IMMUNOGENICITY AND IMMUNE FUNCTION OF THE CELLULAR PRION PROTEIN

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Jeremy Darryl Isaacs

MA (Cantab) MB BS (Lond) MRCP (UK)

Department of Neurodegenerative Disease

Institute of Neurology

University College London

Supervisors:

Dr Graham S Jackson

Professor Daniel M Altmann

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

DECLARATION

I, Jeremy Darryl Isaacs, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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THESIS ABSTRACT

Prion protein (PrP) is the only factor known to be essential in the pathogenesis of the transmissible spongiform encephalopathies (TSEs) or prion diseases. The cellular isoform (PrP^{C}), a GPI-anchored sialoglycoprotein of unknown function, has an identical primary structure to the disease-associated conformer (PrP^{Sc}). Thus, animals are tolerant to PrP^{Sc} and TSEs do not trigger a classical immune response. Vaccine development for human TSEs requires elucidation of the immunodominant human T cell epitopes within PrP. Further, successful immunotherapy requires that the function of PrP^{C} in lymphocytes is understood, as therapeutic targeting of prion protein risks interfering with immune function.

Peripheral blood leukocytes from healthy donors were cultured with PrP sequence peptides to elicit proliferative and cytokine responses. Responses were seen to peptides clustered around the position 129 polymorphism and the C-terminus, in accordance with a predictive algorithm. The substitution of methionine by valine at position 129 altered both epitope immunogenicity and cytokine profile.

Studies in murine T cell activation models demonstrated transcriptional and late surface protein upregulation of PrP^{C}. Memory T cells expressed higher PrP^{C} levels than naïve cells and there was also a strong correlation at both protein and transcriptional levels between expression of PrP^{C} and the regulatory T cell marker, Foxp3. Embryonic deletion of *Prnp* did not lead to deficits in T cell conjugation, proliferation or cytokine production, although memory cell numbers were slightly reduced. In $PrP^{-/-}$ mice regulatory T cells developed normally but may have enhanced suppressor function. However, neither PrP ablation nor anti-PrP monoclonal antibodies altered the phenotype of T cell mediated autoimmune disease.

These findings demonstrate that tolerance to PrP is not complete in humans and raise the prospect of generating protective immunity through vaccination. However, PrP^{C} is a potentially important memory, regulatory and T cell activation antigen, therapeutic disruption of which may precipitate immunopathology.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Aβ	β-Amyloid
AD	Alzheimer Disease
ADEM	Acute Disseminated Encephalomyelitis
AICD	Activation Induced Cell Death
APC	Antigen Presenting Cell
APP	Amyloid Precursor Protein
bp	base pairs
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CFDA	Carboxyfluorescein Diacetate
CHX	Cycloheximide
CJD	Creutzfeldt Jakob Disease
Con A	Concanavalin A
CNS	Central Nervous System
c.p.m.	Counts per minute
CSF	Cerebrospinal Fluid
CWD	Chronic Wasting Disease
DAF	Decay Accelerating Factor
DC	Dendritic Cell
DMSO	Dimethyl Sulphoxide
DP	Double Positive
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic Acid
EEG	Electroencephalogram
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal Calf Serum
FDC	Follicular Dendritic Cell
FFI	Fatal Familial Insomnia
GPI	Glycophosphatidylinositol
GSS	Gerstmann Straussler Scheinker
HEL	Hen Egg Lysosyme
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HSC	Haematopoietic Stem Cell
i/c	intracerebral
IFN	Interferon
IL	Interleukin
i/p	intraperitoneal
LAT	Linker for Activation of T cells
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
mRNA	Messenger RNA
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis

MBP	Myelin Basic Protein
NK	Natural Killer
NMR	Nuclear Magnetic Resonance
OPRI	Octapeptide Repeat Insertion
ORF	Open Reading Frame
OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
PK	Proteinase K
PLP	Proteolipid Protein
PMA	Phorbol Myristate Acetate
PML	Progressive Multifocal Leukoencephalopathy
Prnp	Gene encoding murine PrP
PRNP	Gene encoding human PrP
PrP	Prion Protein
PrP^{C}	Cellular isoform of PrP
PrP^{Sc}	Scrapie (disease associated) isoform of PrP
recPrP	Recombinant PrP
RT-PCR	Reverse Transcriptase PCR
sCJD	Sporadic CJD
SEA	Staphylococcal Enterotoxin A
SEB	Staphylococcal Enterotoxin B
SP	Single Positive
TCR	T Cell Receptor
tg	transgenic
TGF	Transforming Growth Factor
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
TSE	Transmissible Spongiform Encephalopathy
UK	United Kingdom
UV	Ultra Violet
vCJD	variant CJD
VSV	Vesicular Stomatitis Virus
WHO	World Health Organisation

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CHAPTER 1 INTRODUCTION

The prion diseases or transmissible spongiform encephalopathies (TSEs) are invariably fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD) and kuru in humans, scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk and bovine spongiform encephalopathy (BSE) in cattle (Collinge, 2001).

The central player in the pathogenesis of prion disease is prion protein (PrP), a highly conserved 32-kDa GPI-anchored sialoglycoprotein expressed in neurons, glia and a variety of non-neuronal tissues. According to the protein-only or prion hypothesis (Griffith, 1967), the key event in TSE aetiology is the conversion of normal, cellular PrP (denoted PrP^{C}) to an alternate conformation (PrP^{Sc}) (Prusiner, 1982) characterised by increased β-sheet content, resistance to proteases and detergent insolubility (McKinley et al., 1983). This is an entirely post-translational modification in which the primary sequence of the protein is not altered (Stahl et al., 1993). The resultant pathological agent, termed the "prion", or "protein-only infectious particle" is proposed to consist entirely or largely of PrP^{Sc}.

Prions can be generated sporadically, as a result of an as yet uncharacterised stochastic event causing PrP^{C} to PrP^{Sc} conversion, or by dominant mutations in the gene encoding PrP (*PRNP* in humans), producing mutant PrP^{C} that is hypothesised to more readily undergo spontaneous conversion to PrP^{Sc}. However, uniquely among neurodegenerative disorders, prion disease can also be caused through infection with

exogenous prions; the latter inducing host-encoded PrP^C to undergo conformational change, via seeding or template-directed refolding (Jackson and Clarke, 2000).

Although this last process occurs most efficiently within species, for example transmission of sheep scrapie, or kuru due to human endocannibalism, it has become increasingly clear that inter-species transmission of prions is possible and poses a genuine health risk to humans. This has been most dramatically demonstrated in the UK where BSE contamination of beef and beef products has caused the subsequent death of ~160 people from variant CJD (vCJD) (Hill et al., 1997;Bruce et al., 1997;Collinge et al., 1996).

The relationship between prion infection and the immune system is complex. The lack of a clear immune response in prion disease is assumed to be due to tolerance to PrP^{Sc}. Further, the immune system actually contributes to pathogenesis by amplifying prion "load" in lymphoid compartments thereby facilitating efficient neuroinvasion (reviewed in Aguzzi, 2003). This process is at least partly dependent on expression of PrP^C by immune cells (Brown et al., 1999a).

Because prion diseases arise by structural changes in a single protein, PrP is an attractive target for therapeutic intervention. This could be achieved using compounds that specifically bind PrP^C or PrP^{Sc} or by gene silencing of *PRNP*. Alternatively, host immune mechanisms could be adapted to block prion conversion or clear abnormal protein. However, because the constitutive function of PrP^C in the lymphoid system, as in the CNS, remains obscure it is not yet clear how therapies directly or indirectly targeting PrP^C will affect immune function. Further, uncovering the role of PrP^C in the immune system may provide novel insights into the peripheral pathogenesis of prion disease and immune function generally.

In the work presented in this thesis, I have attempted to define the immunogenic regions of human PrP and thus consider the prospects for active immunotherapy and prophylaxis against human prion disease. In tandem I have sought to characterise the expression and function of PrP^C within the immune system itself in order to predict possible side effects of anti-prion therapeutics and to evaluate PrP^C as a therapeutic target in immune-mediated diseases.

1.1 Clinical features of human prion diseases

Human prion diseases can be categorised into those that occur sporadically, those due to dominant mutations in *PRNP*, and those due to infection by exogenous prions. Although these classes have distinct aetiologies, their clinical features show a degree of overlap; for example some *PRNP* mutations cause diseases indistinguishable from classical sporadic CJD, while the inherited prion disease fatal familial insomnia can also occur sporadically. Phenotypic heterogeneity is to a certain extent a reflection of differences in the distribution of neuropathology. However, all prion diseases share a common set of neuropathological features: neuronal loss and vacuolation, spongiform degeneration, astrogliosis, and the presence of abnormal PrP^{Sc} as plaques or fine granular staining on immunohistochemistry, as well as proteinase K (PK)-resistant bands on Western blot of brain homogenate (Ironside, 1998).

1.1.1 Sporadic prion diseases

1.1.1.1 Classical CJD

Sporadic CJD (sCJD) occurs with a uniform worldwide incidence of approximately one case per million per year. Classical forms of sporadic CJD present as a rapidly progressive syndrome characterised by dementia, ataxia and myoclonus (Zerr and

Poser, 2002). The average age of onset is 60 years and average time from onset to death is 5 months (Johnson, 2005). Additional features, such as pyramidal and extra-pyramidal signs, may also be present. The diagnosis is usually made by a combination of clinical assessment, EEG and CSF examination. EEG shows characteristic "pseudoperiodic" complexes in the majority of patients (Steinhoff et al., 2004). CSF analysis is usually normal except for raised levels of certain neuronal and glial proteins. In particular, a raised 14-3-3 is highly predictive of sCJD given an appropriate clinical presentation (Zerr et al., 2000). Neuroimaging may show enhanced signal in the caudate and putamen but is often non-specific (Tschampa et al., 2005). In rare circumstances a brain biopsy is required to confirm the diagnosis.

1.1.1.2 Variants of sporadic CJD

sCJD can give rise to a number of atypical presentations. Disturbed visual perception or cortical blindness is the presenting feature of the "Heidenhain" variant, which accounts for 4-20% of cases of sporadic CJD (Kropp et al., 1999;Rabinovici et al., 2006;Cooper et al., 2005). More rarely, sCJD may present as a phenotype indistinguishable from fatal familial insomnia. This syndrome has in the past been labelled as the "thalamic variant" of CJD, but is now referred to as sporadic fatal insomnia (Gambetti et al., 2003). It accounts for approximately 2% of cases of sporadic CJD.

1.1.2 Inherited forms of prion disease

These are caused by dominant mutations in the prion gene *PRNP*, on chromosome 20. Over 30 different mutations have been reported, the majority being either point mutations or expansions in the N-terminal octapeptide repeat region (OPRI). Cases of

inherited prion disease are rare; in the UK only ~ 85 deaths from inherited forms of prion disease have been reported since 1990 (http://www.cjd.ed.ac.uk/figures.htm).

A remarkable feature of inherited prion diseases is the wide variety of phenotypes that result from mutations in the same gene. Many point mutations and some OPRI cases are associated with syndrome identical or similar to sCJD. However, notable phenotypic exceptions to this are Gerstmann Straussler Scheinker syndrome, most commonly caused by the P102L mutation, which characteristically manifests as a slowly progressive cerebellar ataxia, and fatal familial insomnia (FFI), initially described in association with the D178N mutation. FFI presents with sleep disturbance, stuporous episodes with hallucinations, autonomic dysfunction and seizures. Features generally associated with sCJD, such as myoclonus, may also be present. Neuropathological examination reveals distinctive neuronal loss and astrogliosis in the thalamus (Gambetti et al., 2003).

Interestingly, the correlation between genotype and phenotype is not straightforward with considerable diversity in age of onset and clinical features within pedigrees (Mead, 2006). In particular, the codon 129 polymorphism may influence the phenotype. With respect to the D178N mutation, where methionine is encoded at position 129 of the mutant allele this may cause FFI whereas the identical mutation on a 129V allele is more likely to cause a classical CJD phenotype (Goldfarb et al., 1992). However, this association is not absolute and other as yet unidentified factors also contribute to determining the clinical phenotype. For example a proportion of the phenotypic variation observed in cases of P102L GSS may be due to variable involvement of the wild-type *PRNP* allele (Wadsworth et al., 2006).

1.1.3 Infectious forms of prion disease

1.1.3.1 Kuru

The archetypal infectious human prion disease is kuru, which is seen solely among the Fore tribes of Papua New Guinea. The disease probably originated as a case of sporadic CJD, which was then serially propagated by endocannabilism. The traditional practice of the Fore was to consume recently deceased members of the tribe at funeral feasts. The entire body was consumed at such events, irrespective of the mode of death. In general, male members of the tribe ate the muscle meat, while women and children consumed neural tissue. This led to an epidemic of kuru across a range of villages in which women and children were preferentially affected. During the epidemic, kuru was the leading cause of death among women in some affected villages. The disease was observed by missionaries and subsequently by the Australian colonial government in the 1950s. The practice of endocannibalism was linked to the epidemic and actively discouraged by the administration from the 1950s. This led to a sharp decline in the incidence of the disease. Of note, however, occasional cases were still occurring among the Fore in the late 1990s and early 2000s, at least 40-50 years after the cessation of cannibalism (Collinge et al., 2006).

The clinical syndrome of kuru is characterised by progressive ataxia leading to paralysis, progressive obtundation, coma and death. At post mortem, florid PrP^{Sc} containing amyloid plaques referred to as "kuru plaques" are seen in the brain in the majority of cases (Collins et al., 2001). The extraneural tissue distribution of PrP^{Sc} and infectivity in kuru has yet to be fully elucidated.

The average incubation period of kuru was about 12 years, although age stratified incidence is bimodal, with a peak in the second decade of life among 129MMs and a

peak in middle age among 129MVs (Cervenakova et al., 1999) (J Beck, unpublished observations). Thus, incubation time is directly influenced by the *PRNP* codon 129 polymorphism. This is significant because affected 129MM women usually died before or during their reproductive years, whereas 129MV women usually succumbed to kuru only in middle age. In addition, 129MVs were generally less susceptible to kuru, with several female "long survivors" still alive decades after multiple exposures to prion infected material. Thus, the impact of the codon 129 polymorphism on disease susceptibility was so strong that kuru significantly altered the prevalence of the two alleles in the population. This is the strongest example of balancing selection ever documented in human history and has led to the proposal that the unexpectedly high global prevalence of the 129V allele is due similar epidemics of prion disease in many other populations in human pre-history to which MV heterozygotes would have been relatively resistant (Mead et al., 2003). However, this study remains contentious and its interpretation of the worldwide frequencies of codon 129 genotypes has been challenged (Soldevila et al., 2006;Kreitman and Di Rienzo, 2004).

1.1.3.2 Iatrogenic CJD

Until the appearance of variant CJD, the principal mode of prion infection seen in developed countries was iatrogenic. In a now infamous mishap, deep brain electrodes unwittingly used on a patient with CJD subsequently infected two further individuals despite robust conventional sterilisation between each procedure. In a "proof of principle" experiment, the electrodes were ultimately used to transmit CJD to a chimpanzee (Bernoulli et al., 1977). Further outbreaks of iatrogenic CJD have been caused by use of infected dura mater grafts and cadaveric growth hormone with

occasional cases due to contaminated neurosurgical instruments, corneal transplants and gonadatropin (Brown et al., 2000).

The clinical syndrome of iatrogenic CJD depends on the strain of the inoculum and the route of infection. Prions delivered straight to the brain give rise to a classical CJD phenotype usually within 2 years of exposure. Peripheral infection, such as with growth hormone, typically gives rise to a longer incubation period and is associated with a phenotype closer to that of GSS.

1.1.3.3 Vertical transmission

The age distribution of sporadic CJD means that pregnant women will only very rarely be affected. However, two cases of iatrogenic CJD and one of sCJD presenting during pregnancy have been documented (Bernoulli et al., 1977;Lane et al., 1994;Tamai et al., 1992). In no cases has CJD transmitted to the child (Lane et al., 1994). This is in accordance with data from non-human primates experimentally infected with prions, in which vertical transmission did not occur (Amyx et al., 1981). Maternal transmission has long been proposed as a mechanism underlying the propagation of scrapie in affected sheep herds. Despite the demonstration of PrP^{Sc} in ovine placenta (Race et al., 1998), direct evidence for transplacental transmission is lacking. Similarly, there is no direct evidence that BSE is transmitted from heffer to calf. Studies in mice suggest that although vertical transmission is technically feasible, it occurs as low efficiency (Castilla et al., 2005) likely at least in part due to the inherent resistance of immature mice to prion infection (Ierna et al., 2006). The wider tissue distribution of PrP^{Sc} and younger age range in vCJD has raised concerns that this disease may transmit vertically. However, no such cases have yet been reported, although an unusual type of cerebral palsy has been documented in a child

born to a woman with symptomatic vCJD during pregnancy (Isaacs et al., manuscript in preparation).

1.1.3.4 Variant CJD

The massive epidemic of BSE in UK cattle, estimated to have involved 2 million bovines, led to fears that bovine prions might have entered the food chain and infected humans. These concerns were justified when, in the mid-1990s, a novel prion disease termed variant CJD (vCJD) was identified in young adults (Will et al., 1996). This disease has to date affected approximately 190 people worldwide. The clinical and pathological features of variant CJD are distinct from classical CJD, supporting the notion that it is a novel entity with a strong temporal relationship with, and physicochemical similarities, to BSE (Will et al., 1996;Collinge et al., 1996).

Variant CJD is characterised by a neuropsychiatric prodrome, with prominent depression and other psychological features. Indeed, many patients initially come under the purview of psychiatrists. After several months, progressive cognitive decline ensues, accompanied by ataxia and often additional features such as chorea, pyramidal signs and severe pain (Spencer et al., 2002). The EEG in vCJD does not typically show periodic complexes, and CSF markers used in the diagnosis of sporadic CJD are not reliably elevated. MRI is, however, a useful adjunct to clinical diagnosis; the "pulvinar sign" being present in the majority of individuals (Zeidler et al., 2000) although this finding is not entirely specific to vCJD.

An important feature of vCJD is that PrP^{Sc} accumulates in peripheral tissues, notably those of the lymphoreticular system such as tonsil and appendix (Wadsworth et al., 2001). This is most likely a reflection of the presumed peripheral route of entry and

the tissue tropism of the particular prion strain that causes vCJD. The presence of abnormal PrP has not been demonstrated to have a clinically deleterious effect on the function of these tissues, which remain grossly anatomically normal. PrP^{Sc} can, however, be detected by high sensitivity immunoblot in tonsils from vCJD patients with 100% sensitivity and specificity (Hill et al., 1999;Wadsworth et al., 2001). Proponents of tonsil biopsy argue that it should be included in the routine work up of suspected vCJD cases. However, this opinion is not universally held; the procedure is invasive, carries an operative risk and requires special infection control measures. The current WHO guidelines for diagnosis of vCJD do not afford tonsil biopsy the status of a "gold standard" diagnostic test; this can still only be provided by brain biopsy (www.who.int/bloodproducts/TSE-manual2003.pdf). In practice, however, a patient would be unlikely to have a brain biopsy to confirm a diagnosis of vCJD unless a tonsil biopsy was unavailable or contraindicated.

In rodent models where prions are delivered via a peripheral route (in practise almost always intra-peritoneal or oral) PrP^{Sc} can be detected in the lymphoreticular system several weeks before neuroinvasion occurs. The speed with which prions disseminate around the body following BSE infection in humans has not yet been determined and it is unknown how long prior to the onset of clinical symptoms PrP^{Sc} can be detected in peripheral tissues. Large series of tonsil and appendix specimens taken at routine surgical procedures from otherwise healthy people have been anonymously screened for the presence of PrP^{Sc} (Hilton et al., 2004;Frosh et al., 2004). These have revealed only very small numbers of potentially infected individuals. However, there are methodological limitations to the present studies, and a larger scale screen has been commissioned by the UK Department of Health.

If large numbers of people are harbouring sub-clinical vCJD prions in peripheral tissues, this has two important implications:

1) Further clinical cases of vCJD might occur. Experience from the kuru epidemic indicates that the average incubation time of oral human-to-human transmission is of the order of 10 years (with an upper limit of 40 years or longer (Collinge et al., 2006)). However, experimental models have demonstrated that in the presence of a species barrier, incubation time is significantly prolonged. Thus the first 160 cases of vCJD might merely be drawn from a particularly susceptible section of the population, with larger epidemics to follow.

2) Secondary transmission of vCJD may occur via blood transfusion, surgical and endoscopic instruments, organ donation and possibly through vertical transmission. Indeed, three cases of blood transfusion-related transmission have already been documented (Peden et al., 2004;Llewelyn et al., 2004)(Wroe et al., submitted).

Very little is known about factors governing susceptibility to vCJD. A small cluster was detected in Leicestershire and there is a trend towards increasing incidence in the northern UK, both perhaps explained by regional patterns of meat consumption (Cousens et al., 2001). The only definite association is *PRNP* codon 129 genotype. All cases of clinical vCJD thus far have occurred in individuals drawn from the ~ 40% of the UK population that is 129 methionine homozygous. It remains unclear whether any 129MV or VV individuals will be affected by primary infection with BSE, although two appendix specimens found by Hilton and colleagues to contain PrP^{Sc} were recently reported to have come from 129VV individuals (Ironside et al., 2006).

With respect to secondary transmission of vCJD, possession of one or more PrP 129V alleles may offer less protection than against BSE. One of the cases of transfusion-related infection occurred in a 129MV heterozygote (Peden et al., 2004). However, this individual died of unrelated causes, and PrP^{Sc} was not detected in the CNS. Thus, it remains unknown whether any peripherally infected 129MV or 129VV individuals (~ 50% and 10% of the UK population respectively) will progress to clinical disease within their natural lifespan.

Studies in transgenic mice homozygous for 129V or heterozygous for 129MV human PrP demonstrate that the presence of 129V PrP has a powerful dominant negative effect on susceptibility to BSE (Wadsworth et al., 2004;Asante et al., 2006;Bishop et al., 2006). In contrast, vCJD prions are able to cause neuropathology in 129VV and 129MV hosts. However, in all these experiments disease was initiated by direct intracerebral inoculation of vCJD brain homogenate. Thus, the modifying effects of codon 129 genotype on the peripheral phase of BSE or vCJD infection have not yet been characterised.

A further consideration pertaining to individuals with one or two 129V alleles is whether primary BSE or secondary vCJD infection will propagate as a distinct strain on this genetic background that might not immediately be recognised as vCJD. Indeed, there has been a gradual rise in the number of cases of sCJD in the UK and some other countries in parallel with the emergence of vCJD (Glatzel et al., 2002) (Ladogana et al., 2005). Studies of vCJD infection in transgenic mice expressing human 129V PrP alone or both human 129V and 129M PrP suggest that a distinct neuropathological and possibly clinical entity may occur on these genetic backgrounds (Wadsworth et al., 2004;Asante et al., 2006). However, other

researchers have not replicated this finding (Bishop et al., 2006). Current controversies notwithstanding, it remains prudent to assume that vCJD may induce a modified clinical phenotype in 129MV heterozygotes and 129VV homozygotes. However, despite some differences in PrP^{Sc} strain type, the clinical phenotype of sCJD is not significantly altered by *PRNP* genotype nor is it clear that 129MV and VV individuals with kuru developed a distinct neurological syndrome.

1.2 Host immune responses to prion infection

The major neuropathological hallmarks of prion disease are spongiform degeneration, neuronal loss, astrogliosis and extracellular PrP^{Sc} deposition in the form of amyloid plaques. These severe degenerative changes occur without triggering a classical immune response, either within the CNS or in the periphery. The response of the brain to prion infection has been characterised instead as one of "atypical inflammation", dominated by an unusual pattern of microglial activation, pro-inflammatory signalling and cytokine release (Perry et al., 2002)

1.2.1 Microglial activation

Microglial activation is one of the earliest responses to prion infection. Microglia are attracted to sites of PrP^{Sc} inoculation within a few days via a CCR-5 dependent mechanism (Marella and Chabry, 2004). Glial-derived markers such as L-selectin and myeloid recruitment factors are elevated in mouse scrapie within a few weeks of intracerebral prion inoculation (Lu et al., 2004). Although microglial activation can be detected prior to overt neuronal death (Williams et al., 1997) it appears that it is nonetheless an inflammatory reaction to early synaptic degeneration (Cunningham et al., 2003) rather than a protective response driven by recognition of PrP^{Sc}. Later in

the pre-clinical course a number of immune related genes are upregulated, but again this is likely to contribute further towards pathology rather than being a beneficial immune response by the CNS (Xiang et al., 2004).

1.2.2 Cytokine profiles in prion disease

Pro-inflammatory cytokines including TNF-α and IL-1β have been raised in CSF from two series of patients with CJD (Sharief et al., 1999;Van Everbroeck et al., 2002), although only IL-8 was significantly increased in a recent study (Stoeck et al., 2005b). Similarly, they are upregulated in some (Campbell et al., 1994;Kordek et al., 1996;Williams et al., 1997;Kim et al., 1999;Schultz et al., 2004), but not all (Walsh et al., 2001;Cunningham et al., 2002) experimental models. With the exception of IL-1β, these are probably late responses to neurodegeneration (Baker et al., 1999;Brown et al., 2003). Thus, although an acute phase response can be demonstrated in prion infected brain (Cunningham et al., 2005) this may be mediated by pathways independent of cytokine release. Further, mice deficient in pro-inflammatory cytokines or their receptors either have normal (Mabbott et al., 2000) or modestly prolonged (Schultz et al., 2004) incubation times following intracerebral inoculation, suggesting that cytokine release is generally an ineffectual or harmful response to prion infection.

The major anti-inflammatory signal that has been detected in scrapie-infected brain is TGF-β1, although this is also not upregulated until late in the disease (Tashiro et al., 1998;Baker et al., 1999;Cunningham et al., 2002;Brown et al., 2003), and may in fact contribute towards pathology (Cunningham et al., 2002). Recently, reduced levels of TGF-β2 have been detected in CSF from CJD patients (Stoeck et al., 2005b). Interestingly, IL-$10^{-/-}$ mice have accelerated disease following intracerebral or

intraperitoneal infection, suggesting that the brain attempts an anti-inflammatory response which can defer but not eliminate the neurotoxic effects of prion infection (Thackray et al., 2004a). Raised IL-10 levels have been detected in the CSF of patients with sporadic CJD (Stoeck et al., 2005a) but not in the brain in murine scrapie (Cunningham et al., 2002;Brown et al., 2003).

1.2.3 Complement components in prion disease

Complement protein C1q has been reported to coat PrP^{Sc} amyloid plaques (Kovacs et al., 2004) (reviewed in Mabbott, 2004) and be upregulated in murine scrapie (Dandoy-Dron et al., 1998;Dandoy-Dron et al., 2000;Riemer et al., 2000;Brown et al., 2004), raising the intriguing question as to whether the innate immune system can recognise conformational epitopes produced by polymerized self-proteins. Indeed, C3-inhibited, APP transgenic mice have acceleration of Alzheimer-like pathology (Wyss-Coray and Mucke, 2002). However, whether complement actually has a protective role in prion disease is unclear. Amyloid plaques are not an invariable feature of TSE neuropathology and may not in themselves be neurotoxic (Jeffrey et al., 2004). Furthermore, neither $C1q^{-/-}$ mice nor those unable to form the terminal membrane attack complex have accelerated disease following intracerebral inoculation (Klein et al., 2001;Mabbott et al., 2001;Mabbott and Bruce, 2004). Moreover, there is no evidence that complement is activated by the presence of PrP^{Sc} in the lymphoid system; rather, it appears that complement proteins including C1q and C3b assist in the process of peripheral prion replication (Klein et al., 2001;Mabbott et al., 2001).

1.2.4 Cellular immunity

Cerebrospinal fluid from patients with CJD is typically acellular (Jacobi et al., 2005), suggesting that neutrophil or lymphocyte ingress to the CNS is not a major feature of the disease. There have been reports of T cell infiltration in the brain parenchyma in some experimental prion diseases and patients with vCJD (Betmouni et al., 1996;Lewicki et al., 2003). However, this is most likely a non-specific effect of rapid neurodegeneration, microglial activation and increased CNS MHC class I and II (Duguid and Trzepacz, 1993) and chemokine receptor expression (Burwinkel et al., 2004;Lee et al., 2005). T cells isolated from the brains of mice with scrapie did not secrete IFN-γ or TNF-α or demonstrate cytotoxic activity when cultured with APCs pulsed with PrP sequence peptides (Lewicki et al., 2003), suggesting that even if these infiltrates do represent expansion of specific anti-PrP clonal populations, they have been rendered anergic.

1.2.5 Lymphoreticular phase of prion disease

In variant CJD, sheep scrapie and most rodent models, prions accumulate in the secondary lymphoid organs, typically within follicular dendritic cells (FDCs) of the spleen and lymph nodes (Kitamoto et al., 1991;McBride et al., 1992;Bruce et al., 2001;Wadsworth et al., 2001). Although alterations in splenic morphology have been detected (McGovern et al., 2004), splenic lymphocyte numbers and distribution are not altered (Davies et al., 2004), local macrophages are not activated and no systemic inflammatory response is generated (Xiang et al., 2004). A neurotoxic fragment of PrP (a peptide consisting of residues 106-126) can elicit in vitro changes in leucocyte migration, calcium levels and membrane viscosity (Diomede et al., 1996;Le et al., 2001) and activate dendritic cells (Bacot et al., 2003), but there is no evidence that

PrP^{Sc} produces such effects *in vivo*. Prions invading via the oral route, as in BSE infection of humans, are able to survive proteolytic degradation in the gut and pass through the intestinal lumen seemingly without immune hindrance. Where in the gut prion entry occurs remains unclear, with evidence in favour of elements within Peyer's patches such as lymphocytes and FDCs (Bergstrom et al., 2006;Beekes and McBride, 2000;Heggebo et al., 2000;Heggebo et al., 2002;Herzog et al., 2004) or M cells (Heppner et al., 2001a;Prinz et al., 2003b) but also for the 37/67 kDa Laminin receptor on enterocytes (Morel et al., 2005). Whichever is the case, the relatively low efficiency of oral infection in experimental models suggests that natural defences against prions may exist in the gut (Prinz et al., 2003b).

In experimental prion disease generated by intraperitoneal (i/p) injection of prions, PrP^{Sc} cannot be detected between days 1 and 4 post-inoculation (Beringue et al., 2000), and infectivity is lost in spleens of $PrP^{-/-}$ mice within a few weeks of i/p inoculation (Blättler et al., 1997;Kaeser et al., 2001), suggesting that host mechanisms exist which are capable of degrading PrP^{Sc}, albeit with less efficiency than the process by which PrP^{Sc} converts endogenous PrP^{C}. However the nature of these processes remains unknown, although an anti-PrP antibody response is not thought to be the mechanism by which $PrP^{-/-}$ mice clear a prion inoculum (Kaeser et al., 2001). Splenic tingible body macrophages may be able to ingest PrP^{Sc} (Jeffrey et al., 2000) and depleting macrophages led to a transient increase in PrP^{Sc} levels in the spleen (Beringue et al., 2000). It has been reported that myeloid dendritic cells can degrade PrP^{Sc} when co-cultured with an infected neuronal cell line (Luhr et al., 2002), possibly via cysteine proteases (Luhr et al., 2004). However, it is unclear whether the net contribution of dendritic cells during the peripheral phase of prion infection is protective or contributory. Intestinal dendritic cells are able to take up PrP^{Sc} and may

transport it to lymphoid organs (Huang et al., 2002). Furthermore, $CD205^+$ DCs have been observed to migrate into the CNS from 120 days following i/p scrapie inoculation (Rosicarelli et al., 2005).

1.2.6 Failure of immune defence against prion infection

The failure of the adaptive immune response in prion infection is presumed to be due to sequence homology between PrP^{Sc} and PrP^{C} (Basler et al., 1986), whereby PrP^{Sc} is seen as self protein. Moreover, the structural changes underlying PrP^{C} to ^{Sc} conversion do not appear to generate any novel immunogenic B cell epitopes, as antibody production has never been detected in infected animals (Porter et al., 1973;Kasper et al., 1982). Rodent scrapie infection does not itself appear to downregulate cellular immune responses (Garfin et al., 1978;Kingsbury et al., 1981;Kasper et al., 1982) although PrP 106-126 induces migration of immature dendritic cells but arrests movement of mature DCs (Kaneider et al., 2003). T cells recognising peptides presented by MHC on the surface of immature DCs are rendered anergic (reviewed in Steinman et al., 2003), and this could contribute to the tolerising of T cells to PrP^{Sc}. Indeed, since there is not a clear correlation between thymic PrP^{C} expression and tolerance to PrP (Polymenidou et al., 2004), peripheral mechanisms maintaining tolerance to PrP^{C} and ^{Sc} may exist. Furthermore, although recombinant PrP can be degraded by the 20S proteasome, fewer fragments derive from the structured C terminus than from the unstructured N terminus (Tenzer et al., 2004). The protease-resistant core of PrP^{Sc} is largely C-terminal, suggesting that MHC presentation of products from this region will be particularly inefficient.

The lack of any effective immune response is reinforced by the observation that immunosuppressed individuals are not at increased risk of prion disease.

Furthermore, early animal studies indicated that activating the immune system, with mitogens or concomitant viral infection, actually enhances the efficiency of prion pathogenesis (Dickinson et al., 1978;Ehresmann and Hogan, 1986). Conversely, splenectomy prolongs incubation time (Kimberlin and Walker, 1989;Fraser and Dickinson, 1978). More recent work has demonstrated that peripheral prion accumulation is largely dependent on PrP-expressing splenic FDCs (Brown et al., 1999a;Montrasio et al., 2000), although following high dose i/p challenge non-stromal splenocytes and FDCs may harbour infectivity even if they do not express PrP^{C} (Kaeser et al., 2001). Thus, the relative resistance of neonatal mice to peripheral prion infection is most likely due to a combination of absent FDC PrP^{C} expression and lack of lymphoid follicular differentiation during the first 1-2 weeks of life (Ierna et al., 2006). FDC accumulation of prions in turn requires an intact B cell compartment (Klein et al., 1997), although this is dependent not on B cell expression of PrP, but of trophic factors that sustain FDC differentiation (Klein et al., 1998). The exact role of T cells in prion pathogenesis is unclear. They are not required for effective peripheral amplification in rodent models (Klein et al., 1997). However, whether under certain naturally occurring conditions they are able to effect an immune response and clear infection is not known.

PrP^{Sc} accumulates to high titres in organs undergoing chronic inflammation, although mice with organ-specific inflammation are not more susceptible to infection *per se* (Heikenwalder et al., 2005;Seeger et al., 2005). This has implications for secondary transmission of prions via blood products, surgical instruments and organ donation. This effect may be due to increased local expression of PrP^{C} in the affected organ or because inflammation influences levels of co-factors that facilitate peripheral PrP^{C} to ^{Sc} conversion, such as complement. However, perhaps more likely is that the

inflammation generated in the models used by Heikenwalder and co-workers induced differentiation of local FDCs from stromal precursors thus generating lymphofollicular foci of the kind in which PrP^{Sc} is known to accumulate in the spleen. Nonetheless, recent data suggest that when such forms of inflammation occur naturally they are also associated with increased PrP^{Sc} accumulation (Ligios et al., 2005).

Over-expressing PrP^{C} in T or B cells in mice otherwise deficient in PrP does not permit accumulation of infectivity in lymphoid organs (Raeber et al., 1999b;Montrasio et al., 2001). However, these experiments do not model the effects of varying lymphocyte PrP expression in a system that can fully support peripheral prion replication. Furthermore, lymphocyte PrP^{C} levels in humans are higher than in mouse models in which the roles of the various components of the immune system in prion propagation have been studied (Liu et al., 2001;Holada and Vostal, 2000;Li et al., 2001). Thus, it remains possible that increased PrP^{C} expression levels in B and T (or other) cells, due concomitant infection or inflammation, may influence susceptibility to prion infection in humans.

An additional hazard of concomitant infection is that this may activate tissue-resident macrophages and dendritic cells which may then "capture" and transport incoming prions to the spleen with greater efficiency (Marsh, 1981). Indeed, although PrP^{C} expression within lymphoreticular tissues is required for efficient peripheral replication, capture and transportation of prions from the site of entry to the spleen may be efficiently performed by mobile cells or microparticles irrespective of their own PrP expression status. Thus, priming of dendritic cells, macrophages or lymphocytes, or induction of increased exosome release, by simultaneous

inflammatory stimuli may drastically increase susceptibility following peripheral prion inoculation.

The extent to which an effective immune response may protect humans against prion infection is unknown. The major determinant of susceptibility to vCJD is methionine homozygosity at codon 129 of *PRNP*. However, since this accounts for ~ 40% of the UK population, other genetic and environmental factors are likely to be at play. Initial work suggesting that expression of HLA-DQ7 may be protective against vCJD (Jackson et al., 2001a) was not confirmed when repeated on a larger sample (Pepys et al., 2003). An HLA-mediated effect would most likely be dependent on MHC-restricted presentation of peptidic fragments of foreign or self PrP^{Sc}, and the extent to which the human immune system can do this is unknown. Although a small study in Japan suggested HLA-DQw3 as a susceptibility allele for sporadic CJD (Kuroda et al., 1986), this has not been replicated (Jackson et al., 2001a). In mice, early data suggesting a role for MHC alleles in determining incubation times (Kingsbury et al., 1983) was not replicated in later studies (Mohri and Tateishi, 1989;Lloyd et al., 2001;Stephenson et al., 2000;Manolakou et al., 2001).

1.2.7 Summary

In summary, there is considerable evidence that the host immune system is unable effectively to distinguish PrP^{Sc} from PrP^{C}, and that the principal response of the CNS to prion infection is a deleterious pro-inflammatory reaction to on–going neurodegeneration. Indeed, the specificity of this response is questionable as similar changes, including microglial activation, atypical cytokine profiles, complement activation and T cell infiltration, have all been detected in Alzheimer disease (reviewed in McGeer and McGeer, 2003). This is unsurprising given that

endogenously generated PrP^{Sc} has 100% self sequence and PrP sequence is highly conserved between mammals, making the triggering of a T or B cell response through recognition of foreign or host-generated PrP^{Sc} as non-self highly unlikely. This situation is compounded by the fact that there is almost certainly repeated exposure to animal PrP^{C} in the diets of omnivorous and carnivorous mammals, generating heightened tolerance. Furthermore, the resistance of PrP^{Sc} to proteolysis reduces the likelihood of peptidic fragments being presented to T cells by MHC class I or II. The tantalising prospect of studying prion pathogenesis in a system in which PrP is entirely foreign has not been realised as $PrP^{-/-}$ mice are completely unable to propagate the disease (Weissmann et al., 1993;Prusiner et al., 1993;Manson et al., 1994b).

1.3 Strategies in immunotherapy of prion disease

1.3.1 Stimulating innate immunity

There are conflicting data on whether pharmacological activation of the innate immune system is of benefit in prion disease. A promising result obtained by administration of CpG oligodeoxynucleotides to peripherally infected mice (Sethi et al., 2002) was most likely due to disruption of the splenic FDC differentiation necessary to support prion accumulation, rather than a specific anti-PrP effect. More worryingly, when given to mice for 20 days CpG oligodeoxynucleotides caused haemorraghic ascites and hepatic necrosis (Heikenwalder et al., 2004). Furthermore, Toll-deficient mice have normal incubation times following intraperitoneal infection, suggesting that at least this element of the innate immune system does not have a major role in pathogenesis (Prinz et al., 2003a).

1.3.2 Blocking lymphoreticular amplification

TNF-α and lymphotoxins have been implicated in sustaining differentiation of FDCs, the cell population required for peripheral PrP^{Sc} amplification. Deleting or blocking the effects of these factors can prolong incubation time in experimental models following peripheral but not central prion inoculation (Montrasio et al., 2000;Mabbott et al., 2000;Mabbott et al., 2002). Indeed, neonatal mice in which FDC differentiation has yet to occur are ~ 100-fold less susceptible to peripheral prion infection than mature mice (Ierna et al., 2006). Whether such interventions could be used as post-exposure prophylaxis in humans is unclear, as the required length of treatment and long-term side-effects remain to be established. Furthermore, it is clear that particularly after high-dose inoculation, prions can bypass FDCs and achieve neuroinvasion via other routes (Aucouturier et al., 2001;Shlomchik et al., 2001;Prinz et al., 2002;Oldstone et al., 2002;Race et al., 2000). Similarly, although manipulation of dendritic cells and macrophages, which may be able to clear PrP^{Sc}, appears attractive, the extent to which this would be effective once the disease has entered the CNS is unclear.

1.3.3 Passive immunisation

Antibodies against PrP were initially raised in rabbits by immunisation with preparations enriched for scrapie infectivity (Bendheim et al., 1984;Bode et al., 1985). Attempts to generate antibodies against scrapie fibrils in mice proved more difficult and raised sera reacted only to non-murine sequence (Kascsak et al., 1987). Identification and cloning of PrP in the mid-1980s (Prusiner et al., 1984;Oesch et al., 1985;Basler et al., 1986) allowed antibodies to be generated by immunisation with synthetic protein or peptides (Barry et al., 1988;Harmeyer et al., 1998). PrP knockout

mice subsequently proved to be a useful tool for raising antibodies against PrP from a range of species (Prusiner et al., 1993;Krasemann et al., 1996;Williamson et al., 1996).

