Immunobiology of an alphavirus infection in mice

Mohammed Alsharifi

A thesis submitted for the degree of Doctor of Philosophy of
The Australian National University

November, 2004

Table of contents

Statement

Experiments presented in figure 2.2, figure 4.5, and figure 6.5 were conducted with the great help of Aulikki Koskinen, the research assistant of Prof. Arno Mullbacher. All other experiments described in this thesis represent my own work and were done by me. None of the material presented here has been previously used for the purpose of obtaining a degree

Mohammed Alsharifi
November, 2004

Acknowledgments

Thanks must be bestowed to all members in my supervisory panel, Prof. Arno Müllbacher, Dr. Mario Lobigs, Dr. Eva Lee, and Prof. Robert Blanden. I wish to express my special gratitude to Prof. Müllbacher for the guidance, encouragement, and confidence in my thoughts which made this research enjoyable. I must thank Dr. Lobigs and Dr. Lee for their excellent support and help, and my great appreciation to Prof. Blanden for his valuable critics and views.

Many thanks to everyone in the Molecular Immunology and Immunopathology Group and the Molecular Virology Group at the John Curtin School of medical research. I must acknowledge and thank Dr. Matthias Regner and Aulikki Koskinen for their help. Also, I would like to thank Esmee Weil, Felicity Chivas, and Cathy Woodhams, the friendly admin staff. Many Thanks to Geoffrey Osborne and Sabine Grüninger for their expert help in the FACS lab, and to Anne Prins for preparing nice histology slides. I must thank my friend Daniel Eichner for his help and support.

Finally, I would like to thank those who give without demand and help without reward except looking at my success as their own. To those, you know who you are, I thank your support, your help, your patient, and your prayers.

Abbreviations

2'5'OAS	(2'-5')-oligoadenylate synthetase
7AAD	aminoactinomycin D (fluorescent dye)
129	129T2/Sv mice
Ad-2	Adenovirus-2
Ag	antigens
AICD	activation-induced cell death
apc	allophycocyanin (fluorescent dye)
APC	antigen presenting cells
aSFV	avirulent SFV
asialo GM1	asialo ganglio-N-tetraosylceramide
B6	C57BL/6J mice
β2M	β2-microglobulin
BHK	baby hamster kidney
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester mixed isomers
CMV	Cytomegalovirus
DCs	dendritic cells
DI	defective interfering particles
ds-RNA	double-stranded RNA
ECTV	Ectromelia virus
EMEM	Eagle's minimal essential medium
ER	endoplasmic reticulum
$Fas^{-/-}$	Fas-knockout
Fas.L	Fas ligand
$Fas.L^{-/-}$	Fas.L-knockout
FCS	foetal calf serum
FITC	fluorescein isothiocyanate (fluorescent dye)
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
gzm	granzymes
$gzmA^{-/-}$	gzmA-deficient
$gzmAB^{-/-}$	gzmA and B-deficient

Abbreviations
(continue)

$gzmB^{-/-}$	gzmB-deficient
HBSS	Hanks' balanced salt solution
HBV	hepatitis B virus
HE	hematoxylin and eosin
HSV	Herpes simplex virus
i.c	intracranial injection
IFN	interferons
IFN-I	type I IFN
IFN-IR	IFN-I receptor
$IFN\text{-}IR^{-/-}$	IFN-IR Knockout
IFN-II	type II IFN
IFN-IIR	IFN-γ receptor
$IFN\text{-}\gamma^{-/-}$	IFN-γ knockout
$IFN\text{-}\gamma R^{-/-}$	IFN-γ-receptor knockout
Ii	invariant chain
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p	intraperitoneal
i.v	intravenous
LCMV	Lymphocytic Choriomeningitis virus
LFB	luxol fast blue
Ly49	Lymphocytes antigen 49 complex receptors
MAbs	monoclonal antibodies
MHC	major histocompatibility complexes
MHC-I	MHC class I
MHC-II	MHC class II
MHV	mouse hepatitis virus
MTD	mean times to death
MVE	Murray Valley encephalitis
NK	natural killer

Abbreviations
(continue)

NO	nitric oxide
pDCs	plasmacytoid dendritic cells
perf	perforin
$perf^{-/-}$	perf- deficient
pfu	plaque forming unit
PKR	ds-RNA-dependent protein kinase
RR	Ross River
SCID	sever combined immune deficiency
SFV	Semliki Forest Virus
SIN	Sindbis virus
TAP	transporter associated with antigen processing
Tc	cytotoxic T cells
TCR	T cell receptors
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TNF	tumor necrosis factor
VACV	vaccinia virus
VEE	Venezuelan equine encephalitis virus
Vero	African green monkey kidney
VSV	vesicular stomatitis virus
vSFV	virulent SFV
VZV	varicella zoster virus
wt	wild type

Abstract

This thesis describes some aspects of the immunobiology of the alphavirus Semliki Forest Virus (SFV) infection in the mouse. Both innate and adaptive immune responses are explored.

Chapter 2 describes experiments comparing the immune response to two strains of SFV, virulent and avirulent. Differences between these strains were identified in virus replication in spleen and brain, and their ability to induce IFN-α.

In chapter 3, lethality of vSFV was compared in B6 wild type mice and mice defective in exocytosis and or Fas-mediated pathways of cytolytic activity. Defective mice show more resistance than wild type B6 mice to the lethal vSFV infection. Absence of Tc-mediated cytolytic activity did not affect the outcome of vSFV infection, whereas NK cell-depletion delayed the mean time to death. This indicates a possible NK cell involvement in immunopathology.

In chapter 4, B6 wild type mice and vSFV-resistant mice (perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$x*gld*) were tested for changes in tissue virus titres. Reduced SFV replication was detected in perf$^{-/-}$x*gld* mice compared to all other mouse strains. In naïve perf$^{-/-}$x*gld* mice, expanded population of activated macrophage and elevated expression levels of mRNA of IFN-γ and TNF-α were detected. These factors may be responsible for the observed reduced virus titres.

In chapter 5, the cell surface expressions of activation markers CD69, CD86 and CD25 on lymphocytes were investigated following vSFV and aSFV infections. A generalised and systemic elevation of CD69 and CD86, but not CD25, expression was detected at day 1 postinfection and returned to baseline by day 5 postinfection. This is also a feature of infections with other viruses, such as Adenovirus and Flavivirus. Using interferon receptor knockout mice, it could be concluded that this activation marker expression was mainly due to the action of IFN-I.

In chapter 6, the consequences of the IFN-I mediated expression of activation markers on lymphocytes were investigated. The return to baseline levels of expression was due to down-regulation of cell surface expression than cell turnover via apoptosis. Following this down-regulation, a refractory period of about 9 days was required for a renewed cycle of activation markers to be expressed. Within that period, mice were more susceptible to secondary viral infections. Adoptive transfer of lymphocytes into new host facilitates activation markers re-expression in response to subsequent viral challenge. Therefore, it is possible that a refractory period is required to generate a second systemic IFN response following an acute viral infection.

Chapter 1

Background overview

The immune system has evolved to protect the host from pathogenic infections. It has two main features: recognition and response. Based on these features, the immune response can be divided into an innate arm and adaptive arm. Pathogenic recognition by the innate arm is non-specific, based on non-clonally distributed receptors that recognize certain molecular patterns found in microbes but not in self-tissues. In addition, innate responses occur with same magnitude and kinetics irrespective of repeated encounters with any particular infectious agent. This part of the immune system involves a combination of molecular components such as complement and interferons (IFN), and cellular components such as macrophages, natural killer (NK) cells, and dendritic cells (DCs). These components may also play an important role in the induction of the more specific responses mediated by the adaptive arm of the immune system, which is, by contrast, based on pathogen-specific receptors expressed on cell surfaces of T and B lymphocytes. In addition, the adaptive immune response is characterized by a memory, enabling a more rapid and heightened immune reaction to a subsequent challenge by the same or a cross-reactive pathogen. B cells secrete immunoglobulins, the antigen reactive antibodies responsible for both eliminating extracellular microorganisms, and virus neutralization (the B cell response will not be further discussed). T cells, on the other hand, have two major roles in the adaptive immune response and are mediated by two subsets of T cells. T helper (or $CD4^+$) cells play a regulatory role, which involves providing help to B cells (to make antibodies), and to $CD8^+$T cells (to generate cytotoxic attack). $CD8^+$, or cytotoxic, T (Tc) cells can eradicate intracellular pathogens by generating cytotoxic attacks, and or mediate host protection by the release of cytokines.

If a pathogen is able to breach barrier surfaces such as skin and mucous membranes, it will be faced by a concerted action of both the innate and adaptive arm of the immune system. In fact, the immune response to any given pathogen is one involving án overlapping action of both types. While providing the initial defense against any infectious agents, components of the innate systems play important roles in shaping the more antigen specific adaptive response. For example, DCs at the immature stage are the major IFN producers during viral infection. In addition, DCs function as antigen presenting cells (APC) driving the adaptive response. IFNs, which limit viral replication, are one of the key factors required for DC maturation. At the same time, they act on lymphocytes by inducing the expression of costimulatory molecules and

promote their activation and survival. In addition, NK cells can generate cytotoxic attacks against infected targets, similar to that generated by cytotoxic T cells after their DC-assisted activation.

1.1 Innate immunity

1.1.1 Interferons

Interferon is the term given to a family of inducible glycoproteins that are produced by cells in response to biological stimuli including viruses, bacteria, Mycoplasma, protozoa, cytokines, mitogens, natural and synthetic double-stranded RNA (ds-RNA) and other substances. IFN was discovered as an anti-viral agent in 1957 when Isaacs and Lindenmann reported that influenza virus-infected chick cells produced a secreted factor that mediated a virus-resistant state active against both homologous and heterologous viruses (Isaacs and Lindenmann, 1957). This observation set the stage for subsequent studies that led to the elucidation of many aspects the IFN system. According to the cell types from which they were derived, three types of IFNs were originally recognised: leukocyte (α), fibroblast (β), and immune (γ) (Pestka et al., 1987) (Samuel, 1991). The currently accepted classification divides IFNs, based on amino acid sequence homology, into two groups, type I (IFN-I) and type II (IFN-II). INF-I (IFN-α and IFN-β) are induced by virus infection, whereas IFN-II (IFN-γ) is induced by mitogenic or antigenic stimuli of lymphocytes (NK and T cells) (Samuel, 2001).

Type I IFN

The production of IFN-I is not confined to a specialised cells, and most cells of vertebrates are probably capable of producing IFN-I in response to viral infection (DeMaeyer and DeMaeyer-Guignard, 1998). Macrophages, NK cells, DCs, and fibroblasts were reported to produce IFN-I in response to viral infection or exposure to microbial pathogens (Belardelli and Gresser, 1996) (Cella et al., 1999a; Eloranta et al., 1997; Rodel et al., 1998; Sing et al., 2000). However, by far the most potent producer of IFN-I are plasmacytoid DCs which can produce up to 10^3 fold higher IFN-I *in vitro* than any other cell type following microbial or viral challenge (Asselin-Paturel et al.,

2001; Siegal et al., 1999). Other DCs can also act as a specialised IFN producer during certain viral infections (Diebold et al., 2003).

IFN-I are a diverse group of polypeptides that share a high degree of sequence homology (Kelley et al., 1983), and there are 2 families of IFN-I, IFN-α and IFNβ, present in all mammalian species (Pestka et al., 1987). Both are inducible by viral infections and recognise the same multicomponent cell-surface receptor, the type I IFN receptor (IFN-IR) (Colamonici et al., 1992; Novick et al., 1994). The IFN-α family is a multigenic group of related polypeptides encoded by more than 13 genes in human and more than 10 genes in mice (Foster and Finter, 1998; Walter et al., 1998). IFN-β, in contrast, exists as a single gene in most species, including human and mice.

Anti-viral effects

Viral infections are known to elicit an early induction of IFN-I synthesis that results in cellular resistance to viral infection, inhibition of viral replication, and impediment of viral dissemination (Sen and Ransohoff, 1993; van den Broek et al., 1995). Knockout mice lacking functional IFN-IR (IFN-IR$^{-/-}$) are extremely susceptible to viral infections, such as vesicular stomatitis virus (VSV), Semliki Forest Virus (SFV), vaccinia virus (VACV), and Lymphocytic Choriomeningitis virus (LCMV) (Muller et al., 1994), Sindbis virus (SV) (Ryman et al., 2000), Venezuelan equine encephalitis virus (VEE) (White et al., 2001), and Murray Valley encephalitis (MVE) (Lobigs et al., 2003). High level of IFN-α/β is an important feature of the early cytokine responses to viral infections. Ds-RNA, a known by product of many viral replication strategies, is a potent inducer of IFN-I (Biron, 1998; Malmgaard, 2004). The best-characterised direct anti-viral effect by IFN-I results from the production of two ds-RNA activated enzymes, (2'-5')-oligoadenylate synthetase (2'5'OAS) and ds-RNA-dependent protein kinase (PKR). Production of these enzymes is dependent on JAK-STAT pathway activation by the interaction of IFN-α/β with their specific cell surface receptor (IFN-IR) (reviewed in (Levy and Garcia-Sastre, 2001; Rebouillat and Hovanessian, 1999; Samuel, 2001). This direct effect of IFN-I results in protein synthesis inhibition in infected cells and thus limits the production of progeny viral particles (reviewed in (Jaramillo et al., 1995).

Immunoregulatory effects

In addition to their anti-viral effect, IFN-I plays an important role in modulating both innate and adaptive immunity. The generation of activated T and B lymphocytes requires the recruitment and maturation of APCs. These APCs include B cells, monocyte/macrophages, and the most potent APC DCs. Immature (Plasmacytoid) DCs have been identified to be the major IFN-I producers (Asselin-Paturel et al., 2001; Siegal et al., 1999). IFN-I itself plays an important role in DCs maturation and survival (Luft et al., 1998). In vitro treatment of immature DCs with IFN-I induces phenotypic and functional maturation, as evident CD83, MHC-I and II, CD40, CD80, and CD86 upregulation (Gallucci et al., 1999; Luft et al., 1998). This effect of IFN-I on DCs is mediated by only few other cytokines, such as interleukin-15 (IL-15) (Mattei et al., 2001). Production of IL-15 itself is induced by IFN-I, and IL-15 activating effect on DC is reduced in the absence of IFN-IR (Mattei et al., 2001).

To successfully control a viral infection, virus-infected cells need to be eliminated prior to viral progeny formation and release. A successful anti-viral cell-mediated response depends on the ability of infected cells to present the viral antigens in conjunction with major histocompatibility complexes (MHC) molecules (Zinkernagel and Doherty, 1974). IFN-I has the capacity to upregulate MHC protein expression, thereby promoting the anti-viral responses (Boehm et al., 1997). In addition, IFN-I induces proliferation of memory phenotype lymphocyte subsets and prolongs the survival of activated T cell populations. During viral infections, T cell proliferation, in particular, the $CD44^{hi}$ subset of $CD8^{+}$T cell is induced by IFN-I (Tough et al., 1996), an effect mimicked by poly I:C, an inducer of IFN-I. In addition, *in vitro* treatment with IFN-I prevented the death of *in vivo* activated T cells without inducing their proliferation (Marrack et al., 1999). Therefore, IFN-I contributes to the large numbers of antigen specific and non-specific T cells that are induced during LCMV viral infection (Butz and Bevan, 1998; Murali-Krishna et al., 1998; Yang and Welsh, 1986).

The ability of IFN-I to induce maturation of DCs indicates their adjuvant activity. IFN-I regulates B cell functions such as Ab secretion and isotype switching (Stark et al., 1998). Induction of isotype switching by IFN-I may be mediated by their effect on DCs (Le Bon et al., 2001) as well as their influence on T cell responses (Demeure et al., 1994). Co-injection of IFN-I with a soluble protein antigen enhances *in vivo* T cell

priming (Gallucci et al., 1999). IFN-I enhances T helper type 1 (Th1)-type responses by inhibiting the secretion of typical T helper type 2 (Th2)-type cytokines (interleukin (IL)-4 and IL-5) but promoting Th1-type cytokine production (IFN-γ) in $CD4^+$T cells (Brinkmann et al., 1993; Demeure et al., 1994). Such effect of IFN-I is also linked to isotype switching as the increased production of IFN-γ subsequently promotes switching of B cells to synthesise IgG2a isotype antibodies (Sinigaglia et al., 1999). In addition, IFN-I promotes IgM, IgA, but not IgG1 antibody production by B cells (Estes et al., 1998).

Moreover, IFN-I mediates activation of NK cell proliferation and cytotoxicity *in vivo* (Biron et al., 1999). However, IFN-I does not stimulate NK cell proliferation directly. They fail to elicit NK cell proliferation *in vitro* and exert anti-proliferative effects at high concentrations (Jewett and Bonavida, 1995). IFN-I influence on NK cell responses is dependent on secondary cytokines such as IL-15 (Waldmann and Tagaya, 1999). In addition, it has been shown that, during viral infections, IFN-I-mediated activation of NK cells is selective and does not extend to IFN-γ expression (Nguyen et al., 2000). Furthermore, IFN-I may be crucial for anti-viral responses mediated by macrophages as it synergises with tumor necrosis factor (TNF) to induce inducible nitric oxide synthase (iNOS) and iNOS-dependent pathways of defences mediated by macrophage (MacMicking et al., 1997).

Type II IFN

IFN-γ (IFN-II) is a potent activator of the antimicrobial functions of phagocytes, and it has an essential role in resistance to many pathogenic bacteria, fungi and intracellular parasites (reviewed in (Trinchieri, 2003)). It is primarily an immunoregulatory cytokine (Landolfo et al., 1988), exclusively produced by cells of the immune system, most importantly antigen specific $CD8^+$ T cells and $CD4^+$ Th1 cells during the adaptive phase of the immune response (reviewed in (Boehm et al., 1997; Lertmemongkolchai et al., 2001), but also NK and NKT cells during the innate phase (reviewed in (Biron, 1999; Sen, 2001)). In addition, other cell types, for example macrophages, DCs, and γδT cells have also been reported to express IFN-γ under certain conditions (Airoldi et al., 2000; Munder et al., 1998; Schindler et al., 2001; Stober et al., 2001).

In contrast to the large number of IFN-I genes and proteins found in humans and mice, there is only one IFN-γ gene and protein (Samuel, 2001). IFN-γ exerts its effect through its species-specific cellular receptor, IFN-γR (or IFN-IIR) (Shtrichman and Samuel, 2001). It plays an important role in both innate and adaptive immunity. A protective role for IFN-γ has been demonstrated during several viral infections, including ectromelia virus (ECTV) (Karupiah et al., 1993), influenza A virus (Karupiah et al., 1998), herpes simplex virus (HSV) (Cantin et al., 1999), hepatitis B virus (HBV) (Guidotti et al., 2000), and LCMV (Nansen et al., 1999). In addition, knockout mice lacking functional IFN-IIR are susceptible to some viral infections, notably VACV, and LCMV but not others such as VSV, and SFV (Muller et al., 1994). Most of the anti-viral activity of IFN-γ is related to its ability to activate macrophages and the resultant iNOS pathway (Guidotti and Chisari, 2000; Reiss and Komatsu, 1998). In addition, IFN-γ can directly recruit and activate NK cells, and T cells to perform their effector functions (Guidotti and Chisari, 2000).

Furthermore, IFN-γ upregulates the expression of MHC genes both class I (MHC-I) and class II (MHC-II) and, thus, increases Ag-presentation during viral infection (Boehm et al., 1997; Rosa et al., 1983; Rosa et al., 1986) and/or makes cells more susceptible to cytolytic T cell lysis. This increases the efficiency of cellular immune responses against viral infections and several viruses, including cytomegalovirus (CMV) and varicella zoster virus (VZV), antagonise IFN-γ-inducible MHC molecular expression (Abendroth et al., 2000; Miller et al., 1998).

1.1.2 Macrophages

Macrophages are long-lived phagocytic cells derived from bone-marrow monocytes, and circulate in the blood or reside in different organs and tissues (reviewed in (Guidotti and Chisari, 2000). Macrophages phagocytose foreign material as well as participating in the adaptive immune responses as an important APC for T lymphocytes (Gordon, 1998; Laskin and Pendino, 1995). In vitro studies indicated that macrophages are less efficient than dendritic cells, but more efficient than B cells in terms of antigen presentation (Kulkarni et al., 1991). They discriminate between self and foreign

antigens by expressing receptors for molecules not normally expressed on the cells of vertebrates, such as carbohydrates (Fraser et al., 1998). In addition, they express receptors for Ab (Fc-γ receptors) and complement. Therefore, the coating of microorganism with Abs, complement, or both, enhances phagocytosis and enables Ag-specific Ab-dependent cellular cytotoxicity (Kurane et al., 1984; Macfarlan et al., 1977) reviewed in Aderem and Underhill, 1999).

Macrophages are a key component of the initial immune response against most pathogens, including bacteria and viruses (Blanden, 1982; Russell, 1995a). It is believed that macrophages are activated by virus infection or NK-, NKT- or T cell–derived cytokines. IFN-γ is a very important priming factor required for macrophage activation, which must be accompanied by TNF to fully activate macrophages (reviewed in (Mosser, 2003). TNF, which functions as a second signal, can be secreted by macrophages themselves as a result of ligation of their toll-like receptors by microbial products such as LPS. In addition, many other cytokines, including IL-4, IL-12, M-CSF, GM-CSF, and TNF-α can activate macrophages (reviewed in (Guidotti and Chisari, 2000; Mosser, 2003)). Activated macrophages have been implicated in the infertility and autoimmunity in mice rendered genetically double deficient of both the Fas and exocytosis mediated pathways of cytotoxicity (see later) (Spielman et al., 1998). Inactivating the expanded population of activated macrophages in these mice resulted in pancreas recovery and restored their fertility. Prostaglandin E can transform macrophages from activated into an inactivated state (Strassmann et al., 1994).

Once activated, macrophages synthesise cytokines (e.g. IFN-I, TNF-α) and NO, which have direct anti-viral activity, as well as other cytokines that have immunoregulatory functions (e.g. IL-1, IL-6, IL-8, IL-10, IL-12, IL-18, GM-CSF) (Dinarello, 1999; Laskin and Pendino, 1995). *In vitro* studies indicate that IL-1, TNF-α, and NO can inhibit the replication of HSV (Wildy et al., 1982), VACV (Keller et al., 1985), VSV (LeBlanc et al., 1990), and mouse hepatitis virus (MHV) (Keller et al., 1988). NO has also been shown to inhibit the replication of poliovirus, rhinovirus, MCMV, ECTV (reviewed in (Reiss and Komatsu, 1998), coxsackie virus (Saura et al., 1999), and HBV (Guidotti et al., 2000). Other macrophage-derived cytokines (e.g. IL-12) inhibit *in vivo* the replication of a number of viruses, including HBV (Cavanaugh et al., 1997), HSV (Carr et al., 1997), and encephalomyocarditis virus (Ozmen et al., 1995). Similarly, IL-

18 treatment of mice controls VACV (Tanaka-Kataoka et al., 1999) and HSV (Fujioka et al., 1999) infections. Most of these anti-viral activities are secondary and probably due to the ability of IL-12 and IL-18 to induce IFN-γ, since they do not display direct anti-viral properties by themselves.

1.1.3 Natural killer cells

Natural killer (NK) cells develop in bone marrow and mature cells are present in peripheral blood and spleen, with low numbers in lymph nodes (Biron and Brossay, 2001). They migrate to inflamed tissues in response to different chemoattractants (Moretta et al., 2002). These cells are a principal component of the innate immune responses to many infectious agents, including most viruses (reviewed in (Biron, 1999)), and recently were identified to have a critical role in innate immune surveillance of tumors, particularly B cell lymphomas (Street et al., 2004).

NK cell-effector function depends on a characteristic target-recognition system, which is based on the balance of inhibitory and activating signals delivered through a range of surface receptors (Cerwenka and Lanier, 2001b; Delves and Roitt, 2000). Killer cell immunoglobulin-like receptors (KIRs), lymphocytes antigen 49 complex (Ly49) receptors, and CD94/NKG2A receptors recognise major histocompatibility complex (MHC) class-I molecules and deliver inhibitory signals (reviewed in (Cerwenka and Lanier, 2001a). Activating signals can be delivered by a group of highly NK cell specific activating receptors, NKp30, NKp44, and NKp46, in addition to that of Ly49d, Ly49H, and NKG2D (Moretta et al., 2001). The activating signal is usually overridden by the inhibitory signals dependent on recognising MHC-I molecules, expressed by all nucleated cells (Delves and Roitt, 2000). Thus, NK cells kill those target cells that have lost or express insufficient amount of MHC-I molecules, which occurs frequently in tumor and virus-infected cells (Moretta et al., 2002). In addition to their receptors, NK cells in some mouse strains express cell surface phenotypic markers such as NK1.1 (Koo et al., 1984), and or DX5 (Ortaldo et al., 1999). In addition, asialo ganglio-N-tetraosylceramide (asialo GM1) is expressed at high levels on NK cells of all vertebrate species (Trinchieri, 1989).

NK cells can exert their effector function through generating cytotoxic attacks using either or both cytotoxic pathways: granule exocytosis and Fas-mediated pathways (Trapani and Smyth, 2002). These pathways are also used by cytotoxic T cells and will be discussed later. NK cell cytotoxicity can be induced by IFN-I, which is present at high and systemic levels during viral infection (Biron, 1999; Orange and Biron, 1996; Welsh, 1978b). Another effector function of NK cells involves the secretion of immunoregulatory cytokines such as IFN-γ and TNF-α (Trinchieri, 1989). The role of NK cell-produced IFN-γ in anti-viral defence may be related to inhibition of viral replication, induction of iNOS, and activating macrophages (reviewed in (Biron et al., 1999), as discussed earlier.

In general, peak NK cell responses occur within the first several hours to days after primary infections, whereas adaptive T and B cell responses take more than a week to develop (Biron et al., 1999). Elevated NK cell-mediated cytotoxicity has been observed as a result of different viral infections including LCMV (Welsh, 1978a), SFV (Mullbacher and King, 1989), VACV (Karupiah et al., 1990), and others (reviewed in (Biron et al., 1999). In addition, the importance of NK cells in the anti-viral immune response was illustrated in many *in vivo* depletion studies. *In vivo* depletion of NK cells with antisera to asialo GM1 or monoclonal antibodies (MAbs) to NK1.1 resulted in enhanced MCMV growth in spleen, lung, and liver (Bukowski et al., 1983; Orange et al., 1995; Welsh et al., 1991; Welsh et al., 1994). A similar effect was also seen during infections with VACV and MHV, but not LCMV (Bukowski et al., 1983).

1.2 T cell immunity

T cells play a major role in adaptive immune responses. They express antigen-binding cell-surface receptors, T cell receptors (TCR), which have a certain amount of specificity and reactivity to their respective antigens (Ag). T cells recognize Ag in the context of MHC molecules on cell surfaces. There are two types of MHC molecules: MHC-I expressed by all nucleated cells of vertebrates, and MHC-II expressed only on a limited number of cell types in mouse on DC, macrophages, B cells and endothelial cells predominantly.

T cells can be classified into two subpopulations, $CD8^+$T and $CD4^+$T cells, which provide different functions. $CD8^+$T cells play a major role in eliminating intracellular pathogens by means of inducing apoptosis in their target cells following the recognition of foreign Ags in the context of MHC-I molecules. In contrast, $CD4^+$T cells recognize Ag presented in the context of MHC-II molecules and play a coordinating role, helping both $CD8^+$T and B cells. APC, such as DCs and macrophages, may represent the linking point between innate and adaptive responses, as they are important components of the innate and adaptive immune system.

1.2.1 MHC restriction

MHC molecules occupy a central stage in the generation of specific immune responses as they form complexes with pathogen-derived Ag to be recognized by TCRs. Self Ags are also presented by MHC molecules, but T cells with self Ag-reactive TCRs are eliminated during the development of T cell repertoire, and will not be discussed here. Ag presentation in the context of MHC molecules is an important requirement for T cell activation and is called MHC-restriction (Zinkernagel and Doherty, 1974) (Zinkernagel and Doherty, 1979; Zinkernagel et al., 1976). It involves both classes of MHC molecules: class I and class II. MHC-I (H-2K, D, and L in mouse, HLA-A, B, and C in humans) molecules present Ags of principally endogenous origin to be recognized by $CD8^+$T cells. In addition, MHC-I molecules function as a 'self-marker' (Ljunggren and Karre, 1990) recognized by inhibitory NK cell receptors (Brennan et al., 1996). In contrast, MHC-II (I-A and I-E in mouse, HLA-DP, DQ, and DR in humans) molecules present Ags of predominantly exogenous source to be recognized only by $CD4^+$T cells.

MHC-I and the presentation of endogenous Ag

MHC-I molecules are expressed on the surfaces of almost all nucleated cells (Hood et al., 1983; Steinmetz et al., 1982), and each molecule consists of a heavy chain and a light chain (reviewed in (Jones, 1997)). The heavy chain, called the α-chain, is a transmembrane glycoprotein consisting of cytoplasmic tail, transmembrane region, and extracellular region (reviewed in (Jones, 1997; Klein and Sato, 2000). The α-chain is non-covalently linked to a soluble protein known as β2-microglobulin (β2M) that forms the light chain (Jones, 1997). Crystallographic studies have revealed that the

extracellular portion of the α-chain consists of three domains; α1, α2, and α3 (Bjorkman et al., 1987b; Stern and Wiley, 1994). On the distal surface of MHC-I molecules, α1 and α2 domains form the binding groove for the antigenic peptide (Bjorkman et al., 1987a). β2M associates with the α3 domain and supports the membrane-distal α1 and α2 domains, resulting in minimal contact between α1, α2 and α3 domains. CD8 molecules, the cell marker for $CD8^+$T cells, are responsible for the exclusive recognition of MHC-I molecules. CD8 molecules bind to α3 domain and contact β2M without causing conformational changes in the peptide-binding site (Gao et al., 1997). MHC-I molecules are assembled in the endoplasmic reticulum (ER), and β2M proteins appear to be crucial for their cell surface stability (reviewed in (Jones, 1997)). Surface expression of MHC-I heavy chains in cell lines lacking β2M is minimal, and nascent MHC-I α chains accumulate in the ER (Arce-Gomez et al., 1978; Ploegh et al., 1979). Mice lack β2M expression as a result of gene knockout do not express any detectable MHC-I molecules and have no mature $CD8^+$ T cells (Zijlstra et al., 1990).

MHC-I molecules are loaded with peptides in the ER before being exported via the Golgi apparatus to the cell surface (Klein and Sato, 2000). Self proteins in the cytosol are degraded into peptides by proteasomes (Heemels and Ploegh, 1995; Khan et al., 2001; Reits et al., 2000; Schubert et al., 2000; Yewdell, 2002). Since viruses replicate within infected cells, viral mRNAs are transcribed into proteins in the cytosol, and some viral proteins are degraded in the proteasomes (Klein and Sato, 2000). These peptides generated in the cytosol are transported into the ER by the transporter associated with antigen processing (TAP), which is a member of the ABC-transporter family (Androlewicz et al., 1993; Neefjes et al., 1993; Shepherd et al., 1993). The optimal length of peptide transported by TAP in human and mice is similar, being 8-13 amino acids (Cresswell et al., 1994; Momburg et al., 1994). Antigenic peptides transported into the ER by TAP are loaded onto newly synthesized MHC-I molecules. This process is tightly regulated by ER-resident chaperones, such as calnexin, calreticulin, tapasin, and ERP57 (reviewed in (Antoniou et al., 2003; Pamer and Cresswell, 1998). These peptide-loaded MHC-I molecules are transported from the ER to the cell surface through the Golgi apparatus (Spiliotis et al., 2000).

Interestingly, some APCs have the ability to present exogenous antigen by MHC-I to prime $CD8^+$T cells, in addition to their ability of presenting exogenous antigen by

MHC-II molecules to $CD4^+$T cells (Kovacsovics-Bankowski et al., 1993; Sigal et al., 1999); (Norbury et al., 1995) (Albert et al., 1998; Norbury et al., 1997). This phenomenon is called 'cross-priming' (Bevan, 1976a; Bevan, 1976b).

MHC-II and the presentation of exogenous Ag

MHC-II molecules are expressed mainly on the surface of professional APCs, which include DCs, macrophages, and B cells, and on endothelial cells exposed to IFN-γ (reviewed in (Hansen and Sachs, 1989); and (Robinson and Kindt, 1989)). Antigen presentation by MHC-II molecules depends on endocytosis (or phagocytosis) of extracellular proteins into endosomes by APCs.

Although the overall structure of MHC-II molecules is similar to that of MHC-I molecules featuring a peptide-binding groove, they are of heterodimers formed by association of two chains α and β (Stern and Wiley, 1994). Each chain consists of two extracellular domains, transmembrane region, and a cytoplasmic tail. The membrane distal domain of each chain, α1 and β1 domains, form the peptide-binding groove (Klein and Sato, 2000). This peptide-binding groove binds longer peptides compared to MHC-I molecules (Hunt et al., 1992). MHC-II molecules assemble in the ER with a third transmembrane protein, the invariant chain (Ii) (Bakke and Dobberstein, 1990). The complex of MHC-II and Ii leaves the ER by way of the Golgi apparatus into primary lysosomes, which fuse with the endosomes to form peptide-loading compartments (reviewed in (Bakke and Dobberstein, 1990; Klein and Sato, 2000; Lotteau et al., 1990; Pieters et al., 1993). Enzymes brought into this compartment by lysosomes degrade the engulf proteins and the bulk of the Ii. This process leaves behind a small fragment, CLIP (MHC-II- associated invariant chain peptide), within the peptide-binding groove of the αβ heterodimer (reviewed in (Turk et al., 2001). Antigenic peptides are loaded into MHC-II molecules with the help of HLA-DA molecules, which are class II-like heterodimeric proteins synthesised in the ER (Kelly et al., 1991; Roche, 1995). Ag-loaded MHC-II molecules are exported and expressed on the cell surface where they can be recognized by $CD4^+$T cells (Klein and Sato, 2000).

1.2.2 T cell receptors

TCRs control key events in the life of T cells including their development in the thymus, and their differentiation into effector populations. TCR consists of heterodimers of polypeptide chains of either α-β or γ-δ. A small population of T cells, which is not discussed here, express γδTCR (de Wolf-Peeters and Achten, 2000), whereas the majority of T cells express αβTCR. The TCR complex is usually assembled in the ER and contains a single TCRαβ heterodimer and three signaling dimers, CD3δε, CD3γε and ζζ. The TCR heterodimer is responsible for Ag binding, whereas intracellular signal transduction is initiated by signaling dimers (reviewed in (Call and Wucherpfennig, 2004)).

The T cell response to a particular Ag is affected by TCR affinity to that particular Ag, the number TCRs engaged, and the duration of TCR engagement (Constant and Bottomly, 1997; Germain and Stefanova, 1999; Labrecque et al., 2001; Lyons et al., 1996; McKeithan, 1995). Using a transgenic mouse model to study $CD4^+$T cell differentiation into Th1 and Th2, Rogers and Croft have shown that both affinity and duration of binding to APC determines the pattern of cytokines secreted by Th cells (Rogers and Croft, 1999). Differentiation of $CD4^+$T cells towards a Th2 pattern of cytokine expression resulted from moderate stimulation, while a very high level of stimulation induces differentiation into cells expressing the Th1 cytokines (Rogers and Croft, 1999). In short-term experiments, however, the Th1 differentiation required lower levels of stimulation than the Th2 one, and it is still unclear how different signal strengths generated from different TCR binding affinities to different Ags are processed by naïve $CD4^+$T lymphocytes to derive the two differentiation pathways in the periphery. In contrast, TCR binding affinity in thymic selection of T cells is relatively well understood (reviewed in (Ashton-Rickardt and Tonegawa, 1994; Miller and Basten, 1996; von Boehmer et al., 2003). Immature T cells ($CD4^-CD8^-$) migrate from the bone marrow to the thymus where they begin to express CD4 and CD8 molecules and rearrange their TCR α and β genes (Lo, 1992; Mondino et al., 1996). These CD4 and CD8 molecules together with TCR heterodimers are used in both positive and negative intra-thymic selection. Cells that fail to recognize self-peptide-MHC molecule complexes will be eliminated during the positive selection process. The positively selected cells contain cells with high reactivity to self peptide-MHC complexes; these cells are eliminated in a negative selection process. The result of both positive and

negative selection is the large peripheral repertoire of T cells that can recognize MHC molecules bound to virtually any possible foreign peptide but tolerate self peptide-MHC complexes.

1.2.3 T cell populations

T cells are pivotal in the immune system, exerting effector function to eliminate pathogens and contribute to immunoregulation. Peripheral T lymphocytes can be separated on the bases of their expression of the cell-surface marker CD4 and CD8 (Macphail and Stutman, 1987; Matsubayashi et al., 1989). T cell activation is restricted by their ability to recognise Ags bound to MHC molecules with the $CD4^+$ subset, or Th cells, recognises Ags bound to MHC-II molecules, whereas the $CD8^+$ subset, or Tc cells, recognises Ags presented by MHC-I molecules. Some $CD4^+$T cells, however, can mediate killing activity (Kaplan et al., 1984), and some $CD8^+$Tcells do secret cytokine to mediate regular activity on other immune cells (Callan et al., 2000). Therefore, the classification of $CD4^+$T cells as helper and $CD8^+$T cells as Tc is not absolute.

$CD4^+$T cells

$CD4^+$T cells are primarily responsible for co-ordinating the immune response through direct interaction with other cells and by secreting cytokines. They collaborate with B cells in the generation of effective humoral immunity as they induce antibody isotype switching and enhance antibody production. In addition, they play an important role in the induction of the cellular immune response by providing help for $CD8^+$T cell activation. Generally, based on cytokine secretion patterns, Th cells can be divided into two subsets; Th1 and Th2. Th1 cells secrete IL-2, TNF-α, and IFN-γ, while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 (reviewed in (Del Prete, 1998; Mosmann and Coffman, 1989)). T cells expressing both Th1 and Th2 cytokines have also been described in some studies and designated as Th0 cells (Maggi et al., 1988; Paliard et al., 1988). These cells mediate intermediate effects, depending on the ratio of cytokine produced and the nature of the responding cells, and they probably represent a heterogenous population of partially differentiated effector cells consisting of multiple discrete subsets.

Th1 and Th2 responses are antagonistic to each other and one subtype is usually dominant in a response to any particular pathogen in any one circumstance. Most intracellular pathogens, including viruses, intracellular bacteria, protozoa, and fungi, induce Th1 responses which regulate the cellular immune response. On the other hand, most extracellular pathogens induce Th2 responses that enhance humoral responses. Th2 cells are also predominant in allergic diseases (Parronchi et al., 1991). Both subtypes are defined as populations of cells, since it has been demonstrated that most of these cells secrete only one cytokine per cell at any one time (Karulin et al., 2000). The differentiation of T cell precursors into Th1 and Th2 may depend on cytokine environment, Ag presentation site, physical form and dose of Ag, and type of adjuvant (reviewed in (Del Prete, 1998)). IFN-I is a crucial factor during viral infection that induces Th1 differentiation leading to Tc cell responses as illustrated by the absence of Tc cell responses during LCMV infection in IFN-IR knockout mice (Muller et al., 1994).

$CD4^+$T cells play an important role in anti-viral responses as both Th1-derived cytokines, such as TNF-α and IFN-γ, and Th cell-dependent anti-viral Abs have been shown to be important in the control of some viral infections, such as influenza virus (Eichelberger et al., 1991; Graham et al., 1994; Scherle et al., 1992), VSV (Maloy et al., 2000), and VACV (Kagi et al., 1995b; Spriggs et al., 1992). The importance of Th cells in the induction of the anti-viral Tc cell response varies with different viruses as absence of Th cells significantly reduces the Tc cell response to LCMV, VSV, ECTV, and VACV ((Battegay et al., 1996; Leist et al., 1989), whereas deficiency of Th cells does not cause impairment of the Tc cell response to Sendai virus (Hou et al., 1995). In addition, cytolytic $CD4^+$T cells can be generated using different experimental protocols (reviewed in (Kagi et al., 1996)). Their MHC-II-restricted killing activities appear to be mainly mediated by the Fas-dependent pathway. Cytolytic $CD4^+$T clones have been established from peripheral blood monocytes of individuals during Dengue virus infection (Kurane, I., et al., 1995 and 1998), or following immunisation with JE vaccine (Mullbacher et al., 2003).

$CD8^+$T cells

Tc lymphocytes express CD8 molecules and recognise Ag presented by MHC-I molecules. They have been implicated as critical effector cells during viral infections (Blanden, 1974; Reddehase et al., 1987; Yap and Ada, 1978). Their anti-viral activity is attributed to their ability of generating cytotoxic attack against virus-infected cells, in addition to their ability of secreting cytokines (reviewed in (Mullbacher, 2003)). $CD8^+$T cells can secrete cytokines such as, IFN-γ and TNF-α, as well as chemokine which, in addition to their anti-viral effect, function to recruit and/or activate the microbicidal activities of effector cells such as macrophages and neutrophils (reviewed in (Harty and Bevan, 1999)). In addition, their cytokine profile appears similar to that described for $CD4^+$T cells and they can also be subdivided into two subsets, Tc1 and Tc2 (reviewed in (Karupiah, 1998)). The most investigated $CD8^+$T cells effector function, however, is their ability to generate cytotoxic attack, which requires direct contact with their targets. Activated Tc cells are able to induce cytolysis of target cells by two distinct molecular pathways ((Berke, 1995; Kagi et al., 1994a; Lowin et al., 1994): the granule exocytosis and the Fas-mediated pathways. These two pathways also mediate target cell killing by NK cells.

Granule exocytosis

Granule exocytosis is arguably the principal cytolytic pathway used by Tc cells to eliminate virus-infected cells. Granules of activated Tc cells contain various proteins, including a membrane-disrupting protein named perforin (perf), and a number of structurally related serine proteases known as granzymes (gzm) (reviwed in (Trapani and Smyth, 2002). Generation of lytic granules is an important process in the differentiation of T cells into cytolytic effector cells. Upon Ag encounter, the effector cell secretes the granule contents into the space between target and effector cell, in a $Ca2^+$-dependent process that is associated with extensive cytoskeleton reorganisation (reviewed in (Griffiths, 1995)). The role of granule exocytosis in the immune response during viral infections has been extensively investigated using mice carrying targeted null mutations in key elements of the cytolytic machinery, including perf, gzmA and B (Kagi et al., 1999; Matloubian et al., 1999; Mullbacher et al., 1996; Mullbacher et al., 1999a; Mullbacher et al., 1999b; Ramshaw et al., 1997; Simon et al., 1997).

Perf plays a pivotal role in granule-mediated cytotoxicity with Tc cells and NK cells from perf-deficient ($perf^{-/-}$) mice being defective in their ability to induce both membrane damage and apoptosis *in vitro* (Kagi et al., 1994a; Kojima et al., 1994); (Lowin et al., 1994). Perf is usually released as monomers which polymerise in the plasma membranes of target cells, forming pores (Liu et al., 1995). This action of perf has considerable analogy to the complement component C9 (Shinkai et al., 1988; Young et al., 1986), and can cause osmotic damage of target cells (Lichtenheld et al., 1988; Shinkai et al., 1988). However, apoptotic death rather than osmotic damage is considered to be the main feature of target cell death, and is characterised by chromatin condensation, extensive membrane blebbing, and DNA fragmentation (Shi et al., 1992a; Shi et al., 1992b). This is explained by the synergistic action of perf with other lytic granule constituents, gzms.

Gzms are lymphocyte granule serine proteases belonging to the trypsin family (Odake et al., 1991), located in cytoplasmic granules of Tc and NK cells (Pasternack et al., 1986), and released directly into the immunological synapse between the effector and the target cell (Stinchcombe et al., 2001). A total of 11 highly homologous (with 38-67% homology to gzmB) gzms have been found in human and rodent cytolytic cells (Haddad et al., 1991), and they contain the catalytic triad (His-57, Asp-102, and Ser-195) of the trypsin family serine proteases (Kam et al., 2000). Although gzms can diffuse through pores formed by perf, they can also enter the cytoplasm of target cells independently of perf, either via receptor-mediated endocytosis or through macropinocytosis (reviewed in(Trapani and Smyth, 2002)). The two most studied gzms are gzmA and B. The participation of gzmA (Beresford et al., 2001; Fan et al., 2002) (Fan et al., 2003a; Fan et al., 2003b; Pinkoski and Green, 2003) and gzmB (Metkar et al., 2003; Pardo et al., 2002; Sharif-Askari et al., 2001; Smyth and Trapani, 1995; Sutton et al., 2000; Trapani and Smyth, 1993; Yang et al., 1998) in cytotoxic cell-mediated killing have been investigated extensively. GzmB triggers an endogenous cell death cascade by activating key pro-apoptotic target cell caspases ((Daniels et al., 1999; Fernandes-Alnemri et al., 1996; Song et al., 1996; Srinivasula et al., 1996). Studies using gzmB-deficient ($gzmB^{-/-}$) mice have illustrated its crucial role for rapid induction of apoptosis (Heusel et al., 1994). GzmA, a tryptic protease, has been shown to act through a distinct pathway, characterised by delayed kinetics of apoptosis induction (Beresford et al., 1999; Beresford et al., 2001; Shi et al., 1992a; Shresta et al., 1999).

Mice deficient in both gzmA and B ($gzmAB^{-/-}$) are severely defective in both early and late DNA fragmentation (Shresta et al., 1999; Simon et al., 1997). In addition, the presence of gzmA enhances significantly DNA fragmentation by gzmB (Beresford et al., 1999; Nakajima et al., 1995). This synergistic effect has been illustrated by the increasing susceptibility to ECTV infection in $gzmA^{-/-}$, $gzmB^{-/-}$, and $gzmAB^{-/-}$ mice (Mullbacher et al., 1999b). Furthermore, the ability of cytotoxic cells from $gzmB^{-/-}$ mice to induce rapid target-cell DNA fragmentation is markedly reduced (Heusel et al., 1994; Shresta et al., 1995). In contrast, cytotoxic cells from $gzmA^{-/-}$ mice have normal cytotoxic responses *in vitro* (Ebnet et al., 1995; Shresta et al., 1997), but these mice can no longer restrict the spread of HSV in neurons (Pereira et al., 2000), and are unable to clear ECTV (Mullbacher et al., 1996). Interestingly, perf and/or gzms appear to contribute to the mortality and morbidity during MVE virus infection in mice (Licon Luna et al., 2002). Similarly, the granule exocytosis pathway was reported to contribute to virus infection-induced autoimmune diseases such as coxsackie virus-induced myocarditis (Gebhard et al., 1998) and HSV-1-induced keratitis (Chang et al., 2000).

Fas pathway

Tc cells can also generate cytotoxic attack by up-regulating their surface expression of Fas ligand (Fas.L) (also known as CD178). Engagement of the target cell death receptors Fas (also termed APO-1 or CD95) by Fas.L expressed on effector Tc cells results in classical caspase-dependent apoptosis (Itoh et al., 1991; Lowin et al., 1994; Nagata and Golstein, 1995; Rouvier et al., 1993; Stalder et al., 1994). Fas.L has been shown to be homologous to membrane TNF (Suda et al., 1993), and can be rapidly expressed on $CD8^+$ and $CD4^+$T cells following TCR engagement (Carter and Dutton, 1995; Ramsdell et al., 1994; Suda et al., 1995). This Fas.L induction appears to involve the activation of src-family and other tyrosine kinases, which requires RNA and protein synthesis and the presence of calcium, and is inhibited by cyclosporine A (Anel et al., 1994; Anel et al., 1995).

Fas belongs to the TNF receptor family, and possesses structural similarity with TNF receptor-1, a conserved extracellular region and a cytoplasmic domain essential for the induction of apoptosis (check ref (Nagata and Golstein, 1995; Tartaglia et al., 1993). Fas is constitutively expressed in the thymus (Watanabe-Fukunaga et al., 1992), in all murine (Andjelic et al., 1994; Drappa et al., 1993; Nishimura et al., 1995) and human

(Debatin et al., 1994) thymocyte subsets. In the periphery, Fas expression can be induced by activation on both T and B cells (Watanabe-Fukunaga et al., 1992) (Watanabe et al., 1995). Induction of Fas expression upon TCR triggering requires the product of the *TDAG51* gene (Park et al., 1996). Fas expression on T cells occurs at a late stage of activation (2-3 days) (Miyawaki et al., 1992), rendering them susceptible to activation-induced cell death (AICD), which has been shown to be Fas-dependent (Alderson et al., 1995; Dhein et al., 1995; Ju et al., 1994; Sytwu et al., 1996; Zheng et al., 1995). Outside the immune system, Fas is constitutively expressed in several organs such as the liver (Watanabe-Fukunaga et al., 1992).

