The role of the acquired immune response in virus clearance and neuropathology in Semliki Forest virus infection

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise indicated and all work of other authors is duly acknowledged.

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Abstract

Viral encephalitis is a serious and important human and animal health problem, as exemplified by West Nile encephalitis and HIV-related dementia. There is a need for better understanding of the pathogenesis of virus encephalitis. Experimentally, Semliki Forest virus (SFV) is an excellent mouse model of viral encephalitis and virus-induced demyelination. SFV is an alphavirus of the *Togaviridae.* In mice it is neuroinvasive and neurotropic. Following infection of susceptible mouse strains, clearance of infectious brain virus is coincident with inflammatory cell infiltration and is followed by demyelination. There is some existing evidence to suggest that CNS demyelination observed following SFV infection has an immune aetiology and based on transient depletion studies that $CD8^+$ T-cells are likely to be the main effectors of this.

This thesis examines the role of acquired immune responses in SFV encephalitis. The role of components of the acquired immune response in mediating clearance of infectious virus and virus RNA and in the pathogenesis of demyelination was examined using mice with genetic deletions affecting components of acquired immune responses. This included mice deficient in $CD4^+$ and $CD8^+$ T-cell responses and in specific T-cell mediators IFNγ, perforin and Fas. Infectious virus was determined by plaque assay, the presence of virus RNA by quantitative, real-time PCR and the neuropathology by standard histopathology and immunohistochemistry.

Analysis of inflammatory infiltrates in the SFV infected CNS demonstrated a rapid influx of macrophages and NK cells and a >40-fold increase in T-lymphocytes, predominantly $CD8^+$ cells. Mice lacking $CD8^+$ T-cells showed no difference in their ability to clear infectious virus from the brain, but had a slower clearance of virus RNA. Adoptive transfer of $CD8^+$ T-cells to SFV infected SCID mice demonstrated that $CD8^+$ T-cells mediated the demyelinating lesions. Mice lacking $CD4^+$ T-cells were unable to generate good antibody responses and were unable to clear infectious virus. Transfer of anti-SFV hyperimmune (HI) serum to SFV infected SCID mice lowered virus RNA to levels comparable to those in immunocompetent (BALB/c) mice. However, antibody alone was not sufficient to eliminate virus RNA and infectious virus reappeared once antibody levels dropped.

$IFN\gamma R^{-/-}$ mice were found to have slower clearance of virus RNA compared to wild-type mice but IFNγ was not necessary for the development of demyelinating lesions. A protective role for IFNγ was demonstrated in SFV infection; recombinant IFNγ transiently protected SFV infected $IFN\alpha/\beta R^{-/-}$ mice. Neither perforin nor Fas was necessary for clearance of infectious virus or viral RNA. SFV infected Fas knock-out mice had increased CNS demyelination.

In summary this thesis demonstrates that in SFV infection, $CD8^+$ T-cells are the main component of the CNS inflammatory response; $CD8^+$ T-cells mediate the lesions of demyelination; clearance of infectious virus is mediated by antibody but antibody alone is insufficient to clear all virus RNA; $CD8^+$ T-cells and the IFN-γ system contribute to the elimination of virus RNA. It is likely that both antibody and $CD8^+$ T-cells are required to eliminate SFV infection.

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Abbreviations

AICD	Activation Induced Cell Death
APC	Antigen Presenting Cell
BBB	Blood Brain Barrier
BHK	Baby Hamster Kidney
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T-Lymphocyte
DAB	Diaminobenzidine
DC	Dendritic Cell
DI	Defective Interfering
DMN	Demyelination
DNA	Deoxyribonucleic Acid
dNPT	Deoxy-Nucleoside Triphosphate
dsDNA	Double Stranded DNA
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic Reticulum
FACS	Fluorescent-Activated Cell Sorting
FasL	Fas Ligand
FITC	Fluorescein Isothiocyanate
F/T	Flow Through Cells by IP Inoculation

G	Gram
GAS	IFNγ Activated Sites
GFP	Green Fluorescent Protein
GMEM	Glasgow's Minimal Essential Media
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	Hyperimmune
HRP	Horse Radish Peroxidase
HSV	Herpes Simplex Virus
IC	Intracranial
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INF	Inflammation
IP	Intraperitoneal
IRF	Interferon Regulatory Factor
KO	Knockout
LB	Luria-Bertani
LCMV	Lymphocytic Choriomeningitis Virus
mAb	Monoclonal Antibody
MACS	Magnetically Activated Cell Sorter
MCC	Microcystic Change
MCP	Monocyte Chemoattractant Protein
MHC	Major Histocompatibility Complex
MHV	Murine Hepatitis Virus
MHVR	Murine Hepatitis Virus Receptor

NBCS	Newborn calf serum
NFκB	Nuclear Factor κB
NGS	Normal Goat Serum
NK	Natural Killer
ns	Non Structural
NT	Neutralisation Titre
OAS	Oligo Adenylate Synthetase
OD	Optical Density
PBS	Phosphate Buffered Saline
PBST	Phosphate buffered saline containing Tween 20
PCR	Polymerase Chain Reaction
PE	Phycoerthrin
PFU	Plaque forming units
PI	Post-Infection
PID	Post-Infection Day
PIW	Post-Infection Week
PKR	Protein Kinase R
PRNA	Plaque Reduction Neutralisation Assay
q-PCR	Quantitative Real-Time PCR
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute medium
SCID	Semliki Forest Virus
sPBS	Sterile PBS

sPBSA	Sterile PBS Containing Bovine Serum Albumin
ss	Single Stranded
STAT	Signal Transducers and Activators of Transduction
SV	Sindbis Virus
TGF	Transforming Growth Factor
TLR	Toll Like Receptor
TMB	Tetramethylbenzidine
TMEV	Theiler's Murine Encephalitis Virus
TNF	Tumour Necrosis Factor
T-reg	T Regulatory Cell
VLP	Virus Like Particles
VZV	Varicella Zoster Virus
WHO	World Health Organisation
WNV	West Nile Virus

Chapter 1: Introduction

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Introduction

Semliki Forest virus infection of mice offers a laboratory model of central nervous system (CNS) virus infection allowing study of CNS immune responses. This thesis examines the role of acquired immune responses in the clearance of SFV and in the pathogenesis of the neuropathological changes observed following SFV infection.

This chapter will introduce the biology of SFV and alphaviruses, before setting out the components of the anti-viral immune response. The relatively inaccessible CNS is targeted by a number of infectious agents, but not always successfully. The CNS has a variety of immune mechanisms to counter such infections, the mechanisms and limitations of these defence mechanisms will be discussed. Persistence of viral infection is a complicated topic, and this chapter sets out to define what is meant by persistence. A number of viral infections in mice result in CNS persistence. The issues involved in this will be examined.

Finally, the pathogenesis of SFV will be discussed, especially the known roles of different components of the immune in clearing SFV from the CNS and in inducing CNS pathology.

Alphaviruses

SFV is a member of the genus *Alphavirus,* which is grouped into 6 antigenic complexes based on amino acid sequence (Table 1). These 6 complexes share about 45% amino acid identity in divergent structural proteins and about 60% in the more conserved non-structural proteins (Figure 1). SFV is most closely related to Chikungunya and Mayaro alphaviruses (Table 1). Alphaviruses have a worldwide and geographically distinct distribution. SFV is found naturally in sub-Saharan Africa and is spread by mosquitoes, *aides africanus and aides aegypti.* The original isolate was found in the *aides abnormalis* mosquitoes in the Semliki forest in Uganda, from which it is named (Smithburn & Haddow, 1944).

The Alphavirus' natural cycle is between mosquitoes and birds or small mammals. The infection of humans and larger mammals, which does occur and can lead to severe and fatal disease, is outside the usual transmission cycle of these viruses. The vertebrate host for SFV is unknown, but infections have been reported in horses, monkeys and man. SFV was associated with an epidemic of equine encephalitis in Senegal and death of a laboratory worker was associated with SFV infection; SFV was isolated from the cerebrospinal fluid (CSF). However, it is unclear if SFV was the cause of death (Robin, 1974; Willems, 1979).

Other alphaviruses cause important health and economic problems. In the 1985 Venezuelan Equine encephalitis outbreak in Columbia, an estimated 50,000 horses were infected and up to 8% may have died. More importantly there were an estimated 75,000 humans infected with 300 fatalities (Rivas, 1997). More recently, (2005-2006) a Chikungunya virus outbreak has been reported in islands of the Indian Ocean and in India. It is estimated that over 200,000 people were infected. This virus causes acute fever, prolonged fatigue and crippling arthritis (WHO, 2006). SFV infection of mice represents a model system of alphavirus infection that has greatly contributed to our understanding of alphavirus pathogenesis and studies continue to add to the body of knowledge concerning encephalitis and immune response to CNS viruses.

Antigenic Complex	Virus	Clinical Syndrome	Transmission cycle	Geographic Distribution
Eastern equine encephalitis (EEE)	**EEE virus**	Encephalitis	Mosquito-Bird	N America, Canada, Caribbean
			Mosquito-Bird/Mammal	Central/S America, Caribbean
Venezuelan equine encephalitis (VEE)	**VEE virus**	Febrile illness, encephalitis	Mosquito/biting insects-Equine	Mexico, Central America, Trinidad, Colombia, Venezuela, Peru, Ecuador
Sindbis	**WEE virus**	Encephalitis	Mosquito-Bird/hare	N America, Argentina, Brazil, Uruguay
	Sindbis virus	Febrile illness, rash, arthritis	Mosquito-Bird	Europe, Asia, Australia, Africa
Semliki Forest	**SFV virus**	Febrile illness, encephalitis	Mosquito-Mammal/Bird	Africa, Asia
	Chikungunya virus	Febrile illness, rash, arthritis	Mosquito-Human	Africa, Asia
	Mayaro	Febrile illness, rash, arthritis	Mosquito-Primate/mammal	Central/S America, Caribbean
	Ross River virus	Febrile illness, rash, arthritis	Mosquito-Mammal/Human	Australia, Oceania
Barmah Forest	**Barmah Forest virus**	Febrile illness, rash, arthritis	Mosquito-Mammal	Africa
Middleburg			Unknown	Africa

Table 1. Characteristics of selected alphaviruses (data adapted from (Tsai, 2002))

SFV Biology

SFV virions have a (+) RNA genome surrounded by an icosahedral capsid which is enveloped by a lipid bilayer derived from the host cell. The outermost surface of the virus is almost entirely covered by heterodimers of glycoproteins E1 and E2, arranged in inter-connective trimers, which form an outer shell. The trimers are anchored in the envelope by an E2 cytoplasmic domain that is associated with the nucleocapsid. The (+) RNA genome is 11-12 Kb in size with a poly-A tail and a 5' cap. The 5' two thirds of the genome encode non-structural (ns) proteins and the structural proteins are encoded in the 3' end (Figure 1).

The cell surface receptor for SFV is unknown. SFV is taken up into endocytic vesicles. The fusion of the virion and endosome is mediated by the E1 glycoprotein (Omar & Koblet, 1988). After fusion, the viral genome is released into the host cell's cytoplasm and the virus genome (+) RNA functions as an mRNA. NS proteins are translated as polyproteins. Replicase activity is associated with the ns polyproteins. Replication occurs via a negative strand intermediate, giving rise to a full length genomic RNA for incorporation into new virions. A subgenomic message is translated to give the structural proteins. Envelope glycoproteins are transported to the cell membrane where they associate with capsid proteins prior to virus budding.

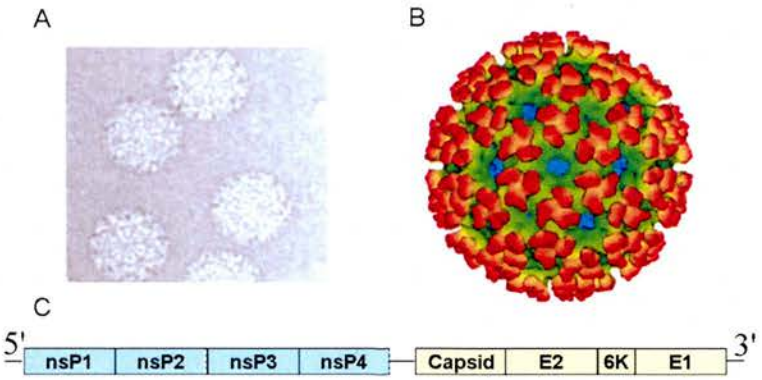

Figure 1. A - Electron microscope pictures of SFV; **B** – icosahedral reconstruction from cryo-electron micrographs and **C** - structure of SFV genome (Mancini, 2000). ns – non-structural; E – envelope.

Recent advances in biotechnology have seen the development of a virus based expression system of virus-like particles (VLPs). VLPs mimic virus structure but are

non-infectious and can express foreign genes. The SFV genome has been modified to produce this system. The benefit of these viral vectors is that they retain their natural tropism while allowing a high and transient level of foreign gene expression. Possible applications of this technology include vaccines, cancer therapy and gene therapy. VLPs are generated by the co-transfection of cells with three plasmids. The first codes for SFV replicase and a foreign gene of interest, the second for the SFV capsid protein and the third for the SFV envelope protein (Smerdou & Liljestrom, 1999). VLPs are identical to SFV particles, except in their RNA content, which only contains the replicase gene, linked to the foreign gene transcript. The use of VLP encoding green fluorescent protein (GFP) allows the visualisation of infected cells without the need for immunostaining (Fazakerley, 2006).

Innate Immune Response

Cytokines

Cytokines are soluble mediators secreted from cells that act in an autocrine and paracrine manner to increase or decrease expression of membrane proteins (including cytokine receptors) and induce cellular proliferation and secretion of effector molecules. The actions of cytokines serve to control infection and induce, sustain or decrease an inflammatory response and cytokines have both protective and pathogenic potential (Stoll, 2000). Local production of cytokines has a pivotal influence on early immune responses. T-cells and macrophages are major producers of cytokines. Cytokines can be classified based on structural homology, however, it is more useful to group them based on their effects, such as pro-inflammatory (interferon γ, TNFα, IL-12 and IL-1) and anti-inflammatory (IL-10 and transforming growth factor [TGF]-β).

Type I interferons

Type I interferons (IFNs) are cytokines first discovered in the cellular response to viruses and are the first line of antiviral defence (Isaacs & Lindenmann, 1957). Type I IFNs are induced by the presence of double stranded (ds) RNA, a required intermediate of RNA virus replication. Upon infection, dsRNA can activate cellular sensors of infection including retinoic acid inducible gene I, melanoma differentiation-associated gene-5, protein kinase (PK) R and toll-like receptor (TLR) 3. These cellular sensors activate transcription factors, such as nuclear factor (NF)-κB, activating transcription factor-2 /c-jun and IFN regulatory factor (IRF)-3 through phosphorylation cascades. These transcription factors bind to the IFN-β promoter, inducing gene expression (Maniatis, 1998). Activated IRF-3 is also initiates expression of IFN-α4 (Au, 1993) which with IFNβ provides the initial wave of the IFN response. Released IFNs act in both an autocrine and paracrine manner to induce anti-viral mechanisms by binding to the type-I IFN receptor. Signal transduction via the signal transducers and activators of transcription (STAT)

signalling pathway initiates transcription of hundreds of interferon-stimulated genes including, 2'-5' oligoadenylate synthetase (OAS)/RNaseL which targets and degrades ssRNA, thereby inhibiting viral replication and PKR, which leads to the inhibition of protein synthesis (Der, 1998c).

There are many type I IFNs (α and β are main types) all of which bear structural homology and bind a single IFN receptor. They are found on chromosome 9 in humans and chromosome 4 in mice. There is only one type II IFN – gamma, which signals through a type II receptor and is found on chromosome 12 in humans and 10 in mice. Type I and II were originally grouped together due to their ability to interfere with viral infection (Isaacs & Lindenmann, 1957).

Chemokines

These glycoproteins are chemotactic cytokines and augment the immune response by their potent activity as leukocyte chemoattractants in inflammatory responses and also by inducing cellular chemotaxis, extravasation and adhesion molecule expression. There are three subclasses, based on their expression of cysteine residues; CXC chemokines - which predominantly recruit monocytes; CC chemokine - which predominantly recruit neutrophils and C chemokine, lymphotactin, that attracts T-cell precursors to the thymus.

Macrophages

Macrophages are long lived phagocytic cells that phagocytose infected cells, cellular debris and pathogens. They effectively present antigen to T-cells as well as being an important source of pro-inflammatory cytokines (IL-1, IL-6 and tumour necrosis factor [TNF]-α) which can inhibit viral replication and affect the type of T-helper response that develops (Laskin & Pendino, 1995). As well as releasing pro-inflammatory mediators that attract and activate other immune cells they also produce reactive oxygen species such as hydrogen peroxide which have direct, chemical anti-microbial functions. An uncontrolled pro-inflammatory macrophage response has the potential to cause great tissue damage to the host.

Natural Killer (NK) Cells

NK cells constitute a major component of the innate immune system that has a crucial role in the early control of many virus infections. They are large, granular lymphocytes with an activating receptor that recognises surface carbohydrate on 'altered cells' and inhibitory receptors that recognizes major histocompatibility complex (MHC) class I alleles, thereby preventing attack of 'normal' cells.

NK cells are cytotoxic and granules in their cytoplasm contain the cytolytic protein perforin and proteases known as granzymes, which are released onto target cells and when endocytosed induce apoptosis. NK cells are activated in response to IFNs or macrophage-derived cytokines. They serve to non-specifically contain viral infections while the adaptive immune response is generating antigen-specific cytotoxic T lymphocytes (CTLs) that can later clear the infection.

Acquired Immune Response

Along with initial clearance and early control of infection, the combined effects of cytokines and chemokines of the innate immune response is to attract cells of the specific immune response to the area of infection, increase antigen processing and presentation and provide the cytokine environment to effect the differentiation of the T-cell response.

T-cell Response

The T-cell response is comprised of two main subpopulations, $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T-cells. T-cells recognise antigen presented on cell-surface MHC molecules. Therefore, T-cells can only detect virus taken up, processed and presented by antigen presenting cells (APCs) or by an infected cell. $CD4^+$ T-cells recognise exogenous antigens presented on MHC class II molecules that are expressed on dendritic cells (DCs), macrophages and B-cells. Upon activation they secrete essential cytokines and bind cell surface ligands, activate B-cells, $CD8^+$ T-cells and induce immunoglobulin (Ig) class switching. $CD8^+$ T-cells have a strong

anti-viral role, and recognise endogenous antigen, for example viral peptides, which are presented in the groove of the MHC class I molecule (which are ubiquitously expressed on almost all cells).

Helper cells contain two subsets; TH-1 cells that promote cell-mediated immunity (secrete IFNγ and TNFβ) and TH-2 cells that promote antibody-mediated immunity (secrete IL-4, 5, 10 and 13). $CD4^+$ T-cells are essential for the majority of B-cell responses and the development of B-cell memory (Gray, 1994). A $CD8^+$ T-cell primary response can develop without $CD4^+$ T cells but the response does not last as long and $CD8^+$ T-cell recall (memory) is seriously impaired in mice lacking $CD4^+$ T-cells (Bourgeois 2003).

$CD8^+$ T-cells can recognise early intracellular viral protein production before virus budding occurs, allowing an early anti-viral response. When CTLs recognise an infected cell they release lytic granules containing perforin and granzyme directly onto the infected cell, inducing apoptosis. $CD8^+$ T-cells can also induce cell death by Fas ligation and can also control further virus infection by IFN-γ and TNF-α secretion.

IFN-γ

IFN-γ, the only type II interferon, is a potent anti-viral cytokine. IFNγ receptors (IFNγR) are expressed on most nucleated cells. In the CNS, glial and neuronal expression of IFNγR has been demonstrated in separate studies (Rubio & de Felipe, 1991; Torres, 1995; Robertson, 2000). IFNγ is secreted by activated NK cells, T_H1 $CD4^+$ cells and $CD8^+$ T-cells, and is consequently expressed later in infection than type I IFNs (Mokhtarian, 1996). Similarly to type I IFNs, IFNγR predominantly signals through the STAT pathway which induces gene expression by STAT homodimers, binding to IFNγ activated sites (GAS) elements. Genes solely upregulated by IFNγ include those for class II transactivator (upregulates MHC class-I and II) and proteins involved in antigen processing and presentation (Der, 1998b). The increased expression of these proteins serves to increase the speed of the anti-viral response by more efficient presentation of pathogens. As well as its

antiviral properties IFNγ is an important pro-inflammatory role and is a potent activator of macrophages and has a major role in the regulation of the T-helper cell differentiation (summarised Figure 2).

Fas and Fas ligand

Fas and Fas ligand (FasL) are members of the TNF receptor and TNF families, respectively. FasL, which is upregulated on CTLs, initiates apoptosis in the target cell by binding to Fas. Fas is expressed on a variety of cell types, whereas FasL is expressed predominantly on activated lymphocytes. Fas ligation by FasL initiates a signalling cascade resulting in binding and activation of caspases. Caspases are a group of cysteine proteases, enzymes with a crucial cysteine residue that can cleave other proteins after an aspartic acid residue. Caspases are essential in cells for apoptosis. Caspase activation eventually leads to DNA degradation, membrane blebbing, and other hallmarks of apoptosis (summarised Figure 2). This receptor-ligand pair also plays an important role in the homeostasis of the immune system known as activation-induced cell death (AICD). AICD is mediated by upregulation of FasL on activated T-cells which limits potentially damaging accumulation of cytokine secreting activated T-cells after antigen elimination and also inactivates potentially autoreactive cells. The importance of this interaction for the maintenance of lymphocyte homeostasis is demonstrated in the generalized lymphoproliferative disorder associated with natural loss-of-function mutations of Fas (*lpr*) and its ligand (*gld*). Mice homozygous for *lpr* or *gld* mutation develop remarkably similar, progressive non-malignant lymphoproliferative diseases characterized by severe lymphadenopathy and an autoimmune disease similar to systemic lupus erythematosus (Watanabe-Fukunaga, 1992; Takahashi, 1994). The importance of Fas induced-apoptosis in the CTL response is not known, while FasL is expressed on killer T-cells its role often seems redundant in the immune response to viruses (Kagi, 1995a; Murray, 1998a).

Perforin and Granzyme

Perforin is secreted in a cytolytic granule with granzyme (A and B) from the CTL and is then endocytosed by the target cell. The synergistic action of these effectors activates an apoptotic cascade which eliminates the infected cell, summarised in Figure 2 (Trapani & Smyth, 2002b). Perforin has a pore-forming capacity in the cell membrane, but this is not thought to be its mode of action, as sub-lytic quantities of perforin increases granzyme delivery far more than other pore forming molecules. The presence of perforin allows the granzymes to act on the target cell. Granzyme B is a potent activator of caspase and of caspase independent apoptosis. It directly activates pro-apoptotic protein which results in the leakage of proapoptotic mitochondrial mediators (cytochrome C) into the cytosol (Sutton, 2000). Granzyme A cannot activate caspases directly but mediates its action by direct cleavage of nuclear proteins, thus facilitating the formation of ssDNA breaks (Beresford, 1999). The actions of both granzymes are dependent on perforin, because perforin deficient mice fail to kill virus infected cells *in vivo* (Kagi, 1995b).

T-regulatory cells

The concept of suppressor T-cells went out of fashion in the 1980s, however several studies have found that T-cells can suppress immune responses in autoimmune disorders, demonstrating unequivocally that these cells have a role in inhibiting immune responses (Penhale, 1975; Fukuma, 1988; Powrie & Mason, 1990). Furthermore, a role for T-regulatory (T-regs) cells was found in infectious diseases such as Leishmania major infection (Belkaid, 2002). T-regulatory cells can be divided into two subsets, firstly naturally occurring T-regs ($CD4^+CD25^+FoxP3^+$), which make up a subset of normal T-cells (5%). T-regs develop in the thymus and when they recognise their MHC/antigen they mediate their action in a cell-contact dependent manner (Baecher-Allan, 2001; Shevach, 2002). The second subset is made of inducible T-regs ($CD4^+CD25^+$). These develop in response to cytokine secretion (IL-10 or TGF-β) and then secrete these anti-inflammatory cytokines (Groux, 1997b; Jonuleit, 2002). It is unclear whether these are a distinct subset from

normal T-cells or if their suppressor activity represents a stage of development of normal T-cells. T-regs have been found to play a role in some virus infections such as hepatitis C and herpes simplex virus (HSV) infection (Rouse, 2006b).

Antibody Response

When a B-cell recognises the antigen for which it has specificity, antigen is taken up by binding of cell surface immunoglobulin (Ig). Most B-cell antigens require that the B-cell receives stimulation from a T-helper cell by antigen/MHC II presentation on the surface of the B-cell. The T-cell provides cell-cell and cytokine signals that allow B-cells to clonally expand and develop into antibody secreting plasma cells. B-lymphocytes secrete soluble antibody into the blood and lymph. Antibody can neutralise virus particles, so preventing binding or entry of the virus into cells, as well as tagging viruses for phagocytosis by opsonisation. Antibodies bind to viral antigen present on the host cell surface and can lead to the cell being lysed by the classical complement pathway or being killed by natural killer cells (Guidotti & Chisari, 2001).

Large IgM immunoglobulin molecules are produced early in the immune response. Following class-switching other isotypes are then secreted that have different tissue specific functions. IgG is the main immunoglobulin isotype elicited following virus infection and importantly this smaller molecule can cross into the CNS during virus infections as the permeability of the blood brain barrier (BBB) increases. This feature allows IgG antibody to extend its actions to the immunospecialised brain tissue (Parsons & Webb, 1982b; Coutelier, 1988a).

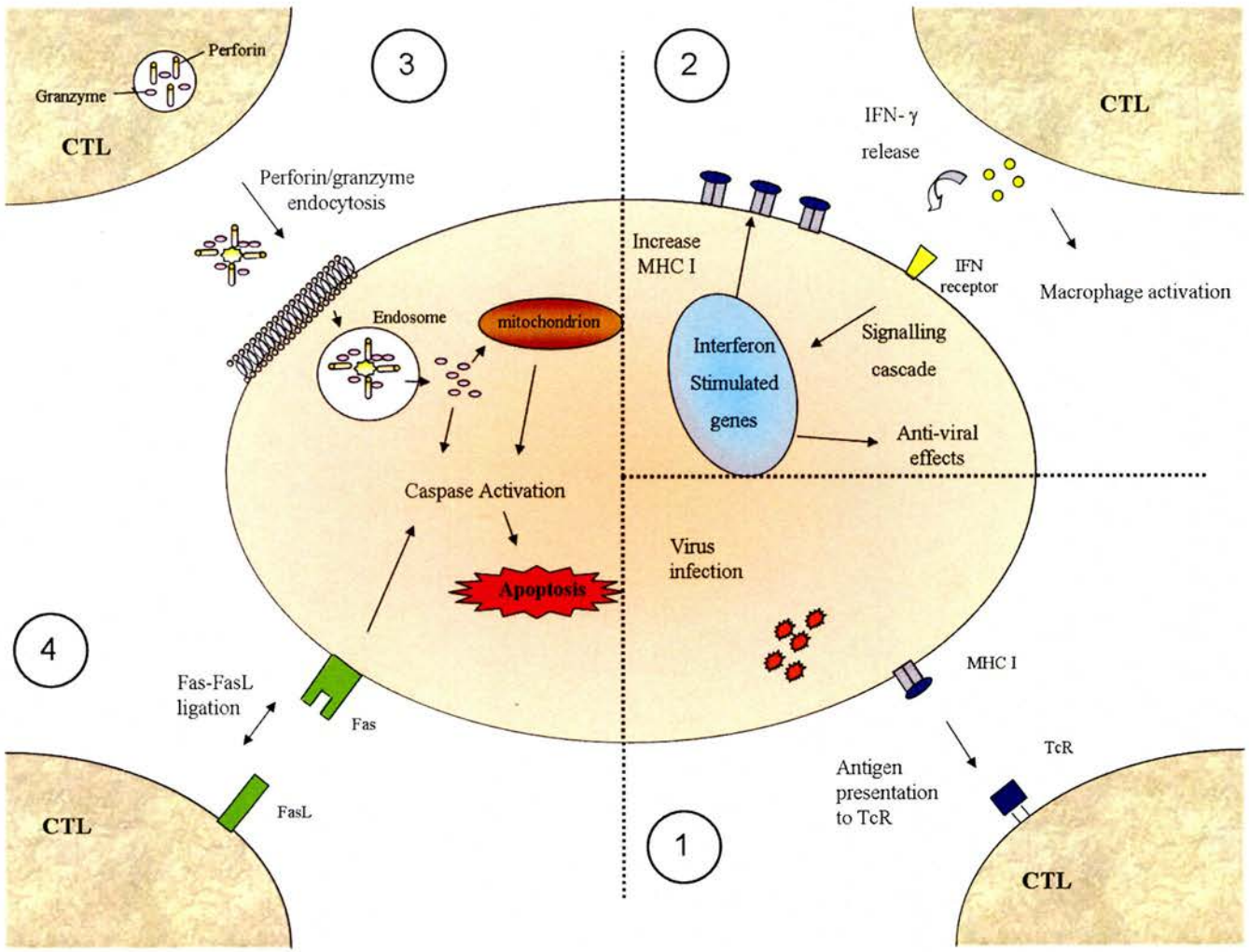

Figure 2 Action of the $CD8^+$ T-cell on a virus infected cell. **1.** Virus infected cell presents antigen to $CD8^+$ T-cell TcR via MHC class I. **2.** IFNγ is released from CTL and binds to IFNγR on target/surrounding cells. IFNγ binding initiates a signalling cascade, stimulating IFN stimulated genes resulting in upregulation of anti-viral genes and MHC I. **3.** Granzyme/perforin released from the CTL is endocytosed by the target cell. Granzymes cleave caspases (especially caspase-3), which in turn activates caspase-activated DNase. This enzyme degrades DNA, thus inducing apoptotic cascades. GrB causes changes to the membrane permeability of the mitochondria, causing the release of cytochrome c (one of the parts of caspase-9). **4.** Fas ligation by FasL initiates a signalling cascade resulting in binding and activation of caspase-8 which is released into the cytosol, where it cleaves other effector caspases, eventually leading to DNA degradation, membrane blebbing, and other hallmarks of apoptosis. TcR – T-cell receptor; GrB – granzyme

CNS immune responses

The CNS has long been considered to be an immunoprivileged site. In health few leukocytes, antigen presenting cells or antibodies are found there. There is also a very low level of MHC molecule expression in the normal CNS. Unlike the peripheral structural lymphoid tissue such as lymph nodes, there is no lymphoid architecture within the CNS. Along with this apparent lack of immune function, tight endothelial cell junctions form the BBB which encloses the brain. The BBB itself has little endocytosis capacity and few adhesion molecules. Suppression or limitation of the immune response in the CNS might be expected, as neurons are terminally differentiated and destruction of such cells by an immune response could have severe effects. Additionally, the brain is enclosed in the rigid bony cranium and thus any inflammatory swelling could cause great or even fatal damage, in contrast to inflammatory swelling in almost all other tissues.

Studies of the CNS in different pathological states have revealed its ability to mount a significant immune response. Resident CNS cells have been shown to produce chemokines (Owens, 1994) which are pivotal to leukocyte recruitment. Leukocytes in their activated form can traffic across the BBB into the CNS (Wekerle, 1986) and activated astrocytes can locally synthesise complement components, as those synthesised outside the CNS are prevented from crossing the BBB (Levi-Strauss & Mallat, 1987; Morgan, 1997). Inflammation compromises the BBB increasing its permeability, allowing some proteins such as particular antibody isotypes to cross into the CNS. Antibody can also be secreted intrathecally from plasma cells that have trafficked into the CNS (Parsons & Webb, 1989).

The healthy, uninfected CNS can express TGF-β1 and IL-12 (Morgan, 1993; Park & Shin, 1996). In response to alphavirus infection, IL-1α/β, IL-6 and TNFα mRNA levels rise rapidly in the CNS (Wesselingh, 1994). Both IL-6 and TNFα have antiviral activity that helps induce an immune response, by attracting circulating lymphocytes. However, TNFα can have deleterious effects in the CNS affecting neuronal function, and therefore may play a direct role in the immunopathogenesis of

some CNS disorders. TNFα has been shown to be directly destructive to oligodendrocytes and also to mediate demyelination *in vitro* (Selmaj & Raine, 1988). There is little evidence of early IFNγ production. Neurons can produce IFNγ but it is possible this cytokine is used in a developmental rather than anti-viral capacity (Neumann, 1997).

The role of chemokines in CNS inflammation has been demonstrated through the use of various knockout (KO) mice, with deficiencies in lymphocyte recruitment to the CNS (Mennicken, 1999). Chemokines can be synthesised and secreted by CNS cells, principally astrocytes and microglial cells, these cells can also respond to chemokines by way of receptor expression (Asensio & Campbell, 2001). Monocyte chemoattractant protein (MCP)-1, stromal derived factor -1 and fractaline are constitutively expressed at low levels in the uninfected CNS. In CNS viral infections, the chemokine profile (IFN induced protein -10, MCP-1 and Regulated upon Activation, Normal T-cell Expressed, and Secreted [RANTES]) results in lymphocyte and monocyte recruitment. Neuronal production of chemokines has been demonstrated in CNS virus infection, thus neurons can help recruit inflammatory cells to fight CNS virus infection (Patterson, 2003).

$CD8^+$ T-cell interactions with infected cells are restricted to cells expressing MHC class I. T-cell binding to their cognate MHC-peptide complex allows initiation of the perforin-granzyme pathway or a Fas-FasL interaction. It has long been observed that MHC molecules are only found at a very low level in the healthy CNS (Lassmann, 1991). The ability of cells of the CNS to express MHC molecules has been demonstrated through experimental infection with neurotropic viruses. Endothelial, ependymal, meningeal, oligodendrocyte, astrocyte and microglial cells can rapidly upregulate MHC class I in response to infection. MHC class I proteins can be detected in neurons and cell surface expression of MHC class I has also been observed, however these neurons were electrically silent and hence terminally damaged (Joly, 1991a; Neumann, 1997; Kimura & Griffin, 2000). MHC I is not though to be expressed on an infected but otherwise healthy neuron. Expression of MHC class II molecules is much more limited in the CNS, and only microglia have

been shown to express this molecule (Morris, 1997; Redwine, 2001). Although MHC is expressed in the brain, given the constitutive expression of anti-inflammatory cytokines, it is likely that the lytic actions of CTLs are very limited.

The literature supports the view that the CNS can mount an effective immune response to pathogens, however the CNS is a specialised tissue and consequently, virus infection and the immune response to it, comes at a cost. Common pathological features to most viral CNS infection are mononuclear cell infiltration, perivascular cuffing, astrocytosis and microgliosis. Such features are commonly seen in the flavivirus encephalitides (Johnson, 1985). Other pathological features are specific to individual viruses, such as the negri bodies (cytoplasmic inclusions containing rabies virus antigen) in pyramidal neurons and Purkinje cells during rabies infection. This is the main morphological change observed in rabies, the limited inflammatory response and cell destruction belies the invariably fatal outcome of this disease. Paralytic polio is characterised by atrophy and necrosis of motor neurons in anterior horn cells of the spinal cord, causing the flaccid paralysis typical of this disease (Esiri & Kennedy, 1997). A more widespread effect is seen in HSV encephalitis where there is widespread necrosis of temporal lobes, and acute swelling and inflammation (Adams & Miller, 1973).

Demyelinating lesions are found in many CNS infections, where loss of the myelin sheath surrounding axons affects conductance of nerve impulses. Myelination in the CNS is mediated by oligodendrocytes, and a single oligodendrocyte can myelinate several axonal internodes (Blakemore, 1982). Therefore the death of a single oligodendrocyte can affect signal conductance in several axons. There are a number of possible ways that CNS infection can cause demyelination. The most obvious example is by direct virus killing of oligodendrocytes, as occurs in progressive multifocal leukoencephalopathy (Johnson, 1983). Oligodendrocytes can also be damaged inadvertently by a bystander effect; the immune response to the oligodendrocyte or adjacent infected cells releases factors resulting in oligodendrocyte destruction. This is thought to occur in mouse hepatitis virus (MHV) infection of the CNS (Houtman & Fleming, 1996; Wu, 2000c).

Severe combined immunodeficiency (SCID) mice, that lack both B- and T-cells, do not have demyelinating lesions following MHV infection but the transfer of $CD4^+$ or $CD8^+$ T-cells to these mice can initiate demyelinating lesions, perhaps through stimulation of macrophages. $CD8^+$ T-cell mediated cytolysis of oligodendrocytes can occur if oligodendrocytes are infected and express peptide on MHC class I. Data suggests that this may occur in SFV, as antibody depletion of $CD8^+$ T-cells abrogates demyelinating disease in SFV infected mice (Subak-Sharpe, 1993). Indirect damage to oligodendrocytes by macrophages and pro-inflammatory cytokines can also damage myelin. Myelin released from damaged axons can be taken up and presented to activated T-cells which have entered the CNS to control the virus infection. The presentation of self peptides alongside viral peptides can result 'epitope spreading' and an autoimmune response (Miller, 1997).

Autoimmune demyelinating disease can also be induced in mice by the injection of myelin components. This experimental autoimmune encephalitis (EAE) provides a frequently studied model of Multiple Sclerosis. Demyelination and paralytic episodes are associated with $CD4^+$ T-cell infiltration into the CNS. Epitope spreading resulting in diversification of the auto-immune response to other CNS components can occur in chronic, relapsing-remitting EAE (Lehmann, 1993). Certain strains of mice are resistant to EAE, however this tolerance can be broken by alphavirus infection (Mokhtarian & Swoveland, 1987). There are two main schools of thought as to why SFV infection breaks tolerance to EAE in C57Bl/6 mice. The first hypothesis is that viral infection triggers production of antibodies and mononuclear cells that cross-react with self-proteins, in a process called molecular mimicry, resulting in an autoimmune response. However this theory is not supported by the findings of Miller *et al* (1997). Using T-cell proliferation assays, they looked for cross-reactive responses between Theiler's murine encephalomyelitis virus (TMEV, another virus that causes immune-mediated demyelination) and myelin epitopes. A TMEV specific CTL response was detectable within 7 days, but those involving myelin did not appear until 50 days post-infection. They suggested that the inflammatory responses in certain hosts have the capacity to lead to chronic, organ-

specific autoimmune disease. Tolerance to certain CNS antigens may be slowly broken via epitope spreading, due to a chronic pro-inflammatory response, making C57Bl/6 more susceptible to EAE following CNS viral infection (Miller, 1997).

Persistence of RNA viruses in the CNS

There are three requirements for a virus to persist, firstly it must infect cells without causing cytolysis, it must be able to maintain its genome in the cell and lastly it must be able to avoid detection by the host's immune system (Ahmed, 1997).

In relation to viral infection, persistence is a broad term and encompasses many meanings. It can be defined as maintenance of the genome only within the host cell, such as the persistence of HSV within neurons. Alternatively, in persistent measles virus infection of neurons the virus exhibits limited replication and there is an absence of virus particles. Persistence also describes the high infectious burden of LCMV infection where many cells and tissues are infected and productively produce virus.

Latency, where there is transcription of a limited number of virus genes and no virus particles, can only truly occur with DNA viruses. The exact mechanisms of how viral latency is established and maintained within the cell are not fully understood. RNA reversiviruses, reverse transcribe their genome to make pro-viral DNA which can persist in the nucleus, but other RNA viruses cannot persist intra-cellularly in this manner. The larger DNA genome can also encode other mechanisms to allow persistence. For example, human cytomegalovirus encodes immunomodulating proteins that target MHC class I for degradation, prevent MHC I transport to the cell surface and prevent peptide loading (Campbell, 1999). RNA viruses do persist intracellularly but use other mechanisms, such as restricted virus replication, avoidance of the immune response by infection of immunoprivileged sites and induction of tolerance to achieve this goal (Figure 3).