However, neither these nor antibodies raised in $PrP^{+/+}$ animals can distinguish PrP^{C} from PrP^{Sc}. Attempts to generate PrP^{Sc}-specific antibodies have been confounded by the 100% sequence homology between it and PrP^{C} and have therefore relied on the former having a structural epitope not present in PrP^{C}. However, although antibodies that recognise PrP^{Sc} but not PrP^{C} have been described these have been low affinity IgMs (Korth et al., 1997), or were raised against simple structural motifs that are most likely present in other proteins (Paramithiotis et al., 2003), limiting their *in vivo* specificity and therapeutic potential. An alternative approach based on PrP^{Sc} having an affinity for PrP^{C} involves grafting PrP sequence corresponding to the putative binding site onto an existing antibody (Moroncini et al., 2004). Although such antibodies recognise PrP^{Sc} and not PrP^{C} it seems likely that they will also bind other proteins and produce off-target effects if used *in vivo*. Furthermore, it is not clear that the proteinase K resistant material recognised by these antibodies is the only or major neurotoxic species. Ligating this entity with antibody may therefore prove ineffectual or drive the equilibrium between pathogenic and more benign forms of PrP^{Sc} in a deleterious direction. For all the above reasons anti-prion effects have largely been reported for antibodies that bind both PrP^{C} and PrP^{Sc}.

Anti-PrP antibodies were initially shown to clear neuronal cells of prion infectivity (Enari et al., 2001;Peretz et al., 2001). Subsequently, mice were protected from peripheral prion inoculation by constitutive transgenic expression of anti-PrP antibodies (Heppner et al., 2001b). The most spectacular result in this field was

achieved when mice repeatedly treated with high dose anti-PrP were completely protected from disease following peripheral prion infection (White et al., 2003). Similar results were obtained whether the antibodies had initially been raised to recombinant PrP in α (analogous to PrP^{C}) or β (analogous to PrP^{Sc}) conformation. Therapy targeting the initial inoculum would likely have to be administered within the first few days following exposure. However, in this study treatment was begun 7 or 30 days following prion infection, suggesting an effect on host-encoded PrP^{C} or PrP^{Sc}. A more modest effect was achieved by low dose antibody administered immediately after infection and therefore primarily directed against the initial inoculum (Sigurdsson et al., 2003). However, in all these studies the antibodies had no effect against prions inoculated directly into the brain or after onset of clinical signs or the results of administering the antibodies under these conditions were not reported.

Although the exact mechanism of action is unknown, these observations suggest that anti-PrP antibodies ligate PrP^{C} and block its conversion to PrP^{Sc}, either by steric hindrance of refolding or by blocking a binding site for incoming PrP or a necessary co-factor. Alternatively anti-PrP may bind PrP^{Sc}, unveiling it as foreign and enabling clearance mechanisms (such as uptake by dendritic cells able to degrade PrP^{Sc}) via its Fc receptor. Clearly, peripherally administered antibodies cannot penetrate the CNS or the necessary clearance mechanisms do not operate in this immunologically privileged site. However it is not clear whether achieving CNS penetration by antibodies will be beneficial. Intracerebral administration of anti-PrP mAbs recognising epitope 95-105 to mice resulted in acute neurodegeneration (Solforosi et al., 2004). Interestingly, this was not observed with mAbs against epitope 133-157. Because this neurotoxic effect requires crosslinking of PrP by bivalent IgG, this could be overcome by use of small monovalent miniantibodies (Donofrio et al., 2005).

1.3.4 Active immunisation

The principal obstacle to establishing durable protective immune responses to prions is tolerance to PrP. Unlike neural antigens to which tolerance can be spontaneously or experimentally broken such as myelin basic protein (MBP) and β-amyloid (Aβ), PrP^C is expressed in the thymus and throughout the lymphoid system. The most likely consequence of this pattern of expression is deletion of auto-PrP reactive T cell clones in the thymus by negative selection. However, peripheral mechanisms of control are also likely to be involved as there is not a clear relationship between thymic PrP^C expression level and tolerance to PrP (Polymenidou et al., 2004) and T cell tolerance to PrP can be broken in wild-type mice with appropriate adjuvants. Intriguingly, no such mechanisms have been demonstrated; in particular the deletion of regulatory T cells as an adjunctive strategy to vaccination has not been reported. The lack of any known disease caused by anti-PrP autoimmunity and the absence of a classical immune response to the presence of PrP^{Sc} in the periphery or the CNS further suggests that tolerance to PrP is extensive.

Generating anti-PrP antibodies and T cell responses (Bainbridge and Walker, 2003;Gregoire et al., 2004;Khalili-Shirazi et al., 2005) in $PrP^{-/-}$ mice is relatively straightforward, although caution is required in predicting immunodominant epitopes from $PrP^{-/-}$ systems as PrP processing and presentation and the responding T and B cell repertoire will differ when PrP is a ubiquitously expressed self, as opposed to entirely foreign, antigen (Gregoire et al., 2005). Unsurprisingly, breaking tolerance in wild-type models has proved more difficult. PrP is highly conserved between different mammals, although occasional non-conserved sequence may be recognised as non-self and trigger a Th1 response (Stoltze et al., 2003).

However, protection against established prion infection, even if confined to the periphery, will require breaking of tolerance to self-PrP. The standard test of efficacy in anti-prion therapeutics in rodent models is incubation time following peripheral or intracerebral scrapie inoculation. A key factor limiting the applicability of such studies is whether vaccine delivery begins before or after exposure to prions. Most studies have examined the effects of vaccination prior to peripheral transmission. Generally, the degree of protection against experimental scrapie afforded by immunisation protocols has been disappointing. Sigurdsson and colleagues achieved a delay in incubation time of 16 days in CD-1 mice when immunised with recombinant murine PrP prior to peripheral scrapie infection and 12 days when immunised 24 hours after scrapie inoculation. However, there was a correlation between anti-PrP antibody titre and protection (Sigurdsson et al., 2002). A similar degree of protection was provided by immunisation with the neurotoxic PrP peptide 105-125 prior to oral infection (Schwarz et al., 2003). Vaccination of hamsters with PrP peptides 119-146 or 142-179 ameliorated intracerebral pathology but did not significantly extend incubation time (Magri et al., 2005). In this study peptide 105-128 was not as effective as the two more C-terminal fragments.

PrP sequence peptides selected principally on the basis of putative MHC binding motifs elicited strong T cell and IgG responses in mice and rats after immunisation in CFA (Souan et al., 2001b;Souan et al., 2001a). Further, vaccination with these peptides led to a reduction in PrP^{Sc} level in prion-infected tumours grafted onto the backs of mice. However, the generalisability of this finding is limited as presumably T cell and antibody entry into these lesions is unimpeded by a blood brain barrier. Indeed, when C57BL/6 mice were inoculated with scrapie following immunisation with these peptides, there was no protective response additional to the administration

of CFA alone (Tal et al., 2003). Immunisation with CFA alone extended incubation time after intraperitoneal and intracerebral infection by 19 and 24 days respectively. Why CFA should provide modest protection following peripheral or central prion inoculation is unknown.

Recently, DNA immunisation with a PrP expressing construct fused to a lysosome targeting signal was shown to induce anti-PrP antibody and CD4 and CD8 responses in wild-type mice, although the immunogenic epitopes within the vaccine (which consisted of the entire murine *Prnp* ORF) were not elucidated (Fernandez-Borges et al., 2006). The vaccine delayed onset of clinical scrapie following i/c inoculation from 57 to 138 dpi. However, whereas control mice remained alive for 10 further weeks before succumbing to terminal scrapie, vaccinated animals had an accelerated course progressing from early scrapie to terminal disease within 2 weeks. Thus, the protective effect of vaccination may not have been an immune mediated reduction in PrP^{Sc} or infectivity but an alteration in the ability of the brain to tolerate prion infection.

Because many prion diseases are spread via the oral route, mucosal vaccination may be an effective preventive strategy. Nasal vaccination with recombinant mouse PrP conferred only marginal protection against oral prion infection in BALB/c mice (Bade et al., 2006). A more promising result was obtained by oral vaccination with Salmonella expressing a PrP construct, but nevertheless only 30% of mice were protected against prion infection via oral gavage (Goni et al., 2005). This result may relate to genetic differences between outbred CD-1 mice. Alternatively, since protection correlated with fecal anti-PrP IgA titre it is possible that vaccine delivery or *in vivo* expression of the PrP construct was sub-optimal in most animals.

Why vaccination strategies have thus far failed to provide robust protection in standard scrapie challenge models is unclear. A drawback of antibodies raised against recombinant PrP (rec-PrP) or synthetic PrP peptides is that they may not recognise native cell-surface PrP^C (Polymenidou et al., 2004;Gregoire et al., 2005;Heppner and Aguzzi, 2004). It may be that epitopes present in rec-PrP are obscured when PrP^C adopts its native conformation. Alternatively, critical structural epitopes for protection may not be present in recombinant PrP, or production of antibodies against protective epitopes may require T cell help that is absent under ordinary circumstances in $PrP^{+/+}$ animals. Indeed, in $PrP^{-/-}$ mice, sera raised against PrP peptides 23-52, 98-127 and 143-172 recognised cell surface PrP, whereas antibodies generated in an identical manner in wild type animals exhibited no native PrP^C binding (Gregoire et al., 2005). Furthermore, the antibody response in $PrP^{+/+}$ mice was dominated by the IgG2b sub-class, with relatively little IgG1 production compared to $PrP^{-/-}$ animals (Gregoire et al., 2005).

In the study by Schwarz and colleagues, modest protection was provided by immunisation with PrP 105-125, whereas antibodies raised to PrP 159-211 by immunisation with recombinant PrP 90-230 were ineffectual. Vaccination with PrP 31-50 and 211-230 was ineffective against scrapie (Tal et al., 2003), despite these peptides having produced antibody responses in a previous study (Souan et al., 2001b). In contrast anti-PrP mAb 6H4, shown to be effective when expressed transgenically, recognises the epitope 144-152, and ICSM18 and ICSM35, raised to recombinant PrP in $PrP^{-/-}$ mice and which block PrP^{Sc} replication after passive transfer, recognise epitopes 143-153 and 93-105 respectively. Fab D18, the most effective antibody at clearing PrP^{Sc} from infected N2a cells in the study by Peretz and colleagues, binds PrP in the region 132-156. Comparing the effects of antibodies with

different epitopes is difficult as absolute quantitation of serum anti-PrP levels is not provided in the papers by Schwarz and Tal. Thus any lack of efficacy may simply be due to a poor B cell response. However, it may be that antibody-based therapeutics will only work *in vivo* if particular PrP epitopes are targeted.

At present it is not clear which part of the PrP sequence provides the most immunogenic T cell epitope. In C57BL/6 mice, immunisation with peptides spanning residues 158-172 with CpG induced IFN-γ producing responders as effectively as in $PrP^{-/-}$ animals, whereas other peptides were ineffectual (Gregoire et al., 2005). This epitope can also induce IL-4 production, albeit less efficiently than IFN-γ, in wild type mice after immunisation with CpG (Rosset et al., 2004). The cytokine profiles induced by stimulation of T cells from wild type animals following immunisation with PrP have not otherwise been addressed.

In the studies by Souan and colleagues robust *ex vivo* T cell responses were produced by immunisation with peptides 131-150 and 211-230 in NOD (H2g7) and C57BL/6 (H2b) mice and 211-230 in A/J (H2a) mice, whereas PrP 182-202 and 211-230 produced T cell responses in Lewis rats (Souan et al., 2001b;Souan et al., 2001a). Interestingly, a rat T cell line responsive to 182-202 was found to be predominantly $CD4^+$ with 16% expressing Vβ16. However, a minority of animals immunised with PrP 182-202 developed hair loss and severe skin inflammation 8 to 12 months post-immunisation (Souan et al., 2001a).

To facilitate non-autoinflammatory T cell help and generate appropriate antibodies, the optimal immunogen may need to contain two (or more) epitopes and adjuvants to "fine tune" the T and B cell responses separately. However, given the diversity of MHC and TCR repertoire between species it is likely that immunodominant T cell

epitopes will differ between humans and rodents. Attempts at protection through adoptive transfer of anti-PrP T cells, which would be most easily generated in $PrP^{-/-}$ animals, have not been reported.

Lack of efficacy due to low anti-PrP titre, poor binding to native PrP^{C} or irrelevant specificity may be partially overcome through novel vaccination strategies such as using PrP-displaying retroviruses, although this technique did not produce an efficient anti-PrP IgM to IgG switch in $PrP^{+/+}$ mice (Nikles et al., 2005). A variety of adjuvants have also been employed (reviewed in Heppner and Aguzzi, 2004). Anti-PrP antibodies have also been generated by immunising wild-type mice with novel vaccines consisting of PrP cross-linked to a heat shock protein (Koller et al., 2002), two identical 13-mer PrP sequence peptides complexed with tetanus toxoid fragments (Bainbridge et al., 2004) and a complex of 8 identical 10-mer peptides from human PrP sequence linked by series of branching lysine residues (Arbel et al., 2003). However, none of these protocols has yet been shown to provide protection *in vivo*.

An alternative strategy is to use PrP^{Sc} or recombinant PrP refolded *in vitro* into an amyloidogenic conformation (β-recPrP) as an immunogen. This does not circumvent tolerance mediated by primary PrP sequence, but may provoke a direct B cell response to a conformational epitope not present in PrP^{C} or recPrP in its native conformation (α-recPrP). When used as an immunogen in $PrP^{-/-}$ mice β-recPrP produces a Th1-skewed T cell response and antibodies predominantly of the IgG2b subtype, whereas α-recPrP elicits a Th2 response with production of IgG1 antibodies (Khalili-Shirazi et al., 2005). Studies on the efficacy of vaccination with recPrP in α or β conformation are ongoing in our laboratory.

Using CpG oligodeoxynucleotides rather than Freund's as an adjuvant may also enhance Th1 responses to PrP peptides in $PrP^{+/+}$ animals (Rosset et al., 2004). However, it remains unclear whether a T cell response is a pre-requisite to protection against prions and if so, whether a Th1 or Th2 skewed response is preferable. As in the recent Aβ vaccine trial, inducing a T cell response may cause catastrophic autoimmune side effects (see below) and it is proposed that a Th2 dominant response is less likely to produce a T cell driven inflammatory autoimmune syndrome (Gelinas et al., 2004).

Although the exact mechanism by which anti-Aβ immunotherapy clears amyloid plaques in AD is unclear, there appears to be a consensus that anti-prion immunotherapy will require induction of anti-PrP antibodies, irrespective of the underlying T cell response. Indeed, some immunisation protocols may be able to induce anti-PrP antibodies without triggering a T cell response (Rosset et al., 2004). However, it is not yet clear which IgG subclass offers most effective protection, although in the study by White et al, anti-PrP mAbs of the IgG1 or IgG2b type had similar efficacy when administered passively (White et al., 2003).

1.3.5 Strain interference

β-recPrP represents a more tractable immunogen than PrP^{Sc}, as the latter carries risks of transmitting prion disease and has not yet been purified to the exclusion of other proteins. Nonetheless, there has been interest in using prion strains of low pathogenicity as prophylactic agents against more virulent agents. The intriguing but as yet unexplained phenomenon of "strain interference" involves establishing infection with a prion strain with a prolonged incubation time that then mediates protection against subsequent infection by a more pathogenic strain (Dickinson et al.,

1972;Dickenson et al., 1975;Manuelidis, 1998;Bartz et al., 2004). If the incubation time of the initial "slow" strain exceeds the natural lifespan of the animal this could provide complete protection against superinfection by more pathogenic "fast" strains (Manuelidis and Lu, 2003). It is not known whether this effect is due to priming of the immune system, appropriation of all available PrP^C (or some other necessary co-factor) by the slow strain or direct interference of one strain by the other. Attempts to resolve this using immunodeficient mice have so far been inconclusive (Manuelidis and Lu, 2003). Of note, the protective effect is enhanced if administration of the slow strain precedes that of the fast by several weeks (Dickinson et al., 1972). The recent recapitulation of this phenomenon in cultured GT1 cells (Nishida et al., 2005) suggests that protection is mediated within individual infected cells. Whatever the underlying mechanism, safety considerations mean that this phenomenon does not currently represent a realistic preventive or therapeutic strategy in humans. Indeed, concerns remain that recombinant PrP may under some circumstances be able to initiate prion disease (Legname et al., 2004), possibly limiting its use as a vaccine. Nevertheless, an understanding of the molecular events underlying strain interference could assist development of novel anti-prion therapeutics.

1.3.6 Potential hazards of breaking tolerance to PrP

The dangers, if any, of breaking tolerance to PrP remain unclear. The only adverse side effect of anti-PrP vaccination so far reported is dermatitis with mononuclear invasion and destruction of hair follicles in rats several months after immunisation with PrP 182-202 (Souan et al., 2001a). PrP^C is expressed in skin (Lemaire-Vieille et al., 2000;Ford et al., 2002b) but whether this reaction was the result of specific breakdown of tolerance to dermal PrP^C is unclear. As demonstrated in the Aβ vaccine trial (see below), breaking tolerance to neural proteins implicated in

neurodegeneration is clearly not without risk. Rats immunised with β-synuclein developed autoimmune encephalomyelitis and uveitis due to activation of auto-reactive T cell clones (Mor et al., 2003) and EAE was induced in C57BL/6 mice by vaccination with Aβ (Furlan et al., 2003). In contrast, rodents have been immunised with α-synuclein and other neural antigens without obvious side effects (Masliah et al., 2005;Mor and Cohen, 2006). The expression pattern of PrP is much wider than these proteins, suggesting that autoimmune side effects will not be restricted to the CNS but could involve a systemic inflammatory syndrome.

However, if properly regulated T cell responses in neurodegenerative diseases may be beneficial rather than destructive. According to the theory of "protective autoimmunity" (Schwartz and Kipnis, 2005), infiltration of the CNS by T cells primed to recognise myelin antigens can be beneficial, through mechanisms such as cytokine and neurotrophin release, microglial activation and clearance of amyloid. This has been exploited in models of Alzheimer (Frenkel et al., 2005) and Parkinson (Benner et al., 2004) disease and spinal cord (Hauben et al., 2000), optic nerve (Moalem et al., 1999), motor nerve (Angelov et al., 2003) and head injury (Kipnis et al., 2003). However, there are no published data on such techniques in prion disease.

1.3.7 Immunotherapy in humans – lessons from Alzheimer Disease

The degree to which humans are tolerant to PrP is unknown. It seems likely that anti-PrP T cells will undergo deletion due to PrP expression in the thymus and repeated exposure to animal PrP in food may further enhance tolerance. However, the demonstration of T cell responses to the predominantly neuronal protein APP and its amyloidogenic Aβ 1-40 or 1-42 peptide fragments (Trieb et al., 1996;Giubilei et al.,

2003;Monsonego et al., 2003;Baril et al., 2004), suggests that tolerance to PrP may also not be complete, although it is likely to be tighter. The importance of defining T cell epitopes in self proteins that are used as vaccines was dramatically illustrated when 6% of AD patients in a clinical trial of Aβ vaccination developed meningoencephalitis presumed to be driven by auto-aggressive T cell invasion of the CNS (Orgogozo et al., 2003;Nicoll et al., 2003). The cause of this side effect is not entirely clear, and may relate to a Th1-dominant response driven by the adjuvant QS-21 (Gandy and Walker, 2004;Cribbs et al., 2003). However, an option for future vaccine development in AD may be to focus on the N-terminal fragment of Aβ that contains the B cell epitope but from which the immunodominant C-terminal T cell epitope has been deleted (Schenk, 2002).

The effects of generating an anti-PrP T cell response in humans are unknown; this may be necessary to elicit a protective response or conversely produce catastrophic autoimmune side effects. Whichever is the case, defining the immunodominant epitopes, if any, of PrP in humans, and characterising the immune response to them, must be a priority.

1.3.8 Summary

Despite the clear success of passive immunisation, attempts at generating active immunity have been of limited efficacy and have concentrated on pre- rather than post-exposure prophylaxis. Further, even passive immunotherapy has been effective only in abrogating prion infection before CNS penetration has occurred, limiting its applicability for symptomatic disease. This reflects both tight tolerance to PrP and the inability of immune effector cells and components to penetrate the CNS. Future work will need to focus on the mechanisms by which tolerance to PrP is maintained and the

development of strategies to break this without precipitating adverse autoimmunity. Delivery of safe anti-prion agents, be these antibodies, T cells or drugs, to the CNS is an absolute priority for drug development in this field.

1.4 Function of PrP^C in the immune system

1.4.1 Structure and function of PrP

Prion protein is encoded by a single gene composed of two exons (three in mice), the entire open reading frame being contained within exon 2. The prion gene (*PRNP* in human, *Prnp* in mice) is found on human chromosome 20, with the synteneic region on mouse chromosome 4.

Newly synthesised PrP is trafficked to the ER where the N-terminal signal peptide (residues 1-22) is cleaved. Further post-translational modifications include attachment of the GPI-anchor after cleavage of the C-terminal signal peptide, formation of a disulphide bond linking residue Cys 179 to Cys 214 and addition of oligosaccharide chains at Asn 180 and/or Asn 197 (Ermonval et al., 2003). The number of sites at which these sugar groups are added determines whether the final PrP molecule is un-, mono- or diglycosylated; all three glycoforms are present albeit at variable ratios in tissues where PrP^C is expressed. PrP then enters the Golgi apparatus where modifications to the carbohydrate moieties are made. It is then mostly trafficked to the cell surface where its GPI anchor allows it preferentially to enter lipid raft domains.

The conformation of the cellular isoform was first established by NMR measurements made on the recombinant mouse protein (Riek et al., 1996). Since then NMR measurements on recombinant proteins from several species (James et al., 1997;Zahn

et al., 2000;Calzolai et al., 2004;Lysek et al., 2005;Calzolai et al., 2005;Gossert et al., 2005) have shown that they have essentially the same conformation. The mature PrP^C species consists of an N-terminal region of about 100 amino acids which is unstructured in the isolated molecule and a C-terminal segment, also around 100 amino acids in length.

Within the unstructured region there is a segment of five repeats of an eight-amino acid sequence (the octapeptide-repeat region) which has a tight binding site for a single Cu^{2+} ion with a dissociation constant (K_d) of 10^{-14}M (Jackson et al., 2001b). A second tight copper site ($K_d = 10^{-13}$M) is present downstream of the octapeptide-repeat region but before the structured C-domain (Jackson et al., 2001b). The remaining ~100 amino acids at the C-terminus is folded into a series of three α-helices and a small two-strand β-sheet and is stabilised by the disulphide bond (Riek et al., 1996).

Much effort has been devoted to identifying potential binding partners and ligands for PrP^C. A variety of techniques have been employed, including yeast-2-hybrid systems (Spielhaupter and Schatzl, 2001) and proteomics-based approaches (Strom et al., 2006). Thus far putative PrP^C ligands include Synapsin 1b, grb2, Pint1, Caveolin1, CK2, STI1, Bcl-2, Laminin and Laminin receptor, glycosaminoglycans, N-CAM, GFAP, Bip, Hsp60, Nrf2, aplp1 and nucleic acids (reviewed in Lee et al., 2003). From this list it is clear that either the principal receptor for PrP^C remains obscure or PrP^C is a nodal point in a variety of cellular pathways with multiple binding partners and functions.

PrP^C is highly expressed in the CNS, and as this is the major site of prion pathology most interest has focussed on defining the role of PrP^C in neurons. Strangely for a

highly conserved protein, PrP^C appears to be functionally redundant as $PrP^{-/-}$ mice have a grossly normal neurological phenotype (Bueler et al., 1992;Prusiner et al., 1993;Manson et al., 1994a), even when neuronal PrP^C is knocked out post-natally (Mallucci et al., 2002). In neurons, PrP^C is clustered at the synapse (Sales et al., 1998;Herms et al., 1999), and this has led to speculation that it may play a role in neural transmission. Indeed, $PrP^{-/-}$ mice have subtle abnormalities in synaptic transmission (Collinge et al., 1994;Colling et al., 1996;Mallucci et al., 2002), hippocampal morphology (Colling et al., 1997), circadian rhythms (Tobler et al., 1996), cognition (Coitinho et al., 2003;Criado et al., 2005) and seizure threshold (Walz et al., 1999). Other postulated neuronal roles for PrP^C include copper-binding (Brown et al., 1997;Jackson et al., 2001b), as an anti- (Kuwahara et al., 1999;Bounhar et al., 2001;Chiarini et al., 2002) and conversely, pro-apoptotic (Paitel et al., 2002) protein, as a signalling molecule (Mouillet-Richard et al., 2000;Spielhaupter and Schatzl, 2001) and in supporting neuronal morphology and adhesion (Mange et al., 2002;Santuccione et al., 2005).

An important consideration is whether PrP^C has a single function in all tissues in which it is expressed, or whether it has multiple tissue-specific roles. If the major role of PrP^C in neurons relates to neurotransmission, the protein must have additional functions as PrP^C is expressed in many non-excitable cells, including glia (Moser et al., 1995;Brown et al., 1998), and in numerous lymphoid and non-lymphoid organs (Bendheim et al., 1992;Ford et al., 2002b). Functional heterogeneity in PrP^C could be mediated by different glycosylation patterns between tissues. For example, in human PBMCs the unglycosylated form is underrepresented (Li et al., 2001).

1.4.2 Expression of PrP^C during lymphoid and myeloid ontogeny

Although the precise function of PrP^C in the immune system remains obscure it is clear that PrP expression during lymphoid and myeloid differentiation is regulated. In mice, fetal thymocyte PrP^C expression is high at day 15 of gestation, and declines thereafter (Kubosaki et al., 2001). However, post-natal PrP expression remains higher on thymocytes, particularly $CD4^-$ $CD8^-$ cells than on mature murine splenocytes (Kubosaki et al., 2001;Liu et al., 2001). Interestingly, mice with 50-fold over-expression of lymphoid PrP undergo premature thymic atrophy in which thymocyte maturation appears arrested at the $CD4^-$ $CD8^-$ stage and γδ cells are overrepresented (Jouvin-Marche et al., 2006). However, secondary lymphoid organs in these mice do not seem to be affected.

PrP expression has been detected in bone marrow haematopoietic stem cells and may define a particular population of murine haematopoietic stem cells with long term repopulation potential (Zhang et al., 2006). Human $CD34^+$ stem cells in bone marrow express PrP, but this is downregulated when these cells undergo granulocyte differentiation as monitored by acquisition of CD15 (Dodelet and Cashman, 1998). Similarly, $CD43^+$ $Gr\text{-}1^+$ granulocyte precursors in murine bone marrow express PrP^C, unlike mature neutrophils (Liu et al., 2001).

In contrast, myeloid differentiation leads to upregulation of PrP. Maturation of murine $CD14^+$ monocytes by culture with IFN-γ leads to upregulation of PrP^C within 24 hours (Dürig et al., 2000) and maturation of human monocytes to DCs upregulates PrP (Burthem et al., 2001).

Studies in mice show a trend towards downregulation of PrP with lymphoid maturation, and murine T lymphocyte expression during quiescence is low (Liu et al.,

2001). PMA stimulation of murine bone marrow cells to induce B cell maturation leads to a reduction in surface PrP^C expression (Liu et al., 2001), although suppression of PrP in lymphoid maturation is usually less than on granulocyte differentiation. However, in humans and sheep, which unlike mice are susceptible to naturally occurring prion diseases, PrP expression on mature blood and lymphoid cells remains high although unlike those of humans, ovine platelets do not express PrP (Herrmann et al., 2001;Halliday et al., 2005). Indeed, in humans lymphocytes from umbilical cord blood express lower levels of PrP^C than those from adults (Li et al., 2001) with levels increasing further with ageing (Politopoulou et al., 2000).

1.4.3 PrP^C expression in mature immune cells

PrP^C has been detected on T and B lymphocytes, NK cells, platelets, monocytes, dendritic cells and follicular dendritic cells (Burthem et al., 2001;Li et al., 2001;Cashman et al., 1990;Barclay et al., 1999;Dodelet and Cashman, 1998;Antoine et al., 2000;Dürig et al., 2000;Holada and Vostal, 2000;Politopoulou et al., 2000;Herrmann et al., 2001;Brown et al., 1999a;Thielen et al., 2001). As with mice, PrP^C expression on human erythrocytes and granulocytes is absent or low in comparison to myeloid and lymphoid cells (Cashman et al., 1990;Barclay et al., 1999;Dodelet and Cashman, 1998;Antoine et al., 2000;Holada and Vostal, 2000;Herrmann et al., 2001).

PrP^C expression increases during human NK cell differentiation, with particularly high levels on $CD56^+$ $CD3^+$ NKT cells (Dürig et al., 2000). PrP^C expression may be somewhat higher in peripheral blood T cells than in B lymphocytes, while $CD8^+$ cells express slightly more PrP^C than $CD4^+$ cells (Dürig et al., 2000;Politopoulou et al.,

2000). It has also been reported that PrP^C expression is higher in $CD45RO^+$ memory compared to $CD45RA^+$ naïve T lymphocytes (Li et al., 2001).

In mice, mitotic lymphocytes and those circulating to non-lymphoid organs have higher PrP^C expression than resting splenocytes (Ford et al., 2002b). A study of PBMCs from sheep showed that PrP^C expression is higher in $CD21^+$ cells, proposed to be B cells that circulate within secondary lymphoid tissues, than in other B lymphocytes (Halliday et al., 2005). Conversely, human tonsillar T and B lymphocytes appear to have lower PrP^C expression than peripheral blood cells, an effect that is not due to increased intracellular sequestration of PrP^C (Antoine et al., 2000).

Gene expression microarrays have revealed murine *Prnp* also to be upregulated in certain types of regulatory T cell (Huehn et al., 2004), via a Stat 6-dependent mechanism during IL-4 driven Th0 to Th2 differentiation (Chen et al., 2003), and in $CD8^+$ memory T cells (Goldrath et al., 2004). Hence, PrP^C may be more important in certain types of functionally differentiated lymphocyte with particular cytokine profiles and homing properties.

1.4.4 PrP expression during T cell activation

PrP^C is upregulated within a few hours in T cells following mitogenic activation with Con A, PHA or anti-CD3 antibodies (Cashman et al., 1990;Mabbott et al., 1997;Li et al., 2001;Kubosaki et al., 2003). This effect is not modulated by extracellular copper levels (Kubosaki et al., 2003). Cashman and colleagues detected a rise in surface PrP by FACS after 6 hours culture of human PBMCs with Con A. PrP levels plateaued at a 3.5-fold increase after 3 days and remained constant for 7 days of culture. Similar results were observed in murine splenocytes activated with Con A (Mabbott et al.,

1997). Interestingly, upregulation of PrP on human tonsillar and peripheral blood lymphocytes does not occur on activation with PMA/Ionomycin, suggesting that signalling via elements of the TCR may be required to induce changes in PrP transcription (Antoine et al., 2000). In contrast to the relative ease of PrP^C upregulation in T cells, treatment with LPS does not increase PrP^C expression on B cells (Kubosaki et al., 2003). Thus although the functional significance of increased PrP expression in activated T cells remains unclear, PrP is not a universal lymphoid activation antigen.

Intracellular pathways linking T cell activation with increased *PRNP* mRNA have not been characterised. Regulation of *PRNP* expression *in vivo* is poorly understood. The *PRNP* promoter does not contain a TATA box, but a GC-rich region with SP1 transcription factor binding sites reminiscent of housekeeping genes is present (Basler et al., 1986;Bredesen et al., 1989;Fischer et al., 1996). Recently, sequencing and *in silico* analysis has identified putative binding sites for transcription factors NF-IL6, MyoD, MZF-1, MEF2, Oct1, MyT1 and NFAT (Mahal et al., 2001;Premzl et al., 2005). Heat shock elements identified in the *PRNP* promoter (Mahal et al., 2001;Shyu et al., 2002) have been shown to interact with HSTF-1 and heat shock increases *Prnp* mRNA and protein levels in neurons (Shyu et al., 2002). Mechanisms that constitutively degrade *PRNP* mRNA, and which may be inhibited by cell activation have not been elucidated.

1.4.5 Lymphoid and myeloid PrP expression and prion disease pathogenesis

The inducibility of PrP^C expression in lymphocytes and myeloid cells is of importance to the pathogenesis of orally transmitted prion diseases such as vCJD, as these cells

are present at possible sites of entry, such as tonsil and gut-associated lymphoid tissue. Scrapie resistant sheep have been reported to have fewer PrP^C expressing microglia and CD14 monocytes in peripheral blood (Herrmann et al., 2006). Further, PrP^C expression in PBMCs may be higher in scrapie susceptible sheep than in those with resistant genotypes (Halliday et al., 2005), although this is not a universal finding (Thackray et al., 2004b). In rodent models, infectivity has been recovered from splenic T and B cells and DCs, although not consistently from circulating lymphocytes (Kuroda et al., 1983;Raeber et al., 1999a;Aucouturier et al., 2001;Aucouturier and Carnaud, 2002). An activated lymphocyte, DC or monocyte carrying increased surface PrP^C as a result of concomitant infection or inflammation might be more readily infected than a PrP^C low naïve cell. Adenoviral infection and mitogens have been demonstrated to reduce incubation time in mice experimentally inoculated with scrapie, although the mechanisms underlying this effect have not been fully elucidated (Dickinson et al., 1978;Ehresmann and Hogan, 1986).

One possible clue is provided by inoculation of mice with immune complexes or vesicular stomatitis virus, which increases PrP immunoreactivity in germinal centres of the spleen, ascribed to increased surface PrP^C on FDCs (Lotscher et al., 2003). However, total spleen *Prnp* mRNA was not increased when measured by real-time RT-PCR. Post-transcriptional processing of PrP in lymphoid cells is not well understood and it is unclear what mechanisms could account for a significant increase in surface PrP without *de novo* transcription. Indeed, near-complete inhibition of PrP upregulation on cell activation in the presence of cycloheximide has been observed (Dürig et al., 2000). Platelets can increase surface PrP^C expression ~ 2.5-fold within 2 hours of activation (Holada et al., 1998), probably by redistributing PrP^C from alpha-granules to the cell membrane (Starke et al., 2005). However, surface PrP^C levels

decline thereafter (Holada et al., 1998), presumably because the short half life of surface PrP means that it is removed before *de novo* translation can compensate. Moreover, such rapid "degranulation" mechanisms have not been demonstrated for PrP^C in lymphoid cells and are unlikely to account for the changes described by Lotscher and colleagues, which were observed some days following viral or immune complex inoculation.

Discrepancies between *Prnp* mRNA and protein levels have previously been noted (Ford et al., 2002a), suggesting that post-transcriptional or translational processing of PrP^C may differ between different cell populations and in response to as yet unidentified signals. However, in the experiment by Lotscher and colleagues although germinal centre PrP^C staining increased by approximately 6-fold, total spleen PrP^C was only 2-fold increased. Indeed, it seems most likely that immune recognition of conventional pathogens will activate host cells to upregulate PrP^C at the transcriptional level in the same manner as crude mitogens. Nevertheless, the possibility remains that viruses and other immune stimuli can induce changes to post-transcriptional processing of PrP mRNA or protein in lymphoid cells that significantly alter surface PrP^C availability.

Another possible explanation for the increased PrP in this model is that the effect of viruses or immune complexes is to activate macrophage or dendritic cell migration to the spleen resulting in increased "delivery" of PrP (and presumably other host proteins) to FDCs. Such a phenomenon may explain the decrease in incubation time produced by inoculating hamsters with vaccinia virus 2 hours prior to i/p scrapie infection (Marsh, 1981). This was associated with increased infectivity in splenic macrophages, which following vaccinia-induced activation may have collected the

prion inoculum and transported it to the spleen with greater efficiency than in non-vaccinia treated animals (Marsh, 1981).

Whatever the explanation for the increased germinal centre PrP^C described by Lotscher, this phenomenon was dependent on C1q expression. FDCs may use surface C1q to assist in trapping of immune complexes. Therefore C1q may be required for downstream activation induced effects on PrP expression. However, since both macrophages and FDCs can produce C1q (Schwaeble et al., 1995) this does not conclusively identify the ultimate source of the increased PrP^C. Nonetheless, a possible sequence of events involves FDCs trapping prions via a complement-dependent mechanism and then being permissive to PrP^{Sc} accumulation due to their own PrP^C expression. Any condition that upregulates the rate limiting factor in this process is likely to increase susceptibility to prion infection.

1.4.6 Localisation of PrP in T cells

The localisation of PrP^C in lipid rafts in neurons (Vey et al., 1996;Naslavsky et al., 1997;Madore et al., 1999) has attracted considerable interest as a means of identifying functions and binding partners of PrP. Available evidence suggests that the microdomain environment in which PrP^C is found in lymphocytes is similar to that in neurons. Fluorescence microscopy and gold immunolabelling reveals that PrP is present on the cell surface of lymphocytes in clusters (Mattei et al., 2004;Stuermer et al., 2004). When T cell lysates are subjected to sucrose density gradient centrifugation, PrP^C is largely found in the Triton insoluble fraction (Mattei et al., 2002;Stuermer et al., 2004). In human CEM T cells PrP^C co-localises and co-immunoprecipitates with ganglioside GM3 (Mattei et al., 2002), and to a lesser extent

GM1 (Hugel et al., 2004) and with Fyn, but not Src (Mattei et al., 2004). In Jurkat cells PrP^C co-immunoprecipates with Reggie-1, Fyn and Lck (Stuermer et al., 2004).

Following activation with anti-CD3 and anti-CD28 PrP^C co-immunoprecipitates with Zap70 (Mattei et al., 2004). In Jurkat lymphocytes PrP^C has been seen to co-localise with CD3 in caps induced by hypothermia (Wurm et al., 2004). However, although PrP accumulates at sites of T cell-DC contact during MHC-peptide stimulation, it does not co-localise with CD3, LFA-1, CD43, LAT or Thy-1 (Ballerini et al., 2006). Thus, these observations might reflect a non-specific clustering effect of activation on lipid raft components rather than a specific role for PrP^C within the immune synapse.

In neurons, PrP^C is endocytosed from the cell surface within minutes, and enters early endosomes, from whence a large proportion is quickly recycled to the cell surface (Sunyach et al., 2003). Endocytosis and recycling of PrP^C appears to be a rapid process in which PrP^C molecules cycle through the cell with a transit time of ~60 minutes. A fraction of the endocytosed PrP^C is transported to other organelles, such as late endosomes, and degraded (Peters et al., 2003). Indeed, during each passage, up to 5% of the internalised PrP^C undergoes proteolytic cleavage near residue 110 (Harris et al., 1993). Pulse chase labelling experiments have shown that the half life of PrP^C in murine splenocytes is similar to that in neurons; about 1.5-2 hours (Parizek et al., 2001).

These observations raise the possibility that PrP^C may exert some intracellular function through being constitutively endocytosed (reviewed in Harris, 2003;Prado et al., 2004;Campana et al., 2005). Interestingly, internalisation of a recombinant PrP-Fc fusion protein by monocytes (via a PrP N-terminal dependent pathway), leads to increased tyrosine phosphorylation of Syk and Pyk2 and activation of ERK1 and 2

and Akt kinase (Krebs et al., 2005). Incubation of T cells for >10 minutes with anti-PrP mAbs results in internalisation of a large proportion of surface PrP^C (together with reggie-1 and reggie-2) into limp-2 positive endosomes (Stuermer et al., 2004). However, to what extent and via what mechanism constitutive endocytosis of PrP^C occurs in lymphocytes is not clear. Caveolae are not present in lymphocytes, implying that PrP^C internalisation would require clathrin-mediated endocytosis or other clathrin-independent mechanisms. Being GPI-anchored, PrP^C would likely have to associate with a transmembrane protein in order to interact with adaptor proteins required for clathrin-mediated endocytosis. Presumably, rapid internalisation of PrP^C would affect the type of signal that it could transduce and its ability to contribute to protein-protein and cell-cell interactions at the cell surface.

A further mechanism of PrP^C trafficking that may apply in lymphocytes is release into the extracellular milieu or onto other cells. PrP has been detected in secretory granules of epithelial cells in a number of tissues (Fournier et al., 2000). PrP^C appears to be constitutively shed from lymphocytes (Parizek et al., 2001) and endothelial cells (MacGregor et al., 1999;Starke et al., 2002) and from activated platelets in exosomes and microvesicles (Perini et al., 1996;Robertson et al., 2006), which may explain the presence of soluble PrP^C in the plasma (MacGregor et al., 1999;Volkel et al., 2001a). Microparticles bearing PrP immunoreactivity are shed from T cells undergoing VP-16 induced apoptosis (Gidon-Jeangirard et al., 1999) and from apoptotic endothelial cells (Simak et al., 2002;Starke et al., 2002). Recently, PrP^C and PrP^{Sc} have been identified in exosomes released from epithelial and neuroglial cell lines *in vitro* (Fevrier et al., 2004), revealing a possible novel pathway of prion spread. PrP^C molecules have previously been shown to transfer between cells, although this required PMA or Con A activation of the donor or recipient cells and cell-cell contact (Liu et al., 2002).

Whether PrP^C shed from lymphocytes in soluble form, exosomes, or other microparticles mediates some function in the immune system is unclear. However, it should be noted that only one third of PrP^C present is human blood is contained on or within its cellular components (MacGregor et al., 1999), the remainder is split into soluble and microparticle-associated PrP^C.

1.4.7 Effects of PrP^C ligation in lymphocytes

An alternative approach to elucidating the function of PrP^C has been to mimic the effects of a putative PrP ligand by cross-linking surface PrP^C with anti-PrP antibodies. Using this technique neuronal PrP^C was demonstrated to activate Fyn in a caveolin-1 dependent mechanism (Mouillet-Richard et al., 2000). In lymphocytes, this can induce clustering of PrP in caps containing Thy1, reggie-1, reggie-2, CD3, F-actin, fyn, lck, LAT and GM1, increased release of reactive oxygen species (ROS) and increased phosphorylation of Src family kinases (but not Fyn) and ERK1/2 (Schneider et al., 2003;Hugel et al., 2004;Stuermer et al., 2004). The effects on ROS may be mediated by NAPDH oxidase and those on ERK1/2 by NAPDH oxidase and MEK1/2 (Schneider et al., 2003). Cross-linking PrP^C in CEM cells modulates ionophore-induced calcium entry and intracellular release (Hugel et al., 2004). Furthermore, in Jurkat cells PrP^C cross-linking alone induces calcium fluxes, although less marked than those produced by anti-CD3 (Stuermer et al., 2004). However, these experiments have been conducted *in vitro* over short time periods and the long-term effects of such treatment are not known.

