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

6.1 General introduction

6.1.1 Live vector vaccines

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

Table 6.1: Current live attenuated bacterial licensed vaccines

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

Table 6.2: Recombinant bacterial vector vaccine candidates

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

6.1.2 *Salmonella* as vaccine vectors

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

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

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

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

Table 6.3: Characteristics of live vaccines developed against typhoid fever

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

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

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

6.2.1 Introduction

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

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

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

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

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

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

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

6.2.2 Results

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

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

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

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

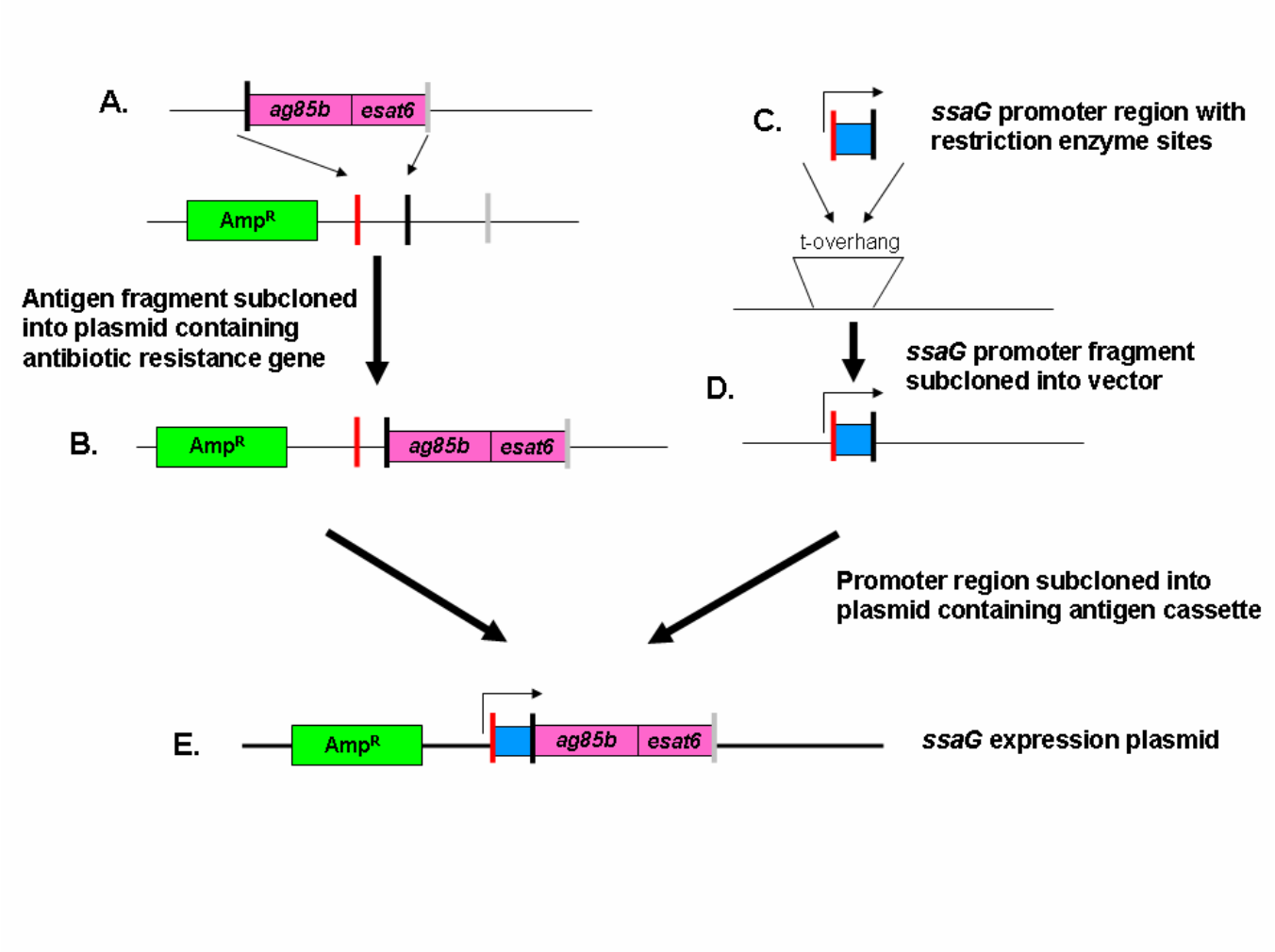
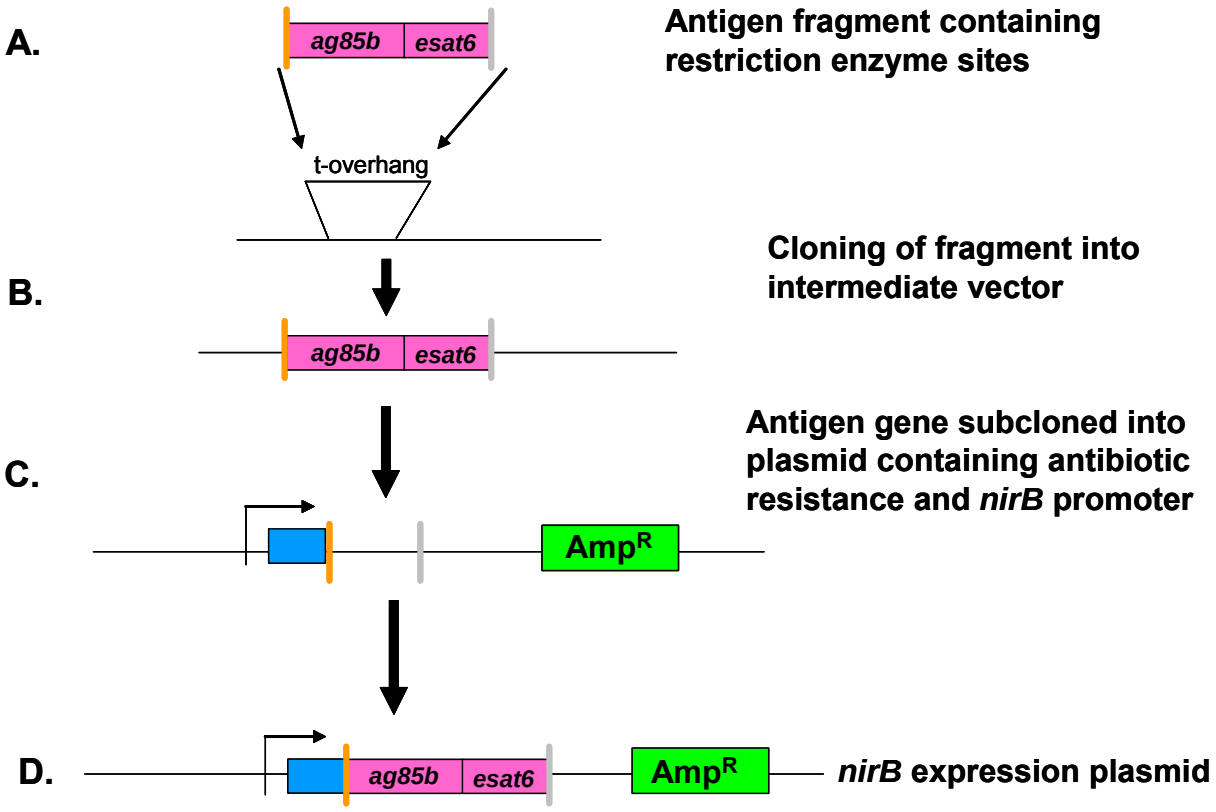


Figure 6.2: Schematic diagram showing the construction of the plasmid used to express Ag85B-ESAT6 via the *nirB* promoter.

