifying FluMist, a new intranasal, live influenza vaccine', *Cleve Clin J Med* 70(9): 801-6.

Mukherjee, J., Nussbaum, G., Scharff, M. D. and Casadevall, A. 1995a 'Protective and nonprotective monoclonal antibodies to Cryptococcus neoformans originating from one B cell', *J Exp Med* 181(1): 405-9.

Mukherjee, J., Scharff, M. D. and Casadevall, A. 1995b 'Variable efficacy of passive antibody administration against diverse Cryptococcus neoformans strains', *Infect Immun* 63(9): 3353-9.

Murphy, E. A., Davis, J. M., Brown, A. S., Carmichael, M. D., Van Rooijen, N., Ghaffar, A. and Mayer, E. P. 2004 'Role of lung macrophages on susceptibility to respiratory infection following short-term moderate exercise training', *Am J Physiol Regul Integr Comp Physiol* 287(6): R1354-8.

Murray, P. J., Aldovini, A. and Young, R. A. 1996 'Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines', *Proc Natl Acad Sci U S A* 93(2): 934-9.

Murray, P. J., Wang, L., Onufryk, C., Tepper, R. I. and Young, R. A. 1997 'T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection', *J Immunol* 158(1): 315-21.

Murthy, A. K., Chambers, J. P., Meier, P. A., Zhong, G. and Arulanandam, B. P. 2007 'Intranasal vaccination with a secreted chlamydial protein enhances resolution of genital Chlamydia muridarum infection, protects against oviduct pathology, and is highly dependent upon endogenous gamma interferon production', *Infect Immun* 75(2): 666-76.

Mustafa, A. S., Oftung, F., Amoudy, H. A., Madi, N. M., Abal, A. T., Shaban, F., Krands, I. R. and Andersen, P. 2000a 'Multiple epitopes from the Mycobacterium tuberculosis ESAT-6 antigen are recognized by antigen-specific human T cell lines', *Clinical Infectious Diseases* 30: S201-S205.

Mustafa, A. S., Shaban, F. A., Abal, A. T., Al-Attiyah, R., Wiker, H. G., Lundin, K. E., Oftung, F. and Huygen, K. 2000b 'Identification and HLA restriction of naturally derived Th1-cell epitopes from the secreted Mycobacterium tuberculosis antigen 85B recognized by antigen-specific human CD4(+) T-cell lines', *Infect Immun* 68(7): 3933-40.

Mustafa, A. S., Shaban, F. A., Al-Attiyah, R., Abal, A. T., El-Shamy, A. M., Andersen, P. and Oftung, F. 2003 'Human Th1 cell lines recognize the Mycobacterium tuberculosis ESAT-6 antigen and its peptides in association with frequently expressed HLA class II molecules', *Scandinavian Journal of Immunology* 57(2): 125-134.

Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R. T., Linder, T., Spyr, C. and Steffen, R. 2004 'Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland', *N Engl J Med* 350(9): 896-903.

Nagler-Anderson, C. 2001 'Man the barrier! Strategic defences in the intestinal mucosa', *Nat Rev Immunol* 1(1): 59-67.

Nardelli-Haefliger, D., Roden, R. B., Benyacoub, J., Sahli, R., Kraehenbuhl, J. P., Schiller, J. T., Lachat, P., Potts, A. and De Grandi, P. 1997 'Human papillomavirus type 16 virus-like particles expressed in attenuated Salmonella typhimurium elicit mucosal and systemic neutralizing antibodies in mice', *Infect Immun* 65(8): 3328-36.

Nathan, C. 2006 'Neutrophils and immunity: challenges and opportunities', *Nat Rev Immunol* 6(3): 173-82.

Nayak, A. R., Tinge, S. A., Tart, R. C., McDaniel, L. S., Briles, D. E. and Curtiss, R., 3rd 1998 'A live recombinant avirulent oral Salmonella vaccine expressing pneumococcal surface protein A induces protective responses against Streptococcus pneumoniae', *Infect Immun* 66(8): 3744-51.

Nicholson, S., Bonecini-Almeida Mda, G., Lapa e Silva, J. R., Nathan, C., Xie, Q. W., Mumford, R., Weidner, J. R., Calaycay, J., Geng, J., Boechat, N., Linhares, C., Rom, W. and Ho, J. L. 1996 'Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis', *J Exp Med* 183(5): 2293-302.

Nochi, T. and Kiyono, H. 2006 'Innate immunity in the mucosal immune system', *Curr Pharm Des* 12(32): 4203-13.

O'Leary, J. G., Goodarzi, M., Drayton, D. L. and von Andrian, U. H. 2006 'T cell- and B cell-independent adaptive immunity mediated by natural killer cells', *Nat Immunol* 7(5): 507-16.

Ogra, P. L. 1971 'Effect of tonsillectomy and adenoidectomy on nasopharyngeal antibody response to poliovirus', *N Engl J Med* 284(2): 59-64.

Ogra, P. L., Faden, H. and Welliver, R. C. 2001 'Vaccination strategies for mucosal immune responses', *Clin Microbiol Rev* 14(2): 430-45.

Ohl, M. E. and Miller, S. I. 2001 'Salmonella: a model for bacterial pathogenesis', *Annu Rev Med* 52: 259-74.

Oliveira, M. L., Areas, A. P., Campos, I. B., Monedero, V., Perez-Martinez, G., Miyaji, E. N., Leite, L. C., Aires, K. A. and Lee Ho, P. 2006 'Induction of systemic and mucosal immune response and decrease in Streptococcus pneumoniae colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A', *Microbes Infect* 8(4): 1016-24.

Olsen, A. W. and Andersen, P. 2003 'A novel TB vaccine; strategies to combat a complex pathogen', *Immunol Lett* 85(2): 207-11.

Olsen, A. W., Hansen, P. R., Holm, A. and Andersen, P. 2000 'Efficient protection against Mycobacterium tuberculosis by vaccination with a single subdominant epitope from the ESAT-6 antigen', *European Journal of Immunology* 30(6): 1724-1732.

Olsen, A. W., van Pinxteren, L. A. H., Okkels, L. M., Rasmussen, P. B. and Andersen, P. 2001 'Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85B and ESAT-6', *Infection and Immunity* 69(5): 2773-2778.

Olsen, A. W., Williams, A., Okkels, L. M., Hatch, G. and Andersen, P. 2004 'Protective effect of a tuberculosis subunit vaccine based on a fusion of antigen 85B and ESAT-6 in the aerosol guinea pig model', *Infect Immun* 72(10): 6148-50.

Olsen, I., Boysen, P., Kulberg, S., Hope, J. C., Jungersen, G. and Storset, A. K. 2005 'Bovine NK cells can produce gamma interferon in response to the secreted mycobacterial proteins ESAT-6 and MPP14 but not in response to MPB70', *Infect Immun* 73(9): 5628-35.

Orme, I. M., Roberts, A. D., Griffin, J. P. and Abrams, J. S. 1993 'Cytokine secretion by CD4 T lymphocytes acquired in response to Mycobacterium tuberculosis infection', *J Immunol* 151(1): 518-25.

Ortaldo, J. R. and Young, H. A. 2003 'Expression of IFN-gamma upon triggering of activating Ly49D NK receptors in vitro and in vivo: costimulation with IL-12 or IL-18 overrides inhibitory receptors', *J Immunol* 170(4): 1763-9.

Ottenhoff, T. H., Kumararatne, D. and Casanova, J. L. 1998 'Novel human immunodeficiencies reveal the essential role of type-I cytokines in immunity to intracellular bacteria', *Immunol Today* 19(11): 491-4.

Ouhara, K., Komatsuzawa, H., Shiba, H., Uchida, Y., Kawai, T., Sayama, K., Hashimoto, K., Taubman, M. A., Kurihara, H. and Sugai, M. 2006 'Actinobacillus actinomycetemcomitans outer membrane protein 100 triggers innate immunity and production of beta-defensin and the 18-kilodalton cationic antimicrobial protein through the fibronectin-integrin pathway in human gingival epithelial cells', *Infect Immun* 74(9): 5211-20.

Pacheco, L. G., Zucconi, E., Mati, V. L., Garcia, R. M., Miyoshi, A., Oliveira, S. C., de Melo, A. L. and Azevedo, V. 2005 'Oral administration of a live Aro attenuated Salmonella vaccine strain expressing 14-kDa Schistosoma mansoni fatty acid-binding protein induced partial protection against experimental schistosomiasis', *Acta Trop* 95(2): 132-42.

Panes, J. and Granger, D. N. 1998 'Leukocyte-endothelial cell interactions: molecular mechanisms and implications in gastrointestinal disease', *Gastroenterology* 114(5): 1066-90.

Parida, S. K., Huygen, K., Ryffel, B. and Chakraborty, T. 2005 'Novel bacterial delivery system with attenuated Salmonella typhimurium carrying plasmid encoding Mtb antigen 85A for mucosal immunization: establishment of proof of principle in TB mouse model', *Ann N Y Acad Sci* 1056: 366-78.

Park, H. S., Costalonga, M., Reinhardt, R. L., Dombek, P. E., Jenkins, M. K. and Cleary, P. P. 2004 'Primary induction of CD4 T cell responses in nasal associated lymphoid tissue during group A streptococcal infection', *Eur J Immunol* 34(10): 2843-53.

Partidos, C. D. 2000 'Intranasal vaccines: forthcoming challenges', *Pharm. Sci. Technol. Today* 3(8): 273-281.

Pasetti, M. F., Barry, E. M., Losonsky, G., Singh, M., Medina-Moreno, S. M., Polo, J. M., Ulmer, J., Robinson, H., Sztein, M. B. and Levine, M. M. 2003 'Attenuated Salmonella enterica serovar Typhi and Shigella flexneri 2a strains mucosally deliver DNA vaccines encoding measles virus hemagglutinin, inducing specific immune responses and protection in cotton rats', *J Virol* 77(9): 5209-17.

Pathak, S. K., Basu, S., Basu, K. K., Banerjee, A., Pathak, S., Bhattacharyya, A., Kaisho, T., Kundu, M. and Basu, J. 2007 'Direct extracellular interaction between the early secreted antigen ESAT-6 of Mycobacterium tuberculosis and TLR2 inhibits TLR signaling in macrophages', *Nat Immunol* 8(6): 610-8.

Peakman, T., Crouzet, J., Mayaux, J. F., Busby, S., Mohan, S., Harborne, N., Wootton, J., Nicolson, R. and Cole, J. 1990 'Nucleotide sequence, organisation and structural analysis of the products of genes in the nirB-cysG region of the Escherichia coli K-12 chromosome', *Eur J Biochem* 191(2): 315-23.

Pedrosa, J., Saunders, B. M., Appelberg, R., Orme, I. M., Silva, M. T. and Cooper, A. M. 2000 'Neutrophils play a protective nonphagocytic role in systemic Mycobacterium tuberculosis infection of mice', *Infect Immun* 68(2): 577-83.

Peppoloni, S., Ruggiero, P., Contorni, M., Pizza, M., Rappuoli, R., Podda, A., Del Giudice, G. 2003 'Mutants of the Escherichia coli heat-labile enterotoxin as safe and strong adjuvants for intranasal delivery of vaccines', *Expert Rev. Vaccines.* 2(2): 285-293.

Perez, E., Samper, S., Bordas, Y., Guilhot, C., Gicquel, B. and Martin, C. 2001 'An essential role for phoP in Mycobacterium tuberculosis virulence', *Mol Microbiol* 41(1): 179-87.

Perretti, M., Wheller, S. K., Harris, J. G. and Flower, R. J. 1996 'Modulation of ICAM-1 levels on U-937 cells and mouse macrophages by interleukin-1 beta and dexamethasone', *Biochem Biophys Res Commun* 223(1): 112-7.

Perry, L. L., Feilzer, K., Portis, J. L. and Caldwell, H. D. 1998 'Distinct homing pathways direct T lymphocytes to the genital and intestinal mucosae in Chlamydia-infected mice', *J Immunol* 160(6): 2905-14.

Perry, M. and Whyte, A. 1998 'Immunology of the tonsils', *Immunol Today* 19(9): 414-21.

Pethe, K., Alonso, S., Biet, F., Delogu, G., Brennan, M. J., Locht, C. and Menozzi, F. D. 2001 'The heparin-binding haemagglutinin of M. tuberculosis is required for extrapulmonary dissemination', *Nature* 412(6843): 190-4.

Petrovska, L., Lopes, L., Simmons, C. P., Pizza, M., Dougan, G. and Chain, B. M. 2003 'Modulation of dendritic cell endocytosis and antigen processing pathways by Escherichia coli heat-labile enterotoxin and mutant derivatives', *Vaccine* 21(13-14): 1445-54.

Philipp, W. J., Schwartz, D. C., Telenti, A. and Cole, S. T. 1998 'Mycobacterial genome structure', *Electrophoresis* 19(4): 573-6.

Pizza, M., Giuliani, M. M., Fontana, M. R., Monaci, E., Douce, G., Dougan, G., Mills, K. H., Rappuoli, R. and Del Giudice, G. 2001 'Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants', *Vaccine* 19(17-19): 2534-41.

Pollock, J. M. and Andersen, P. 1997 'Predominant recognition of the ESAT-6 protein in the first phase of interferon with Mycobacterium bovis in cattle', *Infect Immun* 65(7): 2587-92.

Porcelli, S. A. and Modlin, R. L. 1999 'The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids', *Annu Rev Immunol* 17: 297-329.

Porgador, A., Staats, H. F., Itoh, Y. and Kelsall, B. L. 1998 'Intranasal immunization with cytotoxic T-lymphocyte epitope peptide and mucosal adjuvant cholera toxin: Selective augmentation of peptide-presenting dendritic cells in nasal mucosa-associated lymphoid tissue', *Infection and Immunity* 66(12): 5876-5881.

Powrie, F., Carlino, J., Leach, M. W., Mauze, S. and Coffman, R. L. 1996 'A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells', *J Exp Med* 183(6): 2669-74.

Powrie, F. and Maloy, K. J. 2003 'Immunology. Regulating the regulators', *Science* 299(5609): 1030-1.

Pym, A. S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., Griffiths, K. E., Marchal, G., Leclerc, C. and Cole, S. T. 2003 'Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis', *Nature Medicine* 9(5): 533-539.