These effects seem generally stimulatory, yet anti-PrP antibodies can also block activation of human T cells by Con A (Cashman et al., 1990) or anti-CD3 (Li et al., 2001) and murine TCR tg T cells by MHC-peptide (Ballerini et al., 2006).

Interestingly, some of these effects may be epitope specific; for example, anti-PrP mAbs block anti-CD3-induced proliferation only when certain PrP epitopes are targeted (Li et al., 2001). They may also be mitogen specific as Cashman and colleagues reported that inhibition of PHA-induced proliferation with anti-PrP was not as reproducible as for Con A stimulation (Cashman et al., 1990).

The mechanism by which these blocking effects occur remains unknown. PrP^C is present in or near the immunological synapse and shows enhanced co-localisation with the TCR in lipid rafts, upon cross-linking (Stuermer et al., 2004). It also co-localises with MHC class II in DCs (Burthem et al., 2001). Thus, the blocking effects of anti-PrP could be mediated via the T cell or APC. Indeed, in a mixed lymphocyte reaction, anti-PrP inhibited proliferation when PrP expression was restricted to either DCs or T cells (Ballerini et al., 2006). This effect was maintained when Fab was used in place of total IgG to eliminate PrP cross-linking and reduce the degree of steric hindrance (Ballerini et al., 2006). Thus, the effects of anti-PrP on T cell activation may not be simply be due to mechanical interference but mediated via destabilisation of the immune synapse or altered signalling.

The relationship between these observations and the normal function of PrP^C remains unclear. Most of these experiments involved cross-linked surface PrP^C even though there is no evidence that any potential PrP ligand need be polyvalent. Similar effects have been observed upon cross-linking of other GPI-anchored proteins (Ilangumaran et al., 2000), thus may not inform on the specific function of PrP^C.

1.4.8 Immunological phenotype of $PrP^{-/-}$ mice

$PrP^{-/-}$ mice have been reported to have normal MHC class I and II expression, DC maturation and numbers of haematopoietic stem cells, $CD4^+$, $CD8^+$ and B cells

(Bueler et al., 1992;Kubosaki et al., 2001;Ballerini et al., 2006;Zhang et al., 2006), suggesting that they are not grossly immunodeficient. Spontaneous tumours or greater susceptibility to infections than $PrP^{+/+}$ mice have not been reported. Further, $PrP^{-/-}$ mice have proved a useful tool for production of anti-PrP mAbs of all major IgG classes by immunisation with PrP^{Sc} or recPrP (Prusiner et al., 1993;Krasemann et al., 1996;Williamson et al., 1996;Khalili-Shirazi et al., 2005), suggesting that they are able to mount effective B cell responses and provide T cell help where appropriate.

Based on these data it is perhaps unsurprising that $PrP^{-/-}$ mice kept in pathogen-poor facilities do not spontaneously develop immunopathology; this may become manifest only following focused immunological challenge such as infection or immunisation. Published data on *in vivo* immune responses in $PrP^{-/-}$ animals is limited. Mice lacking both PrP and its downstream partial homologue Doppel have grossly normal CD8 expansion following LCMV infection and specific antibody production after infection with LCMV or VSV (Genoud et al., 2004).

However, there is a lack of consenus from knockout experiments as to whether PrP^{C} is required for optimal T cell activation *in vitro*. In the initial report of the Zurich I PrP knockout mouse, splenocyte proliferation to Con A was normal (Bueler et al., 1992), whereas Mazzoni recently described reduced proliferation to Con A and PHA in Zurich I splenocytes (Mazzoni et al., 2005). Splenocytes from the knockout line derived in Edinburgh were reportedly hyporesponsive to Con A (Mabbott et al., 1997); conversely, Liu and colleagues reported normal responses to Con A and plate-bound anti-CD3 in T cells from the same strain (Liu et al., 2001). Kubosaki and colleagues reported delayed IL-2 mRNA upregulation in $PrP^{-/-}$ splenocytes treated with Con A only when this was done in the presence of a copper chelator (Kubosaki

et al., 2003). Recently another group reported normal proliferation to Con A in $PrP^{-/-}$ splenocytes, but a deficient IFN-γ response to Con A and PMA/Ionomycin that was at least partially corrected by transfection of $PrP^{-/-}$ cells with *Prnp* (Bainbridge and Walker, 2005).

In an attempt to resolve these discrepancies, Ballerini and co-workers studied mixed lymphocyte reactions in which PrP was deleted from either the T cells or DCs (Ballerini et al., 2006). Interestingly, $PrP^{-/-}$ T cells responded normally to $PrP^{+/+}$ DCs, whereas $PrP^{-/-}$ DCs were less efficient than $PrP^{+/+}$ DCs at stimulating $PrP^{+/+}$ T cells. Thus, discrepancies in the literature on $PrP^{-/-}$ T cell proliferation may relate to the degree to which this was dependent on APC-T cell conjugation. Interestingly, in Ballerini's experiment, $PrP^{+/+}$ T cells cultured with $PrP^{-/-}$ DCs made equivalent amounts of IL-2 even though proliferation was poor. This suggests that the role of PrP in DCs in this context is to facilitate T cell mitosis downstream of IL-2 production.

Absence of PrP may affect other mature immune functions. In zymosan induced peritonitis, the leukocyte infiltrate in $PrP^{-/-}$ mice contained more monocytes and significantly fewer neutrophils than in wild type animals (de Almeida et al., 2004). Based on work in $PrP^{-/-}$ mice or cells derived from them, PrP^{C} has been proposed to down-regulate phagocytosis by macrophages (de Almeida et al., 2004) but also to be involved in the phagocytic machinery used by Brucella abortus to invade macrophages (Watarai et al., 2003), although this finding has been challenged (Fontes et al., 2005).

Perhaps the most striking abnormality thus far detected through use of PrP knockout mice is that $PrP^{-/-}$ HSCs display impaired self-renewal, albeit after multiple rounds of

transplantation into lethally irradiated $PrP^{+/+}$ recipients (Zhang et al., 2006). That PrP may be required for cell growth under harsh conditions is supported by gene expression microarray data suggesting that it is upregulated in $CD8^+$ cells undergoing homeostatic proliferation upon transfer into lymphopaenic mice (Goldrath et al., 2004). The molecular programme underlying lymphoid repopulation is remarkably similar to that of memory differentiation, in which increased *Prnp* and surface PrP expression have also been observed (Goldrath et al., 2004;Li et al., 2001). Moreover, $PrP^{+/+}$ TCR tg T cells transferred into peptide challenged $PrP^{-/-}$ mice showed reduced numbers of mitoses (Ballerini et al., 2006). Thus, the role of PrP in T cell expansion and differentiation may be mediated either by the cell itself or supporting populations from other lineages. However, the mechanisms underlying these observations remain elusive. If $PrP^{-/-}$ lymphocytes, or $PrP^{+/+}$ cells in a PrP null environment, suffer premature senescence, via what pathway is this mediated? Is there failure of specific signalling proteins known to be activated by PrP cross-linking, or is a separate mechanism involved?

Although studies to date have revealed subtle immunological abnormalities in $PrP^{-/-}$ mice, none has yet involved post-natal knockout of lymphoid PrP. In a Cre-lox dependent, conditional *Prnp* deletion model developed by Mallucci and co-workers, PrP deletion was restricted to neurons only (Mallucci et al., 2002). In the tetracycline-responsive system used by Tremblay and colleagues, although adult mice remained well for >380 days following doxycycline-mediated suppression of PrP expression, neuronal PrP expression was not entirely abolished and lymphoid PrP expression prior to and following doxycycline treatment was not reported (Tremblay et al., 1998). Therefore, a compensatory mechanism operating in lymphocytes,

myeloid cells and their precursors in all $PrP^{-/-}$ mice thus far generated cannot be excluded.

A detailed molecular analysis of the downstream effects of PrP deletion has not been undertaken. Gene expression arrays in $PrP^{-/-}$ fibroblasts showed changes in expression of a number of genes, with marked downregulation of lipid raft protein CD44 and certain signalling molecules (Satoh et al., 2000). However, such studies have not been performed in lymphocytes.

1.4.9 PrP ablation versus ligation

In reviewing the available data on PrP^{C} function in the immune system, it is worth considering that embryonic deletion and antibody ligation of PrP are not necessarily modelling similar processes. There is increasing evidence that GPI-anchored proteins have signalling functionality in lymphocytes, principally through association with Src family non-receptor tyrosine kinases (Ilangumaran et al., 2000), with which PrP is known to associate. While loss of PrP alone may alter lipid raft composition and obviate any PrP-specific function, it is unlikely to impede the ability of other lipid raft components to signal via these pathways. Anti-PrP treatment is likely to induce additional effects, through disruption of protein-protein interactions in lipid rafts or excessive signalling via PrP, and possibly disrupting or enhancing key pathways shared with other GPI-anchored proteins. This is supported by discrepancies between the effects on T cell physiology of embryonic PrP deletion compared to anti-PrP mAb administration (Ballerini et al., 2006). Similarly, embryonic deletion of Thy-1 produces different effects on immune function to treatment of cells with anti-Thy-1 antibodies (Haeryfar and Hoskin, 2004).

1.4.10 Summary

A definitive function for PrP^C in the immune system, as in neurons, remains to be established. PrP^C appears to be robustly upregulated in T cell activation but its expression in T cells is not a pre-requisite for proliferation. Rather, PrP^C may be important in mediating pro-survival signals in cells undergoing multiple mitoses in stressful conditions, such as lymphopaenia or rapid memory cell expansion. Detailed analysis of immune responses in $PrP^{-/-}$ mice should provide further insights into the role of PrP^C but may require generation of conditional knockout models.

A greater understanding is also required of the effects of targeting PrP in the periphery as part of a therapeutic strategy as the consequences of ligation may be distinctly different from the phenotype produced by genetic ablation. Anti-prion agents that bind surface PrP^C may induce considerable changes in T cell physiology by disrupting lipid raft constituents, enhancing or blocking PrP signalling or endocytosis or by labelling bound cells or soluble PrP for elimination. Cross-linking surface PrP^C in T cells induces striking physiological changes reminiscent of T cell activation, but has also been shown to interfere with mitogenic activation. Thus it remains unclear whether PrP targeting will predispose the immune system to over-activation or hyporesponsiveness. Furthermore, preferential deletion or senescence of PrP^{high} immune cells may disrupt immune homeostasis. If high PrP^C expression is a particular characteristic of HSCs required for long term renewal, or specialised lymphocytes such as memory and regulatory cells, the effects on immune function may be profound. Interference in the function of key components in the immune system may cause severe immunopathology rendering such therapies unsuitable for long term use, despite the fact that anti-PrP therapy following peripheral exposure may need to be life-long. To date, immunisation with self-PrP or transgenic

expression of anti-PrP antibodies (Heppner et al., 2001b) have not been reported to cause immunopathology in mice. However, as discussed above, dermatitis with mononuclear cell invasion and destruction of hair follicles has been described in Lewis rats several months after immunisation with PrP 182-202 (Souan et al., 2001a).

GPI-anchored proteins such as the Campath-1 antigen (CDw52) have either proved tractable therapeutic targets in themselves or modifiers of agents directed against other cell surface constituents (Nagajothi et al., 2004). Thus, interest in the immune function of PrP^C extends beyond its role in prion disease. As an activation antigen and lipid raft component it may be a potential target for immunomodulatory therapy in other diseases. Further, PrP upregulation has been detected in a number of tumour lines (Du et al., 2005;Liang et al., 2006;Diarra-Mehrpour et al., 2004) and implicated in tumour invasion and metastasis (Pan et al., 2006). Because of the intense interest in PrP as the central mediator of prion diseases, many of the tools for studying the contribution of this protein to immune function are readily available. A serendipitous benefit of the tragedy of BSE and vCJD may be a lasting contribution to immunology and immunotherapeutics.

1.5 Conclusions and Aims

PrP represents an attractive target for therapeutic intervention in prion disease because it is the only substrate that is known to be essential for pathogenesis. Immunotherapy offers certain advantages over conventional therapeutics because the toxic effects of standard pharmaceuticals are avoided, and active vaccination may provide lifelong protection or a cure. However, there are certain obstacles to the development of active immunisation against prion disease. Principally it will be necessary to break tolerance to PrP. This has not been straightforward in animal models, and results of

protection studies in rodents have thus far been disappointing. Critically, the extent of tolerance to PrP in humans has never previously been considered, either as a marker of susceptibility to CJD or in relation to vaccine development.

Despite being subject to intense scrutiny, the normal function of PrP has yet to be definitively established. Work to date has not included a thorough description of the expression of the protein in specialised subsets of T lymphocyte. However, anti-PrP based therapeutics, if administered systemically, are likely to disrupt the physiological function of cells that express high levels of PrP. Thus, further work is required to characterise the expression and function of PrP within the immune system.

In the work presented here I have attempted to bring closer the goal of active vaccination against human prion disease by characterising the auto-PrP T cell repertoire in healthy individuals. In tandem I have studied the expression of PrP in T lymphocytes and the effects of PrP deletion and ligation on the immune system to better understand the function of the protein and predict the effects of it being therapeutically targeted.

CHAPTER 2 MATERIALS AND METHODS

2.1 Generic materials and methods

2.1.1 Human volunteers

Healthy human volunteers (n=28) were recruited to take part in the experiments described. All participants gave full informed consent. All work described here using human subjects was approved by the Hammersmith Hospital Research Ethics Committee (protocol 2003/6663).

2.1.2 Mice

Prnp$^{-/-}$ mice were originally made by Charles Weissman on a C57BL/6 x Sv129 background as described (Bueler et al., 1992). Two lines derived from the original Zurich I mouse were used here. The "F5" colony had been crossed onto the FVB/N background for 5 generations, and was housed in the MRC Prion Unit facility. These mice had been assessed by Charles Rivers as >96% homologous with wild type FVB/N (E Asante, personal communication). The "F10" colony used for some experiments had been crossed onto the FVB/N background for 10 generations and assessed by Charles Rivers Maxblast testing as being >99% FVB/N (E Asante, personal communication). Five breeding pairs of F10 mice were transferred to the animal facility at the Hammersmith Hospital for the work described here. All mice from the first litters produced by the transferred mice were tail biopsied and genotyped as below to confirm their knockout status. All experiments on PrP$^{-/-}$ mice used wild-type FVB/N mice (originally obtained from Harlan, UK) kept in the Hammersmith Hospital facility as controls. In all experiments comparing PrP knockout with wild-type mice age matched adult mice were used.

In some experiments wild-type C57BL/6 mice (originally obtained from Harlan, UK) from the Hammersmith facility were used. For EAE induction with PLP, female SJL mice were purchased from commercial suppliers (Harlan, UK) and kept in the Imperial College facility. Splenocytes or purified T cells from FVB/N mice carrying a human HLA-DR1 transgene (Altmann et al., 1995) were used in two experiments studying the biological effects of anti-PrP antibodies. Double transgenic C57BL/6 HLA-DR15 and MBP-TCR (line 7) mice were originally made in the Altmann laboratory as described (Ellmerich et al., 2005). These mice are prone to spontaneous autoimmune demyelination. However, all mice used here were free from clinical disease (score 0) at the time of sacrifice. All mice were housed in accordance with institutional and UK Home Office requirements.

2.1.3 Peptides

A library of 14-mer peptides overlapping by 7 amino acids spanning residues 23 to 225 of the human PrP sequence, plus a 13-mer consisting of residues 219 to 231 were synthesised by the Advanced Biotechnology Centre (ABC) (Imperial College London, UK). The two peptides spanning the M/V polymorphism at position 129 were synthesised in both alternate forms;129-methionine and 129-valine. Peptides were dissolved in PBS or DMSO. Peptides were coded 1 (most N-terminal) to 29 (most C-terminal). Peptide codes, positions, sequences and the number of donors with whose PBMCs each peptide was cultured in the first round of assays in Chapter 3 are shown in Table 2.1.

For EAE induction in SJL mice, a peptide corresponding to mouse PLP 139-151 was synthesized by the ABC. For EAE induction in FVB/N mice and for *in vitro* activation of splenocytes from line 7 mice, a peptide corresponding to mouse MBP

85-99 was synthesized by the ABC.

Peptide	Position	Sequence	No. of donors
1	23-36	KKRPKPGGWNTGGS	14
2	30-43	GWNTGGSRYPGQGS	14
3	37-50	RYPGQGSPGGNRYP	14
4	44-57	PGGNRYPPQGGGGW	14
5	51-64	PQGGGGWGQPHGGG	15
6	58-71	GQPHGGGWGQPHGG	15
7	65-78	WGQPHGGGWGQPHG	15
8	72-85	GWGQPHGGGWGQPH	15
9	79-92	GGWGQPHGGGWGQG	15
10	86-99	GGGWGQGGGTHSQW	15
11	93-106	GGTHSQWNKPSKPK	17
12	100-113	NKPSKPKTNMKHMA	17
13	107-120	TNMKHMAGAAAAGA	18
14	114-127	GAAAAGAVVGGLGG	20
15	121-134	VVGGLGGYMLGSAM	21
16	128-141	YMLGSAMSRPIIHF	21
17	135-148	SRPIIHFGSDYEDR	20
18	142-155	GSDYEDRYYRENMH	20
19	149-162	YYRENMHRYPNQVY	19
20	156-169	RYPNQVYYRPMDEY	19
21	163-176	YRPMDEYSNQNNFV	19
22	170-183	SNQNNFVHDCVNIT	19
23	177-190	HDCVNITIKQHTVT	18
24	184-197	IKQHTVTTTTKGEN	19
25	191-204	TTTKGENFTETDVK	20
26	198-211	FTETDVKMMERVVE	18
27	205-218	MMERVVEQMCITQY	21
28	212-225	QMCITQYERESQAY	20
29	219-231	ERESQAYYQRGSS	21
15V	121-134*	VVGGLGGYVLGSAM	20
16V	128-141*	YVLGSAMSRPIIHF	20

Table 2.1 Codes, positions and sequences of human PrP peptides and number of donors with whose PBMCs each peptide was cultured in the first round of assays in Chapter 3. * denotes peptides with valine at position 129.

2.1.4 Isolation of human PBMCs

PBMCs were isolated from anticoagulated blood by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, Dorset, UK), according to the manufacturers instructions. All samples were processed within one hour of venesection. The buffy coat was extracted by aspiration with a Pasteur pipette and washed three times in PBS. PBMCs were resuspended in tissue culture medium and counted by eosin exclusion prior to downstream application. In some cases PBMCs were suspended in "freeze medium" (90% fetal calf serum, 10% DMSO) and stored at -80°C prior to use.

2.1.5 Preparation of single cell suspensions from lymphoid organs

Mice were sacrificed by CO_2 intoxication or cervical dislocation. Under asepsis, spleens and where relevant, thymus and peripheral lymph nodes (axillary, inguinal and mesenteric) were dissected from freshly culled animals. Organs were placed directly into HL-1 (Cambrex) or RPMI (Invitrogen) culture medium. Single cell suspensions were made by extruding cells from spleens by needle dissection or by mashing tissues through a 70 μm filter. In some experiments splenocytes were suspended for up to 5 minutes in red cell lysis buffer (0.15 M NH_4Cl, 100 mM $KHCO_3$, 100 μM $Na_2EDTA.2H_2O$). Splenocytes were washed and resuspended in 5 ml medium for counting by Eosin exclusion.

2.1.6 Preparation of murine PBMCs for downstream applications

Blood was aspirated by cardiac puncture immediately following sacrifice. Blood samples were anticoagulated with heparin, washed and then incubated for 5 minutes with red cell lysis buffer. Cells were washed again and resuspended in 5 ml medium for counting by Eosin exclusion.

2.1.7 DNA extraction from mouse tail biopsies

Tail biopsies were digested by overnight incubation at 55°C in 400 µl tail lysis buffer with 1 ug/ml Proteinase K. 1.5 ml tubes containing tail digests were placed on ice and 160 µl Protein precipitation solution (Promega) was added per tube. After five minutes on ice tubes were spun for 5 minutes at maximum speed in a bench-top microcentrifuge. Supernatants were transferred to fresh 1.5 ml tubes and 450 µl isopropranol added per tube. Tubes were shaken to precipitate DNA after which tubes were spun as before. Supernatants were discarded and pellets air dried for 30 minutes. 200 µl 75% ethanol was added per tube and tubes spun as before. Supernatants were discarded and pellets air dried before being dissolved in 200 µl TE buffer per tube. DNA samples were then kept at 4°C.

2.1.8 Confirmation of *Prnp* knockout status by PCR

The PCR for discrimination between wild-type *Prnp* and the Zurich I $PrP^{-/-}$ knockout construct was previously designed in the MRC Prion Unit as a duplex reaction with a common forward primer (P10), a wild-type mouse *Prnp* reverse primer (P4) and a neomycin cassette reverse primer (P3). Primers were purchased from Sigma and had the following sequences:

P3: ATT CGC AGC GCA TCG CCT TCT ATC GCC

P4: CCT GGG AAT GAA CAA AGG TTT GCT TTC AAC

P10: GTA CCC ATA ATC AGT GGA ACA AGC CCA GC

Tubes for polymerase chain reaction were set up on ice with a final reaction volume of 20 µl as follows:

2.5 µl 10x buffer

2.5 µl 15 mM $MgCl_2$

1.25 µl 2% Tween

5 µl dNTPs

0.125 µl Taq

1 µl 10 µM P3 primer

1 µl 100 µM P4 primer

1 µl 50 µM P10 primer

1 µl DNA

Polymerase chain reaction was then performed on a PTC-100 thermocycler (Peltier) with cycle conditions as follows:

(1) 94°C for five minutes

(2) 94°C for 30 seconds, then 58.5°C for 45 seconds, then 72°C for 90 seconds

(3) repeat (2) 33 times

(4) 72°C for 10 minutes

PCR products were run out on a 1.5% agarose gel at 110-120V for 40 minutes and visualised by Ethidium bromide fluorescence under UV light. Hyperladder I (Bioline) was used as a size marker. Knockout bands were identified as an ~ 800 bp band, readily discriminated from the larger (~ 1000 bp) wild type *Prnp* band (Figure 2.1).

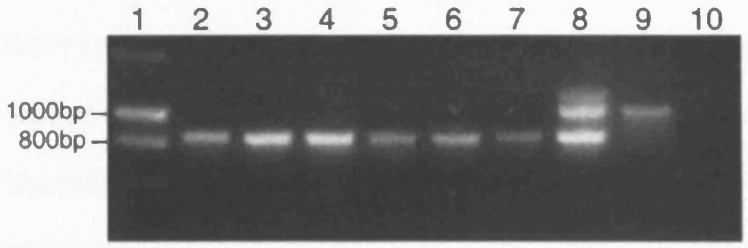

Figure 2.1 PCR to discriminate *Prnp*$^{-/-}$ from wild-type mice

Lane 1, Hyperladder I; lanes 2-7, samples from *Prnp*$^{-/-}$ mice; lane 8, sample from heterozygous *Prnp*$^{+/-}$ mouse; lane 9, sample from FVB/N wild-type mouse; lane 10, H_20.

2.1.9 Mitogens and antigens

The following were used as mitogenic stimuli: SEA (Sigma), SEB (Toxin Technologies), Concanavalin A (Sigma), PMA (Sigma) and Ionomycin (Sigma). Hen egg lysosyme (HEL) and ovalbumin (OVA) were purchased from Sigma. Mouse spinal cord homogenate was made by homogenising spinal cords from freshly culled adult FVB/N and 129/Sv x B6 mice into PBS, followed by freezing to -80°C and lyophilisation. Lyophilised material was then dissolved in PBS at 20 mg/ml.

2.1.10 Negative selection of murine T and B lymphocytes

Splenocytes were suspended at $1x10^8$/ml in PBS with 0.1% BSA (Sigma). T or B cells were negatively selected using the appropriate kit for "untouched" T or B isolation (Dynal) according to the manufacturer's instructions. Purity of isolated T and B cells was determined by flow cytometry to be > 96%. For the functional Treg assay the "untouched" $CD4^+$ T cell isolation kit (Dynal) was used.

2.1.11 *In vitro* culture of human and murine cells

After counting, cells were placed in 96-well plates at indicated densities, except for line 7 activation studies where 24-well plates were used. Antigens, mitogens, antibodies and/or inhibitors were added as described for individual experiments. Human PBMCs were cultured in RPMI (Invitrogen,) or IMDM (Cambrex) supplemented with 5% non-autologous human AB serum (Cambrex), 1% L-glutamine (Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen), or in HL-1 serum-free medium (Cambrex) supplemented with 1% L-glutamine and 0.5% Penicillin-Streptomycin. Murine splenocytes or lymphocytes were cultured in HL-1 supplemented as above except where stated. All incubations were performed at 37°C with 5% CO_2.

2.1.12 Antibodies

In-house anti-PrP mouse IgG1 monoclonal ICSM18 (D-Gen Ltd) was used for *in vivo* EAE studies and *in vitro* blocking studies. A mouse IgG1 mAb without murine antigen specificity from the MOPC21 clone (Sigma) was used as a control in functional assays and *in vivo* studies. Functional grade anti-CD3 and anti-CD28 for *in vitro* T cell activation were purchased from eBioscience. Biotinylated anti-mouse CD25 (clone 7D4) was purchased from BD for the Treg isolation protocol.

For flow cytometry, ICSM18 was FITC-conjugated using the FluoroTag-FITC kit (Sigma) according to the manufacturer's instructions. FITC-conjugated mouse IgG1 (eBioscience) was used as a control for ICSM18-FITC. Other fluorophor-conjugated antibodies (and isotype controls) were purchased from eBioscience as follows. FITC-conjugated: anti-mouse CD4, anti-mouse Qa2, anti-mouse Thy1.2. PE conjugated: anti-human CD44, anti-human CD62L, anti-mouse CD4, anti-mouse CD25, anti-

mouse CD44, anti-mouse CD45RB, anti-mouse CD62L, anti-mouse CD69, anti-mouse OX40, anti-mouse ICOS, anti-mouse Foxp3. PECy5 conjugated: anti-human CD4, anti-mouse CD4, anti-mouse CD8. APC conjugated: anti-mouse Foxp3, anti-mouse CD25. Fluorophor-conjugated antibodies (and isotype controls) were also purchased from BD as follows: FITC-conjugated: anti-mouse CD3. PE conjugated: anti-human CD4, anti-mouse $I^{A/E}$. PECy5 conjugated: anti-human CD8, anti-human CD25.

2.1.13 Flow cytometry

All analytical flow cytometry was performed on single or double-laser FacsCalibur machines (Becton Dickinson) and data was analysed using CellQuest software. At least 10,000 events were acquired in each assay. For analysis, an initial gate was drawn around viable lymphocytes using forward (FSC) and side (SSC) scatter characteristics. Further gates were then applied to identify $CD4^+$ or $CD8^+$ cells. Expression of markers of interest was then defined using dot plots or histograms. For analysis of PrP expression, the geometric mean values for PrP and an isotype control (measuring auto-fluorescence and non-specific binding) were obtained. PrP expression was defined as the difference between these two values (Δ geometric mean). In some experiments, cells were split into PrP^{high} and ^{low} populations. In human PBMCs, PrP expression is not normally distributed but bimodal with PrP^{high} and ^{low} peaks, facilitating discrimination between populations on a histogram (Figure 2.2). In murine lymphocytes, PrP expression has a single peak. The cut off between PrP^{high} and ^{low} murine lymphocytes was determined using a FITC-conjugated IgG1 antibody control for ICSM18. Quadrants were applied to a dot plot of isotype antibody binding against that of the antibody for the T cell marker of interest (eg. CD62L). The horizontal bar was applied at a point on the FL1 axis such that < 1% of

cells were above the line. When the quadrants were applied in the same position for plots of ICSM18 binding against the same T cell marker, cells above the line were considered PrP^{high} and those below PrP^{low}.

For analysis of purity of negatively isolated T and B cells, dot plots of CD3 against MHC class II binding were constructed. For analysis of purity of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ cells, dot plots of CD4 against CD25 binding were constructed. Quadrants were then applied to ascertain the proportions of single and double positive and negative cells in each sample.

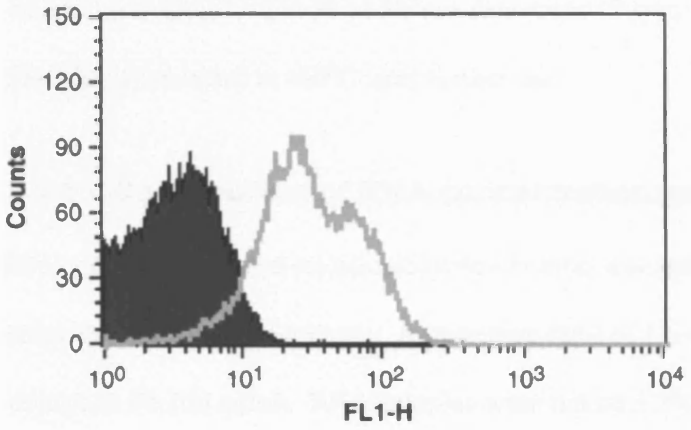

Figure 2.2 Flow cytometry histogram of human PBMCs
Cells are labelled with FITC-conjugated anti-PrP (grey line) or isotype control (filled black line). Two peaks of PrP staining are seen, corresponding to PrP^{high} and ^{low} populations.

2.1.14 RNA extraction from murine splenocytes

Cells were lysed in Trizol (Invitrogen), using 1 ml for $>1x10^6$ cells and 100 µl per $1x10^5$ cells for samples containing less than $1x10^6$ cells. After lysis, samples were stored at -80°C pending RNA isolation. After thawing, 200 µl chloroform (BDH) was

added per 1 ml Trizol. Tubes were shaken for 15 seconds and left at room temperature for 2-3 minutes. Samples were then spun for 15 minutes at 12,000 g in a bench-top centrifuge at 4°C. This separated the sample into a lower red, phenol-chloroform phase and a colourless upper phase. The latter was transferred to a fresh tube to which 500 µl isopropranol was added per 1 ml Trizol. Samples were left at room temperature for 10 minutes and then spun for 10 minutes as above. The supernatant was discarded and the RNA pellet washed with 1 ml 75% ethanol per 1 ml Trizol. After vortexing briefly samples were spun at 7,500 g for 5 minutes as above. The supernatant was removed and the pellet air dried for 10-15 minutes, after which it was dissolved in 30 µl RNase-free water (Sigma) by incubation at 55-60°C. Samples were stored at -80°C until further use.

2.1.15 Confirmation of RNA concentration, purity and integrity

RNA concentration and nucleic acid/protein ratio was analysed using a Nanodrop spectrophotometer (Nanodrop). A minimum ratio of 1.6 was accepted. RNA was diluted to 60-200 ng/ml. RNA samples were run on 1.5% agarose gel and visualised by Ethidium bromide fluorescence under UV light to confirm the presence of intact 18S and 28S ribosomal bands without genomic DNA contamination.

2.1.16 cDNA preparation

This was performed on ice in a 50 µl reaction volume consisting of the following reagents:

5x RT buffer (Invitrogen)	10 µl
50 µM random primers (Invitrogen)	2.5 µl
100 mM dNTP mix (Amersham Biosciences)	2.5 µl

50 mM $MgCl_2$ (Bioline)	5 µl
100 mM DTT (Invitrogen)	5 µl
40 U/µl RNase OUT (Invitrogen)	2.5 µl
200 U/µl Super Script III RNase HRT (Invitrogen)	1.25 µl
250 pg RNA	1.25-5 µl
RNase free water	12-16.25 µl

After mixing, samples were incubated on a PTC-100 thermocycler (Peltier) at 50°C for 50 minutes, followed by 85°C for 5 minutes before being cooled to 4°C prior to immediate use or storage at -20°C.

2.1.17 Quantitative real time polymerase chain reaction

Quantitative analysis of *Prnp*, *Foxp3* and *18S* transcript levels was performed using Taqman assays on an Mx3000P real time PCR system (Stratagene). PCR primers with TaqMan MGB probes (FAM dye-labeled) for *Prnp*, *Foxp3* and *18S* were obtained (Applied Biosystems). Values for each transcript were obtained by running monoplex PCR reactions (in triplicate) for each sample. Reactions were set up on ice in a 20 µl volume as follows:

TaqMan universal PCR master mix (Applied Biosystems)	10 µl
20x "Assays-on-Demand" mix (*Prnp*, *Foxp3* or *18S)* (Applied Biosystems)	1 µl
cDNA	2 µl
RNase free water	7 µl

PCR cycling conditions were as follows:

(1) 50°C for 2 minutes

(2) 95°C for 10 minutes

(3) 95°C for 15 seconds followed by 60°C for 1 minute

(4) 49 repeats of (3)

2.1.18 Standard curves for RT-PCR primers

Standard curves were constructed by running PCR reactions on a series of 5-fold serial dilutions of cDNA. The PCR amplification signal was expressed as a ΔR value, reflecting the intensity of reporter dye emission for each sample at the end of each cycle minus baseline signal during the initial PCR cycles. The threshold was set at a ΔR of 2411.55 (in the linear region of ΔR against cycle number in a semi-log plot). Efficiencies of the PCR reactions for *Prnp*, *Foxp3* and *18S* were compared by plotting cycle number at which the threshold value was obtained (C_T) against log (dilution). Efficiencies of the *Prnp* and *18S* standard curves were 99.7% and 98% respectively, while the *Foxp3* PCR had an efficiency of 76.6%.

2.1.19 $\Delta\Delta C_T$ method for relative quantitation of *Prnp* transcription

Mean C_T values for *Prnp* and *18S* were obtained for each experimental sample. The abundance of *Prnp* transcript in each sample was then calculated relative to *18S* quantity using the $\Delta\Delta C_T$ method. *18S* is a housekeeping gene expressed at high levels that is proposed not to alter significantly with cell activation. It can therefore be used as an indicator of the number of cells that were represented in each sample and thus allow comparison of *Prnp* (or any other gene of interest) expression between samples in which the exact starting amount of cDNA cannot be calculated with accuracy. For each sample the amount of *Prnp* transcription was first normalised to its *18S* value using the equation:

$$\Delta C_T\ (Prnp) = C_T\ (Prnp) - C_T\ (18S)$$

One sample (S_0) was then used as a "baseline" value, typically, a timepoint 0 sample in an activation assay or a $CD4^+$ $CD25^-$ sample in the regulatory T cell study. *Prnp* transcription in all other experimental samples (S_X) was then calculated relative to this value using the equation:

$$Prnp\ (S_X) = 2^{-(\Delta C_T\ (S_X) - \Delta C_T\ (S_0))}$$

2.1.20 Standard curve method for quantitation of *Foxp3* transcription

Because the efficiencies of the *Foxp3* and *18S* real time reactions were divergent, the $\Delta\Delta C_T$ method could not be used to calculate relative *Foxp3* abundance. Instead, relative transcript quantities for each sample were calculated from C_T values using the standard curve equation for each primer. *Foxp3* expression in each sample was then normalised to *18S* expression, allowing direct comparison between samples.

2.1.21 Statistical analysis

All statistical analysis was performed using GraphPad InStat (GraphPad Software, San Diego, CA, USA). The following tests were used: 't' test, paired 't' test, ANOVA with Bonferroni or Tukey-Kramer post-test correction, Mann-Whitney U test. Where appropriate, results were compared as two by two contingency tables using Fisher's exact test. All line graphs and bar charts were initially constructed using Microsoft Excel. All error bars represent standard deviations unless stated otherwise.

2.2 Specific methods for experiments in chapter 3

2.2.1 Culture of human PBMCs with PrP sequence peptides

After isolation and washing PBMCs were cultured in RPMI, IMDM, or HL-1 medium supplemented as above. PBMCs were suspended in flat-bottomed 96 well plates at a density of $5x10^5$ cells per well in 200 µl culture medium. PrP peptides were added to triplicate wells at a final concentration of 50 µg/ml. Control wells contained cells and tissue culture medium only. Where peptides were dissolved in DMSO, triplicate wells containing the same final concentration of vehicle were used as controls. In the first round of assays, sufficient PBMCs were harvested from 14 donors for all 31 peptides to be tested. For the remaining 7 donors, PBMCs were cultured with selected peptides. Each peptide was cultured with PBMCs from at least 14 donors (Table 1).

96 well plates were incubated for 6 days at 37°C, 5% CO_2 and were then pulsed overnight with 1 µCi [^{3}H]-thymidine per well. Plates were harvested and counted with a β-counter (Wallac, Turku, Finland). Stimulation indices (S.I.) were obtained by dividing the mean counts per minute in the peptide treated triplicate wells by the mean counts per minute (c.p.m.) in the relevant control triplicates. A response was considered positive when the S.I. was >2 and the c.p.m. in at least two of the three peptide treated wells were >2 x the mean of the control wells.

2.2.2 Cytokine quantification

For the initial cytokine analysis, unused PBMCs were frozen at -80°C until required. After thawing, cells were washed and cultured in supplemented IMDM as above with or without PrP peptides at 50 µg/ml. In the extended cytokine analysis, fresh *ex vivo* PBMCs were used. On day 6, 100 µl of culture medium was removed from each well

and replaced with 100 µl of new medium containing 1 µCi [^{3}H]-thymidine. Aspirated 100 µl culture medium samples from triplicate control and peptide treated wells were pooled and stored at –20°C. After overnight incubation, plates were harvested and peptides eliciting positive responses identified as above. Cytokine levels were then quantified in culture medium from control and positive peptide treated wells using the Cytometric Bead Array (CBA) Human Th1/Th2 cytokine kit II (BD Biosciences, Oxford, UK), according to the manufacturer's instructions. CBA assays were provided as a service by the Imperial College London flow cytometry core or performed by Dr Rebecca Ingram in the Altmann laboratory.

2.2.3 *PRNP* codon 129 allele discrimination and HLA-typing

DNA was extracted from whole EDTA-anticoagulated blood using the Nucleon kit (Amersham Biosciences, Little Chalfont, UK).

HLA typing was performed by the Oxford Radcliffe Hospitals Transplant Immunology Laboratory by polymerase chain reaction with sequence-specific primers (PCR-SSP) using a 192 primer mix modification of the Phototyping set for HLA-A,B,Cw,DRB1,DRB3/4/5 and DQB1 (Bunce et al., 1995). PCR conditions were also modified as previously described (Bunce et al., 1999).

PRNP codon 129 genotype was determined by real time PCR using allele discrimination on an ABI SDS 7000 thermal cycler (Applied Biosystems, Foster City, CA, USA) using standard conditions. Amplification primers were as follows: forward 5'-tca gtg gaa caa gcc gag taa g-3', reverse 5'-cat agt cac tgc cga aat gta tga t-3' and allele discrimination probes were 129M 5'-6-FAM-cgg cta cat gct gg-MGB-3' and 129V 5'-VIC-cgg cta cgt gct gg-MGB-3'.

2.2.4 *In silico* HLA binding prediction

The TEPITOPE algorithm (Vaccinome, Kearny, NJ, USA) predicts epitopes that may be able to bind up to 25 HLA-DR molecules offering a wide cross-section of human HLA class II specificities. The amino acid sequence of human PrP 23-231 (129M and 129V) was entered into TEPITOPE using standard protocols (Bian et al., 2003;Bian and Hammer, 2004). Stringency was set at 3% and sequences of 9-mer epitopes were obtained.

2.3 Specific methods for experiments in chapter 4

2.3.1 Activation of wild-type FVB/N splenocytes

Splenocytes prepared from four 13 week old female FVB/N mice were placed in round bottomed 96-well plates in supplemented HL-1 medium at a density of $3x10^5$ cells per well. Anti-CD3 and anti-CD28 were added to certain wells at 0.6 μg/ml and 2 μg/ml respectively. Control (non-activated) wells contained cells and medium alone. Plates were incubated at 37°C, 5% CO_2 for indicated periods.

2.3.2 Preparation of activated FVB/N splenocytes for flow cytometry

Plates were spun down and cells resuspended in 100 μl/well RPMI supplemented with 1% FCS. ICSM18-FITC, anti-CD25-APC, anti-CD69-PE and anti-CD4-PECy5, or isotype controls were added to wells. Plates were incubated at 4°C for 30 minutes, washed in PBS and resuspended in 200 μl/well Fix/Perm buffer (eBioscience) for at least 30 minutes at 4°C. Cells were washed in Permeabilisation buffer (eBioscience) prior to analysis by flow cytometry.

2.3.3 Activation of DR15/anti-MBP-TCR transgenic (line 7) splenocytes

Splenocytes prepared from adult line 7 mice were placed in 24-well plates in supplemented HL-1 medium at a density of $2x10^6$/ml. MBP 85-99 peptide was added to certain wells at 2 μg/ml while control wells contained cells and medium alone. In some experiments, Cycloheximide (Sigma) was added to wells at 10 μg/ml. Plates were incubated at 37°C, 5% CO_2 for indicated periods. Cells were then harvested by repetitive pipetting at indicated timepoints for RNA extraction or flow cytometry.

2.3.4 Preparation of activated line 7 splenocytes for flow cytometry

Cells were placed in FACS tubes (approximately $5x10^5$ cells per tube) and washed in PBS, usually supplemented with 0.1% BSA. ICSM18-FITC, anti-CD4-PE or anti-CD4-PECy5, anti-CD69-PE, anti-CD25-PE, anti-ICOS-PE, anti-OX40-PE, anti-CD44-PE, anti-Qa2-FITC or anti-Thy1.2-FITC or isotype controls were added. Tubes were incubated at 4°C for 30-40 mins and then washed in PBS. Cells were then analysed immediately or treated with CellFix (Becton Dickinson) and stored at 4°C for up to 24 hours pending analysis by flow cytometry.

