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).

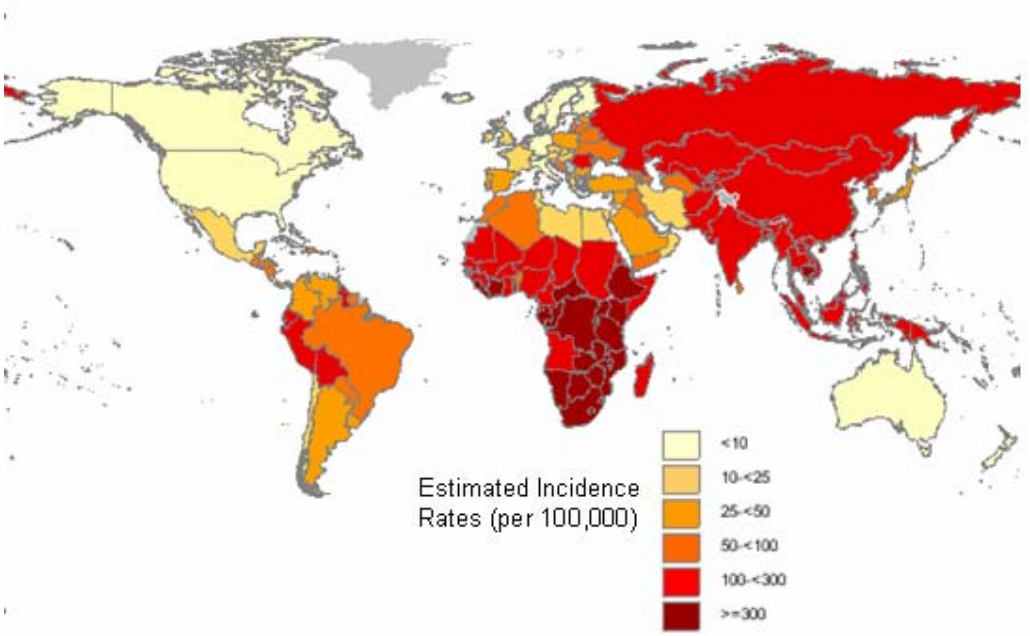

Figure 1.1: Tuberculosis incidence rates 2005 (WHO 2005).

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).