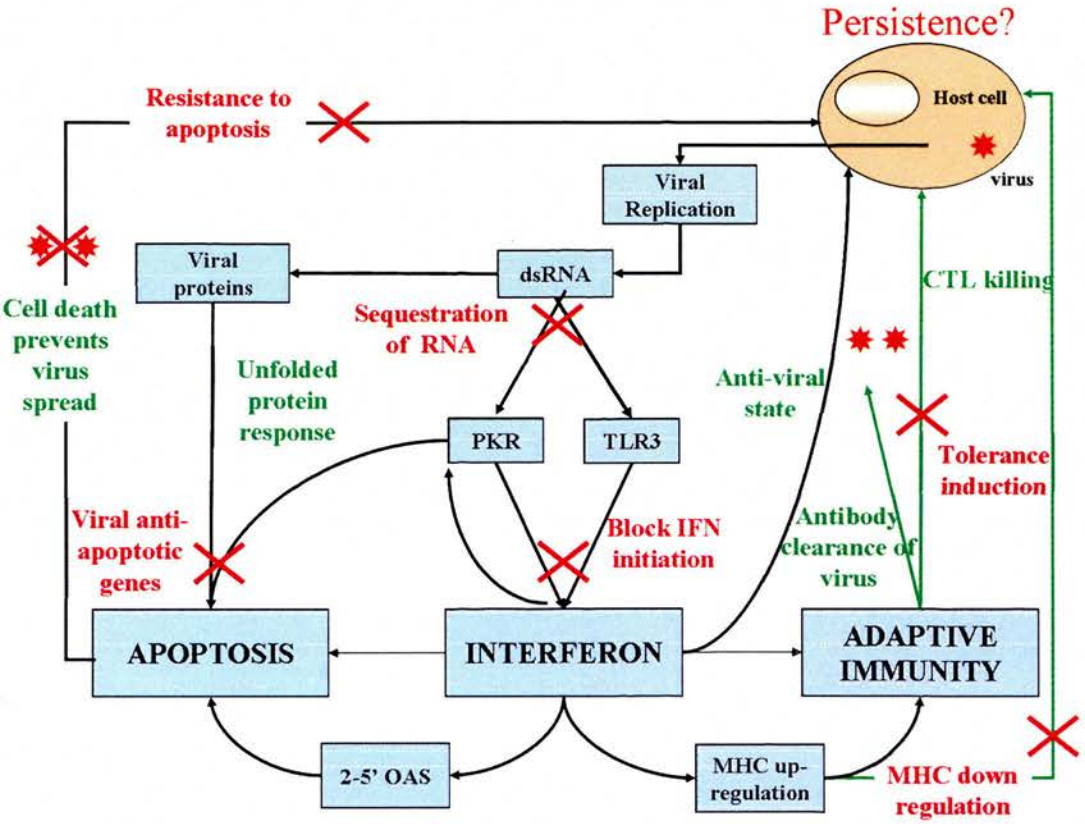

Figure 3. Host cell responses to virus, and virus mechanisms to allow persistence. Black arrows indicate innate immune mechanisms; green arrows indicate adaptive immune mechanisms and red crosses are points at which viruses can suppress these responses predisposing to persistence.

Deciding what constitutes sterilising immunity (the complete clearance of all infectious material) is a complicated matter. There are many sensitive techniques for detecting viruses and each detects different virus products. Infectious virus is detected by infectivity assay, virus proteins by immunoblotting, virus nucleic acid by *in situ* hybridisation and virus genome by polymerase chain reaction (PCR). The presence of one viral product however does not necessarily indicate active infection. For example, if virus RNA can still be detected by sensitive techniques such as real-time quantitative PCR or nested PCR, this does not necessarily mean that there is still infectious or even replicating virus present. The RNA detected could be 'junk' RNA that is non-functional but has yet to be cleared from the CNS, and is not able to replicate to produce infectious virus particles. On the contrary, during chronic measles infection, the virus changes to a form that does not produce virus particles but only replicates non-structural genes within neurons, but the virus does persist.

The CNS is susceptible to persistent infection for a number of reasons. It is inaccessible to some immune mediators, few trafficking lymphocytes enter the CNS, and the BBB restricts the entry of humoral components such as complement or antibody. The longevity of neurons predisposes these cells to persistent infection, if the host cell survives for a long time the virus does not need to infect other cells. Such is the case in varicella-zoster virus infection of trigeminal ganglion cells in humans which can last 80 years or more (Gilden, 1983). The typical effectors against virus infection are MHC class I restricted CTLs. The presence of neurons in the CNS that do not express MHC predisposes the CNS to virus persistence (Joly, 1991b). Selective pressures also protect essential neurons, for example the destruction of neurons in the area of the brain controlling respiration (respiratory centre) would be fatal and therefore such cells do not readily undergo apoptosis (Allsopp & Fazakerley, 2000). The anti-inflammatory cytokine TGF-β is constitutively expressed in the CNS and is further upregulated in response to infection (Ranges, 1987; Stitz, 1991; Wesselingh, 1994). Evolutionary balancing of the potential damage of immune mediators against the benefit of virus clearance appears to have selected a system which can predispose to virus persistence when the potential for CNS damage is too great.

Research on neurotropic viruses has provided a wealth of information on host defence mechanisms, CNS immunity, and chronic viral infection and CNS pathology. Several viruses can establish persistent infection in the CNS and key examples of such viruses are discussed below. These viruses have been extensively researched to delineate the CNS immune response to these viruses, their cellular tropism and the mechanisms employed that prevent their elimination.

Theiler's Murine Encephalomyelitis virus (TMEV)

TMEV is a single stranded (+) RNA virus of the family *Picornaviridae*. The TMEV genome is infectious and about 8Kb in length. TMEV is a natural pathogen of mice which is transmitted by the faecal-oral route and normally persists as an asymptomatic infection of the gut. In naturally occurring infections, the virus occasionally enters the CNS and a persistent infection of spinal cord white matter

occurs. In laboratory studies, direct intracranial inoculations (IC) are used to study TMEV persistence. TMEV has been categorised into two subgroups, firstly the highly neurovirulent GDVII and FA strains; in rare survivors of this infection, the virus is cleared from the CNS. The second group contains the Daniels and BeAn TMEV strains which can cause CNS persistence.

In susceptible mouse strains, IC inoculation of TMEV causes a biphasic disease. The first phase which lasts for about 2 weeks is characterised by acute encephalitis, infection of grey and white matter in the brain and spinal cord. In the transition from acute to persistent infection, there is clearance of infectious virus and virus RNA from the brain but continued infection in the spinal cord. During the chronic phase the virus persists in macrophages and glial cells, resulting in a demyelinating disease of spinal cord white matter tracts. There is debate over which cells in the spinal cord harbour virus because TMEV is a lytic virus, and infected cells often undergo apoptosis after infection, making it difficult to identify them. Viral antigen is present in macrophages, but it is possible that this represents phagocytosed virus particles rather than infection of phagocytes (Lipton, 1995; Zheng, 2001). Glial cells, including astrocytes and oligodendrocytes in the spinal cord have also been shown to be virally infected (Aubert, 1987; Simas & Fazakerley, 1996). All mice infected IC with avirulent TMEV have acute infection but only susceptible (SJL) mice develop persistent infection (Lipton, 1975).

The initial wave of TMEV infection is controlled by massive influx of T-cells and in resistant mice there is complete viral clearance by post-infection day (PID) 21. Resistant mice can be made susceptible to demyelinating disease and acute virus clearance can be abrogated by depletion of T-cell subsets (Rodriguez, 1991; Borrow, 1992b). In susceptible mice, demyelinating lesions are characterised by B and T lymphocyte infiltration. Direct cytolytic damage to oligodendrocytes is thought to be partly responsible for demyelination, but the anti-TMEV $CD4^+$ T-cell response is also involved (Gerety, 1994). The acquired immune response therefore has a contradictory role; it is essential for virus clearance but in susceptible mice exacerbates pathological changes when virus persists. SCID mice die from

overwhelming encephalitis but do not have demyelination, indicating an immune aetiology for demyelination (Rodriguez, 1996).

MHC I genes are an important determinant of resistance to infection. In part, resistance maps to the MHC Class I gene H2, in particular to H-2D. The CTL response of resistant mice is H-2D^b restricted and virus is efficiently cleared without the development of any neuropathological changes. Transgenic expression of TMEV genes in resistant mice inactivates virus specific CTLs, and following infection in these mice, virus persists and demyelination occurs (Lin, 2002). Susceptible mice mount an H-2K restricted response. If the H-2D presentation is inactivated in resistant mice, the remaining H-2K restricted CTLs are unable to mount an efficient responses (Azoulay-Cayla, 2001). The reasons for such poor H-2K restricted CTL response are not known, but it has been suggested that H-2K is poorly expressed in the CNS, resulting in inefficient CTL mediated virus clearance in the brain (Altintas, 1993).

The genome of the virus itself also plays a role in determining persistence. Neurovirulent TMEV (GDVII and FA strains) do not persist in mice surviving infection (Lipton, 1980). Therefore a determinant of persistence is encoded in the BeAn and DA strains. Recombinant GDVII virus expressing capsid from the DA virus has the ability to persist (McAllister, 1990; Tangy, 1991). Persistence is linked to the ability of DA to infect the white matter and establish a restricted infection of oligodendrocytes. In contrast, neurovirulent viruses preferentially infects neurons, leading to more severe disease, but this virus can be fully cleared from the CNS (Jarousse, 1994).

The cell type that harbours TMEV in persistent infections is still controversial. Macrophages are infectious, but can only produce few virus particles as they have a block in (-) strand RNA synthesis (Cash, 1988; Clatch, 1990). TMEV viral RNA is detectable by *in situ* hybridization techniques in macrophages late in infection. It has been demonstrated that the antigen burden is predominantly contained in this cell type but only a low infectious load (Aubert, 1987). Therefore macrophages may harbour infection but perhaps have a block in virion assembly accounting for the low

number of virus particles. However, phagocytosis of infected cells will cause macrophages to contain viral antigens and this makes true viral infection of macrophages difficult to identify. Evidence for persistence of virus in myelin or oligodendrocytes comes from studies on two mouse strains that have mutations in the genes encoding myelin basic protein and myelin structural protein. These mutations confer resistance to TMEV infection, indicating that myelin or oligodendrocytes allow virus to persist (Bihl, 1997). Astrocytes have also been cited as the cell responsible for TMEV persistence. These cells have been shown to effectively replicate the virus and produce infectious virions with minimal CNS cytotoxicity (Zheng, 2001). TMEV persistence requires a combination of host and virus factors, with a poor host CTL response combined with virus cell tropism for white matter, resulting in virus persistence.

Mouse Hepatitis virus (MHV)

Mouse hepatitis virus, a natural pathogen of mice, is a member of the *Coronaviridae*. MHV is a pleomorphic, single stranded (ss) RNA, enveloped virus with surface projections. There are many biotypes of MHV with different cellular tropism and virulence. The John Howard Mueller (JMH) strain of MHV and MHV-4 strains are neurotropic. JMH MHV infection is characterised by a biphasic disease and immune responses. In acute infection there is encephalomyelitis with demyelination which is quickly resolved. The second phase of the disease is persistent infection with demyelination and no infectious virus is detectable in these chronic infections.

During the acute phase of MHV infection, a rapid influx of T-cells, in particular $CD8^+$ T-cells, controls the acute infection (Williamson & Stohlman, 1990) and comprises up to 50% of virus specific T-cells. $CD4^+$ T-cells do not appear to have a direct anti-viral role but are essential for both the $CD8^+$ T-cell and antibody response. $CD8^+$ T-cells use both perforin and IFNγ to control virus replication. Perforin preferentially inhibits viral replication in microglia and astrocytes (Lin, 1997). IFNγ controls oligodendroglia infection (Parra, 1999a) and also increases MHC I expression, an essential function to allow more expression of viral peptides to permit

CTLs to efficiently clear virus. The cellular immune response is not capable of achieving sterile immunity and is surpassed by the humoral response as the virus moves into its persistent phase. Persistence of viral RNA in glial cells has been reported up to 1 year PI. This is consistent with the ongoing demyelination and recovery that is observed with infection in these persistently infected mice (Knobler, 1982a).

The retained $CD8^+$ T-cells lose their *ex-vivo* cytolytic activity in the chronic phase of infection, while maintaining their IFN-γ secretory capacity (Ramakrishna, 2004). Virus induced TNF-α production decreases, indicating reduced AICD, which might account for increased CTL numbers. This loss of cytolytic potential could benefit the host by limiting long-term CNS pathological changes, while contributing to anti-viral functions. T-cells may become exhausted or their cytolytic activity down-regulated in order to prevent immunopathological changes at the cost of allowing persistent viral infection.

The distinct mechanism that induces cytolytic unresponsiveness has yet to be identified. $CD8^+$ and $CD4^+$ MHV specific T-cells are retained in the CNS and the percentage of virus-specific CD8 T-cells (as demonstrated by tetramers) remains constant (Bergmann, 1999). Myelin loss in the chronic phase of disease is consistent with an ongoing immune response. The role of virus in retaining CTLs has been investigated, where the continued presence of MHV RNA in the spinal cord was shown to be necessary for the retention of virus specific T-cells (Marten, 2000).

The presence of serum antibody and specific anti-viral plasma cells are delayed in MHV-4 infection and antibody levels do not rise until infectious virus clears (PID 14-21). In the chronic phase of this infection, antibody titres increase and remain stable during virus persistence (Tschen, 2002b). Humoral immunity alone is required for control of persistent virus infection as demonstrated in studies on μMT mice (that cannot secrete antibody). These mice can control infection in the acute phase, indicating a redundant role for antibody in the early stages of disease. Following initial control during acute infection, the virus reactivates in the absence of antibody to levels similar to those present in the acute phase, indicting that cellular

immunity is not effective in controlling persistent infection. The transfer of neutralising antibody prevents the re-activation of virus in μMT mice, further underlining the importance of humoral immunity in persistent viral infection (Lin, 1999). The specificity of the antibody response changes over the course of the persistent infection. For example, plasma cells specific for viral proteins S and N decline; however, serum neutralising antibody titres do not fall as plasma cells have specificity for other MHV viral antigens (Tschen, 2002a).

MHV is able to rapidly evolve during the course of CNS infection and whether this ability to change contributes to viral persistence is of interest (Adami, 1995). Accumulation of viral quasispecies with deletions in the S protein hypervariable domain was associated with increased loads of infectious virus in the CNS following infection of neonatal mice. CTL escape of virus epitopes was demonstrated in suckling C57Bl/6 mice, which could contribute the increased viral loads through increased viral fitness (Pewe, 1996). Despite these findings, the relevance of mutations in MHV in infection of naive adult mice to viral persistence appears to be negligible (Bergmann, 1998).

It is possible that viral persistence is a trade off between effective viral clearance and limiting immunopathological damage. Virus is neutralised and kept under control by antibody (and possibly by IFNγ) but a low level of infection continues. Balancing the need to eliminate virus from the CNS but also limit immunopathology may favour viral persistence.

Lymphocytic Choriomeningitis virus (LCMV)

LCMV is a member of the *Arenaviridae* Family. It is a non-cytopathic, ambisense, enveloped RNA virus. LCMV causes asymptomatic, chronic infections of rodents worldwide. The outcome of transmission of arenaviruses from rodent to humans varies from asymptomatic infection to severe haemorrhagic disease. In the adult mouse, the virus replicates in macrophages at the site of entry (often the lung) before causing widespread infection of multiple tissues. There is a biphasic immune response, with an initial NK cell expansion giving way to a massive $CD8^+$ T-cell

response. At the peak of the immune response, 50% of all $CD8^+$ T-cells in the spleen are specific for LCMV (Murali-Krishna, 1998).

Adult mice infected IC with LCMV succumb to a fatal choriomenigitis after 6-8 days. Virus infection stimulates several immune effector mechanisms but virus clearance is primarily dependent on CTL. If these cells fail to generate or are deleted, progression from acute to persistent infection occurs. MHC class I expression is necessary for CTL mediated virus clearance and as noted, neurons typically do not express MHC. Nevertheless, following LCMV infection, CTLs have been shown to mediate clearance of infected neurons albeit slower than conventional MHC expressing cells, but virus clearance still resulted (Oldstone, 1986; Tishon, 1993).

In order to persist, a pathogen must avoid detection and elimination by the immune system. CTLs are an important means for clearance of virus. One such mechanism to evade detection is for viral antigen not to be recognised as non-self, and hence not alert the immune system to its presence. LCMV accomplishes this successfully via infection of the neonatal rodent. If LCMV infection occurs while the immune system is still developing, virus is presented as self antigen and virus specific T-cells are negatively selected and deleted in the thymus and so do not appear in the periphery (Pircher, 1989). Cells infected with LCMV do not undergo apoptosis and in the absence of specific immunity the outcome is persistence. Infected neonatal mice become congenital carriers, and have a neuronal infection in addition to leptomeninges and choroid plexus infection. Persistently infected mice shed virus in their body fluids, leading to virus dissemination. Other immune mechanisms are not affected by this specific tolerance, as shown by rejection of skin grafts and the presence of high levels of antibody against LCMV in carrier mice (Buchmeier & Oldstone, 1978). The 'deleted' LCMV-specific CTLs can be reconstituted in a tolerant mouse resulting in efficient removal of virus (Ahmed, 1987; Jamieson, 1987). It has been suggested that the CTLs rather than being deleted are functionally impaired and that adoptive transfer clears persistent virus, allowing regeneration of the specific LCMV-CTL response (Oldstone, 2006).

Infection of adult mice with LCMV normally resolves after acute infection, however, persistence can occur after a high dose of inoculum or after infection with certain LCMV variants. The clone 13 strain produces a generalised immune suppression and persistent infection, whereas mice infected with the Armstrong strain rapidly clear infection and are immunocompetent. A mutation in the clone 13 immunosuppression strain was found where a uracil to cytosine transition at position 855 of the S gene, resulted in a phenylalanine to leucine alteration at amino acid 260 of the envelope glycoprotein, thereby altering cell tropism (Ahmed, 1988; Salvato, 1991). The mechanism of immunosuppression was found to be linked to infection of splenic, antigen presenting cells, which are consequently killed by LCMV specific CTLs, leading to functional suppression of the LCMV specific CTL response (Borrow, 1995).

More recent studies with LCMV clone 13 infected mice (Brooks, 2006; Ejrnaes, 2006) have demonstrated that virus persistence is caused by T-cell immunosuppression mediated by elevated IL-10 levels. IL-10 is an anti-inflammatory cytokine which can inhibit a broad spectrum of cellular immune response, by suppressing the function of APCs and T cells, hence inhibiting pro-inflammatory cytokine production, co-stimulation, MHC class II expression, and chemokine secretion (Moore, 1993). In both studies, the *in vivo* blockade of IL-10 led to resolution of LCMV infection. The infection of IL-$10^{-/-}$ mice with clone 13 led to the generation of a robust T-cell response which rapidly cleared virus. Both studies indicated that DCs were the likely source of the IL-10 production, the reason for this is unclear, preferential infection of DCs by clone 13 LMCV strain was suggested or alternatively that DCs are skewed towards a TH-2 subtype after infection with this strain and thus are induced to secrete IL-10.

Viral persistence has also been examined in mice following acute infection which were deemed to have cleared all virus by conventional assays. Viral cDNA was detected in spleen tissue of apparently healthy mice post-infection. It is not known if this viral DNA has biological activity. It remains possible that low-level protein expression can occur (Klenerman, 1997). More recently, Ciurea *et al* (1999)

demonstrated that mice with an intact immune response which appear to have resolved an acute viral infection, continued to express low-level infection in the form of viral genome, viral antigen and replicative virus in peripheral organs. Whether persistence occurs after acute LCMV infection in order to maintain immunological memory remains to be answered (Ciurea, 1999a).

Sindbis Virus (SV)

SV, an enveloped (+) RNA, is an alphavirus of the *Togaviridae*. SV is closely related to Western equine encephalitis an important cause of mosquito-borne encephalitis in the Americas (Weaver, 1997). In the mosquito, SV replicates in a non-lytic manner and persists (Stevens, 1970). After IC inoculation of mice with neuroadapted SV, the virus replicates primarily in neurons of the brain and the spinal cord. Infectious virus is cleared in immunocompetent mice in 6-8 days without the development of neurological disease (Jackson, 1988). Weanling mice succumb to fatal encephalitis within 4 days (Griffin, 1976).

The antibody response is crucial to infectious virus clearance. Antibody secreting plasma cells are found indefinitely in the CNS of immunocompetent mice following SV infection (Tyor, 1992b). Antibody immunodeficient mice are persistently infected but the transfer of hyperimmune (HI) serum clears infectious virus. When passively transferred antibody decays, virus infectivity then returns indicating the need for permanent antibody secretion to control virus persistence (Levine, 1991; Levine & Griffin, 1992). The role of T-cells has been examined in mice lacking B-cells. Infectious virus is cleared from the spinal cord and parts of the brain, but persists in cortical neurons in the absence of antibody (Kimura & Griffin, 2000; Binder & Griffin, 2001c). IFNγ is necessary for the partial, T-cell mediated clearance observed. While virus infectivity is effectively cleared by the humoral immune response, the role of the immune response in the clearance of virus genetic material is less clear.

SCID mice have persistent infection (of brain and spinal cord) after SV infection, and despite lacking both humoral and cellular immunity, no neurological deficits are

seen in these animals following infection. Persistence was not thought to occur in immunocompetent mice, but using reverse transcriptase PCR, it was discovered that although infectious virus had been cleared, viral RNA was still present in BALB/c mice up to 17 months PI (Levine, 1991; Levine & Griffin, 1992). A number of possible mechanisms could contribute to the restriction of the cytolytic effect.

Soluble factors have been shown to contribute to the establishment of persistent alphavirus infection *in vitro*. SFV infected L929 cells pre-treated with IFNα readily establish a persistent infection rather than a lytic, virus-induced death (Meinkoth & Kennedy, 1980). Defective interfering (DI) particles, i.e. virus particles that are missing part of their genome, have been suggested to have a role in the establishment of virus persistence *in vitro*. SV treated BHKs normally succumb to infection but a small fraction survive along with SV DI particles, it was concluded DI particles were required to establish a persistent infection with wild-type, standard SV virus (Weiss, 1980). Their efficacy *in vivo* has also been demonstrated; co-administration of SFV DI particles with virulent SFV4 to mice prevented lethal encephalitis and results in virus persistence (Atkinson, 1986).

The cellular differentiation state of SV infected cells has also been suggested to be a determinant in persistence. SV causes a lytic infection in rodent neuroblastoma cells but persistent infection occurred in neurons grown with nerve growth factor. The difference between these two cell types included the expression of genes involved in cellular differentiation (Levine, 1991). The Bcl-2 family are anti- and pro-apoptotic proteins that regulate cell death by blocking or promoting mitochondrial outer membrane permeability. Pro-apoptotic proteins are expressed in cells with extended lifespans, such as neurons. Micro-injection of a vector expressing Bcl-2 into isolated neurons prevented growth factor withdrawal triggered apoptosis in these cells (Allsopp, 1993). SV kills non-neuronal cells by inducing apoptosis, for example rat AT-3 and BHK cells have lytic SV infections. However, when AT-3 cells expressed recombinant Bcl-2, these cells survived with persistent infection (Levine, 1993). It is not known how Bcl-2 regulates the lytic potential of SV or if Bcl-2 or an analogue is expressed in the adult nervous system. Further investigation is required to determine

if Bcl-2 is upregulated upon virus infection and whether other cellular genes are involved. Neurons are described as immunoprivileged, as their terminally differentiated state necessitates a different immune surveillance to other cells in order to prevent immune mediated apoptosis. Neurons express very low levels MHC and as noted, are refractory to apoptosis. The preferential infection of these cells by SV, is in itself a mechanism for viral persistence (Jackson, 1987; Mauerhoff, 1988).

Evolution of the viruses may play a role in determining persistence. SV was isolated from two groups of persistently infected SCID mice; one group had been treated with anti-SV HI serum. The neurovirulence of the isolates was then tested in weanling BALB/c mice. Viral mutants from both groups of mice were neurovirulent, resulting in 80-100% mortality. All neurovirulent strains contained Gln to His substitution in the E2 envelope glycoprotein. This change was not antibody-induced as mutants occurred in both untreated and anti-SV HI serum treated SCID mice (Levine & Griffin, 1993).

As noted, antibody has been shown to have a role in mediating SV persistence. Humoral immunity is important for protection against viral infection and neutralization of extracellular virus, but clearance of virus from infected cells is thought to be mediated solely by cellular immunity. However, in a SCID mouse model of persistent alphavirus encephalomyelitis, adoptive transfer of anti-SV HI serum resulted in clearance of infectious virus and viral RNA positive cells (as detected by *in situ* hybridisation) from the nervous system, whereas adoptive transfer of sensitized T lymphocytes had no effect on viral replication. Three monoclonal antibodies to two different epitopes on the E2 envelope glycoprotein mediated viral clearance. Treatment of alphavirus-infected, primary cultured rat neurons with these monoclonal antibodies to E2 resulted in decreased viral protein synthesis, followed by gradual termination of mature infectious virion production. Thus, antibody appears to mediate clearance of alphavirus infection from neurons by restricting viral gene expression, and while neurons become persistently infected, no productive virus is released (Levine, 1991; Levine & Griffin, 1992). It has not been demonstrated if antibody clears virus from neurons. HI serum prevents the detection of infectious

virus in SCID mice, but it has not been determined whether this represents sterilising immunity.

Pathogenesis of SFV

SFV infection

In natural infection, following peripheral inoculation, normally by a mosquito, SFV A7(74) infects local cells such as muscle or Langerhans cells before multiplying and spreading to target tissues. A high titre viraemia is detectable by 24 hr (Fazakerley, 1993). SFV appears to infect endothelial cells of the BBB (Soilu-Hanninen, 1994) and crosses this barrier to infect cells of the CNS. The BBB is leaky following CNS infection from about days 3 to 10 post-infection as demonstrated by the presence of albumin and IgG in the CSF fluid (Parsons & Webb, 1982c). Once in the CNS SFV is known to predominantly infect neurons and oligodendrocytes (Pathak, 1983).

Neurovirulence and age related virulence

All strains of SFV are neuroinvasive but they differ in their virulence. L10 is virulent, and in both neonatal and adult mice, perivascular spread of virus from small foci to large areas of infection occurs, and many neurons die. In adult mice infected with virulent strains of SFV, virulence is unaffected and the virus becomes widespread in the CNS, however most infected neurons are not destroyed. Infection with avirulent SFV A7(74) results in restricted replication in the adult mouse CNS, with just small, scattered perivascular foci of infection detectable. Adult mice are able to clear infectious A7(74) virus from the CNS by PID 10. In stark contrast, mice under 12 days of age die from SFV A7(74) infection, virus replication is unrestricted in the CNS and like L10, infection is rapidly fatal (Fazakerley, 1993). This age-related virulence is attributed to the maturity of the cells in the CNS rather than the immune response, as SFV A7(74) infection is not fatal in immunodeficient (SCID, *nu/nu*) adult mice and causes only small foci of infections in their CNS (Oliver & Fazakerley, 1998). That the adult neuron is refractory to apoptosis is not surprising, the death of vital virally infected neurons would not benefit the animal if the neuron is critical for normal brain function. The ability to limit productive infection is also age-related. In adult mice with A7(74) infection administration of

gold compounds (which induce smooth membrane production) converts the focal infection to a fatal pan-encephalitis (Pathak, 1983). The immaturity of neurons in the developing brain offers cells with the physiological capacity to replicate virus and produce viral proteins but also the susceptibility to undergo apoptosis.

Immune response to SFV

SFV infection rapidly induces a type I IFN response and the level of IFN α/β expression correlates with that of the viraemia (Bradish, 1975). Administration of IFN α/β induces an antiviral state locally and systemically, which reduces alphavirus infectivity (Bradish & Titmuss, 1981). MHC expression is induced by IFN in response to infection and during SFV CNS infection MHC class I and blood vessel-associated MHC Class II expression correlates with areas of infection (Morris, 1997). Furthermore, the administration of anti-type I IFN antibodies enhances viral replication, demonstrating the importance of the anti-viral activity of type I IFN (Fauconnier, 1969). However, the most compelling evidence for the protective role of type I IFN in alphavirus infection has come from the use of type I IFN receptor knockout mice (IFNα/βR$^{-/-}$). The type I IFN family is encoded by many genes and is currently unfeasible to knock out. However, the removal of a receptor subunit results in an unresponsive type I IFN system (Muller, 1994). IFNα/βR$^{-/-}$ mice inoculated with SFV succumb to infection within 72hrs. These mice do not die from panencephalitis as occurs in neonates but from multiple organ failure (Figure 4). These results demonstrate the important and necessary role of type I IFN in protection from alphavirus infection (Grieder & Vogel, 1999; Ryman, 2000b; Breakwell & Fazakerley, 2007a).

Another effect of a defective type I IFN system was that the virus was observed in many different tissues, such as the exocrine pancreas, a feature not seen in infection of wild-type mice. It appears removing the type I IFN system reveals the true tropism of alphaviruses (Ryman, 2000a).

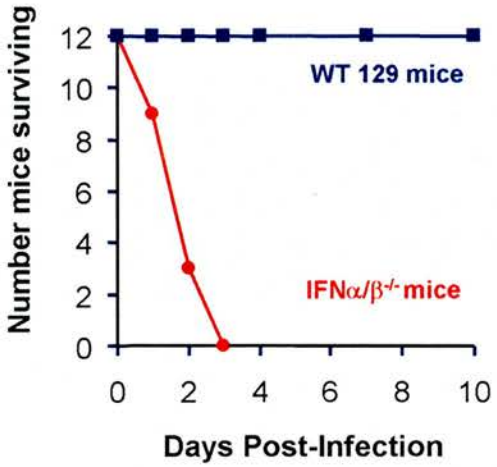

Figure 4. Survival time in WT-129 and IFNα/β-$R^{-/-}$ mice following SFV infection. Data from Fazakerley (Breakwell & Fazakerley, 2007b).

The role of NK cells in alphavirus infection has not been fully examined. It is thought that NK cells infiltrate the CNS early in infection (Hickey, 1999) as indicated by an early peak in lymphocytes. As yet, there is no evidence of either a protective or pathogenic role for NK cells in alphavirus infections (Hirsch, 1981; Griffin & Hess, 1986). The use of Beige mice (an inbred strain naturally deficient in NK cells) did not demonstrate any role for NK cells in alphavirus infection (Fazakerley, 1985).

After peripheral inoculation of alphavirus there is an up-regulation of MHC class I expression on epidermal cells and of class II expression, along with co-stimulatory molecules, on Langerhans cells (Johnston, 1996). Mononuclear cell infiltrates forming perivascular cuffs (T-cells, B-cells and NK cells) are detectable within the CNS from about 3 days post-infection with SFV. The sites of these infiltrates correspond with the areas of infection (Morris, 1997). T-cells of activated phenotypes, and perhaps other cells, can cross the BBB into the CNS and are aided by the up-regulation of adhesion molecules. A preliminary study of SFV encephalitis determined that the ratio of CD4:CD8 is approximately 1:1 in the infected CNS (Trefgarne, 1995); blood normally has a ratio of 2:1. The elevated levels of CD8 T-cells in the CNS suggest they play a role in alphavirus infection.

Oligodendrocytes, the cells that produce myelin in the CNS, are known to be inducible for expression of MHC Class I, and it is possible that infected oligodendrocytes are targeted by CTL lytic mechanisms, resulting in the demyelination seen in SFV. T-cells have been shown to be necessary for demyelination to occur. *Nu/nu* mice that have no T-cells and no Ig class switching develop a persistent CNS infection with SFV (Fazakerley & Webb, 1987; Amor, 1996). The exact role of $CD4^+$ and $CD8^+$ T-cells in CNS immune responses are not known. The only study on the role of T-cells SFV infection was by using antibody depletion of $CD4^+$ and $CD8^+$ T-cells (Subak-Sharpe, 1993). Depletion of $CD8^+$ T-cells prevented demyelinating lesions from forming and removal of both $CD8^+$ and $CD4^+$ subsets completely ablated all pathological changes (Figure 5). This system however was limited in that antibody mediated $CD8^+$ T-cell depletion was temporary and by PID 21, $CD8^+$ T-cells numbers increased resulting in some demyelination. In order to examine the role of $CD8^+$ T-cells alone, infections of $CD8^+$ T-cell knockout mice or use of $CD8^+$ T-cell adoptive transfer is required. The above evidence supports an immunopathogenic role for $CD8^+$ T-cells in alphavirus CNS infection but direct evidence has yet to be shown.

In alphavirus infection, there is clear evidence of immunopathological changes, which raises the question: does the damage caused by the immune response outweigh its benefits? One of the best models in which to attempt to answer this question is SCID mice that are naturally deficient in both B and T cells. SCID mice infected with avirulent SFV A7(74) – die at about 22 days post-infection, with widespread infection including cells in skeletal muscle, myocardium, brain and spinal cord. The cause of death in SCID mice is unknown, but could include a shock-like syndrome or an excessive build-up of virus in the peripheral organs. In the CNS there is very little evidence of inflammation or demyelination, and virally-positive cells are morphologically normal (Amor, 1996). Immunocompetent mice infected with SFV have inflammation and demyelination in the CNS.

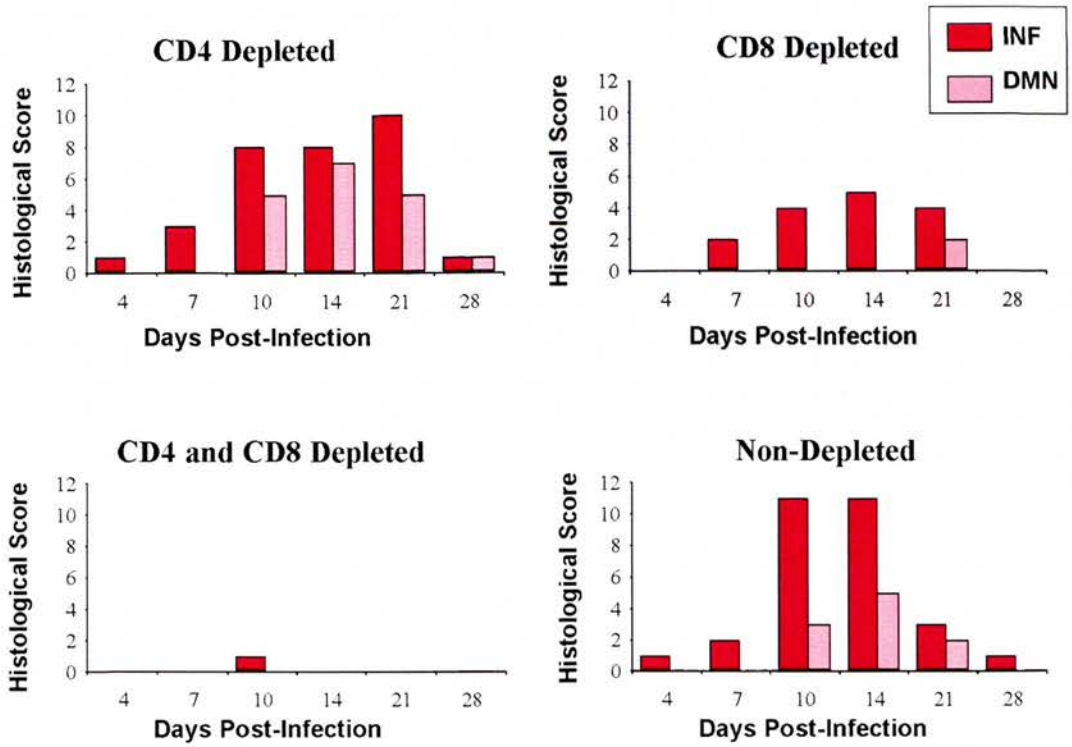

Figure 5. Histological scores from SFV infected mice depleted of $CD8^+$ and/or $CD4^+$ T-cells (Subak-Sharpe, 1993).

$CD8^+$ T-cells also possess non-lytic mechanisms that have an important role in infections of non-renewable cells. $CD8^+$ T-cells are important producers of IFNγ, a potent anti-viral cytokine. IFNγ is produced later in infection than Type I IFN and is an important mediator of MHC upregulation and cellular activation. Binder *et al* used a SV construct that expressed IFNγ, to investigate the antiviral effects of IFNγ. Infection of SCID mice with the SV construct showed reduced brain virus titres and clearance of infectious virus in the spinal cord, compared to persistent infection in SCID mice infected with wild-type SV (Binder & Griffin, 2001d). It could be speculated that the protective potential of $CD8^+$ T-cells in spinal cord appears to come at the price of causing inflammatory demyelination within the CNS.

Following SFV infection there is a rapid neutralizing IgM response which is detected by PID 4. This is followed by an IgG2a (PID 6) and a slow rising IgG1 response (PID 8). From PID 4-10, the BBB is leaky enabling antibody to enter the CNS. Intrathecal antibody production has also been detected. Antibody and plasma cells

are detectable in the CSF and brain for many months after infection (Parsons & Webb, 1982f).

The specificity of antibody appears to be mainly against alphavirus envelope proteins E1 and E2. E2 forms the most distal part of the envelope heterodimers and so is most exposed. There are several neutralising antibodies to this protein. Amino acid changes to E2 affect the efficiency of antibody binding and virulence. The E2 protein is not conserved between different alphaviruses and consequently, antibodies to the E2 protein are not cross-reactive. E1 and capsid are more conserved structural proteins in alphaviruses and so there is cross-reactivity of antibody to E1 between alphaviruses. Although E1 has 7 known epitopes, these are not normally exposed, therefore anti-E1 antibody cannot normally bind to virus. Antibody to capsid is also cross-reactive, but like E1, capsid proteins are not exposed under normal circumstances.

Antibody can clear virus, at least in peripheral (non-CNS) tissues, via the reticuloendothelial system in a complement-dependent manner (Jahrling, 1983). Intraperitoneal (IP) transfer of immune serum protects mice that would normally succumb to infection (Griffin & Johnson, 1977). In immunocompetent mice there is clearance of infectious virus (as determined by plaque assay); in *nu/nu* mice (which only have IgM antibody) there is only peripheral clearance of infectivity, and in SCID and μMT mice which have no antibody, there is neither peripheral nor CNS clearance of infectious virus (Figure 6). When neutralising and non-neutralising IgG was transferred to SCID mice, this reduced brain virus titres and cleared the virus (Amor, 1996). The mechanism by which this operates with non-neutralising antibody is not clear but similar results have been reported with SV (Schmaljohn, 1982). However it has not been demonstrated if antibody alone can bring about 'sterilising' immunity i.e. completely clear virus as detected by infectivity assay and PCR.

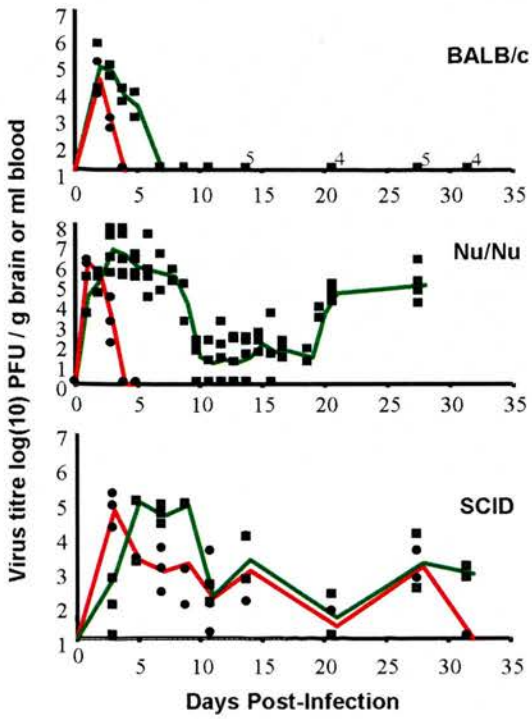

Figure 6. Infectious SFV titres in the blood and CNS of immunocompetent (BALB/c), T-cell deficient (*nu/nu*) and B and T-cell deficient (SCID) mice. Each point represents the sampling of a single animal. The red line indicate mean infectious virus load in the blood, green line represents mean infectious virus load in the brain (Amor, 1996)

While antibody has been shown to be crucial for viral clearance, other work suggests that it may also have an immunopathogenic role. The detection of antibodies to myelin oligodendrocyte glycoprotein and myelin basic protein following SFV infection led to the suggestion antibodies that these autoantibodies had a role in the demyelination process (Smith-Norowitz, 2000). Mokhtarian *et al* observed an increase in B-cells in the recovery period of SFV infection, when demyelination is still occurring, and suggested that antibody takes over from CTLs as mediators of myelin pathology (Mokhtarian, 2003).

Hypothesis

Different specific immune effector mechanisms or combinations of these are responsible for control of infectious virus, eradication of virus and the generation of demyelinating lesions in the brains of SFV infected mice.

Aims

The aim of this thesis is to identify the contributions of different components of the acquired immune response to the control and eradication of virus and to the generation of demyelinating lesions in the CNS of SFV infected mice.

