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.