2.3.5 Comparison of proliferation, cytokine production and conjugation between $PrP^{+/+}$ and $^{-/-}$ splenocytes

Splenocytes from three wild-type FVB/N and three $PrP^{-/-}$ F5 (five generations crossed to FVB/N) mice (all males age 9-10 weeks) were prepared in parallel for *in vitro* experiments as described below.

2.3.6 Footpad immunisations of FVB/N $PrP^{+/+}$ and $^{-/-}$ mice

Ovalbumin (OVA) and hen egg lysozyme (HEL) were emulsified in CFA at a concentration of 1 mg/ml. 50 µl of emulsion was injected into the left footpad as below. Four FVB/N $PrP^{+/+}$ and five F10 (ten generations crossed to FVB/N) $PrP^{-/-}$ mice (all females age 9 weeks) received OVA. Four FVB/N $PrP^{+/+}$ and four F10 $PrP^{-/-}$ mice (all males age 12-18 weeks) received HEL. 10 days later, mice were sacrificed by cervical dislocation and the left popliteal nodes dissected aseptically and placed in RPMI. Single cell suspensions were made by mashing lymph nodes through 70 µm cell strainers. After counting by eosin exclusion cells were placed in round-bottomed 96 well plates in supplemented HL-1 at a density of $3x10^5$ cells per well with HEL or OVA at indicated concentrations as described below.

2.3.7 Stimulation of FVB/N $PrP^{+/+}$ and $^{-/-}$ splenocytes or lymphocytes

Cells were cultured in 96-well plates in supplemented HL-1 at a density of $3x10^5$ cells per well with mitogens (SEA, SEB, Concanavalin A, PMA/Ionomycin, anti-CD3 & CD28) or antigens (HEL or OVA) at indicated concentrations (three wells per concentration). After 48 hours, 50 µl of culture medium was aspirated from each well. Aspirates from triplicate wells were pooled and frozen at -20°C pending cytokine analysis with the CBA mouse Th1/Th2 cytokine kit I (BD Biosciences, Oxford, UK) by the Imperial College flow cytometry service. Wells were topped-up with 50 µl HL-1 containing 1 µCi [^{3}H]-thymidine and incubated overnight before being harvested for, or frozen pending, analysis of isotope uptake with a β-counter.

2.3.8 Staining of FVB/N $PrP^{+/+}$ and $^{-/-}$ T and B cells with fluorescent dyes

T and B cells were isolated with beads as described. T cells were suspended at $5x10^6$/ml in HL-1 medium containing 1 μM CFDA (Molecular Probes). B cells were suspended at $5x10^6$/ml in HL-1 medium containing 10 μM Snarf-1 (Molecular Probes). Cells were incubated at 37°C for 30 minutes, then washed in HL-1 medium. CFDA-stained cells were rested for 1 hour at 37°C, 5% CO_2 prior to further use.

2.3.9 T and B cell conjugation with superantigen

CFDA-stained T cells and Snarf-1-stained B cells at were mixed at a ratio of 1:2 in HL-1 medium to a total cellular density of $5x10^6$/ml. Staphylococcal enterotoxin A (SEA) (Sigma) was added at a final concentration of 5 μg/ml. Cells were incubated at 37°C, 5% CO_2 in the dark for 2-6 hours as indicated. At the end of incubation an equal of volume of 2x CellFix (BD) was gently laid over the cell suspension. Tubes were placed at 4°C for 15 minutes, then washed in PBS. Pellets were gently resuspended in FACSFlow (BD) for detection by flow cytometry.

2.3.10 FACS detection of T-B cell conjugates

Gating was applied to eliminate dead cells and non-lymphocytes. CFDA-stained T cells were detected in the FL-1 channel (green) and Snarf-1-stained B cells in the FL-2 channel (red). Conjugates were seen as a double positive population (figure 2.3). The efficiency of conjugate formation was measured as a percentage of total acquired T cells ie. total number of conjugates/(total number of conjugates + total number of unconjugated T cells) x100.

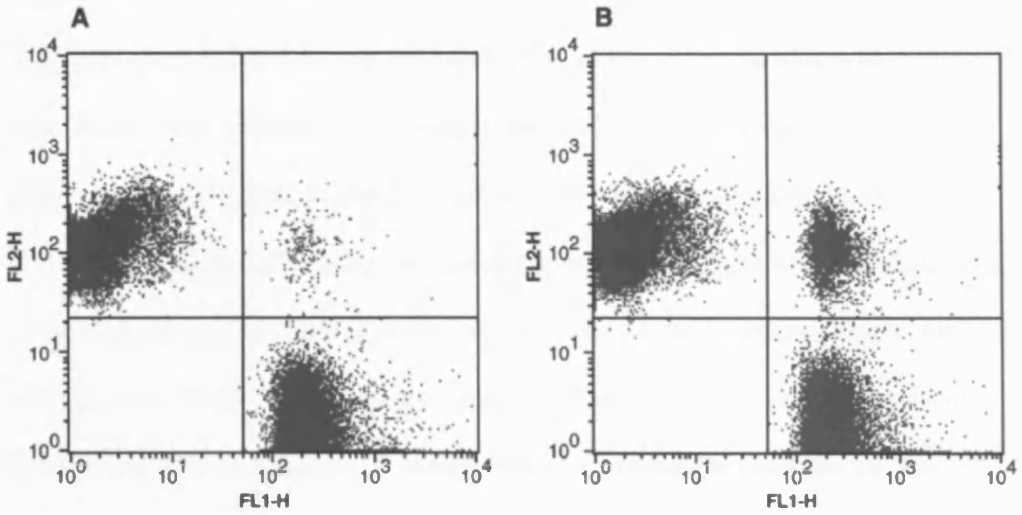

Figure 2.3 Flow cytometry dot plots of mixed T cells and B cells with (B) and without (A) superantigen. CFDA-stained T cells are bright in the FL1 channel (lower right quadrants) while Snarf-1 stained B cells are bright in the FL2 channel (upper left quadrants). In the presence of superantigen an appreciable number of T-B cell conjugates are formed, detected as double positive events (B, upper right quadrant).

2.4 Specific methods for experiments in Chapter 5

2.4.1 Assessment of $CD4^+$, $CD8^+$ and Treg numbers in FVB/N $PrP^{+/+}$ and $^{-/-}$ mice

Thymi and inguinal, axillary and mesenteric lymph nodes were dissected from four FVB/N $PrP^{+/+}$ and four F10 $PrP^{-/-}$ mice (all males age 10 weeks). Spleens were dissected from four further mice of each genotype (all males age 11-12 weeks). Inguinal and axillary nodes from individual mice were pooled. Single cell suspensions were made by mashing organs through 70 μm cell strainers. After

washing and counting by eosin exclusion cells were placed in round bottomed 96-well plates in RPMI supplemented with 1% FCS at a density of $1x10^6$ cells per well. Anti-CD4-FITC, anti-CD25-PE, anti-CD8-PECy5 or isotype controls were added to each well. Plates were incubated for 30 minutes at 4°C. After washing 200 µl Fix/Perm solution (eBioscience) was added to each well. Plates were incubated overnight at 4°C. After washing, cells were resuspended in permeabilisation buffer (eBioscience) containing 0.5 µg Fc-block (eBioscience) per well. After incubation at 4°C for 15 minutes, anti-Foxp3-APC or isotype control was added to each well. Plates were incubated at 4°C for a further 30 minutes prior to washing and analysis by flow cytometry.

2.4.2 Assessment of memory cell numbers in spleens from $PrP^{+/+}$ and $^{-/-}$ mice

$1x10^6$ splenocytes from eight FVB/N $PrP^{+/+}$ and eight F10 $PrP^{-/-}$ mice (all males age 11-22 weeks) were placed in FACS tubes or wells of a round bottomed 96-well plate. Anti-CD4-FITC, anti-CD8-PECy5, anti-CD44-PE, anti-CD45RB-PE, anti-CD62L-PE or isotype controls were added and cells incubated at 4°C for 40-45 minutes. After washing cells were immediately analysed by flow cytometry or treated with CellFix (BD) and stored at 4°C overnight prior to analysis.

2.4.3 Correlation of expression of PrP with memory markers and CD25 in C57/BL6 splenocytes

$5x10^5$ splenocytes from three adult female C57BL/6 mice were placed in FACS tubes. ICSM18-FITC, anti-CD4-PECy5, anti-CD8-PECy5, anti-CD25-PE, anti-CD44-PE, anti-CD45RB-PE or isotype controls were added and tubes incubated at 4°C for 30-45 minutes. Splenocytes from a further three adult female mice were treated identically

but stained with ICSM18-FITC, anti-CD4-PECy5 and anti-CD62L-PE. After washing cells were analysed immediately by flow cytometry.

2.4.4 Analysis of PrP expression in human PBMCs

Cryopreserved PBMCs from three donors were thawed, washed and resuspended in IMDM supplemented as above for counting by eosin exclusion. 4 x 10^5 cells were placed in FACS tubes, to which anti-CD4-PE or -PECy5, or anti-CD8-PECy5, plus anti-CD44-PE, antiCD62L-PE or anti-CD25-PECy5 (or isotype controls), plus ICSM18-FITC (or isotype control) were added. Tubes were incubated at 4°C for 45 minutes followed by washing in PBS supplemented with 0.1% BSA and immediate acquisition on a flow cytometer.

2.4.5 Expression of PrP in Tregs in FVB/N mice

1x10^6 splenocytes from four 10-13 week old FVB/N mice were placed in wells of a round bottomed 96-well plate in RPMI supplemented with 1% FCS. ICSM18-FITC, anti-CD4-PECy5, anti-CD25-APC or isotype controls were added. Cells were incubated at 4°C for 30 minutes. After permeabilisation with FixPerm solution (eBioscience) cells were kept overnight at 4°C. After washing, anti-Foxp3-PE or isotype control was added to each well and cells further incubated at 4°C for 30 minutes. Cells were washed and analysed immediately by flow cytometry.

2.4.6 Isolation of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ splenocytes for comparison of *Prnp* and *Foxp3* expression

Splenocytes from six 12-20 week old female C57BL/6 mice were suspended at 1x10^7/ml in PBS with 0.1% BSA. Anti-CD4-PECy5 and anti-CD25-PE were added and cells incubated at 4°C for 30-45 minutes. After washing, cells were resuspended

in FACS sort buffer (Ca^{2+}/Mg^{2+}-free PBS supplemented with 1 mM EDTA, 25 mM HEPES, 1% FCS) at $1x10^7$/ml. FACS sorting was provided as a service by the MRC CSC flow cytometry core. Briefly, after gating round lymphocytes by FSC and SSC characteristics, $CD4^+$ cells were identified by fluorescence in the FL3 channel. Gates were drawn around $CD25^+$ and $CD25^-$ fractions, distinguished by high or low fluorescence in the FL2 channel, respectively. These were then collected simultaneously into separate tubes. Retrieved cells were dissolved in Trizol and RNA extracted as described above.

2.4.7 Functional studies of $PrP^{+/+}$ and $^{-/-}$ FVB/N murine Tregs

These experiments were performed in collaboration with Dr Oliver Garden in the Regulatory T cell Laboratory, Hammersmith Hospital, Imperial College. Briefly, single cell suspensions were made as above from spleens of 6-12 week old $PrP^{+/+}$ and $^{-/-}$ FVB/N mice. $CD4^+$ cells were negatively isolated using beads (Dynal) according to the manufacturer's instructions. These were further fractionated into $CD25^+$ and $CD25^-$ populations by incubation with biotinylated anti-CD25 followed by Streptavidin MicroBeads (Miltenyi Biotech). $CD4^+$ $CD25^+$ T cells were then positively selected from $CD4^+$ $CD25^-$ cells on MiniMACS magnetic columns (Miltenyi Biotech). The purity of retrieved cells was determined by flow cytometry to be > 84% for $CD4^+$ $CD25^-$ cells and > 92% for $CD4^+$ $CD25^+$ cells. There was no significant difference in the purity of $PrP^{+/+}$ compared to $PrP^{-/-}$ cells. Purified $CD4^+$ $CD25^+$ T cells ($1x10^5$/well) were cultured, in 96-well round-bottomed plates, with $CD25^-$ T cells at indicated ratios in the presence of Epoxy DynaBeads (1 bead/5 cells; Dynal) coated with anti-CD3 and anti-CD28 mAb. After 3 days, incorporation of tritiated thymidine over 16 hours was measured as above.

2.5 Specific methods for experiments in chapter 6

2.5.1 Inhibition of MHC-peptide induced proliferation with ICSM18

Splenocytes from 16 week old line 7 mice were seeded onto a 96-well plate at a density of $3x10^5$ cells per well. MBP 85-99 peptide (2 μg/ml) was added to half the wells. Either anti-PrP monoclonal antibody (ICSM18) or a murine IgG1 control antibody were added to triplicate wells at 50, 10, 2 and 0 μg/ml. The plate was incubated as above for 48 hours, followed by overnight thymidine pulsing for assessment of proliferation.

2.5.2 Inhibition of SEB induced proliferation with ICSM18

Splenocytes from a 7 week old male DR1 tg mouse were placed onto a round bottomed 96-well plate at a density of $3x10^5$ cells per well in RPMI supplemented with 10% fetal calf serum (Invitrogen), 1% L-glutamine and 1% Penicillin-Streptomycin. SEB (1 μg/ml) was added to each well. ICSM18 or mouse IgG1 control mAb was added to quadruplicate wells at 100, 10, 1, 0.1, or 0 μg/ml. The plate was incubated as above for 48 hours, followed by overnight thymidine pulsing for assessment of proliferation.

2.5.3 Inhibition of anti-CD3 and anti-CD28 induced proliferation with ICSM18

T cells were isolated using beads (Dynal) from the spleen of a 7 week old male DR1 transgenic mouse. Cells were placed onto a round bottomed 96-well plate at a density of $1x10^5$ cells per well. Anti-CD3 (0.01 μg/ml) and anti-CD28 (0.1 μg/ml) were added to each well. ICSM18 or mouse IgG1 control mAb was added to triplicate wells at 10, 1, 0.1, 0.01, or 0 μg/ml. The plate was incubated as above for 48 hours,

followed by overnight thymidine pulsing for assessment of proliferation.

2.5.4 Induction of Experimental Autoimmune Encephalomyelitis

An emulsion was prepared by mixing 2 mg/ml PLP 139-151 or MBP 85-99 or 20 mg/ml mouse spinal cord homogenate with an equal volume of Complete Freund's Adjuvant (Sigma) supplemented with 1 mg/ml M. Tuberculosis and M. Butyricum (Difco Microbiology, Detroit) in an 8:1 ratio. The mixture was vortexed for 30 minutes, then sonicated for 30 minutes. Mice received 100 µl emulsion subcutaneously to opposite flanks on days 0 and 7. 200ng Pertussis toxin (Sigma) was administered intraperitoneally on days 0, 2, 7 and 9. From day 9, mice were monitored daily by weighing and scoring for signs of EAE. Mice were sacrificed where body weight fell 20% below initial weight for 24 hours, or where mice had an EAE score of 4 for 3 consecutive days.

2.5.5 Treatment of mice with ICSM18 following EAE induction

Mice received 250 µg ICSM18 or IgG1 isotype control antibody (Sigma) intraperitoneally on post induction days 2, 4, 8 and 15 (early treatment) or 6, 8, 10, 12 and 14 post induction (late treatment).

CHAPTER 3 *IN SILICO* AND *IN VITRO* DETERMINATION OF THE IMMUNODOMINANT T CELL EPITOPES IN HUMAN PRP

3.1 Introduction

As discussed, a notable feature of TSE infection is the lack of a classical immune response, either in the brain or in the periphery, where PrP^{Sc} may accumulate in the early stages of the disease (Aucouturier and Carnaud, 2002). The "blindness" of the immune system to the pathogenic agent is most likely due to its inability to distinguish PrP^{Sc} from PrP^{C} which is a ubiquitously expressed cell-surface sialoglycoprotein (Stahl et al., 1987) of uncertain function (Lasmezas, 2003). The remodelling event, by which PrP^{C} to PrP^{Sc} conversion occurs, involves no change in primary structure producing a pathogenic species consisting entirely of self-sequence (Stahl et al., 1993).

The degree of tolerance to PrP in humans, and whether this might influence susceptibility to prion disease, is not known. PrP^{C} is expressed in the thymus and animals are tolerant to PrP under normal circumstances, suggesting that most T cells recognising PrP epitopes undergo thymic deletion. There is no clear HLA association in sporadic CJD, although small patient numbers have precluded large-scale association studies, and an initial report that HLA-DQ7 might protect against vCJD was not confirmed when repeated on a larger sample (Jackson et al., 2001a;Pepys et al., 2003). Whether some individuals are capable of initiating an immune response against foreign or self-generated PrP^{Sc} remains unknown.

The widespread contamination of beef and beef products with BSE suggests that a substantial proportion of the UK population has been exposed to bovine prions.

Although only ~ 160 people have so far been diagnosed with vCJD in the UK, the number of individuals who are incubating sub-clinical or carrier states (Hill et al., 2000) of the disease remains unknown. The possibility of further peaks of vCJD incidence coupled with the risks of secondary transmission, mean that there is an urgent requirement for effective anti-prion therapeutics. Indeed, concern that vCJD prions may be transmissible via blood transfusion appears to have been realised (Llewelyn et al., 2004;Peden et al., 2004)(Wroe et al., submitted).

Recent developments in Alzheimer disease have demonstrated that immunotherapy may be effective in neurodegenerative diseases characterised by protein misfolding by raising an immune response against the key pathogenic protein species (Schenk et al., 1999;Hock et al., 2003). The major obstacle to effective immunisation against prion infection is tolerance to PrP. Several strategies have been employed to counter this, thus far with limited success in experimental models (Schwarz et al., 2003;Goni et al., 2005;Magri et al., 2005;Sigurdsson et al., 2002;Bade et al., 2006). Whether such a vaccine should incorporate a T cell epitope or simply stimulate a B cell response against PrP is unknown. Breaking T cell tolerance to self-proteins as a therapeutic strategy may be a pre-requisite to an effective response, but can also be hazardous, as demonstrated in a recent Alzheimer disease vaccination trial (Orgogozo et al., 2003;Nicoll et al., 2003).

Furthermore, full length recombinant PrP may not be an appropriate immunogen in humans due to concerns that *de novo* prion infectivity may be generated by such molecules (Legname et al., 2004). Immunogenic peptide fragments of PrP represent a safer alternative for vaccine development. Therefore, rational design of an anti-prion vaccine requires knowledge of the dominant linear T and B cell epitopes in PrP, yet

these have not previously been studied in humans. Studies in PrP knockout mice, in which PrP is entirely foreign, indicate that antigenic processing of, and immune responses to, PrP will occur where tolerance is obviated (Bainbridge and Walker, 2003;Gregoire et al., 2004;Khalili-Shirazi et al., 2005). Further, wild type mice will generate T cell responses against ovine (Stoltze et al., 2003) and even murine PrP where it is administered with appropriate adjuvants (Rosset et al., 2004;Souan et al., 2001b).

In the work presented here, I analysed the T cell response of healthy volunteers to peptides representing the human PrP sequence to identify potential T cell epitopes. I demonstrate that T cell tolerance to PrP is not complete and propose that there are distinct immunogenic regions in PrP that could be exploited for vaccine development.

3.2 Results

3.2.1 *In silico* epitope prediction

When the full length human PrP (residues 23-231) sequence with either methionine or valine at position 129 is analysed by TEPITOPE at a stringency of 3%, the programme generates 13 predicted 9-mer epitopes (Table 3.1). Interestingly, a number of these span the position 129 polymorphism. In contrast, the N-terminal region of PrP from positions 23 to 109 is not predicted to generate any HLA-binding epitopes.

Predicted epitope	Position	HLA DR allele(s)
MKHMAGAAA	109-117	B1*0101
		B1*0102
		B1*0804
		B1*1307
VVGGLGGYM	121-129 [129M]	B1*0301
VGGLGGYML	122-130 [129M]	B1*0101
		B1*0102
VGGLGGYVL	122-130 [129V]	B1*0101
		B1*0102
		B5*0101
LGGYMLGSA	125-133 [129M]	B1*0802
		B1*0804
		B1*0806
		B1*1501
		B1*1502
LGGYVLGSA	125-133 [129V]	B1*0802
		B1*0804
		B1*0806
		B1*1501
		B1*1502
YMLGSAMSR	128-136 [129M]	B1*0301
YVLGSAMSR	128-136 [129V]	B1*0301
LGSAMSRPI	130-138	B1*0102
		B1*0402
		B1*0701
MHRYPNQVY	154-162	B1*1501
		B1*1502
YSNQNNFVH	169-177	B1*0405
FVHDCVNIT	175-183	B1*0301
		B1*0421
VKMMERVVE	203-211	B1*0301
		B1*1305
		B1*1321

Table 3.1 Epitopes from the human PrP sequence predicted by TEPITOPE

3.2.2 T cell proliferation assay

PBMCs from 21 healthy donors were then cultured with up to 29 peptides spanning the full length of human PrP 129M. Proliferative responses were considered positive where the S.I. was >2 (Figure 3.1A). Positive responses were seen to peptides spanning residues 23-36 (peptide 1), 107-120 (peptide 13), 114-127 (peptide 14), 121-134 (peptide 15), 128-141 (peptide 16), 170-183 (peptide 22), 177-190 (peptide 23), 184-197 (peptide 24), 191-204 (peptide 25), 198-213 (peptide 26), 205-220 (peptide 27), 212-227 (peptide 28), 219-231 (peptide 29).

Altogether there were 22 positive responses out of 513 peptide assays, elicited by 13 out of the 29 peptides (44.8%). The most immunogenic peptides were 14 and 15, to which respectively 4/20 (20%) and 4/21 (19%) donors made responses (Figure 3.1B). Overall, 21 of the positive responses (95.5%) were clustered in two regions of the protein: 107-141 (peptides 13 to 16) and 170-231 (peptides 22 to 29) (Figure 3.1B and C). These peptides accounted for 236 of the 513 total assays (46%) of which 8.9% were positive, whereas in the remaining 277 stimulations using peptides outside these two regions, there was only one positive response, to peptide 1. Thus peptides within regions PrP 107-141 or 170-231 were significantly more immunogenic than those spanning the rest of the protein sequence (Fisher's exact test $p < 0.0001$). Indeed, no responses at all were generated by peptides 2 to 12, covering approximately 40% of the PrP sequence (Figure 3.1A-C).

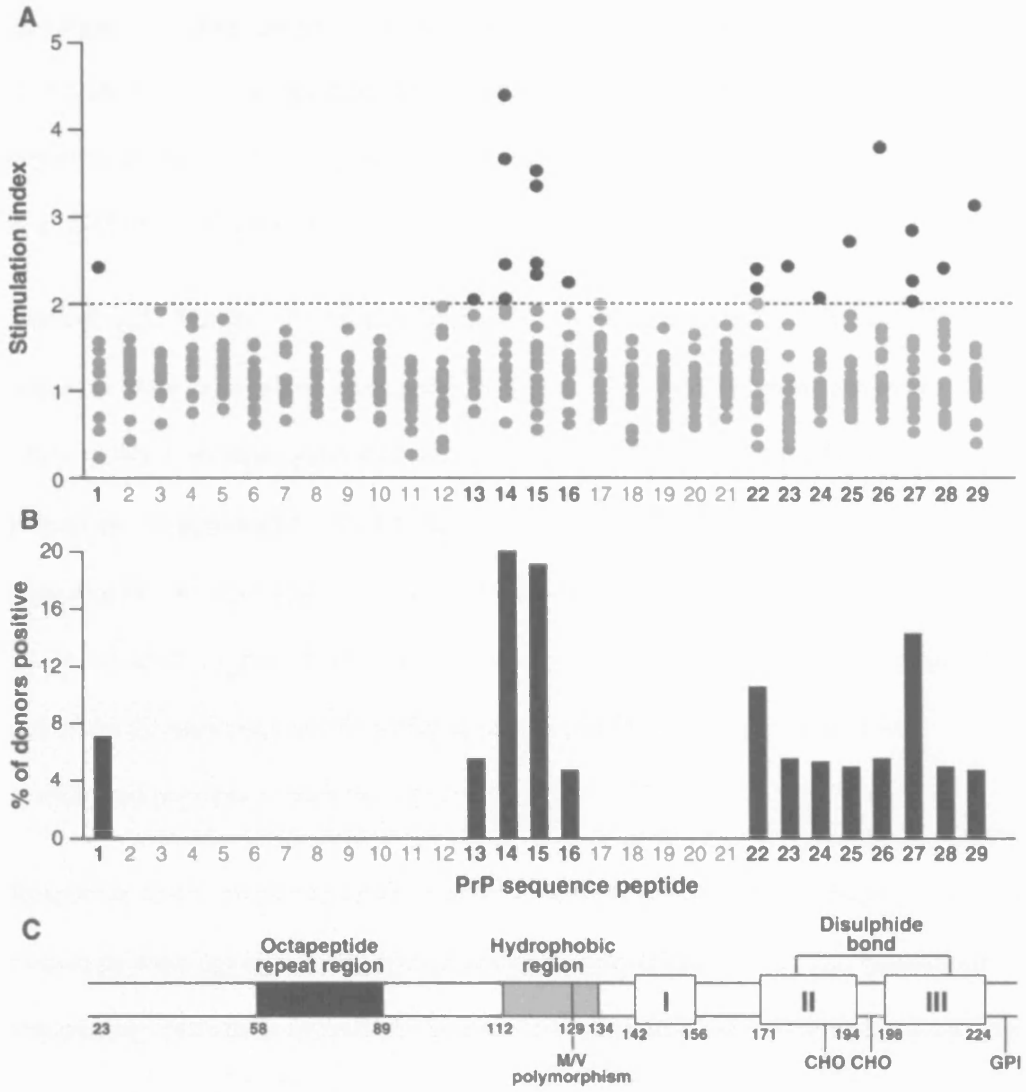

Figure 3.1 Results of human PBMC stimulations with PrP peptides

A. Stimulation indices (S.I.) for each peptide (arranged in N to C-terminal order) elicited by 7-day culture with fresh ex vivo PBMCs from up to 21 donors. Each point represents the S.I. for an individual donor for that peptide. S.I. < 2 (grey) is considered negative; >2 (black) is considered positive.

B. Percentage of donors responding to each peptide. All but one positive response occurred within two regions, 107-141 and 170-231.

C. Linear representation of human PrP aligned to the position of the N-terminal residue of peptides in A and B and demonstrating the principal regions of the protein. I, II and III correspond to the C-terminal α-helices and CHO to glycosylation sites.

Interestingly, 8 of the 10 epitopes predicted by TEPITOPE from the human PrP 129M sequence were represented among the 13 peptides that elicited responses *in vitro*. Thus, putative epitopes generated *in silico* had high predictive value for *in vitro* responses. In agreement with the TEPITOPE predictive algorithm, the N-terminal region of PrP was not immunogenic, with the exception of a single response to PrP 23-36. Overall, peptides spanning epitopes generated by TEPITOPE accounted for 9 out of the 22 responses to PrP 129M sequence (40.9%), although none of the synthesised peptides completely spanned PrP 169-177.

Responses to one or more peptide were seen in 9 out of 21 donors. However, positive responses were not evenly distributed among donors (Figure 3.2). The majority of responding individuals generated responses to between 5.0 and 14.9% of the peptides with which their PBMCs were challenged, while 2 responded to >15%.

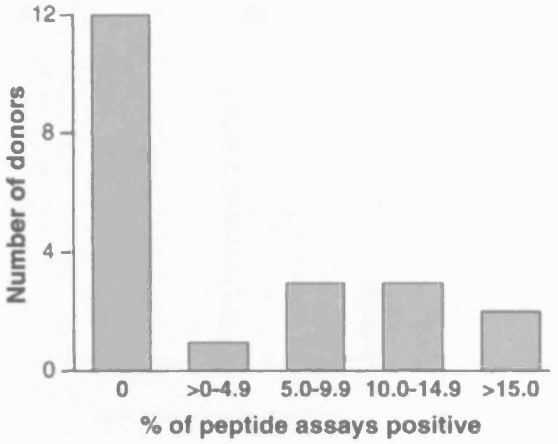

Figure 3.2 Percentage of peptides to which donors made positive responses

Most responders generated responses to 5.0-14.9% of peptides, while 2 out of 21 responded to >15.0%.

In addition, PBMCs from 20 of the donors were cultured with peptides spanning PrP 121-134 and 128-141 with Valine at position 129. Only one response was positive out of 40 individual peptide assays. This was to peptide 16V (Figure 3.3A and B). Positive responses were generated to peptide 15 by 4 out of 21 donors compared to 0 of 20 donors challenged with peptide 15V. Thus the epitope PrP 121-134 appeared to be more immunogenic with methionine at position 129 compared to valine, although this observation did not achieve statistical significance (Fisher's exact test $p = 0.11$).

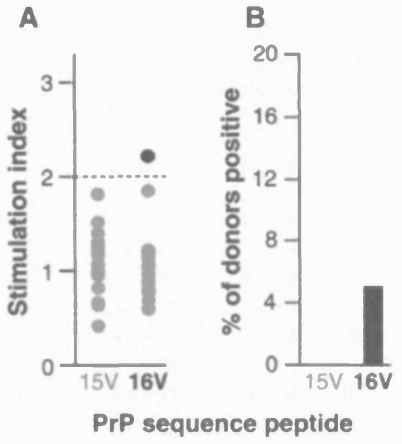

Figure 3.3 Results of stimulations with 129V-spanning peptides

A. Stimulation indices for peptides spanning position 129V elicited by 7-day culture with fresh ex vivo PBMCs from 20 donors. Each point represents the S.I. for an individual donor for that peptide. S.I. < 2 (grey) is considered negative; >2 (black) is considered positive.

B. Percentage of donors responding to 129V-spanning peptides. There was one positive response to peptide 16V.

3.2.3 Role of *PRNP* codon 129 genotype

Four of the 10 MM homozygotes and 5 of the 8 MV heterozygotes responded to one or more peptide (Table 3.2). No responses were made by any of the 3 VV individuals. Responses to 129M spanning peptides were seen in 3/8 129MV heterozygotes and 1/10 129MM homozygotes (Fisher's exact test p = 0.27). The single response to a 129V-spanning peptide was in an MM donor.

Donor	HLA-DR	HLA-DQ	*PRNP* 129	No. of peptides assayed	Peptides eliciting positive responses	No. of 129V peptides assayed	Peptides eliciting positive responses
A	4/52, 13/53	8, 6	MM	29	1, 13	2	
B	4/53, 7/53	2, 8	MV	29		2	
C	4/52, 13/53	8, 6	MV	29	15, 27, 29	2	
D	4/52, 13/53	8, 6	MM	29	24, 25, 26, 28	2	16V
E	14/52, 11/52	7, 5	MV	16	15, 16, 27	2	
F	1/51, 15	5	MV	12		2	
G	13/52	7, 6	MM	16		2	
H	103/52, 17	2, 7	MV	29	14, 27	2	
I	13/52, 17	2, 6	MM	29	14	2	
J	13/51, 15/52	6	MM	6		0	
K	8/51, 15/52	4, 6	MV	29	14, 22, 23	2	
L	4/52, 11/53	8, 7	MM	29		2	
M	7/53N, 7/53	9, 2	MV	24		2	
N	17/52, 11/52	2, 7	MV	17	15	2	
O	17/52, 11	2, 7	VV	29		2	
P	1/53, 4	7, 5	VV	29		2	
Q	4/51, 15/53	6, 8	MM	29		2	
R	17/51, 15/52	2, 5	MM	29		2	
S	7/52, 11/53N	7, 9	MM	29		2	
T	14/52, 17/52	2, 5	MM	16	14, 15, 22	2	
U	7/53, 9/53	2	VV	29		2	

Table 3.2 HLA class II and *PRNP* genotypes and assay results for each donor

3.2.4 Role of HLA type

Responses to peptides were seen in individuals with a range of HLA genotypes (table 3.2). The sample size was too small to allow an association with any particular genotype to emerge. However, responses to peptide 114-127 were only seen in individuals expressing HLA-DR52 (4 out of 15 $DR52^{+}$, 0 out of 6 $DR52^{-}$, Fisher's exact test $p = 0.28$).

3.2.5 Cytokine profiles

The highest number of positive responses to PrP peptides was made by PBMCs from donor D. Cryopreserved PBMCs from this donor were cultured with peptides 24, 25, 28 and 16V to which this individual had previously made a response, as well as peptides 15, 16 and 15V. Positive responses, as judged by S.I. > 2, were seen to peptides 16, 24, 25, 28 and 16V, with negative responses to 15 and 15V. Despite similar stimulation indices, cytokine production elicited by *in vitro* culture differed between PrP peptides. Whereas peptides 16 and 28 elicited very strong IL-4 and IL-6 responses, peptides 24, 25 and 16V did not induce strong cytokine production (Figure 4A i, iii, iv). These responses were associated with modest IFN-γ secretion (Figure 4A ii) and there was no significant TNF-α, IL-2 or IL-10 response to any of the peptides (data not shown). Interestingly, peptide 16 elicited a distinct cytokine profile compared to its 129V polymorph, 16V.

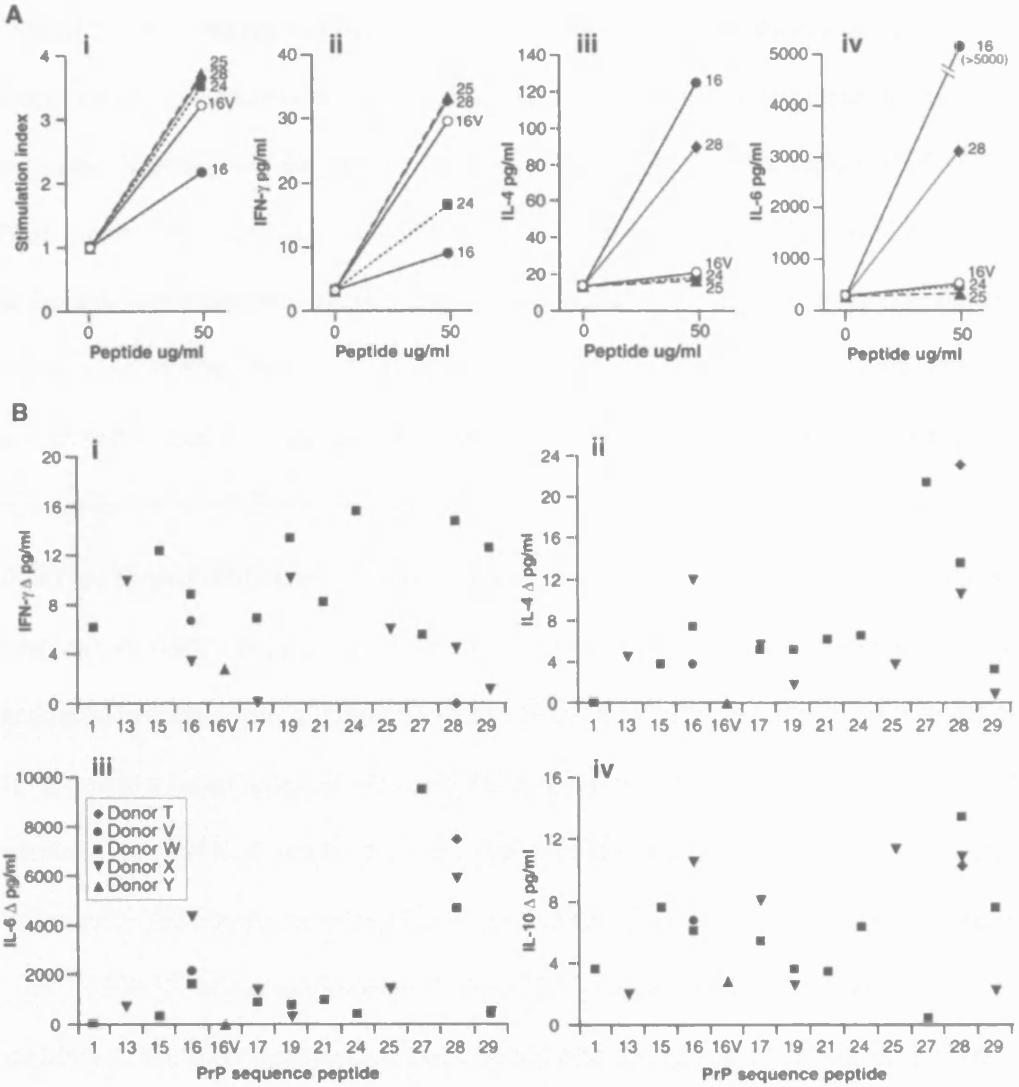

Figure 3.4 Cytokine profiles associated with PrP peptides

A. (i) Stimulation index and (ii) Interferon-γ (IFN-γ), (iii) IL-4 and (iv) IL-6 secretion in response to 6-day culture of cryopreserved PBMCs from donor D with indicated PrP sequence peptides.

B. Δ secretion (peptide-treated minus untreated control) of (i) IFN-γ, (ii) IL-4, (iii) IL-6 and (iv) IL-10 in response to 6-day culture of fresh ex vivo PBMCs from five donors with indicated PrP sequence peptides.

Further cytokine analysis of responses was then undertaken to obtain a more comprehensive understanding of the nature and functionality of responses to PrP epitopes. Responses to the peptide panel were analysed by repeat culture of PBMCs from three of the individuals shown in Table 3 (donors A, N and T), as well as an additional seven donors. Positive responses to one or more peptides were observed in 5 of these additional assays. In accordance with previous observations, there were no positive responses to N-terminal peptides other than PrP 23-36, confirming the lack of immunogenicity within this region. Again only modest IFN-γ production was observed (Figure 4B(i)) and no significant quantities of TNF-α or IL-2 were detected (data not shown). There was once again considerable heterogeneity in cytokine levels produced in response to different peptides. Peptides 16 and 28 induced high levels of IL-6, and to a lesser extent IL-4, as well as low levels of IL-10 (Figure 4B(ii)-(iv)). A similar pattern of IL-6 and IL-4 release was seen in response to peptide 27, although this peptide did not stimulate any IL-10 production. Peptide 16V was again divergent from its PrP 129M variant in being IL-4 and IL-6 silent (Figure 4B(ii)-(iv)). The majority of the other peptides were associated with production of IFN-γ, IL-4, IL-6 and IL-10 at low levels.

3.3 Discussion

A notable feature of vCJD is the accumulation of PrP^{Sc} in lymphoreticular tissues including tonsil, appendix and terminal ileum (Wadsworth et al., 2001;Joiner et al., 2005). Extra-neural PrP^{Sc} has also been detected, albeit at lower titres, in some cases of sporadic CJD (Glatzel et al., 2003). Despite this, no systemic acute phase or immune response has been detected in any human prion disease or experimental model (Aucouturier and Carnaud, 2002;Cunningham et al., 2005). Although T cells

have been detected in prion infected brain, lymphocytes from scrapie infected mice have not been demonstrated to possess anti-PrP cytolytic activity or secrete cytokines on stimulation with PrP peptides (Lewicki et al., 2003). Thus, the "atypical inflammation" (Perry et al., 2002) that ensues in the prion infected CNS is largely secondary to rapid neurodegeneration. The failure of adaptive immunity against prions during the lymphoreticular and neural phases of the disease has been ascribed at least partly to T cell tolerance to PrP^{C}, a ubiquitously expressed self protein with which PrP^{Sc} has 100% sequence identity. However, the extent of tolerance to PrP in humans has not previously been studied.

To my knowledge this is the first time that human auto-reactive T cell responses to PrP have been examined. I found that a significant minority of individuals made responses to PrP peptides and that these reproducibly cluster between residue 107 and the C-terminus. The peptides that elicited responses were remarkably close to those identified using an epitope prediction algorithm. This combined *in silico* and *in vitro* approach suggests that despite being a self protein to which tolerance is assumed to be robust, human PrP is an antigen that is tractable to epitope discovery techniques and hence rational vaccine design.

The major predisposing factor for vCJD is methionine homozygosity at codon 129 of PRNP. However, since this genotype accounts for 40% of the UK population and to date ~ 160 people have been affected by vCJD in the UK, other susceptibility factors must be involved. An initial report postulating that HLA-DQ7 might confer protection against vCJD (Jackson et al., 2001a) was not confirmed when repeated on a larger but not completely overlapping sample (Pepys et al., 2003). With the current small number of affected cases, firm conclusions about HLA association will be

difficult to draw unless any protective effect is extremely strong. In this study, T cell responses to PrP peptides were seen in individuals with a variety of HLA genotypes, suggesting that the ability to generate protective immunity may not be restricted to particular HLA types, although only individuals expressing HLA-DR52 responded to PrP 114-127.

Of particular interest, however, is the finding that the polymorphic residue 129 resides within a major human T cell epitope, with nearly 20% of donors making a response to PrP 121-134. Moreover, my data is highly suggestive of PrP 121-134 with methionine at position 129 being more immunogenic than PrP 121-134 with 129-valine.

Whether the ability to induce an immune response against this region of PrP might be protective against prion disease remains unclear. My data suggest that *PRNP* 129MV or VV individuals are preferentially able to induce a response to 129M-containing sequence although the current study was not sufficiently powered to demonstrate this conclusively. If confirmed this might provide a basis for differential susceptibility to infectious BSE prions. Furthermore, even among MM homozygotes there must be additional susceptibility factors. The possibility that some individuals are able to mount an effective immune response against invading PrP^{Sc}, by HLA-restricted presentation of peptidic fragments corresponding to the immunogenic epitopes identified in this study, cannot be excluded.