Objectives

- Compare virus clearance (infectious virus and virus RNA) in $CD8^+$ T-cell and $MHCII^{-/-}$ KO mice with wild-type mice
- Transfer immune $CD8^+$ T-cells or immune splenocytes depleted of $CD8^+$ T-cells to SFV infected SCID mice and assess CNS histological changes and virus clearance
- Compare the phenotype of immune cells present in the CNS of SFV infected C57Bl/6 mice and BALB/c mice
- Determine if T-regulatory cells are present in the CNS of SFV infected mice
- Compare virus clearance and CNS histology following SFV infection in $IFN\gamma R^{-/-}$, $perforin^{-/-}$ and Fas^{lpr} mice with wild-type mice
- Determine if recombinant IFNγ can protect SFV infected $IFN\alpha/\beta^{-/-}$ mice
- Raise and characterise high titre HI SFV serum
- Perform passive transfer of HI SFV serum to SFV infected SCID mice

- Examine the ability of HI SFV serum to clear virus (infectious and RNA) from selected tissues of SFV infected SCID mice

Chapter 2:

Materials and Methods

Contents Page

Mice

Mice were infected at 4 and 6 weeks of age (summary of strain details in Table 2). BALB/c, C57Bl/6 and WT129 immunocompetent mice were used. Mice with targeted genetic deletions were also studied: MHC class $II^{-/-}$ mice have a mutation in the A_β complex of the MHC Class II gene, the lack of MHC II expression results in near complete elimination of $CD4^+$ T-cells (Cosgrove, 1991b); Fas^{lpr} mice have a spontaneous mutation in the *Tnfrsf6* lpr gene and are deficient in Fas (Gilkeson, 1991); $Perforin^{-/-}$ mice have a targeted mutation in the gene encoding perforin 1 and are deficient in perforin (Kagi, 1994); IFN-γ receptor $^{-/-}$ ($IFN\gamma R^{-/-}$) mice have a targeted mutation in their IFNγ receptor and cannot respond to IFNγ (Huang, 1993); the CD8 molecule is encoded by two genes Lyt-2 and Lyt-3, CD8 knockout (CD8a) mice have disruption in the Lyt-2 gene and as a result do not have $CD8^+$ T-cells (Fung-Leung, 1991c); SCID mice have a natural mutation in the Prkdc gene, this mutation causes developmental arrest of B and T-cells resulting in a B and T-cell deficiency (Bosma, 1983a). SCID mice were on a C.B-17 genetic background; genetically this is closely related to the BALB/c strain.

All animal experiments were approved by The University of Edinburgh Ethical Review Committee and carried out under the authority of a Home Office Project License. All animals were kept in HEPA filtered cages, with environmental enrichment, 12 hr light dark cycle and food and water *ad libitum*.

Name	Deficiency	Background	Source
IFNα/βR$^{-/-}$	Disruption in IFNα/β receptor	WT-129	In house
MHCII$^{-/-}$	Do not express MHC Class II, no CD4^{+} T-cells	C57Bl/6	In house
CD8a	Disruption of CD8 *Lyt2* gene, no CD8^{+} T-cells	C57Bl/6	Charles River
Fas^{lpr}	Do not express functional FAS protein	C57Bl/6	In house
IFNγR$^{-/-}$	Disruption in IFNγ receptor	WT-129	In house
Perforin $^{-/-}$	Do not make perforin	C57Bl/6	In house
SCID	Mutation in *Prkdc,* no B- or T-cells	CB-17/BALB	In house
WT-129	None		In house
BALB/c	None		Harlan
C57Bl/6	None		In house

Table 2. Summary of mouse strains and models used.

Tissue sampling

Mice were euthanized by an overdose of CO_2 or sampled following perfusion. The chest cavity was opened to expose the heart, the right atrium snipped and heparin applied to prevent clotting. Whole blood was collected and separated by centrifugation, serum collected and stored at –80 °C. Brains were removed and bisected along the midline. One half of each brain was used for virus titration, the other half for histology or virus RNA titre. Heart, pancreas and spleen were also sampled in some experiments. Mice were sacrificed at various days post-inoculation, details given in results chapter.

Perfusion

Mice were deeply anaesthetised by halothane inhalation. The chest cavity was opened to expose the heart, the right atrium snipped and heparin applied to prevent

clotting. An 18-gauge butterfly needle was inserted into the left ventricle and sterile phosphate buffered saline (sPBS) was slowly perfused through the animal until the fluid ran clear.

BHK-21 cell tissue culture conditions

Baby hamster kidney (BHK) 21 cells (clone 13 - ECACC No. 85011433) were maintained in 10% newborn calf serum (NBCS - Invitrogen) and tryptose phosphate broth (Invitrogen) in Glasgow's Minimal Essential Media (10% GMEM, Gibco) supplemented with 1% L-glutamine (2 mM, Merck BDH), 100 u/ml penicillin and 100 μg/ml streptomycin (Merck BDH). At confluence, cells were washed with sPBS and resuspended by 5 min incubation with 5 ml trypsin/EDTA (ethylenediaminetetraacetic acid, Invitrogen). Cells were transferred to a universal and pelleted by centrifugation, 5 min at 400 *g*, and washed in 10% GMEM and pelleted as before. Cells were resuspended in 10 ml of 10% GMEM and counted on a haemocytometer by trypan blue (Sigma) exclusion. T175 flasks were reseeded at $3\text{-}5x10^6$ cells in 40 ml of 10% GMEM. Cells were maintained in this fashion until passage number 30.

Virus and virus titration

The avirulent strain A7(74) of SFV was passaged in BHK-21 cells. Mice were inoculated IP with 0.1 ml or with 0.02 ml IC of sPBS in 0.75% bovine serum albumin (sPBSA) containing $5x10^3$ or $1x10^3$ PFU virus, respectively. BHK-21 cells were used to titrate infectious virus in blood and tissue samples by a standard plaque assay. The BeAn strain of TMEV strain was used to raise irrelevant sera. Mice were inoculated IP with 0.1 ml 0.75% PBSA containing $5x10^3$ PFU of TMEV BeAn. SFV VLPs expressing GFP were kindly provided by Audrey Graham. Approximately 80,000 SFV-GFP VLPs were inoculated IC in 0.02 ml 0.75% PBSA.

Plaque assay

The estimated weight of a half brain was 0.15 g. Brain tissue was homogenised in 1.35 ml sPBS to make a final volume of 1.5 ml (1:10 weight-volume homogenate). Other tissues used in plaque assays were diluted to a 1:10 weight-volume homogenate. Homogenates were diluted in sPBSA to give ten-fold serial dilutions. 6-well plates were seeded with BHK-21 cells at $3x10^5$ per well in 10% GMEM and incubated overnight. When monolayers were sub-confluent, media was removed from the wells and 400 µl of a serial dilution of homogenate/virus was added in duplicate. Plates were placed on a rotator at room temperature in a sealed humid box. After 1 hr, 2 ml of media containing GMEM with 2% NBCS, 1% L-glutamine (2 mM), 100 u/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES and 13% agar was added to 6-well plates and incubated for 48 hr at 37°C in a 5% CO_2. Wells were fixed with neutral buffered formalin (Sigma) for 1 hr before removing agar plug and staining with 0.1% toluidine blue (Sigma). Wells with between 2-70 plaques were counted.

To calculate PFU per ml:

$$\frac{\text{Average no. of plaques}}{\text{Amount of inoculum x dilution factor}}$$

RNA extraction

Half brain samples were submerged in RNAlater (Sigma), an RNA stabilization reagent which inhibits the activity of RNases and preserves RNA in tissues. Tissues were removed from RNAlater and 80–100 mg of tissue cut for homogenisation. Tissues were homogenised in an RNase treated glass homogeniser or disposable 1.5 ml plastic homogeniser (Anachem). RNA was extracted using the RNeasy Lipid kit (Qiagen) according to manufacturer's instructions with optional on-column DNase step. RNA was stored at -80°C in RNase/DNase free distilled water. RNA quantity was determined by spectrophotometer (NanoDrop® when available) and was analysed for quantity

RNA quality assessment

The quality on the RNA was analysed using the Agilent RNA 6000 Nano Assay.

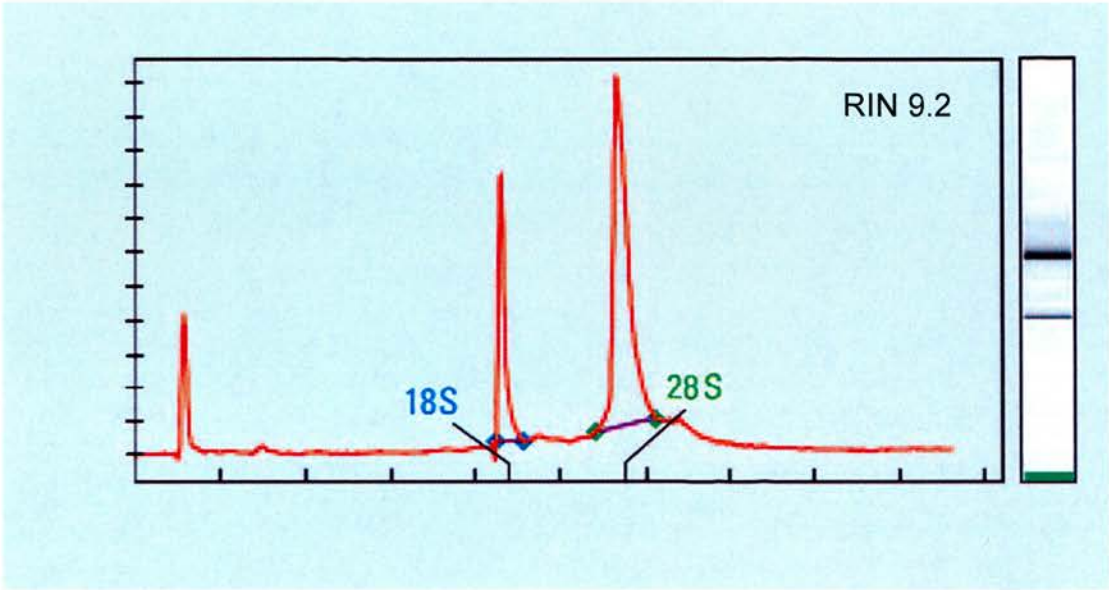

Figure 7. Electropherogram and gel-like image of RNA band from Agilent 2100 Bioanalyser. On electropherogram two large peaks represent 18S and 28S ribosomal RNA peaks and two bands on gel image.

The Agilent 2100 Bioanalyser uses a combination of microfluidics, capillary electrophoresis, and fluorescent dye that binds to nucleic acids to evaluate both RNA concentration and integrity. Size and mass information is provided by the fluorescence of RNA molecules as they move through the channels of the chip. The instrument software automatically compares the peak areas from unknown RNA samples to the combined area of the six RNA 6000 Ladder RNA peaks to determine the concentration of the unknown samples. The RNA integrity number (RIN) software extension tool measures RNA quality and grades it on a quantitative scale of 1 to 10. The RIN software algorithm was developed from samples including approximately 1300 total RNA samples from various tissues from three mammalian species (human, mouse and rat), all with varying levels of integrity. Sample integrity is determined by the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products. This enables interpretation of an electropherogram, comparison of samples and repeatability of experiments. Using

this system the RIN of all samples was determined and only those of greater than 7.5 were used.

Plasmid 'standard' preparation

The PCR standard was prepared from a pGEM plasmid containing the SFV structural genes. This plasmid, pSFV-1 had a size of 8194 bp. pSFV-1 had been previously transformed into DH5α (Invitrogen) *Escherichia coli*. The transformed bacteria were plated onto Luria-Bertani (LB) ampicillin plates (15% agar). Colonies were picked the next day and grown overnight in 5 ml of LB (amp) on a shaker. Plasmid DNA was purified with QIAprep miniprep kits (Qiagen).

Linearisation

The construct had previously been confirmed by DNA sequencing but to check plasmid identity, pSFV-1 was linearised with EcoRI restriction enzyme. 0.5 μg of plasmid DNA was added to 2 μl EcoRI buffer, 2 μl 10X BSA, 1 μl EcoRI and made up to 20 μl with nuclease free H_2O. The plasmid-restriction enzyme mix was heated in water bath at 37°C for 1 hr and then run on a 1% agarose gel with ethidium bromide (0.5 μg/ml).

Following confirmation of its identity by the pSFV-1 plasmid was linearised with Spe1. 2 μg plasmid were added to 2 μl buffer 2, 2 μl 10X BSA, 1 μl Spe1 and made up to 20 μl volume with nuclease free water H_2O. The plasmid-restriction enzyme mix was heated in water bath at 37°C for 2 hr. Linearised DNA was purified with Jet Quick (Genomed) and resuspended in 30 μl of water pre-heated to 65°C. The linearised plasmid was then run on a 1% agarose gel with ethidium bromide (0.5 μg/mL).

In vitro transcription

RNA was synthesized from linearised pSFV-1 plasmid using the following reaction mix:

Spe 1 cut plasmid	25 µl
10X SP6 buffer	5 µl
rNTP mix (10 mM ATP, CTP & UTP, 5 mM GTP)	5 µl
H_2O	7.5 µl
Recombinant RNasin Ribonuclease Inhitibor (60 units)	1.5 µl
SP6 RNA polymerase (50 U/µl)	1 µl
Total Volume	**50 µl**

The reaction mixture was incubated at 37°C for 1 hr and then 65°C for 20 min before being cleaned up on an RNeasy kit column. RNA was suspended in 30 µl of H_2O. To quantify and assess the quality of the RNA, the *in vitro* transcript was run on a 0.7% agarose gel and quantified by both spectrophotometer and Agilent Bioanalyser.

First strand complementary (c) DNA synthesis

In 0.2 ml thin-walled PCR tubes (Axygen, UK) the following were added: 1 µl Oligo $(dT)_{12\text{-}18}$ primer (Invitrogen), 5 µg total RNA, 1 µl 10 mM dNTPs (Promega) and DNase/RNase free dH_2O to make a final volume of 12 µl. An express thermohybaid cycler (Hybaid, USA) was used to heat samples for 5 min at 65°C, before chilling tubes on ice for at least 1 min. The following were added to the tubes (on ice); 4 µl 5X first strand buffer (Invitrogen), 2 µl 0.1 M DTT (Invitrogen), 1 µl RNasin Recombinant Ribonuclease Inhibitor (Promega) and tubes were incubated at 42°C for 2 min. After incubation 1 µl of superscript II RNase-H reverse transcriptase enzyme (Invitrogen) was added and tubes incubated at 50 min at 42°C. Samples were inactivated by heating at 70°C for 15 min before storage at –20°C.

Real Time – quantitative PCR (q-PCR)

The q-PCR protocol used was developed in the Fazakerley laboratory (Fragkoudis, 2002; McKimmie, 2005). Synthesised cDNA diluted 1:5 in nuclease-free water was amplified with primers targeting a 173 base pair fragment of the E1 structural gene of SFV (Table 3) using a Rotor-Gene Version 5 PCR machine (Corbett Research). PCR reactions were carried out in 0.1 ml PCR tubes (Corbett Research). The final volume of the reaction mixture was 20 µl which contained: 0.4 µl of both primers (50 pM) and dNTPs, 2.8 µl $MgCl_2$ (25 mM), 2 µl PCR Buffer, 11.15 µl DNase/RNase free distilled water, 0.7 µl of 1:100 diluted SYBR Green (Biogene), 0.15 µl Taq Faststart (Roche) and 2 µl sample cDNA. Non template controls included water instead of sample cDNA. An initial denaturation step of 95°C for 10 min was followed by 40 cycles of: 30 sec at 95°C, 20 sec at 62°C and 20 sec at 72°C. A standard curve was generated from a series of 10-fold dilutions pSFV-1 of known concentration. A melt curve was generated following the completion of the PCR amplification by increasing the temperature of the samples gradually. This results in the dissociation of the SYBR Green I from the amplicon and a consequent decrease in fluorescence. The characteristic melting peak of the amplicon allows it be distinguished from artefacts. Analysis was carried out using rotorgene software. Samples were normalised against total RNA as measured by the Agilent RNA 6000 Nano Assay and spectrophotometer (NanoDrop® for later experiments). In earlier experiments when the NanoDrop® was not available samples were also checked for levels of the housekeeping gene β-actin. cDNA was amplified with β-actin primers (Table 3), targeting a 202 bp of β-actin gene, using the same reaction mixture and reaction conditions as before. Data from SFV q-PCR was normalised against the levels of β-actin expressed. In all instances where this calculation was carried out there was no change in statistical significance between groups. Therefore measuring the RNA by spectrophotometer and normalising to total RNA was sufficient. The results of the q-PCR are represented as 'SFV RNA copies/5 µg total RNA'. As a 1/50 dilution of the product of the first strand cDNA synthesis reaction (to which 5 µg total RNA was added) was used per q-PCR reaction, the SFV RNA copies/5 µg

total RNA was determined by taking the mean SFV RNA copies for a sample run by q-PCR and multiplying by 50.

Primer Name	Primer Sequence
SFV E1 forward	5'-CGCATCACCTTCTTTTGTG-3'
SFV E1 reverse	5'-CCAGACCACCCGAGATTTT-3'
β-actin forward	5'-CGTTGACATCCGTAAAGACC-3'
β-actin reverse	5'-CTGGAAGGTGGACAGTGAG-3'

Table 3. Details of nucleotide sequences of primers used.

Production of hyperimmune (HI) serum

Forty BALB/c mice (mixed sex) age 4-6 weeks were inoculated (day 0) with $5x10^3$ PFU of SFV A7(74) and boosted with repeat inoculations at 14 and 21 days. As a specificity control, 40 BALB/c mice were inoculated with $5x10^3$ PFU of BeAn TMEV strain. Both groups of mice were sampled at day 28 and sera collected.

Enzyme Linked Immunosorbant Assay (ELISA)

Detection of anti-SFV immunoglobulin

Mice were bled by cardiac puncture. Heparinised blood was separated by centrifugation, plasma pooled and stored at –20°. Microtitre plates (4 HBX, Thermo Electron Corp.) were coated overnight at 4°C with band purified SFV A7(74) diluted in $NaHCO_3$ pH 9.6 buffer at 1:800 dilution. A diluent volume of 100 µl was chosen as this gave the most consistent results. All samples were assayed in triplicate. Plates were washed 3 times with PBS and 0.05% Tween 20 (PBST - Sigma) and blocked with neat CAS block (Sigma) for 10 min at room temperature. Diluent for serum, secondary antibody and tertiary antibody was 0.8% CAS block and 0.8% rabbit serum in PBST. Plates were washed as before, diluted serum samples were added along with relevant controls: no serum; irrelevant serum and immune serum. The

microtitre plate with sera was incubated at room temperature for 90 minutes and then washed as before. Horseradish peroxidase (HRP) conjugated secondary antibody (A-4416 – full details of antibodies in Table 4) was added at a 1:500 dilution and incubated for 2 hr at room temperature. Plates were then washed as before. Colour development was observed by adding 100 µl of tetramethyl benzidine (Sigma) substrate for 15 min, development was stopped with 0.5 M hydrochloric acid and plates were read at 450 nm on an microplate reader (Model MRX, Dynex Technologies).

Isotyping of serum

As before, plates were coated with virus and blocked with neat CAS block. Diluent for serum, secondary antibody and tertiary antibody was 0.8% CAS block and 0.8% rabbit serum in PBST. Bound test antibodies were detected with secondary antibodies to individual immunoglobulin isotypes (incubation 30 min). An HRP conjugated tertiary antibody (A-9917) diluted at 1:5000 was used to detect bound isotype specific antibodies (A-9917, 15 min at room temperature). The reaction was developed with TMB as detailed previously.

Isotype	Specificity	Dilution	Source	Cat. Number
Secondary Ab				
Gt.IgG [HRP]	Mouse IgG	1:500	Sigma	A-4416
Gt.IgG	Mouse IgM	1:1000	Sigma	M-6157
Gt.IgG	Mouse IgA	1:1000	Sigma	M-6032
Gt.IgG	Mouse IgG1	1:1000	Sigma	M-5532
Gt.IgG	Mouse IgG2a	1:1000	Sigma	M-5657
Gt.IgG	Mouse IgG2b	1:1000	Sigma	M-5782
Gt.IgG	Mouse IgG3	1:1000	Sigma	M-5907
Tertiary Ab				
Rb.IgG [HRP]	Goat IgG	1:5000	Sigma	A-9917

Table 4. Antibodies used for ELISA development. **Ab** – antibody, **Gt** – Goat, **Rb** - Rabbit, **HRP** – horse radish peroxidase.

Plaque reduction neutralisation assay (PRNA)

A 96 well plate was seeded with $1.5x10^4$ BHK cells/well in 100 µl of 10% GMEM and incubated overnight at 37°C in 5% CO_2. Two-fold dilutions of sera were made starting at 1:10 dilution. Sera were diluted in 50 µl 2% GMEM containing 10% guinea pig complement. Each sample was tested in triplicate. 50 µl of virus (100 PFU/well) were added to sera dilutions and incubated at 37°C in 5% CO_2 for 1 hr. Controls included normal mouse serum, no serum (virus only) and no virus/no sera control. Media was removed from the BHK monolayer and serum-virus mix (100 µl) added and incubated for 48hrs. The monolayer was fixed with neutral buffered formalin for 1 hr and stained with toluidine blue for 30 min. Monolayers were washed and wells examined for cytopathic effect. The neutralization titre was taken as reciprocal of the highest serum dilution that inhibits cytopathic effect.

Histology

After immersion, fixation in 10% neutral buffered formalin for 48 hr, tissues were processed for paraffin wax embedding. Paraffin embedded slides were soaked in clearene (Surgipath) for 2 x 7 min, before soaking in 100% ethanol followed by 95% ethanol for 5 min each. Slides were incubated in 0.1% luxol fast blue (Sigma) overnight at 50°C in a sealed humid chamber. Slides were rehydrated through a series of graded alcohols (95, 70, 50 and 30% ethanol) for 1 min each, washed in dH_2O, destained briefly in 0.05% lithium carbonate and 70% ethanol, stained in 0.1% Cresyl Violet Acetate (Sigma), dehydrated in ethanol (30%, 50%, 70%, 95% and 100%) and cleared in clearene for 15 min before mounting in vectamount (Vector).

Brain sections were scored for inflammation according to the following criteria: 0, no inflammatory cell infiltrate; 1, a few inflammatory cells; 2, numerous scattered inflammatory cells with an occasional perivascular cuff; 3, many perivascular cuffs; 4 many perivascular cuffs with extensive perivascular infiltration and large clusters of inflammatory cells. The extent of demyelination was determined according to the following scoring system: 0, no demyelination; 1, a small area of myelin loss; 2, >1 small area of myelin loss; 3, several small areas of myelin loss or a few medium sized lesions; 4, several medium sized lesions or one or more large lesions. Micro-cystic change (MCC) was scored as following; 0, no MCC; 1, small area of MCC; 2, a few areas of MCC; 3, several areas of MCC; 4, many and larger areas of MCC.

Examples of uninfected and SFV infected brain tissue stained with luxol fast blue are shown in Figure 8.

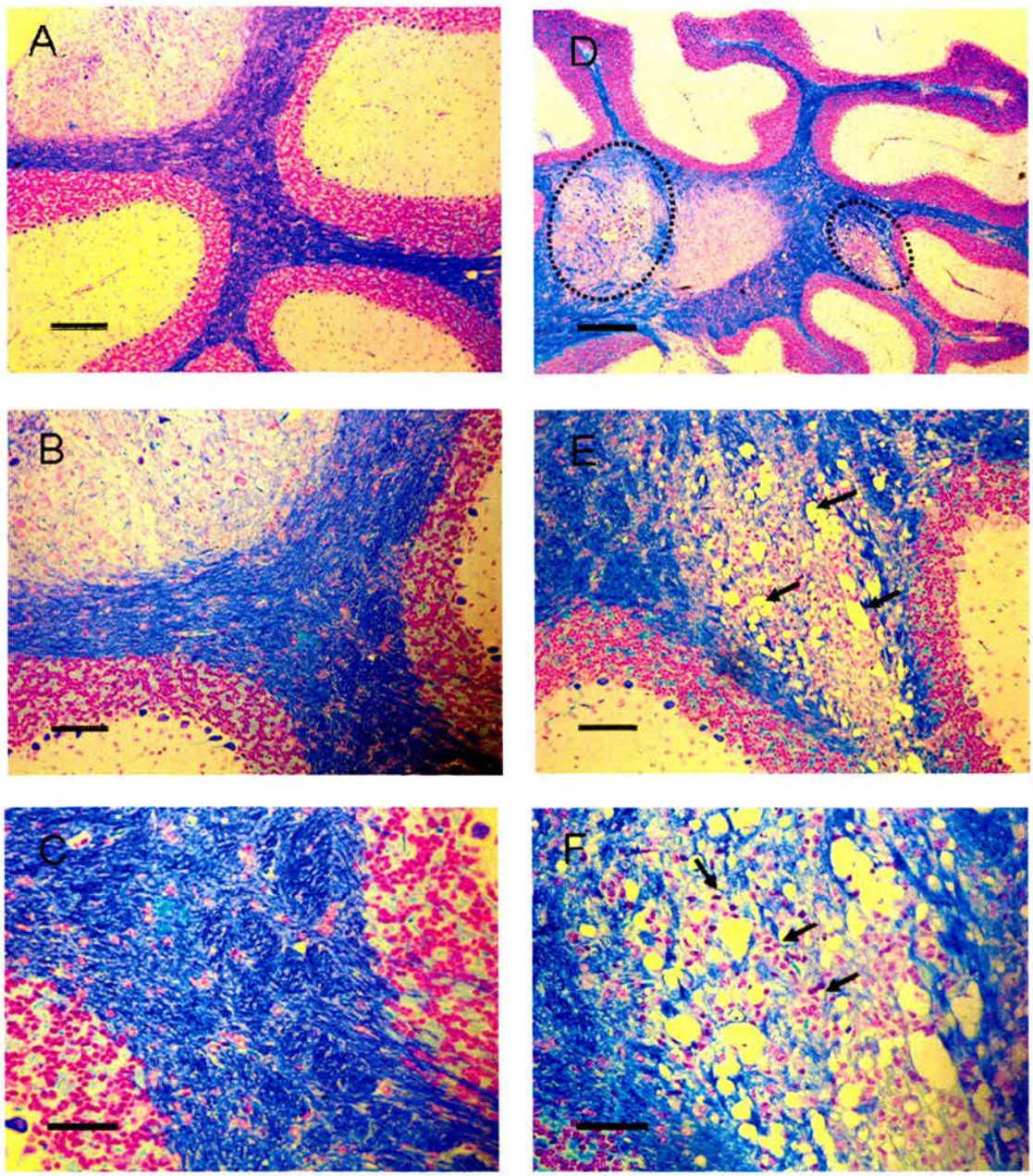

Figure 8. LFB and CFV stained CNS of BALB/c mice. Areas of the cerebellum from uninfected mice (**A, B** & **C**) and infected mice (**D, E** and **F**) sampled 14 days after SFV A7(74) infection. Demyelination (dashed circles - **D**), microcystic change (arrows - **E**) and mononuclear cell infiltrates (arrows - **F**) are visible. Bars represent - 400 microns **D,** 200 microns **A**, 50 microns **B** & **E** and 20 microns **C** & **F**.

Immunostaining for SFV proteins

Slides with paraffin tissue sections were soaked in clearene (Surgipath) for 2 x 7 min, 100% ethanol for 5 min, 0.3% H_2O_2 for 10 min and then passed through an alcohol gradient 95 – 30% ethanol for 1 min each before equilibrating in 0.85% NaCl for 5 min. Slides were then heated in the microwave in antigen unmasking solution (Vector) at 750 W for 12 min and allowed to cool in the same solution for 20 min. Proteinase K was then added, 37°C for 15 min. All incubations were performed in a humid chamber. To stop the reaction slides were soaked in EDTA/glycine/PBS for 5 min. After two 5 min washes in PBS, 10% normal goat serum (NGS) was added for 30 min to block any reactivity to goat antibodies. After decanting the NGS from the sections Rabbit anti-SFV, antibody was added (1:400 dilution, in 2% NGS in PBS) for 1.5 h. Slides were washed three times in PBS for 5 min. Biotinylated Goat anti-Rabbit IgG (1:500 dilution in 2% NGS in PBS) was then added to slides for 1 h. Slides were washed as before. Slides were incubated with VECTASTAIN® ABC kit (Vector Laboratories) for 30 min and again washed. DAB (Diaminobenzidine) solution (Sigma) was added until the sections turn light brown by eye (approx 20 - 40 sec for SFV staining). This was then washed off in distilled water. Slides were counterstained briefly with haematoxylin (Vector 'QS') and rinsed in water. Slides were dehydrated through an alcohol gradient, 30 – 100% Ethanol for 30 sec each, and soaked in Clearene for 15 min before mounting.

Adoptive transfer of antibody

SCID mice, age 4-6 week, were inoculated IP with $5x10^3$ PFU SFV A7(74). At 4 days post-infection, SCID mice received a passive transfer of 0.1 ml of HI SFV serum; control groups received either 0.1 ml of TMEV d28 serum or PBS. Passive transfers were repeated every 3 days until PID 19. At post-infection day 21, 42 and 63 mice were perfused under terminal anesthesia and sampled.

Isolation of mouse splenocytes

After removal mouse spleens were placed in a bijou bottles containing 5 ml of RPMI media (on ice). Spleens were transferred to a petri dish containing 2ml of RPMI media with 10% foetal calf serum, 1% L-glutamine (2 mM), 100 u/ml penicillin, 100 μg/ml streptomycin and 25 mM HEPES (10% RPMI). Any fat was removed from the spleens and splenocytes teased out with a scalpel. The cell preparation was transferred to universals and centrifuged at 400 *g* for 5min. The supernatant was removed and the cells resuspended. To lyse erythrocytes, 1 ml of sterile water was added, followed immediately by 9 ml of PBS with thorough mixing. Debris was allowed to settle, the cell suspension removed to a universal, without disturbing debris. Cells were spun as before, supernatant removed and resuspended in 5 ml 10% RPMI.

Isolation of lymphocytes from mouse brain

The mouse was perfused with sPBS. The brain was removed and placed in a bijou bottle with 1 ml RPMI (on ice). The tissue was cut into small pieces and 500 μl of Collagenase Type IV (8 mg/ml, Worthington Biochemicals, NJ) and 100 μl of DNase (10mg/ml, Sigma) was added, and incubated at 37°C for 30 min. The tissue was triturated through a 23 gauge needle to make a single cell suspension. The cell suspension was transferred to a 20 ml universal. The brain homogenate was centrifuged (5 min 500 *g*) to pellet the cells. Percoll (Sigma) was diluted with 10X PBS to make a 90% solution. All subsequent dilutions were made using 1X PBS. The cell pellet was resuspended in 4-5 ml of 30% Percoll and transferred to a 15 ml conical tube (Falcon), and carefully underlayed with 2 ml of 70% Percoll. The brain suspension was spun (850 *g* for 20 min). The top layer of myelin debris was pipetted off. Mononuclear cells, which band at the interface between the 30% and 70% Percoll layers, were removed and transferred to a universal with 5 volumes 10% RPMI. Cells were centrifuged (10 min at 680 *g*) and the supernatant removed and the cells resuspended in 200 μl of 10% RPMI and counted.

Separation of splenocytes by MACS

Cells were separated on a LS column using a VarioMACS™ separator according to the manufacturer's instructions (Miltenyi Biotec). Briefly, two universals containing $3.5x10^8$ splenocytes were incubated with 350 µL of CD8a Microbeads for 15 min on ice, washed in sPBSA (0.5% BSA) and magnetically separated on a prepared MACS column. Flow-through cells were collected and saved. After the column was removed from the magnet $CD8^+$ cells were eluted and collected in a fresh tube. Flow-through cells were spun down, resuspended in 2.5 ml of sPBSA and passed through a second freshly prepared column. Flow-through cells were collected and the column taken off the magnet and $CD8^+$ cells collected in a fresh tube. Both sets of flow-through cells were pooled and both sets of $CD8^+$ cells were pooled. Cells were spun down and counted and $1x10^6$ cells aliquots taken for fluorescent-activated cell sorting (FACS) analysis.

Staining of lymphocytes for FACS analysis

Up to $1x10^6$ cells were added per FACS tube (Falcon) and pelleted. 25 µl of diluted antibody (concentration determined by titration prior to use) was added to cells and incubated for 20 min at 4°C in the dark (Details Table 5). Cells were washed twice by adding 2 ml PBS (1% foetal calf serum) and spun at 200 *g* for 5 min, the supernatant was then poured off and the tubes blotted to remove excess liquid. Cells were resuspended by tapping. Cells were fixed by addition of 400 µl of 1% Formal Saline to each tube. Samples were stored in the dark at 4°C. Data were acquired on a FACSCaliber cytometer (BD Biosciences) and analyzed by using FLOWJO software (Treestar). Gates were set using previously established forward and side scatter parameters which were based on mouse mononuclear cells extracted from the brain of the EAE mouse model (Steve Anderton, University of Edinburgh, Figure 9). Gates were verified by back-gating to show the position of cells positive for monocyte markers such as CD45/Mac1 (macrophages) and CD3/CD4 ($CD4^+$ T-cells).

Isotype	Specificity	Dilution	Source	Code
Rat IgG2a κ	Mouse CD4 [PE]	1:400	BD Biosciences	553048
Rat IgG2a κ	Mouse CD8 [FITC]	1:100	BD Biosciences	553030
Rat IgM κ	Mouse pan-NK [APC]	1:100	eBioscience	17-5671
Rat IgG2b κ	Mouse CD45 [FITC]	1:200	eBioscience	11-0451
Rat IgG2b κ	Mouse CD11b [APC]	1:500	eBioscience	17-0112
Rat IgG2a κ	Mouse CD19 [PE]	1:400	BD Biosciences	557399
Rat IgG1 λ	Mouse CD25 [APC]	1:200	eBioscience	17-0251
Hamster IgG	Mouse CD3e [FITC]	1:200	eBioscience	11-0031
Rat IgG2a	Mouse FoxP3 [FITC]	1:50	eBioscience	11-5773

Table 5. Antibody details and dilutions used for FACS staining. **PE** - phycoerthrin; **FITC** - fluorescein isothiocyanate; **APC** – allophycocyanin.

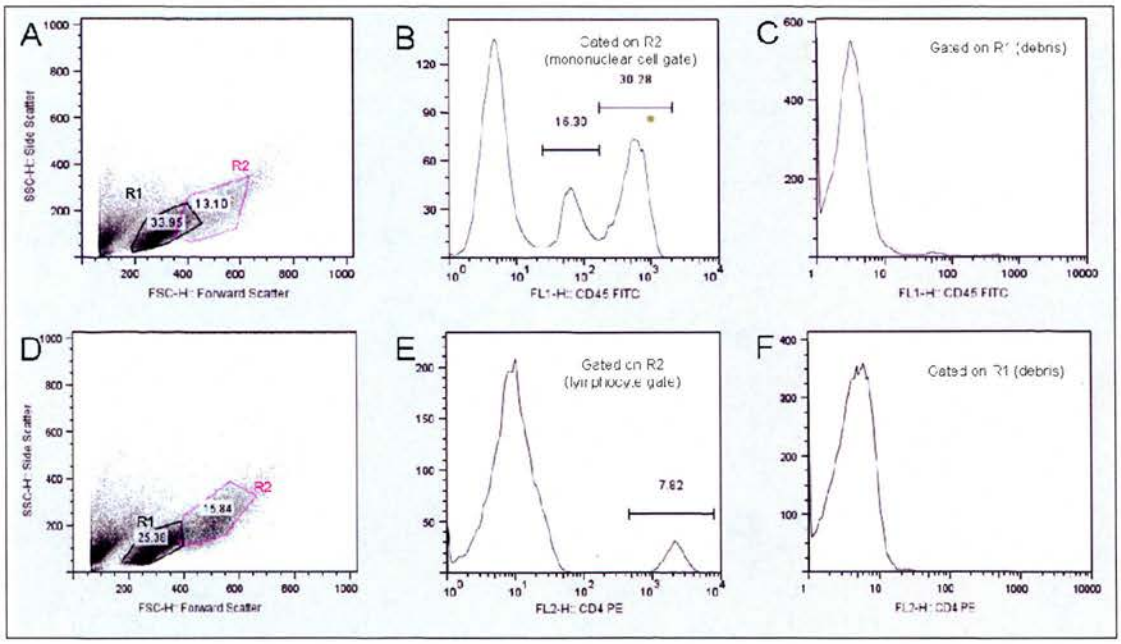

Figure 9. Examples of gates used to analyse mononuclear cells from the mouse brain. **A** Forward and side scatter plot of mononuclear cells isolated from the brain, gate R1 shows debris and gate R2 was used to select macrophages/microglia; **B** CD45 expression in macrophages/microglia selected in gate R2; **C** low or no expression of CD45 in gate R1 (debris); **D** Forward and side scatter plot of mononuclear cells isolated from the brain, gate R1 shows debris and gate R2 was used to select lymphocytes; **E** CD4 expression in lymphocytes selected in gate R2 and **F** absence of CD4 staining in gate R1 (debris). Bars indicate percentage positive cells

Intracellular staining of lymphocytes for FoxP3

Mouse lymphocytes were intracellularly stained for FoxP3 (Table 5) using the Foxp3 Staining Buffer Set (cat. 00-5523) following the manufacturer's instructions (eBioscience). Briefly, $1x10^6$ cells were added to each FACS tube. Cells were stained for surface molecules CD4, CD8 and CD25 following the Staining of lymphocytes for FACS analysis protocol. Cells were washed in cold PBS, pelleted, resuspended and 1 ml of freshly prepared Fixation/Permeabilization working solution (eBioscience) was added to each sample. Cells were incubated at 4°C for at least 30 min in the dark. Cells were washed twice by adding 2 ml 1X Permeabilization buffer (eBioscience) followed by centrifugation and decanting of supernatant. 50 µl of diluted fluorochrome conjugated antiFoxp3 antibody or isotype control (concentration determined by titration prior to use by Steve Anderton) in 1X Permeabilization buffer was added to cells and incubated at 4°C for at least 30 minutes in the dark. Cells were washed with twice with 2 ml 1X Permeabilization buffer before being resuspended in 100 µl volume Flow Cytometry Staining Buffer (eBioscience). Data were acquired on a FACSCaliber cytometer (BD Biosciences) and analyzed by using FLOWJO software (Treestar).

Adoptive transfer of lymphocytes

Magnetically sorted splenocytes from PID 7 SFV infected BALB/c mice were transferred to recipient PID 7 SFV infected SCID mice (Details Table 6). A 1:1 ratio for cell transfer was used. For example, a mean of $4.5x10^6$ $CD8^+$ splenocytes were collected from each BALB/c mouse so this number of cells was transferred IP into each recipient mouse. For IC inoculation a maximum of $1x10^6$ $CD8^+$ splenocytes in a maximum volume of 20 µl was transferred into each recipient SCID mouse. At PID 14 all mice were sampled.

Group	Number of cells and transfer volume
CD8 IP	≈ **4.5x10^6** $CD8^+$ cells in **0.3ml** sPBSA (x5)
CD8 IC	≤ **1x10^6** $CD8^+$ cell in **20μL** sPBSA (x5)
F/T	≈ **4.5x10^7** flow-through cells **250μL** sPBSA (x5)
PBS	**0.3ml** of sPBSA (x5)

Table 6. Details of number, type and volumes of magnetically sorted splenocytes adoptively transferred. **CD8 IP** - $CD8^+$ selected splenocytes by IP inoculation; **CD8 IC** - $CD8^+$ selected splenocytes by IC inoculation; **F/T** - flow-through cells by IP inoculation ; **PBS** - PBS only

Chapter 3:

The role of $CD4^+$ and $CD8^+$ T-cells in SFV infection

Contents Page

Introduction

Avirulent SFV A7(74) infection causes a transient CNS infection and clearance of virus is co-incident with the appearance of demyelinating lesion in susceptible mouse strains (Suckling, 1978; Kelly, 1982). On a BALB/c background, SCID mice, naturally deficient in B and T cells and *nu/nu* mice, naturally deficient in T-cells, cannot clear SFV infection in the CNS but do not develop demyelinating lesions (Fazakerley, 1983). In the CNS of SCID and *nu/nu* mice virally positive cells are morphologically normal, indicating an immune aetiology for demyelination in SFV infection. Reconstitution of *nu/nu* mice with splenocytes from immunocompetent mice restores demyelinating disease (Fazakerley & Webb, 1987), while depletion of $CD8^+$ T-cells from immunocompetent BALB/c mice prevents the formation of demyelinating lesions (Subak-Sharpe, 1993).

