Analysis of the CD4+ T cell immune response to Anthrax Lethal Factor

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Abstract

The Gram-positive bacterium *Bacillus anthracis* is the causative agent of the potentially fatal illness, anthrax. A major determinant of *B. anthracis* pathogenicity is a binary toxin composed of Protective Antigen (PA) and one of two subunits; Lethal Factor (LF) or Edema Factor (EF). Translocation by PA into the host cell cytosol allows LF, which is a zinc metalloprotease, to inactivate the mitogen-activated protein kinase (MAPK) pathway. The expected consequences for the immune response include subversion of antigen presentation and T cell priming. In contrast to this, long term CD4+ T cell responses to LF were identified following natural human anthrax infection and vaccination, indicating that this toxin component is a principle *B. anthracis* antigen which may confer protective immunity. It has been observed that polymorphism in the HLA class II alleles at the DR and DQ loci affects susceptibilty to infectious disease outcome. In order to map the human response within a defined genetic background, transgenic mice expressing individual HLA heterodimers in the absence of endogenous MHC class II were utilised. HLA-DR1, HLA-DR15, HLA-DR4, HLA-DQ8 and HLA-DQ6 (the latter generated as part of this project) transgenic mice were compared in terms of response magnitude to LF and HLA expression levels. This was correlated with survival following live anthrax challenge. Immunodominant epitopes within LF were elucidated for all HLA-transgenic lines. Immunogenicity in the transgenic model was shown to be primarily restricted to epitopes from domains II and IV. Dominant epitopes, which were common to all HLA types, were identified in domain II. HLA-DR specific epitopes were also identified. T cell responses to cryptic epitopes, revealed following immunisation with the individual peptides, were compared to the immunodominant epitope hierarchy. A peptide epitope of LF was identified with a strong

relative binding affinity for HLA-DR15, making it a candidate therapeutic for ‘MHC blockade’ strategies. This epitope was tested for therapeutic blockade in a humanised transgenic mouse model which develops spontaneous paralysis with central nervous system (CNS) pathology similar to human multiple sclerosis (MS). Disease is characterised by an autoimmune response to a DR15 restricted epitope of myelin basic protein (MBP). The LF peptide was found to prevent induction of antigen-specific T cell responses, presumably by HLA class II, reducing the inflammatory response to self-peptide both *in vitro* and *in vivo.*

Abbreviations

BLS	Bare Lymphocyte Syndrome
bp	Base pairs
B2m	β2-microglobulin
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium
CMG2	Capillary Morphogenesis Gene 2
CD	Cluster of Differentiation
ConA	Concavalin A
CNS	Central Nervous System
EF	Edema Factor
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Hprt1	Hypoxanthine phosphoribosyltransferase 1
HAT	Hypoxanthine Aminopterin Thymidine medium
HBSS	Hank's Buffered Salt Solution
HT	Hypoxanthine Thymidine medium
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
LF	Lethal Factor
MAPK	Mitogen Activated Protein Kinase

MBP	Myelin Basic Protein
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
MS	Multiple sclerosis
NFAT	Nuclear Factor of Activated T cells
NF- κβ	Nuclear Factor-κβ
PA	Protective Antigen
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
Rpl-13a	Ribosomal protein L-13a
rpm	Revolutions per minute
SFU	Spot forming unit
SEB	Staphylococcal Enterotoxin B
Sdha	Succinate dehydrogenase complex, subunit A
TLR	Toll Like Receptor
TAE	Tris-Acetate
TNF	Tumour Necrosis Factor
TEM8	Tumour Endothelial Marker 8
TMB	3,3,5,5-Tetra-methylbenzidine
Tbp	TATA binding protein
TfrC	Transferrin receptor C
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

1 Introduction

1.1 *Bacillus anthracis*

1.1.1 Microbiology and taxonomy

Bacillus anthracis is a gram positive bacteria which is the causative agent of the infectious disease anthrax (Duesbery and Vande Woude 1999a; Mourez 2005). *B. anthracis* bacteria are identified by their susceptibility to penicillin, gamma phage induced lysis, and non-motile behaviour, colonies of *B. anthracis*, grown on nutrient or blood agar are nonhemolytic and generally show a characteristic white or gray 'ground glass' appearance (Figure 1.1.1C) (HPA, 2010).

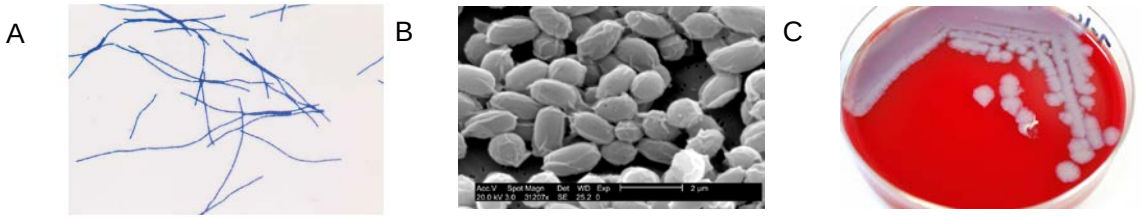


Figure 1.1.1 Microbiological identification of *B. anthracis.*
(A) Gram stain of long chained *B. anthracis* vegetative bacteria (Dixon *et al* 1999). (B) Scanning electron micrograph (SEM) at a magnification of 31,207X depicting spores of the Sterne strain, (C) *B. anthracis* colonies, grown on blood agar showing the classical 'ground-glass' morphology, both taken from the centers for disease control and prevention (CDC) public health image library (PHIL) (www.phil.cdc.gov).

However, phenotypic characteristics may vary between strains and haemolytic, motile, bacteria which are resistant to phage lysis, have been identified in *B. anthracis* isolates (Klee *et al* 2006; Marston *et al* 2006; Turnbull 1999). There have also been reported instances of *B. anthracis*

clinical isolates which have developed resistance to penicillin (Bradaric and Punda-Polic 1992; Lalitha and Thomas 1997). The bacteria are obligate pathogens, upon entry to host tissues anthrax spores germinate and replicate rapidly, forming chains of rod-shaped vegetative bacteria, 1 to 10 μm in length (Figure 1.1.1A) (Mock and Fouet 2001). Following exposure to oxygen the bacteria, which are facultive anaerobes, produce oval spores (Figure 1.1.1B), which are highly resistant to adverse environmental conditions (Mock and Fouet 2001).

B. anthracis belongs to the group one or *Bacillus cereus* group of bacteria, which contains seven related species; *B. anthracis*, *B. cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, and *Bacillus medusa* (Bavykin *et al* 2004; Read *et al* 2003). *B. anthracis*, *B. cereus*, and *B. thuringiensis* are genetically closely related species of medical and economic importance which exhibit different phenotypes; the insect pathogen *B. thuringiensis* carries plasmid genes encoding for crystal protein toxins, which have been widely exploited in agriculture as insecticidal agents (Schnepf *et al* 1998), while *B. cereus* is an opportunistic human pathogen frequently isolated as a food contaminant (Drobniewski 1993). The functional distinguishing characteristics of these bacilli lie in the plasmid genes; *B. cereus*, and *B. thuringiensis* demonstrate horizontal spread of these genes (Helgason *et al* 1998), the associated high level of genetic diversity and low clonality led Helgason to suggest that *B. cereus*, *B. anthracis* and *B. thuringiensis* should be grouped in the same species (Helgason *et al* 2000; Read *et al* 2003). However, in contrast to the other members of the *B. cereus* group, *B. anthracis* is highly monomorphic, showing low levels of genetic diversity, which necessitates detailed analysis to discriminate between *B. anthracis* isolates (Keim *et al* 1997; Keim *et al* 2000; Radnedge *et al* 2003). This high level of clonality is presumably related to the episodic

nature of the *B. anthracis* reproductive cycle, as bacterial cells proliferate rapidly, then sporulate upon the death of the host, remaining quiescent in the environment until entering the next host, resulting in limited opportunities for accumulating DNA mutations (Keim *et al* 2000).

Genome sequencing of single-nucleotide polymorphisms (SNPs), multiple locus sequence typing (MLST) and multiple locus variable number tandem repeat analysis (MLVA) have been used to elucidate the phylogenetic relationship between *B. anthracis* strains and other members of the *B. cereus* group (Pearson *et al* 2004; Van Ert *et al* 2007b). *B. anthracis* appears to have arisen from the *B. cereus* group relatively recently in the evolutionary past, with the acquisition of the two virulence plasmids, pXO1 and pXO2 (Keim *et al* 1997). Of the three major *B. anthracis* lineages, the A branch (which includes the Ames and Vollum strains), diverged during the mid-Holocene epoch, coinciding with an upsurge in the domestication of animals and human population movement (Van Ert *et al* 2007a). This may explain the greater diversity of strains within the A lineage, which shows a wider global dispersal than strains from the B and C branches, as the *B. anthracis* reproductive cycle mandates that a greater host density would drive accumulation of mutations, influencing the evolution of new *B. anthracis* species (Pearson *et al* 2004; Smith *et al* 2000; Van Ert *et al* 2007b).

1.1.2 Epidemiology

Anthrax is an acute zoonotic disease that affects primarily herbivorous mammals (Dixon *et al* 1999). Anthrax spores persist in many parts of the world, and anthrax infection is endemic or hyperendemic in a number of countries, particularly in Turkey, much of sub-Saharan Africa, Southern Asia and much of South America (Figure 1.1.2). Natural occurring cases of human

anthrax infection are usually confined to agricultural workers and individuals in contact with the skins and fibers of infected animals, which historically gave rise to the alternative names of woolsorters or ragpickers disease (Brachman 1980).

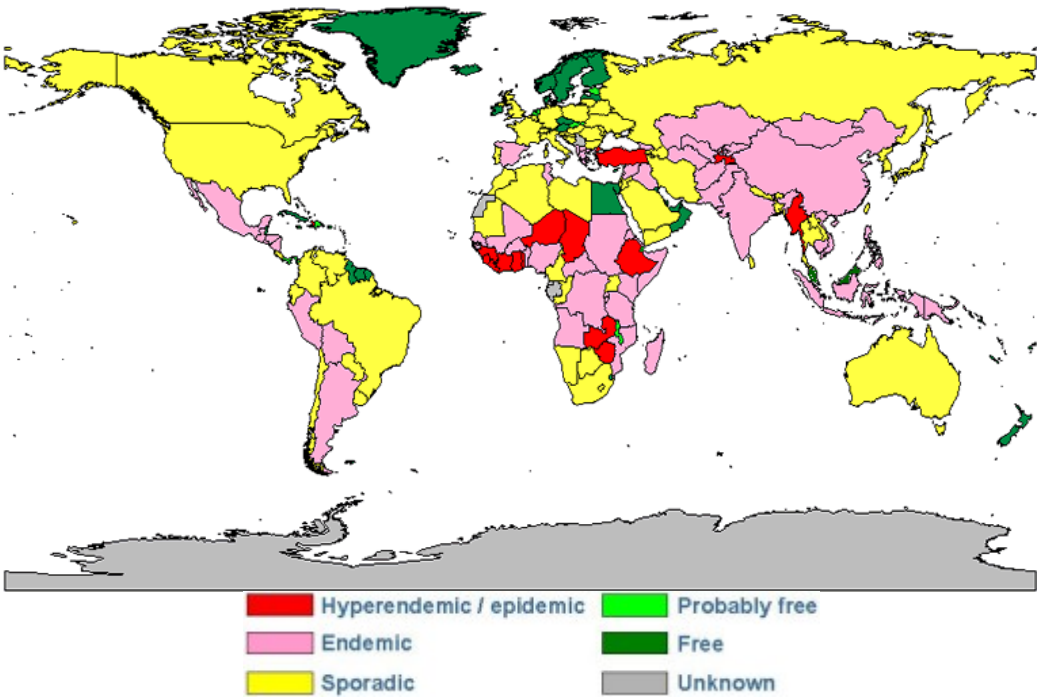


Figure 1.1.2 Geographical distribution of anthrax infection in humans and domesticated animals.
The disease is predominantly endemic or hyperendemic in developing countries. Taken from the World Anthrax Data Site (www.vetmed.lsu.edu/whocc/mp_world.htm).

Although outbreaks of anthrax in humans have been recorded throughout history (Dirckx 1981; Sternbach 2003), the causative agent was first identified in 1877 by Robert Koch. Examination of the anthrax lifecycle allowed Koch to identify the crucial role both the spore and bacilli played in the transmission and development of the disease. By inoculating healthy animals with anthrax bacilli cultured *in vitro*, Koch was able to induce disease, thereby developing the Koch's postulates germ theory regarding the transmission of infectious disease (Sternbach 2003). Further

evidence was subsequently provided by Louis Pasteur; in 1881 he protected cattle from challenge with a virulent strain of anthrax by inoculation with attenuated live anthrax bacteria, developing the first vaccine containing attenuated live organisms (Turnbull 1991).

During the twentieth century the focus of attention shifted from the importance of anthrax as an agricultural disease, which at one point during the eighteenth century killed approximately half of the sheep in Europe (Dirckx 1981), towards its potential as a biological weapon. In addition to agents such as *Francisella tularensis*, *Yersinia pestis* and *Burkholderia pseudomallei*, *B. anthracis* was the focus of several countries biological weapons programs during the First and Second World Wars (Christopher *et al* 1997). The properties which make *B. anthracis* particularly attractive as a bioweapon include the high case fatality rate (Jernigan *et al* 2002; Meselson *et al* 1994), and the formation of spores which are resistant to drying, heat, and ultraviolet or ionising radiation (Mock and Fouet 2001). The physically resilient nature of the spores was illustrated by the aerosol release of anthrax on Gruinard Island near Scotland in 1942, which resulted in viable spores persisting in the environment until the island was decontaminated with formaldehyde in 1986 (Christopher *et al* 1997; Manchee *et al* 1983). Weaponisation of anthrax was further pursued by both American and Soviet scientists during the cold war (Christopher *et al* 1997). The high case fatality rate of anthrax infection was demonstrated in 1979, when the accidental aerosol release of *B. anthracis* spores from a military facility in Sverdlovsk in the Soviet Union resulted in an estimated 96 cases of inhalational anthrax, 64 of which were fatal (Meselson *et al* 1994).

More recently, concerns have surfaced regarding the use of *B. anthracis* as a bioterrorism agent (Henderson 1999). In 1993, the Aum Shinrikyo group attempted to disseminate anthrax spores in Tokyo, although analysis of the spores indicated that they belonged to a non-virulent Sterne 34F2 strain (Keim *et al* 2001). A more lethal incident occurred in 2001, when anthrax spores were deliberately disseminated in the United States mail, resulting in a total of 22 cases of anthrax, with 11 cases of cutaneous disease and 11 cases of inhalational anthrax (Jernigan *et al* 2002). The fatality rate associated with the different routes of infection was demonstrated by the survival of all 11 cutaneous cases, compared to a 45% fatality rate in patients with inhalational disease, despite appropriate antibiotic therapy (Guarner *et al* 2003; Jernigan *et al* 2002). The Federal Bureau of Investigation (FBI) suspect that these attacks were carried out by a US government researcher at Fort Detrick (Bhattacharjee and Enserink 2008; Enserink and Bhattacharjee 2008).

In 2009 and 2010 an outbreak of 31 cases of anthrax in heroin users was identified in Scotland and England, resulting in 11 deaths (Booth *et al* 2010). These cases represent an unusual aetiology, as rather than the classical cutaneous disease, the drug users presented with disease acquired through a route previously described as injectional, involving ‘skin-popping’ or subcutaneous infection, leading to soft-tissue oedema and septic shock (Booth *et al* 2010; Ringertz *et al* 2000). The source of the anthrax infection has yet to be determined, however it is suspected that the heroin was contaminated during the production or handling stage, as much of Europe’s heroin originates from regions in Pakistan, Afghanistan, and Iran, where anthrax is endemic (Ringertz *et al* 2000). These recent cases illustrate that anthrax still remains an environmental hazard in many parts of the developing world.

1.1.3 Clinical aetiology

Exposure to the endospores of *B. anthracis* is responsible for a range of disease symptoms, depending upon the point of entry to the body (Ascenzi *et al* 2002). The three recognized forms; inhalational, cutaneous and gastrointestinal anthrax, are each capable of progressing to lethal systemic infections, but all three are associated with widely varying fatality rates.

1.1.3.1 Cutaneous anthrax

Cutaneous anthrax represents the most prevalent form of anthrax, accounting for 95% of all anthrax infections in the United States (Brachman 1980; Dixon *et al* 1999). While most cases of cutaneous anthrax arise from physical exposure of spores to cuts or abrasions in the skin, mechanical transmission by biting arthropods has also been reported (Bradaric and Punda-Polic 1992; Turell and Knudson 1987). Following a variable incubation period of between 1 and 10 days, early skin lesions usually takes the form of a pruritic papule, progressing to an ulcerated vesicle complete with localized oedema (Bell *et al* 2002; Freedman *et al* 2002). The name of the disease is derived from the Greek word ‘anthrakis’ meaning ‘coal’, referring to the characteristic black eschar which forms as the skin surrounding the oedema becomes necrotic (Figure 1.1.3) (Mourez 2005; Spencer 2003; Sternbach 2003). Cutaneous anthrax is usually self-limiting, and lesions resolve without scarring (Bell *et al* 2002; Dixon *et al* 1999). Although infection is fatal in approximately 20% of untreated cases, appropriate antibiotic therapy reduces the risk of death to less than 1% (Spencer 2003).

A  B 

Figure 1.1.3 Cutaneous anthrax infection.
(A) Localised oedema, figure taken from (Freedman *et al* 2002) and (B) black eschars, figure taken from (Ascenzi *et al* 2002), are both characteristic of cutaneous anthrax infection.

1.1.3.2 Gastronintestinal anthrax

Gastronintestinal anthrax infections are relatively rare, and appear to follow the ingestion of spores or vegetative bacteria in contaminated meat (Beatty *et al* 2003; Sirisanthana and Brown 2002). The clinical presentation takes the form of either intestinal or oropharyngeal anthrax, and the incubation period for both forms is typically between 1 and 6 days (Beatty *et al* 2003). The orophapharyngeal form is characterized by an ulcerated oropharynx and regional lymphadenopathy and is associated with a mortality rate of approximately 13% (Sirisanthana *et al* 1984). The intestinal form of the disease is characterized by the ulceration and acute inflammation of the gastronintestinal tract (Abramova *et al* 1993); initial symptoms of nausea, vomiting and fever are followed by severe abdominal pain, hematemesis and bloody diarrhea which may progress to septicemia (Sirisanthana and Brown 2002). Without treatment, intestinal anthrax results in death in 25% to 60% of cases (Beatty *et al* 2003; Sirisanthana and Brown 2002).

1.1.3.3 Inhalational anthrax

Inhalation anthrax is characterised by a rapid onset of disease (Dixon *et al* 1999); following a prodromal phase of 2-5 days after exposure to the spores, the initial symptoms of mild fever, myalgia and non-productive cough progress to severe respiratory distress, necrotizing pneumonic lesions, septic shock, coagulopathy, widened mediastinum and in many cases, haemorrhagic infection of the thoracic lymph nodes (hemorrhagic mediastinitis) (Abramova *et al* 1993; Bell *et al* 2002; Guarner *et al* 2003; Jernigan *et al* 2001). Inhalation disease may also be associated with haemorrhagic meningitis which is characterized by the presence of blood in the cerebrospinal fluid (Abramova *et al* 1993; Ebrahimi *et al* 2009). Inhalational anthrax is the most severe form of the disease and can result in fatality rates approaching 100% if it is not treated appropriately (Jernigan *et al* 2002; Meselson *et al* 1994). The relatively low mortality rate of 45% in the United States during the 2001 outbreak was attributed to rapid diagnosis and aggressive antibiotic therapy (Jernigan *et al* 2001).

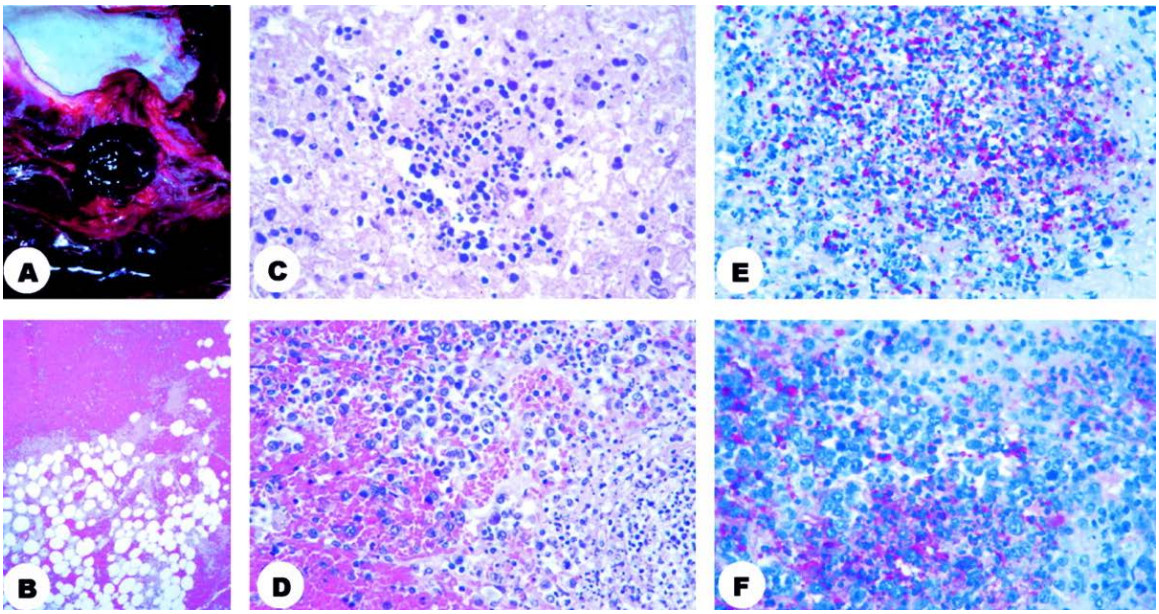


Figure 1.1.4 Tissue damage inflicted upon human hosts during the course of inhalational anthrax infection.

The mediastinal compartment (A) contains enlarged, hemorrhagic lymph nodes. A micrograph of the mediastinal tissues (B) shows edematous, hemorrhagic soft tissue damage, whilst micrographs of the mediastinal lymph nodes show pyknotic nuclei involved in (C) necrosis and (D) hemorrhage. Associated with the host tissue damage, micrographs of the mediastinal lymph nodes also show (E) abundant fragments of *B. anthracis* cell wall and (F) *B. anthracis* capsule. Original magnifications: (B) x5; (C), (D), (E), and (F) x40. Figure taken from (Guarner *et al* 2003).

1.1.4 Prophylaxis and treatment

Rapid treatment, especially of inhalational and gastrointestinal forms of anthrax, is critical to improving prognosis of patients. Prior to the advent of antibiotics, intravenous administration of anti-anthrax horse serum, arsenic derivatives such as neoarsphenamine and sulphonamides were all used in the treatment of anthrax (Baillie 2006) Current treatment focuses upon the administration of antibiotics acting as a central aspect of the chemotherapy, with Anthrax Immune Globulin Intravenous (Human) (AIGIV) (Pittman *et al* 2005) as an adjunctive therapy

directed against the anthrax toxins (HPS, 2010). Front-line antibiotics used in the treatment of anthrax include β-lactams (penicillin G), tetracyclines (doxycycline) and quinolones (ciprofloxacin) (Bell *et al* 2002; Inglesby *et al* 2002), although *B. anthracis* isolates have been identified which display resistance to these classes of antibiotics (Bradaric and Punda-Polic 1992; Chen *et al* 2004; Choe *et al* 2000; Pomerantsev *et al* 1992). Even after 30 days of continuous antibiotic treatment, infection can still occur due to the persistence of dormant spores, therefore the recommended treatment for inhalational anthrax involves 60 days of antibiotic therapy combined with post-exposure vaccination (Fellows *et al* 2001; Friedlander *et al* 1993b; Inglesby *et al* 2002; Meselson *et al* 1994).

Over the past forty years two anthrax vaccines have been widely used in humans. The United Kingdom licensed anthrax vaccine precipitated (AVP), and the US licensed anthrax vaccine adsorbed (AVA or Biothrax), are derived from cell-free culture filtrates of *B. anthracis* (Baillie 2006; Brey 2005; Leppla *et al* 2002). The AVA vaccine is produced from a filtrate of the nonencapsulated, nonproteolytic strain V770-NP1-R, which is adsorbed onto aluminium hydroxide (Baillie 2001). The UK vaccine, is produced from an alum precipitated filtrate of the avirulent, nonencapsulated Sterne strain 34F2 (Baillie 2006; Brey 2005). Pre-exposure vaccination has been proven to offer protection from virulent strains of *B. anthracis* (Fellows *et al* 2001; Pitt *et al* 1999), although post-exposure vaccination was only effective in combination with antibiotic therapy (Friedlander *et al* 1993b).

Protective immunity is induced by administering the vaccines subcutaneously, in the case of AVA as six initial doses at 0, 2, and 4 weeks and 6, 12 and 18 months (Brey 2005; Leppla *et al*

2002), while AVP is administered as a primary series of four vaccinations, at 0, 3, 6 and 32 weeks (Baillie *et al* 1999), annual booster vaccinations are then required for both vaccines. This extensive vaccination regimen, in combination with reported adverse reaction rates of 11% for the UK vaccine (Enstone *et al* 2003), and up to 60% for the US vaccine (Brey 2005), has prompted the design of more effective and safe sub-unit based vaccines (Baillie 2006). Second generation anthrax vaccines currently under development are based upon the administration of the immunogenic anthrax toxins, specifically recombinant protective antigen (rPA). Human clinical trials have proven that these rPA based vaccines are capable of eliciting robust cellular and humoral immune responses, whilst avoiding the adverse reactions associated with older filtrate based vaccines (Brown *et al* 2010; Campbell *et al* 2007; Gorse *et al* 2006).

In addition to delivering the purified toxin, produced in an *Escherichia coli* expression system, by intramuscular injection (Williamson *et al* 2005), the effectiveness of attentuated viral and bacterial expression systems and DNA vaccines to deliver subunit vaccines to the immune system have also been explored. The PA expressing *Salmonella enterica* serovar Typhimurium can induce protective immunity in mice against challenge with virulent anthrax strains (Garmory *et al* 2003; Osorio *et al* 2009). Expression of PA peptides by viral vectors based on influenza virus, rabies virus, and vaccinia virus, induced the production of neutralising antibody responses to PA (Langley *et al* 2010). A similar protective effect occurred with DNA vaccines, introducing genes encoding anthrax antigens such as PA and Lethal Factor (LF) into host cells, *in vivo* expression of these genes stimulated a protective immune response without the requirement for administration of protein or peptide antigens (Galloway and Baillie 2004).

Although DNA vaccines have been used to elicit protective immunity targeting other anthrax antigens, most notably the LF protein (Galloway *et al* 2004; Price *et al* 2001), the vast majority of approaches described to date have focused upon PA as the sole vaccine immunogen. The identification and characterisation of additional vaccine targets is an important stage in the development of rational anthrax vaccines.

1.2 Anthrax genome and toxins

1.2.1 Anthrax virulence factors

The main virulence factors of *B. anthracis* are encoded by genes found on two large plasmids (Gold *et al* 2004; Mourez 2005). The structural genes which code for toxin production are carried on plasmid pXO1 (184.5 kbp) (Gen Bank accession number NC001496) and the formation of a poly-γ-D-glutamic acid capsule, is directed by genes found on plasmid pXO2 (95.3 kbp) (Gen Bank accession number NC 002146) (Duesbery and Vande Woude 1999a). Three of the genes found on pXO1; pagA, lef and cya, are responsible for encoding the three toxins associated with pathogenicity of *B. anthracis*; Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF) respectively (Koehler *et al* 1994; Okinaka *et al* 1999). The pXO1 plasmid also encodes the *pagR* repressor and the *atxA* gene, which acts as a transcriptional regulator of the toxins; all of these genes are located in a 44.8 kbp pathogenicity island, which is flanked by two inverted repeat elements (Ivins *et al* 1986). Strains lacking either of the plasmids, such as the Pasteur (pXO1- pXO2+) or Sterne strains (pXO1+ pXO2-) have proven to be attenuated (Fouet *et al* 1994). The pXO1 plasmid appears to be stabilized by a plasmid encoded type 1 topoisomerase (Green *et al* 1985), as the spontaneous loss of the pXO2 plasmid occurs more readily than that of pXO1, in the absence of any apparent selective pressure (Makino *et al* 1989; Uchida *et al* 1993).

1.2.1.1 Poly-γ-D-glutamic acid capsule

The *capB*, *capC*, *capA*, *capD* and *capE* genes carried on the pXO2 plasmid encode the synthesis of membrane-associated enzymes, which govern the polymerization, transport and attachment of

the γ-linked D-glutamic acid residue polymers forming the anthrax capsule (Bourgogne *et al* 2003; Drysdale *et al* 2004). The *cap* genes are transcribed as a single operon under the control of the pXO2 transcriptional regulators *acpA* and *acpB*, expression of these two genes is in turn regulated by the pXO1 gene *atxA* (Uchida *et al* 1997). Although it is possible to mediate capsule synthesis in pXO1- pXO2+ strains by the over-expression of the *acpA* gene (Record and Wallis 1956), it appears that the expression of the *cap* operon is highly dependent upon the action of all three transcriptional regulators.

The capsule itself is composed of glutamic acid residues that are γ-linked to form large 215kDa homopolymers (Makino *et al* 1989). These polymers are poorly immunogenic, and inhibit phagocytosis of the bacteria by host macrophages (Makino *et al* 2002; Uchida *et al* 1993). The *capD* gene, which is a homologue of the *dep* gene found in *B. subtillis*, is capable of depolymerisation of the capsule, releasing low molecular weight glutamic acid polymers into the extracellular milieu (Makino *et al* 2002). These peptide fragments appear to have a role in evasion of the immune system, as strains lacking the *capD* gene are attenuated, and virulence can be restored by co-injection of the glutamic acid polymers (Guidi-Rontani *et al* 1999a). The products of the *cap* genes therefore represent important virulence factors, which are required for the dissemination of the bacilli into host tissues.

1.2.1.2 Germination operon

In addition to the genes which code for toxin production, the pathogenicity island on the pXO1 plasmid also contains a tricistronic operon, *gerX*, which lies between the *pagA* and *atxA* genes, and is comprised of, *gerXB, gerXA* and *gerXC* (Guidi-Rontani *et al* 1999a). These genes are

transcribed approximately 3 hours after the initiation of sporulation, and encode three proteins; GerXB, GerXA and GerXC, which show sequence similarities to germination proteins found in *B. subtilis*, *B. cereus* and *B. megaterium* (Guidi-Rontani *et al* 1999b). The conserved nature of these proteins across the *Bacillus* species, in addition to the diminished germination rate in strains lacking the *gerX* operon (Popova *et al* 2006), indicates the importance of these genes in regulating *B. anthracis* spore germination.

1.2.1.3 Secondary virulence factors

In addition to the toxins; PA, LF, EF and the capsule, other less well characterized virulence or virulence enhancing factors may contribute towards the bacterial pathogenesis of *B. anthracis*. Several *B. anthracis* haemolytic factors have been identified as potent accelerators of syndecan ectodomain shedding, which disrupts cellular endothelial integrity, leading to oedema and haemorrhage (Klichko *et al* 2003). These factors include; anthralysin O (AnlO) (a pore-forming cholesterol-dependent hemolysin), anthralysin B (AnlB) (a sphingomyelienase) and anthralysin A (AnlA) (a phosphatidyl choline-preferring phospholipase C) (Klichko *et al* 2003; Read *et al* 2003). The AnlO and AnlB proteins show structural similarity to *Staphylococcus aureus* and *Pseudomonas aeruginosa* virulence factors which induce ectodomain shedding, whilst AnlA shows homology to the *B. cereus* cereolysin A (ClnA) (Popova *et al* 2006). The action of LF upon MAPK signalling pathways has also proven capable of stimulating syndecan shedding, but at a much lower level compared to the AnlO, AnlB and AnlA hemolysins (Abramova *et al* 1993; Guarner *et al* 2003).

Disseminated intravascular coagulopathy (DIC) is a feature of anthrax infection which involves depletion of coagulation factors, disregulated haemostasis, a decrease in the levels of circulating thrombocytes, activation of the fibrinolytic pathways and haemorrhaging (Ezzel and Wilhelmsen 1993; Himsworth and Argue 2009). This massive haemostatic imbalance may explain the characteristically impaired blood clotting seen after death in anthrax infection (Alam *et al* 2006). Both LF and EF have been implicated in this process; EF is capable of interfering with thrombin induced platelet aggregation (Kau *et al* 2005), while LF is involved in the downregulation of arachidonic acid induced platelet aggregation (Chung *et al* 2008b; Chung *et al* 2006). However, the anthrax metalloproteases Npr599 (a thermolysin like enzyme) and InhA1 (which shows sequence homology with the *B. thuringiensis* immune inhibitor A) have also been found to play a major role in the process, acting upon plasma proteins and extracellular matrix molecules, specifically von Willebrand factor (VWF) and its endogenous regulator; a disintegrin and metalloproteinase with thrombospondin motifs 13 (ADAMTS13), which are key mediators of platelet recruitment (Chung *et al* 2009). InhA1 is also capable of inducing high levels of the acute phase protein (APP) Plasminogen activator inhibitor-1 (PAI-1) in the liver, which impairs fibrinolysis (Petosa *et al* 1997), further contributing to the coagulopathy seen during anthrax infection.

The Phospholipase C (PLC) family contains enzymes which are capable of cleaving the phosphate group from phospholipids; these enzymes are known to contribute to the pathogenesis of bacterial species, such as *L. monocytogenes* through interfering with cellular signaling, degrading tissues and mucous membranes and aiding in the bacterial evasion of phagocytic immune cells (Smith *et al* 1995). Early studies by Smith and Zwartouw demonstrated that *B.*

anthracis released phospholipases (Smith and Zwartouw 1956), however, it was only recently that the genes *plcB, smcA,* and *plcA* which respectively encode the *B. anthracis* phospholipases; phosphatidylcholine phospholipase C (PI-PLC), sphingomyelinase (SMase) and phosphatidylinositol-specific phospholipase C (PI-PLC) were identified (Read *et al* 2003). These PLC enzymes differ in their substrates; PC-PLC cleaves phosphatidylcholine, while SMase cleaves sphingomyelin and PI-PLC cleaves phosphatidylinositol, although all three contributed to macrophage associated growth of *B. anthracis* with a level of redundancy which indicated the importance of establishing infection in this cell type (Heffernan *et al* 2006). While PI-PLC alone was found to affect the ability of dendritic cells to respond to Toll Like Receptor (TLR) ligand stimulation through the cleavage of glycosylphosphatidylinositol (GPI) anchored proteins which are essential for dendritic cell activation (Zenewicz *et al* 2005).

1.2.1.4 PA

The mature 83 kDa PA protein, which consists of 735 amino acid residues is organised into four domains (Molloy *et al* 1992). The N-terminal domain I contains the furin recognition region, Arg^{164}-Lys^{165}-Lys^{166}-Arg^{167}; cleavage at this site by the host calcium dependent serine endoprotease, releases the smaller PA_{20} fragment (Petosa *et al* 1997). Cleavage of the PA_{83} protein appears to be essential for oligomerisation of the remaining PA_{63} monomers into heptamers, and association of the hydrophobic subdomain I with EF or LF (Mogridge *et al* 2001; Singh *et al* 1991). Domain III has a ferridoxin-like fold which plays a role in the oligomerisation of PA_{63} monomers (Petosa *et al* 1997). The main function of domain II involves pore formation, the large 2β2-2β3 loop arising this domain projects outward from each PA_{63} monomer making up the soluble heptamer bound to the cell surface, to form a transmembrane 14 stranded β-barrel

structure (Krantz *et al* 2006). Endocytosis of this complex is followed by acidification of the endosome, allowing bound LF and EF to translocate through the pore towards the neutral cytosol, driven by the transmembrane proton gradient (Varughese *et al* 1999). The carboxy terminus domain IV features a prominent immunoglobulin like fold and a flexible loop between the 4β8 and 4β9 strands of the domain which is required for PA binding to receptors on the host cell surface (Little *et al* 1988). Monoclonal antibodies which recognize the amino acid residues 679 to 693 of this site are capable of interfering with the binding of PA to host cells (Uerbach and Wright 1955). PA remains the most extensively characterized of the anthrax toxins, and was an early focus of work to identify an antigen which provided protective immunity to anthrax (Firoved *et al* 2005).

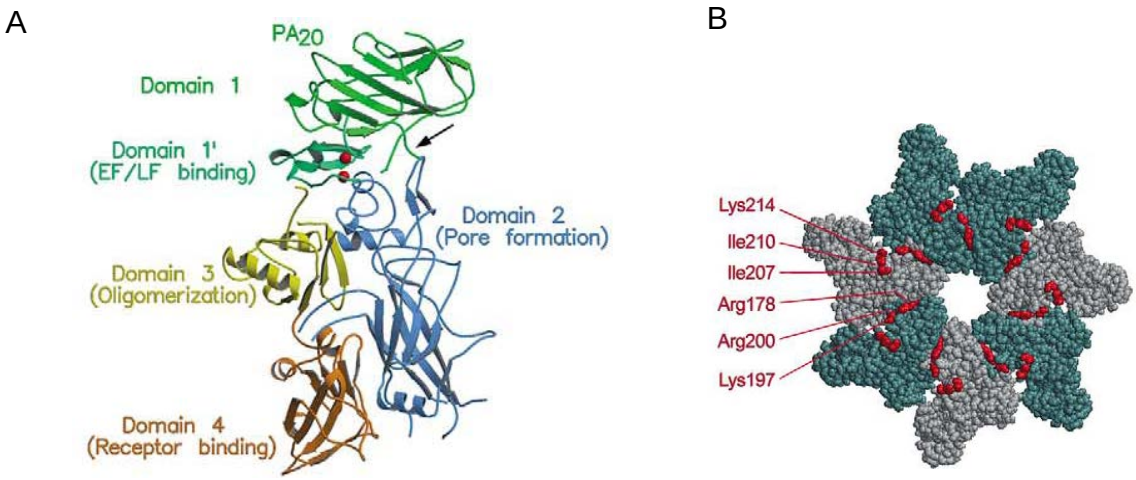


Figure 1.2.1 Crystal structure of the PA toxin.

The monomeric PA_{83} protein (A) is shown demonstrating the four domains, including the PA_{20} fragment and subdomain I, the proteolytic cleavage site separating these is marked with an arrow, Ca^{2+} ions are shown as red spheres. The heptameric PA pore structure (B) is shown with grey and green representation of the individual PA_{63} monomers, residues Arg^{178}, Lys^{197}, Arg^{200}, Ile^{207}, Ile^{210}, and Lys^{214}, which have been suggested to play a role in LF binding, are all shown Figures adapted from (Mourez *et al* 2002; Petosa *et al* 1997).

1.2.1.5 EF

EF is a 767 amino acid residue long 89kDa calcium and calmodulin (CaM) dependent adenylate cyclase, composed of three protein domains, responsible for converting ATP to cyclic AMP (cAMP), which regulates protein kinase A (PKA) activity (Drum *et al* 2002; Leppla 1982). The binding of four cAMP molecules to the regulatory subunits of PKA (PKAR) causes dissociation of the inactive PKA tetramer activating the remaining two catalytic subunits of PKA (PKAC) (Drum *et al* 2002; Leppla 1982). The elevation of intracellular cAMP interrupts osmotic homeostasis, resulting in fluid pooling in the extracellular spaces, which is responsible for the oedema observed in anthrax infected tissues (Maldonado-Arocho *et al* 2006; Xu *et al* 2007). Anthrax derived EF is notable for having a specific activity approximately 1000 fold higher than that of the endogenous adenylate cyclises found in mammalian cells (Leppla 1982). EF is also capable of upregulating the expression of anthrax toxin receptors upon the surface of host monocyte derived cells (Semenova *et al* 2007). This has interesting implications, as for more than 40 years the major vaccines used to protect against anthrax have been the AVP and AVA vaccines, which are filtered supernatant from the Stern strain of anthrax, comprising of PA with smaller amount of LF and EF (Baillie *et al* 1999). Repeated exposure to EF in the vaccine form might therefore be expected to increase anthrax toxin receptors expression, providing more sites for PA binding, which would result in an increased rate of toxin internalization during anthrax infection.

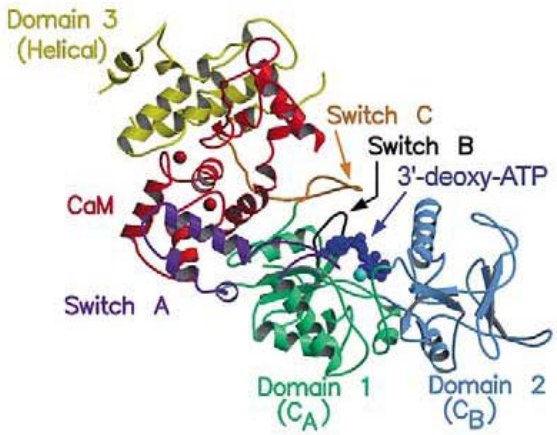


Figure 1.2.2 Crystal structure of the three globular domains which comprise the EF protein.
Ca^{2+} ions are shown as red spheres, also shown are switches A, B and C, which undergo large conformational changes upon CaM binding, and a non-cyclisable nucleotide analogue, 3'-deoxy-ATP (3'dATP) in the active site. Figure taken from (Ascenzi *et al* 2002).

1.2.1.6 LF

LF is a zinc dependent metalloprotease, composed of four domains which follow a predominantly helical structure (Pannifer *et al* 2001c). Following the AAAGGAG ribosome-binding sequence, the upstream ATG start codon begins a continuous 3631bp open reading frame which encodes the 809 amino acids composing the LF precursor protein (Gen Bank accession number M30210) (Bragg and Robertson 1989; Duesbery and Vande Woude 1999a). The first 99bp of the open reading frame encodes a 33 amino acid signal peptide, which is homologous to leader peptides for other proteins secreted by members of the *Bacillus* genus (Bragg and Robertson 1989). The first 300 amino acids of the α-helical domain I, of the 776 amino acid long mature LF protein, share extensive homology with that of the EF protein (Lacy *et al* 2002). In both EF and LF these amino terminal regions are critical for binding to the amino terminus subdomain I of the PA protein (Lacy *et al* 2002). Analysis of the LF binding motif has

confirmed that only the first 254 amino acids of LF are required for PA binding and translocation into the host cell (Arora and Leppla 1993).

The structure of the 90.2 kDa mature protein reveals a clear distinction between the PA binding NH_2 terminus and the catalytic COOH terminus (Pannifer *et al* 2001c). In contrast to the amino terminus, insertional mutagenesis performed in the carboxyl terminus, by Quinn *et al*, has confirmed that PA binding is unaffected, while toxicity is abrogated, indicating a role for this region in substrate catalysis (Quinn *et al* 1991).

The helix bundle which makes up domain III is inserted into domain II, and is believed to have arisen from a series of imperfect repeated duplications of a structural element comprising domain II (Ascenzi *et al* 2002). Domain III is characterised by the presence of the four repeats of the amino acids 293 to 300 of domain II, deletions in this region abrogated the catalytic activity of LF, indicating that this region is essential to the stability and function of the LF catalytic site (Quinn *et al* 1991). Domain II itself resembles the adenosine diphosphate (ADP)-ribosylating toxin, found in the *Bacillus cereus* vegetative insecticidal toxin 2 (VIP2), although its active site has undergone mutation (Pannifer *et al* 2001c). Mutagenesis studies indicate that several domain II residues; Leu^{253}, Lys^{254}, Arg^{491}, Leu^{514} and Asn^{516} are involved in substrate recognition and binding (Liang *et al* 2004). Along with domain III, the domain II protein appears to have become involved in substrate recognition and binding (Pannifer *et al* 2001c).

The final domain IV contains the zinc protease active site (Klimpel *et al* 1994). This domain is predominantly α-helical, with the exception of a 4-stranded β sheet, which is a characteristic of

other zinc proteases (Klimpel *et al* 1994; Pannifer *et al* 2001c). Analysis of the carboxyl terminus amino acid sequence revealed that the amino acid residues 686 to 692 (HEFGHAV) contained a classical binding motif conserved across the thermolysin-like metalloproteases (Klimpel *et al* 1994). The active site lies within a deep cleft formed by domains II, III and IV. A zinc ion is coordinated tetrahedrally to domain IV by three of the active site residues; Glu^{687} which acts as a ligand for a zinc bound catalytic water molecule, and the residues His^{686} and His^{690}, which directly coordinate the zinc ion itself (Hammond and Hanna 1998; Pannifer *et al* 2001c). Tonello *et al,* also suggested a role for Glu^{735}, which aids in the co-ordination of the zinc ion (Tonello *et al* 2004).

Whilst the majority of the active site residues share similarity with the thermolysin family of metalloproteases, an essential tyrosine residue has also been identified which is absent in the thermolysins. The domain IV residue Tyr^{728} has been implicated in catalysis, acting to protonate the departing amino terminal of the active site substrate (Pannifer *et al* 2001c; Tonello *et al* 2004). The Tyr^{728} position in LF shows similarity to the essential Tyrosine residue present in the clostridial metalloprotease neurotoxins, specifically the Tyr^{366} found in *Clostridium botulinum* neurotoxin type A (BTX-A) and the Tyr^{375} found in *Clostridium tetani* tetanus neurotoxin (TeNT) (Rigoni *et al* 2001; Rossetto *et al* 2001; Tonello *et al* 2004). The phenolic OH group of the domain IV Tyr^{728} residue, in addition to the conserved thermolysin-like binding motif appears to be critical to the catalytic action of LF upon the host substrate.

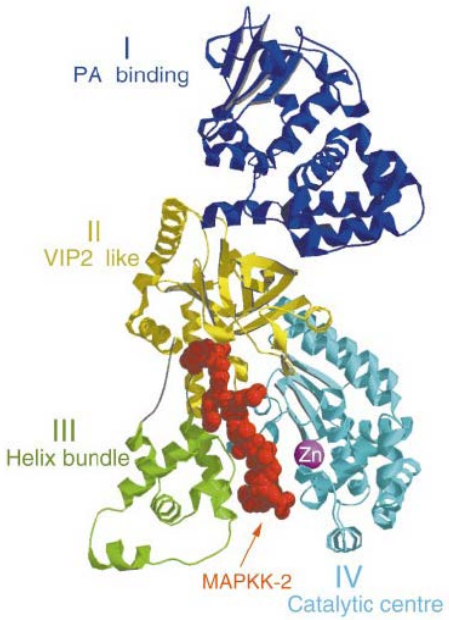


Figure 1.2.3 Stereo ribbon representation of the four domains which compose the LF protein.
The substrate (here shown as MAPKK-2) and Zn^{2+} ion are also pictured, lying within the active site cleft made by the groove between domains II and III, and the catalytic domain IV. Figure taken from (Chopra *et al* 2003)

Within the cell, LF exerts its toxic effect through the highly specific proteolytic cleavage of the amino terminal extension of Mitogen-Activated Protein Kinase Kinases (MAPKKs) or MKK family members (Duesbery *et al* 1998; Liu *et al* 2007b). This cleavage is performed by the catalytically active C terminal domain IV of the LF protein (Figure 1.2.3). MKKs are the central components in a three stage signaling cascade which is initially activated when the cell is stimulated by extracellular factors. In the case of anthrax infection this stimulus is reported to be the peptidoglycan found in the cell wall of *B. anthracis* (Liu *et al* 2007b), but may also include osmotic stress, pro-inflammatory cytokines and growth factors. This induces activation of the Mitogen-Activated Protein Kinase Kinase Kinases (MKKK or MEKK) family members. The most well characterised members of this family are MEKK1 and Raf (Lange-Carter *et al* 1993),

although members include; MEKK2, MEKK3, MEKK4 (Gerwins *et al* 1997; Hagemann and Blank 2001), tumor progression locus 2 (Tpl2) (Salmeron *et al* 1996), apoptosis signal regulating kinase 1 (Ask1) (Ichijo *et al* 1997) and TGFβ activated kinase (TAK1) (Yamaguchi *et al* 1995). The MEKK family members then phosphorylate the serine and threonine residues of MKKs; once activated these dual-specificity kinases are then responsible for the reversible dephosphorylation of both the serine and tyrosine residues on Mitogen Activated Protein Kinases (MAPKs) (Roux and Blenis 2004).

LF acts to disrupt the activity of three of the four families of MAPKs (Turk 2007); Extracellular Signal Regulated Kinases (ERK1 and 2, which are activated by MKKs 1 and 2, and are responsible for regulating cellular differentiation and proliferation) (Liu *et al* 2007b), c-Jun N-terminal kinases (JNK1, 2 and 3, which are activated by MKKs 4 and 7, and are known as Stress Activated Protein Kinases) (Liu *et al* 2007b), and the p38 isoforms (α, β, γ and δ which are activated by MKKs 3, 4 and 7 and are involved in regulating cell differentiation and apoptosis) (Roux and Blenis 2004) (Figure 1.2.4). Only the ERK5 (activated by MKK 5) signaling transduction pathway is not disrupted by the activity of LF within the cell (Duesbery and Vande Woude 1999a).

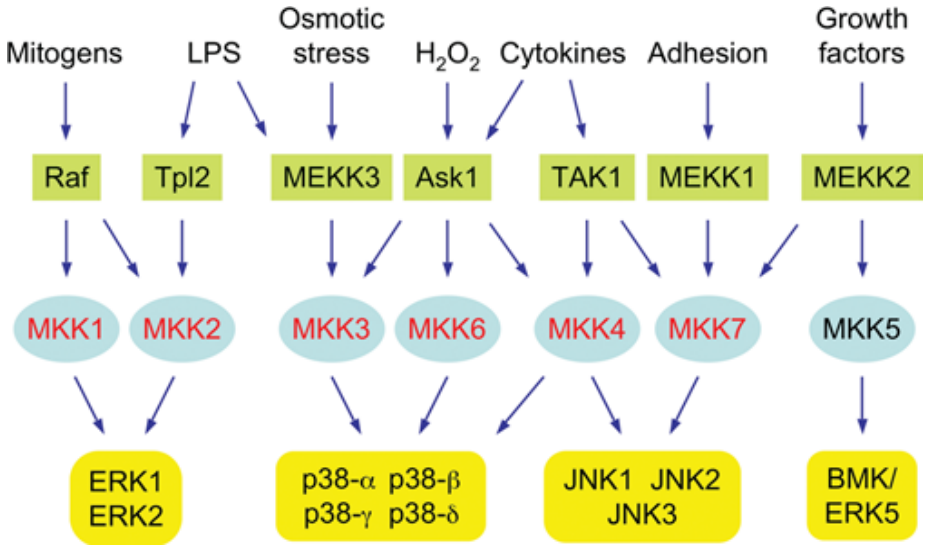


Figure 1.2.4 Mammalian MKKKs, MKKs, and their downstream MAPKs targets.
Various external stimuli induce the activation of specific MKKK signalling pathways. The MKK's targeted by LF, which are shown in red, affect the three of the four MAPK families; ERK1 and 2, the p38 isoforms and the JNKs. Only the MKK5 relationship with ERK5 or big MAPK (BMK) is unaffected by the action of LF. Figure taken from (Turk 2007).

LF acts to preferentially cleave proline residues (Hammond and Hanna 1998). Alignment of the amino acids which comprise the NH_2 terminal of the MKK families, demonstrate that they show a limited homology within the first 20 amino acids, centering around two proline residues, separated by one or two amino acids and preceded by a sequence of basic residues, (Chopra *et al* 2003; Duesbery and Vande Woude 1999b; Vitale *et al* 2000). Cleavage at these conserved proline rich regions, which precede the catalytic kinase domains, disrupts or removes a D-site (also known as a DEJL motif or D-domain), which is a protein interaction site, essential for high affinity binding to MAPKs (Vitale *et al* 2000). LF cleaves the D-sites of MKK1, MKK2, MKK3 and MKK6 once, while MKK4 is cleaved twice and MKK7 contains three putative cleavage sites (Bardwell *et al* 2004; Vitale *et al* 2000). Only the action of MKK5 is not disrupted by LF

cleavage, the reason for this may lie in the lack of homology the hydrophobic N-terminal domain of MKK5 shows to the other MKK D-sites; the MKK5 MAPK binding region instead consists of a stretch of negatively charged residues which are resistant to LF cleavage (Bardwell *et al* 2004; Seyfried *et al* 2005). These MKK D-sites therefore appear to represent the major target of LF substrate recognition and catalysis, and disruption of these regions interfere with MKK binding interaction to MAPK, causing the LF mediated inhibition of the MAPK signaling pathway (Vitale *et al* 2000).

1.2.2 Mechanism of toxin entry into the host cell

Upon entry to the body, anthrax spores germinate in the extracellular space (Guidi-Rontani 2002). Although generally regarded as an extracellular pathogen, *B. anthracis* does show a transient intracellular phase (Ruthel *et al* 2004); systemic infection depends upon the bacilli gaining access to host cells, specifically antigen presenting cells (APCs), which then carry the bacilli to the lymph nodes allowing them to infiltrate the lymphatic and circulatory systems (Bradley *et al* 2001; Chen *et al* 2007; Scobie *et al* 2003). Survival and multiplication of the bacilli within the host is dependant upon the subversion of immune responses by the anthrax virulence factors, most specifically the action of the toxins upon the host cells (Figure 1.2.5).

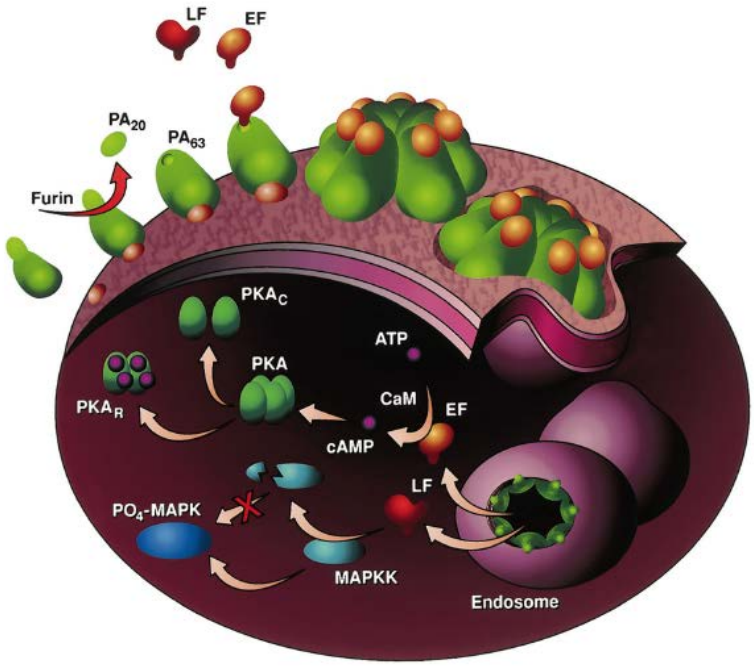


Figure 1.2.5 Internalisation of the anthrax toxins.
Upon binding to either ANTXR1/TEM8 or ANTXR2/CMG2, the PA_{83} protein is cleaved by cellular furin proteases and the smaller PA_{20} fragment released. The PA_{63} fragment forms a ring-shaped heptameric pre-pore, which can bind molecules of LF and EF. The complex is then internalized in a LRP6-dependent manner. The endocytic vesicles are subsequently acidified, initiating a conformational change of the PA heptamer converting it into a mature pore, which allows the entry of toxins into the cytoplasm. Once within a cell anthrax toxin has two distinct modes of action depending upon whether PA complexed with LF or EF. EF is a calmodulin-dependent adenylate cyclase, while LF proteolytically inactivates MAPK kinases, which blocks the activation of the MAPK pathway. Figure taken from (Duesbery and Vande Woude 1999a).

Cellular entry of the toxins is mediated by the secretion of the PA protein, which binds to at least two cell surface receptors; anthrax toxin receptor 1 (ANTXR1), also known as tumour endothelial marker 8 (TEM8) and anthrax toxin receptor 2 (ANTXR2), also known as capillary morphogenesis gene 2 (CMG2) (Bradley *et al* 2001; Petosa *et al* 1997; Scobie *et al* 2003). Both

ANTXR1 and ANTXR2 bind to their cognate ligands through interactions with metal ion dependent adhesion sites (MIDAS) within their von Willebrand factor A domains (Bradley *et al* 2003; Scobie *et al* 2005). The *in vivo* toxicity of anthrax toxin has been found to be predominantly mediated by ANTXR2/CMG2 rather than ANTXR1/TEM8, due to both the high affinity PA displays for the ANTXR2/CMG2 receptor and the higher levels of this receptor expressed upon cell populations within the host (Hu and Leppla 2009; Liu *et al* 2009; Scobie *et al* 2005). Binding to these receptors enables the host protease, furin, to cleave the 83 kD PA molecule (Petosa *et al* 1997). The resulting N-terminus 20 kD fragment is released into the extracellular space, whilst the proteolytically active 63kD PA proteins oligomerise, giving rise to a heptameric ring-like structure which is engulfed by the cell, forming a vesicle with the LF or EF toxins bound to the inner surface (Duesbery and Vande Woude 1999a; Lacy *et al* 2002). The EF and LF toxins bind to the PA heptamers through a homologous N-terminus region, to form Edema Toxin (ET), or Lethal Toxin (LT) (Abrami *et al* 2003). This complex is subsequently transported into the cytoplasm via a raft dependent, clathrin-mediated, endocytosis (Friedlander *et al* 1993a). The lumen of this vesicle acidifies, inducing a conformational change in PA, resulting in the release of LT or ET into the cytosol of the cell (Wei *et al* 2006). Association of the heptamer with the cell surface co-receptor, lipoprotein receptor-related protein 6 (LRP-6), is also essential to the endocytosis of the anthrax toxin complex into the cell (Duesbery and Vande Woude 1999a).

1.3 Role of the immune system in anthrax infection and LF induced pathogenesis.

B. anthracis secretes three toxins which, in combination, are capable of affecting host innate, inflammatory, and adaptive immune mediators through a variety of mechanisms. In particular, the combination of PA and LF forming LT, can impair the function of innate immune cells such as monocytes, macrophages, and neutrophils, while the suppression of phagocytic cells such as macrophages and dendritic cells (DCs) subvert the antigen presentation pathways involved in the generation of adaptive immunity. LT is also capable of acting directly upon adaptive immune cells, down-regulating the MAPK signalling cascade involved in T and B cell gene expression, cell proliferation, survival and apoptosis. The secretion of cytokines and inflammatory mediators by many of the innate and adaptive immune cells are also affected by the action of LT. This manipulation of the host immune response at various levels allows the massive bacteremia seen in systemic anthrax infection.

1.3.1 Complement

The complement system consists of interactive serum enzymes which are involved in mediating inflammation, damaging the membranes of pathogens and opsonising antigenic particles for recognition by cells of the innate and adaptive immune systems. These proteases remain inactive as zymogens, until locally activated. Activation of complement can proceed by three distinct routes; the classical pathway which is initiated by the recognition of the IgM and IgG classes of antibodies bound to cell surface antigens, the alternative pathway which is initiated by direct recognition of microbial cell surface components such as polysaccharides, and the mannose-binding lectin pathway which is initiated by the binding of mannose present on the pathogen cell

surface (Janeway *et al* 2005). All three pathways lead to the production of a C3 convertase which cleaves the C3 protein, giving rise to two proteolytic products C3a and C3b (da Silva *et al* 1967; Muller-Eberhard *et al* 1967). C3a is a potent chemoattractant, whilst the cell associated C3b, which can be directly recognised by phagocytic cell receptors, can form a component of C5 convertase (Janeway *et al* 2005). This proteolytic enzyme cleaves C5 into the chemoattractant C5a and C5b, which remains attached to the cell surface, initiating the formation of the membrane attack complex (MAC) with C6, C7, C8 and C9, which forms a membrane pore, causing microbial cell lysis (Gotze and Muller-Eberhard 1970; Janeway *et al* 2005).

