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Determining the role of follicular dendritic cells in TSE agent neuroinvasion

Laura McCulloch

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, The Roslin Institute and R (D) SVS, University of Edinburgh

March 2011
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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 Dr Neil Mabbott and Professor John Hopkins. No part of this work has been, or will be submitted for any other degree, or professional qualification.

Laura McCulloch

March 2011
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# Abbreviations

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<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex method</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
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<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob disease</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>CR2</td>
<td>Complement receptor 2</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CWD</td>
<td>Chronic wasting disease</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>d</td>
<td>Days</td>
</tr>
<tr>
<td>d.p.i</td>
<td>Days post injection</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-assisted cell sorting</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>FFI</td>
<td>Fatal familial insomnia</td>
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<td>FSE</td>
<td>Feline spongiform encephalopathy</td>
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<td>GSS</td>
<td>Gerstmann Sträussler-Scheinker disease</td>
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<tr>
<td>h</td>
<td>Hours</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>ic</td>
<td>Intracerebral</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>ILF</td>
<td>Isolated lymphoid follicle</td>
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<td>Inguinal lymph node</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
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<tr>
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<td>Immunoglobulin</td>
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<td>Kbp</td>
<td>Kilobase pairs</td>
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<td>KiloDaltons</td>
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<tr>
<td>LoxP</td>
<td>Locus of crossover P</td>
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<tr>
<td>LT</td>
<td>Lymphotoxin</td>
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<tr>
<td>LtβR-Ig</td>
<td>Lymphotoxin β receptor-human immunoglobulin fusion protein</td>
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<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MBM</td>
<td>Mammalian meat and bone meal</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<td>PAb</td>
<td>Polyclonal antibody</td>
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<tr>
<td>PAP</td>
<td>Peroxidase-anti-peroxidase</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PET</td>
<td>Paraffin-embedded tissue</td>
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<td>PK</td>
<td>Proteinase K</td>
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<td>Prnp</td>
<td>Murine PrP gene</td>
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<td>PRNP</td>
<td>Human PrP gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Normal form of the host PrP protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Disease-associated PrP protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>Scrapie-specific form of the host PrP protein</td>
</tr>
<tr>
<td>sCJD</td>
<td>Sporadic CJD</td>
</tr>
<tr>
<td>SCID</td>
<td>Severely combined immunodeficient</td>
</tr>
<tr>
<td>SCS</td>
<td>Sub-capsular sinus</td>
</tr>
<tr>
<td>SRM</td>
<td>Specified risk material</td>
</tr>
<tr>
<td>TBM&lt;sub&gt;s&lt;/sub&gt;</td>
<td>Tingible body macrophages</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible spongiform encephalopathy</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cellular adhesion molecule-1</td>
</tr>
<tr>
<td>vCJD</td>
<td>Variant CJD</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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Abstract

Transmissible spongiform encephalopathies (TSEs), such as scrapie and variant Creutzfeldt-Jakob disease are infectious, fatal, neurodegenerative diseases. Following peripheral infection TSE agents usually accumulate in lymphoid tissues before spreading to the central nervous system. In mice, follicular dendritic cells (FDCs) expressing the host prion protein (PrP<sup>C</sup>) are essential for scrapie agent accumulation in lymphoid tissues. The accumulation of the scrapie agent on FDCs is critical for the efficient spread of infection to the brain. However, it is unknown whether FDCs themselves actively replicate the scrapie agent, or simply accumulate it following production by other cell types such as neurones, lymphocytes or other stromal cell populations. To definitively address this issue a transgenic mouse model was created in which PrP<sup>C</sup> is switched on or off exclusively on FDCs.

Expression of cre-recombinase (Cre) under the action of cell-specific gene promoters can be used to induce or delete the expression of a target gene in specific cell populations. In this model, Cre expression is driven by the complement receptor type 2 gene (Cr2/CD21) which is expressed by FDCs and mature B lymphocytes. Characterisation of the CD21-cre mouse line was achieved by crossing with a ROSA26 reporter strain. The CD21-cre mouse line was subsequently crossed with floxed-PrP mouse lines to produce compound transgenic mouse lines in which PrP<sup>C</sup> expression was switched on or off, only in FDCs. Cre expression by B lymphocytes was eliminated by γ-irradiation and grafting recipient mice with Cre-deficient bone marrow. Immunohistochemical analysis confirmed the expression PrP<sup>C</sup> had been switched on or off exclusively on FDCs. Subsequently, the mice were challenged with scrapie by intra-peritoneal injection to determine the precise role of FDCs in the accumulation of scrapie in lymphoid tissues.

Switching off PrP<sup>C</sup> expression exclusively on FDCs prevented the accumulation of TSE agent specific disease-associated PrP<sup>Sc</sup> in the spleen after i.p inoculation. Conversely, in mice in which PrP<sup>C</sup> was expressed only on FDC, successful replication of the agent occurred on the FDC network in the spleen. Taken together, these data show PrP<sup>C</sup>-expressing FDCs alone are sufficient to support the accumulation of the scrapie agent within lymphoid tissues. Furthermore, these data suggest FDC replicate the TSE agent and do not simply accumulate it following synthesis by other cell types.
CHAPTER 1

Introduction

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1.5 Thesis aims

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1.6.1 Cre-LoxP model
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1. Introduction

1.1 The transmissible spongiform encephalopathies (TSEs)

1.1.1 TSE diseases

The Transmissible spongiform encephalopathies (TSEs) are fatal diseases of the central nervous system (CNS) which affect various mammalian species, including humans (Table 1.1). These include Creutzfeldt-Jakob disease (CJD) and kuru in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle. TSE diseases have a varied aetiology and can be sporadic, familial or acquired. These diseases are characterised by typical pathology in the CNS including spongiform change or vacuolation (Dickinson, Meikle et al. 1968), activation of glial cells, and abnormal accumulation of a host protein, in the brains of TSE-affected animals (Bolton, McKinley et al. 1982; Bruce, McBride et al. 1989). TSE diseases are difficult to study as various factors can impact on pathogenesis including host genotype (Dickinson, Meikle et al. 1968; Bruce, McConnell et al. 1991), route of inoculation (Eklund, Kennedy et al. 1967; Kimberlin and Walker 1979) and the strain of TSE used (Bruce and Fraser 1991). Additionally, in experimental studies the pathogenesis of TSE disease can exceed the lifespan of the infected animal which can complicate interpretation of incubation period data (Dickinson, Fraser et al. 1975). These barriers contribute to the many unknowns still present in TSE research including that the nature of the TSE agent itself, which is still as of yet not fully understood.
Scrapie is the earliest known TSE and affects sheep and goats and most of the knowledge we have of TSE disease has come from experimental transmission of scrapie into mice. In sheep, the disease is characterised by scratching and nibbling at skin leading to severe wool loss and skin damage, uncoordination and recumbancy and results in severe weight loss and inevitably death. Scrapie is a centuries old disease, with the first traceable mention of the disease in a German publication from 1750 (Leopoldt 1750; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). Scrapie has so far been found in twelve EU member states including Britain, France and Germany, 3 candidate member states and Canada, US, Brazil and Japan. However two of the largest sheep producing countries of the world- Australia and New Zealand- remain scrapie-free.

Scrapie has been known to be infectious since its earliest traceable publication however the mechanisms of transmission and the nature of the infectious agent are still not fully understood to this day (Leopoldt 1750; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). The first definitive experiments to show scrapie transmission were carried out by Cuillé and Chelle in 1936. Animals were observed for 18 months to ensure they were scrapie-free before intraocular, subcutaneous, epidural or intracranial (ic) inoculation with brain and spinal cord homogenates from affected animals. They successfully transmitted scrapie to some, but not all of the animals inoculated, with incubation times between one and two years depending on the route of inoculation (Cuillé and Chelle 1936; Cuillé and Chelle 1938; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). Some infected homogenates were
passed through a bacterial exclusion filter prior to inoculation. These innocula successfully transmitted disease showing that the agent was not microbial (Cuillé and Chelle 1938; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). These experiments demonstrated for the first time that scrapie was transmissible by various routes, the disease has a long incubation period and showed differences in susceptibility between sheep. Transmission between species was also achieved by experimental inoculation of goats with sheep brain and spinal cord homogenates (Cuillé and Chelle 1939; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). Furthermore, accidental transmission of scrapie occurred via a new louping-ill vaccine prepared from brain, spinal cord and spleen homogenates of virus-infected sheep, attenuated with 0.35% formalin. This accidental transmission demonstrated that the spleen was also a source of scrapie-infected material and that the scrapie agent was resistant to treatment with formalin (Gordon 1946). The route of transmission of natural scrapie is still not fully understood. However it is known that disease can occur via both horizontal transmission, i.e. from sheep to sheep (Brotherston, Renwick et al. 1968), and lateral transmission, i.e. from ewe to lamb (Foster, Hunter et al. 1996; Foster, McKenzie et al. 2006).

Resistance to scrapie was known to be hereditary by the end of the 18th (Comber 1772) and beginning of the 19th (Thaer 1821; von Richthofen 1827; Schneider, Fangerau et al. 2008a) centuries, however effective genetic breeding selection methods in sheep were only applied recently by the European Commission when the genetics of scrapie were understood more fully (regulation 2001; regulation 2004; regulation 2007). In the 1960’s, studies by Dickinson et al looking at ME7 scrapie incubation times in inbred mouse strains led to the discovery of the involvement of
the scrapie incubation period or “Sinc” gene in scrapie pathogenesis (Dickinson and Mackay 1964; Dickinson, Meikle et al. 1968). This group were also carrying out studies using the scrapie isolate SSBP/1 in sheep and concluded that the resistance of some sheep to SSBP/1 scrapie must be due to a single gene with a fully dominant allele for susceptibility (Dickinson, Stamp et al. 1968). In the 1980’s, a 33-35 KDa protein, named the prion protein or PrP, was discovered to be the main component of scrapie-associated fibrils (SAF; (Oesch, Westaway et al. 1985; Hope, Morton et al. 1986). Subsequently, it was discovered that the Sinc gene encoded the prion protein (PrP) and confirmed the involvement of the normal cellular host prion protein (PrP<sup>C</sup>) in TSE pathogenesis (Hunter, Hope et al. 1987). This discovery led to many studies of PrP sequence polymorphisms and their relevance to scrapie incubation time as well as much research into the role of PrP<sup>C</sup> in health and disease.

1.1.3 Bovine spongiform encephalopathy

Bovine spongiform encephalopathy (BSE) is hypothesised to have emerged after cattle were fed on a diet containing mammalian meat and bone meal (MBM) derived from the rendered carcasses of scrapie-infected sheep (Wilesmith, Ryan et al. 1991; Anderson, Donnelly et al. 1996). Cattle were not considered to be natural hosts of TSE disease however, the first outbreak of BSE was discovered in the 1980’s (Wells, Scott et al. 1987). The development of BSE in cattle led to many subsequent cross species transmissions of TSE disease. Many animal feed products were contaminated with cattle-derived products containing infective material, which led to the development of feline spongiform encephalopathy (FSE) in domestic and exotic cats (Wells, Scott et al. 1987; Wyatt, Pearson et al. 1991), and novel TSE disease in other
exotic animals including nyala, kudu and oryx (Wells, Scott et al. 1987; Jeffrey and Wells 1988; Kirkwood, Wells et al. 1990). This led to the mass culls of many infected cattle and the enforcement of new laws preventing the inclusion of MBM in cattle feed, the prevention of specified risk material (SRM) entering the food chain, the ban of mechanically recovered meat and meat from cattle over 30 months of age entering the food chain (regulation 2001; regulation 2004; regulation 2007).

1.1.4 Creutzfeldt-Jakob disease (CJD) and human TSEs

Human TSEs can be inherited, sporadic or iatrogenic. Genetic TSEs account for around 10% of human prion diseases. These include Gerstmann Sträussler-Scheinker disease (GSS), familial Creutzfeldt-Jakob disease (fCJD) and fatal familial insomnia (FFI) which are all associated with inherited mutations in the prion protein gene PRNP (Creutzfeldt 1920; Jakob 1921a; Jakob 1921b; Gerstmann, Straussler et al. 1936; Hsiao, Baker et al. 1989; Kretzschmar, Honold et al. 1991; Goldfarb, Petersen et al. 1992; Medori, Tritschler et al. 1992).

Sporadic CJD accounts for at least 85% of all human TSEs and occurs by as of yet unknown mechanisms, which lead to abnormal conformations of the normal host protein PrP\textsuperscript{C}. Possible hypotheses for the onset of sporadic TSE include acquired mutations of the PRNP gene or abnormal cellular metabolism of PrP\textsuperscript{C} which subsequently leads to conversion to the disease associated conformation (Westaway, DeArmond et al. 1994).
Iatrogenic, or peripherally acquired, human TSE diseases include kuru and variant CJD (vCJD). Kuru was discovered in 1957 and was transmitted during cannibalistic rituals of the natives of the Eastern Highlands of Papa-New Guniea (Zigas and Gadusek 1957). Kuru was restricted to this geographical location and was all but eradicated by raising awareness to prevent disease transmission. vCJD was first identified shortly after the BSE outbreak and most likely occurred via the consumption of BSE contaminated meat (Will, Ironside et al. 1996; Bruce, Will et al. 1997). As of the 9th March 2011, 171 deaths from vCJD have been reported in the U.K. with 4 patients currently living with probable vCJD (http://www.cjd.ed.ac.uk/figures.htm). Furthermore, the more recent discovery of transmission of vCJD via contaminated blood products, along with the lack of a reliable ante-mortem diagnostic test or cure, has determined that these peripherally acquired TSEs remain a current public health issue (Llewelyn, Hewitt et al. 2004; Peden, Head et al. 2004; Wroe, Pal et al. 2006).

1.2 The TSE agent and TSE pathogenesis

1.2.1 The nature of the TSE agent

The exact nature of the TSE agent is yet to be determined and has been a source of controversy and debate. TSE diseases were originally thought to be caused by a virus due to the transmission of disease and the occurrence of multiple strains of each TSE. Due to their long incubation periods TSEs were considered to be a slow-virus, reproducing much more slowly than typical virus strains. However, a TSE-associated virus could not be detected by electron microscopy. Supporters of the virus
hypothesis suggested it may be extremely small, similar to some very small plant viruses such as Tobacco mosaic virus (Diringer, Gelderblom et al. 1983; Van Everbroeck, Pals et al. 2002).

This belief developed into the virino hypothesis, which stated that a very small nucleic acid, or some other information encoding molecule, is a component of the TSE agent (Dickinson and Outram 1988; Farquhar, Somerville et al. 1998). In support of this theory, a recently published paper claims to have found a 25 nm virion as the cause of TSE diseases (Manuelidis 2007) with the presence of tubovesicular structures found in TSE-affected brains subsequently associated with the presence of this virion (Liberski, Sikorska et al. 2008). As of yet, TSE disease specific nucleic acids have not been identified (Alper, Haig et al. 1966; Brown, Rohwer et al. 1986; Taylor 1993; Somerville, Birkett et al. 1997). However the existence of defined strains of TSE agents which are reproducible over multiple passages in mice has yet to be fully explained (Bruce and Fraser 1991; Bruce 1993).

In 1982, Stanley Prusiner published a paper stating that the TSE agent that caused scrapie contained no genetic material and consisted entirely of protein (Prusiner 1982). This conclusion was reached as the scrapie agent appeared to be resistant to inactivation by treatments that destroy nucleic acids such as ultra-violet light, ionising radiation, dry heat and nucleases (Alper, Haig et al. 1966; Prusiner 1982; Fraser 1987; Taylor 1993; Somerville, Birkett et al. 1997; Taylor, Fernie et al. 1998). Instead, proteinases, which destroy proteins, could either abolish or reduce infectious properties of scrapie (Prusiner 1982). Prusiner suggested that the infectious agent was the proteinaceous infectious particle, or prion, a novel type of protein-only pathogen.
that lacks nucleic acid (Prusiner 1982). Fractions of scrapie-infected hamster brain were purified leading to the identification of a protein, termed PrP (Prion Protein), that was directly proportional to the titre of infectivity and was resistant to digestion by proteinase K (PK; (Bolton 1982; McKinley, Bolton et al. 1983). By testing for mRNA to PrP, it was found that PrP was a normal host protein (PrP\(^{C}\); (Chesebro, Race et al. 1985) and that an abnormal form of this protein, PrP\(^{Sc}\) conferred disease (Meyer, McKinley et al. 1986). It was hypothesised that in prion pathogenesis, abnormal PrP\(^{Sc}\) can act as a template for converting normal PrP\(^{C}\) to the pathological variant (Rogers, Yehiel et al. 1993). Additionally, the recent \textit{de novo} creation of infectious PrP\(^{Sc}\) \textit{in vitro} seems to conclusively show that the TSE infectious agent is composed solely of protein (Legname, Baskakov et al. 2004; Barria, Mukherjee et al. 2009). The prion hypothesis can account for why no disease specific genetic material has thus far been identified and why viral inactivation treatments do not affect disease pathogenesis. The presence of TSE strains is a major factor in favour of the virino hypothesis, however it was proposed that TSE strain variation could be due to different conformations of PrP\(^{Sc}\) or differences in glycosylation of either the host PrP\(^{C}\) or the infecting PrP\(^{Sc}\) (Prusiner 1991; Ermonval, Mouillet-Richard et al. 2003). However, TSE infectivity has been shown in some models without the presence of PrP\(^{Sc}\) (Lasmézas, Deslys et al. 1997; Barron and Manson 2003; Barron, Campbell et al. 2007). Therefore, although there is compelling evidence for the prion hypothesis, clarification is required for some remaining phenomena of TSE pathogenesis.

1.2.2 The prion protein

Although the exact nature of the TSE agent is still controversial, PrP\(^{C}\) plays an important role in disease pathogenesis as PrP\(^{+/}\) mice are resistant to TSE disease
PrP C is a host encoded sialoglycoprotein expressed predominantly on neurons but also on many other cell types, including cells of the immune system (Kretzschmar, Prusiner et al. 1986; Caughey 1988). PrP C is encoded by a single gene in rodents (Prnp), ruminants (PrP or Prnp) and man (PRNP) (Oesch, Westaway et al. 1985; Westaway and Prusiner 1986). The mouse gene has three exons and two introns, with exon 3 containing the entire protein coding region (Prusiner, Scott et al. 1998). The primary translation product in mouse has 254 amino acid (aa) residues, during posttranslational maturation the protein is processed to a length of approx. 210 aa (Hegde, Mastrianni et al. 1998a; Hegde, Voigt et al. 1998b) forming a flexible, unstructured N-terminal domain and a globular, highly structured C-terminal domain (Fig 1.1).

After transcription and translation, the NH2-terminal signal peptide targets the protein to the endoplasmic reticulum (ER). This signal peptide is subsequently cleaved into the ER lumen (Hegde, Mastrianni et al. 1998a; Hegde, Voigt et al. 1998b). After the attachment of a glycosylphosphatidylinositol (GPI) anchor, PrP C protein is further modified through complex carbohydrates as it is transported along the ER-Golgi towards the plasma membrane at the cell surface. The PrP C protein matures as it is transported along the ER-Golgi plasma membrane pathway from an immature form, which is sensitive to endoglycosidase H, to a mature form which is resistant to endoglycosidase H (Sarnataro, Campana et al. 2004). PrP C becomes associated with lipid rafts in the ER and studies have shown that this is essential for the correct folding and glycosylation of PrP C. Furthermore, association with rafts allows PrP C to be exported from the ER to the Golgi. (Sarnataro, Campana et al. 2004; Campana, Sarnataro et al. 2005).
Figure 1.1 Structure of the mouse Prnp gene and PrpC protein

a. Outline of the structure of the mouse Prnp gene. Prnp contains three exons and two introns. Exon 3 contains the open reading frame (ORF) which codes for the entire PrpC protein.

b. Outline of the structure of PrpC protein adapted from (Linden, Martins et al. 2008). The protein consists of a number of domains with specific functional attributes. It contains and N-terminal signalling peptide (SP) from aa 1-22 which targets the protein to the endoplasmic reticulum. The octapeptide repeat region (OR) is between aa 51-91 and is thought to have a role in copper binding (Hornshaw, McDermott et al. 1995; Jackson, Murray et al. 2001; Cerpa, Varela-Nallar et al. 2005). PrpC contains both a charged cluster (CC) and a hydrophobic core (HC). PrpC has two asparagine residues which are modified by N-linked glycans (CHO) at positions 180 and 196 and this results in un-, mono- and di-glycosylated isoforms (Stimson, Hope et al. 1999). PrpC also has a glycosyl phosphatidylinositol (GPI) anchor signalling peptide which allows the addition of a GPI anchor within the ER (Stahl, Borchelt et al. 1987).
PrP$^C$ has a short half-life and is recycled via endosomes or degraded in lysosomes. As part of normal processing, PrP$^C$ can be proteolytically cleaved into to fragments, with the N-terminal fragment secreted and the C-terminal fragment membrane-bound.

Expression levels of Prnp from microarray data from various mouse tissues and cell lines can be seen in Fig 1.2. The normal cellular function of PrP$^C$ is uncertain and three distinct lines of PrP$^{-/-}$ mice show normal development and have no overt neurological phenotype (Bueler, Fischer et al. 1992; Manson, Clarke et al. 1994a; Prusiner, Scott et al. 1998; Lasmézas 2003). This suggests that either PrP$^C$ is not an essential protein or that the genetic loss of PrP$^C$ expression can be compensated for by other factors. Some proposed functions of PrP$^C$ include the maintenance of circadian rhythm (Tobler, Gaus et al. 1996), synaptic transmission (Collinge, Whittington et al. 1994; Colling, Collinge et al. 1996; Mallucci, Ratte et al. 2002) anxiety modulation (Nico, de-Paris et al. 2005), cognition (Coitinho, Roesler et al. 2003) and seizure thresholds (Walz, Amaral et al. 1999) as small changes in these functions have been observed in the PrP$^{-/-}$ lines.

PrP$^C$ has the ability to bind copper molecules through its sequence of octapeptide repeats and has therefore also been implicated in copper metabolism (Hornshaw, McDermott et al. 1995; Jackson, Murray et al. 2001; Lasmézas 2003; Cerpa, Varela-Nallar et al. 2005). More recent work claims that binding of copper ions may promote protein aggregation and consequently may be involved in pathogenesis (Shiraishi 2006). Additionally, PrP$^C$ has been named as a signal transduction protein (Mouillet-Richard, Ermonval et al. 2000; Spielhaupter and Schätzl 2001), and has been suggested to have roles in both pro-apoptotic signalling via an associated
increase in caspase 3 activity (Paitel, Alves da Costa et al. 2002) and anti-apoptotic via binding to the anti-apoptotic molecule Bcl-2 (Kurschner and Morgan 1995; Kuwahara, Takeuchi et al. 1999; Bounhar, Zhang et al. 2001; Chiarini, Freitas et al. 2002; Paitel, Alves da Costa et al. 2002). The hypothesised functions of PrP<sub>C</sub> are varied and as of yet it has proved difficult to distinguish its exact role within the CNS.

PrP<sub>C</sub> is also expressed on many cells of the immune system including B and T lymphocytes, natural killer cells, platelets, monocytes, dendritic cells, mast cells and FDCs (Cashman, Loertscher et al. 1990; Barclay, Hope et al. 1999; Brown, Stewart et al. 1999; Holada and Vostal 2000; Li, Liu et al. 2001; Thielen, Antoine et al. 2001; Haddon, Hughes et al. 2009). Many of the putative functions of PrP<sub>C</sub> in the CNS would not be required in cells of the immune system, however animal and human models have reported up- and down-regulation of PrP<sub>C</sub> in certain immune conditions and differences in immune cell function when PrP is ablated. Maturation of DCs and monocytes has been reported to up-regulate PrP<sub>C</sub> expression (Dürig, Giese et al. 2000; Burthem, Urban et al. 2001; Ballerini, Gourdain et al. 2006), whereas down-regulation has been reported upon activation of B and T lymphocytes in mice (Kubosaki, Yusa et al. 2001; Liu, Li et al. 2001). PrP<sub>C</sub> is up-regulated in some functionally differentiated lymphocytes including a population of regulatory T lymphocytes (Huehn, Siegmund et al. 2004) and in memory CD8 T lymphocytes (Li, Liu et al. 2001; Goldrath, Luckey et al. 2004). PrP<sub>C</sub> has also been linked to fixation of complement component within the immune system (Mitchell, Kirby et al. 2007). Furthermore, PrP<sub>C</sub> is released in response to mast cell-mediated allergic inflammation (Haddon, Hughes et al. 2009). PrP<sub>C</sub> appears to have an active functional role within cells of the immune system, however PrP<sub>C</sub> deficiency has no impact on expression
levels of both MHC Class I and II, maturation of DCs, and numbers of CD4\(^+\) and CD8\(^+\) T lymphocytes and B lymphocytes are no different to that seen in WT counterparts (Bueler, Fischer et al. 1992; Kubosaki, Yusa et al. 2001; Ballerini, Gourdain et al. 2006; Zhang, Steele et al. 2006). Recent studies have shown that PrP\(^C\) deficient T lymphocytes are more susceptible to oxidative stress (Aude-Garcia, Villiers et al. 2011) which is in agreement with previous publications demonstrating a neuroprotective role against oxidative stress for PrP\(^C\) expression by neurones (Mitteregger, Vosko et al. 2007). This suggests PrP\(^C\) expression may have protective role in cells of both the CNS and immune system.

1.2.3 Distinguishing PrP\(^C\) from PrP\(^Sc\)

In TSE diseases, the conformational change of PrP\(^C\) to PrP\(^Sc\) has been shown to be a post-translational event, which involves changes in the content of \(\alpha\)-helices and \(\beta\)-sheets (Rogers, Yehiely et al. 1993). Normal PrP\(^C\) has a structure consisting of around 40% \(\alpha\)-helix and around 3% \(\beta\)-sheet. However, after its conformational change, PrP\(^Sc\) contains only 30% \(\alpha\)-helix and around 45% \(\beta\)-sheet (Pan, Baldwin et al. 1993; Rogers, Yehiely et al. 1993). This increase in \(\beta\)-sheet content of PrP appears to confer some of the disease causing properties of PrP\(^Sc\). For example, increased \(\beta\)-sheet content has been linked to protease resistance, insolubility in detergent and a propensity of the protein to aggregate (Meyer, McKinley et al. 1986; Somerville, Merz et al. 1986; Caughey, Dong et al. 1991).
Figure 1.2 BioGPS expression profiles of Prnp in the mouse

Expression profiles of Prnp from BioGPS database (http://biogps.gnf.org), which stores microarray data of steady state mRNA expression in various murine tissues and cell lines. In agreement with published data, Prnp is ubiquitously expressed at low levels, however higher levels of expression are found within the CNS.
This latter characteristic is seen as fibrils of PrP\textsuperscript{Sc} forming in the brains of diseased animals (Meyer, McKinley et al. 1986; Pan, Baldwin et al. 1993; Prusiner, Scott et al. 1998). The partial proteinase resistance of PrP\textsuperscript{Sc} is thought to be conferred by a 27-30 KDa core which remains after treatment with proteinase (Bolton, Meyer et al. 1985). PrP\textsuperscript{Sc} deposition has been shown in numerous studies to be inseparable from infectivity (Bolton 1982; McKinley, Bolton et al. 1983) although this is not true for all TSEs as seen in studies using the P102L strain of GSS (Barron and Manson 2003; Barron, Campbell et al. 2007; Piccardo, Manson et al. 2007). Nevertheless, the deposition of PrP\textsuperscript{Sc} is widely accepted as a marker of infectivity.

Current antibodies available to detect PrP cannot distinguish between the cellular form, PrP\textsuperscript{C}, and the disease-associated form PrP\textsuperscript{Sc}. New antibodies are being developed which bind exclusively to PrP\textsuperscript{Sc} but are yet to be shown to be reliable for research application (Korth, Stierli et al. 1997; Paramithiotis, Pinard et al. 2003; Moroncini, Kanu et al. 2004; Jones, Wight et al. 2009; Petsch, Muller-Schiffmann et al. 2011). However, the biochemical properties of PrP\textsuperscript{Sc}, such as protease resistance and in solubility in detergents, allow PrP\textsuperscript{Sc} to be distinguished from PrP\textsuperscript{C} experimentally. For the histological detection of PrP\textsuperscript{Sc}, tissue sections can be fixed to nitrocellulose membrane and treated with proteinase K (PK) in the PET blot method (see section 2.4.3). PK denatures native cellular PrP\textsuperscript{C} leaving the PK-resistant PrP\textsuperscript{Sc} core intact (Schulz-Schaeffer, Tschoke et al. 2000). All PrP subsequently detected by immunolabelling can be confirmed to be PrP\textsuperscript{Sc}. PK digestion can also be used on tissue homogenates before immunoblotting. PK treatment of uninfected samples will result in no protein bands being detectable on a blot. Whereas, PK treatment of
1.2.4 Characteristics of TSE pathogenesis in the central nervous system and TSE strains

TSE infections are difficult to diagnose as some clinical symptoms are similar to other neurological diseases. The only definitive diagnosis can be made post-mortem via analysis of the brain. A TSE-infected brain shows characteristic neuropathology. As mentioned previously, PrP<sup>Sc</sup> deposition can be used as a marker of infectivity and is seen at high levels in a brain at end-stage disease. In addition, there will be spongiform change, which is vacuolation in the brain tissue (Mikol 1999; Van Everbroeck, Pals et al. 2002). The areas in which this vacuolation occurs vary between TSEs but each member of the TSE family shows a distinct lesion profile upon transmission to experimental mice, which can be used to determine the type of TSE infection. (Fraser and Dickinson 1973). The final neuropathological characteristics are gliosis, which can occur due to the hypertrophy and/ or hyperplasia of the glial cells in the brain, and neuronal loss (Mikol 1999). Astrocytes are glial cells that provide trophic, metabolic and structural support to neurones. They additionally have roles in neuronal-glial communication, synaptic signalling, regulation of blood flow and can influence neuronal precursors or stem cells within the adult CNS (Seifert, Schilling et al. 2006). Astrocytosis is a common feature of many CNS pathologies, including TSE disease, and is thought to be a neuroprotective response to CNS injury (Liedtke, Edelmann et al. 1996). However, their exact involvement in the pathology/ protection of the CNS in TSE disease is currently
uncertain. Astrocytosis of TSE infected brain is commonly detected via glial fibrillary acid protein (GFAP), an intermediate filament (IF) superfamily member expressed by astrocytes (Schiffer, Giordana et al. 1986). Increased numbers of activated microglia are also seen within TSE-affected brains. Microglia are considered the macrophages of the brain and also respond to many CNS pathological conditions. Their transformation from the resting state to an activated state involves enlargement of cells and changes in morphology, up regulation of cell surface molecules and proteins, and increases in the numbers of membrane ruffles and projections involved in phagocytosis (Block, Zecca et al. 2007). Microglia are detected via the expression of ionised calcium-binding adaptor molecule-1 (Iba-1), which is constitutively and specifically expressed by microglia and macrophages (Ohsawa, Imai et al. 2004; Ladeby, Wirenfeldt et al. 2005). Detection of activated astrocytes and microglia using the previously described markers, in addition to spongiform change and deposition of the disease associated prion protein, provide useful markers to allow post-mortem diagnosis of TSE-affected brains.

Different TSE strains can be distinguished by their neuropathological characteristics and disease incubation periods in lines of inbred mice. Serially passaged strains of scrapie injected directly into the CNS via ic injection result in highly reproducible disease incubation times with all animals succumbing to clinical disease within a matter of days of each other and can be used to distinguish scrapie strains (Dickinson, Meikle et al. 1968; Dickinson and Meikle 1971). Additionally, the pattern of neuropathology in specific scoring areas of the brain can be used to create a lesion profile. Animals from the same inbred strain infected with the same strain of scrapie
give a characteristic distinctive lesion profile (Bruce, McBride et al. 1989). These methods of neuropathological characterisation can be used to distinguish TSE strains in inbred mice. Indeed, these methods were used to confirm that vCJD in humans was the same strain of TSE as that which causes BSE in cattle (Bruce, Will et al. 1997).

An additional method of characterising TSE strains is via the intensity and migration of PrP glycoform bands after immunoblotting. TSE strains show differences in the ratios of unglycosylated, monoglycosylated and diglycosylated isoforms of PrPSc, which migrate at different speeds using gel electrophoresis (Parchi, Castellani et al. 1995; Collinge, Sidle et al. 1996; Somerville, Chong et al. 1997; Somerville 1999). These ratios are characteristic to different strains and this method is used regularly to distinguish scrapie from atypical scrapie (Baron, Biacabe et al. 2007).

The TSE agent strain used in this thesis is the ME7 scrapie strain. This was originally derived from the spleen of a Suffolk sheep with natural scrapie that was intra-gastrically inoculated into mice. The ME7 strain was isolated after serial passages through inbred mouse lines (Zlotnik and Rennie 1965). ME7 is thought to be a prevalent strain of natural sheep scrapie as it is frequently isolated from pooled natural scrapie samples (Bruce, Boyle et al. 2002). It has been used in many experiments within the Neuropathogenesis Division and therefore the incubation period and neuropathology are well characterised for various inbred mouse lines. The pathogenesis of the ME7 strain includes a stage of replication within the lymphoid tissues followed by centripetal spread to other lymphoid tissues, peripheral nerves and subsequent CNS disease. This strain provides a useful model for peripherally
acquired TSE diseases which also have a stage of lymphoreticular replication prior to
neuroinvasion and CNS disease. This is similar mechanism of pathogenesis to many
natural scrapie infections of sheep and vCJD in humans (van Keulen, Schreuder et al.
1996; Hill, Butterworth et al. 1999; Bruce, Brown et al. 2000; Bruce, McConnell et al.
2001; Joiner, Linehan et al. 2002). For these reasons, ME7 provides a useful tool to
model these peripherally-acquired TSE diseases in the lymphoid tissues.

However, other TSE diseases show extra-neural pathology but with a different
mechanism of pathogenesis. Diseases such as sCJD initiate within the CNS followed
by dissemination of the agent to peripheral nerves and extra-neural pathology
(Kitamoto, Mohri et al. 1989; Head, Northcott et al. 2003; Zanusso, Ferrari et al.
2003; Head, Ritchie et al. 2004). This is known as centrifugal spread and the ME7
scrapie strain would therefore not be a relevant model for this mechanism of
pathogenesis.

1.2.5 The host immune response to TSE disease

It is considered that TSE infection does not generate a specific host immune response
and no inflammation is seen in an infected brain. On considering the prion
hypothesis, this may not be surprising, as the infectious agent appears to be an
abnormally-folded form of a normal host protein. There is no PrP Sc specific antibody
response (Clarke and Haig 1966; Marsh, Pan et al. 1970; Porter, Porter et al. 1973)
and deficiencies in circulating antibody or the Fc-γRI have no effect on pathogenesis
(Klein, Kaeser et al. 2001). It is probable that the immune system is tolerized to the
infectious TSE agent via its close resemblance to the normal cellular form of PrP
(Klein, Kaeser et al. 2001). This would occur via deletion of PrP-specific lymphocytes in early development of the immune system repertoire as an immune response against such an agent could provoke an autoimmune response. Furthermore, there is also a lack of a notable cell-mediated immune response against the TSE agent or PrP\textsuperscript{Sc} however, this is not due to any immunosuppressive effects caused by the agent (Kingsbury, Smeltzer et al. 1981).

However, in some but not all TSE strains, the TSE agent appears to undergo a stage of replication in the lymphoid tissue before disease progression to the CNS. Early experiments infecting mice with scrapie demonstrated accumulation and replication of the TSE agent within the spleen before neuroinvasion (Fraser and Dickinson 1970; Clarke and Haig 1971). Most natural sheep scrapie strains (van Keulen, Schreuder et al. 1996) and vCJD (Hilton, Fathers et al. 1998; Hill, Butterworth et al. 1999; Bruce, McConnell et al. 2001) are thought to require this replication stage for disease propagation, demonstrating that this could be an important area to target for early intervention in these diseases. More recent studies have shown that TSE infection has subtle effects on the immune system suggesting there may be consequences in immune function and a subtle immune response in response to infection in peripheral lymphoid tissues. Changes have been reported in the morphology of infected follicular dendritic cells (Jeffrey, McGovern et al. 2000; McGovern, Brown et al. 2004; McGovern and Jeffrey 2007; McGovern, Mabbott et al. 2009a). FDCs are cells found in the B lymphocyte follicles of peripheral lymphoid tissue which are discussed in more detail in Section 1.3.3. The FDC maturation cycle and the ability of infected mice to elicit an efficient germinal centre response are also reported to be affected in the spleens of scrapie-affected mice (Jeffrey, McGovern et al. 2000; McGovern, Brown et al. 2004; McGovern and Jeffrey 2007; McGovern, Mabbott et al. 2009a).
However it is important to remember that not all TSEs appear to require this stage of pathogenesis as is seen in BSE (Somerville, Birkett et al. 1997) and atypical or Nor98 scrapie (Buschmann, Biacabe et al. 2004; Vidal, Tortosa et al. 2008).

