Influence of the Immune System on Peripherally Acquired Transmissible Spongiform Encephalopathy Infection with Special Reference to the Role of the Follicular Dendritic Cell

Karen L.Brown

A thesis submitted in partial fulfilment of the requirements of the University of Edinburgh for the degree of Doctor of Philosophy.

The programme of research was carried out at the Neuropathogenesis Division (Unit)
The Roslin Institute and R (D) SVS, University of Edinburgh

June 2008

Table of Contents

Declaration

I declare that the work presented in this thesis is my own, except where stated. All experiments were designed by myself, in collaboration with my supervisors Professor Moira Bruce, Professor David Gray and Dr Neil Mabbott, unless otherwise stated. No part of this work has been, or will be submitted for any other degree, or professional qualification.

Karen L.Brown

June 2008

Acknowledgements

I would like to express my thanks to my supervisors **Moira Bruce**, **David Gray** and **Neil Mabbott** for their help and support during this thesis. I would particularly like to acknowledge **Neil Mabbott**, who took over as supervisor from Moira Bruce, for his support, guidance and critical reading of the manuscript. Thank-you all for your support.

I would like to acknowledge the contribution to the field of TSE research of a number of individuals including; **Alan Dickinson**, **Hugh Fraser** and **Moira Bruce**. In particular **Hugh Fraser's** work involving ionising radiation which provided the basis for many of these studies presented here.

I would like to acknowledge past members of my group including **Hugh Fraser** for mentorship and friendship, **Moira Bruce** for her support and guidance over a number of years, **Diane Ritchie** for her contribution to some of the confocal microscopy techniques and **Patricia McBride** for helpful discussion.

To **Jean Manson** for her support and critical reading of the manuscript which has been extremely helpful, thank-you.

To **Christine Farquhar** for helpful discussion and friendship.

To **Robert Somerville** for assistance with the analysis of the titrations, thank-you.

I would also like to express my gratitude and thanks to the following people for excellent technical support during the course of this project:

To all of the **NPU animal facility staff** for their care and dedication to the laboratory animals used in these studies. Without their dedication the studies would not have been possible.

To **Irene McConnell**, **Mary Brady**, **Lorraine Gray**, **Simon Cumming** and **Val Thomson** for assistance with some of the experimental animal techniques.

To the NPU histology group: **Anne Coghill, Sandra Mack, Aileen Boyle, Sandra Coupar and Gillian McGregor** for excellent histological preparation of samples.

To **Sandra Coupar** for her constant support, morale boosting, friendship and general encouragement during this preparation of this thesis.

To **Gwen Wathne** for assistance with some of the animal studies, PET blotting and IHC. Thank-you very much.

To **Jill Sales** (BIOSS, University of Edinburgh) for excellent advice and assistance with statistical analysis

To **Debbie Brown** for assistance with confocal microscopy.

To **Aileen Boyle** for carrying out some additional lesion profiling, thank-you.

I would like to take this opportunity to thank **everyone at NPU** who I have not mentioned here it is a real pleasure to work with you all.

Finally to all my friends who have been very supportive over the years but in particular to **Lorraine and Ian Paterson** for their constant support, friendship and laughter over the years, thank-you very much.

To my **mother** for doing some last minute baby-sitting during the final stages of this manuscript, thank-you very much.

To my wonderful, inspirational children **Rachel, Sophie** and **John (Jr)** for everything they do, you are all fantastic, thank-you.

Finally a huge thank-you to my husband **John** for his continued love, support and patience! Thanks for getting me through the last few months. I could not have done it without you

****

I would like to dedicate this thesis to the memory of my late father Ian Miller (1941-2000).
A truly inspirational person, thank-you

Abbreviations

ABC	**Avidin-Biotin Complex method**
BSE	**Bovine spongiform encephalopathy**
C4	**complement component C4**
CJD	**Creutzfeldt-Jakob Disease**
CR1	**complement receptor 1**
CR2	**complement receptor 2**
CWD	**Chronic Wasting Disease**
DNP-KLH	**dinitrophenyl keyhole limpet haemocyanin**
d.p.i	**days post injection**
ELISA	**Enzyme Linked Immunosorbent Assay**
FDC	**Follicular Dendritic Cell**
FFI	**Fatal Familial Insomnia**
h	**hours**
i.c.	**intracerebral**
i.p.	**intraperitoneal**
i.v.	**intravenous**
Ig	**immunoglobulin**
IHC	**immunohistochemistry**
kDa	**KiloDaltons**
Ltβ	**Lymphotoxin β**
LtβR-Ig	**Lymphotoxin β receptor-human immunoglobulin fusion protein**
M	**molar**

N	**normal**
PHA	**phytohaemaglutinin**
PNA	**peanut agglutinin**
Prnp	**murine PrP gene**
PRNP	**human PrP gene**
PrP^{c}	**normal form of the host PrP protein**
PrP^{Sc}	**disease specific form of the host PrP protein**
sCJD	**sporadic CJD**
SCID	**severely combined immunodeficient**
SRBC	**sheep red blood cell**
TME	**transmissible mink encephalopathy**
TNFR1	**tumour necrosis factor 1**
TSE	**transmissible spongiform encephalopathy**
vCJD	**variant CJD**

Abstract

The Transmissible Spongiform Encephalopathies (TSEs) or "prion" diseases are a group of fatal neurodegenerative diseases the aetiology of which is not fully understood. These diseases are characterised by a number of pathological changes in the central nervous system (CNS) including; vacuolation of the neuropil, gliosis and deposition of PrP^{Sc}; the abnormal form of the host glycoprotein PrP. Although the major pathology in these diseases is associated with the CNS the immune system is central to the pathogenesis of many natural and experimental TSEs including natural scrapie in sheep, chronic wasting disease in free ranging and captive deer and variant CJD (vCJD) in humans. Unlike many infectious diseases where deficiencies in immune function are opportunistic for the invading pathogen a competent immune system is required for efficient TSE infection via peripheral routes. As infection of the lymphoid tissues in many TSEs can occur many months before the detection of infectivity in the CNS, the determination of those cells in the lymphoid system has been the focus of much research and a number of studies now point towards the importance of the follicular dendritic cell (FDC), a long-lived radio resistant cell, in TSE pathogenesis. The involvement of FDCs in peripheral TSE pathogenesis relates to the inability of ionising radiation to influence pathogenesis, the association of PrP protein with FDCs in both uninfected and infected lymphoid tissues, and the demonstration that TSE pathogenesis is severely impaired in mice devoid of these cells. The aims of this thesis were to further understand the role of FDCs in the pathogenesis of a range of mouse-adapted experimental TSE strains and to determine if peripherally acquired TSE infections are influenced by host age or by stimulation of the immune system. Using chimaeric mouse models where a mismatch in the

expression of PrP protein between FDCs and lymphoid/myeloid cells was produced, further evidence for a critical role for in the pathogenesis of the ME7 TSE strain was produced. Although these findings produced strong evidence that FDCs were important for the ME7 strain the possibility that different TSE strains may target different cell types in the peripheral lymphoid system was explored using a range of mice with specific immunological defects. Infection of these mice with several experimental TSE strains showed that the presence of mature FDCs was also important for the pathogenesis of the strains tested. Clinical cases of vCJD have been confined almost exclusively to young adults, although the reasons behind this apparent age-related susceptibility are not fully understood. The capacity of the immune system to mediate immune responses to pathogens declines with age as a result of impaired lymphocyte and FDC function. As FDCs are critically involved in the pathogenesis of many TSEs, including vCJD, it was hypothesised that an aging immune system may impair disease pathogenesis. Peripheral infection of senescent mice failed to produce clinical disease during lifespan, although evidence of disease transmission, was detected in a proportion of aged mice. These findings demonstrate that this inefficient disease transmission, as a consequence of age, may lead to considerable levels of sub-clinical disease within the population. Finally the influence of immune system stimulation, by the generation of a humoral immune response, on peripheral TSE pathogenesis was investigated. These findings demonstrated that immunisation can influence pathogenesis, but only during the early stages of infection prior to spread to the CNS. These data imply that modulation of the immune system does not alter TSE pathogenesis once disease has been initiated in the CNS. Finally, these studies have found some preliminary

evidence that TSE infection may induce FDC activation suggesting that TSE infection may influence the immune response. Together, these data show that a functional immune system and specifically, the presence of mature FDCs, are central to the pathogenesis of peripherally acquired TSE infections.

Chapter 1
Introduction and background

1.1Transmissible spongiform encephalopathies

1.1.1 The Transmissible Spongiform Encephalopathies

TSEs are invariably fatal and are characterised by pathological changes in the central nervous system including vacuolation (Dickinson et al, 1968; Fraser, 1976), gliosis and the accumulation of an abnormal form of the host sialoglycoprotein, PrP (Bolton et al, 1982) (Bruce et al, 1989b) (McBride et al, 1992).

The diseases that are collectively known as the transmissible spongiform encephalopathies (TSEs) are arguably some of the most intriguing and perplexing diseases known to man. Over the last 50 years these diseases have been the subject of extensive debate on their origins and aetiology. The nature of these unique agents still remains controversial despite extensive research throughout the world. The characteristics of the TSEs however provide many barriers to understanding and researching these diseases. These barriers include; the prolonged incubation periods of disease, which in some cases can exceed the animal's lifespan (Dickinson et al, 1975); the lack of an ante-mortem diagnostic test, the resistance to standard de-contamination methods (Taylor, 2000) and the uncertainty over the nature of the TSE agent (Chesebro, 1998). In addition, there are a number of factors that control the transmission of these diseases including; genotype of the host (Dickinson et al, 1968) (Dickinson & Outram, 1988) (Bruce et al, 1991) (Hunter, 2007), route of infection, (Eklund et al, 1967) (Kimberlin & Walker, 1979) infectious dose, age of host (Outram et al, 1973) (Ierna et al, 2006) and infecting strain.

1.1.2 History of TSEs

Much of the understanding of the TSEs today originates from the study of natural scrapie (Hunter, 2007). Scrapie, a natural disease of sheep and goats, is the most studied member of the TSEs. Scrapie was first recognised over 300 years ago, although it is likely it has existed for many hundreds of years. The term "scrapie"is derived from a characteristic clinical sign; pruritus. Other names for the disease have included "traben" (to trot) and "tremblante" (to tremble); again both of these terms represent clinical symptoms of infection. Although most of the scientific research on the nature of this disease has occurred in the last century there were reports in the literature of its potentially infective nature as early as 1759 (Leopoldt, 1759). This was recorded in a German publication "A shepherd must isolate such an animal (with scrapie) from healthy stock immediately because it is infectious and can cause serious harm to the flock" (Leopoldt, 1759).

During the late 18th and early 19th century several theories on the causal nature of the disease were put forward. These ranged from the supernatural to the more plausible where the possibility of an infective agent was suggested. In addition, in 1886 it was suggested that a "hereditary factor" may influence susceptibility to these diseases (Dammann, 1886)

In 1899 a French scientist (Besnoit & Morel, 1898) presented findings on the lack of transmissibility of scrapie. In these studies brain and blood from scrapie infected sheep were inoculated into healthy sheep; after only a few months observation he reported that the disease could not be transmitted. This time period of observation

would now be considered far too short to observe clinical signs of scrapie as the incubation periods of disease can range from approximately 7 months to many years. (Foster & Dickinson, 1988; Hunter et al, 1997) (Hunter, 2007). In 1936 however the first demonstration of the scrapie agent's infective nature was demonstrated (Cuille & Chelle, 1936).

In 1946 at the National Veterinary Medical Association of Great Britain and Ireland Annual Congress, evidence of scrapie transmission as a result of louping ill vaccine was presented (Gordon, 1946). Louping–ill is a viral disease spread by the tick *Ixodes ricinus.* This inadvertent discovery was suggested as early as 1936 where farmers, whose sheep had received a vaccination for Louping-ill approximately 2 years earlier, noted disease symptoms now known to be consistent with those of natural scrapie. The components of the vaccine had been treated with 10% formalin which inactivated the louping ill virus but not the scrapie agent. This was possibly the first demonstration that TSE agents were resistant to standard decontamination methods with formalin (Taylor, 2000).

The next TSE to be identified was Creutzfeldt-Jakob disease (CJD), which was first described in the early 1920's by Hans Gerhard Creutzfeldt (Creutzfeldt, 1920) and Alfons Maria Jakob (Jakob, 1921). They described a rare neurodegenerative condition which was initially called "spastic pseudosclerosis". Creutzfeldt-Jakob disease is recognised in different forms; sporadic, iatrogenic, familial (Gambetti et al, 2003) and more recently as (new) variant CJD (Will et al, 1996). Extensive research in the 20th century and the discovery of a number of other similar conditions

affecting both humans and animals led to the classification of this group of diseases as transmissible spongiform encephalopathies or “prion” diseases (Bolton et al, 1982). A summary of the key discoveries and the diseases that comprise the “TSEs” are provided in Table 1.1

Table 1.1: A summary of TSEs and the year of discovery

TSE	Abbreviation	Year	Reference
Classical scrapie in small ruminants (sheep and goats)	Scrapie,	1732	Not defined
Sporadic Creutzfeldt–Jakob disease in humans	sCJD	1920/21	(Creutzfeldt, 1920) (Jakob, 1921)
Gerstmann–Sträussler–Scheinker syndrome in humans	GSS	1936	(Gerstmann et al, 1936)
Kuru in humans	Kuru	1957	(Zigas & Gadusek, 1957).
Transmissible mink encephalopathy in captive mink	TME	1965	(Hartsough & Burger, 1965)
Iatrogenic Creutzfeldt–Jakob disease in humans	iCJD	1974	(Duffy et al, 1974)
Chronic wasting disease in deer and elk	CWD	1980	(Williams & Young, 1980)
Fatal familiar insomnia in humans	FFI	1986	(Lugaresi et al, 1986)
Bovine spongiform encephalopathy in cattle	BSE	1987	(Wells et al, 1987)
BSE (feline spongiform encephalopathy) in domestic cats	FSE	1990	(Leggett et al, 1990)
Familial Creutzfeldt–Jakob disease in humans	CJD, fCJD	1991	(Goldfarb et al, 1991a)
New variant of Creutzfeldt–Jakob disease in humans	vCJD	1996	(Will et al, 1996)
vCJD and BSE are the same agent strain	vCJD/BSE	1997	(Bruce et al., 1997)
Atypical form of TSE in small ruminants	atypical scrapie, Nor98	2003	(Benestad et al, 2003)
Atypical form of BSE in cattle	BASE-1	2003	(Yamakawa et al, 2003)
BSE in goats	BSE	2003	(Eloit et al, 2005)

1.1.3 Nature of the TSE agent

The virus hypothesis

Over the years the TSES have been classified under a number of terms including that of the "slow virus diseases or infections". The concept of "slow viruses" was originally put forward by the Icelandic physician and scientist Dr Bjorn Sigurdsson as a result of his research on Maedi-Visna (Sigurdsson et al, 1957). Maedi is a slowly progressive interstitial pneumonia of adult sheep while Visna affects the central nervous system causing encephalomyelitis; both these conditions are caused by the same virus (Gudnadottir, 1974). The properties of these diseases; long incubation periods of disease, a protracted course of illness and targeting to the central nervous system and lymphoid tissues shared many similarities with scrapie. In later years Maedi-Visna was shown to be caused by a Lentivirus, a subgroup of the Retroviruses. Other examples of viral infections with prolonged incubation periods include; human immunodeficiency virus infection (HIV) and the canine distemper virus.

Previous studies using UV irradiation (Alper et al, 1966) (Alper et al, 1978) suggested that critical target was not nucleic acid (Alper et al, 1966). However, it is possible that shielding of the critical target molecule of the infectious agent by other molecules may have influenced this resistance (Diringer et al, 1994; Diringer et al, 1983). Using target theory calculations, some workers have concluded that the maximum genome size for the TSE agent would be very small (Alper et al, 1978) (Bellinger-Kawahara et al, 1988.). Interestingly, the inactivation spectrum for scrapie obtained from these studies appeared similar to intact tobacco mosaic virus, a well-

characterised RNA virus (Diringer et al, 1983). Despite these comparisons, no conventional viruses have been detected. Although, it is still conceivable that such an agent; either a DNA or a RNA virus is yet to be discovered.

The virino hypothesis

The virino hypothesis is based on the theory that a very small nucleic acid or some form of informational molecule forms part of the scrapie specific genome (Dickinson & Outram, 1988). This theory was first postulated (Dickinson & Outram, 1988) in an attempt to characterise the unique properties of the TSE agents. Although attempts to isolate a classical virus from these diseases have failed, this hypothesis was based on the possibility that the virino nucleic acid is "virus like" and encapsulated by a protein coat, possibly an informational hybrid between the agent's genome and protective host proteins. Although research has failed to define whether the TSE agent is a "virino" it is possible that encapsulation of the virino nucleic acid has "prevented" the detection of a specific informational molecule for these diseases.

Much of the evidence for the virino hypothesis is based on the existence of a number of defined TSE strains (Bruce, 1993; Bruce & Fraser, 1991; Bruce et al, 1992; Bruce et al, 1991). The reproducibility of these strains, following multiple passage within the same species and on occasion through other species, suggests that some form of informational molecule controls these defined characteristics (Bruce & Dickinson, 1987). In addition mutation of some experimental TSE strains has been observed supporting the hypothesis that some form of informational molecule may be present

(Bruce & Dickinson, 1987). Indeed, "virino" like agents exist in the form of plant viroids, which are plant pathogens with a very small genome (Gross et al, 1977).

The prion hypothesis

In the 1960s Tikvah Alper and colleagues suggested that the causal agent of scrapie did not contain a nucleic acid molecule (Alper et al, 1967; Alper et al, 1966). This suggestion was based on studies using ionising and ultraviolet radiation which the researchers found to have no effect on the transmissibility of scrapie. As most conventional viruses containing DNA or RNA would have been inactivated by this treatment it was assumed that the scrapie agent was not a conventional virus. The "protein only" hypothesis was first suggested in 1967 by J.S Griffith (Griffith, 1967) in an attempt to explain Tikvah Alper's findings. He suggested that the infectious agent was in-fact a protein which was capable of replicating within the body.

This theory was further extrapolated by the researcher Stanley Prusiner who was awarded the Nobel Prize in 1997 for research into the origin and causal agent of the TSEs where he identified an unusual class of infectious particles termed "prions". His research constituted a novel theory in that the TSE agent was composed almost completely, if not entirely, of infectious protein molecules termed "prions" (**pro**tei**n**aceaus particles) (McKinley et al, 1983). The term prion was first published

in 1982 (Prusiner, 1982). This theory was based partly on the fact that research had failed to uncover a conventional informational molecule.

The protein-only hypothesis states that the infectious agent or "prion" is identical to the abnormal form of PrP^{Sc} (Prusiner, 1982). The mechanism of infection is thought to occur via recruitment of the normal form of the protein, PrP^{c}, from newly infected cells (Prusiner, 1982). A number of studies have produced evidence which favour the prion hypothesis. These include the generation of infectivity in neuroblastoma cells (Race et al, 1988) and the cell free conversion of PrP^{c} into PrP^{Sc} by Caughey and colleagues (Caughey et al, 1995)

The precise mechanism by which PrP^{c} is converted to PrP^{Sc} is not fully understood although several models to explain this process have been produced, including; the template assisted model (Harrison et al, 1999; Huang et al, 1996) and the nucleation-polymerisation model (Caughey et al, 1995; Thirumalai et al, 2003). Perhaps some of the most controversial or compelling data supporting the protein only hypothesis was provided by studies which demonstrated that mammalian prions synthesised from PrP^{c} under appropriate conditions were capable of producing disease in transgenic mice over-expressing PrP, but not in wild-type mice (Legname et al, 2004) although subsequent passage of brain material from the transgenic mice produced disease in wild-type mice. Criticisms of the failure of this study to produce disease in wild-type mice were explained by; the structure of the converted PrP and "priming" or sensitisation of the animal to infection by the over expression of PrP in the transgenic model (Legname et al, 2004)

Notably, other researchers have demonstrated the conversion of PrP^{c} into the abnormal protease resistant form, PrP^{Sc}. In these studies the acquisition of proteinase K resistance could be demonstrated, but in contrast to the studies described above this acquisition of proteinase K resistance did not result in the generation of infectivity (Hill et al, 1999a). Moreover, some studies have shown that transmission of infectivity can occur in the absence of detectable PrP^{Sc} (Lasmezas *et al.*, 1997; (Barron et al, 2007; Lasmezas et al, 1997).

More recently, the detection of abnormal PrP in the absence of clinical symptoms of disease or infectivity has been demonstrated in transgenic mice inoculated with brain

material from a human case of Gerstmann-Straussler-Scheinker syndrome (GSS) (Piccardo et al, 2007)

Although it is widely accepted that both the normal and abnormal forms of PrP are intrinsic to the disease process, the protein only-hypothesis still remains controversial within the field of TSE research. One of the major criticisms of this hypothesis is that the existence of strains would require some form of informational molecule (Bruce & Dickinson, 1987). However, it has been suggested that the molecular basis for strain variation results from conformational alterations in structure of PrP^{Sc} which results in strain variation (Bessen & Marsh, 1994) (Collinge et al, 1996).

1.1.4 Genetic influences on TSE pathogenesis

The influence of host genetics controls a number of factors including; resistance or susceptibility to specific TSE strains or agent, length of disease incubation period and pathological changes in the central nervous system (Dickinson & MacKay, 1964; Goldmann et al, 1994) (Hunter, 2007).

In the 1960s studies by Alan Dickinson and others led to the discovery of a gene which was responsible for the control of scrapie incubation periods in sheep and mice (Dickinson et al, 1968). This gene was designated *Sinc* in mice and *Sip* in sheep. Subsequent studies following the discovery of PrP protein (Hope et al, 1986) have confirmed that S*inc* and the gene encoding PrP protein, (*Prnp)* are the same (Hunter et al, 1987) (Hunter et al, 1992; Moore et al, 1998). In mice, two alleles of this gene (*Prnp*) have been recognised (designated a and b), encoding proteins that

differ by two amino acids, at codons 108 and 189. In humans the PrP gene is defined as *PRNP*. The influence of host genetics can be demonstrated below in data shown below (Table 1.2) which demonstrates the differences in incubation period in two lines of inbred mice challenged with the ME7 or 22A strains of scrapie (Dickinson et al, 1968).

Table 1.2. Influence of PrP (*Sinc*) genotype on TSE incubation period in mice.

	Mouse strain	
	C57BL	VM
Genotype	P*rnp*^{a} *Sinc*^{S7}	P*rnp*^{b} *Sinc*^{p7}
Agent	mean incubation period in days	
ME7	165	299
22A	443	205

Extracted from Dickinson *et al* 1968. Data demonstrates the influence of the PrP gene. *(Dickinson et al, 1968)*

In studies of natural sheep scrapie the influence of host genetics has a profound effect on the susceptibility to infection (Goldmann et al, 1994) (Hunter et al, 1996) (Elsen et al, 1999) (Tranulis, 2002) (Hunter, 2007). Indeed so great was the influence of genetics that the disease was originally considered to be a genetic condition (Parry, 1962.; Parry, 1983.)

Prior to the discovery of PrP protein and the determination that genetics influenced natural scrapie pathogenesis it was clear that some breeds of sheep were more susceptible to infection than others. Initial studies carried out in the 1950's using a range of sheep breeds provided evidence for breed specific susceptibility (Hunter,

2007) These "susceptible" breeds included Herdwick, Swaledale and South Country Cheviot. In order to understand the influence on genetics on the susceptibility to natural scrapie the development of specific lines was initiated, using scrapie challenge to select susceptible/resistant lines (Hunter, 2007). The most widely studied flock produced from these initial studies is the NPU Cheviot sheep flock which was initiated in 1961. From these studies two lines of sheep, a positive and negative line were produced based on their susceptibility to the experimental strain SSBP-1 (Scrapie Sheep Brain Pool-1) (Pattison & Millson, 1961). Analysis of the PrP gene in sheep has determined that polymorphisms at codons 136, 154 and 171 have profound effects on the susceptibility to infection (Foster & Dickinson, 1988; Goldmann et al, 1994). The first codon known to be polymorphic was codon 171 where either arginine (R) or glutamine (Q) could occur. Subsequently two other codons were found to be important: 136 with valine (V) or alanine (A) and 154 with histidine (H) or arginine (R). The presence of the VRQ/VRQ genotype in some breeds, notably Cheviot, generally denotes susceptibility to natural scrapie (Hunter et al, 1997) (Hunter, 2007). The incidence of natural scrapie in the NPU Cheviot flock (described above) is extremely high, with almost all animals with the susceptible genotype developing natural scrapie in their lifetime (Hunter, 2007). Interestingly this susceptibility may vary between breeds. In Suffolk sheep for example, the ARQ/ARQ genotype confers susceptibility whereas this same genotype in Cheviot sheep confers resistance suggesting that some other factors may also contribute to TSE susceptibility (Hunter, 2007). The understanding of the effects of these polymorphisms on the susceptibility to infection has lead to the introduction of the national scrapie plan. The

aim of the national scrapie plan is to reduce the frequency of susceptible genotypes, theoretically reducing the frequency of infection within the sheep population.

In familial human TSEs the presence of specific mutations on the PrP gene are clearly associated with disease (Gambetti et al, 2003; Goldfarb et al, 1991a). However, controversy exists over whether the presence of these mutations alone cause disease or if their presence influences susceptibility to infection. One of the most common mutations associated with familial CJD, the E200K, occurs at codon 200 in the PrP gene and has been most widely studied in a cluster of cases occurring amongst Libyan Jews (Goldfarb et al, 1991b). The presence of clinical variability in individuals with this mutation, and the variation in the penetrance in different geographical cluster areas however may argue against a genetic condition.

Moreover, transgenic mice carrying a mutation which has been closely linked to the development of Gerstmann–Struässler–Scheinker (GSS) disease in humans (Gerstmann et al, 1936), do not develop clinical symptoms of disease during lifespan, suggesting the presence of this mutation alone is not sufficient to produce disease (Manson et al, 1999; Piccardo et al, 2007).

In both sCJD and vCJD a common polymorphism, with either methionine or valine present at codon 129 of the *PRNP* gene, appears to influence susceptibility to infection and incubation period of disease. Analysis of this polymorphism within the population demonstrates that approximately 38% of Europeans are homozygous for the methionine allele, 51% are heterozygous, and 11% are homozygous for the valine

allele (Gambetti et al, 2003). In sCJD a number of sub-types appear to exist where there is variation between genotype and disease susceptibility (Gambetti et al, 2003). In contrast, all clinical cases of vCJD studied so far have occurred in individuals homozygous for methionine at codon 129 (Will et al, 1996). The discovery of vCJD in an asymptomatic individual heterozygous for this polymorphism at codon 129 suggests that future cases of vCJD may occur in these individuals. This individual received a blood transfusion from a vCJD infected donor (Llewelyn et al, 2004). Indeed, analysis of survivors of the kuru epidemic has shown that most elderly survivors of these traditional mortuary feasts were heterozygous at codon 129 (Mead et al, 2003).

1.1.5 The role of PrP protein

PrP is a host encoded glycoprotein which can be divided into two distinct forms based on their biochemical characteristics (Hope et al, 1986). PrP^{c}, the normal form of this protein, is located on the cell membrane where it is attached via a C-terminal glycosyl-phosphatidyl-inositol (GPI) anchor (Stahl et al, 1987). There are two *N*-glycosylation sites on the polypeptide chain (Asn 181 and Asn 197). PrP^{c} is readily digested by proteinase K and can be liberated from the cell surface by the enzyme phosphatidyl inositol-specific phospholipase C (PIPLC), which cleaves the phosphatidyl inositol glycolipid anchor.

The abnormal, or disease specific form is termed PrP^{Sc} (Hope et al, 1986), although it has also been described as PrP^{res} (to denote the **res**istant form) or PrP^{d} (to denote the **d**isease specific form) (Gonzalez et al, 2003). An intermediate form designated PrP*

has also been postulated, following the conversion of PrP^{c} to PrP^{Sc} in conjunction with Protein X (Kaneko et al, 1997). In contrast to PrP^{c}, PrP^{Sc} is detergent insoluble and partially degraded by proteinase K (Somerville et al, 1986). The proteinase-K-resistant component of the PrP^{Sc} isoform is thought to consist of a 27–30-kDa core (Bolton et al, 1985). In terms of conformation PrP^{c} has a high alpha-helical content (Pan et al, 1993) while PrP^{Sc} folds into a structure that is primarily beta-sheet in content (Caughey, 1991). PrP is highly conserved among mammals and widely expressed in early embryogenesis (Manson et al, 1992).

Although the accumulation of PrP^{Sc} is a feature of TSE infection the function of PrP^{c} remains to be fully defined. Mice homozygous for disrupted PrP genes appear developmentally and behaviourally normal (Bueler et al, 1993; Manson et al, 1994b; Weissmann & Flechsig, 2003). PrP protein is essential for TSE agent replication as transgenic mice devoid of the PrP gene ($PrP^{-/-}$ mice) are resistant to TSE infection (Bueler et al, 1993; Manson et al, 1994a; Manson et al, 1994b). Studies in $PrP^{-/-}$ mice have demonstrated that PrP may be important for the regulation of circadian rhythms (Tobler et al, 1996)

As PrP^{c} is localised to synaptic membranes (Herms et al, 1999) roles in synaptic transmission and neuronal excitability have been postulated and electrophysiological studies have suggested that long term potentiation (Johnston et al, 1998) and GABA receptor mediated inhibition may be impaired in PrP deficient mice (Collinge et al, 1994), although variability in these findings have been reported (Herms et al, 1995).

PrP^c has been shown to bind copper (Todorova-Balvay et al, 2005; Viles et al, 1999) with the major copper binding site identified at the N terminal region. The binding of copper to PrP^c has been shown to be highly pH dependent (Pappalardo et al, 2004). The functional relevance of copper binding by PrP^c is not fully understood but it is thought it may function in a number of ways including; the regulation of copper homeostasis in the synapse (Herms et al, 1999). PrP^c has also been implicated as a neuroprotective molecule, especially in relation to pro-oxidative insults (Brown et al, 1998; Brown et al, 1997a; Herms et al, 1999). Studies in PrP deficient mice have demonstrated a significant reduction in the viability of PrP deficient cerebellar granule neurons in comparison with wild-type mice following exposure to hydrogen peroxide (Brown et al, 1998).

In the immune system PrP^c is expressed on the surface of FDCs (McBride et al, 1992) (Brown et al, 2000a; Ritchie et al, 1999)and at much lower levels on the surface of lymphocytes (Cashman et al, 1990; Mabbott et al, 1996; Mabbott et al, 1997). Within the immune system the role of PrP protein remains to be elucidated, although a possible function in T cell activation (Mabbott et al, 1997) and in the fixation of complement (Mitchell et al, 2007) have been postulated.

1.2 TSE strains

1.2.1 Generation of experimental TSE strains

A number of distinct TSE strains have been isolated in mice from a range of natural scrapie, BSE and CJD sources. In the 1960's studies in goats (Pattison & Millson, 1961; Pattison & Smith, 1963) experimentally infected with an experimental source of natural scrapie designated SSBP/1 (Pattison & Millson, 1961) produced a number of experimental scrapie strains (Chandler, 1961; Dickinson & Outram, 1988). The history of SSBP/1 began in 1945 following the preparation of a brain pool from 3 sheep of cheviot origin with symptoms of natural scrapie. Serial passage of this brain pool was continued in cheviot sheep with very little deviation in disease characteristics during these passages. In the goat transmission studies the source of the SSBP/1 inoculum was obtained from the 18th passage of this brain pool (Pattison & Millson, 1961; Pattison & Smith, 1963).

Following infection of the goats with the SSBP-1 brain pool, two distinct types of clinical symptoms "drowsy" and "scratching" were observed in the affected goats. Subsequent passage in goats of brain material from each distinct type produced the same distinct clinical symptoms as before. In- fact these different characteristics were maintained through a number of passages in goats leading the researchers to conclude that two different scrapie strains had been isolated from the original transmission studies (Chandler, 1961; Chandler, 1962; Pattison & Millson, 1961; Pattison & Smith, 1963)

Subsequent studies in mice (Chandler, 1961) (Chandler, 1962) (Dickinson et al, 1986) determined that the "two distinct" strains isolated from the "drowsy" and "scratching" goat studies actually contained a mixture of strains. Although initial studies to transmit the "scratching" isolate to lines of inbred mice were unsuccessful, the "drowsy" or "chandler" isolate successfully transmitted to mice (Chandler, 1961) (Chandler, 1962). Further transmission studies in mice determined that this isolate contained the 139A, 79A and 79V scrapie agent strains, while the "scratching" strain contained the 22C and 22H strains (Dickinson et al, 1986). Further passage of the "drowsy" or "chandler" isolate at the Rocky Mountain Laboratory (Montana, USA) led to the designation of the RML isolate, although it is believed that this may in-fact be a mixture of both the 139A and 79A strains.

Interestingly, sub-passage in mice of the SSBP/1 material used to conduct the original studies in goats produced the 22A, 22C, 22H, 22F and 22L strains but not the 139A, 79A or 79V strains (Dickinson et al, 1986). A number of possibilities for this phenomenon exist. Potentially the goats that produced the "drowsy" line were naturally infected with another scrapie strain. Alternatively, as the goats used in the studies were outbred, some genetic component may have led to the selection of different strains.

The majority of the studies described in this thesis have used the ME7 strain of scrapie (Zlotnik & Rennie, 1965). This strain was isolated as a result of a series of experiments which began with the intragastric transmission, to mice, of pooled spleen material from a number of cases of Suffolk sheep with natural scrapie (Zlotnik & Rennie, 1965). These initial attempts to transmit this spleen pool intragastrically

resulted in clinical and neurological symptoms of disease primarily in the group initially termed ME.$_7$ (Zlotnik & Rennie, 1965). Subsequent passage of brain material from these brains led to the eventual isolation of the strain that is now referred to as ME7. Notably the experimental ME7 scrapie strain has often been isolated from sources of natural sheep scrapie in the past (Bruce et al, 2002). The ME7 scrapie strain is particularly useful due to its extensive use in many studies at this unit and elsewhere, providing essential information on the pathogenesis of this strain. In addition, this strain is known to target peripheral lymphoid tissues (Bruce et al, 2000; Fraser et al, 1992b)behaving similarly to natural scrapie and vCJD (Bruce et al, 2001).

1.2.2 Human TSE infections

Kuru

In the 1950's "kuru" was identified by several researchers notably Carlton Gadjusek, and Michael Alpers (Alpers, 1965; Zigas & Gadusek, 1957) This disease was found almost exclusively amongst the Fore tribe of the eastern highlands of Papua New Guinea. This particular tribe participated in endocannibalism; a ritualistic form of cannibalism involving the consumption of deceased relatives (Gajdusek, 1963; (Alpers, 1965). This traditional practice was later shown to be responsible for the transmission of kuru (Collinge et al, 2006; Mathews et al, 1968). In addition to the oral consumption of infected tissues, other ritualistic practices, such as coating the body with the remains of the deceased, may have contributed to the epidemic (Hornabrook & Moir, 1970; Mathews et al, 1968). Transmission of TSEs via the skin

(scarification) is known to be an effective route of disease transmission (Taylor et al, 1996) (Mohan et al, 2004). The kuru epidemic reached its peak in the 1960's with an estimated 1,100 victims of the disease. The disproportionate ratio of female to male victims was later explained by the predominant consumption of brain material, containing high levels of infectivity, by women and infants (Gajdusek & Alpers, 1966), in comparison to the male members of the tribe who mainly consumed muscle from the deceased (Hornabrook & Moir, 1970).

The cause of this disease epidemic proved extremely difficult to identify and various possibilities were considered; including heavy metal poisoning and toxins from plants or animal sources being suggested as potential causes of the condition. Notably, some similarities with natural scrapie were suggested in 1959 (Hadlow, 1959). In addition it was believed initially that this disease may have been purely a genetic condition with no infectious component. This possibility was eventually ruled out due to the high frequency of disease within the population; such a lethal genetic condition would have been removed from the gene pool and could not have been maintained in the population at such high levels. In addition, the occurrence in individuals who were not genetically related also disproved this theory (Hornabrook & Moir, 1970). Initial transmission studies to mice from CNS and peripheral tissues of victims of kuru were deemed negative (Gajdusek & Gibbs, 1964), however these studies were terminated after a period of only 3 months which clearly would not have been long enough to produce disease in rodent models. The transmission of kuru to primates in the 1960's (Gajdusek et al, 1966) demonstrating its infectious nature, and

the similarity of the brain pathology to natural scrapie (Hadlow, 1959) placed the disease within the group of diseases that are now know as TSEs

Creutzfeldt-Jakob disease (CJD)

CJD was first recognised in the 1920's (Creutzfeldt, 1920; Jakob, 1921) and is now defined in several forms: Classical or Sporadic CJD, Variant CJD, familial CJD and iatrogenic CJD.

Sporadic CJD

Sporadic CJD (sCJD) occurs worldwide at a rate of approximately 1 case per million/annum. The disease is generally confined to older individuals with an average age of onset approximately 68 years, although there have been some cases in younger individuals (Murray et al, 2008). The duration of the clinical stage of sCJD is generally much shorter than that of vCJD with a median duration of illness of 4-5 months compared to 13-14 months for vCJD. In addition the clinical and pathological characteristics differ for the two forms (Gambetti et al, 2003).

Variant CJD

In 1996 a "new variant" of CJD was described in 10 individuals with a clinical and pathological phenotype distinct from that of sCJD (Will et al, 1996). Since the identification of variant CJD (vCJD) there have been almost 200 cases of the disease worldwide, the majority of which have occurred in the United Kingdom (UK) (for current figures see: http://www.cjd.ed.ac.uk). Epidemiological and transmission studies have produced compelling evidence to show that this disease has been caused

by the consumption of BSE contaminated foodstuffs (Brown et al, 1997b). During the BSE epidemic in the 1980's it was estimated that almost 500,000 infected cattle were slaughtered for consumption; suggesting that the number of people exposed to potentially infective doses through food may have been extremely high (Valleron et al, 2001). This factor and the clinical and pathological similarities of vCJD with kuru suggested that the route of transmission was most likely through oral transmission (ingestion) of BSE contaminated food. In addition, transmission of brain material from vCJD affected individuals into mice produces a "signature" undistinguishable from that of BSE (Bruce et al, 1997). The components of this signature include; incubation period in specific mouse strains (Bruce et al, 1997), lesion profiles in brain and PrP^{Sc} glycoform analysis (Somerville et al, 2005). One of the most striking and distressing features of vCJD is the young age of those affected with a mean age of death of 29 years (Boelle et al, 2004; Ghani et al, 2000). The reasons for this apparent age related susceptibility are not fully understood although it is possible that age related immune-system dysfunction may contribute to this phenomenon. This will be discussed in detail in chapter 4.

In addition to the differences in clinical symptoms of disease between vCJD (Ironside, 2003) and sCJD (Gambetti et al, 2003) there are notable differences in the targeting of infection within the body. In vCJD, like many cases of natural scrapie, the peripheral lymphoid becomes infected early in infection (Hill et al, 1999a; Hill et al, 1999b; Hill et al, 1997). In contrast infectivity has not been detected in lymphoid tissues of sCJD (Hill et al, 1999b). The recent transmission of vCJD to 4 individuals, most probably via blood transfusion (all four cases had received transfusions of non-leucodepleted red blood cells between 1996 and 1999) has raised concerns over the

possibility of a secondary epidemic of vCJD within the population (Llewelyn et al, 2004; Peden et al, 2004; Wroe et al, 2006).

Notably, there have been no recorded cases of sCJD arising from blood transfusion. This may relate to the differences in targeting between sCJD and vCJD where infection of the peripheral lymphoid organs may contribute to the dissemination of infectivity into the bloodstream.

Genetic or Familial CJD

Familial CJD is classified into many haplotypes based on the *PRNP* mutation and codon 129 (and other polymorphic codons) on the mutant allele (Gambetti et al, 2003; Goldfarb et al, 1991a). A great deal of controversy surrounds the aetiology of the familial forms of these diseases. The existence of these mutations is thought to pre-dispose these individuals to the disease. The clinical and pathological symptoms of this form of the disease can vary depending on the mutation present and the age of onset is generally less that of those with sporadic CJD (Gambetti et al, 2003).

1.2.3 Natural TSE infections of animals

Bovine spongiform encephalopathy

In 1986 a new disease in cattle, with neuropathology consistent with a spongiform encephalopathy, was identified (Wells et al, 1987). This disease was termed bovine spongiform encephalopathy (BSE). The cause of this disease was later related to the feeding of cattle with ruminant derived meat and bone meal contaminated with a

TSE agent (Taylor, 2000; Wilesmith, 1993). It is thought that changes in the rendering process used to produce meat and bone meal allowed the survival of TSE infectivity. Although the nature of the infecting agent remains controversial one of the most plausible theories is that BSE arose from a cattle adapted scrapie agent, although strain typing studies have failed to demonstrate any similarities with pre-existing scrapie strains, perhaps with the exception of the experimental strain CH1641 (Hope et al, 1999). Other theories include the possibility that some form of rare natural spongiform encephalopathy, present within the cattle population, was somehow amplified as a result of the modified rendering process (Priola & Vorberg, 2004). BSE has spread to a number of species, including domestic and large cats, exotic ungulates (Nyala and Kudu) and humans as vCJD (Bruce, 1997). Perhaps a more provocative hypothesis is the possibility that BSE in cattle might have arisen in the UK after the incorporation in animal food products of human remains imported from India (Colchester & Colchester, 2005).

Strikingly, transmission studies to panels of inbred mouse lines demonstrated that a single strain of agent was responsible for BSE in cattle which was indistinguishable from the infecting strain responsible for vCJD, FSE and the cases in the exotic ungulates (Bruce et al, 1997). More recently, the identification of a possible natural field transmission of the BSE agent to goats in has been documented in France (Eloit et al, 2005).

Atypical TSEs in ruminants: Nor98, atypical scrapie and BASE

In 1998 an atypical form of scrapie, termed Nor98, was detected in Norwegian sheep (Benestad et al, 2003). The characteristics of Nor98 include its unusual

neuropathology and western blot banding pattern which demonstrates an unusual band of low molecular weight (mass approximately 10–12 kDa). This is in contrast to that of "classical" scrapie where the disease specific form of PrP is detected as three bands between 18 and 29 kDa corresponding to the un-, mono- and di-glycosylated disease specific forms of PrP (Baron et al, 2007). Active surveillance of small ruminants throughout the European Union (EU) to detect these "atypical" cases of scrapie has since identified the presence of atypical forms of scrapie in a number of countries including France, Belgium and Germany (Baron et al, 2007; Buschmann et al, 2004). Notably, a number of these cases were identified in sheep with genotypes normally associated with resistance to classical scrapie or BSE, such as those with the A136 H154 Q171 or A136 R154 R171 genotypes (Buschmann et al, 2004). Interestingly, retrospective analysis of an unusual case of scrapie detected in 1989 indicate that this may have been a case of Nor98 scrapie suggesting that this strain may have been present in certain breeds of sheep for many years (Bruce et al, 2007). Initial suggestions that these atypical forms may not have been TSEs have been ruled out following the transmission of 10 French atypical isolates and three Norwegian Nor98 cases to transgenic mice over-expressing the ovine PrP (V_{136} R_{154} Q_{171} allele) of the sheep PrP gene (Arsac et al, 2007).

Since the introduction of active surveillance methods for the detection of TSEs in ruminants a number of atypical TSE cases have also been identified in cattle. These include the identification of a novel TSE in cattle from Italy with unusual pathology in the form of amyloid plaques in the CNS (Casalone et al, 2004). This has since led to the definition of a new form of BSE termed: bovine amyloidotic spongiform encephalopathy (BASE).

Following this initial discovery, other variations in the BSE phenotype have since been reported in Japan (Nakamitsu et al, 2006; Yamakawa et al, 2003) and other European countries (Biacabe et al, 2004; Buschmann et al, 2006). Although the BASE isolates appear to have different molecular and neuropathological phenotypes from that of classical BSE transmission of brain material from atypical BSE cases has produced striking results. Transmission to wild-type mice produced a molecular and neuropathological ”signature” indistinguishable from that of classical BSE, whereas the characteristics of the BASE strain were preserved in transmissions to transgenic mice expressing bovine PrP (Capobianco et al, 2007). Interestingly the glycoform patterns of the BASE isolates closely resemble those found in some cases of sCJD (Casalone et al, 2004).

1.3 The central nervous system in TSE pathogenesis

1.3.1 Clinical and pathological characteristics of experimental TSEs

In the brain pathological signs of disease include vacuolation, gliosis and the deposition of the abnormal form of PrP protein (Bruce et al, 1989b). The presence of vacuolation is one of the most defining pathological features of TSE infection and is thought to result from neuronal loss (Jeffrey et al, 1994b). Almost without exception, vacuolation of the grey matter is a feature of all TSE infections although the intensity and targeting varies between strains (Fraser & Dickinson, 1973). Indeed the scoring

system used to construct a "lesion profile" is based on the intensity of vacuolar change within 9 defined grey matter and 3 defined white matter areas (Dickinson et al, 1968; Fraser & Dickinson, 1968). The specific pathology observed in brain is dependent not only on TSE strain but also on the PrP genotype of the host. For example; infection of CVF1 mice, an F1 cross between C57BL (*Prnp*^{a}) and VM (*Prnp*^{b}) mice, with the ME7 strain of scrapie produces a severe pathology in the pyramidal cell layer of the CA1 layer of the hippocampus (Scott & Fraser, 1984). This pathology is not observed in the C57BL or VM mouse strains when challenged with ME7.

In addition, the deposition of amyloid, consisting of PrP^{Sc} in the form of amyloid plaques is a feature of a number of strains including; kuru (Collinge et al, 2006), the experimental strain 87V (Bruce, 1984; Bruce & Fraser, 1975) and also in variant CJD (Will et al, 1996). In a number of neurodegenerative diseases inflammation in the CNS is a key feature (Perry, 2007). This is characteristically displayed in multiple sclerosis where T cells and macrophages infiltrate the brain resulting in degeneration of myelin and axons. In both acute and chronic neurodegeneration conditions, including TSE disease, activated microglia can be detected in the CNS (Betmouni et al, 1996; Perry, 2007). In studies using the ME7 strain of scrapie activated microglia could be detected from 12-13 weeks after intracerebral challenge (Betmouni et al, 1996) coincident with behavioural changes in the infected mice (Perry, 2004). Although the exact cause of TSE associated neurodegeneration is not fully understood, accumulation of PrP^{Sc} in the CNS is thought to contribute to these pathological changes. PrPSc has been shown to have neurotoxic effects on cultured

cells of neuronal origin (Salmona et al, 1997; Selvaggini et al, 1993). The topological distribution of PrP^{Sc} correlates with vacuolation in many TSEs (Bruce, 1984; Bruce et al, 1989b) although this is not the case in all TSEs. Recently the accumulation of PrP in the absence of vacuolar changes has been demonstrated in a case of human GSS and in subsequent transmission studies to mice (Piccardo et al, 2007).

Other potential mechanisms for TSE associated neurodegeneration have included a potential role for the complement system (Gasque et al, 2000), specifically complement component C5 (Mabbott & Bruce, 2004). In certain neurodegenerative conditions such as multiple sclerosis overproduction of complement may contribute to neurodegeneration and inflammation (Kirschfink, 1997; Niculescu et al, 2004). However studies in complement component C5 deficient mice found no effects on neuropathology or incubation period of disease suggesting that C5 is not involved in TSE pathogenesis (Mabbott & Bruce, 2004).

1.3.2 Cellular targeting of PrP^{Sc} in the CNS

Following infection, PrP^{Sc} accumulates in the CNS although there are dramatic differences in the distribution and appearance of PrP^{Sc} between TSE strains. In addition there are several pathological forms of PrP^{Sc} found within infected brain which include, amyloid plaques, diffuse granular staining of the neuropil and accumulation around neurons (Bruce et al, 1989b; Jeffrey et al, 1994a; McBride et al, 1998). For example, with the 87V strain of scrapie amyloid plaques are frequent and abnormal accumulations of PrP in brain are precisely targeted to areas of vacuolation (Bruce & Fraser, 1975; Jeffrey et al, 1994a) whereas in the ME7 strain of scrapie fewer amyloid plaques are present and PrP pathology is more widely distributed throughout the brain(Bruce et al, 1989b; Bruce et al, 1994b). In addition to host PrP genotype influencing pathology, in the form of vacuolar changes, the deposition of PrP in the brain also appears to be controlled by the PrP gene (Manson et al, 1994b). In conjunction with the lesion profile, the specific targeting of PrP^{Sc} in the CNS is extremely useful in the diagnosis of TSEs.

1.4 The peripheral lymphoid system in TSE pathogenesis

1.4.1 The importance of the lymphoid system in TSE pathogenesis

Although the main consequence of TSE infection is CNS degeneration, the peripheral lymphoid system plays an important role in pathogenesis (Bruce et al, 2000; Eklund et al, 1967; Fraser & Dickinson, 1978; Mabbott et al, 1998). Following infection by peripheral route (e.g. intraperitoneal or oral) most experimental TSE agents replicate in spleen and lymph nodes prior to neuroinvasion (Kimberlin & Walker, 1979; Kimberlin & Walker, 1989). During this period there are no clinical signs of disease and indeed, the detection of infectivity in spleen precedes that of the CNS by many weeks or longer (Dickinson and Fraser 1969; Dickinson 1975; Dickinson and Outram 1975).

Notably, some TSEs do not appear to involve or depend on the peripheral lymphoid system for pathogenesis. These TSEs include sCJD in humans which does not appear to target or infect lymphoid tissues (Gambetti et al, 2003), BSE in cattle (with exception of targeting to the distal ileum) (Fraser et al, 1988), and in some classical and atypical strains of natural scrapie there appears to be no involvement of lymphoid tissues.

This is contrast to sheep experimentally infected with BSE where there is extensive lymphoid system involvement (Houston et al, 2000) (Foster et al, 2001a); Hunter et al., 2002 ; (Hunter, 2003; Hunter et al, 2002). In addition, humans affected with variant CJD have extensive lymphoid tissue involvement with significant levels of infectivity being detected in lymph nodes and tonsil

(Bruce et al, 2001; Hill et al, 1999a; Hill et al, 1999b).

In studies involving the surgical removal of the spleen (splenectomy) significant prolongation of incubation period was observed with both the ME7 and 139A strains (Fraser et al, 1992b; Fraser & Dickinson, 1970; Fraser & Dickinson, 1978; Kimberlin & Walker, 1989) although this was dependent on route of infection and time of splenectomy. When mice were splenectomised prior to or up to 42 days after intraperitoneal injection with ME7 a significant prolongation of incubation was observed (Fraser & Dickinson, 1978). Splenectomy after this timepoint had no effect on incubation period, although it is likely that the spread of disease to the spinal cord would have been initiated by this stage of infection. In studies involving subcutaneous infection with the ME7 strain the effect on incubation period was generally not significant, although under normal circumstances this route of infection results in high levels of infectivity in spleen. In addition, intragastric infection of splenectomised mice with the 139A strain (Kimberlin & Walker, 1989) did not result in prolongation of incubation period. It is likely that some routes of infection, for example the intraperitoneal route, depend more on the presence of the spleen than other routes, such as the oral or subcutaneous route. These effects are almost certainly related to the sequential infection of specific lymphoid tissues which may vary depending on the route of infection. In addition to route of infection, the effect of splenectomy is also strain and species dependent. For example, splenectomy has no effect on the intraperitoneal infection with 263K in hamsters (Kimberlin & Walker, 1989).

1.4.2 Initial attempts to identify the cellular targets for TSE agent replication in the peripheral lymphoid system

The recognition that lymphoid tissues contained high levels of infectivity and the effects of splenectomy on peripheral pathogenesis demonstrated the important role of these tissues in TSE pathogenesis. A series of experiments conducted by Fraser and colleagues (Fraser & Farquhar, 1985; Fraser & Farquhar, 1987) (Fraser et al, 1989) (Fraser et al, 1992b) suggested that the cell type or types critical for scrapie pathogenesis were post-mitotic, long-lived and radiation resistant. In these studies mice infected with scrapie via a number of peripheral routes were subjected to a variety of sub-lethal and lethal doses of ionising radiation. Mice receiving lethal doses of radiation were subsequently reconstituted with isologous bone marrow. Thorough analysis of the results of these extensive studies (approximately 70 experiments in total) concluded that there was no evidence that irradiation altered incubation period of disease. In addition, irradiation was found to have no effect on the levels of infectivity in lymphoid tissues suggesting that the cells involved in replication or accumulation of infectivity were unaffected by ionising radiation.

Studies attempting to elucidate the cellular basis for TSE infectivity in lymphoid tissues found that isolation of spleen pulp contained lower levels of infectivity than whole spleens (Lavelle et al, 1972). Extrapolation of these studies (Clarke & Kimberlin, 1984a) by the separation of scrapie infected spleens into “stromal” or “pulp” fractions found that the greatest levels of infectivity (approximately 10 fold higher) were within the stromal fraction. It is likely that the stromal fractions would have represented splenic capsule, trabeculae and other cell types (described as fixed

cells) which may have included dendritic cells, some macrophage populations and significantly follicular dendritic cells (FDCs). The pulp fraction is likely to have contained lymphocytes, red blood cells, polymorphonuclear cells and possibly some macrophage populations.

1.4.3 Cellular targeting of the normal and disease specific forms of PrP in the peripheral lymphoid system

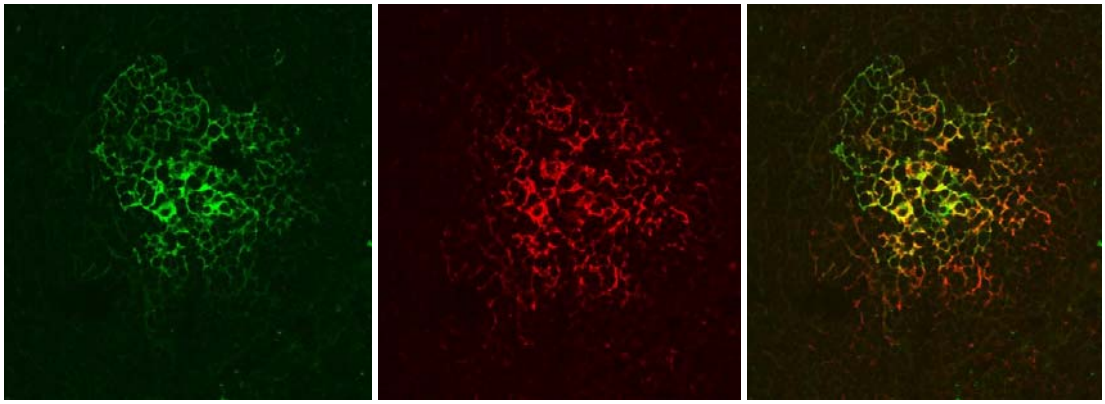
Although it cannot be assumed that cellular expression of PrP means that these cells participate in replication, it seems likely that the localisation of PrP^{c} or accumulations of PrP^{Sc} in specific cell types indicate an involvement in some aspect of the disease process. The most widely used methods for detecting PrP protein in tissues are by western blot and by immunohistochemistry (IHC). Although western blot can be used to distinguish between PrP^{c} and PrP^{Sc}, IHC analysis can precisely identify which cells are associated with PrP providing important information on the targeting of infection in disease pathogenesis.

In mice, PrP protein is associated with FDCs in both uninfected and TSE infected mice (McBride et al, 1992; Ritchie et al, 1999). FDCs are non-lymphoid cells, which are found within germinal centres of secondary lymphoid follicles where they trap antigens in the form of antigen antibody complexes (Kosco et al, 1992).

PrP^{Sc} has been also been detected in association with FDCs in the lymphoid tissues of sheep with natural scrapie (van Keulen et al, 1996), in elk with chronic wasting

disease (Sigurdson et al, 1999) and humans with vCJD (Hill et al, 1997). Whilst PrP on FDCs can be detected by IHC (figure 1.1), lymphocytes are also known to express PrP on their cell surface (Cashman et al, 1990; Mabbott et al, 1997) although only at lower levels which can only be demonstrated by flow cytometry.

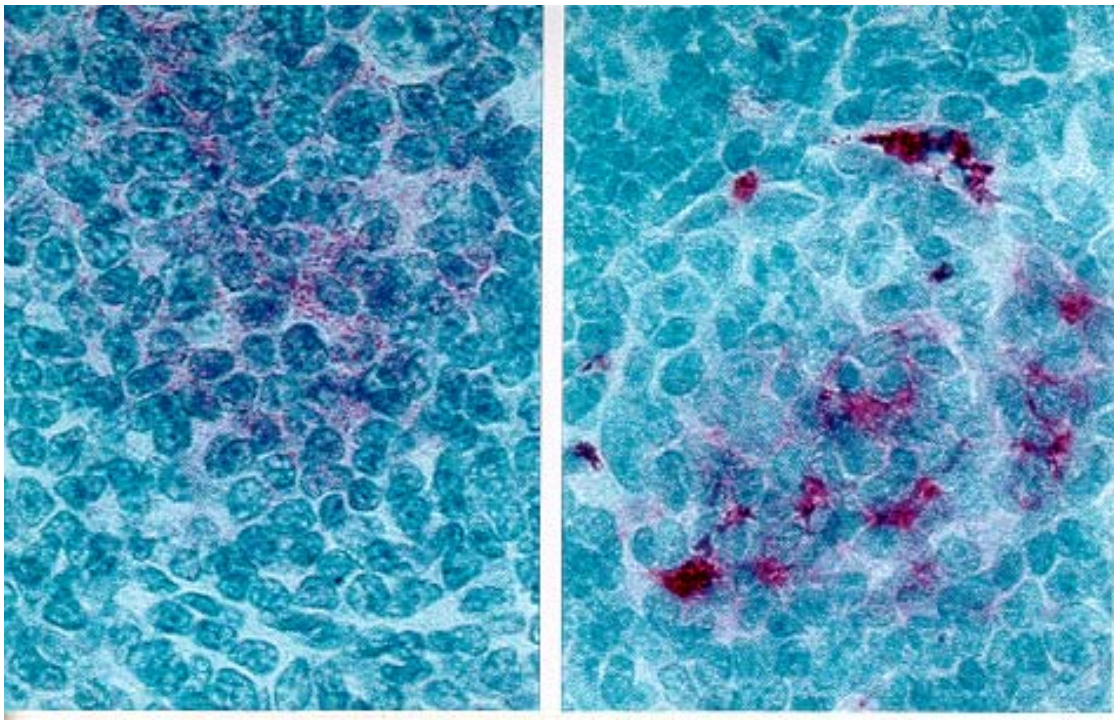
Figure 1.1.Confocal analysis showing co-localisation between PrP and FDCs. Uninfected spleen immunolabelled for PrP protein (green) and FDCs (red). Analysis demonstrates co-localisation (yellow). (From Brown *et al* 2000)



Although there are no specific antibodies which can discriminate between PrP^{c} and PrP^{Sc} immunohistochemically, the appearance of PrP in infected mice is quite different to that of uninfected mice. In spleen from uninfected mice, PrP labelling is very diffuse (figure 1.2) whereas the labelling in infected mice is much more intense and often associated with cells which have the morphology of macrophages.

Figure 1.2. Immunohistochemical detection of PrP protein using the PrP specific polyclonal antibody (1B3) in spleen from ME7 infected and uninfected (normal brain injected). In normal mice accumulations are diffuse, whereas in infected mouse spleen aggregates of PrP can be detected. Image from Brown *et al* 2000.

Uninfected **ME7 scrapie infected**



Analysis of spleen from ME7 scrapie infected mice using electron microscopy has revealed that PrP^{Sc} in the infected spleen is associated with the plasmalemma (Jeffrey et al, 2000) of FDCs and also found within tingible body macrophages (Jeffrey et al, 2000). PrP labelled cells with the appearance of macrophages can also be visualised in close association to FDCs by confocal microscopy (Brown et al, 2000a).

1.4.4 PrP in other extraneural tissues

PrP has also been detected immunocytochemically in several other tissues including, kidney, pancreas, salivary gland, adrenal, liver and thymus (McBride et al, 1992) (Brown et al, 2000a) although it is unclear what the function of PrP is in these tissues. In all of these tissues PrP is localised to specific regions in both uninfected and scrapie infected mice but labelling is more intense in scrapie infected mice. PrP is found in the cuboidal epithelium of proximal and distal tubules of kidney, pancreatic islets of Langerhans and mucous (but not serous) secreting cells of salivary gland and in the adrenal cortex, most prominently in the zona fasiculata (Brown et al, 2000a). Low levels can sometimes be detected in a proportion of liver hepatocytes. In the thymus, PrP labelling is associated with cells in the thymic medulla (Brown et al, 2000a; McBride et al, 1992). Although these have not been specifically identified, their morphology and distribution suggests that they are interdigitating dendritic cells. In these tissues, therefore, PrP appears to be associated with a range of cell types performing different functions, which include secretion, metabolic synthesis and reabsorption.

1.4.5 Effects of immunodeficiency on TSE pathogenesis.

In most infectious diseases a deficient immune system is often the catalyst for effective transmission of disease. For example, immunosupressed individuals, e.g. those with human immunodeficiency virus infection, are highly susceptible to bacterial and viral infections, such as the influenza virus.

Paradoxically, the pathogenesis of peripherally acquired TSE infection appears to be dependent on a functional immune system. Severely combined immunodefcient (SCID) mice lack B and T lymphocytes and carry a mutation which blocks the rearrangement of immunoglobulin and T cell receptor genes; resulting in a profound deficiency of both T and B lymphocytes. In addition to their defects in lymphocyte maturation they lack mature follicular dendritic cells (FDCs), although these cells are still present in lymphoid tissues in an undifferentiated form (Kapasi et al, 1993; Kapasi et al, 1998). As FDCs are dependent on B and T lymphocytes for their maturation these cells remain in an immature state in SCID mice.

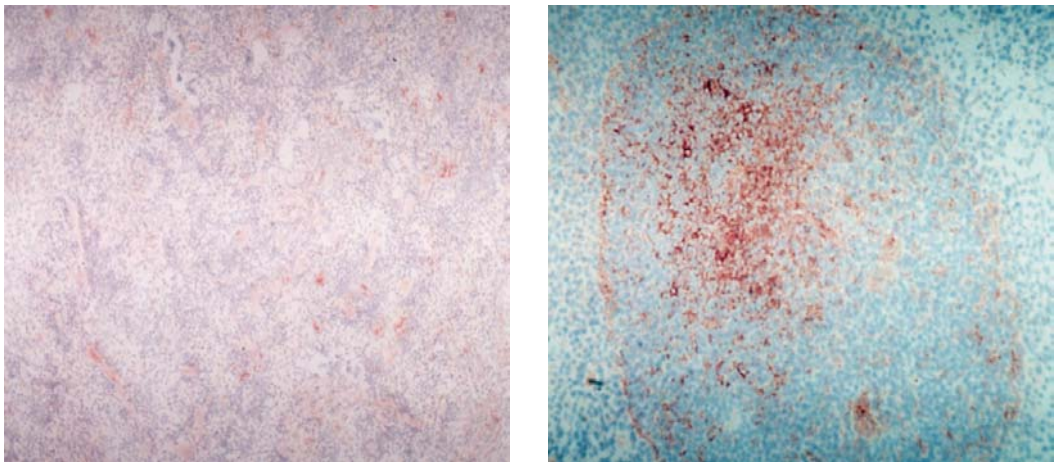
SCID mice are resistant to peripheral infection with moderate doses of the ME7 strain (Fraser et al, 1996; Lasmezas et al, 1996; O'Rourke et al, 1994) although, the effect of the scid mutation on TSE pathogenesis can be completely reversed following isologous bone marrow reconstitution which also leads to maturation of FDCs (Fraser et al, 1996) (figure 1.3). SCID mice are also resistant to challenge with the BSE agent using a combination of peripheral and intracerebral (i.c.) infection (Brown et al, 1997). As SCID mice are fully susceptible to direct i.c. infection with mouse passaged TSE agents these results produced strong evidence that an intact

lymphoid system is essential for effective cross species infection. It is possible that some form of "obligatory" processing of infectivity occurs in the peripheral lymphoid system prior to neuroinvasion when TSE agents are transmitted across species.

Interestingly, further studies using a range of challenge doses of the ME7 strain found that SCID mice were fully susceptible to high dose peripheral challenge, although there was no detectable infectivity or accumulation of PrP^{Sc} in spleen. The striking effects of dose on the pathogenesis of scrapie in SCID mice most probably result from direct infection of the peripheral nerves in lymphoid tissues at high dose, with neuroinvasion occurring independently of lymphoid tissue involvement.

Figure 1.3 Resident FDCs can be induced to mature in SCID mice following bone marrow transplantation from isologous donors. Images show expression of the MFG-E8 receptor upon FDCs as recognised by the monoclonal antibody FDC-M1 (Kranich et al, 2008)

SCID no bone marrow **SCID + bone marrow**



1.5 Involvement of Follicular dendritic cells in TSE pathogenesis.

1.5.1 The origin of FDCs

Follicular dendritic cells are a specialised population of cells found within the primary and secondary follicles of the lymphoid tissues (Kosco-Vilbois et al, 1997; Kosco et al, 1992). FDCs have the ability to trap and retain antigens in the form of antigen-antibody complexes on the surface of their extensive cytoplasmic processes for long periods of time. The presence of these antibody complexes are thought to play a pivotal role in the development of B cell memory. FDCs express complement receptor CR1 and CR2 which are expressed as soon as primary follicles occur (Ierna et al, 2006). These receptors bind C3 coated immune complexes, enhancing and maintaining the immune response.

The origin of FDCs has been the subject of much debate. Most studies point to a stromal/fibroblastic and not a haematopoietic origin, although the subject remains controversial. Initial studies conducted by Humphrey and Sundaram (Humphrey et al, 1984) found that transfer of allogenic bone marrow into irradiated recipient mice detected only FDCs of host origin. Critics of these studies suggested that failure to detect donor derived FDCs was a result of low turnover of host cells. Further studies in SCID mice found that host FDC maturation could be induced by reconstitution with lymphocytes or other haematopoietic sources (bone marrow) suggesting that FDCs are derived from the host and not the donor (Kapasi et al, 1993; Kapasi et al, 1998).

The most prominent studies suggesting that FDC could be derived from bone marrow were conducted by Kapasi and colleagues (Kapasi et al, 1998). In these studies newborn SCID mice were reconstituted with rat bone marrow. Several weeks later FDCs of rat origin were detected using the monoclonal antibody ED-5 which recognises only FDCs of rat origin. However, controversy still exists over the possibility that FDCs can be derived from the bone marrow or other haematopoietic sources. It is possible that the expression of the ED-5 marker was in-fact transferred from the bone marrow derived cells to the host, resident precursor FDCs. A recent study where human cord blood was grafted into recipient RAG-2 mice found no evidence of human FDC development in lymphoid tissues although murine FDCs from the recipient of the graft were induced to mature in the presence of lymphocytes of human origin (Traggiai et al, 2004).

1.5.2 Follicular dendritic cells and the germinal centre micro-environment

FDCs are found within the organs of the secondary lymphoid tissues which include the spleen, lymph nodes and the Peyer's patches of the gut. In an inactive state (before exposure to antigen) FDCs are found within the primary follicle in these lymphoid tissues and following exposure to antigen form germinal centres which contain FDCs, B cells, and helper CD4+ T cells. FDCs secrete chemokines which result in the recruitment of these cells to form the germinal centre (Kosco-Vilbois, 2003; Kosco-Vilbois et al, 1997; Kosco et al, 1992). A number of studies of germinal centers *in vivo* indicate that the germinal centre is a dynamic environment facilitating many important processes; including immunoglobulin (Ig) class switching, rapid B cell proliferation, production of B memory cells, selection of somatically mutated B cells with high affinity receptors, apoptosis of low affinity B cells and affinity

maturation of B memory cells (Kosco-Vilbois, 2003; Kosco et al, 1992). A major function of FDCs is to trap and retain immune complexes on their extensive cytoplasmic processes in order to maintain and perpetuate immunological memory (Ahmed & Gray, 1996; Gray, 2002; Gray & Matzinger, 1991; Gray & Skarvall, 1988). FDCs are thought to trap these immune complexes via cell surface receptors including the complement and complement receptors, CR2 (CD21) and CR1 (CD35) and through the Fc receptors, FcRIIb (CD32) and FcRII (CD23) (Balogh et al, 2001; Qin et al, 2000). In the germinal centre activated FDCs express a number of markers which are not expressed on these cells in their naïve state. These receptors include the vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Videm & Albrigtsen, 2008).

1.5.3 The use of chimaeric mouse models to elucidate the role of FDCs

Although previous studies provided strong evidence that FDCs were involved in TSE pathogenesis (Fraser et al, 1996; Fraser & Farquhar, 1987; Fraser et al, 1989) the data generated could not exclude the potential involvement of other cell types, particularly B lymphocytes. In studies attempting to elucidate the role of these cells chimaeric mouse models were produced where FDCs but not lymphocytes would carry the PrP gene and vice-versa (Brown et al, 1999).

The production of these models was achieved by exploiting the unique characteristic of FDCs; namely their radiation resistance and the fact that these cells are not derived from the bone marrow. Although the ultimate origin of FDCs remains

controversial it is generally accepted that FDCs are not replaced from the bone marrow in adult mice.

To construct the chimaeric models described above, lethally irradiated $PrP^{+/+}$ or $PrP^{-/-}$ deficient and unirradiated $SCIDPrP^{+/+}$ or $SCIDPrP^{-/-}$ mice were reconstituted with bone marrow from $PrP^{+/+}$ or $PrP^{-/-}$ mice. Bone marrow grafting produces lymphocytes of donor origin whereas the FDCs are derived from the host.

In studies with the ME7 strain of scrapie neuroinvasion and replication of infectivity in spleen only occurred when FDCs expressed PrP, irrespective of the expression of PrP on surrounding lymphocytes (Brown et al, 1999). Moreover, the expression of PrP on FDCs in uninfected or ME7 infected mice was only detected when FDCs carried the PrP gene (Brown et al, 1999).