Quiding-Jarbrink, M., Nordstrom, I., Granstrom, G., Kilander, A., Jertborn, M., Butcher, E. C., Lazarovits, A. I., Holmgren, J. and Czerkinsky, C. 1997 'Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric, and nasal immunizations. A molecular basis for the compartmentalization of effector B cell responses', *J Clin Invest* 99(6): 1281-6.

Ramarathinam, L., Shaban, R. A., Niesel, D. W. and Klimpel, G. R. 1991 'Interferon gamma (IFN-gamma) production by gut-associated lymphoid tissue and spleen following oral Salmonella typhimurium challenge', *Microb Pathog* 11(5): 347-56.

Ramon, G. 1924 'Sur la toxine et sur I'anatoxine diphtheriques', *Ann. Inst. Pasteur* 38: 1-10.

Raulet, D. H. 2004 'Interplay of natural killer cells and their receptors with the adaptive immune response', *Nat Immunol* 5(10): 996-1002.

Raupach, B. and Kaufmann, S. H. 2001 'Immune responses to intracellular bacteria', *Curr Opin Immunol* 13(4): 417-28.

Ravn, P., Demissie, A., Eguale, T., Wondwosson, H., Lein, D., Amoudy, H. A., Mustafa, A. S., Jensen, A. K., Holm, A., Rosenkrands, I., Oftung, F., Olobo, J., von Reyn, F. and Andersen, P. 1999 'Human T cell responses to the ESAT-6 antigen from Mycobacterium tuberculosis', *Journal of Infectious Diseases* 179(3): 637-645.

Reis e Sousa, C. 2004 'Activation of dendritic cells: translating innate into adaptive immunity', *Curr Opin Immunol* 16(1): 21-5.

Reljic, R., Clark, S. O., Williams, A., Falero-Diaz, G., Singh, M., Challacombe, S., Marsh, P. D. and Ivanyi, J. 2006 'Intranasal IFNgamma extends passive IgA antibody protection of mice against Mycobacterium tuberculosis lung infection', *Clin Exp Immunol* 143(3): 467-73.

Renshaw, P. S., Lightbody, K. L., Veverka, V., Muskett, F. W., Kelly, G., Frenkiel, T. A., Gordon, S. V., Hewinson, R. G., Burke, B., Norman, J., Williamson, R. A. and Carr, M. D. 2005 'Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6', *Embo J* 24(14): 2491-8.

Renshaw, P. S., Panagiotidou, P., Whelan, A., Gordon, S. V., Hewinson, R. G., Williamson, R. A. and Carr, M. D. 2002 'Conclusive evidence that the major T-cell antigens of the Mycobacterium tuberculosis complex ESAT-6 and CFP-10 form a tight, 1 : 1 complex and characterization of the structural properties of ESAT-6, CFP-10, and the ESAT-6-CFP-10 complex - Implications for pathogenesis and virulence', *Journal of Biological Chemistry* 277(24): 21598-21603.

Rharbaoui, F., Bruder, D., Vidakovic, M., Ebensen, T., Buer, J. and Guzman, C. A. 2005 'Characterization of a B220+ lymphoid cell subpopulation with immune modulatory functions in nasal-associated lymphoid tissues', *J Immunol* 174(3): 1317-24.

Rice, G. E., Munro, J. M., Corless, C. and Bevilacqua, M. P. 1991 'Vascular and nonvascular expression of INCAM-110. A target for mononuclear leukocyte adhesion in normal and inflamed human tissues', *Am J Pathol* 138(2): 385-93.

Roach, D. R., Briscoe, H., Saunders, B., France, M. P., Riminton, S. and Britton, W. J. 2001 'Secreted lymphotoxin-alpha is essential for the control of an intracellular bacterial infection', *J Exp Med* 193(2): 239-46.

Robles, C. P. and Pollack, S. B. 1989 'Asialo-GM1+ natural killer cells directly suppress antibody-producing B cells', *Nat Immun Cell Growth Regul* 8(4): 209-22.

Rodriguez-Monroy, M. A., Rojas-Hernandez, S. and Moreno-Fierros, L. 2007 'Phenotypic and Functional Differences between Lymphocytes from NALT and Nasal Passages of Mice', *Scand J Immunol* 65(3): 276-88.

Rodriguez, A., Tjarnlund, A., Ivanji, J., Singh, M., Garcia, I., Williams, A., Marsh, P. D., Troye-Blomberg, M. and Fernandez, C. 2005 'Role of IgA in the defense against respiratory infections IgA deficient mice exhibited increased susceptibility to intranasal infection with Mycobacterium bovis BCG', *Vaccine* 23(20): 2565-72.

Roetynck, S., Baratin, M., Johansson, S., Lemmers, C., Vivier, E. and Ugolini, S. 2006 'Natural killer cells and malaria', *Immunol Rev* 214: 251-63.

Rogers, H. W. and Unanue, E. R. 1993 'Neutrophils are involved in acute, nonspecific resistance to Listeria monocytogenes in mice', *Infect Immun* 61(12): 5090-6.

Roland, K. L., Tinge, S. A., Killeen, K. P. and Kochi, S. K. 2005 'Recent advances in the development of live, attenuated bacterial vectors', *Curr Opin Mol Ther* 7(1): 62-72.

Romagnani, S. 1992 'Induction of TH1 and TH2 responses: a key role for the 'natural' immune response?' *Immunol Today* 13(10): 379-81.

Rook, G. A., Dheda, K. and Zumla, A. 2005 'Immune responses to tuberculosis in developing countries: implications for new vaccines', *Nat Rev Immunol* 5(8): 661-7.

Rook, G. A. and Hernandez-Pando, R. 1996 'The pathogenesis of tuberculosis', *Annu Rev Microbiol* 50: 259-84.

Rook, G. A., Seah, G. and Ustianowski, A. 2001 'M. tuberculosis: immunology and vaccination', *Eur Respir J* 17(3): 537-57.

Rose, M. L., Birbeck, M. S., Wallis, V. J., Forrester, J. A. and Davies, A. J. 1980 'Peanut lectin binding properties of germinal centres of mouse lymphoid tissue', *Nature* 284(5754): 364-6.

Ruedl, C., Fruhwirth, M., Wick, G. and Wolf, H. 1994 'Immune response in the lungs following oral immunization with bacterial lysates of respiratory pathogens', *Clin Diagn Lab Immunol* 1(2): 150-4.

Ruegg, C., Postigo, A. A., Sikorski, E. E., Butcher, E. C., Pytela, R. and Erle, D. J. 1992 'Role of integrin alpha 4 beta 7/alpha 4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering', *J Cell Biol* 117(1): 179-89.

Rydstrom, A. and Wick, M. J. 2007 'Monocyte Recruitment, Activation, and Function in the Gut-Associated Lymphoid Tissue during Oral Salmonella Infection', *J Immunol* 178(9): 5789-801.

Salam, M. A., Katz, J., Zhang, P., Hajishengallis, G. and Michalek, S. M. 2006 'Immunogenicity of Salmonella vector vaccines expressing SBR of Streptococcus mutans under the control of a T7-nirB (dual) promoter system', *Vaccine* 24(23): 5003-15.

Salata, R. A., Sanson, A. J., Malhotra, I. J., Wiker, H. G., Harboe, M., Phillips, N. B. and Daniel, T. M. 1991 'Purification and characterization of the 30,000 dalton native antigen of Mycobacterium tuberculosis and characterization of six monoclonal antibodies reactive with a major epitope of this antigen', *J Lab Clin Med* 118(6): 589-98.

Salmi, M., Granfors, K., MacDermott, R. and Jalkanen, S. 1994 'Aberrant binding of lamina propria lymphocytes to vascular endothelium in inflammatory bowel diseases', *Gastroenterology* 106(3): 596-605.

Salmi, M., Kalimo, K. and Jalkanen, S. 1993 'Induction and function of vascular adhesion protein-1 at sites of inflammation', *J Exp Med* 178(6): 2255-60.

Salmond, R. J., Luross, J. A. and Williams, N. A. 2002 'Immune modulation by the cholera-like enterotoxins', *Expert Rev Mol Med* 2002: 1-16.

Samanich, K., Belisle, J. T. and Laal, S. 2001 'Homogeneity of antibody responses in tuberculosis patients', *Infect Immun* 69(7): 4600-9.

Sambrook, J., Fritsch, E.F. & Maniatis, T.; Hrsg. 1989 *Molecular Cloning - A Laboratory Manual*, 2nd Edition: Cold Spring Habour Laboratory Press, New York.

Samsom, J. N., Annema, A., Groeneveld, P. H., van Rooijen, N., Langermans, J. A. and van Furth, R. 1997 'Elimination of resident macrophages from the livers and spleens of immune mice impairs acquired resistance against a secondary Listeria monocytogenes infection', *Infect Immun* 65(3): 986-93.

Sanchez-Rodriguez, C., Estrada-Chavez, C., Garcia-Vigil, J., Laredo-Sanchez, F., Halabe-Cherem, J., Pereira-Suarez, A. and Mancilla, R. 2002 'An IgG antibody response to the antigen 85 complex is associated with good outcome in Mexican Totonaca Indians with pulmonary tuberculosis', *International Journal of Tuberculosis and Lung Disease* 6(8): 706-712.

Santambrogio, L. and Strominger, J. L. 2006 'The ins and outs of MHC class II proteins in dendritic cells', *Immunity* 25(6): 857-9.

Santana, M. A. and Esquivel-Guadarrama, F. 2006 'Cell biology of T cell activation and differentiation', *Int Rev Cytol* 250: 217-74.

Santosuosso, M., McCormick, S., Zhang, X., Zganiacz, A. and Xing, Z. 2006 'Intranasal boosting with an adenovirus-vectored vaccine markedly enhances protection by parenteral Mycobacterium bovis BCG immunization against pulmonary tuberculosis', *Infect Immun* 74(8): 4634-43.

Santosuosso, M., Zhang, X., McCormick, S., Wang, J., Hitt, M. and Xing, Z. 2005 'Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen', *J Immunol* 174(12): 7986-94.

Satoskar, A. R., Stamm, L. M., Zhang, X., Okano, M., David, J. R., Terhorst, C. and Wang, B. 1999 'NK cell-deficient mice develop a Th1-like response but fail to mount an efficient antigen-specific IgG2a antibody response', *J Immunol* 163(10): 5298-302.

Savransky, V., Rostapshov, V., Pinelis, D., Polotsky, Y., Korolev, S., Komisar, J. and Fegeding, K. 2003 'Murine lethal toxic shock caused by intranasal administration of staphylococcal enterotoxin B', *Toxicol Pathol* 31(4): 373-8.

Sayeh, E., Sterling, K., Speck, E., Freedman, J. and Semple, J. W. 2004 'IgG antiplatelet immunity is dependent on an early innate natural killer cell-derived interferon-gamma response that is regulated by CD8+ T cells', *Blood* 103(7): 2705-9.

Schafer, R. and Eisenstein, T. K. 1992 'Natural killer cells mediate protection induced by a Salmonella aroA mutant', *Infect Immun* 60(3): 791-7.

Scharton-Kersten, T. and Scott, P. 1995 'The role of the innate immune response in Th1 cell development following Leishmania major infection', *J Leukoc Biol* 57(4): 515-22.

Scharton-Kersten, T. M. and Sher, A. 1997 'Role of natural killer cells in innate resistance to protozoan infections', *Curr Opin Immunol* 9(1): 44-51.

Scharton, T. M. and Scott, P. 1993 'Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice', *J Exp Med* 178(2): 567-77.

Schriever, F., Korinth, D., Salahi, A., Lefterova, P., Schmidt-Wolf, I. G. and Behr, S. I. 1997 'Human T lymphocytes bind to germinal centers of human tonsils via integrin alpha4/VCAM-1 and LFA-1/ICAM-1 and -2', *Eur J Immunol* 27(1): 35-9.

Senaldi, G., Yin, S., Shaklee, C. L., Piguet, P. F., Mak, T. W. and Ulich, T. R. 1996 'Corynebacterium parvum- and Mycobacterium bovis bacillus Calmette-Guerin-induced granuloma

formation is inhibited in TNF receptor I (TNF-RI) knockout mice and by treatment with soluble TNF-RI', *J Immunol* 157(11): 5022-6.

Serbina, N. V. and Flynn, J. L. 1999 'Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of Mycobacterium tuberculosis-infected mice', *Infect Immun* 67(8): 3980-8.

Serbina, N. V., Salazar-Mather, T. P., Biron, C. A., Kuziel, W. A. and Pamer, E. G. 2003 'TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection', *Immunity* 19(1): 59-70.

Sereinig, S., Stukova, M., Zabolotnyh, N., Ferko, B., Kittel, C., Romanova, J., Vinogradova, T., Katinger, H., Kiselev, O. and Egorov, A. 2006 'Influenza virus NS vectors expressing the mycobacterium tuberculosis ESAT-6 protein induce CD4+ Th1 immune response and protect animals against tuberculosis challenge', *Clin Vaccine Immunol* 13(8): 898-904.

Shea, J. E., Hensel, M., Gleeson, C. and Holden, D. W. 1996 'Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium', *Proc Natl Acad Sci U S A* 93(6): 2593-7.

Shevach, E. M. 2000 'Suppressor T cells: Rebirth, function and homeostasis', *Curr Biol* 10(15): R572-5.

Shi, L., North, R. and Gennaro, M. L. 2004 'Effect of growth state on transcription levels of genes encoding major secreted antigens of Mycobacterium tuberculosis in the mouse lung', *Infect Immun* 72(4): 2420-4.

Shimoda, M., Nakamura, T., Takahashi, Y., Asanuma, H., Tamura, S., Kurata, T., Mizuochi, T., Azuma, N., Kanno, C. and Takemori, T. 2001 'Isotype-specific selection of high affinity memory B cells in nasal-associated lymphoid tissue', *Journal of Experimental Medicine* 194(11): 1597-1607.

Siegel, J. P., Sharon, M., Smith, P. L. and Leonard, W. J. 1987 'The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities', *Science* 238(4823): 75-8.

Sirard, J. C., Niedergang, F. and Kraehenbuhl, J. P. 1999 'Live attenuated Salmonella: a paradigm of mucosal vaccines', *Immunol Rev* 171: 5-26.