The Fas pathway is similar to granule exocytosis pathway in that maximal expression of the Fas.L requires an initial activation signal by the TCR (Zheng et al., 1998). The Fas pathway is believed to be associated with immune regulation, particularly in immune system homeostasis (Alderson et al., 1995; Ju et al., 1995; Nagata and Golstein, 1995; Rathmell et al., 1995; Rouvier et al., 1993; Russell, 1995b)). Mice with a loss-of-function mutation in the Fas gene (*lpr*), and Fas.L gene (*gld*) suffer from progressive expansion of peripheral lymphoid populations, resulting in massive lymphadenopathy, splenomegaly and lymphocyte infiltration in organ such as liver and kidney (Watanabe-Fukunaga et al., 1992) (reviewed in (Nagata, 1999)). The *lpr*, which is an insertion of transposon into intron 2 of *Fas* gene, does allow the expression of minute amounts of functional protein (Chu et al., 1993). In contrast, *gld*, which is a point mutation in the extracellular region of Fas.L that affects the binding to Fas, has been considered a null allele (Takahashi et al., 1994). In addition, Fas.L-knockout ($Fas.L^{-/-}$) (Karray et al., 2004), and Fas-knockout ($Fas^{-/-}$) (Adachi et al., 1995) mice suffer similar lymphadenopathy and splenomegaly to that seen in *lpr* and *gld* mice. This may be related to the role of the Fas pathway in AICD, which serves to down-regulate the expansion of activated T cell clones following their response to Ags (Waring and Mullbacher, 1999).

It was generally believed that the granule exocytosis pathway is the primary mechanism for the elimination of intracellular pathogens including viruses, whereas the Fas pathway of cytotoxicity plays an immunoregulatory role (Kagi et al., 1995a; Nagata, 1997; Rouvier et al., 1993). Recent evidence, however, suggests that in many virus infections both pathways are operative as mice defective in either cytolytic pathway

show delayed and reduced mortality when infected with MVE (Licon Luna et al., 2002), and the concerted action of both pathways is mandatory for the development of LCMV infection-induced hepatitis (Balkow et al., 2001). In addition, Tc cells from perf$^{-/-}$ mice can lyse their target cells via Fas-Fas.L interaction (Simon et al., 2000). In addition, Ag-dependent release of IFN-γ by perf-deficient Tc cells increases the expression of Fas on target cells facilitating their killing in Fas/Fas.L-dependent pathway (Mullbacher et al., 2002). Therefore, both cytotoxic pathways are operative during viral infections. Fas-mediated apoptosis, and not granule exocytosis-mediated apoptosis, is implicated in MHC-I-restricted killing of neurons by virus immune CD8^{+}T cells (Medana et al., 2000). This is supported by the ability of Fas.L to protect neurons against granule exocytosis-mediated killing (Medana et al., 2001). This finding suggests that cytolytic effector cells, such as NK and Tc cells, may use only the Fas/Fas.L pathway to eliminate neurons infected with neurovirulent viruses.

1.2.4 T lymphocyte activation

Given the ability of Tc cells to generate tissue destruction, their activation must be tightly regulated. Naïve Tc cell precursors have no effector function and must undergo an activation process requiring 1-3 days for maximal activity. This activation process requires Ag recognition mediated by the TCR. However, signalling through TCR alone is not sufficient by itself to initiate the maturation pathway of naïve T cells. A second, costimulatory signal delivered to T cell, simultaneous with TCR ligation, is required. This concept of two signals required for lymphocytes activation was originally developed for B cells (Bretscher and Cohn, 1970), before it was adapted for T cells (Lafferty and Cunningham, 1975). Cells providing the costimulatory signals required for naïve T cells priming are rare. Only so-called professional APC, like B cells, macrophages and dendritic cells, have the ability to present Ag and provide costimulatory signals. Once activated, T cells do not need costimulatory signals to generate their effector functions whether this be cytotoxicity or cytokine production. Importantly, the same signals that activate T cells also stimulate their proliferation, and virus immune T cells can expand several orders of magnitude in response to viral infections (reviewed by (Doherty and Christensen, 2000)).

Costimulatory molecules

Upon Ag uptake, APC upregulate several membrane-associated accessory molecules important for T cell priming. Both CD80 (also known as B7.1) and CD86 (also known as B7.2) are expressed predominantly on APC. CD86 has been implicated as the predominant costimulatory ligand during initial Ag encounter (Lenschow et al., 1993), whereas CD80 plays a role in modulating the strength of the ongoing immune response (Hathcock et al., 1994; Lenschow et al., 1994; Miller et al., 1995). Early studies indicated that CD80 and CD86 influence Th cell phenotype differentiation with CD80 promoting the Th1 phenotype and CD86 the Th2 phenotype (Kuchroo et al., 1995; Lenschow et al., 1995), but this is still controversial (MacPhee et al., 2001). Both CD80 and CD86 can bind and induce signals through CD28, which is constitutively expressed on T cells and appears to be the most important costimulatory molecule (Green et al., 1994; Vesosky and Hurwitz, 2003). Ligation of CD28 enhances T cell/APC interaction and facilitates TCR engagement and signalling (Michel et al., 2001). The effects of CD28 ligation include, an increase in IL-2 production, upregulation of CD25 (the IL-2 receptor) expression (Jenkins et al., 1991; Martin et al., 1986), as well as upregulation of anti-apoptotic molecules like Bcl-xL, protecting from AICD (Boise et al., 1995). CD152 (also known as CTLA-4), which is upregulated following T cell activation, can also bind to CD80 and CD86. CD152 binding to CD80 or CD86, however, has an inhibitory effect on T cell activation (Krummel and Allison, 1995; Krummel and Allison, 1996). In addition, CD154 has much higher binding affinity for CD80 and CD86 compared to CD28 (Linsley et al., 1994). Mice deficient in CD152 suffer from lymphoproliferative disorder and die within 4 weeks of birth (Waterhouse et al., 1995). *In vitro* study, investigating the costimulatory effect of CD152 on Th cell differentiation, showed that CD152 engagement promotes Th1 differentiation, probably by inhibiting IL-4 secretion (Kato and Nariuchi, 2000).

Other costimulatory molecules include ICOS and PD-1, members of the CD28 family of molecules (reviewed in (Sharpe and Freeman, 2002)). Beside the CD28 family, molecules such as LFA-1, 4-1BB, OX40, CD40, CD2 and others do provide adhesion and costimulatory effects (reviewed in (Vesosky and Hurwitz, 2003; Watts and DeBenedette, 1999)). Among these different molecules, CD69 has been used as an activation marker. Many different cell types including T, B, NK cells, and monocytes

express CD69, which is a member of the NK cell gene complex family of signal transducing receptors (Marzio et al., 1997). In addition, CD69 is one of the earliest induced cell-surface Ag expressed by T cells following activation, and may play a role in their differentiation (reviewed in (Ziegler et al., 1994)). CD69, CD86 and CD25 expression levels on B and T cells have been used as an indication for their activation (Braun et al., 2002; Hakamada-Taguchi et al., 1998; Lowenthal et al., 1985; Tough et al., 1996).

Ag presenting cells

APC can uptake exogenous protein, via phagocytosis or receptor-mediated endocytosis, and present it in the context of MHC-II molecules. As they express costimulatory molecules, they have the ability to activate naïve T cells. Among them, DCs are the most efficient APCs (Bhardwaj et al., 1992; Bhardwaj et al., 1993; Croft et al., 1992; Macatonia et al., 1995; Steinman and Witmer, 1978; Van Voorhis et al., 1983). The frequency of naïve antigen reactive T cell-clones is low to any given MHC-II/peptide T cell determinant (Banchereau and Steinman, 1998), and less than 1% of the total T cell pool even at the hight of the immune response to a viral infection (Tough and Sprent, 1994; Tripp et al., 1995). Infected cells normally lack the costimulatory molecules required for the clonal expansion or proliferation of T cells, cytokine production, and effector cells development. DCs provide the bridge between Ag-presentation as well as providing costimulation required for T cell activation. DCs are present in most tissues to capture and present Ag, after which they upregulate the expression of costimulatory molecules, and migrate to lymphoid tissues (reviewed in (Banchereau and Steinman, 1998; Carbone and Heath, 2003)). Therefore, naïve T cells survey the entire body for their reactive Ag by migrating through secondary lymphoid tissues screening Ag presented by DC.

Considering the intracellular parasitic nature of viruses and their dependence on host protein synthesis machinery for replication, all viral proteins are potentially accessible to the endogenous MHC-I presentation pathway and to surveillance by $CD8^+$T cells (Harty et al., 2000). Therefore, activation of $CD8^+$T cells during viral infection requires the infection of APC, or DC, but not all viruses do infect DCs. A process called cross-

priming (Bevan, 1976a; Bevan, 1976b), or cross-presentation (reviewed in (Heath and Carbone, 2001)) overcomes the requirement of DCs infection in order to activate $CD8^+$T cells. Bone-marrow-derived host APC, particularly DCs can efficiently take up material from apoptotic cells and present antigens derived from this material on MHC-I (den Haan et al., 2000). Relatively small molecules (3-20KD) have been demonstrated to be able to escape the endocytic system of DCs into the cytoplasm where they gain access to cytosolic antigen-processing machinery (MHC-I Ag presenting pathway) (Rodriguez et al., 1999). Recently, Bevan has suggested that DCs can present Ag via MHC-I in the absence of $CD4^+$T cell help, if there is a danger signal in the form of inflammatory cytokines (Bevan, 2004). In the absence of an inflammatory signal, a signal from $CD4^+$T cells to DCs is crucial to induce MHC-I Ag presentation for $CD8^+$T cell priming. In addition, ligation of CD40 via CD40L on activated $CD4^+$T cells may play an important role in DCs activation (Ludewig et al., 1995) and stimulate secretion of IL-12, an inducer of Th1 responses (Macatonia et al., 1995). These CD40-associated features indicate that DCs may be the crucial link that provides $CD4^+$T cell help for $CD8^+$T cell activation, (reviewed in (Bevan, 2004)).

Mouse DCs consist of a large number of subpopulations which can be identified according to their cell surface expression of CD8α, CD4, CD11b, Gr-1 and B220 (reviewed in (Carbone and Heath, 2003; Shortman and Liu, 2002)). Classification on the basis of CD4 and CD8α expression suggested at least 3 distinct subpopulations of DCs namely $CD4^+$, $CD4^-CD8^-$ (DN), and $CD8^+$ (Shortman and Liu, 2002). Despite the controversy as to whether these populations constitute stable DC subsets or differentiation stages, it is thought that these subsets vary in their cytokine production potential, and, possibly, in their function. It has been argued that $CD8^+\alpha$ DC are uniquely able to synthesise IL-12 and promote Th1 responses while other subsets fail to make IL-12 and promote Th2-dominated immunity (Maldonado-Lopez et al., 1999; Pulendran et al., 1999).

Immature DC, tissue-resident, express endocytic receptors for pathogens, such as the mannose receptors, DEC-205 and DC-SIGN (Lanzavecchia and Sallusto, 2001). Upon activation, the phagocytic ability is downregulated, and cytokine receptor and adhesion molecule expression is altered such that activated DC can migrate into secondary lymphoid organs (Cumberbatch and Kimber, 1992). Mature DC secrete cytokines that

attract naïve as well as activated T cells (Adema et al., 1997; Tang and Cyster, 1999), and are located in the T cell areas of the secondary lymphoid tissues (Ingulli et al., 1997). The expression of MHC molecules (Cella et al., 1997; Pierre et al., 1997), and the costimulatory molecules CD80 and CD86 (Caux et al., 1994; Sallusto and Lanzavecchia, 1994; Turley et al., 2000) is upregulated. These changes enable mature DC to prime T cell responses.

DC maturation is a prerequisite for the induction of T cell responses. Immature DCs, particularly plasmacytoid dendritic cells (pDCs), produce 1000-fold higher IFN levels than other cell types, and are responsible for the systemic IFN-I responses during many virus infections (Le Bon and Tough, 2002). IFN-I produced by human monocyte-derived DCs or mouse DCs (derived from spleen or bone marrow) has been shown to exert autocrine stimulatory effects on the respective DC populations (Luft et al., 1998) (Mattei et al., 2001). It has, also, been shown that IFN-I induced during influenza virus infection resulted in sustained presentation of MHC-I/viral peptide complexes to T cells by human DCs (Cella et al., 1999b). Exposure to IFN-I induces expression of costimulatory molecules such as CD80 and CD86 on DCs facilitating communication with T cells during activation (Gallucci et al., 1999; Ito et al., 2001; Santini et al., 2000). Other activating signals have been reported to be induced, including IL-15, IL-1 and TNF-a as well as products generated by cells stimulated with bacterial products (Koide et al., 1987; Mattei et al., 2001; Roake et al., 1995; Witmer-Pack et al., 1987).

1.2.4 Cytotoxicity and immunopathology

Both types of killer cells (NK and Tc) use granule exocytosis and Fas-mediated pathways to generate their cytolytic attack. The granule exocytosis pathway is the principal killing pathway for limiting viral infection in the periphery (Kagi et al., 1994b). NK cells activity can be increased by cytokines like IL-2 and IFN-γ. Their granules are preformed, and they can kill their targets within minutes after stimulation by activating receptors (reviewed in (Russell and Ley, 2002)). Elevated NK-cytolytic activity is present in the spleen early (1-5 days) after viral infections (Biron et al., 1999; Karupiah et al., 1992; Mullbacher and King, 1989a). In contrast, naïve $CD8^+$T cells have no cytolytic activity and they must undergo an activation process that requires

TCR ligation and costimulation, which then leads to the expression of granule components, including perforin and gzms (reviewed in (Russell and Ley, 2002)). Signals that activate $CD8^+$T cells also stimulate their proliferation, and virus-immune $CD8^+$T cells can expand several orders of magnitude in response to viral infection (Doherty and Christensen, 2000). Considering the requirements of activation and proliferation of Tc cells to generate their effector function, maximal cytolytic activity is reached at about 4 days after peak viral load (Mims and Blanden, 1972). $CD8^+$T cell-mediated cytotoxicity following ECTV infection was highest 5-6 days post-infection. A response to LCMV infection was first detected at day 6 which peaked at day 8 post-infection (Zinkernagel and Doherty, 1979).

The Fas pathway of cytotoxicity is similar to that of granule exocytosis mediated killing in that maximal expression of the Fas.L requires an initial activation of the effector cell (Zheng et al., 1998). *In vivo* experiments suggest that the granule exocytosis pathway is principally involved in target cell lysis via MHC-I Ag presentation pathway and that Fas-pathway dominates MHC-II Ag presentation pathway, especially those executed by the $CD4^+$T cells (Graubert et al., 1997; Ju et al., 1994; Schulz et al., 1995). Recently, however, the Fas-mediated pathway was shown to be the prominent killing pathway by $CD8^+$T cells against infected neurons (Medana et al., 2000). An important difference between Fas- and exocytosis-initiated pathways is the speed of the cytotoxic event. Once formed, granules can be reoriented and released within minutes of TCR stimulation (Kupfer et al., 1985). In contrast, very little ligand (e.g., Fas.L) is stored, even in activated cells. Therefore, maximal activity requires the synthesis of new ligand, which requires 1-2 h after TCR stimulation. Induction of new ligand continues as long as there is TCR stimulation, and Fas.L is cleared from the surface either by proteolysis or endocytosis, depending on the effector cell. This clearance of Fas.L from the surface occurs with a half-life of 2-3 h (Nguyen and Russell, 2001). The long half-life of Fas.L on the surface allows effector cells to continue to execute cytotoxic activity in the absence of TCR stimulation and leads to the phenomenon of bystander killing, which means that cells in contact with effector cells that express Fas can be killed even though they do not express nominal Ag recognised by the TCR (Wang et al., 1996).

Despite the important role of both NK and Tc cells in eliminating viral-infected cells, their cytolytic activity itself can increase the severity of the infection, as it may lead to extensive tissue damage. In fact, immune-mediated cytolytic activity, particularly by Tc cells, as a result of viral infection has been associated with tissue injury (Balkow et al., 2001; Doherty and Zinkernagel, 1974; Guidotti et al., 1999; Kagi et al., 1996; Wang et al., 2003). Both LCMV and SFV infections are models for virus-induced Tc cell-mediated immunopathology. Infection of immunosuppressed, e.g. severe combined immune deficiency (SCID) and cyclophosphamide-treated mice with the neurotropic strain LCMV-Armstrong is not lethal compared to lethal encephalitis observed in immunocompetent mice, which is characterised by marked cerebral infiltration of $CD4^+$T and $CD8^+$T cells (Doherty et al., 1990). Adoptive transfer of virus-immune splenocytes into Cy-immunosuppressed mice-infected with LCMV resulted in fatal encephalitis, and depletion of $CD8^+$T cells but not $CD4^+$T cells abrogated the ability of the adoptive transferred cells to induce the fatal outcome (Dixon et al., 1987). Similarly, investigating the immunopathological aspects during SFV infection revealed that demyelination is predominantly mediated by $CD8^+$T cells in CBA/C3H and BALB/c mice (Fazakerley, 2002; Fazakerley and Webb, 1987a; Subak-Sharpe et al., 1993).

1.3 Alphaviruses

1.3.1 Members and distribution

The genus *Alphavirus*, of the family Togaviridae, consists of about 30 members which are defined as enveloped single-stranded positive-sense RNA viruses. They have a wide host range, but the principal vertebrate hosts are birds, rodents, and primates, with infection transmitted by mosquitos (Johnston and Peters, 1996). Alphaviruses, as a genus, are globally widely distributed, and some members are serious human and animal pathogens. In humans, the severity of alphaviruses infections is extremely variable ranging from asymptomatic infection (most infections) to severe encephalitis and death. Others are characteristically associated with an acute arthropathy. Thus, members of this genus can be classified according to their associated disease as shown in table 1.1. The geographic distribution of different viruses within the genus

Alphavirus is restricted, and human pathogenic alphaviruses can be divided into old world viruses (e.g. Sindbis (SIN) and Ross River (RR)) that cause diseases characterised by rash and arthritis, and new world viruses (e.g. Eastern, Western, and Venezuelan equine encephalitis viruses) that cause encephalitis (Griffin, 1996). In Australia, RRV is endemic and epidemic in tropical and temperate regions with Aedes mosquitoes the main vectors. Although unknown to be fatal, the discomfort and loss of productivity from joint symptoms persist for weeks and occasionally even years.

All alphaviruses have similar morphology and structure, and share a common replication strategy. SIN and SFV have been studied extensively, and are the best-characterized alphaviruses. Both SIN (reviewed in (Irusta and Hardwick, 2004)), and SFV (reviewed in (Atkins et al., 1999; Fazakerley, 2002)) can cause lethal encephalitis in mice and have been used as a model for viral neuropathogenesis. The laboratory strains of SFV and SIN are considered safe for humans (Strauss and Strauss, 1994).

1.3.2 Structure and replication

Alphaviruses are simple enveloped viruses having a regular virion structure (reviewed in (Schlesinger and Schlesinger, 1996; Strauss and Strauss, 1994)). The virion consists of one copy of RNA encapsidated in an icosahedral protein shell composed of the capsid protein, which is surrounded by a lipid envelope derived from the host plasma membrane. The envelope bilayer is penetrated by heterodimers of the viral glycoproteins E1 (50 kDa) and E2 (50 kDa) (reviewed in (Strauss and Strauss, 1994). In the case of SFV, a third protein, E3 (10 kDa) is peripherally bound to E1-E2 heterodimers. E1 is required for the membrane fusion between the envelope and target membrane (Gibbons et al., 2003; Gibbons and Kielian, 2002; Haag et al., 2002; Wahlberg and Garoff, 1992), while E2 mediates virus particle attachment to the cell surface (Smith et al., 1995; Strauss and Strauss, 1994). The vast host range of alphaviruses indicates that these viruses must use a variety of different molecules as receptors. In mammalian cells, laminin receptor has been identified as the major receptor for SIN (Wang et al., 1992). MHC molecules were reported to be receptors for SFV (Helenius et al., 1978), but they are not essential for infectivity (Oldstone et al., 1980).

Despite the uncertainty regarding receptor type, alphaviruses have been shown to enter cells via receptor-mediated endocytosis (Helenius et al., 1980; Marsh and Helenius, 1989). Exposure to low pH in the endosomes results in dissociation of the E1-E2 heterodimers and concomitant trimerization of the E1 subunits (Wahlberg et al., 1992). The rearrangement of viral glycoproteins triggers E1-membrane fusion activity, and allows the nucleocapsid to be released into the cytoplasm (reviewed in (Garoff et al., 1994; Kielian, 1995). In the cytoplasm, the binding of nucleocapsid to ribosomes mediates viral RNA uncoating (Wengler and Wurkner, 1992).

The genomic RNA (about 12 kb) contains a 5'-terminal cap and a 3'-terminal poly(A) tail. It codes for nine functional proteins derived from two open reading frames via polyprotein precursors (Figure 1.1) (Kaariainen and Soderlund, 1978). The genome is divided into two major regions: a non-structural domain, encoding the non-structural or replicase proteins, and forms the 5'-terminal two-thirds of the RNA, and a structural domain, encoding the structural proteins of the virus at the 3'-terminal one-third (Figure 1.1). Following its release into the cytoplasm, the positive-sense genomic viral RNA functions as mRNA for translation of the viral nonstructural proteins as a ploy protein (P1234) and as a template for the synthesis of the complementary minus strand. After translation, this polyprotein is cleaved into four proteins designated as nsP1, nsP2, nsP3 and nsP4 (reviewed in (Kaariainen and Ahola, 2002)). The nsP4 is the first to be cleaved, and forms a complex with P123 to initiate minus strand RNA synthesis. Uncleaved polyprotein P123 synthesises plus strand, but it is generally cleaved into nsP1, nsP2 and nsP3 which in conjunction with nsP4 form the complexes that mediates the synthesis of both a new genomic RNA and a 26S subgenomic RNA, from the minus strand template.

Structural proteins are translated from the 26S subgenomic RNA as a polyprotein containing capsid, P62 (the precursor of E3, E2, and 6K), and E1 proteins (Strauss and Strauss, 1994). Processing of this polyprotein to produce the structural proteins is initiated by the autocatalytical release of the capsid protein. This capsid cleavage exposes the N-terminal sequence of the P62 which functions as a signal sequence for the polyprotein translocation into the ER (Garoff et al., 1990; Melancon and Garoff, 1987). Translocation of P62 usually results in 6K protein insertion, and the C-terminal

of 6K mediates the translocation of E1 (Melancon and Garoff, 1986). Signalase in the lumen of the ER mediate appropriate cleavages of the polyprotein into P62 and E1. These proteins form dimers and traveled through the Golgi complex to the cell surface. During transport via the secretory pathway, host cellular enzymes mediate cleavage of P62 into E3 and E2, as well as modification of the N-glycans from high mannose-type to complex ones (de Curtis and Simons, 1988; Green et al., 1981). In addition, the glycoproteins are palmitoylated during the transport (Bonatti et al., 1989; Schmidt, 1982).

Virus particle assembly is initiated by the encapsidation of the genomic RNA (Figure 1.2). The capsid protein recognizes and binds the genomic RNA via specific sequences, the packaging signal. The packaging signals in alphaviruses are not conserved, and they are located in the nsP2 and nsP1 genes in SFV and SIN, respectively (Frolova et al., 1997; Weiss et al., 1989). During viral progeny formation, smaller than normal particles in sizes have been reported for SFV (Barrett et al., 1984) and SIN (Johnston et al., 1975), and have been referred to as defective interfering (DI) particles. Finally, the completion and release of virus particles by budding is driven by specific interactions between the nucleocapsid and the cytoplasmic tail of the spike proteins E2 at the plasma membrane (Gaedigk-Nitschko and Schlesinger, 1991; Sjoberg and Garoff, 2003; Suomalainen et al., 1992).

1.3.3 Semliki Forest Virus

Strains

SFV was first isolated from mosquitoes captured in the Semliki Forest in Uganda in 1942 (Smithburn and Haddow, 1944), with the original isolate, designated L10, being neurovirulent for adult mice causing lethal encephalitis (reviewed in (Atkins et al., 1999)). Subsequently, non-lethal an avirulent strain, designated A7, was isolated in 1961 from mosquitoes in Mozambique (McIntosh et al., 1961). Most laboratory strains of SFV are derived from these two isolates, and they can be divided according to lethality in adult mice into virulent (vSFV), and avirulent (aSFV) (reviewed in (Fazakerley, 2002)). The vSFV strains, such as V13, Osterrieth, and SFV4, were

derived from L10, whereas The aSFV strains, such as A7(74), and A8, were derived from A7 (Bradish et al., 1971; Glasgow et al., 1991; Liljestrom et al., 1991; Henderson et al., 1967). Studies comparing vSFV and aSFV strains have shown that both are neuroinvasive with lethality being dependent on virulence factor and age of animals. While both strains are lethal to neonatal mice, they differ in the extent of replication and the associated neuronal damage in older mice (Fleming, 1977; Gates et al., 1985; Pusztai et al., 1971; Woodward et al., 1977).

SFV was isolated in 1987 from patients, suffering from fever, severe persistent headaches, myalgia, and arthralgia, in the Central African Republic, where it was also isolated from locally caught mosquitoes (Mathiot et al., 1990). Furthermore, seroconversion is common in laboratory workers, and there was one fatal meningoencephalomyelitis, reported in 1979, associated with the vSFV, Osterrieth strain, presence in the brain (Willems et al., 1979). Experimentally, various strains of SFV, capable of infecting voles, mice, guinea pigs, rabbits, and rats, have been used (Bradish et al., 1971; Seamer et al., 1967).

Virulence

Both vSFV and aSFV are neuroinvasive, and have been shown to infect and probably enter the brain by breaching the blood-brain barrier through infecting the cerebral endothelial cells (Fazakerley, 2002; Pathak and Webb, 1974; Soilu-Hanninen et al., 1994). Within the CNS, the infection spreads from an initial perivascular foci that affect oligodendrocytes and glial cells (Balluz et al., 1993). However, the extent of replication and the associated neuronal damage among SFV strains differ according to their virulence factor. The vSFV spreads rapidly around the brain, precluding an effective immune response, resulting in fatal encephalitis (Fazakerley et al., 1993). This occurs irrespective of the age of the mice. In contrast, the severity of CNS infection with aSFV depends on the age of the mice (Fleming, 1977). In neonatal mice, the infection is rapid and fatal, similar to that seen with vSFV infection. In older mice, however, aSFV becomes less able to spread in the CNS and the infection becomes asymptomatic (reviewed in (Fazakerley, 2002)). Generally, the age-dependent asymptomatic infection with the aSFV is believed to be a function of CNS cell maturity. The aSFV is able to

replicate productively in highly active maturing neurons (Oliver et al., 1997). As neurons mature (after 2 weeks), aSFV replication appeared restricted (Fazakerley et al., 1993; Oliver and Fazakerley, 1998; Oliver et al., 1997; Pathak and Webb, 1978). The differences of aSFV replication have been linked to changes in neuron membranes during development. Pre-infection treatment with gold salt, which induces smooth membrane production in the neurons, restores lethality of A7 in mice as well as unrestricted replication in the brain (Gates et al., 1984; Scallan and Fazakerley, 1999).

Both the vSFV (L10 and SFV4) and the aSFV (A7) strains have been sequenced and compared for identification of virulence factors (Glasgow et al., 1994; Santagati et al., 1994; Santagati et al., 1995; Santagati et al., 1998; Tarbatt et al., 1997; Tuittila et al., 2000). These studies have found that the untranslated region at the 3' end of the aSFV genome is 334 nucleotides longer than that of vSFV, but it is not an important determinant of virulence. Differences in the E2 gene, the nsP3 gene, and the 5' untranslated region of SFV genome may be involved in virulence. Virulence in these studies was assessed by lethality in mice without testing possible differences in the immune response. It is possible that vSFV and aSFV strains induce different immune responses, as serum obtained 6 h after vSFV infection show higher plaque inhibitory effect compared to serum obtained after aSFV infection (Fleming, 1977).

Immunopathology

Virus-induced demyelination has been reported in mice infected with avirulent strains of SFV (Chew-Lim et al., 1977). Since virulent strains are lethal to adult mice, avirulent strains, particularly A7, was used in almost all of the work investigating this aspect of SFV infection. However, any mutation that restricts replication of virulent strains in the CNS and allows survival, results in demyelination (Atkins and Sheahan, 1982; Atkins et al., 1985; Glasgow et al., 1991). Demyelination peaks 14-21 days post-infection at a time when infectious virus has been cleared from the CNS in CBA/C3H and BALB/c mice (Fazakerley, 2002; Fazakerley and Webb, 1987b; Subak-Sharpe et al., 1993). Using such mice, depletion studies have indicated a predominant immunopathological role of $CD8^+$T cells. The implication of $CD8^+$T cell in immunopathology is dependent on the genetic background. B-cell-deficient mice of C57BL/6J (B6) background show no white matter vacuolation (or demyelination)

during avirulent SFV infection despite elevated numbers of $CD8^+$T cells in the brain parenchyma (Smith-Norowitz et al., 2000). In fact, Mullbacher and Blanden in 1978 reported a difference in the induction of $CD8^+$T cells-mediated cytolytic responses to alphaviruses (Mullbacher and Blanden, 1978). In that study, splenocytes from both B6 and BALB/c mice failed to lyse SFV-infected macrophages above background levels compared to high killing activity detected for CBA/C3H splenocytes.

Aims of this thesis.

As indicated earlier, the immune response to viral infections is complex and usually involves the interplay of many components of both innate and adaptive responses. Experiments described in this thesis were aimed at contributing to a greater understanding of the immune responses during viral infection, in general, and the alphavirus infections of SFV, in particular.

In chapter 2, infections of vSFV and aSFV in adult mice were compared. A difference in their ability to induce IFN-I was identified as well as a difference in the efficiency of virus growth in spleen and brain.

As SFV infection, particularly aSFV, has been associated with $CD8^+$T cell-mediated immunopathology, experiments described in chapter 3 were aimed to explore the protective and/or detrimental role of cytolytic effector pathways in SFV infection by comparing vSFV lethality in B6 and syngeneic knockout mice. While perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$ mice showed increased resistance to lethal vSFV infection, perf$^{-/-}$x*gld* mice showed the most significant resistance which was associated with reduced virus replication. Mechanisms for the resistance to SFV of this particular strain were explored in chapter 4.

In view of my finding that SFV strains differ in their ability to induce IFN-I, both vSFV and aSFV infections were employed in chapters 5 and 6 to explore the immunoregulatory effect of IFN-I during viral infection. Such an effect was investigated by measuring the expression of the lymphocyte activation markers CD69, CD86, and CD25.

Table 1.1 Alphaviruses and their association with disease (adapted from (Johnston and Peters, 1996)).

Disease	Epidemics
Acute arthropathy	
Chikungunya (CHIK)	+
Mayaro (MAY)	+
O'nyong-nyong (ONN)	+
Igbo Ora	+
Ross River (RR) (epidemic polyarthritis)	+
Sindbis	+
Ockelbo (Pogosta and Karelian fevers)	+
Babanki	
Barmah Forest (BF)	
Systemic febrile illness (encephalitis)	
Semliki Forest (SFV)	
Venezuelan equine encephalitis (VEE)[a]	+
Everglades (EVE)	
Mucambo (MUC)	
Tonate (TON)	
Primarily encephalitis	
Eastern equine encephalitis (EEE)[a]	+
Western equine encephalitis (WEE)[a]	+
Highlands J (HJ)[b]	
No recognized human disease	
Aura (AURA)	
Bebaru (BEB)	
Buggy Creek	
Cabassou (CAB)	
Fort Morgan (FM)	
Getah (GET)[a]	
Kyzylagach (KYZ)	
Middelburg (MID)	
Ndumu (NDU)	
Pixuna (PIX)	
Sagiyama (SAG)	
Una (UNA)	
Whataroa (WHA)	

[a]Also causes equine epizootics.
[b]Rare.

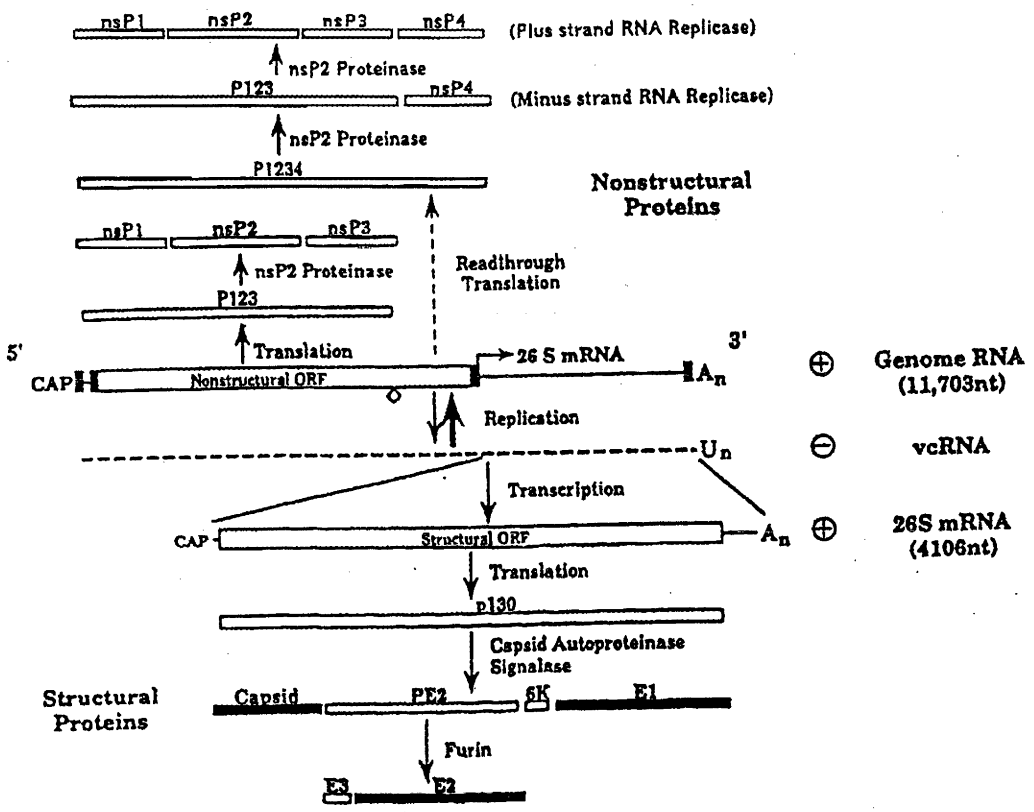

Figure 1.1. Genome organisation of alphaviruses. The genomic RNA serves as a mRNA for the translation of non-structural proteins (nsP). These nsPs function as RNA replicase using the genomic RNA as a template for the generation of minus-strand RNA, which then used as template for the generation of subgenomic RNA, coding the sutructural proteins, and the complete plus-strand RNA, used in nucleocapsidation (adapted from Strauss and Strauss, 1994).

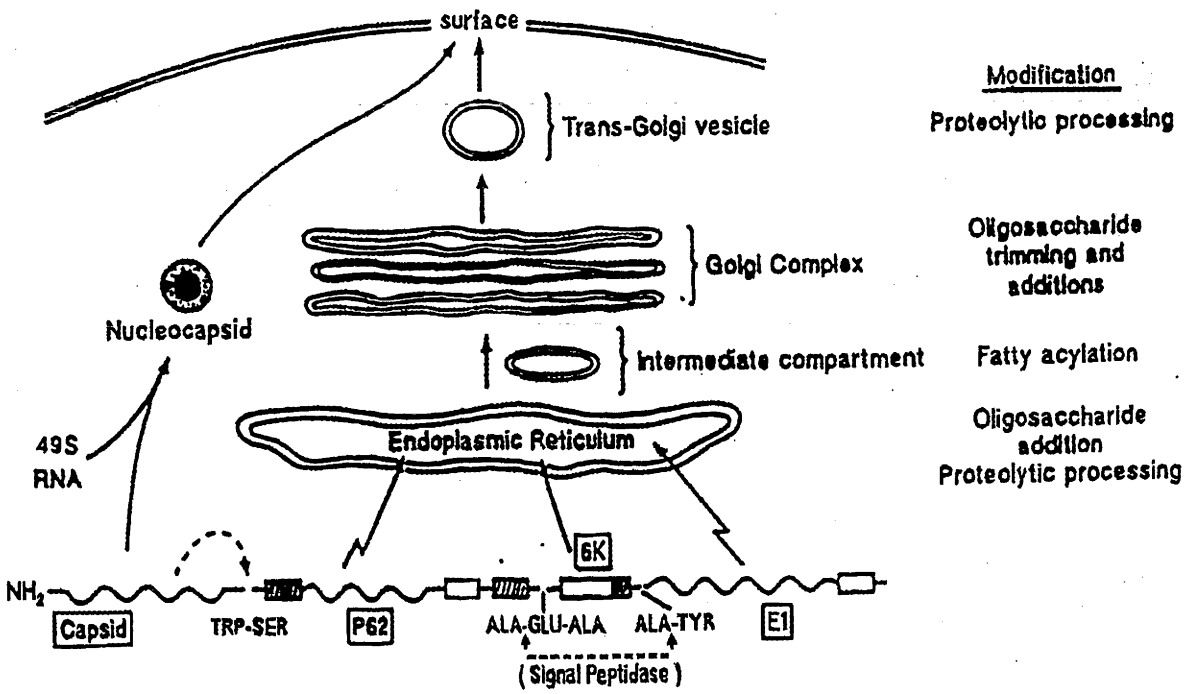

Figure 1.2. The formation of an alphavirus virion. The structural proteins are translated from the subgeneomic RNA as a polyprotein. The capsid protein is released cotranslationally by autoproteolysis and assembles to encapsidate the genomic RNA. Other structural protein are translocated into the ER, modified, and transported to the cell surfase (adapted fromSchlesinger and Schlesinger, 1996).

Chapter 2

A comparison between vSFV and aSFV infections in adult mice

2.1 Summary

Ten-week-old B6 mice were infected with virulent and avirulent SFV and lethality, peripheral replication, and IFN-I induction assessed. In addition, the effect of simultaneous infection with both strains of SFV on lethality was investigated. Both strains are neuroinvasive. vSFV, but not aSFV, results in acute infection and early death characterised by very high virus titres in the brain. The LD50 for vSFV infection < 1 pfu, whereas no mortality occurs with 10^7 pfu aSFV. Mixed infections comprising both strains showed that aSFV protects from lethal vSFV infection in a dose dependent manner. High serum levels of IFN-α were detected following vSFV infection compared to aSFV. This difference in peripheral IFN-I induction may explain the limited replication of vSFV in the spleen compared to that of aSFV.

2.2 Introduction

SFV is a positive stranded RNA virus of the genus alphavirus, member of the Togaviridae family (Strauss and Strauss, 1994). SFV was first isolated from mosquitoes in Uganda in 1944 (Smithburn and Haddow, 1944), with the original isolate, designated L10, being neurovirulent for adult mice causing lethal encephalitis (Atkins et al., 1999). Subsequently, non-lethal an avirulent strain, designated A7, was isolated from mosquitoes in Mozambique (McIntosh et al., 1961). Most laboratory strains of SFV are derived from these two isolates (Atkins et al., 1999).

Previous work used mainly young mice (5-week-old or younger) to compare the two SFV strains for their neurovirulence and lethality (Fleming, 1977; Gates et al., 1985; Pusztai et al., 1971; Woodward et al., 1977). Following peripheral infection, these studies found that SFV replicates in muscles leading to high-titre viremia detectable within 24 h post-infection. Both vSFV and aSFV strains are neuroinvasive, and have been shown to infect and most likely enter the brain by breaching the blood-brain barrier through the infection of the cerebral endothelial cells (Fazakerley, 2002; Pathak and Webb, 1974; Soilu-Hanninen et al., 1994). Within the CNS, the infection spreads from an initial perivascular foci that affects oligodendrocytes and glial cells (Balluz et al., 1993). However, the extent of replication and the associated neuronal damage among SFV strains differ according to their virulence factor. The vSFV spreads rapidly around the brain, resulting in fatal encephalitis (Fazakerley et al., 1993). This occurs irrespective of the age of the mice and so rapidly that an effective immune response is unable to be generated. In contrast, the severity of CNS infection with aSFV varies depending on the age of the mice (Fleming, 1977). In neonatal mice, the infection is rapid and ultimately fatal, whereby the virus is able to spread around the brain, similar to that seen with vSFV infection. With increase age, aSFV becomes less able to spread in the CNS and the infection becomes asymptomatic. In fact, there is a sharp age-boundary for virulence: mice infected at 12 days of age or less die, whereas those infected at 14 days of age or older survive (Fleming, 1977; Oliver and Fazakerley, 1998). This age-related virulence may be dependent on the maturity of cells of the CNS, as aSFV replicates efficiently in highly active maturing neurons but not in mature neurons (reviewed in (Fazakerley, 2002)).

Furthermore, SFV, like most RNA viruses, generates DI particles during *in vitro* passage at high-multiplicity of infection (Barrett et al., 1984; Bruton and Kennedy, 1976). These DI particles have been shown to inhibit virus replication, delay the time of death, completely protect animals, or convert the infection from lethal to persistent when administered either intraperitoneally or intranasally at the same time as vSFV (Atkinson et al., 1986; Barrett et al., 1984; Dimmock and Kennedy, 1978).

In the present study, 10-week-old mice were used to compare both vSFV and aSFV in terms of lethality, peripheral replication, and IFN-I induction. In addition, the effect of simultaneous infection with both strains of SFV on lethality was investigated.

2.3 Material and methods

2.3.1 Animals

Wild type (wt) C57BL/6 (B6) mice of either sex were bred under specific-pathogen-free conditions and supplied by the Animal Breeding Facilities at the John Curtin School of Medical Research, Canberra.

2.3.2 Viruses and cells

Vero (African green monkey kidney), BHK (baby hamster kidney) cells were maintained in Eagle's minimal essential medium (EMEM) plus nonessential amino acids and 5% foetal calf serum (FCS) and incubated at 37^0 C in humidified condition with 5% CO_2.

Virulent SFV and aSFV, used in this study, are most likely to be V13 and A7, respectively (as they were originally obtained from Dr I. Marshal in the early 1980s (Woodward et al., 1977)). Working stocks, of vSFV and aSFV, were prepared by infecting semi-confluent BHK cell monolayers at a multiplicity of infection of 0.5 plaque forming unit (pfu) per cell. Infected cells were incubated for 24h, culture supernatants harvested, centrifuged at 1200x g for 4 minutes to remove cell debris, and stored in single-use aliquots at -70° C. Titres were 5x 10^7 pfu/ml for vSFV and 1x 10^8

pfu/ml for aSFV. Titres were determined by plaque assay on Vero cells. Intravenous (i.v) infections were carried out using a final volume of 150µl/mouse.

2.3.3 Plaque assay

Virus was titrated by plaque formation on semi-confluent monolayers of Vero cells as previously described (Licon Luna et al., 2002), Samples were serially diluted in Hanks' balanced salt solution (HBSS), pH 7.6 containing 0.2% BSA on ice, and cells infected in duplicate with 0.1 ml aliquots of the diluted sample. Adsorption was for 1 h at 37°C followed by the addition of an agar overlay medium. After 48h incubation at 37°C, cells were fixed with 5% paraformaldehyde (BDH chemicals, Aust.) for 1h. Over-layers were removed, and fixed cells were stained with 0.2% crystal violet in H_2O. The added stain was aspirated and plaques counted.

For virus determination of infected mouse tissues, animals were sacrificed at a given time post-infection, tissues were aseptically removed, snap-frozen in liquid nitrogen or dry ice, and stored at -70°C. 10% (weight/volume) tissue suspensions in ice-cold HBSS (pH 7.6) were homogenized, clarified by centrifugation (18,000 x g for 5 min at 4°C), and supernatants were stored in aliquots at -70°C. The limit of virus detection in tissues and serum of infected mice by plaque titration were 10^2 pfu/g or ml, respectively. As a control, tissues from non-infected animals were homogenised in the presence of a known amount of virus. Plaques counted for these samples indicated no loss of infectivity after homogenisation.

2.3.4 Serum IFN-α levels

Serum samples were collected from vSFV and aSFV (10^3 pfu/mouse) infected 10-week-old females B6 mice at 0.5, 1, 2, 3, 4, and 5 days post-infection using 2 individual mice/time-point. IFN-α levels were tested in these serum samples using sandwich ELISA kit according to the manufacturer instructions (USBiological, USA). Briefly, 100 µl samples were tested for IFN-α concentration using duplicates of the undiluted, 1/10 and 1/100 dilutions. Samples were incubated for 1 h, washed twice, and anti-mouse IFN-α antibody was, then, added and incubated at room temperature (all incubations carried out at this temperature). After 24 h, plate was washed 4 times and HRP-conjugate was added and incubated for 1h. Then, plate was washed 4 times and

TMB substrate was added (100 µl/well) and incubated for 15 min in the dark. Reaction was stopped with 100µl of stop solution (provided), and absorbance at 405nm was measured. Serum IFN-α concentrations were estimated and expressed as pg/ml. In each experiment, a standard curve measuring 0-500 pg/ml was generated from the provided mouse IFN-α. The detection limit of IFN-α concentration in tested samples was 12.5 pg/ml.

2.4 Results

2.4.1 Lethality of vSFV versus aSFV

10-week-old female B6 mice were infected i.v with different doses of either vSFV or aSFV and survival was monitored for a 21-day period. The vSFV strain was very lethal with 60% mortality at a dose of 10^0 pfu/mouse, and 100% mortality at 10^1 or more pfu/mouse (figure 2.1). This compares with 100% survival when aSFV at 10^7 pfu/mouse was used (figure 2.1).

2.4.2 Tissue viral titres

In order to understand the difference between both SFV strains, virus titres in brain, spleen, and serum were estimated at different times post-infection. Pre-designated groups of mice were infected i.v with 10^3 pfu of either vSFV or aSFV. Mice were sacrificed at given time point, and virus titres determined by plaque assay as pfu/g for brain, and spleen, or pfu/ml for serum (figure 2.2). Both SFV strains were detectable at day 1 post-infection in all tested tissues. Virus titres differed in both brain and spleen between the two strains. The vSFV replicated to high titres in the brain reaching $>10^8$ pfu/g by day 5 post-infection (figure 2.2.A). A similar observation was previously described (reviewed in (Fazakerley, 2002)). This compares with brain titres of $5x10^4$ pfu/g when aSFV was used (figure 2.2.A). This low brain titre is a distinct feature of aSFV infection where replication is restricted in neurons, as has been shown previously by a number of studies (Fleming, 1977; Gates et al., 1985; Pusztai et al., 1971; Woodward et al., 1977). By contrast the data presented here for spleen titres indicated a different picture than that seen in the brain (figure 2.2.B). Despite similar titres for both strains detected at day 1 post-infection, they differed at day 3 with aSFV replicating more preferentially whereby reaching more than 10^6 pfu/g compared to vSFV titres

which did not increase above the detected titre for day 1 post-infection. This difference in spleen titres seen for vSFV and aSFV was not affected by virus level in the blood as limited serum titres detected for both strains at day 3 post-infection (figure 2.2.C).

2.4.3 Serum IFN-α levels

Serum was collected from mice infected with 10^3 pfu/mouse vSFV or aSFV at various times post-infection and individually tested for the concentration of IFN-α using sandwich ELISA as described in methods. As shown in figure 2.3, elevated IFN-α levels, induced by vSFV infection, were first detected 12 h post-infection, peaked at 24 h (2900 ± 100 pg/ml) and declined to background levels by day 3 post-infection. Using the same dose of aSFV, significant levels of serum IFN-α were detectable at 24 h post-infection, peaking at 48 h (875 ± 25 pg/ml) and then declined. Peak levels were substantially lower than that observed with vSFV. Increasing the dose of aSFV by 4 logs to 10^7 pfu produced qualitatively and quantitatively similar results to that observed with 10^3 pfu vSFV.

2.4.4 Protection against lethal vSFV by non-lethal aSFV

Adoptive transfer of immune cells (total splenocytes) from aSFV-infected mice has been reported to protect against lethal vSFV (Kraaijeveld et al., 1986). Ten-week-old B6 mice were immunised with 10^3 pfu/mouse aSFV. Five months later, these mice were challenge with either 1 or 10^3 pfu vSFV. The survival of these mice was compared to same age unprimed mice (figure 2.4). As shown before, unprimed mice succumbed to an infection with 10^3 pfu vSFV with 100% mortality and 20% death rate with 1 pfu. In comparison, no mortality was observed in mice primed with aSFV (10^3 pfu/mouse) with either challenging dose.