In addition, replication of infectivity in spleen only occurred in mice with PrP expressing FDCs which was again, not dependent on the PrP status of the graft. No replication of infectivity was detected in spleens of PrP deficient mice even when reconstituted with PrP expressing bone marrow (figures 1.5 and 1.6).

Although these studies produced compelling evidence for a critical role for FDCs in TSE pathogenesis similar studies by another group using the RML strain found different results from those described above (Blattler et al, 1997). In these studies, replication of infectivity could be detected in spleens of PrP deficient mice grafted with PrP expressing bone marrow or fetal liver (Blattler et al, 1997). These studies

led the researchers to initially conclude that cells of haematopoietic origin (most probably lymphocytes) and not FDCs were critical for TSE pathogenesis. The contrasting results of these studies led to the suggestion that different TSE strains may target different cell types as they are known to do in the CNS (experimental studies are described in chapter 4). Alternatively, it was suggested that technical differences in the way the studies were carried out; for example different doses, could explain these contrasting results.

Figure 1.5. Immunolabelling for PrP (using the 1B3 antibody) or FDC networks in (using the FDC-M1 antibody) in spleens from uninfected mice 28 days post bone marrow reconstitution. FDC networks are well developed in all of the spleens (lower panel) but PrP protein associated with FDCs is only present when the recipient of the graft carries a functional PrP gene (upper panel). Scale bar represents 100μm. From Brown *et al* 1999

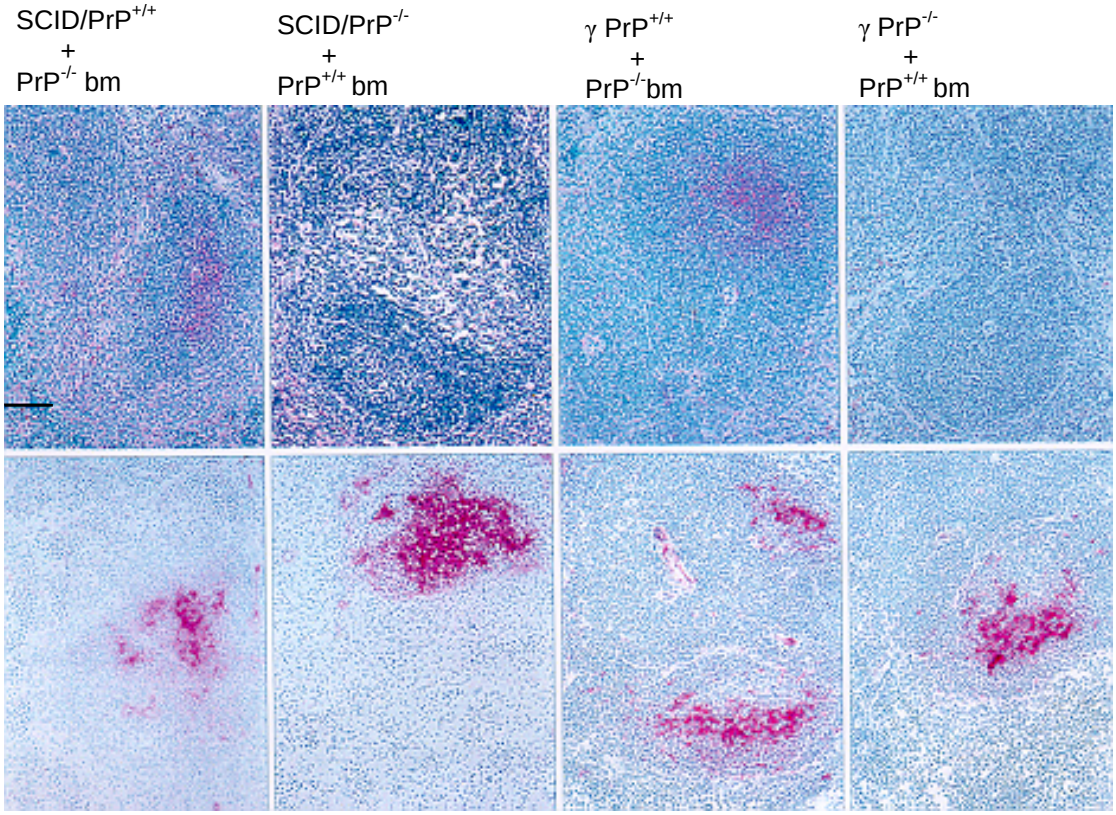
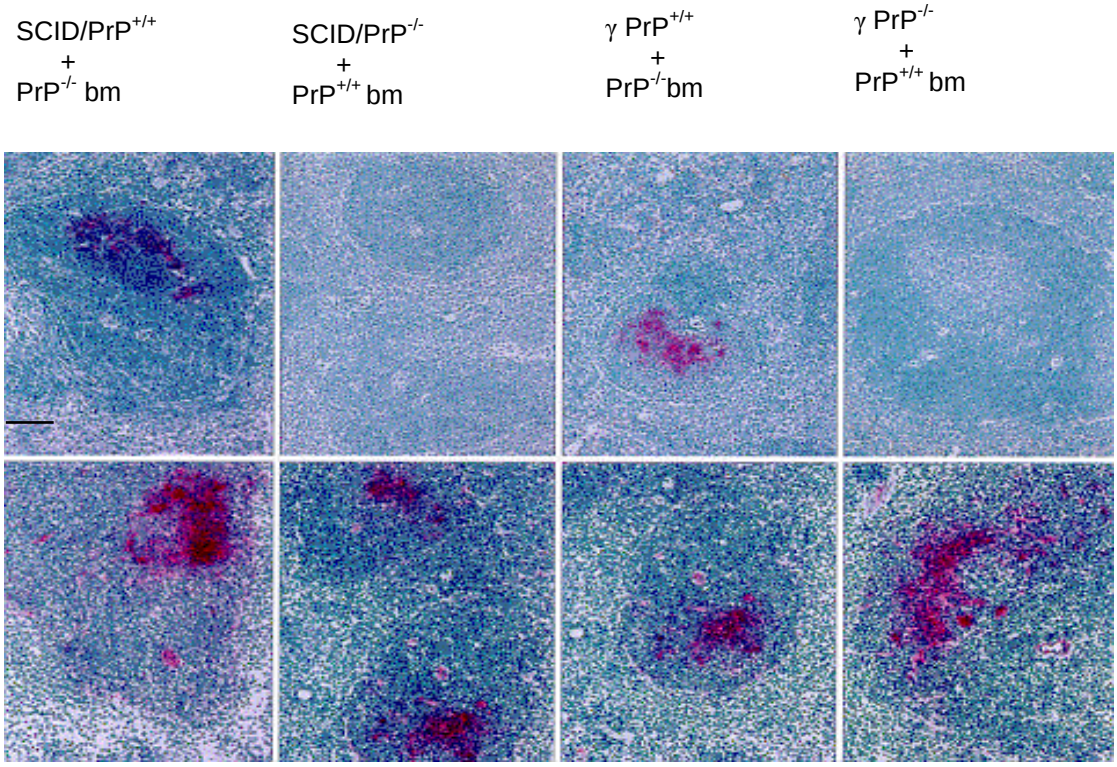


Figure 1.6. Immunolabelling for PrP (using the 1B3 antibody) or FDC networks in (using the FDC-M1 antibody) spleens from bone marrow reconstituted mice 10 weeks post ME7 infection. FDC networks are well developed in all spleens (lower panel) but PrP associated with FDCs (lower panel) is only present when the recipient of the graft carries a functional PrP gene. This labelling is more intense and granular than in uninfected spleen. Scale bar represents 100µm.From Brown *et al* 1999



1.5.4 The use of transgenic mice to determine the key cell types involved in TSE pathogenesis in lymphoid tissues.

Transgenic mice lacking specific cell types have been used to further explain the role of specific cell types in the peripheral pathogenesis of TSEs. The presence of the tumour necrosis factor α (TNF-α), which signals through TNF-receptor 1/p55 (TNF-R1) (Tkachuk et al, 1998) is essential for the maturation of FDCs. In studies with the RML strain disease pathogenesis was severely impaired in transgenic mice deficient in B lymphocytes (Klein et al, 1997; Klein, 1998), whereas transgenic mice lacking FDCs as a result of deficiencies in tumour necrosis factor signalling appeared to be fully susceptible to infection. As a result of these findings it was initially concluded that B cells were critical for TSE pathogenesis (Klein et al, 2001; Montrasio et al, 2000). There were a number of difficulties in interpreting the above studies; firstly, B cell deficient mice also lack mature FDCs and secondly; no information on the levels of infectivity in lymphoid tissues of the TNFR1$^{-/-}$ mice were given. Without knowing if the lymphoid tissues of the TNFR1$^{-/-}$ mice contained infectivity the possibility exists that neuroinvasion in these mice could have occurred in the absence of lymphoid tissue involvement as is known to happen in high dose infection of immunodeficient mice (Fraser et al, 1996). In studies carried out with the ME7 strain of scrapie however, TNF-α deficient mice, were almost resistant to peripheral infection and did not have detectable levels of infectivity in spleen (Mabbott et al, 2000b).

1.5.5 Effects on disrupted lymphotoxin signalling on peripheral TSE pathogenesis following the temporary inactivation of FDCs

Tumor necrosis factor (TNF) family members such as TNFα and lymphotoxin are important in the development and maintenance of follicular dendritic cell function via membrane bound lymphotoxin (LTα/β), whose signal is mediated through the LTβ receptor (LTβR) (Endres et al, 1999). The lymphotoxin signalling pathway can be disrupted via the administration of LT beta R-Ig (Tumanov et al, 2003). This results in the de-differentiation of FDCs within 72hours, an effect which is maintained for approximately 28 days. This technique has been used in a number of studies providing a unique opportunity to examine the role of FDCs at specific points during peripheral infection.

When LTβR signalling was disrupted, via a single dose of LTβR-Ig, prior to, or up until 42 days post intraperitoneal infection with the ME7 strain; incubation period of disease was significantly extended (Mabbott et al, 2000a) (Mabbott et al, 2003). Moreover, accumulation of infectivity and PrP^{Sc} in spleen was also impaired by this treatment. Similar results were also seen with the RML TSE strain (Montrasio et al, 2000). Treatment with LTβR-Ig prior to oral ME7 scrapie infection blocked PrP^{Sc} accumulation in Peyer's patches and mesenteric lymph nodes and prevented neuroinvasion (Mabbott et al, 2003). However, treatment 14 days after oral inoculation did not alter incubation period or susceptibility to infection, suggesting that neuroinvasion may have already occurred by this time (Mabbott et al, 2003).

1.5.6 Effect of complement depletion on TSE pathogenesis

The Nobel prize was awarded in 1919 to Jules Bordet for his discovery of the complement system. The complement system is a complex enzyme cascade made up of numerous serum glycoproteins that normally exist in an in-active, proenzyme form (Carroll, 2004). These proteins circulate in an inactive form, but in response to the recognition of molecular components of microorganisms, one of two pathways is activated to respond to the invading micro-organism (Nizet, 2007). These pathways comprise the classical and alternative pathway. The classical pathway is triggered by activation of the C1-complex either by antibody binding of C1q to IgM or IgG or by directly binding C1q to the surface of the pathogen. The central component of both the complement system is C3. C3 is an abundant serum protein which contains an unusual internal thiolester bond. In native C3 this bond is stable but this thiolester bond can become highly reactive as a result of conformational changes in the C3 protein structure. Generally the activation of C3 comes about as a result of proteolytic cleavage of the C3 molecule into 2 biologically active fragments. Initiation of the alternative pathway relies on the generation of an activated C3 molecule. There are various ways in this initiating activated C3 can be generated. These include proteolysis by enzymes derived from bacteria themselves and, via blood clotting enzymes, injury. However activation of C3 can occur spontaneously.

One of the most characteristic features of FDCs is their ability to trap and retain antigen for extended periods of time (Humphrey et al, 1984) (Kosco-Vilbois et al, 1997; Kosco et al, 1992). This trapping of immune complexes can be mediated via the complement system or via other receptors, such as Fc receptors which are

expressed on the surface of the FDC. However, depletion of Fc-gamma receptors or circulating immunoglobulins had no effect on the peripheral pathogenesis of the RML scrapie isolate (Klein et al, 2001). However the effects of complement depletion on the peripheral pathogenesis of both the RML and ME7 scrapie agents are striking (Klein et al, 2001; Mabbott et al, 2001). In mice temporarily depleted of complement component C3 via the administration of cobra venom factor; incubation period of disease was significantly extended in comparison with control mice (Mabbott et al, 2001). Moreover incubation period was also extended in transgenic mice with deletions in C1q or with dual deletions of Factor B and C2 (Klein et al, 2001). The most significant prolongation of incubation period was seen in the C1q deficient mice (Klein et al, 2001; Mabbott et al, 2001). The results of these studies suggest that the activation of specific complement components may be involved in the initial trapping of TSE in lymphoreticular organs early after infection. Further studies investigating the effect of complement C5 deficiency on pathogenesis found no effect on incubation period of disease suggesting the functional aspects of C5 do not impact on TSE pathogenesis (Mabbott & Bruce, 2004).

1.6 Transport of infectivity and neuroinvasion

1.6.1 Transport of infectivity from sites of infection to peripheral lymphoid tissues

Although it appears that FDCs play a key role in accumulating or replicating infectivity within the germinal centres of the lymphoid follicles how TSE agents are transported to these cells is not fully understood.

Many TSE agents, including BSE, natural scrapie, CWD and vCJD, are acquired via oral exposure (most probably ingestion) to infectious agents. Other potential routes of TSE infection in natural situations may include infection via the skin, for example in transmission of kuru; skin scarification in experimental models of infection is an efficient method of transmission of disease (Taylor et al, 1996).

In the gastrointestinal tract a number of cell-types; including dendritic cells (DC), have been postulated as potential candidate cells for the transport of infectivity (Huang et al, 2002; Huang & MacPherson, 2004; Raymond & Mabbott, 2007). Migratory DCs differ from FDCs in their haematopoietic origin and their mobility, while FDCs reside within the germinal centre and are thought to be of a stromal rather than bone marrow origin.

There have been a number of studies investigating the possible role of conventional DCs in transportation of TSE agent infectivity. In vitro studies suggest that the PrP fragment 106–126 can act as a chemoattractant for monocyte-derived DCs (Le et al, 2001). DCs have been shown to acquire PrP^{Sc} *in vitro* and a sub-population of migrating DCs can acquire and transport PrP^{Sc} from the gut lumen through the lymphatics to lymphoid tissue (Huang et al, 2002; Huang & MacPherson, 2004). In these studies the uptake of PrP^{Sc} from the gut lumen was restricted to DCs and not detected in other lymph cells or cell-free lymph (Huang et al, 2002). Interestingly pathogenesis studies investigating the role of DCs in transmission of TSE agent infectivity demonstrated contrasting findings. In one study the presence of infected $CD11c^{+}$ splenic DCs alone resulted in neuroinvasion when they were injected

systemically into immunodeficient RAG-1 mice (Aucouturier et al, 2001). However, it is possible that the high dose of TSE agent used in these studies may have bypassed the need for lymphoreticular system involvement, as is known to occur in SCID mice (Fraser et al, 1996). However in a more recent study, (Raymond & Mabbott, 2007) $CD11c^{+}$ splenic DCs were unable to efficiently transmit TSE disease directly to the peripheral nervous system (PNS) of FDC-deficient $TNFR1^{-/-}$mice (Raymond & Mabbott, 2007). These findings differ significantly from the study described above (Aucouturier et al, 2001). In another study, migration of dendritic cells into the brain was demonstrated following infection with a mouse-adapted GSS strain was detected (Rosicarelli et al, 2005). More recently studies in mice with alterations in DC migration and tissue localisation found some minor effects on the transmission of infectivity via subcutaneous but not oral routes. These studies however did appear to find evidence that DCs may contribute to neuroinvasion as injection of infected live DCs produced shorter incubation periods than identical studies using dead DCs (Levavasseur et al, 2007)

In the skin, Langheran cells (LC) have been postulated as potential candidates for the transport of TSE infectivity to the lymphoid tissues. LCs play an important role in the immune defences of the skin where they trap antigen and transport it to the skin-draining lymphoid organs, where the recruitment of antigen-specific T cells occurs. LCs require specific stimulation for their migration in sites in the skin to the draining lymph node. One such pathway is the CD40-CD40L signalling pathway which is involved in the regulation of this process. Studies in transgenic mice deficient in CD40 ligand found that transmission of infectivity via the skin was unaffected in

these mice, and was in-fact significantly shorter than wild-type mice (Mohan et al, 2005a). Results of these studies show that neuroinvasion via the skin was not dependent on migratory LCs and the fact that LCs can degrade PrP^{Sc} in-vitro may suggest that LCs may contribute to the "clearing" of infectivity (Mohan et al, 2005b) following infection in a similar way to macrophages (Beringue et al, 2000). Moreover a role for conventional DCs in TSE agent pathogenesis has been described as disease pathogenesis is impaired in mice where splenic DCs were depleted (Raymond et al, 2007).

1.6.2 Mechanisms of neural spread from the peripheral lymphoid system to the CNS

Understanding how TSE agents infect the brain (neuroinvasion) has been of critical importance as determining the key events in this process may allow the development of therapeutic approaches to these diseases. The vast majority of experimental TSE strains can be transmitted to rodents via peripheral routes which have provided opportunities to determine the mechanisms of transport and spread of infectivity.

Initial studies investigating the neural spread of the 139A strain following intraperitoneal, intravenous or subcutaneous infection found that there appeared to be a common neuroinvasive pathway from visceral lymph nodes and spleen along visceral autonomic nerves to the mid thoracic cord (Kimberlin et al, 1987; Kimberlin & Walker, 1989). Once replication of infectivity was initiated in the thoracic cord there was a gradual spread of infection throughout the CNS. In some models, for example the 263K strain in hamsters, neuroinvasion appears to occur very rapidly,

with extremely short incubation periods of approximately 65days. In this model splenectomy has no effect on the incubation period of disease following intraperitoneal infection suggesting that the spleen is not critical for neuroinvasion in this model (Kimberlin & Walker, 1986).

Although it is accepted that the spread of infection from peripheral sites to the brain occurs primarily along neural routes some studies have suggested that haematogenous spread may also occur in TSE infection. This has been primarily demonstrated in the 263K hamster model where infectivity in blood could be detected for at least 40 days after intraperitoneal infection (Diringer, 1984)

In more recent studies mapping of the deposition of PrP^{Sc} in hamsters following oral 263K infection found that PrP^{Sc} accumulated, in a defined temporal sequence, in sites that accurately reflected known autonomic and sensory relays. This data confirmed that the infectious agent primarily uses synaptically linked autonomic ganglia and efferent fibers of the vagus and splanchnic nerves to invade initial target sites in the brain and spinal cord (Beekes et al, 1996; Beekes et al, 1998) (Beekes & McBride, 2000) (McBride et al, 2001).

1.7. The influence of the immune system on TSE pathogenesis

1.7.1 The host response to TSE infection

Previous studies carried out many years ago failed to find evidence of a classical immune response to scrapie infection (Chandler, 1959; Clarke, 1968; Clarke & Haig, 1966; Pattison et al, 1964). One hypothesis for this phenomenon was that infection with the scrapie agent resulted in immunosupression, although little evidence has

been found to support this (Clarke, 1968; Gardiner & Marucci, 1969; Garfin et al, 1978a; Garfin et al, 1978b).

Although there is no evidence for a classical immune response to TSE infection (Chandler, 1959; Clarke & Haig, 1966), studies investigating immunoglobulin (Ig) levels in sheep from flocks with a high incidence of natural scrapie found that these were greatly elevated during the clinical phase of disease (Collis et al, 1979). Further studies in sheep experimentally infected with the SSBP/1 strain of scrapie found similar results (Collis & Kimberlin, 1983) and demonstrated that IgG, specifically IgG2 was the major Ig sub-class present in the sheep sera. At the time of these studies very little was known about the importance of the peripheral lymphoid system, or of cells such as FDCs in TSE pathogenesis. It may be that these alterations in Ig levels are the result of a TSE induced immune system dysfunction, perhaps in cells such as FDCs, which are involved in the regulation of immune responses, such affinity selection of B cells. However, it has been suggested that the increase in immunoglobulin levels in sheep with natural scrapie is a result of pruritus leading to infection and the generation of an immune response (Nora Hunter personal communication)

1.7.2. Effects of age on pathogenesis

Early studies on the influence of age on TSE pathogenesis found that neonatal mice were partially resistant to peripheral infection with doses of infectivity that normally produced disease in adult mice with 100% efficiency (Ierna et al, 2006 ; Outram et al, 1973). In addition, incubation periods in those neonatal mice that did develop

disease had a wider incubation period range than those of the older animals. Susceptibility to scrapie was restored approximately one week after birth (Outram et al, 1973). In more recent studies this effect on pathogenesis was again observed in neonatal mice with the increase in susceptibility with age correlated with the first immunohistochemical detection of PrP^c, on maturing follicular dendritic cell networks (Ierna et al, 2006) (table 1.3)

Table 1.3. Neonatal mice are less susceptible to peripheral TSE challenge

Age at time of inoculation	Susceptibility to infection
0-1day	approximately 33%
10 days	100%

Extracted from Outram *et al* 1973 and Ierna *et al* 2006

Interestingly, lymphoid tissues are more mature at birth in sheep, cattle and humans than in mice, suggesting that in species *in utero* infection with scrapie-like agents is theoretically possible in these species. The effect of age appears to be a widespread phenomenon and includes BSE in cattle and natural scrapie of sheep. Epidemiological evidence indicates that most cattle developing BSE were infected as calves (Arnold & Wilesmith, 2004). Similarly, it has been suggested that young lambs are more susceptible than adults to natural infection with scrapie (Elsen et al, 1999). In addition, since vCJD was first identified, clinical disease has occurred predominantly in young adults, suggesting that age-related factors and/or levels of

exposure to BSE may influence susceptibility. In mammals it is widely accepted that ageing leads to a gradual decline in immune function (Koch et al, 2006) and the term "immunosenescence" can be used to describe these collective alterations in immune function. Ageing leads to increased rates of mortality in response to infection; for example, pneumonia is more prevalent in the elderly than in younger adults (El-Solh et al, 2001). The effects of age on immune function can also be demonstrated in responses to vaccination which are notably less effective in the old. Aged humans are also more susceptible to cytomegalovirus (Koch et al, 2006) and influenza infection. In addition re-current infections such as *Varicella zoster* (Laube, 2004) are more prevalent in aged individuals. Ageing is also associated with increased rates of some cancers which is thought to be linked to immune system dysfunction (Shurin et al, 2007). As cases of vCJD have occurred almost exclusively in young adults it is possible that the decline in immune and in FDC function in older individuals may provide some explanation for the incidence of clinical vCJD in young adults. The potential influences of old age (senescence) on TSE pathogenesis will be discussed in detail in chapter 5.

Thesis Aims

Chapter 3. To further elucidate the role of follicular dendritic cells (FDCs) in the peripheral pathogenesis of the ME7 strain of scrapie.

Chapter 4. To determine the cell type or types in the peripheral lymphoid system that are important for the peripheral pathogenesis of a range of experimental TSE strains.

Chapter 5. To determine the influence of an ageing immune system, using senescent mice, on the peripheral pathogenesis of the ME7 strain of scrapie.

Chapter 6. To determine if the generation of a humoral immune response alters or influences the peripheral pathogenesis of the ME7 scrapie strain.

Chapter 2

Materials and Methods

2.1. Mouse strains

A range of inbred mouse strains were used in these studies. Mouse lines were maintained in a conventional animal facility with a 12 hour (h) light and 12 h dark cycle under specific pathogen free conditions at the Neuropathogenesis unit (NPU) with access to food and water at all times. In some cases immunodeficient mouse lines were maintained in positive pressure isolators or in individually ventilated cages (IVC).

Mice were normally used between 6 and 8 weeks of age and individual experiments were age-matched. In some studies aged mice were used as part of the experimental design.

All mice used in these studies were individually identified from ear punches which relate to individual mouse records. All experiments were conducted under an appropriate home office project licence (PPL 60/2528, PPL 60/3324 held by Karen Brown) within the regulations of the Animals (Scientific Procedures) Act 1986. All individual experiments were scrutinised by the Neuropathogenesis Unit's ethical review process prior to commencement.

2.1.1 Immunocompetent wild-type mouse strains

C57BL/Dk

This mouse strain has been extensively used at the Neuropathogenesis Unit (NPU), Edinburgh for nearly 40 years. The line C57BL/Dk, is a sub-strain of C57BL and carries the a allele of the PrP gene (*Prnp*). The C57BL/Dk line, hereafter referred to as C57BL has been used extensively in TSE transmission studies and as indicator mice in incubation period bioassays to determine scrapie infectivity titres.

129/Ola and PrP deficient lines of mice

The 129/Ola mouse strain was used as a background strain for the generation of *Prnp*$^{-/-}$ mice. The PrP deficient mouse model (*Prnp*$^{-/-}$ mice) was generated on the 129/Ola mouse background by insertion of a neomycin resistance cassette into exon 3 of the *Prnp* gene (Manson et al, 1994a).

C57BL/6

C57BL/6 is a widely used inbred strain. In these studies this line was used as experimental controls for the TNF receptor 1 deficient (TNFR1$^{-/-}$) (Rothe et al, 1993) and the B cell deficient (μMT) (Kitamura et al, 1991) mice as these transgenic lines were produced on this genetic background.

CB20 (CB20$^{+/+}$ congenic BALB/c)

CB20 mice were obtained as a kind gift from Professor John Ansell, Edinburgh University. These mice were used as experimental controls for the severe combined immunodeficient (SCID) mouse line in our TSE transmission studies.

2.1.2 Immunodeficient mouse lines: SCID, SCID-*Prnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ mice

SCID (BALB/c CB17 *scid/scid*)

SCID mice have an autosomal recessive mutation (scid) which is located on chromosome 16. Mice carrying this mutation fail to produce detectable immunoglobulin as a result of impaired B and T cell production and are severely immunodeficient (Bosma et al, 1983). SCID mice were obtained as a kind gift from Professor John Ansell, University of Edinburgh.

SCID-*Prnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ mice

The SCID-*Prnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ mouse lines were originally produced as described by Brown *et al* (Brown et al, 1999), by crossing SCID mice (C.B-17 *scid/scid*, MHC type H-2^{d}) with *Prnp*-deficient mice (129/Ola *Prnp*$^{-/-}$ mice, MHC type H-2^{b}) (Manson et al, 1994a). Full details are provided in appendix I.

2.1.3 Genetically altered immunodeficient mouse lines: µMT and TNF-R1$^{-/-}$

The µMT mouse line (Kitamura et al, 1991), which lacks mature B lymphocytes, and as a consequence follicular dendritic cells, was obtained as a kind gift from Imperial College London. This mouse line was originally produced by gene targeting in mouse embryonic stem cells by disrupting membrane exons of the gene encoding the mu-chain constant region (Kitamura et al, 1991). Tumour necrosis factor receptor 1 deficient mice (TNFR1$^{-/-}$) were obtained from Imperial College London, UK. This mouse line was originally produced by gene targeting in embryonic stem cells (Rothe et al, 1993) Mice deficient in TNFR1 fail to develop mature FDC networks although affinity maturation of lymphocytes, in the absence of germinal centre development, still occurs.

2.1.4 Husbandry of mice with immunodeficiencies

Breeding of SCID mice was carried out in positive pressure isolators to minimise the risk of infection to the breeding stock. Experimental mice were maintained in open housing or in IVC cages. In the case of the SCID-*Prnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ mouse lines breeding was initially performed in open housing, however mice were highly susceptible to intercurrent infections. To counteract this problem breeding pairs were

reconstituted with histocompatible bone marrow from 129/Ola mice via intravenous (i.v.) injection.

2.2 Generation of SCID-*Prnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ mouse lines

2.2.1 Breeding strategies for selection of the SCID-*Prnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ mouse lines

To produce the SCID-*Prnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ mouse lines, SCID mice were crossed with PrP deficient mice. The resulting progeny were crossed together (F1 cross) and mice from the subsequent F2 generation selected for the SCID phenotype, MHC type H-2^{b} and either the *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ genotype.

2.2.2 ELISA for the detection of serum immunoglobulin

Progeny from the F2 generation (2.2.1) were analysed for the SCID phenotype by quantitating total serum immunoglobulin by ELISA. Mice were bled from the tail under halothane anaesthesia. A portion of tail was removed using a sterile scalpel and 50-100µl of blood removed for serum production. Tails were cleaned and sealed using a veterinary sealant "vet-seal" (Jorgensen Laboratories, Inc). Tail tips from each animal were retained for genotype analysis. Serum was produced by allowing blood samples to remain at room temperature for 30 minutes, followed by storage at 4º C for approximately 24 hours. Serum was removed and stored at -40ºC until required. For detection of immunoglobulin levels sera was diluted (1/1000) in carbonate bicarbonate buffer (0.5 M, pH 5: Sigma) and adsorbed onto flat bottomed microwell plates by overnight incubation at 4°C. Additional positive (CB20 serum)

and negative (SCID serum) control samples were also adsorbed onto the plates. Following incubation, plates were blocked with 0.01 M PBS (pH 7.5) containing 1 % bovine serum albumin (BSA). Immunoglobulin was detected using a horseradish peroxidase conjugated rabbit anti-mouse Ig (DAKO, Cambridgeshire, UK) diluted 1/1000 in PBS. Plates were incubated for 3 h at room temperature and washed 3 times. Bound peroxidase activity was measured by incubation with o-phenylenediamine as a substrate (Sigma, UK), stopping the reaction with 3N HCL. Optical density (OD) was measured at 490 nm using a V-max kinetic microplate reader (Molecular devices, CA, USA).

2.2.3 Flow cytometry and selection of H-2 type

Mice identified as having a SCID phenotype were bled from the tail as described in 2.2.2 and 150-250µl of blood collected into heparinised tubes to prevent clotting. Blood samples were pelleted by centrifugation and red cell lysis performed by subjecting the cells to distilled water shock for 10 seconds, stopping the reaction with an equal volume of a balanced salts solution. Cell suspensions were labelled with antibodies directed against mouse H-2D^{b} or H-2D^{d} histocompatibilty types (BD Biosciences, UK) as described in appendix II. Animals identified as having a histocompatibility type H-2D^{b} were genotyped as described below. Animals with a histocompatibility type H-2D^{bd} or H-2D^{d} were humanely sacrificed.

2.2. 4 Southern blot analysis for selection of P*rnp* genotype

Mice identified as having both a SCID phenotype and histocompatibility type H-2D^{b} were subjected to genotype analysis by Southern blot analysis (appendix I).

Previously collected tail samples were used to prevent further procedures. Mice with a *Prnp*$^{+/+}$ genotype were designated SCID-PrP$^{+/+}$ and mice with a *Prnp*$^{-/-}$ genotype were designated SCID-PrP$^{-/-}$. Breeding pairs of each strain were constructed and lines of inbred mice of each strain produced for experimental studies

2.3 Production and characterisation of chimaeric mouse models

2.3.1 Whole body gamma irradiation.

For the production of bone marrow chimaeras in immunocompetent mice lethal doses of ionising radiation were used. The standard lethal dose for immunocompetent mice was 9.5 Grays (Gy). The time of exposure was calculated from previously established radiation dosimetry and adjusted accordingly for ½ life loss. Mice were placed (3/holding cage) on a rotating turntable in an irradiator (Gravatom Engineering Systems Ltd, UK) and exposed to ionising radiation from a Caesium^{137}source for the appropriate time. Mice were returned to sterile filter top cages with free access to food and water and the cages aseptically sealed to minimise the possibility of infection.

2.3.2 Bone-marrow reconstitution

Immunocompetent 129/Ola or PrP$^{-/-}$mice were lethally irradiated, as described above, approximately 18 h prior to bone marrow transfer. SCID-*Prnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ did not require irradiation prior to bone marrow challenge. Bone marrow from the femurs and tibias of adult 129/Ola or PrP$^{-/-}$ mice was prepared as a single cell suspension to an approximate concentration of 1-3 x10^{7}cells/ml in Hanks Balanced

Salts solution (HBSS, Invitrogen, UK). Recipient mice were warmed on a heat pad for approximately 30 minutes prior to cell transfer to ensure successful intravenous (i.v.) injection. Mice were anaesthetised with halothane and 0.1ml injected i.v. into the tail vein, if this was unsuccessful mice were injected intraperitoneally (i.p.). For the reconstitution of SCID mice with CB20 bone marrow the methods described above were used.

2.3.3 Maintenance and characterisation of bone marrow chimaeras

Following bone marrow transfer mice were maintained for a period of 28 days before commencing further experimental studies. This time period was shown to be essential for successful reconstitution (Brown et al, 1999) (Brown et al, 2000b). Irradiated mice were maintained in filter top cages and cleaned in a laminar flow cabinet to minimise the chances of infection. Bone marrow reconstituted immunodeficient mice were maintained in conventional open housing. To determine the success of the reconstitution immunodeficient mice were bled before and after bone marrow reconstitution (28 days post transfer), as described previously. Serum was produced to quantitate immunoglobulin levels. Further characterisation to establish the success of the reconstitution was carried out using flow cytometry.

2.4 TSE strains and experimental TSE challenge

2.4.1 Experimental TSE strains

In the majority of these studies the ME7 scrapie stain was used (Zlotnik & Rennie, 1965). This strain has been used extensively at NPU and has been well characterised. For the study of the peripheral lymphoid system this strain is particularly useful as it has extensive involvement of these tissues (Fraser et al, 1992b). These studies also used the 79A and 139A strains, which were derived from the "chandler" isolate as described in chapter one and chapter four (Chandler, 1961; Chandler, 1962; Dickinson et al, 1986). The BSE derived strain 301C was also used (Bruce et al, 1994a; Bruce et al, 2002). According to experimental design of the study uninfected control tissues, brain or spleen were used as control tissue homogenates for injection.

2.4.2 Routes of TSE agent inoculation

Intracerebral injection

Mice were anaesthetised with halothane and 20µl of tissue homogenate injected intracerebrally (through the mid-temporal cortex) using a 1ml syringe fitted with a 26 gauge (G) needle. Following recovery individually coded mice were returned to labelled cages (5-6 per cage). A range of TSE strains were used for intracerebral inoculation.

Oral dosing

For oral infection mice were housed individually in cages containing a single food pellet inoculated with 50µl of a 10% ME7 brain homogenate. Following successful

ingestion of the pellet mice were coded and returned to their holding cages for the duration of the study.

Intraperitoneal injection

For intraperitoneal (i.p.) infection with TSE agents or normal brain homogenates mice were injected into the abdomen with 20µl of tissue homogenate using a 1ml syringe fitted with a 26G needle. Anaesthetic was not normally used.

2.4.3 Clinical scoring of TSE and incubation period of disease

Following TSE challenge mice were monitored daily for general health status and assessed weekly using a previously established formal TSE clinical scoring system (Dickinson et al, 1968). In all studies mice were coded to avoid the introduction of experimental bias in the scoring system. Although there can be variation in clinical disease symptoms, depending on the combination of TSE strain and mouse strain, the characteristic signs of clinical TSE infection may include; lethargy, hyperactivity, ataxia, pruritus, gait effect and aggression. Non-specific symptoms in the animal may be observed for a few weeks prior to the development of definitive neurological signs which normally occur during the last 2-3 weeks of the incubation period.

Following formal scoring mice were coded as follows:

1: normal

2: "possibly affected": evidence of some abnormalities but not necessarily TSE

+: "definitely affected": animal has clinical TSE

g: animal has gait abnormality but not clinical TSE

G: animal has clinical TSE score and gait abnormality

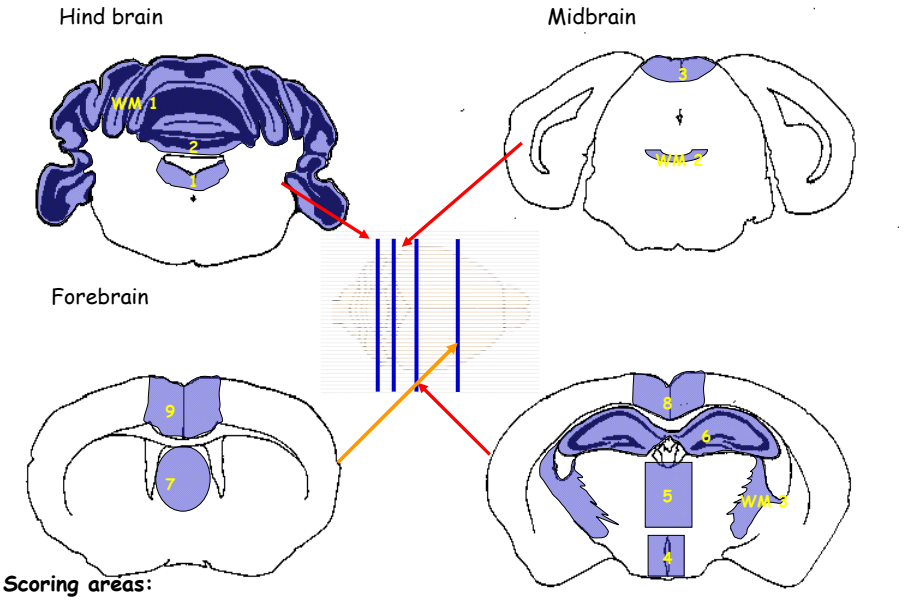
According to established criteria mice were culled when they received scores of "definitely affected" in two consecutive weeks, or in two out of three consecutive weeks. In cases where symptoms were severe, or the clinical period of disease was short, mice were culled following one "definitely affected" score to prevent unnecessary suffering. Once the clinical end-point was defined animals were humanely destroyed and tissues collected for histology, serial passage or further analysis. The incubation period for each mouse was defined as the time in days between challenge and the clinical end-point. Mice that did not develop TSE disease were maintained to a pre-determined time point consistent with the aims of the experiment

2.4.4 Histopathological diagnosis of TSE

Following clinical TSE diagnosis brains were fixed in 10% formal saline for approximately 48 h trimmed coronally into 5 defined brain areas and processed in a tissue processor (figure 2.1). In some cases brains were fixed in periodate-lysine paraformaldehyde solution for a period of 18 h. Brain areas were embedded in paraffin wax and 6μm sections cut on a microtome. After overnight drying in a 37ºC incubator sections were counterstained with haematoxylin and eosin and coverslipped with DPX mountant (BDH, UK.) For pathological confirmation of TSE, vacuolation in the brain was scored on a scale of 0-5 in the following grey-matter (G1-G9) and white-matter (W1-W3) areas (Fraser & Bruce, 1973): G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor

cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, Decoction of superior cerebellar peduncles; W3, cerebral peduncles (figure 2.1) (Fraser & Dickinson, 1967; Fraser & Dickinson, 1968).

Figure 2.1 Sectioning of brain and scoring areas for confirmation of positive TSE pathology as defined by Fraser and colleagues.



2.4.5 Infectivity measurement by bioassay

At various time points after challenge or at the terminal stage of disease mice were sacrificed and spleen taken to determine infectivity levels. For bioassay of scrapie infectivity in spleen individual, whole or half spleens were prepared as a 10 % (wt/vol) homogenate in physiological saline and 20µl injected i.c. into groups of up to 12 C57BL indicator mice. The titre of scrapie infectivity in each spleen was determined from the mean incubation period response curves for scrapie-infected spleen tissue (Dickinson & Fraser, 1969).

2.5 Immune system manipulation

2.5.1. Ageing mouse models

In all mammals ageing results in immune system defects termed "immunosenescence". To model the effects of ageing on TSE infection we used wild-type C57BL mice which were aged to 20 months under conventional open housed facilities. This is a well characterised wild-type mouse model of senescence and extensive studies have shown that mice of this age have characteristic defects in FDC function (Aydar et al, 2003). In studies involving aged mice additional mice were included to allow for age related infections etc. Typically 30% more mice were required in comparison with young animals.

2.5.2 Antigenic challenge of TSE infected mice:Sheep red blood cell (SRBC) immunisation

Mice were antigenically challenged at an early (6 weeks) or at a late (27 weeks), stage (27 weeks) following i.p. injection with 20 µl of an ME7 or normal brain homogenate.

Sheep red blood cells in Alsevers solution (E and O laboratories, UK) were used. Cell suspensions were washed 3 times in sterile saline for 10 minutes at 3000 rpm to remove diluent. Cells were re-suspended in saline and concentration adjusted to 1 x 10^8/ml. Suspensions were aliquoted in 5ml volumes and frozen at -70ºC until required. Prior to antigenic challenge mice were bled from the tail as previously described to establish baseline serum immunoglobulin levels. ME7 challenged or normal brain injected mice received an i.p. injection containing 1x 10^7 SRBC in 0.1ml of saline. Additional ME7 or normal brain injected mice were injected intraperitoneally with saline. Mice were boosted with SRBC or saline 3 weeks after the primary challenge.

2.5.3 Antigenic challenge of TSE infected mice with DNP-KLH.

Dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) (Calbiochem-Novabiochem Corp. La Jolla, CA, USA) was prepared for antigenic challenge by mixing equal volumes of DNP-KLH solution (5mg/ml: Calbiochem) with 9% potassium alum (Sigma A7167, Aluminium potassium sulphate). Solutions were agitated until the formation of a precipitate and an equal volume of medium (HBSS, Invitrogen, UK) added to stop the reaction. The pH was adjusted to pH7 by the addition of IM NaOH

and the solution washed four times by centrifugation in phosphate buffered saline (PBS). The concentration of DNP-KLH was adjusted to 1 mg/ml. For antigenic challenge mice (ME7 or normal brain injected C57BL mice) were injected i.p. with 100 µg/100 µl DNP-KLH or an equivalent volume of saline at 3 timepoints following challenge (10 20, or 30 weeks). Mice were bled and boosted with antigen as described in 2.5.2. Estimation of mouse Ig levels were performed using the radial immunodiffusion assay (The Binding Site, Birmingham, UK) as previously described (Mancini et al, 1965).

2.6 Ex-vivo analysis of experimental tissues

2.6.1 Histological preparation of tissues and cells for analysis

Cryostat sectioning and fixation of cryostat sections

Spleens were snap frozen in liquid nitrogen and stored in liquid nitrogen until required. Tissues were embedded in OCT medium (leica) and 5-20µm thick sections prepared using a cryotome (Reichart). Glass slides "Superfrost plus" with a permanent positive charge (to attract frozen tissue sections thus binding them to the slide) were used throughout (Microm, UK). Sections were air dried overnight and then used immediately or frozen at -40ºC for a maximum of 4 weeks. Cryostat sections were fixed in dried distilled acetone for 10 minutes and then air dried for a minimum of 30 minutes at room temperature. Fixed sections were used immediately.

Paraffin sectioning

Paraffin sections of brain and spleen were cut using a microtome (Leica) and sections floated on a water bath heated to 37ºC before collection onto "Superfrost plus" glass slides. Slides were incubated for up to 72 hours in a 37ºC oven before being used for histopathological or immunohistochemical (IHC) studies.

Vibratome Sectioning

Vibratome sectioning was used in some of the double labelling studies to construct serial images by confocal microscopy. In most cases, tissues for confocal microscopy were prepared by perfusion fixation using periodate-lysine-paraformaldehyde (PLP) (0.1 M periodate; 0.075M dl-Lysine; 2% paraformaldehyde in 0.05 M phosphate buffer pH, as previously described (Brown et al, 2000a). Perfusion fixation was used to improve tissue morphology by increasing efficacy of fixation and to reduce background labelling by the removal of red blood cells. Following perfusion (Brown et al, 2000a) tissues were immersion fixed in PLP for a period of 6 hours and sectioned. Where immersion fixation in PLP was used, tissues were fixed for a period of 18 hours. For sectioning, tissues were mounted onto plastic blocks and secured with "superglue". Tissues were placed in the microtome water bath containing 0.1M phosphate buffer and 100-200 μm sections cut. Sections were stored at 4º C for a maximum period of 24 hours and immunolabelled with a range of antibodies (Appendix II).

2.6.2 Immunohistochemical analysis of brain and lymphoid tissues for PrP^{Sc}

For the detection of PrP protein in brain, spleen or ileum, tissues were immersed in PLP fixative for approximately 18 h, processed in a tissue processor and embedded in paraffin wax. Sections (thickness, 6 μm) were de-waxed and pre-treated to enhance PrP immunostaining using a hydrated autoclaving technique by immersion in water and autoclaving on a standard sterilisation cycle (121°C for 15 minutes) (Yokoyama et al, 1996). Sections were immediately cooled in running water and subsequently immersed in formic acid (98%) for 5 min. Sections were then stained with the PrP-specific rabbit polyclonal antiserum 1B3 (Bruce et al, 1989b) (Appendix II) or the PrP-specific mouse monoclonal antiserum 6H4 (Oesch et al, 1985) Prionics, Zurich, Switzerland). All immunolabelling for the above antisera was carried out using alkaline phosphatase coupled to the avidin-biotin complex (Vector Laboratories). Vector Red (Vector Laboratories) was used as a substrate. All sections were counterstained with haematoxylin to distinguish cell nuclei.

2.6.3 Immunohistochemical analysis of frozen tissues

Spleen sections were fixed in acetone as described in 2.6.1. Sections were blocked appropriately and primary antibody applied at a pre-determined dilution. A range of primary antibodies and their specificity is described in appendix II. Following the addition of primary antibody, biotin conjugated species specific secondary antibodies was (Stratech Scientific, UK) applied. Bound antibody was detected using the alkaline phosphatase conjugated streptavidin ABC kit (DAKO) and vector red as the substrate (Vector Laboratories, UK). Sections were counterstained with haematoxylin as described previously. In some cases fluorescent microscopy was

used employing the use of a range of species specific secondary antibodies coupled to a range of fluorescent dyes, appendix II. Sections were mounted using fluorescent mounting medium (Dako, UK) and examined using a Zeiss LSM5 confocal microscope (Zeiss, UK).

2.6.4 Paraffin embedded tissue (PET) immunoblotting

This method was developed to allow the detection of PrP^{Sc} in paraffin sections (Schulz-Schaeffer et al, 2000) affording better morphology than can normally be obtained from conventional histoblot methods which use cryostat sections. For the detection of PrP^{Sc} in spleen and brain, tissues were fixed in PLP for approximately 18-24 h and embedded in paraffin wax following tissue processing. Serial sections (thickness 6 μm) were mounted on polyvinylidine difluoride membranes (PVDF, 0.45 μm-pore; Bio-Rad, Hemel, Hempstead, UK) and incubated for 24 hours in an incubator set at 55°C. PET blotting was carried out as previously described (Schulz-Schaeffer et al, 2000). Briefly, PVDF membranes were de-waxed and digested with 20μg/ml of proteinase K (10mM Tris-HCl pH 7.8, 100 mM NaCl and 0.1% Brij) for 16 h at 55°C. Membranes were then washed in TBS/Tween (10 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.5% Tween) before being denatured in 3M guanidine isothiocyanate (10 mM Tris-HCl pH 7.8) for 10 minutes. Membranes were blocked in 2% casein and PrP was detected with the PrP specific rabbit polyclonal antiserum 1B3 (Bruce et al, 1989b) (Appendix II) in blocking buffer for 2 h, followed by alkaline phosphatase-conjugated goat anti-rabbit antiserum (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA), and bound alkaline

phosphatase activity detected with SigmaFast™ NBT/BCIP solution (Sigma). PET blots were assessed using an Olympus dissecting microscope.

2.6.5 Quantification in lymphoid tissues

Quantification of FDC networks and germinal centres in were carried out microscopically by determining the amount and intensity of abnormal PrP accumulation and peanut agglutinin binding (PNA) in spleen. This was carried out in experiments investigating the effects of antigenic challenge on TSE pathogenesis. To carry out the quantification each section was captured using light or immunofluorescent microscopy and the area covered by the appropriate antibody marker measured using Image Pro Plus (Media Cybernetics, Wokingham, UK).

2.6.6 Preparation of cells for flow cytometry

Spleens were collected into FACs buffer (PBS pH 7.4 containing 0.1% BSA, 0.1% sodium azide and 0.02% EDTA) and dissociated by sieving through 100µm cell strainers (BD Biosciences, UK). Cell suspensions were then centrifuged at 1800rpm for 10 minutes and re-suspended in1ml of FACS buffer. Cells were counted using trypan blue exclusion and adjusted to a concentration of 1 $x10^6$ cells in each 50µl /test sample. For the preparation of lymphocytes from blood, red blood cells were firstly removed by subjecting heparinised blood samples to ammonium chloride lysis in 0.85% ammonium chloride lysis solution.

2.6.7 Antibody labelling and analysis of cells by flow cytometry

Cells were prepared as described in section 2.6.6. For cell labelling round bottomed 96 well plates (Nunc, Fisher Scientific, UK) were used and 50 µl of cells suspension containing 1 x10^6 cells was dispensed in each well. Cells were labelled with a range of antibodies: the directly conjugated antibodies B220 (CD45R), CD3 and CD4 antibodies (CALTAG med systems) and the H-2D^b and H-2D^d antibodies (Pharmingen, BD Biosciences). For the determination of PrP protein expression the 1B3 antibody was used (Appendix II) (Farquhar et al, 1989) All antibodies were titrated prior to use to determine the appropriate antibody titre. Cells were incubated with appropriately diluted antibodies for 40 minutes on ice and then washed 3 times with FACS buffer. Cells were re-suspended in 300 µl of FACS buffer and transferred to FACS analysis tubes (BD Biosciences, UK) for analysis. All samples were analysed immediately following labelling and flow cytometry conducted on a FACSCalibur™ (Becton Dickinson, San Jose, CA, USA) and data analysed using CELLQuest™ software (Becton Dickinson, San Jose, CA, USA).

Chapter 3

The involvement of follicular dendritic cells (FDCs) in the peripheral pathogenesis of the ME7 scrapie agent strain

3.1 Summary

TSEs are primarily considered neurodegenerative diseases where the major pathology is confined to the CNS. Although these diseases have devastating effects on the CNS, the lymphoid system plays a critical role in disease pathogenesis in many natural and experimental TSEs. The understanding of the critical role that the lymphoid system plays in pathogenesis is the result of research spanning several decades; primarily focusing on the role and involvement of specific lymphoid tissues system in TSE pathogenesis and then in the involvement of specific cell types within these tissues. The conclusion of much of this research points to a critical role for the follicular dendritic cell (FDC) a long-lived, radiation resistant cell. More recently, the use of chimaeric and immunodeficient mouse models has produced firm evidence for a pivotal role for FDCs in pathogenesis and in this chapter the role of FDCs is further investigated in immunodeficient and chimaeric mouse models using peripheral routes of infection.

In these studies novel lines of SCID mice were generated to allow the transplantation of haematopoietic cells from PrP deficient mice ($PrP^{-/-}$) mice permitting the development of resident PrP expressing FDCs. Peripheral infection of these models shows that neuroinvasion is dependent on PrP expression on FDCs but *not* on lymphocytes providing further evidence for a critical role for FDCs in pathogenesis. Further studies in radiation chimaeric mice also find that neuroinvasion is dependent on PrP expressing FDCs although these studies demonstrate an unexpected finding in that incubation periods in peripherally challenged mice irradiated and bone marrow grafted with PrP deficient bone marrow are significantly shortened in comparison with control mice.

3.2 Introduction

The hallmark of most TSE infections is neurodegeneration (Fraser & Dickinson, 1967; Fraser & Dickinson, 1968) (Bruce, 1981) encompassing widespread neuronal loss (Jeffrey et al, 1994b), gliosis and the deposition of PrP within the CNS (Bruce et al, 1989b); (Bruce et al, 1994b). However, the peripheral lymphoid system is targeted many weeks or months before the detection of infectivity or the appearance of pathological changes in the CNS (Kimberlin & Walker, 1979; Kimberlin & Walker, 1989). This involvement of the immune or lymphoid system in this "silent" phase, before clinical symptoms of disease become apparent highlights the importance of defining the events and cells involved as it is at this stage that therapies may be most useful. The focus of much research has been in the determination of the key cells involved in TSE pathogenesis and in the contribution of a fully functional immune system in TSE pathogenesis (Fraser et al, 1992b).

Following infection by peripheral routes, replication of infectivity in lymphoid tissues, precedes that of the CNS and reaches a plateau level, which is maintained for the duration of the disease. The time taken for infectivity to reach a plateau in lymphoid tissues is dependent on the route of infection (Kimberlin & Walker, 1979) and strain of TSE used (Bruce, 1985). In some experimental TSE models, for example the 79A scrapie agent strain, a very rapid replication phase in lymphoid tissues occurs following peripheral infection (Fraser, 1996), however in other strains, such as ME7 scrapie agent strain, replication of infectivity in lymphoid tissues rises less rapidly (Fraser, 1996).

Many studies have focused on defining the cell types which support replication in peripheral lymphoid tissues and most point to a central role for the follicular dendritic cell (FDC) (Brown et al, 1999; Mabbott et al, 2000a; Montrasio et al, 2000) Mabbott *et al*., 2000b; (Mabbott et al, 2002; Mabbott et al, 2000b; Mabbott et al, 2003), a long-lived radiation resistant cell found within the germinal centres of secondary lymphoid tissues. Within the germinal centre, FDCs interact with antigen activated B lymphocytes resulting in the generation of plasma cells and memory B cells. One of the major functions of FDCs is to trap and retain antigen, in the form of immune complexes, which are presented to B cells in order to generate and maintain immunological memory (Kosco et al, 1992). In contrast, some studies suggest that retention of immune complexes are not required for germinal-centre development or memory B-cell maintenance. Instead, it is thought that FDCs support B-cell proliferation and differentiation in a non-specific manner (Haberman & Shlomchik, 2003). Studies using ionising radiation found that lethal doses of radiation had no effect on the pathogenesis of disease or on the ability to replicate infectivity in spleen suggesting that a long-lived radiation resistant cell was involved (Fraser & Farquhar, 1987). One of the unique characteristics of FDCs is their resistance to doses of radiation exceeding those normally considered lethal to mice. In studies carried out by Humphrey and Grennan in 1982 the radio-resistance of FDCs has been shown to be as great as 18.5Gy and further studies in mouse models have substantiated these findings (Balogh et al, 2001).

Further evidence for the involvement of the FDC in peripheral scrapie pathogenesis came from the close association of PrP protein with FDCs in both normal and scrapie

infected mice (Brown et al, 2000a; McBride et al, 1992; Ritchie et al, 1999) and ultra structural studies of infected spleen demonstrate that abnormal PrP accumulates within the extracellular spaces between FDC processes (Jeffrey et al, 2000). In addition studies, studies investigating the co-localisation of PrP protein in both normal and scrapie infected spleen by confocal microscopy confirm that FDCs and PrP are co-localised (figure 3.1).

Figure 3.1 Accumulation of PrP protein in spleen

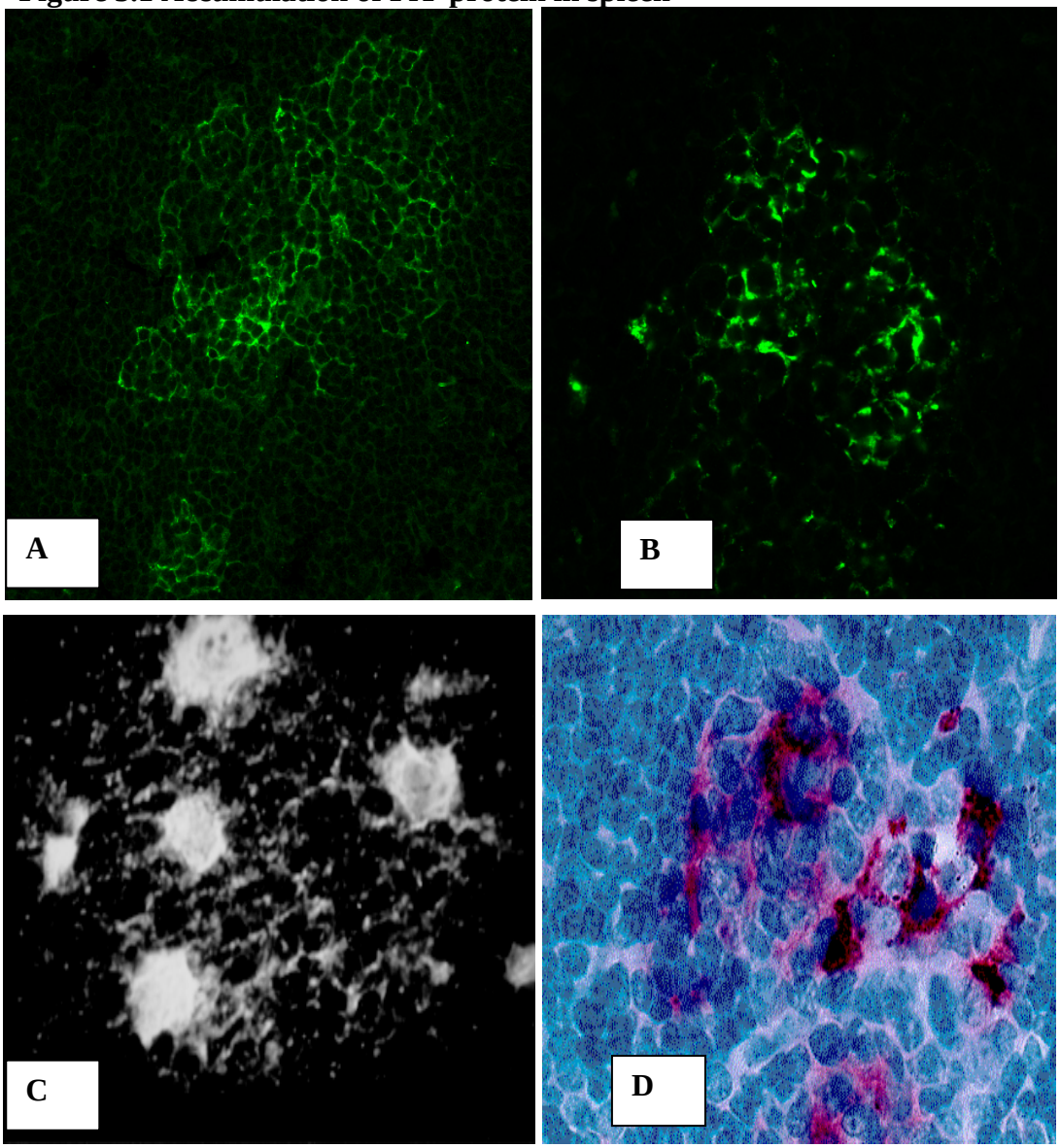


Figure 3.1 Immunohistochemical detection of PrP protein within the germinal centre in association with FDCs. All tissues were fixed in PLP fixative. Sections A-C are 100µm thick vibratome sections analysed by confocal microscopy, section (D) is a 6µm thick paraffin section All labelling performed using the 1B3 antibody (Farquhar et al, 1989) (A) Detection of PrP^c in spleen (green) from an uninfected C57BL mouse where robust, diffuse staining is extensively associated with the FDC network. (B) Detection of PrP (green) in spleen at 7 weeks post ME7 infection. At this stage dramatic differences in labelling can be observed with less diffuse labelling in the germinal centre and more "punctuate" forms which may represent accumulations of PrP within tingible body macrophages. (C-D) Similarly detection of PrP in terminally affected mouse spleen also appears in a more aggregated form within the germinal centre in association with the FDC network but also with cells that have the morphology of macrophages. Images are selected from the following publications (Brown et al, 2000a; Ritchie et al, 1999)

The use of mice with immunological defects, such as severely combined immunodeficient (SCID) mice (Bosma et al, 1983), has proved extremely useful in defining the importance of specific cell types in TSE pathogenesis. SCID mice have a mutation (scid) which results in the absence of mature B and T lymphocytes, and as a consequence, only immature FDCs are present (Kapasi et al, 1993; Kapasi et al, 1998). In 1983 a novel mutation in the C.B-17 inbred strain (BALB/c.C57BL/Ka-*Igh-1*^{b}/Icr N17F34) was described by Melvin Bosma. This finding was the result of a chance observation during a routine serum immunoglobulin quantitation study where mice were initially found to lack detectable IgM, IgG1, and IgG2a. This defect was demonstrated to be a heritable trait under the control of a recessive mutation (Bosma, et al., 1983). The mutant locus, designated *SCID* for severe combined immunodeficiency, has been mapped to mouse chromosome 16 (Bosma et al, 1983).

SCID mice are resistant to peripheral infection with BSE and experimental scrapie (Lasmezas et al, 1996; O'Rourke et al, 1994)Lasmezas *et al.*, 1996; Fraser *et al.*, 1996; (Brown et al, 1997b; Fraser et al, 1996). In addition, replication of infectivity in the spleen of SCID mice is impaired although this can be reversed following bone marrow reconstitution (Fraser et al, 1996), which allows FDC maturation and lymphoid reconstitution (Kapasi et al, 1993). Interestingly, the resistance to peripheral infection in SCID mice, and other immunological knockout mice (Brown et al unpublished, Chapter 4), can be overcome by the use of high doses of infectivity (Fraser et al, 1996), probably as the result of direct peripheral nerve infection as replication of infectivity does not occur in these spleens (Fraser et al, 1996). While

studies in SCID mice produced strong evidence for a critical role for FDCs in pathogenesis (Fraser et al, 1996), these studies were unable to exclude the involvement of lymphocytes in pathogenesis. A more conclusive role for FDCs in scrapie replication was provided by studies in chimaeric mice. In these studies murine bone marrow chimaeras were produced where mice carried the PrP gene, essential for disease replication, in FDCs but not in lymphocytes and *vice versa*. Using these models we found strong evidence that replication of the ME7 strain of scrapie, and progression of disease to the CNS depended on the presence of mature PrP expressing FDCs and was independent of the expression of PrP on lymphocytes (Brown et al, 2000b; Brown et al, 1999).

The involvement of the immune system in TSE infection highlights the importance of defining the key events and cell types involved in the peripheral lymphoid system prior to the infection of the CNS, as the use of therapeutic and preventative strategies may be more relevant in early infection. In these studies the involvement of the follicular dendritic cell in the pathogenesis of the ME7 scrapie agent strain is further investigated using immunodeficient and chimaeric mouse models. Two novel lines of SCID mice were produced to further investigate the role of follicular dendritic cells following peripheral (intraperitoneal) infection). In addition, the pathogenesis of the ME7 strain of scrapie in radiation chimaeras, where there is a mismatch between the PrP genotype of the FDCs and surrounding bone marrow derived cells, is discussed. A preliminary study investigating the effects of haematopoietic cell graft and TSE agent strain on the replication of agent infectivity in spleen of PrP deficient mice is also discussed in this chapter.

3.3 Results

3.3.1. Production of two new lines of SCID mice for use in studies investigating the peripheral pathogenesis of the ME7 scrapie agent.

The chance observation of impaired lymphocyte and consequently immunoglobulin production in an inbred line of C.B-17 mice led to the development of the SCID mouse line which has been used extensively in the study of the immune system. The ability to reconstitute this mouse model with bone marrow to stimulate the development of resident, FDC precursor cells, has produced unique opportunities to study the separate role of FDCs and lymphocytes with respect to PrP protein expression. In planning these studies however the major histocompatibility type of the mouse was considered to avoid rejection of the bone marrow graft. Although it is possible to graft xenogenic bone marrow into SCID mice the use of sub-lethal irradiation is normally required to destroy specific cell types that may result in rejection of the graft (Sakai et al, 1997). In these studies the aim was to primarily reconstitute the lymphoid but not the *myeloid* compartment (which is unaffected in SCID mice) without the requirement for irradiation. In-fact SCID mice have a radiation repair defect which makes these mice more susceptible to the effects of ionising radiation leading to subsequent difficulties in establishing required radiation dosage (Chang et al, 1993; Ogiu et al, 2002). Therefore it was critical to match H-2 haplotype of the bone marrow donor mice (129Ola/Prn-p$^{-/-}$ or 129Ola/Prn-p$^{+/+)}$ with the recipient. In mice the major histocompatibility type is defined by H-2. SCID mice carry the H-2^{d} haplotype whereas 129Ola/Prn-p$^{-/-}$ or 129Ola/Prn-p$^{+/+}$ mice are H-2^{b}. Therefore to allow reconstitution with PrP deficient bone marrow the production of

two new lines of SCID mice was achieved by crossing SCID mice with PrP deficient mice and selecting at the F2 generation; SCID phenotype, H-2^b type and $PrP^{+/+}$ or $PrP^{-/-}$ deficient genotype by Southern blot analysis**.** These mice were designated SCID-$PrP^{+/+}$ (Figure 3.2) or SCID-$PrP^{-/-}$ (not shown). Details of mouse line production are in Appendix I.

Figure 3.2 The SCID-PrP$^{+/+}$ and SCID-PrP$^{-/-}$ mouse lines

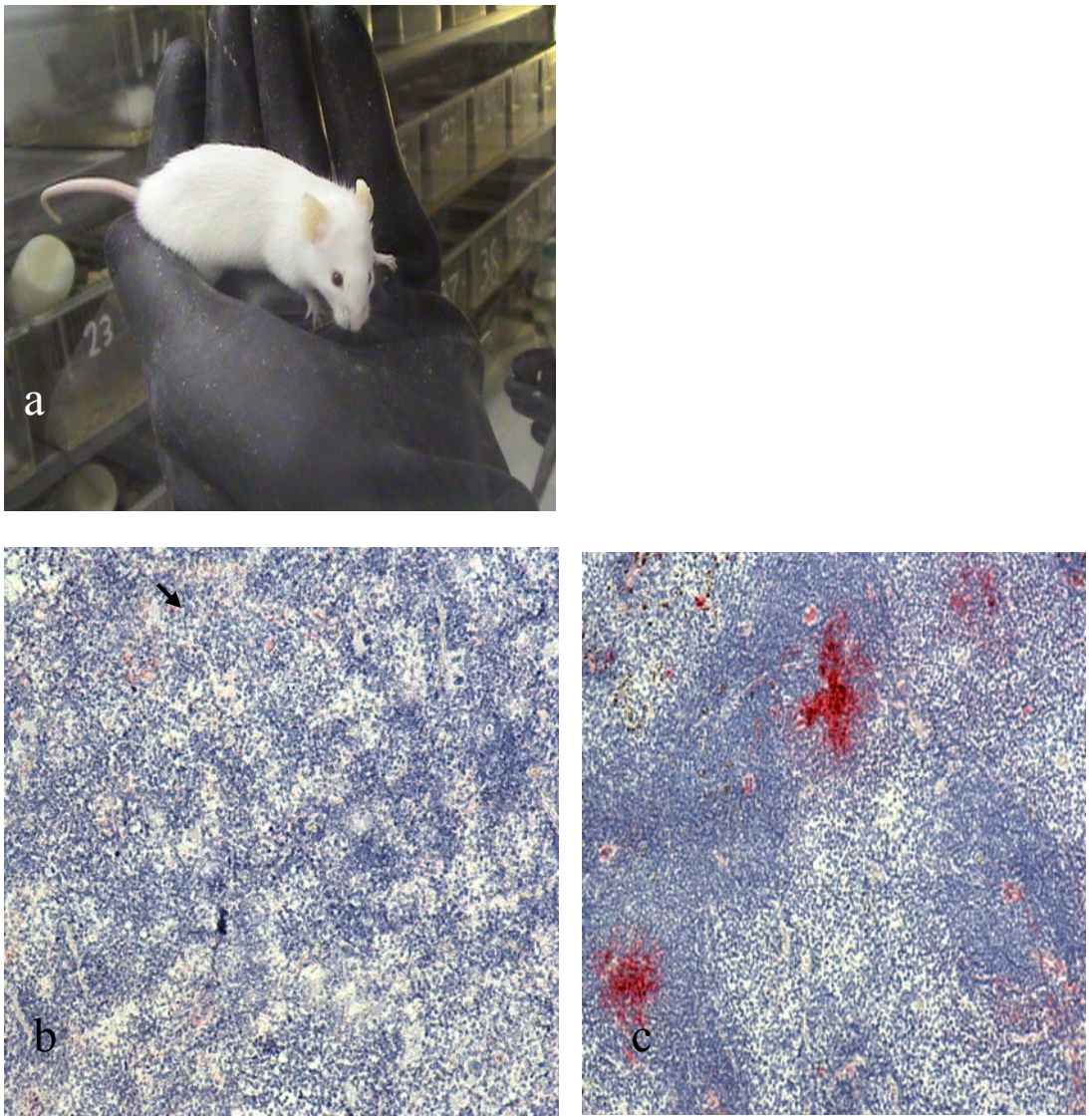


Figure 3.2 The SCID-PrP$^{+/+}$ and SCID-PrP$^{-/-}$ mouse lines

(a) Picture of a SCID-PrP$^{+/+}$ mouse. This line of mice is now maintained in germ-free conditions for breeding purposes. The SCID-PrP$^{-/-}$ line was not available to photograph as they are no longer maintained at our unit, but are available as embryos. (b) Cryostat section of SCID-PrP$^{-/-}$ spleen immunolabelled for FDCs using the FDC-M1 antibody (red). FDC networks are not visible but some labelling can be detected which is thought to represent immature FDCs (Ierna et al, 2006) (c) SCID-PrP$^{-/-}$ spleen reconstituted with 129Ola/Prn-p$^{+/+}$ bone marrow showing organised lymphoid follicles and extensive FDC network labelling. Spleen was collected 30 days post reconstitution. Sections counterstained with haematoxylin.

3.3.2 TSE disease following peripheral infection with the ME7 scrapie agent is not dependent on PrP expressing lymphocytes

The SCID phenotype is characterised by lack of immunoglobulin production as a result of B and T cell deficiency (Bosma et al, 1983). However, a small percentage of scid mice exhibit what is termed a "leaky" phenotype (Bosma et al, 1983; Bosma et al, 1988); the result of aberrant lymphocyte development resulting in the production of immunoglobulin. In published studies this defect is thought to occur in approximately 15% of young adult mice (Bosma et al, 1988) although data from our unit suggests the figure may be lower (unpublished observation). As the presence of this phenotype could have implications for bone marrow reconstitution of lymphoid populations (possibly affecting the interpretation of results) all SCID-$PrP^{+/+}$ mice were screened for "leakiness" by bleeding from the tail prior to bone marrow reconstitution or scrapie infection (Brown et al, 2000b). Of the 106 SCID-$PrP^{+/+}$ animals tested by ELISA for serum immunoglobulin (Ig), 6 had levels of Ig above baseline levels normally recognised for SCID mice and were excluded from the study. SCID$PrP^{+/+}$ mice were reconstituted with 129Ola/Prn-$p^{-/-}$ or 129Ola/Prn-$p^{+/+}$ (hereafter referred to in this chapter as $PrP^{-/-}$or $PrP^{+/+}$ respectively in this chapter) expressing bone marrow and maintained under standard husbandry conditions for a period of 28 days. Analysis of FDC and lymphocyte development following reconstitution previously demonstrated that this is the minimum time period for successful reconstitution (Brown et al, 2000b).