Research by O'Brian *et al* was the first to show that strains of *B. anthracis* could be readily opsonised by both complement and antibodies specific for cell wall antigen (O'Brien *et al* 1985). The role of complement in the immune response to anthrax was further demonstrated by research utilizing complement deficient mice. Both A/J and DBA/2J strains of mice are susceptible to the Sterne strain of anthrax (Welkos *et al* 1986); this was found to be associated with a shared deficiency in complement, which was identified as a reduction in the levels of C5 enzyme activity (Welkos and Friedlander 1988). Depletion of complement in C57BL/6 mice also renders this normally resistant strain highly susceptible to infection with the Sterne strain of anthrax (Harvill *et al* 2005). Conjugation of a complement protein to PA increases protective IgG responses and enhances B cell differentiation into plasma and memory cells, through binding of the C3d protein to the complement receptors CD21/CD35, which are expressed on B cells and follicular dendritic cells (Kolla *et al* 2007). However the importance of complement in generating protective immunity against anthrax has been called into question by recent research which discovered that toxin neutralisation is antibody, but not complement, dependent (Abboud

et al 2010; Harvill *et al* 2008). It appears the complement system plays a negligible role in mediating protection against anthrax infection.

1.3.2 Phospholipase

The phospholipase family of enzymes hydrolyse phospholipids into fatty acids and lipophilic residues. There are four major classes of phospolipase, determined by their catalytic action; the phospholipase A, (subdivided into A_1 and A_2) and B enzymes cleave acyl chains, while phospholipases C and D cleave phosphate groups and are also known as phosphodiesterases.

The phospholipase A_2 (PLA_2) group of enzymes recognizes the *sn*-2 acyl chain of phospholipids, catalysing the hydrolysis of bonds at that position and generating lysophospholipids and the free fatty acid, arachidonic acid (Dennis 1994). Arachidonic acid plays a key role in the production of eicosanoids, a class of compounds which include the pro-inflammatory mediator prostaglandin and leukotrienes (Bingham, III and Austen 1999). The PLA_2 enzymes can be divided into two major groups; the high molecular weight intracellular phospholipases ($cPLA_2$), and the low molecular weight secreted, or extracellular phospholipases ($sPLA_2$) (Mukherjee *et al* 1994). In mammals, eleven distinct members of the $sPLA_2$ group have been identified (Six and Dennis 2000). The type IIA $sPLA_2$ secreted by innate immune cells, particularly macrophages, shows a high bactericidal efficiency against Gram-positive bacteria, binding strongly to the negatively charged surface of the bacteria and rapidly hydrolyzing the membrane phospholipids (Koduri *et al* 2002). Initial studies suggested that secretion of PLA_2 was implicated in the LT mediated cytolysis of host immune cells (Lin *et al* 1996; Shin *et al* 1999). More recently, research has shown that both the germinated spores of *B. anthracis* and the encapsulated vegetative bacteria

are susceptible to the bactericidal activity of type IIA $sPLA_2$ (Gimenez *et al* 2004; Piris-Gimenez *et al* 2005). However, LT is capable of down-regulating the release of the type IIA $sPLA_2$ by altering the capacity of the transcription factor, nuclear factor κ light chain enhancer of B cells (NF-κB) to stimulate phospholipase promoter activity (Gimenez *et al* 2004; Raymond *et al* 2010). The mechanism for this down-regulation remains undefined, however it appears to involve the LT mediated inhibition of the p38 MAPK isoforms which act to phosphorylate and transactivate NF-κB sub-units (Raymond *et al* 2010). A recent study by Raymond *et al* indicated that ET is also capable of inhibiting type IIA $sPLA_2$ expression, through a different mechanism which involves a sequential augmentation of cAMP levels and the activation of PKA (Raymond *et al* 2007).

1.3.3 Neutrophils

Neutrophils are short-lived, terminally differentiated, phagocytic cells of the innate immune system. Their recruitment to sites of infection within the tissue is governed by the expression of chemokines and endothelial adhesion molecules. TNF and IL-1 secreted in the tissues stimulate the expression of selectins and chemokines at the endothelial surface. Neutrophils loosely attach to E-selectin or P-selectin molecules, which are inducibly expressed at the surface of the endothelium (Janeway *et al* 2005). These low affinity interactions are then strengthened by the stable adhesion of the lymphocyte function associated antigen 1 (LFA-1) and macrophage 1 antigen (Mac-1) integrins, expressed on the neutrophil surface, to vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) ligands (Springer 1994). The affinity of these integrins for their ligands and the subsequent extravasation of neutrophils into the inflamed tissues are promoted by the release of chemokines. Chemokines are classified

into four structural groups, based upon the conserved amino terminus cysteine residues: α (CXC), β (CC), γ (C), δ (CX3C) (Baggiolini *et al* 1997; Rollins 1997). Along with the complement protein C5a, and the bacterial peptide formyl methionine (f-met), these chemotactic agents can stimulate directed migration of the neutrophils along a gradient, leading to an accumulation of these innate immune cells in the infected tissues (Janeway *et al* 2005). Elevated levels of peripheral neutrophils were detected in inhalational cases from the 2001 anthrax release in the USA (Jernigan *et al* 2001), although this was not associated with an increased migration of neutrophils to the primary site of infection (Guarner *et al* 2003). This suggests a dysregulation in the chemotactic migration of neutrophils towards sites of infection and inflammation, rather than LF induced apoptosis or necrosis of these lymphocytes (Crawford *et al* 2006; During *et al* 2005). During *et al* demonstrated that LT is capable of inhibiting neutrophil migration along a chemoattractant gradient (During *et al* 2005). These findings are in conflict with earlier research by Wade *et al*, in which exposure to LT stimulated neutrophil migration and chemotaxis (Wade *et al* 1985). During *et al* proposed that this effect was due to differing exposure periods - the length of exposure to the toxin determines the level of paralysis induced in the neutrophils (During *et al* 2005). The inhibition of chemotaxis in these cells is associated with a marked reduction in filamentous actin assembly and establishment of cell polarity (During *et al* 2005; During *et al* 2007). The mechanism governing this inhibition appears to depend upon LF blockade of phosphorylation in both p38 MAPK isoforms and heat shock protein 27 (Hsp27), which impairs actin assembly by sequestering actin monomers and preventing their transport to the leading edge of the motile cell (During *et al* 2007). Recent research has suggested that the inhibitory effect is not limited to LT, as ET demonstrates an ability to impair chemoattractant induced actin assembly through a currently unidentified pathway. ET and LT in combination also

show synergistic inhibitory effects, downregulating the surface expression in neutrophils of the adherence receptor CD11b/CD18, which impairs the extravasation of neutrophils into tissues (Szarowicz *et al* 2009).

Neutrophils are capable of killing microbes by oxygen dependent and independent mechanisms. Both mechanisms involve the formation of a phagosome which fuses with intracellular lysosomes to form a phagolysosome. Within the phagolysosome nicotinamide adenine dinucleotide phosphate (NADPH) is capable of the catalytic conversion of oxygen to reactive oxygen intermediates (ROI), or superoxide radicals, which dismutase to potent reactive oxygen species (ROS) such as hydrogen peroxide. In combination with the enzyme myeloperoxidase (MPO), the hydrogen peroxide is capable of converting halide ions into reactive hypochlorous acid. This respiratory burst, in addition to the production of free radicals through the action of inducible nitric oxide synthase (iNOS), produces microbicidal substances which are capable of killing both phagocytosed and extracellular microbes. Xu *et al* reported that the minimal effect of LT upon JNK signalling, in combination with the blockade of ERK and p38 MAPK signalling, upregulates the production of superoxide in neutrophils (Xu *et al* 2008). The differential effects upon MAPK signalling therefore produce an enhanced proinflammatory response in neutrophils. This is in contrast to previous studies which found that the effect of LT upon MAPK signaling and the EF mediated elevation of intracellular cAMP both inhibited the NADPH mediated production of superoxide (Crawford *et al* 2006; O'Brien *et al* 1985; Wright and Mandell 1986). However, Xu *et al* noted that the discrepancy may be explained by a longer exposure to LT, which could enhance the effects of differential MAPK signaling (Xu *et al* 2008). The contradictory effects of anthrax toxins can also be attributed to the use of human peripheral

neutrophils in these studies, as, unlike murine neutrophils, human neutrophils are capable of producing α defensins (Eisenhauer and Lehrer 1992). These small antimicrobial peptides, found in human neutrophil granules, can reversibly inhibit LT activity, allowing neutrophils to kill the vegetative anthrax bacteria (Kim *et al* 2005; Mayer-Scholl *et al* 2005). The lack of α defensin expression in human myeloid precursor cells may parallel the murine neutrophil susceptibility, indicating a temporal susceptibility to LT effects in human neutrophils (Xu *et al* 2008).

Formation of the phagosome is also associated with the discharge of cytoplasmic granules into the vacuole; the two predominant types of granule are azurophil and specific (Bainton *et al* 1971). The azurophil granules, which are produced first, contain neutral proteinases capable of killing and digesting microbes, while the specific granules replenish membrane components, limiting the damage inflicted by free radicals upon host tissue (Bretz and Baggiolini 1974; Dewald *et al* 1975; Segal 2005). The proteinases contained within the azurophil granules include lysozyme, elastase, and collagenase (Bretz and Baggiolini 1974; Ohlsson *et al* 1977). In addition to the secretion of antimicrobial products through degranulation, activated neutrophils also release neutrophil extracellular traps (NETs), which consist of chromatin and granule proteins such as elastase (Brinkmann *et al* 2004). Neutrophil elastase is a broad-specificity serine protease, capable of cleaving elastin, which under normal physiological conditions plays an important role in killing phagocytosed and extracellular bacteria (Belaaouaj *et al* 1998). Exposure to LT causes the secretion of elevated levels of elastase from activated neutrophils, leading to the proteolytic degradation of antithrombin, which controls the activity of coagulation cascade proteases such as thrombin (Chung *et al* 2008a; Fang *et al* 2010). In addition to the cleavage of fibrinogen by anthrax proteases, this has a profound effect upon host haemostasis,

leading to the uncontrolled bleeding and coagulopathy characteristic of DIC (Chung *et al* 2008a). LT is therefore capable of inducing the release of high levels of proinflammatory elastase from neutrophils, mediating lethality and the systematic spread of anthrax bacteria within the host.

1.3.4 Macrophages

Macrophages, especially alveovar macrophages, have long been considered the 'Trojan horse' responsible for the uptake and transport of *B. anthracis* to lymph nodes, promoting the rapid spread of disease within the host (Guidi-Rontani 2002). Studies of the modulatory effect LT has upon macrophages, and their precursor cells, monocytes, have produced conflicting findings. Pickering *et al* reported that *in vitro* and *in vivo* levels of TNFα and IL-12 were elevated following macrophage phagocytosis of *B. anthracis* spores (Pickering *et al* 2004; Pickering and Merkel 2004) This is in contrast to studies which have found that exposure to LT interferes with the secretion of pro-inflammatory cytokines, inhibiting transcription of TNFa, IL-1β, IL-1α, IL-1RA, and IL-6 in macrophage cell lines (Erwin *et al* 2001). This was mirrored by studies which found that the inhibition of the MKK1 signaling pathway by LT is associated with a reduction in the secretion of TNFα, IL-1β, IL-6, and IL-8 by non-human primate alveolar macrophages (Ribot *et al* 2006). Furthermore, exposure of macrophage cell lines to sublytic concentrations of LT causes the cleavage of MKK2 and 3 within the cell and decreases the levels of TNFα produced by the cells in response to stimulation with LPS or IFNγ (Pellizzari *et al* 1999). The MAPK signalling pathways downstream of the MKK families are responsible for controlling the transcription and synthesis of pro-inflammatory cytokines and chemokines, particularly TNFα, IL-8 (and the murine homologue KC) and regulated upon activation, normal T cell expressed and secreted (RANTES) (also known as CCL5) and the production of antimicrobial substances such

as iNOS in macrophages and monocytes (Bergman *et al* 2005; Dang *et al* 2004; Pellizzari *et al* 1999; Pellizzari *et al* 2000). Cleavage of these pathways by LT has multiple effects upon the phosphorylation and activation of the transcription factors NF-κB, interferon regulatory factor 3 (IRF3), Ets-like gene-1 (Elk-1) and early growth response protein-1 (Egr-1), all of which play a crucial role in the transcription of inflammatory mediators (Bergman *et al* 2005; Dang *et al* 2004; Geppert *et al* 1994; Xu *et al* 2001).

Macrophages play a role in trafficking of antigens to the regional lymph nodes; the alveolar macrophages migrate rapidly to the draining lymph nodes following exposure of the lung to pathogenic bacteria such as *Streptococcus pneumoniae* (Kirby *et al* 2009) and these phagocytic cells have been shown to readily engulf anthrax spores (Ribot *et al* 2006). Therefore it appears that although the spores are capable of inducing an early burst of proinflammatory cytokines released from macrophages, upon germination the vegetative bacteria releases LT in an attempt to inhibit the innate defences, promoting the survival of nascent bacteria and their spread through the host.

A prominent effect of LT is the induction of the selective apoptosis of activated macrophages by disrupting the regulation and expression of pro-survival genes (Friedlander *et al* 1993a). Initial studies suggested that this apoptotic pathway was activated by the LT induced cleavage of the p38 MAPK isoform required for phosphorylation and activation of the NF-κB transcription factor responsible for activating genes which prevent apoptosis of activated macrophages (Park *et al* 2002). Macrophages and monocytes detect *B. anthracis* pathogen associated molecular patterns (PAMPs) such as lipoteichoic acid (LTA), in combination with host derived danger

signals, through the action of pattern recognition receptors (PPRs), specifically the toll like receptor (TLR) superfamily members TLR2 and TLR4 (Hsu *et al* 2004; Hughes *et al* 2005). The involvement of TLR signaling in the response to *B. anthracis* is further supported by the increased susceptibility of macrophages deficient in myeloid differentiation factor 88 (MyD88), or its downstream protein TRAF6, to *B. anthracis* infection and LT induced apoptosis (Hsu *et al* 2004; Hughes *et al* 2005; Okugawa *et al* 2010). As MyD88 is a crucial adaptor protein of the TLR superfamily, the inflammatory pathway which activates the MAPK signaling cascade and ultimately the transcription of anti-apoptotic genes by NF-κB, is profoundly inhibited (Hughes *et al* 2005).

Work by Popov *et al* conversely indicates the role of p38 independent pathways in the induction of macrophage apoptosis, as exposure of murine macrophage cell lines to LT initiates the activation of a number of caspase family members (Popov *et al* 2002). These cysteine proteases play a crucial role in the induction of apoptosis and necrosis, and the caspase 1 family member is known to act as the upstream regulator of IL-1β and IL-18, triggering release of these pro-inflammatory cytokines (Cordoba-Rodriguez *et al* 2004). Recent research exploring the sensitivity of murine and rat macrophages from different genetic backgrounds to LT induced apoptosis, have revealed that a member of the nucleotide binding domain leucine rich repeat (NLR) superfamily is crucial to the activation of the caspase family members and susceptibility to LT mediated apoptosis (Boyden and Dietrich 2006; Newman *et al* 2010). The NLR protein 1 (NLRP1) plays a key role in the formation of a cytosolic multi-protein complex, the inflammasome, which regulates the production of caspase 1, and downstream processing and subsequent release of IL-1β and IL-18 (Boyden and Dietrich 2006; Cordoba-Rodriguez *et al*

2004; Nour *et al* 2009). Unlike humans which have a single *nlrp1* gene, mice possess three paralogues; *nlrp1a, nlrp1b* and *nlrp1c*. Transfection of human fibroblasts with plasmids encoding the LT susceptible murine *nlrp1b* gene caused the heterologous expression of NLRP1b in association with caspase 1, conferring susceptibility to LT mediated death (Liao and Mogridge 2009). The highly polymorphic nature of *nlrp1b* may explain the variation in susceptibility of murine macrophages to anthrax challenge (Muehlbauer *et al* 2007). Whilst the action of NLRP1b does initiate the apoptosis of activated macrophages, Terra *et al* have advanced the theory that this represents a facet of host defense rather than pathogen spread (Terra *et al* 2010). NLRP1b mediated cell death may counteract the immunosuppressive effect of LT, promoting the release of pro-inflammatory cytokines, especially IL-1β, and the subsequent infiltration of immune cells to the site of anthrax infection. This appears to explain the studies which found that murine strains with macrophages susceptible to LT mediated cell death were more resistant to anthrax spore challenge (Boyden and Dietrich 2006; Welkos *et al* 1989; Welkos and Friedlander 1988).

1.3.5 Dendritic cells

DCs are pleiotropic cells, which, along with macrophages, play a central role in bridging the innate and adaptive immune responses. The majority of DCs are divided into the conventional myeloid DCs (mDC), and the rarer plasmacytoid DCs (pDC) (Cella *et al* 1999; Grouard *et al* 1997; Olweus *et al* 1997). In contrast to follicular dendritic cells (FDC), these cells are derived from hemopoietic bone marrow progenitors. FDCs are of mesenchymal rather than hematopoietic origin, and are found in the germinal centers of lymphoid follicles (Humphrey *et al* 1984). Unlike pDCs and mDCs they do not express major histocompatibility complex (MHC) class II molecules at the cell surface; instead antigens complexed to antibodies or complement

are displayed at the cell surface for recognition by B lymphocytes (Humphrey *et al* 1984; Yoshida *et al* 1993).

The majority of immature DCs fulfill a sentinel function in the periphery, forming networks in the epithelia of the skin, gastrointestinal and respiratory systems. Under normal physiological conditions, expression of the P-selectin glycoprotein ligand allows immature circulating DCs to bind to the endothelium and extravasate into tissues (Robert *et al* 1999). Once within the periphery, DCs may remain for up to two weeks before leaving tissue via the lymphatics, although the turnover of DCs in the mucosa is much faster, with a reported rate of 2-3 days (Liu *et al* 2007a; Ruedl *et al* 2000).

Within the periphery, immature DCs expressing basal levels of MHC class II and co-stimulatory molecules, monitor the extracellular milieu through antigen uptake involving phagocytosis, receptor mediated endocytosis and macropinocytosis (Sallusto *et al* 1995). Following stimulation by cytokines or recognition of PAMPs such as lipopolysaccharide (LPS) by DC PPRs there is a change in the pattern of chemokine receptor expression (Roake *et al* 1995; Sozzani *et al* 1998). DCs downregulate the expression of the tissue retaining chemokine receptors, CXCR1, CCR1, CCR2 and CCR5, limiting the ability of DCs to migrate in response to pro-inflammatory chemoattractants, and upregulate CCR7 and CD11c, which allow the DC to home to secondary lymphoid organs (Dieu *et al* 1998; Lin *et al* 1998; Roake *et al* 1995; Vecchi *et al* 1999). Within the lymph node or spleen the mature DC, which has massively upregulated the expression of both peptide loaded MHC class II molecules and co-stimulatulory molecules at the cell surface, is capable presenting antigen to cognate T cells.

The ‘Trojan horse’ role that macrophages play in *B. anthracis* infection is echoed by DCs, which endocytose and transport spores to the regional lymph nodes, allowing the systemic spread of infection (Brittingham *et al* 2005). The central role for DCs in the transit of anthrax to the lymph nodes is supported by the finding that lung DCs phagocytose anthrax spores more efficiently than alveolar macrophages (Cleret *et al* 2006). The rapid transport of intact germinating spores from the site of infection to the thoracic lymph nodes, within lung DCs, in inhalational anthrax infections, presumably mediates the lethality of this route of infection (Cleret *et al* 2007).

Uptake of the spores by human monocyte derived dendritic cells (MDDCs) stimulates the expression of a mature DC phenotype; $CD83^{bright}$, $HLA\text{-}DR^{bright}$, $CD80^{bright}$, $CD86^{bright}$, $CD40^{bright}$, which, in concert with a weak activation of the ERK and strong activation of the p38 MAPK isoforms, stimulates lymph node homing of DCs (Brittingham *et al* 2005). The spores also cause the rapid release of proinflammatory cytokines such as TNFα, IL-6, IL-8, IL-1β and IL-12 from human MDCCs (Pickering *et al* 2004). However, upon germination of the vegetative bacteria, the associated exposure to LT cleaves the MAPK signaling pathways, particularly ERK and p38 MAPK, inhibiting the secretion of IL-1α, IL-12, TNFα and IL-6 by murine DCs, and the secretion of TNFα, IL-6 and IL-8 by human MDDCs and primary blood DCs (Agrawal *et al* 2003; Brittingham *et al* 2005; Cleret *et al* 2006). In contrast, human pDCs appear to respond equally poorly to both *B. anthracis* spores and vegetative bacteria, releasing negligible amounts of pro-inflammatory cytokines in response to either (Hahn *et al* 2008). This may be related to the major role mDCs play in antigen presentation compared to pDCs, especially within the respiratory system (Vermaelen *et al* 2001).

Agrawal *et al* further demonstrated that exposure of both murine DCs and human MDDCs to LT down-regulated the expression of the co-stimulatory molecules, CD40, CD80 and CD86; LT is therefore capable of impairing the efficient priming of both naïve and antigen specific CD4+ T cells (Agrawal *et al* 2003). The spore induced lymph node homing of DCs, coupled with the subsequent LT mediated interference in APC induced T cell priming, appears to allow successful infection through ensuring transport to secondary lymphoid organs, while preventing the induction of adaptive immunity in CD4+ T cells.

While Agrawal *et al* reported that murine DCs are resistant to LT induced apoptosis (Agrawal *et al* 2003), Alileche *et al* found that the susceptibility of murine DCs to LT induced apoptosis and necrosis was dependent upon the genetic background of the mouse strain (Alileche *et al* 2005). In addition to the lethal effect upon DCs, cytokine release also showed a comparable discrepancy between genetic backgrounds following LT exposure; both Balb/c and C57BL/6 strains showed a reduction in IL-12, IL-6 and TNFα, but only C57BL/6 mice showed elevated levels of IL-1β (Chou *et al* 2008). Human MDDCs display a susceptibility to LT mediated death similar to DCs from resistant mouse strains such as C57BL/6 (Alileche *et al* 2005). The genetic backgrounds responsible for DCs susceptible to LT killing, also produced similarly susceptible macrophages, indicating a shared mechanism governing LT cytotoxicity in both cell populations (Alileche *et al* 2005; Reig *et al* 2008). This mechanism in DCs may be related to the induction of the NLRP1b dependent pathway in susceptible strains, although there is also evidence that the induction of a caspase 1 independent pathway, triggered by the LT impairment of the ERK signaling pathway is capable of causing cell death in both genetically susceptible and resistant strains alike (Reig *et al* 2008). This susceptibility to LT induced cell death appears to occupy a narrow window during

the DC development, with immature DCs in peripheral tissues more susceptible than mature DCs trafficking anthrax spores to the lymph nodes (Reig *et al* 2008).

Recent research has suggested that ET may also aid LT in promoting anthrax infection of DCs. Exposure to ET can aid in the migration of infected DCs, as in addition to the down-regulation of dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN), a marker of immature DCs, and upregulation of DC maturation markers CD83 and CD86, through increased cAMP levels, ET is also capable of promoting DC migration towards the lymph node homing chemokine macrophage inflammatory protein 3β (MIP-3β) through the same mechanism (Maldonado-Arocho and Bradley 2009). This co-operation extends to the suppression of cytokine release from DCs; while LT is capable of inhibiting TNFα and ET can decrease levels of IL-12p70 and TNFα, LT and ET together have a synergistic effect upon the decrease in TNFα levels (Tournier *et al* 2005). The effect of LT and ET upon DC cytokine release may guide T cell differentiation towards a Th2 subset, rather than promoting a Th1 immune response, allowing *B. anthracis* to evade appropriate host immune responses.

1.3.6 Adaptive immunity

The majority of anthrax vaccination studies have focused upon the role of antibody-based humoral protective immunity (Little *et al* 1990; Little *et al* 1997; Pitt *et al* 1999; Wild *et al* 2003). Despite this however, there has been relatively little research exploring the effect anthrax virulence factors, and especially LT, has upon the immunoglobulin (IgG) producing B cells. LT is known to potently inhibit B cell ERK, JNK and p38 MAPK signalling pathways, impairing proliferation in response to TLR2, TLR4, CD40 and B cell receptor (BCR) stimulation, although

there is no evidence of LT induced apoptosis, even at high doses (Fang *et al* 2006). There is also a decrease in the levels of IgM and IgG produced by LT exposed B cells, both *in vitro* and *in vivo* (Agrawal *et al* 2003; Fang *et al* 2006). It remains unclear whether the reduction in Ig levels reflect a direct effect of LT upon the mechanism of Ig production or if it is due to the reduced B cell proliferative response (Fang *et al* 2006). This direct inhibition of both B cell proliferation and antibody production represents an effective mechanism of LT for subverting the strongly protective humoral immune response associated with host resistance to infection.

In addition to the indirect effect LT mediates through the disruption of antigen presentation, LT has a direct effect upon human and murine T cells. The MAPK cascades are critical in controlling T-cell activation, as these pathways are involved in the activation of the T cell receptor (TCR) dependent nuclear factor of activated T cells (NFAT) families and the activating transcription factor 2 (ATF2) (Hunter and Karin 1992). The five NFAT family members act synergistically with the nuclear subunit activation protein 1 (AP-1) as transcription factors regulating the expression of inducible genes vital to the adaptive immune response (Hunter and Karin 1992). The regulation of NFAT, AFT2 and AP-1 activation is controlled at multiple levels by MAPK phosphorylation. In this way, the action of LT upon the MKKs blocks JNK, ERK and p38 mediated T cell proliferation (Comer *et al* 2005; DeSilva *et al* 1998; Paccani *et al* 2005).

The NFAT transcription factors are involved in the regulation of genes encoding IL-2 and the IL-2 Receptor (IL-2R) (Jain *et al* 1995), which are essential for the clonal expansion of antigen specific T cells (Hunter and Karin 1992). LT induced impairment of T cell proliferation is associated with the reduced production of the Th1 and Th2 cytokines IL-2, IFNγ, TNFα and IL-5

and the downregulation of the activation markers, CD69 and CD25 (Fang *et al* 2005; Paccani *et al* 2005). This blockade of T cell activation is potent enough to inhibit the co-stimulation *ex vivo* of LT exposed CD4+ T cells, by α-CD3 and α-CD28 antibodies (Comer *et al* 2005). Due to its role in the perturbation of PKA dependent intracellular signalling pathways, ET also has an indirect effect upon NFAT transcriptional regulation, interfering with the calcium dependent signalling, but only when present at much higher concentrations than LT (Paccani *et al* 2005).

The antagonistic effect of ET upon PKA in T cells strengthens the effects of LT upon MAPK cascades, as ET is capable of both phosphorylating a negative regulatory residue of Raf, which lies upstream of the MAPK pathway, and phosphorylating a positive regulatory residue of Rap1 which inhibits Ras initiation of the MAPK cascade (Paccani *et al* 2007). The synergistic effect of LT and EF upon the MAPK pathways suppresses T cell chemotaxis in response to CXCL12 (also known as stromal cell derived factor 1α or SDF-1α) (Paccani *et al* 2007). This may be mediated through inhibiting the phosphorylation of both regulatory components such as paxillin and structural components such as actin, of the cell cytoskeleton (Howe 2004; Huang *et al* 2004). The suppression of T cell chemotaxis presumably blocks the trafficking of both naïve and effector memory T cells to infected tissues, which in combination with the decreased proliferative response, prevents the effective induction of an adaptive immune response.

The elevation of intracellular cAMP by ET appears to skew the differentiation of naïve CD4+ T cells towards a Th2 subset, inhibiting the TCR dependent activation of Akt1, a protein essential for the development of a Th1 subset, whilst enhancing the activation of the guanine nucleotide exchanger Vav1 and the stress kinase p38 which are involved in Th2 differentiation (Paccani *et*

al 2009). Along with the LT and ET effect upon DC cytokine release which promotes preferential Th2 differentiation, this represents both direct and indirect consequences of anthrax toxins upon the lineage commitment of naïve CD4+ T cells. IFNγ is one of the hallmark cytokines produced by the Th1 subset, and is involved in upregulating both adaptive and innate immune functions (Schroder *et al* 2004). The importance of an IFNγ CD4+ T cell response in mediating protection from anthrax infection *in vivo* (Glomski *et al* 2007), in addition to the increased survival and *B. anthracis* bactericidal activity of IFNγ activated macrophages (Gold *et al* 2004), suggests that survival of *B. anthracis* within the host depends in part, upon the suppression of IFNγ release. Conversely this also indicates that the induction of an IFNγ producing memory T cell population should be an important goal of any future *B. anthracis* vaccine design.

Recent research has suggested an important role for a specialized subset of T cells, natural killer T (NKT) cells in the generation of PA specific, LT neutralizing antibodies by B cells (Devera *et al* 2010). The CD1d restricted type I invariant NKT (iNKT) cells, which express an invariant murine Vα14 Jα18 TCR or a human Vα24 Jα28 TCR provide B cell help, in addition to the production of pro-inflammatory cytokines such as IFNγ, TNFα and granulocyte macrophage colony stimulating factor (GM-CSF) (Kronenberg 2005). Surprisingly iNKT cells appear to express higher levels of the PA receptors TEM-8/ANTRX1 and CMG-2/ANTRX1 than other hematopoietic cells (Joshi *et al* 2009). As NKT cells represent less than 1% of the circulating T lymphocytes in humans (although this percentage varies substantially between individuals) (Baev *et al* 2004; Kenna *et al* 2003) this may denote a disproportionate focus upon this cell population by anthrax toxins. This preferential targeting of iNKT cells is supported by the

downregulation of the activating receptor NKG2D on iNKT cells but not NK cells in the presence of LT (Joshi *et al* 2009). LT is capable of mediating a variety of effects upon iNKT cells through the cleavage and inactivation of MKK2 which impacts upon the phosphorylation of downstream ERK1 and 2 (Joshi *et al* 2009; Khan *et al* 2010). Inhibition of the ERK pathway impairs CD1d mediated antigen presentation to iNKT cells, in addition to an observed reduction in endosomal trafficking of antigen loaded MHC class II molecules, this has major implications for the induction of both iNKT and CD4+ Th adaptive immune responses to *B. anthracis* (Agrawal *et al* 2003; Khan *et al* 2010).

1.4 Large Scale epitope mapping

1.4.1 Human Leucocyte Antigens (HLA)

Until recently the majority of anthrax vaccines focused upon eliciting long-lived memory B cells and plasma cells. These cells produce antibodies involved in toxin neutralisation or opsonisation of the infectious agent. However, recent work by Glomski *et al* suggests that humoral immunity alone may not be sufficient to provide protection against live anthrax infection, instead, IFNγ producing CD4+ T cells appear to play a key role in mediating protection (Glomski *et al* 2007). During this period the Altmann lab became interested in efforts to understand the CD4+ T cell immunology of the response to anthrax. This was part of a major initiative from National Institutes of Health–National Institute of Allergy and Infectious Diseases (NIH-NIAID), to generate a database of pathogen-derived epitopes (Sette *et al* 2005). This would be used both as a basic biology resource for other experimentors in the research community, as a dataset for refinement of predictive epitope algorithms, and as a resource for vaccine design. Several initiatives during this period have explored the potential of targeting the stimulation of protective T cells through T cell epitope vaccines: examples have included tuberculosis, malaria, and human immunodeficiency virus (HIV) (Gilbert *et al* 2006; Hanke *et al* 1998; McShane *et al* 2002). T cells are capable of generating antigen-specific responses to peptides presented in the context of self MHC molecules. In humans, the genes encoding the Human Leucocyte Antigens (HLA) are found on the short arm of chromosome 6 (Campbell and Trowsdale 1993), a region containing 252 expressed genes, many of those characterised being of immune related functions (Horton *et al* 2004; Trowsdale 2005). The HLA class II cell surface glycoproteins are involved in peptide presentation to CD4+ cells, and HLA class I to CD8+ cells, while the HLA class III

genes primarily code for other aspects of the immune system, such as complement proteins, TNF cytokines and heat shock proteins (Gay *et al* 1987; Townsend and Bodmer 1989). The region closest to the centromere contains the genes coding for the class II isoforms which are expressed on the surface of professional APCs; DQ, DR and DP (Campbell and Trowsdale 1993). These MHC class II molecules are heterodimers composed of two transmembrane glycoproteins; the α (33-35kDa) and β (26-28kDa) chains, each of which fold into two extracellular domains; α1, α2, and β1, β2 (Stern *et al* 1994). These chains exist in many different allelic forms, with the polymorphic variation arising from both chains (in the case of HLA-DP and DQ). The HLA region has the highest polymorphism amongst all human loci (Trowsdale 2005).

1.4.2 HLA class II and humanised transgenics

When defining immunogenic pathogen derived peptide epitopes relevant to human disease and the induction of immunity, it is appropriate to consider the role played by HLA polymorphisms (Trowsdale 2005). Different MHC class II molecules may vary in their peptide binding specificity and so present different peptides of a given antigen, which will have an effect upon the T cell repertoire activated in the immune response. The development of HLA-transgenic immunocompetent mouse models have advanced studies of HLA associated disease susceptibility, allowing the elucidation of human responses to relevant antigenic stimuli (Shirai *et al* 1995). These models have provided a defined genetic background, in which the response of T cells to antigen presentation by a single MHC isoform can be mapped. The genes encoding the MHC in the mouse, corresponding to the HLA, are found on chromosome 17 and are known as the histocompatibility antigen 2 (H-2) region (Takada *et al* 2003). Historically, the nomenclature of the H-2 alleles has, as with HLA alleles, been revised and refined as DNA based techniques

have allowed finer distinctions between allotypes to be made (Klein *et al* 1983). The H-2 region is identified by its haplotype (H-2^b, H-2^d, H-2^k, H-2^f, H-2^q or H-2^s) (Klein *et al* 1990) which is transmitted between each generation with relatively limited recombination within haplotypes, resulting in homozygous inbred laboratory strains (Bukara *et al* 1985). The class II gene complex is known as the I region and consists of two loci; I-A and I-E, each of which encode the α and a β chains which are expressed at the cell surface (Klein *et al* 1990). The cell surface expression of these endogenous MHC class II can be disrupted by targeted knock-out mutations (Cosgrove *et al* 1991; Grusby *et al* 1993; Kontgen *et al* 1993). Due to the central role played by MHC class II molecules in the development of T cells, this disrupts the thymic education of CD4+CD8- αβ T cells, drastically depleting the levels of single positive CD4 T cells in the periphery (Grusby *et al* 1991). The abrogation of MHC class II expression has a profound effect upon the cell composition of the adaptive immune system. In contrast to αβ T cells, γδ T cell development is unaffected (Grusby *et al* 1993), the number of CD8+ T cells also appears to increase in the periphery in a compensatory manner (Stephens *et al* 2007).

The development of a CD4+ αβT cell population and the normal composition of the T cell compartment in peripheral lymphoid organs can be rescued in MHC class II deficient mice by inducing the expression of human HLA genes in professional APCs (Boyton and Altmann 2002b; Yamamoto *et al* 1994a). A number of transgenic models have been generated which express common HLA alleles. In these models a species matched HLA/CD4 interaction has been found to improve the T cell response to HLA restricted peptides (Altmann *et al* 1995), however, early studies which suggested that a species barrier would block murine TCR and human derived MHC interactions (Woods *et al* 1994) have been contradicted, as these transgenic models have

been shown capable of presenting peptides to murine T cells and directing an adaptive immune response (Yamamoto *et al* 1994a). The transgenic models show a high degree of consistency with human responses to given epitopes, eliciting HLA restricted T cell responses which are reminiscent of those seen in humans of a comparable HLA type (Cheuk *et al* 2002; Shirai *et al* 1995; Tishon *et al* 2000).

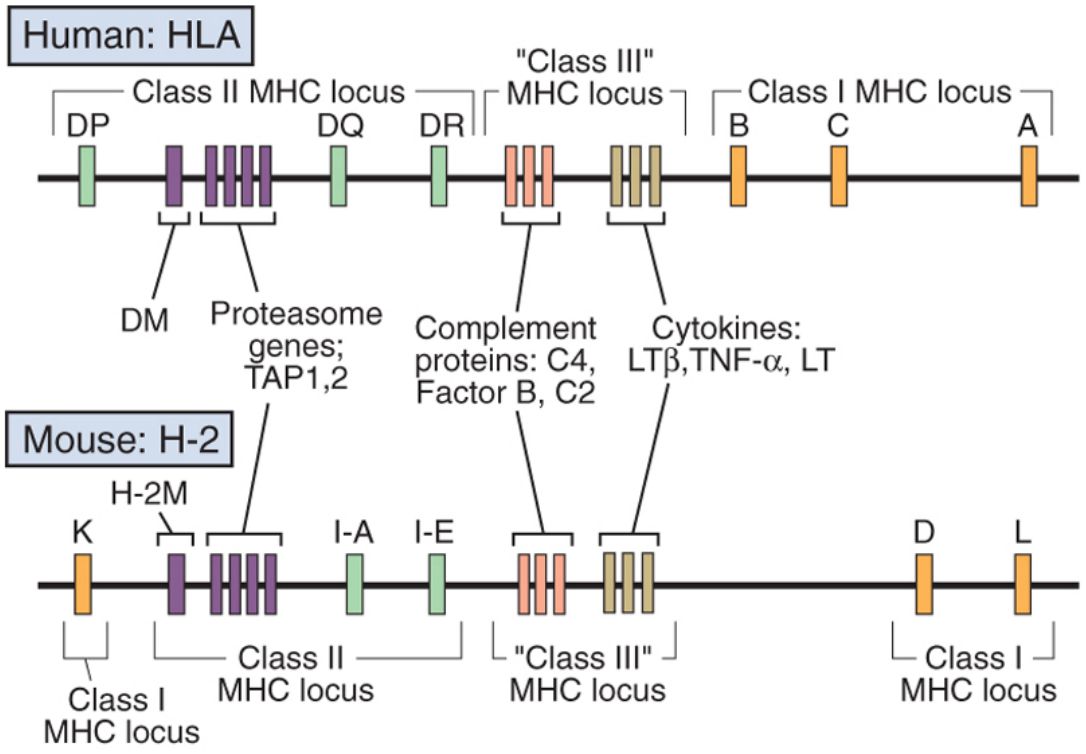


Figure 1.4.1 Schematic representation of human and mouse MHC loci.

Whilst the genes encoding the MHC molecules are the most polymorphic genes present in the genome of both mice and humans, the basic organization within this locus shows similarities between the two species. The mouse homologues of the antigen presenting molecules HLA-DR and HLA-DQ are H-2E and H2-A respectively, whilst the genes which encode the mouse homologue of the human HLA-DP molecule have been lost. The relatively non-polymorphic HLA-DM and its mouse homologue H-2M, do not bind peptides, but are instead involved in the efficient loading of peptides onto the class II antigen presenting molecules. The genes which encode these molecules lie within the MHC class II locus along with genes encoding proteasomes and transporter associated with antigen processing (TAP) complexes, which are involved in the production and transport of peptides. Figure adapted from (Abbas *et al* 2000).

1.4.3 HLA-DQ and HLA-DR transgenic models

The MHC class II molecules are among the most polymorphic glycoproteins known in nature (Reche and Reinherz 2003), with 1353 human HLA class II alleles currently identified (Robinson *et al* 2011). Within an individual the DQA and B genes both show extensive polymorphism (Auffray *et al* 1983; Schenning *et al* 1984; Trowsdale *et al* 1985). The DR subregion derives its vast polymorphism from the DRB products, of which there are nine, only four of which are expressed as functional genes (DRB1, DRB3, DRB4 and DRB5), these gene products are variably expressed in different haplotypes (Trowsdale 2005). Antigen presentation is further complicated by the fact that DQ molecules are expressed at a lower level on the cell surface than DR molecules, which therefore tend to dominate presentation to CD4+ T cells (Gorga *et al* 1987). The vast majority of individuals are heterozygous across the HLA region. It can be calculated that for such individuals, if one takes into account the DP, DQ and DR molecules encoded from each chromosome, as well as the potential for HLA-DQ mixed pairs and DR/DQ mixed pairs, the cell-surface a single human APC may express 14 different class II molecules, each presenting repertoires of bound peptides to CD4+ T cells.

There is strong linkage disequilibrium over a considerable distance within the HLA region, but most notably perhaps between the DQ and DR loci of the HLA class II region, (Carrington *et al* 1994). This has been demonstrated for example in the DQ6/DR15 haplotype; a relatively common haplotype in Caucasians (Klitz *et al* 2003) which has merited extensive research due to its association with several diseases. A number of autoimmune diseases exhibit strong associations with HLA class II alleles. The DRB1*1501/DQB1*0602 haplotype is associated with susceptibility to multiple sclerosis in many populations (Weinshenker *et al* 1998), and also

with Goodpasture's disease (Phelps *et al* 1996). Furthermore the haplotype has been associated with a risk of developing lupus nephritis in systemic lupus erythematosus (Marchini *et al* 2003). The DQA1*0102/DRB1*1501/DQB1*0602 haplotype has been implicated with a predisposition to narcolepsy (Kwon *et al* 1995). Females expressing the DQA1*0102/DQB1*0602 heterodimer show an increased risk of developing cervical neoplasia when exposed to certain serotypes of the human papilloma virus (Sanjeevi *et al* 1996). Conversely, this haplotype has also been linked to a negative disease correlation: DQA1*0102/DQB1*0602 confers resistance to insulin-dependent diabetes mellitus (IDDM) (Ettinger *et al* 1998)

Particular DR/DQ allelic combinations tend to persist in the general human population, with very little recombination due to the strong linkage disequilibrium in the MHC class II alleles of HLA region (Carrington *et al* 1994). This, in combination with the natural genetic heterogeneity involved in antigen presentation to CD4+ T cells, can make it difficult to distinguish the immunological effects of individual DR alleles from the effects of their linked DQ alleles. Transgenic models provide a method of dissecting out the different epitopes which may be processed and presented by the distinct DQA1*0102/DQB1*0602 and DRA1*0101/DRB1*1501 MHC class II molecules. These models also present an opportunity to investigate the divergence in epitopes presented by HLA products common in the human population, such as DRA1*0101/DRB1*0101 (Collins *et al* 2000) and widely characterised HLA alleles such as DQA1*0301/DQB1*0302 which is associated with some of the most prevalent autoimmune diseases, such as rheumatoid arthritis (Nabozny *et al* 1996), IDDM (Ettinger *et al* 1998) and multiple sclerosis (Weinshenker *et al* 1998).

Stimulation of long lasting T cell protection by a vaccine requires the inclusion of peptide epitopes capable of generating a robust T cell response. Design of a vaccine which induces this type of immunity is complicated by the allelic diversity inherent in the MHC class II molecules presenting these peptide epitopes. It is therefore important to determine which epitopes elicit immune responses to a particular infectious agent, across a range of HLA molecules.

1.4.4 MHC class II presentation of exogenous peptides

The MHC plays a pivotal role in regulating the cellular immune response. T cell activation involves the recognition of peptides derived from protein antigens presented by the MHC molecules. Whilst the sensitivity of antigen recognition by B cells correlates with a high affinity binding to structural epitopes, this paradigm does not hold true for the TCR, which can bind its cognate linear peptide/MHC ligand with variable affinity. The efficiency of T cell antigen recognition is dependent upon optimum kinetics of the TCR/peptide/MHC interaction, allowing serial engagement and triggering of many TCR by a few peptide MHC complexes (Valitutti *et al* 1995). Subtle changes in affinity can influence T cell effector function (Boyton and Altmann 2002a).

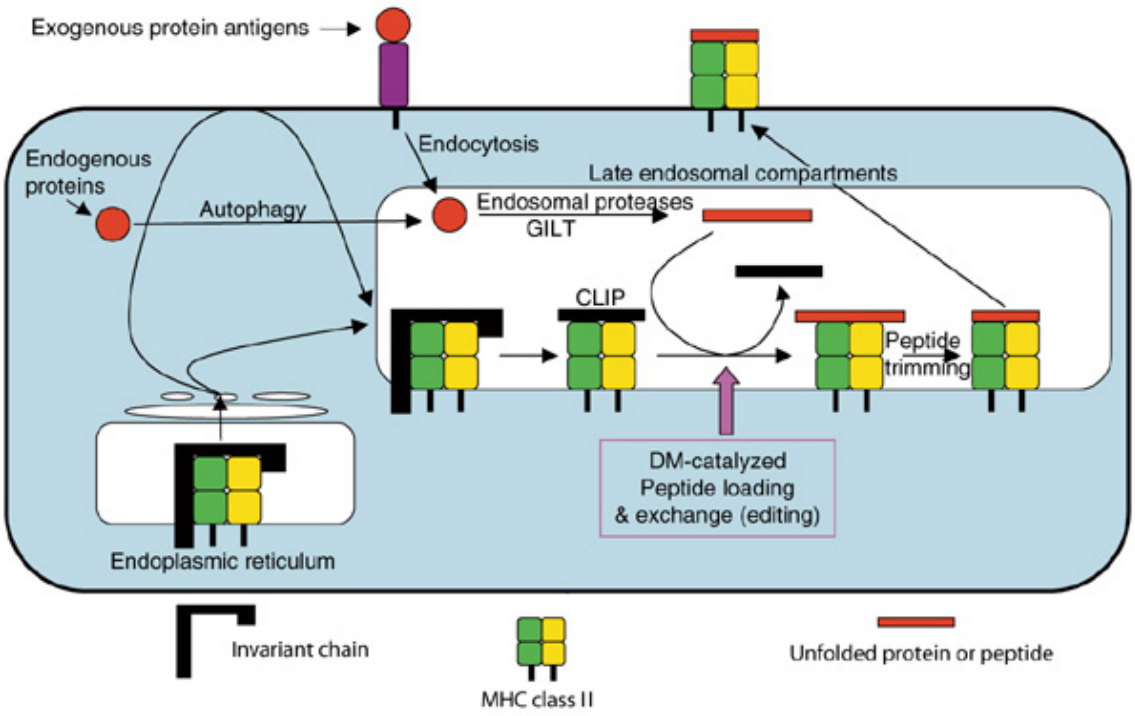


Figure 1.4.2 Pathway of MHC class II antigen processing and presentation.
Following internalisation of exogenous antigens into the endosomal pathway, fragmentation of proteins occurs, catalysed by both lysosomal proteases and the disulfide reductase GILT. MHC class II molecules assemble in the endoplasmic reticulum associated with Ii, Once within the endosomal compartment, Ii is released through protease cleavage events, leaving the CLIP fragment occupying the peptide-binding groove. The release of CLIP is catalysed by the action of HLA-DM, which also edits the repertoire of peptide/MHC class II complexes transported to the cell surface for recognition by $CD4^+$ T cells. Figure taken from (Jensen 2007).

Exogenous proteins constitute the primary source of peptide ligands for MHC class II molecules, although class II restricted presentation of endogenously derived antigens also occurs under certain circumstances, most notably the autophagy of cytosolic antigens (Moreno *et al* 1991). Variations in the antigen processing and presentation pathway are capable of impacting upon the epitope hierarchy, and MHC class II peptide loading compartments vary depending upon both the cell type and the cell specific differences in the trafficking of class II molecules. MHC class

II restricted antigen processing involves certain key stages; internalisation of the peptide antigen and proteolytic processing, the biosynthesis and delivery of class II chains to peptide loading compartments and the subsequent formation of peptide/MHC complexes for expression at the cell surface.

Endocytosis of exogenous antigen can occur through the capture of large particles by phagocytosis, and fluid phase uptake by pinocytosis. Greater efficency of endocytosis is achieved in both cases through receptor mediated uptake of antigen facilitated by a number of specific high affinity endocytic receptors. For macrophages and DCs these include C-type lectin receptors such as DC-SIGN and Dectin-1, the human Fcγ receptors (FcγR) CD16, CD32 and CD64, and integrins such as CD11b/CD18 (Aderem and Underhill 1999; Engering *et al* 2002; Fanger *et al* 1996). B cells meanwhile primarily utilise the clonally restricted antigen specific BCR (Cheng *et al* 1999). Following the endocytosis of exogenous antigen, proteins become localised in the endocytic pathway, which is a complex network of compartments. These compartments can be divided into early endosomes, which are weakly proteolytic, late endosomes, which are highly acidic, and lysosomes, which have a low pH and are rich in hydrolytic acid (Tjelle *et al* 1996). As foreign antigens move through the endocytic pathway they are exposed to gradual proteolysis, and the increasingly acidic conditions influence the unfolding of proteins (Tjelle *et al* 1996). Both the intracellular membrane-bound late endosomes, and phagosomes, are capable of fusing with the enzyme containing lysosomal vesicles. The degradation of protein antigens within these vesicles is an active process involving a number of proteases, although it has proven difficult to precisely define the specific proteases involved in generating peptides. The lysosomal proteases implicated in the MHC class II pathway include

the papain-like cathepsins S, L, B, F, G, H and D, together with the unrelated cysteine protease asparaginyl endoprotease (AEP) (Hsieh *et al* 2002; Hsing and Rudensky 2005). The endosomal protein, γ-interferon inducible lysosomal thiol reductase (GILT), which is constitutively expressed in APCs, has also been implicated in the oxidation of the disulfide linkages found in extracellular antigens, influencing the rate and efficiency with which these proteins are unfolded and cleaved (Arunachalam *et al* 2000; Maric *et al* 2001; Phan *et al* 2000). These enzymes play different roles in processing depending upon the antigen; AEP is instrumental in the processing of epitopes from a tetanus toxoid C fragment which elicits T cell responses (Manoury *et al* 1998), whilst also destroying the immunodominant epitope within the autoantigen Mylein Basic Protein (MBP) (Manoury *et al* 2002). The cathepsins, which are thiol and aspartyl proteases with broad substrate specificities, are capable of both positively and negatively regulating the generation of peptide epitopes by differential cleavage at cysteine and aspartic amino acid residues (Hsieh *et al* 2002). The action of a variety of proteases may therefore contribute towards the hierarchy of epitopes, influencing immunodominance patterns by releasing peptides from their intact antigens or cleaving peptides which contain prominent protease cleavage sites within their critical MHC binding regions (Brown *et al* 2003; Thai *et al* 2004). In contrast to B cell structural epitopes, T cells recognise short lengths of processed amino acids, however, antigens which present a high degree of tertiary structure, or exist within large molecular complexes, are capable of sequestering T cell epitopes from extensive proteolysis and hence release (Dai *et al* 2002). Although it has also been observed that localisation of an antigenic peptide within an extended, unstructured surface domain may conversely promote immunodominance of that epitope (Landry 2000; Thai *et al* 2004).

As membrane glycoproteins, the MHC class II α and β chains are synthesised in the endoplasmic reticulum (ER) (Owen *et al* 1981; Stern *et al* 1994). Following biosynthesis they engage a type II membrane protein known as the MHC class II associated invariant chain (Ii) and the ER resident chaperone protein calnexin (Anderson and Cresswell 1994; Owen *et al* 1981; Shackelford *et al* 1983). Multiple isoforms of Ii exist and are derived through alternative splicing (p41 and p43) or alternative translational initiation (p35 and p43) (O'Sullivan *et al* 1987). Following the release of calnexin from this complex, the Ii, which is a trimer composed of three 30-kD subunits, each capable of binding to a newly synthesised class II heterodimer, forms a nonomeric structure consisting of three class II heterodimers and three Ii molecules (Roche *et al* 1991). This association has multiple functions, allowing the MHC class II molecules to stay in the ER, and overriding premature transport until the molecules are correctly assembled (Lotteau *et al* 1990). Nascent class II dimers are also structurally unstable, capable of dissociating rapidly to their α and β monomers, and it may be possible that Ii acts to stabilise the class II molecule (Roche *et al* 1991; Sadegh-Nasseri and Germain 1991). There is also evidence that targeting signals contained within the cytoplasmic domain of the Ii function to direct the complexes towards the endocytic pathway (Lotteau *et al* 1990). Finally, the most important function of the Ii chain resides within the region of amino acid residues 81-104 of Ii which make up the class II associated invariant chain peptide (CLIP) domain (Bijlmakers *et al* 1994; Freisewinkel *et al* 1993; Riberdy *et al* 1992). This region of the Ii binds to and protects the MHC class II peptide-binding groove, preventing the premature loading of ER resident peptides. Despite these functions, it has been observed that Ii deficient cells, whilst unable to effectively present certain peptide epitopes efficiently, are still capable of generating mature cell surface MHC class II, able to present

peptides from a variety of proteins, suggesting that the importance of the Ii chain varies according the antigen available (Kenty and Bikoff 1999).

From the ER, the MHC II/Ii complexes are transported through the cis and medial Golgi apparatus to the trans Golgi apparatus, allowing the addition of complex oligosaccharides and sialic acid to the vesicles (Bakke and Dobberstein 1990; Cresswell 1994). Following transport from the Golgi region, the subset of vesicles of the endocytic pathway which are known as MHC class II compartments (MIIC) or class II vesicles (CIIV), fuse with late endosomes (Cresswell 1994). Within these heterogeneous multivesicular and multilaminar MIIC/CIIV vesicles, the Ii chain undergoes directional proteolysis from C terminus to N terminus, resulting in the CLIP domain alone remaining bound to the MHC molecule (Pieters *et al* 1991). This occurs via a series of defined intermediates; αβ–Iip22, αβ–Iip10, and αβ–CLIP, cleavage of these intermediaries can be initiated by AEP, but are primarily controlled by cathepsins S and L (Manoury *et al* 2003; Nakagawa *et al* 1998; Nakagawa *et al* 1999; Riese *et al* 1996). Removal of CLIP is accomplished through the actions of the MHC class II like chaperone HLA-DM. HLA-DM and its mouse homologue H-2M, catalyse the removal of CLIP by binding directly to the MHC/CLIP complex and disrupting hydrogen bonds (Denzin and Cresswell 1995; Narayan *et al* 2007). This induces a conformational change, allowing the release of CLIP and subsequent binding of antigenic peptide. Intriguingly, CLIP dissociation from class II molecules occurs at different rates, depending upon the HLA allele involved, suggesting that the allele specific presentation of epitopes may be affected before the MHC/peptide complexes even reaches the APC surface (Stebbins *et al* 1995). A subpopulation of APCs, which includes B cells, DCs and thymic epithelial cells, also express HLA-DO (Douek and Altmann 1997; Hornell *et al* 2006;

Karlsson *et al* 1991). This MHC class II intracellular accessory protein and its mouse homologue H-2O, appear to act as negative regulators of HLA-DM, modulating the dissociation of CLIP and binding of exogenous peptides to MHC class II (Denzin *et al* 1997; van Ham *et al* 1997). Collectively the action of these accessory molecules may affect the extent to which peptide antigens bind to the MHC class II molecules. HLA-DM appears to play a role in editing the peptide repertoire, exchanging peptides with high off-rates which interact poorly, for peptides with low off-rates which bind efficiently to the peptide binding groove (Kropshofer *et al* 1996). While HLA-DO may skew the HLA-DM catalysed MHC class II peptide loading towards highly acidic late endosomal compartments, which are prefentially targeted by antigens taken up through BCR-mediated endocytosis, thereby affecting the peptide repertoire (van Ham *et al* 2000).

In parallel with the classical pathway described, which requires newly synthesized class II molecules, an alternative pathway of antigen presentation also exists. This pathway involves mature class II molecules expressed at the cell surface which recycle through endosomes, becoming available for peptide binding (Salamero *et al* 1990). This recycling pathway involves early endosomal compartments, with a less acidic intracellular pH and weaker proteolytic activities to the classical antigen processing pathway (Griffin *et al* 1997; Lindner and Unanue 1996; Pinet *et al* 1994; Pinet and Long 1998). It has been observed that peptides which are not easily accessible without extensive proteolytic processing are presented more efficiently by the classical antigen presentation pathway, involving newly synthesized class II molecules (Griffin *et al* 1997; Zhong *et al* 1997). In contrast, more superficial determinants, which require less processing, can be loaded directly onto class II molecules recycled from the cell surface (Griffin

et al 1997). Therefore the route and efficiency with which epitopes are presented to the APC surface also appears to play a role in shaping the immunodominance hierarchy.

1.4.5 MHC binding affinities and the epitope hierarchy

The cellular processing and presentation of multideterminant protein antigens generates a large number of potential epitopes, but only a relatively limited number of these linear amino acid sequences are capable of generating an immune response. The observed unpredictability of this immunodominance hierarchy has led to considerable controversy over the role endosomal antigen processing, accessory molecules such as HLA-DM, the binding of MHC molecules to peptides, and the TCR repertoire and precursor frequency all play in shaping the epitope hierarchy and determining the immunogenicity of antigens. (Sant *et al* 2005). Recent studies suggest however that immunodominance hierarchies are largely independent of events related to antigen processing and are instead crucially influenced by the affinity of the peptide epitope for the MHC class II molecules involved in presentation (Lazarski *et al* 2005; Weaver *et al* 2008; Weaver and Sant 2009).

The polymorphism inherent in human HLA class II alleles play a crucial role in restricting the number of possible potential epitopes generated following the intracellular processing of antigens. The extracellular α1 and β1 domains of the MHC class II molecules form pockets which accommodate specific peptidic side chains, and the polymorphisms inherent in the MHC molecules can be primarily found in positions corresponding to the amino acid residues lining sites within these peptide binding pockets.

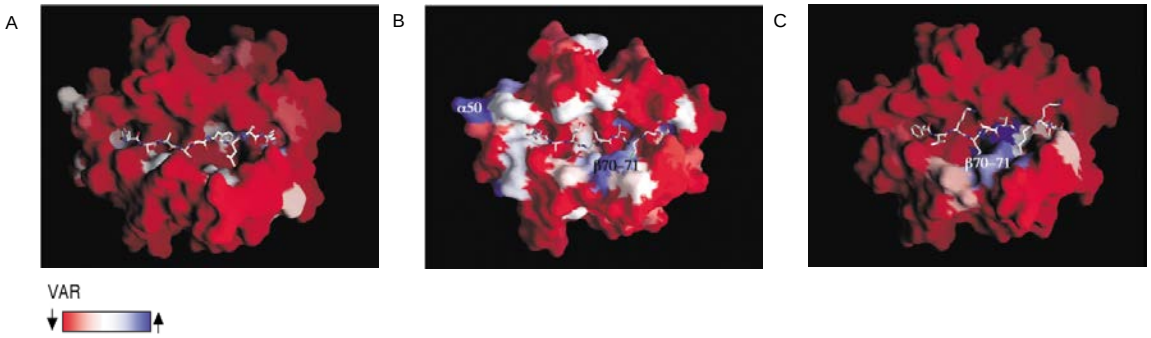


Figure 1.4.3 The 3D surface structure of MHC class II molecules, showing sequence variability in the extracellular domains.

A nonamer peptide ligand, YAASSYLSL which is derived from the human Ig λ chain, is shown mapped onto the binding grooves of (A) HLA-DP, (B) HLA-DQ, and (C) HLA-DR alleles. Variability in the peptide and TCR contact residues are represented on a colour scale, red indicates non-variable regions, blue indicates variable regions, while white regions indicate areas of intermediate variability. Figure taken from (Reche and Reinherz 2003).

The allelic differences found in the MHC class II molecules influence the peptide binding specificity of the various class II MHC alleles, which therefore determines the allele-specific differences in peptides presented by APCs, impacting the epitope hierarchy. MHC class II molecules bind to peptide antigens in a semi-promiscuous fashion, however the peptidic side chains which act as anchor residues, impose structural constraints upon the number of peptides which can be functionally presented by each HLA allele (Hammer *et al* 1993). Alleles which are related share a similar core sequence, and thus may display overlapping peptide binding repertoires (Malcherek *et al* 1995; Sette *et al* 1989), whilst different HLA classes show distinct levels of variability in their contact residues (Figure 1.4.3).

The extended conformation of peptides bound to MHC class II molecules places up to twelve peptide residues in contact with the peptide binding groove (McFarland and Beeson 2002), with nine core residues directly binding within the groove (Carson *et al* 1997). Pockets in these peptide binding grooves accommodate some of the anchoring peptidic side chains, allowing the remaining peptides to interact with TCRs. Binding pockets are numbered along the peptide relative to a large N-terminal hydrophobic pocket, and the peptides adopt a polyproline type II-like conformation (Jardetzky *et al* 1996). Peptide binding motifs for the majority of human and murine MHC class II molecules follow a P1, P4, P6, P9 pattern, in which the side chains of the amino acids located at these positions insert into prominent binding pockets formed by the class II molecule, with smaller, shallow pockets at P3 and P7 (Figure 1.4.4) (Fremont *et al* 2002; Lee *et al* 2001; Li *et al* 2000; Liu *et al* 2002; Southwood *et al* 1998; Stern *et al* 1994). The individual binding pockets show preference for specific residues, for example, in the common allelic variant DRB1*0101, the P1 position, which in most class II molecules comprises the deepest binding pocket, shows a strong preference for large hydrophobic residues such as Tyrosine, Tryptophan, Phenylalanine, Leucine, and Isoleucine (Southwood *et al* 1998). While smaller residues, such as Glycine, Alanine, Serine, and Proline are preferentially presented by the P6 pocket, and the P4 and P9 pockets tend to show a weak preferences for aliphatic residues (Hammer *et al* 1992; Southwood *et al* 1998). There is also a growing appreciation for the influence that polymorphic flanking residues at the C-terminus of the binding groove may have upon peptide binding (Bartnes *et al* 1999; Parry 2008). By contrast, the comparable N-terminal flanking position of P1 shows a negligible contribution to diversity in peptide binding due to its conserved nature (Parry 2008).