Furthermore, previous investigators argued that both strains of SFV have similar tropism but variable rates of replication, at least in neurons (Balluz et al., 1993; Fazakerley et al., 1993). Therefore, it was of interest to investigate the effect of mixed infections on the virulence of vSFV. Figure 2.5 shows that aSFV protects the mice when given simultaneously with otherwise lethal doses of vSFV. Mice were protected from 1 pfu vSFV infection by aSFV in a dose dependent manner (figure 2.5.A). Administration of 10^7 pfu aSFV and increasing doses of vSFV lead to an increased mortality (figure 2.5.B).

2.4.5 Role of IFN-I in protection against lethal vSFV infection

To address if IFN-I induced by aSFV (10^7 pfu/mouse) infection was responsible for the protection from vSFV, poly I.C, the IFN-I inducer, was injected intraperitoneally at the same time as infection with vSFV (10^3 pfu/mouse) and survival of these mice was monitored (figure 2.6). Poly I.C treatment affected the lethality of vSFV in a dose dependent manner. Injection of 0.1 mg/mouse poly I.C increased the survival rate from 0% to 20%, doubling the dose to 0.2 mg/mouse increased the survival rate to 50%, suggesting a possible IFN-I mediated cause for the protective effect of aSFV in susceptibility to vSFV infection.

2.5 Discussion

The data presented here describe experiments comparing the two strains of SFV in regard to their virulence, tissue tropism, replication capacity and ability to induce IFN-I. The two strains are vastly different in their virulence. Virulent SFV has a LD_{50} of < 1 pfu, conversely 10^7 pfu aSFV does not result in mortality in 10-week-old B6 mice. Similar results have been reported previously (Fleming, 1977).

When virus load was determined in various tissues over an 8-day period post-infection with 10^3 pfu of either strain of SFV, peripheral replication was higher with aSFV than virulent. This was especially prominent in the spleen but was also evident in blood. However, the reverse was true for virus titres in the brain. At day 1 post-infection, vSFV could be detected in brain tissue and increased till the death of animals. This contrasts with aSFV which was present in the brain at day 3 but declined to undetectable levels by day 7 post-infection. Brain titres were above that which possibly could have been due to blood borne virus. Thus both strains of virus have the ability to cross the blood-brain barrier but only vSFV replicates to high titres. Replication of aSFV is either controlled by the hosts' immune response or replication is self-limiting in the CNS tissues. Earlier work by Barrett and colleagues suggest that defective interfering particles may be responsible for the different growth characteristics of the two strains of SFV (Barrett et al., 1984). Therefore, the protective effect of co-infection of aSFV on vSFV-mediated mortality may be due to defective interfering particles in the preparation of aSFV stock.

Surprisingly, the vSFV strain was the superior inducer of IFN-I in the periphery, which may explain vSFV limited replication in the spleen compared to that of aSFV. This effect may predominantly be due to IFN-α which appears not to be operative in brain where IFN-β is active (Sandberg et al., 1994). However, more complex immune mediated mechanisms of the innate immune system cannot be ruled out, and the role of IFN-II and early NK cell responses have in limiting aSFV but not vSFV replication in brain is not known.

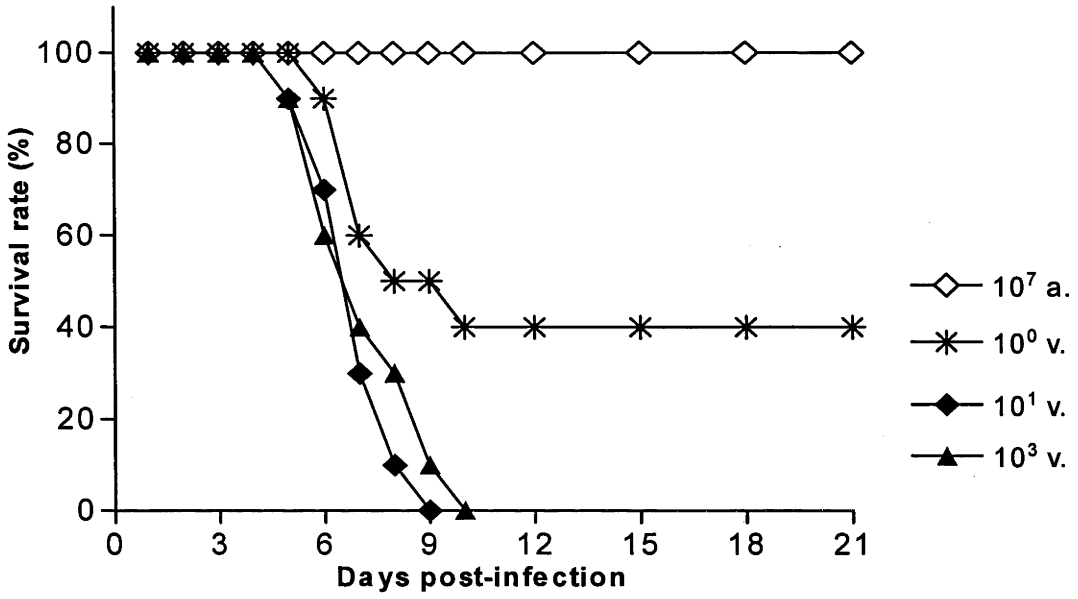

Figure 2.1: Mortality of 10-week-old B6 mice given vSFV or aSFV. Mice were given 1-10^3 pfu vSFV or 10^7 pfu aSFV i.v and mortality recorded for 21 days. No death was recorded beyond day 10 post-infection.

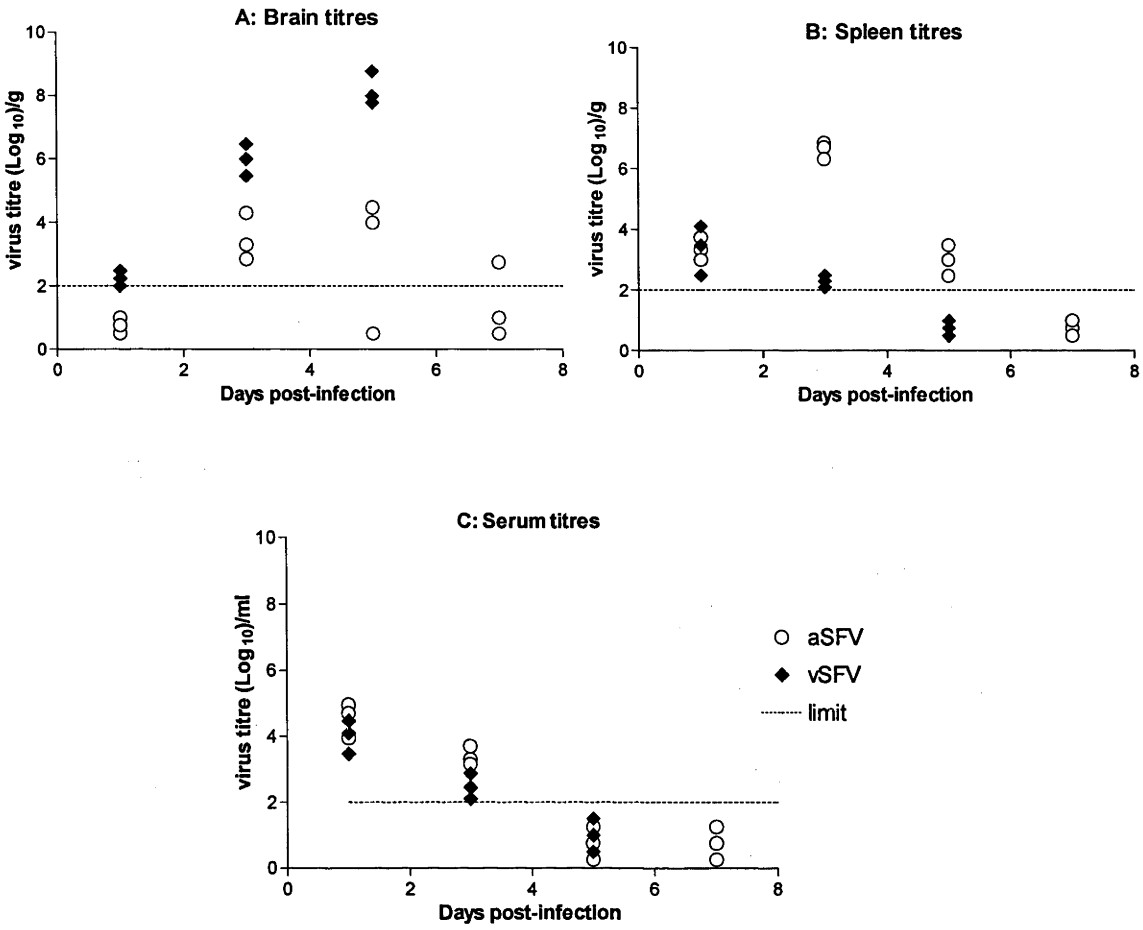

Figure 2.2: Virus titres in brain (A), spleen (B), and serum (C) after infection with 10^3 pfu aSFV or vSFV. Three individual animals per strain were assayed 1-7 days post-infection. Detection limit was 10^2 pfu/g tissue or ml serum.

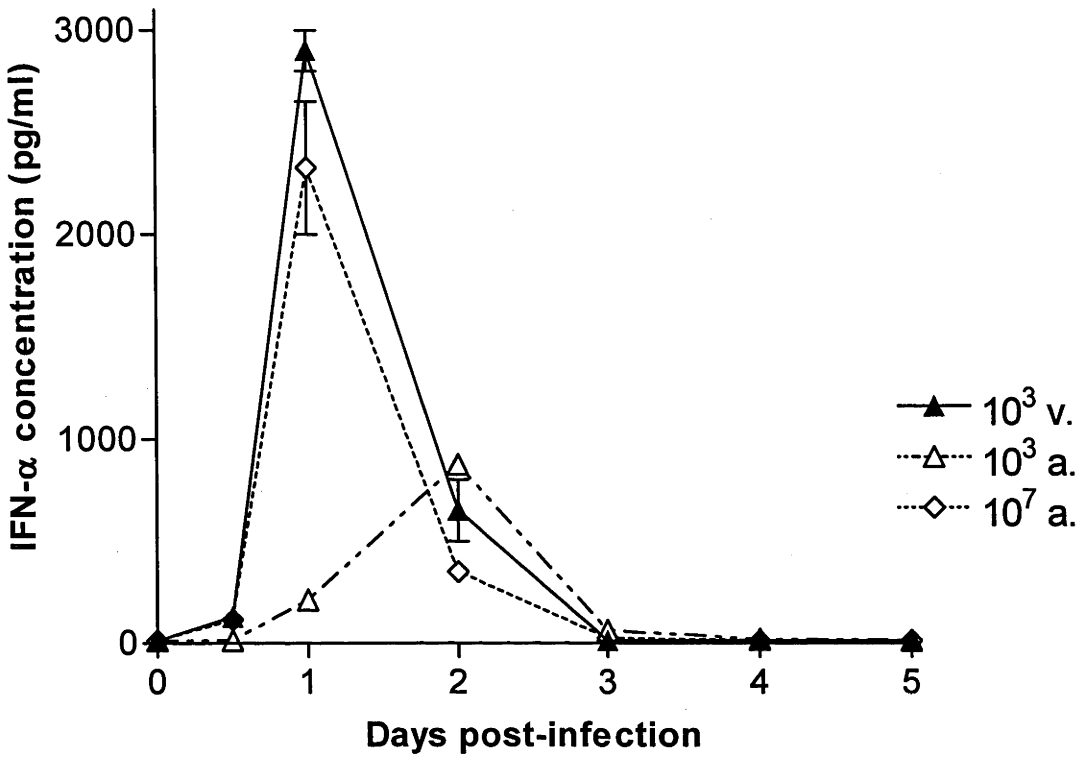

Figure 2.3: Serum IFN-α levels were tested following infection with either vSFV (10^3 pfu) or aSFV (10^3 or 10^7 pfu). Two mice per day (per group) were tested individually for their respected serum IFN-α concentrations and mean results were expressed as pg/ml. The detection limit is 12.5 pg/ml.

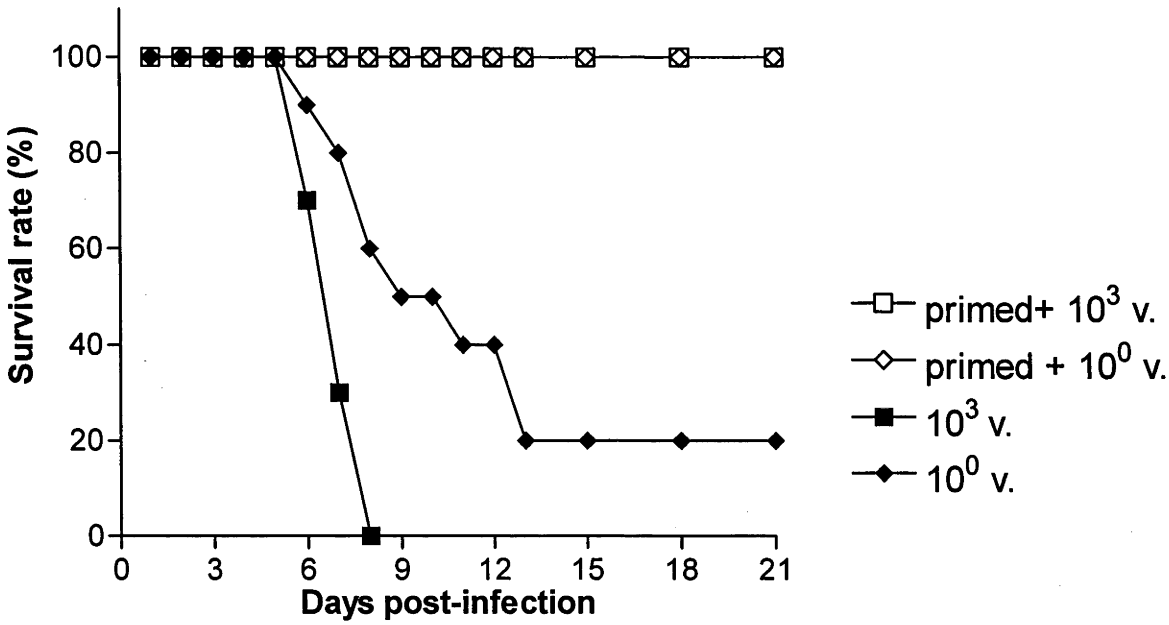

Figure 2.4: Early non-lethal aSFV infection protects against late lethal vSFV. Mice primed with aSFV (10^3 pfu/mouse) were compared to naïve mice in their sensitivity to vSFV using infection with either 1 or 10^3 pfu/mouse.

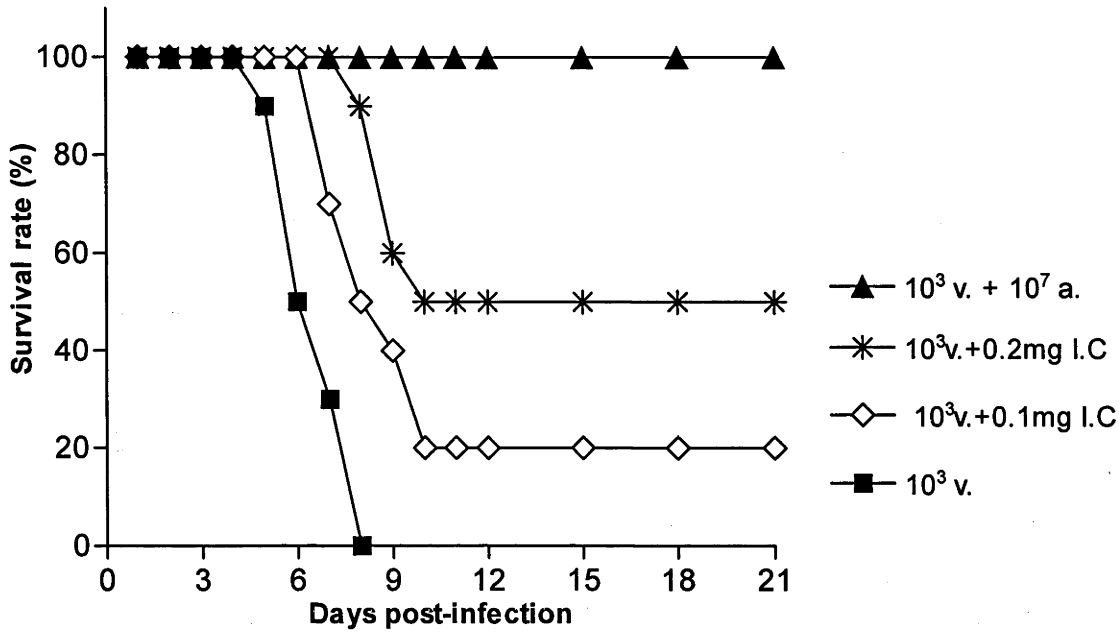

Figure 2.6: The role of IFN-I inducer in protection from vSFV. 10^3 pfu vSFV was combined with i.p injection of poly I.C (0.1 or 0.2 mg/mouse). Survival of these mice monitored for 21 days and compared to that seen during either doses of vSFV alone, or mixed vSFV and aSFV infections.

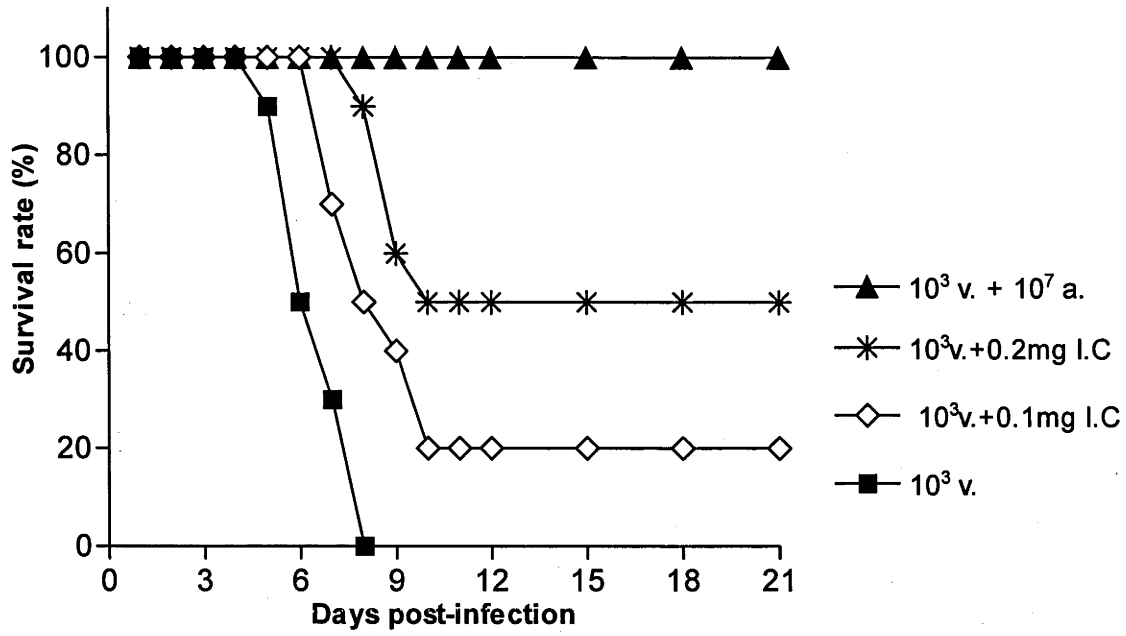

Figure 2.6: The role of IFN-I inducer in protection from vSFV. 10^3 pfu vSFV was combined with i.p injection of poly I.C (0.1 or 0.2 mg/mouse). Survival of these mice monitored for 21 days and compared to that seen during either doses of vSFV alone, or mixed vSFV and aSFV infections.

Chapter 3

NK cell-mediated immunopathology

3.1 Summary

NK and Tc cells are prime effector populations in the antiviral response of the host. Tc cells are essential for recovery from many viral diseases but may also be responsible for immunopathology. The role of NK cells in recovery from viral infections is less well established. I have studied the acute vSFV infection of the CNS in B6 mice, which spares Tc cells and is mainly controlled by NK cells. Results presented in this chapter show that mice with defects in the Fas and/or granule exocytosis pathways of cytotoxicity were more resistant to lethal vSFV infection than wt B6 mice. On the other hand, mice defective in IFN-γ response are more sensitive than wt mice, whereas mice lacking the Tc cell compartment (β/2 microglobulin) exhibit susceptibility similar to wt mice. The additional findings that depletion of NK cells significantly delayed the MTD but did not prevent mortality in SFV infected B6 mice suggests that cytolytic activity of NK cells is detrimental while IFN-γ production is beneficial for recovery from SFV infection. This study illustrates, for the first time, a NK cell-mediated immunopathological outcome to an acute viral infection executed primarily via the Fas and exocytosis pathways.

3.2 Introduction

Efficient early control of viral infections is determined by viral tissue tropism and rate of replication, and the host's ability to mount an effective immune response. Cellular cytotoxicity, in particular that of NK and Tc cells, is central to the early antiviral immune response. Both effector cells, can execute apoptotic processes in virus infected targets via either Fas-mediated or granule exocytosis pathways (Trapani and Smyth, 2002) in addition to the secretion of cytokines such as IFN-γ, TNF-α, and interleukins (Boehm et al., 1997; Vassalli et al., 1992). The Fas-mediated pathway of cytotoxicity involves the binding of Fas.L expressed by activated Tc and NK cells to Fas receptors on target cells and leads to apoptosis of target cells via the caspase cascade (Kagi et al., 1996). The granule exocytosis pathway involves secretion of perf and at least two gzms, gzmA and gzmB (Trapani and Smyth, 2002), contained within cytoplasmic granules of effector cells (Russell and Ley, 2002). Granzymes may enter target cells independent of perf by receptor-mediated endocytosis, but must act in concert with perf in the cytoplasm to cause cell death via caspase-dependent or –independent apoptosis (Russell and Ley, 2002; Trapani and Smyth, 2002). Immune mediated cytolytic activity as a result of viral infection has been associated with tissue injury (Balkow et al., 2001; Doherty and Zinkernagel, 1974; Guidotti et al., 1999; Kagi et al., 1996; Wang et al., 2003).

Since vSFV is lethal to adult mice, aSFV was used in almost all of the work investigating immunopathological aspects during this viral infection. Demyelination, peaking 14-21 days post-infection with aSFV, is predominantly mediated by $CD8^+$T cells in CBA/C3H ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mice (Fazakerley, 2002; Fazakerley and Webb, 1987; Subak-Sharpe et al., 1993). However, cytotoxic $CD8^+$T cells responses to alphaviruses have so far only been associated with the MHC class I allele $H\text{-}2D^k$ (Mullbacher and Blanden, 1978), raising the question by which mechanism demyelination occurs in infected BALB/c mice in the absence of a responder H-2 allele. Additional support for a lack of $CD8^+$T cell involvement in alphavirus infection of some inbred mouse strains comes from B-cell-deficient mice of the $H\text{-}2^b$ haplotype (B6 mice). These mice show no white matter vacuolation during avirulent SFV infection despite elevated numbers of $CD8^+$T cells in the brain parenchyma (Smith-

Norowitz et al., 2000). In addition, T cell depletion (both $CD4^+$ and $CD8^+$) in B6 mice does not alter the outcome of lethal vSFV infection (Doherty, 1973). This together with the observation that perf deficiency does not lead to increased susceptibility of B6 mice to aSFV infections (Kagi et al., 1995b), lead to the assumption that death during vSFV infections is the result of neuronal damage caused by extensive viral replication rather than immunopathology (Atkins et al., 1999).

The possibility that other effector cells, such as NK cells, which have been implicated before in the control of various virus infections (Biron et al., 1999), contribute to SFV-induced pathology has not been excluded. In fact, it was shown that SFV infection leads to the activation of high levels of NK cell-mediated cytotoxicity (Mullbacher and King, 1989b). Maximal NK activity is observed early during infection and prior to T and B cell responses (Biron et al., 1999). Thus, the possible involvement of NK cell-mediated cytolytic activity(ies) in immunopathology during vSFV infections has been investigated.

3.3 Material and methods

3.3.1 Viruses and cells

Vero, BHK, the NK cell-sensitiveYAC-1 thymoma, the NK cell-resistant P815 mastocytoma, and the methylcholanthrane-induced fibroblast lines MC57 ($H\text{-}2^b$), and L929 ($H\text{-}2^k$) were maintained in EMEM plus nonessential amino acids and 5% FCS and grown at 37^0 C in humidified condition with 5% CO_2.

Working stocks for vSFV and aSFV were prepared by infecting semi-confluent BHK cell monolayers at a multiplicity of 0.5 pfu per cell. Infected cells were incubated for 24 h, culture supernatants harvested, centrifuged at 1200 x g for 4 min to remove cell debris, and stored in single-use aliquots at -70° C. Titres determined by plaque assay on Vero cells were 5×10^7 pfu/ml for vSFV and 1×10^8 pfu/ml for aSFV. Animal infections were i.v with a final volume of 150µl/mouse.

3.3.2 Animals

CBA/H (H-2^k), C57BL/6 (H-2^b) (B6) mice, B6 syngeneic mice deficient in the exocytosis pathway involving gene knockouts of perforin ($perf^{-/-}$) (Kagi et al., 1994a), gzm A ($gzmA^{-/-}$) (Ebnet et al., 1995), gzm B ($gzmB^{-/-}$) (Heusel et al., 1994), gzm A and B ($gzmAB^{-/-}$) (Simon et al., 1997), or gzm A and B plus perforin ($perfxgzmAB^{-/-}$) (Mullbacher et al., 1999b), and syngeneic mice deficient in the Fas-mediated cytolytic pathway involving either a loss-of-function mutations in the Fas receptor (CD95, Fas) (lpr) (Samelson et al., 1986) or Fas ligand (Fas-L) (gld) (Takahashi et al., 1994), or Fas receptor ($Fas^{-/-}$) gene knockout by targeted mutation (Adachi et al., 1995), were used. Mice deficient in both pathways were either Fas and gzm A and B negative ($FasxgzmAB^{-/-}$) (Rode M, 2004), or deficient in perforin and Fas-L ($perf^{-/-}$xgld). $Perf^{-/-}$ xgld mice were generated by cross breeding mice heterozygous for perforin and homozygous for the Fas.L mutation, as previously described (Licon Luna et al., 2002). In addition, I used β-2 microglobulin targeted mutant mice ($\beta2M^{-/-}$) (Koller et al., 1990) that lack cell surface expression of MHC-I, the thymic selecting element for $CD8^+$T cells. In addition, interferon (IFN)-γ knockout (IFN-$\gamma^{-/-}$) mice (B6 genetic background) (Tagawa et al., 1997), and interferon (IFN)-γ-receptor knockout (IFN-$\gamma R^{-/-}$) (Huang et al., 1993) mice and their wild-type 129T2/Sv (129) strain were used.

Mice were bred under specific pathogen-free conditions and supplied by the Animal Breeding Facilities at the John Curtin School of Medical Research, Canberra.

3.3.3 ^{51}Cr Release Cytotoxic Assay

Assays for both NK cells-mediated (Mullbacher and King, 1989b) and Tc cell-mediated cytotoxicity (Mullbacher and King, 1989a) have been previously described. In brief, mice were infected with 1 pfu vSFV and splenocytes harvested at predetermined time pi tested for NK cell–mediated cytotoxicity on ^{51}Cr labelled YAC-1 and P815 target cells. For Tc cell–mediated cytotoxicity assays B6 and CBA mice were infected with 10^3 pfu aSFV. Memory splenocytes were restimulated *in vitro* with SFV-infected syngeneic splenocytes. Control alloreactive Tc cells were generated by co-culture of either B6 or CBA splenocytes with 2000 rad irradiated CBA or B6 stimulator cells, respectively, for 5 days at a responder to stimulator ratio of 3:1.

3.3.4 *In vivo* NK cell-depletion

B6 mice were injected i.p with 30 µl rabbit anti-asialo GM1 antiserum (Wako) at -4, -2, 0 (day of infection), 2, 5, and 8 days post-infection. NK cell-mediated cytotoxicity of splenocytes from treated and untreated mice was measured at day 3 pi to test for successful NK cell depletion.

3.3.5 Statistical analysis

All statistical analyses were conducted using GraphPad InStat software. Fisher's exact test was used to compare survival rates and the Mann Whitney test was used to compare mean times to death (MTD).

3.4 Results

3.4.1 Dose response of vSFV in wild-type B6 mice

Results presented in chapter 2 showed that survival rates of B6 mice infected i.v with 10^3 to 1 pfu vSFV were dose-dependent with 40% survival in the group infected with 1 pfu/mouse, and no surviving mice in groups infected with doses of 10 pfu or higher.

To assess the infectivity in mice of 1 Vero cell pfu vSFV, 3 groups of 10 females B6 mice (4 weeks old) were injected intracranially (i.c) with 10^{-1}, 10^{-2}, and 10^{-3} pfu/mouse of vSFV. This resulted in 100%, 60%, and 10% mortality, respectively (figure 3.1), and indicates that 1 Vero cell pfu is equivalent to 10^2-10^3 mouse infectious doses. Accordingly, 1 Vero cell pfu was used as the standard dose to compare the lethality of vSFV infection in B6 and syngeneic mice with deficiencies in cytolytic pathways.

3.4.2 Mortality of cytolytic effector pathway defective mice to vSFV infection

10-week-old female B6, perf$^{-/-}$, gzmA$^{-/-}$, gzmB$^{-/-}$, gzmAB$^{-/-}$, perfxgzmAB$^{-/-}$, lpr, gld, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$xgld mice were infected i.v with 1 pfu vSFV. Clinical signs associated with vSFV infection, such as ruffled fur, hunched posture, impaired movement leading to paralysis were similar in B6 and all defective mice, with the exception of perf$^{-/-}$xgld mice which showed no paralysis. The survival of these mice

was monitored for a 21-day period. The cumulative results from a number of experiments are shown in table 3.1. The survival rate of B6 mice was 30%. All mice defective in one of the cytolytic effector functions showed higher survival rates where a defect in the granule exocytosis pathway ($gzmA^{-/-}$, $gzmB^{-/-}$, $perf^{-/-}$, $gzmAB^{-/-}$) increased the resistance to SFV marginally and a defect in the Fas pathway (gld, and $Fas^{-/-}$) resulted in a significant increase in survival (P=0.012 and 0.014, respectively) relative to B6 mice. The discrepancy between the 'loss of function' mutant lpr and 'knockout' mutant $Fas^{-/-}$ strains in susceptibility to SFV infection is most likely due to "leakiness" in the Fas defect in lpr mice (Nagata and Suda, 1995). Mice defective in both granule exocytosis- and Fas- mediated pathways of cytotoxicity (FasxgzmAB$^{-/-}$, and perf$^{-/-}$xgld) were the least susceptible to vSFV infection; the survival results were 65% and 78%, respectively, and the increase in survival was significant relevant to B6 mice. A significant increase in MTD in groups of $Fas^{-/-}$ (10 days), FasxgzmAB$^{-/-}$ (9.3 days), and perf$^{-/-}$xgld (12.2 days) in comparison to B6 mice (7.3 day) was also observed (table 1).

3.4.3 Tc cell- mediated cytotoxic activity to vSFV infection

To asses whether the increase in survival rates seen in cytolytic function deficient mice was due to impaired cytolytic activity of Tc cells, $\beta2M^{-/-}$ mice which lack $CD8^+$ T cells were infected with vSFV and their survival monitored for 21 days. $\beta2M^{-/-}$ mice were similar to B6 mice in their sensitivity to vSFV infection, with an almost identical MTD and survival rate (Table 3.1). This strongly indicates that $CD8^+$ Tc cells are not responsible for the higher mortality rate of B6 mice relative to mice with defects in one or more of the cytolytic effector pathways of NK and Tc cells.

In addition, the level of $CD8^+$ Tc cell cytotoxic activity induced as a result of SFV infection in B6 ($H\text{-}2^b$) mice was examined. Mice were infected with aSFV 2 weeks prior to *in vitro* boosting with aSFV-infected syngeneic splenocytes for 5 days to optimise induction of cytolytic Tc cell activity. SFV-immune B6 effectors and control CBA ($H\text{-}2^k$) SFV-immune effectors together with anti- $H\text{-}2^b$ and anti- $H\text{-}2^k$ alloreactive control effectors were assayed on SFV infected and uninfected MC57 ($H\text{-}2^b$) and L929 ($H\text{-}2^k$) target cells using a 6h ^{51}Cr release assay (Table 3.2). My data confirm previously published data (Mullbacher and Blanden, 1978) that B6 mice do not respond to SFV infections with a cytotoxic T cell response under conditions that generate cytotoxic responses in CBA ($H\text{-}2^k$) mice. This is further evidence that $CD8^+$Tc cells are

not involved in cytolytic effector molecule-mediated immune pathology during SFV infections.

3.4.4 NK cell-mediated cytotoxic activity

To asses the strength of the NK cell response, B6 mice were infected i.v with 1 pfu vSFV 1-5 days prior to testing their splenocytes *in vitro* for cytolytic activity on YAC-1 and P815 target cells using a 6h ^{51}Cr release assay. SFV infection induced high NK cell-mediated cytotoxicity against YAC-1 targets, which peaked by day 3 post-infection (figure 3.2.A). No cytotoxic activity was detected on the NK cell resistant P815 targets.

3.4.5 NK cell-depletion and its effect on vSFV infection

Since mice defective in cytolytic effector molecules show increased resistant to vSFV infection, and a detrimental effect of $CD8^+$ Tc cells in SFV infection could be excluded, NK cell-mediated cytotoxicity was investigated to determine if it is responsible for the immune-mediated increase in mortality. B6 mice were depleted of NK cells by multiple injections of anti-asialo GM1 anti-serum. Depletion of the NK cell population in spleens was monitored using the ^{51}Cr release assay on YAC-1 target cells at day 3 after SFV infection. Depletion reduced lysis of YAC-1 targets below that obtained with naïve splenocytes (Fig 3.2.B). B6 (n=8) mice were subjected to the NK cell-depletion protocol and infected with vSFV (1 pfu). Survival of these mice was monitored and compared to that of untreated control mice (Figure 3.3). B6 mice showed an average survival time of 6 ± 0.8 days compared to 11.1 ± 2.1 for NK cell-depleted mice (P= 0.0139). Interestingly, death in NK cell-depleted mice was not associated with hindlimb paralysis, which is a consistent clinical feature associated with death in B6 mice from vSFV infection. The overall mortality of NK cell-depleted mice, however, was higher compared to that of untreated B6 mice (88% and 50%, respectively).

3.4.6 Role of IFN-γ in vSFV infection

To address whether reduced levels of IFN-γ in NK cell-depleted SFV infected mice played a role in their increased mortality relative to infected B6 mice, I compared the mortality of IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice following vSFV infection to that of their wild type counterparts B6 and 129/sv, respectively. Groups (n=20) of 10-week-old female wt B6, wt 129/sv, IFN-$\gamma^{-/-}$, and IFN-$\gamma R^{-/-}$ mice were i.v injected with 1 pfu/mouse vSFV. Survival of these mice was monitored for 21 days. Five percent of IFN-$\gamma^{-/-}$

compared to 35% B6 mice, and 55% of IFN-γR$^{-/-}$ compared to 90% of 129 mice survived (figure 3.4). IFN-γ$^{-/-}$ mice were significantly less resistant to vSFV than B6 mice (P= 0.0436), and IFN-γR$^{-/-}$ mice were significantly less resistant to vSFV than 129 mice (P= 0.0310). 129 mice were significantly more resistant to vSFV infection compared to B6 mice (P= 0.0008).

3.5 Discussion

CD8^{+}T cells-mediated immunopathogensis during aSFV infection resulting in demyelination has been reported in CBA/C3H and BALB/c mice (Fazakerley and Webb, 1987; Subak-Sharpe et al., 1993). B cell-deficient B6 mice, however, show no white matter vacuolation despite elevated CD8^{+}T cells levels (Smith-Norowitz et al., 2000). In addition, SFV-infected B6 mice are non-responders in Tc cell assay with respect to SFV-infected target cell lysis (Mullbacher and Blanden, 1978). It was, thus, of interest to investigate if cytolytic effector molecules are at all involved in recovery or immunopathogenesis during SFV infection in B6 mice. The lethality due to vSFV infection as an indicator of such an involvement was utilised and mice defective in cytolytic effector function were found to be more resistant to challenge with vSFV. This clearly suggests an immunopathological aetiology in SFV-infected B6 mice. In comparing the role played by molecules involved in the Fas versus granule exocytosis pathways of cytotoxicity, defects in the Fas pathway exhibit a stronger phenotype. This is not entirely unexpected, given that SFV has been reported to infect a wide variety of cells especially neurons and oligodendrocytes (Fazakerley, 2002), and given the dominant role of Fas-mediated cytotoxicity in neuronal apoptosis (Medana et al., 2000). Furthermore, Fas.L expressed on neurons provides protection against perf attack (Medana et al., 2001). The latter is consistent with our finding that mice defective in the Fas.L (gld) have a significantly shorter survival time than Fas$^{-/-}$ mice (Table 1).

Perforin has been shown to play a crucial role in the recovery of mice from some (Kagi et al., 1994; Mullbacher et al., 1999a; Riera et al., 2000) but not other (Elkon et al., 1997; Kagi et al., 1995b; Wang et al., 2004) viral infections, and in some cases the lack of perf was beneficial in recovery (Licon Luna et al., 2002; Mullbacher et al., 1999a).

Hence this data demonstrates that lack of perf and gzms (perfxgzmAB$^{-/-}$) significantly augments survival from vSFV infection and those single gene knockouts in the granule exocytosis pathway result in a trend towards increased survival relative to B6 mice (Table 3.1). Interestingly, mice lacking both Fas- and granule exocytosis-mediated cytotoxicity (FasxgzmAB$^{-/-}$, and perf$^{-/-}$xgld) were most resistant to the low dose of vSFV infection, demonstrating redundancy, in this case in the pathogenic potential, of the two cytotoxic pathways. Nevertheless, some double-deficient mice did succumb to SFV infection suggesting multiple determinants of disease outcome, which most likely involve the interplay of immune-mediated and virus-mediated pathology. The combined involvement of both cytolytic pathways in immunopathology has also been shown in LCMV associated viral hepatitis (Balkow et al., 2001) and Murray Valley encephalitis (Licon Luna et al., 2002).

Cellular cytotoxicity in response to viral infections generally involves an early NK cell response followed by Tc cell activation (Biron et al., 1999; Mullbacher and King, 1989b). With a MTD of 7 days in B6 mice after vSFV infection, it is unlikely that Tc cell are responsible for the observed mortality. This indicates that NK cell-mediated cytotoxicity may be responsible for the immunopathology during vSFV infections.

Mice defective in CD8^{+}T cells, β2M$^{-/-}$ mice, were as sensitive as B6 mice to vSFV infection (Table 1) confirming earlier work showing that CD8^{+}T cell depletion did not alter the outcome of vSFV infection (Doherty, 1973). In addition, early observations that B6 mice are Tc cell nonresponders in regard to lysis of target cells infected with SFV (Mullbacher and Blanden, 1978), a result which was confirmed here (Table 2), also support the conclusion that Tc cell-mediated is not involved in immunopathology. This conclusion is further supported by the fact that B cell-deficient B6 mice did not show myelin injury despite the presence of elevated numbers of CD8^{+}T cells in the CNS (Smith-Norowitz et al., 2000).

SFV is a potent inducer of NK cell lytic activity with peak cytolytic activity at days 2-3 post infection ((Fig 2) and (Mullbacher and King, 1989b)). The crucial experiment that implicated NK cells in immunopathology during vSFV infection was the effect of NK cell-depletion on the outcome of vSFV infection. Depletion of NK cells by intraperitoneal administration of anti-asialo GM1 serum significantly extended the

average survival time following vSFV infection. This indicates that NK cells contribute to the early death in B6 mice. However, since vSFV is a cytopathic virus replicating to high titres in the brain of infected mice (Pusztai et al., 1971), depletion of NK cells is not expected to prevent virus-mediated cytopathology. The increased mortality of NK cell-depleted mice indicates that anti-asialo GM1 treatment may have compromised a beneficial effector function of NK cells. Activated NK cells secrete cytokines with direct as well as indirect anti-viral activity, including IFN-γ. The present data on mortality and MTD of mice deficient in IFN-γ or lacking the IFN-γ receptor does support the notion that the increased mortality following NK cell-depletion may be due to decreased IFN-γ secretion. A more definitive approach to illustrate the beneficial role of IFN-γ and the detrimental effect of cell-mediated cytotoxicity during vSFV infection would require the generation of Fas, gzmA, gzmB, and IFN-γ quadruple-deficient mice: these would be predicted to be more sensitive to SFV infection than B6 mice and display a delayed MTD. In conclusion, this is the first study indicating to the role of the cytotoxic activity of NK cells in immunopathology during a viral infection, with the Fas pathway playing the critical role. It will be important to re-examine other models of viral infection with immunopathological manifestations to see if part of the aetiology is NK cell-mediated.

Table 3.1: Mortality and mean time to death of mice defective in granule exocytosis- and/or fas-mediated cytolytic effector pathways, or $\beta 2M^{-/-}$ mice as a consequence of vSFV infection.

Strain	Number	% Survival* (P value)@	MTD ± S.D.# (P value)&
B6	39	30.7	7.3 ±1.5
gzmA$^{-/-}$	33	36 (0.628)	6.9 ±0.9 (0.691)
perf$^{-/-}$	43	42 (0.361)	7.7 ±2.2 (0.706)
gzmB$^{-/-}$	44	45 (0.184)	7.7 ±1.5 (0.302)
gzmAB$^{-/-}$	52	46 (0.194)	8.3 ±1.9 (0.078)
lpr	42	50 (0.113)	7.7 ±1.6 (0.435)
perfxgzmAB$^{-/-}$	45	56 (0.028)	6.9 ±1.5 (0.532)
gld	39	62 (0.012)	7.3 ±1.4 (0.742)
Fas$^{-/-}$	29	62 (0.014)	10 ±3.2 (0.008)
FasxgzmAB$^{-/-}$	31	65 (0.007)	9.3 ±2.7 (0.033)
perf$^{-/-}$xgld	23	78 (0.001)	12.2 ±2.2 (0.001)
β2M$^{-/-}$	38	29 (1)	7.3 ±1.2 (0.549)

* Percentage survival rates after i.v infection with 1 pfu vSFV

@ P values obtained from Fisher Exact test comparing survival rates, significant difference is underlined.

Mean time to death ± standard deviation

& P values obtained from Mann-Whitney test comparing MTD, significant difference is underlined.

Table 3.2: Lack of SFV-immune cytolytic T cell response in B6 mice

Effectors	E:T	% Specific targets cell lysis ± SEM*			
		MC57-mock	MC57-SFV	L929-mock	L929-SFV
B6.anti-SFV#	30	3.3 ± 2.0	2.5 ± 0.9	15.9 ± 1.6	11.4 ± 1.5
	10	1.1 ± 0.3	1.0 ± 0.8	7.4 ± 0.5	7.4 ± 1.3
	3	0.3 ± 1.4	0.3 ± 0.6	5.1 ± 1.2	2.6 ± 0.9
B6.anti-CBA	30	4. 5 ± 1.6	4.7 ± 1.5	37.2 ± 2.7	29.1 ± 2.9
	10	1.3 ± 1.3	1.3 ± 1.3	28.6 ± 1.7	16.6 ± 1.3
	3	0.2 ± 0.2	0.0 ± 0.3	19.2 ± 0.9	5.1 ± 1.2
CBA.anti-SFV#	30	7.3 ± 1.0	8.1 ± 1.3	11.2 ± 0.7	32.5 ± 2.5
	10	3.0 ± 0.4	2.9 ± 0.1	8.3 ± 0.6	17.5 ± 0.5
	3	1.4 ± 2.6	2.3 ± 2.6	4.3 ± 2.6	7.4 ± 0.9
CBA.anti-B6	30	39.8 ± 2.8	35.8 ± 1.2	13.7 ± 3.5	14.7 ± 2.1
	10	20.9 ± 3.6	17.4 ± 0.7	6.4 ± 0.3	8.9 ± 2.2
	3	7.7 ± 1.7	9.7 ± 1.5	3.3 ± 0.9	4.1 ± 0.7

* Percent ^{51}Cr release of target cells over a 6 h period. Mean of triplicates with SEM given.

Secondary responses generated *in vitro* 2 wk after priming as described (Mullbacher and Blanden, 1978).

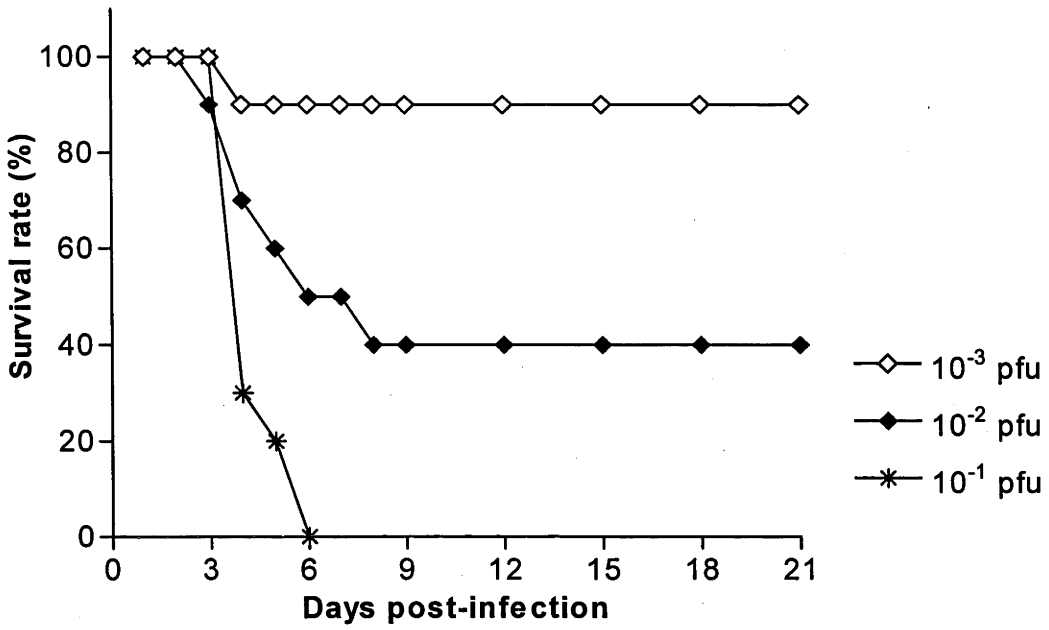

Figure 3.1: Dose response to vSFV infection in B6 mice where groups of 4-week-old mice (n 10) infected i.c with 10^{-1}-10^{-3} pfu/mouse. Mice were monitored for mortality for 21 days.

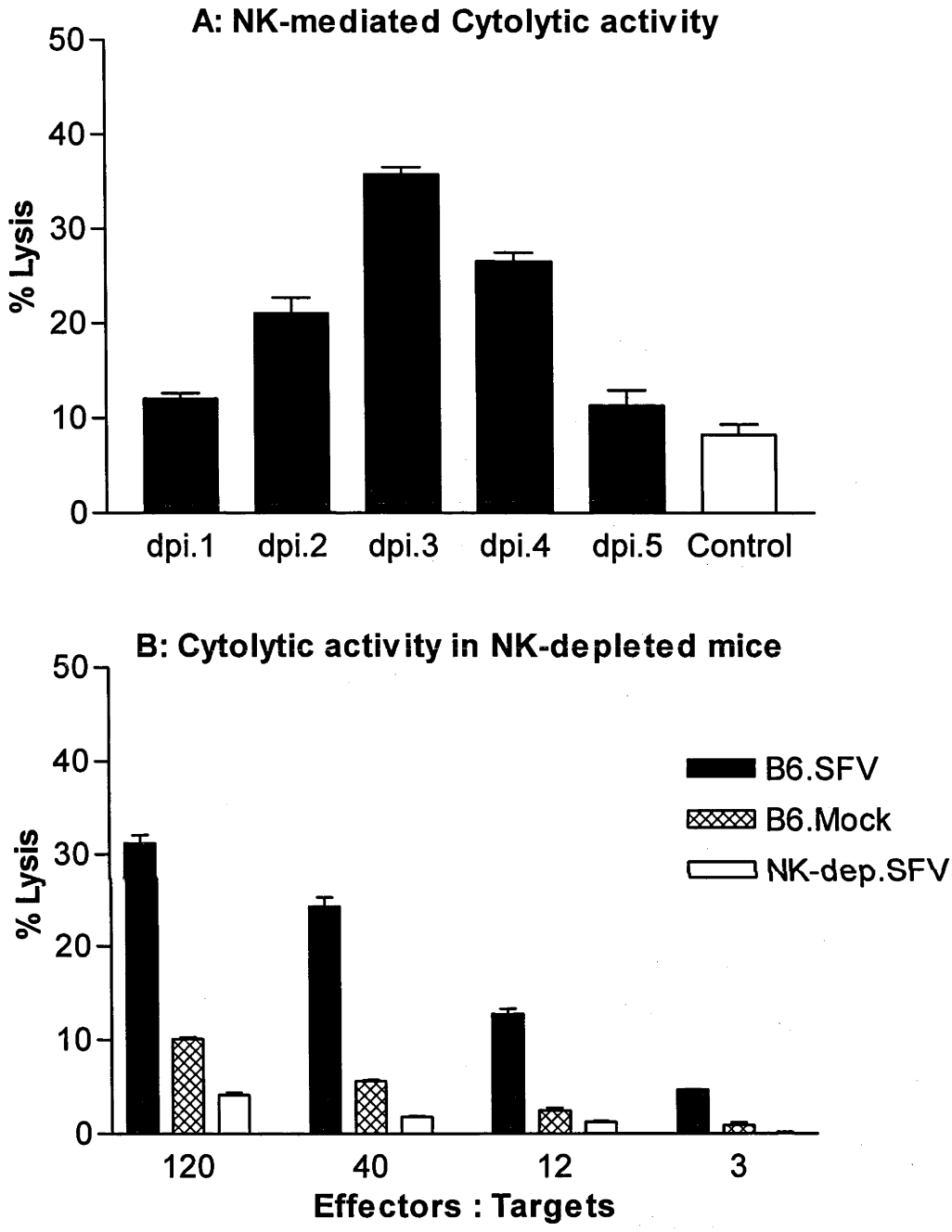

Figure 3.2: NK cell activity in SFV infections. (A) NK-mediated cytotoxicity against YAC-1 cells using 6h ^{51}Cr release assay. (B) NK-mediated cytotoxicity diminished in NK-depleted mice tested at day 3 postinfection.