Sixma, T. K., Kalk, K. H., van Zanten, B. A., Dauter, Z., Kingma, J., Witholt, B. and Hol, W. G. 1993 'Refined structure of Escherichia coli heat-labile enterotoxin, a close relative of cholera toxin', *J Mol Biol* 230(3): 890-918.

Skeiky, Y. A., Alderson, M. R., Ovendale, P. J., Guderian, J. A., Brandt, L., Dillon, D. C., Campos-Neto, A., Lobet, Y., Dalemans, W., Orme, I. M. and Reed, S. G. 2004 'Differential

immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein', *J Immunol* 172(12): 7618-28.

Skjot, R. L., Brock, I., Arend, S. M., Munk, M. E., Theisen, M., Ottenhoff, T. H. and Andersen, P. 2002 'Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family', *Infect Immun* 70(10): 5446-53.

Skjot, R. L., Oettinger, T., Rosenkrands, I., Ravn, P., Brock, I., Jacobsen, S. and Andersen, P. 2000 'Comparative evaluation of low-molecular-mass proteins from Mycobacterium tuberculosis identifies members of the ESAT-6 family as immunodominant T-cell antigens', *Infect Immun* 68(1): 214-20.

Smith, D. A., Parish, T., Stoker, N. G. and Bancroft, G. J. 2001 'Characterization of auxotrophic mutants of Mycobacterium tuberculosis and their potential as vaccine candidates', *Infect Immun* 69(2): 1142-50.

Smith, H. R., Heusel, J. W., Mehta, I. K., Kim, S., Dorner, B. G., Naidenko, O. V., Iizuka, K., Furukawa, H., Beckman, D. L., Pingel, J. T., Scalzo, A. A., Fremont, D. H. and Yokoyama, W. M. 2002 'Recognition of a virus-encoded ligand by a natural killer cell activation receptor', *Proc Natl Acad Sci U S A* 99(13): 8826-31.

Snapper, C. M. and Mond, J. J. 1993 'Towards a comprehensive view of immunoglobulin class switching', *Immunol Today* 14(1): 15-7.

Snapper, C. M. and Paul, W. E. 1987 'Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production', *Science* 236(4804): 944-7.

Snapper, C. M., Yamaguchi, H., Moorman, M. A. and Mond, J. J. 1994 'An in vitro model for T cell-independent induction of humoral immunity. A requirement for NK cells', *J Immunol* 152(10): 4884-92.

Sonoda, K. H., Exley, M., Snapper, S., Balk, S. P. and Stein-Streilein, J. 1999 'CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site', *J Exp Med* 190(9): 1215-26.

Sorensen, A. L., Nagai, S., Houen, G., Andersen, P. and Andersen, A. B. 1995 'Purification and characterization of a low-molecular-mass T-cell antigen secreted by Mycobacterium tuberculosis', *Infect Immun* 63(5): 1710-7.

Spit, B. J., E. G. J. Hendriksen, J. P. Bruijintjes, and C. F. Kuper 1989 'Nasal lymphoid tissue in the rat.' *Cell Tissue Research* 255: 193-198.

Stanley, S. A., Raghavan, S., Hwang, W. W. and Cox, J. S. 2003 'Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system', *Proceedings of the National Academy of Sciences of the United States of America* 100(22): 13001-13006.

Steffen, B. J., Breier, G., Butcher, E. C., Schulz, M. and Engelhardt, B. 1996 'ICAM-1, VCAM-1, and MAdCAM-1 are expressed on choroid plexus epithelium but not endothelium and mediate binding of lymphocytes in vitro', *Am J Pathol* 148(6): 1819-38.

Steinman, R. M. and Hemmi, H. 2006 'Dendritic cells: translating innate to adaptive immunity', *Curr Top Microbiol Immunol* 311: 17-58.

Stenger, S. 2001 'Cytolytic T cells in the immune response to mycobacterium tuberculosis', *Scand J Infect Dis* 33(7): 483-7.

Stenger, S., Hanson, D. A., Teitelbaum, R., Dewan, P., Niazi, K. R., Froelich, C. J., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C., Porcelli, S. A., Bloom, B. R., Krensky, A. M. and Modlin, R. L. 1998 'An antimicrobial activity of cytolytic T cells mediated by granulysin', *Science* 282(5386): 121-5.

Stenger, S., Mazzaccaro, R. J., Uyemura, K., Cho, S., Barnes, P. F., Rosat, J. P., Sette, A., Brenner, M. B., Porcelli, S. A., Bloom, B. R. and Modlin, R. L. 1997 'Differential effects of cytolytic T cell subsets on intracellular infection', *Science* 276(5319): 1684-7.

Stevens, T. L., Bossie, A., Sanders, V. M., Fernandez-Botran, R., Coffman, R. L., Mosmann, T. R. and Vitetta, E. S. 1988 'Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells', *Nature* 334(6179): 255-8.

Stitz, L., Baenziger, J., Pircher, H., Hengartner, H. and Zinkernagel, R. M. 1986 'Effect of rabbit anti-asialo GM1 treatment in vivo or with anti-asialo GM1 plus complement in vitro on cytotoxic T cell activities', *J Immunol* 136(12): 4674-80.

Stokes, M. G., Titball, R. W., Neeson, B. N., Galen, J. E., Walker, N. J., Stagg, A. J., Jenner, D. C., Thwaite, J. E., Nataro, J. P., Baillie, L. W. and Atkins, H. S. 2006 'Oral administration of a Salmonella-based vaccine expressing Bacillus anthracis protective antigen confers protection against aerosolized B. anthracis', *Infect Immun*.

Storkus, W. J., Alexander, J., Payne, J. A., Dawson, J. R. and Cresswell, P. 1989 'Reversal of natural killing susceptibility in target cells expressing transfected class I HLA genes', *Proc Natl Acad Sci U S A* 86(7): 2361-4.

Stratford, R., McKelvie, N. D., Hughes, N. J., Aldred, E., Wiseman, C., Curtis, J., Bellaby, T., Bentley, M., Hindle, Z., Brennan, F. R., Chatfield, S. N., Dougan, G. and Khan, S. A. 2005 'Optimization of Salmonella enterica serovar typhi DeltaaroC DeltassaV derivatives as vehicles for

delivering heterologous antigens by chromosomal integration and in vivo inducible promoters', *Infect Immun* 73(1): 362-8.

Streeter, P. R., Berg, E. L., Rouse, B. T., Bargatze, R. F. and Butcher, E. C. 1988 'A tissue-specific endothelial cell molecule involved in lymphocyte homing', *Nature* 331(6151): 41-6.

Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J. and Russell, D. G. 1994 'Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase', *Science* 263(5147): 678-81.

Szabo, M. C., Butcher, E. C. and McEvoy, L. M. 1997 'Specialization of mucosal follicular dendritic cells revealed by mucosal addressin-cell adhesion molecule-1 display', *J Immunol* 158(12): 5584-8.

Tacket, C. O., Sztein, M. B., Losonsky, G. A., Wasserman, S. S., Nataro, J. P., Edelman, R., Pickard, D., Dougan, G., Chatfield, S. N. and Levine, M. M. 1997 'Safety of live oral Salmonella typhi vaccine strains with deletions in htrA and aroC aroD and immune response in humans', *Infect Immun* 65(2): 452-6.

Tacket, C. O., Sztein, M. B., Wasserman, S. S., Losonsky, G., Kotloff, K. L., Wyant, T. L., Nataro, J. P., Edelman, R., Perry, J., Bedford, P., Brown, D., Chatfield, S., Dougan, G. and Levine, M. M. 2000 'Phase 2 clinical trial of attenuated Salmonella enterica serovar typhi oral live vector vaccine CVD 908-htrA in U.S. volunteers', *Infect Immun* 68(3): 1196-201.

Takata, S., Ohtani, O. and Watanabe, Y. 2000 'Lectin binding patterns in rat nasal-associated lymphoid tissue (NALT) and the influence of various types of lectin on particle uptake in NALT', *Arch Histol Cytol* 63(4): 305-12.

Takatsu, K. and Kariyone, A. 2003 'The immunogenic peptide for Th1 development', *Int Immunopharmacol* 3(6): 783-800.

Talham, G. L., Jiang, H. Q., Bos, N. A. and Cebra, J. J. 1999 'Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system', *Infect Immun* 67(4): 1992-2000.

Tamura, S., Iwasaki, T., Thompson, A. H., Asanuma, H., Chen, Z., Suzuki, Y., Aizawa, C. and Kurata, T. 1998 'Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection', *J Gen Virol* 79 (Pt 2): 291-9.

Tanghe, A., Content, J., Van Vooren, J. P., Portaels, F. and Huygen, K. 2001 'Protective efficacy of a DNA vaccine encoding antigen 85A from Mycobacterium bovis BCG against Buruli ulcer', *Infection and Immunity* 69(9): 5403-5411.

Tanghe, A., Denis, O., Lambrecht, B., Motte, V., van den Berg, T. and Huygen, K. 2000 'Tuberculosis DNA vaccine encoding Ag85A is immunogenic and protective when administered by intramuscular needle injection but not by epidermal gene gun bombardment', *Infect Immun* 68(7): 3854-60.

Tanigawa, M., Bigger, J. E., Kanter, M. Y. and Atherton, S. S. 2000 'Natural killer cells prevent direct anterior-to-posterior spread of herpes simplex virus type 1 in the eye', *Invest Ophthalmol Vis Sci* 41(1): 132-7.

Testi, R., D'Ambrosio, D., De Maria, R. and Santoni, A. 1994 'The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells', *Immunol Today* 15(10): 479-83.

Testi, R., Phillips, J. H. and Lanier, L. L. 1989 'Leu 23 induction as an early marker of functional CD3/T cell antigen receptor triggering. Requirement for receptor cross-linking, prolonged elevation of intracellular [Ca++] and stimulation of protein kinase C', *J Immunol* 142(6): 1854-60.

Tew, J. G., Wu, J., Fakher, M., Szakal, A. K. and Qin, D. 2001 'Follicular dendritic cells: beyond the necessity of T-cell help', *Trends Immunol* 22(7): 361-7.

Thole, J. E., Janson, A. A., Cornelisse, Y., Schreuder, G. M., Wieles, B., Naafs, B., de Vries, R. R. and Ottenhoff, T. H. 1999 'HLA-class II-associated control of antigen recognition by T cells in leprosy: a prominent role for the 30/31-kDa antigens', *J Immunol* 162(11): 6912-8.

Thorbecke, G. J., Amin, A. R. and Tsiagbe, V. K. 1994 'Biology of germinal centers in lymphoid tissue', *Faseb J* 8(11): 832-40.

Tite, J. P., Gao, X. M., Hughes-Jenkins, C. M., Lipscombe, M., O'Callaghan, D., Dougan, G. and Liew, F. Y. 1990 'Anti-viral immunity induced by recombinant nucleoprotein of influenza A virus. III. Delivery of recombinant nucleoprotein to the immune system using attenuated Salmonella typhimurium as a live carrier', *Immunology* 70(4): 540-6.

Tjarnlund, A., Rodriguez, A., Cardona, P. J., Guirado, E., Ivanyi, J., Singh, M., Troye-Blomberg, M. and Fernandez, C. 2006 'Polymeric IgR knockout mice are more susceptible to mycobacterial infections in the respiratory tract than wild-type mice', *Int Immunol* 18(5): 807-16.

Trinchieri, G. 1989 'Biology of natural killer cells', *Adv Immunol* 47: 187-376.

Tsudo, M., Goldman, C. K., Bongiovanni, K. F., Chan, W. C., Winton, E. F., Yagita, M., Grimm, E. A. and Waldmann, T. A. 1987 'The p75 peptide is the receptor for interleukin 2 expressed on large granular lymphocytes and is responsible for the interleukin 2 activation of these cells', *Proc Natl Acad Sci U S A* 84(15): 5394-8.

Tsunetsugu-Yokota, Y., Ishige, M. and Murakami, M. 2007 'Oral Attenuated Salmonella Enterica Serovar Typhimurium Vaccine Expressing Codon-Optimized HIV Type 1 Gag Enhanced Intestinal Immunity in Mice', *AIDS Res Hum Retroviruses* 23(2): 278-86.

Turner, A. K., Beavis, J. C., Stephens, J. C., Greenwood, J., Gewert, C., Thomas, N., Deary, A., Casula, G., Daley, A., Kelly, P., Randall, R. and Darsley, M. J. 2006 'Construction and phase I clinical evaluation of the safety and immunogenicity of a candidate enterotoxigenic Escherichia coli vaccine strain expressing colonization factor antigen CFA/I', *Infect Immun* 74(2): 1062-71.

Tzschaschel, B. D., Klee, S. R., de Lorenzo, V., Timmis, K. N. and Guzman, C. A. 1996 'Towards a vaccine candidate against Shigella dysenteriae 1: expression of the Shiga toxin B-subunit in an attenuated Shigella flexneri aroD carrier strain', *Microb Pathog* 21(4): 277-88.

Unger, W. W. J., Hauet-Broere, F., Jansen, W., van Berkel, L. A., Kraal, G. and Samsom, J. N. 2003 'Early events in peripheral regulatory T cell induction via the nasal mucosa', *Journal of Immunology* 171(9): 4592-4603.

Vajdy, M., Singh, M., Kazzaz, J., Soenawan, E., Ugozzoli, M., Zhou, F., Srivastava, I., Bin, Q., Barnett, S., Donnelly, J., Luciw, P., Adamson, L., Montefiori, D. and O'Hagan, D. T. 2004 'Mucosal and systemic anti-HIV responses in rhesus macaques following combinations of intranasal and parenteral immunizations', *AIDS Res Hum Retroviruses* 20(11): 1269-81.

Valdivia, R. H. and Falkow, S. 1997 'Fluorescence-based isolation of bacterial genes expressed within host cells', *Science* 277(5334): 2007-11.

van Crevel, R., Ottenhoff, T. H. and van der Meer, J. W. 2002 'Innate immunity to Mycobacterium tuberculosis', *Clin Microbiol Rev* 15(2): 294-309.