1.3 Lymphoreticular pathogenesis of the TSE agent

1.3.1 TSEs and the immune system

In many cases of TSE infection after peripheral exposure to the TSE agent, PrP$^{\text{Sc}}$ deposition is seen in the gut-associated lymphoid tissue (GALT), including the Peyer’s patches and mesenteric lymph nodes long before the deposition in the CNS (Kimberlin and Walker 1979; Andreoletti, Berthon et al. 2000). Early experiments using splenectomy of mice prior to scrapie inoculation were shown to prolong disease incubation period, suggesting that the spleen may be an important site for agent replication (Fraser and Dickinson 1970; Clarke and Haig 1971; Fraser 1978). However, splenectomy soon after inoculation had no significant effect on disease pathogenesis (Fraser and Dickinson 1970). Therefore, it was proposed that a functionally insignificant amount of the TSE agent remains in the spleen, while the majority replicates in other tissues (Fraser and Dickinson 1970).

Immunodeficient mice have also been used to demonstrate the necessity of lymphoreticular replication of certain TSE strains. SCID (Severe-Combined Immunodeficient) mice have no B or T lymphocytes or FDCs and are resistant to scrapie infection (Fraser, Brown et al. 1996). However, these cell types can be repopulated by grafting with wild type (WT) bone marrow, and doing so restores
susceptibility to disease (Fraser, Brown et al. 1996). These early experiments, amongst others, gave the first evidence that the immune system could have an important role in early disease pathogenesis.

### 1.3.2 The role of lymphocytes in TSE pathogenesis

SCID mice with no B- or T-lymphocytes are refractory to scrapie infection (Fraser, Brown et al. 1996). Early experiments investigating a role for B and T lymphocytes in TSE agent replication used immunodeficient mice. Thymectomy of mice before or after scrapie inoculation had no effect on scrapie incubation time or neuroinvasion, suggesting T-lymphocytes were not involved in TSE pathogenesis (Fraser 1978). This was confirmed using T lymphocyte-deficient mice with genetic deletions of CD4, CD8, perforin, β2μ or TCR-α which again showed no differences in disease pathogenesis, confirming that T-lymphocytes have no role in TSE agent replication (Klein, Frigg et al. 1997).

To investigate a possible role for B lymphocytes β2μ deficient mice with no B lymphocytes were inoculated with scrapie. These mice did not accumulate PrPSc or infectivity in their spleens and did not develop disease and B lymphocytes were suggested to replicate the TSE agent (Klein, Frigg et al. 1997). As PrPC expression is essential for TSE transmission (Büeler, Aguzzi et al. 1993; Sakaguchi, Katamine et al. 1995). Expression of PrPC exclusively by B lymphocytes was not sufficient to allow scrapie pathogenesis in the lymphoid tissue (Montrasio, Cozzio et al. 2001). Experiments using SCID mice and RAG−/− mice, which have no lymphocytes but express PrPC equivalent to WT mice, were reconstituted with Prnp+/+ or Prnp−/− bone
marrow before inoculation with scrapie. Expression of PrP\textsuperscript{C} by lymphocytes was shown to be irrelevant to TSE pathogenesis in this model (Klein, Frigg et al. 1998). This has since been confirmed with similar chimeric mouse models and with various strains of the TSE agent (Brown, Stewart et al. 1999; Loeuillet, Lemaire-Vielle et al. 2010). These experiments suggested that B lymphocyte products, rather than the cells themselves, were essential in TSE pathogenesis; or that pathogenesis may involve other cells dependent on B lymphocyte-derived signals for their development and/or function. However, it is possible that B lymphocytes may assist in the spread of the TSE agent to other lymphoid organs. PrP\textsuperscript{d} was found associated with CD21\textsuperscript{+} B lymphocytes in the blood of scrapie-affected sheep. Therefore it is possible that although B lymphocytes may not actively replicate the TSE agent, they may still have a role in pathogenesis via aiding the dissemination of the agent post-replication in other cells.

1.3.3 The role of FDCs in TSE pathogenesis

Initial studies examining TSE agent infectivity within the lymphoid tissues found that the stromal component of the spleen, which would have contained FDCs, contained more infectivity than the pulp (Clarke and Kimberlin 1984). FDCs are long-lived and mitotically inactive, making them resistant to ionising radiation which identifies them as a candidate for TSE agent replication as irradiation of mice has no effect on TSE pathogenesis (Fraser 1987). The expression of PrP\textsuperscript{C} has been shown to be essential for TSE pathogenesis and FDCs have relatively high amounts of PrP\textsuperscript{C} on their surface in uninfected mice (Brown, Stewart et al. 1999; Ritchie, Brown et al. 1999). Immunohistochemical analysis of TSE-infected lymphoid tissue in mice shows strong
immunolabelling of PrP^Sc associated with the FDCs, which can be used as a biochemical marker of TSE infectivity (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Ritchie, Brown et al. 1999; Bruce, Brown et al. 2000). Furthermore, labelling of the disease-associated PrP^d is also found in FDCs of sheep with natural scrapie (van Keulen, Schreuder et al. 1996), deer and elk with chronic wasting disease (Sigurdson, Williams et al. 1999) and humans with vCJD (Hill, Zeidler et al. 1997). Electron microscopy of infected lymphoid tissue showed high accumulation of PrP^Sc on the FDC surface and around the dendrites (Jeffrey, McGovern et al. 2000).

Mice with genetic deficiencies in tumour necrosis factor (TNF)-α have a lack of terminally differentiated FDCs but do have functional and mature T and B lymphocytes (Pasparakis, Alexopoulou et al. 1996). TNFα^-/- mice are resistant to TSE infection after peripheral exposure (Mabbott, Williams et al. 2000a). The TNF-signalling pathway can be disrupted treatment with a TNF-R1 homologue that causes a temporary dedifferentiation of FDCs but no disruption to lymphocyte populations. These TNF-depleted mice have increased disease incubation times on infection with ME7 scrapie, suggesting a role for FDCs in the peripheral replication of the scrapie agent (Mabbott, McGovern et al. 2002). However, a few mice in this study did develop disease after the same incubation as the control mice raising the question of whether FDCs are the only cell involved in TSE agent replication (Mabbot 2002). TNF-α is also an important cytokine for macrophage activation, meaning that TNF depleted mice would show some deficiencies in this process. It is possible that macrophages are involved in taking up and degrading the infective agent early after inoculation. In this instance, disease may have occurred in some of the TNF depleted
mice due to the lack of early sequestering and degradation of the agent by macrophages (Mabbott, McGovern et al. 2002). It is also possible that the agent may be able to replicate in unactivated macrophages as the TNF-induced degradation processes, such as lysosome activation and superoxide formation, will not have been activated. This was demonstrated by Prinz et al who inoculated TNFR1⁻/⁻ mice with Rocky Mountain Laboratory (RML) scrapie strain and noted prion protein accumulation associated with some macrophages in the absence of FDC networks (Prinz, Montrasio et al. 2002).

Signalling through the lymphotoxin (LT) β Receptor (LTβR) on FDCs is required for maintenance of a mature FDC network and blocking this signalling results in their dedifferentiation to an immature state. These mice still retain functional lymphocytes, macrophages and DCs (Mackay and Browning 1998). FDCs can be dedifferentiated temporarily using LTβR-Ig, which acts as a soluble decoy receptor, binding LTβ ligands and preventing their interaction with LTβR. The blockade of LTβR-signalling before inoculation with ME7 scrapie can significantly extend the survival time of the mouse and reduces disease susceptibility (Mabbott, Mackay et al. 2000b). However, some animals eventually succumbed to clinical disease, therefore it may be that infectivity can persist in some other cell type until the FDC network reforms and the agent can begin to replicate (Mabbott, Mackay et al. 2000b). LTβR signalling can also be disrupted completely using knockout mice and studies have looked at the differences between LTα and LTβ deficient mice. These mice have similar reductions in their FDC populations, but LTα knockout mice appear to be more susceptible to RML scrapie than LTβ knockouts (Oldstone, Race et al. 2002). This suggests the possible involvement of another, unidentified LTβ-dependent cell type.
which may also have a role in lymphoid replication of the TSE agent. Alternatively, 
LTβ may have additional effects on FDCs, distinct from those shared with LTα, that 
as of yet are unknown.

The CXCR5 receptor on B lymphocytes responds to stimulation via CXCL13 
secreted by FDCs and is important in organising the structure of the follicle. A 
deficiency in CXCR5 disrupts the follicle structure, placing FDCs adjacent to the 
peripheral nerve endings found near the central arterioles of the spleen (Voigt 2000). 
These mice were inoculated with RML scrapie and the speed of neuroinvasion was 
found to be greatly increased when compared to the wild type (Prinz, Heikenwalder et 
al. 2003). This suggested that FDCs replicate the TSE agent prior to neuroinvasion 
and the speed of neuroinvasion can be increased by manipulating distance between 
the FDCs and nerve endings. As a control, this was repeated in CXCR5⁻/⁻ mice with 
their FDCs depleted. These mice did not develop scrapie, confirming that the 
hastened disease progression was due to the displacement of the FDCs (Prinz, 
Heikenwalder et al. 2003).

These studies provided strong evidence that FDCs are responsible for replicating the 
TSE agent in the lymphoid tissues. However, FDCs are specialised in taking up and 
retaining proteins on their surface, therefore, it is uncertain whether the FDCs are 
actively replicating the TSE agent or instead are accumulating agent after replication 
in other cells. This question was addressed using chimeric mice which have 
mismatches in PrP⁰ expression between their FDCs and their bone marrow-derived 
cells. SCID mice have no FDCs due to their lack of B cells but FDC populations of 
host genotype can be restored by grafting with donor bone marrow (Humphrey,

Inoculating these PrP<sup>C</sup> chimeric mice with ME7 scrapie showed that scrapie pathogenesis was dependent on FDC expression of PrP<sup>C</sup>, regardless of expression status of bone marrow-derived cells. This has been confirmed by recent studies which have also shown PrP<sup>C</sup>-expressing bone marrow derived cells are not responsible for replicating the TSE agent (Loeuillet, Lemaire-Vielle et al. 2010). From this it appears that FDCs themselves actively replicate the TSE agent. However, non-bone marrow-derived cells such as neural, epithelial and stromal cells within the lymphoid tissue retained expression of PrP<sup>C</sup> in this model. Therefore, this does not exclude the possibility that FDCs simply acquire PrP<sup>Sc</sup> from other cell types rather than replicating the agent themselves. To definitively determine the role of FDCs in scrapie pathogenesis an FDC-specific model is required that allows the role of these cells to be assessed in isolation from all other cell types within the lymphoid tissue.

1.3.4 The role of macrophages in TSE pathogenesis

Macrophages were also investigated as a possible cellular host for TSE agent replication. Tingible body macrophages (TBMs) reside in the follicles of lymph nodes where their main role is the clearance of proteins and apoptotic cells, many of which are produced in the germinal centre reaction during an immune response (Swartzendruber and Congdon 1963). Electron microscopical analysis of infected lymphoid tissue found PrP<sup>Sc</sup> within TBMs of scrapie-affected sheep and mice (Jeffrey, McGovern et al. 2000; Jeffrey, Martin et al. 2001; Ryder, Dexter et al. 2009) and was also present in TBMs of lymphoid tissues of vCJD patients (Hilton, Ghani et al. 2004). Additionally, in vitro incubation of the scrapie agent with peritoneal
macrophages led to an association of the agent with macrophages (Carp and Callaghan 1981) and incubation with synthetic PrP peptide 106-126 was found to slightly stimulate macrophages causing up-regulation of PrP\(^\text{C}\) and TNF\(\alpha\) production (Zhou, Xu et al. 2008). However incubating ME7 scrapie with macrophages prior to inoculation, led to an increased incubation period in mice in comparison to non-macrophage incubated ME7 (Carp and Callaghan 1982). This indicated that some of the scrapie had been inactivated or degraded by the macrophages and therefore macrophages would not be an adequate host cell for agent replication. Additionally, PrP\(\text{Sc}\) detected in macrophages was usually associated with lysosomes suggesting macrophages were degrading the scrapie agent (Jeffrey, Martin et al. 2003; McGovern and Jeffrey 2007; McGovern, Mabbott et al. 2009a). To further confirm these findings, depletion of splenic macrophages using clodronate just before or after inoculation with scrapie leads to decreased incubation period and accelerated disease progression. Immunolabelling of PrP\(\text{Sc}\) within TBM using a panel of antibodies that bind different epitopes of PrP determined that PrP\(\text{Sc}\) found within TBM was truncated with a loss of 23-90 aa from the N-terminus (Jeffrey, Martin et al. 2003; McGovern, Mabbott et al. 2009a). Evidence suggests that macrophages do take up the TSE agent but degrade and destroy it and therefore macrophages would not be an adequate host cell for replication to occur in.

1.3.5 The role of dendritic cells in TSE pathogenesis

Dendritic cells (DCs) are bone marrow-derived cells of a distinct origin and function to that of FDCs. They form the body’s immune surveillance by patrolling the periphery and sampling antigen in their local environment, subsequently migrating to
the draining lymph node and presenting it to T lymphocytes. Therefore, DCs have also been investigated as a candidate cell for the uptake and replication of the TSE agent in the lymphoid tissues. *In vitro* studies have shown that rat DCs are able to take up and retain scrapie for up to 72 hours (Huang, Farquhar et al. 2002) and PrP fragment 106-126 has been reported to be chemoattractant to immature DCs (Kaneider, Kaser et al. 2003). DCs have been shown to take up scrapie agent from the gut and transport it to the lymph node (Huang, Farquhar et al. 2002). This was confirmed by recent studies which showed DC-like cells containing PrPSc are present in the villus lacteals, submucosal lymphatics and sinuses of mesenteric lymph nodes 1.5-24 h post oral inoculation with scrapie, further suggesting that DCs transport the scrapie agent to the lymphoid tissue (Jeffrey, González et al. 2006). Selective depletion of CD11c+ DCs prior to oral inoculation with scrapie resulted in prolonged incubation periods, further supporting a role for DCs in the uptake and transport of the TSE agent (Raymond, Aucouturier et al. 2007). However, in this model, some animals eventually succumbed to disease suggesting that the TSE agent is able to utilise other cell types. Evidence so far supports a role for DCs in the uptake of the TSE agent, which is thought to be aided by complement opsonisation of the TSE agent (Flores-Langarica, Sebti et al. 2009) and subsequent transportation to the lymphoid tissues. However ionising radiation, which would deplete all DCs, has no effect on scrapie pathogenesis (Fraser and Farquhar 1987; Fraser, Farquhar et al. 1989) and bone marrow chimeric models have shown that TSE agent pathogenesis is not dependent on PrPc expressing bone marrow-derived cells (Klein, Frigg et al. 1998; Brown, Stewart et al. 1999; Loeuillet, Lemaire-Vielle et al. 2010). Therefore it appears that DCs do not replicate the TSE agent but may be involved in initial localisation of the agent to the lymphoid tissue after some routes of peripheral
exposure. DCs may also be involved in the dissemination of the TSE agent after replication on the FDC networks and subsequent neuroinvasion as CD11c⁺ splenic DCs were shown to be able to transport the TSE agent to the CNS in mouse scrapie 139A (Aucouturier, Geissmann et al. 2001).

1.3.6 The role of complement in TSE pathogenesis

The complement system is a cascade of heat labile serum proteins that can interact with pathogens to mark them for destruction. Complement can initiate an immune response by inducing inflammation, recruiting phagocytic cells, opsonising pathogens and forming immune complexes for clearance and lysis of certain pathogens and cells. An additional function of complement is its importance in the binding of antigenic immune complexes to the FDC surface via its CD21 and CD35 complement receptors (van der Berg, Yoshida et al. 1995). It is known that C3 is essential for the follicular localisation of antigen in an immune response therefore, complement may also be important in the localisation of the TSE agent to the follicle via the binding of the TSE agent to the FDC network via its expression of complement receptors.

The importance of complement in scrapie pathogenesis was investigated by both genetic deletion of C3 (Klein, Kaeser et al. 2001) and temporary removal of plasma C3 using cobra venom factor, which inhibits antigen localisation to the follicle (Mabbot 2001). Both of these studies showed increased incubation time and delayed neuroinvasion with intra-peritoneal (ip) inoculation of RML and ME7 scrapie respectively. However, infectivity titres in the spleen were similar to WT control mice, suggesting that although C3 may be involved in localising the TSE agent to the
lymphoid tissue it may not be important in the subsequent replication and 
neuroinvasion of the agent. Deletions of complement components associated with the 
terminal pathway, such as C5, have no effect on TSE pathogenesis, suggesting that 
this complement pathway has no role in the pathogenesis of the TSE agent (Mabbott 
2004). Furthermore, genetic deletions of complement components appear to have no 
effect on TSE-associated neuropathology, suggesting that neurodegeneration in TSE 
infection is not complement-mediated (Mabbott, Bruce et al. 2001).

Immune complexes, can also bind to FDCs via their Fc receptors, however depletion 
of these receptors had no effect on RML scrapie pathogenesis (Klein, Kaeser et al. 
2001). Therefore the localisation of the TSE agent to the FDC is unlikely to be 
mediated via immunoglobulins. The C1q complement component binds antigen 
complexes and can mediate their uptake directly via its receptor, CD35, or by 
activating C4 (Klickstein, Barbashov et al. 1997). Recent studies have shown that 
PrPSc can directly bind C1q via covalent interactions and activate complement via the 
classical pathway (Mitchell, Kirby et al. 2007). In addition, it is thought that FDCs 
themselves can synthesise C1q aiding immune complex formation and localisation of 
antigen to the follicle (Schwaeble, Schafer et al. 1995). These studies suggest that 
complement components, especially C1q, have a role in the localisation of the TSE 
agent to the lymphoid tissue.
1.4 The origin and function of follicular dendritic cells

1.4.1 Function of the FDC network

Follicular dendritic cells (FDCs) are currently thought to be the cells in the lymphoid tissue responsible for replicating the TSE agent. Prior to initiation of an immune response, FDCs are found within the primary follicle of secondary lymphoid tissues where they form networks of long dendrites and maintain the structural integrity of the B lymphocyte follicle (Heinen, Bosseloir et al. 1995). After exposure to antigen, primary follicles form germinal centers which contain FDCs, B lymphocytes, helper CD4+ T lymphocytes and TBMs. FDCs aid this process via the secretion of chemokines which result in the recruitment of these cells to form the germinal centre (Kosco, Pflugfelder et al. 1992; Kosco-Vilbois, Bonnefoy et al. 1997; Kosco-Vilbois 2003). The structure of the germinal centre ensures the FDCs have close contact with large numbers of B lymphocytes, which is essential for their function. FDCs are specialised to capture antigen in the form of immune complexes but are non-phagocytic and have no class II MHC expression (Gray and Skarvall 1988; Kosco, Pflugfelder et al. 1992; Maeda, Matsuda et al. 1995; van der Berg, Yoshida et al. 1995). Instead these immune complexes are retained on their surface for long periods of time and are thought to be important in B lymphocyte development, affinity maturation, antibody class switching and maintenance of B lymphocyte memory (Gray and Skarvall 1988; Gray, Kosco et al. 1991; Kosco, Pflugfelder et al. 1992; Heinen, Bosseloir et al. 1995). This occurs via positive selection of anti-apoptotic signals to B cells with highest antigen affinity. To allow immune complex capture, FDCs express complement receptors CR2 (CD21) and CR1 (CD35) and FcγRIIb on
their surface and may additionally have complement receptor 3 (CR3) and FcεRII (Qin, Wu et al. 2000; Balogh, Aydar et al. 2001). Fc receptors have high affinity for the Fc fragment of immunoglobulin molecules and allow FDCs to bind immune complexes of antigen opsonised by IgG. This is aided by the expression of the complement receptors. CD21 has a high affinity for the complement fragments C3dg and C3d, whereas CD35 binds the complement fragments C3b and C4b, allowing FDCs to bind antigens opsonised by these (Liu, Xu et al. 1997). Other possible surface markers found on FDCs include some usually found on B cells, including CD20, CD22, CD24 and CD45 however, this is difficult to determine by immunohistochemistry due to FDC close proximity to B lymphocytes in vivo (Heinen, Bosseloir et al. 1995; van der Berg, Yoshida et al. 1995). During the germinal centre response FDCs also up-regulate the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Kosco, Pflugfelder et al. 1992; Heinen, Bosseloir et al. 1995; Nielsen 2000; Videm and Albrigtsen 2008).

FDCs produce IL-6, which is important for the organisation and maintenance of the germinal centre reaction (Kosco-Vilbois, Bonnefoy et al. 1997; Kopf, Herren et al. 1998). This IL-6 is thought to amplify production of complement component C3 by local TBM s, which may subsequently increase the formation of immune complexes and attachment of these to the FDCs (Kopf, Herren et al. 1998). FDCs also produce the chemokine CXCL13, which attracts B cells to the follicle via their CXCR5 receptor (Kosco, Pflugfelder et al. 1992; Heinen, Bosseloir et al. 1995; Kosco-Vilbois, Bonnefoy et al. 1997).
FDCs have recently been discovered to aid the uptake of apoptotic B cells in the germinal centre by tingible body macrophages (TBMs). FDC-M1 or monoclonal antibody (MAb) clone 4C11 is considered to be a useful marker for FDCs as its expression is restricted to FDCs and TBMs. However until recently the epitope recognised by this MAb was unknown (Kosco, Pflugfelder et al. 1992). Kranich et al noted that milk fat globule epidermal growth factor 8 knockout (Mfge8\(^{-/-}\)) mice had no FDC-M1\(^{+}\) FDC networks in the lymphoid tissues although the FDCs were detectable by CD21/35 immunolabelling. They confirmed that MFGE-8 is the same antigen as the epitope recognised by MAb FDC-M1 and is secreted by the FDCs to opsonise apoptotic B cells in the germinal centre. This mediates the clearance of apoptotic B cells by TBMs and is essential for the prevention of autoimmune disorders (Hanayama, Tanaka et al. 2004; Kranich, Krautler et al. 2008). Secretion of MFGE-8 is possibly driven by signalling through the lymphotoxin \(\beta\) receptor (LT\(\beta\)R) as MFGE-8 levels are greatly reduced in LT\(\beta\)R\(^{-/-}\) mice (Kranich, Krautler et al. 2008).

1.4.2 The origin and development of the FDC network

The origin of FDCs is controversial but the predominant hypothesis is that they are non-bone marrow-derived cells and instead are derived from stromal cell precursors. One reason for this opinion is that FDCs share many surface markers with fibroblasts and can form ectopically under chronic inflammatory conditions, for example in the inflamed synovium of rheumatoid arthritis patients (Heinen, Bosseloir et al. 1995; Maeda, Matsuda et al. 1995). Initial experiments showed that animals which were irradiated and reconstituted with bone marrow consistently had FDCs of host origin (Humphrey, Grennan et al. 1984). This was confirmed in later studies using SCID
mice which have no FDCs. Grafting these mice with donor bone marrow can indirectly restore FDC networks via B-lymphocyte-derived signals. Work by Kapasi et al showed that newly generated FDCs in SCID mice were consistently of host genotype and not that of donor bone marrow (Kapasi, Burton et al. 1993). This procedure was subsequently used to create mice with mismatches in PrP^C expression with the newly generated FDCs consistently of host PrP^C expression status and not that of donor bone marrow, further suggesting FDCs are not bone marrow-derived cells (Brown, Stewart et al. 1999). Another experiment used transplantations of human cord blood into RAG-2⁻/⁻ mice, which also have no B or T lymphocytes or FDCs. This resulted in the generation of murine, and not human, FDCs even though animal was repopulated with human blood cells (Traggiai, Chicha et al. 2004). FDC-derived cell lines have been shown to express many surface proteins relating them to bone marrow stromal cell progenitors and the expression of α-smooth muscle actin on FDC cell lines specifically related them to myofibroblasts (Munoz-Fernandez, Blanco et al. 2006).

In contrast, some studies argue that the FDC precursor is derived from the bone marrow and not the stromal cell population (Mebius, van Tuijl et al. 1998). Various authors have suggested bone marrow-derived cells such as CD5⁻ B-1B lymphocytes or antigen transporting cells such as DCs can act as inducer cells for the formation of the FDC networks (Szakal and Tew 1992; Wen, Shinton et al. 2005). Kapasi et al reconstituted SCID mice with donor bone marrow and found cells expressing donor antigens and the FDC marker FDC-M1 in the newly formed lymphoid tissues (Kapasi, Qin et al. 1998). However, FDCs are known to be able to acquire proteins on their surface that they themselves do not express, therefore it is possible that FDCs
acquired these antigens from donor B lymphocytes, rather than the FDC networks developing from donor bone marrow precursors (Gray, Kosco et al. 1991; Denzer, van Eijk et al. 2000).

A recent study by Murakami et al proposed to have found a cell type that induces the formation of FDC networks. CD19−CD11c−CD35−B220+ inducer cells were separated from splenocytes, and injected intradermally along with stromal like CD45−CD35+ cells to successfully induce the formation of lymphoid tissue-like structures. Furthermore, GFP-tagged inducer cells demonstrated that these inducer cells gain expression of the FDC marker FDC-M1 in the newly formed follicles. This was confirmed by adoptive transfer of GFP-tagged inducer cells into naïve C57BL/6J-Jcl mice resulting in GFP+ FDCs in the reticulum of the spleen (Murakami, Chen et al. 2007). The inducer cells were able to retain immune complexes on their surface both in-vitro and in-vivo, a major distinguishing feature of FDCs. These data strongly suggest a role for these CD19−CD11c−CD35−B220+ inducer cells in the formation of the FDC network. The development of the FDC network is also dependent on B cell-derived lymphotoxins (Chaplin and Fu 1998), especially the membrane-bound LT. LTα−/− or LTβ−/− mice, have defects in the formation of lymph nodes and no Peyer’s patches (Chaplin and Fu 1998). TNF−/− mice also had a similar phenotype, suggesting this is also involved in FDC development (Chaplin and Fu 1998). It has since been confirmed that signalling through the p55 TNFR on FDC is essential for formation of the FDC network and maintenance of FDC function (Victoratos, Lagnel et al. 2006). Furthermore, these signals are not just required for initial FDC development, continual signalling via LTα1β2 secreted from B cells is also required for
maintenance of the FDC network structure as blocking this signal using a soluble LTβ receptor causes the networks to dedifferentiate (Mackay and Browning 1998).

1.4.3 FDC-independent TSE agent replication

From the work mentioned previously, it seems certain that FDCs have a role in TSE pathogenesis, however some conflicting results have shown that they may not always be required. Some naturally occurring TSE diseases appear to be restricted to the CNS, with no PrP Sc deposition or infectivity found in the extraneural tissues. For example, no extraneural disease was detected in sCJD patients (Hill, Butterworth et al. 1999) until a method of concentrating PrP Sc to increase the sensitivity for western blot was used and PrP Sc was subsequently detected in some of the samples tested (Glatzel, Abela et al. 2003). This is in contrast to vCJD where infectivity and PrP Sc deposition have been reported in various tissues including the spleen and tonsils (Hill, Butterworth et al. 1999). Additionally, disease appears to be restricted to the CNS in cattle naturally infected with BSE (Somerville, Birkett et al. 1997). In contrast, PrP Sc has been detected in the PP of the distal ileum in cattle experimentally infected with disease (Terry, Marsh et al. 2003) and experimental inoculation of BSE into sheep has also resulted in PrP Sc deposition in the lymphoid tissues (Lemzi, Ronzon et al. 2006). Atypical forms of BSE and scrapie have recently been described and there also appears to be no involvement of the lymphoid tissues with these TSE strains (Buschmann, Biacabe et al. 2004; Benestad, Arsac et al. 2008). A case of atypical scrapie with a concurrent, non-related inflammation in the brain was recently described. This animal was detected as having atypical scrapie by active surveillance and also had virus induced, non-purulent encephalitis which had led to the formation
of ectopic lymphoid follicles in the brain tissue. Examination tissues from this animal showed that there was no association of PrP<sup>Sc</sup> with any draining lymph nodes or ectopic follicles found in the brain tissue (Vidal, Tortosa et al. 2008). This lack of PrP<sup>Sc</sup> accumulation on ectopic follicles in the brain further demonstrate the lack of lymphoreticular involvement of the atypical scrapie strain as inflammatory conditions in various organs which cause the formation of FDC-containing ectopic follicles, have previously shown to be a favourable environment for PrP<sup>Sc</sup> accumulation (Heikenwalder, Zeller et al. 2005). Furthermore, PrP<sup>Sc</sup> accumulation in inflamed excretory organs such as the kidney or mammary glands is thought to aid spread of scrapie through shedding of the TSE agent in urine or milk respectively (Heikenwalder, Zeller et al. 2005; Ligios, Sigurdson et al. 2005; Ligios, Cancedda et al. 2007; Konold, Moore et al. 2008). These studies suggest that there may be variations in TSE strain cellular targeting.

Recent evidence has suggested that the FDCs themselves might not be essential for PrP<sup>Sc</sup> replication but that any stromal cell with some FDC-like functions could theoretically support prion replication. Heikenwalder et al created a model in which TSE agent replication occurred in complete Freund's adjuvant (CFA) -induced granulomas in the skin following ip inoculation. Analysis of the granuloma showed a complete lack of commonly used FDC markers and FDC-specific, M<sub>fge8</sub> mRNA but relatively high levels of PrP<sup>C</sup>. However, administration of the LTβR-Fc, which has been shown to dedifferentiate FDCs, reduced TSE agent accumulation in the granulomas (Mackay and Browning 1998; Heikenwalder, Kurrer et al. 2008). Furthermore, these stromal cells expressed high levels of PrP<sup>C</sup> and organised lymphocytes within the granuloma into follicle-like structures. Analysis of this model

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led to the conclusion that that scrapie replication was occurring on a radio-resistant fibroblastic cell which did not express any FDC-specific markers and that this occurred in a LTβ-dependent manner (Heikenwalder, Kurrer et al. 2008). This is in accordance with the finding that unaltered, mouse derived fibroblast cell lines can be infected with RML scrapie in vitro and are sufficient to sustain PrPSc replication (Vorberg, Raines et al. 2004). Muscle cell lines have also been shown to support prion replication with finding of PrPSc in the muscle tissue of a sporadic CJD patient confirming this (Kovacs, Lindeck-Pozza et al. 2004; Dlakic, Grigg et al. 2007). Therefore it may be possible that any stromal cell with an FDC-like function has the capacity to support TSE agent replication.

Natural and experimental strains of TSE disease show variation in pathogenesis thought to be due to genetic or strain properties, but there are also reported differences between groups using the same agent strain. For example, Klein et al inoculated TNFR1−/− mice, which have no mature FDCs with RML scrapie and observed no difference in disease pathogenesis between the TNF deficient mice and their wild type counterparts, suggesting that FDCs are not involved in scrapie pathogenesis (Klein, Frigg et al. 1997). This was further confirmed by Prinz et al who also found that TNF−/− mice developed scrapie at the same time as their WT counterparts. Furthermore, although PrPSc was absent in the spleen, they found high accumulation in the lymph nodes of these animals (Prinz, Montrasio et al. 2002). However, they also discovered PrPSc accumulation in macrophages in the lymph nodes of these mice, suggesting that unactivated macrophages may act as a replication site in the absence of FDCs (Prinz, Montrasio et al. 2002). Studies by Oldstone et al using the RML scrapie strain in LTα−/− and LTβ−/− mice analysed the role of FDCs after oral and i.p. scrapie inoculation. In
these studies, all of the LTα−/− mice developed disease, whereas the lack of FDCs led to impaired scrapie pathogenesis in the LTβ−/− mice. It was suggested that scrapie replication may be dependent on some other unknown cell type dependent on LTβ (Oldstone, Race et al. 2002). However, it should be mentioned that high doses of RML Scrapie were used for inoculation in these studies, so it may be that the need for an amplification stage in the spleen in unnecessary for neuroinvasion to proceed after high dose of infection. The discrepancies between various experiments using same strains have been put down to various factors such as TSE agent load and route of inoculation, however the reasons behind these differences will not be fully understood until the true nature of the TSE agent is discovered.

1.6 Thesis aims

From studies published so far, it has been determined that PrPc expressing FDCs are required for the successful pathogenesis of many TSE agent strains within the lymphoid tissue (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, McGovern et al. 2002; Prinz, Montrasio et al. 2002; Mabbott, Young et al. 2003). However in the models used so far, it is possible that FDCs acquire accumulation of PrPsc on their surface after scrapie agent replication on other neuronal, stromal or endothelial cells within the lymphoid tissue. To definitively determine the role of FDCs in scrapie pathogenesis, a model is required where PrPC expression can be manipulated exclusively on the FDC network.

The aims of this thesis were to create and characterise transgenic mouse models that allow the manipulation of PrPC expression specifically on FDCs and subsequently to
infect these animals with scrapie to determine the specific role of the FDCs in TSE pathogenesis within the lymphoid tissue. The hypothesis was explored that if FDCs are simply accumulating the TSE agent on their surface after replication on another cell type, the accumulation of the TSE agent will occur on PrP\(^C\) deficient FDCs when all other cell types within the lymphoid tissue retain PrP\(^C\) expression. If however, the FDCs actively replicate the TSE agent in the lymphoid tissue, accumulation of PrP\(^d\) will not occur on the PrP\(^C\) deficient FDCs. Additionally, if FDCs are responsible for replicating the TSE agent within the lymphoid tissue, then PrP\(^C\) expression exclusively on FDCs should be sufficient to allow replication of the TSE agent within the lymphoid tissue.

1.7 Transgenic mouse lines used in this thesis

1.7.1 Cre-LoxP Model

To create a model which allows manipulation of PrP\(^C\) specifically on FDCs, the Cre-LoxP system of targeted gene deletion was used. The Cre-LoxP system is a model that can be used to generate transgenic animals in which gene expression is tissue- or cell-type specific. This occurs through the use of the Cre-recombinase enzyme that cuts out or inverts any DNA that is flanked by LoxP sites i.e. “floxed” DNA. In non-Cre expressing cells the target DNA remains unchanged. This system can be made specific by inserting the Cre-recombinase after a tissue- or cell type- specific promoter (Kos 2004). LoxP is short for Locus of crossover P and these sites are 34bp DNA sequences that are used to flank the target DNA. They contain an 8bp core that determines the “directionality” of the target DNA and this is flanked by 13bp pallindromic sequences. Cre-recombinase is a 38 KDa cyclisation recombination
recombinase enzyme that creates the recombination of the DNA between LoxP sites. It is a member of the integrase family of recombinases and is coded for by bacteriophage P1 (Kos 2004). When Cre is activated, it creates a transient DNA-protein covalent linkage to bring the two LoxP sites together and catalyses a site-specific recombination event. Depending on the orientation of the two LoxP sites, the DNA will either be inverted or excised. If the LoxP sites are inverted, i.e. are in opposite directions, the DNA segment between undergoes inversion and the two LoxP sites remain in their position in the DNA. However if the two LoxP sites are in the same direction, Cre excises the target DNA segment and a single LoxP site remains (Kos 2004).