Bone marrow reconstituted and unreconstituted SCID$PrP^{+/+}$ mice were injected i.p. with a 1% ME7 brain homogenate (a dose of approximately 1x $10^{4.5}$ intracerebral

ID_{50} units). In addition, a group of C57BL mice were infected i.c. to act as a control on the source of inoculum. All of the C57BL mice infected mice developed disease within the expected range demonstrating high infectivity titre in the original inoculum. In the unreconstituted SCID-$PrP^{+/+}$ mice twenty-two mice died before the first positive TSE case in the control mice and were excluded from the analysis. The vast majority of these mice that died intercurrently were affected by thymic tumour a common occurrence in SCID mice (Ogiu et al, 2002). At the time of conducting these studies mice were maintained under standard SPF conditions which almost certainly contributed to intercurrent losses, although the development of thymic tumours may not have been prevented even if mice had been maintained under germ-free conditions. Of the remaining animals only 5 survived long enough to be included in the analysis, of these, one animal had confirmed TSE pathology the rest were pathologically negative. Although one animal developed disease this is not an unexpected finding. In previous studies peripheral infection of SCID mice using the same infectious dose generally results in disease in 10-15% of animals despite the absence of infectivity or the accumulation of abnormal PrP in lymphoid tissues (Fraser et al, 1996).

Strikingly, TSE disease occurred in 100% of the bone marrow reconstituted SCID-$PrP^{+/+}$ mice (table 3.1, figure 3.3), and no differences in incubation period were observed between the mice reconstituted with the PrP deficient or PrP expressing bone marrow. Although some intercurrent losses also occurred in the bone marrow reconstituted mice (mainly thymic tumour development), the incidence of disease in mice surviving past the first positive TSE case was 100% in both experimental

groups. These findings demonstrate that expression of PrP protein on lymphocytes is not required for neuroinvasion of the ME7 strain of scrapie and provide further evidence for a critical role for FDCs in pathogenesis. Immunohistochemical analysis of spleen sections from uninfected and scrapie infected spleen (not shown) demonstrate that the expression of PrP on FDCs is not dependent on PrP expression on lymphocytes (Figure 3.4).

Table 3.1 Disease incidence and incubation period (*or survival*) in SCID-PrP$^{+/+}$ mice following peripheral infection with ME7.

Mouse model	TSE incidence	Mean incubation (days)±SEM *or survival*
SCID-PrP$^{+/+}$	1/5	304 (*244,259, 286,303*)
SCID-PrP$^{+/+}$ + PrP$^{-/-}$ bm	15/15	322± 7
SCID-PrP$^{+/+}$ +PrP$^{+/+}$ bm	13/13	328± 7

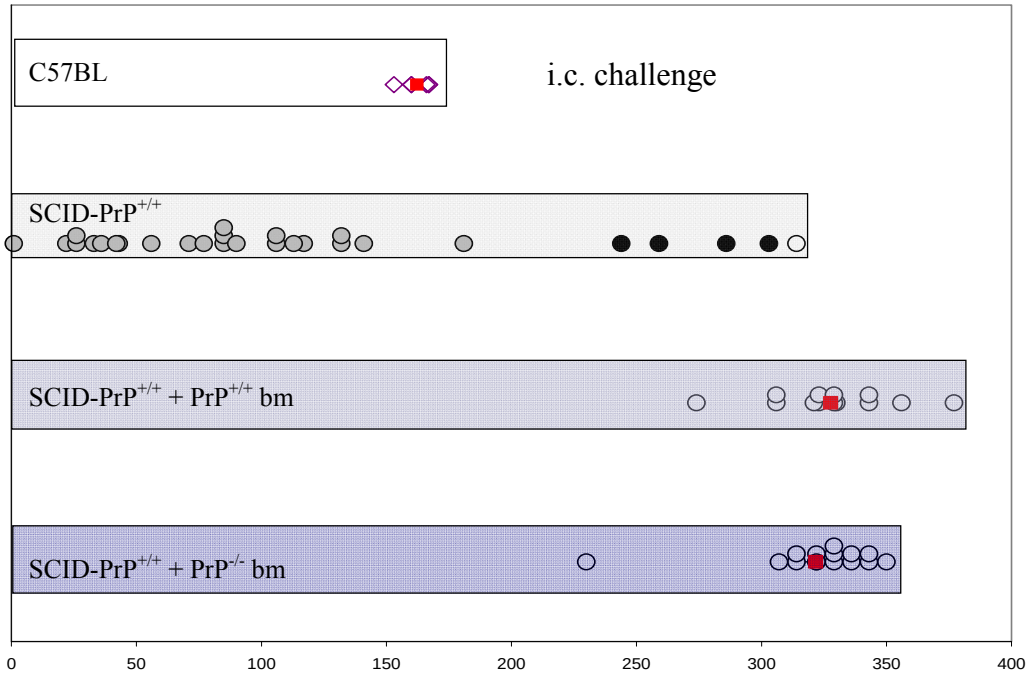


Figure 3.3 Disease pathogenesis in bone marrow reconstituted SCID-PrP$^{+/+}$ mice
Incubation periods of disease or survival following intraperitoneal challenge with 20µl of a 1% ME7 brain homogenate. Intracerebrally challenged mice were also included as a control on the infectious titre of the original inoculum. Open symbols represent mice with clinical and pathological signs of disease. Closed symbols (black) represent clinically and pathologically negative unreconstituted SCID-PrP$^{+/+}$ mice that survived to the endpoint of disease and included in the analysis. Closed symbols (grey) represent SCID-PrP$^{+/+}$ mice that died intercurrently but are not included in the analysis.

Figure 3.4 Expression of PrP protein on FDCs in uninfected SCID-PrP$^{+/+}$ mice is not dependent on PrP expression on lymphocytes

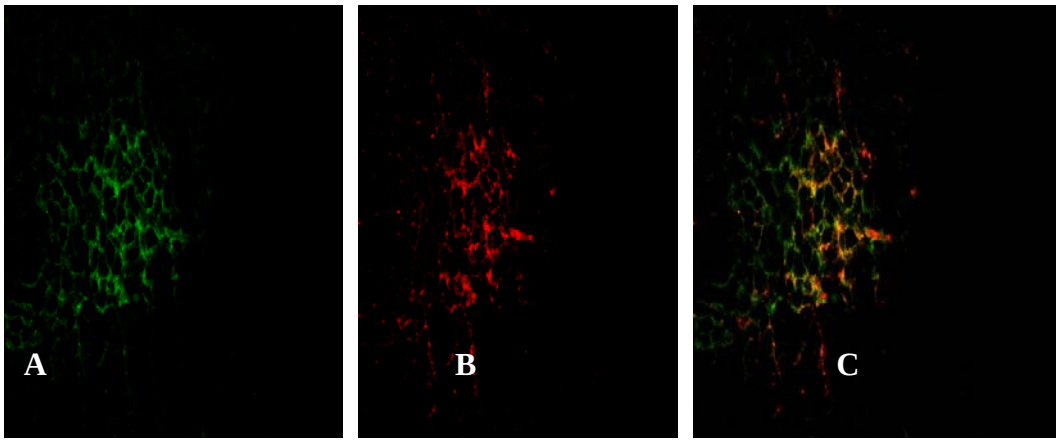


Figure 3.4 (a-c) Co-localisation of PrP protein (green) and FDCs (red) in spleen from uninfected SCID-PrP$^{+/+}$ mice reconstituted with PrP$^{-/-}$ bone marrow. Spleen collected 30 days post bone marrow reconstitution.Co- localisation of PrP in association with FDCs (orange/yellow) occurs independently of the expression of PrP on lymphocytes.

3.3.3 Disease pathogenesis is altered in irradiated mice with PrP chimaeric immune systems.

In mice, ionising radiation doses of approximately 950-1000 rads (9.5-10Gy) are considered lethal (1 Gy =100rads) and mice receiving such doses in the absence of haematopoietic cell reconstitution (bone marrow or fetal liver) invariably die within a short period of time (usually 7-10days) (Kretchmar & Congdon, 1967). However, one of the features of follicular dendritic cells is their resistance to radiation, surviving doses which are lethal to most dividing cells (such as lymphocytes) (Humphrey et al, 1984; Kinet-Denoël et al, 1982). This unique characteristic of FDCs provides opportunities to create chimaeric immune systems, using lethal irradiation and bone marrow reconstitution, where host FDCs differ from that of the haematopoietic cell graft. In previous studies these techniques were used to produce mice where the PrP status of the FDCs differed from that of the surrounding lymphocytes by grafting bone marrow from PrP deficient mice into PrP-expressing mice and *vice-versa*. In our previous studies we found strong evidence that PrP expressing follicular dendritic cells, and *not* bone marrow derived cells (lymphocytes or myeloid cells), were critical for TSE pathogenesis (Brown et al, 1999). In addition to providing strong evidence for a critical role for FDCs these previous studies produced a novel finding in that incubation periods in peripherally challenged 129Ola/Prn-$p^{+/+}$ mice reconstituted with 129Ola/Prn-$p^{-/-}$ bone marrow were significantly *shorter* than non-irradiated or 129Ola/Prn-$p^{+/+}$mice irradiated and grafted with isologous (129Ola/Prn-$p^{+/+}$) bone-marrow (Table 3.2). For the purpose of this chapter 129Ola/Prn-$p^{+/+}$ mice are denoted $PrP^{+/+}$ while 129Ola/Prn-$p^{-/-}$ mice are referred to as $PrP^{-/-}$.

To further investigate the role of FDCs in TSE pathogenesis and to determine if this unexpected alteration in pathogenesis could be repeated a second experiment was set up. Conditions of this study were kept as similar to that of the first experiment for example; inoculum from the same experimental source as that of the original study was used. In this second study almost identical results were obtained, and, as in the previous study mice reconstituted with $PrP^{-/-}$ bone marrow had significantly shorter incubation periods than mice that were not irradiated ($p= 0.04$). In-fact, incubation periods in the $PrP^{+/+}$ mice reconstituted with $PrP^{-/-}$ bone marrow were *also* significantly shorter than those of the $PrP^{+/+}$ mice irradiated and reconstituted with isologous ($PrP^{+/+}$) bone marrow ($p= 0.0025$). There was no significant difference in incubation periods between $PrP^{+/+}$ mice reconstituted with isologous bone marrow and unreconstituted $PrP^{+/+}$ mice suggesting that the effects on pathogenesis are not a consequence of the irradiation and reconstitution procedure but most probably related to the mis-match in PrP status between the host and the bone marrow graft (table 3.2 and 3.3).

Although similar incubation periods for each model were obtained in the second study greater variation in incubation periods were observed in almost all groups (table 3.2), this variation is reflected in the standard errors and significance values obtained (table 3.2 and 3.3). A number of factors may account for this variation including infectious titre of starting inoculum. Notably, during the incubation period of disease in the second study the NPU mouse colony became infected with the pinworm *Syphacia oblevata*. As a consequence the entire NPU mouse colony was treated with the bis-acridine compound, piperazine, for a period of 6 weeks. It is

possible that the effects of this treatment may have resulted in these minor variations in incubation period observed in this study.

Table 3.2. Disease incidence and incubation periods in $PrP^{+/+}$ radiation chimaeric mice following infection with 20 µl of a 1% ME7 scrapie brain homogenate.

	i ntracerebral inoculation			**intraperitoneal inoculation**		
mouse model	Exp	scrapie incidence	incubation period	Exp	scrapie incidence	incubation period
$PrP^{+/+}$ + ***$PrP^{-/-}$***	1	8/8	153 ±3	1	10/10	239 ±3
	2	9/9	164 ±3	2	9/9	235 ±6
$PrP^{+/+}$ + ***$PrP^{+/+}$***	1	9/9	157 ±2	1	11/11	252 ±2
	2	8/8	164 ±3	2	10/10	261 ±2
$PrP^{+/+}$	1	9/9	161 ±2	1	10/10	256 ±3
	2	7/7	165 ±5	2	10/10	256 ±7

Table 3.3. Statistical analysis of scrapie incubation periods in radiation chimaeric mice

P values (student's t-test 2 tailed, unpaired) obtained from comparison of incubation periods from i.p or i.c. challenged $PrP^{+/+}$ mice reconstituted with $PrP^{+/+}$ or $PrP^{-/-}$ bone marrow. Statistically significant values are represented in italics. Incubation periods in i.c. challenged $PrP^{+/+}$ mice reconstituted with $PrP^{-/-}$ bone marrow were significantly shorter than un-irradiated control mice in the first but not the second experiment.

	Model	**$PrP^{+/+}$ + $PrP^{-/-}$**	**$PrP^{+/+}$ + $PrP^{-/-}$**	**$PrP^{+/+}$ + $PrP^{+/+}$**
Route	*compared with* **Experiment**	*$PrP^{+/+}$ no bm*	*PrP + $PrP^{+/+}$*	*$PrP^{+/+}$ no bm*
i.p.	**1**	*0.0002*	*0.0019*	0.22
	2	*0.04*	*0.0024*	0.48
i.c.	**1**	*0.0076*	0.07	0.16416
	2	0.843397	0.59	0.877197

3.3.4 Immunohistochemical detection of PrP protein and FDCs in irradiated mice with chimaeric immune systems and assessment of reconstitution efficacy by flow cytometry.

Following irradiation and bone marrow reconstitution mice were maintained in filtered caging and cleaned under sterile laminar flow conditions to minimise exposure to infection for 28 days. This timescale has previously been shown to be the optimum time for reconstitution of specific cellular population, including the maturation of r;esident FDCs (Brown et al, 2000b; Kapasi et al, 1998). After this time radiation chimaeric mice were infected with the ME7 strain of scrapie intraperitoneally or intracerebrally. Some animals were sacrificed prior to infection to assess efficacy of reconstitution using immunohistochemical (IHC) analysis with the FDC-M1 antibody which recognises the MFG-E8 marker (Kranich et al, 2008) (Figure 3.4). High levels of MFG-E8 staining were detected in all groups. There appeared to be greater numbers of FDC networks in the mice subjected to irradiation and reconstitution, although clearly quantification studies would be required to validate this suggestion. Flow cytometry of lymphocyte populations from radiation chimaeric mice prior to infection with the ME7 scrapie agent showed that successful reconstitution had occurred (figure 3.4 f and g).

To determine the expression of PrP protein in radiation chimaeric mice animals from each group were sacrificed 10 weeks post i.p. challenge and spleens from all groups analysed for PrP protein and FDC networks by IHC (figure 3.5). For detection of PrP the 6H4 and 1B3 antibodies were used. In previous studies the 6H4 antibody has only detected accumulations of PrP by either IHC of FACS analysis, suggesting that this antibody may be useful in discriminating between the normal and infection

specific form of the protein. Abundant accumulations of PrP^{Sc} were detected in germinal centres in spleen from all groups of mice with both antibodies demonstrating that the lack of PrP expression on bone marrow derived cells had no impact on the expression of PrP on FDCs.

Figure 3.4 Successful bone marrow reconstitution of mice subjected to ionising radiation

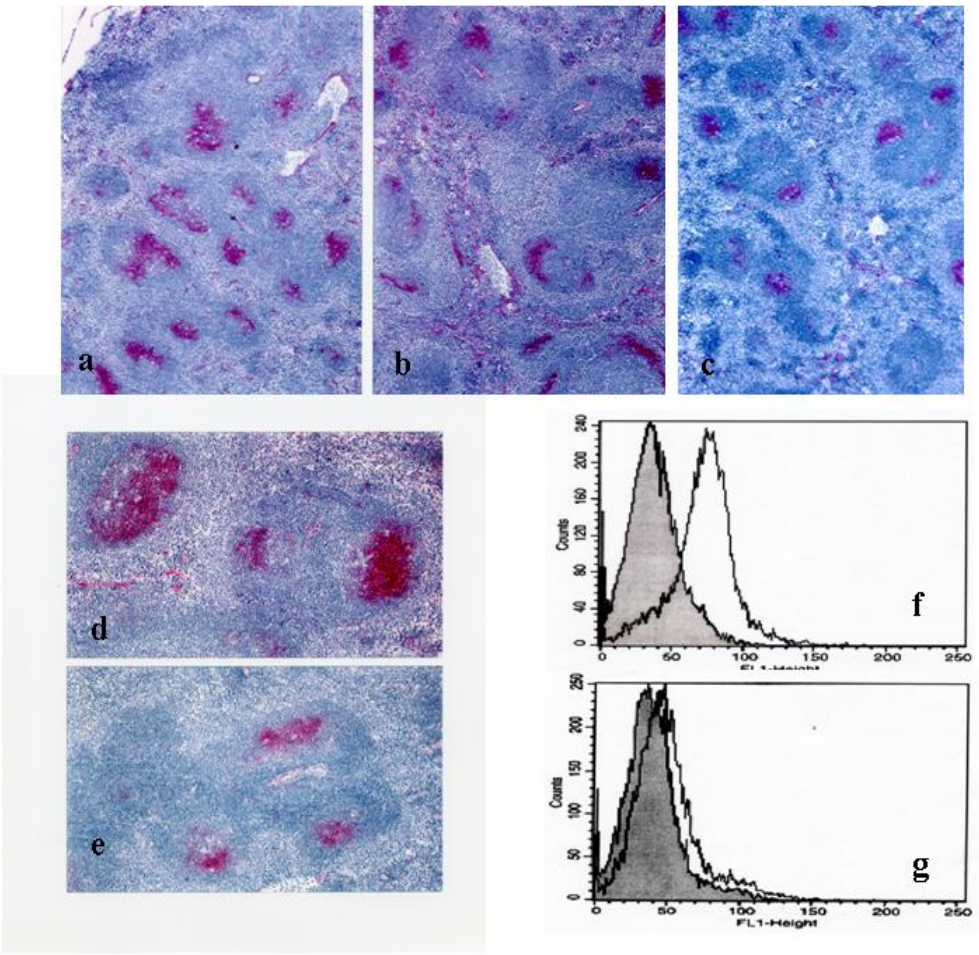


Figure 3.4 Immunohistochemical detection of MGF-E8 positive FDC networks in uninfected radiation chimaeric mice 30 days post bone marrow reconstitution. (a-c magnification X40, d-e magnification x100) (a) $PrP^{+/+}$ mice reconstituted with $PrP^{-/-}$ bone marrow (b) $PrP^{+/+}$ reconstituted with $PrP^{-/-}$ (c) $PrP^{+/+}$ mice with no bone marrow graft. Robust networks are detected in all mouse models demonstrating that effective bone marrow reconstitution occurred post irradiation. Notably, FDC size and number did appear to be increased in irradiated and reconstituted mice particularly in the mice reconstituted with $PrP^{-/-}$ bone marrow (d) in comparison with unreconstituted mice (e). Detection of PrP^{c} on splenic lymphocytes from radiation chimaeric mice 30 days post irradiation and reconstitution using flow cytometry and the 1B3 antibody (f)$PrP^{+/+}$ mice reconstituted with $PrP^{+/+}$ bone marrow demonstrating low levels of expression on lymphocytes (g) $PrP^{+/+}$ reconstituted with $PrP^{-/-}$ bone marrow showing little PrP^{c} expression above control sample labelling. Shaded area represents FL1 intensity in cells labelled with FITC labelled secondary antibody (goat anti-rabbit FITC) alone as a control.

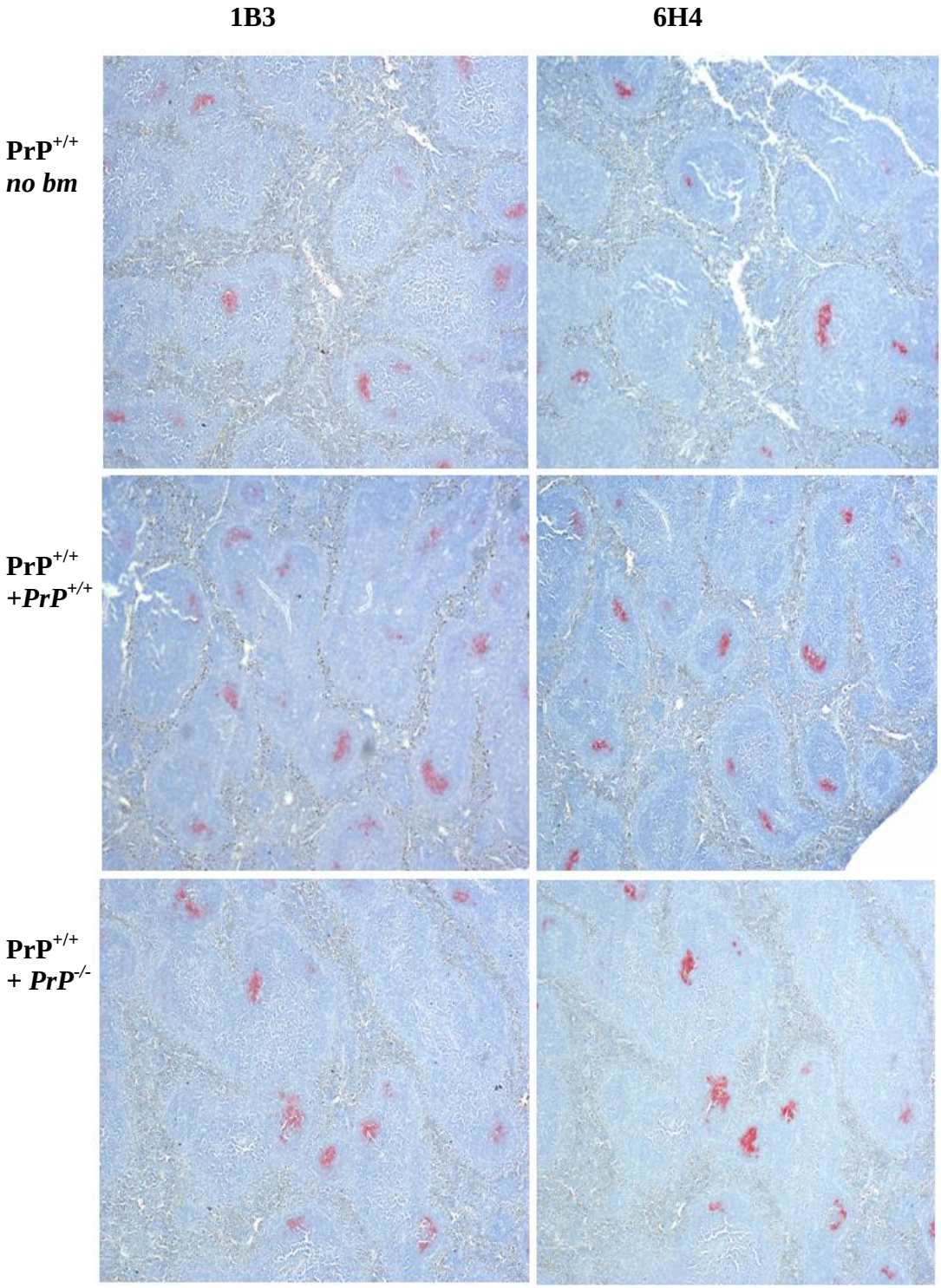


Figure 3.5 Immunohistochemical detection of PrP^{Sc} in radiation chimaeric mice
At 10 weeks post i.p. ME7 infection with the ME7 scrapie agent detection of PrP^{Sc} was carried out in PLP fixed sections of spleen. PrP^{Sc} was detected using both the 1B3 and 6H4 antibodies in all of the mouse models demonstrating that PrP accumulation on FDCs is not dependent on the expression of PrP protein on lymphocytes.

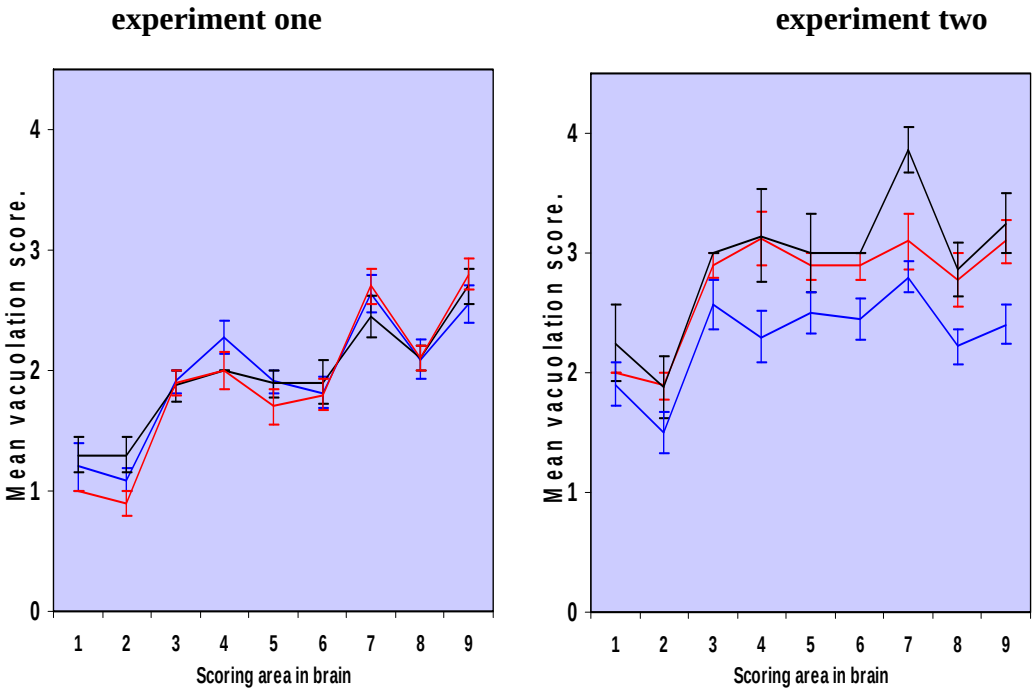
3.3.5. Analysis of lesion profiles in the brains of scrapie infected mice with chimaeric immune systems

Confirmation of disease in TSE infected rodents is based on a combination of a clinical scoring system with defined endpoints of disease and pathological assessment (Fraser & Dickinson, 1967; Fraser & Dickinson, 1968). Once positive clinical disease is established animals are humanely destroyed and brains examined histopathologically for the presence of vacuolation in defined areas within the brain. Severity of vacuolation in these defined areas is assessed and a lesion profile established (Fraser & Dickinson, 1967; Fraser & Dickinson, 1968).

To further investigate differences in pathogenesis in radiation chimaeric mice lesion profiles generated from animals in each of the experimental groups ($PrP^{+/+}$ + $PrP^{-/-}$, $PrP^{+/+}$ + $PrP^{+/+}$ and non-irradiated $PrP^{+/+}$) were compared (figure 3.6). Lesion profiles generated from the i.p. infected mice were examined. As previously discussed $PrP^{+/+}$ mice irradiated and reconstituted with $PrP^{-/-}$ bone marrow had significantly shorter incubation periods following peripheral challenge than non-irradiated $PrP^{+/+}$ mice or $PrP^{+/+}$ mice reconstituted with isologous bone marrow. Lesion profiles from the peripherally challenged mice in the first experiment were almost identical. However, in the second study comparison of the severity of lesion profiles between groups showed lower levels of vacuolation in the $PrP^{+/+}$ mice irradiated and reconstituted with $PrP^{-/-}$ bone marrow. It should be noted that severity of pathology was generally higher in the second study although the reasons for this are not understood. To assess differences in the severity of the pathology statistical analysis of these lesion profiles was carried out on individual mice from each group.

The area under the lesion profile (AUC) was estimated using the trapezoidal rule, for those mice that had valid scores in at least eight of the regions and a one-way analysis of variance carried out. $PrP^{+/+}$ mice receiving $PrP^{-/-}$ bone marrow had a significantly lower mean AUC than the other two groups (Mann-Whitney: $P < 0.001$).

Figure 3.6 Lesion profiles in the brains of radiation chimaeric mice



Comparison	Mean difference	Standard error of difference
$PrP^{+/+}$ bm v. $PrP^{-/-}$ bm	5.50	1.08
No bm (Control) v. $PrP^{-/-}$ bm	3.75	1.04
$PrP^{+/+}$ bm v no bm (control)	1.75	1.11

Figure 3.6 Lesion profiles in the brains of radiation chimaeric mice
Lesion profiles and statistical significance (experiment two) in $PrP^{+/+}$ mice (red line), $PrP^{+/+}$ +$PrP^{+/+}$ bm (black line) and $PrP^{+/+}$ +$PrP^{-/-}$ bm (blue line) following i.p. infection with the ME7 scrapie agent. Vacuolation in the brain was scored on a scale of 0 to 5 in the following gray-matter (G1 to G9) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex. There was no difference in severity of vacuolation between groups in experiment one while in experiment two mice reconstituted with $PrP^{-/-}$ bone marrow appeared to have lower levels of vacuolation. However, in experiment two levels of vacuolation in control and $PrP^{+/+}$ mice reconstituted with $PrP^{+/+}$ bone marrow appear higher than the same groups in experiment one, although the reasons for this difference are not understood. Statistical analysis showed strong evidence ($P < 0.001$) of a difference in the mean AUC between the $PrP^{+/+}$ mice receiving $PrP^{-/-}$ bone marrow and the other groups in experiment two. The means of the control $PrP^{+/+}$ mice and those receiving $PrP^{+/+}$ bone marrow are similar whereas the $PrP^{-/-}$ deficient mice have a significantly lower mean AUC than the other two groups. The mean differences and standard errors of the three pair-wise comparisons are shown above.

3.3.6 Analysis of infectivity levels in spleen from PrP mice reconstituted with PrP expressing or PrP deficient fetal liver

Studies using bone marrow chimaeric and immunodeficient mice have strongly suggested that replication of the ME7 scrapie agent strain in lymphoid tissues is dependent on mature PrP expressing FDCs and not PrP expressing lymphocytes (Brown et al, 2000b; Brown et al, 1999; Bruce et al, 2000; Mabbott et al, 2000a). However these findings are in contrast to those obtained elsewhere, which suggested that PrP expressing bone marrow derived cells (most probably B lymphocytes) and not FDCs were required for replication of the RML scrapie isolate in spleen (Klein et al, 1997; Klein, 1998). Although the studies were very similar a comparison of experimental design revealed several technical differences in methodology; namely the use of a different scrapie strain, the RML scrapie isolate and the use of fetal liver as the source of haematopoietic cell graft as opposed to bone marrow. In these studies PrP deficient mice irradiated and grafted with PrP expressing fetal liver and then infected with the RML scrapie isolate had high levels of infectivity in spleen (Klein et al, 1997; Klein, 1998). Similar studies carried out in our laboratory with ME7 infected $PrP^{-/-}$ mice grafted with $PrP^{+/+}$ bone marrow found only trace levels of infectivity in spleen (Brown et al, 1999). The possibility that different TSE strains may target different cell types in the lymphoid system is examined in detail in chapter 4 although a preliminary study using two different TSE strains and two different sources of haematopoietic cell grafts is presented here.

To establish firstly if the source of haematopoietic cell graft may have contributed to these results radiation chimaeric mice were constructed by lethally irradiating $PrP^{-/-}$

mice and grafting with either $PrP^{-/-}$ or $PrP^{+/+}$ fetal liver. These mice were then infected i.c. with 20 µl of a 1% brain homogenate from mice terminally affected with either the 79A or ME7 scrapie agent strain. Spleens were collected 10 weeks post challenge for immunohistochemical analysis and infectivity bioassay. The RML "chandler" isolate (Chandler, 1961) while not a classically cloned strain is may contain a mixture of the 79A and 139A scrapie agent strains (Chandler, 1961). As the RML scrapie isolate was not available for these studies the 79A strain was used.

Scrapie agent infectivity bioassay of spleens from the 79A scrapie agent infected mice produced no disease in indicator mice (table 3.4) while bioassay of spleen mice infected with the ME7 scrapie agent produced disease in a very small proportion of mice, although these represent trace levels of infectivity and are not consistent with replication. Likewise, immunohistochemistry was unable to detect the presence of PrP protein in spleens of $PrP^{-/-}$ mice reconstituted with $PrP^{+/+}$ bone marrow (figure 3.7). As these results are almost identical to those of previously published studies using bone marrow as the source of haematopoietic cell graft this suggests that source of haematopoietic graft does not appear to influence the replication of infectivity in lymphoid tissues.

Table 3.4. Incidence of disease and incubation periods in C57BL bioassay mice following intracerebral inoculation with 20 µl of a 10% homogenate of spleen from PrP deficient mice irradiated and reconstituted with $PrP^{+/+}$ or $PrP^{-/-}$ bone marrow and infected intracerebrally with a 1% ME7 or 79A brain homogenate. Spleens for bioassay were collected 10 weeks post challenge.

mouse model	**scrapie agent strain**	**spleen number**	**scrapie incidence**
$PrP^{-/-}$ + $PrP^{+/+}$	79A	1	0/12
		2	0/12
	ME7	1	1/11
		2	3/11
$PrP^{-/-}$ + $PrP^{-/-}$	79A	1	0/12
		2	0/12
	ME7	1	0/11
		2	0/12

Figure 3.7 Detection of PrP^{Sc} in chimaeric PrP deficient mice

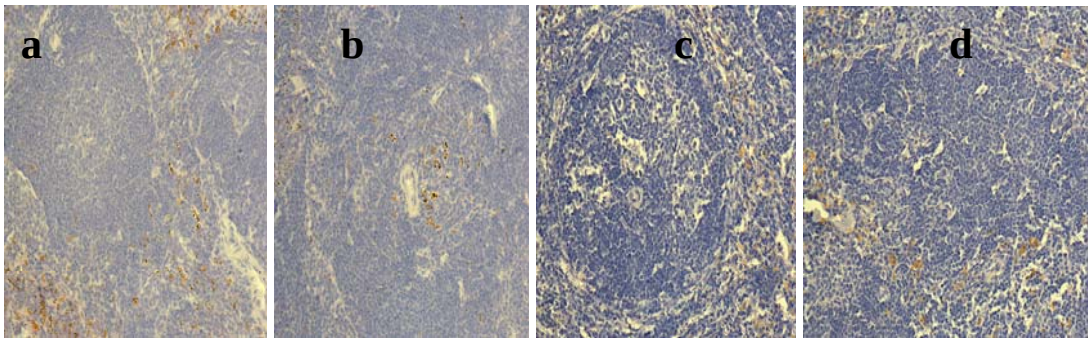


Figure 3.7 Detection of PrP^{Sc} in chimaeric PrP deficient mice PrP^{Sc} (red) is not detected in association with FDCs in germinal centres of $PrP^{-/-}$ mice irradiated and reconstituted with $PrP^{+/+}$ bone marrow when infected with either the 79A (a) or ME7(b) scrapie agent strains. $PrP^{-/-}$ mice were reconstituted with $PrP^{-/-}$ bone marrow as controls and also infected with the 79A (c) or ME7 strains of scrapie (d). Original magnification X200. The 1B3 antibody was used for this labelling (Farquhar et al, 1989)

Discussion

The congenital syndrome known as severe combined immunodeficiency (SCID) is characterised by a loss of both B and T cell immunity. It was first characterised in human infants (Glanzmann & Riniker, 1950) and later in Arabian foals (McGuire & Poppie, 1973). It has also been recognised in mice (Bosma et al, 1983), and mice homozygous for this mutation are hypogammaglobulinaemic and deficient in immune functions mediated by T and B lymphocytes. As a consequence the maturation of resident precursor FDCs is impaired in SCID mice, although resident FDCs can be induced to mature following haematopoietic reconstitution using bone marrow or fetal liver (Brown et al, 1997; Brown et al, 2000b; Fraser et al, 1996; Kapasi et al, 1993; Kapasi et al, 1998). Although the origin of FDCs remains the subject of much controversy it is widely accepted that FDCs are *not* derived from the bone marrow but develop, in the presence of B and T lymphocytes, from stromal precursor cells (Humphrey et al, 1984). An elegant series of studies using bone marrow chimaeras showed that FDCs always originated from the recipient and never from the bone marrow graft itself (Humphrey et al, 1984). Critics of this study argued that since FDCs are radioresistant, their presence in recipient animals may have inhibited donor FDCs from developing. To overcome the possibility that mature FDCs may compete with the putative development of FDC precursor cells from the bone marrow newborn SCID mice were grafted with bone marrow or fetal liver from rats (Kapasi et al, 1998). Surprisingly, FDCs of rat origin were subsequently detected in lymphoid tissues suggesting that in the newborn mouse, but not in the adult, FDCs could be replaced from the bone marrow. Despite these interesting findings the vast majority of studies suggest that FDCs are not derived from the bone marrow but are

derived from a stromal origin (Balogh et al, 2001; Humphrey et al, 1984; Lindhout & de Groot, 1995; Tew et al, 1990). However in the studies presented here these findings of potential transfer of FDCs were not dismissed and grafting of haematopoietic cells was only conducted in adult mice.

Following reconstitution and subsequent FDC maturation neuroinvasion occurred in SCID-$PrP^{+/+}$ mice irrespective of the source of the bone marrow graft. Although it is generally accepted that FDCs are critical for pathogenesis (Brown et al, 1999) this work provides further evidence in support of these findings and is further supported from the findings in the radiation chimaeric mice. Interestingly the expression of PrP protein in association with FDCs was not impaired in SCID-$PrP^{+/+}$mice reconstituted with $PrP^{-/-}$ bone marrow or in radiation chimaeric mice where the source of the bone marrow graft was of $PrP^{-/-}$ origin. It has not been determined if FDCs themselves produce PrP and although these data may provide evidence in support of this, the contribution of other PrP expressing cells, such as peripheral nerves which are not affected by the radiation procedure, cannot be ruled out. As a major function of the FDC is to acquire and retain immune complexes and other proteins on their cell surface (Kosco-Vilbois et al, 1997; Kosco et al, 1992) it is possible that PrP may be transported to the FDCs and not produced by the cells themselves. In studies investigating the expression of PrP in the neonatal and post-natal spleen (Ierna et al, 2006) although PrP^{c} expression occurred coincidently with FDC-M1 network development, there was greater co-localisation with PrP^{c} and the FDC-M2 marker (which recognises immune complex trapping via the localisation of complement component C4) at14 days of age than with the FDC-M1 marker (Ierna et al, 2006).

The highly reproducible incubation periods of distinct TSE strains are one of the hallmarks of TSE infection (Bruce & Fraser, 1991; Bruce et al, 1992; Dickinson & MacKay, 1964; Dickinson et al, 1968). In experimental studies attempts to modulate pathogenesis have been carried out using a variety of compounds. Significant prolongation of incubation period in scrapie affected mice and hamsters has been achieved using a number of sulphated polyanions (Ehlers & Diringer, 1984; Farquhar & Dickinson, 1986; Ladogana et al, 1992). Other studies have found effects on the conversion/accumulation of PrP in cell culture using heparan sulphate (Schonberger et al, 2003) and Congo red derivatives (Ingrosso et al, 1995). Moreover, administration of Congo red derivatives to scrapie infected hamsters delays incubation period of disease (Ingrosso et al, 1995). These findings are extremely important as they may represent potential approaches for treating TSE infections. The ability to shorten incubation period and understanding the effects and mechanisms involved is also important. In the studies presented in this chapter, significantly shorter incubation periods were found in peripherally infected $PrP^{+/+}$reconstituted with $PrP^{-/-}$ bone marrow, in two separate, but identically performed, studies. It is not understood why these incubation periods in these chimaeric mice are shorter. A number of possibilities exist including the effects of the irradiation procedure, the generation of an immune response as a result of mismatch between the PrP status of the lymphocyte and the FDCs or CNS related effects from the replacement of cells such as microglia.

Treatment of mice with lethal doses of ionising radiation results in the destruction of actively dividing cells such as lymphocytes. In addition, lethal doses of radiation administered to mice result in breach of blood brain barrier following irradiation (Nordal & Wong, 2005) and other effects have been documented including the induction of astrocyte gliosis following exposure to ionising radiation (Hwang et al, 2006). However, the effects on pathogenesis in these studies were confined to mice reconstituted with $PrP^{-/-}$ bone marrow suggesting the effect is related to the origin of the bone marrow graft and not specifically the irradiation procedure itself. In addition previous studies using ionising radiation found no effect on pathogenesis (Fraser & Farquhar, 1985; Fraser et al, 1989), although in these studies the bone marrow graft would always have been sourced from PrP expressing mice (Fraser et al, 1989).

It is known that mitogenic stimulation with phyto-haemagluttinin (PHA) and administration of modified BCG vaccine prior to infection with experimental strains of scrapie can shorten incubation period of disease (Dickinson et al, 1978; Kimberlin & Cunnington, 1978a), most probably as a result of immune system stimulation. It is possible that a similar effect has occurred in these experiments. As the effects on pathogenesis were only observed in mice reconstituted with $PrP^{-/-}$ bone marrow it may be that an immune response was generated from the mismatch in PrP status between host FDCs and surrounding lymphocytes resulting in germinal centre formation. It is possible that increased replication of infectivity, following increased germinal centre formation in lymphoid tissues, resulted in more efficient neuroinvasion. However, there was no significant difference in incubation periods between SCID-$PrP^{+/+}$ mice grafted with $PrP^{-/-}$ or $PrP^{+/+}$ bone marrow suggesting that

the effects in the radiation chimaeric mice may be in part related to the destruction of PrP expressing cells within the host and the subsequent replacement with PrP deficient cells.

In addition to replacement of lymphoid cells by bone marrow reconstitution, cells of the myeloid lineage such as monocytes, macrophages, dendritic cells and natural killer cells are also replaced. Depletion of macrophages by the administration of substances toxic to these cells results in shortened incubation period of disease (Beringue et al, 2000). Although bone marrow grafting would replace macrophage/monoctye populations it is possible the lack of PrP expression or perhaps lower numbers of these cells post re-population may have contributed to the effects on pathogenesis.

In TSE infection and other neurodegenerative conditions it is thought that microglial activation contributes to disease pathology (Betmouni et al, 1996; Giese et al, 1998; Perry et al, 2003; Perry, 2004; Perry, 2007; Williams et al, 1995). Notably microglia are derived from the bone marrow and studies in radiation chimaeric mice infected with the RML scrapie isolate demonstrate extensive recruitment of microglia from the bone marrow during the incubation period of disease (Priller et al, 2006). In these studies the bone marrow derived microglia appeared to replace endogenous microglial cells coincident with the deposition of PrP^{Sc} in the CNS (Priller et al, 2006). Like the results presented here, no difference in incubation period was observed between the non-irradiated mice and the $PrP^{+/+}$ mice reconstituted with isologous bone marrow (although bone marrow chimaeras reconstituted with $PrP^{-/-}$

bone marrow were not used in these studies). It is therefore possible that the recruitment of $PrP^{-/-}$ deficient microglia into the CNS in the $PrP^{+/+}$ mice reconstituted with $PrP^{-/-}$ bone marrow may have had some influence on incubation period, potentially as a result of mismatch in PrP expression between these cells and endogenous host cells. Microglia are considered effectors of the innate immune response in the brain and function in a similar way to tissue macrophages by inducing immune responses. Indeed the generation of an immune response at a late stage of infection exacerbates disease possibly via increased migroglial activation in the CNS (Combrinck et al, 2002; Perry, 2004). Further studies investigating microglial activation in these models as a reason for altered pathogenesis are required to explore this suggestion further.

Although results obtained in the second study were strikingly similar to the first experiment a wider range of incubation periods in each of the groups was obtained. Interestingly during the incubation period of disease in this study the colony became infected with the pinworm *Syphacia oblevata*. To eradicate this parasite the entire colony was treated with the bis-acridine compound piperazine. These may be important considerations to bear in mind especially when immunological assessments are being carried out. However, the wider range of incubation periods obtained in the second study may be attributed to the treatment of mice with piperazine during the incubation period of disease. Studies in cell culture using a scrapie infected cell line suggest that piperazine may impair accumulation of PrP^{Sc} in these cells (May et al, 2003) suggesting it may also have affects on pathogenesis. Alternatively it is possible the variation in incubation periods is simply related to

starting titre of inoculum as incubation periods of the i.c. injected mice found those in the second study were longer throughout all groups.

TSEs are invariably fatal and it is generally assumed and accepted that the pathological changes in the CNS e.g. vacoulation, gliosis and the deposition of abnormal PrP are the causal factors in the death of the animal or individual. In the studies with the radiation chimaeric mice a less severe pathology (in terms of vacoular changes) was observed in the peripherally challenged $PrP^{+/+}$ mice reconstituted with $PrP^{-/-}$ bone marrow, although this was only found in the second study. As this finding was only found in one experiment the validity of the findings must be questioned and may simply represent variation in pathology as a result of a number of factors. Animals in the second study were treated with piperazine during the incubation period of disease which may have had some implications by altering incubation period of disease. There is evidence that longer incubation periods of disease may produce more severe pathology in some models (Bruce, 1996). It is possible that the variation in incubation periods in the second study, possibly as a result of the piperazine treatment, may have produced this more severe vacuolation. However the contribution of vacuolar changes to the development of clinical disease is an interesting area of research and one that is not completely understood. Studies in transgenic mice with inducible PrP expression found that lack of PrP expression in microglia led to lower levels of neuro-degeneration in the retina with the RML isolate of scrapie (Kercher et al, 2007). The occurrence of clinical symptoms of disease in the absence of vacuolar changes has been described in aged mink of the Chediak -Higashi strain (Marsh et al, 1976). The Chediak-Higashi Syndrome (CHS)

is documented in humans and several animal species and results in increased susceptibility to infection as a result of granulocyte abnormalities. As TSEs have highly reproducible incubation periods the ability to modulate infection is extremely significant. In future studies it will be important to establish the exact mechanisms by which incubation period may be shortened; for example by infection or immunisation. In addition, the contribution of pathology to clinical symptoms of disease is also intriguing and understanding the conditions where clinical disease can occur with less severe pathology may be significant especially for diagnosis.

Although previously published studies (Brown et al, 1999) and the findings here produce strong evidence for FDC involvement in pathogenesis other studies carried out elsewhere suggested that B lymphocytes were critical for pathogenesis (Klein et al, 1997; Klein, 1998). There were several technical differences in these studies, notably the use of fetal liver as a source of cell graft and the RML scrapie isolate as source of TSE infection as opposed to the ME7 scrapie agent strain which was used here. No differences were found between the 79A and ME7 scrapie agent strains, and the use of fetal liver instead of bone marrow produced no difference in infectivity levels in spleen from those described previously (Brown et al, 1999). Thus, differences in haematopoietic cell graft used in the two studies cannot account for the discrepancies in findings between the studies.

In summary, the work presented in this chapter has provided further evidence for a role for FDCs in the pathogenesis of the ME7 scrapie agent strain. Despite these findings what is not understood is whether FDCs are actively involved in other TSE

strains models. This issue is addressed in chapter 4 through the use of a range of TSE agent strains and mice with specific immunological defects (chapter 4). The significant shortening in pathogenesis in the radiation chimaeric mice is interesting and while these findings are not fully understood it seems likely that some form of immune system influence has contributed to these effects. The effects of immune system stimulation are further investigated using immunisation (chapter 6) and the influence of an ageing immune system with reference to effects of age on FDC function are addressed in chapter 5.

Chapter 4

The cellular targeting of a range of TSE agent strains in the peripheral lymphoid system

4.1 Summary

Although TSE infection results in dramatic pathological changes in the central nervous system (CNS), replication of infectivity also occurs in spleen and lymph nodes early in the disease. Previous studies using chimaeric and immunodeficient mice demonstrated that mature follicular dendritic cells (FDCs) within the spleen and lymphoid system support scrapie agent replication and are critical for neuroinvasion following moderate dose peripheral challenge with ME7 scrapie (chapter 3). Despite a wealth of evidence supporting the involvement of FDCs in the pathogenesis of the ME7 agent strain it is not know if these cells are involved in the peripheral pathogenesis of other TSE agent strains. As different TSE agent strains target different cell types in the CNS it is possible that these differences exist in the peripheral lymphoid system also. The hypothesis that different TSE agent strains may target different cell types of the immune system was tested by peripherally infecting a range of immunodeficient mice with 4 distinct experimental TSE agent strains; ME7, 79A, 139A and the BSE derived strain 301C. The mouse strains used were μMt which lack B cells and mature FDCs, $TNFR1^{-/-}$ which lack mature FDCs but have functional B and T lymphocytes and SCID mice which lack mature FDCs and B and T lymphocytes. Results indicated that neuroinvasion and replication of infectivity in spleen with all TSE agent strains tested appeared to be dependent on the presence of mature FDCs. However some variations in pathogenesis between TSE agent strains were observed; notably the development of extensively prolonged incubation periods with the 79A/139A strains and in some of the 301C infected mice, primarily at high dose. The significance of this is not fully understood but may suggest some alternative less efficient mechanism of pathogenesis which may occur

in the in the absence of mature FDCs. These effects were not apparent following peripheral infection with the ME7 scrapie agent strain suggesting that different mechanisms of peripheral pathogenesis may exist between TSE agent strains.

4.2 Introduction

Previous studies have focused on defining the cell types which support TSE replication in peripheral lymphoid tissues and most point to a central role for the follicular dendritic cell (FDC) (Fraser et al, 1992b; McBride et al, 1992) (Bruce et al, 2000; Mabbott et al, 1998)a long-lived radiation resistant cell (Humphrey et al, 1984) found within secondary lymphoid tissues (Kosco-Vilbois et al, 1997). A major function of FDCs is the trapping of immune complexes on their extensive cytoplasmic processes (Williams & Nossal, 1966). More conclusive evidence for a critical role for FDCs in scrapie replication has been provided by studies in chimaeric and immunodeficient mice (Brown et al, 1999) (chapter 3). In these studies chimaeric and immunodeficient mice were created where PrP was expressed on FDCs or lymphocytes and *vice versa*. Using these chimaeric and immunodeficient models strong evidence was obtained that replication of the ME7 strain on the spleen, and progression of disease to the CNS depended on the presence of mature PrP expressing FDCs (Brown et al, 1999) (chapter 3).

However, studies carried out elsewhere using the RML scrapie isolate suggested that B cells or bone marrow derived cells, and not FDCs, were critical for replication of infectivity and neuroinvasion (Blattler et al, 1997; Klein et al, 1997; Klein, 1998). Firstly, studies in radiation chimaeric mice infected with RML found strong evidence that replication of agent infectivity depended on cells of a haematopoietic origin and not upon FDCs (Blattler et al, 1997). However studies carried out at this unit (Brown et al, 1999), found little evidence of infectivity in similar radiation chimaeric mice when infected with ME7 (Brown et al, 1999). The possibility that the differences

between haematopoietic cell source (HSC), used in the production of the chimaeric mice, could influence the ability to replicate infectivity in spleen was tested in chapter 3 using ME7 and 79A. However no differences were observed discounting this possibility. In a further series of studies carried out by the same researchers (Klein et al, 1997; Klein, 1998), peripheral challenge of immunological knockout mice with RML only resulted in clinical disease in mice with normal B cell populations (Klein et al, 1997; Klein, 1998). B cell deficient mice (Kitamura et al, 1991), which also indirectly lack mature FDCs, were resistant to disease, however T cell deficient mice were fully susceptible. In addition, tumour necrosis factor receptor 1 deficient ($TNFR1^{-/-}$) mice (Rothe et al, 1993), which lack FDCs but have normal T and B cell populations, were fully susceptible to peripheral infection suggesting that B cells and not FDCs were important for neuroinvasion of the RML scrapie isolate. In these studies spleen infectivity levels in spleen were not provided from the $TNFR1^{-/-}$ mice therefore it is impossible to determine if normal replication of infectivity occurred in these spleens. However in studies carried out at this unit TNF-α deficient mice (Pasparakis et al, 1996), which have similar defects to the $TNFR1^{-/-}$ mice, were almost completely resistant to infection with moderate doses of ME7 scrapie (Brown et al, 1999). These discrepancies in results between the studies, which were carried out under very similar conditions, are difficult to interpret however a number of differences between the studies exist (summarised in figure 4.1).

One of the major differences between the studies is in that of the TSE strain. In the studies carried out in this unit the ME7 scrapie strain was used whereas the other

studies used the RML scrapie isolate. The RML (Rocky Mountain Laboratory) isolate was originally isolated from a pool of natural scrapie (SSBP/1) following passage in goats (discussed in detail in chapter 1). This passage in goats produced two distinct lines of phenotype in the goats a “scratching” and “drowsy” line. Sub-passage in mice of the “drowsy” isolate (Chandler, 1961) produced the “Chandler” isolate and RML represents sub-passage of this “chandler” isolate at the Rocky Mountain Laboratory (Montana, USA). Extensive sub-passage of the “drowsy goat” isolate in mice by Alan Dickinson and others (Dickinson et al, 1986) at our unit produced the 139A and the 79A strains (Dickinson et al, 1986). Although studies suggest that RML scrapie isolate and 139A scrapie agent strain are the same agents. RML has never been “cloned” by limiting dilution analysis, therefore it remains possible that the RML scrapie isolate may contain both the 139A and 79A scrapie agent strains since both were derived from the original “drowsy” goat isolate.

Figure 4.1 Summary of findings from studies in PrP radiation chimaeric mice

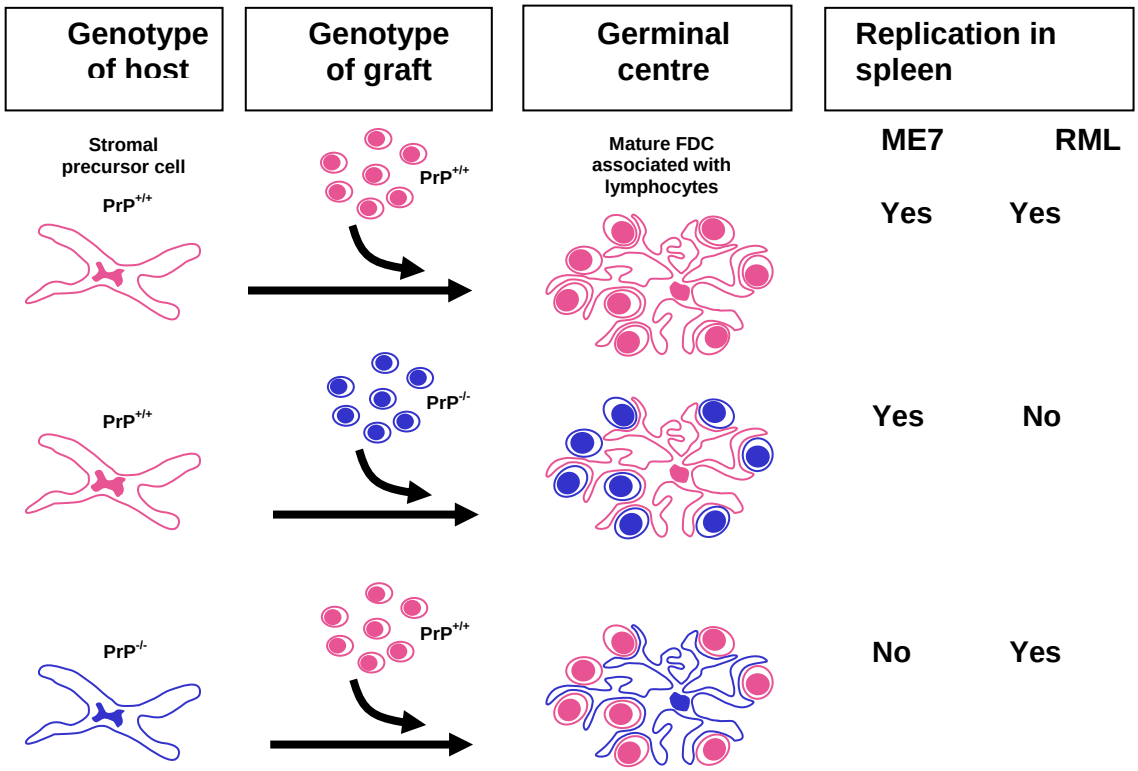


Table 4.1 Summary of differences in the design of the studies investigating the cellular involvement in peripheral lymphoid system.

	RML studies	**ME7 studies**
	Blattler et al 1997	*Brown et al 1999*
Dose	1% dilution 100µl given i.p. (*high*)	1% dilution 20µl given i.p. (*moderate*)
TSE strain	RML	ME7 (cloned)
Haematopoietic Cell source	fetal liver/ bone marrow	bone marrow

In the CNS dramatic differences in pathology exist between natural and experimental strains; for example in brains of mice infected with the ME7 strain, vacuolation and PrP deposition is widespread within the CNS (Bruce et al, 1989b). In contrast the 87V scrapie agent strain produces vacuolation and PrP deposition within defined areas for example the CA2 region of the hippocampus (Bruce et al, 1989b). The infecting TSE agent strain is not the only factor controlling vacoulation and PrP deposition; PrP genotype also plays an important role (Bruce et al, 1991; Dickinson et al, 1968). In the CV mouse strain (an F1 cross between the C57BL S*inc*^{s7} or *Prnp-a* and VM S*inc*^{p7} or *Prnp-b* genotype), infection with the ME7 scrapie strain results in destruction of the pyramidal cell layer of the CA1 region of the hippocampus by day 170 of a 250 day incubation period (Scott & Fraser, 1984) whereas in ME7 infected C57BL mice there is no specific pathology associated with this area of the CNS. In addition, in natural and acquired TSEs there is great strain-specific variation between the pathology and cellular targeting in the CNS (Fraser, 1993).

If TSE strains target different cell types in the lymphoid system this may have implications for therapeutic approaches and risk assessments of disease transmission. To attempt to elucidate these potential differences a range of TSE agent strains were used (79A, 139A, ME7 and the BSE derived strain 301C) to peripherally and intracerebrally challenge a range of mice (μMT, SCID, TNFR1$^{-/-}$). Mice with a range of immunodeficiencies (figure 4.2) were then challenged with these agent strains and disease pathogenesis compared. An important factor, which was considered in the design of these studies, was the potential effect that dose may have following peripheral exposure. In previous studies carried out at this unit peripheral infection of

SCID mice with high doses of the scrapie agent produced terminal disease in the absence of agent replication in spleen (Fraser et al, 1996). However at lower doses SCID mice were mainly refractory to disease (Fraser et al, 1996) (Table 4.2). These findings suggested that infection of the CNS following exposure to high doses of the scrapie agent may have occurred via direct peripheral nerve infection (Fraser et al, 1996) without the requirement for agent replication within lymphoid tissues. Therefore in the studies presented here a range of peripheral challenge doses were used. In summary the aims of the studies presented in this chapter are to determine the requirement of FDCs, B cells and T cells following peripheral exposure.

Figure 4.2 Summary of immune system defects in the mouse strains used in these studies

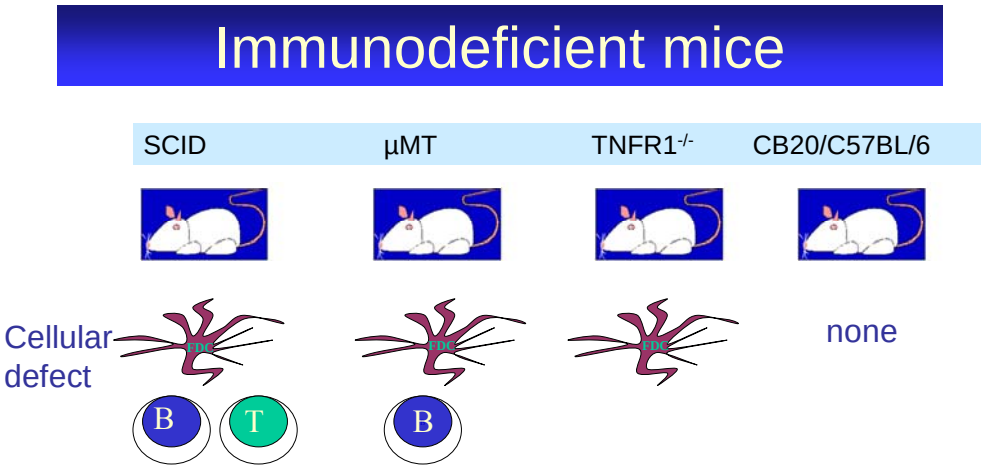


Figure 4.2. µMT mice were produced by gene targeting techniques (Kitamura et al, 1991). The subsequent B cell deficiency in these mice is a consequence of arrested B cell maturation at the stage of pre-B cell maturation. µMT mice lack B cells and mature FDCs. Several members of the TNF ligand and receptor families control development and organisation of secondary lymphoid tissues. Transgenic mice deficient in the tumour necrosis factor 1 receptor ($TNFR1^{-/-}$) mice do not have mature follicular dendritic cells (FDCs) and are unable to form germinal centres (GC) (Karrer et al, 2000; Rothe et al, 1993; Victoratos et al, 2006). SCID mice, described extensively in chapter 3, are derived from an inbred line of C.B-17 mice and are not produced using transgenic technology. As described in chapter 3, the occurrence of a "leaky" phenotype may complicate interpretation of results therefore all animals used in these studies were tested for the presence of a "leaky" phenotype and those mice with detectable serum Ig excluded from the study.

4.3 Results

4.3.1 Effect of dose on the development of peripherally routed ME7 scrapie agent in SCID mice

In previous work conducted by Fraser et al (Fraser et al, 1996) it was noted that SCID mice were almost fully susceptible at to peripheral exposure with a high dose of the ME7 scrapie agent (10% w/v brain homogenate), but almost resistant to infection when the infectious dose was reduced (1%). In these studies, despite the development of terminal disease in SCID mice at high dose, scrapie agent accumulation and replication did not occur in spleen suggesting that neuroinvasion followed exposure to a high dose of the scrapie agent in the absence of lymphoid system involvement. To further investigate these findings a titration was performed in SCID and immunocompetent control CB20 mice using the ME7 scrapie strain (Table 4.2). This showed that at high dose almost all SCID mice are fully susceptible to infection but almost resistant at lower doses (Table 4.2). This finding has significance for interpretation of results because in the studies using the RML scrapie isolate the peripheral challenge dose was estimated to be 5 times greater than that used in the studies with the ME7 strain which may have had some influence on pathogenesis.

Table 4.2 Effect of scrapie agent dose on disease susceptibility following peripheral (i.p.) infection of SCID and CB20 mice.

Inoculum Concentration	Mouse Strain CB20		Mouse Strain SCID	
	Incidence x/y	incubation ±SEM*	Incidence x/y	incubation (range)
10%	6/6	273± 3	7/8	(239-386)
1%	5/5	297±10	1/5	(309)
0.1%	6/6	327± 4	0/6	_
0.01%	4/6	(329-369)	0/9	_
0.001%	0/4		Not done	
0.0001%	0/6		Not done	

***= standard error of the mean**

4.3.2 The peripheral pathogenesis of the ME7, 79A, 139A and 301C strains in immunodeficient mice

Immunodeficient (SCID, μMT and TNFR1$^{-/-}$) and immunocompetent (CB20/C57BL/6) mice were injected with four experimental TSE strains (79A, 139A, ME7 and the BSE derived strain 301C) by the intracerebral route (i.c.) (using a 1% TSE brain homogenate) or intraperitoneal (i.p.) routes using 3 different doses (10%, 1% and 0.1%). These challenge doses correspond to a high (10%), moderate (1%) and low (0.1%) dose challenge. As the studies presented here involved the use of 5 different mouse strains in 20 experiments, each involving peripheral challenge at 3 separates doses and also i.c. challenge, it was important to ensure that the inoculum used for each TSE strain was pooled before use.

Peripheral infection of all of the immunocompetent mouse strains produced 100% incidence of disease at all dilutions tested (table 4.3). An exception to this was the 301C strain where only 15/16 and 13/16 mice developed disease at the 1% and 0.1% dilutions and in the 0.1% ME7 infected CB20 mice where only 9/13 mice developed disease (Table 4.3 Figure). It is possible this represents a lower starting titre of the original inoculum. In the μMt, TNFR1$^{-/-}$ and SCID mice the highest overall incidence of disease was observed at the 10% dilution with almost all mice developing disease. At the 1% and 0.1% dilutions the incidence of disease was greatly reduced in all of the immunodeficient mice. Unfortunately in SCID mice, the incidence of intercurrent illness was high and survival at the lower dilutions was poor although some analysis was possible with the 79A and 139A strains. These findings from the high dose infection groups support original observations obtained from the ME7 strain where

SCID mice were susceptible to high dose peripheral infection but mainly resistant at lower doses (Fraser et al, 1996). However in these studies only 2/10 SCID mice developed clinical and pathological disease following high dose ME7 infection. The reasons for this are unclear but as only 9/13 CB20 mice developed disease at the 0.1% dilution it is possible this represents lower infectious titre of starting inoculum when compared to previous experiments.

One of the most striking differences observed in these studies were the extensively prolonged incubation periods of disease in both TNFR1$^{-/-}$ and μMT mice infected with 79A or 139A agent strains. This was also recorded with the 301C agent strain although disease incidence was much lower in general with this strain, perhaps suggesting lower titre of starting inoculum. High dose infection of TNFR1$^{-/-}$ mice with 79A produced prolonged incubation periods of disease in almost all mice whereas infection of μMT mice produced a mixture of short and long incubation periods. However at lower doses all of the incubation periods were prolonged in both the 79A and 139A in both μMT and TNFR1$^{-/-}$ mice. This is in comparison with the ME7 strain where the majority of animals developing disease did so within the incubation period anticipated for the ME7 strain (figure 4.3-4.6). In previous studies of ME7 infected SCID mice the 87A strain was isolated following sub-passage of SCID spleen suggesting that inefficient replication or processing of infectivity in the lymphoid system may lead to the selection of minor strains. In response to this original finding lesion profiling was conducted from all of the TNFR-1 mice infected with ME7 which demonstrated the presence of the ME7 strain in these animals (figure 4.7).

Table 4.3. Pathogenesis of a range of TSE strains in immunodeficient mice.

			scrapie incidence/incubation period ±SEM									
			C57BL/6		**TNFR-1$^{-/-}$**		**µMT**		**CB20**		**SCID**	
TSE Agent	***dose***	***Cell Defect***	***none***		***FDCs***		***B cells/FDCs***		***none***		***B/T cells/FDCs***	
TSE strain												
ME7	10-1		15/15	256±1	11/13	225-380	14/17	210-308	15/15	307±8	2/10	193, 193
	10-2		15/15	290±3	2/12	330,343	4/12	291-324	14/14	340±5	*	
	10-3		14/14	313±4	1/13	267	2/20	312, 336	9/13	343-398	*	
139A	10-1		14/14	205±1	12/15	171-394	10/16	175-483	18/18	216±2	12/16	185-212
	10-2		14/14	222±3	5/15	233-518	4/16	316-400	17/17	233±1	0/13	
	10-3		14/16	239±2	4/15	240-455	1/16	286	17/17	247±4	0/10	
79A	10-1		16/16	215±2	6/11	231-524	11/15	175-601	14/14	226±5	9/16	171-270
	10-2		14/14	228±3	3/13	285, 371,517	3/15	286,287,505	18/18	244±1	1/8	255
	10-3		16/16	241±2	4/15	393-553	0/16	314	12/12	260±3	0/8	
301C 313	10-1		16/16	258±6	6/13	246-432	12/16	223-490	16/16	322±7	3/11	238, 308,
	10-2		15/16	320±7	0/16	-	3/15	217,595	15/15	362±6	0/5	
	10-3		13/16	357±5	0/13	-	1/16	400	16/16	383±5	*	

* there were no positive SCID mice in this group and no mice surviving until the first positive CB20 case.

Figure 4.3 Incubation period range in immunodeficient mice injected i.p. with the ME7, 79A, 139A and 301C TSE agent strains.

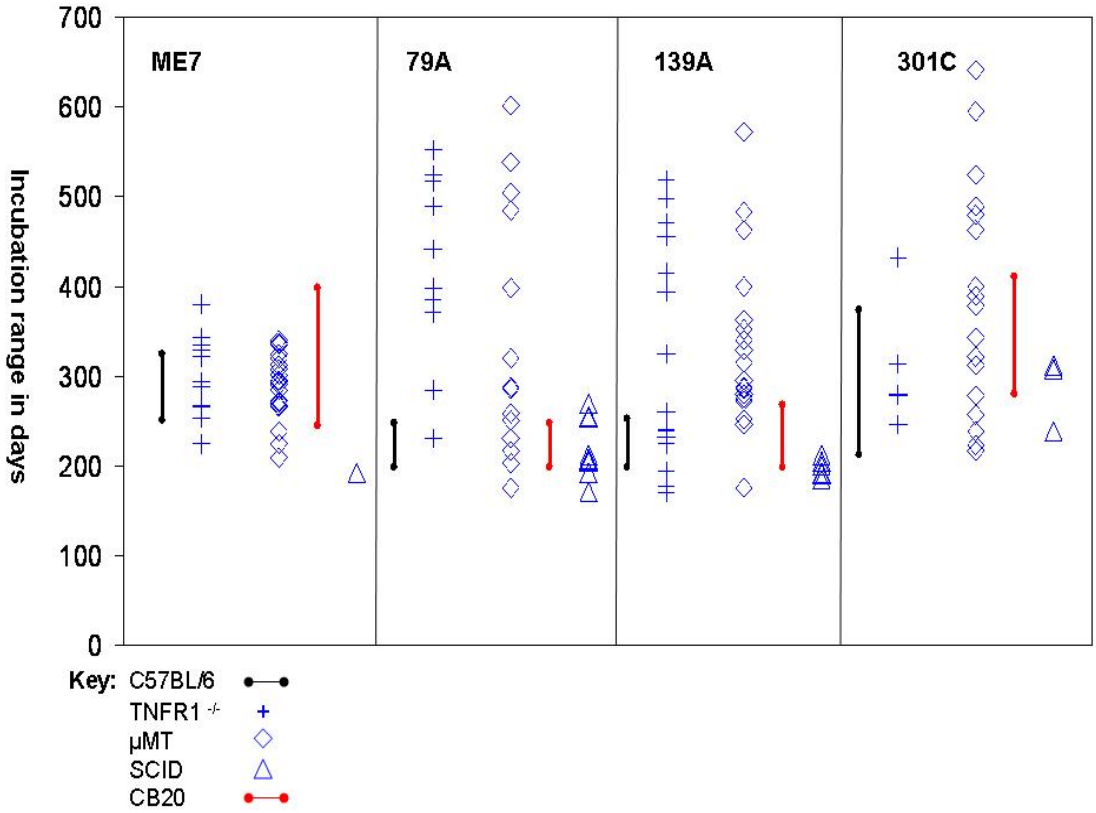


Figure 4.3 Graph showing incubation period (*y* axis) range (days) in TNFR1$^{-/-}$, µMT and SCID mice following i.p. infection with the ME7, 79A, 139A and 301C strains. Incubation periods shown represent all of the mice with clinical and positive TSE disease from all of the dilution groups. The graph also displays the shortest and longest incubation periods in the control C57BL/6 (●—●) and CB20 (●—●) for comparison. The most prolonged incubation periods are observed in the TNFR1$^{-/-}$ and µMT mice infected with the 139A and 79A agent strains. Although there is also evidence of incubation period prolongation in these mouse strains with the 301C agent strain, a wide range of incubation periods was also obtained for the control C57BL/6 and CB20 mice. Incubation periods of SCID mice with each of the TSE strains were shorter than, or within the range of the controls. An exception to this was the SCID mice infected with 79A agent strain where slightly longer incubation periods were observed in some SCID mice.