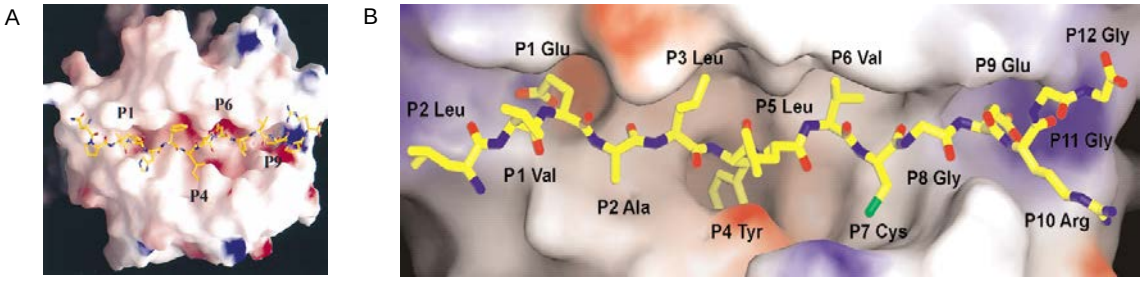


Figure 1.4.4 The 3D surface structure of MHC class II/peptide complexes as presented to T cells.
(A) The structure of the HLA-DR15/MBP_{85-99} peptide complex, from asparagine at P3 to arginine at P11. The hydrophobic P1 and large P4 pockets are occupied by valine and phenylalanine, respectively, serving as the primary MHC anchor residues of the MBP peptide, while histidine, phenylalanine, and lysine, at positions P2, P3 and P5, respectively, exist as prominent, solvent exposed residues, important for TCR recognition of the peptide, figure adapted from (Smith *et al* 1998). (B) The structure of the HLA-DQ3/$InsulinB_{9-23}$ peptide complex. The peptide side chains glutamic acid, tyrosine and glutamic acid are found in deep P1, P4 and P9 pockets, respectively, while the valine and cysteine residues occupy shallow, shelf-like positions found at positions P6 and P7, respectively, figure adapted from (Lee *et al* 2001). Nonpolar regions in the models are represented as white, positively charged areas as blue, and electrostatically negative regions as red.

The variation in structural features due to divergent residues in the peptide binding pockets of MHC molecules give rise to differences in the binding motifs, impacting upon the peptides presented by both DR and DQ subtypes. The P1 binding pocket is strongly conserved and plays a major role as the primary anchor position in DR molecules, while P1 appears relatively degenerate in the DQ molecules and their murine I-A homologue (Reizis *et al* 1996; Reizis *et al* 1997). This occurs due to the polar rather than hydrophobic characteristics of residues within the DQ P1 pocket, allowing this pocket to accommodate a broad range of residues, which leads to a more restricted role in dictating critical peptide binding anchors (Reizis *et al* 1996). Instead, the P4 and P9 pockets play a prominent role in peptide binding in DQ molecules (Kwok *et al* 1996). Similarities in the structure and relative importance of anchor residues have also been described

between the DR and DQ subtypes, such as those observed in the DRB1*0403 and DQB1*0301 molecules, which show extensive parity, particularly in the P4 and P9 binding pockets, and are both positively correlated with protection from the autoimmune disease IDDM (Cucca *et al* 2001). The role different binding pockets play in governing susceptibility and resistance to disease can be illustrated by the relative contributions of the DQ and DR peptide binding motifs towards the development of IDDM. The DQA1*0102/DQB1*0602 haplotype encodes an asparagine at the β57 position, this negative residue is assocated with a low risk for IDDM across different ethnic groups (Sanjeevi *et al* 1997; Todd *et al* 1987). While the increased susceptibility of DQA1*0301/DQB1*0302 individuals to IDDM can be linked to the presence of a positively charged alanine, valine or serine residue at the same β57 position, increasing the affinity of the P9 pocket for the negatively charged amino acids found within a prominent T cell epitope of the insulin B chain (Lee *et al* 2001; Todd *et al* 1987). In contrast, it is the presence of a glycine residue at β86 which allows the high affinity binding of large aromatic side chains to the P1 pocket in the DRB1*0405 and DRB1*0401 molecules, confering an increased susceptibility to IDDM in individuals expressing these MHC molecules (Cucca *et al* 2001). The peptidic anchor positions can therefore have a dramatic influence upon binding capacity, which impacts upon the epitope hierarchy in an allele specific manner. The wide range of polymorphisms inherent in the DR/I-E and DQ/I-A subtypes of MHC class II molecules increase the diversity of peptides presented to TCRs, shaping the T cell repertoire whilst at the same time accommodating common pathways of antigen presentation and recognition. The MHC subtypes achieve this through binding peptides in a broadly similar fashion, whilst employing a divergent hierarchy of anchor specificies.

In addition to the peptidic binding preferences of individual MHC pockets, the epitope hierarchy also appears to rely upon the promiscuity and specificity of the epitopes. Contrasting studies have found that multiple common DR alleles, which include DRB1*0101, DR5*0101, DRB1*1501, DRB1*0401, DRB1*1302, DRB1*0701 and DRB1*0901, share overlapping peptide repertoires, allowing the recognition of the same epitope in the context of different HLA-DR alleles (Hammer *et al* 1993; Hill *et al* 1994; Rothbard and Taylor 1988; Sinigaglia *et al* 1988). Promiscuous binding of determinants to different HLA types has also been observed in the immune response to MBP (Valli *et al* 1993) and HIV (Berzofsky *et al* 1991) antigens, indicating that promiscuity can play an important role in determining immunodominance to both self and non-self derived antigens. Broadly degenerate MHC class II peptidic epitopes, as well as the polymorphisms inherent in class II alleles, therefore clearly have potential implications for epitope based vaccine design.

Inclusion of epitopes which are capable of binding to a wide range of allelic products from a single MHC locus, or even to different MHC molecules within an individual, in prophylatic or therapeutic vaccines, may provide population coverage irrespective of ethnic bias. The inclusion of these epitopes may also allow HLA-restricted CD4+ T cells to be directed against cryptic or sub-dominant regions of an antigen. In contrast to immunodominant peptide epitopes, cryptic or sub-dominant epitopes prime T cell responses only when made available as peptides, and not as part of an intact protein which requires processing. Previous studies have suggested that inclusion of these cryptic determinants in vaccines may allow the adaptive immune response to avoid suppressive determinants and exploit conserved antigenic regions of pathogens (Amante *et al* 1997; Oscherwitz *et al* 2009; Rathore *et al* 2005; Xu *et al* 2000).

1.4.6 Immune Epitope Database

In the past 200 years since Pasteur developed the first anthrax vaccine containing attenuated live organisms (Turnbull 1991), the anthrax vaccines in clinical and agricultural use have largely been empirically derived. This historically included the non-encapsulated, live 34F2 strain vaccine introduced by Sterne in 1939 (Baillie 2006), the Soviet era non-encapsulated, live STI-1 strain vaccine (Shlyakhov and Rubinstein 1994), and has continued with the cell culture filtrate based AVA and AVP vaccines in use today (Baillie 2006; Baillie *et al* 1999). The sub-optimal immune responses to these vaccines (Skowera *et al* 2005), in combination with documented side effects (Brey 2005; Enstone *et al* 2003), has prompted the need for rationally designed, subunit vaccines. To elicit effective CD4+ T cell responses to *B. anthracis*, these vaccines should contain peptide epitopes which provoke a robust response. Recent research into subunit vaccines has demonstrated the important role epitope identification plays in the development of more effective and less reactogenic vaccines against bacterial pathogens such as *Y.pestis* (Li *et al* 2009) and *F. tularensis* (Yu *et al* 2010).

The minimal sequences which are capable of eliciting an immune response are known as epitopes, and effective incorporation of these epitopes into a vaccine stimulates a cellular immune response directed at conserved, immunogenic regions of pathogen derived antigens. The development of immune epitope databases has provided researchers with an invaluable tool, enabling the selection of predicted or experimentally determined epitopes which generate the most effective immune response to a pathogen, in a specific population or individual (Kim *et al* 2010; Tong and Ren 2009).

The Immune Epitope Database (IEDB) represents a centralized immunological database of B and T cell epitope information and related analysis tools, curated by the La Jolla Institute for Allergy and Immunology (LIAI), and the NIAID, as part of a NIH project (Vita *et al* 2006; Zhang *et al* 2008). Peptidic epitope data for humans, non-human primates, rodents, and other animal species is available for infectious diseases, allergens and autoimmune diseases, with a specific emphasis upon NIAID category A, B, and C priority pathogens and NIAID emerging and re-emerging infectious diseases (Vita *et al* 2010; Zhang *et al* 2008). The work described within this thesis, analyzing one of the major immunogenic proteins of *B. anthracis*, forms a component of the NIH funded large scale epitope mapping project. Under this scheme, awards were made to 14 laboratories to undertake work that would lead to the better characterization of CD4 and CD8 epitopes of pathogens on the NIAID list of category A-C microbial pathogens.

1.5 Aims of this thesis

Vaccination strategies in anthrax infection have been largely dominated by studies of PA. However, it has recently become clear that PA based vaccines are unable to elicit protection from all strains of *B. anthracis* (Wang and Roehrl 2005). As such, incorporating a broader range of antigens may represent a more successful vaccination strategy. Recent research by the Altmann lab has indicated that LF rather than PA may represent a major target for immunity in naturally exposed individuals (Ingram *et al* 2010b).

The main focus of work within this thesis therefore involved defining the immunodominant and cryptic CD4+ T cell epitopes relevant for the presentation of antigen from the LF toxin found in *B. anthracis*. This was achieved using transgenic mouse lines which express human HLA class II alleles HLA-DR1, HLA-DR15, HLA-DR4, HLA-DQ8 and HLA-DQ6. The epitopes identified using the responses of transgenic mouse lines were compared with the responses of naturally infected and vaccinated humans and the relative HLA binding affinities of the LF peptides. HLA class II polymorphisms influence the onset and severity of pathology, therefore the survival of the transgenic mice following live anthrax challenge was also compared with the magnitude of the response to LF, and to HLA expression levels.

Having identified an exceptionally strong HLA-DR15-binding peptide from LF, work was undertaken to identify the effects of this peptide on the progression of disease in a DR15-restricted transgenic mouse model which develops spontaneous paralysis with central nervous system (CNS) pathology similar to human multiple sclerosis (MS).

2 Materials and Methods

2.1 Materials

2.1.1 Solutions and buffers

Annexin V binding buffer

0.01M Hepes/NaOH pH 7.4

0.14M NaCl

2.5mM $CaCl_2$

Anti-CD3/CD28 buffer

2mM EDTA

0.1% BSA

PBS pH 7.4

ELISA blocking buffer

1% BSA

PBS, pH 7.4

ELISA wash buffer

0.05% Tween 20

PBS pH 7.4

ELISpot wash buffer

0.1% Tween 20

PBS pH 7.4

FACS Buffer

0.02% sodium azide

10% FCS

PBS

HEPES buffer

10mM HEPES

50mM NaCl pH 7.5

HLA binding buffer

10 mM phosphate

150mM NaCl

1mM n-dodecyl β-D-maltoside

10 mM citrate pH 6.0

HLA binding neutralisation buffer

450mM Tris HCl pH 7.5

0.3% BSA

1mM n-dodecyl β-D-maltoside

Mammalian Cell Lysis Buffer

50mM Tris-HCl (pH 8.0)

100mM EDTA

100mM NaCl

1% SDS

MTT Lysis buffer

10% sodium dodecyl sulphate

45% dimethylformamide

adjusted to pH 4.5 with glacial acetic acid

Phosphate Buffered Solution (PBS)

137mM NaCl

2.7mM KCl

10mM Na_2HPO_4

2mM KH_2PO_4

Plasmid Elution Buffer

1.25M NaCl

50mM Tris·Cl, pH 8.5

15% isopropanol (v/v)

Plasmid Equilibration Buffer

750mM NaCl

50mM MOPS, pH 7.0

15% isopropanol (v/v)

0.15% Triton X-100 (v/v)

Plasmid Injection Buffer

7.5mM Tris

0.1mM EDTA

Embryo grade H_2O (Sigma)

Plasmid Lysis Buffer

200mM NaOH

1% SDS (w/v)

Plasmid Resuspension Buffer

50mM Tris·Cl, pH 8.0

10mM EDTA

100μg/ml RNase A

Plasmid Wash Buffer

1.0M NaCl

50mM MOPS, pH 7.0

15% isopropanol (v/v)

Protein elution buffer

150mM imidazole

50mM sodium phosphate

300mM NaCl pH 7.0

SOC medium

2% Tryptone

0.5% Yeast extract

10mM NaCl

2.5mM KCl

10mM $MgCl_2$

20mM glucose

Tris-acetate buffer (TAE)

2M Tris

1.56M Acetic Acid

0.05M EDTA pH 8.0

Tris/Borate/EDTA buffer (TBE)

89mM Tris-HCl pH 7.8

89mM Borate

2mM EDTA

2.1.2 Antibodies

Table 2.1.1 Antibodies used in this study

Antibody Name	Specificity	Clone	Isotype	Flurochrome	Supplier
TAL 4.1	HLA-DQA1	WT46	IgG2b	Alexa Fluor 546	Santa Cruz
Leu10	HLA-DQB1	SK10	IgG1κ	FITC	Becton Dickinson
TÜ169	HLA-DQ1 dimers	TÜ169	IgG2aκ	FITC	Pharmingen
M5/114.15.2	I-A/I-E	M5/114.15.2	IgG2bκ	PE	Becton Dickinson
17.3.3	I-Eβ	17.3.3	IgG2aκ	FITC	Becton Dickinson
AF6.120.1	I-Aα	AF6.120.1	IgG2aκ	FITC	Becton Dickinson
25-9-17	I-Aβ	25-9-17	IgG2aκ	FITC	Becton Dickinson
B220/CD45R	B220	RA3-6B2	IgG2aκ	PE	Becton Dickinson
B220/CD45R	B220	RA3-6B2	IgG2aκ	FITC	Serotec
CD19	CD19	4G7	IgG1κ	PE	Becton Dickinson
GK1.5	CD4	GK1.5	IgG2bκ	PE	EBioscience
L3T4	CD4	L3T4	IgG2bκ	APC-H7	Pharmingen
53-6.7	CD8	53-6.7	IgG2aκ	PE	Becton Dickinson
53-6.7	CD8	53-6.7	IgG2aκ	FITC	Becton Dickinson
NK-1.1	NK	PK136	IgG2aκ	PE-Cy7	Pharmingen
CD3e	CD3 ε chain	500A2	IgG2κ	Pacific blue	Pharmingen
IFNγ	IFNγ	XMG1.2	IgG1	FITC	Serotec
IFNγ	IFNγ	XMG1.2	IgG1κ	Alexa Fluor 700	Pharmingen
IL-17A	IL-17A	TC11-18H10	IgG1κ	Alexa Fluor 647	Pharmingen
IL-10	IL-10	JES5-16E3	IgG2b	PE	Pharmingen
Fc block	CD16/CD32 (Fcγ III/II Receptor)	2.4G2	IgG2bκ	Purified	Pharmingen

2.1.3 Primers used in this study

Table 2.1.2 Genomic primers used in this study

Specificity	Sense primer sequence (5'----->3')	Antisense primer sequence (5'----->3')
DRA1*0101	CTCCAAGCCCTCTCCCAGAG	ATGTGCCTTACAGAGGCC
DRB1*0101	TTCAATGGGACGGAGCGGGTG	CTGCACTGTGAAGCTCTC
DRB1*1501	TGAAAGCGGTGCGTGCTG	CACCCGCTCCGTCCCGTTGAA
DQA1*0301	TACCAGTTTTACGGTCCCTC	GGTAGCAGCGGTAGAGTTG
DQB1*0302	GCATGTGCTACTTCACCAACG	CACCGTGTCCACCGACGCCCG
DQA1*010201	TAATGTCCTCCCTTTATTCA	AGGAAATCTGAAGACACCCA
DQB1*0602	TTCCAGATCCTGGTGATG	GTAGGCAGTTTCCTCAG
TCR-Vα3.1Jα40	ATTCAGGTAGAGGCCTTGTCCA	GAGGTAGTATCCGTAGCACAG
TCR-Vβ2.1Jβ2.1	GATGGCAACTTCCAATGAGGGCT	CGCCCCGCTAGTTAGATCTC
I-E/I-A	TCCGCAGGGCATTTCGTGTA	AGGGAGGTGTGGGTCTCCGG
Neo cassette	TCCGCAGGGCATTTCGTGTA	GAGGATCTCGTCGTGACCCA

Table 2.1.3 qPCR primers used in this study

Specificity	Sense primer sequence (5'----->3')	Antisense primer sequence (5'----->3')	Hydrolysis probe sequence (5'----->3')
CD3e	CATTGAATACAAAGTCTCCATCTCA	ACCAGGTGCTTATCATGCTTCT	TGACGTGCCCTCTAGACAGTGACGAGA
T-bet	ACTTTGAGTCCATGTACGCATCT	AGGATACTGGTTGGATAGAAGAGGT	CACCTGGACCCAACTGTCAACTGCTT
GATA3	TACCACCTATCCGCCCTATGT	ACACACTCCCTGCCTTCTGT	ACAGCTCTGGACTCTTCCCACCCAG
RORγt	GTCTGCAAGTCCTTCCGAGAG	ATCTCCCACATTGACTTCTG	CTGCGACTGGAGGACCTTCTACGGC
Foxp3	ATAGTTCCTTCCCAGAGTTCTTCC	ATGGTAGATTTCATTGAGTGTCCTC	CACCTATGCCACCCTTATCCGATGG
LAG3	CTCAAGGTTCTGGGTCTGGA	ACTTGGCAATGAGCAAAGAAG	CCCTCTGACAGTGTACGCTGCTGAAG
GAPDH	GAGAAACCTGCCAAGTATGATGAC	AGACAACCTGGTCCTCAGTGTAG	TCAAGAAGGTGGTGAAGCAGGCATC
Hprt1	AACCAGGTTATGACCTAGATTTGTTT	CAAGTCTTTCAGTCCTGTCCATAAT	CCTAATCATTATGCCGAGGATTTGGAA
Tbp	CAGTGCCCAGCATCACTATTT	GCATCCTCTGAATATCTCCTTAGAA	CATGGTGTGTGAAGATAACCCAGAACA
B2m	CTACTGGGATCGAGACATGTGAT	TGTGTACATTGCTATTTCTTTCTGC	TGCTCTGAAGATTCATTTGAACCTGCT
Rpl-13a	CCTACCAGAAAGTTTGCTTACCTG	GATCTGCTTCTTCTTCCGATAGTG	TGACAGCCACTCTGGAGGAGAAACG
YWHAZ	AACTTAACATTGTGGACATCGGATA	TGGATGACAAATGGTCTACTGTGTA	TGAAGCAGAAGCAGGAGAAGGAGGG
TfrC	AATGGTAACTTAGACCCAGTGGAG	ATTAGCATGGACCAGTTTACCAGA	TCCCGAGGGTTATGTGGCATTCAGT
Sdha	CTGGTGTGGATGTCACTAAGGAG	CACCTGTCCCTTGTAGTTAGTGG	CACTGTGCATTACAACATGGGTGGG

2.1.4 LF peptides used in this study

Table 2.1.4 The amino acid sequence of the peptide panel covering domains I to IV of LF. Each peptide is composed of 20 amino acids overlapping by 10mers.

Domain	Amino Acid Sequence Number	Amino Acid Sequence
I	31-50	VQGAGGHGDVGMHVKEKEKN
	41-60	GMHVKEKEKNKDENKRKDEE
	51-70	KDENKRKDEERNKTQEEHLK
	61-80	RNKTQEEHLKEIMKHIVKIE
	71-90	EIMKHIVKIEVKGEEAVKKE
	81-100	VKGEEAVKKEAAEKLLEKVP
	91-110	AAEKLLEKVPSDVLEMYKAI
	101-120	SDVLEMYKAIGGKIYIVDGD
	111-130	GGKIYIVDGDITKHISLEAL
	121-140	ITKHISLEALSEDKKKIKDI
	131-150	SEDKKKIKDIYGKDALLHEH
	141-160	YGKDALLHEHYVYAKEGYEP
	151-170	YVYAKEGYEPVLVIQSSEDY
	161-180	VLVIQSSEDYVENTEKALNV
	171-190	VENTEKALNVYYEIGKILSR
	181-200	YYEIGKILSRDILSKINQPY
	191-210	DILSKINQPYQKFLDVLNTI
	201-220	QKFLDVLNTIKNASDSDGQD
	211-230	KNASDSDGQDLLFTNQLKEH
	221-240	LLFTNQLKEHPTDFSVEFLE
	231-250	PTDFSVEFLEQNSNEVQEVF
	241-260	QNSNEVQEVFAKAFAYYIEP
	251-270	AKAFAYYIEPQHRDVLQLYA
	261-280	QHRDVLQLYAPEAFNYMDKF
	271-290	PEAFNYMDKFNEQEINLSLE
	281-300	NEQEINLSLEELKDQRMLSR
II	287-306	LSLEELKDQRMLSRYEKWEK
	297-316	MLSRYEKWEKIKQHYQHWSD
	307-326	IKQHYQHWSDSLSEEGRGLL
III	317-336	SLSEEGRGLLKKLQIPIEPK
	327-346	KKLQIPIEPKKDDIIHSLSQ
	337-356	KDDIIHSLSQEEKELLKRIQ
	347-366	EEKELLKRIQIDSSDFLSTE
	357-376	IDSSDFLSTEEKEFLKKLQI
	367-386	EKEFLKKLQIDIRDSLSEEE
	377-396	DIRDSLSEEEKELLNRIQVD
	387-406	KELLNRIQVDSSNPLSEKEK
	397-416	SSNPLSEKEKEFLKKLKLDI

II	407-426	EFLKKLKLDIQPYDINQRLQ
	417-436	QPYDINQRLQDTGGLIDSPS
	427-446	DTGGLIDSPSINLDVRKQYK
	437-456	INLDVRKQYKRDIQNIDALL
	447-466	RDIQNIDALLHQSIGSTLYN
	457-476	HQSIGSTLYNKIYLYENMNI
	467-486	KIYLYENMNINNLTATLGAD
	477-496	NNLTATLGADLVDSTDNTKI
	487-506	LVDSTDNTKINRGIFNEFKK
	497-516	NRGIFNEFKKNFKYSISSNY
	507-526	NFKYSISSNYMIVDINERPA
	517-536	MIVDINERPALDNERLKWRI
	527-546	LDNERLKWRIQLSPDTRAGY
	537-556	QLSPDTRAGYLENGKLILQR
	547-568	NLENGKLILQRNIGLEIKDVQI
	558-584	IGLEIKDVQIIKQSEKEYIRIDAKVVP
IV	574-593	EYIRIDAKVVPKSKIDTKIQ
	584-603	PKSKIDTKIQEAQLNINQEW
	594-613	EAQLNINQEWNKALGLPKYT
	604-623	NKALGLPKYTKLITFNVHNR
	614-633	KLITFNVHNRYASNIVESAY
	624-643	YASNIVESAYLILNEWKNNI
	634-653	LILNEWKNNIQSDLIKKVTN
	644-663	QSDLIKKVTNYLVDGNGRFV
	654-673	YLVDGNGRFVFTDITLPNIA
	664-683	FTDITLPNIAEQYTHQDEIY
	674-693	EQYTHQDEIYEQVHSKGLYV
	684-703	EQVHSKGLYVPESRSILLHG
	694-713	PESRSILLHGPSKGVELRND
	704-723	PSKGVELRNDSEGFIHEFGH
	714-733	SEGFIHEFGHAVDDYAGYLL
	724-743	AVDDYAGYLLDKNQSDLVTN
	734-753	DKNQSDLVTNSKKFIDIFKE
	744-763	SKKFIDIFKEEGSNLTSYGR
	754-773	EGSNLTSYGRTNEAEFFAEA
	764-783	TNEAEFFAEAFRLMHSTDHA
	774-793	FRLMHSTDHAERLKVQKNAP
	784-803	ERLKVQKNAPKTFQFINDQI
	794-809	KTFQFINDQIKFIINS

2.2 Methods

2.2.1 Transforming competent cells

Ligated inserts for DQA1*010201 and DQB1*0602 were cloned from the Supercos I vector (Stratagene, USA) generated from the ROF-NL cell line. This is a B-lymphoblastoid cell line prepared by Epstein-Barr virus (EBV) transformation from an HLA-DR15, HLA-DR4, HLA-DQ6 and HLA-DQ8 individual with narcolepsy and cataplexy (Lock *et al* 1988). Fifty nanograms of the construct were transformed into 100µl competent DH5α -TI^{R} *Escherichia coli* cells (Invitrogen, UK) by incubating together on ice for 30 minutes then heat-shocking at 42°C for 45 seconds. The cells were then transferred to ice and 250µl of room temperature SOC medium was added to the solution, which was incubated at 37°C for 1 hour with constant agitation. 10µl of cells from each transformation were spread out onto LB agar plates (Merck Sharp & Dohme, Ltd., UK) containing 50µg/ml of kanamycin (Sigma-Aldrich, USA) and cultured at 37°C for 48 hours. Cells which had acquired expression of the neo genes from the plasmid vector were resistant to the presence of the kanamycin antibiotic.

2.2.2 Isolation and purification of plasmid DNA

DNA was isolated from plasmids using the Invitrogen Maxiprep Kit following the manufacturer's protocol. Briefly, individual *E.coli* colonies were picked and inoculated into 500ml of LB broth (Merck Sharp & Dohme, Ltd., UK) containing 100µg/ml of kanamycin and cultured at 37°C with constant agitation for 24 hours. The cells were pelleted by centrifuging at 50,000xg for 10 minutes, then resuspended in 10ml plasmid resuspension buffer. 10ml plasmid lysis buffer was added to this and mixed by gentle inversion. Following 5 minutes incubation at

room temperature 10 ml plasmid precipitation buffer was added to each tube and mixed by gentle inversion. The solution was centrifuged at 50,000xg for 10 minutes and the supernatant was loaded onto HiPure Maxicolumns (Invitrogen, UK) which had been washed with 30ml plasmid equilibration buffer. Unbound contaminants were washed through the columns with 60ml plasmid wash buffer. Then the DNA was collected from the columns by washing with 15ml elution buffer, 10ml isopropanol (VWR International Ltd., UK) was added to each sample and the DNA was pelleted by centrifuging at 50,000xg for 30 minutes. The DNA was washed by resuspension in 5ml of 70% ethanol (VWR International Ltd., UK) and centrifugation at 50,000xg for 5 minutes. The pellet was air-dried and resuspended in molecular grade H_2O (Sigma-Aldrich, USA) The optical density and concentration of the DNA was determined using an Eppendorf Biophotometer® spectrophotometer. The 260/280nm ratios were approximately 1.9 and the DNA concentrations were ≥2000µg/ml. For DQα the plasmid DNA was cleaved using a 50µl NotI endonuclease restriction digest which contained; 20µl Plasmid, 5µl NotI, 3µl of 100mM NaCl, 50mM Tris HCl, 10mM $MgCl_2$ and 1mM dithiothreitol in 22µl H_2O. For DQβ the plasmid DNA was cleaved using a 80µl NotI and HpaI endonuclease double restriction digest which contained; 27µl Plasmid, 4µl NotI, 4µl HpaI, 8µl of 50mM potassium acetate, 20mM Tris-acetate, 10mM Magnesium acetate, and 1mM dithiothreitol in 22µl H_2O (all restriction endonucleases and appropriate reagents were obtained from New England Biolabs, USA). The restriction digests were incubated at 37°C for 12 hours, the DNA was then separated out by running on a 0.8% agarose gel (Sigma-Aldrich, USA) in TAE buffer with 10µl ethidium bromide (Sigma-Aldrich, USA) run in TAE buffer at 0.5 volts/cm^{-1} for 10 hours using a flat-bed tank and a ECPS 3000/150 Electrophoresis Constant Power Supply (both from Pharmacia Biotech, USA). The bands were visualized and excised from the gel utilising the UV GelDoc-It™ Imaging

System (UVP, UK). The DNA was then purified following the manufacturers protocol for the Anachem Geneclean II Kit. Briefly, the gel was incubated with 4M sodium iodide (Sigma-Aldrich, USA) at 55°C for 1 minute, then 5µl of glassmilk beads were added to each sample and incubated at room temperature for 30 minutes with constant agitation. The glassmilk was pelleted by centrifuging at 15,000xg for 5 seconds, then washed twice in 30% ethanol by centrifugation at 15,000xg for 5 seconds. The pellets were air-dried and resuspended in 10µl injection buffer. The solution was applied to 0.22µm spin filters (Millipore, UK) and centrifuged at 15,000xg for 30 seconds. The purity and concentration of the collected DNA was determined by running on a 2% agarose gel in TAE buffer with 5µl ethidium bromide in TAE buffer at 10 volts/cm^{-1} for 40 minutes. Following purification 4µl of 66.9µg/ml DQα and 80µl of 5.2µg/ml DQβ constructs were given to Dr Godwin at the Embryonic Stem Cell Facility, Hammersmith Hospital for microinjection into C57BL/6 x CBA F1 fertilized oocytes to generate strain founders.

2.2.3 Genomic DNA extraction

5mm tail snips were lysed overnight at 56°C in 400µl of mammalian cell lysis buffer and 10µl of Proteinase K (Amresco, USA) at 100µg/ml. Following complete lysis, samples were agitated with 200µl of saturated NaCl (Sigma-Aldrich, USA), and centrifuged for 15 minutes at 15,000xg. The supernatants were transferred into 600µl of isopropanol, and the DNA precipitated by gentle agitation. DNA was recovered by centrifuging for 10 minutes at 9000xg, the pellet was then washed in 1ml 70% ethanol and centrifuged for 10 minutes at 9000xg. The pellet was air-dried and resuspended in molecular grade H_2O to 100ng/µl at a 260/280nm ratio of approximately 1.8.

2.2.4 Genomic PCR

All primers were designed using GeneFisher software and synthesised by Sigma-Genosys. The 25µl PCR reaction mixtures containing; 2.5µl of 10x PCR Buffer (Invitrogen, UK), 0.1µl of Taq DNA polymerase (Invitrogen, UK), 1.25µl of 5' sense primer at 100µg/ml, 1.25µl of 3' anti sense primer 100µg/ml, 5µl of $MgCl_2$ at 15mM (Invitrogen, UK), 7.4µl of molecular grade H_2O (Sigma-Aldrich, USA), 5µl of a 10mM dNTP mix (25mM each of ATP, TTP, CTP, GTP) (Invitrogen, UK), and 2.5µl of 40ng/µl genomic DNA per sample, were amplified on a PTC-200, Peltier thermal cycler PCR block (Bio-Rad Labs., USA), using the appropriate primers (Table 2.1.2) and PCR programme (Table 2.2.1, Table 2.2.2, Table 2.2.3, Table 2.2.4);

Table 2.2.1 PCR program for DRA1*0101, DRB1*0101 and DRB1*1501

1 cycle	95°C for 5 minutes
32 cycles	94°C for 1 minute 55 ^{o}C for 1 minute 72 ^{o}C for 1 minute
1 cycle	72 ^{o}C for 8 minutes

Table 2.2.2 PCR program for DQA1*0301 and DQB1*0302

1 cycle	95°C for 5 minutes
32 cycles	94°C for 1 minute 57 ^{o}C for 1 minute 72 ^{o}C for 1 minute
1 cycle	72 ^{o}C for 8 minutes

Table 2.2.3 PCR program for DQA1*010201

1 cycle	95°C for 5 minutes
30 cycles	94°C for 1 minute 50 °C for 1 minute 72 °C for 1 minute
1 cycle	72 °C for 8 minutes

Table 2.2.4 PCR program for DQB1*0602

1 cycle	95°C for 5 minutes
30 cycles	94°C for 1 minute 56 °C for 1 minute 72 °C for 45 seconds
1 cycle	72 °C for 8 minutes

2.2.5 Electrophoresis

All genomic PCR products were electrophoresed on a 2% agarose gel, run in TAE buffer. The samples (14µl of PCR product plus 1µl loading buffer (Bioline, UK)) were loaded into each well. The first well of each gel always contained 5µl of a 10Kb molecular weight marker (Bioline, UK). Samples were electrophoresed at 10 volts/cm^{-1} for 40 minutes. The gels were visualized on a UV GelDoc-It™ Imaging System using Labworks™ software.

2.2.6 RNA Extraction

Tissues were immersed in RNA later (Invitrogen, UK), (the volume of RNA later was at least ten times that of the tissues), cells were pelleted by centrifugation at 300xg and added to 500µl of

trizol (Sigma-Aldrich), these were immediately stored at -80°C until RNA extraction. Tissues were transferred to 500µl of trizol then homogenised using a disposable RNA free plastic pestle in a 1.5ml Eppendorf tube. 200µl of chloroform was added to each tube of either homogenised tissue or cells, and shaken vigorously for 15 seconds. The tubes were incubated for 3 minutes at room temperature then centrifuged at 12000xg for 15 minutes at 4°C. The top colourless aqueous layer was pipetted into a fresh eppendorf and 500µl of isopropanol was added, the resulting mixture was incubated for 10 minutes at room temperature, this was then centrifuged at 12000xg for 15 minutes at 4°C. The supernatant was discarded and the pellet washed in 1ml of 75% ethanol, this was then centrifuged at 8000xg for 5 minutes at 4°C. The supernatant was discarded and the pellet was air dried for 30 minutes before being resuspended in 30ul nuclease free water (Qiagen) and 1.5ul RNAse out (Invitrogen, UK). The RNA was incubated at 58°C for 10 minutes, then resuspended to 200ng/µl at a 260/280nm ratio of approximately 2.0. The integrity of the RNA was determined by electrophoresis on a 1.5% agarose gel with 0.5 µg/ml ethidium bromide, run in TBE buffer at 10 volts/cm^{-1} for 40 minutes and visualised as previously described to check for the presence of the 18S and 28S bands. The RNA was stored at -80°C until use.

2.2.7 cDNA synthesis

cDNA was synthesised by reverse transcription; 1µl of 250ng/µl random primers (Invitrogen, UK), 1µl of 10mM dNTPs (2.5mM each of ATP, TTP, CTP, GTP) (Invitrogen, UK), 6µl RNase free water and 5µl of RNA at 200ng/µl were incubated at 65°C for 5 minutes. The reaction mixture was cooled on ice for 1 minute. 1µl of 100mm DTT (Invitrogen, UK), 4µl of 5x first strand buffer (Invitrogen, UK), 1µl of 200U/ul Superscript III (Invitrogen, UK) and 1µl RNase

out (Invitrogen, UK) was then added to the mixture, and incubated for 1 hour at 50°C, followed by 15 minutes at 70°C. The cDNA was stored at -20°C until used.

2.2.8 qPCR

The relative expression of target genes was determined using qPCR, the primers and hydrolysis probes were designed using Primer3 software and ordered from SigmaGenosys. The hydrolysis probe's dual fluorescence label was 5'-FAM 3'-TAMRA, and the primers or probe designed to cross exon boundaries. The 20µl qPCR reaction mixtures containing; 10µl of 2x TaqMan Gene Expression Mastermix, 1µl of '5 sense primer at 10µM, 1µl of 3' anti sense primer 10µM, 2µl of hydrolysis probe at 1µM, 5.75µl of nuclease free water, and 0.25µl of 50ng/µl cDNA per sample, were amplified on a Mx3000p machine (Stratagene), using the appropriate primers (Table 2.1.3) and qPCR programme (Table 2.2.5);

Table 2.2.5 qPCR program for all primers and probes

1 cycle	50°C for 2 minutes 95°C for 10 minutes
50 cycles	95°C for 15 seconds 60°C for 15 seconds 72°C for 15 seconds

The efficiency and sensitivity was determined for each of the qPCR primers and probes using a linear standard curve generated from a five fold serial dilution of cDNA from stimulated cells. The qPCR amplication signals were expressed as Ct values, which reflect the intensity of the

reporter dye FAM's emission of each sample minus the baseline signal established in the initial cycles of PCR. The TaqMan gene expression assay was run in triplicate for each sample, and the threshold value was set at 1000, which was used in all subsequent experiments. The reference genes GAPDH, Hprt1, Tbp, B2m, Rpl-13a, YWHAZ, TfrC, and Sdha were run on each of the tissues and cells to determine the two most suitable reference genes in each case. All expression levels were normalised to these two reference genes by the relative expression software tool 2008 (REST) (Corbett Life Science Pty, Ltd), which is derived from the $2^{-\Delta\Delta CT}$ formula (efficiency of target $^{\Delta \text{ Ct target}}$ divided by the efficiency of reference $^{\Delta \text{ Ct reference}}$) (Pfaffl 2001; Pfaffl *et al* 2001). The results were expressed as a fold expression increase or decrease, relative to control.

2.2.9 Humanised transgenic mice

The groups of HLA class II transgenic mice, HLA-DRA1*0101/HLA-DRB1*0101 (Altmann *et al* 1993), HLA-DRA1*0101/HLA-DRB1*0401 (Ito *et al* 1996), HLA-DRA1*0101/HLA-DRB1*1501 (Ellmerich *et al* 2004; Ellmerich *et al* 2005), HLA-DQA1*0301-DQB1*0302 (Boyton *et al* 1998), HLA-DQA1*0102/HLA-DQB1*0602, and the HLA-TCR transgenics, HLA-DRA1*0101/HLA-DRB1*1501/TCR-Vα3.1Jα40/TCR-Vβ2.1Jβ2.1 (Ellmerich *et al* 2004; Ellmerich *et al* 2005) on an H2-Ab^{00} background (Cosgrove *et al* 1991) were used for *in vivo* experiments in age-, sex-, and weight-matched groups. All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by local ethical review.

2.2.10 Immunisation of transgenic models

Mice were immunized intraperitoneally or subcutaneously in the hind footpad or flank, with ≤100µl of antigen, or a control of PBS, emulsified in Titremax gold (Sigma-Aldrich, USA), complete freunds adjuvant (CFA) or incomplete freunds adjuvant (IFA) by syringe extrusion using 27G needles and 1ml syringes.

2.2.11 Collection of blood and tissue samples from transgenic models

Blood samples were either taken from peripheral veins or post-mortem by cardiac puncture into 10µl of 5000 I.U./ml sodium heparin (CP Pharmaceticals Ltd., UK) and the red blood cells were lysed with 1ml Erythrocyte Lysis Buffer (Qiagen, UK). The cells were washed twice in PBS by centrifuging at 300xg for 5 minutes. Mice were sacrificed by cervical dislocation or intraperitoneal injection of 500µl of sodium pentobarbital. The local draining popliteal lymph nodes were removed and disaggregated into a single cell suspension by passing through 0.7µm cell strainers. Spleens were removed and the capsids disrupted using 21G needles and 1ml syringes. The resulting cell suspensions were stained with 0.2% Trypan Blue (Sigma-Aldrich, USA) and counted on Glasstic® Slides (Hycor, Biomedical Inc., USA) using a CK2 inverted phase contrast light microscope.

2.2.12 LF protein and peptide expression and purification

Recombinant full-length LF and individual domains were produced in an *E. coli* expression system by T.B. Huwar at the University of Maryland, Baltimore. In brief, the cysteine residue at position 687 was replaced with glutamic acid to produce a biologically inactive form of LF. The

gene sequence of LF was codon optimized for expression in *E. coli* (GenScript, USA) to allow for the high AT nucleotide content of the protein. Using the pQE30 expression system (Qiagen, Germany) the full length LF and LF domain sequences (Table 2.1.4) were cloned and expressed from *E. coli* as recombinant N-terminal histidine-tagged proteins. Bacterial pellets were disrupted using a French press, and the target proteins recovered by centrifuging for 20 minutes at 45000x*g* at 4°C. These were then incubated with Talon metal affinity resin (Clontech, USA) to bind the N-terminal histidine tag. The proteins were eluted from this resin at 4°C by washing with protein elution buffer. The proteins were dialyzed against HEPES buffer, using a 10000 molecular weight cut-off dialysis cassette (Pierce, Thermo Fisher Scientific, USA), to a final endotoxin level of <4 EU/mg. A synthetic peptide panel, HPLC purified to a purity of ≥98% purity, comprising of 20mer amino acids overlapping by 10 amino acids encompassing the full-length sequence of LF were obtained from a commercial supplier (Abgent, USA) (Table 2.1.4). All peptides were resuspended in DMSO at 25mg/ml.

2.2.13 LF Epitope mapping in transgenic models

Ten days after subcutaneous immunisation with 12.5µg of recombinant full-length LF in Titremax adjuvant, lymph node cells were challenged with 25µg/ml of either recombinant full-length LF, the 4 domains which comprise the LF protein, or the overlapping 20mer peptides covering the full-length LF sequence (Table 2.1.4). This generated a map of the entire LF protein sequence. To confirm the immunodominant epitopes identified by this large scale mapping, mice were then immunized subcutaneously with 12.5µg of the individual LF peptides in Titremax adjuvant. After 10 days the lymph node cells were challenged *in vitro* with 25µg/ml of the recombinant full-length LF and the immunising and two flanking LF peptides. To generate a

map of the cryptic epitopes, mice were immunised with pools of non-overlapping LF peptides comprising domains I or III, (each pool containing ≤6 peptides) in Titremax adjuvant. After 10 days the lymph node cells were challenged with 25µg/ml of the individual LF peptides.

2.2.14 Lymphocyte Proliferation Assay

Cells were resuspended at $3.5x10^6$ cells/ml in HL-1 media (1% L-Glutamine, 1% Penicillin Streptomycin, 2.5% β-Mercaptoethanol) and 100µl/well was plated out in triplicate on 96 well Costar tissue culture plates (Corning Incorporated, USA). The cells were stimulated with 100µl/well of; appropriate antigen, positive controls of 5µg/ml Con A (Sigma-Aldrich, USA) or 25ng/ml of SEB (Sigma-Aldrich, USA) or negative controls of medium alone. The plates were incubated at 37°C, 5% CO_2 for 5 days. Eight hours prior to harvesting, 1µCi/well of [^{3}H]-Thymidine (GE Healthcare, UK) was added. The cells were harvested onto fiberglass filtermats (PerkinElmer, USA) using a Harvester 96® plate harvester (Tomtec, USA) and counted on a Wallac Betaplate scintillation counter (EG&G Instruments, Netherlands). Results were expressed as either delta counts per minute (Δcpm) (cpm of stimulated cells minus cpm of negative control cells) or stimulation index (SI) (cpm of stimulated cells divided by cpm of negative control cells). An SI of ≥2.5 was considered to indicate a positive proliferation response.

2.2.15 Generation of antigen specific T cell lines

Cells were cultured in HL-1 media (1% L-Glutamine, 1% Penicillin Streptomycin, 2.5% β-Mercaptoethanol) at $3.5x10^6$ cells/ml with 25 µg/ml antigen on Costar 12 well plates (Corning Incorporated, USA). After 7 days incubation at 37°C, 5% CO_2 the T cell blasts were fused with a

BW7 CD4 cell line (gift from the Frelinger lab, University of Carolina at Chapel Hill). Prior to the fusion both cell lines had been washed in 1x HBSS (Invitrogen, UK) by centrifuging at 500xg for 10 minutes, to remove all serum. The T cell blasts and BW7 CD4 cells were both resuspended at a concentration of $2x10^6$ cells/ml in 1 ml of 1x HBSS. Fusion was then induced by adding 700 µl of PEG 1500 (Sigma-Aldrich, USA) pre-warmed to 37˚C, drop-wise, with constant gentle agitation. 20 ml of 1x HBSS, pre-warmed to 37˚C, was then added drop-wise to the cell lines with gentle agitation. The cells were centrifuged at 300xg for 5 minutes and the pellet gently resuspended to a concentration of $2x10^6$ cells/ml in RPMI-1640 medium. The cell suspension (100 µl/well) was plated out on flat bottomed Costar 96 well plates at four dilutions, neat, 1:3, 1:9 and 1:27. The cells were incubated at 37˚C, 5% CO_2 for 48 hours, then 100 µl/well of RPMI-1640 containing1x HAT (Sigma-Aldrich, USA) was added to each well. Following 10 days incubation at 37˚C, 5% CO_2 wells showing clonal growth were transferred to 1 ml of 1x HAT on a Costar 24 well plate. After 14 days growth in HAT supplemented medium the cells were transferred into RPMI-1640 containing 1x HT medium (Sigma-Aldrich, USA) and after 14 days growth they were transferred into HL-1 medium (1% L-Glutamine, 1% Penicillin Streptomycin, 2.5% β-Mercaptoethanol).

2.2.16 ELISpot assay

INFγ ELISpots (Diaclone) were used to measure the function of antigen-specific T cell populations directly *ex vivo*. The method was adapted from the manufacturers protocol, briefly, 96 well hydrophobic polyvinyldene difluoride membrane-bottomed plates (MAIP S 45; Millipore) were pre-wet with 50µl/well of 70% ethanol for 30 seconds. The plates were washed twice with 100µl/well of PBS, then coated with 100µl/well of anti-INF-γ monoclonal antibody at

4°C overnight. This was followed by blocking with 100µl/well of 2% skimmed milk for 2 hours at room temperature. The plates were washed once with 100µl/well of PBS, then 100µl/well of antigen was added, in triplicate, to appropriate wells. For each assay a media only negative and a positive control of ConA (5µg/ml) or SEB (25ng/ml) were included. Wells were seeded with 100µl of $2x10^6$cells/ml in HL-1 media (1% L-Glutamine, 1% Penicillin Streptomycin, 2.5% β-Mercaptoethanol) and plates were incubated for 72 hours at 37°C with 5% CO_2. The plate contents were then discarded and plates were incubated with 100µl/well of ELISpot wash buffer for 10 minutes at 4°C. Plates were washed twice with 100µl/well of ELISpot wash buffer, and then incubated for 1 hour 30 minutes at room temperature with 100µl/well biotinylated anti-INF-γ monoclonal antibody. Plates were washed twice with 100µl/well of ELISpot wash buffer, and then incubated for 1 hour at room temperature with 100µl/well of streptavidin-alkaline phosphatise conjugate. Plates were washed three times with 100µl/well of ELISpot wash buffer. Then 50µl/well of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) was added and spot formation monitored visually. The plate contents were then discarded and plates were washed with water, then air-dried and incubated overnight at 4°C to enhance spot clarity. Spots were counted using an automated ELISpot reader (AID), and results were expressed as delta spot forming cells per 10^6 cells ($\Delta SFC/10^6$) ($SFC/10^6$ of stimulated cells minus $SFC/10^6$ of negative control cells). The results were considered positive if the $\Delta SFC/10^6$ was more than two standard deviations above the negative control.

2.2.17 Quantification of cytokine levels by Cytometric Bead Array (CBA)

The Th1/Th2/Th17 CBA kit and the IL-1β CBA flexset kit (both Becton Dickinson, USA) use bead array technology to detect multiple cytokine proteins in samples. The methods were

adapted from the manufacturers protocol, briefly, samples were diluted using an appropriate volume of CBA assay diluent. The lyophilized standards were reconstituted with CBA assay diluent, and after 15 minutes at room temperature a serial dilution of the standards was prepared. Aliquots of appropriate antibody-conjugated cytokine capture beads were added to all samples and standards, and the IL-1β CBA flexset samples and standards were incubated for 1 hour at room temperature. The PE detection reagent was added to the Th1/Th2/Th17 CBA samples and standards, these were then incubated for 2 hours at room temperature. The PE detection reagent was added to the IL-1β CBA samples and standards and they were incubated for a further 1 hour at room temperature. After which, all samples and standards were washed with 1ml of CBA wash buffer by centrifuging at 200xg for 5 minutes. The beads were then resuspended in 300µl of CBA wash buffer and the Th1/Th2/Th17 CBA beads were acquired on the FACSCaliber, the IL-1β CBA flexset beads were acquired on the FACSARIA II. All samples were analysed using FCAP array software.

2.2.18 Quantification of cytokine secretion by ELISA

Levels of murine IFNγ, IL-17A, IL-10, TNFα, and human IL-6 were measured in supernatants removed following cell stimulation for 72 or 120 hours. The method was adapted from the manufacturers protocol (IFNγ, IL-17A and IL-10 Mabtech, TNFα, and IL-6 R&D). Briefly, 96 well high-binding ELISA plates (Costar) were coated overnight at 4°C with 100µl of; 1µg/ml of IFNy capture antibody in binding buffer (0.1 M NaHCO3, pH 8.3), 1 µg/ml of IL-17A capture antibody in PBS pH 7.4, 0.8 µg/ml of TNFα capture antibody in PBS pH 7.4, 2 µg/ml of IL-10 capture antibody in PBS pH 7.4 or 2 µg/ml of IL-6 capture antibody in PBS pH 7.4. Plates were washed 3 times with 200µl/well of PBS pH 7.4, and then blocked with 200µl/well of ELISA

blocking buffer for at least 1 hour at room temperature. Following 3 washes with 200µl/well ELISA wash buffer, 100µl/well of samples were plated in triplicate and appropriate purified recombinant human and mouse cytokines were serially diluted in ELISA blocking buffer on the plate to determine a standard curve. Plates were incubated at 4°C overnight, and then washed 3 times with 200µl/well of ELISA wash buffer. Plates were then incubated at room temperature for 1 hour with 100µl/well of; 0.5 µg/ml IFNγ detection antibody in blocking buffer, 0.5 µg/ml IL-17A detection antibody in blocking buffer, 0.2 µg/ml IL-10 detection antibody in blocking buffer, 0.4 µg/ml TNFα detection antibody in blocking buffer, or 0.2 µg/ml IL-6 detection antibody in ELISA blocking buffer. Plates were washed 3 times with 200µl/well of ELISA wash buffer and then incubated for 30 minutes at room temperature with 100µl/well of streptavidin-HRP diluted 1:1000 in ELISA blocking buffer. Plates were washed 3 times with 200µl/well of ELISA wash buffer, then incubated with 100µl/well of TMB (3,3,5,5-Tetra-methylbenzidine), development of the ELISA was monitored by eye, and the enzymatic reaction was halted by adding 100µl/well of 1M sulfuric acid. The plates were read immediately in a ELISA reader (µQuant BIO-Tek Instruments Inc., software KCJunior;) at 450 nm (wavelength) and 630 nm (reference wavelength). The cytokine concentration in the samples was extrapolated from the standard curve and expressed as delta cytokine levels/ml (cytokine levels in stimulated samples minus cytokine levels in negative control wells).

2.2.19 Antibody Conjugation

Following the manufacturer's instructions, monoclonal antibodies were conjugated to a fluorochrome using the Invitrogen Monoclonal Antibody Labeling Kit. Briefly, 100µg of the monoclonal antibody and isotype matched control in 0.1M sodium bicarbonate were conjugated

for 1 hour, to an Alexa Fluor 546 reactive dye with gentle agitation. The antibodies were loaded onto a spin column packed with size-exclusion purification resin in PBS pH 7.2, 2mM sodium azide. The columns were then spun at 80xg for 5 minutes to collect the labeled antibodies, whose absorbance was measured by spectrophotometer at 280nm (A_{280}) and 558nm (A_{558}). Both the monoclonal antibody and the isotype matched control were labeled with 2 moles of Alexa Fluor 546 dye per mole of antibody. Titrations were then carried out to determine the optimum antibody concentration using the EBV transformed B lymphoblastoid cell line PGF, which constitutively expresses HLA class II (Horn *et al* 1988); (Lee *et al* 1987).

2.2.20 Cell surface staining for Flow Cytometry

Cells were resuspended at 1 x 10^6 in 100µl of FACS buffer, containing the required antibody (Table 2.1.1). Commercially labelled antibodies were used at a concentration of 10µg/ml and the Alexa Fluor 546 labelled antibodies were diluted by a factor of 1/20. The cells were stained for 45 minutes at 4°C, then, following two washes in FACS buffer carried out by centrifuging at 300xg for 5 minutes, they were resuspended in 200µl of 4% Paraformaldehyde (Sigma-Aldrich, USA). The cells stained with directly conjugated antibodies were enumerated on a FACScan (Becton Dickinson, UK). The data was analysed using Flowjo 7.2.2. software.

2.2.21 Intracellular cytokine staining

Cells were resuspended at 1x10^7/ml in HL-1 media (1% L-Glutamine, 1% Penicillin Streptomycin, 2.5% β-Mercaptoethanol) in V-bottom 96-well plates (Costar). As a negative control, cells were incubated in the presence of medium only and as a positive control cells were

incubated in the presence of 10ng/ml PMA and 500ng/ml ionomycin (Sigma). Cells were incubated for either 8 or 24 hours at 37°C and 5% CO_2, in the presence of 10µg/ml Brefeldin A for the final 6 hours. The plates were then washed twice with FACS buffer carried out by centrifuging at 400g for 5 minutes. The cells were then resuspended in 100µl of FACS buffer with appropriate cell surface antibodies and 100µl Fc block (Table 2.1.1). The cells were stained for 45 minutes at 4°C, then washed twice in ice cold PBS, by centrifuging at 300xg for 5 minutes, they were then resuspended in 500µl of commercial fixation/permeabilization buffer (ebioscience) and incubated at 4°C for 6 hours. The cells were then washed once in 100µl of commercial permeabilization buffer (ebioscience), by centrifuging at 300xg for 5 minutes. The cells were then resuspended in 100µl of permeabilization buffer with appropriate intracellular antibodies (Table 2.1.1) and incubated at 4°C for 45 minutes. The cells were washed once in 100µl of permeabilization buffer, by centrifuging at 300xg for 5 minutes, then resuspended in 200µl of 4% Paraformaldehyde. The cells were enumerated on a FACSARIAII (Becton Dickinson, UK). The data was analysed using Flowjo 7.2.2. software.

2.2.22 Competitive HLA binding assay

Competitive ELISAs were used to determine the relative binding affinity of LF peptides to HLA-DR molecules (Texier *et al* 2000). These assays were performed by B. Mallie at the Commissariat a l'Energie Atomique, France. Briefly, the HLA-DR molecules were immunopurified from homozygous EBV-transformed lymphoblastoid B cell lines by affinity chromatography. The HLA-DR molecules were diluted in HLA binding buffer and incubated for 24 to 72 hours with an appropriate biotinylated reporter peptide (Table 2.2.6), and a serial dilution of the competitor LF peptides. A control of unlabeled reporter peptides was used as a

reference peptide to assess the validity of each experiment. 50µl of HLA binding neutralisation buffer was added to each well and the resulting supernatants were incubated for 2 hours at room temperature in ELISA plates (Nunc, Denmark) previously coated with 10µg/ml of the monoclonal antibody L243. Bound biotinylated peptide was detected by addition of streptavidin-alkaline phosphatase conjugate (GE Healthcare, Saclay, France) and 4-methylumbelliferyl phosphate substrate (Sigma-Aldrich, France). Emitted fluorescence was measured at 450 nm post-excitation at 365 nM on a Gemini Spectramax Fluorimeter (Molecular Devices, St. Gregoire, France). LF peptide concentration that prevented binding of 50% of the labeled peptide (IC_{50}) was evaluated, and data expressed as relative binding affinity (ratio of IC_{50} of the LF competitor peptide to the IC_{50} of the reference peptide which binds strongly to the HLA-DR molecule).

Table 2.2.6 Reference peptides with IC_{50} values

HLA-DR molecule	Reference peptide	IC_{50} value
HLA-DRB1*0101	$HA_{306–318}$ PKYVKQNTLKLAT	4Nm
HLA-DRB1*0401	$HA_{306–318}$ PKYVKQNTLKLAT	8Nm
HLA- DRB1*1501	$A3_{152–166}$ EAEQLRAYLDGTGVE	48nM

2.2.23 Peptide binding prediction *in silico*

The regions of the LF domains sequence which bind to HLA-DRB1*0101, HLA-DRB1*0401 and HLA- DRB1*1501 were predicted using the IEDB T cell epitope prediction tool, which is based on the consensus approach. This method averages the ranking of independent algorithms; Sturniolo, SMM-align and average relative binding (ARB) (Wang *et al* 2010, Nielsen *et al* 2007, Bui *et al* 2005 and Sturniolo *et al* 1999) by comparing the score of a peptide against the scores of

five million 15mer peptides randomly selected from the SWISSPROT database, to generate the rank for the consensus method. The smaller the consensus percentile rank the higher the predicted binding affinity.

2.2.24 LF Epitope mapping in human patients

Cells were isolated from human peripheral blood samples and stimulated as described by Ingram *et al* 2010. In brief, sodium heparinised blood was collected from 8 Turkish patients treated for cutaneous anthrax infection within the last 8 years with full informed consent (Ericyes University Ethical Committee) (Table 2.2.7A), 8 volunteers routinely vaccinated every 12 months for a minimum of 5 years with the UK Anthrax Vaccine Precipitated (AVP) vaccine (UK Department of Health under approval by the Convention on Biological Diversity Independent Ethics Committee for the UK Ministry of Defence) (Table 2.2.7B), and 5 age-matched healthy controls with no known exposure to anthrax antigens (Ethics REC reference number 08/H0707/173) (Table 2.2.7C). PBMCs were prepared from the blood using Accuspin tubes (Sigma, Dorset, UK) centrifuged at 800*g* for 30 minutes, after which the cells were removed from the interface and washed twice in AIM-V serum free media. Cells were counted for viability and resuspended at $2x10^6$ cells/ml, then stimulated with recombinant full-length LF, individual LF domains or peptides at an in-well concentration of 25µg/ml, for quantification of IFNγ by ELISpot assay.

Table 2.2.7 HLA class II type, age and sex in addition to clinical details or vaccination schedule of human donors.

Details are shown for (A) cutaneous anthrax patients, (B) AVP vaccinees and (C) healthy controls. Representation of common HLA alleles is similar in the 3 groups of donors.

A

HLA class II						Sex	Age	Lesion site	Clinical severity	Time post infection (days)	Duration of infection (days)
DR1		DRB3/4/5		DQB1							
11	13	52	-	6	7	M	24	Right arm	Mild	523	20
4	-	53	-	8		M	48	Left hand	Severe	211	20
4	14	52	53	5	8	M	33	Right & left arms	Severe	909	40
15	-	51	-	6	-	M	23	Left forearm	Mild	1026	24
8	11	52	-	7	-	M	19	Right wrist	Severe	132	30
11	13	52	-	6	7	M	30	Anterior neck	Toxemic shock	1648	60
4	14	52	53	5	-	M	30	Left wrist	Mild	132	25
15	13	51	52	6	-	F	18	Left forearm	Mild	924	15

B

HLA class II						Sex	Age	Time since first dose (days)	Time since last dose (days)	Number of boosters
DR1		DRB3/4/5		DQB1						
11	15	51	52	6	7	M	33	2019	314	4
11	15	51	52	6	7	M	29	2286	26	6
11	13	52	-	6	7	M	38	1866	182	4
15	7	51	53	2	6	M	29	1992	243	4
103	17	52	-	2	5	M	27	1519	244	3
1	13	52	-	5	6	M	38	3618	244	7
11	15	51	52	6	7	M	32	2088	50	5
1	-	-	-	5	-	M	37	4052	244	9

C

HLA class II						Sex	Age
DR1		DRB3/4/5		DQB1			
13	14	52	-	6	5	F	29
11	16	51	52	5	7	M	22
1	4	53	-	5	8	F	34
7	11	52	53	2	7	M	35
1	4	53	-	5	7	F	31

2.2.25 Quantification of cytokine levels by Luminex

The levels of IL-1β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-17 and TNFα were measured in the supernatants removed from the negative control and LF domain stimulated wells of the human IFNγ ELISpot assay carried out in Ingram *et al* 2010, on cells isolated from Turkish patients naturally infected with anthrax, AVP vaccinees and healthy controls. The supernatants were diluted by 1:1 with AIM-V media. The bead assay, based on a capture sandwich immunoassay method, was adapted from the manufacturers protocol for a Bioplex assay (Bio-Rad, Hercules, CA, USA), briefly, a mixture of antibodies to the cytokines, coupled to internally dyed beads, were incubated with the samples and a standard curve generated by serial dilution of reconstituted standard. The plates were washed twice with commercial Luminex wash buffer to remove unbound protein, and biotinylated detection antibodies were added. Streptavidin-phycoerythrin was then added, and the beads were read using the Luminex 200 system (Luminex Corporation, Austin, TX, USA). The individual dyed bead populations as well as the fluorescent signal on the bead surface were detected. This allowed identification of each cytokine and reported the level of target protein in the well, extrapolated from the standard curve.