Virus clearance and pathological changes attributable to T-cells are observed in a number of CNS viral infections. The pathogenicity of an immune response can outweigh the benefits, for example the $CD8^+$ T-cell response induced during LCMV infection causes a fatal choriomenigitis before virus is cleared (Baenziger, 1986b; Dixon, 1987c). Whereas following TMEV infection, the $CD8^+$ lymphocyte response in C57Bl/6 mice efficiently clears virus with the help of $CD4^+$ T-cells without pathological changes, the same virus in SJL mice results in a poor immune response, slow virus clearance and immune mediated demyelinating lesions (Rodriguez, 1991). It is evident that T-cells can play a role in both the clearance and the pathology of CNS viral infections.

To elucidate the separate roles of $CD4^+$ and $CD8^+$ T-cells in SFV infection, mice deficient in either $CD4^+$ or $CD8^+$ lymphocytes were infected with SFV A7(74). Subsequent CNS neuropathology and ability to clear infectious virus and virus RNA were compared in immunodeficient mice and wild-type mice. The two strains of mice used were, mice with a mutation of the CD8 alpha (Lyt-2) chain (CD8a) that do not have $CD8^+$ lymphocytes and mice with a disruption in the MHC class II Aβ gene ($MHCII^{-/-}$) which do not express MHC class II and have no $CD4^+$ T-cells, both strains were on a C57Bl/6 background (Grusby, 1991; Cosgrove, 1991a).

During the course of investigation into the pathological changes induced by SFV it was observed that mice on a C57Bl/6 genetic background showed little or no

demyelination or microcystic change in the CNS following infection. To determine the possible cause of the lack of CNS pathology in C57Bl/6 mice, it was investigated whether cells in the CNS were permissive to infection using SFV virus-like particles (VLPs) expressing GFP to visualise infected cells.

To get around the minimal demyelination in mice with a C57Bl/6 genetic background, adoptive transfers of $CD8^+$ immune splenocytes to SFV infected SCID mice on the BALB/c background which is susceptible to demyelination, were undertaken specifically to analyse the role of $CD8^+$ T-cells in demyelination. To further examine the cellular immune response in the CNS, the phenotype of inflammatory cells in the CNS during infection was examined by FACS.

Objectives

- Compare virus clearance (infectious and RNA) in $MHCII^{-/-}$ mice with wild-type mice
- Compare virus clearance (infectious and RNA) in $CD8^+$ T-cell KO mice with wild-type mice
- Transfer immune $CD8^+$ splenocytes or immune splenocytes depleted of $CD8^+$ cells to SFV infected SCID mice
- Assess neuropathological changes in brains of SCID mice that receive immune splenocyte transfers
- Determine virus clearance in SCID mice that receive immune splenocyte transfers
- Determine if SFV virus-like particles can infect cells in the CNS of C57Bl/6 mice
- Examine the phenotype of cells present in the CNS of SFV infected C57Bl/6 mice
- Compare the phenotype of cells present in the CNS of SFV infected C57Bl/6 mice and BALB/c mice

- Determine if T-regulatory cells are present in the CNS of SFV infected mice

Results

MHC II$^{-/-}$ mice do not clear infectious virus

To examine the role of $CD4^+$ T-cells in virus clearance, C57Bl/6 MHC $II^{-/-}$ mice (n=20) and wild-type C57Bl/6 mice (n=20) aged 4-6 weeks (mixed sexes) were infected with SFV A7(74) and sampled between PID 3 and 21. MHC $II^{-/-}$ mice showed increased susceptibility to SFV infection, with 30% dying prior to sampling. The titres of infectious virus in the brains of both mouse strains were examined between PID 3 and 10 (Figure 10A). Infectious virus was detectable in all (4/4) MHC $II^{-/-}$ mice at PID 3 and 7 but was not detectable in any mice (0/4) by PID 10. Virus was detectable in 100% (4/4) C57Bl/6 mice at PID 3, 75% (3/4) at PID 7 and in none (0/4) at PID 10. MHC $II^{-/-}$ mice showed slightly higher (not significant) levels of infectious virus compared to C57Bl/6 mice at PID 3 (Log_{10} 4.5 and 4.3, respectively) and PID 7 (Log_{10} 5 and 3.5, respectively). Amor *et al* demonstrated that in *nu/nu* mice virus clearance was observed at PID 10 but infectious virus was once again detected at later time points (Amor, 1996). In order to determine if the virus clearance observed in $MHCII^{-/-}$ mice was enduring, further mice were infected and sampled at a later time point. A group of $MHCII^{-/-}$ mice (n=10) and wild-type mice (n=10) were infected and sampled at PID 28 (Figure 10B). 66% (2/3) of $MHCII^{-/-}$ mice had infectious virus detectable at PID 28 compared with 0% (0/4) of C57Bl/6 mice. During the course of this experiment mouse hepatitis virus (MHV) was detected in the animal facility and, although unlikely, it is possible that the mice used in this study were infected. This could have affected the results. The MHV outbreak precluded any further studies.

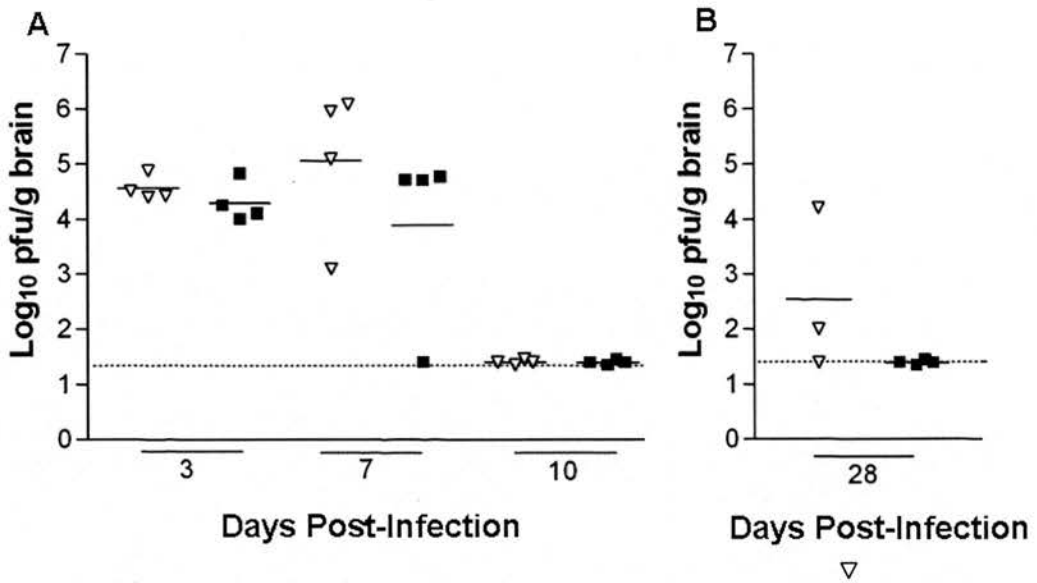

Figure 10. Infectious virus titres in the brains of SFV infected MHCII-/- mice (▽) and C57Bl/6 mice (■) at PID 3-10 (A) and PID 28(B). Horizontal lines indicate the mean of the groups and the dashed line indicates the limit of detection.

Levels of SFV RNA were determined at days 14, 21 and 28 in SFV infected MHCII$^{-/-}$ and C57Bl/6 mice (Figure 11). At PID 14, the virus RNA levels were similar (Log_{10} 4.8 and 5.3, respectively). To allow statistical analysis the data from the two later time points were combined, virus RNA levels were significantly higher in MHC II$^{-/-}$mice ($p=0.004$ by Mann-Whitney test) at later time points compared to wild-type mice.

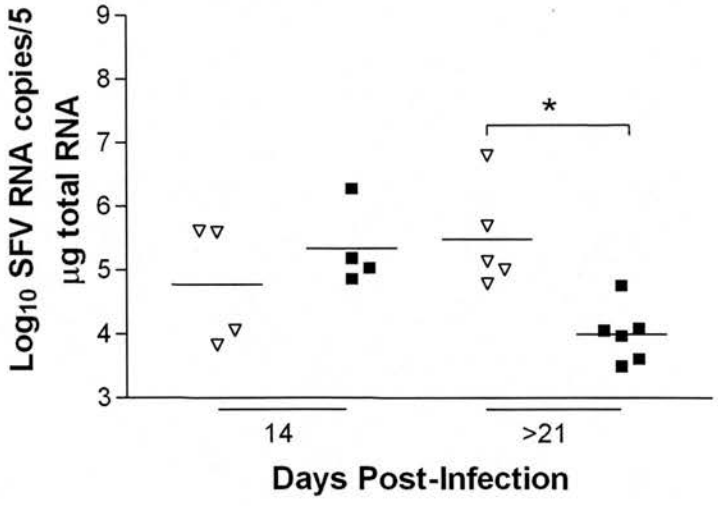

Figure 11. SFV RNA copies in the brains of MHCII$^{-/-}$ mice (▽) and C57Bl/6 mice (■), as measured by q-PCR. Horizontal lines indicate the mean of the groups and dashed line indicates the limit of detection. * indicates significant difference ($p<0.05$). Data were analysed by Mann-Whitney test. <21 days post-infection is combined data from PID 21 and 28.

The MHCII$^{-/-}$ mice had no detectable infectious virus at PID 10, which was unexpected. B-cell responses to most pathogens are CD4 dependent. In the absence of CD4^{+} T-cells, immunoglobulin class switching will not occur (Stavnezer, 1996). Anti-SFV IgG is necessary for infectious virus clearance in the CNS (Amor, 1996) and mice deficient in MHC II would be expected to have very little IgG and hence be unable to clear CNS infection. Sera taken at PID 10 were isotyped by an indirect ELISA to determine their composition (Figure 12). At PID 10 there was a weak IgM response in the MHCII$^{-/-}$ mice compared with C57Bl/6 mice and HI SFV serum (positive control). This was unexpected as an equivalent IgM titre was previously noted in *nu/nu* mice compared with wild-type mice (Amor, 1996). However, this may be explained as only free IgM (not complexed with virus) will be detectable by ELISA so any infectious virus present in the blood will bind IgM hence reducing the titre detectable. The only other immunoglobulin isotype detectable above background ('no serum' and 'no secondary' controls) in MHCII$^{-/-}$ mice was IgG2b. There was a clear difference between the levels of antibody isotypes detected between the wild-type and mutant mice, with a higher OD reading in C57Bl/6 observed in 4/6 of the immunoglobulin isotypes tested. The lack of CD4^{+} T-cells in MHCII$^{-/-}$ mice, as expected does affect isotype switching, but low levels of IgG2b antibody were present.

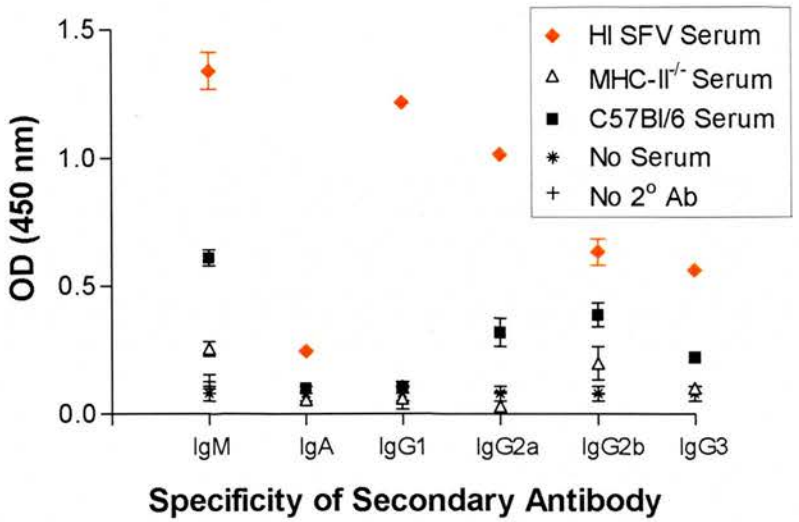

Figure 12. Comparison of antibody isotypes specific for SFV present in PID 10 serum from MHCII$^{-/-}$ and C57Bl/6 mice. Controls included HI SFV serum, no sera and no secondary antibody (2° Ab). Bound antibody was detected by ELISA using isotype specific antibodies. Points represent the mean of three replicates; error bars (where visible) represent SEM.

To determine the role of CD4$^+$ T-cells in the neuropathological changes observed in the CNS following SFV infection, paraffin embedded sections from MHC II$^{-/-}$ and C57Bl/6 mice were stained with luxol fast blue, counterstained with cresyl violet and scored for inflammatory, demyelination and microcystic changes; details of pathology scoring system are given in chapter 2. MHCII$^{-/-}$ and wild-type C57Bl/6 mice had low levels of inflammatory infiltrates in the CNS between PID 10-21 but very few demyelinating lesions or microcystic changes (Figure 13). No significant differences in pathology (as tested by Mann-Whitney) were observed between MHCII$^{-/-}$ mice and wild-type mice. Frustratingly mice on the C57Bl/6 background appear to be a poor model in which to study SFV neuropathology. It is therefore not possible to conclude if CD4$^+$ T-cells are necessary for the development of demyelination and microcystic changes in the CNS following SFV infection due to the low incidence of these pathological features on the C57Bl/6 genetic background.

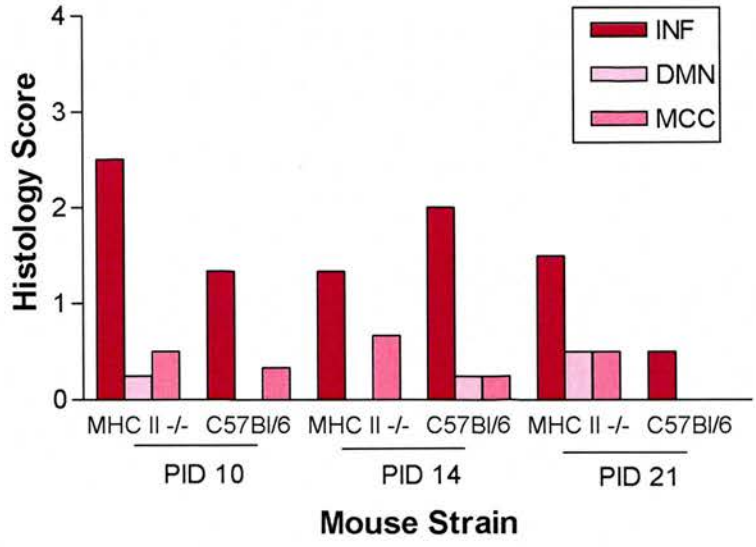

Figure 13. Pathological scores in SFV infected C57Bl/6 and MHCII$^{-/-}$ mice between PID 14 and 21. Results are the mean scores for at least 12 sections from at least 4 mice. INF – inflammation, DMN – demyelination and MCC – microcystic change.

Mice lacking CD8$^+$ T-cells have slower clearance of virus RNA

To determine the role of CD8$^+$ T-cells in virus clearance, CD8a mice (n=30) and wild-type (C57Bl/6) mice (n=30) aged 4-6 weeks of mixed sex were infected IP with SFV A7(74). The amount of infectious virus and virus RNA in the brain were determined at various time points post-infection. Infectious virus was titred in brain tissue by a standard plaque assay on BHK-21 cells (Figure 14). CD8a mice cleared infectious virus at the same rate as immunocompetent wild-type mice (there was no significant difference by Mann-Whitney test). At PID 3 all (6/6) CD8a mice and all but one (5/6) wild-type mice had a high virus titre. At PID 7 5/6 CD8a and 5/5 wild-type mice had detectable virus. At PID 10 infectious virus was not detectable in any (0/6) CD8a mice or wild-type mice.

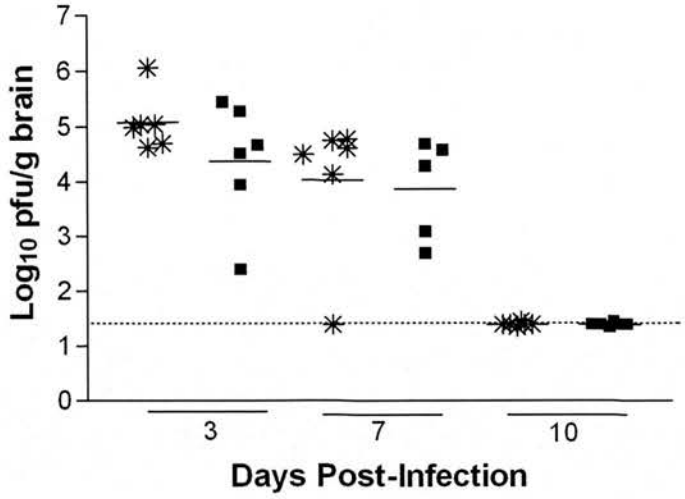

Figure 14. Infectious virus titres in the brains of SFV infected CD8a mice (✳) and C57Bl/6 mice (■), as measured by plaque assay. Horizontal lines indicate mean of the groups and the dashed line indicates the limit of detection.

The levels of SFV virus RNA were determined in CD8a and C57Bl/6 mice at various time-points after infectious virus clearance (PID 14 and 21) by q-PCR (Figure **15**). Virus RNA was detectable in 100% of CD8a mice at both time points, in 100 % (5/5) of C57Bl/6 mice at PID 14 and 80% (4/5) C57Bl/6 at PID 21. Levels of virus RNA in the CD8a mice were significantly higher than in to C57Bl/6 mice at both PID 14 and PID 21 ($p=0.0303$ and $p=0.0025$, respectively by Mann-Whitney test).

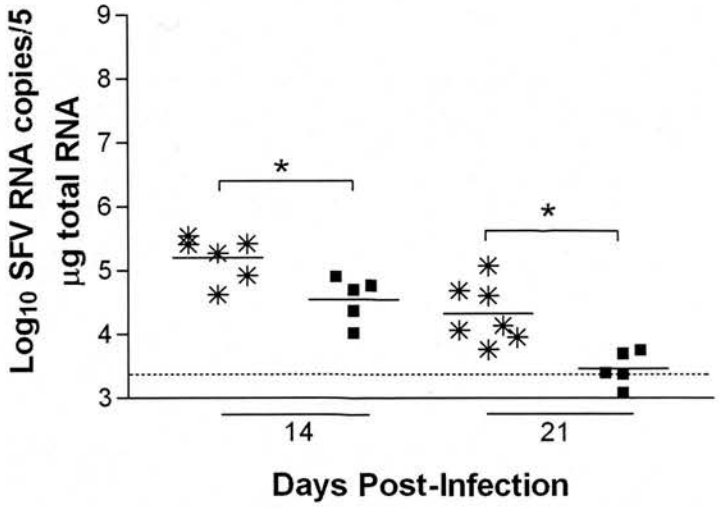

Figure 15. Titres of SFV RNA in the brains of SFV infected CD8a mice (✳) and C57Bl/6 mice (■), as measured by q-PCR. * indicates significant difference ($p<0.05$). Data were analysed by Mann-

Whitney test. Horizontal bars indicate the mean of the groups, dashed line indicates the limit of detection.

To determine if the difference in virus RNA clearance between CD8a and C57Bl/6 mice continued beyond 21 days (3 weeks) a second group of CD8a mice (n=12) and C57Bl/6 mice (n=12) were infected (mice aged 4-6 weeks, mixed sexes) and mice sampled at 6, 9 and 12 weeks (n=4/time-point). All mice had low levels of virus RNA (near or below the limit of detection) in the brain, as determined by q-PCR (Figure 16). There was a small decrease in both CD8a (mean virus copies 3.8) and C57Bl/6 mice virus RNA titre (mean virus copies 3.7) from 6 to 12 weeks post-infection (mean virus copies 3.6 and 3.5 respectively). CD8a mice had a slightly higher mean virus RNA load at each time point but there was no significant difference between the two groups at any time point.

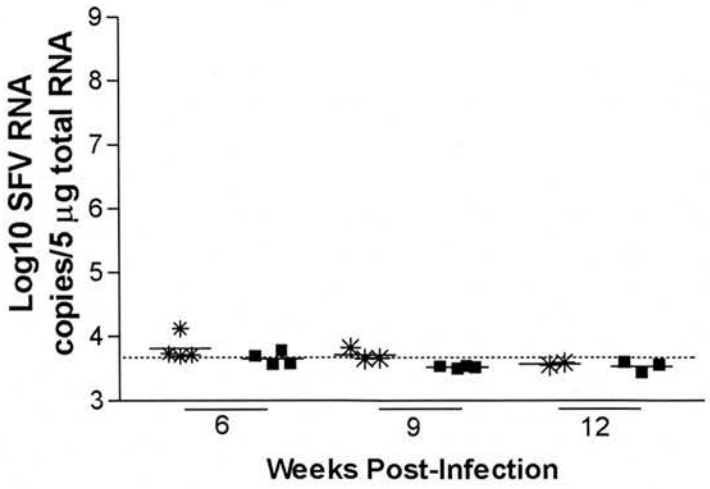

Figure 16. Long term clearance of SFV RNA in the brains of CD8a mice (✳) and C57Bl/6 mice (■), as measured by q-PCR. Horizontal bars indicate the mean of the groups, dashed line indicates the limit of detection.

To determine if $CD8^+$ T-cells have a role in inducing SFV neuropathological changes, paraffin embedded brain sections from CD8a and C57Bl/6 mice (PID 14 and 21) were stained with luxol fast blue (myelin stain) and counterstained with cresyl violet. Sections were scored for inflammation, demyelination and microcystic change. The wild-type C57Bl/6 and the CD8a mice both showed low levels of CNS inflammation but no obvious demyelination or microcystic change at either time point (data not shown).

Adoptive Transfer of SFV immune cells to SFV infected SCID mice

Subak-Sharpe *et al* used rat anti-CD4 and anti-CD8 monoclonal antibodies to transiently deplete these subsets in BALB/c mice and noted a reduction in demyelination in $CD8^+$ T-cell depleted mice (Subak-Sharpe, 1993). It had been hoped that studies on the CD8a mice would confirm this finding in a more robust system, however due to the low incidence of microcystic changes and demyelinating lesions on the C57Bl/6 background it was not possible to determine the role of $CD8^+$ and $CD4^+$ cells in mediating these changes. In an alternative approach it was decided to utilise SCID mice on a BALB/c background and to examine the role of $CD8^+$ T-cells in CNS pathology and virus clearance by carrying out adoptive transfers of immune cells from SFV infected BALB/c mice in to SFV infected SCID mice. BALB/c (n=15) and SCID mice (n=20) were infected IP with SFV A7(74) and at PID 7 spleens were sampled from the BALB/c mice. $CD8^+$ cells were positively selected using a magnetically activated cell sorter (MACS). The $CD8^+$ selected cells or flow through cells were adoptively transferred to SFV infected SCID mice (5 mice/group) at a 1:1 ratio. For example, a mean of $4.5x10^6$ $CD8^+$ splenocytes were collected per BALB/c mouse and this number of cells were transferred IP into each recipient SCID mouse. One group of SCID mice received $1x10^6$ $CD8^+$ splenocytes IC and a control group received PBS (n=5/group). SCID mice were sampled at PID 14 and the tissues assayed for infectious virus titre, virus RNA titre and pathological changes (Figure 17).

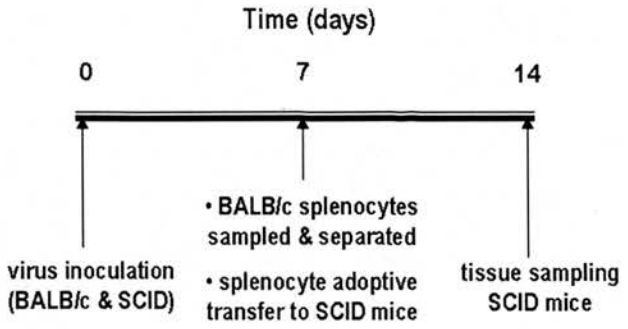

Figure 17. Timeline for raising, and adoptive transfer of immune splenocytes from SFV infected BALB/c/ mice to SCID mice.

Purity of cell population separated by MACS

After splenocytes were sampled from the BALB/c mice they were separated on a LS column using a VarioMACS™ separator according to the manufacturer's instructions (Miltenyi Biotec). Splenocytes from PID 7 SFV infected BALB/c mice were isolated, counted and mixed with anti-CD8a microbeads (Miltenyi Biotec). Stained cells were put through a LS MACS column on a VarioMACS™ cell separator. Flow-through cells were collected and saved and $CD8^+$ cells eluted from the column. Flow-through cells were then passed through a second MACS column and $CD8^+$ cells eluted and pooled with the first population of eluted $CD8^+$ cells. $1x10^6$ cells from each population (as well an unseparated control) were stained with anti-CD4-PE and CD8-FITC and analysed by FACS to assess the cell populations present (Figure 18). Unseparated splenocytes contained 20% $CD4^+$ T-cells and 10.5% $CD8^+$ T-cells. There was a small population of CD4 and CD8 double-positive cells. Flow-through cells had an increased proportion of $CD4^+$ cells (21%) and a decreased $CD8^+$ cell population (3.8%). The positively selected cells had a very low CD4 population (0.6%) and were highly enriched for $CD8^+$ cells (97%).

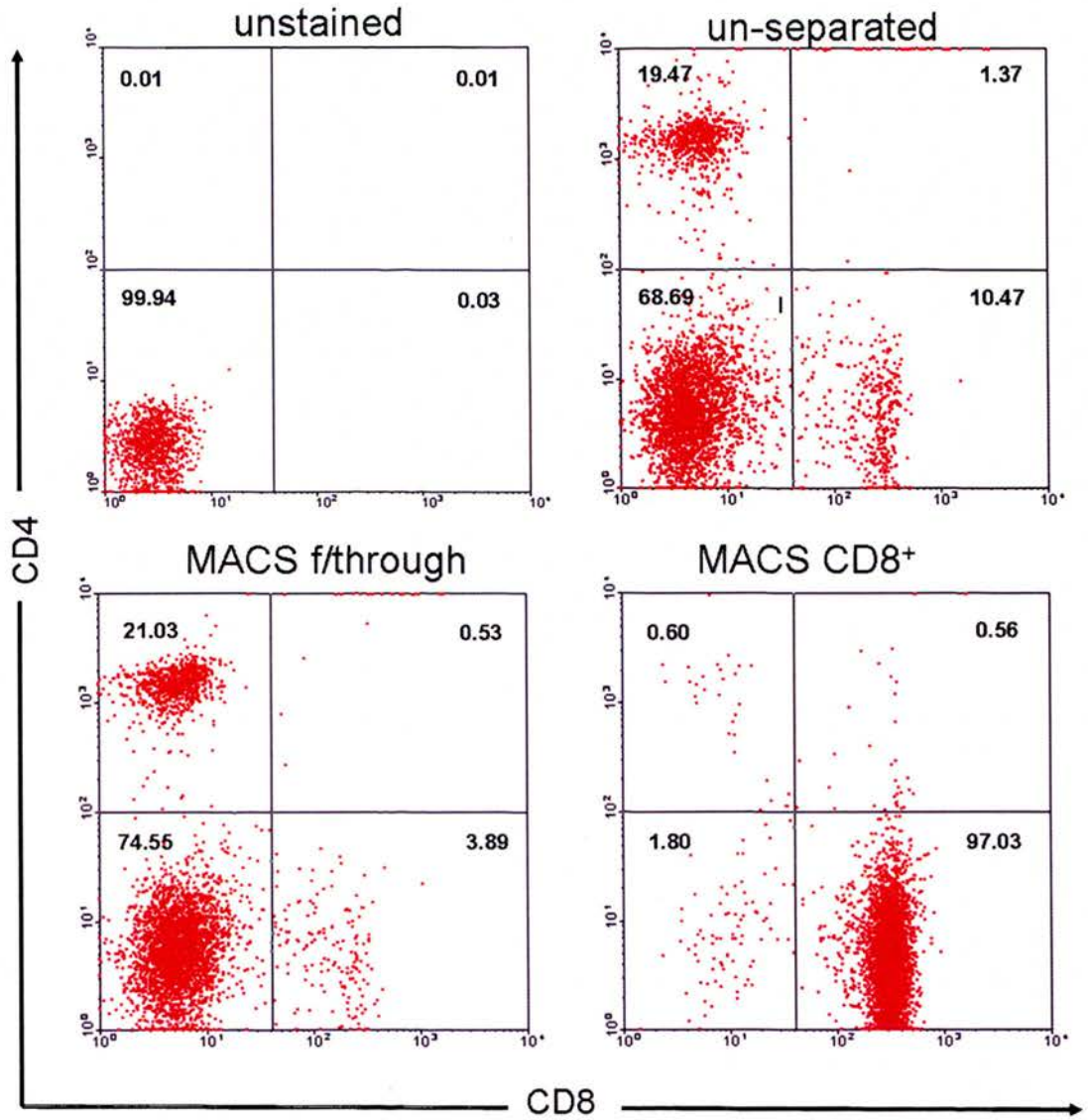

Figure 18. $CD4^+$ and $CD8^+$ staining of splenocytes from SFV infected BALB/c mice at PID 7 before (unstained and unseparated) and after (MACS f/through and MACS $CD8^+$) $CD8^+$ cell selection on a MACS. Numbers are events in a quadrant expressed as a percentage of the lymphocyte gate. MACS f/through – MACS flow-through cells; MACS $CD8^+$ - positively selected $CD8^+$ cells.

SFV infected SCID mice that receive $CD8^+$ splenocytes do not clear infectious virus or virus RNA

Three groups of SFV infected SCID mice received immune transfers at PID 7. One group received $CD8^+$ selected splenocytes by IP inoculation ($CD8^+$ IP), a second group received $CD8^+$ selected splenocytes by IC inoculation ($CD8^+$ IC) and a third group received flow-through cells by IP inoculation (F/T). A control group received PBS only. Of the $CD8^+$ IP mice, 3/5 showed hind-limb paralysis and all showed piloerection, hunched posture and visible weight loss. F/T mice also had

piloerection, hunched posture, immobility and visible weight loss. $CD8^+$ IC mice and PBS control mice had ungroomed coats but otherwise, no outward signs of illness.

Levels of infectious virus were assayed in the heart tissue of the SCID mice at PID 14 (Figure 19). Mice that received $CD8^+$ T-cells (IP or IC) had low infectious virus titres. A low level (Log_{10} 2.4 – 2.8 pfu/g) of infectious virus was detectable in 40% (2/5) mice in both groups. F/T mice had no (0/4) infectious virus and 100% (5/5) mice that received PBS had significantly higher levels of infectious virus than all other groups (p=0.0079 $CD8^+$IP; p= 0.0079, $CD8^+$IC and p=0.0159 F/T).

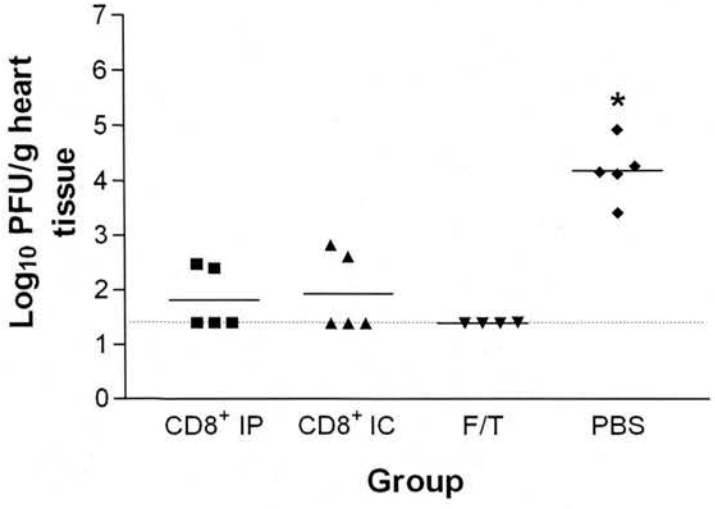

Figure 19. Infectious virus titres at PID 14 in heart tissue of SFV infected SCID mice that received immune cell adoptive transfers. * indicates significant difference ($p<0.05$) with all other groups. Data analysed by Mann-Whitney test. Horizontal lines indicate the mean of the groups and the dashed line indicates the limit of detection. Column titles represent the immune cells transferred. F/T – flow-through splenocytes from MACS, PBS – control PBS transfer.

In SCID mice that received adoptive transfers, the number of copies of SFV RNA in the brain tissue was assayed at PID 14 (Figure 20). The $CD8^+$ IP group had the highest mean SFV copies (7.8 Log_{10} SFV copies/5 μg RNA), which was significantly higher than the F/T group, that received splenocytes depleted of $CD8^+$ T-cells (p=0.0317 data analysed by Mann-Whitney test). There was also a significant difference in virus RNA levels between the F/T group and the control mice which received no cell transfers (p=0.0317).

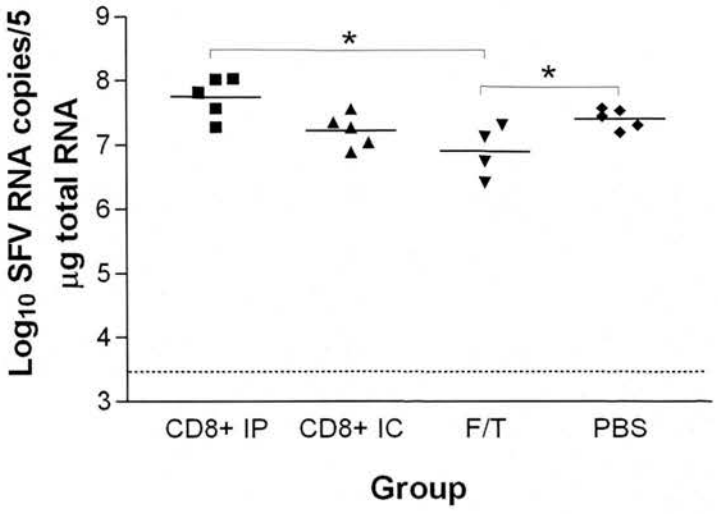

Figure 20. Levels of SFV RNA at PID 14 in the brains of SFV infected SCID mice that received immune cell adoptive transfers. * indicates significant difference ($p<0.05$). Data analysed by Mann-Whitney test. Horizontal lines indicate the mean of the groups and the dashed line indicates the limit of detection. Column titles represent the immune cells transferred. **F/T** – flow-through splenocytes from MACS, **PBS** – control PBS transfer.

To determine if antibody was being secreted in mice that received adoptive transfers, sera from all groups ($CD8^+$ IP[x3], $CD8^+$ IC [x2], F/T [x3] and PBS [x2]) were tested for neutralisation activity by plaque reduction neutralisation assay. There was no protection of BHK-21 cell monolayers by the sera in any of the groups (data not shown); the level of neutralising antibody was below $1x10^4$ pfu neutralised/ml (limit of detection).

CNS pathology in mice that have received adoptive transfers

PID 14 brain sections from the SCID mice that received adoptive transfers were fixed and processed for paraffin sectioning. To examine virus spread, immunostaining for SFV antigen was carried out. The average number of virus-positive cells per section was determined from 6 brain sections per mouse (Figure 21). The $CD8^+$ IP group had the highest mean number of virus positive cells/brain section (mean 20 cells/section). This correlated with the virus RNA load in the brain of the $CD8^+$ IP mice. F/T mice, CD8 IC mice and PBS mice had similar numbers of SFV^+ cells/section. It was unexpected that the $CD8^+$ IP group that received adoptive transfers should have more virus-positive cells (not significant by Mann-Whitney

test) than control mice, as these SFV infected SCID mice have no B or T-cells and received only PBS.

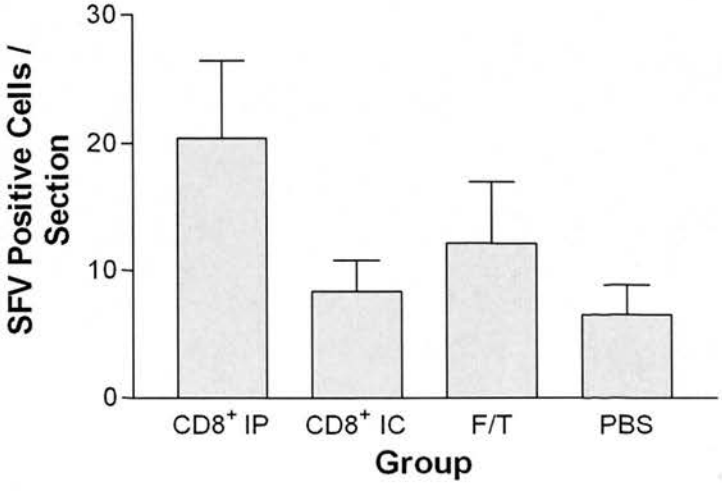

Figure 21. SFV-positive cells per section of brain (detected by immunostaining) at PID 14 in SFV infected SCID mice that had received immune cells by adoptive transfer. Error bars indicate the SEM. Column titles represent the immune cells transferred and the method of transfer. F/T – flow-through splenocytes from MACS, PBS – control PBS transfer.

Histological scoring for inflammation (INF), demyelination (DMN) and microcystic change (MCC) was also carried out on paraffin sections of the brain stained with luxol fast blue (myelin stain) and counterstained with cresyl violet (nuclear stain). In mice that received PBS, some virus infected areas of the brain had swollen nuclei (Figure 23A) but otherwise, as expected, these sections showed little pathology (0.25 – INF, 0.25 – MCC). In contrast, mice receiving adoptive transfers had inflammatory infiltrates, microcystic changes and lesions of demyelination. There was little difference in their mean histological scores (Figure 22) between the 3 groups of SFV infected SCID mice that received splenocytes ($CD8^+$IP, $CD8^+$IC and F/T). Demyelination although observed infrequently was present in both F/T and $CD8^+$ mice. An example of a demyelinating lesion from a $CD8^+$ IC mouse is shown in Figure 23B. The composition of inflammatory infiltrates appeared to differ between the groups that received $CD8^+$ T-cells and F/T. $CD8^+$ IP mice had more mononuclear cell infiltrates (dense, round purple cells) whereas the F/T mice had a greater number of macrophage-like cells (pale purple, elongated and or dumbbell shaped cells) in inflammatory regions (Figure 23C & D, respectively). Inflammatory

lesions were present in both $CD8^+$ IP and F/T group which contained microcystic change, many SFV positive cells and necrotic areas (Figure 23E & F). Conversely, SFV positive cells were also found in mice without any inflammatory response (Figure 23G – $CD8^+$ IP, Figure 23H – F/T).

At the group level, INF was significantly greater ($p<0.05$) in all groups compared to PBS. The levels of DMN and MCC was significant in the $CD8^+$IC ($p=0.03$) and F/T ($p= 0.0286$) groups compared to PBS group (data analysed by Mann-Whitney test). There was much intra-group variation, especially in the mice that received $CD8^+$ cells IP (Table 7). For example, $CD8^+$ IP mouse 1 had a high pathological score (INF 4, DMN 1.8, MCC 3.1) whereas mouse 3 had almost no pathological changes (INF 0.9, DMN 0, MCC 0.1). This difference could indicate natural variation in responses to the virus or, more likely, differences in the efficacy of the adoptive transfers. If few cells were successfully transferred, a low pathological score might be expected, as observed in the PBS control group.

The experiment was designed to elucidate the role of $CD8^+$ T-cells in demyelination and the key finding is that $CD8^+$ cells are sufficient to generate these lesions. No conclusions can be drawn about the role of CD4 T-cells alone. It is possible that $CD4^+$ T-cells can initiate demyelination, perhaps mediated by macrophages, but as a sizable $CD8^+$ population was also present in the flow-through, it cannot be excluded that $CD8^+$ cells were also responsible for the demyelination in this group.

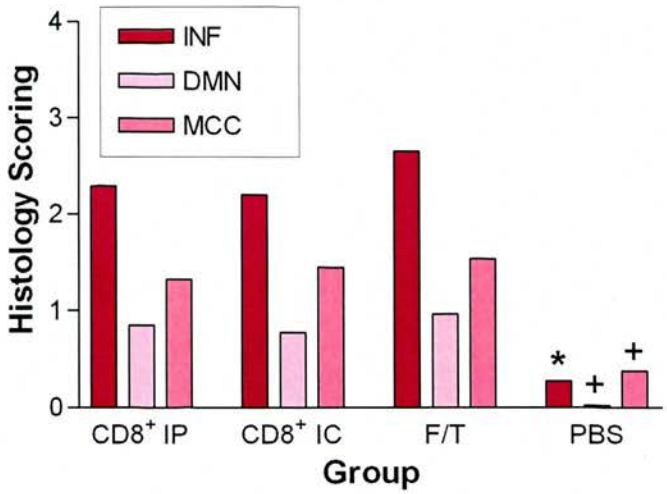

Figure 22. Mean histological scores at PID 14 in SFV infected SCID mice that had received immune cell adoptive transfers. Results are the means scores for at least 12 brain sections from at least 4 mice. * indicates $p<0.05$ with all other groups; $^{+}$ indicates significant difference ($p<0.05$) with $CD8^{+}$IC and F/T groups. Data analysed by Mann-Whitney test. **INF** – inflammation; **DMN** – demyelination and **MCC** – microcystic change.