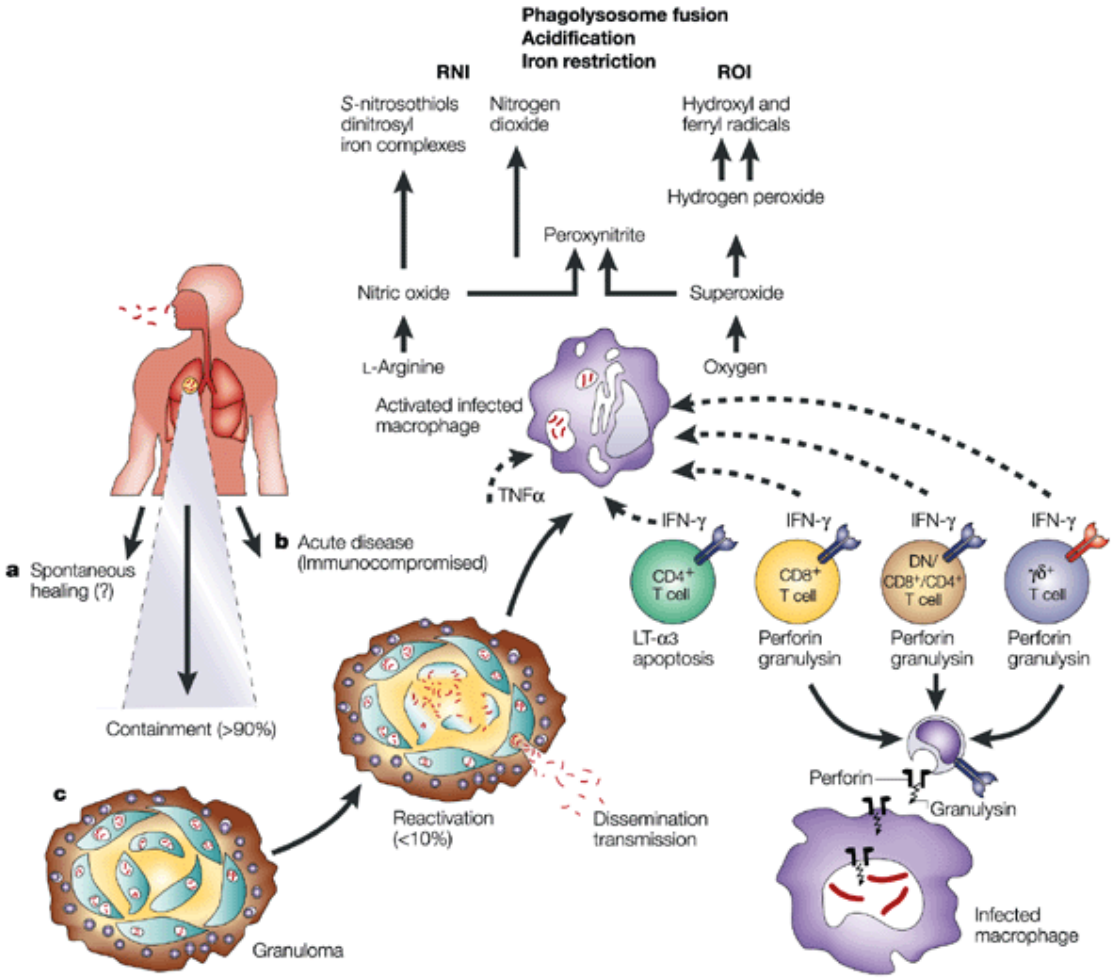

Figure 1.2: Main features of tuberculosis: from infection to host defence.

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*.

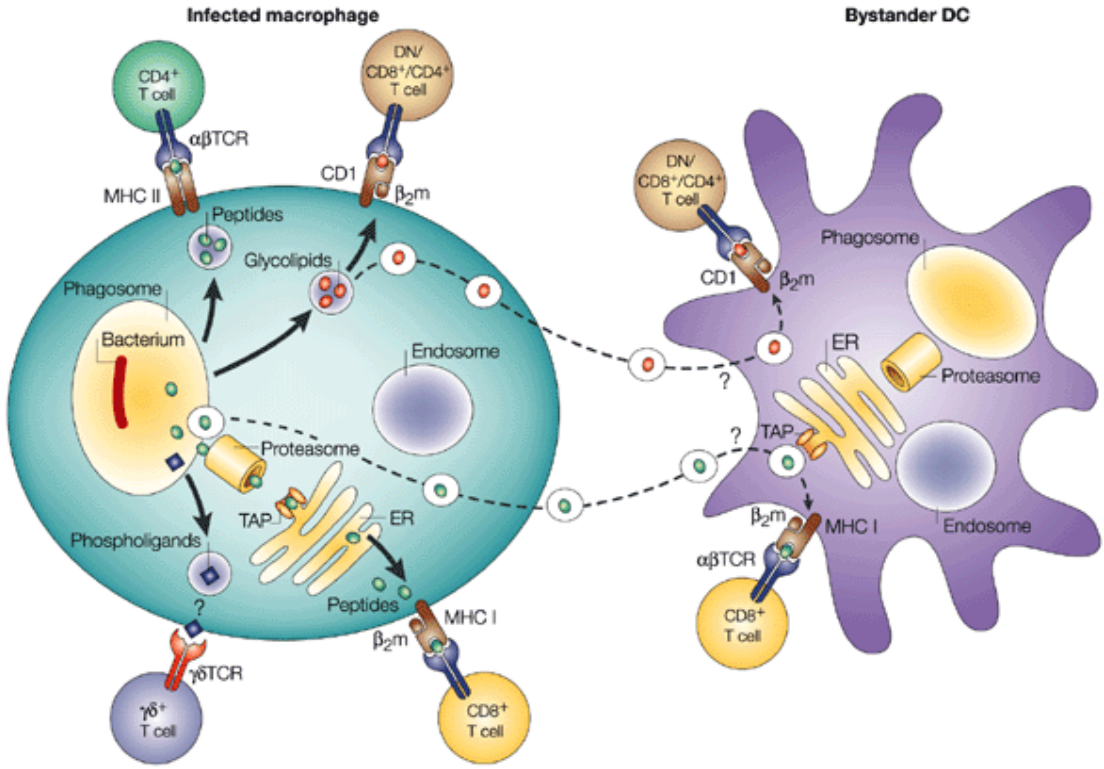

Figure 1.3: Antigen processing/presentation pathways and activation of different T-cell subsets. The different T-cell-processing pathways that result in activation of distinct T-cell populations in the immune response against *Mycobacterium tuberculosis* are shown. This pathogen preferentially resides 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.

