2 Materials and Methods

2.1 Materials

2.1.1 Reagents

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

2.1.2 Bacterial strains

Table 2.1: Bacterial strains used during study

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

2.1.3 Plasmid vectors

Table 2.2: Plasmid vectors used during study

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

2.1.4 Oligonucleotide primers

Table 2.3: Details of primers used during study

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

2.1.5 Proteins for immunisations

Table 2.4: Proteins used in immunisations

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

2.1.6 ELISA antibodies

Table 2.5: Antibodies used for ELISAs

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

2.1.7 Immunofluorescence antibodies

Table 2.6: Antibodies for Immunofluorescence staining

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

2.1.8 Antibodies for FACS

Table 2.7: Antibodies for FACS analysis

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

2.1.9 Mice

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

2.2 Methods

2.2.1 Bacterial growth conditions

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

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

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

1.2.1.2 Growth conditions for *M. tuberculosis*

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

2.2.2 Animal methods

2.2.2.1 Preparation for inoculation/immunisation

2.2.2.1.1 Oral

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

2.2.2.1.2 Intranasal

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

2.2.2.1.3 Intraperitoneal

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

2.2.2.1.4 Intravenous

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

2.2.2.1.5 BCG immunisation

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

2.2.2.2 Experimental *M. tuberculosis* infections

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

2.2.2.3 Determination of pathogen burden

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

2.2.2.4 Natural Killer Cells (NKC) depletion

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

2.2.3 Molecular Methods

2.2.3.1 Plasmid DNA extraction

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

2.2.3.2 Agarose gel electrophoresis

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

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

2.2.3.3 DNA digestion

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

2.2.3.4 DNA ligation

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

2.2.3.5 Oligonucleotides

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

2.2.3.6 Polymerase chain reaction (PCR)

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

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

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

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

Table 2.8: PCR Mixture for Platinum SuperMix (Invitrogen)

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

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

2.2.3.6.1 Characterisation of PCR products

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

2.2.3.7 Precipitation of DNA

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

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

2.2.3.8 DNA sequencing

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

2.2.3.9 Preparation of electrocompetent cells

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

2.2.3.10 Electroporation

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

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

2.2.3.11 Identification of bacteria

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

2.2.3.12 Red Recombinase "one step" mutagenesis

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

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

2.2.3.13 Transduction of *Salmonella* with P22 phage

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

2.2.4 ELISA methods

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

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

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

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

2.2.4.3 ELISA for IgA in sera and lung and nasal washes

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

2.2.5 Measurement of cellular responses

2.2.5.1 Isolation of lymphocytes

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

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

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

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

2.2.5.3 Lymphocyte preparation for FACS

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

2.2.6 Protein overexpression

2.2.6.1 Protein gel separation

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

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

2.2.6.2 Western blot analysis

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

2.2.6.3 Coomassie blue stain

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

2.2.6.4 Colony blot analysis

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

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

2.2.6.5 Opti-4CN stain (Bio-Rad)

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

2.3 Tissue staining methods

2.3.1 Sectioning

2.3.1.1 NALT removal

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

2.3.1.2 Frozen sectioning

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

2.3.2 Immunofluorescent staining

2.3.2.1 Single immunofluorescent staining

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

2.3.2.2 Double or triple immunofluorescent staining

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

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

2.3.3 Histology

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

2.4 Statistical analysis

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