4.3.3 The peripheral pathogenesis of the ME7 scrapie strain in immunodeficient mice

Peripheral challenge of immunocompetent C57BL/6 and CB20 mice produced 100% incidence of disease at all dilutions with the exception of the CB20 mice infected with the 0.1% dilution where only 9/13 mice developed disease (figures 4.4, 4.5, 4.6). Peripheral infection of SCID, μMT and TNFR1$^{-/-}$ with the ME7 strain deficient mice produced the following results. At the highest dose (10%) the incidence of clinical and pathological disease was in 2/10 in SCID 14/17 in μMT and 11/13 in TNFR1$^{-/-}$ mice. These results obtained with the TNFR1$^{-/-}$ and μMT mice at high dose are very similar to those obtained previously with SCID mice (Fraser et al, 1996) where a high proportion of mice were susceptible to infection at high dose. Moreover, at high dose the majority of incubation periods in the TNFR1$^{-/-}$ and μMT mice occurred within the incubation period range of the ME7 strain. At high dose several μMT mice and one TNFR1$^{-/-}$ mouse had shorter incubation periods than those of the immunocompetent control mice, an occurrence that has been observed previously. At the 1% and 0.1% doses the incidence of disease was greatly reduced in the μMT and TNFR1$^{-/-}$ mice and those mice that did develop disease did so with similar incubation periods to those of the immunocompetent mice.

Disease incidence in SCID mice was lower than obtained previously with the ME7 strain although 3 mice that died within the incubation period range had pathological signs of disease. Notably only 9/13 CB20 mice succumbed to disease at the 0.1% dilution perhaps suggesting that the starting titre may have been slightly lower. At the lower dilution groups no SCID mice survived long enough to be included in the analysis.

Figure 4.4 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 10% dilution of the ME7 scrapie agent.

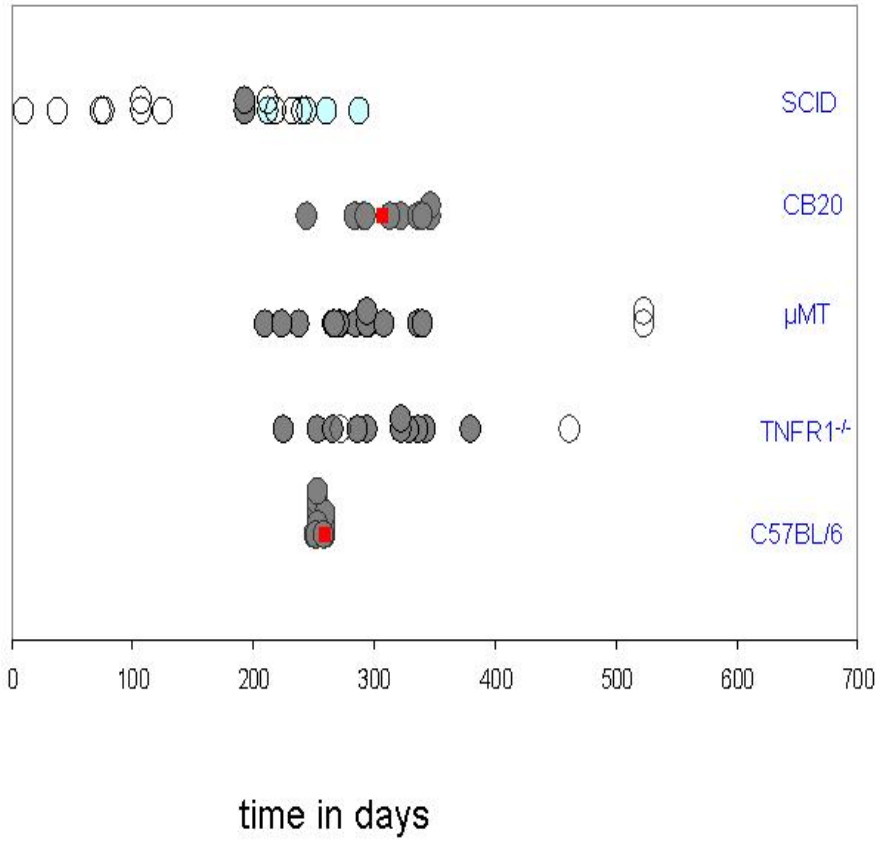


Figure 4.4. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 10% ME7 scrapie brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without clinical disease but with pathological disease in brain (●). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.5 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 1% dilution of the ME7 scrapie agent.

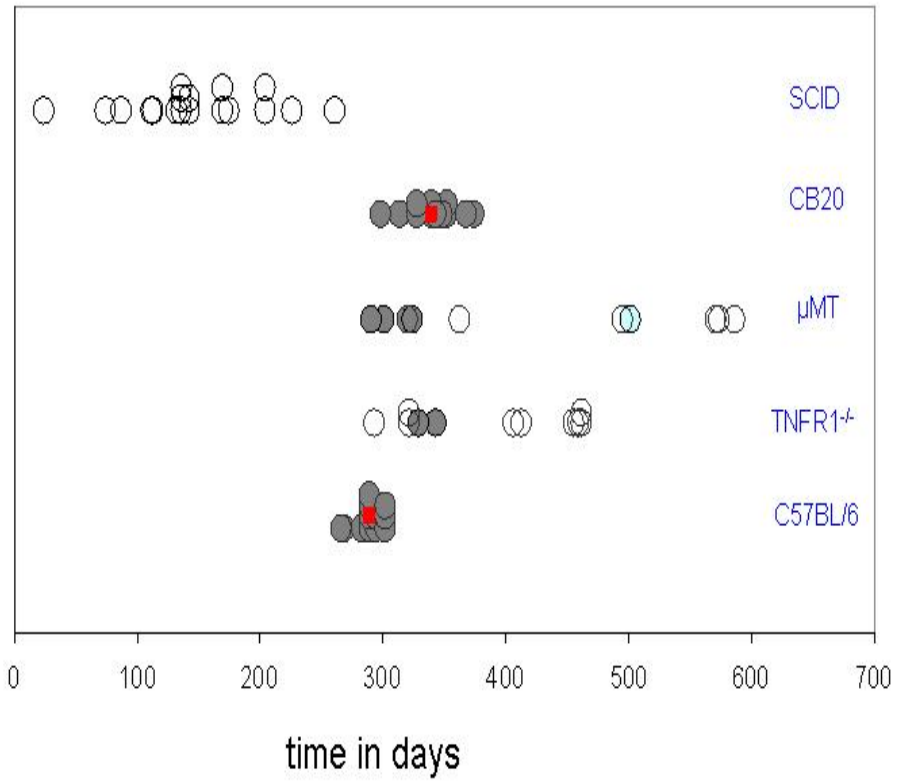


Figure 4.5. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 1% ME7 scrapie brain homogenate. Animals with both clinical and pathological disease in brain(●). Animals without clinical disease but with pathological disease in brain (◐). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.6 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 0.1% dilution of the ME7 scrapie agent.

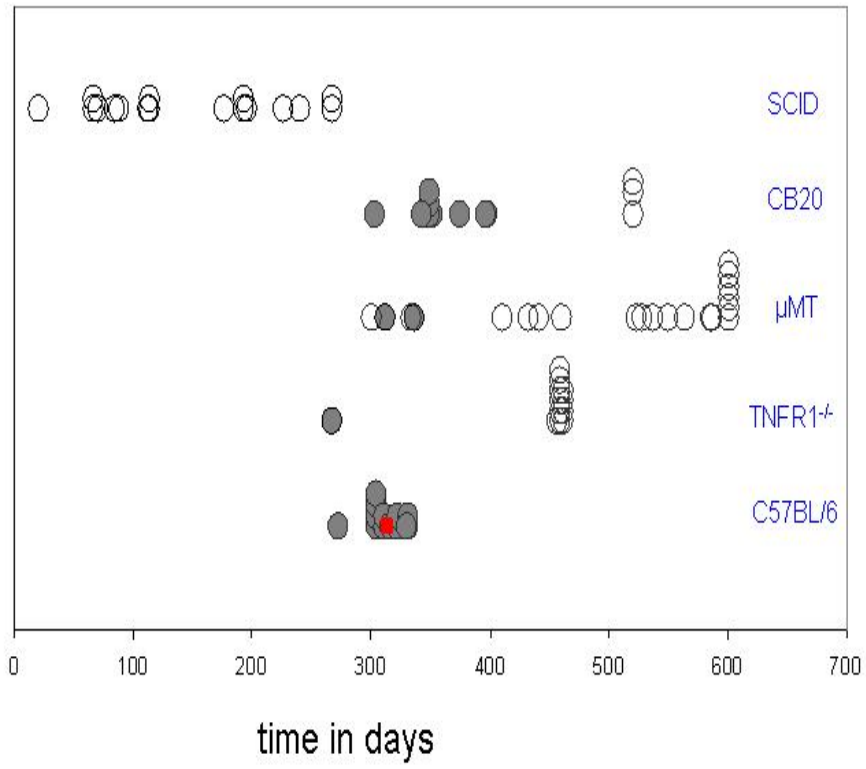


Figure 4.6. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 0.1% ME7 scrapie brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without clinical disease but with pathological disease in brain(◐). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.7 Lesion profiles of TNFR1$^{-/-}$ and C57BL/6 mice infected i.p. with ME7.

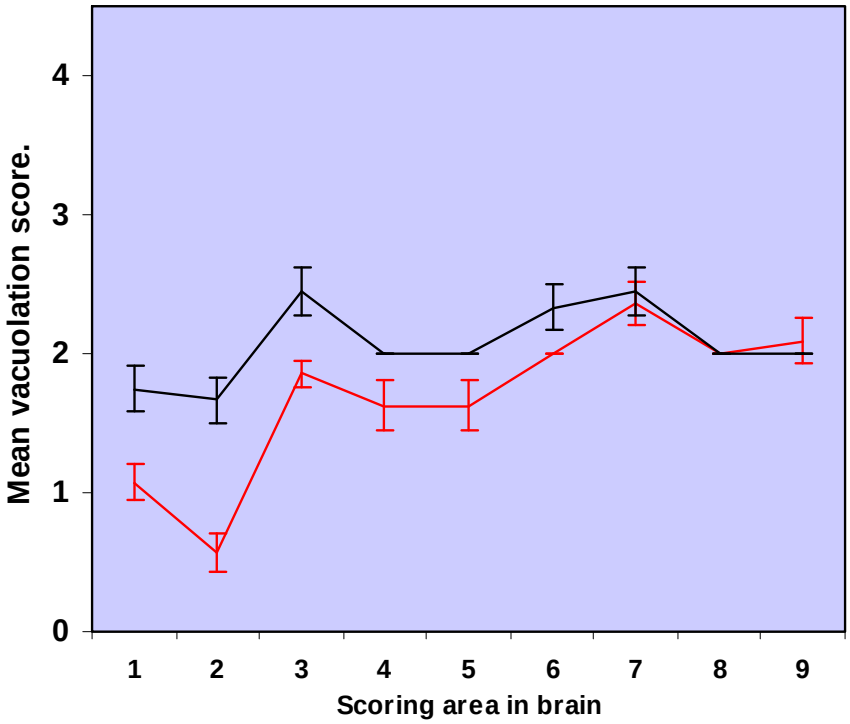


Figure 4.7. Assessment of the characteristic spongiform pathology in the brains of mice terminally affected with the ME7 scrapie agent strain The establishment of lesion profiles of terminally affected C57BL/6 and TNFR1$^{-/-}$ infected at the 10% dose was carried out. Analysis demonstrated that lesion profiles from the TNFR1$^{-/-}$ mice were comparable with that of the ME7 strain. As can be seen from the graph lower levels of pathology are observed in almost all of the areas examined in the C57BL/6 mice (red line) in comparison with the TNFR1$^{-/-}$ mice (black line).

4.3.4. The peripheral pathogenesis of the 139A scrapie strain in immunodeficient mice

Immunocompetent C57Bl/6 and CB20 mice were fully susceptible to infection throughout all the dilutions. At high dose, challenge of TNFR1$^{-/-}$ and μMT mice produced clinical and pathological disease in 12/15 and 13/16 mice respectively (figures 4.8, 4.9, 4.10). At high dose the majority of the TNFR1$^{-/-}$ mouse had incubation periods within the range of C57BL/6 mice, although two animals had much longer incubation periods. However infection of μMt mice at high dose produced a much wider range of incubation periods with all mice developing clinical and pathological signs of disease. At high dose infection 3 TNFR1$^{-/-}$ and 2 μMT mice developed disease before the C57BL/6 mice. At the 1% dilution disease incidence was similar in both TNFR1$^{-/-}$ and μMT mice, although the majority of the incubation periods were greatly prolonged (one μMT mouse developed clinical and pathological signs of disease at 570 days). At the 0.1% dilution (figure 4.10) the highest incidence of disease was in the TNFR1$^{-/-}$ mice with 4/15 mice developing disease (one was pathologically positive but clinically negative) and only one TNFR1$^{-/-}$ mouse developed disease within the incubation period range of the C57BL/6 mice. Infection of SCID mice at high dose produced almost 100% incidence of disease (7/8). As with the TNFR1$^{-/-}$ and μMT mice a number of SCID mice (n=4) developed disease before the first positive case in the CB20 control mice.

Figure 4.8 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 10% dilution of the 139A scrapie agent.

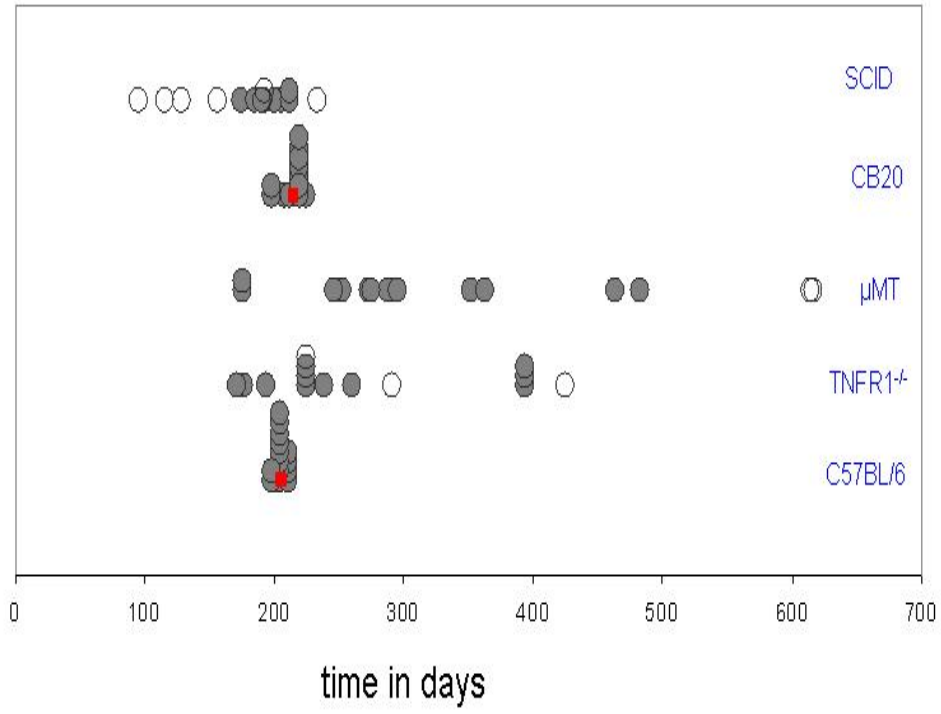


Figure 4.8. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 10% 139A scrapie brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without clinical disease but with pathological disease in brain (◐). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.9 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 1% dilution of the 139A scrapie agent.

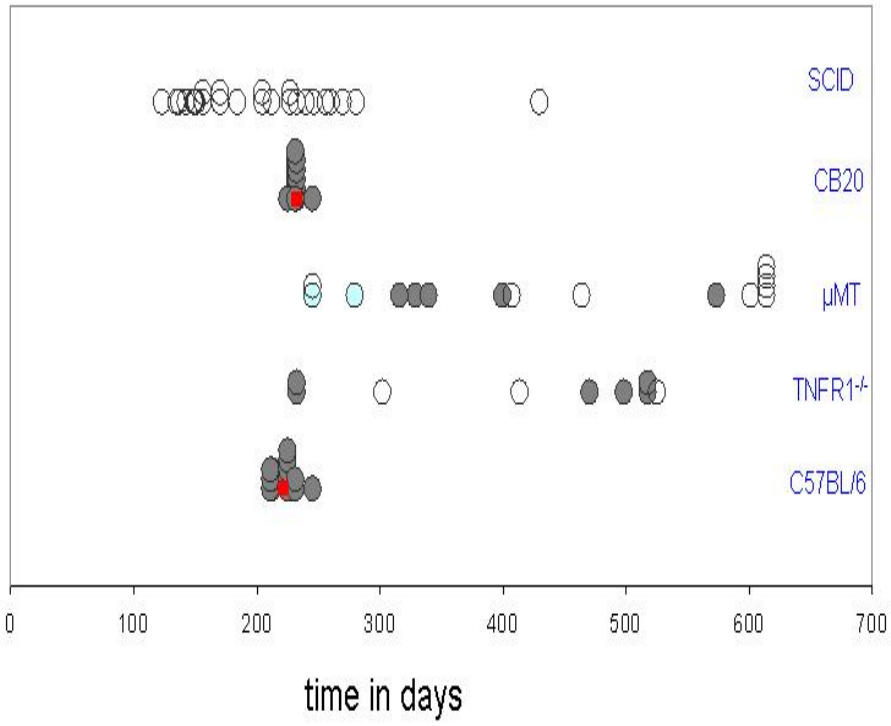


Figure 4.9. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 1% 139A scrapie brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without clinical disease but with pathological disease in brain (●). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.10 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 0.1% dilution of the 139A scrapie agent.

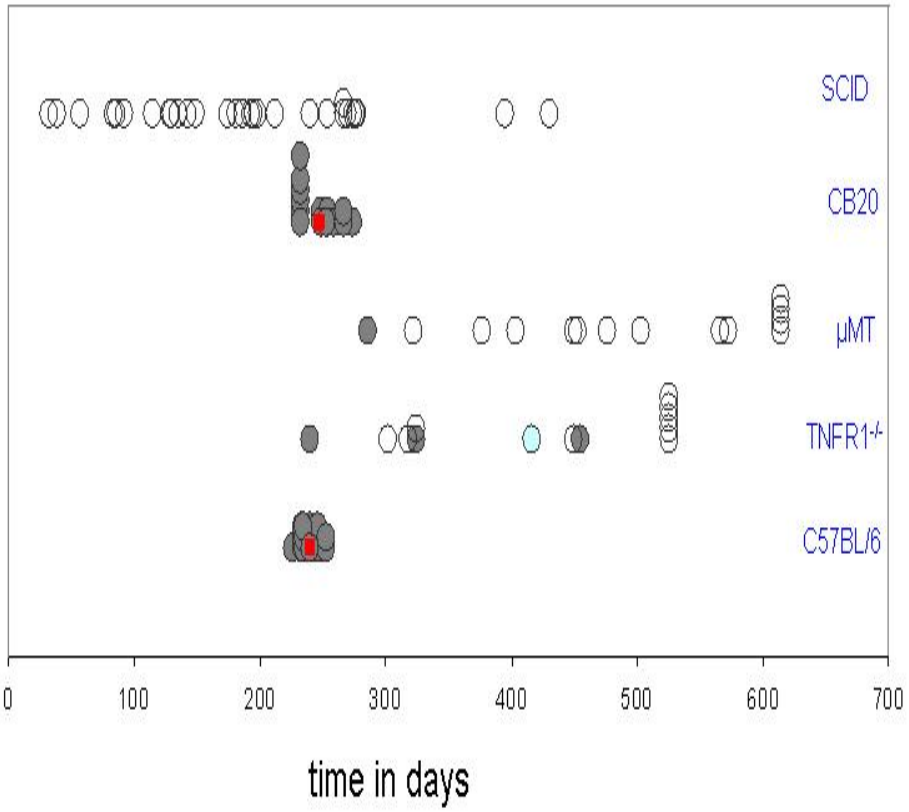


Figure 4.10. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 0.1% 139A scrapie brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without clinical disease but with pathological disease in brain (◐). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (▬)

4.3.5 The peripheral pathogenesis of the 79A scrapie strain in immunodeficient mice

Immunocompetent C57BL/6 and CB20 mice were fully susceptible to infection at all dilutions. At high dose challenge of TNFR1$^{-/-}$ and μMT mice produced disease in 6/11 and 11/15 mice respectively. However, only one TNFR1$^{-/-}$ mouse had an incubation period within the range of C57BL/6 mice, the remaining mice developing disease with much protracted incubation periods (figures 4.11, 4.12, 4.13). All of these mice had clinical and pathological signs of disease. Infection of μMt mice at high dose produced some incubation periods within the range of the C57BL mice but as with the TNFR1$^{-/-}$ mice, a number of mice had very long incubation periods, all with clinical and pathological signs of disease. At the lower dilutions (1% and 0.1%) the TNFR1$^{-/-}$ and μMT mice that developed disease had very prolonged incubation periods. At the 0.1% dilution nearly 30% of TNFR1$^{-/-}$ mice developed disease again with very prolonged incubation periods. Only one μMT mouse had evidence of disease at the 0.1% dilution although this animal was killed intercurrently without clinical disease. Infection of SCID mice at high dose produced high incidence of disease although there were no mice surviving past 260 days in the 10% dilution group. Therefore it is not known if longer incubation periods could have developed in these mice if more animals had survived. At the lower dilutions only one SCID mouse developed disease in the 1% dilution group at 255 days, again survival was poor in these mice with the longest surviving animal at just under 300days.

Figure 4.11 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 10% dilution of the 79A scrapie agent.

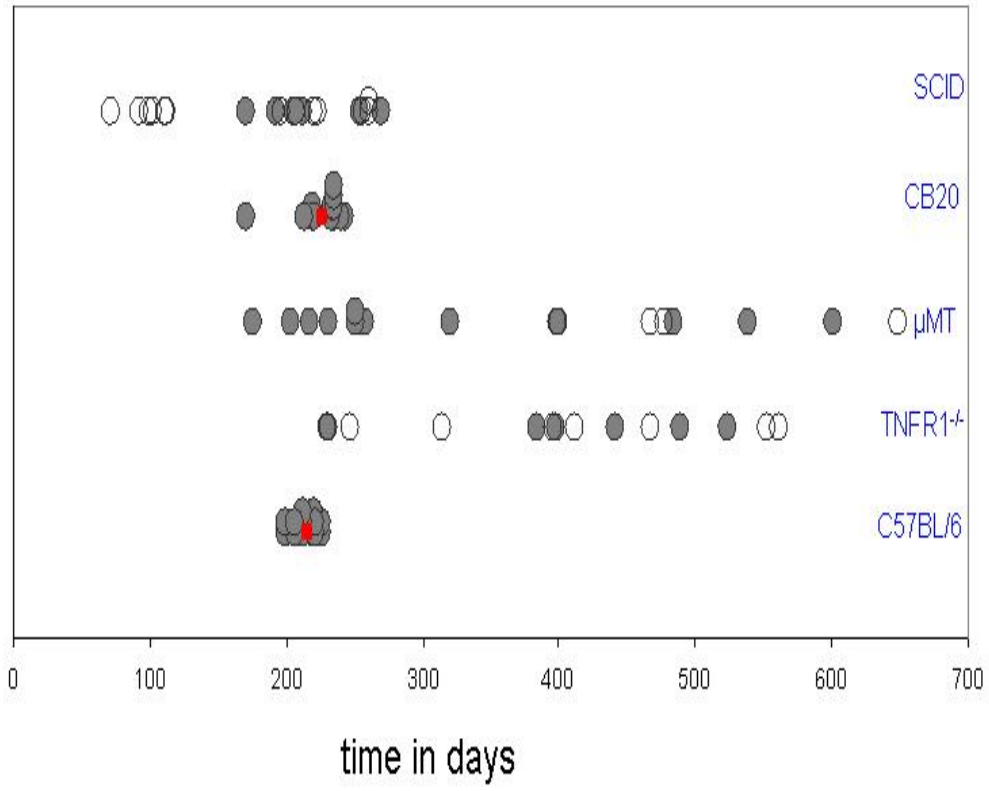


Figure 4.11. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 10% 79A scrapie brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.12 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 1% dilution of the 79A scrapie agent.

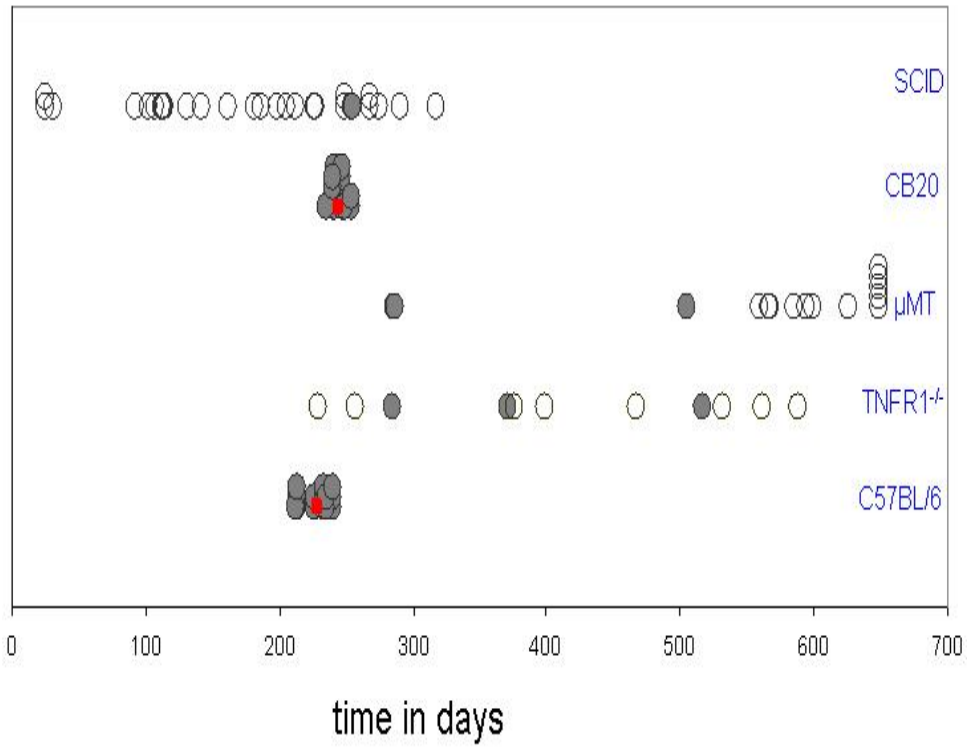


Figure 4.12. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 1% 79A scrapie brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.13 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 0.1% dilution of the 79A scrapie agent.

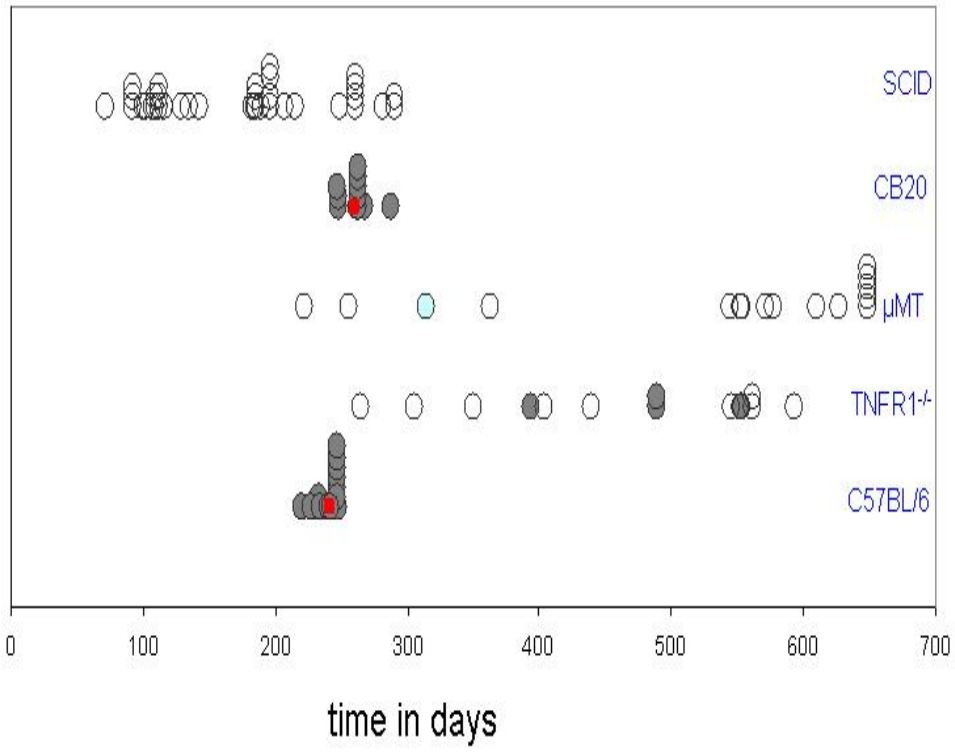


Figure 4.13. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 0.1% 79A scrapie brain homogenate. Animals with both clinical and pathological disease (●) in brain. Animals without clinical disease but with pathological disease in brain (○). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

4.3.6. The peripheral pathogenesis of the 301C TSE agent in immunodeficient mice

Immunocompetent CB20 mice were fully susceptible to infection with 301C at all dilutions however incidence of disease was not complete at the 1% (15/16) and 0.1% (13/16) groups in the C57BL/6 (figures 4.14, 4.15, 4.16). As found with all the other TSE strains the highest incidence of disease in the immunodeficient mice occurred at the highest infection group (10%).

There were some similarities with the 301C strain and those of the 139A and 79A strain in that some very prolonged incubation periods were obtained; primarily at the highest dilution (10%). However unlike the 79A and 139A strains few immunodeficient mice developed disease at the lower dilutions with the 301C strain. At the 1% and 0.1% dilution there was no clinical disease in any of the $TNFR1^{-/-}$ mice although one $TNFR1^{-/-}$ mouse in the 1% dilution group had positive vacuolar pathology in brain. Two μMT mice had clinical and pathological signs of disease at the 1% dilution, with one animal developing clinical signs of disease almost 600 days after original infection. At the 0.1% dilution only one μMT mouse developed clinical disease and this was within the range of the C57BL/6 mice. These findings demonstrate that 301C strain requires a functional immune system for effective pathogenesis and while some prolongation of incubation periods were recorded it is possible this may reflect lower starting titre of inoculum as control C57Bl/6 were not fully susceptible to infection at the lower dilution groups (1% and 0.1%).

Figure 4.14 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 10% dilution of the 301C TSE agent.

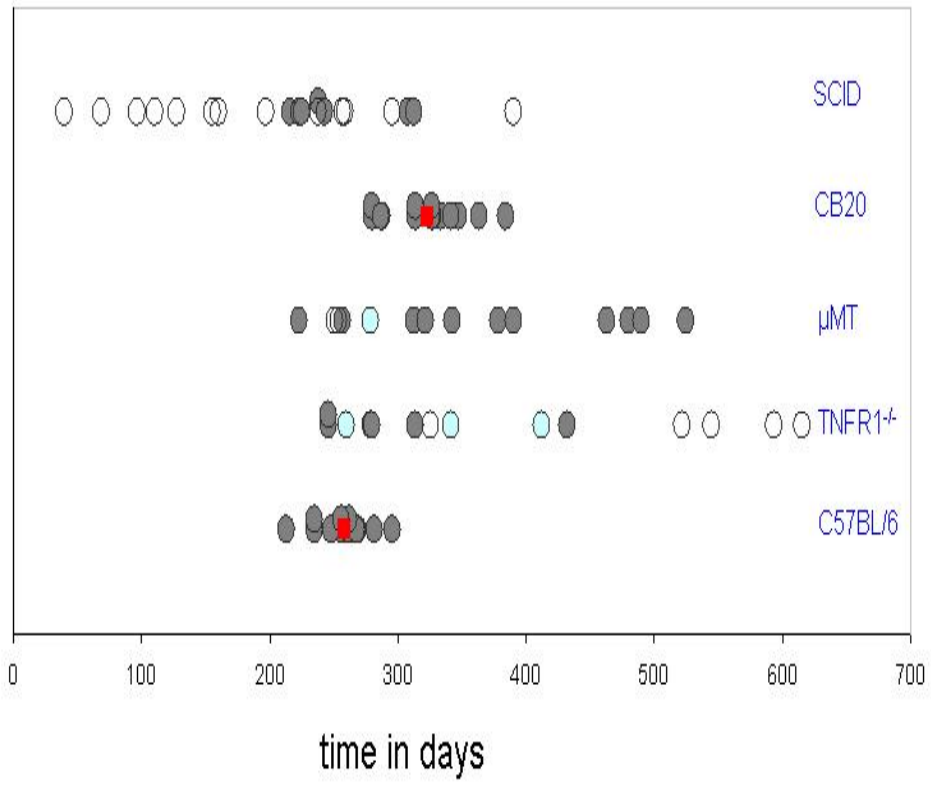


Figure 4.14. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 10% 301C TSE brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without clinical disease but with pathological disease in brain (◐). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.15 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 1% dilution of the 301C TSE agent.

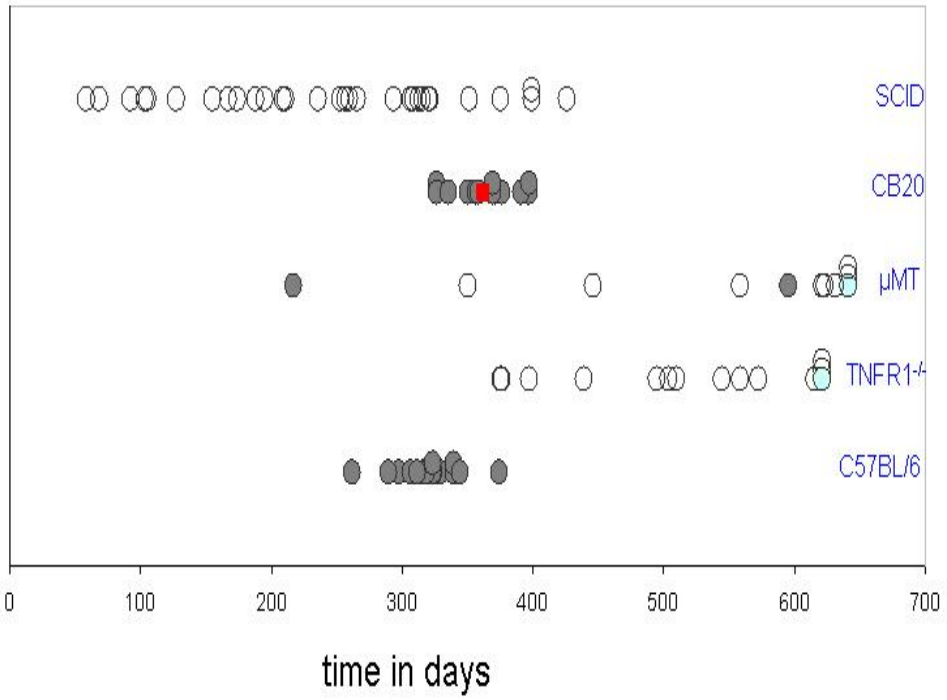


Figure 4.15. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 1% 301C TSE brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without clinical disease but with pathological disease in brain (◐). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

Figure 4.16 Disease pathogenesis in immunocompetent and immunodeficient mice following i.p. exposure to a 0.1% dilution of the 301C TSE agent.

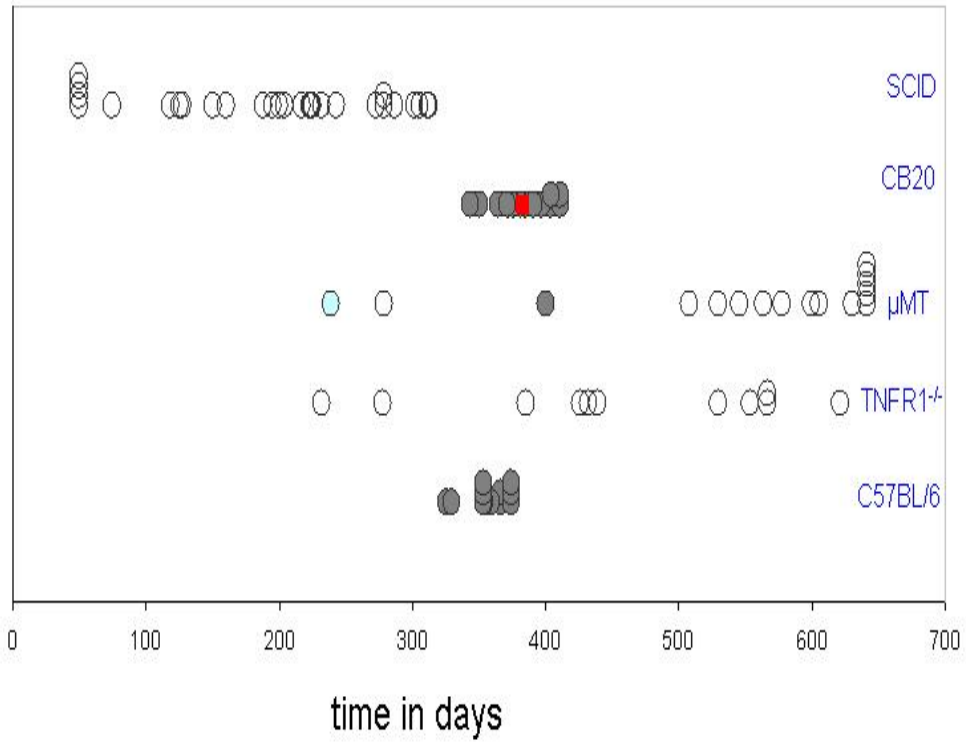


Figure 4.16. Disease pathogenesis in immunocompetent (CB20and C57BL/6) and immunodeficient (SCID, TNFR1$^{-/-}$ and µMT) mice following i.p. injection with a 0.1% 301C TSE brain homogenate. Animals with both clinical and pathological disease in brain (●). Animals without clinical disease but with pathological disease in brain (◐). Animals without disease (○). Mean incubation periods in groups with 100% disease incidence (■)

4.3.7 Disease pathogenesis following intracerebral infection of immunodeficient mice with the ME7, 79A, 139A and 301C strains.

In this study all of the immunodeficient mice were injected i.c. with the TSE agent strains to determine if these immune system defects had any implications for development of TSE disease itself within the CNS.

As expected, after i.c. injection all SCID, μMT and TNFR1$^{-/-}$ mice were fully susceptible to infection indicating that their immunological defects had no direct effect on the development of disease in the CNS (table 4.4). Following i.c. injection of μMt and TNFR1$^{-/-}$ mice shorter incubation periods of disease were observed with all of the TSE agents with the exception of μMT mice infected with the ME7 agent strain. In the SCID mice however only the ME7 agent strain infected and 301C agent strain injected animals had sorter incubation periods; no differences in incubation period were observed following i.c. challenge of SCID mice with the 79A and 139A agent strains.

Some ME7 agent strain infected SCID mice were killed prior to the development of clinical disease due to intercurrent illness. While analysis of these brains from these animals showed they were pathologically positive it is difficult to predict when these mice would have developed terminal disease.

Table 4.4. Pathogenesis of a range of TSE agent strains in immunodeficient mice following intracerebral injection

TSE strain		**scrapie incidence/incubation period**			
	CB20	**SCID**	**C57BL**	**TNFR1**	**µMT**
Cell Defect	***none***	***FDCs B/T cells***	***none***	***FDCs***	***B cells/FDCs***
TSE strain					
ME7					
incidence	9/9	3/3	9/9	10/10	13/13
incubation	186±1	114±3*	175±5	181±2	160±0.1
139A					
incidence	15/15	8/9	7/7	10/10	12/12
incubation	155±2	154±2	180±5	172±1	171±1
79A					
incidence	7/7	18/18	12/12	10/10	12/12
incubation	165±2	166±2	183±1	172±1	171±1
301C					
incidence	8/8	15/15	7/7	11/11	5/5
incubation	196±1	182±3	183±1	179±3	168±1

*** All mice were path positive but were culled with intercurrent illness.**

4.3.8 TSE agent infectivity levels in spleen of immunodeficient mice following i.p. or i.c. injection with the ME7, 79A, 139A and 301C TSE agent strains

TSE agent infectivity bioassay of spleen, collected at the terminal endpoint of disease was performed from all of the mouse and TSE strain combinations in the original pathogenesis studies. All of the spleens collected were from terminally affected animals and all taken from the 10% infection groups. The purpose of these assays was to determine if spleens from μMT or TNFR1$^{-/-}$ mice with clinical and pathological signs of disease contained TSE agent infectivity. Additional bioassays were performed from intracerebrally ME7 challenged μMt and C57BL/6 mice collected 5 and 10 weeks post challenge. As the i.c. route of infection represents an efficient way of routing infection to the spleen (Fraser & Dickinson, 1970; Kimberlin & Walker, 1979; Millson et al, 1979) these assays were performed to establish if replication of infectivity was occurring in spleen at these early stages of infection. To accurately measure the infectivity titre in spleen, titrations of spleen from terminally affected C57BL mice infected with each of the TSE agent strains was also carried out to produce a dose response curve for each TSE agent strain.

Bioassay of spleen from C57BL/6 mice terminally affected with the 139A, ME7 and 79A TSE agent strains produced 100% incidence of disease in all of the recipient assay mice demonstrating a high titre of agent infectivity in these spleens. Bioassays of spleens from terminally affected, peripherally infected μMT or TNFR1$^{-/-}$ mice showed that low, but consistent, levels of infectivity could be detected in spleen in all the TSE strains tested. However levels of infectivity in spleen from μMT mice were undetectable at 5 and 10 weeks post infection in comparison with immunocompetent

C57BL/6 spleen where high levels of infectivity were detected (table 4.5 and figure 4.17).

In previous studies the isolation of the 87A strain of scrapie was observed following bioassay of SCID spleen. To determine if this had occurred in these studies lesion profiles were constructed from brains of bioassay mice following passage of TNFR1-/- or μMT spleen. However lesion profiles of these brains demonstrated the presence of the ME7 scrapie agent strain (figure 4.18).

Table 4.5. Infectivity bioassay of spleen from control and immunodeficient mice originally infected i.c. or i.p with the ME7, 139A, 79A and 301C strains. Bioassay in C57BL mice showing disease incidence/mean incubation period (or incubation range)

TSE strain/original route of inoculation/time of collection of bioassay spleen												
TSE strain	**ME7**			**ME7**		**139A**		**79A**		**301C**		
route	**i.c.**	**i.c.**		**i.p.**		**i.p.**		**i.p**		**i.p**		
time of collection	**5wpi**	**10wpi**		**terminal**		**terminal**		**terminal**		**terminal**		
Spleen Origin												
C57BL/6	7/7 205±3 8/8 206 ±6	11/11 190±2 9/9 196±2		11/11 12/12	204±8 203±3	9/9 8/8	180±2 177±3	12/12 6/6	182±3 175±0.5	9/9 10/10	216±2 200±3	
$\mu MT^{-/-}$	1/11(418) 0/12	0/10 1/12 (242)		9/10 7/12	(204-512) (253-399)	7/11 6/12	(215-454) (189-370)	9/12 10/11	(202-258) (215-250)	5/12 2/12	(292-505) (351, 502)	
TNFR-$1^{-/-}$	NT NT	NT NT		5/12 NT	(313-524)	1/12* NT	(345)	1/12 3/12	(214)* (243, 236, 393)	5/12 8/12	(292-505) (242-263)	

* animal was clinically negative but had positive vacuolar pathology

Figure 4.17 Infectivity titres in spleen from immunocompetent or immunodeficient mice

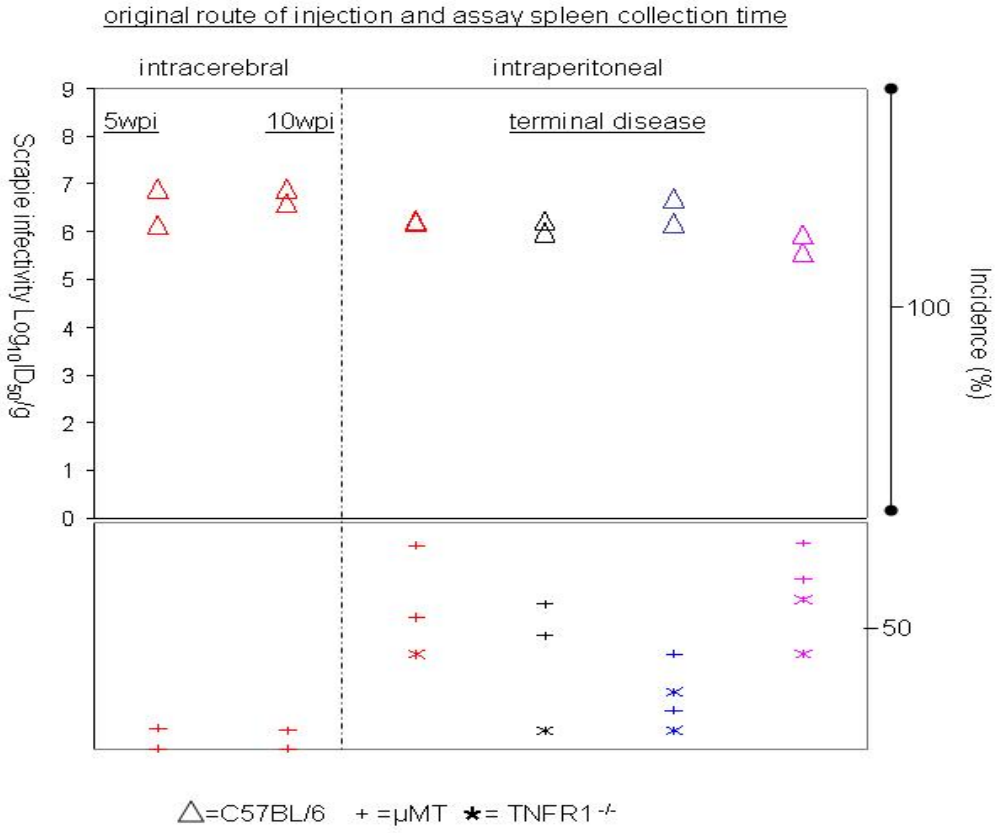


Figure 4.17. Infectivity titres in spleen from immunocompetent or immunodeficient mice Estimation of infectivity titres in spleen ME7 (red), 139A (black), 301C (blue) or 79A strains (pink). Bioassay of spleen from immunocompetent C57BL/6 mice produced 100% incidence of disease in all assay mice with all of the TSE strains and all of the timepoints sampled. None of the spleens assayed from the immunodeficient mice produced 100% incidence of disease in the assay mice however there were clear differences between disease incidence in assay mice depending on the original route of inoculation with a greater incidence obtained from spleen collected from peripherally challenged immunodeficient mice.

Figure 4.18 Analysis of lesion profiles in the brains of recipient mice following sub-passage of spleen from immunodeficient mice

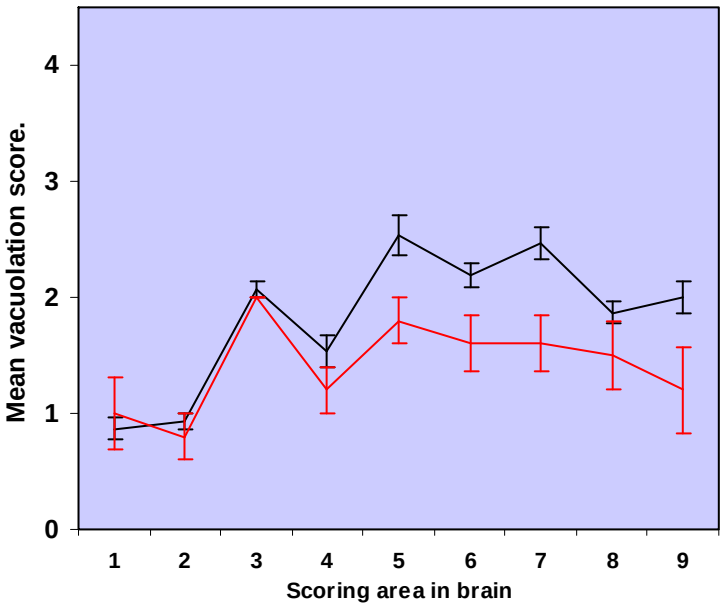


Figure 4.18. As described previously sub-passage of spleen from ME7 infected SCID mice resulted in the isolation of the 87A agent strain. In these studies the establishment of lesion profiles of terminal C57BL mice following bioassay of spleen from µMT (-) and TNFR1$^{-/-}$ (-) was conducted to determine if this had occurred in these bioassays. However, analysis showed that the TSE strains isolated from these spleens had histopathology consistent with that of the ME7 agent strain.

4.3.9 Immunolocalisation of PrP protein in imunodeficient mice following infection with the ME7, 139A and 79A TSE agent strains

Immunohistochemical (IHC) analysis of spleen was performed at 10 weeks post i.c. and i.p. injection of C57BL/6, TNFR1$^{-/-}$ and μMT mice with the 139A, 79A and ME7 TSE agent strains (figure 4.19, figure 4.20, table 4.6) (Farquhar et al, 1989). IHC and PET blot analysis of spleens from terminally affected with the ME7 scrapie agent strain was also undertaken (figure 4.19).

IHC analysis of spleen from all of the i.p. or i.c. infected immunodeficient TNFR1$^{-/-}$ or μMT mice failed to detect PrP protein with the exception of some very limited labelling in spleen from mice infected i.c. with the ME7 (n=1) or the 139A (n=1) TSE agent strain. These localisations of PrP protein were only detected in association with one or two cells in these spleens the cellular specificity of which is unknown (figure 4.20, table 4.6). In addition, as the polyclonal antibody used here recognises both the normal and disease specific forms of the protein (Farquhar et al, 1989) it is impossible to determine whether these foci of labelling represents the disease specific form of PrP (PrP^{Sc}). Further analysis by PET blotting (Schulz-Schaeffer et al, 2000) to discriminate between PrP^{c} and PrP^{Sc} is required.

IHC and PET blot analysis of spleen from terminally affected μMT and control C57BL/6 mice found robust accumulations of PrPSc in association with FDCs in the C57BL/6 mice but failed to detect PrP^{Sc} in the μMT mice (figure 4.19).

Figure 4.19. Immunolocalisation of PrP in spleen at the terminal stage of disease in i.p. ME7 scrapie infected C57BL/6 and µMT mice.

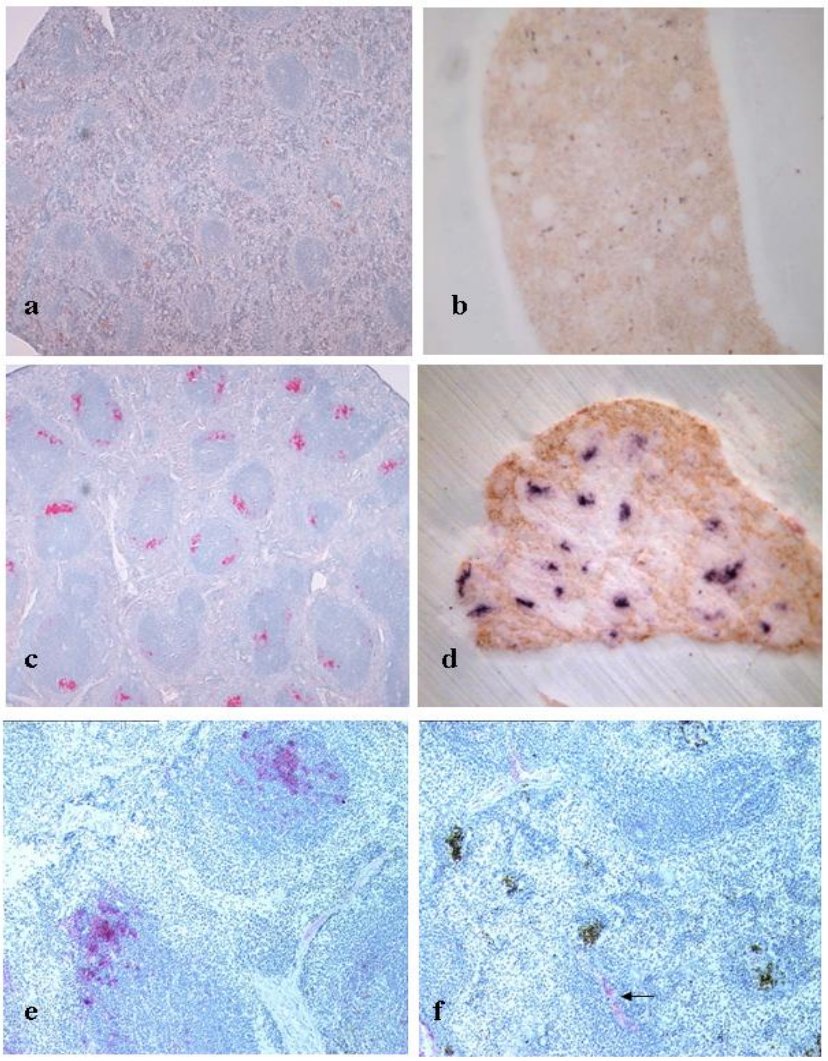


Figure 4.19. IHC and PET blot analysis of spleens from C57BL/6 or µMT mice infected with the ME7 strain of scrapie. Both the µMT and C57BL/6 mice developed terminal disease following i.p. challenge with ME7 (a,b). No PrP^{Sc} can be detected by IHC (a) or PET blot analysis (b) in µMT mice. Heavy deposits of haemosiderin can be detected within the µMt spleen (a) (c)PrP^{Sc} (red) is localised to FDCs in terminal C57BL/6 mice. (d)PET blots from terminal C57BL/6 mice show abundant PrP^{Sc} (dark blue). (d) IHC analysis using the FDC-M1 antibody to detect FDCs only detects labelling in C57BL mice (e). No labelling for FDCs in GCs can be detected in µMT mice (f) however some foci of labelling can be seen (black arrow). similar results have been found in immunodeficient and neonatal mice with this marker(Ierna et al, 2006)and is thought to represent immature FDCs. (figure a, c original magnification X40, figures b,d original magnification x20, figures e,f original magnification X100).

Figure 4.20 Immunolocalisation of PrP in spleen at 10 weeks post infection.

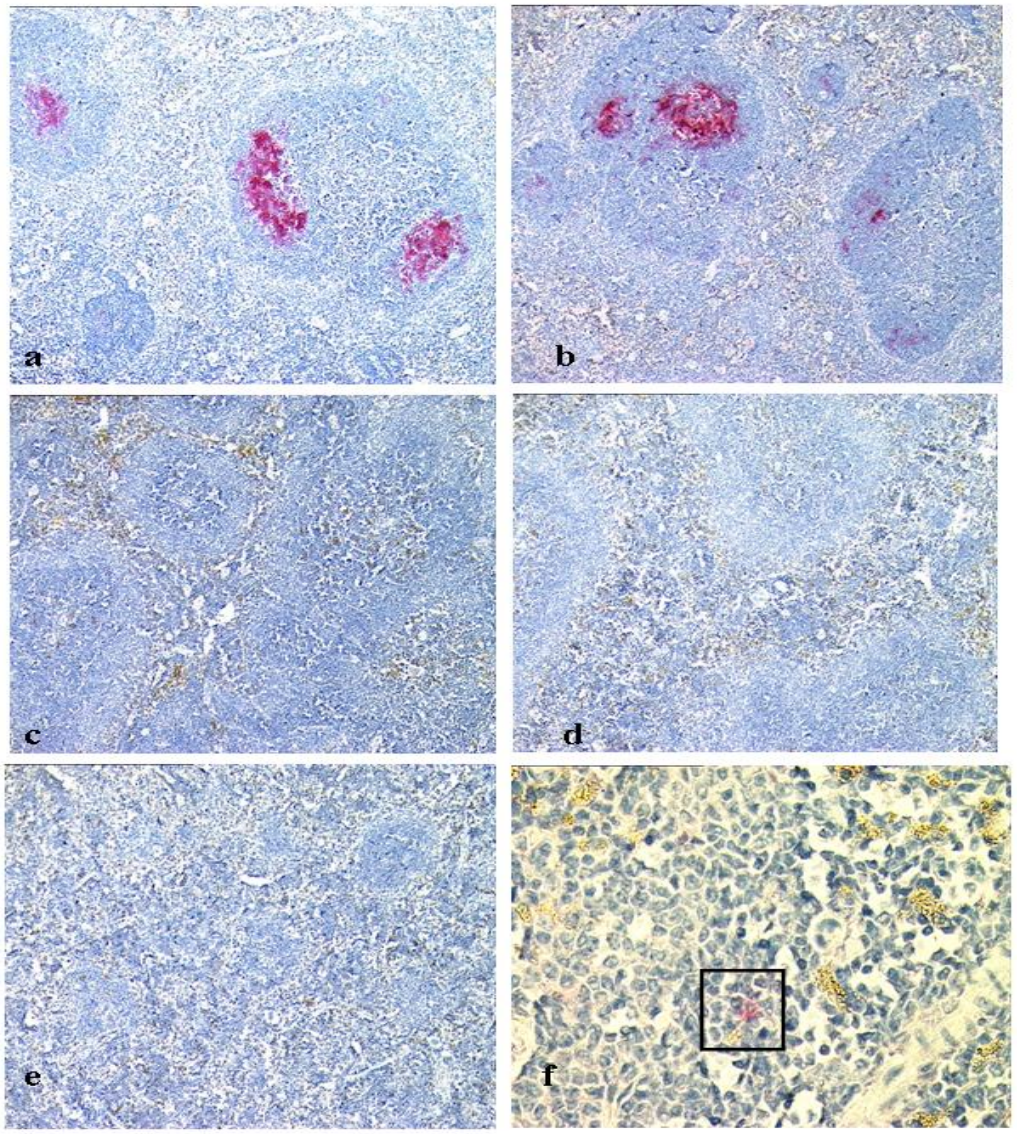


Figure 4.20 High levels of PrP^{Sc} accumulation in spleens from i.p. infected C57BL/6 mice collected 10 weeks after exposure (a,b). (a) 79A agent strain infected C57BL/6 mouse (b) 139A agent strain infected C57BL/6 mouse. PrP^{Sc} is undetectable in spleen from peripherally challenged $TNFR1^{-/-}$ mice at 10 weeks post infection with either the (c) 79A or (d)139A agent strains. PrP^{Sc} is also undetectable in spleen from an i.p. 79A agent strain infected μMT mouse (e). Isolated foci of probable PrP^{Sc} labelling in spleen from a $TNFR1^{-/-}$ mouse infected with the 139A agent strain (f). (a-e) original magnification X100, (f) original magnification X600.

Table 4.6 Summary of IHC localisation of PrP in the spleen at 10wpi

	TSE agent strain					
	ME7		**79A**		**139A**	
	Route		**Route**		**Route**	
Mouse strain	**i.c.**	**i.p**	**i.c.**	**i.p.**	**i.c.**	**i.p.**
μMT	-	-	-	-	NT	NT
$TNFR1^{-/-}$	*	-	-	-	*	-
C57BL/6	+	+	+	+	+	+

NT: not tested

+: Detection of PrP in spleen in association with FDCs.

*** small foci of PrP detected in association with single cells but not FDCs**

Discussion

The primary aim in this chapter was to determine the role of FDCs and/or other cell types in the pathogenesis of a range of TSE agent strains following peripheral exposure. This work also aimed to determine the effect of infectious dose on disease pathogenesis with a range of TSE agent strains.

Although these studies demonstrate some variations in the peripheral pathogenesis of different TSE agent strains, the *major* finding of these studies is that neuroinvasion in the absence of FDCs is severely impaired following peripheral exposure to a range of TSE strains. As TNFR1$^{-/-}$ mice lack mature FDCs but have normal B and T cell populations this would suggest that B cells are not directly responsible for neuroinvasion in the TSE strains tested and show that functional FDCs are required for efficient pathogenesis. This finding is also supported by studies in μMT mice, which lack B cells and as a result functional FDCs.

There was a profound effect of dose on these immunodeficient mice as demonstrated previously in studies with SCID mice (Fraser et al, 1996) and the highest incidence of disease was observed at high dose in all of the immunodeficient mouse strain and agent combinations demonstrating that this effect is not specific to the ME7 strain of scrapie. At the lower dilutions incidence of disease was greatly reduced in all of the TSE and mouse strain combinations as previously observed with ME7.

In the studies presented here the most notable differences were observed between the ME7 strain and the 79A and 139A strains, with perhaps the most extreme differences between ME7 and 79A agent strains. The experimental strain termed “ME7” was

originally isolated from spleen from a natural scrapie case (Zlotnik & Rennie, 1965). ME7 is often used as a "prototype" for the study of natural scrapie and vCJD due to its extensive involvement in the lymphoid system and studies in radiation chimaeric and immunodeficient mice have shown that this strain depends on functional FDCs for neuroinvasion and replication in spleen

With the ME7 strain the development of disease in the immunodeficient (SCID, μMT and TNFR1$^{-/-}$) mice occurred predominantly within the incubation period range normally associated with this TSE strain agent. Following infection with moderate and lower doses of infectivity (1% and 0.1% dilutions) clinical and pathological disease only occurred in a few animals, again with incubation periods within the range of the C57BL/6 mice.

A feature of the 79A and 139A strains in μMT and TNFR1$^{-/-}$ mice was the extensively prolonged incubation periods of disease, notably these prolonged incubations were not observed with the ME7 strain suggesting these effects on pathogenesis may be a feature of the these strains, although a few prolonged incubation periods were also observed with 301C agent strain. In-fact, in the 79A agent strain most of the incubation periods in the immunodeficient mice that developed disease were much extended, even at the highest dilutions. The situation is less clear in the 139A strain where a combination of short and long incubation periods were observed. Prolonged incubation periods were not seen in SCID mice with the 79A or 139A agent strains although only a few 79A infected SCID mice survived beyond the incubation periods of the CB20 mice. However, a proportion of

139A infected SCID mice survived until almost 420 days without developing clinical or pathological signs of disease. Both the 79A and 139A strains of scrapie were originally derived from a pool of natural sheep scrapie brains following passage through goats and then subsequent passage in inbred strains of mice. One of the characteristics of the 79A strain of scrapie is its rapid replication of infectivity in lymphoid tissues following infection by a variety of routes. As early as 20 days post infection high levels of infectivity can be detected in spleen and lymph nodes of 79A scrapie infected mice. Preliminary analysis of lesion profiling from immunodeficient mice infected with the 79A and 139A agent strains suggests that there are no alterations in lesion profiling with either of these strains.

Based on the results presented here it is possible that subtle differences in pathogenesis exist between TSE agent strains in the peripheral lymphoid system with perhaps the greatest differences observed between the ME7 and 79A strains. With the ME7 strain it seems likely that at high dose direct peripheral nerve infection occurs, without lymphoid system involvement producing a high incidence of disease, with similar incubation periods to those of the control mice. However with the 139A and particularly the 79A strains seems there may be alternative mechanisms to peripheral nerve infection. These alternative routes may represent inefficient routes of pathogenesis resulting in the extensively prolonged incubation periods observed with these TSE strains. Although both the μMt and TNFR1$^{-/-}$ mouse strains lack functional FDCs μMT mice have functional T cells and TNFR1$^{-/-}$ mice functional B and T cells. This “alternative” mechanism may disseminate infection to the CNS via haematogenous routes, possibly following low level replication or sequestration of

infectivity by cells other than FDCs, e.g. macrophage or lymphocyte populations. The fact that prolonged incubation periods were not observed in 139A infected SCID mice, which lack B and T lymphocytes, may support these suggestions. However such a mechanism does not appear to occur with the ME7 agent strain which may rely solely on replication upon FDCs and subsequent peripheral nerve infection to infect the CNS. While results suggest there may be differences in pathogenesis between TSE strains further analysis of this data is required to explore these possibilities further.

Transmission of primary BSE from a number of sources shows remarkable uniformity demonstrating that the BSE agent contained a single major agent strain. In these transmissions to mice, the 301C and 301V strains were isolated. The 301C agent strain was isolated from mice of the (*Sinc*^{s7}or *Prnp*^{a}) and the 301V agent strain from inbred mice of the *Sinc*^{p7} or *Prnp*^{b} genotype (Bruce et al, 2002). One of the most unique features of these primary BSE transmissions is a 100 day difference in mean incubation period between two *Sinc*^{s7} mouse strains, RIII and C57BL (Bruce et al, 1997). However, passage of brain from either *Sinc*^{s7}strain results in the isolation of the 301C agent strains demonstrating that this anomaly in incubation period is not a result of the selection of different strains (Bruce et al, 2002). The availability of this mouse adapted BSE strain provided the opportunity to investigate the lymphoreticular involvement of a BSE derived strain using mouse models. There were some similarities with the 301C strain and those of the 139A and 79A strain in that some very prolonged incubation periods were obtained. However unlike the 79A and 139A strains few immunodeficient mice developed disease at the lower dilutions

with the 301C strain. These findings demonstrate that 301C strain requires a functional immune system for effective pathogenesis and while some prolongation of incubation periods were recorded it is possible this may reflect lower starting titre of inoculum as control C57Bl/6 were not fully susceptible to infection at the lower dilution groups.

The hypothesis that different TSE strains may have different cellular targets in the peripheral lymphoid system was partly generated by the discrepancies in studies carried out in our unit with the ME7 agent strain and those carried out elsewhere with the RML isolate. Although the results presented in this chapter show some variation between strains, especially between the ME7 and 79A agent strains the presence of mature FDCs remains a requisite for efficient pathogenesis with all of the strains tested although results suggest that other cell types or mechanisms may participate in the absence of these cells. In-fact, studies published within the last few years provide support for the role of FDCs in the peripheral pathogenesis of the RML isolate (Montrasio et al, 2000). Studies using the lymphotoxin β receptor/immunoglobulin fusion protein to temporarily de-differentiate FDCs show that the accumulation of the scrapie agent in the spleen and subsequent neuroinvasion were impaired following infection with either the ME7 agent strain (Mabbott et al, 2000a) or the RML scrapie isolate (Montrasio et al, 2000) demonstrating a critical requirement for mature FDCs in the pathogenesis of both the ME7 and RML agents.

When mouse passaged TSE strains are injected i.c. into immunocompetent mice the peripheral lymphoid system becomes infected shortly after infection, most probably via haematogenous spread of disease. Indeed the i.c. method of infection is an efficient way of routing infection to the lymphoid tissues (Fraser & Dickinson, 1970; Kimberlin & Walker, 1979). In previous studies where SCID mice were infected i.c. with the ME7 strain of scrapie mice were fully susceptible to infection demonstrating that this route of infection does not require the presence of a competent immune system, at least with mouse passaged strains (O'Rourke et al, 1994); (Lasmezas et al, 1996); (Fraser et al, 1996); (Brown et al, 1997). An interesting observation in the studies presented here is the shorter i.c. incubation periods in all of the μMt and TNFR1$^{-/-}$ mice (with the exception of the ME7 agent strain infected μMT mice) with all of the TSE agent strains tested, although incubation periods in SCID mice were only shorter with the 301C and ME7 agent strains. In these instances it is possible that immune system deficiencies may impact on this route of infection. One possibility is that lack of sites of replication sites or less PrP-expressing cells in lymphoid tissues in immunodeficient mice may mean more infectivity is concentrated in the CNS and not sequestered by the peripheral lymphoid system following infection.

It would seem logical that infection via peripheral routes would be the most efficient way of routing infection to lymphoid tissues however infection via intracerebral routes is an effective way of initiating infection in the spleen. Paradoxically, infectious titre increases more rapidly following i.c. infection as opposed to i.p. infection (Fraser & Dickinson, 1970; Kimberlin & Walker, 1979). Studies by

Millson *et al* (Millson et al, 1979) demonstrated that a proportion of infectivity is transported to the spleen via haematogenous routes whereas i.p. routing of infection most probably infects the spleen via uptake and transport of infectivity there by cells such as dendritic (Huang et al, 2002; Huang & MacPherson, 2004; Mabbott & MacPherson, 2006). As only small amounts of infectivity are disseminated via the i.c. route this may be a more precise way of determining the ability of the spleen to replicate infection. Bioassay of spleens from peripherally infected immunodeficient mice at the terminal stage of disease showed low but consistent levels of infectivity in many of the spleens assayed, although none of these spleens produced 100% incidence of disease in assay mice. The highest incidence of disease obtained following spleen bioassay was from the 79A infected μMT spleen although the relevance of this is not clear. In contrast, spleen bioassay from i.c. ME7 infected C57BL6 and μMT mice found that while C57BL/6 mice had high infectivity levels, infectivity levels were almost undetectable in μMT spleens. Similar findings were obtained in previous bioassays of spleen from i.p. and i.c. infected SCID mice where levels of infectivity were much higher in i.p. infected animals (Fraser et al, 1996). It is possible infection via i.p. routes may inadvertently "contaminate" organs within the peritoneum, such as the spleen, and studies suggest that infectivity may be sequestered in lymphoid tissues for several months following intraperitoneal infection (Karen Brown unpublished). Alternatively, as the spleens from the i.p. injected animals were collected at the terminal stage of the disease it is possible that the infectivity detected is a result of retrograde spread of infectivity to the spleen from the CNS at this stage of infection.