Incomplete tolerance to neuronal proteins is not an unexpected finding. Healthy individuals without multiple sclerosis routinely have T cell responses to myelin antigens (Davies et al., 2005). Further, a number of studies have demonstrated T cell reactivity to APP or Aβ peptides (Trieb et al., 1996;Giubilei et al., 2003;Monsonego

et al., 2003;Baril et al., 2004). However, unlike APP, PrP is highly expressed within the immune system itself, where it has been proposed to play a role in T cell activation (Cashman et al., 1990;Mattei et al., 2004).

Although the mechanisms underlying tolerance to PrP have not been fully elucidated, in $PrP^{-/-}$ mice where PrP expression is directed to specific organs by insertion of PrP transgenes under specific promoters, tolerance is readily induced by targeted expression in lymphocytes and other extra-neural tissues (Polymenidou et al., 2004). Expression of PrP^{C} is higher in human lymphocytes compared to those of rodents (Holada and Vostal, 2000). Consequently, I anticipated that T cell tolerance to PrP in humans would be tighter than to other neural proteins, most likely due to PrP^{C} expression in the thymus and other lymphoid organs and repeated exposure to animal PrP in food. However, I found T cell responses to PrP peptides in a significant proportion of the subjects in the study, suggesting that auto-PrP reactive clonal deletion may not be complete. Despite this, there is no evidence for autoimmune disease in these individuals due to spontaneous breakdown of tolerance to PrP, analogous to the presence of anti-myelin T cell responses in healthy individuals. Thus, for certain epitopes in some individuals, peripheral suppressor mechanisms such as regulatory T cells or low affinity antigen presentation may help maintain tolerance to PrP. Indeed, Polymenidou and colleagues found no relationship between the ability of mice to produce antibodies to PrP and thymic PrP^{C} expression level, implying a role for peripheral tolerance mechanisms (Polymenidou et al., 2004).

Although tolerance to PrP has been successfully broken in rodents, this has generally required use of powerful adjuvants or novel immunisation strategies (reviewed in Heppner and Aguzzi, 2004). T cell responses have been demonstrated to a variety of

self-PrP sequence epitopes in rodents (Table 3.3). Systematic studies by Aucouturier and colleagues suggest that the only significantly immunogenic epitope of self-PrP in C57BL/6 mice resides within PrP 158-172 (Gregoire et al., 2005;Gregoire et al., 2004;Rosset et al., 2004). However, peptides spanning 158-172 did not induce a significant antibody response in vivo. In this model, the major B cell epitope was 98-127. In contrast, another group used a variety of PrP sequence peptides selected on the basis of putative MHC binding motifs to elicit both strong T cell and IgG responses in rats and C57BL/6, NOD and A/J mice (Souan et al., 2001b;Souan et al., 2001a).

Sequence	Responder lymphocyte species/strain	Reference
Mo 31-50	C57BL/6	(Souan et al., 2001b)
Mo 131-150	NOD, C57BL/6, A/J	(Souan et al., 2001b)
Mo 151-170	C57BL/6	(Souan et al., 2001b)
Mo 156-172	C57BL/6	(Gregoire et al., 2004)
Mo 158-172	C57BL/6	(Rosset et al., 2004)
Mo 158-172	C57BL/6	(Gregoire et al., 2005)
Mo 182-202	NOD	(Souan et al., 2001b)
Mo 211-230	NOD, C57BL/6, A/J	(Souan et al., 2001b)
Ra 118-137	Lewis rat	(Souan et al., 2001a)
Ra 182-202	Lewis rat	(Souan et al., 2001a)
Ra 211-230	Lewis rat	(Souan et al., 2001a)

Table 3.3 T cell responses induced by native PrP immunisation in wild-type PrP expressing animals

The relevance for humans of epitopes revealed by rodent studies is constrained by the considerable sequence diversity between human and rodent MHC class II. My data suggest that human PrP 158-172 does not contain a T cell epitope although the immunogenic regions I identified do have some overlap with those described by Souan and colleagues. Similar proteolytic processing of PrP between species may

account for reduced tolerance towards certain common regions. The structured C-terminal portion of PrP is underrepresented among human proteosome digests (Tenzer et al., 2004), thus it may be less frequently presented during thymic T cell selection, allowing some escape of T cells recognising C-terminal epitopes.

Cytokine responses to PrP peptides were heterogeneous, being characterised either by striking induction of IL-6, and to a lesser extent IL-4, or a weak mixed cytokine response. Importantly, epitopes spanning the residue 129 polymorphism were associated with distinct patterns of cytokine production. Responses to PrP 128-141 [129M] were consistently associated with high levels of IL-6 and IL-4 production which were absent in the responses to PrP 128-141 [129V]. This is the first time that the residue 129 polymorphism has been implicated in determining the quality of the immune response to PrP.

IL-6 can be produced by T cells, but is also released by monocytes and B cells (Naka et al., 2002) which will also be present in a PBMC culture. Could PrP peptides drive IL-6 expression by non-T cells in culture? PrP 106-126 has previously been demonstrated to induce IL-6 production by human monocyte-derived dendritic cells (Bacot et al., 2003). Furthermore, in sporadic CJD, plasma IL-6 levels may be elevated (Volkel et al., 2001b). However, where peptides induced very high levels of IL-6 production this was coupled to IL-4 release, suggesting that whatever its source, IL-6 was driving a Th2 dominant T cell response. Indeed, IL-6 is required for IL-4 production by T cells undergoing Th0 to Th2 differentiation (Rincon et al., 1997;Diehl et al., 2002). The overall pattern of cytokine responses suggests that PrP 128-141 [129M] and 205-225 may be able to drive a Th2 differentiated response, PrP

23-36 and 128-141 [129V] give rise to a limited Th1 response while other PrP epitopes are associated with a weak Th0 profile.

It is still not clear to what extent a primed T cell response to PrP can protect against prion infection. Vaccination of A/J mice with PrP 31-50 or 211-230 in CFA led to a reduction in PrP^{Sc} level in prion-infected N2a tumour grafts (Souan et al., 2001b). However, when C57BL/6 mice were inoculated with scrapie following immunisation with these peptides, there was no protective response additional to the administration of CFA alone (Tal et al., 2003).

Other vaccination studies have tended to concentrate on generating anti-PrP antibody responses (Sigurdsson et al., 2002;Schwarz et al., 2003;Magri et al., 2005;Bade et al., 2006). However, anti-PrP antibodies raised by vaccination or administered passively (White et al., 2003) have thus far only been effective against disease restricted to extra-neural tissues, presumably due to poor blood brain barrier penetration. Activation of other components of the immune system, especially those capable of safe CNS penetration may therefore be a pre-requisite to effective immunotherapy.

The dangers, if any, of breaking tolerance to PrP are uncertain. The only adverse side effect of anti-PrP vaccination so far reported is dermatitis with mononuclear cell invasion and destruction of hair follicles in Lewis rats several months after immunisation with PrP 182-202 (Souan et al., 2001a). The importance of defining T cell epitopes in self proteins that are used as human vaccines was dramatically illustrated when 6% of AD patients in a clinical trial of Aβ vaccination developed meningoencephalitis driven by T cell invasion of the CNS (Orgogozo et al., 2003). The cause of this side effect is not entirely clear, and may relate to a Th1-dominant response driven by the adjuvant QS-21 (Cribbs et al., 2003). However, an option for

future vaccine development in AD may be to focus on the N-terminal fragment of Aβ that contains the B cell epitope but from which the immunodominant C-terminal T cell epitope has been deleted (Schenk, 2002).

These unexpected adverse effects demonstrate that mouse models with homogeneous MHC class I and II expression, and/or incomplete recapitulation of human expression patterns of the target autoantigen, may not be sufficient to model the effects of breaking tolerance to self proteins in humans. This study suggests that the immunodominant auto-epitopes in human PrP reside between residue 107 and the C-terminus, and that the residue 129 polymorphism quantitatively and qualitatively influences the immune response to PrP. Furthermore, PrP epitopes recognized by donor T cells *ex vivo* were closely matched to predicted epitopes based on known HLA binding motifs. The optimal model for assessing a vaccine based on these sequences would be one with humanised PrP and HLA class II expression, such as a double transgenic mouse. Whether a robust T cell response to these epitopes will result in harmful autoimmunity or be a prerequisite for protection from prion disease remains to be determined. However, elucidation of these immunodominant epitopes should help refine rational vaccine design for human prion diseases.

CHAPTER 4 EXPRESSION AND FUNCTION OF THE CELLULAR PRION PROTEIN IN T CELL ACTIVATION

4.1 Introduction

Constitutive expression of PrP^C has previously been documented in human peripheral blood lymphocytes and, albeit at a lower level, in mouse thymocytes and lymphocytes (Holada and Vostal, 2000;Liu et al., 2001;Li et al., 2001). However, it is also clear that levels of PrP^C in the lymphoid system are not static and change in response to cell activation or maturation. In particular, work from a number of groups has demonstrated that PrP^C expression is highly inducible during T cell activation (Cashman et al., 1990;Mabbott et al., 1997).

In the work described here I have re-examined the kinetics of PrP expression during T cell activation. I have determined the speed and magnitude of PrP upregulation at both transcriptional and surface protein levels in response to specific peptide-MHC stimulation of T cells. I have also addressed the question as to whether upregulation of surface protein on T cell activation is a general feature of GPI-anchored proteins or a specific property of PrP^C. Using both specific MHC-peptide and polyclonal T cell activation, I have investigated the temporal relationship between PrP^C upregulation and that of other classical activation markers.

Despite being robustly upregulated in T cell activation, it is far from clear that PrP^C has a functional role in activation, with conflicting reports in the literature on the proliferative response of $PrP^{-/-}$ lymphocytes to mitogens. To resolve these difficulties I stimulated splenocytes from $PrP^{+/+}$ and $^{-/-}$ mice with a variety of mitogens to assess proliferative potential and measured cytokine production in cells cultured with the T cell mitogen Concanavalin A. I also examined whether including a phase of *in vivo* T

cell activation reveals any difference in the antigen-specific T cell response in $PrP^{-/-}$ mice. Finally, because PrP^C has been claimed to accumulate at the immunological synapse (Ballerini et al., 2006) and to co-localise both with MHC class II and the TCR (Burthem et al., 2001;Stuermer et al., 2004), I examined whether the efficiency of superantigen induced T cell-APC conjugation is affected by the absence of PrP^C.

The findings presented here raise important questions about the role of PrP^C in the immune system and the possible effects of ligating or ablating lymphoid PrP^C as part of a therapeutic strategy.

4.2 Results

4.2.1 PrP^C, CD69 and CD25 upregulation in activated CD4 splenocytes

I stimulated splenocytes from FVB/N mice with soluble anti-CD3 and anti-CD28 and observed the expression of PrP, CD69 and CD25 in $CD4^+$ lymphocytes during the first 72 hours of activation. The first marker to be upregulated was CD69, with approximately 70% of CD4 cells expressing this marker within 4 hours of activation, rising to over 80% by 24 hours and over 90% by 48 hours (Figure 4.1A). This was followed by CD25, which was expressed by ~ 25% of $CD4^+$ cells after 8 hours stimulation, ~ 50% after 24 hours stimulation and universally expressed by 48 hours (Figure 4.1B). PrP expression was measured using the delta geometric mean (see Section 2.1.13). PrP was first significantly elevated from baseline by 24 hours with an approximately 9-10-fold increase in expression at 72 hours compared to starting basal levels and non-activated cells cultured in parallel (Figure 4.1C).

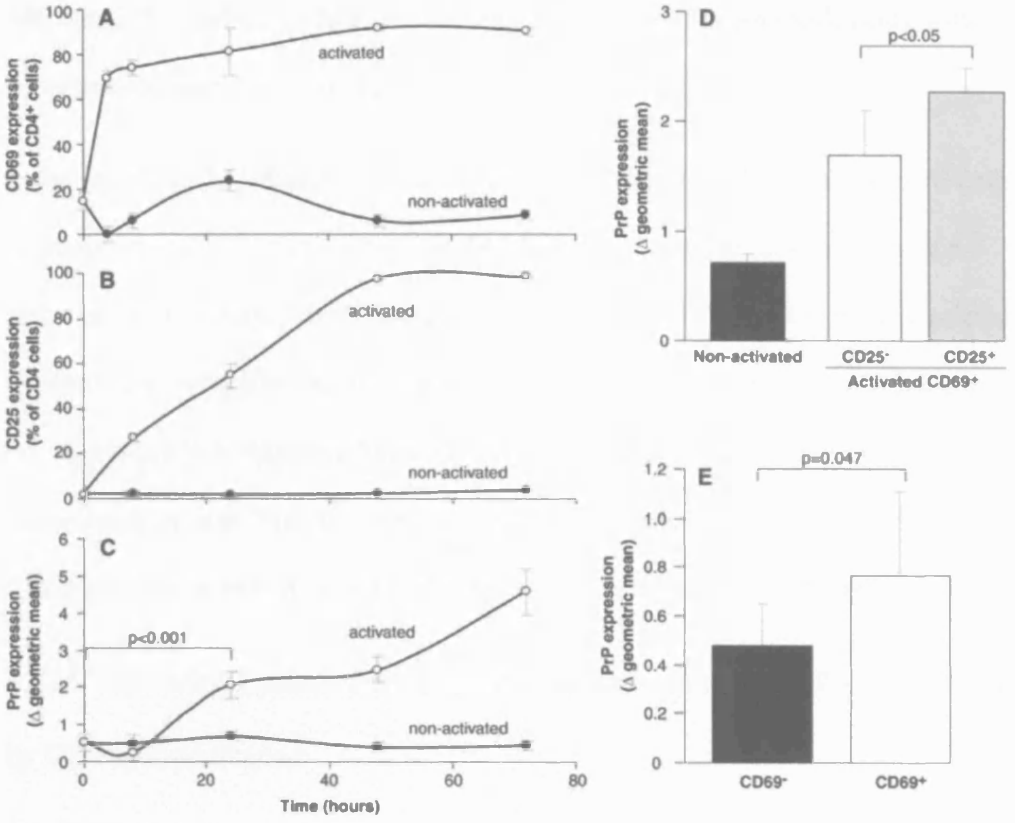

Figure 4.1 PrP, CD25 and CD69 expression in activated T cells

Surface expression of CD69 (A), CD25 (B) and PrP (C) in murine $CD4^+$ cells stimulated with anti-CD3 and anti-CD28. In activated cells, PrP is first significantly elevated from baseline at 24 hours (ANOVA with Bonferroni multiple comparisons post test) (C). PrP expression at 24 hours is higher in cells co-expressing CD25 (ANOVA with Tukey-Kramer multiple comparisons post test) (D). In fresh *ex vivo* splenocytes, PrP expression is higher in cells co-expressing CD69 (paired t test) (E).

At 24 hours the rise in PrP expression was greater in the $CD69^+$ $CD25^+$ population, compared to the $CD69^+$ $CD25^-$ population (Figure 4.1D). Thus, I concluded that PrP^C is an activation antigen, but that it is more slowly upregulated than CD69 and CD25

following CD3 and CD28 ligation. Further, PrP upregulation proceeds faster with, but is not dependent on, co-expression of CD25.

There was no appreciable change in expression of PrP or CD25 in non-activated cells cultured in parallel (Figure 4.1B-C). CD69 expression did vary somewhat in non-activated cells, perhaps reflecting the ease with which this marker can be upregulated (Figure 4.1A). However these changes were modest and not sustained. Interestingly, PrP expression was slightly higher in $CD4^+$ $CD69^+$ compared to $CD4^+$ $CD69^-$ cells immediately *ex vivo* (Figure 4.1E), suggesting that T cells constitutively expressing CD69, perhaps as a result of activation *in vivo*, have higher basal PrP expression.

4.2.2 Transcriptional and translational upregulation of PrP in TCR tg CD4 lymphocytes

To characterise the expression of PrP during a more physiological model of *in vitro* T cell activation I used splenocytes from transgenic mice expressing human HLA-DR15 and a TCR specific for MBP 85-99 (line 7). Approximately 97% of the mature $CD4^+$ lymphocytes from these animals carry the transgenic TCR and recognise this myelin epitope (Ellmerich et al., 2005). *Ex vivo* culture with a peptide spanning this sequence therefore results in uniform activation of T cells via conjugation with MHC class II expressing APCs. When cultured with MBP 85-99 (2 μg/ml) I saw reproducible increases in *Prnp* mRNA from line 7 splenocytes as measured by real time RT-PCR (Figure 4.2A). Mean *Prnp* expression rose 6-fold in the first 8 hours of culture, although due to variability between experiments it was only first significantly increased compared to baseline at 24 hours. However, *Prnp* expression at 8 hours was significantly greater in activated compared to non-activated cells. By 48 hours, *Prnp* expression was on average ~50-fold increased compared to baseline.

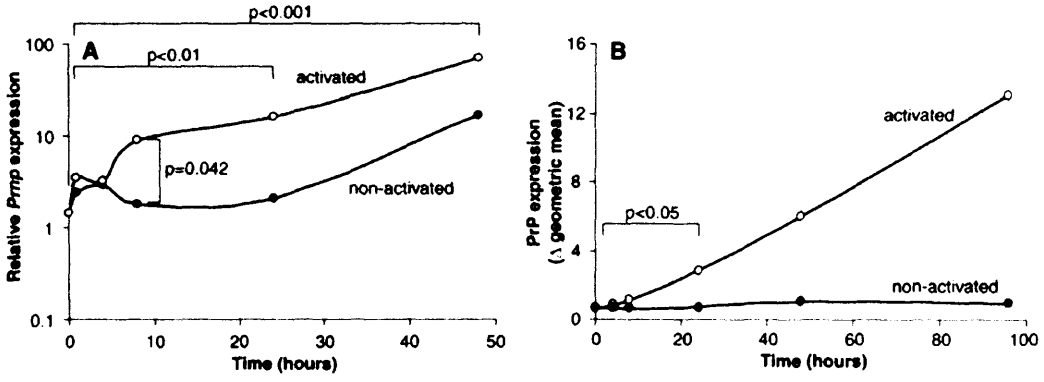

Figure 4.2 PrP mRNA and surface protein expression during T cell activation

Prnp expression in TCR tg splenocytes stimulated with MHC-peptide (A). *Prnp* levels are significantly elevated compared to unstimulated cells at 8 hours (paired t test) and compared to *ex vivo* levels at 24 hours (log transformed values ANOVA with Bonferroni multiple comparisons post test). At 48 hours Prnp levels are ~50 times greater than baseline (log transformed values ANOVA with Bonferroni multiple comparisons post test). Surface PrP expression in $CD4^+$ cells activated with specific MHC-peptide (B). Levels were first significantly elevated over baseline at 24 hours (ANOVA with Bonferroni multiple comparisons post test).

These changes were reflected in surface PrP levels, which demonstrated an upwards trend at 8 hours and were first significantly increased at 24 hours (Figure 4.2B). Interestingly, after this initial lag phase of ~ 8 hours, surface PrP increased in a broadly linear fashion with a ~20-fold increase on baseline expression detectable at 96 hours.

As a control, splenocytes from line 7 mice were cultured in medium alone. No significant changes in surface PrP were seen (Figure 4.2B). However, some changes

in *Prnp* level were documented (Figure 4.2A). These were marginal during the first 24 hours of *in vitro* culture, with a significant but small increase by 48 hours.

4.2.3 PrP upregulation in activated line 7 TCR tg CD4 lymphocytes co-expressing classical activation markers

To analyse the distribution of the increased PrP expression I compared PrP with activation markers CD69, CD25, ICOS and OX40 in TCR tg cells cultured for 48 hours with or without MBP 85-99. Despite robust increases in the percentage of $CD4^+$ T cells classed as PrP^{high} (from ~ 5% in non-activated cells to ~ 50% after activation), relatively few cells expressed these inducible markers in response to activation in this model (CD69 13.3%, CD25 8.2%, ICOS 8.6%, OX40 15.1%).

Indeed, appreciable numbers of $CD4^+$ T cells not expressing CD25, CD69, ICOS or OX40 on peptide stimulation also upregulated PrP levels (Figure 4.3A). However, co-expression of these markers was associated with more efficient PrP upregulation (Figure 4.3A). This was particularly marked for OX40 and CD25, with a less marked effect for CD69 or ICOS co-expression. Thus, although only a minority of cells that became PrP^{high} also expressed other activation markers (Figure 4.3B), those that did so, particularly OX40 and CD25, were more likely to show increased PrP levels. Further, PrP expression levels in activated $CD4^+$ $OX40^+$ cells were significantly greater than in activated $OX40^-$ cells (Figure 4.3C).

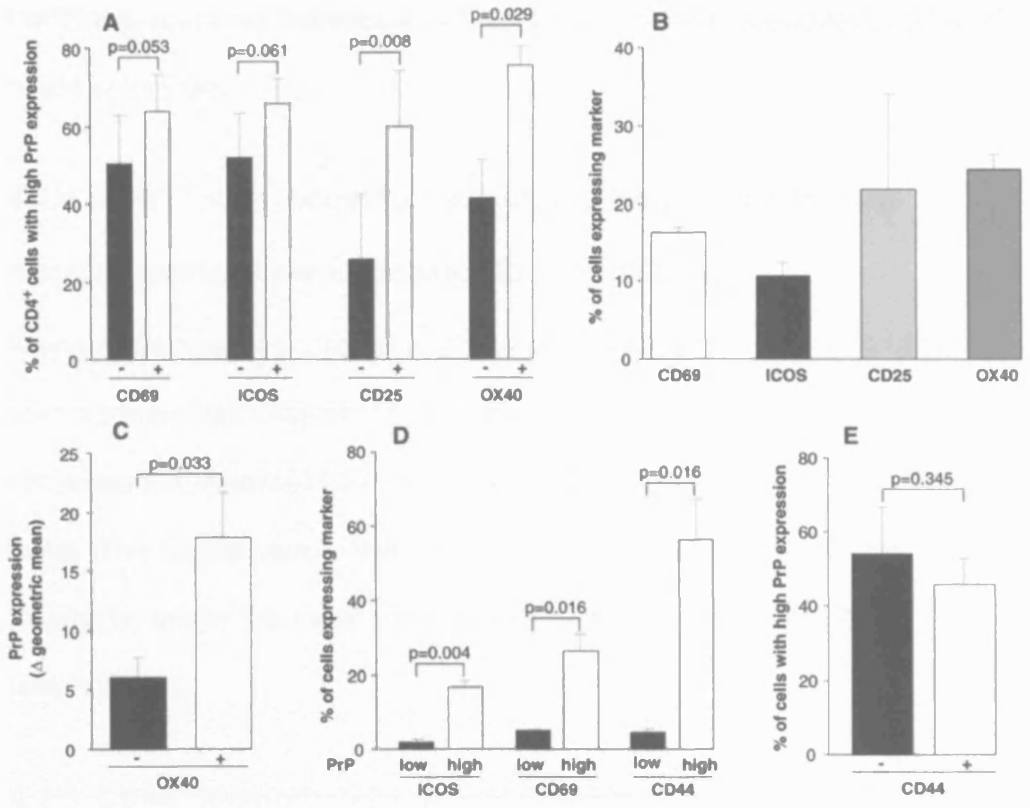

Figure 4.3 Correlation of PrP expression with other activation antigens

(A) Comparison of percentage of cells with high PrP expression between $CD4^+$ cells that do or do not co-express CD69, ICOS, CD25 or OX40 after specific MHC-peptide stimulation for 48 hours. Cells that co-express these markers on activation are more likely to be PrP^{high}. P values obtained using paired t test. In this model only a minority of activated PrP^{high} cells co-express these other markers (B). On activation, $CD4^+$ cells co-expressing OX40 have higher surface PrP levels than $OX40^-$ cells (paired t test) (C). (D) Comparison of ICOS, CD69 and CD44 expression between PrP^{high} and ^{low} cells cultured without stimulation for 48 hours. A significantly higher percentage of the PrP^{high} cells also expressed CD69, CD44 and ICOS (paired t test) compared to PrP^{low} cells. (E) Comparison of

PrP^{high} cell numbers between $CD4^+$ $CD44^+$ and $CD44^-$ populations after 48 hours activation.

4.2.4 $CD4^+$ T cells constitutively expressing activation and memory markers are enriched with PrP^{high} cells

Where splenocytes were cultured for 48 hours without peptide, only ~ 5% of $CD4^+$ T cells expressed high levels of PrP. However, of the PrP^{high} cells a significantly higher percentage also expressed CD69, CD44 and ICOS compared to PrP^{low} cells (Figure 4.3D). This suggests that, as with the wild type cells examined above, $CD4^+$ T cells forming the *in vivo* "activated-memory" pool may be enriched with a PrP^{high} population.

4.2.5 $CD44^+$ memory cells do not preferentially increase PrP expression on T cell activation

In contrast to the activation markers described above, after peptide stimulation the percentage of $CD4^+$ cells that are PrP^{high} did not differ between $CD44^+$ and $CD44^-$ populations (Figure 4.3E). Thus, it appears that although PrP expression and memory status correlate during quiescence, the upregulation of PrP induced by activation occurs at least as efficiently in the naïve $CD4^+$ lymphocyte population.

4.2.6 Upregulation on T cell activation is not a general property of GPI-anchored proteins

One possible explanation for the rise in PrP expression is that this is a general feature of GPI-anchored proteins during T cell activation and not a specific property of PrP^C. I therefore compared surface PrP expression with that of the GPI-anchored proteins Thy1 and Qa2 during *ex vivo* stimulation of line 7 splenocytes. Although there was a

~ 30% increase in surface Qa2 after 48 hours, this was much less than the > 7-fold increase observed for PrP, while there was no appreciable change in the level of Thy1 (Figure 4.4). After correcting for multiple comparisons, only the change in PrP expression was significant. Thus, it appears that the robust upregulation of PrP during T cell activation is specific and not brought about through general redistribution of GPI-anchored proteins.

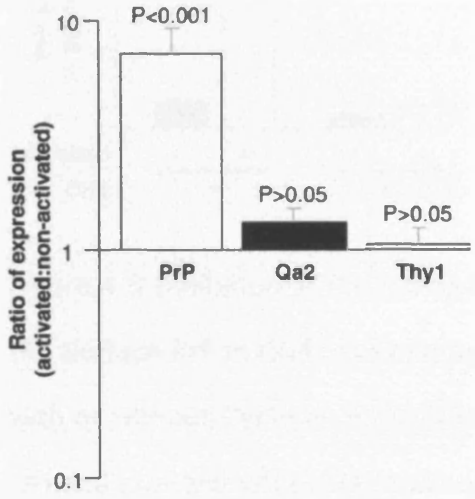

Figure 4.4 Change in surface expression of PrP, Qa2 and Thy1 on T cell activation

Only PrP expression is significantly increased by activation (log transformed values, ANOVA with Bonferroni multiple comparisons post test) when line 7 splenocytes are activated for 48 hours *in vitro*.

4.2.7 Surface PrP upregulation requires *de novo* protein synthesis

To ensure that the increased surface PrP seen on T cell activation required *de novo* protein synthesis, I conducted the *ex vivo* stimulation in the presence of the ribosomal toxin Cycloheximide. This almost completely inhibited the increase in PrP seen at 24 hours, even though *Prnp* upregulation was not entirely abolished (Figure 4.5A-B).

This implies that surface changes in PrP in activated lymphocytes are dependent on new protein synthesis and cannot be mediated by cycling of pre-formed protein to the surface.

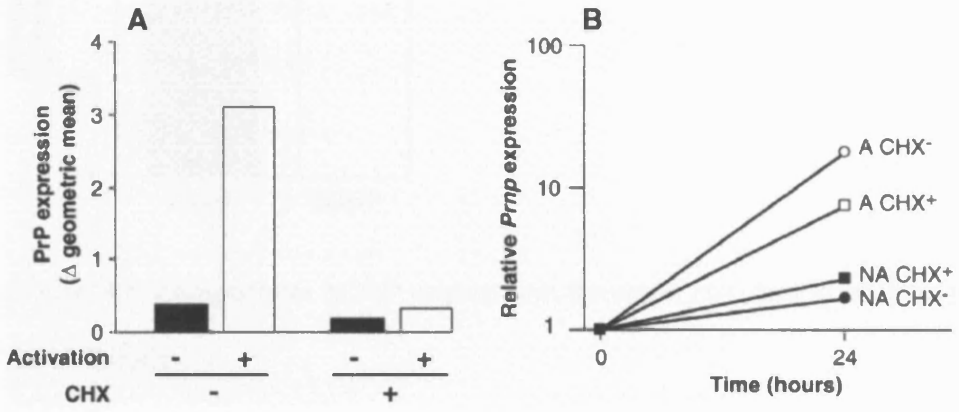

Figure 4.5 Inhibition of PrP upregulation by Cycloheximide

(A) Surface PrP in $CD4^+$ cells stimulated with MHC-peptide for 24 hours with or without Cycloheximide (CHX). (B) *Prnp* mRNA levels in activated (A) and non-activated (NA) splenocytes cultured for 24 hours with or without CHX.

4.2.8 Comparison of PrP^C expression in lymphoid and circulating $CD4^+$ cells

These experiments were performed using $CD4^+$ splenocytes. However, I wondered whether these might differ from circulating PBMCs with respect to PrP expression. A comparison of surface PrP demonstrated that line 7 $CD4^+$ splenocytes have marginally higher PrP expression than their counterparts in the peripheral blood (Figure 4.6), although this was not statistically significant (paired t test).

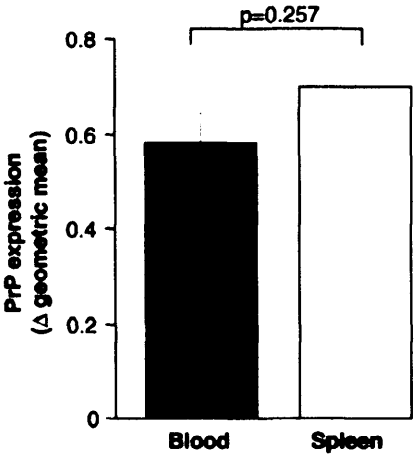

Figure 4.6 Comparison of PrP expression between circulating and splenic $CD4^+$ T cells

4.2.9 $PrP^{-/-}$ lymphocytes demonstrate normal proliferation and cytokine production

Having observed robust PrP upregulation in activated lymphocytes, I then determined the extent to which PrP is required for lymphocyte proliferation and cytokine production. Splenocytes from wild type FVB/N mice and $PrP^{-/-}$ mice crossed for 5 generations into the FVB/N strain (F5 colony) were cultured with Con A, SEA, SEB, anti-CD3 and anti-CD28 and PMA and Ionomycin. Over a range of concentrations, there were no consistent differences in proliferation between $PrP^{+/+}$ and $^{-/-}$ splenocytes (Figure 4.7A-E). The only significant difference was the response to 0.5 μg/ml Con A, which was slightly higher in $PrP^{-/-}$ splenocytes (ANOVA with Bonferroni multiple comparisons post test, $p<0.01$). I then measured cytokine levels in culture medium from Con A stimulated splenocytes. Apart from an excess of IL-2 produced by $PrP^{-/-}$ cells in response to 1 μg/ml Con A, there were no differences in cytokine levels (Figure 4.8A-E).

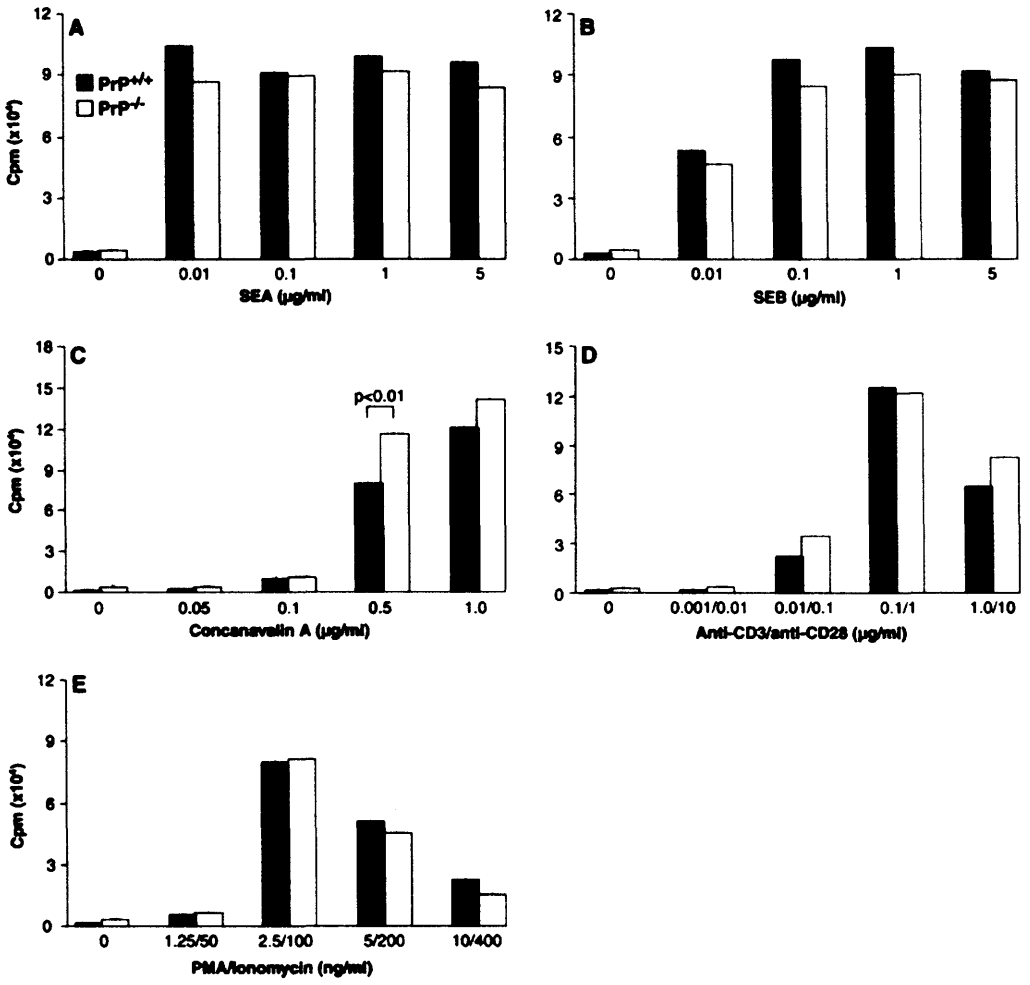

Figure 4.7 Proliferation of $PrP^{+/+}$ and $^{-/-}$ splenocytes in response to mitogens.

The only statistically significant difference after 64 hours stimulation was the response to 0.5 µg/ml Con A, which was slightly higher in $PrP^{-/-}$ splenocytes (ANOVA with Bonferroni multiple comparisons post test).

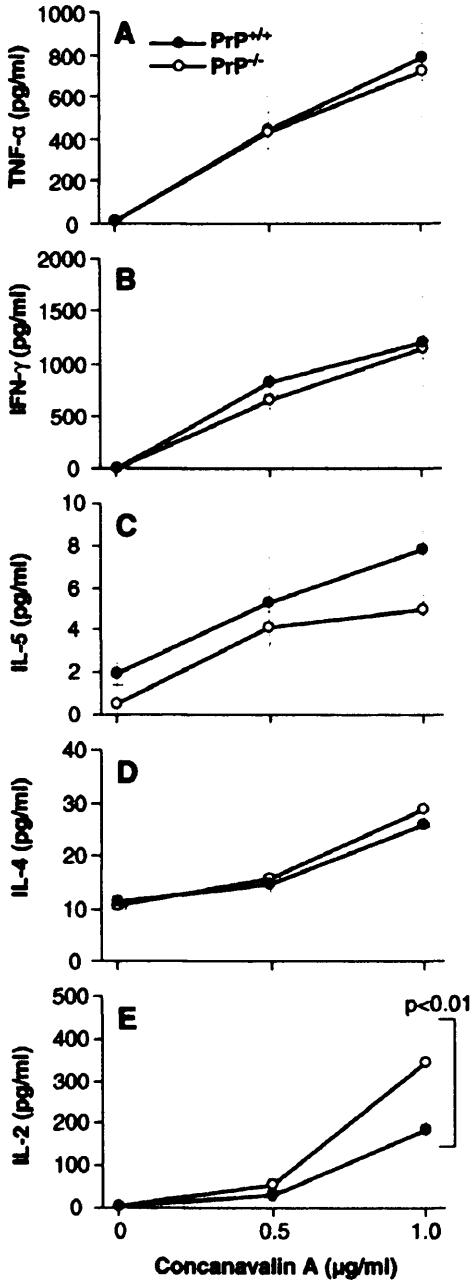

Figure 4.8 Cytokine production in $PrP^{+/+}$ and $^{-/-}$ splenocytes cultured with Con A

Responses were not significantly different except for greater IL-2 production by $PrP^{-/-}$ cells in response to 1 μg Con A (ANOVA with Bonferroni multiple comparisons post test) (E).

4.2.10 $PrP^{-/-}$ lymphocytes have normal *ex vivo* responses to antigens following immunisation

These observations were made *in vitro* and may have missed an essential role played by PrP^{C} *in vivo*. I therefore repeated an *ex vivo* proliferation assay, but preceded this with an *in vivo* step. $PrP^{-/-}$ mice crossed for 10 generations into the FVB/N strain and wild type FVB/N mice were immunised in the footpad with 50 μg HEL or OVA in CFA. 10 days later, mice were sacrificed and lymphocytes from draining (popliteal) nodes were restimulated with the same antigen. After a 3 day incubation period, proliferation of $PrP^{+/+}$ and $PrP^{-/-}$ lymphocytes was equivalent (Figure 4.9A-B). Thus, I concluded that PrP is not required for full T cell activation either *in vitro* or *in vivo*.

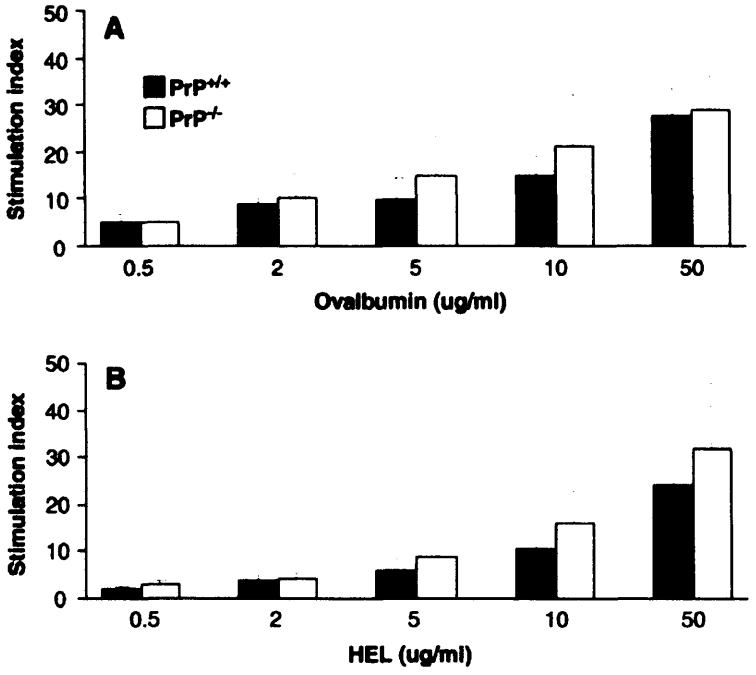

Figure 4.9 Proliferative responses of $PrP^{+/+}$ and $^{-/-}$ lymphocytes to recall antigens

Proliferation of popliteal lymph node lymphocytes from $PrP^{+/+}$ and $^{-/-}$ FVB/N mice immunised in the footpad with OVA (A) or HEL (B), restimulated *in vitro* with the same antigen at indicated concentrations.

4.2.11 Absence of PrP does not affect T cell-APC conjugation

If PrP^{C} indeed plays a role in the immune synapse, lack of expression might be expected to influence T cell-APC conjugate formation. I modelled this in a polyclonal system by using the superantigen SEA to cross-link the TCR Vβ chain with MHC class II on APCs. T and B cells (as a source of APC) from wild type FVB/N and F5 $PrP^{-/-}$ mice were purified. T cells were labelled green with CFDA and B cells with the red dye Snarf 1. Upon addition of SEA, T and B cell conjugates were detected by flow cytometry as red-green double positive events. I saw no significant difference in the proportion of $PrP^{+/+}$ or $^{-/-}$ T cells conjugating to syngeneic B cells in the presence of SEA (Figure 4.10); around ~ 10% of T cells in each group formed conjugates at 2 and 4 hours. $PrP^{+/+}$ cells showed slightly, but not significantly, greater de-conjugation at 6 hours, perhaps reflecting quicker downregulation of the TCR or MHC class II.

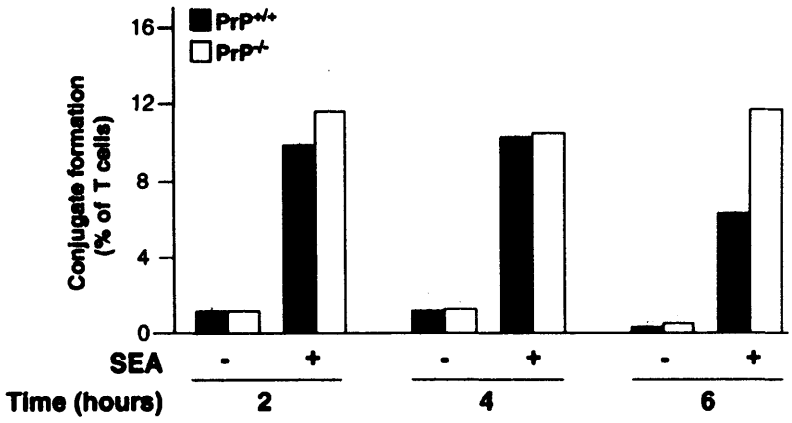

Figure 4.10 $PrP^{+/+}$ and $^{-/-}$ T cell-B cell conjugation induced by SEA

T and B cells were mixed at a 1:2 ratio with or without SEA (5 μg/ml) for indicated time periods.