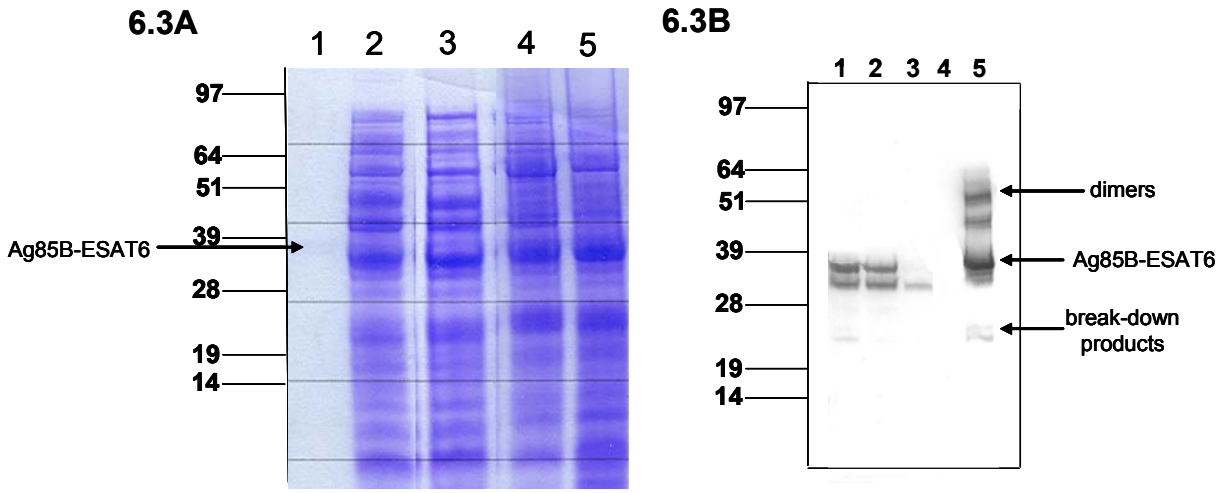
(A) The *ag85B-esat6* gene (pink symbol) was amplified from pMCT6 using primers containing NcoI (orange line) and BamHI (grey line) sequences. (B) The fragment was then cloned into t-overhang sites within the pTOPO vector. (C) The NcoI/BamHI antigen fragment was removed from the intermediate vector and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



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

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

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



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

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

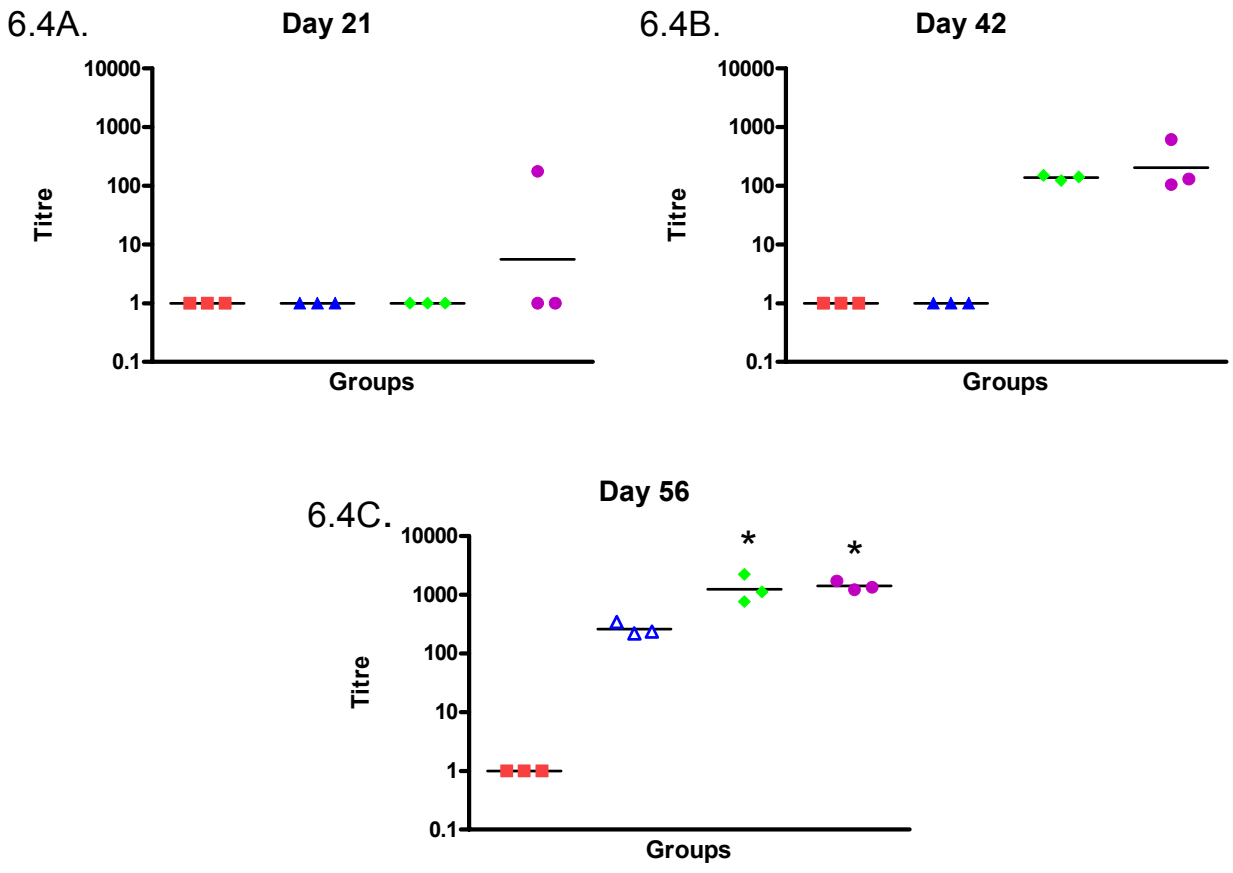
Table 6.4: Recombinant SL3261 immunisation regimen (3 mice/group)