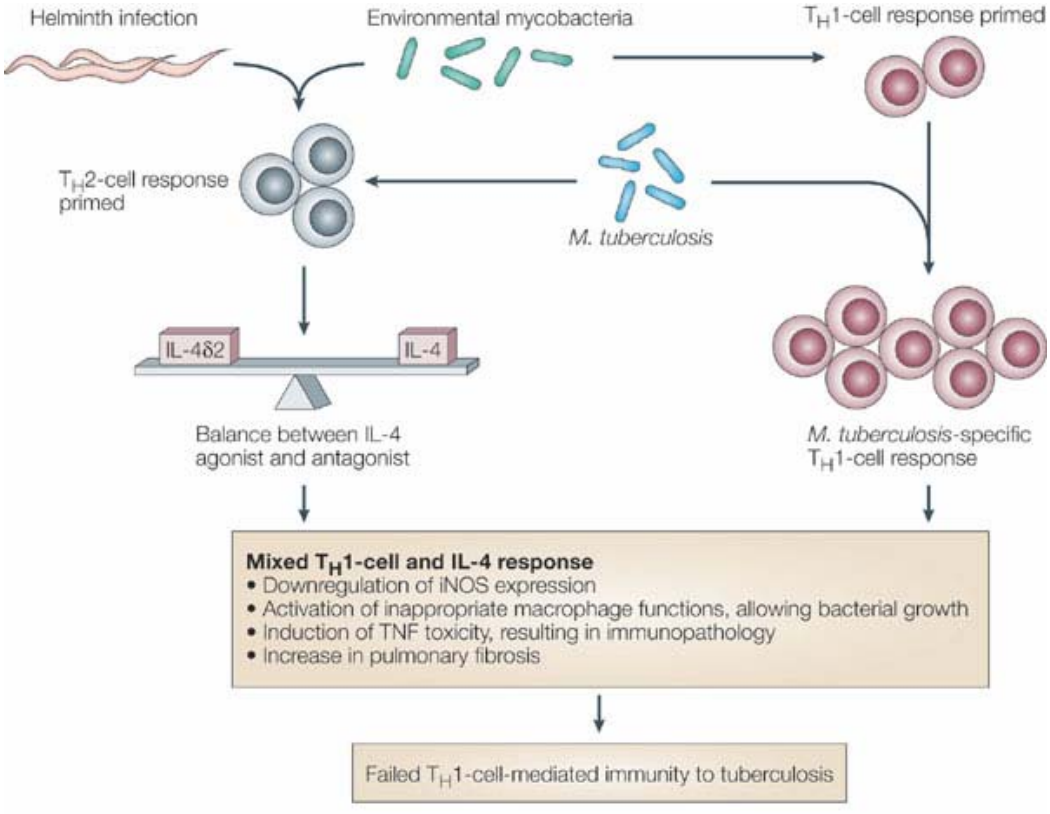

Figure 1.4: Events that might lead to failure of BCG in developing countries.

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).