2.2.26 Live *B. anthracis* challenge

10 mice per transgenic strain (HLA-DR1, HLA-DR4, HLA-DR15, HLA-DQ8 and HLA-DQ6) plus 10 control C57BL6 mice were challenged intra-peritoneally with 10^5 – 10^6 colony forming units of *B. anthracis* STI strain. These assays were carried out by E.D. Williamson at Defence Science Technology Laboratory, Porton Down. The animals were monitored daily for 20 days post-infection, and post-mortem spleens were homogenized in 1ml of PBS prior to plating out at

a range of dilutions onto L-agar plates. The colonies were counted after 24 hours culture at 37°C, and the mean bacterial count per spleen was determined.

2.2.27 MTT assay of cytotoxicity and cell viability

The cytotoxicity effect of the peptides was estimated by measurement of the rate of mitochondrial metabolism of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 100µl of control and treated cells in HL-1 media (1% L-Glutamine, 1% Penicillin Streptomycin, 2.5% β-Mercaptoethanol) were seeded at 1x10^{5} cells/well in 96-well plates and 10µl of MTT (5mg/ml) were added to each well. Following 4 hours of incubation at 37°C, 100µl of MTT lysis buffer was added to each well and the blue formazan crystals were dissolved by pipetting. The plates were read with a microplate reader using a test wavelength of 595nm and a reference wavelength of 655nm.

2.2.28 Annexin V apoptosis assay

The FITC Annexin V assay was used to quantitatively determine the percentage of cells actively undergoing apoptosis. The method was adapted from the manufacturers protocol (BD, Pharmingen), briefly, 100µl of 1x10^{6}/ml spleen cells were incubated at 37°C for 24 hours with 100µl of peptides at 25µg/ml, cells were washed twice with cold PBS, then resuspended in 100µl of 1x Annexin V binding buffer. 2µl of FITC Annexin V and 2µl of PI was added to the cells and incubated for 15 minutes at room temperature in the dark. 400µl of 1x binding buffer was added to each tube and cells were analysed on FACS Calibur within 1 hour.

2.2.29 Anti-CD3/CD28 bead stimulation of T cells

T cells were stimulated by anti-CD3/CD28 Dynabeads (Invitrogen, UK); the method was adapted from the manufacturer's protocol. Briefly, 400µl of beads were vortexed for 30 seconds, then resuspended 1:1 in anti-CD3/CD28 bead buffer, and vortexed for 5 seconds. The beads were run through a MS MACS column on a miniMACS separator (both Miltenyi Biotech) for 1 minute and the resulting supernatant discarded. The MS MACS column was removed from the magnet and the washed beads were resuspended in 400µl of HL-1 (1% L-Glutamine, 1% Penicillin Streptomycin, 2.5% β-Mercaptoethanol). A 96-well plate was seeded with 100µl of $3.5x10^6$cells/ml in HL-1 media (1% L-Glutamine, 1% Penicillin Streptomycin, 2.5% β-Mercaptoethanol) and the Dynabeads were added to the wells at a bead to cell ratio of 1:10. The plates were then incubated and harvested according to the Lymphocyte Proliferation Assay protocol as described previously.

2.2.30 PEGlyation of LF peptides

LF_{61-80} RNKTQEEHLKEIMKHIVKIE and $LF_{457-476}$ HQSIGSTLYNKIYLYENMNI were commercially synthesised to 98% purity by Genscript, USA, using solid phase peptide synthesis (SPPS) with an α-amino protecting group Fmoc-8-Amino-3,6-Dioxaoctanoic (Fmoc) acid coupled to a mini-Poly Ethylene Glycol (PEG) spacer (Figure 2.2.1). The presence of this short spacer improved the pharmacokinetics of the peptides whilst avoiding the reduction in receptor binding affinity generally seen with the insertion of longer PEG spacers.

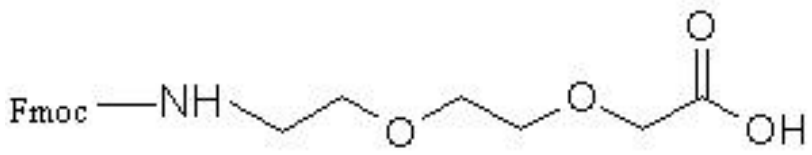


Figure 2.2.1 Structure of amino terminal coupled mini-PEG spacer

2.2.31 Effect of peptide immunization on the induction of EAE

Experimental Autoimmune Encephalomyelitis (EAE) was induced in the HLA-TCR transgenic mice by subcutaneous injection in the flank with 150µg $MBP_{85–99}$ peptide emulsified in 100µl of CFA, this was followed immediately afterwards and 24 hours later by 100µl of 200ng *Bordetella pertussis* toxin given by intraperitoneal injection. On days seven, eight and nine, mice were immunized by intraperitoneal injection with 100µl of LF_{61-80} or $LF_{457-476}$ emulsified in IFA.

2.2.32 Effect of peptide immunization on the development of spontaneous paralysis

Transgenic mice were immunized by intraperitoneal injection with 100µl of LF_{61-80} or $LF_{457-476}$ emulsified in IFA.

2.2.33 Monitoring of paralysis

Mice were monitored and scored daily for paralysis using the scoring system: 0, normal; 1, limp tail; 2, impaired righting reflex or gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, total limb paralysis; 6, moribund.

3 HLA restricted responses to LF

3.1 Introduction

The HLA class II molecules show broad variability in terms of their expression levels (Gorga *et al* 1987), this disparity can be attributed to differences not only in the coding regions of DR, DQ, and DP but also in the promoters and upstream regulatory regions (Andersen *et al* 1991). It has been observed that although this large family of related genes are usually expressed in concert (Amaldi *et al* 1989), there are inter-allelic polymorphisms in the nucleotide sequences of the regions which govern the individual transcriptional regulation of these genes (Singal and Qiu 1996). The demonstrable difference in promoter strengths, which affects the constitutive and inducible expression of MHC class II molecules (Andersen *et al* 1991), can be seen in the levels of cell surface expression of DR, which are up to 25 times those of DQ (Gorga *et al* 1987). The promoter driving the expression of HLA-DR in classical antigen presenting cells is stronger than those which drive the expression of either HLA-DQ or HLA-DP; as such, HLA-DR is often considered a functionally dominant molecule for antigen presentation. However, there are examples in the infectious disease literature in which antigen presentation through both HLA-DQ and HLA-DR appear to be of importance, but lead to differential responses (Angyalosi *et al* 2001).

While there exists a wide body of literature which seeks to describe the characteristics associated with the immune response of individual HLA alleles within transgenic models (Chen *et al* 2002; Cheng *et al* 1996; Fugger *et al* 1994; Gonzalez-Gay *et al* 1996; Ito *et al* 1996; Rosloniec *et al*

1997), there is currently a dearth of experimental data which considers the differential impact HLA polymorphisms have upon cellular immunity to pathogen derived antigens. The clearly demonstrated influence of allotypic murine H2 haplotypes upon the presentation of antigens and activation of T cells (Charlton *et al* 1997; Robinson *et al* 1991; Taub *et al* 1992), suggests that a similar hierarchical effect may be observed within the HLA repetoire. Work within this chapter utilised the humanised transgenic mouse strains, developed within the Altmann lab, to explore the impact HLA polymorphism has upon T cell responses to the anthrax toxin LF and susceptibility to live *B. anthracis* infection. During the course of this project a HLA-DQA1*010201/HLA-DQB1*0602 transgenic mouse model was also developed and evaluated.

3.2 Results

3.2.1 Generation and evalution of a HLA-DQ6 transgenic line

Following micro-injection of the DQA1*010201 and DQB1*0602 full length constructs into fertilised oocytes, tail biopsies from the resulting litters were genotyped using oligonucleotides specific to the transgene constructs. The expression of the DQ hetrodimer at the cell surface was analysed by staining peripheral blood cells from the transgenic line with the TAL4.1 DQA1 specific antibody, the Leu10 DQB1 specific antibody and the TÜ169 DQ dimer specific antibody. In a representative sample (Figure 3.2.1), HLA-DQ6 transgenic cells which were gated on CD45R/B220 and stained with the the Leu10 DQB1 specific antibody displayed a shift from the isotype control (Figure 3.2.1B), this was compared with the Leu10 DQB1 specific staining of human peripheral B cells from a DRB1*1501/DQB1*0602 individual (Figure 3.2.1C).

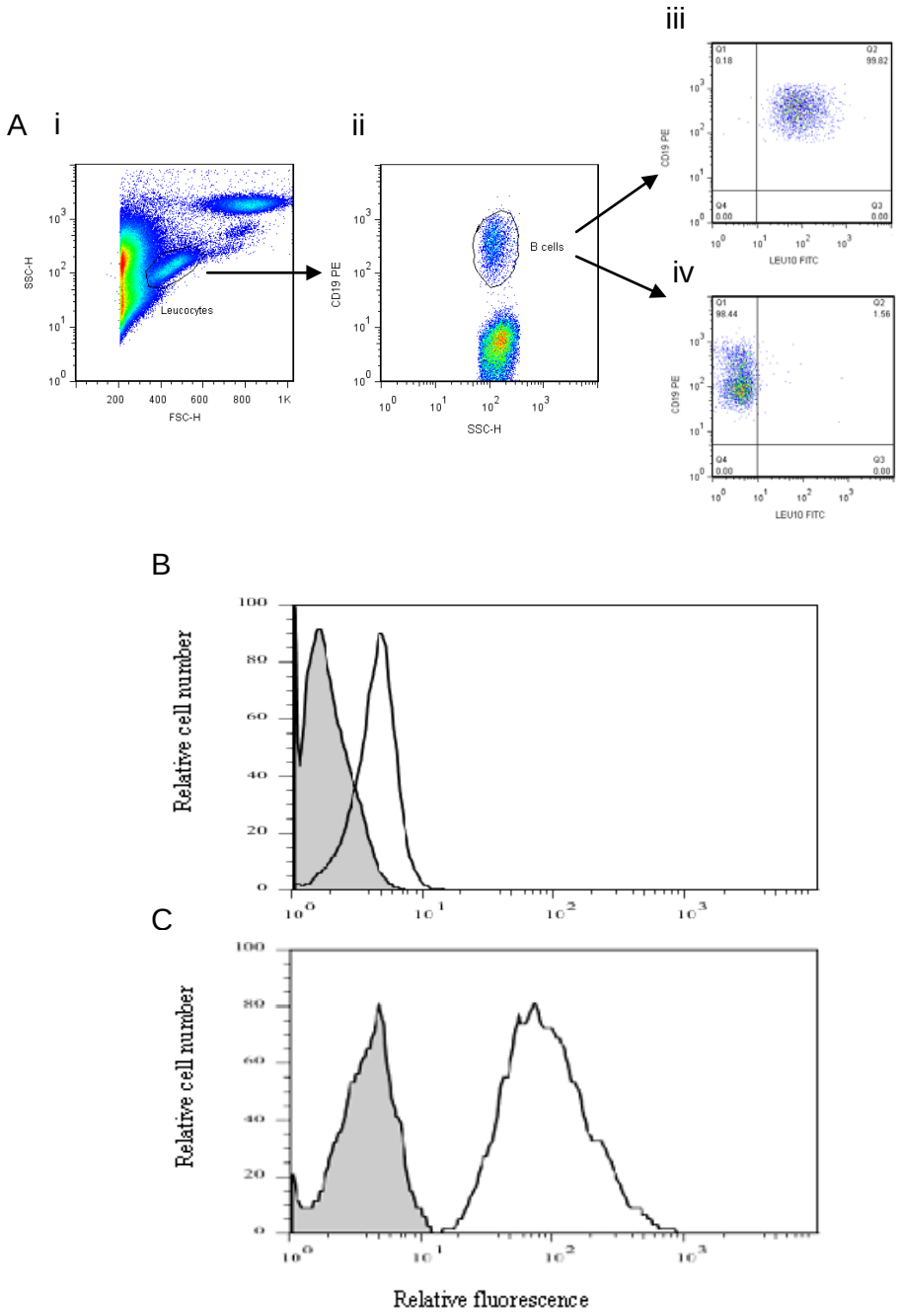


Figure 3.2.1 Cell surface expression of DQB1 determined by Leu10 antibody staining

Typical gating strategy for flow cytometry data (A), demonstrating leucocyte gates (i), B cell gates (ii), double positive cells (iii) and isotype controls (iv). Fluoresecence histograms are shown for the Leu10 staining of cells gated on CD45R/B220 from HLA-DQ6 transgenics (B), and cells gated on CD19 taken from a HLA-DR15/DQ6 human (C). The isotype control is shown as a filled grey histogram.

In common with the other HLA transgenics within the Altmann lab, this line was crossed to the $A\beta^{0/0}$ line (Cosgrove *et al* 1991) which carries an introduced mutation in the second exon of the Aβ gene and the $H2^b$ haplotype carried by the background C57BL/6 strain in this line is also constitutively unable to express the Eα protein due to a natural mutation in the Eα gene promoter (Mathis *et al* 1983). This is designed to prevent the expression of the mouse MHC class II molecles at the cell surface, impairing the thymic education of CD4+ T cells, so in mice which do not express the human HLA molecule, the CD3+ population should consist predominately of CD8+ T cells. In those mice which are capable of expressing the human HLA molecule, the CD4+ T cell population should be rescued, and a normal phenotype observed. This was determined by staining peripheral blood cells from tail bleeds with anti-CD4 and anti-CD8 specific monoclonal antibodies. The DQ6 transgenic line showed CD4+ staining characteristic for individuals expressing MHC class I and II molecules (Figure 3.2.2). In order to examine the abrogation of the mouse MHC class II, peripheral blood cells from the DQ transgenic line were stained with haplotype specific antibodies for Aα and Aβ (Figure 3.2.3). The H2 staining indicated that in the DQ transgenic line the α and β chains of the DQ homologue I-A, are not expressed at the cell surface. Therefore the CD4+ T cells were responding to presentation by transgenic rather than endogenous MHC class II molecules.

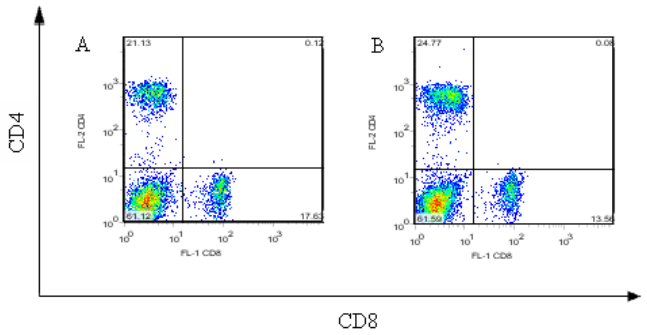


Figure 3.2.2 Representative dot plots of peripheral T cell staining.

All cells were stained with anti-CD4 and anti-CD8. The staining of CD4+CD8- and CD4-CD8+ populations from (A) C57Bl/6 mice with endoengous $A\beta^{+/+}$ cells was equivalent to the staining of CD4+CD8- and CD4-CD8+ T cell populations from the DQ6 transgenic strain.

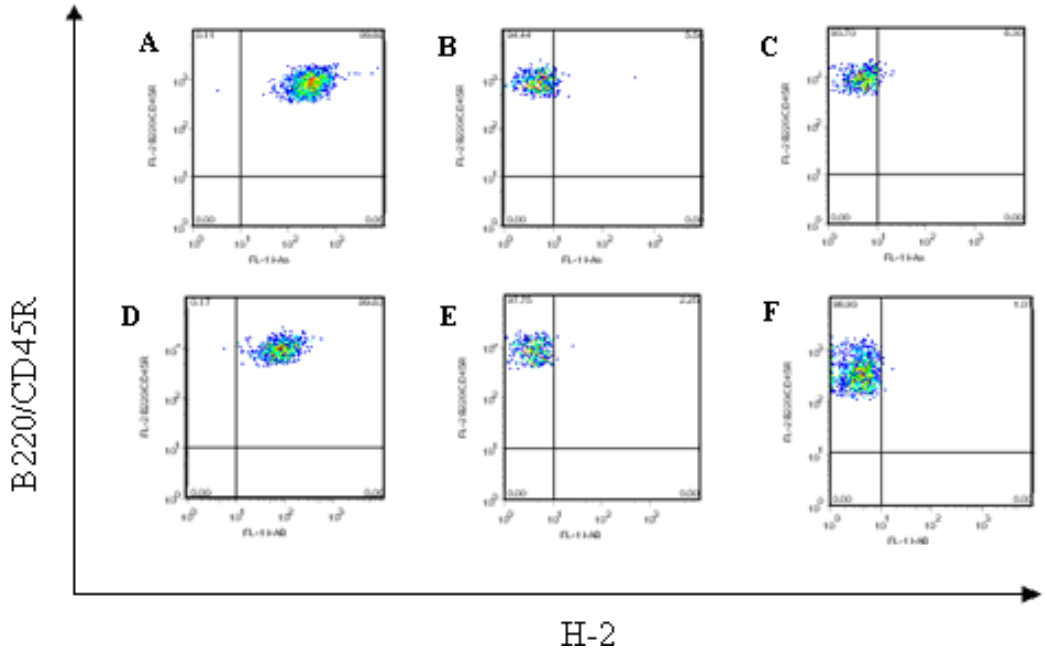


Figure 3.2.3 Representative dot plots of endogenous mouse MHC class II staining in the HLA-DQ6 transgenic strain.

All cells were stained with B220/CD45R and different H-2 antibodies, for analysis the cells were gated on B220/CD45R. (A) Staining of wild-type $A\beta^{+/+}$ cells with AF6.120.1 Aα specific antibody, (B) Staining of mouse MHC class II knock out $A\beta^{0/0}$ cells with AF6.120.1 Aα specific antibody, (C) Staining of DQ transgenic cells with AF6.120.1 Aα specific antibody, (D) Staining of wild-type $A\beta^{+/+}$ cells with 25-9-17 Aβ specific antibody, (E) Staining of mouse MHC class II knock out $A\beta^{0/0}$ cells with 25-9-17 Aβ specific antibody, (F) Staining of DQ transgenic cells with 25-9-17 Aβ specific antibody.

The DQA1*010201/DQB1*0602 MHC class II heterodimer has been implicated in the CD4+ T cell immune response to the self-peptide myelin basic protein (MBP) derived from the myelin sheath of neurons within the CNS (Madsen *et al* 1999b). Taking advantage of the documented ability of this HLA product to present MBP to autoreactive T cells, the HLA-DQ6 transgenics were immunised with the MBP_{85-99} peptide and the *ex-vivo* global recall response to the MBP peptide compared to the naïve response to a control ovalbumin protein (p=0.0184), indicating that the HLA transgenics were immunocompetent (Figure 3.2.4).

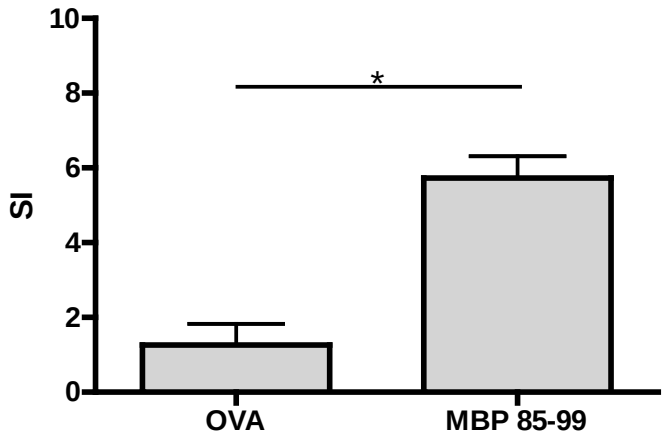


Figure 3.2.4 Antigen specific proliferative response of HLA-DQ6 transgenic leucocytes.
HLA-DQ6 transgenic mice (n=3), were immunised with the MBP_{85-99} peptide emulsified in Titremax, ten days later cells were isolated from the spleen and cultured in with 25μg/ml MBP_{85-99} or the control ovalbumin protein. The proliferation of the splenocytes, expressed as SI, demonstrated a significant difference between the recall response to the MBP peptide and the naïve response to the ovalbumin protein (p=0.0184, paired t-test) (± standard deviation).

3.2.2 HLA restricted responses to *B. anthracis* LF

The HLA class II transgenics HLA-DRA1*0101/HLA-DRB1*0101, HLA-DRA1*0101/HLA-DRB1*0401, HLA-DRA1*0101/HLA-DRB1*1501, HLA-DQA1*0301/DQB1*0302, HLA-DQA1*0102/HLA-DQB1*0602, on an H2-A$\beta^{0/0}$ background (Altmann *et al* 1993; Boyton *et al* 1998; Cosgrove *et al* 1991; Ellmerich *et al* 2004; Ellmerich *et al* 2005; Ito *et al* 1996), were immunised with the LF protein and the *ex-vivo* local draining lymph node and spleen cell responses analysed (Figure 3.2.5). It was apparent that the proliferative response of the lymph nodes cells (Figure 3.2.5A) followed a hierarchy within the HLA strains which was echoed by the secretion of the pro-inflammatory cytokine IFNγ by spleen (Figure 3.2.5B) and lymph node (Figure 3.2.5C) derived cells. Both the DR15 ($p<0.05$, Dunn's multiple comparison) and DR1 ($p<0.05$, Dunn's multiple comparison) transgenic lymph node cells demonstrated a significantly higher proliferative response to LF stimulation than the DQ6 transgenics. The IFNγ secretion by DR15 transgenics was higher than that observed in DR4 transgenics in both the spleen ($p<0.001$, Bonferroni's multiple comparison) and lymph node ($p<0.001$, Bonferroni's multiple comparison) responses to the LF protein. The DR15 transgenic strain also demonstrated significantly higher levels of IFNγ secretion than the DQ8 transgenics in the responses of both the spleen ($p<0.05$, Bonferroni's multiple comparison) and lymph node ($p<0.01$, Bonferroni's multiple comparison) derived cells. The magnitude of the IFNγ DR1 cellular response to the LF protein is significantly higher than the DR4 response in cells from both the spleen ($p<0.001$, Bonferroni's multiple comparison) and lymph node ($p<0.05$, Bonferroni's multiple comparison). Whilst there was no disparity between the DQ8 and DR4 response to LF in the lymph node cells, the IFNγ secretion by DQ8 splenocytes was significantly higher than that of the DR4 transgenics ($p<0.05$,

Bonferroni's multiple comparison). These results indicated that the LF response in the transgenic mice occurs both locally and globally and follows an HLA hierarchy.

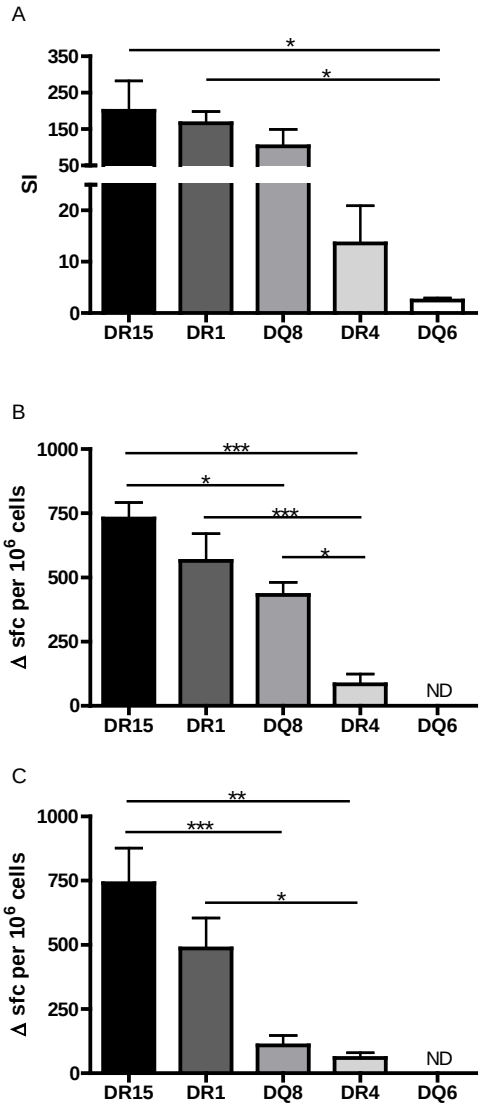


Figure 3.2.5 Antigen specific responses of the HLA transgenic strains to the LF protein.
The proliferative response of lymph nodes (A) expressed as SI for HLA-DR15 (n=4), DR1 (n=4), DR4 (n=4), DQ8 (n=4) and DQ6 (n=5) demonstrated a significant difference between strains (p=0.0042, Kruskal-Wallis). A significant difference between strains in terms of the IFNγ production, assayed by ELISpot, was observed in both spleen (B) ($p<0.0001$, One-way ANOVA) (n=5) and lymph nodes (C) (p=0.0002, One-way ANOVA) (n=5), (± standard deviation).

The proliferative and IFNγ responses delineate a clear HLA hierarchy in the transgenic strains response to LF. However, in order to gain a fuller understanding of the HLA transgenic strains response to LF we also considered the cytokines secreted following immunisation with the LF protein and *ex-vivo* local draining lymph node restimulation with the protein (Figure 3.2.6). The release of the cytokines TNFα and IL-2 was higher in the HLA-DR1 than the DQ6 transgenics, ($p<0.05$, Bonferroni's multiple comparison, for both cytokines). The levels of IL-4, IL-6, IL-10 and IL-17A were not significantly elevated in the responses of any of the HLA strains to LF, which suggests that the murine T cell recall response to this protein does not involve Th2 or Th17 subsets and is instead skewed towards the Th1 subset (the relatively low levels of IL-2 production may have been due to the length of incubation of the cell cultures, after 5 days there may have been upregulation of IL-2 receptors on activated leucocytes, with subsequent binding and uptake of this cytokine).

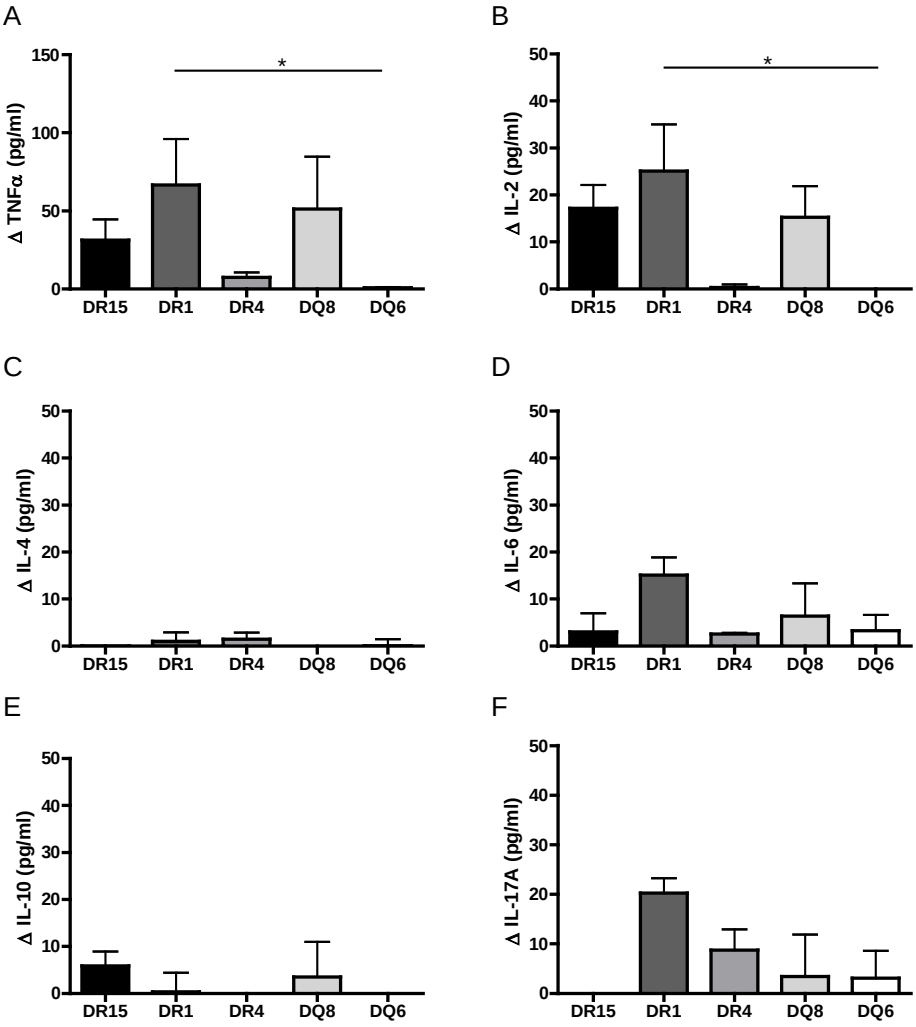


Figure 3.2.6 Cytokine secretion of the HLA transgenic strains in response to the LF protein.
Murine cytokine levels were quantified in the supernatants recovered from LF stimulated lymph nodes cells from all HLA transgenic strains, there was a significant difference between strains in the levels of (A) TNFα (p=0.0092, Kruskal-Wallis) (n=5 for all strains), and (B) IL-2 (p=0.0203, One-way ANOVA) (n=5 for all strains). There was no significant difference between strains in terms of (C) IL-4 for HLA-DR15 (n=4), DR1 (n=5), DR4 (n=3), DQ8 (n=3) and DQ6 (n=5), (D) IL-6 (n=5 for all strains), (E) IL-10 (n=5 for all strains), and (F) IL-17A (n=5 for all strains) (±SEM).

The susceptibility of the different strains of HLA transgenics was assessed using *in vivo* challenge with live *B. anthracis* (Figure 3.2.7). Previous work had established that the C57Bl/6

background strain was susceptible to challenge with the STI strain, hence its use as a control strain.

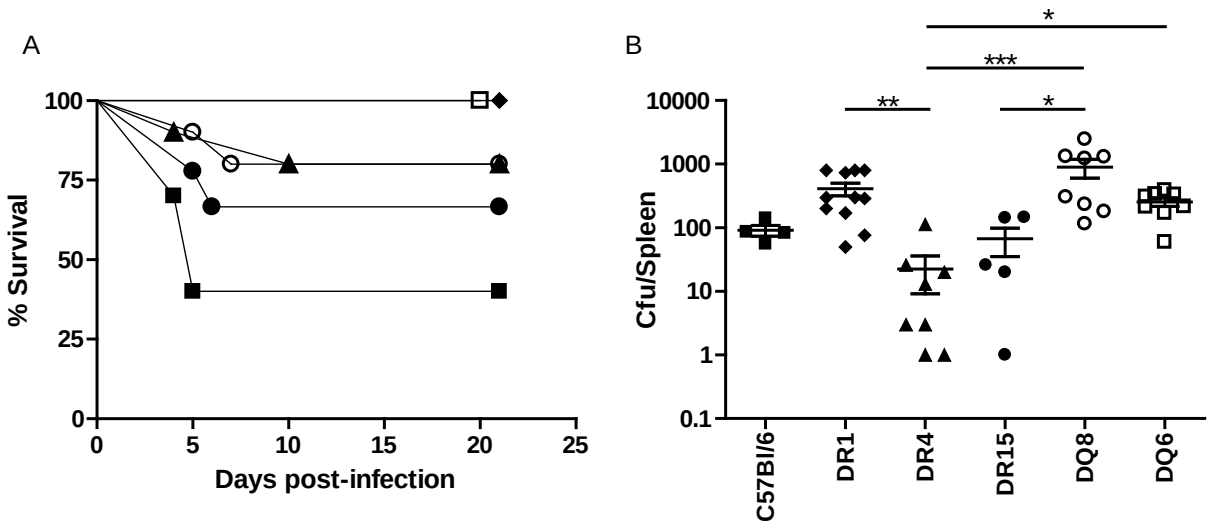


Figure 3.2.7 Resistance to *B. anthracis* challenge amongst HLA transgenic strains.
Groups of HLA-DR1 (♦ n=11), DR4 (▲ n=10), DR15 (● n=10), DQ8 (○ n=10), DQ6 (□ n=10) and C57Bl/6 (■ n=10) mice were challenged with 10^5 or 10^6 cfu *B. anthracis* STI strain by the i.p. route and monitored for 21 days. (A) The impact of infection upon survival was described using Kaplan Meier estimation. (B) Spleens were recovered from surviving mice at day 21; HLA-DR1 (♦ n=11), DR4 (▲ n=8), DR15 (● n=5), DQ8 (○ n=8), DQ6 (□ n=8) and C57Bl/6 (■ n=4) and a mean bacterial count per spleen determined following culture of *B. anthracis* for 24 hours. There was a significant difference between strains in cfu/spleen ($p<0.0001$, Kruskal Wallis) (mean ± SEM). This work was carried out in collaboration with DSTL, Porton Down.

Both the DR1 and DQ6 mice were found to be fully resistant to *B. anthracis* challenge, while the DQ8 and DR4 transgenics showed an elevated mortality rate. The highest mortality rate amongst the HLA strains was found in the DR15 transgenics, with only C57Bl/6 displaying a higher susceptibility. Following Kaplan Meier estimation it was evident that only the DR1 ($p<0.05$) and DQ6 ($p<0.05$) transgenic strains showed a significantly elevated survival rate compared to the

C57Bl/6 control strain. The bacterial loads recovered from the spleens showed that the DR1 transgenics had a higher bacterial burden than the DR4 transgenics ($p>0.01$, Dunn's multiple comparison) and the DQ8 transgenics showed a higher bacterial burden than both the DR4 ($p>0.001$, Dunn's multiple comparison) and the DR15 transgenics ($p>0.05$, Dunn's multiple comparison). However, interpretation of these results is complicated by the higher challenge dose received by the DR1 and DQ8 transgenics (10^6 cfu), rather than the 10^5 cfu received by the DQ6, DR15, DR4 and C57Bl/6 strains. It is possible to directly compare the bacterial burden of the resistant DQ6 strain with that of the relatively susceptible DR4 strain, which was significantly higher ($p>0.05$, Dunn's multiple comparison). Along with a lack of significant difference between any of the HLA strains and the susceptible C57Bl/6 strain in terms of bacterial counts, indicates that susceptibility to infection was not linked to the ability to control or clear the bacterial infection *in vivo*.

3.3 Discussion

Expression of the class HLA II molecules by human APC is complex, making interpretation of patterns of epitope presentation to CD4+ T cells highly challenging. Of the classical class II products, dimers of DP, DQ and DR are each expressed (Gorga *et al* 1987), and in view of the polymorphism of these loci, most individuals will express different alleles of each of these inherited from each parent. When one factors in also the fact that the HLA-DR region encodes from 1-3 different co-expressed DRB genes, that DQA and DQB products from different haplotypes can pair in cis or trans and that there is potential for inter-isotype mixed pairs, notably DRαDQβ, it is clear that any human APC may be presenting peptides to CD4+ T cells using up to 14 different class II products. This means that it is virtually impossible to delineate patterns of peptide epitope/HLA class II complex presentation in human immunity except by resorting to relatively demanding experiments such as characterizing individual T cell clones or immunizing HLA transgenic mice.

Murine I-A molecules and their human homologue, HLA-DQ, display a low affinity between homologous chains, which results in a low efficiency of pairing and an increased promiscuity, allowing for the generation of mixed isotype pairs with I-E and HLA-DR respectively (Scott *et al* 1996). This promiscuity has also been observed in inter-species pairings as mixed I-A/DQ pairs have been well-documented (Chen *et al* 2002; Gonzalez-Gay *et al* 1996). Despite the higher affinity between homologous chains and there being only 75% homology between DRα and Eα (Lawrance *et al* 1989), transgenic lines have also been generated which express inter-species DRα/I-Eβ molecules (Chen *et al* 2002). Previous in-depth analysis of DQ transgenics has determined that DQ dimers are unlikely to complex with both species and isotype mismatched

chains, as DQ $A\beta^{0/0}$ transgenics do not display any pairings between human DQα and mouse Eβ (Cheng *et al* 2003; Taneja *et al* 1997) (Figure 3.3.1). Altmann *et al* also noted that in spite of shared similarities in the amino acid residues surrounding their peptide-binding grooves, DQβ and Eβ showed low homology (Altmann *et al* 1993). Therefore, to disprove both the existence of intra-isotopic I-Aα/I-Eβ or inter-species DRα/I-Eβ or DQ/I-A pairs all of the HLA transgenic lines were characterised using the M5/114.15.2 antibody which detects the expression of both I-A and I-E sub-region-encoded molecules, for the HLA-DQ transgenics, the AF6.120.1 $A\alpha^{b}$ specific antibody and the 25-9-17 $A\beta^{b}$ specific antibody were utilised, and for the HLA-DR transgenics the 17-3-3 $E\beta^{b}$ specific antibody was employed.

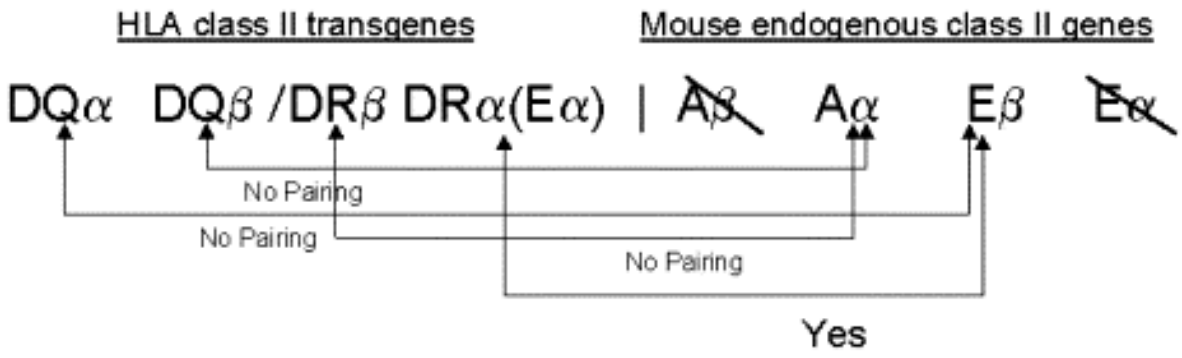


Figure 3.3.1 The possible complexing of DQ and DR molecules with endogenous I-A and I-E to form inter-species pairs.
DQ $A\beta^{0/0}$ transgenic mice are incapable of expressing mouse endogenous class II molecules, while DR $A\beta^{0/0}$ transgenic mice may express either Eαβ or DrαEβ molecules at the cell surface. (Taken from Cheng *et al* 2003).

In common with the other HLA strains utilized within this study, in the newly generated DQ6 transgenic line, thymic selection of the CD4+ T cell population was rescued by class II expression (Figure 3.2.2) and proven to be immunocompetent in its presentation of not only MBP_{85-99}, but also related myelin epitopes (Kaushansky *et al* 2009). While the background of

this strain precludes the formation of I-Eα molecules, it was considered possible that the APCs were expressing endogenous class II by forming intra-isotopic pairs (AαEβ), these mismatched heterodimers are known to be functional and capable of presenting antigen (Malissen *et al* 1986), however the formation of intra-isotopic pairs in the DQ6 transgenic line was disproved by the complete absence of either Aα or Aβ at the cell surface (Figure 3.2.3). Alternative postulates suggest that there may have been a contribution of $H2^k$ haplotype MHC sequences which had somehow not been excluded during the crossing onto the $A\beta^{0/0}$ strain which followed the initial microinjection of the constructs into C57BL/6 x CBA F1 fertilised oocytes, or that endogenous class II is complexing with the transgenic HLA, but at levels which are not detectable by H2 staining of peripheral leucocytes. Both Lawrence and Chen have reported that interspecies dimers are typically expressed on the cell surface at one-tenth the level of dominant homologous pairs (Chen *et al* 2002; Lawrance *et al* 1989), it is therefore possible that these pairs are expressed in the thymus, influencing the formation of the T cell repertoire in these mice. Although these possibilities have not been fully explored within this study, it may be interesting in further studies to breed the HLA transgenic mice onto a more recently generated $MHCII^{\Delta/\Delta}$ line (Madsen *et al* 1999a) which has been proven to lack the ability to express all classical endogenous MHC class II genes.

Previous work has established a hierachy in the response by inbred mouse strains to infection with *B. anthracis* (Welkos *et al* 1986). The work contained within this thesis represents the first known attempt to determine the role HLA polymorphisms play in the response to anthrax infection and the immune response to the LF toxin. The HLA transgenic strains demonstrated a skewing towards a Th1 subset in the recall response to LF, with a pro-inflammatory cytokine profile of TNFα, IL-2 and a high level of IFNγ secreted by responder cells. Following exposure

to bacterial products TNFα is involved in the activation of the MAPK pathway and the upregulation of transcription factors which are involved in cell proliferation and survival, and the release of IL-2 also regulates the expansion of antigen specific T cells (Jain *et al* 1995). In light of the action LT has upon the upstream elements of the MAPK pathway, and the NFAT associated with the reduction in the production of the cytokines IL-2, IFNγ and TNFα (Comer *et al* 2005; DeSilva *et al* 1998; Paccani *et al* 2005), it is interesting to consider the preferential activation of this subset upon exposure to the LF protein, which is incapable of causing PA mediated cellular intoxication. Glomski and Gold have both suggested that the survival of the *B. anthracis* bacteria within the host is dependent upon a rapid suppression of Th1 cytokines, notably IFNγ (Glomski *et al* 2007; Gold *et al* 2004). The strong proliferative and IFNγ responses which follow a clear HLA hierarchy (DR1=DR15>DQ8>DR4>DQ6) and the TNFα and IL-2 responses, which were dominated by DR1, tend to suggest that DR1 and DR15 alleles would confer an advantage in the immune response against anthrax. However, challenge with live *B. anthracis* revealed that although DR1 transgenics are resistant, the DR15 strain is highly susceptible to infection, while the DQ6 strain which showed the lowest magnitude of response to the LF protein was equal to DR1 transgenics in resistance to infection. These results illustrate the importance of exercising caution when attempting to develop a hierachical model of relative susceptibility to infection based upon the affinity particular HLA polymorphisms have for presenting antigenic peptides. In order to gain a comprehensive picture of how HLA class II restriction impacts upon the immune response to anthrax we focussed upon mapping the immunodominant epitopes of this protein.

4 LF immunodominant epitope mapping

4.1 Introduction

There are currently two anthrax vaccines in widespread use, the U.K.-licensed AVP and the U.S.-licensed AVA. Both of these vaccines are produced from a filtered culture supernatant of *B. anthracis* strains (Baillie 2006; Brey 2005; Leppla *et al* 2002), containing the toxin PA and variable amounts of the two enzymatically active toxin subunits, LF and EF, which together form a tripartite exotoxin (Baillie *et al* 1999). Previous vaccination strategies against anthrax have largely focused upon the role of antibody-based humoral immunity; the few studies concerning cellular immunity have concentrated primarily upon analysing the T cell response to PA (Kwok *et al* 2008; Laughlin *et al* 2007). Despite this, it is known that both PA and LF are capable of conferring protective immunity in human and animal vaccination studies (Baillie *et al* 1999; Fellows *et al* 2001; Pitt *et al* 1999). Recent work by our lab has demonstrated that both individuals receiving the AVP vaccine, and those naturally infected with live anthrax, generate strong CD4+ T cell responses to LF (Figure 4.1.1). A corresponding response to PA was seen only in naturally infected individuals. This suggests that, in addition to LF comprising a major target of T cell immunity in both infected and vaccinated individuals, the AVP vaccine generates a less robust, long lasting T cell memory response, compared to infection (Ingram *et al* 2010b). In combination with the onerous vaccination schedule, these inherent shortcomings indicate that there is an urgent need for a rationally designed, effective vaccine against anthrax.

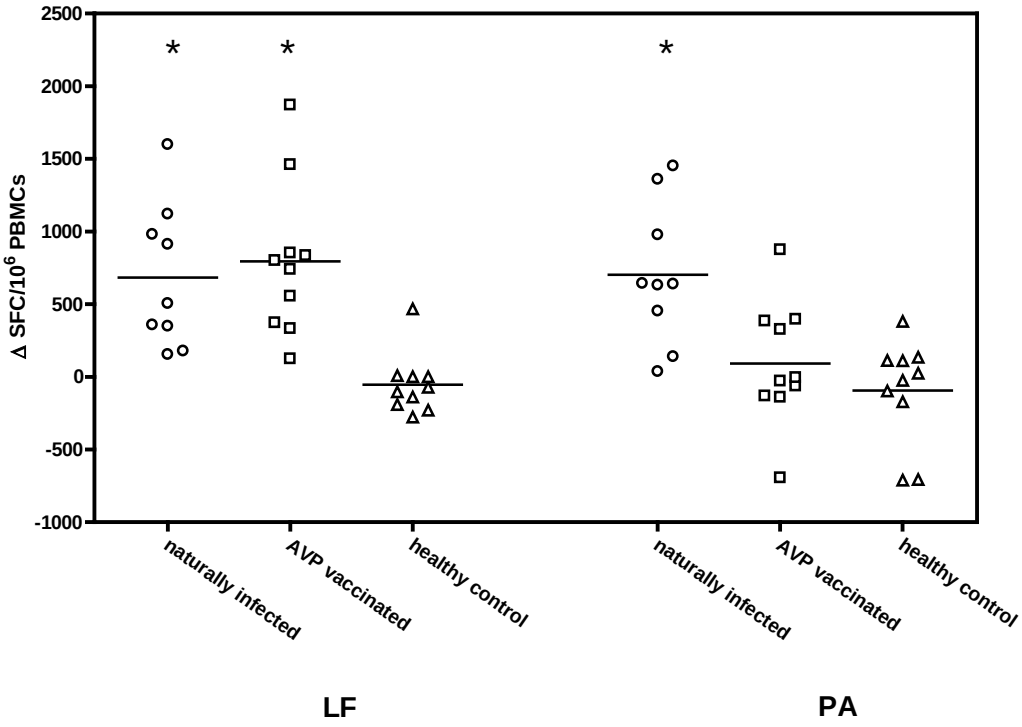


Figure 4.1.1 T cell IFNγ release in response to anthrax toxins PA and LF.
Responses were analysed in naturally infected (○), AVP-vaccinated (□), and healthy control (Δ) individuals. ELISpot results were quantified as the number of spots above background (ΔSFU)/10^6 PBMCs, and indicated that responses to LF were elevated in both naturally infected and vaccinated individuals compared to healthy controls *(*$p<0.001$ Mann-Whitney U test). Figure taken from Ingram *et al* 2010 (Ingram *et al* 2010b).

Pathogen derived proteins contain a relatively small number of immunodominant epitopes that bind to and are presented by the MHC class II complexes, eliciting T cell responses. The protective efficacy of a protein vaccine can be enhanced by identifying these immunodominant epitopes and incorporating them into a targeted vaccine. The rational selection of epitopes for use in the design of vaccines requires a detailed understanding of the T cell immunity evoked by the relevant antigens from a target organism. There is a current lack of work mapping the epitope recognition of anthrax antigens and correlating these responses with protection. Work described

in this chapter aimed to characterise T cell responses to the immunodominant epitopes derived from the anthrax protein, LF. The response to whole protein, domains and peptides was analysed using a panel of HLA-transgenic mice, cohorts of AVP vaccinated and naturally infected humans, and the relative HLA binding affinities of the peptide sequences.

4.2 Results

4.2.1 Intracellular cytokine production by leucocytes in response to LF

IFNγ is both an innate and adaptive effector molecule, being a critical component of the pro-inflammatory immune response secreted by NK cells and T cells which is capable of increasing the production of reactive nitrogen and oxygen species and complement, as well as up-regulating complement and Fc receptors (Schroder *et al* 2004). IFNγ has also been associated with the activation of phagocytic cells and up-regulation of antigen presentation, promoting a Th1 response, which augments the clearance of bacterial pathogens, including *B. anthracis* (Glomski *et al* 2007). Secretion of IFNγ has a proven role in protecting macrophages from LF induced cell death and mediating protection from anthrax pathogenicity (Glomski *et al* 2007; Gold *et al* 2004; Kang *et al* 2005). The induction of an LF antigen specific cellular response, encompassing IFNγ secretion, appears to be a vital step in providing protective immunity against anthrax. Following re-exposure to LF antigens, intracellular cytokine staining was performed to establish the cell populations which predominantly secrete IFNγ (Figure 4.2.1). The IFNγ release in response to LF antigens was found to be dominated by CD4+ T cells compared to CD8+ T cells ($p<0.001$) or NK cells ($p<0.001$). Corresponding human studies have also indicated the role CD4+ T cells play in the response to LF antigens (Ingram *et al* 2010b).

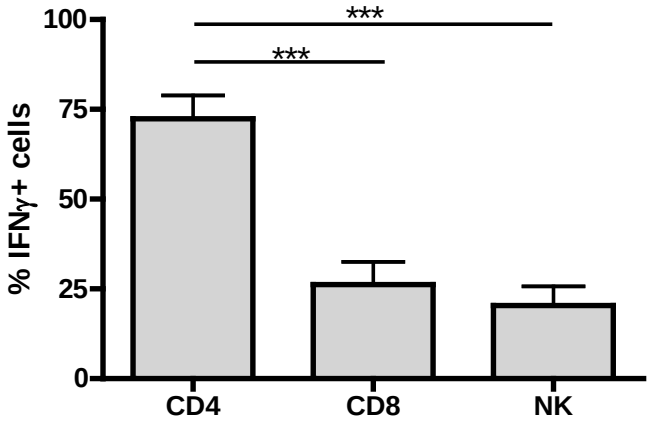


Figure 4.2.1 Intracellular cytokine production by leucocyte populations in response to LF antigens.
Ten days after LF immunisation, HLA-DR4 transgenic *ex-vivo* spleen cells were stained with anti-CD4, anti-CD8 and anti-NK1.1 then stained intracellularly with anti-IFNγ and analysed by flow cytometry. Results were expressed as the percentage of each cell population which was IFNγ+, following arcsin transformation of the data the responses were compared using repeated measures ANOVA with Bonferroni post hoc testing. The IFNγ is predominantly derived from CD4+ T cells ($p>0.0001$ repeated measures ANOVA) (± standard deviation) (n=5).

Subcutaneous immunisation with LF protein mediates an antigen specific T cell response in the local draining lymph nodes as well as a systemic T cell response in the spleen, this was demonstrated in the previous chapter for both proliferative responses and IFNγ secretion (Figure 3.2.5). The IFNγ responses of CD4+ T cells from these secondary lymphoid organs were evaluated (Figure 4.2.2), revealing that although T cells from both the spleen and lymph node were antigen specific, distinct local and systemic T cell responses emerge following immunisation with protein antigens. CD4+ T cells taken from the spleen do not show a dose related response to LF antigens (Figure 4.2.2A), whilst a statistically significant relationship was seen between LF antigen dose and IFNγ release by cells from the local draining lymph nodes (Kruskal wallis, $p=0.0164$) (Figure 4.2.2B). Mapping the T cell responses to epitopes from LF was therefore carried out on cells isolated from the local draining lymph nodes.

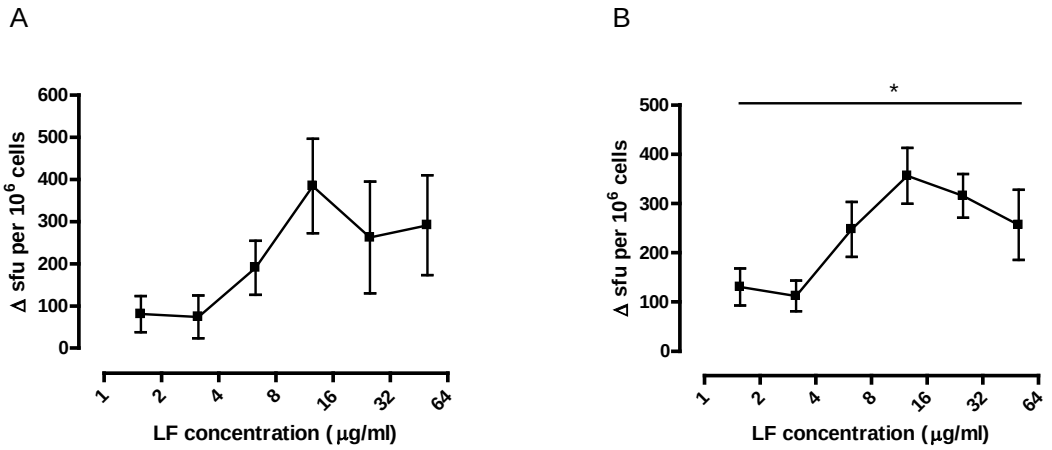


Figure 4.2.2 Magnitude of the IFNγ response of peripheral lymphoid organs to increasing concentrations of LF.

An *ex vivo* ELISpot assay was used to measure production of IFNγ by HLA-DR15 restricted CD4+ T cells from (A) spleens (n=5) (± standard deviation) and (B) lymph nodes (n=6) (Kruskal wallis, p=0.0164). Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs (± standard deviation).

4.2.2 Mapping of LF immunodominant epitopes

The LF protein is composed of four structural domains (Pannifer *et al* 2001c). Subcutaneous immunisation of HLA transgenics with whole LF, followed by restimulation with each of the four individual domains, indicated that both the HLA-DR (Figure 4.2.3A) and HLA-DQ (Figure 4.2.3B) restricted T cell responses were directed primarily towards epitopes which reside within domain II of LF. This was confirmed as an antigen specific response by stimulating HLA-DR4 restricted T cells from sham-immunised transgenics with the LF domain proteins (Figure 4.2.3C); in contrast to the LF immunised transgenics, there was no response to either the full length LF or the domains. Surprisingly, the *in silico* prediction of HLA-DR restricted T cell LF

epitopes (Figure 4.2.3D), showed a negligible difference in the number of epitopes present in domains I, II and IV, with only the small α-helical bundle composed of 79 aa residues, which comprises domain III, containing a lower number of predicted epitopes. This underlined the importance of verifying predicted epitopes using *in vivo* epitope mapping.

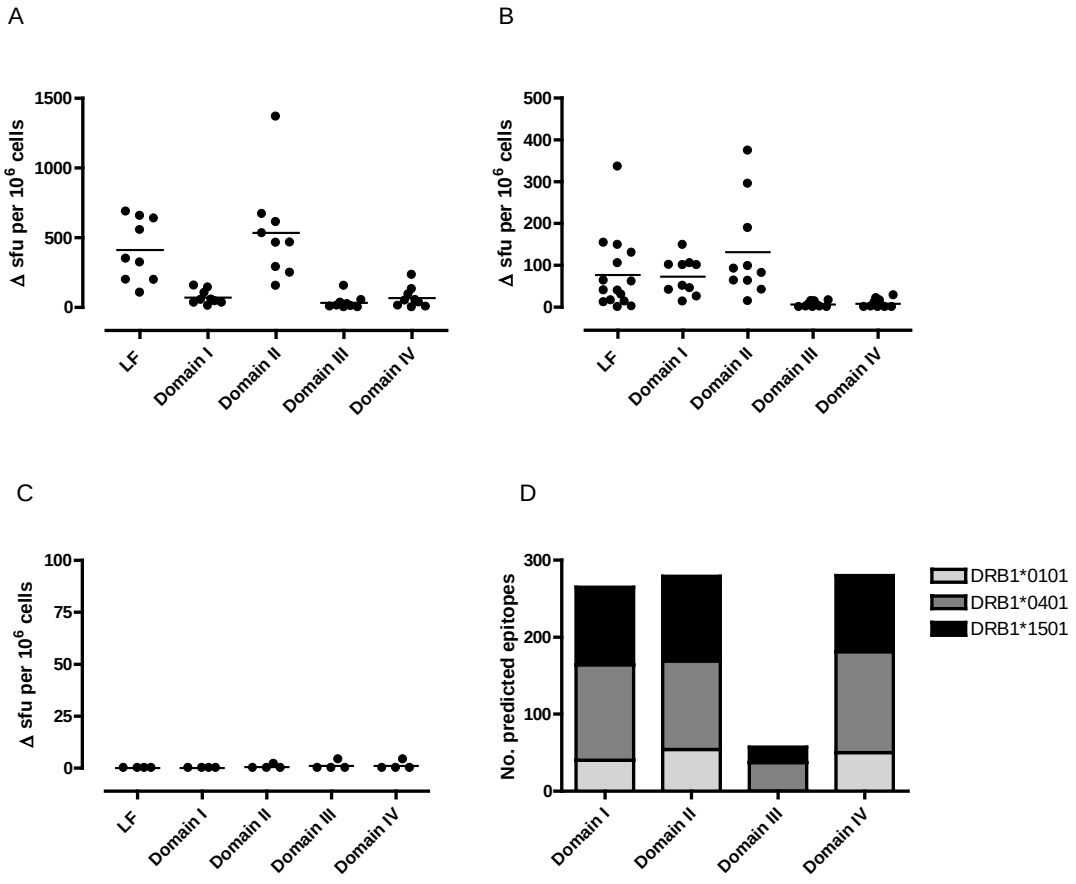


Figure 4.2.3 Analysis of the HLA-DR and DQ restricted response to whole LF protein and individual LF domains.

The T cell response of (A) DR15 (n=9) and (B) DQ6 (n=10) transgenics immunised with whole LF, or (C) sham immunised HLA-DR4 transgenics (n=4), were determined by IFNγ specific ELISpot stimulation with LF and domains. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation. The IEDB T cell epitope prediction tool was used to predict peptides from each LF domains which bind strongly to DR1 (light grey), DR4 (dark grey) and DR15 (black) for presentation to CD4+ T cells (D).

The immunodominant CD4+ T cell response to the peptides which encompass the whole LF protein were mapped by immunising HLA transgenics with the whole protein and restimulating with the individual peptides (Figure 4.2.4, Figure 4.2.5 and Figure 4.2.6). These epitopes were confirmed by immunising with the individual peptide and restimulating with the immunising peptide and flanking peptides (Figure 4.2.8 and Figure 4.2.9). An antigen specific T cell response to the individual peptides which make up the domain proteins was indicated by the response of cells from sham immunised HLA-DR4 transgenic mice (Figure 4.2.7). These cells showed a negligible IFNγ response to the LF peptide panel, which was in agreement with the sham immunised response to the whole domains (Figure 4.2.3C).

The T cell epitope mapping revealed that the immunodominant epitopes were largely localized to domains II and IV of LF (Table 4.2.1). The response to epitopes from domain II was common to all transgenics, whilst peptides from domain IV appeared to be predominantly presented by HLA-DR. The lack of response to peptides from domain III, in addition to the responses to the whole domains and *in silico* predictions (Figure 4.2.3D) confirmed that domain III does not contain any immunodominant epitopes. In contrast, the response to peptides from domain I was dominated by both HLA-DR1 and HLA-DQ8 transgenics (Table 4.2.1), indicating that peptides from this domain were available for presented by both HLA-DR and DQ molecules.