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

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

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

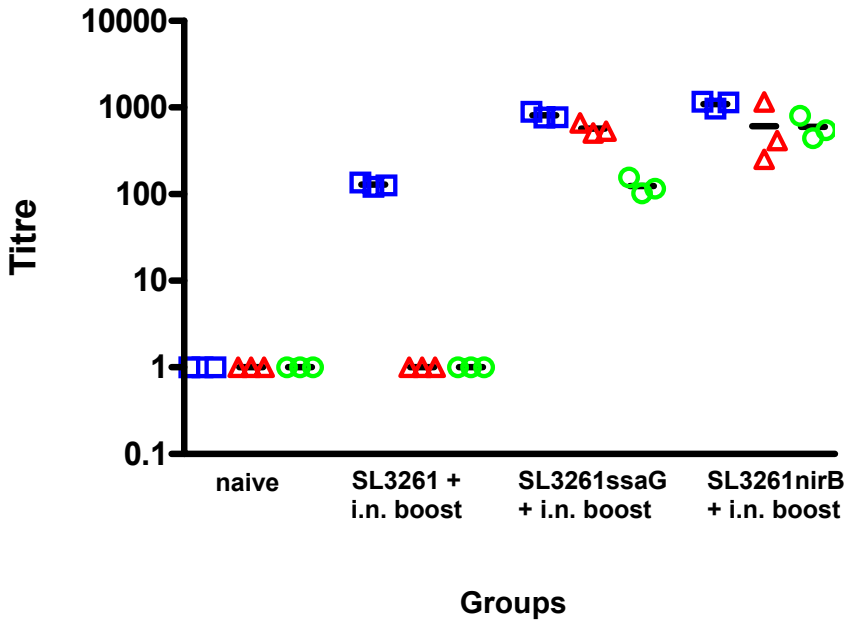
As outlined in Table 6.4, intragastric administration of recombinant SL3261 strains; SL3261*ssaG* and SL3261*nirB*, into Balb/c mice was performed at day 0 (immunisation) and day 35 (boost) with a final intranasal boost of 10µg Ag85B-ESAT6 + 1µg LT (day 49). Mice were inoculated with approximately 5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



6.2.2.3 IgG1/IgG2a profile after recombinant *Salmonella* immunisation

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

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



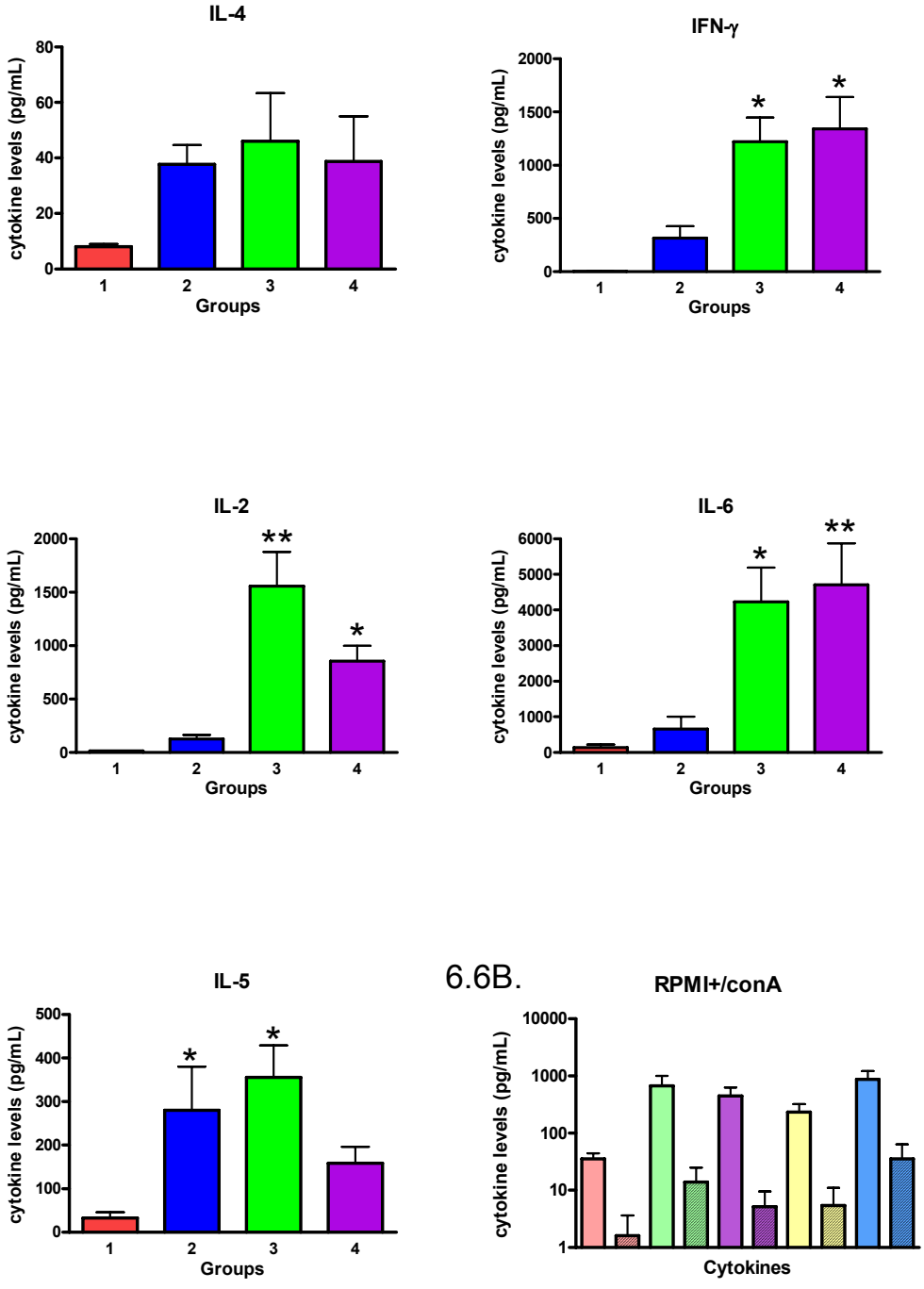
6.2.2.4 Intragastric priming with recombinant SL3216 strains plus an intranasal Ag85B-ESAT6 boost and cytokine responses

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

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

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

6.6A.


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

6.6B.