1.7.2 The *CD21-Cre* mouse line

The *CD21-cre* mouse line was chosen to create a model which allows manipulation of PrP<sup>C</sup> expression exclusively on FDCs. CD21 is also known as complement receptor 2 (CR2) and was thought to be expressed solely on FDCs and mature B lymphocytes (Reynes, Aubert et al. 1985; Liu, Xu et al. 1997; Takahashi, Kozono et al. 1997). There are two types of CD21- a long and a short isoform- which are expressed on FDCs and B cells respectively. CD21 has a high affinity for the complement fragments C3dg and C3d and on FDCs, will bind antigens opsonised by these (Liu, Xu et al. 1997). On B lymphocytes, CD21 acts as a co-receptor for the B cell receptor (BCR), where the binding of specific antigen opsonised by complement components decreases the threshold level for signalling through the BCR. However, in humans, expression of CD21 has also been reported on a subpopulation of immature thymocytes (Tsoukas and Lambris 1988; Wagner and Hansch 2006), peripheral T
lymphocytes (Morgan, Marchbank et al. 2005) and on cervical epithelium (Sixbey, Lemon et al. 1986). Within the mouse, expression has also been reported on a small population of CD4⁺ T lymphocytes within the MLN, activated granulocytes and mast cells (Gray and Skarvall 1988; Gray, Kosco et al. 1991; Andrasfalvy, Prechl et al. 2002; Heggebo, Gonzalez et al. 2003; Llewelyn, Hewitt et al. 2004). The expression of CD21 appears to more widespread than initially reported, however all non-FDC expression of CD21 is found on bone marrow derived cells. This is confirmed using data from the BioGPS database which stores microarray data of gene expression profiles from various mouse and human tissues. Analysis of CD21 (Cr2) expression using the BioGPS database confirms there is no non-lymphoid expression of CD21 reported (Fig 1.3). To restrict Cre expression in the CD21-cre mouse exclusively to FDCs, animals are lethally γ-irradiated and reconstituted with non-Cre expressing bone marrow.
Figure 1.3 BioGPS expression profiles of Cr2 in the mouse

Expression profiles of Cr2 from BioGPS database (http://biogps.gnf.org) which stores microarray data from various murine tissues and cell lines. In agreement with publication data, Cr2 expression is restricted to the lymphoid tissues on FDCs and bone marrow-derived cells.
# CHAPTER 2

## Materials and methods

### 2.1 Production of mouse lines

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### 2.2 Characterisation of $CD21$-cre$ROSA26$ animals

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### 2.3 Characterisation of $CD21$-cre$PrP^{fl/}$ and $CD21$-cre$PrP^{stop/}$ mouse lines

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</table>
2. Materials and methods

2.1 Production of mouse lines

2.1.1 CD21-cre mice

CD21-cre mice were kindly provided by Klaus Rajewski (CBR Institute for Biomedical Research) and were created by targeted insertion of CD21/35 bacterial artificial chromosome (BAC) clone containing a Cre expression cassette. Homologous recombination of Cre into the BAC was confirmed by Southern Blot and used for microinjection into fertilized oocytes (Kraus, Alimzhanov et al. 2004). CD21-cre mice are on a C57BL/6 background.

2.1.2 CD21-creROSA26 mice

The CD21-creROSA26 reporter strain was created at the Roslin Institute by crossing the CD21-cre mice with a ROSA26 reporter strain (a kind gift from Yuko Fujiwara and Stuart H. Orkin, Howard Hughes Medical Institute, Harvard Med School, Massachusetts, USA). The ROSA26 mice have a floxed 1.3-kb BglII–EcoRI ‘‘stopper’’ fragment from plasmid cAct-XstopXnZ (Fig 2.1), followed by the LacZ gene, a β-galactosidase coding sequence, inserted into the ROSA26 locus. The ROSA26 locus is ubiquitously expressed during embryonic development and in adult cells. Cell-specific expression of Cre causes removal of the floxed stop cassette and β-galactosidase expression is switched on in that cell type. (Mao, Fujiwara et al. 1999). The progeny of this cross were genotyped and selected for Cre and LacZ (ROSA26)
expression by PCR analysis of genomic DNA from tail snips. Age matched, Cre-negative ROSA26 litter mates were used to control for background $\beta$-galactosidase staining.

Fig 2.1 CD21-creROS2A6 mice
Cre is expressed under the $Cr2$ promoter region. Expression of Cre causes excision of a floxed stop cassette, switching on expression of the LacZ gene which is a $\beta$-galactosidase coding region.

2.1.3 CD21-crePrPfl/fl mice

The CD21-crePrPfl/fl mouse strain was created by crossing the CD21-cre mice with mice which has the $Prnp$ ORF flanked by loxP sites i.e. “floxed” (termed PrP fl/fl) (Tuzi, Clarke et al. 2004). The ORF of the murine Prnp gene is contained within exon 3. The PrP fl/fl mice are on a 129/Ola background and have loxP sites flanking Prnp exon 3 created by homologous recombination with a PrP fl/fl targeting vector. Cell-specific Cre activation removes exon 3 of Prnp thus switching off PrpC expression. The progeny mice were genotyped and selected for Cre and $Prnp^{fl}$ expression.

Figure 2.2 CD21-crePrPfl/fl mice
Cre is expressed under the $Cr2$ promoter region. Expression of Cre causes excision of the floxed exon 3 of the Prnp gene, which contains the protein coding region. Consequently, PrpC expression is switched off in Cre-expressing cells.
2.1.4 CD21-crePrP\(\text{fl}/\text{c}\) mice

Non promoter-specific recombination of the floxed Prnp ORF was detected in the CD21-crePrP\(\text{fl}/\text{c}\). Multiple generations of the CD21-cre line crossed with the floxed line on a mixed genetic background may have led to the Cr2 promoter losing control of Cre expression. As a consequence, these mice were not used for subsequent scrapie experiments. To compensate for this, the CD21-cre mice were bred onto a Prnp null background by crossing with a PrP\(^{+/-}\) mouse (Manson, Clarke, et al. 1994). This PrP\(^{+/-}\) mouse is on a 129/Ola background and was created by gene targeting a PrP DNA fragment construct containing a neomycin resistance gene into exon 3 of the Prnp gene, disrupting the protein coding region for PrP\(^{C}\). These CD21-crePrP\(^{+/-}\) mice were then crossed with the PrP\(\text{fl}/\text{c}\) line. Animals were selected by genotyping for Cre, Prnp\(^{null}\) and Prnp\(^{\text{fl}}\) and the resulting CD21-crePrP\(\text{fl}/\text{c}\) mice produced were used in subsequent scrapie experiments. Age matched Cre-negative littermates were used as controls and bone marrow donors in experiments, where indicated.

2.1.5 CD21-crePrP\(\text{stop}/\text{c}\) mice

The CD21-crePrP\(\text{stop}/\text{c}\) strain was created by crossing CD21-crePrP\(^{+/-}\) mice with PrP\(\text{stop}/\text{c}\) mice (Tuzi, Clarke et al. 2004). The PrP\(\text{stop}\) mice contain a floxed \(\beta\)-geo cassette inserted into intron 2 of the Prnp gene, upstream of the coding region. Insertion of this \(\beta\)-geo cassette prevents the expression of PrP\(^{C}\), however site specific expression of Cre removes the cassette, switching on PrP\(^{C}\) expression. The PrP\(\text{stop}/\text{c}\) mice are also on a 129/Ola background. Animals were selected by genotyping for Cre, Prnp\(^{null}\) and Prnp\(^{\text{stop}}\) and the resulting CD21-crePrP\(\text{stop}/\text{c}\) mice were used in subsequent
scrapie experiments. Age-matched Cre-negative littermates were used as controls and bone marrow donors in experiments, where indicated.

![Diagram](image)

**Figure 2.3 CD21-crePrP\textsuperscript{stop/-} mice**

Cre is expressed under the Cr2 promoter region. Expression of Cre causes excision of a floxed stop cassette inserted before exon 3 of the Prnp gene, which contains the protein coding region. Consequently, PrP\textsuperscript{C} expression is switched on in Cre-expressing cells.

2.1.6 Genotyping animals

Tail snips of CD21-creRosa26 mice were lysed in 800μl of proteinase K (PK)-containing buffer (0.3 M sodium acetate, 0.1% SDS, 0.1M Tris pH8, 1 mM EDTA, 7mg/ml PK) and cleaned by addition of 600μl phenol chloroform followed by centrifugation. The supernatants were removed and added to 600μl of isopropanol and 20μl of 3M sodium acetate to pellet DNA. DNA pellets were then cleaned by centrifugation with 70% ethanol and resuspended in 100μl distilled water. Resulting DNA samples were analysed for Cre and LacZ expression using the primers in the table below (Table 2.1). PCR products were viewed by running on a 1% agarose gel, containing 0.001% ethidium bromide (Sigma).

For CD21-crePrP\textsuperscript{fl/fl}, CD21-crePrP\textsuperscript{+/−}, CD21-cre PrP\textsuperscript{fl/−}, CD21-crePrP\textsuperscript{stop/−} strains, DNA was prepared from tailsnips, earpunches, blood, tail or spleen using a DNeasy
blood and tissue kit (Qiagen, Crawley, UK) according to the manufacturer’s instructions.

*CD21-cre* PrP^{fl/fl} DNA samples were genotyped for *Cre* and Prp{fl} using primers listed in Table 2.1. PCR product for Cre samples were viewed by running on a 1% agarose gel containing 0.001% ethidium bromide producing a band of 786 base pairs (bp).

Prn{fl} product was run on a 2% agarose gel containing 0.001% ethidium bromide resulting in a band of 210 bp. Prn{WT} could also be detected as a band of 167 bp which demonstrated whether the sample was homozygous or heterozygous for Prp{fl}.

Animals were subsequently genotyped for Prn{fl} with or without recombination of the floxed exon 3 (Prn{fl(R)}). To do this, the Prn{fl} primers were used with the addition of an extra primer, *vitro Cre B*, to detect any recombination of the floxed exon. Full recombination resulted in one band of 344 bp whereas partial recombination resulted in both the recombined band of 344 bp and the Prn{fl} band at 210 bp. Prn{WT} was also detected in as a band of 167 bp as in the Prn{fl} PCR.

*CD21-cre* PrP^{/-} DNA samples were genotyped for *Cre* as previously described. Samples were also genotyped for Prn{null} and Prn{WT} using primers listed in Table 2.1. PCR product was run on a 1% agarose gel containing 0.002% gelred (Biotium, Cambridge Biosciences Ltd, Cambridge, UK) and produced bands of 1.2 Kbp and 600 bp respectively.
CD21-cre PrP
\(^{fl/}\) DNA samples were genotyped for Cre, Prnp\(^{WT}\) and Prnp\(^{null}\) as above and also Prnp\(^{fl(R)}\) as in the genotyping of CD21-crePrP\(^{fl/}\) DNA samples with primers listed in Table 2.1.

CD21-cre PrP\(^{stop/}\) DNA samples were genotyped for Cre, Prnp\(^{WT}\) and Prnp\(^{null}\) as previously described. In addition, these samples were genotyped for Prnp\(^{stop}\) to detect the floxed STOP cassette and any recombination of the floxed STOP DNA using the primers listed in Table 2.1. This produced a band of 1 Kbp for the floxed stop cassette (Prnp\(^{stop}\)) and a band of 840bp for recombined Prnp\(^{stop}\) (Prnp\(^{stop(R)}\)).

2.1.7 Production of bone marrow chimeras

Where indicated, mice were lethally $\gamma$-irradiated with 950 rads from a Caesium\(^{137}\) source (Gravatom Engineering Systems Ltd, UK). Mice were reconstituted with bone marrow from age and sex matched animals 24 h post-irradiation. Bone marrow preparations were made as single cell suspensions in Hank’s balanced salt solution (HBSS; GIBCO Life Technologies, Paisley, UK) from the femurs and tibias of selected donor mice. Bone marrow suspensions contained between 3x \(10^7\) - 4x \(10^7\) viable cells per 1ml. Mice were reconstituted by injection of 0.1ml of bone marrow suspension into the tail vein. Animals were left for 100 days before experimental analysis to allow removal of long-lived B lymphocyte populations and replacement with the donor bone marrow.
2.1.8 Animal Housing

All protocols using experimental mice were approved by the Neuropathogenesis Unit’s Protocols and Ethics Committee and conducted according to the regulations of the Home Office. Experimental work was carried out under project license number 60/3983 held by Dr. Karen Brown to investigate immune system influences on TSE pathogenesis. All mouse strains used were kept in a conventional animal housing unit under specific pathogen free conditions. Bone marrow chimeric mice were housed in individually ventilated cages.

2.2 Characterisation of CD21-creROSA26 animals

2.2.1 Detection of β-galactosidase

Tissues from CD21-creROSA26 animals were dissected into ice cold phosphate buffered saline pH 7.4 (PBS) and fixed for one hour in LacZ fixative [paraformaldehyde-glutaraldehyde (2% paraformaldehyde, 0.2% gluteraldehyde, PBS), 0.02% Nonidet P40, 0.01% sodium deoxycholate, 5mM EGTA, 2mM MgCl₂]. Tissues were then given three 20 minute washes in LacZ wash buffer (0.02% Nonidet P40, 0.01% Sodium deoxycholate, 2mM MgCl₂, 1X PBS pH 7.4). After washing, tissues were incubated in 15% sucrose in PBS overnight followed by a further overnight incubation in 30% sucrose in PBS. Tissues were subsequently embedded in Tissue-Tek® O.C.T. Compound™ (Bayer PLC, Newbury, UK) and frozen using isopentane at the temperature of liquid nitrogen. Serial sections of 8µm were cut on a cryostat and processed further for X-gal staining and immunohistochemistry.
To detect β-galactosidase, sections were incubated in *LacZ* staining solution [1X PBS pH 7.4, 1mg/ml X-gal (Glycosynth, Warrington, UK), 0.02% Nonidet P40, 0.01% sodium deoxycholate, 2mM MgCl₂, 5mM potassium ferricyanide, 5mM potassium ferrocyanide] at 37°C overnight and protected from light. Staining reaction was stopped by washing for 5 min in *LacZ* wash buffer followed by distilled water for 1 min. Sections were counterstained with nuclear fast red (Vector Laboratories, Peterborough, UK), dehydrated and mounted in Pertex (CellPath, Powys, UK).

### 2.2.2 Immunohistochemistry (IHC) of frozen tissues

Sections (thickness 8μm) were cut from tissues fixed for β-galactosidase detection (Section 2.2.1) using a cryostat. Sections were fixed in acetone for 10 minutes before addition of a species specific normal serum block followed by primary antibodies as listed in Table 2.2. For light microscopy, a species specific biotin-conjugated secondary antibody was added. This was detected by alkaline phosphatase (AP) coupled to the avidin–biotin complex (Vector Laboratories) using Vector Red as a substrate (Vector Laboratories) and counterstained with haematoxylin. Light microscopy was carried out on a Nikon Eclipse E800 microscope (Nikon U.K. Ltd, Surrey, U.K.) For fluorescent microscopy, species specific secondary antibodies conjugated to alexa-fluor 488, 594 or 647 were added and detected by confocal microscopy. All fluorescent microscopy was carried out on a Zeiss LSM5 confocal microscope (Zeiss, Welwyn Garden City, UK)
<table>
<thead>
<tr>
<th>PCR</th>
<th>Forward Primer(s)</th>
<th>Reverse Primer(s)</th>
<th>Product Size</th>
</tr>
</thead>
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<tr>
<td>Cre</td>
<td>CreScreen 1 CGAGTGATGAGGGTTCC GCAAGAACC</td>
<td>CreScreen 3 GCTAAGTGCCCTTCT CTACACCTGC</td>
<td>786 bp</td>
</tr>
<tr>
<td>LacZ</td>
<td>LACZ1ROSA26 TACACACGGGATGG TTCCG</td>
<td>LACZ2ROSA26 GTGGTGGTTATGCC GATCGC</td>
<td>300 bp</td>
</tr>
<tr>
<td>Prnp\text{fl}</td>
<td>3'orfR1 GCCGACATCAGTCC ACATAG 5'orfR1 GGTGACGCCTG ACTTTTC</td>
<td>Prnp\text{fl} 210 bp Prnp\text{WT} 167 bp</td>
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</tr>
<tr>
<td>Prnp\text{fl(R)}</td>
<td>Vitro CreB AATGGTTAATTCTTC GTTAAGGAT 3'orfR1 and 5'orfR1 As previous</td>
<td>Prnp\text{fl(R)} 344 bp Prnp\text{fl} 210 bp Prnp\text{WT} 167 bp</td>
<td></td>
</tr>
<tr>
<td>Prnp\text{null}</td>
<td>Null A1 GCCATCACGAGATT CGATT Null A2 ATCCCAAGCATCAG AAGATG</td>
<td>Prnp\text{null} 1.2 Kbp</td>
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</tr>
<tr>
<td>Prnp\text{WT}</td>
<td>PrP 44 TCATCCACGATCAG GAAGATGAG PrP45 ATGGCGAACCTTGG CTACTGGCTG</td>
<td>Prnp\text{WT} 600 bp</td>
<td></td>
</tr>
<tr>
<td>Prnp\text{stop}</td>
<td>STP1 ACAATATGGTGATGG CTGATTATG STP2 ATGATGATTGAACA AGATGGATTG</td>
<td>Prnp\text{stop} 1Kbp Prnp\text{stop(R)} 840 bp Prnp\text{WT} 750Kbp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WTS1 TACACGAAGTCCGG GATAG WTS2 GGCAGAGGCTAAGG ACAACA</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 2.1 Primers used in PCR genotyping of animals

Details of oligonucleotide primers used for PCRs to genotype transgenic animals during breeding to produce mouse lines detailed in section 2.1.
2.2.3 Characterisation of spleen and blood cells from CD21-creROSA26 animals by flow cytometry

Single cell suspensions of spleens were prepared by passing cells through a cell strainer in 1ml of fluorescence-activated cell sorting (FACS) buffer [GIBCO 1xPBS pH 7.4 (Invitrogen, Paisley, UK) 2% Foetal calf serum (Sigma, Dorset, UK)]. Blood was collected into heparinised tubes to prevent clotting and added to FACS buffer. Cell suspension were spun at 2000 rpm for 10 min at 4°C and resulting cell pellets were re-suspended in red blood cell lysis buffer (Sigma) for 5 minutes. Viable cells were counted in a 1:10 dilution in trypan blue (GIBCO) and adjusted to 1 X 10^6 viable cells per 50 μl. Samples of 1x10^6 cells were blocked using 1μl Seroblock FcR rat anti-mouse CD16/32 (AbD Serotech, Oxford, UK) and incubated for 30 minutes with an appropriate dilution of primary antibody (Table 2.3) at 4°C.

Samples labelled with directly conjugated primary antibodies were transferred to FACS tubes for analysis. Samples labelled with biotin-conjugated primary antibodies were incubated for 30 min in a 1:200 dilution of streptavidin-conjugated alexa 594 or 488. Samples labelled with unconjugated primary antibodies were incubated with a 1:200 dilution of the species specific secondary antibody conjugated to a fluorochrome. To detect intracellular antigens, such as β-galactosidase, samples were permeabilised using a BD fixation/Permeabilisation kit (BD bioscience, Oxford, UK). After labelling cell surface antigens, cells were added to BD Fixation/Permeabilisation solution. Cells were incubated in the primary antibody for 30 minutes according to the manufacturer’s instructions. Species specific, isotype controls were used in all experiments to detect any background fluorescence. A
FACS Scan flow cytometer (Becton Dickinson, Oxford, UK) was used to analyse cells with the lymphoid cells gated for by forward and side scatter.

2.3 Characterisation of CD21-crePrP\(^{0/-}\) and CD21-crePrP\(^{stop/-}\) mouse lines

2.3.1 PCR analysis of bone marrow chimeric mice

Blood, tail and spleen samples were taken from transgenic mouse lines and snap frozen until DNA extraction was performed. DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Crawley, UK) according to the manufacturer’s instructions. Samples were genotyped for Cre, Prnp\(^{null}\), Prnp\(^{0/\text{R}}\) and Prnp\(^{\text{stop}}\) as described previously (2.1.5). Differences in gene expression between blood and spleen/ tail were used to demonstrate the chimera status of animals and confirm whether specific Cre-mediated DNA recombination was occurring in stromal, haematopoietic or both compartments.

2.3.2 Immunohistochemistry of frozen tissues

Tissues were snap frozen in liquid nitrogen, embedded in Tissue-Tek\textsuperscript{®} O.C.T. Compound\textsuperscript{TM} (Bayer PLC, Newbury, UK) and 8\(\mu\)m sections were cut using a cryostat. IHC analysis was carried out for light and fluorescent microscopy as previously described (2.2.2) using the antibodies listed in Table 2.2. Light and fluorescent microscopy was carried out as detailed previously (2.2.2)
2.3.3 ImageJ analysis of confocal images

Confocal images were analysed and converted to data using the program ImageJ (http://rsbweb.nih.gov/ij/index.html). This program processes images in Java to create data that were used to analyse the total area of specific immunostaining and the co-localisation of proteins. The accuracy of analysing images in this way was previously validated by comparing manual counts to automated computer generated data (Inman, Rees et al. 2005). This method of analysis has considerable advantages because it ensures consistency over analysis of images and is faster and more accurate than observational analysis. Furthermore, it allows statistical analysis and data to be extracted from images to prove true co-localisation of proteins as opposed to co-localisation due to the random association of fluorochromes on an image.

ImageJ was used to determine and compare the area occupied by FDC networks in spleen sections from each of the transgenic mouse lines when compared to wild type mice. To do this spleen sections were stained using the rat-anti-mouse CD35 antibody clone 8C12 as described previously (2.3.2). Five areas of each spleen were imaged using the Zeiss confocal microscope at x200 magnification. ImageJ was used to convert images to grayscale and measure the number of pixels positively labelled with the CD35 antibody. This enabled calculation of the average area of FDC per image.

Co-localisation of proteins was also assessed using ImageJ. Firstly, the multiple colour backgrounds macro (Appendix I) was used over 2 images from each of the animals. A line is drawn through the image and the macro measures the intensity of the colour over that line. This enables a threshold for background staining to be determined for each colour in the image. The multiple colour analysis macro
(Appendix II) is then used to measure the number of black and coloured pixels in the image, using the previously estimated threshold to eliminate the count of any background. These values were then used to work out the total area of staining for each antibody used and determine the amount of co-localisation.

2.3.4 Fluorescence-activated cell sorting (FACS) analysis

Flow cytometry was used to determine PrP$^C$ expression status of CD21$^+$ lymphocytes. Cells were extracted from both spleen and blood to demonstrate the chimera status of animals. Single cell suspensions from spleen and blood were prepared as described previously (2.2.3) and labelled with 1B3 and CD21/35 (Table 2.3). CD21$^+$ B lymphocytes were gated and assessed for PrP$^C$ expression status using a FACS Scan flow cytometer (Becton and Dickinson).

2.3.5 In-vivo analysis of immune complex trapping by FDCs

Animals were given intra-peritoneal injections of 100 μl of preformed rabbit peroxidise-anti-peroxidase (PAP) immune complex (Sigma) and culled 48 h later. Spleens were snap frozen and 8μm sections were cut on a cryostat. Sections were blocked with normal goat serum and incubated with a goat-anti-rabbit antibody conjugated to alexa-fluor 555. After washing, sections were blocked with normal mouse serum and then incubated with biotin-conjugated rat-anti-CD35. Immunolabelling was detected using a streptavidin-alexa 488 conjugate. Images were taken on the Zeiss confocal microscope and were analysed for co-localisation using the ImageJ macro described previously (2.3.3).
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<th>Antigen</th>
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<th>Source</th>
<th>Secondary Antibody</th>
<th>Source</th>
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<td>PrP&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Rabbit anti-PrP Polyclonal 1B3</td>
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<td>Complement component C4 bound to FDC surface</td>
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Table 2.2 Detection antibodies used for immunohistochemical analysis of frozen sections
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<th>Control Antibody</th>
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<td>Goat anti Rabbit IgG-FITC</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

**Table 2.3 Detection antibodies used for flow cytometric analysis**  
Details of antibodies used for immunolabelling of lymphocytes isolated from spleen and blood for detection by flow cytometry
2.4 Experimental analysis of scrapie infected animals

2.4.1 Inoculation with the scrapie agent

All animals were injected with the ME7 strain of the scrapie agent either intra-peritoneally (ip) or intra-cranially (ic). This strain was originally isolated from a spleen of a Suffolk sheep with natural scrapie and was passaged into Moredun random-bred mice via intra-gastric inoculation followed by passage via ic inoculation in Moredun random bred mice (Zlotnik and Rennie 1963). This was subsequently passaged nine times via ic inoculation in C57BL/Dk mice at the Neuropathogenesis Unit in Edinburgh. This strain has been extensively used as a mouse model of TSE disease and pathogenesis has been thoroughly characterised in inbred mouse lines (Bruce, Boyle et al. 2002). For ic inoculation, mice were anaesthetised with 3% fluorine gas in oxygen. Mice were injected in the right mid temporal cortex with 20 μl of 1% (wt/vol) scrapie-infected brain homogenate in physiological saline. For i.p. inoculation, 20μl of 1% (wt/vol) scrapie-infected brain homogenate was injected into the peritoneal cavity.

2.4.2 Immunohistochemical analysis of paraffin-embedded tissues

Spleens and brains from scrapie-infected mice were fixed in 2% periodate-lysine-paraformaldehyde (PLP) (0.1M sodium periodate, 0.075M D-L lysine, 2% paraformaldehyde in 0.05M PBS) and embedded in paraffin wax. Sections of 6μm in thickness were cut on a microtome. For detection of PrP in brains and spleens, sections were deparaffinised and autoclaved at 121°C for 15 minutes. Sections were
immersed in 98% formic acid for 10 minutes followed by incubation with primary antibodies 1B3 or 6H4 (Table 2.4) overnight at room temperature. Biotin-conjugated, species specific, secondary antibodies were incubated for one hour. PrP in the spleen was detected using avidin-biotin complex conjugated to AP (Vector Labs) and visualised using Vector Red. PrP in the brain was detected using avidin-biotin complex conjugated to horseradish peroxidise (HRP, Vector Labs) which was visualised using 3,3’-diaminobenzadine (DAB, Sigma). Spleens were also labelled for FDCs and B lymphocytes using antibodies listed in Table 2.4 and visualised using AP-Vector red. Brains were also labelled for activated astrocytes and microglia and visualised using AP-Vector red (Table 2.4).

2.4.3 Paraffin-embedded tissue (PET) blots

Antibodies used to detect PrPSc are polyclonal and can detect both PrP^C and PrP^Sc. To ensure labelling in infected spleens was the disease-associated PrPSc, paraffin-embedded tissue blots were used. During this procedure, treatment with PK destroys any cellular PrP^C, leaving only the pK-resistant PrPSc if present. Section of 6 μm thickness were cut onto nitrocellulose membrane Trans-blot transfer medium (Bio-Rad Laboratories, Hertfordshire, U.K.) and dried overnight at 55°C. Sections were deparaffinised and incubated overnight in 20 μg/ml pK (Sigma) in PK digest buffer (10 mM/0.01 M Tris pH 7.8, 100 mM/0.01 M Sodium Chloride, 0.1% Brij). Protein was denatured in 3 mol/L guanidine isothiocyanate (GndSCN) for 10 minutes at room temperature followed by a block with 2% Casein western blotting reagent (Roch, Welwyn Garden City, U.K.) in Tris-buffered saline-Tween (TBS-tween; 10 mM/0.01 M Tris pH 7.8, 100 mM/0.01 M NaCl₂, 0.5% Tween 20). Primary polyclonal rabbit-
anti PrP, antiserum 1B3 (Neuropathogenesis Unit, Edinburgh) was added in a 1/4000 dilution in blocking buffer and incubated for 2 hours at room temperature. Bound 1B3 was detected using goat anti-rabbit conjugated to AP (Jacksons Laboratories) and visualised using Nitro-blue tetrazolium chloride- 5-bromo, 4-chloro, 3’-indolyphosphate P-toluidine salt (NBT/BCIP) tablets (Sigma) dissolved in distilled water. Sections were imaged using a Lecia WLD MZ8 stereo light microscope (Lecia, Milton Keynes, UK)
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary antibody</th>
<th>Pre-treatment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>Rabbit anti-PrP Clone 1B3</td>
<td>Autoclave 121°C and Formic acid</td>
<td>Roslin Institute (Farquhar, Somerville et al. 1989)</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>Mouse anti-PrP Clone 6H4</td>
<td>Autoclave 121°C and Formic acid</td>
<td>Prionics, Zurich, Switzerland</td>
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<td>Citrate buffer</td>
<td>BD Biosciences PharMingen</td>
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<tr>
<td>B lymphocytes (B220)</td>
<td>Rat anti-CD45R Biotin conjugated Clone RA36A2</td>
<td>None</td>
<td>Caltag Laboratories</td>
</tr>
<tr>
<td>Microglia</td>
<td>Iba1</td>
<td>Citrate buffer</td>
<td>Abcam, Cambridge, U.K.</td>
</tr>
<tr>
<td>Activated astrocytes</td>
<td>Glial fibrillary acidic protein (GFAP) Polyclonal</td>
<td>None</td>
<td>Dako, Camebridgeshire, UK</td>
</tr>
</tbody>
</table>

Table 2.4 Detection antibodies used for immunohistochemical analysis of paraffin-embedded sections
Details of antibodies used for immunolabelling of tissue sections from paraffin embedded tissues.
CHAPTER 3

Characterisation of the CD21-cre mouse using a ROSA26 reporter strain

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3.1 Abstract

Follicular dendritic cells (FDCs) are considered to be important sites of scrapie agent accumulation in lymphoid tissues after peripheral exposure. However, models used so far have not been able to distinguish the role of the FDCs from that of all other stromal and lymphoid cells found within the lymphoid tissue. The Cr2 gene encodes complement receptor 2 (CR2) also known as CD21, is reported to be expressed only on FDCs and mature B lymphocytes. In this chapter, the CD21-cre mouse was investigated as a possible tool for creating an FDC-specific transgenic mouse model. This was achieved by crossing the CD21-cre mouse line with the ROSA26 reporter line, resulting in β-galactosidase expression in cells where Cre is activated. To restrict Cre expression exclusively to FDCs, animals were lethally γ-irradiated and reconstituted with non-transgenic WT bone marrow. Cellular localisation of Cre expression in tissues was determined by the histological detection of β-galactosidase expression. Cre was successfully activated in FDCs and B lymphocytes in the CD21-creROSA26 mouse line. Irradiation and reconstitution of CD21-creROSA26 mice with WT bone marrow successfully restricted Cre expression to the FDCs in lymphoid tissues. Data from the CD21-creROSA26 model suggests that the CD21-cre mice reconstituted with WT bone marrow are a useful tool to manipulate gene expression exclusively on FDCs.
3.2 Introduction

After peripheral infection, for example oral or ip exposure, many TSE agents undergo a stage of intense replication in the spleen and lymph nodes accompanied by high deposition of PrP^Sc (Fraser and Dickinson 1970; Clarke and Haig 1971; van Keulen, Schreuder et al. 1996). FDCs reside in the B cell follicles of peripheral lymphoid tissue and express relatively high amounts of PrP^C on their surface, expression of which is essential for scrapie pathogenesis (Klein, Frigg et al. 1998; Brown, Stewart et al. 1999). In the lymphoid tissue of scrapie affected mice strong immunolabelling for PrP^Sc is associated with FDCs (McBride, Eikelenboom et al. 1992; Bruce, Brown et al. 2000). To confirm this finding, analysis of infected lymphoid tissue at the ultrastructural level showed high accumulation of PrP^Sc on the FDC surface and around the dendrites (Jeffrey, McGovern et al. 2000). Studies using mice depleted of FDCs have shown significant increases in incubation time, delayed pathogenesis and reduced susceptibility (Mabbot 2000; Montrasio 2000; Mabbot 2002; Oldstone 2002; Mabbott, Young et al. 2003). However, in the models used so far, there has been no way to dissociate PrP^C expression on FDCs from stromal, neural and lymphoid cells within the lymphoid tissue. Therefore it is possible that FDCs themselves do not actively replicate the TSE agent, but instead accumulate it on their surface after replication on another cell type. Thus, to definitively determine the role of the FDCs in peripheral TSE pathogenesis, a model is required where manipulation of PrP^C expression is exclusively restricted to the FDC network.

The Cre-loxP system can be used to generate transgenic animals in which gene expression is tissue- or cell type-specific. This occurs through the use of a Cre-
recombinase (Cre) that cuts out or inverts any DNA that is flanked by *loxP* sites i.e. “floxed” DNA. Whereas, in non-Cre expressing cells the target DNA remains unchanged. This system can be made specific by inserting Cre after a tissue- or cell type- specific promoter (Kos 2004). In this chapter, the *CD21-cre* mouse (Kraus, Alimzhanov et al. 2004) is tested as a possible method of manipulating gene expression exclusively on FDCs. In this strain, Cre is inserted after the *Cr2* promoter, which encodes CD21. CD21 is considered to be restricted to FDCs and mature B lymphocytes. (Reynes 1985; Liu 1997; Takahashi, Kozono et al. 1997; Heggebo, Press et al. 2002). However, in humans, expression of CD21 has also been reported on a subpopulation of immature thymocytes (Tsoukas and Lambris 1988; Wagner and Hansch 2006), peripheral T lymphocytes (Fox, Jewell et al. 2006) and on human cervical epithelium (Sixbey, Lemon et al. 1986). Expression has also been reported within the mouse on a population of CD4⁺ T lymphocytes found within the mesenteric lymph node, activated granulocytes and mucosal mast cells (Gray and Skarvall 1988; Gray and Matzinger 1991; Andrasfalvy, Prechl et al. 2002; Heggebo, Gonzalez et al. 2003). In this study, to overcome expression of Cre in lymphocytes, the mice will be exposed to lethal γ-irradiation, which will deplete host bone marrow-derived cells, and subsequently reconstituted with donor, non-Cre-expressing, bone marrow. As a consequence, all Cre-expressing host-derived lymphocytes are depleted and replaced with the non-transgenic WT donor lymphocytes restricting Cre-expression to the FDCs.

Next, to determine the cellular localisation of Cre activation in *CD21-cre* mice, this line was crossed with a Rosa reporter strain. The *ROSA26* line contains a floxed STOP cassette in front of the *LacZ* gene, which contains a β-galactosidase coding
region (Mao, Fujiwara et al. 1999). In cells which activate Cre, the STOP cassette is
removed by the activity of Cre and β-galactosidase expression is switched on.
Expression of β-galactosidase can be detected using X-gal, a substrate of β-
galactosidase. Digestion of X-gal by β-galactosidase produces a blue product which
can be used to histologically locate where β-galactosidase is expressed. Lymphoid
and a large variety of non-lymphoid tissues from both the CD21-creROSA26 line and
CD21-creROSA26 animals that were lethally γ-irradiated and reconstituted with non
transgenic bone marrow from WT mice were then assessed to determine the cellular
sites of Cre expression. This analysis confirmed that Cre is activated in the FDCs and
mature B lymphocytes in CD21-cre mice and this expression is restricted to the FDC
network after irradiation and reconstitution with WT bone marrow. Therefore it was
concluded that CD21-cre mice will be a useful tool to manipulate gene expression
exclusively in FDCs.
3.3 Results

3.3.1 Production of CD21-creROSA26 mice

To determine the cellular localisation of Cre expression in the CD21-cre mouse line, CD21-cre mice were first crossed with the ROSA26 reporter line which induces the expression of β-galactosidase in cells which activate Cre. Animals were genotyped by PCR and chosen based on expression of the Cre and Rosa (LacZ) transgenes. To restrict Cre expression to FDCs, animals were given whole body, lethal, ionising radiation to remove all host lymphocytes and were subsequently reconstituted with non-transgenic, WT bone marrow (termed WT → CD21-creROSA26 mice). Cellular expression of Cre driven by the Cr2 promoter was assessed in animals with and without bone marrow reconstitution (Fig. 3.1). In the CD21-creROSA26 animals, expression of Cre induces production of the bacterial enzyme β-galactosidase in Cre expressing cells. An X-gal stain for β-galactosidase expression is used to locate β-galactosidase and assess Cre activity. Animals expressing only the ROSA26 transgene were used to control for any background β-galactosidase staining that may be present.

3.3.2 Irradiation of CD21-creROSA26 mice followed by reconstitution with WT bone marrow successfully removes Cre expression in CD21+ B-lymphocytes

Cellular sites of Cre expression in tissues from CD21-creROSA26 mice was assessed using an X-gal stain to detect β-galactosidase expression. X-gal (5-bromo, 4-chloro, 3-indolyl β-D-galactopyranoside) is a substrate of β-galactosidase. Tissue sections
Figure 3.1 Confirmation of genotype of $CD21$-cre$ROSA26$ mice

DNA was extracted from the tails of $CD21$-cre$ROSA26$, $ROSA26$ and WT $\rightarrow CD21$-cre$ROSA26$ mice. A PCR analysis was used to confirm the presence of $Cre$ and $LacZ$ transgenes. This analysis confirmed that $CD21$-cre$ROSA26$ (lanes 1-6) and WT $\rightarrow CD21$-cre$ROSA26$ (lanes 9-14) mice contained both the $Cre$ and $LacZ$ transgenes. As anticipated $ROSA26$ mice (lanes 7-8) lacked the $Cre$ transgene.
were immersed in X-gal solution which in Cre-expressing cells is catalysed by β-galactosidase to form a blue product that can be visualised using light microscopy.

B-lymphocytes and FDCs are closely compacted within the B lymphocyte follicle of lymphoid tissues. For this reason, it is difficult to distinguish β-galactosidase labelling of B lymphocytes in the follicle from that of FDC networks. However, labelling in the follicles of CD21-creROSA26 mice which received irradiation and reconstitution with WT bone marrow was greatly reduced in comparison to labelling in follicles of untreated CD21-creROSA26 animals (Fig 3.2). Therefore it can be concluded that irradiation and reconstitution with WT bone marrow successfully removes Cre-expressing CD21+ B lymphocytes and restricts Cre expression to the FDC networks in the lymphoid tissues.