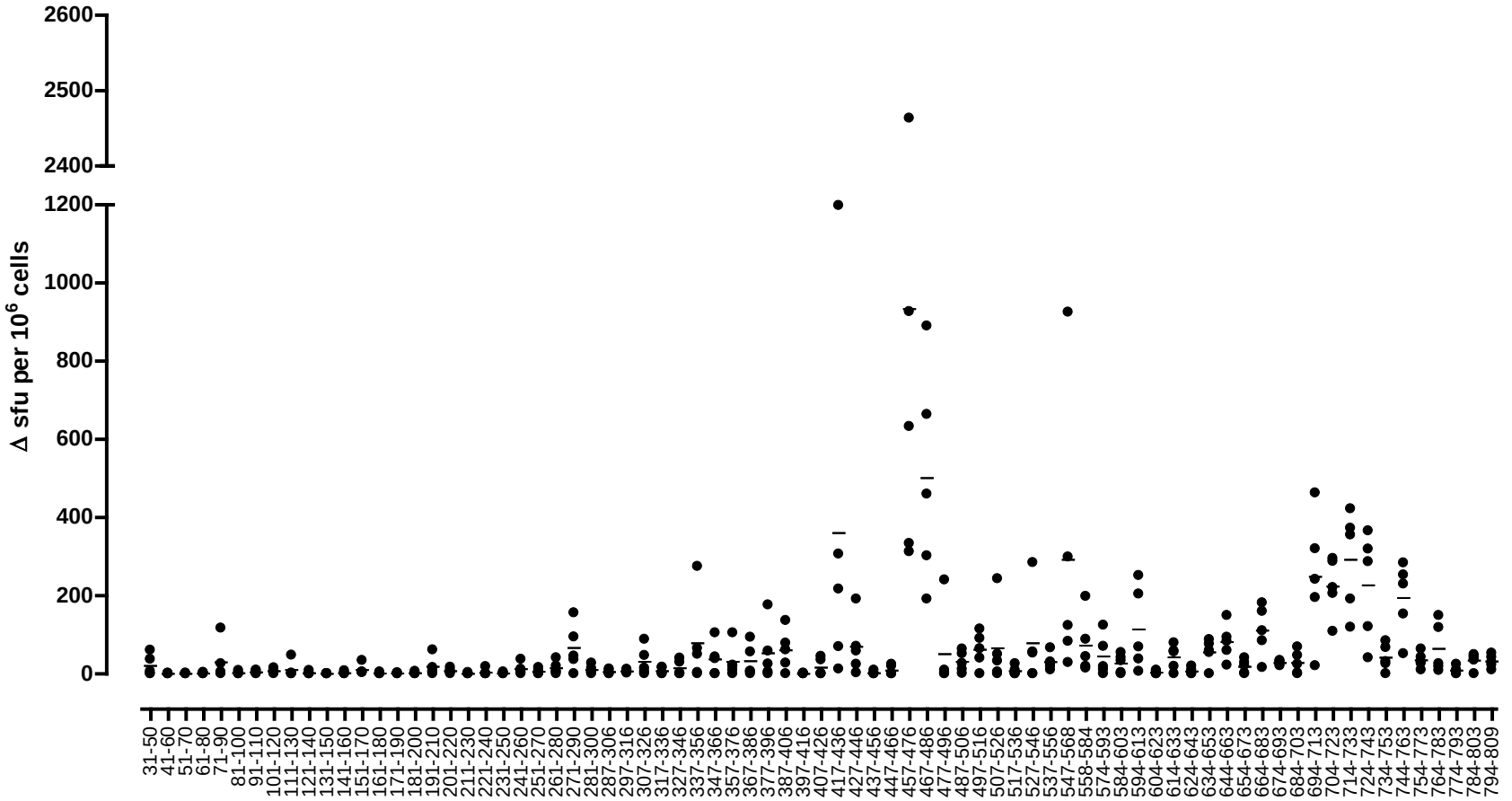


Figure 4.2.4 *Ex vivo* mapping of the HLA-DR15 restricted CD4+ T cell epitopes of LF.

HLA-DR15 transgenic mice were immunised with LF protein emulsified in Titremax, ten days later cells were isolated from the draining lymph node and cultured in an IFNγ specific ELISpot plate with LF peptide for 72 hours. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation (n=5 for each data point).

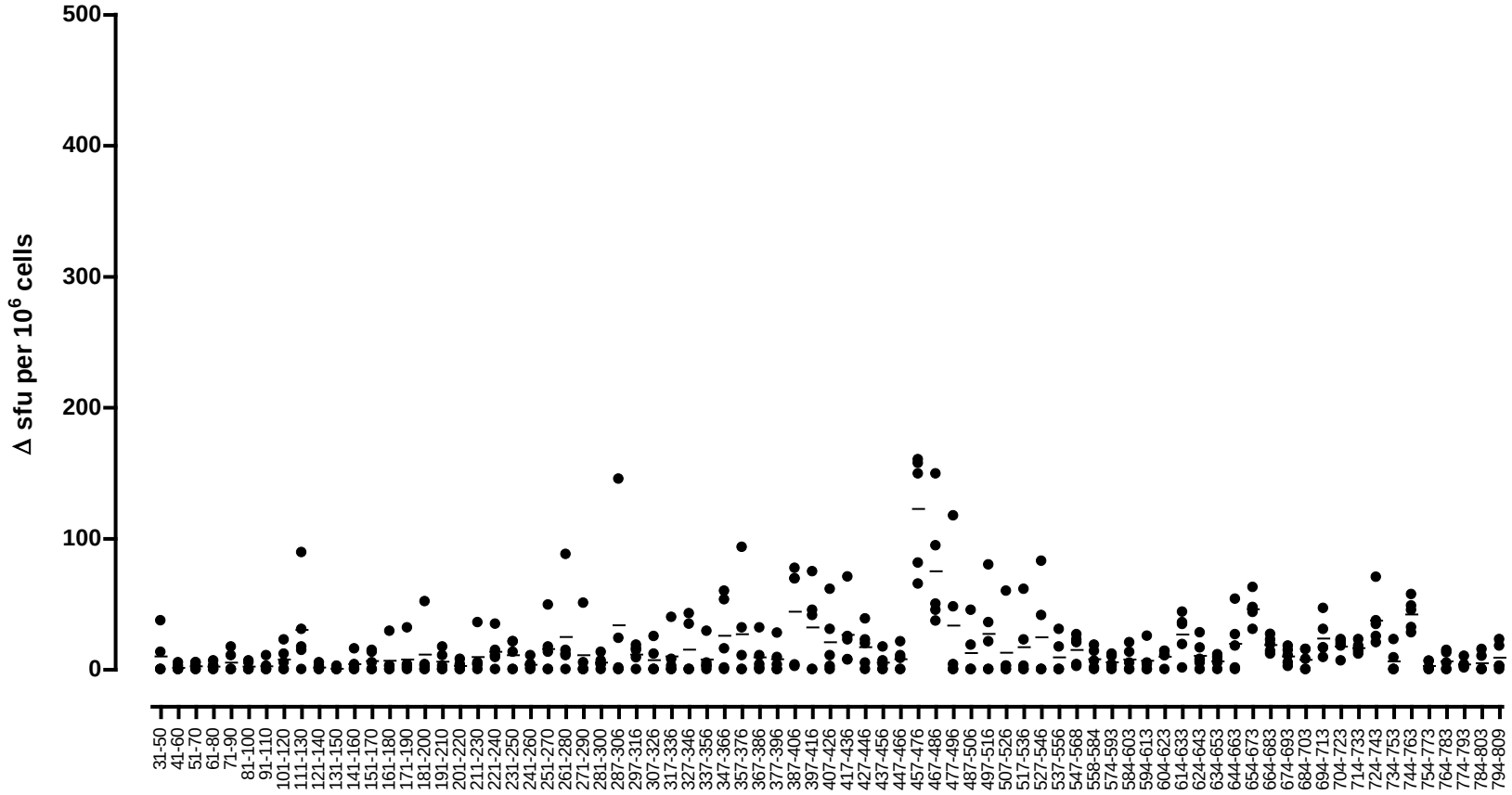


Figure 4.2.5 *Ex vivo* mapping of the HLA-DR4 restricted CD4+ T cell epitopes of LF.

HLA-DR4 transgenic mice were immunised with LF protein emulsified in Titremax, ten days later cells were isolated from the draining lymph node and cultured in an IFNγ specific ELISpot plate with LF peptide for 72 hours. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation (n=5 for each data point).

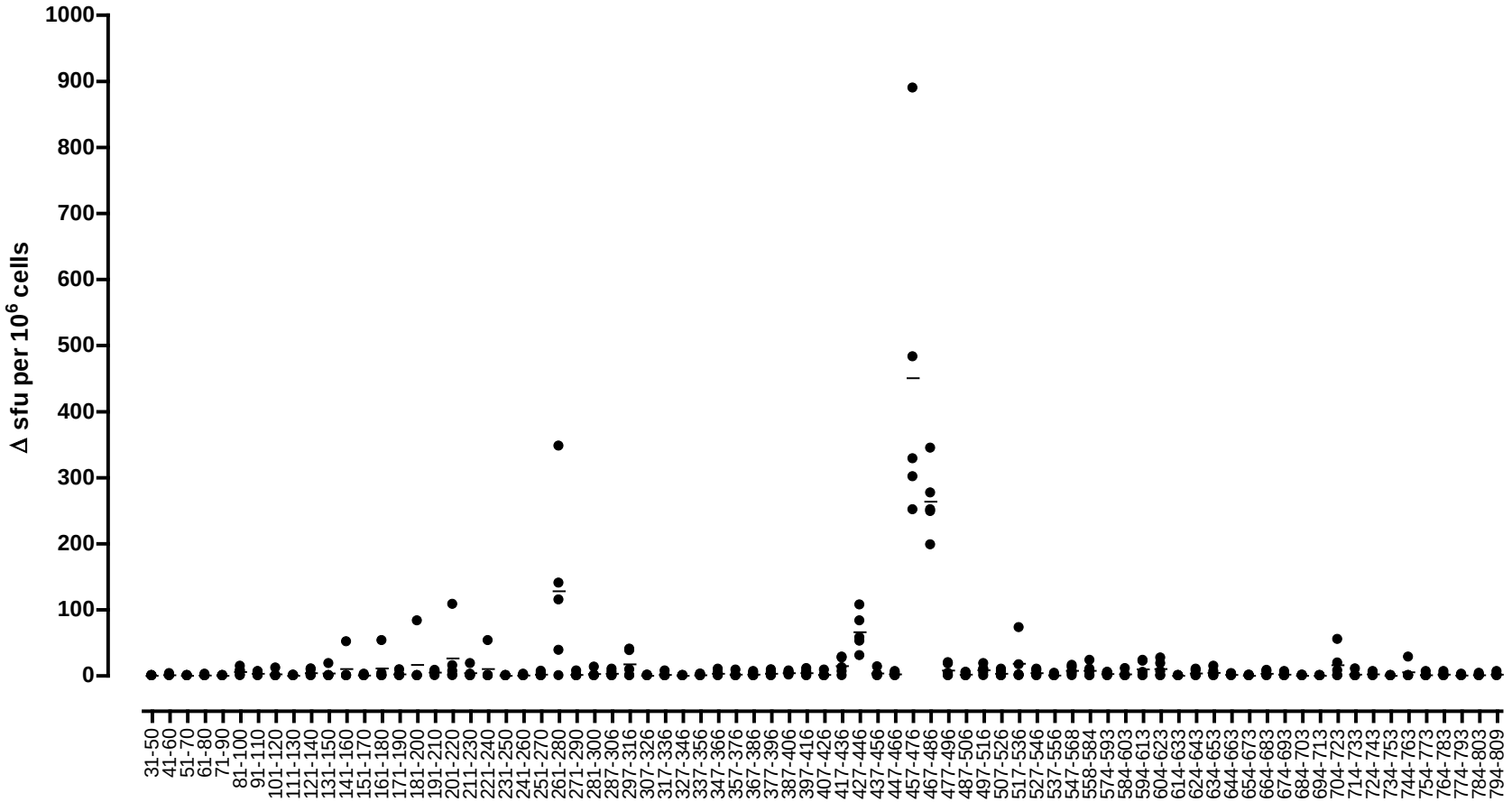


Figure 4.2.6 *Ex vivo* mapping of the HLA-DQ6 restricted CD4+ T cell epitopes of LF.

HLA-DQ6 transgenic mice were immunised with LF protein emulsified in Titremax, ten days later cells were isolated from the draining lymph node and cultured in an IFNγ specific ELISpot plate with LF peptide for 72 hours. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation (n=5 for each data point).

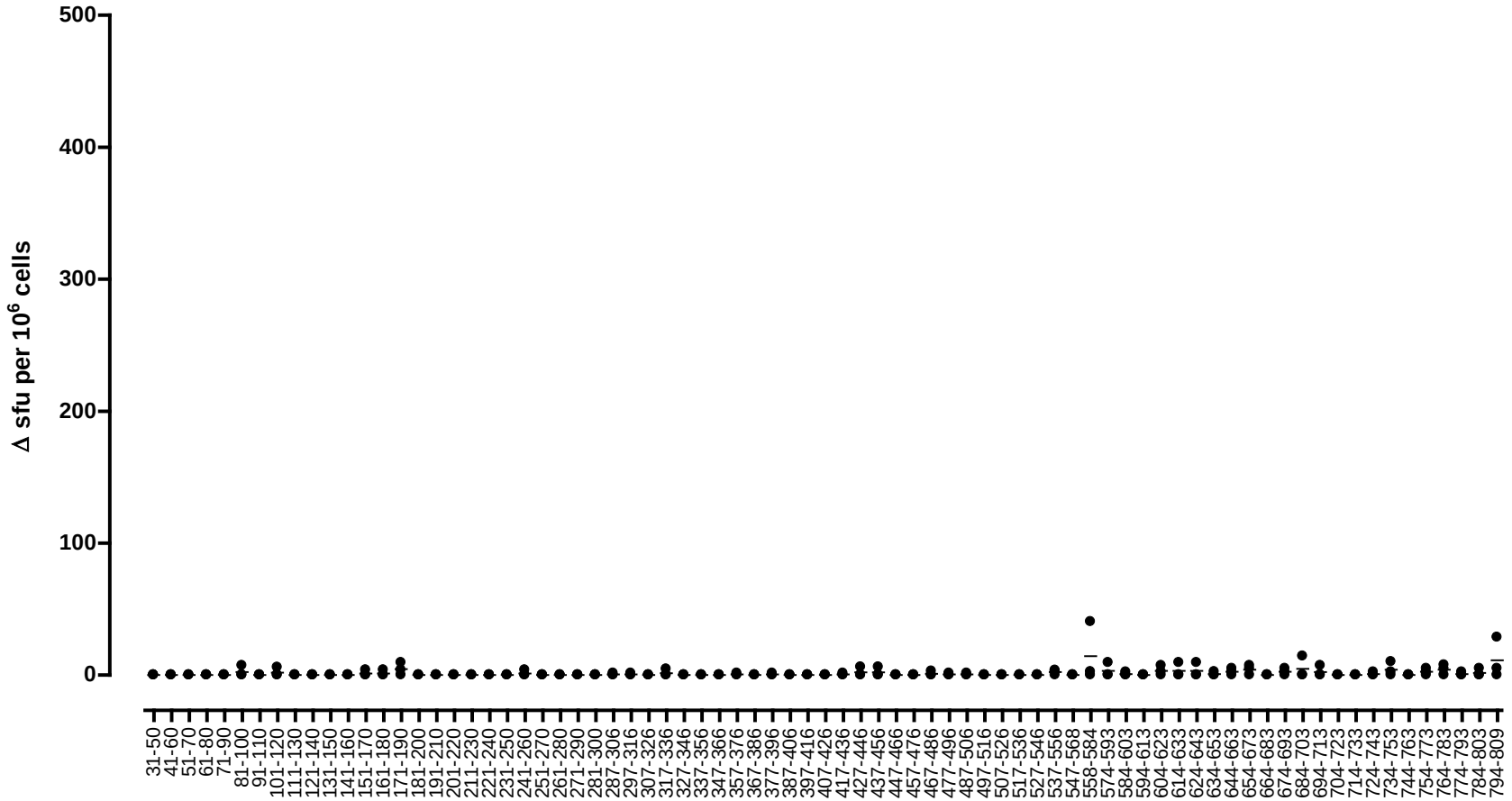


Figure 4.2.7 *Ex vivo* mapping of the HLA-DR4 restricted CD4+ T cell response in sham immunised mice.

HLA-DR4 transgenic mice were immunised with a control of PBS emulsified in Titremax, ten days later cells were isolated from the draining lymph node and cultured in an IFNγ specific ELISpot plate with LF peptide for 72 hours. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation (n=3 for each data point).

The allelic diversity of MHC class II molecules varies their peptide binding specificity, resulting in the presentation of different peptides of a given antigen, shaping the T cell repertoire activated in the immune response. This diversity was observed in the range of LF peptides presented by the HLA transgenics. Along with the allele specific epitopes, fine mapping of the recall response following single peptide immunisation also revealed that the peptides $LF_{457-476}$ and $LF_{467-487}$, from domain II, were both immunodominant and immunoprevalent epitopes (also known as promiscuous epitopes), capable of eliciting a T cell response from all HLA-DR and DQ transgenics. Whilst the T cell responses to the peptide $LF_{547-567}$, from domain VI, appeared to be HLA-DR restricted, as only T cells from the DR transgenics; DR15, DR4 and DR1, not the DQ transgenics DQ8 and DQ6 responded to this peptide.

This form of fine mapping uncovered several antigenic peptides which did not elicit a T cell response following individual peptide immunisations. These peptides, which for HLA-DR15 included $LF_{271-290}$, $LF_{417-436}$, $LF_{497-516}$, $LF_{537-556}$, $LF_{614-633}$, $LF_{664-683}$, $LF_{674-693}$, $LF_{704-723}$ and $LF_{744-763}$ (Figure 4.2.8), and for HLA-DQ6 included; $LF_{201-220}$, $LF_{261-280}$, $LF_{297-316}$ and $LF_{427-446}$ (Figure 4.2.9) demonstrated the importance of fine mapping in identifying epitopes, as they proved incapable of inducing an IFNγ specific T cell response within this context.

A number of peptides presented by HLA-DR15, $LF_{427-446}$, $LF_{547-568}$, $LF_{558-584}$ and $LF_{694-713}$ (Figure 4.2.8) were shown to lead to a T cell recall response only when presented as peptide alone, not within the context of the whole protein during fine epitope mapping.

Conversely, fine mapping also revealed a class of peptides which are capable of inducing a T cell response to the peptide, as well as to the whole native protein. These heteroclitic epitopes, which for HLA-DR15 included; $LF_{457-476}$, $LF_{467-486,}$ $LF_{594-613}$, $LF_{644-663}$, $LF_{714-733}$, and $LF_{724-743}$, (Figure 4.2.8) and for HLA-DQ6 included $LF_{457-476}$ (Figure 4.2.9) proved efficient at inducing T cell immunity following initial administration as either intact protein or peptide.

The limitations of fine epitope mapping appear to lie in the lack of discrimination between separate or concurrent epitopes within overlapping peptides. This was demonstrated by responses to the overlapping peptides, $LF_{457-476}$ and $LF_{467-486}$ in HLA-DR15 transgenics (Figure 4.2.8); following immunisation with each individual peptide, the recall response was directed towards both peptides. It is therefore unclear whether these represent one or two distinct epitopes. In contrast, it was discovered that immunisation with $LF_{714-733}$ did provoke a response to the immunising peptide plus the overlapping peptide $LF_{724-743}$, whilst immunisation with $LF_{724-743}$ did not provoke a response to $LF_{714-733}$, but to $LF_{724-743}$ plus $LF_{734-753}$ instead (Figure 4.2.8). This suggests that $LF_{714-733}$ and $LF_{724-743}$ represent separate epitopes.

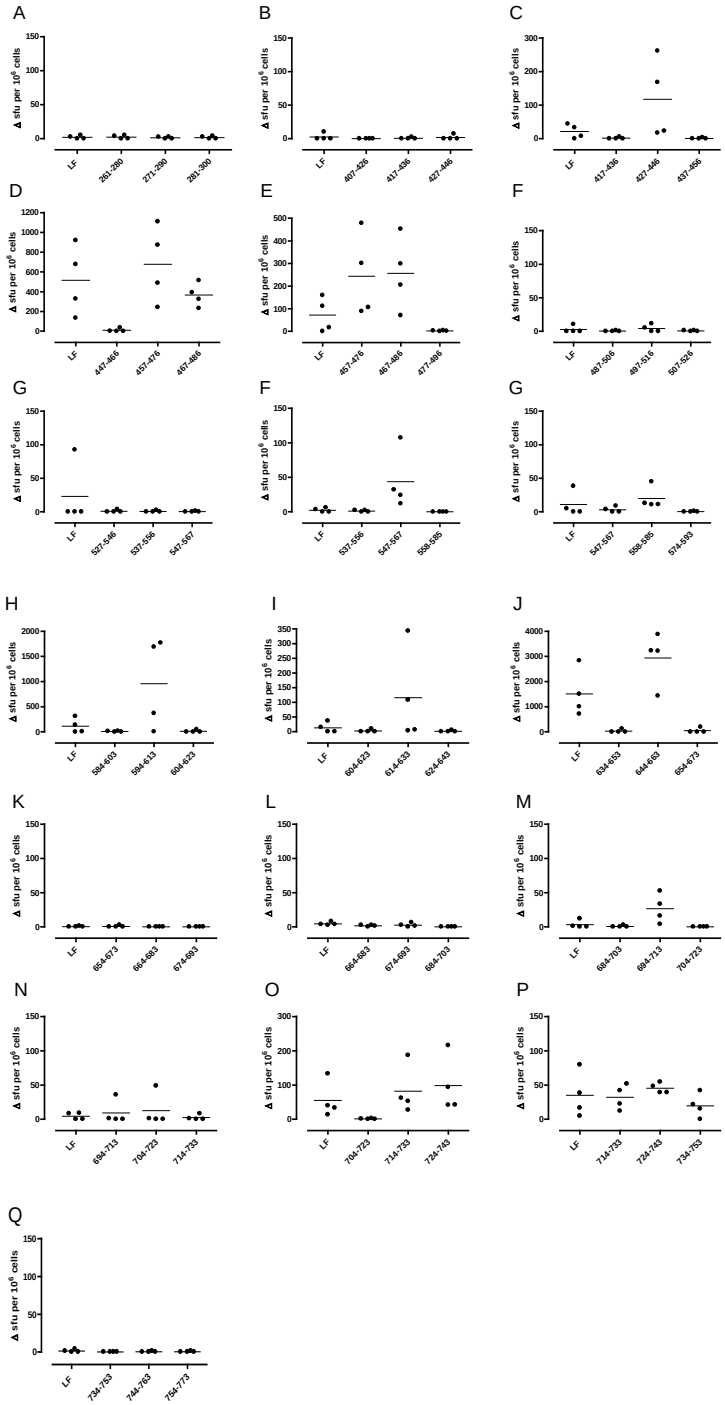


Figure 4.2.8 Fine mapping of previously identified HLA-DR15 restricted T cell epitopes. Confirmation of antigenic peptides was performed by immunisation of HLA-DR15 transgenics with the identified peptide. Cells from the draining lymph node were cultured in an IFNγ specific ELISpot plate with LF peptides and protein for 72 hours. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation (n=4 for each data point).

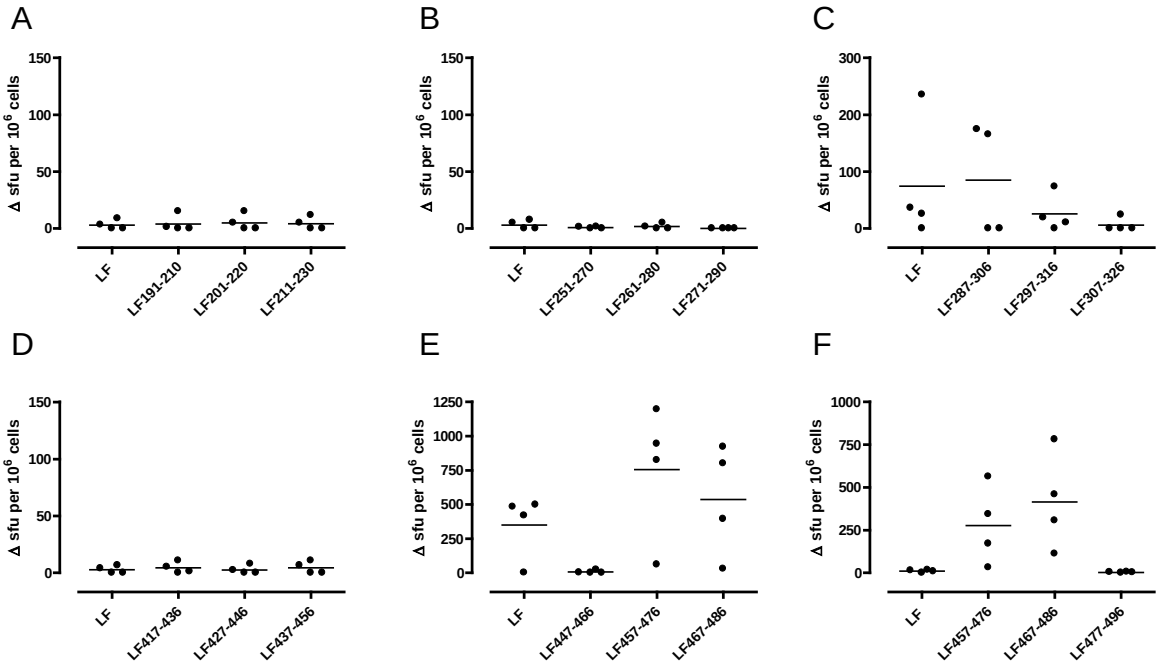


Figure 4.2.9 Fine mapping of previously identified HLA-DQ6 restricted T cell epitopes. Confirmation of antigenic peptides was performed by immunisation of HLA-DQ6 transgenics with the identified peptide. Cells from the draining lymph node were cultured in an IFNγ specific ELISpot plate with LF peptides and protein for 72 hours. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation (n=4 for each data point).

Table 4.2.1 HLA DR and DQ-restricted LF epitopes confirmed by single peptide immunisation and restimulation

	Domain I	Domain II	Domain III	Domain IV
DR1	LF 151YVYAKEGYEPVLVIQSSEDY 170 LF 161 VLVIQSSEDYVENTEKALNV 180 LF 171 VENTEKALNVYYEIGKILSR 190 LF 241 QNSNEVQEVFAKAFAYYIEP 260 LF 251 AKAFAYYIEPQHRDVLQLYA 270 LF 261 QHRDVLQLYAPEAFNYMDKF 280	LF 457 HQSIGSTLYNKIYLYENMNI 476 LF 467 KIYLYENMNINNLTATLGAD 486		LF 547 LENGKLILQRNIGLEIKDVQI 567 LF 558 IGLEIKDVQIIKQSEKEYIRIDAKVVP 585 LF 574 EYIRIDAKVVPKSKIDTKIQ 593 LF 604 NKALGLPKYTKLITFNVHNR 623 LF 614 KLITFNVHNRYASNIVESAY 633 LF 674 EQYTHQDEIYEQVHSKGLYV 693
DR4		LF 457 HQSIGSTLYNKIYLYENMNI 476 LF 467 KIYLYENMNINNLTATLGAD 486		LF 547 LENGKLILQRNIGLEIKDVQI 567 LF 614 KLITFNVHNRYASNIVESAY 633 LF 654 YLVDGNGRFVFTDITLPNIA 673 LF 724 AVDDYAGYLLDKNQSDLVTN 743 LF 744 SKKFIDIFKEEGSNLTSYGR 763
DR15		LF 427 DTGGLIDSPSINLDVRKQYK 446 LF 457 HQSIGSTLYNKIYLYENMNI 476 LF 467 KIYLYENMNINNLTATLGAD 486		LF 547 LENGKLILQRNIGLEIKDVQI 567 LF 558 IGLEIKDVQIIKQSEKEYIRIDAKVVP 585 LF 594 EAQLNINQEWNKALGLPKYT 613 LF 644 QSDLIKKVTNYLVDGNGRFV 663 LF 694 PESRSILLHGPSKGVELRND 713 LF 714 SEGFIHEFGHAVDDYAGYLL 733 LF 724 AVDDYAGYLLDKNQSDLVTN 743
DQ8	LF 61 RNKTQEEHLKEIMKHIVKIE 80 LF 71 EIMKHIVKIEVKGEEAVKKE 90 LF 101 SDVLEMYKAIGGKIYIVDGD 120 LF 141 YGKDALLHEHYVYAKEGYEP 160 LF 151 YVYAKEGYEPVLVIQSSEDY 170 LF 181 YYEIGKILSRDILSKINQPY 200 LF 221 LLFTNQLKEHPTDFSVEFLE 240	LF 261 QHRDVLQLYAPEAFNYMDKF 280 LF 271 PEAFNYMDKFNEQEINLSLE 290 LF 387 KELLNRIQVDSSNPLSEKEK 406 LF 457 HQSIGSTLYNKIYLYENMNI 476 LF 467 KIYLYENMNINNLTATLGAD 486		LF 594 EAQLNINQEWNKALGLPKYT 613
DQ6		LF 457 HQSIGSTLYNKIYLYENMNI 476 LF 467 KIYLYENMNINNLTATLGAD 486		

The mapping of the CD4+ T cell epitopes relevant for the presentation of antigen, from the LF toxin found in *B. anthracis*, was primarily achieved using transgenic mouse lines which express human HLA class II alleles. These models have provided a defined genetic background, in which the response of T cells to antigen presentation by a single MHC isoform can be mapped. The epitopes identified were compared with the CD4+ immunity produced by LF antigens in naturally infected and vaccinated individuals, as well as healthy controls (manuscript in preparation). Cells were stimulated with a peptide library, prepared in a matrix comprising six peptides per pool, so that each peptide occurred in

two pools but no peptides occurred in the same two pools. This allowed the determination of responses to individual peptides.

In the AVP vaccinated individuals the immunodominant response was broad and encompassed eleven epitopes. Of these peptides, LF_{41-60}, $LF_{417-436}$, $LF_{437-456}$, and $LF_{584-603}$ did not induce any response in any of the HLA transgenics. $LF_{337-356}$ is a cryptic epitope, which was identified in the HLA-DQ8 transgenics (Chapter 5). The T cell responses to the domain VI peptide $LF_{547-567}$ were confirmed as HLA-DR, but not DQ, restricted immunodominant epitopes (Table 4.2.1), $LF_{674-693}$ has been confirmed as an immunodominant epitope in both HLA-DR1 and HLA-DR15 transgenics (Table 4.2.1). The remaining peptides were all identified as immunodominant epitopes which each elicited a T cell response in a single HLA transgenic strain. $LF_{101-120}$ was confirmed as an immunodominant epitope in HLA-DQ8 transgenics (Table 4.2.1), $LF_{574-593}$ was confirmed as an immunodominant epitope in HLA-DR1 transgenics (Table 4.2.1), $LF_{654-673}$ was confirmed as an immunodominant epitope in HLA-DR4 transgenics (Table 4.2.1), and $LF_{694-713}$ was identified as an immunodominant epitope in HLA-DR15 transgenics (Table 4.2.1).

In the Turkish naturally infected individuals, however, the T cell response was focused upon four LF peptides. In parallel with the epitope hierarchy identified in the AVP vaccinees, a peptide epitope, $LF_{281-300}$, was uncovered which had not induced a response in any of the HLA transgenics (which of course have coverage of a minority of the common alleles). The three remaining peptides were all identified as immunodominant

epitopes which elicited a T cell response in the HLA transgenics. The domain II peptide, $LF_{467-487}$, had previously been identified as an immunodominant and immunoprevalent epitope, capable of eliciting a T cell response from all the HLA transgenics (Table 4.2.1). $LF_{694-713}$ and $LF_{714-733}$ had both been identified as immunodominant epitopes in HLA-DR15 transgenics (Table 4.2.1) unusually $LF_{694-713}$ was the only epitope also identified as an epitope in the human AVP vaccinated cohort.

Both naturally infected and vaccinated individuals showed robust T cell responses to LF antigens. Although the response in AVP vaccinated individuals was broader and included more epitopes than in the naturally infected individuals, it appears that the magnitude of the T cell response in naturally infected individuals is higher in relation to the naïve controls than the vaccinated individuals (Ingram *et al* 2010b). The human T cell responses not only consisted of a Th1 driven IFNγ response, but also encompassed a broad pro-inflammatory cytokine profile, which indicated that domain IV was the primary focus of the immune response (Figure 4.2.10).

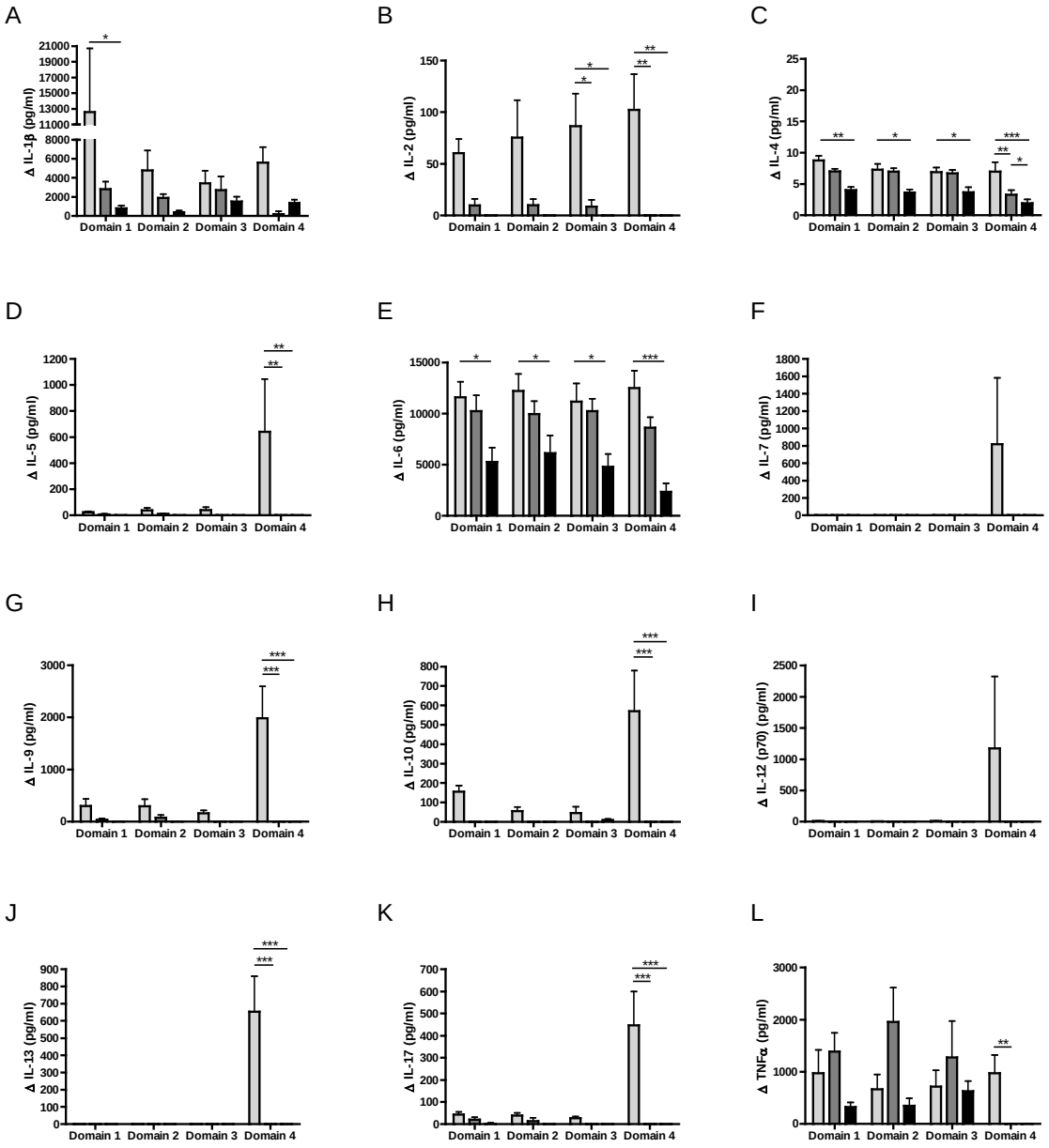


Figure 4.2.10 Evaluation of the cytokine profile produced in response to LF domains.

Human cytokine levels were quantified in supernatants recovered from the LF domain stimulated ELISpot assays from; naturally infected individuals (light grey bars, n=8), AVP-vaccinated individuals (dark gray bars, n=8), and healthy naïve controls (black bars, n=5) (±SEM). Responses were compared using two-way ANOVA with Bonferroni post hoc testing.

Levels of IL-5, IL-9, IL-10, IL-13, IL-17 and TNFα were significantly elevated in response to domain IV alone. The levels of IL-5 (Figure 4.2.10D) were increased in naturally infected individuals compared to vaccinees ($p<0.01$) and healthy controls ($p<0.01$). IL-9 levels (Figure 4.2.10G) were increased in naturally infected individuals compared to vaccinees ($p<0.001$) and in naturally infected individuals compared to healthy controls ($p<0.001$). Levels of IL-10 (Figure 4.2.10H) were elevated in naturally infected individuals compared to vaccinees ($p<0.001$), and in naturally infected individuals compared to healthy controls ($p<0.001$). Levels of IL-13 (Figure 4.2.10J) were increased in naturally infected individuals compared to vaccinees ($p<0.001$), and in naturally infected individuals compared to healthy controls ($p<0.001$). Levels of IL-17A (Figure 4.2.10K) were elevated in naturally infected individuals compared to vaccinees ($p<0.001$) and in naturally infected individuals compared to healthy controls ($p<0.001$). Levels of TNFα (Figure 4.2.10L) were increased in naturally infected individuals compared to vaccinees ($p<0.01$).

In contrast, levels of IL-6 and IL-4 were increased in response to all four domains. Levels of IL-4 (Figure 4.2.10C) were increased in naturally infected individuals compared to vaccinees in response to domain IV ($p<0.01$), there was an elevated level of the cytokine in naturally infected individuals compared to healthy controls in response to domain I ($p<0.01$), domain II ($p<0.05$), domain III ($p<0.05$) and domain IV ($p<0.001$), levels were also elevated in vaccinees compared to healthy controls in response to domain IV ($p<0.001$). Levels of IL-6 (Figure 4.2.10E) were elevated in naturally infected individuals compared to healthy controls in response to domain I ($p<0.05$), domain II ($p<0.05$),

domain III ($p<0.05$) and domain IV ($p<0.001$). Levels of IL-2 (Figure 4.2.10B) were increased in naturally infected individuals compared to vaccinees in response to domain III ($p<0.05$) and domain IV ($p<0.01$), levels were also increased in naturally infected individuals compared to healthy controls in response to domain III ($p<0.05$) and domain IV ($p<0.01$). Levels of the pro-inflammatory cytokine, IL-1β (Figure 4.2.10A) were significantly elevated in naturally infected individuals compared to healthy controls ($p<0.05$) only in response to domain I.

There was a significant increase in the levels of the Th2 cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 produced in response to stimulation with domain IV in infected individuals. In addition to the increase in IL-10 there was also a significant increase in the levels of IL-9, indicating a role for the Th9 subset in response to stimulation with domain VI in these individuals. Elevated levels of IL-6, IL-17A, and TNFα following exposure to domain IV, also suggested the involvement of both Th17, and Th1 subsets.

4.2.3 HLA binding of immunodominant epitopes

The limited number of epitopes derived from protein antigens that can provoke an immune response depend not only upon the efficiency with which an epitope is generated by cellular processing and the existence of an peptide/MHC specific TCR repertoire, but also upon the capacity of epitopes to bind to MHC molecules. This has a profound effect in determining the immunodominance hierarchy of the individual peptides. The binding capacity of LF peptides to the HLA-DR alleles; DR1, DR4, and DR15 was determined using competitive ELISA with the lab of Dr Bernard Maillere. Data was expressed as

relative activity, defined as the ratio of the IC_{50} of the LF peptide to the IC_{50} of an allele specific reference peptide which binds strongly to the HLA II complexes.

The HLA binding affinity studies revealed a number of epitopes which bound strongly to MHC class II molecules. Although it has been suggested that the strength of peptide MHC binding affinity is correlated with immunogenicity, several epitopes were identified which showed strong binding to all three HLA-DR alleles, but were not found to provoke an immune response in any of the HLA transgenics or human cohorts. These peptides which included; $LF_{91\text{-}110}$, $LF_{307\text{-}326}$, $LF_{447\text{-}466}$, $LF_{497\text{-}516}$, $LF_{507\text{-}526}$, $LF_{527\text{-}546}$, $LF_{684\text{-}704}$, $LF_{764\text{-}783}$, $LF_{784\text{-}803}$, and $LF_{794\text{-}809}$ (Table 4.2.2), were capable of binding strongly to the MHC peptide binding cleft, but incapable of generating a strong *in vivo* CD4+ T cell response.

The binding specificities of different HLA alleles governed the relative epitope binding affinities, allele specific epitopes such as $LF_{287–306}$ and $LF_{774\text{-}773}$ were identified which showed strong binding affinities to only one or two of the HLA alleles (Table 4.2.2).

Table 4.2.2 Binding affinities of LF epitopes to HLA-DR alleles, relative to their allele specific reference peptide.

Domain	LF aa	DR1	DR4	DR15
I	81 VKGEEAVKKEAAEKLLELVP 100	4	>1 250	65
	91 AAEKLLEKVPSDVLEMYKAI 110	85	31	0.2
	101 SDVLEMYKAIGGKIYIVDGD 120	5	305	2
	111 GGKIYIVDGDITKHISLEAL 130	122	61	6
	171 VENTEKALNVYYEIGKILSR 190	1	81	0.2
	181 YYEIGKILSRDILSKINQPY 200	69	19	43
	191 DILSKINQPYQKFLDVLNTI 210	3,944	566	13
	221 LLFTNQLKEHPTDFSVEFLE 240	909	6	193
	231 PTDFSVEFLE QNSNEVQEVF 250	17	3	335
	241 QNSNEVQEVFAKAFAYYIEP 260	0.1	137	1
	251 AKAFAYYIEPQHRDVLQLYA 270	115	51	8
II	261 QHRDVLQLYAPEAFNYMDKF 280	146	100	0.3
	271 PEAFNYMDKFNEQEINLSLE 290	2156	2	463
	281 NEQEINLSLEELKDQRMLSR 300	85	559	11
	287 LSLEELKDQRMLSRYEKWEK 306	31	>1 250	18
	387 KELLNRIQVDSSNPLSEKEK 406	146	0	1
	407 EFLKKLKLDIQPYDINQRLQ 426	218	500	0.5
	447 RDIQNIDALLHQSIGSTLYN 466	1	9	1
	457 HQSIGSTLYNKIYLYENMNI 476	115	55	0.02
	467 KIYLYENMNINNLTATLGAD 486	0.2	0.2	0.1
	497 NRGIFNEFKKNFKYSISSNY 516	23	6	99
	507 NFKYSISSNYMIVDINERPA 526	0.3	0.2	44
	527 LDNERLKWRIQLSPDTRAGY 546	0.1	0.3	7
III	307 IKQHYQHWSDSLSEEGRGLL 326	73	2	67
	327 KKLQIPIEPKKDDIIHSLSQ 346	0.2	750	14
	347 EEKELLKRIQIDSSDFLSTE 366	267	9	0.2
	367 EKEFLKKLQIDIRDSLSEEE 386	135	188	8
IV	547 LENGKLILQRNIGLEIKDVQI 567	0	7	0.04
	558 IGLEIKDVQIIKQSEKEYIRIDAKVVP 585	1	919	1
	574 EYIRIDAKVVPKSKIDTKIQ 593	3	15	156
	594 EAQLNINQEWNKALGLPKYT 613	104	43	1
	604 NKALGLPKYTKLITFNVHNR 623	0	10	3
	614 KLITFNVHNRYASNIVESAY 633	35	3	5
	624 YASNIVESAYLILNEWKNNI 643	42	331	3
	634 LILNEWKNNIQSDLIKKVTN 653	345	217	1
	644 QSDLIKKVTNYLVDGNGRFV 663	12	15	0.2
	654 YLVDGNGRFVFTDITLPNIA 673	123	0.3	2
	674 EQYTHQDEIYEQVHSKGLYV 693	1	237	13
	684 EQVHSKGLYVPESRSILLHG 703	2	99	64
	694 PESRSILLHGPSKGVELRND 713	7	22	17
	714 SEGFIHEFGHAVDDYAGYLL 733	5	46	0.04
	724 AVDDYAGYLLDKNQSDLVTN 743	1,380	10	0.1
	734 DKNQSDLVTNSKKFIDIFKE 753	>2 563	>1 250	10
	744 SKKFIDIFKEEGSNLTSYGR 763	4	0.2	1
	754 EGSNLTSYGRTNEAEFFAEA 773	>2 563	992	20
	764 TNEAEFFAEAFRLMHSTDHA 783	0.2	1	0.1
	774 FRLMHSTDHAERLKVQKNAP 793	5	1	>208
	784 ERLKVQKNAPKTFQFINDQI 803	1	22	21
	794 KTFQFINDQIKFIINS 809	9	6	1

In contrast, previously defined immunodominant LF epitopes, such as the domain II peptides $LF_{457-476}$ and $LF_{467-487}$, which were identified in all HLA transgenic strains, (and in the case of $LF_{467-487}$ also recognised by naturally infected individuals), showed relatively broad binding to all HLA-DR alleles examined (Table 4.2.2). The HLA-DR allele specific epitope, $LF_{547-568}$, which was also recognised by the human vaccinee cohort as an immunodominant epitope, showed strong binding to all HLA-DR alleles (Table 4.2.2). The domain IV peptides $LF_{694–713}$ and $LF_{714-733}$ which were both identified as HLA-DR15 restricted epitopes, recognised in the context of natural infection (and, in the case of $LF_{694–713}$ in vaccination) in humans, bound strongly to all three HLA-DR alleles (Table 4.2.2).

A number of key immunodominant epitopes identified in both transgenics and human cohorts, showed a strong HLA binding affinity, and domain IV especially appeared to be rich in epitopes which induced a strong *in vivo* response (Table 4.2.1) as well as epitopes which bound strongly to HLA alleles (Table 4.2.2). However, examining the relationship between strength of peptide-MHC binding affinity and immunogenicity reveals a lack of significant correlation between the strength of the binding affinity and the magnitude of the CD4+ T cell response to any of the LF peptides (Figure 4.2.11). The absence of any relationship held true regardless of whether all LF peptides were examined or the data were enriched for immunodominant peptides or peptides which bound strongly to the HLA alleles.

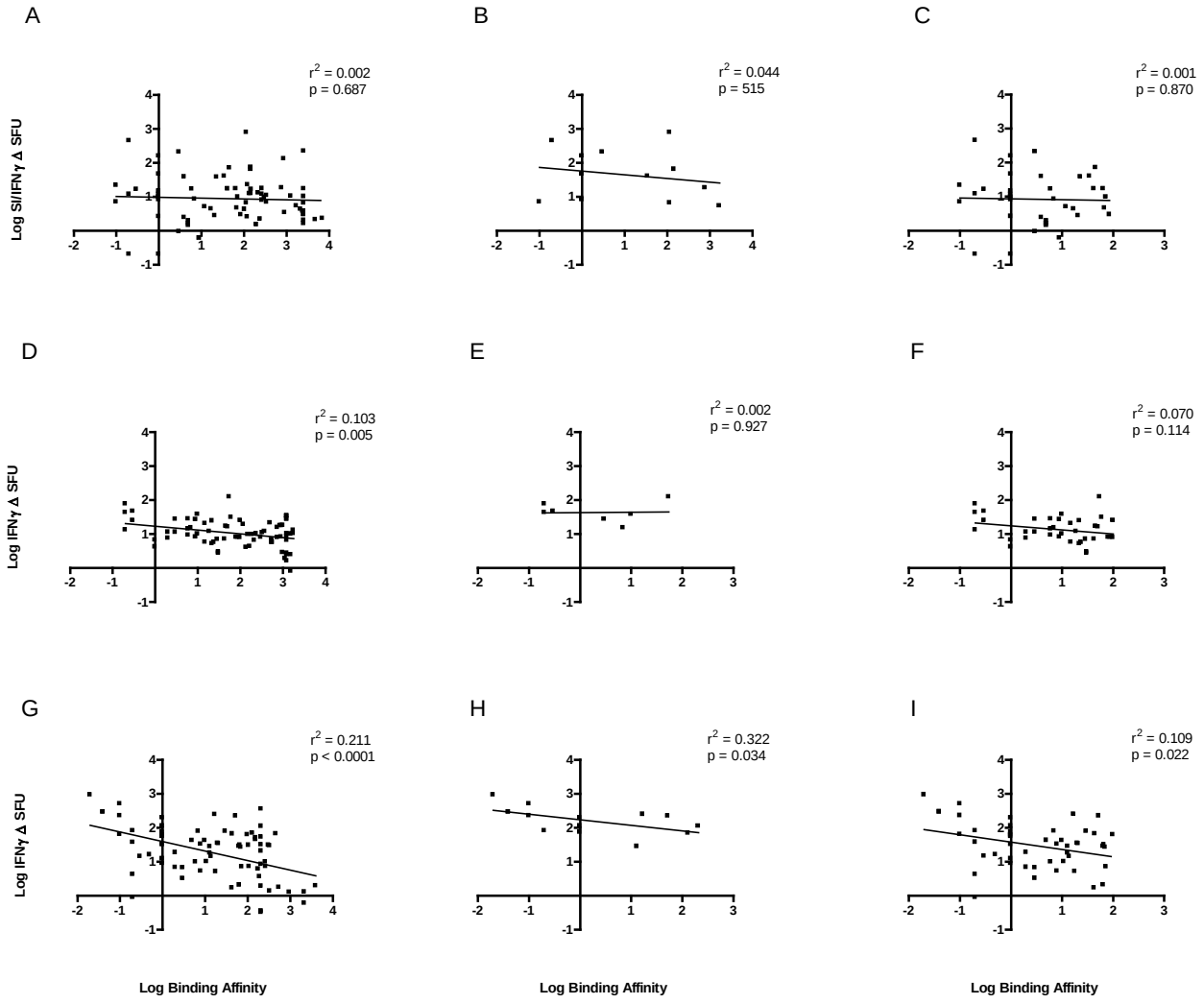


Figure 4.2.11 HLA-DR alleles were examined for a relationship between the binding affinity and the *in vivo* response to LF peptides.

The DR1 restricted response to; (A) all LF peptides, (B) immunodominant peptides, (C) peptides which were strong MHC binders, the DR4 restricted response to; (D) all LF peptides, (E) immunodominant peptides, (F) peptides which were strong MHC binders, the DR15 restricted response to; (G) all LF peptides, (H) immunodominant peptides, (I) peptides which were strong MHC binders, showed that there was no significant correlation between the strength of the binding affinity and the magnitude of the CD4+ T cell response to any of the LF peptides. Values were logged along the x and y axis and analysed by linear regression.

4.3 Discusion

Antigen presentation through both HLA-DR and DQ is important in the induction of immunity and the allelic diversity inherent in these class II molecules influences susceptibility to infection (Kwok *et al* 2000). The reductionist use of transgenic models in this study was intended as a means of eliciting HLA restricted T cell responses to immunogenic epitopes of the LF toxin secreted by *B. anthracis*. It was anticipated that the nature of these models would indicate epitopes which would induce a similar recall response to those seen in humans of a similar HLA type. Despite the presence of many potential peptide epitopes within the LF toxin, the elicited T cell response indicates that immunodominant LF epitopes are concentrated in domains II and IV. It is interesting to note that the T cell epitope mapping reveals a very different pattern to that of the B cell epitope mapping performed by Nguyen *et al*, who found that the majority of structural B cell epitopes clustered in domains I and III (Nguyen *et al* 2009a). This emphasises the importance of mapping the cellular as well as humoral response to complex protein antigens such as LF.

The variation in expression levels demonstrated by HLA class II molecules (Gorga *et al* 1987) would be expected to complicate the context in which the T cells encounter the presented LF epitopes. However, across the transgenic lines used in this study, which represented five distinct HLA alleles, the T cell response showed broad similarities. This was most evident in the response to domain II, which produced immunogenic responses in both HLA-DR and DQ transgenic mice following stimulation with either the whole domain (Figure 4.2.2), or the individual peptides $LF_{457-476}$ and $LF_{467-487}$, (Table 4.2.1)

which dominate the T cell response to this domain. These immunodominant epitopes therefore also comprise 'public specificities' or promiscuous epitopes which are efficiently processed and presented by APCs, to a peptide-MHC specific TCR repertoire, in all HLA transgenics.

The C-terminal domain II shows structural homology with the ADP-ribosyltransferase found in the *Bacillus cereus* VIP2 toxin. Along with domains III and IV it forms the active site which is involved in substrate recognition and binding (Pannifer *et al* 2001b). The amino terminus of MAPK kinases fit into the LF groove which contains several conserved long chain aliphatic residues (Liang *et al* 2004). These residues occur in three distinct clusters; the first is composed of Ile^{298}, Ile^{300}, Ile^{485}, Leu^{494}, and Leu^{514}, the second cluster of residues contains Ile^{322}, Ile^{343}, Leu^{349}, Leu^{357}, and Val^{362} which lie at the end of the catalytic groove. The final cluster of aliphatic residues lies close to the domain IV groove; Leu^{450}, Ile^{467}, Leu^{677}, Leu^{725}, and Leu^{743} (Liang *et al* 2004). Both of the immunodominant epitopes $LF_{457\text{-}467}$ and $LF_{467\text{-}487}$ overlap two of the aliphatic residues, Ile^{467} and Ile^{485} which may have an effect upon the substrate binding of MAPK kinases.

Domain IV is the catalylically active center of the LF toxin (Ascenzi *et al* 2002), its protein folds contain a sequence which shares similarity with the zinc-dependant metalloproteases found in the bacterial peptidase thermolysin and in *C. tetani* and *C. botulinum* (Mock and Roques 2002). The binding groove created by domains II, III and IV is responsible for holding the 16 amino acid long peptide which makes up the tail of the MAPK kinase family (Pannifer *et al* 2001a), and it has been observed that mutations

in the sequence coding for domain IV eliminates the peptidase activity of LF, abrogating its toxicity (Mock and Roques 2002). The putative zinc binding site [HEFGHAV] which occurs between the amino acid residues LF_{686} and LF_{690} (Duesbery *et al* 1998; Hammond and Hanna 1998) was only a feature of the HLA-DR1 transgenic response to $LF_{674-693,}$ this is surprising given that these important residues for zinc binding and catalysis as well as cytotoxicity are conserved across this class of metalloprotease (Klimpel *et al* 1994), and might be expected to represent a major immune target. The immunodominant epitopes identified within domains II and IV therefore appear to comprise essential residues of LF which are critical for efficient catalytic activities and the execution of substrate cleavage.

None of the transgenics showed CD4+ T cell responses against either the whole domain III or the individual peptides from this domain. The helix bundle which makes up domain III is inserted into domain II, and is believed to have to have arisen from repeated duplications of a structural element of domain II (Ascenzi *et al* 2002). Although these domains share elements of their structure and function the CD4+ T cell response to each is very different. Domain III appears to be a hidden or infrequent target of the immune response. The subdominant or cryptic responses to LF were explored in Chapter 5.

Domain I binds with high affinity to the proteolytically active 63kD PA heptamers which are responsible for the membrane-translocation of the anthrax toxins (Friedlander *et al* 1993a). Over the first 250 residues, this domain shares significant sequence identity (35%) and similarity (55%) with domain I of EF (Quinn *et al* 1991). The N-terminal

sequence of both toxins contain a common domain for PA binding, which in LF has been shown to be sufficient to act alone as a carrier for delivery of heterologous proteins across membranes in the presence of PA (Arora and Leppla 1993). The sequence homology between the two toxins within domain I was demonstarated by Nguyen *et al*, who found that immunisation with LF induced antibodies which were cross-reactive with EF (Nguyen *et al* 2009b). One of the most important cross-reactive epitopes $LF_{265-274}$ (which corresponds to $EF_{257-268}$), overlapped with the HLA-DR1 restricted T cell epitopes $LF_{251-270}$ and $LF_{261-280}$, indicating that these epitopes are capable of inducing a neutralising antibody response to both LF and EF as well as the T cell response, making them interesting candidates for inclusion in a polyepitopic anthrax vaccine.

Of the five HLA strains challenged with domain I peptides, only the HLA-DR1 and DQ8 transgenics showed CD4+ T cell responses to peptide epitopes from this domain. In contrast to domain IV peptides, epitopes in this domain are presented in the context of both DR and DQ, although there appears to be minimal overlap in the actual peptides presented. This appears unusual as when when immune responses are analysed in the context of normal presentation on human APC, the DR promoter is so much stronger than the DQ promoter that the vast majority of human T cell clones analysed tend to be HLA-DR restricted (Kwok *et al* 2000). It may be that by enabling the study of the DQ-restricted repertoire in the absence of this DR-restricted dominance, the transgenic lines are allowing the delineation of a part of the repertoire that would normally be inaccessible through being dwarfed compared with the other responses.

Most vaccine strategies against anthrax have concentrated upon PA, however our lab has identified LF as a major target of T cell immunity in humans (Ingram *et al* 2010b). This is surprising given that the amount of LF released by *B. anthracis* is one-sixth that of PA (Fish *et al* 1968). It was discovered that the magnitude of the CD4+ T cell response to LF antigens was greater in naturally infected individuals than in vaccinees (Ingram *et al* 2010b). The robust, long lasting T cell immunity to LF, particularly domain IV, identified in naturally infected individuals is in contrast to the expected response to LF exposure, especially in the context of infection. The cleavage of MAPK kinases by LF inhibits the evolutionarily conserved MAPK signal transduction pathway, which has multiple downstream consequences for both the innate and adaptive immune systems. The resulting immune subversion might be expected to impair the T cell memory of *B. anthracis* in survivors of natural infection.

A number of epitopes were identified in human vaccinees which were not present in the naturally infected individuals. Of the vaccine specific epitopes, only $LF_{694\text{-}713}$ showed overlap with the epitope repertoire identified in the naturally infected individuals. Whilst it might be expected that some epitopes present in the context of vaccination would be lost upon infection (Vogel *et al* 2002), the repertoire of the immune response detected after immunization differed substantially from the immune response after infection. It is unclear whether this represents the differential antigen processing of pathogen associated proteins experienced in vaccination in contrast to infection, or if it represents an artefact of the repeated AVP vaccinations which may have served to skew the cytokine

environment present during the induction of the immune response, impacting upon the T cell epitope repertoire (Siew *et al* 1998).

Taking into account all the HLA-DP, DQ and DR products, as well as inter and intra isotypic mixed pairs, a heterozygous human can present peptides for CD4+ T cell recognition on up to fourteen different class II molecules. It is therefore interesting to note that, despite the immunogenetic heterogeneity seen in human populations, which along with differences in exposure to the antigen, might be expected to complicate the epitopes recognised by the human cohorts studied, compared to the HLA transgenics, the $LF_{467\text{-}487}$ peptide was one of the main targets of a strong CD4+ T cell response amongst the naturally infected individuals.

Humans exposed to LF following cutaneous anthrax infection thus generate robust long-term T cell memory to *B. anthracis* epitopes, in many cases several years after the initial infection event. These naturally infected individuals T cell response showed significantly elevated levels of the pro-inflammatory cytokines associated with Th1, Th2, Th9 and Th17 subsets compared to vaccinees and naïve controls, in response to domain IV (Figure 4.2.10). Stimulation of naïve CD4+ T cells to enter the complex differentiation program leading to the development of particular Th cell subsets can be critical in determining the outcome of infection. The Th1 subset, which plays a major role in co-ordinating cell-mediated immunity and the Th2 subset which has been associated with the maturation of activated B cells into antibody-producing plasma cells, thus promoting the development of humoral responses, have been joined by the recently described Th9 and Th17 subsets

(Mosmann and Coffman 1989; Murphy and Stockinger 2010; Zhu *et al* 2010). The Th9 subset has been implicated in inflammatory responses, while Th17 cells promote the recruitment and activation of phagocytes and complement the function of Th1 cells through the release of pro-inflammatory cytokines (Dardalhon *et al* 2008; Veldhoen *et al* 2006; Veldhoen *et al* 2008; Zhou and Littman 2009). The inhibitory effects of both LT and ET upon expression of the activation markers CD25 and CD69 and the secretion of the pro-inflamatory cytokines IL-2, IL-5, TNFα, and IFNγ by human T cells has been described *in vitro* (Fang *et al* 2005; Paccani *et al* 2005). Murine lymphocytes have also demonstrated impaired TCR mediated cell activation and CD4+ T cell production of the cytokines IL-3, IL-4, IL-5, IL-6, IL-10, IL-17, TNFα, IFNγ and GM-CSF following exposure to LT and ET (Comer *et al* 2005). However, the cellular immunity we have identified within the naturally infected humans indicates that, although *in vitro* exposure to ET has been implicated in immune deviation towards both the Th2 and Th17 pathways (Paccani *et al* 2011; Paccani *et al* 2009), the human immune response against LF encompasses a broad range of Th subsets with little or no helper T cell polarization. This lack of skewing following repeated exposure to LF antigens echos the work of Mark Peakmans lab, who found that there was an equal balance of Th1 and Th2 reponder cells in the recall immunity to anthrax (Allen *et al* 2006). It is however important to note that this group analysed the T cell responses of a population of AVP vaccinated individuals to the anthrax vaccine preparation itself, which contains variable amounts of PA, LF and EF. The use of luminex to analyse the cytokines released by human T cells gives a fuller picture of the cellular response to anthrax antigens than the IL-2, IL-4, IL-13 and IFNγ ELISpots utilised by Allen *et al*, however, further work using multiparameter flow

cytometer analysis would be needed to delineate the existence of several distinct subsets of LF antigen specific Th cells.