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

6.2.3 Discussion

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

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

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

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

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

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

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

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

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

6.3.1 Introduction

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

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

6.3.2 Results

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

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

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

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

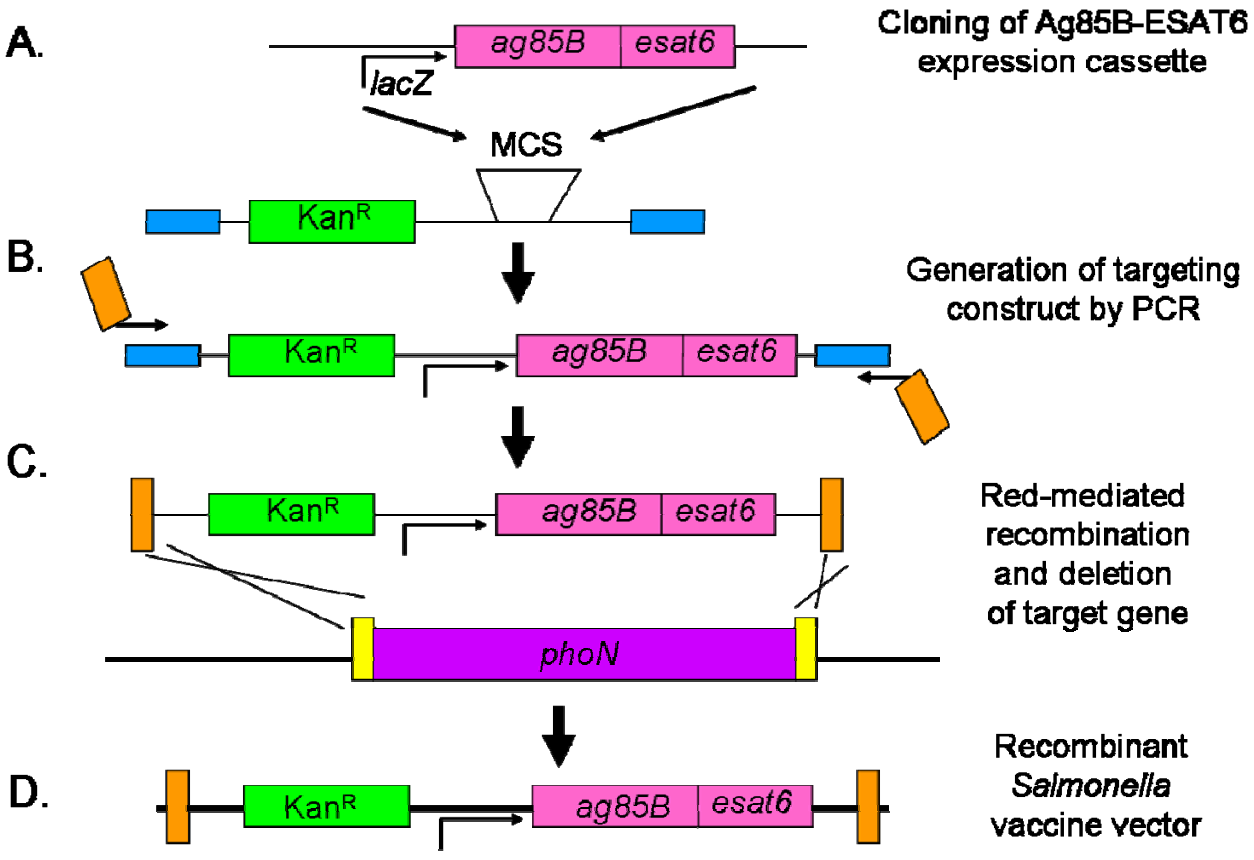
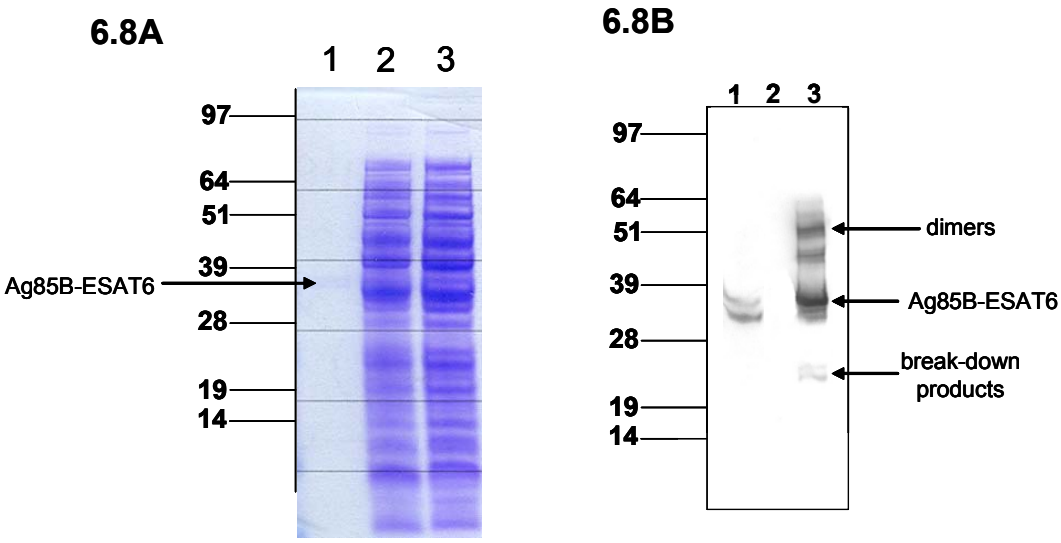


Figure 6.8: Coomassie gel and western blot analysis of recombinant chromosomal *Salmonella* construct.

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



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

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

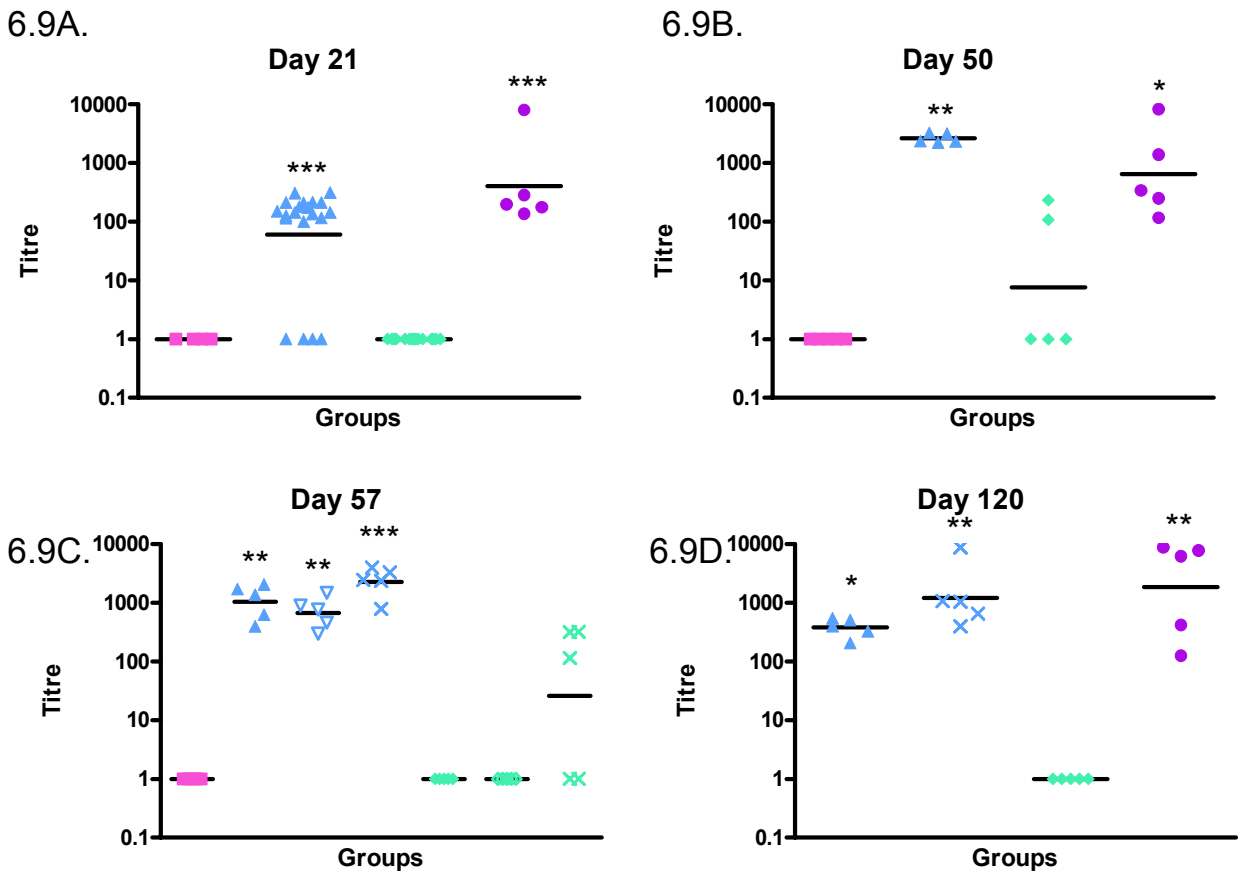
shows the serum anti-Ag85B-ESAT6 responses achieved when one dose of Ag85B-ESAT6 plus LT was administered intranasally and was considered a positive control group as this regimen has already been shown to induce significant anti-Ag85B-ESAT6 titres (see Chapter 3). As with Groups 2, 2a, 2b and 2c the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