The existence of strains is perhaps one of the most intriguing characteristics of the TSEs. There are at least 20 experimental TSE strains most of which were originally isolated from natural scrapie in sheep and goats (Pattison & Smith, 1963). A number of factors are responsible for the definition of a TSE strain. These include; incubation period of disease, distribution and severity of vacuolar pathology, inactivation properties, and PrP^{Sc} glycoform profile (Somerville et al, 2005) and stability and preservation of strain characteristics upon passage within the same genotype from which the strain was isolated. Perhaps one of the most intriguing aspects of the TSEs is the ability of certain TSE strains, for example 87A, to mutate under certain conditions (Bruce & Dickinson, 1987). In previous studies using SCID mice the 87A strain was detected in a bioassay animal with a very prolonged incubation period following passage of spleen from an ME7 challenged SCID mouse (Fraser et al, 1996). This raised the possibility that minor strains may be selected perhaps following inefficient processing of infectivity in these immunodeficient spleens. In the studies presented here a similar prolongation of incubation periods in bioassay animals was observed although histopathological examination demonstrated the presence of the ME7 strain.

In most natural TSEs such as chronic wasting disease (CWD) (Sigurdson et al, 2002) and in the majority of experimental strains PrP^{Sc} is found in association with FDCs (McBride et al, 1992) (Brown et al, 2000a; Ritchie et al, 1999). As anticipated PrP^{Sc} was readily detected in immunocompetent mice with all of the TSE strains tested. Small foci of PrP labelled single cells were detected in spleen from $TNFR1^{-/-}$ mice 10 weeks post i.c. infection with the ME7 and 139A agent strains. PrP was not detected

in i.p. challenged TNFR1$^{-/-}$ mice or in any of the μMT mice infected i.p. or i.c. with any of the strains. Although studies using PET blotting are incomplete, it is possible that in the absence of mature FDCs PrP^{Sc}, may accumulate in association with other cell types. As this work was carried out using the PrP specific polyclonal antibody 1B3 antibody (Farquhar et al, 1989) which recognises both the normal abnormal forms of the PrP protein it is possible this is localisation of PrP^{c}, although the localisation of PrP^{c} in uninfected spleen normally produces a very diffuse labelling. Further work is required to explore these findings further using co-localisation studies with a range of cellular markers or possibly ultrastructural studies. It is known that dendritic cells (Huang et al, 2002; Huang & MacPherson, 2004) and tingible body macrophages can acquire PrP^{Sc} (Jeffrey et al, 2000; McGovern et al, 2004) so it is possible that this labelling represents accumulations in either of these cell types.

In conclusion these findings presented here demonstrate the importance of FDCs to a range of TSE agent strains. While these findings are unable to account for the marked differences between the original studies conducted with the ME7 and RML agents these data show that there are some differences between TSE strains following peripheral infection.

This chapter and the previous chapter (chapter 3) highlight the importance of the immune system and indeed the presence of functional FDCs to TSE pathogenesis not just with the ME7 strain but with a range of strains. These findings are taken forward in chapter 5 where the influence of a senescent immune system is examined. The

effects of an immune response on the peripheral pathogenesis of the ME7 strain are examined in chapter 6.

Chapter 5

The influence of an ageing immune system on peripheral TSE pathogenesis

5.1 Summary

Since the identification of variant CJD (vCJD) there have been almost 200 cases of the disease worldwide, the majority of which have occurred in the United Kingdom (UK). The disease has been confined almost exclusively to young adults although the reasons behind this apparent age-related susceptibility are not fully understood. The capacity of the immune system to mediate immune responses to pathogens declines with age as a result of impaired lymphocyte and follicular dendritic cell (FDC) function. As FDCs are critically involved in the pathogenesis of many TSEs, including vCJD, an ageing immune system may impair disease pathogenesis.

To examine the potential age related influences on TSE infection, mice were aged to approximately two thirds of their natural lifespan (600 days) and challenged peripherally with the ME7 strain of scrapie. None of the aged mice developed clinical disease; however, vacuolar degeneration in brain was detected in 33% of intraperitoneally and 42% of orally challenged mice. In addition, infectivity levels in lymphoid tissues from aged mice were significantly lower than those of the young mice demonstrating that peripheral disease pathogenesis in aged mice is inefficient. These data suggest that the impaired pathogenesis in aged mice coincides with reduced immune function and provides strong evidence that immunosenescence may influence susceptibility to vCJD infection.

5.2 Introduction

In most bacterial or viral infections deficiencies in immune function may increase susceptibility to infection. Paradoxically, a declining or dysfunctional immune system may prevent or impair peripheral infection with TSE agents as demonstrated in studies with immunodeficient mice (Fraser et al, 1996; O'Rourke et al, 1994).

In mammals it is widely accepted that ageing leads to a gradual decline in immune function. The term "immunosenescence" can be used to describe these collective alterations in immune function. Ageing leads to increased rates of mortality in response to infection (Gavazzi & Krause, 2002; Vasto et al, 2007); for example, pneumonia is more prevalent in the elderly than in younger adults (El-Solh et al, 2001). The effects of age on immune function can also be demonstrated in responses to vaccination which are notably less effective in the old (Aspinall et al, 2007). In addition, aged individuals are often associated with an increased risk of developing re-current infections such as *Varicella zoster* (Gunawardena & Attygalle, 2008). Ageing is also associated with increased rates of some cancers (Fuller, 2006).

Ageing has effects on a range of immune functions, including FDC function. In humans involution of the thymus is thought to occur around puberty (Ferone et al, 2000) although it is thought that functional thymic tissue may still be present in humans until at least age sixty. Within the thymus, progenitor T cells derived from the bone marrow are transferred to secondary lymphoid tissues such as the spleen where they are located within the periarteriolar lymphatic sheath (PALS) (Anderson & Jenkinson, 2001). Studies in mice and humans have shown that as ageing

progresses fewer T cells are produced in the thymus and transferred to secondary lymphoid tissues such as the spleen. Studies in young mice adoptively transferred with haematopoietic stem cells or bone marrow from aged mice failed to generate normal T cell populations suggesting that the age associated defects result from the maturation of progenitor cells (Mu & Thoman, 2000). In cellular immunity there are significant decreases in mature and naïve T lymphocytes with increasing age (Linton & Dorshkind, 2004; Pawelec & Larbi 2008). This age-associated decline in T cell function is preceded by involution of the thymus.

Aging also has effects on B cell development (Frasca et al, 2005) which include impaired B cell migration (Labrie et al, 2005) from the bone marrow to secondary lymphoid tissues such as the spleen (Dumont et al, 1984). It has been suggested that this may be the result of diminished signalling in age which controls the migration of these cells to organs such as the spleen. In age there are deficiencies in the immune response which consist of a shortened humoral response where the production of antibodies are of lower affinity (Frasca et al, 2005; Labrie et al, 2005) and despite the existence of isotype switching in the aged, the proportion of IgM antibody produced is much higher in aged individuals. It is not fully understood if these effects in the immune response are directly attributed to specific B cell associated defects (Frasca et al, 2005; Rogerson et al, 2003) or if they are a consequence of the effects of ageing on other cell types.

The impact of ageing on conventional dendritic cell (DC) function has been considered although not comprehensively (Agrawal et al, 2007; Shurin et al, 2007).

In terms of DC number in ageing animals studies have shown a reduction in Langerhan cell numbers in skin (Pinto et al, 2003) and oral mucosa (Schwartz et al, 1983) in aged mice. Another study found increased numbers of DCs in thymus and spleen in an aged model of lupus nephritis. This age-dependent increase in DC number has been postulated to contribute to autoimmunity (Agrawal et al, 2007).

Follicular dendritic cells (FDCs), as previously described, are critically involved in TSE pathogenesis (Brown et al, 1999; Bruce et al, 2000; Mabbott et al, 1998)(chapter 3). Significantly, a number of studies have demonstrated that ageing has intrinsic effects on these cells (Aydar et al, 2003; Aydar et al, 2004; Balogh et al, 2001). FDCs reside within the secondary lymphoid follicle of the germinal centre where they are involved in a number of immune functions; notably the trapping immune complexes on the surface of the surface of the FDC reticulum (Kosco-Vilbois et al, 1997; Kosco et al, 1992). A number of studies have demonstrated functional aberrations in FDCs in aged mice. Studies using 600 day old mice (approximately two thirds of the natural lifespan of the laboratory mouse) show that the expression of number of receptors critical for the regulation of the normal immune response are sparsely expressed or absent on the surface of FDCs in the ageing germinal centre (Aydar et al, 2003; Aydar et al, 2004; Szakal et al, 2002). Within the germinal centre FcγRII/III is highly expressed on FDCs in the germinal centre. The expression of this receptor is critical for the conversion of trapped immune complexes into a form that supports the development of the normal antibody response and in maintaining the structure of the FDC reticulum. One of the most widely understood effects of ageing is the reduction in immune complex trapping

(Szakal et al, 2002) which can be demonstrated by the reduction in labelling with the FDC-M2 antibody, which recognises complement component C4 upon FDCs (Taylor et al, 2002).

In humans effects on the gastrointestinal tract as a result of age have been documented (Phillips & Powley, 2007). These changes include the age associated decline in the numbers Peyers patches (PP) within the ileum of the small intestine. In addition, studies in senescent mice have shown that functional aspects of Peyers patches (PP) in the ileum are diminished in aged mice (Kato et al, 2003). Further studies in sheep have demonstrated that there is an age-related decline in the number of PPs in the ileum which may impact on TSE disease susceptibility (St Rose et al, 2006). In studies with the ME7 scrapie strain neuroinvasion from the intestine was severely impaired (Mabbott et al, 2003) in mice treated with a fusion protein containing the soluble lymphotoxin β receptor domain linked to the Fc portion of human immunoglobulin (LTβR-Ig). This treatment results in the temporary de-differentiation of FDCs in lymphoid tissues, including PPs. In further studies mice lacking both PPs and mesenteric lymph nodes or PPs alone failed to develop disease following oral transmission of the ME7 strain of scrapie (Glaysher & Mabbott, 2007). Thus demonstrating that mature FDCs are required for efficient neuroinvasion from the gut suggesting that an age-associated decline in the function of gut associated lymphoid tissues may impact on TSE pathogenesis.

In addition to effects on FDC function, studies have demonstrated that age-related neuronal losses occur in both the myenteric plexus and submucosal plexus of the

intestine (Phillips & Powley, 2007). It appears this loss of neurons, which is specific to cholinergic neurons, is instigated in adulthood and continues throughout life. Studies have shown that this neuronal loss is more prominent in the distal gastrointestinal tract (Phillips & Powley, 2007).

Many TSEs, including bovine spongiform encephalopathy (BSE) in cattle (Wells et al, 1987), are acquired by peripheral exposure to infection. BSE has spread to a number of other species, including humans (as vCJD) (Will et al, 1996) also by a peripheral (dietary) route (Bruce et al, 1997). During the BSE epidemic in the UK it was estimated that almost 500,000 infected cattle were slaughtered for consumption; suggesting that the number of people exposed to potentially infective doses through food may have been extremely high (Valleron et al, 2001)

Despite this probable widespread exposure to the BSE agent, cases of vCJD have occurred almost exclusively in young adults (Boelle et al, 2004; Valleron et al, 2001). It is therefore possible that the decline in immune and in FDC function in older individuals may provide some explanation for the incidence of clinical vCJD in young adults.

To test this hypothesis a well characterised murine model of senescence was used to model the effects of ageing with relation to effects on peripherally acquired TSE infection. In this murine model extensive characterisation of immune function has been carried out and it has been established that FDC function is impaired in aged mice at approximately 20 months (600 days) (Aydar et al, 2003). In the studies

presented in this chapter the spleen was used as a model for lymphoid tissue involvement, primarily because of the extensive background work that has been carried out on the role of spleen in TSE pathogenesis at this unit and elsewhere. In addition, the spleen was used for analysis of infectivity levels in lymphoid tissues mainly because of the availability of standard dose response curves based on spleen, thus enabling the calculation of accurate infectivity levels from these tissues. However, it is recognised that the gut associated lymphoid tissues (GALT) are equally important in their contribution to TSE agent neuroinvasion from the gut and these tissues have been collected from both aged and young animals in these studies for future analysis.

5.3 Results

5.3.1 Effect of age on the peripheral pathogenesis of the ME7 strain of scrapie.

To determine the influence of age on TSE pathogenesis aged (600 day old) and young adult (42-56 day old) C57BL mice were inoculated with the ME7 scrapie strain intracerebrally (i.c.) or using one of two peripheral routes; the oral and intraperitoneal (i.p.) routes. The ME7 scrapie strain was used in these studies as, like vCJD in humans (Hill et al, 1999a; Hill et al, 1999b; Hill et al, 1997) this experimental strain has extensive lymphoid system involvement with agent accumulation upon FDCs (Brown et al, 1999; McBride et al, 1992) (Brown et al, 2000b) (Bruce et al, 2000; Mabbott et al, 2000a; Mabbott et al, 2002; Mabbott et al, 2000b; Mabbott et al, 2003).

Both aged and young mice were fully susceptible to direct i.c. challenge with no differences in clinical signs or incubation period of disease (Table 5.1, 5.2). Young adult mice were 100% susceptible to ME7 infection by both peripheral routes (Table 5.1, 5.2) and all had clinical and neuropathological features of scrapie as defined by the presence of vacuolar changes in specific brain areas (9 Grey matter areas and 3 white matter areas). None of the aged mice developed clinical signs of TSE disease however positive vacuolar pathology in brain was detected in 3/9 of i.p. infected mice and 5/12 of orally infected mice (table 5.1, table 5.2). Statistical analysis of the number of aged mice developing vacuolar pathology in comparison with the young mice was assessed using a Fisher's Exact test. In the i.p. infection group 12/12 young mice showed positive vacuolar pathology compared to 3/9 in the older age group. This difference in the proportion of aged mice developing vacuolar pathology was highly significant ($P < 0.001$). In the orally challenged mice, 12/12 young mice

showed positive vacuolar pathology compared to 5/12 in the older age group. This difference in proportion was highly significant as assessed by a Fisher's Exact test (P = 0.002).

Table 5.1. TSE incubation periods (*or survival range*[a]), incidence of clinical disease and vacuolar pathology in aged (600 days) and young (42-56 days)C57BL mice following i.p., oral or i.c. ME7 challenge.

Mouse model	Challenge route	Clinical disease incidence	Vacuolar Pathology in brain	Mean incubation period ±sem in days (*or survival range*)
Aged C57BL	i.p.	0/9[a]	3/9	*247-323* (P < 0.001)
	Oral	0/12[a]	5/12	*282-366* (P = 0.002)
	i.c.	3/3	3/3	179±7.6
Young C57BL	i.p.	12/12	12/12	274±5.2
	Oral	12/12	12/12	301±3.9
	i.c.	6/6	12/12	174±3

a: Only aged mice that died after the first positive TSE case in the young mice were included in the analysis.

Table 5.2. Survival times (days)[a], clinical disease, presence of vacuolar pathology and detection of PrP^{Sc} in brain and spleen in individual, aged (600 day old) C57BL mice following i.p. or oralME7 infection. Collective results are displayed for the young mice.

Challenge route	survival (or mean incubation period ±SEM)	Clinical disease	Vacuolar pathology in brain	PrP^{Sc} in brain	PrP^{Sc} in spleen
i.p.					
aged	247	no	no	yes	no
	247	no	no	no	no
	254	no	no	yes	-[b]
	261	no	no	no	no
	275	no	no	no	no
	282	no	no	yes	no
	303	no	yes	yes	yes
	310	no	yes	yes	yes
	323	no	yes	yes	yes
young	274 ± 5	12/12	12/12	12/12	12/12
Oral					
aged	282	no	no	yes	yes
	289	no	yes	yes	no
	289	no	no	yes	no
	295	no	no	yes	no
	295	no	no	yes	no
	295	no	no	yes	yes
	310	no	yes	yes	yes
	310	no	no	yes	yes
	310	no	yes	yes	yes
	310	no	no	yes	yes
	338	no	yes	yes	yes
	366	no	yes	yes	yes
young	301 ±4	12/12	12/12	12/12	12/12
i.c.					
aged	179 ±8	3/3	3/3	-	-
young	174 ±3	6/6	6/6	-	-

a: Only aged mice that died after the first positive TSE case in the young mice were included in the analysis

b: Not done/Not available

5.3.2 Severity of TSE pathology is reduced in aged mice

One of the most defining characteristics of TSE infection is the presence of vacuolar changes in the brain. The presence and severity of these vacuolar changes in 9 specific areas in the brain can be used to construct a "lesion profile" (Fraser & Dickinson, 1967). Although none of the orally or intraperitoneally challenged aged mice developed clinical signs of TSE disease positive vacuolar pathology was detected in 3/9 of i.p. infected aged mice and 5/12 of orally infected aged mice. To determine if the severity of pathology in brain in aged mice was reduced, a comparison of the lesion profiles from these aged mice that died within the same time period as the young mice with clinical scrapie was carried out (figure 5.1).

For each mouse brain the extent of vacuolation was assessed in each of nine regions, with scoring done on a scale of 0 to 3. The area under the lesion profile (AUC) was estimated using the trapezoidal rule, for those mice that could be scored in at least eight of the nine the brain regions (total number of aged and young mice: n = 14 i.p route; n = 15 oral route n=9). For each route of infection (i.c., oral or i.p.) the differences in median AUC between the aged and young animals was tested using a two-tailed Mann-Whitney U-test.

These analyses suggested that the severity of the vacuolar pathology was significantly lower in the aged mice following exposure to the scrapie agent by i.p. injection (a, $P = 0.022$, $n = 3$) and oral exposure (b, $P = 0.001$, $n = 5$). In contrast, when young and aged mice were injected with the scrapie agent directly into the brain by i.c. injection, each group was fully susceptible to disease and developed

clinical scrapie with similar incubation periods (table 5.1, table 5.2). However, analysis of the magnitude of the spongiform pathology in the aged brains (n = 3) revealed it was significantly lower on average when compared to the young mice (P = 0.024, n = 6; two-tailed Mann-Whitney U test) (figure 5.1).

Figure 5.1 Severity of Vacuolation is reduced in Aged mice

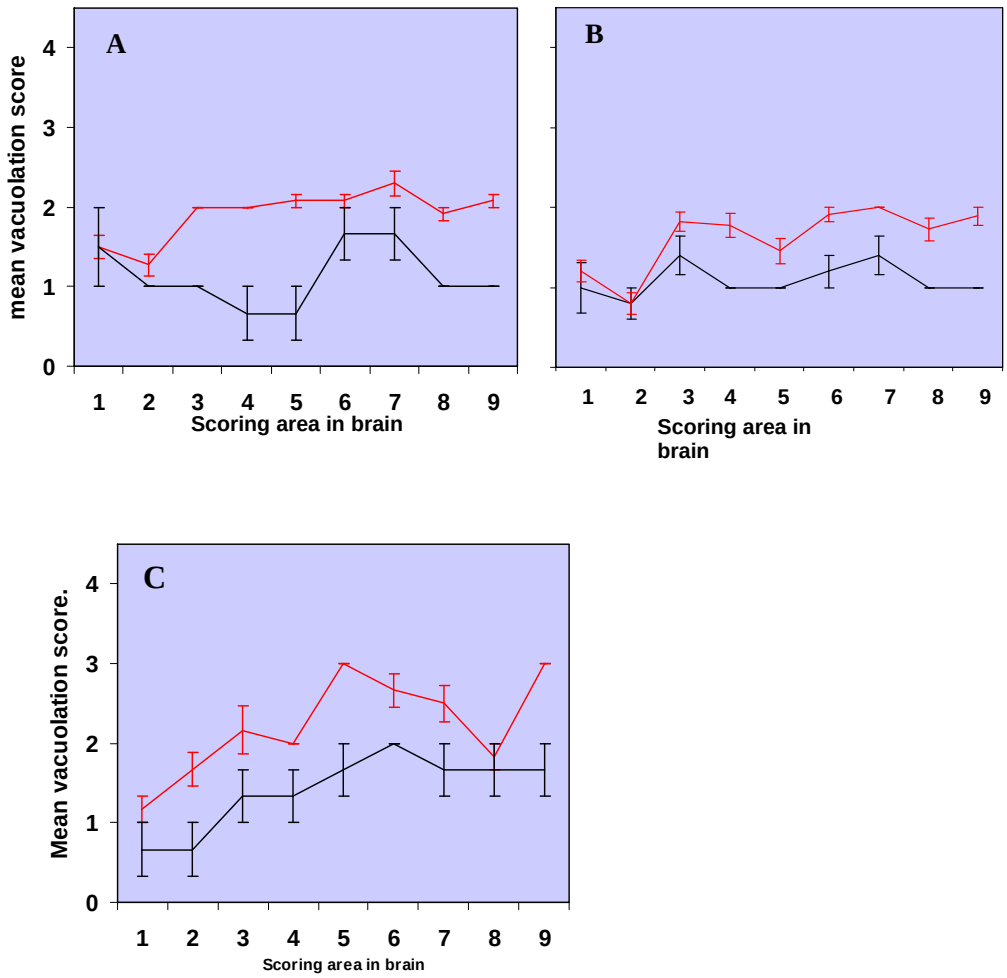


Figure 5.1. **Effect of host age on TSE-specific neuropathology in the brain**. (a-c) Lesion profiles are shown from aged (black line) and young (red line) from mice infected by the (a) i.p. (b), oral and (c) i.c. routes. Pathological assessment of the spongiform change (vacuolation) in the brains of mice with positive vacuolar pathology has been constructed to form a lesion profile. Vacuolation was scored on a scale of 0-5 in nine grey matter areas: 1, dorsal medulla; 2, cerebellar cortex; 3, superior colliculus; 4, hypothalamus; 5, thalamus; 6, hippocampus; 7, septum; 8, retrosplenial and adjacent motor cortex; 9, cingulate and adjacent motor cortex. Each point represents mean vacuolation score ± SEM for groups of 3-12 mice. To determine if the severity of the vacuolation was significantly altered in aged mice, the median differences in the areas under the lesion profile curves (AUC) between the aged and young mice were tested using a two-tailed Mann-Whitney U-test.

5.3.3 Detection of PrP in brain and spleen of scrapie affected aged and young mice

A defining characteristic of TSE infection is the accumulation of PrP^{Sc} in brain and lymphoid tissues following infection. During TSE disease, the deposition of PrP^{Sc} in the brain typically precedes the vacuolar changes (Bruce et al, 1989a). As only a proportion of aged mice developed vacuolar pathology the detection of PrP in brain was used to determine the presence of TSE disease in the brains of the peripherally-exposed aged mice despite the absence of positive vacuolar pathology. Large accumulations of PrP^{Sc} were detected in the brains of all clinically scrapie-affected young mice after i.p. injection (figure. 5.2), oral exposure and i.c. injection. Within the brains of the clinically negative aged mice, PrP^{Sc} was detected in most brains from the i.p. injected mice (six of nine mice; figure 5.2) and all the orally exposed mice (details in table 5.2).

Further analysis revealed that there was considerable variation in the detection of PrP^{Sc} in the spleens of the clinically negative aged survivors (figure 5. 2 and table 5.2). All the peripherally exposed aged mice that displayed positive vacuolar pathology (except one orally exposed animal) had detectable PrP^{Sc} in their brains and spleens. In contrast, the aged mice that did not show signs of vacuolar pathology had detectable PrP^{Sc} in the brain but not in the spleen (Fig. 5.2). The reasons for these differences are uncertain. In mice deficient in FDCs neuroinvasion can occur in some mice by an FDC-independent process such as direct uptake by peripheral nerves (Bruce et al, 1996; Bruce et al, 2000; Fraser et al, 1996), implying direct transfer of the TSE agent to the nervous system in some aged mice.

Figure 5.2. Immunohistochemical localisation of PrP^{Sc} in spleen and brain from i.p. ME7 scrapie infected aged and young mice at the terminal stage of disease

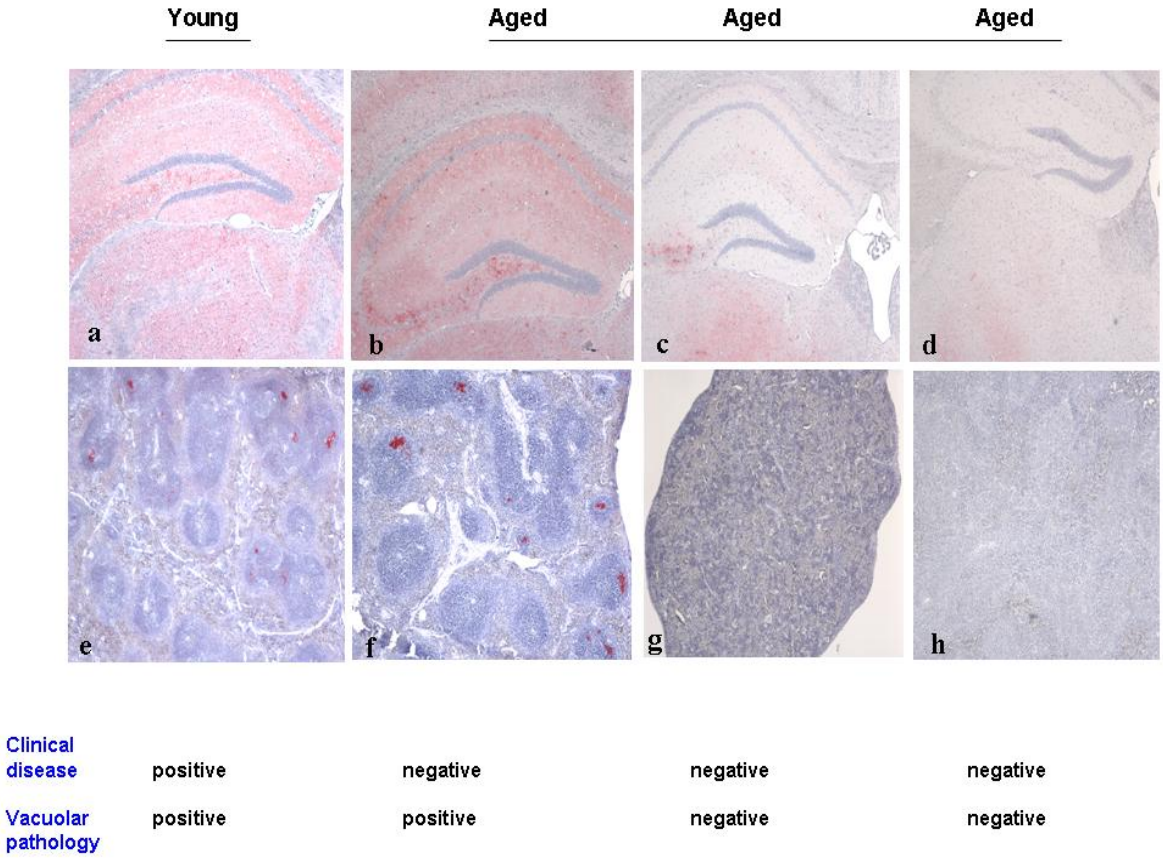


Figure 5.2. Immunohistochemical detection of PrP^{Sc} (red) in brains from terminally scrapie affected young mice and aged mice that remained free of the clinical signs of disease after i.p. injection with the ME7scrapie agent. (a) Large PrP^{Sc} accumulations (red) were detected in brains of all clinically scrapie-affected young mice. (b-d) In the brains of the clinically-negative survivors, six of nine aged mice had evidence of PrP^{Sc} in their brains, although vacuolar pathology was only found in three of these mice. Original magnification X40. (e) High levels of PrP^{Sc} were detected in association with FDCs in spleens from clinically scrapie-affected young mice (e, red). However, there was considerable variation in the detection of PrP^{Sc} in the spleens of the clinically negative aged mice (f-h). (f) All the peripherally exposed aged mice that displayed positive vacuolar pathology in the brain had detectable levels of PrP^{Sc} in their spleens. (g-h) PrP^{Sc} was undetectable in the spleens of aged mice that did not show histopathological signs of vacuolar pathology in their brains. Original magnification X40.

5.3.4 Effect of age on infectivity levels in spleen

Scrapie agent infectivity levels in spleen at 5, 7, 10 and 15 weeks post i.p. injection were measured by bioassay in C57BL indicator mice. As many TSE agents are known to replicate rapidly in lymphoid tissues early in infection these timepoints were chosen to provide information on the efficiency of the early agent replication in lymphoid tissues.

Infectivity levels were significantly lower in spleens from aged mice and in some cases undetectable (table 5.3) At all timepoints infectivity levels in aged spleens were greatly reduced except at 10 weeks post challenge where one of the aged spleens contained similar levels to those of the young mice. At 15 weeks post challenge both spleens assayed from aged mice produced 100% incidence of disease in the indicator mice. However, the levels of infectivity present still represent a reduction of approximately 2 $\log_{10}$ i.c.ID_{50} units/g in comparison with the young mice (table 5.3, figure 5.4).

Table 5.3. Infectivity titres in spleens taken from aged(20 month+) and young (6-8 weeks+) C57BL mice at 5, 7,10 and 15 weeks following intraperitoneal challenge with the ME7 strain of scrapie. Assay spleens prepared as a 10% homogenate in physiological saline and 20µl injected i.c. into C57BL indicator mice.

	time post challenge (weeks)	spleen number	incidence	mean ± SEM (days)	estimated titre (log_{10} i.c.ID_{50} units/g)[a]
	5	1	3/5	*185,241,262*[a]	1.4
		2	7/12	*217-354*[a]	1.6
	7	1	0/12	-	UD[b]
		2	10/10	223±6	5.2
Aged	10	1	7/7	184±2	7.2
		2	0/8	-	UD[b]
	15	1	9/9	226±8	5.1
		2	11/11	216±6	5.6
	5	1	11/11	181±3	7.4
		2	7/7	187±5	7.1
	7	1	11/11	178±2	7.5
		2	12/12	188±2	7
Young	10	1	12/12	182±5	7.3
		2	12/12	181±3	7.4
	15	1	8/8	174±3	7.7
		2	12/12	172±3	7.8

a:Incubation period range or individual incubation periods are represented in assays where TSE incidence was less than 100%. When one or more animals survived, the maximum titre was assumed to be lower than the titre representative for the longest incubation period observed; all titres are approximate.

b: outwith the detection limit of the assay.

Figure 5.3 Scrapie agent infectivity titres in spleen from aged and young mice following i.p. injection with the ME7 scrapie agent.

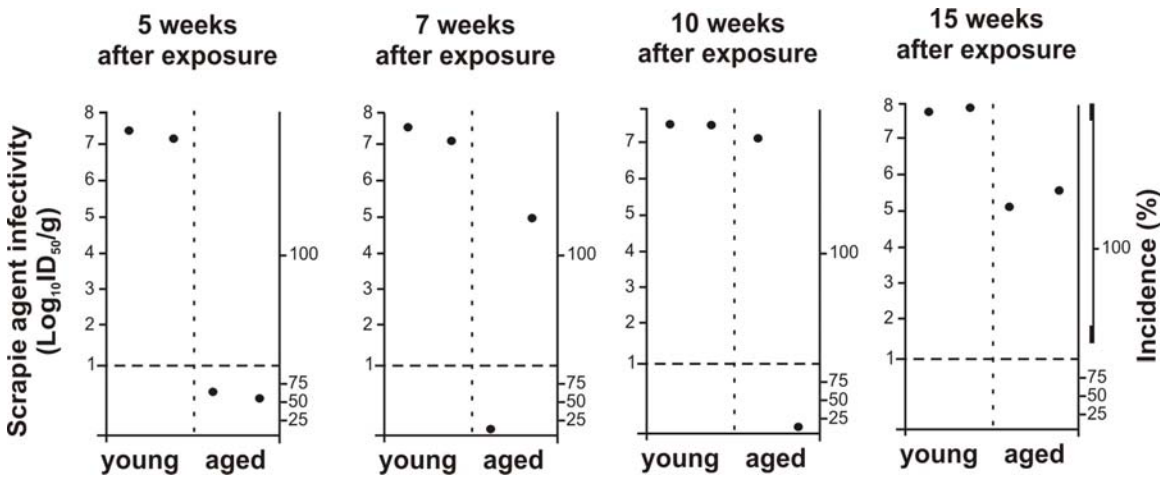


Figure 5.3. Scrapie agent infectivity titres in spleens from young and aged mice collected 5, 7, 10 and 15 weeks after i.p. exposure were determined by transmission into groups of indicator C57BL/Dk mice. Each point represents data for individual spleens. Data below the horizontal line indicate disease incidence in the recipient mice below 100% and considered to contain trace levels of infectivity. High levels of agent infectivity were detected in the spleens of young adult mice as soon as 5 weeks after exposure. However, significantly lower agent infectivity levels were detected in the spleens of aged mice ($P < 0.001$, Linear mixed model using the REML directive in Genstat 10th edition. Fixed effects tested using F-tests with appropriately modified degrees of freedom), with many containing trace or undetectable levels.

5.3.5 Effect of age on PrP^{Sc} accumulation in spleen

At 5, 7, 10 and 15 weeks post oral or intraperitoneal inoculation spleen (n=2) and gut (ileum) (n=2) was collected from aged and young mice for immunohistochemical (IHC) and PET blot analysis for the detection of PrP^{Sc} (figure 5.4). In each instance tissues were fixed in PLP fixative and processed in a tissue processor. This method of preparation has been previously found to give optimum detection of PrP and good tissue morphology (Brown et al, 2000a).

The precise nature of the TSE agent is uncertain, but PrP^{Sc}, a proteinase K (PK)-resistant form of the cellular prion protein, PrP^{c}, co-purifies with agent infectivity in diseased tissues (Bolton et al, 1982). In this study, PrP^{Sc} is used to describe the disease-specific PrP accumulations detected by immunohistochemistry. Within 10 weeks after peripheral exposure of young adult mice to the ME7 scrapie agent, strong accumulations of PrP^{Sc} and agent infectivity accumulate within the spleen and are sustained until the terminal stages of disease (Brown et al, 1999; Bruce et al, 2000; Mabbott et al, 2003). This early accumulation within lymphoid tissues is critical for efficient neuroinvasion (Brown et al, 1999; Bruce et al, 2000; Mabbott et al, 2003). Heavy PrP^{Sc} accumulations were detected in the spleens of all young adult mice at 5 weeks (not shown) and 10 weeks after intraperitoneal (i.p.) or oral infection (not shown) with the ME7 scrapie agent (Fig. 5.4) and were maintained until the terminal stages of disease. The distribution of PrP^{Sc} within these tissues was consistent with accumulation upon FDCs (McBride et al, 1992) (Brown et al, 1999; Bruce et al, 2000; Mabbott et al, 2003). PET immunoblot analysis of adjacent sections confirmed that these PrP accumulations were PrP^{Sc} (Fig. 5.4). Although gut (ileum) was collected

from all of the aged and young mice as detailed above analysis of these tissues remains incomplete and is not presented in this thesis.

Figure 5.4. PrP^{Sc} Localisation in spleen from aged and young mice following i.p. injection with the ME7 scrapie agent

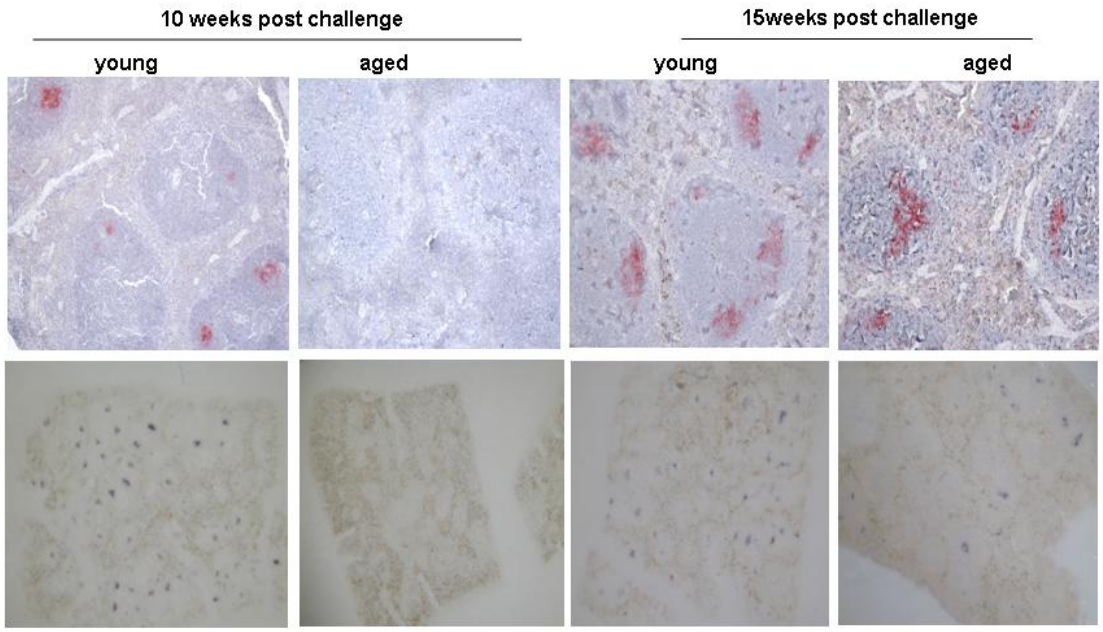


Figure 5.4 Effect of host age on TSE agent accumulation upon in the spleen. (**a**) High levels of PrP^{Sc} were detected in association with FDCs in spleens from young mice from 5 weeks (not shown) and 10 weeks after i.p. injection with the scrapie agent (upper row, red). In contrast, PrP^{Sc} was not detected in the spleens of age mice until 15 weeks after i.p. exposure (upper row, red). Analysis of adjacent sections by PET immunohistoblot confirmed the presence of proteinase-K-resistant PrP^{Sc} (lower row,/black). Upper row, magnification X100; lower row, X20.

5.3.6 Determination of FDC functionality during the TSE disease incubation period

The monoclonal antibody FDC-M2 labels complement component C4 on the surface of FDCs (Taylor et al, 2002). The expression of C4 is a result of interaction between trapped immune complexes and complement. Thus the expression of this marker on FDCs can be used to demonstrate the ability of FDCs to trap immune complexes.

To establish functionality of FDCs during incubation period of disease in both orally and intraperitoneally infected mice immunolabelling for the FDC-M2 marker was carried out at 5, 7, 10 and 15 weeks post i.p. or oral ME7 scrapie agent infection. At all timepoints post infection C4 expression on FDCs was reduced in all of the aged spleens examined (figure 5.5), however in some animals labelling more similar to that of young mice could be observed. These variations suggest that the effect of age on FDC function is not a complete effect and in some cases FDCs may retain some functionality. However, in general, FDCs in spleen from both orally or i.p. infected aged mice appeared atrophied with little evidence of C4 expression on these cells.

Mice express three Fc receptors for IgG: FcγRI, FcγRII, and FcγRIII (Qin et al, 2000) (Balogh et al, 2001). FcγRIIB is a monomeric inhibitory receptor and highly expressed on FDCs in secondary follicle germinal centres. It is thought the function of FcγRIIB expression on FDCs in germinal centres is important for immune complex retention and the conversion of these immune complexes to a highly immunogenic form (Qin et al, 2000). The presence of these Fc receptors on FDCs was determined by IHC to further assess their functionality. At 10 weeks post

infection abundant labelling was detected in spleen sections from young mice. In the aged animals labelling was absent in spleen from most mice, although a few positive follicles were detected (figure 5.5).

Figure 5.5 Expression of C4 upon FDCs in ME7 infected aged and young mice

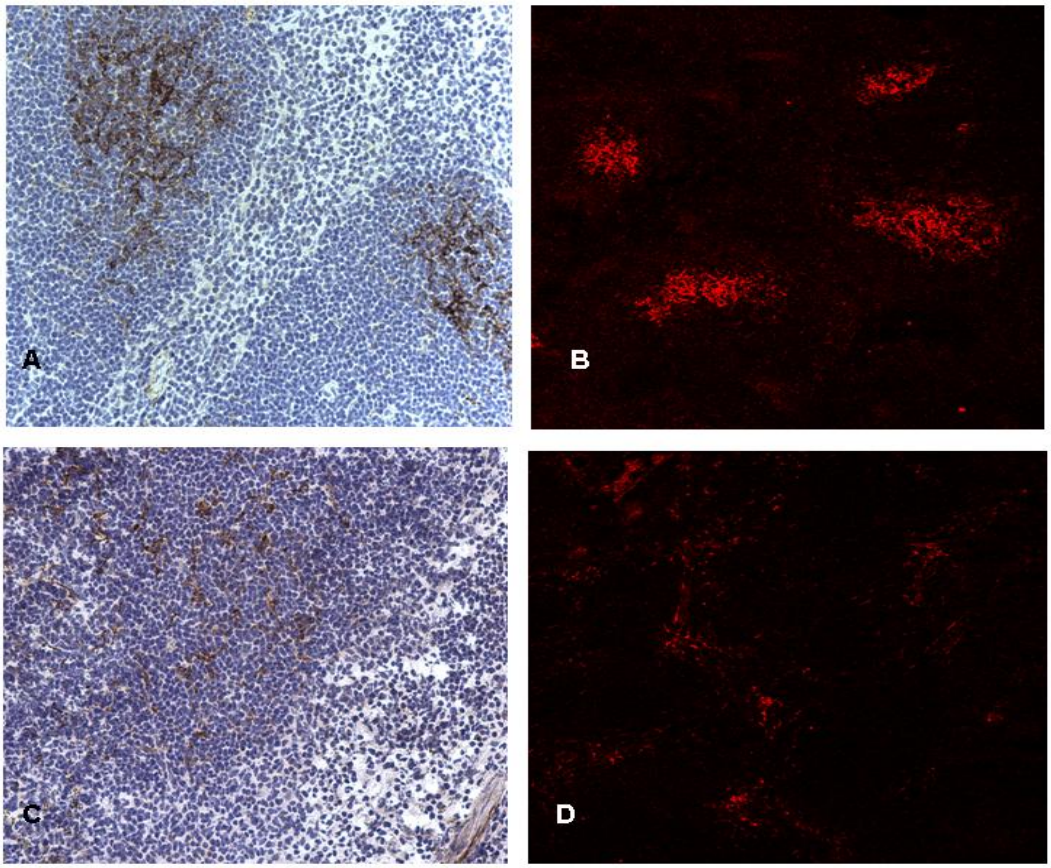


Figure 5.5 Immunohistochemical detection of complement component C4 using monoclonal antibody FDC-M2 at 10 weeks post ME7 infection. In young mice (A, B) extensive labelling of this marker within the germinal centre can be observed using confocal analysis (B) (original magnification X200). In the majority of aged mice (C, D) FDC-M2 labelling is dramatically reduced or absent (original magnification X200). Light microscopy images (C) show much lower levels of FDC-M2 labelling in aged mice and when present appears in a condensed punctuate form suggesting that aberrant and impaired immune complex trapping occurs upon FDCs from aged mice. (D) Sparse labelling for C4 is similarly detected using confocal analysis of spleens from aged mice labelled with the FDC-M2 antibody.

Figure 5.6 Expression of the FcγRII/III receptor in spleens of ME7 infected aged and young mice

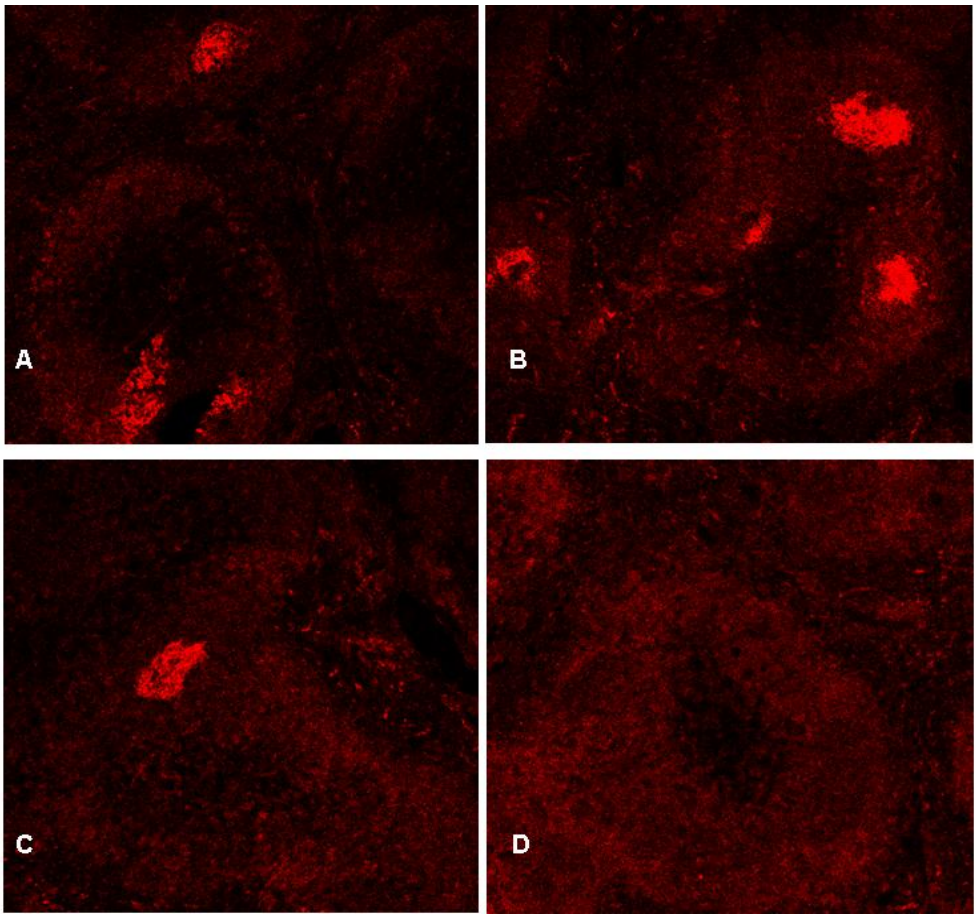


Figure 5.6. Immunohistochemical detection of the expression of the FcγRII/III receptor using monoclonal antibody 2.4G2 (anti-FcγRII/III, PharMingen) at 10 weeks post infection in i.p. infected young (A, B) and aged mice (C, D). Abundant expression of the FcγRII/III marker can be detected in spleen from young mice (A, B), whereas the marker is almost undetectable in aged animals (C,D) with the exception of some mice where typically one or two follicles displayed labelling for this marker(C). Original magnification for all panels X200.

5.3.7 FDC functionality appears to be reduced in aged mice

In murine models of immunosenescence the decline in the secondary antibody response is thought to be directly related to FDC function (Aydar et al, 2003). This dysfunction is thought to be related to the reduced expression of specific markers, including FcγRII/III, which contribute to the development and maintenance of the normal immune response. Here, the reduced susceptibility to TSE infection in aged mice appears to correlate with a decline in FDC function as demonstrated by immunohistochemical analysis of lymphoid tissues from uninfected aged, mice (figure 5.7). In aged mice FDC-M2, which recognises complement component C4 expression upon FDCs, was greatly reduced in all spleens examined (n=5) reflecting the reduced retention of trapped immune complexes on these FDC-networks. In young mice however (n=5) abundant FDC-M2 labelling could be detected (Fig. 5.7).

Interestingly detection of complement receptors 1 and 2 (CD21/CD35) was readily detected in both aged and young mice, suggesting that the expression of this marker is not impaired in aged mice (Aydar et al, 2003). Peripheral nerves were detected immunohistochemically using an anti- tyrosine hydroxylase marker. Innervation was not reduced in aged mice and in-fact there was some suggestion that greater levels of innervation were present in spleens from these aged mice (figure 5.8).

Double immunofluorescent labelling for PrP protein and FDC-M2 in aged spleen found considerable variation between aged mice in the detection of normal PrP. In some germinal centres where FDC-M2 labelling was more abundant PrP^c was readily

detected (figure 5.10, b) whereas the labelling was reduced or undetectable in other germinal centres where the localisation of FDC-M2 was reduced (figure 5.9, c).

Figure 5.7 Detection of C4 in association with FDCs in aged and young mice.

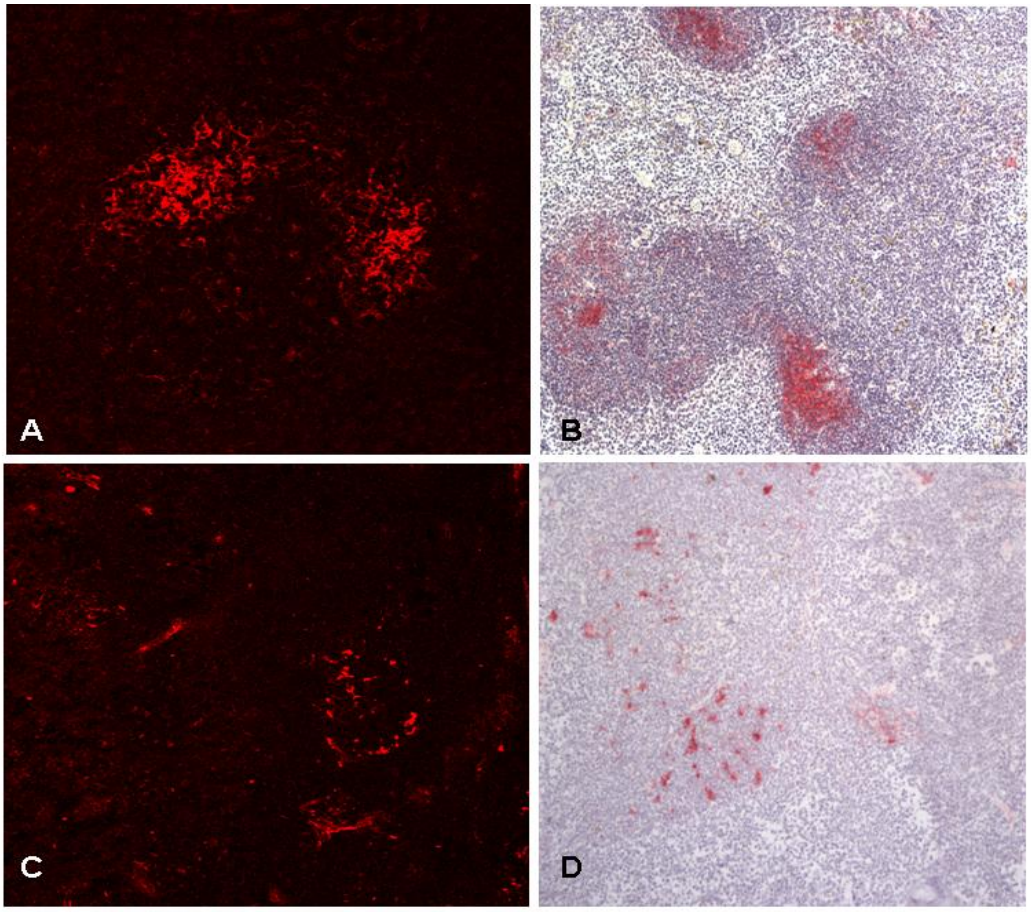


Figure 5.7 Analysis of spleen from uninfected young mice shows abundant FDC-M2 labelling using confocal (A) and light microscopy (B). In aged mice (C,D) there was some variation in the level and appearance of this labelling between animals and within spleens although the overall level of labelling was reduced in all of the spleens examined in comparison with that of the young mice. In most aged spleens, FDC-M2 labelling appeared atrophied and sparsely distributed. Original magnification X200.

Figure 5.8 Effect of host age on CR2/CR1 status and peripheral nerve detection in the spleen.

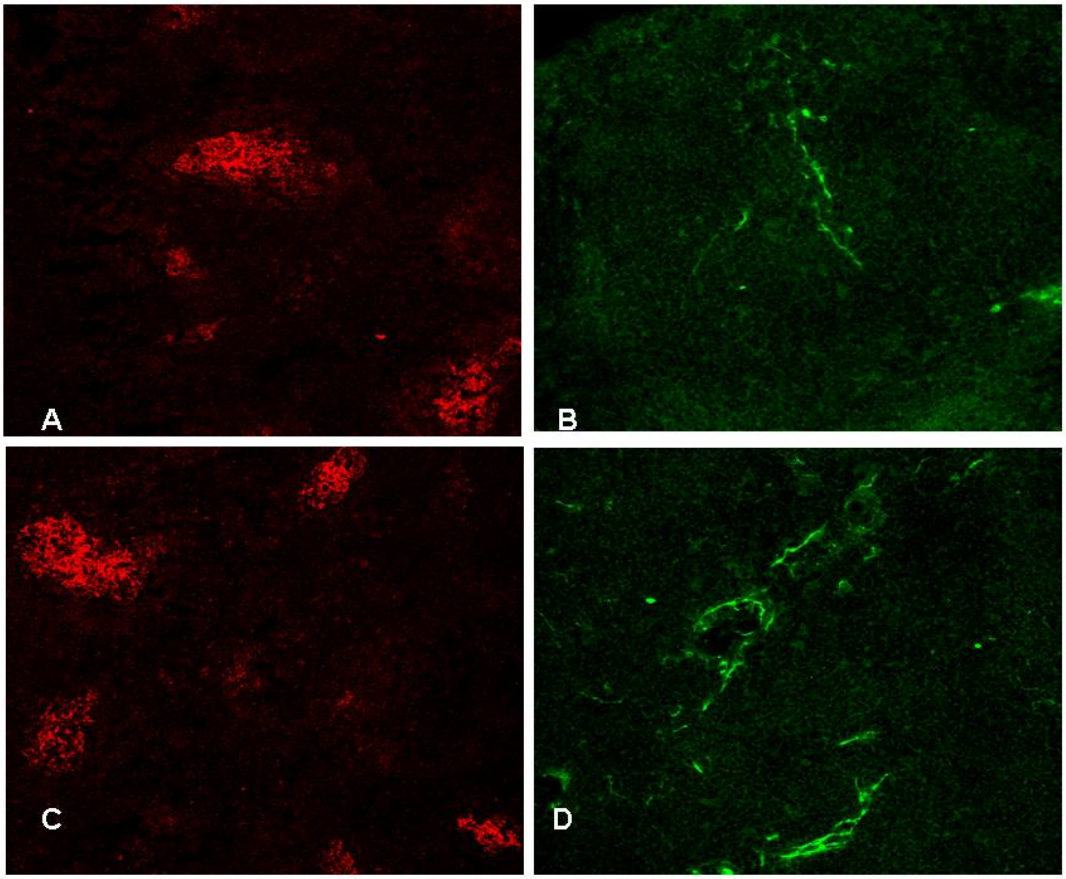


Figure 5.8 Unlike C4 expression which is dramatically reduced in aged mice (Figure 5.5) there was no difference in the detection of CR2/CR1 in spleens from young (A) or aged mice (C) (original magnification X200). To assess the effect of age on innervation of the spleen the immunohistochemical detection of peripheral nerves was performed using the noradrenergic marker tyrosine hydroxylase. Immunohistochemical analysis demonstrated the presence of tyrosine hydroxylase positive sympathetic nerves (green) in the spleens of both young (B) and aged mice (D). In-fact, there was some indication that spleens of aged mice were more highly innervated than those of the young mice. Original magnification X200.

Figure 5.9 PrP^c expression is reduced in the spleens of aged mice with disrupted FDC networks

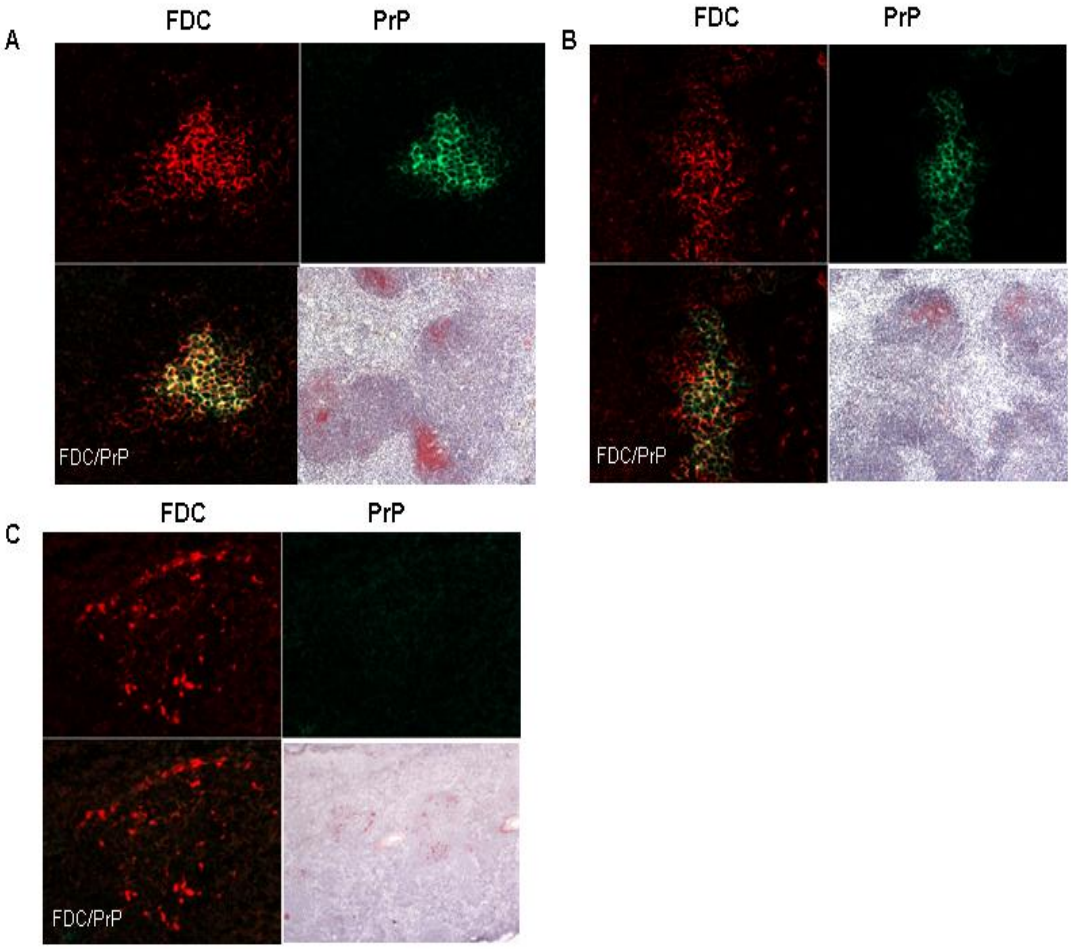


Figure 5.9. (a) High levels of cellular PrP^c expression (green) and complement component C4 (red) co-localized upon FDCs in spleens of young mice. (b) In some spleens from aged mice, levels of PrP^c and complement C4 were similar to those observed in spleens from young mice. (c) In most spleens from aged mice, immunolabelling for PrP^c and complement C4 was greatly reduced and appeared atrophied and sparsely distributed within the follicle. (original magnification X400) Light level images from each section are provided to demonstrate the labelling within each section (original magnification X100)

5.4 Discussion

In the majority of naturally acquired and experimental TSEs extensive lymphoid tissue involvement occurs. In many experimental TSEs high levels of infectivity and PrP^{Sc} can be detected in tissues such as spleen and lymph nodes several weeks after peripheral infection. In these models, infection in the lymphoid tissues precedes the first detection of disease within the CNS by many weeks or months. In vCJD (Hill et al, 1999b), but not sCJD (Gambetti et al, 2003), PrP and infectivity accumulates in lymphoid tissues of affected individuals suggesting that a functional immune system may be critical for the pathogenesis of vCJD (Bruce et al, 2001). This paradox between vCJD and sCJD is not fully understood but may be the result of strain specific tissue tropism; for example some experimental TSE strains such as the ME7 agent strain and the 79A agent strain are known to extensively target lymphoid tissues (Fraser, 1996) whereas the 87V agent strain (Bruce, 1985) has very little lymphoid tissue involvement. The two forms of CJD differ in other ways including clinical presentation and neuropathology (Ironside et al, 2002) but one of the most striking differences between vCJD and sCJD is the age distribution of cases (Ghani et al, 2000). In vCJD the median age of death is approximately 29 years, whereas it is 68 years for sCJD. A number of hypothesis exist to explain this predominance of young people with vCJD including exposure to vaccination (Minor et al, 2000) and preferential dietary exposure (Boelle et al, 2004). However, an epidemiological study to consider the effect of diet found that dietary exposure alone could not explain this phenomenon. In fact, this study predicted that if dietary exposure was the major factor in the age distribution of cases the cumulated proportion of cases over 40 years

should have been 48% when in-fact the proportion of cases was only 10% (Boelle et al, 2004).

In these studies aged mice infected with ME7 either orally or by i.p. injection failed to develop clinical disease whereas aged mice developed clinical disease following direct CNS infection with similar incubation periods to those of the young mice. However, subsequent analysis of brain found that 33% of i.p. and 42% of orally infected mice had positive vacuolar changes in brain. Analysis of the severity of vacuolation in specific brain regions from these aged mice found that it was significantly lower than that of the young mice that died within the same time period. As these mice were almost 1000 days old at the time of death it is possible that could they have survived longer they may have eventually developed clinical signs of disease; albeit with prolonged incubation periods to those of the young mice. Alternatively, it is possible that age associated changes in the CNS may modulate the clinical symptoms of disease observed or perhaps produce lower levels of pathology than those observed in younger mice. As aged mice developed clinical disease following i.c. infection this seems a less likely possibility; although the severity of pathology was significantly reduced in these mice. Similarly, mink of the Chediak-Higashi phenotype, infected with transmissible mink encephalopathy (TME) displayed lower levels of vacuolar pathology in the aged but not the young mink of the same phenotype (Marsh et al, 1976).

Following exposure to many TSEs, including the ME7 agent strain, deposition of the disease specific form of PrP (PrP^{Sc}) in the CNS precedes vacuolar changes in the CNS (Bruce et al, 1989b). Indeed, accumulation of PrP in lymphoid tissues occurs very early in infection in a number of experimental (Bruce et al, 1996; McBride et al, 1992)and naturally acquired infections for example chronic wasting disease (CWD) (Sigurdson et al, 2002). In these studies the detection of PrP^{Sc} in brain and lymphoid tissues was used to establish the level and presence of TSE disease in the absence of clinical symptoms of disease or vacuolar brain pathology.

Significantly all of the orally challenged mice had evidence of PrP^{Sc} accumulation in brain although there was no evidence of clinical disease and only a proportion of these animals had positive vacuolar scores. These findings may have important implications since the most likely original route of vCJD transmission was via the oral route (Bruce et al, 1997), raising the possibility that many people may be infected with this disease in the absence of any clinical disease symptoms. There was considerable variation in the detection of PrP^{Sc} in the spleen of aged mice. All of the aged mice with positive vacuolar brain pathology (with the exception of one orally challenged animal) had accumulation of PrP^{Sc} in both brain and spleen. As PrP^{Sc} is localised to the surface of FDCs in lymphoid tissues this may suggest that the pathogenesis of disease in these mice was more similar to that of the young adult mice, possibly as a result of a more functional immune system at the time of infection. In contrast, in the aged mice where there was evidence of PrP^{Sc} accumulation in brain but not in spleen it is possible that infection of the CNS has occurred in the absence of lymphoid system involvement, possibly via direct

peripheral nerve infection as is known to occur in immunodeficient mice after high dose challenge (Fraser et al, 1996). The reasons for these differences are uncertain. In mice deficient in FDCs neuroinvasion may occur in some mice by an FDC-independent process such as direct uptake by peripheral nerves (Fraser et al, 1996), implying direct transfer of the TSE agent to the nervous system in some aged mice. Alternatively, it is plausible that the TSE agent initially accumulated upon FDCs facilitating subsequent neuroinvasion, but was cleared from the spleen as the status of the FDCs declined.

These data have important implications for the development of reliable pre-clinical diagnostics, as tests based on the detection of the TSE agent in blood or lymphoid tissues may be less sensitive when used on the elderly. In addition surveillance methods attempting to estimate levels of potential disease within the population such as analysis of tonsil, appendix or other tissues (Hill et al, 1999b; Hill et al, 1997; Hilton et al, 2002; Hilton et al, 2004) (Ironside et al, 2006) may be largely redundant in the elderly due to the lack of PrP accumulation in these tissues

The mouse-passaged ME7 scrapie agent strain was used in these studies as its pathogenesis in mice appears to closely resemble that of natural sheep scrapie (Hunter, 2007), chronic wasting disease (Sigurdson et al, 1999) and vCJD in the natural host (Brown et al, 1997; Hill et al, 1997) in that TSE agent accumulation occurs in lymphoid tissues before neuroinvasion (Bruce et al, 2001). As TSE agents are known to replicate rapidly in lymphoid tissues early in infection (Collis & Kimberlin, 1989; Fraser et al, 1992b; Fraser & Dickinson, 1978; Fraser, 1996;

Kimberlin & Walker, 1979; Kimberlin & Walker, 1989; Kimberlin & Walker, 1990)several timepoints in early infection were chosen to provide information on the efficiency of disease replication in lymphoid tissues and on the accumulation of PrP^{Sc} in spleen. The analysis of these tissues was especially important as it was impossible to predict at the outset of the study if aged animals would survive to the clinical endpoint of disease.

Infectivity levels in aged spleen were significantly lower than those of the young spleens and in some cases undetectable. At 15 weeks post challenge both spleens assayed produced 100% incidence of disease in the indicator mice. However, the levels of infectivity present still represented a reduction of approximately 2 $\log_{10}$ i.c.ID_{50} units/g in comparison with the young mice. Similarly, PrP^{Sc} in spleen was not detected by PET (histoblot) or immunohistochemistry until 15 weeks after intraperitoneal or oral challenge. This suggests that disease pathogenesis in aged mice is delayed as a result of inefficient disease transmission.

In murine models of immunosenescence, the decline in the secondary antibody response is thought to be directly related to FDC function (Aydar et al, 2003; Szakal et al, 2002). This dysfunction is thought to be related to a reduction in the expression of specific markers, including FcγRII/RIII, which contribute to the development and maintenance of the normal immune response (Qin et al, 2000). Here, studies in this chapter show that the reduced susceptibility to TSE infection in aged mice correlates with a decline in FDC function as demonstrated by immunohistochemical analysis of lymphoid tissues from uninfected aged mice. In these aged mice the association of

complement component C4 with FDCs was greatly reduced reflecting the amount and location of trapped immune complexes on the FDC-network. In young mice however abundant localisation of C4 on FDCs could be detected. The complement receptors CR1/CR2 was readily detected in both aged and young mice, suggesting that the expression of this marker is not impaired in aged mice (Aydar et al, 2003). Double immunofluorescent labelling for PrP protein and C4 in aged spleen found considerable variation between aged mice in the detection of normal PrP. In some germinal centres where C4 labelling was more abundant PrP^c was readily detected whereas the labelling was reduced or undetectable in other germinal centres where the localisation of FDC-M2 was reduced (figure 3). As PrP^c expression (Brown et al, 1999; Klein, 1998)and opsonising complement components (Klein et al, 2001; Mabbott et al, 2001) are critical for the accumulation of TSE agents upon FDCs, it is likely their reduced association with FDCs in the spleens provides some explanation for the reduction in infectivity levels and in the reduced accumulation of PrP^{Sc} in lymphoid tissues.

The assessment of C4 and expression of the FcγRII/III receptors were also assessed in infected animals from 5-15 weeks post challenge. As demonstrated previously, C4 labelling was greatly reduced in uninfected aged mice in comparison with younger animals. In addition abundant labelling of the FcγRII/III marker was detected in infected young mice although the expression of this marker was barely detectable in aged mice infected with the ME7 scrapie strain. Interestingly, the expression of the IgG Fc receptor FcγRIIB (Qin et al, 2000) is not found in primary follicles, but only in mature germinal centres normally in response to a humoral immune response

(Aydar et al, 2003). The expression of FcγRII/III on FDCs in GCs is critical for immune complex trapping upon FDCs and in the conversion of these immune complexes to a highly immunogenic form (Qin et al, 2000) (Aydar et al, 2003). In uninfected aged and young mice FcγRII/III was only sparsely detected in a few follicles irrespective of age suggesting that scrapie infection has enhanced the expression of FcγRII/III in the young mice indicating that scrapie infection may provoke some form of immune response. If infection with scrapie agents does indeed provoke an immune response it is possible the lack of expression of this marker in infected aged mice is due to impaired replication of infectivity by the FDCs themselves (as demonstrated by splenic infectivity measurement). Indeed, studies in aged mice find that FcγRII/III labelling on aged FDCs is dramatically reduced following immunisation (Aydar et al, 2004). Further studies are required to establish the significance of this finding but it may suggest that TSE infection elicits some form of immune response within the host leading to this greater expression of FcγRII /III within the germinal centre.

Although the findings presented here show that the effects of age on pathogenesis *coincide* with FDC dysfunction, it is not possible to determine from these studies if the FDCs themselves are responsible for these effects on pathogenesis. It remains possible that this impaired pathogenesis is related to defects in other cell types, such as lymphocytes. In future studies the creation of chimaeric models where a mismatch between aged FDCs and young lymphoid cells are created and *vice-versa* may be extremely useful in elucidating which cells directly contribute to these

defects in TSE pathogenesis, the design of these potential studies is described in chapter 7.

Although these findings show that increasing age results in impaired TSE pathogenesis previous studies have shown that neonatal mice are also less susceptible to peripheral TSE infection than adults (Ierna et al, 2006; Outram et al, 1973). Similarly, this resistance correlates with an absence of FDCs in the spleens of neonatal mice at the time of exposure with the onset of disease susceptibility coinciding with the development and maturation of FDCs during the first 10 days post birth (Ierna et al, 2006).