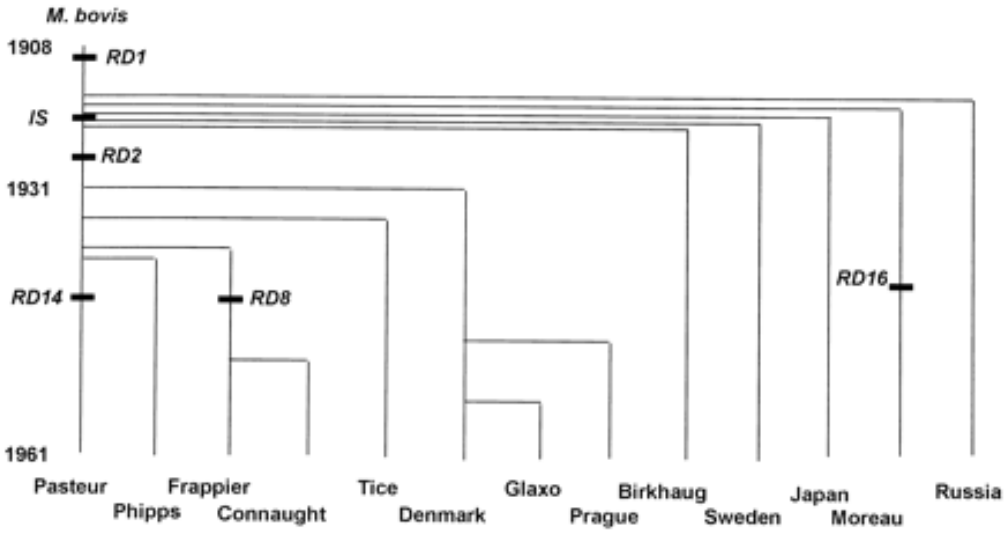

Figure 1.5: BCG historical genealogy incorporating genetic differences previously noted and newly detected genetic deletions.

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).

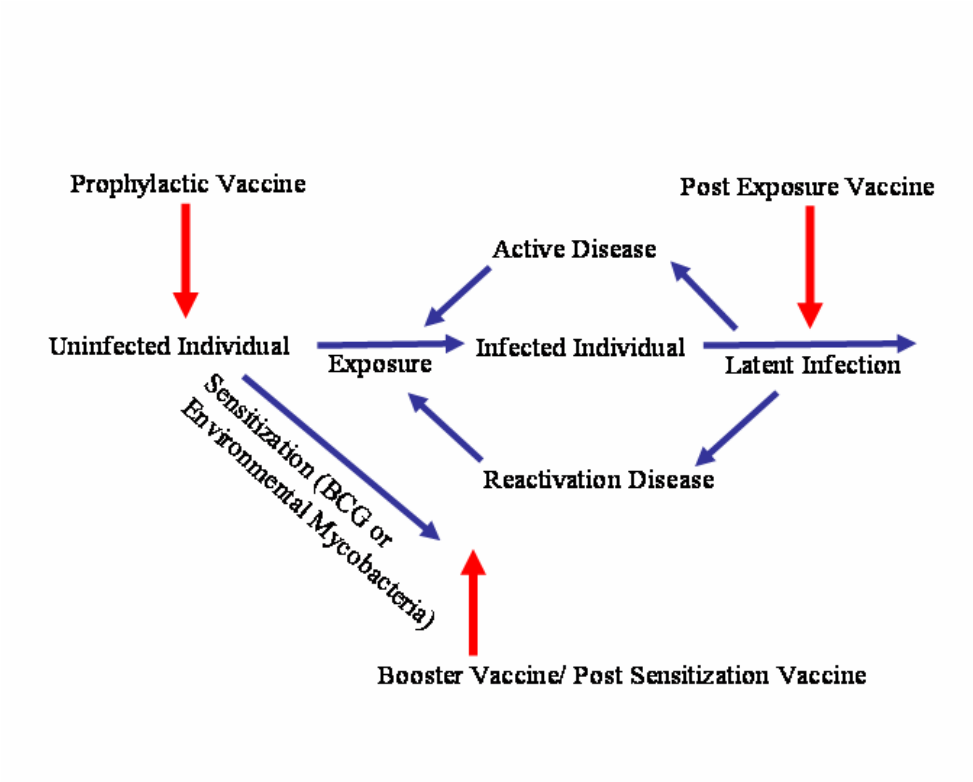

Figure 1.6: Different tuberculosis vaccination strategies.

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.