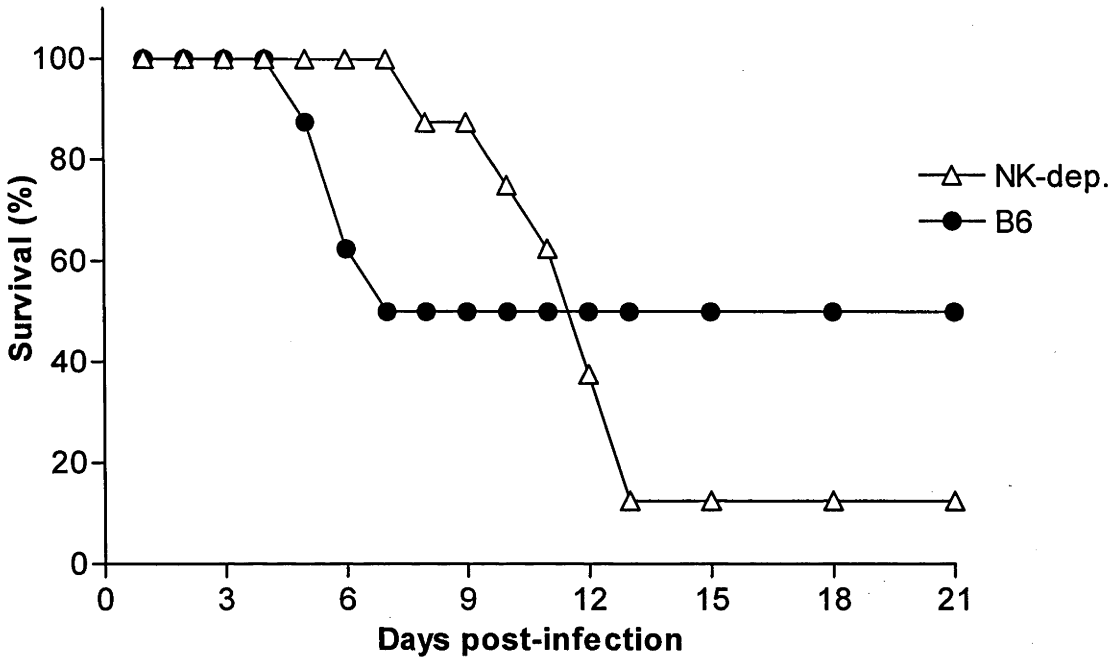

Figure 3.3: Survival of NK depleted (NK-dep.) (n 8) was compared to that of wt B6 mice (n 8). Mice were 10-week-old, infected with to 1 pfu/mouse vSFV, and monitored for mortality for 21 days.

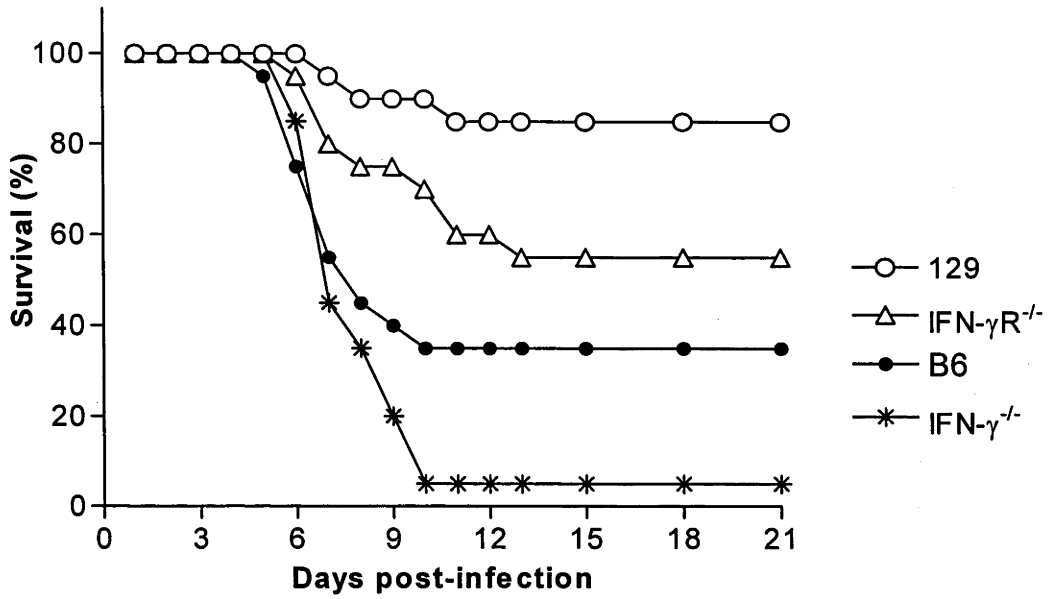

Figure 3.4: IFN-γ increases resistant to vSFV infection. Survival of IFN-$\gamma^{-/-}$, and IFN-$\gamma R^{-/-}$ mice and their respective wt counterparts B6, 129 mice (n 20) was compared during vSFV infection. Mice were 10-week-old, infected with 1 pfu/mouse vSFV, and monitored for mortality for 21 days.

Chapter 4

Restricted SFV replication in perf$^{-/-}$x*gld* mice

4.1 Summary

Previously, perfxgzmAB$^{-/-}$ mice (defective in granule exocytosis), *gld* and Fas$^{-/-}$ mice (defective in Fas.L/Fas-mediated killing), and FasxgzmAB$^{-/-}$ and perf$^{-/-}$x*gld* mice (defective in both pathways) have shown significant resistance to vSFV infection (1 pfu/mouse) compared with B6 mice. In this chapter, I investigated viral replication in different tissues of infected mice and found reduced virus titres in tissues harvested from perf$^{-/-}$x*gld* mice. Thus, I investigated the underlying factors restricting viral replication in perf$^{-/-}$x*gld* mice, and found naïve mice to have a remarkable increase in macrophages, with RT-PCR of splenocytes showing increased levels of macrophage activating cytokines (IFN-γ and TNF-α) in their spleens. Reconstituting Fas$^{-/-}$ mice with thioglycolate-activated macrophages resulted in restricted viral replication compared to non-reconstituted Fas$^{-/-}$ mice. Therefore, the expanded population of macrophages in perf$^{-/-}$x*gld* mice may play a role in increasing their resistance to vSFV infection.

4.2 Introduction

The positive-stranded RNA alphavirus, SFV, replicates in muscles following peripheral infection leading to high-titre plasma viremia detectable within 24 h (Amor et al., 1996; Fazakerley et al., 1993). SFV is neuroinvasive and has been shown to infect endothelial cells, causing blood-brain barrier damage (Fazakerley, 2002; Pathak and Webb, 1974; Soilu-Hanninen et al., 1994). SFV strains can be classified into vSFV and aSFV (Atkins et al., 1999); both are lethal in weanling mice, however, they differ in their lethality in adult mice. Infection of adult mice with aSFV is mostly asymptomatic, characterised by low virus titres in the brain and demyelination that peaks at 14-21 days post-infection (Atkins et al., 1999; Fazakerley et al., 1993). In comparison, vSFV infection is characterised by high virus titres in the brain, and rapid and lethal encephalitis. Although not a human pathogen, infection in mice with aSFV has been investigated as a model for multiple sclerosis and experimental allergic encephalitis (Butt et al., 1996; Fazakerley and Webb, 1987a). Such studies have shown lesions of demyelination during aSFV infection to be immune-mediated, particularly $CD8^+$T cell-dependent in BALB/c and CBA/H mice (Fazakerley and Webb, 1987a; Fazakerley and Webb, 1987b; Subak-Sharpe et al., 1993), and B cell-mediated in C57BL/6 (B6) mice (Smith-Norowitz et al., 2000). Interestingly, cytotoxic T (Tc) cell responses against SFV in mice are limited to particular major histocompatibility complex (MHC) class I antigens and not elicited in B6 mice (Mullbacher and Blanden, 1978).

In addition to secretion of cytokines, Tc and NK cells can use at least two pathways to generate cytotoxic attacks against target cells (Henkart, 1994; Rouvier et al., 1993) ; the Fas-mediated pathway requires the interaction of the Fas.L on cytotoxic cells with the Fas receptor on target cells (Depraetere and Golstein, 1997; Mullbacher, 2003; Nagata, 1997), whereas the granule exocytosis pathway of cytotoxicity involves the secretion of at least two types of effector molecules, perforin and gzms (Russell and Ley, 2002). Both gzmA and gzmB have been shown to be important in controlling viral infections (Mullbacher et al., 1999). In chapter 3, I compared the susceptibility of mice deficient in one or both cytotoxic pathways of NK and Tc cells to infection with vSFV to that of wt B6 mice. I found that a deficiency with Fas and /or perforin pathways of cytotoxicity increased survival rates of groups of mice. In addition, I showed that absence of Tc cell activity as a result of a deficiency in β2 microglobulin ($\beta 2M^{-/-}$ mice) did not adversely

impact on survival rate or MTD. Here, I describe the results of investigating the underlying factors associated with the significant reduction of disease in mice deficient in Fas- and/or granule exocytosis-mediated cytotoxicity following infection with SFV.

4.3 Material and methods

4.3.1 Viruses and cells

Vero and BHK cells were maintained in EMEM plus nonessential amino acids and 5% FCS and grown at 37^0 C in humidified condition with 5% CO_2.

Working stocks for vSFV and aSFV were prepared by infecting semi-confluent BHK cell monolayers at a multiplicity of 0.5 pfu/cell. Infected cells were incubated for 24 h, culture supernatants harvested, centrifuged at 1200 x g for 4 min to remove cell debris, and stored in single-use aliquots at -70° C. Virus titres were determined by plaque assay on Vero cells and ranged from 5 x 10^7 pfu/ml for vSFV to 1 x 10^8 pfu/ml for aSFV.

4.3.2 Animals

B6 mice, syngeneic mice deficient in granzymes A and B plus perforin (perfxgzmAB$^{-/-}$) (Mullbacher et al., 1999), and syngeneic mice deficient in the Fas-mediated cytolytic pathway involving either a loss-of-function mutation in the Fas ligand (*gld*) (Takahashi et al., 1994), or Fas receptor gene knockout (Fas$^{-/-}$) (Adachi et al., 1995) were used. Mice deficient in both pathways of cytotoxicity were either Fas and gzm A and B negative (FasxgzmAB$^{-/-}$) (Rode M, 2004), or deficient in perforin and Fas.L (perf$^{-/-}$x*gld*). Perf$^{-/-}$ x*gld* mice were generated by cross-breeding mice heterozygous for perforin and homozygous for the Fas.L mutant as previously described (Licon Luna et al., 2002). Throughout this study, 10-week-old female mice were used.

Mice were bred under specific pathogen-free conditions and supplied by the Animal Breeding Facilities at the John Curtin School of Medical Research, Canberra.

4.3.3 Plaque assay

Virus was titrated by plaque formation on semi-confluent monolayers of Vero cells as previously described (Licon Luna et al., 2002). Briefly, samples were serially diluted on ice in HBSS, pH 7.6, containing 0.2% BSA, and cells inoculated in duplicate with 0.1 ml aliquots of the diluted samples. Adsorption was for 1 h at 37°C followed by the addition of an agar overlay medium. After 48 h incubation at 37°C, cells were fixed with 5% paraformaldehyde (BDH chemicals, Aust.) for 1 h. Over-layers were removed, and fixed cells were stained with 0.2% crystal violet in ddH_2O. The stain was aspirated and plaques counted.

For virus determination in infected mouse tissues, animals were sacrificed at a given time post-infection, tissues were aseptically removed, snap-frozen in liquid nitrogen or dry ice, and stored at -70°C. 10% (weight/volume) tissue suspensions in ice-cold HBSS (pH 7.6) containing 0.2% BSA were homogenized, clarified by centrifugation (18,000 x g for 5 min at 4°C), and supernatants were stored in aliquots at -70°C. The limit of virus detection by plaque assay in tissues and serum samples of infected mice was 10^2 pfu/g or ml, respectively. As a control, brain, spleen, and muscle tissues from uninfected animals were homogenised in the presence of a known amount of virus. Plaque titration indicated no loss of viral infectivity due to tissue components released during homogenisation.

4.3.4 Flow-Cytometric Analysis

Spleens from uninfected B6, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$x*gld* mice were harvested and single cell suspensions prepared. After red cell lysis, splenocytes were suspended in PBS + 1% FCS at 10^7 cell/ml. Splenocyte constitutions were tested for the percentages of T, B, NK, and macrophage cells using anti-CD3 allophycocyanin (apc)-conjugated, anti-CD4 fluorescein isothiocyanate (FITC)-conjugated, anti-CD8 FITC-conjugated, anti-B220 FITC-conjugated, anti-NK1.1 FITC-conjugated, and anti-F4/80 FITC-conjugated antibodies (PharMingen). 1 x 10^6 cells were stained with 5 µl of a 1/10 dilution of antibody stocks in PBS. Cells were incubated for 30 min, washed, and analysed by FACS. Dead cells were labelled by the addition of 5 µl of 7-aminoactinomycin D (7AAD) (20 µg/ml) (Sigma). Fc receptors were blocked by the addition of 5 µl of a 1/10 dilution of anti-mouse CD16/CD32 (Fcγ III/II receptor)

(PharMingen). This antibody and the 7AAD were added prior to the addition of cell marker-specific antibodies.

4.3.5 Extraction of RNA, reverse transcription, DNA amplification and visualization

Total RNA was extracted from spleens of uninfected mice using TRIzol (Invitrogen) in accordance with the manufacturer's instructions. Reverse transcription using 1 µg total RNA was with 200 U Molony murine leukemia virus reverse transcriptase (M-MLV RT) (Promega) in the presence of 2.5 mM dNTPs (Astral Scientific), 20 U RNase inhibitor (Invitrogen), 1 µg/reaction of random primers, 50 mM Tris-HCl (pH8.8), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol (DTT) in a volume of 25 µl. Reverse transcription was carried out for 1 h at 37°C followed by heating at 94°C for 5 min to inactivate the transcriptase. The resulting cDNA was diluted with DNase- and RNase-free water (Sigma-Aldrich) to a final volume of 200 µl.

PCR was performed for the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), IFN-γ, and TNF-α (Yoshida et al 2000). Amplification of 5 µl of cDNA was achieved using 25µl reaction that contains 0.5 U of Taq polymerase (ABgene), magnesium-free buffer [75 mM Tris, 40 mM $(NH4)_2SO_4$, 0.1% (v/v)Tween 20] (10x reaction buffer; ABgene), 2 mM $MgCL_2$ (ABgene) and 2.5 mM dNTPs. The reaction mix was incubated at 94°C for 1 min. The reaction was cycled 30x at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min using Thermocycler (Biorad). These reactions were carried in the presence of 5 pmol of sense and anti-sense primers using the following primers:

GAPDH	sense (5'-GGTGAAGGTCGGTGTGAACGGA-3')
	antisense (5'-TGTTAGTGGGGTCTCGCTCCTG-3')
IFN-γ	sense (5'-CCTCATGGCTGTTTCTGGCTGTTA-3')
	antisense (5'- CATTGAATGCTTGGCGCTGGACC-3')
TNF-α	sense (5'-GAAAGCATGATCCGCGACGTGGA-3')
	antisense (5'-TACGACGTGGGCTACAGGCTTG-3').

PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The resultant bands were visualized with an ultraviolet transilluminator and compared among B6, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$x*gld* mice.

4.3.6 Histology

Midsagittal plane bisected brains were fixed in 10% normal buffered formalin and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin (HE) and luxol fast blue (LFB), and tissues were examined for morphology.

4.3.7 Macrophage transfer

10-week-old female Fas$^{-/-}$ mice were injected with thioglycolate (0.03 mg/mouse) intraperitoneally (i.p). Four days later, cells were harvested from the peritoneal cavity by rinsing 3x with PBS. Cells were washed twice, resuspended in PBS to a final concentration of 1 x 10^8 cell/ml, and 200 μl (2 x 10^7 cell) were injected i.v into naïve Fas$^{-/-}$ mice. 2 h after transfer, macrophage-enriched (Fas$^{-/-}$+Mθ) and naive Fas$^{-/-}$ mice were infected with vSFV (1 pfu/mouse). Tissues were harvested at day 3 and 5 post-infection and virus titers were determined by plaque assay.

4.4 Results

4.4.1 Growth of vSFV in tissues of mice defective in Fas- and/or granule exocytosis-mediated pathways of cytotoxicity

B6 mice deficient in perforin, gzmA, gzmB, or gzmA&B are marginally more resistant to low dose of vSFV infection relative to wt mice. This resistance increases significantly in mice lacking functional Fas (Fas$^{-/-}$), Fas.L (*gld*), perforin plus gzmA and B (perfxgzmAB$^{-/-}$), or Fas plus gzmA and B (FasxgzmAB$^{-/-}$). Mice deficient in perforin plus Fas.L (perf$^{-/-}$x*gld*) are most resistant to vSFV infection and represent a third category of SFV susceptibility. This is reflected in ~20% mortality, compared to ~70% mortality in wt B6 mice (chapter 3). These results were interpreted in terms of a deleterious effect of cytotoxic Fas- and granule exocytosis-mediated pathways of cytotoxic effector (NK or Tc) cells in disease outcome of SFV CNS infection.

Groups of mice (B6, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$x*gld*) were infected i.v with 1 pfu vSFV, monitored daily, and clinical symptoms recorded. At 2 day intervals, mice were sacrificed, serum, spleen, muscle, and brain harvested, and virus titres were determined. When the kinetics of SFV replication in extraneural and brain

tissues of mice defective in granule exocytosis and/or Fas-mediated pathways of cytotoxicity were compared to that in B6 wt mice, the pattern of SFV growth in perf$^{-/-}$ x*gld* mice differed from that of all other mice strains investigated (figure 4.1). The most striking observation was the generally much lower or undetectable presence of SFV in all tissues and serum of perf$^{-/-}$x*gld* mice. Whilst SFV was present in serum of wt, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, and FasxgzmAB$^{-/-}$ mice on 2 and 4 days post-infection, with titres ranging from 10^2-10^5 pfu/ml, virus was undetectable in perf$^{-/-}$x*gld* mice. SFV was also present in some spleens harvested at 2 and 4 days post-infection from all mouse strains other than perf$^{-/-}$x*gld* mice. Given that tissue titres were often not significantly higher than the corresponding virus titres in serum, the contribution of virus-containing blood to titres in the tissue samples cannot be excluded.

SFV replication in muscle did not yield high virus titres (< 10^5 pfu/g). SFV was present in most mouse strains on all days sampled with the exception of perf$^{-/-}$x*gld* mice where it was found only on day 8 post-infection. The highest virus titres were obtained in the brains of B6 wt and most mutant mice. Again in perf$^{-/-}$x*gld* mice brain titres of SFV were either undetectable or low. In addition, the observed clinical sings associated with vSFV infection indicated that the most severe clinical signs such as morbidity and hind limb paralysis were mostly correlated to the virus titre in the brains. This was observed in groups of B6 wt, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, and FasxgzmAB$^{-/-}$ mice on day 6 and 8 post-infection. In contrast, perf$^{-/-}$x*gld* mice did not show clinical signs of morbidity and CNS infection.

To confirm the reduced or lack of SFV replication in perf$^{-/-}$x*gld* mice, groups of B6 wt and perf$^{-/-}$x*gld* mice were infected i.v with 10^3 pfu of aSFV, brain, spleen, muscle, and serum titres were determined at days 2, 4, 6, and 8 post-infection (figure 4.2). Avirulent SFV grew to significantly higher titres in spleen on day 2 and 4 post-infection than vSFV, reached the CNS but was cleared from the brain between 6 and 8 days post-infection without reaching titres > 10^5 pfu or causing death in B6 wt mice. The low virus titres in the brain are a distinct feature of aSFV infection where replication in neurons is restricted (Fazakerley et al., 1993) (chapter 2). Replication of aSFV in muscle of B6 wt mice resulted in slightly higher titres on day 2 post-infection but presence in this tissue was of shorter duration to that of vSFV. The magnitude and duration of viremia did not markedly differ between aSFV and vSFV in B6 wt mice (figure 4.2.D and figure 4.1.D).

A comparison of growth of aSFV in B6 wt and perf$^{-/-}$x*gld* mice clearly demonstrates reduced growth in the double-deficient mouse strain in all tissues tested although viremia on day 2 post-infection is comparable.

These results suggest that the absence of clinical symptoms in, and increased survival rate of, perf$^{-/-}$x*gld* mice during vSFV infection is the result of restrict viral replication rather than reduced immune-mediated pathology due to defective cytolytic activity.

4.4.2 Natural antibodies

Mice suffering from generalised lymphoproliferative disease (*gld* mice) produce large amounts of IgG and IgM antibodies (Nagata and Suda, 1995). These antibodies, present before viral infection, could play a role in reducing virus growth. To test this possibility, serum samples from uninfected gld, perf$^{-/-}$x*gld*, perfxgzmAB$^{-/-}$, and B6 wt mice were assessed for their ability to neutralise vSFV *in vitro*. Serum dilutions were incubated with a fixed amount of vSFV at 37°C for 1 h, and neutralizing activity determined by plaque assay. No virus neutralizing effect was apparent (Table 4.1). Therefore, any involvement for these antibodies in the reduction of virus growth in strains with high serum levels of IgG and IgM is unlikely.

4.4.3 Splenocyte subsets

Mice with defect in the Fas pathway develop lymphadenopathy and splenomegaly (Cohen and Eisenberg, 1991; Nagata and Suda, 1995). The lymphoproliferative disease alters the ratio of NK, macrophages, T, and B cells in spleens which in turn would influence the immune response to a viral infection. Table 4.2 shows the cellular composition of spleens from B6 wt, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$ x*gld* mice. PerfxgzmAB$^{-/-}$ mice were comparable to B6 wt mice in terms of percentages of T (CD3^{+}), B (B220^{+}), NK (NK1.1^{+}) cells, and macrophages (F4/80^{+}) in spleen. The percentage of double positive (CD3^{+}B220^{+}) splenocytes was low in wt and perfxgzmAB$^{-/-}$ mice. In contrast, an elevated percentage of CD3^{+}B220^{+} cells, which is characteristic of a defect in the Fas pathway, was detected in *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$x*gld* mice. The percentage of macrophages in spleens of B6, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, and FasxgzmAB$^{-/-}$ mice was comparable but significantly elevated in perf$^{-/-}$x*gld* mice, as has

been previously reported (Spielman et al., 1998). In addition, a slight increased in the percentage of NK cells in spleens of perf$^{-/-}$x*gld* mice was observed compared to the other mouse strains.

4.4.4 Histology

Perf$^{-/-}$x*gld* mice have been shown previously to suffer from autoimmune diseases. High number of activated macrophages have been found in pancreas and ovaries, the later is thought to be responsible for female infertility (Spielman et al., 1998). Therefore, I investigated whether expanded macrophages in perf$^{-/-}$x*gld* are responsible for any histological differences in their brains compared to B6 wt mice. Abundant monocytes were present in the meninges of naïve perf$^{-/-}$x*gld* mice compared to naïve B6 mice (figure 4.3).

4.4.5 RT-PCR analysis for IFN-γ and TNF-α expression

High levels of TNF-α and IFN-γ in brain of SJL mice were associated with low viral titres during SFV infection. In comparison, low levels of TNF-α and IFN-γ were associated with high viral titres in B6 mice (Mokhtarian et al., 1996). This suggests that these cytokine, directly or indirectly, influence SFV replication in mice. Therefore, mRNA expression levels for both IFN-γ, and TNF-α were studied in naïve B6 and cytolytic effector function deficient mice. IFN-γ mRNA was not detectable in naive B6 and perfxgzmAB$^{-/-}$ mice but RNA transcripts were clearly present in *gld*, Fas$^{-/-}$, and FasxgzmAB$^{-/-}$, and elevated levels detected in perf$^{-/-}$x*gld* mice (figure 4.4). Furthermore, in perf$^{-/-}$x*gld* mice elevated TNF-α mRNA levels were detected in comparison to all other strains of mice (figure 4.4).

4.4.6 Transfer of activated macrophages restricts SFV replication *in vivo*

Increased number of activated macrophages in perf$^{-/-}$x*gld* mice have been implicated in immunopathology (Spielman et al., 1998). I investigated the effect of expanded activated macrophage populations on viral replication in these mice. My aim was to transfer macrophages from perf$^{-/-}$x*gld* mice into wt B6 strain and then study their effect on viral

replication. Peritoneal rinsing was used to harvest macrophages; about 4 x 10^6 cells were harvested per perf$^{-/-}$x*gld* mouse compared to about 1 x 10^6 cells/B6 mouse. The availability of homozygotes perf$^{-/-}$x*gld* obtained from our breeding program proved to be insufficient to permit peritoneal macrophage isolation in sufficient numbers for transfer. I therefore used thioglycolate-activated macrophages or transfer.

Fas$^{-/-}$ mice were used because of their delayed MTD compared to B6 wt mice (chapter 3). Macrophage-enriched (Fas$^{-/-}$+Mθ) and control Fas$^{-/-}$ mice were infected with vSFV (1 pfu/mouse) and tissue titres were determined at day 3 and 5 post-infection. As shown in figure 4.5, high virus titres were detected in almost all tissues harvested from control mice. This compares with little or no virus detectable in almost all tissues harvested from Fas$^{-/-}$&Mθ mice. These data illustrate the ability of activated macrophages to ameliorate SFV infection, and suggest that activated macrophages are at least in part responsible for the increased resistance of perf$^{-/-}$x*gld* mice.

4.4.7 Infection with high dose of vSFV

Death of mice from SFV infection is usually the result of brain damage (Atkins et al., 1999). If activated macrophages protect perf$^{-/-}$x*gld* mice by preventing viral replication and, possibly, brain invasion, such protection would be dependent on the dose of infection. To test this, B6 and perf$^{-/-}$x*gld* mice (6 mice per group) were infected i.v with 10^3 pfu/mouse vSFV. This dose of infection led to 100% mortality in both perf$^{-/-}$x*gld* and B6 mice (figure 4.6). However, perf$^{-/-}$xgld mice showed a delayed MTD (~10 days) compared to B6 mice (~7 days). Statistically this delay was significant (P= 0.013, Mann-Whitney test). In addition, death in perf$^{-/-}$x*gld* mice was not associated with hind limb paralysis compare to death seen in B6 mice suggesting less neuronal damage in the absence of cytolytic activity, and was similar to NK-depleted mice (chapter 3).

4.5 Discussion

Chapter 3 describes experiments which show that perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$x*gld* mice are significantly more resistant to vSFV infection compared to wt B6 mice. Experiments in this chapter were aimed at investigating whether clinical vSFV infection differs among these mouse strains. Clinical symptoms such as ruffled fur, hunched posture and hind-limb paralysis before death are similar in B6, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, and FasxgzmAB$^{-/-}$ mice. In these strains, the observed severe symptoms, such as paralysis, are clearly associated with high virus titres in the brains. Although a cytopathic effect due to SFV per se cannot be excluded, the impaired cytolytic activity of mice defective in the Fas or exocytosis mediated pathways of cytolysis and the associated reduced damage in the CNS may be responsible for their increased resistance to SFV infection. Contrary to all other strains, no clinical signs were observed in perf$^{-/-}$x*gld* mice. This is associated with restricted viral replication. Resistance to Flavivirus infection with MVE has also been reported in perf$^{-/-}$x*gld* mice (Licon Luna et al., 2002). In this instance it was speculated that the increase in resistance was due to lack of cytolytic activity of effector T and NK cells and prevention of blood brain barrier damage. However, the results shown here suggest that, in perf$^{-/-}$x*gld* mice, the abundance of activated macrophages and the elevated expression levels of IFN-γ and TNF-α may have limited vSFV infection.

IFN-γ induces the expression of MHC-I molecules (Rosa et al., 1986), and MHC-I molecules itself have been reported to inhibit Sindbis virus replication (Hahn et al., 1999). RNA transcripts for IFN-γ were detected in naive *gld*, Fas$^{-/-}$, FasxgzmAB$^{-/-}$, and perf$^{-/-}$x*gld* mice. Since virus titres in tissues harvested from *gld*, Fas$^{-/-}$, and FasxgzmAB$^{-/-}$ mice were comparable to that of B6 samples, any role for IFN-γ in increasing anti-viral resistance through induction of MHC-I molecules seems unlikely. Yet, IFN-γ must play a crucial role in resistance to SFV infection since wt mice of the 129 strain became susceptible as a result of IFN-γ receptor deficiency (chapter 3). This is supported by circumstantial evidence from SJL mice where low SFV titres in brains are associated with high levels of TNF-α and IFN-γ compared to B6 mice where high SFV titres in the brains are associated with low levels of TNF-α and IFN-γ (Mokhtarian et al., 1996). IFN-γ is required for macrophage activation, but must be accompanied by TNF to achieve full

macrophage activation (reviewed in (Mosser, 2003)). In addition, a synergistic action of both IFN-γ and TNF-α has been reported to mediate an anti-viral effect *in vitro* (Wong and Goeddel, 1986). Elevated levels of RNA transcripts for both IFN-γ and TNF-α detected in spleens of naive perf$^{-/-}$x*gld* mice, which is accompanied by the presence of activated macrophages in large numbers, may have contributed to resistance in these mice.

Although thioglycolate-activated macrophages have the ability to inhibit SFV replication in Fas$^{-/-}$ mice, they may have different properties from those found in perf$^{-/-}$x*gld* mice. Therefore, confirmation for the role of macrophages in resistance of perf$^{-/-}$x*gld* mice to SFV infection will require further investigations. If activated macrophages play an important role in increasing perf$^{-/-}$xgld mouse resistance to SFV infection, their role may include an ability to inhibit SFV replication as well as protecting the blood brain barrier. Transferring macrophages from perf$^{-/-}$x*gld* mice into B6 mice could prove to be a vital experiment illustrating such a role for macrophages in perf$^{-/-}$x*gld* mice. Since many attempts to isolate enough macrophages from perf$^{-/-}$x*gld* mice for adoptive transfer were unsuccessful, macrophage inactivation or depletion studies in these mice may resolve these questions.

Table 4.1. Neutralization assay, natural Abs did not affect infectivity of vSFV in perf$^{-/-}$ x*gld*.

Mice strains	pfu detected for each serum dilution		
	1:05	1:10	1:20
B6	$8.8x10^2$	$1.1x10^3$	$1.2x10^3$
	$9.6x10^2$	$1.2x10^3$	$9.4x10^2$
perfxgAB$^{-/-}$	$1.1x10^3$	$1x10^3$	$9.3x10^2$
	$1.2x10^3$	$9.6 x10^3$	$1.2x10^3$
gld	$8.9x10^2$	$1.1x10^3$	$9.2x10^2$
	$1x10^3$	$9.5x10^3$	$9.4x10^3$
perf$^{-/-}$x*gld*	$9.2x10^2$	$1x10^3$	$1.1x10^3$
	$1.1x10^3$	$8.6x10^2$	$9.2x10^3$
Mock	$1.2x10^3$	$9x10^2$	$1.1x10^3$

Neutralisation activity of serum from uninfected B6, perfxgzmAB$^{-/-}$, *gld*, and perf$^{-/-}$x*gld* mice on vSFV. 2 different serum samples from each strain were tested with no effect detected on the infectivity of vSFV. Mock samples represent incubation of virus stock alone under similar condition before testing infectivity.

Table 4.2. Lymphocyte constitution showing more macrophages in the spleen of perf$^{-/-}$ x*gld* mice compared to all other strains.

	Cell markers (%±SE)						
Strain	**CD3**	**B220**	**CD3+B220**	**CD4**	**CD8**	**NK1.1**	**F4/80**
B6	34.3±2.2	51.3±0.7	4±0.1	22.1±1.1	13.2±0.1	4.6±0.2	7.2±0.5
perfxgzmAB$^{-/-}$	36.9±3.9	52.3±3.3	3.1±0.3	22.3±2.8	13.7±1.3	4.1±0.1	6.1±0.1
gld	44.2±3.5	60.6±2.2	27.9±2.1	16.9±0.4	8.6±0.1	5.3±0.5	8.5±1.7
Fas$^{-/-}$	47.1±1.8	72.1±0.1	30.2±5.4	11.6±0.3	7.4±0.4	3.9±0.6	5.9±0.9
FasxgzmAB$^{-/-}$	43.1±8.7	67.2±2.6	25.3±6.6	12.1±1.6	6.9±0.2	4.9±0.2	7.7±1.2
perf$^{-/-}$xgld	41.6±5.5	62.1±2.5	21.1±4.2	13.1±1.2	8.4±0.9	6.2±1.8	17.5±4.1

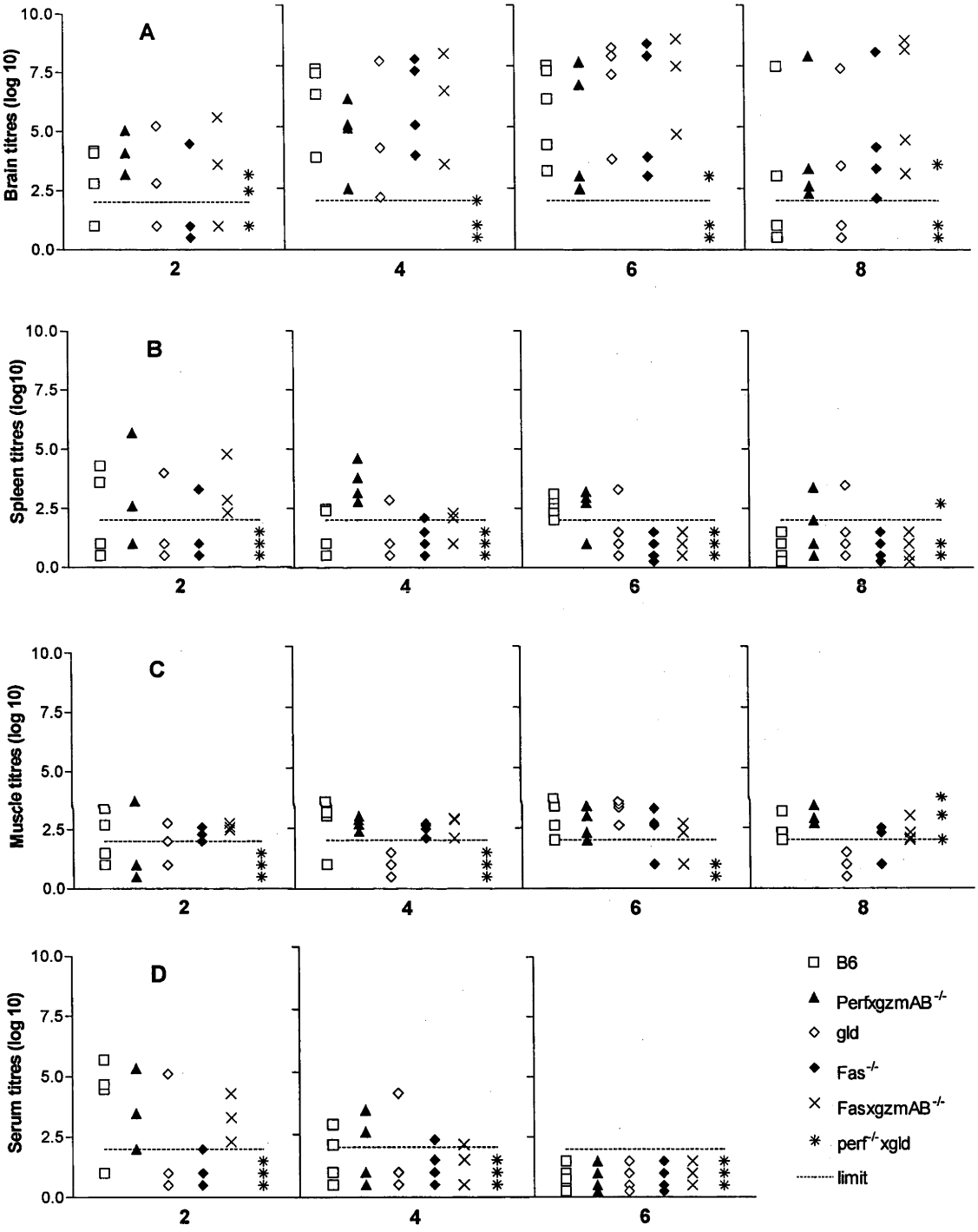

Figure 4.1. Virus titres detected in brain, spleen, muscle, and serum samples at days 2, 4, 6, and 8 post-infection with vSFV (1 pfu/mouse). While B6, perfxgzmAB$^{-/-}$, *gld*, Fas$^{-/-}$, and FasxgzmAB$^{-/-}$ mice show comparable titres in tested tissues; perf$^{-/-}$x*gld* mice show restricted viral replication. Virus titres expressed as pfu (log10)/gm or ml of brain, spleen, and muscle or serum, respectively.

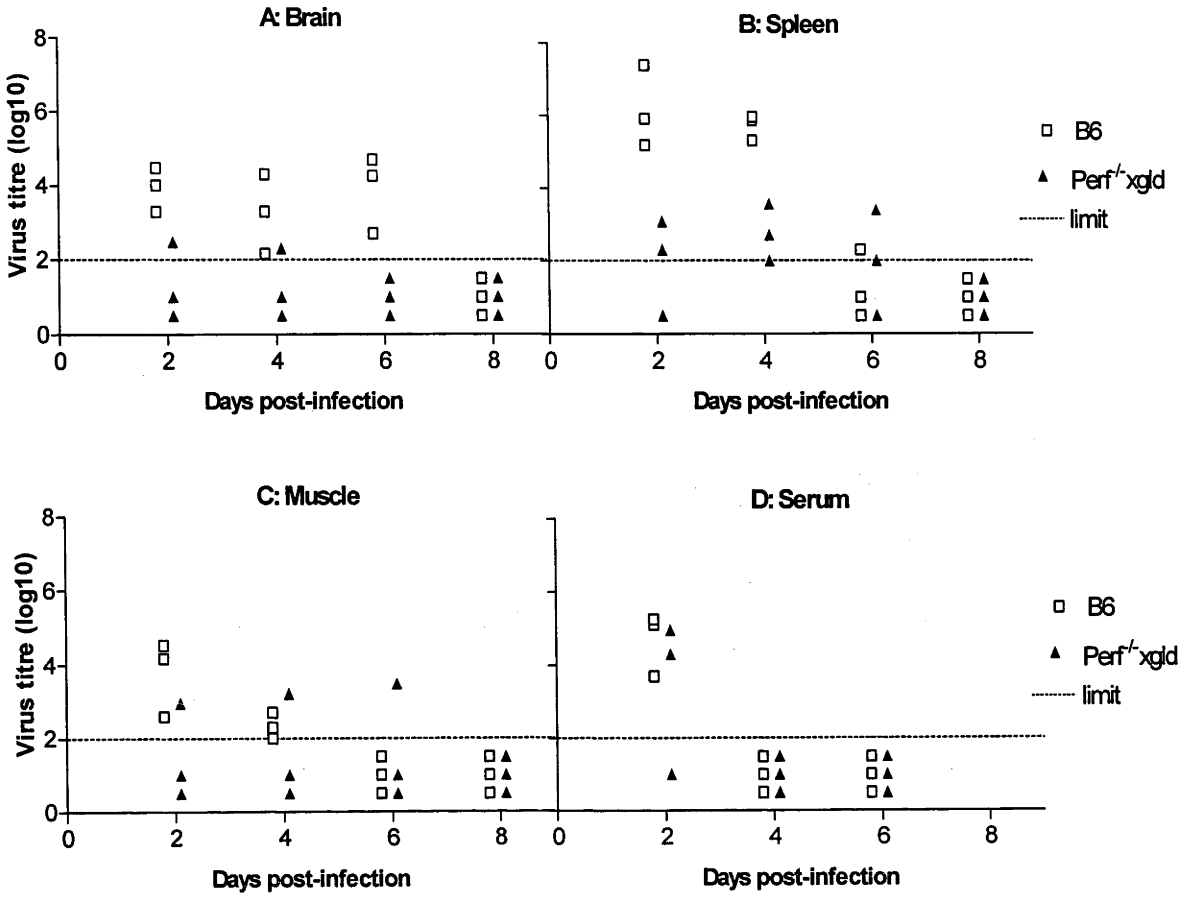

Figure 4.2. Tissue titres of aSFV are compared between B6 and perf$^{-/-}$xgld mice following infection with 10^3 pfu/mouse. Titres detected in tissue samples from perf$^{-/-}$xgld mice confirm the restricted viral replication previously detected in these mice when vSFV where used. Virus titres expressed as pfu (log10)/gm or ml of brain, spleen, and muscle or serum, respectively.

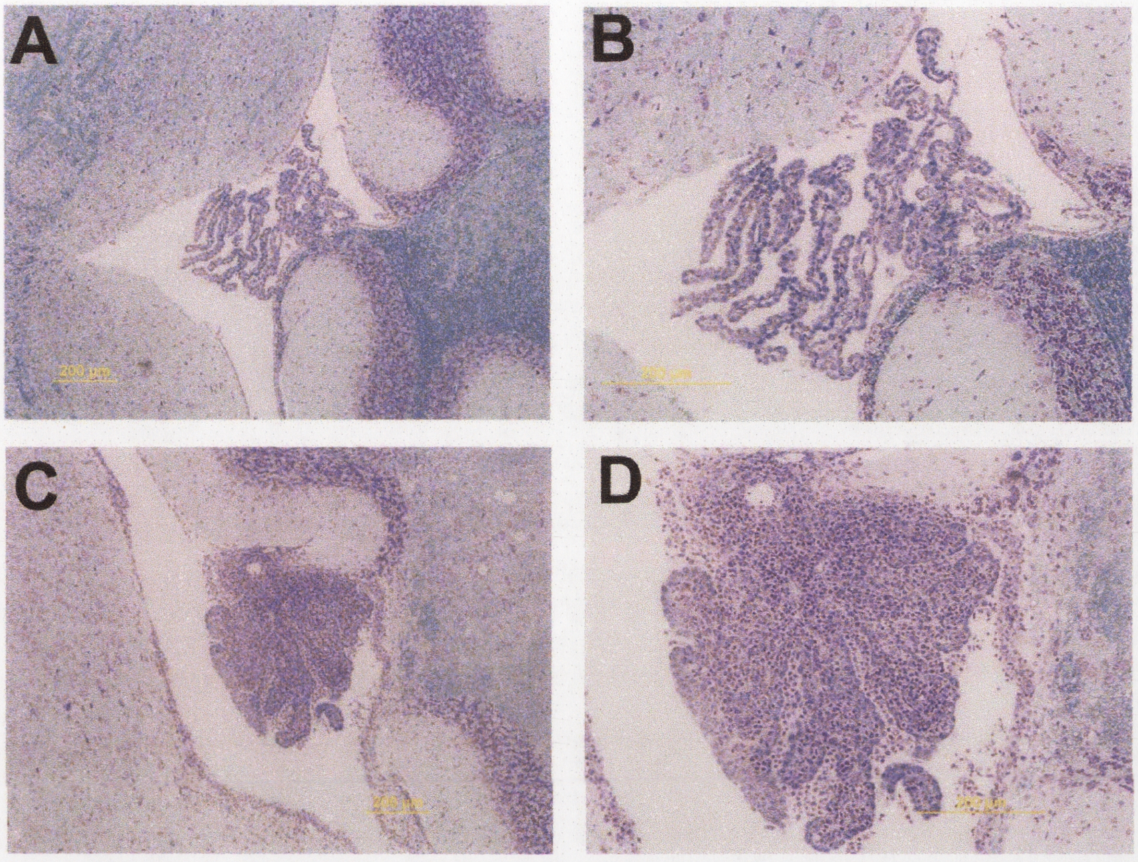

Figure 4.3. Brains from naïve B6 and perf$^{-/-}$x*gld* mice are compared for monocytes infiltration. A & B show clear choroid plexus in the brain of B6 mice, whereas C & D show monocyte infiltration in the same area in the brain of perf$^{-/-}$xgld mice.

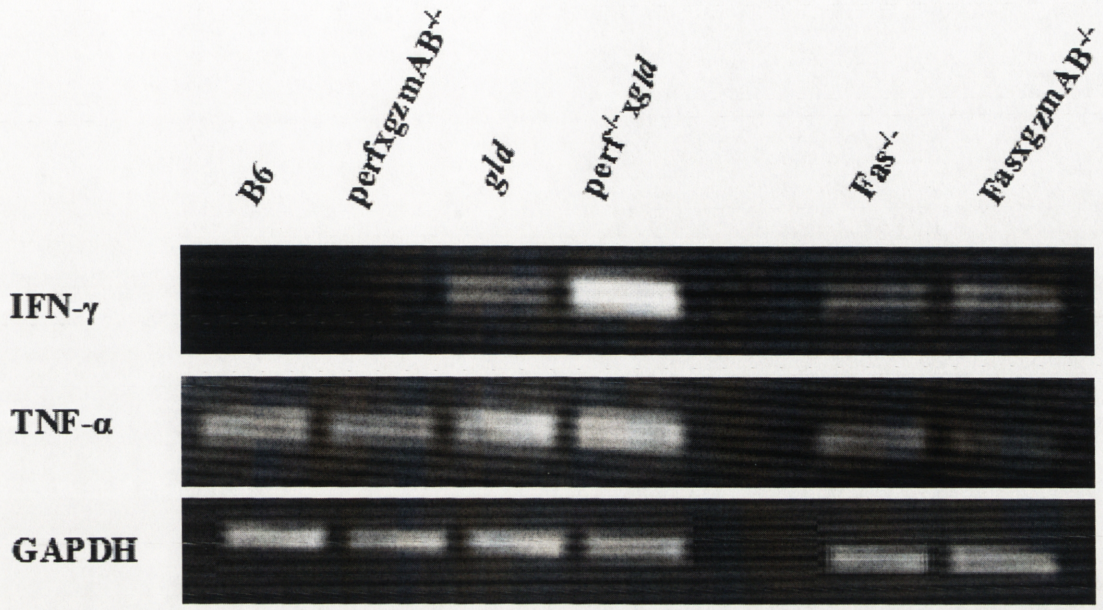

Figure 4.4. Elevated mRNA levels of IFN-γ and TNF-α in the spleen of naïve perf$^{-/-}$x*gld* mice compared to all other strains. GAPDH is a housekeeping gene.