An early inflammatory response to LF caused by the nod like receptor Nlrp1b inflamasome activation is associated with an increased resistance to *B. anthracis* spore challenge (Terra *et al* 2010). The role of LT in the disruption of the MAPK signalling pathways, with its consequences for the apoptosis of antigen presenting cells, specifically the lysis of dendritic cells and macrophages, might be expected to subvert host immunity and promote the establishment of a systemic anthrax infection. Furthermore, it would be predicted that survivors of natural infections with *B. anthracis* would have impaired T cell memory of the pathogen.

However, in mice which possess LT sensitive alleles of the Nlrp1b gene, the inflamasome-mediated cell lysis promotes a pro-inflammatory response predominantly driven by IL-1β which recruits monocytes and PMN's to the site of LT exposure to resolve infection. Recent work by us shows that humans exposed to LT following cutaneous anthrax infection generate robust long-term, T cell memory to *B. anthracis* epitopes. We propose that in addition to resolving infection, the cell infiltration and cytokine milleu seen in early inflammation are crucial in driving antigen presentation and T cell priming which results in broad Th cell subset responses to LF, several years after the initial infectious event.

Immunodominance is influenced by multiple factors; the presentation of a peptide/MHC class II complex to a T cell repertoire is one of these critical parameters in developing a immune response directed towards a few key antigens. A discrepancy between the DR and DQ restricted responses occurred following challenge with domain IV peptides, epitopes from this domain were overwhelmingly presented in the context of HLA-DR. This was demonstrated by the CD4+ T cell response to $LF_{547-567}$, which was a feature of the response in HLA-DR1, DR4 and DR15 transgenics. The ability of this domain to readily provoke a recall response in CD4+ T cells from all of the HLA-DR but not DQ transgenics indicates a preferential association of the LF antigen with specific HLA alleles. A number of immunodominant epitopes identified within LF also showed broad HLA binding characteristics, most notably the domain II epitopes $LF_{457-476}$ and $LF_{467-487}$ which showed strong binding across a range of HLA-DR molecules as well as the preponderance of epitopes from domain IV which were presented by HLA-DR. The strength of HLA binding does not however appear to predict the immunodominance of the peptide epitope. This was surprising as a number of studies have described a strong correlation between the affinity of binding and the ability of a peptide to be presented by a particular MHC molecule resulting in an immunodominant T cell response (Buus *et al* 1987; Lazarski *et al* 2005; Schaeffer *et al* 1989; Weaver *et al* 2008). There is, however, still a lack of concensus regarding the mechanisms which govern the immunodominance of certain epitopes. Work by our collaborators in John Robinsons lab upon the immunodominance of epitopes within PA (Musson *et al* 2003) and the *Y. pestis* capsular protein fraction 1 (Caf1) (Musson *et al* 2006) demonstrated the importance of both the classical and recycling presentation pathways in determining the epitope hierarchy.

Peptides which require extensive proteolytic processing are presented by newly synthesized class II molecules through the classical antigen presentation pathway (Griffin *et al* 1997; Zhong *et al* 1997), while determinants which require less processing, can be presented through the recycling presentation pathway, which allows peptides to be loaded directly onto class II molecules recycled from the cell surface (Griffin *et al* 1997). It is not clear whether this sequential processing plays an important role in determining the immunodominance of epitopes within LF, and it is evident that further work examining the precise mechanisms which govern the immunodominance of the epitopes identified within this chapter is required.

A polyepitopic vaccine in which each epitope is promiscuous, or covers a number of HLA alleles, increases the chance that each individual in a genetically heterogeneous population acquires immunity to multiple epitopes from a pathogen, thus giving increased protection. Within this chapter we have identified the LF domains which are rich in CD4+ T cell epitopes, and the individual peptide epitopes which are capable of eliciting a T cell response from all HLA alleles assayed.

5 LF cryptic epitope mapping

5.1 Introduction

Protein antigens typically possess a large number of possible immunogenic peptide sequences, only a few of which contribute to the development of a T cell response against the whole protein antigen. This subset of epitopes can be surprisingly limited; in the case of Leishmania the initial immune response is directed towards a single immunodominant epitope (Pingel *et al* 1999; Reiner *et al* 1993). Epitopes which are capable of triggering this kind of potent T cell response, following *in vivo* priming, are classified as immunodominant. In contrast, epitopes which do not elicit a T cell response against the whole protein antigen, but are immunogenic in peptide form, are termed cryptic. These cryptic peptides (which may also be referred to as subdominant determinants), have proven important in a number of immunological contexts. Determinant spreading has been observed, during the course of certain autoimmune diseases, from a limited number of immunodominant epitopes to numerous cryptic epitopes later in disease (Sercarz 1998; Tuohy *et al* 1998). Seen in this light, cryptic epitopes which might otherwise be deemed a lab artefact only seen on priming with synthetic peptide, can be seen as part of a dynamic response, unveiled in a context dependent manner. Development of autologous transgenic T cells which are specific for cryptic and dominant epitopes derived from self antigens has demonstrated, during studies of self-non-self discrimination, that immune tolerance may be limited to dominant epitopes (Cibotti *et al* 1992; Moudgil and Sercarz 1993). This failure to develop tolerance towards cryptic epitopes, which are uncovered during

the course of autoimmune reactions, may be related to an increased severity of disease over time (Lehmann *et al* 1992; Vanderlugt *et al* 2000; Yu *et al* 1996). The incorporation of cryptic epitopes into vaccines, principally against cancer antigens has also been explored in vaccine studies (Melief *et al* 1996; Moudgil and Sercarz 1994; Slingluff, Jr. 1996). The identification of cryptic epitopes and incorporation into vaccines, alongside immunodominant T cell epitopes, may have a major advantage in aiding the development of an immune response against a pathogenic organism. The T cell repertoire of the immune response following immunisation and infection has proven to differ in regard to immunodominant epitopes (Sewell *et al* 1999; Vogel *et al* 2002), and our own studies in humans have demonstrated that exposure to live anthrax bacteria uncovers cryptic epitopes within LF, which were not identified as T cell determinants following protein antigen vaccination (Ingram *et al* 2010b). This suggests that cryptic epitopes may play an essential role in directing the immune response to pathogens *in vivo*. In the case of the protozoan parasite *Trypanosoma cruzi*, immunisation with cryptic or subdominant determinants contribute to an artificially broadened T cell repertoire which provides increased protection against infection, compared to immunisation with the single immunodominant epitope (Dominguez *et al* 2011). The immunodominance of certain pathogen derived epitopes can therefore be detrimental as they can lead to a restriction in the breadth of the immune response. Individuals immunised with vectors which encode the HIV proteins Gag, Pol and Nef, respond to a single epitope per protein, this concentration upon such a limited number of immunodominant epitopes has a negative impact upon protection against HIV (Watkins *et al* 2008). Thus polyepitopic vaccines, which contain multiple epitopes, both cryptic and immunodominant, may act to

circumvent the mechanisms allowing pathogens to escape recognition by the immune system.

Work described in this chapter attempted to uncover cryptic epitopes derived from domains I and III of the anthrax protein, LF. The T cell responses were analysed using a panel of HLA-transgenic mice and the relative HLA binding affinities of the relevant peptide sequences.

5.2 Results

5.2.1 Mapping of LF cryptic epitopes

The T cell epitope mapping carried out in the previous chapter revealed that immunodominant epitopes were largely localized to domains II and IV of LF. However, the *in silico* prediction of HLA-DR restricted T cell LF epitopes carried out using the IEDB T cell epitope prediction tool and the HLA binding affinity studies, suggested that a number of epitopes existed in both domains I and III, which had not been uncovered by mapping the immunodominant epitopes. Domain III was of specific interest due both to its 'silent' nature which was common to all HLA alleles during the immunodominant epitope mapping and the role that this region may play in dictating the stability and function of the LF catalytic site (Quinn *et al* 1991).

As a proof of principle, the cryptic CD4+ T cell responses to the peptides which encompass LF domain I were mapped by immunising HLA-DR4 transgenics with one of four pools of non-overlapping LF peptides and restimulating with the individual peptides (Figure 5.2.1). The transgenics immunised with the pool comprising LF_{51-70}, LF_{91-110}, $LF_{131-150}$, $LF_{171-190}$, $LF_{211-230}$, and $LF_{251-270}$ did not show any response to these peptides upon restimulation. However transgenics immunised with pools comprising LF_{41-60}, LF_{81-100}, $LF_{121-140}$, $LF_{161-180}$, $LF_{201-220}$, and $LF_{241-260}$ demonstrated a strong T cell recall response to $LF_{161-180}$, while pools comprising LF_{61-80}, $LF_{101-120}$, $LF_{141-160}$, $LF_{181-200}$, $LF_{221-240}$, and $LF_{261-280}$ elicited a T cell response to both $LF_{181-200}$ and $LF_{221-240}$. The transgenics immunised with the pool which included LF_{31-50}, LF_{71-90}, $LF_{111-130}$, $LF_{151-170}$, $LF_{191-210}$ and

$LF_{231-250}$ demonstrated a strong T cell recall response to the epitopes $LF_{111-130}$ and $LF_{231-250}$, however, unlike the other pools, the peptides within this pool were capable of inducing a T cell response to the peptide, as well as to the whole native protein. It remains unclear exactly which cryptic epitope from the pool is capable of causing this heteroclitic response; while $LF_{111-130}$ or $LF_{231-250}$ may be responsible, there remains the possibility that one of the peptides which did not produce a T cell response was capable of inducing a response to the whole protein instead.

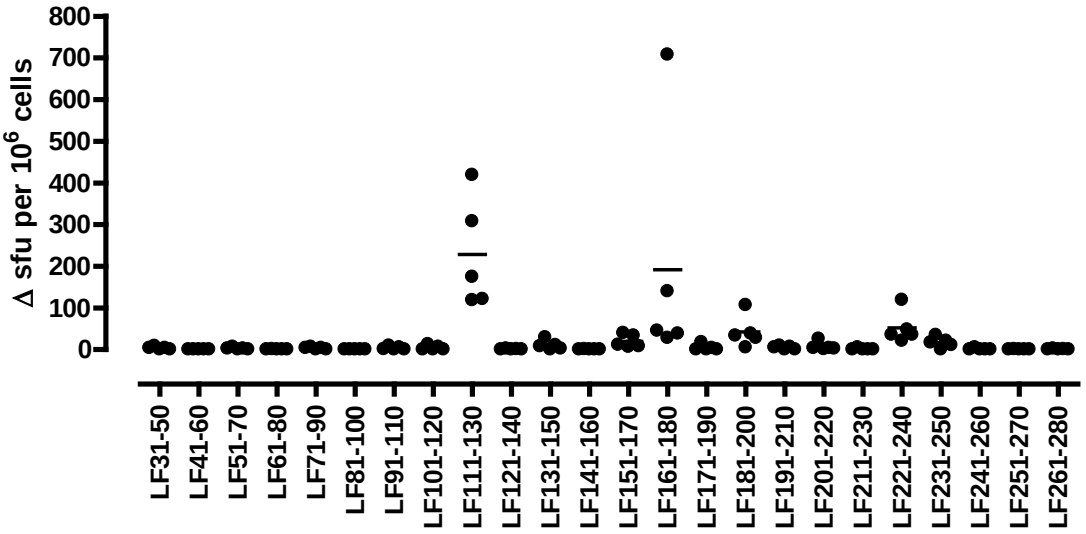


Figure 5.2.1 *Ex vivo* mapping of the HLA-DR4 restricted cryptic CD4+ T cell epitopes of LF domain I.

HLA-DR4 transgenic mice were immunised with pools of non-overlapping LF peptides comprising domains I emulsified in Titremax. After 10 days, cells were isolated from the draining lymph node and cultured in an IFNγ specific ELISpot plate with 25μg/ml of the individual LF peptides for 72 hours. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation (n=5).

The cryptic CD4+ T cell responses to the peptides which encompass LF domain III were mapped in HLA-DR and DQ transgenic strains by immunising the animals with one of two distinct pools of non-overlapping LF peptides and restimulating with the individual peptides (Figure 5.2.2). A T cell recall response to the whole native protein as well as to the individual peptides was only induced in one peptide pool, which was HLA-DR1 restricted, this pool comprised LF $_{287\text{-}306}$, $LF_{307\text{-}326}$, $LF_{327\text{-}346}$, $LF_{347\text{-}366}$, $LF_{367\text{-}386}$ and $LF_{387\text{-}406}$. The HLA-DR1 allele demonstrated a strong T cell recall response to the peptides $LF_{287\text{-}306}$, $LF_{307\text{-}326}$, $LF_{327\text{-}346}$, $LF_{347\text{-}366}$, and $LF_{387\text{-}406}$ (Figure 5.2.2A), while the HLA-DR4 restricted cryptic epitopes were identified as $LF_{307\text{-}326}$ and $LF_{387\text{-}406}$ (Figure 5.2.2B). Only one HLA-DR15 restricted cryptic epitope was identified; $LF_{317\text{-}336}$ (Figure 5.2.2C) The HLA-DQ alleles showed a divergence in their ability to present cryptic epitopes, while no HLA-DQ6 restricted cryptic epitopes were identified (Figure 5.2.2E), $LF_{337\text{-}356}$, $LF_{377\text{-}396}$ and $LF_{387\text{-}406}$ were all identified as HLA-DQ8 restricted cryptic epitopes (Figure 5.2.2D).

HLA-DR1 alleles therefore present the highest number of cryptic epitopes, derived from domain III of LF, which are capable of eliciting a strong T cell response. The cryptic epitope $LF_{307\text{-}326}$ was presented predominantly by HLA-DR alleles, both HLA-DR1 and DR4 transgenics exhibited T cell responses to this peptide, while $LF_{387\text{-}406}$ was the only cryptic epitope identified which was restricted by both HLA-DR and DQ alleles.

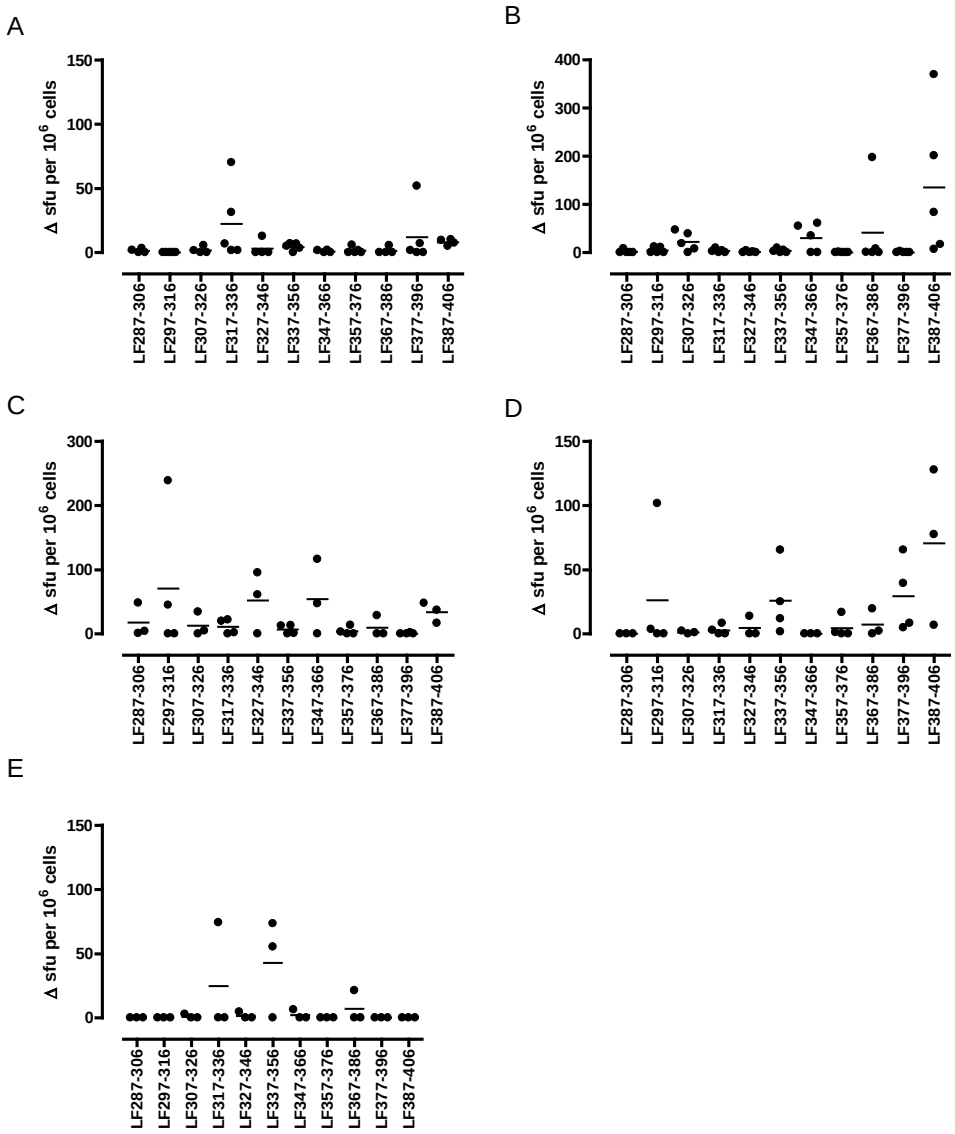


Figure 5.2.2 *Ex vivo* mapping of the HLA DR and DQ-restricted cryptic CD4+ T cell epitopes of LF domain III.

Transgenic mice; (A) HLA-DR1, (B) HLA-DR4, (C) HLA-DR15, (D) HLA-DQ8 and (E) HLA-DQ6 were immunised with pools of non-overlapping LF peptides comprising domain III emulsified in Titremax. After 10 days, cells were isolated from the draining lymph node and cultured in an IFNγ specific ELISpot plate with 25µg/ml of the individual LF peptides for 72 hours. Results are given as the number of spots above background (ΔSFU)/10^6 PBMCs with mean ± standard deviation (n=3-5).

5.2.2 HLA binding of cryptic epitopes

The HLA binding affinity of the cryptic LF epitopes, determined using competitive ELISA, to HLA-DR1, DR4, and DR15 revealed a lack of consensus between the alleles regarding the strength of peptide MHC binding affinity and immunogenicity (Table 5.2.1). Cryptic epitopes were identified which bound weakly to an allele (as in the case of $LF_{317-336}$ to HLA-DR15), even though they were capable of generating a strong *in vivo* CD4+ T cell response. Equally, peptides which bound strongly to an allele ($LF_{347-366}$ to HLA-DR4 and DR15) had not been identified as a cryptic epitope *in vivo* for these alleles. The peptide $LF_{387-406}$ exhibited strong binding affinity to HLA-DR4 and DR15 and weak binding to HLA-DR1, but was efficiently presented as a cryptic epitope *in vivo* by both HLA-DR1 and DR4.

Table 5.2.1 Binding affinities of cryptic LF epitopes to HLA-DR alleles, relative to their allele specific reference peptide.

Domain	LF aa	DR1	DR4	DR15
I	111 GGKIYIVDGDITKHISLEAL 130	122	61	6
	161 VLVIQSSEDYVENTEKALNV 180	1702	29	958
	181 YYEIGKILSRDILSKINQPY 200	69	19	43
	221 LLFTNQLKEHPTDFSVEFLE 240	909	6	193
	231 PTDFSVEFLE QNSNEVQEVF 250	17	3	335
III	287 LSLEELKDQRMLSRYEKWEK 306	31	>1250	18
	307 IKQHYQHWSDSLSEEGRGLL 326	73	2	67
	317 SLSEEGRGLLKKLQIPIEPK 336	149	>1788	268
	327 KKLQIPIEPKKDDIIHSLSQ 346	0.2	750	14
	337 KDDIIHSLSQEEKELLKRIQ 356	267	88	30
	347 EEKELLKRIQIDSSDFLSTE 366	267	9	0.2
	377 DIRDSLSEEEKELLNRIQVD 396	267	88	30
	387 KELLNRIQVDSSNPLSEKEK 406	146	0	1

An interesting pattern was however identified in the HLA-DR4 restricted domain I cryptic epitopes, it appeared that the epitopes identified in this domain all bound strongly to HLA-DR4 (Table 5.2.1). The relationship between the strength of peptide-MHC binding affinity and immunogenicity was examined (Figure 5.2.3) and intriguingly, although all the cryptic epitopes were strong binders, within this group, a decrease in binding affinity was correlated with an increase in the IFNγ T cell response to the peptide (r^2=0.809, p=0.038). It became clear upon further examination of all LF domain I peptides (Figure 5.2.4A) and following enrichment of the data for peptides which bound strongly (Figure 5.2.4B) or weakly (Figure 5.2.4C) to HLA-DR4 alleles, that the significant correlation between the low binding affinity and the magnitude of the CD4+ T cell response was only exhibited by cryptic epitopes.

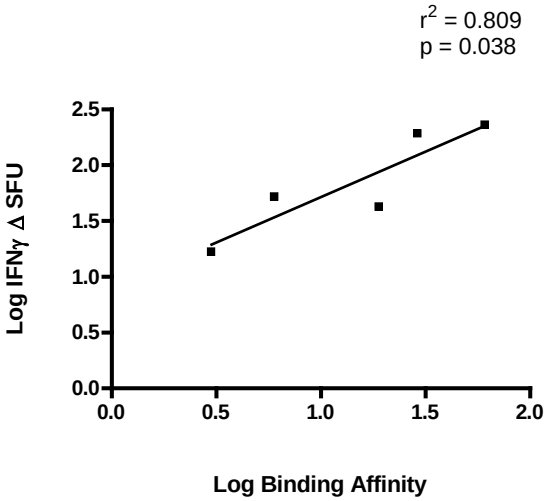


Figure 5.2.3 HLA-DR4 alleles were examined for a relationship between the binding affinity and the *in vivo* response to cryptic epitopes from LF domain I.

The DR4 restricted response to cryptic epitopes showed that there was a significant correlation between the strength of the binding affinity and the magnitude of the CD4+ T cell response to the cryptic epitopes from LF domain I (r^2=0.809, p=0.038). Values were logged along the x and y axis and anlysed by linear regression.

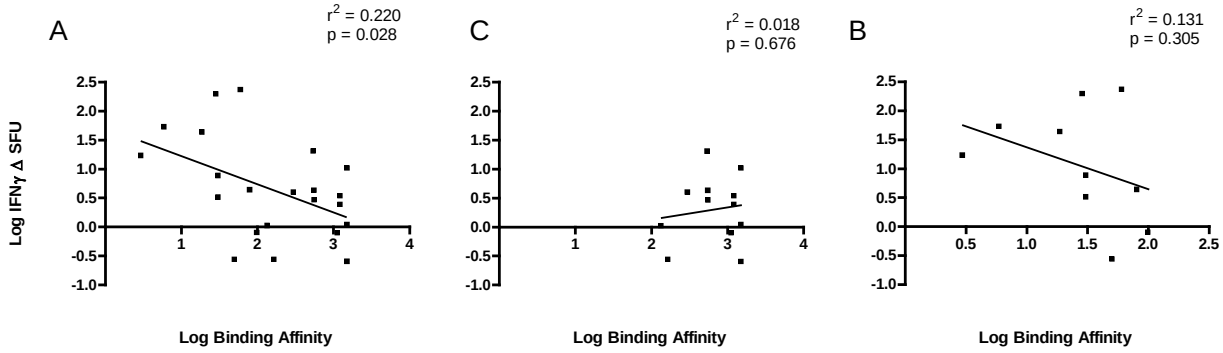


Figure 5.2.4 HLA-DR4 alleles were examined for a relationship between the binding affinity and the *in vivo* response to domain I LF peptides.
The DR4 restricted response to; (A) all domain I LF peptides, (B) peptides which were strong MHC binders, and (C) peptides which were weak MHC binders, showed that there was no significant correlation between the strength of the binding affinity and the magnitude of the CD4+ T cell response to the LF peptides from domain I. Values were logged along the x and y axis and analysed by linear regression.

In contrast to domain I, the relationship between the strength of the peptide-MHC binding affinity to the HLA-DR alleles and the immunogenicity of the cryptic epitopes identified within domain III reveals a lack of significant correlation between the binding affinity and the magnitude of the CD4+ T cell response (Figure 5.2.5). Enrichment of the data for peptides which bound strongly to HLA-DR1 alleles (Figure 5.2.6B) indicated instead that there was a significant correlation between the strength of binding affinity and the magnitude of the CD4+ T cell response to the HLA-DR1 restricted peptides in domain III (r^2=0.994, p=0.049).

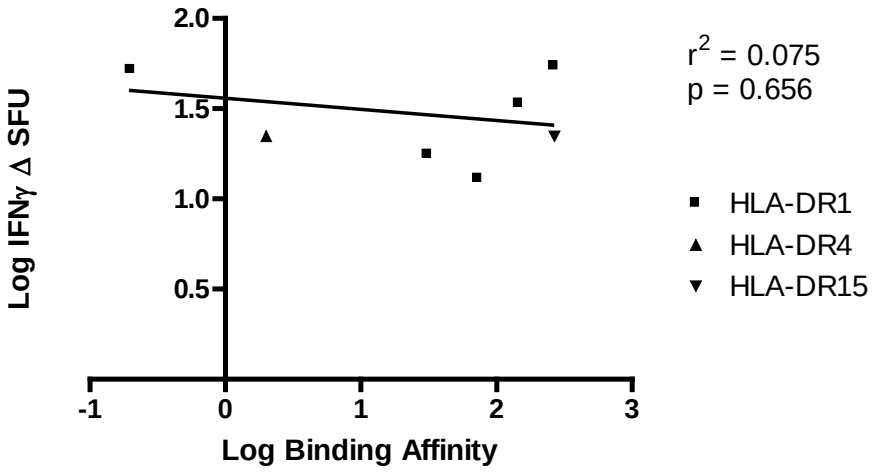


Figure 5.2.5 HLA-DR alleles were examined for a relationship between the binding affinity and the *in vivo* response to cryptic epitopes from LF domain III.

The HLA-DR1, DR4 and DR15 restricted response to cryptic epitopes showed that there was no significant correlation between the strength of the binding affinity and the magnitude of the CD4+ T cell response to the cryptic epitopes from LF domain III. Values were logged along the x and y axis and analysed by linear regression.

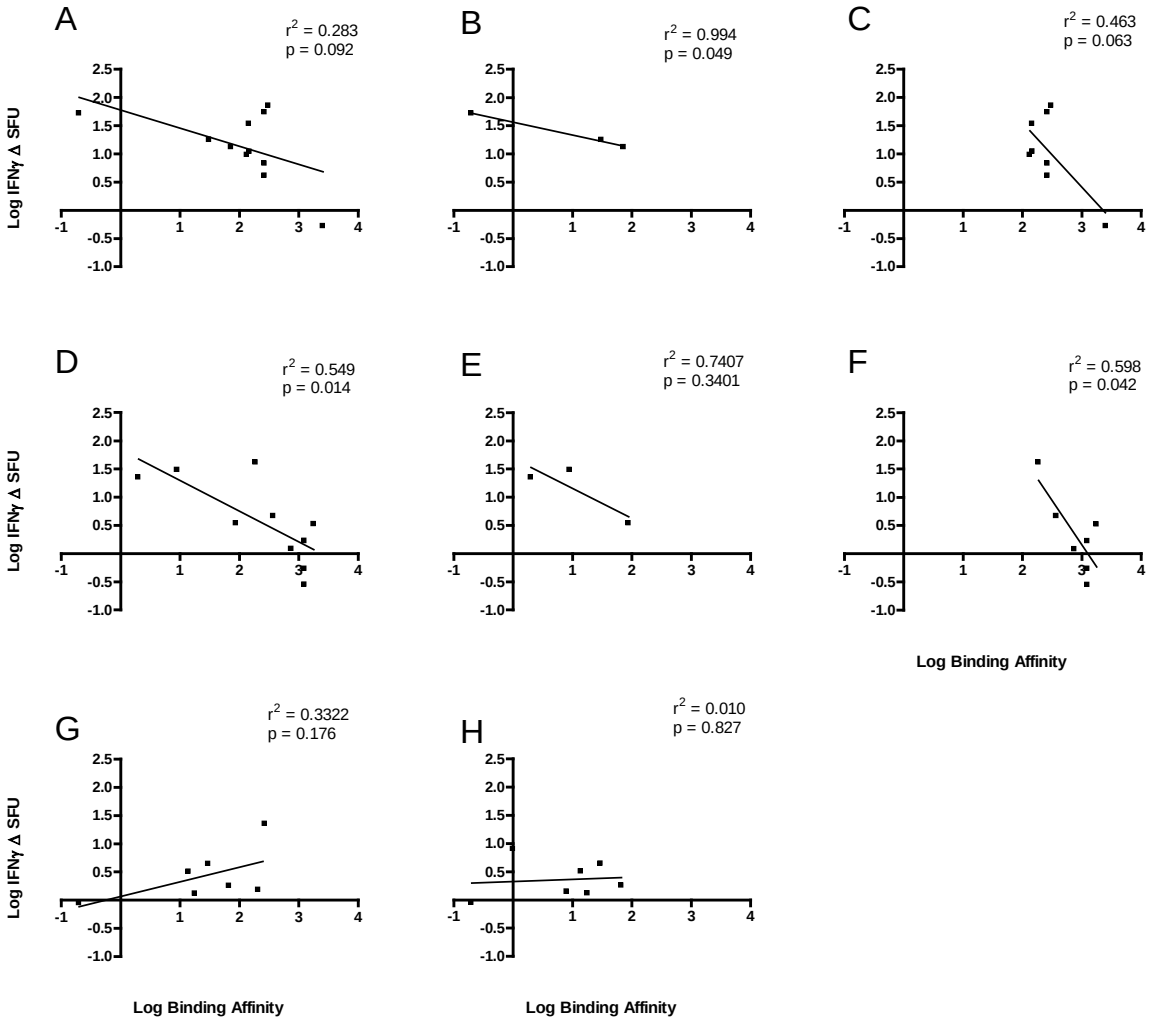


Figure 5.2.6 HLA-DR alleles were examined for a relationship between the binding affinity and the *in vivo* response to LF peptides from domain III.

The DR1 restricted response to; (A) all domain III LF peptides, (B) peptides which were strong MHC binders, (C) peptides which were weak MHC binders, the DR4 restricted response to; (D) all domain III LF peptides, (E) peptides which were strong MHC binders, (F) peptides which were weak MHC binders, the DR15 restricted response to; (G) all domain III LF peptides, (H) peptides which were strong MHC binders, indicated that there was a significant correlation between the strength of the binding affinity and the magnitude of the CD4+ T cell response to those HLA-DR1 restricted peptides which showed strong MHC binding (r^2=0.994, p=0.049). Values were logged along the x and y axis and a linear regression.

5.3 Discusion

Efforts to explain the mechanisms which govern immunodominance and crypticity have focused primarily upon role played by antigen processing and presentation. It has been suggested that intrinsic features within the protein molecule reduce the availability of particular epitopes, and efficient endosomal antigen processing may be hampered by flanking amino acids which either promote the enzymatic destruction of the determinant or hinder processing through slow or incomplete unfolding of the protein molecule (Moudgil *et al* 1996; Moudgil *et al* 1998). This is supported by studies which have indicated that immunodominant epitopes tend to cluster within regions which are either within or adjacent to protease-sensitive loops (Carmicle *et al* 2007; Dai *et al* 2002). The non-classical MHC class II heterodimer DM has also been implicated in directing the cryptic and immunodominant fate of epitopes (Nanda and Bikoff 2005; Nanda and Sant 2000), although it remains unclear whether this is due to the DM induced editing of the epitope repertoire to favour peptides which display a higher binding affinity for MHC molecules (Kropshofer *et al* 1996). Recent studies, which have focused on the critical role peptide binding plays in determining dominance hierarchies, have also demonstrated a direct correlation between immunodominance and the stability of the MHC/peptide complex (Lazarski *et al* 2005; Weaver *et al* 2008).

The initial screen of domain I of LF for HLA-DR4 restricted cryptic epitopes revealed the presence of several unique epitopes, which had not been identified during the mapping of immunodominant epitopes within this domain. $LF_{111-130}$, $LF_{161-180}$, $LF_{181-200,}$ $LF_{221-240}$ and $LF_{231-250}$ were all capable of eliciting a strong T cell response following administration in

peptide form (Figure 5.2.1). These cryptic epitopes, in direct contrast to the immunodominant epitopes identified in the preceeding chapter, showed a significant correlation between relative binding affinity and the magnitude of the IFNγ response. Unexpectedly this correlation indicated that, although all the cryptic epitopes exhibited strong binding affinities to HLA-DR4, within this group a weak binding affinity was ultimately associated with an increased IFNγ response (Figure 5.2.3). This result was not reproduced when all domain I peptides were analysed nor when the results were enriched for peptides which showed strong or weak binding to HLA-DR4 (Figure 5.2.4). The cryptic epitopes identified in domain III also did not follow this pattern, as the HLA-DR restricted epitopes showed no correlation between relative binding affinity and the magnitude of the IFNγ response (Figure 5.2.5). Following analysis of all peptides and enrichment for strong and weak binders (Figure 5.2.6) it was evident that only those domain III peptides which demonstrated a strong binding affinity for the HLA-DR1 allele displayed a significant correlation between binding affinity and the magnitude of the IFNγ response. Signifying that, in this context, a high relative binding affinity was associated with an increased IFNγ response.

These contradictory results suggest that the cryptic epitopes which exist within domains I and III of the LF protein are influenced to a great extent by the nature of the MHC class II molecules responsible for peptide presentation. The MHC class II molecules are among the most polymorphic glycoproteins known in nature (Reche and Reinherz 2003), with 1353 human HLA class II alleles currently identified (Robinson *et al* 2011). They play a crucial role in restricting the number of possible potential epitopes generated following

the intracellular processing of antigens. MHC polymorphism is concentrated in the region encoding the peptide-binding groove, giving rise to a distinct set of epitopes which bind in a preferential manner to each individual HLA allele. Murine studies have identified epitopes whose dominance or crypticity was dependent upon the H-2 haplotype involved in presentation (Grewal *et al* 1995; Moudgil *et al* 1998; Sinha *et al* 2007). It is therefore plausible that the different patterns of peptide binding affinity displayed by these cryptic epitopes reflect the highly individual requirements of each MHC peptide binding cleft, which are nevertheless capable of inducing high avidity T cell responses.

The cryptic epitopes which were identified in domain III were presented by HLA-DR1, DR4, DR15 and HLA-DQ8 (Figure 5.2.2). The majority of these cryptic epitopes were unique to a particular HLA allele, however $LF_{307-326}$ and $LF_{387-406}$ were of particular interest as they were presented by HLA-DR1 and DR4, and HLA-DR1, DR4 and DQ8, respectively. A desirable goal in the construction of epitope string vaccines is the incorporation of epitopes which are promiscuously presented by a wide range of HLA alleles, allowing for coverage of a widely heterogeneous population. In addition to the promiscuous immunodominant epitopes identified in Chapter 4, these promiscuous cryptic epitopes represent an attractive vaccine target.

During the T cell mapping of domain I in HLA-DR4 transgenics, the peptide pool which contained $LF_{111-130}$ and $LF_{231-250}$ was also capable of inducing a response which encompassed the whole native LF protein. This was also discovered in the HLA-DR1 restricted peptide pool which contained the domain III cryptic epitopes $LF_{287-306}$, LF_{307-}

$_{326}$, $LF_{327-346}$, $LF_{347-366}$ and $LF_{387-406}$. Immunisation with peptides which also elicit a T cell response to the peptide in the context of the whole protein has been correlated with survival following infection in the case of murine studies of peptides derived from the Nef protein of HIV (Zhang *et al* 2009). While this indicates that these peptides are a good candidate for inclusion in an epitope string vaccine, unfortunately, as a function of the methods used in this study of cryptic LF epitopes, it is not possible to differentiate the individual peptides which were capable of inducing a response to the whole protein. More in depth mapping of the cryptic epitopes, with immunisation of the individual peptides would be required to resolve this issue.

Previous studies which identified B cell epitopes within LF indicated that domain III was extremely antigenic and represented an important target for a neutralizing antibody response (Lim *et al* 2005; Nguyen *et al* 2009a). The disparity between these results and the T cell immunodominant epitope mapping carried out within our work, highlights the differences between the T cell and B cell epitope repertoire. In terms of cryptic epitopes however, six of the eight T cell epitopes identified within domain III overlapped the structural B cell epitopes identified by Nguyen *et al* (Figure 5.3.1). The secondary structure consisted of an alpha helix for all four of the B cell epitopes, and the antibodies to epitopes $LF_{345-365}$, $LF_{383-394}$ and $LF_{361-374}$ bound to tandem repeats within the substrate recognition domain. CD4+ T cells play a crucial role in directing the adaptive immune response, not only through promoting the expansion of cytotoxic CD8+ T cells and maintaining the memory T cell population, but also through promoting B cell differentiation into plasma cells to produce neutralizing antibodies and assist memory B

cells for a swift recall response. The identification of cryptic CD4+ T cell epitopes which may also be involved in initiating a neutralizing antibody response against LF allows both the humoral and cellular immune system to provide robust protection to anthrax infection following immunisation.

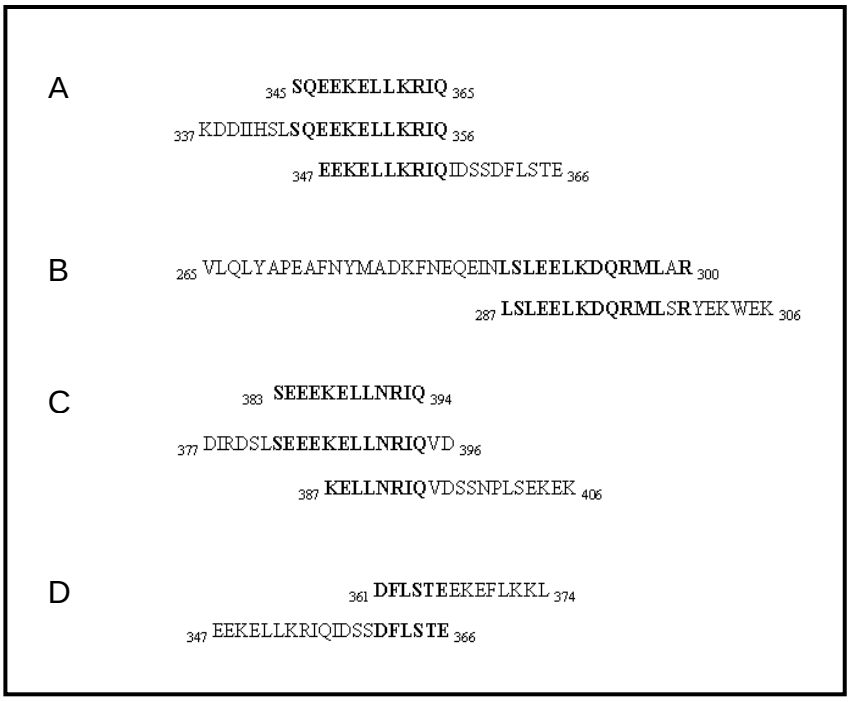


Figure 5.3.1 Comparison of cryptic T cell epitopes and B cell epitopes.
The B cell epitope $LF_{345-365}$ overlaps the cryptic T cell epitopes $LF_{337-356}$ and $LF_{347-366}$ (A). The B cell epitope $LF_{265-300}$ overlaps the cryptic T cell epitope $LF_{287-306}$ (B) (although it should be noted that there is a single amino acid substitution of alanine for serine in the epitope identified by Nguyen *et al*). The B cell epitope $LF_{383-394}$ overlaps the cryptic T cell epitopes $LF_{377-396}$ and $LF_{387-406}$ (C). The B cell epitope $LF_{361-374}$ overlaps the cryptic T cell epitopes $LF_{347-356}$ (D). Overlapping regions are displayed in bold.

6 LF peptide blockade in autoimmunity

6.1 Introduction

In the following chapter I describe a series of experiments undertaken to investigate whether the very high binding affinity to HLA-DR15 identified for the LF epitope, $LF_{457-476}$ can be exploited as a blockade agent in HLA-DR15 presented responses involved in multiple sclerosis.

6.1.1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease characterised by the inflammation and progressive demyelination of the central nervous system (CNS). MS is the most common cause of neurological disability in young adults, with recurrent inflammations leading to eventual neurodegeneration (Compston 1997; Compston 1999). The prevalence of MS in the United Kingdom is approximately 100 per 100,000, and in common with other autoimmune diseases, there is a preponderance of female cases (Baum and Rothschild 1981; Forbes and Swingler 1999; Rothwell and Charlton 1998; Swingler and Compston 1986; Williams and McKeran 1986). The course of disease is variable, but the majority of patients present with either relapsing-remitting MS (RRMS) or primary-progressive MS (PPMS) (Lublin and Reingold 1996; Weinshenker *et al* 1989). RRMS is characterised by acute episodes of neurological symptoms or relapses, interspersed by periods of complete or partial recovery (Lublin and Reingold 1996). A high proportion of patients presenting with RRMS convert to secondary–progressive MS

(SPMS) within 10 years of the first neurological episode, this form of MS is characterised by progressive disability in the absence of relapses (Confavreux *et al* 1980). PPMS is distinguished by a continual progression to disability without remission, from the onset of disease (Lublin and Reingold 1996). PPMS and SPMS, unlike RRMS, are both associated with decreased responsiveness to immunotherapy (Hafler 2004).

6.1.2 Immunopathogenesis of MS

The accumulation of inflammatory cells within the brain and spinal cord of patients with intermittent neurological episodes was first identified in the nineteenth century (Charcot 1868). The aetiology of the disease is still poorly understood, but MS is often thought to be mediated by self-reactive CD4+ T cells which have escaped the induction of tolerance and become activated in the periphery. Under normal physiological conditions the trafficking of lymphocytes into the CNS is low, but increased expression of VCAM-1 at the blood-brain barrier (BBB) is believed to mediate cellular entry to the CNS, binding to the α4 integrins expressed by activated lymphocytes in the periphery, and facilitating their migration across the tight junctions of the brain endothelium (Steffen *et al* 1994; Vajkoczy *et al* 2001). Subsequent release of proinflammatory cytokines such as IFNγ and TNFα, and chemokines such as RANTES, CXCL10 and IL-8, activate resident antigen presenting microglia, and recruit other immune cells, including monocytes, CD8 T cells and B cells, from the peripheral blood to the CNS (Aloisi *et al* 1998; Olson and Miller 2004).

The presumed targets of the auto-reactive CD4+ T cells are self-peptides derived from the CNS, in particular the myelin sheath of neurons. These target autoantigens include MBP, myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated oligodendrocyte basic protein (MOBP) and αB-crystallin. The presence of CD4+ T cells which are reactive to these epitopes has been identified in both human MS and experimental autoimmune encephalomyelitis (EAE), a commonly used animal model of MS, induced by immunization of animals with encephalitogenic antigens (Ben-Nun and Cohen 1982; Chou *et al* 2004; Hafler *et al* 1987; Holz *et al* 2000; Mendel *et al* 1995; Ota *et al* 1990; Pelfrey *et al* 1993). The role of CD4+ T cells in MS is supported by observations that adoptive transfer of encephalitogenic MBP specific CD4+ T cells into healthy naïve animals can induce EAE in the recipients (Mokhtarian *et al* 1984; Zamvil *et al* 1985; Zamvil *et al* 1986). Subsequent studies have revealed that injection of autoreactive antibodies and CD8+ T cells, can also induce large-scale demyelination and induction of EAE (Schluesener *et al* 1987; Sun *et al* 2001).

The autoreactive CD4+ T cells were initially accepted to drive disease down an IL-12 mediated Th1 pathway, characterised by the production of IFNγ and TNFα (Leonard *et al* 1995; Leonard *et al* 1997; Waldburger *et al* 1996). Recent data, however, has suggested a primary role for Th17 cells in the induction of pathogenesis in EAE (Komiyama *et al* 2006; Langrish *et al* 2005). This Th subset differentiates in the presence of TGF-β, IL-21 and IL-6, and is maintained by the production of IL-23, which is associated in mice with the expression of the transcription factor, retinoid orphan receptor γt, (RORγt), (or in humans, with the ortholog, RORC variant 2), driving Th17 lineage commitment (Bettelli

et al 2006; Ivanov *et al* 2006; Veldhoen *et al* 2006; Wilson *et al* 2007). The cytokines IL-23 and IL-12 are related and share a p40 subunit, knockout mice which are deficient in this subunit, or in the cytokine receptors IL-23R and IL-12R-β1, have been shown to develop less severe EAE. In contrast to mice deficient in the IL-12 specific subunit p35 (IL-12$\alpha^{-/-}$), which show increased susceptibity to EAE, knockout mice specific for the IL-23 subunit P19 (IL-23$\alpha^{-/-}$) also show the reduced risk of developing EAE associated with knocking out the p40 subunit (Becher *et al* 2002; Cua *et al* 2003; Gran *et al* 2002). This inhibition of IL-23, the upstream modulator of the pathogenic IL-17 producing Th17 subset, demonstrated that these cells are crucial to susceptibility to EAE.

In addition to IL-17A and IL-17F, Th17 cells also produce IL-6, IL-21, IL-22, TNFα and GMCSF. These cytokines, specifically IL-17A and IL-22, have been implicated in the trafficking and migration of innate immune cells such as neutrophils, which may contribute to the recruitment of inflammatory cells to the CNS (Laan *et al* 1999; Ye *et al* 2001). However, the central role of Th17 in EAE and MS pathogenesis has been challenged by the finding that Th1 cells have a preferential ability to breach the BBB to enter the non-inflamed CNS (O'Connor *et al* 2008). O'Connor's research suggests that the IFNγ+IL-17- Th1 cells are critical in establishing lesions in the CNS, the subsequent demyelination and associated pathology then promote infiltration of the Th17 cells.

Like other autoimmune diseases, MS is a disease of complex aetiology comprising environmental components and contributory effects of a large number of genes, only a small number of which have been identified. The genetic component is well-illustrated by

family studies: close relatives of individuals with MS show an increased risk of developing the disease, with monozygotic twins having a 30% concordance rate in terms of developing the disease (Mumford *et al* 1994; Sadovnick *et al* 1993). The strongest genetic risk factor for MS lies in the HLA class II region, in particular the β-chain alleles; DRB1*1501, DRB5*0101 and DQB1*0602 which are characterized as the old class II serotype HLA-DR2 (Barcellos *et al* 2002). This serotype is most closely associated with MS in Northern European Caucasian populations (Hillert 1994; Hillert *et al* 1994; Olerup and Hillert 1991). Although recent studies have implicated the HLA-DR rather than the HLA-DQ loci as a risk factor (Lincoln *et al* 2005; Oksenberg *et al* 2004), it has proven difficult to identify the specific gene product that predisposes individuals to the disease, given the high degree of linkage disequilibrium in this part of the class II region.

The spontaneously autoimmune transgenic strain disease 'Line 7' mice previously generated in the Altmann lab express the human MHC class II allele HLA-DRA1*0101/DRB1*1501, in the absence of mouse MHC class II (Ellmerich *et al* 2005; Ellmerich *et al* 2004). They also express a human Vα3.1Jα40 and Vβ2.1Jβ2.1 TCR from the MS-patient-derived T-cell clone Ob.1A12. This transgenic mouse model develops spontaneous demyelination and neurodegeneration similar to human MS, making it an optimal model for investigating immunotherapies in MS, as it does not introduce the inherent bias towards certain epitopes which is attached to the induction of EAE by immunisation with defined auto-antigens. The disease is driven by a response to an HLA-DR15 restricted immunodominant epitope of MBP; the amino acids 85-99 of MBP (ENPVVHFFKNIVTPR), of which the core motif HFFK is recognised by both auto-

reactive T cells and antibodies isolated from MS patients (Wucherpfennig *et al* 1997). In common with MS and EAE, the spontaneous disease is characterised by determinant spreading; as the inflammatory demyelination progresses in the transgenic mouse model, the T cell response spreads from one or a limited number of dominant epitopes to other self-peptides derived from the CNS (Ellmerich *et al* 2004). This leads to the infiltration of the CNS by autoreactive CD4+ T cells (Figure 6.1.1).

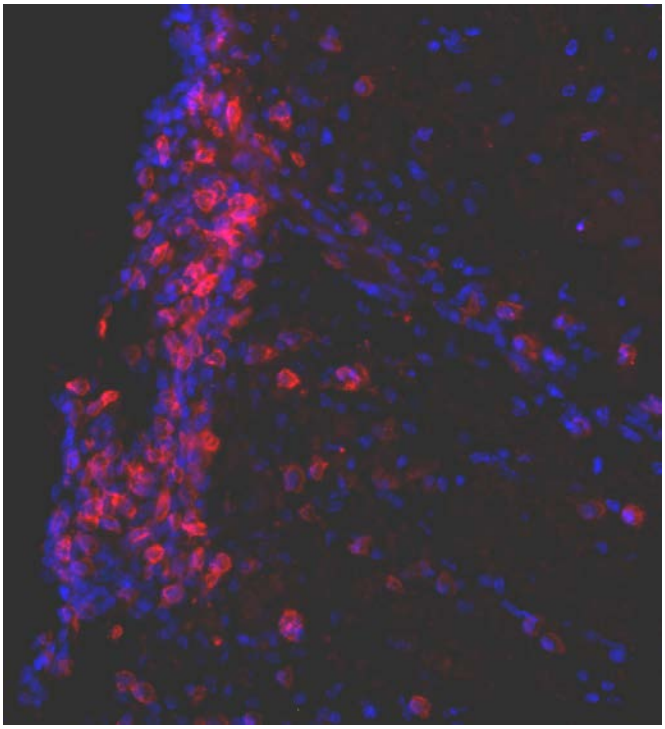


Figure 6.1.1 Immunofluorescence staining of spinal cord section.
An inflammatory focus, located within a spinal cord section taken from a Line 7 transgenic mouse in the acute stages of spontaneous demyelinating disease, demonstrating extensive lymphocytic infiltrate of CD4+ Tcells can be seen in the two-colour overlay of DAPI (blue) and CD4 (red), original magnification, x10. Image courtesy of Daniel Lowther.

6.1.3 Immunotherapy in MS

The autoimmune responses to CNS antigens in MS have been targeted with a range of different therapeutic approaches. Current therapies are palliative, delivering a short-term reduction in symptoms or retarding progression to disability. These disease modifying therapies (DMT), such as the beta interferons (IFNβ), have proven to be effective at modulating inflammatory demyelination in MS. However, in common with a number of other DMTs, it remains unclear precisely how IFNβ exerts its effects; mechanisms may include regulating T cell activation and proliferation, induction of apoptosis in autoreactive T cells, shifting the cytokine profile away from a proinflammatory Th1 type response, or inhibiting matrix metalloproteinase (MMP) assisted lymphocyte migration across the BBB (Nicoletti *et al* 2000; Revel *et al* 1995; Rudick and Ransohoff 1995; Stuve *et al* 1996).

In contrast to IFNβ, humanised or fully human therapeutic monoclonal antibodies interact with their targets by binding to, blocking or interfering with the signaling of specific antigens. This specificity makes antibody therapy attractive as a means of modulating the autoimmune responses seen in MS. Antibodies currently used in MS therapy include Alemtuzumab (also known as Campath) which interferes with the signaling of CD52, leading to the depletion of T and B cells, NK cells, macrophages, DCs and granulocytes (Moreau *et al* 1996). Rituximab (Rituxan) targets CD20 molecules, enabling the selective depletion of mature B cells (Hauser *et al* 2008). The blockade of CD49d, the α4 subunit of the very late antigen 4 (VLA-4) expressed on lymphocytes, by Natalizumab or Tysabri prevents binding to the VCAM-1 expressed at the BBB, inhibiting cellular trafficking

across the CNS (Tubridy *et al* 1999). The proposed mode of action of Daclizumab or Zenapax involves the blockade of CD25 (IL-2Rα), which both inhibits the proliferation of activated T cells (Bielekova *et al* 2004), and expands a population of regulatory $CD56^{bright}$ NK cells (Bielekova *et al* 2006). Although the effect of Daclizumab upon CD25, which is transiently expressed upon activation of naïve CD4+ T cells, may also inhibit the survival of CD25+ FoxP3+ Tregs, raising the risk of autoimmune complications (Oh *et al* 2009)

This range of target antigens indicates different points of therapeutic intervention in the treatment of MS, underscoring the complex nature of the disease. The relatively large size of antibodies, which acts to limit their ability to cross the intact BBB and thus gain access to many of their proposed sites of action, combined with the need for repeated administration by injection, and the host anti-globulin and anti-idiotype responses, restrict their use in the treatment of chronic diseases such as MS.

Glatiramer acetate (GA, also known as Copolymer-1, or Copaxone) was the first non-interferon treatment approved for use in MS patients, it is a random synthetic copolymer composed of the amino acids L-glutamic acid, L-lysine, L-alanine and L-tyrosine (Teitelbaum *et al* 1971). GA is capable of inhibiting the progression of both EAE and human MS, decreasing the frequency of relapses in RRMS patients (Johnson 1996; Teitelbaum *et al* 1971). In common with IFNβ, there have been several proposed mechanisms of action for GA. Unlike IFNβ however, which exerts a non-specific effect, GA appears to affect cells which are specific for myelin antigens such as MBP. The

random nature of the amino acids which comprise GA show promiscuous binding to several different MHC class II alleles, allowing the occupation of the antigen-binding cleft on MHC molecules, competing for MHC binding with other antigens (Ben-Nun *et al* 1996; Fridkis-Hareli *et al* 1994; Fridkis-Hareli *et al* 2001; Fridkis-Hareli and Strominger 1998; Teitelbaum *et al* 1996). The GA/MHC complexes which can compete with MBP/MHC complexes for binding to MBP-specific T cells show a limited ability to cross the BBB, but there is evidence that these complexes preferentially induce CD4+ Th2 cells capable of crossing the BBB and exerting anti-inflammatory effects within the CNS (Aharoni *et al* 2000; Chen *et al* 2001).

Another potential mode of action proposed for GA focused upon its action as an altered peptide ligand (APL). APLs are auto-antigenic peptides which have been modified in such a way that the MHC binding efficiency remains but a number of the TCR binding amino acids have been changed, allowing the APL to be presented in the context of the MHC to antigen specific T cells without initiating a pathogenic response. The self-peptide specific T cell clones are antagonized by APLs, leading to possible anergy, bystander suppression, or skewing the cytokine production from a Th1 to a Th2 profile produced by a subset of GA reactive T cells *in vivo*.

Activating peripheral T cells and breaking self-tolerance is one of the key steps in the initiation of an autoimmune reaction. The pathological mechanisms required to initiate this reaction in MS remain unclear and environmental cues, such as infectious organisms, have been suggested as potential triggers (Gautam *et al* 1998; Mokhtarian *et al* 1999;

Wucherpfennig and Strominger 1995). It is unclear to what extent the role of infections may be either through molecular mimicry or by altering the neuroinflammatory environment. Other examples of mimicry have been invoked; treatment with the extracellular IgV-like domain of the milk protein butyrophilin (BTN), prior to immunization with encephalogenic MOG epitopes suppresses the development of EAE and also ameliorates pre-existing disease (Stefferl *et al* 2000). Foreign antigens may therefore be capable not only of initiating disease in genetically susceptible individuals, but also conversely, in certain circumstances, of interacting with autoreactive T cells to treat and prevent autoimmune diseases through a form of molecular mimicry.

Modulating the activation of the T cells by blocking the binding of self-reactive peptides to MHC class II molecules presents an attractive DMT. Incubating APCs with an excess of competitor epitope can inhibit peptide binding to purified MHC class II molecules *in vitro*, thereby interfering with T cell activation (Babbitt *et al* 1986). In the 1990s, the notion of MHC blockade as a therapeutic option for autoimmunity became a major research theme in a number of labs and pharmaceutical companies. *In vivo* administration of a competitor peptide in concert with an immunogenic self-peptide has shown that it is also possible to block the priming of encephalitogenic T cells (Adorini *et al* 1988b; Adorini *et al* 1991a; Adorini *et al* 1991b).

The blockade experiments, in which the premise was competition for class II sites through high affinity binding, were somewhat different from the APL experiments; in the latter case, MHC binding may be the same as the wild-type self-peptide, but TCR-

binding residues are changed to give modulated signalling. Although the inhibition of autoimmune responses with an APL have proven to be a viable strategy in modulating a range of autoimmune diseases it remains difficult to interpret the precise mechanism of action involved. It is possible that, in addition to MHC blockade, the APL mechanism may include generation of CD4+CD25+FoxP3+ Treg cell populations or skewing of the pro-inflammatory Th1 or Th17 response towards a distinct, non-pathogenic Th2 phenotype, both of which are capable of mediating bystander suppression (Boehm *et al* 2009; Illes *et al* 2004; Nicholson *et al* 1995; Nicholson *et al* 1997; Stern *et al* 2005). Previous work has proven that it is possible to inhibit the induction of EAE using a competitor peptide entirely unrelated to the immunogenic self-peptide, which has a core sequence with a high binding affinity for the MHC peptide binding cleft involved in presentation of disease related self-peptides (Lamont *et al* 1990). This targets the MHC class II alleles capable of binding and presenting the disease related epitopes. These susceptible alleles, which have an inherent preference for binding and presenting specific amino acids at distinct binding pockets, may explain the strong genetic risk factor for MS involving the HLA β-chain alleles; DRB1*1501, DRB5*0101 and DQB1*0602.

Previous epitope mapping described in this thesis (see Chapter 4), identified the peptide ^{457}HQSIGSTLYNKIYLYENMNI^{476} ($LF_{457-476}$) from the lethal factor of anthrax, as an epitope capable of binding extremely strongly to the HLA-DR15 allele (HLA-DRA1*0101/DRB1*1501). Work described in this chapter assessed the capability of $LF_{457-476}$ to interfere with the binding and subsequent presentation by APCs of self-peptides involved in the induction of autoimmunity, thereby retarding the progression to

disease, within the context of the Line 7 transgenics backcrossed onto Foxp^{DTR} mice (Kim *et al* 2007).

6.2 Results

6.2.1 Effect of LF derived peptide upon the response of autoreactive leucocytes to HLA-DR15-restricted auto-antigens

Leucocytes isolated from the spleens of HLA-TCR transgenic mice which have developed moderate paralysis (characterized as score 1-2 disease) were stimulated with MS-related epitopes alone or in co-culture with MS-related epitopes plus the pathogen derived epitope $LF_{457-476}$. The strong proliferative and pro-inflammatory cytokine response of the naïve splenocytes to MS-related epitopes reveal that the cells have previously been sensitized to myelin epitopes *in vivo*, following cell infiltration of the CNS (Ellmerich *et al* 2005). The proliferative response of the leucocytes to the primary MS-related epitope MBP_{85-99} (Figure 6.2.1A) and the spread epitope αB-crystallin ($\alpha Bc_{161-176}$) (Figure 6.2.1D) was significantly reduced following co-culture with $LF_{457-476}$.

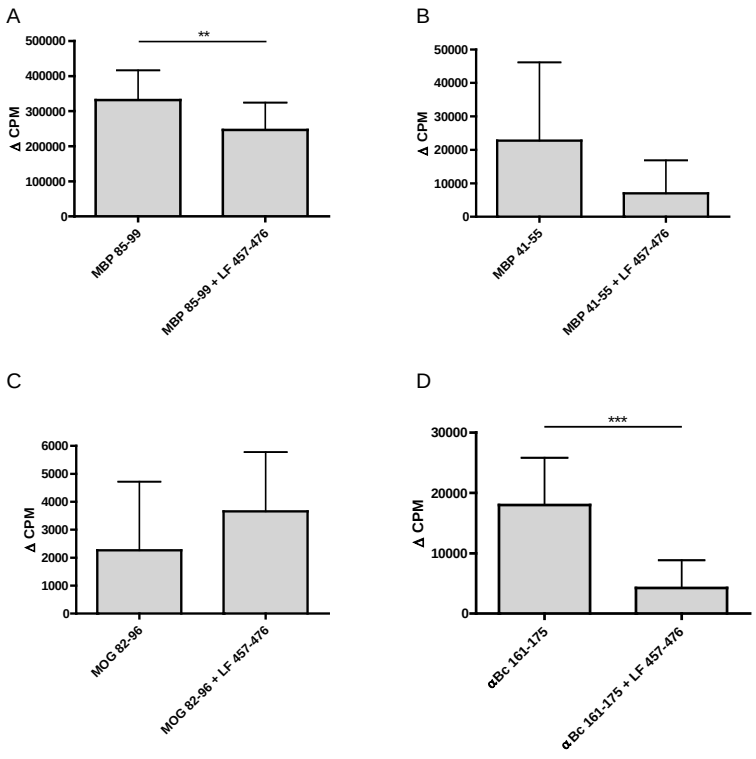


Figure 6.2.1 Proliferation of naïve, disease score 1-2, HLA-TCR transgenic *ex vivo* spleen cells in response to HLA-DR15-restricted epitopes.
Responses were analysed following stimulation with 25µg/ml MS-related epitope alone or 25µg/ml MS-related epitope plus 25µg/ml $LF_{457-476}$; (A) MBP_{85-99} (paired t-test, p=0.0012), (B) MBP_{41-55}, (C) MOG_{82-96} and (D) $\alpha Bc_{161-176}$ (paired t-test, p=0.0002), (n=3) (± standard deviation).