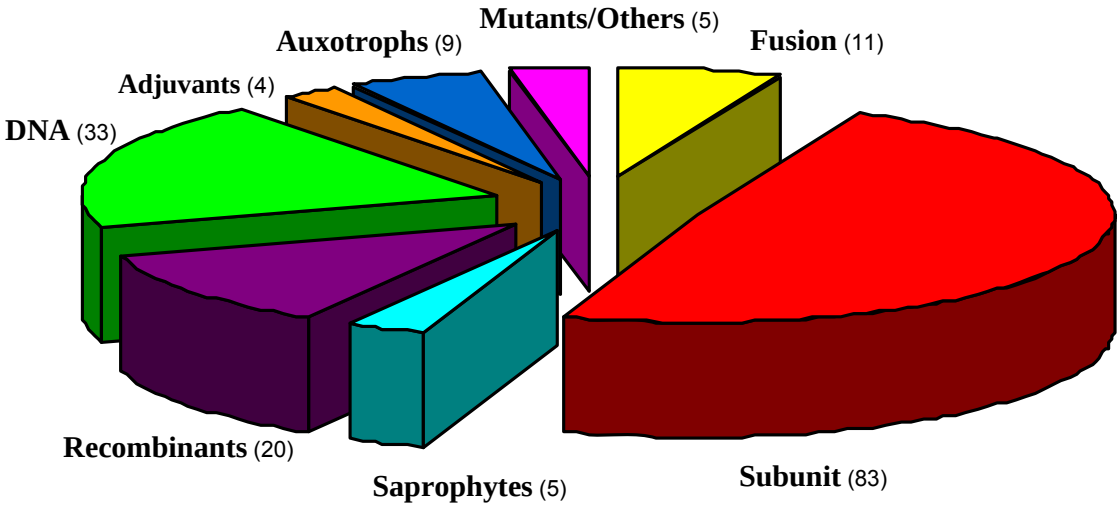

Figure 1.7: Tuberculosis vaccines currently undergoing screening.

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).

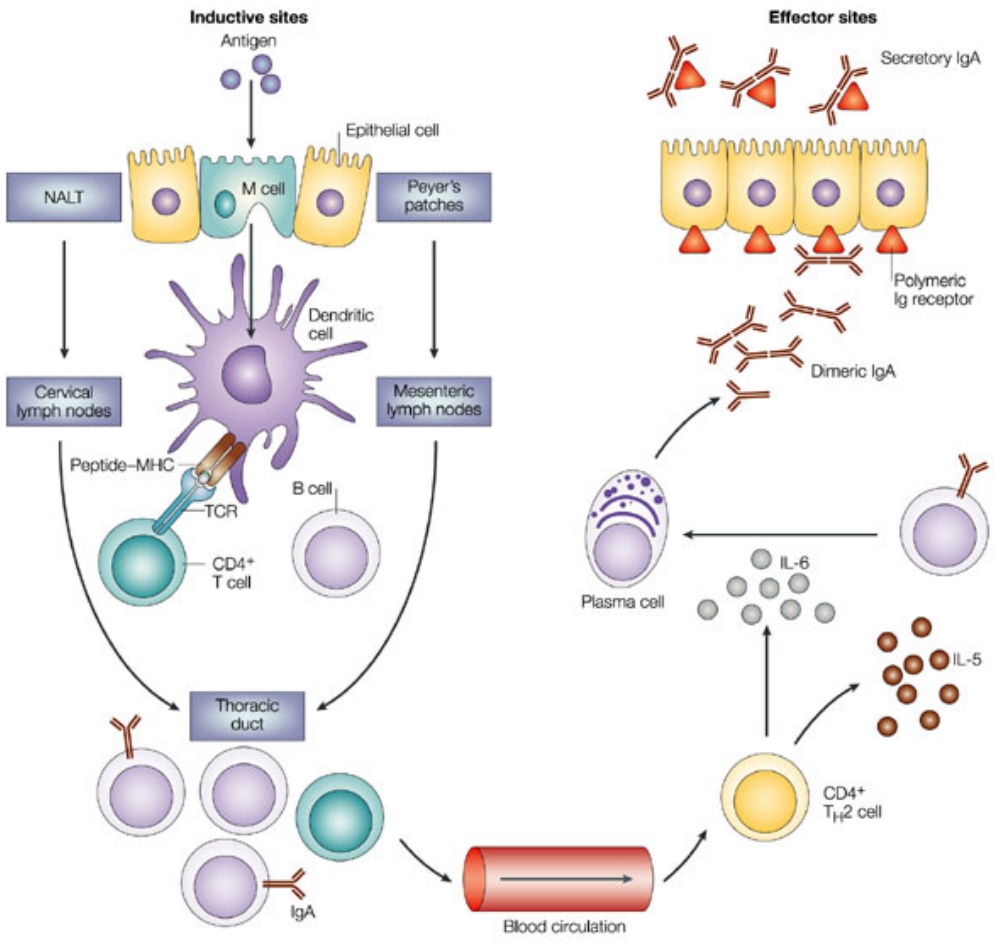

Figure 1.8: The CMIS

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).

