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

3.1 Introduction

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

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

3.1.1 The tuberculosis fusion antigen, Ag85B-ESAT6

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

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

3.1.2 Intranasal immunisation and *M. tuberculosis*

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

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

3.2 Results

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

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

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

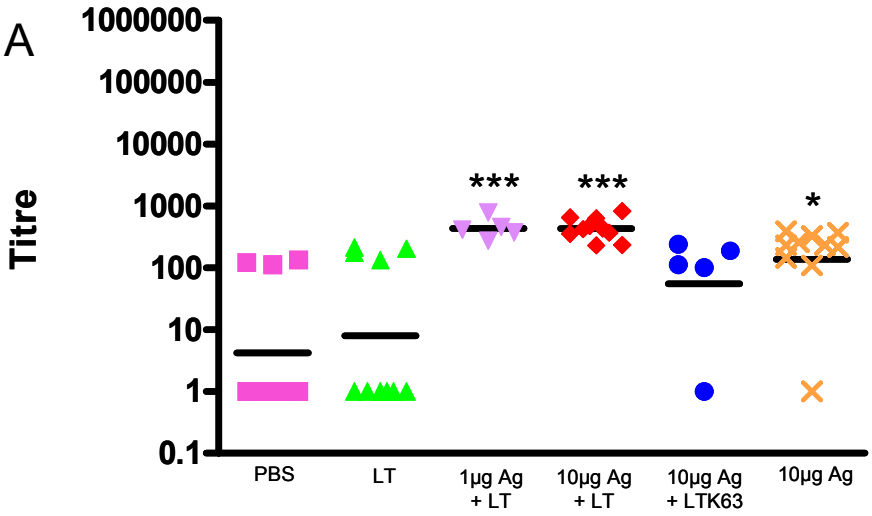
Day	Group	Immunised antigen	Procedure	Route
0	1	naïve	immunisation	i.n.
	2	1µg LT		i.n.
	3	1µg Ag85B-ESAT6 + 1µg LT		i.n.
	4	10µg Ag85B-ESAT6 + 1µg LT		i.n.
	5	10µg Ag85B-ESAT6 + 10µg LTK63		i.n.
	6	10µg Ag85B-ESAT6		i.n.
21	All groups		sample bleed	
28	1	naïve	boost	i.n.
	2	1µg LT		i.n.
	3	1µg Ag85B-ESAT6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