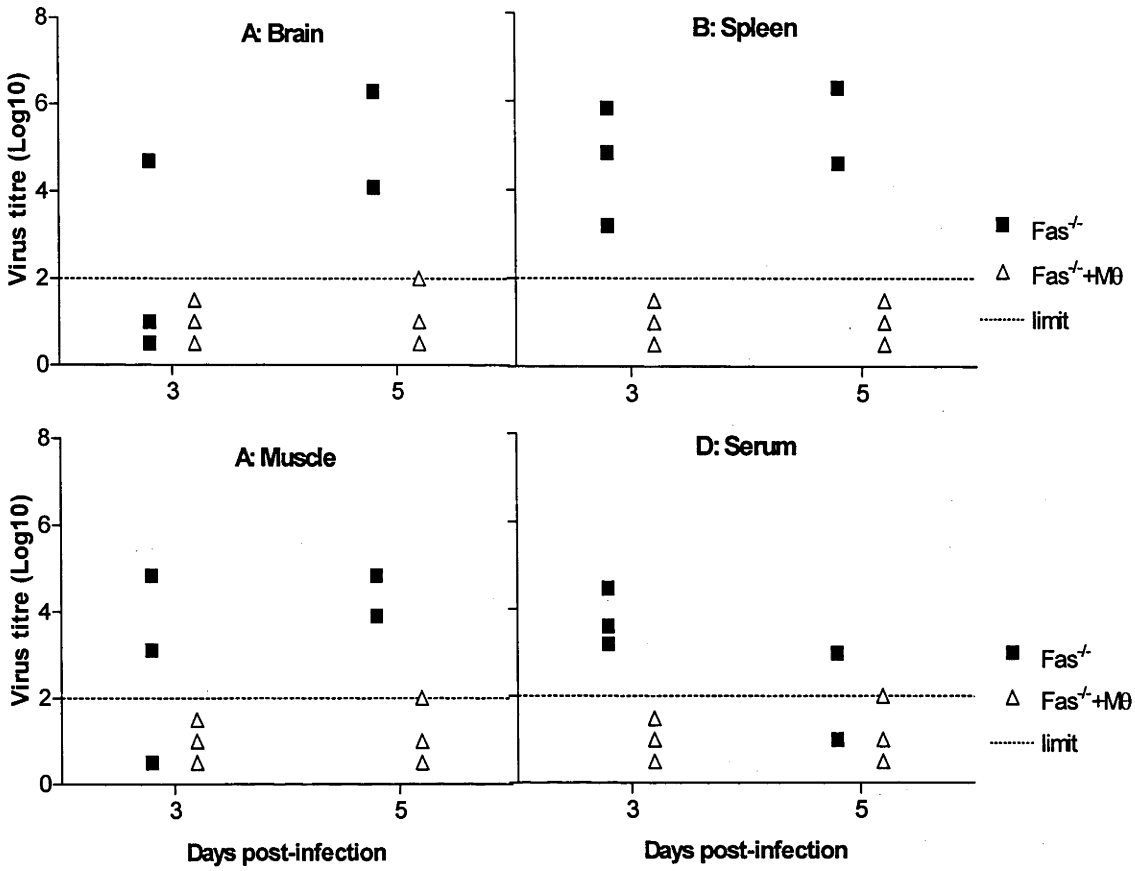

Figure 4.5. The effect of activated macrophages on SFV replication. Macrophage-enhanced and control Fas$^{-/-}$ mice were infected with vSFV (1 pfu), and tissue titres were estimated at day 3 and 5 post-infection. Virus titres expressed as pfu (log10)/gm or ml of brain, spleen, and muscle or serum, respectively.

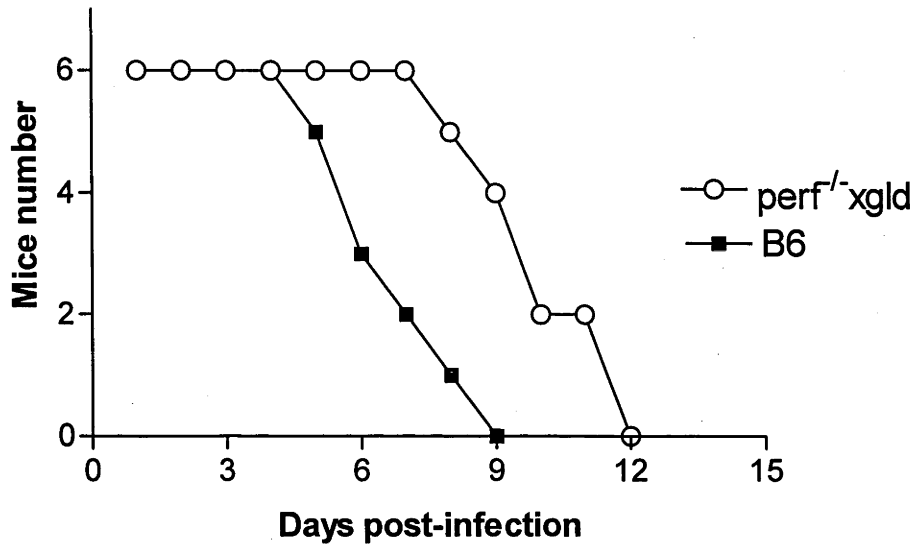

Figure 4.6: Survival of B6 and perf$^{-/-}$x*gld* mice to vSFV (10^3 pfu) infection were compared.

Chapter 5

IFN-I mediated lymphocyte activation during viral infection

5.1 Summary

In this chapter, profiles of cell surface lymphocyte activation markers for both $CD3^+$T cells and B ($B220^+$) cells, after infection with different doses of vSFV and aSFV, was investigated. The data indicate that the cell surface expression levels of the activation markers, CD69 and CD86, were dependent on virus dose and virulence. High dose of avirulent and low dose of virulent SFV resulted in more than 80% of T and B cells expressing activation markers within 24 h. These decline to base levels over the next 4 days. This pattern of cell surface marker expression appears to be a general phenomenon in response to viral infections and is not restricted to SFV. The very early "activation" of such a high percentage of lymphocytes is mediated by cytokines, particularly IFN-I. Inactivated virus does not produce a similar pattern of activation marker expression. No increase in CD25 expression was detected following viral infection. However, when lymphocytes were treated with mitogens *in vitro,* CD25 expression level was elevated.

5.2 Introduction

Interferons were discovered on the basis of their antiviral activity (Isaacs and Lindenmann, 1957). Two types of IFNs can be distinguished: IFN-I and IFN-II (Muller et al., 1994). IFN-II (IFN-γ) is structurally unrelated to IFN-I, encoded by a single gene (Gray and Goeddel, 1982), and secreted by NK and T cells exclusively (Battistini et al., 1990; Foster, 1997). IFN-γ is primarily involved in immunoregulation (Boehm et al., 1997). On the other hand, IFN-I (IFN-α, IFN-β) are encoded by a family of over 20 genes, (Muller et al., 1994) and can be secreted by a large spectrum of cell types (Battistini et al., 1990; Foster, 1997). All IFN-I subtypes bind to a common cell surface receptor (Darnell, 1997; Darnell et al., 1994), and play an important role in host protection following infection by a variety of viruses (Lobigs et al., 2003; Muller et al., 1994; Ryman et al., 2000).

Virus-infected cells synthesise IFN-I within hours of infection, and can act in an autocrine and paracrine manner to limit replication and spread of virus (Deonarain et al., 2000). In addition to their antiviral properties, IFN-I exerts regulatory effects on cellular and humoral immune responses. IFN-I stimulates T helper functions in both mouse (Evans and Ozer, 1987) and human (Brinkmann et al., 1993), can directly support survival of activated T cells (Marrack et al., 1999; Tough et al., 1996), and induces bystander cytotoxic T cell proliferation during viral infections, particularly memory-T cells (Tough et al., 1996). Moreover, IFN-I stimulates antibody production *in vitro* (Le Bon et al., 2001; Rodriguez et al., 1983), and can act as an adjuvant for vaccination against influenza virus (Proietti et al., 2002). However, the role of IFN-I in immunoregulation during viral infection *in vivo* has received little attention.

Lymphocyte activation can be measured by the expression of several cell surface molecules such as CD69, CD86, and CD25. CD69 is the first cell surface antigen expressed, following activation, on T, B, NK cells, monocytes, and most other hematopoietic lineages (reviewed in (Ziegler et al., 1994). CD80 and CD86 are cell surface molecules that provide co-stimulation for T cell activation by interaction with CD28 (Hakamada-Taguchi et al., 1998), and both molecules can be expressed on various APCs such as DCs, macrophages, and B cells (Hathcock et al., 1994). CD25 is the α chain of the IL-2 receptor, and can be expressed on the surface of activated B and

T cells following Ag recognition (Lowenthal et al., 1985). *In vitro* treatment of B cells with IFN-I upregulates the expression of CD69, CD86, and CD25 (Braun et al., 2002).

In this chapter, I investigate the magnitude and kinetics of lymphocyte activation associated with an acute cytopathic viral infection in mice. I found that more than 80% of splenocytes become activated one day post-infection with splenocyte activation markers expression returning to background levels by day 5 post-infection. This massive level of lymphocyte activation was a general phenomenon associated with viral infections, and mediated by IFN-I.

5.3 Materials and methods

5.3.1 Viruses and cells

Vero and BHK cells were maintained in EMEM plus nonessential amino acids and 5% FCS, and incubated at 37^0 C in humidified condition with 5% CO_2.

Working stocks for vSFV and aSFV were prepared by infecting semi-confluent BHK cells monolayers at a multiplicity of infection of 0.5 pfu/cell. Infected cells were incubated for 24h, culture supernatants harvested, centrifuged at 1200x g for 4 minutes to remove cell debris, and stored in single-use aliquots at -70° C. Titres were 5x 10^7 pfu/ml for vSFV and 1x 10^8 pfu/ml for aSFV- based on plaque-titration on Vero cells. Stocks of Adenovirus (Ad-2) (Hayder and Mullbacher, 1996) and WNV (Wang et al., 2003) were prepared as described.

Inactivated preparations of vSFV and aSFV were obtained by γ-irradiation (1.3 GRad) using a Cobalt-60 source (CSIRO-Canberra). Loss of inactivity of virus replication was confirmed by plaque assay.

5.3.2 Mice

Wild type, C57BL/6 (B6) and 129-2SV mice were used in addition to 129 syngeneic mice deficient in IFN receptors: IFN-α-receptor knockout (IFN-$IR^{-/-}$) (Muller et al., 1994), IFN-γ-receptor knockout (IFN-$IIR^{-/-}$) (Huang et al., 1993), and double receptors

knockout (IFN-I&IIR$^{-/-}$). Mice were bred under specific pathogen-free conditions and supplied by the Animal Breeding Facilities at the John Curtin School of Medical Research, Canberra. Only females 10 wk of age were used through out.

5.3.4 Flow-Cytometric Analysis

Spleens from infected and control mice were harvested and single cell suspensions prepared. RBC lysis buffer (Ph 7.2), consists of 90% 0.16M NH4Cl and 10% 0.17M Tris-base (Ph 7.65), was used to lysis red cells. Splenocytes were incubated in RBC lysis buffer (1 ml/spleen) for 3 minute at room temperature. Following incubation, splenocytes were washed twice in PBS+1%FCS and resuspended at 1 x 10^7 cell/ml. Lymphocyte subpopulations were estimated using fluorescent-conjugated CD3, CD4, CD8 and B220 antibodies (PharMingen). Activation status was assessed using CD69 (for T and B cells) and CD86 (for B cells) antibodies (PharMingen). 1x10^6 cells were stained with 5µl of 1/10 antibody dilution in PBS. Cells were incubated for 30 minutes, washed, and analysed by FACS. Dead cells were labelled with 7AAD (Sigma), using 5µl of 1/100 dilution per sample (final concentration is 1µg/ml). Fc receptors were blocked by the addition of mouse CD16/CD32 (Fcγ III/II receptor) antibody (PharMingen). This antibody and 7AAD were added prior to the addition of cell subpopulation and activation marker specific antibodies.

5.4 Results

5.4.1 Activation markers

Cell surface expression of CD69, CD25, and CD86 has been used as an indication of lymphocyte activation *in vitro* (Braun et al., 2002). To evaluate the *in vivo* activation status of splenocytes, B6 mice were infected with aSFV (10^7 pfu/mouse) and expression levels of the markers were determined 1 day postinfection by FACS. As shown in figure 5.1, CD69 and CD86 expressions are elevated on both B220$^+$B and CD3$^+$T cells. Both CD4$^+$ and CD8$^+$ T cell subpopulations showed a CD69 profile similar to that seen on CD3$^+$ cells. Thus, CD3$^+$T cells were used throughout this study representing both CD4$^+$T and CD8$^+$T cells. As background levels of CD86 were higher on CD3$^+$T cells than on B220$^+$ B cells, CD86 will only be used as an indicator for

activation of B cells. No increase of CD25 expression was observed on any leukocyte cell type. This contrasts with observations from *in vitro* studies (Braun et al., 2002). However, when splenocytes from naïve mice were treated *in vitro* with B (LPS) or T (ConA) cell mitogens CD25 expression was induced on the surface of $B220^+$ cells by LPS (figure 5.3.A) and on $CD3^+$ cells by ConA treatment (figure 5.3.B).

5.4.2 Lymphocyte activation during SFV infection

Groups of B6 mice were infected with either 1 or 10^3 pfu vSFV or 1, 10^3, or10^7 pfu aSFV i.v. The percentages of B and $CD3^+$ cells with elevated expression of lymphocyte activation markers CD69 and CD86 as shown in Fig 5.2 were measured over a 5 day period (Fig 5.4). Kinetics and expression levels of CD69 on B and T cells as well as CD86 on B cells were very similar. 1 pfu of aSFV did not result in significant up-regulation of lymphocyte activation markers. On the other hand, the same dose of vSFV induced expression of activation markers on both B and T cells with peak numbers at day 3 post-infection and returning to background levels by day 5 post-infection. Increasing the doses of either SFV strain increased the number of lymphocytes with activation markers and accelerated their appearance. Infection with inactivated SFV did not result in generalized lymphocyte activation (Fig 5.4.D).

5.4.3 Systemic feature of lymphocyte activation

To investigate whether lymphocyte activation during SFV infection was systemic or restricted to the spleen, B6 mice were infected with 10^7 pfu aSFV and activation markers on lymphocytes from spleen (figure 5.5.A), lymph node (figure 5.5.B), and blood (figure 5.5.C) were determined at day 1 post-infection. Expression of CD69 was induced on both B and T cells to similar levels on cells from all tissues tested. CD86 expression mirrored that of CD69.

5.4.4 General viral infections and lymphocyte activation

To address whether this generalised pattern of lymphocyte activation is a feature of any anti-viral immune response, I tested lymphocyte activation using two SFV unrelated viruses, Ad-2 a DNA virus and the Flavivirus WNV, a single stranded RNA virus similar to SFV. Figure 5.6 shows that i.v infection with either of these two viruses

induce similar patterns and magnitude of lymphocyte activation as seen with SFV, whereby an elevated expression of CD69 and CD86 was detected on up to 80% of splenocytes at day 1 post-infection. This elevated expression of CD69 and CD86 decreased with time and reached background levels by day 5 post-infection. Thus, general lymphocyte activation appears to be an intrinsic property of the hosts' early immune response to viral infections.

5.4.5 Role of IFN-I in lymphocyte activation

The early occurrence of systemic lymphocyte activation after low doses of viral infections suggests the possibility that the hosts' early interferon type-I response may be responsible. To test this assumption I compared the magnitude of virus-induced lymphocyte activation in mice deficient in type-I and/or type-II interferon receptor(s) (IFN-IR$^{-/-}$, IFN-IIR$^{-/-}$, and IFN-I&IIR$^{-/-}$) with their wt 129 counterpart. Mice were infected with 10^7 pfu aSFV and lymphocyte activation estimated at day 1 postinfection. A generalized elevated expression of CD69 on T cells was observed in both wt 129 (figure 5.7.A) and IFN-IIR$^{-/-}$ (figure 5.7.B) mice. On the other hand, CD69 expression on T cells from IFN-IR$^{-/-}$ (figure 5.7.C) and IFN-I&IIR$^{-/-}$ (figure 5.7.D) mice was substantially lower. This dramatically reduced level of expression was also seen for CD69 on B cells and CD86 on B cells (figure 5.7) as compared to wt control mice. The magnitude of activation marker expression was similar after infection with 10^3 pfu of vSFV or with 10^7 pfu of aSFV (figure 5.4). Given that vSFV elicits a much stronger IFN-I response than aSFV (chapter 2) these results are consistent with the interpretation that IFN-I is the predominant mediator for the induction of CD69 and CD86 expression on lymphocytes *in vivo*.

5.5 Discussion

The data described in this chapter demonstrates that *in vivo* infection with viruses causes a generalised activation of lymphocytes as indicated by an elevated expression of CD69 and CD86 on both B and T ($CD4^+$ and $CD8^+$) cells. This cell marker expression is rapid, virus dose and strain dependent, and appears to be a feature of most viral infections (also occurs with poxvirus infection, Müllbacher personal

communication). Activation marker levels decline to background levels at about 5 days post-infection. This observed activation is clearly related to viral replication since inactivated SFV did not induce any detectable level of activation at early days post-infection despite the administration of high doses (5×10^7 pfu equivalent/mouse). This also eliminated any possible involvement for FCS and other cellular debris possibly present in the viral preparation in the detected lymphocyte activation.

The systemic and generalised activation of tested lymphocytes at very early time postinfection suggest the involvement of early released cytokines. Two pieces of evidence strongly indicate a major role of IFN-I in this lymphocyte activation. Firstly, when IFN-R knockout mice were used, only IFN-IR$^{-/-}$ mice but not IFN-IIR$^{-/-}$ mice lacked this generalised lymphocyte activation when infected with viruses. These mice, in general, are more sensitive and die very early after viral infection (Lobigs et al., 2003; Muller et al., 1994; Ryman et al., 2000), thus lymphocyte activation were only tested at day 1 pi. Secondly, the strength and kinetics of the observed activation marker expression correlated with the ability of the individual SFV strain to induce IFN-I. As shown in chapter 2, vSFV is a more potent inducer of IFN-I than aSFV. Earlier and higher levels of activation marker expression were induced by lower virus doses of vSFV compare to aSFV.

IFN-I exerts, in addition to their important antiviral properties, pleiotropic effects including inhibition of cell growth, anti-tumour action, involvement in homeostasis, and regulatory effects on cellular and humoral responses (reviewed in (De Maeyer and De Maeyer-Guignard, 1998). However, the importance of immunoregulation by IFN-I during viral infection has not been fully addressed. This is probably because the early general concept scientists tended to believe was that IFNs antiviral effect must be far more important than any immunoregulatory role, which if occurs is likely to be local (reviewed in (De Maeyer and De Maeyer-Guignard, 1982)). Data presented in this chapter illustrate a generalised phenomenon characterised by massive lymphocyte activation during early days following viral infection. The biological significant of this phenomenon, however, is at present not clear, especially what the host may benefit by having 80% of lymphocytes in an activated state, irrespective of their antigen specificity. One possibility is that CD69 and CD86 expressions are by products of lymphocytes attaining an anti-viral state, which certainly is an advantage to the host

reducing viral dissemination. In this context the lack of CD25 expression may be significant, by not rendering the lymphocytes susceptible to IL-2 mediated proliferation. Thus "true activation" may also require CD25 upregulation, which may only be triggered by ligation of the clonally expressed antigen receptors.

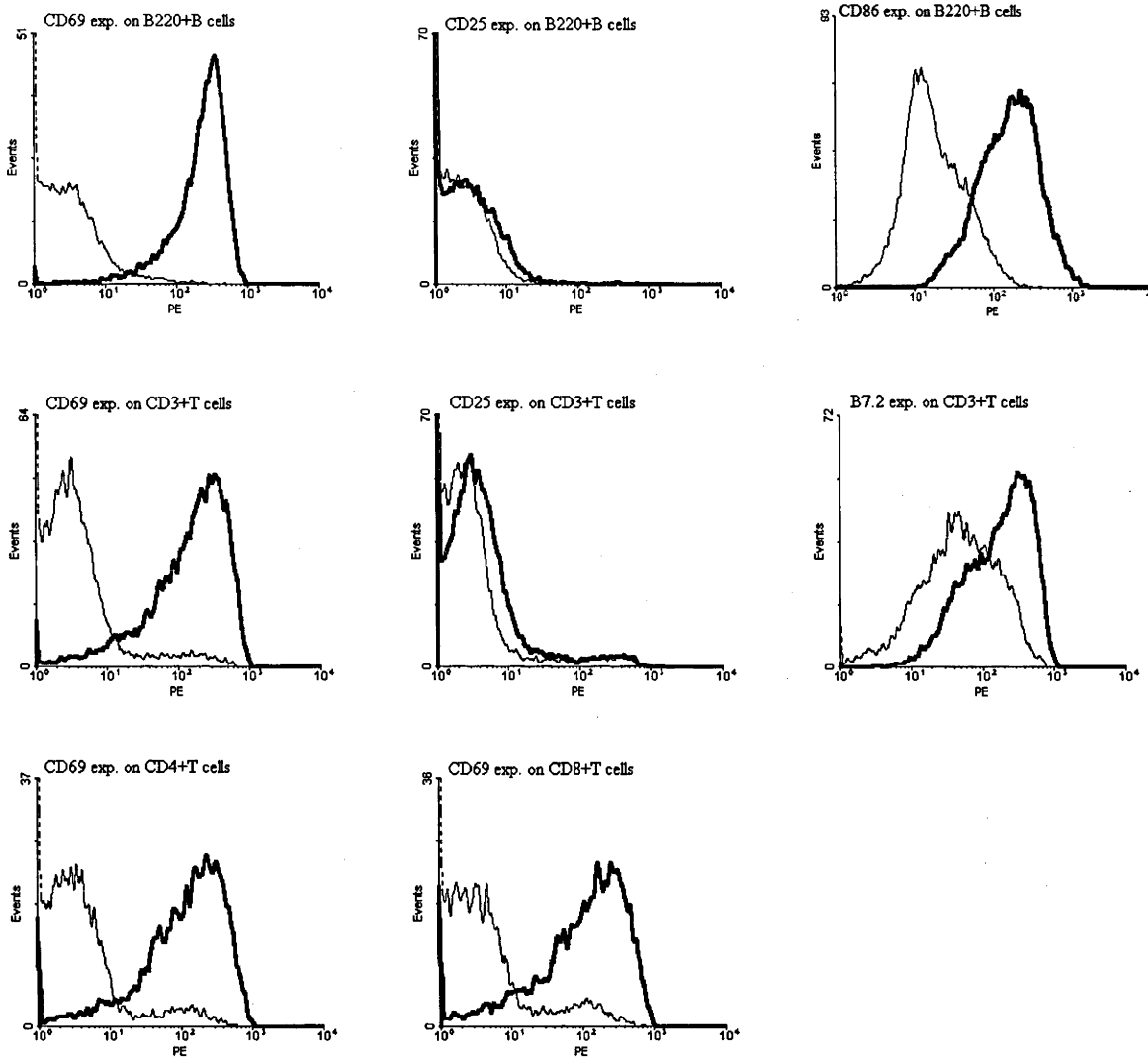

Figure 5.1: CD69 and CD86 expression levels were induced on both $B220^+$B and $CD3^+$T cells following *in vivo* infection with aSFV. CD25 expression level was not induced on either cell type. Both $CD4^+$ and $CD8^+$T cells show comparable level of CD69 expression to that seen in $CD3^+$T cells.

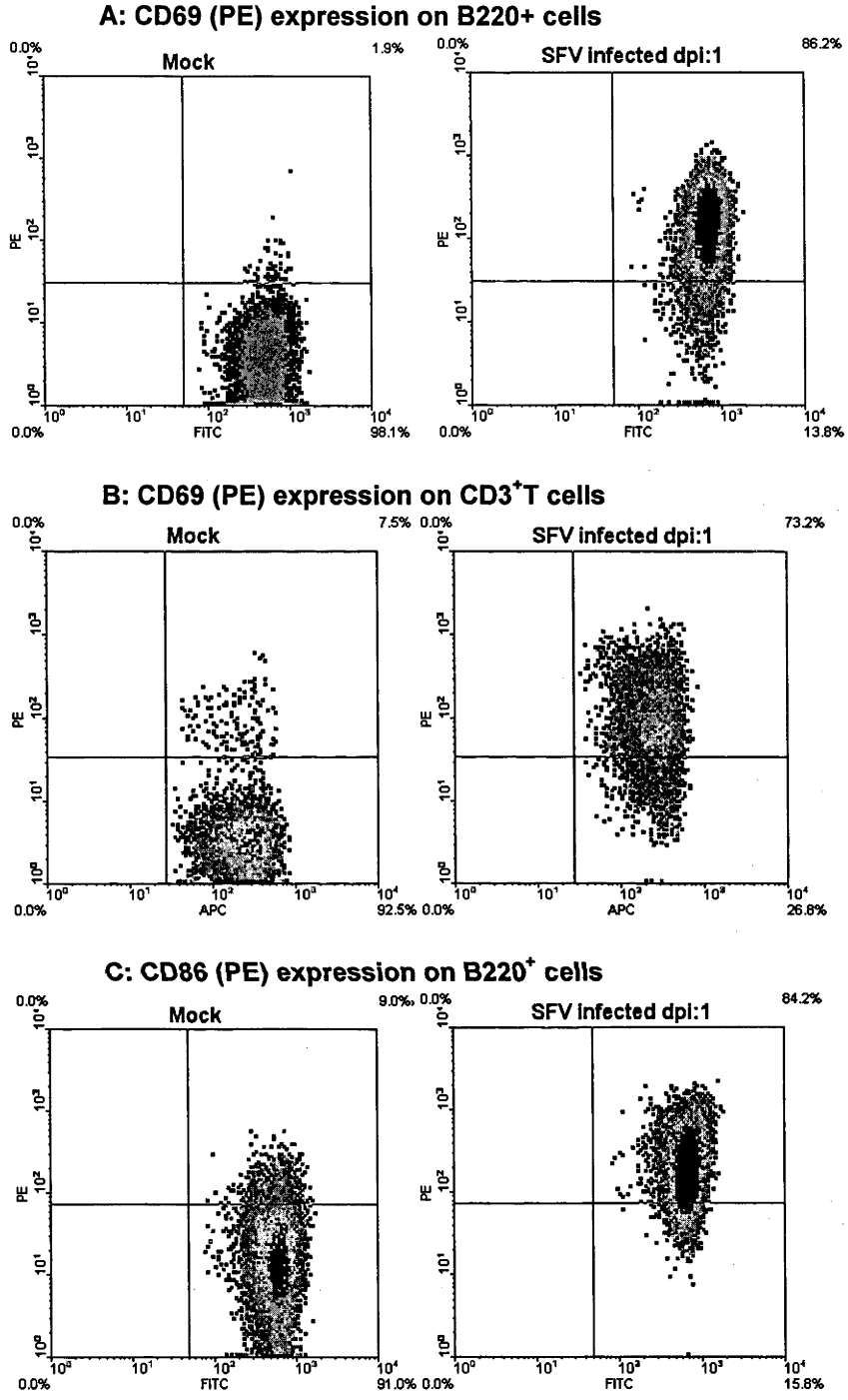

Figure 5.2: Examples for calculating the percentages of activated $B220^+$B cells and $CD3^+$T cells following SFV infection.

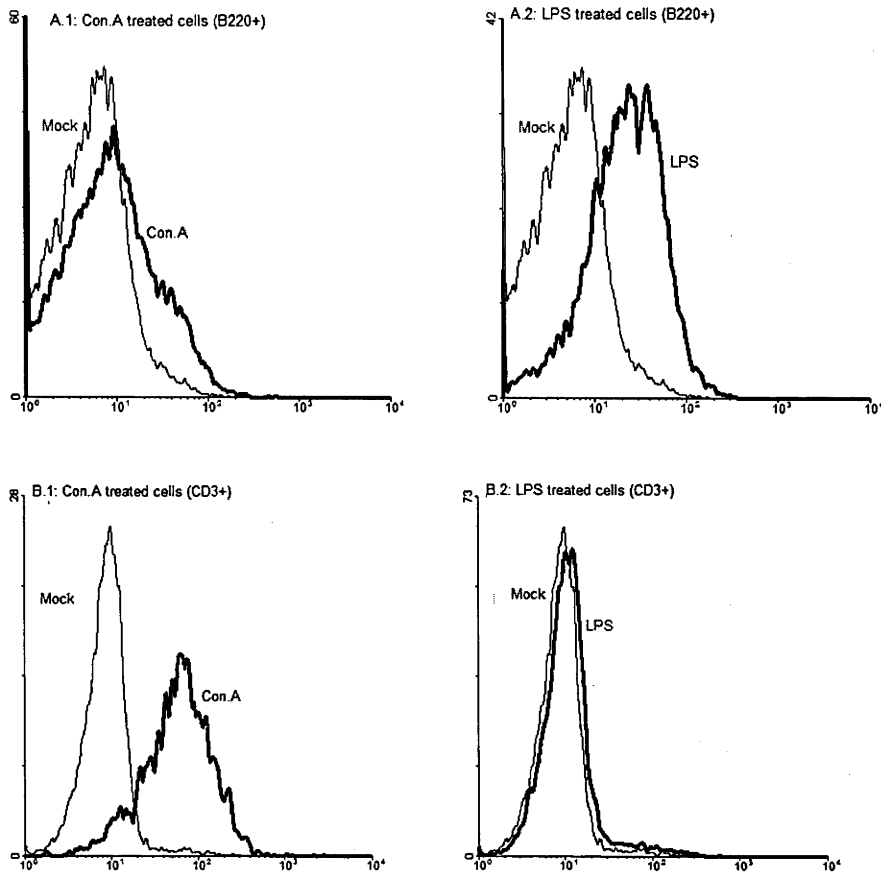

Figure 5.3: CD25 expression after in vitro activation with mitogens Con.A (T cell mitogen) or LPS (B cell mitogen). A) CD25 expression on $B220^+$ B cells following both Con.A (A.1) and LPS (A.2) treatment. B) CD25 expression on $CD3^+$ T cells following both Con.A (B.1) and LPS (B.2) treatment.

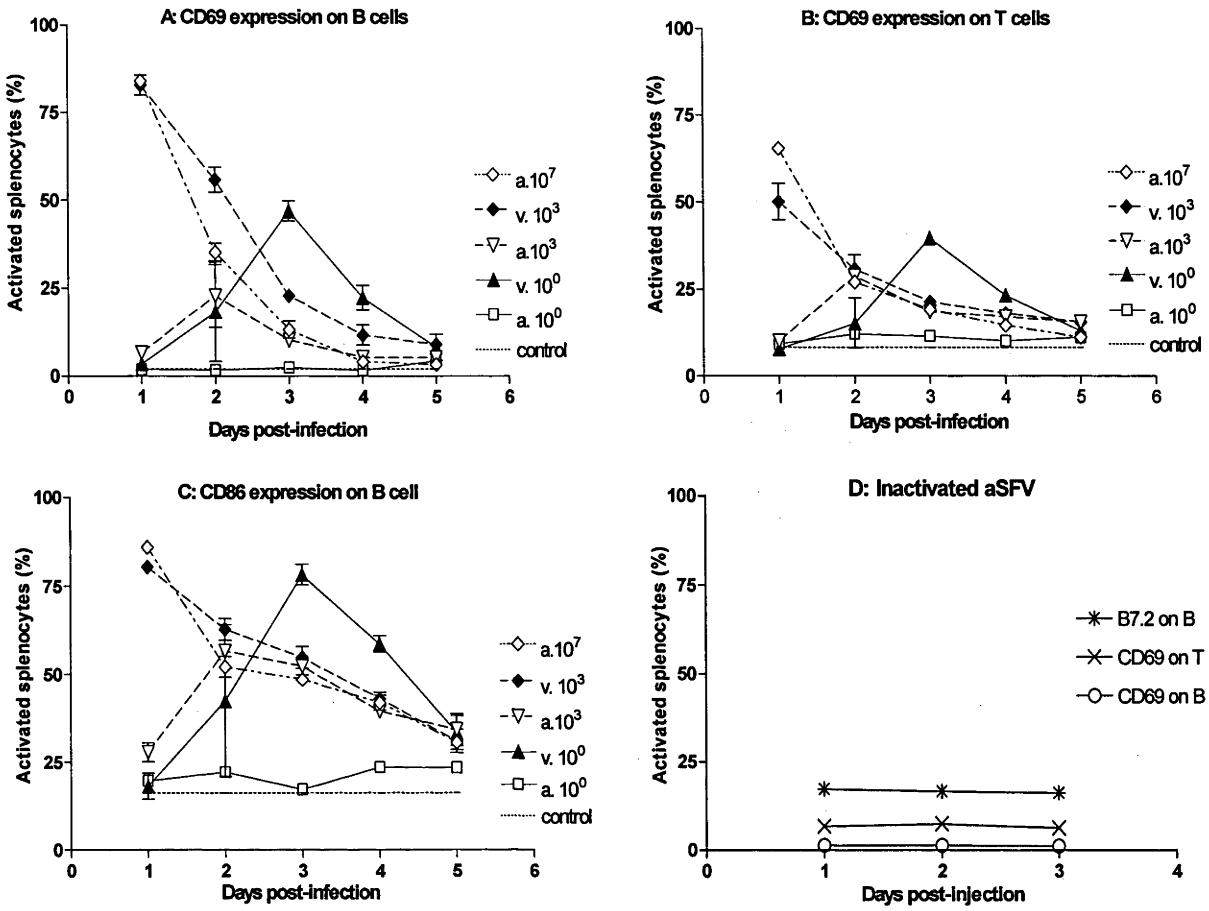

Figure 5.4: Time course of lymphocyte activation as a result of SFV infection. Percentage of activated lymphocytes detected at different times after vSFV and aSFV infection using different doses. (A) Expression of CD69 on $B220^+$ B cells. (B) Expression of CD69 on $CD3^+$ T cells. (C) Expression of CD86 on $B220^+$ B cells. (D) Expression of CD69 and CD86 on T and B cells after vaccination with inactivated aSFV.

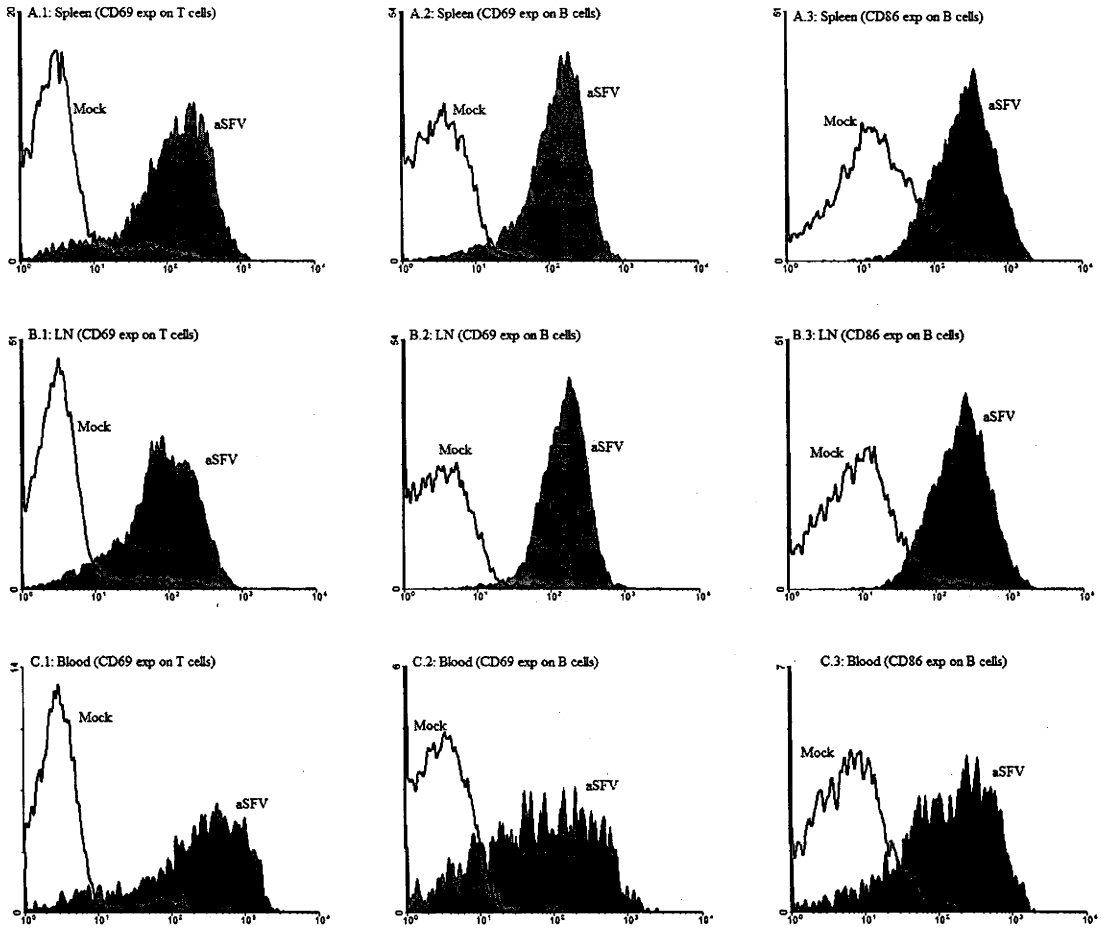

Figure 5.5. Lymphocyte activation is systemic during SFV infection. B6 mice infected with 10^7 pfu/mouse aSFV and lymphocytes from spleen (A), lymph nodes (B), and blood (C) were tested at day 1 postinfection for CD69 expression on T cell (A.1, B.1, and C.1) and B cells (A.2, B.2, and C.2), and CD86 expression on B cells (A.3, B.3, and C.3).

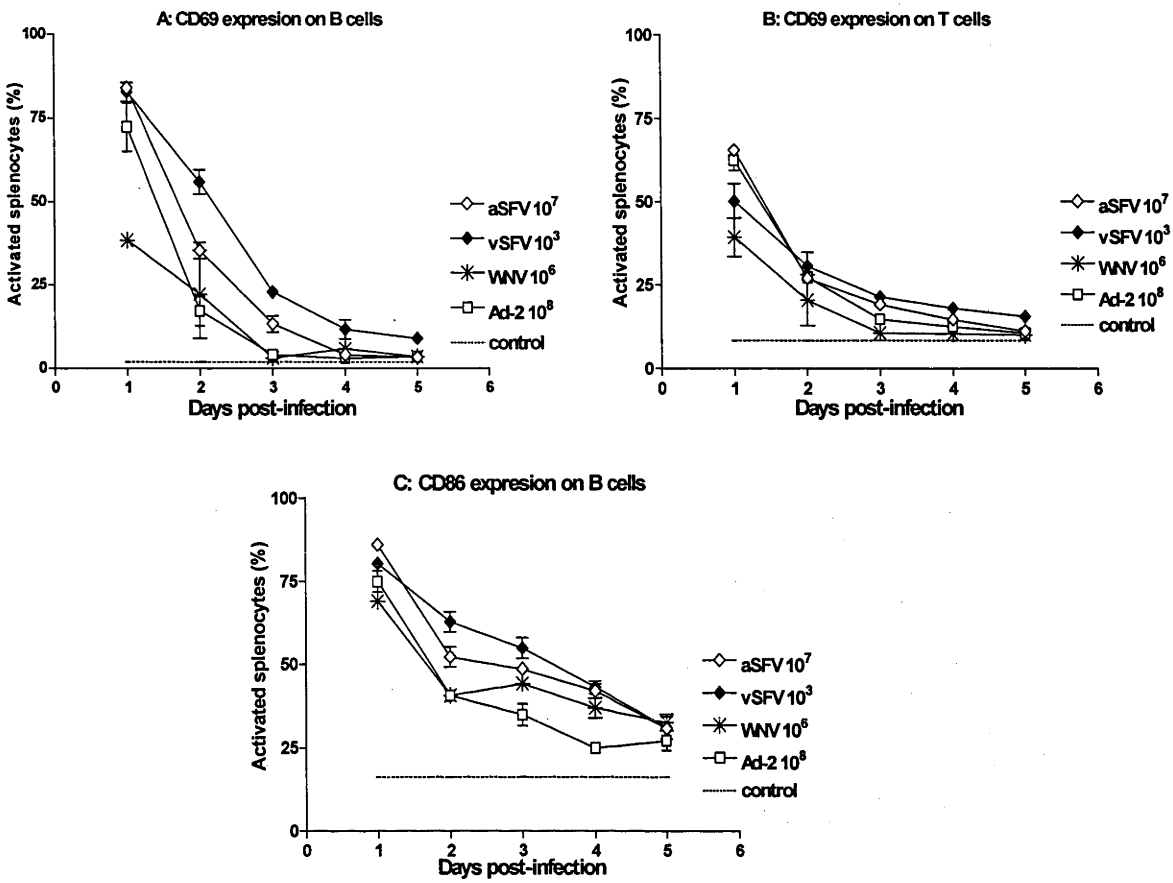

Figure 5.6. Percentage of activated lymphocytes after different viral infection, (A) CD69 expression on $B220^+$ B cells, (B) CD69 expression on $CD3^+$T cells. (C) CD86 expression on $B220^+$ B cells.

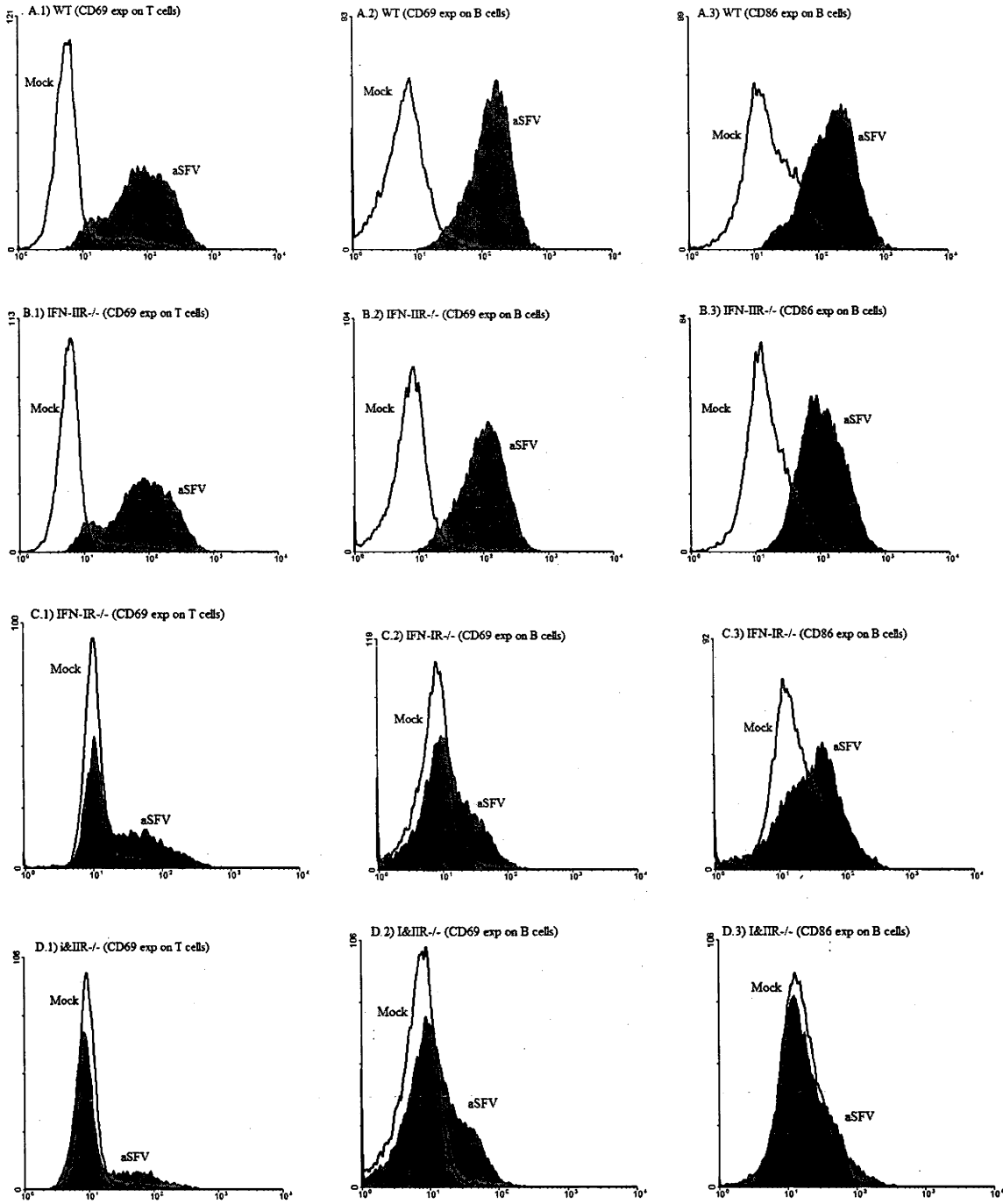

Figure 5.7: Histograms comparing lymphocyte activation in following aSFV (10^7 pfu/mouse) infection in wt (129) mice (A), IFN-IIR$^{-/-}$ (B), IFN-IR$^{-/-}$ (C), and IFN-I&IIR$^{-/-}$ (D) using CD69 expression on T cells (1) and on B cells (2), and CD86 expression on B cells (3). In wt mice the activation markers expression levels markedly increased following aSFV infection (shaded) compared to mock (open). This detected expression was abolished in IFN-IR$^{-/-}$ and IFN-I&IIR$^{-/-}$. IFN-IIR$^{-/-}$ mice show similar activation levels to that detected for wt.

Chapter 6

Fate of lymphocytes following primary viral infection

6.1 Summary

The decrease in activated cells was investigated and was found to be not a result of cell loss, but rather down-regulation of activation markers. This was associated with the inability to display a similar activated phenotype in response to a subsequent viral challenge, despite the animal's susceptibility to the second infection. As these cells were responsive to subsequent viral challenge when transferred to a new host, it is possible that a refractory period is required to generate a second systemic IFN response following an acute viral infection.

6.2 Introduction

As a result of viral infections, type I IFNs are rapidly induced to provide the first line of defence by protecting cells from viral replication and to modulate the immune response (Levy and Garcia-Sastre, 2001). Although all virus infected cells can produce IFN-I within hours of infection, immature DCs, particularly plasmacytoid dendritic cells (pDCs), produce 1000-fold higher IFN-I levels than other cell types, and are responsible for a systemic IFN-I responses to many viruses (Le Bon and Tough, 2002). Mature DCs are the professional APC to stimulate T cells (reviewed in (Cella et al., 1997). These cells are characterised by a decreased level of endocytic activity compared to immature DCs (reviewed in (Steinman and Swanson, 1995), with IFN-I playing a role in their maturation (Luft et al., 1998; Montoya et al., 2002), probably mediated via the induction of IL-15 (Mattei et al., 2001). IFN-I produced by human monocyte-derived DCs or murine DCs (derived from spleen or bone marrow) has been shown to exert an autocrine stimulatory effect on the respective DC populations (Luft et al., 1998). It has been shown that IFN-I induced-during influenza virus infection resulted in sustained expression of MHC-I molecules presenting viral peptide by human DCs (Cella et al., 1999). In addition, exposure to IFN-I may induce the expression of costimulatory molecules such as CD80, and CD86 on DC, which are engaged in communication with T cells during activation (Gallucci et al., 1999; Ito et al., 2001; Santini et al., 2000). Therefore, IFN-I may serve as a link between innate and adaptive immunity through stimulation of both antigen presenting cells and Ag-reactive effector cell populations (T and B cells).

In chapter 5, I have described massive generalised lymphocyte activation-marker expression during viral infections. This expression was IFN-I-mediated and induced on the surface of more than 80% of splenocytes. The percentages of lymphocytes expressing activation markers decreased with time to reach background levels at day 5 postinfection (chapter 5). Attrition of bystander $CD8^{+}$T cells, particularly of the memory phenotype, has been reported during viral infection in response to IFNs (McNally et al., 2001). In addition, activated T cells were shown to have reduced induction capabilities of IFN-sensitive genes compared to naïve cells when both populations were subjected to *in vitro* treatment with IFN-α (Dondi et al., 2003). Thus,

it was of interest to investigate the fate of activated cells and their responsiveness to subsequent viral challenge.

6.3 Material and methods

6.3.1 Viruses and cells

Vero and BHK cells were maintained in EMEM plus nonessential amino acids and 5% FCS, and incubated at 37^0 C in humidified condition with 5% CO_2.

Working stocks for vSFV and aSFV were prepared by infecting semi-confluent BHK cells monolayers at a multiplicity of infection of 0.5 pfu/cell. Infected cells were incubated for 24h, culture supernatants harvested, centrifuged at 1200x g for 4 minutes to remove cell debris, and stored in single-use aliquots at -70° C. Titres were 5x 10^7 pfu/ml for vSFV and 1x 10^8 pfu/ml for aSFV- based on plaque-titration on Vero cells. Stocks of Ad-2 (Hayder and Mullbacher, 1996) and WNV (Wang et al., 2003) were prepared as described.

6.3.2 Mice

Wild type B6 mice were bred under specific pathogen-free conditions and supplied by the Animal Breeding Facilities at the John Curtin School of Medical Research, Canberra. Only females 10-week-old were used through out.