The levels of the pro-inflammatory cytokines INFγ (Figure 6.2.2A), TNFα (Figure 6.2.2B) and IL-17A (Figure 6.2.2C) released from leucocytes in response to the MS-related epitope MBP_{85-99}, were significantly reduced following co-culture with $LF_{457-476}$. Although the levels of IL-10, which is capable of acting in an anti-inflammatory capacity, were high in response to culture of cells with MBP_{85-99} alone, co-culture with $LF_{457-476}$ did not alter the levels of IL-10 produced (Figure 6.2.2D).

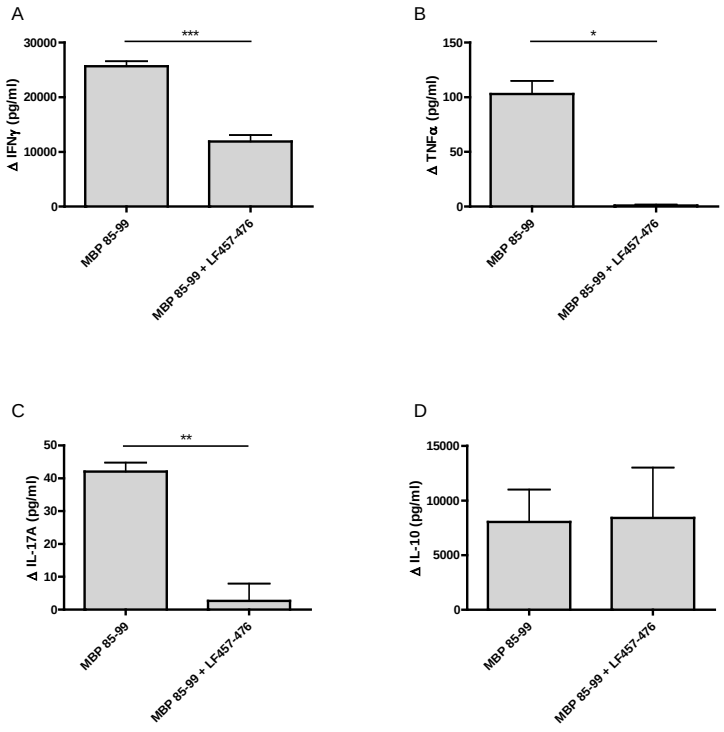


Figure 6.2.2 Cytokine release of naïve, disease score 1-2, HLA-TCR transgenic *ex vivo* spleen cells, in response to HLA-DR15-restricted epitopes.

Responses were analysed following stimulation with 25µg/ml MS-related epitope alone or 25µg/ml MS-related epitope plus 25µg/ml $LF_{457-476}$; (A) IFNγ (paired t-test, p=0.0006), (B) TNFα (paired t-test, p=0.0119), (C) IL-17A (Wilcoxon signed rank test, p=0.0039), and (D) IL-10, (n=3) (± standard deviation).

6.2.2 Effect of whole recombinant LF upon the response of auto-reactive HLA-TCR transgenic leucocytes to HLA-DR15-restricted auto-antigens

The effect of the whole LF protein upon the response to the primary MS-related epitope MBP_{85-99} was evaluated by stimulation of leucocytes isolated from the spleens of HLA-TCR transgenic mice which have developed moderate paralysis (characterized as score 1-2 disease), with MBP_{85-99} alone or MBP_{85-99} plus whole LF. In contrast to co-culture with $LF_{457-476}$, the whole LF protein has a synergistic effect, with a significant increase in the

proliferative response of cells co-cultured with both MBP_{85-99} and whole LF (Figure 6.2.3A). The addition of whole LF to cells cultured with MBP_{85-99} had no effect upon the levels of cytokines IFNγ, TNFα, IL-17A and IL-10 quantified in culture supernatant. This indicates that any inhibitory effect of $LF_{457-476}$ is dependent upon its availability as an individual peptide rather than as part of the whole LF protein.

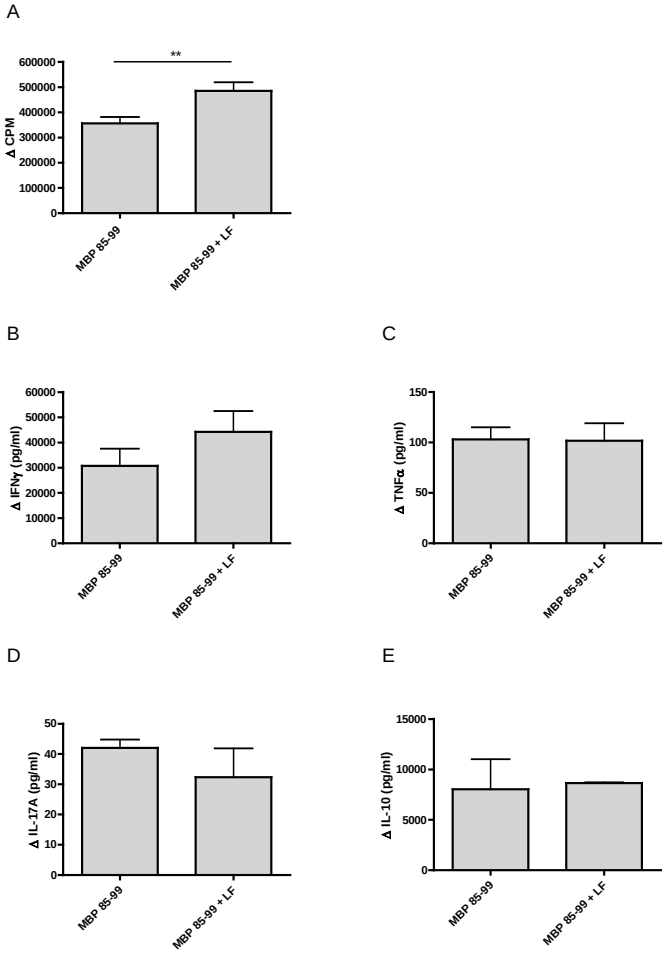


Figure 6.2.3 Response of naïve, disease score 1-2, HLA-TCR transgenic *ex vivo* spleen cells to the MS-related epitope, $MBP_{85-99,}$ and whole LF.

Responses were analysed following stimulation with 25µg/ml of MBP_{85-99} alone or 25µg/ml of MBP_{85-99} plus 25µg/ml of whole LF. (A) Proliferation (paired t-test, p=0.0077), and cytokine release of; IFNy (B), TNFa (C), IL-17 (D) and IL-10 (E), (n=3) (± standard deviation).

6.2.3 Dose dependent inhibition by $LF_{457-476}$ of autoreactive HLA-TCR transgenic T cells to stimulation with HLA-DR15-restricted auto-antigens

A dose dependent relationship between $LF_{457-476}$ and the inhibition of MBP_{85-99} induced stimulation was established by incubating auto-reactive T cell lines derived from HLA-TCR transgenic leucocytes (Figure 6.2.4) and *ex vivo* cells (Figure 6.2.5) with a single dose of MBP_{85-99} and increasing concentrations of $LF_{457-476}$. The T cell line shows that with increasing concentration of LF peptide the levels of the pro-inflammatory cytokine IFNγ released in response to auto-antigen are significantly reduced (Figure 6.2.4B). The proliferative response of the T cell line decreased (Figure 6.2.4A) but did not approach levels of significance, this, coupled with the fluctuations in the levels of IL-17A (data not shown) indicated that T cell lines were not appropriate models to investigate the inhibition of MBP_{85-99} stimulation by $LF_{457-476}$.

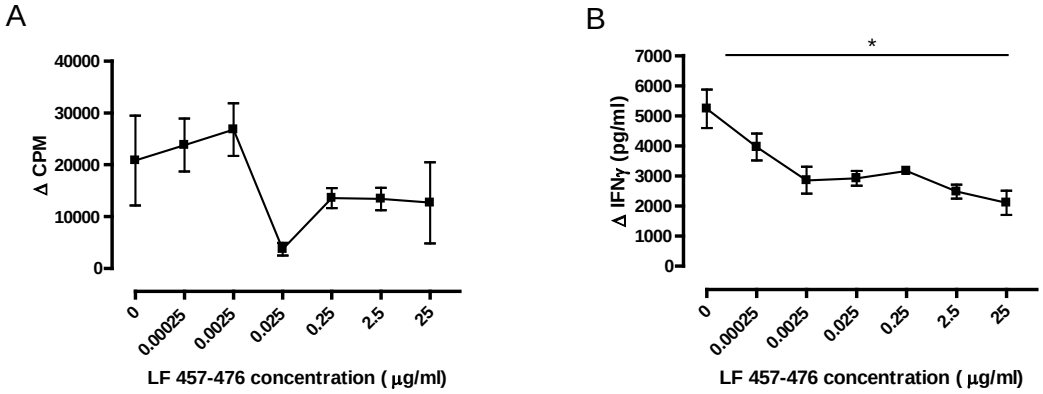


Figure 6.2.4 Inhibition of the proliferation and cytokine release from an MBP_{85-99} reactive HLA-TCR transgenic T cell line in response to MS-related epitopes.

Following co-culture with 0.0025μg/ml of MBP_{85-99} and a rising concentration of $LF_{457-476}$, the T cell response was expressed as proliferation (A) and release of the pro-inflammatory cytokine IFNγ (B) (Spearmans rank order correlation, p=0.0238) (± SEM of 3 independent experiments).

Ex vivo leucocytes taken from the spleens of HLA-TCR transgenic mice which had developed moderate paralysis (characterized as score 1-2 disease) were simulated with a low dose of MBP_{85-99} and increasing concentrations of $LF_{457-476}$. The proliferative response of cell (Figure 6.2.5A) was significantly reduced, with Dunn's multiple comparison indicating that co-culture with 25μg/ml of $LF_{457-476}$ resulted in reduced proliferation compared to that produced following co-culture with 0.00025μg/ml ($p<0.01$) or 0.025μg/ml ($p<0.01$) of $LF_{457-476}$. The levels of pro-inflammatory cytokines released by *ex vivo* leucocytes in response to MBP_{85-99} stimulation were also reduced following co-incubation with higher concentrations of $LF_{457-476}$. Co-culture with 25μg/ml of $LF_{457-476}$ led to a significant decrease in the levels of IFNγ released (Figure 6.2.5B) compared to levels produced following co-culture with 0.00025μg/ml of $LF_{457-476}$ (Dunn's multiple comparison, $p<0.01$). The levels of IL-17A (Figure 6.2.5C) released following co-culture with 25μg/ml of $LF_{457-476}$ were significantly lower than the levels produced following co-culture with 0.00025μg/ml of $LF_{457-476}$ (Dunn's multiple comparison, $p<0.01$).

IL-10 is known to play a role in regulating the development and course of EAE (Bettelli *et al* 1998); if the decrease in pro-inflammatory cytokines was dependent upon an $LF_{457-476}$ induced release of IL-10, levels of IL-10 might be expected to inversely correspond to those of IL-17A and IFNγ. There was a significant difference in the levels of IL-10 released by *ex vivo* leucocytes stimulated by MBP_{85-99} in the presence of concentrations of $LF_{457-476}$ (Figure 6.2.5D), however Dunn's multiple comparison indicates that low (0.00025μg/ml) ($p<0.01$) and high (25μg/ml) ($p<0.05$) doses of $LF_{457-476}$, both reduced

the IL-10 levels from those produced following stimulation with 0.025µg/ml of $LF_{457-476}$. Although the cytokine is produced in increased quantities by cells exposed to 0.025µg/ml of $LF_{457-476}$, compared to 0.00025µg/ml of $LF_{457-476}$, there is a decrease in IL-10 as the concentration of the LF peptide rises to 25µg/ml.

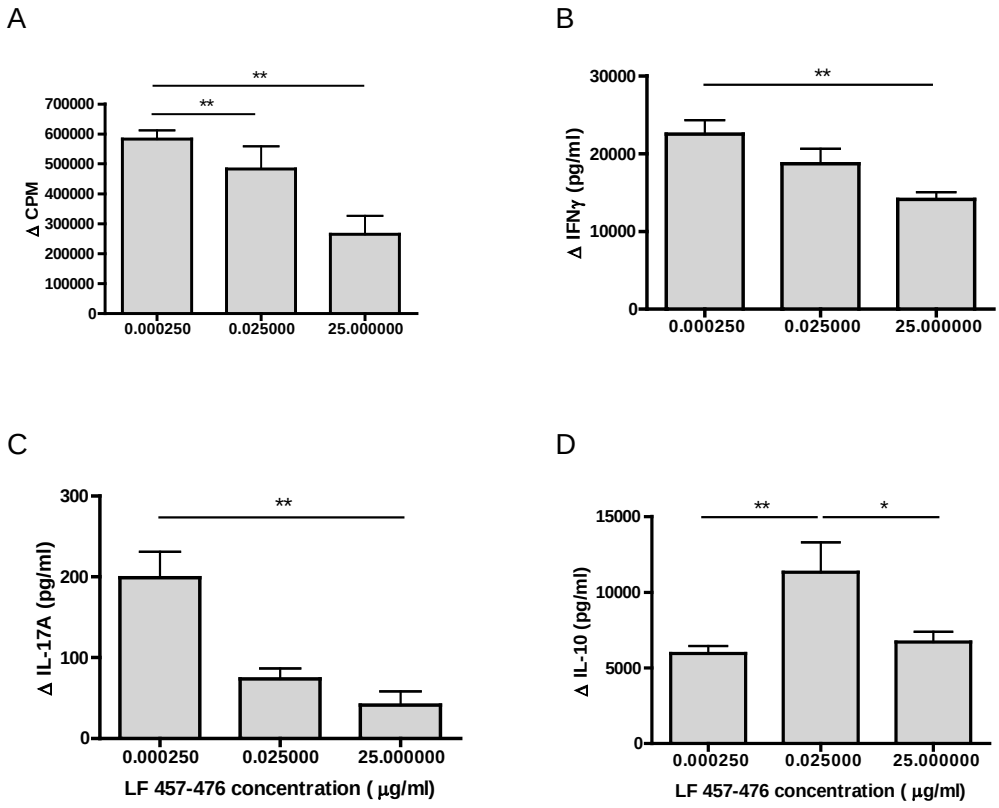


Figure 6.2.5 Inhibition of the proliferation and cytokine release of HLA-TCR transgenic *ex vivo* cells in response to MS-related epitopes.

Cells were cultured with 0.0025µg/ml of the primary MS-related epitope MBP_{85-99} and 0.00025µg/ml, 0.025µg/ml, or 25µg/ml of $LF_{457-476}$. The response was expressed as (A) proliferation (Friedman test p=0.0003), and cytokine release of; (B) IFNy (Friedman test p=0.0013), (C) IL-17A (Friedman test p=0.0007) and (D) IL-10 (Friedman test p=0.0003) (± standard deviation) (n=3).

6.2.4 Stabilisation of $LF_{457-476}$ *in vivo* and specificity of the $LF_{457-476}$ mechanism of inhibition

In addition to the stage of disease progression at which treatment is initiated, the *in vivo* peptide stability is vitally important in influencing the efficacy of peptide DMTs. Proteins and peptides can be rapidly eliminated from the circulation by proteolytic degradation, renal filtration or neutralizing antibody reactions, leading to inefficient presentation of peptide immunotherapeutics by APCs *in vivo*, which can limit their efficacy. Methods of prolonging the peptides presence in circulation include: incorporation into drug delivery vehicles such as liposomes (phospholipid capsules) for slow release, conjugation to natural or synthetic polymers, or manipulating amino acid residues that are susceptible to proteolytic degradation (Monfardini and Veronese 1998; Werle and Bernkop-Schnurch 2006). The most successful strategy involves covalently conjugating poly ethylene glycol (PEG) which is an uncharged, hydrophilic, non-toxic polymer, to therapeutically useful peptides (Harris and Chess 2003). This strategy of PEGylation can shield the peptides from proteolytic enzymes and increase the size of the peptide, altering the peptides pharmacokinetic and pharmacodynamic properties, whilst avoiding the inherent toxicity which is associated with many other drug delivery vehicles (Veronese and Pasut 2005).

$LF_{457-476}$ peptide, and an irrelevant control peptide, LF_{61-80}, selected due to its lack of known relative binding affinity to any HLA-DR alleles, were coupled to mini-PEG spacers (described in 2.2.30). The proliferative response to MBP_{85-99} of leucocytes isolated from the spleens of mice which have developed moderate paralysis (characterized as score 1-2 disease), was significantly reduced following co-incubation

with either $LF_{457-476}$ (Dunn's multiple comparison, $p<0.05$) or PEG-$LF_{457-476}$ (Dunn's multiple comparison, $p<0.001$) (Figure 6.2.6). The control LF_{61-80} peptide as well as the PEGylated version of this peptide exerted no significant effect upon the proliferative response to MBP_{85-99}. This also indicated that the PEG-$LF_{457-476}$ effect upon the proliferation was not attributable to a toxic effect exerted by the mini-PEG spacer.

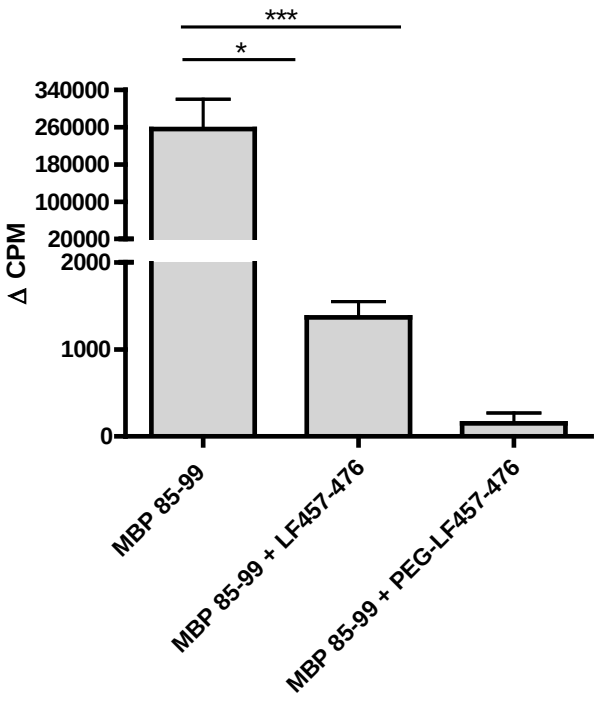


Figure 6.2.6 Inhibition of the proliferation of naïve, disease score 1-2, HLA-TCR transgenic *ex vivo* spleen cells following culture with HLA-DR15-restricted epitopes.
Responses were analysed following stimulation with 0.0025μg/ml of MBP_{85-99} alone or with 0.0025μg/ml of MBP_{85-99} plus 25μg/ml of either $LF_{457-476}$ or PEGylated $LF_{457-476}$ (Kruskal-Wallis, $p<0.0001$) (± standard deviation) (n=3).

The *in vivo* stability of the PEGylated $LF_{457-476}$ was determined by analyzing the proliferative response of an $LF_{457-476}$ reactive HLA-TCR transgenic T cell line presented with peptide/MHC class II complexes by APCs from HLA-TCR transgenic mice (Figure

6.2.7). Immediately following immunization with PEG-$LF_{457-476,}$ the ability of *ex vivo* APCs to stimulate the T cell line remained high, until at five days following immunization the levels of proliferation had significantly reduced from those seen on day one (Bonferroni's multiple comparison, $p<0.01$) and day three (Bonferroni's multiple comparison, $p<0.01$). This indicated that by day five following immunization the PEGylated peptide had been cleared from circulation, and as such was no longer bound to MHC class II products for presentation by APCs. Based upon this, the optimum time between immunizations of PEG-$LF_{457-476}$ was determined to be 3 days, in order to maintain a constant level of peptide in the bloodstream.

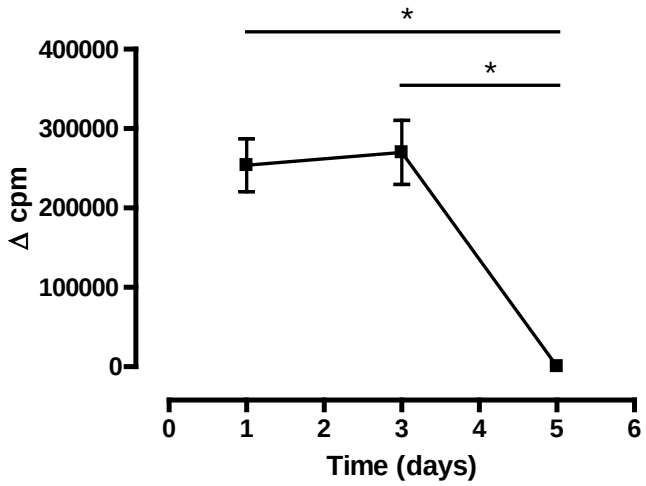


Figure 6.2.7 Proliferation of an $LF_{457-476}$ reactive HLA-TCR transgenic T cell line following stimulation with $LF_{457-476}$ presenting cells.

The response of the T cell line following presentation with *ex vivo* irradiated peptide/MHC class II complexes from the spleens of transgenic mice was determined one, three and five days after PEG-$LF_{457-476}$ immunisation. Proliferation reduced over 5 days following immunisation (one-way ANOVA $p=0.0012$) (± SEM of 3 independent experiments).

The possibility that $LF_{457-476}$ inhibited the MS epitope specific response through a non-specific mechanism was examined through the use of $LF_{457-476}$ in non-specific stimulation and cytotoxicity assays. T cells from HLA-TCR transgenic spleens were stimulated with beads coated with anti-CD3 and anti-CD28, mimicking stimulation by antigen-presenting cells (Figure 6.2.8A). Co-culture with either $LF_{457-476}$, or PEG-$LF_{457-476}$ did not have any effect upon the proliferative response of the T cells, indicating that $LF_{457-476}$ dependent inhibition of stimulation was linked to the presentation of epitopes by APCs. Furthermore both $LF_{457-476}$ and PEG-$LF_{457-476}$ failed to inhibit the stimulation of T cells from HL-DR1 transgenics with the mitogen ConA (Figure 6.2.8B). As HLA-DR1 shows a low binding affinity for $LF_{457-476}$, this indicated that the peptide's mechanism of action depended upon presentation by APCs which were capable of binding strongly to the peptide. The percentage of dead and apoptotic cells as determined by the Annexin V assay (Figure 6.2.8D) was not significantly elevated by exposure to either $LF_{457-476}$ or a control peptide LF_{61-80} which shows a low binding affinity for HLA-DR15, in both the PEGylated and non-PEGylated forms. The MTT assay (Mosmann 1983), determining levels of formazan in the presence of rising concentrations of either $LF_{457-476}$ or LF_{61-80}, in both the PEGylated and non-PEGylated forms also indicated that these peptides have no effect upon the mitochondrial metabolism within the cells (Figure 6.2.8C). Both these assays demonstrate that the inhibitory mechanism of $LF_{457-476}$ is not dependent upon a cytotoxic effect.

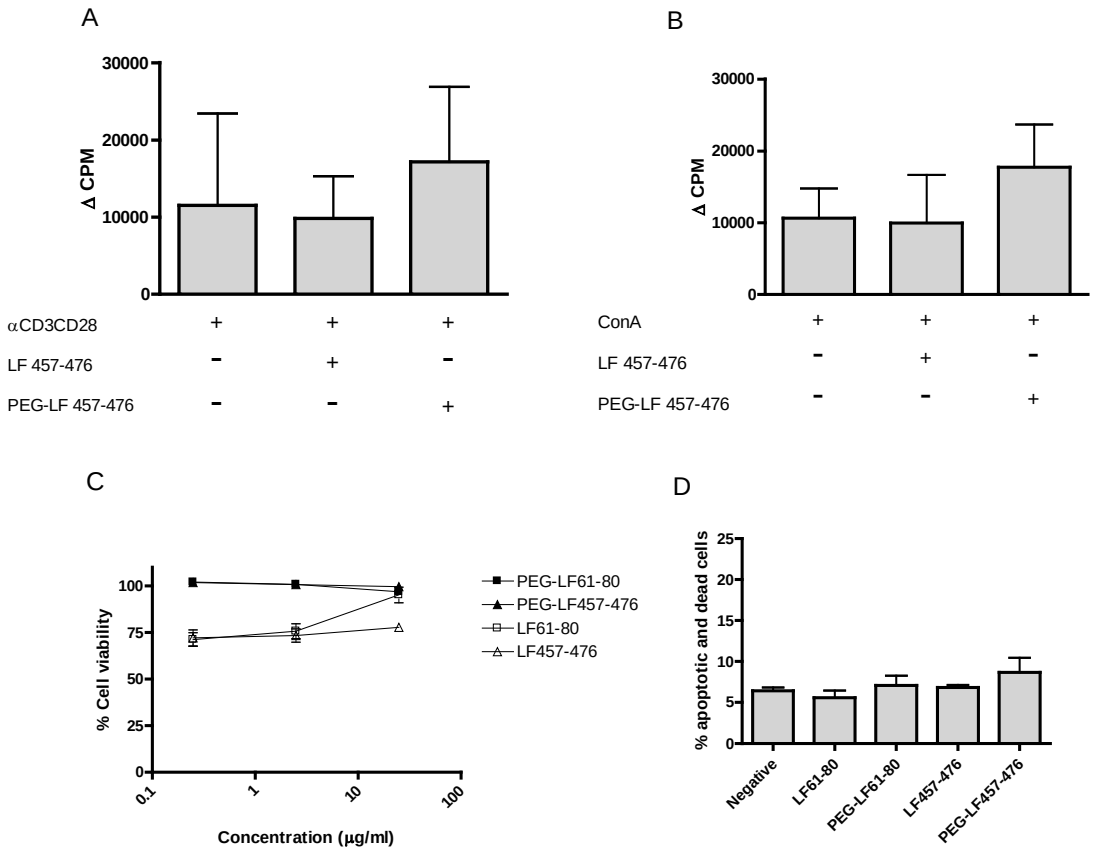


Figure 6.2.8 Specificity of the mode of action of $LF_{457-476}$.

Proliferation of naïve, *ex vivo* HLA-TCR transgenic spleen cells following non-specific stimulation and co-culture with $LF_{457-476}$ were analysed following (A) stimulation of T cells from HLA-TCR transgenics with αCD3CD28 beads and co-culture of $LF_{457-476}$ or PEG-$LF_{457-476}$ or (B) stimulation of T cells from HLA-DR1 transgenics with the mitogen ConA and co-culture of $LF_{457-476}$ or PEG-$LF_{457-476}$. In both experiements, n=3 per group (± standard deviation). (C) Cell viability was assessed through culture of naïve, *ex vivo* HLA-TCR transgenic spleen cells with a rising concentration of 0.25µg/ml, 2.5µg/ml and 25µg/ml of both PEGylated and non-PEGylated $LF_{457-476}$ and LF_{61-80}. The rate of mitochondrial metabolism within the cells was measured by the cleavage of MTT. Data are presented as a percentage of the absorbance obtained in untreated cultures (± SEM of 3 independent experiments). Cells cultured with 25µg/ml of both PEGylated and non-PEGylated $LF_{457-476}$ and LF_{61-80} were assayed for cytoxicity using Annexin V staining, the percentages of necrotic and apoptotic cells were compared to those of cells in untreated cultures (± SEM of 3 independent experiments).

6.2.5 Effect of $LF_{457-476}$ immunisation upon the inhibition of spontaneous paralysis in HLA-TCR transgenic mice

The effect of the $LF_{457-476}$ peptide was examined *in vivo* on the development of spontaneous paralysis in the HLA-TCR transgenic models (Figure 6.2.9). At three months of age (spontaneous paralysis develops at 4-5 months of age within this model (Ellmerich *et al* 2005)), groups of transgenic mice were immunised with either PEG-$LF_{457-476}$ or the control peptide PEG-LF_{61-80}. The initial onset of clinical disease symptoms was observed in the PEG-LF_{61-80} immunised group at day 17, while the onset of any disease symptoms in the PEG-$LF_{457-476}$ immunised group was delayed until day 32. Although the clinical signs were mild in both groups, a significant reduction in the severity of the disease score was observed in the PEG-$LF_{457-476}$ immunised group compared to the control PEG-LF_{61-80} immunised group, Mann Whitney ($p<0.0001$).

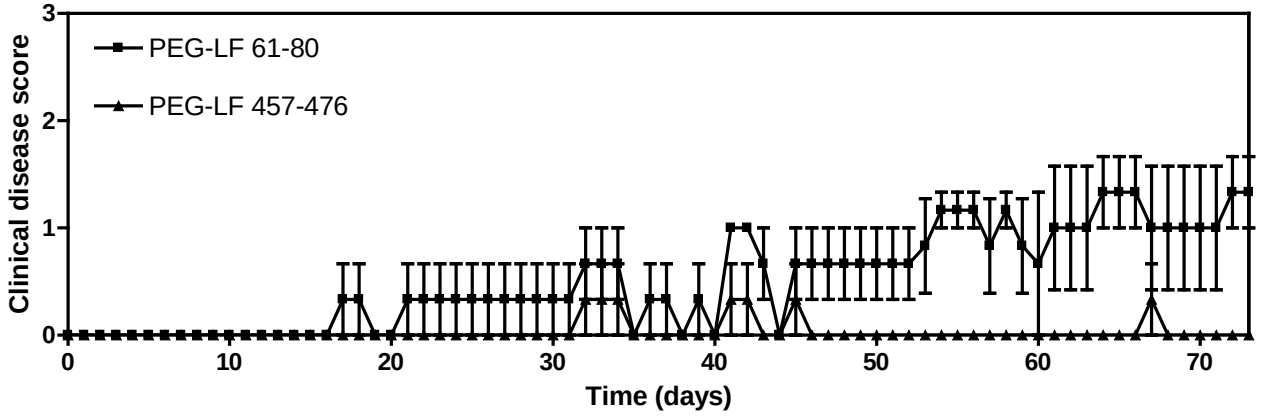


Figure 6.2.9 Development of spontaneous paralysis in peptide immunised HLA-TCR transgenics. Age- and sex- matched groups of transgenic mice were immunised with either PEG-$LF_{457-476}$ or the control peptide PEG-LF_{61-80} on day 0 and every third day for 73 days. The effect upon the inhibition of spontaneous paralysis in transgenic mice was observed through daily clinical scores. (± SEM) (n=3). Mann Whitney ($p<0.0001$).

The manner in which cell populations were affected by PEG-$LF_{457-476}$ immunisation was analysed by flow cytometry of peripheral leucocytes taken at timepoints during the development of spontaneous paralysis in the PEG-$LF_{457-476}$ immunised HLA-TCR transgenics (Figure 6.2.10). The secretion of both pro-inflammatory cytokines IFNγ (Figure 6.2.10A) and IL-17A (Figure 6.2.10B) by CD4+ T cells were significantly reduced over time, while the lower levels of IFNγ (Figure 6.2.10C) and IL-17A (Figure 6.2.10D) produced by the CD8+ T cells in response to MBP_{85-99} were unaffected by administration of $LF_{457-476}$ over time. Interestingly NK cells showed a reduction in the levels of IFNγ (Figure 6.2.10E) but not IL-17A (Figure 6.2.10F) following repeated immunisation with the LF peptide.

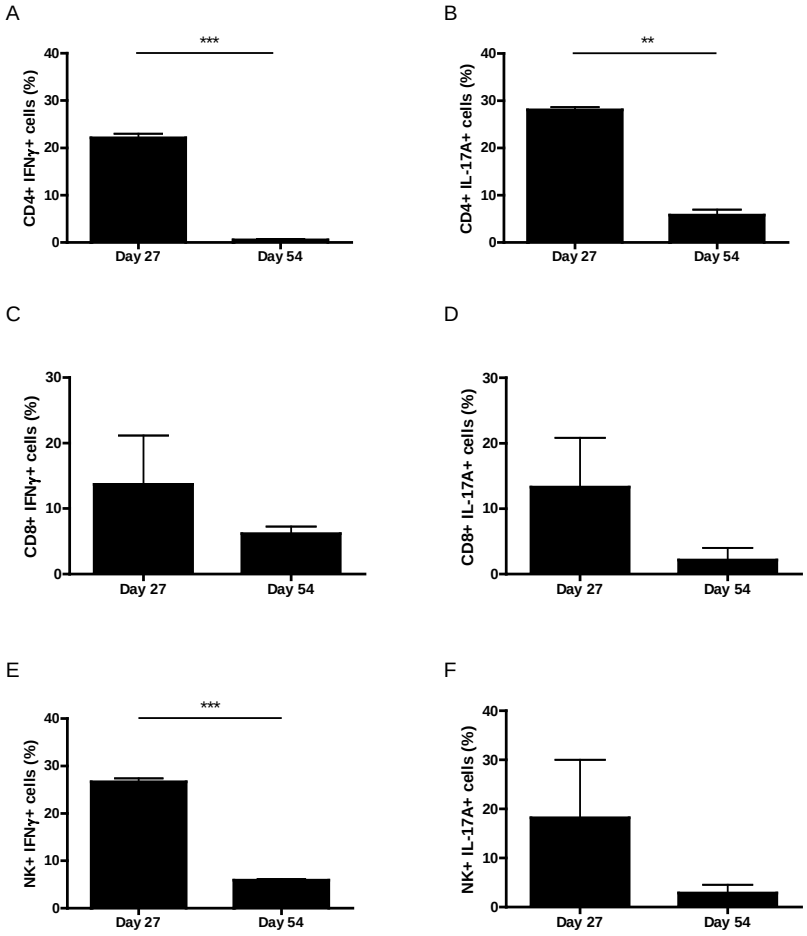


Figure 6.2.10 Frequency of leucocytes secreting pro-inflammatory cytokines from HLA-TCR transgenics analysed at days 27 and 54 of PEG-$LF_{457-476}$ immunisation.
Peripheral leucocytes were stimulated with 0.0025μg/ml of MBP_{85-99}, then stained with anti-CD4, anti-CD8 and anti-NK1.1, stained intracellularly with anti-IL-17A and anti-IFNγ and analysed by flow cytometry. Results were expressed as the mean frequency of (A) CD4+ T cells secreting IFNγ (p=0.0004 paired T test), (B) CD4+ T cells secreting IL-17A (p=0.002 paired T test), (C) CD8+ T cells secreting IFNγ, (D) CD8+ T cells secreting IL-17A, (E) NK cells secreting IFNγ (p=0.0003 paired T test) and (F) NK cells secreting IL-17A (± standard deviation) (n=3).

IFNγ+ IL-17A+ double positive CD4+ T cells have been observed in elevated numbers in inflamed tissues in autoimmune diseases (Annunziato *et al* 2007; Kebir *et al* 2009), to explore whether these cytokines were produced by different populations of CD4+ T cells

or by a single population of IFNγ+ IL-17A+ double positive CD4+ T cells, the effect of PEG-$LF_{457-476}$ immunisation upon the induction double positive CD4+ T cells in response to MBP_{85-99} stimulation was examined (Figure 6.2.11). The levels of IFNγ+ (Figure 6.2.11A) and IL-17A+ (Figure 6.2.11C) single positive cells were low and remained unaffected by PEG-$LF_{457-476}$ immunisation, while the frequency of IFNγ+ IL-17A+ double positive CD4+ T cells was reduced by administration of PEG-$LF_{457-476}$ (Figure 6.2.11B).

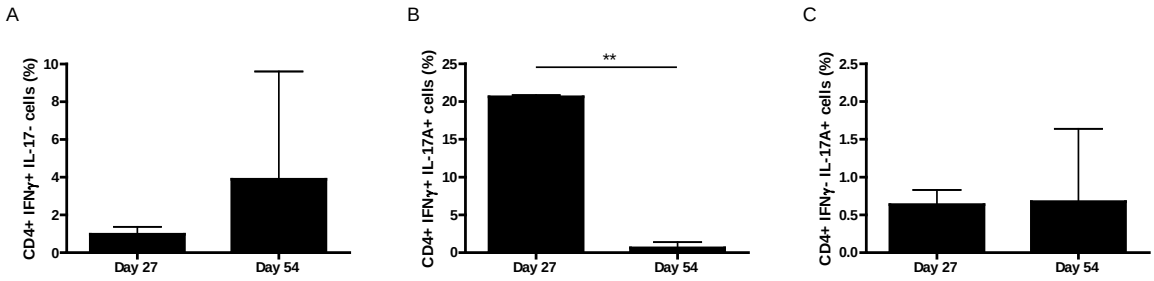


Figure 6.2.11 Frequency of CD4+ T cells secreting IL-17A, IFNγ or both cytokines from HLA-TCR transgenics analysed at days 27 and 54 of PEG-$LF_{457-476}$ immunisation.
CD4+ T cells stimulated with 0.0025μg/ml of MBP_{85-99} were analysed by flow cytometry for secretion of (A) IFNγ, (B) IFNγ and IL-17A (p=0.0049 paired T test) and (C) IL-17A. Results were expressed as the mean frequency of CD4+ cells in the leucocyte population, (± standard deviation) (n=3).

To quantitatively analyse the effect of 73 days of administration of PEG-LF_{61-80} and PEG-$LF_{457-476}$ the cytokine profile produced by the supernatants of splenocytes from immunised transgenics, following three days of MBP_{85-99} stimulation, were harvested and the expression level of a range of cytokines measured by ELISA. The levels of the pro-inflammatory cytokines IFNγ, TNFα, IL-6 and IL-9 produced in response to MBP_{85-99}

were unaffected by the administration of either LF_{61-80} or $LF_{457-476.}$ The levels of IL-17A (Figure 6.2.12C) were significantly reduced in splenocytes taken from PEG-$LF_{457-476}$ immunised group compared to the PEG-LF_{61-80} immunised control group. Conversely, the levels of IL-10 (Figure 6.2.12D) were significantly higher in the PEG-$LF_{457-476}$ immunised group than in the PEG-LF_{61-80} immunised group.

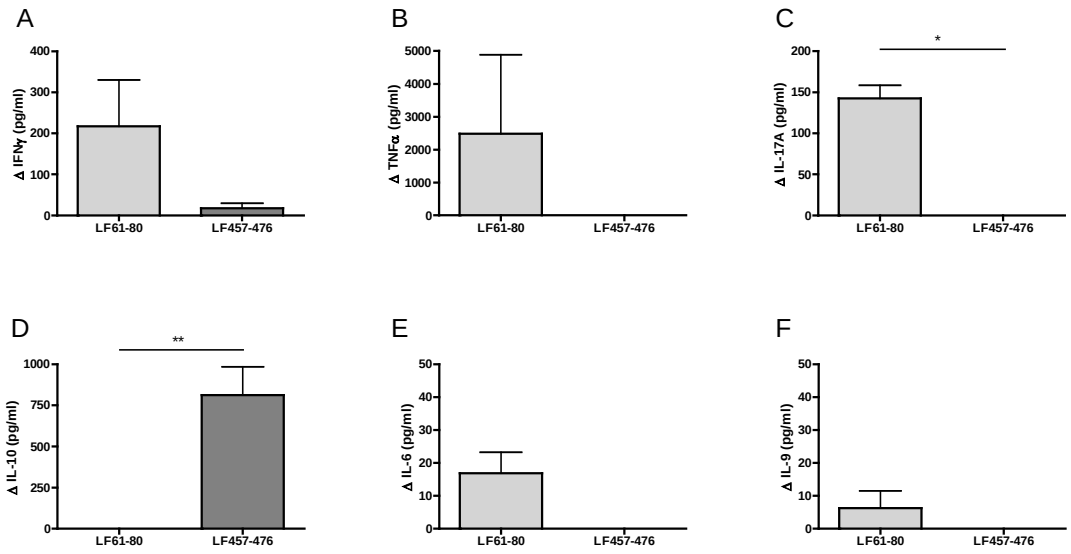


Figure 6.2.12 Cytokine release by e*x-vivo* spleen cells from PEG-LF_{61-80} and PEG-$LF_{457-476}$ immunised HLA-TCR transgenics undergoing spontaneous paralysis.

Following culture of the cells with 0.0025μg/ml of MBP_{85-99}, supernatants were assessed by ELISA for the production of the following cytokines; (A) IFNγ, (B) TNFα, (C) IL-17A (p=0.0158, paired t-test), (D) IL-10 (p=0.0059, unpaired t-test), (E) IL-6 and (F) IL-9, n=3 per group (± standard deviation).

Expression of the Th1, Th2, Th17, natural (nTreg) and inducible (iTreg) lineage-specific transcription factors T-bet, GATA-3, ROR-γt, FoxP3 and LAG-3, respectively, along with myelin-associated oligodendrocyte basic protein (MOBP, which is expressed exclusively in the CNS and plays a role in stabilizing the myelin sheath (Holz *et al* 2000; Yamamoto *et al* 1994b)) and the T cell co-receptor CD3ε polypeptide chain, were determined in the CNS of control peptide or PEG-$LF_{457-476}$ immunised transgenics. The expression levels of T-bet (Figure 6.2.13A) and CD3ε (Figure 6.2.13F) were both significantly reduced in $LF_{457-476}$ immunised group compared to the LF_{61-80} immunised group in the murine spinal cord. The cerebellum (Figure 6.2.14) and cortex (Figure 6.2.15) did not show any variation in the levels of the genes following administration of the LF peptides. This demonstrates that CD3+ T cells, specifically those of a Th1 lineage, infiltrated the spinal cord of the control, but not the $LF_{457-476}$ immunised HLA-TCR transgenics.

Taken together, these results indicate that immunisation with $LF_{457-476}$ reduced the infiltration of Th1 cells into the spinal cord tissues, which had an effect upon the onset and severity of spontaneous paralysis in the HLA-TCR transgenics. This is consistant with the heavy infiltration of lymphocytes into the spinal cord which characterises the early stages of disease within this model (Ellmerich *et al* 2005). It is interesting that the splenocytes from mice immunised with $LF_{457-476}$ showed a reduction in the amount of IL-17A, but not IFNγ, produced in response to stimulation with MBP_{85-99}, IFNγ is primarily identified as a Th1 associated cytokine, whilst in contrast, IL-17A is associated with the Th17 subset. The increase in IL-10 levels in the $LF_{457-476}$ immunised transgenics

meanwhile, might suggest skewing towards a Th2 or Treg cytokine profile was involved in the control of the inflammatory Th1 response. However, although elevations IL-10 have been associated with Treg and Th2 lineage cells inducing remission in autoimmune conditions (Barrat *et al* 2002; Bettelli *et al* 1998), in this case, there is no evidence of involvement of cells of a either a GATA-3, nTreg or iTreg lineage.

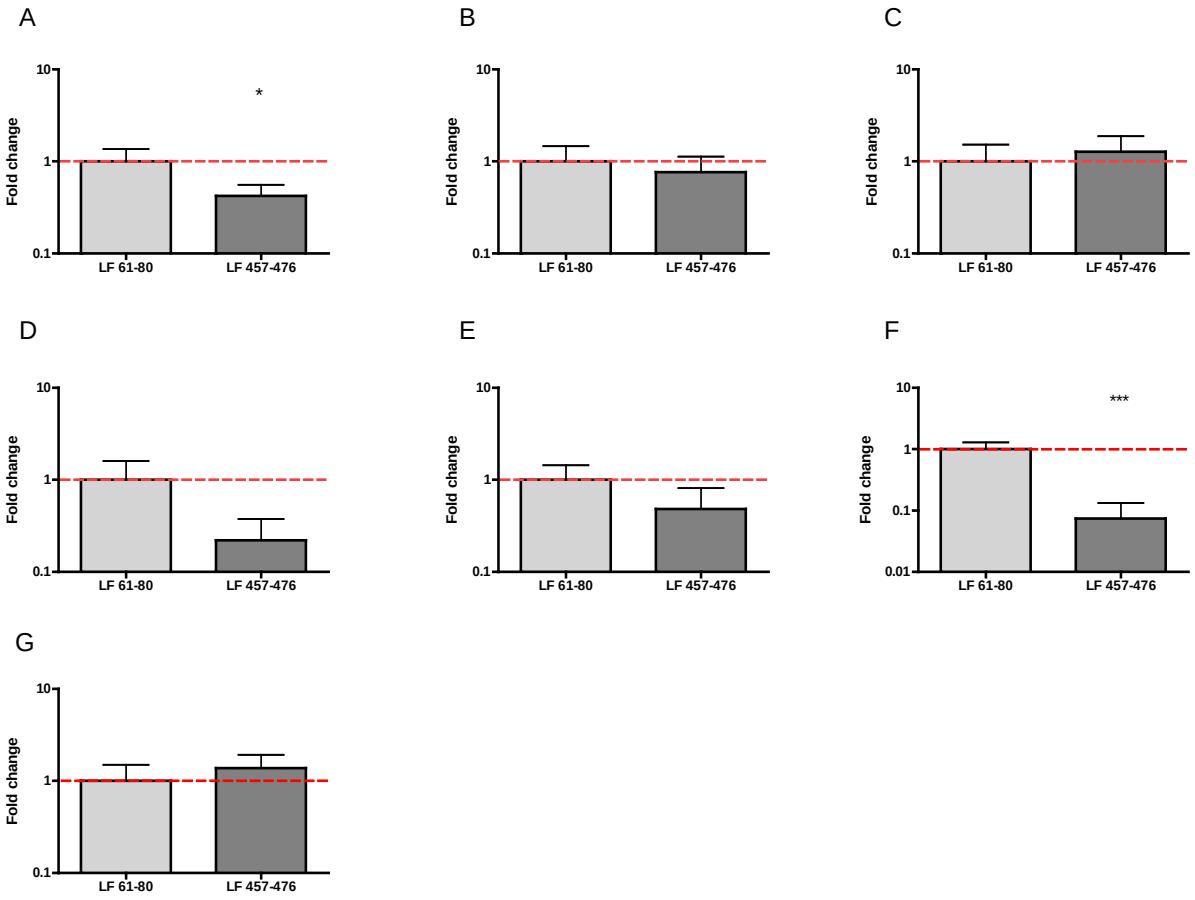


Figure 6.2.13 Quantitative PCR analysis of the spinal cord from PEG-$LF_{457-476}$ and PEG-LF_{61-80} immunised HLA-TCR transgenics undergoing spontaneous paralysis.

The expression levels of the following genes in spinal cord were determined by qPCR; (A) *tbet* (REST analysis p=0.046), (B) *gata3*, (C) *rorγt*, (D) *foxp3*, (E) *lag3*, (F) *cd3e* (REST analysis p=0.001) and (G) *mobp*. Levels were normalised to the reference genes GAPDH and Rpl-13a, and data are expressed as a fold expression increase or decrease, relative to levels in the LF_{61-80} injected control goup, n=3 per group (± SEM).

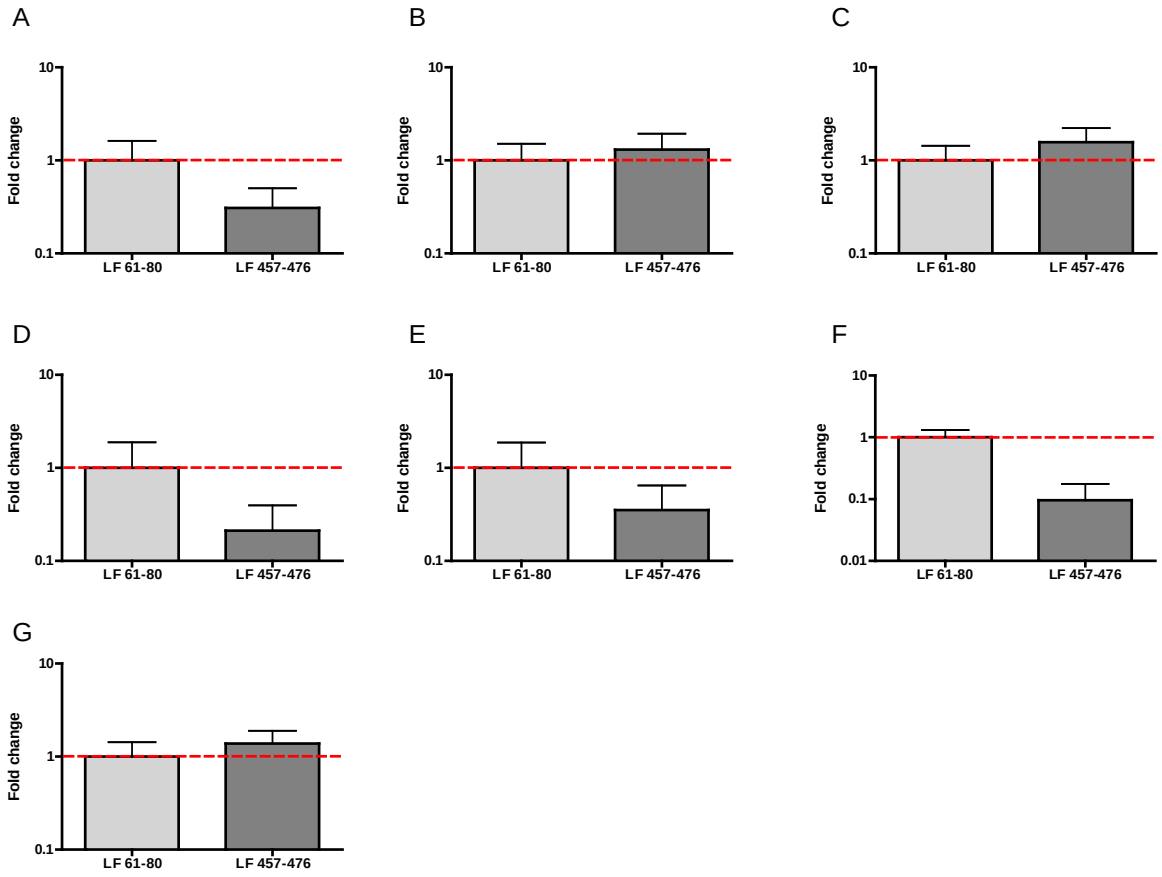


Figure 6.2.14 Quantitative PCR analysis of the cerebellum from PEG-$LF_{457-476}$ and PEG-LF_{61-80} immunised HLA-TCR transgenics undergoing spontaneous paralysis.

The expression levels of the following genes in cerebellum were determined by qPCR; (A) *tbet*, (B) *gata3*, (C) *rorγt*, (D) *foxp3*, (E) *lag3*, (F) *cd3e* and (G) *mobp*. Levels were normalised to the reference genes GAPDH and Rpl-13a, and data are expressed as a fold expression increase or decrease, relative to levels in the LF_{61-80} injected control goup, n=3 per group (± SEM).

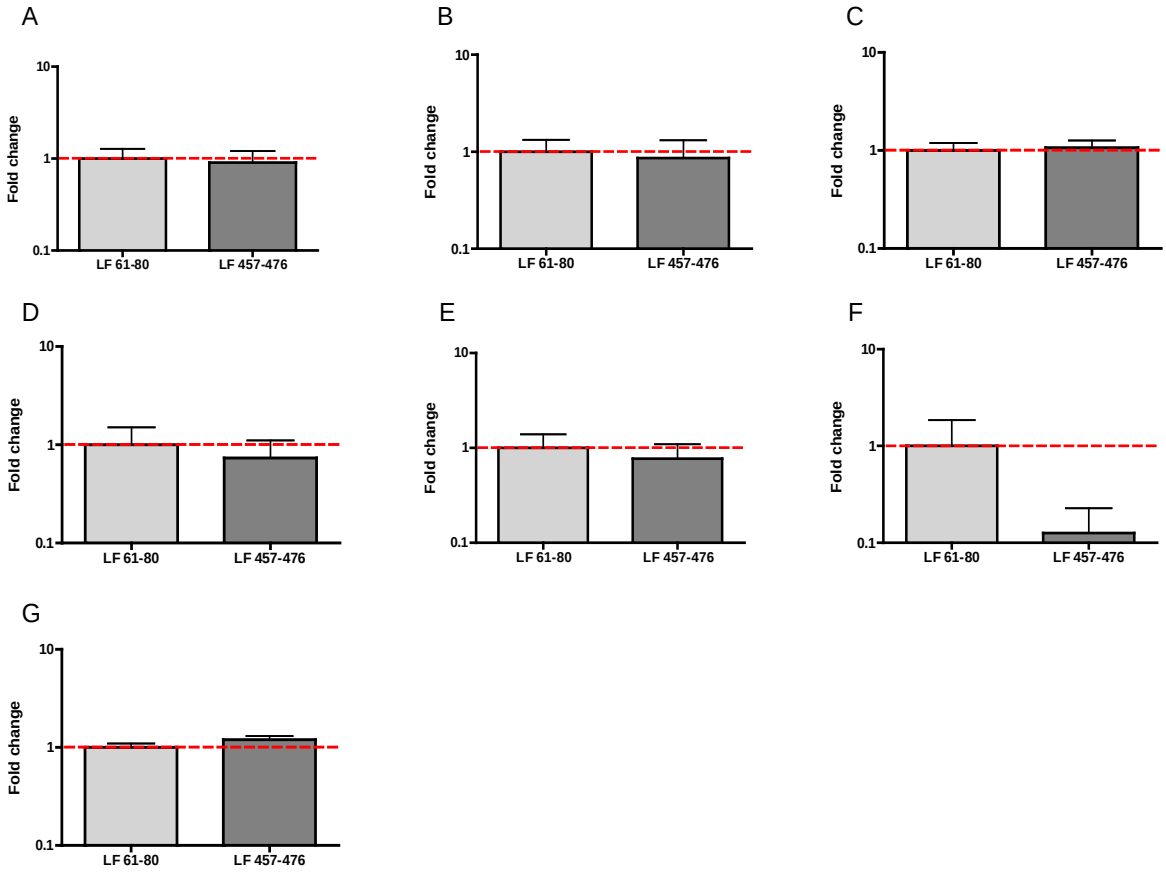


Figure 6.2.15 Quantitative PCR analysis of the cortex from PEG-$LF_{457-476}$ and PEG-LF_{61-80} immunised HLA-TCR transgenics undergoing spontaneous paralysis.

The expression levels of the following genes in cortex were determined by qPCR; (A) *tbet*, (B) *gata3*, (C) *rorγt*, (D) *foxp3*, (E) *lag3*, (F) *cd3e* and (G) *mobp*. Levels were normalised to the reference genes GAPDH and TfrC, and data are expressed as a fold expression increase or decrease, relative to levels in the LF_{61-80} injected control goup, n=3 per group (± SEM).

6.2.6 Effect of $LF_{457-476}$ immunisation upon the inhibition of EAE development in transgenic mice

There is evidence that peptide immunotherapy is effective in blocking the early stages of disease progression in autoimmune conditions such as type 1 diabetes (Tisch *et al* 1999). However, the efficacy of the treatment may be limited by the stage at which administration of treatment begins, as at later stages of disease the frequency of pathogenic T cells is high, and the proinflammatory milieu already well established. It is therefore important to establish whether prospective peptide based immunotherapies such as the $LF_{457-476}$ peptide are capable of suppressing the activaton of autoreactive T cells during active disease. To address this question EAE was induced in the HLA-TCR transgenics by active immunisation with the MS-related epitope MBP_{85-99}, the mice were then treated with the PEGylated $LF_{457-476}$ or LF_{61-80} peptides.

In contrast to the effect of the $LF_{457-476}$ peptide upon the development of spontaneous paralysis in the HLA-TCR transgenic models (Figure 6.2.9), the onset and severity of the clinical disease symptoms (Figure 6.2.16A), and the percentage weight loss (Figure 6.2.16B), which is also used as a surrogate marker of EAE disease severity, were identical in both peptide immunised groups. The course of the MBP_{85-99} induced EAE was therefore unaffected by the administration of either PEG-$LF_{457-476}$ or the control peptide PEG-LF_{61-80}.

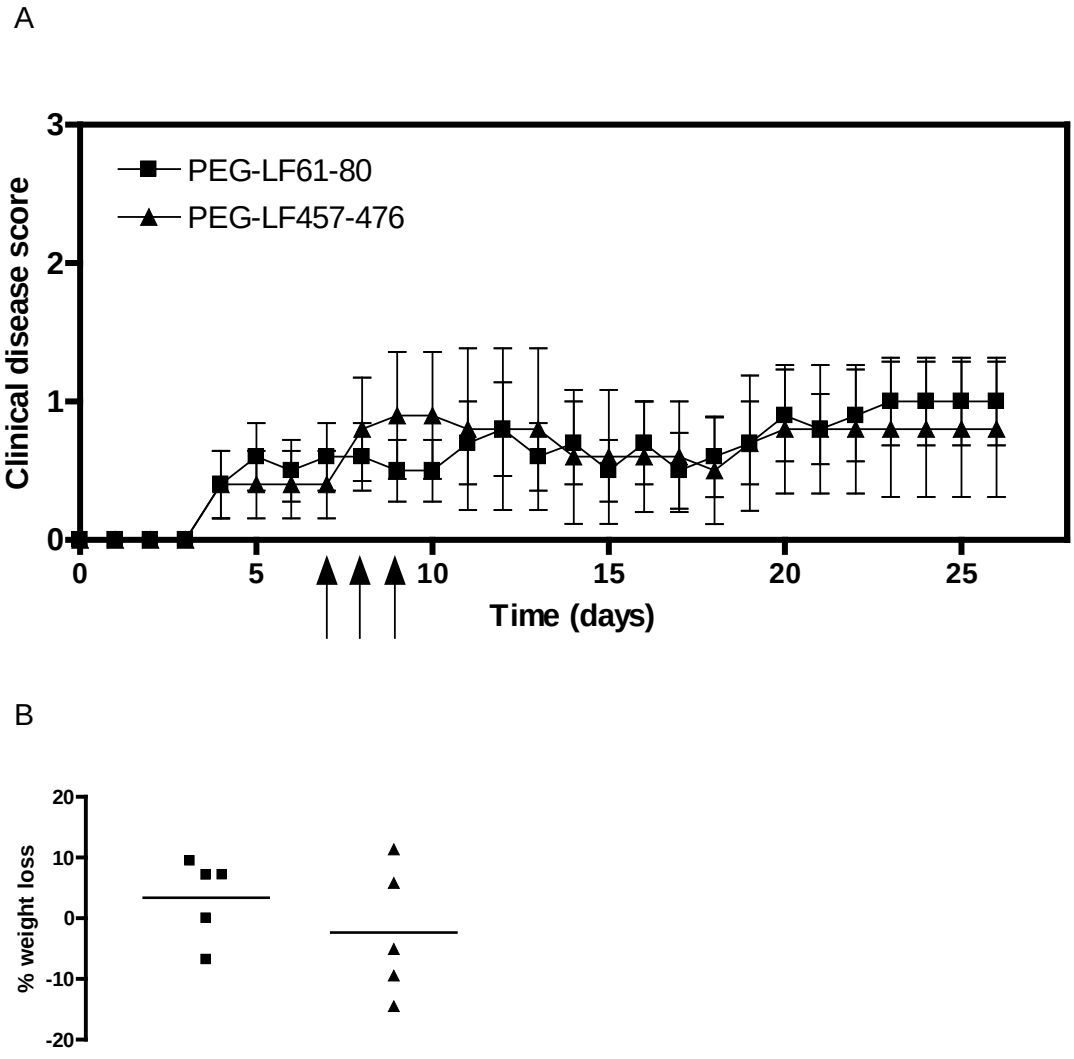


Figure 6.2.16 Development of EAE in peptide immunised HLA-TCR transgenics.

(A) EAE was induced in age- and sex- matched groups of transgenic mice by immunisation with $MBP_{85–99}$ and *B. pertussis* toxin on day 0 and *B. pertussis* toxin on day 1. On days 7, 8 and 9 mice were immunised with either PEG-LF_{61-80} or PEG-LF457-476. The effect upon the development of EAE in transgenic mice was observed over 26 days through daily clinical scores. (B) The weight loss was determined for PEG-LF_{61-80} (■) and PEG-LF457-476 (▲) immunised groups at the end of the 26 days and expressed as a percentage relative to each animal's initial body weight (mean ± SEM) (n=5).