4.3 Discussion

The principle function of T cells is to become activated in response to specific antigen presented by MHC on an APC. The major effects of this are cytokine production and cell division, followed by apoptosis. This process is complex, with multiple proteins involved in the cascade of signalling following TCR engagement. The multiplicity of genes involved in T cell activation has recently been glimpsed using microarray technology (Hess et al., 2004;Chtanova et al., 2005). Some microarray experiments have detected increased PrP transcripts during T cell differentiation (Chen et al., 2003;Goldrath et al., 2004) and direct measurement of surface PrP by FACS has shown it to be upregulated on T cell activation (Cashman et al., 1990;Mabbott et al., 1997). However, the exact role of PrP^C in T cell function remains obscure. The aim of the experiments described in this chapter has been to confirm and extend existing observations of the expression and function of PrP^C expression during T cell activation. This has facilitated prediction of what model systems might be required to

firmly establish the function of PrP^C and the effects of targeting it as part of a therapeutic strategy.

Although the kinetics of PrP expression during T cell activation have been partially elucidated in human and mouse lymphocytes, the precise relationship between *Prnp* transcription and PrP translation during this process has not been determined. Further, the relationship between PrP upregulation and expression of other activation markers has not previously been studied. Moreover, with the exception of recent work by Ballerini and colleagues (Ballerini et al., 2006), previous studies have employed polyclonal activation using non-specific mitogens. Thus, observed effects will have been modified by the presence of cells of differing proliferative potential and by activation of non-T cells in the culture. In the work described here, I sought to resolve these outstanding questions using a combination of polyclonal and monoclonal stimulation of murine T lymphocytes. I then asked whether T cell activation would proceed normally in the absence of PrP, an issue on which there is conflicting published data.

I followed PrP mRNA and surface protein levels for the first 48 and 96 hours respectively of activation in TCR transgenic cells. These cells represent an essentially monoclonal population that is specifically activated via the TCR by peptide presented by MHC class II on B cells and DCs in the splenocyte culture. Although necessarily conducted *in vitro* this more closely resembles physiological T cell activation as it obligatorily involves MHC-peptide-TCR conjugation and allows analysis of a clonal population of cells responding identically to their cognate epitope.

In these experiments PrP mRNA increased 6-fold after 8 hours of activation, with surface PrP on $CD4^+$ cells increasing to a similar degree after 24 hours. The overall

increase in PrP mRNA was approximately 50-fold at 48 hours, with surface protein increasing 20-fold by 96 hours. This difference may be accounted for by degradation of mRNA prior to translation, or trafficking of PrP to cell compartments other than the surface membrane.

The kinetics of surface PrP upregulation in the line 7 TCR tg model were similar to those observed with polyclonal stimulation of wild type murine $CD4^+$ cells, with significant increases only detectable after 24 hours, followed by further rises up to at least 72-96 hours. PrP upregulation does not therefore appear to be dependent on specific MHC-peptide-TCR signalling but at a minimum may require non-specific TCR stimulation. Indeed, others have not been able to demonstrate PrP upregulation when cells were treated with PMA (Antoine et al., 2000). Thus, it appears that peptide-MHC, anti-CD3 and Con A will reliably upregulate PrP, whereas PMA may not be so effective. This suggests that upregulation of PrP is linked to T cell signalling events upstream of protein kinase C and calcineurin.

A potential limitation of this work is that I performed the quantitative PCR using only one internal control, 18S. Endogenous control genes may be subject to changes in expression level during T cell activation. However, work by others in the Altmann laboratory has shown that 18S expression, to a far greater extent than either HPRT or GAPDH, remains stable during T cell activation (S Ellmerich and G Kagaba, unpublished observations), and it has been reported that 18S, in contrast to GAPDH and β-actin, remains a reliable indicator of cell number during T cell activation (Bas et al., 2004)). An additional concern is that in these experiments total 18S and *Prnp* were measured, whereas only the T cells were activated. Granulocytes, for example, do not express as much PrP as lymphoid cells. An artefactual elevation in *Prnp* could

then be generated by a change in the proportion of cells in culture over time. However, this would have to be an enormous change in cell populations over a 48 hour period, which is unlikely. Moreover, the vast majority of non red cells in a mouse splenocyte culture are B and T lymphocytes which have similar PrP expression levels. These concerns could have been addressed by including a second endogenous control that was specific for T cells, such as CD2. However, we had not been able to optimise the CD2 real time primers prior to performing these experiments. Alternatively, we could have purified T cells and added back irradiated B cells as APCs.

In addition, *Prnp* mRNA estimations were made on the total culture, whereas I only studied surface PrP expression on $CD4^+$ T cells. Thus a certain amount of the increase in *Prnp* mRNA may have been accounted for by upregulation in non-CD4 cells, such as $CD8^+$ or NK lymphocytes. However, approximately 35% of splenocytes from line 7 mice are $CD4^+$, while only about 15% are $CD8^+$ (Ellmerich et al., 2005). Furthermore, although $CD8^+$ lymphocytes from line 7 mice do proliferate in response to MBP 85-99 in vitro, the degree of proliferation is approximately 10-fold less than that induced in $CD4^+$ cells, and requires IL-2 supplementation (Ellmerich et al., 2005), which was not used here.

Finally, I also saw changes in *Prnp* expression in cells that were cultured *ex vivo* without peptide stimulation. This might be because *Prnp* transcription is modestly altered by changes in the cell's external milieu, such as the transition from the spleen to *ex vivo* culture in serum free medium. Indeed, the *Prnp* promoter may contain a heat shock element (Mahal et al., 2001;Shyu et al., 2002) and other transcription factor binding sites through which such a signal could be mediated. Alternatively,

mRNA stabilisation mechanisms may be altered during preparation of the culture. However, these changes are not of the same magnitude as those induced by T cell activation and do not lead to appreciable increases in surface PrP expression.

This work demonstrates that under identical activation conditions, other GPI-anchored proteins are not robustly upregulated. Thus, PrP upregulation cannot be explained by non-specific effects of T cell activation on GPI synthesis and trafficking. The kinetics of PrP mRNA upregulation and the specificity of surface PrP upregulation suggest that PrP levels during T cell activation are regulated via specific preformed transcription factors. Indeed, the *PRNP* promoter contains a putative NFAT binding site (Premzl et al., 2005) and PrP mRNA expression is increased during Th0 to Th2 differentiation (Chen et al., 2003), a process that is dependent on induction of NFATc2 expression by IL-6 (Diehl et al., 2002).

The observed increase in surface PrP is dependent on *de novo* synthesis, as activation in the presence of cycloheximide blocked surface upregulation, a phenomenon previously observed in monocytes/DCs that upregulate PrP in response to IFN-γ (Dürig et al., 2000). Thus, although release to the surface of preformed PrP^C has been demonstrated in activated platelets (Holada et al., 1998), such mechanisms can be discounted as likely sources of increased surface expression in activated T cells.

Interestingly, the dynamics of this process in our models were different from those reported by Cashman and colleagues (Cashman et al., 1990). This group measured specific PrP mRNA using semi-quantitative Northern blotting in human PBMCs and found reduced *PRNP* signal compared to α-actin after 2 days stimulation with Con A. Using FACS they first noticed an increase in surface PrP after 6 hours, whereas I did not detect significantly increased surface PrP until 24 hours. The presence of a lag

phase might reflect a longer signalling pathway in our models, which require TCR ligation and signalling that may take 2-4 hours to optimise. Alternatively, murine T cells may differ from human PBMCs in their speed of intracellular signalling, as I did not see faster surface upregulation following anti-CD3 and anti-CD28 compared to MHC-peptide stimulation. However, my results are in general agreement with those of Cashman, Mabbott and others in observing PrP upregulation over a matter of hours rather than minutes. Cashman described stabilisation of surface PrP expression level by 72 hours with levels remaining elevated for a further 4 days. I did not continue *in vitro* stimulation for this period, but saw no slowing in the rate of upregulation up to 96 hours in the line 7 experiments.

Surprisingly, the relationship of PrP^C expression to that of other activation markers has not previously been studied. Following anti-CD3 and anti-CD28 stimulation of wild type $CD4^+$ splenocytes, PrP was upregulated more slowly than CD69 or CD25. In this model nearly all $CD4^+$ cells had become $CD69^+$ prior to upregulation of PrP. PrP upregulation proceeded in both CD25 positive and negative cells at 24 hours, but was significantly greater in $CD25^+$ cells. These observations place PrP as a late T cell activation antigen. In the line 7 stimulation experiments PrP level was positively correlated with co-expression of CD69, CD25, ICOS and OX40. However, in this TCR tg model, relatively few activated $CD4^+$ cells expressed these well characterised antigens, whereas PrP was robustly upregulated. Thus, it appears that co-expression of other activation markers is not a pre-requisite to upregulation of PrP. Consequently, PrP may be of particular use in studying genetically modified cells that have lost the propensity to upregulate conventional activation markers.

The dissociation of proliferation and PrP upregulation from CD25, CD69, ICOS and OX40 expression in the line 7 experiments raises interesting questions about TCR function in this model. T cells in this system have a human Vβ transgene which may be able to mediate some but not all of the normal signals that follow TCR ligation. The failure of CD25 upregulation, for example, suggests that activated T cells in this model may be relatively insensitive to, and poor producers of, IL-2. This in turn might underlie a failure of T cell regulation in these auto-immune encephalomyelitis prone animals (Malek and Bayer, 2004). Although PrP upregulation proceeds faster in $CD25^+$ T cells, substantial increases were also observed in the absence of CD25 expression, in both wild-type and TCR tg cells. Thus, PrP is regulated by different mechanisms than those controlling other activation markers.

An important consideration is whether PrP upregulation during T cell activation is a necessary event for this process to be fully completed, or merely a consequence of it without any functional significance for proliferation. I attempted to address this issue using PrP knockout mice. These lines were initially generated to test the prion hypothesis – that cellular PrP is necessary for prion replication and pathogenesis. However, they also provide an opportunity to study the effects of deleting PrP and thus inform on the function of the protein. The two most widely used $PrP^{-/-}$ mice were made using slightly different approaches (reviewed in Weissmann and Flechsig, 2003). The first described was the Zurich I mouse (Bueler et al., 1992) followed by the Edinburgh *Prnp*$^{-/-}$ strain (Manson et al., 1994a). Interestingly, the Zurich I construct retains the 5'-UTR and first two codons of *Prnp*. This explained why we detected *Prnp* mRNA in samples from our $PrP^{-/-}$ mice using the Applied Biosystems assays-on-demand *Prnp* primers (G Kaur, unpublished observations), which hybridise within the 5'-UTR (exactly where remains proprietary information). Applied

Biosystems supply a 25bp context sequence that lies between the hybridisation points of the forward and reverse primers. This was synthesised and used as an internal primer to amplify the product from the *Prnp* real time RT-PCR. Sequencing of this amplicon showed it to be identical to *Prnp* mRNA positions 87-118, which are retained in the Zurich I construct (G Kaur, unpublished observations). Thus, I was unable to use splenocytes from our $PrP^{-/-}$ mice as a negative control for the qPCR detection method.

The neuronal phenotype of $PrP^{-/-}$ mice has been extensively characterised. However, relatively little work has been undertaken on the function of the immune system in the absence of PrP. This consists largely of polyclonal *ex vivo* stimulation of lymphocytes, and results have been conflicting. More recently some groups have used PrP deficient mice or cell lines to model more complex processes such as peritonitis or intra-cellular parasitosis (de Almeida et al., 2004;Watarai et al., 2003;Fontes et al., 2005). Such work has also produced comparatively modest, and indeed conflicting, results. Although Lindquist's group recently demonstrated impaired regenerative potential in $PrP^{-/-}$ haematopoietic stem cells, this was brought out by extensive manipulation of these cells under extremely harsh *in vivo* conditions (Zhang et al., 2006).

In the first round of experiments presented here, I used splenocytes from $PrP^{-/-}$ mice descended from the orginal Zurich I line. This colony had been subject to only five generations of crossing into the FVB/N strain, and therefore cannot be considered totally isogenic to wild type FVB/N mice. However, it can be used for elucidation of major phenotypic differences. Initially, I stimulated splenocytes from these and wild type FVB/N mice with a range of agents capable of polyclonal stimulation – anti-CD3

and CD28, Con A, PMA/Ionomycin, SEB and SEA. In addition, I measured cytokine levels in the culture medium of cells stimulated with the highest concentrations of Con A. These experiments did not reveal any clear differences between $PrP^{+/+}$ and $^{-/-}$ splenocytes. If anything, $PrP^{-/-}$ mice made slightly better proliferative and IL-2 responses to Con A, in contrast to previous reports of hyporesponsiveness (Mabbott et al., 1997;Bainbridge and Walker, 2005). Our data are generally consistent with those of Bueler et al, who in reporting the phenotype of the initial Zurich I mouse, describe normal responses to Con A stimulation (Bueler et al., 1992).

Although not essential for mitogen-induced proliferation I wondered, based on the localisation of PrP in lymphocyte lipid rafts and its co-immunoprecipitation with key proximal signalling molecules (Mattei et al., 2002;Mattei et al., 2004;Hugel et al., 2004;Stuermer et al., 2004), if PrP^{C} was implicated in function of the immunological synapse. This is a reorganisation of molecules immediately surrounding the TCR-MHC interaction that facilitates signalling between T cell and APC (Dustin, 2005). Successful immunological synapse formation is contingent on conjugation between an individual T cell and APC. As a model for this I used MHC class II presentation by B cells of the superantigen SEA to T cells. T-B cell conjugation caused by SEA was identical between PrP deficient and wild-type cells. Although the superantigen-induced immunological synapse may differ in some respects from conventional MHC-peptide-TCR ligation, it is similar in being integrin dependent (Morgan et al., 2001). Ballerini and colleagues recently reported that despite its presence at the synapse during antigen-driven T-DC conjugation, PrP did not co-localise with integrin LFA-1, nor with CD3, CD43, LAT or Thy-1 (Ballerini et al., 2006).

A number of caveats may be pertinent to evaluation of the experiments comparing $PrP^{+/+}$ and $^{-/-}$ lymphocytes described here. First, it is possible that the F5 $PrP^{-/-}$ mice are not sufficiently homologous to the FVB/N strain to allow genuine comparison. Thus, differences due to absence of PrP may be obscured by differences due to non-identical genetic background. Second, as with prior work on PrP and T cell activation, cells were stimulated with non-specific mitogens that activate a variety of cells and a selection of T cell clones. Third, a protein's effects may only be mediated *in vivo* where its natural ligand is available. Thus experiments performed *in vitro* to detect effects of specific protein ablation may produce false negative results.

I sought to overcome these limitations by using the newly available F10 colony established by the MRC Prion Unit. This consists of $PrP^{-/-}$ mice that have been backcrossed with the FVB/N strain for 10 generations. Using these animals I repeated the proliferation experiment using a specific antigen and introducing an *in vivo* priming stage by immunising mice in the footpad with the same antigen 10 days prior to lymphocyte harvesting. Once again, no difference in *ex vivo* proliferation could be demonstrated.

My failure to demonstrate a significant effect of embryonic PrP deletion on T cell conjugation, proliferation or cytokine production brings into question whether the previously reported hypoproliferation in $PrP^{-/-}$ lymphocytes is really due to loss of PrP. The only group to have found a deficit in Zurich I mice was Mazzoni and colleagues (Mazzoni et al., 2005). However, this work was performed on mice >10 generations backcrossed onto a BALB/c background. Further, Mabbott and colleagues' data on the Edinburgh 129/Ola $PrP^{-/-}$ mouse (Mabbott et al., 1997) could

not be replicated by Liu et al (Liu et al., 2001). There are a limited number of explanations for these conflicting results:

1) All differences between $PrP^{+/+}$ and $^{-/-}$ mice so far reported are due to inadequate matching of knockout and control strains.

2) Some mouse strains, eg. FVB/N can compensate for the loss of PrP, whereas others eg. BALB/c cannot. This might reflect differing basal levels of PrP^{C} expression in T cells.

3) $PrP^{-/-}$ T cells have normal proliferative potential but $PrP^{-/-}$ APCs are deficient at providing co-stimulatory signalling. Thus, only experiments in which T cell activation is largely or entirely APC-dependent will demonstrate differences between $PrP^{+/+}$ and $^{-/-}$ lymphocytes. This hypothesis is supported by recent work by Ballerini and colleagues (Ballerini et al., 2006) but can only be fully resolved by crossing a TCR tg line onto a $PrP^{-/-}$ background.

4) Proliferation of individual effector $PrP^{-/-}$ lymphocytes is normal, but differences are due to altered *ratios* of T and non-T cells or naïve, memory and regulatory T cells due to an effect of PrP deletion on cell ontogeny or survival.

5) Proliferation of individual effector $PrP^{-/-}$ lymphocytes is normal, but differences are due to altered *function* of regulatory T cells.

In conclusion, embryonic deletion of PrP does not significantly alter basic T cell functions, such as conjugation with APCs, proliferation and cytokine production. In work presented elsewhere in this thesis, I have attempted to address the remaining unresolved questions in points (4) and (5) above, namely to determine whether PrP is

important in the ontogeny and function of key sub-classes of T cell – those mediating immunological memory and regulation.

Finally, what can we say about the function of PrP^C in T cell activation from this work? The fact that it is not significantly upregulated until several hours of activation suggests that it is not required for the most proximal events. This is supported by my failure to demonstrate a role for the protein in T cell-APC conjugation, proliferation or cytokine production. What more distal processes might require PrP^C? The majority of T cells undergo apoptosis following activation. PrP^C has been implicated in both pro- and anti-apoptotic pathways in neurons (Kuwahara et al., 1999;Bounhar et al., 2001;Chiarini et al., 2002;Paitel et al., 2002;Paitel et al., 2003). Preliminary work suggests that $PrP^{-/-}$ T cells do not show major alterations in propensity to activation-induced cell death (data not shown), however this requires formal validation. Alternatively, an immune response may fail if effector cells prematurely enter senescence. There is evidence that $PrP^{-/-}$ HSCs show reduced repopulative potential (Zhang et al., 2006) and that $PrP^{+/+}$ T cells undergo fewer mitoses when antigen challenged in $PrP^{-/-}$ hosts (Ballerini et al., 2006). *Prnp* is modestly upregulated during $CD8^+$ T cell repopulation of a lymphopaenic compartment (Goldrath et al., 2004). Thus, PrP^C may be implicated in the medium to long-term requirement of T cells and other effector and supporting immune cell populations to remain "replication" competent in situations of physiological stress. Further manipulation of $PrP^{-/-}$ animals and development of conditional knockout models will assist in validating this hypothesis.

These data also suggest that gene silencing of *PRNP* is unlikely to have a major effect on the proximal arms of the T cell response to activation, although embryonic deletion

cannot entirely model the effects of post-natal knockdown. However, we can be less certain about the effects of antibody or drug targeting of PrP^C. Agents that preferentially bind PrP^{high} cells and label them for removal may have a devastating impact on the immune response. Alternatively, they may result in blockade or augmentation of PrP^C-mediated signalling. Fully predicting the effects of this requires elucidation of the normal function of the protein, or at least modelling the effects of ligating it with a putative ligand. To date, experiments in this area have been limited to short term *in vitro* treatment of lymphocytes with anti-PrP mAb. Longer term and *in vivo* administration of anti-PrP mAbs and other PrP-specific compounds will be required to determine the effects on immune function and consequently, the safety of such agents.

CHAPTER 5 EXPRESSION AND FUNCTION OF PRP^C IN MEMORY AND REGULATORY T CELLS

5.1 Introduction

As already discussed, PrP^C is constitutively expressed in mammalian lymphoid cells and can be induced to increase its expression by T cell activation. However, little is known about how constitutive expression levels vary between mature lymphocyte subclasses. The aim of the work presented here was to further investigate the distribution and function of PrP^C expression within different populations of T lymphocyte. By dissecting out expression patterns I hoped to shed more light on the putative role of the protein. In addition, characterising the exact way in which the immune system uses PrP^C will help design anti-prion therapies. For example, as discussed earlier, drugs that specifically or non-specifically bind the normal prion protein may interfere with its function. The extent to which this will precipitate immunopathology depends on which elements in the lymphoid system express the highest amounts of PrP or are particularly functionally dependent on PrP^C expression.

Furthermore, one possible explanation for the previously reported hyporesponsiveness of $PrP^{-/-}$ splenocytes to mitogens is that researchers have not been comparing identical cell populations. Swift proliferative responses to antigenic stimulation are provided by memory cells, whereas regulatory T cells can suppress this proliferation. Thus, changes in the relative numbers or function of these classes of lymphocyte might bias an experiment where the readout is proliferation of a population of unfractionated lymphocytes.

5.1.1 Specialisation of peripheral lymphocytes into memory cells

Lymphocyte maturation occurs in the thymus, where TCR expression is determined and T cells with potentially autoreactive specificities are deleted. A major marker of maturation is the co-expression of CD4 and CD8 antigens, followed by downregulation of one of these, leaving single positive CD4 and CD8 cells. These mediate helper functions via MHC class II restricted antigen presentation ($CD4^+$), or cytotoxic activity in response to peptides presented by MHC class I ($CD8^+$). However, once in the periphery, further differentiation of mature T cells can occur. Following initial exposure to antigen, naïve T cells proliferate to expand the pool of effector cells. After this response, a proportion of cells avoid activation induced cell death to become memory cells. These are primed for rapid helper or cytotoxic functions on repeat exposure to their cognate antigen. Memory differentiation involves changes in surface expression of a number of key markers, in particular upregulation of CD44 and, in certain subsets, downregulation of CD62L. In mice downregulation of CD45RB occurs, whereas human memory T cells switch CD45 isoform from RA to RO. To provide effective defence, memory cells must patrol potential sites of repeat encounter with antigen, such as major peripheral organs, sites of injury, and lymph nodes. This gives rise to further differentiation, for example $CCR7^+$ central memory cells circulate between secondary lymphoid organs while $CCR7^-$ effector memory cells patrol peripheral non-lymphoid tissues (Sallusto et al., 1999).

One group has reported higher surface PrP expression in $CD45RA^+$ compared to $CD45RO^+$ PBMCs (Li et al., 2001). However, it is not clear that these cells were co-stained for CD4, although non-specific fractionation techniques were employed. Thus, non T cells may have been included in either the RA^+ or RO^+ populations,

potentially skewing the results. Further, T cells bearing other phenotypic memory markers have not been examined for PrP expression.

5.1.2 Regulatory T cells

The possible existence of naturally occurring T cells with the capacity to suppress auto-immunity was raised in the 1970s by Penhale, who reported spontaneous thyroiditis in thymectomised rats that could be abrogated by transfer of lymphocytes from healthy donors (Penhale et al., 1976). Later workers including Sakaguchi and Mason extended the range of organ specific auto-immune diseases that could be induced in rodents by thymectomy in early post-natal life and further observed that disease reversal required transfer of a subset of $CD4^+$ cells (Fowell and Mason, 1993). These phenomena were subsequently linked to loss and then reconstitution of a population of $CD4^+$ T cells that develop in, and then exit, the thymus and constitutively express CD25 (reviewed in Sakaguchi, 2005). A similar population has been identified among human PBMCs. These cells, termed regulatory T cells (or Tregs) have been shown to efficiently suppress effector T cell functions, including proliferation and cytokine release, both *in vitro* and *in vivo*. However, the exact mechanism by which this is mediated remains unclear, with conflicting data on the necessity of cell-cell contact or anti-inflammatory cytokines. The phenotypic identification of Tregs has been assisted by the discovery that they express the forkhead family transcription factor Foxp3, the only major class of T cell to do so.

Microarray analysis has revealed PrP to be transcriptionally upregulated in certain classes of regulatory T cell (Huehn et al., 2004). However, PrP expression in regulatory T cells has not been directly determined. The identification of CD25 and Foxp3 as Treg markers has facilitated identification and isolation of constitutive

regulatory T cells using flow cytometry or bead-based techniques. This provided an opportunity to define PrP expression in regulatory T cells and to assess the ontogeny and function of Tregs in $PrP^{-/-}$ animals.

5.2 Results

5.2.1 $PrP^{-/-}$ mice have essentially normal numbers of $CD4^+$ and $CD8^+$ T cells

First, I assessed the relative numbers of $CD4^+$ and $CD8^+$ cells in wild type and $PrP^{-/-}$ mice (10 generations crossed to FVB/N) in spleen, peripheral (inguinal and axillary) and mesenteric lymph nodes. No major differences in $CD4^+$ and $CD8^+$ numbers were seen (Figure 5.1A-C), although $PrP^{-/-}$ mice had a slightly higher percentage of $CD8^+$ cells in peripheral lymph nodes, producing a marginally reduced CD4:8 ratio (Figure 5.1D). In thymus, relative numbers of double negative, double positive and single positive cells were equivalent (Figure 5.1G-H). However, I found that more $PrP^{-/-}$ $CD4^+$ $CD8^+$ thymocytes expressed CD25 than the equivalent population in $PrP^{+/+}$ mice. CD25 is expressed by a proportion of double negative immature thymocytes; failure to downregulate this marker during the double positive stage may indicate arrest or delay in T cell maturation. However, numbers of $CD4^+$ $CD8^+$ $CD25^+$ cells were small and final numbers of mature single positive cells were similar, suggesting that there is not a significant problem with thymocyte maturation in $PrP^{-/-}$ mice. I concluded that $PrP^{-/-}$ mice have grossly unpeturbed lymphoid ontogeny.

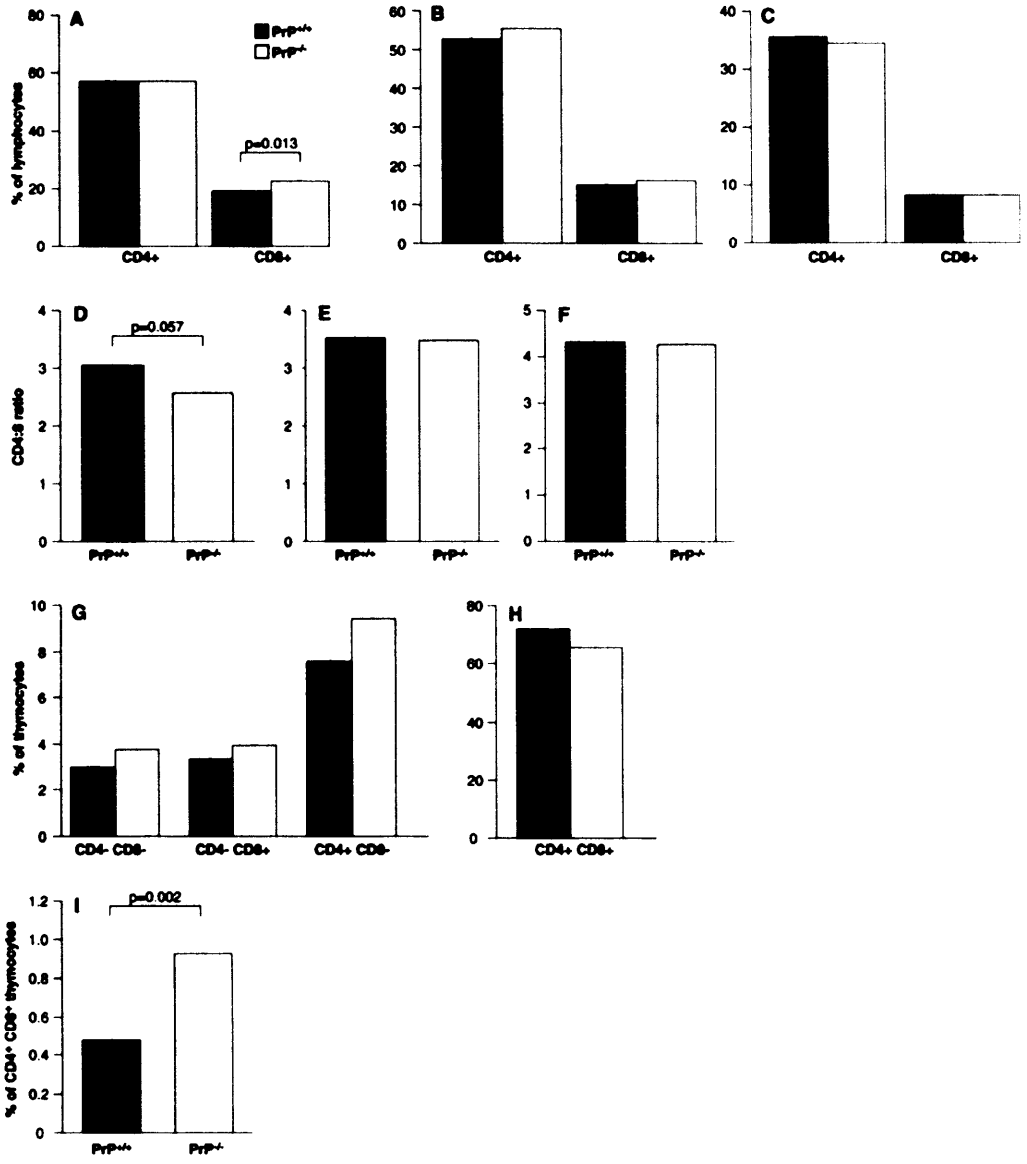

Figure 5.1 Relative numbers of $CD4^+$ and $CD8^+$ T cells in $PrP^{+/+}$ and $^{-/-}$ mice

$CD4^+$ and $CD8^+$ T cell numbers in peripheral (A) and mesenteric (B) lymph nodes and spleen (C) from $PrP^{+/+}$ and $^{-/-}$ FVB/N mice. $PrP^{-/-}$ mice had slightly higher proportion of CD8 cells in peripheral lymph nodes (t test) (A), producing a reduced CD4:8 ratio (Mann-Whitney test) (D). CD4:8 ratios in mesenteric nodes (E) and spleen (F) were identical. In thymus, percentages of double negative, single positive cells (G) and double

positive (H) and were equivalent. (I) More $PrP^{-/-}$ $CD4^+$ $CD8^+$ thymocytes expressed CD25 than the equivalent population in $PrP^{+/+}$ mice (t test).

5.2.2 Surface PrP^C expression level correlates with memory T cell markers

I then examined PrP^C expression in wild-type murine splenocytes and human PBMCs to determine which cells harboured the highest expression levels. Splenocytes from adult C57BL/6 mice were stained for CD4, for a selection of memory markers and for PrP. The cut-off between PrP "low" and "high" populations was determined by an isotype control for the anti-PrP mAb. The proportion of cells in each group expressing phenotypic memory markers was then determined. I found that PrP^{high} cells are highly enriched with respect to expression of a memory phenotype ($CD62L^{low}$, $CD44^{high}$, $CD45RB^{low}$), whereas PrP^{low} cells represent a more heterogeneous population with a mixture of memory and naïve cells (Figure 5.2A). Further, a direct comparison of PrP expression between $CD62L^{low}$ and ^{high} subsets revealed this to be significantly higher in the $CD62L^{low}$ (memory) population (Figure 5.2B). Interestingly, in these fresh *ex vivo* cells, only a minority of those with a memory phenotype were PrP^{high}, although this was higher than the proportion of non-memory cells with high PrP expression (Figure 5.2C). Thus, in mice the small PrP^{high} $CD4^+$ fraction represents a highly selected subpopulation of the memory cell pool; whether PrP^{high} and PrP^{low} memory cells are functionally different remains unclear.

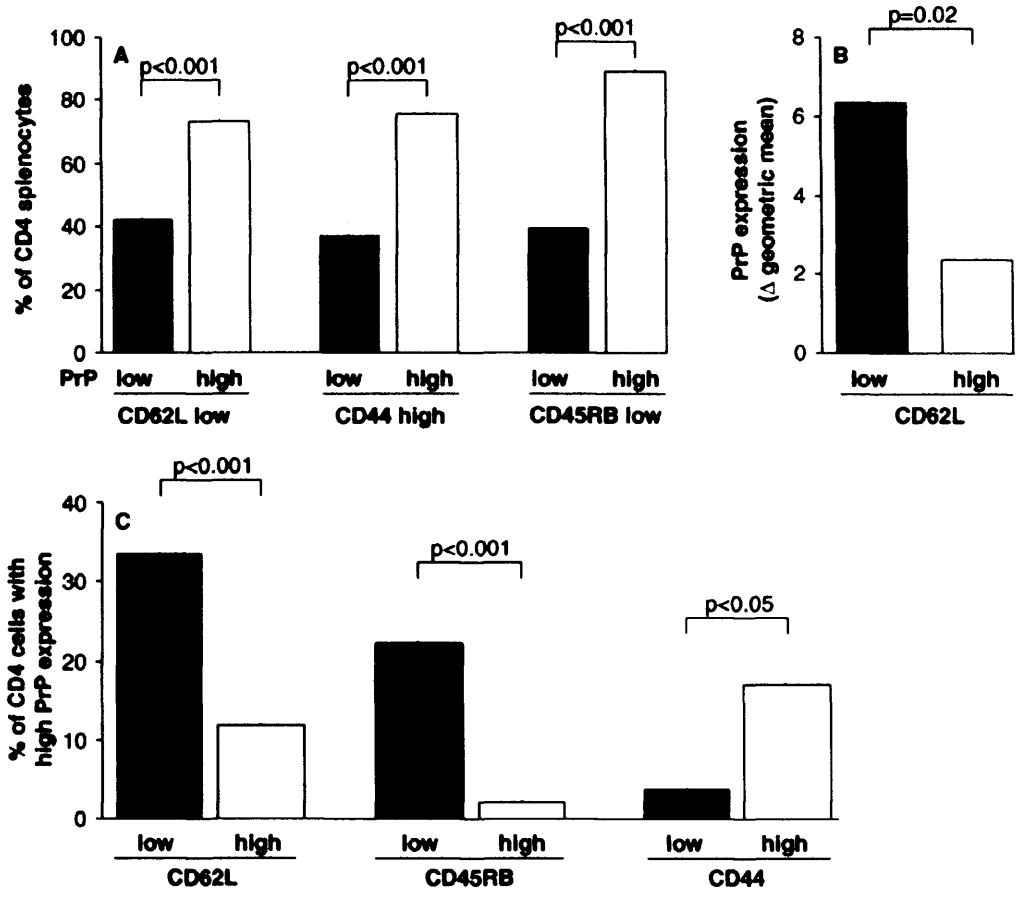

Figure 5.2 Correlation of PrP expression with memory T cell markers

(A) Comparison of memory marker expression between PrP^{low} and ^{high} C57BL/6 $CD4^+$ cells. The PrP^{high} population has a significantly greater proportion of cells with a memory phenotype (ANOVA with Bonferroni multiple comparisons post test). (B) PrP expression is significantly higher in the $CD4^+$ $CD62L^{low}$ compared to the $CD62L^{high}$ population (paired t test). (C) Proportion of cells in memory and naïve populations with high PrP expression. Only a minority of cells with a memory phenotype were PrP^{high}, although for each memory marker this was higher than the percentage of PrP^{high} naïve cells (ANOVA with Bonferroni multiple comparisons post test).

5.2.3 PrP^C expression in human $CD4^+$ and $CD8^+$ T cells correlates with the $CD44^{high}$ but not the $CD62L^{low}$ memory phenotype

I then assessed PrP^C expression in human memory T lymphocytes. PBMCs from healthy donors (n=3) were stained for CD4 or CD8, CD44 or CD62L and PrP. After gating on viable $CD4^+$ or $CD8^+$ populations, cells were split into CD44 or $CD62L^{high}$ and ^{low}. The mean PrP expression in each group was determined and the ratio of expression in memory to naïve cells calculated. $CD44^{high}$ $CD4^+$ and $CD8^+$ memory cells expressed about twice as much PrP as $CD44^{low}$ naïve cells (Figure 5.3A-B). However, CD62L downregulation did not correlate with PrP expression (Figure 5.3A-B). Thus, in humans the CD44 memory antigen correlates with PrP expression, as already reported for CD45 isoforms (Li et al., 2001), whereas CD62L and PrP expression are not related.

Using ICSM18 to detect surface PrP in human T lymphocytes, two peaks corresponding to PrP^{low} and ^{high} populations are frequently visible (see Figure 2.2). The proportion of PrP^{high} cells in the three donors studied ranged from 20-42% for $CD8^+$ cells and from 15-38% for $CD4^+$ cells. The $CD4^+$ and $CD8^+$ PrP^{high} fractions are overwhelmingly $CD44^{high}$ (>90%) compared to the PrP^{low} population (Figure 5.3C-D). However, as in mice, the $CD4^+$ and $CD8^+$ PrP^{high} populations do not contain all the $CD44^{high}$ cells. Thus, they represent a selected memory cell sub-population of uncharacterised functional significance. In contrast, CD62L expression does not differ substantially between PrP^{high} and ^{low} populations (Figure 5.3C-D), in keeping with the results above.

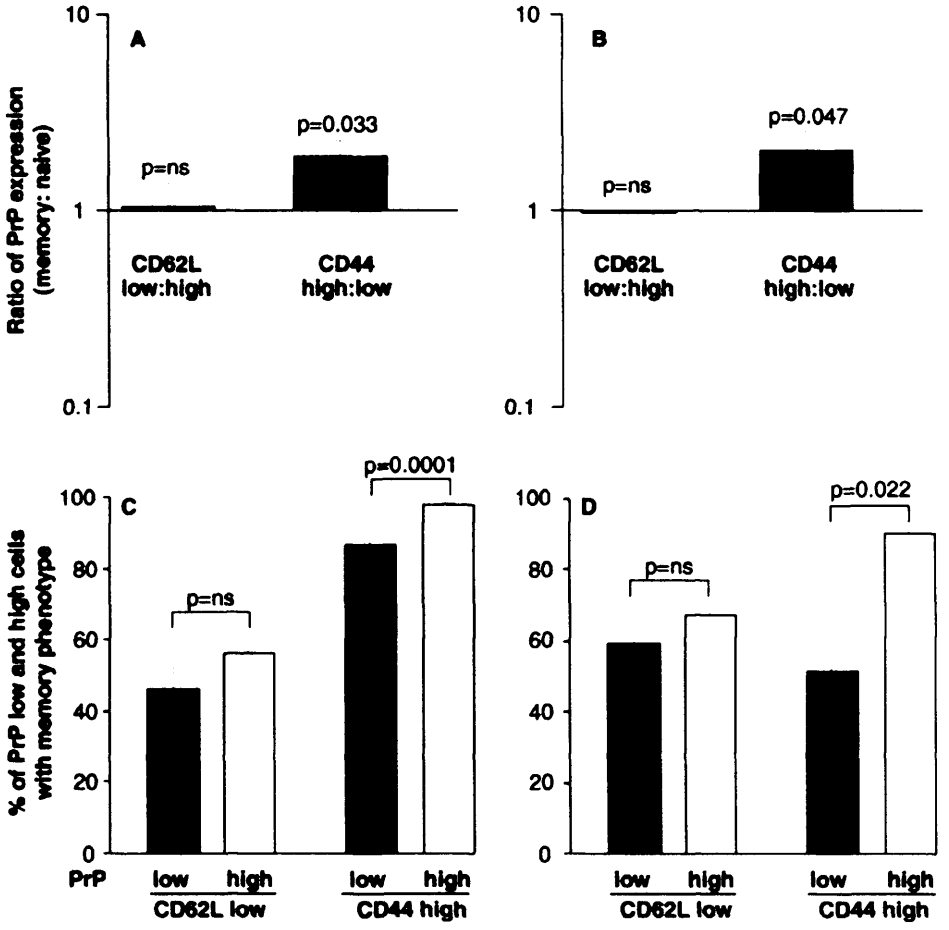

Figure 5.3 Correlation of PrP with memory markers in human PBMCs

Ratio of surface PrP expression between memory and naïve $CD4^+$ (A) and $CD8^+$ (B) PBMCs from three human donors. High expression of CD44 is associated with significantly greater PrP expression, whereas CD62L levels do not correlate with PrP expression (log transformed values, one sample t test). Comparison of memory marker expression between PrP^{low} and ^{high} $CD4^+$ (C) and $CD8^+$ (D) PBMCs. The PrP^{high} fractions contain significantly greater numbers of $CD44^{high}$ cells compared to the PrP^{low} population (t test), whereas numbers of $CD62L^{low}$ cells do not differ between the two groups.

5.2.4 $PrP^{-/-}$ mice have reduced numbers of memory T cells

Because high expression of PrP appeared to be a property of memory cells in mice and humans, I then examined whether PrP deficient animals had normal numbers of memory T cells. Splenocytes from wild type and $PrP^{-/-}$ mice (10 generations backcrossed to FVB/N) were stained for CD4 or CD8 and for memory markers CD44, CD62L and CD45RB. I found that $PrP^{-/-}$ mice had slightly but significantly fewer $CD4^+$ T cells expressing high levels of CD44 and low levels of CD62L, with no difference in numbers of $CD45RB^{low}$ cells (Figure 5.4A). Similarly, $PrP^{-/-}$ mice were slightly deficient in $CD8^+$ $CD44^{high}$ lymphocytes, although there was no significant difference in the numbers of $CD8^+$ $CD62L^{low}$ cells (Figure 5.4B). Thus, I concluded that PrP may be required for the maintenance of a full memory T cell compartment, although it is not essential for memory T cell development.