Van de Verg, L., Herrington, D. A., Murphy, J. R., Wasserman, S. S., Formal, S. B. and Levine, M. M. 1990 'Specific immunoglobulin A-secreting cells in peripheral blood of humans following oral immunization with a bivalent Salmonella typhi-Shigella sonnei vaccine or infection by pathogenic S. sonnei', *Infect Immun* 58(6): 2002-4.

van Egmond, M., Damen, C. A., van Spriel, A. B., Vidarsson, G., van Garderen, E. and van de Winkel, J. G. 2001 'IgA and the IgA Fc receptor', *Trends Immunol* 22(4): 205-11.

van Helvoort, J. M., Samsom, J. N., Chantry, D., Jansen, W., Schadee-Eestermans, I., Thepen, T., Mebius, R. E. and Kraal, G. 2004 'Preferential expression of IgG2b in nose draining cervical lymph nodes and its putative role in mucosal tolerance induction', *Allergy* 59(11): 1211-8.

Van Vooren, J. P., Drowart, A., De Bruyn, J., Launois, P., Millan, J., Delaporte, E., Develoux, M., Yernault, J. C. and Huygen, K. 1992 'Humoral responses against the 85A and 85B antigens of Mycobacterium bovis BCG in patients with leprosy and tuberculosis', *J Clin Microbiol* 30(6): 1608-10.

VanCott, J. L., Staats, H. F., Pascual, D. W., Roberts, M., Chatfield, S. N., Yamamoto, M., Coste, M., Carter, P. B., Kiyono, H. and McGhee, J. R. 1996 'Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages, and derived cytokines following oral immunization with live recombinant Salmonella', *J Immunol* 156(4): 1504-14.

Velin, D., Fotopoulos, G., Luthi, F. and Kraehenbuhl, J. P. 1997 'The nasal-associated lymphoid tissue of adult mice acts as an entry site for the mouse mammary tumor retrovirus', *J Exp Med* 185(10): 1871-6.

Vindurampulle, C. J., Cuberos, L. F., Barry, E. M., Pasetti, M. F. and Levine, M. M. 2004 'Recombinant Salmonella enterica serovar Typhi in a prime-boost strategy', *Vaccine* 22(27-28): 3744-50.

Vordermeier, H. M., Cockle, P. C., Whelan, A., Rhodes, S., Palmer, N., Bakker, D. and Hewinson, R. G. 1999 'Development of diagnostic reagents to differentiate between Mycobacterium bovis BCG vaccination and M. bovis infection in cattle', *Clin Diagn Lab Immunol* 6(5): 675-82.

Vordermeier, H. M., Venkataprasad, N., Harris, D. P. and Ivanyi, J. 1996 'Increase of tuberculous infection in the organs of B cell-deficient mice', *Clin Exp Immunol* 106(2): 312-6.

Vuola, J. M., Ristola, M. A., Cole, B., Jarviluoma, A., Tvaroha, S., Ronkko, T., Rautio, O., Arbeit, R. D. and Reyn, C. F. 2003 'Immunogenicity of an inactivated mycobacterial vaccine for the prevention of HIV-associated tuberculosis: a randomized, controlled trial', *Aids* 17(16): 2351-5.

Waldeyer-Hartz, H. W. G. 1884 'Ueber den lymphatischen Apparat des Pharynx', *Deutsche medizinische Wochenschrift* 10: 313.

Walzer, T., Dalod, M., Vivier, E. and Zitvogel, L. 2005 'Natural killer cell-dendritic cell crosstalk in the initiation of immune responses', *Expert Opin Biol Ther* 5 Suppl 1: S49-59.

Wang, C. H., Lin, H. C., Liu, C. Y., Huang, K. H., Huang, T. T., Yu, C. T. and Kuo, H. P. 2001 'Upregulation of inducible nitric oxide synthase and cytokine secretion in peripheral blood monocytes from pulmonary tuberculosis patients', *Int J Tuberc Lung Dis* 5(3): 283-91.

Wang, J., Thorson, L., Stokes, R. W., Santosuosso, M., Huygen, K., Zganiacz, A., Hitt, M. and Xing, Z. 2004 'Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis', *J Immunol* 173(10): 6357-65.

Wang, M., Ellison, C. A., Gartner, J. G. and HayGlass, K. T. 1998 'Natural killer cell depletion fails to influence initial CD4 T cell commitment in vivo in exogenous antigen-stimulated cytokine and antibody responses', *J Immunol* 160(3): 1098-105.

Ward, S. J., Douce, G., Figueiredo, D., Dougan, G. and Wren, B. W. 1999 'Immunogenicity of a Salmonella typhimurium aroA aroD vaccine expressing a nontoxic domain of Clostridium difficile toxin A', *Infect Immun* 67(5): 2145-52.

Warnock, R. A., Askari, S., Butcher, E. C. and von Andrian, U. H. 1998 'Molecular mechanisms of lymphocyte homing to peripheral lymph nodes', *J Exp Med* 187(2): 205-16.

Warnock, R. A., Campbell, J. J., Dorf, M. E., Matsuzawa, A., McEvoy, L. M. and Butcher, E. C. 2000 'The role of chemokines in the microenvironmental control of T versus B cell arrest in Peyer's patch high endothelial venules', *J Exp Med* 191(1): 77-88.

Warren, H. S., Kinnear, B. F., Phillips, J. H. and Lanier, L. L. 1995 'Production of IL-5 by human NK cells and regulation of IL-5 secretion by IL-4, IL-10, and IL-12', *J Immunol* 154(10): 5144-52.

Weinstein, P. D. and Cebra, J. J. 1991 'The preference for switching to IgA expression by Peyer's patch germinal center B cells is likely due to the intrinsic influence of their microenvironment', *J Immunol* 147(12): 4126-35.

Weissman, D. and Fauci, A. S. 1997 'Role of dendritic cells in immunopathogenesis of human immunodeficiency virus infection', *Clin Microbiol Rev* 10(2): 358-67.

Weltzin, R., Kleanthous, H., Guirakhoo, F., Monath, T. P. and Lee, C. K. 1997 'Novel intranasal immunization techniques for antibody induction and protection of mice against gastric Helicobacter felis infection', *Vaccine* 15(4): 370-6.

Wetzel, S. A. and Parker, D. C. 2006 'MHC transfer from APC to T cells following antigen recognition', *Crit Rev Immunol* 26(1): 1-21.

WHO 2005 'Tuberculosis: Interactive Maps': WHO.

— 2007a 'JOINT HIV/TUBERCULOSIS INTERVENTIONS'.

— 2007b 'Tuberculosis' *Fact Sheet No 104*: WHO.

Wiker, H. G. and Harboe, M. 1992 'The antigen 85 complex: a major secretion product of Mycobacterium tuberculosis', *Microbiol Rev* 56(4): 648-61.

Wilder, J. A., Koh, C. Y. and Yuan, D. 1996 'The role of NK cells during in vivo antigen-specific antibody responses', *J Immunol* 156(1): 146-52.

Wiley, J. A., Hogan, R. J., Woodland, D. L. and Harmsen, A. G. 2001 'Antigen-specific CD8(+) T cells persist in the upper respiratory tract following influenza virus infection', *J Immunol* 167(6): 3293-9.

Wiley, J. A., Tighe, M. P. and Harmsen, A. G. 2005 'Upper respiratory tract resistance to influenza infection is not prevented by the absence of either nasal-associated lymphoid tissue or cervical lymph nodes', *J Immunol* 175(5): 3186-96.

Williams, A., Reljic, R., Naylor, I., Clark, S. O., Falero-Diaz, G., Singh, M., Challacombe, S., Marsh, P. D. and Ivanyi, J. 2004 'Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs', *Immunology* 111(3): 328-33.

Wolvers, D. A., Coenen-de Roo, C. J., Mebius, R. E., van der Cammen, M. J., Tirion, F., Miltenburg, A. M. and Kraal, G. 1999 'Intranasally induced immunological tolerance is determined by characteristics of the draining lymph nodes: studies with OVA and human cartilage gp-39', *J Immunol* 162(4): 1994-8.

Woolard, M. D., Hodge, L. M., Jones, H. P., Schoeb, T. R. and Simecka, J. W. 2004 'The upper and lower respiratory tracts differ in their requirement of IFN-gamma and IL-4 in controlling respiratory mycoplasma infection and disease', *J Immunol* 172(11): 6875-83.

Wu, H. Y., Nahm, M. H., Guo, Y., Russell, M. W. and Briles, D. E. 1997a 'Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with Streptococcus pneumoniae', *J Infect Dis* 175(4): 839-46.

Wu, H. Y., Nguyen, H. H. and Russell, M. W. 1997b 'Nasal lymphoid tissue (NALT) as a mucosal immune inductive site', *Scandinavian Journal of Immunology* 46(5): 506-513.

Wu, H. Y., Nikolova, E. B., Beagley, K. W. and Russell, M. W. 1996 'Induction of antibody-secreting cells and T-helper and memory cells in murine nasal lymphoid tissue', *Immunology* 88(4): 493-500.

Wu, H. Y., Russell, M. W. 1997 'Nasal lymphoid tissue, intranasal immunization, and compartmentalization of the common mucosal immune system.' *Immunol. Res.* 16(2): 187-201.

Xu, D., McSorley, S. J., Chatfield, S. N., Dougan, G. and Liew, F. Y. 1995 'Protection against Leishmania major infection in genetically susceptible BALB/c mice by gp63 delivered orally in attenuated Salmonella typhimurium (AroA- AroD-)', *Immunology* 85(1): 1-7.

Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P. and Russell, D. G. 1994 'Intracellular trafficking in Mycobacterium tuberculosis and Mycobacterium avium-infected macrophages', *J Immunol* 153(6): 2568-78.

Yanagita, M., Hiroi, T., Kitagaki, N., Hamada, S., Ito, H. O., Shimauchi, H., Murakami, S., Okada, H. and Kiyono, H. 1999 'Nasopharyngeal-associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell-regulated IgA responses for the inhibition of bacterial

attachment to epithelial cells and subsequent inflammatory cytokine production', *J Immunol* 162(6): 3559-65.

Yang, Q., Goding, S. R., Hokland, M. E. and Basse, P. H. 2006 'Antitumor activity of NK cells', *Immunol Res* 36(1-3): 13-25.

Yang, X., Hinnebusch, B. J., Trunkle, T., Bosio, C. M., Suo, Z., Tighe, M., Harmsen, A., Becker, T., Crist, K., Walters, N., Avci, R. and Pascual, D. W. 2007 'Oral vaccination with salmonella simultaneously expressing Yersinia pestis F1 and V antigens protects against bubonic and pneumonic plague', *J Immunol* 178(2): 1059-67.

Yasukawa, H., Imaizumi, T., Matsuoka, H., Nakashima, A. and Morimatsu, M. 1997 'Inhibition of intimal hyperplasia after balloon injury by antibodies to intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1', *Circulation* 95(6): 1515-22.

Yeo, S. G., Tumang, J. R. and Rothstein, T. L. 2006 'Characteristic features of B cells in murine cervical lymph nodes', *Acta Otolaryngol* 126(1): 56-61.

Yokoyama, W. M. and Scalzo, A. A. 2002 'Natural killer cell activation receptors in innate immunity to infection', *Microbes Infect* 4(15): 1513-21.

Yoshimoto, T. and Paul, W. E. 1994 'CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3', *J Exp Med* 179(4): 1285-95.

Young, H. A. and Ortaldo, J. 2006 'Cytokines as critical co-stimulatory molecules in modulating the immune response of natural killer cells', *Cell Res* 16(1): 20-4.

Yu, Y. Y., Kumar, V. and Bennett, M. 1992 'Murine natural killer cells and marrow graft rejection', *Annu Rev Immunol* 10: 189-213.

Yuan, D., Bibi, R. and Dang, T. 2004 'The role of adjuvant on the regulatory effects of NK cells on B cell responses as revealed by a new model of NK cell deficiency', *Int Immunol* 16(5): 707-16.

Zenner, H. P. and Brunner, F. X. 1988 'Immunological aspects of tonsils and of tonsillitis', *Acta Otolaryngol Suppl* 454: 70-4.

Zhu, D., Barniak, V., Zhang, Y., Green, B. and Zlotnick, G. 2006 'Intranasal immunization of mice with recombinant lipidated P2086 protein reduces nasal colonization of group B Neisseria meningitidis', *Vaccine* 24(26): 5420-5.

Zitvogel, L., Terme, M., Borg, C. and Trinchieri, G. 2006 'Dendritic cell-NK cell cross-talk: regulation and physiopathology', *Curr Top Microbiol Immunol* 298: 157-74.

Zuercher, A. W. 2003 'Upper respiratory tract immunity', *Viral Immunology* 16(3): 279-289.

Zuercher, A. W., Coffin, S. E., Thurnheer, M. C., Fundova, P. and Cebra, J. J. 2002 'Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses', *Journal of Immunology* 168(4): 1796-1803.

Zuercher, A. W., Horn, M. P., Wu, H., Song, Z., Bundgaard, C. J., Johansen, H. K., Hoiby, N., Marcus, P. and Lang, A. B. 2006 'Intranasal immunisation with conjugate vaccine protects mice from systemic and respiratory tract infection with Pseudomonas aeruginosa', *Vaccine* 24(20): 4333-42.

Zurbriggen, R., Metcalfe, I. C., Gluck, R., Viret, J. F. and Moser, C. 2003 'Nonclinical safety evaluation of Escherichia coli heat-labile toxin mucosal adjuvant as a component of a nasal influenza vaccine', *Expert Rev Vaccines* 2(2): 295-304.

Zwilling, B. S., Kuhn, D. E., Wikoff, L., Brown, D. and Lafuse, W. 1999 'Role of iron in Nramp1-mediated inhibition of mycobacterial growth', *Infect Immun* 67(3): 1386-92.

8 Appendix

Figure 8.1: Scatter plots of cell populations early after intranasal immunisation.

Innate immune cell composition of NALT and CLN 5, 24 and 72 hours after intranasal immunisation. NALT and CLN cells were isolate from immunised Balb/c mice. Single cell suspensions (1 x 10^6/sample) were stained with flurochrome-labelled mAbs and analysed by flow cytometry. Figures 8.1A and B show $CD11c^+$ and $F4/80^+$ cells in the NALT 5 and 24 hours after immunisation, respectively with Figures 8.1C and D showing the same cells both in the CLN after both 24 and 72 hours. Figures 8.1E-G show $DX5^+$ and $Ly6G^+$ cells in the NALT, 5 (5.1E), 24 (5.1F) and 72 (5.1G) hours post immunisation and Figures 8.1H-J show these time-points in the CLN. Plots shown are from ten individual representative mice, and the mean values are indicated. Numbers in the upper plots refer to percentages of cells within leukocyte gate.