Although the reasons associated with the predominance of vCJD cases in young people may be complex, our data demonstrates that the pathogenesis of peripherally acquired TSE agents, such as vCJD, in older humans is likely to be much less efficient than that of young individuals. However, this data also demonstrates that inefficient disease transmission in aged individuals may result in significant levels of sub-clinical disease within the older population presenting further implications for iatrogenic spread of infection. These findings suggest that this impaired pathogenesis in aged mice correlates with FDC function and provides strong evidence that immunosenescence may influence susceptibility to vCJD infection.

Chapter 6

The effect of immunisation on TSE pathogenesis

6.1 Summary

Although TSE infections are thought of primarily as neurodegenerative diseases, the immune system is critically involved in the pathogenesis of most experimental and natural TSEs. However it is uncertain whether this infection of lymphoid tissues results in any associated immune system dysfunction or immune system activation. The main aims in this chapter were to investigate the effects of TSE infection on the immune system and to determine how the immune system influences TSE pathogenesis. Here two distinct T cell dependent antigens were used to immunise mice and study their effects on TSE pathogenesis in separate studies. Immunisation of mice with sheep red blood cell antigen (SRBC) at 6 and 9 weeks post i.p. injection significantly prolonged the disease incubation period ($p=0.033$). However immunisation with DNP-KLH at 10 and 13 weeks post infection significantly shortened incubation period ($p=0.001$). This finding was highly significant. Immunisation with DNP-KLH at a mid-point of infection and a pre-clinical timepoint had no effect on incubation period of disease suggesting that these effects on pathogenesis are limited to the early stages of infection. An unexpected finding was the sudden death of a third of the ME7 scrapie infected mice following boosting with SRBC antigen at a late stage of infection. No deaths or adverse effects occurred in any of the other groups suggesting that some form of immune system dysfunction at this late stage of infection may have contributed to the deaths of these mice or accelerated the onset of clinical TSE disease. Together these findings demonstrate that manipulation of the immune system during scrapie infection mice can alter disease pathogenesis.

6.2 Introduction

The majority of TSE infections are most likely acquired by peripheral exposure; for example by ingestion of infectious material in food or via transmission through the skin or mucous membranes. BSE in cattle (Wells et al, 1987), CWD in deer and elk (Sigurdson et al, 1999) and vCJD (Bruce et al, 1997) and kuru in humans (Collinge et al, 2006) are all thought to be transmitted via the oral route. The occurrence of BSE in cattle almost certainly arose from the feeding of TSE contaminated meat and bone meal derivates, this contamination has been attributed to changes in rendering practices in the early 1980s permitting the survival of TSE infectivity. A number of theories exist for the origin of BSE and most suggest it arose from scrapie in small ruminants (Priola & Vorberg, 2004), or from a previously unrecognised form of TSE in cattle, although some more controversial theories suggest BSE may have a human origin (Colchester & Colchester, 2005). BSE has since been transmitted to a number of species including humans as variant CJD; with no deviation in strain characteristics even when passaged through other species (Bruce et al, 2002).

CWD has been reported in captive and free-ranging deer and elk from north-eastern Colorado and south-eastern Wyoming. In CWD the epidemiology is not fully understood but it is thought that horizontal transmission, via oral exposure, is the most probable mechanism for infection of free- ranging animals (Sigurdson et al, 2002). Indeed, experimental studies in orally dosed mule deer have shown that PrP^{Sc} accumulates in lymphoid tissues of the alimentary tract, in association with FDCs, as early as 42 days after infection (Sigurdson et al, 1999).

The ritualistic practice of endocannabalism (Zigas & Gadusek, 1957); Gadusek, 1963; (Alpers, 1965) (Gajdusek, 1963) amongst the Fore tribe of Papua New Guinea resulted in the transmission of "kuru" (Gajdusek & Alpers, 1966). The majority of affected individuals were women and adolescents who predominantly consumed brain and human offal ; males consumed mainly muscle which is not normally associated with significant levels of infectivity (Hornabrook & Moir, 1970) (Bosque et al, 2002; Collinge et al, 2006). Although most cases of kuru were probably transmitted orally, epidemiological evidence suggests that kuru may also have been parenterally transmitted via the skin or mucous membrane; by the smearing of ash onto the skin from the remains of the deceased or by handling contaminated tissues (Collinge et al, 2006). In natural scrapie, infection via the skin or mucous membranes is also thought to be an important route of disease transmission (Hunter, 2007). Indeed experimental studies in mice have shown that infection via the skin (scarification) is an efficient route of transmission (Taylor et al, 1996)

In vCJD high levels of infectivity and the accumulation of PrP^{Sc} can be detected in lymphoid tissues in association with FDCs. This involvement of the immune system and the probable dissemination of infection, or cells associated with infectivity into the bloodstream has resulted in four cases of vCJD in individuals who received vCJD contaminated blood products (Llewelyn et al, 2004; Peden et al, 2004; Wroe et al, 2006). It is not understood which cell types participate in dissemination of infection in blood and although FDCs are associated with high levels of infectivity these cells are non-mobile and remain within the germinal centre. The most likely candidates for dissemination of infectivity are lymphocytes. These cells, through their intimate

associations with FDCs, may disseminate infectivity into the bloodstream from lymphoid tissues which are known to contain infectivity (Bruce et al, 2001). In contrast, there is no evidence of disease transmission via blood in sporadic CJD (Wilson et al, 2000); however there is no recognized involvement of the lymphoid tissues with sCJD (Hill et al, 1999b).

Previous studies carried out many years ago failed to find evidence of a classical immune response to scrapie infection (Chandler, 1959; Clarke & Haig, 1966; Pattison et al, 1964). One hypothesis for this phenomenon was that infection with the scrapie agent resulted in immunosupression, although little evidence has been found to support this (Clarke, 1968; Gardiner & Marucci, 1969; Garfin et al, 1978a; Garfin et al, 1978b). This lack of immune response to infection is perhaps not unexpected since the infectious agent is thought to be at least partly composed of PrP^{Sc} (Somerville & Dunn, 1996 ; Somerville et al, 1986) the infectious isoform of the host protein, PrP (Bolton et al, 1982; Bolton et al, 1985; McKinley et al, 1983). When these original studies were conducted PrP protein and its abnormal infectious isoform had not been discovered (Bolton et al, 1982; Hunter et al, 1992). Although there is no evidence for a classical immune response to TSE infection (Chandler, 1959; Clarke & Haig, 1966), studies investigating immunoglobulin (Ig) levels in sheep from flocks with a high incidence of natural scrapie found that these were greatly elevated during the clinical phase of disease(Collis et al, 1979). Further studies in sheep experimentally infected with the SSBP/1 strain of scrapie found similar results (Collis & Kimberlin, 1983) and demonstrated that IgG, specifically IgG2a was the major Ig sub-class present in the sheep sera. However, evidence now suggests that

the increased levels of IgG2a in these scrapie affected animals were in-fact the indirect result of animals suffering from pruritus (Nora Hunter, personal communication).

In a number of infectious diseases, modulation of the host's immune system by the infectious agent occurs as a result of infection. These diseases include Chagas' disease, caused by the intracellular protozoan parasite Trypanosoma cruzi (Medina et al, 2007), where molecules released by the infection influence the host's immune system. The effects of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infection on the immune system are also well documented (Boasso & Shearer, 2008; Kottilil et al, 2006; Moss et al, 2000; Tompkins & Tompkins, 2008; Zhang et al, 2004). In a murine model of HIV germinal centre and FDC network formation were impaired as was immunoglobulin isotype switching (Poudrier et al, 2001). Furthermore defects in T helper (TH1) lymphocyte responses have been noted in tuberculosis (Wu et al, 2008). The detection of any alterations in immune function or some form of immune response during TSE infection would not only be fundamentally important but could aid the development of future diagnostic tests for TSE infection. In addition, any TSE associated immune system dysfunction may have implications for human and animal health especially if many individuals in a population are infected with a TSE such as vCJD.

Studies in mice with immunodeficiencies have demonstrated that the presence of a functional immune system is a requisite for efficient peripheral pathogenesis of disease. A number of studies have focused on the effects and relevance of

immunodeficiency or absence of specific cell types such as FDCs in pathogenesis. However, what is little understood is the effect of modulating the immune system by generating an immune response either at the time of infection or during incubation period of disease. Studies carried out in the 1970's showed that a single treatment with phytohaemagluttinin (Dickinson et al, 1978) or modified BCG vaccine (Kimberlin & Cunnington, 1978a) prior to infection could significantly shorten incubation period of TSE disease in rodents. At the time of conducting these studies the implications of these findings in relation to the role of FDCs and the immune system in pathogenesis was not fully understood. Based on our current understanding of FDC involvement in TSE infection (Brown et al, 2000a; Brown et al, 2000b; Brown et al, 1999; Mabbott et al, 1998; Mabbott et al, 2002; Mabbott et al, 2000b; Mabbott et al, 2003) it seems likely these effects on pathogenesis would have been the result of germinal centre activation leading to increased levels of agent replication in lymphoid tissues and more efficient neuroinvasion.

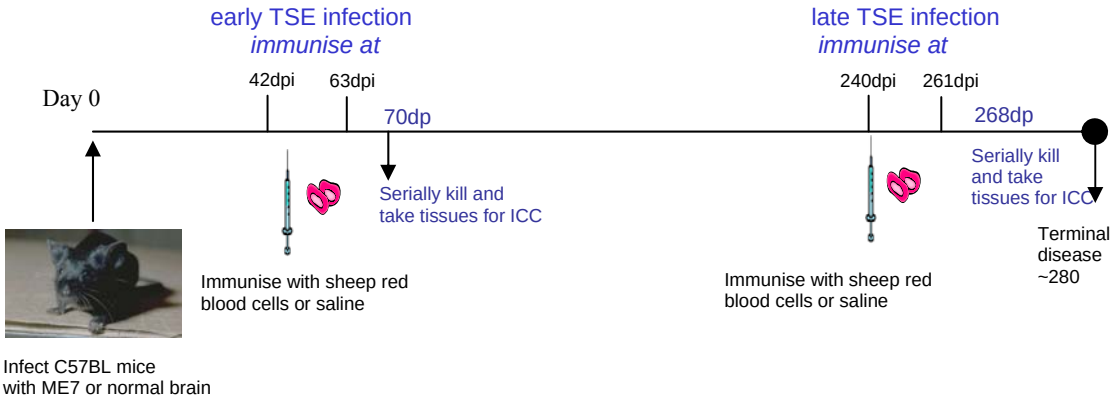
In this chapter the aims are to (1) determine the effects of the immune system on pathogenesis by generating a humoral immune response at several timpoints during pathogenesis, (2) to determine if there are any assoicated alterations in immune function for example in germinal centre development in lymphoid tissues and (3) to determine if immunisation alters levels of agent infectivity in lymphoid tissues. If immunisation alters efficacy of infection or levels of infection within lymphoid tissues this may have important implications in determining risk factors in the transmission of TSE infection for example in vCJD infection.

In the first series of experiments, sheep red blood antigen (SRBC) was used to immunise mice at an early and late stage of infection to determine the effects of immunisation on pathogenesis and to assess immune function in TSE infected animals. However, despite its widespread use, SRBC antigen may not be an ideal T-dependent immunogen because of differences in the ability of different donor sheep to stimulate antibody responses. In the second series of experiments, a hapten-protein carrier system (dinitrophenyl-keyhole limpet haemocyanin: DNP-KLH), was used to immunise mice at three separate timepoints during the incubation period of disease. Dinitrophenyl (DNP) has been used extensively as a model immunogen in the study of immunology and much of our current understanding of the antibody response to antigens has been derived using these systems.

6.3 Results

6. 3.1 Disease pathogenesis in scrapie infected mice following SRBC immunisation

Figure 6.1 Experimental design



ME7 or normal brain (NB) infected mice were immunised with SRBC antigen at the timepoints shown above. Animals were immunised at an early and late stage of infection and a proportion of animals from each group left until the clinical endpoint of disease to determine whether immunisation had any effects on incubation period at specific timepoints. Immunisation at an early infection timepoint was carried out to determine the effects of immunisation before the spread of infection to the CNS (neuroinvasion) had occurred. In addition at this stage active replication of agent infectivity would be occurring in lymphoid tissues; although infectivity would not yet have reached a plateau at this stage. Immunisation at a late (pre-clinical) stage of infection was carried out to determine effects on pathogenesis when high levels of TSE associated pathology would be present in the CNS.

In the early immunisation group mean incubation periods in immunised mice were significantly longer (284±7 p=0.033) than those of the ME7 challenged mice which were given saline (261±7). Notably the incubation periods in the mice given saline as control were slightly shorter than anticipated for this agent strain. In these studies, ME7 infected mice alone were not included so it is not known if injection with saline alone affected pathogenesis. In the animals immunised at the late stage of infection, there were no differences in incubation periods between immunised mice (263±3 p= 0.65) and the saline control group (271±7). However following immunisation at late stage infection, approximately one third of the scrapie infected mice died (7/23 animals) unexpectedly (all of the mice died within a 24 hour period)following boosting with SRBC antigen. No deaths or other clinical effects were recorded in the immunised uninfected mice or in the scrapie infected mice given saline instead of the SRBC immunisation. In addition, no adverse effects were recorded following antigenic challenge at the early stage of scrapie infection. As these adverse effects were not anticipated, mice were returned to their holding cages immediately following immunisation and only checked later that day; therefore it can only be estimated that the mice died within an 24 hour time period following boosting. To establish if clinical status of the mice had any relevance to these unexpected deaths; analysis of the TSE scoring records was carried out. This revealed that of the 7 animals that died only 1 had a "definite" scrapie score and another 2 had a "probably affected" score. The remaining 4 animals had been allocated a clinically normal score prior to boost immunisation with SRBC.

Figure 6.2 ME7 pathogensis following immunisation with SRBC antigen

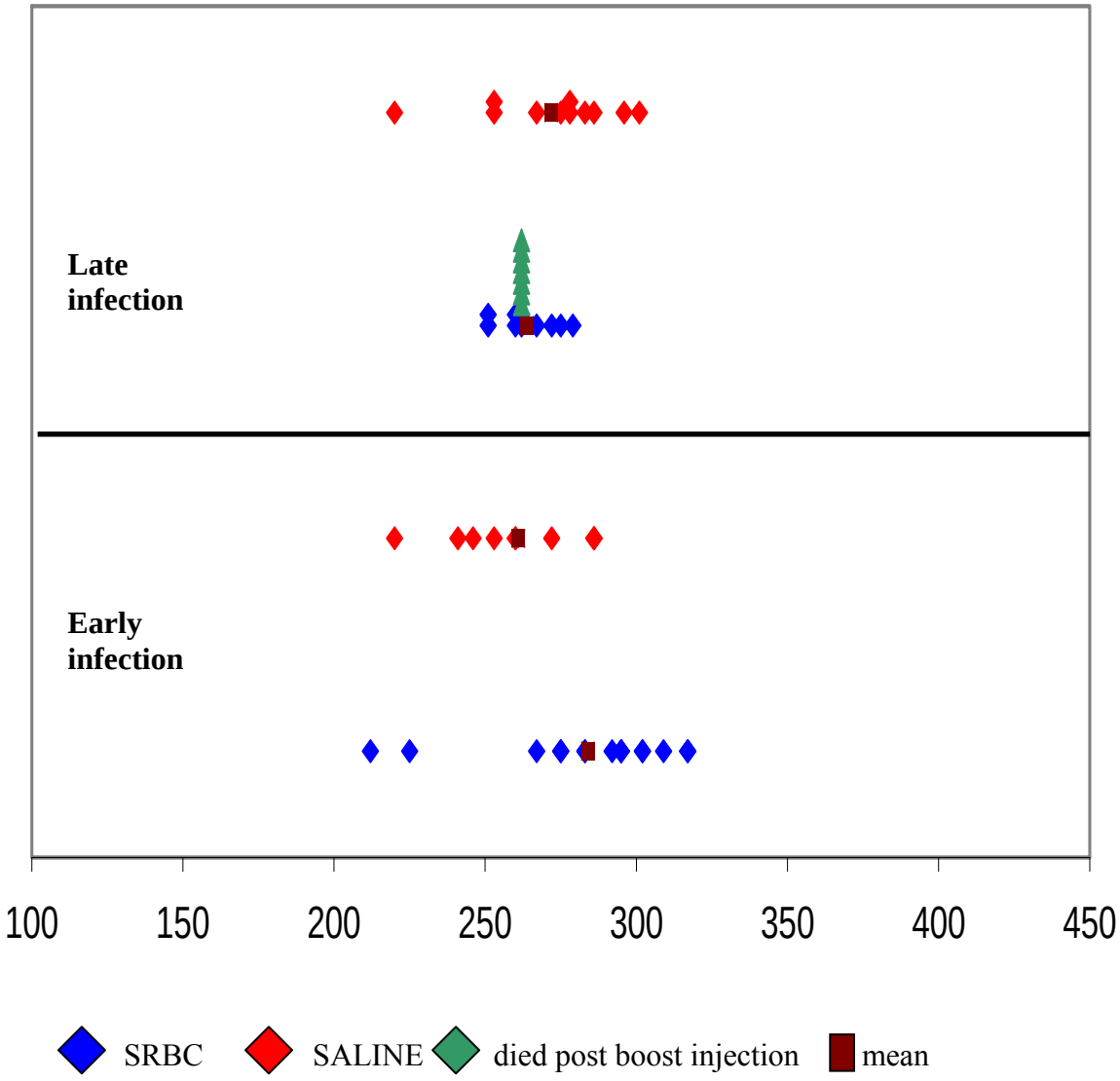


Figure 6.2 Effect of immunisation on the pathogenesis of the ME7 strain of scrapie. Mean incubation periods of mice immunised at an early stage of infection (42 and 63 days) were signifcantly longer than the saline control (p=0.033). Following immunisation with SRBC antigen at the late stage of infection (240 and 261 days) no significant difference in incubation period was observed between the immunised and non-immunised mice although 7/23 mice died suddenly following the boost immunisation. These animals are excluded from the incubation period analysis.

6.3.2 Effect of immunisation with SRBC antigen on PrP accumulation in the spleen

The accumulation of the disease specific form of PrP protein (PrP^{Sc}) in lymphoid tissues is a characteristic of many natural and experimental TSE infections. As antigenic challenge stimulates the number and size of germinal centres and FDC networks in lymphoid tissues this may enhance PrP^{Sc} accumulation in the spleens of scrapie-affected mice. Immunohistochemistry (IHC) and PET blot analysis was used to assess the numbers of PrP^{Sc} postive FDCs in the spleen (Schulz-Schaeffer et al, 2000) (Figure 6.3). Data is presented as number of PrP^{Sc} positive FDCs in spleen/individual animal (two spleen sections from each animal were quantified) and also as the mean number of PrP^{Sc} positive FDCs in spleen in each experimental group. Statistical analysis was carried out using the student's t-test (two-tailed, unpaired), based on comparison of mean counts from individual animals. Comparison of PrP^{Sc} labelling one week post boosting (268dpi) from the pre-clincal infection groups found almost identical numbers of PrP positive FDCs in SRBC immunised mice (mean number of 38 PrP^{Sc} positive FDCs per ½ spleen section) compared with the saline injected mice (a mean number of 40 PrP^{Sc} positive FDCs per ½ spleen section) p=0.81. Quantification of PrP positive FDCs from the mice which died suddenly found that there was a mean number of 51 PrP postive FDCs per ½ spleen section. However it is not possible to determine if this figure represents a significant increase in PrP positive FDCs as due to the sudden death of the immunised mice equivalent ME7 saline controls were not collected at the same time. Data is not presented from the early infection immunisation timepoints due to small sample number (only one spleen was available from the ME7 immunised group and

two from the non-immunised group). However there was a mean numbers of 92 PrP positive FDCs in spleen from the immunised animal in comparison with a mean of 29 PrP postive FDCs in the non-immunised infected animals (n=2) (p=0.09) suggesting that immunisation at this early stage of infection may have increased numbers of PrP positive FDCs in spleen.

Figure 6.3 Quantification of PrP^{Sc} positive FDCs following immunisation of scrapie infected mice

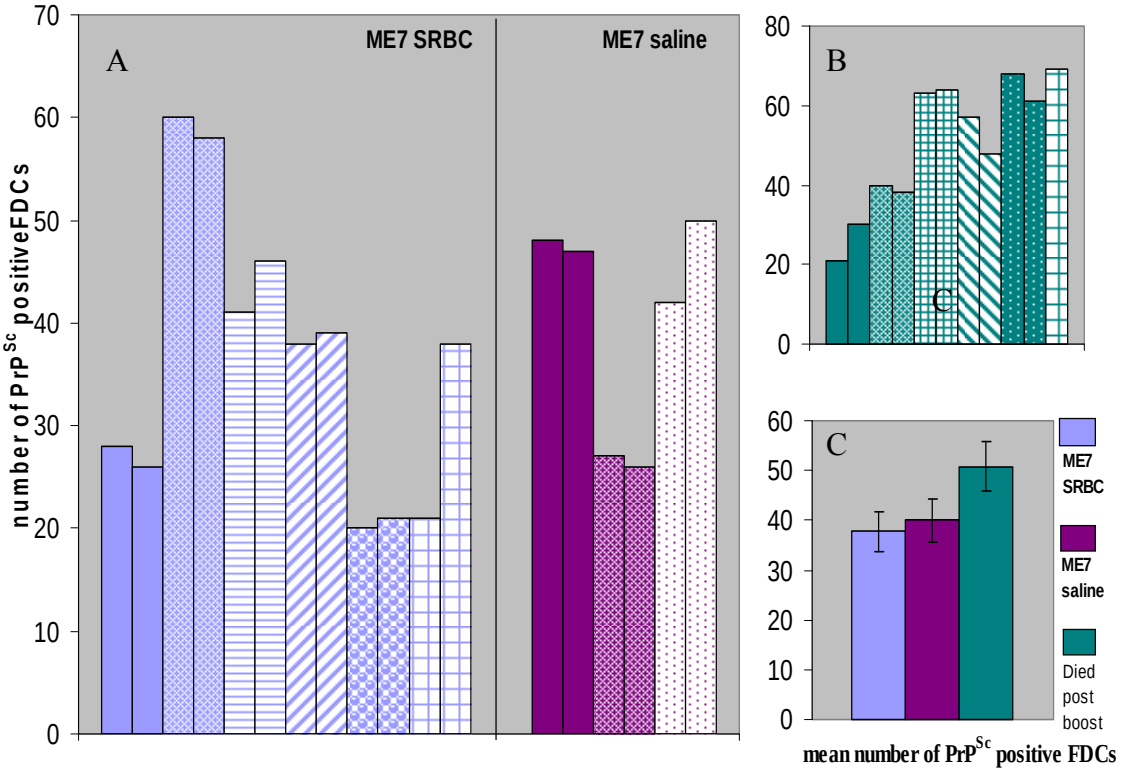


Figure 6.3 Estimation of PrP^{Sc} positive FDCs in spleen sections from individual mice, labelled. PrP^{Sc} positive FDC networks were detected using the PET blot method and the numbers of PrP^{Sc} positive FDCs in each spleen section (2 from each mouse) quantified. (a) Number of PrP^{Sc} positive FDCs in spleen from ME7 infected mice immunised with SRBC or saline at the late stage of infection. Spleens were taken 1 week post boosting with SRBC or saline (268 days post infection). (b) PrP^{Sc} positive FDCs in mice which died suddenly post SRBC boost immunisation. (c) Mean numbers of PrP^{Sc} positive FDCs in spleen from animals which died suddenly and those taken 1 week post boosting. Statistical analysis was carried out using the student's t-test and based on comparison of average counts from individual animals. There was no significant difference in the number of PrP positive FDCs one week post boost between infected mice immunised with SRBC or saline (p=0.81).

Figure 6.4 Accumulation of PrP^{Sc} in spleen following immunisation

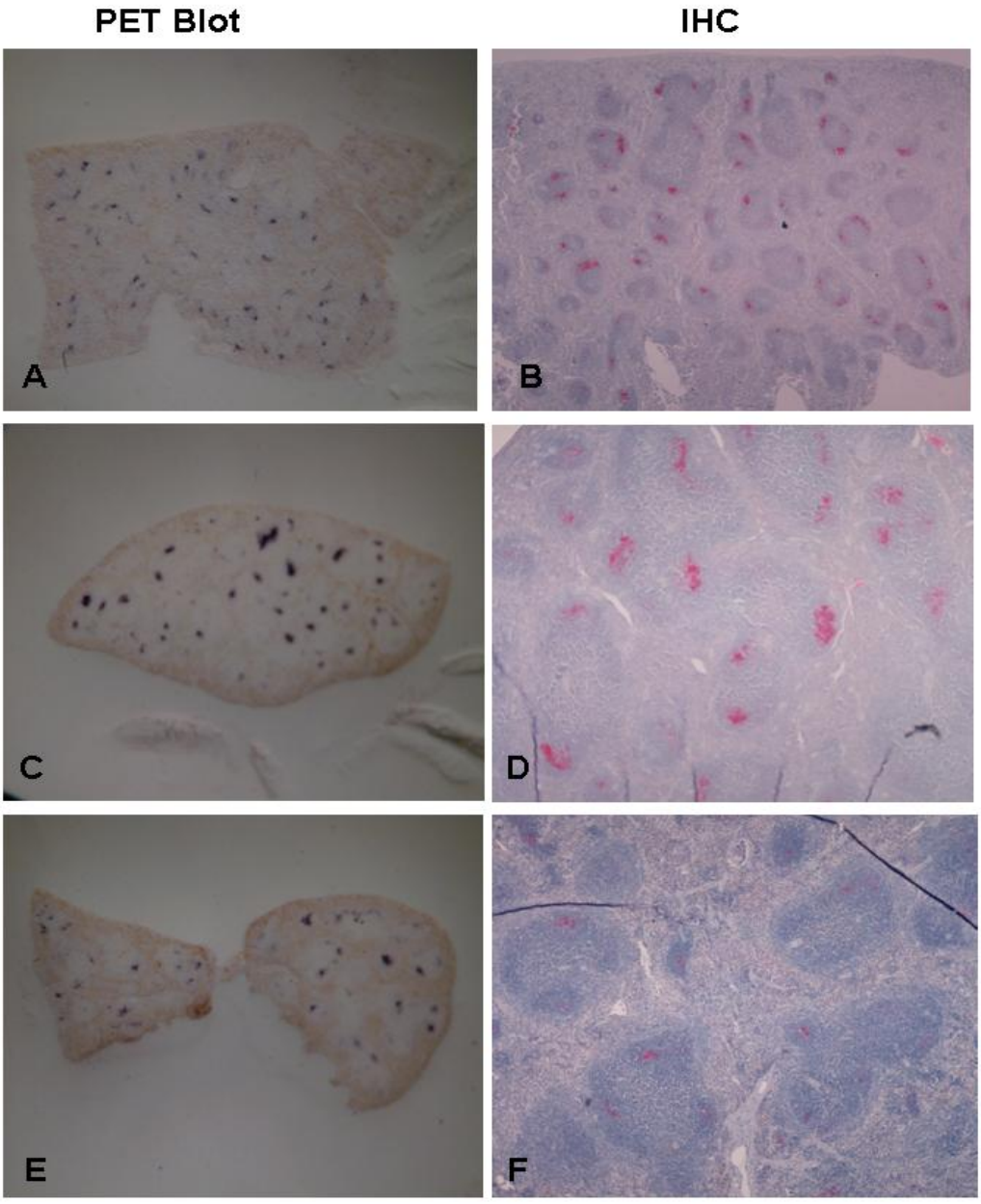


Figure 6.4 Immunohistochemical detection of PrP^{Sc} at the late stage of infection using the monoclonal antibody 6H4. A, B shows spleen sections from animals that died immediately following boost immunisation. C, D are from mice immunised and boosted with SRBC antigen and collected at the terminal stage of disease and the E,F shows sections from mice injected with saline and collected at the terminal stage of disease. Quantification of PrP^{Sc} positive germinal centres in PET blots from SRBC immunised mice demonstrates that there is no difference in numbers of PrP^{Sc} positive germinal between SRBC and saline immunised ME7 infected mice.

6.3.3 Immunoglobulin (Ig) levels in the serum of ME7 infected mice following immunisation with SRBC antigen.

One of the aims of this study was to determine whether antigen-specific immune responses were altered in TSE infected mice. However, attempts to measure specific Ig responses to SRBC antigen were unsuccessful; despite many attempts at this procedure. Therefore to provide some estimate of Ig levels in sera from infected and uninfected mice following SRBC immunisation a radial immunodiffusion (RID) assay was performed (Mancini et al, 1965) using sera from uninfected (n=4) and infected mice (n=6) collected one week post boost immunisation at a late stage of infection (268 dpi). The RID assay is based on the principle of complexing antigen and antibody to produce a visible precipitin ring. In these studies the mouse BINDARID™ kit was used. The RID assay is performed in (ready-to-use) agar plates, containing specific anti-IgG subclass antibodies. Test samples, standard and control sera, are also prepared and added to the plates. After 72 hours incubation at room temperature the diameters of the control and sample rings were measured and values calculated using the standard reference table provided. Immunoglobulin sub-class measurements were performed and the results displayed in figure 6.5. There was no significant differences in the total Ig levels in any of the Ig sub-classes between the ME7 infected and uninfected mice following immunisation. However, levels of IgM in 3 of the 6 ME7 infected mice exceeded the upper detection limit of the assay and these individual values are presented in figure 6.5. In-fact 4 of the 6 mice had higher IgM levels than those of the uninfected mice. Clearly more studies are required to further investigate these differences and these planned studies will also include measurement of Ig levels in non-immunised TSE infected mice for comparison.

Figure 6.5 Serum immunoglobulin levels as determined by RID in ME7 infected or uninfected mice following immunisation

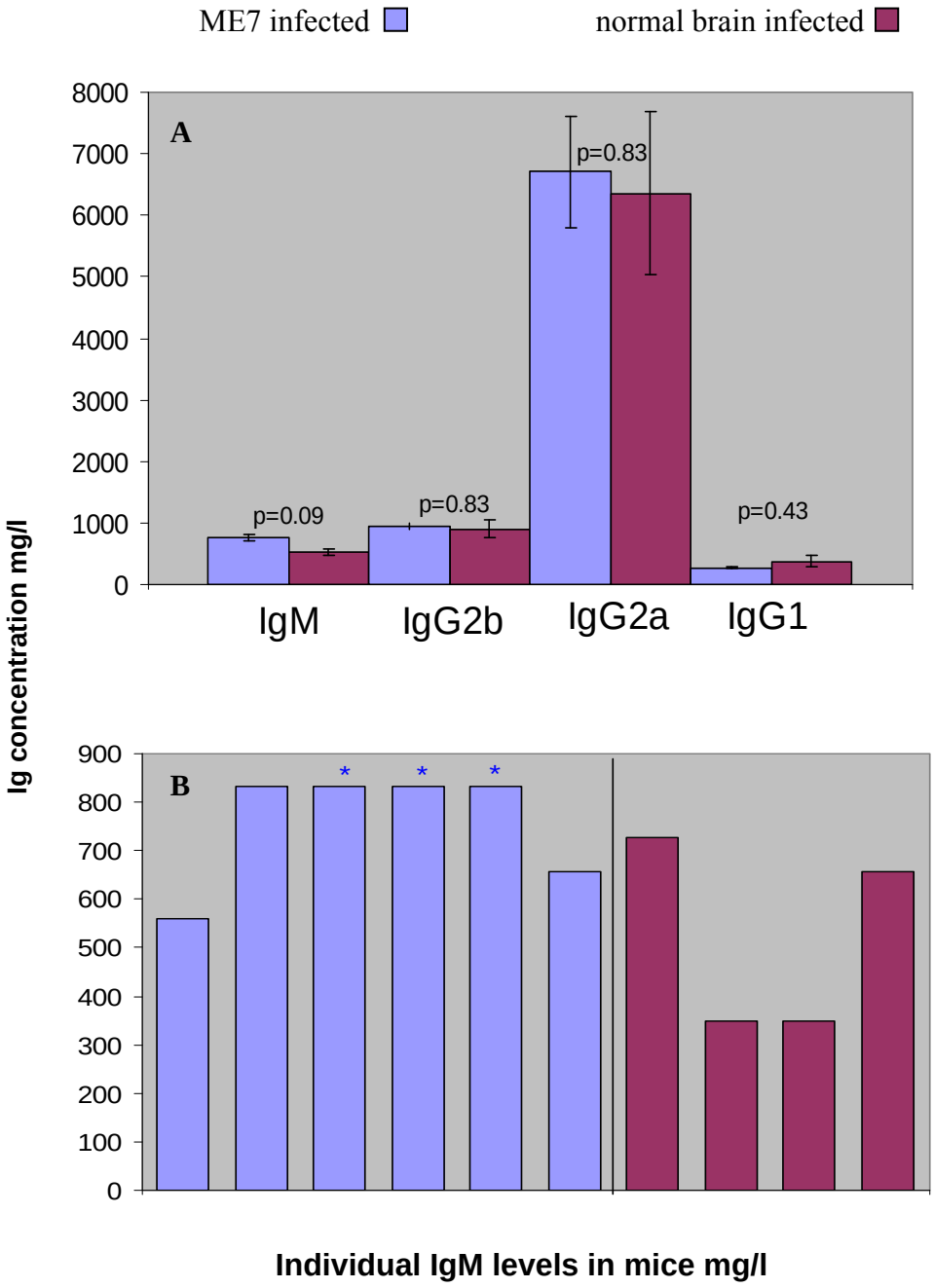


Figure 6.5 RID measurement of total serum IgM, IgG2b, IgG2a and IgG1 levels in sera of SRBC immunised ME7 infected (blue bars) or normal brain (maroon bars) injected mice. Sera samples were collected 7 days post boost immunisation with SRBC at a late stage of infection. (A) Mean serum Ig levels in SRBC immunised ME7 (n=6) and uninfected (NB) mice (n=4). (B) Individual serum IgM levels in infected or uninfected mice following boost immunisation * 3/6 mice had levels of IgM exceeding the upper detection limit of the assay (>833mg/l).

6.3.4 Analysis of PNA germinal centres in uninfected and scrapie infected mice following immunisation with SRBC antigen

Peanut agglutinin (PNA), isolated from *Arachis hypogaea* (peanuts), is a 10,000 molecular weight lectin composed of four identical subunits of approximately 27,000 Daltons each. PNA (PNA) (Reisner et al, 1979). Mature B cells, but not immature B cells, within active germinal centres exhibit high levels of binding by PNA (Kosco-Vilbois, 2003). The enumeration of PNA positive germinal centres in infected and non-infected mice following immunisation was carried out to determine any effects of TSE infection on the formation of germinal centres.

PNA positive germinal centre numbers in immunised ME7 infected or normal brain injected mice were quantified, one week post boost immunisation at an early and late stage of infection (figure 6.6). In both the early and late immunisation groups there were more PNA positive germinal centres in the immunised ME7 infected mice compared with the NB injected controls, although there was considerable variation between some animals in the same experimental groups. To display this variation, data is presented as germinal centre counts from individual animals (two spleen sections from each animal were counted) and the mean number of germinal centres in spleen in each experimental group. Statistical analysis was carried out using the student's t-test and based on comparison of average germinal centre counts from individual animals. At the early stage of infection the ME7 infected mice had a mean number of 13.1 germinal centres per ½ spleen section compared to 3.1 GC in the NB injected mice ($p=0.08$). Similarly, at the late stage of infection the ME7 infected mice ($n=6$) had a mean number of 13.3 GC per ½ spleen section compared with 6.9

in the control mice (n=7) (p=0.09). Analysis of GC numbers at the early stage of infection from non-immunised ME7 infected (n=3) and uninfected mice (n=3) also demonstrated greater numbers of GC in infected spleen, although this difference was not statistically significant (p=0.48) (data not shown).

Although quantification studies failed to demonstrate significant differences between germinal centre numbers in uninfected and ME7 scrapie infected spleen, germinal centre size appeared greater in ME7 scrapie infected mice. Further studies are clearly required using larger sample sizes to establish if indeed germinal number and/or size is increased in TSE infection. In addition, in ME7 infected mice high levels of singly labelled PNA positive cells could be visualised throughout the splenic white pulp (figure 6.7). It is possible this represents distribution of mature B cells throughout the white pulp in these scrapie infected spleens although the significance of this is not understood, again further studies are required to validate this observation.

Figure 6.6 Estimation of PNA positive germinal centre number in spleen following immunisation of ME7 infected or uninfected mice

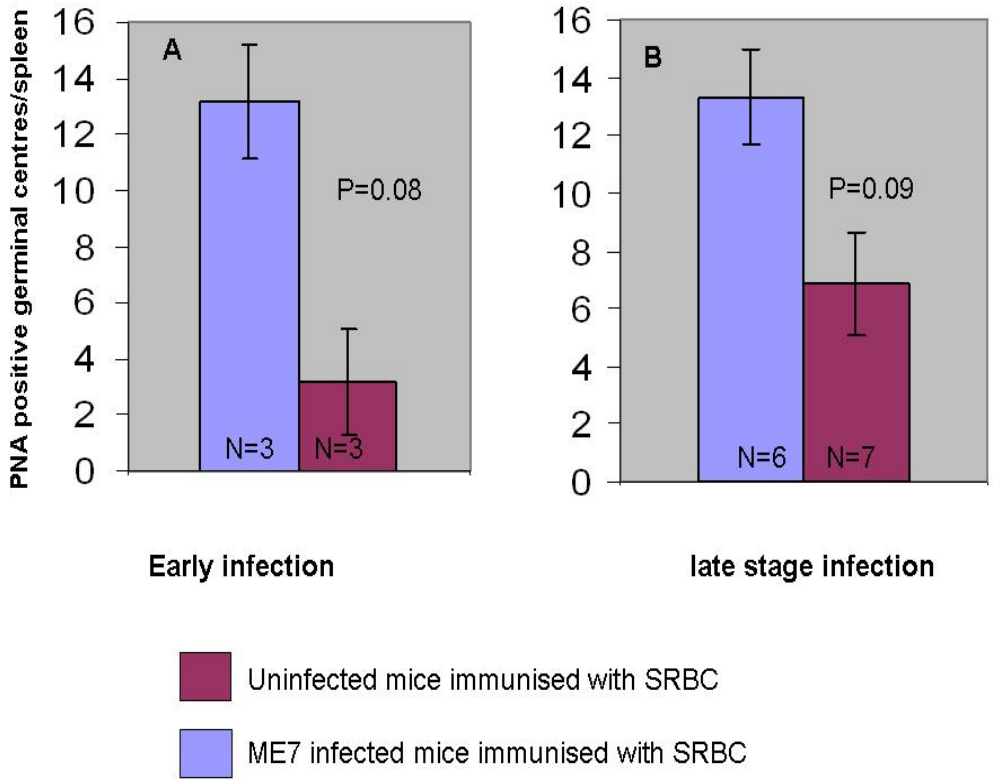


Figure 6.6. PNA positive germinal centre numbers in spleen in uninfected or ME7 infected spleen 7 days post boost immunisation with sheep red blood cells (SRBC) at the early (A) or late stage of infection (B). Mean numbers of PNA positive GCs in spleen from animals in each experimental group are displayed. Statistical analysis was carried out using the student's t-test and based on comparison of average counts from individual animals.

Figure 6.7 Immunohistochemical detection of PNA positive germinal centres following immunisation

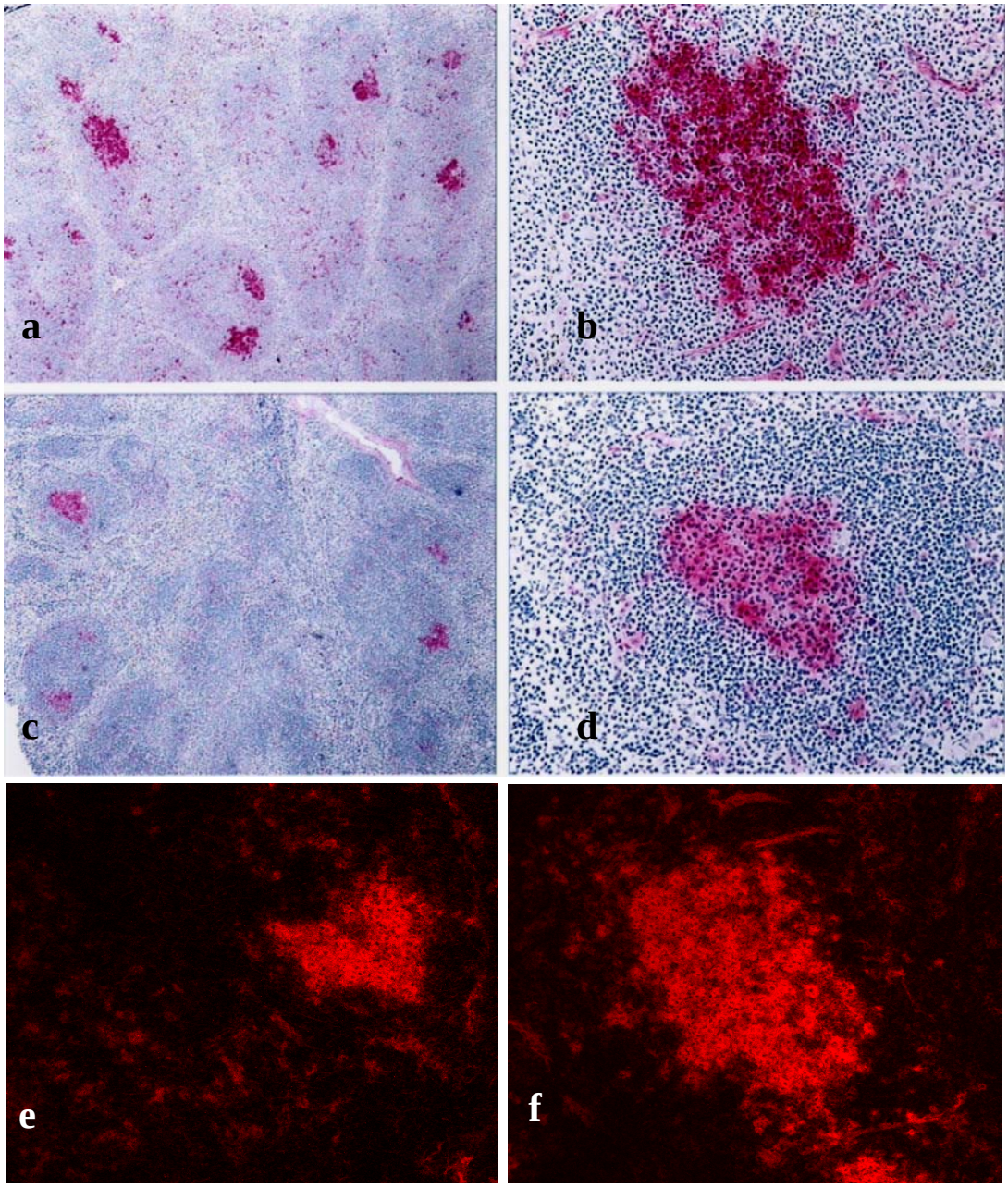
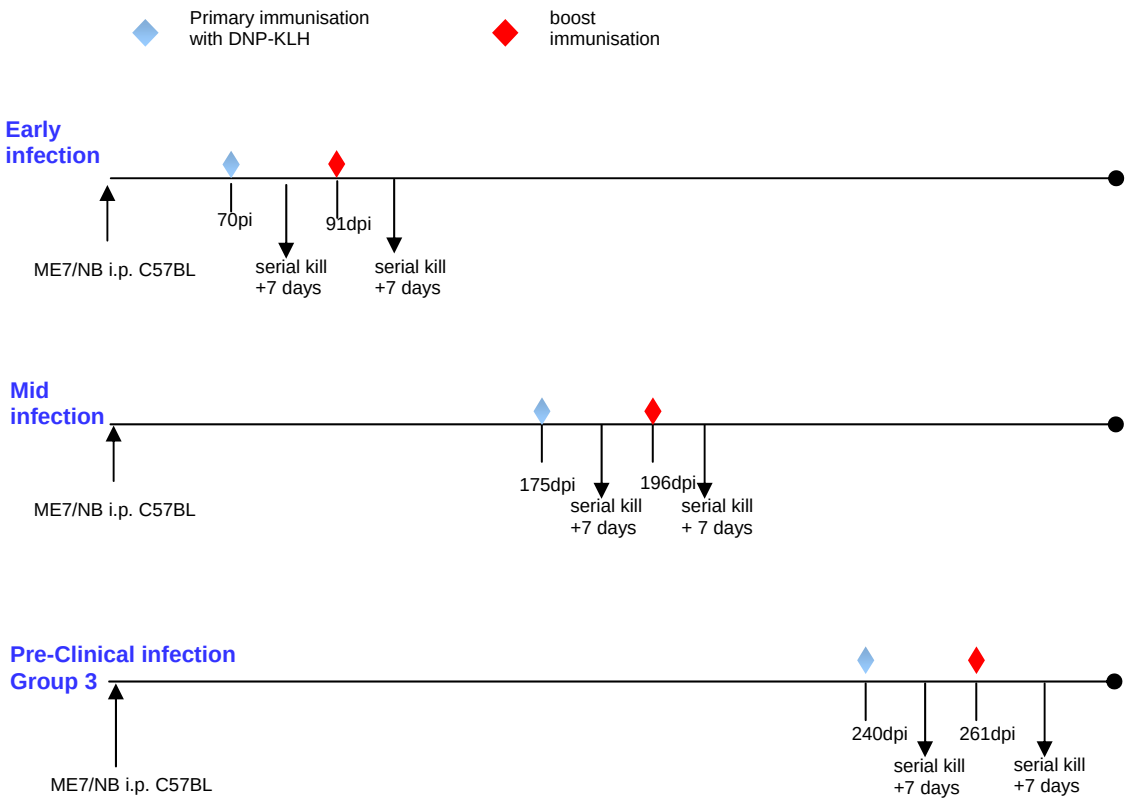


Figure 6.7 Immunohistochemical detection of germinal centres in spleen by labelling with peanut agglutinin (PNA). Spleens from uninfected (normal brain injected) or ME7 infected mice were collected one week post boost immunisation with sheep red blood cells (SRBC). (at 268 days post ME7 or NB challenge). Although quantification studies failed to demonstrate significant differences between GC numbers in uninfected (c,d and e) and ME7 infected spleen (a, b and f) GC size appeared greater in ME7 infected mice. In addition in infected mice high levels of singly labelled PNA positive cells could be visualised throughout the splenic white pulp although the significance of this is not understood.

6. 3.5 Effect of immunisation with dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH) on scrapie pathogenesis.

Figure 6.8 Experimental design



Following the results obtained with the SRBC immunisations another immunisation study was set up, this time using a different immunogen: dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH.) The main reasons for this change of immunogen were concerns over variability in immunogenic properties that can occur with SRBC antigen and difficulties in the detection of SRBC specific Ig sub-class by ELISA. As shown above in figure 6.8. the effects of immunisation were investigated at three timepoints 1) an early timepoint (70 days post injection (dpi) where high levels of

infectivity would be present in spleen but neuroinvasion would not have occurred 2) a mid infection timepoint where infection into the CNS would have occurred but there would be no clinical signs of disease and minimal CNS pathology and 3) a late pre/early clinical timepoint where some early clinical symptoms may be present but major pathological changes (deposition of PrP and vacuolar changes) would be present. Mice were injected i.p. with ME7 scrapie agent strain (1% dilution of terminal ME7 scrapie brain homogenate) and then immunised with 100 µg of DNP-KLH in alum in 100 µl PBS given i.p (see section 2.5.3) at the times shown in figure 6.8. A secondary immunisation with 100 µg of DNP-KLH was given 3 weeks after primary immunisation. From each immunisation timepoint tissues were collected 7 days after primary and boost immunisation to determine the effects of a primary or secondary immune response on infectivity levels in the spleen. Groups of immunised and control (non-immunised) mice were maintained until the endpoint of disease to determine the effects of immunisation on disease pathogenesis.

Mice immunised at an early stage of infection had significantly shorter incubation periods in comparison with non-immunised mice ($p=0.001$) (figure 6.9, figure 6.10). There were no effects on pathogenesis in mice immunised at the middle stage of infection although this is perhaps not surprising as infection would have already been established in the CNS at this stage. Indeed studies involving splenectomy (Kimberlin & Walker, 1989) or manipulation of FDCs in the lymphoid system (Mabbott et al, 2003) are only effective early in peripheral infection and not in the later stages of infection. Because of the adverse effects obtained in the SRBC study where a high proportion of mice died suddenly following boost immunisation this

raised some issues in relation to home office licencing with this procedure (the use of experimental animals in the UK is subject to the regulation of the animals (scientific procedures) act of 1986). As a result it was not permitted to boost immunise any animals with suspected or confirmed clinical signs of disease and such animals were excluded from the study. Thus it is not known if boost immunising these animals would have produced the same effect as obtained with the SRBC antigen.

Figure 6.9 Effect of immunisation with DNP-KLH on the pathogenesis of peripherally acquired ME7 scrapie

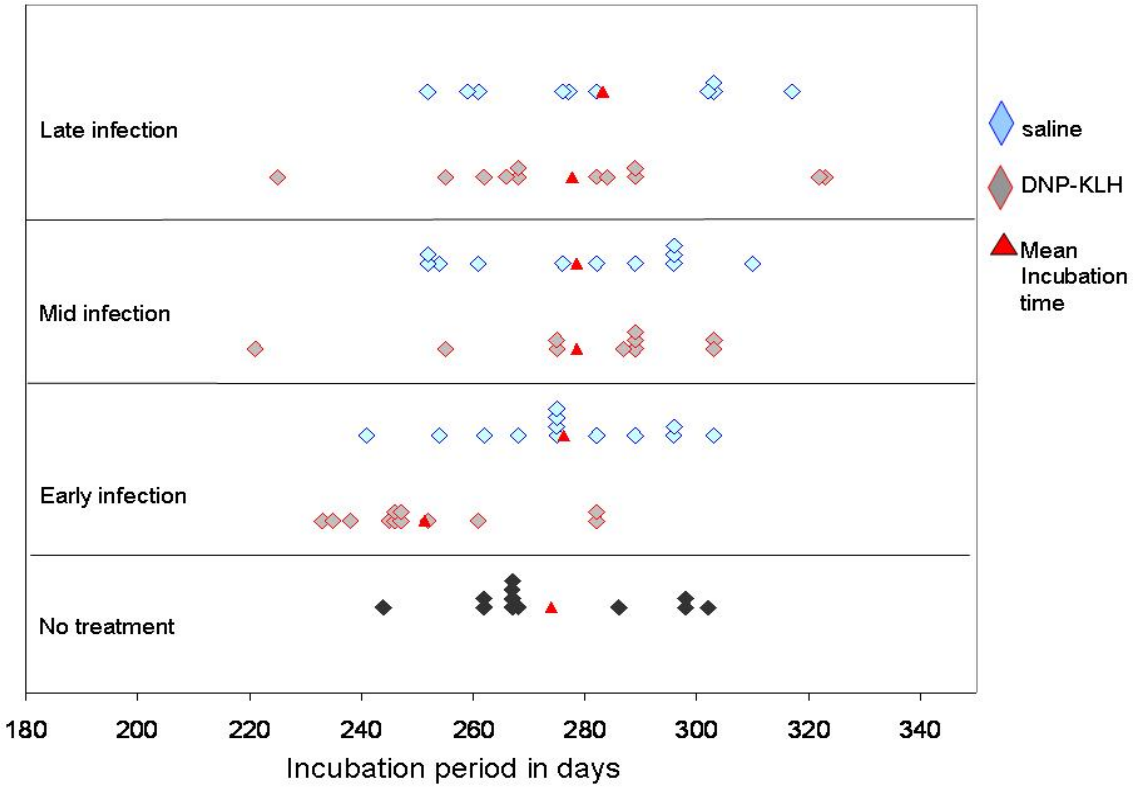


Figure 6.9 Pathogenesis of the ME7 strain of scrapie in mice immunised with DNP-KLH or saline at an early, mid and late stage of infection. Untreated, ME7 infected mice are also displayed for comparison.

Table 6.1. TSE disease incubation periods in mice immunized with DNP-KLH or saline and untreated mice at 3 separate points of infection. Statistical analysis using the student's t-test was performed (two-tailed unpaired) *p* values represent statistical comparison of individual incubation periods obtained from ME7 infected DNP-KLH immunised mice and saline treated mice at each immunisation timepoint. Values that are significant at the 5% level are shown in italics.

	Early infection	**Mid Infection**	**Pre-clinical**
DNP immunised	**251±5** ***p=0.001***	**278±7** **P=0.99**	**278±** **P=0.61**
Saline	**276±5**	**278±6**	**283±7**
No treatment	**274±6**	**274±6**	**274±6**

Figure 6.10 Boxplots of incubation period data obtained from ME7 infected DNP-KLH or saline immunised and untreated control ME7 infected mice at an early, mid and late point during incubation period of disease.

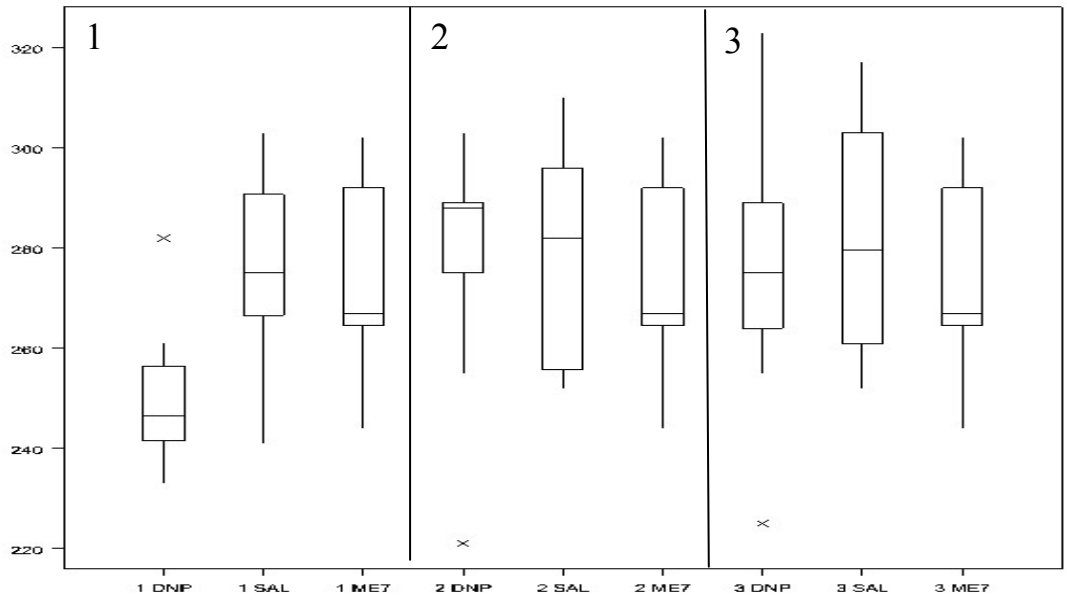


Figure 6.10 Boxplots showing incubation periods in days (y-axis) in immunised and non-immunised mice at an early (1), mid (2) and late (3) timepoint post infection. The highest and lowest data points are demonstrated by 'whiskers' with the line through the middle of the median value. The box itself contains the middle half of the data. Unusual data points are shown by a ×. The coding along the *x*-axis refers to: DNP= mice immunised with DNP-KLH, SAL= mice given saline as control immunisation, ME7= mice without any treatment to act as controls for the DNP-KLH and saline groups.

6.3.6 Effects of immunisation with DNP-KLH on splenic infectivity levels and the accumulation of PrP^{Sc} in spleen.

As immunisation stimulates germinal centre formation and increases the size of FDC networks (Kosco-Vilbois et al, 1997) it is possible that immunisation may likewise increase infectivity levels in lymphoid tissues or blood.

Levels of infectivity were measured in spleen collected one week post primary and boost immunisation from the early immunisation group (spleens collected at 11 and 14 weeks post infection). Spleen was also collected for assay from the other timepoints but the results of these assays are not complete and therefore not included in this thesis. In addition, blood samples from these studies have also been collected from all the immunisation timepoints and it is proposed to measure infectivity levels in these blood samples.

Infectivity levels in spleen from DNP-KLH immunised mice one week post primary immunisation ranged from 5.5–6.0 i.c.ID_{50}/g, and 5.3-6.5 i.c.ID_{50}/g in the saline control mice. Infectivity titres following boost immunisation ranged from 6.0-6.9 i.c.ID_{50}/g in spleen from the DNP-KLH immunised mice in comparison with 5.7-6.1 i.c.ID_{50}/g in the saline control mice. Two of the three spleens from the DNP-KLH boost immunised mice contained levels of infectivity that were greater than those of the saline injected animals. Although it appeared that levels of infectivity were increased in spleen following boost immunisation statistical analysis was carried out to establish whether these were significant. A linear mixed model was fitted to the data with treatment (saline or DNP-KLH) and immunisation (primary or secondary)

included as fixed effects, and mouse sample as a random effect. The parameters of the model were estimated using the REML directive in Genstat 10th edition. The estimated mean incubation period in the DNP boost immunisation group was lower than in the saline group (197.1 ± 4.7 compared to 208.8 ± 4.6) however this difference was not significant at the 5% level (figure 6.11 (B)).

The deposition or accumulation of PrP^{Sc} in association with FDCs in lymphoid tissues is a characteristic of many TSE infections. Immunohistochemical (IHC) of spleen was carried out to determine if immunisation produced any subsequent increase in PrP^{Sc} in association with FDCs (figure 6.12). IHC analysis for PrP^{Sc} using the monoclonal antibody 6H4 antibody in spleen sections collected one week post boosting with DNP-KLH was carried out at the early stage of infection. Spleens were collected 98 dpi (14 weeks post infection) at the same timepoint for the infectivity bioassay (results shown in figure 6.11). Although there appeared to be greater levels of PrP^{Sc} in spleen from the DNP-KLH immunised mice quantification remains to be carried out on these tissues and it is difficult to determine at this stage if immunisation increases PrP^{Sc} in spleen.

Figure 6.11 Infectivity levels in spleen following primary and boost immunisation with DNP-KLH immunogen.

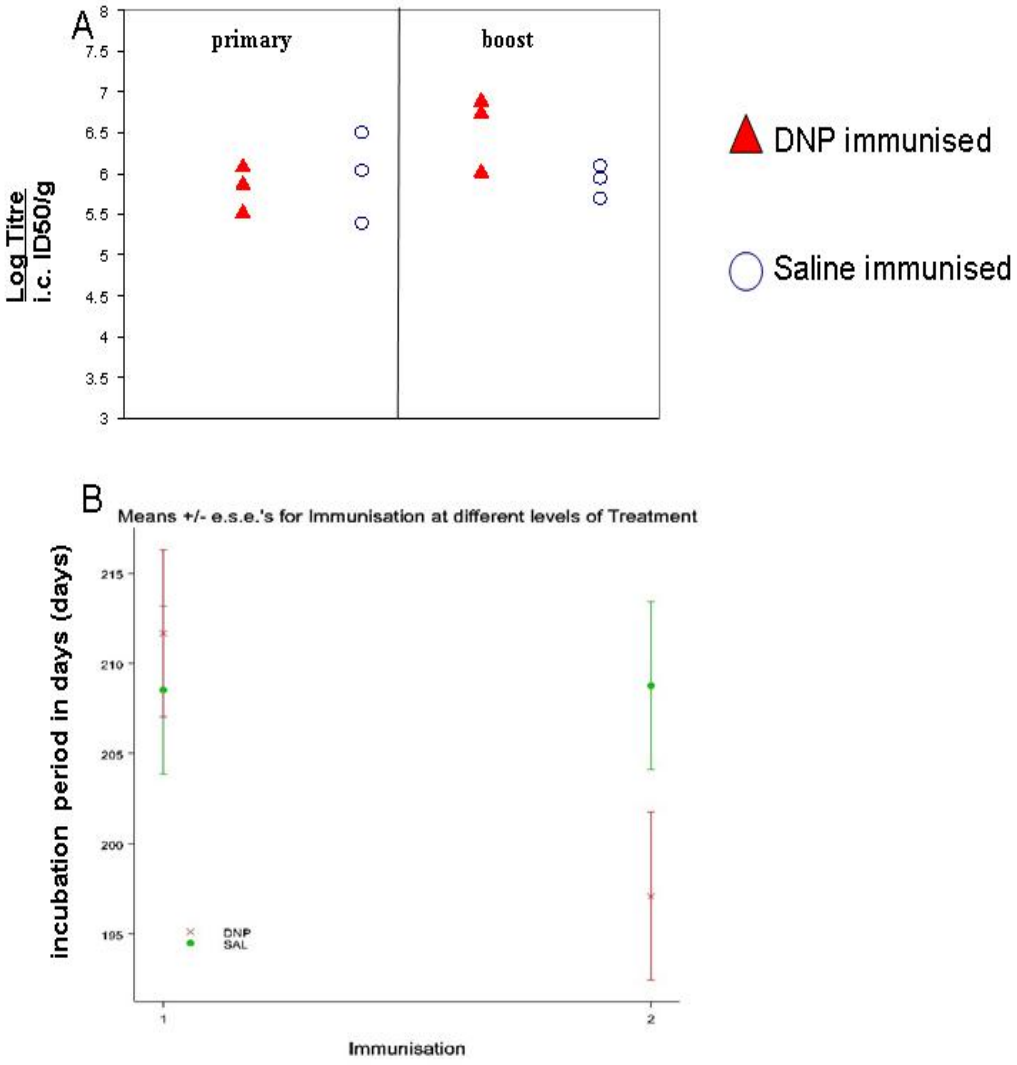


Figure 6.11 (A) Infectivity titres (log i.c.ID_{50}/g) in spleen from C57BL mice immunised and boosted with DNP-KLH at an early stage of infection (primary boost spleens collected 77 dpi and secondary boost spleens 98 dpi). (B) Statistical analysis of the difference in the mean incubation periods in bioassay mice following bioassay assay of spleen from DNP or saline immunised mice at 98dpi. In the original study animals had received either saline (negative control) or DNP as either a primary or secondary immunisation. There was no statistically significant difference in infectivity levels in spleen from DNP immunised (red line) or saline control mice (green line) at either the primary boost immunisation points (full details of the analysis are given in appendix III)

Figure 6.12 Immunohistochemical detection of PrP^{Sc} in DNP-KLH immunised mice

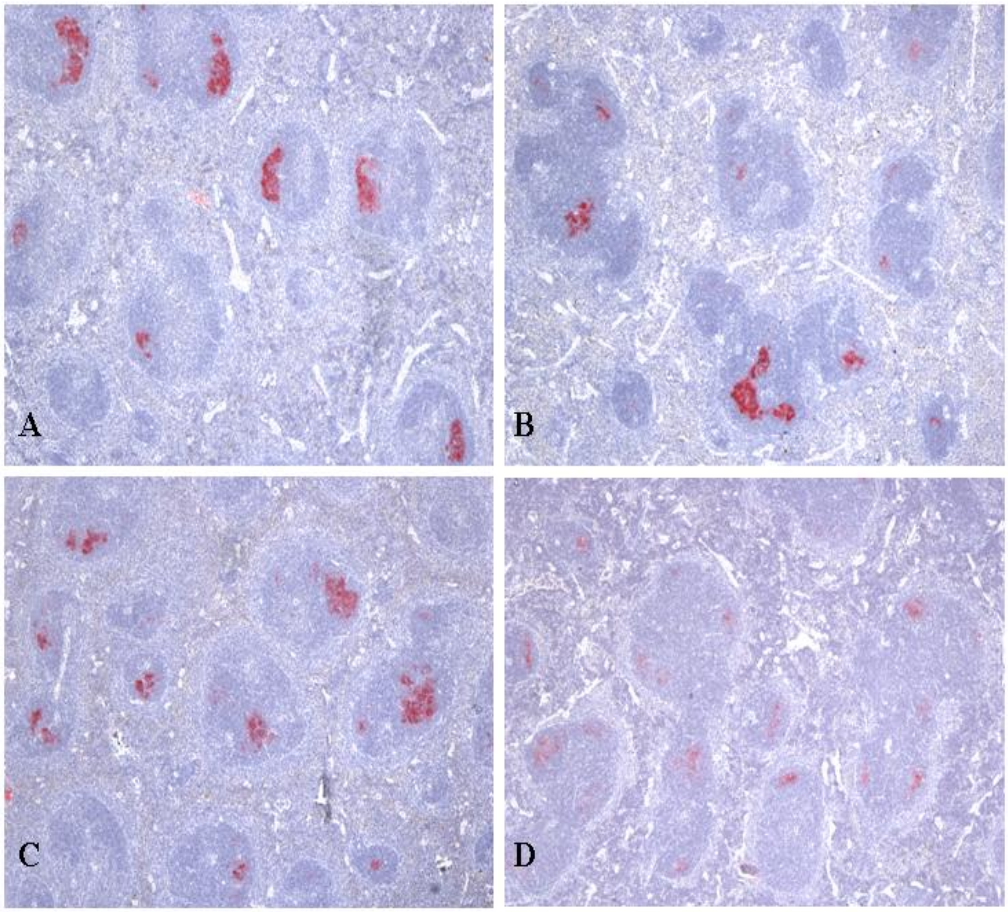


Figure 6.12 Immunohistochemistry for PrP^{Sc} using the monoclonal antibody 6H4 antibody in spleen sections collected one week post boosting with DNP-KLH (a,b) or saline (c,d). Spleens were collected 98 dpi (14 weeks post infection). Spleens were collected at the same timepoint for the infectivity bioassay (results are discussed in Fig 6.13). Although there appeared to be greater levels of PrP^{Sc} in spleen from the DNP-KLH immunised mice quantification remains to be carried out on these tissues.

6.4 Discussion

As the immune system is critical to a number of experimental and naturally acquired TSE infections it would seem logical that manipulation of the immune system may impact on TSE agent pathogenesis. In this chapter immunisation of mice infected with the ME7 scrapie strain produced some quite dramatic effects on pathogenesis, but notably only at specific points following infection. These data demonstrate the intrinsic involvement of the immune system, and the state of immune system activation to TSE pathogenesis. Implications from the findings obtained in this chapter are presented in this discussion.

TSE infections contradict much of what we understand about the nature of many "conventional" infectious diseases. In most viral or bacterial infections deficiencies in the immune system as a result of senescence (Linton & Dorshkind, 2004) or immunosupression increase opportunities for infection (El-Solh et al, 2001). Paradoxically, it is likely that individuals with immune system deficiencies may be less likely to develop peripherally acquired TSE infections such as vCJD than individuals with more competent immune systems. Extensive studies in immunodeficient and transgenic mice demonstrate the importance of the immune system and FDCs for efficient pathogenesis (Fraser et al, 1996) (Bruce et al, 2000). Indeed findings presented in chapter 5 (Brown *et al* submitted for publication) demonstrate that aged individuals are less likely to develop peripherally acquired infections such as vCJD, almost certainly as the result of age related immune system deficiencies and dysfunctional FDCs.

Immune responses to antigen either following infection or by immunisation (vaccination) lead to the generation of a humoral immune response (the production of antibody molecules in response to an antigen) (Ahmed & Gray, 1996; Gray & Matzinger, 1991). There are a number of steps in the generation of this response which culminate in the production of memory B cells which act to initiate a rapid, heightened secondary response upon encountering the original antigen (Gray, 2002). Follicular dendritic cells (FDCs) are pivotal to the generation and maintenance of the humoral immune response which is facilitated via the capture and retention of immune complexes on FDCs (Kosco-Vilbois et al, 1997; Kosco et al, 1992). These trapped immune complexes are considered to be processed into high immunogenic IC-coated bodies (iccosomes) which contribute to the generation of these high-affinity recall responses (Szakal et al, 1988) (Victoratos et al, 2006). The generation of a humoral immune response leads to the development of germinal centres (Kosco-Vilbois et al, 1997); unique microenvironments formed as a result of antigenic stimuli which contain FDCs, proliferating B lymphocytes and tingible body macrophages which ingest remnants of apoptosing B cells (Kosco-Vilbois et al, 1997). As FDCs are critical for efficient neuroinvasion (Brown et al, 2000b; Brown et al, 1999; Bruce et al, 2000; Mabbott et al, 2000a; Mabbott et al, 2000b) subsequent increase in FDC cell numbers and germinal centres may impact on peripheral TSE pathogenesis. This possibility was investigated in this chapter by eliciting an immune response during the disease incubation period.

One of the most unexpected findings from the studies presented in this chapter was the sudden deaths in the ME7 infected mice immunised with SRBC antigen at a late

stage of infection. This is the first time that such an effect has occurred following antigenic challenge of TSE infected mice. There were no deaths in any of the other groups at this timepoint, nor were there any adverse effects recorded when infected mice were immunised at the early stage of infection. The exact cause of these sudden deaths has not been established, although it is possible these effects are the result of some form of exaggerated (hyper-immune) response to antigen either as a direct result of long-term infection of lymphoid tissues, evoking some form of TSE induced immune system dysfunction, or as a secondary result of neural dysfunction. Indeed, ultrastructural studies of ME7 infected spleen demonstrate increased FDC hypertrophy and an increased retention of electron-dense material at the FDC plasma membrane in comparison with uninfected mice; these effects were further increased in mice immunised and boosted with antigen (McGovern et al, 2004). Moreover, in these published studies increased numbers of of morphologically mature plasma cells were also observed ultrastructurally within the GC of scrapie-infected mice (immunised or non-immunised) than in uninfected controls mice (McGovern et al, 2004).

In the studies presented here, measurement of specific and total immunoglobulin levels by RID (Mancini et al, 1965) found some indication of raised IgM levels in SRBC immunised mice in comparison with immunised uninfected mice. This finding was not statistically significant but may reflect the small sample size available in the study. It is proposed to further investigate these findings and sera are available from the animals immunised with DNP-KLH (unfortunately time restraints meant that the measurement of specific immune responses to DNP-KLH immunisation could not be

completed). In addition, the number of germinal centres were increased in SRBC immunised TSE infected mice compared with non-immunised scrapie infected mice at both an early and late stage of infection. Here the findings were not statistically significant. Although only a small number of tissues have been studied so far, it may be that TSE infection alone generates some form of persistent immune response leading to the presence of long-term germinal centres in lymphoid tissues (McGovern et al, 2004) (McGovern & Jeffrey, 2007), as is known to occur following viral infection (Bachmann et al, 1996) . Although these findings require substantiation it may be that some form of TSE associated FDC dysfunction results in these B cell associated effects, perhaps in immunoglobulin class switching or in affinity maturation in the germinal centre, as previously suggested (McGovern et al, 2004). The possibility that TSE infection may induce some form of increased germinal centre formation and exaggerated immune response to antigen is extremely important. This may have serious implications for TSE infected individuals receiving routine immunisations which may exacerbate TSE pathogenesis. In addition, these finding may impact on therapeutic strategies against TSE infection involving vaccination with anti-PrP antibodies (Heppner et al, 2001; Sigurdsson et al, 2002) especially if these are carried out during the clinical phase of infection. However, there did not appear to be a correlation between clinical symptoms of disease and sudden death in these studies as only one of the seven mice that died had clinical signs of disease.