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

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

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

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

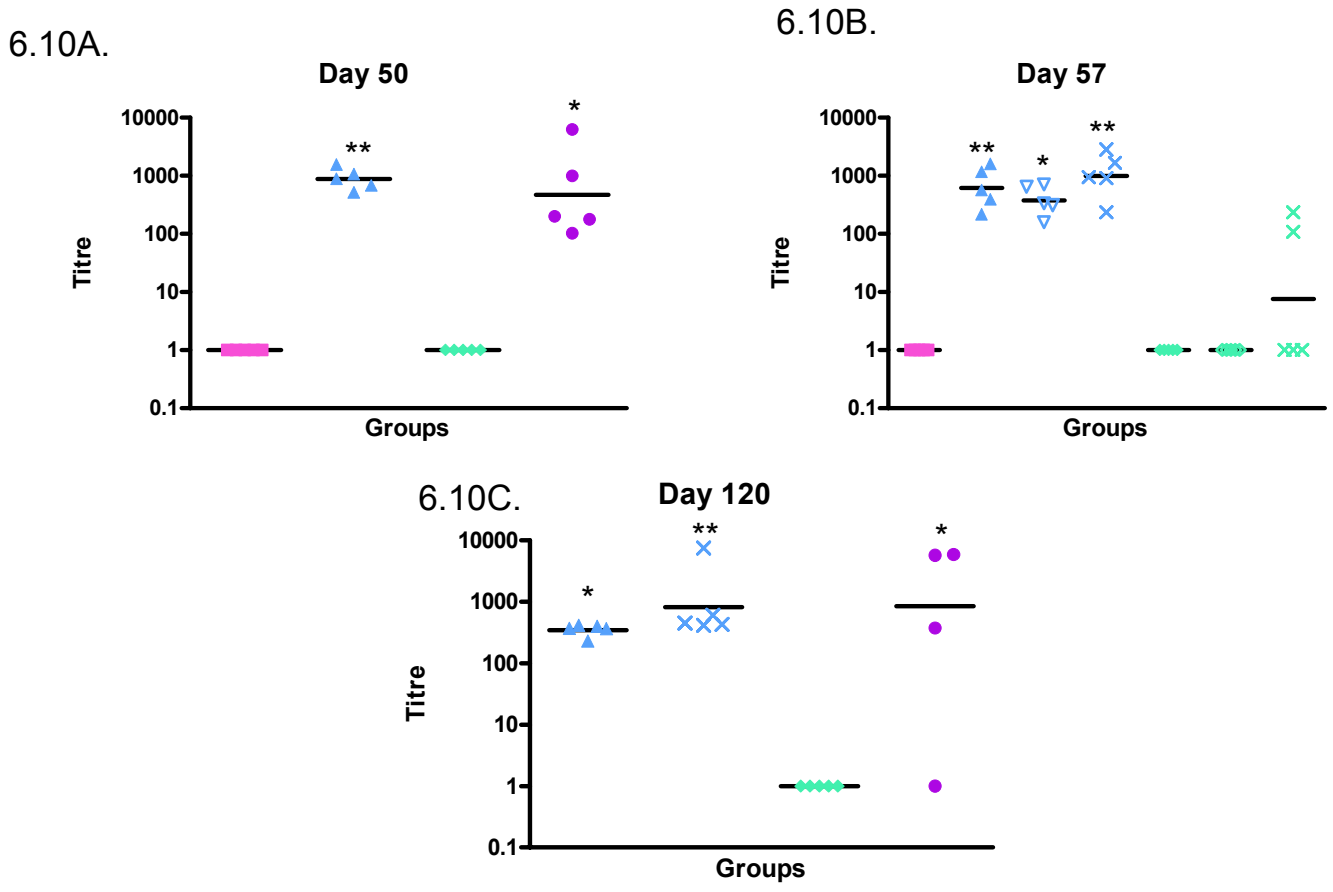


6.3.2.2.1 Serum IgG antibody responses

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

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

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

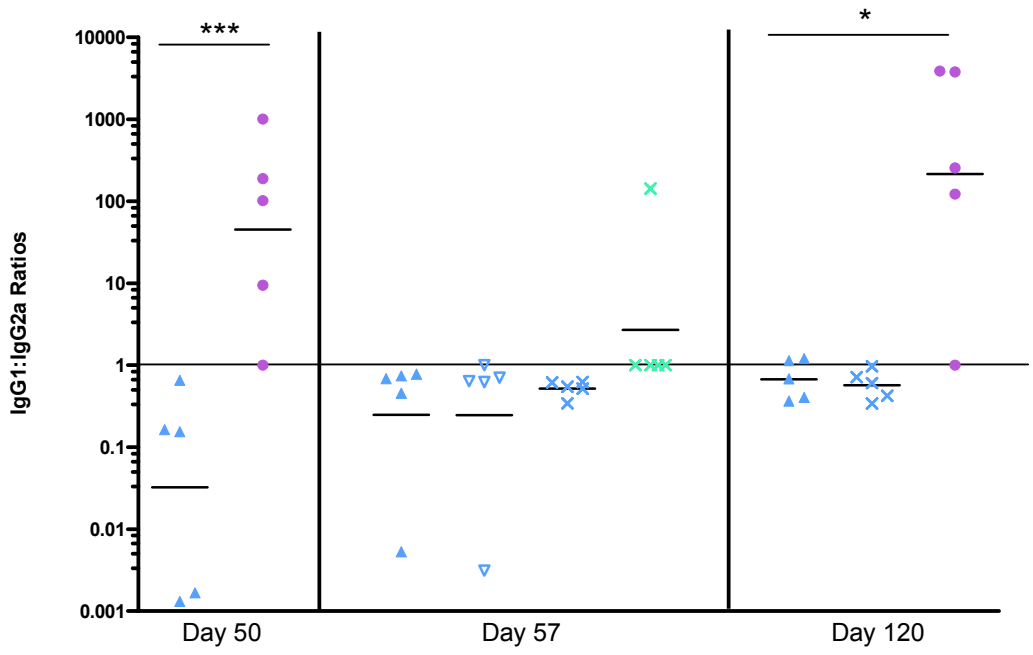


6.3.2.2.2 IgG sub-class responses

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

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

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

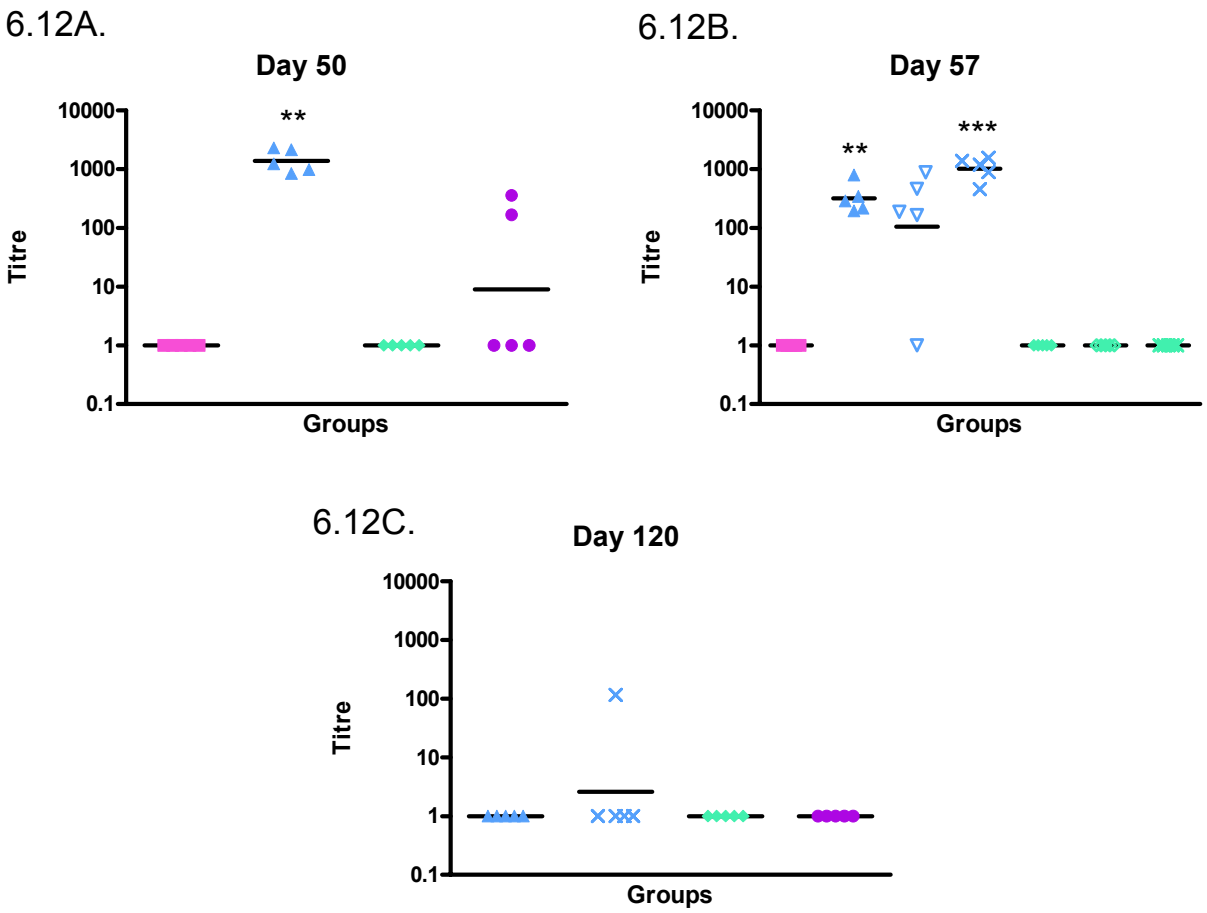


6.3.2.2.3 Serum IgA responses.

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

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

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

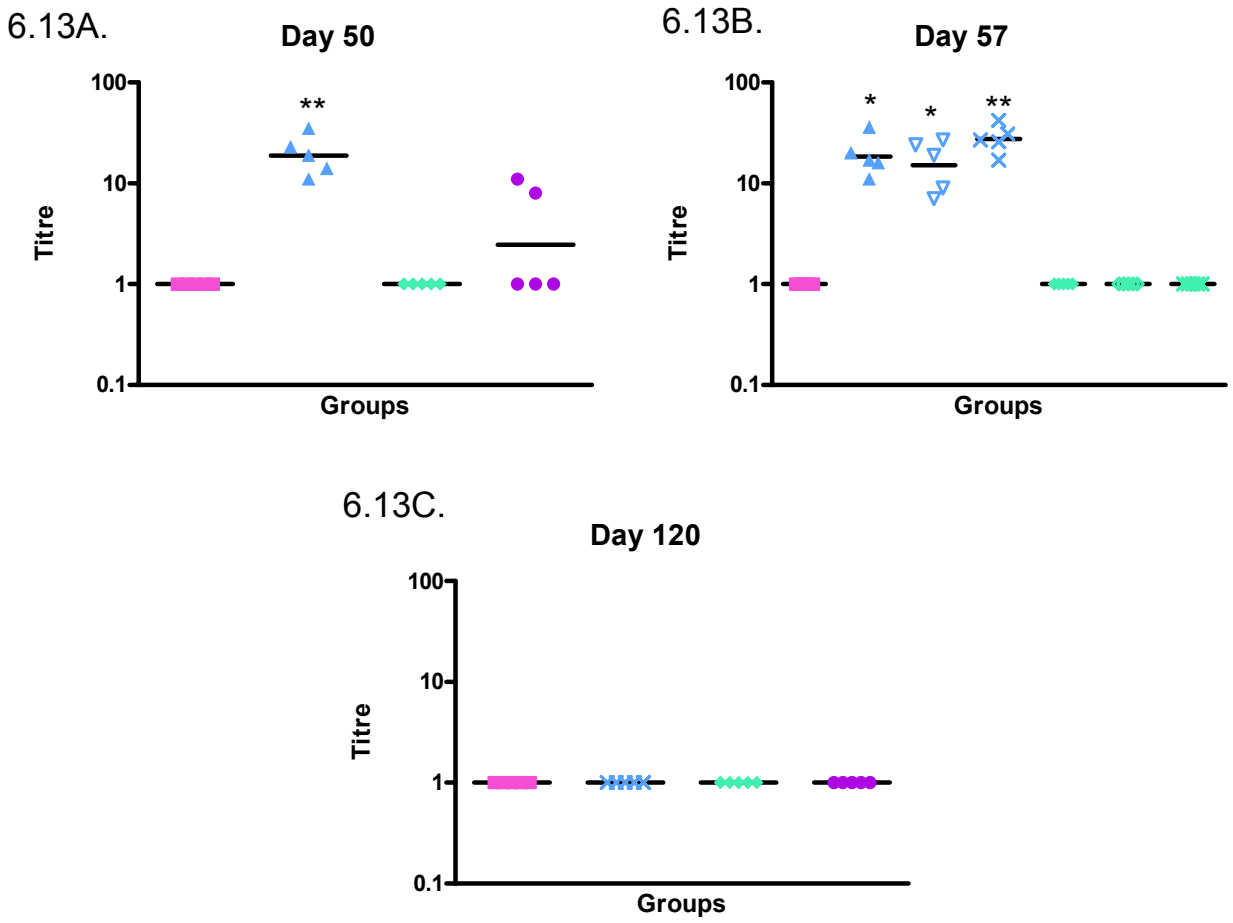