6.3.3 Flow-Cytometric Analysis

Spleens from infected and control mice were harvested and single cell suspensions prepared. RBC lysis buffer (Ph 7.2), consists of 90% 0.16M NH4Cl and 10% 0.17M Tris-base (Ph 7.65), was used to lysis red cells. Splenocytes were incubated in RBC lysis buffer (1 ml/spleen) for 3 minute at room temperature. Following incubation, splenocytes were washed twice in PBS+1%FCS and resuspended at 1 x 10^7 cell/ml. Lymphocyte subpopulations were estimated using fluorescent-conjugated CD3, and B220 antibodies (PharMingen). Activation status was assessed using CD69 (for T and

B cells) and CD86 (for B cells) antibodies (PharMingen). 1 x 10^6 cells were stained with 5µl of 1/10 antibody dilution in PBS. Cells were incubated for 30 minutes, washed, and analysed by FACS. Dead cells were labelled with 7AAD (Sigma), using 5µl of 1/100 dilution per sample (final concentration is 1µg/ml). Fc receptors were blocked by the addition of mouse CD16/CD32 (Fcγ III/II receptor) antibody (PharMingen). This antibody and 7AAD were added prior to the addition of anti-cell markers (CD3, B220, CD69, and CD86) antibodies.

6.3.4 Irradiation, reconstitution, and adoptive transfer

Splenocytes at a concentration of 5 x 10^7 cell/ml in PBS were labelled with the cell tracker CFSE at a final concentration of 100 µM. After 5 minutes incubation at room temperature, 10 volumes of PBS containing 2% FCS were added and cells were centrifuged and washed 3 times with PBS/FCS. CFSE labelled lymphocytes were resuspended in PBS at a concentration of 2.5 x 10^8 cell/ml. 10-week-old irradiated (650 Rad) B6 mice, 24 h after irradiation, were reconstituted with 5 x 10^7 CFSE-labelled cells/mouse. Similarly, 5 x 10^7 CFSE-labelled cells/mouse were adoptively transferred into non-irradiated naïve B6 mice.

6.3.5 Lymphocyte responsiveness to a second infection

Mice were infected with 10^7 pfu aSFV and splenocytes harvested at day 5 post-infection. After RBC lysed (described above), splenocytes were labelled with CFSE (described above), and 5 x 10^7 CFSE labelled cells were transferred into naïve mice. 2 h later, mice were infected with Ad-2 virus (2 x 10^8 IU/mouse). Activation levels were estimated a day later and compared between naïve and CFSE labelled cells.

6.5 Results

6.5.1 The fate of activated lymphocytes

The disappearance of activation markers by day 5 post-infection on lymphocytes may be the result of two possibilities. One is the loss of activated cells due to activation induced cell death (AICD) (Dhein et al., 1995; Ju et al., 1995) and/or attrition caused by

IFN-I exposure (McNally et al., 2001), and replenishment by haematopoiesis; the other is a reversion of the activation to a quiescent state of the lymphocytes. To differentiate between these two possibilities I used sublethally irradiated mice reconstituted with CFSE-labelled lymphocytes to monitor their fate after viral infections.

Twenty four hours after sub-lethal irradiation (650 rads), B6 mice were reconstituted with CFSE-labelled syngeneic splenocytes and infected with 10^7 pfu aSFV or mock infected and the activation profiles of CFSE-labelled cells were monitored. The CFSE profile at day 1 post-infection (day 2 post cell transfer) (figure 6.1.A) and day 5 post-infection (day 6 post cell transfer) (figure 6.1.C) is similar in both mock and aSFV infected mice. CD69 expression on CFSE-labelled splenocytes was markedly elevated at day 1 post-infection in aSFV-infected mice (figure 6.1.B), and returned to background level by day 5 post-infection (figure 6.1.D).

Some CFSE-labelled splenocytes were shown to have divided following transfer into both uninfected and infected recipients (figure 6.1.C). B cells were the least dividing cell population both at day 1 and 5 post-infection (figure 6.2.A &B). A portion of $CD4^+$T cells underwent at least one division by day 5 postinfection (figure 6.2.C &D). $CD8^+$T cells constituted the main dividing splenocyte population with 2 and 4 divisions on days 1 (figure 6.2.E) and 5 (figure 6.2.F) post-infection, respectively.

6.5.2 Induction of activation markers after a second unrelated viral infection

Sublethally irradiated mice reconstituted with CFSE labelled splenocytes were initially infected with aSFV (1 x 10^7 pfu/mouse). Four days later, these mice were challenged with WNV (2 x 10^7 pfu/mouse). 24 h after the second infection, CD69 expression was tested and compared on splenocytes from mice receiving the initial aSFV infection only, and mice subjected to the subsequent infection with WNV. CD69 expression levels on splenocytes of mice receiving the subsequent WNV infection did not increase above the level detected on splenocytes from mice infected with aSFV (figure 6.1.F). In addition, CFSE profiles for splenocytes obtained from mice infected with aSFV alone or followed by WNV were similar (figure 6.1.E). Therefore, 5 days after activation by a

primary infection, splenocytes are refractory to activation by a subsequent viral challenge with an unrelated virus.

6.5.3 Lymphocytes reactivation by secondary viral infection

To assess at what stage previously activated lymphocytes regain their ability to respond to a secondary viral infection, and to avoid the proliferation detected for some lymphocyte, I adoptively transferred CFSE-labelled splenocytes into B6 mice and evaluated their activation pattern after infections with an unrelated virus at different time intervals after the primary infection. Twenty four h after transfer, mice were infected with 1 x 10^7 pfu/mouse aSFV (primary infection). Either 6 or 9 days later, mice were infected with 2 x 10^8 IU/mouse Ad-2 virus (subsequent infection). The recovery rate and the activation profile of CFSE-labelled cells were assessed 24 h after the second infection.

CD69 expression induced by aSFV infection by day 1 post-infection (figure 6.3.A) had returned to background levels on day 7 post-infection (figure 6.3.C). At this time point, an Ad-2 infection (figure 6.3.D) was unable to induce re-expression of activation markers. However, an Ad-2 infection 9 days after the initial aSFV infection induced CD69 expression significantly (figure 6.3.F) compared to splenocytes from mice infected only with aSFV (figure 6.3.E). The level of CD69 expression induced by the Ad-2 infection 9 days after the initial infection with aSFV was comparable to that seen in control mice, infected only with Ad-2 (figure 6.3.B). Expression of CD86 was similar to that seen for CD69 (figure 6.4). Changing the order of infections, using Ad-2 as a primary infection and aSFV as a secondary infection, did not change the outcome. 6 days after the primary Ad-2 infection, mice were infected with aSFV. The secondary infection of aSFV failed to induce activation markers expression above levels detected for Ad-2 infection alone, which was at background levels (figure 6.5 and figure 6.6).

The numbers of CFSE labelled cells recovered from spleens throughout this experiment were monitored to see if activated cells were preferentially lost (i.e undergoing apoptosis) compared to non-activated transferred cells. As shown in figure 6.7, the loss of CFSE-labelled cells as a result of activation is comparable to that of non-activated cells. Generally, there was a decrease in the percentage of CFSE-labelled cells

recovered from spleens of infected mice at day 7 and 10 post-infection. However, the level of reduction was similar in non-infected mice to that of infected mice.

6.5.4 IFN-I mediated antiviral state and lymphocyte activation to subsequent infection

To ascertain whether IFNs induced by the initial infection generated an anti-viral state responsible for preventing a subsequent infection from becoming established and thus preventing renewed IFN-I secretion, Mice were tested to see if they expressed increased resistance to a lethal viral infection. B6 mice were infected with 2 x 10^8 iu/mouse Ad-2. This dose of Ad-2 virus induces similar level of activation to that of 10^7 pfu aSFV (chapter 5). Ad-2 infected mice were challenged with a lethal dose of vSFV (10^3 pfu/mouse) at day 6 post-infection. Ad-2 infected mice were more susceptible than their naïve counterpart (figure 6.8). This indicates that the inability of the second viral infection to activate lymphocytes was not due to the persistence of a general anti-viral state induced by the primary infection, but rather reflects a time requirement for cellular signal reprogramming in lymphocytes or regeneration of IFN-secretory capabilities.

6.5.5 Lymphocyte responsiveness to second infection

To investigate whether the refractory period in lymphocytes to undergo a renewed activation process is caused by impaired IFN-I synthesis inhibited responsiveness to IFN-I of previously activated lymphocytes, mice were infected with 10^7 pfu aSFV and splenocytes were harvested at day 5 post-infection, labelled with CFSE, and transferred into naïve mice. Two hours later, mice were infected with Ad-2 virus. Expressions of activation markers of CFSE-labelled (transferred) and unlabelled (resident) lymphocytes were determined 1 day after Ad-2 infection. As shown in figure 6.9, Ad-2 infection induced cell surface expression of both CD69 and CD86 on both naïve and CFSE labelled cells. This result proves the ability of previously activated cells to respond to renewed viral infection. Thus, it is possible that lymphocyte unresponsiveness in the same host is related to the absence of activation factor: IFN-I. This needs to be further investigated. The most likely explanation for this detect refractory period in activation marker expression is due to exhaustion of IFN-I production.

6.6 Discussion

In chapter 5, I have shown that virus infections in mice lead to a rapid (within 24h) expression of activation markers on lymphocytes. Up to 80% of all lymphocytes including B, $CD4^+$ and $CD8^+$T cells, in spleen, lymph nodes and blood are affected. This generalised lymphocyte activation is primarily mediated by IFN-I. Reversion to background levels of activation marker expression occurs at around day 5 post-infection but does not necessarily coincide with virus clearance. Data obtained by adoptive transfer studies using fluorescent labelled splenocytes expressing activation markers clearly show that the observed disappearance of activation marker-positive cells is not due to cell turnover via apoptosis, but rather that the individual cells undergo some sort of a cellular deprogramming of the maturation pathway. Thus, it is distinct from AICD, which may be operative only when the maturation pathway is initiated via antigen reactive receptor signalling but not when induced by IFN.

Of potential clinical interest is the observation that re-expression of activation markers as a result of an infection with a second, unrelated virus does not occur within a window of 5-9 days after the primary infection, at a time when activation markers are already back to baseline levels. A number of possibilities that may be responsible for such a refractory period in activation marker expression have been studied. Firstly, I investigated the hypothesis that the anti-viral state induced by the interferon response during primary viral infection persisted for an extended time period and thus prevented the second viral infection to establish and induce renewed IFN-I release. This, however, appears unlikely as mice become less resistant, rather than more resistant to lethal vSFV infection when infected during the refractory period induced by an unrelated virus. Secondly, I investigated the possibility that the refractory period was due to the need of lymphocytes to undergo de-programming to revert to a more naïve phenotype and that such deprogramming required 7-8 days to be accomplished. However, when lymphocytes were taken at the height of the refractory period, transferred into naïve animals and tracked during viral infection (and consequent high IFN-I-production), renewed expression of activation markers was not suppressed. Thus, it reasonable to conclude that the refractory period for re-expression of activation markers on lymphocytes is due to the failure of the animals to respond to a secondary infection with an adequate IFN-I production. It is known that immature DC, particularly pDCs,

are the principal cell type responsible for the systemic IFN-I release after a viral infection (Cella et al., 1999), with IFN-I play a major role in their maturation (Montoya et al., 2002). Thus, it is most likely that the refractory period for re-expression of activation markers is due to a lack of DC with capabilities to respond to a new viral infection. Infected DC cells may either have been eliminated by the ongoing innate and adaptive immune response and not yet replaced, or were undergoing differentiation to a non-IFN-I producing phenotype.

The biological significance of this generalized lymphocyte activation is not yet known. One can only assume that an early induction of an anti-viral state, especially on circulating lymphocytes, would reduce their susceptibility to be infected by the virus and thus also reduce dissemination. The consequence of which is an increased susceptibility to a secondary infection during the refractory period. In fact, increased susceptibility to secondary infections, particularly bacterial infections (reviewed in (Beadling and Slifka, 2004)), is a well-known clinical observation and may at least in part be due to the phenomena described here.

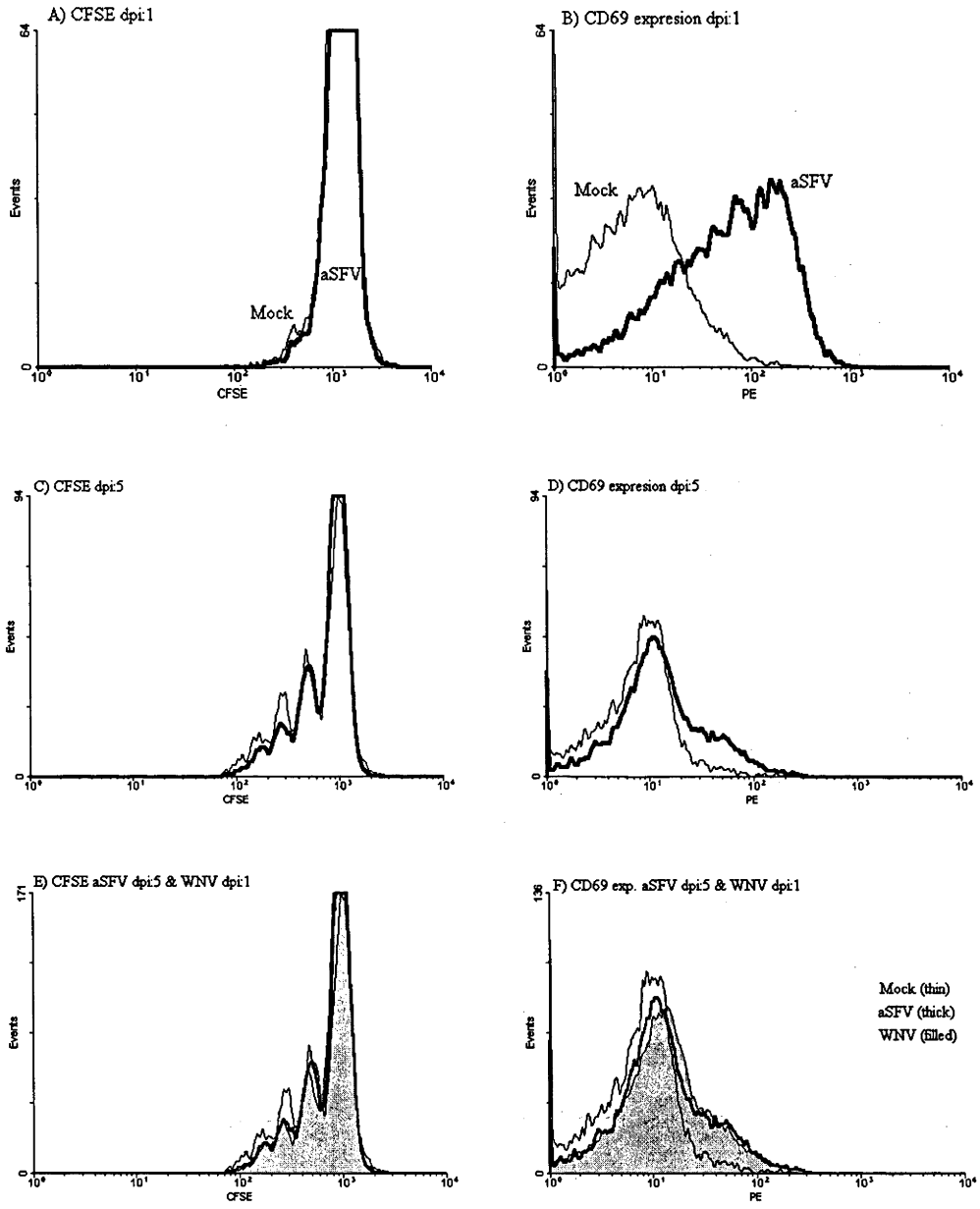

Figure 6.1. Fate of activated splenocytes. CFSE profiles appeared similar in un-infected and aSFV infected mice whether tested at day1 post-infection (dpi:1) (A) and day 5 post-infection (dpi:5) (C), and when WNV administered 4 days after the initial aSFV infection (E). Expression of CD69 induced on CFSE labelled cells following aSFV infection at dpi:1 (B), and return to background level at dpi:5 (D). 2nd infection with WNV did not affect the expression of CD69 (F).

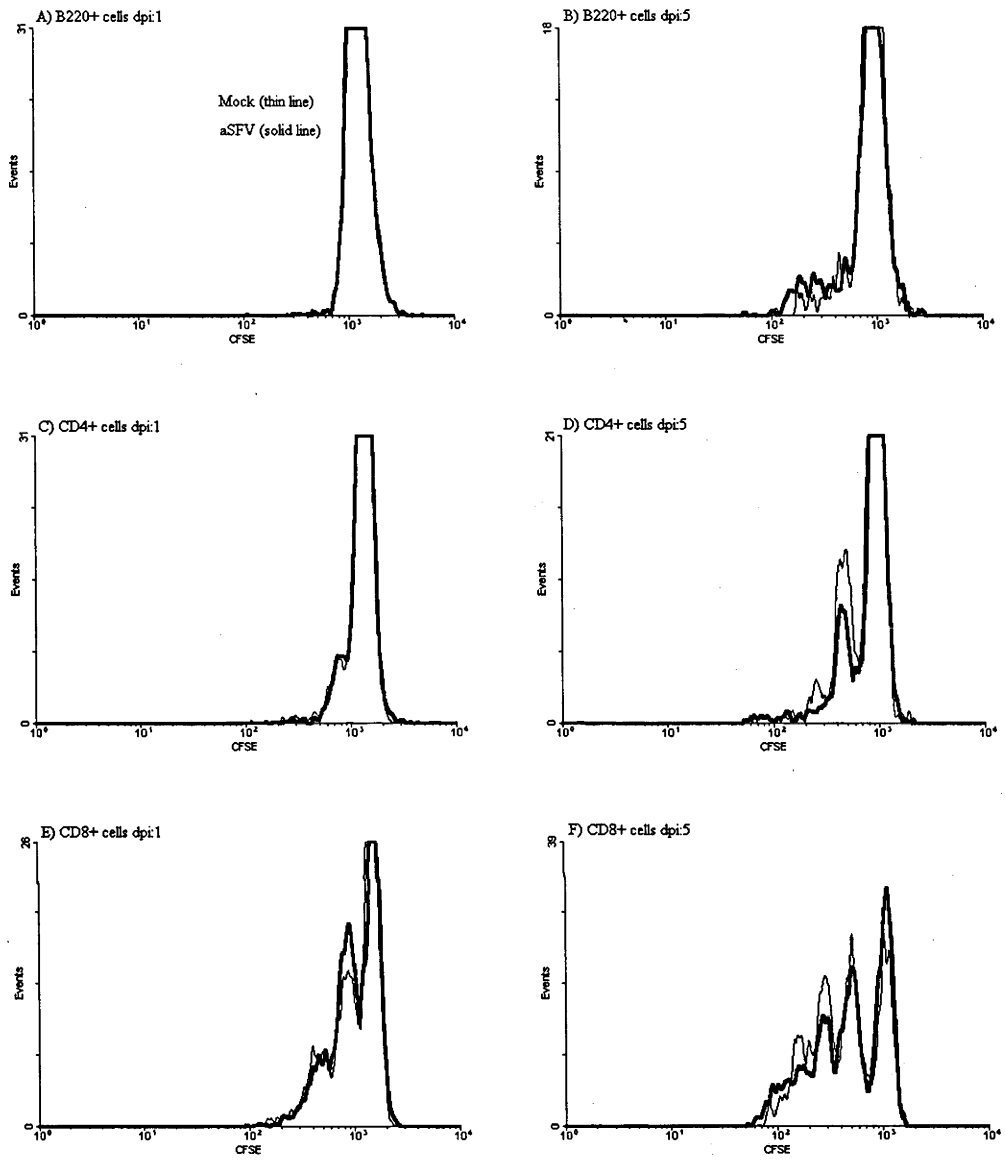

Figure 6.2. CFSE profiles of different cell types in mock and aSFV infected mice following reconstitution. No clear division detected for B cells in both mock and infected mice at day 1 post-infection (dpi:1) (A) and day 5 post-infection (dpi:5) (B), very little $CD4^{+}$T cells divided by day 1 post-infection (C) with very clear one division at day 5 (D), 2 division seen for $CD8^{+}$T cells at day 1 postinfection (dpi:1) (E) and about 4 division at day 5 post-infection (dpi:5) (F).

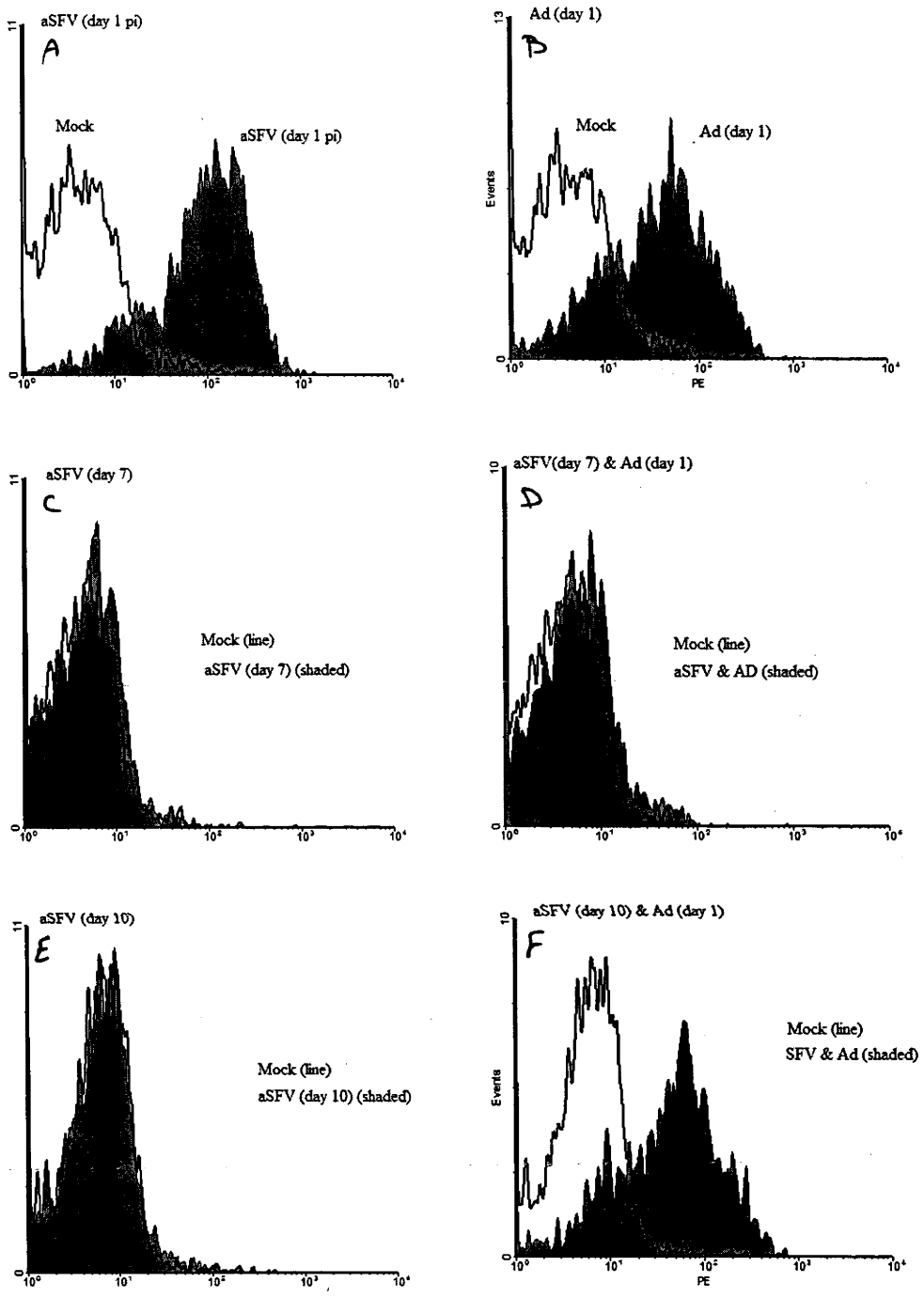

Figure 6.3. Fate of activated splenocytes investigated in B6 mice using adoptive transfer of CFSE labelled cells. CD69 expression on CFSE labelled cells induced after aSFV infection at day 1 post-infection and return to background levels at day 7 post-infection. CD69 expression was not induced by a secondary Ad-2 infection at day 7, but it was induced by day 10. As control, level of CD69 expression induced by Ad-2 infection at day 1 post-infection tested simultaneously at day 7 and 10.

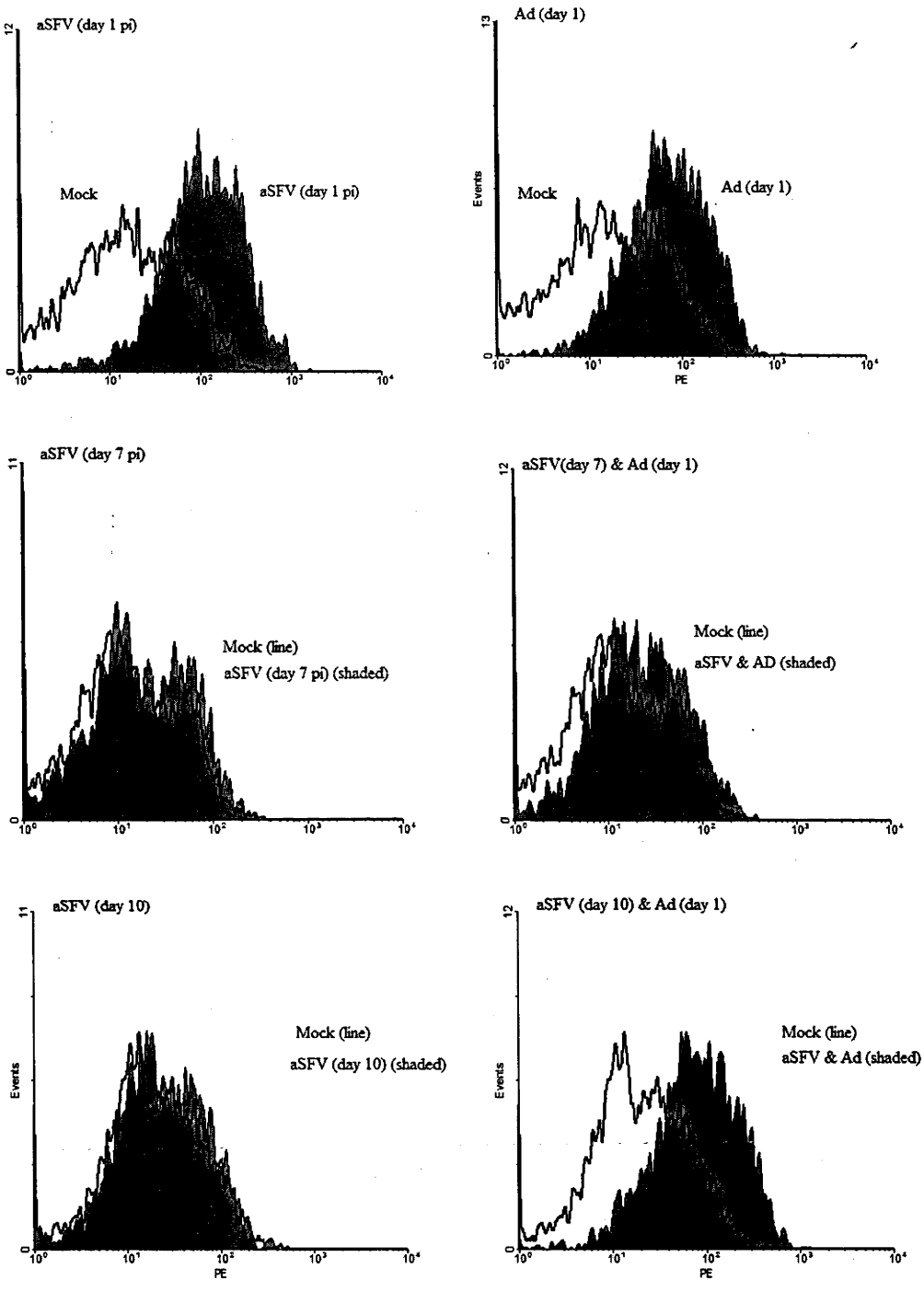

Figure 6.4. Fate of activated splenocytes investigated in B6 mice using adoptive transfer of CFSE-labelled cells. CD86 expression on CFSE-labelled cells induced after aSFV infection at day 1 post-infection and return to background levels at day 7 post-infection. CD86 expression was not induced by a secondary Ad-2 infection at day 7, but it was induced by day 10. As control, level of CD86 expression induced by Ad-2 infection at day 1 post-infection tested simultaneously at day 7 and 10.

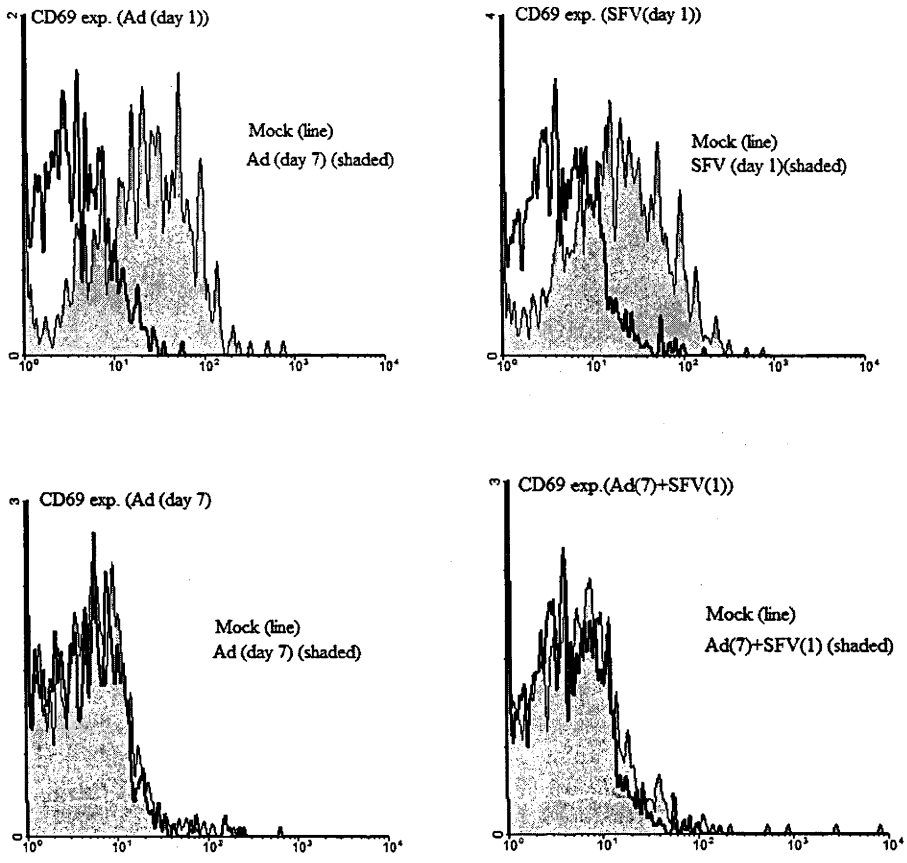

Figure 6.5. Fate of activated splenocytes investigated in B6 mice using adoptive transfer of CFSE labelled cells. CD69 expression on CFSE labelled cells induced after Ad-2 infection at day 1 post-infection and return to background levels at day 7 post-infection. CD69 expression was not induced by a secondary aSFV infection at day 7, despite the ability of aSFV to induce CD69 expression in naïve mice, tested simultaneously.

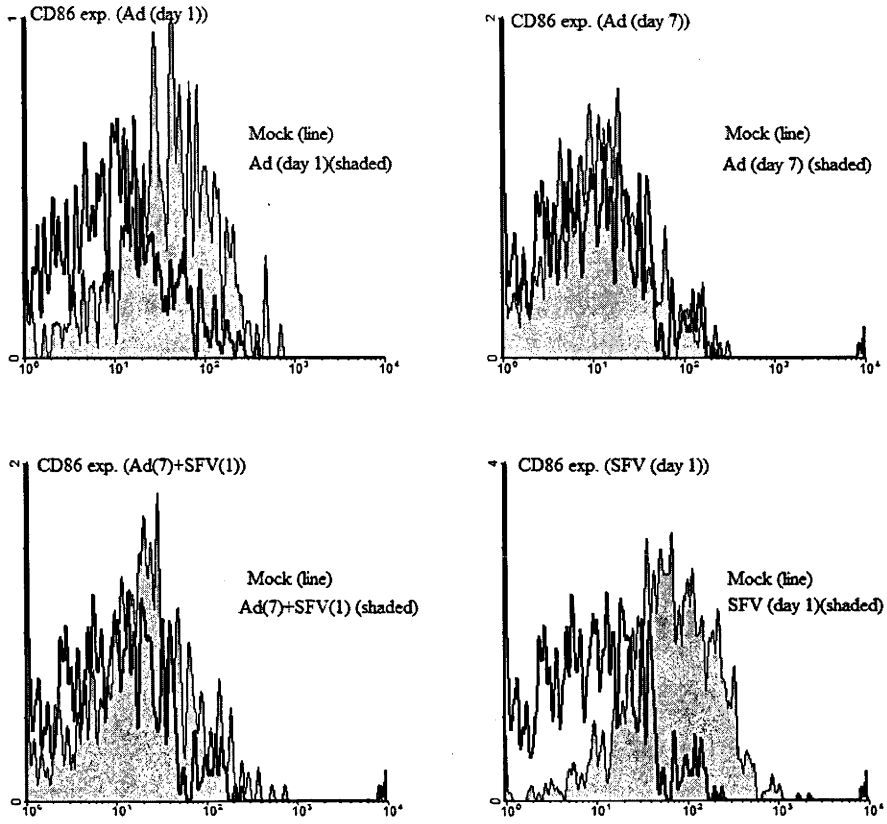

Figure 6.6. Fate of activated splenocytes investigated in B6 mice using adoptive transfer of CFSE labelled cells. CD86 expression on CFSE labelled cells induced after Ad-2 infection at day 1 post-infection and return to background levels at day 7 post-infection. CD86 expression was not induced by a secondary aSFV infection at day 7, despite the ability of aSFV to induce CD86 expression in naïve mice, tested simultaneously.

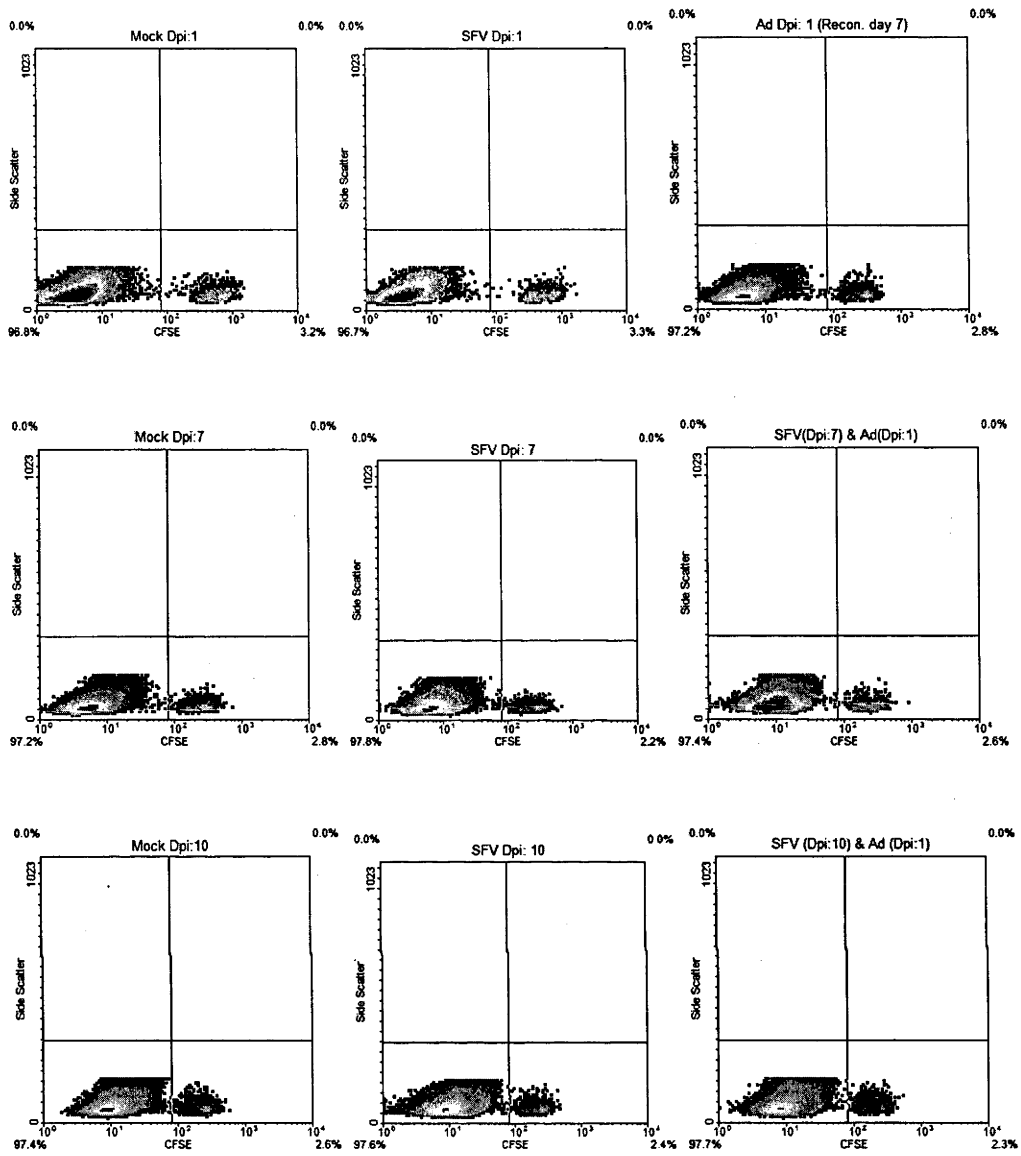

Figure 6.7. Fate of activated splenocytes investigated in B6 mice after adoptive transfer of CFSE-labelled cells. Similar percentages of CFSE labelled cells were detected in mock and infected mice for each tested day indicating no loss of activated lymphocytes from circulation.

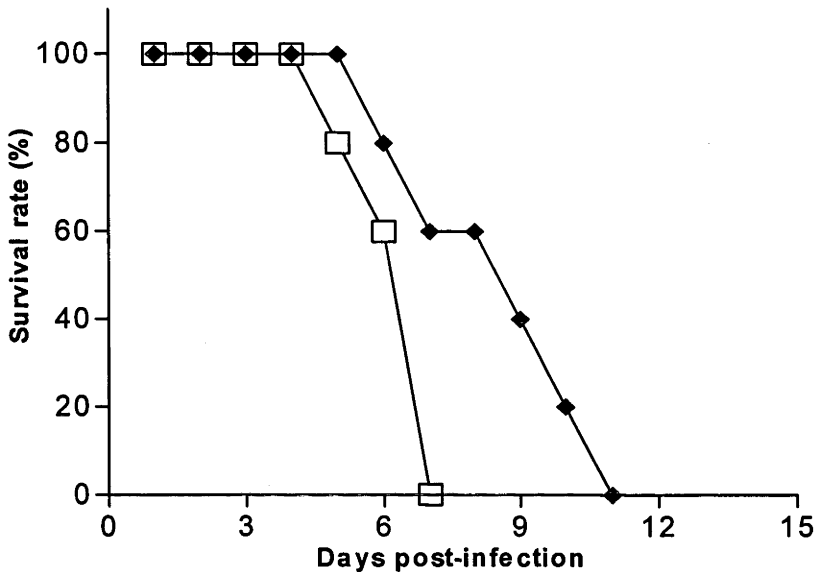

Figure 6.8. Ad-2 infected mice challenged 7 days later with vSFV using 10^3 pfu/mouse. Survival was monitored and compared with naïve mice receiving same dose of vSFV. 5 female 10-week-old B6 mice were used per group.

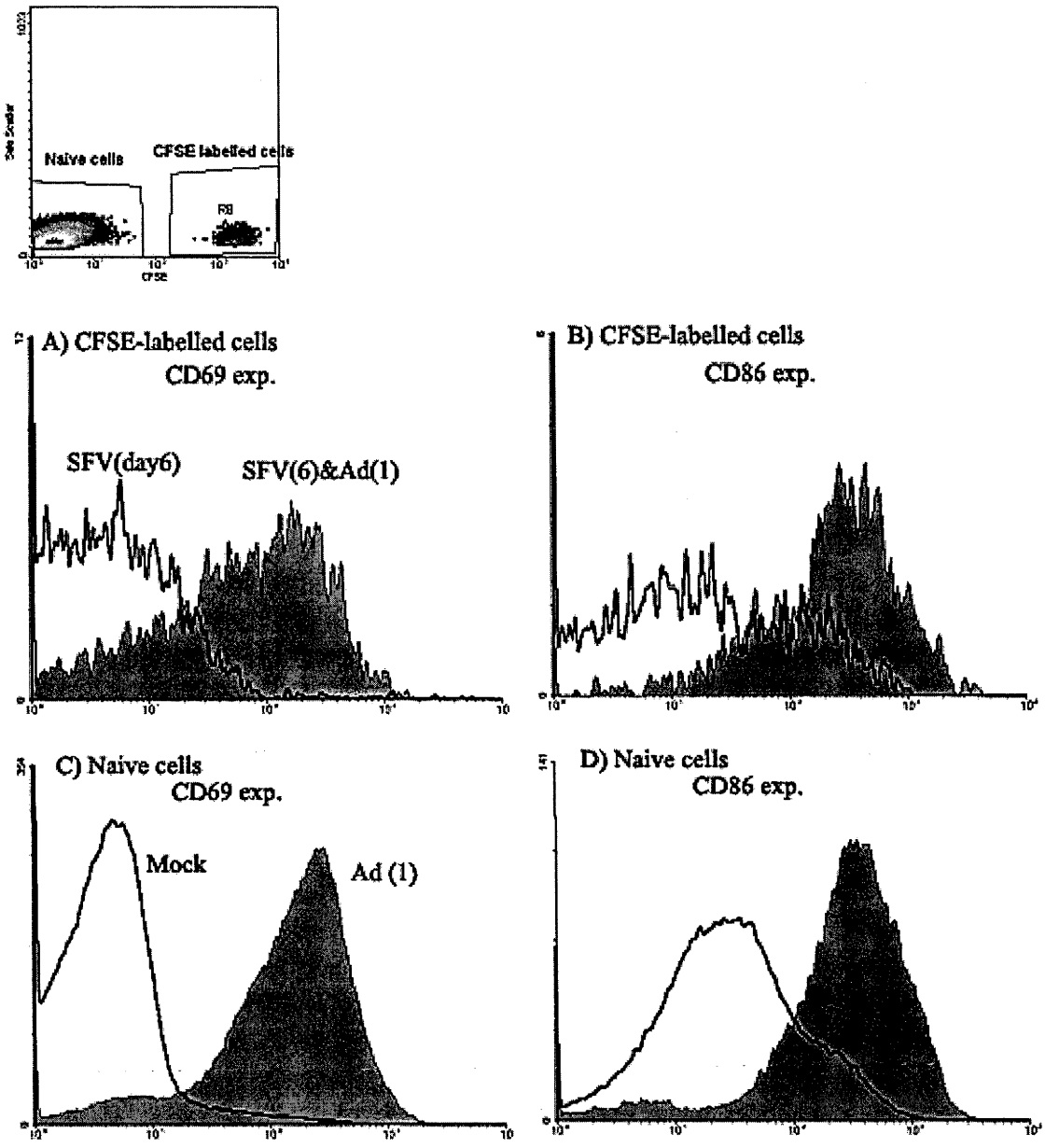

Figure 6.9. Activation markers expressions were assayed on cell surface of naïve and aSFV-primed (6 days), CFSE-labelled, cells. Induced expressions of both CD69 and CD86 on were detected at day 1 post Ad-2 infection on both CFSE-labelled (A & B) and naïve (C & D) cells.

Chapter 7

Concluding remarks

The experiments described in this thesis were aimed to provide a better understanding of the innate and adaptive immune responses during viral infections in general, and the alphavirus SFV infection in particular. SFV strains are classified as either virulent or avirulent. The experiments described in chapter 2 were aimed at comparing infections with vSFV and aSFV in adult mice (10-week-old). The clinical symptoms are similar to that described in literature for SFV infection in young mice. Infection with vSFV is characterised by high virus titre in the brain associated with an early death (MTD about 7 days). In comparison, aSFV infection is asymptomatic with restricted replication in the brain, but aSFV replicates to high titres in spleen which is not seen with vSFV. Replication of aSFV in mature neurons is restricted compared to vSFV, and vSFV brain titres reach lethal threshold before immune response intervention (Atkins et al., 1999). Physiological changes occurring during neuron maturation does contribute to restricting aSFV replication in neurons (reviewed in (Fazakerley, 2002)). Different mouse strains show varying levels of sensitivity to SFV infection, which was attributed to differing induction levels of cytokines, in particular IFN-γ and TNF-α (Mokhtarian et al., 1996). Therefore, lethality of vSFV and aSFV may be influenced by their differential ability to induce cytokines. The ability of vSFV infection, compared to aSFV, to induce high levels IFN-α may be responsible for the reduced vSFV replication in spleen. Lethality as a result of vSFV infection is mainly due to virus replication in the brain. Secretion of IFN-β, preferentially expressed in the CNS over IFN-α (Sandberg et al., 1994), may be protective, whereas IFN-α has marked CNS toxicity (Akwa et al., 1998; McLaurin et al., 1995). Thus, lethality during vSFV infection, which results from neurotropism and high replication levels in the brain, may be aided by restricted induction of IFN-α. Conversely, clearance of aSFV from the brain is unlikely the result of restricted viral replication and adaptive immune response but also IFN-β induction. Therefore, it will

be necessary to investigate differences in IFN-α and β induction in the brain following vSFV and aSFV to obtain a broader appreciation of the immunobiology induced by the two SFV strains.

Previous studies have shown that the aSFV infection-associated demyelination is principally mediated by $CD8^+$T cells in CBA/C3H and BALB/c mice (Fazakerley and Webb, 1987; Subak-Sharpe et al., 1993), and B cell mediated in B6 mice (Smith-Norowitz et al., 2000). Cytotoxic activities of NK and Tc cells are mediated by granule exocytosis and Fas-mediated pathways. Mice defective in cytolytic effector molecules, at the gene level, show increased resistance to lethal vSFV infection compared to wild type B6 mice. Mice defective in Fas-mediated pathway show more resistance than those defective in the exocytosis pathway. These data clearly suggest an immunopathological aspect of lymphocyte-mediated cytotoxicity in SFV-infected B6 mice, with Fas-mediated pathway playing a major role. This finding is supported by the ability of SFV to infect a wide variety of cells especially neurons and oligodendrocytes (Fazakerley, 2002), and the dominant role of Fas-mediated cytotoxicity in neuronal apoptosis (Medana et al., 2001). The absence of Tc cell-mediated cytolytic activity in $\beta2M^{-/-}$ mice has no effect on the outcome of vSFV infection, whereas NK cells depletion results in delayed MTD. In $\beta2M^{-/-}$ mice, absence of MHC-I molecules does not result in elevated NK-mediated cytolytic activity during other viral infection, such as LCMV (Zajac et al., 1995). Since no Tc cell-mediated cytolytic activity is induced after alphavirus infection in B6 mice ((Mullbacher and Blanden, 1978) and chapter 3), data presented here suggest the involvement of NK cell-mediated cytolytic activity in the immunopathology during SFV infection. Non-cytolytic activity of NK cells, however, must play an important role in controlling SFV infection, as vSFV infection

was more lethal in NK cell-depleted mice despite the delayed MTD. IFN-γ is a possible candidate for the non-cytolytic anti-viral effect since mice with defective IFN-γ response show increased sensitivity to lethal vSFV infection compared to their wt counterparts. These data illustrate the requirement for non-cytolytic anti-viral response to control viral infection in tissues where unrepairable damage by cytolytic activity increases the severity of infection. Investigating lethality of vSFV in mice with a combination of defects in cytolytic effector molecules and IFN-γ, such as of $Fas^{-/-}$ x$gzmAB^{-/-}$x$IFN\text{-}\gamma^{-/-}$, could give additional evidence supporting this conclusion. Such mice are expected to have delayed MTD with a high death rate in response to vSFV infection.