Figure 8.1A: $CD11c^+$ and $F4/80^+$ cells in NALT 5hrs after immunisation

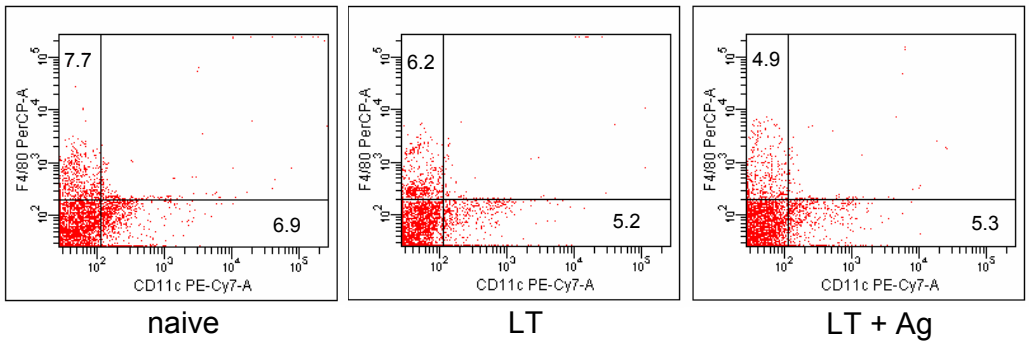


Figure 8.1B: $CD11c^+$ and $F4/80^+$ cells in NALT 24hrs after immunisation

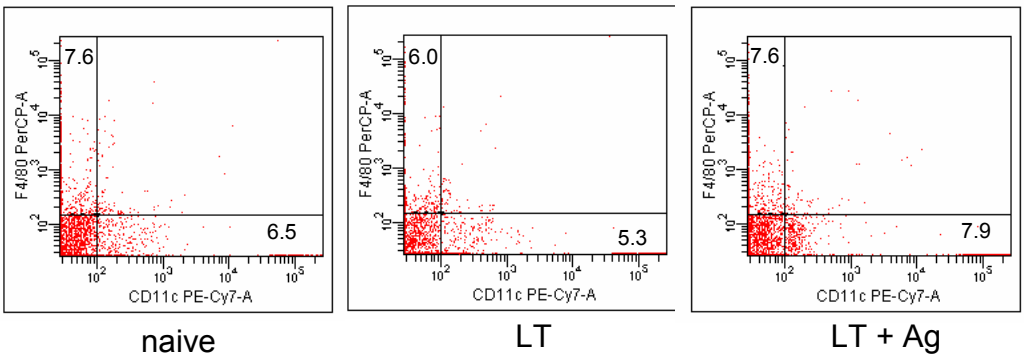


Figure 8.1C: CD11c$^+$ and F4/80$^+$ cells in CLN 24hrs after immunisation

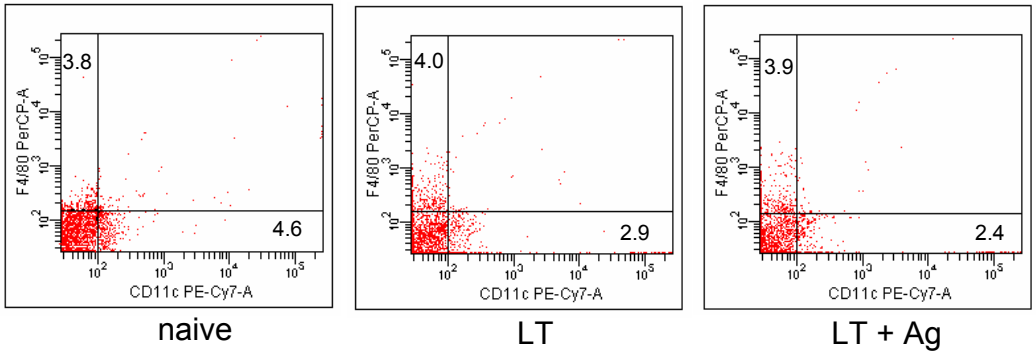


Figure 8.1D: CD11c$^+$ and F4/80$^+$ cells in CLN 72hrs after immunisation

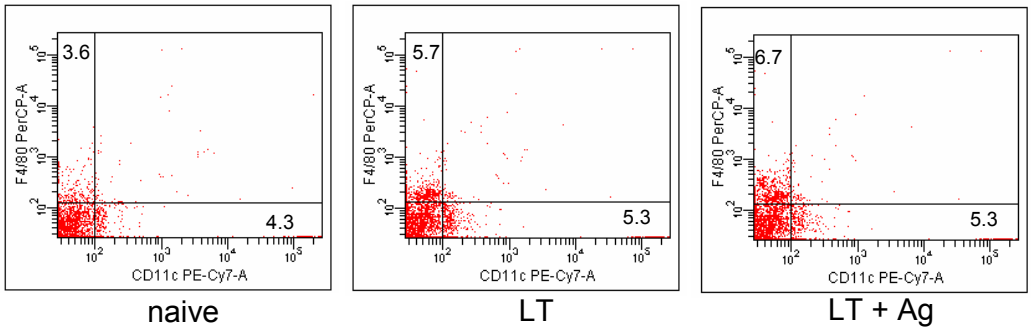


Figure 8.1E: DX5$^+$ and Ly6G$^+$ cells in NALT 5hrs after immunisation

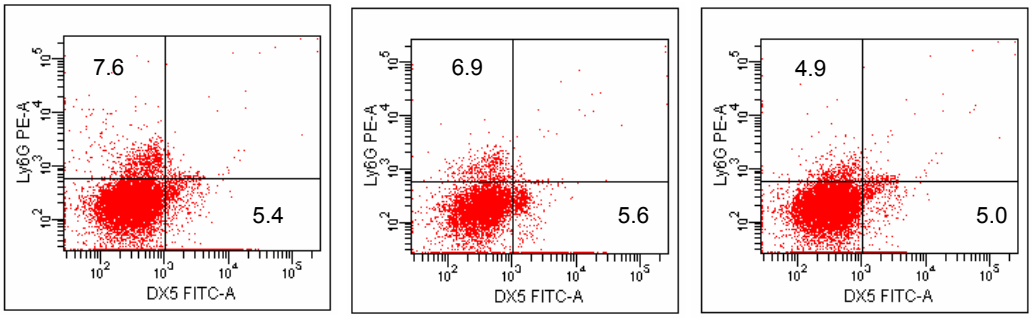


Figure 8.1F: DX5$^+$ and Ly6G$^+$ cells in NALT 24hrs after immunisation

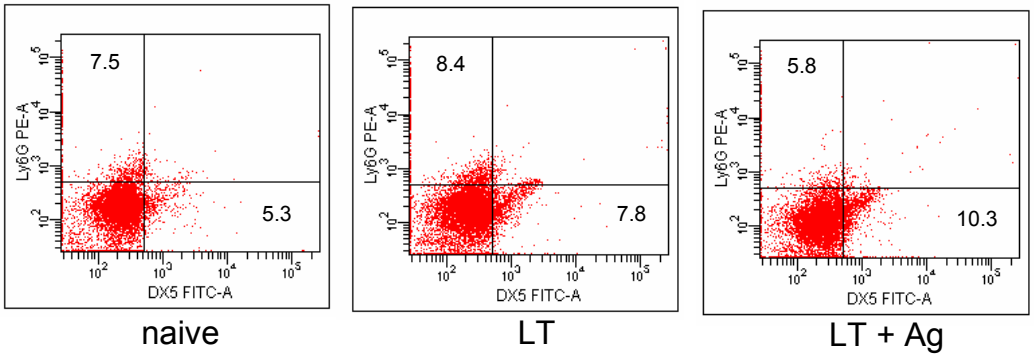


Figure 8.1G: $DX5^+$ and $Ly6G^+$ cells in NALT 72hrs after immunisation

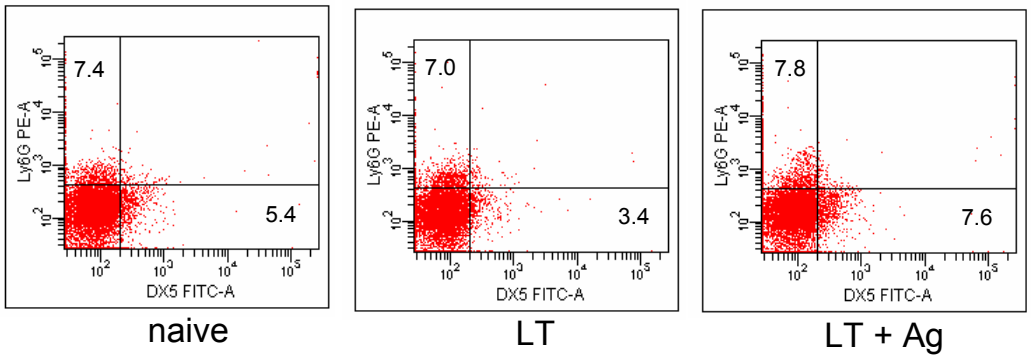


Figure 8.1H: $DX5^+$ and $Ly6G^+$ cells in CLN 5hrs after immunisation

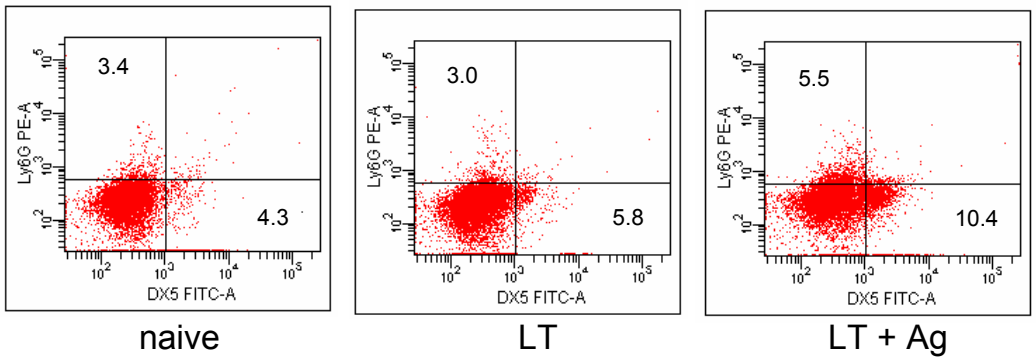


Figure 8.1I: $DX5^+$ and $Ly6G^+$ cells in CLN 24hrs after immunisation

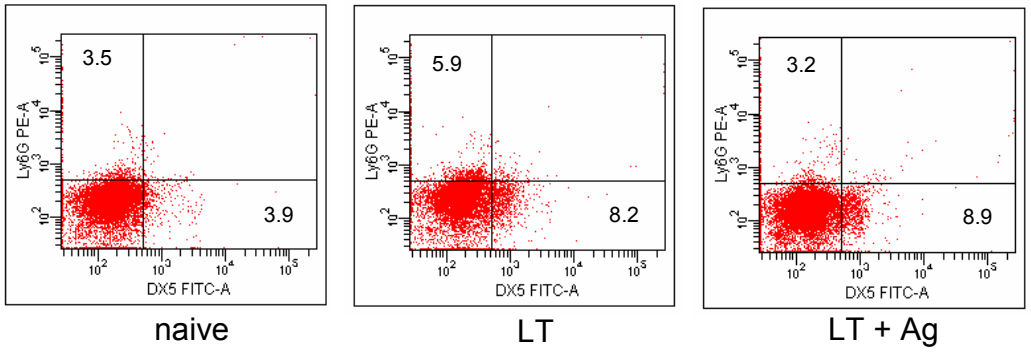


Figure 8.1J: $DX5^+$ and $Ly6G^+$ cells in CLN 72hrs after immunisation

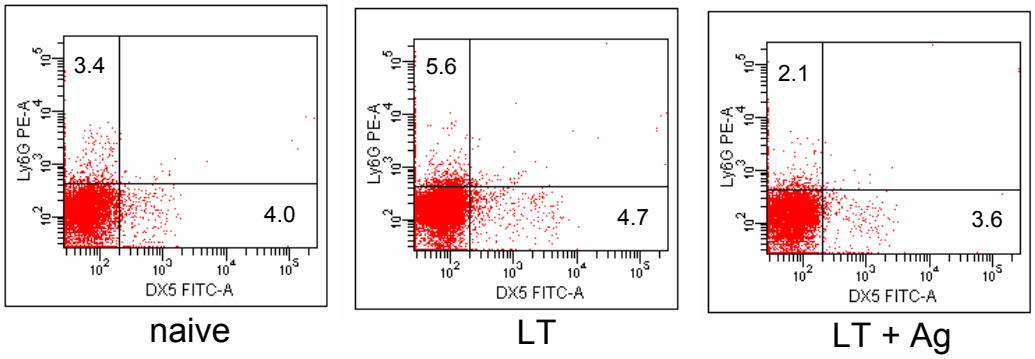


Figure 8.2: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT) Balb/c were compared. Figure 8.2A represents staining of frozen sections for CD11c in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and CD11c (red). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES).