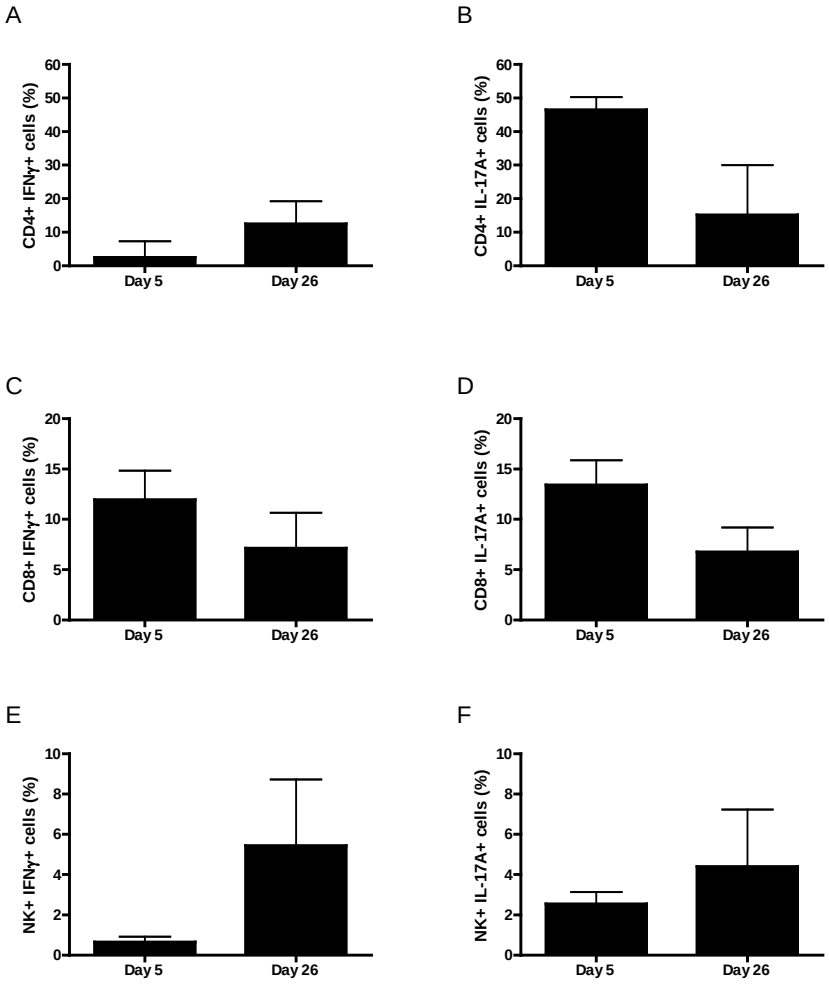


Figure 6.2.17 Frequency of leucocytes secreting pro-inflammatory cytokines from HLA-TCR transgenics analysed at days 5 and 26 following EAE induction.

Peripheral leucocytes were stimulated with 0.0025μg/ml of MBP_{85-99}, then stained with anti-CD4, anti-CD8 and anti-NK1.1, stained intracellularly with anti-IL-17A and anti-IFNγ and analysed by flow cytometry. Results were expressed as the mean frequency of (A) CD4+ T cells secreting IFNγ, (B) CD4+ T cells secreting IL-17A, (C) CD8+ T cells secreting IFNγ, (D) CD8+ T cells secreting IL-17A, (E) NK cells secreting IFNγ and (F) NK cells secreting IL-17A (± standard deviation) (n=5).

The cell populations affected by PEG-$LF_{457-476}$ immunisation were analysed by flow cytometry of peripheral leucocytes taken at timepoints during the development of EAE in

the peptide immunised HLA-TCR transgenics (Figure 6.2.17). The secretion of the pro-inflammatory cytokines IFNγ and IL-17A produced by NK cells and CD4+ or CD8+ T cells in response to MBP_{85-99} were unaffected by the administration of $LF_{457-476}$ following induction of EAE.

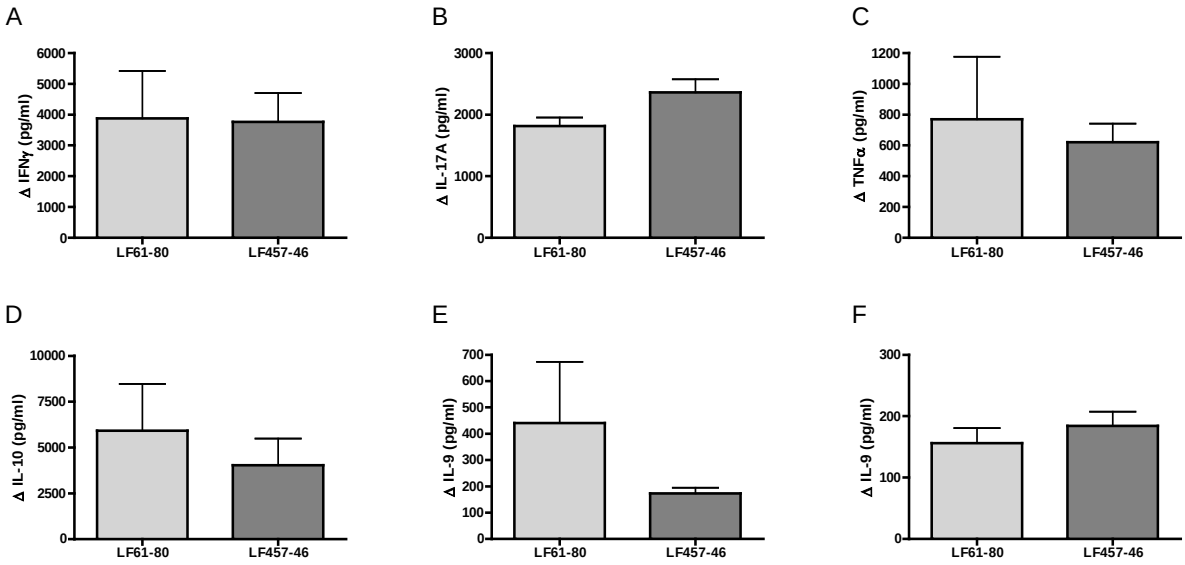


Figure 6.2.18 Cytokine release by e*x-vivo* spleen cells from PEG-LF_{61-80} and PEG-$LF_{457-476}$ immunised HLA-TCR transgenics undergoing EAE.
Following culture of the cells with 0.0025μg/ml of MBP_{85-99}, supernatants were assessed by ELISA for the production of the following cytokines; (A) IFNγ, (B) TNFα, (C) IL-17, (D) IL-10, (E) IL-6 and (F) IL-9, n=5 per group (± standard deviation).

Following three days of MBP_{85-99} stimulation, the production of the pro-inflammatory cytokines IFNγ, TNFα, IL-17A, IL-6, IL-9 and the anti-inflammatory cytokine IL-10 by splenocytes harvested from immunised HLA-TCR transgenics, were were unaffected by the prior administration of either LF_{61-80} or $LF_{457-476}$ in the HLA-TCR EAE model (Figure 6.2.18).

Expression levels of the Th1, Th2, Th17, nTreg and iTreg lineage-specific transcription factors T-bet, GATA-3, ROR-γt, FoxP3 and LAG-3, respectively, along with MOBP and CD3ε, were determined in the CNS of HLA-TCR transgenics immunised with control peptide or PEG-$LF_{457-476}$ during the course of EAE induction. The expression levels of these genes in the spinal cord (Figure 6.2.19) and cerebellum (Figure 6.2.20) of the mice were unaffected by administration of either LF_{61-80} or $LF_{457-476}$. In the cortex, however, the expression level of the Th2 transcription factor, GATA-3, was significantly reduced in the PEG-$LF_{457-476}$ immunised compared to the control group (Figure 6.2.21B).

It therefore appears that administration of the $LF_{457-476}$ peptide has no effect upon the onset and severity of EAE and the cytokine profile of cells in response to stimulation with a primary MS-related epitope.

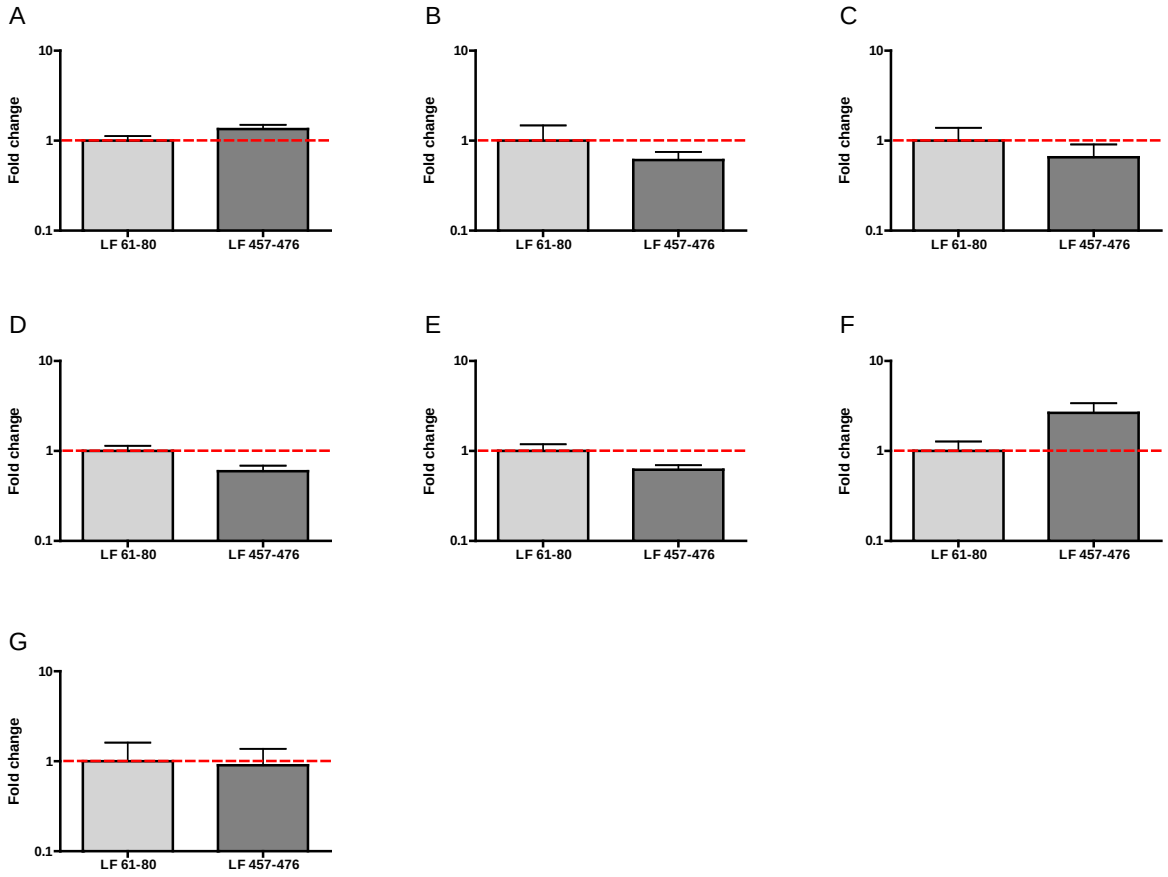


Figure 6.2.19 Quantitative PCR analysis of the spinal cord from transgenics with MBP_{85-99} induced EAE immunised with PEG-$LF_{457-476}$ and PEG-LF_{61-80}.

The expression levels of the following genes in spinal cord were determined by qPCR; (A) *tbet*, (B) *gata3*, (C) *rorγt*, (D) *foxp3*, (E) *lag3*, (F) *cd3e* and (G) *mobp*. Levels were normalised to the reference genes GAPDH and Rpl-13a, and data are expressed as a fold expression increase or decrease, relative to levels in the LF_{61-80} injected control goup, n = 5 per group (± SEM).

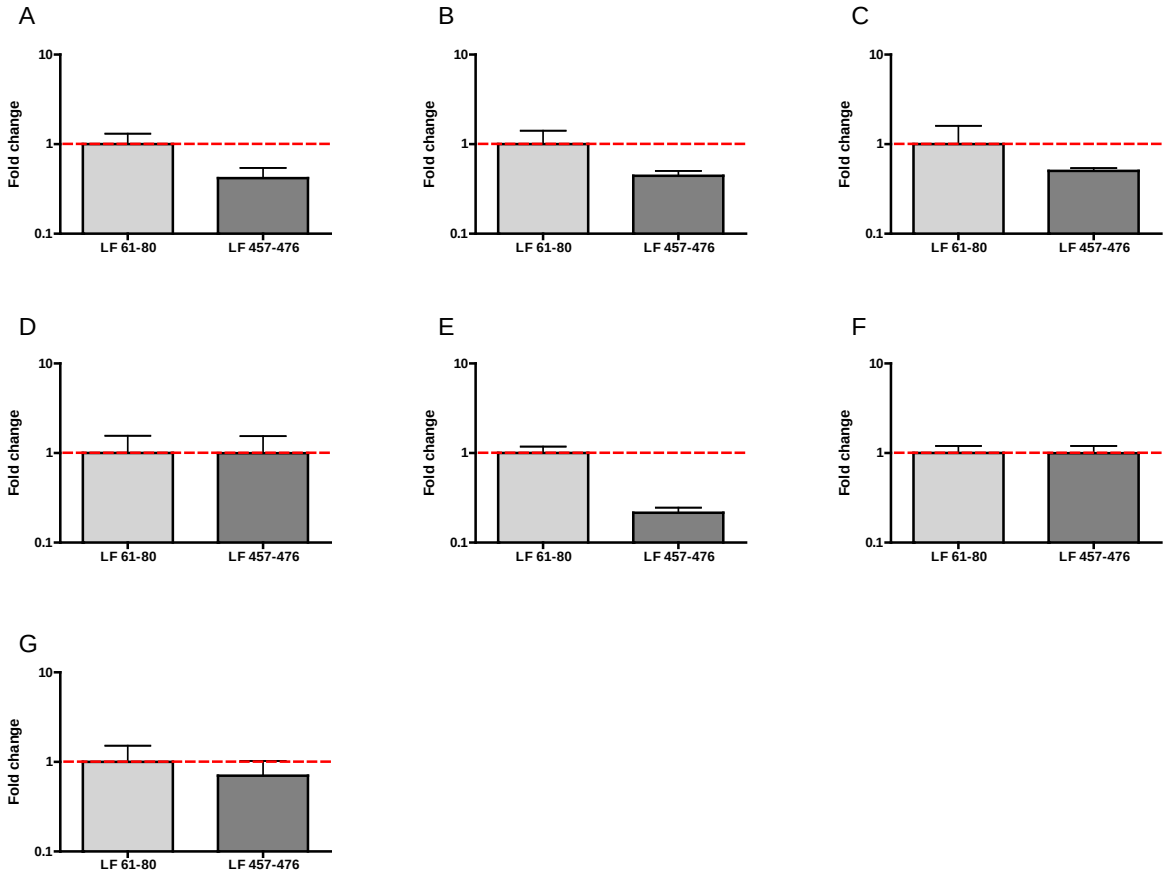


Figure 6.2.20 Quantitative PCR analysis of the cerebellum from transgenics with MBP_{85-99} induced EAE immunised with PEG-$LF_{457-476}$ and PEG-LF_{61-80}.

The expression levels of the following genes in cerebellum were determined by qPCR; (A) *tbet*, (B) *gata3*, (C) *rorγt*, (D) *foxp3*, (E) *lag3*, (F) *cd3e* and (G) *mobp*. Levels were normalised to the reference genes GAPDH and Rpl-13a, and data are expressed as a fold expression increase or decrease, relative to levels in the LF_{61-80} injected control goup, n = 5 per group (± SEM).

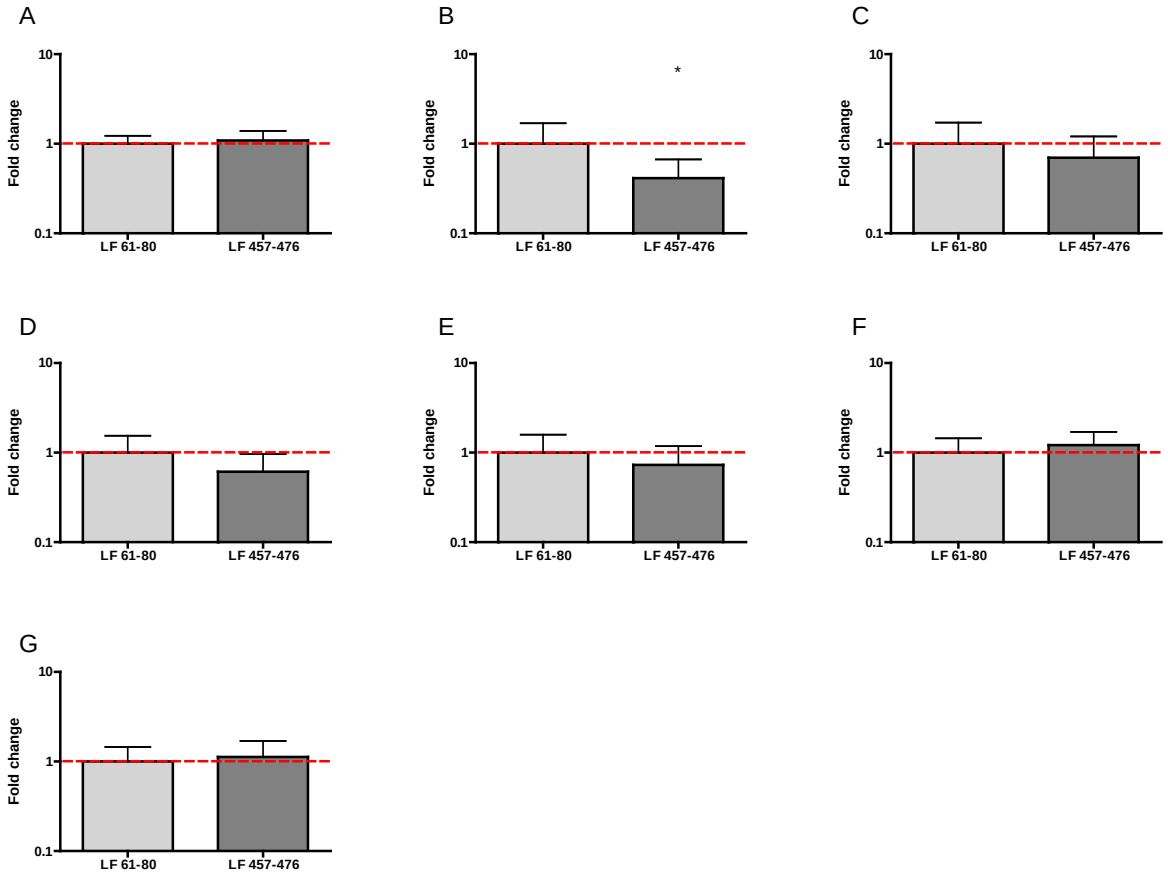


Figure 6.2.21 Quantitative PCR analysis of the cortex from transgenics with MBP_{85-99} induced EAE immunised with PEG-$LF_{457-476}$ and PEG-LF_{61-80}.

The expression levels of the following genes in cortex were determined by qPCR; (A) *tbet*, (B) *gata3* (p = 0.025), (C) *rorγt*, (D) *foxp3*, (E) *lag3*, (F) *cd3e* and (G) *mobp*. Levels were normalised to the reference genes GAPDH and TfrC, and data are expressed as a fold expression increase or decrease, relative to levels in the LF_{61-80} injected control group, n = 5 per group (± SEM).

6.3 Discussion

In the majority of studies of immunomodulatory peptides, disease induction by encephalitogenic peptides has been prevented by coinjection of either nonhomologous or close analogues of the encephalitogenic peptides into EAE models. This disease model, which involves the co-administration of self-antigen and competitor peptide, raises the possibility of antigen-specific, rather than MHC-specific, mechanisms of disease prevention. Use of the HLA-TCR transgenic spontaneous disease model, in contrast, allows the investigation of the effect of immunomodulatory molecules independent of disease induction, possibly even allowing for treatment before disease induction.

To obtain evidence for MHC blockade *in vitro*, an *ex vivo* experimental model was first established to detect complexes of naturally processed antigenic peptides generated and expressed by MHC class II molecules on the surface of APCs. $LF_{457-476}$ is capable of blocking the activation of self peptide-specific, class II-restricted *ex-vivo* cells following restimulation with the encephalitogenic epitopes, MBP_{85-99} and αB-crystallin$_{161-176}$ (Figure 6.2.1). There is a tendancy towards immunodominance of the MBP_{85-99} peptide in the responses of both MS patients and murine models of EAE (Ota *et al* 1990; Sakai *et al* 1988; Wucherpfennig *et al* 1997). Eli Sercarz first observed the phenomenon of epitope spread within the EAE model following the injection of an immunodominant myelin epitope, leading to activation of T cells against other antigens found within the CNS (Lehmann *et al* 1992). In the HLA-TCR transgenic model, used in these experiments, disease progression is associated with epitope spreading to a number of antigens, released

possibly as a consequence of local tissue damage (Ellmerich *et al* 2004), including the small heat shock protein αB-crystallin (van Noort *et al* 1995). Both the αB-crystallin and the MBP sequences are strongly conserved between humans and mice (Frederikse *et al* 1994; Larson *et al* 1995), the ability of $LF_{457-476}$ to restrict the T cell response to epitopes contained within these sequences indicates that it may be capable of inhibiting the *in vivo* response to these antigens.

Interestingly, exposure to the whole LF protein did not have an inhibitory effect upon either the proliferative or cytokine response to MBP_{85-99} (Figure 6.2.3). The LF protein acts as a MAPK inhibitor, disrupting the activity of three of the four families of MAPKs, including the p38 MAPK isoforms (Turk 2007), which have been the target of attempts to develop a small molecule inhibitor due to their role in the downstream regulation of TNFα and IL-1β signalling in autoimmune diseases such as rheumatoid arthritis (RA) (Cohen *et al* 2009). In combination with the inability of $LF_{457-476}$ to inhibit the MS epitope specific response through non-specific mechanisms (Figure 6.2.8), this indicates that the $LF_{457-476}$ dependent inhibition of stimulation was strongly linked to the binding and presentation of this epitope by DR15 molecules.

APL's and small molecule inhibitors have been found to work in part by altering the cytokine production of T cells responding to self antigen (Adorini *et al* 1988b; Gaur *et al* 1997; Windhagen *et al* 1995), $LF_{457-476}$ is capable of reducing the production of the pro-inflamatory cytokines IFNγ, TNFα and IL-17A (Figure 6.2.2) released in response to MBP_{85-99} by *ex-vivo* cells. $LF_{457-476}$ is also capable of reducing the production of these

cytokines in a dose dependent manner in ex-vivo cells and IFNγ in T-cell hybridoma lines (Figure 6.2.4, Figure 6.2.5 and Figure 6.2.7). Previous studies which focused upon peptide-based immunotherapy have demonstrated that the development of autoimmune diseases such as IDDM can be prevented by immunisation with small peptide inhibitors, correlating with the induction of Th2 cells and Tregs (Tian *et al* 1996; Tisch *et al* 1998). Immunisation with peptides may elicit Th2 cell differentiation and effector function by a number of possible mechanisms, which are not mutually exclusive. Multiple immunisations with high doses of peptide emulsified in IFA may directly induce differentiation of Th2 cells that down-regulate Th1 cell development and function or trigger clonal deletion and anergy of Th1 cells which in turn facilitates the expansion of Th2 cells (Yip *et al* 1999). The production of anergic T cell clones which can suppress immune responses *in vivo* is associated with a strong production of the anti-inflammatory cytokine IL-10 (Buer *et al* 1998; Chai *et al* 1999). The production of CD4+CD25+ Treg populations, which have proven capable of inhibiting the development of diseases such as autoimmune colitis (Cottrez *et al* 2000; Groux *et al* 1997), mediate their suppressive activities via the release of the anti-inflammatory cytokines IL-10 and TGFβ (Sakaguchi *et al* 1995). IL-10 also plays a role in regulating the development and course of EAE (Bettelli *et al* 1998). The levels of this cytokine produced *in vivo* in the HLA-TCR model, however, suggests that while the cytokine could play a role in $LF_{457-476}$ induced control of inflammation, skewing towards an IL-10 producing Th subset is not the only or primary mechanism involved in regulating the inhibition of the proliferative and pro-inflammatory response to MBP_{85-99}. It is interesting that, although the peptide blockade might have been expected to act through the absence of a response, due to the

inaccessibility of the peptide groove, a shifted cytokine response was observed. This presumably occurs because a certain, reduced amount of the MBP peptide still binds to the MHC molecule, representing a reduced half-life.

In vivo evidence suggests that progression to disease may be ameliorated by administration of $LF_{457-476}$ (Figure 6.2.9). The levels of the anti-inflamatory cytokine IL-10 produced by HLA-TCR spleen cells were significantly greater in the $LF_{457-476}$ peptide immunised rather than control LF_{61-80} immunised group which, contrary to the *in vitro* evidence, suggests a prominent role for Th2 or Treg cells in controlling the development of a demyelinating disease (Figure 6.2.12). It is therefore interesting that expression of the Th1 lineage-specific transcription factor T-bet, and the T cell co-receptor CD3ε polypeptide chain, were both significantly reduced in the $LF_{457-476}$ immunised group compared to the LF_{61-80} immunised group in the murine spinal cord indicating inability of Th1 cells to infiltrate the CNS (Figure 6.2.13), but there was no change in the Th2, Th17, nTreg and iTreg lineage-specific transcription factors, or in the levels of MOBP (which could indicate remyelination within the CNS, which is a prominent feature in EAE lesions, particularly in the early stages of the disease). Infiltration of the control spinal cord with CD3+ T cells of a Th1 lineage is consistent with the early stages of disease in these HLA-TCR transgenics (Ellmerich *et al* 2005), but immunisation with $LF_{457-476}$ reduced infiltration of the Th1 cells into the spinal cord tissues, which was associated with an increase in the time to onset of clinical symptoms and reduction in the severity of spontaneous paralysis. Notably, stimulation with MBP_{85-99} caused the splenocytes from mice immunised with $LF_{457-476}$ to secrete a reduced amount of IL-17A, but not IFNγ,

compared to controls (Figure 6.2.12). Since IFNγ is primarily identified as a Th1 associated cytokine and IL-17A is associated with the Th17 subset, this would appear to contradict the quantatitive PCR results. However, the $LF_{457-476}$ induced amelioration may be mediated by a pathway dependent upon T-bet transcription, rather than specific cytokine profile and T helper subset. Yang *et al* have posited that the encephalitogenic potential of CD4+ T cells lies primarily in the expression of this specific transcription factor rather than in the expression of either IL-17A or IFNγ (Yang *et al* 2009). $LF_{457-476}$ therefore may act upon this pathway, inhibiting the induction of pathogenic T cells expressing the T-bet transcription factor. The analysis of peripheral leucocytes also demonstrates that repeated administration of $LF_{457-476}$ skews the MBP_{85-99} reactive CD4+ cells away from an IFNγ+IL-17A+ double positive population (Figure 6.2.11), (this is also linked to a reduction in the frequency of IL-17A+ NK cells, which suggests that $LF_{457-476}$ exerts an unforseen effect upon this cell population). Recent studies have reported that the majority of CD4+ cells observed in the CNS, during actively induced EAE, produced both IL-17 and IFNγ (Axtell *et al* 2006; Ivanov *et al* 2006; Suryani and Sutton 2007). Although previous work by Dan Cua has also shown that either IL-17A+ or IFNγ+ producing T cells are equally capable of driving a pathogenic autoimmune response (Luger *et al* 2008), it appears that inflammatory IFNγ+IL-17A+ Tcells cross the BBB more efficiently than single positive cells in MS patients (Kebir *et al* 2009), supporting a role for $LF_{457-476}$ in preventing the induction of these pathogenic T cell clones. Over a decade ago it was found that IFNγ deficient mice are susceptible to EAE induction (Ferber *et al* 1996), this was followed by the discovery that myelin specific IL-17 producing T cells were capable of transferring EAE (Langrish *et al* 2005), and IL-17

mRNA levels were elevated in the CFS of MS patients during clinical relapses (Matusevicius *et al* 1999), but that IL-17 deficient mice were still capable of developing EAE (Komiyama *et al* 2006). There is currently still a lack of concensus regarding the role Th1 and Th17 cells play in mediating the inflammatory response in the CNS. However, it appears that cytokine production by myelin specific T cells is not the determining factor for T cell encephalitogenicity, rather it is T-bet which plays a key role in regulating the inflammatory nature of CD4+ T cells. $LF_{457-476}$ therefore, in common with the established DMT, IFNβ (Drulovic *et al* 2009), is able to decrease levels of this transcription factor, which correlates with a decreased disease severity in the HLA-TCR transgenics.

Epidemological studies indicate that there is an environmental component to the risk of developing MS. In the Northern hemisphere the prevalence of MS shows a North-South gradient, this is paralleled by a South-North gradient seen in the Southern hemisphere (Forbes and Swingler 1999). The underlying mechanism of this environmental risk is unknown, however the amino acid residues of MBP_{84-101} are known to share extensive homology with common pathogens such as influenza virus, Epstein Barr virus, herpes viruses, papilloma virus, and pseudomonas bacteria (Wucherpfennig and Strominger 1995). This molecular mimicry may act as a possible mechanism in the induction of autoimmunity, mimicking sequestered self-epitopes such as MBP and cross-activating auto-reactive T and B cells. Tolerance to myelin epitopes could thus be broken by an immune response to a microbial sequence. As various critical contact residues governing binding to MHC molecules and TCRs are present in these microbial sequences it is

tempting to view the binding capacity of these epitopes in the same role as the random co-polymer, GA, which binds efficiently to murine H-2A molecules and to their human counterparts, MHC class II DR (specifically DR1, DR15 and DR4), preferentially occupying the peptide binding cleft and preventing the presentation of self-peptides to T cells (Fridkis-Hareli and Strominger 1998).

Attempts to identify the primary MS susceptibility gene in the HLA region have been complicated by the extensive linkage disequilibrium across the β-chain alleles of DRB1*1501, DRB5*0101 and DQB1*0602 which have been identified as the strongest genetic risk factor (Barcellos *et al* 2002). There is inconclusive evidence either for or against a primary role for DQB1*0602 in MS susceptibility (Allen *et al* 1994; Boon *et al* 2001; Spurkland *et al* 1991). In comparison, a primary role for HLA-DR (especially DRB1*1501) (Lincoln *et al* 2005; Oksenberg *et al* 2004), is consistent with both the pathogenesis of a T cell mediated autoimmune response against MBP_{85-99}, and the therapeutic potential of GA which has a high binding affinity for DR but not DQ molecules (Fridkis-Hareli *et al* 1994). The docking of peptides within the antigen binding cleft is governed by the anchor residue preferences of the binding pockets, and the crystal structure of DRB1*1501 differs from other, related, DRβ chains due to the presence of a large hydrophobic pocket at P4 in the peptide-binding groove. This pocket displays a high affinity for aromatic amino acids and accommodates the primary anchor residues for peptide binding to this MHC molecule (Smith *et al* 1998). During the immune response to MBP_{85-99}, the P4 pocket is occupied by phenylalanine (Li *et al* 2000), correspondingly, the P4 pocket may be occupied by tyrosine when complexed to $LF_{457-476}$, which acts as an

important primary anchor, accounting for the high affinity binding to this peptide. Unfortunately, it appears that the LF peptide is only capable of displacing self peptides following repeated administration prior to the advent of spontaneous demyelinating disease, administration of the LF peptide within the context of a classic EAE induction did not result in the amelioration of disease. This suggests that once tolerance has been broken and the disease has progressed beyond the induction of T cell clones specific for self-antigen, $LF_{457-476}$ peptide blockade has a limited effect upon the pathogenic milleu.

7 Discussion and future work

Despite the preponderance of studies which focus upon the importance of PA in developing vaccination strategies against anthrax infection, it has lately become clear that LF has potential as a potent human immunogen. Recent research has indicated that LF rather than PA may represent a major target not only for antibody responses but also T cell immunity in naturally exposed individuals (Brenneman *et al* 2011; Ingram *et al* 2010b). LF immunisation has also proven capable of providing protection against lethal toxin challenge in mice, and protection against aerosol spore challenge in rabbits (Galloway *et al* 2004; Hermanson *et al* 2004). Therefore analysis of the antigen specific T cell response to LF, and identification of a range of immunogenic epitopes for incorporation into an epitope string vaccine may represent a more successful vaccination strategy.

The cellular immune response does not recognise all the possible epitopes which may be generated from a protein antigen, instead focusing upon a relatively restricted number of determinants. A wide range of hypotheses, such as the existence of the so-called ‘Type A’ and ‘Type B’ T cells, which respond differently to the same peptide (Lovitch *et al* 2003; Lovitch *et al* 2007; Pu *et al* 2002), have been advanced to explain the mechanisms which govern the immunodominance and crypticity of these T cell epitopes. However, the major variables which have been accepted to influence the epitope hierarcy include; the efficiency with which the prospective epitope is generated by cellular processing, the capacity the peptide shows for binding to the MHC molecule which governs its

presentation upon the APC cell surface, and the existence of a T cell repetoire which is capable of recognising the MHC/epitope complex presented by the APC (Adorini *et al* 1988a; Buus *et al* 1987; Schaeffer *et al* 1989; Shastri *et al* 1985). The work contained within this thesis has attempted to elucidate the cellular immune response to the anthrax toxin LF and, in particular, determine a hierarcy for the epitopes derived from this protein.

7.1 Mapping the immune responses to LF

7.1.1 CD4+ T cell LF epitopes

Antigen presentation through both HLA-DR and DQ is important in the induction of immunity and the allelic diversity inherent in these class II molecules influences susceptibility to infection (Kwok *et al* 2000). The use of transgenic models in this study was intended to elicit HLA restricted T cell responses to immunogenic epitopes of the LF toxin secreted by *B. anthracis*. It was anticipated that the nature of these models would indicate epitopes which would induce a similar recall response to those seen in humans of a comparable HLA type. The CD4+ T cell response across the five distinct HLA alleles examined in this study, indicates that, although there are allele specific differences, (epitopes from domain IV were overwhelmingly presented in the context of HLA-DR, while domain I epitopes were only presented by the HLA-DR1 and DQ8 alleles), the immunodominant LF epitopes are concentrated in domains II and IV. Recent work by Baillie *et al* has characterised the immunodominant and protective regions of the protein, revealing that domains II and IV stimulated strong IgG antibody responses in AVP-

immunized humans and anthrax infected A/J mice (Baillie *et al* 2010). They also found that the levels of toxin neutralising antibodies, which have been previously proven to correlate with protection against lethal spore challenge (Baillie *et al* 2008), are stimulated by domain I and II immunisation. However, neither domain III or IV proved capable of inducing production of toxin neutralising antibodies (Baillie *et al* 2010). This is intriguing, as previous work had suggested the presence of a linear binding sites for toxin neutralising antibodies within domains III and IV (Albrecht *et al* 2007; Lim *et al* 2005; Nguyen *et al* 2009a). Indeed, the work by Nguyen *et al* indicated that domain III contained a number of structural B cell epitopes, making it extremely antigenic within this context (Nguyen *et al* 2009a). These contradictory results may reflect the nature of the conformational epitopes which, unlike linear CD4+ T cell epitopes, can be lost when domains are expressed as individual recombinant proteins. In contrast Baillie *et al* did find that T cells from human AVP vaccinees proliferated in response to stimulation domains II and IV (Baillie *et al* 2010). This was mirrored by the work performed by our lab (Ingram *et al* 2010b), which indicated the presence of immunodominant epitopes in both domains in AVP-vaccinated individuals and naturally exposed humans. Furthermore, the human T cell response indicated that domain IV was the primary focus of the immune response, with a broad response to the LF domain encompassing Th1, Th2, Th17 and Th9. It is therefore interesting that our work with the HLA transgenics demonstrated that epitopes from this domain were overwhelmingly presented in the context of HLA-DR rather than DQ, this is illustrated by the ribbon diagram representation of the epitope position in LF (Figure 7.1.1 and Figure 7.1.2). This preferential association of LF antigens with specific HLA alleles can be seen not only in

the CD4+ T cell response to $LF_{547-567}$, which was a feature of the response in HLA-DR1, DR4 and DR15 transgenics, but also in the response to $LF_{694-713}$, which stimulates T cells from both AVP-vaccinated and naturally exposed humans, as well as HLA-DR15 transgenics. The response to domain II meanwhile was dominated by $LF_{457-476}$ and $LF_{467-487}$, which produced immunogenic responses in both HLA-DR and DQ transgenic mice following stimulation with either the whole domain, or the individual peptides (Figure 7.1.1 and Figure 7.1.2). The promiscuous immunodominant epitope, $LF_{467-487}$, was a feature of the naturally exposed human response but not the AVP vaccinee response. The ability of this epitope to readily provoke a recall response in CD4+ T cells indicates an important role in anthrax immunogenicity, and illustrated the finding that in Turkish naturally infected individuals the T cell response was focused upon a limited number of LF peptides while in the AVP vaccinated individuals the immunodominant response was broader, encompassing eleven epitopes. Presumably, recognition of these missing epitopes, which were present in the context of vaccination but lost upon infection were not essential to generating a robust T cell response.

The T cell repertoire following immunisation and live anthrax infection therefore differs in regard to the epitopes uncovered, a finding which has been replicated in simian immunodeficiency virus (SIV) infection and immunisation (Vogel *et al* 2002). The possibility that infection works to skew the epitopes which are preferentially processed or presented, uncovering infection specific epitopes, raises the possibility that epitopes which are not immunodominant, but rather cryptic, may be relevant to the design of epitope string vaccines. Within this study several cryptic epitopes capable of eliciting a

strong T cell response following administration in peptide form alone, were identified. The unveiling of cryptic epitopes such as the domain III peptides $LF_{307-326}$ and $LF_{387-406}$ which were presented by a variety of HLA-DR and DQ alleles, suggests not only that regions which appear devoid of immunodominant epitopes may instead contain cryptic epitopes, but also that the epitopes presented are influenced to a great extent by the nature of the MHC class II molecules. The polymorphism inherent in the MHC peptide binding cleft plays a crucial role in restricting the immunodominant and cryptic epitopes presented in an HLA-allele specific manner. Although a number of epitopes such as $LF_{467-487}$, which is recognised by all HLA transgenics as well as naturally infected humans, showed strong relative binding across a range of HLA-DR molecules, it was found that the strength of HLA binding does not adequetly predict the immunodominance or crypticity of the LF peptides. The underlying mechanisms which govern the epitope hierarchy as yet remain unclear, however the work contained within this thesis along with recent research (Baillie *et al* 2010; Ingram *et al* 2010a; Oscherwitz *et al* 2009), indicates that identifying cryptic as well as immunodominant epitopes located within the anthrax toxins may be relevant in the attempt to elicit protective immune responses towards *B. anthracis*.

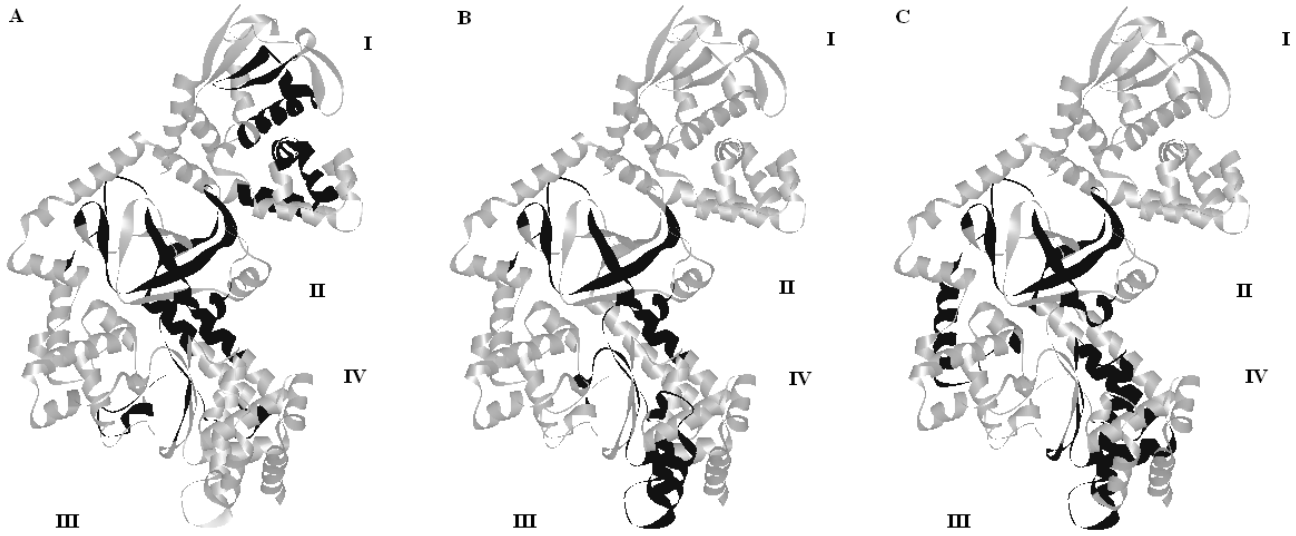


Figure 7.1.1 HLA-DR restricted immunodominant epitopes superimposed onto the LF crystal structure.

The immunodominant epitopes identified in HLA-DR1 (A), DR4 (B) and DR15 (C) are superimposed on the LF crystal structure (Protein Data Bank accession code 1J7N). Roman numerals indicate the structural domains.Ribbon diagrams were generated using the Accelrys discovery studio client 2.5 program, courtesy of Louise Kim.

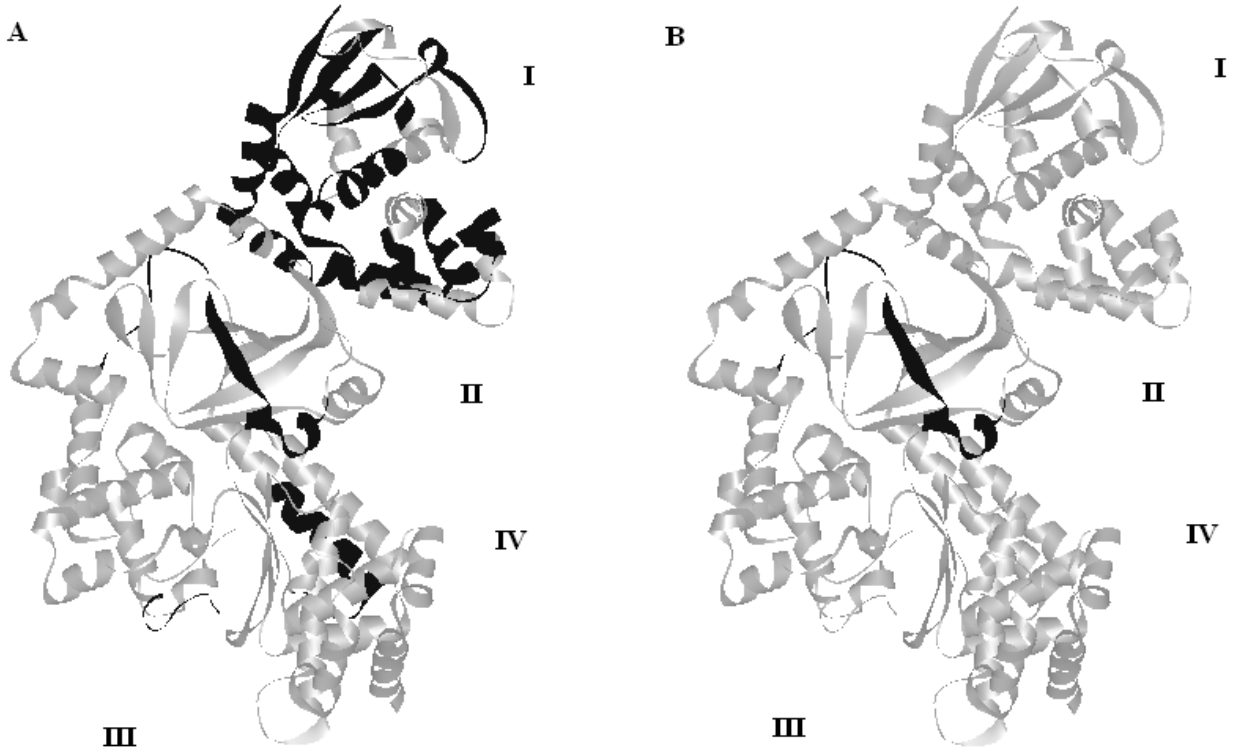


Figure 7.1.2 HLA-DQ restricted immunodominant epitopes superimposed onto the LF crystal structure.

The immunodominant epitopes identified in HLA-DQ8 (A) and DQ6 (B) are superimposed on the LF crystal structure (Protein Data Bank accession code 1J7N). Roman numerals indicate the structural domains. Ribbon diagrams were generated using the Accelrys discovery studio client 2.5 program, courtesy of Louise Kim.

7.1.2 Effect of exposure to anthrax antigens upon immune cell populations

Research by Friedlander *et al* was the first to establish the cytotoxic effect LF has upon host immune cells, preferentially targeting activated macrophages (Friedlander 1986). Further research has revealed that not only macrophages, but also DCs and B cells, are more sensitive to LF intoxication than T cells, with both CD4+ and CD8+ cells proving more robust against LF intoxication (Hu and Leppla 2009). The toxin sensitivity of the phagocytic APCs would be expected to impact upon the presentation of antigens from *B. anthracis* allowing the bacteria to evade the host immune response (Agrawal *et al* 2003; Hu and Leppla 2009). However Terra *et al* described a role for the host Nalp1b/Nlrp1b inflammasome as part of a protective host immune response, mediating macrophage and DC sensitivity to lethal toxin and stimulating an inflammatory reaction at the site of LF production which rapidly recruits lymphocytic cells (Terra *et al* 2010). In this manner LF related cytotoxicity may be related to the host rather than the pathogen response, which possibly explains the long lived, robust, adaptive immunity to LF antigens in naturally infected individuals, which was observed by our lab (Ingram *et al* 2010b), and described within this thesis.

It might therefore be expected that an inverse relationship exists between toxin sensitivity and resistance to infection, and several studies have documented this effect (Beall and Dalldorf 1966; Jones, Jr. *et al* 1967; Welkos *et al* 1986; Welkos and Friedlander 1988). However, more recent work has added complexity to this model (Banks *et al* 2005; Cote *et al* 2008), indicating that it is not merely a targeting of myeloid cells which induces cell

cytoxicity, but that the ANTXR mediated uptake of the anthrax toxins is intimately involved. ANTXR2/CMG2 shows both a high affinity for PA, and higher levels of cell surface expression relative to ANTXR1/TEM8 (Hu and Leppla 2009; Liu *et al* 2009; Scobie *et al* 2005). DCs, macrophages and B cells however display a higher sensitivity to LF induced toxicity, but express significantly less ANTXR2/CMG2 compared to both CD4+ and CD8+ T cells (Hu and Leppla 2009). The reason for this lack of correlation between a high level of anthrax toxin sensitivity and the mechanism governing the entry of the toxin into the cell remains unclear. Work carried out by R. Ingram (personal communication) indicates that expression of ANTXR1/TEM8 upon myeloid cells is significantly lower following anthrax exposure, which intriguingly, might present either a pathogen or host mediated adaption to infection.

7.2 Efficacy of an epitope based vaccine against *B. anthracis* challenge

The UK AVP vaccine, which contains both PA, and lower levels of LF, stimulates LF specific antibodies (Baillie *et al* 2010; Guidi-Rontani *et al* 1999b; Weiner and Hanna 2003), while exposure to natural infection results in a faster, more robust antibody response to LF than PA (Brenneman *et al* 2011). In combination with the fact that LF is able to boost the magnitude of PA-specific antibody responses following co-administration (Pezard *et al* 1995; Price *et al* 2001), and that LF truncate proteins are capable of conferring protection against *B. anthracis* aerosol challenge (Galloway *et al* 2004; Hermanson *et al* 2004), this suggests that LF represents the most potent immunogen of the anthrax toxins. The primary importance of humoral immunity in mediating protection against anthrax has been questioned by recent studies which have suggested that IFNγ producing CD4+ T cells play an important role in long lasting immunity (Glomski *et al* 2007; Ingram *et al* 2010b). In addition, induction of memory CD4+ T cells may feedback not only to cellular immunity, but also aid in the production of toxin neutralising antibodies, Ig class switching and B cell affinity maturation.

As a proof of principle, to determine whether the immunodominant T cell epitopes identified within LF could be incorporated into an epitope sting vaccine which was capable of conferring protection against lethal anthrax challenge, a fusion protein was generated in collaboration with the Baillie lab. The LF fusion protein construct consisted of the T cell epitopes, $LF_{101-120}$, $LF_{151-170}$, $LF_{261-280}$, $LF_{467-486}$, $LF_{547-568}$, $LF_{574-593}$, $LF_{614-633}$, $LF_{654-673}$, $LF_{674-693}$, $LF_{714-733}$, $LF_{724-743}$, and $LF_{744-763}$. These epitopes, which are known to

be HLA-DQ8 restricted, were fused to amino acids 865-1120 from C. *tetani* which constituted a toxin helper domain (Figure 7.2.1).

**MKNLDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKA
IHLVNNESSEVIVHKAMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSIISSM
KKHSLSIGSGWSVSLKGNNLIWTLKDSAGEVRQITFRDLPDKFNAYLANKWVFITIT
NDRLSSANLYINGVLMGSAEITGLGAIREDNNITLKLDRCNNNNQYVSIDKFRIFCKA
LNPKEIEKLYTSYLSITFLRDFWG**SDVLEMETYKAIGGKIYIVDGDYVVYAKEGYEPVLV
IQSSEDYQHRDVLQLYAPEAFNYMETDKFKIYLYENMETNINNLTATLGADLENGKLILQ
RNIGLEIKDVQIEYIRIDAKVVPKSKIDTKIQKLITFNVHNRYASNIVESAYYLVDGNGRFV
FTDITLPNIAEQYTHQDEIYEQVHSKGLYVAVDDYAGYLLDKNQSDLVTNSKKFIDIFKE
EGSNLTSYGRSEGFIHEFGHAVDDYAGYLL

Figure 7.2.1 The protein sequence of the LF fusion protein construct with the tetanus toxin C fragment in bold.

As the genome of both these organisms contained a high AT nucleotide content, the Baillie lab codon optimised the string in order to enhance the levels of expression within the resulting vaccine, this was then cloned into a pQE-30 expression plasmid. Groups of HLA-DQ8 transgenic mice were primed, and then boosted twice with either the LF fusion construct or a peptide pool consisting of the LF epitopes expressed in the fusion protein. These mice, plus a naïve unimmunised group, were then challenged with live *B. anthracis*.

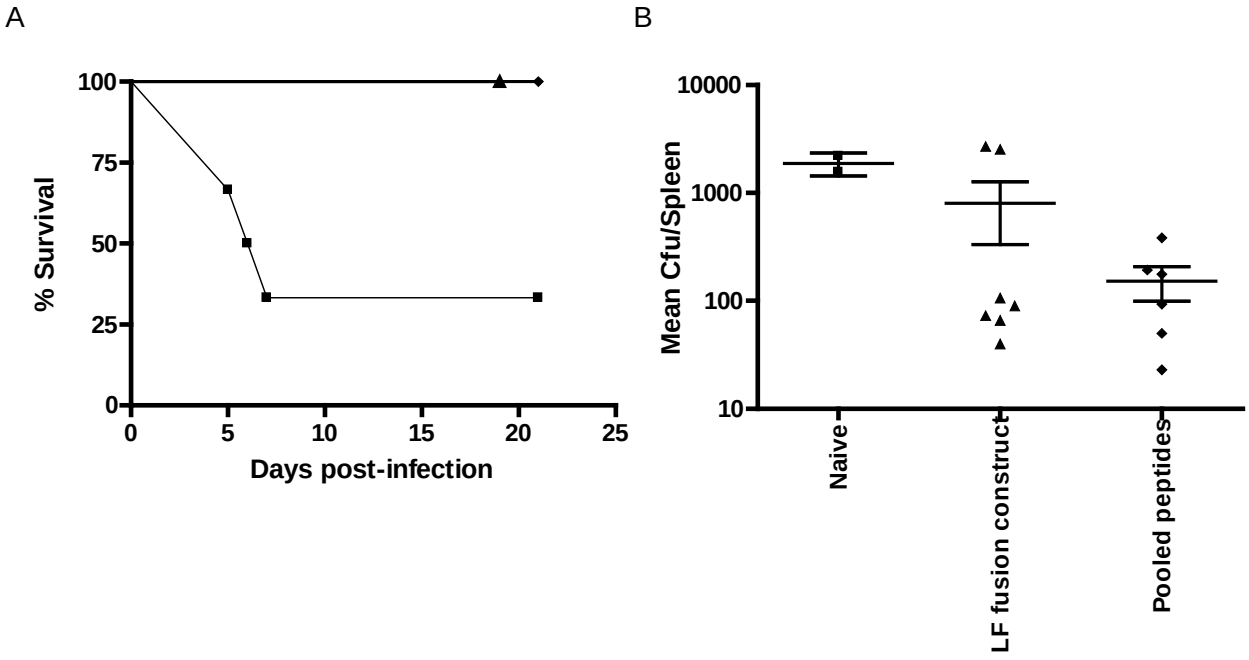


Figure 7.2.2 Efficacy of LF epitope based vaccine against *B. anthracis* challenge

Groups of HLA-DQ8 transgenic mice were immunised 3 times, on days 0, 14 and 35 by the intra-peritoneal route, with either an LF fusion construct comprising a tetanus toxin helper domain (aa 865-1120) and 12 confirmed HLA-restricted LF epitopes (♦ n=7), or a peptide pool of the LF epitopes expressed in the fusion protein (▲ n=6), a naïve unimmunised group was also included in the experiment (■ n=6). All groups were challenged with 10^6 cfu *B. anthracis* STI by the intra-peritoneal route, on day 77, and monitored daily for survival. The impact of infection upon survival was described using Kaplan Meier estimation (A). Spleens were recovered from surviving mice at day 21, (LF fusion protein (♦ n=7), peptide pool (▲ n=6) and naïve mice (■ n=2)), and a mean bacterial count per spleen determined following culture of *B. anthracis* for 24 hours. There was no significant difference between groups in term of cfu/spleen (mean ± SEM). This work was carried out in collaboration with DSTL, Porton Down.

The immunised HLA-DQ8 transgenics were fully protected against challenge with 10^6 cfu *B.anthracis*. Following Kaplan Meier estimation the naïve, unimmunised group showed a significantly lower survival rate than either the pooled peptde ($p>0.01$) or the fusion protein ($p>0.01$) immunised groups (Figure 7.2.2A). The bacterial loads recovered from the spleens of surviving mice showed that, although the immunised mice appeared to have cleared the infection more successfully than the naïve mice, there was no significant difference between groups in terms of bacterial burden (Figure 7.2.2B). The high degree of protection against anthrax infection observed in both the immunised groups indicated, not only that the LF fusion protein was capable of conferring the same protective affect as the individual peptides, but also validated the immunoprotective effects of the epitopes identified within this study. Further work to attribute this protection to a specific response, through both cellular and humoral markers, would be of merit in determining the potential of the LF fusion protein as a future anthrax vaccine candidate.

7.3 LF MHC peptide blockade

Although the extensive linkage disequilibrium across the β-chain alleles of DRB1*1501, DRB5*0101 and DQB1*0602 has complicated attempts to to identify the primary MS susceptibility gene in the HLA region, recent studies have implicated the HLA-DR loci as a definitive risk factor (Barcellos *et al* 2002; Lincoln *et al* 2005; Oksenberg *et al* 2004). This is consistent not only with the T cell mediated autoimmunity against the self-antigen MBP_{85-99}, but also with the action of therapeutic peptides which have a high binding affinity for DR but not DQ molecules (Fridkis-Hareli *et al* 1994). This made the Altmann lab 'Line 7' mice which express the HLA-DR15 allele, particularly valuable in investigating immunotherapeutic agents which act to prevent or treat the inflammatory demyelination seen in the mouse model of MS.

The action of competitor epitopes which modulate the activation of the T cells by blocking the binding of self-reactive peptides, such as MBP_{85-99}, to MHC class II molecules, has been investigated in previous studies (Adorini *et al* 1991b; Adorini *et al* 1991a; Adorini *et al* 1988b; Babbitt *et al* 1986). The blockade experiments described in this thesis explored the possibility of using a peptide with high HLA binding affinity properties, such as $LF_{457-476}$, as a competitor peptide blocking the binding and presentation of self-antigens. It was apparent that this model was capable both *in vitro* and *in vivo* of reducing the inflammatory CD4+ T cell response to self-reactive peptides. Furthermore an *in vivo* mode of action was proposed which involved the reduced infiltration of the spinal cord tissues with immunopathogenic T cells of a Th1 lineage.

However, the increased time to onset of clinical symptoms and reduction in the severity of spontaneous paralysis, along with associated immune markers, was only observed in mice which received the $LF_{457-476}$ competitor peptide prior to the onset of the disease. In mice which received the peptide within the context of a classic EAE induction, after self-peptide has been administered, the disease progressed unabated and immune markers generally associated with the amelioration of disease were not observed. This suggests that the competitor peptide model would face serious challenges in the clinical setting, as patients are typically only diagnosed following the onset of symptoms, at which point tolerance has already been broken. Furthermore, the HLA-DR15 serotype is most closely associated with MS in Northern European Caucasian populations (Hillert *et al* 1994; Hillert 1994; Olerup and Hillert 1991), whereas other populations displayed a higher risk factors associated with other HLA alleles (Dekker *et al* 1993; Serjeantson *et al* 1992). This limits the applicability of this peptide inhibitor to a subset of MS patients within the general population or necessitates the development and assessment of competitor peptides which bind strongly across a range of HLA alleles.

7.4 Future work

- The epitope mapping described within this thesis identifed epitopes which are presented across a broad range of HLA class II alleles, as well as those which showed more conservative HLA binding properties. Considering that the majority of individuals are heterozygous in the HLA class II region, there is a high likehood of developing epitope vaccine strategies which are relevant to a wide number of individuals within the general population. However, it must be recognised that any epitope vaccine which is based upon genetic characteristics must cover individuals from different ethnic backgrounds in which a variety of different HLA alleles are prevalent. Therefore it would be prudent to analyse a wider range of HLA allele restricted responses to the epitopes already identified.

- Infection-specific cryptic epitopes have been identified in the human response to LF, the possibility that infection skews the CD4+ T cell epitopes that are preferentially processed or recognized by specific HLA alleles should be investigated further in the HLA transgenic strains following exposure to anthrax infection. The findings open several vistas of possible fundamental immunology and translational relevance. If epitopes generated during infection are different to those seen after protein immunisation, the premise must be that B. anthracis gene products are able to subvert or modulate antigen processing in fairly subtle ways. It will be important to characterise these pathways and define the gene products

and targets involved. Furthermore, if epitope recognition can be infection-context dependent, this will need to be taken into account in designing T cell vaccines for this or other diseases.

- The existence of cryptic epitopes within regions which contain no immunodominant epitopes indicates that fine mapping of the entire LF protein would uncover further cryptic epitopes in all domains.

- A substantial number of cryptic heteroclitic epitopes, which are capable of eliciting a T cell response to the peptide in the context of the whole protein, were identified within this study. In depth mapping of the cryptic epitopes, with immunisation as individual peptides, rather than as pools, could help pinpoint the exact epitopes capable of inducing this response.

- T cell responses to the signal sequence of LF were not analysed, as it is generally accepted that since the mature LF protein would be expected to lack this sequence it is of limited immunological relvance within the host. However, parallel work by our collaborators in the Robinson lab has unexpectedly identified HLA-DR1 restricted epitopes in the signal sequence of the *Y. pestis* protein Caf1 (Musson *et al* 2010). This suggests that the signal sequence is available for antigen presentation following cleavage from the mature protein, and while it remains unclear whether this is a common phenomenon among bacterial toxins, there is an

obvious benefit in determining whether the signal sequence of LF contains any previously overlooked CD4+ T cell epitopes.

- It was not possible within the scope of this study to delineate the minimal epitope sequence required to elicit a T cell response. It would be interesting to establish whether overlapping peptides, such as LF457-476 and LF467-486 represented separate or concurrent epitopes.

- The proof of principle experiments which proved that the LF fusion protein was capable of conferring protection against live B. anthracis challenge must be expanded by further work to analyse the associated cellular markers such as the magnitude of the IFNγ producing CD4+ T cell response as well as the humoral markers, such as the levels of toxin neutralising antibodies and LF specific IgG induced. The next generation of anthrax vaccines should also be designed to cover a wide range of HLA alleles, therefore any future LF fusion protein must be validated in a number of HLA transgenic strains.

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