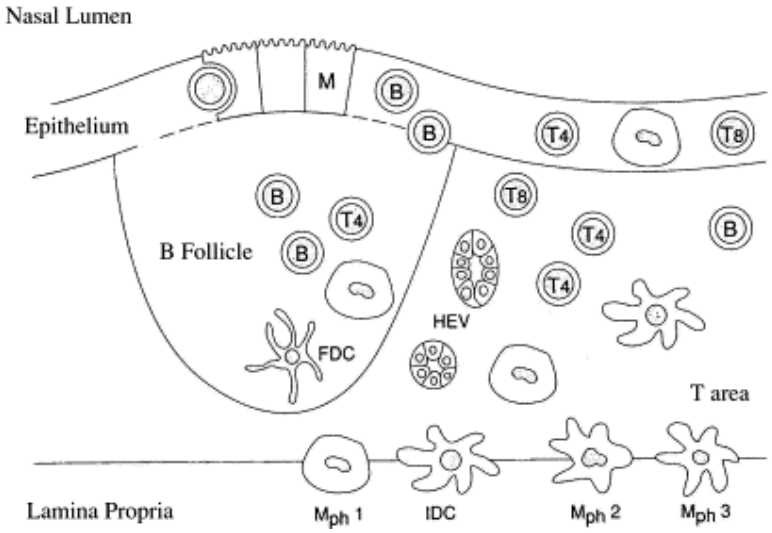

Figure 1.9: Schematic representation of compartments and cellular composition of NALT.
B, B cell; T4, CD4-positive T cell; T8, CD8-positive T cell; $M_{ph}1$, $M_{ph}2$, $M_{ph}3$, macrophages positive for ED1, ED2 or ED3 (macrophage surface antigens), respectively; IDC, interdigitating dendritic cell;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

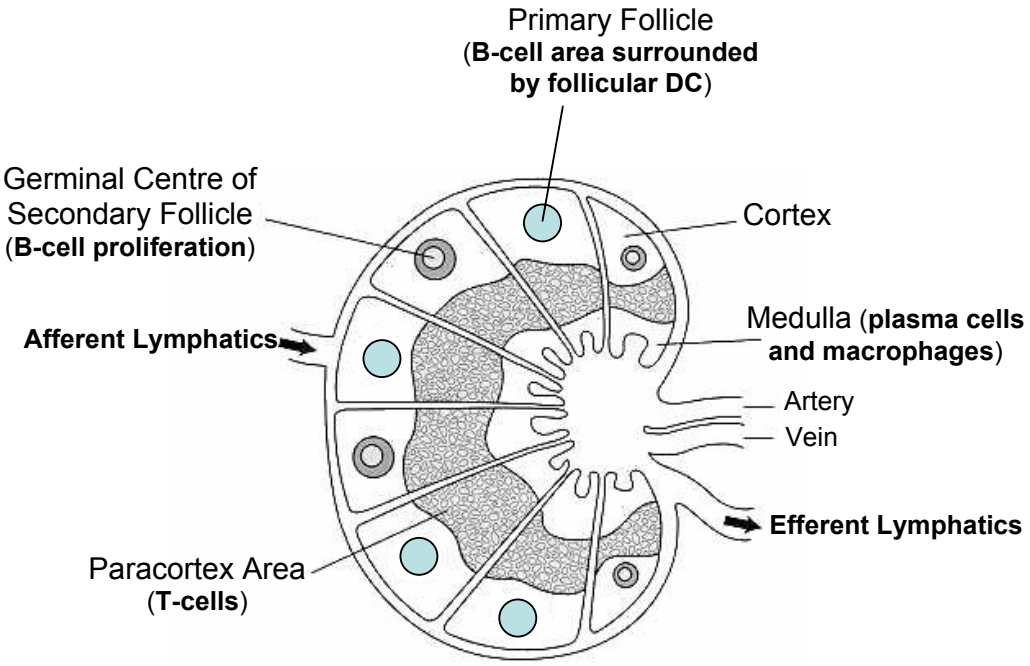

Figure 1.10: Diagram of a lymph node

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).