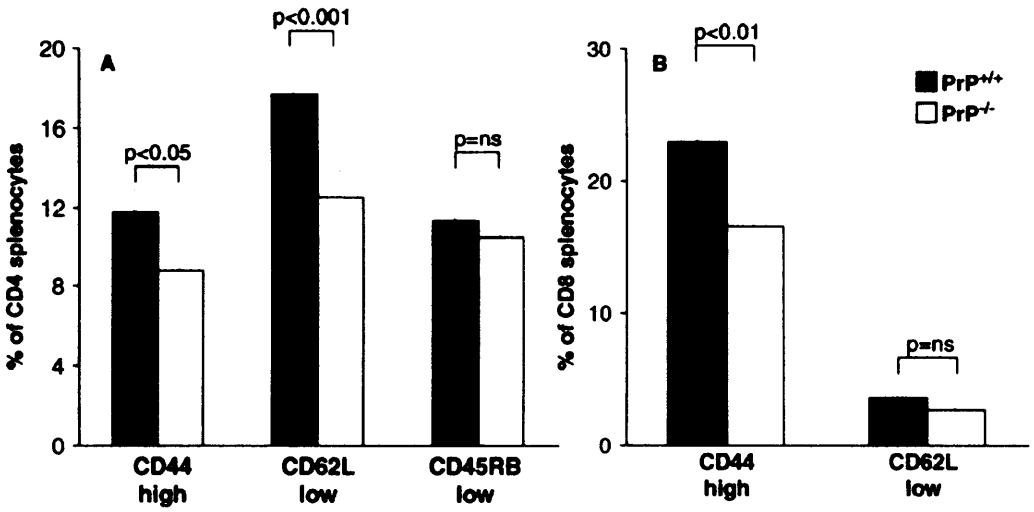

Figure 5.4 Relative numbers of memory cells in $PrP^{+/+}$ and $^{-/-}$ mice
Comparison of percentage of $CD4^+$ (A) and $CD8^+$ (B) splenocytes expressing phenotypic memory markers between $PrP^{+/+}$ and $^{-/-}$ FVB/N mice. $PrP^{-/-}$ mice have significantly fewer $CD4^+$ T cells expressing high levels of CD44 and low levels of CD62L (ANOVA with Bonferroni multiple

comparisons post test), with no significant difference in numbers of CD45RB^{low} cells (A). PrP$^{-/-}$ mice are deficient in CD8^{+} CD44^{high} lymphocytes (ANOVA with Bonferroni multiple comparisons post test), with no significant difference in the numbers of CD8^{+} CD62L^{low} cells (B).

5.2.5 Correlation of PrP and CD25 expression in CD4^{+} T cells

The reduced numbers of memory cells among PrP$^{-/-}$ splenocytes offers one explanation as to why some investigators have reported reduced proliferation in mitogen stimulated PrP$^{-/-}$ splenocytes. An alternative explanation is that PrP$^{-/-}$ mice harbour different numbers or altered function of suppressor cells. I thus sought to characterise the expression of PrP in regulatory T cells and to determine if their number and function were normal in PrP deficient animals. I initially observed that in C57BL/6 mice the ~ 10% of CD4^{+} splenocytes constitutively expressing CD25 contained more PrP^{high} cells than the CD4^{+} CD25^{-} effector pool (Figure 5.5A). I also observed in two human donors that the 0.5% of CD4^{+} cells with the highest CD25 expression had ~ 50-75% higher PrP expression than CD25^{low} and negative cells (Figure 5.5B).

5.2.6 CD4^{+} CD25^{+} Foxp3^{+} Tregs express high levels of surface PrP

To examine the relationship between PrP expression and regulatory cell phenotype more directly, I used flow cytometry to identify regulatory T cells from spleens of wild-type FVB/N mice by staining for surface CD25 and intracellular Foxp3. Approximately 7% of CD4^{+} splenocytes expressed these two markers. I co-stained for PrP and calculated PrP expression level as the delta geometric mean. I observed approximately 10-fold higher surface PrP expression in CD4^{+} CD25^{+} Foxp3^{+} T regs than in non-regulatory CD4^{+} T cells (Figure 5.6).

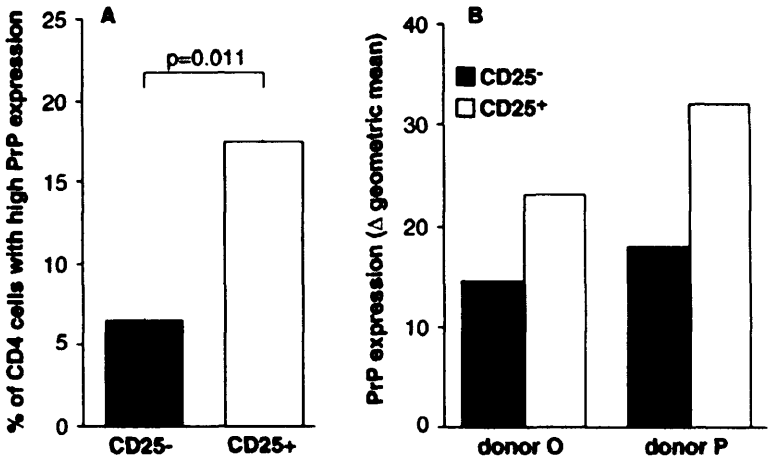

Figure 5.5 Correlation between PrP and CD25 in $CD4^+$ lymphocytes

(A) PrP expression in $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ C57BL/6 splenocytes. A significantly greater proportion of $CD4^+$ $CD25^+$ cells are PrP^{high} (t test).

(B) PrP expression in $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ PBMCs from two human donors.

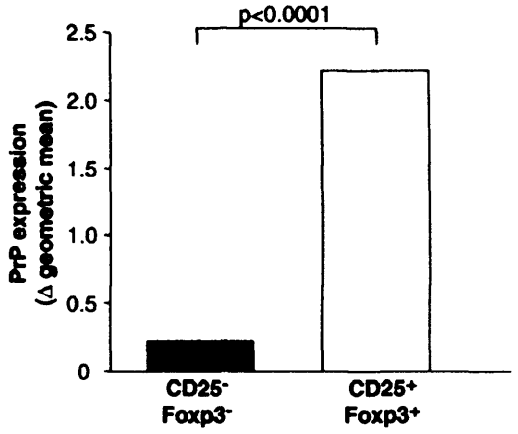

Figure 5.6 Correlation of PrP with CD25 and Foxp3 in murine splenocytes

Comparison of PrP expression between $CD4^+$ $CD25^-$ $Foxp3^-$ and $CD4^+$ $CD25^+$ $Foxp3^+$ T cells from FVB/N mice. Cells with a Treg phenotype have significantly higher surface PrP expression (t test).

5.2.7 PrP is transcriptionally upregulated in Tregs

To confirm these findings at the transcriptional level, I used FACS sorting to separate $CD4^+$ T cells from C57BL/6 mice into CD25 positive and CD25 negative fractions. I then determined *Foxp3* and *Prnp* mRNA expression in these two populations by real time RT-PCR. The effective separation of Tregs from other T cells using this method was validated by finding that the $CD4^+$ $CD25^+$ fraction contained ~ 100 times more *Foxp3* mRNA than the $CD4^+$ $CD25^-$ population, suggesting that I had indeed discriminated Tregs from effector cells (Figure 5.7A). I then found that *Prnp* expression was ~ 4.5-fold higher in $CD4^+$ $CD25^+$ cells than in $CD4^+$ $CD25^-$ cells (Figure 5.7B). Thus, I concluded that *Prnp* is preferentially expressed by Tregs.

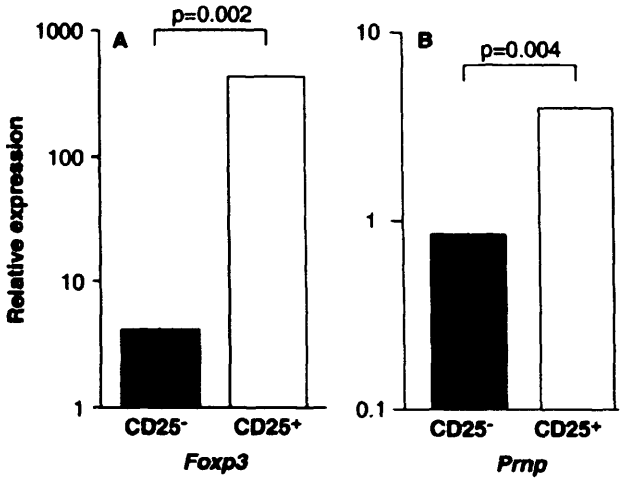

Figure 5.7 Expression of *Prnp* and *Foxp3* in murine $CD4^+$ splenocytes

Analysis by real time RT-PCR of *Foxp3* (A) and *Prnp* (B) transcription in $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ splenocytes from C57BL/6 mice. The $CD4^+$ $CD25^+$ fraction contains significantly more *Foxp3* and *Prnp* mRNA than the $CD4^+$ $CD25^-$ population (Mann-Whitney test).

5.2.8 $PrP^{-/-}$ mice have normal numbers of Tregs and Foxp3 expression levels

These data raise the question as to whether Treg ontogeny or function will be affected by the absence of PrP. Using flow cytometry, I determined the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in thymus, spleen, mesenteric and non-mesenteric lymph nodes from $PrP^{+/+}$ and $^{-/-}$ mice. I found no deficit in Treg number in $PrP^{-/-}$ mice, indicating that these cells do not require PrP for their thymic development or maintenance in the periphery (Figure 5.8A). The level of Foxp3 expression was also no different between $PrP^{+/+}$ and $^{-/-}$ mice, although in both strains $CD4^+$ $CD25^+$ thymocytes expressed slightly higher Foxp3 than peripheral Tregs (Figure 5.8B).

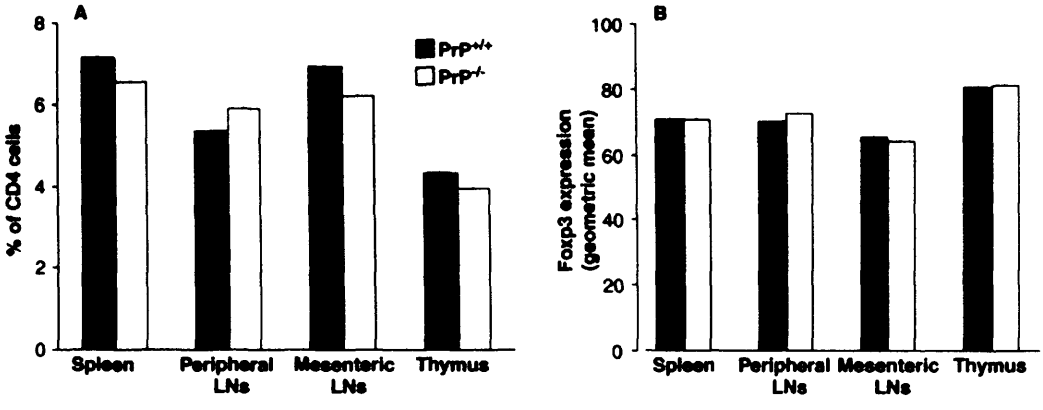

Figure 5.8 Regulatory T cell numbers and Foxp3 expression in $PrP^{+/+}$ and $^{-/-}$ mice

(A) Percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in thymus and peripheral lymphoid tissues from $PrP^{+/+}$ and $^{-/-}$ FVB/N mice. (B) Foxp3 expression levels determined by flow cytometry in $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in thymus and peripheral lymphoid tissues from $PrP^{+/+}$ and $^{-/-}$ FVB/N mice.

5.2.9 $PrP^{-/-}$ Tregs have enhanced suppressor function

Finally I determined whether Treg function would be affected by embryonic deletion of PrP. $CD4^+$ lymphocytes were purified from spleens of $PrP^{+/+}$ and $^{-/-}$ mice and further split into $CD25^+$ and $CD25^-$ fractions. $CD4^+$ $CD25^-$ cells were stimulated with anti-CD3 and anti-CD28 coated beads in the presence of an increasing number of syngeneic or congeneic $CD4^+$ $CD25^+$ cells. This resulted in almost complete suppression of proliferation at high ratios of regs:effectors (Figure 5.9A-D). Interestingly, $PrP^{-/-}$ T regs had greater suppressive capacity than $PrP^{+/+}$ T regs, irrespective of the genotype of the effector population (Figure 5.9E-F). This suggests a further explanation for the hyporesponsiveness of $PrP^{-/-}$ lymphocytes to certain mitogens. Thus, I concluded that PrP^C may have an important role in controlling excessive suppression by Tregs; conversely, deletion of PrP may enhance the function of Tregs where this is beneficial, such as in averting autoimmunity.

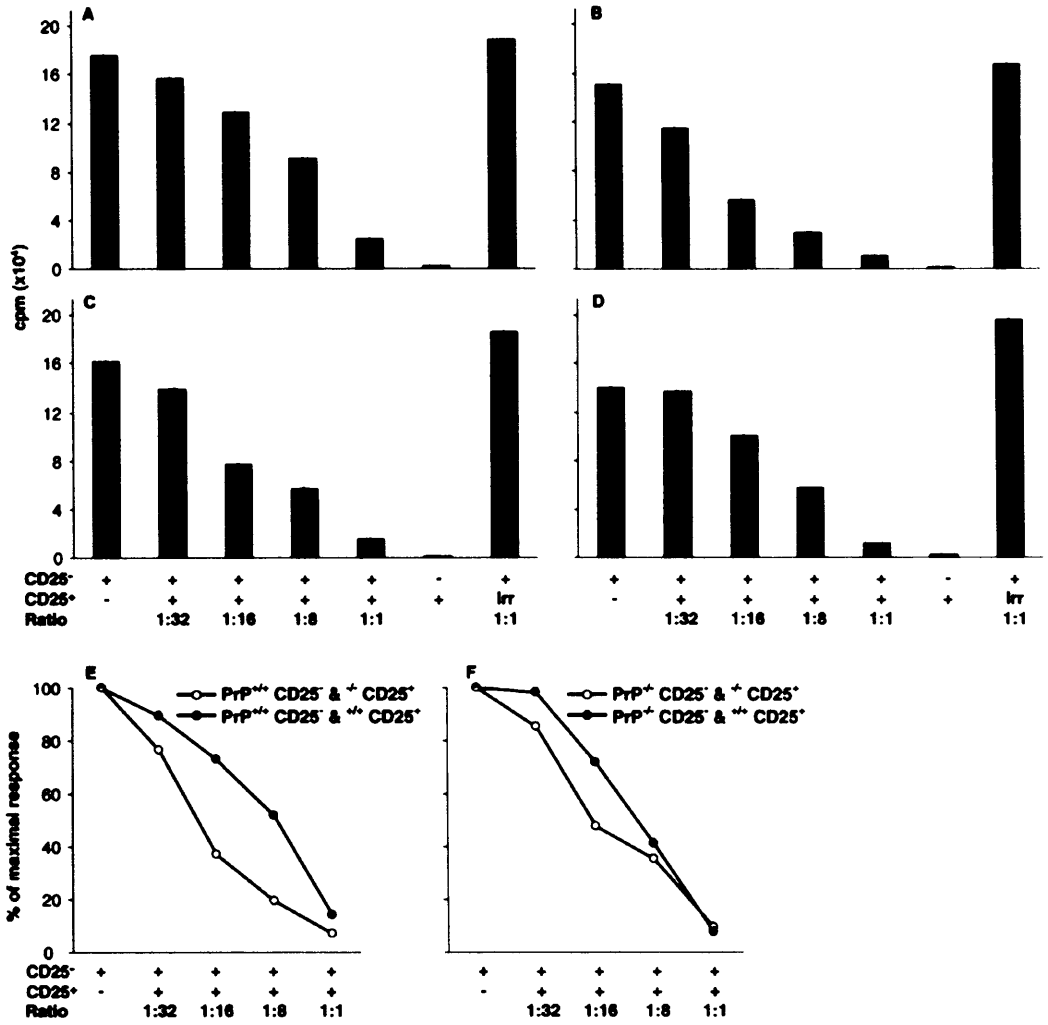

Figure 5.9 Functional assay of $PrP^{+/+}$ and $^{-/-}$ Treg suppression

Suppression of anti-CD3 and anti-CD28 mediated proliferation of $CD4^+$ $CD25^-$ T cells by co-culture with increasing numbers of $CD4^+$ $CD25^+$ Tregs. Irradiated Tregs (Irr) have no suppressor function. (A) Syngeneic culture of $PrP^{+/+}$ $CD4^+$ $CD25^-$ with $PrP^{+/+}$ $CD4^+$ $CD25^+$ cells. (B) Congeneic culture of $PrP^{+/+}$ $CD4^+$ $CD25^-$ with $PrP^{-/-}$ $CD4^+$ $CD25^+$ cells. (C) Syngeneic culture of $PrP^{-/-}$ $CD4^+$ $CD25^-$ with $PrP^{-/-}$ $CD4^+$ $CD25^+$ cells. (D) Congeneic culture of $PrP^{-/-}$ $CD4^+$ $CD25^-$ with $PrP^{+/+}$ $CD4^+$ $CD25^+$ cells. (E-F) Proliferation shown as percentage of maximal response ($CD4^+$ $CD25^-$ cells cultured

alone), demonstrates that $PrP^{-/-}$ Tregs have enhanced suppressor function irrespective of genotype of $CD4^+$ $CD25^-$ effector cells.

5.3 Discussion

This work confirms and extends previous observations on the expression of PrP by memory T cell subsets. Using flow cytometry, I demonstrated that PrP expression in murine $CD4^+$ and $CD8^+$ memory cells defined by high expression of CD44, low expression of CD45RB or low expression of CD62L is significantly higher than in naïve cells with the converse phenotypes. In addition, high CD44 expression correlates closely with high PrP expression in human $CD4^+$ and $CD8^+$ PBMCs. This confirms a previous observation regarding differential expression of PrP between CD45RA and RO expressing PBMCs (Li et al., 2001). However, in humans CD62L expression does not correlate with PrP levels. Why this memory marker is different from CD44 and CD45 isoforms in humans but not mice remains unclear. One possibility is that specific memory markers are dependent on PrP for their expression, and that this varies by species. Interestingly, deletion of PrP results in marked downregulation of CD44 in mouse fibroblasts and brain tissue (Satoh et al., 2000). Another possibility is that CD62L downregulation alone correlates poorly with memory cell status in human PBMCs. Certainly, the relationship between CD62L expression and $CD8^+$ memory cell differentiation is a complex one, in which surface CD62L is initially lost but then regained once antigen clearance has been achieved (reviewed in Gourley et al., 2004). Furthermore, CD62L is a lymph node homing receptor associated with the central memory phenotype (Sallusto et al., 1999). Thus it is likely that memory lymphocytes will be present in both $CD62L^{low}$ and ^{high} fractions,

perhaps explaining why human CD62L^{low} cells do not clearly have higher PrP expression.

These observations raise important questions about the function of PrP. Why should memory cells upregulate this protein? The role of memory cells involves complex trafficking and signalling, in which PrP could play a part, perhaps as an adhesion or signalling molecule. When does PrP upregulation occur? Do certain cells upregulate PrP after they have differentiated into memory lymphocytes? Or is it a reflection of previous activation, following which PrP is not downregulated? If so, it appears that only a proportion of memory cells maintain high PrP levels while the majority return to low constitutive expression.

Thus, the situation is complex in that although the PrP^{high} T cell compartment is very highly enriched with memory lymphocytes, there are memory T cells that maintain low constitutive PrP expression. It remains unclear exactly which memory cells require high PrP and whether they are functionally different from their PrP^{low} counterparts. Analysis of PrP expression in memory cells in different organs and functional studies of PrP^{high} and ^{low} memory T cells would address this issue. It may be that PrP behaves similarly to CCR7 and CD27 in that it is only expressed by certain memory cells with particular functions and homing properties.

I sought to address this question by studying memory cell numbers in PrP$^{-/-}$ mice. Overall numbers of CD4^{+} and CD8^{+} T cells were not consistently different. However, we found that PrP$^{-/-}$ mice had a slight but significant deficiency of CD4^{+} CD44^{high}, CD4^{+} CD62L^{low} and CD8^{+} CD44^{high} memory cells. As in previous studies, relative numbers of thymocyte classes were similar to wild type, suggesting that PrP does not play an indispensable role in thymic ontogeny and lymphocyte maturation. The

deficiency in memory cell number suggests, however, that PrP is important in directing peripheral lymphocyte homeostasis. No such effect is seen with respect to regulatory T cells, numbers of which were equal in $PrP^{+/+}$ and $^{-/-}$ mice, as discussed below.

A marker that was upregulated in parallel with PrP would help determine whether it is loss of the PrP^{high} memory population seen in wild type animals that accounts for the slightly reduced memory cell numbers in $PrP^{-/-}$ mice. This modest deficiency does not appear to be sufficient to cause immunodeficiency in a pathogen-poor environment. However, more robust manipulation of $PrP^{-/-}$ mice and adoptive transfer of memory lymphocytes into congeneic hosts may reveal such an altered phenotype.

How might PrP^{C} contribute to lymphocyte homeostasis and maturation in the periphery? Memory T cells are derived from responding clones following an immune response. Whether they are randomly selected or pre-determined to avoid AICD remains controversial. However, Fas-mediated AICD is the essential mechanism by which non-memory lymphocytes are deleted at the close of an effector response. An inability to resist Fas-mediated apoptosis would lead to a deficiency of memory lymphocytes. $PrP^{-/-}$ neurons are more susceptible to Bax-mediated cell death (Bounhar et al., 2001;Roucou et al., 2003). However, there is no published work on the relationship between Fas and PrP^{C} in lymphocytes.

Another possibility is that $PrP^{-/-}$ naïve cells are less able to differentiate into memory cells because they are defective in certain aspects of activation. Memory cells are generated from the pool of activated lymphocytes following an immune response; if fewer daughter cells are generated, then fewer memory cells will result (Hou et al., 1994). As described in Chapter 4 above, $PrP^{-/-}$ cells proliferate normally in response

to short term polyclonal activation *in vitro*. I was not able in the work described here to fully ascertain the effects of PrP deletion on the size of specific antigen-driven clonal expansion, although the HEL and OVA immunisation experiments suggest that this is normal. However, it may be that after repeated rounds of stimulation in vivo, $PrP^{-/-}$ T cells are less efficient at continued cell division. This hypothesis might be addressed by comparing telomere length in mature $CD4^+$ or $CD8^+$ lymphocytes from age-matched $PrP^{+/+}$ and $^{-/-}$ mice. Such "exhaustion" of cell division could also explain the gradual failure of $PrP^{-/-}$ haematopoietic stem cells on multiple rounds of adoptive transfer into myeloablated $PrP^{+/+}$ hosts (Zhang et al., 2006).

Interestingly, the molecular programme underlying haemostatic proliferation in lymphopaenia is very similar to that involved in memory cell differentiation (Goldrath et al., 2004). Indeed, *Prnp* mRNA is modestly upregulated in $CD8^+$ memory cells and progressively upregulated in naïve $CD8^+$ cells entering a lymphopaenic host (Goldrath et al., 2004). Thus, via a common but as yet undetermined mechanism, PrP^C may be important both to situations where multiple mitoses are required to repopulate an immunodeficient compartment and in maintenance of a memory pool in immunocompetent hosts. This might be further elucidated by studying the mature lymphocyte population generated on repopulating a lymphopaenic host with naïve $PrP^{-/-}$ CD4 or CD8 lymphocytes.

A further consideration that may underlie a deficit in memory cell formation is whether lymphocytes mature normally in the absence of PrP. In $PrP^{-/-}$ mice, I observed a statistically significant increase in the numbers of DP thymocytes continuing to express CD25. The biological significance of this result is unclear. Although numbers of mature T cells may differ slightly between $PrP^{+/+}$ and $^{-/-}$ mice, I

did not find any gross perturbation of thymocyte maturation as numbers of single and double positive cells were equivalent. The persistence of CD25 expression in a small number of cells through the double positive stage may imply a block on forward maturation, or the persistence of immature cells that under normal circumstances ought to be disposed of, presumably via apoptotic cell death. However, the proportions of $CD25^+$ double positive thymocytes were extremely small; 0.48% versus 0.93%. Thus this seems unlikely to signal a genuine failure of thymic maturation. In contrast, mice with ~ 50-fold overexpression of lymphoid PrP have thymic abnormalities characterised principally by premature involution and interrupted thymocyte maturation at the $CD4^-$ $CD8^-$ $CD44^-$ $CD25^+$ (DN3) stage (Jouvin-Marche et al., 2006). On balance, my data and that of Zhang and Jouvin-Marche suggest that PrP may be involved in a negative feedback loop in thymic differentiation but supply a positive signal in peripheral differentiation.

A further mechanism that could explain the reduced numbers of memory T cells in $PrP^{-/-}$ animals is that trafficking of these cells to the spleen is PrP dependent. This could have been resolved by comparing memory cell numbers in peripheral lymph nodes and among circulating PBMCs. Alternatively, an effect of PrP deletion may be downregulation of surface memory cell markers, rather than failure of memory cell differentiation. As previously mentioned, PrP deletion results in reduced CD44 expression in fibroblasts and brain (Satoh et al., 2000). The same effect may apply to T cells, resulting in an apparent loss of memory cell numbers. The loss of CD44 expression from a proportion of memory cells may in itself affect function as CD44 is important for lymphocyte adhesion and homing. I did, however, also observe a reduction in $CD4^+$ $CD62L^{low}$ memory cells, arguing against a selective effect of PrP deletion on CD44 expression.

This work is the first in which PrP expression has been directly quantified in regulatory T cells. Using CD25 and Foxp3 staining to identify Tregs by FACS, I demonstrated a 10-fold increase in surface PrP expression compared to non-regulatory $CD4^+$ splenocytes. Further, using real time RT-PCR in cells split by FACS sorting into $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ fractions, I demonstrated that cells bearing a regulatory phenotype have increased *Prnp* mRNA. One possible criticism of this experiment is that the $CD4^+$ $CD25^+$ fraction may have contained numerous activated effector cells, which also upregulate *Prnp*. However, I and others have observed that among fresh *ex vivo* splenocytes from untreated wild type mice the percentage of $CD4^+$ $CD25^+$ cells expressing Foxp3 is > 80% (data not shown). In other words, the vast majority of $CD4^+$ $CD25^+$ cells in such conditions are genuine regulatory T cells and not activated effectors. Further, I confirmed that the sorted $CD4^+$ $CD25^+$ fraction was predominantly a regulatory T cell population by comparing *Foxp3* expression with that in the $CD25^-$ pool.

Thus, I propose that PrP^C can be considered a marker of regulatory T cells, although unlike Foxp3 it is not exclusively expressed at high levels by Tregs. PrP^C therefore has more in common with CD25, GITR, CD40 and CTLA-4, in that it is both an inducible marker in activated effector T cells and constitutively expressed by Tregs. The precise function of PrP^C in regulatory T cells, as in memory cells, remains unclear.

I attempted to resolve this by examining Treg number and function in $PrP^{-/-}$ mice, in which regulatory T cells have not previously been characterised. I found that $PrP^{-/-}$ mice have normal numbers of regulatory T cells in spleen, mesenteric nodes and non-

mesenteric lymph nodes. Furthermore, Foxp3 expression level as measured by flow cytometry did not differ between $PrP^{+/+}$ and $^{-/-}$ Tregs.

Most interesting was the observation that $PrP^{-/-}$ Tregs have greater suppressive capacity than wild type Tregs. This was an unexpected finding, as we had hypothesised that the increased *Prnp* expression in Tregs would be important in sustaining their suppressive function. However, it is becoming increasingly clear that Treg function is finely controlled and subject to numerous modifying signals, such as TLR-8 signalling (Peng et al., 2005). Thus, PrP^{C} may be required to avoid excessive suppression, which if unchecked might result in inadequate control of infections and failure to reject tumours. The latter does not appear to be a spontaneous feature of $PrP^{-/-}$ mice; however, the former has not been tested using a wide range of pathogens. Aguzzi and co-workers reported normal $CD8^{+}$ expansion and antibody production after VSV and LCMV infection in mice lacking PrP or both PrP and Doppel (Genoud et al., 2004), however full data on the lethality of these infections in $PrP^{-/-}$ mice was not given. Another group has reported modest changes in leukocyte infiltrate during zymosan induced peritonitis (de Almeida et al., 2004), although the implications of this for mouse survival are unclear. Work on viral infection of $PrP^{-/-}$ neurons has not considered the role of systemic immunity in handling these pathogens (Thackray and Bujdoso, 2002;Baj et al., 2005).

A possible criticism of our data is that the $PrP^{+/+}$ Treg fraction may not have been as pure as the $^{-/-}$ population. Failure to remove contaminating effectors from the Treg fraction would result in reduced suppressive potential. We checked the purity of all fractions used in this experiment, and this was equivalent between $PrP^{+/+}$ and $^{-/-}$ preparations. However, this experiment will require repetition to confirm these initial

results. Further studies of PrP deletion and reintroduction in the regulatory T cell compartment are also required to determine the physiological importance of this observation.

These data offer two further explanations for the previously reported hyporesponsiveness of $PrP^{-/-}$ lymphocytes to mitogens. First, $PrP^{-/-}$ mice may be slightly deficient in memory T cells. Second, their Tregs may have better suppressive function than those of $PrP^{+/+}$ mice. These differences may be better compensated for in some strains of mice than others. We made these observations in FVB/N $PrP^{-/-}$ mice yet, as shown in the previous chapter, these mice have normal proliferative and cytokine responses. Ideally, these observations should now be confirmed in $PrP^{-/-}$ mice in which hypoproliferation to mitogens has been documented, ie. those made on or bred onto a 129/Ola or BALB/c background.

How might PrP^{C} both control the activity of Tregs and contribute to the maintenance of memory T lymphocytes? Could PrP^{C} mediate a pro-survival signal into memory lymphocytes and also an inhibitory signal into Tregs? This question could be definitively answered if the natural ligand of PrP^{C} is identified. One possible candidate is the adaptor protein Grb2 (Spielhaupter and Schatzl, 2001), postulated to have a binding site involving PrP residues 100-109 (Lysek and Wuthrich, 2004). Grb2 is intimately involved in linking proximal events following T cell activation with distal signalling pathways, and in particular shows an affinity for LAT (Norian and Koretzky, 2000;Zhang et al., 2000;Clements et al., 1999). LAT is a key player in T cell activation that also negatively regulates T cell homeostasis (Malissen et al., 2005) and has recently been shown to be important in development of Tregs (Koonpaew et al., 2006). It is conceivable therefore, that loss of PrP^{C} may lead to

subtle alterations in lymphocyte signalling affecting both Treg function and long term memory lymphocyte viability via a common mechanism. Although direct evidence of a relationship between LAT and PrP is lacking, treatment of lymphocytes with anti-PrP mAbs results in formation of surface "caps" into which LAT mobilises (Stuermer et al., 2004). Further, preliminary experiments suggested that tyrosine phosphorylation of LAT is reduced in polyclonally activated $PrP^{-/-}$ splenocytes (data not shown), although this requires confirmation.

Because of its expression and possible function in regulatory and memory T cells PrP^{C} may be an attractive target for immunomodulatory therapy in diseases characterised by excessive immune activation such as autoimmunity or transplant rejection. Manipulation of regulatory T cells is being developed as a means of treating a variety of autoimmune diseases. Further work is required to see if treating these cells with anti-PrP mAbs, for example, enhances their suppressive function. This represents another possible explanation for the reported ability of anti-PrP mAb to block T cell activation *in vitro*.

From these observations can the effects of ligating PrP^{C} *in vivo* be predicted? An extreme possibility is that Tregs and a sub-population of memory T cells will be targeted for destruction, especially if a depleting antibody is used. This could result in major, potentially life-threatening immunopathology. Loss of memory cells is more likely to produce immunodeficiency whereas depletion of Tregs will induce autoimmunity. However, some perturbations of the immune system, such as HIV infection or treatment with lymphocyte depleting antibodies, can trigger both opportunistic infection and autoimmunity (Zandman-Goddard and Shoenfeld, 2002;Coles et al., 2006).

Another possibility is that the normal function of PrP^C will be disrupted. This might result in excessive suppression of immune responses leading to uncontrolled infections and tumours. Alternatively, PrP signalling may be enhanced, producing the reverse effects. As discussed earlier, using currently available $PrP^{-/-}$ mice to model these effects has its limitations, as these mice are kept in pathogen-poor environments and may have been able to adapt to germline PrP depletion. Further, excessive signalling or "gain of misfunction" effects of PrP ligation cannot be predicted using these animals.

These constraints notwithstanding, it may still be possible to use existing tools such as anti-PrP mAbs and $PrP^{-/-}$ mice to determine the role of PrP during a major immunopathological challenge *in vivo*. In the final set of experiments presented in this thesis, I studied the effect of PrP ligation or depletion on immune function in an archetypal autoimmune disease model, experimental autoimmune encephalomyelitis (EAE).

CHAPTER 6 MODIFYING ROLE OF PRP IN A MODEL OF AUTOIMMUNE DISEASE

6.1 Introduction

The upregulation of PrP^C in activated T cells and those with memory and regulatory function raises the question as to whether ligation of PrP in such cells may modulate the immune response. As discussed, PrP^C co-localises with numerous proteins associated with T cell signalling and activation, and antibodies directed against surface PrP have myriad effects *in vitro* including suppression of T cell activation (Hugel et al., 2004;Stuermer et al., 2004;Schneider et al., 2003;Li et al., 2001;Ballerini et al., 2006;Cashman et al., 1990). In the work described here, I first attempted to confirm previously published observations on the blocking effects of anti-PrP mAbs on T cell activation *in vitro*.

Further, aside from their ability to block peripheral prion replication (White et al., 2003), the effects of anti-PrP administration *in vivo* have not been considered in detail. Indeed, little is known about the *in vivo* effects of anti-PrP antibodies on immune function. This is of major interest for two reasons. First, anti-PrP mAbs are envisaged as therapeutic agents in the treatment of prion infection, thus it is important to model their biological effects *in vivo*. Second, the upregulation of PrP in cells mediating immune memory, regulation and effector responses suggests that manipulation of lymphoid PrP expression or function may have potential as a therapeutic strategy in other diseases.

In the work described here I attempted to characterise the effects of PrP ligation *in vivo*, using an archetypal autoimmune disease, experimental autoimmune encephalomyelitis (EAE). EAE has been used as a model of autoimmune

demyelination for over 50 years. It is usually initiated by raising a T cell response against myelin antigens by immunisation with self proteins in adjuvant, or adoptive transfer of anti-myelin T cells. These then cross the blood brain barrier and initiate an inflammatory response in the CNS characterized by a cellular infiltrate, extensive destruction of myelin, oedema and axonal and ultimately neuronal loss. EAE has principally been developed as a model of multiple sclerosis and as such has been instrumental in demonstrating the essential relationship between auto-aggressive T cell clones and inflammatory demyelination. Important phenomena such as epitope spread have also been characterized using this model. However, recently EAE has been criticized as being a rather poor model of MS which, it is claimed, is a more complex multiphasic disease in which non-inflammatory or secondary neurodegeneration occurs which cannot be resolved with immune based therapies (Chaudhuri and Behan, 2004;Sriram and Steiner, 2005). These concerns notwithstanding, I chose to study the role of PrP in EAE pathogenesis because it is a well characterized T cell driven disease with which the Altmann laboratory has considerable experience (Boyton et al., 2005;Ellmerich et al., 2005;Takacs et al., 1997;Elliott et al., 1996). I initially sought to modulate a well characterized form of EAE using anti-PrP mAbs. Finally, I induced EAE in $PrP^{-/-}$ mice to assess whether disease phenotype would be altered in the absence of PrP.

6.2 Results

6.2.1 ICSM18 specifically inhibits MHC-peptide but not anti-CD3 driven T cell proliferation

Anti-PrP antibodies have previously been reported to block stimulation of human PBMCs with anti-CD3 and Con A (Cashman et al., 1990;Li et al., 2001) and to

disrupt allogeneic mixed lymphocyte reactions and MHC-peptide driven murine T cell proliferation (Ballerini et al., 2006). I stimulated splenocytes from line 7 TCR tg mice with MBP 85-99, in the presence of ICSM18 or an isotype control. This demonstrated firstly that neither ICSM18 nor the IgG1 isotype were immunogenic (Figure 6.1A). I found that ICSM18 specifically inhibited T cell activation but only at a high concentration (Figure 6.1A).

To determine if ICSM18 had a general inhibitory effect on T cell activation I next examined its effects on SEB-induced proliferation of splenocytes from FVB/N mice carrying a human DR1 transgene. Dendritic and B cells from these animals express human DR1, for which SEB has a higher affinity than native murine MHC class II. In contrast to MHC-peptide induced T cell proliferation, both ICSM18 and the control isotype antibody were able to inhibit SEB-induced activation (Figure 6.1B). Thus, the mechanism of action of anti-PrP may differ according to the mitogenic agent used, with a specific role only in obviating MHC-peptide driven proliferation.

Finally, to assess whether the specific effect of ICSM18 could be mediated in the absence of APCs, I stimulated purified T cells from DR1 tg FVB/N mice with anti-CD3 and anti-CD28 in the presence of ICSM18 or an isotype control. Again, ICSM18 did not have a specific effect (Figure 6.1C), suggesting that it may only be able specifically to disrupt T cell activation that is entirely APC dependent. Both antibodies had a strong suppressive effect at the same concentration (10 μg/ml), suggesting that the mechanism of suppression in this experiment was competing out or steric inhibition of the anti-CD3 mAb.

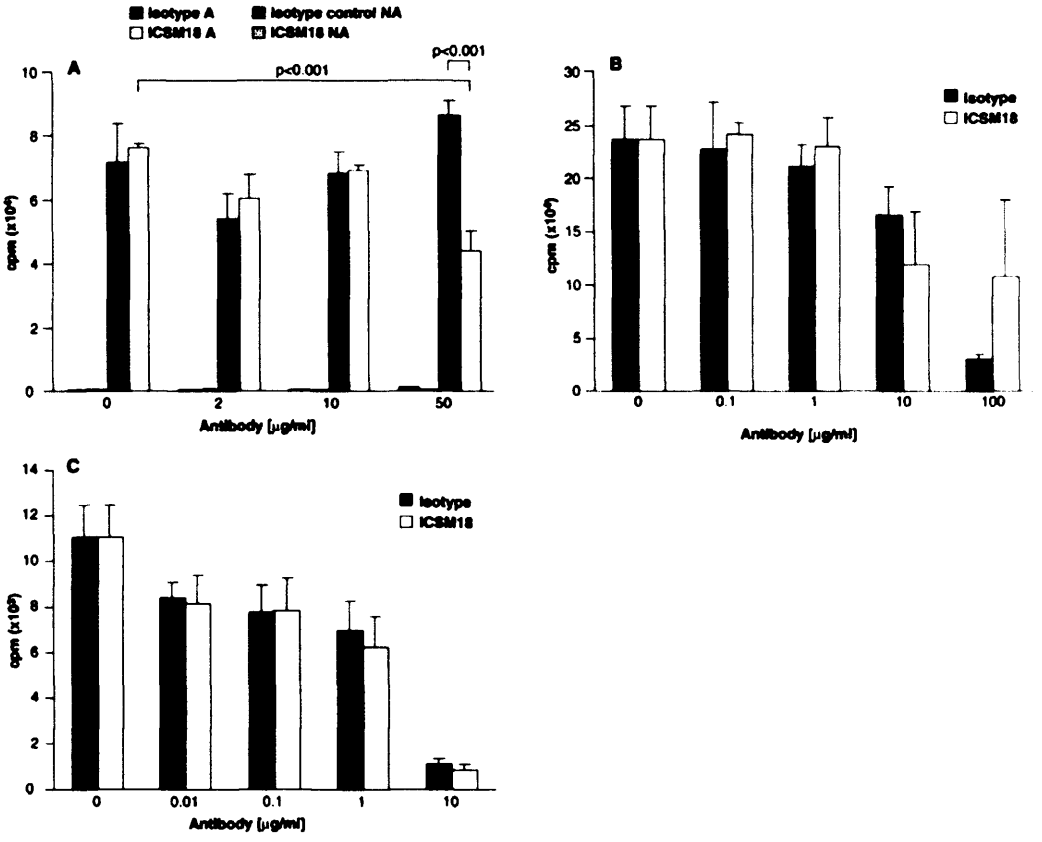

Figure 6.1 Effect of anti-PrP mAb on T cell proliferation

(A) Effect of indicated concentrations of ICSM18 and IgG1 isotype control on proliferation of TCR tg splenocytes cultured with specific peptide (A) or with medium alone (NA). ICSM18 specifically inhibits T cell activation at 50 µg/ml (ANOVA with Bonferroni multiple comparisons post test). (B) Effect of indicated concentrations of ICSM18 or IgG1 isotype control on activation of DR1 tg splenocytes with SEB (1 µg/ml). (C) Effect of indicated concentrations of ICSM18 or IgG1 isotype control on activation of T cells with anti-CD3 (0.01 µg/ml) and anti-CD28 (0.1 µg/ml).

6.2.2 ICSM18 administration does not alter the phenotype of EAE induced by immunisation of SJL mice with PLP 139-151

Next I attempted to demonstrate an immunosuppressive effect of ICSM18 *in vitro*, by administering it to mice with EAE. EAE was induced in SJL mice by immunisation with PLP 139-151. ICSM18 or an isotype control antibody was administered to mice either early in the disease course (Figure 6.2A-B) or after a delay (Figure 6.2C-D). Neither protocol resulted in any modulation of disease incidence or severity as assessed by body weight and clinical score (Figure 6.2A-D).