6.3.2.2.4 Mucosal immune responses.

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

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

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



6.3.2.3 Evaluation of cytokine responses after recombinant *Salmonella* vaccination

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

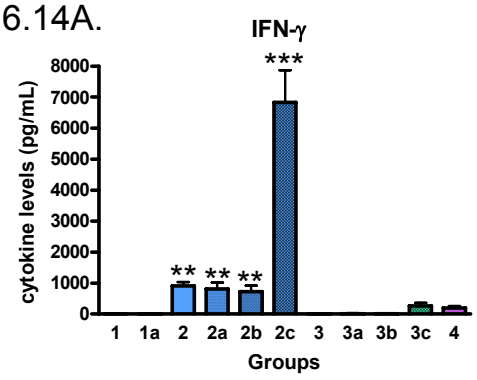
To analyse the T-helper type of immune response induced in the different groups of immunised mice, I evaluated the cytokines secreted in cell culture supernatants derived from splenocytes after *in vitro* restimulation with the specific antigen (purified Ag85B-ESAT6 protein). CBA was used to determine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

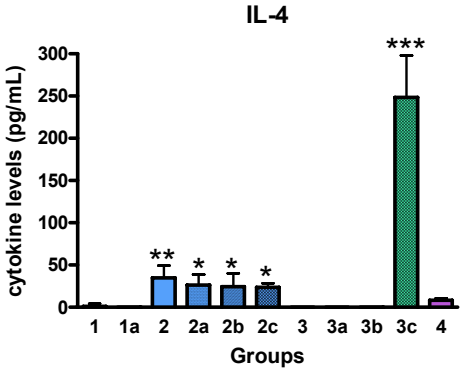
1a, 3, 3a, 3b and 3c) as well as the positive control group (group 4) on days 50 and 57. In fact, the increase in IFN-γ secretion by mice primed with SL3261mycolacZ and boosted