Group	Number	INF	DMN	MCC
$CD8^{+}$ IP	1	4	1.8	3.1
	2	3	1	1.5
	3	0.9	0	0.1
	4	2.3	1	1.1
	5	1.4	0	0.8
$CD8^{+}$ IC	1	2.3	0.8	18
	2	2.6	1.3	1.5
	3	2	0.4	0.5
	4	2.4	0.5	1.5
	5	2.1	1	2
F/T	1	3.6	2	3.1
	2	1.4	0.5	0.9
	3	2.8	0.6	1
	4	3.3	0.8	1.2
PBS	1	0.3	0	0.4
	2	0.3	0	0.3
	3	0.5	0	0.6
	4	0.6	0.1	0.5
	5	0.1	0	0.1

Table 7. Individual histological scores for SFV infected SCID mice at PID 14 that had received immune cell adoptive transfers. Results are the means scores for at least 12 sections. INF – inflammation, DMN – demyelination and MCC – microcystic change.

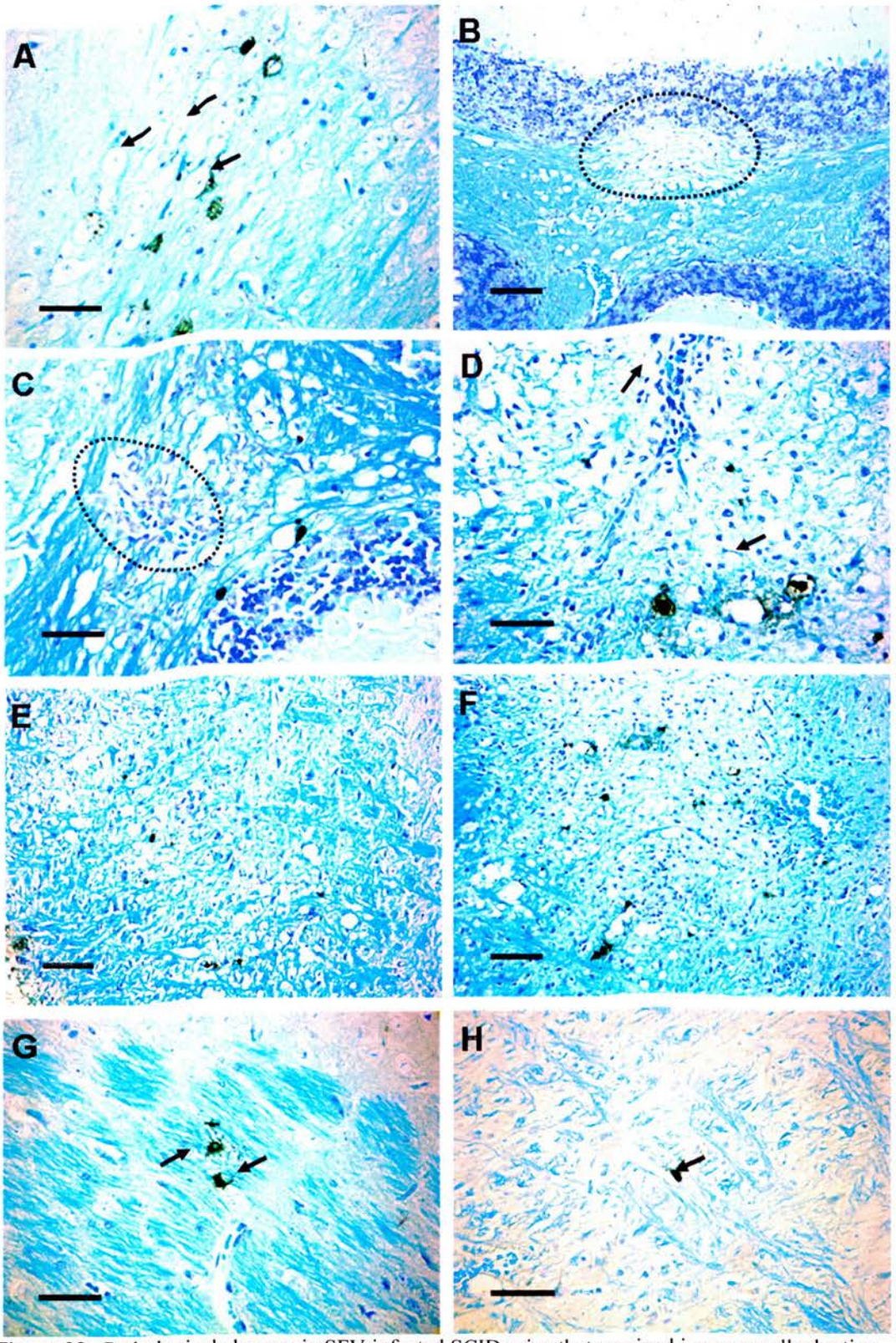

Figure 23. Pathological changes in SFV infected SCID mice that received immune cell adoptive transfer. Sections of paraffin embedded brain tissue were immunostained for SFV antigen (black) and then stained with luxol fast blue and cresyl violet. **A** swollen nuclei (arrows) in SFV infected control mice; **B** DMN (circle) in cerebellum of $CD8^+$ IC; **C** mononuclear cell infiltrates in $CD8^+$ IP (circle); **D** macrophage (arrows) and mononuclear cell infiltrates in F/T; **E** necrotic lesions in $CD8^+$ IP; **F** necrotic lesions in F/T; **G** SFV infected cells (arrows) with no inflammatory response in $CD8^+$ IP; **H** SFV infected cell (arrow) with no inflammatory response in F/T. **$CD8^+$ IC** – mice that received $CD8^+$ cells IC; **$CD8^+$ IP** mice that received $CD8^+$ cells IP; **F/T** mice that received F/T cells IP; **DMN** demyelination. Bar represents: 20 microns **A, C, D, G** & **H**; 50 microns **E** & **F**, 100 microns **B**.

Virus-like particles can successfully infect cells of the CNS in C57Bl/6 mice

Data shown earlier in this chapter demonstrated that mice with a C57Bl/6 genetic background showed little or no demyelination or microcystic change in the CNS following SFV infection. Other strains of mice (BALB/c, WT-129) typically have demyelinating lesions from PID 14 to 21 when inoculated IP with SFV. Previous reports have shown demyelination in C57Bl/6 mice; however these mice may have had a slightly different genetic background due to differences between breeding stocks or suppliers (Sheahan, 1983; Mokhtarian & Swoveland, 1987). One possible explanation for the lack of demyelination in C57Bl/6 mice would be more efficient virus clearance from the brain; if C57Bl/6 mice have less viral spread in the CNS, perhaps due to a more efficient immune response, this could limit or prevent demyelination. Such is the case in TMEV infection where depletion of the protective response of $CD4^+$ or $CD8^+$ T-cells makes previously resistant C57Bl/6 mice susceptible to demyelinating disease (Rodriguez, 1991). Another possible explanation for the lack of demyelination would be that C57Bl/6 mice lack a receptor that allows productive CNS infection. SJL mice are resistant to MHV infection of the CNS whereas BALB/c mice succumb to CNS infection with a few days. Resistance to MHV in SJL mice has been linked to a polymorphism in the MHV receptor (MHVR) biliary glycoprotein 1; the virus has 10 to 30-fold less affinity for biliary glycoprotein 1b (MHVR2) than for biliary glycoprotein 1a (MHVR1) allele expressed in susceptible BALB/c mice (Boyle, 1987; Ohtsuka & Taguchi, 1997a). If SFV does not productively or efficiently infect CNS cells or a subpopulation of CNS cells, for example oligodendrocytes, in C57Bl/6 mice fewer pathological changes would be expected.

SFV has been detected in the CNS of C57Bl/6 by plaque assay; however a high titre viraemia could contribute to virus detected in the brain. To determine whether oligodendrocytes in C57Bl/6 mice were susceptible to SFV infection, C57Bl/6 mice (n=4) were inoculated IC with approximately 80,000 SFV-GFP VLPs and sampled at PID 5 and 14. VLPs are structurally identical to virus but they contain a modified genome which can express foreign genes. The use of VLPs encoding GFP allows microscopic visualisation of infected cells without the need for immunostaining. In a

previous study it was demonstrated that direct IC inoculation of SFV-GFP VLPs into the BALB/c mouse brain resulted in widespread infection of cells, predominantly oligodendrocytes in the major white matter tracts of the brain (Fazakerley, 2006).

VLP infected cells (EGFP-positive) were clearly visible at PID 5 and were most evident along the white matter tracts (Figure 24A and B). Areas of infection were also visible in areas away from the white matter (Figure 24C). The higher power image (Figure 24D) clearly shows VLP infection of oligodendrocytes. The cell body fluoresces brightly as do all the processes extending from the oligodendrocytes, which are myelinating surrounding axons. A later time point in infection (PID 14) was also examined to determine if virus was cleared rapidly in C57Bl/6 mice (Figure 24E and F). The fluorescence was weaker than at earlier time points, but VLP infected cells were still visible. This experiment demonstrated that the white matter tract cells including oligodendrocytes in the CNS of C57Bl/6 mice are permissive for infection with SFV or SFV-like particles and that resistance of this cell population to CNS infection is not the reason for the low levels of demyelination observed in this mouse strain. Most likely these mice differ to BALB/c mice in the quality or quantity of their immune response.

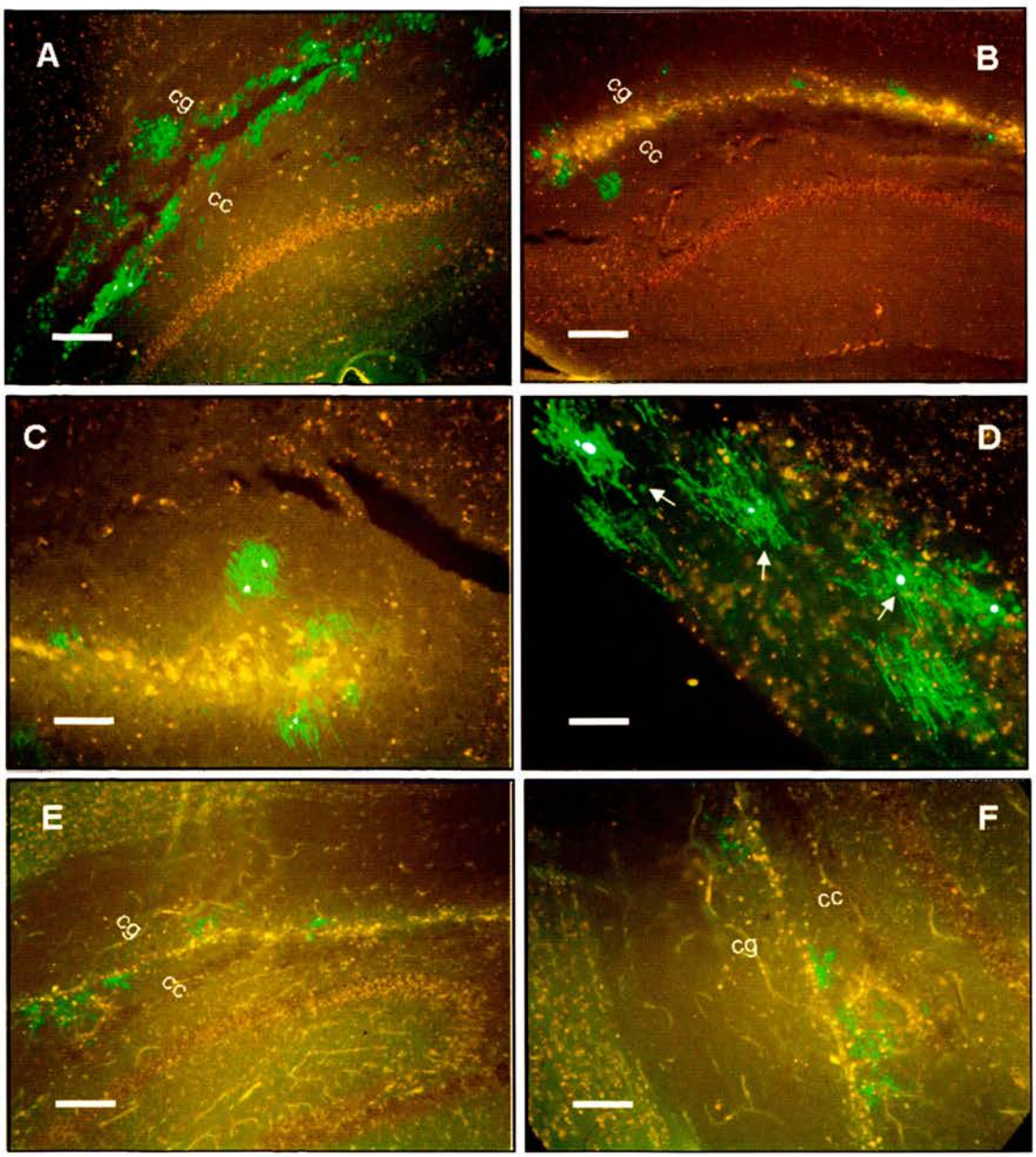

Figure 24. SFV-EGFP VLP infections in the CNS of C57Bl/6 mice at PID 5 (**A-D**) and PID 14 (**E** and **F**). C57Bl/6 mice were inoculated IC with 80,000 VLP and sampled at PID 5 and 14. Many of the infected cells (bright green) have the typical morphology of oligodendrocytes (shown by arrow); characterised by many extending processes. **A,B, E** and **F** show infected cells in the white matter tracts of the corpus callosum (cc) and cingullum (cg), **C** and **D** show infected cell in the cortex. Bars represent 100 microns **A**, **B** & **E;** 50 microns **C** & **E** and 20 microns **D**.

The phenotype of CNS inflammatory cells in SFV infection

FACS experiment 1

In this chapter the roles of $CD4^+$ and $CD8^+$ T-cells were examined to elucidate their contribution, beneficial or damaging, in SFV infection. In order to examine the CNS cellular inflammatory response to SFV as a whole, the phenotype of lymphocytes extracted from the CNS of SFV infected mice were analysed by FACS. The kinetics of lymphocyte entry into the CNS, the magnitude of immune responses and the quality of the responses in different mouse strains (C57Bl/6 mice and BALB/c) were studied.

For FACS experiment 1, C57Bl/6 mice (n=20, age 4-6 weeks, mixed sexes) were inoculated with $5x10^3$ pfu SFV A7(74) IP. Five mice were sampled at 3, 6, 8 and 15 days post-infection. These time points were chosen to allow observations prior to inflammatory infiltration (PID 3), at peak lymphocyte infiltration of the CSF (Parsons & Webb, 1982i) and after infectious virus has cleared (PID 15). Brains from perfused animals were treated with collagenase and DNase and disrupted by trituration through decreasing needle sizes. Mononuclear cells were then prepared from a 30:70% discontinuous Percoll gradient after centrifugation (see Materials and Methods for full details). Cells were stained for FACS analysis with antibodies to CD4 (T helper cell marker), CD8 (cytotoxic T-cell marker), CD19 (B-cell marker), CD25 (T-cell subset marker), CD45 (activation marker) and CD11b (Mac1 - macrophage marker). These markers allow recognition of T-cell subsets ($CD4^+$ and $CD8^+$) and B-cells (CD19). Infiltrating macrophage (CD11b $CD45^{hi}$) and resident microglia (CD11b $CD45^{lo}$) can be distinguished by their differential expression of CD45 (Sedgwick, 1991b). An anti-CD25 antibody was included; CD25 is a putative marker of T-reg cells, but is also an activation marker of T-cells. Cells double-staining for CD4 and CD25 may represent a regulatory population. It was of particular interest to observe if T-reg cells were present in this model system of virus encephalitis. After FACS analysis, cells were presented as the total number of cells/brain and not as a proportion of the total number of cells. This method was chosen as the total cell population increases in the inflamed brain, therefore representing cell populations as a percentage of the total can be misleading. Using

the total number of cells/brain allows data between days to be compared. This method does assume that cell isolation efficiencies in all samples are equal.

At PID 3, low levels of $CD4^+$ and $CD8^+$ cells were detected, but microglia were, as expected, the most common cell type detected (Figure 25). By PID 6 there was a 3.5-fold increase in microglial cells, a 20-fold increase in macrophages and a >25-fold increase in $CD4^+$ and $CD8^+$ T-cells. A detectable population of $CD4^+CD25^+$ T-cells was present (Figure 25C). The peak of inflammation was at PID 8 when there were increases in all cell types. The greatest increase was in $CD8^+$ T-cells (10-fold increase from PID 6 to a mean of 17,100 cells/brain) levels at twice those of $CD4^+$ T-cells (mean 7,800 cells/brain). PID 8 was the first day that $CD19^+$ cells were stained, a mean cell count/ brain of 1,500, which was low compared to other cell types. At PID 15 there was a decrease in most cell types, macrophages decreased 2.5-fold and microglia had a 1.3-fold decrease. At PID 15, both $CD4^+$ and $CD8^+$ T-cells had the same mean cell count/brain (decreases of 2.8-fold and 4-fold, respectively). $CD19^+$ cells were the only population to remain constant at this time point.

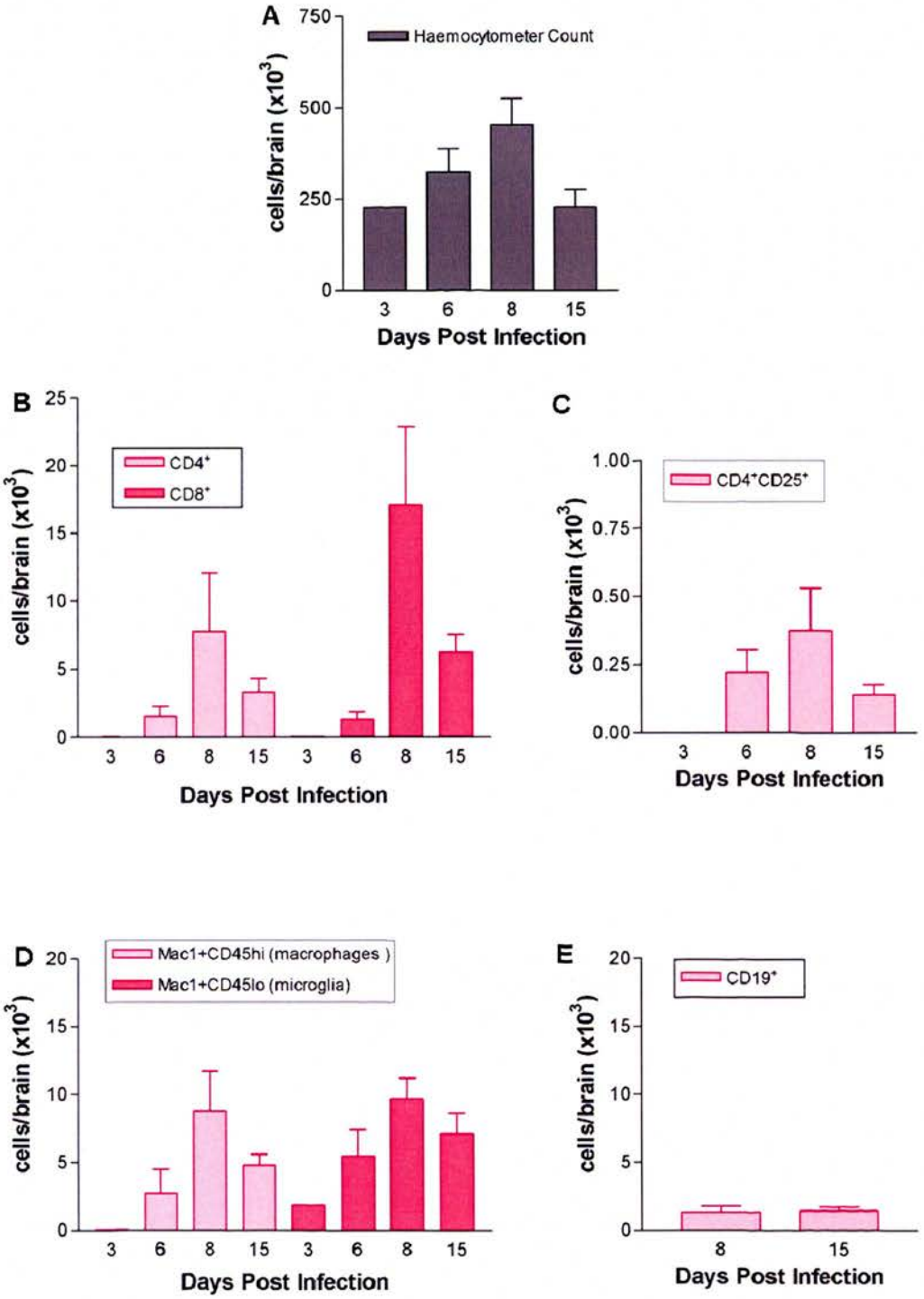

Figure 25. Phenotype and mean number of cells in the CNS of C57Bl/6 mice (n=5) infected with SFV. Cells were surface stained with labelled antibody and analysed by FACSCalibur. **A-** Haemocytometer count; **B-** CD4 and CD8 staining of lymphocytes; **C-** CD4 and CD25 staining of lymphocytes; **D-** Mac1 and CD45 staining of lymphocytes; **E-** CD19 staining of lymphocytes. Error bars represent the SEM.

FACS experiment 2

The earlier studies in this chapter highlighted differences in CNS pathology between SFV infected BALB/c and C57Bl/6 mice. These changes could be related to differences in immune responses. The phenotype of inflammatory cells isolated from the CNS of two strains of SFV infected mice were compared by FACS analysis. Pan-NK marker Dx5 and T-reg cell marker FoxP3 were included in this experiment. NK cells are lymphocytes of the innate immune system but possess the cytolytic capacity of $CD8^+$ T-cells. They can rapidly kill virus infected cells via perforin/granzyme release and are an important source of cytokines such as IFNγ. It is not known if these cells are important in SFV infection, but an early peak of white blood cells is detected in the CSF at PID 6 and this could be NK cells (Parsons & Webb, 1982e). The detection of naturally occurring T-regs is currently defined by the expression of FoxP3 (Hori, 2003). The Foxp3 gene is a member of the forkhead/winged-helix family of transcriptional regulators and this gene appears to function as the master regulator in the development and function of regulatory T cells.

Mice (C57Bl/6, n=20 and BALB/c, n=20) were inoculated with $5x10^3$ pfu SFV A7(74) IP and 5 mice from each group were sampled at 3, 6, 8 and 14 days post-infection. Mouse brain lymphocytes were extracted using a neuronal dissociation kit (Miltenyi Biotec). This resulted in improved tissue homogenisation and increased cell yield per brain, but also increased the debris collected, which resulted in increased autofluorescence.

The cell yield per brain in BALB/c mice (as quantified by haemocytometer) was high at days 3, 6 and 8 but reduced at day 14 (Figure 27B) which was similar to the kinetics observed in C57Bl/6 mice in experiment 1 (Figure 25A). In contrast, the cell yield for C57Bl/6 mice showed the lowest number of cells/brain at PID 8 (

Figure **26**A). Cell isolates from different animals were kept separate but samples from the same time point were processed together as a batch. It is possible that something happened to the C57Bl/6 day 8 samples to reduce the yield during processing. This could have been disruption of Percoll layers during separation in the centrifuge. As a result the cells/brain counts for all C57Bl/6 cell types at PID 8 are most probably low, though their relative percentages at this time point remain correct.

The CD8a stain did not work in this second experiment so the $CD4^-CD3^+$ population was analysed as a substitute; this population will include the $CD8^+$ T-cells. The $CD4^-$ $CD3^+$ population is unlikely to be exactly the same $CD8^+$ population. However, the former showed the same dynamics as the $CD8^+$ T-cells in experiment 1 suggesting it was a reasonable substitute.

Very few cells were detectable at PID 3 in both groups of mice. The exceptions to this are microglia, which are resident in the CNS, and a small population of NK cells. By PID 6 there was an increase in infiltrating cell populations in both mouse strains. Notably in the C57Bl/6 mice between PID 3 and 6 there was a huge increase in macrophages (70-fold) and a 5-fold increase in NK cells. The BALB/c mice, as in C57Bl/6 mice in experiment 1, had the highest cells/brain count for all cell types at PID 8. However, the cells/brain counts for C57Bl/6 mice in experiment 2 were low or stable at PID 8, as discussed previously this may have been an artefact. Interestingly in these mice at this time point, the $CD4^+$ $CD25^+$ population still increased. At PID 14 in the BALB/c mice, all cell populations contracted except $CD4^+$ T-cells which remained stable.

T-regs are defined phenotypically as $CD4^+CD25^+$ cells that express the FoxP3 gene. To investigate if such cells are present during viral CNS infections, pooled cells from 5 mice at PID 6, 8 and 14 were stained for CD4 and CD25 followed by intracellular staining for FoxP3. PID 3 was not used as a $CD4^+CD25^+$ population was not detectable at this early time-point. The dot plot of the FoxP3 staining from PID 8 in C57Bl/6 and BALB/c mice is shown in

Figure 26F and Figure 27F, respectively. A very small percentage of FoxP3 positive cells were detected, it is not possible to say whether these represent T-reg cells without a more extensive study and the inclusion of a positive control. The dot plot from PID 8 is also representative of the results at PID 6 and PID 14.

Taking into account the problems with cell extraction and the anti-CD8 antibody, it would have been desirable to repeat this study; however an MHV outbreak in the animal facilities precluded any further animal work.

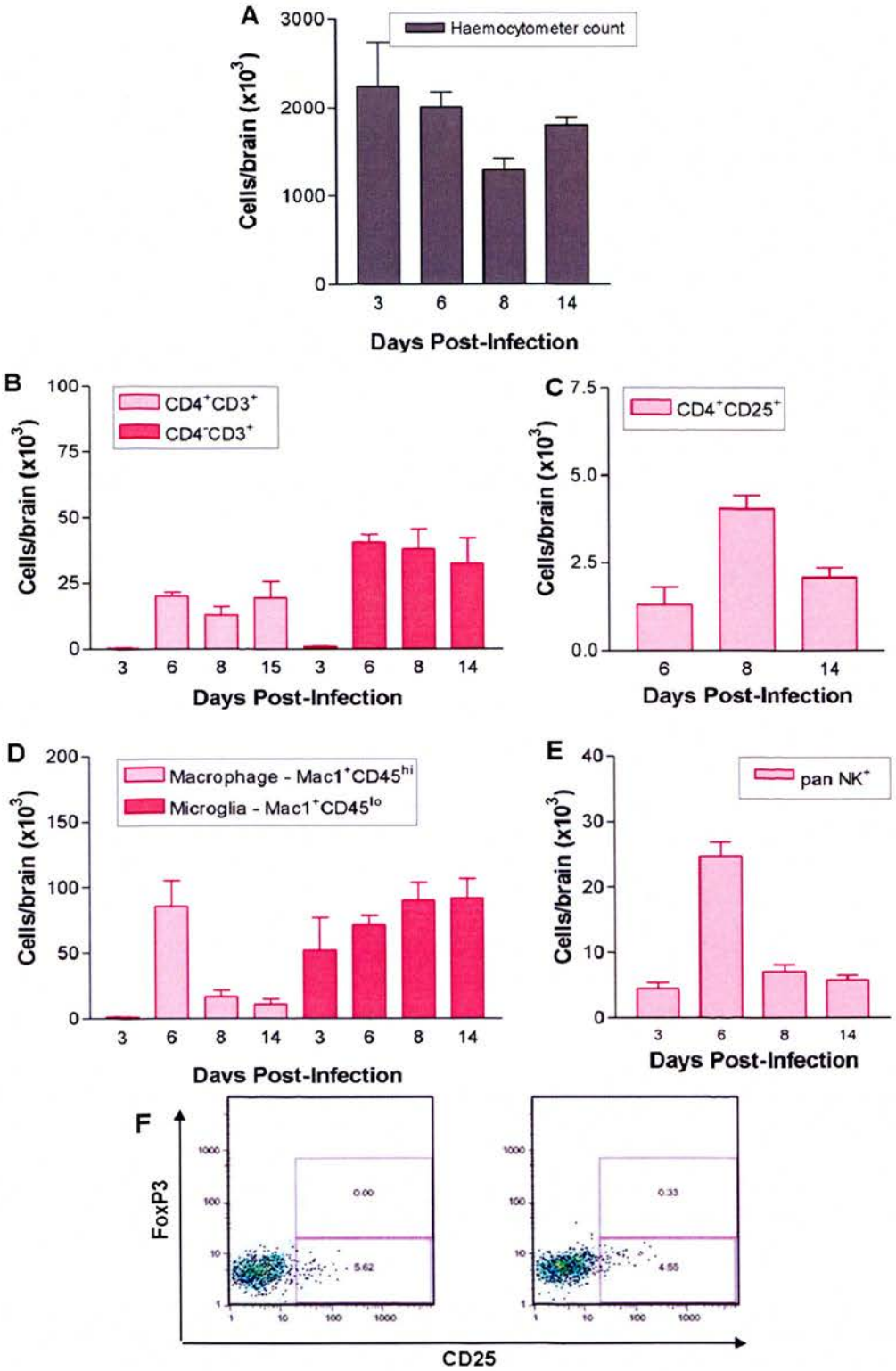

Figure 26. Phenotype and mean number of cells/brain in the CNS of C57Bl/6 mice (n=5/time point) infected with SFV. Cells were surface stained with labelled antibody and analysed by FACSCalibur. **A-** Haemocytometer count, **B-** CD3 and CD4 staining of lymphocytes, **C-** CD4 and CD25 staining of lymphocytes, **D-** Mac1 and CD4 staining of lymphocytes, **E-** Dx5 staining (pan NK marker) of lymphocytes, **F-** Dot plot of isotype control (left) and FoxP3 (right) with CD25 staining of $CD4^+$ lymphocytes. Error bars represent the SEM

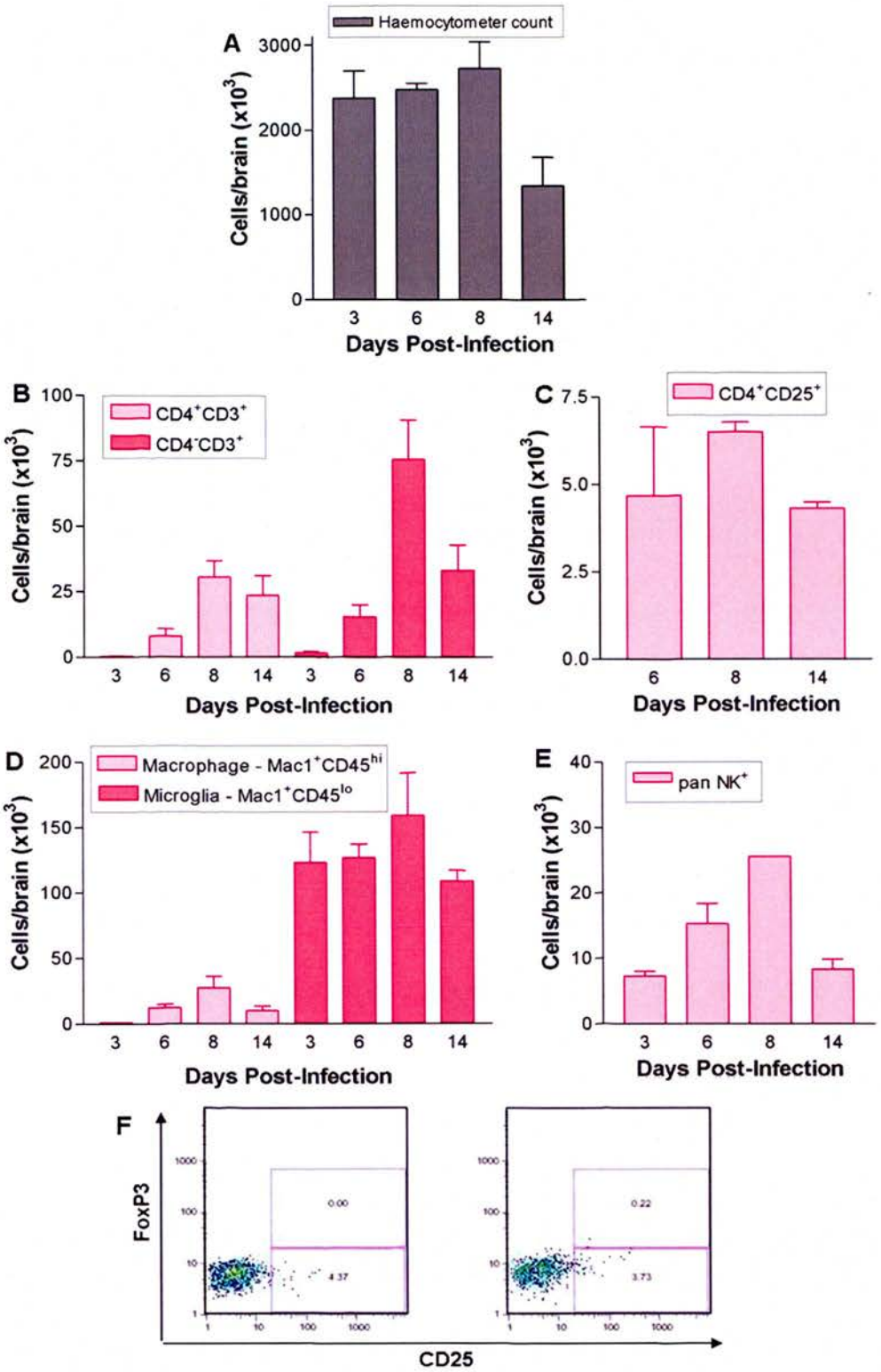

Figure 27. Phenotype and mean number of cells in the CNS of BALB/c mice (n=5/time point) infected with SFV. Cells were surface stained with labelled antibody and analysed by FACSCalibur. **A-** Haemocytometer count, **B-** CD3 and CD4 staining of lymphocytes, **C-** CD4 and CD25 staining of lymphocytes, **D-** Mac1 and CD4 staining of lymphocytes, **E-** Dx5 staining (pan NK marker) of lymphocytes, **F-** Dot plot of isotype control (left) and FoxP3 (right) with CD25 staining of $CD4^+$ lymphocytes. Error bars represent the SEM

Summary of findings

- Mice lacking $CD4^+$ T-cells are unable to make a complete antibody response to SFV
- Mice lacking $CD4^+$ T-cells are unable to clear infectious virus or virus RNA from the brain
- Mice lacking $CD8^+$ T-cells show no difference in their ability to clear infectious virus from the brain compared to wild-type mice
- Mice lacking $CD8^+$ T-cells are significantly slower at clearing of SFV RNA from the brain compared to wild-type mice
- $CD8^+$ T-cells are sufficient to mediate the demyelinating lesions observed in SFV infected SCID mice that receive T-cells by adoptive transfer
- SFV can successfully infect cells in the CNS of C57Bl/6 mice

Discussion

To investigate the roles of different elements of the immune response in infectious disease, a multitude of animal models have been studied. In mice these include the naturally occurring mutations of *SCID* and *nu/nu* to genetically engineered disruption or insertion of specific genes. The possibilities to investigate the role of a particular immune mechanism in disease are sometimes numerous. Caution must be used in comparing results between studies, not only to allow for mouse strain and pathogen differences, but also to recognise that genetic changes which may be anticipated to give similar results may not do so. For example, following LCMV infection of β2 microglobulin KO ($\beta_2m^{-/-}$) mice and CD8a mice, CD8a mice survive persistently infected without brain immunopathology whereas 80% of $\beta_2m^{-/-}$ mice die. This disparity is due to the haplotype ($H\text{-}2^d$) of the $\beta_2m^{-/-}$ mice. In the absence of β_2m mice of this haplotype can still present a limited number of epitopes and in the case of LCMV, a fatal immunopathology develops (Fung-Leung, 1991b; Cook, 1995). In contrast the CD8a mice have no functional CTLs and do not develop immunopathology. For the discussion of this chapter, comparison of results is, where possible, limited to studies using the same mouse strains used in this thesis. Select examples of other mice have been included, where their inclusion aids understanding of the T-cell response in viral CNS infection.

The role of $CD8^+$ T-cells in CNS viral infections

The role of CD8 T-cells in SFV infection was examined by infecting CD8a mice and adoptively transferring $CD8^+$ splenocytes from SFV infected immunocompetent mice to SFV infected SCID mice. The former studied events in the absence of $CD8^+$ T-cells but on otherwise intact adaptive immune system; the latter studied events in the absence of an adaptive immune system except for the presence of activated $CD8^+$ lymphocytes. The aim of these studies was to address the following questions about the role of $CD8^+$ T-cells in SFV encephalitis: *Are $CD8^+$ T-cells sufficient to clear virus? Are $CD8^+$ T-cells sufficient to cause neuropathology? Are $CD8^+$ T-cells required to clear virus? Are $CD8^+$ T-cells required to cause neuropathology?*

Are $CD8^+$ T-cells sufficient to clear virus? 2/5 mice that received activated $CD8^+$ splenocytes (either IP or IC) had infectious virus in peripheral tissues 14 days post-

infection compared with 5/5 control SCID mice that received PBS. When $RAG1^{-/-}$ mice (which lack B- and T-cells) were inoculated with MHV and received transfers of immune $CD8^+$ T-cells, infectious virus loads were reduced but not cleared (Wu, 2000a). Further work using expanded MHV $CD8^+$ T cell clones (Yamaguchi, 1991; Stohlman, 1995) and IC adoptive transfer into infected immunocompetent BALB/c mice, increased the efficiency of virus clearance, but complete clearance was still not achieved. In TMEV infection, resistance to persistent infection is associated with, amongst others, MHC class I genes. It has been demonstrated that $CD8^+$ T-cells have an important role in virus clearance (Borrow, 1992a), however the transfer of $CD8^+$ T-cells alone into TMEV infected SCID mice is insufficient to clear virus infection (Rodriguez, 1996). In SFV infection the $CD8^+$ T-cells had an antiviral function, but alone were not sufficient to control viral spread. $CD8^+$ T-cells inoculated directly IC were more effective at clearing CNS virus RNA, but both groups of mice that received $CD8^+$ T-cells IC had detectable peripheral infectious virus. The inability of $CD8^+$ T-cells alone to clear virus is in keeping with the role of these cells in other neurotropic viruses.

Are $CD8^+$ T-cells sufficient to cause neuropathology? SFV infected SCID mice that received $CD8^+$ cells had lesions of demyelinating disease whereas control mice (that received PBS only) did not. This demonstrates the $CD8^+$ T-cells alone are sufficient mediate demyelination in SFV infection. The number of virus infected cells/brain section was also highest in the $CD8^+$ IP mice. It is possible that the increase in virus-positive cells is caused or initiated by $CD8^+$ T-cells. For example, infected cells lysed by $CD8^+$ T-cells could allow virus release into an antibody free environment, augmenting viral spread. The increase in virus spread could also account for the high amount of viral RNA in mice receiving $CD8^+$ cells IP. The increase in pathological changes observed in $CD8^+$ IP is consistent with previous work by Subak-Sharpe *et al* which demonstrated that antibody mediated depletion of $CD8^+$ T-cells in SFV infected BALB/c mice abrogated CNS demyelination. (Subak-Sharpe, 1993). The mechanism of demyelination was not investigated in this thesis but it is likely, given the propensity of SFV to replicate in oligodendrocytes that demyelination results from $CD8^+$ T-cell mediated destruction of infected oligodendrocytes.

In another neurotropic virus infection, LCMV, $CD8^+$ T-cells mediate a fatal immunopathology. When immune CTLs were transferred into LCMV infected,

irradiated or immunosuppressed wild-type mice, neuropathology and fatal disease occurred within 4 days. In contrast, transfer of the same cells to uninfected mice had no effect (Baenziger, 1986a; Dixon, 1987b).

Similarly, the pathogenic potential of $CD8^+$ T-cells in the CNS has been demonstrated in MHV infection. $CD8^+$ T-cells alone can mediate demyelination as demonstrated in $Rag1^{-/-}$ mice given $CD8^+$ enriched splenocyte transfers (Wu, 2000b). Conversely, IC transfer of CTL clones specific for MHV into BALB/c mice did not cause visible brain pathology (Stohlman, 1995). This could be due to rapid clearance of infectious virus from the CNS in this system, whereas $CD8^+$ T-cells administered IP take longer to track into the brain, allowing further virus spread in the CNS which makes the infection more difficult to clear and immune pathology more likely. The balance between immune response and virus persistence is an important determinant in whether demyelination occurs. This is elegantly demonstrated in TMEV infection where resistance and susceptibility to CNS virus persistence and CNS demyelination are genetically determined. In resistant mice (C57Bl/6) virus is rapidly cleared by $CD8^+$ T-cells and there is no brain pathology. In susceptible (SJL) mice there is a slow, weak $CD8^+$ T-cell response (Dethlefs, 1997b). BALB/cByJ mice can be made susceptible to TMEV infection by irradiation but the transfer of $CD8^+$ T-cells from a non-irradiated mice (resistant) confers protection (Nicholson, 1996). Both the strength and the specificity of the CTL response play a part in prevent or initiating demyelination in TMEV infection.