Alternatively, it is possible that these effects were a secondary result of neural dysfunction at late stage infection. Notably, anecdotal evidence exists to suggest that

peripheral infections in those with neurodegenerative conditions such as Alzheimer's disease excarbate disease symptons changes and induce "sickness" behaviours (Cunningham et al, 2005; Perry, 2004). In neurodegenerative conditions such as TSE diseases or Alzheimer' disease where there are are high levels of activated microglia in the brain (Betmouni et al, 1996; Williams et al, 1995), systemic infection or inflammation, leading to increased synthesis of inflammatory cytokines by these microglia, may influence progression of neurodegenerative disease. Moreover, adminstration of the T cell independent antigen lipopolysaccharide (LPS) to ME7 infected mice at an early clinical stage of infection demonstrated behavioural and physiological changes in LPS dosed animals (Combrinck et al, 2002). It is therefore possible that the sudden deaths of the animals in this study were directly attributed to effects of the immunisation on the CNS.

The blood-brain barrier (BBB) is a membranic structure that acts primarily to protect the brain from chemicals in the blood, whilst permitting essential metabolic function. The breakdown of the BBB is a characteristic of a number of infectious and neurodegenerative conditions including; dengue fever (Chaturvedi et al, 1991), bacterial meningitis, multiple sclerosis, ishchaemia (Marsala et al, 2004), Alzheimers disease (Perry et al, 1997) (Leake et al, 2000) and in TSE infection (Perry et al, 1997; Williams et al, 1995). Activation of the innate immune system by bacterial infections in individuals affected by multiple sclerosisis is thought to be linked to relapse induction (Correale & Farez, 2007). In the studies presented here only ME7 infected mice immunised at a late stage of infection with SRBC suffered adverse effects

suggesting that TSE associated blood brain barrier breach may have further contributed to the adverse effects on the mice.

The abnormal form of the PrP protein is known to at least partially co-purify with infectivity (Somerville et al, 1986) and its deposition in tissues can be used biochemically (Farquhar et al, 1994) and immunohistochemically (McBride & Beekes, 1999) to track the spread of infectious agent throughout the host. Preliminary studies using basic quantitative methods suggest that there is no significant difference between numbers of PrP^{Sc} positive FDCs in immunised mice compared with non-immunised infected mice at the terminal stage of infection; however biochemical studies will be required for quantitative estimations of $PrP^{Sc.}$

Two separate experiments were carried out to determine the influence of immunisation on peripheral ME7 pathogenesis. The first experiment used SRBC antigen as immunogen the second, the hapten-protein carrier DNP-KLH. Both of these immunogens are T cell dependent antigens, requiring the involvement of T lymphocytes (T Helper) cells to generate antibody production by B cells (Ahmed & Gray, 1996). T cell independent antigens on the other hand can provoke antibody production by B cells in the absence of T cell involvement (Gray & Matzinger, 1991). In these studies mice were immunised at an early or a late timepoint with SRBC and at an early, mid and late timepoint with DNP-KLH. In the first study incubation periods of mice immunised with SRBC at 6 and 9 weeks post ME7 infection were significantly *longer* than the unimmunised mice, $p=0.033$. In the second study however incubation periods in mice immunised with DNP-KLH at 10

and 13 weeks post infection were significantly *shorter* than those of the saline immunised mice or mice infected with ME7 alone (p=0.001). It is not clear why these differences in pathogenesis have occurred. The effects on pathogenesis with the SRBC antigen, while still significant were less so than those obtained from the DNP-KLH immunisation study. Although both SRBC and DNP-KLH function as T cell dependent antigens it is possible some subtle difference between the antigens resulted in these differences in pathogenesis between the studies. It is known that some variability in immunogenic properties can occur with SRBC antigen. Alternatively these differences may be related to the timing of the immunisations as the primary DNP-KLH immunisation was given 4 weeks later than the SRBC immunisation. In planning the studies it was originally intended to use DNP-KLH at identical timepoints to those used previously however technical difficulties meant these immunisations were delayed. Previous studies investigating the replication kinetics of a number of TSE strains found that levels of infectivity reach a plateau at approximately 10 weeks with the ME7 strain (Kimberlin & Walker, 1979). It is therefore possible that immunising before maximum levels of infectivity in lymphoid have been reached, results in a delay in the spread of infectivity to the CNS; perhaps as a result of the generation and subsequent infection of newly formed germinal centres as a result of immunisation. Alternatively, it is possible that immunisation using SRBC antigen at these timepoints resulted in some form of infectivity "clearance" from lymphoid tissues by macrophages. In previously published studies clearance of infectivity by macrophages from lymphoid tissues is known to prolong incubation periods of disease (Beringue et al, 2000). Although both antigens evoke similar immune system functions it may be that SRBC antigen provokes a more

pronounced innate response to antigen resulting in macrophage activation and subsequent "clearance" of infectivity from the lymphoid system (Beringue et al, 2000). It is also possible that higher levels of these cell types were present earlier in incubation period, perhaps in response to the initial infection. At 10 weeks post infection however, levels of infectivity and accumulation of PrP^{Sc} in GCs would have reached a plateau in spleen. It is possible that immunisation at this stage could have shortened incubation period of disease by enhancing spread of infection into the CNS as a result of increased FDC replication of infectivity in lymphoid tissues already "saturated" with infectivity. There were no effects on pathogenesis in mice immunised at the middle stage of infection although this is perhaps not surprising as neuroinvasion would have occurred at this stage. Indeed studies involving splenectomy (Fraser & Dickinson, 1970) or manipulation of FDCs in the lymphoid system are only effective early in infection (Mabbott et al, 2003). These findings are important as they suggest that therapies aimed at manipulating cells such as FDCs may only be effective early in peripheral infection. In comparison with the study involving SRBC immunisation no adverse effects were obtained following immunisation at late stage infection with DNP-KLH. The differences obtained from the studies are not fully understood but may be related to the use of two different immunogens.

The establishment of specific tissue involvement and infectivity levels within those tissues from TSE infected humans and animals is immensely important in determining risk of disease transmission. TSE agents are unique in their resistance to inactivation provoking concerns over iatrogenic transmission of infectivity via

surgical procedures and blood transfusion. It is well established that infectivity accumulates in the peripheral lymphoid system and transmission of infection via blood has been demonstrated in experimental blood transfusion studies in sheep (Houston et al, 2000; Hunter et al, 2002) and more recently in human to human transmission of vCJD via infected blood products (Llewelyn et al, 2004; Peden et al, 2004; Wroe et al, 2006). As immunisation has direct effects on FDC activation and germinal centre formation (Kosco-Vilbois et al, 1997) it is possible this could alter levels of infectivity in lymphoid tissues potentially disseminating more infection into the blood. Although, it is not understood if the presence of infectivity in blood is a result of the presence of infected cells or via the passage of blood through infected lymphoid tissues. On a cellular level the most likely candidates for infection of blood are B lymphocytes which form close contact with FDCs within the germinal centre (Kosco-Vilbois et al, 1997), although the presence of FDC precursor cells has also been described in blood (Kapasi et al, 1998). Infectivity bioassay of spleen found that there were no statistical differences in infectivity levels between non-immunised and immunised mice following primary or secondary response at the early stage of infection. However, levels of infectivity were higher in 2 of 3 spleens from the immunised boost group in comparison with the saline control group. Although not a significant result this still represents an important finding but may require larger sample numbers to determine if there are increased levels of infectivity in lymphoid tissues or blood following immunisation.

In conclusion, these findings presented here suggest that immunisation has effects on the pathogenesis of disease. It would have been interesting, and highly relevant, to

immunise mice prior to TSE infection to determine how an activated immune system at the time of infection would impact on pathogenesis and disease susceptibility. Moreover, the effects of immunisation on mice at the late stage of TSE infection have not been observed before and may give some indication that infection results in some form of immune system dysfunction. These findings will be examined in more detail in future research.

Chapter 7

General discussion and opinions on future directions

7.1 Introduction

The unique properties of TSE agents produce many barriers to understanding and researching these diseases. These barriers include; the prolonged incubation periods of disease, which in some cases can exceed the animal's lifespan (Dickinson et al, 1975); the lack of a reliable ante-mortem diagnostic test, and the uncertainty over the nature of the TSE agent. Moreover, the lack of treatments and the fatal nature of these diseases demonstrate the importance of research into these devastating diseases.

In many naturally acquired and experimental TSEs, transmission of infection by peripheral routes involves the lymphoid system (Fraser & Dickinson, 1970), and although high levels of infectivity can be detected in lymphoid tissues early in infection (Fraser, 1996) there are no clinical or outward signs that the animal or individual is infected. The clinical symptoms of disease that eventually arise from TSE infection are almost certainly the result of pathological changes within the central nervous system (CNS) which includes vacuolation, gliosis and the deposition of the abnormal form of PrP, PrP^{Sc} (Bruce et al, 1989b) As many naturally acquired TSEs are thought to arise from infection by peripheral routes, for example ingestion of infectivity (Glaysher & Mabbott, 2007) or via the skin (Mohan et al, 2005a) or mucous membranes, understanding the key events and influence in the peripheral system before infection in the CNS is initiated may lead to the development of intervention strategies or treatments for these diseases. In addition, understanding the influences of the immune system on pathogenesis may allow the assessment of risk factors that influence disease.

One of the main aims of this thesis has been to further elucidate the role of follicular dendritic cells (FDCs) in the pathogenesis of the ME7 strain of scrapie and to understand what cell types or mechanisms may be important in the peripheral pathogenesis of other TSE strains. The aims of this thesis have also been to understand how the immune system, and alterations in immune function, such as those caused by increasing age or immunisation, can influence the pathogenesis of these diseases The findings from these studies and the future areas of research are presented in this discussion.

7.2. The role of follicular dendritic cells in TSE pathogenesis

A wealth of evidence now suggests that the FDC is critical for neuroinvasion and replication of TSE agent infectivity. Initial studies investigating the cellular association of infectivity in spleen found that stromal cell populations (FDCs are thought to arise from a stromal origin) contained the highest levels of infectivity (Clarke & Kimberlin, 1984b). Studies involving ionising radiation produced evidence that a radiation resistant cell type was critical for neuroinvasion and replication of infectivity in spleen (Fraser et al, 1992b) and in the last decade a number of studies in SCID mice, which lack FDCs, produced further evidence for a role for FDCs (Fraser et al, 1996; Lasmezas et al, 1996; O'Rourke et al, 1994). In addition the localisation of PrP protein to FDCs by IHC and ultrastructural methods demonstrated the potential involvement of these cells in TSE infection (Brown et al, 2000a; Jeffrey et al, 2000; McBride et al, 1992). More recently, chimaeric mouse models were created with a mismatch between PrP expression on FDCs and on

lymphoid/myeloid cells and vice versa. In these studies neuroinvasion and replication of infectivity was dependent on PrP expressing FDCs and was not dependent on PrP expression on lymphoid cells (Brown et al, 1999). These studies were extended in this thesis to include the use of two new lines of SCID mice which were created on an H-2^b background to allow the grafting of bone marrow from PrP deficient mice (chapter 3). This design allowed the production of bone marrow chimaeras which permitted the development of FDCs from SCID-$PrP^{+/+}$ mice in the presence of isologous $PrP^{+/+}$ or $PrP^{-/-}$ bone marrow, creating a mis-match between the PrP status of the surrounding lymphocytes and that of the host FDCs. Results from these studies demonstrated that peripherally routed scrapie was dependent on PrP expressing FDCs, irrespective of the PrP status of the lymphocytes in the bone marrow graft (Chapter 3.3.3). These findings have provided further evidence in support of the critical involvement for the FDC in pathogenesis the pathogenesis of scrapie.

7.2.1 Disease pathogenesis in radiation (bone marrow) chimaeras

Analysis of data obtained from radiation (bone marrow) chimaeras, created to determine which cells were critical for peripheral TSE pathogenesis, produced some intriguing results. A significant shortening of incubation period was observed in $PrP^{+/+}$ (129/Ola) mice reconstituted with bone marrow from $PrP^{-/-}$ mice, in comparison with unirradiated and reconstituted mice. A second identical experiment produced similar results (Chapter 3.3.2).

There may be several explanations for these findings. One possibility is that the lack of PrP expression on cells in the peripheral lymphoid system (for example on lymphocytes, macrophages etc) is related to these shortened incubation periods. Sequestration or degradation of infectivity (thus reducing available infectivity in the lymphoid tissues) is known to occur by macrophages, indeed depletion of these cells shortens incubation periods of disease (Beringue et al, 2000). If these mechanisms require the cellular expression of PrP this "sequestration" of agent infectivity may not have occurred in these chimaeric mice where the majority of lymphoid and myeloid cells would have been replaced by PrP deficient cells from the donor.

The possibility that the irradiation procedure itself contributed to these effects on pathogenesis has been discounted as the shortened incubation periods were not observed in $PrP^{+/+}$ mice reconstituted with isologous $PrP^{+/+}$ bone marrow suggesting these findings are related to the mismatch between the PrP status of the recipient and the graft. Alternatively, and perhaps a more plausible explanation, is the possibility that the creation of a chimaeric immune system where the PrP status of the FDCs differed from the resulting lymphocytes may have resulted in an immune response either to the mismatch of the graft or to the inoculum itself which is likely to have contained high levels of PrP^{Sc}. It is known that PrP deficient mice mount a humoral immune response to PrP protein (Heppner et al, 2001) therefore similar effects may have occurred here to either the cells expressing PrP^{c} or to PrP^{Sc} in the inoculum. The possibility that stimulation of the immune system may influence pathogenesis is considered in more detail later in this discussion (7.5), but these results have similarities with previously published work which showed that mitogenic stimulation

shortens incubation period of disease (Dickinson et al, 1978) (Kimberlin & Cunnington, 1978a).

Further studies investigating germinal centre formation and expression of markers related to FDC activation such as the FcγRII/RIII (Aydar et al, 2004; Qin et al, 2000), VCAM-1 and MAdCAM-1 antigens which are only expressed on activated FDCs (Qin et al, 2000) may be considered to establish if there are differences in FDC activation between these chimaeric mice and those of unirradiated or mice irradiated with isologous bone marrow in both uninfected and infected mice. In addition sera is available from these mice therefore it may be possible to use ELISA methods to determine whether a specific humoral immune response occurred in these mice.

7.2.2 Do FDCs produce PrP protein?

Although data from our previous studies demonstrates the importance of FDCs in pathogenesis (Brown et al, 1999; Bruce et al, 2000; Mabbott et al, 1998; Mabbott et al, 2000b) it is not fully understood if the expression of PrP protein on these cells is a result of production of PrP by the cells themselves or whether PrP is somehow transported to these cells and retained on the cell surface in a similar way to other antigens, for example MHC-class II (Denzer et al, 2000). In addition, PrP^c is attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor and it is known that GPI-anchored proteins can be transferred from cell to cell (Low, 1998). Within the germinal centre B cells and FDCs are intimately associated therefore it is possible

that transfer of PrP^c from these B cells, which are known to express PrP (Mabbott et al, 1997), could occur.

An intrinsic mechanism of FDCs is their ability to trap and retain antigen-antibody complexes for many weeks or months leading to the maintenance and stimulation of immunological memory (Gray & Skarvall, 1988); (Gray & Matzinger, 1991); (Ahmed & Gray, 1996; Gray, 2002). The facilitation of immune complex trapping on FDCs is partly mediated by complement component C4 and can be recognised by the antibody marker FDC-M2 (Taylor et al, 2002). The reactivity of this monoclonal antibody FDC-M2 with FDCs is dependent on trapped immune complexes that are capable of fixing complement on the FDC cell surface. Therefore it is possible that in the uninfected animal, PrP protein is somehow transported to the FDCs and retained and trapped on the extensive network processes of the cells. The detection of disease specific PrP on FDCs following infection may therefore result from the conversion of trapped PrP^c on the cell surface by the infectious agent or it may be that the abnormal form of PrP is somehow transported to the FDCs following infection.

Several previous studies have demonstrated that a specialised population of macrophages, tingible body macrophages, found within the germinal centre can ingest and internalise PrP^{Sc} in infected mice and sheep (Jeffrey et al, 2000; McGovern et al, 2004). In addition, studies have demonstrated that dendritic cells can acquire and transport PrP^{Sc} (Huang et al, 2002; Huang & MacPherson, 2004; Raymond & Mabbott, 2007) or macrophages (Beringue et al, 2000) suggesting that

these cells could potentially transfer PrP to these cells. In addition to trapping and retraining of immune complexes on FDCs it is known that proteins can be sequestered by these cells and expressed on their surface for long periods of time. An example of this is the expression of MHC-class 2 antigens which are not considered to be produced by the FDCs themselves (Denzer et al, 2000).

Alternatively, cell surface expression of PrP on FDCs may be the result of the production of the protein by the FDCs themselves and the subsequent expression of PrP^{Sc} on these cells, the result of infection of the FDC itself with TSE agents. There are several previous findings which lend support to the possibility that FDCs themselves produce PrP; firstly the studies involving ionising radiation (Fraser & Farquhar, 1987), where no effect on pathogenesis or infectivity levels in spleen was observed. Furthermore our studies (Brown et al, 2000b; Brown et al, 1999) using radiation chimaeras where mice were subjected to lethal doses of radiation and subsequently grafted with $PrP^{+/+}$ or $PrP^{-/-}$ bone marrow found no difference in the expression levels of PrP in uninfected or ME7 infected spleen. As some cells types, including nerve cells are resistant to radiation (Maruyama et al, 1967) it remains possible that PrP is somehow sequestered to the FDCs. In a study mapping the development of FDCs and specific lymphoid cells in SCID mice following bone marrow reconstitution the development of mature FDCs was found to coincide with the expression of PrP protein (Brown et al, 2000b). Similarly in a study of the development of FDCs and lymphocytes in neonatal mice PrP expression was found to coincide with FDC development (Ierna et al, 2006).

The determination of whether FDCs express PrP protein is of great importance. If PrP is not produced by the FDC itself identifying the key events in the transport of this protein will be critical for development of therapeutic or preventative strategies in these diseases. Several approaches which aim to elucidate whether FDCs produce PrP are being considered in our unit and elsewhere. These include the generation of transgenic mice with PrP expression restricted to the FDCs (Neil Mabbott, personal communication).

Recently, published studies describe methods for the isolation of highly enriched populations of murine FDCs (Sukumar et al, 2006); (Sukumar et al, 2008) and these techniques could be useful in elucidating whether FDCs produce PrP protein. In SCID mice FDC precursor cells exist within lymphoid tissues which can be recognised by the FDC-M1 antibody (Balogh et al, 2001; Ierna et al, 2006); although there is no detectable expression of PrP on these cells. In studies carried out previously, and those presented in this thesis (chapter 3), the expression of PrP^{c} on FDCs was not dependent on PrP expressing lymphocytes. The two novel lines of SCID mice (described in 3.3.2): SCID-$PrP^{+/+}$ and SCID-$PrP^{-/-}$ could potentially be used to isolate FDCs for *in-vitro* studies using co-culture regimes with $PrP^{-/-}$ or $PrP^{+/+}$ lymphocytes. If successful, this could permit the development of FDCs from PrP expressing or PrP deficient mice without the complication of other PrP expressing cells such as nerve cells. These cultures may help elucidate if the transfer of PrP from lymphocytes can occur. These cultures could likewise be used in infection studies in order to measure the relative contribution of FDCs and/or lymphocytes in the accumulation of PrP^{Sc} on FDCs.

7.3 Do different TSE strains target different cell types in the peripheral lymphoid system?

The existence of strains remains one of the most widely debated and perhaps controversial aspects of TSE research (Bruce & Dickinson, 1987; Bruce et al, 1991). The existence of strains implies that some form of informational molecule must exist to denote the unique and often extreme difference between TSE strains (Bruce & Dickinson, 1987). Extensive studies in mice using limiting dilution analysis where isolated strains are diluted to the lowest dilution capable of producing disease has resulted in over 20 experimental TSE strains (Bruce et al, 1991; Chandler, 1961; Dickinson et al, 1968; Zlotnik & Rennie, 1965). The vast majority of these strains were derived from a pool of natural scrapie brains although some of the experimental strains such as 301C and 301V are derived from BSE (Bruce et al, 2002). These defined strains, can be identified by their lesion profile (assessment of vacuolar changes in defined brain areas) and patterns of PrP^{Sc} deposition in the CNS. For example the ME7 strain of scrapie produces widespread deposition of PrP throughout the CNS whereas with the 87V strain targets only specific areas with distinct characteristics such as the deposition of PrP plaques (Bruce et al, 1989b).

Studies using mice with chimaeric immune systems produced compelling evidence for a critical role for FDCs with the ME7 strain of scrapie (Brown et al, 1999) however similar studies carried out elsewhere with the RML scrapie isolate produced contrasting results suggesting that bone marrow derived cells were critical for pathogenesis (Blattler et al, 1997). This led to the hypothesis that different scrape

strains may target different cell types as they are known to do in the CNS. To address this possibility mice with specific cell type deficits were challenged with a range of TSE agent strains (79A, 139A, ME7 and 301C) (Chapter 4 section 4.3.1).

In setting up these studies important consideration was given to the effect of dose on disease pathogenesis. Previous studies in SCID mice with the ME7 agent strain demonstrated that these mice were almost fully susceptible to peripheral infection at high dose but almost completely resistant at lower doses (Fraser et al, 1996). This susceptibility to infection at high dose occurred independently of the lymphoid system suggesting that neuroinvasion probably occurred via direct peripheral nerve infection. In these studies (chapter 4 section 4.3.1) high dose infection of immunodeficient mice produced disease in the majority of mice with all the TSE strains tested suggesting that this effect is not specific to the ME7 agent strain. At lower doses much lower incidence of disease was observed in all of the immunodeficient mice with all of the TSE strains. Despite the detection of trace levels of infectivity in many of these spleens (Chapter 4) these levels were not consistent with agent replication. In summary, the results from these studies showed that all of the TSE strains tested appeared to depend on the presence of mature FDCs for efficient neuroinvasion. In addition, these studies demonstrate the importance of considering dose when designing studies which aim to elucidate the requirement of specific immune system cells for neuroinvasion as failure to do so could potentially lead to misinterpretation of results. In-fact, the original studies involving the RML isolate used a 5 fold higher dose for infection of mice which may have impacted on result interpretation (Blattler et al, 1997).

However, some subtle differences in the peripheral pathogenesis between the TSE agent strains tested was observed. Most notable was the development of extensively prolonged incubation periods with the 79A/139A strains. Furthermore some of the 301C infected immunodeficient mice showed prolonged incubation periods; primarily at high dose. The significance of this is not fully understood but may suggest evidence of an alternative, less efficient mechanism of pathogenesis which may occur in the in the absence of mature FDCs. While pathological assessment of these mice with prolonged incubation periods show no difference in TSE agent strain characteristics, investigation of the targeting of PrP^{Sc} in brain may prove useful in determining whether neuroinvasion occurred via a neuroanatomical route.

7.4. The effect of age on pathogenesis

7.4.1 The influence of senescence on the peripheral pathogenesis of the ME7 agent strain.

In mammals senescence has wide ranging effects on immune function and in mice FDC function is impaired suggesting that this may have implications for TSE pathogenesis. In the studies in this thesis old mice were used to model immunosenescence in humans in an attempt to establish the effect that host age may have on peripheral TSE pathogenesis.

The identification of a “new variant” of CJD in 1996 is almost certainly the result of peripheral (ingestion) exposure of the UK human population to the BSE agent (Bruce et al, 1997; Will et al, 1996). However, despite probable widespread exposure to the BSE agent there have been relatively few cases of variant CJD; and almost without

exception, all have occurred in young individuals (mean age of death approximately 29 years) (Valleron et al, 2001).

Initial research suggested that dietary preference in the young (e.g. consumption of foods such as burgers containing high levels of mechanically recovered meat) may have contributed to this high incidence of disease in young people however epidemiologic analysis has failed to substantiate a link between dietary preference alone and incidence of disease (Boelle et al, 2004). Although the reasons behind this apparent age-related susceptibility are not fully understood the possibility that age related effects on the immune system may influence peripheral disease pathogenesis were considered in this thesis.

Using a murine model of senescence where defects in immune and FDC function are well documented we found that TSE neuroinvasion from the periphery was severely impaired. None of the aged mice developed clinical disease following peripheral exposure; however TSE specific pathology (vacuolar changes and/or the accumulation of PrP^{Sc} in brain) was detected in a high proportion of peripherally infected animals. In contrast, aged mice infected intracerebrally developed clinical disease with similar incubation periods to the young mice, but with significantly lower levels of vacuolar pathology in the CNS.

The findings from these studies suggest that significant levels of "sub-clinical" or pre-clinical infection may exist within the population presenting further implications for iatrogenic spread of disease. In addition, alterations in the severity of pathology

and in the presentation of clinical symptoms of disease may occur in aged individuals which could have significance for the diagnosis of disease in the aged (discussed in more detail in 7.4.2). In addition recent studies where lymphoid tissues (tonsil and appendix) were sampled to assess potential levels of disease in the UK population may have little relevance in older individuals (Hilton et al, 2002; Ironside et al, 2006). Studies in senescent mice showed that only a few peripherally exposed mice had PrP^{Sc} in lymphoid tissues despite the presence of PrP^{Sc} in brain in a high proportion of these mice.

In the studies presented in Chapter 4 (section 4.3.1) it appears that high dose infection of immunodeficient mice results in neuroinvasion without the requirement for lymphoid system involvement. It would be interesting to test this hypothesis in aged mice where there are effects on FDC and immune function, as it may be that neuroinvasion, following high dose infection, may readily occur in aged mice.

7.4.2 Effects of age on TSE agent transmission via different routes of exposure.

In recent years four cases of variant CJD have occurred (Llewelyn et al, 2004)Peden *et al.,* 2004; (Wroe et al, 2006), almost certainly via the transfusion of blood or blood products to these individuals. These cases represent the first recorded cases of human to human transmission of variant CJD and a number of other individuals who have received blood products from donors known to be infected with vCJD are being monitored.

Although variant CJD has manifested almost without exception in young individuals, three of the four cases of vCJD as a result of blood transfusion have occurred in older individuals (Llewelyn et al, 2004); Peden *et al.,* 2004; Hewitt *et al*., 2006; HPA 2007). The first case (Llewelyn et al, 2004), a 62 year old methionine homozygote at codon 129 of the human prion protein gene (*PRNP*), had received a non-leucodepleted red cell component from a donor later confirmed as suffering from vCJD. The recipient developed clinical symptoms of disease almost 7 years after the blood transfusion. The second case, an elderly individual (Peden *et al.,* 2004), died 5 years after transfusion without any clinical symptoms of vCJD, and was found to have PrP^{Sc} in the spleen and one lymph node (but not in the brain) at post mortem. This individual was a codon 129 *PRNP* heterozygote and this case is thought to represent pre- or sub-clinical infection. The third case, initial presentation at 29 years and confirmed clinical diagnosis at 31 yeas of age (Wroe et al, 2006), developed symptoms of vCJD approximately 6 years after receiving a transfusion of red blood cells from a donor who developed vCJD about 20 months after this blood was donated. This individual subsequently died almost 9 years after receiving the transfusion aged 32 years (Wroe *et al*., 2006). The fourth case (an 83year old individual) (HPA 2007) developed symptoms of vCJD 8.5 years after receiving a transfusion of red blood cells from a donor who developed vCJD about 17 months after this blood was donated. In the 3rd and 4th cases the same donor donated the vCJD-implicated blood. All of the four cases had received transfusions of non-leucodepleted red blood cells between 1996 and 1999.

The occurrence of these vCJD cases in these older individuals following blood transfusion may suggest that route of infection may influence susceptibility to infection e.g. older people might be less susceptible to BSE via the oral route than they are to vCJD via the intravenous route. It has been suggested that adaption of the BSE agent following secondary passage in humans may result in increased efficacy of transmission. However, passage in mice of vCJD brain from the first blood transfusion case (Llewellyn et al., 2004) into mice (Bishop et al, 2008) demonstrates no significant alteration in transmission characteristics or efficacy. These findings suggest that the major factor in these 4 cases of vCJD transmission via blood may be the route of transmission and not alterations in agent strain efficacy following passage through humans (Bishop et al, 2008).

As described in chapter 5 the effects of age on the immune system have dramatic effects on peripherally (oral or i.p.) acquired ME7 scrapie providing strong evidence that the predominance of vCJD cases in the young may be related to immune function. As vCJD is known to target lymphoid tissues the presence of infectivity in blood is perhaps not surprising, and the ability to transmit infection via blood from sheep infected with BSE or natural scrapie confirms that blood may represent a significant risk factor in disease transmission (Houston et al, 2000 Hunter et al, 2002). However it must be recognised that not all TSEs have lymphoid tissue involvement, for example sCJD, and in these diseases the risk of transmission of disease via blood may be minimal. Indeed there have been no recorded cases of sCJD transmission via the transfusion of blood or blood products (Gambetti et al., 2003).

As previously described, studies have shown that the intravenous (i.v.) route is almost as efficient as the i.c. route of infection (Kimberlin & Walker, 1979) (approx. 100 times more efficient than other peripheral routes) therefore it is possible that the i.v. route of infection may influence the age-range of susceptibility. To test this hypothesis, it is proposed to compare TSE disease pathogenesis, using an experimental model which has extensive lymphoid tissues involvement in aged in young mice using the i.v. and other peripheral routes to establish how these routes influence susceptibility to disease in relation to age.

7.4.3 Effect of age on cross-species TSE agent transmission.

In addition to investigating the effect of route on pathogenesis in aged mice it will be important to establish if age alters susceptibility to TSE infection across a species barrier. Previous studies in SCID mice have shown that neuroinvasion is severely impaired following infection with BSE (Brown et al, 1997) suggesting that similar effects may occur in aged mice.

These studies will be important in determining if aged individuals are less susceptible to cross-species infection and may determine if sub-clinical disease is present in aged individuals in the absence of disease symptoms. The occurrence of sub-clinical disease in the human population, (possibly as a result of inefficient disease transmission associated with age) may represent a significant risk factor in terms of iatrogenic disease transmission.

7.4.4 Effects of age on the central nervous system and possible implications for TSE pathogenesis.

Although aged mice were fully susceptible to i.c. infection with the ME7 agent severity of vacuolation was significantly lower in the aged mice in comparison with the young mice; although both aged and young mice had similar incubation periods of disease. In addition, anecdotal evidence from assessment of these animals suggested that clinical symptoms following i.c. infection may be subtly altered from those of the young mice. The reasons for this less severe pathology are unclear but similar findings have been observed in TME infected aged mink of the Chediak-Higashi (CH) phenotype, which has an abnormality in granulocyte production (Marsh et al, 1976). Infection of young mink of the same phenotype, or non-CH mink, produced high levels of vacuolation in comparison with the aged mink.

There are extensive effects of ageing on the CNS which include neuron loss (Cowen & Gavazzi, 1998). Although the major findings from the studies in the aged mice relate predominantly to effects on the immune system these alterations in pathology and possibly clinical presentation of disease following i.c. infection of aged mice may have important implications; for example in diagnosis. Precise comparison of clinical symptoms of disease between aged and young mice may determine if there are alterations in the clinical presentation of disease between young and aged mice. In addition, it is not known if this alteration in vacuolar pathology may impact on agent infectivity levels in CNS tissues and further analysis of CNS tissues from aged and young mice may be useful in establishing whether any such differences exist.

7.4.5 Functional effects of age on the immune and the nervous system

A number of studies have demonstrated that the function of FDCs is impaired in mice of from approximately 600 days old. This represents approximately two thirds of the natural lifespan of the laboratory mouse. These studies have shown that FDCs from these aged mice are atrophied and are unable to effectively trap immune complexes leading to impaired responses to antigen (Aydar et al, 2003; Aydar et al, 2004). Although the findings presented in chapter 5 show that the effects of age on pathogenesis *coincide* with FDC dysfunction, it is not possible to determine from these studies if the FDCs themselves are responsible for these effects on pathogenesis. It remains possible that this impaired pathogenesis is related to defects in other cell types, such as lymphocytes.

To elucidate the effects of age on pathogenesis chimaeric mice, where a mismatch in aged FDCs and young lymphocytes and *vice- versa* could be created. As one of the characteristics of FDCs is their resistance to high doses of radiation it is possible to use whole body irradiation to ablate dividing cells such as lymphocytes, while resident FDCs are unaffected by the effects of the irradiation. Following the radiation procedure grafting of suitable cell populations e.g. bone-marrow can be carried out to create a chimaeric immune system. As FDCs are not replaced from the bone marrow, dividing cells such as lymphocytes would be derived from the donor whereas FDCs would be of a host origin. These chimaeric mice could be used in TSE infection studies to determine whether defects in FDCs result in the impaired TSE pathogenesis in aged mice as described in chapter 5. Alternatively, *in-vitro* cultures could be obtained to determine FDC function within these cultures. Similar studies

where *in-vitro* cultures of aged FDCs and young lymphocytes and *vice-versa* were created found that defects in FDCs and not in lymphocytes were responsible for impaired immune responses (Aydar et al, 2003).

In addition to examining how age influences susceptibility to TSE infection it may be important to determine at what age these defects in FDC function become apparent. This could be achieved by examining FDC function in mice at specific timepoints during lifespan and then using these data to inform the design of the TSE pathogenesis studies to determine if defects in FDC function correlate with impaired pathogenesis.

Preliminary studies of uninfected aged mice demonstrate no difference in innervation in spleen between aged and young mice using the marker tyrosine hydroxylase to detect noradrenergic sympathetic innervation, suggesting that the impaired pathogenesis in these aged mice was not directly related to lack of peripheral nerves. In-fact, there was some suggestion of greater levels of innervation in aged mice, as demonstrated previously in SCID mice (Mitchell et al, 1997). Indeed studies have shown that the relative positioning of germinal centres and FDCs to peripheral nerves in the spleen influences pathogenesis (Prinz et al, 2003). However further studies investigating innervation perhaps in a range of lymphoid tissues from aged and young mice may be considered as little information exists with regard to the innervation of lymphoid tissues in senescence

7.5 Immune system influences on TSE pathogenesis

7.5.1 Influence of immune system stimulation on pathogenesis

Unlike most infectious agents a functional immune system is critical for the peripheral pathogenesis of TSEs. This paradox led to the hypothesis that immune system stimulation, in the form of immunisation, may have effects on pathogenesis. Some studies were performed in the 1970s and 1980s although these focused on the single administration of mitogenic substances such as PHA (Dickinson et al, 1978) or modified BCG vaccine (Kimberlin & Cunnington, 1978b). At the time of conducting these studies little was known about the role of the immune system or indeed the involvement of FDCs in TSE pathogenesis. In the studies presented in this thesis (Chapter 6, 6.3.1 and 6.3.6) the aim was to emulate the generation of a primary and secondary immune response in a similar way to generating immunity via vaccination and study the influence this may have on TSE pathogenesis.

As TSEs have highly reproducible and consistent disease incubation periods the modulation of pathogenesis may have wide ranging implications. The demonstration that incubation period of disease can be significantly shortened by immunising with DNP-KLH at an early stage of infection may have implications for the assessment of risk in TSE infection. Immunisation with DNP-KLH at a mid and preclinical stage of infection had no effect on incubation period of disease, however at this stage of infection it is almost certain that TSE disease would have been initiated in the CNS. Thus these data suggest that the spread and progression of infection within the CNS cannot be modulated by immune system stimulation at this stage of infection.

7.5.2 Can immunisation alter susceptibility to TSE infection?

What is not understood however is whether a humoral immune response to a non-TSE related antigen can alter susceptibility to infection. Following antigenic challenge germinal centre formation and maturation of FDCs to form secondary follicles occurs (Kosco-Vilbois et al, 1997), therefore it is possible that the increased numbers of FDCs and germinal centres may enhance neuroinvasion and increased susceptibility to infection. The hypothesis that immunisation may increase susceptibility to infection is currently being tested in studies at this unit; a summary of the design is shown in figure 7.1.

Figure 7.1 Determining the effects of immunisation on the susceptibility to orally or i.p. routed ME7.

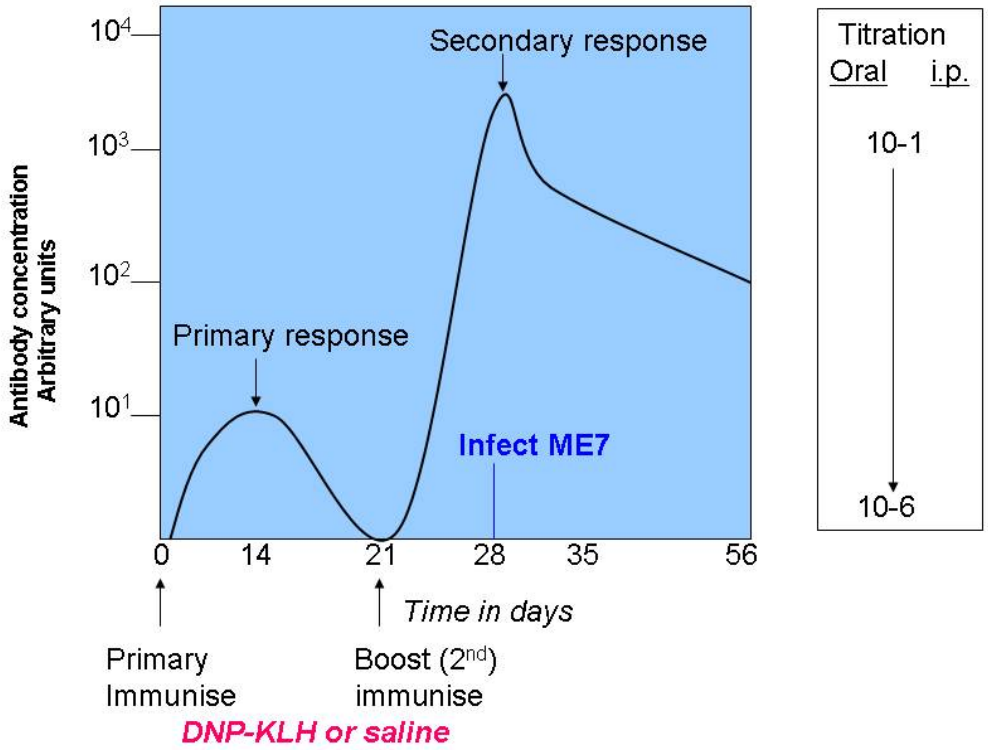


Figure 7.1 Infection of mice with ME7 by the oral or intraperitoneal routes was designed to coincide with the height of the secondary immune response following boosting with the immunogen DNP-KLH. Tissues and sera were collected from groups of immunised or non-immunised uninfected mice just prior to infection to assess the effectiveness of the immune response to the immunogen DNP-KLH.

7.5.3 Can immunisation alter susceptibility to cross species TSE infection?

During the height of the BSE epidemic it was estimated that almost 500,000 infected cattle were slaughtered for consumption in the UK (Valleron et al, 2001) although only 201 clinical cases of vCJD have been recorded (the majority of which have been in the UK). Variant CJD appears to have increased exponentially following its identification in 1996 to a peak of cases in 2000 (Ironside et al, 2002). However, what is not fully understood is whether further cases will develop in the future, perhaps in a similar way to that of the kuru epidemic where incubation periods in some individuals were estimated to be as long as 56 years (Collinge et al, 2006). Risk factors for development of vCJD susceptibility are not fully understood, however all current clinical cases of vCJD have occurred exclusively in individuals homozygous for methionine at codon 129 of the PrP gene (Ironside et al, 2002). Moreover, young age appears to be as risk factor as discussed in chapter 5 and it appears that effects on the immune system, and specifically on FDCs, with increasing age may account for this predominance of vCJD cases in the young (Chapter 5; 5.3.1). Although the use of mouse adapted experimental strains are extremely useful in determining how immune responses influence TSE pathogenesis it is not known how immunisation may alter pathogenesis or susceptibility to infection in cross species infection, for example in the transmission of BSE to humans. If immunisation increases susceptibility to infection in cross species infection this may provide useful information in establish risk factors for the transmission of BSE or other TSEs to humans.

7.5.4 Can immunisation exacerbate TSE infection?

One of the most unexpected findings of this thesis was the sudden death of a third of the ME7 scrapie infected mice following boost immunisation with sheep red blood cell antigen (SRBC) at a late, pre-clinical stage of infection (Chapter 6; 6.3.1). These effects did not occur in uninfected mice boosted with antigen or in ME7 infected mice immunised at an early stage of infection. The reasons for these sudden deaths are unclear but it is possible the effects may be directly attributed to some form of hyper immune response at this late stage of infection, perhaps as a result of some associated immune system dysfunction. Indeed, ultrastructural studies of spleens from ME7 infected mice demonstrate increased FDC hypertrophy and an increased retention of electron-dense material at the FDC plasma membrane in comparison with uninfected mice (McGovern *et al.,* 2004); these effects were further increased in mice immunised and boosted with antigen (McGovern et al, 2004). In addition, recent studies of natural sheep scrapie find that FDCs are hypertrophied implying some form of FDC dysfunction (McGovern & Jeffrey, 2007). Perhaps a potential way to test the relative contribution of the immune system to the sudden deaths in these animals would be to conduct similar immunisations in TSE infected immunodeficient mice where the ability to generate an immune response is impaired.

Alternatively, these effects on the mice may be the result of TSE associated neural dysfunction in the CNS at this stage of infection. Notably, anecdotal evidence exists to suggest that peripheral infections in those with neurodegenerative conditions such as Alzheimer's disease exacerbate the clinical signs of disease (Cunningham et al, 2005; Perry, 2004). In a number of neurodegenerative conditions including the TSEs

and in Alzheimer's disease where there are high levels of activated microglia in the brain (Betmouni et al, 1996; Williams et al, 1995), systemic infection or inflammation, leads to increased synthesis of inflammatory cytokines by these microglia. Therefore it may be appropriate to examine the effect of immunisation on the expression of these markers in the CNS.

7.5.5 Does TSE infection alter immune function or generate an immune response in the host?

Unlike most infectious diseases, TSE agents do not elicit a classical immune response in the host (Clarke, 1968; Clarke & Haig, 1966). This lack of humoral response is most likely the result of the inability of the host to recognise PrP^{Sc} as a foreign antigen due to the widespread expression of PrP^{Sc} within the host. However, the PrP protein is immunogenic since studies in PrP deficient mice immunised with recombinant PrP demonstrate specific antibody responses (Heppner et al, 2001). Despite this lack of a classical immune response during TSE disease there may be some evidence that TSE infection induces some form of immune system activation in the infected host. The expression of FcγRII/III on FDCs is normally associated with the development of secondary follicles in germinal centres in response to antigenic stimulation (Aydar et al, 2004; Qin et al, 2000). An observation in chapter 5 was the demonstration of high levels of FcγRII/III expression on FDCs in ME7 infected young in contrast this marker was expressed at much lower levels in spleen of uninfected young mice. This may suggest that TSE infection evokes some form of immune response resulting in the activation of FDCs, as has been demonstrated recently (McGovern & Jeffrey, 2007). The rationale behind using this marker was to

provide comparison between aged and young mice in terms of FDC functionality. The lack of expression of FcγRII/III in ME7 in uninfected and scrapie infected aged mice is most probably related to defects in the immune system of the aged animals that result in sparse expression of FcγRII/III within the germinal centre. Indeed studies in aged mice find that FcγRII/III labelling on aged FDCs is dramatically reduced following immunisation (Aydar et al, 2004). These observations may be very significant and should be extended to include morphometric analysis of this marker in uninfected and TSE infected mice. The analysis of the expression of other markers related to FDC activation such as should also be considered including the VCAM-1 and MAdCAM-1 antigens which are expressed upon activated FDCs (Kosco-Vilbois, 2003). Further approaches could include analysis of markers of FDC activation in cultured FDCs (Sukumar et al, 2008) or in dissected FDCs using laser micro dissection microscopy for molecular analysis. Further observations from chapter 6 suggest that germinal centre number and size may be increased in TSE infected animals which also support the possibility that TSE infection evokes FDC activation. Indeed there is now some evidence that FDC function may be altered during TSE infection (McGovern et al, 2004; McGovern & Jeffrey, 2007).

It is not fully understood if immune responses in TSE infected individuals are altered although this is clearly an important question to address. Serum samples were collected from scrapie infected and uninfected mice immunised with DNP-KLH (as described in chapter 6) and it is proposed to measure antigen specific Ig levels at specific timepoints from an early stage of infection to the terminal stage of disease.

Unfortunately results from these studies were not available at this time for inclusion in this thesis.

7.6. Natural and experimental TSEs with minimal lymphoid system involvement

There are some characteristics of TSE infection that are common to all of these diseases for example; resistance to standard methods of de-contamination, prolonged incubation period of disease and pathological changes in the CNS. However there are some important differences that exist between these diseases, notably in the targeting of infection to the peripheral lymphoid system. One of the most defining characteristics of many naturally acquired and experimental TSEs is the targeting of infection and the deposition of PrP^{Sc} in lymphoid tissues in association with follicular dendritic cells (McBride *et al.*, 1992, Brown *et al*., 1999, Bruce *et al*., 2000). In addition, extensive studies using experimental strains have shown that the presence of an intact immune system and the presence of functional FDCs are important for pathogenesis (Brown *et al*., 1999; Mabbott *et al*., 2001). In many TSE infections the lymphoid system plays a key role although there are a number of exceptions notably in sporadic CJD, in cattle infected with BSE and in sheep with certain PrP genotypes infected scrapie (Hunter, 2007).

In natural or classical scrapie PrP^{Sc} targeting to lymphoid tissues appears to vary according to the host genotype. Generally, susceptible (VRQ) ovine genotypes have greater amounts of PrP^{Sc} deposition, in both scrapie and BSE, while those genotypes associated with resistance generally have lower levels of lymphoid involvement.

However, genotype might not be the only factor determining lymphoid involvement since sheep of "resistant" ARQ/ARQ genotypes have lymphoid system involvement when infected experimentally with BSE (Foster *et al.*, 2001b). In addition, it has been reported that some "susceptible" sheep genotypes lack detectable PrP^{Sc} in lymphoid tissues (Jeffrey et al, 2002).

Moreover, although BSE in cattle appears to have minimal lymphoid involvement (Fraser et al, 1992a), once passaged into other species e.g. sheep (Foster et al, 2001b) (Hunter et al, 2002); and humans as vCJD (Will et al, 1996), extensive lymphoid system involvement occurs (Bruce et al, 2001) suggesting that the host *and* the infecting strain may influence the involvement of the lymphoid system.

In recent years there has been great interest and concern following the confirmation of several cases of "atypical" scrapie termed Nor98 (Benestad et al, 2003) with all of the confirmed cases to date occurring in sheep with genotypes normally associated with resistance to classical scrapie. Atypical or Nor98 scrapie was originally discovered in Norway in 1998 but has since been detected in small ruminants in most European countries (Buschmann et al, 2004). The characteristics of this new strain differ greatly from classical scrapie and include; alterations in clinical presentation and differences in biochemical characteristics which include a characteristic band of apparent low molecular weight (11 kDa) following western blot analysis. Notably, cases of atypical/Nor98 scrapie also appear to lack lymphoid system involvement which have led to some suggestions of similarities of this strain to that of sCJD.

In addition, epidemiological analysis of 60 cases of atypical scrapie and 20 cases of "classical" scrapie demonstrate that 60% of the atypical cases were older than 5 years in comparison with the classical scrapie cases where almost all of the cases were aged 3-5 years (Lühken et al, 2007).

In addition some experimental mouse passaged strains, such as the 87V agent strain, appear to minimally involve the lymphoid system (Bruce, 1985). The reasons for this lack of lymphoid system involvement with these strains are unclear but perhaps suggest some tissue specific agent tropism which is under the direct control of the host.

Sporadic CJD (sCJD) is associated with elderly individuals, with a few exceptions (Murray et al, 2008) although the converse occurs with vCJD where the predominance of cases is in the young. In sCJD the mean age at onset of the symptoms is 65 years with an average clinical duration of 4 months (Gambetti et a., 2003). Sporadic CJD has been classified into a number of sub-types, the most common of these sub-types represent individuals who are MM homozygous or MV heterozygous at codon 129 of the PrP gene and carry PrP^{Sc} type 1 (Gambetti et al, 2003). In contrast to vCJD where there is extensive lymphoid system involvement, there does not appear to be targeting to the lymphoid system in sCJD, and unlike vCJD there are no recorded cases of disease transmission from sCJD infected blood donors. The lack of lymphoid system involvement with sCJD is not fully understood but is likely to represent strain-specific tropism perhaps in a similar way to the experimental strain 87V which also lacks lymphoid tissue involvement. Alternatively

it may be possible that age-associated immune dysfunction may prevent infection of lymphoid tissues in a similar way to that observed with the aged mice (chapter 5). However, recent studies in transgenic mice over-expressing human PrP, failed to find evidence of disease in lymphoid tissues of these mice infected with sCJD (Béringue et al, 2008) suggesting that sCJD does not target the lymphoid system. The cause of sCJD remains unresolved and as a result it is difficult to provide estimates of the probable incubation period length for this disease. It is possible that the development of sCJD in old age may reflect very prolonged incubation periods of disease in a similar way to that observed with some individuals with Kuru (Collinge et al, 2006).

The recognition that the lymphoid system is not involved in all TSE infections is important, particularly in determining the risk of iatrogenic infection and in relation to the design of therapies that may interfere with components of the lymphoid system these approaches may have little relevance to those diseases with minimal lymphoid system involvement.

7.7. Diagnosing and treating TSEs: where we are now.

TSEs are fatal diseases for which there is currently no cure. During the last quarter of a century extensive advances in the fundamental understanding of these diseases have been made but despite this no reliable ante-mortem diagnostic test exists for these diseases. No classical immune response exists in TSE infection precluding the use of standard antibody responses to infection. The disease specific form of the PrP protein, PrP^{Sc}, is considered a marker for disease, although studies show that TSE disease can exist and be perpetuated in the absence of this marker (Barron et al,

2007; Lasmezas et al, 1997). Also, studies have shown that the disease specific form of PrP may not be transmissible (Piccardo et al, 2007). Nevertheless, the presence of PrP in the majority of TSE infections is considered diagnostic for disease. In infected humans and animals the presence of PrP^{Sc} is detected in tissues of the CNS and in many, but not all, TSEs, in lymphoid tissues also. Despite this involvement of the peripheral lymphoid system the detection of PrP^{Sc} in blood remains to be accurately and reliably achieved, meaning that confirmation of disease is currently restricted to invasive biopsies and normally only considered when the presence of disease is strongly suspected. In the vast majority of suspected human and animal TSEs (for example testing in abattoirs) definitive diagnosis of infection is carried out after death (Grassi et al, 2008). Confirmation of human TSE infection is based on a number of factors (Ironside et al, 2002). In the last few years the use of the protein misfolding cyclic amplification (PMCA) technique has demonstrated that this method may be an extremely powerful tool for the diagnosis of TSE infection due to its apparent sensitivity (Grassi et al, 2008). Thus it may detect the presence of disease in tissues or fluids (blood) where very low levels of infectivity are present perhaps in the very earliest stages of infection where treatments may be most appropriate. However the technique remains to be fully validated for use as a diagnostic test and the lack of an early diagnostic test means that treatment of these diseases is often limited to the late stages of infection (Grassi et al, 2008).

Infection of the lymphoid tissues occurs many weeks or months before infection of the CNS or clinical signs of disease are detected, and this asymptomatic phase may offer opportunities for intervention. A number of studies demonstrate that

neuroinvasion following infection from a number of peripheral routes can be impaired using treatments to de-differentiate FDCs *in-situ* (Mabbott et al, 2000a; Mabbott et al, 2002; Mabbott et al, 2003; Montrasio et al, 2000). More recently *in-vivo* depletion of conventional dendritic cells was shown to impair neuroinvasion suggesting that these cells may also play a role in TSE pathogenesis (Raymond et al, 2007).

However the use of these treatments may be most appropriate in early stage infection as shown in studies involving oral ME7 infection where de-differentiation of FDCs after 2 weeks of infection had no effect on pathogenesis (Mabbott et al, 2003). These treatment show promise however for prophylaxis or early treatment of TSEs e.g. in iatrogenic transmission in the case of inadvertent cases of infection; such as needle stick injuries or potential infection via surgery or blood transfusion

The administration of pentosan polysulphate (PPS), a sulphated, semi-synthetic, polyanion, to mice with experimental scrapie and BSE (Farquhar et al, 2003) shows promise in prolonging incubation period of disease. More recently, treatments with PPS show that disease can be significantly extended in humans with vCJD, over the range of those individuals not treated with PPS. One individual with vCJD is still alive 7 years after administration of this compound (Christine Farquhar, personal communication).

The effects of TSE infection on the CNS include neuron loss and subsequent vacuolation, deposition of PrP^{Sc} and gliosis. It is thought these effects on the CNS

lead to the development of clinical disease and eventual death of the individual. In recent years the potential of cell replacement strategies for a number of neurodegenerative conditions has been demonstrated (Pluchino et al, 2005). These strategies include the replacement of neuronal cells into the damaged CNS using stem cells or less frequently embryonic cells (Pluchino et al, 2005). The use of cell replacement strategies has been explored in TSE infection and shows some potential in ameliorating pathology (Brown et al, 2001) and potentially extending incubation period of disease at a late pre-clinical stage of infection (Brown *et al* unpublished) The use of these cell replacement strategies demonstrate a potential use in the late stage treatment of TSEs, and could potentially be used in combination with other treatments such as the sulphated polyanions to potentially extend lifespan and ameliorate the clinical signs of TSE disease.

7.8. Conclusions

Data presented in this thesis provide further evidence for a critical role for the FDC not just in the pathogenesis of the ME7 strain (Chapter 3), but in a range of TSE strains (Chapter 4). These findings may be important in the development of therapeutic strategies. If differential targeting of cells in the peripheral lymphoid occurs this would require strain dependent approaches to therapies. Although these results show that FDCs are critical for neuroinvasion in a range of TSE agent strains some subtle differences in pathogenesis was observed, particularly between the 139A/79A agent strains and the ME7 agent strain. These findings suggest that alternative, less efficient routes of neuroinvasion can be exploited by some strains (chapter 4). The dependence of mature FDCs for the pathogenesis of the BSE

derived strain 301C was also demonstrated which has relevance to the transmission of vCJD, since BSE and vCJD are considered to be the same agent strains. Further evidence that neuroinvasion, following high dose infection, can occur in the absence of lymphoid system involvement was obtained from studies in immunodeficient mice (Chapter 4). These findings may have implications for naturally acquired infections which demonstrate that neuroinvasion can still occur in the absence of a functional immune system if the infecting dose is high enough. One of the most poignant aspects of vCJD infection is the young age of those affected. The determination that age impairs neuroinvasion in mouse models provides some explanation for this age related distribution of vCJD cases most likely as a result of impaired FDC function in aged mice (Chapter 5). The ability to modulate pathogenesis was demonstrated in studies where mice were immunised at several points in infection. These findings demonstrated that immunisation may only impact on pathogenesis during the early stages of infection, before infection of the CNS occurs. Finally, these studies have found some preliminary evidence that TSE infection may induce FDC activation demonstrating that TSE infection does result in some form of immune response. Together these data show that a functional immune system and specifically, the presence of mature FDCs, are central to the pathogenesis of peripherally acquired TSE infections.

Bibliography

Agrawal A, Agrawal S, Gupta S (2007) Dendritic cells in human aging. *Experimental Gerontology* **42**(5)**:** 421-426

Ahmed R, Gray D (1996) Immunological memory and protective immunity: understanding their relation. *Science* **272:** 54-60

Alper T, Cramp WA, Haig DA, Clarke MC (1967) Does the agent of scrapie replicate without nucleic acid ? *Nature* **214**(5090)**:** 764-766

Alper T, Haig DA, Clarke MC (1966) The exceptionally small size of the scrapie agent. *Biochemical and Biophysical Research Communications* **22**(3)**:** 278-284

Alper T, Haig DA, Clarke MC (1978) The scrapie agent: evidence against its dependence for replication on intrinsic nucleic acid. *Journal of General Virology* **41**(3)**:** 503-516

Alpers M (1965) Epidemiological changes in kuru, 1957 to 1963. *NINDB monograph no 2 Slow, latent and temperate virus infections, National Institute of Neurological Diseases and Blindness, Bethesda, MD, USA* 65-82

Anderson G, Jenkinson EJ (2001) Lymphostromal interactions in thymic development and function. *Nature Reviews in Immunology* **1**(1)**:** 31-40

Arnold ME, Wilesmith JW (2004) Estimation of the age-dependent risk of infection to BSE of dairy cattle in Great Britain. *Preventative Veterinary Medicine* **66:** 35-47

Arsac J-N, Andreoletti O, Bilheude J-M, Lacroux C, Benestad SL, Baron T (2007) Similar biochemical signatures and prion protein genotypes in atypical scrapie and Nor98 cases, France and Norway *Emerging Infectious Diseases* **13**(1)**:** 58-65

Aspinall R, Del Giudice G, Effros R, Grubeck-Loebenstein B, Sambhara S (2007) Challenges for vaccination in the elderly. *Immunity & Ageing* **4**(1)**:** 9

Aucouturier P, Geissmann F, Damotte D, Saborio G, Meeker H, Kascsak R, Kascsak R, Carp R, Wisniewski T (2001) Infected splenic dendritic cells are sufficient for prion transmission to the CNS in mouse scrapie. *Journal of Clinical Investigation* **108**(5)**:** 703-708

Aydar A, Balogh P, Tew JG, Szakal AK (2003) Follicular dendritic cells in aging, a "bottle-neck" in the humoral immune response *Ageing Research Reviews* **3**(1)**:** 15-29

Aydar Y, Wu J, Song J, Szakal AK, Tew JG (2004) FcRII expression on follicular dendritic cells and immunoreceptor tyrosine-based inhibition motif signaling in B cells. *European Journal of Immunology* **34**(1)**:** 98-107

Bachmann MF, Odermatt B, Hengartner H, Zinkernagel RM (1996) Induction of long-lived germinal centres associated with persisting antigen after viral infection. *Journal of experimental medicine* **183:** 2259-2269

Balogh P, Aydar Y, Tew JG, Szakal AK (2001) Ontogeny of the follicular dendritic cell phenotype and function in the postnatal murine spleen. *Cellular Immunology* **214**(1)**:** 45-53

Baron T, Biacabe AG, Arsac JN, Benestad S, Groschup MH (2007) Atypical transmissible spongiform encephalopathies (TSEs) in ruminants. *Vaccine* **25**(30)**:** 5625-5630

Barron RM, Campbell SL, King D, Bellon A, Chapman KE, Williamson RA, Manson JC (2007) High titres of TSE infectivity associated with extremely low levels of PrPSc in vivo. *Journal of Biological Chemistry***:** M704329200

Beekes M, Baldauf E, Diringer H (1996) Sequential appearance and accumulation of pathognomonic markers in the central nervous system of hamsters orally infected with scrapie. *Journal of General Virology* **77**(8)**:** 1925-1934

Beekes M, McBride PA (2000) Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie. *Neuroscience Letters* **278**(3)**:** 181-184

Beekes M, McBride PA, Baldauf E (1998) Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie. *Journal of General Virology* **79:** 601-607

Bellinger-Kawahara CG, Kempner E, Groth D, Gabizon R, Prusiner SB (1988) Scrapie prion liposomes and rods exhibit target sizes of 55,000 Da. *Virology* **164**(2)**:** 537-541

Benestad SL, Sarradin P, Thu B, Schonheit J, Tranulis MA, Bratberg B (2003) Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. *Veterinary Record* **153:** 202 - 208

Beringue V, Demoy M, Lasmézas CI, Gouritin B, Weingarten C, Deslys J-P, Andreux J-P, Couvreur P, Dormont D (2000) Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis. *The Journal of Pathology* **190**(4)**:** 495-502

Béringue V, Le Dur A, Tixador P, Reine F, Lepourry L, Perret-Liaudet A, Haïk S, Vilotte JL, Fontés M, Laude H (2008) Prominent and Persistent Extraneural Infection in Human PrP Transgenic Mice Infected with Variant CJD. *PLoS ONE*(1)**:** e1419

Besnoit MM, Morel C (1898) Note sur les lésions nerveuse de la tremblante du mouton. *Rev Vét (Toulouse)* **23:** 397-400

Bessen RA, Marsh RF (1994) Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. *Journal of Virology* **68**(12)**:** 7859-7868

Betmouni S, Perry VH, Gordon JL (1996) Evidence for an early inflammatory response in the central nervous system of mice with scrapie. *Neuroscience* **74**(1)**:** 1-5

Biacabe AG, Laplanche J-L, Ryder S, Baron T. (2004) Distinct molecular phenotypes in bovine prion diseases *EMBO Reports* **5**(1)**:** 110-115

Bishop MT, Ritchie DL, Will RG, Ironside JW, Head MW, Thomson V, Bruce M, Manson JC (2008) No major change in vCJD agent strain after secondary transmission via blood transfusion. *PLoS ONE* **3**(8)**:** e2878

Blattler T, Brandner S, Raeber AJ, Klein MA, Voigtlander T, Weissmann C, Aguzzi A (1997) PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain. *Nature* **389**(6646)**:** 69-73

Boasso A, Shearer GM (2008) Chronic innate immune activation as a cause of HIV-1 immunopathogenesis. *Clinical Immunology* **126**(3)**:** 235-242

Boelle PY, Cesbron JY, Valleron AJ (2004) Epidemiological evidence of higher susceptibility to vCJD in the young. *BMC infection diseases* **4**(26)**:** 7

Bolton DC, McKinley MP, Prusiner SB (1982) Identification of a protein that purifies with the scrapie prion. *Science* **218:** 1309-1311

Bolton DC, Meyer RK, Prusiner SB (1985) Scrapie PrP 27-30 is a sialoglycoprotein. *Journal of Virology* **53**(2)**:** 596-606

Bosma GC, Custer RP, Bosma MJ (1983) A severe combined immunodeficiency mutation in the mouse. *Nature* **301:** 527-530

Bosma GC, Fried M, Custer RP, Carroll AM, Gibson DM, Bosma MJ (1988) Evidence of functional lymphocytes in some leaky SCID mice. *Journal of Experimental Medicine* **167:** 1016-1033

Bosque PJ, Ryou C, Telling G, Peretz D, Legname G, DeArmond SJ, Prusiner SB (2002) Prions in skeletal muscle. *Proceedings of the National Academy of Sciences* **99**(6)**:** 3812-3817

Brown DR, Schmidt B, Kretzschmar HA (1998) Effects of copper on survival of prion protein knockout neurons and glia. *Journal of Neurochemistry* **70**(4)**:** 1686-1693

Brown DR, SchulzSchaeffer WJ, Schmidt B, Kretzschmar HA (1997a) Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Experimental Neurology* **146**(1)**:** 104-112

Brown KL, Brown J, Ritchie DL, Fraser J (2001) Fetal cell grafts provide long-term protection against scrapie induced neuronal loss. *Neuroreport* **12:** 77-82

Brown KL, Ritchie DL, McBride PA, Bruce ME (2000a) Detection of PrP in extraneural tissues. *Microscopy research and technique* **50:** 40-45

Brown KL, Stewart K, Bruce ME, Fraser H (1997) Severely combined immunodeficient (SCID) mice resist infection with bovine spongiform encephalopathy. *Journal of General Virology* **78:** 2707-2710

Brown KL, Stewart K, Ritchie D, Fraser H, Morrison WI, Bruce ME (2000b) Follicular dendritic cells in scrapie pathogenesis. *Archives of Virology* **16:** 13-21

Brown KL, Stewart K, Ritchie DL, Mabbott NA, Williams A, Fraser H, Morrison WI, Bruce ME (1999) Scrapie replication in lymphoid tissues depends on prion protein expressing follicular dendritic cells. *Nature Medicine* **5**(11)**:** 1308-1312

Bruce M, Chree A, McConnell I, Brown K, Fraser H (1996) Transmission and strain typing studies of scrapie and bovine spongiform encephalopathy. *Transmissible Spongiform Encephalopathies:Prion Diseases***:** 259

Bruce M, Chree A, McConnell I, Foster J, Pearson G, Fraser H (1994a) Transmission of bovine spongiform encephalopathy and scrapie to mice - strain variation and the species barrier. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **343**(1306)**:** 405-411

Bruce ME (1981) Serial studies on the development of cerebral amyloidosis and vacuolar degeneration in murine scrapie. *Journal of Comparative Pathology* **91**(4)**:** 589-597

Bruce ME (1984) Cerebral amyloid plaques in murine scrapie. *Age and Ageing* **13**(3)**:** 185-186

Bruce ME (1985) Agent replication dynamics in a long incubation period model of mouse scrapie. *Journal of General Virology* **66:** 2517-2522

Bruce ME (1993) Scrapie strain variation and mutation. *British Medical Bulletin* **49**(4)**:** 822-838

Bruce ME, Boyle A, Cousens S, McConnell I, Foster J, Goldmann W, Fraser H (2002) Strain characterization of natural sheep scrapie and comparison with BSE. *Journal of General Virology* **83**(3)**:** 695-704

Bruce ME, Brown KL, Mabbott NA, Farquhar CF, Jeffrey M (2000) Follicular dendritic cells in TSE pathogenesis. *Immunology Today* **21**(9)**:** 442-446

Bruce ME, Dickinson AG (1987) Biological evidence that scrapie agent has an independent genome. *Journal of General Virology* **68:** 79-89

Bruce ME, Dickinson.A.G., Fraser H (1989a) Mutation and asymmetrical vacuolation with a particular strain of scrapie. *Unconventional Virus Diseases of the Central Nervous System***:** 426-435

Bruce ME, Fraser H (1975) Amyloid plaques in the brains of mice infected with scrapie: morphological variation and staining properties. *Neuropathology and Applied Neurobiology* **1:** 189-202

Bruce ME, Fraser H (1991) Scrapie strain variation and its implications. *Current Topics in Microbiology and Immunology* **172:** 125-138

Bruce ME, Fraser H, McBride PA, Scott JR, Dickinson AG (1992) The basis of strain variation in scrapie. *Prion diseases of humans and animals***:** 497-508

Bruce ME, McBride PA, Farquhar CF (1989b) Precise targeting of the pathology of the sialoglycoprotein, PrP, and vacuolar degeneration in mouse scrapie. *Neuroscience Letters* **102**(1)**:** 1-6

Bruce ME, McBride PA, Jeffrey M, Scott JR (1994b) PrP in pathology and pathogenesis in scrapie-infected mice. *Molecular Neurobiology* **8**(2-3)**:** 105-112

Bruce ME, McConnell I, Fraser H, Dickinson AG (1991) The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis. *Journal of General Virology* **72:** 595-603

Bruce ME, McConnell I, Will RG, Ironside JW (2001) Detection of variant Creutzfeldt-Jakob disease infectivity in extraneural tissues. *Lancet* **538:** 208-209

Bruce ME, Nonno R, Foster J, Goldmann W, Di Bari M, Esposito E, Benestad SL, Hunter N, Agrimi U (2007) Nor98-like sheep scrapie in the United Kingdom in 1989. *Veterinary Record* **160**(19)**:** 665-666

Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ (1997) Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* **389**(6650)**:** 498-501

Bueler H, Aguzzi A, Sailer A, Greiner R-A, Autenreid P, Aguet M, Weissmann C (1993) Mice devoid of PrP are resistant to scrapie. *Cell* **73:** 1339-1347

Buschmann A, Biacabe AG, Ziegler U, Bencsik A, Madec JY, Erhardt G, Lühken G, Baron T, Groschup MH (2004) Atypical scrapie cases in Germany and France are identified by discrepant reaction patterns in BSE rapid tests. *Journal of Virological Methods* **117**(1)**:** 27-36

Buschmann A, Gretzschel A, Biacabe AG, Schiebel K, Corona C, Hoffmann C, Eiden M, Baron T, Casalone C, Groschup MH (2006) Atypical BSE in Germany: Proof of transmissibility and biochemical characterisation. *Veterinary Microbiology* **117**(2-4)**:** 103-116

Capobianco R, Casalone C, Suardi S, Mangieri M, Miccolo C, Limido L, Catania M, Rossi G, Fede GD, Giaccone G, Bruzzone MG, Minati L, Corona C, Acutis P, Gelmetti D, Lombardi G, Groschup MH, Buschmann A, Zanusso G, Monaco S, Caramelli M, Tagliavini F (2007) Conversion of the BASE prion strain into the BSE strain: The Origin of BSE? *PLoS Pathogens* **3**(3)**:** e31

Carroll MC (2004) The complement system in regulation of adaptive immunity *Nature Immunology* **5:** 981-986

Casalone C, Zanusso G, Acutis P, Ferrari S, Capucci L, Tagliavini F, Monaco S, Caramelli M (2004) Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. *Proceedings of the National Academy of Sciences* **101:** 3065-3070

Cashman NR, Loertscher R, Nalbantoglu J, Shaw I, Kascsak RJ, Bolton DC, Bendheim PE (1990) Cellular isoform of the scrapie agent protein participates in lymphocyte-activation. *Cell* **61**(1)**:** 185-192

Caughey B, Kocisko DA, Raymond GJ, Lansbury PT (1995) Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state. *Chemistry & Biology* **2**(12)**:** 807-817

Caughey BW (1991) Secondary Structure Analysis of the Scrapie-Associated Protein PrP 27-30 in Water by Infrared Spectroscopy. *Biochemistry* **30**(31)**:** 7672-7680

Chandler RL (1959) Attempts to demonstrate antibodies in scrapie disease. *Veterinary Record* **71:** 58

Chandler RL (1961) Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet* **277**(7191)**:** 1378-1379

Chandler RL (1962) Encephalopathy in mice. *Lancet* **279**(7220)**:** 107-108

Chang C, Biedermann KA, Mezzina M, Martin Brown J (1993) Characterization of the DNA Double Strand Break Repair Defect in scid Mice. *Cancer Research* **53**(6)**:** 1244-1248

Chaturvedi UC, Dhawan R, Khanna M, Mathur A (1991) Breakdown of the blood-brain barrier during dengue virus infection of mice. *Journal of General Virology* **72**(4)**:** 859-866

Chesebro B (1998) BSE and Prions: Uncertainties about the agent. *Science* **279**(5347)**:** 42-43

Clarke MC (1968) The antibody response of scrapie-affected mice to immunisation with sheep red blood cells. *Research in Veterinary Science* **9**: 595-597

Clarke MC, Haig DA (1966) Attempts to demonstrate neutralising antibodies in the sera of scrapie-affected animals. *Veterinary Record* **78:** 647-648

Clarke MC, Kimberlin RH (1984a) Pathogenesis of mouse scrapie: distribution of agent in the pulp and stroma of infected spleens. *Veterinary Microbiology* **9:** 215-225

Clarke MC, Kimberlin RH (1984b) Pathogenesis of mouse scrapie:Distribution of agent in the pulp and stroma of infected spleens. *Veterinary Microbiology* **9:** 215-225

Colchester ACF, Colchester NTH (2005) The origin of bovine spongiform encephalopathy: the human prion disease hypothesis. *Lancet* **366**(9488)**:** 856-861

Collinge J, Sidle KCL, Meads J, Ironside J, Hill AF (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* **383**(6602)**:** 685-690

Collinge J, Whitfield J, McKintosh E, Beck J, Mead S, Thomas DJ, Alpers MP (2006) Kuru in the 21st century: an acquired human prion disease with very long incubation periods. *Lancet* **367**(9528)**:** 2068-2074

Collinge J, Whittington MA, Sidle KCL, Smith CJ, Palmer MS, Clarke AR, Jefferys JGR (1994) Prion protein is necessary for normal synaptic function. *Nature* **370**(6487)**:** 295-297

Collis SC, Kimberlin RH (1983) Further studies on changes in immunoglobulin G in the sera and CSF of Herdwick sheep with natural and experimental scrapie. *Journal of Comparative Pathology* **93**(2)**:** 331-338

Collis SC, Kimberlin RH (1989) Polyclonal increase in certain igg subclasses in mice persistently infected with the 87V-strain of scrapie. *Journal of Comparative Pathology* **101**(2)**:** 131-141

Collis SC, Kimberlin RH, Millison GC (1979) Immunoglobulin G concentrations in the sera of herdwick sheep with natural scrapie. *Journal of Comparative Pathology* **89:** 389-396

Combrinck MI, Perry VH, Cunningham C (2002) Peripheral infection evokes exaggerated sickness behaviour in pre-clinical murine prion disease. *Neuroscience* **112**(1)**:** 7-11

Correale J, Farez M (2007) Monocyte-derived dendritic cells in multiple sclerosis: The effect of bacterial infection. *Journal of Neuroimmunology* **190**(1-2)**:** 177-189

Cowen T, Gavazzi I (1998) Plasticity in adult and ageing sympathetic neurons. *Progress in Neurobiology* **54**(3)**:** 249-288

Creutzfeldt HG (1920) Uber eine eigenartige herdformige Erkankung des Zentralnervenssystems. *Z Ges Neurol Psychiat* **57:** 1-18

Cuille JU, Chelle PL (1936) La maladie dite tremblante du mouton, est-elle inoculable? *Comptes rendu de l'Academie des Sciences* **203:** 1552- 1554

Cunningham C, Wilcockson DC, Campion S, Lunnon K, Perry VH (2005) Central and Systemic Endotoxin Challenges Exacerbate the Local Inflammatory Response and Increase Neuronal Death during Chronic Neurodegeneration. *Journal of Neuroscience* **25**(40)**:** 9275-9284

Dammann C (1886) Achzehente Vorlesung. *Zucht and Aufzucht***:** 1146-1170

Denzer K, van Eijk M, Kleijmeer MJ, Jakobson E, de Groot C, J. Geuze H (2000) Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. *Journal of Immunology* **165**(3)**:** 1259-1265

Dickinson AG, Fraser H (1969) Genetical control of the concentration of ME7 scrapie agent in mouse spleen. *Journal of Comparative Pathology* **79**(3)**:** 363-366

Dickinson AG, Fraser H, McConnell I, Outram GW (1978) Mitogenic stimulation of the host enhances susceptibility to scrapie. *Nature* **272**(5648)**:** 54-55

Dickinson AG, Fraser H, Outram GW (1975) Scrapie incubation time can exceed natural lifespan. *Nature* **256**(5520)**:** 732-733

Dickinson AG, MacKay JMK (1964) Genetical control of the incubation period in mice of the neurological disease, scrapie. *Heredity* **19:** 279−288

Dickinson AG, Meikle VM, Fraser H (1968) Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. *Journal of Comparative Pathology* **78 (3):** 293-299

Dickinson AG, Outram GW (1988) Genetic aspects of unconventional virus infections: the basis of the virino hypothesis. *Ciba Foundation symposium* **135:** 63-83

Diringer H (1984) Sustained viremia in experimental hamster scrapie. Brief report. *Archives of Virology* **82:** 105-109

Diringer H, Beekes M, Oberdieck U (1994) The nature of the scrapie agent: The Virus theory. *Annals of the New York Academy of Sciences* **724**(1)**:** 246-258

Diringer H, Gelderblom H, Hilmert H, Ozel M, Edelbluth C, Kimberlin RH (1983) Scrapie infectivity, fibrils and low molecular weight protein. *Nature* **306**(5942)**:** 476-478

Duffy P, Wolf J, Collins G, DeVoe AG, Streeten B, Cowen D (1974) Possible person-to-person transmission of Creutzfeldt-Jakob disease. *New England Journal of Medicine* **290**(12)**:** 692-693

Dumont FJ, Barrois R, Jacobson EB (1984) Migration of peripheral T and B cells into the thymus of aging (NZB × SJL)F1 female mice. *Cellular Immunology* **83**(2)**:** 292-301

Ehlers B, Diringer H (1984) Dextran sulphate 500 delays and prevents mouse scrapie by impairment of agent replication in spleen. *Journal of General Virology* **65:** 1325-1330

Eklund CM, Kennedy RC, Hadlow WJ (1967) Pathogenesis of scrapie virus infection in the mouse. *Journal of Infectious Disease* **117:** 15-22

El-Solh AA, Sikka P, Ramadan F, Davies J (2001) Etiology of Severe Pneumonia in the Very Elderly. *American Journal of Respiratory Critical Care Medicine* **163**(3)**:** 645-651

Eloit M, Adjou K, Coulpier M, Fontaine JJ, Hamel R, Lilin T, Messiaen S, Andreoletti O, Baron T, Bencsik A, Biacabe AG, Beringue V, Laude H, Le Dur A, Vilotte JL, Comoy E, Deslys JP, Grassi J, Simon S, Lantier F, Sarradin P (2005) BSE agent signatures in a goat. *Veterinary Record* **156**(16)**:** 523-b-524

Elsen JM, Amigues Y, Schelcher F, Ducrocq V, Andreoletti O, Eychenne F, Tien Khang JV, Poivey JP, Lantier F, Laplanche JL (1999) Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. *Archives of Virology* **144**(3)**:** 431-445

Endres R, Alimzhanov MB, Plitz T, Futterer A, Kosco-Vilbois MH, Nedospasov SA, Rajewsky K, Pfeffer K (1999) Mature follicular dendritic cell networks depend on expression of Lymphotoxin beta receptor by radioresistant stromal cells and of Lymphotoxin beta and tumor necrosis factor by B cells. *Journal of Experimental Medicine* **189**(1)**:** 159-168

Farquhar CF, Dickinson AG (1986) Prolongation of scrapie incubation period by an injection of dextran sulphate 500 within the month before or after infection. *Journal of General Virology* **67:** 463-473

Farquhar CF, Dornan J, Somerville RA, Tunstall AM, Hope J (1994) Effect of sinc genotype, agent isolate and route of infection on the accumulation of protease-resistant prp in noncentral nervous-system tissues during the development of murine scrapie. *Journal of General Virology* **75:** 495-504

Farquhar CF, McConnell I, Cumming S, Prescott RJ, Boyle A, Barclay GR, Pepper DS, Turner ML, Bruce ME (2003) Pentosan polysulphate has prophylactic potential for transmissible spongiform encephalopathies. In *New Perspectives for Prion Therapeutics* pp 71-78. Paris

Farquhar CF, Somerville RA, Ritchie LA (1989) Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *Journal of Virological Methods* **24**(1-2)**:** 215-221

Ferone D, Pivonello R, Van Hagen PM, Waaijers M, Zuijderwijk J, Colao A, Lombardi G, Bogers AJJC, Lamberts SWJ, Hofland LJ (2000) Age-related decrease of somatostatin receptor number in the normal human thymus. *American Journal of Physiology and Endocrinology Metabolism* **279**(4)**:** E791-798

Foster JD, Dickinson AG (1988) Genetic-control of scrapie in cheviot and suffolk sheep. *Veterinary Record* **123**(6)**:** 159

Foster JD, Parnham D, Chong A, Goldmann W, Hunter N (2001a) Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats. *Veterinary Record* **148:** 165 - 171

Foster JD, Parnham DW, Hunter N, Bruce M (2001b) Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. *Journal of General Virology* **82**(10)**:** 2319-2326

Frasca D, Riley RL, Blomberg BB (2005) Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans. *Seminars in Immunology* **17**(5)**:** 378-384

Fraser H (1976) The pathology of a natural and experimental scrapie. [Review]. *Frontiers of Biology* **44:** 267-305

Fraser H (1993) Diversity in the neuropathology of scrapie-like diseases in animals. *British Medical Bulletin* **49**(4)**:** 792-809

Fraser H, Brown KL, Stewart K, McConnell I, McBride P, Williams A (1996) Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone-marrow. *Journal Of General Virology* **77:** 1935-1940

Fraser H, Bruce ME, Chree A, McConnell I, Wells GA (1992a) Transmission of bovine spongiform encephalopathy and scrapie to mice. *Journal of General Virology* **73:** 1891-1897

Fraser H, Bruce ME, Davies D, Farquhar CF, McBride PA (1992b) The lymphoreticular system in the pathogenesis of scrapie. In *Prion Diseases of Humans*

and Animals, pp 308-317. Edited by S.B. Prusiner, J.Collinge, J.Powell and B Anderton.Chichester: Ellis Horwood.