Figure 8.2A:

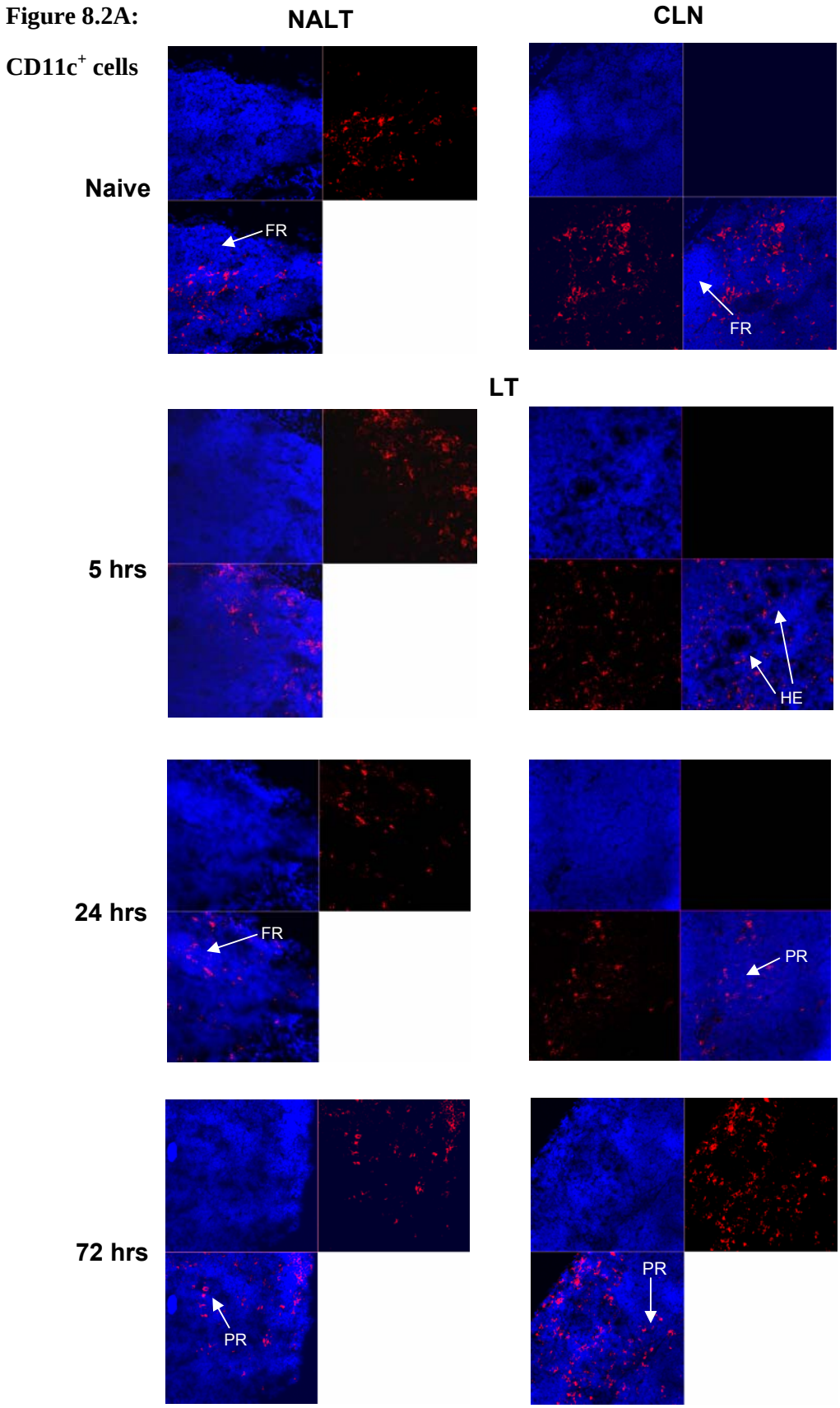

NALT
CLN
CD11c+ cells
Naive
FR
FR
LT
5 hrs
HE
24 hrs
FR
PR
72 hrs
PR
PR

Figure 8.2: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT) Balb/c were compared. Figure 8.2B represents staining of frozen sections for F4/80 in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and F4/80 (green). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES).

Figure 8.2B:

F4/80^{+} cells

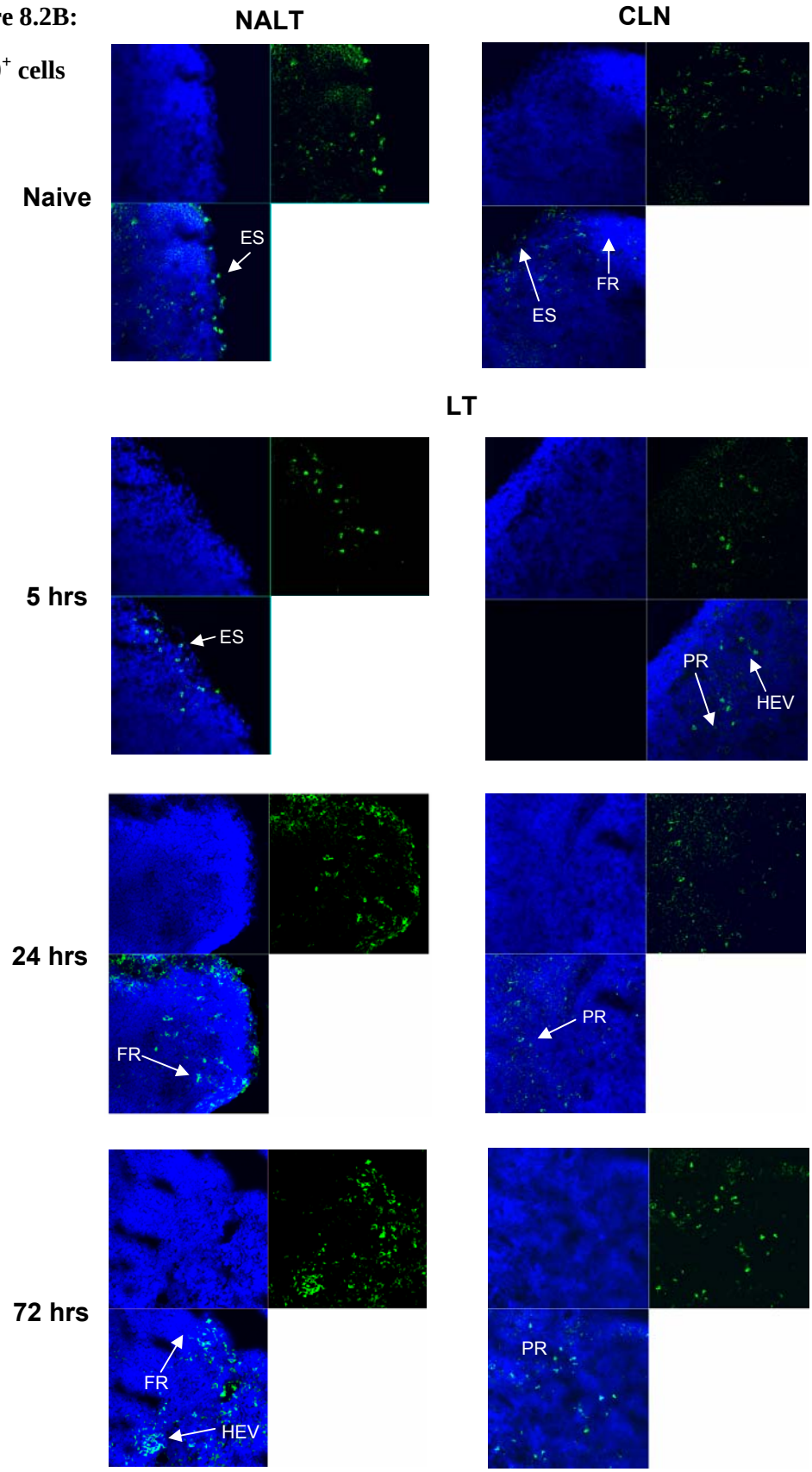


Figure 8.2: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT) Balb/c were compared. Figure 8.2C represents staining of frozen sections for Ly6G in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and Ly6G (red). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES).

Figure 8.2C:

Ly6G$^+$ cells

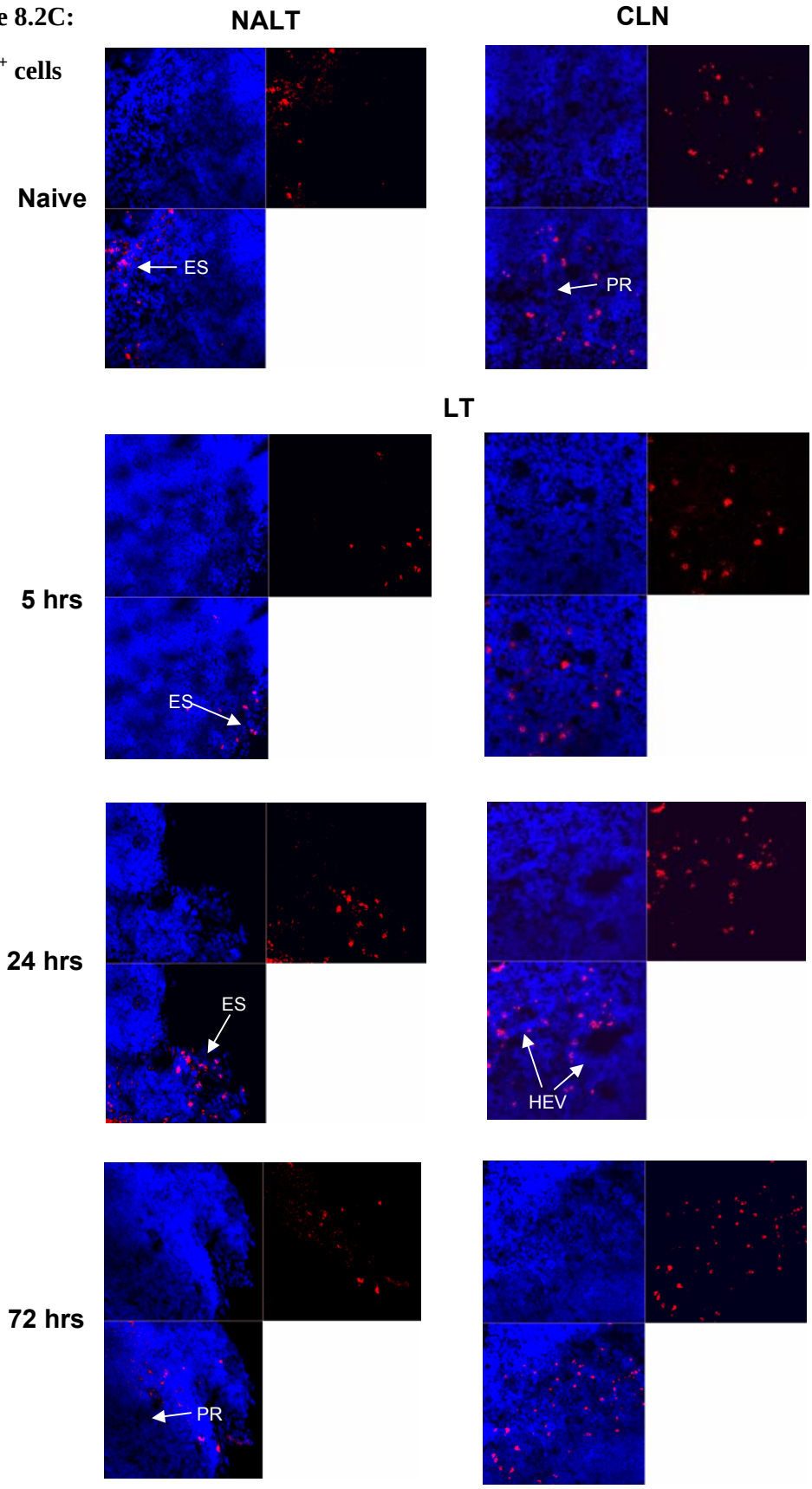


Table 8.1: Activation marker expression on NALT and CLN innate immune cells from immunised Balb/c mice.

Percentage expression of activation markers MHCII and VCAM-1 on $CD11c^+$ and $F4/80^+$ cells (Table 8.2A) and CD25 and CD69 on $DX5^+$ and CD69 on $Ly6G^+$ cells (Table 8.2B) of NALT and CLN from immunised Balb/c mice. Numbers represent the mean percentage of expression ± SD of groups of ten individual mice, from two independent experiments. The mean percentage values indicating the frequency of either, $CD25^+$, $CD69^+$, $MHCII^+$ or $VCAM\text{-}1^+$ cells, were calculated individually for each gated cell population expressing the corresponding surface marker ($DX5^+$, $Ly6G^+$, $CD11c^+$ or $F4/80^+$). The * indicates significant values of $p < 0.05$ and **, $p < 0.01$ as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Black represents those percentage values not significantly different to those seen in naïve mice; red indicates values significantly increased, and blue shows percentages significantly decreased in comparison to negative control mice.

Table 8.2A.