The work presented in this chapter has shown that $CD8^+$ T-cells alone are sufficient to produce lesions of demyelination following SFV infection. The adoptive transfer performed here was a small study and would benefit from increased group numbers, as it appears not all adoptive transfers were successful, as was evident from the huge range in pathological changes in the CD8 IP mice (Table 7).

To address the following questions: *Are $CD8^+$ T-cells required to clear virus? Are $CD8^+$ T-cells required to cause neuropathology?* Mice lacking $CD8^+$ lymphocytes (C57Bl/6 CD8a mice) were infected with SFV. However, as there were almost no demyelinating lesions detectable in wild-type C57Bl/6, unlike in other strains of mice (129 and BALB/c), this study was unable to determine if the lack of $CD8^+$ T-cells had an affect on demyelination. CD8a mice cleared infectious virus at the same rate as wild-type mice, however the rate of virus RNA clearance was significantly

slower (CD8a titres were higher at PID 14 and 21). It can be concluded that $CD8^+$ T-cells are not required for infectious virus clearance but they accelerate the clearance of virus RNA. The data are consistent with these from another alphavirus, SV, where CD8a mice also have a delay in virus RNA clearance but not infectious virus clearance (Kimura & Griffin, 2000). The difference in virus RNA clearance at 2 and 3 weeks between CD8a and wild-type mice did not persist at later time points (6 and 12 weeks PI).

A more prominent protective role for $CD8^+$ T-cells is apparent in MHV infected CD8a mice. CD8a mice are more susceptible to MHV infection (70% mortality at PID 12 compared to 20 % in wild-type) and mice surviving to PID 12 are all positive for infectious virus, whereas wild-type mice clear infection by this time. Similarly to SFV, MHV infected CD8a mice did not show increased CNS pathology (Lane, 2000). The importance of $CD8^+$ T-cells in reducing MHV infection is corroborated by delayed virus clearance in mice depleted of CD8 T-cells (Pearce, 1994) and $\beta 2m^{-/-}$ mice (Gombold, 1995).

Murray *et al* examined neuropathology in TMEV infected mice lacking $CD8^+$ T-cells, on both susceptible (SJL and PLJ) and resistant (C57Bl/6) backgrounds. CD8a mice on a C57Bl/6 background developed persistent infection and demyelinating disease. Susceptible CD8a mice (SJL and PLJ background) showed no difference in the extent of demyelinating disease however virus titres increased in CD8a SJL mice but not in PLJ mice (Murray, 1998d). $CD8^+$ T-cells contribute to resistance to TMEV and either their absence or the increased presence of virus allows demyelination to occur. In this system, in contrast to SFV, $CD8^+$ T-cells prevent demyelination by controlling virus; demyelination is enhanced in the absence of $CD8^+$ T-cells indicating that theses cells are not required to produce these lesions. LCMV infected CD8a mice also cannot clear infection. CD8a mice have a high virus titre following IC LCMV infection; however some pathological changes still occur in the absence of $CD8^+$ T-cells (Fung-Leung, 1991a). This has been attributed to $CD4^+$ T-cell mediated cytotoxicity via Fas ligation (Zajac, 1996).

The data from the CD8a mice demonstrates a function of the $CD8^+$ T-cells not evident in the adoptive transfer of $CD8^+$ splenocytes alone. The presence of $CD8^+$ T-cells aids the reduction of virus RNA in co-operation with the rest of the adaptive

immune response. The q-PCR allows more subtle changes in virus load to be observed than are detectable by plaque assay. Changes in infectious virus clearance and in neuropathology, observed in other neurotropic viruses, were not evident in SFV infected CD8a mice.

The role of CD4$^+$T-cells in CNS viral infections

MHCII$^{-/-}$ mice were used to compare levels of virus clearance and neuropathology to C57Bl/6 mice. Mice lacking CD4$^+$ T-cells appeared to clear infectious virus at the same rate as wild-type mice, but sampling at a later time point (PID 28) demonstrated that infectious virus was still present. As for wild-type C57Bl/6 mice, MHCII$^{-/-}$ mice had few pathological changes in their CNS. B-cell CD4$^+$ T-cell independent responses to viruses are also known to occur (Markowitz, 1993). This was not observed in SFV infection where the antibody response was weaker without CD4$^+$ T-cells present, as demonstrated by the low levels of SFV specific antibody in the MHC II$^{-/-}$ mice. A primary CD8$^+$ T-cell response can also develop in a CD4 independent manner (Bourgeois, 2002; Janssen, 2003). However, *Nu/nu* mice which lack CD4$^+$ and CD8$^+$ T-cells similarly cannot clear infectious virus in the CNS and do not develop demyelinating disease (Fazakerley & Webb, 1987). This indicates that the CD8$^+$ T-cell response that develops in MHC II$^{-/-}$ mice is not sufficient to clear infectious virus. CD4$^+$ T-cells are required directly or indirectly (via antibody isotype switching) for clearance of SFV.

Unlike in SFV infection, following MHV infection CD4$^+$ T-cells were found to have a pivotal role in accelerating neuropathology; MHC II$^{-/-}$ mice had significantly less inflammation and demyelination than C57BL/6 mice. In this model system MHC II$^{-/-}$ mice had a significant reduction in the number of activated macrophages/microglial cells in the brain compared to wild-type mice, suggesting a role for CD4$^+$ T-cells in myelin destruction.

In persistent TMEV infection, CD4$^+$ T-cells protect against neurological deficits and as with SFV are essential for virus clearance. Murray *et al* investigated the role of CD4$^+$ T-cells in both resistant and susceptible MHCII$^{-/-}$ mice following TMEV infection. Resistant mice lacking CD4$^+$ T-cells had virus persistence and demyelination during the chronic stages of disease. Susceptible mice lacking CD4$^+$

T-cells had drastically increased demyelination. Thus both strains of MHCII$^{-/-}$ mice had increased neurological deficits (Murray, 1998c).

The flow-through cells adoptively transferred to the SFV infected SCID mice, were enriched for CD4$^+$ T-cells, however they still only amounted to 21% of the cells present. Histological studies showed many macrophage-like cells present in the CNS of mice that received flow-through cells. The role of macrophages in viral infection is not addressed here, but a critical role in both innate immunity and neurodegeneration has been suggested for macrophages in alphavirus infection (Carmen, 2006). It is not possible to reach conclusions about the role of CD4$^+$ T-cells alone in SFV infection from the adoptive transfer study described here. The inclusion of an adoptive transfer of immune CD4$^+$ positively selected splenocytes to SFV infected SCID mice would be necessary to determine if CD4$^+$ T-cells alone are pathogenic. This would also be required to determine whether CD8 T-cells are required for pathogenesis (since CD4$^+$ T-cells may also mediate demyelination) and to determine the extent to which CD4$^+$ cells can clear virus in the absence of B-cells, antibody and CD8$^+$ T-cells.

SFV can productively infect the CNS of C57Bl/6 mice

Many of the commercially available KO mice are on the C57Bl/6 background. It is apparent that the virus induced neuropathology in these mice is not the same as that observed in mice on a BALB/c or WT-129 background (Gates, 1984). Earlier work with SFV A7(74) infection of C57Bl/6 mice conflicts with the results presented here, as demyelinating lesions were evident in C57Bl/6 mice (Mokhtarian & Swoveland, 1987). These differences could be due to subtle changes in the mouse strain due to different suppliers; additionally different viral stocks may cause different pathology.

Differences in susceptibility to viral CNS infection between mouse strains have been studied a great deal. As noted C57Bl/6 mice are resistant to TMEV infection and demyelinating disease, whereas SJL/J mice succumb. This difference in susceptibility has been accounted for by the efficacy of the CTL response and this resistance is in part genetically determined by the H2 locus (Rodriguez, 1996; Lin, 2002). In MHV infection, allelic differences in the viral receptor can account for differing susceptibility to infection between SJL mice (resistant) and BALB/c (Dveksler, 1993; Ohtsuka & Taguchi, 1997b). Using VLPs we inoculated C57Bl/6

mice IC to determine if a productive infection can occur in the oligodendrocytes of these mice. This was indeed the case, with many VLP cells visible in white matter tracts in CNS sections at both PID 5 and 14. There was an abundance of virally infected cells at early time points and lower levels at PID 14, indicating that virus is not cleared rapidly. It must be noted that immunostaining was not performed for viral proteins; and so the remaining fluorescence could be from GFP alone. In conclusion, the oligodendrocytes of C57Bl/6 mice can become infected with SFV; the reason for difference in demyelination compared to BALB/c or 129 mice must therefore have another explanation, perhaps a more efficient antibody or less cytolytic effector response.

The phenotype of cells present in the CNS of SFV infected mice

In order to directly study the magnitude and quality of the cellular immune response in the CNS, the phenotype of the inflammatory infiltrate in C57Bl/6 mice was investigated by FACS analysis. Microglia are resident in the CNS, and upon stimulation by trauma, inflammation or infection they up-regulate markers such as CD4, MHC class I and CD45 (but are still distinguishable from $CD45^{hi}$ macrophages) and proliferate (Sedgwick, 1991a). The 5-fold increase in microglia (CD11b $CD45^{lo}$) observed between day 3 and day 8, the peak of CNS inflammation is consistent with this. Very low numbers of macrophages are present in the resting CNS. At the peak of infection (PID8) there was an approximately 200-fold increase in macrophage (CD11b $CD45^{hi}$) numbers in the CNS and their subsequent decrease was equally efficient.

For a specialised tissue lacking lymphoid architecture, the influx of T-cells into the CNS over the course of infection was impressive. There was an increase of 165-fold for $CD4^+$ T-cells and 185-fold for $CD8^+$ T-cell, demonstrating that when required, these cells efficiently enter the CNS. Like the macrophage population their disappearance was equally rapid. At PID 8 there were twice as many $CD8^+$ T-cells as $CD4^+$ T-cells, highlighting the importance of these effectors in viral infection.

The B-cell population within the CNS was examined by $CD19^+$ staining. The $CD19^+$ population at PID 8 and 15 was small but stable unlike the other cell populations it did not contract. Subpopulations of B-cells that mature into antibody secreting

plasma cells down-regulate CD19 and these cells would not have been detected in this study. It has been suggested that antibody secreting plasma cells remain within the CNS for several months following SFV infection (Parsons & Webb, 1982g). It would be of interest to stain for this cell type to confirm if plasma cells are indeed present in the CNS following SFV infection. The presence of antibody secreting plasma cells has implications for virus persistence and whether persistent virus is kept under constant control by antibody.

Taking into account the procedural problems of the experiments comparing the inflammatory cell phenotype in C57Bl/6 and BALB/c mice, overall there did not appear to be any difference in the magnitude or the kinetics of cellular infiltrates between the mouse strains. The reasons for different SFV induced CNS pathology between these mice strains may not be related to the immune response or, more likely, may be due to more subtle immune differences such as the T-cell epitopes recognised or the cytokine balance.

The phenotyping study allowed investigation of the presence of T-regs and differences between mouse strains. The presence of T-regs has been observed in EAE. Following EAE induction there is an accumulation $CD4^+CD25^+$ T-cells in the CNS, which is co-incident with disease resolution. The detected $CD4^+CD25^+$ T-cells secrete IL-10 and resemble peripheral T-regs. CNS derived T-regs could mediate activity *in vitro*, as EAE was exacerbated when these cells were depleted and their transfer to mice with EAE caused disease resolution (McGeachy, 2005).

T-regs have been shown to be present in a number of chronic viral infections. Patients infected with hepatitis C virus who develop a chronic infection have a population of specific T-regs that controls the immune response to hepatitis C. However it is unclear whether these T-regs are the cause or the result of chronic infection (MacDonald, 2002; Sugimoto, 2003). The presence of T-regs in the model virus for immunological studies, LCMV, has yielded little information. The depletion of $CD4^+CD25^+$ cells has no effect on the LCMV specific immune response and *ex vivo* functional studies of these cells has not shown regulatory activity (Rouse, 2006a).

We looked for similar cells in viral encephalitis at PID 6, 8 and 14. A population of $CD4^+CD25^+$ cells was present; however the number of cells positive for FoxP3 (the

current marker for T-regs) was very low and without further experiments it was not possible to say whether this indicated the presence of T-reg cells. As mouse strain affects CNS pathology, it was thought this could be due to a difference in T-regs. SFV infection resolves rapidly, co-incident with the activation of the adaptive immune response which induces an effective antibody response that rapidly neutralises virus. It is possible that T-regs do not have a role in this infection as the virus is cleared rapidly.

There is more than one sort of T-reg and not all express FoxP3. A number of T-regs secrete IL-10 and anti-inflammatory cytokines (Groux, 1997a). To further investigate whether T-regs have a role in controlling the immune response to viral encephalitis it would be necessary to deplete the $CD4^+$ $CD25^+$ subset from SFV infected mice and observe the effect of this depletion on the strength and pathogenicity of the immune response.

The studies here demonstrate that $CD4^+$ T-cells are required for virus clearance. While virus RNA is cleared at a slower rate in the absence of $CD8^+$ T-cells, virus clearance is not affected in the long-term. Therefore $CD8^+$ T-cells contribute but are not required for CNS virus clearance. In contrast $CD8^+$ T-cells are sufficient to induce demyelination in the CNS following SFV infection, but further investigation is necessary to if they are required.

Chapter 4:

The role of selected $CD8^+$ T-cell mediators in SFV infection

Contents Page

Introduction

Previous published studies using antibody depletion of T-cell subsets suggest that $CD8^+$ T-cells have a role in the pathogenesis of demyelinating lesions found in SFV infection (Subak-Sharpe, 1993) and the studies in chapter 3 confirm that these immune cells alone are sufficient; however it is not known which specific $CD8^+$ T-cell mediators cause these lesions.

In general perforin-induced apoptosis is the predominant mechanism for CTL elimination of virus infected cells (Kagi, 1995e). CTLs specifically recognise a MHC-peptide complex on an infected cell and secrete a cytolytic granule containing perforin and granzymes (A and B); this is then endocytosed by the target cell where it activates apoptosis eliminating the infected cell (Trapani & Smyth, 2002a). A second mechanism, the upregulation of FasL on CTLs also allows these cells to initiate an apoptosis cascade on target cells, by binding to Fas. FasL can be targeted to virus infected cells after TcR engagement with MHC on infected cells (Lynch, 1995). Neurons protect themselves against perforin mediated lysis by the expression of FasL which binds to Fas on the attacking CTL and transmits an inhibitory signal, thereby preventing granule exocytosis (Medana, 2001). Apoptosis in neurons can be induced if Fas is expressed on the neuron, however this needs to be coincident with MHC I expression, which is thought only to be expressed on damaged neurons (Medana, 2000; Redwine, 2001). Other cells of the CNS are vulnerable to perforin-induced damage; in SFV infection demyelination has been proposed to occur as a result of immune mediated damage of infected oligodendrocytes (Fazakerley & Walker, 2003).

CTLs also secrete IFNγ, which can stimulate release of cytotoxic factors from macrophages, upregulate MHC expression on many cell types and induce anti-viral defences within cells (Farrar & Schreiber, 1993). This non-cytolytic mechanism for virus clearance is of particular interest in the CNS. Terminally differentiated neurons, which are relatively resistant to apoptosis, can respond to IFNγ and this may be an important mechanism for clearing virus (Neumann, 1997; Allsopp & Fazakerley, 2000). IFNγ can be synthesised by neurons (Olsson, 1994) and been

shown to be effective in clearing viruses from oligodendrocytes (Parra, 1999b). IFNγ levels peak relatively late in SFV infection (PID 6), whereas type I IFNs (IFN α and β) are essential for early virus control (Muller, 1994; Mokhtarian, 1996). Some anti-viral genes are upregulated by type I or type II IFNs, and some respond to both.

The role of $CD8^+$ T-cell mediators, IFNγ, perforin and Fas, was examined to see which specific mediators were crucial for virus clearance and which for generating the lesions of demyelination. IFNγ, perforin and Fas^{lpr} KO mice were infected with SFV and virus clearance (infectious and virus RNA) and neuropathology were compared to wild-type mice. The ability of IFNγ to substitute for the protective type I (α/β) IFN response was also studied.

Objectives

- Compare virus clearance and CNS histology following SFV infection in $IFN\gamma R^{-/-}$ mice with wild-type mice
- Determine if recombinant IFNγ can protect SFV infected $IFN\alpha/\beta^{-/-}$ mice
- Compare virus clearance and CNS histology in $perforin^{-/-}$ mice with wild-type mice following SFV infection
- Compare virus clearance and CNS histology in Fas^{lpr} mice with wild-type mice following SFV infection

Results

Mice lacking a functional IFNγ system are slower at clearing viral RNA in the short term than wild-type mice

To determine the role of IFNγ in SFV clearance, IFNγR$^{-/-}$ (129 genetic background) and wild-type (WT-129) mice aged 4-6 weeks (mixed sex) were infected IP with SFV A7(74). Inoculate for this experiment was titred at a later date and found to be lower than originally thought (log_{10} 2x10^2 compared to log_{10} 5x10^3 pfu/ml). Infectious virus in the brain was determined at PID 3 and 7 (Figure 28). The WT-129 mice had a higher mean titre of infectious virus at both time points however these differences were not significant.

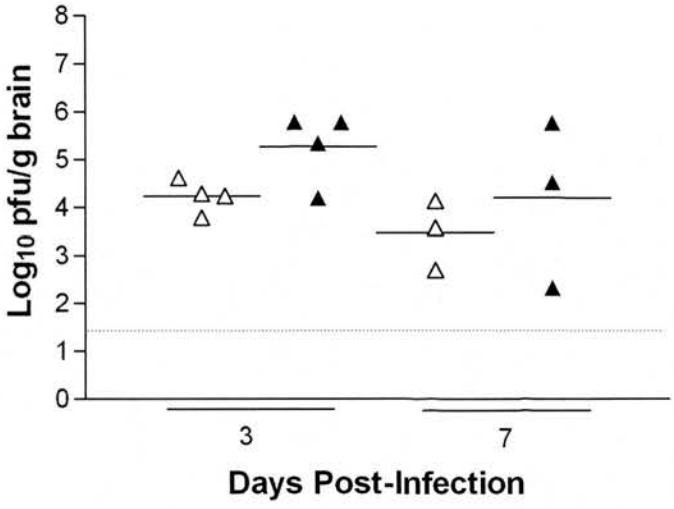

Figure 28. Infectious virus measured by plaque assay in SFV infected IFNRγ$^{-/-}$(△) and WT-129(▲) mice at PID 3 and 7. Horizontal bars indicate mean of the group, dashed line indicates the limit of detection for the assay.

To assess virus clearance at later time points, copies of SFV RNA in the brain were titred by q-PCR at 2, 3, 4, 12 and 24 weeks PI (Figure 29); infectious virus is generally not detectable after PID 10. The mean number of virus RNA copies at each time point was higher for IFNγR$^{-/-}$ mice compared to wild-type mice. The difference between the groups was significant at post-infection week (PIW) 2

(p=0.0286, by Mann-Whitney test). The difference between the group mean of the mutant and wild-type mice decreased over time (Log_{10} 0.76 at PIW 2 to Log_{10} 0.2 at PIW 24). This indicates that IFNγ has a role in the clearance of viral RNA, as mice lacking functional IFNγ clear virus RNA from the CNS more slowly. The effect is similar to that observed in CD8a mice (Chapter 3, Figure 14).

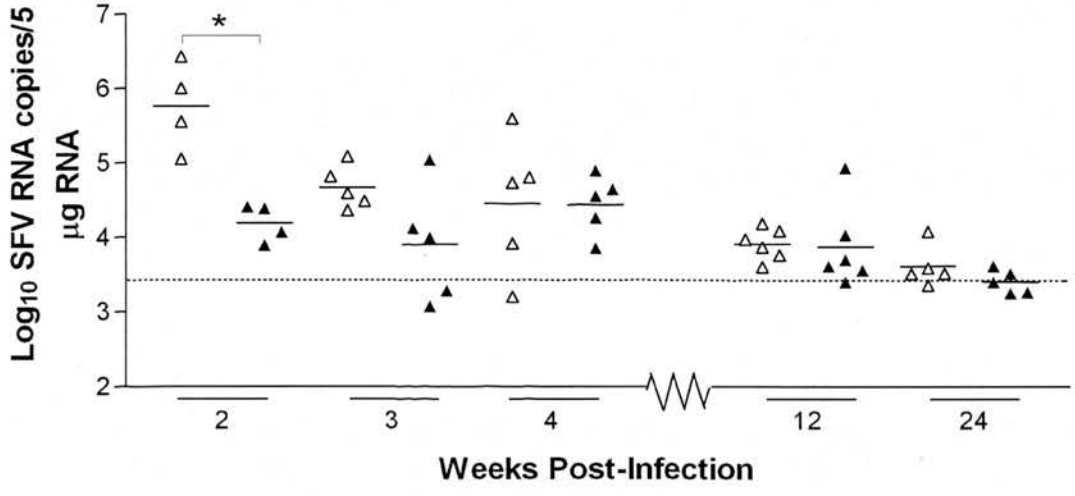

Figure 29. SFV RNA copies measured by quantitative PCR in brain tissue at 2 – 24 weeks PI in IFNγR$^{-/-}$(△) and WT-129(▲) mice infected with SFV A7(74). * indicates significant difference ($p<0.05$), data were analysed by Mann-Whitney test. Horizontal bars indicate mean of the group, dashed line indicates the limit of detection for the assay.

IFNγR$^{-/-}$ mice display the same neuropathology as wild-type mice during SFV infection

To examine CNS histology a stain for myelin (luxol fast blue) and a nuclear counterstain (cresyl fast violet) were used. Sections (5µm paraffin processed) were scored coded on a scale of 1 to 4 for inflammation (cell infiltration), demyelination (loss of luxol fast blue staining) and microcystic change (tissue vacuolation and destruction) using the criteria described in histological techniques in the materials and methods chapter.

The scoring of histology sections is quite subjective, and the sections scored only represent a snapshot of the histological changes in the CNS. There was very little difference between the mean scores of the two experimental groups (no significant

difference by Mann-Whitney test ,Figure 30). WT-129 mice had a higher mean inflammation score (2.15) compared to IFNγ-R$^{-/-}$ mice but the demyelination and microcystic change scores were similar. These results show that IFNγ is not required for demyelination, inflammation or microcystic change in SFV infected mice.

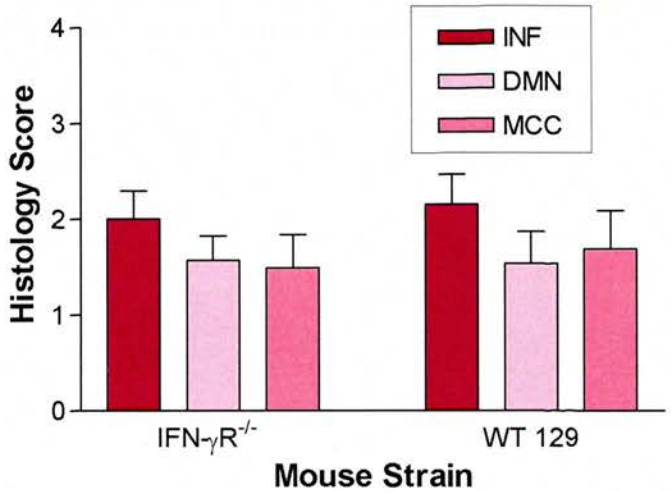

Figure 30. Pathology scores in SFV infected IFNγR$^{-/-}$ mice and WT-129 mice at PID 14 and 21 (data combined). Results are the means scores for at least 9 sections from at least 7 mice. Error bars represent the SEM. **INF** – inflammation, **DMN** – demyelination and **MCC** – microcystic change.

Recombinant IFNγ offers some protection to SFV infected IFNα/β receptor knockout mice

There are many type I IFNs, all of which bear structural homology and bind a single IFN receptor. There is only one type II IFN, IFNγ. IFNs bind to their cognate receptors and initiate a signalling cascade, involving the JAK family of tyrosine kinases and STAT family of transcription factors, which leads to the transcriptional induction of the IFN-stimulated genes (ISGs). This results in transcriptional shutdown, inhibition of cellular protein synthesis and RNA degradation. ISGs can be stimulated by Type I or Type II IFNs. ISGs that are preferentially induced by IFNα/β but not IFNγ include 2'5'-oligoadenylate synthetase 1, MxA and MxB genes. ISGs preferentially induced by IFNγ include IFN regulatory factor-1, IFN inducible protein-30, and class II transactivator. ISGs that are responsive to all IFNs include MHC Class I genes, STAT 1 and guanylate binding protein (Der, 1998a).

Type I IFNs are crucial in early virus control during SFV infection as they are upregulated within hours of infection. IFNγ, which is produced by T-lymphocytes and NK cells, acts later in infection and following SFV infection its levels peak at PID 6 (Mokhtarian, 1996).

Type I IFN receptor knock-out (IFNα/βR$^{-/-}$) mice have a 100% mortality rate following infection with SFV highlighting the importance of this innate defence in anti-viral protection (Muller, 1994) and demonstrating that an intact IFNγ system is insufficient for protection. In contrast, IFNγR$^{-/-}$ mice cleared infectious virus at the same rate as wild-type mice (Figure 28) and did not show increased mortality, demonstrating that the type II IFN system is not required for protection. It is possible that the failure of the IFNγ system to protect IFNα/βR$^{-/-}$ mice from SFV is related to the timing of this response.

To determine if mice with no functional type I IFN system could be protected by the early presence of IFNγ, SFV infected IFNα/βR$^{-/-}$ mice were given recombinant IFNγ. Two groups of IFNα/βR$^{-/-}$ mice (n=6/group) age 4-6 weeks were infected IP with $5x10^3$ pfu of SFV A7(74), one infected group also received 10^4 IU units of recombinant IFNγ (ProSpec-Tany TechnoGene Ltd) in 0.1 ml PBSA (0.1%) 5 hr post-infection, and every subsequent 24 hr for 6 days. A control (uninfected) group received recombinant IFNγ at the same time as the infected group (Figure 31).

Mice that were infected with SFV but did not receive IFNγ were extremely sick at PID 1 and were euthanised. The two other groups of mice were healthy at this time point. At PID 3 mice that were infected with SFV and had received 3 inoculations of recombinant IFNγ were found dead; uninfected mice that received recombinant IFNγ alone were still healthy at end of the experiment (PID 6). These results indicate that IFNγ offers partial protection against SFV infection, however this was only sufficient to protect mice for 48 hr longer than mice that did not receive recombinant IFNγ. This experiment could be repeated with different timings and concentrations of recombinant IFNγ to determine if the doses and timing alter the level of protection. Nevertheless, the data show that IFNγ can provide protection against this virus.

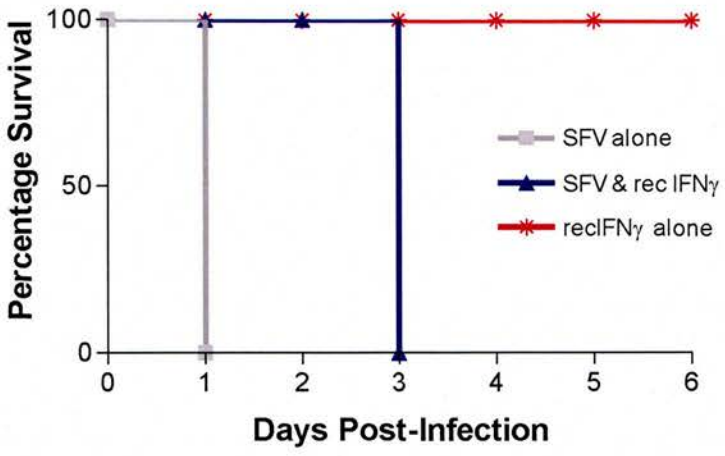

Figure 31. Survival curve showing survival rates of SFV infected IFNα/βR$^{-/-}$ mice and the effects of recombinant IFNγ. IFNα/βR$^{-/-}$ mice were infected with 5x10^4 PFU of SFV (grey square and blue triangle) and one group was also given 10^4 IU units of recombinant IFNγ (blue triangle) at 5 hr PI and every subsequent 24 hr. A third control group of mice received recombinant IFNγ alone (red star). **recIFNγ** - recombinant IFN γ.

Perforin$^{-/-}$ mice do not have reduced SFV infectious virus or virus RNA clearance

To determine the role of perforin in SFV clearance, perforin$^{-/-}$ mice (on a C57Bl/6 background) and C57Bl/6 mice (n=18) aged 4-6 weeks (mixed sex) were infected IP with SFV A7(74). Infectious virus and virus RNA titres were assayed and paraffin sections from the brain were prepared for histological scoring.

Brain sections from mice at PID 14 and 21 were stained with luxol fast blue and cresyl fast violet and scored for pathological changes (Figure 32). Both groups showed low levels of inflammation (scores of between 0.7 – 1.1) and almost no demyelination or microcystic change. There was no significant difference in CNS pathology, between perforin$^{-/-}$ and wild-type mice (analysed by Mann-Whitney test). The lack of microcystic change and demyelination confirms the findings on this genetic background in chapter 3.

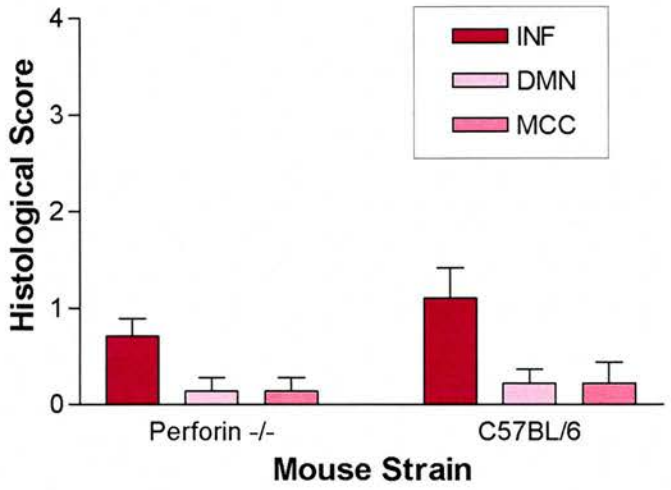

Figure 32. Histological scoring for inflammation, demyelination and microcystic change in the brains of Perforin$^{-/-}$ and C57Bl/6 mice. Results are the means scores for at least 9 sections from at least 7 mice. Error bars represent the SEM. **INF** – inflammation, **DMN** – demyelination and **MCC** – microcystic change.

Infectious brain virus titres were compared in perforin $^{-/-}$ mice (n=8) and C57Bl/6 mice (n=8) to examine if perforin has a role in early virus clearance (Figure 33). The mean virus titres were different between the groups with higher titres at PID 3 and PID 7 in the perforin$^{-/-}$ mice, however these differences were not significant ($p>0.05$, by Mann-Whitney test).

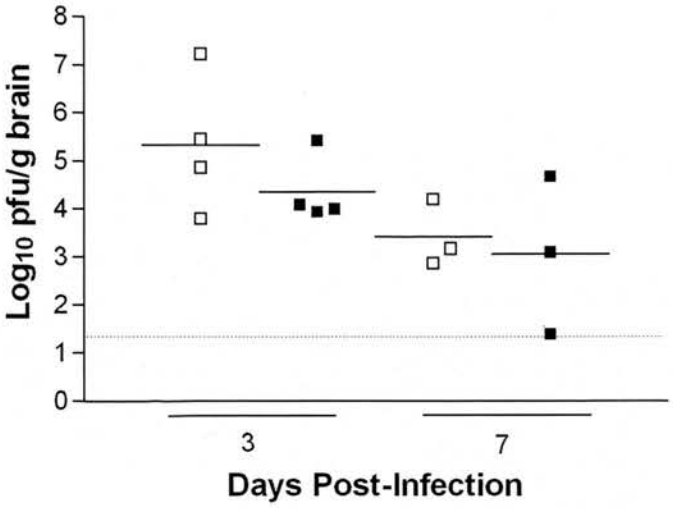

Figure 33. Infectious virus measured by plaque assay in SFV infected perforin$^{-/-}$ (□) and C57Bl/6 (■) mice at PID 3 and 7. Horizontal lines indicate mean of the group, dashed line indicates the limit of detection.

Brain virus RNA levels were also compared by q-PCR between perforin $^{-/-}$ mice (n=8) and C57Bl/6 mice (n=8), after infectious had been cleared at PID 14 and 21 (Figure 34A). Although there was an apparent difference in RNA virus copies at PIW 2 (sample size too small for Mann-Whitney analysis), this result is counterintuitive with the greater clearance occurring in the perforin$^{-/-}$ mice. It is difficult to hypothesise why a lack of perforin might result in greater virus clearance; however the situation was reversed at PIW 3 where wild-type mice had apparently lower virus RNA titres. Repeating this experiment with increased numbers of mice would be necessary to elucidate if there really are differences between these groups. If the data from both time points is pooled, thus comparing virus RNA load between the groups at 2-3 weeks PI, there is no significant different ($p= 0.535$, Mann-Whitney test) between the groups.

Virus RNA clearance was examined over a longer period of time. Perforin$^{-/-}$ mice (n=10) and C57Bl/6 mice (n=10) were infected and virus RNA titred at PIW 6 and 12 (Figure 34B). There was no significant difference in virus titre between the groups at either time point (as analysed by Mann-Whitney test). The group means at both time points were on or near the limit of detection of the assay.

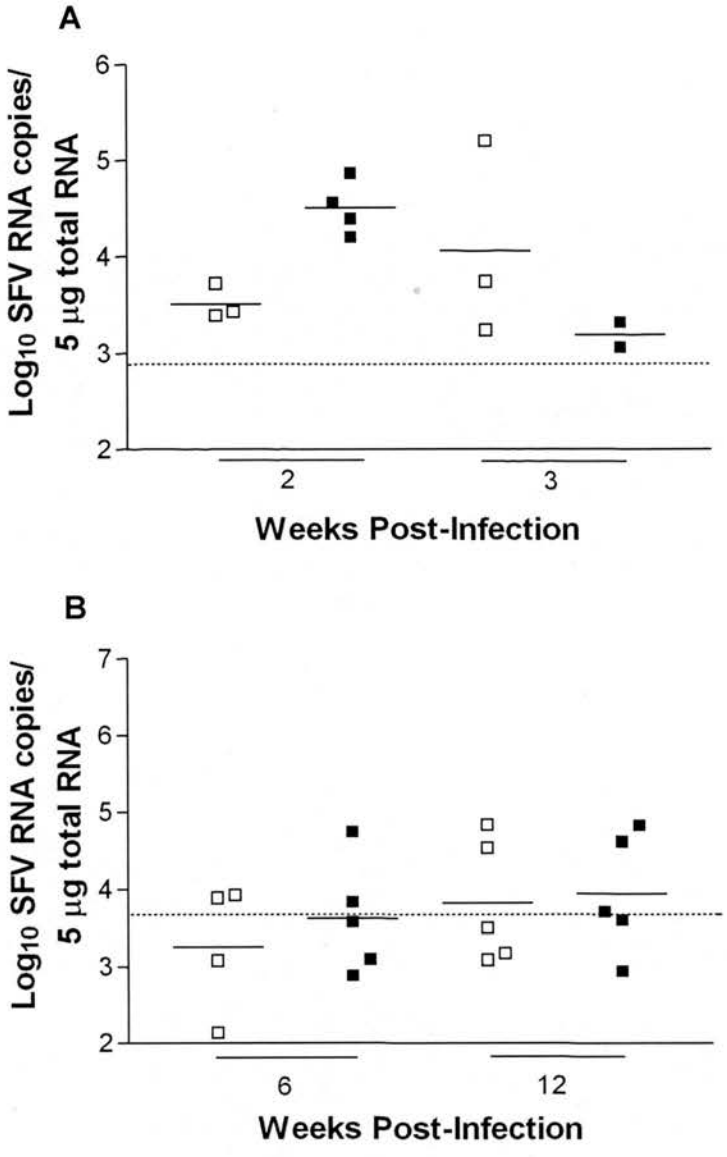

Figure 34. **A** - Virus RNA load of SFV in SFV infected perforin$^{-/-}$ mice (□) and C57Bl/6 (■) mice at PID 14 and 21. **B** - Long term clearance of SFV virus RNA from SFV infected perforin$^{-/-}$ (□) and C57Bl/6 (■) mice. Horizontal bars indicate mean of the group, dashed line indicates the limit of sensitivity for the assay.

FAS^{lpr} mice

The third CD8$^+$ T-cell mediator investigated was Fas, which when bound by its cognate ligand initiates programmed cell death. The role of Fas in neuropathology and in virus clearance in SFV infection was investigated in mice with a mutation in their Fas gene (Fas^{lpr}, n=30) and C57Bl/6 mice (n=30), age 4-6 week (mixed sex).

Mice lacking Fas showed no significant difference (as analysed by Mann-Whitney test) from wild-type mice in their clearance of infectious virus , although 50% (2/4) of the Fas^{lpr} mice sampled on PID 7 cleared infectious virus as compared to 25% (1/4) of C57Bl/6 mice. By PID 10, 100% (5/5) of mice from both groups had no infectious virus detectable.

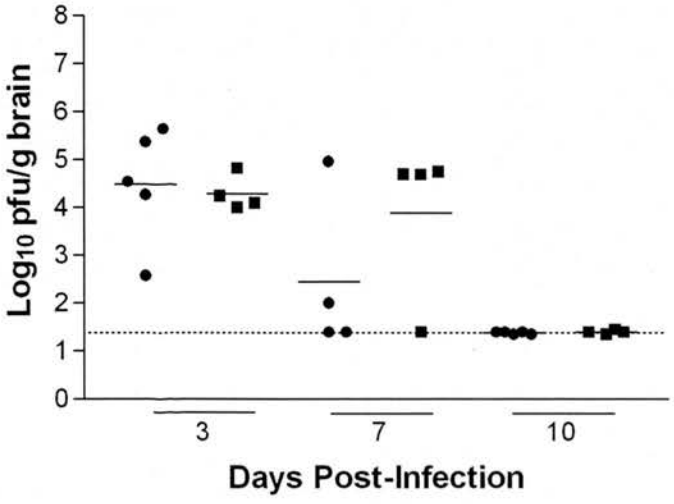

Figure 35. Infectious virus measured by plaque assay in SFV infected FAS^{lpr} mice (●) and C57Bl/6 mice (■) at PID3 and 7. Horizontal lines indicate mean of the group, dashed line indicates the limit of detection.

Virus RNA levels in SFV infected Fas^{lpr} mice at PIW 2 to 4 were quantified by q-PCR (Figure 36). Virus RNA levels decreased with similar kinetics in both the mutant and wild-type mice, with no significant difference between the groups (as analysed by Mann-Whitney test). Consistent with findings in the wild-type mice, virus RNA was still detectable 4 weeks post-infection in the Fas^{lpr} mice.

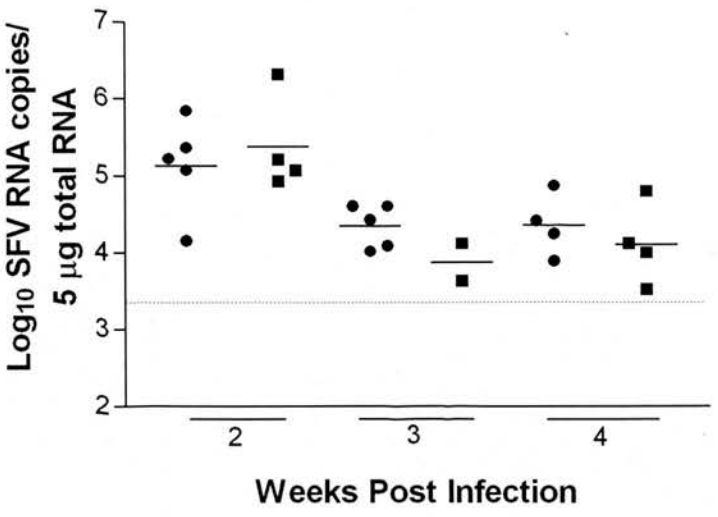

Figure 36. Clearance of SFV RNA from the CNS of FAS^{lpr} mice (●) and C57Bl/6 mice (■) at PID 14, 21 and 28. Horizontal bars indicate mean of the group, dashed line indicates the limit of sensitivity for the assay.