Fraser H, Dickinson AG (1967) Distribution of experimentally induced scrapie lesions in the brain. *Nature* **216:** 1310-1311

Fraser H, Dickinson AG (1968) The sequential development of the brain lesion of scrapie in three strains of mice. *Journal of Comparative Pathology* **78:** 301-311

Fraser H, Dickinson AG (1970) Pathogenesis of Scrapie in the Mouse: the Role of the Spleen. *Nature* **226** (5244)**:** 462-463

Fraser H, Dickinson AG (1973) Scrapie in mice: Agent-strain differences in the distribution and intensity of grey matter vacuolation. *Journal of Comparative Pathology* **83**(1)**:** 29-40

Fraser H, Dickinson AG (1978) Studies of the lymphoreticular system in the pathogenesis of scrapie: the role of spleen and thymus. *Journal of Comparative Pathology* **88**(4)**:** 563-573

Fraser H, Farquhar CF (1985) Are mitotically dormant, radiation insensitive cells involved in the peripheral pathogenesis of scrapie. *Neuropathology and Applied Neurobiology* **11**(1)**:** 71

Fraser H, Farquhar CF (1987) Ionising radiation has no influence on scrapie incubation period in mice. *Veterinary Microbiology* **13**(3)**:** 211-223

Fraser H, Farquhar CF, McConnell I, Davies D (1989) The scrapie disease process is unaffected by ionising radiation. *Progress in Clinical & Biological Research* **317:** 653-658

Fraser H, McConnell I, Wells GAH, Dawson M (1988) Transmission of bovine spongiform encephalopathy to mice. *Veterinary Record* **123**(18)**:** 472

Fraser JR (1996) Infectivity in extraneural tissues following intraocular scrapie infection. *Journal of General Virology* **77:** 2663-2668

Gajdusek DC (1963) Kuru. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **57**(3)**:** 151-169

Gajdusek DC, Alpers M (1966) Kuru in childhood: Disappearance of the disease in the younger age group. *The Journal of Pediatrics* **69**(5, Part 2)**:** 886-887

Gajdusek DC, Gibbs CJ (1964) Attempts to demonstrate a transmissible agent in kuru, amyotrophic lateral sclerosis, and other sub-acute and chronic nervous system degenerations of man. *Nature* **204**(4955)**:** 257-259

Gajdusek DC, Gibbs CJ, Alpers M (1966) Experimental transmission of a kuru-like syndrome to Chimpanzees. *Nature* **209**(5025)**:** 794-796

Gambetti P, Kong Q, Zou W, Parchi P, Chen SG (2003) Sporadic and familial CJD: classification and characterisation. *British Medical Bulletin* **66**(1)**:** 213-239

Gardiner AC, Marucci AA (1969) Immunological responsiveness of scrapie infected mice. *Journal of Comparative Pathology* **79:** 233-235

Garfin DE, Stites DP, Perlman JD, Cochran SP, Prusiner SB (1978a) Mitogen stimulation of splenocytes from mice infected with scrapie agent. *Journal of Infectious Disease* **138**(3)**:** 396-400

Garfin DE, Stites DP, Zitnik LA, Prusiner SB (1978b) Suppression of polyclonal B cell activation in scrapie-infected C3H/HeJ mice. *Journal of Immunology* **120**(6)**:** 1986-1990

Gasque P, Dean YD, McGreal EP, VanBeek J, Morgan BP (2000) Complement components of the innate immune system in health and disease in the CNS. *Immunopharmacology* **49**(1-2)**:** 171-186

Gavazzi G, Krause KH (2002) Ageing and infection. *The Lancet Infectious Diseases* **2:** 659-666

Gerstmann J, Sträussler E, Scheinker I (1936) Über eine eigenartige hereditär-familiäre Erkrankung des Zentralnervensystems. Zugleich ein Beitrag zur Frage des vorzeitigen lokalen Alterns. . *Zeitschrift für die gesamte Neurologie und Psychiatrie,* **154:** 736-762

Ghani AC, Ferguson NM, Donnelly CA, Anderson RM (2000) Predicted vCJD mortality in Great Britain. *Nature* **406**(6796)**:** 583-584

Giese A, Brown DR, Groschup MH, Feldmann C, Haist I, Kretzschmar HA (1998) Role of microglia in neuronal cell death in prion disease. *Brain Pathology* **8**(3)**:** 449-457

Glanzmann E, Riniker P (1950) Essentielle Lymphocytophtose. Ein neues Krankeitsbild aus der Sauglingspathologie. *Annals of Paediatrics* **174:** 1-5

Glaysher BR, Mabbott NA (2007) Role of the GALT in scrapie agent neuroinvasion from the intestine. *Journal of Immunology* **178**(6)**:** 3757-3766

Goldfarb LG, Brown P, McCombie WR, Goldgaber D, Swergold GD, Wills PR, Cervenakova L, Baron H, Gibbs CJJ, Gajdusek DC (1991a) Transmissible familial Creutzfeldt-Jakob disease associated with five, seven, and eight extra octapeptide coding repeats in the PRNP gene. *Proceedings of the National Academy of Sciences* **88**(23)**:** 10926-10930

Goldfarb LG, Brown P, Mitrova E, Cervenakova L, Goldin L, Korczyn AD, Chapman J, Galvez S, Cartier L, Rubenstein R, Gajdusek DC (1991b) Creutzfeldt-Jakob disease associated with the PRNP codon 200LYS mutation - an analysis of 45 families. *European Journal of Epidemiology* **7**(5)**:** 477-486

Goldmann W, Hunter N, Smith G, Foster J, Hope J (1994) PrP genotype and agent effects in scrapie - change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. *Journal of General Virology* **75:** 989-995

Gonzalez L, Martin S, Jeffrey M (2003) Distinct profiles of PrPd immunoreactivity in the brain of scrapie and BSE-infected sheep: implications for differential cell targeting and PrP processing. *Journal of General Virology* **84**(5)**:** 1339-1350

Gordon WS (1946) Louping-ill, Ttick-born Fever and Scrapie. *Veterinary Record* **58**(47)**:** 516

Grassi J, Maillet S, Simon S, Morel N (2008) Progress and limits of TSE diagnostic tools (review). . *Veterinary Research* **39:** 33

Gray D (2002) A role for antigen in the maintenance of immunological memory *Nature Reviews Immunology* **2:** 60-65

Gray D, Matzinger P (1991) T cell memory is short-lived in the absence of antigen. *Journal of Experimental Medicine* **174:** 969-974

Gray D, Skarvall H (1988) B cell memory is short-lived in the absence of antigen. *Nature* **336:** 70-73

Griffith JS (1967) Nature of the Scrapie Agent: Self-replication and Scrapie. *Nature* **215**(5105)**:** 1043-1044

Gross HJ, Domdey H, Sanger HL (1977) Comparative oligonucleotide fingerprints of three plant viroids. *Nucleic Acids Research* **4**(6)**:** 2021-2028

Gudnadottir M (1974) Visna-maedi in sheep. *Progress in medical virology Fortschritte der medizinischen Virusforschung Progres en virologie medicale* **18**(0)**:** 336-349

Gunawardena I, Attygalle T (2008) Re-infection with primary varicella zoster in older people. *Age Ageing* **37**(2)**:** 235-

Haberman AM, Shlomchik MJ (2003) Reassessing the function of immune-complex retention by follicular dendritic cells. *Nature Reviews in Immunology* **3**(9)**:** 757-764

Hadlow WJ (1959) Scrapie and kuru. *The Lancet* **274**(7097)**:** 289-290

Harrison PM, Chan HS, Prusiner SB, Cohen FE (1999) Thermodynamics of model prions and its implications for the problem of prion protein folding. *Journal of Molecular Biology* **286**(2)**:** 593-606

Hartsough GR, Burger D (1965) Encephalopathy of mink. I. Epizootiologic and clinical observations. *Journal of Infectious Disease* **115:** 387-392

Heppner FL, Arrighi I, Kalinke U, Aguzzi A (2001) Immunity against prions? *Trends in Molecular Medicine* **7**(11)**:** 477-479

Herms J, Tings T, Gall S, Madlung A, Giese A, Siebert H, Schurmann P, Windl O, Brose N, Kretzschmar H (1999) Evidence of presynaptic location and function of the prion protein. *Journal of Neuroscience* **19**(20)**:** 8866-8875

Herms JW, Kretzschmar HA, Titz S, Keller BU (1995) Patch-clamp analysis of synaptic transmission to cerebellar Purkinje cells of prion protein knockout mice. *European Journal of Neuroscience* **7**(12)**:** 2508-2512

Hill AF, Antoniou M, Collinge J (1999a) Protease-resistant prion protein produced in vitro lacks detectable infectivity. *Journal of General Virology* **80**(1)**:** 11-14

Hill AF, Butterworth RJ, Joiner S, Jackson G, Rossor MN, Thomas DJ, Frosh A, Tolley N, Bell JE, Spencer M, King A, AlSarraj S, Ironside JW, Lantos PL, Collinge J (1999b) Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet* **353**(9148)**:** 183-189

Hill AF, Zeidler M, Ironside J, Collinge J (1997) Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. *Lancet* **349:** 99-100

Hilton DA, Ghani AC, Conyers L, Edwards P, McCardle L, Penney M, Ritchie D, Ironside JW. (2002) Accumulation of prion proteins in tonsil and appendix: review of tissue samples. *British Medical Journal*, Vol. 325, pp. 633-634.

Hilton DA, Ghani AC, Conyers L, Edwards P, McCardle L, Ritchie D, Penney M, Hegazy D, Ironside J (2004) Prevalence of lymphoreticular prion protein protein accumulation in UK tissue samples. *Journal of Pathology* **203**(3)**:** 733-739

Hope J, Morton LJD, Farquhar CF, Multhaup G, Beyreuther K, Kimberlin RH (1986) The major protein of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). *EMBO* **5:** 2591-2597

Hope J, Wood SC, Birkett CR, Chong A, Bruce ME, Cairns D, Goldmann W, Hunter N, Bostock CJ (1999) Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH1641. *Journal of General Virology* **80**(1)**:** 1-4

Hornabrook RW, Moir DJ (1970) KURU : Epidemiological Trends. *Lancet* **296**(7684)**:** 1175-1179

Houston F, Foster JD, Chong A, Hunter N, Bostock CJ (2000) Transmission of BSE by blood transfusion in sheep. *Lancet* **356**(9234)**:** 999-1000

Huang F-P, Farquhar CF, Mabbott NA, Bruce ME, MacPherson GG (2002) Migrating intestinal dendritic cells transport PrPSc from the gut. *Journal of General Virology* **83**(1)**:** 267-271

Huang F-P, MacPherson GG (2004) Dendritic cells and oral transmission of prion diseases. *Advanced Drug Delivery Reviews* **56**(6)**:** 901-913

Huang Z, Prusiner SB, Cohen FE (1996) Scrapie prions: a three-dimensional model of an infectious fragment. *Folding and Design* **1**(1)**:** 13-19

Humphrey JH, Grennan D, Sundaram V (1984) The origin of follicular dendritic cells in the mouse and the mechanism of trapping of immune complexes on them. *European Journal of Immunology* **14:** 859-864

Hunter N (2003) Scrapie and experimental BSE in sheep. *British Medical Bulletin* **66**(1)**:** 171-183

Hunter N (2007) Scrapie--Uncertainties, biology and molecular approaches. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1772**(6)**:** 619-628

Hunter N, Dann JC, Bennett AD, Somerville RA, McConnell I, Hope J (1992) Are Sinc and the PrP gene congruent? Evidence from PrP gene analysis in *Sinc* congenic mice. *Journal of General Virology* **73:** 2751-2755

Hunter N, Foster J, Chong A, McCutcheon S, Parnham D, Eaton S, MacKenzie C, Houston F (2002) Transmission of prion diseases by blood transfusion. *Journal of General Virology* **83**(11)**:** 2897-2905

Hunter N, Foster JD, Goldmann W, Stear MJ, Hope J, Bostock C (1996) Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. *Archives of Virology* **141**(5)**:** 809-824

Hunter N, Goldmann W, Foster JD, Cairns D, Smith G (1997) Natural scrapie and PrP genotype: Case-control studies in British sheep. *Veterinary Record* **141**(6)**:** 137-140

Hunter N, Hope J, McConnell I, Dickinson AG (1987) Linkage of the scrapie-associated fibril protein (PRP) gene and sinc using congenic mice and restriction fragment length polymorphism anaysis. *Journal of General Virology* **68:** 2711-2716

Hwang S-Y, Jung J-S, Kim T-H, Lim S-J, Oh E-S, Kim J-Y, Ji K-A, Joe E-H, Cho K-H, Han I-O (2006) Ionizing radiation induces astrocyte gliosis through microglia activation. *Neurobiology of Disease* **21**(3)**:** 457-467

Ierna M, Farquhar CF, Outram GW, Bruce ME (2006) Resistance of neonatal mice to scrapie is associated with inefficient infection of the immature spleen. *Journal of Virology* **80**(1)**:** 474-482

Ingrosso L, Ladogana A, Pocchiari M (1995) Congo red prolongs the incubation period in scrapie-infected hamsters. *Journal of Virology* **69**(1)**:** 506-508

Ironside JW (2003) Variant Creutzfeldt-Jakob Disease. *Veterinary Research Communications* **27**(0)**:** 11-13

Ironside JW, Bishop MT, Connolly K, Hegazy D, Lowrie S, Le Grice M, Ritchie DL, McCardle LM, Hilton DA (2006) Variant Creutzfeldt-Jakob disease: prion protein genotype analysis of positive appendix tissue samples from a retrospective prevalence study. *British Medical Journal* **332**(7551)**:** 1186-1188

Ironside JW, Head MW, McCardle L, Knight R. (2002) Neuropathology of variant Creutzfeldt-Jakob disease. *Acta Neurobiologiae Experimentalis*, Vol. 62, pp. 175-182.

Jakob AM (1921) Uber eine der multiplem Sklerose klinisch nahestehenden Erkrankung des Zentralnervensystems (spastiche Pseudosklerose) mit bemerkenswertem anatomischem Befunde. *Med Klin* **13:** 372-376

Jeffrey M, Goodsir CM, Bruce M, McBride PA, Scott JR, Halliday WG (1994a) Correlative light and electron microscopy studies of PrP localisation in 87V scrapie. *Brain Research* **656**(2)**:** 329-343

Jeffrey M, Goodsir CM, Bruce ME, McBride PA, Fowler N, Scott JR (1994b) Murine scrapie-infected neurons in-vivo release excess prion protein into the extracellular-space. *Neuroscience Letters* **174**(1)**:** 39-42

Jeffrey M, McGovern G, Goodsir CM, Brown KL, Bruce ME (2000) Sites of prion protein accumulation in scrapie -infected mouse spleen revealed by electron microscopy. *Journal of Pathology* **191:** 323-332

Johnston AR, Fraser JR, Jeffrey M, MacLeod N, (1998) Synaptic plasticity in the CA1 area of the hippocampus of scrapie-infected mice. *Neurobiology of Disease* **5:** 188-195

Kaneko K, Zulianello L, Scott M, Cooper CM, Wallace AC, James TL, Cohen FE, Prusiner SB (1997) Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. *Proceedings of the National Academy of Sciences* **94**(19)**:** 10069-10074

Kapasi ZF, Burton GF, Shultz LD, Tew JG, Szakal AK (1993) Induction of functional follicular dendritic cell development in severe combined immunodeficiency mice. *Journal of Immunology* **150**(7)**:** 2648-2658

Kapasi ZF, Qin D, Kerr WG, Kosco-Vilbois, Shultz LD, Tew JG, Szakal AK (1998) Follicular dendritic cell precursors in primary lymphoid tissues. *Journal of Immunology* **160:** 1078-1084

Karrer U, Lopez-Macias C, Oxenius A, Odermatt B, Bachmann MF, Kalinke U, Bluethmann H, Hengartner H, Zinkernagel RM (2000) Antiviral B cell memory in the absence of mature follicular dendritic cell networks and classical germinal centers in TNFR1-/- Mice. *Journal of Immunology* **164**(2)**:** 768-778

Kato H, Fujihashi K, Kato R, Dohi T, Fujihashi K, Hagiwara Y, Kataoka K, Kobayashi R, McGhee JR (2003) Lack of oral tolerance in aging is due to sequential loss of Peyer's patch cell interactions. *International Immunology* **15**(2)**:** 145-158

Kercher L, Favara C, Striebel JF, LaCasse R, Chesebro B (2007) Prion protein (PrP) expression differences in microglia and astroglia influence scrapie-induced neurodegeneration in retina and brain of transgenic mice. *Journal of Virology* **81:** 10340-10351

Kimberlin RH, Cole S, Walker CA (1987) Pathogenesis of scrapie is faster when infection is intraspinal instead of intracerebral. *Microbial Pathogenesis* **2:** 405-415

Kimberlin RH, Cunnington PG (1978a) Reduction of scrapie incubation time in mice and hamsters by a single injection of methanol extraction of BCG. *FEBS Microbiology letters* **3:** 169-172

Kimberlin RH, Cunnington PG (1978b) Reduction of scrapie incubation time in mice and hamsters by a single injection of methanol extraction residue of bcg. *FEMS Microbiology Letters* **3**(3)**:** 169-172

Kimberlin RH, Walker CA (1979) Pathogenesis of mouse scrapie : dynamics of agent replication replication in spleen, spinal cord and brain after infection by different routes. *Journal of Comparative Pathology* **89:** 551-562

Kimberlin RH, Walker CA (1986) Pathogenesis of scrapie (strain263K) in hamsters infected intracerebrally, intraperitoneally or intraocularly. *Journal of General Virology* **67:** 255-263

Kimberlin RH, Walker CA (1989) The role of the spleen in the neuroinvasion of scrapie in mice. *Virus Research* **12**(3)**:** 201-211

Kimberlin RH, Walker CA (1990) Intraperitoneal infection with scrapie is established within minutes of injection and is nonspecifically enhanced by a variety of different drugs. *Archives of Virology* **112**(1-2)**:** 103-114

Kinet-Denoël C, Heinen E, Radoux D, Simar LJ (1982) Follicular Dendritic Cells in Lymph Nodes after X-irradiation *International Journal of Radiation Biology* **42**(2)**:** 121-130

Kirschfink M (1997) Controlling the complement system in inflammation. *Immunopharmacology* **38**(1-2)**:** 51-62

Kitamura D, Roes J, Kühn R, Rajewsky K (1991) A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin chain gene *Nature* **350:** 423 - 426

Klein MA, Frigg R, Flechsig E, Raeber AJ, Kalinke U, Bluethmann H, Bootz F, Suter M, Zinkernagel RM, Aguzzi A (1997) A crucial role for B cells in neuroinvasive scrapie. *Nature* **390:** 687-690

Klein MA, Frigg, R., Raeber, A.J., Flechsig, E., Hegyi, I., Zinkernagel, R.M., Weissmann, C. and Aguzzi, A. (1998) PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nature Medicine* **4**(12)**:** 1429-1433

Klein MA, Kaeser PS, Schwarz P, Weyd H, Xenarios I, Zinkernagel RM, Carroll MC, Verbeek JS, Botto M, Walport MJ, Molina H, Kalinze U, Acha-Orbea H, Aguzzi A (2001) Complement facilitates early prion pathogenesis. *Nature Medicine* **7**(4)**:** 488-492

Koch S, Solana R, Dela Rosa O, Pawelec G (2006) Human cytomegalovirus infection and T cell immunosenescence: a mini review. *Mechanisms of Ageing and Development* **127**(6)**:** 538-543

Kosco-Vilbois MH (2003) Are follicular dendritic cells really good for nothing? *Nature Reviews in Immunology* **3**(9)**:** 764-769

Kosco-Vilbois MH, Bonnefoy J-Y, Chvatchko Y (1997) The physiology of mouse germinal center reactions. *Immunology Reviews* **156:** 127-136

Kosco MH, Pflugfelder E, Gray D (1992) Follicular dendritic cell-dependent adhesion and proliferation of B cells i*n-vitro. Journal of Immunology* **148:** 2331-2339

Kottilil S, Shin K, Jackson JO, Reitano KN, O'Shea MA, Yang J, Hallahan CW, Lempicki R, Arthos J, Fauci AS (2006) Innate Immune Dysfunction in HIV Infection: Effect of HIV Envelope-NK Cell Interactions. *Journal of Immunology* **176**(2)**:** 1107-1114

Kranich J, Krautler NJ, Heinen E, Polymenidou M, Bridel C, Schildknecht A, Huber C, Kosco-Vilbois MH, Zinkernagel R, Miele G, Aguzzi A (2008) Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8. *Journal of Experimental Medicine* **205 :** 1293-1302

Kretchmar AL, Congdon CC. (1967) Serine metabolism in radiation chimaeras. *From 1st International Conference on Organ Transplantation and Bone Marrow, Paris, France.*, Pages: 2.

Labrie JE, Borghesi L, Gerstein RM (2005) Bone marrow microenvironmental changes in aged mice compromise V(D)J recombinase activity and B cell generation. *Seminars in Immunology* **17**(5)**:** 347-355

Ladogana A, Casaccia P, Ingrosso L, Cibati M, Salvatore M, Xi Y-g, Masullo C, Pocchiari M (1992) Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. *Journal of General Virology* **73**(3)**:** 661-665

Lasmezas CI, Cesbron JY, Deslys JP, Demaimay R, Anjou KT, Rioux R, Lemaire C, Locht C, Dormont D (1996) Immune System-Dependent and System-Independent Replication Of the Scrapie Agent. *Journal Of Virology* **70**(2)**:** 1292-1295

Lasmezas CI, Deslys J-P, Robain O, Jaegly A, Beringue V, Peyrin J-M, Fournier J-G, Hauw J-J, Rossier J, Dormont D (1997) Transmission of the BSE Agent to Mice in the Absence of Detectable Abnormal Prion Protein. *Science* **275**(5298)**:** 402-404

Laube S (2004) Skin infections and ageing. *Ageing Research Reviews* **3**(1)**:** 69-89

Lavelle GC, Sturman L, Hadlow WJ (1972) Isolation from mouse spleen of cell populations with high specific infectivity for scrapie virus. *Infection and Immunity* **5:** 319-323

Le Y, Yazawa H, Gong W, Yu Z, Ferrans VJ, Murphy PM, Ji Ming W (2001) Cutting edge: The neurotoxic prion peptide fragment PrP106-126 is a chemotactic agonist for the G protein- coupled receptor formyl peptide receptor-like 11,2. *Journal of Immunology* **166**(3)**:** 1448-1451

Leake A, Morris CM, Whateley J (2000) Brain matrix metalloproteinase 1 levels are elevated in Alzheimer's disease. *Neuroscience Letters* **291**(3)**:** 201-203

Leggett MM, Dukes J, Pirie HM (1990) A spongiform encephalopathy in a cat. *Veterinary Record* **127**(24)**:** 586-588

Legname G, Baskakov IV, Nguyen H-OB, Riesner D, Cohen FE, DeArmond SJ, Prusiner SB (2004) Synthetic Mammalian Prions. *Science* **305**(5684)**:** 673-676

Leopoldt J (1759) Nützliche, auf die Erfahrung gegründete Einleitung zu der Landwirtschaft,. *Glogau, Berlin***:** 344-360

Levavasseur E, Metharom P, Dorban G, Nakano H, Kakiuchi T, Carnaud C, Sarradin P, Aucouturier P (2007) Experimental scrapie in 'plt' mice: an assessment of the role of dendritic-cell migration in the pathogenesis of prion diseases. *Journal of General Virology* **88**(8)**:** 2353-2360

Lindhout E, de Groot C (1995) Follicular dendritic cells and apoptosis: Life and death in the germinal centre. *The Histochemical Journal* **27**(3)**:** 167-183

Linton PJ, Dorshkind K (2004) Age-related changes in lymphocyte development and function. *Naure Immunology* **5**(2)**:** 133-139

Llewelyn CA, Hewitt PE, Knight RSG, Amar K, Cousens S, Mackenzie J, Will RG (2004) Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* **363**(9407)**:** 417-421

Low MG (1998) Rapid intercellular transfer of GPI-anchored CD4. *Journal of Laboratory and Clinical Medicine* **131**(3)**:** 189-191

Lugaresi E, Medori R, Montagna P, Baruzzi A, Cortelli P, Lugaresi A, Tinuper P, Zucconi M, Gambetti P (1986) Fatal familial insomnia and dysautonomia with selective degeneration of thalamic nuclei
New England Journal of Medicine **315:** 997-1003

Mabbott NA, Brown KL, Bruce ME (1996) Lymphocyte-Activation and the Cellular-Form Of the Prion Protein, Prpc. *Immunology* **89**(S1)**:** AA410

Mabbott NA, Brown KL, Manson J, Bruce ME (1997) T-lymphocyte activation and the cellular form of the prion protein. *Immunology* **92**(2)**:** 161-165

Mabbott NA, Bruce ME (2004) Complement component C5 is not involved in scrapie pathogenesis. *Immunobiology* **209**(7)**:** 545-549

Mabbott NA, Bruce ME, Botto M, Walport MJ, Pepys MB (2001) Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie
Nature Medicine **7:** 485 - 487

Mabbott NA, Farquhar CF, Brown KL, Bruce ME (1998) Involvement of the immune system in TSE pathogenesis. *Immunology Today* **19:** 201-203

Mabbott NA, Mackay F, Minns F, Bruce ME (2000a) Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. *Nature Medicine* **6:** 719-720

Mabbott NA, MacPherson GG (2006) Prions and their lethal journey to the brain. *Nature Reviews in Microbiology* **4**(3)**:** 201-211

Mabbott NA, McGovern G, Jeffrey M, Bruce ME (2002) Temporary blockade of the tumor necrosis factor receptor signaling pathway impedes the spread of scrapie to the brain. *Journal of Virology* **76:** 5131-5139

Mabbott NA, Williams A, Farquhar CF, Pasparakis G, Kollias G, Bruce ME (2000b) Tumor necrosis factor alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie. *Journal of Virology* **74:** 3338-3344

Mabbott NA, Young J, McConnell I, Bruce ME (2003) Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility. *Journal of Virology* **77**(12)**:** 6845-6854

Mancini G, Carbonara AO, Heremans JF (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* **2**(3)**:** 235-254

Manson J, West JD, Thomson V, McBride P, Kaufman MH, Hope J (1992) The prion protein gene: a role in mouse embryogenesis? *Development* **115**(1)**:** 117-122

Manson JC, Clarke AR, Hooper ML, Aitchison L, McConnell I, Hope J (1994a) 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Molecular Neurobiology* **8:** 121-127

Manson JC, Clarke AR, McBride PA, McConnell I, Hope J (1994b) PrP gene dosage determines the timing but not the final intensisty or distribution of lesions in scrapie pathology. *Neurodegeneration* **3:** 331-340

Manson JC, Jamieson E, Baybutt H, Tuzi NL, Barron R, McConnell I, Somerville R, Ironside J, Will R, Sy MS, Melton DW, Hope J, Bostock C (1999) A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. *EMBO* **18**(23)**:** 6855-6864

Marsala M, Vanicky I, Tokumine J, Kakinohana O, Marsala J, Hari Shanker S, Jan W (2004) Blood-brain barrier changes in global and focal cerebral ischemia. In *Blood-Spinal Cord and Brain Barriers in Health and Disease*, pp 385-394. San Diego: Academic Press

Marsh RF, Sipe J, Morse S, . , Hanson R (1976) Reduced spongiform degeneration in aged mink of the Chediak-Higashi genotype. *Laboratory Investigation* **34**(4)**:** 381-386

Maruyama Y, Mylrea MM, Logothetis J (1967) Neuropathy following irradiation: an unusual late complication of radiotherapy. *American Journal of Roentgenology* **101**(1)**:** 216-219

Mathews J, Glasse R, Lindenbaum S (1968) Kuru and cannibalism. *The Lancet* **292**(7565)**:** 449-452

May BCH, Fafarman AT, Hong SB, Rogers M, Deady LW, Prusiner SB, Cohen FE (2003) Potent inhibition of scrapie prion replication in cultured cells by bis-acridines *Proceedings of the National Academy of Sciences* **100**(6)**:** 3416-3421

McBride PA, Beekes M (1999) Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie. *Neuroscience Letters* **265**(2)**:** 135-138

McBride PA, Eikelenboom P, Kraal G, Fraser H, Bruce ME (1992) PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie infected mice. *Journal of Pathology* **168:** 413-418

McBride PA, Schulz-Schaeffer WJ, Donaldson M, Bruce M, Diringer H, Kretzschmar HA, Beekes M (2001) Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibers of the splanchnic and vagus nerves. *Journal of Virology* **75**(19)**:** 9320-9327

McBride PA, Wilson MI, Eikelenboom P, Tunstall A, Bruce ME (1998) Heparan sulfate proteoglycan is associated with amyloid plaques and neuroanatomically targeted PrP pathology throughout the incubation period of scrapie-infected mice. *Experimental Neurology* **149**(2)**:** 447-454

McGovern G, Brown KL, Bruce ME, Jeffrey M (2004) Murine scrapie infection causes an abnormal germinal centre reaction in the spleen. *Journal of Comparative Pathology* **130**(2-3)**:** 181-194

McGovern G, Jeffrey M (2007) Scrapie-Specific Pathology of Sheep Lymphoid Tissues. *PLoS ONE* **2**(12)**:** e1304

McGuire TC, Poppie MJ (1973) Hypogammaglobulinemia and thymic hypoplasia in horses: a primary combined immunodeficiency disorder. *Infection and Immunity* **8**(2)**:** 272-277

McKinley MP, Bolton DC, Prusiner SB (1983) A protease-resistant protein is a structural component of the Scrapie prion. *Cell* **35**(1)**:** 57-62

Mead S, Stumpf MPH, Whitfield J, Beck JA, Poulter M, Campbell T, Uphill JB, Goldstein D, Alpers M, Fisher EMC, Collinge J (2003) Balancing selection at the prion protein gene consistent with prehistoric kurulike Epidemics. *Science* **300**(5619)**:** 640-643

Medina FA, Cohen AW, de Almeida CJ, Nagajyothi F, Braunstein VL, Teixeira MM, Tanowitz HB, Lisanti MP (2007) Immune dysfunction in caveolin-1 null mice following infection with Trypanosoma cruzi (Tulahuen strain). *Microbes and Infection* **9**(3)**:** 325-333

Millson GC, Kimberlin RH, Manning EJ, Collis SC (1979) Early distribution of radioactive liposomes and scrapie infectivity in mouse tissues following administration by different routes. *Veterinary Microbiology* **4:** 89-99

Minor PD, Will RG, Salisbury D (2000) Vaccines and variant CJD. *Vaccine* **19**(4-5)**:** 409-410

Mitchell B, Kendall M, Adam E, Schumacher U (1997) Innervation of the thymus in normal and bone marrow reconstituted severe combined immunodeficient (SCID) mice. *Journal of Neuroimmunology* **75:** 19-27

Mitchell DA, Kirby L, Paulin SM, Villiers CL, Sim RB (2007) Prion protein activates and fixes complement directly via the classical pathway: Implications for the mechanism of scrapie agent propagation in lymphoid tissue. *Molecular Immunology* **44**(11)**:** 2997-3004

Mohan J, Brown KL, Farquhar CF, Bruce ME, Mabbott NA (2004) Scrapie transmission following exposure through the skin is dependent on follicular dendritic cells in lymphoid tissues. *Journal of Dermatological Science* **35**(2)**:** 101-111

Mohan J, Bruce ME, Mabbott NA (2005a) Neuroinvasion by scrapie following inoculation via the skin is independent of migratory Langerhans cells. *Journal of Virology* **79**(3)**:** 1888-1897

Mohan J, Hopkins J, Mabbott NA (2005b) Skin-derived dendritic cells acquire and degrade the scrapie agent following in vitro exposure. *Immunology* **116**(1)**:** 122-133

Montrasio F, Frigg R, Glatzel M, Klein MA, Mackay F, Aguzzi A, Weissmann C (2000) Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. *Science* **288**(5469)**:** 1257-1259

Moore RC, Hope J, McBride PA, McConnell I, Selfridge J, Melton DW, Manson JC (1998) Mice with gene targeted prion protein alterations show that Prnp, Sinec and Prni are congruent. *Nature Genetics* **18**(2)**:** 118-125

Moss RB, Jensen FC, Carlo DJ (2000) Insights into HIV-specific immune function: implications for therapy and prevention in the new millennium. *Clinical Immunology* **95**(2)**:** 79-84

Mu XY, Thoman ML (2000) Aging affects the regeneration of the CD8+ T cell compartment in bone marrow transplanted mice. *Mechanisms of Ageing and Development* **112**(2)**:** 113-124

Murray K, Ritchie DL, Bruce M, Young CA, Doran M, Ironside JW, Will RG (2008) Sporadic Creutzfeldt Jakob disease in two adolescents. *Journal of Neurology Neurosurgery and Psychiatry* **79**(1)**:** 14-18

Nakamitsu S, Miyazawa T, Horiuchi M, Onoe S, Ohoba Y, Kitagawa H, Ishiguro N (2006) Sequence variation of bovine prion protein gene in Japanese cattle (Holstein and Japanese Black). *The Journal of Veterinary Medical Science* **68**(1)**:** 27-33

Niculescu T, Weerth S, Niculescu F, Cudrici C, Rus V, Raine CS, Shin ML, Rus H (2004) Effects of complement C5 on apoptosis in experimental autoimmune encephalomyelitis. *Journal of Immunology* **172**(9)**:** 5702-5706

Nizet V (2007) Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. *Journal of Allergy and Clinical Immunology* **120**(1)**:** 13-22

Nordal RA, Wong CS (2005) Molecular targets in radiation-induced blood-brain barrier disruption. *International Journal of Radiation Oncology.Biology.Physics* **62**(1)**:** 279-287

O'Rourke KI, Huff TP, Leathers CW, Robinson MM, Gorham JR (1994) SCID mouse spleen does not support scrapie replication. *Journal of General Virology* **75:** 1511-1514

Oesch B, Westaway D, Wälchli M, McKinley MP, Kent SBH, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissmann C (1985) A cellular gene encodes scrapie PrP 27-30 protein. *Cell* **40**(4)**:** 735-746

Ogiu T, Ishii-Ohba H, Kobayashi S, Nishimura M, Shimada Y, Tsuji H, Ukai H, Watanabe F, Suzuki F, Sado T (2002) Development and molecular analysis of thymic lymphomas induced by ionizing radiation in Scid mice. *International Congress Series* **1236:** 157-161

Outram GW, Dickinson AG, Fraser H (1973) Development maturation of susceptibility to scrapie in mice. *Nature* **241:** 536-537

Pan K, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohen FE, Prusiner SB (1993) Conversion of {alpha}-Helices {beta}-Sheets Features in the Formation of the scrapie Prion Proteins. *Proceedings of the National Academy of Sciences* **90**(23)**:** 10962-10966

Pappalardo G, Impellizzeri G, Campagna T (2004) Copper(II) binding of prion protein's octarepeat model peptides. *Inorganica Chimica Acta* **357**(1)**:** 185-194

Parry HB (1962.) Scrapie: a transmissible and hereditary disease of sheep. *Heredity* **17:** 75- 105

Pasparakis M, Alexopoulou L, Episkopou V, Kollias G (1996) Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *Journal of Experimental Medicine* **184**(4)**:** 1397-1411

Pattison I, H., Millson GC, Smith K (1964) An examination of the action of whole blood, blood cells or serum on the goat scrapie agent. *Research in Veterinary Science* **5:** 116-121

Pattison IH, Millson GC (1961) Scrapie produced experimentally in goats with special reference to the clinical syndrome. *Journal of Comparative Pathology* **71:** 101 - 109

Pattison IH, Smith K (1963) Experimental scrapie in goats: a modification of incubation period and clinical response following pre-treatment with normal goat brain. *Nature* **200**(4913)**:** 1342-1343

Pawelec G, Larbi A Immunity and ageing in man (2008)*Experimental Gerontology* **43** (1) 34-38

Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW (2004) Preclinical vCJD after blood transfusion in a PRNP. *Lancet* **364**(9433)**:** 527-529

Perry HV, Newman T, Cunningham C (2003) The impact of systemic infection on the progression of neurodegenerative disease. *Nature Reviews* **4:** 103-112

Perry VH (2004) The influence of systemic inflammation on inflammation in the brain: implications for chronic neurodegenerative disease. *Brain, Behavior, and Immunity* **18**(5)**:** 407-413

Perry VH (2007) Stress primes microglia to the presence of systemic inflammation: Implications for environmental influences on the brain. *Brain, Behavior, and Immunity* **21**(1)**:** 45-46

Perry VH, Anthony DC, Bolton SJ, Brown HC (1997) The blood-brain barrier and the inflammatory response. *Molecular Medicine Today* **3**(8)**:** 335-341

Phillips RJ, Powley TL (2007) Innervation of the gastrointestinal tract: Patterns of aging. *Autonomic Neuroscience* **136**(1-2)**:** 1-19

Piccardo P, Manson JC, King D, Ghetti B, Barron RM (2007) Accumulation of prion protein in the brain that is not associated with transmissible disease. *Proceedings of the National Academy of Sciences* **104**(11)**:** 4712-4717

Pinto A, De Filippi R, Frigeri F, Corazzelli G, Normanno N (2003) Aging and the hemopoietic system. *Critical Reviews in Oncology/Hematology* **48**(Supplement 1)**:** S3-S12

Pluchino S, Zanotti L, Deleidi M, Martino G (2005) Neural stem cells and their use as therapeutic tool in neurological disorders. *Brain Research Reviews* **48**(2)**:** 211-219

Poudrier J, Weng X, Kay DG, Paré G, Calvo EL, Hanna Z, Kosco-Vilbois MH, Jolicoeur P (2001) The AIDS disease of CD4C/HIV transgenic mice shows impaired germinal centers and autoantibodies and develops in the absence of IFN-[gamma] and IL-6. *Immunity* **15**(2)**:** 173-185

Priller J, Prinz M, Heikenwalder M, Zeller N, Schwarz P, Heppner FL, Aguzzi A (2006) Early and rapid engraftment of bone marrow-derived microglia in scrapie. *Journal of Neuroscience* **26**(45)**:** 11753-11762

Prinz M, Heikenwalder M, Junt T, Schwarz P, Glatzel M, Heppner FL, Fu Y-X, Lipp M, Aguzzi A (2003) Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion. *Nature* **425**(6961)**:** 957-962

Priola SA, Vorberg I (2004) Identification of possible animal origins of prion disease in human beings. *Lancet* **363**(9426)**:** 2013-2014

Prusiner SB (1982) Novel Proteinaceous Infectious Particles Cause Scrapie *Science* **216**(4542)**:** 136-144

Qin D, Wu J, Vora KA, Ravetch JV, Szakal AK, Manser T, Tew JG (2000) Fc{gamma} Receptor IIB on Follicular Dendritic Cells Regulates the B Cell Recall Response. *Journal of Immunology* **164**(12)**:** 6268-6275

Race RE, Caughey B, Graham K, Ernst D, Chesebro B (1988) Analyses of frequency of infection, specific infectivity, and prion protein biosynthesis in scrapie-infected neuroblastoma cell clones. *Journal of Virology* **62**(8)**:** 2845-2849

Raymond CR, Aucouturier P, Mabbott NA (2007) In Vivo depletion of CD11c+ cells impairs scrapie agent neuroinvasion from the intestine. *Journal of Immunology* **179:** 7758-7766

Raymond CR, Mabbott NA (2007) Assessing the involvement of migratory dendritic cells in the transfer of the scrapie agent from the immune to peripheral nervous systems. *Journal of Neuroimmunology* **187**(1-2)**:** 114-125

Reisner Y, Biniaminov M, Rosenthal E, Sharon N, Ramot B (1979) Interaction of peanut agglutinin with normal human lymphocytes and with leukemic cells. *Proceedings of the National Academy of Sciences* **76**(1)**:** 447-451

Ritchie DL, Brown KL, Bruce ME (1999) Visualisation of PrP protein and follicular dendritic cells in uninfected and scrapie infected spleen. *Journal of Cellular Pathology* **1:** 3-10

Rogerson BJ, Harris DP, Swain SL, Burgess DO (2003) Germinal center B cells in Peyer's patches of aged mice exhibit a normal activation phenotype and highly mutated IgM genes. *Mechanisms of Ageing and Development* **124**(2)**:** 155-165

Rosicarelli B, Serafini B, Sbriccoli M, Lu M, Cardone F, Pocchiari M, Aloisi F (2005) Migration of dendritic cells into the brain in a mouse model of prion disease. *Journal of Neuroimmunology* **165**(1-2)**:** 114-120

Rothe J, Lesslauer W, Lotscher H, Lang Y, Koebel P, Kontgen F, Althage A, Zinkernagel R, Steinmetz M, Bluethmann H (1993) Mice lacking the tumour

necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. *Nature* **364:** 798-802

Sakai R, Maruta A, Yanoma S, Shimizu A, Harada M, Nakmura Y, Kodama F (1997) Effect of sublethal total body irradiation on acute graft-versus-host disease and graft-versus-leukemia effect in SCID mice *Bone Marrow Transplantation* **20**(3)**:** 183-189

Salmona M, Forloni G, Diomede L, Algeri M, De Gioia L, Angeretti N, Giaccone G, Tagliavini F, Bugiani O (1997) A Neurotoxic and Gliotrophic Fragment of the Prion Protein Increases Plasma Membrane Microviscosity. *Neurobiology of Disease* **4**(1)**:** 47-57

Schonberger O, Horonchik L, Gabizon R, Papy-Garcia D, Barritault D, Taraboulos A (2003) Novel heparan mimetics potently inhibit the scrapie prion protein and its endocytosis. *Biochemical and Biophysical Research Communications* **312**(2)**:** 473-479

Schulz-Schaeffer WJ, Tschöke S, Kranefuss N, Dröse W, Hause-Reitner D, Giese A, Groschup MH, Kretzschmar HA (2000) The paraffin-embedded tissue blot detects PrP^{Sc} early in the incubation time in prion diseases *American Journal of Pathology* **156**(1)**:** 51-56

Schwartz JL, Weichselbaum R, Frim SR (1983) The effect of aging on the density and distribution of oral mucosal langerhans cells. *Experimental Gerontology* **18**(1)**:** 65-71

Scott JR, Fraser H (1984) Degenerative hippocampal pathology in mice infected with scrapie. *Acta Neuropathologica* **65**(1)**:** 62-68

Selvaggini C, Degioia L, Cantu L, Ghibaudi E, Diomede L, Passerini F, Forloni G, Bugiani O, Tagliavini F, Salmona M (1993) Molecular characteristics of a protease-resistant, amyloidogenic and neurotoxicpeptide homologous to residues 106-126 of the prion protein. *Biochemical and Biophysical Research Communications* **194**(3)**:** 1380-1386

Shurin MR, Shurin GV, Chatta GS (2007) Aging and the dendritic cell system: Implications for cancer. *Critical Reviews in Oncology/Hematology* **64**(2)**:** 90-105

Sigurdson CJ, Barillas-Mury C, Miller MW, Oesch B, Keulen LJMv, Langeveld JPM, Hoover EA (2002) PrPCWD lymphoid cell targets in early and advanced chronic wasting disease of mule deer. *Journal of General Virology* **83**(10)**:** 2617-2628

Sigurdson CJ, Williams ES, Miller MW, Spraker TR, O'Rourke KI, Hoover EA (1999) Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (Odocoileus hemionus). *Journal of General Virology* **80**(10)**:** 2757-2764

Sigurdsson B, Pálsson' PA, Grímsson H (1957) Visna, a demyelinating transmissible disease of sheep. *Journal of Neuropathology* **39:** 519-528

Sigurdsson EM, Brown DR, Daniels M, Kascsak RJ, Kascsak R, Carp R, Meeker HC, Frangione B, Wisniewski T (2002) Immunization Delays the Onset of Prion Disease in Mice. *American Journal of Pathology* **161**(1)**:** 13-17

Somerville RA, Dunn AJ (1996) The sssociation Between PrP and infectivity in scrapie and BSE infected mouse brain. *Archives Of Virology* **141**(2)**:** 275-289

Somerville RA, Hamilton S, Fernie K (2005) Transmissible spongiform encephalopathy strain, PrP genotype and brain region all affect the degree of glycosylation of PrP^{Sc}. *Journal of General Virology* **86**(1)**:** 241-246

Somerville RA, Merz PA, Carp RI (1986) Partial copurification of scrapie-associated fibrils and scrapie infectivity. *Intervirology* **25**(1)**:** 48-55

Stahl N, Borchelt DR, Hsiao K, Prusiner SB (1987) Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell* **51**(2)**:** 229-240

St Rose SG, Hunter N, Matthews L, Foster JD, Chase-Topping ME, Kruuk, LEB, Shaw, DJ, Rhind, SM, Will RJ and Woolhouse M (2006). Comparative evidence for a link between Peyer's patch development and susceptibility to transmissible spongiform encephalopathies. *BMC Infectious Diseases* **6**, 5

Sukumar S, Shikh M, Tew J, Szakal A (2008) Ultrastructural study of highly enriched follicular dendritic cells reveals their morphology and the periodicity of immune complex binding. *Cell and Tissue Research* **332:** 89-99

Sukumar S, Szakal AK, Tew JG (2006) Isolation of functionally active murine follicular dendritic cells. *Journal of Immunological Methods* **313**(1-2)**:** 81-95

Szakal AK, Aydar Y, Balogh P, Tew JG (2002) Molecular interactions of FDCs with B cells in aging. *Seminars in Immunology* **14**(4)**:** 267-274

Szakal AK, Kosco MH, Tew JG (1988) A novel in vivo follicular dendritic cell-dependent iccosome-mediated mechanism for delivery of antigen to antigen-processing cells. . *Journal of Immunology* **140:** 341-353

Taylor DM (2000) Inactivation of Transmissible Degenerative Encephalopathy Agents: A Review. *The Veterinary Journal* **159**(1)**:** 10-17

Taylor DM, McConnell I, Fraser H (1996) Scrapie infection can be established through skin scarification in immunocompetent but not immunodeficient mice. *Journal of General Virology* **77:** 1595-1599

Taylor PR, Pickering MC, Kosco-Vilbois MH, Walport MJ, Botto M, Gordon S, Martinez-Pomares L (2002) The follicular dendritic cell restricted epitope, FDC-M2, is complement C4; localization of immune complexes in mouse tissues. *European Journal of Immunology* **32**(7)**:** 1883-1896

Tew JG, Kosco MH, Burton GF, Szakal AK (1990) Follicular dendritic cells as accessory cells. *Immunological Reviews* **117**(1)**:** 185-211

Thirumalai D, Klimov DK, Dima RI (2003) Emerging ideas on the molecular basis of protein and peptide aggregation. *Current Opinion in Structural Biology* **13**(2)**:** 146-159

Tkachuk M, Bolliger S, Ryffel B, Pluschke G, Banks TA, Herren S, Gisler RH, Kosco-Vilbois MH (1998) Crucial role of tumor necrosis factor receptor 1 expression on non-hematopoietic cells for B cell localization within the splenic white pulp. *Journal of Experimental Medicine* **187**(4)**:** 469-477

Tobler I, Gaus SE, Deboer T, Achermann P, Fischer M, Rulicke T, Moser M, Oesch B, McBride PA, Manson JC (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature* **380**(6575)**:** 639-642

Todorova-Balvay D, Simon S, Créminon C, Grassi J, Srikrishnan T, Vijayalakshmi MA (2005) Copper binding to prion octarepeat peptides, a combined metal chelate affinity and immunochemical approaches. *Journal of Chromatography B* **818**(1)**:** 75-82

Tompkins MB, Tompkins WA (2008) Lentivirus-induced immune dysregulation. *Veterinary Immunology and Immunopathology* **123**(1-2)**:** 45-55

Traggiai E, Chicha L, Mazzucchelli L, Bronz L, Piffaretti J-C, Lanzavecchia A, Manz MG (2004) Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* **304**(5667)**:** 104-107

Tranulis MA (2002) Influence of the prion protein gene, Prnp, on scrapie susceptibility in sheep. *APMIS* **110**(1)**:** 33-43

Tumanov AV, Kuprash DV, Nedospasov SA (2003) The role of lymphotoxin in development and maintenance of secondary lymphoid tissues. *Cytokine & Growth Factor Reviews* **14**(3-4)**:** 275-288

Valleron A-J, Boelle P-Y, Will R, Cesbron J-Y (2001) Estimation of epidemic size and incubation time based on age characteristics of vCJD in the United Kingdom. *Science* **294**(5547)**:** 1726-1728

van Keulen LJ, Schreuder BE, Meloen RH, Mooij-Harkes G, Vromans ME, Langeveld JP (1996) Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. *Journal of Clinical Microbiology* **34**(5)**:** 1228-1231

Vasto S, Colonna-Romano G, Larbi A, Wikby A, Caruso C, Pawelec G (2007) Role of persistent CMV infection in configuring T cell immunity in the elderly. *Immunity & Ageing* **4**(1)**:** 2

Victoratos P, Lagnel J, Tzima S, Alimzhanov MB, Rajewsky K, Pasparakis M, Kollias G (2006) FDC-Specific Functions of p55TNFR and IKK2 in the Development of FDC Networks and of Antibody Responses. *Immunity* **24**(1)**:** 65-77

Videm V, Albrigtsen M (2008) Soluble ICAM-1 and VCAM-1 as Markers of Endothelial Activation. *Scandinavian Journal of Immunology* **67**(5)**:** 523-531

Viles JH, Cohen FE, Prusiner SB, Goodin DB, Wright PE, Dyson HJ (1999) Copper binding to the prion protein: Structural implications of four identical cooperative binding sites. *Proceedings of the National Academy of Sciences* **96**(5)**:** 2042-2047

Weissmann C, Flechsig E (2003) PrP knock-out and PrP transgenic mice in prion research. *Br Med Bull* **66**(1)**:** 43-60

Wells GAH, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, Dawson M, Bradley R (1987) A novel progressive spongiform encephalopathy in cattle. *Veterinary Record* **121**(18)**:** 419-420

Wilesmith JW (1993) BSE: Epidemiological approaches, trials and tribulations. *Preventive Veterinary Medicine* **18**(1)**:** 33-42

Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, P.G.Smith (1996) A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* **347:** 921-925

Williams AE, Ryder S, Blakemore WF (1995) Monocyte recruitment into the scrapie-affected brain. *Acta Neuropathologica* **90**(2)**:** 164-169

Williams ES, Young S (1980) Chronic wasting disease of captive mule deer: a spongiform encephalopathy. *Journal of Wildlife Diseases* **16**(1)**:** 89-98

Williams GM, Nossal GJV (1966) Ontogeny of the immune response: The development of the follicular antugen-trapping mechanism. *Journal of Experimental Medicine* **124**(1)**:** 47-56

Wilson K, Code K, Ricketts MN (2000) Risk of acquiring Creutzfeldt-Jakob disease from blood transfusions: systematic review of case-control studies *British Medical Journal* **321**(7252)**:** 17-19

Wroe SJ, Pal S, Siddique D, Hyare H, Macfarlane R, Joiner S, Linehan JM, Brandner S, Wadsworth JDF, Hewitt PE, Collinge J (2006) Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. *Lancet* **368**(9552)**:** 2061-2067

Wu B, Huang C, Kato-Maeda M, Hopewell PC, Daley CL, Krensky AM, Clayberger C (2008) IL-9 is associated with an impaired Th1 immune response in patients with tuberculosis. *Clinical Immunology* **126**(2)**:** 202-210

Yamakawa K, Hagiwara K, Nohtomi Y, Nakamura M, Nishijima Y, Higuchi Y, Sato T, Sata T (2003) Atypical proteinase K-resistant prion protein (PrPres) observed in an apparently healthy 23-month-old Holstein steer. *Journal of Infectious Disease* **56:** 221-222

Yokoyama T, Momotani E, Kimura K, Yuasa N (1996) Immunoreactivity of specific epitopes of PrP^{Sc} is enhanced by pretreatment in a hydrated autoclave. *Clinical and Diagnostic Laboratory Immunology* **3**(4)**:** 470-471

Zhang Z-Q, Schleif WA, Casimiro DR, Handt L, Chen M, Davies M-E, Liang X, Fu T-M, Tang A, Wilson KA, McElhaugh M, Carella A, Tan C, Connolly B, Hill S, Klein H, Emini EA, Shiver JW (2004) The impact of early immune destruction on the kinetics of postacute viral replication in rhesus monkeys infected with the simian-human immunodeficiency virus. *Virology* **320**(1)**:** 75-84

Zigas V, Gadusek DC (1957) Kuru: clinical study of a new syndrome resembling paralysis agitans in natives of the Eastern Highlands of Australian New Guinea. *Medical Journal of Australia* **2:** 245-254

Zlotnik I, Rennie JC (1965) Experimental transmission of mouse passaged scrapie to goats, sheep, rats and hamsters. *Journal of Comparative Pathology* **75**(2)**:** 147-156

Appendix I. Generation of SCID-P*rnp*$^{+/+}$ and SCID-*Prnp*$^{-/-}$ mouse lines

---

Step 1

SCID x PrP$^{-/-}$ F2 generation (224 animals)

Selection of SCID phenotype by ELISA

Ig producing	**SCID phenotype**
189	**36**

Step 2 Selection of H-2 type by flow cytometry(n=23 due to death/exclusion)

H-2d	H-2bd	**H-2b**
1	10	**11**

Step 3 *Selection of PrP$^{+/+}$ or PrP$^{-/-}$ genotype using southern blot analysis**

PrP$^{+/+}$ 2 (animals 130b and 134b*) > SCID-P*rnp*$^{+/+}$ (SCID-PrP$^{+/+}$) line

PrP$^{-/-}$ 2 (animals 91b and 158b) > SCID-*Prnp*$^{-/-}$ (SCID-PrP$^{-/-}$) line

Step 4: Lines established and used experimentally at the 7th inbred generation.

** animal 134b was a heterozygous phenotype*

**from original production of 224 animals two pairs of each genotype selected for each line*

Appendix II. List of primary antibodies used in this thesis

List of primary antibodies

Name	**Specificity**	**Antibody type**	**Reference or commercial source**
6H4	PrP 27-30	Mouse monoclonal	Prionics, Switzerland.
1B3	Normal and disease specific forms of the PrP protein	Rabbit Polyclonal	(Farquhar *et al.*, 1989) produced at the Neuropathogenesis unit
FDC-M1	MFG-E8	Rat monoclonal	Pharmingen, BD Biosciences, UK. (Kranich *et al.*, 2008)
FDC-M2	Complement component C4	Rat monoclonal	ImmunoKontact, UK. (Taylor *et al.*, 2002)
Tyrosine Hydroxylase	Noradrenergic sympathetic innervation	Rabbit polyclonal	Chemicon, UK
CR2/CR1 (clone 7G6).	CD21/CD35 Complement receptors 1 and 2	Rat monoclonal	Pharmingen, BD Biosciences, UK.
Fcγ II/III Receptor (clone 2.4G2)	CD16/CD32	Rat monoclonal	Pharmingen,BD Biosciences, UK.
$H\text{-}2D^{d}$ PE	$H\text{-}2D^{d}$ Clone 34-2-12	mouse monoclonal	Pharmingen, BD Biosciences, UK.
$H\text{-}2D^{b}$ FITC	$H\text{-}2D^{b}$ Clone KH95	mouse monoclonal	Pharmingen, BD Biosciences, UK.

References.

Farquhar, C. F., Somerville, R. A. & Ritchie, L. A. (1989). Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *Journal of Virological Methods* 24, 215-221.

Kranich, J., Krautler, N. J., Heinen, E., Polymenidou, M., Bridel, C., Schildknecht, A., Huber, C., Kosco-Vilbois, M. H., Zinkernagel, R., Miele, G. & Aguzzi, A. (2008). Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8. *Journal of Experimental Medicine*, jem.20071019.

Taylor, P. R., Pickering, M. C., Kosco-Vilbois, M. H., Walport, M. J., Botto, M., Gordon, S. & Martinez-Pomares, L. (2002). The follicular dendritic cell restricted epitope, FDC-M2, is complement C4; localization of immune

complexes in mouse tissues. *European Journal of Immunology* 32, 1883-1896.

Appendix III: Analysis of bioassay data from Chapter 6 figure 6.11

Bioassay results (incubation periods) were available for samples from three mice for each of four treatment combinations. In the original study animals had received either saline (negative control) or DNP as either a primary or secondary immunisation.

A linear mixed model was fitted to the data with treatment (saline or DNP) and immunisation (primary or secondary) included as fixed effects, and mouse sample as a random effect. The parameters of the model were estimated using the REML directive in Genstat 10th edition.

```
  77  VCOMPONENTS [FIXED=Treatment*Immunisation; FACTORIAL=9] RANDOM=Sample;
INITIAL=1; CONSTRAINTS=none
  78  REML [PRINT=model,components,waldTests; PSE=differences; FMETHOD=automatic;
MVINCLUDE=*;\
  79    METHOD=AI; MAXCYCLE=20] Incubation
```

REML variance components analysis

Response variate:	Incubation
Fixed model:	Constant + Treatment + Immunisation + Treatment.Immunisation
Random model:	Sample
Number of units:	132

Estimated variance components

Random term	component	s.e.
Sample	51.4	32.4

Residual variance model

Term	Factor	Model(order)	Parameter	Estimate	s.e.
Residual		Identity	Sigma2	145.6	18.8

Tests for fixed effects

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment	0.82	1	0.82	8.0	0.391
Immunisation	2.39	1	2.39	8.0	0.161
Treatment.Immunisation	2.55	1	2.55	8.0	0.149

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment.Immunisation	2.55	1	2.55	8.0	0.149

Table of predicted means for Constant

206.5 Standard error: 2.32

Table of predicted means for Treatment

Treatment	DNP	SAL
	204.4	208.6

Standard errors

Treatment	DNP	SAL
	3.3	3.3

Table of predicted means for Immunisation

Immunisation	1	2
	210.1	202.9

Standard errors

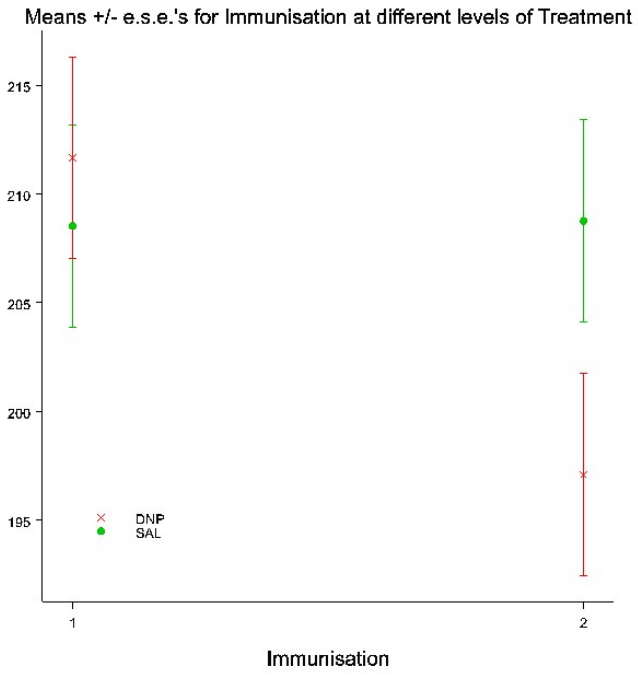
Immunisation	1	2
	3.3	3.3

Table of predicted means for Treatment.Immunisation

Immunisation	1	2
Treatment		
DNP	211.7	197.1
SAL	208.5	208.8

Standard errors

Immunisation	1	2
Treatment		
DNP	4.6	4.7
SAL	4.6	4.6



The estimated mean incubation period in the DNP secondary immunisation group is lower than in the saline group (197.1 ± 4.7 compared to 208.8 ± 4.6) but this difference is not significant at the 5% level.