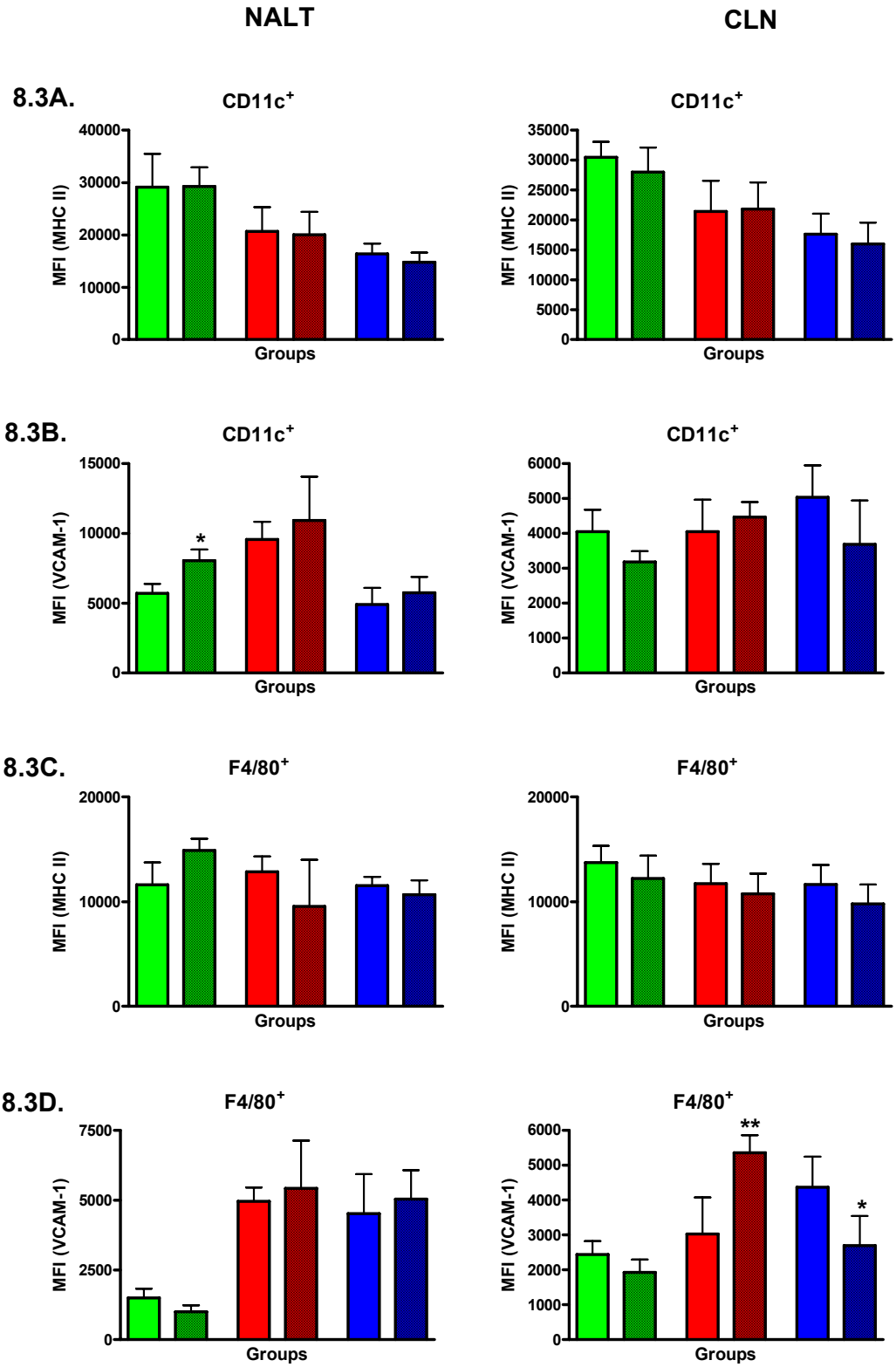
			5 hrs	24 hrs	72 hrs	5 hrs	24 hrs	72 hrs
Leukocyte	**Tissue**	**Immunisation**	**MHC II**			**VCAM-1**		
$CD11c^+$	NALT	Naive	4.6 ± 1.0	4.5 ± 0.4	4.4 ± 0.2	3.1 ± 0.8	3.2 ± 0.5	3.4 ± 0.4
		LT	4.2 ± 1.1	4.0 ± 0.8	4.1 ± 0.4	2.6 ± 0.7**	4.4 ± 0.5**	3.1 ± 0.4**
		LT + Ag	4.0 ± 0.8	5.5 ± 0.8**	4.0 ± 0.3	2.6 ± 0.6**	4.0 ± 1.1	4.0 ± 0.8**
$CD11c^+$	CLN	Naive	2.3 ± 0.2	2.1 ± 0.3	2.1 ± 0.2	1.7 ± 0.1	1.6 ± 0.3	1.8 ± 0.3
		LT	2.9 ± 0.3**	1.7 ± 0.3**	2.6 ± 0.2**	1.9 ± 0.3	1.4 ± 0.2	2.5 ± 0.5**
		LT + Ag	2.6 ± 0.4	1.5 ± 0.3**	2.9 ± 0.4**	1.7 ± 0.1	1.2 ± 0.2**	2.4 ± 0.4*
$F4/80^+$	NALT	Naive	5.5 ± 1.1	5.4 ± 0.8	5.3 ± 0.5	3.8 ± 0.5	3.5 ± 0.6	3.8 ± 0.6
		LT	5.9 ± 1.2	5.9 ± 1.3	5.7 ± 0.6	3.1 ± 0.3**	3.3 ± 0.6	3.8 ± 0.9
		LT + Ag	4.7 ± 0.8	4.4 ± 0.3	4.8 ± 0.3	2.9 ± 0.2**	3.9 ± 0.7	4.4 ± 0.8
$F4/80^+$	CLN	Naive	2.5 ± 0.3	2.6 ± 0.1	2.3 ± 0.3	1.6 ± 0.1	1.7 ± 0.2	1.6 ± 0.1
		LT	2.7 ± 0.3	2.2 ± 0.1**	2.8 ± 0.2**	2.1 ± 0.2**	1.4 ± 0.1**	2.5 ± 0.5**
		LT + Ag	2.1 ± 0.4	2.0 ± 0.2**	2.7 ± 0.3*	2.3 ± 0.4**	1.2 ± 0.1**	2.2 ± 0.3**

Table 8.2B.

			5 hrs	24 hrs	72 hrs	5 hrs	24 hrs	72 hrs
Leukocyte	**Tissue**	**Immunisation**	**CD25**			**CD69**		
$DX5^+$	NALT	Naive	2.3 ± 0.3	2.1 ± 0.4	2.0 ± 0.2	1.6 ± 0.1	1.4 ± 0.3	1.4 ± 0.4
		LT	1.7 ± 0.1**	2.3 ± 0.2	1.5 ± 0.1	1.2 ± 0.1**	1.4 ± 0.4	1.1 ± 0.2
		LT + Ag	2.0 ± 0.2	4.3 ± 1.1**	3.1 ± 1.1**	1.1 ± 0.1**	1.3 ± 0.5	2.3 ± 0.4**
$DX5^+$	CLN	Naive	1.3 ± 0.4	1.1 ± 0.2	1.0 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
		LT	1.5 ± 0.3	1.9 ± 0.6**	1.5 ± 0.4**	2.5 ± 0.8**	0.9 ± 0.3	0.9 ± 0.2
		LT + Ag	3.8 ± 1.2**	1.6 ± 0.5*	1.1 ± 0.1	3.0 ± 1.1**	0.6 ± 0.1	0.7 ± 0.1
$Ly6G^+$	NALT	Naive				2.2 ± 0.2	2.3 ± 0.5	2.2 ± 0.5
		LT				1.2 ± 0.1**	3.1 ± 1.1**	2.6 ± 0.5
		LT + Ag				0.8 ± 0.1**	1.9 ± 0.2	2.7 ± 0.6
$Ly6G^+$	CLN	Naive				0.7 ± 0.1	0.8 ± 0.2	0.8 ± 0.1
		LT				0.5 ± 0.1	1.9 ± 0.3**	1.2 ± 0.4*
		LT + Ag				0.9 ± 0.3	1.0 ± 0.1	0.6 ± 0.1

Figure 8.3: Mean Fluorescence Intensity (MFI) of activation markers on innate immune cell populations early after intranasal immunisation.

Cells were isolated from the NALT and CLN of control (PBS immunised) and LT immunised Balb/c mice, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $CD11c^+$ and $F4/80^+$ expression as well as MHC II and VCAM-1 expression (Figure 8.3A $CD11c^+/MHCII^+$; 8.3B $CD11c^+/VCAM\text{-}1^+$; 8.3C $F4/80^+/MHCII^+$ and 8.3D $F4/80^+/VCAM\text{-}1^+$). $DX5^+$ populations were examined for CD25 and CD69 expression with $Ly6G^+$ cells examined for CD69 expression (Figure 8.3E $DX5^+/CD25^+$; 8.3F $DX5^+/CD69^+$ and 8.3G $Ly6G^+/CD69^+$). For analysis, gates were set on the innate subset marker positive ($CD11c^+$, $F4/80^+$, $DX5^+$ and $Ly6G^+$) cells and the MFI of MHC II, VCAM-1, CD25 and CD69 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$ as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, red at 24 hours and blue at 72 hours. Block colours represent naïve (PBS immunised) animals and, hatched lines show animal's intranasally immunised adjuvant alone.


NALT
CLN
8.3A.
CD11c+
MFI (MHC II)
Groups
8.3B.
CD11c+
MFI (VCAM-1)
Groups
8.3C.
F4/80+
MFI (MHC II)
Groups
8.3D.
F4/80+
MFI (VCAM-1)
Groups

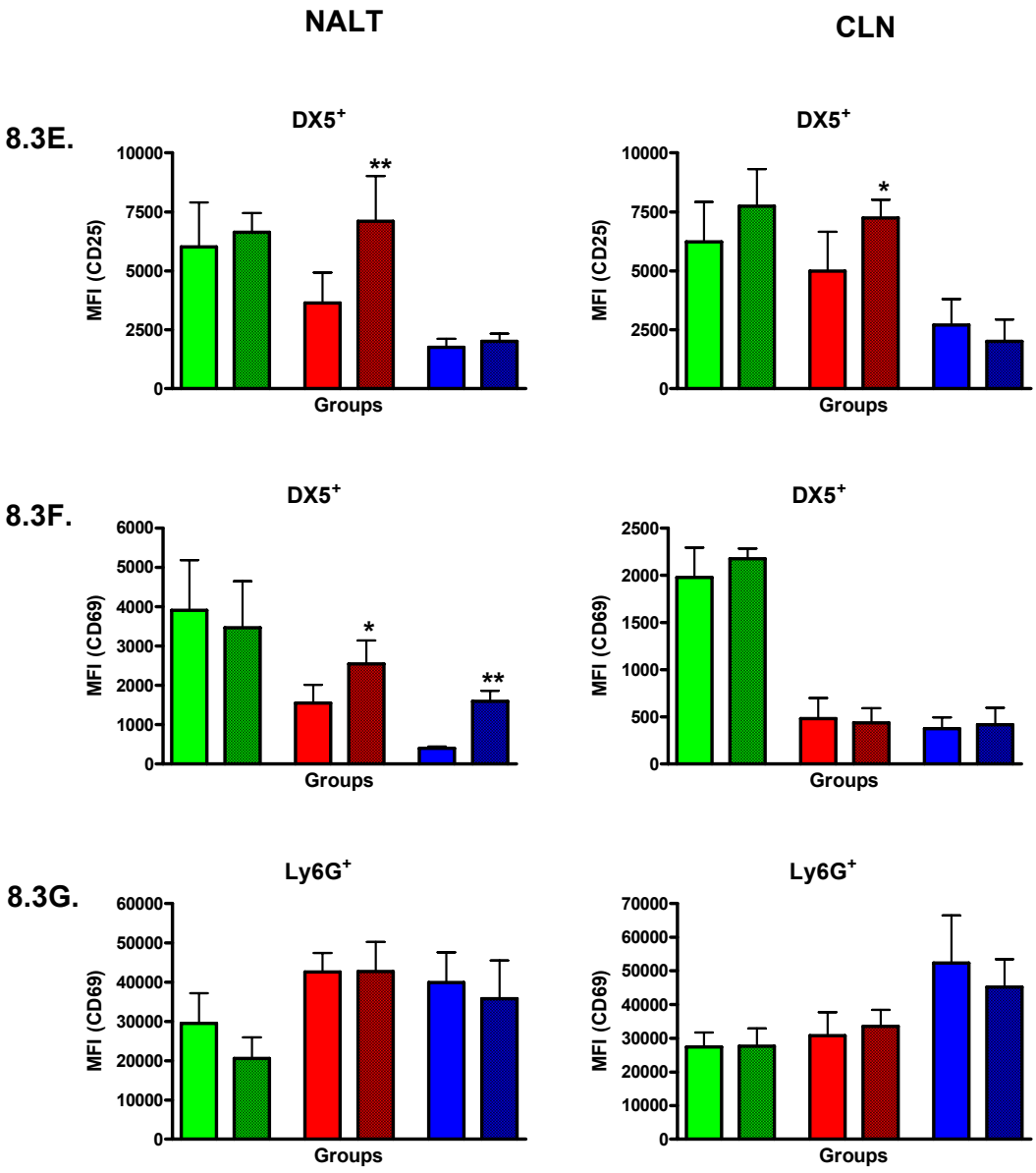

NALT
CLN
8.3E.
DX5+
MFI (CD25)
Groups
8.3F.
MFI (CD69)
8.3G.
Ly6G+

Figure 8.4: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 8.4A represents staining of frozen sections for MAdCAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and MAdCAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 8.4A:

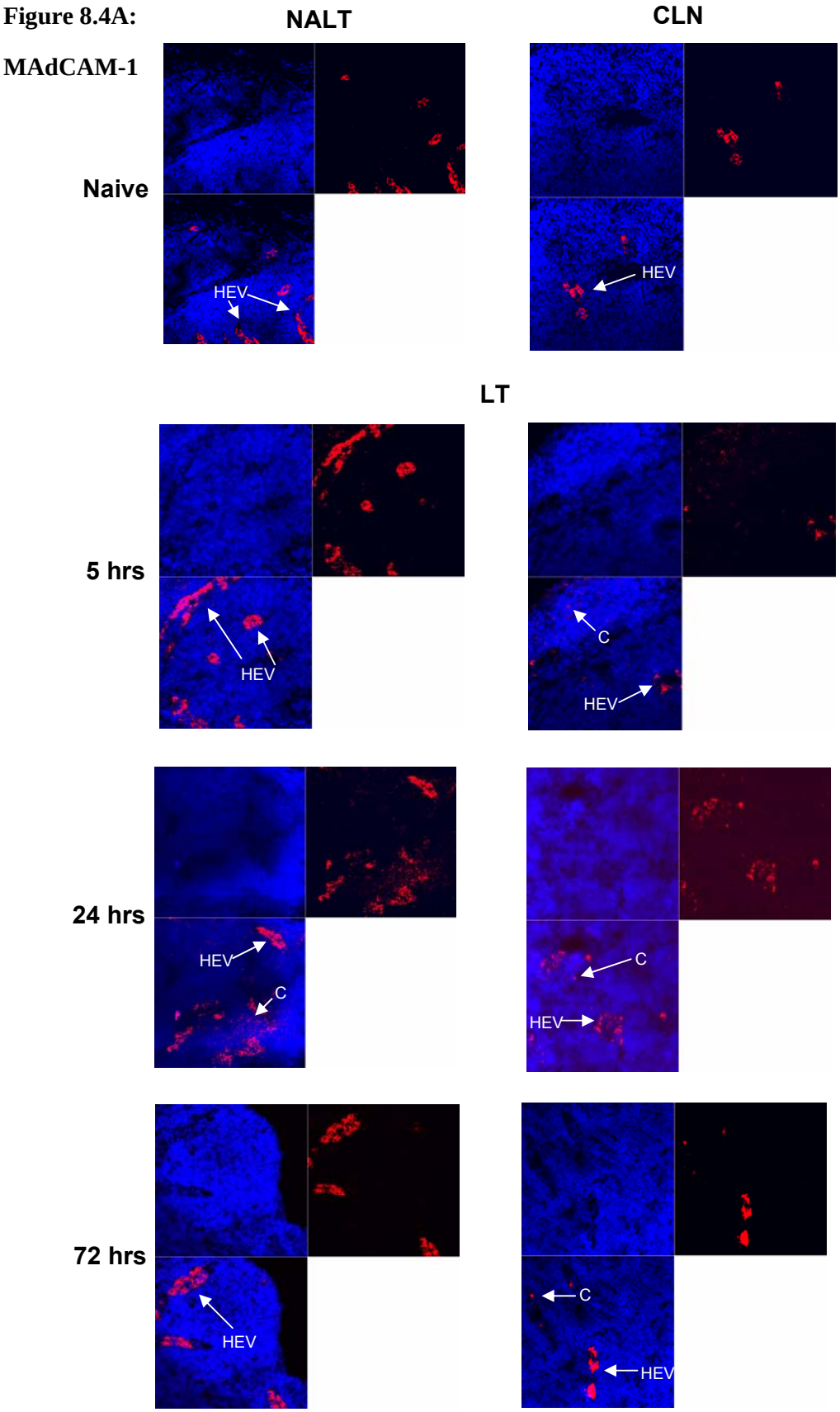

NALT
CLN
MAdCAM-1
Naive
HEV
HEV
LT
5 hrs
HEV
C
HEV
24 hrs
HEV
C
C
HEV
72 hrs
HEV
C
HEV