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

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

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

3.1A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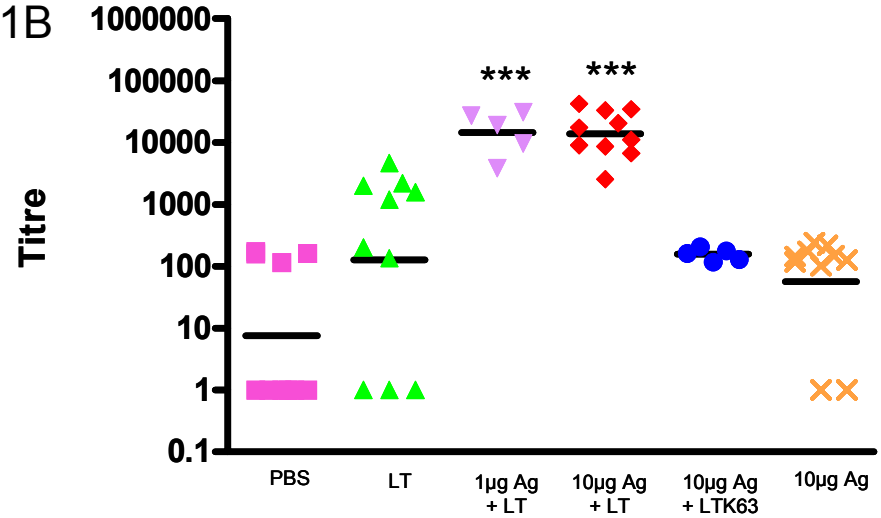
3.1B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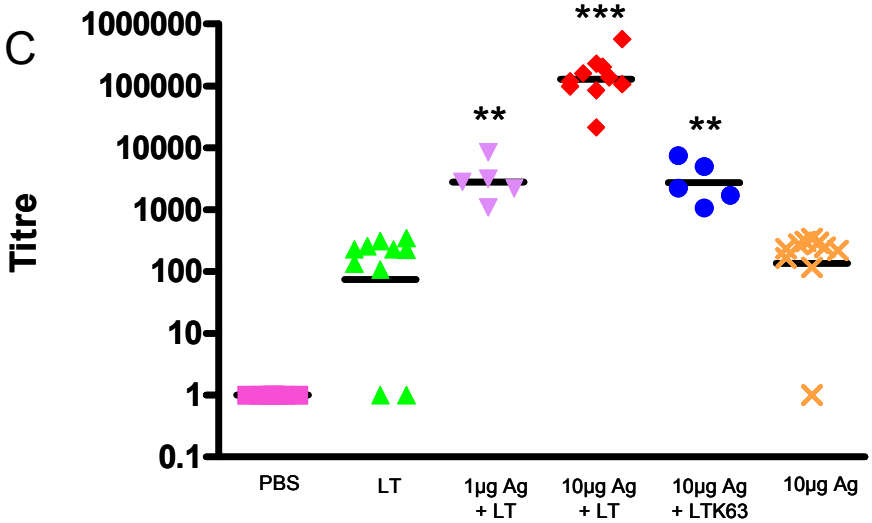
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PBS
LT
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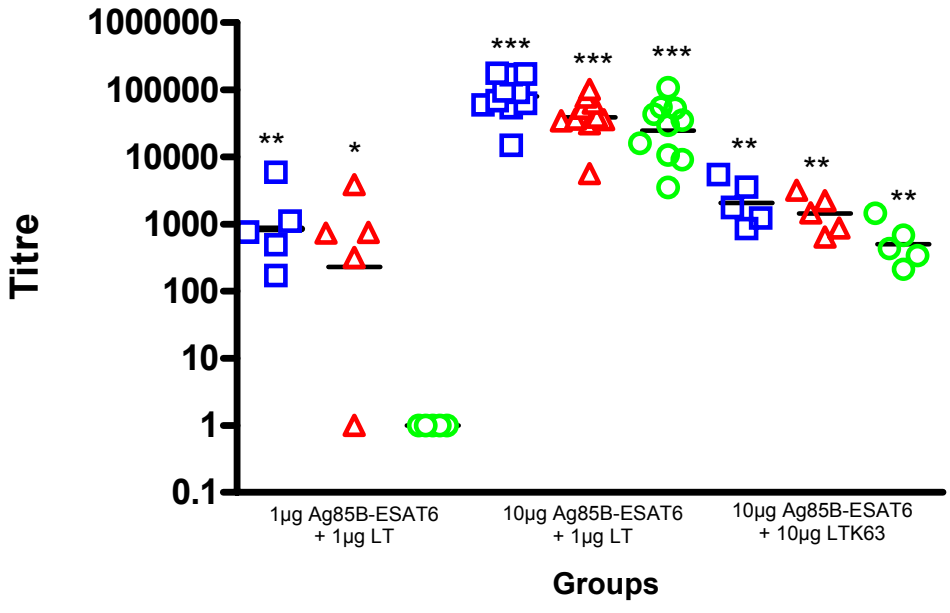
10µg Ag + LT
10µg Ag + LTK63
10µg Ag