Comparing vSFV lethality in B6 and syngeneic knockout mice showed that perfx$gzmAB^{-/-}$, *gld*, $Fas^{-/-}$, Fasx$gzmAB^{-/-}$, and $perf^{-/-}$x*gld* mice were significantly more resistant than wt B6 mice. Tissue virus titres were comparable in B6, perfx$gzmAB^{-/-}$, *gld*, $Fas^{-/-}$, and Fasx$gzmAB^{-/-}$ mice. Lethality of vSFV infection in these mice is associated with high virus titres in the brain. However, when both pathways of cytotoxicity are defective such as in $perf^{-/-}$x*gld* mice, one would expect elevated tissue virus titres. By contrast, $perf^{-/-}$x*gld* mice show restricted viral replication. IFN-γ and TNF-α have been reported to synergise in generating anti-viral activity *in vitro* (Wong and Goeddel, 1986). In addition, early induction of both cytokines was associated with increased resistance of SJL mice to SFV infection compared to B6 mice (Mokhtarian et al., 1996). The detection of elevated mRNAs levels for both cytokines in naïve $perf^{-/-}$xgld mice suggests that these cytokines may be implicated in reduced SFV replication but requires further investigation at the protein levels. In addition, both IFN-γ and TNF-α are associated with macrophage activation, and an expanded population of activated

macrophages has been detected in perf$^{-/-}$xgld mice. Thus, the possible roles of macrophages, IFN-γ and TNF-α in restricting viral replication in perf$^{-/-}$x*gld* mice need to be established. *In vivo* macrophage inactivation has been previously used to address their involvement in autoimmunity (Spielman et al., 1998). A similar approach may be appropriate to address the role of an expanded population of activated macrophages in restricting viral replication in pef$^{-/-}$x*gld* mice.

Viral infections are known to elicit an early induction of IFN-I synthesis, which result in cellular resistant to viral infection, inhibition of viral replication, and impediment of viral dissemination (Sen and Ransohoff, 1993) (van den Broek et al., 1995). In addition, *In vitro* treatment of B cells with IFN-I upregulates the expression of CD69, CD86, and CD25 (Braun et al., 2002). Experiments described in chapter 5 demonstrate that *in vivo* viral infections cause an elevation of cell surface expression of CD69 and CD86, but not CD25, on more than 80% of both B and T cells. This generalised lymphocyte-activation markers expression is systemic, a feature of most viral infections, and mainly mediated by IFN-I. The increased expression of CD69 and CD86 may be a by-products of the anti-viral effects mediated by IFN-I that help decrease viral dissemination. CD86, and CD69 function as costimulatory molecules aiding lymphocytes activation. Thus, it is possible that by inducing the expression of costimulatory molecules, IFN-I may help to reduce the threshold required for lymphocyte activation. Simultaneous administering of inactivated virus and an unrelated live virus, and testing the antibody response to the inactivated virus may test this possibility. The function of CD25, however, is different to that of CD86 and CD69. CD25 is the α chain of the IL-2 receptor, and can be expressed on the surface of activated B and T cells following Ag recognition (Lowenthal et al., 1985). *In vivo* cytokine microenvironment must be much

more tightly controlled compared to *in vitro* systems. Induction of CD25 expression can increase lymphocytes susceptibility to IL-2 mediated proliferation, which will not be beneficial at such massive scale. In this context the lack of CD25 expression may be significant, and "true lymphocyte activation" may also require CD25 upregulation, which may only be triggered by ligation of the clonally expressed antigen receptors.

Generally, during acute viral infection a high level of activated cells was detected at very early days postinfection peaking at day 1 postinfection and returning to baseline levels by day 5 postinfection. As shown in chapter 6, the disappearance of activation marker-positive cells is not due to cell turnover via apoptosis but rather down-regulation of cell surface expression. Re-expression of activation markers as a result of an infection with a second, unrelated, virus does not occur within a window of 5-9 days after the primary infection. There are a number of possibilities which could be responsible for this refractory period required for marker re-expression. Firstly it could be due to a lasting anti-viral state by the initial IFN-I, secondly unresponsiveness of lymphocytes, and thirdly an impairment of IFN-I induction. The anti-viral state mediated by IFN-I, induced by the primary infection, did not protect the animals from a lethal secondary viral infection within the refractory period making it unlikely that persistence of anti-viral state prevents a secondary infection. In addition, lymphocytes are responsive to a second IFN exposure when adoptively transferred to a new host. Therefore, it is reasonable to conclude that the refractory period for re-expression of activation markers on lymphocytes is due to the animals' failure to respond to a secondary infection with an adequate IFN-I production. This conclusion highlights the importance to study immature DC, the principal IFN-I producer after a viral infection (Cella et al., 1999), in

terms of fate, IFN-I secretion capabilities, and replacement following primary acute virus infection.

The biological significance of this phenomenon is not yet clear. What is the benefit to the host to have the majority of its lymphocytes in a partially activated state irrespective of their antigen reactivity remains to be investigated. However, the phenomenon of massive lymphocyte activation following acute viral infection accompanied by a refractory period may be of potential clinical interest since it can be associated with an increased susceptibility to a secondary infection, particularly bacterial infections. One example is the striking association between influenza and respiratory syncytial virus (RSV) infections and the subsequent increased cases of sepsis (reviewed in (Beadling and Slifka, 2004)). Sepsis is mainly due to secondary bacterial infection and can count for more than 215000 deaths per year in the USA. In fact, increased susceptibilities to Staphylococcal enterotoxin B and LPS have been reported, in mice, at day 7-8 following influenza or LCMV infection (Nansen et al., 1997; Nguyen and Biron, 1999; Zhang et al., 1996). These studies argue that increased expression of TNF, IL-2, and IFN-γ is responsible for the increased severity of the bacterial infection. IFN-I enhances Th1- type responses by inhibiting the secretion of Th2-type cytokines (IL-4 and IL-5) but promoting Th1-type cytokine production (IFNγ) in $CD4^+$T cells (Brinkmann et al., 1993; Demeure et al., 1994). Thus, prophylactic antibiotic treatment during viral infection may avert such scenarios.

References

Abendroth A., Slobedman B., Lee E., Mellins E., Wallace M. and Arvin A. M. (2000) Modulation of major histocompatibility class II protein expression by varicella-zoster virus. *J Virol* **74**, 1900-7.

Adachi M., Suematsu S., Kondo T., Ogasawara J., Tanaka T., Yoshida N. and Nagata S. (1995) Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nat Genet* **11**, 294-300.

Adema G. J., Hartgers F., Verstraten R., de Vries E., Marland G., Menon S., Foster J., Xu Y., Nooyen P., McClanahan T., Bacon K. B. and Figdor C. G. (1997) A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* **387**, 713-7.

Aderem A. and Underhill D. M. (1999) Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* **17**, 593-623.

Airoldi I., Gri G., Marshall J. D., Corcione A., Facchetti P., Guglielmino R., Trinchieri G. and Pistoia V. (2000) Expression and function of IL-12 and IL-18 receptors on human tonsillar B cells. *J Immunol* **165**, 6880-8.

Akwa Y., Hassett D. E., Eloranta M. L., Sandberg K., Masliah E., Powell H., Whitton J. L., Bloom F. E. and Campbell I. L. (1998) Transgenic expression of IFN-alpha in the central nervous system of mice protects against lethal neurotropic viral infection but induces inflammation and neurodegeneration. *J Immunol* **161**, 5016-26.

Albert M. L., Sauter B. and Bhardwaj N. (1998) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86-9.

Alderson M. R., Tough T. W., Davis-Smith T., Braddy S., Falk B., Schooley K. A., Goodwin R. G., Smith C. A., Ramsdell F. and Lynch D. H. (1995) Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med* **181**, 71-7.

Amor S., Scallan M. F., Morris M. M., Dyson H. and Fazakerley J. K. (1996) Role of immune responses in protection and pathogenesis during Semliki Forest virus encephalitis. *J Gen Virol* **77 (Pt 2)**, 281-91.

Andjelic S., Drappa J., Lacy E., Elkon K. B. and Nikolic-Zugic J. (1994) The onset of Fas expression parallels the acquisition of CD8 and CD4 in fetal and adult alpha beta thymocytes. *Int Immunol* **6**, 73-9.

Androlewicz M. J., Anderson K. S. and Cresswell P. (1993) Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner. *Proc Natl Acad Sci U S A* **90**, 9130-4.

Anel A., Buferne M., Boyer C., Schmitt-Verhulst A. M. and Golstein P. (1994) T cell receptor-induced Fas ligand expression in cytotoxic T lymphocyte clones is blocked by protein tyrosine kinase inhibitors and cyclosporin A. *Eur J Immunol* **24**, 2469-76.

Anel A., Simon A. K., Auphan N., Buferne M., Boyer C., Golstein P. and Schmitt-Verhulst A. M. (1995) Two signaling pathways can lead to Fas ligand expression in CD8+ cytotoxic T lymphocyte clones. *Eur J Immunol* **25**, 3381-7.

Antoniou A. N., Powis S. J. and Elliott T. (2003) Assembly and export of MHC class I peptide ligands. *Curr Opin Immunol* **15**, 75-81.

Arce-Gomez B., Jones E. A., Barnstable C. J., Solomon E. and Bodmer W. F. (1978) The genetic control of HLA-A and B antigens in somatic cell hybrids: requirement for beta2 microglobulin. *Tissue Antigens* **11**, 96-112.

Ashton-Rickardt P. G. and Tonegawa S. (1994) A differential-avidity model for T-cell selection. *Immunol Today* **15**, 362-6.

Asselin-Paturel C., Boonstra A., Dalod M., Durand I., Yessaad N., Dezutter-Dambuyant C., Vicari A., O'Garra A., Biron C., Briere F. and Trinchieri G. (2001) Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* **2**, 1144-50.

Atkins G. J. and Sheahan B. J. (1982) Semliki forest virus neurovirulence mutants have altered cytopathogenicity for central nervous system cells. *Infect Immun* **36**, 333-41.

Atkins G. J., Sheahan B. J. and Dimmock N. J. (1985) Semliki Forest virus infection of mice: a model for genetic and molecular analysis of viral pathogenicity. *J Gen Virol* **66 (Pt 3)**, 395-408.

Atkins G. J., Sheahan B. J. and Liljestrom P. (1999) The molecular pathogenesis of Semliki Forest virus: a model virus made useful? *J Gen Virol* **80 (Pt 9)**, 2287-97.

Atkinson T., Barrett A. D., Mackenzie A. and Dimmock N. J. (1986) Persistence of virulent Semliki Forest virus in mouse brain following co-inoculation with defective interfering particles. *J Gen Virol* **67 (Pt 6)**, 1189-94.

Bakke O. and Dobberstein B. (1990) MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell* **63**, 707-16.

Balkow S., Kersten A., Tran T. T., Stehle T., Grosse P., Museteanu C., Utermohlen O., Pircher H., von Weizsacker F., Wallich R., Mullbacher A. and Simon M. M. (2001) Concerted action of the FasL/Fas and perforin/granzyme A and B pathways is mandatory for the development of early viral hepatitis but not for recovery from viral infection. *J Virol* **75**, 8781-91.

Balluz I. M., Glasgow G. M., Killen H. M., Mabruk M. J., Sheahan B. J. and Atkins G. J. (1993) Virulent and avirulent strains of Semliki Forest virus show similar cell tropism for the murine central nervous system but differ in the severity and rate of induction of cytolytic damage. *Neuropathol Appl Neurobiol* **19**, 233-9.

Banchereau J. and Steinman R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392**, 245-52.

Barrett A. D., Cubitt W. D. and Dimmock N. J. (1984a) Defective interfering particles of Semliki Forest virus are smaller than particles of standard virus. *J Gen Virol* **65 (Pt 12)**, 2265-8.

Barrett A. D., Guest A. R., Mackenzie A. and Dimmock N. J. (1984b) Protection of mice infected with a lethal dose of Semliki Forest virus by defective interfering virus: modulation of virus multiplication. *J Gen Virol* **65 (Pt 11)**, 1909-20.

Battegay M., Bachmann M. F., Burhkart C., Viville S., Benoist C., Mathis D., Hengartner H. and Zinkernagel R. M. (1996) Antiviral immune responses of mice lacking MHC class II or its associated invariant chain. *Cell Immunol* **167**, 115-21.

Battistini A., Affabris E., Fiorucci G., Coccia E. M., Romeo G., Marziali G. and Rossi G. B. (1990) Spectrum of biological activity of interferons. *Ann Ist Super Sanita* **26**, 227-53.

Beadling C. and Slifka M. K. (2004) How do viral infections predispose patients to bacterial infections? *Curr Opin Infect Dis* **17**, 185-91.

Belardelli F. and Gresser I. (1996) The neglected role of type I interferon in the T-cell response: implications for its clinical use. *Immunol Today* **17**, 369-72.

Beresford P. J., Xia Z., Greenberg A. H. and Lieberman J. (1999) Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity* **10**, 585-94.

Beresford P. J., Zhang D., Oh D. Y., Fan Z., Greer E. L., Russo M. L., Jaju M. and Lieberman J. (2001) Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J Biol Chem* **276**, 43285-93.

Berke G. (1995) The CTL's kiss of death. *Cell* **81**, 9-12.

Bevan M. J. (1976a) Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* **143**, 1283-8.

Bevan M. J. (1976b) Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. *J Immunol* **117**, 2233-8.

Bevan M. J. (2004) Helping the CD8(+) T-cell response. *Nat Rev Immunol* **4**, 595-602.

Bhardwaj N., Friedman S. M., Cole B. C. and Nisanian A. J. (1992) Dendritic cells are potent antigen-presenting cells for microbial superantigens. *J Exp Med* **175**, 267-73.

Bhardwaj N., Young J. W., Nisanian A. J., Baggers J. and Steinman R. M. (1993) Small amounts of superantigen, when presented on dendritic cells, are sufficient to initiate T cell responses. *J Exp Med* **178**, 633-42.

Biron C. A. (1998) Role of early cytokines, including alpha and beta interferons (IFN-alpha/beta), in innate and adaptive immune responses to viral infections. *Semin Immunol* **10**, 383-90.

Biron C. A. (1999) Initial and innate responses to viral infections--pattern setting in immunity or disease. *Curr Opin Microbiol* **2**, 374-81.

Biron C. A. and Brossay L. (2001) NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol* **13**, 458-64.

Biron C. A., Nguyen K. B., Pien G. C., Cousens L. P. and Salazar-Mather T. P. (1999) Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* **17**, 189-220.

Bjorkman P. J., Saper M. A., Samraoui B., Bennett W. S., Strominger J. L. and Wiley D. C. (1987a) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* **329**, 512-8.

Bjorkman P. J., Saper M. A., Samraoui B., Bennett W. S., Strominger J. L. and Wiley D. C. (1987b) Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**, 506-12.

Blanden R. V. (1974) T cell response to viral and bacterial infection. *Transplant Rev* **19**, 56-88.

Blanden R. V. (1982) Role of macrophages in elimination of viral infection. In *Self-defence mechanisms: role of macrophages*. (Mizune D. et al. ed.). Tokyo: University of Tokyo press, *pp*. 269-278.

Boehm U., Klamp T., Groot M. and Howard J. C. (1997) Cellular responses to interferon-gamma. *Annu Rev Immunol* **15**, 749-95.

Boise L. H., Minn A. J., Noel P. J., June C. H., Accavitti M. A., Lindsten T. and Thompson C. B. (1995) CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* **3**, 87-98.

Bonatti S., Migliaccio G. and Simons K. (1989) Palmitylation of viral membrane glycoproteins takes place after exit from the endoplasmic reticulum. *J Biol Chem* **264**, 12590-5.

Bradish C. J., Allner K. and Maber H. B. (1971) The virulence of original and derived strains of Semliki forest virus for mice, guinea-pigs and rabbits. *J Gen Virol* **12**, 141-60.

Braun D., Caramalho I. and Demengeot J. (2002) IFN-alpha/beta enhances BCR-dependent B cell responses. *Int Immunol* **14**, 411-9.

Brennan J., Mahon G., Mager D. L., Jefferies W. A. and Takei F. (1996) Recognition of class I major histocompatibility complex molecules by Ly-49: specificities and domain interactions. *J Exp Med* **183**, 1553-9.

Bretscher P. and Cohn M. (1970) A theory of self-nonself discrimination. *Science* **169**, 1042-9.

Brinkmann V., Geiger T., Alkan S. and Heusser C. H. (1993) Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. *J Exp Med* **178**, 1655-63.

Bruton C. J. and Kennedy S. I. (1976) Defective-interfering particles of Semliki Forest Virus: structural differences between standard virus and defective-interfering particles. *J Gen Virol* **31**, 383-95.

Bukowski J. F., Woda B. A., Habu S., Okumura K. and Welsh R. M. (1983) Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. *J Immunol* **131**, 1531-8.

Butt A. M., Tutton M. G., Kirvell S. L., Amor S. and Jenkins H. G. (1996) Morphology of oligodendrocytes during demyelination in optic nerves of mice infected with Semliki Forest virus. *Neuropathol Appl Neurobiol* **22**, 540-7.

Butz E. A. and Bevan M. J. (1998) Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* **8**, 167-75.

Call M. E. and Wucherpfennig K. W. (2004) The T Cell Receptor: Critical Role of the Membrane Environment in Receptor Assembly and Function. *Annu Rev Immunol.*

Callan M. F., Fazou C., Yang H., Rostron T., Poon K., Hatton C. and McMichael A. J. (2000) CD8(+) T-cell selection, function, and death in the primary immune response in vivo. *J Clin Invest* **106**, 1251-61.

Cantin E., Tanamachi B. and Openshaw H. (1999) Role for gamma interferon in control of herpes simplex virus type 1 reactivation. *J Virol* **73**, 3418-23.

Carbone F. R. and Heath W. R. (2003) The role of dendritic cell subsets in immunity to viruses. *Curr Opin Immunol* **15**, 416-20.

Carr J. A., Rogerson J., Mulqueen M. J., Roberts N. A. and Booth R. F. (1997) Interleukin-12 exhibits potent antiviral activity in experimental herpesvirus infections. *J Virol* **71**, 7799-803.

Carter L. L. and Dutton R. W. (1995) Relative perforin- and Fas-mediated lysis in T1 and T2 CD8 effector populations. *J Immunol* **155**, 1028-31.

Caux C., Vanbervliet B., Massacrier C., Azuma M., Okumura K., Lanier L. L. and Banchereau J. (1994) B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J Exp Med* **180**, 1841-7.

Cavanaugh V. J., Guidotti L. G. and Chisari F. V. (1997) Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. *J Virol* **71**, 3236-43.

Cella M., Engering A., Pinet V., Pieters J. and Lanzavecchia A. (1997a) Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388**, 782-7.

Cella M., Jarrossay D., Facchetti F., Alebardi O., Nakajima H., Lanzavecchia A. and Colonna M. (1999a) Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* **5**, 919-23.

Cella M., Salio M., Sakakibara Y., Langen H., Julkunen I. and Lanzavecchia A. (1999b) Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* **189**, 821-9.

Cella M., Sallusto F. and Lanzavecchia A. (1997b) Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol* **9**, 10-6.

Cerwenka A. and Lanier L. L. (2001a) Ligands for natural killer cell receptors: redundancy or specificity. *Immunol Rev* **181**, 158-69.

Cerwenka A. and Lanier L. L. (2001b) Natural killer cells, viruses and cancer. *Nat Rev Immunol* **1**, 41-9.

Chang E., Galle L., Maggs D., Estes D. M. and Mitchell W. J. (2000) Pathogenesis of herpes simplex virus type 1-induced corneal inflammation in perforin-deficient mice. *J Virol* **74**, 11832-40.

Chew-Lim M., Suckling A. J. and Webb H. E. (1977) Demyelination in mice after two or three infections with avirulent Semliki Forest virus. *Vet Pathol* **14**, 62-72.

Chu J. L., Drappa J., Parnassa A. and Elkon K. B. (1993) The defect in Fas mRNA expression in MRL/lpr mice is associated with insertion of the retrotransposon, ETn. *J Exp Med* **178**, 723-30.

Cohen P. L. and Eisenberg R. A. (1991) Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu Rev Immunol* **9**, 243-69.

Colamonici O. R., Pfeffer L. M., D'Alessandro F., Platanias L. C., Gregory S. A., Rosolen A., Nordan R., Cruciani R. A. and Diaz M. O. (1992) Multichain structure of the IFN-alpha receptor on hematopoietic cells. *J Immunol* **148**, 2126-32.

Constant S. L. and Bottomly K. (1997) Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol* **15**, 297-322.

Cresswell P., Androlewicz M. J. and Ortmann B. (1994) Assembly and transport of class I MHC-peptide complexes. *Ciba Found Symp* **187**, 150-62; discussion 162-9.

Croft M., Duncan D. D. and Swain S. L. (1992) Response of naive antigen-specific CD4+ T cells in vitro: characteristics and antigen-presenting cell requirements. *J Exp Med* **176**, 1431-7.

Cumberbatch M. and Kimber I. (1992) Dermal tumour necrosis factor-alpha induces dendritic cell migration to draining lymph nodes, and possibly provides one stimulus for Langerhans' cell migration. *Immunology* **75**, 257-63.

Daniels M. A., Schober S. L., Hogquist K. A. and Jameson S. C. (1999) Cutting edge: a test of the dominant negative signal model for TCR antagonism. *J Immunol* **162**, 3761-4.

Darnell J. E., Jr. (1997) STATs and gene regulation. *Science* **277**, 1630-5.

Darnell J. E., Jr., Kerr I. M. and Stark G. R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415-21.

de Curtis I. and Simons K. (1988) Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. *Proc Natl Acad Sci U S A* **85**, 8052-6.

De Maeyer E. and De Maeyer-Guignard J. (1982) Immuno-modulating properties of interferons. *Philos Trans R Soc Lond B Biol Sci* **299**, 77-90.

De Maeyer E. and De Maeyer-Guignard J. (1998) Type I interferons. *Int Rev Immunol* **17**, 53-73.

de Wolf-Peeters C. and Achten R. (2000) gammadelta T-cell lymphomas: a homogeneous entity? *Histopathology* **36**, 294-305.

Debatin K. M., Suss D. and Krammer P. H. (1994) Differential expression of APO-1 on human thymocytes: implications for negative selection? *Eur J Immunol* **24**, 753-8.

Del Prete G. (1998) The concept of type-1 and type-2 helper T cells and their cytokines in humans. *Int Rev Immunol* **16**, 427-55.

Delves P. J. and Roitt I. M. (2000) The immune system. First of two parts. *N Engl J Med* **343**, 37-49.

DeMaeyer E. and DeMaeyer-Guignard J. (1998) Interferons. In *The Cytokine Handbook (3rd edition)*. (Thompson A.W. ed.). Sydney. Academic Press. *pp*.491-561.

Demeure C. E., Wu C. Y., Shu U., Schneider P. V., Heusser C., Yssel H. and Delespesse G. (1994) In vitro maturation of human neonatal CD4 T lymphocytes. II. Cytokines present at priming modulate the development of lymphokine production. *J Immunol* **152**, 4775-82.

den Haan J. M., Lehar S. M. and Bevan M. J. (2000) CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* **192**, 1685-96.

Deonarain R., Alcami A., Alexiou M., Dallman M. J., Gewert D. R. and Porter A. C. (2000) Impaired antiviral response and alpha/beta interferon induction in mice lacking beta interferon. *J Virol* **74**, 3404-9.

Depraetere V. and Golstein P. (1997) Fas and other cell death signaling pathways. *Semin Immunol* **9**, 93-107.

Dhein J., Walczak H., Baumler C., Debatin K. M. and Krammer P. H. (1995) Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature* **373**, 438-41.

Diebold S. S., Montoya M., Unger H., Alexopoulou L., Roy P., Haswell L. E., Al-Shamkhani A., Flavell R., Borrow P. and Reis e Sousa C. (2003) Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* **424**, 324-8.

Dimmock N. J. and Kennedy S. I. (1978) Prevention of death in Semliki Forest virus-infected mice by administration of defective-interfering Semliki Forest virus. *J Gen Virol* **39**, 231-42.

Dinarello C. A. (1999) Cytokines as endogenous pyrogens. *J Infect Dis* **179 Suppl 2**, S294-304.

Dixon J. E., Allan J. E. and Doherty P. C. (1987) The acute inflammatory process in murine lymphocytic choriomeningitis is dependent on Lyt-2+ immune T cells. *Cell Immunol* **107**, 8-14.

Doherty P. C. (1973) Quantitative studies of the inflammatory process in fatal viral meningoencephalitis. *Am J Pathol* **73**, 607-22.

Doherty P. C., Allan J. E., Lynch F. and Ceredig R. (1990) Dissection of an inflammatory process induced by CD8+ T cells. *Immunol Today* **11**, 55-9.

Doherty P. C. and Christensen J. P. (2000) Accessing complexity: the dynamics of virus-specific T cell responses. *Annu Rev Immunol* **18**, 561-92.

Doherty P. C. and Zinkernagel R. M. (1974) T-cell-mediated immunopathology in viral infections. *Transplant Rev* **19**, 89-120.

Dondi E., Rogge L., Lutfalla G., Uze G. and Pellegrini S. (2003) Down-modulation of responses to type I IFN upon T cell activation. *J Immunol* **170**, 749-56.

Drappa J., Brot N. and Elkon K. B. (1993) The Fas protein is expressed at high levels on CD4+CD8+ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRL lpr/lpr. *Proc Natl Acad Sci U S A* **90**, 10340-4.

Ebnet K., Hausmann M., Lehmann-Grube F., Mullbacher A., Kopf M., Lamers M. and Simon M. M. (1995) Granzyme A-deficient mice retain potent cell-mediated cytotoxicity. *Embo J* **14**, 4230-9.

Eichelberger M., Allan W., Zijlstra M., Jaenisch R. and Doherty P. C. (1991) Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *J Exp Med* **174**, 875-80.

Elkon K. B., Liu C. C., Gall J. G., Trevejo J., Marino M. W., Abrahamsen K. A., Song X., Zhou J. L., Old L. J., Crystal R. G. and Falck-Pedersen E. (1997) Tumor necrosis factor alpha plays a central role in immune-mediated clearance of adenoviral vectors. *Proc Natl Acad Sci U S A* **94**, 9814-9.

Eloranta M. L., Sandberg K., Ricciardi-Castagnoli P., Lindahl M. and Alm G. V. (1997) Production of interferon-alpha/beta by murine dendritic cell lines stimulated by virus and bacteria. *Scand J Immunol* **46**, 235-41.

Estes D. M., Tuo W., Brown W. C. and Goin J. (1998) Effects of type I/type II interferons and transforming growth factor-beta on B-cell differentiation and proliferation. Definition of costimulation and cytokine requirements for immunoglobulin synthesis and expression. *Immunology* **95**, 604-11.

Evans S. S. and Ozer H. (1987) Enhancement of a human antibody response in vitro mediated by interaction of interferon-alpha with T lymphocytes. *J Immunol* **138**, 2451-6.

Fan Z., Beresford P. J., Oh D. Y., Zhang D. and Lieberman J. (2003a) Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell* **112**, 659-72.

Fan Z., Beresford P. J., Zhang D. and Lieberman J. (2002) HMG2 interacts with the nucleosome assembly protein SET and is a target of the cytotoxic T-lymphocyte protease granzyme A. *Mol Cell Biol* **22**, 2810-20.

Fan Z., Beresford P. J., Zhang D., Xu Z., Novina C. D., Yoshida A., Pommier Y. and Lieberman J. (2003b) Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. *Nat Immunol* **4**, 145-53.

Fazakerley J. K. (2002) Pathogenesis of Semliki Forest virus encephalitis. *J Neurovirol* **8 Suppl 2**, 66-74.

Fazakerley J. K., Pathak S., Scallan M., Amor S. and Dyson H. (1993) Replication of the A7(74) strain of Semliki Forest virus is restricted in neurons. *Virology* **195**, 627-37.

Fazakerley J. K. and Webb H. E. (1987a) Semliki Forest virus induced, immune mediated demyelination: the effect of irradiation. *Br J Exp Pathol* **68**, 101-13.

Fazakerley J. K. and Webb H. E. (1987b) Semliki Forest virus-induced, immune-mediated demyelination: adoptive transfer studies and viral persistence in nude mice. *J Gen Virol* **68 (Pt 2)**, 377-85.

Fernandes-Alnemri T., Armstrong R. C., Krebs J., Srinivasula S. M., Wang L., Bullrich F., Fritz L. C., Trapani J. A., Tomaselli K. J., Litwack G. and Alnemri E. S. (1996) In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc Natl Acad Sci U S A* **93**, 7464-9.

Fleming P. (1977) Age-dependent and strain-related differences of virulence of Semliki Forest virus in mice. *J Gen Virol* **37**, 93-105.

Foster G. R. (1997) Interferons in host defense. *Semin Liver Dis* **17**, 287-95.

Foster G. R. and Finter N. B. (1998) Are all type I human interferons equivalent? *J Viral Hepat* **5**, 143-52.

Fraser I. P., Koziel H. and Ezekowitz R. A. (1998) The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. *Semin Immunol* **10**, 363-72.

Frolova E., Frolov I. and Schlesinger S. (1997) Packaging signals in alphaviruses. *J Virol* **71**, 248-58.

Fujioka N., Akazawa R., Ohashi K., Fujii M., Ikeda M. and Kurimoto M. (1999) Interleukin-18 protects mice against acute herpes simplex virus type 1 infection. *J Virol* **73**, 2401-9.

Gaedigk-Nitschko K. and Schlesinger M. J. (1991) Site-directed mutations in Sindbis virus E2 glycoprotein's cytoplasmic domain and the 6K protein lead to similar defects in virus assembly and budding. *Virology* **183**, 206-14.

Gallucci S., Lolkema M. and Matzinger P. (1999) Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* **5**, 1249-55.

Gao G. F., Tormo J., Gerth U. C., Wyer J. R., McMichael A. J., Stuart D. I., Bell J. I., Jones E. Y. and Jakobsen B. K. (1997) Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2. *Nature* **387**, 630-4.

Garoff H., Huylebroeck D., Robinson A., Tillman U. and Liljestrom P. (1990) The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. *J Cell Biol* **111**, 867-76.

Garoff H., Wilschut J., Liljestrom P., Wahlberg J. M., Bron R., Suomalainen M., Smyth J., Salminen A., Barth B. U., Zhao H. and et al. (1994) Assembly and entry mechanisms of Semliki Forest virus. *Arch Virol Suppl* **9**, 329-38.

Gates M. C., Sheahan B. J. and Atkins G. J. (1984) The pathogenicity of the M9 mutant of Semliki Forest virus in immune-compromised mice. *J Gen Virol* **65 (Pt 1)**, 73-80.

Gates M. C., Sheahan B. J., O'Sullivan M. A. and Atkins G. J. (1985) The pathogenicity of the A7, M9 and L10 strains of Semliki Forest virus for weanling mice and primary mouse brain cell cultures. *J Gen Virol* **66 (Pt 11)**, 2365-73.

Gebhard J. R., Perry C. M., Harkins S., Lane T., Mena I., Asensio V. C., Campbell I. L. and Whitton J. L. (1998) Coxsackievirus B3-induced myocarditis: perforin exacerbates disease, but plays no detectable role in virus clearance. *Am J Pathol* **153**, 417-28.

Germain R. N. and Stefanova I. (1999) The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu Rev Immunol* **17**, 467-522.

Gibbons D. L., Erk I., Reilly B., Navaza J., Kielian M., Rey F. A. and Lepault J. (2003) Visualization of the target-membrane-inserted fusion protein of Semliki Forest virus by combined electron microscopy and crystallography. *Cell* **114**, 573-83.

Gibbons D. L. and Kielian M. (2002) Molecular dissection of the Semliki Forest virus homotrimer reveals two functionally distinct regions of the fusion protein. *J Virol* **76**, 1194-205.

Glasgow G. M., Killen H. M., Liljestrom P., Sheahan B. J. and Atkins G. J. (1994) A single amino acid change in the E2 spike protein of a virulent strain of Semliki Forest virus attenuates pathogenicity. *J Gen Virol* **75 (Pt 3)**, 663-8.

Glasgow G. M., Sheahan B. J., Atkins G. J., Wahlberg J. M., Salminen A. and Liljestrom P. (1991) Two mutations in the envelope glycoprotein E2 of Semliki Forest virus affecting the maturation and entry patterns of the virus alter pathogenicity for mice. *Virology* **185**, 741-8.

Gordon S. (1998) The role of the macrophage in immune regulation. *Res Immunol* **149**, 685-8.

Graham M. B., Braciale V. L. and Braciale T. J. (1994) Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. *J Exp Med* **180**, 1273-82.

Graubert T. A., DiPersio J. F., Russell J. H. and Ley T. J. (1997) Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. *J Clin Invest* **100**, 904-11.

Gray P. W. and Goeddel D. V. (1982) Structure of the human immune interferon gene. *Nature* **298**, 859-63.

Green J., Griffiths G., Louvard D., Quinn P. and Warren G. (1981) Passage of viral membrane proteins through the Golgi complex. *J Mol Biol* **152**, 663-98.

Green J. M., Noel P. J., Sperling A. I., Walunas T. L., Gray G. S., Bluestone J. A. and Thompson C. B. (1994) Absence of B7-dependent responses in CD28-deficient mice. *Immunity* **1**, 501-8.

Griffin D. E. (1996) Alphaviruses. In *Fields Virology (3rd edition)*. (edited by Fields B.N., Knipe D.M., Howley P.M., et al.). Philadeophia. Lippincott - Raven Publishers. *pp.* 917-949.

Griffiths G. M. (1995) The cell biology of CTL killing. *Curr Opin Immunol* **7**, 343-8.

Guidotti L. G., Borrow P., Brown A., McClary H., Koch R. and Chisari F. V. (1999) Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte. *J Exp Med* **189**, 1555-64.

Guidotti L. G. and Chisari F. V. (2000) Cytokine-mediated control of viral infections. *Virology* **273**, 221-7.

Guidotti L. G., McClary H., Loudis J. M. and Chisari F. V. (2000) Nitric oxide inhibits hepatitis B virus replication in the livers of transgenic mice. *J Exp Med* **191**, 1247-52.

Haag L., Garoff H., Xing L., Hammar L., Kan S. T. and Cheng R. H. (2002) Acid-induced movements in the glycoprotein shell of an alphavirus turn the spikes into membrane fusion mode. *Embo J* **21**, 4402-10.

Haddad P., Jenne D., Tschopp J., Clement M. V., Mathieu-Mahul D. and Sasportes M. (1991) Structure and evolutionary origin of the human granzyme H gene. *Int Immunol* **3**, 57-66.

Hahn Y. S., Guanzon A., Rice C. M. and Hahn C. S. (1999) Class I MHC molecule-mediated inhibition of Sindbis virus replication. *J Immunol* **162**, 69-77.

Hakamada-Taguchi R., Kato T., Ushijima H., Murakami M., Uede T. and Nariuchi H. (1998) Expression and co-stimulatory function of B7-2 on murine CD4+ T cells. *Eur J Immunol* **28**, 865-73.

Hansen T. H. and Sachs D. H. (1989) The major histocompaticility comples. In *fundamental immunology*. (Paul W.E, et.). New York: Raven Press, *pp.* 445-87.

Harty J. T. and Bevan M. J. (1999) Responses of CD8(+) T cells to intracellular bacteria. *Curr Opin Immunol* **11**, 89-93.

Harty J. T., Tvinnereim A. R. and White D. W. (2000) CD8+ T cell effector mechanisms in resistance to infection. *Annu Rev Immunol* **18**, 275-308.

Hathcock K. S., Laszlo G., Pucillo C., Linsley P. and Hodes R. J. (1994) Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J Exp Med* **180**, 631-40.

Hayder H. and Mullbacher A. (1996) Molecular basis of immune evasion strategies by adenoviruses. *Immunol Cell Biol* **74**, 504-12.

Heath W. R. and Carbone F. R. (2001) Cross-presentation in viral immunity and self-tolerance. *Nat Rev Immunol* **1**, 126-34.

Heemels M. T. and Ploegh H. (1995) Generation, translocation, and presentation of MHC class I-restricted peptides. *Annu Rev Biochem* **64**, 463-91.

Helenius A., Kartenbeck J., Simons K. and Fries E. (1980) On the entry of Semliki forest virus into BHK-21 cells. *J Cell Biol* **84**, 404-20.

Helenius A., Morein B., Fries E., Simons K., Robinson P., Schirrmacher V., Terhorst C. and Strominger J. L. (1978) Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus. *Proc Natl Acad Sci U S A* **75**, 3846-50.

Henderson D. W., Peacock S. and Randles W. J. (1967) On the pathogenesis of Semliki forest virus (SFV) infection in the hamster. *Br J Exp Pathol* **48**, 228-34.

Henkart P. A. (1994) Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *immunity* **1**, 343-6.

Heusel J. W., Wesselschmidt R. L., Shresta S., Russell J. H. and Ley T. J. (1994) Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell* **76**, 977-87.

Hood L., Steinmetz M. and Malissen B. (1983) Genes of the major histocompatibility complex of the mouse. *Annu Rev Immunol* **1**, 529-68.

Hou S., Mo X. Y., Hyland L. and Doherty P. C. (1995) Host response to Sendai virus in mice lacking class II major histocompatibility complex glycoproteins. *J Virol* **69**, 1429-34.

Huang S., Hendriks W., Althage A., Hemmi S., Bluethmann H., Kamijo R., Vilcek J., Zinkernagel R. M. and Aguet M. (1993) Immune response in mice that lack the interferon-gamma receptor. *Science* **259**, 1742-5.

Hunt D. F., Henderson R. A., Shabanowitz J., Sakaguchi K., Michel H., Sevilir N., Cox A. L., Appella E. and Engelhard V. H. (1992) Characterization of peptides

bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* **255**, 1261-3.

Ingulli E., Mondino A., Khoruts A. and Jenkins M. K. (1997) In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. *J Exp Med* **185**, 2133-41.

Irusta P. M. and Hardwick J. M. (2004) Neuronal apoptosis pathways in Sindbis virus encephalitis. *Prog Mol Subcell Biol* **36**, 71-93.

Isaacs A. and Lindenmann J. (1957) Virus interference. I. The interferon. *Proceedings of the Royal Society of London (Biological Sciences)* **147**, 258-267.

Ito T., Amakawa R., Inaba M., Ikehara S., Inaba K. and Fukuhara S. (2001) Differential regulation of human blood dendritic cell subsets by IFNs. *J Immunol* **166**, 2961-9.

Itoh N., Yonehara S., Ishii A., Yonehara M., Mizushima S., Sameshima M., Hase A., Seto Y. and Nagata S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**, 233-43.

Jaramillo M. L., Abraham N. and Bell J. C. (1995) The interferon system: a review with emphasis on the role of PKR in growth control. *Cancer Invest* **13**, 327-38.

Jenkins M. K., Taylor P. S., Norton S. D. and Urdahl K. B. (1991) CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol* **147**, 2461-6.

Jewett A. and Bonavida B. (1995) Interferon-alpha activates cytotoxic function but inhibits interleukin-2-mediated proliferation and tumor necrosis factor-alpha secretion by immature human natural killer cells. *J Clin Immunol* **15**, 35-44.

Johnston R. E. and Peters C. J. (1996) Alphaviruses. In *Fields Virology (3rd edition)*. (edited by Fields B.N., Knipe D.M., Howley P.M., et al.). Philadeophia. Lippincott - Raven Publishers. *pp.* 843-899.

Johnston R. E., Tovell D. R., Brown D. T. and Faulkner P. (1975) Interfering passages of Sindbis virus: concomitant appearance of interference, morphological variants, and trucated viral RNA. *J Virol* **16**, 951-8.

Jones E. Y. (1997) MHC class I and class II structures. *Curr Opin Immunol* **9**, 75-9.

Ju S. T., Cui H., Panka D. J., Ettinger R. and Marshak-Rothstein A. (1994) Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. *Proc Natl Acad Sci U S A* **91**, 4185-9.

Ju S. T., Panka D. J., Cui H., Ettinger R., el-Khatib M., Sherr D. H., Stanger B. Z. and Marshak-Rothstein A. (1995) Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature* **373**, 444-8.

Kaariainen L. and Ahola T. (2002) Functions of alphavirus nonstructural proteins in RNA replication. *Prog Nucleic Acid Res Mol Biol* **71**, 187-222.

Kaariainen L. and Soderlund H. (1978) Structure and replication of alpha-viruses. *Curr Top Microbiol Immunol* **82**, 15-69.

Kagi D., Ledermann B., Burki K., Seiler P., Odermatt B., Olsen K. J., Podack E. R., Zinkernagel R. M. and Hengartner H. (1994a) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369**, 31-7.

Kagi D., Ledermann B., Burki K., Zinkernagel R. M. and Hengartner H. (1995a) Lymphocyte-mediated cytotoxicity in vitro and in vivo: mechanisms and significance. *Immunol Rev* **146**, 95-115.

Kagi D., Ledermann B., Burki K., Zinkernagel R. M. and Hengartner H. (1996) Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu Rev Immunol* **14**, 207-32.

Kagi D., Odermatt B. and Mak T. W. (1999) Homeostatic regulation of CD8+ T cells by perforin. *Eur J Immunol* **29**, 3262-72.

Kagi D., Seiler P., Pavlovic J., Ledermann B., Burki K., Zinkernagel R. M. and Hengartner H. (1995b) The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. *Eur J Immunol* **25**, 3256-62.

Kagi D., Vignaux F., Ledermann B., Burki K., Depraetere V., Nagata S., Hengartner H. and Golstein P. (1994b) Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* **265**, 528-30.

Kam C. M., Hudig D. and Powers J. C. (2000) Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. *Biochim Biophys Acta* **1477**, 307-23.

Kaplan D. R., Griffith R., Braciale V. L. and Braciale T. J. (1984) Influenza virus-specific human cytotoxic T cell clones: heterogeneity in antigenic specificity and restriction by class II MHC products. *Cell Immunol* **88**, 193-206.

Karray S., Kress C., Cuvellier S., Hue-Beauvais C., Damotte D., Babinet C. and Levi-Strauss M. (2004) Complete loss of Fas ligand gene causes massive lymphoproliferation and early death, indicating a residual activity of gld allele. *J Immunol* **172**, 2118-25.

Karulin A. Y., Hesse M. D., Tary-Lehmann M. and Lehmann P. V. (2000) Single-cytokine-producing CD4 memory cells predominate in type 1 and type 2 immunity. *J Immunol* **164**, 1862-72.

Karupiah G. (1998) Type 1 and type 2 cytokines in antiviral defense. *Vet Immunol Immunopathol* **63**, 105-9.

Karupiah G., Chen J. H., Mahalingam S., Nathan C. F. and MacMicking J. D. (1998) Rapid interferon gamma-dependent clearance of influenza A virus and protection from consolidating pneumonitis in nitric oxide synthase 2-deficient mice. *J Exp Med* **188**, 1541-6.

Karupiah G., Coupar B. E., Andrew M. E., Boyle D. B., Phillips S. M., Mullbacher A., Blanden R. V. and Ramshaw I. A. (1990) Elevated natural killer cell responses in mice infected with recombinant vaccinia virus encoding murine IL-2. *J Immunol* **144**, 290-8.

Karupiah G., Fredrickson T. N., Holmes K. L., Khairallah L. H. and Buller R. M. (1993) Importance of interferons in recovery from mousepox. *J Virol* **67**, 4214-26.

Karupiah G., Ramsay A. J., Ramshaw I. A. and Blanden R. V. (1992) Recombinant vaccine vector-induced protection of athymic, nude mice from influenza A virus infection. Analysis of protective mechanisms. *Scand J Immunol* **36**, 99-105.

Kato T. and Nariuchi H. (2000) Polarization of naive CD4+ T cells toward the Th1 subset by CTLA-4 costimulation. *J Immunol* **164**, 3554-62.

Keller F., Schmitt C. and Kirn A. (1988) Interaction of mouse hepatitis virus 3 with Kupffer cells explanted from susceptible and resistant mouse strains. Antiviral activity, interleukin-1 synthesis. *FEMS Microbiol Immunol* **1**, 87-95.

Keller F., Wild M. T. and Kirn A. (1985) In vitro antiviral properties of endotoxin-activated rat Kupffer cells. *J Leukoc Biol* **38**, 293-303.

Kelley K. A., Kozak C. A., Dandoy F., Sor F., Skup D., Windass J. D., DeMaeyer-Guignard J., Pitha P. M. and DeMaeyer E. (1983) Mapping of murine interferon-alpha genes to chromosome 4. *Gene* **26**, 181-8.

Kelly A. P., Monaco J. J., Cho S. G. and Trowsdale J. (1991) A new human HLA class II-related locus, DM. *Nature* **353**, 571-3.

Khan S., de Giuli R., Schmidtke G., Bruns M., Buchmeier M., van den Broek M. and Groettrup M. (2001) Cutting edge: neosynthesis is required for the presentation of a T cell epitope from a long-lived viral protein. *J Immunol* **167**, 4801-4.

Kielian M. (1995) Membrane fusion and the alphavirus life cycle. *Adv Virus Res* **45**, 113-51.

Klein J. and Sato A. (2000) The HLA system. First of two parts. *N Engl J Med* **343**, 702-9.

Koide S. L., Inaba K. and Steinman R. M. (1987) Interleukin 1 enhances T-dependent immune responses by amplifying the function of dendritic cells. *J Exp Med* **165**, 515-30.

Kojima H., Shinohara N., Hanaoka S., Someya-Shirota Y., Takagaki Y., Ohno H., Saito T., Katayama T., Yagita H. and Okumura K. (1994) Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity* **1**, 357-64.

Koller B. H., Marrack P., Kappler J. W. and Smithies O. (1990) Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. *Science* **248**, 1227-30.

Koo G. C., Peppard J. R. and Mark W. H. (1984) Natural killer cells generated from bone marrow culture. *J Immunol* **132**, 2300-4.

Kovacsovics-Bankowski M., Clark K., Benacerraf B. and Rock K. L. (1993) Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc Natl Acad Sci U S A* **90**, 4942-6.

Kraaijeveld C. A., Benaissa-Trouw B. J., Harmsen M. and Snippe H. (1986) Adoptive transfer of immunity against virulent Semliki Forest virus with immune spleen cells from mice infected with avirulent Semliki Forest virus. *Arch Virol* **91**, 83-92.

Krummel M. F. and Allison J. P. (1995) CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **182**, 459-65.

Krummel M. F. and Allison J. P. (1996) CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* **183**, 2533-40.

Kuchroo V. K., Das M. P., Brown J. A., Ranger A. M., Zamvil S. S., Sobel R. A., Weiner H. L., Nabavi N. and Glimcher L. H. (1995) B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* **80**, 707-18.

Kulkarni A. B., Mullbacher A. and Blanden R. V. (1991) Functional analysis of macrophages, B cells and splenic dendritic cells as antigen-presenting cells in West Nile virus-specific murine T lymphocyte proliferation. *Immunol Cell Biol* **69 (Pt 2)**, 71-80.

Kupfer A., Dennert G. and Singer S. J. (1985) The reorientation of the Golgi apparatus and the microtubule-organizing center in the cytotoxic effector cell is a prerequisite in the lysis of bound target cells. *J Mol Cell Immunol* **2**, 37-49.

Kurane I., Hebblewaite D., Brandt W. E. and Ennis F. A. (1984) Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. *J Virol* **52**, 223-30.

Labrecque N., Whitfield L. S., Obst R., Waltzinger C., Benoist C. and Mathis D. (2001) How much TCR does a T cell need? *Immunity* **15**, 71-82.

Lafferty K. J. and Cunningham A. J. (1975) A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci* **53**, 27-42.

Landolfo S., Gariglio M., Gribaudo G., Jemma C., Giovarelli M. and Cavallo G. (1988) Interferon-gamma is not an antiviral, but a growth-promoting factor for T lymphocytes. *Eur J Immunol* **18**, 503-9.

Lanzavecchia A. and Sallusto F. (2001) Regulation of T cell immunity by dendritic cells. *Cell* **106**, 263-6.

Laskin D. L. and Pendino K. J. (1995) Macrophages and inflammatory mediators in tissue injury. *Annu Rev Pharmacol Toxicol* **35**, 655-77.

Le Bon A., Schiavoni G., D'Agostino G., Gresser I., Belardelli F. and Tough D. F. (2001) Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* **14**, 461-70.

Le Bon A. and Tough D. F. (2002) Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol* **14**, 432-6.

LeBlanc P. A., Heath L. S. and Um H. D. (1990) Activated macrophages use different cytolytic mechanisms to lyse a virally infected or a tumor target. *J Leukoc Biol* **48**, 1-6.

Leist T. P., Kohler M. and Zinkernagel R. M. (1989) Impaired generation of anti-viral cytotoxicity against lymphocytic choriomeningitis and vaccinia virus in mice treated with CD4-specific monoclonal antibody. *Scand J Immunol* **30**, 679-86.

Lenschow D. J., Ho S. C., Sattar H., Rhee L., Gray G., Nabavi N., Herold K. C. and Bluestone J. A. (1995) Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. *J Exp Med* **181**, 1145-55.