Brain sections from Fas^{lpr} mice and C57Bl/6 mice were scored for neuropathological changes at PID 14. High levels of inflammatory infiltrates were present at a (mean histological score-2.6) in Fas^{lpr} mice as well as low levels of demyelination (mean score – 1.1) and microcystic change (mean score – 0.9). C57Bl/6 mice had a lower level of inflammation, significantly less demyelination ($p=0.03$, Mann-Whitney test) and very little microcystic change. The lack of Fas appears to have increased the inflammation and demyelination relative to that observed in the C57Bl/6 background strain. This was an unexpected finding as no other knock-out strain (CD8a, MHC II$^{-/-}$ and perforin$^{-/-}$) on the C57Bl/6 background showed any increase in neuropathology.

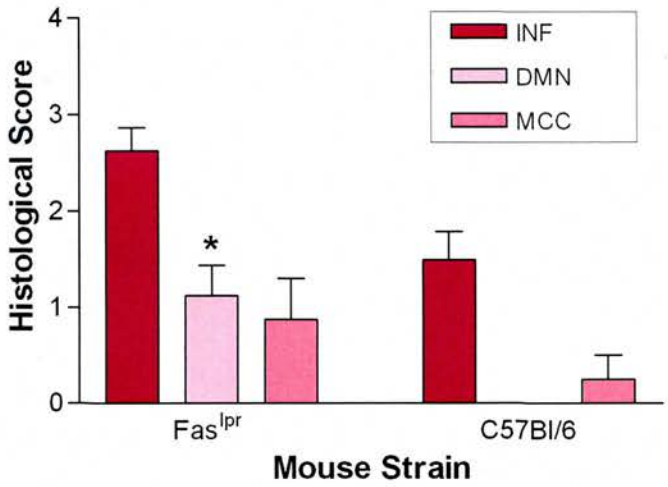

Figure 37. Histology scoring for inflammation, demyelination and microcystic change in Fas^{lpr} mice and C57Bl/6 mice. Results are the means scores for at least 6 sections from at least 4 mice. * indicates significant difference ($p<0.05$), data were analysed by Mann-Whitney test. Error bars represents the SEM. **INF** – inflammation, **DMN** – demyelination and **MCC** – microcystic change.

Summary of findings

- IFNγR$^{-/-}$ mice show no difference in their ability to clear infectious virus from the brain as compared to wild-type mice
- IFNγR$^{-/-}$ mice have slower clearance of SFV RNA from the brain as compared to wild-type mice
- IFNγ is not required for the development of demyelinating brain lesions in SFV infected mice
- Recombinant IFNγ transiently protects SFV infected IFNα/βR$^{-/-}$ mice
- Neither perforin nor Fas are required for clearance of infectious virus or viral RNA from the brain
- SFV infected Fas^{lpr} mice have increased CNS demyelination compared to wild-type mice

Discussion

The findings of the previous chapter demonstrated that mice on a C57Bl/6 background that lack $CD8^+$ T-cells are significantly slower than controls at clearing virus RNA from the brain. $CD8^+$ T-cells have previously been shown to be required for demyelination in the CNS of BALB/c mice (Subak-Sharpe, 1993) and chapter 3 showed that these cells alone provided a sufficient immune response to generate these immunopathological lesions To separately analyse the role of the $CD8^+$ T-cell mediators IFNγ, perforin and Fas in SFV clearance and CNS pathogenesis, mice deficient for these effectors were studied.

IFNγ is a potent anti-viral cytokine, in other CNS virus infections, such as vesicular stomatitis (Indiana) virus and measles virus, where it is essential for virus clearance (Finke, 1995; Komatsu, 1996a; Patterson, 2002a). In the present study during the first few weeks PI mice lacking a functional IFNγ system were slower at clearing viral RNA from their brain. However, by 3 and 6 months post-infection mice lacking a functional IFNγ system and wild-type mice had low levels of or no virus RNA detectable above background levels indicating that IFNγ was not required to establish clearance or very low levels of virus RNA. The results in SFV infected IFNγ knockout mice also showed that IFNγ is not necessary for the inflammation, demyelination and microcystic changes observed. This supports data from Keogh *et al*, where SFV A7(74) infected $IFN\gamma R^{-/-}$ mice showed increased neuronal necrosis at PID 7 after which pathological changes were similar to those of wild-type mice with no differences observed in the severity of demyelination (Keogh, 2003). In contrast in a neurovirulent alphavirus infection, neuroadapted SV in C57Bl/6 mice, a pathogenic role for IFNγ was evident. Mortality was reduced to 10% in $IFN\gamma R^{-/-}$ compared to 93% in C57Bl/6 mice. The authors suggest that the pathogenic effect of this cytokine is possibly a result of its regulatory effect skewing towards a TH1 response or recruitment and activation of macrophages that can secrete toxic mediators and directly damage neurons (Rowell & Griffin, 2002).

The role of IFNγ in clearance of alphaviruses was highlighted by the construction of a recombinant SV expressing IFNγ. This virus was inoculated into SCID mice; virus

was cleared from neurons in the spinal cord and brainstem but not from cortical neurons, this is a similar pattern of infection as seen in μMT mice. Wild-type mice typically clear SV, so it can be concluded that antibody is required for clearance and IFNγ alone is insufficient (Binder & Griffin, 2001b).

The absence of IFNγ in other CNS infections has more severe consequences than those seen in SFV infection. TMEV infection of IFNγR$^{-/-}$ mice, on a genetic background normally resistant to virus persistence, results in persistent infection. IFNγ protects neurons against TMEV induced damage (Rodriguez, 2003). MHV infected IFNγR$^{-/-}$ mice show increased mortality and clinical signs. A cell specific effect is also evident, as there is increased MHV antigen in oligodendroglia (Parra, 1999c). The anti-viral state induced by IFNγ is not the only function observed in MHV. MHC upregulation is suboptimal in the absence of IFNγ and this deficiency affects MHC I mediated effector mechanisms, such as perforin mediated lysis (Bergmann, 2003).

Recombinant IFNγ temporarily protected the SFV infected IFNα/βR$^{-/-}$ mice, as these mice lived to PID 3 whereas mice that did not receive the recombinant cytokine succumbed to infection within 24 hrs. Recombinant IFNγ has also been shown to be protective in measles virus encephalitis. 50% IFNγR$^{-/-}$ mice which normally succumb to fatal measles virus CNS disease, were protected by recombinant cytokine transfer (Patterson, 2002b). In SFV infection, recombinant IFNγ protection was short-lived and mice receiving IFNγ died after 3 days. This was not due to a toxic effect of the recombinant IFNγ, as mice receiving IFNγ alone remained healthy until the end of the experiment at PID 6.

There are many possible reasons why the mice that received IFNγ were not fully protected. IFNγ can initiate some but not all of the same response as type I IFNs. It is likely that in order to induce a fully protective response, many genes associated with type I IFNs must be upregulated. Administering IFNγ alone may not have induced certain genes required for protection. However, this was a small study with a single concentration of recombinant IFNγ and doses well-spaced out in time. This dose was similar to that used by others (Clark, 1987). This study was not

comprehensive enough to conclude that a type I IFN response is required however, it can be concluded that IFNγ can induce a protective response against SFV A7(74).

Perforin is a major effector mechanism of $CD8^+$ T-cells. $Perforin^{-/-}$ mice infected with SFV do not have any difference in the rate of infectious virus or virus RNA clearance. This is in contrast to other CNS virus infections where perforin has been shown to have an important role in virus clearance and protection. TMEV infected MHC $I^{-/-}$ or $perforin^{-/-}$ mice, on a genetic background resistant to TMEV persistent infection are susceptible to persistent infection and ongoing demyelination but have no neurological signs. This indicates that in TMEV infection and $CD8^+$ T-cells, perforin, are required for virus clearance and for the development of neurological deficits (Murray, 1998a). Following IC inoculation with LCMV, $perforin^{-/-}$ mice have milder disease and delayed mortality compared to wild-type mice. Following IP LCMV infection (which is cleared in wild-type mice) $perforin^{-/-}$ mice cannot clear virus (Kagi, 1995c). Expression of perforin in the absence of granzyme or Fas, demonstrates that perforin alone is insufficient to clear LCMV; perforin works synergistically with both granzyme A/B and Fas (Rode, 2004a).

West Nile virus (WNV) infected mice show CTL dependent virus clearance with mortality of about 30% in wild-type mice; virus is cleared in surviving mice. Knocking out perforin increases mortality to 78% and virus persists in mice surviving infection. WNV differs from the other CNS viruses discussed above due to the high cytopathic effect of WNV for neurons (Shrestha, 2006). Removing perforin allows the full pathogenic potential of the virus to be realised, therefore in this case the anti-viral benefits of perforin outweigh the risk potential immune mediated as perforin protects against direct virus mediated injury.

It has been shown that effector mechanisms in $CD8^+$ T-cells work synergistically, for example MHV infected mice require IFNγ for virus clearance and upregulation of MHC class I. However perforin works with IFNγ, leading to more effective clearance of MHC I expressing cells (Bergmann, 2003). Therefore knocking out single CD8 effectors may have a much less deleterious effect than the combined results of a double knock-out. The use of such double knock-outs would be of interest in SFV to determine the combined effects of $CD8^+$ T-cell mediators on virus

clearance and neuropathogenesis. Unfortunately, the perforin$^{-/-}$ mice are on the C57Bl/6 which is not susceptible to SFV induced demyelinating lesions (chapter 3). To investigate the role of perforin in SFV neuropathogenesis a perforin$^{-/-}$ mouse on a demyelination susceptible background (BALB/c, 129) is required.

C57Bl/6 FAS^{lpr} mice infected with SFV did not show any deficiencies in virus clearance (infectious or virus RNA) compared to wild-type mice. However, in mice lacking Fas demyelinating lesions were observed at PID 14. The control group of wild-type C57Bl/6 mice had no demyelination, neither did the CD8a or perforin$^{-/-}$ mice on this background.

TMEV infected Fas^{lpr} and FasL^{gld} KO(C57Bl/6) mice maintain resistance to persistent CNS infection indicating that the Fas system not essential for TMEV clearance (Murray, 1998a). LCMV is also cleared without measurable assistance from Fas/FasL (Kagi, 1995d). As noted previously, the role of Fas may be less important than other mediators such as IFN or perforin, however with (IP) LCMV infection, mice deficient in Fas and granzyme A/B did not survive acute infection whereas mice with perforin and Fas could control virus replication (Rode, 2004b). A combined role for perforin and Fas in virus clearance is also suggested in MHV infection (Parra, 2000).

CTL expansion after activation is controlled by AICD which is primarily regulated by FasL upregulation on activated T-cells. It is possible that in knocking out Fas expression in SFV infection, anti-SFV CTL populations did not contract and their increased numbers led to an increased size of the inflammatory infiltrates and the lesions of demyelination. The increase in CNS pathology observed requires further investigation to determine whether this is caused by a dysregulation of AICD induced by the lack of a functional Fas system. The data presented here demonstrates as in other CNS virus infections, Fas/FasL is not essential in SFV clearance. Whether it does have a role that is masked by functional redundancy remains to be elucidated.

This chapter aimed to examine the roles of IFNγ, perforin and Fas in both virus clearance and in modulating CNS neuropathology. It was shown that IFNγ increases the rate at which SFV RNA is cleared from the CNS and that recombinant IFNγ can

protect in IFNα/β$^{-/-}$ mice. However, IFNγ is not required for demyelination. Perforin was not required for virus clearance and its role in the development of demyelination lesions could not be answered due to the background strain of the perforin$^{-/-}$ mice. Fas was also not required for virus clearance but these studies highlighted that Fas may have a separate role in modulating CNS pathology by controlling numbers of activated T-cells.

Chapter 5:

The role of antibody in clearance of SFV from the CNS

Contents

Page

Introduction

Non-cytolytic mechanisms for virus clearance in the CNS have long been of interest, as they allow protection of terminally differentiated neurons from both cytolytic and toxic immune mediators while preventing the spread of the pathogen. The antibody response is an example of an immune mediator that can mediate its action in a non-lytic manner, and the importance of antibody in CNS virus clearance has long been of interest. Immunoglobulin class switching in B-cells is reliant on $CD4^+$ T-cell help and therefore the role of B-cells cannot be considered in isolation, as they are dependent on T-cells to attain full functionality.

Previous research in this laboratory (Fragkoudis, 2002) investigated the clearance of SFV in B-cell deficient (μMT) mice. These mice were unable to clear infectious virus or reduce virus RNA levels in the brain, demonstrating the importance of B-cells in virus clearance from this organ. Whereas this can most likely be ascribed to antibody, the results must be interpreted with more caution since abnormalities in dendritic and T-cell functions have also been observed in μMT mice. For example, following MHV infection of μMT mice deficiencies in T-cell virus specific response were detected (Bergmann, 2001). To focus on the role on antibody alone in SFV clearance and persistence, the adoptive transfer of HI serum to SFV infected SCID mice was investigated. SCID mice are homozygous for the *Prkdc*^{scid} mutation and lack both T and B cells due to a defect in V(D)J recombination (Bosma, 1983b). SCID mice are a good model to study the role of the acquired immune response in SFV infection. Avirulent SFV (A7(74)) establishes a persistent infection in this mouse strain (Amor, 1996). Passive transfer of anti-SFV antibody to SFV infected SCID mice can clear infectious virus from the blood and the brain; IgG is necessary for this function. A non-neutralising monoclonal antibody (mAb) also was able to reduce brain virus titres, by mechanisms unknown, but not viraemia in SCID mice. This work is supported by similar studies on another alphavirus, SV (Levine & Griffin, 1992). Using passive transfers of antibody to SV infected SCID mice, Levine *et al* demonstrated the crucial role of antibody in virus clearance and suggested a role for antibody in inhibiting virus replication in neurons.

Neither of these studies (Levine & Griffin, 1992; Amor, 1996) addresses whether antibody alone can bring about sterilising immunity, the removal of all infectious virus. Infectious virus, or material capable of giving rise to infectious virus, could be present in a tissue even when no infectivity is detectable by plaque assay. Firstly, the infectivity assay does not detect material capable of giving rise to infectious virus and secondly, virus neutralisation can occur after sampling, during tissue homogenisation, giving a false impression of virus clearance.

To address these questions the SFV SCID model was used. The intention was to administer HI SFV serum to reduce brain virus infectivity to zero and then assay brains for infectivity and virus RNA weeks after the last serum transfer, to allow time for clearance of transferred antibody. HI SFV serum was given at 4 days PI and repeat inoculations every 3 days. This ensured a high titre neutralising antibody in SFV infected SCID mice, as the half-life of passively administered anti-SFV antibodies is approximately 4 days (Fazakerley, 1985). This system is more representative of the enduring antibody response seen in immunocompetent mice, and measures both persistence of infectivity and virus RNA.

Objectives

- Raise high titre HI SFV serum
- Characterise HI SFV serum
- Passive transfer of HI SFV serum to SFV infected SCID mice
- Examine ability of HI SFV serum to clear infectious virus from selected tissues of SFV infected SCID mice
- Examine ability of HI SFV serum to clear virus RNA from the CNS of SFV infected SCID mice
- Determine if infectious virus can be detected after treatment with HI SFV serum
- As a comparison, determine the rate of virus RNA clearance from SFV infected wild type (BALB/c) mice

Results

Antibody Transfer Experiment 1

The first experiment set out to determine the effect of passive intraperitoneal antibody transfer on virus RNA load in the CNS of SFV infected SCID mice. SCID mice (n=8) were inoculated with SFV (day 0). From PID 4 - 19 SCID mice received six transfers of HI SFV serum. Mice were perfused and tissues sampled at PID 21.

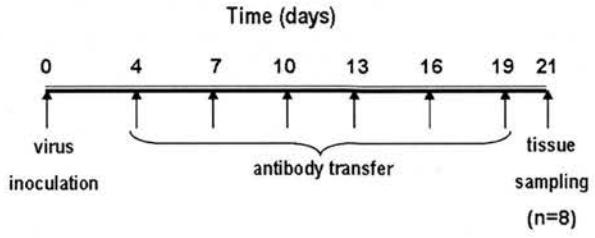

Figure 38. Time course of experiment 1 showing times of virus inoculation, antibody transfers and tissue sampling.

Production of HI SFV serum

The first step was to raise HI SFV serum for the passive transfer experiment. Forty BALB/c mice (mixed sex) age 4-6 weeks were inoculated (day 0) with $5x10^3$ PFU of SFV A7(74) and boosted with repeat inoculations at 14 and 21 days. As a specificity control, 40 BALB/c mice were inoculated with $5x10^3$ PFU of TMEV strain BeAn. At day 28 mice were euthanised and sera were collected from both groups of mice, pooled and characterized by ELISA. As a positive control, serum was prepared from BALB/c mice given a single SFV inoculation of SFV and sampled at PID 7 (SFV d7 serum).

ELISA development

In order to characterise the HI sera an ELISA was developed. To determine the optimum amount of coating antigen required, antigen (sucrose gradient purified SFV) was diluted 2-fold across a 96-well plate. HI SFV serum (1:100) or TMEV d28 serum (1:100) or PBS alone was added. Two dilutions of the secondary antibody (HRP conjugated, anti-mouse IgG) were tested. From this experiment

optimum dilutions of antigen and secondary antibody were selected (Figure 39). The antigen clearly dilutes out, showing specificity of the reaction. Conservation of the coating antigen was an important consideration; the process of purification is difficult and time consuming. Choosing an antigen dilution with the lowest concentration that would give a clear result was necessary. A dilution of 1:800 for virus coating, and a dilution of 1:500 for the secondary antibody were chosen as optimum concentrations.

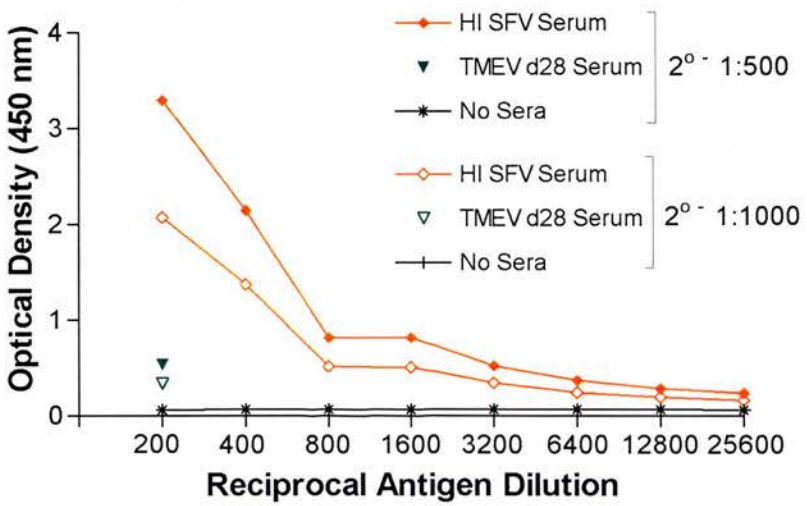

Figure 39. Titration of coating antigen (band purified SFV) for antibody binding was determined by ELISA. Microtitre plate was coated with serial dilutions of antigen. 2° - secondary antibody dilution. Points represent mean of three replicates.

To determine the isotypes of antibody present in the HI sera, goat antibodies specific to different mouse immunoglobulins were used and detected with a HRP labelled (rabbit anti-goat) antibody (Figure 40).

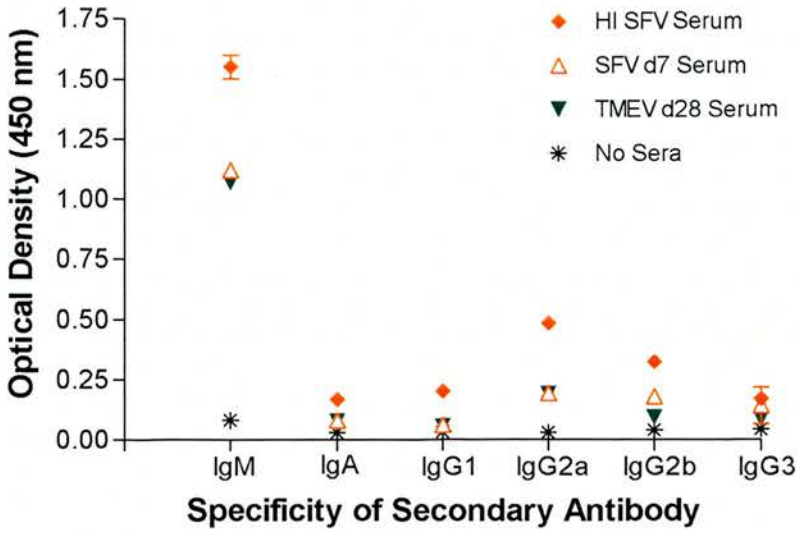

Figure 40. Comparison of antibody isotypes specific for SFV present in HI SFV serum. Controls included TMEV d28 serum, SFV d7 serum and no sera. Bound antibody was detected by ELISA using isotype specific antibodies. Points represent the mean of three replicates; error bars (many too small to be visible) represent SEM.

The high level of anti-SFV IgM observed was expected in SFV d7 serum but not in HI SFV sera (as class switching should have occurred). IgM is a large pentameric molecule and is known to be 'sticky', and to bind non-specifically to the microtitre plates. This can be seen by the high OD reading with not only the HI SFV serum but also the TMEV d28 serum. The higher OD reading for the HI SFV serum, compared with controls, suggests however that there is a specific IgM anti-SFV activity in the HI SFV serum.

The difference in IgG2a levels between HI SFV sera and controls is specific and would be expected in HI serum (Coutelier, 1988b). The differences in ODs between the isotypes are not comparable as each isotype will bind to the microtitre plate with a different efficiency giving a different background value and each isotype specific antibody will have a different avidity for its target antibody.

Using 2-fold serial dilutions of the HI SFV, SFV d7 and TMEV d28 sera, the IgG2a response was examined in more detail (Figure 41).

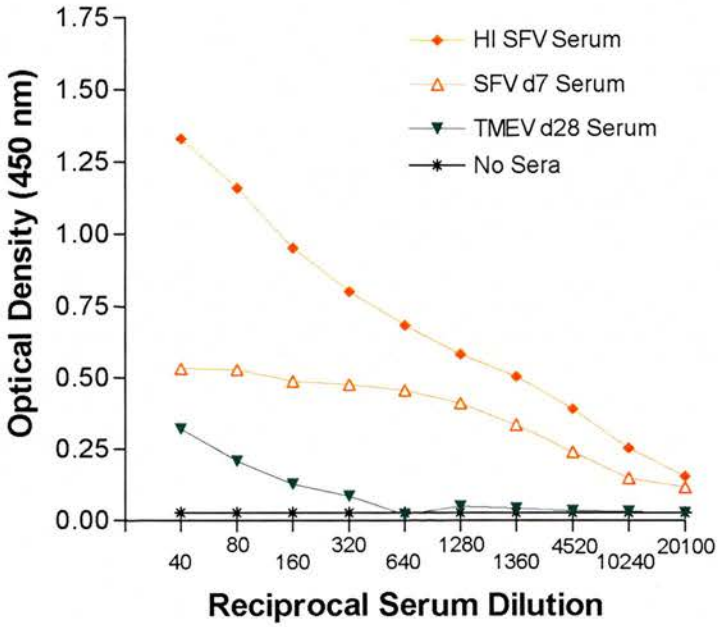

Figure 41. Anti-SFV IgG titres in the HI SFV, d7 SFV and TMEV d28 sera. Serial dilutions of sera were added to microtitre plate coated with a 1:800 dilution of band purified SFV. Antibody binding was detected by HRP labelled goat anti-mouse IgG. Each point represents the mean of three replicates.

TMEV d28 serum contains antibody that binds non-specifically to the microtitre plate, this signal titrates to zero at a 1/640 serum dilution. The IgG detected in SFV d7 serum only titrates out after an initial plateau suggesting that between 1/40 and 1/640 the assay is saturated. The d7 serum titrates out at 1/1280 – 1/20100 dilutions, this would be consistent with a high titre of antibody, perhaps of one specific IgG subclass, which is saturating the assay. This would be expected, as some class switching should have occurred by PID 7 but IgG titres would not have peaked (Amor, 1996). The HI SFV serum has a high OD at low serum dilutions. As the HI SFV serum is diluted, the OD drops correspondingly, indicating that there is a high level of IgG in the HI SFV serum and that it is specific for SFV. The combined ELISA results demonstrate that the HI serum raised is specific to SFV and that it contains IgM, IgA, IgG1, IgG2a and IgG2b.

Infectious virus and virus RNA is reduced by HI serum transfer

For the passive transfer, three groups (n=8) of SCID mice were infected with $5x10^3$ pfu SFV A7(74). At 4 days post-infection, one group of mice received a passive transfer of 0.1 ml of HI SFV serum; control groups received either 0.1 ml of TMEV d28 serum or PBS. Passive transfers were repeated every 3 days until PID 19. At PID 21 all mice were perfused and tissues sampled. Figure 38 outlines the time course of experiment 1.

To determine virus infectivity heart and brain tissue were titrated by standard plaque assay on BHK-21 cells. Virus was below the limit of detection in both brain and heart tissue in 100% (8/8) of the SCID mice that received HI SFV serum (Figure 42). Virus was also below the limit of detection in 100% (6/6) immunocompetent BALB/c mice at the same time point. Mice in control groups that had received either PBS or TMEV d28 serum had detectable virus in the brain and heart, these titres were significantly greater than mice that received HI SFV serum and immunocompetent BALB/c mice ($p<0.01$ as analysed by Mann-Whitney test, Figure 42). The range of virus titres was from 3.9 $\log_{10}$ PFU/g to 6.2-$\log_{10}$ PFU/g.

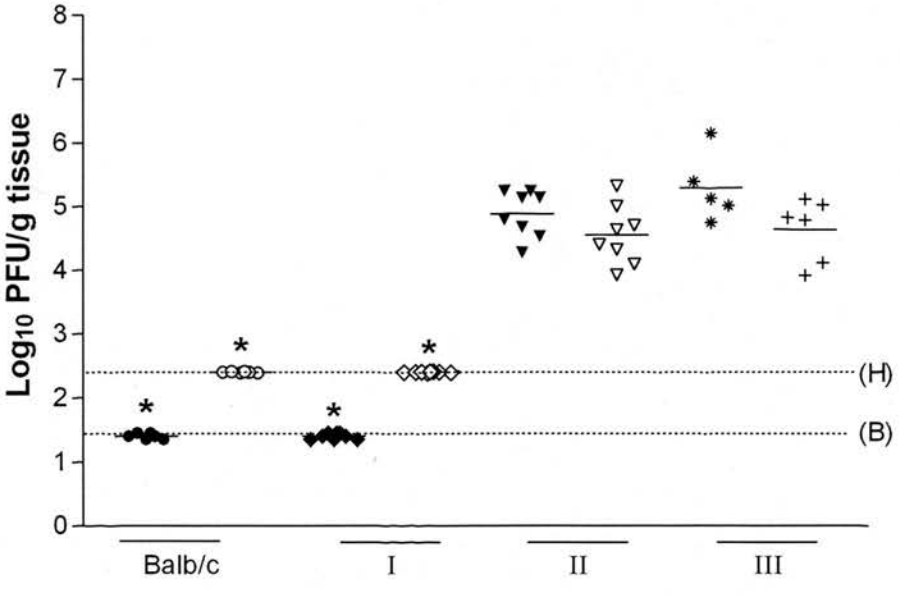

Figure 42. Infectious titres at three weeks post-infection in SFV infected BALB/c mouse brain (•) and heart (o) (n=6); **I** – SFV infected SCID mice that received HI SFV serum, brain (♦) and heart (◊)

(n=8); **II** – SFV infected SCID mice that received TMEV d28 serum, brain (▼) and heart (▽) (n=8) and **III** – SFV infected SCID mice that received PBS, brain (✷) and heart (+) (n=6). * indicates significant difference ($p<0.01$) with control groups (II and III); data were analysed by Mann-Whitney test. Dotted line indicates the limit of detection of the assay for heart (H) and brain (B). Horizontal bars indicate the mean of each group.

Levels of virus RNA were measured in the brains of the SFV infected SCID mice that received HI SFV serum, as well as in infected BALB/c mice and the two control groups that received TMEV d28 serum or PBS using q-PCR. Virus RNA was detectable in all groups at 3 weeks post-infection (Figure 43). Mice that had received HI SFV serum had a significantly lower level of SFV RNA than control groups that had received PBS ($p=0.0007$, Mann-Whitney) or TMEV d28 serum ($p=0.0016$). The difference between the mice that received HI SFV serum and BALB/c mice was not significant ($p=0.2844$).

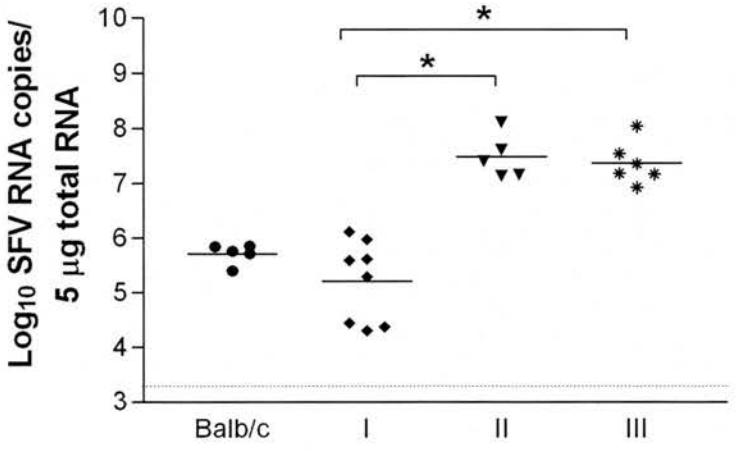

Figure 43. SFV RNA copies measured by quantitative PCR in brain tissue at 3 weeks post-infection in SFV infected BALB/c mice (●); **I** – SFV infected SCID mice that received HI SFV serum (♦); **II** – SFV infected SCID mice that received TMEV d28 serum (▼) and **III** – SFV infected SCID mice that received PBS (✷). There was no significant difference between BALB/c mice and group I; group I were significantly different to groups II and III; *indicates significant difference ($p<0.01$); data were analysed by Mann-Whitney test. The dotted line indicates the limit of detection of the assay. Horizontal bars indicate the means of each group.

Antibody Transfer Experiments 2 and 3

In experiment 1, passive transfer of HI serum reduced the infectivity titre to undetectable by 3 weeks (Group I, Figure 42) however virus RNA was still

detectable in these mice (Group I, Figure 43) and even in BALB/c mice. In both groups RNA levels were approximately 100-fold greater than in the negative controls (dotted line, Figure 43). This virus specific RNA could represent 'junk' RNA incapable of forming infectious virus, but alternatively it could be potentially infectious genomes. Although the plaque assays at 3 weeks PI did not detect virus (Figure 42), these mice may still have had antibody in their system. Only virus in excess of neutralising antibody in the homogenised tissue can be detected by plaque assay, therefore any remaining infectious virus could be rendered undetectable by remaining anti-SFV antibody. The same considerations apply to the BALB/c mice. To determine if anti-SFV HI serum had cleared all infectious virus from the mice and established a sterile state the experiment was repeated with a longer interval between the cessation of antibody transfers and sampling.

In experiment 2 HI sera transfers were carried out as before, with 10 mice each receiving 6 doses of HI sera every 3 days from 4 days PI. Two mice were again sampled at day 21. The remaining mice were sampled at day 42 (Figure 44). Following exactly the same design in a 3rd experiment mice were sampled at 21, 42 and 63 days post-infection (Figure 44). To control for virus contamination, either in the animal unit or during RNA processing, a control group of uninfected SCID mice were included in experiment 3. These mice were kept under the same conditions as the infected mice. For both experiment 2 and 3 it was necessary to produce new batches of HI serum. This serum was raised in exactly the same way as in experiment 1. HI SFV serum from the second experiment will be referred to as HI-(2) and mice that received this serum R-(2), HI SFV serum from the third experiment HI-(3) and the recipient mice of this serum R-(3).

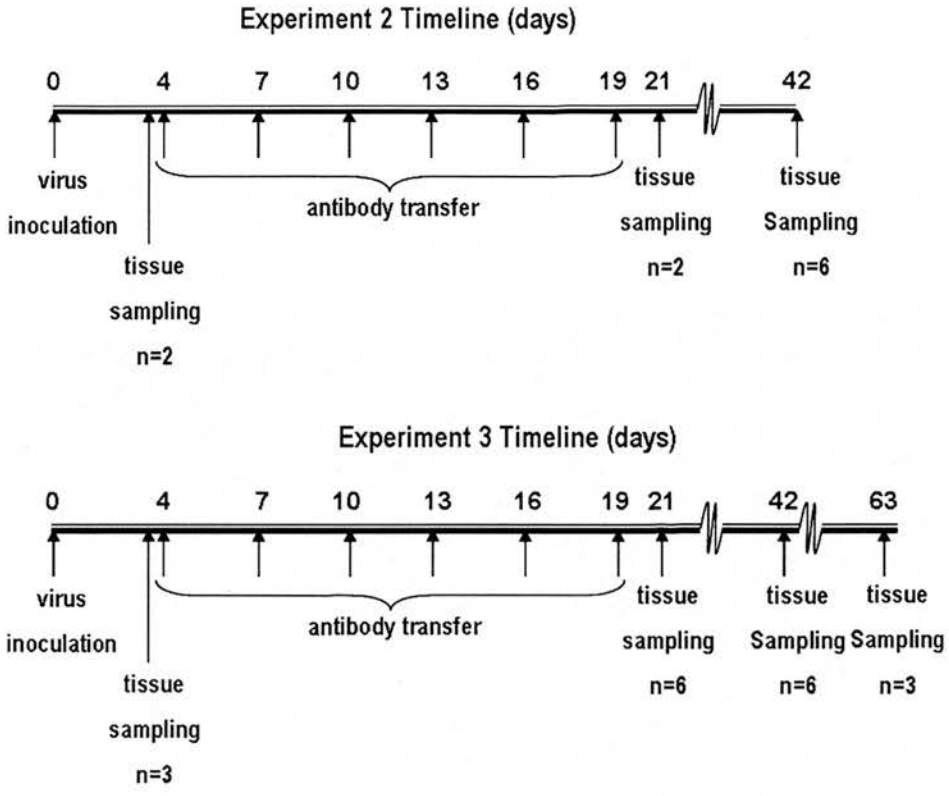

Figure 44. Time course of infection, antibody transfer and tissue sampling for experiments 2 and 3.

The antibody isotypes present in HI-(2) and (3) sera were compared by ELISA (Figure 45). As for experiment 1 HI serum, high OD values were observed with IgM but as shown by the TMEV d28 serum and the 'no serum' controls; a considerable proportion of this was non-specific. For both HI-(2) and HI-(3) the biggest difference above background was seen in IgG1 and IgG2a, which was expected in HI sera, as class switching should have occurred. The results show that there were slightly but consistently higher levels of all isotypes in HI-(3).

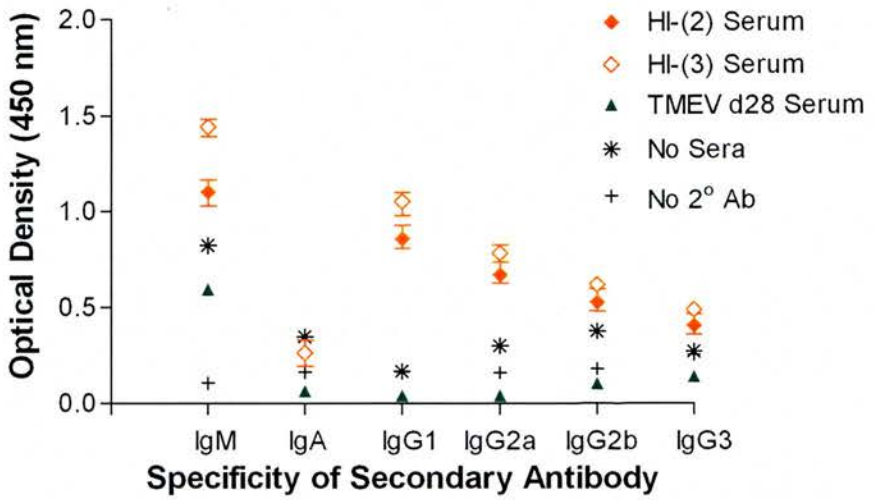

Figure 45. Comparison of the antibody isotypes specific for SFV in HI-(2) and HI-(3). Controls included TMEV d28 serum, no sera and no secondary antibody (2° Ab). Serum levels were measured by ELISA using isotype specific antibodies. Points represent the mean of three replicates, error bars indicate the SEM.

To determine if recipient SCID mice had detectable anti-SFV antibodies at the time of sampling, sera were tested by ELISA (Figure 46). Three weeks after the last HI-sera transfer (PID 42) both R-(2) and R-(3) mice, had significantly increased levels of anti-SFV IgG ($p<0.05$) compared with uninfected mice. At PID 42, the R-(3) mice had higher levels of anti-SFV antibody (mean OD - 0.41) compared to R-(2) mice (mean OD - 0.26). Furthermore, the R-(3) mice sampled at PID 63 had marginally higher levels of antibody than the R-(2) mice sampled at PID 42. These differences correlate with the higher titres of antibodies in the HI-(3) sera than in the HI-(2) sera (Figure 45 and Figure 46)

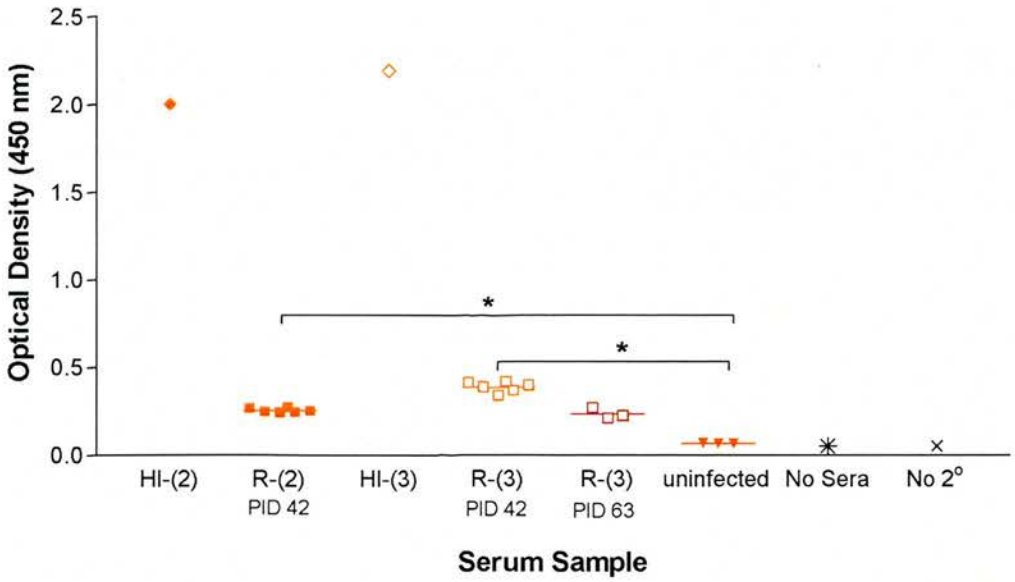

Figure 46. Anti-SFV IgG levels in the sera of SCID mice receiving HI serum. HI serum and serum from uninfected mice were included for comparison. No sera and no secondary antibody (2°) controls were included. R-(2) and (3) indicates sera from SFV infected SCID mice that received HI-(2) and -(3) respectively. Horizontal bars indicate the mean of each group. No 2°- no secondary antibody. Each point indicates the mean of three replicates, * indicates significant difference ($p<0.05$); data were analysed by Mann-Whitney test.

To determine if the sera from the sampled R-(2) and R-(3) mice had neutralising activity, a plaque neutralisation reduction assay (PRNA) was carried out to determine the neutralisation titre (NT). The NT of the HI SFV sera were also determined (Table 8, Figure 47). Consistent with the ELISA, HI-(2) serum had a lower NT than HI-(3), $1.29x10^6$ pfu/ml serum compared to $1.54x10^7$ pfu/ml serum. Similarly, all R-(2) mice had a lower mean NT than R-(3) mice at PID 42, five serum samples from each group were tested.

Serum	NT/ml serum
HI-(2)	$1.3x10^6$ pfu
HI-(3)	$1.5x10^7$ pfu
R-(2)	$1.1x10^4$ pfu
R-(3)	$3.3x10^4$ pfu

Table 8. NT for HI sera (**HI**) and sera from recipient mice (**R**) at PID 42. Expressed as number of pfu neutralised by 1 ml of serum.