with Ag85B-ESAT6 plus LTK63 (group 2c) was significantly higher when compared to those just immunised with SL3261mycolacZ alone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

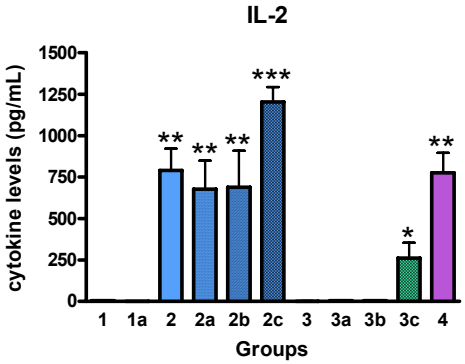
Figure 6.14: Ag85B-ESAT6-specific cytokine responses in mice immunised with *Salmonella* live vector vaccines.

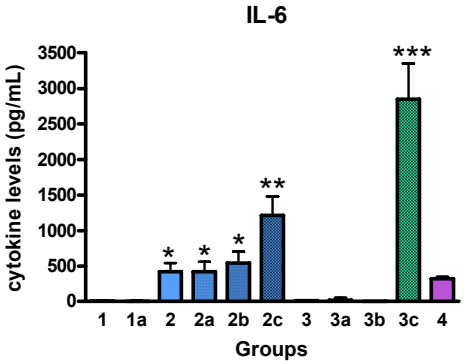
(A) Mice were immunised as described in Table 6.5 with spleens harvested on day 50 and 57, (C) and 120. Cytokine responses were measured upon *in vitro* stimulation with Ag85B-ESAT6 for 36-42 hours. (B) Cells were also stimulated with conA (positive control) and $RPMI^+$ media (negative control). Columns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

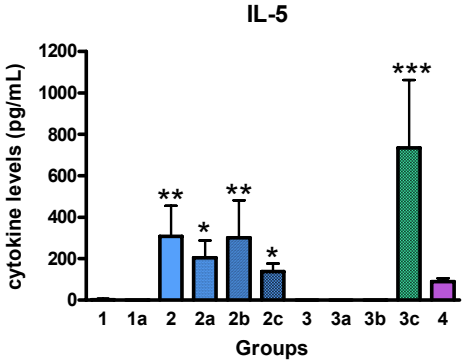
6.14A.


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

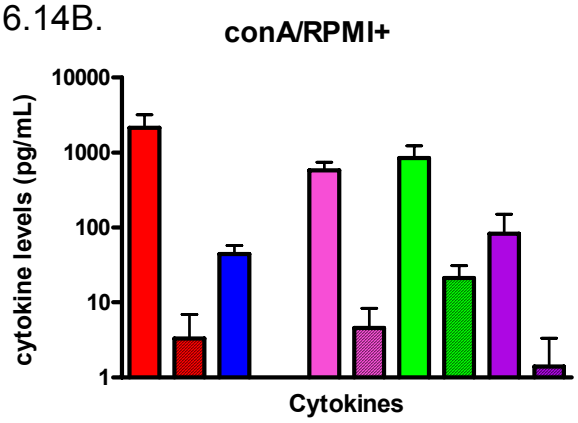

IL-4
cytokine levels (pg/mL)
300
250
200
150
100
50
0
1 1a 2 2a 2b 2c 3 3a 3b 3c 4
Groups