Single cell labelling of β-galactosidase was detected in the villi and isolated lymphoid follicles (ILFs) of the large and small intestine in tissues from CD21-creROSA26 mice which did not receive any pre-treatment (Fig 3.5). This labelling was presumed to be intra-epithelial B lymphocytes and B lymphocytes in the capillaries, lymphatics and follicles of the intestine. However monoclonal antibodies against B lymphocyte markers were not sensitive enough to detect single cells after an X-gal stain. This type of single cell labelling was also demonstrated in the thymus and lung and was thought to be circulating lymphocytes in the capillaries of these organs. In CD21-creROSA26 mice which received the irradiation and bone marrow reconstitution, labelling of single cells within these tissues was no longer detected. Therefore it can be concluded that irradiation and reconstitution with WT bone marrow successfully removes Cre-expressing CD21+ circulating B lymphocytes.
Figure 3.2 Irradiation of CD21-creROSA26 mice and reconstitution with wild type bone marrow successfully removes Cre expressing B lymphocytes
Sections of lymphoid tissues from CD21-creROSA26 and CD21-creROSA26 given irradiation and WT bone marrow (WT → CD21-creROSA26) to remove all the transgenic CD21⁺ B lymphocytes were stained for β-galactosidase (blue) expression using an X-gal staining solution and were counterstained with nuclear fast red (pink). Lymphoid tissues from Rosa mice containing the LacZ reporter gene insert but no Cre transgene were stained as negative controls. In all lymphoid tissues from Cre expressing animals, staining of β-galactosidase is found in the B-lymphocyte follicles which contain both FDCs and CD21⁺ B lymphocytes. The levels of staining in lymphoid tissues from animals which received the irradiation and bone marrow graft is greatly reduced in comparison to those that did not receive this pre-treatment. This suggests that β-galactosidase, and therefore Cre expression, has been removed from CD21⁺ B lymphocytes in the lymphoid tissue. This implies that WT →CD21-creROSA26 mice are a useful model to study FDC-restricted gene expression.
3.3.3 Cre is activated by the Cr2 promoter in FDCs and CD21⁺ B lymphocytes

The cellular localisation of Cre expression was assessed in the lymphoid tissues, including the spleen, mesenteric, axillary and inguinal lymph nodes, thymus, Peyers patches and isolated ILFs of the gut. CD21 expression was initially reported to be restricted to CD21⁺ mature B lymphocytes and FDCs however, expression has since been reported in other bone marrow-derived cells in mice, including T lymphocytes, activated granulocytes and mast cells, however this expression was postulated to be removed by the irradiation and bone marrow reconstitution (Gray and Skarvall 1988; Gray and Matzinger 1991; Andrasfalvy, Prechl et al. 2002; Llewelyn, Hewitt et al. 2004). To determine whether Cre-mediated DNA recombination occurred in other cell types, various non-lymphoid tissues were also analysed. These included the heart, lung, liver, kidney, large intestine, small intestine, pancreas, tongue, epithelium (ear), muscle, sciatic nerve, spinal cord and brain. Results describing the detection of β-galactosidase activity in all tissues is summarised in Table 3.1

β-galactosidase activity was detected on FDC networks within the B lymphocyte follicles of all lymphoid tissues in CD21-creROSA26 mice, both with and without pre-treatment of lethal irradiation and reconstitution with WT bone marrow (Fig 3.2). Subsequently, an X-gal stain followed by immunolabelling of B lymphocytes and/or FDCs was used to determine cellular location of the β-galactosidase. The β-galactosidase labelling was found in areas of the follicle which also showed positive immunolabelling for FDC and FDC/B lymphocyte markers demonstrating that Cre recombinase is effectively activated under the Cr2 promoter in FDCs and CD21⁺ B lymphocytes (Fig. 3.3)
Figure 3.3 Cre is expressed within B lymphocyte follicles and FDC networks in CD21-creROSA26 mice
Spleen sections from CD21-creROSA26, WT → CD21-creROSA26 and ROSA26 mice, were stained for β-galactosidase (blue) and B lymphocytes (red) or FDCs (red). Immunohistochemical detection of B lymphocytes using anti-B220 monoclonal antibodies and FDCs using anti CD35 monoclonal antibodies occurs in the same area of the follicle as β-galactosidase detected by an X-gal stain. This demonstrates that Cre is successfully expressed under the CD21 promoter in FDCs and CD21-expressing B lymphocytes. Scale bar 100μm.
Table 3.1 Expression patterns of $\beta$-galactosidase in $CD21$-cre$ROSA26$ mice
Summary of $\beta$-galactosidase staining found in tissues from 6 untreated $CD21$-cre$ROSA26$ and 6 $WT \rightarrow CD21$-cre$ROSA26$ mice. Staining was concluded to be B-lymphocyte staining if it was present in the untreated group but absent in the group which received the irradiation and bone marrow. Staining was concluded to be background if it was also present in the same regions in tissues from $ROSA26$ mice. Examples of this can be seen in Figure 3.5
3.3.4 Insertion and expression of the Cre transgene under the Cr2 promoter has no toxic effect on CD21 expressing cells

Cre toxicity is a reported phenomenon that can occur in some Cre transgenic lines. In these cases, Cre recombinase targets sequences of DNA that are similar to its 24 bp target LoxP sites causing mis-recombination, DNA damage and death of the Cre-expressing cells (Schmidt-Supprian and Rajewsky 2007). Immunohistochemistry and flow cytometry was used to determine whether insertion of the Cre transgene has any adverse effects on CD21-expressing cells. Sections of spleen from CD21-creROSA26 and WT → CD21-creROSA26 mice were labelled with antibodies against FDCs and B lymphocytes and compared to spleen sections from WT and ROSA26 controls. Immunohistochemical analysis showed no differences in FDC or B lymphocyte labelling between the transgenic animals and their WT counterparts (Fig. 3.4). In addition, CD21+ B lymphocytes were isolated from spleen and counted by FACS analysis. FACS analysis of spleens from each mouse group showed no differences in either the number of CD21+ or CD19+ B lymphocytes (Fig. 3.5).

3.3.5 Detection of β-galactosidase expression in non-lymphoid tissues

Unexpectedly, β-galactosidase expression was detected in a population of cells in the ganglia of the myenteric and submucosal plexi of the large and small intestine. This staining only occurred in a small number of ganglia per section, however it was observed consistently in all of the animals (Fig 3.7). Practical difficulties were encountered in determining the precise nuclei with which the X-gal staining was associated with, possibly due to differential cellular packaging of β-galactosidase. Therefore, it could not be determined from the X-gal staining, whether this Cre-
Figure 3.4 Expression of Cre under the Cr2 promoter has no effect on FDCs and CD21⁺ B lymphocyte numbers

Immunohistochemical detection of B lymphocytes (B220, CD19, CD1d, red) and FDCs (CD35, C4, red) in spleens from double transgenic mice, with and without irradiation and reconstitution with WT bone marrow, ROSA26 and C57BL/6 lines. There is no observed difference in immunolabelling of B lymphocytes and FDCs of transgenic mice in comparison to C57BL/6 WT control line using a panel of anti-B lymphocyte and anti-FDC monoclonal antibodies. Scale bar 100 μm. Sections counterstained with haematoxylin, blue.
Figure 3.5 Expression of Cre under the Cr2 promoter has no effect on CD21\(^+\) B lymphocyte numbers

Detection of CD21 and CD19 on splenic lymphocytes from CD21-creROSA26 and C57BL/6 mice. No difference was observed in the numbers of CD19\(^+\) splenic B lymphocytes in CD21-creROSA26 mice (B) in comparison to WT controls (A). There is also no difference in CD21 expression between the transgenic line (D) and WT control (C). These data suggest that insertion and expression of Cre under the Cr2 promoter has no toxic effect on B lymphocytes numbers or the expression of CD21 or CD19.
Fig 3.6 β-galactosidase detection in cells other than FDCs
Sections from various tissues of CD21-creROSA26, WT →CD21-creROSA26 and ROSA26 transgenic animals were stained for β-galactosidase (blue) and counterstained with nuclear fast red (pink).

a. Background β-galactosidase staining was found in some tissues from Rosa animals containing no Cre transgene. These included the capsule of the spleen (A), the tubules and glomeruli of the kidney (B), paneth cells in the crypts of the intestines (C), the thymus (D), the brush borders of the bronchiole of the lung (E) and villi of large intestine (F), Brunner’s glands in the duodenum and the uro-genital tract of both male and female animals (epididymus pictured H) Original magnification D x100 A, E, F, G, H x200 B, C x400

b. Single cell β-galactosidase staining was detected in the lung (A), villi of intestine (B) and thymus (C) of CD21-creROSA26 mice but not in WT →CD21-creROSA26 mice. These were concluded to be circulating CD21+ Cre-expressing B lymphocytes which were removed after irradiation and replaced with non-Cre-expressing WT bone marrow derived CD21+ B lymphocytes. Original magnification C, x100 A, B x400
expression was in neuronal or glial cell populations within the ganglia. To clarify this, immunohistochemistry was attempted using an anti-β-galactosidase antibody along with an antibody against a pan-neuronal marker which recognises a neuronal protein named protein gene product 9.5 (PGP 9.5), to examine whether expression of these proteins were co-localised. Unfortunately, anti-β-galactosidase immunostaining using this antibody was not achieved even on positive control spleen and lymph node sections tested. An alternative method using an X-gal stain followed by immunohistochemistry for PGP 9.5 was tried instead (Fig 3.7). Although both staining of PGP 9.5 and X-gal occurred within the ganglia, it did not provide any further information on the cellular location of the X-gal staining.

The sciatic nerve and spinal cord did not show any activation of Cre. However β-galactosidase staining occurred in a population of large cells in the brain (Fig 3.7.). As with the co-localisation analysis on the plexi of the intestine, immunolabelling with anti-β-galactosidase antibodies was unsuccessful, therefore it is uncertain whether this staining is occurring within neuronal or glial cell populations. However, as irradiation and reconstitution with WT bone marrow did not remove this staining from both the intestine and the brain, it is likely that the Cre activation was within host-derived neurones rather than glial cells.
Fig 3.7 β-galactosidase labelling occurs in a population of cells in the plexi of intestine and the brain

A population of cells in the ganglia of both the submucosal (A) and myenteric (B) plexi of the intestine were found to express β-galactosidase (blue). This only occurred in a few ganglia per section of intestine, however it appeared consistently in each of the 12 animals analysed. Co-localisation analysis using anti-β-galactosidase antibodies and neuronal antibodies was unsuccessful (data not shown). Staining for β-galactosidase using an X-gal staining solution followed by immunolabelling of nerves using the monoclonal antibody PGP 9.5 (Red) (C) was not accurate enough to determine if this β-galactosidase was expressed in a neuronal or glial cell population within the ganglia.

Transverse (D) and longitudinal (E) sections of spinal cord were both clear of β-galactosidase labelling, as was the sciatic nerve (F). However a population of cells in the brain which appeared in the optic tract (G), hippocampus, (H) and corpus collosum (I) also expressed β-galactosidase. Sections are counterstained with nuclear fast red (pink). Original magnification A, C x100, B, D, E, F, G, H, I x200.
3.4 Discussion

In this chapter, the CD21-cre mouse was investigated as a possible model for restricting gene expression to FDCs. This was achieved by crossing the CD21-cre mouse with a ROSA26 reporter strain which allows the detection of Cre activity via the production of β-galactosidase. The characterisation studies undertaken on this mouse line indicate that the CD21-cre model will be a useful tool to manipulate PrP<sup>C</sup> expression specifically on FDCs and to study the role of the FDC network in TSE pathogenesis. Cre-expression under the Cr2 promoter did not appear to affect lymphoid tissue structure as determined by immunolabelling of B lymphocytes and FDCs using a panel of monoclonal antibodies against various cell-specific markers. This is in accordance with Kraus et al (2004), who reported that insertion and expression of Cre under the Cr2 promoter does not interfere with B lymphocyte development or numbers (Kraus, Alimzhanov et al. 2004).

The detection of β-galactosidase expression using the ROSA26 reporter strain has shown that Cre-mediated DNA recombination is occurring efficiently on both FDCs and B lymphocytes. Although DNA recombination on B lymphocyte subsets was not examined, previous studies suggest that mature B lymphocytes express the highest levels of CD21, while immature and pro- and pre- B lymphocytes express low levels or none, respectively (Takahashi, Kozono et al. 1997). Studies on the CD21-cre line by Victoratos et al indicate that efficiency of this DNA recombination is 75% on B lymphocytes and 96% in FDCs (Victoratos, Lagnel et al. 2006). This was reflected in this characterisation study as all CD21-creROSA26 animals showed efficient expression of β-galactosidase in the FDCs and B lymphocytes in a variety of lymphoid tissues tested.
Irradiation and reconstitution with WT bone marrow successfully restricted Cre expression to the FDC networks within the lymphoid tissue. The animals were lethally $\gamma$-irradiated (950 rads) and reconstituted with age and sex matched bone marrow 24 h later. Animals were maintained for 100 days post bone marrow transfer before analysis of tissues. Previous studies have shown that a minimum of 28 days is required for efficient re-differentiation of the FDC networks and repopulation of lymphocytes from donor bone marrow (Brown, Stewart et al. 1999). However, populations of long-lived B lymphocytes have also been reported, therefore animals were left for 100 days post irradiation and bone marrow transfer to ensure removal of the majority of host derived lymphocytes (Miller and Cole 1967).

Unfortunately FACS analysis of B lymphocytes using anti-$\beta$-galactosidase monoclonal antibodies was attempted for both cell surface and intracellular $\beta$-galactosidase but was unsuccessful. Fluorescent immunolabelling of tissue sections using the same antibodies also did not work. This is possibly because all tissues taken were fixed specifically for staining with X-gal in fixative which included detergents to permeabilise tissues. However when comparing levels of X-gal staining between $CD21$-$creROSA26$ animals with $CD21$-$creROSA26$ animals which were irradiated and given bone marrow, staining was greatly reduced in the irradiated animals suggesting that expression of $\beta$-galactosidase by B lymphocytes had been removed.

Staining of $\beta$-galactosidase in non-lymphoid tissues was also detected, for example in the kidney, lung, and intestine (Table 3.1). This is thought to be Cre-activation on circulating B lymphocytes as this staining was absent in $CD21$-$creROSA26$ animals which were irradiated and re-grafted with WT bone marrow. In agreement with this,
Kraus et al performed analysis of Cre-mediated DNA recombination on B lymphocyte populations isolated from various tissues and found intermediate levels of recombination in the kidney and gut samples, confirming the presence of Cre-mediated DNA recombination in CD21⁺ B lymphocytes found in non-lymphoid tissues (Kraus, Alimzhanov et al. 2004). As the irradiation and reconstitution with WT bone marrow removes all Cre expressing lymphocytes, CD21-expressing cells within these tissues should have no impact on the specificity of the model. Expression of CD21 has also been reported on activated granulocytes and mast cells in mice, however again, any Cre expression in these cell types will be eliminated by the irradiation and bone marrow reconstitution pre-treatment (Gray and Matzinger 1991; Ahmed and Gray 1996).

β-galactosidase expression on a small subset of cells in the thymus was also consistently detected. A population of immature human thymocytes has been reported to express CD21 and additionally in the mouse, CD21 expressing activated T lymphocytes have been reported (Gray and Skavall 1988; Tsoukas and Lambris 1988; Llewelyn, Hewitt et al. 2004). However, a study analysing CD21 expression in the mouse found that CD21 expressing cells in the thymus always co-localised with B lymphocyte markers, and suggested that these were the rare thymic B lymphocyte pool (Takahashi, Kozono et al. 1997). Again, this labelling was eliminated after irradiation and reconstitution with WT bone marrow.

Expression of β-galactosidase was detected in a population of neuronal or glial cells in the brain. β-galactosidase labelling was detected in large cells of the hippocampus, corpus colossom, dentate gyrus, coroid plexus and occurs diffusely throughout the
cortex. As monoclonal antibodies against β-galactosidase did not work on these tissues, it was not possible to do double immunolabelling and co-localisation analyses to determine which subset of cells were expressing Cre. This Cre-mediated DNA recombination was still present in animals which received irradiation and bone marrow reconstitution. The manipulation of gene expression in these cells in CD21-cre mice should have no impact in the early stages of TSE agent accumulation in the spleen as the agent does not reach the brain until the late stages of the incubation period. However, this may need to be taken into account for any animals that are left to progress to CNS disease as manipulation of PrP expression in these neurones may affect disease outcome.

Expression of β-galactosidase was also found in a population of cells within the ganglia of the myenteric and submucosal plexi of both the large and small intestine. For reasons stated previously, it was not possible to determine whether this staining occurred in a neuronal or glial cell population. These cells are reasonably close to the sites of initial prion accumulation in oral inoculation. Expression of β-galactosidase was only found in a few ganglia of these plexi per section of intestine but was expression was consistent in most of the animals. Sciatic nerve and spinal cord did not show any labelling for β-galactosidase.

A study by Gonzalez et al investigated the role of the enteric nervous system in oral inoculations with the murine BSE strain 301V. They observed strong labelling for PrP on both the myenteric and submucosal plexus of the inoculated mice, however, much of this was removed after PK treatment indicating that it was mainly the cellular PrP$^C$ that was being detected (Gonzalez, Terry et al. 2005). Due to the detection of
Cre-mediated DNA recombination within the intestine, the ip route was chosen as the route of TSE agent exposure in future studies to avoid possible effects of Cre-mediated DNA recombination in enteric ganglia.

In conclusion, these data show the CD21-cre model will be a useful tool to manipulate Prnp expression exclusively on FDCs to determine the role of FDCs in scrapie pathogenesis after ip inoculation. Cre-mediated DNA recombination occurred efficiently on the FDC networks and mature B lymphocytes. Furthermore, irradiation and repopulation with non-Cre-expressing bone marrow successfully eliminated the expression of Cre on B-lymphocytes within the lymphoid tissues.
CHAPTER 4

Characterisation of the CD21-crePrP^{fl/-} mouse line

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4.1 Abstract

Data from the CD21-creRosa26 model (Chapter 3) suggests that CD21-cre mice reconstituted with WT bone marrow are a useful tool to manipulate gene expression exclusively on FDCs. PrP<sup>C</sup> expressing FDCs are considered to be important sites of scrapie agent accumulation in lymphoid tissues after peripheral exposure. However, models used so far have not been able to definitively distinguish the role of the FDCs from that of all other stromal, lymphoid and neural cells found within the lymphoid tissue. For this reason, it is possible that rather than actively replicating the scrapie agent, FDCs may simply accumulate the agent after replication on another cell type. To generate a model which may be used to determine the specific role of FDCs in peripheral scrapie pathogenesis the CD21-cre mouse line was crossed with a line where the coding region of PrP<sup>C</sup> is flanked by loxP sites (PrP<sup>fl/fl</sup>) allowing PrP<sup>C</sup> to be switched off under control of the Cr2 promoter. The progeny CD21-crePrP<sup>fl</sup> mice were fully characterised to determine within which cells PrP<sup>C</sup> was switched off and ensure that the transgenes had no additional effects on the lymphoid tissues that could have an impact on scrapie pathogenesis. Characterisation of the CD21-crePrP<sup>fl</sup> mouse demonstrated that PrP<sup>C</sup> was efficiently and exclusively switched off on Cre-expressing FDCs. Furthermore, insertion and expression of the transgenes had no adverse effects on the microarchitecture of the lymphoid tissue or the number, area or functionality of FDCs. These data suggest that the CD21-crePrP<sup>fl</sup> mouse will be a useful tool to study the role of FDCs in scrapie pathogenesis.
4.2 Introduction

Expression of the cellular prion protein, PrPC, is essential for efficient scrapie pathogenesis to occur (Bueler, Fischer et al. 1992; Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997; Klein, Frigg et al. 1998; Brown, Stewart et al. 1999; Wroe, Pal et al. 2006). In the lymphoid tissue of scrapie affected mice strong immunolabelling for PrPSc is associated with FDCs (McBride 1992; Bruce, Brown et al. 2000; Jeffrey, McGovern et al. 2000). Previous studies have shown that expression of PrPC on FDCs is essential for scrapie pathogenesis to occur after peripheral exposure (Brown, Stewart et al. 1999). Previous data suggests that FDCs are responsible for replicating the scrapie agent within the lymphoid tissue prior to neuroinvasion and CNS disease. However in the models used so far, there has been no way to dissociate PrPC expression on FDCs from all other stromal, neural and lymphoid cells within the lymphoid tissue. Therefore it is possible that FDCs themselves do not actively replicate the TSE agent, but instead accumulate it on their surface after replication on another cell type. Data in this thesis (Chapter 3) shows that the CD21-creROSa26 mouse efficiently activated Cre-mediated DNA recombination in the FDCs and this could be made specific by lethal γ-irradiation and reconstitution with non-Cre expressing bone marrow. Therefore this model was crossed with a transgenic floxed PrP line to allow PrPC to be switched off exclusively on FDCs.

The coding region for PrPC is contained within exon 3 of the Prnp gene. In PrPfl/fl mice, exon 3 of Prnp is flanked by loxP sites i.e. “floxed” exon 3 (Tuzi, Clarke et al. 2004). This mouse strain was crossed with the CD21-cre mouse to allow PrPC to be switched off under control of the Cr2 promoter. Based on data from the CD21-
creROSA26 model, the CD21-crePrP\textsuperscript{fl} mouse together with lethal \(\gamma\)-irradiation and reconstitution with non-Cre-expressing bone marrow, should allow PrP\textsuperscript{C} to be switched off exclusively on FDCs. In this chapter, the CD21-crePrP\textsuperscript{fl} line was characterised to determine if PrP\textsuperscript{C} was successfully switched off on the FDC networks and that the transgenes had no additional affects on the lymphoid tissue. Analysis of the CD21-crePrP\textsuperscript{fl} line showed that PrP\textsuperscript{C} was efficiently switched off on Cre-expressing FDCs however expression remained present on other non-CD21-expressing cells in the lymphoid tissues such as the nerves. Furthermore, there were no differences in the microarchitecture and the number, area and function of FDCs in the spleen. From these data, it can be concluded that the CD21-cre PrP\textsuperscript{fl} line will be a useful tool to study scrapie pathogenesis when PrP\textsuperscript{C} has been switched off exclusively on FDCs.
4.3 Results

4.3.1 Production of CD21-cre PrP^{fl/fl} mouse line

To switch off PrP C in FDCs the CD21-cre line was crossed with the PrP^{fl/fl} line, and progeny selected by screening for expression of Cre and Prnp^{fl} by PCR. Progeny mice were then bred together, further selecting for Cre and Prnp^{fl} expression until sufficient CD21-crePrP^{fl/fl} mice were obtained for use in characterisation studies. During this process, the results of the genotyping indicated there was a problem and DNA was extracted from a number of tissues to assess where Prnp^{fl} DNA was being recombined in the animals. In CD21-crePrP^{fl/fl} animals, the PCR results should show partial recombination of Prnp^{fl} DNA in the lymphoid tissues due to the presence of CD21{\textsuperscript{+}} B lymphocytes and FDCs, and also a band for un-recombined Prnp^{fl} due to the presence of non-CD21-expressing cells in the tissues. This is termed “partial recombination” of Prnp^{fl} DNA. In the first mouse analysed, partial recombination of Prnp^{fl} DNA was found in the spleen, mesenteric lymph node (MLN) and inguinal lymph node (ILN) as expected. However partial recombination was also detected in the liver, kidney, brain and ear but not in the heart or lung (Figure 4.1). This suggested that Cre-mediated DNA recombination was not occurring under control of the Cr2 promoter. Tissues analysed from animals taken from subsequent rounds of breeding showed full recombination of the Prnp^{fl} DNA with no un-recombined Prnp^{fl} present (Figure 4.1). This spontaneous recombination of the Prnp^{fl} DNA resulted in a Prnp deficient mouse as all Prnp^{fl} DNA was recombined to remove exon 3 of the Prnp gene, switching off PrP C expression in all cell types. As a consequence, this line could no longer be used in subsequent scrapie experiments.
4.3.2 Production of CD21-crePrP\textsuperscript{fl/null} mouse line

To compensate for the spontaneous recombination occurring in the PrP\textsuperscript{fl/fl} homozygous mice, the CD21-cre line was subsequently bred as Prnp\textsuperscript{flxed} heterozygotes on a PrP\textsuperscript{c/-} background. To achieve this, the CD21-cre line was first crossed with a PrP\textsuperscript{c/-} line. The resulting CD21-creXPrP\textsuperscript{c/-} mice were then crossed with the PrP\textsuperscript{fl/fl} line and CD21-crePrP\textsuperscript{fl/-} progeny were used in subsequent experiments.

To switch off PrP\textsuperscript{C} exclusively in FDCs, CD21-crePrP\textsuperscript{fl/-} animals were lethally $\gamma$-irradiated and given bone marrow from age- and sex-matched Cre negative littermates (termed Cre\textsuperscript{-ve} $\rightarrow$ CD21-crePrP\textsuperscript{fl/-}). Experimental control groups were also produced. These included, CD21-crePrP\textsuperscript{fl/-} mice given bone marrow from Cre\textsuperscript{+ve} littermates, (termed Cre\textsuperscript{+ve} $\rightarrow$ CD21-crePrP\textsuperscript{fl/-}), in which PrP\textsuperscript{C} will be switched off in both FDCs and CD21\textsuperscript{+} B lymphocytes. Also, PrP\textsuperscript{fl/-} mice were given bone marrow from CD21-cre PrP\textsuperscript{fl/-} mice, (termed Cre\textsuperscript{+ve} $\rightarrow$ PrP\textsuperscript{fl/-}), in which PrP\textsuperscript{C} is switched off on CD21\textsuperscript{+} B lymphocytes only. The final control group was PrP\textsuperscript{WT/-} mice given PrP\textsuperscript{WT/-} bone marrow. The copy number of the Prnp gene has been shown to have a strong influence in scrapie incubation period, with PrP\textsuperscript{WT/-} heterozygous mice having almost double the incubation time of PrP\textsuperscript{WT/WT} homozygotes (Manson, Clarke et al. 1994b) Therefore, this control group was included due to the CD21-crePrP\textsuperscript{fl/-} experimental line having half levels of PrP\textsuperscript{C} expressed.
Figure 4.1 Spontaneous recombination of Prnpfl DNA in CD21-crePrPfl/fl mice

DNA was extracted from a variety of tissues from CD21-crePrPfl/fl mice and genotyped by PCR to determine in which tissues Prnpfl DNA was recombined. Tissues analysed included liver (1), kidney (2), heart (3), lung (4), spleen (5), ILN (6), MLN (7), brain (8), ear (9) and gut (10). PCR results show a band of 167 bp for Prnp WT (c), a band of 210 bp for Prnpfl (a), and a band of 344 bp for Prnpfl(R) recombined DNA. Heterozygous PrPfl/WT or PrPfl/- animals have bands at 167 bp and 210 bp (b). Homozygous PrPfl/fl animals with partial recombination of the Prnpfl DNA show bands at 210bp and 344 bp (d) whereas heterozygous PrPfl/WT or PrPfl/- animals with partial recombination of the Prnpfl DNA show all three bands (e).

Tissues from the first animal assessed, CD21-crePrPfl/fl 1, showed partial recombination of Prnpfl DNA in the spleen and lymph nodes as expected, but also in the liver and kidney. Tissues from an animal taken after subsequent rounds of breeding, CD21-crePrPfl/fl 2, showed full recombination of Prnpfl DNA in all tissues. This demonstrates that the Cr2 promoter has lost control of Cre-mediated recombination of Prnpfl DNA. These animals are now PrP-/- as all Prnpfl DNA has been recombined.
Bone marrow chimera status of experimental animal groups was confirmed by PCR analysis (Fig 4.2). DNA was extracted from blood, tail and spleen and was genotyped for both Cre and Prnp<sup>fl</sup> with or without Cre-mediated recombination of the Prnp<sup>fl</sup> DNA. This analysis confirmed that the host/ donor genotypes were as expected and also that Prnp<sup>fl</sup> DNA was only recombined in tissues where both the Cre transgene and CD21-expressing cells were present.

4.3.3 Insertion and expression of transgenes and bone marrow reconstitution have no effect on lymphoid tissue microarchitecture

In addition to confirming that Cre-mediated recombination was restricted to CD21-expressing cells and was occurring efficiently, it was also essential to ensure that there were no other changes to the lymphoid tissue that could mediate an effect on scrapie pathogenesis. Spleens from all four groups of experimental mice were immunolabelled to detect B lymphocyte subsets, T lymphocytes, FDCs and dendritic cells and compared tissues from WT animals (Fig 4.3). No differences could be seen in intensity or location of immunolabelling for each cell type between WT and experimental spleens. It can be concluded that neither expression of the transgenes nor irradiation and bone marrow reconstitution has any significant effect on the microarchitecture of the lymphoid tissues. Furthermore, spleen sections taken from CD21-crePrp<sup>fl</sup>Cre<sup>-ve</sup>Prp<sup>fl</sup> and WT 129/Ola mice were immunolabelled using an anti-CD35 MAb to detect the FDC networks. FDC networks in the spleen were manually counted and the area of FDC networks in the spleen was measured using ImageJ image analysis software (Inman, Rees et al. 2005). This quantification demonstrated that there were no significant differences in the number or area of FDC
a. The Cre transgene was present in the spleen and tail of CD21-crePrP\textsuperscript{fl}\textsuperscript{-} mice but was only present in the blood if Cre\textsuperscript{+ve} donor bone marrow had been given. PrP\textsuperscript{fl}\textsuperscript{-} mice had no Cre transgene detected in tail DNA, however Cre was present in the spleen and blood due to Cre\textsuperscript{+ve} bone marrow given.

b. Tissues from all animals contained Prnp\textsuperscript{fl} and Prnp\textsuperscript{-} bands. However, recombined Prnp\textsuperscript{fl(R)} DNA was only detected in tissues where both the Cre transgene and CD21-expressing cells are present.

Figure 4.2 Genotypes of CD21-crePrP\textsuperscript{fl}\textsuperscript{-} animals used for characterisation and subsequent scrapie experiments
DNA was extracted from blood (B), spleen (S), and tail (T) from 6 animals from each of the transgenic lines to be used in subsequent scrapie experiments. One example of genotype results for Cre\textsuperscript{-ve} → CD21-crePrP\textsuperscript{fl}\textsuperscript{-}, Cre\textsuperscript{+ve} → CD21-crePrP\textsuperscript{fl}\textsuperscript{-} and Cre\textsuperscript{+ve} → CD21-crePrP\textsuperscript{fl}\textsuperscript{-} lines is shown on the above gels.
networks or differences in the number or area of FDC networks between transgenic mice and WT controls with \( P=0.221 \) and 0.091 respectively using a one-way ANOVA test (Fig 4.4). Taken together, these results show that insertion of the Cre transgene under the Cr2 promoter has no adverse effects on the number and size of the FDC networks, or the general microarchitecture of the surrounding lymphoid tissue.

### 4.3.4 PrP\(^C\) immunolabelling is removed on Cre-expressing FDCs

Spleens from all experimental and control lines were immunolabelled to detect PrP\(^C\) expression to ensure that PrP\(^C\) protein was specifically removed from the FDC network in animals that expressed the Cre transgene. Within the lymphoid tissues, PrP\(^C\) can be detected on FDCs and peripheral nerves using immunolabelling. Therefore, to determine if removal of PrP\(^C\) was specific to the FDC network, spleen sections were fluorescently immunolabelled for PrP\(^C\), FDCs and peripheral nerves. Co-localisation of fluorescent labelling was then quantified using Image J software. Animals which expressed Cre showed removal of PrP\(^C\) specifically on the FDC networks but not on the peripheral nerves (Fig 4.5). Quantification of co-localised pixels showed that the co-localisation detected was significant co-localisation (\( P=5.4 \times 10^{-30} \) \(*\) and \( 6.5 \times 10^{-22} \) \(**\), Fig 4.6) and not background due to random association of pixels. Animals with PrP\(^C\) switched off on FDCs had statistically significantly lower levels of PrP\(^C\) staining in comparison to non-Cre expressing spleens (\( P< 1.0 \times 10^{-23} \) and \( 9.0 \times 10^{-24} \), Fig 4.6).
Figure 4.3 Insertion and expression of the transgenes and irradiation and bone marrow reconstitution has no effect on the microarchitecture of peripheral lymphoid tissues

Frozen spleen sections from all experimental transgenic mouse lines were immunolabelled for B lymphocyte subsets (CD45R and CD1d), T lymphocytes (CD3), FDCs (C4 and CD21/35), classical DCs (CD11c) and marginal zone cells (MADCAM-1 and CD1d). Comparison of sections from transgenic animals with WT controls showed no differences in the number or location of cell subsets within the spleen. Scale bar on fluorescent images 100 μm. Scale bar on light microscopy images 500 μm and sections counterstained with haematoxylin, blue.
Fig 4.4 Expression of transgenes has no effect on the number or area of FDC networks in the spleen

Frozen spleen sections from WT, PrP^fl/^- and CD21-crePrP^fl/^- animals were immunolabelled for FDCs using an anti CD35 monoclonal antibody. For each mouse line, 4 spleen sections 50 μm apart were immunolabelled and 4 images of 900.47 μm^2 were taken per section.

**a.** FDC networks were manually counted for each image and the average number of FDC networks per image was calculated. No significant difference was observed in the number of FDC networks per 900.47μm^2 of spleen in transgenic animals in comparison to WT controls using a one-way anova (p=0.221)

**b.** The area of FDC networks per image was determined using ImageJ. This uses the total number of positive immunolabelled pixels per image to calculate a percentage area of a 900.47μm^2 of spleen positive for FDC network immunolabelling. No significant difference was observed in the percentage area of FDC immunolabelling in transgenic spleens in comparison to WT controls using a one-way anova (p=0.091)
4.3.5 Insertion and expression of transgenes and irradiation and bone marrow reconstitution have no effect on the distance between FDC networks and peripheral nerves in the spleen

Previous studies have shown that the distance between peripheral nerves and FDC networks can influence scrapie incubation period after peripheral inoculation (Glatzel, Heppner et al. 2001; Prinz, Heikenwalder et al. 2003). Therefore immunolabelling was used to further characterise the microarchitecture of lymphoid tissues of transgenic mice by measuring the distance between peripheral nerves and FDCs in the spleen. Frozen sections were immunolabelled for FDCs and peripheral nerves and LSM image browser software was used to measure the distance between them (Fig 4.7). Comparison of the average distance between FDCs and the nearest peripheral nerve showed there was no statistically significant difference in the distance between FDCs and peripheral nerves between transgenic spleens in comparison to WT counterparts (P=0.765, Fig4.7).

4.3.6 Insertion and expression of transgenes has no effect on immune complex trapping function of FDCs

A major function of FDCs in the immune system is to trap and retain antigen-containing immune complexes. In scrapie infection, removal of complement components C3 or C1q can significantly delay incubation period, suggesting that complement-mediated binding of scrapie agent to FDC networks is one possible method of scrapie agent localisation to the follicle (Cardone and Pocchiari 2001; Klein, Kaeser et al. 2001; Mabbott, Bruce et al. 2001; Mabbott 2004; Sim, Kishore et al. 2007). To ensure that FDCs in transgenic animals were not functionally
Fig 4.5 PrP\textsuperscript{C} expression is removed specifically on FDC networks in animals which express Cre

Immunolabelling of PrP\textsuperscript{C} (blue) on FDC networks \(\triangle\) (CD35/red) and peripheral nerves \(\equiv\) (Tyrosine hydroxylase/green) in spleen sections from transgenic mouse lines. Animals were lethally \(\gamma\)-irradiated, received donor bone marrow 24 h later and were culled 100 days post reconstitution to allow replacement of all host-derived lymphocytes. Spleen sections from 6 animals from each line were labelled and analysed. \textit{CD21}-crePrP\textsuperscript{fl/-} animals which received Cre\textsuperscript{-ve} (A) or Cre\textsuperscript{+ve} (B) bone marrow had no PrP\textsuperscript{C} labelling on their FDC networks however PrP\textsuperscript{C} labelling was detected on the peripheral nerves. These data demonstrate that expression of PrP\textsuperscript{C} has been removed specifically on the Cre-expressing FDCs. PrP\textsuperscript{fl/-} animals which received Cre\textsuperscript{+ve} bone marrow (C) and PrP\textsuperscript{WT/-} animals which received PrP\textsuperscript{WT/-} bone marrow (D) show PrP\textsuperscript{C} labelling on both FDC networks and peripheral nerves. Scale bar 100 \(\mu\text{m}\).
**Fig 4.6** Expression of Cre in FDCs under the Cr2 promoter specifically removes PrP<sup>C</sup> immunolabelling on FDC networks

Frozen spleen sections were immunolabelled for PrP<sup>C</sup>, FDC and peripheral nerves. For each mouse line, spleens were taken from 6 animals. Two sections, 50μm apart, were immunolabelled for each spleen and 4 images per section were taken. This resulted in the analysis of 48 images for each mouse line using ImageJ image analysis software. For each image the number of pixels of each colour were counted using the multiple colour analysis macro allowing values to be obtained for total number of red (FDC) blue (PrP<sup>C</sup>) and magenta (PrP<sup>C</sup> co-localised with FDC) pixels per image. These values allowed the calculation of the average percentage of FDC area co-localised with PrP<sup>C</sup>. In *CD21-crePrP<sup>fl</sup>/* animals, which have Cre-expressing FDCs this value is almost 0 and is significantly lower than PrP<sup>fl</sup>/* or PrP<sup>WT</sup>/* animals which do not have Cre-expressing FDCs. These data show that PrP<sup>C</sup> has been switched off specifically on the Cre-expressing FDCs.
a. 

\[ \text{Cre}^{\text{ve}} \rightarrow CD21-\text{crePrP}^{\text{flc}} \]

b. 