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

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

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

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



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

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

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

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

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

3.2.4 Strong pro-inflammatory cytokine responses are induced following immunisation

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

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

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

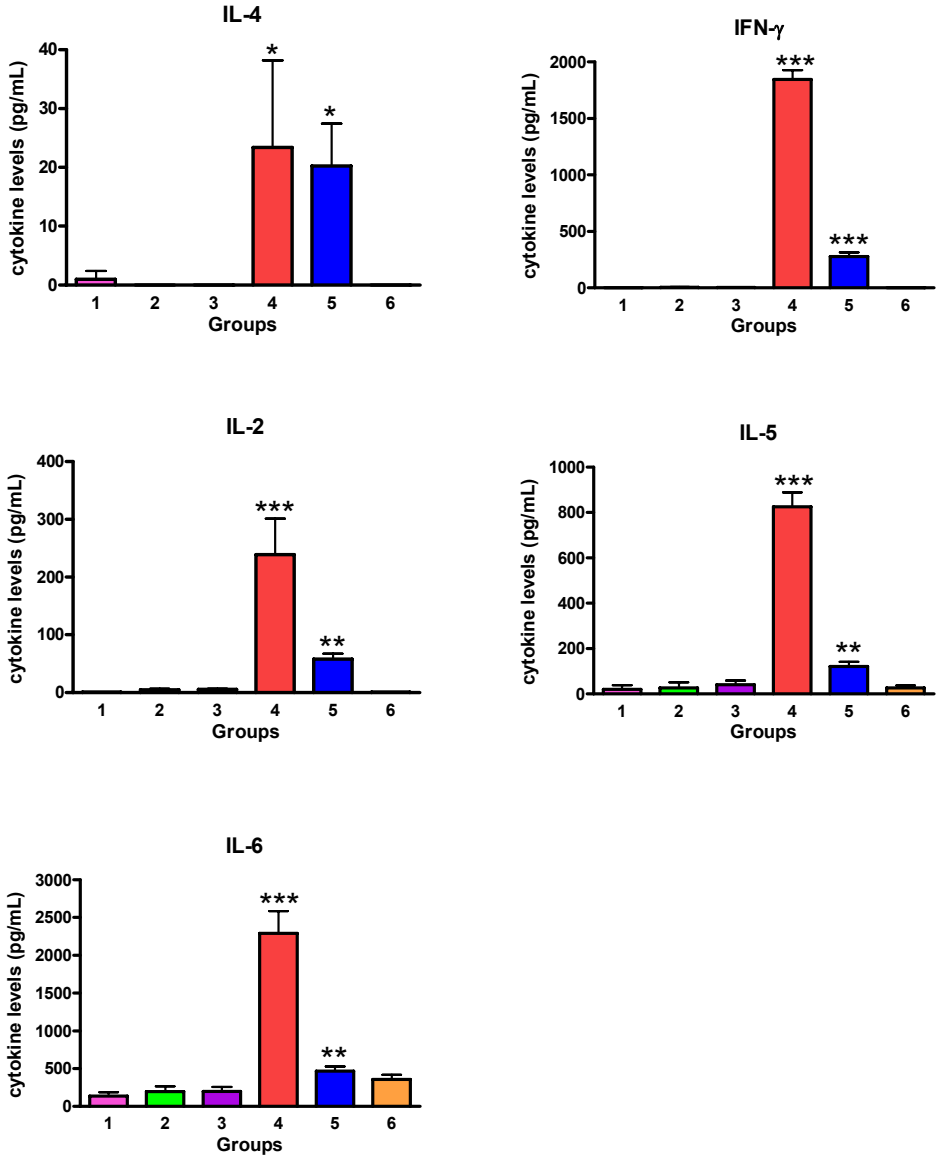
Supernatants from stimulated splenocytes of immunised Balb/c mice (see Table 3.1 for details) were analysed for cytokine levels using CBA after 24-48 hours. Stimulants used in the T-cell assay included Ag85B-ESAT6, ESAT-6, Ag85B peptide, and the positive control as concavalinA (all at 5µg/mL) and, the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

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

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

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

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



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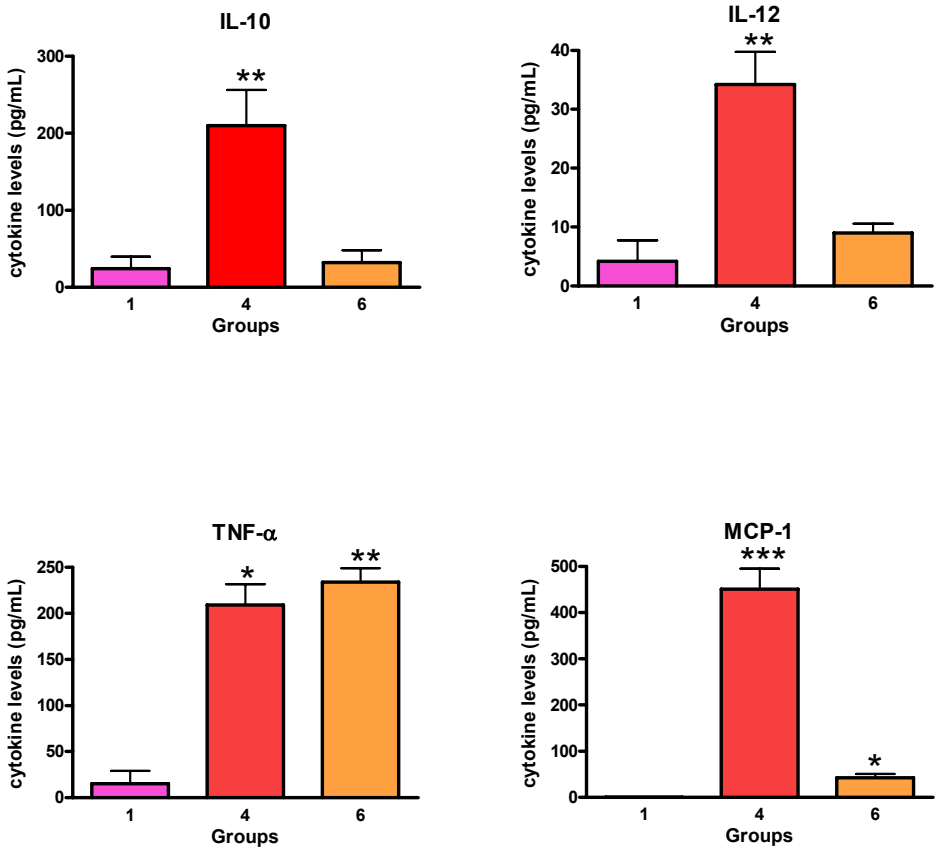

3.3 Discussion

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

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

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

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

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

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

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

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

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

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