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

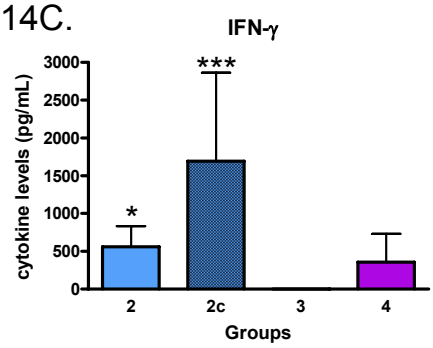

IL-6
cytokine levels (pg/mL)
3500
3000
2500
2000
1500
1000
500
0
1 1a 2 2a 2b 2c 3 3a 3b 3c 4
Groups

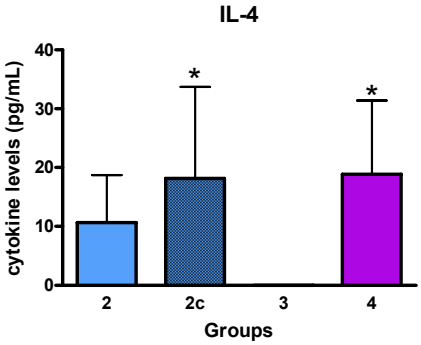

IL-5
cytokine levels (pg/mL)
1200
1000
800
600
400
200
0
1 1a 2 2a 2b 2c 3 3a 3b 3c 4
Groups

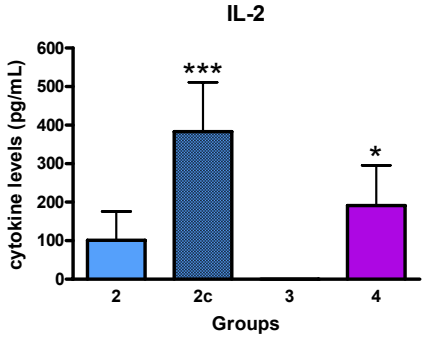
6.14B.

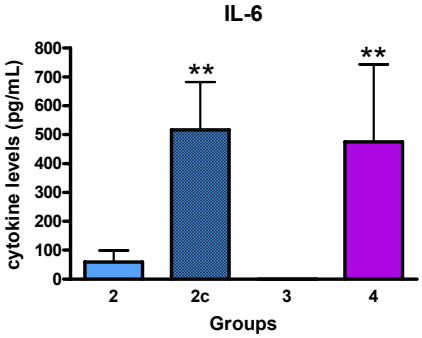

conA/RPMI+
cytokine levels (pg/mL)
10000
1000
100
10
1
Cytokines

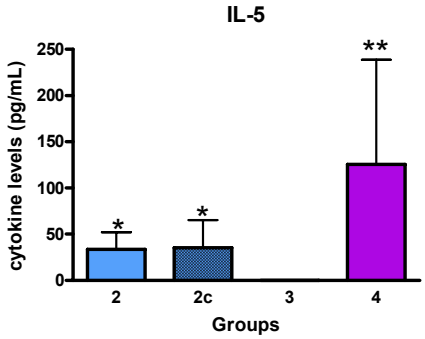
6.14C.


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


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


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


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

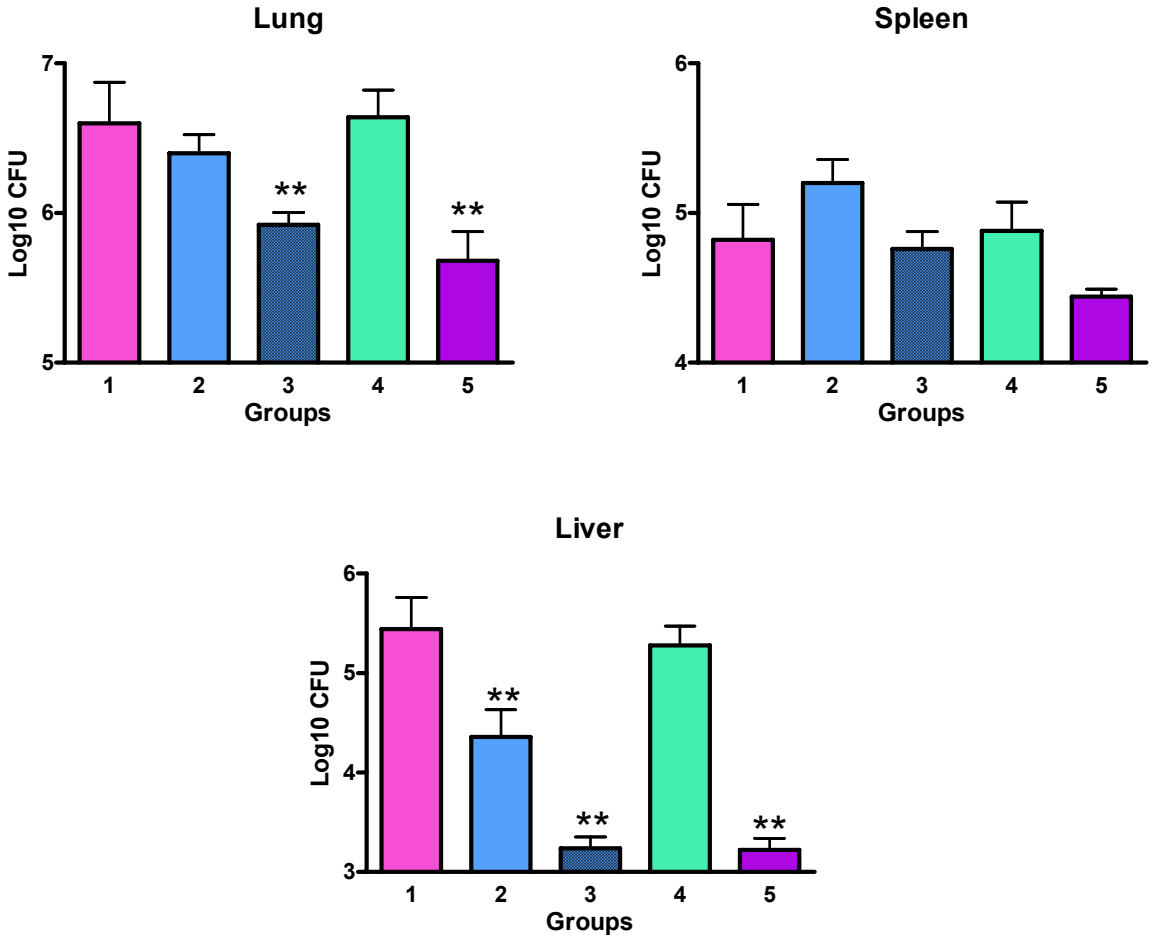

IL-5
cytokine levels (pg/mL)
250
200
150
100
50
0
*
*
**
2
2c
3
4
Groups

6.3.2.4 Protective efficacy of the recombinant *Salmonella* vaccine candidate

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

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

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



6.3.3 Discussion

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

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

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

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

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

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

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

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

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

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

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

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