Distance between peripheral nerves and FDCs in the spleen

\[ \text{Cre}^{\text{ve}} \rightarrow CD21-\text{crePrP}^{\text{flc}} \]

\[ \text{Cre}^{\text{ve}} \rightarrow \text{PrP}^{\text{flc}} \]

\[ \text{PrP}^{\text{WTc}} \rightarrow \text{PrP}^{\text{WTc}} \]

Fig 4.7 Insertion and expression of the transgenes has no effect on the distance between peripheral nerves and FDCs in the spleen

\[ \text{a.} \quad \text{Frozen spleen sections from transgenic and WT lines were immunolabelled for FDC networks (CD35/red) and peripheral nerves (Tyrosine hydroxylase/green). For each mouse line, spleens were taken from 6 animals. Two sections, 50 μm apart, were immunolabelled for each spleen and 4 images per section were taken. This resulted in 48 images analysed for each mouse line. Scale bar 50 μm.} \]

\[ \text{b.} \quad \text{The distance between the FDC networks and peripheral nerves was measured using LSM image browser software (Zeiss). The average distance between nerves and FDCs was calculated for each mouse line. Analysis using a one-way anova test determined there was no significant difference in the distance between the FDC networks and peripheral nerves in the spleens of transgenic lines in comparison to WT controls (p=0.765).} \]
impaired, immune complex trapping was assessed and compared with that of WT animals. Transgenic and WT control lines were injected with pre-formed rabbit PAP immune complexes. Animals were culled 24 hours later and spleens were harvested for analysis. Frozen sections were stained for rabbit immunoglobulin (Ig) and co-localisation of rabbit Ig on FDC networks was measured using ImageJ software as described previously. There were no observed differences in levels of immune complex trapping by FDCs between transgenic animals and WT controls (P=0.085, Fig 4.8). Uninjected control animals showed no immunolabelling of rabbit Igs on the FDC networks. These data show that insertion and expression of Cre under the Cr2 promoter has no effect on FDC immune complex trapping function.
Figure 4.8 Insertion and expression of transgenes has no effect on immune complex trapping by FDCs

Transgenic and WT mice were injected with preformed rabbit peroxidise-anti-peroxidase (PAP) immune complexes (n=4). Spleens were harvested 24 h later for analysis. Uninjected controls for each line (n=2) were also collected.

a. Frozen sections were immunolabelled for FDCs (CD35/green) and rabbit Ig (red) to detect co-localisation of PAP on FDC networks. Original magnification x 200.

b. Images were analysed for co-localisation using an ImageJ macro described previously (Fig 4.6). For each animal, two spleen sections 50 μm apart were immunolabelled and 5 images were taken per section. A total of 40 images per mouse line for PAP-injected, and 20 images per mouse line for uninjected controls, were analysed for co-localisation. There is no significant difference in the percentage area of FDC co-localised with PAP between WT and transgenic lines. From this data, it can be concluded that insertion and expression of the transgenes has no functional effect on FDC immune complex trapping.
4.4 Discussion

The CD21-cre line was crossed with a PrP^{fl/fl} line to create a model where PrP^C could be switched off exclusively on FDCs. In this chapter the CD21-crePrP^{fl} line was extensively characterised to determine if it would be an efficient tool to study the role of FDCs after peripheral scrapie exposure. The characterisation studies carried out on this line indicate that PrP^C was switched off specifically on Cre-expressing FDCs and remained present on other non-Cre-expressing cells within the lymphoid tissue such as the nerves. Furthermore, insertion and expression of the transgenes had no additional effects on the lymphoid tissue that could influence scrapie pathogenesis.

The first breeding strategy used to create this line with homozygous PrP^{fl/fl} resulted in the Cr2 promoter losing control of Cre expression and all floxed Prnp DNA within the animal was recombined, resulting in a PrP deficient animal. As multiple generations of mice were produced containing both the Cre and the floxed Prnp transgenes, it is possible that Cre was expressed in the germline cells. If this occurs and both the Cre and loxP transgenes are inherited from one parent, this can result in Cre being expressed in all tissues and complete recombination of the floxed DNA throughout the animal (Schmidt-Supprian and Rajewsky 2007). To compensate for this, the CD21-cre line was crossed with a PrP^{+/+} line and resulting CD21-crePrP^{+/+} progeny were crossed in one generation with the PrP^{fl/fl} line to create CD21-crePrP^{fl/-} line. By eliminating generations of mice which had both the Cre and loxP transgenes present, the problem of Cre-mediated DNA recombination in the entire animal was also eliminated. Therefore the CD21-crePrP^{fl/-} line was used in subsequent characterisation and scrapie infection experiments.
PrP$^C$ expression was successfully switched off in the FDCs of Cre-expressing animals. PCR analysis of DNA extracted from spleens confirmed the presence of the Cre transgene and recombination of the Prnp$^{fl}$ transgenes. Immunolabelling of spleen sections confirmed that PrP$^C$ was removed on FDCs but present on non-CD21 expressing cells such as peripheral nerves, leaving little/no PrP$^C$ detectable by immunolabelling. This is in agreement with previous studies using the CD21-cre line, which measured 96% of floxed DNA was recombined in CD21-cre mouse line crosses (Victoratos, Lagnel et al. 2006).

Characterisation of the lymphoid tissues of transgenic experimental lines was essential to ensure that any differences in scrapie pathogenesis were due to removal of PrP$^C$ on the FDC and not due to changes in the lymphoid tissues caused by the insertion of the transgenes. Depletion of CD11c$^+$ cells and B lymphocytes has been shown to delay the onset of peripherally acquired scrapie (Klein, Frigg et al. 1997; Raymond, Aucouturier et al. 2007). Therefore, various cell subsets were immunolabelled on spleen sections from transgenic experimental and WT lines to determine if there were any differences in microarchitecture caused by insertion and expression of the transgenes. No observable difference could be seen in the number or location of B lymphocytes, T lymphocytes, dendritic cells or marginal zone cells in the spleen.

The presence of mature FDC networks in the spleen is also essential for efficient scrapie pathogenesis and changes in the number or cellular location of FDCs can alter scrapie pathogenesis (Brown, Stewart et al. 1999; Mabbott, Williams et al. 2000a; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Prinz, Heikenwalder et
al. 2003). Analysis of FDC networks in the spleens of transgenic mice showed there were no differences in the number and area of FDC networks in comparison to WT controls. The function of the FDC networks in transgenic mice was also analysed by measuring their ability to trap pre-formed immune complexes. No measureable differences in the level of immune complex trapping on FDCs of transgenic lines in comparison to WT controls was detected indicating that insertion and expression of the transgenes had no effect on FDC function. The distance between peripheral nerves and FDC networks can also influence scrapie incubation period (Glatzel, Heppner et al. 2001; Prinz, Heikenwalder et al. 2003). This was measured in spleen sections from transgenic experimental lines and WT controls and no significant difference was measured in the distance between the FDC networks and nerves.

Taken together, these data show that the insertion and expression of the transgenes in experimental mice has no effects on the microarchitecture of the spleen or the number or distribution of various cell subsets found within. These data confirm previous studies using the CD21-cre mice which did not report any toxic effects caused by the Cre transgene and showed no differences in FDC and B lymphocyte numbers or location in the spleen (Kraus, Alimzhanov et al. 2004; Victoratos, Lagnel et al. 2006; Schenten, Kracker et al. 2009). Additionally, characterisation of the PrP<sup>−/−</sup> line has shown no overt phenotype even though PrP<sup>C</sup> is ubiquitously expressed (Manson, Clarke et al. 1994a).

Characterisation of the CD21-crePrP<sup>0/0</sup> mouse line has shown that PrP<sup>C</sup> is efficiently removed in Cre-expressing FDCs but remains present on other cell types within the lymphoid tissues. Furthermore, insertion and expression of the transgenes have no
additional effects on the cellular composition or structure of the lymphoid tissue. Therefore the CD21-crePrP^{fl/fl} mouse line is an effective model to study the role of FDCs after peripheral exposure to scrapie. This line will be used to determine if PrP^{C} expressing FDCs are required for efficient scrapie agent replication in the spleen (Chapter 5).
CHAPTER 5

Effect of FDC-restricted PrP ablation on TSE agent accumulation in the spleen

5.1 Abstract

5.2 Introduction

5.3 Results

5.3.1 Experimental design

5.3.2 Effect of FDC-restricted PrP ablation on TSE agent accumulation in the spleen at 35 days post-exposure

5.3.3 Effect of FDC-restricted PrP ablation on TSE agent accumulation in the spleen at 70 days post-exposure

5.3.4 FDC-restricted PrP ablation has no effect on TSE disease when infection is established directly in the CNS

5.3.5 No PrPSc accumulation upon the FDC network in spleens of clinically-scrapie-affected mice with FDC-restricted PrP ablation

5.4 Discussion
5.1 Abstract

After peripheral exposure to TSE agents, PrP<sub>Sc</sub> and infectivity usually accumulates on FDCs in the lymphoid tissues. Models used so far have not been able to definitively distinguish the role of the FDCs from that of all other stromal, lymphoid and neural cells found within the lymphoid tissue. For this reason, it is possible that rather than actively replicating the scrapie agent, FDCs may simply accumulate the agent after replication on another cell type within the lymphoid tissue. Characterisation of the CD21-crePrP<sup>0/-</sup> model (Chapter 4) confirmed that CD21-crePrP<sup>0/-</sup> mice reconstituted with WT bone marrow switch off PrP<sup>C</sup> expression exclusively on FDCs. These mice were inoculated ip with the ME7 scrapie agent to determine if FDCs simply acquire the TSE agent on their surface.

After initial localisation of the scrapie agent to the FDC network, animals with Cre-expressing, PrP<sup>C</sup> deficient FDCs were unable to replicate the scrapie agent in the lymphoid tissue when inoculated ip with ME7 scrapie. In the absence of replication, PrP<sub>Sc</sub> was scavenged from the FDC network by TBMs and possibly degraded. Furthermore, animals with Cre-expressing, PrP<sup>C</sup> deficient FDCs inoculated ic with ME7 scrapie were susceptible to scrapie infection and had positive neuropathology and PrP<sub>Sc</sub> deposition in their brains. However, spleens from these animals remained free from PrP<sub>Sc</sub> accumulation. In conclusion, these data show that PrP<sup>C</sup>-expressing FDCs actively replicate the TSE agent in the periphery prior to neuroinvasion and do not simply accumulate infectivity on their surface after replication on another cell type.
PrP\textsuperscript{C} expression by FDCs is considered essential for effective scrapie pathogenesis after peripheral exposure. Furthermore, heavy accumulations of TSE-agent-specific disease-associated PrP\textsuperscript{Sc} occur upon the FDC networks in lymphoid tissues of scrapie-affected mice (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Jeffrey, McGovern et al. 2000). Previous data suggest that FDCs have a role in replicating the scrapie agent within the lymphoid tissue, however as of yet, there has not been a suitable model which has been able to exclusively address this issue (Brown, Stewart et al. 1999; Montrasio 2000; Mabbott, Williams et al. 2000a; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Mohan, Bruce et al. 2005). As the main function of FDCs within the B lymphocyte follicles is to pick up and retain antigen-containing immune complexes on their surfaces, the possibility remains that FDCs are simply acquiring the scrapie agent after replication on another lymphoid, stromal or neural cell types within the lymphoid tissues. Furthermore, FDCs have been shown to acquire expression of proteins that they themselves do not express, such as class II MHC, via exosomes (Gray, Kosco et al. 1991; Thery, Regnault et al. 1999; Denzer, van Eijk et al. 2000).

To definitively determine the role of FDCs in scrapie pathogenesis, the \textit{CD21-cre} mouse line was crossed with the \textit{Prnp}\textsuperscript{fl} mouse line to create a model in which PrP\textsuperscript{C} expression was switched off exclusively on CD21-expressing cells. These animals were subsequently irradiated and reconstituted with non-Cre-expressing bone marrow to restrict the removal of PrP\textsuperscript{C} expression exclusively to the FDC network. Characterisation of this compound transgenic model (Chapter 4) showed that PrP\textsuperscript{C}
expression was efficiently and specifically removed only on the FDC networks of CD21-crePrP^{0/} mice. Furthermore, no adverse effects on the status or function of FDC networks, or in the general microarchitecture of the lymphoid tissue were observed. These data confirmed that the CD21-crePrP^{0/} model would be a useful tool to determine the role of PrP^{C}-expressing FDCs in scrapie pathogenesis and elucidate whether FDCs simply accumulate TSE agent on their surfaces after replication on another cell type. As host expression of PrP^{C} is essential for TSE agent pathogenesis (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997), if FDCs themselves actively replicate the TSE agent, then efficient pathogenesis of TSE agent in peripheral lymphoid tissues should not be possible when PrP^{C} expression is removed exclusively on the FDC network. If however FDCs simply acquire the TSE agents, then in the absence of PrP^{C} expression, heavy accumulation of PrP^{Sc} would still be detected.

CD21-crePrP^{0/} mice were inoculated ip with ME7 scrapie and culled at 35 or 70 dpi. Further animals were also inoculated ic with ME7 scrapie to ensure that transgenic lines were susceptible to TSE infection. Spleens were harvested and detection of PrP^{Sc} was used to assess the pathology of scrapie. PrP^{Sc} has been shown to co-purify with TSE agent infectivity and is considered by many to be the sole component of the TSE infectious agent (Bolton, McKinley et al. 1982).

Experiments demonstrated that in animals with PrP ablation specifically in FDCs, the scrapie agent, as detected by PrP^{Sc} immunolabelling, initially located to the FDC network in the follicle. However at later time points PrP^{Sc} was found only within the TBMs. This is in contrast to control animals which retained Prmp^{+/-} FDCs, where
PrP$^\text{Sc}$ located to the FDC network in the follicle and accumulation increased over time suggesting replication of the scrapie agent. These data confirm that PrP$^\text{C}$-expressing FDCs actively replicate the TSE agent. When PrP$^\text{C}$ is ablated specifically on FDCs, no accumulation of PrP$^\text{Sc}$ occurs on the FDC surface, demonstrating that FDCs do not acquire the scrapie agent after replication on another cell type. Instead, TBM in the follicle clear PrP$^\text{Sc}$ from the FDC network and possibly degrade it.
5.3 Results

5.3.1 Experimental design

To determine the role of FDCs in TSE agent replication, the \textit{CD21-crePrP}^{fl-} mouse line which has PrP\textsuperscript{C} switched off specifically on FDCs was inoculated ip with ME7 scrapie. Animals were aged to 8 weeks, lethally $\gamma$-irradiated and given donor bone marrow 24 h later. To restrict the PrP ablation specifically to FDCs, \textit{CD21-crePrP}^{fl-} mice were given bone marrow from Cre-negative littermates. However other transgenic host/bone marrow combinations were also produced as control lines and these are summarised in Table 5.1. Animals were used in subsequent experiments at 100 d post bone-marrow reconstitution to allow for efficient replacement of host bone-marrow derived cells with donor counterparts. Mice from each group were inoculated with 20 $\mu$l of a 1\% (wt/vol) scrapie brain homogenate. Animals were then culled at 35 and 70 days post-inoculation (dpi) and tissues were collected to assess the cellular sites of PrP\textsuperscript{Sc} accumulation in the spleen. A summary of experimental design can be found in Figure 5.1.

Cellular sites of PrP\textsuperscript{Sc} accumulation in the spleen were determined by immunolabelling of PrP. Current antibodies used to detect PrP cannot distinguish between the normal cellular isoform of the prion protein, PrP\textsuperscript{C}, and the scrapie-associated, relatively PK-resistant isoform, PrP\textsuperscript{Sc}. For this reason, the disease-specific PrP detected by immunohistochemistry in spleens of infected animals is termed PrP\textsuperscript{d}. All immunohistochemistry was carried out using the rabbit anti-PrP polyclonal antibody (PAb) 1B3 and confirmed using the mouse anti-PrP monoclonal (MAb)
Table 5.1 Summary of experimental lines used in scrapie experiments

<table>
<thead>
<tr>
<th>Host Genotype</th>
<th>Donor Genotype</th>
<th>PrP&lt;sup&gt;C&lt;/sup&gt; switched off</th>
<th>Nomenclature</th>
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<tr>
<td>CD21-crePrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
<td>PrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
<td>FDCs only</td>
<td>Cre&lt;sup&gt;-ve&lt;/sup&gt; → CD21-crePrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
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<tr>
<td>CD21-crePrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
<td>CD21-crePrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
<td>FDCs and B lymphocytes</td>
<td>Cre&lt;sup&gt;-ve&lt;/sup&gt; → CD21-crePrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
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<tr>
<td>PrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
<td>CD21-crePrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
<td>B lymphocytes only</td>
<td>Cre&lt;sup&gt;-ve&lt;/sup&gt; → PrP&lt;sup&gt;fl&lt;/sup&gt;-</td>
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Figure 5.1 Summary of experimental design
antibody 6H4. As both antibodies gave similar results, only immunohistochemistry images from 1B3 labelling are shown.

Treatment with PK digests the cellular \( \text{PrP}^C \) leaving PK-resistant core of \( \text{PrP}^\text{Sc} \) intact (Manson, Clarke et al. 1994b). \( \text{PrP}^C \) and \( \text{PrP}^\text{Sc} \) can be distinguished on histological sections using the PET blot method. This method uses PK treatment of tissue sections on nitrocellulose membrane prior to immunolabelling with anti-PrP antibodies, to destroy \( \text{PrP}^C \). Therefore any PrP detected by this method can be confirmed as the scrapie-associated \( \text{PrP}^\text{Sc} \). Detection of \( \text{PrP}^d \) and \( \text{PrP}^\text{Sc} \) have been confirmed by many studies to be reliable markers of TSE disease and in most cases correlate closely with presence of TSE agent infectivity (Bolton, McKinley et al. 1982; McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009).

5.3.2 Effect of FDC-restricted PrP ablation on TSE agent accumulation in the spleen at 35 days post-exposure

Animals were culled at 35 dpi with the ME7 scrapie agent and spleens were harvested for analysis of the cellular sites of \( \text{PrP}^\text{Sc} \) accumulation. In all spleens with Cre-deficient, \( \text{PrP}^C \) expressing FDCs, accumulation of the abnormal, disease-associated \( \text{PrP}^d \) occurred in the germinal centres of the spleen (Figs 5.2). This \( \text{PrP}^d \) was located to the FDC network as shown by immunolabelling of serial sections for \( \text{PrP}^d \), FDCs and B lymphocytes (Fig 5.3). However, as animals are only expressing half copy
Figure 5.2 PrP\textsuperscript{d} immunolabelling in the spleen at 35 dpi with the ME7 scrapie agent

Four animals from each experimental line were culled at 35 dpi with ME7 scrapie and spleens were immunolabelled to detect PrP\textsuperscript{d} (using PAb 1B3, red). All spleens with Cre-deficient, PrP\textsuperscript{C} expressing FDCs (I-P) show PrP\textsuperscript{d} immunolabelling at low levels on the FDC networks (▲) and tingible body macrophages (TBM, *) in the follicles. Spleens from some animals with Cre-expressing, Prnp\textsuperscript{-/-} FDCs (A-H) also show immunolabelling on the FDC network, however this is detected at a lower frequency with only 2/8 animals (C, H) with positive PrP\textsuperscript{d} immunolabelling in the FDC networks. Some of these animals instead show PrPd labelling located only within the TBM of the follicle (A). Scale bar on main figure100 μm. Scale bar on x 1000 images 20μm. Sections counterstained with haematoxylin, blue.
Fig 5.3 Immunohistochemical analysis of PrP, FDC and B lymphocytes in spleens of mice taken at 35 dpi after exposure to the ME7 scrapie agent

Serial sections from spleens were immunolabelled for PrP (PAb 1B3, red), FDCs and CD21+ B lymphocytes (MAb 7G6, red) and B lymphocytes (MAb CD45R, red) to determine if PrPd detected co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with Cre-deficient, PrPC-expressing FDCs (G-L), PrPd (▲) is localised to the FDC networks (→) as shown in serial sections. PrPd location in spleens with Cre-expressing, PrPc deficient FDCs (A-F) was variable at 35 dpi. PrPd was present on the FDCs of some follicles but in others was found exclusively within TBMs (*) in the B lymphocyte follicles. Scale bar 100 μm. Sections counterstained with haematoxylin, blue.
**Figure 5.4** PET blot analysis of PrP\textsuperscript{Sc} accumulation in spleens taken at 35 dpi with the ME7 scrapie agent

Paraffin-embedded sections on nitrocellulose membrane were treated with PK to remove any native PrP\textsuperscript{C}, then immunolabelled (PAb 1B3, blue/black) to detect any remaining PrP\textsuperscript{Sc}. One representative example from each transgenic line is shown. A, Cre\textsuperscript{−}→CD21-crePrP\textsuperscript{fl/−}; B, Cre\textsuperscript{+}→CD21-crePrP\textsuperscript{fl/−}; C, Cre\textsuperscript{+}→PrP\textsuperscript{fl/−}; D, PrP\textsuperscript{fl/−}→PrP\textsuperscript{fl/−}. Scale bar 500 μm.

Due to the low levels of PrP\textsuperscript{d} detected in infected tissue at this stage by immunohistochemistry (Fig 5.2), PrP\textsuperscript{Sc} is not readily detected after PK treatment in any of the transgenic experimental lines. Therefore it is not confirmed that the PrP\textsuperscript{d} detected by immunohistochemistry in these tissues is PrP\textsuperscript{Sc} at this timepoint.
number levels of Prnp and were culled early in the incubation period, the magnitude of PrPd accumulation was insufficient to be confirmed as PrPSc by the PET blot method (Fig 5.4). Therefore it could not be confirmed that PrPd detected by immunohistochemistry was indeed scrapie-associated PrPSc.

In animals with Cre-expressing, PrP C-deficient FDCs, spleen sections taken at 35 dpi showed variable deposition of PrPd in the follicles. Only 3 out of 8 animals had detectable PrPd by immunohistochemistry (Figs 5.2). This deposition was located to the FDCs in some animals, but only found within TBMs within the follicle in others (Fig 5.3). As with animals with PrP C-expressing FDCs, levels of PrPd accumulation were too low to be confirmed by the PET blot method as PrPSc (Fig 5.4).

5.3.3 Effect of FDC-restricted PrP-ablation in TSE agent accumulation in the spleen 70 days post-exposure

Animals were culled at 70 dpi with ME7 scrapie and spleens were harvested for analysis of the cellular sites of PrPSc accumulation. In all spleens with Cre-deficient, PrP C-expressing FDCs, accumulation of the abnormal, disease-associated PrPd occurred in the germinal centres of the spleen at a greater intensity than that seen in spleens from animals culled at 35 dpi (Figs 5.5). This PrPd was located on the FDC network as shown by immunolabelling of serial sections for FDCs and B lymphocytes (Fig 5.6). Furthermore, the PrPd accumulation detected upon the FDCs was confirmed to be PK-resistant, scrapie agent-associated PrPSc using the PET blot method (Fig 5.7). From these data it can be concluded that high levels of the scrapie
Figure 5.5 PrP\textsuperscript{d} immunolabelling in the spleen at 70 dpi with the ME7 scrapie agent

Four animals from each experimental line were culled at 70 dpi with ME7 scrapie and spleens were immunolabelled to detect PrP\textsuperscript{d} (PAb 1B3, red) all spleens with Cre-deficient, PrP\textsuperscript{C}-expressing FDCs (I-P) show PrP\textsuperscript{d} immunolabelling on the FDC networks (△) within the follicles at an increased level than that seen in spleens from animals culled at 35 dpi. Spleens from animals with Cre-expressing, PrP\textsuperscript{C} deficient FDCs (A-H) no longer show any labelling on the FDC networks as was detected in spleens from D35 animals. Instead, PrP\textsuperscript{d} is detected only within TBMs (∗) in the follicle. This suggests the scrapie agent cannot replicate on PrP\textsuperscript{C} deficient FDCs and any PrP\textsuperscript{Sc} from inocula has been cleared from FDC networks and sequestered by TBMs in the follicle. Scale bar on main figure 100 μm. Scale bar on x 1000 magnification images 20 μm. Sections counterstained with haematoxylin, blue.
Fig 5.6 Immunohistochemical analysis of PrP, FDC and B lymphocytes in spleens of mice taken at 70 dpi with the ME7 scrapie agent

Serial sections from spleens were immunolabelled for PrP (PAb 1B3, red), FDCs and CD21^+ B lymphocytes (MAb 7G6, red) and B lymphocytes (MAb CD45R, red) to determine whether the PrP\textsuperscript{d} detected was co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with Cre-deficient, PrP\textsuperscript{C}-expressing FDCs (G-L), PrP\textsuperscript{d} (▲) is localised to the FDC networks (→) as shown in serial sections. PrP\textsuperscript{d} location in spleens with Cre-expressing, PrP\textsuperscript{C} deficient FDCs (A-F) was found exclusively within TBMs (*) and didn’t co-localise with FDC networks in the follicle. Scale bar 100 μm. Sections counterstained with haematoxylin, blue.
Figure 5.7 PET blot analysis of PrP<sub>Sc</sub> accumulation in spleens taken at 70 dpi with the ME7 scrapie agent
Paraffin-embedded sections on nitrocellulose membrane were treated with PK to remove any native PrP<sup>C</sup>, then immunolabelled (PAb 1B3, blue/black) to detect any remaining PrP<sup>Sc</sup>. One representative example from each transgenic line is shown A, Cre<sup>−ve</sup> → CD21-crePrP<sup>fl/−</sup>; B, Cre<sup>−ve</sup> → CD21-crePrP<sup>fl/−</sup>; C, Cre<sup>−ve</sup> → PrP<sup>fl/−</sup>; D, PrP<sup>fl/−</sup> → PrP<sup>fl/−</sup>. Scale bar 500 µm

In spleens with Cre-deficient, PrP<sup>C</sup>-expressing FDCs (C and D), some PrP labelling (►) can be detected in the follicles after treatment with PK and can therefore be confirmed as the disease-associated PrP<sup>Sc</sup>. In spleens with Cre-expressing, PrP<sup>C</sup>-deficient FDCs (A and B), immunohistochemistry had shown PrP<sup>d</sup> was present only within the TBMs (Fig 5.5). At the low levels of PrP<sup>d</sup> observed within the TBMs, PrP<sup>d</sup> is not readily detected after PK treatments. Therefore it is not confirmed that the PrP<sup>d</sup> detected in these tissues by immunohistochemistry is PrP<sup>Sc</sup>.  

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agent are able to accumulate on PrP<sup>C</sup>-expressing FDCs as expected. In addition, as no significant differences are seen between levels of PrP<sup>Sc</sup> in Cre<sup>+</sup>→ PrP<sup>f/-</sup> spleens and PrP<sup>WT/-</sup>→ PrP<sup>WT/-</sup> spleens, it can be concluded that switching off PrP<sup>C</sup> specifically on CD21<sup>+</sup> B lymphocytes in Cre<sup>+</sup>→ PrP<sup>f/-</sup> mice has no detectable impact on TSE agent accumulation upon the FDCs.

In animals with Cre-expressing, PrP<sup>C</sup> deficient FDCs, spleen sections at 70 dpi no longer showed deposition of PrP<sup>d</sup> on the FDC network. Instead if PrP<sup>d</sup> was detected at all, it was only found within TBMns (Figs 5.5). Indeed, immunolabelling of serial sections for FDCs and B lymphocytes confirmed that the PrP<sup>d</sup> immunolabelling did not co-localise with FDC networks (Fig 5.6). The, levels of PrP<sup>d</sup> observed within the TBMns were too low to be confirmed by the PET blot method as PrP<sup>Sc</sup> (Fig 5.7). From these data it can be concluded that PrP<sup>C</sup>-expressing FDCs are essential for the scrapie agent to replicate within the lymphoid tissues. Without active replication of the agent by PrP<sup>C</sup>-expressing FDCs, the scrapie agent appeared to be scavenged by TBMns in the follicle and possibly degraded. This suggests that PrP<sup>C</sup>-expressing FDCs actively replicate the TSE agent and do not simply acquire it from other PrP<sup>C</sup>-expressing cells present in the lymphoid tissue.

5.3.4 FDC-restricted PrP<sup>C</sup> ablation has no effect on TSE disease when infection is established directly in the CNS

Some transgenic animals with Cre-expressing, PrP<sup>C</sup> deficient FDCs were inoculated with the ME7 scrapie agent directly into the brain by ic injection to determine whether the nervous system in these animals was still susceptible to TSE disease. 129/Ola WT
Fig 5.8 Neuropathology within the brains of ic injected, clinically scrapie-affected mice

Serial brain sections were immunolabelled to compare the characteristic neuropathological hallmarks of TSE disease: spongiform change (vacuolation, H+E), deposition of the abnormal disease associated PrPSc (PAb 6H4, brown) and proliferation and activation of astrocytes (GFAP+ cells, red) and microglia (IBA-1+ cells, red) within the brain.

The levels of vacuolation, deposition of PrPSc and accumulation of activated astrocytes and microglia were slightly lower in the brains of transgenic animals in comparison to WT controls. However all characteristic hallmarks of TSE neuropathology are present.

These data confirm that transgenic animals are equally susceptible to TSE disease as WT counterparts. Therefore, any differences in TSE pathogenesis observed within the peripheral lymphoid tissues are due to specific removal of PrPC expression on the FDCs and not a general resistance to TSE disease. Scale bars 500 μm. Sections counterstained with haematoxylin.
Figure 5.9 Mean duration of incubation periods of clinically-scrapie-affected mice inoculated ic with the ME7 scrapie agent

Incubation period of disease in transgenic and WT control lines after ic inoculation with the ME7 scrapie agent. Each bar represents the mean +/- the standard error of the mean for groups of 3-5 mice.

129/Ola and C57BL/6x129/Ola WT mouse lines which are Prnp\(^{+/+}\) have incubation periods of approximately 160 d after ic inoculation with the ME7 scrapie agent. Transgenic CD21-crePrP\(^{0/-}\) lines have an incubation period of around 280 d. This is in line with the disease incubation observed in 129/Ola Prnp\(^{+/+}\) mice which also express half copy number levels of PrP\(^{C}\).
mice were also inoculated ic as controls. Following infection, both WT and transgenic animals developed clinical TSE disease with positive spongiform pathology in the brain. The classical hallmarks of TSE infection in the brain are deposition of disease-associated PrP\textsuperscript{Sc}, gliosis and spongiform pathology with vacuolation. Immunolabelling confirmed PrP\textsuperscript{d}, microglia (IBA-1\textsuperscript{+} cells) and activated astrocytes (GFAP\textsuperscript{+} cells) were all present in the brains of WT and transgenic animals. These data confirm that \textit{CD21-crePrP\textsuperscript{fl/-}} transgenic mice were fully susceptible to TSE disease when injected directly into the brain (Fig 5.8). However the neuropathology in the brains of transgenic animals appeared to be at lower than that found in WT controls, possibly due to the transgenic mice only expressing half copy number levels of \textit{Prnp} or due to mouse strain differences. Transgenic \textit{CD21-crePrP\textsuperscript{fl/-}} animals all succumbed to the clinical signs of scrapie around 280 days post-ic inoculation with the ME7 scrapie agent (Fig 5.9). This is in line with previous transmissions of the ME7 scrapie agent into 129/Ola \textit{Prnp} \textsuperscript{+/-} mice undertaken at this institute, which also express half copy number expression levels of \textit{Prnp} (Manson, Clarke et al. 1994b).

These data demonstrate that the transgenic lines used are equally susceptible as WT mice to the ME7 scrapie agent when infection was established directly in the brain by ic injection. Furthermore, insertion and expression of the \textit{Cre} and \textit{Prnp\textsuperscript{fl}} transgenes also appeared to have had no detectable effect on the neuropathology in the CNS.
5.3.5 No PrP\textsuperscript{Sc} accumulation upon FDCs in the spleens of clinically-scrapie-affected mice with FDC-restricted PrP\textsuperscript{C} ablation

Following exposure to the ME7 scrapie agent, high levels of PrP\textsuperscript{Sc} are maintained on FDCs for the duration of the incubation period; even after ic exposure (Brown, Stewart et al. 1999). The spleens from the clinically-scrapie-affected, ic injected mice were also assessed to determine the cellular sites of PrP\textsuperscript{Sc} accumulation. As anticipated, spleens from PrP\textsuperscript{C}-expressing WT animals showed heavy accumulation of PrP\textsuperscript{d} in the follicles as determined by immunolabelling (Fig 5.11). This deposition was confirmed to be PrP\textsuperscript{Sc} by the PET blot method (Fig 5.12). However, in spleens with Cre-expressing, PrP\textsuperscript{C} deficient FDCs, little to no PrP\textsuperscript{d} could be detected. Where PrP\textsuperscript{d} was detected it appeared to be mostly associated with TBMs rather than the FDC networks (Fig 5.11). Furthermore, this pattern of immunolabelling could not be confirmed as PrP\textsuperscript{Sc} by PET blots (Fig 5.12). This suggests that although animals with Cre-expressing, PrP\textsuperscript{C} deficient FDCs are susceptible to ME7 infection in the CNS, animals with terminal clinical disease are still unable to replicate the TSE agent in the periphery when FDCs do not express PrP\textsuperscript{C}. This confirms that even at clinical disease without PrP\textsuperscript{C}-expressing FDCs, the scrapie agent is unable to accumulate upon FDCs in the lymphoid tissue and is instead cleared by neighbouring TBMs.
Figure 5.11 PrP<sup>d</sup> immunolabelling in the spleens of clinically-scrapie-affected mice inoculated with the ME7 scrapie agent

Animals were inoculated with ME7 scrapie and left to develop terminal disease. Spleens were immunolabelled to detect PrP<sup>d</sup> (PAb 1B3, red). Spleens from WT animals with Cre-deficient, PrP<sup>C</sup>-expressing FDCs (A-D) show intense PrP<sup>d</sup> immunolabelling on the FDC networks (▲) within the B lymphocyte follicles. In contrast, spleens from transgenic animals with Cre-expressing, PrP<sup>C</sup> deficient FDCs (E-K) show no PrP<sup>d</sup> labelling on the FDC networks. Instead, the only PrP<sup>d</sup> detected is found within TBM (▲) in the follicle. This suggests the scrapie agent is unable to replicate on PrP<sup>C</sup> deficient FDCs even when animal has clinical TSE disease. Scale bar 100 μm. Sections counterstained with haematoxylin, blue.
Figure 5.12 PET blots analysis of PrP\textsuperscript{Sc} accumulation in spleens from clinically-scrapie-affected mice ic inoculated with the ME7 scrapie agent

Paraffin-embedded sections on nitrocellulose membrane were treated with PK to remove any native PrP\textsuperscript{C}, then immunolabelled (PAb, 1B3, blue/black) to detect any remaining PrP\textsuperscript{Sc}. Labelling in ic injected transgenic animals B, Cre\textsuperscript{+ve} → CD21\textsuperscript{-}crePrP\textsuperscript{fl/-}; C, Cre\textsuperscript{+ve} → CD21\textsuperscript{-}crePrP\textsuperscript{fl/-} was compared with that of A, 129/Ola WT animals. Scale bar 500 μm

In WT spleens with Cre-deficient, PrP\textsuperscript{C}-expressing FDCs (A), intense PrP\textsuperscript{Sc} labelling (►) can be confirmed in the B lymphocyte. However, in transgenic spleens with Cre-expressing, PrP\textsuperscript{C} deficient FDCs (B and C), immunohistochemistry had shown PrP\textsuperscript{d} was present only within the TBMs (Fig 5.10). This pattern of immunolabelling is not readily detected after PK treatment and therefore could not be confirmed to be the disease-associated PrP\textsuperscript{Sc}.
5.4 Discussion

The CD21-crePrPfl/- mouse line, with irradiation and reconstitution with non-Cre-expressing bone marrow, was previously shown to allow the removal of PrP\(^C\) specifically on FDCs with no other adverse effects on the lymphoid tissues (Chapter 4). In this chapter the CD21-crePrPfl/- mouse line was injected ip with the ME7 scrapie agent to determine if PrP\(^C\)-expressing FDCs simply acquire the scrapie agent after replication on another cell type within the lymphoid tissue. As host expression of PrP\(^C\) is essential for TSE agent pathogenesis (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997), if FDCs themselves actively replicate the TSE agent, then PrP\(^Sc\) accumulation should not be possible when PrP\(^C\) expression is removed exclusively on the FDC network. In contrast, if FDCs only acquire TSE agents, high levels of PrP\(^Sc\) will accumulate upon the FDC, even in the absence of PrP\(^C\) expression.