Figure 8.4: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 8.4B represents staining of frozen sections for PNAd in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and PNAd (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 8.4B :

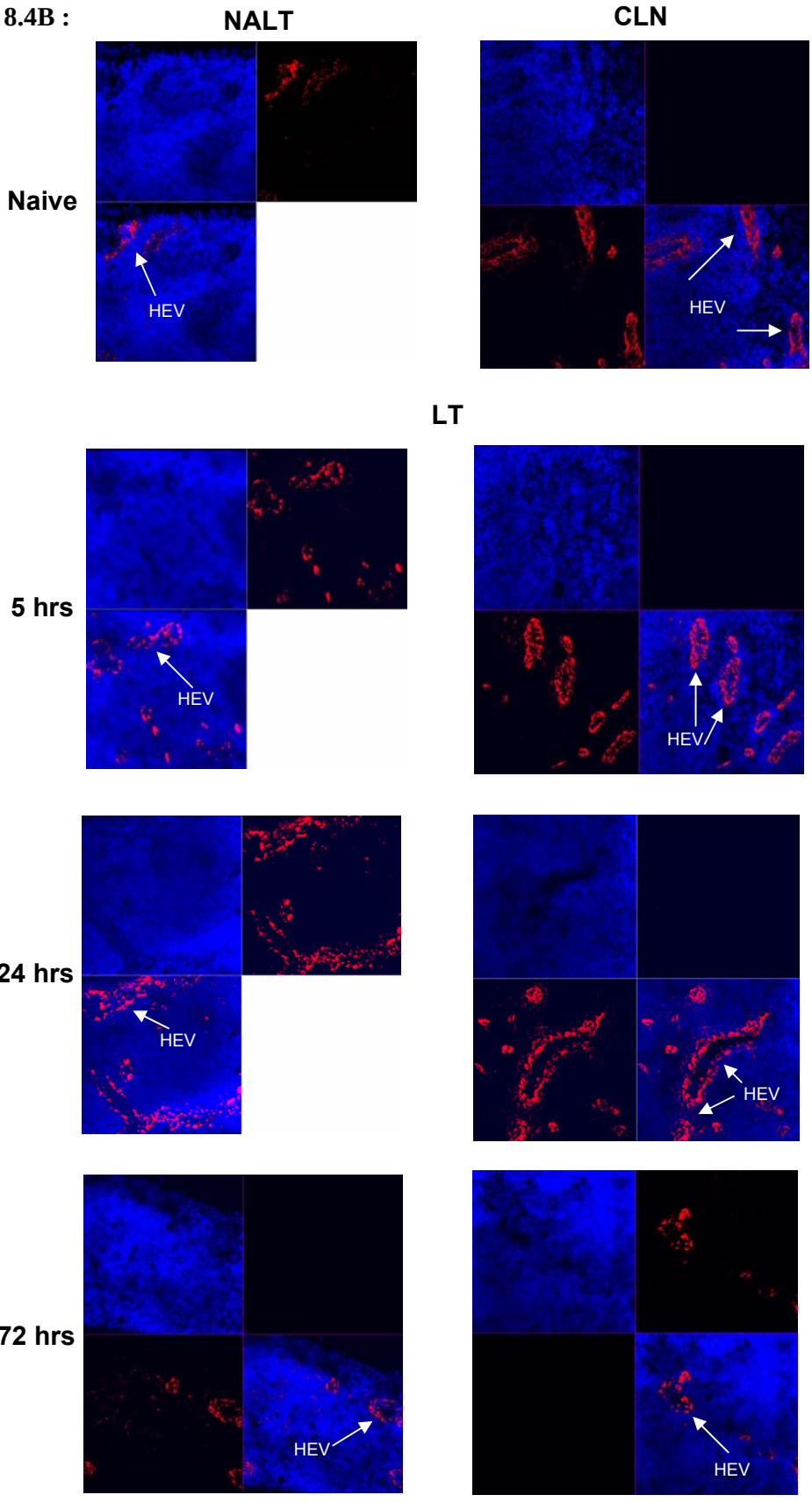

NALT
CLN
PNAd
Naive
HEV
HEV
LT
5 hrs
HEV
HEV
24 hrs
HEV
HEV
72 hrs
HEV
HEV

Figure 8.4: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 8.4C represents staining of frozen sections for ICAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and ICAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 8.4C :

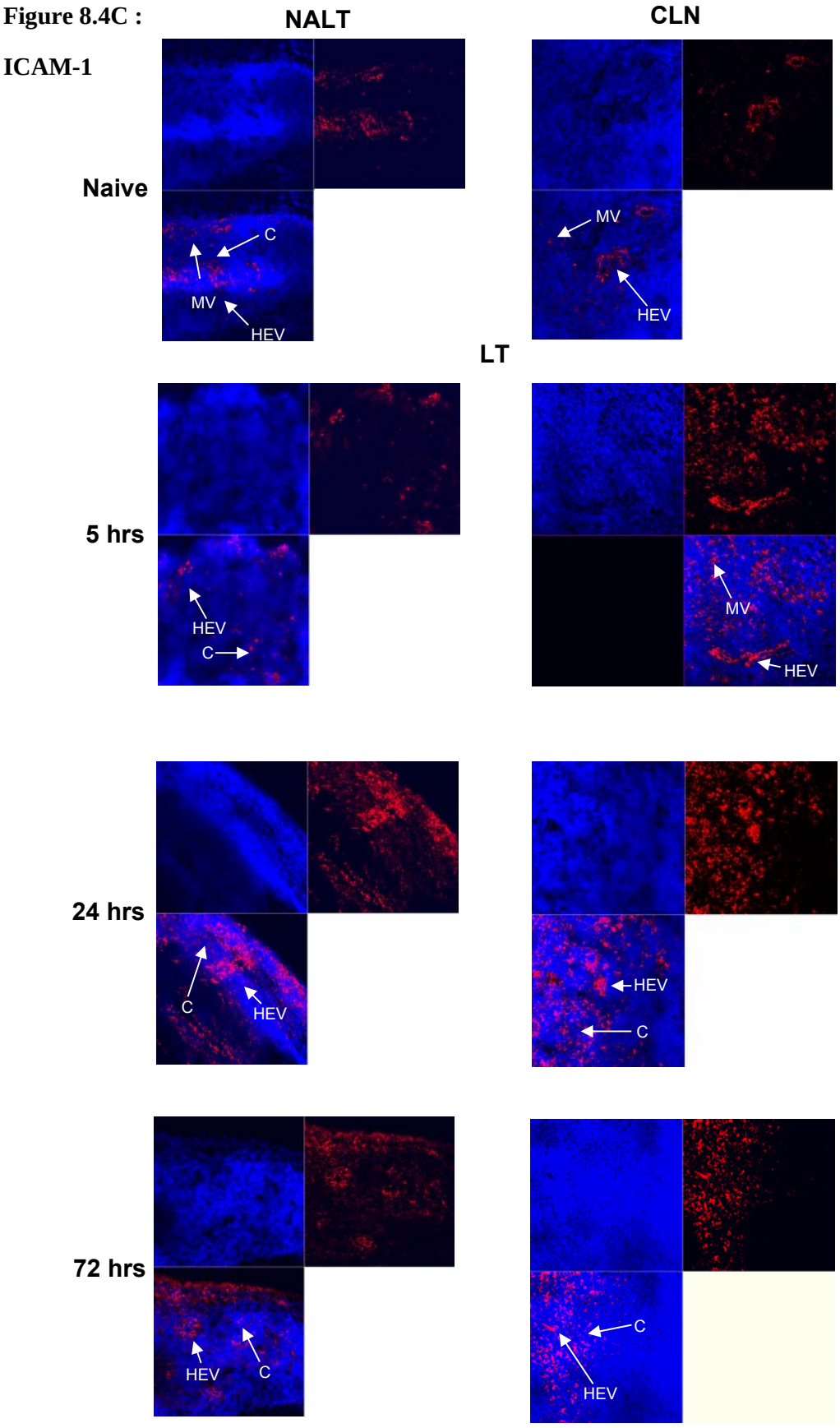

NALT
CLN
ICAM-1
Naive
C
MV
HEV
MV
HEV
LT
5 hrs
HEV
C
MV
HEV
24 hrs
C
HEV
HEV
C
72 hrs
HEV
C
C
HEV

Figure 8.4: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 8.4D represents staining of frozen sections for VCAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and VCAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 8.4D :

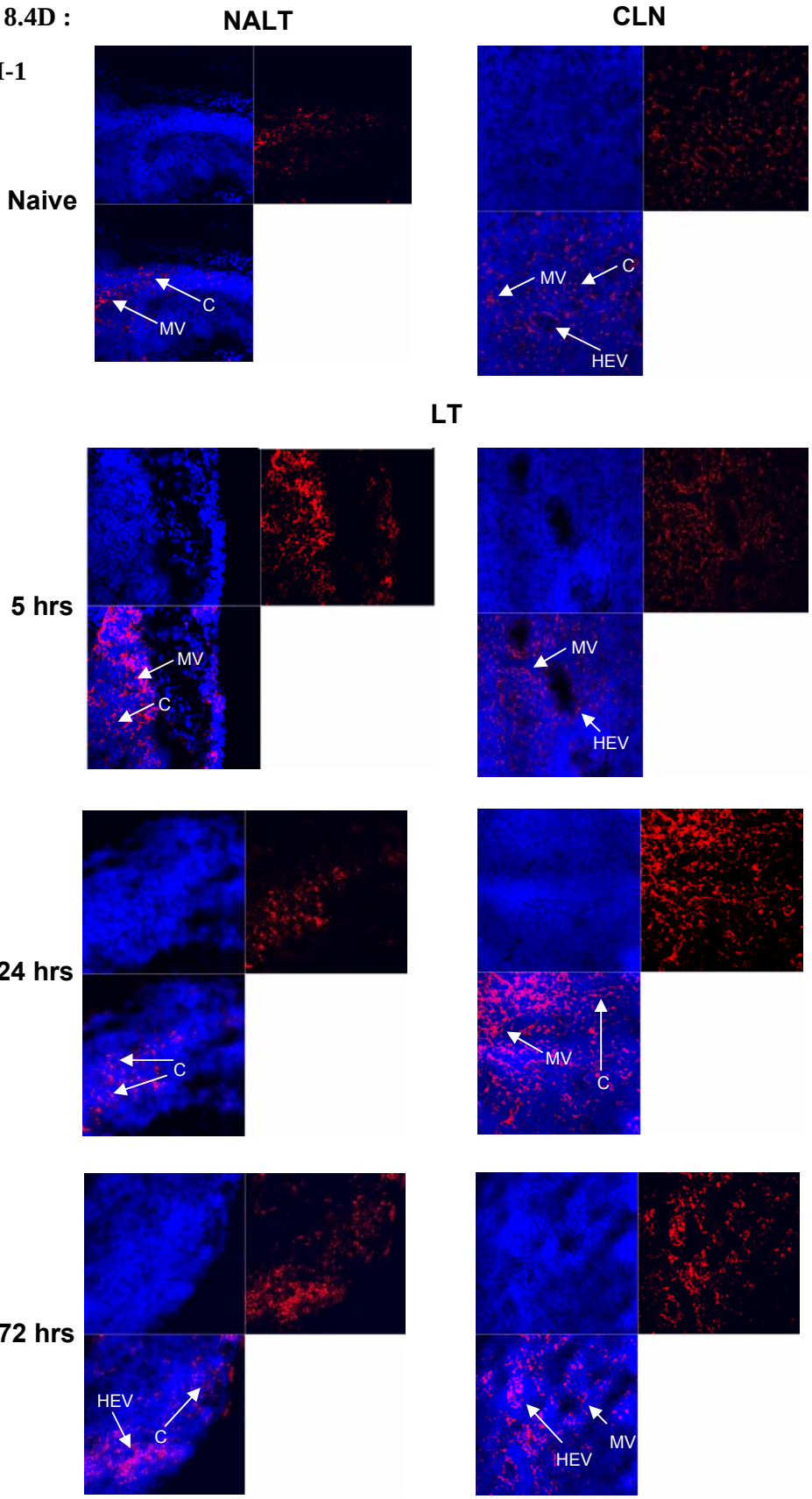

NALT
CLN
VCAM-1
Naive
C
MV
MV
C
HEV
LT
5 hrs
MV
C
MV
HEV
24 hrs
C
MV
C
72 hrs
HEV
C
HEV
MV

Figure 8.5: Immunohistochemical evaluation of GC in both CLN and NALT from immunised Balb/c mice.

Balb/c mice were immunised with either PBS (naïve) or 1μg LT. Figure 8.5 reveals the presence of GC in the NALT and CLN of immunised mice at 5, 24 and 72 hours post intranasal immunisation. Triple immunolabeling of B220-positive cells (green), PNA binding cells (red) and cell nuclei by Hoechst (blue) is shown for all images. White arrows indicate GC, or lack of in naïve animals. There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 8.5: GC