Lenschow D. J., Sperling A. I., Cooke M. P., Freeman G., Rhee L., Decker D. C., Gray G., Nadler L. M., Goodnow C. C. and Bluestone J. A. (1994) Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen. *J Immunol* **153**, 1990-7.

Lenschow D. J., Su G. H., Zuckerman L. A., Nabavi N., Jellis C. L., Gray G. S., Miller J. and Bluestone J. A. (1993) Expression and functional significance of an additional ligand for CTLA-4. *Proc Natl Acad Sci U S A* **90**, 11054-8.

Lertmemongkolchai G., Cai G., Hunter C. A. and Bancroft G. J. (2001) Bystander activation of CD8+ T cells contributes to the rapid production of IFN-gamma in response to bacterial pathogens. *J Immunol* **166**, 1097-105.

Levy D. E. and Garcia-Sastre A. (2001) The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. *Cytokine Growth Factor Rev* **12**, 143-56.

Lichtenheld M. G., Olsen K. J., Lu P., Lowrey D. M., Hameed A., Hengartner H. and Podack E. R. (1988) Structure and function of human perforin. *Nature* **335**, 448-51.

Licon Luna R. M., Lee E., Mullbacher A., Blanden R. V., Langman R. and Lobigs M. (2002) Lack of both Fas ligand and perforin protects from flavivirus-mediated encephalitis in mice. *J Virol* **76**, 3202-11.

Liljestrom P., Lusa S., Huylebroeck D. and Garoff H. (1991) In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J Virol* **65**, 4107-13.

Linsley P. S., Greene J. L., Brady W., Bajorath J., Ledbetter J. A. and Peach R. (1994) Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* **1**, 793-801.

Liu C. C., Walsh C. M. and Young J. D. (1995) Perforin: structure and function. *Immunol Today* **16**, 194-201.

Ljunggren H. G. and Karre K. (1990) In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* **11**, 237-44.

Lo D. (1992) T-cell tolerance. *Curr Opin Immunol* **4**, 711-5.

Lobigs M., Mullbacher A., Wang Y., Pavy M. and Lee E. (2003) Role of type I and type II interferon responses in recovery from infection with an encephalitic flavivirus. *J Gen Virol* **84**, 567-72.

Lotteau V., Teyton L., Peleraux A., Nilsson T., Karlsson L., Schmid S. L., Quaranta V. and Peterson P. A. (1990) Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* **348**, 600-5.

Lowenthal J. W., Tougne C., MacDonald H. R., Smith K. A. and Nabholz M. (1985) Antigenic stimulation regulates the expression of IL 2 receptors in a cytolytic T lymphocyte clone. *J Immunol* **134**, 931-9.

Lowin B., Hahne M., Mattmann C. and Tschopp J. (1994) Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* **370**, 650-2.

Ludewig B., Graf D., Gelderblom H. R., Becker Y., Kroczek R. A. and Pauli G. (1995) Spontaneous apoptosis of dendritic cells is efficiently inhibited by TRAP (CD40-ligand) and TNF-alpha, but strongly enhanced by interleukin-10. *Eur J Immunol* **25**, 1943-50.

Luft T., Pang K. C., Thomas E., Hertzog P., Hart D. N., Trapani J. and Cebon J. (1998) Type I IFNs enhance the terminal differentiation of dendritic cells. *J Immunol* **161**, 1947-53.

Lyons D. S., Lieberman S. A., Hampl J., Boniface J. J., Chien Y., Berg L. J. and Davis M. M. (1996) A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* **5**, 53-61.

Macatonia S. E., Hosken N. A., Litton M., Vieira P., Hsieh C. S., Culpepper J. A., Wysocka M., Trinchieri G., Murphy K. M. and O'Garra A. (1995) Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* **154**, 5071-9.

Macfarlan R. I., Burns W. H. and White D. O. (1977) Two cytotoxic cells in peritoneal cavity of virus-infected mice: antibody-dependent macrophages and nonspecific killer cells. *J Immunol* **119**, 1569-74.

MacMicking J., Xie Q. W. and Nathan C. (1997) Nitric oxide and macrophage function. *Annu Rev Immunol* **15**, 323-50.

Macphail S. and Stutman O. (1987) L3T4+ cytotoxic T lymphocytes specific for class I H-2 antigens are activated in primary mixed lymphocyte reactions. *J Immunol* **139**, 4007-15.

MacPhee I. A., Turner D. R., Yagita H. and Oliveira D. B. (2001) CD80(B7.1) and CD86(B7.2) do not have distinct roles in setting the Th1/Th2 balance in autoimmunity in rats. *Scand J Immunol* **54**, 486-94.

Maggi E., Del Prete G., Macchia D., Parronchi P., Tiri A., Chretien I., Ricci M. and Romagnani S. (1988) Profiles of lymphokine activities and helper function for IgE in human T cell clones. *Eur J Immunol* **18**, 1045-50.

Maldonado-Lopez R., De Smedt T., Michel P., Godfroid J., Pajak B., Heirman C., Thielemans K., Leo O., Urbain J. and Moser M. (1999) CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J Exp Med* **189**, 587-92.

Malmgaard L. (2004) Induction and regulation of IFNs during viral infections. *J Interferon Cytokine Res* **24**, 439-54.

Maloy K. J., Burkhart C., Junt T. M., Odermatt B., Oxenius A., Piali L., Zinkernagel R. M. and Hengartner H. (2000) CD4(+) T cell subsets during virus infection. Protective capacity depends on effector cytokine secretion and on migratory capability. *J Exp Med* **191**, 2159-70.

Marrack P., Kappler J. and Mitchell T. (1999) Type I interferons keep activated T cells alive. *J Exp Med* **189**, 521-30.

Marsh M. and Helenius A. (1989) Virus entry into animal cells. *Adv Virus Res* **36**, 107-51.

Martin P. J., Ledbetter J. A., Morishita Y., June C. H., Beatty P. G. and Hansen J. A. (1986) A 44 kilodalton cell surface homodimer regulates interleukin 2 production by activated human T lymphocytes. *J Immunol* **136**, 3282-7.

Marzio R., Jirillo E., Ransijn A., Mauel J. and Corradin S. B. (1997) Expression and function of the early activation antigen CD69 in murine macrophages. *J Leukoc Biol* **62**, 349-55.

Mathiot C. C., Grimaud G., Garry P., Bouquety J. C., Mada A., Daguisy A. M. and Georges A. J. (1990) An outbreak of human Semliki Forest virus infections in Central African Republic. *Am J Trop Med Hyg* **42**, 386-93.

Matloubian M., Suresh M., Glass A., Galvan M., Chow K., Whitmire J. K., Walsh C. M., Clark W. R. and Ahmed R. (1999) A role for perforin in downregulating T-cell responses during chronic viral infection. *J Virol* **73**, 2527-36.

Matsubayashi Y., Zenita K., Morioka A., Iwashiro M., Masuda T., Uchino H., Fujita T. and Kuribayashi K. (1989) Characterization of a CD4(L3T4)-positive cytotoxic T cell clone that is restricted by class I major histocompatibility complex antigen on FBL-3 tumor cell. *Immunobiology* **180**, 33-46.

Mattei F., Schiavoni G., Belardelli F. and Tough D. F. (2001) IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation. *J Immunol* **167**, 1179-87.

McIntosh B. M., Brookworth C. and Kokernot R. H. (1961) Isolation of Semliki Forest Virus from *Aedes (Aedimorphus) argenteopunctatus* (Theobald) collected in Portugese East Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **55**, 386-393.

McKeithan T. W. (1995) Kinetic proofreading in T-cell receptor signal transduction. *Proc Natl Acad Sci U S A* **92**, 5042-6.

McLaurin J., Antel J. P. and Yong V. W. (1995) Immune and non-immune actions of interferon-beta-Ib on primary human neural cells. *Mult Scler* **1**, 10-9.

McNally J. M., Zarozinski C. C., Lin M. Y., Brehm M. A., Chen H. D. and Welsh R. M. (2001) Attrition of bystander CD8 T cells during virus-induced T-cell and interferon responses. *J Virol* **75**, 5965-76.

Medana I., Li Z., Flugel A., Tschopp J., Wekerle H. and Neumann H. (2001) Fas ligand (CD95L) protects neurons against perforin-mediated T lymphocyte cytotoxicity. *J Immunol* **167**, 674-81.

Medana I. M., Gallimore A., Oxenius A., Martinic M. M., Wekerle H. and Neumann H. (2000) MHC class I-restricted killing of neurons by virus-specific CD8+ T lymphocytes is effected through the Fas/FasL, but not the perforin pathway. *Eur J Immunol* **30**, 3623-33.

Melancon P. and Garoff H. (1986) Reinitiation of translocation in the Semliki Forest virus structural polyprotein: identification of the signal for the E1 glycoprotein. *Embo J* **5**, 1551-60.

Melancon P. and Garoff H. (1987) Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. *J Virol* **61**, 1301-9.

Metkar S. S., Wang B., Ebbs M. L., Kim J. H., Lee Y. J., Raja S. M. and Froelich C. J. (2003) Granzyme B activates procaspase-3 which signals a mitochondrial amplification loop for maximal apoptosis. *J Cell Biol* **160**, 875-85.

Michel F., Attal-Bonnefoy G., Mangino G., Mise-Omata S. and Acuto O. (2001) CD28 as a molecular amplifier extending TCR ligation and signaling capabilities. *Immunity* **15**, 935-45.

Miller D. M., Rahill B. M., Boss J. M., Lairmore M. D., Durbin J. E., Waldman J. W. and Sedmak D. D. (1998) Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. *J Exp Med* **187**, 675-83.

Miller J. F. and Basten A. (1996) Mechanisms of tolerance to self. *Curr Opin Immunol* **8**, 815-21.

Miller S. D., Vanderlugt C. L., Lenschow D. J., Pope J. G., Karandikar N. J., Dal Canto M. C. and Bluestone J. A. (1995) Blockade of CD28/B7-1 interaction prevents epitope spreading and clinical relapses of murine EAE. *Immunity* **3**, 739-45.

Mims C. A. and Blanden R. V. (1972) Antiviral action of immune lymphocytes in mice infected with lymphocytic choriomeningitis virus. *Infect Immun* **6**, 695-8.

Miyawaki T., Uehara T., Nibu R., Tsuji T., Yachie A., Yonehara S. and Taniguchi N. (1992) Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. *J Immunol* **149**, 3753-8.

Mokhtarian F., Wesselingh S. L., Choi S., Maeda A., Griffin D. E., Sobel R. A. and Grob D. (1996) Production and role of cytokines in the CNS of mice with acute viral encephalomyelitis. *J Neuroimmunol* **66**, 11-22.

Momburg F., Roelse J., Hammerling G. J. and Neefjes J. J. (1994) Peptide size selection by the major histocompatibility complex-encoded peptide transporter. *J Exp Med* **179**, 1613-23.

Mondino A., Khoruts A. and Jenkins M. K. (1996) The anatomy of T-cell activation and tolerance. *Proc Natl Acad Sci U S A* **93**, 2245-52.

Montoya M., Schiavoni G., Mattei F., Gresser I., Belardelli F., Borrow P. and Tough D. F. (2002) Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* **99**, 3263-71.

Moretta A., Bottino C., Mingari M. C., Biassoni R. and Moretta L. (2002) What is a natural killer cell? *Nat Immunol* **3**, 6-8.

Moretta A., Bottino C., Vitale M., Pende D., Cantoni C., Mingari M. C., Biassoni R. and Moretta L. (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol* **19**, 197-223.

Mosmann T. R. and Coffman R. L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* **7**, 145-73.

Mosser D. M. (2003) The many faces of macrophage activation. *J Leukoc Biol* **73**, 209-12.

Mullbacher A. (2003) Cell-mediated cytotoxicity in recovery from poxvirus infections. *Rev Med Virol* **13**, 223-32.

Mullbacher A. and Blanden R. V. (1978) Murine Cytotoxic T-Cell Response to Alphavirus is Associated Mainly with *H-2D*^{k}. *Immunogenetics* **7**, 551-561.

Mullbacher A., Ebnet K., Blanden R. V., Hla R. T., Stehle T., Museteanu C. and Simon M. M. (1996) Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. *Proc Natl Acad Sci U S A* **93**, 5783-7.

Mullbacher A., Hla R. T., Museteanu C. and Simon M. M. (1999a) Perforin is essential for control of ectromelia virus but not related poxviruses in mice. *J Virol* **73**, 1665-7.

Mullbacher A. and King N. J. (1989a) Differential target cell susceptibility to SFV-immune cytotoxic T-cells. *Arch Virol* **107**, 97-109.

Mullbacher A. and King N. J. (1989b) Target cell lysis by natural killer cells is influenced by beta 2-microglobulin expression. *Scand J Immunol* **30**, 21-9.

Mullbacher A., Lobigs M., Hla R. T., Tran T., Stehle T. and Simon M. M. (2002) Antigen-dependent release of IFN-gamma by cytotoxic T cells up-regulates Fas on target cells and facilitates exocytosis-independent specific target cell lysis. *J Immunol* **169**, 145-50.

Mullbacher A., Lobigs M. and Lee E. (2003) Immunobiology of mosquito-borne encephalitic flaviviruses. *Adv Virus Res* **60**, 87-120.

Mullbacher A., Waring P., Tha Hla R., Tran T., Chin S., Stehle T., Museteanu C. and Simon M. M. (1999b) Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. *Proc Natl Acad Sci U S A* **96**, 13950-5.

Muller U., Steinhoff U., Reis L. F., Hemmi S., Pavlovic J., Zinkernagel R. M. and Aguet M. (1994) Functional role of type I and type II interferons in antiviral defense. *Science* **264**, 1918-21.

Munder M., Mallo M., Eichmann K. and Modolell M. (1998) Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. *J Exp Med* **187**, 2103-8.

Murali-Krishna K., Altman J. D., Suresh M., Sourdive D. J., Zajac A. J., Miller J. D., Slansky J. and Ahmed R. (1998) Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8**, 177-87.

Nagata S. (1997) Apoptosis by death factor. *Cell* **88**, 355-65.

Nagata S. (1999) Fas ligand-induced apoptosis. *Annu Rev Genet* **33**, 29-55.

Nagata S. and Golstein P. (1995) The Fas death factor. *Science* **267**, 1449-56.

Nagata S. and Suda T. (1995) Fas and Fas ligand: lpr and gld mutations. *Immunol Today* **16**, 39-43.

Nakajima H., Park H. L. and Henkart P. A. (1995) Synergistic roles of granzymes A and B in mediating target cell death by rat basophilic leukemia mast cell tumors also expressing cytolysin/perforin. *J Exp Med* **181**, 1037-46.

Nansen A., Jensen T., Christensen J. P., Andreasen S. O., Ropke C., Marker O. and Thomsen A. R. (1999) Compromised virus control and augmented perforin-mediated immunopathology in IFN-gamma-deficient mice infected with lymphocytic choriomeningitis virus. *J Immunol* **163**, 6114-22.

Neefjes J. J., Momburg F. and Hammerling G. J. (1993) Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science* **261**, 769-71.

Nguyen K. B., Cousens L. P., Doughty L. A., Pien G. C., Durbin J. E. and Biron C. A. (2000) Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox. *Nat Immunol* **1**, 70-6.

Nguyen T. and Russell J. (2001) The regulation of FasL expression during activation-induced cell death (AICD). *Immunology* **103**, 426-34.

Nishimura Y., Ishii A., Kobayashi Y., Yamasaki Y. and Yonehara S. (1995) Expression and function of mouse Fas antigen on immature and mature T cells. *J Immunol* **154**, 4395-403.

Norbury C. C., Chambers B. J., Prescott A. R., Ljunggren H. G. and Watts C. (1997) Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur J Immunol* **27**, 280-8.

Norbury C. C., Hewlett L. J., Prescott A. R., Shastri N. and Watts C. (1995) Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* **3**, 783-91.

Novick D., Cohen B. and Rubinstein M. (1994) The human interferon alpha/beta receptor: characterization and molecular cloning. *Cell* **77**, 391-400.

Odake S., Kam C. M., Narasimhan L., Poe M., Blake J. T., Krahenbuhl O., Tschopp J. and Powers J. C. (1991) Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins. *Biochemistry* **30**, 2217-27.

Oldstone M. B., Tishon A., Dutko F. J., Kennedy S. I., Holland J. J. and Lampert P. W. (1980) Does the major histocompatibility complex serve as a specific receptor for Semliki Forest virus? *J Virol* **34**, 256-65.

Oliver K. R. and Fazakerley J. K. (1998) Transneuronal spread of Semliki Forest virus in the developing mouse olfactory system is determined by neuronal maturity. *Neuroscience* **82**, 867-77.

Oliver K. R., Scallan M. F., Dyson H. and Fazakerley J. K. (1997) Susceptibility to a neurotropic virus and its changing distribution in the developing brain is a function of CNS maturity. *J Neurovirol* **3**, 38-48.

Orange J. S. and Biron C. A. (1996) Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J Immunol* **156**, 4746-56.

Orange J. S., Wang B., Terhorst C. and Biron C. A. (1995) Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J Exp Med* **182**, 1045-56.

Ortaldo J. R., Mason A. T., Winkler-Pickett R., Raziuddin A., Murphy W. J. and Mason L. H. (1999) Ly-49 receptor expression and functional analysis in multiple mouse strains. *J Leukoc Biol* **66**, 512-20.

Ozmen L., Aguet M., Trinchieri G. and Garotta G. (1995) The in vivo antiviral activity of interleukin-12 is mediated by gamma interferon. *J Virol* **69**, 8147-50.

Paliard X., de Waal Malefijt R., Yssel H., Blanchard D., Chretien I., Abrams J., de Vries J. and Spits H. (1988) Simultaneous production of IL-2, IL-4, and IFN-gamma by activated human CD4+ and CD8+ T cell clones. *J Immunol* **141**, 849-55.

Pamer E. and Cresswell P. (1998) Mechanisms of MHC class I--restricted antigen processing. *Annu Rev Immunol* **16**, 323-58.

Pardo J., Balkow S., Anel A. and Simon M. M. (2002) Granzymes are essential for natural killer cell-mediated and perf-facilitated tumor control. *Eur J Immunol* **32**, 2881-7.

Park C. G., Lee S. Y., Kandala G. and Choi Y. (1996) A novel gene product that couples TCR signaling to Fas(CD95) expression in activation-induced cell death. *Immunity* **4**, 583-91.

Parronchi P., Macchia D., Piccinni M. P., Biswas P., Simonelli C., Maggi E., Ricci M., Ansari A. A. and Romagnani S. (1991) Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. *Proc Natl Acad Sci U S A* **88**, 4538-42.

Pasternack M. S., Verret C. R., Liu M. A. and Eisen H. N. (1986) Serine esterase in cytolytic T lymphocytes. *Nature* **322**, 740-3.

Pathak S. and Webb H. E. (1974) Possible mechanisms for the transport of Semliki forest virus into and within mouse brain. An electron-microscopic study. *J Neurol Sci* **23**, 175-84.

Pathak S. and Webb H. E. (1978) An electron-microscopic study of avirulent and virulent Semliki forest virus in the brains of different ages of mice. *J Neurol Sci* **39**, 199-211.

Pereira R. A., Simon M. M. and Simmons A. (2000) Granzyme A, a noncytolytic component of CD8(+) cell granules, restricts the spread of herpes simplex virus in the peripheral nervous systems of experimentally infected mice. *J Virol* **74**, 1029-32.

Pestka S., Langer J. A., Zoon K. C. and Samuel C. E. (1987) Interferons and their actions. *Annu Rev Biochem* **56**, 727-77.

Pierre P., Turley S. J., Gatti E., Hull M., Meltzer J., Mirza A., Inaba K., Steinman R. M. and Mellman I. (1997) Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* **388**, 787-92.

Pieters J., Bakke O. and Dobberstein B. (1993) The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J Cell Sci* **106 (Pt 3)**, 831-46.

Pinkoski M. J. and Green D. R. (2003) Granzyme A: the road less traveled. *Nat Immunol* **4**, 106-8.

Ploegh H. L., Cannon L. E. and Strominger J. L. (1979) Cell-free translation of the mRNAs for the heavy and light chains of HLA-A and HLA-B antigens. *Proc Natl Acad Sci U S A* **76**, 2273-7.

Proietti E., Bracci L., Puzelli S., Di Pucchio T., Sestili P., De Vincenzi E., Venditti M., Capone I., Seif I., De Maeyer E., Tough D., Donatelli I. and Belardelli F. (2002)

Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. *J Immunol* **169**, 375-83.

Pulendran B., Smith J. L., Caspary G., Brasel K., Pettit D., Maraskovsky E. and Maliszewski C. R. (1999) Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci U S A* **96**, 1036-41.

Pusztai R., Gould E. A. and Smith H. (1971) Infection patterns in mice of an avirulent and virulent strain of Semliki Forest virus. *Br J Exp Pathol* **52**, 669-77.

Ramsdell F., Seaman M. S., Miller R. E., Picha K. S., Kennedy M. K. and Lynch D. H. (1994) Differential ability of Th1 and Th2 T cells to express Fas ligand and to undergo activation-induced cell death. *Int Immunol* **6**, 1545-53.

Ramshaw I. A., Ramsay A. J., Karupiah G., Rolph M. S., Mahalingam S. and Ruby J. C. (1997) Cytokines and immunity to viral infections. *Immunol Rev* **159**, 119-35.

Rathmell J. C., Cooke M. P., Ho W. Y., Grein J., Townsend S. E., Davis M. M. and Goodnow C. C. (1995) CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature* **376**, 181-4.

Rebouillat D. and Hovanessian A. G. (1999) The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. *J Interferon Cytokine Res* **19**, 295-308.

Reddehase M. J., Mutter W., Munch K., Buhring H. J. and Koszinowski U. H. (1987) CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. *J Virol* **61**, 3102-8.

Reiss C. S. and Komatsu T. (1998) Does nitric oxide play a critical role in viral infections? *J Virol* **72**, 4547-51.

Reits E. A., Vos J. C., Gromme M. and Neefjes J. (2000) The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature* **404**, 774-8.

Riera L., Gariglio M., Valente G., Mullbacher A., Museteanu C., Landolfo S. and Simon M. M. (2000) Murine cytomegalovirus replication in salivary glands is controlled by both perforin and granzymes during acute infection. *Eur J Immunol* **30**, 1350-5.

Roake J. A., Rao A. S., Morris P. J., Larsen C. P., Hankins D. F. and Austyn J. M. (1995) Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J Exp Med* **181**, 2237-47.

Robinson M. A. and Kindt T. J. (1989) Major histocompatibility complex antigens and genes. In Fundamental immunology. (Paul W.E. ed.). New York: Raven Press, pp. 489-539.

Roche P. A. (1995) HLA-DM: an in vivo facilitator of MHC class II peptide loading. *Immunity* **3**, 259-62.

Rode M B. S., Sobek V, Brehm R, Martin P, Kersten A, Dumrese T, Stehle T, Müllbacher A, Wallich R, Simon MM. (2004a) Perforin and Fas act together in the induction of apoptosis and are both critical in the clearance of lymphocytic choriomeningitis virus infection. *J Virol* **in press**.

Rode M B. S., Sobek V, Brehm R, Martin P, Kersten A, Dumrese T, Stehle T, Müllbacher A, Wallich R, Simon MM. (2004b) Perforin and Fas act together in the induction of apoptosis and are both critical in the clearance of lymphocytic choriomeningitis virus infection. *journal of virology* **accepted**.

Rodel J., Groh A., Vogelsang H., Lehmann M., Hartmann M. and Straube E. (1998) Beta interferon is produced by Chlamydia trachomatis-infected fibroblast-like synoviocytes and inhibits gamma interferon-induced HLA-DR expression. *Infect Immun* **66**, 4491-5.

Rodriguez A., Regnault A., Kleijmeer M., Ricciardi-Castagnoli P. and Amigorena S. (1999) Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol* **1**, 362-8.

Rodriguez M. A., Prinz W. A., Sibbitt W. L., Bankhurst A. D. and Williams R. C., Jr. (1983) alpha-Interferon increases immunoglobulin production in cultured human mononuclear leukocytes. *J Immunol* **130**, 1215-9.

Rogers P. R. and Croft M. (1999) Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J Immunol* **163**, 1205-13.

Rosa F., Hatat D., Abadie A., Wallach D., Revel M. and Fellous M. (1983) Differential regulation of HLA-DR mRNAs and cell surface antigens by interferon. *Embo J* **2**, 1585-9.

Rosa F. M., Cochet M. M. and Fellous M. (1986) Interferon and major histocompatibility complex genes: a model to analyse eukaryotic gene regulation? *Interferon* **7**, 47-87.

Rouvier E., Luciani M. F. and Golstein P. (1993) Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. *J Exp Med* **177**, 195-200.

Russell D. G. (1995a) Of microbes and macrophages: entry, survival and persistence. *Curr Opin Immunol* **7**, 479-84.

Russell J. H. (1995b) Activation-induced death of mature T cells in the regulation of immune responses. *Curr Opin Immunol* **7**, 382-8.

Russell J. H. and Ley T. J. (2002) Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* **20**, 323-70.

Ryman K. D., Klimstra W. B., Nguyen K. B., Biron C. A. and Johnston R. E. (2000) Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. *J Virol* **74**, 3366-78.

Sallusto F. and Lanzavecchia A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **179**, 1109-18.

Samelson L. E., Davidson W. F., Morse H. C., 3rd and Klausner R. D. (1986) Abnormal tyrosine phosphorylation on T-cell receptor in lymphoproliferative disorders. *Nature* **324**, 674-6.

Samuel C. E. (1991) Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology* **183**, 1-11.

Samuel C. E. (2001) Antiviral actions of interferons. *Clin Microbiol Rev* **14**, 778-809, table of contents.

Sandberg K., Eloranta M. L. and Campbell I. L. (1994) Expression of alpha/beta interferons (IFN-alpha/beta) and their relationship to IFN-alpha/beta-induced genes in lymphocytic choriomeningitis. *J Virol* **68**, 7358-66.

Santagati M. G., Itaranta P. V., Koskimies P. R., Maatta J. A., Salmi A. A. and Hinkkanen A. E. (1994) Multiple repeating motifs are found in the 3'-terminal non-translated region of Semliki Forest virus A7 variant genome. *J Gen Virol* **75 (Pt 6)**, 1499-504.

Santagati M. G., Maatta J. A., Itaranta P. V., Salmi A. A. and Hinkkanen A. E. (1995) The Semliki Forest virus E2 gene as a virulence determinant. *J Gen Virol* **76 (Pt 1)**, 47-52.

Santagati M. G., Maatta J. A., Roytta M., Salmi A. A. and Hinkkanen A. E. (1998) The significance of the 3'-nontranslated region and E2 amino acid mutations in the virulence of Semliki Forest virus in mice. *Virology* **243**, 66-77.

Santini S. M., Lapenta C., Logozzi M., Parlato S., Spada M., Di Pucchio T. and Belardelli F. (2000) Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *J Exp Med* **191**, 1777-88.

Saura M., Zaragoza C., McMillan A., Quick R. A., Hohenadl C., Lowenstein J. M. and Lowenstein C. J. (1999) An antiviral mechanism of nitric oxide: inhibition of a viral protease. *Immunity* **10**, 21-8.

Scallan M. F. and Fazakerley J. K. (1999) Aurothiolates enhance the replication of Semliki Forest virus in the CNS and the exocrine pancreas. *J Neurovirol* **5**, 392-400.

Scherle P. A., Palladino G. and Gerhard W. (1992) Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *J Immunol* **148**, 212-7.

Schindler H., Lutz M. B., Rollinghoff M. and Bogdan C. (2001) The production of IFN-gamma by IL-12/IL-18-activated macrophages requires STAT4 signaling and is inhibited by IL-4. *J Immunol* **166**, 3075-82.

Schlesinger S. and Schlesinger M. J. (1996) Togaviridae: The viruses and their replication. In *Fields Virology (3rd edition)*. (edited by Fields B.N., Knipe D.M., Howley P.M., et al.). Philadeophia. Lippincott - Raven Publishers. *pp*. 825-841.

Schmidt M. F. (1982) Acylation of viral spike glycoproteins: a feature of enveloped RNA viruses. *Virology* **116**, 327-38.

Schubert U., Anton L. C., Gibbs J., Norbury C. C., Yewdell J. W. and Bennink J. R. (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404**, 770-4.

Schulz M., Schuurman H. J., Joergensen J., Steiner C., Meerloo T., Kagi D., Hengartner H., Zinkernagel R. M., Schreier M. H., Burki K. and et al. (1995) Acute rejection of vascular heart allografts by perforin-deficient mice. *Eur J Immunol* **25**, 474-80.

Seamer J., Randles W. J. and Fitzgeorge R. (1967) The course of Semliki Forest virus infection in mice. *Br J Exp Pathol* **48**, 395-402.

Sen G. C. (2001) Viruses and interferons. *Annu Rev Microbiol* **55**, 255-81.

Sen G. C. and Ransohoff R. M. (1993) Interferon-induced antiviral actions and their regulation. *Adv Virus Res* **42**, 57-102.

Sharif-Askari E., Alam A., Rheaume E., Beresford P. J., Scotto C., Sharma K., Lee D., DeWolf W. E., Nuttall M. E., Lieberman J. and Sekaly R. P. (2001) Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. *Embo J* **20**, 3101-13.

Sharpe A. H. and Freeman G. J. (2002) The B7-CD28 superfamily. *Nat Rev Immunol* **2**, 116-26.

Shepherd J. C., Schumacher T. N., Ashton-Rickardt P. G., Imaeda S., Ploegh H. L., Janeway C. A., Jr. and Tonegawa S. (1993) TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective. *Cell* **74**, 577-84.

Shi L., Kam C. M., Powers J. C., Aebersold R. and Greenberg A. H. (1992a) Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. *J Exp Med* **176**, 1521-9.

Shi L., Kraut R. P., Aebersold R. and Greenberg A. H. (1992b) A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J Exp Med* **175**, 553-66.

Shinkai Y., Takio K. and Okumura K. (1988) Homology of perforin to the ninth component of complement (C9). *Nature* **334**, 525-7.

Shortman K. and Liu Y. J. (2002) Mouse and human dendritic cell subtypes. *Nat Rev Immunol* **2**, 151-61.

Shresta S., Goda P., Wesselschmidt R. and Ley T. J. (1997) Residual cytotoxicity and granzyme K expression in granzyme A-deficient cytotoxic lymphocytes. *J Biol Chem* **272**, 20236-44.

Shresta S., Graubert T. A., Thomas D. A., Raptis S. Z. and Ley T. J. (1999) Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity* **10**, 595-605.

Shresta S., Heusel J. W., Macivor D. M., Wesselschmidt R. L., Russell J. H. and Ley T. J. (1995) Granzyme B plays a critical role in cytotoxic lymphocyte-induced apoptosis. *Immunol Rev* **146**, 211-21.

Shtrichman R. and Samuel C. E. (2001) The role of gamma interferon in antimicrobial immunity. *Curr Opin Microbiol* **4**, 251-9.

Siegal F. P., Kadowaki N., Shodell M., Fitzgerald-Bocarsly P. A., Shah K., Ho S., Antonenko S. and Liu Y. J. (1999) The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284**, 1835-7.

Sigal L. J., Crotty S., Andino R. and Rock K. L. (1999) Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* **398**, 77-80.

Simon M. M., Hausmann M., Tran T., Ebnet K., Tschopp J., ThaHla R. and Mullbacher A. (1997) In vitro- and ex vivo-derived cytolytic leukocytes from granzyme A x B double knockout mice are defective in granule-mediated apoptosis but not lysis of target cells. *J Exp Med* **186**, 1781-6.

Simon M. M., Waring P., Lobigs M., Nil A., Tran T., Hla R. T., Chin S. and Mullbacher A. (2000) Cytotoxic T cells specifically induce Fas on target cells, thereby facilitating exocytosis-independent induction of apoptosis. *J Immunol* **165**, 3663-72.

Sing A., Merlin T., Knopf H. P., Nielsen P. J., Loppnow H., Galanos C. and Freudenberg M. A. (2000) Bacterial induction of beta interferon in mice is a function of the lipopolysaccharide component. *Infect Immun* **68**, 1600-7.

Sinigaglia F., D'Ambrosio D. and Rogge L. (1999) Type I interferons and the Th1/Th2 paradigm. *Dev Comp Immunol* **23**, 657-63.

Sjoberg M. and Garoff H. (2003) Interactions between the transmembrane segments of the alphavirus E1 and E2 proteins play a role in virus budding and fusion. *J Virol* **77**, 3441-50.

Smith T. J., Cheng R. H., Olson N. H., Peterson P., Chase E., Kuhn R. J. and Baker T. S. (1995) Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy. *Proc Natl Acad Sci U S A* **92**, 10648-52.

Smithburn K. C. and Haddow A. (1944) Semliki Forest Virus. I. Isolation and pathogenic properties. *Journal of Immunology* **49**, 141-157.

Smith-Norowitz T. A., Sobel R. A. and Mokhtarian F. (2000) B cells and antibodies in the pathogenesis of myelin injury in Semliki Forest Virus encephalomyelitis. *Cell Immunol* **200**, 27-35.

Smyth M. J. and Trapani J. A. (1995) Granzymes: exogenous proteinases that induce target cell apoptosis. *Immunol Today* **16**, 202-6.

Soilu-Hanninen M., Eralinna J. P., Hukkanen V., Roytta M., Salmi A. A. and Salonen R. (1994) Semliki Forest virus infects mouse brain endothelial cells and causes blood-brain barrier damage. *J Virol* **68**, 6291-8.

Song Q., Burrows S. R., Smith G., Lees-Miller S. P., Kumar S., Chan D. W., Trapani J. A., Alnemri E., Litwack G., Lu H., Moss D. J., Jackson S. and Lavin M. F. (1996) Interleukin-1 beta-converting enzyme-like protease cleaves DNA-dependent protein kinase in cytotoxic T cell killing. *J Exp Med* **184**, 619-26.

Spielman J., Lee R. K. and Podack E. R. (1998) Perforin/Fas-ligand double deficiency is associated with macrophage expansion and severe pancreatitis. *J Immunol* **161**, 7063-70.

Spiliotis E. T., Manley H., Osorio M., Zuniga M. C. and Edidin M. (2000) Selective export of MHC class I molecules from the ER after their dissociation from TAP. *Immunity* **13**, 841-51.

Spriggs M. K., Koller B. H., Sato T., Morrissey P. J., Fanslow W. C., Smithies O., Voice R. F., Widmer M. B. and Maliszewski C. R. (1992) Beta 2-microglobulin-, CD8+ T-cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses. *Proc Natl Acad Sci U S A* **89**, 6070-4.

Srinivasula S. M., Fernandes-Alnemri T., Zangrilli J., Robertson N., Armstrong R. C., Wang L., Trapani J. A., Tomaselli K. J., Litwack G. and Alnemri E. S. (1996) The Ced-3/interleukin 1beta converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2alpha are substrates for the apoptotic mediator CPP32. *J Biol Chem* **271**, 27099-106.

Stalder T., Hahn S. and Erb P. (1994) Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. *J Immunol* **152**, 1127-33.

Stark G. R., Kerr I. M., Williams B. R., Silverman R. H. and Schreiber R. D. (1998) How cells respond to interferons. *Annu Rev Biochem* **67**, 227-64.

Steinman R. M. and Swanson J. (1995) The endocytic activity of dendritic cells. *J Exp Med* **182**, 283-8.

Steinman R. M. and Witmer M. D. (1978) Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* **75**, 5132-6.

Steinmetz M., Winoto A., Minard K. and Hood L. (1982) Clusters of genes encoding mouse transplantation antigens. *Cell* **28**, 489-98.

Stern L. J. and Wiley D. C. (1994) Antigenic peptide binding by class I and class II histocompatibility proteins. *Structure* **2**, 245-51.

Stinchcombe J. C., Bossi G., Booth S. and Griffiths G. M. (2001) The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* **15**, 751-61.

Stober D., Schirmbeck R. and Reimann J. (2001) IL-12/IL-18-dependent IFN-gamma release by murine dendritic cells. *J Immunol* **167**, 957-65.

Strassmann G., Patil-Koota V., Finkelman F., Fong M. and Kambayashi T. (1994) Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J Exp Med* **180**, 2365-70.

Strauss J. H. and Strauss E. G. (1994) The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58**, 491-562.

Street S. E., Hayakawa Y., Zhan Y., Lew A. M., MacGregor D., Jamieson A. M., Diefenbach A., Yagita H., Godfrey D. I. and Smyth M. J. (2004) Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. *J Exp Med* **199**, 879-84.

Subak-Sharpe I., Dyson H. and Fazakerley J. (1993) In vivo depletion of CD8+ T cells prevents lesions of demyelination in Semliki Forest virus infection. *J Virol* **67**, 7629-33.

Suda T., Okazaki T., Naito Y., Yokota T., Arai N., Ozaki S., Nakao K. and Nagata S. (1995) Expression of the Fas ligand in cells of T cell lineage. *J Immunol* **154**, 3806-13.

Suda T., Takahashi T., Golstein P. and Nagata S. (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75**, 1169-78.

Suomalainen M., Liljestrom P. and Garoff H. (1992) Spike protein-nucleocapsid interactions drive the budding of alphaviruses. *J Virol* **66**, 4737-47.

Sutton V. R., Davis J. E., Cancilla M., Johnstone R. W., Ruefli A. A., Sedelies K., Browne K. A. and Trapani J. A. (2000) Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J Exp Med* **192**, 1403-14.

Sytwu H. K., Liblau R. S. and McDevitt H. O. (1996) The roles of Fas/APO-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor transgenic mice. *Immunity* **5**, 17-30.

Tagawa Y., Sekikawa K. and Iwakura Y. (1997) Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. *J Immunol* **159**, 1418-28.

Takahashi T., Tanaka M., Brannan C. I., Jenkins N. A., Copeland N. G., Suda T. and Nagata S. (1994) Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* **76**, 969-76.

Tanaka-Kataoka M., Kunikata T., Takayama S., Iwaki K., Ohashi K., Ikeda M. and Kurimoto M. (1999) In vivo antiviral effect of interleukin 18 in a mouse model of vaccinia virus infection. *Cytokine* **11**, 593-9.

Tang H. L. and Cyster J. G. (1999) Chemokine Up-regulation and activated T cell attraction by maturing dendritic cells. *Science* **284**, 819-22.

Tarbatt C. J., Glasgow G. M., Mooney D. A., Sheahan B. J. and Atkins G. J. (1997) Sequence analysis of the avirulent, demyelinating A7 strain of Semliki Forest virus. *J Gen Virol* **78 (Pt 7)**, 1551-7.

Tartaglia L. A., Ayres T. M., Wong G. H. and Goeddel D. V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74**, 845-53.

Tough D. F., Borrow P. and Sprent J. (1996) Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science* **272**, 1947-50.

Tough D. F. and Sprent J. (1994) Turnover of naive- and memory-phenotype T cells. *J Exp Med* **179**, 1127-35.

Trapani J. A. and Smyth M. J. (1993) Killing by cytotoxic T cells and natural killer cells: multiple granule serine proteases as initiators of DNA fragmentation. *Immunol Cell Biol* **71 (Pt 3)**, 201-8.

Trapani J. A. and Smyth M. J. (2002) Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* **2**, 735-47.

Trinchieri G. (1989) Biology of natural killer cells. *Adv Immunol* **47**, 187-376.

Trinchieri G. (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**, 133-46.

Tripp R. A., Hou S., McMickle A., Houston J. and Doherty P. C. (1995) Recruitment and proliferation of CD8+ T cells in respiratory virus infections. *J Immunol* **154**, 6013-21.

Tuittila M. T., Santagati M. G., Roytta M., Maatta J. A. and Hinkkanen A. E. (2000) Replicase complex genes of Semliki Forest virus confer lethal neurovirulence. *J Virol* **74**, 4579-89.

Turk V., Turk B. and Turk D. (2001) Lysosomal cysteine proteases: facts and opportunities. *Embo J* **20**, 4629-33.

Turley S. J., Inaba K., Garrett W. S., Ebersold M., Unternaehrer J., Steinman R. M. and Mellman I. (2000) Transport of peptide-MHC class II complexes in developing dendritic cells. *Science* **288**, 522-7.

van den Broek M. F., Muller U., Huang S., Zinkernagel R. M. and Aguet M. (1995) Immune defence in mice lacking type I and/or type II interferon receptors. *Immunol Rev* **148**, 5-18.

Van Voorhis W. C., Valinsky J., Hoffman E., Luban J., Hair L. S. and Steinman R. M. (1983) Relative efficacy of human monocytes and dendritic cells as accessory cells for T cell replication. *J Exp Med* **158**, 174-91.

Vassalli P., Grau G. E. and Piguet P. F. (1992) TNF in autoimmune diseases, graft-versus-host reactions, and pulmonary fibrosis. *Immunol Ser* **56**, 409-30.

Vesosky B. and Hurwitz A. A. (2003) Modulation of costimulation to enhance tumor immunity. *Cancer Immunol Immunother* **52**, 663-9.

von Boehmer H., Aifantis I., Gounari F., Azogui O., Haughn L., Apostolou I., Jaeckel E., Grassi F. and Klein L. (2003) Thymic selection revisited: how essential is it? *Immunol Rev* **191**, 62-78.

Wahlberg J. M., Bron R., Wilschut J. and Garoff H. (1992) Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein. *J Virol* **66**, 7309-18.

Wahlberg J. M. and Garoff H. (1992) Membrane fusion process of Semliki Forest virus. I: Low pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into cells. *J Cell Biol* **116**, 339-48.

Waldmann T. A. and Tagaya Y. (1999) The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol* **17**, 19-49.

Walter M. R., Bordens R., Nagabhushan T. L., Williams B. R., Herberman R. B., Dinarello C. A., Borden E. C., Trotta P. P., Pestka S. and Pfeffer L. M. (1998) Review of recent developments in the molecular characterization of recombinant alfa interferons on the 40th anniversary of the discovery of interferon. *Cancer Biother Radiopharm* **13**, 143-54.

Wang K. S., Kuhn R. J., Strauss E. G., Ou S. and Strauss J. H. (1992) High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J Virol* **66**, 4992-5001.

Wang R., Rogers A. M., Ratliff T. L. and Russell J. H. (1996) CD95-dependent bystander lysis caused by CD4+ T helper 1 effectors. *J Immunol* **157**, 2961-8.

Wang Y., Lobigs M., Lee E. and Mullbacher A. (2003) CD8+ T cells mediate recovery and immunopathology in West Nile virus encephalitis. *J Virol* **77**, 13323-34.

Wang Y., Lobigs M., Lee E. and Mullbacher A. (2004) Exocytosis and Fas mediated cytolytic mechanisms exert protection from West Nile virus induced encephalitis in mice. *Immunol Cell Biol* **82**, 170-3.

Waring P. and Mullbacher A. (1999) Cell death induced by the Fas/Fas ligand pathway and its role in pathology. *Immunol Cell Biol* **77**, 312-7.

Watanabe D., Suda T. and Nagata S. (1995) Expression of Fas in B cells of the mouse germinal center and Fas-dependent killing of activated B cells. *Int Immunol* **7**, 1949-56.

Watanabe-Fukunaga R., Brannan C. I., Copeland N. G., Jenkins N. A. and Nagata S. (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356**, 314-7.

Waterhouse P., Penninger J. M., Timms E., Wakeham A., Shahinian A., Lee K. P., Thompson C. B., Griesser H. and Mak T. W. (1995) Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* **270**, 985-8.

Watts T. H. and DeBenedette M. A. (1999) T cell co-stimulatory molecules other than CD28. *Curr Opin Immunol* **11**, 286-93.

Weiss B., Nitschko H., Ghattas I., Wright R. and Schlesinger S. (1989) Evidence for specificity in the encapsidation of Sindbis virus RNAs. *J Virol* **63**, 5310-8.

Welsh R. M., Brubaker J. O., Vargas-Cortes M. and O'Donnell C. L. (1991) Natural killer (NK) cell response to virus infections in mice with severe combined immunodeficiency. The stimulation of NK cells and the NK cell-dependent control of virus infections occur independently of T and B cell function. *J Exp Med* **173**, 1053-63.

Welsh R. M., Jr. (1978a) Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. I. Characterization of natural killer cell induction. *J Exp Med* **148**, 163-81.

Welsh R. M., Jr. (1978b) Mouse natural killer cells: induction specificity, and function. *J Immunol* **121**, 1631-5.

Welsh R. M., O'Donnell C. L. and Shultz L. D. (1994) Antiviral activity of NK 1.1+ natural killer cells in C57BL/6 scid mice infected with murine cytomegalovirus. *Nat Immun* **13**, 239-45.

Wengler G. and Wurkner D. (1992) Identification of a sequence element in the alphavirus core protein which mediates interaction of cores with ribosomes and the disassembly of cores. *Virology* **191**, 880-8.

White L. J., Wang J. G., Davis N. L. and Johnston R. E. (2001) Role of alpha/beta interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an attenuating mutation in the 5' untranslated region. *J Virol* **75**, 3706-18.

Wildy P., Gell P. G., Rhodes J. and Newton A. (1982) Inhibition of herpes simplex virus multiplication by activated macrophages: a role for arginase? *Infect Immun* **37**, 40-5.

Willems W. R., Kaluza G., Boschek C. B., Bauer H., Hager H., Schutz H. J. and Feistner H. (1979) Semliki forest virus: cause of a fatal case of human encephalitis. *Science* **203**, 1127-9.

Witmer-Pack M. D., Olivier W., Valinsky J., Schuler G. and Steinman R. M. (1987) Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J Exp Med* **166**, 1484-98.

Wong G. H. and Goeddel D. V. (1986) Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons. *Nature* **323**, 819-22.

Woodward C. G., Marshall I. D. and Smith H. (1977) Investigations of reasons for the avirulence of the A7 strain of Semliki Forest virus in adult mice. *Br J Exp Pathol* **58**, 616-24.

Yang H. and Welsh R. M. (1986) Induction of allospecific and virus-specific memory cytotoxic T cells during acute arenavirus infections. *Med Microbiol Immunol (Berl)* **175**, 137-9.

Yang X., Stennicke H. R., Wang B., Green D. R., Janicke R. U., Srinivasan A., Seth P., Salvesen G. S. and Froelich C. J. (1998) Granzyme B mimics apical caspases. Description of a unified pathway for trans-activation of executioner caspase-3 and -7. *J Biol Chem* **273**, 34278-83.

Yap K. L. and Ada G. L. (1978) The recovery of mice from influenza A virus infection: adoptive transfer of immunity with influenza virus-specific cytotoxic T lymphocytes recognizing a common virion antigen. *Scand J Immunol* **8**, 413-20.

Yewdell J. (2002) To DRiP or not to DRiP: generating peptide ligands for MHC class I molecules from biosynthesized proteins. *Mol Immunol* **39**, 139-46.

Young J. D., Liu C. C., Leong L. G. and Cohn Z. A. (1986) The pore-forming protein (perforin) of cytolytic T lymphocytes is immunologically related to the

components of membrane attack complex of complement through cysteine-rich domains. *J Exp Med* **164**, 2077-82.

Zajac A. J., Muller D., Pederson K., Frelinger J. A. and Quinn D. G. (1995) Natural killer cell activity in lymphocytic choriomeningitis virus-infected beta 2-microglobulin-deficient mice. *Int Immunol* **7**, 1545-56.

Zhang W. J., Sarawar S., Nguyen P., Daly K., Rehg J. E., Doherty P. C., Woodland D. L. and Blackman M. A. (1996) Lethal synergism between influenza infection and staphylococcal enterotoxin B in mice. *J Immunol* **157**, 5049-60.

Zheng L., Fisher G., Miller R. E., Peschon J., Lynch D. H. and Lenardo M. J. (1995) Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature* **377**, 348-51.

Zheng L., Trageser C. L., Willerford D. M. and Lenardo M. J. (1998) T cell growth cytokines cause the superinduction of molecules mediating antigen-induced T lymphocyte death. *J Immunol* **160**, 763-9.

Ziegler S. F., Ramsdell F. and Alderson M. R. (1994) The activation antigen CD69. *Stem Cells* **12**, 456-65.

Zijlstra M., Bix M., Simister N. E., Loring J. M., Raulet D. H. and Jaenisch R. (1990) Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. *Nature* **344**, 742-6.

Zinkernagel R. M. and Doherty P. C. (1974) Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**, 701-2.

Zinkernagel R. M. and Doherty P. C. (1979) MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv Immunol* **27**, 51-177.

Zinkernagel R. M., Dunlop M. B., Blanden R. V., Doherty P. C. and Shreffler D. C. (1976) H-2 compatibility requirement for virus-specific T-cell-mediated cytolysis. Evaluation of the role of H-2I region and non-H-2 genes in regulating immune response. *J Exp Med* **144**, 519-32.