Scrapie agent accumulation in lymphoid tissues was determined by immunolabelling for PrP\(^d\) and PrP\(^Sc\). Previous studies have shown that PrP\(^Sc\) co-purifies with infectivity in tissues from scrapie-affected animals and has been confirmed in many studies to be a reliable marker of TSE disease in the lymphoid tissue (Bolton, McKinley et al. 1982; McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009). Abnormal, pathological accumulations of PrP detected by immunohistochemistry were termed PrP\(^d\), as the antibodies used cannot distinguish between PrP\(^C\) and PrP\(^Sc\). Any PrP\(^d\) present was subsequently verified to be PrP\(^Sc\) if immunolabelling was still detected after PK treatment using the PET blot method (Section 2.4.3). Previous studies have
shown that PrPSc and infectivity accumulates in the lymphoid tissues long before
detection in the CNS and deposition on the FDC network can be seen easily at 4
weeks post peripheral inoculation (Brown, Stewart et al. 1999). For this reason,
animals were culled at 35 and 70 dpi to assess the early pathogenesis of the ME7
scrapie agent in transgenic and control lines. However, as animals in this study are
expressing only half copy number levels of Prnp, less deposition of PrPSc would be
expected on the FDC networks than seen in equivalent studies using Prnp+/+ animals.

Animals with Cre-deficient, PrPc-expressing FDCs had detectable PrPd in the spleen
at 35 dpi and this accumulation intensified by 70 dpi suggesting active replication of
the scrapie agent upon the PrPc-expressing FDCs. This is in agreement with previous
studies which suggested that PrPc-expressing FDCs are essential for replicating the
scrapie agent (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999;
Mabbott, Mackay et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown,
Wathne et al. 2009). However in animals with Cre-expressing, PrPc deficient FDCs,
deposition was only seen on occasional FDC networks at 35 dpi. By 70 dpi PrPd
could no longer be found on the FDC networks and is instead located within TBMs in
the follicle. These data suggest that the scrapie agent initially locates to the FDCs
network independently of PrPc expression on the FDCs. Previous data indicates that
the scrapie agent is opsonised by complement components and is trapped on the FDC
network as an immune complex bound by complement and Fc receptors (Klein,
Kaeser et al. 2001; Mabbott, Bruce et al. 2001; Zabel, Heikenwalder et al. 2007).
With no PrPc present on the FDCs, data in this chapter shows that the scrapie agent is
unable to replicate and is cleared from the FDC network by TBMs in the surrounding
follicle. Previous studies have shown that TBMs are capable of taking up immune-complex coated portions of FDC membrane, also known as iccosomes, therefore this is a possible mechanism by which TBMs remove the scrapie agent from the FDC surface (Szakal and Tew 1992; Sandberg, Al-Doujaily et al. 2011). As Cre-expressing, PrP\(^c\) deficient FDCs have no deposition of PrP\(^\text{Sc}\) on their surface, it can be concluded that FDCs do not accumulate the TSE agent from the other cells in the lymphoid tissue, such as neurones, stromal cells and lymphocytes which retain PrP\(^c\) expression in this model.
CHAPTER 6

Characterisation of the \(CD21\text{-}crePrP^{\text{stop/}}\) mouse line

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6.1 Abstract

The CD21-cre mouse line has been used to switch off PrP^{C} expression specifically on FDCs and subsequently used to show that PrP^{C} expressing FDCs are required for replication of the scrapie agent in the lymphoid tissues (Chapter 4 and 5). To complement this model, the CD21-cre mouse line was subsequently crossed with a line which contains a floxed β-geo stop cassette inserted before the coding region of PrP^{C}. This CD21-crePrP^{stop/-} line allows PrP^{C} expression to be switched on under control of the Cr2 promoter. In the absence of Cre expression, the floxed stop cassette prevents transcription of the open reading frame of the Prnp gene. However in CD21-expressing cells, Cre-mediated recombination removes the stop cassette and PrP^{C} expression is switched on. Irradiation and reconstitution of CD21-crePrP^{stop/-} mice with non-Cre-expressing bone marrow allows PrP^{C} expression to be switched on exclusively on CD21-expressing FDCs. CD21-crePrP^{stop/-} mice were fully characterised to determine within which cells PrP^{C} was switched on and to ensure that the transgenes had no additional effects on lymphoid tissue microarchitecture that could have an impact on scrapie pathogenesis. Characterisation of the CD21-crePrP^{stop/-} mouse demonstrated that PrP^{C} was efficiently and exclusively expressed on FDCs under control of the Cr2 promoter. Furthermore, insertion and expression of the transgenes had no adverse effects on the microarchitecture of the lymphoid tissue. These data suggest that the CD21-crePrP^{stop/-} mouse will be a useful model to determine if PrP^{C} expression exclusively on FDCs is sufficient to support replication of the scrapie agent in the lymphoid tissue.
6.2 Introduction

Previous work in this thesis has shown that if PrP$^C$ expression is removed specifically on the FDCs, the scrapie agent is unable to replicate in the spleen and accumulate on FDC surface (Chapter 5). In this chapter, a transgenic mouse line was created to allow PrP$^C$ expression to be switched on exclusively on the FDC network, which would subsequently be used in Chapter 7 to determine if PrP$^C$ expression on FDCs alone was sufficient to allow scrapie agent replication in the spleen.

The coding region for PrP$^C$ is contained within exon 3 of the Prnp gene (Chapter 1, Fig 1.1). In PrP$^{stop/-}$ mice, the coding region of Prnp is preceded by a β-geo stop cassette flanked by loxP sites (Tuzi, Clarke et al. 2004). The PrP$^{stop/-}$ mouse line was crossed with the CD21-cre mouse to allow PrP$^C$ to be switched on under control of the Cr2 promoter (Fig 2.3). Based on data from the CD21-creROSA26 and CD21-crePrPfl/- lines (Chapter 3 and 4, respectively) the CD21-crePrPstop/- line, together with lethal γ-irradiation and reconstitution with non-Cre-expressing bone marrow, should restrict PrP$^C$ expression exclusively to FDCs. In this chapter, the CD21-crePrPstop/- line was characterised to determine if PrP$^C$ was successfully switched on specifically on the FDC networks and also that insertion of the transgenes had no additional effects on the lymphoid tissue. Analysis of the CD21-crePrPstop/- line confirmed that PrP$^C$ was efficiently expressed on Cre-expressing FDCs. Furthermore, no PrP$^C$ expression was detected on non-CD21-expressing cells in the lymphoid tissues such as the nerves. In addition, there were no differences in the microarchitecture of the lymphoid component of the spleen which could have potentially impacted on scrapie pathogenesis. From these data, it can be concluded that the CD21-cre PrP$^{fl}$ line will
be a useful tool to study scrapie pathogenesis when PrPC expression has been switched off exclusively on FDCs.
6.3 Results

6.3.1 Production of $CD21$-crePrP$^{\text{stop}^-}$ mouse line

The $CD21$-crePrP$^{\text{stop}^-}$ mouse line, which was created during previous work to make the $CD21$-crePrP$^{\text{floxed}^-}$ mouse line (Section 2.1.3 and 4.3.2), was crossed with PrP$^{\text{stop}^-}$ line to create the $CD21$-cre PrP$^{\text{stop}^-}$ mice. The PrP$^{\text{stop}^-}$ line contains a floxed stop cassette inserted into the $Prnp$ gene prior to exon 3 which contains the ORF (Tuzi, Clarke et al. 2004). In the absence of Cre, cells in this animal cannot express PrP$^C$ due to the presence of the stop cassette before the protein coding sequence in exon 3. However in cells which express CD21, Cre recombinase is expressed, the stop cassette is removed and PrP$^C$ expression is switched on. The $Prnp$ stop allele is lethal at homozygosity (Tuzi, Clarke et al. 2004), therefore, no attempts were made to create a homozygous $CD21$-crePrP$^{\text{stop}^-}\text{stop}$ mouse line.

To restrict PrP$^C$ expression exclusively to FDCs, $CD21$-crePrP$^{\text{stop}^-}$ animals were lethally $\gamma$-irradiated and given bone marrow from age- and sex-matched Cre negative littermates (termed Cre$^{-ve}$ → $CD21$-crePrP$^{\text{stop}^-}$). Experimental control groups were also produced as in the $CD21$-crePrP$^{\text{floxed}^-}$ experimental lines. Firstly, $CD21$-crePrP$^{\text{stop}^-}$ mice given bone marrow from Cre$^{+ve}$ littermates, (termed Cre$^{+ve}$ → $CD21$-crePrP$^{\text{stop}^-}$ mice), in which PrP$^C$ will be switched off in both FDCs and CD21$^+$ B lymphocytes. Also, PrP$^{\text{fl}^-}$ mice were given bone marrow from $CD21$-cre PrP$^{\text{stop}^-}$ mice, (termed Cre$^{+ve}$ → PrP$^{\text{stop}^-}$), in which PrP$^C$ expression is switched on in CD21$^+$ B lymphocytes only. The final control group was PrP$^{\text{WT}^-}$ mice given PrP$^{\text{WT}^-}$ bone marrow. The copy number of the $Prnp$ gene has been shown to have a strong
influence in scrapie incubation period, with PrP\textsuperscript{WT/-} heterozygous mice having almost double the incubation time of PrP\textsuperscript{WT/WT} homozygotes (Tew and Mandel 1979). Therefore, this control group was included due to the \textit{CD21-crePrP\textsuperscript{fl/-}} experimental line having half copy number levels of PrP\textsuperscript{C}.

Bone marrow chimera status of experimental animal groups was confirmed by PCR analysis (Fig 6.1). DNA was extracted from blood, tail and spleen and was genotyped for both expression of \textit{Cre} and \textit{Prnp\textsuperscript{stop}} with and without Cre-mediated recombination of the \textit{Prnp\textsuperscript{stop}} DNA. This analysis confirmed that the host/ donor genotypes were as expected and also that recombination of the \textit{Prnp\textsuperscript{stop}} DNA only occurred in tissues which contained both Cre expression and CD21-expressing cells.

\textbf{6.3.2 Insertion and expression of transgenes and bone marrow reconstitution have no effect on lymphoid tissue microarchitecture}

In addition to confirming that Cre-mediated recombination was restricted to CD21-expressing cells and was occurring efficiently, it was also essential to ensure that there were no other changes to the lymphoid tissue that could mediate an effect on scrapie pathogenesis. Spleens from all four groups of experimental mice were immunolabelled to detect B lymphocyte subsets, T lymphocytes, FDCs and classical dendritic cells and compared with immunolabelling of spleens from WT animals (Fig 6.2)
Figure 6.1 Genotypes of \(CD21\text{-}cre\)Pr\(P^{\text{stop/\text{-}}}\) animals used for characterisation and subsequent scrapie experiments

DNA was extracted from blood (B), spleen (S), and tail (T) from 6 animals from each of the transgenic lines to be used in subsequent scrapie experiments. One example of genotype results for Cre\(^{\text{+ve}}\rightarrow CD21\text{-}cre\)Pr\(P^{\text{stop/\text{-}}},\) Cre\(^{-\text{ve}}\rightarrow CD21\text{-}cre\)Pr\(P^{\text{stop/\text{-}}}\) and Cre\(^{\text{+ve}}\rightarrow PrP^{\text{stop/\text{-}}}\) lines is shown on the above gel.

**a.** The Cre transgene was present in the spleen and tail of CD21\text{-}crePr\(P^{\text{stop/\text{-}}}\) mice but was only present in the blood if Cre\(^{\text{+ve}}\) donor bone marrow had been given. Pr\(P^{\text{stop/\text{-}}}\) mice had no Cre transgene detected in tail DNA, however Cre was present in the spleen and blood due to Cre\(^{\text{+ve}}\) bone marrow given.

**b.** Tissues from all animals are heterozygous for Pr\(n^{\text{stop/\text{-}}}\) however, recombination of the Pr\(n^{\text{stop}}\) DNA (Pr\(n^{\text{stop(R)}}\)) was only present in tissues that contain both the Cre transgene and CD21-expressing cells. Control DNA was from Pr\(n^{\text{WT}}\) (a), Pr\(n^{\text{stop/\text{-}}}\) (b) and Pr\(n^{\text{stop(R)}}\) DNA extracted from tails.
Figure 6.2 Insertion and expression of the transgenes and irradiation and bone marrow reconstitution has no effect on the microarchitecture of peripheral lymphoid tissues as determined by immunolabelling

Frozen spleen sections from all experimental transgenic mouse lines were immunolabelled for B lymphocyte subsets (CD45R and CD1d), T lymphocytes (CD3), FDC (C4, red and CD21/35, red), DCs (CD11c) and marginal zone cells (MADCAM-1). Comparison of sections from transgenic animals with WT controls showed no differences in the number or location of cell subsets within the spleen. Scale bars on fluorescent images 100 μm. Scale bars on light microscopy images 500 μm and sections counterstained with haematoxylin, blue.
No differences could be seen in intensity or location of immunolabelling for each cell type between WT and experimental spleens. It can be concluded that neither expression of the transgenes nor irradiation and bone marrow reconstitution has any significant effect on the microarchitecture of the lymphoid tissue.

6.3.3 PrP$^C$ immunolabelling is present on Cre-expressing FDCs

Spleens from all experimental and control lines were immunolabelled for PrP$^C$ expression to determine within which cells PrP$^C$ protein expression was switched on. Within the lymphoid tissues, PrP$^C$ can be detected on FDCs and peripheral nerves using immunolabelling. Therefore, to determine if PrP$^C$ expression was switched on specifically on the FDC network, spleen sections were fluorescently immunolabelled for PrP$^C$, FDCs via CD35 expression and tyrosine hydroxylase (TH) expressing peripheral nerves. Co-localisation of fluorescent labelling was then quantified using ImageJ image analysis software. Animals which expressed Cre showed PrP$^C$ immunolabelling present on the FDC networks but this was absent on the Cre-deficient peripheral nerves (Fig 6.3). Quantification of co-localised pixels showed that the co-localisation detected was true co-localisation and not background due to random association of pixels ($p=1.2 \times 10^{-26*}$, $9.1 \times 10^{-25**}$ and $1.3 \times 10^{-23***}$, Fig 6.4). Animals with PrP$^C$ expression switched on specifically on FDCs had statistically significantly higher levels of PrP$^C$ staining in comparison to non-Cre expressing spleens ($p<1.1 \times 10^{-25}$, Fig 6.4).
Fig 6.3 PrP^C is switched on specifically on FDC networks of animals which express Cre

Immunolabelling of PrP^C (blue) on FDC networks (CD35/red) and peripheral nerves (TH/green) in spleen sections from transgenic mouse lines. Animals were lethally γ-irradiated, received donor bone marrow 24 h later and were culled 100 days post reconstitution to allow replacement of all host-derived lymphocytes. Spleen sections from 6 animals from each line were immunolabelled and analysed. CD21-crePrPstop/- animals which received Cre^-ve (A) or Cre^+ve (B) bone marrow had detectable PrP^C labelling on the FDC networks however PrP^C labelling was not present on the peripheral nerves. This demonstrates that expression of PrP^C has been switched on specifically on the Cre-expressing FDCs. PrPstop/- animals which received Cre^+ve bone marrow (C) showed no PrP^C immunolabelling on FDCs or nerves. B lymphocyte expression of PrP^C is not detectable by immunolabelling. PrPWT/- animals which received PrPWT/- bone marrow (D) show PrP^C labelling on both FDC networks and peripheral nerves. These data demonstrate that PrP^C has been switched on specifically on the FDCs of animals which express Cre under the Cr2 promoter. Scale bar 100 μm.
Co-localisation of PrP<sup>C</sup> on FDC networks

**Fig 6.4 Expression of Cre in FDCs under the CD21 promoter removes PrP<sup>C</sup> immunolabelling on FDC networks**

Frozen spleen sections were immunolabelled for PrP<sup>C</sup>, FDC and peripheral nerves. For each mouse line, spleens were taken from 6 animals. Two sections, 50 μm apart, were immunolabelled for each spleen and 4 images per section were taken. This resulted in the analysis of 48 images for each mouse line using ImageJ image analysis software. For each image the number of pixels of each colour were counted using the multiple colour analysis macro allowing values to be obtained for total number of red (FDC) blue (PrP<sup>C</sup>) and magenta (PrP<sup>stop</sup> co-localised with FDC) pixels per image. These values allowed the calculation of the average percentage of FDC area co-localised with PrP<sup>C</sup>. In CD21-crePrP<sup>stop</sup> animals and PrP<sup>WT</sup> animals, which have Cre-expressing FDCs this value is significantly higher than PrP<sup>stop</sup> animals which do not have Cre-expressing FDCs. These data show that PrP<sup>C</sup> has been switched on specifically on the Cre-expressing FDCs as anticipated.
6.3.4 Insertion and expression of transgenes and irradiation and bone marrow reconstitution have no effect on the distance between FDC networks and peripheral nerves in the spleen

Previous studies have shown that the distance between peripheral nerves and FDC networks can influence scrapie incubation period after peripheral inoculation (Glatzel, Heppner et al. 2001; Prinz, Heikenwalder et al. 2003). Therefore immunolabelling was used to further characterise the microarchitecture of lymphoid tissues of transgenic mice by measuring the distance between peripheral nerves and FDCs in the spleen. Frozen sections were immunolabelled for FDCs and peripheral nerves and LSM image browser software was used to measure the distance between them (Fig 6.5). Comparison of the average distance between FDCs and the nearest peripheral nerve showed there was no statistically significant difference in the distance between FDCs and peripheral nerves between transgenic spleens in comparison to WT counterparts (p=0.932, Fig 6.5).
Fig 6.5 Insertion and expression of the transgenes has no effect on the distance between peripheral nerves and FDCs in the spleen

**a.** Frozen spleen sections from transgenic and WT lines were immunolabelled for FDC networks (CD35/red) and peripheral nerves (TH/green). For each mouse line, spleens were taken from 6 animals. Two sections, 50 μm apart, were immunolabelled for each spleen and 4 images per section were taken. 48 images were analysed for each mouse line. Scale bar 50 μm.

**b.** The distance between the FDC networks and peripheral nerves was measured using LSM image browser software (Zeiss). The average distance between nerves and FDCs was calculated for each mouse line. Analysis using a one-way anova test determined there was no significant difference in the distance between the FDC networks and peripheral nerves in the spleens of transgenic lines in comparison to WT controls (p=0.932).
6.4 Discussion

The CD21-cre line was crossed with a PrPstop/- line to create a model where PrP\textsuperscript{C} expression could be switched on exclusively on FDCs. In this chapter the CD21-crePrP\textsuperscript{stop/-} line was characterised to establish if this mouse model would be an efficient tool to determine whether PrP\textsuperscript{C} expression exclusively on FDCs is sufficient to allow replication of the scrapie agent within the lymphoid tissue. The characterisation studies carried out on this line indicate that PrP\textsuperscript{C} was switched on specifically on Cre-expressing FDCs and not on other non-Cre-expressing cells within the lymphoid tissue such as the nerves. Furthermore, insertion and expression of the transgenes had no additional effects on the lymphoid tissue that could influence scrapie pathogenesis.

Homozygous expression of the Prnp\textsuperscript{stop} allele is embryonically lethal. Therefore CD21-crePrP\textsuperscript{/-} mice were crossed once with the PrP\textsuperscript{stop/-} mice and selected CD21-crePrP\textsuperscript{stop/-} progeny were used in subsequent characterisation. Prnp expression levels have previously been shown to have a crucial influence on scrapie incubation period (Manson, Clarke et al. 1994a). Therefore, in subsequent scrapie infection experiments (Chapter 7), PrP\textsuperscript{WT/-} mice will be used as a control line for the half copy number expression levels of PrP\textsuperscript{C} present on the FDCs of the CD21-crePrP\textsuperscript{stop/-} mice.

Characterisation of the CD21-crePrP\textsuperscript{stop/-} mice showed that PrP\textsuperscript{C} expression was efficiently and exclusively expressed on FDCs. PCR analysis of DNA extracted from spleens confirmed the presence of the Cre transgene and recombination of the Prnp\textsuperscript{stop} transgene. Furthermore, immunolabelling of spleen sections confirmed that PrP\textsuperscript{C} was
expressed on FDCs but not present on non-CD21 expressing cells such as peripheral nerves. Previous studies using the CD21-cre line, which measured 96% of floxed DNA was recombined in FDCs of CD21-cre mouse line crosses (Victoratos, Lagnel et al. 2006). This type of analysis was not carried out on CD21-crePrP<sup>stop/-</sup> mice, however PCR and immunohistochemical analysis showed efficient recombination of the floxed DNA.

Depletion of various lymphoid cell subsets other than FDCs has been shown to delay the onset of peripherally acquired scrapie (Klein, Frigg et al. 1997; Raymond, Aucouturier et al. 2007). Therefore, characterisation of the lymphoid tissues of transgenic mice was essential to ensure that the only factor affecting scrapie pathogenesis in CD21-crePrP<sup>stop/-</sup> mice was the expression of PrP<sup>C</sup>. Various cell subsets were immunolabelled on spleen sections from transgenic experimental and WT lines to determine if insertion of the transgenes or irradiation had any effect on lymphoid tissue microarchitecture. No observable difference could be seen in the number or location of B lymphocytes, T lymphocytes or classical dendritic cells or in the integrity of the marginal zone in the spleen.

Changes in the number and maturation status of the FDC networks in the lymphoid tissue has been shown to have a critical influence on peripheral scrapie pathogenesis (Brown, Stewart et al. 1999; Mabbott, Williams et al. 2000a; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Prinz, Heikenwalder et al. 2003). Analysis of the CD21-crePrP<sup>0/-</sup> line (Chapter 4) showed there were no significant differences in the number, area and function of FDC networks of transgenic mice in comparison to WT controls. It was not considered to be essential to repeat these extensive studies on
FDCs of CD21-crePrPstop mice, however immunolabelling of spleen sections from this line showed no observable differences in the presence of various FDC markers in comparison to WT controls. The positioning of FDC networks within the spleen with regard to their proximity to peripheral nerves can also influence the incubation period of peripherally acquired scrapie (Glatzel, Heppner et al. 2001; Prinz, Heikenwalder et al. 2003). This was measured in spleen sections from transgenic experimental lines and WT controls and no significant difference was measured in the distance between the FDC networks and nerves.

These data show that neither the insertion and expression of the Cre or Prnpstop transgenes nor irradiation and bone marrow reconstitution in experimental mice, has any significant effect on the microarchitecture of the spleen or the number or distribution of various cell subsets found within. Previous studies using the CD21-cre mice also did not report any toxic effects caused by the Cre transgene and showed no differences in FDC and B lymphocyte numbers or location in the spleen (Kraus, Alimzhanov et al. 2004; Victoratos, Lagnel et al. 2006; Schenten, Kracker et al. 2009). Additionally, characterisation of the PrP-/- line has shown no overt phenotype even though PrP is ubiquitously expressed (Manson, Clarke et al. 1994a).

FDCs have the ability to acquire proteins on their surface which they themselves do not express, for example Class II MHC (Gray, Kosco et al. 1991; Denzer, van Eijk et al. 2000). Although PrP has been shown to be present on the FDC surface via immunolabelling and electron microscopy, it is possible that FDCs acquire the expression of PrP from other cell types within the lymphoid tissues. Switching on Prnp expression exclusively in FDCs has determined that FDCs themselves do
actively express relatively high levels of PrP\textsuperscript{C} and are not acquiring this expression from other cells. This is in agreement with previous studies using bone marrow chimeric mice. Mice were lethally \(\gamma\)-irradiated and reconstituted with PrP\textsuperscript{\textminus/\textminus} bone marrow, however host derived FDCs remained PrP\textsuperscript{\textplus/\textplus} suggesting that FDCs themselves expressed PrP\textsuperscript{C} (Brown, Stewart et al. 1999).

Characterisation of the CD21-crePrP\textsuperscript{\textstop/\textslant/\textendash} mouse line has shown that PrP\textsuperscript{C} is efficiently switched on in Cre-expressing FDCs but is not present on other cell types within the lymphoid tissues. Furthermore, insertion and expression of the transgenes have no additional effects on the cellular composition or structure of the lymphoid tissue. Therefore the CD21-crePrP\textsuperscript{\textstop/\textslant/\textendash} mouse line is an effective model to determine if PrP\textsuperscript{C} expression exclusively by FDCs is sufficient to allow scrapie agent replication in the lymphoid tissues (Chapter 7). This will allow the role of the FDCs in scrapie pathogenesis to be fully investigated and to establish whether FDCs themselves actively replicate the scrapie agent.
CHAPTER 7

Pathogenesis of ME7 Scrapie in mice with PrP\textsuperscript{C} expression switched on exclusively on FDCs

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7.4 Discussion 176
7.1 Abstract

Data in this thesis (Chapter 5) has shown that PrP\textsuperscript{C} deficient FDCs are unable to replicate the scrapie agent. Without PrP\textsuperscript{C}–expressing FDCs, PrP\textsuperscript{Sc} appears to be removed from the FDCs by TBMs in the follicle and possibly degraded. In this Chapter experiments are designed to determine if PrP\textsuperscript{C} expression exclusively by FDCs in a Prnp\textsuperscript{-/-} animal is sufficient to support replication of the scrapie agent in the lymphoid tissue.

The CD21-crePrP\textsuperscript{stop/-} transgenic mouse model was extensively characterised (Chapter 6) and it was confirmed that in this model, PrP\textsuperscript{C} expression was switched on exclusively and efficiently on FDCs. Furthermore, the insertion and expression of the transgenes had no adverse effects on the cellular composition or microarchitecture of the lymphoid tissues that could potentially impact on scrapie pathogenesis. CD21-crePrP\textsuperscript{stop/-} mice and control lines were inoculated ip with ME7 scrapie and culled at various time points after inoculation to assess disease progression. By 35 dpi all animals with PrP\textsuperscript{C} expression exclusively in FDCs, or in FDCs and CD21\textsuperscript{+} B lymphocytes, showed low levels of PrP\textsuperscript{d} immunolabelling on the FDC network. At later time points, immunolabelling of PrP\textsuperscript{d} was more intense and could be confirmed as PrP\textsuperscript{Sc} using the PET blot method. These data show that PrP\textsuperscript{C} expression exclusively on FDCs is sufficient to support replication of the scrapie agent in the lymphoid tissues. Therefore it can be concluded that the FDCs themselves are actively replicating the scrapie agent and not acquiring it after replication on another cell type.
7.2 Introduction

Heavy accumulations of TSE-agent-specific disease-associated PrP\textsuperscript{Sc} occur upon the FDC networks in lymphoid tissues of scrapie-affected mice after peripheral exposure (McBride 1992; Brown, Stewart et al. 1999; Bruce 2000; Jeffrey, McGovern et al. 2000). FDCs are thought to have a role in replicating the scrapie agent within the lymphoid tissue, however as of yet, there has not been a suitable model which has been able to specifically assess the role of the FDCs in isolation from all other stromal, neural and lymphoid cells within the lymphoid tissue (Brown, Stewart et al. 1999; Montrasio 2000; Mabbott, Williams et al. 2000a; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Mohan, Bruce et al. 2005). Previous data in this thesis (Chapter 5) has shown that PrP\textsuperscript{C} ablation specifically on FDCs prevents the accumulation of the scrapie agent on the FDC surface in the lymphoid tissue. This suggests that FDCs are not simply accumulating the FDCs on their surface after replication on another cell type within the lymphoid tissue and instead actively replicate the scrapie agent themselves.

In this Chapter it was determined whether PrP\textsuperscript{C} expression exclusively on FDCs was sufficient to support scrapie agent replication within the lymphoid tissue. To address this issue, the CD21-cre mouse line was crossed with the Prnp\textsuperscript{stop/-} mouse line to create a model in which PrP\textsuperscript{C} expression was switched on exclusively on CD21-expressing cells. These animals were subsequently irradiated and reconstituted with non-Cre-expressing bone marrow to restrict PrP\textsuperscript{C} expression exclusively to the FDC network. Characterisation of this compound transgenic model (Chapter 6) showed that PrP\textsuperscript{C} was efficiently and specifically expressed on the FDC networks of CD21-
crePrPstop/- mice. Furthermore, no adverse effects on the status of FDC networks, or in the general microarchitecture of the lymphoid tissue were observed. These data confirmed that the CD21-crePrPstop/- model would be a useful tool to determine the role of PrP^C-expressing FDCs in scrapie pathogenesis and determine whether if PrP^C expression on exclusively on FDCs is sufficient to support scrapie agent replication in the peripheral lymphoid tissues. As host expression of PrP^C is essential for TSE agent pathogenesis (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997), if FDCs themselves actively replicate the TSE agent, then PrP^C expression exclusively on the FDC network should be sufficient to enable TSE agent replication in the lymphoid tissues.

CD21-crePrPstop/- mice were infected ip with the ME7 scrapie agent and culled at 35, 70 or 105 dpi. PrP^C expression in the CNS is essential for efficient transmission and neuropathology of the scrapie agent (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997). As CD21-crePrPstop/- mice have no PrP^C expression in the CNS, it is not anticipated that these animals could develop clinical TSE disease (Tuzi, Clarke et al. 2004). For this reason, no animals were left beyond 105 dpi. However to determine if this was the case, some transgenic animals were inoculated ic with the ME7 scrapie agent directly into the brain. Spleens were harvested from all time points and the cellular sites of PrP^Sc accumulation were determined. PrP^Sc has been shown to co-purify with TSE agent infectivity and is considered by many to be the sole component of the TSE infectious agent and therefore was used as a biochemical marker of disease in this study (Bolton, McKinley et al. 1982).
In animals with PrP\textsuperscript{C} expression switched on specifically in FDCs, the scrapie agent, as detected by PrP\textsuperscript{Sc} immunolabelling, located to the FDC network by 35 dpi. The deposition of PrP\textsuperscript{Sc} on the FDC increases at later time points, suggesting that the scrapie agent is efficiently replicating on the FDC networks. However, when PrP\textsuperscript{C} is switched on only in CD21\textsuperscript{+} B lymphocytes, PrP\textsuperscript{Sc} is found only within the TBMs. These data show that PrP\textsuperscript{C} expressing FDCs are responsible for replicating the scrapie agent within the lymphoid tissue. Without PrP\textsuperscript{C} expression on FDCs the scrape agent was unable to replicate within the lymphoid tissues and is instead taken up by TBMs.
7.3 Results

7.3.1 Experimental design

To determine if PrP\textsuperscript{C} expression exclusively on FDCs is sufficient to support TSE agent replication in the lymphoid tissues, the \textit{CD21-crePrP\textsuperscript{stop/-}} mouse line was injected ip with ME7 scrapie. Animals were aged to 8 weeks, lethally \(\gamma\)-irradiated and given donor bone marrow 24 h later as indicated. To restrict PrP\textsuperscript{C} expression specifically to FDCs, \textit{CD21-crePrP\textsuperscript{stop/-}} mice were given bone marrow from Cre-negative littermates. However, other transgenic host/bone marrow combinations were also produced as control lines and these are summarised in Table 5.1. Animals were used in experiments at 100 d post bone-marrow reconstitution to allow for efficient replacement of host bone-marrow derived cells with donor counterparts. Mice from each group were then injected ip with 20 \(\mu\)l of a 1\% (wt/vol) scrapie brain homogenate. Animals were then culled at 35, 70 and 105 days post-inoculation (dpi) and tissues were collected to assess the cellular sites of PrP\textsuperscript{Sc} accumulation in the spleen. As animals have no PrP\textsuperscript{C} expression in the CNS, no animals were expected to develop clinical disease. For this reason, no animals were left beyond the 105 dpi time point. However, to determine if this was true, some \textit{CD21-crePrP\textsuperscript{stop/-}} animals were infected with scrapie directly into the CNS via ic injection alongside 129/Ola WT controls. A summary of experimental design can be found in Figure 7.1.

The cellular sites of PrP\textsuperscript{Sc} accumulation in the spleen were determined by immunolabelling for PrP. Immunohistochemical analysis was carried out using the
### Table 5.1 Summary of experimental lines used in scrapie experiments

<table>
<thead>
<tr>
<th>Host Genotype</th>
<th>Donor Genotype</th>
<th>PrP&lt;sup&gt;C&lt;/sup&gt; switched on</th>
<th>Nomenclature</th>
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<tbody>
<tr>
<td>CD21-crePrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
<td>PrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
<td>FDCs only</td>
<td>Cre&lt;sup&gt;-ve&lt;/sup&gt; → CD21-crePrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
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<tr>
<td>CD21-crePrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
<td>CD21-crePrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
<td>FDCs and B lymphocytes</td>
<td>Cre&lt;sup&gt;-ve&lt;/sup&gt; → CD21-crePrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
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<td>PrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
<td>CD21-crePrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
<td>B lymphocytes only</td>
<td>Cre&lt;sup&gt;-ve&lt;/sup&gt; → PrP&lt;sup&gt;stop&lt;/sup&gt;-</td>
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<td>PrP&lt;sup&gt;WT&lt;/sup&gt;-</td>
<td>PrP&lt;sup&gt;WT&lt;/sup&gt;-</td>
<td></td>
<td>PrP&lt;sup&gt;WT&lt;/sup&gt;- → PrP&lt;sup&gt;WT&lt;/sup&gt;-</td>
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**Figure 5.1 Summary of experimental design**
rabbit anti-PrP PAb antibody 1B3 and confirmed using the mouse anti-PrP MAb 6H4. As both antibodies gave similar results, only immunohistochemistry images from 1B3 immunolabelling are shown. Treatment with PK digests the cellular PrP\textsuperscript{C} leaving the PK-resistant core of PrP\textsuperscript{Sc} intact (Manson, Clarke et al. 1994b). This property of PrP\textsuperscript{Sc} can be used to distinguish PrP\textsuperscript{C} and PrP\textsuperscript{Sc} on histological specimens by using the paraffin-embedded tissue (PET) blot method. This method was used to confirm if the disease specific PrP (PrP\textsuperscript{d}) detected by immunohistochemistry were scrapie-associated PrP\textsuperscript{Sc}. Detection of PrP\textsuperscript{d} and PrP\textsuperscript{Sc} have been confirmed by many studies to be reliable markers of the ME7 scrapie agent and in most cases correlate closely with presence of TSE agent infectivity (Bolton, McKinley et al. 1982; McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009).

**7.3.2 Pathogenesis of ME7 scrapie at 35 dpi in mice with PrP\textsuperscript{C} expression exclusively on FDCs**

Animals were culled at 35 dpi with the ME7 scrapie agent and spleens were harvested for analysis of the cellular sites of PrP\textsuperscript{Sc} accumulation. In spleens with PrP\textsuperscript{C} expression exclusively on Cre-expressing FDCs, and in PrP\textsuperscript{WT/\textminus} control spleens, accumulation of the abnormal, disease-associated PrP\textsuperscript{d} occurred in the germinal centres of the spleen (Figs 7.2). This PrP\textsuperscript{d} was located on the FDC network as shown by immunolabelling of serial sections for PrP\textsuperscript{d}, FDCs and B lymphocytes (Fig 7.3). A
Figure 7.2 PrP\textsuperscript{d} immunolabelling in the spleen at 35 dpi with the ME7 scrapie agent

Four animals from each experimental line were culled at 35 dpi with the ME7 scrapie agent and spleens were immunolabelled to detect PrP\textsuperscript{d} (PAb 1B3, red). One animal was lost to an intracurrent death.

All spleens with PrP\textsuperscript{C} expression exclusively in Cre-expressing FDCs (A-G) or PrP\textsuperscript{WT/-} mice with PrP\textsuperscript{C} expressing FDCs (L-O), show PrP\textsuperscript{d} immunolabelling at low levels on the FDC networks (\(\blacktriangle\)) in the follicles. However, spleens from animals with PrP\textsuperscript{C/-}-deficient FDCs (H-K) do not show immunolabelling on the FDC network. In these animals, the only positive PrP\textsuperscript{d} immunolabelling is found within the TBMs (\(\ast\)).