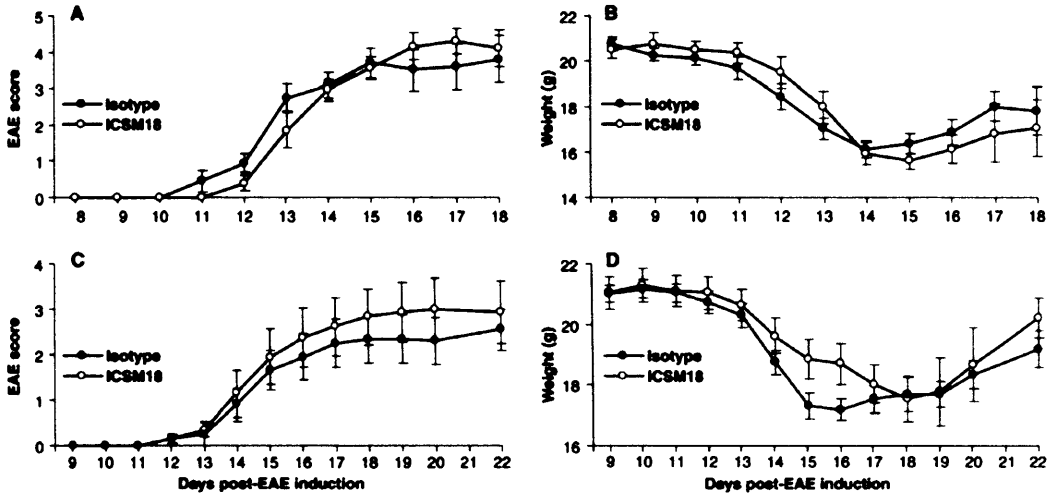

Figure 6.2 Effects of anti-PrP mAb on PLP 139-151-induced EAE in SJL mice

(A) Mean EAE score and (B) body weight in mice receiving "early" mAb treatment (days 2, 4, 8 and 15 post induction). (C) Mean EAE score and (D) body weight in mice receiving "late" mAb treatment (days 6, 8, 10, 12, 14 post induction). Error bars indicate S.E.M.

6.2.3 EAE induction in $PrP^{-/-}$ mice results in only minor alterations in phenotype

Finally I tested whether the phenotype of EAE would be modified by the complete absence of PrP. On the basis of their shared H2q MHC class II allele with the SWR strain I assumed that MBP 85-99 was the immunodominant MBP epitope in FVB/N mice (Cross et al., 1991), which have previously been shown to be susceptible to disease induced by whole MBP (Baker et al., 2000). However, I was unable to induce disease by immunizing $PrP^{+/+}$ and $PrP^{-/-}$ FVB/N mice with MBP 85-99 in CFA (data not shown). Instead, I used whole mouse spinal cord homogenate emulsified in CFA. This induced only mild self-limiting clinical disease in 2/10 $PrP^{+/+}$ and 2/11 $PrP^{-/-}$ mice. Interestingly, the two sick $PrP^{-/-}$ animals developed signs earlier and had higher maximum scores than the two sick $PrP^{+/+}$ mice (Table 6.1). However, with such small numbers of affected animals it is not possible to determine whether this result reflects a genuine role for PrP in protecting against EAE or is a chance finding.

Mouse strain	Number of sick animals	Days to clinical disease	Highest EAE score	Maximum weight loss
$PrP^{+/+}$	2/10	16, 19	1, 1	9%, 6%
$PrP^{-/-}$	2/11	14, 14	1.5, 2	8%, 11%

Table 6.1 Incidence and severity of EAE in $PrP^{+/+}$ and $PrP^{-/-}$ FVB/N mice immunised with mouse spinal cord homogenate

6.3 Discussion

In the work presented here, I sought to utilise my and others' observations on PrP expression in activated T cells to modulate T cell function. Initially I attempted to confirm work in which T cell activation was blocked by anti-PrP antibodies (Cashman et al., 1990;Li et al., 2001;Ballerini et al., 2006). I found that ICSM18 had a specific, if modest, inhibitory effect on MHC-peptide driven clonal T cell expansion. However, I also found that both ICSM18 and the IgG1 isotype control could inhibit SEB induced proliferation in HLA-DR1 tg splenocyte cultures.

Further, in contrast to previous studies, ICSM18 had no specific effect on T cells activated with anti-CD3. The use of purified T cells in this experiment eliminates the possibility of an inhibitory effect on T cell activation being mediated via B cells or other APCs. The ability of both ICSM18 and an IgG1 with no specificity for mouse antigens to inhibit anti-CD3 induced proliferation may simply reflect competing out of the stimulating antibodies by any IgG1 antibody added to the culture at sufficient concentration. The absence of B cells may also have decreased the efficacy of soluble anti-CD3 by preventing cross-linking, facilitating its interference by ICSM18 or the isotype control mAb.

These data suggest firstly that the specific inhibitory effects of anti-PrP may be APC dependent and therefore not adequately modelled using systems in which the mitogen is relatively APC independent. Secondly, anti-PrP may have a specific effect only on certain types of immunological synapse or stimulation. Thus, ICSM18 could specifically suppress proliferation of TCR tg mouse splenocytes activated with MHC-peptide, but not superantigen-mediated proliferation, which is also entirely dependent on T cell-APC conjugation.

These data are in partial agreement with Ballerini and colleagues, who recently reported the ability of anti-PrP mAb SAF83 to block proliferation in a mixed lymphocyte reaction whether PrP was present on both T cells and DCs, on DCs alone, or on T cells alone (Ballerini et al., 2006). Similar findings were also reported for MHC-peptide stimulation of TCR tg T cells, as performed here. Although statistical analysis is not provided, inspection of Ballerini's figures suggests that SAF83 was most effective where PrP was expressed on both APC and T cell. Thus, blockade of PrP may be particularly effective when APC-T cell conjugation is required for T cell stimulation, as in peptide-MHC-TCR interactions, and relatively ineffective when APCs are not involved, as in stimulation with anti-CD3 or mitogens.

These data imply an important role for PrP at the immunological synapse albeit only where T-APC conjugation is mediated by MHC-peptide-TCR binding. However, as discussed in Chapter 4 above, although PrP may accumulate at the immunological synapse during T cell activation, its loss does not affect the stability of B-T cell conjugation. A specific role for anti-PrP in disrupting MHC-peptide driven T cell proliferation therefore requires that the effects of ligation of surface PrP are distinct from those of embryonic deletion, ie. observed *in vitro* effects of anti-PrP mAbs may be due to alteration rather than loss of PrP function. How then might ligation of surface PrP interfere with T cell activation if not through non-specific effects such as steric hindrance of the mitogen or mechanical interference with conjugation? First, anti-PrP may have a specific effect on destabilising the immune synapse, via secondary downregulation of key adhesion or signalling molecules on either the T cell or the APC. Ballerini and colleagues documented accumulation of PrP at sites of T cell-DC contact but were unable to resolve its localisation to the centre or periphery of, or complete exclusion from, the immunological synapse (Ballerini et al., 2006).

An explanation for the lack of co-localisation of PrP with CD3, LFA-1, CD43, LAT or Thy1 in this work is that anti-PrP itself is responsible for disrupting interactions between these molecules and PrP with critical effects on the integrity of the synapse. Second, even if T cell-APC conjugation proceeds normally in the presence of anti-PrP, the myriad changes in T cell physiology induced by anti-PrP treatment may be inhibitory rather than stimulatory. For example, recruitment of signaling proteins such as LAT and Fyn by antibody-bound PrP may sequester them away from the T cell activation pathway where they are key players. Anti-PrP may also induce an inhibitory signal in APCs, perhaps resulting in downregulation of key co-stimulatory molecules. Third, all inhibitory effects of anti-PrP antibodies thus far reported may be due to triggering of apoptosis, as has been demonstrated in neurons (Solforosi et al., 2004).

That ICSM18 required a much higher concentration than SAF83 to achieve suppression may be due to differences in cell density and TCR avidity between experiments. Alternatively, the lack of a major effect in my *in vitro* blocking experiments may reflect a need for mAb cross linking. Although the presence of bystander B cells in the line 7 splenocyte culture should have facilitated this, use of plate-bound rather than soluble ICSM18 may have increased its efficacy. However, the necessity for PrP crosslinking has recently been challenged by Ballerini and colleagues who successfully inhibited T cell proliferation using monovalent anti-PrP Fabs (Ballerini et al., 2006).

Alternatively, the epitope recognised by ICSM18 (PrP 143-153) may not efficiently mediate a suppressive signal in T cells. I could have excluded an epitope specificity issue by repeating this experiment with a range of anti-PrP mAbs recognising

different parts of the PrP sequence. However, Ballerini and colleagues reported that both SAF83 (which has a poorly defined binding site said to be a structural epitope located within PrP 126-164) and whole and Fab fragments of SAF61 (which recognises PrP142-153) could inhibit allogeneic mixed lymphocyte reactions (Feraudet et al., 2004;Ballerini et al., 2006).

Biological effects of PrP mAbs have previously been demonstrated to be epitope-specific. A mAb recognising a C-terminal epitope was able to block anti-CD3 induced T cell proliferation whereas one possessing an N-terminal binding site was less effective (Li et al., 2001). Similarly, antibodies recognizing PrP 95-105 caused neuronal apoptosis on direct inoculation into mouse brain, whereas anti-PrP 133-157 did not (Solforosi et al., 2004). These studies raise important questions about whether PrP's normal signaling pathway is mediated by a ligand binding to particular domains of the protein. This might result in onward signaling via PrP itself or through stabilization of a ligand allowing it to interact with an adjacent molecule. Various C-terminal regions of PrP have been proposed as key binding sites for incoming PrP^{Sc} or the elusive "protein X" proposed to be the essential co-factor in PrP^{C} to PrP^{Sc} conversion (Piening et al., 2006). Deletion of residues 32-80 from PrP does not abolish its ability to generate PrP^{Sc} and clinical disease (Fischer et al., 1996). In contrast, the minimum regions of PrP^{C} required to mediate its normal function are unknown. However, Zhang and colleagues recently found that transfection of $PrP^{-/-}$ stem cells with *Prnp* could improve their long term proliferative potential, whereas introducing truncated PrP with a deletion of residues 23-72 had no effect (Zhang et al., 2006).

ICSM18 is able to block peripheral prion accumulation *in vivo* (White et al., 2003), for which it presumably must bind native PrP^{C} or PrP^{Sc}. I thus sought to use it to modulate effector T cell function *in vivo*. For this I chose to study EAE, a model of autoimmune demyelination caused by auto-aggressive anti-myelin T cells. Such cells are clonally expanded and activated *in vivo* by immunisation with self myelin antigens. I studied PLP-induced disease in SJL mice, a well characterised EAE model. I was not able to modify disease by treatment with intraperitoneal ICSM18. This may reflect poor penetration of appropriate tissues, inadequate dosing or the severity of the disease model, rather than a complete lack of biological effect. Again, epitope selection may be critical, PrP 143-153 being unable to induce an immune modifying signal. Antibodies recognising other regions of PrP may produce an effect and this experiment could usefully be repeated using a range of anti-PrP mAbs, doses and schedules. At the present time, however, the ability of anti-PrP mAbs to modulate immune function *in vivo* remains to be demonstrated.

Finally, I attempted to induce EAE in FVB/N mice to test whether the pathogenesis of this archetypal autoimmune disease caused by autoaggressive T cells was modified in the absence of PrP. A potential difficulty in interpreting data from these experiments is that the critical steps in EAE pathogenesis occur in different tissues, principally lymphoid and neural, many of which express PrP^{C}. For example, autoimmune destruction of myelin is a pre-requisite for clinical disease. Yet, PrP is expressed in the brain at high levels and has been reported to associate, at least *in vitro*, with the key myelin protein αB-crystallin (Sun et al., 2005). Thus, any alteration in the phenotype of EAE in $PrP^{-/-}$ mice could not necessarily be ascribed to absence of PrP in T cells.

Hitherto, only one group has reported the successful generation of disease in the FVB/N strain, using whole Guinea pig MBP (Baker et al., 2000). FVB/N mice express the H2q allele, as do SWR mice in which the immunodominant MBP epitope is 87-99 (Cross et al., 1991). Thus I assumed that MBP 85-99 would induce EAE in FVB/N mice. However, it proved not to be encephalitogenic using our protocol, which has previously produced robust disease in a number of EAE models. Using whole mouse spinal cord homogenate I was able to induce mild disease in a minority of animals of both genotypes. Despite a hint of more aggressive disease in $PrP^{-/-}$ mice there was no significant difference in incidence, progression or severity of disease between $PrP^{+/+}$ and $^{-/-}$ mice. Indeed, based on the observed enhanced suppressor phenotype of $PrP^{-/-}$ Tregs (Chapter 5 above) one might expect EAE in $PrP^{-/-}$ mice to run a more benign course.

These results demonstrate that using appropriate techniques, EAE can be induced in FVB/N mice, albeit with a mild phenotype. EAE has been extensively studied in a range of animals and there is limited benefit in creating new models in previously neglected and generally resistant mouse strains. However, the frequently explosive onset and high mortality of traditional EAE models has led some critics to liken these more to the human disease Acute Disseminated Encephalomyelitis (ADEM), than to MS. Thus, there has been some interest in generation of milder phenotypes which run a more chronic course, and the model created here may merit further investigation for this reason.

Using FVB/N mice, I was not able to demonstrate any significant modulation of disease in the absence of PrP. This suggests that the protein does not play a major role in pathogenesis, at least in this strain. Demonstrating phenotypic differences

between $PrP^{+/+}$ and $^{-/-}$ mice has been a challenging task for other investigators. In a recent paper, differences in haematopoietic stem cells only emerged after 2-3 rounds of transplantation into lethally irradiated recipients (Zhang et al., 2006). A similarly harsh stress may be required to bring out a defect in mature T cell function. My data can only hint at a survival disadvantage in EAE due to embryonic loss of PrP; this may require a model that generates more robust disease to emerge fully. One approach would be to cross $PrP^{-/-}$ mice onto a more EAE-susceptible strain, such as SJL or C57BL/6. Alternatively, other immunological stresses such as infection, toxic shock, or alternative types of autoimmune disease may be required. In summary, it remains to be demonstrated that PrP^{C} is an important player in the various steps in EAE pathogenesis or that ligating it via mAbs holds any promise as a therapeutic application in T cell mediated demyelinating diseases.

CHAPTER 7 DISCUSSION

Despite only modest effects in experimental models, the prospects for active immunisation against prion infection remain promising. The prion hypothesis, although highly reductive, is an extremely powerful paradigm for the development of treatment models because it identifies a single protein as key to the pathogenesis of the disease. In this context, the challenge is to break tolerance to PrP so that effector elements of the host immune system can recognise PrP^{C} and/or PrP^{Sc}, thereby blocking prion replication and allowing clearance of PrP^{Sc}.

I have demonstrated here for the first time that tolerance to autologous PrP in humans is not complete and that distinct regions of the protein contain auto-epitopes. This work brings closer the prospect of a vaccine against human prion disease. An important consideration in taking forward this work is whether raising a T cell response against PrP is an absolute requirement for protection or will, as in the case of Aβ vaccination, precipitate catastrophic autoimmunity. Thus far only one side effect of immunisation with autologous PrP has been reported (Souan et al., 2001a). However, rodents express lower levels of lymphoid PrP than humans. Thus, the effects of PrP ligation by antibody or cytotoxic attack on PrP expressing cells could be profound. In addition, the diversity of human HLA haplotypes contrasts with the restricted repertoire offered by inbred mouse strains. Experiments in isogenic animals will never predict the full range of immune responses to particular antigens that might occur in humans. The appropriate model for taking forward my observations will therefore be one with humanised MHC and PrP expression. In particular, any model used to test vaccination must recapitulate human lymphoid expression levels of PrP^{C}. The tools to generate such a model are readily available. Once constructed, a logical approach would be to immunise mice with whole protein and selected peptides to

generate a T cell response, and then to test the ability of any protocol to delay peripheral and subsequently central prion pathogenesis.

An important consideration in taking forward this work is whether current models for developing anti-PrP based immunotherapy are actually capable of translation into clinically useful therapeutics. With respect to human health, the clinical scenarios in which anti-PrP therapy might be required are:

1) Individuals who have been exposed to prion-infected material eg. blood transfusion, who remain asymptomatic

2) Individuals with pre-symptomatic dominant mutations in PRNP

3) Individuals with symptomatic CJD of any cause

For patients in the first category, blocking of peripheral prion replication will be sufficient. Clearly, development of a vaccine is preferable to repeated doses of antibody, as the latter will be more invasive, expensive and carry the risk of anti-idiotype reactions and generation of neutralising antibodies. The development of adjunctive strategies to slow peripheral prion accumulation and neuroinvasion will also be beneficial. These might include blocking cytokines that facilitate FDC differentiation, blocking key complement factors such as C1q or C3, or interfering with macrophage or dendritic cell migration between sites of prion entry and secondary lymphoid organs.

Where in the periphery might primed anti-PrP T cells or antibodies have an effect? The principal obstacle to T cell-based therapies is that T cells can only see a pathogen when it is presented as an MHC-restricted peptidic fragment. However, any cell that can digest PrP^{Sc} to the degree that it can process peptides for MHC presentation will

most likely have destroyed infectivity in the process. It is precisely those cells that are capable of capturing antigens in their intact form that appear to propagate prions in the periphery ie. FDCs and probably myeloid DCs (reviewed in Mabbott and MacPherson, 2006). Unless some MHC class II presentation of PrP occurs, it seems unlikely that these cells will trigger a T helper response. Similarly, MHC class I presentation would be required for these cells to be recognised as infected by $CD8^+$ T cells and appropriately dealt with.

More promising is the possibility of using primed T cells to trigger an anti-PrP antibody response. However, as Aucouturier's group has demonstrated, the B cell repertoire in $PrP^{+/+}$ animals is profoundly biased against inducing anti-PrP antibodies (Gregoire et al., 2005). The extent to which breaking T cell tolerance will assist this has only been assessed in one published protection study, which reported a correlation between anti-PrP T cell response, antibody production and delayed disease onset (Fernandez-Borges et al., 2006). Researchers reporting vaccination trials in rodent scrapie models should be expected to address the question of whether an anti-PrP T cell response has been triggered and if so whether this qualitatively and quantitatively affects the anti-PrP antibody repertoire.

So why have active immunisation protocols thus far produced only modest protective effects? The ability of anti-PrP monoclonal antibodies to suppress prion replication when transgenetically expressed (Heppner et al., 2001b) or administered passively (White et al., 2003), suggests that the prion hypothesis is valid in attempting immunotherapy ie. that PrP^C is the fundamental substrate of the pathological agent and that ligating it should block infection. If this is true, then active vaccination strategies are failing because the titre or specificity of anti-PrP antibodies produced

are insufficient to completely suppress prion replication. In the study by White and colleagues, ICSM18 or 35 was given at a dose of 2mg (approximately 100mg/kg) twice a week – a high dose compared to many mAb-based regimes used to treat disease in experimental mouse models of Alzheimer disease, autoimmunity or cancer (for examples, see (Levites et al., 2006;Lee et al., 2006;Wilcock et al., 2006;Zhang et al., 2003;Theien et al., 2001)). This work has not been replicated, either by the original researchers, or any other group. When it is, it will be necessary to define the minimum dose required in mice to achieve protection, and whether this differs according to the prion strain and inoculum size. Whether an equivalent dose would be tolerated in humans remains unknown.

ICSM18 and 35 have been demonstrated, here and elsewhere (Polymenidou et al., 2004;Khalili-Shirazi et al., 2005), to bind PrP in its native state, eg. on the surface of lymphocytes or neuronal cell lines. In contrast, most active immunisation protocols are limited in failing to show whether antibodies produced can recognise native PrP^{C}. Such assays should be a minimum requirement for any vaccination regime. There is ample evidence that the anti-PrP antibody repertoire in $PrP^{+/+}$ animals is fundamentally different compared to $PrP^{-/-}$ mice (Gregoire et al., 2005), and therefore the production of antibodies with affinity for native PrP in $PrP^{+/+}$ animals should not be taken for granted.

Attention must also be given to the epitope specificity of antibodies generated by immunisation protocols. Protective effects may be epitope specific, although both ICSM18 and 35, recognising PrP 143–153 and 94–105 respectively, are protective (White et al., 2003). Further, the side effect profile of anti-PrP based therapies may

also depend on which part of the protein they target, and whether they achieve cross-linking of PrP.

Although the relationship between epitope specificity, protection and side effect profile has begun to be addressed for anti-PrP antibodies, no such analysis can yet be provided for T cell responses. This is a major gap in the literature and a block to further development of active vaccination. Hitherto, only one group has reported cytokine responses to stimulation with PrP peptides, albeit in mice (Rosset et al., 2004;Gregoire et al., 2005). In this work, I have shown that PrP epitopes128-141 [129M] and 212-225 may be able to elicit Th2 dominant T cell responses in humans while others generate limited Th1 or Th0 responses, suggesting that the immune repertoire does not respond to all parts of the PrP sequence in the same way. This has important implications for vaccinology, as a Th1 cytokine response is more likely to result in harmful cytotoxicity and inflammation, as suggested by the recent trial of Aβ vaccination in Alzheimer disease.

For patients in categories 2 and 3, blockade of CNS prion accumulation will be essential. Direct intracerebral inoculation of anti-PrP mAbs may be an appropriate rescue therapy for symptomatic individuals, provided that the consequent neuronal apoptosis observed in animal models (Solforosi et al., 2004) is avoided. However, to achieve protection against intracerebral inoculation or established or latent CNS disease through active vaccination represents an unprecedented challenge. Can human and mouse studies of Aβ vaccination provide any clues as to the mechanism of action of vaccines targeting CNS proteins? Currently it remains unclear whether protective effects seen in APP-tg mice were mediated by direct CNS penetration of primed T cells or antibodies, or whether all beneficial effects were mediated by

peripheral mechanisms, such as by generating a sink for Aβ in the systemic circulation (DeMattos et al., 2001).

Since the initial work of Schenk and colleagues (Schenk et al., 1999) numerous groups have attempted to resolve the mechanism of action of Aβ vaccination. Initially it was proposed that anti-Aβ antibodies had been raised that entered the CNS and targeted Aβ plaques for clearance via phagocytosis (Schenk et al., 1999;Bard et al., 2000). However, Aβ clearance in vaccinated APP tg mice is not abrogated when these are crossed onto a FcR-$\gamma^{-/-}$ background in which phagocytosis of immune complexes is deficient (Das et al., 2003b). Although not disproving a role for antibodies raised by immunisation in clearance of Aβ, this work suggests that this may be mediated via FcR-independent mechanisms. Indeed, direct application of anti-Aβ Fab-fragments to APP-tg mouse brain resulted in plaque clearance with similar efficacy to full-length antibody (Bacskai et al., 2002). Thus, antibodies may merely be required to disrupt plaque architecture (Solomon et al., 1997), thereby facilitating non-specific clearance mechanisms. This could equally be achieved by amyloidophilic compounds without requiring breakdown of tolerance to Aβ.

Early studies of Aβ vaccination proposed that protective effects were independent of a T cell response. However, plaque clearance has been observed in immunisation experiments in which a T cell response against Aβ was raised, but antibody production did not occur, implying a direct therapeutic effect of a cellular response against the target antigen (Monsonego et al., 2006). The cases of meningoencephalitis seen following Aβ vaccination in humans demonstrate that unwitting induction of cellular immunity is much more likely when this is applied to a species with HLA heterogeneity. Indeed, considerable heterogeneity in Aβ epitopes

has been demonstrated between inbred mouse strains with different MHC class II alleles (Das et al., 2003a;Monsonego et al., 2006). Hence the importance of defining T cell epitopes in humans prior to attempting immunisation experiments, as I have done for PrP in the work presented here.

These concerns raise the issue of whether the influx of antigen-specific T cells into the CNS following vaccination against neuronal proteins is always undesirable, or conversely can enhance clearance provided that the response is appropriately regulated. This will be determined by the efficacy and safety of such cells entering the CNS and undergoing clonal expansion in an environment in which the normal mechanisms of immune regulation may not apply. The occurrence of PML due to JC virus reactivation in patients taking Natalizumab (Berger and Koralnik, 2005), probably due to blockade of JC-specific T cells traversing the blood brain barrier suggests that pathogen-specific T cells patrol the CNS and prevent infection. Indeed, immunocompromised individuals with a $CD4^+$ T cell deficit, such as those with HIV infection, are at increased risk of CNS infections and tumours. Thus, T cells with specificities for antigens present within the CNS are most likely present within this compartment in humans without necessarily inducing damaging inflammation.

What factors might determine whether a cellular immune response against an auto-antigen will be protective or pathogenic? Recent work has suggested that the relevant parameters determining the safety of the cellular response to Aβ vaccination include the expression level of the target antigen in the CNS, the presence of a high affinity T cell epitope in the immunogen (which is itself dependent on the genetic background of the recipient) and a pro-inflammatory signal, such as IFN-γ, within the CNS (Monsonego et al., 2006). When these conditions are fulfilled, the risks of an

encephalitic response to immunisation are heightened. Indeed, a microarray analysis of pre-immunisation PBMCs from patients in the Aβ vaccine trial suggested that altered expression of a number of genes involved in inflammatory pathways was associated with subsequent development of meningoencephalitis (O'Toole et al., 2005). The HLA types of the 6% of patients who developed encephalitis following Aβ vaccination have not been revealed.

The quality of the cellular immune response is absolutely critical because T cells cannot distinguish normal self from misfolded or aggregated protein in the absence of any change in primary sequence. Thus directing the helper and cytotoxic arms to a non-inflammatory response is critical. In contrast, antibodies can potentially recognise novel structural epitopes in self proteins. However, do misfolded or aggregated self proteins really generate neo-epitopes which the immune system, appropriately primed, can recognise as non-self?

There is evidence that naturally occurring anti-Aβ antibodies are present in human serum (Hyman et al., 2001;Mruthinti et al., 2004;Moir et al., 2005). Further, vaccination with Aβ increases anti-Aβ titres although such antibodies do not appear to cross the blood brain barrier with great efficiency (~ 15% of patients vaccinated with Aβ had detectable anti-Aβ in the CSF) (Bayer et al., 2005;Gilman et al., 2005). Sera obtained following vaccination with Aβ exhibit binding to extracellular Aβ *in situ* which is abrogated by pre-absorption with aggregated $A\beta_{1-42}$ (Hock et al., 2002). Furthermore, immune sera do not bind native APP, or denatured APP, C-terminal derivatives of APP and Aβ, or synthetic mono-, di- or trimeric $A\beta_{1-42}$ (Hock et al., 2002). Naturally occurring human anti-Aβ antibodies can disrupt Aβ fibrillisation and abrogate neurotoxicity *in vitro*, and clinical response in the AN-1792 trial

correlated with antibody production (Gilman et al., 2005), particularly where high titres of antibodies capable of binding native Aβ were generated (Hock et al., 2003).

However, although asserted for aggregated forms of Aβ, evidence in other amyloidoses for the existence of structural neo-epitopes to which the human B cell repertoire can respond is limited. Indeed, with respect to prion disease, it has proved extremely difficult to generate PrP^{Sc} specific antibodies even in $PrP^{-/-}$ mice. Thus the likelihood of these being engineered in a PrP expressing system seems remote. Vaccination protocols must therefore assume that cellular and humoral arms of the immune response will target the normal protein and screen recipient animals carefully for adverse effects in both the periphery and the CNS.

Some authors have asserted that where the target protein is constitutively expressed immunisation might generate a persistent inflammatory process, in contrast to vaccination against conventional pathogens in which effector arms of the immune system can "stand down" once the agent has been cleared (McGavern, 2006). However, it is quite clear from my and others data that healthy individuals harbour T cells that recognise self epitopes without developing autoimmune disease (Danke et al., 2004). Nonetheless, a major challenge in vaccinating against self proteins, as envisaged for prion, Alzheimer and other neurodegenerative diseases, will be achieving immunological homeostasis post-vaccination.

Ultimately, the CNS, however primed, needs actively to degrade the abnormal protein. As discussed, extracellular deposits can be cleared by appropriately primed microglia. More problematic is the clearance of intraneuronal proteins. PrP^{Sc} can be deposited as extracellular amyloid plaques, but these are not an invariable feature. Clinical disease probably requires intraneuronal accumulation of PrP^{Sc}, as PrP

deficient neurons remain undamaged even when surrounding astrocytes accumulate high titres of PrP^{Sc} and infectivity (Mallucci et al., 2003). Indeed, in infected neuronal cells PrP^{Sc} may be associated with particular cytoplasmic organelles (Kristiansen et al., 2005). Targeting intracellular self-protein aggregates is an unprecedented challenge. Vaccination against α-synuclein and Huntingtin has been reported and may offer promise, although the underlying mechanisms of action have not been elucidated (Masliah et al., 2005;Miller et al., 2003).

However, debate remains as to whether breaking tolerance to the key pathogenic protein is required for immunotherapy in neurodegenerative disease. Clearance dependent on a T cell (but not a B cell) response has been demonstrated following mucosal vaccination of APP-tg mice with glatiramer acetate (GA), rather than Aβ (Frenkel et al., 2005). Recently it has been proposed that the protective effects of GA in AD models are mediated by induction of IL-4 producing T cells that cause an alteration in microglial morphology facilitating plaque clearance (Butovsky et al., 2006). These authors argue that breaking T cell tolerance to Aβ is not desirable, and that inducing "protective autoimmunity" (Moalem et al., 1999) by altering the T cell response to myelin antigens through use of GA and similar compounds can enhance innate clearance mechanisms. These strategies, developed in isogenetic rodent models, must surely also carry the risk of initiating EAE when translated to humans, in whom the phenotype of potentially autoagressive anti-myelin T cells entering the CNS, and the risk of epitope spread, will be much less predictable. Nevertheless, T cells that secrete trophic factors and stimulate clearance of damaged cells may represent a means by which lymphocyte influx into neural tissues may be beneficial without targeting the pathogenic protein directly.

The second part of this thesis was concerned with elucidating the normal function of PrP^C in the immune system. I considered this an important objective because

(i) the lymphoid system is well characterised and therefore offers a tractable model for uncovering general aspects of PrP biology

(ii) therapies targeting PrP in the periphery might impact on immune function, especially if considered for long term prophylaxis or treatment

(iii) characterising the role of PrP^C in the lymphoid system might shed light on immune biology generally and offer novel insights into immune function and therapy for autoimmune and infectious diseases.

What can we say definitively about the expression and function of PrP^C in T cells based on data presented here and elsewhere? First, it can be considered an activation antigen. I have shown here that the upregulation of PrP in T cell activation cannot be due simply to redistribution of pre-synthesised protein, but is associated with, and dependent on, increased mRNA abundance, either through upregulated gene expression or (less likely) RNA stabilisation. Second, its upregulation is delayed relative to that of CD69 and CD25, placing PrP^C as a late activation antigen. Experiments in genetically modified T cells in which induction of classical activation markers is inefficient suggest that the mechanisms initiating *Prnp* upregulation are dissociated from those controlling other activation genes including IL-2Rα (CD25). The identification of an NFAT binding site in the *Prnp* promoter suggests that this transcription factor could mediate PrP upregulation during T cell activation. Indeed, increased *Prnp* mRNA has been detected during Th0 to Th2 differentiation (Chen et al., 2003), a process that is NFAT dependent (Diehl et al., 2002).

Third, high PrP^C expression is a feature of memory cells with a $CD44^{high}$, $CD45RB^{low}$ or $CD62L^{low}$ phenotype in mice and a $CD44^{high}$ or $CD45RO^+$, but not a $CD62L^{low}$ phenotype in humans. Fourth, PrP is relatively highly expressed by regulatory T cells. Finally, PrP is not essential for T cell ontogeny, regulatory and memory specialisation, conjugation, proliferation or cytokine production, at least in the FVB/N mouse strain. Further hypotheses about lymphoid and myeloid PrP^C function touched on this work remain to be proven; first that PrP may be functionally important in controlling the suppressive capacity of Tregs; second that it plays a role in lymphocyte homeostasis by influencing a cell's propensity to differentiation or repeated mitoses.

Why does it matter whether or not PrP^C is differentially expressed during T cell activation? PrP expression levels, at least in the CNS, correlate with disease susceptibility in experimental models. Several groups have reported a decrease in scrapie incubation time in mice treated with mitogens or infected in parallel with viruses (Dickinson et al., 1978;Ehresmann and Hogan, 1986;Marsh, 1981). The mechanisms behind these observations have not been characterised, but one possibility is that systemic inflammation due to conventional infection increases lymphoid PrP^C expression, thus rendering the animal more susceptible to peripherally inoculated prions. With respect to vCJD, it is generally assumed that risk factors other than methionine homozygosity at codon 129 of *PRNP* must be genetic. However, environmental factors such as concomitant viral infection at the time of BSE exposure could be powerful determinants of susceptibility and partially explain the geographical variation in vCJD incidence in the UK. Those concomitantly infected with (for example) upper respiratory tract viruses or enteric pathogens may be exquisitely susceptible to propagating an incoming prion infection simply because

they have upregulated PrP^C as part of their immune response to the conventional pathogen. Alternatively, conventional infection may increase susceptibility by upregulating co-factors for prion replication (such as DC migration or complement). These hypotheses cannot readily be tested as no model currently exists in which lymphoid or myeloid PrP expression is dissociated from neuronal expression such that the former can be varied in a controlled way while the latter remains stable at wild-type levels. Yet this is likely to represent the situation in humans being exposed to BSE and other exogenous prions via peripheral routes.

That blood contains prion infectivity has been demonstrated in animal models (Cervenakova et al., 2003;Hunter et al., 2002;Bons et al., 2002;Brown et al., 1999b;Holada et al., 2002;Casaccia et al., 1989), and in three transfusion related incidents in humans (Llewelyn et al., 2004;Peden et al., 2004)(Wroe, et al. submitted). Whether individuals with vCJD become more infectious when their circulating T cells are activated by inflammation or conventional infection remains unclear, but could potentially be modelled in rodents. The relative ease of PrP^C upregulation in lymphocytes may also therefore have implications for the secondary spread of vCJD among humans. Indeed, surgical instruments, a possible vector for iatrogenic transmission, are most likely to be used on individuals undergoing a systemic inflammatory response, thus heightening PrP^C expression in lymphoid tissues.

Embryonic deletion of PrP^C seems to have relatively minor effects on immune function. However, ligating it may induce an abnormal signal ie. a gain of misfunction, which might interfere with normal immune functions, such as T cell activation. More dramatically, targeting cells that preferentially express high levels of PrP^C for removal may result in deletion of key populations of activated, memory and

regulatory T cells, as well as mature myeloid cells. The consequences of this could be catastrophic. There is an urgent need to model this by treating wild type animals with deleting and non-deleting anti-PrP antibodies for long periods of time and during various immune challenges. Unfortunately, such experiments fell outside the time and budgetary constraints of this project.

The identification of PrP^C expression on haematopoietic cells with long term regenerative potential has been proposed as a means of enriching such cells prior to transplantation (Zhang et al., 2006). I did not have the opportunity within this project to assess whether memory or regulatory cells expressing high levels of PrP had different properties from those expressing low PrP. However, this merits further investigation and might identify PrP^C as a marker for other classes of cell with potential therapeutic applications. Indeed, the protective effects of adoptive transfer of regulatory T cells in autoimmune diseases such as EAE (Kohm et al., 2002) and diabetes (Tang et al., 2004;Tarbell et al., 2004) and the improved tumour surveillance mediated by Treg depletion (Lizee et al., 2006), suggest that this cellular population will be a key target for therapeutic development in coming years.

In this work, as elsewhere, proving a definitive function for PrP^C has been difficult. To what extent being GPI-anchored determines the function of PrP^C remains unclear. GPI-anchored proteins are enormously functionally diverse, encompassing adhesion molecules (NCAM, LFA-3), enzymes (acetylcholinesterase, caeruloplasmin), receptors (folate receptor) and elements of the immune response including MHC constituents (Qa2) and complement regulators (DAF). The GPI-anchor is probably more critical for localisation rather than signalling as it is unclear how specific signals might be mediated via common GPI moieties. However, some GPI-anchored proteins

can also function in soluble or transmembrane forms, suggesting that the GPI-anchor is not always essential for function. PrP is probably not exclusively GPI-anchored; soluble PrP is released into the extracellular milieu and a minority of cell surface molecules may exist in a transmembrane form (Harris, 2003). $PrP^{-/-}$ mice into which exclusively transmembrane PrP is re-introduced do not display overt neurological abnormalities, although their immunological phenotype has not been reported (Stewart et al., 2005). Where transmembrane PrP is expressed alongside wild-type PrP a neurodegenerative phenotype is induced, but again, the implications for lymphoid PrP expression and function remain unknown (Stewart et al., 2005). Similarly, mice expressing only non-GPI-anchored PrP have an altered clinical and neuropathological phenotype following scrapie infection, but the effects of this modification on PrP function have not been explored (Chesebro et al., 2005). Thus the centrality of the GPI-anchor to PrP function (as opposed to pathogenicity) remains to be proven.

An interesting feature of research into PrP^{C} is that multiple binding partners of PrP have been identified and yet there is no consensus on the exact role of the protein. The constitutive endocytosis of PrP suggests that it may be able to transduce a signal by being internalised with a bound cargo; alternatively, PrP may bind a ligand and stabilise it at the cell surface to allow it to interact with a signalling molecule. Perhaps PrP^{C} has no specific role but participates in multiple pathways. The fact that many proteins seem to bind PrP^{C} may represent an adaptive mechanism for limiting the availability of cell surface or soluble forms of PrP to prionogenic ligands, be they PrP^{Sc} or the putative protein X. However, dissociating normal ligand interactions of PrP^{C} from those required for prion conversion will be a challenging task in the

absence of a definitive function for the protein which can be measured in a simple assay.

These doubts notwithstanding, it is clear from the association of PrP^C with functionally distinct T cells populations that immune function might be modified by therapies directly or indirectly targeting PrP^C. This is of immediate concern with respect to therapies for prion disease. However, a more distant application of this data would be in developing anti-PrP agents as a means of controlling T cell function in the treatment of diseases characterised by immune dysregulation. At a minimum, anti-PrP antibodies will selectively detect activated, memory and regulatory T cells, due to their higher surface PrP^C expression. Whether such agents will actually alter T cell physiology by mediating a signal through PrP^C or by blocking PrP^C signalling, remains unclear. Further work is required to fully elucidate the effects of PrP ligation in disease models.

The high degree of evolutionary conservation of PrP sequence has led some researchers to propose that its function is likely also to be phylogenetically ancient and perhaps predate the advent of adaptive immunity. The presence of an octapeptide repeat region with the capacity to bind copper has been interpreted as evidence for PrP^C being a component of the innate immune system, able to "pattern-recognise" harmful substances, such as bacterial or viral nucleic acid and proteins, free radicals and metal ions. According to this hypothesis, PrP^C has overlapping functions with the Toll-like receptors, perhaps explaining why $PrP^{-/-}$ mice lack a discernable immune phenotype (McBride, 2005). However, the fact that PrP^C expression is considerably higher on cellular components of the adaptive immune response (eg. lymphocytes)

than those of the innate immune system (eg. neutrophils) suggests that PrP postdates the evolution of these basic immune defences.

In the absence of a definitive function for PrP, we can but speculate on why it has been so tightly conserved during evolution. It has been argued that large-scale prehistoric epidemics of prion disease, to which heterozygotes would have been relatively resistant, could be the driving force behind balancing selection in favour of methionine/valine heterozygosity at codon 129 of *PRNP* (Mead et al., 2003). However, another hypothesis is tenable; that polymorphic variants of PrP^{C} effect different functions in the face of a common, but lethal, conventional pathogen. This could be mediated at the neuronal level if the pathogen is neurotropic or by differential function of 129M and V PrPs within the immune system during the host response to systemic infection. Recent data on herpes simplex and poliovirus infection of $PrP^{-/-}$ neurons is therefore intriguing (Thackray and Bujdoso, 2002;Baj et al., 2005). The availability of mice expressing human *PRNP* with methionine and/or valine at position 129 (Bishop et al., 2006;Asante et al., 2006) will facilitate modelling the effect of this polymorphism on immune function.

Although the physiological function of PrP^{C} remains obscure, it is clear that PrP^{C} expression is regulated during lymphocyte activation and differentiation, and is a particular property of regulatory and memory T cells. Furthermore, PrP^{C} ligation by antibody has been shown to produce myriad effects on T cell physiology. The consequences for the immune system of therapeutics deliberately or accidentally targeting PrP^{C} may therefore be profound. At present it is impossible to predict whether these effects will produce harmful autoimmunity or render the recipient immunocompromised (or both), although data from rats immunised with self-PrP

peptides suggest the former (Souan et al., 2001a). Whether such side-effects will outweigh the potential benefits in a disease that is relentless, lethal, and currently untreatable remains to be seen. However, recent experience in pre-clinical trials (Suntharalingam et al., 2006), and in Alzheimer disease (Orgogozo et al., 2003) and Multiple Sclerosis (Berger and Koralnik, 2005) suggest that we embark on novel immunotherapeutics with caution. In the case of prion disease, the era of effective immune-based therapy is some way off, with the exception of passive transfer of anti-PrP antibodies to peripherally exposed individuals. It seems prudent that alongside the development of much needed anti-prion therapies we further define the function of this extraordinary protein, and model the effects of breaking tolerance to it.

APPENDIX: PUBLICATIONS ARISING FROM WORK IN THIS THESIS

J D Isaacs, R J Ingram, J Collinge, D M Altmann, G S Jackson. The human prion protein residue 129 polymorphism lies within a cluster of epitopes for T cell recognition. *Journal of Neuropathology & Experimental Neurology*, 2006, 65:1059-68.

J D Isaacs, G S Jackson, D Altmann. The role of the cellular prion protein in the immune system. *Clinical & Experimental Immunology*, 2006, 146:1-8.

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