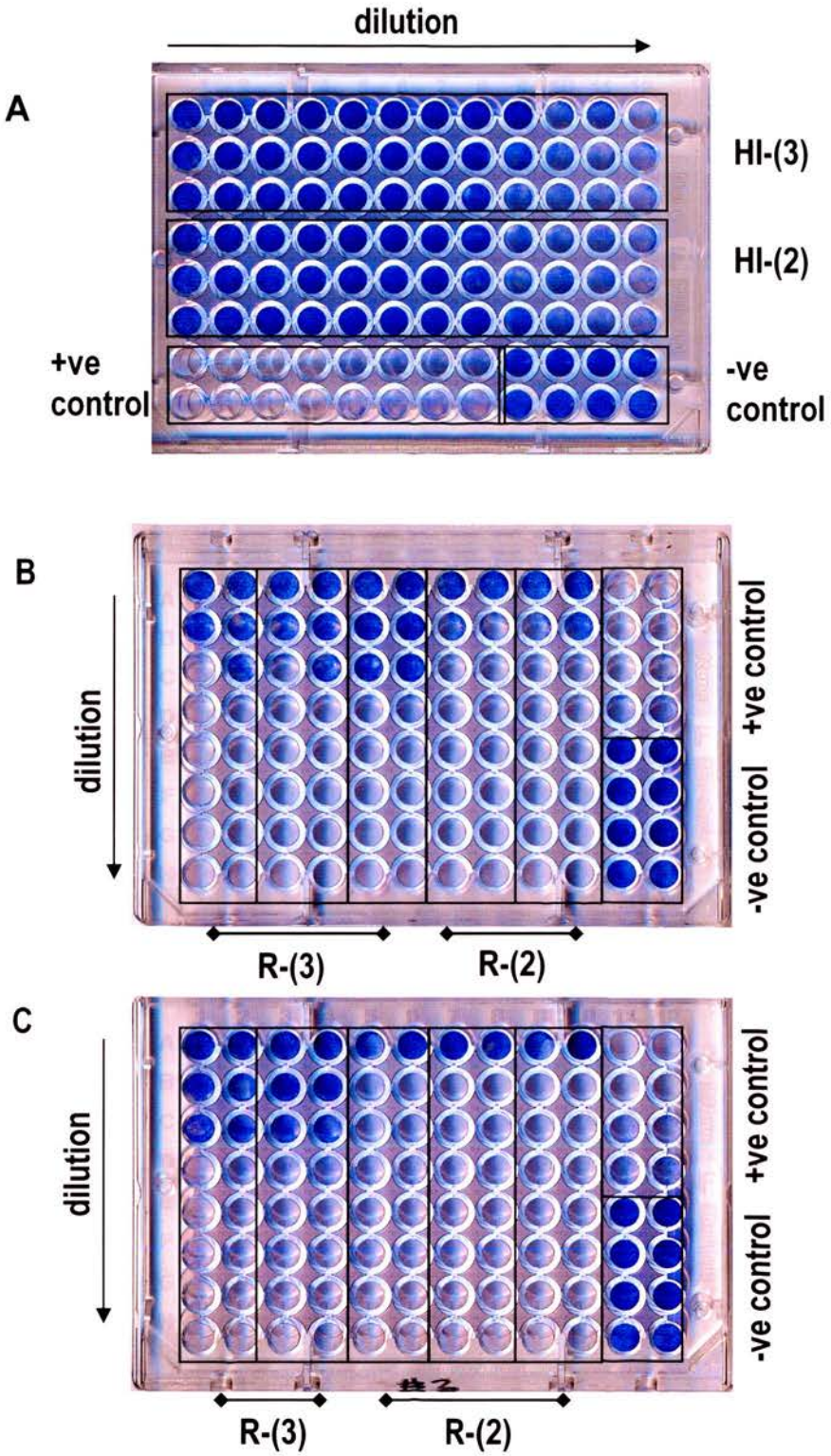

Figure 47. The NT of sera HI-(2) and (3) (**A**) and R-(2) PID 42 and R-(3) PID 42 (**B** and **C**) were determined by PRNA. Each dilution was tested in duplicate or triplicate in 2-fold serial dilutions starting at 1/10. Stained monolayers (dark wells) indicate inhibition of cytopathic effect (neutralisation of virus by anti-sera). The NT of the sera was determined against 100 pfu of SFV (constant virus – varying antibody assay). The reciprocal of the highest dilution that inhibited cytopathic effect was designated the NT titre.

Passive transfer of antibody does not remove all virus from the CNS

In SFV infected SCID mice that had received HI SFV serum, infectious virus titres in the brain were determined by plaque assay. In experiment 2, to verify that a CNS infection had been established, two mice were sampled at PID 4 prior to the first inoculation of HI SFV serum and their brains titrated to determine the titre of infectious virus. As expected these mice had a high titre of brain virus (Figure 48A). Of the six mice sampled at PID 42, three weeks after the last HI SFV serum transfer, 100% (6/6) were positive for infectious virus, with virus titres ranging from 2.2 – 6.1 log10 pfu/g brain. In experiment 3, mice again tested positive for infectious virus at PID 4, prior to the HI SFV serum transfer, and no (0/5) mice were positive for virus at PID 21. At PID 42 (0/6) and at PID 63 (2/3), mice had infectious virus in the brain (Figure 48B). A non-infected control group was included to check for any possible cross contamination, none (0/6) of these mice were positive for virus.

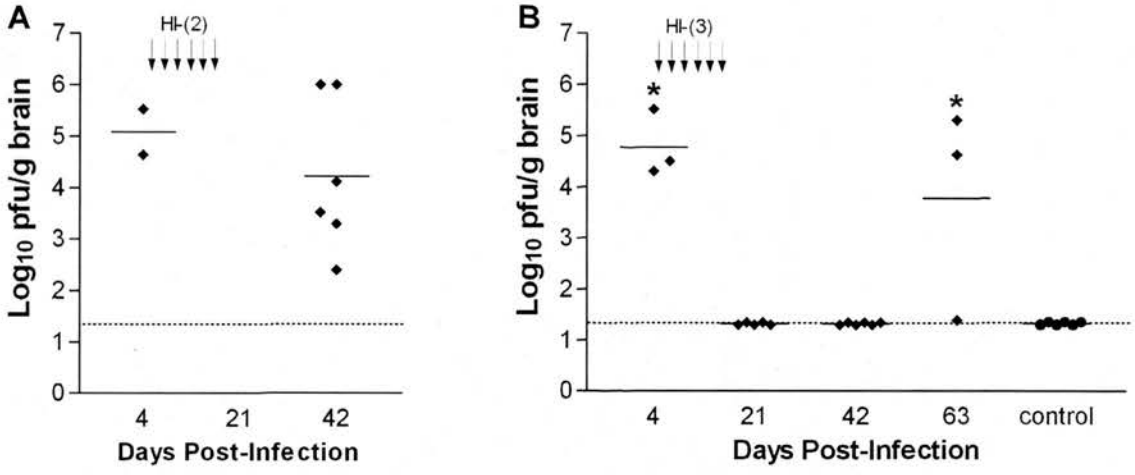

Figure 48. Virus infectivity titres in the brains of SFV infected SCID that received HI SFV serum (♦). Titres were measured by plaque assay in BHK-21 cells. **A** – Experiment 2, mice that received HI-(2); **B** – Experiment 3, mice that received HI-(3), includes uninfected control mice(•). * indicates significant difference ($p<0.05$) with days 21, 42 and control groups; data were analysed by Mann-Whitney test. Horizontal bars indicate group means, dashed line indicates limit of detection. ↓ indicates number and time points of passive transfer of HI-(2) and HI-(3).

Using q-PCR, the number of copies of SFV RNA was quantified in the brains of SFV infected SCID mice that had received HI-(2) or HI-(3) sera (Figure 49). In experiment 2, at days 21 and 42 all (8/8) mice had virus RNA levels above the limit of detection. The virus RNA load increased slightly from PID 21 to PID 42, from a mean of 6.0 to 6.4 $\log_{10}$ SFV copies, when the mice were no longer being given HI SFV serum. As clearly shown in these two experiments, at least some of the RNA remaining in the brains after passive transfer of antibody is capable of perpetuating the infection. The experiment was repeated and the time of sampling was extended. SFV infected SCID mice were given transfer of HI-(3) and sampled at PID 4, 21, 42 and 63 (Figure 44). In experiment 3 a high virus RNA load was detectable at PID 4 in the brain of a single mouse tested (7.0 Log_{10}) confirming the establishment of a CNS infection. The HI-(3) serum transfer reduced the virus RNA copies from 7.0 Log_{10} at day 4 to 5.5 Log_{10} copies at day 21. By PID 42 the virus RNA load had decreased further (mean 4.4 Log_{10}) this reduction was significant ($p= 0.0173$). By PID 63 however the virus RNA load had increased significantly ($p=0.0238$) relative to PID 42. Samples from the control group of uninfected SCID mice were below the limit of detection for the assay and significantly lower than all of the virus infected mice.

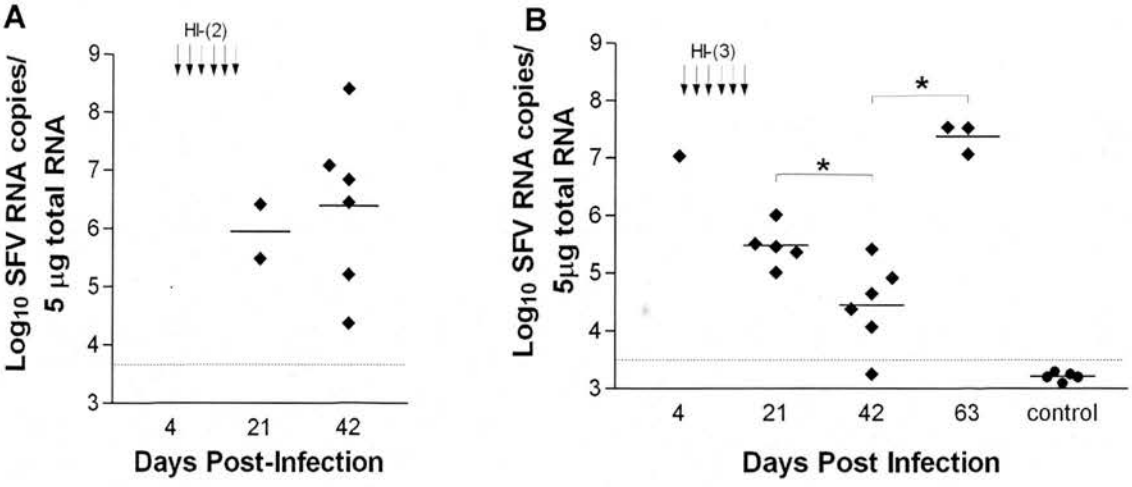

Figure 49. Titres of SFV RNA in the brains of SFV infected SCID mice that received HI SFV serum (♦), as measured by q-PCR. **A** – experiment 2, mice that received HI-(2); **B** – experiment 3, mice that received HI-(3), includes uninfected control (•) mice. * indicates significant difference ($p<0.05$); data were analysed by Mann-Whitney test. Horizontal bars indicate the means of each group, dashed line indicates the limit of detection for the assay. ↓ indicates number and time points of passive transfer of antibodies HI-(2) and HI-(3)

Virus RNA is still detectable 12 weeks post-infection in wild type mice

All 3 passive antibody transfer experiments demonstrated that there could be no infectious virus detectable by plaque assay but high levels of virus RNA in the brain. That virus infectivity was undetectable could be due to the presence of neutralising antibody and also that remaining virus material is RNA in the form of virus replication complexes. As a comparison, the rate of clearance of infectious SFV (by plaque assay) and viral RNA (by q- PCR) from the CNS of immunocompetent (BALB/c) mice was analysed. 15 BALB/c mice, age 4-6 weeks (mixed sex) were infected IP with SFV A7(74). Three mice per group were sampled from weeks 1 to 12 at three week intervals.

In brain tissue, infectious virus was detectable in 66% (2/3) of mice at day 7 only and at no other time point (difference not significant by Mann-Whitney test, Figure 50A). This is consistent with previous data that shows clearance of infectious virus from the blood at PID 3 and from the brain at PID 7. At one week post-infection 7 Log_{10} copies of virus RNA were detectable by q-PCR in SFV infected BALB/c mice. This quickly dropped (4.5 Log_{10} copies virus RNA, difference not significant by Mann-

Whitney test) by 3 weeks post-infection and changed little at later time points. The limit of detection for this assay was 3.7 Log_{10} copies virus RNA; virus RNA was therefore above the limit of detection for the assay in all mice at all time points.

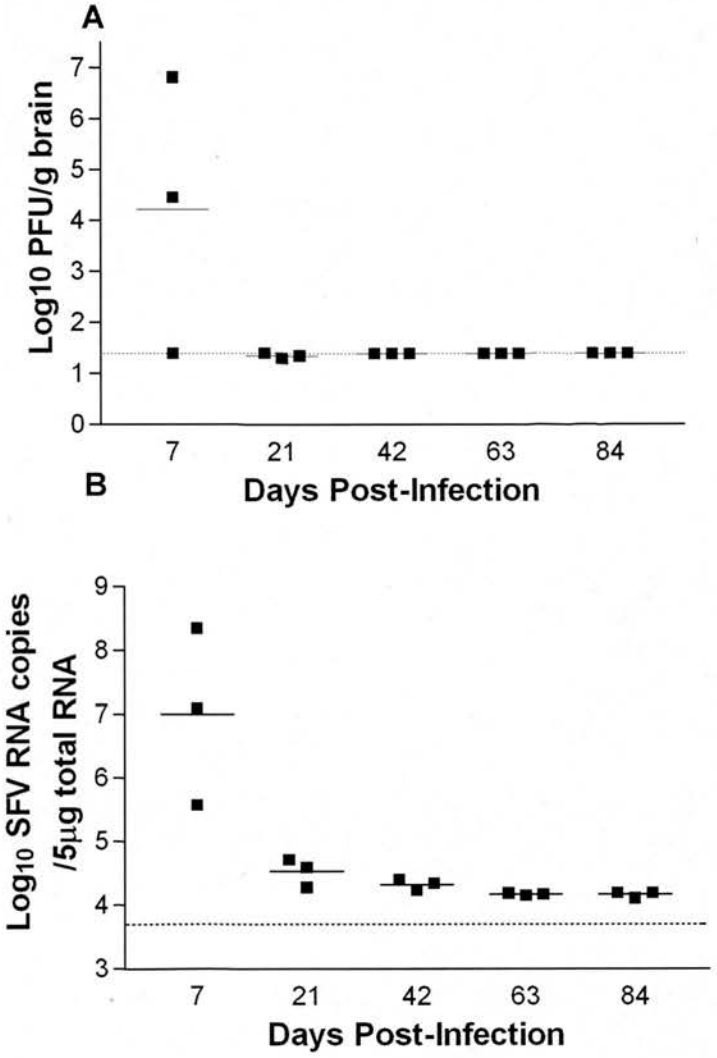

Figure 50. Clearance of infectious virus and virus RNA in SFV infected BALB/c mice (■). **A** infectious virus levels as measured by plaque assay. **B** virus RNA levels as measured by q-PCR. Dashed line indicates the limit of detection, horizontal bars indicate group mean.

Summary of findings

- Immunocompetent mice slowly clear SFV RNA from the brain but it was not established whether sterilising immunity is achieved
- HI SFV serum transfer to SFV infected SCID mice clears infectious virus from the brain
- HI SFV serum transfer to SFV infected SCID mice lowers virus RNA levels to those comparable with infected immunocompetent (BALB/c) mice
- HI SFV serum transfers are not sufficient to bring about sterilising immunity in SFV infected SCID mice

Discussion

Virus infection of the CNS poses a challenge for the immune response. Generally, in virus infected tissues complete clearance of virus requires destruction of virus infected cells by cytotoxic T-cells or apoptosis induction. However this action within the CNS is potentially dangerous as many CNS cells are vital. Limiting the spread of the pathogen by cytotoxicity is not compatible with protecting vital terminally differentiated neurons against cytolytic and toxic immune mediators. The propensity to dampen immune responses, combined with the limited magnitude of the immune response in the CNS offers the opportunity for virus persistence. As a result the CNS has highly specialised immune responses and is generally considered an immunosuppressive environment. Antibody can mediate its action via non-cytolytic mechanisms and has been shown in several neurotropic viruses to be crucial in protection in both the natural course of disease and via adoptive transfers. However, it is not known if antibody alone is sufficient to bring about sterilising immunity in the CNS following SFV infection or if virus can become persistent in the absence of other aspects of the adaptive immune response.

The role of antibody in CNS virus clearance was examined using HI serum transfers to SFV infected SCID mice. Infectious virus was detected by plaque assay and virus RNA copies by q-PCR. Although single transfers of serum have been shown to be effective in clearing infectious virus in SFV (Fazakerley & Webb, 1987) and SV infection (Griffin & Johnson, 1977; Hirsch, 1979), a series of HI serum transfer over several weeks was undertaken as this was considered more representative of the consistently raised titre of neutralising antibody which occurs in immunocompetent mice.

Following HI SFV serum transfer, infectious virus was not detectable by plaque assay and virus RNA levels were decreased to levels comparable to an immunocompetent mouse. Passive antibody transfers have been shown to mediate virus clearance and confer protection against other neurotropic viruses. In TMEV infection, although T-cells are essential for clearance of virus and for prevention of demyelination lesions (Rodriguez, 1996; Kang, 2005), antibody transfer confers partial protection against CNS infection of SCID and *nu/nu* mice. The passive

transfer of antibody to TMEV infected SCID mice was found to prevent virus spread into the grey matter in the CNS, that can cause irreversible damage and death, and limits virus to white matter areas (Fujinami, 1989; Njenga, 1997). Antibody transfer following rabies virus infection also protects, in both susceptible strains of mice and young mice that would normally succumb to infection (Schumacher, 1989). *In vitro* mAb was able to inhibit rabies virus transcription and spread in neurons by unknown mechanisms (Dietzschold, 1992a). This phenomenon has also been reported *in vitro* infections of neuronal cultures with SV (discussed below) (Despres, 1995). SV is closely related to SFV, and similarly HI serum transfers have been shown to be effective in the clearance of infectious virus and virus RNA, whereas transfer of T-cells has no effect on viral replication (Levine, 1991; Binder & Griffin, 2001e).

In experiment 3 following the passive transfer of antibody, no infectious virus was detectable at PID 21 or 42, but by PID 63, six weeks after the last HI serum transfer, virus infectivity was again detectable. Two weeks of HI serum treatment was insufficient to clear all virus from the CNS and the virus RNA detectable at 21 and 42 days PI was not 'junk' RNA but represented infectious genomes. This confirms observations with SV where virus recrudescence occurred after transfers of HI serum and purified mAb to E2 (Levine & Griffin, 1992). This effect was dose dependent. When a high level of mAb was given to SV infected SCID mice, there was no virus reactivation but virus RNA was still detectable by southern hybridisation. It was not determined whether mAb was still present and suppressing infectious SV at this time.

Virus persistence in the face of a neutralising antibody response is seen with other neurotropic viruses. During the acute phase of MHV infection, $CD8^+$ T-cells clear the virus infection but in μMT mice virus reactivation occurs after initial virus clearance (Lin, 1999). T-cells appear to be down-regulated after the acute phase, perhaps to limit cytotoxicity in the CNS; antibody is essential for controlling late virus levels (Ramakrishna, 2002). A single passive transfer of anti-MHV antibody prevents reactivation of infectious virus within the CNS of μMT mice, however immunologic control is not maintained and as antibody titres decline, infectious virus reactivates. The antibody response must be maintained in order to keep infectious MHV below detection.

As with SV, the time it took for infectious virus to become detectable following withdrawal of passive antibody correlated with the amount of anti-SFV IgG and the NT of the HI SFV serum. HI-(3) serum had both a higher NT and detectable anti-SFV IgG than HI-(2) serum and took 6 weeks after the last HI serum transfer for virus to rebound in SFV infected SCID mice. It appears that the higher NT of the sera, the longer the virus is kept under control. However even with increased NT and higher levels of anti-SFV IgG present in HI-(3), virus reactivation still occurred, antibody alone was not able to bring about sterilising immunity.

In immunocompetent mice the immune response to SFV rapidly clears infectious virus, this decrease is paralleled by the decreased levels of virus RNA. When quantified by q-PCR, virus RNA copies dropped by 2.5 $\log_{10}$ from 1 week post-infection to 3 weeks post-infection. After this point virus RNA copies did not drop below the limit of detection of the assay. This corresponds with data from Donnelly *et al* examining long-term effects of SFV infection, where viral RNA was shown to for up to 90 days by PCR (Donnelly, 1997a). Work done on another alphavirus, SV has shown persistence of viral RNA and long term CNS inflammation (Levine & Griffin, 1992; Tyor, 1992c). A persistent low turnover of LCMV and MHV also occurs after acute infection, they are kept in check by antibody response but the immune response is unable to completely clear the virus (Lin, 1999; Ciurea, 1999b). Following the development of the sensitive q-PCR to measure virus copies it would be interesting to extend study of SFV persistence to determine if virus RNA is eventually cleared and if so at what time point.

SFV infected BALB/c mice had similar levels of virus RNA at PID 21 as SCID mice that received passive antibody transfers and showed similar kinetics of infectious virus clearance, this suggests that antibody alone is the main mechanism of virus clearance used in immunocompetent mice. Other data (Chapter 3 & 4) have shown that the presence of $CD8^+$ T-cells and IFNγ can speed up this process, but do not have an effect on the overall decrease in virus RNA in the CNS.

Antibody is generally thought to mediate virus clearance by three mechanisms. Neutralisation, blocking virus binding or entry to target cells; virus opsonisation, the uptake of virus by phagocytes via Fc receptors and complement activation. Another

mechanism has been suggested (Levine, 1991), specifically in the CNS, which is separate from virus neutralisation, where antibody interacts with virus antigen on the infected cell and can affect virus transcription. This novel mechanism has been put forward for a number of viruses. In rabies infection a mAb was found to inhibit RNA transcription in tissue culture, possibly by uptake of the antibody-antigen complex by the cell (Dietzschold, 1992b). In SV infection in rat neuronal culture the addition of mAb to envelope protein E2, which can be detected on neuronal surfaces, decreased cytopathic vacuoles and rough ER associated with virion production and stopped viral replication (Levine, 1991; Despres, 1995). However such viral modulation does not always favour the host. This is the case with measles virus. The application of mAb to measles hemagglutinin in neuronal culture reduced measles virus gene expression by 80% in 6 days reducing both intracellular viral structural proteins and virus specific transcription. As always protection *in vitro* does not necessarily translate into protection *in vivo*, as there are heterogeneous cell types present in the CNS and far more systems interacting than in a homogenous tissue culture flask. *In vivo,* mAb initially protects against acute encephalopathy in newborn Lewis rats, these same animals go on to develop antibody induced acute measles encephalitis (Liebert, 1990; Schneider-Schaulies, 1992). The exact mechanism of how antibody inhibits virus transcription or gene expression is not fully understood. The efficacy of antibody alone in clearing virus is conflicting, measles virus persists in the face of a specific antibody response, antibody clears infectious SV but virus RNA is still detectable and in animals infected with rabies, clearance of infectious virus and virus RNA occurs. If antibody is able to restrict neuronal virus replication in SFV infection, as suggested in SV, it is not sufficient to suppress all virus replication in the CNS. It could be that antibody is insufficient to completely stop virus replication or the actions of antibody are restricted to particular cell types. Further investigation into the cell types that harbour persistent infection following passive antibody transfer is needed to address these questions.

In this chapter the system of HI serum transfer used demonstrated that antibody alone is not sufficient to achieve sterilising immunity in SFV infection; a reservoir of virus persists either as whole virus or intracellular virus genomes that can reactivate after antibody has been cleared from the system. It is possible that a much longer transfer

of serum could prevent viral reactivation, as seen in SV, but it cannot be ruled out that remaining antibody was inhibiting replicating virus. In wild type mice, low levels of virus RNA are still detectable after infection and long term antibody production and raised white cell counts are seen following SFV infection (Parsons & Webb, 1982h). It seems a likely possibility that this is due to virus persistence at a low level that is kept under control by an enduring immune response to the virus.

Chapter 6

Final Discussion

Contents

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Summary of findings

Chapter 3 – The role of $CD4^+$ and $CD8^+$ T-cells in SFV infection

- Mice lacking $CD4^+$ T-cells are unable to make a complete antibody response to SFV
- Mice lacking $CD4^+$ T-cells are unable to clear infectious virus or virus RNA from the brain
- Mice lacking $CD8^+$ T-cells show no difference in their ability to clear infectious virus from the brain compared to wild-type mice
- Mice lacking $CD8^+$ T-cells are significantly slower at clearing of SFV RNA from the brain compared to wild-type mice
- $CD8^+$ T-cells are sufficient to mediate the demyelinating lesions observed in SFV infected SCID mice that receive T-cells by adoptive transfer
- SFV can successfully infect cells in the CNS of C57Bl/6 mice

Chapter 4 – The role of selected $CD8^+$ T-cell mediators in SFV infection

- IFN$\gamma R^{-/-}$ mice show no difference in their ability to clear infectious virus from the brain as compared to wild-type mice
- IFN$\gamma R^{-/-}$ mice have slower clearance of SFV RNA from the brain as compared to wild-type mice
- IFNγ is not required for the development of demyelinating brain lesions in SFV infected mice
- Recombinant IFNγ transiently protects SFV infected IFN$\alpha/\beta R^{-/-}$ mice
- Neither perforin nor Fas are required for clearance of infectious virus or viral RNA from the brain

- SFV infected Fas^{lpr} mice have increased CNS demyelination compared to wild-type mice

Chapter 5 – The role of antibody in clearance of SFV from the CNS

- Immunocompetent mice slowly clear SFV RNA from the brain but it was not established whether sterilising immunity is achieved
- HI SFV serum transfer to SFV infected SCID mice clears infectious virus from the brain
- HI SFV serum transfer to SFV infected SCID mice lowers virus RNA levels to those comparable with infected immunocompetent (BALB/c) mice
- HI SFV serum transfers are not sufficient to bring about sterilising immunity in SFV infected SCID mice

Discussion

This thesis has examined the contributions of different components of the acquired immune response to clearance of both infectious SFV and SFV RNA from the CNS of infected mice and examined whether the immune response, specifically the antibody response, can bring about sterilising immunity. CNS demyelination observed following SFV infection has an immune-mediated aetiology (Fazakerley & Webb, 1987) and the role of components of the acquired immune response in inducing the pathological changes (demyelination and microcystic changes) observed following infection were investigated.

The use of q-PCR for the first time, allowed the accurate detection and quantitation of SFV RNA at low levels. Q-PCR although not more sensitive than standard PCR, the generation of a melting curve authenticates the presence of the specific amplicon and the use of the absolute standard curve allows quantification of virus RNA levels and comparison between samples not achievable with standard PCR (Bustin, 2000).

A limitation of q-PCR is that detection of virus RNA does not necessarily indicate the presence of replicating viral genomes, and consequently care must be taken when interpreting q-PCR data. Data from q-PCR complements virology techniques, such as plaque assay (for infectious virus). An important consideration in interpreting infectivity assay data, such as plaque assays, is that these assays inevitably involve homogenisation of tissues which can result in antibody neutralisation of infectious virus at the time of the assay. Viral persistence is an outcome of several CNS viral infections and q-PCR is a useful tool in determining the level of virus clearance. This study demonstrates a novel use of this technique in studying alphaviruses persistence.

A role for $CD8^+$ T-cells in immune mediated pathology in the CNS

$CD8^+$ T-cells migrate into the CNS in response to infection as demonstrated by the phenotyping of CNS lymphocytes in chapter 3. This thesis demonstrates by the transfer of activated $CD8^+$ T-cells to SFV infected SCID mice that $CD8^+$ T-cells are sufficient to mediate demyelinating lesions present in SFV infected brains. A previous study examining the role of $CD8^+$ T-cells in this process used antibody to deplete $CD8^+$ T-cells (Subak-Sharpe, 1993), however this depletion was transient. The system used here is an improvement on these depletion studies as SCID mice have no B or T lymphocytes and a pure population of $CD8^+$ T-cells was transferred (97%). The results shown here compliment and agree with the finding of Subak-Sharpe; antibody depletion of $CD8^+$ T-cells prevents demyelination in SFV infected immunocompetent mice (Subak-Sharpe, 1993) and adoptive transfer of highly purified population of $CD8^+$ T-cells induced demyelination in SFV SCID mice. The adoptive transfer also substantiates the Morris *et al.* study where $CD8^+$ T-cells are found at sites of demyelination in SFV infection (Morris, 1997).

The role of $CD8^+$ T-cells in causing immunopathological changes is well established in other CNS virus infections. Neurotropic MHV infection of SCID mice results in persistent infection without demyelination, however the transfer of $CD8^+$ T-cells is

sufficient to initiate demyelination (Murray, 1998b). $CD8^+$ T-cells generated in the response to Borna disease virus mediate fatal neuronal destruction and brain tissue loss (Bilzer & Stitz, 1994; Hausmann, 2001) and IC injection of LCMV induces fatal meningitis caused by anti-viral $CD8^+$ T-cells (Baenziger, 1986c; Dixon, 1987a).

$CD8^+$ T-cells are key effectors in virus clearance and activated T-cells are able to cross the BBB. The lytic capacity of $CD8^+$ T-cells makes them effective at clearing virus, as the only way to completely clear a virus infection from tissues is by removal of infected cells. However, this characteristic makes these cells dangerous in the CNS, as exemplified by the fatal encephalitis induced by $CD8^+$ T-cells in LCMV infection.

There is a fine balance between virus clearance and the development of immunopathological changes in the CNS. While a strong $CD8^+$ T-cell response can clear virus, if viral clearance is not executed rapidly, virus can spread further within the CNS and more extensive CNS tissue damage (viral lysis or immune mediated) can occur through direct cell lysis and bystander activation. For example, following TMEV infection differing efficiencies of antigen presentation to $CD8^+$ T-cells in resistant and susceptible mice determines the rate at which virus is cleared and whether CNS damage will occur (Dethlefs, 1997a).

Once $CD8^+$ T-cells are active in the CNS, their response can be regulated, as shown by the down-regulation of $CD8^+$ T-cell activity during the transition from acute to chronic MHV infection. It is not known what causes this down-regulation of the $CD8^+$ T-cell response but it serves to limit the on-going demyelinating disease (Stohlman, 2002b).

It was not possible to establish what $CD8^+$ T-cell mechanism(s) caused the demyelinating lesions observed in SFV infection. IFNγ was not necessary for demyelination, and due to the background strain of the perforin and Fas deficient mice (C57Bl/6) which did not develop demyelination; it was not possible to conclude if these mediators contributed to demyelination in susceptible mouse strains. The effector mechanisms involved in demyelination could be perhaps determined by transfer of $CD8^+$ T-cells from perforin and Fas deficient mice to SFV infected SCID

mice. However, this would require crossing the currently available perforin and Fas deficient mice (on the C57Bl/6 background) onto a BALB/c background.

The data shows that $CD8^+$ T-cells are sufficient to mediate demyelinating lesions in SFV infection. The antibody transfer in SCID mice can reduce infectious virus to undetectable and virus RNA loads to very low and $CD8^+$ T-cells are not required to reduce infectious virus to undetectable and virus RNA loads to very low in immunocompetent mice. The $CD8^+$ T-cell mediated demyelination is a clear example of immunopathology however, whether this response is necessary for complete eradication of the virus remains unclear.

The use of non-cytolytic mechanism by CD8+ T-cells to contribute to virus RNA clearance in the CNS

By using mice genetically deficient in $CD8^+$ T-cells and IFNγ, it was successfully demonstrated that both these effectors contribute to the clearance of virus RNA from the CNS. There was no difference in infectious virus clearance between the knockout mice and wild-type mice, however, the use of q-PCR allowed more subtle differences in virus titres to be observed. The results in this thesis indicate that IFNγ plays a key role in the anti-SFV $CD8^+$ T-cell response and that non-cytolytic mechanisms are involved in clearance of virus RNA from the brain.

$CD8^+$ T-cells can recognise early viral proteins produced intracellularly before virus budding occurs. This recognition allows an early anti-viral response which can result in killing of infected cells or in the release of pro-inflammatory cytokines, notably IFNγ. IFNγ can induce an antiviral state so preventing further viral infection and restricting virus replication. $CD8^+$ T-cell's ability to recognise viral infection early and to non-lytically restrict viral replication (by use of IFNγ) makes them valuable in viral CNS infection.

The roles of $CD8^+$ T-cells and IFNγ in virus RNA clearance are supported by studies on SV infection, where CD8a mice were also found to clear virus RNA at a slower rate (Kimura & Griffin, 2000). The ability of IFNγ to control virus replication was

studied using a SV construct expressing IFNγ, which could clear virus from spinal cord neurons and certain areas of the brain in SCID mice (Binder & Griffin, 2001a).

The importance of non-cytolytic, anti-virus factors of $CD8^+$ T-cells has been demonstrated in other CNS virus infections. Persistent LCMV infection requires IFNγ secretion in order to clear virus (Tishon, 1993). Infectious MHV is not cleared from the brains of mice deficient in IFNγ, they also have increased clinical signs and higher mortality rates than wild-type mice, consistent with persistent infection. Virus antigen was found almost exclusively in oligodendroglia in MHV infected $IFN\gamma R^{-/-}$ mice, demonstrating that IFNγ alone restricts virus replication in this cell type (Parra, 1999d). The neuronal replication of VSV *in vitro* and *in vivo* is restricted by IFNγ induced nitric oxide synthase induction (Komatsu, 1996b).

$CD8^+$ T-cells contribute to SFV clearance in the CNS and the use of non-cytolytic mechanisms by $CD8^+$ T-cell in the CNS seems prudent in the presence of a non-renewable cell population, as it allows antiviral function of $CD8^+$ T-cells to be utilised whilst limiting direct pathology through lytic mediators. Lytic mediators do not appear to have an important role in the clearance of SFV from the CNS, as the absence of perforin and Fas mediated cell lysis did not affect SFV clearance from the brain. Antibody although crucial, is not the only mediator that affects virus clearance. However, it was not established if $CD8^+$ T-cell secretion of IFNγ is essential for complete virus clearance or if it increases the rate at which virus is cleared.

It is known that IFNγ can contribute to virus clearance; however, this is the first study to demonstrate that mice lacking IFNγ have significantly slower clearance of alphavirus RNA from the CNS than wild type mice. It was also demonstrated that $CD8^+$ T-cells are the main source of IFNγ, as CD8a mice also had significantly slower clearance of virus RNA.

Is sterilising immunity ever achieved following CNS virus infection?

The question of whether sterilising immunity occurs in CNS virus infection was addressed in this thesis using two methods. In the first experiment a series of transfers of HI SFV serum to SFV infected SCID mice were carried out to examine the extent to which antibody alone can clear SFV from the CNS. Previous studies examining the role of antibody in viral CNS clearance have used a maximum of three antibody transfers. The advantage of giving six transfers was that it maintained a high titre of neutralizing antibody in the SFV infected SCID mice over a longer period and so was more representative of the enduring immune response seen in immunocompetent mice. It was demonstrated that antibody alone was sufficient to protect mice in SFV infection and to clear infectious virus. However, sterilising immunity was not achieved by HI transfers to SFV infected SCID mice, as virus RNA was still detectable in brain tissue at all time points, and more critically, when antibody titres were allowed to decline, infectious virus was once again detectable.

Antibody was capable of reducing virus RNA levels in the CNS in SFV infected SCID mice to those comparable with immunocompetent mice. Leading on from this observation, a second experiment investigated whether all virus RNA is cleared from the CNS of immunocompetent mice. At 12 weeks post-infection virus RNA was detectable in immunocompetent BALB/c mice. This raises the possibility that sterilising immunity may not be achieved in immunocompetent mice or may take much longer (many months) to be achieved after infectious virus is last detected.

This theory is supported by data from other studies with SFV. Not only was SFV RNA detected by PCR in the CNS for up to 90 days post-infection in immunocompetent mice (Donnelly, 1997b), but persistently raised white cell counts and immunoglobulin titres have been detected in the cerebrospinal fluid up to PID 61 (Parsons & Webb, 1982a; Parsons & Webb, 1982d). This supports the concept that SFV persists in the CNS; a continued immune response to the pathogen may keep virus levels at the threshold of detection. In another study suppression of the immune response to SFV in immunocompetent mice by cyclophosphamide (a chemotherapeutic drug which is cytotoxic to rapidly proliferating cells such as

lymphocytes) administration at 80 weeks post-infection resulted in infectious virus detection in the CNS. No infectious virus was detectable in control mice that did not receive cyclophosphamide. This suggests that over a year after SFV inoculation virus infection is being controlled rather than eliminated by the immune response (Fazakerley, 1985). A similar slow rate of virus clearance occurs with the related alphavirus SV. Virus RNA was detectable by PCR at 6 months post-infection and anti-SV plasma cells, which continually secrete antibody, were detected 1 year post-infection (Tyor, 1992a).

The terminally differentiated cells and specialised immune system of the CNS makes complete virus clearance difficult and therefore the outcome of infection of the CNS with RNA viruses is frequently persistent infection. Possible mechanisms of virus persistence for RNA viruses include infection of immunospecialised sites (CNS), non-lytic replication cycles and antigen expression in the thymus (resulting in tolerance induction). Neo-natal LCMV infection, results in thymic expression of viral antigens during T-cell development and virus specific T-cells are deleted; in the absence of specific immunity persistence occurs. In diseases associated with late complications of measles infection, subacute sclerosing pan-encephalitis and measles inclusion-body encephalitis, virus particles cannot be isolated due to restricted replication of the virus in the CNS, but viral persistence occurs. Transcription of viral genes involved in budding and maturation are reduced but genes for replication and transcription are maintained (Ahmed & Chen, 1999). Even in the face of a specific immune response virus can persist, in the case of MHV infection a strong acute response which clears infectious virus, but antibody secreting plasma cells are still detectable at 90 days PI, viral antigen one year PI and virus RNA two years PI (Knobler, 1982b; Fleming, 1995; Stohlman, 2002a), additionally, demyelinating disease also occurs months following infection indicating persistent infection.

It appears that virus persistence is a trade off for the protection of neurons. For sterilising immunity to be achieved, all replicating virus needs to cleared from all infected cells. The most efficient was to achieve this is to kill infected cells but in the CNS this is not an option as the destruction of irreplaceable key neurons can have fatal consequences. In general CNS virus infections are limited and often result in

persistence rather than clearance of infection. There are a number of factors which contribute to this situation. The immune response as a whole in the CNS is less efficient than in peripheral tissues, because there are no lymphoid organs, limited access for leukocytes, antibody and complement, and limited MHC I expression. Furthermore, neurons are protected from immune-mediated damage by several mechanisms including; the absence of MHC expression, an ability to prevent perforin- mediated damage and that they do not easily undergo apoptosis compared to renewable nerve cells, such as the neurons of the olfactory bulb.

It has been suggested that the continued presence of antigen serves to benefit the host through the continued stimulation of the immune response and the maintenance of memory. Low quantities of antigen can stimulate and help maintain the memory response of B and T lymphocytes resulting in more rapid clearance if subsequent infections should occur (Klenerman, 1997).

To demonstrate if antibody is necessary to control persistent infection the selective depletion of B-cells by murine anti-CD20 antibody could be used. CD20 is a marker found on all mature B-cells. This system has been shown to be effective in the treatment of antibody- mediated autoimmune disorders in humans using Rituximab (human anti-CD20 antibody). Anti-CD20 treatment has been shown to deplete B-cells by both AICD and by directly stimulating apoptosis, however the efficacy of anti-CD20 on all B-cell populations has not been proved (Silverman, 2006). Immunocompetent mice would be infected with SFV and left for 6 months. The B-cell population would then be ablated many months post-infection by antibody administration. Mice would now be sampled for the presence of infectious virus. To ensure the complete elimination of B-cells and antibodies cyclophosphamide could also be administered in conjunction with anti-CD20, as this would inhibit lymphocyte function and prevent clonal expansion of existing lymphocytes. If infectious virus was recoverable after the B-cells had been depleted this would demonstrate that SFV does persist and that infectious virus is controlled in the long-term by antibody.

Whether sterilising immunity can be induced following SFV infection remains to be answered. The data shown here and in previous studies suggests that sterilising

immunity is not achieved, however further evidence is need to answer this question conclusively.

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