These data demonstrate that the scrapie agent is able to localise to the follicles of the spleen independently of PrP\textsuperscript{C} expression as PrP\textsuperscript{d} is present on FDCs of animals expressing PrP\textsuperscript{C} exclusively on the FDCs. If FDCs are PrP\textsuperscript{C/-} deficient, the agent is localised to the follicle but is found mainly within the TBMs. Scale bar on main figure 100 \(\mu\text{m}\). Scale bar on x 1000 magnification images 20\(\mu\text{m}\). Sections counterstained with haematoxylin, blue.
Fig 7.3 Immunohistochemical analysis of PrP<sup>d</sup>, FDC and B lymphocytes in spleens of mice taken at 35 dpi with the ME7 scrapie agent

Serial sections from spleens were immunolabelled for PrP<sup>d</sup> (PAb 1B3, red), FDCs and CD21<sup>+</sup> B lymphocytes (MAb 7G6, blue) and B lymphocytes (MAb B220, red) to determine if PrP<sup>d</sup> detected was co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with PrP<sup>C</sup> expression exclusively on Cre-expressing FDCs (A-F), or with PrP<sup>C</sup>-expressing FDCs in PrP<sup>WT</sup>- mice (J-L), PrP<sup>d</sup> (▲) is localised to the FDC networks (→) as shown in serial sections. PrP<sup>d</sup> location in spleens with PrP<sup>C</sup> deficient, FDCs (G-I) PrP<sup>d</sup> was did not co-localise to the FDC networks but instead was found exclusively within TBMs (★) in the follicles. Scale bar 100 μm. Sections counterstained with haematoxylin, blue.
Figure 7.4 PET blot analysis of PrP<sup>Sc</sup> accumulation in spleens taken at 35 dpi with the ME7 scrapie agent

Paraffin-embedded sections on nitrocellulose membranes were treated with PK to remove any native PrP<sup>C</sup>, then immunolabelled (PAb, 1B3, blue/black) to detect any remaining PrP<sup>Sc</sup>. One representative example from each transgenic line is shown. A, Cre<sup>−ve</sup>→CD21-crePrP<sup>fl/−</sup>; B, Cre<sup>−ve</sup>→CD21-crePrP<sup>fl/−</sup>; C, Cre<sup>−ve</sup>→PrP<sup>fl/−</sup>; D, PrP<sup>fl/−</sup>→PrP<sup>fl/−</sup>). Scale bar 500 μm.

Due to the low levels of PrP<sup>d</sup> detected in infected tissue at this stage by immunohistochemistry (Fig 7.2), PrP<sup>Sc</sup> is not readily detected after PK treatment in any of the transgenic experimental lines, however a few follicles in spleens with PrP<sup>C</sup>-expressing FDCs have a little PrP<sup>d</sup> labelling present (→). Therefore PrP<sup>d</sup> detected in these tissues by immunohistochemistry can be confirmed to be PrP<sup>Sc</sup>.
little immunolabelling of PrP\textsuperscript{d} was still detected in the follicles after PK treatment, however, this was only at very low levels as animals are only expressing half copy number levels of \textit{Prnp} and were culled early in the incubation period (Fig 7.4). These data suggest that in animals with PrP\textsuperscript{C} expression exclusively on FDCs at 35 dpi with the ME7 scrapie agent, the TSE agent can locate to the follicles of the spleen and begin to replicate on the PrP\textsuperscript{C}-expressing FDC networks.

In mice with PrP\textsuperscript{C} deficient FDCs, PrP\textsuperscript{d} was detected in the spleen at 35 dpi (Figs 7.2). However, the PrP\textsuperscript{d} detected did not co-localise to the FDC networks. Instead immunolabelling of PrP\textsuperscript{d} was located exclusively within TBMs within the follicle (Fig 7.3). Analysis of PET blots of spleens from these animals could not confirm the presence of PrP\textsuperscript{Sc} (Fig 7.4).

7.3.3 Pathogenesis of ME7 scrapie at 70 dpi in mice with PrP\textsuperscript{C} expression exclusively on FDCs

At 70 dpi with the ME7 scrapie agent, spleens with PrP\textsuperscript{C} expression exclusively on Cre-expressing FDCs, and in PrP\textsuperscript{WT/-} control spleens, accumulation of the abnormal, disease-associated PrP\textsuperscript{d} occurred in the germinal centres of the spleen at a greater intensity than that seen in spleens from animals culled at 35 dpi (Figs 7.5). This PrP\textsuperscript{d} was located on the FDC network as shown by immunolabelling of serial sections for FDCs and B lymphocytes (Fig 7.6). Furthermore, the PrP\textsuperscript{d} accumulations detected upon the FDC networks were confirmed to be PK-resistant, scrapie agent-associated PrP\textsuperscript{Sc} using the PET blot method (Fig 7.7).
Figure 7.5 PrP<sup>d</sup> immunolabelling in spleen at 70 dpi with the ME7 scrapie agent

Four animals from each experimental line were culled at 70 dpi with ME7 scrapie and spleens were immunolabelled to detect PrP<sup>d</sup> (PAb 1B3, red).

All spleens with PrP<sup>C</sup> expression exclusively in Cre-expressing FDCs (A-G) or PrP<sup>WT/-</sup> mice with PrP<sup>C</sup> expressing FDCs (L-O), show PrP<sup>d</sup> immunolabelling on the FDC networks (▲) within the follicles at a greater level than that seen in spleens from mice culled at 35 dpi. This indicates the scrapie agent is successfully replicating on the PrP<sup>C</sup>-expressing FDCs despite all other cell types within the animal being Pr<sup>np</sup><sup>-/-</sup> (A-D) or only CD21<sup>+</sup> B lymphocytes also expressing PrP<sup>C</sup> (E-G).

Spleens from animals with PrP<sup>C</sup>-deficient FDCs (H-K) do not show any immunolabelling of PrP<sup>d</sup> on the FDC, instead, PrP<sup>d</sup> is detected only within neighbouring TBM's (*). This suggests the scrapie agent is unable to replicate on PrP<sup>C</sup> deficient FDCs and any PrP<sup>d</sup> from innocula has been cleared from FDC networks and sequestered by TBM's in the follicle. Scale bar on main figure 100 μm. Scale bar on x 1000 magnification images 20μm. Sections counterstained with haematoxylin, blue.
**Fig 7.6 Immunohistochemical analysis of PrP\textsuperscript{d}, FDC and B lymphocytes in spleens of mice taken 70 dpi with the ME7 scrapie agent**

Serial sections from spleens were immunolabelled for PrP\textsuperscript{d} (PAb 1B3, red), FDCs and CD21\textsuperscript{+} B lymphocytes (MAb 7G6, red) and B lymphocytes (MAb B220, red) to determine if PrP\textsuperscript{d} detected co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with PrP\textsuperscript{C} expression exclusively on Cre-expressing FDCs (A-F), or with PrP\textsuperscript{C}-expressing FDCs in PrP\textsuperscript{WT/-} mice (J-L), PrP\textsuperscript{d} (▲) is localised to the FDC networks (→) as shown in serial sections. PrP\textsuperscript{d} in spleens with PrP\textsuperscript{C} deficient FDCs (G-I) was not present on the FDC networks but instead was found exclusively within TBM\textsuperscript{s} (*) in the B lymphocyte follicles. Scale bar 100 μm. Sections counterstained with haematoxylin, blue.
Figure 7.7 PET blot analysis of PrP<sup>Sc</sup> accumulation in spleens taken at 70 dpi with the ME7 scrapie agent

Paraffin-embedded spleens sections on nitrocellulose membranes were treated with PK to remove any native PrP<sup>C</sup> and then immunolabelled (PAb 1B3, blue) to detect any remaining PrP<sup>Sc</sup>. One representative example from each transgenic line is shown. 

A, Cre<sup>ve</sup>→ CD21-cre PrP<sup>fl/-</sup>; B, Cre<sup>ve</sup>→ CD21-cre PrP<sup>fl/-</sup>; C, Cre<sup>ve</sup>→ PrP<sup>fl/-</sup>; D, PrP<sup>fl/-</sup>→ PrP<sup>fl/-</sup>). Scale bar 500 μm

In spleens with PrP<sup>C</sup> expression exclusively on Cre-expressing FDCs (A, B), or with PrP<sup>C</sup>-expressing FDCs in PrP<sup>WT/-</sup> mice (D), PrP<sup>d</sup> immunolabelling is present in the follicles after PK treatment (►). Therefore PrP<sup>d</sup> detected in these tissues by immunohistochemistry (Fig 7.5) can be confirmed to be the disease-associated PrP<sup>Sc</sup>. In contrast, spleens from mice with PrP<sup>C</sup> deficient FDCs (C) show no PrP<sup>Sc</sup> in the follicles. This confirms that PrP<sup>C</sup> expressing FDCs actively replicate the TSE agent in the lymphoid tissues.
From these data it can be concluded that PrP\textsuperscript{C} expression exclusively on FDCs is sufficient to support replication of the scrapie agent in peripheral lymphoid tissues, confirming that the FDCs actively replicate the TSE agent. Furthermore, no significant differences were observed in the levels of deposition of PrP\textsuperscript{d} on Cre-expressing, PrP\textsuperscript{C}-expressing FDCs in mice that received Cre\textsuperscript{+ve} or Cre\textsuperscript{-ve} bone marrow. This suggests that PrP\textsuperscript{C} expression on CD21\textsuperscript{+} B lymphocytes is irrelevant to peripheral disease pathogenesis as previously reported (Klein, Frigg et al. 1998; Brown, Stewart et al. 1999; Mohan, Brown et al. 2004).

In spleens from mice with PrP\textsuperscript{C} deficient FDCs, no deposition of PrP\textsuperscript{d} was observed on the FDC networks. Instead, if PrP\textsuperscript{d} was detected at all, it was only found within TBMs (Fig 7.5). Immunolabelling of serial sections for FDCs and B lymphocytes confirmed that PrP\textsuperscript{d} immunolabelling did not co-localise with FDC networks (Fig 7.6). The levels of PrP\textsuperscript{d} observed within the TBMs were too low to be confirmed by the PET blot method as PrP\textsuperscript{Sc} (Fig 7.7). From these data it can be concluded that PrP\textsuperscript{C}-expressing FDCs are essential for the scrapie agent to accumulate within the lymphoid tissues. Without active replication of the agent by PrP\textsuperscript{C} expressing FDCs, the scrapie agent appears to be scavenged by TBMs in the follicle and possibly degraded. Furthermore, these animals with PrP\textsuperscript{C} deficient FDCs had been given Cre\textsuperscript{+ve} bone marrow so have PrP\textsuperscript{C} expression switched on exclusively in CD21\textsuperscript{+} B lymphocytes. These data also confirm that PrP\textsuperscript{C} expression by mature B lymphocytes is not sufficient to support replication of the scrapie agent in the spleen as previously reported (Montrasio, Cozzio et al. 2001).
7.3.4 Pathogenesis of ME7 scrapie at 105 dpi in mice with PrP<sup>C</sup> expression exclusively on FDCs

At 105 dpi with the ME7 scrapie agent in spleens with PrP<sup>C</sup> expression exclusively on Cre-expressing FDCs, and in PrP<sup>WT/-</sup> control spleens, accumulation of the abnormal, disease-associated PrP<sup>d</sup> occurred in the germinal centres of the spleen at a greater intensity than that seen in spleens from animals culled at 70 dpi (Fig 7.8). As previous, PrP<sup>d</sup> was co-localised to the FDC network as shown by immunolabelling in serial sections for FDCs and B lymphocytes (Fig 7.9). Furthermore, PrP<sup>d</sup> accumulation within the germinal centres was confirmed to be the PK-resistant, scrapie-associated PrP<sup>Sc</sup> using the PET blot method (Fig 7.10). These results further supports data from previous time points showing that the PrP<sup>C</sup> expression exclusively on FDCs is sufficient to support scrapie agent replication in the peripheral lymphoid tissues and confirms that PrP<sup>C</sup>-expressing FDCs actively replicate the TSE agent in the lymphoid tissue. Additionally, no significant differences were seen in deposition of PrP<sup>d</sup> in spleens from mice with PrP<sup>C</sup> expression exclusively on Cre-expressing FDCs that received Cre<sup>++</sup> or Cre<sup>-ve</sup> bone marrow indicating that PrP<sup>C</sup> expression on CD21<sup>+</sup> B lymphocytes is irrelevant to peripheral disease pathogenesis of the scrapie agent.

In spleens from mice with Cre-deficient, PrP<sup>C</sup> deficient FDCs, there was no deposition of PrP<sup>d</sup> on the FDC networks (Fig 7.8). Furthermore, levels of PrP<sup>d</sup> detected within the TBMs at 105 dpi were greatly reduced in comparison to that seen at 35 and 70 dpi (Figs 7.2 and 7.5 respectively). Immunolabelling of serial sections for FDCs and B lymphocytes confirmed that the little PrP<sup>d</sup> immunolabelling present
**Figure 7.8 PrP^d immunolabelling in the spleen at 105 dpi with the ME7 scrapie agent**

Four animals from each experimental line were culled at 105 dpi with the ME7 scrapie agent and spleens were immunolabelled to detect PrP^d (PAb 1B3, red). All spleens with PrP^C expression exclusively in Cre-expressing FDCs (A-H) or PrP^WT/- mice with PrP^C expressing FDCs (M-P), show PrP^d immunolabelling on the FDC networks (▲) within the follicles at an increased level than that seen in spleens from animals culled at 70 dpi. This indicates the scrapie agent is continuing to replicate on the PrP^C-expressing FDCs despite all other cell types within the animal being PrP^C-deficient (A-D) or only CD21^+ B lymphocytes additionally expressing PrP^C (E-H).

Spleens from mice with PrP^C deficient FDCs (I-L) show little/no immunolabelling of PrP^d at this time point. PrP^d is detected only within TBMs (*) in one animal. This suggests the scrapie agent is still unable to replicate on PrP^C deficient FDCs and as levels of PrP^d staining within TBMs are reduced in comparison to earlier time points (Fig 7.2 and 7.5), TBMs are most likely degrading the scrapie agent. Scale bar on main figure 100 μm. Scale bar on x1000 magnification images 20 μm. Sections counterstained with haematoxylin, blue.
Serial sections from spleens were immunolabelled for PrP (PAb 1B3, red), FDCs and CD21+ B lymphocytes (MAb 7G6, red) and B lymphocytes (MAb B220, red) to determine if PrP<sup>d</sup> detected was co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with PrP<sup>C</sup> expression exclusively on Cre-expressing FDCs (A-F), or with PrP<sup>C</sup>-expressing FDCs in PrP<sup>WT/-</sup> mice (J-L), PrP<sup>d</sup> (▲) is localised to the FDC networks (→) as shown by serial sections. PrP<sup>d</sup> in spleens with PrP<sup>C</sup> deficient FDCs (G-I), was not present on the FDC networks but instead was found exclusively within TBMs (★) in the follicles. Scale bar 100 μm. Sections counterstained with haematoxylin, blue.
Figure 7.10 PET blot analysis of PrPSc accumulation in spleens taken at 105 dpi with the ME7 scrapie agent

Paraffin-embedded sections on nitrocellulose membranes were treated with PK to remove any native PrPC, then immunolabelled (PAb 1B3, blue/black) to detect any remaining PrPSc. One representative example from each transgenic line is shown A, Cre\textsuperscript{\textasciitilde} → CD21-crePrP\textsuperscript{fl/\textminus}; B, Cre\textsuperscript{\textasciitilde} → CD21-crePrP\textsuperscript{fl/\textminus}; C, Cre\textsuperscript{\textasciitilde} → PrP\textsuperscript{fl/\textminus}; D, PrP\textsuperscript{fl/\textminus} → PrP\textsuperscript{fl/\textminus}). Scale bar 500 μm.

In spleens with PrP\textsuperscript{C} expression exclusively on Cre-expressing FDCs (A, B), or with PrP\textsuperscript{C}-expressing FDCs in PrP\textsuperscript{WT/\textminus} mice (D), PrP\textsuperscript{d} immunolabelling is present in the follicles after PK treatment (\textsuperscript{\blacktriangleright}). Therefore PrP\textsuperscript{d} detected in these tissues by immunohistochemistry (Fig 7.8) can be confirmed to be the disease-associated PrPSc. In contrast, spleens from mice with PrP\textsuperscript{C} deficient FDCs (C) show no PrPSc in the follicles. This confirms that PrP\textsuperscript{C} expressing FDCs actively replicate the TSE agent in the lymphoid tissues.
did not co-localise with FDC networks (Fig 7.9). The levels of PrP\textsubscript{d} accumulation observed within the TBMs were too low to be confirmed by the PET blot method as PrP\textsubscript{Sc} (Fig 7.10). These data confirm that PrP\textsubscript{C}-expressing FDCs are essential for the scrapie agent to replicate within the lymphoid tissues and without active replication by FDCs, the scrapie agent is scavenged by neighbouring TBMs. As levels of PrP\textsubscript{d} immunolabelling within the TBM are reduced over time it appears that the TBMs within the B lymphocyte follicle are degrading the scrapie agent.

7.3.5 TSE disease in the CNS of mice with PrP\textsubscript{C} expression exclusively on FDCs

Previous studies have shown that host expression of PrP\textsubscript{C} is absolutely required for TSE disease to occur. \textit{CD21-crePrP\textsubscript{stop/-}} animals only express PrP\textsubscript{C} on FDCs and mature B lymphocytes and are therefore TSE disease should not be able to progress to the CNS and develop into clinical disease in these animals. For this reason, the 105 dpi time point after ME7 scrapie agent exposure was the final time point included in this experiment as no animals were expected to develop clinical disease. However to ensure that \textit{CD21-crePrP\textsubscript{stop/-}} mice are resistant to TSE disease in the CNS, some animals were injected with the ME7 scrapie agent directly into the CNS via ic injection alongside 129/Ola WT controls. WT animals developed TSE clinical disease with positive neuropathology in the brain with an average incubation period of 158 ± 2 dpi (Chapter 5 Fig 5.10). The classical hallmarks of TSE infection in the brain are deposition of abnormal, disease-associated PrP\textsubscript{d}, gliosis and spongiform pathology (vacuolation). TSE disease in the brains of WT animals was confirmed by positive immunolabelling of PrP\textsubscript{d}, activated astrocytes (GFAP\textsuperscript{+} cells) or microglia (IBA-1\textsuperscript{+} cells) in the brains of transgenic animals in comparison to WT controls.
(Chapter 5 Fig 5.8). Furthermore, lesion profiling of the TSE specific vacuolation in specific areas of the brain is commonly used to characterise TSE disease in mice. Typical neuropathology, characteristic of infection with the ME7 scrapie agent was observed in the brains of control mice (Chapter 5 Fig 5.9). In contrast, CD21-crePrPstop/- mice injected with ME7 scrapie are still alive at 313 dpi at point of writing. This exceeds the mean incubation time of 297 ± 4 dpi in CD21-crePrPfl/- mice (Chapter 5 Fig 5.10), which have half copy level expression of Prnp. When surviving CD21-crePrPstop/- mice are culled due to clinical disease or aging, brains and spleens will be harvested to determine if signs of scrapie pathology are present. However, due to lack of PrPC expression in the CNS, it is not anticipated that these animals will develop clinical TSE disease.
7.4 Discussion

The CD21-crePrPstop/- mouse line, with irradiation and reconstitution with non-Cre-expressing bone marrow, was previously shown to allow PrP<sup>C</sup> expression to be switched on specifically on FDCs without adverse effects on FDC status or the lymphoid tissues (Chapter 6). In this chapter the CD21-crePrPstop/- line was injected ip with the ME7 scrapie agent to determine if PrP<sup>C</sup> expression exclusively on FDCs is sufficient to support scrapie agent replication within the lymphoid tissue and conclusively determine whether FDCs themselves actively replicate the scrapie agent. If FDCs themselves actively replicate the TSE agent, then PrP<sup>C</sup> expression exclusively on the FDC network should be sufficient to support replication of scrapie in the lymphoid tissue.

Immunolabelling of PrP<sup>Sc</sup> in the lymphoid tissue was used to assess the cellular localisation of the scrapie agent. PrP<sup>Sc</sup> has previously been shown to co-purify with TSE agent infectivity in tissues from scrapie-affected animals and many studies have confirmed this to be a reliable marker of TSE disease (Bolton, McKinley et al. 1982; McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009). PrP<sup>Sc</sup> and infectivity accumulates in the lymphoid tissues prior to detection in the CNS and deposition of PrP<sup>Sc</sup> on the FDC network can be seen as early as 4 weeks post peripheral inoculation (Brown, Stewart et al. 1999). In this experiment, animals were culled at 35, 70 and 105 dpi to assess the early pathogenesis of ME7 scrapie in transgenic and control lines. As animals in this study are expressing only half copy number levels of Prnp,
less deposition of PrP\textsuperscript{Sc} would be expected on the FDC networks than seen in previous studies using Prnp\textsuperscript{+/+} animals. No animals were left to progress beyond 105 dpi. Host expression of PrP\textsuperscript{C} has been shown to be essential for effective pathogenesis of the scrapie agent (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997). Therefore, as animals are Prnp\textsuperscript{-/-} except in cells where Cre is expressed (FDCs and/or CD21\textsuperscript{+} B lymphocytes), it is unlikely that CNS disease would ever develop. However, to verify this, some CD21-crePrP\textsuperscript{stop/-} animals were also injected ic with the ME7 scrapie agent along with 129/Ola WT mice as controls.

Animals with Cre-expressing, Prnp\textsuperscript{+//-} FDCs had detectable PrP\textsuperscript{Sc} on the FDCs of the spleen at 35 dpi. This accumulation intensified by 70 dpi and 105 dpi suggesting active replication of the scrapie agent on the PrP\textsuperscript{C}-expressing FDCs. This shows that PrP\textsuperscript{C} expression on FDCs alone is sufficient to support replication of the scrapie agent in the lymphoid tissues. This confirms previous studies which implied that that PrP\textsuperscript{C}-expressing FDCs actively replicate the scrapie agent in the lymphoid tissue (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009).

In animals with PrP\textsuperscript{C} switched on only in CD21\textsuperscript{+} B lymphocytes, little to no PrP\textsuperscript{d} labelling is found in follicle at 35 dpi and by 70 and 105 dpi, the only labelling detected is within the TBMNs in the follicle. Some studies have suggested a role for B lymphocytes in replicating the scrapie agent in the lymphoid tissue (Klein, Frigg et al. 1997). However, others conclude that B lymphocyte involvement is indirect and is due to the dependence of the FDCs on B lymphocyte-derived signals to maintain a
mature and functioning FDC network (Klein, Frigg et al. 1998; Brown, Stewart et al. 1999). Data from the CD21-crePrPstop/- study shows that PrP<sup>C</sup> expression on CD21<sup>+</sup> mature B lymphocytes alone is not sufficient to support scrapie replication. Furthermore, no differences were seen in pathogenesis of scrapie when PrP<sup>C</sup> was expressed on FDCs alone or on both FDCs and CD21<sup>+</sup> B lymphocytes. These data suggest that CD21<sup>+</sup> B lymphocytes do not have an active role in the replication of the scrapie agent within the lymphoid tissue. This is in agreement with data from a previous study which found over-expression of PrP<sup>C</sup> on B-lymphocytes only was not sufficient to support scrapie replication in the lymphoid tissue (Montrasio, Cozzio et al. 2001).

Transgenic animals that were inoculated ic with the ME7 scrapie agent have still not succumbed to clinical TSE disease 313 dpi with the ME7 scrapie agent at time of writing. Previous studies using Prnp<sup>+/−</sup> mice (Tew and Mandel 1979) and data from this thesis using CD21-crePrPflm<sup>−/−</sup> animals (Chapter 5) has shown that following ic injection with the scrapie agent, the incubation period of Prnp<sup>+/−</sup> mice is approximately 300 dpi (Chapter 5 Fig 5.10). CD21-crePrPstop/- mice in this study have progressed beyond this time point without showing signs of TSE disease so it appears that this line will be resistant to disease in the CNS as expected.

These data show that with no PrP<sup>C</sup> present on the FDCs, the scrapie agent is unable to replicate on FDCs after initial localisation and appears to be rapidly cleared from the FDC network by TBMs in the follicle. TBMs are capable of taking up immune-complex coated portions of FDC membranes and therefore have the potential to remove the scrapie agent directly from the FDC surface (Szakal and Tew 1992;
Additionally, previous publications have shown that depletion of macrophages prior to scrapie infection decreases scrapie incubation period and enhances replication in the lymphoid tissues further suggesting a role for macrophages in the degradation of the scrapie agent (Beringue, Demoy et al. 2000). These data further confirm that PrP$^C$ expressing FDCs actively replicate the scrapie agent on their surface and without this replication, the scrapie agent is cleared from the FDC networks by TBMs.
CHAPTER 8

General Discussion

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8. General discussion

8.1 Introduction

Accumulation of the TSE agent in the lymphoid tissue occurs in many cases of natural TSE disease. Many natural scrapie infections of sheep have accumulation of PrP$^{\text{Sc}}$ in the spleen and lymphoid tissues of prior to CNS disease (van Keulen, Schreuder et al. 1996; Andreoletti, Berthon et al. 2000; Heggebo, Press et al. 2000; Heggebo, Press et al. 2002) and deer which have been naturally infected with CWD also show PrP$^{\text{d}}$ and agent infectivity within the lymphoid tissues (Sigurdson, Williams et al. 1999; Fox, Jewell et al. 2006). Furthermore, patients with vCJD show PrP$^{\text{d}}$ and/or infectivity in the appendix and tonsils (Hilton, Fathers et al. 1998; Hill, Butterworth et al. 1999; Bruce, McConnell et al. 2001) and recent evidence has shown transmission of vCJD from contaminated blood products (Llewelyn, Hewitt et al. 2004; Peden, Head et al. 2004; Wroe, Pal et al. 2006; Peden, McCardle et al. 2010). These data, along with the current lack of available treatments and invariable fatality of infected patients, highlight the need to understand the pathogenesis of TSE disease within the lymphoid tissues to determine cellular targets for focused research into prophylactic treatments.

Previous studies investigating the role of the lymphoid tissues in TSE disease have highlighted PrP$^{\text{C}}$-expressing FDCs as an essential requirement for successful pathogenesis of the TSE agent within peripheral lymphoid tissues (McBride, Eikelenboom et al. 1992; Klein, Frigg et al. 1998; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Kaeser, Klein et al. 2001; Mabbott and Bruce 2002; Mabbott, McGovern et al. 2002; Prinz, Montrasio et al. 2002; Mabbott, Young et al. 2002; Peden, Head et al. 2004; Wroe, Pal et al. 2006; Peden, McCardle et al. 2010).
However, in models used so far, there has been no definitive way to exclusively examine the role of PrP<sup>C</sup>-expressing FDCs in isolation from all other stromal, neural and lymphoid cells within the lymphoid tissue. One of the main functions of FDCs is to pick up antigenic protein complexes and retain them on their surface for weeks, months or even years, which is an essential component in the affinity maturation of B lymphocytes and the development of immunological memory (Tew and Mandel 1979; Gray and Skarvall 1988; Gray and Matzinger 1991; Ahmed and Gray 1996). Therefore, it is possible that the PrP<sup>d</sup> observed on the FDC networks of TSE-affected animals is simply due to accumulation of the TSE agent on the FDC surface after replication on another cell type. An FDC-specific model was required to definitively determine whether FDCs themselves actively replicate the TSE agent or if they simply accumulate the agent after replication on another cell type. If FDCs are simply acquiring the TSE agent after replication on another cell type, investigations into which cell is responsible for replicating the TSE agent could provide a new therapeutic target for the prevention or prophylactic treatment of TSE disease.

The aims of this thesis were to create and characterise transgenic mouse models that allow the manipulation of PrP<sup>C</sup> expression specifically on FDCs and subsequently to infect these animals with scrapie to determine the specific role of the FDCs in TSE pathogenesis within the lymphoid tissue. The hypothesis was explored that if FDCs are simply accumulating the TSE agent on their surface after replication on another cell type, the accumulation of the TSE agent will occur on PrP<sup>C</sup>-deficient FDCs when all other cell types within the lymphoid tissue retain PrP<sup>C</sup> expression. If however, the
FDCs actively replicate the TSE agent in the lymphoid tissue, accumulation of PrP\textsuperscript{d} will not occur on the PrP\textsuperscript{C} deficient FDCs. Additionally, if FDCs are responsible for replicating the TSE agent within the lymphoid tissue, then PrP\textsuperscript{C} expression exclusively on FDCs should be sufficient to allow replication of the TSE agent within the lymphoid tissue.

8.2 Critical analysis of the transgenic mouse models used in this study

The creation and characterisation of the FDC-specific transgenic mouse models were a crucial component of this study. Transgenic lines were extensively characterised before use in ME7 infection studies, however some important points regarding conditional gene targeting mice should be noted. The ROSA26 mouse line is a reporter strain which is commonly used to characterise Cre-expression under cell specific promoters (Mao, Fujiwara et al. 1999). Promoter specific activation of Cre induces expression of $\beta$-galactosidase within Cre-expressing cells, which can subsequently be detected histologically using an X-gal stain. However, when analysing results achieved from such reporter strains, it is important to note that individual alleles may have a variable sensitivity to Cre activity as reported by Schmidt-Supprian and Rajewsky (Schmidt-Supprian and Rajewsky 2007). Therefore, the pattern of Cre-mediated DNA recombination will only be identical to that seen in reporter strains if the floxed alleles in compound transgenic mice have the same sensitivity to Cre as those in the reporter strain. In this thesis, the ROSA26 reporter strain was used to provide an initial indication of the cellular locations of Cre-expression, however extensive characterisation was also carried out on the compound transgenic lines achieved by crossing CD21-cre mice with the Prnp-floxed mouse lines.
Cre toxicity is a phenomenon where transgenic expression of Cre is lethal to the cell due to DNA damage and or chromosomal abnormalities. This can occur due to the presence of cryptic or pseudo LoxP sites that are contained within the mouse genome undergoing Cre-mediated DNA recombination (Thyagarajan, Guimarães et al. 2000; Schmidt-Supprian and Rajewsky 2007; Semprini, Troup et al. 2007). Characterisation of the number of CD21+ B lymphocytes (Chapter 3), the lymphoid tissue composition and microarchitecture, and the status and function of the FDCs (Chapters 4 and 6) all showed no apparent effects of toxicity in the Cre-expressing cells.

In the transgenic mouse lines used in this thesis, Cre is inserted after the Cr2 promoter, which encodes CD21. CD21 was considered to be restricted to FDCs and mature B lymphocytes. (Reynes 1985; Liu 1997; Takahashi, Kozono et al. 1997; Heggebo, Press et al. 2002). However, in humans, expression of CD21 has also been reported on a subpopulation of immature thymocytes (Tsoukas and Lambris 1988; Wagner and Hansch 2006), peripheral T lymphocytes (Fox, Jewell et al. 2006; Peden, McCardle et al. 2010) and on human cervical epithelium (Sixbey, Lemon et al. 1986). Within the mouse, expression has also been reported CD4+ T lymphocytes found within the MLN, activated granulocytes and mucosal mast cells (Gray and Skarvall 1988; Gray and Matzinger 1991; Andrasfalvy, Prechl et al. 2002; Heggebo, Gonzalez et al. 2003; Llewelyn, Hewitt et al. 2004). These data indicate that expression of CD21 is more widespread than previously thought. However, all other cells currently reported to express CD21 are derived from the blood. Therefore lethal γ-irradiation and reconstitution with non-Cre expressing bone marrow should still restrict Cre expression to the FDC network.
The CD21-creROSA26 mouse demonstrated that Cre was efficiently expressed in FDCs and mature B lymphocytes and that irradiation and reconstitution with WT bone marrow successfully restricted Cre expression to the FDC networks (Chapter 3). This was a key factor in using the CD21-cre mouse line for FDC specific manipulation of Prnp expression. Characterisation of both the CD21-crePrP$^{fl/+}$ and the CD21-crePrP$^{stop/-}$ lines confirmed the specificity and efficiency of the CD21-cre model by demonstrating efficient removal or expression of PrP$^C$ specifically on the FDC networks (Chapters 4 and 6 respectively) which is in agreement with previous data published using this mouse line showing efficient Cre-mediated DNA recombination in mature B lymphocytes and FDCs (Kraus, Alimzhanov et al. 2004; Victoratos, Lagnel et al. 2006). Previous studies using the CD21-cre line to restrict Cre expression to the FDC network analysed DNA from enriched FDC populations from the spleen and used PCR to assess the DNA recombination in the FDCs (Victoratos, Lagnel et al. 2006). These experiments were not undertaken in this thesis. However, PCR analysis of whole blood, spleen and tail DNA sufficiently showed the correct chimerism between donor bone marrow and host Cre expression status.

A population of Cre-expressing neuronal or glial cells within the ganglia of both the submucosal and myenteric plexi of the intestine and in the brain were also detected (Chapter 3). No literature has reported expression of the CD21 protein within cells of these tissues as of yet. Furthermore, data from the bioGPS database which stores microarray data of gene expression profiles from various mouse and human tissues shows no non-lymphoid expression of Cr2; including samples from intestine and brain (Chapter 1, Fig 1.3 http://biogps.gnf.org). Due to time constraints, it was not
possible to further investigate within which cell population Cre was activated in these tissues. However, the route of inoculation was adapted from oral to ip to take any Cre-mediated manipulation of Prnp in the intestine into consideration. In agreement with CD21-creROSA26 characterisation studies in this thesis, Cre activation in the forebrain of the CD21-cre mouse line has recently been reported (Schmidt-Supprian and Rajewsky 2007). Cre activation in the forebrain has no effect on the early stages of disease incubation that were examined in the ME7 infection studies but a possible effect of Cre-activation in the brain was considered when analysing clinical animal data in this thesis.

The Prnp floxed mouse lines used were created via gene targeting using bacterial artificial chromosomes, which helps to prevent the problems of ectopic- and over-expression of transgenes and disruption to gene function where the transgene has been inserted (Tuzi, Clarke et al. 2004). The PrpBlc model has been used with previous success to switch off PrPC expression exclusively on Schwann cells in the peripheral nerves to determine their role in TSE agent neuroinvasion and achieved a 90% reduction of PrPC levels (Bradford, Tuzi et al. 2009). Western blot quantification of PrPC levels expressed was not carried out in this thesis. However, PCR analysis confirmed recombination of the floxed DNA and quantification of the co-localisation of PrPC on immunohistochemical images showed efficient switching off/on of PrPC on FDC in both mouse lines characterised (Chapters 4 and 6).

The compound transgenic mouse lines used in the ME7 scrapie agent infection studies only expressed half copy number levels of Prnp due to complications in breeding the CD21-crePrpBlc line and the PrnpStop allele being lethal at homozygosity, as discussed
in Chapters 4 and 6, respectively. Previous studies have shown that expression levels of PrP\(^C\) inversely correlate with incubation periods of TSE infection in mice. Over expression of PrP\(^C\) leads to significantly shorter incubation periods (Fischer, Rulicke et al. 1996), whereas half copy number levels of Prnp expression extend the incubation period of disease (Manson, Clarke et al. 1994b). Therefore in the models used in this thesis, although disease incubation period was increased there should be no other differences in disease pathogenesis.

In summary, characterisation of the CD21-creROSA26, CD21-crePrP\(^{fl/}\) and CD21-crePrP\(^{stop/-}\) mouse lines used in this thesis has sufficiently demonstrated gene manipulation specifically on FDCs after irradiation and transfer of WT bone marrow. Furthermore, no adverse effects were observed on the FDC status or general microarchitecture of the lymphoid tissue. Data from characterisation studies demonstrate that the CD21-crePrP\(^{fl/}\) and CD21-crePrP\(^{stop/-}\) mice are robust in vivo models for manipulating expression of Prnp exclusively on FDCs.

### 8.3 Do FDCs express PrP\(^C\)?

The presence of PrP\(^C\)-expressing FDCs has been shown by many studies to be necessary for successful replication of the TSE agent (Brown, Stewart et al. 1999; Mabbott, Williams et al. 2000a; Mabbott, Mackay et al. 2000b; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Mohan, Brown et al. 2004; Ierna, Farquhar et al. 2006; Brown, Wathne et al. 2009). However, it has not been confirmed that FDCs themselves produce the PrP\(^C\) protein that they express on their surface. It is important to determine if FDCs acquire or produce expression of PrP\(^C\) as the mechanisms
involved in this process could have implications on the deposition of PrP<sup>d</sup> seen on the FDC surface in scrapie-affected animals.