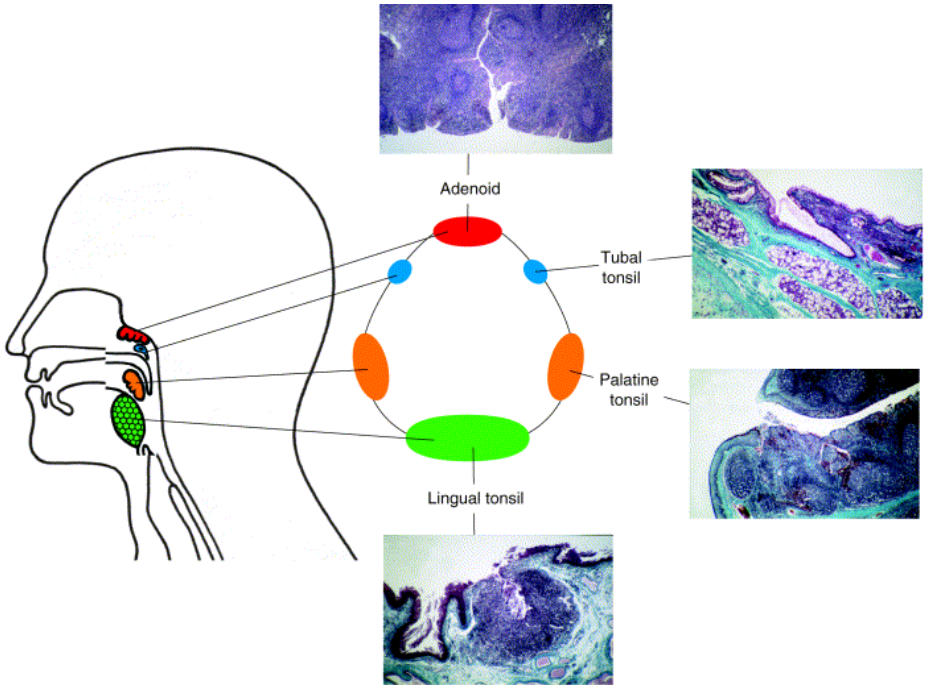

Figure 1.11: Pharyngeal lymphoid tissue of Waldeyer's ring.

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).

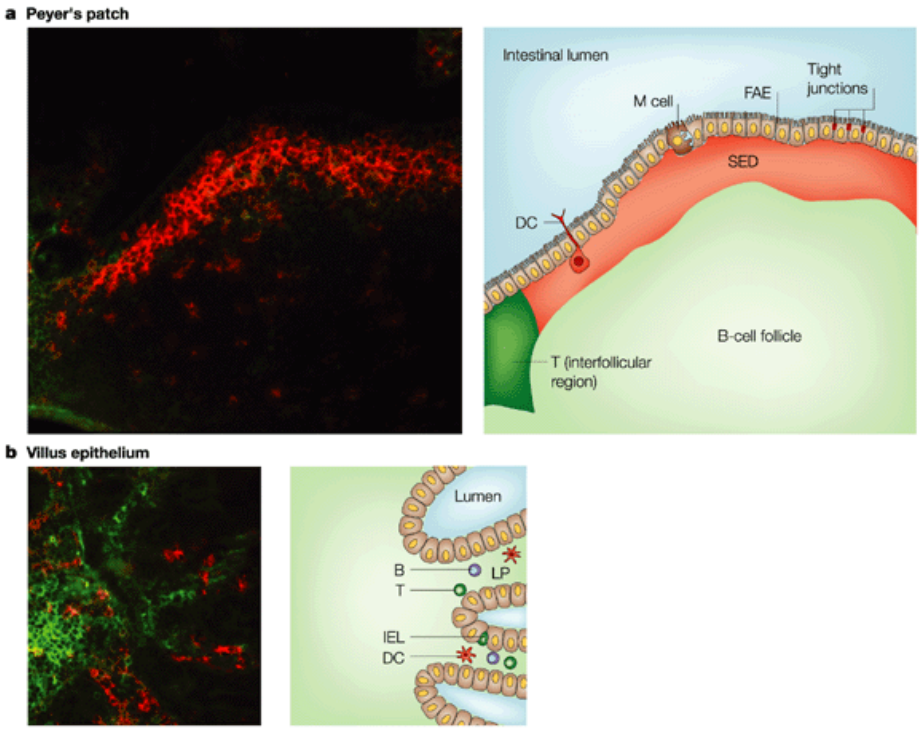

Figure 1.12: Gut associated lymphoid tissue

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.