One of the main roles of the FDC is to trap antigenic protein complexes which they can retain on their surface for long periods of time via expression of complement and Fc receptors (Fang, Xu et al. 1998; Prodeus, Goerg et al. 1998; Wu, Jiang et al. 2000). This is considered essential for affinity maturation of B lymphocytes and the development of immunological memory (Gray and Skarvall 1988; Gray, Kosco et al. 1991; Gray and Matzinger 1991). Furthermore, previous studies have shown that FDCs can acquire proteins, such as MHC class II, on their surface that they themselves do not express (Gray, Kosco et al. 1991; Thery, Regnault et al. 1999; Denzer, van Eijk et al. 2000). For these reasons, it is possible that FDCs acquire expression of PrP<sup>C</sup> rather than producing this protein themselves.

Exosomal transport of membrane proteins is a mechanism cells can use to acquire proteins that they themselves do not express. Exosomes are membrane vesicles of 40-100 nm in diameter, which preferentially contain lipid rafts and GPI-anchored proteins (Stoorvogel, Kleijmeer et al. 2002). This makes exosomal transport a potential mechanism for the transportation of PrP<sup>C</sup> protein which is also GPI-anchored. Neighbouring B lymphocytes in the follicle also express PrP<sup>C</sup> and are a possible candidate for the transfer of PrP<sup>C</sup> to FDCs (Mabbott, Brown et al. 1997). Indeed, previous studies have shown that FDCs can obtain proteins via exosomes from B lymphocytes (Denzer, van Eijk et al. 2000) but also from DCs (Thery, Regnault et al. 1999) and macrophages (Wang, Zhou et al. 2010). Furthermore, in vitro studies have shown that cells can release both PrP<sup>C</sup> and PrP<sup>Sc</sup> on exosomes and
have implicated this mechanism may be involved in the intracellular transfer of the TSE agent (Fevrier, Vilette et al. 2004; Fevrier, Vilette et al. 2005).

However, there is also evidence to support the expression of PrP\(^C\) by FDCs themselves. Studies analysing the development of FDC networks in SCID mice (Bruce, Brown et al. 2000) and neonatal mice (Ierna, Farquhar et al. 2006) found that PrP\(^C\) was detectable in the follicle at the same time as the development of mature FDC networks, suggesting that FDCs themselves express PrP\(^C\). Additionally, studies using bone marrow chimeric mice which had mismatches in PrP\(^C\) expression between stromal and lymphoid components showed that PrP\(^C\) was detected upon FDCs by immunohistochemistry when the donor bone marrow was PrP\(^C\) deficient. This eliminates the possibility that bone marrow-derived cells are donating PrP\(^C\) expression to the FDCs in this model (Brown, Stewart et al. 1999). Furthermore, data from the microarray analysis of gene expression by enriched splenic FDCs show that Prnp is expressed in these cells indicating that they subsequently express the PrP\(^C\) protein (Huber, Thielen et al. 2005; Wilke, Steinhauser et al. 2010). Data from this thesis supports FDCs producing and expressing PrP\(^C\) protein themselves. When PrP\(^C\) was switched off exclusively on FDCs, little to no PrP\(^C\) was detected on the FDC network even though all neighbouring cells in the follicle retained PrP\(^C\) expression (Chapter 4, Figs 4.5 and 4.6). From this evidence, it appears that FDCs actively produce the PrP\(^C\) protein they express. Hypothetically, it may be possible for FDCs to also acquire PrP\(^C\) protein from other cell types. However the model used in this study where PrP\(^C\) expression is switched off exclusively on FDCs suggest that little to no PrP\(^C\) is acquired by the FDCs from other cell types.
The function of PrP$^C$ in the immune system

The function of PrP$^C$ is as of yet not fully understood, and many possible functions of this protein have been suggested. PrP$^{+/−}$ mice have no overt neurological phenotype although small changes in synaptic transmission (Collinge, Whittington et al. 1994; Collinge, Collinge et al. 1996; Mallucci, Ratte et al. 2002), circadian rhythms (Tobler, Gaus et al. 1996), cognition (Coitinho, Roesler et al. 2003) and seizure thresholds (Walz, Amaral et al. 1999) have been reported. Additional suggested functions of PrP$^C$ in neurones are copper binding (Hornshaw, McDermott et al. 1995; Jackson, Murray et al. 2001), cell signalling (Mouillet-Richard, Ermonval et al. 2000; Spielhaupter and Schätzl 2001) and both pro- (Paitel, Alves da Costa et al. 2002) and anti- (Kuwahara, Takeuchi et al. 1999; Bounhar, Zhang et al. 2001; Chiarini, Freitas et al. 2002) apoptotic activity. However, these proposed functions of PrP$^C$ within the CNS do not always have bearing on what PrP$^C$ function in the immune system, and more specifically on the FDCs, truly is. Maturation of DCs and monocytes has been reported to up-regulate PrP$^C$ expression (Dürig, Giese et al. 2000; Burthem, Urban et al. 2001; Ballerini, Gourdain et al. 2006), whereas down-regulation has been reported upon activation of B and T lymphocytes in mice (Kubosaki, Yusa et al. 2001; Liu, Li et al. 2001). PrP$^C$ is up-regulated in some functionally differentiated lymphocytes including a population of regulatory T lymphocytes (Huehn, Siegmund et al. 2004) and in memory CD8 T cells (Li, Liu et al. 2001; Goldrath, Luckey et al. 2004). Immune function in PrP$^{+/−}$ mice has not been addressed to any great extent. Studies carried out so far have shown PrP$^C$ deficiency has no impact on expression levels of both MHC Class I and II, maturation of DCs, and numbers of CD4$^+$ and CD8$^+$ T lymphocytes and B lymphocytes are no different to that seen in WT counterparts.
(Bueler, Fischer et al. 1992; Kubosaki, Yusa et al. 2001; Ballerini, Gourdain et al. 2006; Zhang, Steele et al. 2006). Recent studies have shown that PrP<sup>C</sup> deficient T lymphocytes are more susceptible to oxidative stress (Aude-Garcia, Villiers et al. 2011) which is in agreement with previous publications demonstrating a neuroprotective role against oxidative stress for PrP<sup>C</sup> expression by neurones (Mitteregger, Vosko et al. 2007). However, there is a lack of *in vivo* studies investigating the role of PrP<sup>C</sup> during an active immune response, effects of PrP<sup>C</sup> deficiency on the germinal centre or the role PrP<sup>C</sup> may play specifically on FDCs. If PrP<sup>C</sup> plays a protective role against oxidative stress or anti-apoptotic functions as discussed, FDC may express high levels of PrP<sup>C</sup> due to their functional attributes, for example accumulating large amounts of protein on their surface.

Data from this thesis has shown that removing PrP<sup>C</sup> specifically on FDCs had no effect on the area or number of FDC networks in the spleen (Chapter 4, Fig 4.4). In mice with PrP<sup>C</sup> deficient FDCs, no observed difference was seen in expression of common FDC markers such as the complement receptors CD21/35 and bound complement C4 (Chapter 4, Fig 4.3) or on the ability of FDCs to capture immune complexes (Chapter 4, Fig 4.8) which suggests that PrP<sup>C</sup> function does not impact on these processes. However, these observations are made during steady state conditions where animals are housed in individually ventilated cages in specified pathogen-free conditions. Perhaps an effect of PrP<sup>C</sup> deficient FDCs would only become apparent after immunisation during initiation of a germinal centre response, or during an ongoing immune response. Furthermore, ultrastructural studies of PrP<sup>C</sup> deficient FDCs in comparison to WT controls may also provide indications as to the function of PrP<sup>C</sup> on the FDCs.
8.5 The role of FDCs in TSE pathogenesis

It has now been known for some time that many peripherally acquired TSE infections have a stage of replication in the lymphoid tissue prior to neuroinvasion and CNS disease. Studies investigating which cell type was responsible for replicating the agent have gradually led to the FDC being identified as the main candidate to replicate the TSE agent in the lymphoid tissue. Initial studies identified the stromal component of the spleen as having more TSE agent infectivity than that found in the pulp (Clarke and Kimberlin 1984). Subsequently, it was discovered that ionising radiation had no effect on scrapie pathogenesis indicating that the cell type responsible for replicating the agent was long lived and radio-resistant (Fraser and Farquhar 1987). As investigations into the cell type responsible for replicating the TSE agent continued, the involvement of T lymphocytes (Fraser and Dickinson 1978; Klein, Frigg et al. 1997), B lymphocytes (Klein, Frigg et al. 1998; Montrasio, Cozzio et al. 2001) or macrophages (Carp and Callaghan 1982; Beringue, Demoy et al. 2000) in actively replicating the TSE agent became more unlikely. Many studies showed that SCID mice, which lack T and B lymphocytes and FDCs, were resistant to peripherally acquired TSE infection (O'Rourke, Huff et al. 1994; Fraser, Brown et al. 1996; Lasmezas, Cesbron et al. 1996). As a role for T and B lymphocytes seemed unlikely these studies suggested that FDCs may be responsible for replicating the TSE agent.

FDCs had been shown to have relatively large quantities of PrP\(^C\) on their surface. Furthermore, immunohistochemical analysis of TSE-infected spleen showed high
levels of deposition of PrP\textsuperscript{d} on the FDC surface, which electron microscopy studies confirmed to be located to the FDC plasmalemma (Kitamoto, Muramoto et al. 1991; McBride, Eikelenboom et al. 1992; Brown, Ritchie et al. 2000; Jeffrey, McGovern et al. 2000). Animals with genetic deficiencies in TNF\textsubscript{α} (Mabbott, Williams et al. 2000a) or LT\textsubscript{β} (Oldstone, Race et al. 2002), which have no mature FDC networks, were resistant to peripherally inoculated scrapie infection. Additionally, studies which temporarily dedifferentiated the FDC networks of mice prior to peripheral inoculation with the scrapie agent using the TNF-R1 homologue (Mabbott, McGovern et al. 2002) the LT\textsubscript{β}R-Ig fusion protein (Montrasio 2000; Mabbott, Mackay et al. 2000b) prevented or delayed clinical scrapie disease. These studies provided strong evidence that FDCs were responsible for replicating the TSE agent. However, in all of the above models, in addition to removing the PrP\textsuperscript{C} expressing FDC networks, more general effects on the immune system were also incited, such as impairment of macrophage function (Klein, Frigg et al. 1997; Mabbott, Williams et al. 2000a; Mabbott, McGovern et al. 2002) or removal of FDC networks resulting in no germinal centres (Montrasio 2000; Mabbott, Williams et al. 2000a; Mabbott, McGovern et al. 2002; Oldstone, Race et al. 2002). Therefore it is possible that the effects on TSE pathogenesis could have been due to a more general immunodeficiency and not just the lack of FDCs. Bone marrow chimeric models, which used lethal γ- irradiation and reconstitution with donor bone marrow to create mismatches in PrP\textsuperscript{C} expression status between stromal and bone marrow-derived cells, showed that PrP\textsuperscript{C} expressing FDCs were essential for successful replication of scrapie whereas PrP\textsuperscript{C} expression on lymphocytes was irrelevant (Brown, Stewart et al. 1999). These models did not have a general effect on the immune system as was seen in previous models. However, PrP\textsuperscript{C} expression was retained by other neural, stromal
and epithelial cell types alongside the FDCs. For this reason, the possibility still remained that FDCs simply acquired the scrapie agent after replication on another cell type. An FDC-specific model, which had no impact on normal immune function, was required to definitively determine if FDCs simply acquire the TSE agent rather than actively replicate the agent themselves.

In this thesis, mouse models were created where PrP[^C] could be switched off (Chapter 4) or on (Chapter 6) exclusively on FDCs. Characterisation of these models determined the specificity and efficiency of Cre-mediated recombination of the floxed Prnp genes. Additionally we showed that there were no observed effects on the status or function of the FDC networks and no effects on lymphoid tissue microarchitecture. In animals in which PrP[^C] was switched off exclusively on FDCs that were inoculated ip with the ME7 scrapie agent, the scrapie agent initially localised to the FDC networks, but at later time points was only detectable within the neighbouring TBMs (Chapter 5). This was in contrast to animals which retained PrP[^C] expression on FDCs which showed PrP[^d] accumulation on the FDC network increasing over time. Furthermore, clinically scrapie-affected animals with PrP[^C] deficient FDC that had been injected directly into the CNS also showed no PrP[^d] accumulation on the FDC networks. Again, the only PrP[^d] detected in these animals was found within the TBMs. PrP[^C] deficient FDCs remained free of PrP[^d] accumulation, at early time points and in clinically scrapie-affected animals, despite all other cell types within the lymphoid tissue retaining expression of PrP[^C] and therefore potentially capable of replicating the scrapie agent. It can therefore be concluded that FDCs do not acquire PrP[^d] on their surface after replication on another cell type.
Animals with PrP\textsuperscript{C} expression switched on exclusively in FDCs were also inoculated with the ME7 scrapie agent to determine if PrP\textsuperscript{C} expression exclusively on FDCs was sufficient to support scrapie replication in the lymphoid tissue and therefore determine whether FDCs actively replicate the TSE agent (Chapter 7). Animals with PrP\textsuperscript{C} expression exclusively on FDCs showed deposition of PrP\textsuperscript{d} on the FDC network which increased over time at a comparable level to that observed in PrP\textsuperscript{+/-} hemizygous controls. Whereas control animals with PrP\textsuperscript{C} expression switched on only in B lymphocytes had little/no detectable PrP\textsuperscript{d} labelling and again any labelling present was only found within the TBMs. These data show that PrP\textsuperscript{C} expression exclusively on FDCs is sufficient to support TSE agent replication in the lymphoid tissue.

Taken together, data from these studies shows that FDCs do not acquire PrP\textsuperscript{d} from other cell types within the lymphoid tissue and instead actively replicate the TSE agent. FDCs appear to be responsible for replicating the TSE agent to sufficient levels that allow neuroinvasion and CNS disease. However are the FDCs themselves actually infected with the scrapie agent? Data from this thesis has shown that FDCs do not accumulate PrP\textsuperscript{d} from other cells in the lymphoid tissues and instead actively replicate the scrapie agent themselves. However, ultrastructural analysis shows PrP\textsuperscript{d} accumulations on the FDCs have only ever been observed on the plasmalemma, not within the cytoplasm (Jeffrey, McGovern et al. 2000; McGovern, Mabbott et al. 2009). Furthermore, there is a general belief that although PrP\textsuperscript{d} accumulation on neurones causes cell death by an unknown mechanism, PrP\textsuperscript{d} accumulation on the FDCs does not have an obvious toxic effect. As the scrapie agent is not replicating within the cell itself and replication does not appear to have a pathological effect on
the FDCs, one could argue that FDCs merely provide a platform for replication prior to neuroinvasion and infection of cells within the CNS.

Recent studies investigating the effects of scrapie infection on cells of the immune system have shown that replication of the TSE agent does effect the morphology of the FDCs and therefore may have a subtle effect on immune function that as of yet has not been investigated closely. Studies looking at PrP\textsuperscript{d} accumulation on the FDCs of sheep noted abnormal convolutions in the plasmalemma and abnormal clustering of immune complexes which co-localised with areas of PrP\textsuperscript{d} accumulation on the FDC surface. An increased number of exosomes was also observed around FDCs showing morphological changes due to PrP\textsuperscript{d} accumulation, some of which contained PrP\textsuperscript{d} (McGovern and Jeffrey 2007). Morphological changes to the FDCs leading to the shedding of the PrP\textsuperscript{d} in exosomes may be one possible mechanism of neuroinvasion of the scrapie agent. These observations were confirmed in the FDCs of scrapie-affected mice, however the increased number of exosomes in the follicle was not observed in these models (Jeffrey, McGovern et al. 2000; McGovern, Brown et al. 2004). More recent studies showed that accumulation of PrP\textsuperscript{d} in scrapie-affected mice led to disruptions in the FDC maturation cycle, abnormal ubiquitin in cell membranes and an excessive accumulation of IgGs which could therefore impact on immune function (McGovern, Mabbott et al. 2009a). The transfer of PrP\textsuperscript{d} to mature Ab-producing B lymphocytes has also been noted in the follicles of scrapie-affected sheep (McGovern and Jeffrey 2007). Additional studies have reported changes in B lymphocyte subsets in response to scrapie infection in sheep, which again could impact on immune function (Eaton, Rocchi et al. 2007; Eaton, Anderson et al. 2009). Although PrP\textsuperscript{d} does not appear to be internalised within the FDCs, FDCs actively
replicate the TSE agent and show pathological changes in response to accumulation of
the agent on their surface, which in turn may impact on immune function. For this
reason, FDCs could be considered to be infected with the TSE agent as they are
adversely affected as a consequence.

8.6 FDC-independent TSE pathogenesis

Although data from this thesis and many previous studies show that FDCs have an
important role in replicating the TSE agent in the lymphoid tissue, in some cases TSE
infections appear to be able to bypass the need for replication upon FDCs. Some
naturally acquired infections, for example BSE in cattle and atypical BSE and scrapie,
develop clinical disease in the CNS without any apparent replication in the lymphoid
tissue (Somerville, Birkett et al. 1997; Buschmann, Biacabe et al. 2004; Benestad,
Arsac et al. 2008). Recent experimental studies created granulomas in the skin of
mice prior to inoculation with scrapie (Heikenwalder, Kurrer et al. 2008). Accumulation of PrPSc and infectivity occurred within the granulomas in association
with stromal cells. These stromal cells did not express the characteristic FDC markers
such as C1q, CD35, FDC-M1, FDC-M2 or mRNA for Mfge8 and therefore this study
was used as an example of FDC-independent replication of the scrapie agent.
However, stromal cells within the granuloma were dependent on LT signalling,
expressed high levels of PrPc and organised B lymphocytes within the granuloma into
follicle type structures. Therefore although the commonly used FDC markers were
not present, these cells demonstrated many of the functional abilities FDCs have
within the lymphoid tissue. If these cells were characterised by function they would
possibly be described as FDCs. The lack of FDC markers in an artificially induced
tertiary lymphoid tissue is possibly not a great example of non-FDC associated replication of the scrapie agent as natural disease-induced chronic inflammation with formation of ectopic lymphoid follicles have shown development of PrP \textsuperscript{C} expressing FDCs which are capable of replicating PrP\textsuperscript{Sc} agent upon co-infection with the scrapie agent (Heikenwalder, Zeller et al. 2005; Ligios, Sigurdson et al. 2005).

Evidence from other studies has implied that there may be variations between TSE agent strains regarding whether replication on the FDCs is required prior to neuroinvasion. Studies described previously in this chapter (and thesis) demonstrated that expression of PrP \textsuperscript{C} by the FDCs is essential for successful pathogenesis of the ME7 scrapie agent in mice, whereas PrP \textsuperscript{C} expression on lymphocytes was irrelevant (Brown, Stewart et al. 1999). However, similar studies using the RML scrapie isolate inoculated into Prnp\textsuperscript{-/-} mice reconstituted with Prnp\textsuperscript{+/+} haematopoietic stem cells showed successful neuroinvasion of the scrapie agent despite the FDCs lacking PrP \textsuperscript{C} expression in this model (Blattler, Brandner et al. 1997). This study may demonstrate that different strains of TSE agent have different requirements for replication in the periphery prior to neuroinvasion. However, an important factor to consider is that this study used a high TSE agent dose for inoculation, 100 \mu{l} of a 1\% brain homogenate (Blattler, Brandner et al. 1997) in comparison to 20 \mu{l} of a 1\% brain homogenate used in the ME7 study (Brown, Stewart et al. 1999). Previous experiment using SCID mice have shown that the need for TSE agent replication in the periphery can be overcome by inoculating with a high dose of the TSE agent (Fraser, Brown et al. 1996). Therefore it may be that levels of infectivity in the inocula used in the RML experiments were sufficient to allow neuroinvasion without further replication in the periphery.
With the exception of some natural disease models, such as BSE in cattle, the majority of experimental evidence suggests that FDCs provide a crucial site for peripheral replication of the TSE agent until sufficient levels of infectivity are reached that allow neuroinvasion. Data in this thesis shows, the ME7 scrapie agent, cannot replicate in lymphoid tissues containing PrP\textsuperscript{C} deficient FDC despite all other cell types retaining PrP\textsuperscript{C} expression. However, other TSE strains have not been tested in this thesis, so the prospect of strain differences cannot be completely discounted.

**8.7 Localisation of the TSE agent to the follicle**

Another uncertainty in TSE disease pathogenesis is how the TSE agent localises to the FDC network prior to replication and subsequent neuroinvasion. Data from this thesis has shown that the scrapie agent can localise to PrP\textsuperscript{C} deficient FDCs in the follicles at the same time points as that seen in PrP\textsuperscript{C} expressing FDCs (Chapter 5). This demonstrates that PrP\textsuperscript{C} expression by FDCs is not required for the scrapie agent to initially localise to the FDC network. Furthermore, when PrP\textsuperscript{C} was expressed exclusively on the FDC network, the scrapie agent could again localise to the follicle at the same time points as Prnp\textsuperscript{+/-} controls (Chapter 7). This shows that PrP\textsuperscript{C} expression by other cell types is not required for the transport of the agent to the FDC network. These data suggest that PrP\textsuperscript{C} is neither required as a ligand for the transportation of the scrapie agent to the follicle nor for initial uptake by the FDCs. This is in agreement with previous studies which showed that localisation of the scrapie agent to the follicle was dependent on CD21/35 expression on the stromal compartment of the spleen, whereas expression status of PrP\textsuperscript{C} was irrelevant (Zabel, Heikenwalder et al. 2007).
Some experimental evidence suggests that the TSE agent may use cell-mediated transport to localise to the follicle. Macrophages have been shown to have the ability to take up the PrP\textsuperscript{d} and after peripheral inoculation with scrapie are also capable of harbouring infectivity (Jeffrey, McGovern et al. 2000; Manuelidis, Zaitsev et al. 2000). However, most studies agree that rather than transporting the agent, PrP\textsuperscript{d} is taken up into lysosomes and partially degraded (Jeffrey, Martin et al. 2003; McGovern, Mabbott et al. 2009a). Removal of macrophages leads to increased replication of the TSE agent suggesting they are involved in clearing the agent (Beringue, Demoy et al. 2000; Maignien, Shakweh et al. 2005). DCs are another possible candidate for transport of the TSE agent to the lymphoid tissues. Previous studies have indicated that after oral exposure DCs take up the scrapie agent and transport it to the draining lymphoid tissues (Huang, Farquhar et al. 2002; Huang and MacPherson 2004; Mohan, Hopkins et al. 2005; Raymond, Aucouturier et al. 2007). Recent studies have shown that uptake of the scrapie agent by DCs is facilitated by complement components (Flores-Langarica, Sebti et al. 2009). However, even after the temporary depletion of DCs at the time of exposure, some animals eventually succumbed to disease, therefore it is possible that cell-free transport of the TSE agent to the follicle is also possible (Raymond, Aucouturier et al. 2007).

After peripheral inoculation, the TSE agent may be transported via the blood directly to the spleen, by cell-free mechanisms. Studies investigating which size of PrP\textsuperscript{d} aggregates are most infectious discovered that particles of 300-600 KDa in size contained the most infectivity. Therefore it is likely that infectivity and the TSE agent itself is associated with PrP\textsuperscript{d} fragment of this size. The pathway of protein/antigen processing by the spleen varies in accordance with the size of the antigen. Small
soluble protein antigens, less than 70 KDa, are transported through the spleen via the conduits to the follicle (Pape, Catron et al. 2007; Roozendaal, Mempel et al. 2009). Whereas large antigen, greater than 70KDa, is trapped by sub-capsular sinus (SCS) macrophages in the lymph nodes or marginal zone (MZ) macrophages in the spleen. Antigen is then removed from the SCS/ MZ macrophages by follicular B lymphocytes which then traffic to the follicle where antigen is taken up by FDCs (Carrasco and Batista 2007; Phan, Grigorova et al. 2007; Phan, Green et al. 2009; Roozendaal, Mempel et al. 2009). Therefore, it is likely that cell free infective scrapie agent is taken up by SCS macrophages and trafficked via B lymphocytes to the FDCs. This mechanism of antigen transport has been shown to be dependent on complement receptors (Phan, Grigorova et al. 2007). In agreement with the scrapie agent using this mechanism of transport to locate to the follicle, previous studies have shown that complement components aid scrapie agent localisation to the follicle and that depletion of these factors or their receptors can delay neuroinvasion and clinical disease (Klein, Kaeser et al. 2001; Mabbott, Bruce et al. 2001).

In all of these studies examining the trafficking of the TSE agent to the FDCs, impairment in various mechanisms causes a delay in neuroinvasion but not a prevention of disease. It appears that the TSE agent can use various mechanisms to get to the follicle and replicate on the FDCs and no one mechanism is solely responsible for disease pathogenesis to occur.
8.8 The role of B lymphocytes in TSE pathogenesis

Experimental evidence from previous studies indicated that B lymphocytes may have a role in replicating the scrapie agent in the lymphoid tissue prior to neuroinvasion (Klein, Frigg et al. 1997). However, it was subsequently determined that effects of B cell deficiency on pathogenesis in this model was due to the indirect effect of having a lack of B lymphocyte-derived LTs to maintain mature FDC networks. Subsequent studies have shown that PrP<sup>C</sup> expressing B lymphocytes are not essential for effective pathogenesis of the scrapie agent (Brown, Stewart et al. 1999; Montrasio, Cozzio et al. 2001). This is in agreement with data from this thesis which found that switching off PrP<sup>C</sup> exclusively on CD21<sup>+</sup> B lymphocytes had no effect on PrP<sup>d</sup> deposition in the spleen after inoculation with the ME7 scrapie agent (Chapter 5). Furthermore, PrP<sup>C</sup> expression exclusively by CD21<sup>+</sup> B lymphocytes did not allow replication of the scrapie agent in the spleen (Chapter 7). Data from previous studies and this thesis imply that B lymphocytes do not have a role in replicating the TSE agent in peripheral lymphoid tissue. However, this does not mean that they have no involvement in TSE disease. As discussed in Section 8.7, B lymphocytes may be involved in the initial transportation of the TSE agent from SCS macrophages to the FDC networks via complement-dependent mechanisms (Carrasco and Batista 2007; Phan, Grigorova et al. 2007; Phan, Green et al. 2009; Roozendaal, Mempel et al. 2009). Furthermore, experiments have shown that PrP<sup>d</sup> is transferred from FDCs to the surface membranes of B lymphocytes in the germinal centres of scrapie-affected mice (McGovern and Jeffrey 2007). In addition PrP<sup>d</sup> was found associated with CD21<sup>+</sup> B lymphocytes in the blood of scrapie-affected sheep. Therefore it is possible that B lymphocytes are involved in trafficking the scrapie agent before or after replication on the lymphoid
One experiment that could have been carried out in the studies in this thesis would be to isolate CD21⁺ B lymphocytes from the blood and spleen of scrapie affected animals and measure the levels of PrP labelling by FACS analysis. This could provide data on whether switching PrP⁰ expression on or off specifically on CD21⁺ B lymphocytes, or on CD21⁺ B lymphocytes and FDCs, had any effect on the accumulation of PrPᵈ on the CD21⁺ B lymphocytes. It could be used as one possible method to determine if CD21⁺ B lymphocytes were responsible for trafficking the agent after replication on the FDC networks. Unfortunately there was not sufficient time to carry out these experiments in this thesis, but it may provide a method for addressing this question in future studies.

8.9 The role of tingible body macrophages in TSE pathogenesis

TBMs are large phagocytic cells found in the germinal centres of secondary lymphoid tissues which contain phagocytosed, apoptotic cells known as tingible bodies (Swartzendruber and Congdon 1963). Labelling of PrPᵈ has been found within TBMs after inoculation with scrapie in mice (Jeffrey, McGovern et al. 2000) and sheep (Jeffrey, Martin et al. 2001) and also in lymphoid tissues taken from individuals who have died from vCJD infection (Hilton, Ghani et al. 2004). In this thesis, PrPᵈ was found in association with TBMs when PrP⁰ was switched off on the FDCs after ip inoculation with the ME7 scrapie agent (Chapter 5). In addition, when PrP⁰ was switched on exclusively on CD21⁺ B lymphocytes, PrPᵈ was only found in association with TBMs in the lymphoid tissue (Chapter 7). These data suggest that in the absence of TSE agent replication by the FDC network, TBMs scavenge the PrPᵈ that has located to the FDC network probably via complement dependent mechanisms (See
Section 8.6). Indeed, previous studies have shown that TBMs are capable of taking up portions of immune complex coated membrane from the FDCs (Tew, Kosco et al. 1989; Szakal and Tew 1992). In this thesis, it is not certain whether the PrP\textsubscript{d} labelling found within the TBMs will act as a reservoir of infection, harbouring the scrapie agent and allowing a delayed neuroinvasion, or whether PrP\textsubscript{d} within the TBMs is degraded and infectivity is destroyed. Cre\textsuperscript{+ve} → CD21-\textit{crePrPfl/-} mice, which have PrP\textsubscript{C} switched off exclusively in FDCs, have been ip inoculated with the ME7 scrapie agent and left until clinical disease develops or animals need to be culled due to old age. However, at the time of writing, these animals have not succumbed to clinical disease or age, so as of yet no conclusions can be drawn from these experiments. However, previous studies have shown that PrP\textsubscript{d} found within the TBMs is truncated with a loss of 23-90 aa from the N-terminal region (Jeffrey, Martin et al. 2003; McGovern, Mabbott et al. 2009a). This suggests that the TBMs are degrading the scrapie agent after uptake from the FDCs. In scrapie infections, an initial exponential growth of PrP\textsubscript{d} deposition and infectivity occurs initially in the spleen which is then followed by a plateau. Many suggestions have been made as to why this effect is seen. If TBMs in the follicle are responsible for degrading the scrapie agent, it may be that a balance is eventually reached between replication of the scrapie agent on the FDCs and degradation by the TBMs.

8.10 Conclusions

In conclusion, data from this thesis have confirmed that FDCs play a crucial role in replicating the scrapie agent prior to neuroinvasion. The CD21-\textit{crePrPfl/-} transgenic mouse line enabled PrP\textsubscript{C} expression to be switched off exclusively on the FDC
network after irradiation and transfer of non-Cre-expressing bone marrow. This model was used to determine whether FDCs simply acquire the TSE agent on their surface after accumulation on another cell type. Characterisation of this model showed efficient removal of PrP\textsuperscript{C} specifically on the FDCs with no additional effects on FDC status or function, or on the general microarchitecture of the lymphoid tissue. These animals were infected with the ME7 scrapie agent via ip inoculation. In these animals, PrP\textsuperscript{d} initially located to the FDC networks, however the TSE agent was unable to replicate on the PrP\textsuperscript{C} deficient FDCs and the agent was taken up by neighbouring TBMs and possibly degraded (Chapter 5). These animals were also infected directly into the CNS via ic injection and developed clinical TSE disease with positive neuropathology in the brain. This demonstrated that the transgenic animals were equally susceptible to TSE disease as their WT counterparts and that any effect on disease pathogenesis in the lymphoid tissue was due to PrP\textsuperscript{C} deficient FDCs and not a general resistance to TSE disease. As PrP\textsuperscript{C} deficient FDCs did not show deposition of the TSE agent on their surface which increased as disease progressed, it was concluded that FDCs do not acquire the TSE agent from other stromal, neural or lymphoid cells within the lymphoid tissue which retained PrP\textsuperscript{C} expression in the CD21-crePrP\textsuperscript{f/f} transgenic mouse model. PrP\textsuperscript{C}-expressing FDCs are essential for replicating the scrapie agent in the lymphoid tissue.

Following on from these studies, it was determined whether PrP\textsuperscript{C} expression exclusively on FDCs was sufficient to support replication of the scrapie agent in the lymphoid tissue. The CD21-crePrP\textsuperscript{stop/}\textsuperscript{f/f} transgenic mouse line enabled PrP\textsuperscript{C} to be expressed exclusively on the FDC network after irradiation and transfer of non-Cre-expressing bone marrow. Characterisation of this model demonstrated that PrP\textsuperscript{C} was
efficiently and specifically expressed on the FDC networks and again no additional effects were observed on FDC status or general microarchitecture of the lymphoid tissue. After infection of these animals with the ME7 scrapie agent via ip inoculation, accumulation of PrP$^d$ occurred on FDCs when PrP$^C$ was restricted to the FDC network. Furthermore, this accumulation increased over time suggesting active replication of the scrapie agent by the FDCs. Taken together, data from both of these transgenic mouse models shows that FDCs do not acquire PrP$^d$ and infectivity from other cells within the lymphoid tissue. Furthermore, PrP$^C$ expression exclusively on FDCs is sufficient to support replication of the scrapie agent in the lymphoid tissue. From these data we can conclude that PrP$^C$ expressing FDCs actively replicate the TSE agent in the lymphoid tissue after peripheral exposure to scrapie.


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Appendix I

ImageJ macro - Multiple colour backgrounds

This macro was used to determine background levels of immunolabelling in fluorescently labelled images and to determine threshold levels to use in further image analysis. A line is drawn through the image using ImageJ software, ensuring that line passes through areas of positive bright immunolabelling, areas of positive lower level immunolabelling and background levels of colour present. The intensity of colour for all plots along that line is then plotted out on a graph. This graph allows the determination of a threshold of colour intensity to eliminate background staining from further analysis of images.

// Generic multiple colour macro for assisting setting of thresholds
// Analyses pixel intensities along a line selection
// for a series of greyscale slices in a stack, each corresponding to a colour channel
// Expandable to any number of colour channels (slices)

// Check for RGB image and convert to greyscale stack
// Breaks if image name includes ":(RGB)"
image = getImageID(); selectImage(image); info = getInfo();
if (indexOf(info, "(RGB)" ) > 1) {
    getLine(x1,y1,x2,y2,width);
    run("RGB Split");
    run("Convert Images to Stack");
    makeLine(x1,y1,x2,y2);
}

Greyscales = 255; if (indexOf(info, "pixel: 16") > 1) {Greyscales = 6553}

setLineWidth(1);
getLine(startx, starty, endx, endy, temp);
stack = getImageID();
selectImage(stack); slices = nSlices();

for (i = 0; i<slices; i++) {
    selectImage(stack);
    slice = "slice"+(i+1);
    run("Set Slice...", slice);
    ydata = getProfile();
    Plot.create(slice, "pixels", "intensity");
    Plot.setLimits(0, ydata.length, 0, Greyscales);
    Plot.setLineWidth(1);
    Plot.setColor("red");
    Plot.add("line", ydata);
Appendix II

ImageJ macro- Multiple colour analysis

This macro was used to measure co-localisation of proteins in immunolabelled fluorescent images. Background levels on immunolabelling were excluded from measurements using the multiple colour backgrounds macro (Appendix I) to set a threshold for positive labelling. Each pixel in the image is counted and recorded as black (no labelling), red, green, blue, yellow (red and green together), magenta (red and blue together), cyan (green and blue together) or white (red, green and blue). Quantification of area of image single or double immunolabelled for each protein can then be used to perform statistical analysis of co-localisation present.

// Generic multiple colour co-localisation analysis macro
// Analyses a series of greyscale slices in a stack, each corresponding to a colour channel
// Expandable to any number of colour channels (slices)
// Provides number of pixels above threshold for each combination of colour channels
// Data provided is in bins corresponding to (for three channels):
//
// Bin   Ch 1   Ch 2   Ch 3
// 0     -      -      -      
// 1     +      -      -      
// 2     -      +      -      
// 3     +      +      -      
// 4     -      -      +      
// 5     +      -      +      
// 6     -      +      +      
// 7     +      +      +      

// Set threshold values for each colour channel (each slice)
// Add extra thresholds for more than 5 channels
Threshold = newArray(6);
Threshold[1] = 10;
Threshold[2] = 40;
Threshold[3] = 50;
Threshold[4] = 50;
Threshold[5] = 50;

// Check for RGB image and convert to greyscale stack
// Where Ch 1 = red, Ch 2 = green, Ch 3 = blue
// Breaks if image name includes "(RGB)"
image = getImageID(); selectImage(image); info = getInfo();
if (indexOf(info, "(RGB)") >1) {
    run("RGB Split");
    run("Convert Images to Stack");
}
exit("Draw your ROI and rerun the macro");

stack = getImageID(); selectImage(stack); slices = nSlices();
results = newArray(pow(2, slices));
Greyscales = 255; if (indexOf(info, "pixel: 16")>1) {Greyscales = 65535;}
//run("Measure");

setBackgroundColor(0,0,0);
for (i =0; i<slices; i++) {
    selectImage(stack);
    slice = "slice="+(i+1);
    run("Set Slice...", slice); run("Clear Outside", "slice");
    changeValues(0,Threshold[i+1],0);
    changeValues((Threshold[i+1]+1),Greyscales,pow(2,i));
}
run("Z Project...", "start=1 stop=5 projection='Sum Slices'"); sum = getImageID();
selectImage(stack);
selectImage(sum);
run("Restore Selection");
run("Histogram", "bins=" + pow(2,slices) + " x_min=0 x_max=" + pow(2,slices));